

**ANTIMICROBIAL ACTIVITY PROFILES OF TRADITIONAL FERMENTED MILK  
STARTER CULTURES FROM NORTH-EASTERN NAMIBIA**

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## ABSTRACT

The aim of this study was to identify and examine the antimicrobial properties of Lactic Acid Bacteria (LAB) isolated from fermented milk collected from Ohangwena, Omusati, Oshana, Oshikoto, Zambezi and Kavango regions. Traditional fermented milk in Namibia are produced by spontaneous fermentation using traditional utensils. In this study, thirty homesteads from six regions that produce and process fermented milk were selected and interviewed using semi-structured questionnaires. *Omashikwa* and *Mashini ghakushika* have similar processing method whereby fermentation is achieved by accumulation of milk; mean while *Mabisi* is produced by allowing the milk to ferment naturally. The pH decreased logarithmically, nonlinearly over the fermentation period from  $6.5 \pm 0.002$  from first day of fermentation to  $3.92 \pm 0.001$  over 4 days. There was no significant difference ( $p < 0.05$ ) in the pH values between the three types of fermented milk preparations. Cell free supernatants (CFS) of 180 LAB isolated from traditional fermented milk were evaluated for antimicrobial activities against selected food borne pathogens; *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 14053, *Bacillus cereus* ATCC 10876 *Geotrichum klebahnii* (IKST F. Lab. isolate) *Escherichia coli* ATCC 25922 using the well diffusion method. Twenty LAB isolates that shown the highest inhibitory effects were selected for biochemical identification using API 50 CHL were identified as; *Lactobacillus plantarum* (53%), *Lactobacillus rhamnosus* (29%), *Pediococcus pentosaceus* (6 %), *Lactobacillus paracasei* ssp. *paracasei* (6%) and *Lactococcus lactis* ssp. *lactis* (6%), of which *Pediococcus pentosaceus* showed the most inhibitory effect on all the indicator strains and they have potential to produce bacteriocin *Pediocin* and the most inhibited indicator strain belonged to yeast *Candida famata*. In addition to antimicrobial activities, the major organic acids in all three types of milk were found to be lactic acid

0.265±0.056 mg/L. However, the levels of volatile organic compounds in the naturally fermented samples varied from one sample to another but, butyric, acetic and propionic acid were found in trace amounts. Some of the volatile flavor compounds found in *Omashekwa*, *Mabisi* and *Mashini ghakushika* were acetic acid, 2,3 Butanediol and Lactic acid. Further genetic confirmation of species was carried out using the 16S rDNA sequences. The research outcome addressed the establishment of the newly identified starter cultures and contributes positively to the advancement of national LAB's profiling. The study is also the first attempt that indicates probiotic potential and exceptional preservation properties of LAB from traditional fermented milk produced in North- Eastern Namibia.

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**DECLARATION**

I declare that this dissertation is the product of my own work, that it has not been submitted before for any degree or examination in any other university and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

**DEDICATION**

This is dedicated to my family; Paulus Heita, Fiina Heita and my four sisters; Rachel, Selma, Sarafina and Vistolina and also to my brother Josephat Heita. I want to thank them for their support, prayers and encouragement during the two years I have been toiling as a student, at the University of Namibia.

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**LIST OF ABBREVIATIONS**

°C	Degree Celsius
ANOVA	Analysis of variance
Cfuml-1	Colony forming units per milliliter
CO <sub>2</sub>	Carbon dioxide
d	Day
g/l	Grams per liter
h/hrs	Hours
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HPLC	High performance liquid chromatography
LAB	Lactic acid bacteria
NADH	Nicotinamide Adenine Dinucleotide
pH	Negative logarithm of hydrogen concentration
spp.	Species

## **CHAPTER 1: INTRODUCTION**

Fermented milk is one of the foods which are highly respected and form part of daily intake in Namibia. There are different types of fermented milk in Namibia which have different preparation methods. The diversity of such fermented milk products is derived from the heterogeneity of traditions found in the Namibia, cultural preference, different geographical areas where they are produced, the staple and/or by-products used for fermentation. These types of milk are mostly prepared using natural ingredients without any addition of preservatives.

Fermentation is a relatively efficient, low energy preservation process which increases the shelf life and decreases the need for refrigeration or other form of food preservation technology (Marshall, & Mejia, 2011). It is therefore a highly appropriate technique for use in developing countries and remote areas where access to sophisticated equipment is limited.

Traditional fermentation is a form of food processing where microbes for example, lactic acid bacteria (LAB) are utilized (Chelule *et al.*, 2010). The technology of fermentation has been used for centuries as a form of food preservation technique for perishable food products (Hansen, 2002). Over the years, it became part of the cultural and traditional norm among the indigenous communities in most developing countries, especially in Africa. On this note, the people living in remote areas prefer fermented over unfermented milk due to improved flavor and texture that accompanies fermentation. In addition, these products are an important supplement to the local diet and provide vital elements for growth and good health. This popularity has made fermented foods one of the main dietary components of the developing world.

Fermenting milk assist in the preservation of milk by generating lactic acid and other antimicrobials, production of flavor compounds (e.g. acetaldehyde and diacetyl) and other metabolites (e.g. extracellular polysaccharides) that confer consumer desired organoleptic properties to the product (Chelule, *et al.*, 2010). Fermentation also improves the nutritional value of milk; it releases free amino acids and promotes synthesis of vitamins (Kailasapathy, 2008). Fermented milk contains special therapeutic or prophylactic properties against cancer and lactose intolerance (Mercenier, *et al.*, 2002). Fermentation also, improves food safety through inhibition of pathogens (Adams & Mitchell, 2002) as well as removal of toxic compounds in food. These significant changes cause desirable biochemical effects resulting into development of new aroma, flavor, taste and texture thereby increasing the sensory quality, palatability and acceptability of the product.

### **1.1 African Traditional Fermented Milk**

Traditional fermented milk has a long history in Africa which relies on the indigenous knowledge of the population. Generally, fermentation processes are believed to have been developed over the years by women, in order to preserve food for use during off seasons and also to bring in desirable flavors in food (Rolle & Satin, 2002). The oldest references highlight the fact that the earliest fermented milks have existed for more than 3000 years and doubtlessly go right back to the original domestications of dairy animals (Jashbhai & Nair, 2008). The recipes for fermentation process have been handed down from generation to generation, sometimes changing and giving rise to a new type of fermented milk. Thus, there is enormous diversity of names dedicated to these traditional fermented milks throughout the world. These are all words used to describe a taste, texture and specific acidity based on skills and specific strains. In Namibia there is a wide variety of traditionally fermented milk of which the most common type

is *Omaere* which is commercially found in groceries retail outlets. Other types of fermented milks are less popular due to their low distributed in the formal markets and are only sold in informal open markets. Apart from *Omaere*, *Omashikwa* is also commonly sold in the open markets. *Omashikwa* is a popular fermented milk product among the *Oshiwambo* speaking people of Namibia prepared by natural fermentation of raw milk mixed with clean roots of Sheppard tree (*Bosciaa albitrunca*) and incubated at room temperature ranging from 27 °C to 36 °C for 3-4 days (Bille *et al.*, 2007). This type of milk has a unique effervescent acid taste with a rooty flavour, ropy appearance that slowly whey's off when left undisturbed for some time (Bille *et al.*, 2002; Bille, 2013)

## **1.2. Orientation of the Study**

Few studies on Namibian fermented milk products have been reported that focused on the technological aspects and sensory evaluation (Bille *et al*, 2002; Bille *et al*, 2007; Bille, 2013). No information relating to antimicrobial activities or metabolites of the Namibian fermented milk products starter cultures could be found. This study focused on the antimicrobial characteristics of LAB isolated from traditional fermented milk of North- central and North-eastern Namibia, their potentials for industrial applications with special focus on the possible protective (probiotic) activity and antagonism against food spoilage microorganisms. These important function(s) may render these starter cultures to be considered as important nominee for food safety applications.

### 1.3 Statement of Problem

*Omashikwa*, *Masanza* or *Mabasi* and *Mashini ghakushika* are common fermented milk products in the rural areas of the northern Namibia. Like any other traditional fermented milk in the developed countries, these types of milk serve as substitutes for traditional vegetables, beans or meat supplementing nutrition for low income households and for women and children in rural area (Bille, *et al.*, 2002). In addition, the fermentation is based on spontaneous process due to the growth of the micro flora naturally present in the raw material. Therefore, the quality of the fermentation end product depends on the microbial load and spectrum present in the raw material. Since *Omashikwa*, *Masanza* or *Mabasi* and *Mashini ghakushika* are not prepared uniformly by the rural processors, they have fluctuating qualities due to natural fermentation, uncontrolled fermentation temperature and time. Failure of fermented milk to cause food poisoning in spite of uncontrolled fermentation environments suggests unique chemical properties in these products. Therefore, there is a need to examine the diversity of LAB, the antimicrobial content of the milk and to purify the most predominant LAB in traditional fermented milk from the northern parts of Namibia. Previous antibacterial activity studies have been performed by isolating LAB from fermented products. However, LAB isolated from Namibia traditionally fermented milk has not been intensively studied.

### 1.4 Objectives of the Study

The overall aim of the study was to isolate LAB from traditional fermented sour milk products of Northern part of Namibia (Ohangwena, Omusati, Oshana, Oshikoto, Zambezi and Kavango regions) and to investigate their antimicrobial properties that may have bearing for food safety application to control and eliminate the growth of harmful bacteria in food.

The specific objectives of this research project were:

1. To analyze the physicochemical properties (pH, viscosity and titratable acidity) for the three types traditional fermented milk in Namibia.
2. To isolate different types of lactic acid bacteria using selective media such as Rogosa for *Lactobacillus* spp. and M17 for *Lactococcus* spp. e.t.c.
3. To identify starter culture strains to the species and sub-species level using biochemical and genetic identification methods (API 50 CHL and 16S rDNA).
4. To screen the isolated strains for antimicrobial compounds with the ability to act as a natural competitive substance inhibiting other microorganisms that share the same niche; these include: hydrogen peroxide, organic acids, diacetyl and bacteriocins.
5. To determine the concentration of different organic acids (lactic and acetic acids) using conversional method (titration) and chromatographic method (HPLC).

### **1.5 Significance of the Study**

Fermented milk plays an important role in the diet of low income and the majority of people living in the rural areas in Namibia. This research work provides a baseline data on the antimicrobial activities of Namibian fermented milk products. It focuses on antimicrobial compounds profiling of different starter cultures from fermented milks produced in Namibia which has not been reported previously. In addition, this research identified the types of micro flora in traditional fermented milk to the genus level. Once the identity of the milk fermenting micro flora is known it would be possible to optimize fermentation process through alteration and modification of starter cultures so as to improve the quality of fermented milk products for possible commercialization purpose. Furthermore, knowledge of the starter cultures metallic products would facilitate their ease of extraction and subsequent use in food industries as

preservatives. Alternatively, the isolated LAB could be used as probiotics. Micro flora profiling is significant as it may unravel species' diversity and concentrations in the fermented milk; it may shed light on the organisms' ecological characteristics as they interact with the host and the environment at large.

### **1.6 Limitation of the Study**

Preferably, the study was to include all northern regions in Namibia, but Namibia is a very big country and there are many different cultural groups with different traditional foods and fermented milks. It would be an expensive, time consuming and labor intensive to screen all fermented milk products in Namibia. DNA sequencing facilities are not available in Namibia; genetic identification of the starter cultures was therefore done at Inqaba Biotech Laboratory in South Africa. During the screening and strains identification phases, a large number of isolates was obtained. Therefore, only isolates which showed outstanding results on different tests, in particular, the bacteriocogenic strains which showed protective characteristics were considered for identification on molecular diagnosis' and other antimicrobial tests such as diacetyl, hydrogen peroxide and organic acids using conversional analytical techniques, since screening all isolates was going to be a very expensive and time consuming process.

## **CHAPTER 2 LITERATURE REVIEW**

### **2.1. The Origin of Fermented Milk**

Traditional Fermented milk is defined by Abdelbasset & Djamila, (2008) ; as a dairy product obtained by the spontaneous fermentation of milk, which may have been made from products obtained from milk with or without any modification of their composition, through appropriate process of microorganisms which results in a lowering of the pH with or without coagulation. The traditional fermentations are taking place as a result of the activities of natural flora present in the food or added from the environment. Fermentation of milk is a very ancient practice of man which has been passed down from generation to generation (FAO, 1990).

The definite origin of fermented milks is indefinite but there is no doubt that their consumption dates back to prehistoric times and its believed that fermented milks originated in the Near-East, perhaps before Phoenician era, and spread through central and Eastern Europe (Lefoka, 2009) . Though the composition and microbiology of fermented milks were not known, they were more preferred comparing to raw milk because of their beneficial effects over fresh milk. With these advantages, fermented milk preserved the high quality nutrients present in milks in a relatively stable form (Panesar, 2011). In addition, the fermented milk could be stored at warm temperatures and be safely consumed for several days. Apart from nutritional benefits, fermented milk was also known to contain pharmaceutical benefits. It was prescribed for curing disorders of the stomach, intestines and other illnesses (Gandhi, 2006) . The history of fermented milks is now often described in terms of a sequence of several generations of products (Steinkraus, 2002): the first generation (800 BC until 1900 BC), when the micro flora was not defined and differed from villages to villages. In the early years of milk fermentation, milk was simply allowed to be fermented by its normal micro flora, but the actual process was not

completely understood. Cultures could be maintained by inoculating fresh milk with fermented milk (Kerr & McHale, 2001). This first generation was followed from about 1910 by a second generation of fermented milks in which the micro flora was defined and the fermentation process was controlled (Chandan, & Shah, 2013).

## **2.2 Traditional Fermented Milk**

A wide variety of fermented milk products have been produced in different parts of Africa as a method of milk preservation. The major source of fermented milk is from cow but, but sheep, goat, buffalo, camel and horse milk are also used in some cultures. The fermentation process occurs spontaneously by back-sloping; i.e. inoculation of the raw material with a small quantity of the previously performed successful fermentation. Therefore, back-sloping results in dominance of the best adapted strains (Steinkraus, 2002). It represents a way, be it unconsciously, of using a selected starter culture to shorten the fermentation process and to reduce the risk of fermentation failure. This traditional method of preparing fermented milk is still used in some less sophisticated societies. Modern techniques of milk fermentation, on the other hand, use starter cultures with known characteristics (Chandan, & Shah, 2013). The advantage of modern techniques over the traditional methods is the production of consistent products that are less likely to spoil and are relatively safe.

## **2.3 African Traditional Fermented Milk**

Fermented Milk is a major component of the traditional diet in many regions in Africa. Most of the milk produced is consumed in the house mostly by children and excess is infrequently sold in open markets. Due to limitation of cold storage facilities such as refrigerators in many African countries, milk is stored at ambient temperatures which usually fermented rapidly by the natural flora.

Fermented milk produced in South Africa (*Amasi*) was described by (Beukes *et al.*, 2001). The product is consumed in different regions of Southern African, including Zimbabwe, South Africa and Lesotho. *Amasi* is an unsweetened curd which is traditionally prepared from unpasteurized bovine (cow's) milk which is allowed to ferment spontaneously in a pottery (clay pot) or gourd ("calabash") for two to three days at ambient temperature. Beukes, *et al.* (2001) also reported that clay pots have a better flavor of *Amasi* than calabashes because a calabash needs to be seeded with microbial inoculums before it could be used for the production of fermented milk. The microbial flora responsible for the fermentation is derived from the air, raw milk and walls of the containers. After coagulation, the whey is drained through a plugged hole at the bottom of the container. According to a study which was done by Feresu and Muzondo (1990), the predominant LAB isolated from *Amasi* belongs to *Lactobacillus helveticus*, *Lb. plantarum*, *Lb. delbrueckii* subsp. *lactis*, *Lb. paracasei* subsp. *paracasei* and *Lb. paracasei* subsp. *pseudo-plantarum*.

*Amasi* is reported to have consistency slightly thicker than yoghurt and with a pH between 3.6 and 4.2 (Todorov, *et al.*, 2007). Although normally consumed with thick corn-meal porridge, *Amasi* is also consumed between meals with ground sorghum which is similar to muesli.

## 2.4 Fermentation Process

The term "fermentation" comes from Latin word *fermentum* (to ferment) (Gogineni *et al.*, 2013). The historical definition describes fermentation as the process in which chemical changes in an organic substrate occur as the result of action of microbial enzymes (Bamforth, 2005). The fermentation processes can be classified as spontaneous and induced (Bamforth, 2005). A wide variety of microbes can be responsible for the fermentation process of milk including lactic acid bacteria (LAB), acetic acid bacteria (AAB), yeasts and mycelia fungi (Ross *et al.*, 2002). The

fermentation process of traditional fermented milk depends on the end products of lactic acid bacteria formed during the fermentation of lactose. LAB may be classified as *homofermentative* or *heterofermentative* based on their by-products of sugar (e.g., lactose) fermentation (Rattanachaikunsopon & Phumkhachorn, 2010).

***Homo-fermentative LAB***; Use the Embden-Meyerhof-Parnas's pathway to generate two moles of lactate per mole of glucose and derive approximately twice as energy per mole of glucose as hetero fermentative lactic acid bacteria (Bassyouni *et al.*, 2012). They include *Lactococcus* spp. that is used in dairy starter culture applications, while, other homo-fermentative LAB include yoghurt strains consisting of rods (*Lactobacillus delbruckii* subspecies *bulgaricus*, *Lactobacillus* (*Lb.*) *acidophilus*) and cocci (*Streptococcus salivarius* subsp. *thermophilus*) and thermophilic strains that might be used in cheese (e.g., *Lb. helveticus*) (Gandhi, 2006).

***Hetero-fermentative LAB***; produce equal molar amounts of lactate, carbon dioxide and ethanol from lactose via the hexose monophosphate or pentose pathway (figure 1.1). These type of LAB are rarely used as dairy starter cultures, Although , they are not commonly used in milk and dairy products , if they are allowed to grow significantly in numbers, they can cause defects related to their acid and CO<sub>2</sub> production, such as slits in hard cheeses or bloated packaging in other dairy products. They include *Leuconostoc* spp. (Gram-positive cocci) and Gram-positive rods such as *Lactobacillus brevis*, *Lb. fermentum* and *Lb. reuteri*.

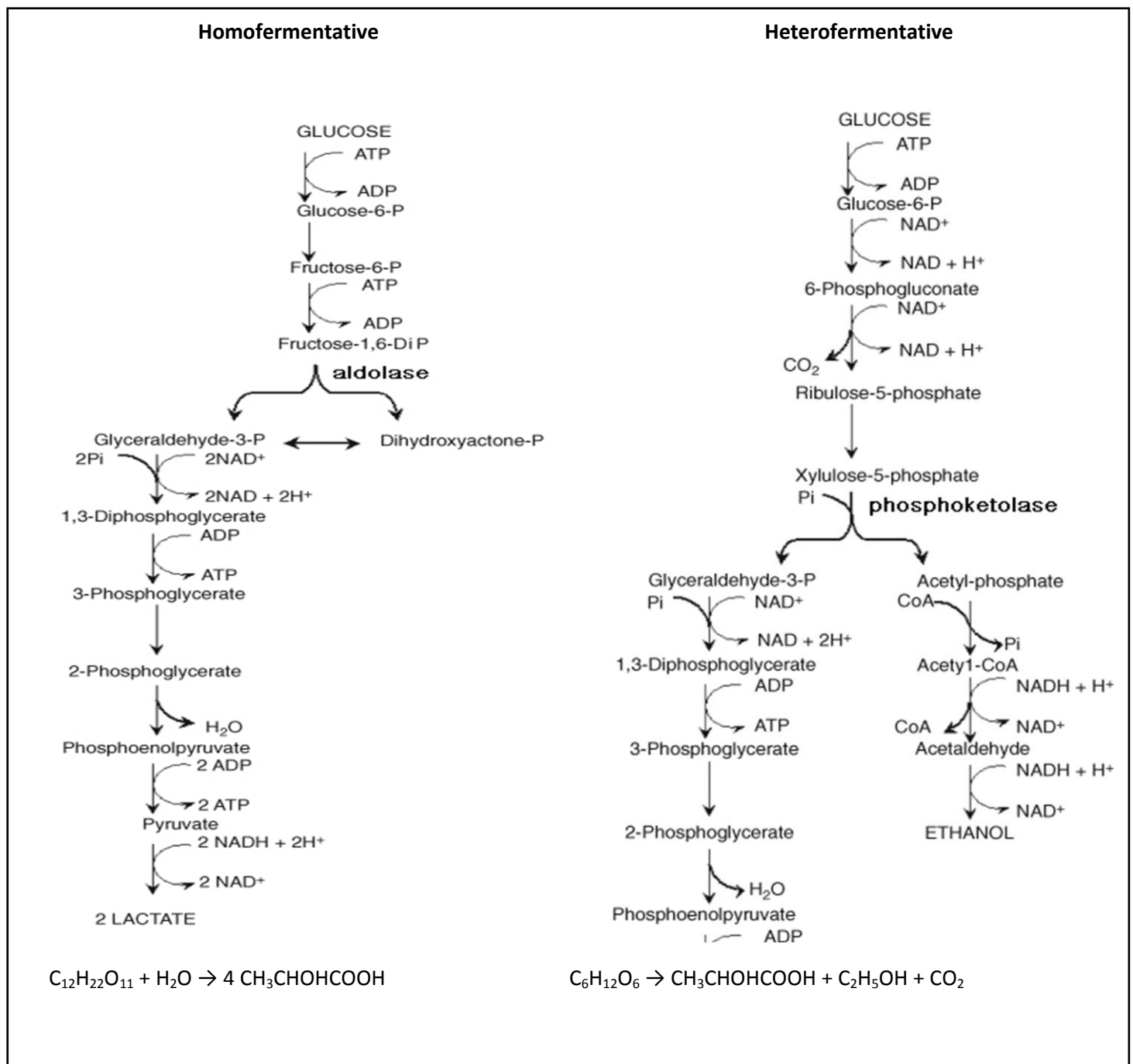


Figure 1.1 Glucose utilization metabolic pathways of LAB (Khalid, 2011)

## 2.5 Lactic Acid Bacteria (LAB)

Lactic acid bacteria (LAB) are a group of gram-positive, non-spore forming, cocci or rods, which produce lactic acid as the major end product during the fermentation of carbohydrates. The group consists of several genera, which include *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* ((Amenu, 2013; Jin *et al.*, 2009; Khalid, 2011). LAB in general are nonpathogenic to man and animals as they are best known for their use as starter cultures in the manufacture of dairy products such as acidophilus milk, yoghurt, buttermilk, cottage cheese, hard cheeses.

### 2.5.1. Classification

The classification of lactic acid bacteria into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures, configuration of the lactic acid produced and ability to grow at high salt concentration (Rattanachaikunsopon & Phumkhachorn, 2010). These genera are grouped together based on similarities in physiology, metabolism and nutritional needs. A primary similarity is that all members produce lactic acid as a major or almost only end product of the fermentation of sugars. It was found that species that belong to *Streptococcus* and *Leuconostoc* produce the least amount of acid; while, the homo-fermentative species of *Lactobacillus* produce the greatest amount of acid (Christiansen, *et al.*, 2005). Hetero-fermentative *Leuconostoc* and *Lactobacillus* species convert glucose to about 50% lactic acid, 25% acetic acid and ethyl alcohol and 25% carbon dioxide (FAO, 1990). This leads to flavor development in fermented milk. Hence, new tools for classification and identification of lactic acid bacteria are in progress (Christiansen, *et al.*, 2005). The most promising for routine methods used are nucleic acid probing techniques, partial rRNA gene sequencing using the polymerase chain reaction and soluble protein patterns.

### **2.5.2. LAB as a Preservative Agent**

Lactic acid bacteria (LAB) are widely known carry out safe metabolic activities (fermentation) in food and are mostly used for food preservation and flavor development (Khalid, 2011). In starter cultures they produce lactic acid during the fermentation process which can prevent the growth of undesired microorganisms. The active metabolites which are produced by LAB are more of interest as nowadays consumers favor food with few chemical preservatives (Amenu, 2013). As a result, there is increased interest in the preservation through LAB because of their safe association with fermented foods. Some of the metabolites produced by LAB are known to have antimicrobial effects, including organic acids, fatty acids, hydrogen peroxide and diacetyl. But more interest is on the LAB which is able to produce specific proteinaceous substances, bacteriocins that inhibit the growth of pathogens, such as *Listeria*, *Clostridium*, *Staphylococcus*, *Bacillus spp.* and *Enterococcus spp.*, therefore enhancing the shelf life of the food (Soomro *et al.*, 2002).

### **2.5.3. Lactic Acid Bacteria as a Probiotics**

Probiotics are defined as “living micro-organisms, which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition (Guarner, & Schaafsma 1988). Beneficial effects related to probiotics include antagonistic effects, competition, enhancement of digestion, strengthening of the immune system, improved level of immunoglobulin A (IgA) and stimulation of vitamin production (Chelule, *et al.*, 2010). Lactic acid bacteria have contributed in increased volume of fermented foods worldwide especially in foods containing health promoting bacteria (Soomro *et al.*, 2002). Food fermentation therefore, is of great economic values and has been accepted to contribute in improving human health.

#### **2.5.4. Lactic Acid Bacteria in Milk Fermentation (Starter Cultures)**

Dairy starter cultures are microorganisms that are purposely added to milk in order to create a desired outcome in the final product. The most common use of starter cultures is for the production of lactic acid from lactose (milk sugar), which in most cases causes or assists in the coagulation of milk protein by lowering its pH value (Hati, *et al.*, 2013). The acid is responsible for development of characteristic body and texture of the fermented milk products, which has an overall effect on flavor and preservation (Hati, *et al.*, 2013). In addition, starter cultures can contribute to aroma and gas production through proteolytic and lipolytic activities and inhibition of undesirable organisms (Adams and Nicolas, 1997). The starter culture may be a pure culture or mixture of known organisms. Each product requires its own special starter and these contain different mixtures of organisms. The natural micro-flora of the milk may be incompetent, uncontrollable and unpredictable or is destroyed altogether by the heat treatments given to the milk. A starter culture can provide particular characteristics in a more controlled and predictable fermentation.

#### **2.5.5. Types of Starter Cultures Used in Dairy Industry**

The lactic acid bacteria used in the dairy fermentation as starter cultures are divided into two groups depending on their optimum growth temperatures. Mesophilic lactic acid bacteria have an optimum growth temperature between 20°C and 30°C. Mesophilic cultures are mostly found in the traditional fermented milk products that originated from Western and Northern European countries (Wouters *et al.*, 2002). On the other hand, thermophilic starter cultures have optimum between 30°C and 45°C, and are mostly found in traditional fermented products from sub-tropical countries.

Several varieties of fermented milks which are traditionally fermented in Africa originate from sub-Saharan countries such as *ergo* in Ethiopia and spread throughout Africa (Amenu, 2013). These products have emerged from spontaneous acidification of raw milk by indigenous organisms. Very often, fermentation occurs as a result of mixed culture originating from the native microflora of the raw materials. Although these organisms have by no means been characterized in detail, they consist largely of thermophilic lactic acid bacteria, probably due to the relatively high incubation temperature determined by the prevailing climate. The first description of milk fermentation by these bacteria can be found in the literature of a few centuries ago (Wauters *et al.*, 2002). This spontaneous fermentation of milk into yoghurt have now been consistently developed in two most frequently used starter bacteria are now classified as *Lactobacillus delbrueckii* subsp. *Bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*, generally shortened as *Lb. bulgaricus* and *S. thermophilus*, respectively, (Amenu, 2013). However, in an industrial scale a particular defined starter culture, which has been developed under controlled conditions, is of first preference so that the qualities of the finished product could be consistently maintained day after day. Therefore, several attempts were made at that time to identify the bacteria dominating the flora in yoghurt like products and were given names such as *Bacillus bulgaricus* and *Diplo-streptococcus*. Moreover, modern methods of gene-technology made it possible for the microbiologists to design and develop starter cultures with specific qualities.

## 2.6 Microbial Compositions of Fermented Milks

The earliest information concerning the microbiological composition of fermented milk products was given at the end of the 19th century. The presence of a diverse range of microorganisms was reported in the early investigations. Metchnikoff, (1907) (as cited by Amenu, (2013) pointed out the presence of *Bacillus bulgaricus*, cocci and yeasts in yoghurt. Grigoroff isolated rod-shaped bacteria called *Bacillus A* from Bulgarian milk in 1905. The presence of lactobacilli, such as *Lactobacillus longus*, *Bacillus lebensis*, exhibiting and not exhibiting granules, *Streptobacillus*, yoghurt *bacillus*, was described by several other investigators. With all the developments that have taken place over the years in food microbiology and the present level of knowledge, it is evident that the micro-flora of fermented milks consists of different strains of lactic acid bacteria belonging to *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Bifidobacterium* species and of minor proportions of yeasts and milk moulds in associated growth. In Africa, the microbial compositions of traditional fermented milk in some countries are really being explored. For instance the microbial composition of *Ergo*, Ethiopian fermented milk was studied by Gonfa, *et al.*, (2001) who reported that *Lactococcus garvieae* and *Lactococcus lactis* sub sp. *lactis* are the main microorganisms involved in the culturing of Ergo.

## 2.7. Antimicrobial Substances Produced by LAB

The Food and Drugs Administration (FDA, 2000) defines antimicrobial agents as “substances used to preserve food by preventing growth of microorganisms and subsequent spoilage, including fungi, molds and rope inhibitors. The traditional function of food antimicrobials is to prolong shelf life and preserve quality through inhibition of spoilage microorganisms. However, antimicrobials have been used gradually more as a primary intrusion for inhibition or

inactivation of pathogenic microorganisms in foods (Davidson & Zivanovic, 2003). Antimicrobials may be classified as traditional or naturally occurring (Davidson & Zivanovic, 2003). The naturally occurring antimicrobials include compounds produced by microorganisms, in milk e.g. LAB on plants, and on animal surfaces. To date they are mostly only proposed for use in foods. Antimicrobials used exclusively to control specific pathogens include nitrite which is used to inhibit the growth of *Clostridium botulinum* in cured meats. In addition, selected organic acid are sprayed on beef carcass surfaces in order to reduce pathogens. Nisin and lysozyme can be used to inhibit growth of *C. botulinum* in pasteurized processed cheese. Lactate and di-acetate are used to inactivate *Listeria monocytogenes* in processed meats (FDA, 2000).

The primary antimicrobial effect exerted by LAB is the production of lactic acid and reduction of pH (Ammor, et al., 2006). In addition, lactic acid bacteria have an important role in the inhibition of food-borne pathogenic and spoilage microorganisms through production of antimicrobial metabolites, classified as low-molecular-mass (LMM) compounds including hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), carbon dioxide (CO<sub>2</sub>) and diacetyl (2,3-butanedione). They also produce, uncharacterized compounds and high-molecular-mass (HMM) compounds like bacteriocins (Mbawala, et al., 2013) all of which can inhibit the growth of some spoilage and pathogenic bacteria in foods.

### **2.7.1. Organic Acids**

A number of organic acids such as; lactic, acetic, citric, orotic, sialic, benzoic, sorbic etc. are known to occur in milk products as a result of hydrolysis of lactose and butterfat (fatty acids), biochemical metabolic processes, or bacterial metabolism (Bensmira & Jiang, 2011). The antagonistic actions of organic acids are believed to be due to the interference with the

maintenance of cell membrane . According to, Urbienė & Leskauskaitė, (2006) found citric acid as the major organic acid in raw milk which disappeared during storage as a result of bacteria actions. However, organic acids such as lactic acid, acetic acid, and formic acid, are major metabolites in fermented milk of which lactic and acetic acid are products of lactose degradation (Bensmira & Jiang, 2011). These acids have been demonstrated to be one of the inhibitory factors. Other acids, such as benzoic and sorbic acids, are present in milk in smaller quantity, though they are important due to their preservative properties.

Urbienė & Leskauskaitė, (2006) reported that, during fermentation of milk, the concentration of some organic acids (lactic, propionic, acetic) increases, while the concentration of the other organic acids (hippuric, orotic, citric) decreases. At low pH, a large amount of lactic acid is in the un-dissociated form, and it is toxic to many bacteria, fungi and Yeasts (Zewge, 2006). However, different microorganisms vary considerably in their sensitivity to lactic acid. At pH 5.0 lactic acid is found to be inhibitory toward spore-forming bacteria but was ineffective against yeasts and moulds.

Acetic and propionic acids are produced by LAB strains through hetero-fermentative pathway and they do interact with cell membranes and cause intracellular acidification and protein denaturation (Rattanachaikunsopon, & Phumkhachorn, 2010). They are more antimicrobial effective than lactic acid due to their higher pKa values (lactic acid 3.08, acetic acid 4.75, and propionic acid 4.87), and higher percent of un-dissociated acids than lactic acid at a given pH (Zewge, 2006). Organic acids have a broad mode of action and inhibit both Gram-negative and Gram-positive bacteria as well as yeasts and moulds (Rattanachaikunsopon, & Phumkhachorn, 2010). The levels and types of organic acids produced during the fermentation process depend on the species of organisms, culture composition and growth conditions.

### 2.7.2. Hydrogen Peroxide

Hydrogen peroxide is produced by LAB in the presence of oxygen as a result of the action of flavoprotein oxidases or nicotinamide adenine dinucleotide reduced (NADH) peroxidase (Enitan, *et al.*, 2011). It may also be as a precursor for the production of bactericidal free radicals such as superoxide ( $O_2^{\cdot -}$ ) and hydroxyl ( $OH^{\cdot}$ ) radicals which can damage DNA (Dike & Sanni, 2010). The antimicrobial effect of  $H_2O_2$  may result from the oxidation of sulfhydryl groups causing denaturing of a number of enzymes, and from the peroxidation of membrane lipids thus the increasing membrane permeability (Figueroa-González *et al.*, 2011).

### 2.7.3. Bacteriocins

Bacteriocins are ribosomal synthesized proteins complexes or peptides with antibacterial activity produced by bacteria which are usually active against genetically closely related species (Xie, *et al.*, 2009). They can inhibit or kill microorganisms that are usually, but not always, closely related to the producer strain (Cladera-Olivera, *et al.*, 2004). Among the Gram positive (+) bacteria, lactic acid bacteria (LAB) have gained particular attention nowadays, due to the production of bacteriocins (Savadogo, 2004). Bacteriocins producing strains of LAB may be very vital in competing with other organisms in the intestine. They consist of a biologically active protein moiety, have a bactericidal mode of action and attach to specific cell receptor (Zacharof & Lovitt, 2012). Thus, these substances can be applied in the food industry as natural preservatives. The use of LAB and of their metabolic products is generally considered as safe (GRAS, Grade One) (Cladera-Olivera, *et al.*, 2004). Therefore, bacteriocins produced by LAB are a subject of intense research due to their antibacterial activity against food borne bacteria. In addition, the application of the produced antimicrobial compounds as a natural barrier against

pathogens and food spoilage caused by bacterial agents has been proven to be efficient (Xie, *et al.*, 2009).

Nisin produced by *Lactobacillus* ALTA 2341 and by *Pediococcus acidilactici lactis subsp. lactis* strains, belongs to the class 1 antibiotics and is the classic example. It prevents *Clostridial* spoilage of processed and natural cheeses, inhibits the growth of some psychrotrophic bacteria in cottage cheeses, extends the shelf life of milk in warm countries, prevents the growth of spoilage *lactobacilli* in beer and wine fermentations and provides additional protection against *Bacillus* and *Clostridial* spores in canned foods. (Soomro *et al.*, 2002).

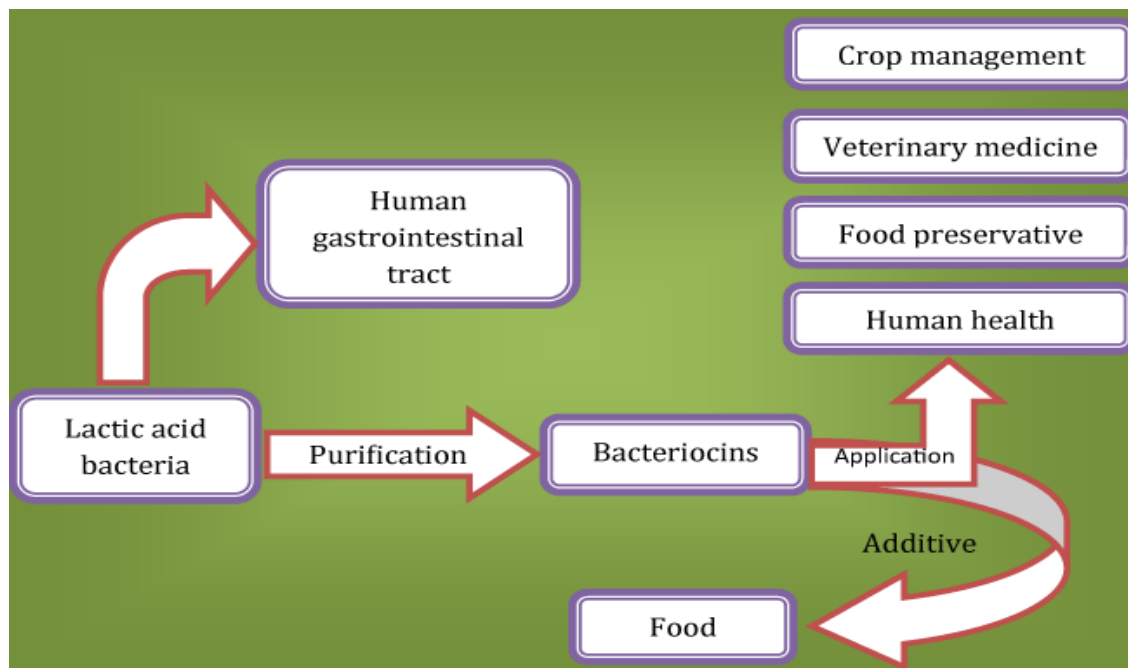


Figure 2.2 Overview of the applications of bacteriocins produced by LAB (Nishant *et al.*, 2011)

#### 2.7.4. Aroma Compounds (Diacetyl)

Hetero-fermentative LAB produces active acetaldehyde by decarboxylation of pyruvate. This product condenses with pyruvate, forming  $\alpha$ -acetolactate which is then converted by  $\alpha$ -acetolactate synthetase to diacetyl. Therefore, Diacetyl (2, 3-butanedione:  $C_4 H_6 O_2$ ) (Fig 2.3) is an acetoinic molecule produced by some strains within all genera of LAB such as *Streptococcus*, *Leuconostoc*, *Lactobacillus*, and *Pediococcus* through citrate fermentation. Diacetyl (2, 3-butanedione) is best known for the buttery aroma that it imparts to fermented dairy products. The antimicrobial effect of diacetyl has been known since the 1930s (Jay, 1982). It inhibits the growth of Gram-negative bacteria by reacting with the arginine-binding protein, thus affecting the arginine utilization (Jay, 1986). In addition, diacetyl may act synergistically with other antimicrobial factors and contributes to combined preservation systems in fermented foods.

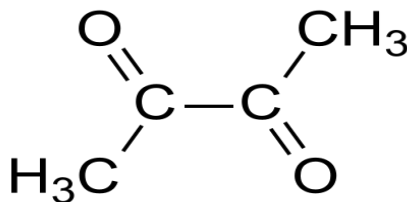


Figure 2.3. The chemical formula of diacetyl

## CHAPTER 3: MATERIALS AND METHODS

### 3.1. The study areas

The area of study covered six regions of the northern eastern part of Namibia: Oshana, Ohangwena, Oshikoto, Omusati and in Kavango and Zambezi. These six regions are the most common regions which are well known for the communal farming activities, especially milking of cow to make sour milk commonly known as *Omashikwa*; *Mashini Ghakushikwa* in Kavango and *Mabisi* in Caprivi. The aim of the study was to establish whether there is a significance difference between the three types of milk exist, in terms of house hold processing methods (Heita & Cheikhyoussef, 2012).

**Ohangwena region:** The Region depends on rain fed agriculture, and it comprised of ten constituencies: Ongenga, Engela, Oshikango, Ondobe, Eenhana, Omundaungilo, Okongo, Ohangwena, Endola and Epembe. Analysis and samples were collected in Ondobe constituency. ([www.parliament.gov.na/constituencies](http://www.parliament.gov.na/constituencies)).

**Omusati region:** There are 12 Constituencies in Omusati Region namely Anamulenge, Elim, Etayi, Ogongo, Okahao, Okalongo, Onesi, Oshikuku, Otamanzi, Outapi, Ruacana and Tsandi ([www.omusatirc.gov.na](http://www.omusatirc.gov.na)). This region has the majority of the dialects among the Oshiwambo people. There are; Aakwambi, Ngandjela, Kwaluudhi, Aambadja, and Aakolonghadhi

**Oshana region:** The name Oshana lends itself well to this region as it describes the most prominent landscape feature in the area namely the shallow, seasonally inundated depressions which highlight the local agro ecological system. The region comprises of three dialect

speaking groups and ten constituencies: Okaku, Okatana, Okatyali, Ompundja, Ondangwa, Ongwediva, Oshakati East, Oshakati West, Uukwiya and Uuvudhiya

**Oshikoto region:** Is located in the north-central part of Namibia. The region is divided into ten constituencies; Eengodi, Guinas, Okankolo, Olukonda, Omuntele, Omuthiyagwiipundi, Onayena, Oniipa, Onyaanya, and Tsumeb

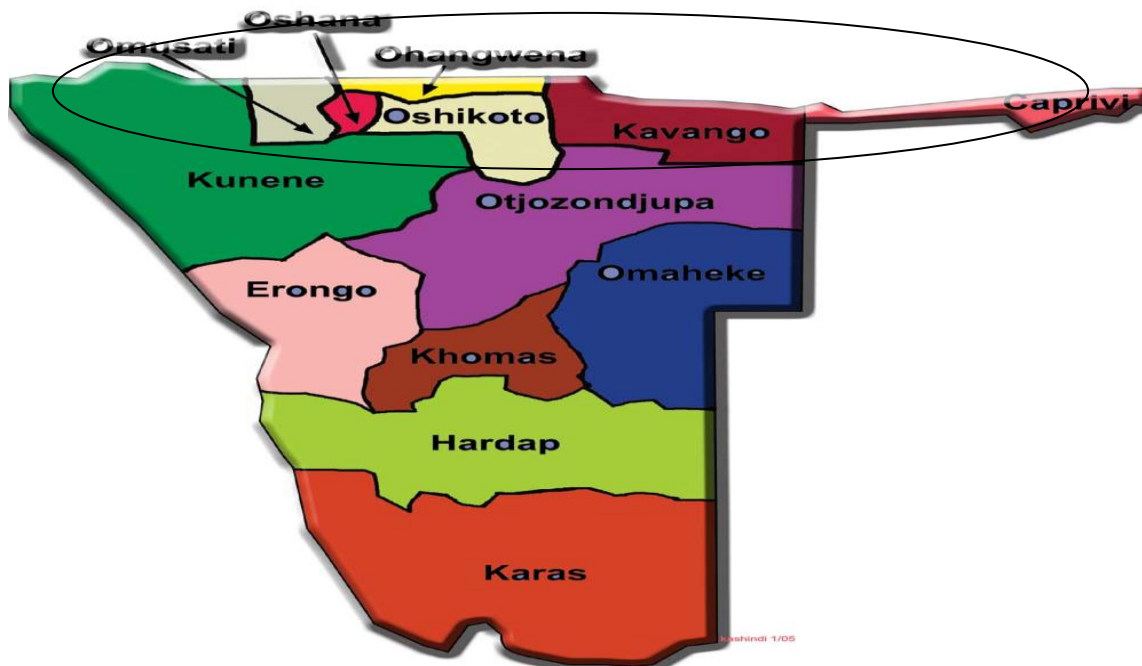


Figure 3.1 The Map of Namibia Showing Study Areas ([www.namibsafari.com](http://www.namibsafari.com))

### 3.2. Data Collection

Preliminary survey was conducted in Ohangwena, Oshana, Omusati and Oshikoto, in addition to Zambezi and Kavango regions to assess production, handling, processing, preservation and consumption of milk and milk products. Interviews were conducted in the local languages. Only a few were done in English, when someone could both understand and speak English. Five

households were randomly selected in each region, who owned milking cows. Thirty individuals were in total interviewed using a semi-structured questionnaire (Heita & Cheikhoussef, 2012).

### **3.3. Samples Collection**

Traditional fermented milk Samples (*Omashikwa*, *Mabisi* and *Mashini Ghamushika* ) were collected in 250 ml sterilized, screw capped bottles. In total 20 samples were collected and analyzed, 13 samples of *Omashikwa* were collected from the four northern regions (Oshana, Ohangwena, Omusati and Oshikoto) while four samples of *Mabisi* were collected from Zambezi region and three samples of *Mashini ghamushika* from Kavango region. All samples were kept on ice and brought to the Biotechnology Laboratory at Department of Chemistry and Biochemistry; at the University of Namibia for analysis. Samples were then kept in a refrigerator at 4 °C until analysis.

### **3.4. Physicochemical Analysis**

#### **3.4.1 pH**

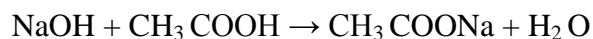
Potentiometric measurements of samples were done in the field upon collection and also after 7 days from the day of collection using a pin electrode of a pH meter (Eutech instrument, ECPH602K, Malaysia) (Omafuvbe & Enyioha, 2011). The readings were done in triplicates and the means were calculated.

#### **3.4.3. Titratable Acidity**

The total acidity of fermented milk was measured as lactic and acetic acid respectively.

**a). Acetic acid** in fermented milk was measured by pipetting 10.00 ml of fermented milk sample, into a 100 ml conical flask 90 ml of sterile distilled water was added to make up the volume to 100 ml and gently mixed. Ten (10) ml of the mixture was then transferred to a conical

flask using a pipette, 3 drops of Phenolphthalein was added and titrated to a faint pink color with 0.1 M NaOH solution. The reaction is given by the equation below (James, 1995).

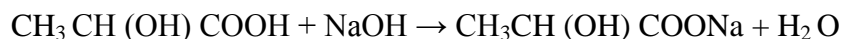


Percentage acetic acid is given by:

$$\% \text{ Acetic acid (M/V)} = T \times 0.6$$

T = Mean titre (in ml) of 0.1 M NaOH solution required to neutralize acidity in 10 ml of diluted fermented milk.

**b). Lactic acid** was measured by titrating 10 ml fermented milk sample in conical flask, to which 1 ml of 0.5 % Phenolphthalein solution was added. The mixture was then titrated with M/9 NaOH solution until faint pink colour. The reaction is illustrated at the equation below (Akabanda et al., 2010).



The percentage of lactic acid was calculated by:

$$\text{Titrate acidity (as \% lactic acid)} = (\text{ml M/9 NaOH used} / 10).$$

### 3.5. Microbiological Analysis

#### 3.5.1 Total Plate Count

Ten (10) milliliters of each sample were aseptically added to 90 ml of sterile buffered peptone water and mixed thoroughly. Serial dilutions ( $10^{-1}$  to  $10^{-8}$ ) were performed and 0.1 ml aliquots of the appropriate dilutions were directly spread plated with a sterile glass rod on 90mm Petri

dishes with Plate count agar (PCA) (Merck) and incubated aerobically at 37 °C for 24 hours for the enumeration of total aerobic mesophilic bacteria.

### **3.5.2 Isolation of LAB**

Isolation was done right after the field work within seven days of arrival of the sample in the laboratory. Firstly, 1ml of sample was mixed with 9 ml of buffered Peptone water (Bio-lab Merck) and serial dilutions were made up to  $10^{-5}$ . Thereafter, enumeration of lactic acid bacteria was carried out whereby 0.1 ml of sample aliquot was spread plated using a sterile glass rod on different selective agar MRS ( DE Mann Rogosa &Sharp ), Rogosa (Bio-lab Merck) and M17 agar (Bio-lab Merck) in triplicates. Incubation was done aerobically at 37 °C for 24 hours. The pH of media were adjusted by using 0.1N NaOH and 0.1N HCl to make an appropriate pH which is required.

Three colonies were then randomly selected from countable different selective media (MRS, Rogosa and M17) agar plate replica. The colonies were purified by successive streaking on appropriate agar media (MRS, Rogosa, M17) before being subjected to characterization. In total 9 colonies from each sample were selected 180 colonies in total which was characterized further. The isolates were transferred to MRS broth, incubated for 24 and some were preserved in 25 % of glycerol until further analysis (Li, *et al.*, 2012).

### **3.5.3 Phenotypic Characterization of LAB**

The isolates were grouped as lactic acid bacteria after examining for their Gram reaction, Cell and Colony morphology and Catalase reaction (Li, *et al.*, 2012). Isolates which were characterized as Lactic acid bacteria were kept as a stock culture in the refrigerator at -20 °C and in a glycerol solution. The isolates were examined for cellular arrangement and morphology by

using a common procedure which differentiates two large groups of bacteria based on their different cell wall constituents. The Gram stain procedure distinguishes between Gram positive and Gram negative groups by coloring these cells red or violet. Gram staining procedure is based on the ability of microorganisms to retain color of the stains used during the gram stain reaction. Gram-negative bacteria are decolorized by the alcohol, losing the color of the primary stain, purple. Gram-positive bacteria are not decolorized by alcohol and will remain as purple. After decolonization step, a counter stain is used to impart a pink color to the decolorized gram-negative organisms. Gram stain was carried out by transferring a loop full of freshly prepared bacteria colony to the surface of a clean glass slide with a drop of sterile distilled water, and spread over a small area. The slide was passed over the flame three times to fix the smear on the slide. The Gram's staining was then completed by:

1. Addition of the primary stain (crystal violet) to the sample/slide for 1 minute. The slides were rinsed with gentle stream of water for a maximum of 5 seconds to remove unbound crystal violet.
2. Then Gram's iodine was added for 1 minute- this is a mordant, or an agent that fixes the crystal violet to the bacterial cell wall.
3. The slide was rinsed with acetone or alcohol for 3 seconds and rinse with a gentle stream of water. The alcohol will decolorize the sample if it is Gram negative, removing the crystal violet.
4. The secondary stain, safranin was added to the slide for 1 minute. The slides were washed with a gentle stream of water for a maximum of 5 seconds.

5. Lastly, The slide were then air dried and observed under light microscope (Olympus BX51, Japan) using oil immersion, observed cell shapes and cell arrangements were recorded.

Some bacteria produce the enzyme catalase which facilitates cellular detoxification. Catalase neutralizes the bactericidal effects of hydrogen peroxide (Wheelis, 2008) and its concentration in bacteria has been correlated with pathogenicity. The catalase test facilitates the detection of the enzyme catalase in bacteria. It is essential for differentiating catalase-positive from catalase-negative. The catalase test is also valuable in differentiating aerobic and obligate anaerobic bacteria, as anaerobes are generally known to lack the enzyme. It was done according to method described by MacFaddin (2000); A drop of 3% solution of hydrogen peroxide ( $H_2O_2$ ) was placed on a clean microscope slide, and 24 hour old culture colony from the MRS, Rogosa and M17 plates were picked using sterile wire loop and the evolution of gas bubbles was indicative of positive test.

### **3.6. Antimicrobial Assay of LAB**

#### **3.6. 1. Microorganisms and Culture Conditions**

*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 14053, *Bacillus cereus* ATCC 10876 *Geotrichum klebahnii* (IKST F. Lab. isolate) were used as test microorganisms or indicator strains. The indicator strains used for determination of inhibitory activity spectra of studied isolates were purchased from the American Type Culture Collection (ATCC) Stock cultures. Bacteria were kept in a refrigerator (4 °C) on nutrient agar slants. *Geotrichum klebahnii* was isolated from Namibian fermented food and beverages samples collected from Karas region in the south of Namibia (Cheikhoussef, 2011).

### 3.6. 2. Agar Well Diffusion Assay

Cell-free culture supernatants for antibacterial assay was prepared by growing the LAB isolates in MRS broth at 37 °C and centrifuged at 13, 000 x *g* for 10 min at 4° C then pH was adjusted to 7 by 1M NaOH to exclude antimicrobial effect of organic acid (Savadogo *et al.*, 2004). The antimicrobial activity of the cell-free culture supernatants of isolated LAB against the indicator organisms was determined by the agar well diffusion assay according to the method described by Lavanya, *et al.*, (2011). Aliquots of supernatants (100 µL) were placed in wells (6 mm diameter) cut in cooled soft nutrient agar plates using a cork bore, previously seeded with 100 µl of the appropriate indicator strains. The plates were first stored in the refrigerator for the LAB supernatant to set then incubated under optimal conditions for growth of the target microorganisms after which they were examined for clear zones around the wells. The diameters of the growth inhibition zones were measured and recorded in millimeter (mm).

### 3.6.3 Hydrogen Peroxide Production

All isolated LAB cultures were inoculated into MRS broth (pH 7.0) and incubated for 24h at 30 °C; the cultures were centrifuged at 3,000 rpm for 20 min at 4 °C. 5 mg/ml Proteinase K was added to cell free extract solution and pH was adjusted to 7.0 by means of 0.1N NaOH to exclude the antimicrobial effect of bacteriocins and organic acid respectively, followed by filtration of the supernatant through a 0.2 µm pore size filter. 25 ml of supernatant of broth cultures of the test organisms was measured into a 100 ml flask. To this was added 25 ml of dilute H<sub>2</sub>SO<sub>4</sub>. This was then titrated with, 0.1 N potassium permanganate (KMnO<sub>4</sub>). Each milliliter of 0.1 N KMnO<sub>4</sub> is equivalent to 1.701mg of H<sub>2</sub>O<sub>2</sub>. A decolorization of the sample was regarded as the end point. The volume of H<sub>2</sub>O<sub>2</sub> produced was then calculated (AOAC, 1990). All

experiments were performed in duplicate and the concentration of H<sub>2</sub>O<sub>2</sub> produced by isolates was calculated thus:

$$\text{H}_2\text{O}_2 \text{ concentration} = \frac{\text{Ml KMnO}_4 \times \text{N KMnO}_4 \cdot \text{M.E} \times 100}{\text{Ml KMnO}_4 \times \text{volume of sample}}$$

Ml KMnO<sub>4</sub> = volume of KMnO<sub>4</sub> used; N KMnO<sub>4</sub> = concentration of KMnO<sub>4</sub> used,

M.E = equivalence factor: ml H<sub>2</sub>SO<sub>4</sub> = volume of H<sub>2</sub>SO<sub>4</sub> added to the sample.

### 3.6.3. Analysis of Volatile Compounds (Diacetyl)

Fermented milk samples were prepared according to the method described by Alonso & Fraga (2001) with few modifications. A total of 10 ml of fermented milk samples were concentrated using a rotary vapor at 40 °C water bath, filtered using a 0.2 µm filter paper. One ml of Di-ethyl ether was added as an internal standard solution to one ml of milk sample filtrate. The volatile compounds were then analyzed using a GC focus coupled to ITQ700 Ion Trap Mass Spectrometers (Thermo Scientific). A total of 1 µl of the extract was injected into the GC, equipped with a capillary column (30 m x 250 µm i.d. x 0.25 µm film thickness). Carrier gas was Helium (He) (1ml/min) and the chromatographic conditions were as follows: initial oven temperature was maintained at 40 °C for 10 min, and subsequently programmed from 40 °C to 120 °C at a rate of 3°C/min and at a rate of 10°C/min from 120°C to 250 °C where it was held for another 5min. Injector T<sup>a</sup>: 250 °C ; Mass range: 30-350 amu ; Solvent Delay: 4 min. ; Electron impact at 70 eV. Identification of the peaks was based on comparison of their mass spectra with the spectra of the library and in addition, in some cases, by comparison of their retention times with those of standard compounds.

### 3.6.5. Analysis of Organic Acids

The fermented milk samples were prepared according to the method described by Friedrich, (2001) whereby few drops of a sample was placed on the lower prism of a refracto-meter and measured and recorded the °Brix (which should be in the range of 11 to 12.5). If the brix was low then 11 then, 10 ml of milk samples were concentrated using a rotary vapor (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) until the brix is in range. The samples were then filtered through 0.45µm syringe filters into vials capped. Four standard organic acids were prepared in 100 ppm, Lactic acid, Acetic acid, Propionic acid and Butyric acid.

Analyses of organic acids were then measured using a HPLC (PerkinElmer Flexar, USA) with a 210nm UV detector, using a C18 5µm 150×4.6mm column. Using (0.05M KH<sub>2</sub>PO<sub>4</sub>) as a mobile phase. Concentrations of organic acids were then calculated using the formula below:

#### *Organic acid concentrations*

##### **1. Response factor (RF) for each acid in the standard**

$$RF = C/A$$

Where *C* is the concentration (ppm) of the organic acid in the standard solution and *A* is the peak area generated (AU).

##### **2. Concentration of each acid in the sample**

$$C_s = RF \times A_s$$

Where *C<sub>s</sub>* is the concentration (ppm) of the organic acid in the sample and *A<sub>s</sub>* is the area of the peak (AU) generated in the sample.

### **3.7. Biochemical Identification of LAB**

The carbohydrate fermentation profiles of the selected 20 LAB isolates were investigated using API 50 CH strips and API CHL medium according to manufacturer's instruction (API system, BioMérieux, France). Overnight cultures of the isolates grown in 10ml MRS broth at 30 °C were washed twice with sterile peptone water and the pellets were re suspended in API 50 CHL medium, using sterile pasture pipettes. With subsequent mixing, homogenized suspensions of the cells in the medium were transferred into each of the 50 wells on the API 50 CH strips. Strips were covered as recommended by the manufacturer instruction and incubated at 30 °C. Changes in color were monitored after 24hrs of incubation. The first strip served as a control well and Esculine hydrolysis (well 25) was revealed by change to darker or black colors, others changed to yellow or no change at all. Results were represented by positive sign (+) while a negative sign (-) was designated for no change. The APIWeb™ V1.2.1. Software (BioMérieux, France) was used according manufactures instruction in interpretation of the results.

### **3.8. Genetic Identification of LAB**

#### **3.8.1 Genomic DNA Isolation**

Genomic DNA was prepared by using the Gentra Puregene kit (Qiagen, USA) with some modifications. Ten ml overnight cultures of LAB were prepared in MRS broths then 2ml of culture were transferred into micro centrifuge tubes to harvest cells for 5 min at 6000 rpm. The pellets were washed with distilled water twice to remove any broth remains then, centrifuged for 5 seconds at 13, 0000× g to pellet cells. The supernatant was removed using a pipette to which 300 µl Cell suspension solutions was added then mixed by pipetting up and down. The tubes were then sonicated using Ultra-sound Sonicator at 35 °C for 10 minutes to break the cells. 1.5 µl

of Lytic Enzyme solution was then added and mixed by inverting 25 times. This was then incubated for 30 minutes at 37 °C. The tubes were centrifuged for 1 min at 13,000 ×g to pellet cells, discard the supernatant using a pipette. Cells were then lysed by addition of 300 µl Cell Lysis solution mixed by pipetting it up and down. The tubes were then incubated for 5 minutes at 80 °C in order to lyse some species. 1.5 µl of RNase A solution was then added and mixed by inverting the tubes 25 times and incubated for 60 minutes at 37 °C. The tubes were then quickly cooled on ice for 1 minute. 100 µl of Protein Precipitation solution was then added and vortexed vigorously for 20 seconds at high speed. Incubation of tubes on ice was then done for samples with high polysaccharide content for 60 minutes. The tubes were then centrifuged for 3 minutes at 13,000 ×g; the precipitated proteins formed a tight pellet. 300 µl isopropanol was pipette into clean 1.5 ml micro-centrifuge tubes and to which the supernatant from previous step were poured in carefully without dislodging the pellet. This was then mixed by inverting the tubes 50 times, then centrifuges for 1 min at 13,000 ×g. The supernatant was then discarded carefully and the tube was drained by inverting on a clean tissues paper. 300 µl of cold 70% ethanol was added to wash the DNA pellet by inverting the tube several times. The tube was then centrifuged for 1 minute at 13,000 ×g. The ethanol was then discarded and the tube was drained on a clean piece of absorbent paper and allowed the tube to air dry for 15 minutes. 100 µl of DNA Hydration solution was added then vortex the tubes for 5 seconds. The tubes were then incubated for 1 hour at 65 °C to dissolve the DNA. Finally, they were incubated at room temperature overnight with gentle shaking and then transferred into storage tubes and stored at – 20 °C. Isolated Genomic DNA from these LAB strains were quantified on Nano-Drop spectrophotometer at 230, 260 and 280nm.

### 3.8.2 Amplification of 16S rDNA Region by Polymerase Chain Reaction (PCR)

DNA templates for PCR amplification were prepared according to the method described by Boontawan, (2010) using a PCR system (Eppendorf, Germany). Amplification of 16S rDNA region of isolates and reference strains was carried out by mixing, 5 µl of genomic DNA with 15 µl PCR mixture given in the Appendix (A) using primers listed in table (3.1). The final reaction mixture was taken to the PCR steps in the final volume of 20 µl. The Oligonucleotide primers were manufactured by Inqaba Biotech (South Africa).

**Table 3.1 Primers used for amplification of 16S rDNA of LAB isolates**

Target name	Primer name	Orientation	oligonucleotides	Reference
16S rDNA	27F LAB	Forward	5'- AGAGTTTGATCCTGG CTCAG-3'	Delacroix- Buchet <i>et al.</i> , 2004
16SrDNA	1492R LAB	Reverse	5'- TACGGYTACCTTGTTA CGACTT-3'	
16S rDNA	F-lac <i>Lactobacillus</i> spp.	Forward	5'- GCAGCAGTAGGGAAT CTTCCA-3'	Walter <i>et al.</i> , 2001
16S rDNA	R- <i>Lactobacillus</i> spp.	reverse	5'- GCATTYCACCGCTAC ACATG-3'	
16S rDNA	<i>Pediococcus</i> spp.	Forward	5'- AGAGTRTGATCMTYG CTWAC-3'	Sukumar & Ghosh , 2010
16S rDNA	<i>Pediococcus</i> spp.	reverse	5'CGYTAMCCTWTTAC GRCT-3'	
16S rDNA	<i>Lactococcus</i> ssp.	Forward	5'- AGAGTTTGATCCTGG CTCAGGA-3'	Delacroix- Buchet <i>et al.</i> , 2004
16S rDNA	<i>Lactococcus</i> spp.	Reverse	5'- GGAGGTGATCCAGCC GC-3'	

### **3.8.3 Separation of Amplified Fragments**

After the completion of PCR reaction, amplified products were separated in a 1 % agarose gel. For this purpose, 1 g agarose was dissolved in 100 ml 0.5x TBE buffer by boiling. Agarose solution was cooled to nearly 40 °C. After cooling, 5µl ethidium bromide solution (10mg/ml) was added. The agarose gel was then poured into the gel casting stand and the combs were placed. When the gel was solidified, the combs were removed. For loading, 5 µl of amplification products were mixed with 2 µl of gel loading dye and 10µl of 100bp lambda was used. Electrophoresis was performed at 170v according to the manufactures instructions for 3 hours. Amplification products were then visualized under florescent UV light.

### **3.8.4. Sequencing**

Unpurified PCR amplified products were sent to Inqaba Biotec, Sunnyside, Pretoria, South Africa for sequencing. Amplicons of 16S rDNA were column purified and subsequently sequenced for ~1.5 kb fragment using a set of primers of the sequences which are known and available with Gene Bank at National Center for Biotechnology Information (NCBI). After sequencing the resulted nucleotide sequences were checked for their homologies with the known ones using BLASTn tool at NCBI to identify these strains on molecular level.

### **3.9. Statistical analysis**

The data collected during the survey were analyzed using descriptive statistics and mean comparison procedure of the Statistical Package for Social Science (SPSS V. 12.0) and GENSTAT Discovery Edition 4. All laboratory experiments were repeated three times except the

well diffusion assay which was done twice. The different samples treatments were compared by performing one-way analysis of variance (ANOVA) on the replicates at 95% level of significance using SPSS12.0 statistical program. Significant results refer to  $p < 0.05$ .

## CHAPTER 4: RESULTS

### 4.1 Milk Handling and Storage

The handling and hygienic practices used before milking in the six regions (Oshana, Ohangwena, Omusati, Oshikoto and Zambezi) were analyzed by means of semi structured questionnaires by looking at the types of equipment used for milking, and all the hygiene practices related to milking.

Cleaning of milking utensils and cow udder before milking is not a common practice in all the regions. In all the six regions, 100% of the farmers do not wash the cow udder before milking' 77.42 % of the entire respondents do not wash their milking utensils before milking. Only few (22.58 %) in all the regions wash the milking equipment used (Table 4.1).

Milking is mostly done during the morning hours. First the calf is allowed to suckle to stimulate the milk. Milking is done using different types of equipment such as plastic buckets or traditional buckets made from wood (*Eholo*), but plastic is more commonly used 61.29 % in comparison to *Eholo* (38.71 %) in the six regions. Eighty (80) % of the respondents from Ohangwena region use the traditional bucket for milking, while 66.67 % of respondents from Kavango region use plastic containers.

The milk is stored at room temperatures in either plastic container with lids or in calabashes where it ferments. The type of equipment used for storage of milk differs from houses to houses; all depends on the preferences and the availability of equipment. According to this survey, plastic containers were mostly used in all regions (67.74%) while only 32.26 % still used traditional equipment, out of these 80 % of respondents from Zambezi region used traditional calabash to ferment milk.

The usage of traditional equipment for milking and fermenting milk is low in all the regions. The trend of the type of equipment used for milking and fermenting; weather modern (plastic) or tradition (wooden) is not constant in the regions i.e. 80 % of respondents in Ohangwena region use traditional utensils for milking but only 20% use traditional calabash to ferment the milk.

**Table 4.1. Milk handling and hygiene practice in Ohangwena, Oshana, Omusati, Oshikoto , Kavango and Zambezi regions**

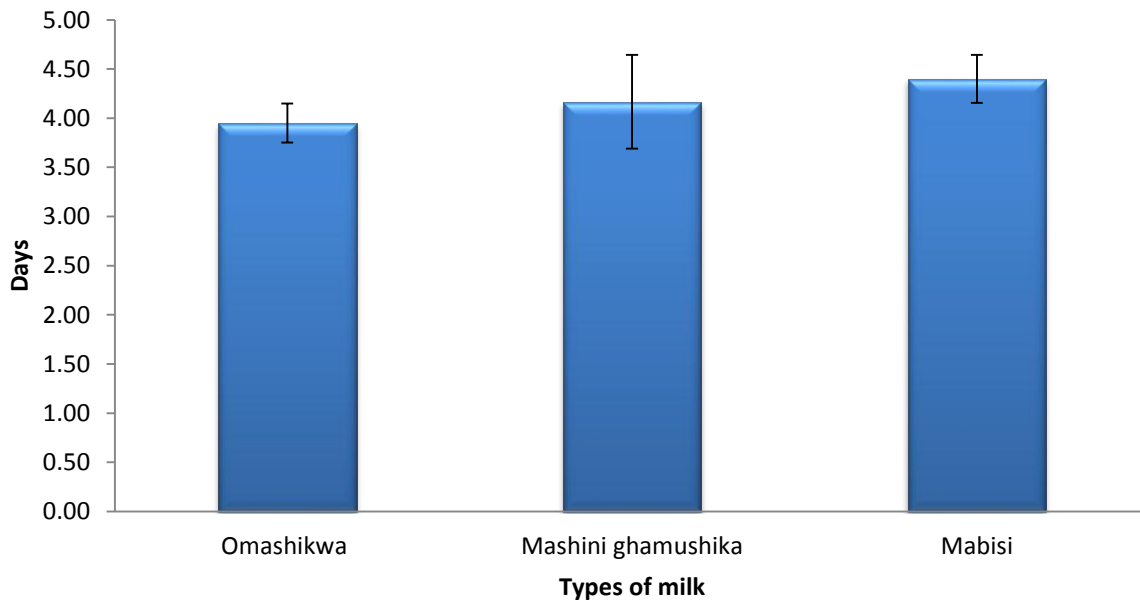
Regions	Washing of milking utensils before milking (%)		Type of equipment used for milking (%)		Type of equipment used for fermenting milk %	
	No	Yes	Plastic	Wood	Calabash	Plastic
Kavango	83.33	16.67	66.67	33.33	16.67	83.33
Ohangwena	80.00	20.00	20.00	80.00	20.00	80.00
Omusati	75.00	25.00	50.00	50.00	25.00	75.00
Oshana	100.00	0.00	66.67	33.33	33.33	66.67
Oshikoto	60.00	40.00	60.00	40.00	20.00	80.00
Zambezi	60.00	40.00	100.00	0.00	80.00	20.00
<b>Grand Average</b>	<b>77.42%</b>	<b>22.58%</b>	<b>61.29%</b>	<b>38.71%</b>	<b>32.26%</b>	<b>67.74%</b>

#### **4.2 Household Processing Method of Traditional Fermented Milk from the Six Regions**

Within the six regions, there are three types of milk products:

***Omashikwa*** is traditional fermented milk produced by the local farmers of the *Oshiwambo* speaking people from the northern part of Namibia. It is commonly found in four regions (*Oshana, Ohangwena, Oshikoto, and Omusati*), ***Mashini ghakushika*** is produced in Kavango region and ***Mabisi*** in the Zambezi region.

The fermentation duration of these products varies, but there is no significance difference at ( $p \geq 0.05$ ) in the fermentation period between the three types of milk (Appendix B). However, *Mabisi* has the longest fermentation period of  $4.40 \pm 0.24$  days (fig 4.1).

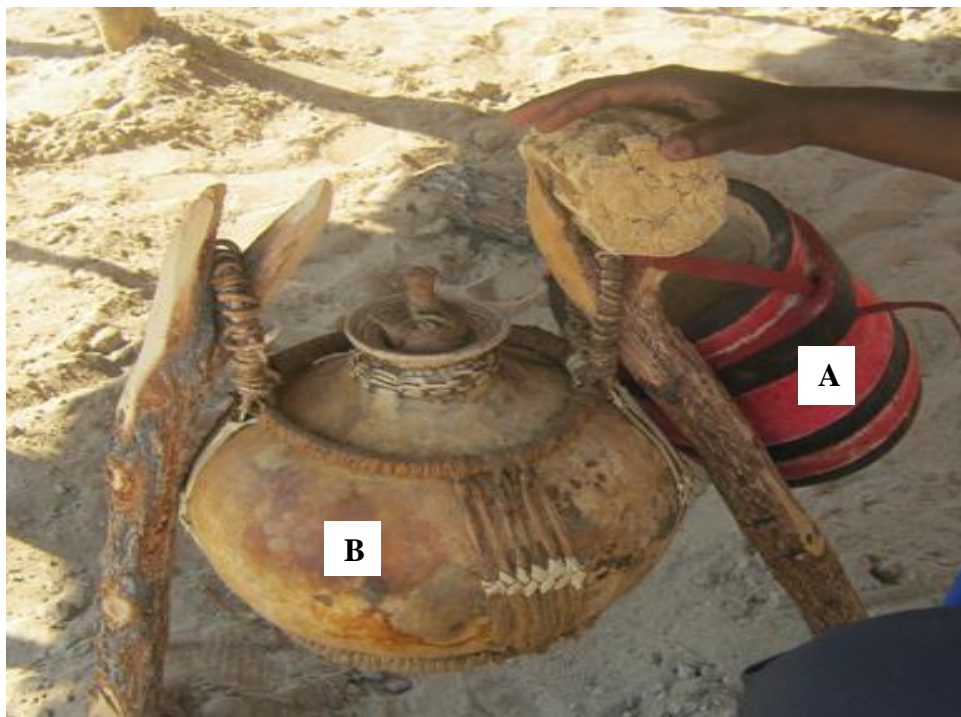


**Figure 4.1.** The fermentation period of *Omashikwa*, *Mashini ghakushika* and *Mabisi*

#### 4.2.1 *Omashikwa*

*Omashikwa* is the traditional fermented buttermilk produced by the local farmers of the *Oshiwambo* speaking people from the northern part of Namibia. It is commonly found in four regions (*Oshana*, *Ohangwena*, *Oshikoto*, and *Omusati*). *Omashikwa* is prepared by accumulation of milk in *Ohupa* (gourd, calabash) (Figure. 1) or in a plastic container with an additions mostly of roots of *Boscia albitrunca* tree ( *omunkuzi*.). However, according to the survey 25 % of respondents from Omusati region and 16.67 % from Oshana (Table. 2) use the root of *Omukwa* (baobab) root instead of *Omunkuzi* root. The milk is allowed to ferment for  $3.95 \pm 0.20$  days at

room temperature 30- 37°C. After fermentation, the roots are removed and fermented milk is churned by shaking the calabash for 2-3 hours until butter granules accumulate on top of the sour milk. Butter is then removed using a wire mesh or hands. The butter granules are then washed using clean cold tap water to remove excess milk. The churned milk (*Omashikwa*) is usually kept in plastic containers with or without lids depending on the availability of the utensils in the household.



**Figure 4.2. (A): The wooden bucket (*Eholo*) used for milking; (B): the calabash (*Ohupa*) which is used to churn the fermented milk.**

**Table4. 2. Type of root used to ferment Omashikwa in Ohangwena, Oshana Omusati and Oshikoto regions**

Regions	Type of root used (%)	
	<i>Omukwa</i>	<i>Omunkuzi</i>
Ohangwena	0.00	100.00
Omusati	25.00	75.00
Oshana	16.67	83.33
Oshikoto	0.00	100.00
<b>Grand Total</b>	<b>10.00%</b>	<b>90.00%</b>

#### 4.2.2 *Mashini ghakushika*

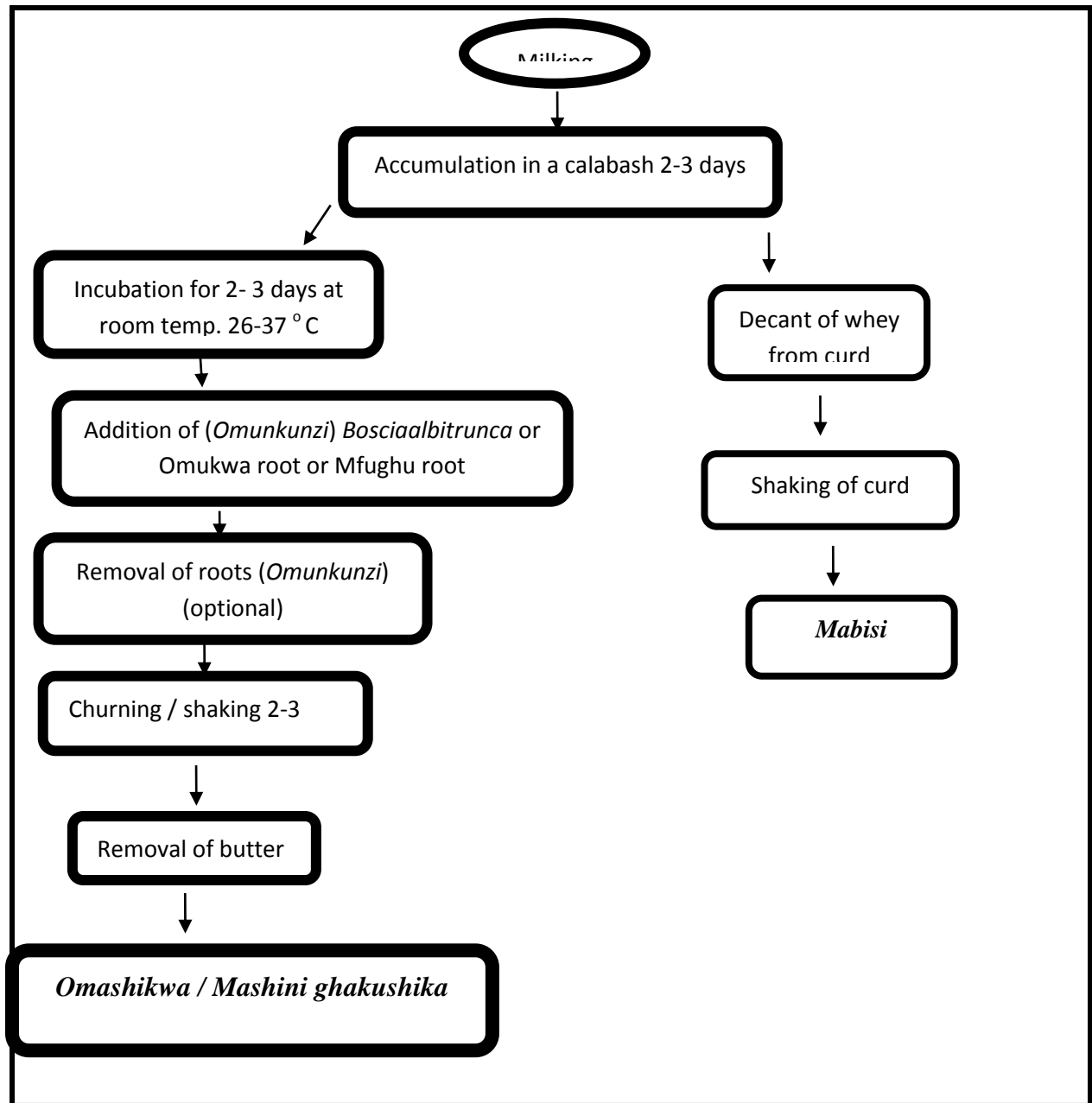
This is the type of fermented milk found in Kavango region. The name means the churned milk. High percentage (83.33 %) of respondents accumulates milk in 20 litters' plastic containers while 16.67 % use calabash which are made of gourd. The milk is allowed to ferment in a presence of root, mostly 66.67 % of respondents use *Omunkuzi* root while 33.33% (Table.4. 3) use *Mfughu* root. The milk is allowed to ferment for  $4.17 \pm 0.48$  days at room temperature of 30 – 37 ° C. The milk is then churned for 2 -3 hours to remove the butter granules, which are then washed with water. The churned milk is then stored in plastic buckets or containers.

**Table4.3. Type of root used to make *Mashini ghakushika***

Region	Type of root	
	<i>Mfughu</i>	<i>Omunkuzi</i>
Kavango	33.33%	66.67%
<b>Grand Total</b>	<b>33.33%</b>	<b>66.67%</b>

### **4.2.3 *Mabisi***

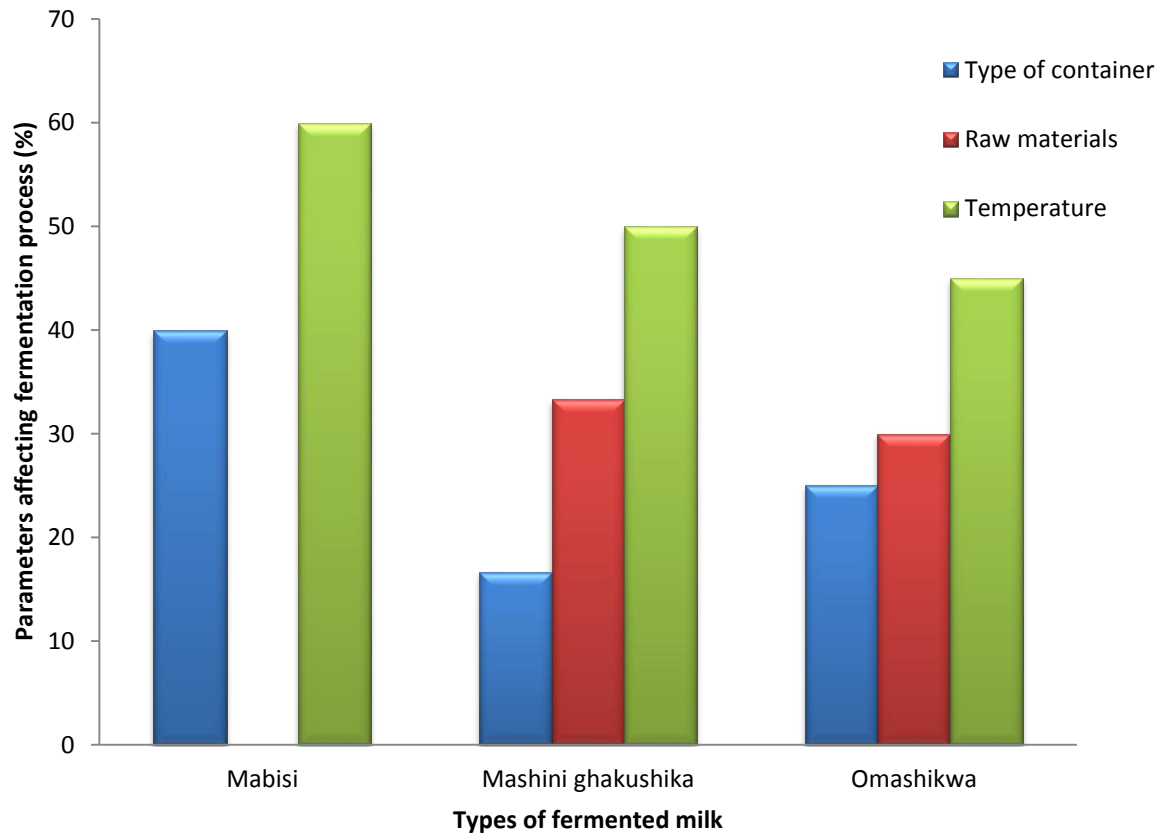
*Mabisi* is the traditional fermented milk which is common in Zambezi region. Before milking in the morning, the cows are let out of the kraal to go feed for a while for about an hour, to acquire energy. *Mabisi* is prepared mostly by accumulating milk in traditional calabash and let the milk to ferment naturally for  $4.4 \pm 0.24$  days at ambient temperature. No starters are added and acidification develops from natural flora of milk. Usually, the container is covered to protect it from dust. Once milk is coagulated, some whey is removed and subsequently, some more fresh milk is added on top. This process is repeated until the container is full of partly-drained curd. The whole process takes generally one week depending on the size of the container. Then, concentrated fermented milk may be shaken before consumption and might keep for up to a week at room temperature.



**Fig 4.3. Flow diagram for the household processing methods of *Omashikwa*, *Mabisi* and *Mashini ghakushika***

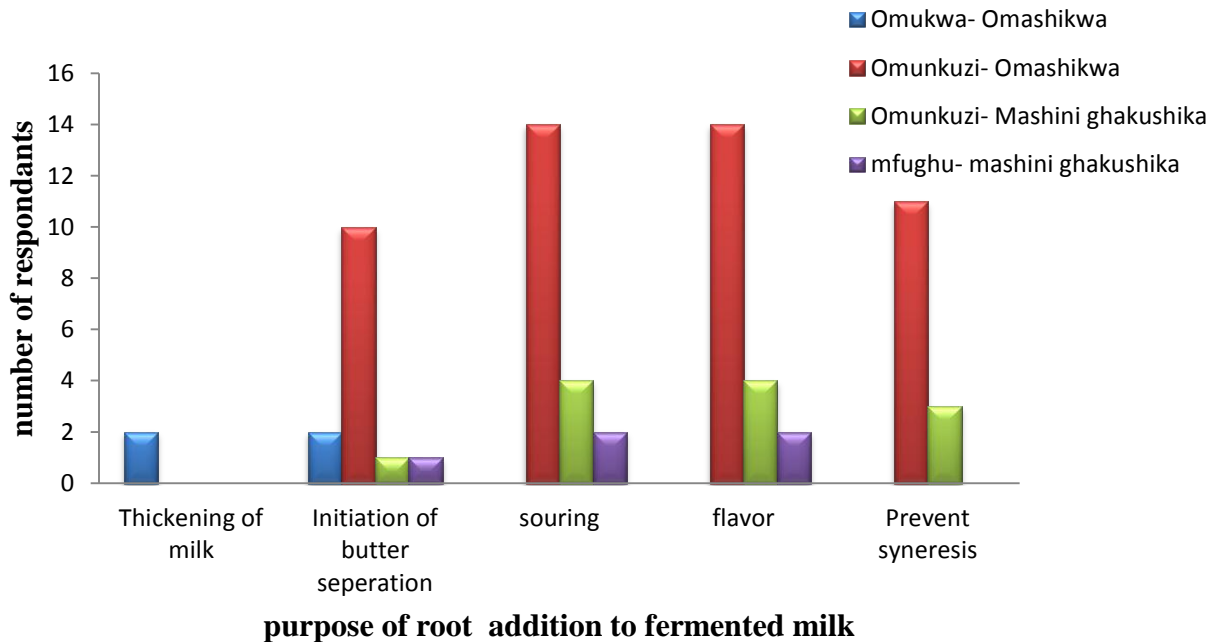
### 4.3 Parameters Affecting Processing Method and Duration of Milk Fermentation

The time required for fermentation of milk depend on different factors like temperature (climatic condition), the cleaning and smoking practices of the equipment used for fermentation and use of starter or previous fermented milk for back sloping was analyzed. The type of container used for fermentation whether plastic or traditional calabash was also analyzed. Temperature was found to be the most factors which affect the fermentation process of the three type's milk; 60 % *Mabisi* 50 % *Mashini ghakushika* and 45 % of *Omashikwa*. According to the respondents if the temperature is relatively higher the milk ferments within a short period, while low temperature results in somewhat long period for fermentation. The second parameter which is found to effect the fermentation 40 % is of *Mabisi*, is the type of container. Traditional containers provide more as starter cultures as the surface of these containers are not smooth hence, some micro organisms might be living there. However, the type of container used is not really reported for *Mashini ghakushika* (16.67 %) and 25 % in *Omashikwa*. The type of raw of material such as the root used also affect the fermentation process. High percentages were reported by respondents that ferment *Mashini ghakushika* (33.33%) and 30 % for *Omashikwa* but 0 % in *Mabisi* since there is no addition of raw materials in the processing method (Figure 4.4).



**Figure 4.4 Parameters that affect the fermentation process of *Omashikwa*, *Mashini ghakushika* and *Mabisi***

The respondents were also asked the motive behind added raw materials to milk before fermentation. Most respondents mentioned more than one reason, thus the general purpose of *Omunkuzi* root in *Omashikwa* is to impart flavor and also to enhance fermentation. The same reason was given for the use of *Omunkuzi* in *Mashini ghamushika* processing. On the other hand, the baobab (*Omukwa*) root is rarely added to *Omashikwa* whose aim is to thicken milk and to prevent syneresis. Moreover, *Mfughu* helps in flavoring and souring of *Mashini ghakushika* with little effect on initiation of butter separation (figure 4.5).



**Figure 4.5. Functions of raw materials added to *Omashikwa* and *Mashini ghakushika***

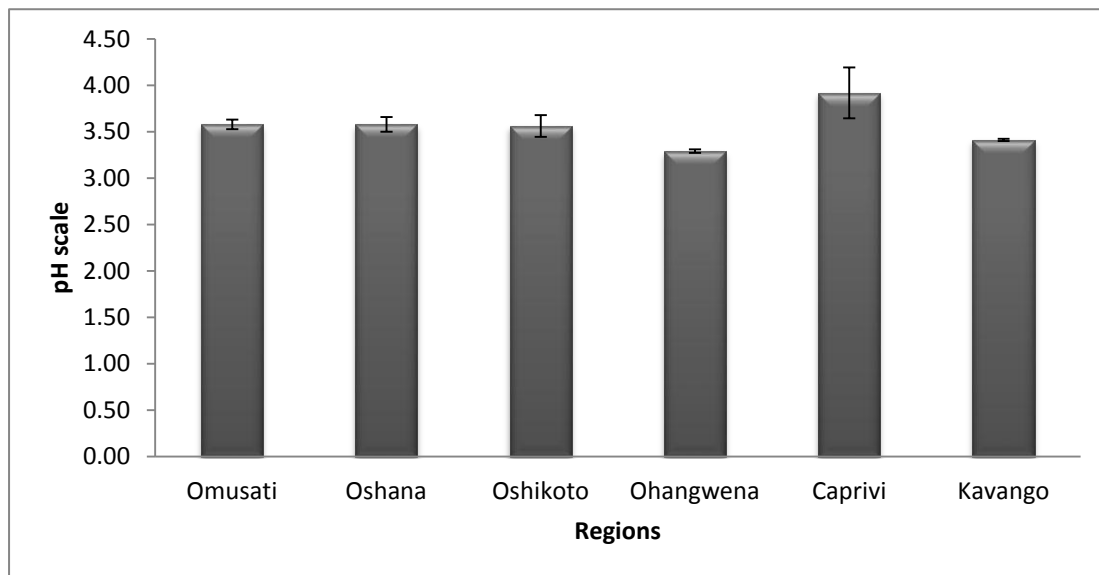
#### **4.5 The Socio-Economic Importance of *Omashikwa*, *Mabisi* and *Mashini ghakushika***

Fermented milk products fulfill multiple purposes in rural developing communities. To own cows is a sign of affluence and pride of being manhood. The household with cows is well respected in the community. In addition, these types of milk are consumed as relish. The beverages, the market value and storage life are improved over that of raw milk. The traditional fermented milk serves as a source of income in some households as they are sold mostly by women in open markets. Milk products are also used as cosmetics by rural people. For example, milk butter oil (ghee) is used as hair and body oil in the rural areas.

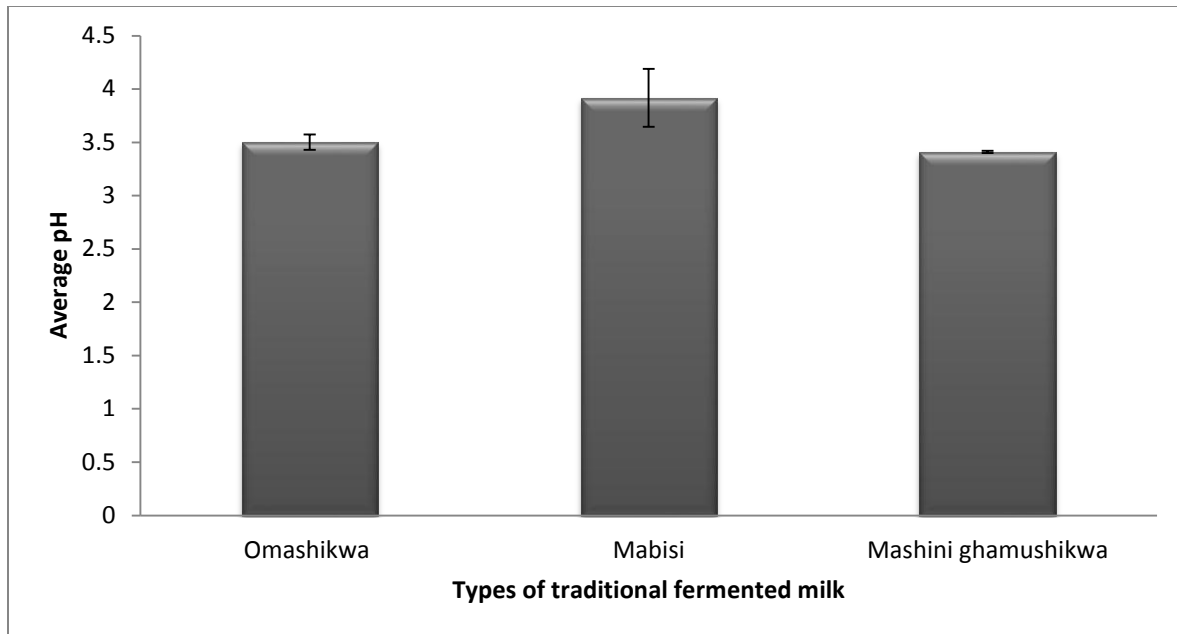
#### 4.6. The Physicochemical Properties of *Omashikwa*, *Mabisi* and *Mashini ghakushika*

##### 4.6.1. pH

The pH of milk samples were measured in the field at the point of collection using a portable pH meter, it was measured in triplicates and means were calculated. The pH of fermented milk was all lower than four. The milk sample collected from Zambezi region had the highest pH of  $3.92 \pm 0.29$  and Ohangwena the lowest with  $3.29 \pm 0.001$  (figure 4.6.a). The milk samples were then grouped into three according to the type of milk i.e. *Omashikwa* or *Mabisi* (Figure 4.6.b). *Mabisi* was found to have high pH of  $3.9 \pm 0.29$  and *Mashini ghakushika* lowest with  $3.41 \pm 0.01$ . In both cases there was a significant difference in the pH values between the regions ( $P < 0.05$ ) (F.pr 0.023) and also between the three types of milk ( $P < 0.05$ ) (F.pr 0.005), but no significant difference between *Omashikwa* and *Mashini ghakushika* ( $P > 0.05$ ) (F.pr 0.151), meaning that the difference is only brought up by *Mabisi*.



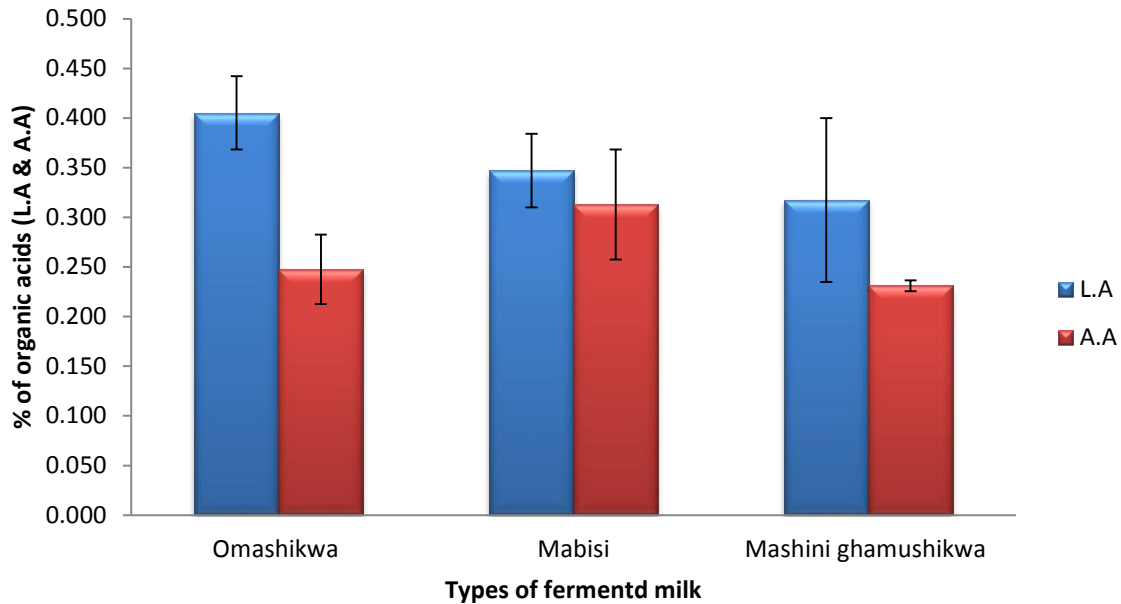
**Figure 4.6.a** pH of fermented milk from different regions at point of collection



**Figure 4.6.b Average pH of *Omashikwa*, *Mabisi* and *Mashini ghakushika***

#### **4.6.2. Titratable Acidity**

Preliminary quantitative estimation of different organic acids (lactic and acetic acids) produced by LAB species in traditional fermented milk was determined by titration of milk using Sodium Hydroxide and phenolphthalein as an indicator. The concentration of lactic acid in all the products was substantially higher than the concentration of acetic acid, with the highest yield of lactic acid produced by *Omashikwa* samples 0.4% (figure 4.7). Acetic acid was in the range of 0.23 to 0.3%. Despite the low concentration of acetic acid, there was no significant difference ( $P > 0.05$ ) in the concentration content between lactic acid and acetic acid produced in all samples but there was a significant difference in *Omashikwa* between lactic acid and acetic acid.



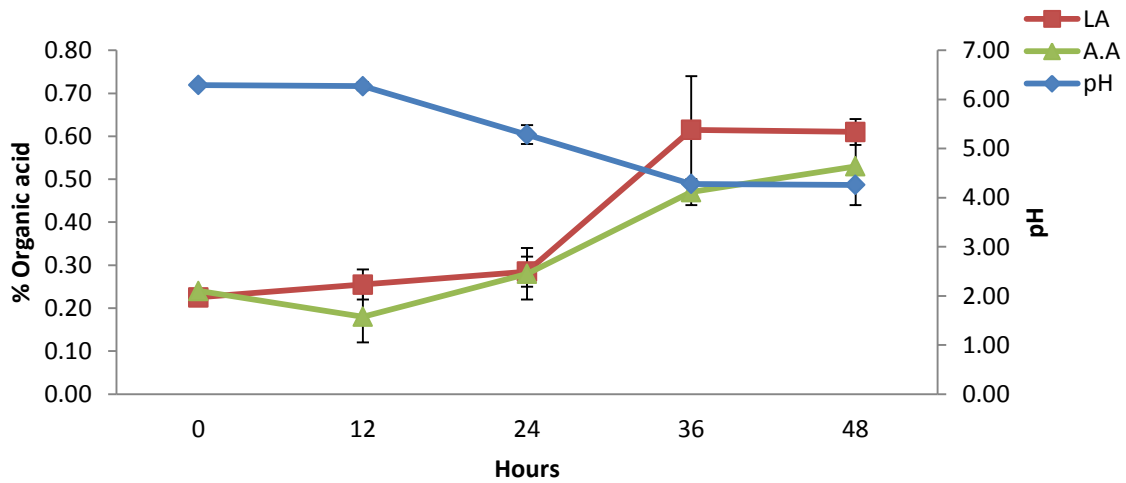
**Figure 4.7 Organic acid % in *Omashikwa*, *Mabisi* and *Mashini ghakushika***

#### 4.6.3. Kinetics of Traditional Milk

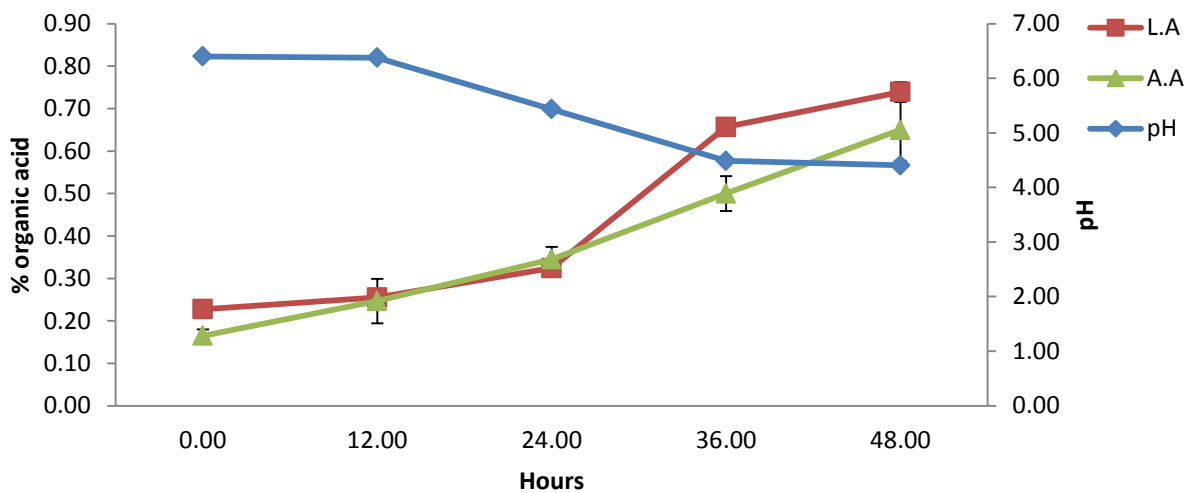
Kinetic model was produced in order to predict how fast the microorganisms can grow and use substrates to produce lactic acid, acetic acid and to see how these relate to the acidity as the milk ferments. This was done by fermenting milk using 2 % starter culture from respective previous fermented milk samples collected from the field. Low fat fresh milk (150 ml) was distributed into sterile bottles, 2 % of starter culture from previous representative regions was added. The pH, lactic acid and acetic acids were measured and recorded at 12 hours intervals.

Initially, the pH of *Mabisi* was 6.3, lactic acid 0.23 % and acetic acid 0.16 %. After 12 hours the pH decreased slowly to 6.27, while organic acids increased substantially. Increment in acid concentration started after 24 h of fermentation and increased linearly until 36 h, after which the production of lactic acid was constant until 48 hours where it slightly increased. The two types of milk had the same trend but Lactic acid was produced more in *Mabisi* than in *Omashikwa*. Acetic acid on the other hand increased at a low pace during the 36 hours of fermentation but its

graph did not come to a stationary phase as in lactic acid. Acetic acid increased throughout the 48 hours of fermentation. Even though the organic acid content increased during 48 hours of fermentation, there was no significant difference ( $P > 0.05$ ) between concentrations for lactic and acetic acids (Figures 4.8 and 4.9) both in *Omashikwa* and *Mabisi*.



**Figure 4.8 the kinetic Fermentation model of *Mabisi***



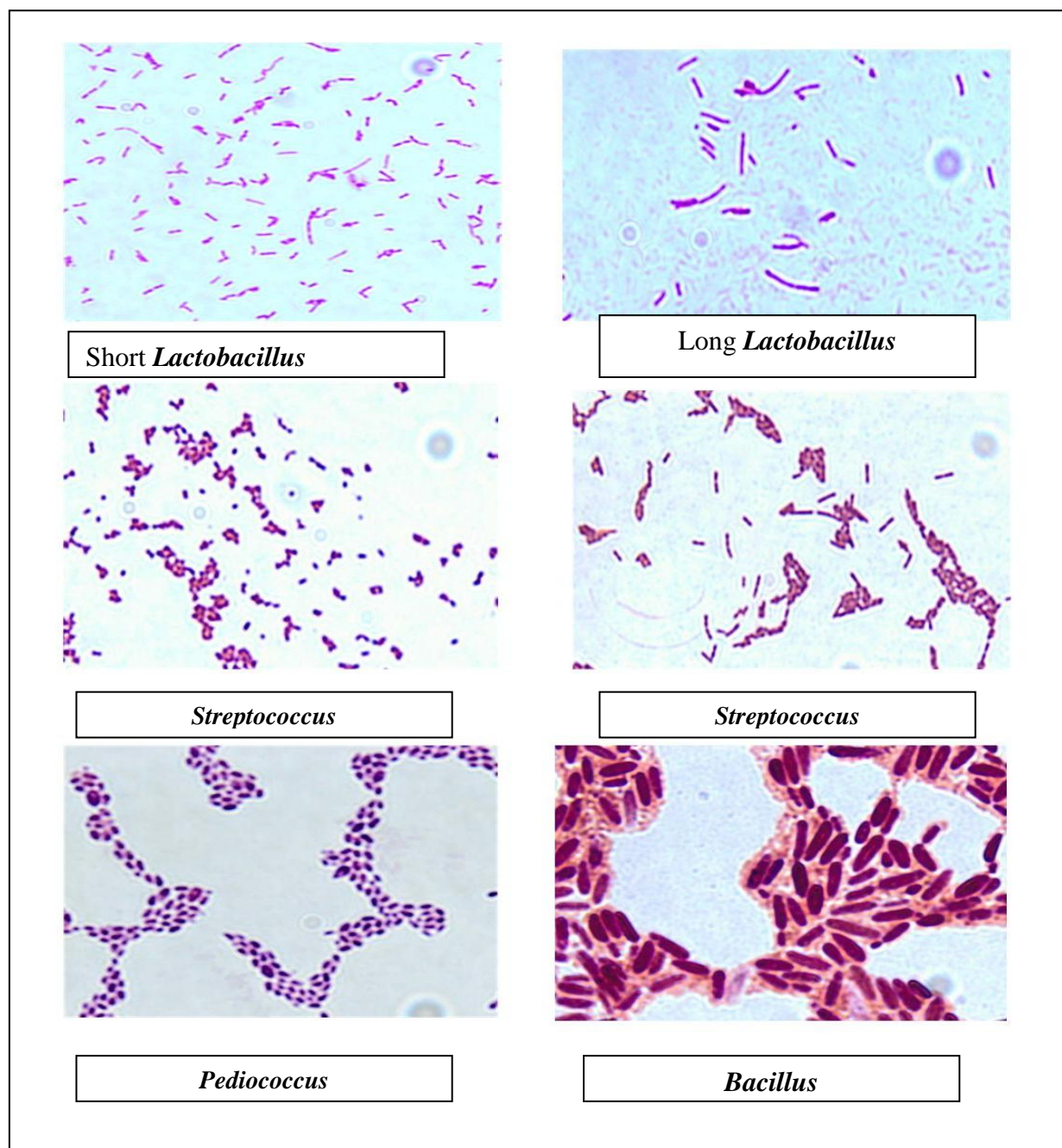
**Figure 4.9 the kinetic Fermentation model of *Omashikwa***

#### 4.7. Isolation and Characterization of LAB

The aim of this section was to isolate and to carry out taxonomic differentiation of strain with potential for bioprocess development of lactic acid production. Approximately 3 colonies were randomly picked from selective plates containing samples from representative regions. These were then propagated twice and streaked on MRS to obtain pure cultures. Sixty strains of lactic acid bacteria (LAB) were isolated on non-selective media (MRS), sixty from Rogosa and sixty from M17 agar. In total 180 LAB were isolated from 20 samples of fermented milk from six regions. These isolates were characterized based on their morphology by microscopy (Figure 4.10) after Gram staining. All of the isolated LAB's were Gram positive and catalase negative, bacillus or cocci with arrangement of singles cells, paired to clusters (table 4.4).

**Table 4.4. Morphological and biochemical characterization of bacteria strains isolated from (*Mabisi, Omashikwa and Mashini ghakushika*) that were collected from six regions in the northern eastern Namibia**

Genus	Characteristics				Total number of isolates
	Cell shape	Cellular arrangement	catalase	Gram	
<i>Lactobacillus</i>	Rods	Short rods, straight edges	-	+	88
<i>Lactobacillus</i>	Rods	Long rods, rounded edges	-	+	45
<i>Streptococcus</i>	cocci	Chains, pairs	-	+	27
<i>Pediococcus</i>	cocci	tetrads and pairs, around	-	+	17
<i>Staphylococcus</i>	cocci	Groups	-	+	6

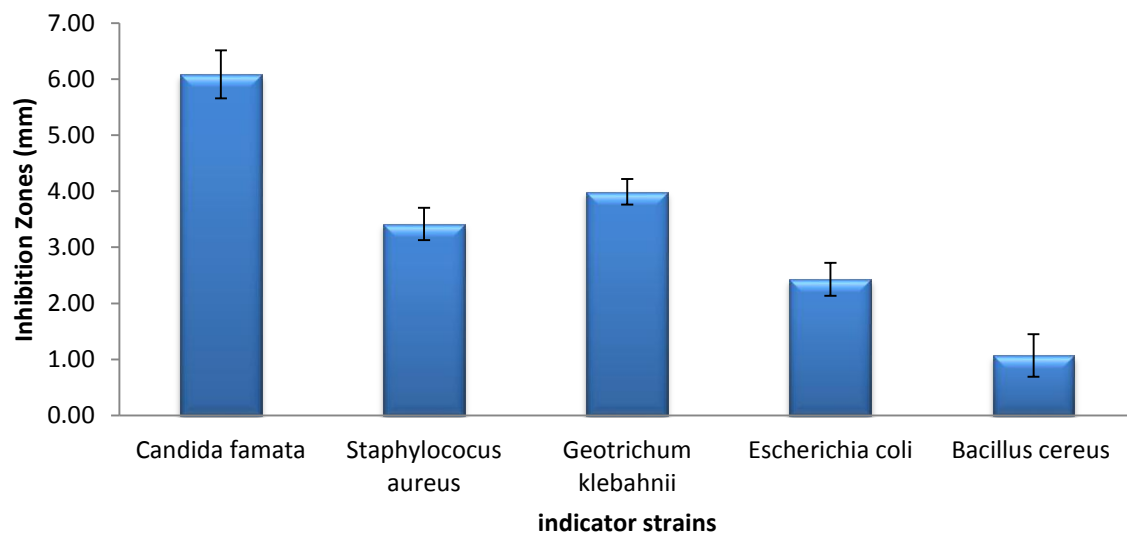


**Figure 4.10. Types of LAB isolated from traditional fermented milk from six regions**

#### 4.8 Determination of Antimicrobial Activities of the LAB

The antimicrobial activity of 180 isolated LAB was carried out using well diffusion method. The degree of inhibition is given in Table 4.5. From a total of 180 lactic acid bacteria isolates that were subjected to antimicrobial activity test against some food borne pathogens, only 20 extracts showed the highest inhibition zones.

The biggest diameters of 9mm were obtained from the extracts of strains: Ms15i3 on *Candida famata*. All LAB extracts showed inhibition zones against *Candida famata*. In contrast, *Bacillus cereus* was the most resistant strain as only few of the extracts showed inhibition zones (figure 4.11). All extracts showed positive results towards *Candida famata* with an average of at least 6.08mm in comparison to gram positive *Bacillus cereus* with an average of 1.07mm. Although with a huge variations in the inhibition zones, statistically, there was no significant difference ( $P > 0.05$ ) on the level of antimicrobial activity (inhibitory zone) of the lactic acid bacteria extracts against the five indicator strains used.



**Figure 4.11 Average of Inhibition zones toward the indicator strains**

**Table 4.5 Zones of inhibition (mm) showing antimicrobial spectrum activity toward some food borne pathogens, using well diffusion assay**

<i>Isolate number</i>	<i>Candida famata</i>	<i>Staphylococcus aureus</i>	<i>Geotrichum klebahnii</i>	<i>Escherichia coli</i>	<i>Bacillus cereus</i>
Ms7i3	8 ±1	2.5 ±0.5	4 ±1	2.5 ±0.5	1±0.5
Ms11i3	6 ±1	5.5 ±0.5	3.5 ±1.5	2 ±2	N/I
Ms12i1	6.5 ±1.5	4.5 ±1.5	3.5 ±0.5	1 ±1	N/I
Ms15i3	9 ±0	2.5 ±2.5	6 ±1	1.5 ±1.5	2.5±0.75
17s1i2	5.5 ±1.5	3.5 ±0.5	2.5 ±2.5	1.5 ±1.5	N/I
17s3i2	5.5 ±0.5	2.5 ±2.5	3.5 ±0.5	3.5 ±0.5	N/I
17s4i3	7.5 ±1.5	2 ±2	3 ±1	1 ±1	4±1.75
17s5i1	7.5 ±0.5	3 ±3	3.75 ±2.25	2.25 ±1.75	N/I
17s7i2	8.75 ±0.25	1 ±1	3 ±1	2 ±2	N/I
17s13i2	7.5±0.5	1.5 ±1.5	3.25±0.75	N/I	3.5±0.75
RS2 I3	4.75 ±2.25	4.5 ±1.5	2.5 ±2.5	4.5±0.5	N/I
Rs7i2	7.25 ±1.75	4.5 ±0.5	6 ±1	1.5 ±1.5	N/I
Rs8i3	5 ±2	4.5 ±0.5	2.5 ±2.5	3.5 ±1.5	N/I
Rs10i2	5 ±1	5.25 ±0.25	4 ±1	4 ±4	5±2
Rs13i3	2.5 ±2.5	3.5 ±0.5	4.5 ±0.5	3 ±3	N/I
Rs16i1	3.5 ±3.5	4 ±1	4.5 ±0.5	4 ±4	N/I
Rs17i2	4 ±4	3.5 ±0.5	5 ±0	3 ±3	3.5±0.5
Rs18i1	2.5 ±2.5	4.5 ±0.5	4.5 ±1.5	4 ±4	N/I
Rs19i2	8.5 ±0.5	2.5 ±2.5	4.5 ±1.5	0.75 ±0.25	2.5±1.75
Rs19i3	7.5 ±0.5	1.5 ±1.5	5 ±1	1 ±1	N/I
Rs20i2	5.5 ±2.5	5 ±0	4.75 ±1.25	4.5 ±0.5	N/I

( F.pr >0.05)	0.399	0.940	0.962	0.198
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\*N/I – No inhibition zone; Ms7i3- M: media; s: sample; 7: sample number; i: isolate; 3: isolate number;  $\pm$  SD triplicates for all experiments

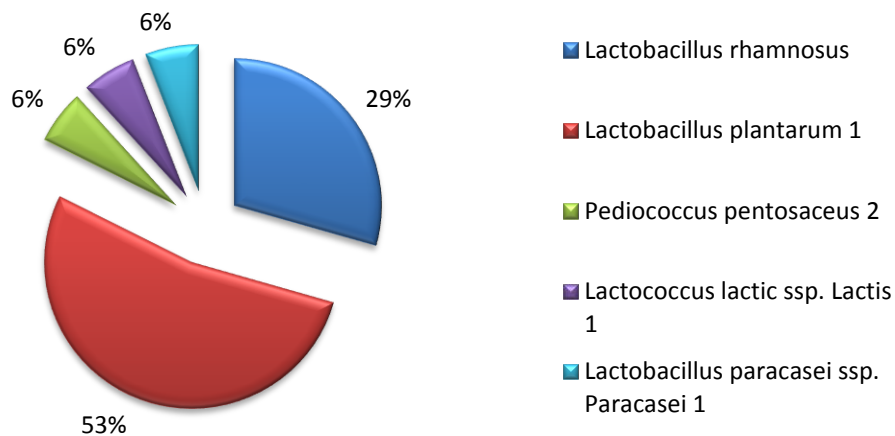


**Figure 4.12. Inhibition zone on foodborne pathogens by lactic acid bacteria isolated from *Omashikwa*, *Mabisi* and *Mashini ghakushika*.**

#### 4.9 Identification of the Inhibitory Substance(s) Producing LAB Strains

Twenty strains among one hundred and eighty lactic acid bacteria isolated from the three types of milk “*Omashikwa*, *Mashini ghakushika*, and *Mabisi*” were selected according to their maximum antimicrobial activity against indicator strains. They were then identified from the production of acids from carbohydrates and related compounds using an API 50 CHL system (BIOMÉRIEUX, France). Table 4.6 shows the LAB species positively identified in which five different species were identified. The predominant species were *Lactobacillus plantarum* (9 strains), *Lactobacillus rhamnosus* (5 strains) , *Pediococcus pentosaceus* 2 (1 strain) ,

*Lactococcus lactis* ssp. *Lactis* 1(1 strains) *Lactobacillus paracasei* ssp. *Paracasei* 1(1 strain) and three un- identified LAB strains . Three LAB species: *L. plantarum*, *L. rhamnosus* and *L. lactis* ssp. *Lactis* were isolated from *Omashikwa*; while four species (*L. plantarum*, *L. paracasei* ssp., *L. rhamnosus* and *Pediococcus pentosaceus*) were isolated from *Mabisi*. *Mashini ghakushika* yielded two LAB species *L. plantarum* and *L. rhamnosus* (Table 4.6)



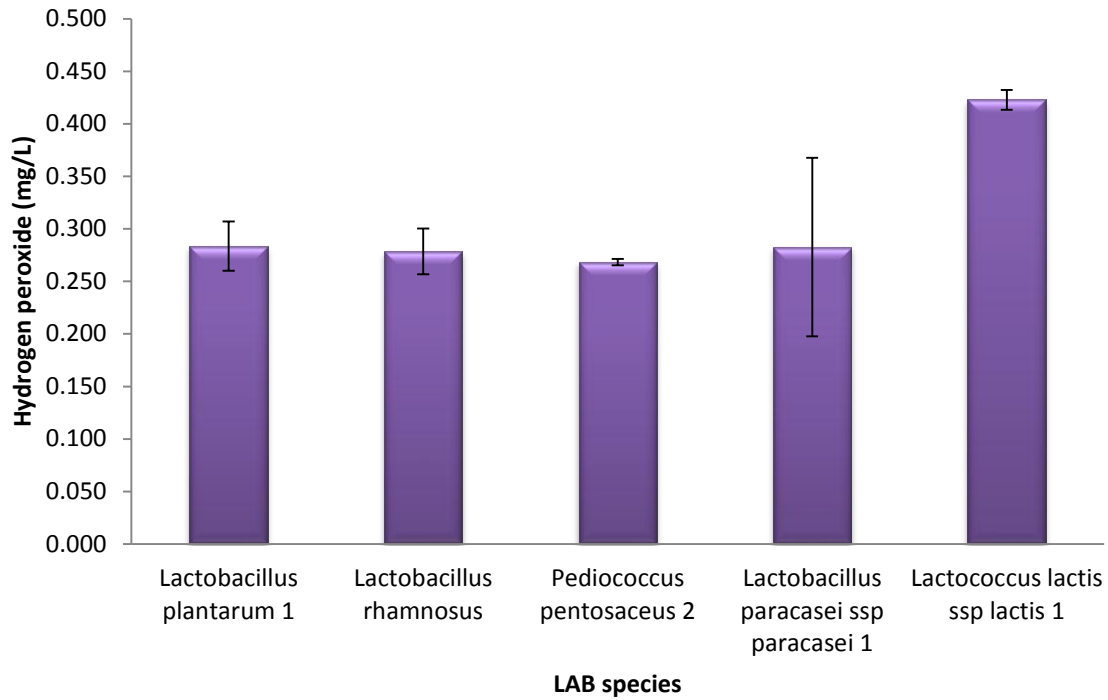
**Figure 4.13** Lactic acid bacteria species in *Omashikwa*, *Mashini ghakushika* and *Mabisi*

**Table 4.6** Distribution of LAB species in *Omashikwa*, *Mabisi* and *Mashini ghakushika*

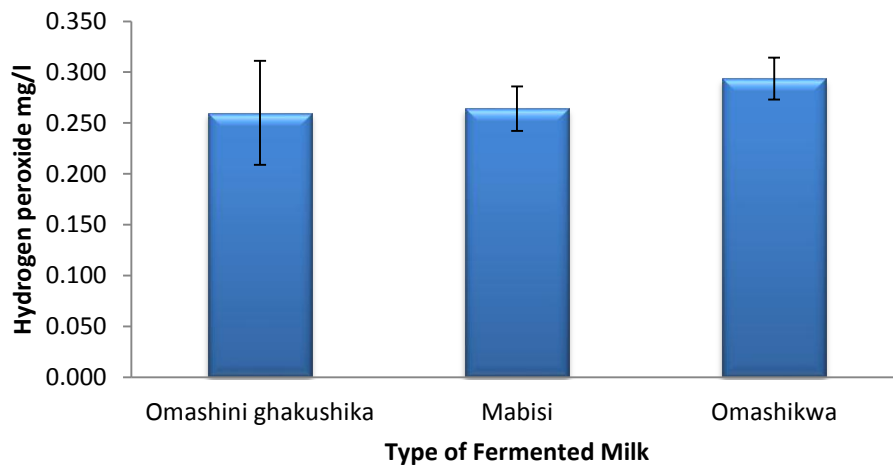
LAB species	Type of fermented milk		
	<i>Omashikwa</i>	<i>Mabisi</i>	<i>Mashini ghakushika</i>
<i>Lactobacillus rhamnosus</i>	5	1	2
<i>Lactococcus lactis</i> ssp. <i>lactis</i> 1	1	-	-
<i>Lactobacillus plantarum</i> 1	6	1	1
<i>Lactobacillus paracasei</i> ssp. <i>paracasei</i> 1	-	1	-
<i>Pediococcus pentosaceus</i> 2	-	1	-

#### 4.10. Hydrogen Peroxide Produced by LAB

The LAB species were screened for the quantitative production of hydrogen peroxide by titration of the cell free supernatants with potassium peroxide. It was observed that *L. lactis* produced the highest quantity of  $H_2O_2$  (0.423 mg/L) compared to all other LAB species used in this work, while *P. pentosaceus* 2 had the lowest yield (0.268 mg/L) (0.268 mg/L) (figure4.14) In spite of the variation in the hydrogen peroxide concentrations produced, statistically there was no significant difference ( $p > 0.05$ ) (F.pr 0.119) between the LAB species. But there is a difference in the hydrogen peroxide production between *L. lactic ssp.* and other LAB species (error bars of the graph) all the LAB species are overlapping except that of *L. lactis ssp.* hence, there is a significant difference). On the other hand, *O ماشيكا* isolates produced more hydrogen peroxide ( $0.294 \pm 0.021$ ) compared to *Mabisi* ( $0.264 \pm 0.022$ ) and *Mashini ghakushika* ( $0.260 \pm 0.051$ ) isolates. However, there was no significance differences  $H_2O_2$  production between the three types of milk (Figure4.15).



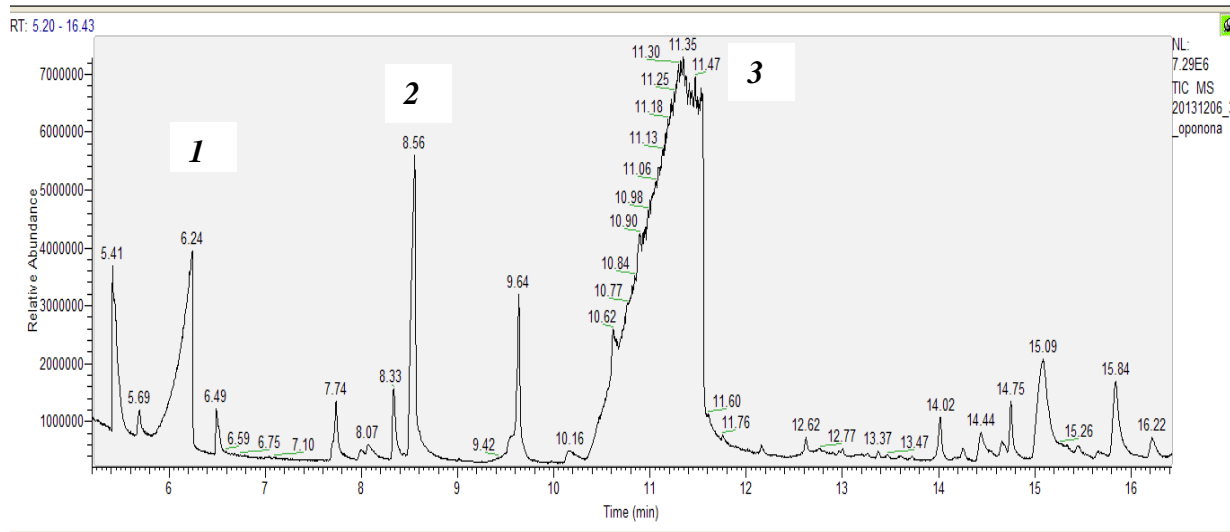
**Figure 4.14 Interspecies Comparison of H<sub>2</sub>O<sub>2</sub> Production**



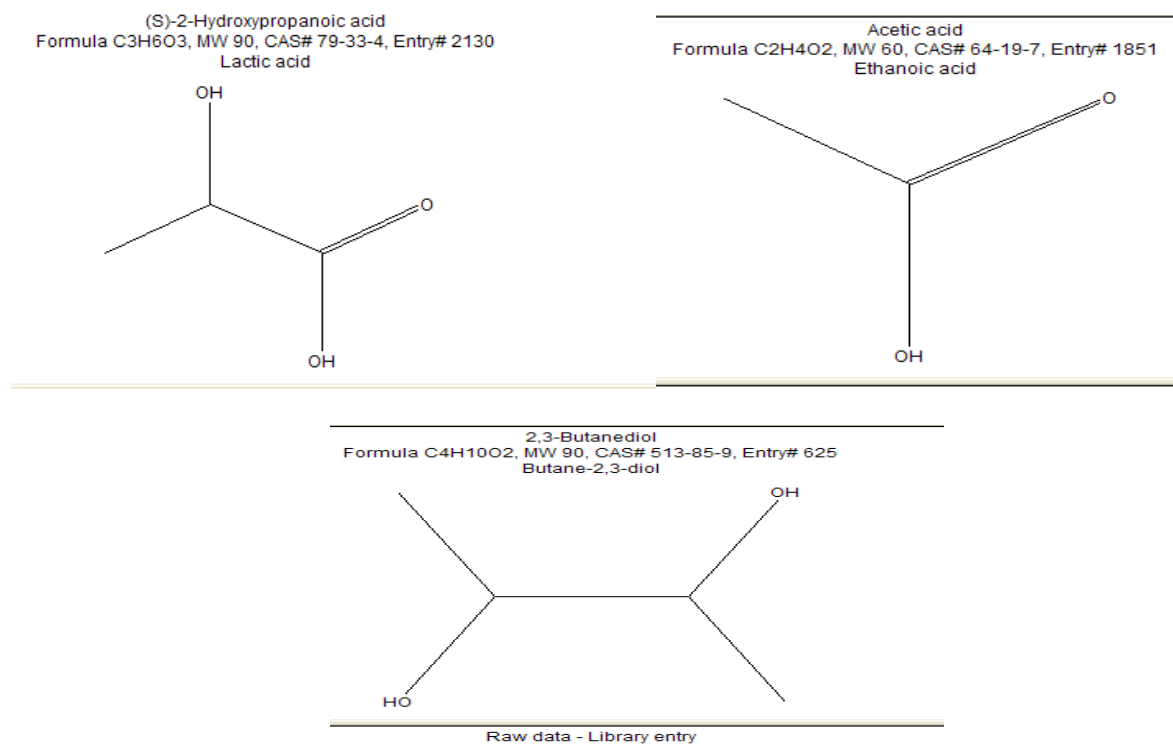
**Figure 4.15 Concentrations of Hydrogen Peroxide produced by *Mashini ghakushika*, *Mabisi* and *Omashikwa***

#### 4.11. Flavor compounds production (Diacetyl)

Gas chromatography coupled with Mass spectrometer (GC-MS) was used to analyze flavor compounds in the three types of traditional fermented milk *Omashikwa*, *Mabisi* and *Mashini ghakushika*. Identification by using GC-MS library showed that the major aroma compounds in all fermented milk samples were; acetic acid, 2,3-butanedione and hydroxypropanoic acid (lactic acid) with retention time (RT) of 6.24 minutes, 7.65 minutes and 11.36 minutes respectively (figure 4.16). The chemical structures of volatile compounds, acetic acid, 2, 3-butanedione and hydroxypropanoic acid is shown in figure 4.17. The buttery, creamy odor of 2, 3-butanedione was found in *Mashini ghakushika* and *Omashikwa* but not in *Mabisi*. Quantification of these compounds was not done since the Mass spectrometry library is limited (demo version).



**Figure 4.16** chromatogram of volatile compounds in fermented milk samples (*Omashikwa*, *Mabisi* and *Mashini ghakushika*) 1.Acetic acid; 2,2,3-butanedione & 3. hydroxypropanoic acid.



**Figure 4.17** Chemical structures of acetic acid, 2,3-butanedione and hydroxypropanoic acid

#### 4.12. Organic Acids Production

HPLC was used to determine non-volatile organic acid contents in traditional fermented milk samples of *Omashikwa*, *Mabisi* and *Mashini ghakushika*. Standards solutions of individual non-volatile and volatile organic acids were separated and mixed to determine the retention times and the response of each organic (figure 4.18). The individual non-volatile organic acids were qualitatively determined by comparison with standard chromatograms and by doping the samples with standard reference acids. A total of four organic acids, including butyric acid, acetic acid, lactic acid and propionic acid were analyzed. The mean values of organic acids, such as butyric acid, acetic acid, lactic acid, and propionic acid determined in *Omashikwa*, *Mabisi* and *Mashini ghakushika* samples were found to be  $0.265 \pm 0.056$ ,  $0.038 \pm 0.003$ ,  $0.456 \pm 0.054$  and  $0.038 \pm 0.009$  mg % respectively (figure 4.19). The major non-volatile organic acid in all three types of milk samples was found to be lactic acid. However, butyric, acetic and propionic acids were present in a very small amounts. Thus there was no significance difference ( $p > 0.05$ ) in butyric, acetic and propionic acid concentration between the three types of milk. Nevertheless there was a significance difference ( $p < 0.05$ ) (F.pr 0.033) in lactic acid concentration among the three types of milk.

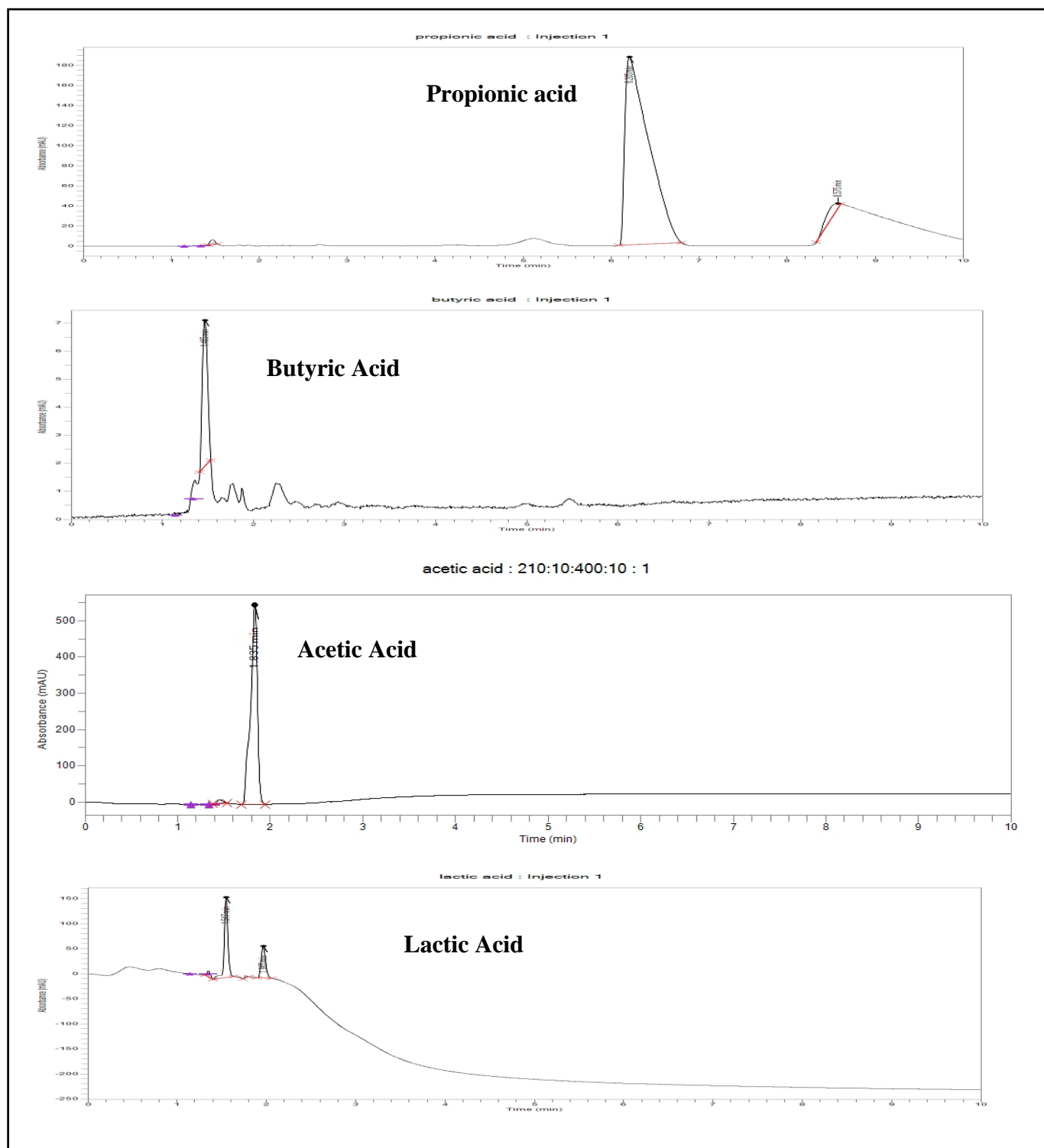
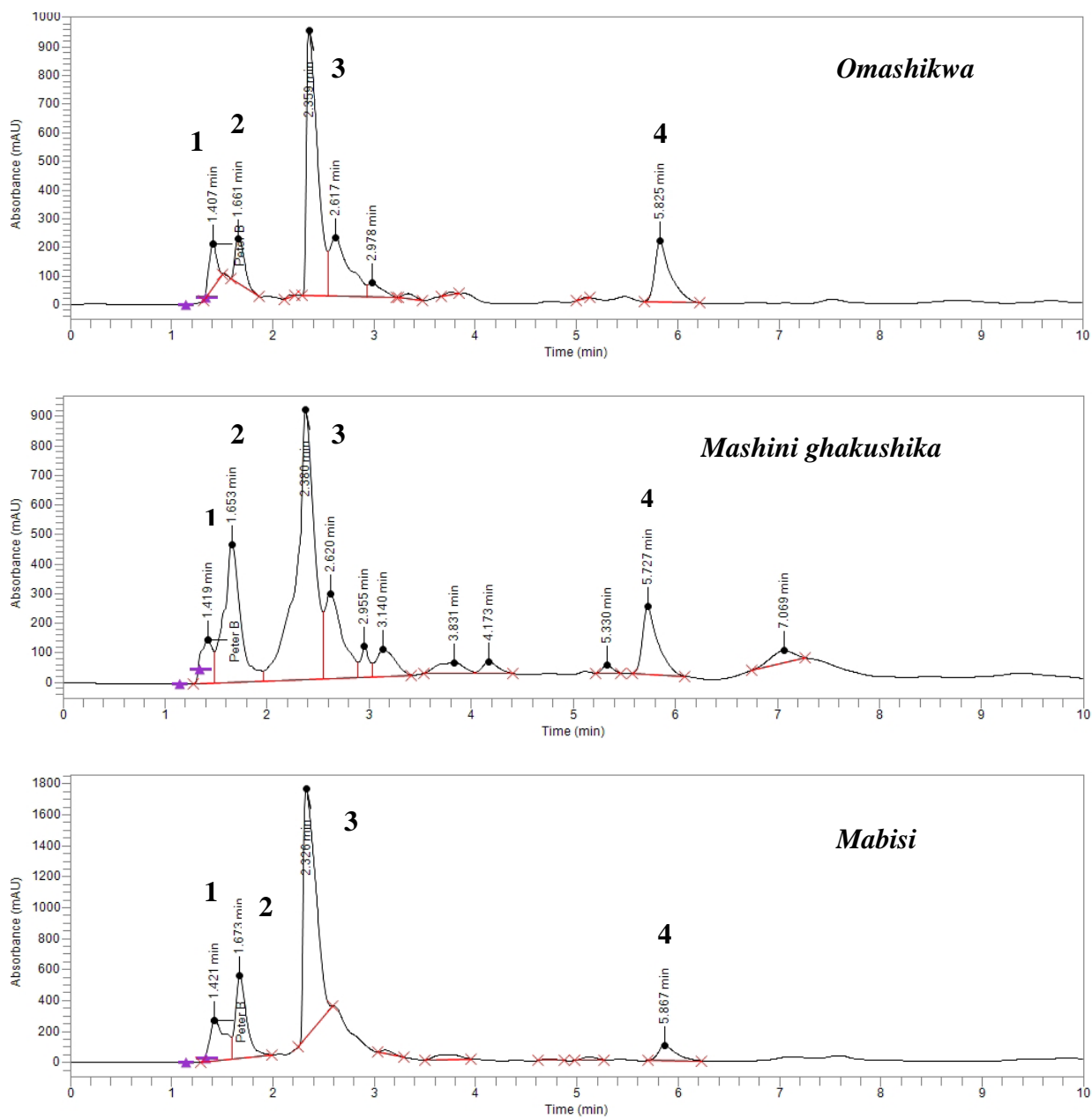
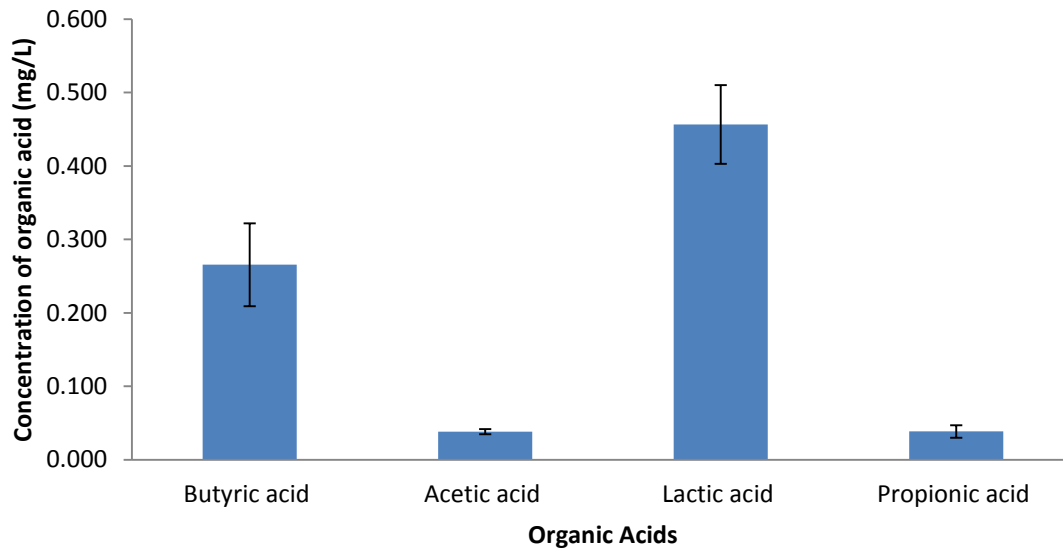


Figure 4.18 Organic Acid Standards Chromatograms

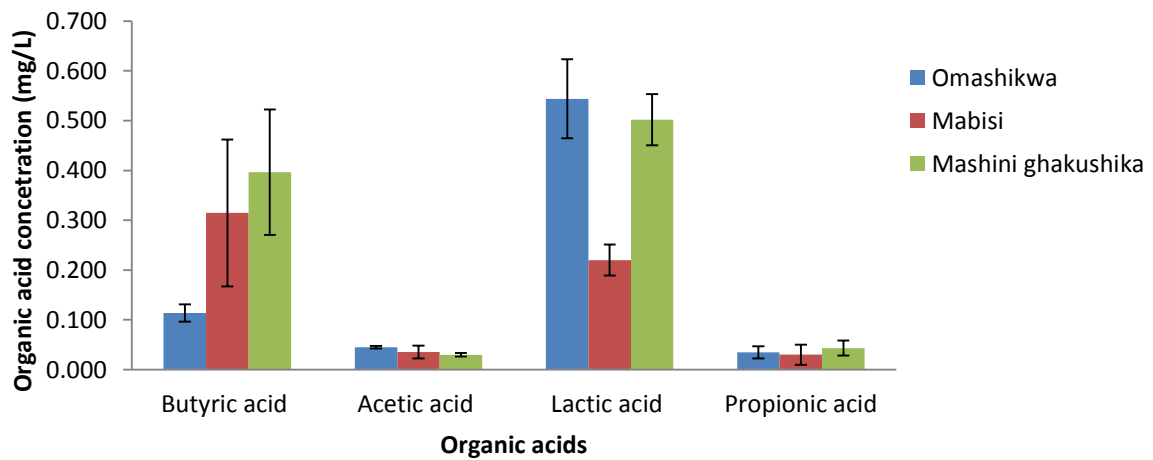


a) Figure 4.19 Organic acid HPLC chromatograms for *Omashikwa*, *Mashini ghakushika* and *Mabisi*

1. Butyric Acid, 2. Acetic Acid, 3. Lactic Acid, 4. Propionic Acid



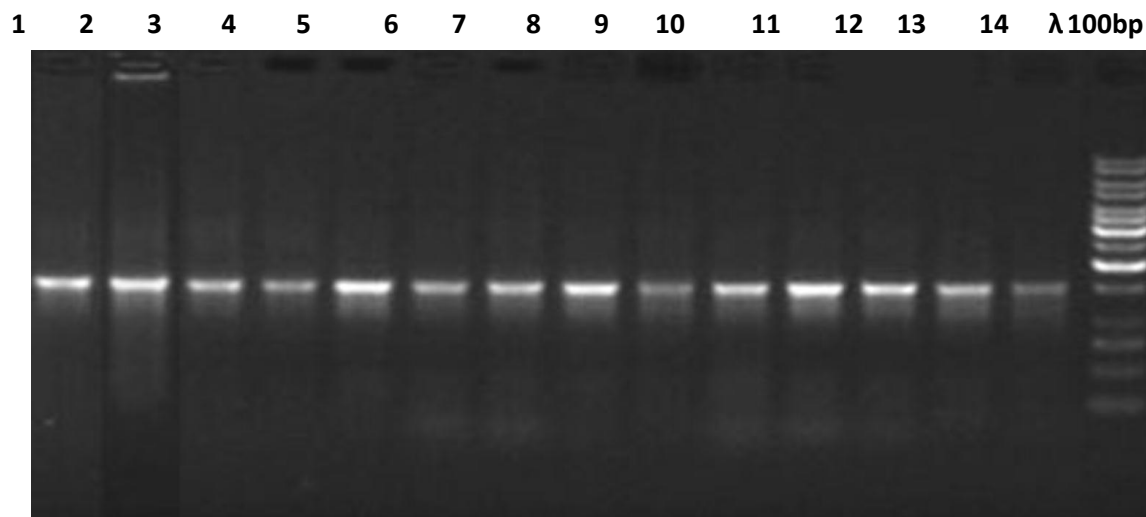
**Figure 4.20** Concentration of organic acid (mg/l) in *Omashikwa, Mabisi & Mashini ghakushika*



**Figure 4.21** Organic acid concentrations in *Mabisi, Mashini ghakushika & Omashikwa*

### 4.13 Amplification of 16S rDNA Region

After DNA isolation the 16S rDNA region was amplified by PCR protocol. Then 10 µl of PCR products were visualized by agarose gel electrophoresis under UV light. The molecular weight of amplified products was around 50 bp (Figure 4.22).



**Figure 4.22 16S rDNA amplification products and standards (λ)**

1. *Lactobacillus rhamnosus*; 2. *Lactococcus lactis* ssp. *lactis* 1; 3. *Lactobacillus plantarum* ; 4. *Lactobacillus plantarum*; 5. *Lactobacillus plantarum*, 6. *Lactobacillus rhamnosus*; 7. *Lactobacillus rhamnosus*, 8. *Pediococcus pentosaceus* 2; 9. *Lactobacillus paracasei* ssp. *paracasei* 1; 10- 14 *Lactobacillus plantarum*.

## CHAPTER 5: DISCUSSION

### 5.1. Household Processing Methods

From the preliminary survey conducted in the study of six regions (Oshana, Ohangwena, Omusati, Oshikoto, Zambezi and Kavango); the house hold processing of milk is of poor quality due to the poor handling and hygienic control that was observed. In all the regions no attempt was made to wash the udder before milking. This may be due to the traditional practice of allowing the calf to suckle before milking. According to Svennersten-Sjaunja (2004), this practice releases the hormone oxytocin which acts on the milk secretory (alveolar) cells, causing release of milk. Furthermore, in all the study regions, milking is practiced in unhygienic way and unclean environment. Hands are not washed before milking, as well as the milking utensils as they believe that washing milking utensils will “wash away” lead to the death of their cows (personal communication).

Traditional milking and holding utensils are on margin as most people are turning onto modern techniques. This is due to the fact that the traditional instruments for milking and fermenting milk are made from the plants which require a lot of rainfall. According to Dieckmann (2013) due to climatic change in Namibia, soil moisture levels are projected to decline dramatically with the cumulative impacts of higher temperature, lower rainfall, lower humidity and higher rates of evaporation. Thus , these changes have severe implications for plant growth and carrying capacity of rangelands throughout Namibia. All these factors have led to use of modern equipment for milking and fermenting milk. In this study high percentage of communal people at least still use traditional wooden bucket for milking than the calabash for fermenting mainly because, the bucket (*Eholo*) is curved from wood which has an overall life span of approximately 20 years if handled with care. While, the calabash is made from gourd and it is

thin and can break easily and it has 2 years life span according to Meekulu Amalia Mbwalu (oral interview).

The household processing methods of *Omashikwa* and *Mashini ghakushika* are similar since they use the raw material (*Omunkuzi*) for flavor and for souring as the main reason behind the root use. Our results are in agreement with those of Bille, (2013); Bille, *et al.*, (2007); the root is believed to add flavor to the product, increases the rate of milk fermentation and helps in churning. Addition of roots is not only done in Namibia. Milton, (2003) stated that the powdered roots of *Boscia albitrunca* have preservative qualities and local tribes use this powder to preserve butterfat. Further experiments showed that the powered root also prevents moulds forming on oranges, tomatoes, bread and potatoes. The processing method of *Mabisi* is completely different from *Omashikwa* and *Mashini ghakushika* as there is no addition of roots. *Mabisi* is also found in Zambia.

## **5.2. Factors Affecting Fermentation**

The time required for fermentation of milk depends on different factors such as temperature (climatic condition), the cleaning and smoking practices of the equipment used for fermentation and use of starter or previous fermented milk for back sloping. Namibia is a semi-arid country hence; the temperature can go up to 39 °C. In this study temperature was found to be the major factor affecting fermentation. Due to high ambient temperatures coupled with the general lack of refrigeration facilities imply that the milk, often containing high initial numbers of bacteria, becomes sour in 12 to 24 hours. It was found that a plastic container takes longer to fermented milk than traditional calabashes. According to Gadagaa, *et al.*, (1999) earthenware pots or calabash are better containers for traditional fermentation of milk. This is because earthenware

pots have micro-pores in their walls, which, if not sterilized, may harbor lactic acid bacteria from the previous fermentation, which then act as inoculums for the next fermentation.

### 5.3. Physicochemical Characteristics

*Omashikwa*, *Mabisi* and *Mashini ghakushika* are found to have pH lower than 4 with *Mabisi* with the highest 3.9 and *Omashikwa* the lowest 3.41. However, the pH values of *Omashikwa* are in the same range with values reported by Bille, *et al.*, (2007) when he found the pH of *Omashikwa* to be 3.25. Furthermore, according to Schoustra, *et al.*, (2013); the pH of *Mabisi* was found to be in the range of 4.0 to 4.5 which is almost close to our results. The lower pH range of our samples could be attributed to the duration period of these samples at the time of collection. Most of the samples collected were a week old at a point of collection. Since the pH of fermented milk dropped with time (hours) this drop could be attributed to a decrease in lactose content and a consequent increase in lactic acid content. Thus, fermentation period and storage condition should be taken into account as other factors to maintain a certain level of required pH, (Ibtisam, & Marowa, 2009). Other factors which could have an effect on the pH of milk is unhygienic preparation method of the fermented milk, the higher the contamination, the lower the pH as molds and yeast would be higher which makes the milk too sour and reduction in shelf life. Titratable acidity is a measure of freshness and bacterial activity in milk. The titratable acidity of milk expressed as Lactic acid and Acetic acid was measured in all three types of milk *Omashikwa*, *Mabisi* and *Mashini ghakushika*. The concentration of lactic acid in all three products was substantially higher than the concentration of acetic acid with *Omashikwa* with the highest. According to Bhekisisa, *et al.*, (2008) the titratable acidity (T.A.) of *Amasi* was found to be 1.4% at pH of 4, though our results were lower than the values reported by Bhekisisa which could be due to the low pH of our samples (lower than 4). Organic acids are important

contributors to the flavors of beverages, as they determine different desirable sensory characteristics (Arrizon *et al.*, 2006) and they occur in dairy products as a result of hydrolysis of butterfat (fatty acids), biochemical metabolic processes or bacterial metabolism Güzel-Seydim *et al.* (2000). Thus, the presence of lactic and acetic acids in fermented foods is advantageous due to their antimicrobial property, thus preventing spoilage by other microorganisms.

The relationship between pH and Titrable acidity of the three types on milk was studied in over the period of 72 hours. Short time after milking, the acidity of milk starts to increase noticeably due to bacterial activity. The degree of bacterial contamination and the temperature at which the milk is kept are the main factors influencing acid formation. Therefore, the amount of acid in milk surely depends on the cleanliness of production and the temperature at which milk is kept. Therefore, it is for this reason; determination of acid in milk is an important factor in judging milk quality. When Lactic acid reaches about 0.3%, the sour taste of milk becomes sensible while at 0.4% acidity, milk is clearly sour, and at 0.6% it precipitates at normal temperature (Torkar & Teger, 2008). Generally, the pH decreased with fermentation time while titratable acidity increased. The pH of *Mabisi* became very acidic, decreasing from 6.3 to about 4.26 and Titratable acidity increased from 0.23 to 0.62 (% lactic acid) while acetic acid increased from 0.24 to 0.47 after 36 hours. The pH increased between 36 and 48 hours while the TA continued to increase over the same period. The titratable acidity of all manufactured fermented milks increased as a result of the performed fermentation process. The gradual increase in titratable acidity during storage was also observed in Sudanese yoghurt (Manhal & Kamal, 2010). The difference in the rate of decrease in pH during yoghurt production is due to production of lactic acid using *Lactobacillus* (Adams & Moss, 1995) and other lactic acid bacteria. Furthermore, the

production of lactic acid after fermentation has the effect of lowering pH and thereby arresting any further development of pathogens and other toxic microorganisms, apart from having lethal and destructive effect on bacteria and arresting bacterial multiplication (Jayeola *et al.*, 2010).

#### 5.4. LAB Identification

Out of the 180 LAB isolates from the three types of fermented milk *Omashikwa*, *Mabisi* and *Mashini ghakushika* most of the genera belonged *Lactobacillus* 88 %, *Lactococcus* 6 %, and *Pediococcus* 6 %. These results showed the heterogeneity in the three types of fermented milk *Omashikwa*, *Mabisi* and *Mashini ghakushika* from different regions or agro climatic zones either in its chemical or microbial composition and quality attributes. This finding was similar with those of Savadogo *et al.* (2004) who reported on fermented milk from Burkina Faso. The *Lactobacillus* was made up of 53 % of *L. plantarum*, 29 % *L. rhamnosus* and 6 % *L. paracasei* ssp. *paracasei*. It is reported that traditional fermented milks in regions with a cold climate favor the growth of mesophilic bacteria such as *Lactococcus* and *Leuconostoc* spp. whereas, in warm regions, thermophilic bacteria like *Lactobacillus* and *Streptococcus* prevailed (Savadogo *et al.*, 2004). This could explain the dominance of thermophilic bacteria in our samples as our samples are from regions with high ambient temperatures ranges between 37 °C to 40 °C, at which the natural fermentation took place. In addition, the dominance of *Lactobacillus* could be due to the fact that the pH of the fermented samples were low then 4 as according to Jayeola *et al.*, (2010 ) who stated that the yoghurt mixture coagulates during fermentation due to the drop in pH. However, the *Streptococci* are responsible for the initial pH drop of the yogurt mix to approximately 5.0 while the *Lactobacilli* are responsible for a further decrease to pH 4.0 *Mabisi* showed diversity of genera with four types of LAB species *L. plantarum*, *L. paracasei* ssp. *paracasei*, *L. rhamnosus* and *Pediococcus pentosaceus*. *Omashikwa* has only three species *L.*

*plantarum*, *L. rhamnosus* and *L.lactis* ssp. *lactis*; these were in agreement with the study reported by Bille (2013) when he found that the main dominant lactic acid bacteria belonged to *Lactobacillus plantarum* (25 %) and 15 % *Lactococcus lactis* ssp. *lactis*. The only difference was the *Lactobacillus rhamnosus* which was found in this study which was not the case with the study made by Bille, (2013). This may be attributed to high environmental temperatures during processing and probably the use of *Omunkuzi* root. The diversity of LAB found in the fermented milk can be used to make the starter culture to make this milk uniform. Such as *Lactococcus lactis* which is already widely used by the dairy industry for the manufacture of fermented milk products. The primary role of *L. lactis* during fermentations is the production of lactic acid from milk sugar lactose. In addition, the LAB isolated have potential to be used as probiotics as some of the lactic acid bacteria can be used in preservative function. Previous studies were able to isolate *Lactobacillus plantarum* from fermented sausages (Parente *et al.*, 2001), naturally fermented Sicilian green olives (Randazzo *et al.*, 2004). *Lactobacillus plantarum* is not only used as a starter culture in cheese making but can as well be used as a probiotic LAB (Gomes *et al.*, 1995; Vinderola *et al.*, 2000). The dominance of *Lactobacillus plantarum* strains in *Omashikwa* and *Mashini ghakushika* could be associated with the root which is added during fermentation, Adebayo-tayo & Onilude (2008) stated that the presence of *L. plantarum* are known to be commonly associated with plant based food fermentations.

### **5.5. Antimicrobial activity**

All the 180 isolates of lactic acid bacteria isolated from the three types of fermented milk *Omashikwa*, *Mabisi* and *Mashini ghakushika* were subjected to inhibitory activity test using Well diffusion method. Only twenty isolates *Lactobacillus plantarum* (9 strains), *Lactobacillus rhamnosus*(5 strains) , *Pediococcus pentosaceus* 2 (1 strain) , *Lactococcus lactis* ssp.*lactis* 1(1

strains) , *Lactobacillus paracasei* ssp. *paracasei* 1(1 strain) and three which were not identified by the API CHL techniques showed inhibition zone on some pathogenic bacteria to varying degree. Similarly, Tadesse *et al.*, (2005) and Cadirici & Citak, (2005) observed varying degree of inhibition of various food borne pathogens by the culture filtrate of lactic acid bacteria, although these inhibitory substances produced by the lactic acid bacteria strains acts differently on the pathogenic reference indicator strains. The most inhibited indicator strain belonged to yeast *Candida famata*, it was the most sensitive pathogenic indicator strain to the inhibitory substance produced by the lactic acid bacteria isolates followed by *Geotrichum klebahnii* and with the *Bacillus cereus* the lowest and *E.coli*. Similar results were reported by Savadago *et al.*, (2004) and Tadesse *et al.*, (2005). *E.coli* strains were the least sensitive to inhibitory substance produced by the lactic acid bacteria as compared to the other indicator strains. The resistance of Gram negative bacteria is attributed to the particular nature of their cellular envelop, the mechanisms of action described for bacteriocins bringing in phenomenon of adsorption. *Pediococcus pentosaceus* species (Ms15i3) showed inhibitory effects on all the indicator strain. This could be due to the fact that *Pediococcus pentosaceus* species have potential to produce bacteriocins *Pediocin* (Savadago *et al.*, 2004). In addition, the *Lactococcus lactis* ssp. *lactis* (Rs10i2) showed varied inhibition zones against all the indicator strains. This could be due to the fact that the isolate can produce Nisin (Soomro *et al.*, 2002) and the primary target of nisin's antimicrobial action is the cell membrane. Nisin has an inhibitory effect against a wide variety of Gram-positive food borne pathogens and spoilage microorganisms (Soomro *et al.*, 2002). Thus, the use of bacteriocin-producing starter cultures may not only contribute to food safety, but also prevent the growth of undesirable autochthonous lactic acid bacteria that produce off-flavor.

## 5.6. Hydrogen peroxide

In addition to the other antimicrobial compounds produced by LAB, hydrogen peroxide was closely examined in this study. The identified 20 LAB isolates were screened for the quantitative production of hydrogen peroxide using titration method. It was observed that *L. lactis* produced the highest quantity of H<sub>2</sub>O<sub>2</sub> (0.423 mg/L) as compared to all other LAB species used in this work, while *P. pentosaceus* 2 had the lowest yield (0.268 mg/L). Similar results were obtained by Enitan *et al.*, (2011), when they found that *L. lactis* produced the highest quantity of Hydrogen peroxide (0.4279 mg/L). The bactericidal effect of hydrogen peroxide has been attributed to its strong oxidizing effect on the bacterial cells and to the destruction of basic molecular structure of the cell protein (Zalan *et al.*, 2005). Therefore, the addition of live cells of hydrogen peroxide producing LAB as starter culture or as an adjunct starter will not only contribute to the flavor and aroma of yoghurt and other dairy products but would be useful for food preservation as well as prevention of growth of food borne pathogens.

## 5.7. Flavor compounds

Flavor is essential characteristic of traditional fermented milk as the sensory characteristics play an important role in product acceptance by consumers. In fermented dairy products, flavor perception is strongly based on the volatile compounds (Kalviainen, *et al.*, 2003). Volatile compounds that have been identified in the three types of fermented milk *Omashikwa*, *Mabisi* and *Mashini ghakushika* are acetic acid, 2,3 Butanediol and Lactic acid. The primary flavor of dairy products arises mainly from the indigenous volatile constituents in cow's milk, which are influenced by pasteurization, fermentation, processing, and storage (McGorin, 2001). A large number of the volatile organic compounds found in three types of traditional fermented milk but our study only focused on the production of Diacetyl. Even though, there was no distinct result

of diacetyl in *Omashikwa*, *Mabisi* and *Mashini ghakushika*, other flavor compounds were detected such as lactic acid which is a prime source of flavor and functionality for many fermented food products and is responsible for the refreshing tart flavor of yoghurt (Panagiotidis & Tzia, 2001). While, 2,3 butanediol which is natural byproduct of fermentation is responsible for the buttery flavor of milk. 2,3 has the same function as diacetyl as it contributes to the delicate, full flavor and aroma of yoghurt and is especially important for products that contain low acetaldehyde concentrations. Even though, the extraction and concentration of volatile flavor components from three types of fermented milk was not quantified in this study positive results were observed similar to other researches that were done on flavor compounds in traditional fermented milk.

### **5.8. Organic Acids Concentration**

The major non-volatile organic acid in traditional fermented milk *Omashikwa*, *Mabisi* and *Mashini ghakushika* milk samples was found to be lactic acid. However, butyric, acetic and propionic acids were present in a very small amounts. A number of factors, such as fermentation environment, types of microorganisms and the state of raw materials can affect the ratio of the contents of organic acids (Choi *et al.*, 2007). During fermentation process of dairy products, around 20-40% of lactose present in milk is transformed into lactic acid, and the content of lactic acid in yogurt is around 0.9%. To produce lactic acid through the biological route, there are various kinds of lactic acid producing microorganisms. Lactic acid bacteria convert lactose into lactic acid through the homo-fermentative pathway (Hofvendahl & Hagerda, 2000). While, Acetic acid is an important compound produced by lactic starter cultures (Alonso & Fraga, 2001), and acetic acid in the concentration range of 0.5 to 18.8 mg/kg has been reported in yoghurt (Alonso & Fraga, 2001), which is similarly to our findings. In addition Butyric acid has

recently been the subject of intensive research due to its purported anti-colon cancer effects. It has also been shown to inhibit the growth of a range of cancer cells. The content of butyric acid in milk fat varies ranging between 3 and 4.6% in milk. The presence of these organic acids results in a low pH. Hence, prevent the growth of pathogenic microorganisms in fermented milk.

## CHAPTER 6: CONCLUSION

The overall aim of the study was to isolate and investigate the antimicrobial properties of LAB isolates from traditional fermented sour milk from northern part of Namibia (Ohangwena, Omusati, Oshana, Oshikoto, Zambezi and Kavango regions) with potential for food safety application to control and eliminate the growth of harmful products in food. Even though this milk are produced under non-hygienic environment with a high possibility of contamination with desirable, pathogenic, non-pathogenic and/or spoilage bacteria, the lactic acid bacteria fermentation can reduce the risk of spoilage and pathogenesis. The culture filtrates from twenty strains of lactic acid bacteria isolated from *Omashikwa*, *Mabisi* and *Mashini ghakushika* exhibited antimicrobial activity against four pathogenic test strains. Lactic acid bacteria and their by-products have been shown to be more effective and flexible in several applications. Most inhibitory substances produced by lactic acid bacteria are safe and effective natural inhibitors of pathogenic and food spoilage bacteria in various foods. Due to the properties, such as broad spectrum, heat stability over a wide range of pH, all the inhibitory substances produced by the isolated strains can effectively be used as a bio-preservative in food with a wide range of pH, even after pasteurization. All the taxonomic determination of the lactic acid bacteria isolates were done by using morphological observations, biochemical tests and molecular method of identification . Hence, further studies can also focus on the characterization of bacteriocins form these types of milk. Also to study more on the flavor compounds which are found in traditional fermented milk as flavor is a crucial characteristic of foods as the sensory characteristics play important role in product acceptance by consumers.

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**APPENDIX 1****A: 1. QUESTIONARRES**

Serial number



**MULTIDISCIPLINARY RESEARCH RENTER (MRC)**  
**SCIENCE, TECHNOLOGY AND INNOVATION DIVISION**  
**INDEGENOUS KNOWLEDGE SYTEMS AND TECHNOLOGY (IKST)**  
**FOOD PROGRAM**

**Pilot study on fermented milks from northern-Eastern regions in Namibia/**

**Omapekapeko kombinga gomahini nga ga otekwa monooli yaNamibia**

**September 2012**

**By**

**Lusia Heita**

Name / edhina: .....

Age/ eemvula.....

Gender/ uukwashike kookantu: .....

Ethnicity / uukwamuhoko: .....

Region / village / oshikandjohogololo/ omukunda: .....

Name (milk) / eedhina lyo mahini: .....

Type of equipment used for milking.....

Do you was milking Equipments' before milking: YES / NO

Type of equipment used for milk storage.....

How often do you wash the milking storage equipment .... ALWAYS /SOMETIMES / NEVER

Ingredients / (additives) / oshike hashi gwedhwamo momahini longithwa mokuninga omahini ngaka?

.....

Processing methods in details / omahini ngaka ohaga etwapo ngiini?

.....

.....

.....

Preservation method / omukalo gwokupungula:

.....

.....

Shelf life/ omihini ohagakwata ethimbo lyithike peni opo ganinge nai:

.....

.....

Does the milk have Social values?

Yes / eeheno	No / aahawe
--------------	-------------

Reasons for choosing that answer / etompelo lyokuhogolola eyamukulo ndjoka?

.....  
 .....  
 .....

**Can anyone drink/ eat this type of milk (i.e. age limit, pregnant, gender )? Keshe gumwe otavulu nga okulya/ okunwa oshikulya shika?**

<b>Yes / eeheno</b>	<b>No / ahawe</b>
---------------------	-------------------

**Reasons if the answer is NO / eetompelo ngele owahogolola ;ahawe:**

.....  
 .....

## **APPENDIX 2.PREPARATIONS OF CHEMICALS AND REAGENTS**

### **1. Real time PCR mixture**

Reagents	Final concentration	1×
Primers Forward	0.3 $\mu$ M	1.2 $\mu$ l
Reverse	0.3 $\mu$ M	1.2 $\mu$ l
Master mix	10 $\mu$ l	10 $\mu$ l
DNA templates	$\leq$ 500 ng	5 $\mu$ l
Nuclease free water	Up to 20 $\mu$ l	2.6 $\mu$ l
Total	20 $\mu$ l	20 $\mu$ l

### APPENDIX 3: CALCULATIONS

#### 1. Response Factor and Retention time of Organic acid Standards

Organic acid standards (100 ppm)	Retention time (R.T) (min)	Area	Response factor (RF)= Concentration / Area (AU) (ppm)
Butyric acid	1.465	20508.4	$4.88 \times 10^{-3}$
Acetic acid	1.605	2,904990	$1.12 \times 10^{-5}$
Lactic acid	1.547	432036.4	$2.3 \times 10^{-4}$
Propionic acid	6.205	3509622.3	$2.85 \times 10^{-5}$

#### Concentration of each acid in the sample

Type of milk	concentration of each acid in the sample $C_s = RF \times$ $A_s$ (area of the peak (AU) generated in the sample)			
	<b>Butyric acid</b> $4.88 \times 10^{-3}$	<b>Acetic acid</b> $1.12 \times 10^{-5}$	<b>Lactic acid</b> $2.3 \times 10^{-4}$	<b>Propionic acid</b> $2.85 \times 10^{-5}$
<i>Mabisi</i>	0.26	0.03145	0.1585	0
<i>Mabisi</i>	0.5931396	0.05909	0.261089	0.06885
<i>Mabisi</i>	0.090932	0.01546	0.240389	0.02104
<i>Omashikwa</i>	0.2368329	0.04442	0.16895	0.01078
<i>Omashikwa</i>	0.1426756	0.03842	0.35695	0.01325
<i>Omashikwa</i>	0.06455	0.0381	0.287376	0.0887
<i>Omashikwa</i>	0.1125522	0.05385	0.416824	0.04725
<i>Omashikwa</i>	0.1354165	0.04533	0.314245	0.03355
<i>Omashikwa</i>	0.229073	0.04905	0.319155	0.015737
<i>Mashini ghakushika</i>	0.415634	0.0348	0.24367	0.06358
<i>Mashini ghakushika</i>	0.1810538	0.0348	0.243785	0.08883
<i>Mashini ghakushika</i>	0.743832	0.0299	0.210231	0.0405
<i>Mashini ghakushika</i>	0.24514	0.01996	0.241685	0.00757

**APPENDIX4ANOVA TABLEs****DURATION OF FERMENTATION BETWEEN 3 TYPES OF MILK**

Analysis of variance

Variate: duration\_of\_fermentation

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
name_of_milk	2	0.8876	0.4438	0.54	0.588
Residual	28	22.9833	0.8208		
Total 30		23.8710			

**ANOVA TABLE: RELATIONSHIP BETWEEN THE TYPE OF EQUIPMENT USED  
FOR FERMENTATION AND DURATION OF FERMENTATION PROCESS**

Analysis of variance

Variate: duration\_of\_fermentation\_period

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
type_of_equipment_used_for_ferme	1	0.0614	0.0614	0.07	0.786
Residual	29	23.8095	0.8210		
Total	30	23.8710			

**ANOVA TABLE . PH OF MILK SAMPLES FROM SIX REGIONS**

Analysis of variance

Variate: ph

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
regions	4	1.04125	0.26031	3.83	0.023
Residual	16	1.08841	0.06803		
Total	20	2.12966			

**ANOVA TABLE. FERMENTED MILK SAMPLES OF THREE TYPES OF MILK  
(OMASHIKWA, MABISI AND MASHINI GHAKUSHIKA)**

Analysis of variance

Variate: pH

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
type_of_milk	2	0.95788	0.47894	7.36	0.005
Residual	18	1.17178	0.06510		
Total 20		2.12966			

Analysis of variance

Variate: C\_f

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
_units_ stratum isolates	19	75.417	3.969	3.53	0.399
Residual	1	1.125	1.125		
Total 20		76.542			

Analysis of variance

Variate: E\_c

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
_units_ stratum isolates	19	36.6429	1.9286	15.43	0.198
Residual	1	0.1250	0.1250		
Total	20	36.7679			

Analysis of variance

Variate: S\_a

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
_units_ stratum isolates	19	29.042	1.529	0.25	0.940
Residual	1	6.125	6.125		
Total	20	35.167			

**Analysis of variance**

Variate: G\_k

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
_units_ stratum					
isolates	19	17.185	0.904	0.20	0.962
Residual	1	4.500	4.500		
Total	20	21.685			

**HYDROGEN PEROXIDE PRODUCTION BY LAB ISOLATES**

Analysis of variance

Variate: Hydrogen\_peroxide\_mg\_L

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
LAB_species	4	0.037314	0.009328	2.14	0.119
Residual	17	0.073936	0.004349		
Total	21	0.111250			

**ANOVA TABLE: ORGANIC ACID CONCETRATION ( HPLC)**

<b>1. Variate: Lacticacid</b>					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
_units_ stratum					
type_of_milk	2	0.2219	0.11095	4.9	0.033
Residual	10	0.22634	0.02263		
Total	12	0.44824			

<b>2. Variate: ButyricAcid</b>					
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
type_of_milk	2	0.15106	0.07553	2.2	0.162
Residual	10	0.34361	0.03436		
Total	12	0.49467			

<b>3. Variate: Aceticacid</b>					
<b>Source of variation</b>	<b>d.f.</b>	<b>s.s.</b>	<b>m.s.</b>	<b>v.r.</b>	<b>F pr.</b>
type_of_milk	2	0.000569	0.000284	2.17	0.164
Residual	10	0.001307	0.000131		
Total	12	0.001876			

<b>4. Variate: Propionic acid</b>					
<b>Source of variation</b>	<b>d.f.</b>	<b>s.s.</b>	<b>m.s.</b>	<b>v.r.</b>	<b>F pr.</b>
type_of_milk	2	0.000837	0.000419	0.4	0.682
Residual	10	0.010539	0.001054		
Total	12	0.011376			

## APPENDIX 5

Table1. Factors affecting fermentation of the three types of milk

Type of milk	factors affecting fermentation		
	Type of container	Raw materials	Temperature
<i>Mabisi</i>	40.00%	0.00%	60.00%
<i>Mashini ghakushika</i>	16.67%	33.33%	50.00%
<i>Omashikwa</i>	25.00%	30.00%	45.00%
<b>Grand Total</b>	<b>25.81%</b>	<b>25.81%</b>	<b>48.39%</b>

Table 2.Milk handling and Hygiene

Regions	Washing of equipments before milking		Washing of udder before milking		Let the calf suckle before milking	
	yes	no	yes	no	yes	no
Oshana	0	6	0	6	6	0
Omusati	1	3	0	4	4	0
Oshikoto	2	3	0	5	5	0
Ohangwena	1	4	0	5	4	1
Kavango	1	5	0	6	5	1
Zambezi	2	3	0	5	4	1

Table 3.Fermentation Conditions

region	Days of fermentation		Factors affecting fermentation		
	mean	error	temperature	container	Raw materials
<b>Oshana</b>	<b>4.167</b>	<b>0.307</b>	<b>2</b>	<b>3</b>	<b>2</b>
<b>Omusati</b>	<b>3.5</b>	<b>0.65</b>	<b>2</b>	<b>0</b>	<b>2</b>
<b>Oshikoto</b>	<b>4.2</b>	<b>0.37</b>	<b>3</b>	<b>0</b>	<b>2</b>
<b>Ohangwena</b>	<b>3.8</b>	<b>0.37</b>	<b>2</b>	<b>3</b>	<b>0</b>
<b>Kavango</b>	<b>4.167</b>	<b>0.477</b>	<b>3</b>	<b>1</b>	<b>2</b>
<b>Zambezi</b>	<b>4.4</b>	<b>0.24</b>	<b>3</b>	<b>2</b>	<b>0</b>

## APPENDIX 6

## API 50 CHL Results

Sample no	API results	% ID
S 2 I1	<i>Lactobacillus rhamnosus</i> <i>Lactobacillus plantarum</i> 1	99.6 0.3
S 5 I 1	<i>Lactobacillus rhamnosus</i> <i>Lactobacillus plantarum</i> 1	98.4 0.8
S9 I3	<i>Lactococcus lactis</i> ssp. <i>lactis</i> 1 <i>Pediococcus pentosaceus</i> 1	94.7 3.0
S 16 I 1	<i>Lactobacillus paracasei</i> ssp. <i>paracasei</i> 1 <i>Lactobacillus plantarum</i> 1	65.0 32.7
S 18 I 1	<i>Lactobacillus rhamnosus</i> <i>Lactobacillus paracasei</i> ssp. <i>paracasei</i> 1	99.9 0.1
S 1 I2	<i>Lactobacillus rhamnosus</i>	U P
S 4 I 1	<i>Lactobacillus rhamnosus</i> <i>Lactobacillus plantarum</i> 1	99.9 0.1
S 7 I 2	<i>Lactobacillus rhamnosus</i> <i>Lactobacillus plantarum</i> 1	99.5 0.3
S 11 I 3	<i>Lactobacillus plantarum</i> 1 <i>Lactobacillus rhamnosus</i>	97.6 2.2
S 17 I 3	<i>Lactobacillus plantarum</i> 1 <i>Lactobacillus rhamnosus</i>	99.9 0.1
S 20 I 1	<i>Lactobacillus plantarum</i> 1 <i>Lactobacillus pentosus</i>	93.6 6.3
S 3 I2	<i>Lactobacillus plantarum</i> 1 <i>Lactobacillus rhamnosus</i>	99.8 0.1
S 8 I 2	<i>Lactobacillus plantarum</i> 1 <i>Lactobacillus pentosus</i>	59.1 40.8
S 10 I 1	<i>Lactobacillus plantarum</i> 1 <i>Lactobacillus pentosus</i>	50.2 49.6
S 12 I 1	<i>Lactobacillus plantarum</i> 1 <i>Lactobacillus pentosus</i>	59.1 40.8
S 13 I 1	<i>Lactobacillus plantarum</i> 1 <i>Lactobacillus pentosus</i>	59.1 40.8
S 15 I2	<i>Pediococcus pentosaceus</i> 2 <i>Lactobacillus brevis</i> 1	99.7 0.2
S 17 I 1	UP	UP
S 19 I1	<i>Lactobacillus plantarum</i> 1	99.9
S 20 I 3	<i>Lactobacillus rhamnosus</i> <i>Lactobacillus plantarum</i> 1	NO %

UP: Un-accepted Profile