ISOLATION AND CHARACTERIZATION OF LACTIC ACID BACTERIA AND THEIR INHIBITORY/SAFETY POTENTIAL IN TRADITIONALLY FERMENTED MILK, *MURSIK* IN KENYA

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DECLARATION AND APPROVAL

Declaration

I declare that this thesis is my original work and has not been presented in this or any other University for any degree.

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Approval

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DEDICATION

To my dear Dad Mr. Joshua Laja for his hard work, the role of a model and an inspiring father. My beloved mother Alice, my siblings: Harry and Adams; my cousin Kwame, my dear wife Jackline and my daughter Mary, thanks for your unconditional support and prayers.

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ABSTRACT

Commercial fermented milks have health benefit potential attributed to pure probiotics used as starter culture. Lactic acid bacteria (LABs) are the probiotics used. LABs promote stabilization of the gastrointestinal microecology of humans by producing secondary metabolites like lactic, acetic and propionic acid, hydrogen peroxide and bacteriocins (bactericidal proteins). These metabolites are health benefiting. *Mursik,* an indigenous fermented milk product is consumed by many Kenyan communities, specifically the Kalenjin. The fermentation of *mursik* is not based on pure cultures, instead it is spontaneous. Whether the types and concentrations of the probiotics in this traditionally fermented milk have the same probiotic potential as those of commercial fermented milk is not documented. This study aimed at isolating *mursik* probiotics and determining their probiotic potential. *Mursik* was obtained from informal women groups and individuals involved in small scale production and marketing in Bomet County, Kenya. Two types of mursik are made in this county; smoked and non-smoked. A total of forty one (41) *mursik* samples were collected and analysed. Type and concentration of the probiotics was determined by pour plate method. The inhibitory/safety potential was determined by acid and bile tolerance, as well using disc diffusion method against *Salmonella enterica* ATCC 13076, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* isolate which were used as standard strains of public health concern. Data was analyzed using analysis of variance (ANOVA) and chi-square tests at $\alpha = 0.05$. 78 % of the samples had a microbial load of $\geq 10^7$ cfu/ml. LABs formed 80 % of the microbial load in *mursik*. The remaining 20 % consisted of coliforms and spore forming bacteria. The main probiotics isolated from mursik were, *Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus brevis* and *Lactobacillus casei.* The metabolites produced by the isolated probiotics in broth demonstrated significant ($P<0.05$) antibacterial effect against the standard strains of public health concern used. The results obtained from this study confirm that probiotics exist in *mursik*, a traditionally fermented milk product and that these probiotics have significant potential against enteric and environmental pathogens that are of public health concern.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	-	Analysis of Variance
BCP	-	Bromocresol purple
CFU	-	Colony-Forming Units
FAO	-	Food and Agriculture Organization
GI	-	Gastrointestinal Infections
GIT	-	Gastrointestinal Tract
H. pylori	-	*Helicobacter pylori*
H_2O_2	-	Hydrogen Peroxide
HIV	-	Human Immunodeficiency Virus
IDF	-	International Dairy Federation
LAB	-	Lactic Acid Bacteria
LGG	-	*Lactobacillus rhamnosus* GG
mg	-	Milligram
mL	-	Millilitre
MRS	-	de Man-Rogosa-Sharpe
PUD	-	Peptic Ulcer Disease
US	-	United States
SD	-	Standard Deviation
Spp	-	Species
SPSS	-	Statistical product for service and solutions
WHO	-	World Health Organization

CHAPTER ONE
INTRODUCTION

1.1 Background Information

In Africa, spontaneous food fermentation has a long history and relies on indigenous knowledge of the majority of the communities. Most of the indigenous food products are informally produced. Many still occur at the household-scale or at small enterprise scale (Elaine & Danilo, 2012). A number of communities in Kenya use traditionally fermented milk. The Kalenjin use *mursik*, the North Kenya pastoralists' *suusa* and the Maasai community, *kule naoto*. These communities have been taking these milk products as traditional food product for a long time because of their attributes of taste, flavour, consistency, colour and shelf life (Mathara et al., 2008; Lore et al., 2005). These products have since found their way to informal urban market sectors of Kenya because of dynamic urbanization, hence, increasing the demand at urban levels.

Mursik is from un-pasteurized whole cow's milk. It is prepared by leaving milk to naturally ferment spontaneously in a gourd (*sotet*) for at least 3-5 days, though a longer period is usually preferred (Mathara, 1999; Muigei et al., 2013). *Mursik* is preferred among the Kalenjin community and the Nilotic people of East Africa because of its aroma and taste. As a traditional food product, it has been associated with therapeutic values towards curing or protecting consumers from ailments such as diarrhoea and constipation (Mathara, 1999). The therapeutic value of fermented milk products is mainly due to the production of secondary metabolites, organic acids (lactic, acetic and propionic acid), hydrogen peroxide and bacteriocins or bactericidal proteins produced by the lactic acid bacteria (Ostad, et al., 2009).

These secondary metabolites not only necessitate desirable effects on food attributes e.g. taste, smell, colour and texture but also inhibit undesirable gastrointestinal and environmental microorganisms (Elaine & Danilo, 2012). Organic acids produced as end products provide an acidic environment unfavourable for growth of many pathogenic and spoilage microorganisms such *Escherichia coli* and *Salmonella enterica* (Parkes et al., 2009). Probiotics are live microorganisms, which, when consumed in appropriate amounts, result in health benefit to the host (Saunders, 2000; Guarner et al., 2005; FAO, 2006). These probiotic properties have been documented from the tests using commercial fermented milk where pure probiotic cultures are used industrially.

The potential benefit following the consumption of fermented dairy products is due to viable lactic acid bacteria (LABs). LABs promote stabilization of the gastrointestinal microecology by producing secondary metabolites (Parvez et al., 2006; World Gastroenterology Organisation, 2009). Most of the lactic acid bacteria consumed in fermented milk products are grouped as part of probiotics. They include *Streptococcus thermophilus, Lactobacillus delbrueckii, Lactococcus lactis and Lactobacillus acidophilus* with the commonest being *Lactobacillus* strains (Abdullah & Osman, 2010). The mechanisms by which probiotics exert their effects is not very clear, but postulated to involve gut pH modification, antimicrobial compounds production, competition for binding and receptor sites, competition for nutrients and growth factors and immune modulator cells stimulation (Vanderpool et al., 2008; Britton & Versalovic, 2008; Wang et al., 2009).

Currently probiotic use is being advocated for as an alternative to conventional drugs used for gastrointestinal infections (WHO, 2010). In Asia probiotics from traditional fermented milk, *kumis* has been applied for therapeutic purposes (Barakat et al., 2011). Probiotics bacteria have demonstared inhibition against gastrointestinal pathogens such as *Helicobacter pylori, Salmonella enteritidis, Salmonella typhimurium, Escherichia coli, Bacillus cereus, Listeria monocytogenes, Clostridium difficile, Campylobacter jejuni* and *Vibrio cholera* have been established (Ashton, 2013; Hoque et al., 2010). Probiotics use, either through food or parentally to reduce the effect of gastrointestinal pathogenic infections has been tried through addition of bio-typed probiotics in yoghurts (Serim, 2004; Chichlowsk et al., 2007; Mengkol, 2011).

Mursik in Kenya has been consumed for a long time but has never been prescribed for therapeutic purposes, however communities consuming it say it's good for diarrhoea. The reason is that there has been no documentation of its properties in the communities that consume it related to health benefits. There is therefore limited information on therapeutic properties of mursik. This study characterized the types and concentration of probiotics present in the *mursik* and determined their probiotic potential against some enteric and environmental pathogens.

1.2 Statement of the Problem

In Sub-Saharan Africa, gastrointestinal infections are prevalent with a rate of 55.7 %. In Kenya diarrhoeal rates are high with a prevalence of 80 %. The diarrhoea incidences are as a result of pathogens of public health concern such as *Salmonella typhimurium*, *Escherichia coli*, *Campylobacter jejuni* and *Vibrio cholera.* Antibiotic–based treatment against these pathogens is 90 % effective. But it is expensive, prone to antibiotic resistance and accumulation of residues in the body. Antibiotic resistance has threatened loss of therapeutic options. Currently the World Health Organization is advocating for alternative disease control strategies, such as use of potential probiotic bacteria. Fermented milks produced commercially are the main source of probiotics. They contain pure culture probiotics of known concentration and type. Traditionally fermented milk products that do not use purified known cultures are expected to have potential probiotic bacteria. *Mursik* in Kenya has been consumed for a long time but has never been prescribed for therapeutic purposes. The communities that consume it only associate it with treatment of diarrhoea. There is no documentation of its properties in the communities that consume it related to health benefits. This study aimed at isolating, characterizing and testing the probiotic potential of probiotics in *mursik.*

1.3 Objectives

1.3.1 General objective

To enhance the use of indigenous fermented milk products in the prevention of gastrointestinal infections by exploiting probiotic potential of the probiotics in traditional fermented milk, *mursik*.

1.3.2 Specific objectives

1. To determine the type and concentration of probiotics in traditionally fermented milk, *mursik*.
2. To isolate and characterize probiotic species in traditionally fermented milk, *mursik*.
3. To determine the probiotic potential of the isolated probiotic *species* from *mursik.*

1.4 Hypothesis

1. The type and concentration of probiotics in *mursik* will be insignificant.
2. The species of probiotics in *mursik* are not the same as those used in commercial fermentations.
3. The probiotic potential of the probiotics in *mursik* is limited.

1.5 Justification

Gastrointestinal infections are major cause of death in children (WHO, 2010). The risks involved include gastroenteritis, peptic ulcer, diarrhoea and dehydration. In 2012, Tabu et al., reported that the prevalence of diarrhoea in Kenya is 80 %. The major causes of diarrhoea are the gastrointestinal pathogens such as *Helicobacter pylori, Campylobacter jejuni, Salmonella enteritidis, Salmonella typhimurium*, *Escherichia coli* and *Vibrio cholera.* Treatment against these gastrointestinal pathogens is mainly through conventional antibiotics. In 2013, World Health Organization reported antibiotic resistance as a global health security. Currently the WHO in collaboration with FAO are advocating for use of alternatives to control gastrointestinal pathogens including through use of probiotics (WHO, 2013). In Kenya the common source of probiotics are yoghurts and commercial fermented milk, though a number of communities in Kenya consume traditionally fermented milk products. In other countries, such as Asian countries use of traditionally fermented milk, *kumis* as probiotic source to control gastrointestinal pathogens has been tried (Pawar et al., 2012). This is not the case in Kenya. While there is a growing body of knowledge on the potential of pure probiotics from fermented milk products in gastrointestinal pathogens control, there is a dearth of literature on evaluation of probiotic potential of *mursik*. This study aimed at isolating, characterizing and testing the probiotic potential of probiotics in *mursik.* Hence contributing towards alternative gastrointestinal infections control.

1.6 Scope and Limitation of the Study

The study was conducted in Silibwet and Chebunyo locations in Bomet and Chepalungu sub-counties respectively, in Bomet County. In this sub-counties dairy farming is carried out on both small scale and large scale that supply milk and milk products to Bomet town and other towns in outside the county.

The study was be limited to small-scale producers of milk and milk products with a production capacity ranging one litre to ten litres.

CHAPTER TWO
LITERATURE REVIEW

2.1 Fermented milk products

Deliberate fermentation of foods by man precedes written history and is possibly the oldest method of preserving perishable foods. In the world there are a large variety of fermented milk with traditional and cultural value. The diversity of such fermented products derives from the heterogeneity of traditions found around the world, cultural preferences, different geographical areas where they are produced (Elaine & Danilo, 2012). In many instances the method of production were unknown and came about by chance, and passed down by cultural and traditional values to subsequent generations. For generations, fermentation has been used as an effective means of extending the storage life of milk in Africa (FAO, 1990). Cow, goat, sheep or camel milk may be used as the raw material. In some cases, a mixture of milk from different animals may be used. During fermentation, the lactic acid bacteria in the milk convert the milk sugar lactose into lactic acid, which gives the product a distinct sour taste. In addition to lactic acid, LABs also produce a number of secondary metabolites, organic acids (acetic and propionic acid), hydrogen peroxide and bacteriocins or bactericidal proteins. Antimicrobial substances, bacteriocins produced inhibit growth of disease-causing bacteria or pathogens of public health concern (Abdullah & Osman, 2010). Microbial safety of raw milk is of concern primarily because milk is a highly nutritious product that readily supports the growth of spoilage microbes (Forouhandeh et al., 2010).

Fermented milk may be safer than fresh milk since the lactic acid and the bacteriocins kill coliforms and other disease-causing bacteria such as *E. coli*. In addition to enhanced microbiological safety, regular drinking of fermented milk offer various health-related benefits to the consumer, such as lowering of cholesterol, boosting of the body's immune system, and protection against diarrhoea and constipation (Chung et al., 2010). Spontaneous fermentation of milk takes advantage of the actions of various species of lactic acid bacteria that occur naturally in milk. (Chamberlain & Subden, 1997; Martin et al., 2004; Sansonnetti, 2008).

Fermented foods of either plant or animal origin, form an integral part of the peoples' diet in all parts of the world. Fermented food plays a very important role in the socioeconomics of developing countries by extending the shelf life of foods with aspects such as wholesomeness, acceptability

and quality. Every country has their own types of fermented food, representing the staple diet and the raw ingredients available in that region (Lore et al., 2005). Kenya is home to a variety of traditional fermented milk products such as *mursik* of the Kalenjin community, *susaa* of the North Kenya pastoralists, *ambere amaruranu* of the Gusii and *kule naoto* of the Maasai community (Muinde, 2011; Muigei et al., 2013). *Mursik* is prepared from cows' milk fermented in ash-treated gourds. Blood may be added to fresh milk before fermentation, or to already fermented milk. The milk (or blood-milk mixture) is heated to boiling point then cooled to ambient temperature. Then the mixture undergoes spontaneous fermentation for three to five days, through the action of lactic acid bacteria, yeast and mould species (Mathara et al., 2008; Muigei et al., 2013). Fermentation makes major contributions to the health benefits of the rural population. The preparation of many indigenous or traditional fermented foods and beverages remains a household art to date (Elaine & Danilo, 2012).

Fermented milk products are an important part of the diet for many communities of Kenya and may protect against some infections. The high concentrations of the lactic acid bacteria in traditional diets, such as *mursik* may play an important biological role in the gastrointestinal tract of humans. Furthermore, probiotics represent an exciting therapeutic advance that should be exploited. Investigation must be undertaken before their role in gastroenterology is clearly delineated. Importantly, each fermented milk being proposed for probiotic use must be studied individually and extensively to determine its efficacy and safety in each disorder for which its use may be considered. Studies have reported that fermented milk products are potential probiotic sources. These probiotic have exhibited health benefiting properties in their control of gastrointestinal pathogens of public health concern (Anuradha & Rajeshwari, 2005; Britton & Versalovic, 2008).

2.1.1 Health benefits of fermented diets.

Fermented foods enjoyed across the globe, conveys health benefits through lactic acid fermentation. The health benefits conveyed are due to transformation of food flavor from plain to a sourness invigorated by colonies of beneficial bacteria and enhanced micronutrients bioavailability (Dunne et al., 1999). Studies on the Nigerian fermented foods such as *ogi* and *kunun-zaki* with *Lactobacillus rhamnosus* and *Lactobacillus reuteri* revealed that they can colonize the vagina, kill viruses, and reduce the risk of infections, including bacterial vaginosis (Lee & Salimen, 1995; Reid, 2002). The potential therapeutic effects of Lactic acid bacteria include their immune-

stimulatory effect, by promoting beneficial bacteria growth in the gastrointestinal tract (GIT) which in turn suppress the growth of pathogens (Britton & Versalovic, 2008). Population increase of the LABs has been predicted to suppress gastrointestinal pathogenic bacteria of public health concern such as *Helicobacter pylori*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Escherichia coli*, *Clostridium difficile*, *Campylobacter jejuni* and *Vibrio cholera* (Reid, 2002; Adebolu et al., 2007; Ashton, 2013).

Lactic acid producing bacteria such as *Lactobacillus acidophilus, L. bulgaricus, L. plantarum, L. caret, L. pentoaceticus, L. brevis and L. themophilus* produces high acidity during fermentation (Dardir, 2012). The high acidity is attributed to many organic acids such as lactic, acetic and propionic acids produced as end products which provide an acidic environment unfavorable for the growth of gastrointestinal pathogenic bacteria. Acids are generally thought to exert their antimicrobial effect by interfering with the maintenance of cell membrane potential, inhibiting active transport, reducing intracellular pH and inhibiting a variety of metabolic functions (Rattanachaikunsopon & Phumkhachorn, 2010). They have a very broad mode of action and inhibit both gram-positive and gram-negative bacteria as well as yeast and moulds (Hoque et al., 2010). One good example is propionic acid produced by propionic acid bacteria, which has formed the basis for some bio-preservative products, given its antimicrobial action against microorganisms including yeast and moulds. Despite their complexity, the whole basis of lactic acid fermentation centers on the ability of lactic acid bacteria to produce organic acids, which then inhibits the growth of other non-desirable organisms. Other compounds are important as they improve particular tastes and aromas to the final products and help the human gastrointestinal micro flora. Although recently studies have suggested that the human gastrointestinal micro flora may contain more than 2000 species of bacteria genera which were previously unknown to be in the GIT (McFall-Ngai, 2007).

The micro flora begins to colonize the gastrointestinal tract of young infants immediately at birth usually through the first diets, and the interaction between host and micro flora continues throughout their entire life. In well recognized and characterized patterns of succession, the micro biota develops in response to exposure, as well as host health and nutrition, especially on locally available diets (Lu et al., 2003). These microbes help break down food in the intestines, aid in the digestion process, help fight off disease, and boost the immune system. A good balance of intestinal flora is very important to the overall health. Predigested foods such as fermented foods help the

gut micro flora to flourish well as they also provide them metabolites for their nutrition (Chichlowsk et al., 2007). Fermentation is not only a way to preserve certain foods, in some cases it actually adds to the nutrient value of it. Fermented foods contain more vitamin C and B-complex vitamins. The bioavailability of these vitamins also increases with fermentation (Chung et al., 2010).

Apart from to the gastrointestinal tract providing a home to numerous microbial inhabitants, the intestinal tract is also an active immunological organ. It comprises of more resident immune cells than anywhere else in the body (Britton & Versalovic, 2008) enhanced by fermented foods. But for a host encountering an overt or an opportunist pathogen or due to a pathological overgrowth of an opportunistic member of the intestinal microbial community gastrointestinal infection occurs. While for a successful infection the infecting agent has to overcome a number of the host defence, such as the mucosal immunity or colonization resistance posed by the normal micro biota and the beneficial diets consumed by an individual (Inna & Brett, 2009).

2.2 Gastrointestinal Infections

Gastrointestinal infections are a major cause of morbidity and mortality. Lopez et al, (2006) reported that severe diarrhoea and dehydration contributes significantly to about 15 % of the annual children under five years mortality rates. The world's gastrointestinal infections prevalence is > 50 % with an estimated prevalence of > 70 % in the developing countries and about 20 – 30 % in developed countries (Suerbaum & Michetti, 2002; WHO, 2010). Studies in developing countries have reported that > 50 percent of children are infected with gastrointestinal infections by the age of 10 years and the prevalence of infections is > 80 % in adults. In developed countries the gastrointestinal rate to children is < less than 1 % (Segal et al., 2001; Mandeville et al., 2009). In Africa, studies have documented the prevalence to be slightly higher, > 85 % (Susan et al., 2009; de Jong et al., 2012). In Kenya diarrhoeal prevalence alone is > 80 % as documented by Tabu et al., (2012), contributing significantly to the gastrointestinal infections burden.

The gastrointestinal pathogens, notably *Helicobacter pylori*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Escherichia coli*, *Bacillus cereus*, *Listeria monocytogenes*, *Clostridium difficile*, *Campylobacter jejuni* and *Vibrio cholerae*, have been studied and shown to cause a variety of human diseases including gastroenteritis, peptic ulcer and diarrhoea (Felley & Michetti, 2003). These gastrointestinal infection pathogens are transmitted through human-to-human transmission,

thus either vertical or horizontal transmission. The vertical transmission is spread from ascendants to descendants within the same family, while horizontal transmission involves contact with individuals outside the family but does not exclude environment contamination via contaminated water, food or directly in young children and adults (Schwarz et al., 2008). The pathogens flourish well in settings characterised by poor sanitation, dirty/contaminated water, substandard and overcrowded housing (Godwin, 1994; Megraud & Lamouliatte, 2003; Ziemniak, 2006). These pathogens are also associated with gastric and colon cancers (Susan et al., 2005; Amanda, 2011). In past years the use of the probiotics either through food or parentally to reduce the effect of gastrointestinal pathogens has been tried industrially by bio fortification of yoghurt with probiotics (Sheu et al., 2002; Serim, 2004; Mengkol, 2011).

2.3 Probiotics and Health

Probiotics generally enhance the intestinal microflora by replenishing suppressed health benefiting bacteria and inhibiting pathogenic flora (Pennacchia et al., 2004; Gawad et al., 2010). Some probiotics including *Lactobacillus GG* actively secrete an antimicrobial substance which inhibits the growth of certain other organisms. Volatile fatty acids produced by the indigenous microflora, of which lactic acid bacteria form a part, are responsible for controlling the colonisation of gut by *S. Sonnei* and Enteropathogenic *E. coli*. Probiotics such as *S. faecalis, C. butyricum* and *B. mesentericus* grow profusely and also accelerate the growth of *bifidobacterium* (Thirabunyanon et al., 2009). *Bifidobacterium* is not only an antagonist of pathogenic organisms, but it also produces glutamine from NH_4^+ and glutamic acid. Glutamine is well known as a major supplement for intestinal epithelium and maintains mucosal integrity (Anuradha & Rajeshwari, 2005).

Secondary metabolites (biomolecules) of probiotic bacteria create a harmful environment for pathogenic *E. coli, Salmonella* and methicillin resistant *S. aureus*, by lowering intestinal pH (Tambekar & Bhutada, 2010a). Butyrate is also required by intestinal epithelial cells as a supplement to stimulate proliferation of normal epithelium (Anuradha & Rajeshwari, 2005). The gastrointestinal pathogens, notable above, cause a variety of human diseases including gastroenteritis, peptic ulcer and diarrhoea. These pathogens are also associated with gastric (Thirabunyanon et al., 2009) and colon cancers (Howe et al., 1992; Havenaar, Brink, & Huis Veld, 1992).

2.3.1 *Helicobacter pylori*

Helicobacter pylori causes chronic gastritis and peptic ulcers (Plummer et al.,2004). Treatment of these has been by antibiotics and the result has been development of resistance by the organism (Malfertheiner et al., 2002; Felley & Michetti, 2003). Alternatively, potential probiotics including, *Enterococcus faecium* and *Lactobacillus fermentum,* isolated from fermented dairy products have been tried and can inhibit the growth of *H. Pylori* (Myllyluoma et al., 2007; Thirabunyanon et al., 2009). Other probiotics that are being used for include *Lactobacillus rhamnosus* GG, *L. rhamnosus*, *Propionibacterium freudenreichii* and *Bifidobacterium lactis. , Lactobacillus acidophilus* and *Bifidobacterium lactis* (Wang et al., 2004).

2.3.2 *Salmonella spp.*

These are major food borne pathogens normally found in many food products. They cause many human diseases such as gastroenteritis, enteric fever, bacteraemia, faecal infections and enterocolitis. Though human salmonellosis is an important international public health and economic issue (Velge et al., 2005). Continual use of antimicrobial agents for treatment of salmonellosis has resulted in the emergence of antibiotic-resistant strains of *Salmonella*. This multi-drug resistance has caused great public health concern (Dechet et al., 2006; Buntin et al., 2008). Thirabunyanon et al. (2009) reported that LABs isolated from dairy products suppressed the growth of *Salmonella typhimurium.* A novel probiotic *Bacillus subtilis (*NC11) strain was protective against *Salmonella enteritidis* infection of intestinal epithelial cells. Similar results were obtained by use of probiotic *Lactobacillus plantarum* isolated from Greek cheese (Fayol-Messaoudi et al., 2007).

2.3.3 *Escherichia coli*

Escherichia coli is known to be the cause of various forms of diarrhoea and is classified into six categories. Enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. Coli* (EAggEC) and diffusely adherent *E. coli* (DAEC) (Miyazaki et al., 2010). Certain strains of EHEC are highly infectious pathogens that produce one or more Shiga toxins which induce gastrointestinal diseases such as diarrhoea, haemorrhagic colitis and life-threatening haemolytic uremic syndrome (HUS) in humans (Mead & Griffin, 1998; LeBlanc, 2003) thus posing serious global health threats. EAggEC infection is associated with childhood and adult diarrhoea such as travellers' diarrhoea, paediatric

diarrhoea and persistent diarrhoea (Miyazaki et al., 2010). Multi-drug resistance has led to limited use of antibiotics for treating *E. coli* infection and alternative therapies such as application of probiotics have been recommended.

In a study by Ostad et al., (2010) probiotic *Lactobacillus acidophilus* strain isolated is reported to inhibit EAggEC adhesion to gastrointestinal epithelial cells, thus preventing pathogenic colonisation and infection. Probiotic strain of *Enterococcus faecium* has demonstrated bactericidal effects on EAggEC by inducing membrane damage and cell lysis (Miyazaki et al., 2010).

2.3.4 *Clostridium difficile*

Approximately one third of cases of antibiotic-associated diarrhoea appear to be caused by *Clostridium difficile* (Fekety, 1997). A study by Gorbach et al., (1997) in adults suggested that orally administered *Lactobacillus rhamnosus* GG significantly reduced relapse after therapy for *C. difficile*-associated antibiotic-induced diarrhoea, while other effective probiotic treatment of *C. Difficile* infection such as *S. boulardii* has been proposed (Parkes et al., 2009; McFarland, 2009).

2.4 Probiotics Mechanism of Actions

Probiotic microorganisms are considered to support the host health. However, the support mechanisms have not been explained (Holzapfel et al., 1998). There are studies with postulates of how probiotics work (Serim, 2004; Mengkol, 2011), with some mechanisms from these studies are trying to explain how probiotics could protect the host from the intestinal disorders (Gibson & Roberfroid, 1995; Isolauri et al., 2002). These mechanisms are postulated as production of antimicrobial substances, completion for nutritional substrates, competitive exclusion, enhancement of intestinal barrier function and immunomodulation.

2.4.1 Production of Antimicrobial Substances

Several probiotic strains have been studied and indicated to produce antimicrobial substances which may exert their effective antagonistic activity alone or synergistically against gastrointestinal pathogens (Spinler et al., 2008; Gaudana et al., 2010). These antimicrobial substances were found to range in size from small molecules to bioactive peptides. Bacteriocins are important ribosomal synthesised antimicrobial peptides. They possess functional therapeutic activity against gastrointestinal pathogenic infection. These bacteriocins have been categorised into four classes. Class-I bacteriocins are small peptides (which are also classified as lantibiotics such as nisin).

Class-II bacteriocins are small, heat stable peptides such as pediocin. Class-III bacteriocins are large, heat-labile proteins such as helveticin J. Lastly, class-IV bacteriocins are complex bacteriocins (Jones & Versalovic, 2009).

2.4.2 Competition for Nutritional Substrates

Intestinal probiotic population in the gastrointestinal tract may increase after consuming certain diets. Thus, competition for nutritional substrates from diets amongst probiotics, gastrointestinal pathogens and micro biota may occur (Chichlowsk et al., 2007). Similarly, biogenic metabolites such as vitamins, fatty acids and bioactive peptides, may act as a growth substrate for selected compounds with different probiotics, intestinal pathogens or micro biota (Stanton et al., 2005).

2.4.3 Competitive Exclusion

On the other hand competitive exclusion may occur, this is whereby there is elimination of pathogens at the adhesion and infection sites of epithelial cells in the human intestine with the probiotics. Some pathogens begins with the binding to intestinal epithelial cells through the interaction between bacterial lectins and carbohydrate moieties of glycoconjugate receptor molecules on the intestinal epithelial cell surface (Vanderpool et al., 2008), hence competition by probiotic strains to bind on the intestinal glycolipids (Mukai et al., 2004) or colonisation of the intestinal cells (Ramiah et al., 2008) may prevent the adhesion of gastrointestinal pathogenic strains.

2.4.4 Enhancement of Intestinal Barrier Function

Pathophysiology of the intestinal pathogenic infection displays a disruption of epithelial barrier function and a loss of tight junction formation in the intestinal epithelium cells. These phenomena can increase the pathogenic or enterotoxin permeability of the mucosa wall (Khailova et al., 2009). Probiotics have been promoted for their enhancement of intestinal barrier function by impeding the translocation and attachment of pathogenic bacteria to the intestinal epithelium (Felley & Michetti, 2003; Hamilton-Miller, 2003; Mennigen et al., 2009).

2.4.5 Immuno-modulation

The role of intestinal epithelial cells is associated with immunomodulation through complex interactions between immune cells and probiotics, triggering a cascade of appropriate innate or adaptive immune defence responses (Vanderpool et al., 2008; Britton & Versalovic, 2008). The production of pro-inflammatory or anti-inflammatory cytokines by human peripheral blood mononuclear cells has been shown to be challenged with some probiotics (Wanget al., 2009). Similarly, with probiotic product consumption innate immunity is enhanced by increasing production of natural killer cell, hence immunomodulation (Martínez-Cañavate et al., 2009).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Introduction

This chapter discusses the study area, sampling and experimental design, and methods for isolation, identification and determination of the probiotic potential of Lactic acid bacteria from *mursik* and data analysis of the obtained data.

3.2 Study Area

Bomet County was purposively selected since it comprises of high production and consumption of fermented and non-fermented cow milk. The County has a prominent peri-urban cow population and thriving cow milk business (Muinde, 2011).

Bomet County has four Sub-Counties (Bomet, Chepalungu, Konoin and Buret). The study areas in the county were Bomet and Chepalungu Sub-Counties. The County covers approximately 1,592.4 Km^2 and it borders Kericho to the North and North East, Narok to the South East, South, and South West, and Nyamira to the North West. The County lies between latitudes -0.78333 (Latitude in decimal degrees) South of the equator and between the longitudes 35.35 (Longitude in decimal degrees) East of Greenwich Meridian. Bomet town is the major economic hub and is about 229 km from the capital city of Kenya, Nairobi.

Dairy farming is carried out in the county on both small scale and large scale that supply milk and milk products to Bomet town and other towns in the county. This provides residents with a source of income to supplement other sources such as horticulture and tea farming. According to the Kenya National Bureau of Statistics (KNBS) (2009), Bomet County has an approximate population density of 367 people per Km squared with an annual growth rate of 2.7 percent.

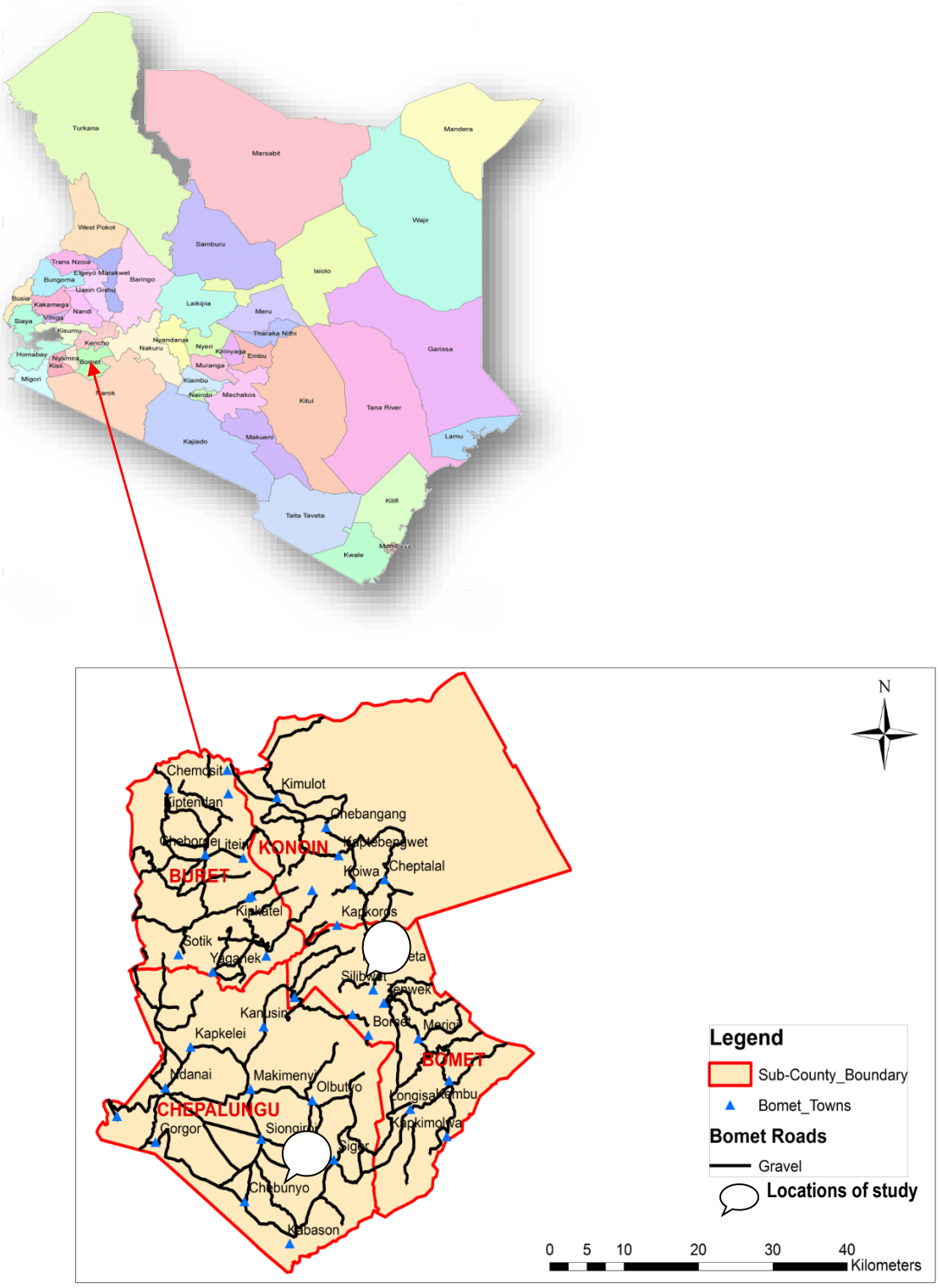

Figure 3.1 Map of Kenya and map of Bomet County (Source: GoK, 2012).

3.1.2 Sampling and Experimental design

The two sub counties (Bomet and Chepalungu) were purposively selected for the study since they comprise of high production of cow's milk. From the two sub counties two locations were further chosen for sample collection. Snowballing technique was used to identify thirteen (13) women groups and thirty one (31) individuals all for the study. Of the thirteen (13) women groups three (3) women groups eligible dropped out of the study due to lack of consent and ten (10) were left in the study.

Two categories of sampling units were used namely; individual *mursik* samples and group *mursik* samples. A representative composite *mursik* sample was taken as one sample. Bulked/group *mursik* was collected at women groups. The *mursik* in the containers was mixed by shaking the container and a cup (normally used to dispense *mursik*) was used to take the sample. *Mursik* was collected from various containers, traditional gourds (*sotets*), metal and plastic jericans in the study area. The sample was then poured gently into a sterile screw-cap glass bottles.

The experimental design was the completely randomized design (CRD). The measured value of a parameter was the mean of TVC, CC and LABs counts of *mursik* samples.

Model: $y_i = \mu + \tau_i + \varepsilon_{ij}$

y_i = individual observation of the i^{th} treatment and the j^{th} replication

μ = is the overall mean.

τ_i = the i^{th} treatment effect

ε_{ij} = the random error component

250ml was collected from 10 groups and 31 individuals each making a total of 41 samples. All the samples in each category were collected aseptically and kept below 10 ^{0}c in a cool box that had ice cubes. The samples were transported to Food Microbiology Laboratory, Egerton University within eight (8) hours and analytical work started immediately.

3.3 Sample Analysis

Total viable counts and coliform counts were used to determine the microbial load of *mursik*, while biochemical methods were used to isolate the lactic acid bacteria (LABs) which were preserved for testing.

3.3.1 Total viable counts (TVC) for microbial load determination

The TVC was to determine the initial microbial load in *mursik* for food safety. TVC was done using pour plate technique. Plate count agar (Fluka) was used. This test was carried out to determine the content of microbial load of *mursik*. One milliliter (ml) of the sample was serial diluted seven-fold using buffered peptone water (CDH).Then 1 ml of the sample *mursik* was diluted in 9 ml of peptone water (ratio of 1:10) up to seven dilutions. Sterile duplicate glass petri dishes were labeled according to the dilution index and pour plated using PCA. One ml of the dilutions was aseptically withdrawn using a sterile 1ml pipette and delivered into an opened and sterile petri dish and closed. The same was done for a duplicate petri dish. This was repeated till all the dilutions were pipetted into their corresponding plates up to 10^{-7}. This was followed by pouring about 15 ml of plate count agar (PCA), which had been autoclaved at 121 ^{0}C for 15 min, cooled and tempered in a water bath at 45 ^{0}C. The sample and the agar were gently mixed by alternate clock and anti-clockwise rotations for about 3 min. and left to solidify on the bench for 30 min. The plates were inverted and incubated at 37 ^{0}C for 48 hours. Because the first dilutions are expected to have heavier growth, they were not used; instead the last three dilutions (10^{-5}, 10^{-6}, and 10^{-7}) were used for total viable counts.

3.3.2 The coliform count (CC)

Coliform counts are used to determine the hygienic conditions under which the product is produced and handled. *E. coli* is the main indicator as it's an enteric organism. MacConkey agar (HIMEDIA) and Violet red bile agar (VRBA) (HIMEDIA) were used to select for lactose fermenters One milliliter (1ml) of *mursik* sample was serial diluted seven-fold using buffered peptone water (CDH) then one ml of the sample *mursik* was diluted in 9 ml of peptone water (ratio of 1:10) up to six dilutions. Sterile duplicate glass petri dishes were labeled according to the dilution index. One ml of the dilutions was aseptically withdrawn using a sterile 1ml pipette and delivered into an opened and sterile petri dish and closed. The same was done for a duplicate petri dish. This was repeated till all the dilutions were pipetted into their corresponding plates up to 10^{-7}. This was followed by pouring about 15 ml of VRBA (HIMEDIA) or MacConkey agar (HIMEDIA), which had been autoclaved at 121 ^{0} C for 15 min, cooled and tempered in a water bath at 45 ^{0} C. The sample and the agar were gently mixed by alternate clock- and anticlockwise rotations for about 3 min. and left to solidify on the bench for 30 min. The plates were inverted and incubated at 37 ^{0}C for 48 hours. The first dilutions were expected to have heavier growth, hence were not used for counting. Instead the last three dilutions (10^{-5}, 10^{-6}, and 10^{-7}) were used for coliform counts.

3.3.3 Isolation of probiotics from *Mursik*

The test was used to determine the quantity of probiotics present in *mursik*. Pour plate technique using specific media was also used to isolate the organisms. Samples were serially diluted, 1 ml of *mursik* sample was transferred aseptically into 9 ml sterile peptone water solution and mixed thoroughly. Serial dilutions of up to10^{-6} – 10^{-7} were subsequently made using sterile peptone water. One ml aliquot of the samples and dilutions were plated into MRS (de-Mann, Rogosa and Sharpe) agar (HIMEDIA) (pH 6.2 and pH 5.5). The plates were incubated at 37 ^{0}C for 2 days under anaerobic conditions (in anaerobe jar using Oxoid anaerogen compact). The use of these medium aimed for isolation and enumeration of lactobacilli. After incubation, individual colonies were selected and transferred into sterile broth mediums and incubated at 37 ^{0}C for 48 hours as described in the International Dairy Federation reference method (IDF 100B: 1991). Thereafter using streak plate technique colonies purification was done. The isolates were examined according to their colony morphology, catalase reaction and gram reaction. Pure LABs colonies were harvested using a sterile cotton swab and suspended in sterile 0.25 molar sucrose solutions in plastic vials with screw caps and stored at – 23 ^{0}C in a deep freezer for further analysis.

3.3.4 Cultural and Morphological Characterization

3.3.4.1 Gram Staining

To differentiate the morphological characteristics of the isolates gram staining was done. The gram reaction of the isolates was determined by light microscopy after gram staining. LABS are known to be gram positive. It means that they give blue-purple colour by gram staining. Cultures were grown in appropriate mediums at 37 ^{0}C for 24 hours under anaerobic conditions. Cells from fresh cultures were used for gram staining. After incubation cultures were transferred aseptically into 1.5 ml eppendorf tubes and centrifuged for 5 minutes at 6000 revolutions per minute (rpm). Then, supernatant was removed and cells were resuspended in sterile water. Gram staining procedure was applied as per Hoque et al. (2010), a bacterial suspension was first heat-fixed on a glass slide. The smear was then flooded with crystal violet for 1 minute before washing off the excess stain. Following this, the smear was covered with Gram's iodine for about 60 seconds and washed. Decolourization was then carried out by adding five drops of 95 % ethyl alcohol for five seconds and the action of alcohol was stopped by rinsing the slide with water. Lastly, the smear was counterstained with safranin for 30 seconds, washed and air dried then observed under a light microscope (Gerhardt et al., 1981).

3.4 Biochemical Analysis

3.4.1 Catalase test

Catalase is an enzyme produced by many microorganisms that breaks down the hydrogen peroxide into water and oxygen and causes gas bubbles. The formation of gas bubbles indicates the presence of catalase enzyme to differentiate the isolates.

$$2\ H_2O_{2\ (l)} \rightarrow 2\ H_2O_{\ (l)} + O_{2\ (g)}$$

Catalase test was performed to isolates in order to see their catalase reactions. For this purpose, two methods were applied. Overnight cultures of isolates were grown on MRS agar at suitable conditions. After 24 hours 3 percent hydrogen peroxide solution were dropped onto randomly chosen colony. Also fresh liquid cultures were used for catalase test by dropping 3 % hydrogen peroxide solution onto 1 ml of overnight cultures. The isolates, which did not give gas bubbles, were chosen because LABs have been reported as catalase negative (Chelikani et al., 2004; Holt et al., 1994).

3.4.2 Gas Production from Glucose

In order to determine the homofermentative and heterofermentative characterization of isolates, CO_2 production from glucose test was applied. Citrate lacking MRS broths and inverted Durham tubes were prepared and inoculated with 1µl of overnight fresh cultures. Then the test tubes were incubated at 35 ^{0}C for 5 days. Gas occurrence in Durham tubes was observed during 5 days which is the evidence for CO_2 production from glucose (Forouhandeh et al., 2010).

3.4.3 Growth at Different Temperatures

LABs are thermophiles and growth at different temperatures was used to confirm thermophiles against mesophilic and psychophilic bacteria. Temperature test media, MRS containing bromocresol purple indicator, was prepared and transferred into tubes as 5 ml. Then fifty µl of overnight cultures inoculated to tubes and incubated for 7 days at 10, 15 and 45 degrees Celsius. During these incubation time cells growth at any temperatures was observed by the change of the cultures, from purple to yellow (Bulut, 2003).

3.4.4 Growth at Different NaCl Concentrations

Isolates were tested for their tolerance against different NaCl concentrations. In the study 4 percent, 6.5 percent, 8 percent and 10 percent NaCl concentrations were selected. Test mediums containing

bromocresol purple indicator were prepared according to the appropriate concentrations and transferred into tubes in 5 millilitres. These tubes were inoculated with 1percent overnight cultures and then incubated at 37 ^{0}C for 7 days. The change of the colour from purple to yellow was proved the cell growth (Bulut, 2003).

3.4.5 Arginine Hydrolysis Test

Arginine MRS medium and Nessler's reagent were used in order to see ammonia production from Arginine. MRS containing 0.3% L-Arginine hydrochloride was transferred into tubes as 5 millilitres and inoculated with 1% overnight cultures. Tubes were incubated at 37 degree Celsius for 24 hours. After incubation, 100 microliter (µl) of cultures transferred onto a white background. The same amount of Nessler's reagent was pipetted on the cultures. The change in the colour was observed. Bright orange colour indicated a positive reaction and was taken as the indication for ammonia production, while yellow indicated the negative reaction implying the strains could not hydrolyze Arginine. The test was used to differentiate heterofermentative *Lactobacillus* from *Leuconostoc*. A negative control, which did not contain Arginine, was also used as negative control (Nikita & Hemangi, 2012).

3.4.6 Carbohydrate Fermentations

Lactic acid bacteria produce acidic products when they ferment certain carbohydrates. The carbohydrate utilization tests are designed to detect the change in pH which would occur if fermentation of the given carbohydrate occurred. Acids lower the pH of the medium which will cause the pH indicator (bromocresol purple) to turn yellow. If the bacteria do not ferment the carbohydrate then the media remains purple.

Isolates were characterized according to their fermentation profiles of ability to ferment 17 different carbohydrates (D (+) Xylose, D (-) Ribose, L (+) Arabinose, Mannitol, D (+) Trehalose, Raffinose, D (+) Galactose, Maltose, Sucrose, D (+) Mannose, Fructose, Lactose, Sorbitol, Glucose, Esculin, Rhamnose and Salicin). Active cells and sugar solutions were prepared separately. For preparation of active cells; isolates were activated in 10 millilitre MRS medium and incubated at 37 degrees Celsius for 24 hours. Then, they were centrifuged 10 minutes at 10000 rpm. Pellets were washed twice and resuspended in MRS without glucose and containing pH indicator bromocresol purple. Each sugar solutions were prepared at a final concentration of 10 percent (w/v), only Salicin was

prepared at concentration of 5 percent. Then the solutions were filter sterilized with filters (0.22 μm pore diameter) (Nikita & Hemangi, 2012).

After preparation steps the procedure was applied. 2 millilitre of sugar solutions were pipetted into each sterile test tube and 8 millilitres of suspended cells were added onto the sugar solutions. Thus, 2 percent final sugar concentration was obtained. All the reactions were performed twice. Also positive and negative controls were used to indicate any contamination. 8 millilitre of suspended cells and 2 millilitre of glucose solution were used as positive control while 10 millilitres of suspended cells was used as negative one. After overnight incubation at 37 degrees Celsius, the turbidity and the colour change from purple to yellow was recorded as positive fermentation results compared with the positive and negative controls. The most useful test for the determination of strain differences is carbohydrate fermentation. Seventeen (with glucose) different carbohydrates were used for identification. They give different fermentation patterns when they are compared.

3.5 Probiotic potential of probiotics Isolated

For the determination of probiotic properties of isolates this major selection criteria included, resistance to low pH, tolerance against bile salt and the antimicrobial activity.

3.5.1 Low pH resistance and bile salt tolerance

One of the most important criteria for selection of probiotic organisms is their ability to survive in the acidic environment of the product and in the stomach, where the pH can reach as low as 1.5. Similarly, the organisms must be able to survive in the bile concentrations encountered in the intestines (Ashton, 2013). Acid and bile tolerance of the Isolated *Lactobacillus* strains was determined according to Tambekar & Bhutada (2010a) method. LAB isolates were inoculated into MRS medium of varying pH, i.e. pH 1.5, 2.0, 3.0 and 3.5; as well as broth with varying concentrations of bile salt (oxgall) 0.5 percent, 1.0 percent, 1.5 percent and 2.0 percent, and incubated at 37 degrees Celsius for 48hours. Then 0.1millilitre inoculums was transferred to MRS agar by pour plate method and incubated at 37 degrees Celsius for 48hours. The growth of LAB on MRS agar plate was used to designate isolates as acid and bile salt tolerant. The growth of LAB isolates in MRS broth containing different concentration of oxgall was measured by a spectrophotometer at 620nm (Srinu et al., 2013).

3.5.2 Antimicrobial activity

The gastrointestinal microflora is a complex ecosystem. Introducing new organisms into this highly competitive environment is difficult. Thus organisms that can produce a product or products that will inhibit the growth or destroy existing organisms in the intestinal milieu have a distinct advantage. The ability of probiotics to establish in the gastrointestinal tract is enhanced by their ability to eliminate competitors. The antagonistic properties of isolated LAB species were determined by modifying the disc diffusion method. Sterile blotting paper discs (6 mm) were dipped into 48 hours incubated *Lactobacillus* sp. culture broth and then placed on solidified Nutrient Agar seeded with 3 hours old culture of test pathogens, which will include *Salmonella enterica* (ATCC 13076) and *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (Isolate). The plates were be kept at 4 degrees Celsius for 1 hour diffusion and then incubated at 37 degrees Celsius for 24hours. Zones of inhibition were measured according to conventional method no NCCLS M2-A7 (Sandhya & Biradar, 2014; Kirby-Baur, 1966).

3.6 Data analysis.

The microbiological counts data obtained were logarithmically transformed to base-10 logarithm of CFUs per millilitre (ml) of the *mursik* samples ($\log_{10}$ cfu/ml) for statistical analysis. The means of CFUs per millilitre and standard deviations were also calculated. Mean comparison was determined using the least significant difference (LSD) procedure.The statistical analysis was done using analysis of variance (ANOVA) and chi-square. The model used for ANOVA was general linear model (GLM) of Statistical product for service and solutions (SPSS) version 20. Significance of the measurements were determined at $\alpha = 0.05$.

CHAPTER FOUR

RESULTS

4.1 Introduction

This chapter presents the results of the study. The report takes the order of initial microbial load results, isolation of lactic acid bacteria, physiological and biochemical characterization, probiotic properties (resistance to low pH, tolerance against bile and antimicrobial activity).

First Objective

4.2 Initial microbiological load

A total of 41 samples were collected. Of 41 sample > 75 % (32 samples) had microbial load $\geq 10^7$ cfu/ml while the rest of the samples had lower than $\geq 10^7$ cfu/ml. Bulked *mursik* from the women groups had 80 % total viable count $\geq 10^7$ cfu/ml. *Mursik* from individual women had 77.4 % TVC $\geq 10^7$ cfu/ml (Table 4.1). The coliforms counts in the samples collected was ≤ 30 cfu/ml. Bulked *mursik* had a higher coliform count ≤ 30 cfu/ml (30 %) than samples from individual women at 26 %. Table 4.1 shows that most of the microorganisms making the TVC in *mursik* are Lactic acid bacteria ($\geq 10^7$cfu/ml). The microorganisms at each level are shown in brackets against the number of isolates. The yeasts and moulds were the least in *mursik*.

The yeast and moulds count were low in all the bulked and individual samples tested with 10 % and 3.2 % respectively (Table 4.1). When the *mursik* samples were analysed for the Lactic acid bacteria count, > 80 % of the samples had lactic acid bacteria count of $\geq 10^7$cfu/ml. 80 % of bulked *mursik* had a LABs count of $\geq 10^7$cfu/ml, while 90.3 % in individual women samples had a LABs count of $\geq 10^7$cfu/ml. Therefore, the average concentration of LABs in *mursik* is 85.2 %.

Table 4.1 Initial microbial load in *mursik* samples (n= 41)

	Samples from individuals (n = 31)			Samples from groups (n = 10)		
Ranges (cfu/ml)	≤ 30	$\leq 10^6$	$\geq 10^7$	≤ 30	$\leq 10^6$	$\geq 10^7$
Tests						
Total Viable Count	**1**	**6**	**24 (77.4%)**	**0**	**2**	**8 (80%)**
Smoked *mursik*	1	4	15	0	2	4
Non smoked *mursik*	0	2	9	0	0	4
Coliform Counts	**8 (25.8%)**	**0**	**0**	**3 (30%)**	**0**	**0**
Smoked *mursik*	1	0	0	1	0	0
Non smoked *mursik*	7	0	0	2	0	0
Yeasts and Molds	**1 (3.2%)**	**2**	**0**	**1 (10%)**	**1**	**0**
Smoked *mursik*	1	0	0	0	0	0
Non Smoked *mursik*	0	2	0	1	1	0
LABs Count	**0**	**3**	**28 (90.3%)**	**0**	**2**	**8 (80%)**
Smoked *mursik*	0	2	18	0	1	5
Non smoked *mursik*	0	2	9	0	1	3

TVC- total viable count, CC-coliform count and LABs count-Lactic acid bacteria count. > 75 % TVC and LABs count had ≥ 10^7 cfu/ml. The microorganisms at each level are shown in brackets against the number of isolates. The yeasts and moulds were the least in mursik.

4.3 Comparisons of the initial load of bacteria in mursik samples

The mean values with their standard deviation (SD) of total viable counts (TVC) of individuals and group (bulked) *mursik* samples either smoke treated or non-smoke treated were compared. The means of the smoke treated mursik samples among the individuals was between 8.72 and 8.81 with an average of 8.75 ± 0.058 $\log_{10}$TVC (cfu/ml) with a coefficient variation of 0.663 percent, while the mean of the smoke treated *mursik* samples among the bulked samples from groups was observed to be between 8.67 and 8.80 with a mean of 8.74 ± 0.053 $\log_{10}$TVC (cfu/ml) with a coefficient variation of 0.606 percent.

When the non-smoke treated *mursik* among individuals and among groups (bulked) samples from was tested, samples from individuals had $\log_{10}$TVC (cfu/ml) ranging from 8.81 to 8.85 with a mean and standard deviation of 8.83 ± 0.017 (coefficient variation = 0.193%), while samples from bulked group *mursik* had $\log_{10}$TVC (cfu/ml) ranging from 8.77 to 8.88 with a mean and standard deviation of 8.83 ± 0.017 (coefficient variation = 0.556%) (Table 4.2).

Table 4.2 further shows that $\log_{10}$ of the total viable counts (TVC) among the smoke treated *mursik* from individuals and groups were significantly ($P< 0.05$) different. At the individual (production) level, the initial load of TVC of smoked *mursik* were different with the initial load of the groups (bulked). When $\log_{10}$ TVC was compared among the non-smoke treated *mursik* from individuals and groups they were insignificantly ($P > 0.05$) different. Table 4.2 shows comparison on total viable counts of *mursik*, non-smoke treated *mursik* had high TVC than smoke treated *mursik*.

Smoke treated *mursik* had low microbial load (TVC and CC) than the non-smoke treated *mursik* (Table 4.3). The $\log_{10}$TVC and log10 of coliform counts between smoke treated and the non-smoke treated *mursik* were significantly ($P = 0.0001$) different. The initial load of both TVC and Coliforms from of *mursik* were different. When the $\log_{10}$ lactic acid bacteria count was determined, it was found that smoke treated *mursik* had a mean of 8.50 ± 0.05 cfu/ml implying that the LABs were 56.09 percent of their TVC and non-smoke treated had a mean of 8.52 ± 0.03 cfu/ml, hence a 45.45 percent of the microbial load from the non-smoke treated mursik was LABs. The coliforms count in non-smoke treated *mursik* had a mean of 7.55 ± 0.19 cfu/ml (5.55 percent) as compared to smoke treated *mursik* which had a lower mean of 4.77 ± 3.69 cfu/ml (1.72 percent). The mean coliforms counts were lower than LABs counts in *mursik*. But from non-smoke treated *mursik* coliforms were high than from smoke treated *mursik* as shown by the difference in the mean values (Table 4.3).

Table 4.2 Total viable counts of individuals and group *mursik* samples (Both smoke treated and non-smoke treated).

	Samples from individuals log_{10}TVC (cfu/ml)	Samples from groups log_{10}TVC (cfu/ml)
Smoke treated *mursik*		
A	8.78 ± 0.02^{abc}	8.80 ± 0.01^{a}
B	8.81 ± 0.04^{abc}	8.79 ± 0.02^{ab}
C	8.72 ± 0.17^{cd}	8.70 ± 0.03^{cd}
D	8.74 ± 0.05^{bc}	8.71 ± 0.02^{c}
E	8.65 ± 0.04^{d}	8.67 ± 0.01^{d}
F	8.79 ± 0.03^{abc}	8.76 ± 0.02^{b}
Mean ± SD of smoke treated *mursik*	**8.75 ± 0.06**	**8.74± 0.05**
% CV	0.663	0.606
	P < 0.05	
Non-smoke treated *mursik*		
G	8.85 ± 0.01^{a}	8.84 ± 0.03^{e}
H	8.81 ± 0.02^{abc}	8.79 ± 0.02^{ab}
I	8.82 ± 0.04^{abc}	8.88 ± 0.03^{f}
J	8.83 ± 0.04^{ab}	8.77 ± 0.02^{ab}
Mean ± SD of non-smoked treated *mursik*	**8.83 ± 0.02**	**8.82 ± 0.05**
% CV	0.193	0.556
	P > 0.05	

Mean values in the same column with the same superscript are not significantly different (P > 0.05). Letters A, B, C, D, E, F, G, H, I and J represents the samples. Smoke treated mursik had high % coefficient variation (CV) of TVC than non-smoke treated mursik.

Table 4.3 Total viable counts (TVC), Coliforms (CC) and lactic acid bacteria counts (LABs) from *mursik*.

	log_{10} TVC (cfu/ml)	log_{10} CC (cfu/ml)	log_{10} LABs Count cfu/ml)
Smoke treated *mursik*			
A	8.80 ± 0.02^{a}	7.30 ± 0.01^{a} (3.55)	8.57 ± 0.26^{a} (65.68)
B	8.79 ± 0.02^{ab}	7.00 ± 0.03^{b} (1.49)	8.54 ± 0.41^{a} (52.23)
C	8.70 ± 0.03^{cd}	7.00 ± 0.03^{b} (1.78)	8.46 ± 0.53^{a} (50.90)
D	8.71 ± 0.02^{c}	0.003 ± 0.01^{c} (0)	8.50 ± 0.42^{a} (58.65)
E	8.67 ± 0.01^{d}	0.01 ± 0.01^{c} (0)	8.45 ± 0.19^{a} (58.21)
F	8.76 ± 0.02^{b}	7.30 ± 0.02^{a} (3.51)	8.46 ± 0.25^{a} (50.88)
Mean ± SD	**8.74 ± 0.05**	4.77 ± 3.69 **(1.72)**	**8.50 ± 0.05 (56.09)**
Non-smoke treated *mursik*			
G	8.84 ± 0.03^{e}	7.70 ± 0.01^{d} (6.88)	8.54 ± 0.31^{a} (48.16)
H	8.79 ± 0.02^{ab}	7.48 ± 0.01^{e} (4.27)	8.53 ± 0.24^{a} (48.34)
I	8.88 ± 0.03^{f}	7.70 ± 0.02^{d} (7.58)	8.48 ± 0.22^{a} (45.45)
J	8.77 ± 0.02^{ab}	7.30 ± 0.01^{a} (3.05)	8.53 ± 0.14^{a} (51.77)
Mean ± SD	**8.82 ± 0.05**	7.55 ± 0.19 **(5.55)**	**8.52 ± 0.03 (48.43)**
	F = 16.51, P < 0.05	**F = 0.05, P > 0.05**	

Mean values in the same column with the same superscript are not significantly different ($\alpha = 0.05$). Mean LABs count of smoke treated mursik were high than non-smoke treated mursik. The % LABs and CC at each level are shown in brackets against the cfu/ml.

Second Objective

4.4 Physiological and Biochemical Characterization

4.4.1 Physiological Characterization

A total of 64 microorganisms were isolated from 41 *mursik* sample. Based on biochemical tests and comparative morphology characterization (Figure 4.1), they were classified into four groups as follows, Lactobacillus (56.1 %), Other LABs (Lactococcus, Enterococcus and Leuconostoc) (25.9 %), coliforms (7.0 %) and, yeast and molds (11.0 %). These results show that *mursik* in this region has a variety of microorganisms, but the predominant species is LABs (82%).

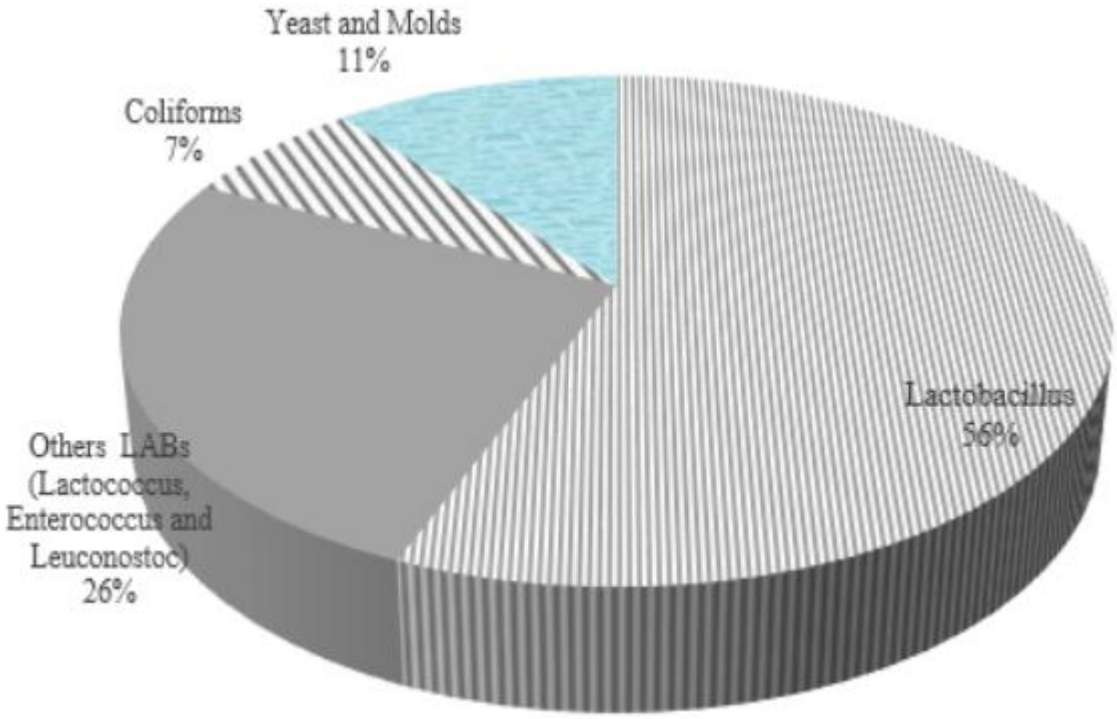

Figure 4.1 Genus Distribution of Microorganisms Isolated from Mursik.

The gram negative and catalase positive strains were regarded as non-LABs. Of 64 isolates, 31 gram positive and rod isolates obtained were selected for further isolation and purification as potential probiotics. A total of 16 isolates remained after purification that were of bacilli morphology with long and rounded ends (Figure 4.2). The isolates were then subjected to the test for catalase reactions (figure 4.3). The isolates, which were deficient of gas bubbles, were chosen since LABs have been reported as catalase negative. Catalase negative isolates were further studied for biochemical reactions. Colony characteristics of the 16 gram positive (figure 4.3), catalase negative and rod shaped isolates revealed a few common characters (Table 4.4), as well as diversity among themselves.

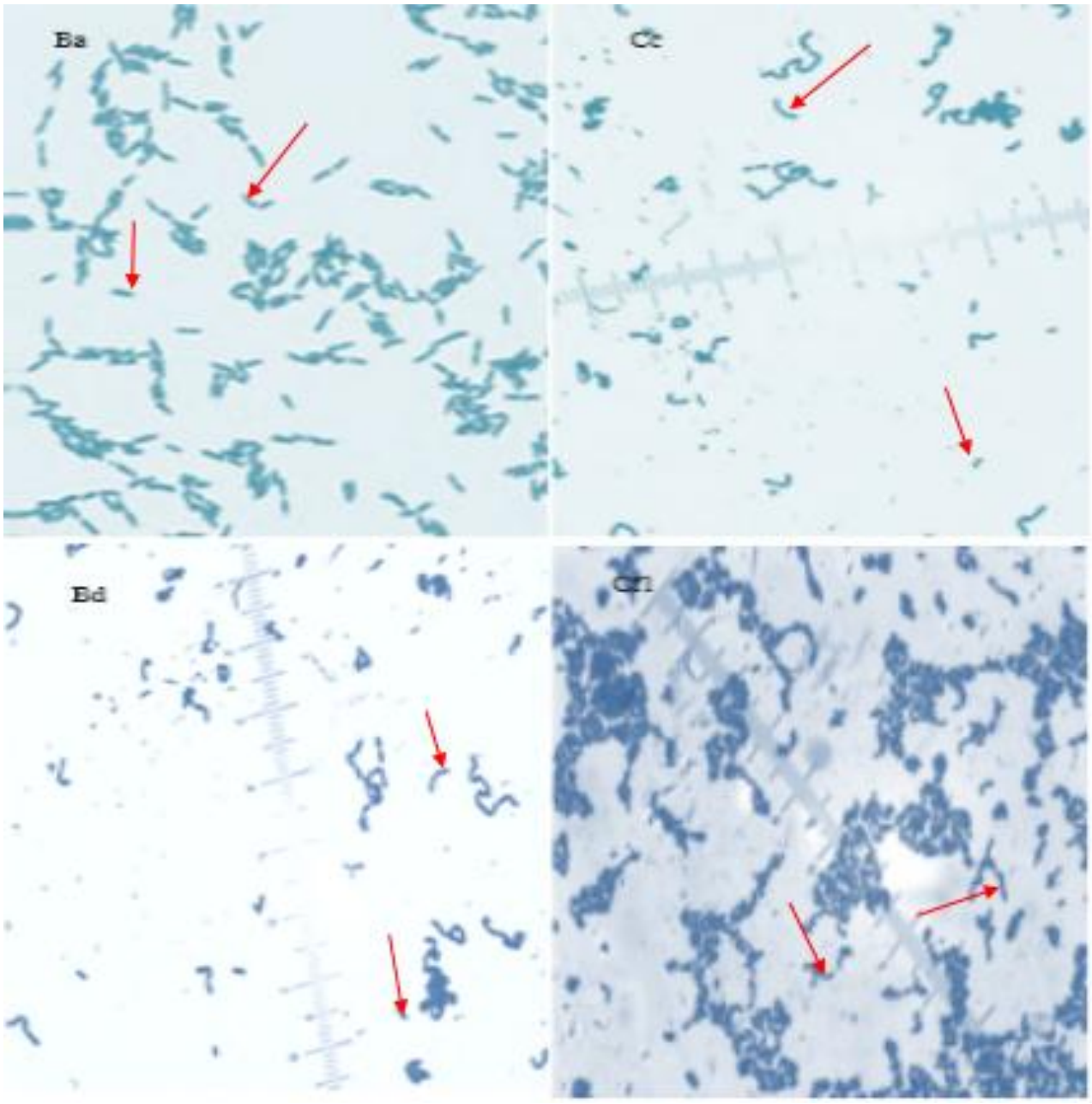

Figure 4.2 Light Microscopic images of gram staining of isolates Ba, Cc, Bd and Cf1. (Arrows indicate the Lactobacillus rods)

Table 4.4 Colony Characterization of isolates from *mursik* samples

***Lactobacillus* Isolates**	**Colony Characterization**					
	Size	**Shape**	**Margin**	**Texture**	**Elevation**	**Opacity**
***Lactobacillus plantarum* (Ba)**	Small	Circular	Regular	Smooth	Raised	Opaque
***Lactobacillus plantarum* (Ba1)**	Small	Circular	Regular	Mucoid	Raised	Opaque
***Lactobacillus fermentum* (Bb)**	Small	Circular	Regular	Mucoid	Raised	Opaque
***Lactobacillus fermentum* (Bb1)**	Medium	Circular	Regular	Mucoid	Raised	Opaque
***Lactobacillus brevis* (Bc)**	Small	Circular	Entire	Smooth	Raised	Opaque
***Lactobacillus brevis* (Bc1)**	Small	Circular	Regular	Smooth	Raised	Opaque
***Lactobacillus casei* (Bd)**	Small	Circular	Regular	Glistering	Raised	Opaque
***Lactobacillus plantarum* (Bd1)**	Small	Circular	Regular	Smooth	Raised	Opaque
***Lactobacillus fermentum* (Be)**	Medium	Circular	Regular	Smooth	Raised	Opaque
***Lactobacillus fermentum* (Be1)**	Medium	Circular	Regular	Smooth	Raised	Opaque
***Lactobacillus casei* (Ca)**	Medium	Lenticular	Undulate	Glistering	Raised	Opaque
***Lactobacillus casei* (Cb)**	Medium	Circular	Entire	Smooth	Raised	Opaque
***Lactobacillus fermentum* (Cc)**	Large	Circular	Entire	Smooth	Raised	Opaque
***Lactobacillus plantarum* (Cd)**	Small	Lenticular	Regular	Mucoid	Raised	Opaque
***Lactobacillus fermentum* (Cf)**	Large	Circular	Regular	Smooth	Raised	Opaque
***Lactobacillus casei* (Cf1)**	Small	Circular	Regular	Mucoid	Raised	Opaque

Letters used in the table refers to isolates from laboratory samples obtained after morphological characterization.

When the LABs were also tested for catalase activity, they were observed as catalase negative.

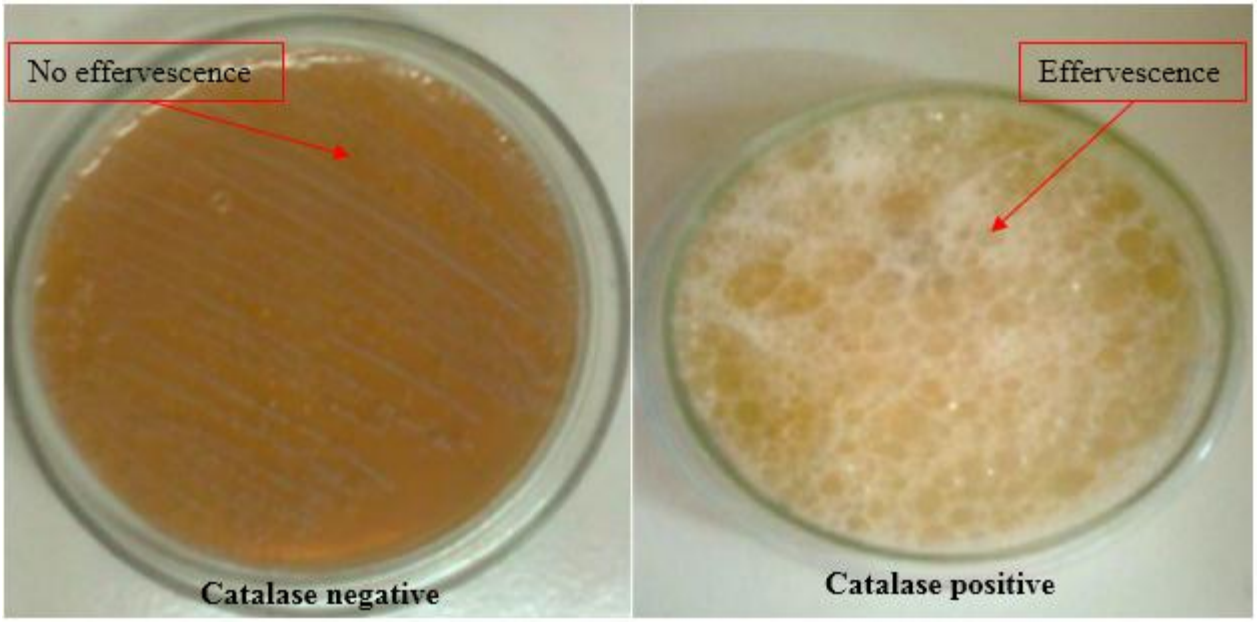

Figure 4.3 Catalase activity (negative and positive) of isolated microorganism in *mursik*

4.4.2 Biochemical Identification of LABs

Glucose fermentation under non-limiting concentrations of glucose and growth factors, amino acids, vitamins and nucleic acid precursors and limited oxygen availability are an important characteristic of Lactic acid bacteria differentiation. Under these conditions, lactic acid bacteria can be divided into two groups: homofermentative, which convert glucose almost quantitatively to lactic acid, and heterofermentative, which ferment glucose to lactic acid, ethanol/acetic acid, and CO_2 (Sharpe, 1979; Forouhandeh, Vahed, Hejazi, & Akbari, 2010).

To test the gas production from citrate lacking MRS broths inoculated with 1µl of overnight fresh cultures and inverted Durham tubes in test tube were observed for 5 days. Gas occurrence in Durham tubes was observed during 5 days which is the evidence for CO_2 production from glucose. LAB Isolates *Lactobacillus plantarum* (Ba), *Lactobacillus plantarum* (Ba1), *Lactobacillus casei* (Bd), *Lactobacillus plantarum* (Bd1), *Lactobacillus casei* (Ca), *Lactobacillus casei* (Cb), *Lactobacillus plantarum* (Cd) and *Lactobacillus casei* (Cf1) showed no gas production while gas production was observed from isolates *Lactobacillus fermentum* (Bb), *Lactobacillus fermentum* (Bb1), *Lactobacillus brevis* (Bc), *Lactobacillus brevis* (Bc1), *Lactobacillus fermentum* (Be),

Lactobacillus fermentum (Be1), *Lactobacillus fermentum* (Cc), and *Lactobacillus fermentum* (Cf) (Table 4.5). This implies that, gas producing isolates were heterofermentative cultures whereas non-gas producing isolates were homofermentative as per gas production on LABs.

Additional measure for the proof of identity of the isolates was the ability to grow at different temperatures. From the results (Table 4.5) of 7 days observation, 9 isolates were observed to grow at temperatures 10°C and 15^0C. *Lactobacillus brevis* (Bc) was unable to grow at 10 °C but exhibited growth at 15 °C. *Lactobacillus casei* (Bd), *Lactobacillus casei* (Cb), and *Lactobacillus casei* (Cf1) isolates were able to grow in the three temperatures (10°C, 15^0C and 45^0C) they were subjected to. Growth at different NaCl concentrations was also observed. All of the isolates have the ability to grow at 4%, 6.5% and 8% NaCl concentration however at 10% NaCl concentration none of the isolates showed the ability to grow.

Arginine hydrolysis test was one more step to follow the identification technique. The purpose was to see if the isolates obtained could use the amino acid Arginine as a source of carbon and energy for growth. The isolates which gave the bright orange were accepted that they can produce ammonia from Arginine. The yellow color indicated negative Arginine hydrolysis. Isolates Bb, Bb1, Bc, Bc1, Be, Be1, Cc and Cf which produced gas were all subjected to Arginine hydrolysis tests. According to this test all these isolates were able to hydrolyze Arginine hence can produce ammonia from Arginine implying that the strains obtained in the study were heterofermentative capable of producing lactic acid, ethanol/acetic acid, and CO_2 (Forouhandeh, Vahed, Hejazi, & Akbari, 2010).

Table 4.5 Growth characteristics of *Lactobacillus* isolates

Lactobacillus Isolates	Biochemical Tests							
	Gas production from glucose	Growth at 10°C	Growth at 15°C	Growth at 45°C	4% NaCl	6.5% NaCl	8% NaCl	10% NaCl
***Lactobacillus plantarum* (Ba)**	-	+	+	-	+	+	+	-
***Lactobacillus plantarum* (Ba1)**	-	+	+	-	+	+	+	-
***Lactobacillus fermentum* (Bb)**	+	-	-	+	+	+	+	-
***Lactobacillus fermentum* (Bb1)**	+	-	-	+	+	+	+	-
***Lactobacillus brevis* (Bc)**	+	-	+	-	+	+	+	-
***Lactobacillus brevis* (Bc1)**	+	+	+	-	+	+	+	-
***Lactobacillus casei* (Bd)**	-	+	+	+	+	+	+	-
***Lactobacillus plantarum* (Bd1)**	-	+	+	-	+	+	+	-
***Lactobacillus fermentum* (Be)**	+	-	-	+	+	+	+	-
***Lactobacillus fermentum* (Be1)**	+	-	-	+	+	+	+	-
***Lactobacillus casei* (Ca)**	-	+	+	-	+	+	+	-
***Lactobacillus casei* (Cb)**	-	+	+	+	+	+	+	-
***Lactobacillus fermentum* (Cc)**	+	-	-	+	+	+	+	-
***Lactobacillus plantarum* (Cd)**	-	+	+	-	+	+	+	-
***Lactobacillus fermentum* (Cf)**	+	-	-	+	+	+	+	-
***Lactobacillus casei* (Cf1)**	-	+	+	+	+	+	+	-

Symbols: + = positive, - =: negative. Letters used in the table refers to isolates obtained after morphological characterization. Trend: Most LABs grow best at salt concentration of 4 – 8 % NaCl.

4.4.2.1 Carbohydrate Fermentation

The most useful test for the determination of strain differences is carbohydrate fermentation. Ability to ferment different carbohydrates was determined on MRS-BCP broth medium, by using the different sugars as carbon source, which was added to the sterile basal medium to get final concentration 1 percent weight per volume. Carbohydrates utilization is evaluated by acidic

products produced when the Bacteria ferment certain carbohydrates. The carbohydrate utilization tests are designed to detect the change in pH which would occur if fermentation of the given carbohydrate occurred. Acids lower the pH of the medium which will cause the pH indicator (Bromocresol purple) to turn yellow. If the bacteria do not ferment the carbohydrate then the media remains purple (Kneifel et al., 2000). If gas is produced as a byproduct of fermentation, then the Durham tube will have a bubble in it. Fermentation of sugar provided to isolates studied which indicates, isolates can utilize provided sugars except rhamnose. According to Bergey's manual many probiotics cannot utilize rhamnose (Holt et al., 1994).In the seventeen (with glucose) different carbohydrates used for identification the *Lactobacillus* Isolates in the study showed fermentation on all the other sugars except on rhamnose hence was in agreement with Holt et al., (1994).

According to the carbohydrate fermentation test results isolates Ba, Ba1, Bd1 and Cd gave positive results with the carbohydrates, galactose, lactose, mannose, Raffinose, salicin, sorbitol, trehalose, Esculin, and gave negative results with xylose, ribose, arabinose, mannitol, raffinose, maltose, sucrose, fructose, lactose, glucose and rhamnose. Bb, Bb1, Be, Be1, Cc and Cf gave positive results with Ribose, Arabinose, Trehalose, Raffinose, Galactose, Maltose, Sucrose, Lactose, Glucose and fructose. Isolates Bc and Bc1 gave positive results with Xylose, Ribose, Arabinose, Mannitol, Galactose, Maltose, Sucrose, Mannose and Fructose. Lastly, isolates Bd, Ca, Cb and Cf1 gave positive results on Ribose, Arabinose, Trehalose, Galactose, Maltose, Mannose, Fructose, Lactose, Glucose, Esculin and Salicin. The isolates gave different fermentation patterns when they are compared. The patterns are showed in Table 4.6.

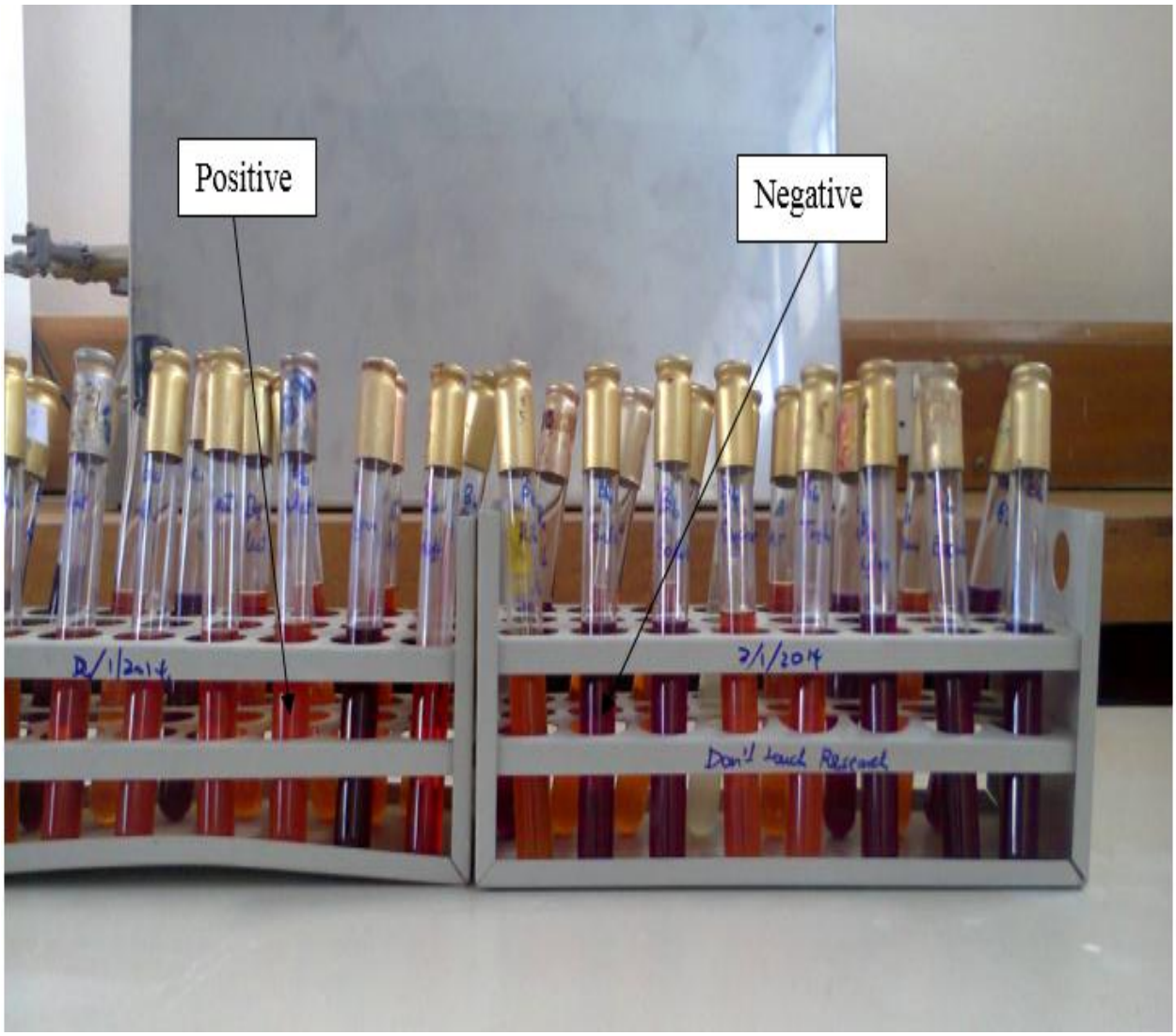

Figure 4.4 Carbohydrate utilization tests for different sugars by isolates, positive (yellow) and negative (purple) Ref. Table 4.6

Table 4.6 Carbohydrate Fermentation Test Results of *Lactobacillus spp.*

Lactobacillus spp.	**15 ^{0}C**	**45 ^{0}C**	**L (+) Arabinose**	**Catalase**	**Fructose**	**D (+) Galactose**	**Glucose**	**Lactose**	**Maltose**	**Mannitol**	**D (+) Mannose**	**Raffinose**	**D (-) Ribose**	**D(-) Salicin**	**Sorbitol**	**Sucrose**	**D (+) Trehalose**	**Rhamnose**	**D (+) Xylose**	**Esculin**	**Arginine**
***Lactobacillus plantarum* (Ba, Ba1, Bd1 and Cd)**	+	-	-	-	-	+	-	+	+	-	+	+	-	+	+	-	+	-	-	+	+
***Lactobacillus fermentum* (Bb, Bb1, Be, Cc and Cf)**	-	+	+	-	+	+	+	+	+	-	-	+	+	-	-	+	+	-	-	-	+
***Lactobacillus brevis* (Bc and Bc1)**	+	+	+	-	+	+	-	-	+	+	+	-	+	-	-	+	-	-	+	-	-
***Lactobacillus casei* (Bd, Ca, Cb and Cf1)**	+	-	+	-	+	+	+	+	+	-	+	-	+	+	-	+	+	-	-	+	-

*Symbols: **+;** resembles positive reaction (yellow), while –; negative reaction (purple). Letters used in the table refers to isolates obtained after morphological characterization.*

When the carbohydrate utilization test results (Table 4.6 above) were compared with the literature information (Table 4.7) isolates Ba, Ba1, Bd1 and Cd were similar as *Lactobacillus plantarum*; Bb, Bb1, Be, Be1, Cc and Cf, *Lactobacillus fermentum*; Bc and Bc1, *Lactobacillus brevis* and; Bd, Ca, Cb and Cf1 were *Lactobacillus casei* (Roos *et al*., 2005; Hammes & Vogel, 1995; Bergey *et al.*, 1989).

Table 4.7 Literature on Carbohydrate Utilization Test Results

Strains	15 °C	45 °C	Glucose (Gas)	Arabinose	Galactose	Lactose	Maltose	Mannose	Raffinose	Salicin	Sorbitol	Trehalose	Esculin	Fructose	Mannitol	Ribose	Sucrose	Xylose	Rhamnose	NO_3^-	Arginine
Lactobacillus plantarum	+	-	-	**D**	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Lactobacillus fermentum	-	+	+	**D**	+	+	+	-	+	-	-	-	-	+	-	+	+	**D**	-	-	+
Lactobacillus casei	+	**d**	-	-	+	**D**	+	+	-	+	+	+	+	+	+	+	+	+	-	-	-
Lactobacillus brevis	+	-	+	+	**w**	**D**	+	**d**	**d**	-	-	-	**D**	+	-	+	**d**	**ND**	-	-	+

Symbols: +: 90% or more strains are positive, -: 90% or more are negative, d: 11-89% of strains are positive, w: weak positive reaction, ND: no data available (Roos et al., 2005; Hammes & Vogel, 1995; Bergey et al., 1989).

Third Objective

4.6 Testing the potential for Probiotic Properties

4.6.1 Acid and Bile Salt Tolerance

When the isolates were screened whether they could survive at an acidic pH range of between 1.5 to 3.5, the observations made showed that *Lactobacillus plantarum, Lactobacillus fermentum* and *Lactobacillus casei* isolates were resistant to pH 1.5, 2.0, 3.0 and 3.5. However for *Lactobacillus brevis* was not resistant to pH 1.5 and 2.0 but was resistant to pH 3.0 and 3.5 (Table 4.8). Lack of resistance to pH 1.5 and 2.0 may be due to the fact that a significant decrease in the viability of strains is often observed at pH 2.0 and below.

Table 4.8: Survival of *Lactobacillus Spp.* at acidic pH range results

Tolerance at acidic pH range				
	pH Range			
***Lactobacillus* Isolate**	**1.5**	**2.0**	**3.0**	**3.5**
Lactobacillus plantarum	+ve	+ve	+ve	+ve
Lactobacillus fermentum	+ve	+ve	+ve	+ve
Lactobacillus brevis	-ve	-ve	+ve	+ve
Lactobacillus casei	+ve	+ve	+ve	+ve

Note: -ve means no growth, + ve means growth present after incubation at 37°C for 48 hours.

The growth of LAB in MRS broth containing different concentrations of bile salts (Oxgall) (0.3 % to 2.0%) was measured at 620 nm. All the LAB strains tested showed survivability at different bile salt concentrations, i.e. from 0.3 % to 2.0 % (Figure4.5). Absorbance patterns are shown in the figure 4.5. All the LAB strains survived and tolerated bile salts (0.3 % -2%). But a marginal decrease in the viability of all the strains was found as bile salt concentrations was increased from 0.3 % to 2.0 % (Figure 4.5).

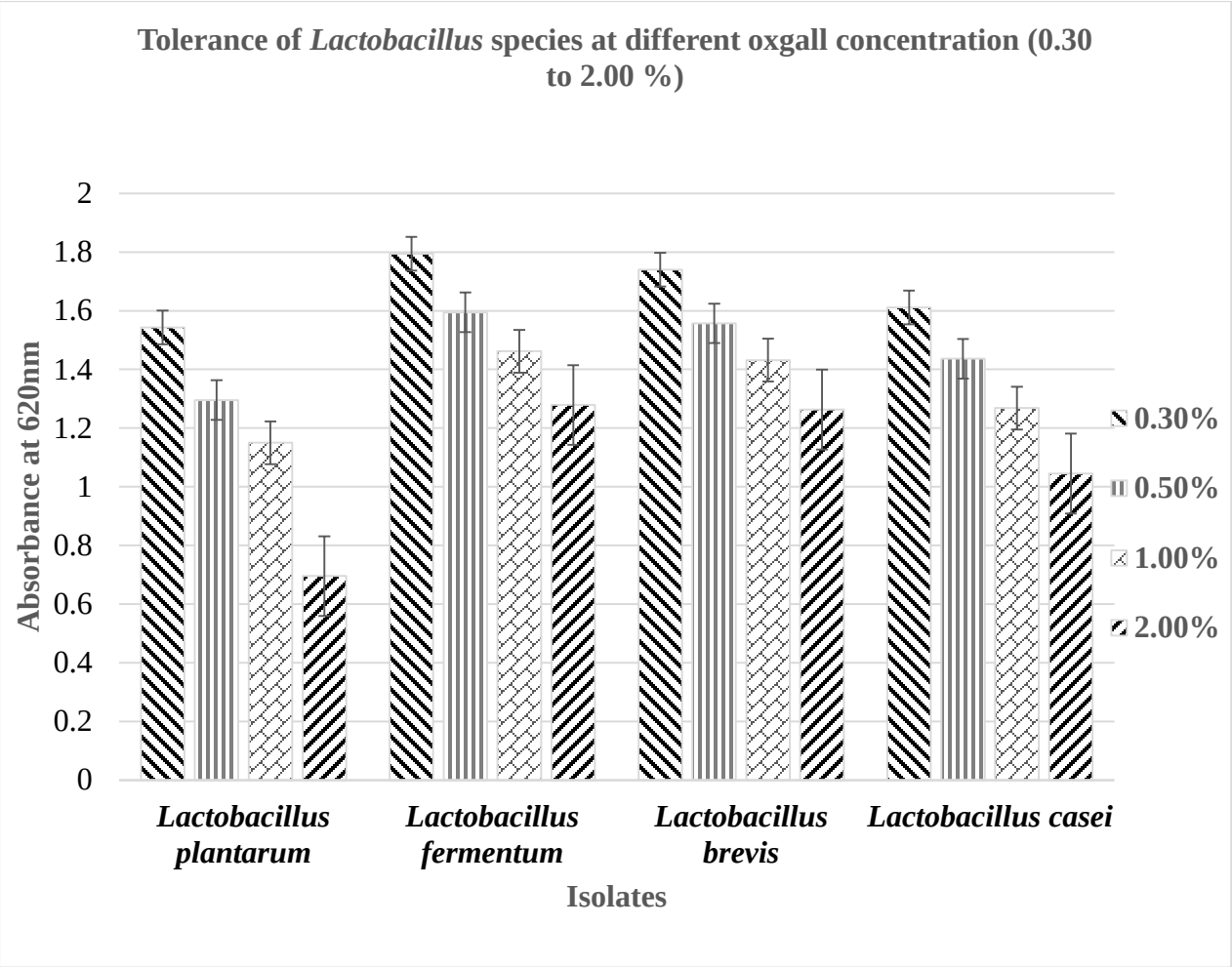

Figure 4.5 Absorbance values of MRS broth inoculated with Lactobacillus species at different oxgall concentration (0.3% to 2.0%) after incubation at 37^{o}C for 24 hours.

Figure 4.5 shows survival and growth of *Lactobacillus* isolates. *Lactobacillus fermentum* isolate showed highest absorbance at 620 nm at different bile salt concentrations (0.3 - 2.0 %) followed by *Lactobacillus brevis*, then *Lactobacillus casei* and lastly *Lactobacillus plantarum*.

4.6.2 Antimicrobial Activity

An important aspect of the function of probiotic bacteria is the protection of the host gastrointestinal microenvironment from invading pathogens. It is generally believed that the resident gastrointestinal micro flora in vivo provides protection for the host against possible colonization by pathogenic bacteria (Mohnl et al., 2007).

Table 4.9 shows the sensitivity against different food borne pathogens (*Salmonella enterica* ATCC 13076, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* Isolate) with varying degree of

zone of inhibition. *Salmonella enterica* was the most sensitive to *Lactobacillus* isolates with mean diameter of 17.1 ± 1.1. The highest diameter of 17.4 ± 0.4 was observed on Lactobacillus casei while the lowest diameter of 15.3 ± 0.1 millimetre for Lactobacillus brevis against *Salmonella enterica.* The highest diameter of 17.1 ± 0.1 millimetre was obtained from *Lactobacillus brevis against Escherichia coli* followed by *Lactobacillus plantarum, Lactobacillus casei and Lactobacillus fermentum* with zones of inhibition at 16.9, 16.6 and 16.5 millimetres respectively. Tests against *Staphylococcus aureus* isolate was observed to exhibit small zones of inhibitions when compared to the other test strains, the zones of inhibition against ranged from 15.6 to 16.9 millimetres with *Lactobacillus casei* showing the least zone of inhibition while *Lactobacillus brevis showing* the highest zone of inhibition against *Staphylococcus aureus.* The positive controls set with erythromycin exhibited inhibition zones as shown in Table 4.8 while negative control (MRS broth) used showed no inhibition zones against the test organisms.

Table 4.9 Average diameter of inhibition zone (mm) ± S.D

	Test microorganisms		
***Lactobacillus* Isolates**	***Salmonella enterica* (ATCC 13076)**	***Escherichia coli* (ATCC 25922)**	***Staphylococcus aureus* (Isolate)**
Lactobacillus plantarum	17.3 ± 1.6^{abc}	16.9 ± 1.2^{ac}	15.7 ± 0.3^{bd}
Lactobacillus fermentum	17.3 ± 0.3^{a}	16.5 ± 0.4^{cd}	15.6 ± 0.5^{bd}
Lactobacillus brevis	15.3 ± 0.1^{b}	17.1 ± 0.1^{a}	16.9 ± 0.1^{abc}
Lactobacillus casei	17.4 ± 0.4^{a}	16.6 ± 0.4^{ac}	15.6 ± 0.4^{bd}
Mean ± SD	**16.83 ± 1.0**	**16.78 ± 0.3**	**15.95 ± 0.6**
% CV	5.94	1.79	3.76
Standard	15.6	15.0	15.2

*Note: Values are means ± standard deviations (**S.D**) of triplicates and diameter of inhibition zone (mm) includes the disc diameter of 6.00 mm. **CV**, Coefficient of Variation. Mean values in the same column with the same superscript are not significantly different (P > 0.05).*

The highest diameter of inhibition zones was observed on *Lactobacillus spp.* against *Salmonella enterica* when compared with zones of inhibition on *Escherichia coli* and *Staphylococcus aureus* test strains. Picture A and B for test microorganism *Salmonella enterica* in figure 4.6 shows the zones of inhibition by different isolate *Lactobacillus spp.* With a range of 5.65 millimeter to 5.8 millimeter, this zones of inhibition were higher as compared to the ones observed in pictures C (range 5.15 to 5.5 mm) and picture D (4.8 mm) for the test strains *Escherichia coli* and *Staphylococcus aureus* respectively.

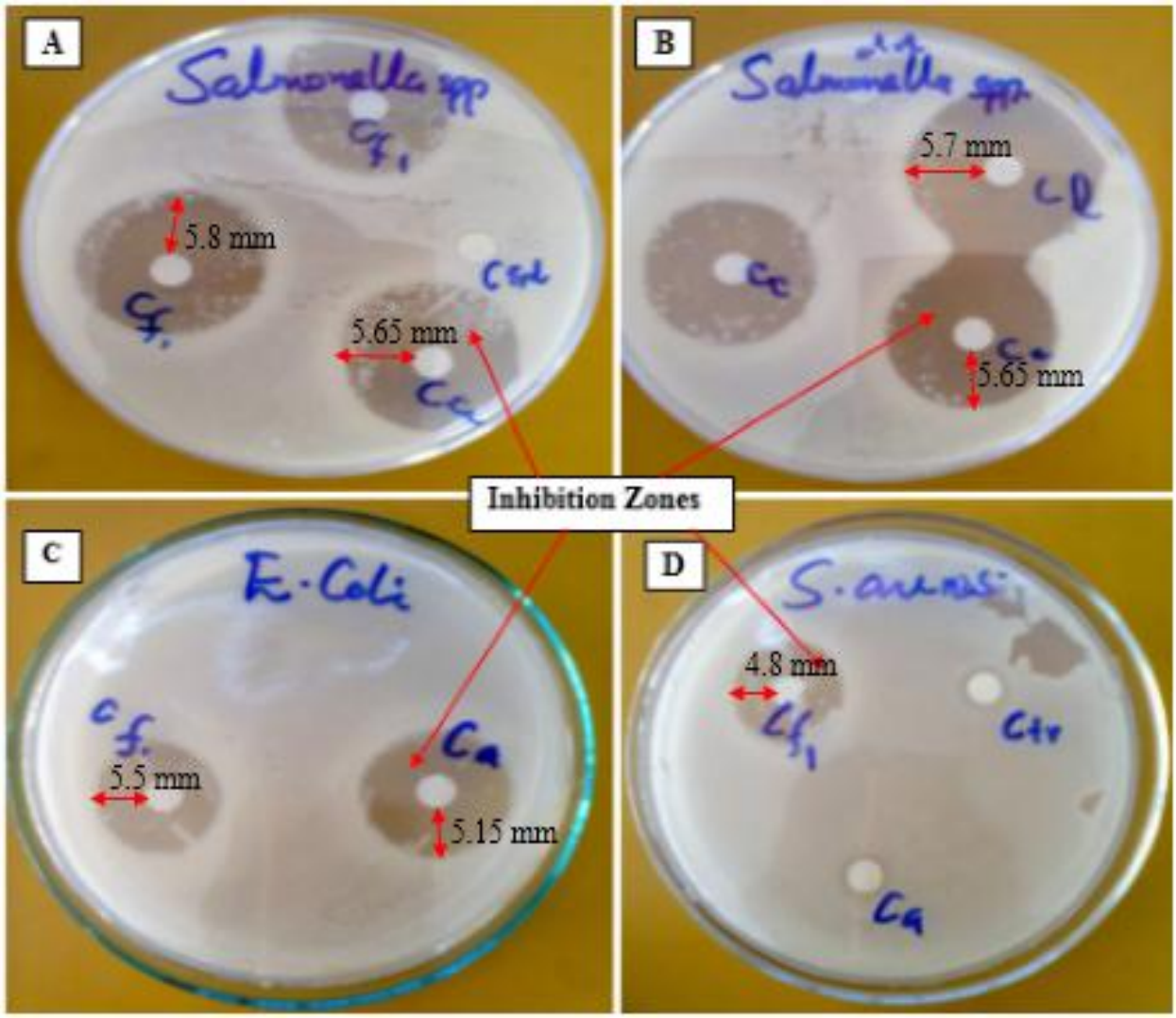

Figure 4.6 Pictures A, B, C and D showing zones of inhibitions against the test strains.

Figure 4.6 outlines pictorials for zones of inhibitions against test strains as obtained in the laboratory. The diameter of inhibition zones (Table 4.7) showed that all of the isolates have antibacterial effect on the indicator microorganisms. The tests were applied three times and the averages of diameters of zones were given.

CHAPTER FIVE

DISCUSSION

5.1 Introduction

This chapter presents the discussion of the study. The discussion takes the order of initial microbial load, comparisons of the initial load, physiological and biochemical characterization and probiotic properties (resistance to low pH, tolerance against bile and antimicrobial activity).

First Objective

5.2 Initial microbiological Load

Mursik from the current study had microbial load of $\geq 10^7$cfu/ml compared to Kenyan standard for fermented (cultured) milks which is put in a range $10^5 – 10^7$ cfu/ml (KEBS, 2013).

Mursik production in most of the households involves either smoke or non-smoke treatment. Of forty one (41) *mursik* samples that were analyzed > 75 % (32 samples) of had microbial load $\geq 10^7$ cfu/ml. Bulked *mursik* from the women groups had 80 % TVC $\geq 10^7$ cfu/ml. *Mursik* from individual women had 77.4 % TVC $\geq 10^7$ cfu/ml (Table 4.1). The difference in the microbial load between the individual and bulked mursik can be associated with environment of spontaneous fermentation as well as the environment where *mursik* is stored. Spontaneous fermentation takes advantage of the actions of various species of lactic acid bacteria that occur naturally in the environment (Mathara et al., 2008). Extrinsic factors that influence the high content of TVC and low content of CC are water, temperature and gaseous environment. The high nature of protein polymer network in *mursik* can thus affect the water activity, crosslinking due to fermentation reduces the activity hence suppressing the growth of yeast, mould and coliforms. The amount of water activity in *mursik* was below a critical water activity to support the growth of coliforms, yeast and moulds but was enough for LABs to thrive and grow. Smoking the gourd was found to favour the growth of LABs in the *mursik* which is associated with increased concentration of carbon monoxide and carbon dioxide. These gases in turn created an anaerobic environment which favoured growth of LABs and suppressed the growth of aerobic organisms such as the yeast, mould and coliforms.

5.3 Comparisons of the initial load

Comparison of the initial microbial load (Table 4.2) showed that bulking of *mursik* allows for an array of microorganisms that when bulked together might compete or suppress others as they strive to grow. Smoke treated *mursik* had lower microbial content (TVC and CC) than the non-smoke treated *mursik.* The $\log_{10}$ TVC and $\log_{10}$ coliform counts between smoke treated and the non-smoke treated mursik samples were significantly (P = 0.0001) lower, because of the environmental microflora, the majority being coliforms, had not gained entry into the *mursik.*

The high TVC could be associated with the high lactic acid bacteria count in *mursik.* Averagely, 50.8 % of the Lactic acid bacteria count was isolated in *mursik* and is mostly associated with the total viable count. The organisms involved are mostly *Lactobacillus spp.* This is also reported in studies done in the Kenya and Sudan by Lore et al. (2005), Mathara (1999) and Tambekar & Bhutada (2010). They reported that the LABs groups of bacteria, especially *Lactobacillus spp.* are commonly encountered in traditional fermented milk. The mean difference between TVC and coliforms can further be associated with the high content of LABs *mursik.* The LABs metabolize the milk via a number of fermentation pathways to produce carbon dioxide, lactic, acetic and propionic acids. These acid in turn lowers the pH of *mursik.* The increased acidity in *mursik* provides unfavorable environment for the growth of the coliforms. Acids exert their antimicrobial effect by interfering with the maintenance of cell membrane potential, inhibiting active transport, reducing intracellular pH and inhibiting a variety of metabolic functions (Rattanachaikunsopon & Phumkhachorn, 2010; Dardir, 2012)

From the current results the $\log_{10}$ LABs count was 8.50 ± 0.05 cfu/ml (56.09 %) for smoked *mursik* and 8.52 ± 0.03 cfu/ml (48.43 %) for non-smoked *mursik. Lactobacillus spp*. and *Bifidobacterium spp*. viable cells in adequate numbers found in fermented milks minimum need to be consumed regularly to transfer probiotic molecules to consumers (Dunne et al., 2001). The LABs count in *mursik* was higher than the standard range set by KEBS of 0.70 percent to 0.90 percent by mass expressed as lactic acid.

Although *mursik* production technology is similar among the Kalenjin people, different households have different practices for *mursik* fermentation. Individual household variations arise from the methods for gourd preparation, *mursik* incubation conditions, the amount of charcoal fines in the gourds, and hygiene and cleaning practices for the gourds (Muigei et al., 2013). This results in variation in the LAB profile and/or composition of other microorganisms that are in *mursik*. Ripening the gourd prior to its use as a fermentation vessel is very important

since it determines the type of LAB which becomes established on its inner surface, hence the LAB culture in *mursik*. These cultures therefore determine the quality of the final *mursik* that is produced using the gourd. Cleaning of the gourd is also important since it affects its microbiological quality. Poor cleaning and storage of the gourd results in establishment of non-desirable microorganisms such as yeast and moulds on its inner surface, and eventually become contaminants in *mursik* (Muigei et al., 2013).

The coliforms are pointer bacteria that give an indication, when present in any food, that there is the possibility of the presence of poor hygienic conditions. They show the hygienic conditions under which the product was produced and handled. The coliforms in the *mursik* could be associated with contaminated containers surfaces, water and the soil including dust and mud (Matofari, 2007). The coliform organisms are found in the soil, on plant materials and can be dispersed into the atmosphere by dust. Rainwater carries the surface contamination to the water sources (Matofari, 2007). Since coliforms are both of faecal and non-faecal origin, they are capable of multiplying outside the surface of containers, hence their presence in *mursik.* This is therefore not indicative of original contamination by coliforms but of improper handling of *mursik*. Transferring of *mursik* from one container to the next during bulking sweep over wide container surfaces, thus collecting the microorganisms from container surfaces. Hence, the reason for their presence in *mursik.*

How these coliforms evade the natural antimicrobial proteins in *mursik* and the organic acids produced after substrate breakdown and other products of microbial synthesis could be expounded as follows: Coliforms are gram-negative rod organisms that can adapt to several survival strategies in any food material. These survival strategies range from temperature evasions, acid tolerances and production of complex patterns or cooperative organizations of colonies. Other strategies genetically controlled (Adams & Moss, 1997). Coliforms, just like other microbes use several gene products to control their movement within fermented milk environment. The coliforms respond to the concentration of chemo-repellants in *mursik* by measuring the fraction of receptors occupied by the signaling molecules. At high concentrations, the chemotactic response vanishes because of receptor saturation. The movement of motile bacteria (chemotaxis) involves changes in the movement of the cell in response to a concentration gradient of certain chemical fields (Matofari, 2007),

Second Objective

5.4 Biochemical Characterization

Production of LABs is important in fermented milk products since they impart health beneficial effects to the consumers of fermented milks. In this study sixty four (64) different microorganisms were isolated in traditional *mursik.* It was established that the traditional *mursik* contained 82 percent LABs (53 isolates), (Lactobacillus, 56.1 % and Other LABs (Lactococcus, Enterococcus and Leuconostoc), 25.9 %), coliforms (7.0 %) and, yeast and molds (11.0 %) (Figure 4.1). The high LABs content in *mursik* may be attributed to several factors which include nutrient content, pH and organic acids, low water activity and gaseous environment which promote LABs growth in *mursik.*

All isolates were initially tested for gram reaction and catalase enzyme reactions. Only gram positive bacteria with catalase negative reactions were observed (Hoque et al., 2010) and the representative isolates were purified by successive streaking on to the same agar substrate. On the basis of Cell Morphology, Colony characterization and all Biochemical tests performed, gram-positive and catalase negative rods were selected for further analysis. Since they formed part of the predominant LABs that were present in *mursik.*

In identification of the isolates or bacteria culture obtained from fermented milk biochemical characterization is a primary feature. Enzyme catalase is common and found nearly in all living organisms that are exposed to oxygen, where it catalyzes the breakdown of hydrogen peroxide to water and oxygen, which are less dangerous/reactive after a normal metabolic activity. (Chelikani, Fita, & Loewen, 2004). When the 16 isolates, *Lactobacillus plantarum* (Ba), *Lactobacillus plantarum* (Ba1), *Lactobacillus fermentum* (Bb), *Lactobacillus fermentum* (Bb1), *Lactobacillus brevis* (Bc), *Lactobacillus brevis* (Bc1), *Lactobacillus casei* (Bd), *Lactobacillus plantarum* (Bd1), *Lactobacillus fermentum* (Be), *Lactobacillus fermentum* (Be1), *Lactobacillus casei* (Ca), *Lactobacillus casei* (Cb), *Lactobacillus fermentum* (Cc), *Lactobacillus plantarum* (Cd), *Lactobacillus fermentum* (Cf) and *Lactobacillus casei* (Cf1) were tested for catalase activity. They were observed as catalase negative (hence, do not show catalase activity) this was in agreement with Bergey's manual on the catalase activity of the lactic acid bacteria, hence a significant character of probiotics (Cheah et al., 2014).

Gas occurrence in Durham tubes was observed during 5 days which is the evidence for CO_2 production from glucose. Of sixteen (16) isolates, eight (8) of them were homofermentative while the rest were heterofermentative (Table 4.5). This indicates that unique fermentation

pathways coordinated by the individual isolates through their genetic makeup. Additional measure for the proof of identity of the isolates was the ability to growth at different temperatures.

From the results of 7 days observation, isolates Lactobacillus *plantarum* (Ba), *Lactobacillus plantarum* (Ba1), *Lactobacillus brevis* (Bc1), *Lactobacillus casei* (Bd), *Lactobacillus plantarum* (Bd1), *Lactobacillus casei* (Ca), *Lactobacillus casei* (Cb), *Lactobacillus plantarum* (Cd), *Lactobacillus fermentum* (Cf) and *Lactobacillus casei* (Cf1) grew at temperatures 10 degrees Celsius and 15 degrees Celsius. *Lactobacillus brevis* (Bc) was unable to grow at 10 °C but exhibited growth at 15 degrees Celsius. Three (*Lactobacillus casei* (Bd), *Lactobacillus casei* (Cb) and *Lactobacillus casei* (Cf1) isolates were exceptional since they showed growth in all the three temperatures (10°C, 15^0C and 45^0C) that were tested under (Table 4.5). This indicates the isolated LABs species were thermophiles capable of growing in an array of temperature. LABs have been reported to exhibit limited biosynthetic ability, requiring preformed amino acids, B vitamins, purines, pyrimidines and typically a sugar as carbon and energy source. A rich medium and temperature range is usually employed when cultivating lactics as compared to mesophilic and psychophilic bacteria. These multiple requirements restrict their habitats to areas where the required compounds are abundant (animals, plants, and other multicellular organisms). Lactic acid bacteria can grow at temperatures from 5 degrees Celsius to 45 degrees Celsius and not surprisingly are tolerant to acidic conditions (Trontel et al., 2010).

Growth at different NaCl concentrations was also observed. The isolates studied proved ability to grow at 4 %, 6.5 % and 8 % NaCl concentration however at 10 % NaCl concentration none of the isolates showed the ability to grow. NaCl resistant isolates can help to survive in simulated gastric fluid. This implies that it may be useful in enhancement of the stability and functional properties of probiotic strains. Property of tolerance NaCl concentrations could be advantageous for probiotic culture for successful colonization in gastrointestinal environment.

Arginine hydrolysis test was one more step to follow the identification technique. The isolates which gave the bright orange were accepted that they can produce ammonia from Arginine. The yellow color indicated negative Arginine hydrolysis. Use of Arginine is accomplished by the enzyme arginine dihydrolase (Nikita & Hemangi, 2012). Isolates Bb, Bb1, BC, Bc1, Be, Be1, Cc and Cf which produced gas were all subjected to Arginine hydrolysis tests. According to this test all these isolates were able to hydrolyze arginine hence can produce ammonia from

arginine indicating that all the isolates under investigation were *Lactobacillus* and not *Leuconostoc* (Nikita & Hemangi, 2012).

5.4.1 Carbohydrate Fermentation

Carbohydrate fermentation by LABs varied among the *Lactobacillus* species and strains, although all the strains tested were unable to ferment rhamnose. LABs species vary in their ability to ferment or not to ferment carbohydrates due to diversity in their genetic potentials. Also carbohydrate fermentation is a complex process which involve activities of different enzymes encoded by gene either located in the plasmid DNA or chromosomal DNA which might be expressed differently due to diversity of the genetic makeup of the isolates hence difference in the fermentation pattern.

Third Objective

5.5 Probiotic Potential

A number of requirements have been established for lactic acid strains to be effective probiotic microorganisms. Required probiotic characteristics include inhibition of entero-pathogenic bacteria, tolerance to acid and bile salts.

5.5.1 Acid and Bile Salt Tolerance

Bile and acid tolerance are important characteristics of the lactic acid bacteria strains. Bile tolerance is necessary for bacterial growth in the small intestines and acid tolerance is required for the bacteria to survive the passage through the stomach as well as to survive in food (Ashton, 2013). Bile acids are synthesized in the liver from cholesterol and sent to the gall–bladder and secreted into the duodenum in the conjugated form (500-700 ml/day). In the large intestine this acids undergo some chemical modifications (deconjugation, dehydroxylation, dehydrogenation and deglucuronidation) due to the microbial activity (Buntin et al., 2008). The strains, resistant to low pH, were later screened for their ability to tolerate the bile salt. Although the bile concentration of the human gastro intestinal tract varies, the mean intestinal bile concentration is believed to be 0.3 percent weight per volume and the staying time is suggested to be 4 hours (Prasad et al., 1998).

All the four (4) *Lactobacillus* isolates were subjected to acidic pH range 1.5 to 3.5 to test their survivability. The isolates differed considerably in their resistance to acid. Table 4.7 indicates there that *Lactobacillus plantarum, Lactobacillus fermentum* and *Lactobacillus casei* isolates were resistant to the test range of pH (1.5 to 2.0) except for the *Lactobacillus brevis* isolate which was not resistant to pH at 1.5 and 2.0. However, all the four strains showed tolerance to

pH 3.0 and 3.5 and gives growth that can be considered as significant character of them acting as probiotics. Because in intestinal tract acidic pH is there and isolates should survive from the entry and to establish in gut. Survival of fermented milk bacteria in presence of acid is significant because in case of human consumption of fermented milk they might colonizes stomach with friendly bacteria (Maragkoudakis et al., 2005). This suggests that bile resistant traits of *Lactobacillus spp.* are crucial for maintaining viability during gastrointestinal transit and are desirable attributes of an orally administered probiotic. Moreover, most of probiotic lactic acid bacteria are consumed in fermented dairy products and milk proteins may provide a protective martrix enhancing and supporting survival of bacteria in the gastric juice of the stomach (Ouwenhand, Tuomola, Tolkko, & Salminen, 2001; Fernandez, Boris, & Barbes, 2003; Tambekar and Bhutada 2010a).

Just like resistance to acidic pH tolerance to bile was considered as a prerequisite for colonization and metabolic activity of bacteria in intestine of the host (Dardir, 2012; Zoumpopoulou et al., 2008). In the study, it was observed that all the Lactic acid bacterial strains survived and tolerated bile salts (Oxgall) concentrations of 0.3 percent to 2.0 percent quite effectively. But a marginal decrease in viability of all the strains was found when the bile salt concentration was increased from 0.3 percent to 2.0 percent (Figure 4.4). The differences in the absorbance values of bile tolerance (at 620nm) between strains in this study might be due to differences in their ability to grow and colonize, as the bile salt concentration was increased from 0.3 percent to 2.0 percent the ability of strains to de-conjugate bile acids was decreasing hence the marginal decrease. Similar to this study marginal decrease in viability has been reported by Barakat et al., 2011 and Shukla et al., 2010).

Among all the *Lactobacillus* isolates, isolate *Lactobacillus fermentum* showed highest absorbance at 620 nm at different bile salt concentrations ranging between 0.3 to 2.0%, implying that it was the most effective strain in de-conjugation by producing high amounts of bile salt hydrolase (BSH) activity on bile acid reducing their toxic effects more in different concentrations as compared to *Lactobacillus plantarum*, *Lactobacillus brevis* and *Lactobacillus casei* isolates. Bile salt hydrolase activity or possession of the bile salt hydrolase homologs in their genetic material has most often been found in organisms isolated from the commensal inhabitants of the gastrointestinal tract such *Lactobacillus*, *Bifidobacterium*, *Enterococcus,* and *Bacteroides spp.* and not in the bacteria isolated from environments from which bile salts are absent (Holzapfel et al., 1998; Tanaka et al., 1999; Rana et al., 2011).

Isolates survival and growth at low pH (1.5 to 2.0) (Table 4.8) suggests that they can survive in extreme condition of intestinal tract while growth in presence of Bile at a range of 1 percent to 4 percent indicates bile tolerance of isolates and the possibility of these organisms to be administered orally. Property of tolerance to bile concentrations and acidic pH could be advantageous for probiotic culture for successful colonization in gastrointestinal environment.

5.5.2 Antimicrobial Activity

All the *Lactobacillus* strains isolated were examined according to their antimicrobial activity. For this purpose, strains were detected against the indicator microorganisms *Salmonella enterica* ATCC 13076, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* Isolate. The culture filtrate from LABs, that was identified to be solely genera of *Lactobacillus* species, isolated from smoke treated and non-smoke treated mursik samples were tested by disc diffusion method to know if the antimicrobial metabolites produced by LABs were extracellular and released into the growth medium. In this test the three indicator microorganisms were inhibited showing the inhibition zones in pictures A, B, C and D, indicating that the inhibitory metabolites produced by the isolates were extracellular and diffusible because inhibition took place by diffusing on the agar layer.

The four (4) *Lactobacillus spp., Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus brevis* and *Lactobacillus casei* from *mursik* are able to inhibit target organisms used. The inhibition of the target organisms varied among the *mursik* LABs. *Salmonella enterica* was the most sensitive to *Lactobacillus* isolates with mean diameter of 17.1 ± 1.1. The highest diameter of 17.4 ± 0.4 was observed on Lactobacillus casei while the lowest diameter of 15.3 ± 0.1 millimetre for *Lactobacillus brevis* against *Salmonella enterica. Escherichia coli* was moderately inhibited in the study with the highest diameter of 17.1 ± 0.1 millimetre was obtained from *Lactobacillus brevis* followed by *Lactobacillus plantarum, Lactobacillus casei and Lactobacillus fermentum* with zones of inhibition at 16.9, 16.6 and 16.5 millimetres respectively. Tests against *Staphylococcus aureus* isolate was observed to exhibit small zones of inhibitions when compared to the other test strains, the zones of inhibition against ranged from 15.6 to 16.9 millimetres with *Lactobacillus casei* showing the least zone of inhibition while *Lactobacillus brevis showing* the highest zone of inhibition against *Staphylococcus aureus.* These varying degree of inhibition could be due to metabolic pathways controlled by the test microorganisms gene expressions such as acid-tolerance response system that protects them against severe acid stress for longer periods, hence showing that *Escherichia coli* (ATCC

25922) and *Staphylococcus aureus* being more tolerant to some organic acids than *Salmonella enterica*.

When mean of the inhibition zones produced by isolates in specific test microorganisms were compared, differences were significant ($p < 0.05$). Likewise significant difference was observed in degree of inhibition between the control and the LAB strains in all cases ($p < 0.05$). Based on the differences in inhibition zones produced by LAB cultures and the control, the highest inhibitory was observed to the test strains *Salmonella enterica* followed by *Escherichia coli* and *Staphylococcus aureus* in that order. Among the three test strains used in this study, *Salmonella enterica* subsp. *enterica serovar enteritidis* (ATCC 13076) was relatively susceptible to the antimicrobial activity of LAB than the *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (Isolate). The differences in antagonistic activity of LABs on tested pathogenic organisms may be due to production of antimicrobial compounds such as Lactic acid, acetic acid or bacteriocins to a varying degree. The antimicrobial effect of lactic acid is due to undissociated form of acid, which penetrates the membrane and liberates hydrogen ion in the neutral cytoplasm thus leading to inhibition of vital cell functions. This might be due to the production of acetic and lactic acids that lower the pH of the medium or competition for nutrients or production of bacteriocins or other antibacterial compounds (Chuayana et al., 2003; Quwehand et al., 2002).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Introduction

This chapter presents the conclusions and recommendations from the research work. This chapter also gives suggestions for further research in this area.

6.2 Conclusion

The study objectives were to determine the microbial load, isolation and characterize LABs as well as determination of probiotic potential of Lactic Acid Bacteria isolated from mursik. To determine the probiotic properties different tests were applied such as resistance to low pH and bile salt and antimicrobial activity tests. Results obtained from current study, the following conclusions can be made; Lactic acid bacteria (LABs) count of smoke treated *mursik* was 56% and non-smoked 48% out of the total samples taken forming a concentration of 80% of the lactic acid bacteria in samples taken. The remaining 20% formed coliforms, yeast and moulds.

The main probiotics isolated from *mursik* include, *Lactobacillus plantarum*, *Lactobacillus fermentum, Lactobacillus brevis* and *Lactobacillus casei.* The metabolites produced by the isolated probiotics in broth demonstrated significant antibacterial effect against the standard strains used. The results obtained from this study confirm that the probiotics in *mursik*, a traditionally fermented milk product have significant potential against enteric and environmental pathogens that are of public health concern.

6.3 Recommendations

Micro biota and its function along the human gut is complex and research needs to continue with the objective of better understanding microbe-microbe interactions as wells microbe-host interactions under both normal and pathological conditions with defined dosage. Future research needs to test adhesion of the LABs to the mucosal surface, clinical studies for human health across different ages on the micronutrient absorption on the gastrointestinal tract upon supplementation with probiotics, technological properties (strain stability, viability in products, bacteriophage resistance) and antibiotic resistance of the LABs from traditional fermented milk.

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APPENDICES

APPENDIX 1: RECIPES FOR CULTURE MEDIA AND BIOCHEMICAL TESTS

1. MRS Broth

Ingredients	g/l
Protease peptone	10.0
Beef extract	10.0
Yeast Extract	05.0
Dextrose	20.0
Polysorbate 80	01.0
Dipotassium phosphate	02.0
Sodium acetate	05.0
Ammonium citrate	02.0
Magnesium sulphate	00.2
Manganese sulphate	0.05

Deionized water 1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was dispensed into test tubes and sterilized by autoclaving at 121°C for 15 min.

2. MRS Agar

Ingredients	g/l
Protease peptone	10.0
Beef extract	10.0
Yeast Extract	05.0
Dextrose	20.0
Polysorbate 80	01.0
Dipotassium phosphate	02.0
Sodium acetate	05.0
Ammonium citrate	02.0
Magnesium sulphate	00.2
Manganese sulphate	0.05

Agar	15.0
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was sterilized by autoclaving at 121°C for 15 min.

3. Modified MRS Broth for Testing the Growth at Different Temperatures

Ingredients	**g/l**
Protease peptone	10.0
Beef extract	10.0
Yeast Extract	05.0
Dextrose	20.0
Polysorbate 80	01.0
Dipotassium phosphate	02.0
Sodium acetate	05.0
Ammonium citrate	02.0
Magnesium sulphate	00.2
Manganese sulphate	0.05
Bromocresol purple	0.04
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3.Medium was dispensed into test tubes and sterilized by autoclaving at 121°C for 15min.

4. Modified MRS Broth for Testing the Growth at Different NaCl Concentrations

Ingredients	**g/l**
Protease peptone	10.0
Beef extract	10.0
Yeast Extract	05.0
Dextrose	20.0
Polysorbate 80	01.0
Dipotassium phosphate	02.0

Sodium acetate	05.0
Ammonium citrate	02.0
Magnesium sulphate	00.2
Manganese sulphate	0.05
Bromocresol purple	0.04
Sodium Chloride (NaCl)	20, 40, 65 for the concentration of 2%, 4% and 6.5%
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3.Medium was dispensed into test tubes and sterilized by autoclaving at 121°C for 15min.

5. Modified MRS Broth for Gas Production from Glucose

Ingredients	**g/l**
Protease peptone	10.0
Beef extract	10.0
Yeast Extract	05.0
Dextrose	20.0
Polysorbate 80	01.0
Dipotassium phosphate	02.0
Sodium acetate	05.0
Ammonium citrate	02.0
Magnesium sulphate	00.2
Manganese sulphate	0.05
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3. Medium was dispensed into test tubes and inverted Durham tubes were distributed to each test tube, and lastly sterilized by autoclaving at 121°C for 15 min.

6. Modified MRS for Carbohydrate Fermentations

Ingredients	**g/l**

Protease peptone	10.0
Beef extract	10.0
Yeast Extract	05.0
Dextrose	20.0
Polysorbate 80	01.0
Dipotassium phosphate	02.0
Sodium acetate	05.0
Ammonium citrate	02.0
Magnesium sulphate	00.2
Manganese sulphate	0.05
Bromocresol purple	0.04
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3.Medium was sterilized by autoclaving at 121°C for 15 min.

7. Arginine MRS

Ingredients	**g/l**
Protease peptone	10.0
Beef extract	10.0
Yeast Extract	05.0
Dextrose	20.0
Polysorbate 80	01.0
Dipotassium phosphate	02.0
Sodium acetate	05.0
Ammonium citrate	02.0
Magnesium sulphate	00.2
Manganese sulphate	0.05
Arginine	01.5
Deionized water	1000ml

All ingredients were dissolved in deionized water and pH was adjusted to 6.3.Medium was sterilized by autoclaving at 121°C for 15 min.

APPENDIX 2: CARBOHYDRATES USED FOR FERMENTATION TESTS

Sugar solutions prepared at concentration 10%

1. D (+) Xylose
2. D (-) Ribose
3. L (+) Arabinose
4. Mannitol
5. D (+) Trehalose
6. Raffinose
7. D (+) Galactose
8. Maltose
9. Sucrose
10. D (+) Mannose
11. Fructose
12. Lactose
13. Sorbitol
14. Glucose
15. Esculin
16. Rhamnose

Sugar solution prepared at concentration 5%

17. D (-) Salicin

APPENDIX 3: RESEARCH APPROVAL LETTER

EGERTON
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OFFICE OF THE DIRECTOR GRADUATE SCHOOL

10th February, 2014.

HM18/3035/11
Chadwick Adongo Digo
Department of HN,
P. O Box 536,
EGERTON

Dear Mr. Digo,

RE: MSc. RESEARCH PROPOSAL APPROVAL- 29TH JANUARY, 2014

This is to inform you that the Board of Post graduate Studies in its meetings held on 29th JANUARY, 2014 Considered and **Approved** your research proposal entitled : *:" Isolation and Characteristic Determination of Probiotic Lactic Acid Bacteria from Traditionally Fermented Milk, Mursick of Kenya."*

With the following corrections;

- Rework on the title to be more clear.
- Make other corrections as indicated in the text
- Rework on the work plan to reflect the current status.

Two copies of the corrected proposal together with certificate of correction form should be forwarded to graduate school not later than thirty days from the date of this letter.

Yours sincerely,

G.K.Maranga
FOR: DIRECTOR, BOARD OF POSTGRADUATE STUDIES

C.c. Dean (HEATH SCIENCES)

DIRECTOR BOARD OF POST GRADUATE STUDIES * 14 FEB 2014 * EGERTON UNIVERSITY P.O. Box 536, EGERTON

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