
**UNIVERSITY OF BOTSWANA**

# Isolation and characterization of Lactic Acid Bacteria and indicator microorganisms of food safety from *Omaere*, a traditionally fermented milk product in Botswana.

**By**

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# DEDICATION

This work is dedicated to my son Rorisang Liam-Leyton Plaatjie and my mother Baikgatlhi Soo Plaatjie.

# DECLARATION

The material contained in this thesis is my own original work except where due reference is made. It has not been and shall not be submitted for the award of any degree at any other institute of higher learning.

…….. ……

Student`s signature Date signed

# CERTIFICATE

This thesis has been approved as meeting the required standards of scholarship of partial fulfilment for the degree of Master of Science (Applied Microbiology), University of Botswana.

Supervisors' signatures Date signed

DR. Leteane: _____ ……..

DR. M.I. Matsheka _____ …….

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AAB | Acetic Acid Bacteria |
| API | Analytical Profile Index |
| BITRI | Botswana Institute for Technology Research and Innovation |
| BLAST | Basic Local Alignment Search Tool |
| DG 18 | Dichloran 18% Glycerol Agar |
| DRBC | Dichloran Rose Bengal Chloramphenicol |
| EMBA | Eosin Methylene Blue Agar |
| FAO | Food and Agriculture Organization |
| GRAS | Generally Recognized as Safe |
| LAB | Lactic Acid Bacteria |
| MRS | Man Rogosa Sharpe |
| NCBI | National Center for Biotechnology Information |
| NFTRC | National Food Technology Research Center |
| NSLAB | Non-Starter Lactic Acid Bacteria |
| dNTP | Deoxyribonucleotide triphosphate |
| PCR | Polymerase Chain Reaction |
| RFLP | Restriction Fragment Length Polymorphism |
| RAPD | Random Amplification of Polymorphic DNA |
| REP | Repetitive Element Palindromic |
| rBST | recombinant bovine somatotropin |
| TBE | Tris-borate buffer |
| WHO | World Health Organization |

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# ABSTRACT

Botswana has many traditional foods and beverages that are prepared by means of natural/spontaneous fermentation. The traditional preparation of these foods is for the most part largely undocumented and the microorganisms involved in the fermentation process unknown. The main objective of this study was to isolate and characterize dominant culturable lactic acid bacteria (LAB) strains associated with the end-product fermentation of *Omaere*, a fermented milk product popular with the Herero tribe in Botswana. In addition to this, the study also aimed to investigate the presence of selected pathogens and microbial indicators of poor sanitary quality in the *Omaere* samples. Twelve *Omaere* samples were collected from domestic households in two geographical regions of Botswana (Ghanzi and North-Western districts of Botswana). MRS agar was used to isolate lactic acid bacteria, and standard microbiological, biochemical and morphological tests were used to categorize the isolates into 14 phenotypic groups based on similar biochemical, physiological and morphological characteristics. These isolates were further identified to species level using the API 50 CH system (bioMerieux, France). The isolates that had low identification percentages using the API 50 CH system, were subjected to 16S rRNA gene sequence analysis to confirm their identity. Bacterial pathogens, fungi and microbial indicators of sanitary quality were isolated using seven selective media; Eosin Methylene Blue Agar (EMBA) for the detection of coliforms, Mannitol Salt Agar (MSA) for isolation of *Staphylococcus aureus*, Sabouraud Dextrose Agar (SDA), Dichloran Glrycerol agar (DG-18) and Dichloran Rose Bengal Chlorotetracycline (DRBC) for isolation of fungi and some yeasts. Corn meal Agar was used for isolation of yeasts while *Bacillus cereus* Agar was used for isolation of *Bacillus cereus*. *Lactobacillus plantarum* was found to be the most dominant species of lactic acid bacteria in the *Omaere* samples, having been isolated from all the samples, with *Lactobacillus rhamnosus* being

the second most dominant LAB species with 58% percentage distribution across samples. Other bacterial species commonly associated with *Omaere* fermentation were *Enterococcus faecium* and *Lactobacillus helveticus* with 25% and 17% distributions, respectively. No pathogens or bacterial indicators of poor sanitary quality specific to the seven selective media used were isolated from the *Omaere* samples. No yeast and fungi were isolated from the traditional fermented milk product.

**KEY WORDS**: Lactic acid bacteria, *Omaere*, Fermentation, Characterization, Identification, Sanitary quality

# CHAPTER 1

## INTRODUCTION

Botswana like other developing countries in Africa has many traditional foods and beverages that are prepared by means of natural/spontaneous fermentation. Popular examples include fermented milk such as sour milk (*madila*), cereal fermented alcoholic and non-alcoholic beverages, slurries and porridges such as *chibuku* and *mageu*, which are not only produced traditionally but commercially as well. *Ting*, a fermented sorghum slurry used in the preparation of porridge, is another popular example of a fermented food product indigenous to Botswana.

Although most consumers of dairy products are known to do so for nutritive purpose, interest in the health benefits associated with the consumption of fermented milk products is growing. These beneficial effects can be seen in the variety of bioactive compounds of lactic acid bacteria (Griffiths and Tellez, 2013). Some of the health benefits that are thought to come with consumption of fermented/probiotic dairy products include; alleviation of lactose intolerance, protection against gastrointestinal infection, anti-carcinogenic effect, alleviation of constipation, anti-hypertensive activity, immune system stimulation and anti-allergenic qualities (Olson, 1990; Urbach, 1995; Wouters *et al*., 2002; Ali, 2010; Panesar, 2011; Griffiths and Tellez, 2013;).

*Omaere* is a traditional fermented food that is widely consumed in Namibia and in the North-Western areas of Botswana by the Ovaherero and Ovambanderu tribes. These tribes are found in local villages/settlements such as Toromoja, Rakops, Makunda, Makakung, regions of Ngamiland and regions of Ghanzi. The traditionally fermented product is made from cow milk, almost in the

same manner as *Madila*; a traditional fermented milk product consumed in various areas of Botswana and the Southern African region. *Madila* is an unsweetened curd with a consistency that is slightly thicker than yoghurt and with an acidic pH (Todorov, 2008). In South Africa and Zimbabwe they call this fermented milk product *Amasi*. In areas where it's most common, *Omaere* is seen as a variation of *Madila* because in the former the curd and whey are not separated as is the case with the latter. This makes the consistency of *Omaere* less dense (lighter) than *Madila* and slightly less acidic.

The fermentation of *Madila* has been found to be dominated by lactic acid bacteria such as *Lactobacillus plantarum*, *Lactobacillus helveticus*, *Lactobacillus lactis* subsp *lactis* and *Leuconostoc lactis* among others (Beukes *et al*., 2001; Todorov, 2008). Like *Madila*, *Omaere* can be consumed with sorghum-meal porridge, maize meal (which the tribes called *Osopa*) and thick sorghum meal. In its raw form *Omaere* is a preference for the Ovaherero and Ovambanderu tribes as a weaning food for infants and toddlers and also exploited in alleviation of diarrhea in young children. There is also a claim that *Omaere* provides energy and enhances libido in adults and therefore is fed to adult males at night to improve their performance (Sekwati-Monang, personal communication with *Omaere* producing communities, 2011).

*Omaere*, like *Madila*, is prepared from raw cow milk. It can be considered to be sour milk that has a higher proportion of whey and the rest being curd. The Ovaherero and Ovambanderu tribes of Botswana and Namibia describe it as milk that has gone bad, ''spoiled milk'', a literal translation used in derivation of the ethnic names of the product. During *Omaere* production, raw cow milk

is allowed to stand for 3 to 5 days at room temperature in a container, either a plastic one or a gourd called Ondjoupa locally. During this process LAB are believed to take part in a fermentation process that occurs in different stages/phases. To enhance the taste and accelerate fermentation, plant material may be added to the milk; leaves are believed to be added for taste while roots and bark can be added to accelerate fermentation. Roots of plants such as *Omunkunzi* (*Boscia albitrunca*) or *Motopi* as is known in Setswana and *Mongana* (*Acacia mellifera*), are popular among some of the Herero communities, with regards to *Omaere* fermentation. This is an optional step, as the milk will still ferment without the plant material, though maybe much slower. The *Motopi* root is believed to also reduce the sourness of the milk and enhances the thickness of *Omaere* and its taste. The root is dried, crushed and the powder added at the bottom of the calabash prior to pouring milk. If at the end of fermentation process the consumer feels it is too sour to the point where you can't enjoy it, fresh raw milk is added as a buffer to the acidity. Figure 1.1 shows a summarized process of the traditional fermentation of *Omaere*, in a flow diagram. (Sekwati-Monang, personal communication with *Omaere* consuming communities, 2011).

The Baherero locals seem to have also learnt the 'science' of what could be termed traditional starter cultures as they believe that one never drinks the entire batch made, but always lives a little behind. To this, raw milk is added to start the fermentation process over again but not from scratch (back slopping).

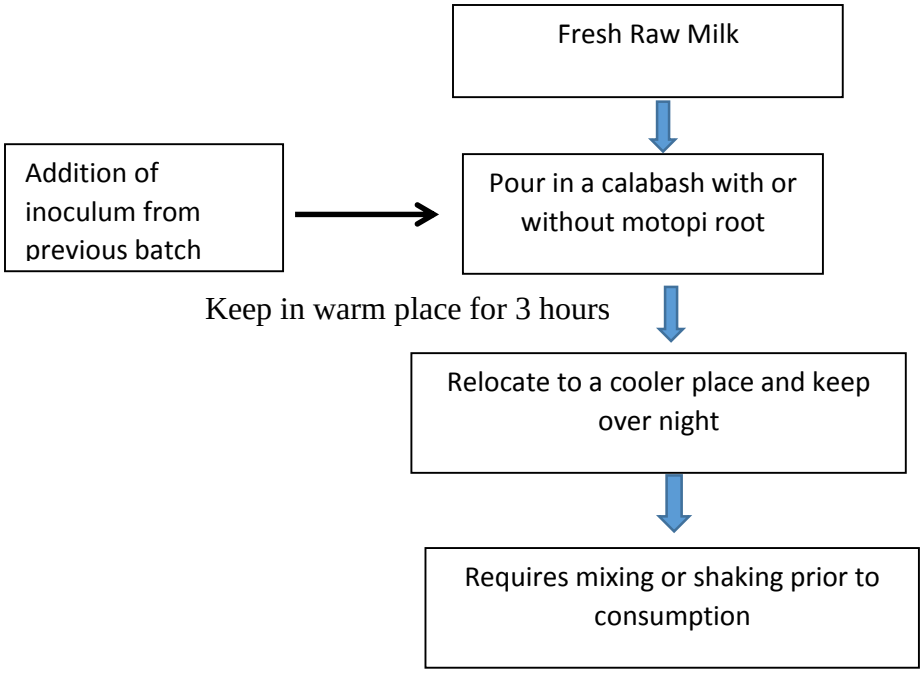


Figure 1.1. Flow chart depicting traditional fermentation of *Omaere.*

### 1.1. Statement of the problem

Food technology and processing is seen as a potential avenue of increasing self-reliance in food production and diversifying the economy of Botswana. Commercialization of indigenous fermented food products is one viable way to help realize these goals. Traditional recipes can be improved and marketed commercially; *Omaere* is one such food product. The problem lies in that there is lack of information and research on the microbial flora and the dynamics involved in fermentation of *Omaere*. Therefore, this research project mainly addresses the characterization of lactic acid bacteria in fermented *Omaere.* In addition, *Omaere* is prepared at household level without any measures of monitoring the sanitary conditions under which it is prepared, hence the

need for identifying some of the indicator microorganisms of sanitary quality/food safety that may be present in the samples collected in this study.

### 1.2. Main Objective

Isolate and characterize dominant culturable lactic acid bacteria strains from samples of *Omaere* collected from two regions in Botswana.

#### 1.2.1. *Specific Objectives*

- Enumerate LAB present in the collected *Omaere* samples.
- Characterize and determine the diversity of LAB strains isolated from *Omaere* samples using biochemical and molecular techniques.
- Identify, isolate, and characterize food safety indicators that may be present in *Omaere* samples.

### 1.3. Significance of the study

The microbial characterization of lactic acid bacteria in *Omaere* will be the first step in the investigation of bacteriocins, which lately have been used to extend shelf life of perishable food products. In addition, the quality and safety of *Omaere* will be ascertained so that it can be used as a weaning food. The characterized LAB could also identify potential starter cultures for the commercial exploitation of this product. It would enable manipulation of the starter cultures, to

improve texture and taste. The local producers, which are mainly small and medium enterprise-based (SME-based), would benefit greatly from this endeavor. Stakeholders such as the National Food Technology Research Center (NFTRC) could be tasked with research related to development of the starter cultures to facilitate commercialization.

### 1.4. Justification of the study

Probiotic bacteria have a long history of association with dairy products and the potential health benefits of probiotics to consumers have been documented in numerous studies (Feresu and Muzondo, 1990; Keller and Jordan, 1990; Mathara *et al.*, 2004). Traditionally fermented milk curd from Southern Africa *Madila/Amasi/Mafi* have been successfully commercialized. *Omaere* as a semi-curd, also has the potential to be ranked under these desirable traditional foods. Determining the LAB flora that contributes to the fermentation of *Omaere* is essential as this fermented dairy product could also serve as a mode of delivery for probiotic bacteria.

# CHAPTER 2

## LITERATURE REVIEW

For many people engaged in subsistence stock farming, traditionally prepared dairy products are important sources of food. Milk has been preserved since early times by fermentation and many of the traditionally fermented milk products were made in Asia, Africa, the Middle East, and northern and eastern Europe. The microbiological characteristics of several fermented milk products have been studied in Indonesia (Yodoamijoyo *et al*., 1983; Hosono *et al.*, 1989), in Zimbabwe (Feresu and Muzondo, 1990), in South Africa (Keller and Jordan, 1990; Beukes *et al*., 2001), in Morocco (Hamama, 1992), in Tanzania (Isono *et al*., 1994). Some of the major fermentation processes are based on the use of lactic acid bacteria, which produce organic acids. The presence of fermentative Lactic acid bacteria is crucial to the intrinsic properties of fermented food products (Thomas, 1985; Tamine and Robinson, 1988; Mckay and Bal780dwin, 1990; Soomro *et al*., 2002).

In today's technological era, the major concern of food science is to identify ways of improving the health of consumers, by providing safe and nutritious foods. To reach this goal, different methods of preservation are exploited in the food industry such as salting, drying, high/low temperature treatment, fermentation, and more recently, pulsed electric field, high pressure and radiation - alone or in combination (Kongo, 2013). Preference of methods depends on the type of raw materials, availability of the method, cost, effectiveness and degree of change it causes to the flavor and nutritional features of the food product. Fermentation, which is also called bio-preservation, is a cheap, widely accessible method that meets today's increasing consumer´s

demand for minimally processed/preserved food products. Fermentation of lactic acid bacteria is indeed one of the oldest and highly efficient forms of non-thermal processing method. Cheese production is based on LAB ability to ferment sugars, especially glucose and galactose, so to produce lactic acid and aroma substances that give typical flavors and tastes to fermented products. (Kongo *et al*, 2007).

The WHO food safety unit prioritized research on fermentation as a technique for preparation/storage of food. This is because of the deaths of one tenth of children under five years due to dehydration, mainly caused by incidences of diarrhoea. The main cause of diarrhoea has been found to be ingestion of food that doesn't meet the standard regarding hygienic conditions. The hygienic standard of a food is based on the processing and handling of the food, as well as on the conditions of the raw materials. Lactic acid fermentation of food has been found to reduce the growth of pathogenic microorganisms in the food (Motarjemi *et al*., 1993).

## 2.0. Lactic acid bacteria

Lactic acid bacteria are a group of phylogenetically diverse Gram-positive bacteria that are considered to have GRAS (Generally Recognized As Safe) status and play an important role in food and feed fermentation and preservation, either as the natural microflora or as starter cultures added under controlled conditions (Stiles and Hastings, 1991). The genera included here are *Lactococcus, Streptococcus, Lactobacillus, Pediococcus, Leuconostoc, Enterococcus, Carnobacterium, Aerococcus, Oenococcus, Tetragenococcus, Vagococcus*, and *Weisella* (Wigley, 1999). These bacteria are widely distributed in nature, and are found in environments rich in

carbohydrate substrates, such as food and feed, but also in human and animal cavities, and in sewage and plant material. Indeed, strains of lactic acid bacteria have been isolated from all these environments (Kandler *et al*., 1986).

Lactic acid bacteria are characterized by some common morphological, metabolic and physiological traits; they are nonmotile, non-spore forming, rod-and coccus-shaped organisms that can produce chiefly lactic acid as the major end-product of fermentation. The physiological properties of LAB make them very attractive to food microbiologists; they have resistance to bacteriophages (Wigley, 1999), proteolytic activity, can ferment lactose and citrate, have a high resistance to freezing and lyophilization, are non-toxic and possess the capacity to adhere to and colonize the digestive mucosa (Ananou *et al*., 2007). Another interesting characteristic of these bacteria that is beneficial to food microbiologists is the ability of the organisms to produce an array of antimicrobial substances. This property makes them ideal in improving the safety and sanitary quality of food.

These substances include antimicrobial active metabolites such as such as organic acids (lactic and acetic) which result in lowering of pH, production of hydrogen peroxide, reuterin, diacetyl, CO2 and antimicrobial peptides known as bacteriocins.

### 2.1. Bacteriocins of Lactic acid bacteria

Delves-Broughton *et al*, (1996), defines Bacteriocins as proteinaceous ribosomally synthesized peptides that have bactericidal or bacteriostatic effects against bacteria usually closely related to the producer organism. Producer organisms are immune to their own bacteriocin(s), a property

mediated by specific immunity proteins (Cotter *et al*., 2006). Bacteriocin producing lactic acid bacteria have received particular interest due to their potential application in the food industry as natural preservatives, given that they have the advantage of being reliable, non-toxic to eukaryotic cells and rapidly digested by proteases within the gastrointestinal tract (Parada *et al*., 2007). Bacteriocins produced by LAB are increasingly becoming popular in research because of their anti-bacterial activity against food-borne spoilage and pathogenic bacteria. Most of the LAB bacteriocins identified so far are small, thermo stable cationic molecules with many attractive characteristics that make them suitable candidates for use as food preservatives. Nisin as a bacteriocin is the classic example, and undoubtedly the most extensively studied bacteriocin, with the ability to prevent spoilage of processed and natural cheeses, extend the shelf life of milk in warm countries, prevent the growth of spoilage lactobacilli in beer and wine fermentations and provide additional protection against *Bacillus* and *Clostridial* spores in canned foods (Soomro *et al*., 2002). Other LAB bacteriocins include lactacins, lactococcins, lactacins, enterocins, pediocins and sakacins.

### 2.2. Uses of Lactic acid bacteria

Lactic acid bacteria are industrially significant organisms renowned for their fermentative ability as well as their health and nutritional benefits (Gilliand, 1990). Species popular in food fermentations belong to the genera *Lactococcus, Streptococcus, Pediococcus, Leuconostoc, Lactobacillus*, and the newly recognized *Carnobacterium*. Once used to retard spoilage and preserve foods through natural fermentations, they have found commercial applications as starter cultures in the dairy, baking, meat, vegetable, and alcoholic beverages industries. They produce various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocins or

bactericidal proteins during lactic fermentations (Lindgren and Dobrogosz, 1990). Not only are these components of the lactic acid bacteria desirable for their effects on food taste, smell, color and texture, but they also inhibit unwanted microflora. (Rattanachaikunsopon and Phumkhachorn, 2010)

The majority of food items contain microorganisms of different types and in different amounts. The group of microorganisms that will dominate depends on several factors, and sometimes microorganisms such as lactic acid bacteria, will outnumber the other organisms and inhibit their growth (Fleming & McFeeters, 1981). Adams (1990) suggested that lactic acid bacteria are inhibitory to many other microorganisms when they are cultured together, and this is the basis of the extended shelf life and improved microbiological safety of lactic-fermented foods. Pathogens responsible for food borne diseases such as *Mycobacterium tuberculosis* and *Brucella spp* have in studies been found to be present in raw milk; and lactic acid fermentation proved in some of those studies to play a role in the elimination of the pathogens in raw milk. This elimination of *M. tuberculosis* in raw, fermented milk appears to be attributable to the resident LAB microflora, through the mechanisms of lactic acid and bacteriocins production (Cotter *et al*., 2006; Mariam, 2014). In a comparative study by Gavrilova *et al*., (2015) antagonistic activity of lactic acid bacteria against five reference strains of *Brucella* was investigated; it was revealed that the six active cultures had pronounced antagonistic activity against *Brucella* of all kinds. Consequently, LAB are being used in the food industry as starter cultures, for enhancement of food safety and extension of shelf-life

### 2.2.1. *Lactic acid bacteria as probiotics.*

Increasing knowledge of lactic acid bacteria physiology, together with new developments in processing technology, is leading to their application beyond the narrow scope of traditional starter culture application. LAB are now being used in new food safety roles that directly benefit human health (Kongo, 2013). Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on host" (the Food and Agriculture Organization/World Health Organization (FAO/WHO). Most probiotics commercially available today belong to the genera *Lactobacillus* and *Bifidobacterium* (Phillip *et al*., 2012). Several mechanisms by which probiotics mediate their health benefits on the host have been suggested, and can be divided into three categories; (i) probiotics that have antimicrobial activity and can exclude or inhibit pathogens; (ii) probiotic bacteria that can enhance the intestinal epithelial barrier; and (iii) probiotic bacteria are believed to modulate the host immune response (Ezendam and Loveren, 2006; Marco *et al*., 2006; Lebeer *et al*., 2008; Lebeer *et al*., 2010).

### 2.2.2. *Lactic acid bacteria in production of dairy and non-dairy products*

Milk is a highly perishable food/raw material and does not have a long shelf life. With this in mind, its transformation to cheese or other form of fermented dairy product, is an ideal way to prolong its shelf life and preserve its valuable nutrients, thus making it available throughout the year. Production of cheese is based on application of defined or undefined lactic acid bacteria starter cultures that are expected to cause a rapid acidification of milk through the production of lactic acid. This in turn lowers pH, which affects a number of aspects of the cheese manufacturing process and in the end cheese composition and quality (Briggiler-Marco *et al*., 2007). Studies

directed to characterize traditional cheeses have found that those made from raw milk harbor a diversity of LAB (Bernardeau *et al*., 2008) depending on geographical region. A few may show interesting technological features that upon optimization may have industrial applications (Buckenhiiskes, 1993).

In a study by Camu *et al*, (2007), an investigation into the dynamics and biodiversity of populations of LAB and AAB involved in Spontaneous Heap Fermentation of Cocoa Beans in Ghana, a non-dairy product, showed that *Lactobacillus plantarum* and *Lactobacillus fermentum* were the most dominant LAB species in the Ghanaian cocoa bean heap fermentations performed. Sourdough is another noteworthy non-dairy product that has a large application for bakery products, for examples the production of sourdough bread, classical bread, snacks, pizza and sweet baked products (De Vuyst and Vancanneyt, 2007). The incorporation of sourdough in baking technological traits, enhances sensorial characteristics, increases shelf life and improves nutritional properties (Arendt *et al*., 2007; Corsetti *et al*., 2000). Studies have shown that the LAB microbiota that is predominant in Sourdough fermentation is represented, principally, by members of the genus *Lactobacillus*. Yeasts are generally counted in lower levels and can even be absent. The major part of the yeasts belong to the genera *Saccharomyces* and *Candida* (Vera, 2012).

### 2.2.3. *Lactic acid bacteria in production of traditional foods in different countries*

*Omaere* is believed to be a variant of *Madila* by some researchers. *Madila* is a popular fermented food product of Botswana and other Southern African countries. The traditional brewing of *Madila* is a slow process that requires patience. Milk is strained using a clean white cloth to remove

exogenous particles, and the clean milk is then kept in a clean covered enamel pail and allowed to ferment spontaneously for one day. The fermented milk is then poured into a cotton sack (*lekuka*) which is then hung on a tree. On fermentation, the milk separates into curd and whey. The *lekuka* allows the whey (tlhoa) to drain off from the fermenting milk, thereby resulting in the formation of a thick sour cream regarded as a delicacy in many Tswana societies. Afterwards, variations in flavor may be made by the addition of various fruits including oranges, mangoes or marula – a Southern African fruit (Ohenhen *et al*., 2013).

Ohenhen *et al*., (2013) investigated the strains of lactic acid bacteria in *Madila*. Characterization of the isolates based on morphological, physiological, biochemical and carbohydrate fermentation tests resulted in identification of six species of lactic acid bacteria; *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, *Lactococcus lactis*, *Lactobacillus fermentum* and *Lactobacillus brevis*. Other non-lactic acid bacteria identified were *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus*. It was observed that the counts of non-lactic acid bacteria reduced progressively as the number of lactic acid bacteria increased.

The rural communities of the Zulu and the Xhosa in South African also ferment milk in this manner to produce what they call *Amasi*, which is their version of *Madila*. It is prepared at home by storing unpasteurized cow's milk in a calabash or a hide sack, and allowing it to ferment. The milk separates out into a thick white substance called *Amasi* and a thin water liquid called *Umlaza.* Traditionally, the required amount of this product is removed from the top of the dish for consumption and more milk is added to the rest of it in the dish. The above mentioned leftover

amount acts as a culture for the fresh milk (back slopping). Commercial *Amasi* preparations involves the use of *Lactococcus lactis* subsp lactis and *Lactococcus. lactis* subsp *cremoris* to culture milk. Pasteurized milk is used to increase the shelf life of the product for up to 21 days (Feresu and Muzondo, 1990).

In Zimbabwe, a neighboring country to Botswana, the rural population also ferment their milk traditionally to make their version of *Madila, (Amasi*). Fresh unpasteurized cow's milk is allowed to stand at ambient temperature, in an earthenware pot loosely covered by a plate. This allows microorganisms inherent in the milk, from the pot, and from the surrounding air to ferment the milk. The fermentation takes one to two days depending on the ambient temperature (20 to 25°C). The fermented milk has an estimated shelf life of three days at ambient temperature (Feresu and Muzondo, 1989). In response to the urban population's desire for fermented milk, the Zimbabwe Dairy Marketing Board also produces a fermented milk called *Lacto,* but on an industrial scale. Milk is standardized, pasteurized and inoculated with 1.2 percent of an imported mesophilic starter culture. The milk is immediately packaged into sachets, left to ferment at ambient temperature for 18 hours, and stored at 5°C ready for the market. The shelf life of refrigerated *Lacto* is 7 days (Feresu and Muzondo. 1990).

A study was carried out to compare the two; it characterized 10 predominant lactic acid bacterial isolates from traditionally fermented milk and four isolates from *Lacto*. All the isolates from traditionally fermented milk belonged to the genus *Lactobacillus*. Seven of the isolates could be identified as belonging to L. *helveticus, L. plantarum, L. delbrueckii* subspecies *lactis* (two

isolates), *L. casei* subsp. *casei* (two isolates) and *L. casei* subsp. *pseudoplantarum*. Three of the isolates could only be identified as either *betabacteria* or *streptobacteria*. The four isolates from *Lacto* were identified as *Lactococcus lactis* (Feresu and Muzondo, 1990).

The Fulani of Burkina Faso, also ferment their milk in calabashes, gourds and clay pots. These containers need to be seeded with a natural microbial inoculum before its use in the production of fermented milk. Containers filled with fresh milk are covered and placed in the house. The milk coagulates, and the whey and proteins are then homogenized (Savadogo *et al*., 2001). Generally, the species identified during investigation of the fermentation process of milk by the Fulani were found to be *Lactobacillus plantarum, Lactococcus lactis* ssp. *lactis*, *Lactobacillus delbrueckii* subsp *lactis*, and *Leuconostoc lactis.*

The origin, development and succession of a particular microbial community in any food are governed by its ecological factors (Deak and Beuchat, 1996), which influence the physiological expression of microbial cells. Thus, strategies for food processing and preservation can be developed on the basis of ecological factors associated with specific foods and beverages (Gould, 1992). Ethiopian *borde* is a spontaneously fermented, low or non-alcoholic cereal beverage. It is produced from a variety of locally available cereal ingredients using traditional techniques. *Borde* fermentation has indicated an association of lactic acid bacteria, composed of heterofermentative *Lactobacillus* strains and homofermentative *Pediococcus* spp, involved from the beginning to the end of *borde* fermentation. A wider diversity of LAB strains are often present at the early period of maize dough fermentation at Phase I than any other phases of *borde* fermentation. The diversity

of species is seen to lower as the fermentation progresses and finally, *W. confusa*, *L. brevis*, *L. viridescens* and *P. pentosaceous* spp were the most significance species (Abegaz, 2006).

In East African countries such as Kenya and Somalia, raw camel milk product or spontaneously fermented milk such as *suusacee* play an important role in the daily diet of pastoralists (Farah *et al*, 2007). *Suusacee* fermentation is spontaneously initiated through continuous utilization of the same fermentation gourds or vessels at ambient temperatures over one to two days. It results in a microflora dominated by lactic acid bacteria probably originating from the vessel surface (Lore *et al*., 2005). Generally, the microflora of raw and fermented camel milk products has been reported as a mix of different species of typical dairy bacteria, e.g. *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus casei, Lactococcus lactis* subsp. lactis, *Enterococcus faecium*, and *Streptococcus thermophilus* (Lore *et al*., 2005; Abdelgadir *et al*., 2008; Khedid *et al*., 2009).

In the Rwandan community, a fermented milk product called *Kivuguto* is popular. The typical flora that creates the traditional sour milk *kivuguto,* is very complex and varies from one location to another. This sour milk has a lot of similarities with some fermented milk products of different tribes across Africa; *Kule-naoto*, a traditional fermented milk of Kenya (Mathara *et al*., 2004), the Masai fermented milk in Northern Tanzania (Isono *et al*., 1994), and a traditional fermented milk of Zimbabwe, *Amasi* (Feresu, 1992).

The dominant lactic acid bacteria of *Kule-naoto* belong to the genus *Lactobacillus*, followed by *Enterococcus, Lactococcus* and *Leuconostoc* (Mathara *et al*., 2004). Isono *et al*. (1994) found that

the major microorganisms of the Masai fermented milk in Northern Tanzania consist of *Lactococcus lactis* ssp. *lactis* and *Lactobacillus confus.* A similar study was carried out on traditional fermented milk of Zimbabwe, *Amasi* (Feresu, 1992). Assays for formulating a starter culture for the production of *Amasi* were conducted with isolates of *Lactococcus lactis* subsp. *lactis biovar lactis*, *Lactobacillus plantarum* and *Leuconostoc mesenteroides* subsp. *mesenteroides)* (Mutukumira *et al*., 1995). In Sudan, a combination of lactic acid bacteria and yeasts is responsible for the fermentation of the traditional fermented milk, *Rob*. The predominant lactic acid bacteria in its fermentation have been found to be *Lactobacillus fermentum*, *Lactobacillus acidophilus*, *Lactococcus lactis* and *Streptococcus salivarius* (Abdelgadir *et al*., 2001). The predominant yeasts have been identified as *Saccharomyces cerevisiae* and *Candida kefyr* (Abdelgadir *et al*., 2001). This association of lactic acid bacteria and yeasts is also found in the fermented milk of Ethiopia, and in the *kefir* and *koumis* milks of Eastern European countries. The taste of these fermented milks displays an alcoholic flavor and is less sour, properties that are not well appreciated in Central and East Africa.

### 2.3. Identification and characterization of Lactic acid bacteria

To characterize new lactic acid bacteria isolates, phenotypic methods which rely on morphological, physiological or biochemical criteria have been widely applied (Montel *et al*., 1991; Kongo *et al*., 2007). Phenotypic characterization classifies LAB on the basis of their cellular morphology, mode of glucose fermentation, growth at different temperatures, lactic acid configuration, the fermentation of various carbohydrates, the methyl esters of fatty acids, and the pattern of proteins in the cell wall or in the whole cell. Unfortunately, the phenotypic methods have limitations such as poor reproducibility, the ambiguity of some techniques, the extensive

logistics for large-scale investigations and their poor discriminatory power (Hammes and Vogel, 1995). Another disadvantage of phenotypic analysis is that the whole information potential of a genome is never expressed, i.e., gene expression is directly related to the environmental conditions (e.g., the growth conditions in the laboratory). All these mentioned issues affect the reliability of phenotype-based methods for culture identification at the genus or species level (Mohania *et al*., 2008).

Molecular (genotypic) methods on the other hand have much greater discriminatory power, all the way to differentiation of individual strains (Cocolin *et al*., 2004; Furet *et al*., 2004; Aymerich *et al*., 2006; Prabhakar *et al*., 2011). Thus, a combination of both phenotypic and genotypic identification techniques are more effective (Temmerman *et al*., 2004; Aquilanti *et al*., 2006). Many different genotyping techniques may be applied as tools for species identification. The major advantages of these DNA-based typing methods lie in their discriminatory power and in their universal applicability. Closely related strains with similar phenotypic characteristics may now reliably be distinguished by DNA-based techniques such as randomly amplified polymorphic DNA (RAPD), RFLP, DGGE and TGGE and amplification rDNA restriction analysis (ARDRA).

### 2.3.1. *Physiological and biochemical tests*

Orla-Jensen achieved the basis of classification of lactic acid bacteria. Their work has had a large impact on the systematics of LAB. It is obvious that there have been several revisions as considerable extent for classification of LAB, but even with these revisions, the basis of

classification remains remarkably unchanged and includes the following physiological and biochemical criteria (Stiles and Holzapfel, 1997).

- Mode of glucose fermentation (homo or hetero-fermentation)
- Growth at certain cardinal temperatures (e.g. 10 °C and 45 °C)
- Range of sugar utilization

Acid Production: LAB ferment carbohydrates to produce lactic acid. The production of this organic acid leads to a decrease in pH. Fermentation of sugars that cause pH decrease is important for clotting of milk. An increase in acidity initiates the desirable reactions and changes such as whey expulsion (there is a correlation between pH and whey expulsion from curd). Additionally, acid production has beneficial effect on formation of texture, aroma and flavor (Ross *et al*., 2000)

Proteolytic Activity: Proteolysis is an important event that occurs during cheese ripening. The lactic acid bacteria involved in the process use the polypeptides generated by milk clotting enzymes and by bacterial cell-wall proteins. Rennet which is the milk clotting enzyme, is responsible for casein degradation. Because of the casein degradation peptides are produced which are transported into the cell. In the cell, peptidases continue degradation to produce smaller peptides and amino acids. It has been known that amino acid composition plays an essential role in the aroma of cheese (Wouters *et al.,* 2002).

An important character used in differentiation is mode of glucose fermentation under standard conditions. Standard conditions refer to non-limiting concentration of glucose, growth factors, limited availability of oxygen. Under these conditions, LAB can be divided into two groups:

1. Homofermentative. Glucose is converted almost quantitatively to lactic acid

2. Heterofermentative. Glucose is fermented to lactic acid, ethanol, acetic acid, and carbon dioxide.

Many heterofermentative lactic acid bacteria have the ability to produce energy through the utilization of arginine in formation of ornithine, NH3, CO2, and ATP. It has been thought that most heterofermentative lactobacilli produce NH3 from arginine, whereas homofermentative lactobacilli and *Oenococcus oeni* do not (Garvie, 1967; Kandler *et al.*, 1986; Tonon and Lonvaud-Funel, 2002). However, Pilone *et al*., (1991) questioned the sensitivity of Nessler's reagent commonly used to detect the low concentrations of ammonia produced. Furthermore, Pilone *et al*., (1991) suggested that some hetero-fermentative lactobacilli are capable of carrying out only the first two biochemical steps, thus only yielding one molecule of NH3 per molecule of arginine.

Biochemical tests such as the analysis of dextran and acid formation from various types of sugar are also part of the identification process of LAB. Fermentation capabilities of selected isolates using 49 types of carbohydrates contained in the API 50 CHL kit determines the biochemical properties of isolates within fermented carbohydrates. The ability of isolates to ferment carbohydrates is indicated by a color change of the basal medium used, where the initial basal media color is purple and the possible positive isolates are expected to change the fermentation

medium to yellow basal medium. This test identifies isolates to genus and species level, using the API 50 CHL system (Biomerieux, Japan) (Bukola *et al.*, 2008; Maqsood *et al*., 2013).

Physiological testing of isolated LAB often includes the influence of temperature and pH towards growth. The growing temperature treatments that are used are usually, 4°C 15o C, 25 o C, 40 o C and 45 o C, while for pH values used were 3.0 ;4.0; 6.0 and 7.0. These tests are often conducted according to the method as described by Hammes and Vogel (1995) and Ray (1996) with slight modifications, varying from one study to another. This type of testing determines whether the isolated LAB could be classified into psychrophylic, mesophylic or thermophylic group.

### 2.3.2. *Molecular methods*

Traditional classification methods of LAB based on morphological, physiological, and biochemical tests are really important for selection, enumeration and biochemical characterization, but they are also quite time-consuming, misleading at times, laborious and often considered inefficient in classifying a culture taxonomically (Klaenhammer and Kullen, 1999; Wattiau *et al*., 2001;). These are considered shortfalls in the current era of modern technology, and to overcome them various methods that use DNA for molecular identification of microbial resources have been developed.

Molecular characterization methods are efficient in differentiating even between closely related species. Polymerase chain reaction based methods (PCR-RFLP, REP-PCR, PCR ribotyping and

RAPD) are mainly used as molecular tools (Bulut, 2003). More convenient and accurate identifications are achievable using nucleotide base sequencing of 16S ribosomal DNA (rDNA), which provides a basis for phylogenetic analysis and identification (Chin *et al*., 2006). Several studies have rapidly and more accurately classified LAB based on 16S rRNA gene sequencing (Kim *et al.*, 2003; Chao *et al.*, 2008). Comparison of rRNA gene sequences is currently considered to be the most powerful and accurate method for determining the degree to which microorganisms are phylogenetically related (Coeuret *et al*., 2003). Initially, reverse transcriptase was used to generate DNA from rRNA, and this DNA was then sequenced. It is now possible to sequence 16S or 23S rDNA molecules by direct PCR sequencing, and this method has generated large sequence databases. Although the species-specific sequences are located in the first half of the 16S rRNA gene (V1-V3 region), identification is more accurate if the whole gene is sequenced (Tannock *et al*, 1999). All bacterial genomes contain this conserved gene, and the small variability in this region is unique and specific to each species (Mohania *et al*, 2008).

## 2.4 Sanitary quality and safety of dairy products

Milk and dairy products can harbor a variety of microorganisms (Kaufmann *et al*., 2002). Where an animal is healthy, the microbiological quality of milk at the time of milking is generally good and can stay so for the first three or four hours at ambient temperatures (Jay *et al*., 2005). Once milk is secreted from the udder though, it can be contaminated from many sources (air, faeces, bedding material, soil, feed, water, equipment, animal hides and people). Food-borne pathogens of concern that are associated with raw milk include *Campylobacter spp*., pathogenic strains of *E. coli, L. monocytogenes, S. aureus* and *Salmonella* spp. (Fox and Cogan, 2004). *B.cereus* and *Salmonella spp* are also pathogens of concern (Oliver *et al*., 2005).

# CHAPTER 3

## METHODS

### 3.0. Sample collection

A total of twelve (12) *Omaere* samples were collected from different households. Ten (10) of the samples were from different areas of the North-western region of Botswana (Thololamoro cattlepost, Legotlhwana cattlepost) while two samples were from Tsootsha village in the Ghanzi region (Farm 9 and Farm 10). Table 3.1 shows the samples collected. Most of the traditionally fermented samples from these regions have plant material added to enhance taste and accelerate fermentation, according to the locals, and are prepared in either *Ondjupa* or plastic container.

Table 3.1. *Omaere* preparation and sample characteristics.

| Sample No. | Sampling place | Additional components | Container of production |
|---|---|---|---|
| | ***North-western Region*** | | |
| OMT 1 | Household 1 | No addition | Gourd (*Ondjupa*) |
| OMT 2 | *Household 2* | No addition | Gourd (*Ondjupa*) |
| OMT 3 | *Household 3* | No addition | Gourd (*Ondjupa*) |
| MAD | *Household 2* | No addition | Gourd (*Ondjupa*) |
| OMS 1 | Legotlhwana cattlepost | *Acacia mellifera* roots (Mongana) | Plastic container |
| OMS 2 | Thololamoro cattlepost | *Acacia mellifera* (Mongana) | Gourd (*Ondjupa*) |
| OMS 3 | Legotlhwana cattlepost | No addition | Plastic container |
| OMS 4 | Legotlhwana cattlepost | *Acacia mellifera* roots (Mongana) | Plastic container |
| OMS 5 | Thololamoro cattlepost | *Acacia mellifera* (Mongana) | Gourd (*Ondjupa)* |
| OMS 6 | Legotlhwana cattlepost | No additions | Plastic container |
| | ***Ghanzi region*** | | |
| OMF 9 | Farm 9 | No additions | Plastic container |
| OMF 10 | Farm 10 | No additions | Plastic container |

All the samples were collected in sterile blue capped bottles and stored at 4 °C. The samples were named using the first two letters of *Omaere* and another letter representing the household/area from where they were collected.

### 3.1 Methodology

The methodology followed in isolation and biochemical characterization was as follows:

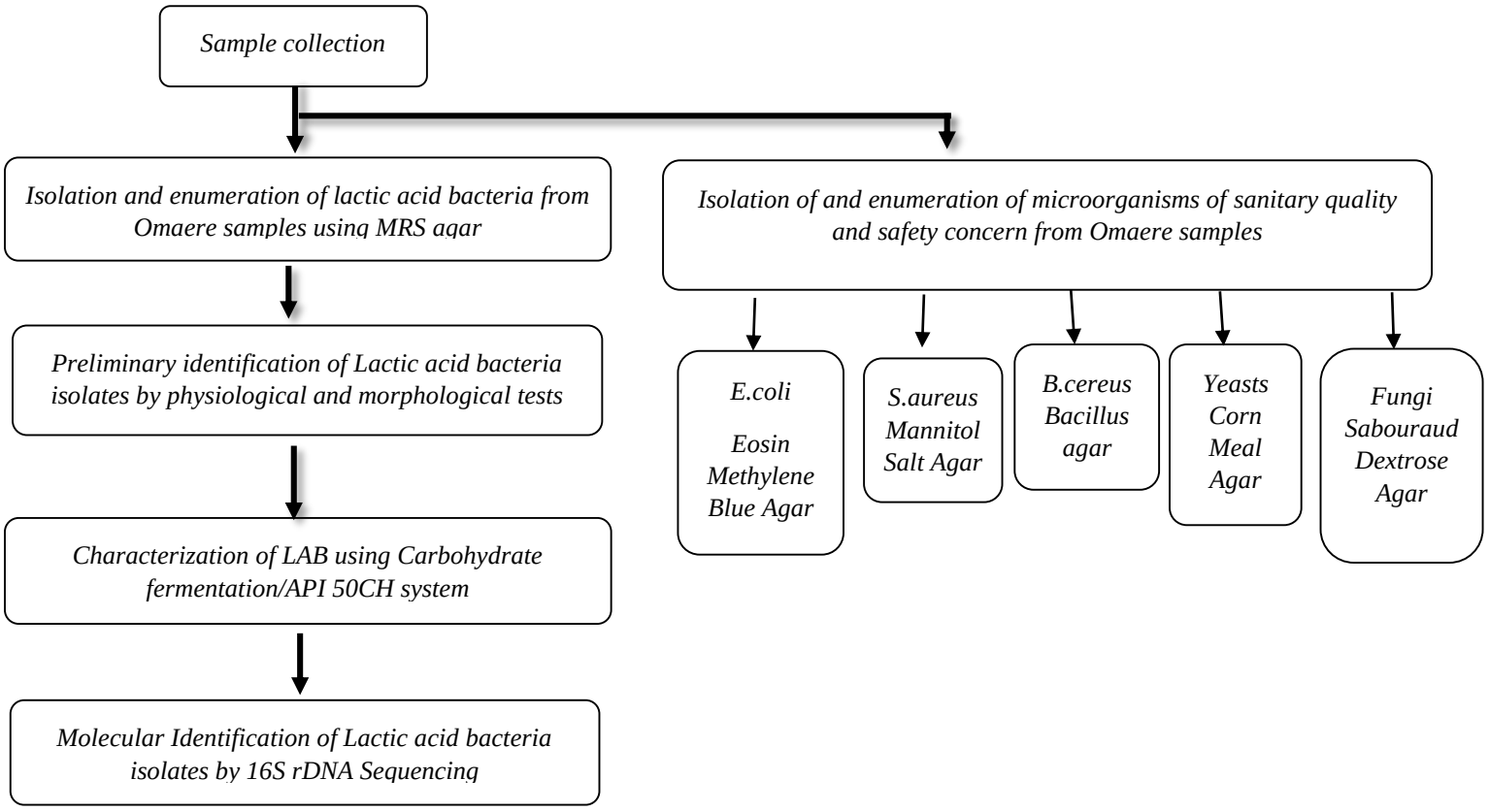


Figure 3.1 Flow diagram of procedures and techniques used to isolate and characterize lactic acid bacteria from *Omaere* samples.

### 3.2. Isolation and enumeration of Lactic acid bacteria from *Omaere* samples

#### 3.2.1 *Dilution strategy*

Ten ml of each sample were aseptically added to 90ml of sterile 0.1% Peptone water diluent to make a 1:10 dilution and mixed thoroughly. This was further diluted to make up to $10^{-7}$ dilutions. One ml aliquots from appropriate dilutions were applied in duplicate plates onto which Man Rogosa Sharpe (MRS) agar (Oxoid Ltd, UK) were poured, homogeneously mixed and allowed to solidify (Pelczar *et al*., 1993). Duplicate plates were incubated anaerobically (using Anaerobic jars together with the Anaerobic Gas pak) and aerobically for 48 hours at 37°C. After incubation, plates with 30–300 colonies were enumerated and the total numbers of viable cells were recorded as colony forming units (cfu/mL) of samples. Colonies with distinct morphological differences (such as color, shape, margin, elevation and size) were selected randomly and purified by repeated streaking on fresh MRS agar plates. This process was repeated once to ensure maximum isolation of LAB. All isolates from both sessions were checked for purity, catalase reaction and Gram`s reaction before stock preparations. Gram-positive, catalase negative bacterial isolates were purified and frozen stocks were made in 20% (v/v) glycerol for future use.

### 3.3 Preliminary identification of Lactic acid bacteria isolates

The isolated LAB strains were identified to genus level on the bases of morphological and physiological tests outlined by Beukes *et al.,* (2001). These included the Gram stain reaction, Catalase test, Growth at different temperatures (4°C, 15°C, and 45°C) and 6.5% NaCl concentration, production of carbon dioxide from glucose, reaction in litmus milk, and production

of ammonia from arginine broth infused with Nessler's reagent. A light microscope was used to confirm the Gram's reaction, cell shape and arrangement.

### 3.3.1. *Catalase test (Nelson and George, 1995)*

A loopful of pure culture was placed onto a clean slide to make a smear of each of the LAB isolates, in order to perform the catalase test (Merch, Germany). Three drops of freshly prepared 3% hydrogen peroxide were added to the smear. A positive test was observed with the development of effervescence, indicating liberation of oxygen from hydrogen peroxide.

### 3.3.2. *Gas production from glucose (Harrigan and McCance, 1976)*

Carbon dioxide production from glucose was tested using the Gibson`s semi-solid medium (HiMedia, Mumbai, India) in tubes, in order to determine whether the isolates were homofermentative or heterofermentative. A drop of fresh isolate from liquid culture was inoculated into the semi-solid medium contained in tubes. To create anaerobic conditions, 2 to 3ml of the molten nutrient agar (M001; Himedia, Mumbai, India) was poured on top of the semi- solid medium. Incubation was carried out at 37°C for 14 days. Heterofermentative lactic acid bacteria produce gas that is marked by the cracking of the Nutrient agar plug, as opposed to homofermentative (Harrigan and McCance, 1976).

### 3.3.3. *Arginine hydrolysis test (Harrigan and McCance, 1976)*

Arginine MRS Broth (Oxoid Ltd, UK) and Nessler's reagent were used in order to determine ammonia production by the isolates from arginine. MRS containing 0.3% L-arginine hydrochloride was transferred into tubes at a volume of 5 ml and inoculated with 0.5ml overnight cultures. Tubes were incubated at 37°C for 7 days. After incubation, 1 ml of cultures were transferred into fresh sterile tubes. The same amount of Nessler's reagent (1 ml) was pipetted into the tubes containing the cultures. The mixture was viewed against a white background. The change in the color with the development of a bright orange color indicated a positive reaction (presence of ammonia) while a deep yellow color indicated a negative reaction (production of lactic acid only). A culture tube which did not contain arginine was used as negative control.

### 3.3.4. *Growth at different temperatures*

Growth at different temperatures were carried out following the methods described by Ayad *et al*., 2001) MRS broth (Oxoid Ltd, UK) was prepared and 10 ml added to test turbes. Then 1ml of overnight cultures inoculated into the tubes and incubated for 3 days at 4 °C, 15 °C, and 45 °C. During the incubation period cell growth at the various temperatures was determined by observing changes in turbidity and sedimentation at the bottom of the tubes.

### 3.3.5. *Growth at 6.5% NaCl concentration*

Isolates were tested for their salt tolerance at 6.5% NaCl concentration. MRS broth supplemented with 6.5% NaCl was prepared and transferred into tubes containing 5 ml of broth. These tubes

were inoculated with 0.5ml overnight cultures and then incubated at 37 °C for 3 days. The changes in turbidity as well as sedimentation at the bottom of the tubes indicated cell growth.

### 3.3.6. *Reaction in Litmus Milk*

To evaluate the reactions in litmus milk, overnight cultures of the isolates were inoculated into tube containing sterile litmus milk (HiMedia, USA) and incubated at 37°C for 14 days (Harrigan and McCance, 1976)**.** Litmus milk determines the metabolic activities of microorganisms against lactose or casein contained in milk. The various metabolic activities such as fermentation of lactose, followed by color change in litmus, coagulation of casein, or peptonization (digestion of casein) and with or without gas formation (MacFaddin, 2000) were recorded.

### 3.3.7. *API 50 CH system*

Identification of bacteria up to the species level was done using API 50 CH kits (Ghanbari *et al*., 2009) following procedures described by the manufacturer. The API 50 CH strips were used in conjunction with API 50 CHL medium for the identification of Lactic acid bacteria and related genera, according to the manufacturer's instructions (bioMerieux, Marcy l' Etoile, France) (Ghanbari *et al*., 2009). Representative bacterial isolates were inoculated into the strips following instructions provided by the manufacturer. The APIs were incubated at 37°C after the wells were filled with the bacterial suspensions to the line mark with the addition of mineral oil top it up in order to create an anaerobic condition. The reaction was observed after 24hrs and 48hrs of incubation. Identification tables were consulted based on color change as positive or negative reactions. API database (Biomerieux SA) and accompanying computer software were used to

interpret the results. Percentage of reliability of identification was used as criteria, with a cut off percentage of 98%, for identification.

### 3.4. Molecular Identification

The samples that could not be identified successfully with the API 50 CH system (The strains with <98%) were analyzed further using molecular methods. The strains were subjected to 16S rRNA gene sequencing.

#### 3.4.1. *Genomic DNA isolation from Lactic acid bacteria isolates*

For genomic DNA isolation a Genomic DNA Isolation kit was used (ZR Fungal/Bacterial DNA MiniPrep™), and the protocol modified to suit the samples as follows: The cultures were activated/resuscitated overnight in 10ml MRS broth and the cells were then harvested by centrifuging for 10 minutes at 12 000 rpm. 50mg (wet weight) of bacterial cells were resuspended in 100 µl of water and added to a ZR Bashing Bead Lysis Tube. The sample was then vortexed at maximum speed for 5 minutes before centrifuging the ZR Bashing Bead Lysis Tube in a microcentrifuge at 10,000 rpm for 1 minute. 400 µl of the supernatant was transferred to a Zymo-Spin IV Spin Filter in a Collection Tube and centrifuged at 7,000 rpm for 1 minute. 1,200 µl of DNA Binding Buffer was added to the filtrate in the Collection Tube from the step above. 800 µl of the mixture was transferred to a Zymo-Spin IIC Column in a Collection Tube and centrifuged at 10,000 rpm for 1 minute.

The flow through from the Collection Tube was discarded and the step repeated. 200 µl DNA Pre-Wash Buffer was added to the Zymo-Spin IIC Column in a new Collection Tube and centrifuged at 10,000 rpm for 1 minute. DNA Wash Buffer was added to the Zymo-Spin IIC Column at a volume of 500 µl and centrifuged at 10,000 rpm for 1 minute. The Zymo-Spin IIC Column was transferred to a clean 1.5 ml microcentrifuge tube and 100 µl of DNA Elution Buffer added directly to the column matrix. Centrifuging was done at 10,000 rpm for 30 seconds to elute the DNA. At the end of the procedure all of the samples were checked to confirm genomic DNA isolation by agarose gel electrophoresis. The process of Genomic DNA isolation using this kit is pictorially summarized in figure 3.2.

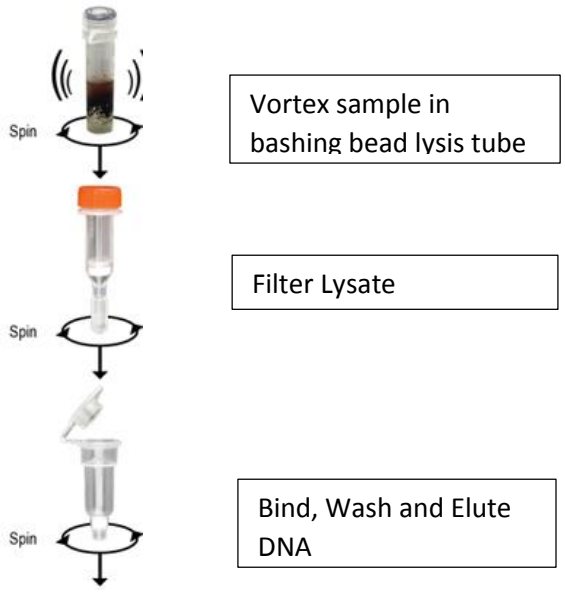


Figure 3.2 Flow diagram showing summary of protocol for Genomic DNA isolation. Adapted from ZR Fungal/Bacterial DNA MiniPrep™

### 3.4.2. *Assessment of the quality of genomic DNA*

*Preparation of Agarose Gel and Loading of Agarose Gel*

The quality of the isolated DNA was evaluated by Agarose gel prepared in the concentration of 0.8%. The agarose gel was prepared by dissolving 0.8g agarose (Sigma-Aldrich) in 100 ml of boiling TAE buffer and then cooled to about 45 °C. Ethidium bromide solution (Sigma-Aldrich) (10mg/ml) was added in a volume of 15 µl. The prepared agarose gel was poured into the gel casting stand and the combs were placed to create wells for loading. 5 µl of eluted product and 2 µl of loading dye were mixed and loaded into wells. A DNA size-marker (Lambda DNA) was loaded into the first well to approximate the concentration of the isolated DNA.

*Electrophoresis of the Products*

The extracted genomic DNA were electrophoresed at a constant voltage of 60 V for 45 minutes in a horizontal mini-gel apparatus (Bio-rad, USA) and the bands on the gel were later visualized in a gel documentation system (Syngene Gene Genius Bio Imaging system, India).

### 3.4.3. *Amplification of 16S rDNA region by PCR*

The DNA template was prepared to ensure approximately 5ng/ml of DNA concentration for each isolate. For the amplification of 16S rDNA region of the isolates S-D-Bact-0011-a-S-17 forward primer and S-G-Lab-0677-a-A-17 reverse primer that were used and are outlined in Table 3.5. A forward primer is complementary to the 5'end of 16S rDNA region, while the reverse primer is complementary to the 3' end of 16S rDNA region. The PCR amplifying procedure was carried out

in a Mastercycler gradient PCR machine (Germany), using the Dream Taq DNA polymerase (Thermo scientific). The 50ul reaction mixture contained 10X Taq buffer at a volume of 5ul, dNTP Mix 10 mM at 1ul, S-D-Bact-0011-a-S-17 primer (10 µM primer) at 2ul , S-G-Lab-0677-a-A-17 primer (10 µM primer) at 2ul, Nuclease free water at 38.75ul, and Taq DNA polymerase (Thermo Scientific) at 1.25U. The following PCR Cycle programme was employed: Pre-denaturation at 94°C for 5 minutes; 35 cycles of denaturation 94°C for 2 minutes; annealing temperature at 57°C for 2minutes; extension at 72°C for 2 minutes and final extension at 72°C for 10 minutes.

*Analysis of PCR Product*

10ul of the PCR product was run on 1.5 % agarose gel to confirm that the PCR product obtained was of the right size. The amplicon was expected to correspond to 700bp. A DNA size-marker (100bp, Thermo Scientific Gene rule) was loaded into the first well to approximate the size of the amplicon. PCR products were electrophoresed using TBE buffer at 80 V for 45 min. The amplification products were then visualized in a gel documentation system (Syngene Gene Genius Bio Imaging system, India).

Table 3.2. Primers used to amplify the 16S rDNA region from *Lactobacillus* spp. from *Omaere.*

| Primer | Primer sequence (5'-3') | Length | Source |
|---|---|---|---|
| S-D-Bact-0011-a-17 | AGAGTTTGATYMTGGCTCAG | 20 bases | Kane *et al* (1993) |
| S-G-Lab-0677-a-A-17 | CACCGCTACACATGGAG | 17 bases | Heilig *et al* (2002) |

### 3.4.4. *PCR Product Purification*

The remaining (40ul) of the PCR product was purified using a Zymo Purification kit (United States of America). Binding buffer was added in 3 X the volume of the amplicon (40ul). The mixture was centrifuged at 10 000rpm for 30 seconds to form a pellet and the supernatant was discarded. To the pellet, 500ul of washing buffer was added and centrifuged for 30 seconds at 10 000 rpm before discarding the supernatant and repeating the step twice. The pure DNA was eluted to a new sterile 1.5 ml eppendorf tube using elution buffer and vacuum dried in preparation for DNA sequencing.

### 3.4.5. *Identification of isolates using partial 16S rRNA gene sequencing*

The purified DNA was sent for sequencing at Inqaba Biotech (South Africa). The resultant nucleotide sequences were checked for homology with known bacterial strains using the BLAST tool at National Centre for Biotechnology Information (NCBI) to identify the strains.

## 3.5. Isolation and enumeration of microorganisms of sanitary quality and food safety concern from *Omaere* samples

A total of seven selective media were used to isolate fungi, yeasts, selected pathogenic bacteria and bacterial species associated with microbial quality.

### 3.5.1 *Isolation and enumeration of indicators of sanitary quality from Omaere samples*

A loopful each sample were spread plated on Eosin Methylene Blue Agar (EMBA; HiMedia, USA). This was carried out to determine if fecal coliforms (*E. coli*) were present in the samples. The presence of *E. coli* would be indicated by a distinctive metallic green sheen.

### 3.5.2 *Isolation and enumeration of Staphylococcus aureus and Bacillus cereus from Omaere samples*

Mannitol Salt Agar (MSA; Oxoid Ltd, UK) and Bacillus cereus agar (Oxoid Ltd, UK) were used to isolate *Staphylococcus aureus* and *Bacillus cereus* respectively. Yellow colonies with yellow zones on MSA would be an indication of a positive test for *S. aureus* while peacock blue colonies on *Bacillus cereus* agar, with precipitate and peacock blue medium would indicate the presence of *B. cereus*.

### 3.5.3 *Isolation and enumeration of Yeasts and Fungi from Omaere samples*

Corn Meal Agar (SigmaAldrich) was used to isolate Yeasts. Aliquots of 0.1ml were plated after serial dilutions up to $10^{-6}$ as outlined by Pelczar *et al*, (1993) and incubated at 25°C and 15°C for over a 48 hour period.

Sabouraud Dextrose Agar (SDA; HiMedia, USA) Dichloran Glrycerol agar (DG-18; Oxoid Ltd, UK) and Dichloran Rose Bengal Chlorotetracycline (DRBC; Oxoid Ltd, UK) were used to isolate different fungal groups. The plating techniques were carried out as outlined in studies by Wehr

and Frank, (2004); Murray *et al*, (2003), and incubated at 15°C and 25°C over a period of 48 hours. Observations were made at 24 hour intervals.

# CHAPTER 4

## RESULTS

### 4.0. Isolation and enumeration of Lactic acid bacteria from *Omaere* samples

The colony counts performed on the *Omaere* samples showed that the lactic acid bacteria load of the *Omaere* samples range from $Log_{10}$ 4 to $Log_{10}$ 7. The lowest value of less than $Log_{10}$ 4 was observed in only one *Omaere* sample (OMT 3) (See Table 4.1). The counts showed no significant difference between the LAB populations across the samples.

Table 4.1. The enumeration and distribution of LAB in *Omaere* samples using MRS agar

| *Omaere* samples | $Log_{10}$ (cfu/ml) |
|---|---|
| OMT1 | 4-5 |
| OMT2 | 4-5 |
| OMT3 | <4 |
| OMS1 | 7 |
| OMS2 | 5-6 |
| OMS3 | 6-7 |
| OMS4 | 6-7 |
| OMS5 | 7 |
| OMS6 | 7 |
| MAD | 5-6 |
| OMF9 | 5-6 |
| OMF10 | 5-6 |

The majority of the *Omaere* samples showed a lactic acid bacterial load range of ($Log_{10}$ 5 to $Log_{10}$-6) and $Log_{10}$ 7. The lowest value of less than $Log_{10}$ 4 was observed in only one sample.

### 4.1. Preliminary identification of isolates

All of the isolates from MRS agar plates were subjected to Gram staining and catalase activity. Microscopic observation showed that 109 isolates were Gram positive and catalase negative. The majority of these, being 96.3 % (105 out of 109), were mostly observed to be bacilli (rod shaped) and either in pairs or chains. The remaining 3.7% (4 isolates) were cocci in chain arrangements. The colonies were smooth, round, with entire margins and whitish colonies on MRS agar. As demonstrated in table 4.2, the 109 isolates were classified into 14 groups. Those that showed the same growth patterns under the set conditions and reactions to tests were considered as being similar. This preliminary identification of the isolates using biochemical tests showed that 71% of the isolates in the 14 groups did not produce gas from glucose fermentation tubes while gas production was observed from the remaining 29% of the isolates. This was an indication that the majority of the isolates were homofermentative.

The Arginine hydrolysis test identified 57% of the isolates as being unable to hydrolyze arginine while the remaining 43% produced ammonia from arginine. These results, together with the Gram stain and catalase activity helped to classify the majority of the isolates as genus *Lactobacillus*. Some of the heterofermentative lactobacilli could be said to have grown as coccobacilli and were not easy to distinguish. The results of the salt tolerance tests indicated that the majority of the isolates tested have the ability to grow at 6.5% NaCl concentration. Only 35.7% of the isolates did not show the ability to grow at this salt concentration.

All isolates were able to grow at 4°C, 15°C and 45°C. However, there was variability in growth at 4°C and 15°C since the amount of growth seen as turbidity of growth medium and bottom sedimentation was smaller than that of growth at 45°C 9

Table 4.2 Morphological and Biochemical characteristics of *Lactobacillus* strains isolated from *Omaere* samples.

| Phenotypic Group | Cell Morphology | Gram' Reaction | Catalase Reaction | $CO_2$a | $NH_3$ | Growth in litmus milk | Growth at Different Temperatures: 4°C | 15°C | 45°C | Growth in 6.5% NaCl | Rep. Isolates |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | Rods | + | - | - | - | Pro ,Pep, LR | ** | ** | *** | ** | OMT1-10 |
| **2** | Rods | + | - | - | - | CU | - | - | *** | - | OMS1-3 |
| **3** | Cocci to ovoid | + | - | - | + | Pro, LF, Pep, GP | * | ** | *** | *** | OMS6-189 |
| **4** | Rods | + | - | + | + | Pro, LF, Pep, GP | - | * | *** | *** | OMS4-149 |
| **5** | Rods | + | - | - | + | Pro, LF, Pep, GP | * | *** | *** | - | OMF10-2 |
| **6** | Rods | + | - | + | + | Pro, LF, Pep, GP | * | - | ** | * | OMF10-7 |
| **7** | Rods | + | - | - | - | Pro, LF, Pep, GP | - | - | *** | - | OMS3-130 |
| **8** | Rods | + | - | + | - | Pro, LF, Pep, GP | ** | *** | *** | *** | OMS3-133 |
| **9** | Rods | + | - | - | - | Pro, LF, Pep, GP | ** | *** | *** | *** | OMS3-136 |
| **10** | Rods | + | - | - | - | Pro, LF, Pep, GP | - | - | - | - | OMF9-4 |
| **11** | Rods | + | - | - | - | Pro, LF, Pep, GP | * | ** | *** | * | MAD-3 |
| **12** | Rods | + | - | - | + | Pro, LF, Pep, GP | - | * | *** | *** | OMS4-147 |
| **13** | Rods | + | - | + | - | Pro, LF, Pep, GP | * | - | - | * | OMS2-124 |
| **14** | Rods | + | - | - | - | Pro, LF, Pep, GP | ** | *** | ** | ** | MAD-15 |

From these tests 14 groups of gram (+) and catalase (-) isolates were found and 8 groups of gram (+) and catalase (+) isolates. Only 3 groups of gram (-) bacteria were profiled with a total of 7 isolates. The majority of the gram-positive, catalase-negative rods, whether homofermentative or heterofermentative, were capable of growing at 15°C and/or 45°C. Growth at 4°C was highly variable. Pro: (Proteolytic activity), LF: (Lactose fermentation), Pep: (Peptonization), GP, (Gas Production), CU: (Citrate Utilization), *: minor growth, **: medium growth, ***: major growth

### 4.1.1. *Identification of Lactic acid bacteria using API CH kit*

A representative from each of the 14 groups determined in the preliminary identification were subjected to the API 50 CH test for identification to the species level. Of the 14 representative isolates from the 14 groups, 6 were identified as good with a reliability of identification of 90% - 97%. Three of the isolates were identified as very good with a range of 98% - 99.8% and 4 of the isolates being excellently identified at a percentage of 99.9 %. The API 50 CH test gave excellent identification of 4 isolates; OMS1-19, OMS4-52, OMT1-4 and OMF10-103; which were identified as *Lactobacillus plantarum*. According to the same API 50 CH tests OMF10-108 was also identified as *Lactobacillus plantarum* and given the rating of very good identification. This gave *L. plantarum* the highest prevalence in the representative isolates, followed by *L. rhamnosus* with 14% (Figure 4.2). Only one isolate could not be conclusively identified as it showed low discrimination between 2 possible species (Table 4.3).

**OMT1-1** **MAD-93**

Figure 4.1. API 50 CH results of two of the LAB isolates tested for carbohydrate fermentation. Color changes show positive results for metabolism of the sugar in that particular well.

A total of 6 (43%) representative isolates had low % of reliability of identification (Figure 4.2). These 6 isolates included OMS3-39 and OMS3-42, which showed good identification as *Lactobacillus plantarum* 1. With a lower % of reliability of identification of 97.0% and 91.7% (Table 4.3), the 16S rDNA region was amplified by PCR for isolates OMS3-39 and OMS3-42 respectively, and sequenced for further identification. Isolate OMS3-45 was identified as *Lactobacillus plantarum* which is considered only good identification according to the cut off percentage. Isolates OMS6-78 and MAD-91 were identified as *Lactobacillus paracasei* with low % reliability of identification (good identification). The 16S rDNA for the isolates was also amplified by PCR and sequenced to confirm their identification. Isolates OMS2-34 and OMF9-96 were excellently identified as *Lactobacillus rhamnosus* with 99.9 % of reliability of identification for both. Figure 4.1 shows the color changes of the API 50 CH test strips for isolates OMT1-1 and MAD-93.

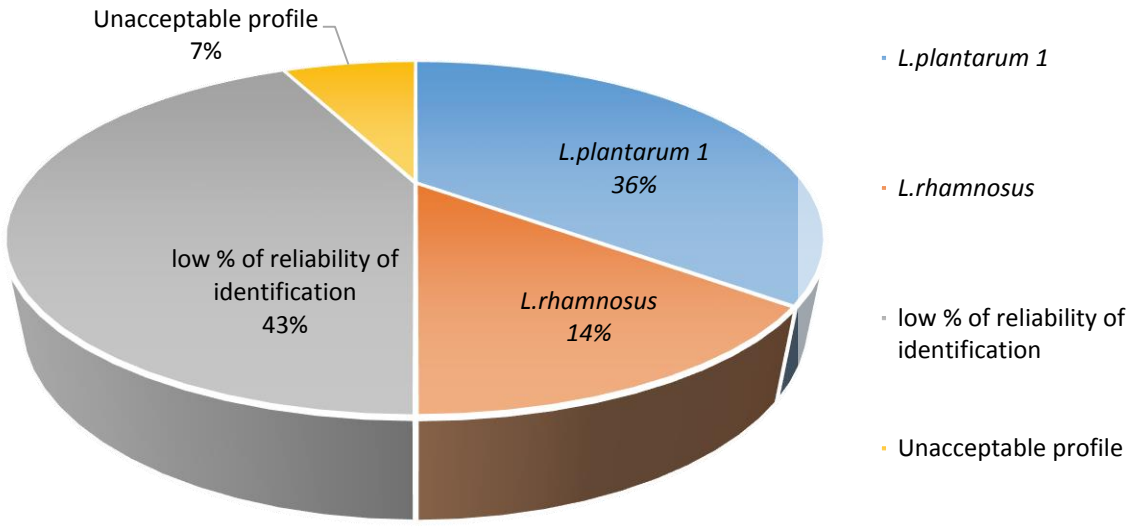


Figure 4.2 Pie chart showing API 50 CH Identification of representative LAB isolates. *Lactobacillus plantarum* 1 showed the highest prevalence at 36%, followed by *Lactobacillus rhamnosus*. Six representative isolates showed low % of reliability of identification and were subjected to further tests.

Table 4.3 Representative isolates from *Omaere* samples identified by API 50 CH kit

| **Isolate no.** | **Species identification** | **% reliability of identification** |
|---|---|---|
| OMT1-5 | *Lactobacillus plantarum 1* | 99.6 |
| OMS 1-19 | *Lactobacillus plantarum 1* | 99.9 |
| OMS 4-52 | *Lactobacillus plantarum 1* | 99.2 |
| OMS 4-54 | *Lactobacillus rhamnosus* | 90.9 |
| OMF10-103 | *Lactobacillus plantarum 1* | 99.9 |
| OMF10-108 | *Lactobacillus plantarum 1* | 98.4 |
| OMS 3-39 | *Lactobacillus plantarum 1* | 97.0 |
| OMS 3-42 | *Lactobacillus plantarum 1* | 91.7 |
| OMS 3 -45 | *Lactobacillus plantarum* | 91.6 |
| OMS 6-78 | *Lactobacillus paracasei* | 94.4 |
| MAD-91 | *Lactobacillus paracasei 1* | 90.1 |
| OMS 2-34 | *Lactobacillus rhamnosus* | 99.9 |
| OMF9-96 | *Lactobacillus rhamnosus* | 99.9 |
| OMT3-12 | *Lactobacillus plantarum* | No % |
| | *Lactobacillus paracasei* | No % |

The most prevalent Lactic acid bacteria was *Lactobacillus plantarum* 1 followed by *Lactobacillus rhamnosus*.

## 4.2. Molecular identification

### 4.2.1. *Genomic DNA isolation from Lactic acid bacteria isolates*

Genomic DNA from the 6 representative isolates that could not be identified by API 50 CH system was successfully extracted (OMS4-54, OMS3-39, OMS3-42, OMS3-45, OMS6-78, and MAD 91). Three of the samples were suspect for DNA degradation and re-extraction was carried out to ensure use of good quality DNA (lanes 4, 5 and 6) (Figure 4.3).

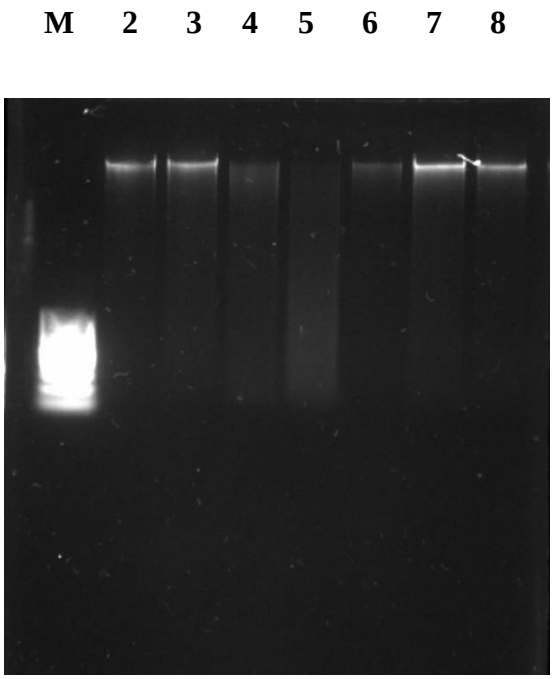


Figure 4.3. Genomic DNA extracted from LAB isolates from *Omaere* samples. In the first lane is Lambda DNA, and in lane 2-7 are the isolates as follows; 2: OMS3-42, 3: OMS3-39, 4: OMS4-54, 5: OMS3-45, 6: MAD-91, 7: OMS6- 78, 8: OMS3-42.

### 4.2.2. *Amplification of 16S rDNA by PCR*

After DNA isolation, the 16S rDNA region was amplified by PCR protocol. Then PCR products were visualized by agarose gel electrophoresis under UV light. The amplified PCR products were found to the give the expected band of approximately 720 bp as shown in Figure 4.4.

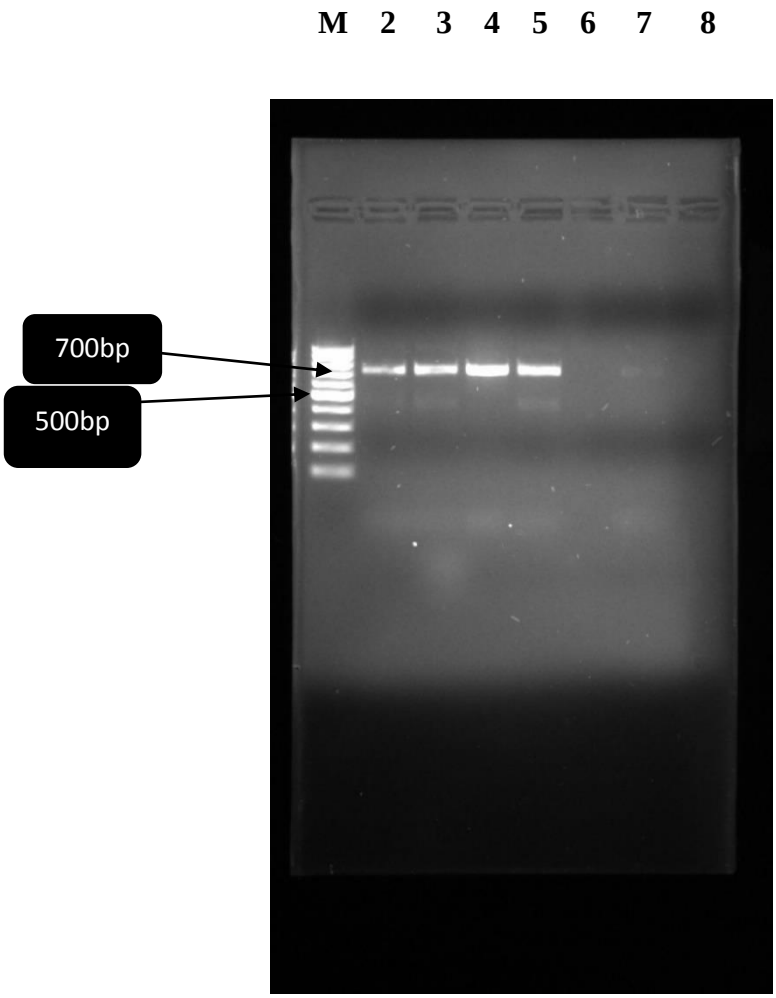


Figure 4.4. Amplified 16S rDNA region for LAB isolates obtained from *Omaere* samples. In lane 1 is the O Gene ruler of 100bp. Lanes 2- 5 have bands estimated at 720bp while lanes 6 -8 are characterized by no bands. In lanes 2-5 are the isolates as follows; 1: OMS3-39, 2: OMS3-42, 3: OMS6-78 and 4: MAD-91.

### 4.2.3. *Identification of isolates using partial 16S rRNA gene sequencing*

The amplicons were sent to Inqaba Biotechnological Industries (South Africa) for sequencing, and the resultant nucleotide sequences were checked for homology with known bacterial strains using the BLAST tool at the National Center for Biotechnology Information (NCBI) to identify the isolates (Appendix). Isolates OMS3-39 and OMS6-78 were identified by API 50 CH as *Lactobacillus plantarum* 1 and *Lactobacillus paracasei* respectively. The BLAST tool identified the two isolates as *Lactobacillus helveticus* and *Enterocuccus faecium* respectively. Their homologies ranged in the high 80`s (Table 4.4). Isolate OMS3-42 and MAD-91 could not be sequenced successfully.

Table 4.4 Isolates with low % of reliability of identification using API test sequenced and identified using BLAST tool.

| Isolate no. | Identification | Accession number | Homology (%) |
|---|---|---|---|
| OMS3-39 | *Lactobacillus helveticus* LB31 | LC164740.1 | 87 |
| OMS6-78 | *Enterococcus faecium HSK5-1* | KU555488.1 | 86 |

The two isolates that were successfully sequenced showed identities of two Lactic acid bacteria respectively, with one being bacilli and the other being of cocci origin; *Lactobacillus helveticus* and *Enterococcus faecium.*

Once identification of the isolates was completed, prevalence rate of the lactic acid bacteria species in each of the twelve *Omaere* samples was calculated. This was deduced from the number of isolates per representative group in a given plate. The representative isolate identified as a specific species was then used to calculate the proportion LAB per sample (Table 4.5 below). This proportion was used to determine percentage distribution of the LAB species across all twelve *Omaere* samples. *L. plantarum* had the highest percentage distribution as it was found present in all the twelve *Omaere* samples, comprising more than 60% of the population in each sample. *L. rhamnosus* had the second highest percentage distribution of 58%, with the proportions in the samples being 30% or less. *E. faecium* is a cocci lactic acid bacteria with percentage distribution of 25% across the samples. *L. helveticus* was found to have the lowest percentage distribution of 17% across the twelve *Omaere* samples.

The results also show that only 6 of the 109 isolates could not be identified as LAB, and were therefore regarded as unknown. These unidentified isolates were found to occur in half of the twelve *Omaere* samples analyzed, and constituted 33% percent or less of the total bacterial microflora found in each of the samples.

Table 4.5. The prevalence rates of different species of LAB in *Omaere* samples.

| Sample no. | *L. plantarum* | *L. rhamnosus* | *L. helveticus* | *E. faecium* | *Unknown* |
|---|---|---|---|---|---|
| OMT 1 | 75% | - | - | - | 25% |
| 0MT 2 | 83% | - | - | 17% | - |
| OMT 3 | 67% | - | - | - | 33% |
| MAD | 70% | 30% | - | - | - |
| OMS 1 | 69% | 23% | - | - | 8% |
| OMS 2 | 81%% | 9% | 10% | - | - |
| OMS 3 | 85% | - | 15% | - | - |
| OMS 4 | 60% | 20% | - | 10% | 10% |
| OMS 5 | 64% | 29% | - | - | 7% |
| OMS 6 | 82% | - | - | 18% | - |
| OMF 9 | 75% | 12.5% | - | - | 12.5% |
| OMF 10 | 87.5% | 12.5 | - | - | - |

*Lactobacillus plantarum* is the most prevalent in all the 12 samples and was found to have 100% distribution, while *Lactobacillus helveticus* showed to be the least prevalent and had the least percentage distribution across samples.

### 4.3. Isolation and enumeration of microorganisms of sanitary quality and food safety concern from *Omaere* samples

Isolation of selected microorganisms and bacteria associated with sanitary quality of the *Omaere* samples was carried out. Their microbial characterization was carried out to check for common food borne pathogens and determine if the samples were exposed to unsanitary conditions during preparation.

The EMBA plates did not show any metallic green colonies, or any other colonies. This indicates that no coliforms (*E. coli* in particular) were isolated (0 CFU/ml). Which means that there is no contamination of environmental or fecal origin. *S. aureus* and *B. cereus* are food pathogens that were investigated to determine the safety of traditionally fermented *Omaere*. *Staphylococcus aureus*, a skin commensal and food borne pathogen was not isolated from the *Omaere* samples. There were no colonies observed on the plates (0 CFU/ml) and the color of the medium remained reddish instead of turning yellow. The Bacillus Agar Base plates (with supplements) also showed no colony growth and retained the initial color of the medium, instead of a peacock green color change, indicating that no *Bacillus cereus* were present in the samples. There were no yeast colonies observed on Corn Meal Agar, used to isolate chlamydospore forming *C. albicans,* associated with the human gut. Observations were made both at 24 hours and 48 hours' time intervals.

Selective media SDA, DG-18 and DRBC were used to isolate for fungi and yeasts. There was no growth observed, so the colony counts reported for all the three media plates at 24 and 48 hour intervals were 0 CFU/ml.

# CHAPTER 5

## DISCUSSION

This study was conducted with the main objective of isolating and characterizing culturable lactic acid bacteria from samples of *Omaere* collected from two regions in Botswana. To characterize these LAB isolates, phenotypic methods that rely on physiological or biochemical criteria were applied. These phenotypic profiling methods often require less sophisticated equipment, but in most of the cases they are insufficient for accurate species identification because of the great number of different LAB species with similar phenotypic characteristics (Temmerman *et al.*, 2004). In the current study the same was observed; phenotypic identification was not sufficient to unambiguously identify the isolates. This could be attributed to the fact that, in addition to being mixed cultures, the isolates are closely related strains. It has been reported that there is a lot of similarity between *L. helveticus* and both *L .plantarum* and *L. rhamnosus*. In previous studies it was found that the identification of *L. helveticus, L. casei* and *L. plantarum* was difficult and uncertain. Wheater, (1955). Sharpe and Wheater (1957) found that *L.helveticus* and *L. casei* var. *rhamnosus* have been confused with each other and with other species in the genus *Lactobacillus*.

### 5.0. Isolation and identification of Lactic Acid Bacteria from *Omaere* samples

Phenotypic (biochemical and physiological) tests were carried out and 14 profiles were determined and isolates allocated the appropriate profile groups. The isolates in these groups could not be unambiguously identified from just the phenotypic tests, and so representative isolates from each group were picked and subjected to the API 50 CH test. Out of the representative isolates that were identified successfully by API 50 CH system, *Lactobacillus plantarum* was found to be the

dominant LAB species in traditional *Omaere* fermentation, followed by *Lactobacillus rhamnosus*. Several studies reported isolation of *Lactobacillus spp*, including *Lactobacillus plantarum*, from fermented milk / dairy products and other non-dairy products. In Ghana and in Ethiopia, studies to investigate fermentation of non-dairy products; cocoa and *borde*, found that all fermentations were characterized by domination of LAB, with high levels from the start in chocolate fermentation (Abegaz, 2006; Camu *et al*., 2007). Noteworthy was the selective isolation of AAB on DMS and of *L. plantarum* on KAA under the conditions used in chocolate fermentation, representing 78% and 72% of the isolates, respectively. Most *L. plantarum* strains were isolated from MRS; almost no enterococci were found on KAA (15%) nor on the other media (Camu *et al*., 2007).

Studies to characterize LAB in fermentation of milk/dairy products have also been carried out. Nyambane *et al.*, (2014) sought to find the microbial diversity in *amabere amaruranu*, a traditionally fermented milk product from Kenya. In their findings *Lactobacillus fermentum* was highly prevalent in *amabere amaruranu* which comprised 20% of the isolates. Mathara *et al*., (2008) also investigated a kenyan milk product known as *kule naoto*, and found that *L. plantarum* was the most dominant *Lactobacilli* involved in fermentation. It was also isolated by Beukes *et al*. (2001) from South African traditional fermented milk products and Lore *et al*. (2005) from *suusac*. These studies all show that *Lactobacillus plantarum* is associated with fermented milk products, therefore supporting the results in the current study.

*Lactobacillus plantarum* is also largely found as the dominant species in the last step of natural food raw- material fermentation, including a variety of vegetables, meat and milk (Kumar and

Murugalatha, 2011). A number of studies have commonly associated this microorganism with plant-based fermentations (Holzapfel, 1997) such as production of lactic acid in pickles and *sauerkraut* (Stiles and Holzapfel, 1997), and the Ethiopian *borde* (Abegaz, 2006). The dominant presence of this organism in fermented milk products was suggested by Mathara *et al*, (2008) to be due to the use of plant materials such as the gourd for fermentation and the adaptation of strains to milk. This is something to consider as well in the current study; that the presence of *Lactobacillus plantarum* as the dominant species could be due to this same reason, seeing as the containers in which the *Omaere* was fermented were either plastic containers or guard, which they call *Ondjupa* in the Northern region (Herero tribe). Another reason that could add to this, which was observed in the current study is the addition of plant material; *Acacia mellifera* roots (Mongana) by the Herero locals. The roots have also been observed to catalyze the speedy fermentation of the product and enhance the taste

The API 50 CH system was able to unambiguously identify seven of the representative isolates that belonged to the groups; 1, 2, 5, 6, 10, 12 and 13. Only one of the isolates, representative of group 11, gave unacceptable profiles and was considered not LAB. The system identified isolates OMS6-78 (group 3) and MAD-91 (group 14) as *Lactobacillus paracasei* and isolates OMS4-54 (group 3) as *Lactobacillus rhamnosus*. The % of reliability of the three isolates mentioned did not meet the set 98% criteria, together with three other representative isolates; OMS3-39, OMS3-42 and OMS3-45 of groups 7,8 and 9 respectively. The six isolates were further analyzed because of the low % of reliability of identification. Their genomic DNA was extracted and their 16S rDNA region amplified using PCR for sequencing. The results found that two of the isolates were *Lactobacillus helveticus* and *Enterococcus faecium*, while the other three that could not be

sequenced successfully suggested that the isolates were mixed cultures and could not be identified conclusively. The difficulty in identification of *Enterococcus faecium* using phenotypic and physiological methods has also been noted by Zuo *et al*., (2014). Successful identification was attained by 16S rRNA gene sequencing. Jose` *et al*., (2007) also carried out a study in which it was found that because LAB have similar nutrition and growth requirements, it proves difficult to identify them by classical methods and some modern methods.

The biochemical tests used to preliminarily identify isolates in the current study remain vital in microbiology today, but should be used simultaneously with modern methods of identification, such as API 50 CH/CHL and genotypic methods of identification. In the current study tests such as the gram stain and the catalase test played an important role in distinguishing gram negatives from gram positives and characterizing LAB with morphology and catalase reaction. The methods that proved insufficient when evaluated in isolation were the arginine hydrolysis test, growth at different temperatures and growth at 6.5% NaCl concentration because of their inconsistency. In studies by Bulut, (2003) and Savadogo *et al*., (2004), all the lactobacilli isolates could grow at 15° C, while in other studies lactobacilli isolates showed variation in growth at 15° C; some grew well while others didn't grow at all (Abdullah and Osman, 2010; Patil *et al*., 2010). The current study found results similar to the latter reports, as growth of the isolates at 15° C showed variation. Five of the groups of isolates identified did not show growth at this temperature and temperatures below 15°C as the turbidity of the medium remained clear. Two of the groups showed very slight turbidity change, which indicated very little growth of cells. In a study by (State the Author) Kadere and Kutima, (2012) all the strains isolated from coconut toddy (*mnazi*) grew at 15°C and 30°C but not at 45°C. Almost 50% of the 15 isolates in the study were able to grow at 6.5% NaCl but none was

able to grow at 18% NaCl. However in the present study, isolates showed growth at 45°C and the majority of them showed ability to grow at 6.5% NaCl. The majority of the isolates in the present study were identified as *L. plantarum*, and according to a study by Papamanoli *et al*., (2002), *L. plantarum* strains and some *L. sakei* and *L. curvatus* strains are capable of growing at 45°C. *L. plantarum* strains are reported to grow at 6.5%-10% NaCl whereas the growth of *L. sakei* and *L. curvatus* was deemed as variable (Papamanoli *et al*., 2002). These results support the findings in the present study, confirming that because of the variability in the growth patterns of LAB at different temperatures and at 6.5% NaCl, it is of great importance that biochemical tests be used complementing one another because they may show variation from one study to another. Kandler and Weiss, (1986) suggested that many of the recently described LAB species do not fit into the traditional classification scheme based on morphology and growth temperatures. Therefore, one should rely on other method of classification such as those based on biochemical and molecular criteria.

### 5.1. The Diversity of Lactic Acid Bacteria strains in the *Omaere* samples

The diversity of lactic acid bacteria was found to be relatively low in the current study, with the genus *Lactobacillus* being the most prevalent and represented by two predominant species. Only one species of *Enterococcus* was identified, after proving difficult to identify phenotypically and physiologically. Molecular tests did show better ability to identify the *Enterococcus* species, but cannot be said to have significantly increased diversity. Calculation of the prevalence rates also showed the distribution pattern of these LAB. *L. plantarum* was the most prevalent in all the samples and showed 100% distribution across the samples *Lactobacillus rhamnosus* was isolated from 58% of the samples while *Enterococcus faecium* was isolated from only 25% of the samples.

*Lactobacillus helveticus* was isolated from the lowest number of samples, but added to the higher rate of lactobacilli prevalence over the enterococci. In a study to investigate fermentation of borde in Ethiopia, it was found that spontaneous fermentation typically results from the competitive activities of different microorganisms whereby strains best adapted and with the highest growth rate will dominate during particular stages of the process (Abegaz, 2006). Because *Omaere* fermentation is also a step by step process, the fact that other Lactic acid bacteria might diminish in numbers from one step to another as fermentation is in progress, cannot be ruled out. In the study by Abegaz, (2006), a wider diversity of LAB strains that are both rods and cocci, were present at the early period of maize dough fermentation at Phase I than any other phases of *borde* fermentation. One could argue as well that during the early stages of *Omaere* fermentation there is a wider diversity of LAB isolates than the last stage of fermentation, which is sampling in this current study.

In another study by Ni *et al*., (2015), Spontaneous fermentation by lactic acid bacteria in silage was shown to be a complex microbial process in which the composition of the dominant microbiota changes at different stages, particularly during the first week of ensiling when major changes in the fermentation products and pH occur (Muyzer and de Waal., 1993). From their investigations it was found that eight LAB species belonging to cocci were found to be present in rice silages, among epiphytic LAB. Cocci LAB species (e.g., *Enterococci, Leuconostocs, Weissella, Pediococci* and *Lactococci*) are known to initiate lactic acid production during the early stage of fermentation. During this process, some heterofermentative cocci produce gas from glucose and create aerobic conditions that are suitable for the development of lactobacilli. In contrast to cocci, lactobacilli are important promoters of lactic acid fermentation for a longer time, particularly

during the latter stage of ensiling. This could also be true for traditional *Omaere* fermentation in the current study.

A study on traditionally fermented milk in Zimbabwe also found that all the isolates from traditionally fermented milk belonged to the genus *Lactobacillus.* Seven of the isolates were identified as belonging to *L. helveticus, L. plantarum, L. delbrueckii* subspecies *lactis* (two isolates), *L. casei* subsp. *casei* (two isolates) and *L. casei* subsp. *pseudoplantarum*. The findings of this study support the results of the current study, in which the significant majority of the isolates were found to be lactobacilli as compared to LAB of cocci morphology.

There were six unknown isolates that could not be identified as LAB. These isolates belonged to the two groups whose representative isolates could not be identified using neither the API 50 CH test nor the 16S rDNA sequencing.

### 5.2. The potential use of isolated LAB strains from *Omaere* as probiotics and starter cultures

In general lactic acid bacteria used in fermentation of milk products are proteolytic and cause coagulation of milk due to the instability of milk protein, and this curd can change the texture of milk products. The Proteolytic system of LAB is involved in casein utilization within LAB cells and is known to contribute to the development of organoleptic properties of fermented milk products (Moulay *et al*., 2006; Yamina *et al*., 2013). The statements above support the findings of the present study, as the results of the Litmus milk tests showed the majority of the isolates to have

proteolytic activity. This is a characteristic that can be exploited to come up with starter cultures manipulated to improve the flavor and texture of *Omaere.*

There has been much recent interest in the use of various strains of LAB as probiotics, because these bacteria, mainly lactobacilli and *bifidobacteria*, may have several therapeutic functions (Berg, 1996; Oberg *et al*., 1998). One of the beneficial health effects related to probiotics is their ability to reduce serum cholesterol levels. In general, the probiotic strains should also have desirable antibiotic resistance and sensitivity patterns, be antagonist toward potentially pathogenic microorganisms and have metabolic activities beneficial to the well-being of the host. Antimicrobial activity is thought to be an important means of probiotic bacteria, and *L. plantarum* strains have been known to produce bacteriocins with antimicrobial activity, and some of these are produced by strains of *L. plantarum* isolated from milk products; plantaracin C from *L. plantarum* isolated from cabrales cheese, plantaracin LC74 from *L. plantarum* LC74 isolated from crude goats milk, and plantaracin AMA-K, produced by *L. plantarum* AMA-K isolated from fermented milk in Gwanda, Zimbabwe (Gonza´lez *et al*., 1994; Rekhif *et al*., 1994, Todorov *et al*., 2007). *Lactobacillus rhamnosus* is another species of the few in the *Lactobacillus* genus that have been used as probiotic organisms in functional foods. One of its strains has also been identified to be flavor enhancing in addition to its probiotic attributes. It can be used as an adjunct during cheese manufacture to reduce adventitious microflora, accelerate cheese ripening, and improve cheese flavor (Klaenhammer *et al*., 2002). Among the genus *Enterococcus, Enterococcus faecium* and *Enterococcus faecalis* are stated to have probiotic traits. *Enterococcus faecium*'s usage on diarrhea treatment is considered to be an alternative for antibiotic use. This probiotic effect of *Enterococcus faecium* on humans has to do with its action on the digestive system, which involves reduction of

the absorption rate of cholesterol from the digestive system (Erginkaya *et al*., 2007).These studies give light to the fact that *L. plantarum* and *E. faecium* strains in the current study could also be considered as potential probiotics if their ability to produce bacteriocins is investigated and proven.

Fermentation with lactic acid bacteria is a cheap and effective food preservation method that can be applied even in more rural/remote places, and leads to improvement in texture, flavor and nutritional value of many food products (Ray, 1992; Wood and Holzapfel, 1995; Wood, 1997; Caplice and Fitzgerald, 1999; Aquilanti *et al.*, 2006; Giraffa *et al*., 2010). *Lactobacillus plantarum* is one of the most important lactic acid bacteria used for starter cultures in meat, cereal and vegetable fermentations (McKay and Baldwin, 1990; Ruiz-Barba *et al*., 1991; Kato *et al*., 1994; Muller *et al.,* 2009). In a study by Palomino *et al.*, (2014), *Lactobacillus plantarum* IMDO 788 was found to be a useful starter in the fermentation of cauliflower and mixed vegetables as it resulted in an accelerated fermentation process. It dominated the process, making it more stable and predictable. In another study, the application of *Lactobacillus plantarum* Lb9 isolated from spontaneous caper berry fermentation as starter culture was evaluated by means of culture-dependent and independent methods. The results obtained showed that *L. plantarum* Lb9 accelerated the fermentation process, dominating the background microbiota present in the raw material until the end of fermentation (Palomino *et al*., 2014). Due to its probiotic traits, *Lactobacillus rhamnosus* GG or *Lactobacillus* GG is the most common microorganism used in dairy products marketed for infant's and children's consumption. *Lactobacillus rhamnosus* GG, is one of the most studied strains and is one of the most common bacteria that is used in probiotic preparations and foods. It was found beneficial to children's health and is widely used with products for infants and children (Yerlikaya, 2014). The studies above show that the two LAB

strains isolated from the *Omaere* samples in the present study have potential to be used as efficient starter cultures and probiotics.

Studies by Soomro *et al.,* (2002) and Daly and Davis, (1998) have shown that research carried out on the fermentation of milk by LAB species such as *L. plantarum, L.helveticus, Enterococcus* sp. and *Lactococcus lactis* is vital in the sense that functional properties in lactic acid bacteria not only improve preservative effect, but also add flavor and taste to the fermented products. Savadogo *et al*., (2004) observed this to be the case in Fulani fermented milk in Burkina Faso. The LAB identified in the present study were also identified in the study by Savadogo *et al*., (2004) and could also have the potential to add taste and flavor to *Omaere*.

### 5.3. Isolation and enumeration of microorganisms of sanitary quality and safety concern from *Omaere* samples

According to Mutukumira *et al*., (1995), the microorganisms present in milk and naturally fermented milk may originate from the animal itself, from the milking equipment and environment, from personnel or from the previous product batch if back-slopping is the technique adopted. In the current study an investigation into the presence of *Escherichia coli* as an indicator for coliforms was done. EMBA was used to isolate for the microorganism. The results showed no growth of *E. coli,* which implies that there was no fecal contamination of the *Omaere* samples, meaning that the preparation of the dairy product was done under good sanitary conditions in all the homesteads from which the samples were collected. Limited hygiene that is often practiced in the preparation of these traditionally fermented products results in the presence of a variety of microorganisms. In

a study by Feresu and Nyati, (1990), the growth and survival of pathogenic and non-pathogenic strains of *E. coli* was determined in traditionally fermented pasteurized and unpasteurized milk and in *Lacto*, an industrially fermented milk in Zimbabwe. *Lacto* inhibited all the three *E. coli* strains and the general trend in the traditionally fermented milk showed that fewer *E. coli* if not none, survived when the fermented milk products were stored at refrigeration temperature. In the present study isolation of the above mentioned organism was carried from *Omaere* samples that had been stored at 4° C during the course of the research, this could also be another reason as to why no *E. coli* was isolated from the samples, which would be supported by the study above.

It is also important to note that lactic acid bacteria exert strong antagonistic activity against many microorganisms, including food spoilage organisms and pathogens (Narayanan and Srivastava, 2004). Lactic acid, an organics acid, is one of the compounds produced by LAB as a result of fermentation metabolism, and can be useful as a pH buffering agent or inhibitor of bacterial spoilage in processed foods (Narayanan and Srivastava, 2004). It is because of the production of lactic acid and bacteriocins by lactic acid bacteria that pathogens and spoilage bacteria may not be found in most fermentation products.

In the present study *Staphylococcus aureus* and *Bacillus cereus* were tested by using MSA and *Bacillus cereus* agar base. The findings showed that none of the two food borne pathogens were isolated from the *Omaere* samples investigated. These two food borne pathogens can be an indicator of poor handling of food by those that prepare it as it can be found naturally on the skin. The absence of these microorganisms, could mean that production of the *Omaere* was done under

good hygiene practices (good sanitary conditions). It could also be attributed to the production of lactic acid or bacteriocin like substances. In a study by Zhang *et al*., (2013), *L. plantarum* was found to produce antimicrobial metabolites that were capable of inhibiting a wide range of LAB and some Gram positive pathogens, including *S. aureus* and *L. monocytogenes*. The metabolites were also found to have activity against gram-negative bacteria (*E. coli* included), which cannot be said for most bacteriocins of lactic acid bacteria that are inefficient when it comes to gram negative bacteria. In another study *L. plantarum* strains gave inhibition zones that measured significantly against *S. aureus*, Bacillus sp, and *E. coli* (Anas *et al.*, 2008). *Lactobacillus plantarum* TF711 isolated from raw Tenerife goat's cheese by Hernández *et al.,* (2005) produced a bacteriocin-like substance; plantaricin TF711. It showed activity against Gram-positive bacteria; *Bacillus cereus, Clostridium sporogenes* and *Staphylococcus aureus*, as well as against the *Enterobacteriaceae, Shigella sonnei* and *Klebsiella pneumoniae*. Considering the studies above, it can be presumed that for the current study, *L. plantarum* in the *Omaere* samples could have produced metabolites that are active against pathogens and spoilage bacteria, both gram negative and gram positive.

It is important to note that there are some interactions between lactic acid bacteria and some of the microorganisms previously mentioned as microorganisms of safety concern. These interactions are sometimes observed in fermentation of milk products, one example is the yeast and LAB interaction. In the current study it could be said that no interactions between LAB strains and other organisms were reported. No yeasts and fungi were found in the samples. In studies of indigenous fermented milk in Zimbabwe and Uganda (Narvhusa and Gadaga, 2003), many samples contained more than one species of yeast, but *Saccharomyces cerevisiae* was most commonly isolated. Other

frequent isolates were other species of *Saccharomyces* and several species of *Candida*. The same cannot be said for the current study; no yeast growth was observed on the Corn meal agar plates at both 24 hours and 48 hours incubation times. This suggests that the fermentation process of the *Omaere* in the current study did not include the action of yeasts.

In another study various fungi isolated from bakeries were inhibited by *L. plantarum* (LB1) and *L. rossiae* (LB5) isolated from raw wheat germ. Organic acids and peptides synthesized during fermentation were responsible for the antifungal activity; formic acid had the highest inhibition activity (Rizzello *et al*., 2010). However, the inhibitory compounds characterized were different, depending upon the LAB strains and flour type used. It could be that the same can be said for the current study; that the organic acids and peptides that are synthesized during the fermentation of *Omaere* could have antifungal activity, therefore inhibiting possible fungal growth in the milk product.

### 5.4. Limitations of the study

- Bacteriocin production by the LAB strains isolated from the *Omaere* samples could not be carried out successfully to determine the probiotic potential of the isolated strains.

### 5.5. Conclusion

The current study was able to isolate and characterize the dominant lactic acid bacteria strains in traditionally fermented *Omaere*. Biochemical tests together with molecular characterization were

implored to identify the dominant LAB as *Lactobacillus plantarum* and *Lactobacillus rhamnosus*, with *Lactobacillus plantarum* being more prevalent than other strains. The majority of the LAB samples isolated showed that they have the ability to grow well at 15°C and 45°C (with the exception of *L. rhamnosus* that is incapable of growing at 15°C and temperatures below that), and in salt concentration of 6.5% (NaCl). The arginine hydrolysis test and the ability to produce gas from glucose showed great variations and therefore could not be used alone as characteristics of identification. Diversity of microflora in the samples was found to be low, and there didn't seem to be a patterned variation between the strains of *L. plantarum* isolated from the *Omaere* samples collected from the North-western region and those collected from the Ghanzi region. The current study gives light to the fact that traditionally fermented *Omaere* may serve as a potential source for the isolation of starter cultures for its large-scale production basis and also as a potential source for isolation of probiotic LAB strains. All these species identified can contribute to the quality of *Omaere* traditional fermented milk by acid, flavor and aroma production. The fact that no organisms of sanitary and safety concern were isolated from these *Omaere* samples, gives an indication that although traditionally fermented at household level, it is safe for consumption.

### 5.6. Recommendations

- Studies should be carried out to isolate and characterize bacteriocins produced by the *Lactobacillus plantarum* strains isolated from traditionally fermented *Omaere*, in order to investigate their potential as biopreservatives in the food industry and as potential probiotics.

- *Omaere* is very popular with the Baherero and other tribes in the North Western areas of Botswana. It is believed that it is highly nutritious, boost the immune system and has aphrodisiac properties. This makes ~~investigating the~~investigating the diversity of lactic acid bacteria strains using molecular techniques for un-culturable strains worthwhile, in order to have a wider knowledge of the stains that can be implored as starter cultures for commercial production.

- *Omaere* production is a step by step process, and therefore the composition of microbial flora should be tracked through the all stages of the production process in order to determine the succession and changes in microbial population during fermentation.

- Identification of the samples that proved difficult to characterize could be done by carrying out streaking for pure cultures and employing molecular techniques for conclusive identification.

More work can be done on the inhibitory metabolites present in *Omaere* samples and their activity against microorganisms of sanitary and safety concern.

# CHAPTER 6

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# APPENDIX

## MEDIA AND SOLUTIONS:

### Arginine Broth (Harrigan and McCance, 1976)

| | |
|---|---|
| Tryptone | 5.0g |
| Yeast extract | 2.5g |
| D-Glucose | 0.5g |
| Dipotassium hydrogen phosphate | 2.0g |
| L-aginine monohydrochloride | 3.0g |
| Distilled water | 1liter |

Dissolve the ingredients in water and sterilize at 121°C for 15 minutes; pH 7.0.

### Gibson`s semi-solid medium (Gibson and Abd-el-Malek, 1945: Stamer et al., 1964)

| | |
|---|---|
| Yeast Extract | 2.5g |
| D-Glucose | 50.0g |
| Manganese sulphate, 0.4% | 10ml |
| Reconstituted skim milk | 800ml |
| Nurient agar | 200ml |

Dissolve the dry ingredients in reconstituted milk, with nutrient agar to semi solidify the media. Distribute the media into test tubes in 10ml. When ready to use dissolve the medium in a water bath of 100 °C. Cool to 45°C and use at that temperature.

**API 50 CHL medium**

| | |
|---|---|
| Polyptone | 10 g (bovine origin) |
| Yeast extract | 5 g |
| Tween 80 | 1 ml |
| Dipotassium phosphate | 2 g |
| Sodium acetate | 5 g |
| Diammonium citrate | 2 g |
| Magnesium sulfate | 0.20 g |
| Manganese sulfate | 0.05 g |
| Bromcresol purple | 0.17 g |
| Demineralized water | 1000 ml |

pH : 6.7-7.1

**20% Glycerol v/v**

20ml Glycerol

80ml H2O

Autoclave and use

**SEQUENCES:**

**Isolate OMS3-39**

>130_S-D-Bact-0011-a-S17_C02_08

GAAAACCCAGGGTTCCCCATGCAGTTCCTCGWTTAMGCCRAGGGCTTTCACAGCARACTTAT
TTTTCAGGGGGGGCTCGCTTTACGCCCAATAAATCCGGACAACGCTTGCCACCTACGTATTA
CCGCGGCTGCTGRCACGWARTTAGCCGWGACTTTCTGGTTGAWTACCGTCAARGAAWGGM
CAGTTACTACYTYTATYCTTCTTCYCCAACAACAGAGYTTTACGATCCGAAAACCTTCTTCAC
TCACGSGGCGTTGCTCCRTCCKACTTGCGTCCATTGCGGAAGATTCCCTAMTGCTGCCTCCCG
TASSAGTTTGGGCCGKGTCTCAGYCCCAATGYGGCCGATCAGCCTCTCAGSTCGGYTATGCA
TCATTGCCCTTGGTAAGCCGTTACCTTACCAAMTAWCTAATGCYCCGCGGRGCCATCCCATA
GCGACAGCCSACASCGCTTTTTAAAAACTACACCATGCGRTTTGKTTTTCTTATCCGGTATTA
SCCACCCTGTTTYCCCAGTGYTATCCCC

>130_S-G-Lab-0677-aA24_H02_23

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ACCGCGGCTGCTGRCACGTARTTAGCCGWGACTTTCTGGTTGATTACCGWMAAAYAATGRC
CAGTTACTACCTCTATTGTTCTTCMCCAACAACAGAGYTTTACGATCCGAAAACCTTCTTCAC
TCACGCGGSGTTGCTCCATCAGACTTGCGTCCATTGCSGAAGATTCCCTACTGCTGCCTCCCG
TAGGAGTTTGGGSCGKGTCTCAGTCCCAATGTGGCCGATMASCCTGTCARKTCGRCTATGCA
TCGTTGCCTGGRWGAGCCGTTACCTTACCAACTAGCTAATGCCCCGCGGGGCCATCCCATAG
MGACACCTYACSCCGCCTTTTATATCTAACCCTGCGRTCTGGTTTGTTATMCGGSATTASCAC
CTGKTTCCAARKGWTATCCCAKACTATGGGGCAGKMTACCCACGTGWTACTCACCCGTYCG
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**Isolate OMS6-78**

>179_S-G-Lab-0677-aA24_D03_12

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CGWCAAGGTAATCCAGTACTCTCATGMTGTTCTTCTCTAACAACAGAKTTTTAAWAMCGAA
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GCCTCCCCCMCGTTTGARCGGTTCATCACATGTGGCCGATAACCTCTCAGG

>179_S-G-Lab-0677-aA24_G02_20

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TACCGAATGCGCTCGCTTTACGCCCAATAAATCCGGAYAACGCTTGCCACCTACGTATTACC
GCGGSTGMTGRCACGWARWTAGCCGTGGCTTTCTGGTTAGAAACCGTCASGSSRTGAACAGT
TACTCTGATGCTTGTTCTTCTCYAACAACAGAGTTTTACGAYCCGAAARCCTTCTTCACTCAC
GCGGSGTTGCTCCRTCAGACTTKCGTCCATTGCCGAAKATTCCCTACTGCTGCCTCCCGTASG
AGTTKGGGSCGKGTCTCAGTCCCAATGTGGCCGATCASCCTCTCAGGTCGACTATGCATCGT
GGCCTTGRTGAACCYTTACCTCCCCAAATAGTTAATGCCCACGGRWCCATCCTRAGCGACCC
CCGAAAGCTCGTCAAATCYAACCATGCGKCTTTWTTGCATACGTATTAGCMCCTGTTCCAAG
TGWTATCCCCTCTTATGGSAWTTTATCCACGTGWTACTCCCCCSKKCGCCTCTTTCTTTTTCT
GTGRACCACCCTCCCTCGGCAAAGAAYCCTCCACATGCTTWTATTTTTTACGCCG