# MICROBIAL ECOLOGY AND DIVERSITY OF SWAZI TRADITIONAL FERMENTED FOODS

**By**

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## PREFACE

The work described in this thesis was carried out in the School of Agricultural, Earth and Environmental Sciences, University of KwaZulu-Natal and Faculty of Health Sciences, University of Swaziland, from February 2014 to November 2016, under the supervision of Dr Muthulisi Siwela and Prof Tendekayi Henry Gadaga.

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As the candidate's supervisors, we agree to the submission of this thesis.

Signed: Date:

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Signed:  Date: 07/12/2016

Prof Tendekayi H Gadaga (Co-supervisor)

## DECLARATION

I, Protus Simatende, declare that the thesis hereby submitted by me for the Philosophiae Doctorate degree in Food Security at the University of KwaZulu-Natal is my own original and independent research work. This thesis or any part of it has not been previously submitted by me for any degree or examination to another faculty or University. The research work reported in this thesis does not contain any person's data, pictures, graphs or other information unless specifically acknowledged as being sourced from those persons.

Signed:  Date: 01/12/2016

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## ABSTRACT

Spontaneously fermented foods are part of the diets and livelihood in many African countries. In Swaziland, several fermented foods are produced at household level. The present study covered the microbial diversity and biochemical aspects of *umcombotsi*, *buganu*, *emasi* and *emahewu*, but focused on *emasi* and *emahewu*, Swazi traditional fermented foods. The methods of preparation of *umcombotsi*, *buganu*, *emasi* and *emahewu* were documented. The lactic acid bacteria (LAB), yeasts, and coliforms were enumerated, and the pH and titratable acidity (TA) were determined in samples collected from households in Hhohho region of Swaziland. Also investigated was the probiotic potential of LAB Swazi fermented foods focussing on *emasi* and *emahewu*. The safety of *emasi* and *emahewu* was evaluated. *Umcombotsi* was mostly prepared by mixing maize meal, un-milled sorghum malt (*magayiwe*), and brown sugar (3 kg) in water (20 L). The initial stage involved the cooking the mixture to gelatinise the starch, followed by fermentation at ambient temperature (25-30$^{o}$C) for about 72 h. The whole preparation process takes about 4 to 5 days. *Emahewu* was prepared by mixing maize meal (1 kg) with water (ca. 5 L) and cooking to make a soft porridge. The cooled porridge was left to ferment at room temperature. Some reported adding sugar or a pilled potato to aid the fermentation process. *Emasi* was prepared by letting raw milk to naturally ferment at room temperature (25-30$^{o}$C) in either metal or plastic containers (buckets) for 2 to 3 days. *Buganu* was prepared from marula fruit (*Sclerocarya birrea*) (*amaganu*) juice and pulp mixed with water (ca. 10 L) and sugar (ca. 2 kg). The mixture was allowed to ferment at ambient temperature for about 3 days, sieved and then served. The home-made *emasi* was found to have an average pH of 4.68 and TA of 0.89%. The LAB counts were 8.25 log cfu/mL. Similarly, *Emahewu*, a maize based non-alcoholic beverage had pH of about 3.62 and TA of 0.43%. This product also had high LAB counts of 8.10 log CFU/mL. The LAB counts in the two products *emasi* and *emahewu* were consistent with

observations for similar African fermented foods. The LAB from *emasi* and *emahewu* were characterised a series of assays, Gram stain, catalase reaction, sugar assimilation tests (using API 50 CH test strips) and sequencing the 16S rDNA. Nine morphologically different isolates were identified from *emasi*, while sixteen (16) were identified from *emahewu*. It was found that *Lactococcus lactis* subsp. *lactis* and *Leuconostoc mesenteroides* were the common strains in *emasi*. *Lactobacillus plantarum, Lactobacillus paracasei* ssp. *paracasei* and *Lactobacillus brevis* were also detected. In *emahewu, Lactobacillus plantarum* were the most common strains, followed by *Leuconostoc mesenteroides* ssp. *mesenteroides*, *Lb. fermentum* and *Lb. brevis, Wessella confusa, Lactobacillus acidophilus* and *Lactococcus lactis*. It was concluded that these findings were consistence with other flora of naturally fermented South African milk where the common genera were *Leuconostoc, Lactococcus* and *Lactobacillus*. The strains in *emahewu* compared well with other similar products like *ting* (South African spontaneously fermented sorghum non-alcoholic porridge) where the main strains were *Lactobacillus plantarum, Lactococcus lactis, Lactobacillus fermentum, Lactobacillus rhamnsosus, Weissella cibaria*, and *Enterococcus faecalis*. Sixteen LAB isolates belonging to *Leuconostoc lactis, Lactobacillus plantarum, Lb. acidophilus, Weissella confusa, Lactococcus lactis* and *Leuconostoc pseudomesenteroides* were tested for the ability to grow at low pH, in the presence of bile, antibiotics, and for their antimicrobial properties. All of the strains were able to survive at pH 3. *Leuconostoc lactis, Lb. plantarum, Lb. acidophilus, L. lactis, Weissella confusa* and *Leuconostoc pseudomesenteroides* grew in the presence of 0.3, 0.5, and 1.0% (w/v) oxgall bile. All of the isolates were sensitivity to ampicillin, amoxicillin and tetracycline. However, *Lb. acidophilus* was resistant to ampicillin, and a strain of *Lb. plantarum* (M9) was moderately susceptible to tetracycline. All of the isolates showed resistance to streptomycin, ciprofloxacin and nalidixic acid, while *Weissella confusa* and *Lb. acidophilus* were moderately susceptible to ciprofloxacin. A neutral supernatant from each of

the actively growing cultures of the isolates inhibited *Salmonella typhimurium* ATCC 13311 and *Escherichia coli* ATCC 25922. The study showed that among the strains tested, *Lb. acidophilus Lb. plantarum* had properties that could be beneficial to the health of consumers.

Occasionally, high numbers of *Enterococcus* spp have been detected in some Southern African fermented foods. The presence of *Enterococcus* spp in foods is associated with poor hygienic practices and may indicate a potential risk to consumers who regularly consume the foods. The *Enterococcus* spp in traditionally fermented milk (*emasi*), non-alcoholic cereal beverage (*emahewu*) were isolated on MacConkey agar. Typical colonies were isolated, purified and phenotypically representative strains were identified by sequencing the 16S rDNA. The colony counts in *emasi* ranged from not detectable to 8.58 log cfu/ml while *emahewu* ranged from not detectable to 6.72 ± 0.10 log cfu/ml. Five isolates from *emasi* were identified as *Enterococcus durans, Enterococcus hirae, and Enterococcus lactis*. One isolate from *emasi* was *Enterococcus durans and Enterococcus lactis*. Despite concerns raised by earlier obseravtions on coliform counts, these observations suggest that the fermented foods maybe safe for consumption since the strains recovered are known to be non pathogenic.

## ACKNOWLEDGEMENTS

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## DEDICATION

To mum and dad, and all members of the family we have lost.

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## ABBREVIATIONS

| | |
|---|---|
| AOAC | Association of Official Analytical Chemists |
| ANOVA | Analysis of Variance |
| AP | Ampicillin |
| API | Analytical Profile Index |
| ATCC | American Type Culture Collection |
| BHI | Brain Heart Infusion |
| CFU | Colony forming units |
| CIP | Ciprofloxacin |
| DGGE | Denaturing Gradient Gel Electrophoresis |
| DNA | Deoxyribonucleic acid |
| FAO | Food and Agriculture Organisation |
| GMP | Good Manufacturing Practices |
| GIT | Gastro Intestinal Tract |
| IKS | Indigenous knowledge systems |
| LAB | Lactic Acid bacteria |
| MRS | de Man, Rogosa and Sharpe |
| NA | Nalidixic acid |
| PCR | Polymerase Chain Reaction |

| | |
|---|---|
| SD | Standard Deviation |
| SPSS | Statistics Package for Social Sciences |
| TA | Titratable acidity |

# CHAPTER 1: INTRODUCTION, THE PROBLEM AND ITS SETTING

## 1.0. Introduction

Fermentation of food is one of the oldest methods of food processing options in several African communities (Blandino et al 2003; Göran 2001). Consumption of fermented foods is thought to contribute to good health attributed to the beneficial effects of the activity of product microflora in the human gut. Several investigations have revealed the important role of fermented food products' microflora in preventing food borne illnesses, especially childhood diarrhoea (Mortarjemi 2002) and enhancing dietary diversity. They are also relatively safe from pathogenic microorganisms due to the inhibitory effects of acid as well as antimicrobial substances produced by some of the microorganisms; hence, they are often used as weaning foods in many African countries. In Swaziland, these fermented foods play a role in stabilizing Swazi homestead Food Security.

The predominant microorganisms in most of these foods are lactic acid bacteria, especially *Lactococcus lactis* (Blandino et al 2003). Schoustra et al (2013), reported that in Munkoyo and Chibwantu, a traditional non-alcoholic fermented beverage popularly consumed in Zambia that, is similar to Swazi *emahewu*, *Lactobacillus* and *Weissella* were the common genera isolated others were *Lactococcus*, *Streptococcus* and *Leuconostoc*. Osvik et al (2013) reported that the majority of the strains in South African *amasi* were in the genus *Lactococcus*, as well as several species of *Lactobacillus*, *Leuconostoc*, and *Enterococcus*. *Amasi* from KwaZulu-Natal in South Africa is a similar product to Swazi *emasi*. In *kule naoto*, Kenyan traditional fermented milk produced by the Maasai, the genus *Lactobacillus* was found to be predominant, especially *Lactobacillus plantarum*, followed by *Lactobacillus fermentum*, *Lactobacillus paracasei* and *Lactobacillus acidophilus* (Mathara et al 2004)).

Other genera that were isolated in *kule naoto* were *Enterococcus*, *Lactococcus* and *Leuconostoc*. Yeasts have also been isolated from some fermented foods especially fermented milk (Gadaga et al 2001). The role of yeasts in milk fermentation has been linked to their ability to utilise milk constituents. For example, *Kluyveromyces marxianus* ferments lactose while other species have proteolytic and lipolytic characteristics (Roostita and Fleet 1996).

Although there are a variety of fermented foods, they can be grouped into four categories, namely, alcoholic, lactic acid, acetic acid and alkali fermented foods (Blandino et al 2003; Steinkraus 2002). Many African cereal grain-based fermented foods are categorised as lactic acid fermented. Examples include mahleu/mahewu (South Africa, Zimbabwe, Lesotho), togwa (Tanzania), kenkey (Ghana), amasi (southern Africa) and motoho (Lesotho). Masarirambi et al (2009) also described some traditional fermented foods in Swaziland. These were fermented maize (*sancoti*), fermented porridges (*incwancwa*), fermented milk (*emasi*), non-alcoholic cereal beverage (e*mahewu*), alcoholic beverage (*umcombotsi*) from sorghum (e*mabele*) or millet (*nyawotsi*), malt distilled spirits (*mankanjane*) and fermented fruit mashes *(buganu*/*marula* wine and papaya beer).

Fermented foods are widely consumed in Swaziland play a great role in the food security of the house hold, especially in rural settings. Notably traditional fermented Swazi foods are prepared from raw materials that are found in homesteads. These fermented foods are relatively cheap to buy compared to commercial products, hence contribute to food security.

In the past, traditional meals of the Swazi consisted of maize porridge supplemented with a side dish, prepared from legumes, vegetables (*umshibo*) and meat (*sithulo*). A lot depended on the seasonal availability of raw materials. Kgaphola and Viljoen (2004) observed that the food habits of the Swazi have been changing over the years, including changes in the numbers of meals and the composition of the meals consumed per day. For example, at the end of the 19th century sour milk (*emasi*) was the core food of the Swazi people. The morning meal consisted of *emasi* or sorghum porridge while the evening meal consisted of *emasi* and porridge prepared from boiled crushed maize (Kgaphola and Viljoen 2004; Jones 1963 (cited by Kgaphola and Viljoen 2000). By mid 1950s, there was now a tendency to take traditional snacks, notably boiled jugo beans, sugar cane, wild fruit, toasted groundnuts and *amahewu* in between meals. This trend however further changed in next two decades, where Swazi were reported have been taking snacks in form of bread, leftovers and fermented porridge *amahewu*. Coupled with the outlined changes and the natural rinderpest outbreak in 1897 that destroyed most of the Swazi cattle, the Swazi diet has changed resulting in the replacement of the staple food milk with maize (Kgaphola and Viljoen 2004) and have compromised the general food security of Swazi. These changes lead to loss of the traditional knowledge and technology associated with these foods. Although many Swazi people consume fermented food regularly, no scientific research has been done to isolate and identify the specific strains of microorganism involved in fermented foods in Swaziland. Little research has been carried out to determine the microbiological and biochemical characteristics of foods in Swaziland. The few documented reports on foods in Swaziland mainly refer to methods of preparation (Masarirambi et al 2009). The proposed study was meant to further advance knowledge on Swazi fermented foods. Some of the fermented products in Swaziland are seasonal (*buganu*/*marula*), therefore, this study concentrated on *emasi* and e*mahewu*. The two products (*emasi* and e*mahewu*) are non-alcoholic and further

fall under the category lactic acid fermented Swazi foods. Yet in other countries in Africa, Europe and Asia extensive research has been done in documenting the technical, artistic, microbial, and biochemical characteristics of fermented foods (Steinkraus 2002).

Preliminary studies on some fermented food in Swaziland have shown that the levels of total coliforms are persistently high (Simatende et al 2015). This could be attributed to contamination during the preparation of the products. The quality of these traditionally prepared products depend on who is preparing the food and how well they adhere to sound personal hygiene and observing good manufacturing practices (GMP) to ensure the safety of these fermented foods. Microorganisms that predominate in a product are influenced by prevailing environment factors, including temperature, soil and water. Traditionally, fermentation is done spontaneously at ambient temperature; hence the quality of the fermented product varies from one fermentation cycle to the next because the types of fermenting microorganisms are not controlled. Improvement in quality is achieved by isolating the predominant, safe microorganisms from the food, sub-culturing them and then using them for controlled fermentations.

### 1.1. Summary of research focus

This current study aimed at documenting the steps and equipment used in the preparation of four products, *emahewu*, *emasi*, *umcombotsi* and *buganu* prepared at household level in Swaziland. The study further focused on the enumeration, isolation and identification of lactic acid bacteria in *emasi* (fermented milk) and *emahewu* (maize based non-alcoholic beverage), Swazi traditional fermented foods. Lactic acid bacteria (LAB) were enumerated,

isolated and identified, from samples that were collected from households in Hhohho region of Swaziland. The probiotic properties of LAB in Swazi fermented foods focusing on *emahewu* were characterised by looking at properties like acid resistance, bile tolerance, susceptibility to antibiotics and antimicrobial activity of LAB. The safety of *emasi* and *emahewu* to consumers was investigated. Some results (Chapter 4) have been published others submitted for publication (Appendix 1). The findings in chapter 5, 6 and 7 manuscripts are under review in peer-reviewed journals (Appendix 1).

## 1.2. Purpose of the study

The purpose of study was to study the ecology of Swazi fermented foods and their contribution to food security by documenting methods of preparation, enumerating, isolating and identifying predominant microorganisms in the products, focusing on *emasi* and *emahewu*.

## 1.3. Hypothesis and study objectives

### *1.3.1. Hypothesis 1*

Each of the traditional Swazi fermented foods, *emahewu*, *emasi*, *umcombotsi*, and *buganu*, is processed according to a specific method that follows logical technical steps. The basis for the processing methods is traditional and/or indigenous knowledge systems (IKS), some of which can be also explained in terms of modern Science principles.

### *1.3.2. Objective 1*

*1.3.2.1. To document the preparation methods of emahewu, emasi, umcombotsi, and buganu produced at house hold levels.*

### *1.3.3. Hypothesis 2*

In a product similar to *emasi* called *kule naoto* (Kenyan traditional fermented milk produced by the Maasai), Mathara et al (2004) reported that genus *Lactobacillus* species (*Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus paracasei* and *Lactobacillus acidophilus*.) were predominant. Others genera that were isolated were Enterococcus, *Lactococcus* and *Leuconostoc*. In another similar product to Swazi *emahewu*, Schoustra et al (2013) reported that in Munkoyo and Chibwantu, (traditionally non-alcoholic fermented beverages prepared and popularly consumed in Zambia), *Lactobacillus* and *Weissella* were the common genera isolated together with *Lactococcus*, *Streptococcus* and *Leuconostoc*. Similar microflora will be found predominant in the Swazi traditional fermented foods.

### *1.3.4. Objective 2*

*1.3.4.1. To enumerate, isolate and identify Lactic Acid Bacteria (LAB) in Swazi fermented foods focusing on emasi and emahewu.*

### *1.3.5 Hypothesis 3*

Some of the microbial types associated with Swazi traditional fermented foods will possess significant probiotic properties, similar to numerous fermented foods whose fermentation processes are largely driven by lactic acid bacteria (LAB).

### *1.3.6. Objective 3*

*1.3.6.1. To characterise the probiotic properties of LAB in Swazi fermented foods focusing on emasi and emahewu.*

### *1.3.7. Hypothesis 4*

Some of the LAB types associated with Swazi traditional foods will be characteristic of probiotic LAB, contribute to the microbiological safety of the fermented products due to several factors, such as competitive advantages over pathogens (e.g. tolerance to acidic environment of the fermented food) and antimicrobial activity factors against pathogens, e.g. production of bacteriocins.

### *1.3.8. Objective 4*

*1.3.8.1. To assess the safety of traditionally fermented Swazi foods (emasi and emahewu).*

## 1.4. Study parameters and general assumptions

The setup followed an experimental design where samples of fermented foods from Hhohho region (one of the four regions that make Swaziland geographically) were conveniently sampled and taken to the laboratory at the University of Swaziland for microbial and biochemical analysis.

## 1.5. Outline of the thesis

The thesis is laid out as follows:

Chapter 1: Introduction, the problem and its setting.

Chapter 2: Literature review.

Chapter 3: Study design and methodology.

Chapter 4: Methods of preparation of Swazi traditional fermented foods.

Chapter 5: The microbial and biochemical aspects of Swazi traditional fermented foods and identity of lactic acid bacteria in the foods, focussing on *emasi* and *emahewu* foods.

Chapter 6: Evaluation of probiotic properties of Lactic Acid bacteria strains isolated from *emahewu*, a Swazi fermented food.

Chapter 7: Determining the safety of traditionally fermented Swazi foods (*emasi* and *emahewu*).

Chapter 8: General Discussion.

Chapter 8: Conclusions and Recommendations.

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# CHAPTER 2: LITERATURE REVIEW

## 2.1. Introduction

Traditional fermented foods are commonly widely consumed globally. The fermentation processes associated with the different products makes the raw materials not only more palatable but also extends the shelf life of the product. Fermentation can improve the nutritional quality of some products due to some desirable changes such as increased nutrient bioavailability and synthesis of some nutrients. Further, fermented foods often reported to be more microbiologically stable and safe and health-promoting compared to unfermented foods. This is attributed to a number of factors, including the low pH of the fermented foods and probiotic activities, such as production of bacteriocins and health-enhencing substances of some of the microbial types, particularly the LAB, associated with the fermented foods. It is hypothesised that by identifying the microorganisms that are involved in fermentation of traditional Swazi foods and characterising their probiotics properties may be beneficial as the isolated probiotic microorganisms could be used in the production of innovative food products with enhanced microbiological stability, safety and health-beneficial properties. This chapter reviews types of traditional fermented foods popular in Swaziland, microorganisms often reported present in traditional fermented foods of Southern Africa, reported probiotic properties of lactic acid bacteria found in fermented foods focusing on Swazi traditional fermented foods and characterisation of probiotic properties of LAB in fermented foods.

## 2.2. Traditional fermented foods

Traditional fermented foods are consumed in many communities in the world. In Africa, including Swaziland, fermented foods are derived from cereals, milk, fruits and wood sap (Gadaga et al 1999; Steinkraus 2002; Masarirambi et al 2009). The fermentation process makes the raw materials more palatable and extends the shelf life of the product. In addition, various nutritional benefits have been reported including improved bioavailability of nutrients and added protection from pathogenic microorganisms due to the low pH. Lactic acid bacteria and yeasts constitute the main groups of desirable microorganisms responsible for fermentation. They are able to break down the sugars or carbohydrates present in the fermented raw material and convert it into different products such as lactic acid, acetic acid, ethanol, diacetyl and acetaldehyde. Some of these microorganisms have been shown to survive the harsh environment in the human gut and offer some beneficial effects to the consumer. They are therefore considered as probiotic microorganisms and include species such as *Lactobacillus rhamnosus* and *Lactobacillus paracasei*. Some of the *Lactobacillus* species that are common in African fermented foods have also been isolated from the human intestinal mucosae. In a study focusing on non-dairy probiotics, Göran (2001) observed that *L. plantarum* is the microorganism frequently associated with lactic acid fermented foods of plant origin.

## 2.3. Types of traditional fermented foods popular in Swaziland

Although there are a variety of fermented foods, they can be grouped into four categories, namely, alcoholic, lactic acid, acetic acid and alkali fermented foods (Blandino et al 2003; Steinkraus 2002). Many African fermented cereal grain-based foods are categorized as lactic acid fermented. Examples include mahleu/mahewu (South Africa, Zimbabwe, Lesotho),

togwa (Tanzania), kenkey (Ghana), amasi (southern Africa) and motoho (Lesotho). Masarirambi et al (2009) described some traditional fermented foods in Swaziland. These were fermented maize (*sancoti*), fermented porridges (*incwancwa*), fermented milk (*emasi*), non-alcoholic cereal beverage (e*mahewu*), alcoholic beverage (*umcombotsi*) from sorghum (e*mabele*) or millet (*nyawotsi*), malt distilled spirits (*mankanjane*) and fermented fruit mashes *(buganu*/*marula* wine and papaya beer). Masarirambi et al (2009) further grouped Swaziland traditional fermented foods under the following categories, cereal-based fermented products, non-alcoholic products (*emahewu, incwancwa* & *singwangwa*), alcoholic beverages (*umcombotsi*), fermented wild fruit products (*emaganu* wine or *buganu*) and fermented milk (*emasi*).

### *2.3.1. Umcombotsi*

Generally, almost all cereal-based alcoholic and non-alcoholic traditionally fermented products in Swaziland according to Masarirambi et al (2009), are prepared either using one of the malt or as combination of the malts; sorghum (*Sorghum bicor*), maize (*Zea mays*), bulrush millet (*Pennisetum typhoideum*) or some finger millet (*Eleusine coracana*). In Swaziland different types of malts are commonly referred to as *umtfombo*.

To prepare the malt the grain undergoes the first step known as steeping where the grains in some Hessian sack or any substitute sack are immersed in water (ambient temperature that range between 10 $^{o}$C to 20 $^{o}$C, but may vary depending on the temperatures in the regions) for a day (Masarirambi et al 2009). The immersion of grains in water is done at home or using nearby streams. After grains are immersed in water for a day, the swollen grains that have

absorbed some water (swollen) are spread on floor to facilitate germination in the next 3 days (Masarirambi et al 2009).

The malt (sorghum) is mixed with maize meal, followed by boiling the mixture to make some thin gruel, and then left over night to ferment. The malt is then milled to fine powder. The malting stage is also referred to as mashing where non-fermentable starch is transformed into sugars that later yeast can ferment. The malt phase is enzymatic in nature and involves some biochemical reactions. No literature is available on the detailed process beyond this stage (Masarirambi et al 2009). Nevertheless, the traditional Swaziland fermented *Umcombotsi* is similar to other traditionally fermented alcoholic beverages such as *Joala* (traditional *Sesotho* or sorghum beer in Lesotho). According to Gadaga et al (2013), sorghum/maize meal together with wheat meal in equal proportions are mixed together before cold water is added to make an opaque solution. This step is followed by adding boiling water to form viscous slurry, and then cooled to temperatures of 30-35 $^{o}$C. In Lesotho a traditional starter (liquid) called *tomoso* is added to the mixture. To retain moisture, the mixture is then covered with a blanket. Under ambient temperature the mixture is left to stand and ferment for 24-48 h. After this stage the mixture is boiled for 2-3 h before cooling to 30-35 $^{o}$C. A starter culture (solid material) referred to as *moroko* (that is made up of spent dregs from other fermentations) is added. This allows the mixture to ferment for an additional 24-48 h (Figure 2.1.). Before the alcoholic beverage is ready to drink, it is filtered to remove the coarse particles.

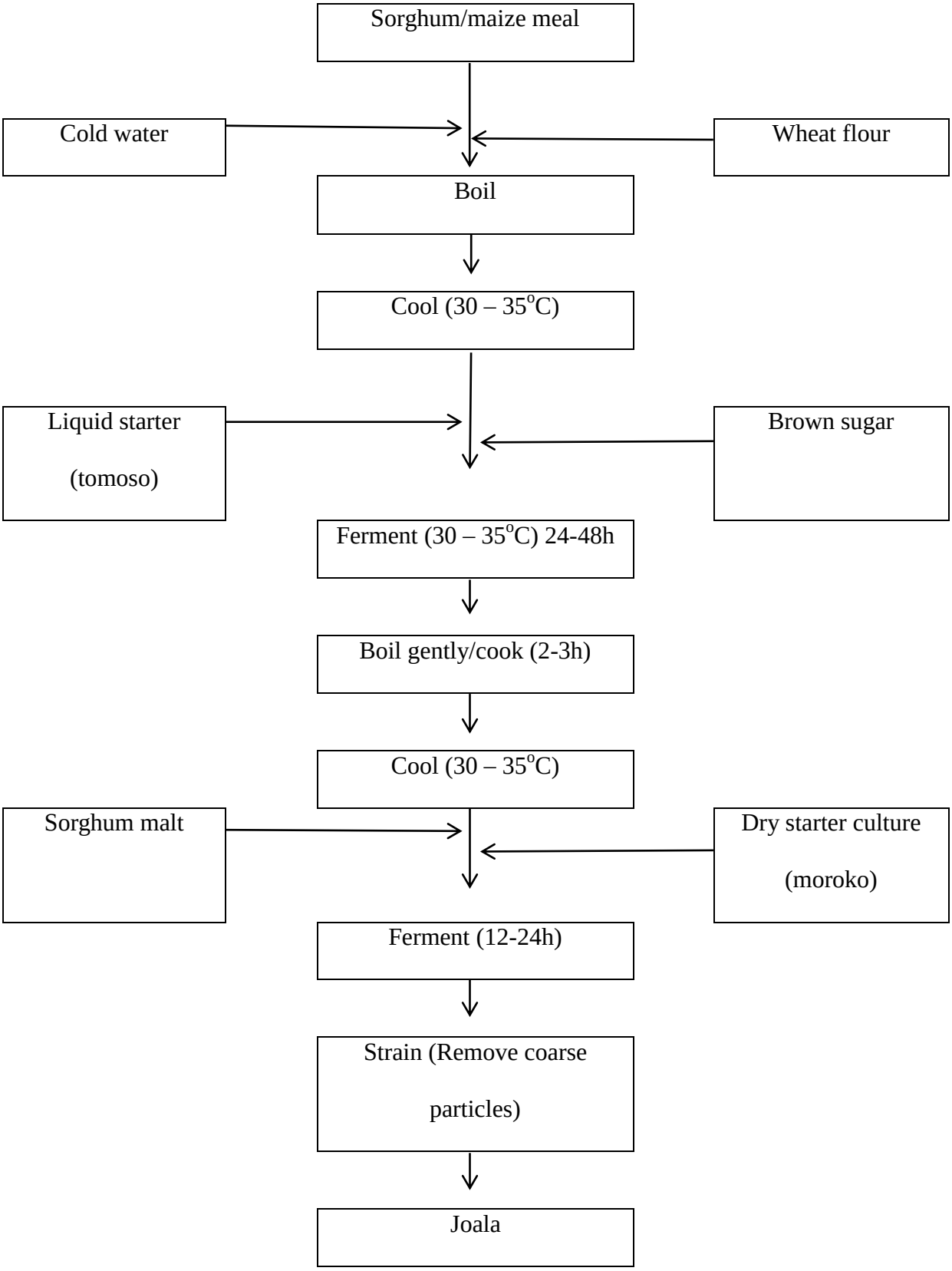


**Figure 2.1.** Traditional preparation of *Sesotho* (sorghum beer) in Lesotho (Gadaga et al 2013).

### *2.3.2. Emahewu*

This a common home prepared non-alcoholic beverage in Sub-Sahara Africa usually made from thick maize porridge (Gadaga et al 1999; Masarirambi et al 2009; Okagbue 1995). It is usually used to wean children in Southern Africa (Simango 1997).

In Swaziland during preparation of *emahewu,* water may be added subject to thickness of the maize porridge (Masarirambi et al 2009). If during preparation they start with thick porridge, the porridge is mashed into sizeable pieces before the addition of water. The common ingredients used are millet malt, sorghum malt or wheat flour. The ingredients are added to the mixture and left to ferment at ambient temperature. The product is ready for consumption in about 24 h. There is no much detailed information documented on preparation of *emahewu* beyond this stage in Swaziland.

Gadaga et al (2013) described the preparation of *Motoho* a non-alcoholic sour porridge prepared in Lesotho by some Basotho (people from Lesotho). *Motoho* is similar product to *emahewu*. To prepare *motoho,* Basotho prefer red type of sorghum and because of the colour of sorghum used the finally product has a brownish colour. The summary of the preparation process is outline in Figure 2.2. One (1) part of red sorghum is mixed to three (3) parts of water to give the solution thin viscosity. A starter culture called *tomoso* is used to make this product. The ratio at which *tomoso* to thin viscous gruel solution is added is at one (1) part *tomoso* to twenty (20) parts of slurry solution. *Motoho* can be ready in 24h after fermentation at ambient temperature (25–30$^{o}$C). In winter due to low temperatures the fermentation

process takes longer and the product will be ready between 48 – 72h. To aid the fermentation process in winter the container where the mixture is put is covered by a blanket. Once fermentation has taken place before the final product is served; it is boiled for 20–30 minutes before cooling it to 25–30$^{o}$C.

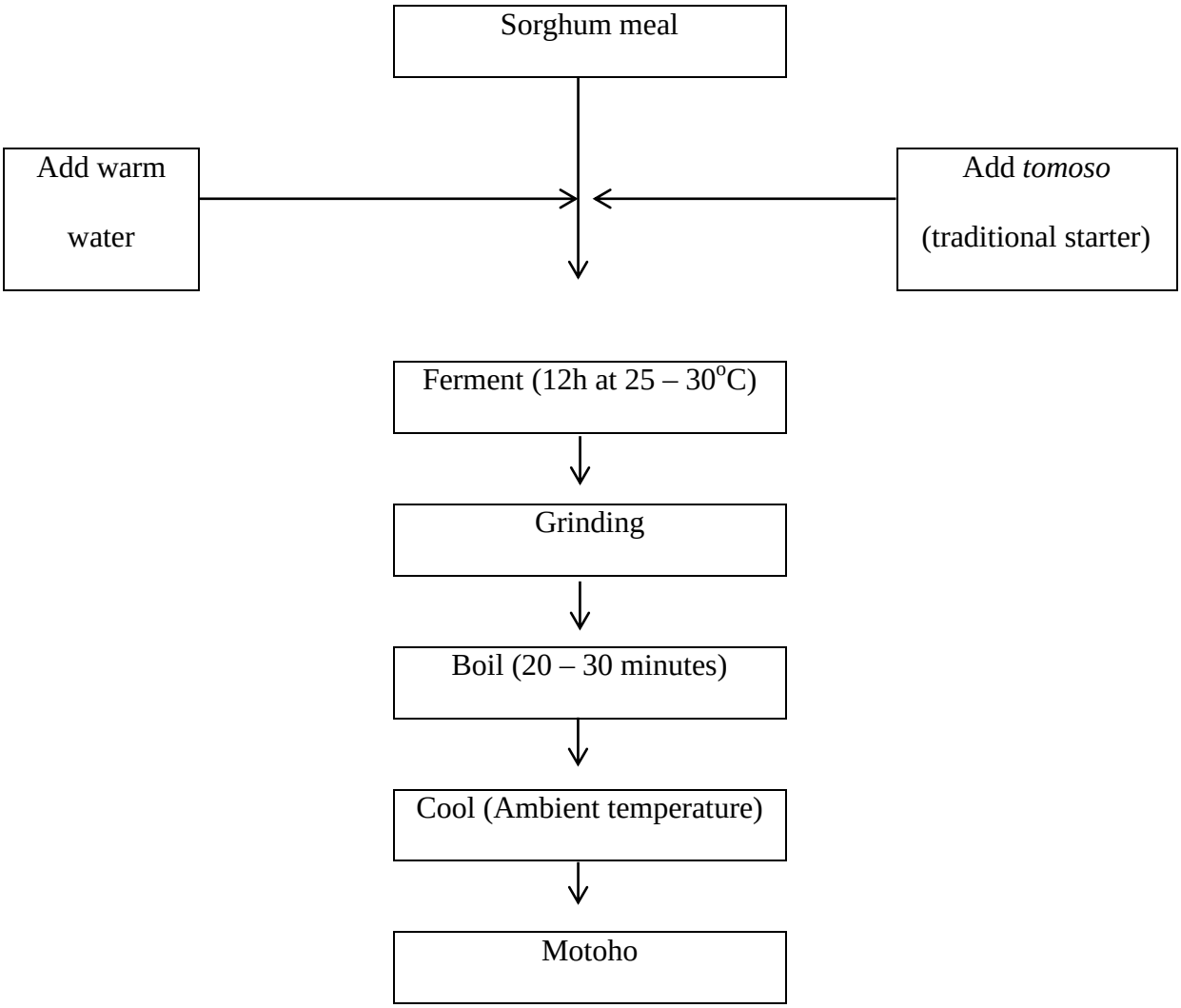


**Figure 2.2.** Traditional preparation of *motoho* in Lesotho (Gadaga et al 2013).

The liquid starter culture (*tomoso*) used in preparing *motoho* is made by mixing a portion of sorghum with very little amounts of warm water. The warmed water is poured to just cover the sorghum meal. This is preceded by fermentation for 24 h. Ocassionally a slopping approach is followed where previous complete fermented material is used (Gadaga et al 2013).

### *2.3.3. Emasi*

*Emasi* a product derived from traditional fermentation of milk is widely made and consumed largely in most of Southern Africa countries (Gadaga et al 1999; Masarirambi et al 2009; Mutukumira et al 1995). *Emasi* is a common traditionally fermented milk product in Southern African region where rearing of cattle is common preoccupation even in rural homes. Cattle are kept as source of rich proteins and in rural homes are a source much needed draught power.

To prepare *emasi*, in Swaziland residents in homestead milk their cows by hand then place the milk in containers and left to ferment for 24-48 h under ambient temperatures (Figure 2.3.). Masarirambi et al (2009), reports that then the whey is separated, while some fermented cream from previous fermented *emasi* is normally added. This is done in order to increase the total solids. The fermentation of *emasi* is achieved by using natural microorganisms in milk to grow and use the milk lactose to produce lactic acid which in turn lowers the pH. In Swaziland during the production of traditionally fermented *emasi*, there have been recorded two types of fermentation that occurs. There is the desirable fermentation process and undesirable fermentation process. The desirable fermentation process refers to the breakdown

of milk sugars by lactic acid bacteria giving a pleasant and acceptable product (Nsibande and Dlamini 2000). The other process refers to fermentation of milk that takes place when non-lactic acid bacteria ferment milk sugars resulting into stale or insipid tasting product. The containers (vessel or calabash gourd) used during the making of *emasi* are known as ***ingula.***

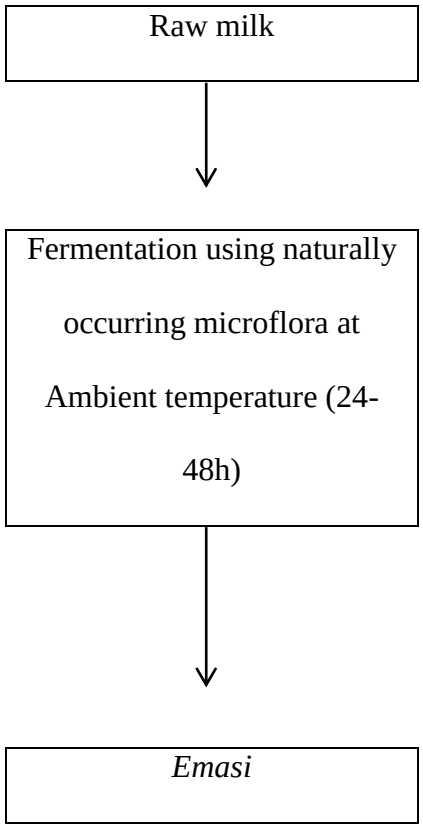


**Figure 2.3.** Traditional preparation of *emasi* in Swaziland (Masarirambi et al 2009)

*Emasi* is similar to traditionally fermented milk called *mafi* (product prepared in Lesotho). The fermentation procedure is no different from that followed in Swaziland. In Lesotho during the preparation of *mafi* the raw milk is allowed to naturally ferment in clay pots for 24-72h at ambient temperature ranging from 25–30$^{o}$C (Gadaga et al 2013). Other product similar to *emasi* and produced with the Southern Africa region is *madila* (traditional

fermented milk prepared in Botswana). Though the procedure is similar with same fundamental fermentation interpretation, there some variation in approach. To prepare *madila* the raw milk is filtered using a strainer before the filtrate is placed in an enamel or metal bucket. To initiate fermentation, the container with milk is left to stand in a warm place of roughly 30$^{o}$C for period of 24 h. This is followed by pouring the sour milk into sack (woven polypropylene), then one-day old fermented milk is added. This procedure of adding one-day old bucket of sour is repeated daily 7 to 8 days to facilitate further fermentation. After 7 of 8 days the bags with fermented milk are hung for a period of 3-4 days and this enables the whey to drain away through the small openings of the woven bag. The final stage involves the mixing of *madila* with fresh milk at the ratio of 4.1 (Gadaga et al 2013). Other traditionally fermented foods with Southern Africa that are similar to *Emasi* are *mukaka wakakora* or *zifa* (Shona) or *amasi* (Ndebele), product prepared in Zimbabwe. *Zifa* is like Swaziland *emasi* is spontaneously fermented raw milk (Mutukumira 1995; Gadaga et al 1999). Produced in Zambia *mabisi* is raw milk traditionally fermented product (Figure 2.4.).

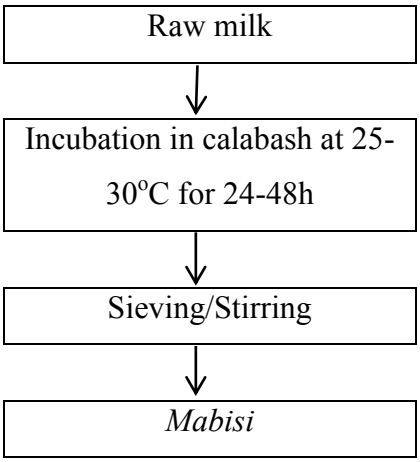


**Figure 2.4.** Traditional preparation of *mabisi* in Southern and Central parts of Zambia (Schoustra et al 2013).

The fermentation of raw milk takes place in calabash over 48h at ambient temperatures (Schoustra et al 2013). During the production of *mabisi*, not cleaned calabashes that previous had *mabisi* are used. This is done so that they provide starter culture for the next raw milk.

### *2.3.4. Buganu*

*Buganu* (*emaganu*) is a season Swaziland alcoholic beverage produced from wild fruits during when marula fruits are available and ripens. The first step begins with harvesting of ripe marula fruits from the wild trees. Some fruits are picked from the ground. To improve on the safety of the final products, the fruits are washed in containers (stainless buckets or plastics) before being pierced and squeezed (Gadaga et al 1999; Masarirambi et al 2009). The capacity of containers used range from 5 to 20 litres in volume. This step is preceded by removal of seeds and pounding of fruits before water is added. The mixture is poured into drums. Fermentation begins in drums that are covered to protect them from entrance of other unwanted foreign materials (Masarirambi et al 2009). The seeds from the fermenting mixture in the drums are squeezed and removed. Then there is a repeat of the procedure described above where fresh fruits are pounded so that the seeds and flesh can be separated, and the mixture poured into the drum. To ensure a good mixture the slurry obtained is stirred and left to ferment for a night. This step where fresh fruits are pounded so that the seeds and flesh can be separated is repeated with new marula fruits, allowed to ferment overnight until white foam gradually forms on the surface (Masarirambi et al 2009). The formation of white foams is referred to as flocculation stage. It occurs because yeasts form low density clumps or flocs that traps carbon dioxide and rises to the surface. The flocculation stage implies that fermentation is almost complete (Hutkins 2006; Masarirambi et al 2009). The flocculation may appear at about day 5, although it may depend on other type of microflora, temperature,

nutrients and oxygen present (Masarirambi et al 2009). On the fifth day, the flocs are removed by filtering leaving a yellowish-brown *buganu* beverage.

*Emaganu* is similar to *mukumbi* (a Zimbabwean traditionally prepared alcoholic beverage from marula fruits) (Mpofu et al 2008). *Mukumbi* is prepared by fermenting a mash and this mash is from ripe marula fruits. Although preparation of *mukumbi* wine vary from one producer to another fermentation of the mash is done by the yeasts.

### *2.3.5. Significance of Swazi traditional fermented foods to household food and nutrition security.*

The traditionally fermented Swazi foods play different roles at household that border on nutritional security. *Emasi* in Swaziland provide some much needed proteins in Swazi diets (Masarirambi et al 2009). *Emasi* contains about 2 ppm diacetyl, a range of volatile and non-volatile organic compounds such as lactic acid, acetaldehyde, acetic acid, succinic acid, citric acid, pyruvic acid and ethanol that makes the product nutritionally acceptable (Mutukumira et al 2008).

The people in Swazi homestead are known to take two to three meals per day (Kgaphola and Viljoen 2004) and fermented Swazi foods make part of the portions eaten daily in homestead. And amongst the common taken traditionally fermented foods is *emahewu* and *emasi*. Other commonly taken fermented foods is the fermented porridges (*incwancwa*), alcoholic

beverage (*umcombotsi*) and fermented fruit mashes *(buganu*/*marula* wine and papaya beer). Although *Buganu* is taken due to its alcohol content it is also nutritious.

Kgaphola and Viljoen (2004) describes Swazi foods under five categories as, cultural super foods, prestige foods, body image foods, sympathetic magic foods and physiological group foods. Cultural super foods refer to those foods that are eaten regularly, the dominant staple foods and normally substantially contribute to the energy intake of the diet. Undoubtedly Swazi traditionally fermented foods such as *emasi* and *emahewu* do contribute among other factors the valuable energy calories. Traditionally in Swaziland food products from sorghum were considered as cultural super food in the Swazi diets (Kgaphola and Viljoen 2004). Sorghum is one of the main ingredients used to prepare Swazi traditionally fermented foods. Mothers in Swaziland usually prefer to feed their young children cultural super food. Kgaphola and Viljoen (2004) further states that a household without maize or sorghum in Swaziland was looked after as starving even though other food items were available. Maize remains a cultural super food in Swaziland. It is an important portion of the daily meals and quite often taken two times in a day.

Prestige foods are those foods left aside for very important occasions. In Swaziland traditionally what makes up this category is beer and meat (Kgaphola and Viljoen 2004). Therefore, Swazi traditionally fermented foods like *buganu* contribute to food security of Swazi culture. In addition, alcoholic beverage (*umcombotsi*) from sorghum (e*mabele*) or millet (*nyawotsi*) too does add up to prestige foods.

Then comes the body image foods category that simply refers to foods that equates to how the body functions (Kgaphola and Viljoen 2004). In Swazi diets milk is regarded as an important health food and sometimes may be used as a purifying medicine on certain ritual occasions. Products prepared from milk like *emasi* does also fall under this category. The sympathetic magic foods do not necessarily have any traditionally fermented Swazi foods that we can refer to it though it covers other foods consumed by Swazi people. The last category is that of physiological group foods. These are foods restricted to a particular physiological group based on age, gender or physiological condition. In Swaziland, traditionally girls were not allowed to eat traditionally fermented *emasi* during their menstruation period (Kgaphola and Viljoen 2004).

## 2.4. Microorganisms often reported present in traditional fermented foods of Southern Africa.

### *2.4.1. Microorganisms reported present in Swazi traditional fermented foods.*

According to Masarirambi et al (2009) concluded that there was no work done on biochemical and microbiological properties of traditional fermented Swazi foods. Therefore, with respect to microorganisms reported present in Swazi traditional fermented foods, the literature review covers microorganisms that have been reported in similar foods mostly with Southern Africa and to some extent other parts of Africa.

#### *2.4.1.1. Umcombotsi*

The traditionally fermented South African sorghum beer called *umqombothi* is similar to Lesotho traditionally fermented beer called *joala* (Gadaga et al 2013). Swazi *umcombotsi* is

similar to South African fermented sorghum beer (*umqombothi*). In traditionally fermented beverages, the fermentation process initially starts with lactic fermentation then followed by alcoholic fermentation (Solange 2014). Therefore, with respect to microorganisms in these fermented beverages we expect microorganisms that participate in lactic acid fermentation and alcoholic fermentation. In *umqombothi* the most prevalent fermenting microorganism were lactic acid bacteria (*Lactotobacillus, leuconostoc, Pediococcus* and *Enterococcus*) and yeasts (*Saccharomyces cerevisiae, Saccharomyces capsularis, Candida ethanolica, Candida haemuloni, Candida sorbophila, Dekkera anomala* and *Dekkera bruxellensis*) (Katongole 2008; Solange et al 2014). In a similar product to *umqombothi,* Doro or Chibuku (Zimbabwe and Zambia), the common microflora during fermentation were *Saccharomyces cerevisiae*, *Lactobacillus plantarum, Lactobacillus delbrueckii, Lactococcus lactis* and *Lactococcus rafanolactis* (Lyumugabe et al 2012).

It is also reported that in African beers prepared with sorghum as one of the ingredients *Saccharomyces cerevisiae* together with lactic acid bacteria do predominate during fermentation process (Lyumugabe et al 2012). In *burukutu*, (a sorghum beer from West Africa - Ghana, Benin and Nigeria) *Saccharomyces cerevisiae* and *Saccharomyces chavelieri* were the common microorganisms responsible for fermentation of sorghum sugars to alcohol (Faparusi et al 1973).

*2.4.1.2. Emahewu*

In a separate study, Madoroba et al (2011) isolated and identified LAB in *ting*, a South African spontaneously fermented sorghum non-alcoholic beverage, and found that the

predominant LAB were *Lactobacillus plantarum*, *Lactococcus lactis*, *Lactobacillus fermentum*, *Lactobacillus rhamnsosus*, *Weissella cibaria*, and *Enterococcus faecalis*. Some Enterobacteriaceae were also isolated. The Swazi *emahewu* samples were prepared from maize meal. Solange et al (2014) elaborate further that in African non-alcoholic beverages the predominant microorganisms were *Lactococcus lactis* subsp. lactis and some yeasts (*Candida haemuloni, Candida sorbophila, Debaryomyces hansenii, Saccharomyces capsularies* and Saccharomyces. On the other hand, the predominant microorganisms in *koko*, Ghanaian spontaneously fermented porridge from millet, were identified as *Weissella confusa* and *Lactobacillus fermentum* (Lei and Jakobsen 2004), while Yousif et al (2010) found that *Lactobacillus fermentum* and *Pediococcus acidilacti* were the predominant strains in *hussuwa*, a Sudanese fermented sorghum food. Also in *gari*, a cassava based fermented food from Benin, *Lactobacillus plantarum* was the most commonly isolated species followed by *Leuconstoc fallax* and *Lactobacillus fermentum* (Kostinek et al 2005). Muyanja et al (2003) also identified the LAB isolated from the spontaneously fermented Ugandan bushera as *Lactobacillus plantarum, L. paracasei* subsp. *paracasei*, *Lb. fermentum*, *Lb. brevis* and *Lb. delbrueckii* subsp. *delbrueckii*. *Streptococcus thermophiles.* Similarly, Munkoyo and Chibwantu, traditionally non-alcoholic fermented beverages popularly consumed in Zambia, *Lactobacillus* and *Weissella* were the common genera isolated (Schoustra et al 2013).

In comparison the main LAB in *ogi*, a Nigerian fermented cereal beverage, were found to be *Lb. plantarum, Lb. casei, Lb. brevis, Lb. fermentum, Lb. delbrueckii, Lb. acidophilus, Leuconostoc mesenteroides* and *Pediococcus acidilacti* (Dike and Sanni 2010). Some of the *ogi* strains were evaluated as starter cultures by inoculating them in maize gruel for making

*ogi*, and *Lb. plantarum* was found to have the highest potential as a starter culture in terms of acid production and increase in LAB counts (Nwachukwu and Ijeoma 2010).

This, therefore, suggests that the *Lactobacillus* genus, in particular *Lb. plantarum*, is typical biota of spontaneously fermented maize and sorghum non-alcoholic beverages and play a key role in defining the attributes of these products. Some strains of *Lb. plantarum* have been found to be amylolytic, breaking down starch in pearl millet slurries (Songre-Outtara et al 2010) and further studies on these *emahewu* strains is needed.

#### *2.4.1.3. Emasi*

The Swazi fermented milk is similar to other naturally fermented products from Southern Africa, in particular *amasi* from South Africa and Zimbabwe. The predominant LAB in *amasi* (traditionally fermented milk in Zimbabwe) was *Lactobacillus helveticus, L. plantarum, L. delbrueckii subsp. Lactis, L. paracasei subsp. paracasei* and *L. paracasei subsp. pseudo-plantarum* (Feresu and Muzondo 1990). Others were genus that were reported found in amasi belong to *Lactococcus.* Mutukumira (1995) observed that in *amasi Lactococcus lactis* subsp. *lactis* was the predominant strain. Slight differences that may be found in the microbial diversity of *amasi* (spontaneously fermented milk produced in Zimbabwe), can be attributed to different types of containers used as well as the environment under which the fermentation is done. Commonly used containers are those of clay pots, metal containers, calabashes and gourds. These containers have been found to impact on the microbial diversity (Kebede et al 2007). These observations also supported with recent research work conducted by Osvik et al (2013) on bacterial diversity of *amasi* (traditional

fermented milk from EkuPindiseni community in KwaZulu-Natal in South Africa) using 16S rRNA and Denaturing Gradient Gel Electrophoresis (DGGE) for identification. The main the strains (in *amasi*) were of the genus *Lactococcus*, as well as several species of *Lactobacillus*, *Leuconostoc*, and *Enterococcus*. In another study on South African naturally fermented milk, the genera *Leuconostoc*, *Lactococcus* and *Lactobacillus* were main flora. In addition, *Lactococcus lactis* subsp. *lactis, Leuconostoc mesenteroides* subsp. *dextranicum, Leuconostoc citreum*, *Leuconostoc lactis*, *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus plantarum* were among the LAB identified in traditionally fermented from South Africa (Beukes et al (2001).

In Zambia they prepare a traditionally fermented milk product called *mabisi* and most abundant LAB that were isolated were *Lactobacillus, Leuconostoc, Lysinibacillus* and *Bacillus* (Schoustra et al 2013). The identity of the LAB in traditionally fermented milk from Southern Africa is comparable to other traditionally fermented milk in other regions of Africa. For instance, in East Africa study by Mathara et al (2004) showed that the genus *Lactobacillus* was predominant in *kule naoto*. *Kule naoto* is Kenyan traditional fermented milk produced by the Maasai. The major LAB in *kule naoto* was the *Lactobacillus* species such as *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus paracasei* and *Lactobacillus acidophilus*. Other Enterococcus, *Lactococcus* and *Leuconostoc* were as well isolated in *kule naoto*.

Study by Saleh (2013) on Laban Zeer (product produced in Egypt but similar to *emasi*) the identification of LAB species in Laban Zeer was as follows; *Leuconostoc mesenteroides*

subsp. *cremoris*, *Lb. rhamnosus*, *Lb. plantarum*, *Lb. paracasei* subsp. *paracasei*, *Lb. delbruekii* subsp. *bulgaricus*, *Lb. curvatus* subsp. *curvatus* and *Lb acidophilus*. The most frequently isolated LAB species were found to be Leuconostoc mesenteroides subsp. cremoris and Lb. rhamnosus.

### *2.4.1.4. Buganu*

The predominant microflora that ferment marula fruits to *buganu* are mainly yeasts. Okagbue and Siwela (2002) isolated and identified yeasts from marula fruits in Zimbabwe and found that the predominant strains were *Aureobasidium pullulans, Geotrichum capitatum, Trichosporon brassicae, Rhodotorula mucilaginosa, Hansenula anomala, Hansenula jadinii* and *Hansenula* sp. In a similar study on identification of yeasts isolated from mukumbi (A Zimbabwean traditional fermented wine from marula fruits), Mpofu et al (2008) observed that *Saccharomyces cerevisiae* were the most predominant followed other yeasts species such as *Pichia anomala*, *Pichia guilliermondii*, *Candida tropicalis* and *Candida intermedia*. In this study the prevalence *Candida tropicalis* raised concern on the safety of the product because *Candida tropicalis* is a human pathogen (Mpofu et al 2008). In a separate study on microbiological changes in marula, Dlamini and Dube (2008) highlighted that during the fermentation of marula fruits to make marula wine (Gwanda, Zimbabwe), most of the yeasts involved in fermentation belong to the genus *Saccharomyces*. These microorganisms occur as natural flora on marula fruits. Once the fruit ripens fermentation naturally occurs as a result of presence of predominant yeasts that can ferment sugars into alcohol.

### *2.4.2. Role of microorganisms in the production of fermented foods with special focus on traditional fermented foods of Southern Africa*

Basically fermentation refer to metabolic process or some chemical reactions that are induced by microorganisms (section 2.4.1) (yeasts and bacteria or enzymes that split cpmplex organic compunds into relatively simple substances (acids, gases or alcohol or carbon dioxide). This leads to development of diversity of flavours, aromas and texture in food, preservation of foodthrough end products such as lactic acid, acohol and acetic acid. Fermentation also brings about enrichment of food with in some cases vitamins, proteins and essential fatty acids (Steinkraus 2002).

Some of the traditionally fermented foods like *buganu/marula* play vital culture role amongst Swazi people and people of Southern Africa. In Swaziland they do have an annual *buganu* ceremony that is attended by the King of the Kingdom of Swaziland and other Royal family members (Masarirambi et al 2009). Traditional fermented beverages like *mukumbi* and *emahewu* apart from being taken as drinks are actually important during the time of social gatherings (weddings, religious ceremonies and funeral) (Mpofu et al 2008; Gadaga et al 1999). In Namibia and South Africa, *marula* play a role socially and culturally amongst the people (Shackleton and Shackleton 2002). Previously in South Africa, first fruit ceremonies at which the first *marula* beer was normally taken used to occur at national and local level.

These ceremonies celebrated were meant to give thanks to the ancestors and to mark the commencement of season of growth and abundance (Shackleton and Shackleton 2002). Just like *buganu* in Swaziland, *marula* in Namibia and South Africa is mainly used to generate income from the sale of beer/wine. Shackleton and Shackleton (2002) points out that while jobs in the formal sector are becoming difficult to get making of *marula* offers unemployed

temporal employments. The other role is linked the oil extracted from kernel that can be used for cooking. The cake left after pressing the oil is either eaten at household or used as supplement for animal feed (Shackleton and Shackleton 2002). Indirectly related to the actual fermented *buganu/marula*, is that other valuable materials are obtained during the process of collecting the fruits and making of the alcoholic beverage. The leaves collected are used as medicine for coughs, stomach pains and diarrhoea/indigestion. Also utilised as medicinal value are the roots. This is because during collection of *marula* fruits the *marula* plant components are simultaneously harvested for other beneficial use.

Mutukumira et al (2008) highlighted that as for *emasi* the process of fermentation in the end give a product that contains roughly 2 ppm diacetyl, also characterised with impressive range of volatile and non-volatile organic compounds. These compounds like lactic acid, acetaldehyde, acetic acid, succinic acid, citric acid, pyruvic acid and ethanol make the product acceptable. Many people in Southern Africa enjoy taking sour milk (*emasi*) owing to the fermentation that has taken place by fermenting LAB. Beukes et al (2001), report agrees with Mutukumira et al (2008), where they pointed out that LAB do perform essential task of preserving a highly nutritious food product. Microorganisms give the fermented *emasi* therapeutic value as it is used to cover for lactose intolerance, and grossly a social value as a way of making income in homestead.

Although there are variations in the preparation of traditionally sorghum beers that are produced in many African countries but one thing is sure that these beers are rich in energy, vitamins B-group (thiamine, folic acid, riboflavin and nicotinic) and vital essential amino

acids like lysine (Lyumugabe et al 2012). *Dolo* and *tchapalo* traditionally fermented opaque sour alcoholic beverage product (Burina Faso and Côte d'Ivoire) have both therapeutic properties and nutritional value. In addition, they are commonly consumed during rural work, admirable festivities and funerals (Solange et al 2014). These products are similar to South African traditionally fermented beer *umqombothi* and Swaziland fermented *umcombotsi*. The *umcombotsi* (popularly consumed by black South African) is used for sale and associated with social gatherings (Solange et al 2014).

### *2.4.3. Probable impact of microorganisms on the quality and safety of fermented foods, with particular focus on microorganisms found in Swazi traditional fermented foods*

Normally fermented foods are known to be safe for consumers regardless of where they are made, be in the developing world like where people who make them may not have any training in microbiology, food hygiene (Steinkraus 2002). This is because in one way desirable microorganisms in fermented foods using substrates overgrows and resist invasion by spoilage, toxic even food poisoning microorganisms. In addition, other undesirable microorganisms just can face difficulties competing with desirable microorganisms. In other cases, the environment is not conducive for growth of undesirable microorganisms due to the production of lactic acid by fermenting microorganisms.

Occasionally in some fermented foods reasonably high numbers of coliforms have been detected. And the presence of coliforms in fermented foods is associated with poor hygienic practices (Mugochi et al 1999) during preparation, handling and storage or sampling. Coliforms make up the family of Enterobacteriaceae. This family also include *E. coli* and

*Samonella* sp. I some instances in fermented foods that involves the use of fruits as materials for preparation, high numbers of coliforms have been suggested to account poor quality of fruits (marula fruits). High numbers of coliforms in fermented foods have been cited before and one of the main reasons for presence of high loads of coliforms was associated to poor hygienic practices. Worrying is due to the fact that there has been reported presence of *Escherichia coli* in some fermented foods that may imply other enteric pathogens may be present (Gran et al 2002). In naturally sour milk that was being prepared in Zimbabwe, Gran et al (2002) reported that 39% of the samples (where the sample size was 31) had more than $10^3$ cfu *E. coli* $ml^{-1}$. The reason given to account for high counts was poor hygiene and handling of milk at the farms. The mean numbers of coliform counts in naturally soured milk were between 9.3 x $10^4$ and 5.7 x $10^4$ cfu/ml. In some other microbiological study on naturally soured milk that is often used as weaning food, out of 12 samples, 2 had enteropathogenic *E. coli* 0157:H7 (Gadaga et al 2013). Other related studies that were conducted to establish if low pH < 4.5, can inhibit growth of pathogens like Salmonella spp. and *Escherichia coli*, it was observed that the numbers of these pathogens that survived at 48h in spontaneously fermented milk were high and unacceptable.

The presence of coliforms in the fermented milk, however, is not unique to *emasi*. Mutukumira (1995) reported that *amasi* had coliforms counts ranging from 3.41 to 6.90 cfu/g. Saleh (2013) enumerated Enterococcus in Laban Zeer and recorded average values of 4.67 log cfu/g, respectively. Poor hygiene could explain the high coliform counts in *emasi*. Special care is therefore needed in preparing the products because it is also consumed by children and those with weak immune systems who may then become susceptible to food poisoning.

Contrary to the above observations Schoustra et al (2013) in their pilot study on *mabisi* (traditionally fermented milk in Zambia) found out that some pathogens (*Listeria innocua, Escherichia coli* and *Staphylococcus epidermidis*) could not disturb the naturally fermenting microorganisms even when it was inoculated at high amounts of $10^6$ microbial counts cells per gram of product. The actual mechanism that rendered predominant LAB in *mabisi* not to be suppressed by the inoculated pathogens is yet to be established. Possible mechanisms could be due to low pH, production of bacteriocins and other anticompetitor toxins. But Katongese (2008) pointed out that in mahewu they could not find any Enterobacteriaceae by the end of processing stage. These finding contradicts the preliminary findings in Swaziland fermented foods, hence the need to establish levels of safety of Swaziland fermented foods with a broad view to identify and characterise coliforms in these foods.

## 2.5. Reported probiotic properties of lactic acid bacteria found in fermented foods focusing on Swazi traditional fermented foods

### *2.5.1. Probiotics*

Probiotics are defined as selected, viable microbial dietary supplements that, when introduced in sufficient quantities; beneficially affect the human organism through their effects in the intestinal tract (Zimmer and Gibson 1998; Holzapfel and Schillinger 2002; Lei and Jakobsen 2004). The FAO/WHO (2002) also define probiotics as live microorganisms which, when administered in adequate amounts confer a health benefit on the host.

Probiotic properties are often studied using different models that involve the use of *in vitro* epithelial cell cultures, laboratory animals, and human volunteers. However, a number of

characteristics are needed to qualify a bacterial species as a probiotic (Gibson and Roberfroid, 1995; Ouwehand et al 2002), i.e. a probiotic microorganism:

- should be non pathogenic, with no relationship to diarrhogenic bacteria
- have strong antagonistic activity against human pathogens
- have a stable genome and not transfer antibiotic resistance genes
- have acid and bile stability
- have resistance to digestive enzymes
- have strong avidity to intestinal surface
- have anti-carcinogenic activity
- possess cholesterol lowering effects
- stimulate immune system without inflammatory effects
- enhance bowel motility
- enhance maintenance of mucosal integrity
- improve bioavailability of food compounds
- produce essential vitamins and enzymes
- possess excellent fermentative activity
- have good sensorial properties
- be tolerant to freeze drying or spray-drying
- have proper growth and viability in food products
- possess phage resistance
- have high stability during long term storage

### *2.5.2. Benefits of probiotics*

The potentially beneficial uses for probiotics include managing lactose intolerance, prevention of colon cancer, lowering cholesterol levels, lowering blood pressure, improving immune function, prevention of infections, improving mineral absorption, reducing inflammation, and repression of pathogenic bacteria. Because lactic acid bacteria (LAB) convert lactose into lactic acid, their ingestion may help lactose intolerant individuals tolerate more lactose than what they would have otherwise (Sanders, 2000). The best known example of a pro-biotic product is yoghurt. Human studies related to the consumption of yoghurt show increased milk digestibility, quicker recovery from certain types of diarrhoea, enhanced immune function, reduction in certain cancers, and possible lowering of blood cholesterol levels (Zanini et al 2007). Many commercial yoghurt types now have added cultures of *Lactobacillus acidophilus* and *Bifidobacteria bifidus* (AB cultures).

Non-diary probiotic products are a relatively new trend, with a few cereal grain-based probiotic products having been reported. Dogik is an improved ogi, a cereal fermented product consumed in Nigeria, which is made using a lactic acid bacteria starter with antimicrobial activities against diarrhogenic bacteria (Okagbue, 1995). Yosa is prepared in some Scandinavian countries, principally Finland (Blandino et al 2003). It is made by fermenting oat bran in water with LAB and *Bifidobacteria* (Salminen and von Wright 1998).

LAB have also demonstrated anti-mutagenic effects thought to be due to their ability to bind with (and therefore detoxify) heterocyclic amines formed in cooked meat (Wollowski et al 2001). LAB can also protect against colon cancer in rodents, though human data is limited

and conflicting (Sanders 2000; Brady et al 2000). Most human trials have found that LAB may exert anti-carcinogenic effects by decreasing the activity of an enzyme called ß-glucuronidase, which can regenerate carcinogens in the digestive system (Brady et al 2000).

Some dairy foods fermented with LAB can produce modest reductions in total and low density lipoprotein cholesterol levels (Sanders 2000). It is hypothesized that probiotic lactobacilli may help correct malabsorption of trace minerals, found particularly in those diets high in phytate content from cereals, nuts and legumes (Famularo et al 2005). These are also the same materials used to produce many African fermented foods. It is therefore important to continue studying the relationship between consumption of these fermented foods and health benefits.

Antibiotics affect an individual's ability to digest food and may result in antibiotic-associated diarrhoea by irritating the bowel directly, changing the gut flora or allowing pathogenic bacteria to grow. Probiotics may be beneficial in restoring the microbial balance in the human gut.

The health benefits imparted by probiotic bacteria are strain specific, and not species or genus specific. No strain will provide all proposed benefits; not even strains of the same species will be effective against defined health conditions (Shah 2007).

### *2.5.3. Types of probiotics*

Lactic acid bacteria are a diverse group of organisms providing considerable benefits to humankind, some as natural inhabitants of the intestinal tract and others as fermentative lactic acid bacteria (Grajek et al 2005). They are important in the dairy industry because they are able to convert lactose into lactic acid, which results in the characteristic sour taste of fermented dairy foods such as yoghurt and sour milk and the low pH acts as a preservative against spoilage and pathogenic bacteria. *Lactobacillus acidophilus* is a well known probiotic and is isolated from the human small intestine. *L. acidophilus* produces enzymes such as proteases, which help digest proteins and lipases to digest fat, improves the digestibility of food, helps in the alleviation of lactose intolerance and inhibits the growth of pathogenic bacteria. Therefore, supplementation with probiotics such as *L. acidophilus can* be beneficial for any person interested in improving nutrition and digestion. Other probiotic LAB include *Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus rhamnosus and Lactobacillus GG.*

Bifidobacteria are some of the major strains of bacteria that make up the gut flora. They are associated with a lower incidence of allergies (Björkstén et al 2004) and also prevent some forms of tumor growth (Guarner and Malagelada, 2003). The various species in this genus include *B. adolescentis, B. angulatum, B. animalis, B. asteroides* and *B. bifidum.* They can be isolated from a variety of materials such as human and animal faeces, sewage and oral cavity. Probiotics naturally present in food are of the Lactobacillus genus.

### *2.5.4. Characterisation of probiotic properties of LAB in fermented foods*

#### *2.5.4.1. Acid resistance*

Acid test is vital for probiotic bacteria. In order for probiotic bacteria to perform their role in the gut they have to overcome amongst others the acid environment in the gastrointestinal tract. These bacteria must withstand highly acidic gastric juice encountered on the way if they are to viably reach the small intestine (Owusu-Kwarteng et al 2015). Some LAB are able to grow after being exposed to medium with pH 2.0 for 3 h. Ramos et al (2013) noted out of the samples of 234 LAB, 51 (34 isolates as *Lactobacillus fermentum*, 10 isolates as *Lactobacillus plantarum*, and 7 isolates as *Lactobacillus brevis*) isolates were able to grow after being subjected to medium with pH 2.0 for 3 h. In addition, it was recorded that out of 48 *Lactobacillus fermentum* isolates, 16 were considered as acid resistant because they were able to grow after exposure to pH 2.5 for 4 h (Owusu-Kwarteng et al 2015). This observation is supported by findings by Yu et al (2013) who also established that some *Lactobacillus plantarum* managed to survive acidic conditions at pH 3.0 for period of incubation ranging from 60 to 180 minutes. At pH 2.0 the survival was greatly affected.

Jose et al (2015) investigated the probiotic characteristics of LAB that are used as feed additives in poultry and other livestock. The genetic identification of all 10 LAB isolates was as follows *Lactobacillus reuteri*, *Lactobacillus rhamnosus* and *Lactobacillus plantarum*. It was reported that the isolates that showed better tolerance to low pH were from dairy origin. In all strains there was decrease in viability after 6 h exposure at pH 2.0. The isolates *Lactobacillus rhamnosus* from dairy source did not grow at pH 2.0 after 6 h incubation in low pH. The isolate *Lactobacillus reuteri* (dairy source) exhibited the best performance at low pH of 2.0 after 6 h of exposure. All isolates of LAB performed well at pH 3 after 6 h exposure. It

was concluded that LAB isolates from dairy source performed or showed better tolerance to acidic environment compared to isolates from rumen source.

Turchi et al (2013) reported that all 42 wild strains of *Lactobacillus plantarum* grew at pH 3.0 in MRS broth but the growth of the strains was decreased at pH 2.0.

*2.5.4.2. Bile tolerance*

Ramos et al (2013) reported that one isolate of *Lactobacillus fermentum*, three isolates of *Lactobacillus plantarum* and two isolates of *Lactobacillus brevis* had the highest tolerance to bile. While all 51 LAB isolates (34 isolates as *Lactobacillus fermentum*, 10 isolates as *Lactobacillus plantarum*, and 7 isolates as *Lactobacillus brevis*) were able to tolerate 0.3% (w/v) oxgall bile salts. The value 0.3% (w/v) oxgall bile salts are actually considered to be the critical concentration when screening for resistant strains (Ramos et al 2013). In addition, it was recorded that out of 48 *Lactobacillus fermentum* isolates, 16 were resistant bile salt (Owusu-Kwarteng et al 2015). Further in the study by Yu et al (2013), all *Lactobacillus plantarum* strains did survive pretty well (> 7.4 log cfu $ml^{-1}$) under oxgall at 0.3%, 0.5% and 1% in MRS broth. It was noted that though the survived quite strongly the viability of *Lactobacillus plantarum* decreased gradually with in oxgall concentration.

Although LAB isolates can survive for 3-6 h of exposure in up to 2% bile salt concentration the performance vary depending on the source of the LAB isolates. LAB (*Lactobacillus plantarum*) isolates from rumen showed the best performance after 6 h exposure in 2% bile

salt. *Lactobacillus rhamnosus* from dairy source had the least tolerance to 2% bile salt. It was therefore concluded that after 6 h exposure in bile salts, LAB isolates from the rumen source exhibited far better tolerance that those from dairy source (Jose et al 2015). Bile salt concentrations of 0.3, 0.5 and 1.0% had no effects on the viability of *Lactobacillus plantarum* cells (Turchi et al 2013).

### *2.5.4.3. Susceptibility to antibiotics*

When exposed to antibiotics like streptomycin, gentamicin and kanamycin, *Lactobacillus fermentum* strains have some resistance towards the listed antibiotics (Owusu-Kwarteng et al 2015). *Lactobacillus plantarum* and *Lactobacillus rhamnosus* GG strain were observed to be sensitive to erythromycin, chloromycetin, penicillin and rifampicin, though resistant to kanamycin, vancomycin, polymyxin B, streptomycin and gentamicin (Yu et al 2013). *Lactobacillus rhamnosus* GG were more susceptible to ampicillin than *Lactobacillus plantarum*.

Further work as demonstrated by (Jose et al 2015) showed that LAB isolates (*Lactobacillus reuteri*, *Lactobacillus rhamnosus* and *Lactobacillus plantarum*) had 100% resistance to streptomycin, gentamycin, ciprofloxacin, vancomycin, kanamycin and nalidixic acid. In addition, 4 dairy isolates of LAB exhibited some resistance to tetracycline while only 1 rumen isolate did show some resistance to tetracycline. Four other rumen isolate had some intermediate performance, while 1 isolate from dairy source was susceptible to tetracycline. With respect to their performance to erythromycin, 3 isolates (dairy source) were resistance, while 5 isolates (rumen source) and 1 isolate (dairy source) exhibited some intermediate

resistance. One isolate from dairy source was however susceptible to the erythromycin (Jose et al 2015). The isolates performed very badly to chloramphenicol and ampicillin. All isolates from rumen were susceptible to chloramphenicol and ampicillin. Two (dairy source) isolates were susceptible to chloramphenicol and ampicillin, while 3 were resistant. Therefore, it was concluded that between the dairy and rumen isolates, the dairy isolates showed better resistance to antibiotics than those from the rumen (Jose et al 2015).

These findings are supported by Turchi et al (2013) where it was found out that out of 42 wild *Lactobacillus plantarum*, all the strains were actually susceptible to chloramphenicol, erythromycin and gentamicin.

*2.5.4.4. Antimicrobial activity*

Some LAB such as *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus brevis* exhibited antagonistic activity towards the following pathogens, *Listeria monocytogenes*, and *Staphylococcus aureus* (Ramos et al 2013). And *Lactobacillus brevis* recorded the highest antagonistic activity towards *Staphylococcus aureus*. Owusu-Kwarteng et al (2015) pointed out that some *Lactobacillus fermentum* strains had some antimicrobial activity towards *Listeria monocytogenes*, and *Staphylococcus aureus* but no activity towards *E. coli* and *Salmonella enteritidis*. *Lactobacillus plantarum* strains did exhibit some antimicrobial activities against *E. coli* and *S. flexneri* CMCC(B), other *Lactobacillus plantarum* (S38) could not exhibit any antimicrobial activities against *E. coli*, while other *Lactobacillus plantarum* strains (S2-5, S56) and *Lactobacillus rhamnosus* GG strain were inhibitory to *E. coli* and *S. flexneri* CMCC(B). There were some *Lactobacillus plantarum*

strains (S2-5, S2-6 and S4-1) that did inhibit *S. typhimurium* S50333. Interesting point to note was by neutralising the pH of culture supernatants to 6.5 using NaOH (1 mol $l^{-1}$) did eliminate the antimicrobial activities of strains towards the chosen pathogens (Yu et al 2013).

Jose et al (2015) observed that none of the following LAB isolates, *Lactobacillus reuteri, Lactobacillus rhamnosus* and *Lactobacillus plantarum* could inhibit the growth of *E. coli*. Nevertheless, the isolates had the highest inhibition zones against *L. monocytogenes*. Three dairy isolates namely *Lactobacillus reuteri* had no effect of *Listeria*. The isolates had relatively an average performance towards *E. aerogenes, S. aureus* and *S. menston* (Jose et al (2015). Generally, those isolates from the rumen had better antimicrobial activity to pathogens than those from dairy source.

## 2.6. Conclusion

The common traditional fermented foods in Swaziland refer to products such as fermented maize (*sancoti*), fermented porridges (*incwancwa*), fermented milk (*emasi*), non-alcoholic cereal beverage (e*mahewu*), alcoholic beverage (*umcombotsi*) from sorghum (e*mabele*) or millet (*nyawotsi*), malt distilled spirits (*mankanjane*) and fermented fruit mashes *(buganu*/*marula* wine and papaya beer. These fermented foods in Swaziland are grouped under the following categories, cereal-based fermented products. Then we have non-alcoholic products that include *emahewu, incwancwa* & *singwangwa*. The third group refer to alcoholic beverages such as *umcombotsi*, fermented wild fruit products (*emaganu* wine or *buganu*) and the last group is fermented milk (*emasi*). Swazi *umcombotsi* is similar to South African fermented sorghum beer (*umqombothi*). To prepare traditionally fermented beverages, the

fermentation process initially starts with lactic fermentation then followed by alcoholic fermentation. During the preparation Swazi *emahewu* the initial stage begins with the making of thick porridge. The common ingredients used are millet malt, sorghum malt or wheat flour. These ingredients are added to the mixture and left to ferment at ambient temperature. The product is ready for consumption in about 24 h. Whereas the fermentation of *emasi* is achieved by using natural microorganisms in milk to grow and use the milk lactose to produce lactic acid and other organic compounds depending on the type microflora which in turn lowers the pH. *Buganu* (*emaganu*) a season Swaziland alcoholic beverage is produced naturally by fermentation of ripe wild marula fruits by natural microflora. The traditionally fermented Swazi foods play different roles at household that border on nutritional security. They are source of income too. Food security and source of income are common recorded roles of traditionally fermented foods in other Southern African Countries. Swazi *umcombotsi* is similar to South African *umqombothi*. In *umqombothi* the most prevalent fermenting microorganism were lactic acid bacteria (*Lactotobacillus, leuconostoc, Pediococcus* and Enterococcus) yeasts (*Saccharomyces cerevisiae, Saccharomyces capsularis, Candida ethanolica, Candida haemuloni, Candida sorbophila, Dekkera anomala* and *Dekkera bruxellensis*). Swazi *emahewu* is similar *ting* (South African fermented non-alcoholic beverage) In *ting* predominant LAB were *Lactobacillus plantarum, Lactococcus lactis, Lactobacillus fermentum*, *Lactobacillus rhamnsosus, Weissella cibaria*, and *Enterococcus faecalis*. Swazi *emasi* is similar to *amasi* (traditionally fermented milk in Zimbabwe). The predominant LAB in *amasi* was *Lactobacillus helveticus, L. plantarum, L. delbrueckii subsp. Lactis, L. paracasei subsp. paracasei and L. paracasei subsp. pseudo-plantarum.* The predominant microflora that ferment marula fruits to *buganu* are mainly yeasts such as *Aureobasidium pullulans, Geotrichum capitatum, Trichosporon brassicae, Rhodotorula mucilaginosa, Hansenula anomala, Hansenula jadinii* and *Hansenula* sp. With respect to

safety of these traditional fermented foods, occasionally in some fermented foods reasonably high numbers of coliforms have been detected. This is associated with poor hygienic practices. The LAB as probiotics in fermented foods can be characterised under acid resistance, bile tolerance, susceptibility to antibiotics and antimicrobial activities. Most LAB can grow after been exposed to pH 3.0 for up to 6 h. Generally, *Lactobacillus plantarum* strains have survived under oxgall salts at 0.3%, 0.5% and 1% in MRS broth after 3-6 h exposure. LAB have variable susceptibility to different antibiotics. Similarly, LAB have variable antimicrobial activities towards different pathogens.

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# CHAPTER 3: THE MATERIALS AND METHODOLOGY


## Abstract

Detailed fermentation stages and other demographic characteristics and practices were documented for *umcombotsi*, *emahewu*, *buganu* and *emasi* that were established using interviews and questionnaires. LAB were cultured on MRS agar (Oxoid, Basingstoke, UK; CM0361). The LAB isolates were identified using API 50 CHL kits and by sequencing the 16S rDNA. The food borne pathogens; *Salmonella typhimurium* ATCC 13311 (Microbiologics, St. Cloud, MN) and *Escherichia coli* ATCC 25922 (Microbiologics, St. Cloud, MN) were revived in sterile Brain Heart Infusion (BHI) broth (Mast Group Ltd DM106D, Merseyside, UK). To characterise the LAB isolates for probiotic potential, they were exposed to low pH (pH 3) for 3 h, LAB strains were inoculated in MRS broth (Oxoid, CM0359, Basingstoke, UK) containing 0.3, 0.5 and 1.0 % (w/v) oxgall (Difco$^{TM}$ 212820, Sparks MD, using Inhibition Diameter Zones (IDZ) method LAB isolates were subjected to different antibiotics and using agar well diffusion assay the LAB supernatants were evaluated for their antimicrobial activity against *Salmonella typhimurium* ATCC 13311 (Microbiologics, St. Cloud, MN) and *Escherichia coli* ATCC 25922 (Microbiologics, St. Cloud, MN). The identity of colonies that grew on MacConkey agar (Oxoid CM0007, Basingstoke, UK) was identified by sequencing the 16S rDNA.

### 3.1. Demographic division of Swaziland and Location of study

Swaziland is divided into four districts, namely; Hhohho, Manzini, Shiselweni and Lubombo (Figure 3.1). The administrative regions straddle over the four agroecological zones, the Highveld, middleveld, lowveld and Lubombo mountains. Hhohho and Mbabane are in the Highveld, while Manzini is in the Middleveld. The escarpment of the Lubombo Mountains dominates the border with Mozambique and South Africa. The climate is temperate in the west, but may reach 40°C in summer in the lowveld (Wikipedia, http://en.wikipedia.org/wiki/Swaziland). Each district is further divided into tinkhundla. Households that were preparing any of the traditional fermented foods from Hhohho region were sampled from tinkhudla/chieftainships that were randomly selected from a list for that district. Because not every household was producing fermented products at the same time, convenience sampling was used. There are 14 tinkhudla in Hhohho region, 11 in Lubombo, 16 in Manzini and 15 in Shiselweni. Out of 14 local administrations (*tinkundla*) in Hhohho 5 were randomly selected and samples were collected.

### 3.2. Sampling of fermented products

Samples of *emasi* and *emahewu* was be collected from households from one region (Hhohho) of Swaziland and ferried in cooler boxes to the laboratory for analysis at the University of Swaziland. Before collecting samples, the purpose of the study was verbally explained to heads of the households, highlighting that the data obtained was for scientific purposes only and no financial benefits should be expected or are implied. The names of individuals were not disclosed and samples were only identified using a code according to region, tinkhudla, household number visited and date of sampling. Samples were only collected from those

households willing to participate. Interviews and questionnaires (Appendix 2) were used to collect data for demographic characteristics and practices.

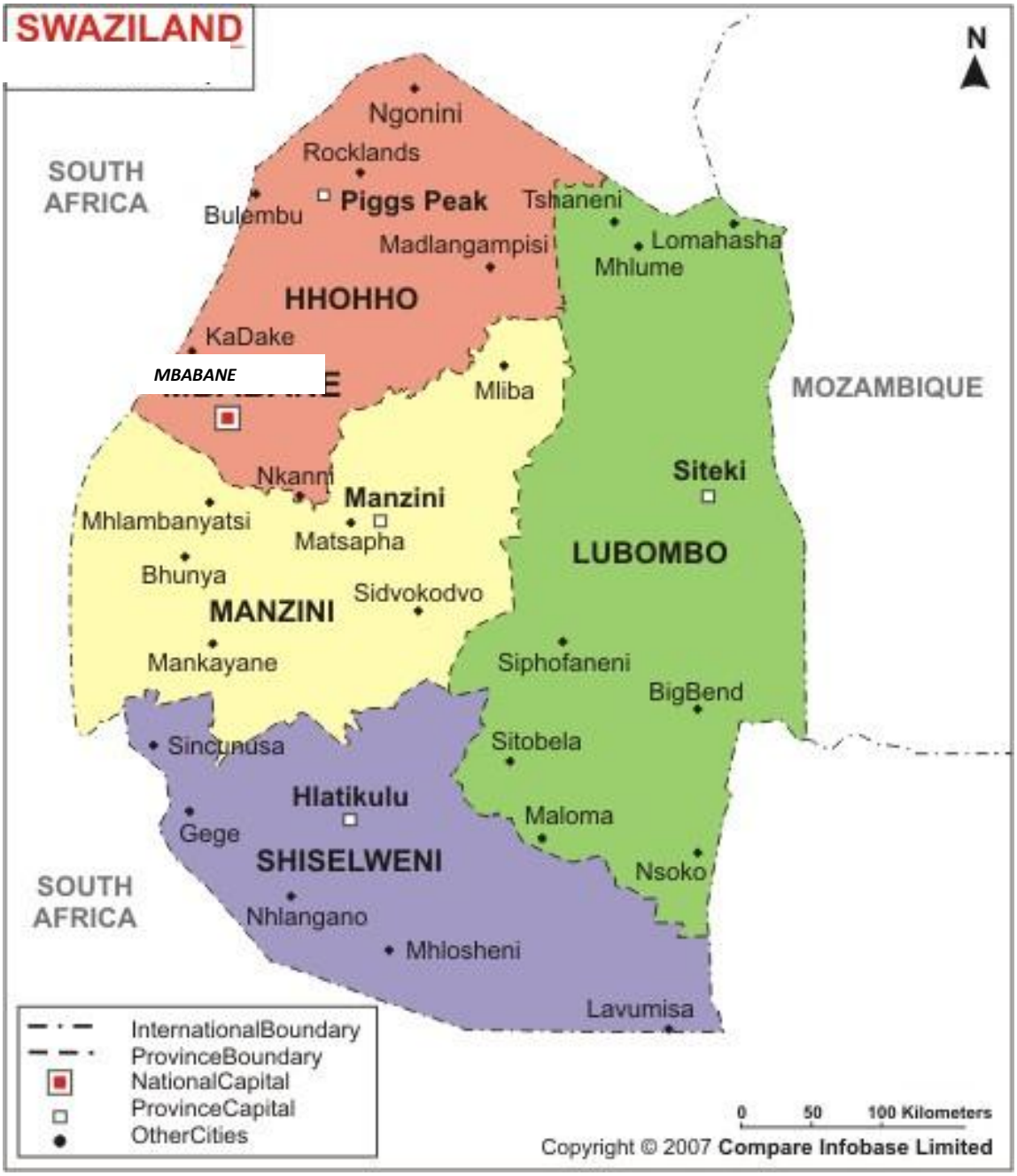


**Figure 3.1.** A map of Swaziland showing the four administrative regions of the country. (Source: http://www.ezmapfinder.com/en/map-50761.html, 31/08/2011)

## 3.3. Enumeration, isolation and identification of lactic acid bacteria in Swazi traditional fermented *emasi* and *emahewu* foods

### *3.3.1. Sampling*

Samples of fermented foods were collected from Hhohho region of Swaziland. The region is divided into 14 local administrations (*tinkundla*) from which 5 were randomly selected and samples were collected and taken to University of Swaziland for analysis (Chapter 5).

### *3.3.2. Microbiological analysis*

#### *3.3.2.1. Culturing and Isolation of LAB strains*

*Emasi* and *emahewu* samples were analysed immediately upon receipt at the laboratory. LAB were cultured on MRS agar (Oxoid, Basingstoke, UK; CM0361) by spreading 0.1ml of appropriate serial dilutions, and incubating anaerobically at 30$^{o}$C for 48 h using Oxoid anaerobic gas generating system (Oxoid; BR0038B) according to the manufacturer's instructions. The details of the procedure are as illustrated in chapter 5.

#### *3.3.2.2. Identification of LAB*

##### *3.3.2.2.1. Identification using API 50 CHL kits*

The frozen LAB isolates were thawed and resuscitated by inoculating into sterile MRS broth (Oxoid) and incubating at 30$^{o}$C for 24h. A portion of the fresh culture was streaked onto

MRS agar (Oxoid), which was then incubated anaerobically for 48h. The pure colonies were picked and inoculated onto API 50 CH (bioMerieux, Marcy l'Etloile, France; Ref 50 300) test strips according to the manufacturer's instructions (Chapter 5).

*3.3.2.2.2. Identification of LAB by sequencing the 16S rDNA*

The identification was done at Inqaba Biotec Laboratories, South Africa. Briefly, DNA was extracted using ZR Fungal/Bacteria DNA Kit™ (Zymo Research) (Chapter 5).

## 3.4. Evaluation of probiotic properties of Lactic Acid bacteria isolates from *Emahewu,* a Swazi fermented food

### *3.4.1. Source of lactic acid bacteria (LAB) isolates*

Nine isolates of LAB previously isolated and identified from emahewu were subjected to several tests for probiotic characterisation (Chapter 5). These isolates included three strains of *Lactobacillus plantarum*, one strain of *Lactobacillus*, one strain of *leuconostoc lactis*, one strain of *Weissella confusa*, one strain of *Lactobacillus acidophilus*, one strain of *Lactococcus lactis*, and one strain of *Leuconostoc pseudomesenteroides*. The identification of these isolates was done at Inqaba Biotec Laboratories, South Africa. Briefly, DNA was extracted using ZR Fungal/Bacteria DNA Kit™ (Zymo Research) (Chapter 5). The cultures were stored frozen at -28$^{o}$C in MRS broth (Oxoid CM0359, Basingstoke, UK) containing 20%

glycerol (L&T Diagnostics CC 090307, Johannesburg, South Africa) until required for the tests (Chapter 6).

### *3.4.2. Revival of LAB*

The frozen isolates were revived by separately inoculating 0.1 ml of thawed concentrated cultures into 9 ml MRS broth (Oxoid CM0359, Basingstoke, UK) and incubating for 24h at 30$^{o}$C (Chapter 6).

### 3.4.3. Revival of pathogens

The pure cultures swabs of food borne pathogens; Salmonella typhimurium ATCC 13311 (Microbiologics, St. Cloud, MN) and Escherichia coli ATCC 25922 (Microbiologics, St. Cloud, MN) were revived in sterile Brain Heart Infusion (BHI) broth (Mast Group Ltd DM106D, Merseyside, UK). Then cultures were incubated for 24 h at 37 $^{o}$C (Chapter 6).

### *3.4.4. Resistance to acid*

To evaluate resistance to low pH, the frozen isolates were revived by separately inoculating 0.1 ml of thawed concentrated cultures into 9 ml MRS broth (Oxoid CM0359, Basingstoke, UK) and incubating for 48 h at 30$^{o}$C. The broth was then centrifuged at 3000 rpm for 15 min to obtain a pellet of cells. The detail of the procedure was as described in Chapter 6.

### *3.4.5. Bile tolerance*

Overnight cultures of LAB strains were inoculated (3 %, v/v) (0.3 ml strain into 10 ml sterile separate MRS broth (Oxoid, CM0359, Basingstoke, UK) containing 0.3, 0.5 and 1.0 % (w/v) oxgall (Difco$^{TM}$ 212820, Sparks MD, USA). Resistance to bile salts was expressed as the surviving colony forming units (cfu) on MRS agar (Oxoid CM0361, Basingstoke, UK) after 3h of incubation at 30$^{o}$C (Chapter 6).

### *3.4.6. Susceptibility to antibiotics*

To determine susceptibility to antibiotics, LAB strain suspensions with turbidity (approx. 3.0 x $10^{8}$ cfu $ml^{-1}$) 0.1 ml were spread plated on MRS agar (Oxoid CM0361, Basingstoke, UK) plates. Disks impregnated with antibiotics; ampicillin 10 µg, amoxicillin 10 µg, tetracycline 30 µg, streptomycin 10 µg, ciprofloxacin 5 µg and nalidixic 30 µg were placed on plates inoculated with different strains. After incubation under aerobic conditions for 24 h at 30$^{o}$C inhibition Diameter Zones (IDZ), including the diameter of the 6 mm disks, were measured (Chapter 6).

### *3.4.7. Antimicrobial activity*

The antimicrobial activity was determined by the agar well diffusion assay. The LAB isolates were revived using MRS broth (Oxoid CM0359, Basingstoke, UK) by incubating for 24 h at 30$^{o}$C. The pathogens Salmonella typhimurium ATCC 13311 (Microbiologics, St. Cloud, MN) and Escherichia coli ATCC 25922 (Microbiologics, St. Cloud, MN) were revived using BHI broth (Mast Group Ltd DM106D, Merseyside, UK) by incubating for 24h at 37$^{o}$C. A portion (1ml) of the inoculated BHI broth (Mast Group Ltd DM106D, Merseyside, UK) was

pour plated on Nutrient agar (Oxoid CM0003, Basingstoke, UK). Wells (6 mm diameter) were made into the Nutrient agar (Oxoid CM0003, Basingstoke, UK) using a sterile cork borer. Cell free supernatant (CFS) (60 µl) obtained by centrifugation of the culture at 3000 rpm for 15 min, pH adjusted to about 6 - 6.5 and filter sterilised (0.45 µm) was added into the wells. The plates were then incubated at 30 $^{o}$C for 24-48 h. The antimicrobial activities of LAB were determined in terms of development of inhibition zones around the wells. This experiment was repeated to ensure reliability of results (Chapter 6).

## 3.5 The safety of Swazi traditional fermented foods, *emasi* and *emahewu focussing on Enterococcus spp.*

### *3.5.1. Sampling*

Samples were collected from 5 *tinkhundla* that were randomly selected from the 14 *tinkhundla* using a lottery system. The samples were collected in sterile screw capped bottles and ferried in a cooler box to the laboratory at the University of Swaziland for analysis (Chapter 7).

### *3.5.2. Microbiological analysis*

#### *3.5.2.1. Isolation of Enterococcus spp*

The fermented samples were analysed immediately upon receipt at the laboratory. Total coliforms were enumerated on MacConkey agar (Oxoid CM0007, Basingstoke, UK) and incubating at 37$^{o}$C for 24-48 h (Chapter 7).

#### *3.5.2.2. Identification of Enterococcus by sequencing the 16S rDNA*

The identification was done at Inqaba Biotec Laboratories, South Africa. Briefly, DNA was extracted using ZR Fungal/Bacteria DNA Kit™ (Zymo Research) (Chapter 7).

## 3.6. Statistical analysis

The experiments were done two times and each measurement was done in duplicate. The means and standard deviations were calculated. Significant differences between the treatments were determined through Analysis of Variance using Statistical Package for Social Sciences (SPSS) for Windows version 20 (SPSS, Chicago, IL). Significant differences were taken for value of $p < 0.05$.

## 3.7. References

Anonymous. (2011). Swaziland political map. http://www.ezmapfinder.com/en/map-50761.html. 31/8/2011.

# CHAPTER 4: METHODS OF PREPARATION OF SWAZI TRADITIONAL FERMENTED FOODS[1]


## Abstract

Fermentation is an age old technique of preserving food in many communities. A wide range of fermented products are prepared by varying the types of raw materials, utensils and fermentation times. Several fermented foods are consumed in Swaziland. A survey of the types of fermented foods, preparation methods and utensils used was done in the Hhohho region of Swaziland. The current study aimed at documenting the preparation methods of *emahewu*, *emasi*, *umcombotsi* and *buganu* at household level. Detailed fermentation steps were documented for *umcombotsi*, *emahewu*, *buganu* and *emasi*. Five constituencies, called *tinkhundla*, were randomly selected from the 14 found in Hhohho region of Swaziland. At each *inkhundla*, households that were known to regularly prepare the fermented foods were identified with the assistance of local community leaders, and interviewed. A semi-structured questionnaire was used for the face-to-face interviews. With respect to preparation procedures and practices, all respondents indicated that they had prepared different fermented foods at one time or another. The most commonly prepared and readily available fermented foods were *umcombotsi* (alcoholic beverage), *emahewu* (non-alcoholic beverage), *buganu* (*marula* wine), and *emasi* (spontaneously fermented milk). Both men and women indicated that they prepared *umcombotsi*, and only women reported that they prepared *emahewu*, *buganu*, and *emasi*. *Umcombotsi* was mainly prepared for sale, while *buganu*, *emahewu* and *emasi* were for sale as well as household consumption. *Umcombotsi* was mostly prepared by mixing maize meal, un-milled sorghum malt (*magayiwe*), and brown sugar (3 kg) in water (20 L). The initial stage involved the cooking the mixture to gelatinise the starch, followed by

fermentation at ambient temperature (25-30$^{o}$C) for about 72 h. The whole preparation process takes about 4 to 5 days. *Emahewu* was prepared by mixing maize meal (1 kg) with water (ca. 5L) and cooking to make a soft porridge. The cooled porridge was left to ferment at room temperature. Some reported adding sugar or a pilled potato to aid the fermentation process. *Emasi* was prepared by letting raw milk to naturally ferment at room temperature (25-30$^{o}$C) in either metal or plastic containers (buckets) for 2 to 3 days. *Buganu* was prepared from marula fruit (*amaganu*) juice and pulp mixed with water (ca. 10 L) and sugar (ca. 2 kg). The mixture was allowed to ferment at ambient temperature for about 3 days, sieved and then served. *Umcombotsi*, *emahewu*, *buganu* and *emasi* were the fermented foods commonly prepared at household level in the Hhohho region, Swaziland. The main ingredient used for preparing *umcombotsi* and *emahewu* was maize meal. Un-milled sorghum malt was also added during preparation of *umcombotsi*. However, typically no malt was added during the preparation of *emahewu*. *Buganu* and *emasi* also play an important role in the diet and socio-economic activities of the population in Swaziland.

[1]Part of this work has been published: Simatende, P., Gadaga, T.H., Nkambule, S.J. and Siwela, M. 2015. Methods of preparation of Swazi traditional fermented foods. J Ethnic Foods. 2: 119-125.

## 4.1. Introduction

Several traditional spontaneously fermented foods are prepared at household level in Africa (Steinkraus 2002; Hesseltine and Wang 1980). Numerous investigations have revealed the important role of this technique including making the raw materials more palatable and extending the shelf life product. Various benefits of fermentation have been reported such as improved bioavailability of some nutrients, destruction of anti-nutritional compounds such as tannins, phytates and polyphenols, as well as inhibition of spoilage and pathogenic microorganisms facilitated by the low pH (Mortarjemi 2002; Blandino et al 2003). Cereals, fruits, and milk are the common raw ingredients used in the preparation. African traditional fermentation technologies are at best an art, hence the fermentation products vary in quality depending on the type of raw materials, types of containers used, and environmental conditions (Gadaga et al 1999; Beukes et al 2001; Kebede et al 2007; Madoroba et al 2009). However, the communities where a specific product is made normally have well known and agreed steps for its preparation. For example, Simango (2002) described *mahewu* prepared in Zimbabwe as maize based cereal gruel with added malt. The malt provides the inoculum and enzymes for spontaneous fermentation. In South Africa, traditional fermented milk (*amasi*) is prepared in several types of containers of varying sizes. Buekes et al (2001) reported that the Xhosa and Zulu people mainly use calabashes to make *amasi*, while the South Sotho use clay pots to make a similar product called *mafi*. The use of clay pots reportedly gives a better flavour to the fermented milk than calabashes. The types of container used, as well as the environmental conditions, contribute to the gradual selection of specific microorganisms that are responsible for the perceived flavour (Beukes et al 2001).

Common traditional fermented foods consumed in Swaziland include non-alcoholic cereal beverage *(emahewu*), spontaneously fermented milk (*emasi),* fermented porridge (*incwancwa),* fermented maize meal (*sancoti*), fermented marula fruit juice and pulp *(buganu*), alcoholic cereal beverage (*umcombotsi*), and malt distilled spirits (*mankanjane*) (Masarirambi et al 2009). The importance of these products to the diet and socio-cultural well-being of the Swazi community is well documented. However, details of the preparation steps have not been systematically studied and recorded. It is important to document the process, quantify the ingredients and identify the key conditions for a successful fermentation in order to replicate the process under standardised conditions and ultimately at industrial level. The current study's aim was to document the steps and equipment used in the preparation of four products, *emahewu*, *emasi*, *umcombotsi* and *buganu* prepared at household level in Swaziland.

## 4.2. Materials and Methods

### *4.2.1. Location of study and selection of households*

The study was conducted in Hhohho region of Swaziland. The region is divided into 14 local administrations called *tinkhundla*. Hhohho region is in the Highveld of the country where the temperatures can range from the very cold in winter to hot in summer. Using a lottery system, 5 *tinkhundla* were selected for the study. At each *inkhundla*, members of the community who were known to prepare fermented foods were identified with the assistance of community leaders, such as the village head (*umphakatsi*) or school teachers. To determine the sample size, convenience sampling was used.

### *4.2.2. Preparation methods*

Using a semi structured questionnaire (Appendix 3), preparation steps and the utensils or equipment used for the different fermented foods were recorded. Demographic characteristics (gender of person preparing the food), purpose of preparing and any cultural restrictions on consumption of the fermented food were also obtained for each of the four products.

## 4.3. Results and Discussion

### *4.3.1. Demographic characteristics and practices*

Twenty five (25) respondents who prepared fermented foods were interviewed. The fermented foods that were commonly prepared were *umcombotsi*, *buganu*, *emahewu*, and *emasi* (Table 4.1.). All respondents indicated that they had prepared all these different products at one time or another, but did not always have them available. Among those preparing *umcombotsi*, 50% were male and 50% female. However, all respondents who prepared *emahewu, emasi* and *buganu* were women.

All respondents who prepared *umcombotsi* reported that it was for sale and a major source of income. Hence 25% were making a new batch of *umcombotsi* every three days, while 50% made every week and 25%, every month. *Umcombotsi* was only consumed by adults, as it has high alcohol content.

**Table 4.1.**

Frequency of responses to demographic and socio-economic issues concerning preparation and consumption of *umcombotsi*, *buganu*, *emahewu* and *emasi* at household level in Hhohho region, Swaziland

| | | Frequency of response (%) (n=25) | | |
|---|---|---|---|---|
| | **Question** | ***umcombotsi*** | ***emahewu*** | ***buganu*** |
| 1. | Gender of respondent preparing product: | | | |
| | • Male | 50 | 0 | 0 |
| | • Female | 50 | 100 | 100 |
| 2. | Marital status: | | | |
| | • Single | 100 | 33.3 | 0 |
| | • Married | 0 | 66.7 | 100 |
| | • Divorced | 0 | 0 | 0 |
| | • Widow | 0 | 0 | 0 |
| 3. | Frequency of preparation: | | | |
| | • Every 3 days | 25 | 33.3 | 0 |
| | • Once a week | 50 | 66.7 | 0 |
| | • Once a month | 25 | 0 | 0 |
| | • Seasonal* | 0 | 0 | 100 |
| 4. | Who consumes product? | | | |
| | • Adults only | 100 | 0 | 100 |
| | • Adults and children | 0 | 100 | 0 |
| 5. | Do you prepare for sale? | | | |
| | • Yes | 100 | 100 | 100 |
| | • No | 0 | 0 | 0 |
| 6. | Is this your major source of income? | | | |
| | • Yes | 100 | 100 | 100 |
| | • No | 0 | 0 | 0 |

*Marula fruits are normally harvested in February-March and *buganu* is prepared during those months.

Equipment and ingredients used in preparing the different fermented foods were also documented. Generally, 210 L metal drums, traditional clay pots and plastic containers were used for preparing *umcombotsi* and *buganu*. Plastic buckets, aluminium pots and clay pots were reported to be used for mixing ingredients for *emahewu* and for fermentation and storage of *emasi*.

### *4.3.2. Ingredients and methods of preparation*

#### *4.3.2.1. Umcombotsi*

Main ingredients used in preparation of *umcombotsi* were found to be similar in the different households. *Umcombotsi* was mainly prepared by mixing maize meal, un-milled sorghum malt (known as *magayiwe or mnandi*), and brown sugar (3 kg) in water (20 L). The common method for preparation involved adding maize meal (5 kg) to water (20 L) to make a slurry and cooking the mixture in 3-legged cast iron pots or metal drums to gelatinize the starch as shown in Figure 4.1. Cooking time was not standardized. It depended on visual and rheological observations made by the person preparing the product. Sorghum malt (*magayiwe*; 1 kg) was then added to the cooled soft porridge, followed by brown sugar (3 kg). The whole mixture was fermented at ambient temperature (ca. 25-30$^{o}$C) for about 72 h to give a brown coloured beverage. As shown in Figure 4.1, the whole preparation process takes about 4 to 5 days. However, one respondent also described a slight modification of the above method which is outlined in Figure 4.2. Thin maize meal porridge was inoculated with strainings (*emashica*) from a previous successful fermentation and the mixture was fermented for 18 to 24 h. This was then followed by a cooking step and cooling. The cooled, soured

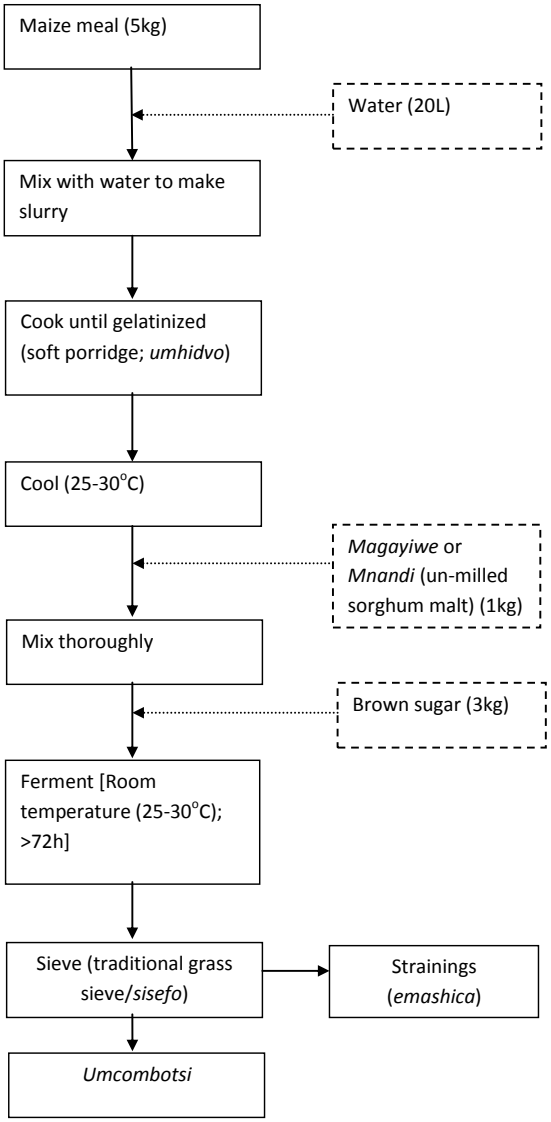


**Figure 4.1.** Commonly practiced traditional preparation of *umcombotsi*, Swazi maize meal and sorghum beer.

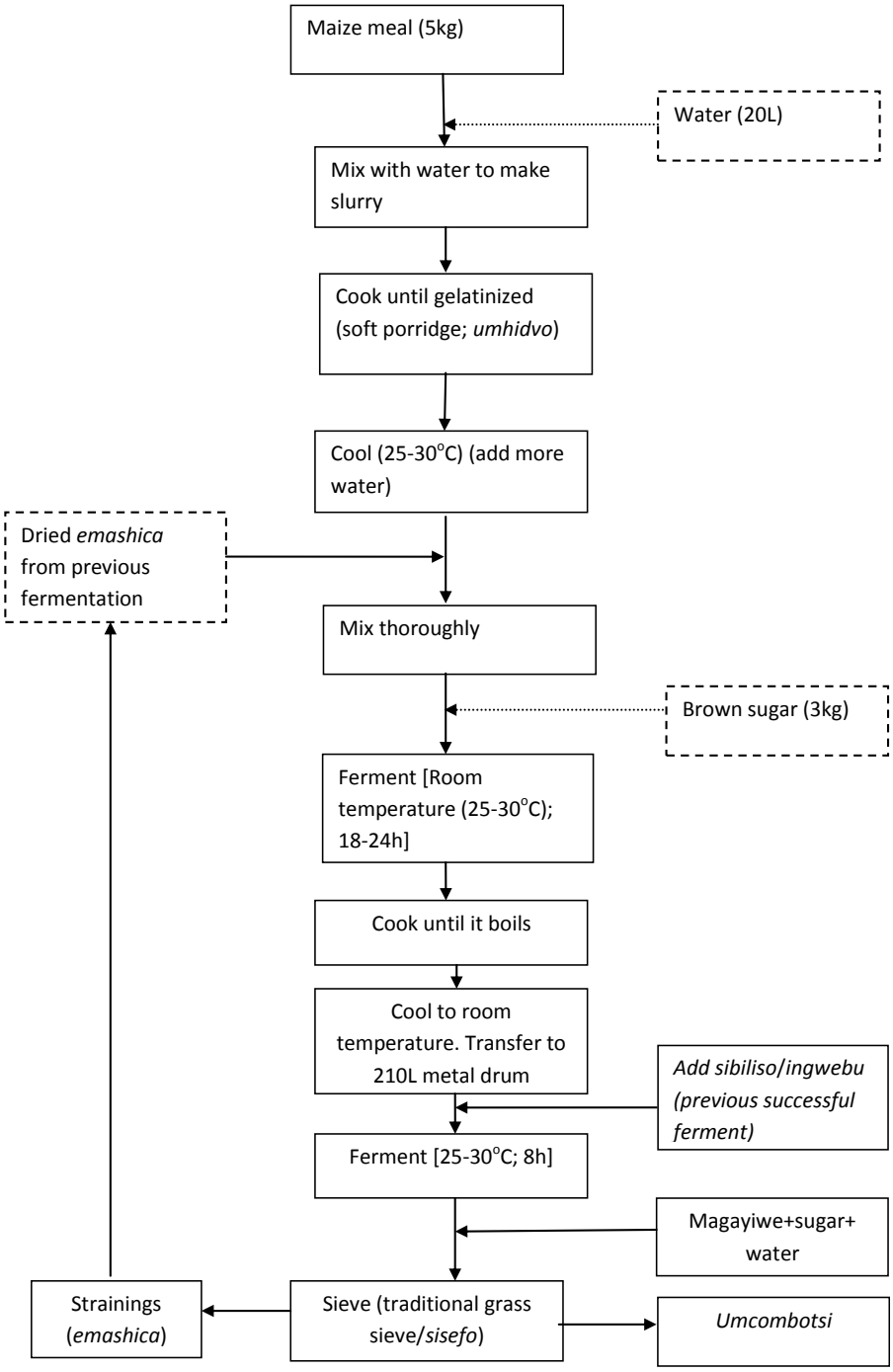


**Figure 4.2.** A less commonly used variation of traditional preparation of *umcombotsi*, Swazi maize meal and sorghum beer.

product was further inoculated with a small amount of previous ferment called *sibiliso* or *ingwebu*, and allowed to ferment for about 8 h and sieving as previously described.

*Magayiwe*, sugar and water can be added after this second fermentation, if needed. Although the actual preparation steps were more than the commonly used method (Figure 4.1.), the fermentation time in the second approach was shorter by about a day. This can be attributed to the back-slopping with *emashica* and *sibiliso* that speeds up the fermentation. Back-slopping adds microorganisms that are well adapted to the fermentation media and the desired pH and organoleptic properties are achieved much quicker. Similar African beers with sorghum malt have been found to have alcohol content ranging from 2 to 4.5% (v/v), and pH of between 3.3 and 4 (Lyumugabe et al 2012).

Figure 4.3A-E shows some of the intermediary products and equipment used during preparation of *umcombotsi.* The brew can be thinned by occasionally adding more water and stirring. Thin thin brew was then sieved (Figure 4.3C) and served. Respondents reported that they obtained malt from supermarkets. Two brands, *magayiwe* and *mnandi*, were commonly used.

The method for preparing *umcombotsi* was in many ways similar to the traditional preparation of *Sesotho joala* reported by Gadaga et al (2013). However, in preparing *joala*, maize meal could be replaced or used together with wheat or sorghum flour. Briefly, this starch base is mixed with water and cooked to make a thin porridge. Homemade liquid starter culture called *tomoso* and brown sugar are then added to kick start the fermentation. After 48 h, the mixture is boiled, which tends to kill most of the mesophilic souring bacteria.


**Figure 4.3A-E**. *Umcombotsi* ingredients and product at different preparation stages, and some utensils used. A = sorghum malt; B = fermenting beverage; C = strainer/sieve with strained malt; D = strained *umcombotsi*; E = containers used during preparation of *umcombotsi*.

This is similar to the second cooking stage for *umcombotsi* shown in Figure 4.3B. After cooling, sorghum malt and a dry starter culture called *moroko* are added and fermented for an additional 24 h. The product is then strained, ready for consumption. The second starter is thought to predominantly contain yeasts as the associated fermentation is alcoholic. *Moroko* may be comparable to dried *emashica* or *sibiliso/ingwebu*. The preparation of *joala* takes 3 - 4 days, similar to *umcombotsi*, but there are more elaborate steps. Preparation of *doro*, a sorghum beer from Zimbabwe, takes 5-7 days (Gadaga et al 1999; Benhura and Chingombe 1989). *Umqombothi* is the Shangaan name given to a similar sorghum beer in South Africa (Novellie 1966; Katongele 2008). The differences in the quality of ingredients, utensils used and preparation times are probably the only distinguishing characteristics of these products.

#### *4.3.2.2. Emahewu*

*Emahewu* is a non-alcoholic beverage which is consumed by all members of the family, including infants. Interviews with women who prepared the product showed that it was prepared by mixing maize meal (1 kg) with water (5 L), or in the same proportions as described for *umcombotsi*. The outline for preparation is shown in Figure 4.4. The thin slurry was then cooked to make a soft porridge called *umhidvo*, cooled, and left to ferment at room temperature. No malt was added but some households added sugar or a pilled potato. *Emahewu* therefore lacks enzymes that come with addition of malt to kick start the fermentation. However, some bacterial inoculum may come with the added potato and may be present on the utensils used during handling. Brown sugar is often added and it provides a readily fermentable substrate for any microorganisms present. In contrast, similar fermented products prepared in other countries, such as *mahewu/mageu in South Africa*, have sorghum

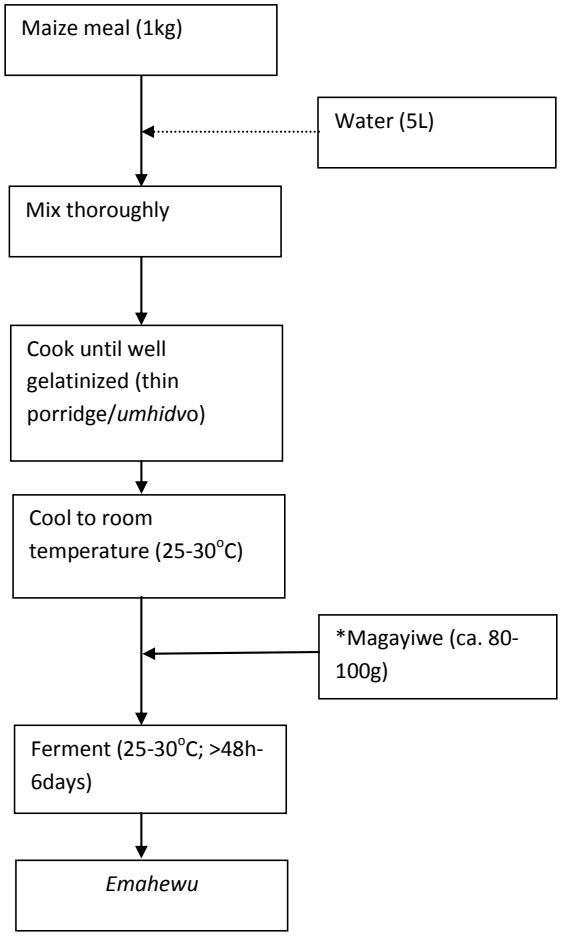


**Figure 4.4.** Traditional preparation of *emahewu* in Swaziland. *Adding malt (*magayiwe*) for *emahewu* can be done to speed up the process, but it is optional.

or millet malt added after the cooking stage and therefore develops low pH and high counts of fermenting microorganisms (Katongele 2008).

The current practice at household level in Swaziland is a cause for concern as the final product may be susceptible to proliferation of harmful microorganisms. Fermentation takes long (2-3 days in summer and up to 5 days in winter). The usual protection due to low pH is not achieved fast enough. Byaruhanga et al (1999) reported that *mageu* in South Africa was prepared by using 8 to 10% (w/v) maize flour as the major solid substrate in water. Wheat flour or maize bran was then added to initiate the lactic acid fermentation. Acceptable *mageu* reportedly contained 0.4–0.5% lactic acid corresponding to an average pH of 3.5 (Schweigart et al 1960). Figure 4.5 shows the utensil used in preparing and storing emahewu.

#### *4.3.2.3. Emasi*

The outline for preparation of *emasi* is shown in Figure 4.6. The fermented milk was prepared by leaving raw milk to spontaneously ferment at room temperature in either metal or plastic containers (buckets). The whey was occasionally decanted to give a thick product similar in consistency to cottage cheese (Figure 4.7). This suggests that *emasi* could have high numbers of fermenting microorganisms and low pH. It was observed that the milk is prepared in metal, plastic or clay pots. This is similar to that practiced in many Southern African countries in preparing *amasi*, *sethemi*, *mafi*, or *madila* (Gadaga et al 1999; Beukes et al 2001; Kebede et al 2007; Gadaga et al 2013; Ohiokpehai and Jagow 1998). The Sotho *mafi* is traditionally prepared by allowing raw milk to ferment spontaneously in clay pots until thick curds form. This may take 2 to 3 days at 25-30$^{o}$C (Gadaga et al 2013). To prepare *madila*, fresh milk is filtered through a strainer and placed in an enamel/metal bucket and fermented in a warm place (30$^{o}$C) for 24 h (Ohiokpehai and Jagow 1998). The soured milk is

**Figure 4.5.** Utensils used in preparing *emahewu* in Swaziland.

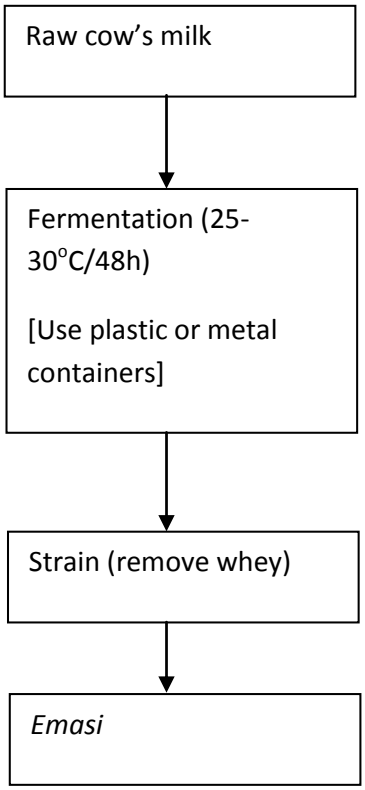


**Figure 4.6.** Traditional preparation of e*masi*, Swazi sour milk

**Figure 4.7.** A clay pot used for preparing *emasi* and the product in a serving dish.

then poured into a woven polypropylene sack and additional one day old soured milk is added each day over a 7 – 8 day period. The bag is hung from a beam for 3 - 4 days during which time the whey drains away through the woven bag. Finally, the fermented milk is removed from the bag and mixed with fresh milk in a ratio of 4:1 before consumption or sale (Ohiokpehai and Jagow 1998).

While the above similarities have been noted, the nature of fermented products varies from one region to another depending on the local indigenous microflora, which in turn reflects the climatic conditions of the area (Akabanda et al 2010).

#### *4.3.2.4. Buganu*

*Buganu* is a potent wine made from the ripe fruits of the marula tree (*Scleroecarya birrea*; *amaganu*), which are yellow in color and are predominantly found in the Lowveld of Swaziland. Women in the villages collect the fruits and make marula beer used in the celebration of the first fruit ceremonies. In the past *buganu* was often first offered to the chief or headman before everyone else and was thought to be important in strengthening social networks between friends, neighbours and relatives (Cunningham 1988). In present day Swaziland, an annual marula festival with a lot of cultural activities, and attended by the King, is held.

The traditional production process for buganu is outlined in Figure 4.8. According to respondents in this study, freshly ripe marula fruits (10 kg) were cleaned thoroughly and

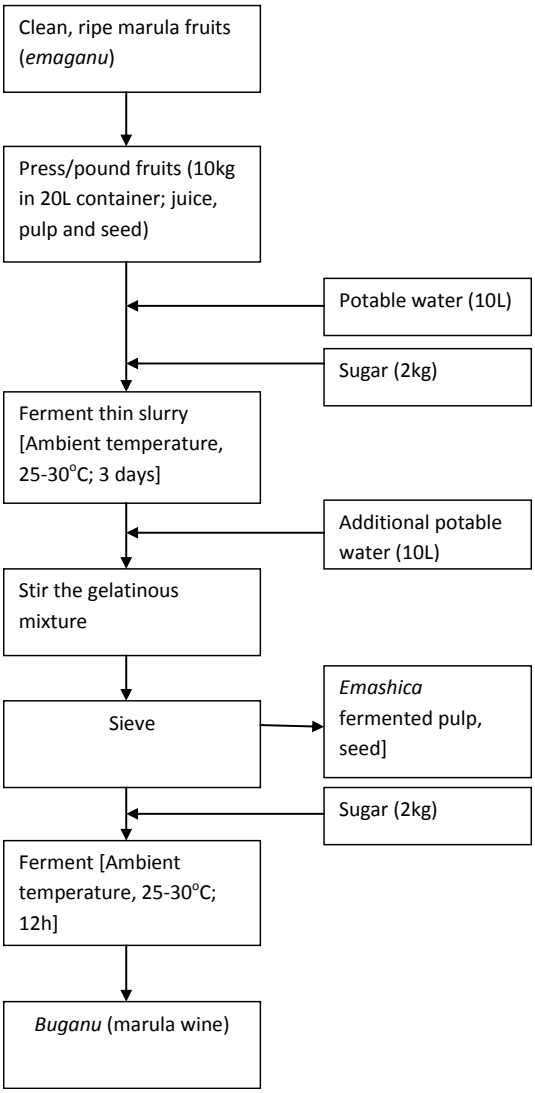


**Figure 4.8.** Traditional preparation of Swazi naturally fermented marula wine, *buganu.*

pounded or pressed to extract the juice (Figure 4.9.). The juice, pulp and seeds from the fruit were transferred to a 20 L container (Figure 4.9C). Plastic buckets were commonly used. Water (10 L) was then added and the mixture stirred. Sugar (2 kg) was also added and mixed, and left to ferment at ambient temperature for about 3 days. During this time the mixture becomes gelatinous in consistency with gas bubbles showing signs of fermentation. The gelatinous mixture was then stirred to make it thin, followed by sieving using a traditional grass sieve or metal mesh as described for *umcombotsi*. Some respondents also reported adding sugar after sieving and allowing further fermentation for 12 h before serving. This production process was generally in agreement with that described by Masarirambi et al (2009). While plastic buckets were being used, it was also noted that traditional clay pots or gourds (*ingula*) could also be used.

*Marula* fruit also plays an important nutritional and socio-economic role in other countries in Southern Africa. For example, Shackleton and Shackleton (2002) reported that over 90% of households in Limpopo province of South Africa collected *marula* fruit mainly to make beer, and also to consume fresh, make into juice and/or process into jam. About 74% of households in the Limpopo study produced between 138 and 311 L of marula wine, which is locally called *vukanyi*, each season. The wine is shared with friends, relatives and neighbours, while a few households prepared it for sale. In the South African study, beer brewing was primarily done by women. Production of a marula alcoholic beverage on an industrial commercial scale has been achieved in South Africa and a commercial wild fruit cocktail, *amarula*, is now available on the market. A similar traditional marula wine is prepared in Zimbabwe and Namibia (Gadaga 1999). Gadaga et al (1999) also reported that both fermentative and non-fermentative yeasts have been isolated from the marula fruits.

**Figure 4.9.** The marula tree (A), marula fruits (B) and fermented marula fruit wine (C).

## 4. 4. Conclusions

*Umcombotsi* (alcoholic beverage), *emahewu* (non-alcoholic beverage), *buganu* (*marula* wine) and *emasi* (spontaneously fermented milk) were the fermented foods commonly prepared at household level in the Hhohho region. The fermented foods were prepared for own consumption as well as for sale. The main ingredient used for preparing *umcombotsi* and *emahewu* was maize meal. Un-milled sorghum malt (*magayiwe* or *mnandi*) was also added during preparation of *umcombotsi*. However, typically no malt was added during the preparation of *emahewu*.

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# CHAPTER 5: THE MICROBIAL AND BIOCHEMICAL ASPECTS OF SWAZI TRADITIONAL FERMENTED FOODS AND IDENTITY OF LACTIC ACID BACTERIA IN THE FOODS, FOCUSSING ON *EMASI* AND *EMAHEWU*


## Abstract

Spontaneously fermented foods are part of the diets and livelihood in many African communities. Several fermented foods are produced at household level in Swaziland. The present study focused on the biochemical aspects, enumeration, isolation and identification of lactic acid bacteria in *emasi* (fermented milk) and *emahewu* (maize based non-alcoholic beverage), Swazi traditional fermented foods. The lactic acid bacteria (LAB) were enumerated, and the pH and titratable acidity (TA) were determined in samples collected from households in Hhohho region of Swaziland. The home-made *emasi* was found to have an average pH of 4.68 and TA of 0.89%. The LAB counts were 8.25 log cfu/mL. Similarly, *Emahewu*, a maize based non-alcoholic beverage had pH of about 3.62 and TA of 0.43%. This product also had high LAB counts of 8.10 log CFU/mL. The LAB counts in all two products were consistent with observations for similar African fermented foods. The LAB from *emasi* and *emahewu* were identified through profiling using Gram stain, catalase reaction, sugar assimilation tests using API 50 CH test strips and through sequencing the 16S rDNA. Nine morphologically different isolates were identified from *emasi*, while sixteen (16) were identified from *emahewu*. It was found that *Lactococcus lactis* subsp. *lactis* and *Leuconostoc mesenteroides* were the common strains in *emasi*. *Lactobacillus plantarum, Lactobacillus paracasei* ssp. *paracasei* and *Lactobacillus brevis* were also detected. In *emahewu, Lactobacillus plantarum* were the most common strains, followed by *Leuconostoc*

*mesenteroides* ssp. *mesenteroides*, *Lb. fermentum* and *Lb. brevis*, *Wessella confusa*, *Lactobacillus acidophilus* and *Lactococcus lactis*. It was concluded that was consistence with other flora of naturally fermented South African milk where the common genera were *Leuconostoc*, *Lactococcus* and *Lactobacillus*. The strains in *emahewu* compares well with other similar products like *ting* (South African spontaneously fermented sorghum non-alcoholic beverage) where the main strains were *Lactobacillus plantarum, Lactococcus lactis, Lactobacillus fermentum, Lactobacillus rhamnsosus, Weissella cibaria,* and *Enterococcus faecalis*. Almost all *lactobacillus* spp. were able to utilise mainly ribose, galactose, D-glucose, D-fructose, D-mannose, N-acetylglucosamide, amygdaline, arbutine, esculine, salicine, cellobiose, maltose, lactose, melibiose, saccharose and trehalose *Lactocococcus* ssp. metabolised carbon source ribose, D-xylose, galactose, D-glucose, D-fructose, D-mannose, N-acetylglucosamide, amygdaline, arbutine, esculine, salicine, cellobiose, maltose, lactose and trehalose. Most of *leuconostoc mesenteroides* ssp. utilised substrate ribose, galactose, D-glucose, D-fructose, D-mannose, cellobiose, maltose, lactose, melibiose, saccharose and trehalose. In general LAB in the current study fermented other carbohydrates such as L-arabinose, rhamnose, mannitol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, melizitose, D-raffinose, starch, B gentiobiose, D-turanose, D-tagatose, D-arabitol, gluconate, 2 ketogluconate and 5 ketogluconate. The metabolism of LAB is this study compares well LAB isolated from Ethiopian naturally fermented buttermilk and from Sudanese fermented Camel's milk. These LAB were able to utilise carbohydrates namely galactose, maltose, glucose, fructose, mannose, lactose, trehalose, meliobiose, xylitol and sorbose. The identity and carbohydrate metabolism of Swazi isolated LAB are in agreement with literature.

## 5.1. Introduction

Fermentation of food is one of the oldest forms of food preservation (Blandino et al 2003). Several studies have shown how this technique helps in preventing foodborne illnesses, including childhood diarrhoea (Mortarjemi 2002). Consumption of fermented foods is thought to contribute to good health owing to the beneficial role of the product microflora to the human gut (Gardiner et al 2002).

Fermented foods can be grouped into four categories, namely; alcoholic, lactic acid, acetic acid and alkali fermented foods (Blandino et al 2003; Steinkraus 2002). Several traditional African fermented cereal grain foods, such as *mahewu* (sour sorghum or maize meal non-alcoholic beverage from South Africa, Zimbabwe, Lesotho), *togwa* (thin sour maize meal porridge; Tanzania), *kenkey* (thick sour maize meal porridge; Ghana), *amasi* (spontaneously fermented milk; southern Africa) and *motoho* (thin sour sorghum porridge or beverage; Lesotho), are largely products of lactic acid fermentation.

Lactic acid bacteria have been found to be the predominant microorganisms in most of these products (Blandino et al 2003). However, yeasts are also important in alcoholic fermented foods (Steinkraus 2002), and may be fortuitous contaminants in fermented milk (Gadaga et al 2001; Roostita and Fleet 1996). In a product similar to *emasi* (*kule naoto*, Kenyan traditional fermented milk produced by the Maasai), Mathara et al (2004) found that genus *Lactobacillus* species (*Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus paracasei* and *Lactobacillus acidophilus*.) were predominant. Other genera that were isolated (from *kule naoto*) were Enterococcus, *Lactococcus* and *Leuconostoc*. Schoustra et al (2013), reported

that in Munkoyo and Chibwantu, (traditionally non-alcoholic fermented beverages popularly consumed in Zambia), *Lactobacillus* and *Weissella* were the common genera isolated together with *Lactococcus*, *Streptococcus* and *Leuconostoc*. *Emasi* and *emahewu* are non – alcoholic lactic acid fermented traditional foods.

The preparation methods of *sancta* (fermented maize meal), *incwancwa* (fermented porridges), *emasi* (fermented milk), e*mahewu* (non-alcoholic cereal beverage), *umcombotsi* (alcoholic sorghum or millet beverage), *mankanjane* (malt distilled spirits), *buganu*/*marula* wine and papaya beer (fermented fruit mashes) were previously outlined (Masarirambi et al 2009; Simatende et al 2015). However, the microbial flora responsible for the fermentation has not been studied. The aim of this study, therefore, was to investigate the microbial diversity, isolate potential probiotic LAB strains and identify LAB in *emasi* and *emahewu*, Swazi traditional fermented foods. Some of the biochemical properties were also investigated. This is an important step in up-grading and possible commercialisation of these products.

## 5.2. Materials and Methods

### *5.2.1. Location of study*

The study was done in the Hhohho region of Swaziland. The region is divided into 14 local administrations called *tinkhundla*. Hhohho region is in the Highveld of the country where the temperatures range from very cold to warm.

### *5.2.2. Sampling*

Samples were collected from 5 *tinkhundla* that were randomly selected from the 14 *tinkhundla* using a lottery system. At each *inkhundla*, a list of members of the community who were known to prepare fermented foods was compiled with the assistance of community leaders, such as the *umphakatsi* or school teachers. Samples of fermented food were then collected from the households that were randomly selected from the list. The samples were collected in sterile screw capped bottles and ferried in a cooler box to the laboratory at the University of Swaziland for analysis.

### *5.2.3. Determination of pH and titratable acidity*

pH was determined using a Hanna Instruments pH meter (HI 8314) after calibrating with buffers at pH4 and pH7. Titratable acidity (TA) was determined using standardized 0.1N NaOH (Rochelle Chemicals, Johannesburg, South Africa) according to AOAC method no. 947.05 (AOAC, 1990).

### *5.2.4. Microbiological analysis*

#### *5.2.4.1. Enumeration of lactic acid bacteria (LAB)*

The fermented samples were analysed immediately upon receipt at the laboratory. Lactic acid bacteria (LAB), yeasts and total coliforms were enumerated on respective selective agar. LAB were enumerated on MRS agar (Oxoid, Basingstoke, UK; CM0361) by spreading 0.1mL of appropriate serial dilutions, and incubating anaerobically at 30$^{o}$C for 48 h.

Anaerobic conditions were created using Oxoid anaerobic gas generating system (Oxoid, Basingstoke, UK, BR0038B) according to the manufacturer's instructions.

#### *5.2.4.2. Isolation and selection of LAB strains*

Colonies with different appearances (colour, shape and size) were picked from the MRS agar (Oxoid) and purified by streaking on a fresh MRS agar (Oxoid) plate. The purification process was repeated until single colonies with distinct appearance were obtained. The pure isolates were tested for Gram and catalase reactions. The cell morphology was observed under the microscope. The isolates that were Gram positive and catalase negative were taken as presumptive lactic acid bacteria. The LAB isolates were stored at -20$^{o}$C in MRS broth (Biolab, Wadeville, South Africa; HG000C87.500) containing 20% (v/v) glycerol until required for further tests.

#### *5.2.4.3. Identification of LAB using API 50 CHL kits*

The frozen LAB isolates were thawed and resuscitated by inoculating into fresh MRS broth (Oxoid) and incubating at 30$^{o}$C for 24h. A portion of the fresh culture was streaked onto MRS agar (Oxoid), which was then incubated anaerobically for 48h. The pure colonies were picked and inoculated onto API 50 CH (bioMerieux, Marcy l'Etloile, France; Ref 50 300) test strips according to the manufacturer's instructions. The sugar fermentation profiles were then used to identify the isolates using API identification software (APIweb).

#### *5.2.4.4. Identification of LAB by sequencing the 16S rDNA*

The identification was done at Inqaba Biotec Laboratories, South Africa. Briefly, DNA was extracted using ZR Fungal/Bacteria DNA Kit™ (Zymo Research). The 16S rDNA target region was amplied using DreamTaq™ DNA polymerase (Thermo Scientific™) and the primers 16S-27F, sequence 5′-AGAGTTTGATCMTGGCTCAG-3′ and 16S-1492R, sequence 5′-CGGTTACCTTGTTACGACTT-3′. PCR products were gel extracted (Zymo Research, Zymoclean™ Gel DNA Recovery Kit), and sequenced in the forward and reverse directions on the ABI PRISM™ 3500xl Genetic Analyser. Purified sequencing products (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™) were analysed using CLC Main Workbench 7 followed by a BLAST search (NCBI) (Altschul et al., 1997).

### *5.2.5. Statistical analysis*

The mean values (± standard deviation) for the pH, TA, and microbial counts for the samples in the different categories were calculated.

## 5.3. Results and Discussion

### *5.3.1. Samples collected*

Samples of *emasi* and *emahewu* were collected from Lobamba (coded L), Mangwaneni (M), Zone 4 (Z4), Motshane (Mot), Mbabane (Mb), Ezulwini (Ez), Mvutjini (Mv) and Ntfonjeni (Nt) areas in Hhohho, Swaziland.

### *5.3.2. eMasi*

#### *5.3.2.1. pH and titratable acidity (TA)*

The average pH in *emasi* was 4.68, and TA was 0.89% (Table 5.1.), which corresponds well with values obtained in other studies for naturally fermented milk. For example, Kebede et al (2007) reported that *sethemi*, South African naturally fermented milk similar to *emasi*, had pH values of about 4.1-4.3. Beukes et al (2001) also reported that the pH in indigenous

**Table 5.1.**

The pH, titratable acidity, and LAB counts of *emasi*, Swazi naturally fermented milk.

| Sample code | pH | Titratable acidity (% Lactic acid) | LAB counts (log CFU/mL) |
|---|---|---|---|
| MOT-emasi | 4.31 | 0.98 | 8.34 |
| Nt-emasi-1 | 4.57 | 0.97 | 8.69 |
| Nt-emasi- | 4.52 | 0.83 | 8.82 |
| L-emasi | 5.03 | 0.82 | 7.30 |
| Mb-emasi 1 | 4.98 | 0.88 | 7.78 |
| Mb-emasi 2 | 4.87 | 0.89 | 8.24 |
| Mb-emasi 3 | 4.62 | 0.90 | 8.36 |
| Mb-emasi 4 | 4.55 | 0.87 | 8.45 |
| **Average** | **4.68±0.25** | **0.89±0.06** | **8.25±0.49** |

fermented milks from South Africa and Namibia ranged from 4.0 to 5.4, with an average of 4.6. *Amasi* produced at household level in Zimbabwe was found to have a mean pH of 3.98 and 0.97% TA (Mutukumira 1995). Gran et al (2003) found that the pH of naturally fermented *amasi* produced by small-holder producers in Zimbabwe was about 4.2 after 48h fermentation. *Nunu* is Ghanaian spontaneously fermented milk with the consistency of

yogurt. The pH of this product was found to be about 3.4 after 48h of fermentation (Akabanda et al 2010). However, the reported TA of 4.5% for *nunu* was uncharacteristically high compared with the values recorded for *emasi*, *amasi* and other similar products in Southern Africa. In comparison, Moyane and Jideani (2013) found that the pH of commercially produced *amasi* in Venda, South Africa, ranged from pH 4.22 to pH 4.34 and the TA ranged from 0.80% to 0.84%, which is close to what was recorded for spontaneously fermented *emasi*.

*5.3.2.2. Enumeration of lactic acid bacteria*

The LAB counts in *emasi* ranged from 7.30 to 8.82 log cfu/ml (Table 5.1.). The LAB counts were very comparable to similar African naturally fermented milk products. For instance, the presumptive LAB counts in indigenous spontaneously *amasi* from South Africa were about $7.7x10^8$ cfu/mL (8.89 log cfu/mL) (Beukes et al 2001). Zimbabwean *amasi* had LAB ranging from 8.29 to 9.88 log cfu/g (Mutukumira 1995), while Nigerian fermented milk, *nono*, was found to have LAB counts of about $9.8 \times 10^6$ cfu/mL (6.99 log cfu/mL). In addition, Egyptian traditional fermented milk, Laban Zeer had LAB counts of up to 7.4 log cfu/g. The Ghanaian *nunu* was also reported to have LAB counts of up to 9 log cfu/mL after 48h fermentation (Akabanda et al 2010). In contrast, Matsheka et al (2013) reported a much lower value of 5.3 log cfu/mL LAB in *madila*, Botswana spontaneously fermented milk.

Other studies on non-African fermented milks showed similar trends on LAB. Traditional natural fermented goat milk collected from households in Haixi region in China had LAB counts of $2.5x10^8 – 3.0x10^9$ (8.4 – 9.5 log cfu/mL) (Zhang et al 2008). The high LAB counts

give a high actively growing dose of microorganisms on consumption, which one requirement for probiotic food.

### *5.3.3. eMahewu*

#### *5.3.3.1. pH and titratable acidity (TA)*

In *emahewu*, the average pH was 3.62, but it ranged from 3.15 to 4.51. The TA was 0.43% (Table 5.2.). A similar product prepared in Zimbabwe, which is also called *mahewu*, had a final pH of 3.0 (Simango 2002). This product had titratable acidity of about 0.9% after 48h fermentation, which is higher than that observed for *emahewu*. The Zimbabwean *mahewu* is made with maize meal and sorghum malt flour, probably resulting in production of higher amounts of organic acids. No sorghum malt is added during preparation of the Swazi *emahewu* (Masarirambi et al 2009; Simatende et al 2015). The pH in *Bushera*, non- alcoholic sorghum based beverage from Uganda, was found to range from 3.7 to 4.5 (Muyanja et al 2003), which is close to the values obtained for *emahewu*. The TA in this product was 0.52%, which tallies with the current study and the pH values obtained.

**Table 5.2.**

The pH, titratable acidity, and LAB counts of *emahewu*, a Swazi non-alcoholic fermented beverage.

| Sample code | pH | Titratable acidity (% Lactic acid) | LAB counts (log CFU/ml) |
|---|---|---|---|
| L-emah-1 | 4.34 | 0.22 | 6.91 |
| L-emah-2 | 4.17 | 0.41 | 7.78 |
| L-emah | 3.28 | 0.46 | 9.30 |
| Lemah-3 | 3.86 | 0.53 | 8.75 |
| Nt-emah | 3.84 | 0.53 | 8.14 |
| Z4-emah-1 | 4.51 | 0.75 | 6.88 |
| L-emah-20 | 2.95 | 0.45 | 8.11 |
| L-emah-21 | 3.09 | 0.40 | 8.67 |
| L-emah-22 | 3.15 | 0.34 | 8.43 |
| Ez-emah | 3.30 | 0.24 | 7.74 |
| Mv-emah | 3.24 | 0.43 | 8.41 |
| **Mean** | **3.62 ± 0.55** | **0.43± 0.14** | **8.10± 0.74** |

*5.3.3.2. Enumeration of lactic acid bacteria*

The LAB counts in *emahew*u ranged from 6.88 to 9.30 log cfu/mL (Table 5.2.). The LAB counts were within the range expected when compared to other studies. Muyanja et al (2003), in their study of *bushera*, found that the LAB counts varied between 7.1 and 9.4 log cfu/mL. LAB counts in home-made *mahewu* from South Africa increased from 3.25 to 6.28 log cfu/mL after 72 hours' fermentation (Katongele 2008).

*Ting* is non-alcoholic beverage prepared in Botswana, made from sorghum meal and malt. The LAB counts in this product were found to range between 8.08 and 10.1 log cfu/g (Sekwati-Monang 2011).

The high LAB counts in home-made *emahewu* therefore explain the significant reduction in pH recorded for the product.

### *5.3.4. Identification of LAB*

The isolates were initially screened as presumptive LAB using the Gram stain, catalase test and microscopic examination. The Gram positive, catalase negative isolates were identified to species level using API 50 CH test strips (bioMerieux SA, Marcy-l'Etoile, France) and by sequencing the 16S rDNA as shown in Table 5.3. & 5.4.

#### *5.3.4.1. Emasi*

Among the nine *emasi* isolates nine identified using the API 50CH kits, four were identified as *Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum*, while two were identified as *Lactococcus lactis*, one as *Lactobacillus plantarum* and the other two as *Lactobacillus brevis* (Table 5.3.). The four *Leuconostoc* isolates were characterised by sequencing the 16S rDNA and were identified as *Leuconostoc pseudomesenteroides* (Table 5.4.), which was in close agreement with the API identification. In a study on South African naturally fermented milk,

**Table 5.3.**

Identification using API 50CHL of lactic acid bacteria isolated from Swazi traditional fermented *emasi* and *emahewu*.

| | ISOLATE CODE | IDENTITY |
|---|---|---|
| **EMASI** | | |
| 1 | L-emasi-1 | *Leuconostoc mesenteroides* ssp. *mesenteroides*/*dextranicum* |
| 2 | L-emasi-5 | *Leuconostoc mesenteroides ssp. mesenteroides* |
| 3 | L-emasi-7 | *Leuconostoc mesenteroides* ssp. *mesenteroides*/d*extranicum* |
| 4 | L-emasi-8 | *Leuconstoc mesenteroides* ssp. *mesenteroides*/*dextranicum* |
| 5 | Mot-emasi-7 | *Lactococcus lactis* ssp. *lactis* |
| 6 | Nt- emas-2 | *Lactococcus lactis ssp. lactis* |
| 7 | Nt-emas2-6 | *Lactobacillus paracasei* ssp. *paracasei* |
| 8 | Nt-emas-5 | Lactobacillus plantarum |
| 9 | Nt-emas-6 | *Lactobacillus brevis* |
| **EMAHEWU** | | |
| 10 | L-emah-1 | *Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum* |
| 11 | L-emah-3 | *Lactobacillus plantarum* |
| 12 | L-emah-5 | *Lactobacillus brevis* |
| 13 | L-emah-6 | *Lactobacillus brevis* |
| 14 | L-emah-7 | *Lactobacillus collinoides* / *Lb. fermentum* |
| 15 | L-emah-8 | *Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum* |
| 16 | L-emah-9 | *Lactobacillus plantarum* |
| 17 | L-emah-13 | *Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum* |
| 18 | L-emah-16 | *Lactobacillus plantarum* |
| 19 | L-emah-18 | *Lactobacillus plantarum* |
| 20 | Mot-emah-4 | *Lactobacillus fermentum* |
| 21 | Mot-emah-6 | *Lactobacillus collinoides* |
| 22 | Nt-emah-2 | *Lactobacillus fermentum* |
| 23 | Nt-emah-6 | *Lactobacillus paracasei* ssp. *paracasei* |
| 24 | S-emah | *Lactobacillus plantarum* |
| 25 | S-emah-5 | *Lactobacillus plantarum* |

**Table 5.4.**

Identification of LAB isolates from Swazi traditional fermented *emasi* and *emahewu* using API 50CH Kit and by sequencing the 16S rDNA.

| | ISOLATE CODE | IDENTITY by API 50 CH Kit | IDENTITY by 16S rDNA* |
|---|---|---|---|
| **EMASI** | | | |
| 1 | L-emasi-1 | *Leuconostoc mesenteroides* ssp. *mesenteroides*/*dextranicum* | *Leuconostoc pseudomesenteroides* |
| 2 | L-emasi-5 | *Leuconostoc mesenteroides ssp. mesenteroides* | *Leuconostoc pseudomesenteroides* |
| 3 | L-emasi-7 | *Leuconostoc mesenteroides* ssp. *mesenteroides*/*dextranicum* | *Leuconostoc pseudomesenteroides* |
| 4 | L-emasi-8 | *Leuconostoc mesenteroides* ssp. *mesenteroides*/*dextranicum* | Not identified |
| 5 | Mot-emasi-7 | *Lactococcus lactis* ssp. *lactis* | Not identified |
| 6 | Nt- emas-2 | *Lactococcus lactis ssp. lactis* | Not identified |
| 7 | Nt-emas2-6 | *Lactobacillus paracasei* ssp. *paracasei* | Not identified |
| 8 | Nt-emas-5 | *Lactobacillus plantarum* | Not identified |
| 9 | Nt-emas-6 | *Lactobacillus brevis* | Not identified |
| 10 | L-emasi-13 | | *Leuconostoc pseudomesenteroides* |
| **EMAHEWU** | | | |
| 11 | L-emah-1 | *Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum* | Not identified |
| 12 | L-emah-3 | *Lactobacillus plantarum* | *Lactobacillus plantarum* |
| 13 | L-emah-5 | *Lactobacillus brevis* | Not identified |
| 14 | L-emah-6 | *Lactobacillus brevis* | Not identified |
| 15 | L-emah-7 | *Lactobacillus collinoides* / *Lb. fermentum* | *Weissella confusa* |
| 16 | L-emah-8 | *Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum* | Not identified |
| 17 | L-emah-9 | *Lactobacillus plantarum* | *Lactobacillus plantarum* |

**Table 5.4. Continued,**

Identification of LAB isolates from Swazi traditional fermented *emasi* and *emahewu* using API 50CH Kit and by sequencing the 16S rDNA

| | ISOLATE CODE | IDENTITY by API 50 CH Kit | IDENTITY by 16S Rdna* |
|---|---|---|---|
| **EMAHEWU** | | | |
| 18 | L-emah-13 | *Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum* | Not identified |
| 19 | L-emah-16 | *Lactobacillus plantarum* | *Lactobacillus plantarum* |
| 20 | L-emah-18 | *Lactobacillus plantarum* | *Leuconostoc lactis* |
| 22 | L-emah-19 | | *Lactobacillus plantarum* |
| 23 | Mot-emah-4 | *Lactobacillus fermentum* | *Lactococcus lactis* |
| 24 | Mot-emah-6 | *Lactobacillus collinoides* | *Lactobacillus acidophilus* |
| 25 | Nt-emah-2 | *Lactobacillus fermentum* | Not identified |
| 26 | Nt-emah-6 | *Lactobacillus paracasei* ssp. *paracasei* | *Leuconostoc pseudomesenteroides* |
| 27 | S-emah | *Lactobacillus plantarum* | Not identified |
| 28 | S-emah-5 | *Lactobacillus plantarum* | Not identified |

*Only representative strains were further identified by molecular method by sequencing 16S rDNA

Buekes et al (2001) reported that the genera *Leuconostoc*, *Lactococcus* and *Lactobacillus* were main flora. The dominant *Lactococci* species in the South African product was *Lactococcus lactis* subsp. *lactis*, while most of the *Leuconostoc* isolates were identified as *Leuconostoc mesenteroides* subsp. *dextranicum* which is similar to the Swazi *emasi*. Other species identified in that study included *Leuconostoc citreum*, *Leuconostoc lactis*, *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus plantarum*.

Mutukumira (1995) observed that *Lactococcus lactis* subsp. *lactis* was the predominant strain isolated from *amasi* spontaneously fermented milk produced in Zimbabwe. The Zimbabwean *amasi* is produced in a similar way to *emasi*, which may explain the similarity in microbial ecology. Slight differences that may be found in the microbial diversity can be attributed to different types of containers used as well as the environment under which the fermentation is done. Clay pots, metal containers, calabashes and gourds are often used and have been found to impact on the microbial diversity (Kebede et al 2007). The current observations also agree with recent work by Osvik et al (2013) who studied the bacterial diversity of *amasi* from EkuPindiseni community in KwaZulu-Natal in South Africa using 16S rRNA and Denaturing Gradient Gel Electrophoresis (DGGE) for identification. The majority of the strains were in the genus *Lactococcus*, as well as several species of *Lactobacillus*, *Leuconostoc*, and *Enterococcus*. However, a study by Mathara et al (2004) showed that the genus *Lactobacillus* was predominant in *kule naoto*, Kenyan traditional fermented milk produced by the Maasai, where the major *Lactobacillus* species was *Lactobacillus plantarum*, followed by *Lactobacillus fermentum*, *Lactobacillus paracasei* and *Lactobacillus acidophilus*. Other genera that were isolated in *kule naoto* were Enterococcus, *Lactococcus* and *Leuconostoc*.

Laban Zeer produced in Egypt seems to have similar flora as *emasi*. Saleh (2013) identified the LAB species in Laban Zeer as *Leuconostoc mesenteroides* subsp. *cremoris*, *Lb. rhamnosus*, *Lb. plantarum*, *Lb. paracasei* subsp. *paracasei*, *Lb. delbruekii* subsp. *bulgaricus*, *Lb. curvatus* subsp. *curvatus* and *Lb. acidophilus*. The most frequently isolated LAB species were found to be *Leuconostoc mesenteroides* subsp. *cremoris* and *Lb. rhamnosus*.

The Swazi fermented milk's microflora is therefore similar to that in other naturally fermented products from Southern Africa, in particular *amasi* from South Africa and Zimbabwe.

### *5.3.4.2. Emahewu*

Sixteen isolates from *emahewu* were identified using the API 50 CH test kit. Among these, six were identified as *Lactobacillus plantarum*, three as *Leuconostoc mesenteroides ssp. mesenteroides*, two as *Lb. fermentum*, two as *Lb. brevis*, and one as *Lb. collinoides* (Table 5.3.). The predominant isolates were therefore *Lactobacillus plantarum* strains. Nine isolates were further characterised by sequencing the 16S rDNA. Four were confirmed as *Lactobacillus plantarum*, while the others were identified as *Leuconostoc lactis*, *Weissella confusa*, *Lactobacillus acidophilus*, and *Lactococcus lactis* (Table 5.4.). In comparison the main LAB in *ogi*, a Nigerian fermented cereal beverage, were found to be *Lb. plantarum, Lb. casei, Lb. brevis, Lb. fermentum, Lb. delbrueckii, Lb. acidophilus, Leuconostoc mesenteroides* and *Pediococcus acidilacti* (Dike and Sanni 2010). Some of the *ogi* strains were evaluated as starter cultures by inoculating them in maize gruel for making *ogi*, and *Lb. plantarum* was found to have the highest potential as a starter culture in terms of acid production and increase in LAB counts (Nwachukwu and Ijeoma 2010). In a separate study, Madoroba et al (2011) isolated and identified LAB in *ting*, a South African spontaneously fermented sorghum non-alcoholic beverage, and found that the predominant LAB were *Lactobacillus plantarum, Lactococcus lactis, Lactobacillus fermentum, Lactobacillus rhamnsosus, Weissella cibaria*, and *Enterococcus faecalis*. Some Enterobacteriaceae were also isolated. The Swazi *emahewu* samples were prepared from maize meal. On the other

hand, the predominant microorganisms in *koko*, Ghanaian spontaneously fermented porridge from millet, were identified as *Weissella confusa* and *Lactobacillus fermentum* (Lei and Jakobsen 2004), while Yousif et al (2010) found that *Lactobacillus fermentum* and *Pediococcus acidilacti* were the predominant strains in *hussuwa*, a Sudanese fermented sorghum food. Also in *gari*, a cassava based fermented food from Benin, *Lactobacillus plantarum* was the most commonly isolated species followed by *Leuconstoc fallax* and *Lactobacillus fermentum* (Kostinek et al 2005). Muyanja et al (2003) also identified the LAB isolated from the spontaneously fermented Ugandan bushera as *Lactobacillus plantarum, L. paracasei* subsp. *paracasei*, *Lb. fermentum, Lb. brevis* and *Lb. delbrueckii* subsp. *delbrueckii*. *Streptococcus thermophiles.* Similarly, Munkoyo and Chibwantu, traditionally non-alcoholic fermented beverages popularly consumed in Zambia, *Lactobacillus* and *Weissella* were the common genera isolated (Schoustra et al 2013).

This, therefore, suggests that the *Lactobacillus* genus, in particular *Lb. plantarum*, is typical biota of spontaneously fermented maize and sorghum non-alcoholic beverages and play a key role in defining the attributes of these products. Some strains of *Lb. plantarum* have been found to be amylolytic, breaking down starch in pearl millet slurries (Songre-Outtara et al 2010) and further studies on these *emahewu* strains is needed.

### *5.3.5 Carbohydrate profile of LAB*

Almost all *lactobacillus* spp. were able to utilise mainly ribose, galactose, D-glucose, D-fructose, D-mannose, N-acetylglucosamide, amygdaline, arbutine, esculine, salicine, cellobiose, maltose, lactose, melibiose, saccharose and trehalose (Tab. 5.5.).

**Table 5.5.**

Carbohydrate fermentation by lactic acid bacteria species isolated from emasi and emahewu

| Substrate | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| O | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Gly | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Ery | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Dara | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Lara | - | - | + | + | - | - | - | + | - | - | - | + | + | - | - | - | + | - | - | - | - | - | - | + | + |
| Rib | + | + | + | + | + | + | + | + | + | - | + | + | + | + | - | + | + | + | + | + | + | + | + | + | + |
| Dxyl | + | + | - | - | + | + | - | - | - | + | - | + | + | + | + | - | + | - | - | - | + | - | - | - | - |
| Lxyl | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Ado | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Mdx | - | - | - | - | - | - | - | - | - | - | - | + | + | - | - | - | - | - | - | - | - | - | - | - | - |
| Gal | + | + | + | + | + | + | + | + | + | - | + | + | + | + | - | + | + | + | + | + | + | + | + | + | + |
| Glu | + | + | + | + | + | + | + | + | + | + | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + |
| Fru | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Mne | + | + | + | + | + | + | + | + | + | + | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + |
| Sbe | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Rha | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + |
| Dul | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Ino | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Man | - | - | - | - | - | + | + | + | + | - | + | - | - | - | - | + | - | + | + | - | - | - | + | + | + |
| Sor | - | - | - | - | - | - | - | + | - | - | + | - | - | - | - | + | - | + | + | - | - | - | - | + | + |
| Mdm | - | - | - | - | - | - | - | + | - | - | + | - | - | - | - | + | - | + | + | - | - | - | - | + | + |
| Mdg | + | + | + | + | - | - | - | - | - | + | - | - | - | - | + | - | + | - | - | - | - | - | - | - | - |
| Nag | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + |

**Table 5.5. continued,**

Carbohydrate fermentation by lactic acid bacteria species isolated from emasi and emahewu

| Substrate | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Amy | - | - | - | - | - | + | + | + | + | - | + | - | - | - | - | + | - | + | + | - | - | - | + | + | + |
| Arb | - | - | - | - | - | + | + | + | + | + | + | - | - | - | - | + | - | - | - | - | - | - | + | + | + |
| Esc | - | - | + | + | + | + | + | + | + | + | + | - | + | - | + | + | + | + | + | - | + | - | + | + | + |
| Sal | - | - | - | - | - | + | + | + | + | + | + | - | - | - | + | + | - | + | + | - | - | - | + | + | + |
| Cel | + | + | + | + | + | + | + | + | + | - | + | - | - | - | - | + | + | + | + | - | + | - | + | + | + |
| Mal | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Lac | + | + | + | + | - | + | + | + | + | - | + | - | - | - | - | + | + | + | + | + | - | + | + | + | + |
| Mel | + | + | + | + | - | - | - | + | + | + | + | + | + | - | + | + | + | + | + | + | - | + | - | + | + |
| Sac | + | + | + | + | + | - | + | + | + | + | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + |
| Tre | + | + | + | + | - | + | + | + | + | + | + | - | - | - | + | + | + | + | + | - | - | - | + | + | + |
| Inu | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Mlz | - | - | - | - | - | - | - | + | - | - | + | - | - | - | - | + | - | + | + | - | - | - | - | + | + |
| Raf | - | + | + | - | - | - | - | + | + | + | + | - | - | - | + | + | + | + | + | + | - | + | - | + | + |
| Amd | - | - | - | - | - | + | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - |
| Glyg | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Xlt | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Gen | + | + | + | + | + | + | + | + | + | - | + | - | - | - | - | + | + | + | + | + | + | - | + | + | + |
| Tur | + | + | + | + | - | - | - | + | - | + | - | - | - | - | + | - | + | - | - | - | - | - | - | + | + |
| Lyx | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Tag | - | - | - | - | - | - | + | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - |
| Dfuc | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Lfuc | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Darl | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | + | + | - | - | - | - | + | + |
| Larl | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

**Table 5.5. continued,**

Carbohydrate fermentation by lactic acid bacteria species isolated from emasi and emahewu

| Substrate | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gnt | - | + | + | + | + | + | + | + | + | - | + | + | + | + | - | + | - | + | + | + | + | + | + | + | + |
| 2kg | + | + | - | + | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| 5kg | - | + | + | - | - | - | - | - | - | - | - | + | + | - | - | - | - | - | - | - | - | - | - | - | - |

Metabolism, + = positive reaction, - = negative reaction.

Abbreviation, *Lc. Lactococcus, Lb. Lactobacillus, L. Leuconostoc*

Identity of LAB and label of sample, 1: *L. mesenteroides* ssp. *mesenteroides/dextranicum* (L-emasi-1); 2: *L. mesenteroides* ssp. *mesenteroides* (L-emasi-5); 3: *L. mesenteroides* ssp. *mesenteroides/ dextranicum* (L-emasi-7); 4: *L. mesenteroides* ssp. *mesenteroides/dextranicum* (L-emasi-8); 5: *Lc. Lactis* ssp. *lactis* (Mot-emasi-7); 6: *Lc. Lactis* ssp. *lactis* (Nt-emas-2); 7: *Lb. paracasei* ssp. *paracasei* (Nt-emas2-6); 8: *Lb. plantarum* (Nt-emas-5); 9: *Lb. brevis* (Nt-emas-6); 10: *L. mesenteroides* ssp. *mesenteroides/dextranicum* (L-emah-1); 11: *Lb. plantarum* (L-emah-3); 12: *Lb. brevis* (L-emah-5); 13: *Lb. brevis* (L-emah-6); 14: *Lb. collinoides/Lb. fermentum* (L-emah-7); 15: *L. mesenteroides* ssp. *mesenteroides/dextranicum* (L-emah-8); 16: *Lb. plantarum* (L-emah-9); 17: *L. mesenteroides* ssp. *mesenteroides/dextranicum* (L-emah-13); 18: *Lb. plantarum* (L-emah-16); 19: *Lb. plantarum* (L-emah-18); 20: *Lb. fermentum* (Mot-emah-4); 21: *Lb. collinoides* (Mot-emah-6); 22: *Lb. fermentum* (Nt-emah-2); 23: *Lb. paracasei* ssp. *paracasei* (Nt-emah-6); 24: *Lb. plantarum* (S-emah); 25: *Lb. plantarum* (S-emah-5);

Type of carbohydrates, O: Control; Gly: glycerol; Ery: erythritol; Dara: D-arabinose; Lara: L-arabinose; Rib: ribose; Dxyl: D-Xylose; Lxyl: L-Xylose; Ado: Adonitol; Ddx: B Methyl-xyloside; Gal: Galactose; Glu: D-Glucose; Fru: D-Fructose; Mne: D-Mannose; Sbe: L-Sorbose; Rha: Rhamnose; Dul: Dulcitol; Ino: Inositol; Man: Mannitol; Sor: Sorbitol; Mdm: α-Methyl-D-mannoside; Mdg: α-Methyl-D-glucoside; Nag: N-Acetylglucosamide; Amy: Amygdaline; Arb: Arbutine; Esc: Esculine; Sal: Salicine; Cel: Cellobiose; Mal: Maltose; Lac: Lactose; Mel: Melibiose; Sac: Saccharose; Tre: Trehalose; Inu: Inuline; Mlz: Melizitose; Raf: D-Raffinose; Amd: Starch; Glyg: Glycogen; Xlt: Xylitol; Gen: B Gentiobiose; Tur: D-Turanose; Lyx: D-Lyxose; Tag: D-Tagatose; Dfuc: D-Fucose; Lfuc: L-Fucose; Darl: D-Arabitol; Larl: L-Arabitol; Gnt: Gluconate; 2kg: 2 Ketogluconate; 5kg: 5 Ketogluconate

*Lactococcoccus* ssp. metabolised carbon source ribose, D-xylose, galactose, D-glucose, D-fructose, D-mannose, N-acetylglucosamide, amygdaline, arbutine, esculine, salicine, cellobiose, maltose, lactose and trehalose. Most of *leuconostoc mesenteroides* ssp. utilised substrate ribose, galactose, D-glucose, D-fructose, D-mannose, cellobiose, maltose, lactose, melibiose, saccharose and trehalose. In general LAB in the current study fermented other carbohydrates such as L-arabinose, rhamnose, mannitol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, melizitose, D-raffinose, starch, B gentiobiose, D-turanose, D-tagatose, D-arabitol, gluconate, 2 ketogluconate and 5 ketogluconate (Tab. 5.5).

The metabolism of carbohydrates by LAB is similar to observations made by Negussie et al (2016) where it was observed that LAB isolated from Ethiopian naturally fermented buttermilk were able to utilise carbohydrates such as galactose, maltose, glucose, fructose, mannose and lactose. The results of the current study are supported by Ashmaig et al (2009) where it was observed that LAB isolated from traditional Sudanese fermented Camel's milk were able to ferment some carbohydrates. The common substrates that were fermented include carbohydrates such as lactose, fructose, galactose, trehalose, melibiose, mannose, xylitol and sorbose.

### *5.3.6. Conclusions*

Emasi and emahewu are fermented foods of Swaziland. Leuconostoc mesenteroides and Lactococcus lactis subsp. lactis were typical strains in emasi, while the Lactobacillus genus especially Lb. plantarum was typical in emahewu. *Emasi* and *emahewu* help in enhancing

dietary diversity and in Swaziland they are popular foods taken by children and adults. There is need to conduct some studies in developing starter cultures.

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# CHAPTER 6: EVALUATION OF PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA STRAINS ISOLATED FROM *EMAHEWU,* A SWAZI TRADITIONAL FERMENTED CEREAL GRAIN BEVERAGE


## Abstract

Several fermented foods are produced at household level in Swaziland. This study focused on characterising probiotic potential of lactic acid bacteria (LAB) strains previously isolated from *emahewu,* a maize based fermented beverage. Sixteen LAB isolates belonging to *Leuconostoc lactis, Lactobacillus plantarum, Lb. acidophilus, Weissella confusa, Lactococcus lactis* and *Leuconostoc pseudomesenteroides* were tested for ability to grow at low pH, in presence of bile, antibiotics, and for their antimicrobial properties. All the strains were able to survive at pH 3. *Leuconostoc lactis, Lb. plantarum, Lb. acidophilus, L. lactis, Weissella confusa* and *Leuconostoc pseudomesenteroides* grew in the presence of 0.3, 0.5, and 1.0% (w/v) oxgall bile. All the isolates were sensitivity to ampicillin, amoxicillin and tetracycline. However, *Lb. acidophilus* was resistant to ampicillin, and a strain of *Lb. plantarum* (M9) was moderately susceptible to tetracycline. All isolates showed resistance to streptomycin, ciprofloxacin and nalidixic acid, while *Weissella confusa* and *Lb. acidophilus* were moderately susceptible to ciprofloxacin. Neutral supernatant from each of the actively growing cultures of the isolates were inhibitory to *Salmonella typhimurium* ATCC 13311 and *Escherichia coli* ATCC 25922. The study showed that among the strains tested, *Lb. acidophilus Lb. plantarum* had properties could be beneficial to the health of consumers.

## 6.1. Introduction

There is increasing use of probiotics as food supplements worldwide (Yu et al 2013). Probiotics are defined as selected, viable microbial dietary supplements that, when introduced in sufficient quantities, beneficially affect the human organism through their effects in the intestinal tract (Holzapfel and Schillinger 2002; Lei and Jakobsen 2004; Zimmer and Gibson 1998). The FAO/WHO (2002) also define probiotics as live microorganisms which, when administered in adequate amounts confer a health benefit on the host. The minimum concentration of probiotic microorganisms required to achieve some therapeutic effects is strain dependent. The minimum dosages of > $10^6$ cfu per ml are recommended (Hawrelak 2002; Lourens-Hattingh and Viljoen 2001).

Some lactic acid bacteria (LAB) strains, due to their probiotic properties have been used as food supplements (Ramos et al 2013). Amongst the widely used probiotic LAB are strains of *Lb. acidophilus*, *Lb. rhamnosus*, *Lb. plantarum* and *Lb. fermentum*. For example, Mathara et al (2008) reported that *L. plantarum* 299v and *Lb. plantarum* Lp 01 were marketed as probiotics. Other LAB that have shown potential as commercial probiotics, include *Lb. plantarum* Lpc-37 and *Lb*. *plantarum* L4 as reported by Yu et al (2013).

Swazi traditionally fermented foods include fermented porridges (*incwancwa*), fermented milk (*emasi*), non-alcoholic cereal beverage (e*mahewu*), and alcoholic beverage (*umcombotsi*) from sorghum (e*mabele*) or millet (*nyawotsi*) (Masarirambi et al 2009; Simatende et al 2015). A previous study showed that *emasi* and *emahewu* are fermented by a diverse group of microorganisms including yeasts and lactic acid bacteria (Simatende et al

**unpublished**). Traditional fermented foods may therefore be reservoirs for LAB strains that have probiotic properties.

The criteria an effective probiotic has to meet to be of any value to human health includes the ability to survive the harsh environment in the human gut (where pH can be as low as 1-2 during fasting and increases to 4-5 after taking of food) and offer some beneficial effects to the consumer (Grajek et al 2005; Turchi et al 2013). They also have to overcome toxicity of bile in the stomach whose concentration can be as high as 0.3% of stomach contents of an average healthy person (Jose et al 2015). The probiotics should be able to colonize the lining of gastro intestinal tract (GIT) by displaying sound surface hydrophobicity and aggregation qualities (Turchi et al 2013). They are required to show some good antioxidative properties at the same time not impacting negatively on the immune system. In addition, they are expected to show some acceptable antimicrobial activities against common pathogens through production of organic acids that lowers the pH, and/or production of bacteriocins and hydrogen peroxide (Jose et al 2015; Owusu-Kwarteng et al 2015; Turchi et al 2013). Furthermore, a good probiotic is expected to exhibit some resistance to commonly used antibiotics.

It has been proposed that probiotics are beneficial to humans through reduction of lactose intolerance, prevention of colon cancer, lowering cholesterol levels, lowering blood pressure, improving immune function, prevention of infections, improving mineral absorption, reducing inflammation, improving the digestibility of food and repression of pathogenic bacteria (Sanders 2000). LAB have also demonstrated anti-mutagenic effects thought to be

due to their ability to bind with (and therefore detoxify) heterocyclic amines formed in cooked meat (Wollowski et al 2001). It is also theorised that probiotic lactobacilli may help correct malabsorption of trace minerals, found particularly in those diets high in phytate content from cereals, nuts and legumes (Famularo et al 2005).

The objective of this study was to evaluate the probiotic properties of LAB previously isolated from *emahewu*, Swazi maize based fermented beverage. This will help in further developing the traditional products into more predictable functional foods.

## 6.2. Material and Methods

### *6.2.1. Source of lactic acid bacteria (LAB) isolates*

Nine isolates of LAB previously isolated and identified from *emahewu* were used for probiotic characterisation. These isolates included four strains of *Lb. plantarum*, one strain of *Lactococcus lactis*, one strain of *Weissella confusa*, one strain of *Lb. acidophilus*, one strain of *leuconostoc lactis*, and one strain of *Leuconostoc pseudomesenteroides*, which were coded as shown in Table 6.1. The cultures were stored frozen at -28 $^{o}$C in MRS broth (Oxoid CM0359, Basingstoke, UK) containing 20% glycerol (L&T Diagnostics CC 090307, Johannesburg, South Africa) until required for the tests.

**Table 6.1**

Name and codes for isolates of LAB.

| Name | Code |
|---|---|
| *Leuconostoc lactis* | M1 |
| *Lactobacillus plantarum* | M2 |
| *Lactobacillus plantarum* | M3 |
| *Lactococcus lactis* | M4 |
| *Leuconostoc pseudomesenteroides* | M5 |
| *Lactobacillus acidophilus* | M6 |
| *Weissella confusa* | M7 |
| *Lactobacillus plantarum* | M8 |
| *Lactobacillus plantarum* | M9 |

### *6.2.2. Source of pathogens*

Pure culture swabs of human pathogens; namely; *Salmonella typhimurium* ATCC 13311 and *Escherichia coli* ATCC 25922 were revived by asceptically suspending each swab in sterile 9 ml Brain Heart Infusion (BHI) broth (Mast Group Ltd MD106D, Merseyside, UK). The cultures were then incubated for 24 h at 37 $^{o}$C. The pathogens were stored frozen at -28 $^{o}$C in BHI broth (Mast) containing 20% glycerol (L&T Diagnostics CC 090307, Johannesburg, South Africa) until required for the tests.

### *6.2.3. Revival of LAB and pathogens*

The frozen LAB isolates were revived by separately inoculating 0.1 ml of thawed concentrated cultures into 9 ml MRS broth (Oxoid) and incubating for 24 h at 30 $^{o}$C. The frozen pathogen isolates were revived by separately inoculating 0.1 ml of thawed concentrated cultures into 9 ml BHI broth (Mast) and incubating for 24 h at 37 $^{o}$C.

### *6.2.4. Assessment of probiotic potential of LAB*

The LAB were assessed for their ability to survive at low pH, growth in the presence of bile, susceptibility to antibiotics and antimicrobial activity against *Salmonella typhimurium* ATCC 13311 and *Escherichia coli* ATCC 25922.

#### *6.2.4.1. Growth at low pH*

After reviving isolates separately into 9 ml MRS broth (Oxoid), the broth was then centrifuged at 3000 rpm for 15 min to obtain a pellet of cells. The supernatant was aseptically decanted and the pellets were washed in phosphate-saline buffer (PBS at pH 7.2) (European Pharmacopeia, 2005). The suspension was again centrifuged at 3000 rpm for 15 min to obtain a pellet. The pellets were re-suspended in MRS broth (Oxoid) adjusted to pH 3 and incubated for 3 hours at 30 $^{o}$C. Viable counts were determined by sub-culturing at 3 h intervals and pour plating serially diluted portions on MRS agar (Oxoid CM0361, Basingstoke, UK). These were anaerobically incubated at 30 $^{o}$C for 48 h.

*6.2.4.2. Bile tolerance*

Overnight cultures of LAB strains were separately inoculated (3%, v/v) (0.3 ml strain into 10 ml sterile MRS broth) (Oxoid) containing 0.3, 0.5 and 1.0% (w/v) oxgall (Difco$^{TM}$ 212820, Sparks MD, USA). At zero minute and after 4 h of incubation at 30$^{o}$C, the cultures from different concentrations of oxgall (Difco), were serially diluted by transferring 1 ml culture into sterile 9 ml quarte strength Ringers solution (Oxoid BR0052G, Basingstoke, UK). Then cultures were spread plated by transferring 0.1 ml on to MRS agar (Oxoid). Resistance to bile salts was expressed as the surviving colony forming units (cfu) on MRS agar (Oxoid) of strains that were exposed to different concentrations of oxgall compared to the control.

*6.2.4.3. Susceptibility to antibiotics*

To determine susceptibility to antibiotics, actively growing LAB strain suspensions with turbidity equivalent to McFarland Standard 1 (approx. 3.0 x $10^{8}$ cfu $ml^{-1}$) (0.1 ml) were separately spread on MRS agar (Oxoid) plates. Disks impregnated with antibiotics; ampicillin 10 µg, amoxicillin 10 µg (inhibitors of bacterial cell wall), tetracycline 30 µg, streptomycin 10 µg (inhibitors of protein synthesis), ciprofloxacin 5 µg and nalidixic acid 30 µg (inhibitors of nucleic acid synthesis) were placed on plates inoculated with the different strains. Inhibition Zones (IZ), including the diameter of the 6 mm disks, was measured after incubation under aerobic conditions for 24 h at 30$^{o}$C. Strains were considered resistant (diameter ≤ 13 mm), moderately susceptible (diameter 14 - 17 mm), or susceptible (diameter ≥ 18 mm), based on IZ. The classification of IZ was based on criteria used by Turchi et al (2013).

*6.2.4.4. Antimicrobial activity*

The antimicrobial activity was determined by the agar well diffusion assay. Thawed LAB isolates were revived separately by transferring 0.1 into sterile 9 ml MRS broth (Oxoid) and incubated for 24 h at 30 $^{o}$C. Actively growing cultures of pathogenic Salmonella typhimurium ATCC 13311 and Escherichia coli ATCC 25922 were obtained by transferring 0.1 ml of thawed culture into sterile 9 ml BHI broth (Mast) and incubated for 24 h at 37 $^{o}$C. A portion (1ml) of the inoculated BHI broth (Mast) was pour plated on Nutrient agar (Oxoid CM0003, Basingstoke UK). Wells (6 mm diameter) were made into the Nutrient agar (Oxoid) using a sterile cork borer. Cell free supernatant (CFS) was obtained by centrifugation of the culture (LAB) at 3000 rpm for 15 min. The pH of the supernatant was adjusted to about 6.5, filter sterilised (using 0.45 µm disk filters) (Prima, V0412101402, Randburg, South Africa), and 60 µl was added into the wells. The plates were then incubated at 30 $^{o}$C for 24-48 h. The antimicrobial activities of LAB were determined in terms of development of inhibition zones around the wells.

### *6.2.5. Statistical analysis*

The experiments were done two times and each measurement was done in duplicate. The means and standard deviations were calculated. Significant differences between the treatments were determined through Analysis of Variance using Statistical Package for Social Sciences (SPSS) for Windows version 20 (SPSS, Chicago, IL). Significant differences were taken for value of $p < 0.05$.

## 6.3. Results and Discussion

### *6.3.1. Growth at low pH*

Seven of the nine tested isolates were able to survive at pH 3 for 3 h (Table 6.2.). The average colony counts for *Lb. plantarum* M2, *Lb. plantarum* M3, *Lb. plantarum* M8 and *Lb. plantarum* M9 at start of experiment and at 3 h were: 9.42 ± 0.03 and 5.37 ± 0.08; 9.38 ± 0.02 and 5.48 ± 0.24; 9.43 ± 0.01 and 5.28 ± 0.07; 9.58 ± 0.06 and 4.35 ± 0.01 log cfu $ml^{-1}$; respectively. Whereas the average log cfu $ml^{-1}$ for *L. lactis* M4, *Lb. acidophilus* M6 and *Leuconostoc pseusomesenteroides* M5 were: 9.43 ± 0.17 and 4.99 ± 0.20; 8.30 ± 0.86 and 3.65 ± 0.12; 9.18 ± 0.19 and 4.32 ± 0.03; respectively. The log cfu $ml^{-1}$ for *Weissella confusa* M7 was 7.30 ± 0.00 at 0 h but the isolate did not grow after 3 h incubation at pH 3. *Lactobacillus acidophilus* M6 had the lowest viable count of 3.65 ± 0.12 cfu $ml^{-1}$ after 3 h, followed by *Leuconostoc pseudomesenteroides* M5 with 4.32 ± 0.03 cfu $ml^{-1}$ and *Lb. plantarum* M9 with 4.35 ± 0.01 cfu $ml^{-1}$. There was a significant decrease ($p < 0.05$) in colony counts for most isolates after 3 h exposure to pH 3.

Most of the isolates (*Leuconostoc lactis* M1, *Lb. plantarum* M2, *Lb. plantarum* M3, *Lb. plantarum* M8, *Lb. plantarum* M9, *L. lactis* M4, *Lb. acidophilus* M6, and *Leuconostoc pseudomesenteroides* M5) were able to survive at pH 3 for 3 hours except *Weissella confusa* M7. The beneficial properties of probiotics can only be realized if LAB are able to survive the low pH in the GIT and transit to the small and large intestines where they adhere and contribute to the functions of the gut flora.

**Table 6.2**

Growth of LAB strains from *emahewu* at pH 3.

| | Average log cfu $ml^{-1}$ ± SD at time (h) [†] | |
|---|---|---|
| Isolate | 0 | 3 |
| *L. plantarum* M2 | 9.42 ± 0.03 | 5.37 ± 0.08* |
| *L. plantarum* M3 | 9.38 ± 0.02 | 5.48 ± 0.24* |
| *L. plantarum* M8 | 9.43 ± 0.01 | 5.28 ± 0.07* |
| *L. plantarum* M9 | 9.58 ± 0.06 | 4.35 ± 0.01* |
| *Lactococcus lactis* M4 | 9.43 ± 0.17 | 4.99 ± 0.20* |
| *L. acidophilus* M6 | 8.30 ± 0.86 | 3.65 ± 0.12* |
| *Weissella confusa* M7 | 7.30 ± 0.00 | No growth |
| *Leuconostoc pseudomesenteroides* M5 | 9.18 ± 0.19 | 4.32 ± 0.03* |

[†]Results are means two experiments ± standard deviations.

Viable counts of strains were compared with that at 0 h, $^{*}p < 0.05$

It is believed that LAB that resist the low pH have a cellular system that transports lactic acid and protons to the cell's exterior (Ramirez-Chavarin et al 2013). In addition some strains are thought to have intrinsic tolerance towards low pH and therefore can pass through the stomach and reach upper part of the intestines. Other studies suggest that ability to survive at low pH by some LAB is due to an increase in ATPase expression when exposed in the presence of acid (Lertworapreecha et al 2011; Reis et al 2016). However, the mechanism is not clearly defined. Finding of the current study are similar to those reported by Turchi et al (2013) who reported that 37 wild *Lb. plantarum* strains from different Italian foods of animal origins were able survive after 30 min at pH 3. Yu et al (2013) observed that *Lb. plantarum* and *Lb. rhamnosus* strains survived at pH 3 after 60 min to 180 min exposure. Similarly, Mathara et al (2008) reported that *Lb. plantarum* tested survived at pH 2 for 2 h exposure, while Jose et al (2015) observed that *Lb. plantarum, Lb. rhamnosus* and *Lb. reuteri* tolerated pH 2 and pH 3. Similar observations were reported Ramirez-Chavarin et al (2013). It appears

therefore that the acid tolerance trait is common in *Lb. plantarum* and *Lb. fermentum* as observed in the current study. Commercial available probiotics also have this ability (Mathara et al 2008).

### *6.3.2. Bile tolerance*

All the isolates survived in the presence of different concentrations of oxgall, i.e. 0.3, 0.5 and 1.0% (v/v) (Table 6.3). There was a significant increase ($P < 0.05$) in colony counts for all isolates after 4 h exposure to 0.3% oxgall.

However, although the bacteria survived at 0.5 and 1% oxgall concentrations, the viable counts did not change significantly. Yu et al (2013) suggested the ability to resist oxgall is related to expression of bile resistance proteins in the bacterial cells. Similarly, Hamon et al (2011) reported that ability to resist oxgall in LAB is related to expression of bile resistance naturally diverse proteins in LAB cells. Sanchez et at (2006), suggested that LAB have enzyme F0F1-ATP synthases. The F0F1-ATP synthases facilitate in pumping the proton during exposure to bile thereby tightly regulating the internal pH (Hamon et al 2011; Lebeer et al 2008; Sanchez et at 2006). Other proposed systems that help *Lb. plantarum* withstand adverse effects of bile salts stresses are the ABC transporters that in a way are major component of efflux systems. These are cellular system components that partake in the transport of harmful-compounds and cell detoxication (Hamon et al 2011; Poolman and Glaasker 1998). Finding of the current study are similar to those reported by Jose et al (2015), Mathara et al (2008) and Turchi et al (2013) where it was found out that *Lb. plantarum* and *Lb. brevis* isolates tolerated effects of 0.3% (w/v) bile.

**Table 6.3.**

Survival of *Leuconostoc lactis, L. plantarum, L. acidophilus*, *Lactococcus lactis Weissella confusa* and *Leuconostoc pseudomesenteroides* in the presence of different concentration of oxgall (log cfu $ml^{-1}$).

| Strains | 0 h[†] | 4 h | | |
|---|---|---|---|---|
| | | 0.3% oxgall | 0.5% oxgall | 1% oxgall |
| *Leuconostoc lactis* M1 | 6.61 ± 0.21 | 6.75 ± 0.15* | 6.94 ± 0.65 | 6.01 ± 0.25 |
| *Lactobacillus plantarum* M2 | 7.85 ± 0.04 | 8.22 ± 0.06* | 8.12 ± 0.01 | 8.14 ± 0.03 |
| *Lactobacillus plantarum* M3 | 7.34 ± 0.10 | 7.24 ± 0.05* | 7.37 ± 0.01 | 7.20 ± 0.12 |
| *Lactobacillus plantarum* M8 | 7.60 ± 0.05 | 7.95 ± 0.01* | 7.70 ± 0.05 | 7.69 ± 0.02 |
| *Lactobacillus plantarum* M9 | 7.62 ± 0.04 | 7.69 ± 0.03* | 7.53 ± 0.02 | 7.57 ± 0.06 |
| *Lactobacillus acidophilus* M6 | 7.92 ± 0.04 | 8.41 ± 0.00* | 8.12 ± 0.03 | 8.13 ± 0.01 |
| *Lactococcus lactis* M4 | 6.50 ± 0.11 | 6.74 ± 0.16* | 6.00 ± 0.15 | 5.70 ± 0.06 |
| *Weissella confusa* M7 | 6.65 ± 0.05 | 6.94 ± 0.16* | 6.55 ± 0.48 | 6.32 ± 0.28 |
| *Leuconostoc pseudomesenteroides* M5 | 6.46 ± 0.14 | 7.14 ± 0.39 | 6.46 ± 0.20 | 6.00 ± 0.11 |

[†]Results are means of two experiments ± standard deviations.

Viable counts of strains at different concentration of oxgall were compared with that at 0 h, $^{*}p < 0.05$

The current results with respect to ability of LAB to tolerate different concentrations of oxgall were also consistent with other studies where it was concluded that LAB survived after 3 – 6 h exposure to 0.3 – 1% oxgall (Owusu-Kwarteng et al 2015; Yu et al 2013). The maximum bile concentration on average in the GIT of health person is 0.3% (Jose et al 2015, Ramos et al 2013). The LAB isolates in this study were able to tolerate oxgall concentration above 0.3% (0.5% to 1%), therefore have the probiotic properties and are likely to survive the harsh conditions of the GIT as probiotics. This is also because it is known that LAB have several strategies to adapt to bile stress (Hamon et al 2011). It appears therefore that the oxgall tolerance trait is common in *Lb. plantarum* and *Lb. acidophilus* as observed in the current study.

### *6.3.3. Susceptibility to antibiotics*

All strains were to some extend susceptible to amoxicillin. Most (8 of the 9) of strains were susceptible to ampicillin except *Lb. acidophilus* M6 that did not show a zone of inhibition (Table 6.4. and 6.5). The following strains were inhibited by tetracycline: *Leuconostoc lactis* M1, *Lb. plantarum* M2, *Lb. plantarum* M3, *L. lactis* M4, *Lb. acidophilus* M6, *Weissella confusa* M7 and *Leuconostoc pseusomesenteroides* M5. However, *Lb. plantarum* M8 *and Lb. plantarum* M9 had zones of inhition of 17 mm and 16.7 mm respectively. This means that *Lb. plantarum* M8 *and Lb. plantarum* M9 were moderately susceptible to tetracycline. All strains were resistant to streptomycin and nalidixic acid and zones of inhibition ranged from 7.5 mm to 13.5 mm for streptomycin, 7 mm to 11.5 mm for nalidixic acid, respectively. The strains *Leuconostoc lactis* M1, *Lb. plantarum* M2, *Lb. plantarum* M3, *Lb. plantarum* M8, *Lb. plantarum* M9 were resistant ciprofloxacin while *L. lactis* M4, *Lb. acidophilus* M6, *Weissella confusa* M7 and *Leuconostoc pseusomesenteroides* M5 were moderately susceptible.

**Table 6.4.**

Inhibition zones (mm) against ampicillin (AP), amoxicillin (A), tetracycline (T), streptomycin (S), ciprofloxacin (CIP) and nalidixic acid (NA) of 9 LAB strains*

| Sample | Strains | AP (10 µg) | A (10 µg) | T (30 µg) | S (10 µg) | CIP (5 µg) | NA (30 µg) |
|---|---|---|---|---|---|---|---|
| Mah 1 | *Leuconostoc lactis* M1 | 30 ± 0.00 | 29 ± 4.24 | 30 ± 0.00 | 9.5 ± 0.71 | 11.5 ± 0.71 | 8 ± 0.00 |
| Mah 2 | *Lactobacillus plantarum* M2 | 24 ± 1.41 | 21 ± 0.00 | 30 ± 1.41 | 10 ± 0.00 | 13 ± 0.00 | 11.5 ± 0.71 |
| Mah 3 | *Lactobacillus plantarum* M3 | 34 ± 1.41 | 26 ± 1.41 | 38.5 ± 2.12 | 13.5 ± 2.12 | 12.5 ± 0.71 | 7 ± 0.00 |
| Mah 8 | *Lactobacillus plantarum* M8 | 37 ± 0.00 | 36.5 ± 2.12 | 17 ± 1.41 | 11.5 ± 2.12 | 7 ± 0.00 | 7 ± 0.00 |
| Mah 9 | *Lactobacillus plantarum* M9 | 28.5 ± 2.12 | 31 ± 1.41 | 16.5 ± 2.12 | 10 ± 4.24 | 11 ± 1.41 | 10.5 ± 2.12 |
| Mah 4 | *Lactococcus lactis* M4 | 34 ± 0.00 | 31.5 ± 0.71 | 35.5 ± 2.12 | 11.5 ± 0.71 | 16.5 ± 0.71 | 7 ± 0.00 |
| Mah 6 | *Lactobacillus acidophilus* M6 | 7.5 ± 0.71 | 29 ± 1.41 | 29 ± 1.41 | 7.5 ± 0.71 | 18.5 ± 2.12 | 7 ± 0.00 |
| Mah 7 | *Weissella confuse* M7 | 30.5 ± 4.95 | 26 ± 2.83 | 28 ± 2.83 | 8 ± 1.41 | 18 ± 1.41 | 8 ± 0.00 |
| Mah 5 | *Leuconostoc pseudomesenteroides* M5 | 31 ± 1.41 | 22 ± 3.54 | 35 ± 2.83 | 9 ± 1.41 | 16.5 ± 2.12 | 8 ± 0.00 |

*R* resistant strains: antibiotic diameter ranges (mm) ≤ 13; *MS* moderately susceptible strains 14 – 17; *S* susceptible ≥ 18 (Turchi et al 2013)

*Results are mean of two experiments ± standard deviation

**Table 6.5.**

The growth pattern of 9 LAB strains to exposure on ampicillin (AP), amoxicillin (A), tetracycline (T), streptomycin (S), ciprofloxacin (CIP) and nalidixic acid (NA) *

| Sample | Strains | AP (10 µg) | A (10 µg) | T (30 µg) | S (10 µg) | CIP (5 µg) | NA (30 µg) |
|---|---|---|---|---|---|---|---|
| Mah 1 | *Leuconostoc lactis* M1 | S | S | S | R | R | R |
| Mah 2 | *Lactobacillus plantarum* M2 | S | S | S | R | R | R |
| Mah 3 | *Lactobacillus plantarum* M3 | S | S | S | MS | R | R |
| Mah 8 | *Lactobacillus plantarum* M8 | S | S | MS | R | R | R |
| Mah 9 | *Lactobacillus plantarum* M9 | S | S | MS | R | R | R |
| Mah 4 | *Lactococcus lactis* M4 | S | S | S | R | MS | R |
| Mah 6 | *Lactobacillus acidophilus* M6 | R | S | S | R | S | R |
| Mah 7 | *Weissella confuse* M7 | S | S | S | R | S | R |
| Mah 5 | *Leuconostoc pseudomesenteroides* M5 | S | S | S | R | MS | R |

*R* resistant strains: antibiotic diameter ranges (mm) ≤ 13; *MS* moderately susceptible strains 14 – 17; *S* susceptible ≥ 18 (Turchi et al 2013)

*Results are mean of two experiments ± standard deviation

Lactobacilli are able to resist antibiotics because they have antibiotics specific genes (Hummel et al 2007). This intrinsic property is displayed during resistance to aminoglycosides, quinolones, glycopeptides, streptomycin, gentamicin and ciprofloxacin. The resistance by LAB strains to aminoglycoside antibiotics (gentamicin and streptomycin) is referred to as intrinsic because it occurs in the absence of cytochrome-mediated electron transport. The electron transport system mediates drug uptake. The LAB are able to resist some antibiotics through varying membrane permeability. In some *Lactobacillus* species have specific antibiotic resistance genes such as tetracycline resistance genes and β-lactam resistance genes. In addition in *Lactobacillus* species some erythromycin resistance genes and streptogramins resistance genes have been reported (Hummel et al 2007; Lin et al 1996). Furthermore Lactobacilli have a mechanism to resist antibiotics because the strains have mediated multidrug resistance transporters (Gueimonde et al 2009). Other previous studies have also pointed out that *Lactobacillus* sp. do possess intrinsic or natural resistance to certain antibiotics (Yu et al 2013).

The findings of the current study are similar to those reported by Gueimonde et al (2009). These researchers reported that *Lactobacillus* species were sensitive to cell wall targeting penicillin and β-lactam (such as penicillin derivatives, amoxicillin, ampicillin, methicillin, oxacillin, carbepenems and monobactams) but quite resistant to cephalosporins (that include cephalothin, cefamandole and cefotaxime). Most Lactobacilli have been shown to exhibit high level of resistance to vancomycin and antibiotics that inhibits nucleic acid synthesis (Gueimonde et al 2009). Hummel et al (2007) too observed very low resistance (< 7%) to erythromycin, chloramphenicol, tetracycline or β-lactam. By contrast, the resistances by LAB to aminoglycoside (gentamicin and streptomycin) and ciprofloxacin were higher than 70%,

indicating that these responses may constitute intrinsic resistances. Furthermore, Jose et al (2015) highlight that all LAB strains (from dairy food and animal rumen) were resistant to streptomycin, ciprofloxacin and nalidixic acid. In addition some LAB isolates from dairy food and animal rumen were susceptible and some intermediately susceptible to tetracycline, while majority of the isolates were susceptible to ampicillin (Jose et al 2015). Other research results are in line with the current results. It was demonstrated previously that *Lb. plantarum* and *Lb. rhamnosus* GG were sensitive to erythromycin, chloromycetin, penicillin and rifampicin. The same strains were resistant to kanamycin, vancomycin, polymyxin B, streptomycin and gentamicin (Mathara et al 2008; Owusu-Kwateng et al 2015; Yu et al 2013). Although the study by Owusu-Kwateng et al (2015) was on *Lactobacillus* of species *fermentum*, the results are similar to the current findings. Some strains of *Lb. fermentum* were resistant to antibiotics that inhibit protein synthesis such as streptomycin, kanamycin and gentamicin. Mathara et al (2008) pointed out that eleven out of sixteen *Lb. plantarum* were sensitive to ampicillin and tetracycline but resistant to streptomycin. The LAB isolates in the current study have shown some resistance against some antibiotics indicating that they may possess some properties that could be beneficial to health of consumers.

### *6.3.4. Antimicrobial activity*

All the LAB strains were to some extent inhibitory against the pathogenic bacteria *Salmonella typhimurium* ATCC 13311 and *Escherichia coli* ATCC 25922 (Table 6.6.). The following strains exhibited highest inhibition zones against *S. typhimurium* ATCC 13311: *Lb. plantarum* M2, *L. lactis* M4 and *Lb. acidophilus* M6. However, *Leuconostoc pseudomesenteroides* M5 exhibited lowest inhibition zones against *S. typhimurium* ATCC 13311. Whereas, strain *Weissella confusa* M7 exhibited highest inhibition zones against *E.*

*coli* ATCC 25922. The following strains exhibited moderate antimicrobial activity towards *E. coli* ATCC 25922: *Leuconostoc lactis* M1, *Lb. plantarum* M2, *Lb. plantarum* M3, *Lb. plantarum* M8, *Lb. plantarum* M9, *L. lactis* M4, *Lb. acidophilus* M6 and *Leuconostoc pseudomesenteroides* M5. The antimicrobial activity of different supernatants from *Leuconostoc lactis* M1, *Lb. plantarum* M2, *Lb. plantarum* M3, *Lb. plantarum* M8, *Lb. plantarum* M9, *L. lactis* M4, *Lb. acidophilus* M6 and *Weissella confusa* M7 marginally decreased when the supernatant was not neutralised to pH about 6.5 using 1M NaOH. The neutralised and not neutralised supernatant of *Leuconostoc pseudomesenteroides* M5 had similar antimicrobial activity against *S. typhimurium* ATCC 13311.

Supernatant from *Lb. plantarum* strains have been reported to exhibit some antimicrobial activities against pathogens due to production of organic acids, production of bacteriocins, analogous bacteriocins and other inhibitory metabolites (Reis et al 2016; Yu et al 2013). The antimicrobial activity of the LAB isolates in the current study might have been due to the presence of bacteriocins, analogous bacteriocins and other inhibitory metabolites.

These findings of the current study are in line with the findings reported by Jose et al (2015) and Yu et al (2013) whereby it was reported that supernatant from *Lb. plantarum* strains had shown some antimicrobial activities against pathogens such as *E. coli* 0157 and *S. flexneri* CMCC(B). Other strains of *Lb. plantarum* exhibited some antimicrobial activity against *S. typhimurium* S50333. Some Lactobacilli isolates have exhitited some antimicrobial activity against pathogens such as *Salmonella menston*, *Listeria monocytogenes*, *Enterobacter aerogenes* and *Staphylococcus aureus* (Jose et al 2015). In previous work, Essid et al (2009)

**Table 6.6.**

Antimicrobial activity profile of LAB strains against *Salmonella typhymurium* and *Escherichia coli*.

| Isolate[†‡] | *Salmonella typhymurium* ATCC 13311 | | *Escherichia coli* ATCC 25922 | |
|---|---|---|---|---|
| | Neutralised supernatant | Not neutralised supernatant | Neutralised supernatant | Not neutralised supernatant |
| *Leuconostoc lactis* M1 | ++ | + | ++ | + |
| *L. plantarum* M2 | +++ | + | ++ | + |
| *L. plantarum* M3 | ++ | + | ++ | + |
| *L. plantarum* M8 | ++ | + | ++ | + |
| *L. plantarum* M9 | ++ | + | ++ | + |
| *Lactococcus lactis* M4 | +++ | + | ++ | + |
| *L. acidophilus* M6 | +++ | + | ++ | + |
| *Weissella confusa* M7 | ++ | + | +++ | ++ |
| *Leuconostoc pseudomesenteroides* M5 | + | + | ++ | + |

[†]+ Inhibition zone ≤ 2 mm, ++ inhibition zone between 3 and 4 mm, +++ inhibition zone between 5 and 7 mm.

[‡]Results are mean two experiments

also observed that different strains of *Lb. plantarum* from salted meat do display different antimicrobial activity towards such pathogen as *S. aureus, S. arizonae, E. coli* and *Pseudomonas aeuroginosa*. The LAB isolates in the current study have shown some inhibitory effect against *Salmonella typhimurium* ATCC 13311 and *Escherichia coli* ATCC 25922 that may suggest that the isolates have some probiotic properties that could be beneficial to health of consumers.

### *6.3.5. Conclusions*

This study indicates that LAB are able to tolerate low pH (3) exposure for 3 h. The LAB isolates exhibited tolerance to different concentration of bile salts (0.3, 0.5 and 1%) for a period ≥ 4 h. Most LAB isolates were found sensitive to antibiotics that inhibit bacterial cell wall synthesis and resistant to antibiotics that inhibit the synthesis of nucleic acids. The different LAB isolates tested in the current study showed different responses (some were resistant and others were susceptible) to antibiotics that inhibit the synthesis of proteins. The LAB have some antimicrobial activity against *S. typhimurium* ATCC 13311 and *E. coli* ATCC 25922. From the properties studied, it can be concluded that LAB have significant probiotic properties that could be beneficial to health of consumers.

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# CHAPTER 7: THE SAFETY OF SWAZI TRADITIONAL FERMENTED FOODS, *EMASI* AND *EMAHEWU*, FOCUSSING ON *ENTEROCOCCUS* SSP.


## Abstract

Occasionally, high numbers of *Enterococcus* spp have been detected in some Southern African fermented foods. The presence of *Enterococcus* spp in foods is associated with poor hygienic practices and may indicate a potential risk to consumers who regularly consume the foods. The *Enterococcus* spp in traditionally fermented milk (*emasi*), non-alcoholic cereal beverage (*emahewu*) were isolated on MacConkey agar. Typical colonies were isolated, purified and phenotypically representative strains were identified by sequencing the 16S rDNA. The colony counts in *emasi* ranged from not detectable to 8.58 log cfu/ml while *emahewu* ranged from not detectable to 6.72 ± 0.10 log cfu/ml. Five isolates from *emasi* were identified as *Enterococcus durans, Enterococcus hirae, and Enterococcus lactis*. One isolate from *emasi* was *Enterococcus durans and Enterococcus lactis*. Despite concerns raised by earlier obseravtions on coliform counts, these observations suggest that the fermented foods maybe safe for consumption since the strains recovered are known to be non pathogenic.

## 7.1. Introduction

Normally fermented foods are known to be safe for consumers regardless of where they are made, including developing world where people who make them may not have any training in microbiology or, food hygiene (Steinkraus 2002). This is because in one way desirable microorganisms in fermented foods overgrows and resist invasion by spoilage, and even food poisoning microorganisms. In other cases the environment is not conducive for growth of undesirable microorganisms due to the production of lactic acid by fermenting microorganisms which lowers the pH of the fermented product below 4.5.

Occassionally high numbers of *Enterococcus* spp have been identified in some fermented foods. In particular *Enterococcus* spp have been reported in milk and milk products (Collison and Jonnes 1999). This genus of lactic acid acid bacteria have been found to be present in some fermented food because they tolerate wide range of unfavourable conditions such as moderate heat treatment (63.5 $^{o}$C for 30 min), low temperatures (psychrotropic behaviour), low pH and have good ability to utilise numerous substrates for growth during harsh conditions (Chajęcka-Wierzchowska et al. 2017; Collison and Jonnnes 1999; Trivedi and Karpišková 2008). Some *Enterococcus* spp are resistant to wide number of antibiotics such as chlorampenical and vancomycin (Oguntoyinbo and Okueso 2013). In some dairy fermented products the presence of *Enterococcus* spp is associated with poor hygiene from the milking of cows to fermentation and other processing stages that are applied on milk as raw materials (Trivedi and Karpišková 2008).

The safety implication of the presence of *Enterococcus* spp in fermented foods is because they have been reported to be opportunistic pathogens in immunocompromised hosts (Chajęcka-Wierzchowska et al. 2017). While some posse's intrinsic resistance to a number of antimicrobial agents and some resistance to antibiotics. In addtion *Enterococcus* spp have the potential to cause blood pressure, headache, urticarial, nausea and vomiting in sensitive people (Trivedi and Karpišková 2008).

Other safety concerns of Swazi traditionally fermented foods (*emasi* and emahewu) may be attributed to presence of coliforms in some fermented foods. High numbers of coliforms have been detected in some fermented foods. The presence of coliforms in fermented foods is associated with poor hygienic practices (Mugochi et al 1999) during preparation, handling and storage or sampling. Coliforms are part of the Enterobacteriaceae family of spoilage and pathogenic microorganisms, including *Escherichia coli* and *Samonella* sp. Gran et al (2002) reported the presence of *Escherichia coli* in amasi produced by small holder farmers in Zimbabwe. In that study 39% of the samples had more than $10^3$ cfu/ml of *Escherichia coli*. The reason given to account for high counts was poor hygiene and poor handling of milk at the farms. The actual numbers of coliform counts in the the fermented milk were between 9.3 x $10^4$ and 5.7 x $10^4$ cfu/ml (Gran et al 2002). In another study on naturally soured milk, out of 12 samples, 2 had enteropathogenic *E. coli* 0157:H7 (cited by Gadaga et al 2013). Other related studies that were conducted to establish if low pH < 4.5, can inhibit growth of pathogens like *Salmonella* spp. and *Escherichia coli*, observed that the numbers that survived after 48h in spontaneously fermented milk were unacceptably high.

Mutukumira (1995) also reported that *amasi* had coliforms counts ranging from 3.41 to 6.90 log cfu/g. Saleh (2013) enumerated Enterococcus in Laban Zeer (an Egyptian traditional fermented milk product) and recorded average values of 4.67 log cfu/g.

However, Schoustra et al (2013) in their pilot study on *mabisi* (traditionally fermented milk in Zambia) found out that naturally fermenting LAB grew normally in the presence of some pathogens (*Listeria innocua*, *Escherichia coli* and *Staphylococcus epidermidis*) even when the inoculum of pathogens was at $10^6$ cfu/g. Possible mechanisms could be due to low pH, production of bacteriocins and other anticompetitor toxins by LAB. Contrarily Katongele (2008) pointed out that in mahewu they could not find any Enterobacteriaceae by the end of processing stage. In some preliminary studies, presumptive poor hygiene indicator microorganisms in Swaziland traditional fermented foods have been isolated. High viable counts were observed on selective MacConkey agar, hence the need to establish levels of safety of Swaziland fermented foods with a broad view to identify and characterise these microorganisms in these foods.

The objective of this study was to evaluate the safety of *emasi* and *emahewu*, Swazi traditional fermented foods. This will help in further developing the safe traditional products.

## 7.2. Materials and Methods

### *7.2.1. Location of study*

The current study was conducted in one of the four regions of Swaziland. Samples wre collected from Hhohho region of Swaziland.

### *7.2.2. Sampling*

Hhohho region is divided into some local administrations called *tinkhundla*. There are 14 *tinkhundla* in Hhohho region. Samples were collected from 5 *tinkhundla* that were randomly selected from the 14 *tinkhundla.* The samples were collected in sterile screw capped bottles and ferried in a cooler box to the laboratory at the University of Swaziland for analysis.

### *7.2.3. Microbiological analysis*

#### *7.2.3.1. Growth of Enterococcus* spp. *on MacConkey agar*

The fermented samples were analysed immediately upon receipt at the laboratory. Total coliforms were enumerated on MacConkey agar (Oxoid CM0007, Basingstoke, UK) using spread plate technique and incubating at 37$^{o}$C for 24-48 h. [MacConkey agar (Oxoid) is selective differential media that inhibits the growth of gram positive bacteria but promotes growth of gram negative bacteria mainly due to presence of crystal violet and bile salts. MacConkey agar (Oxoid) also contains other additives such as neutral red (a pH – indicator) and lactose (a disaccharide) that make it differential. It is primarily used for detection and isolation of members of family enterobacteriaceae and *Pseudomonas*, MacConkey's Agar 2016. The bile salts in MacConkey agar (Oxoid) inhibit most gram positive bacteria, except

*Enterococcus* and some *Staphylococcus* spp. Vlab.amarita.edu. 2016]. Typical coliforms were detected as those with a deep red centre and a translucent halo around them.

### *7.2.3.2. Isolation and selection of viable Enterococcus spp.*

Colonies with deep red centre and a translucent halo around them were picked from MacConkey agar (Oxoid) and purified by streaking on a fresh MacConkey agar (Oxoid) plate. The purification process was repeated until single colonies with distinct appearances were obtained. The isolates from viable microogainsms were stored frozen at -28 $^{o}$C in BHI broth (Mast) containing 20% glycerol (L&T Diagnostics CC 090307, Johannesburg, South Africa) until required for the tests.

### *7.2.3.3. Identification of viable Enterococcus spp by sequencing the 16S rDNA*

The identification was done at Inqaba Biotec Laboratories, South Africa. Briefly, DNA was extracted using ZR Fungal/Bacteria DNA Kit$^{TM}$ (Zymo Research). The 16S rDNA target region was amplied using DreamTaq$^{TM}$ DNA polymerase (Thermo Scientific$^{TM}$) and the primers 16S-27F, sequence 5′-AGAGTTTGATCMTGGCTCAG-3′ and 16S-1492R, sequence 5′-CGGTTACCTTGTTACGACTT-3′. PCR products were gel extracted (Zymo Research, Zymoclean$^{TM}$ Gel DNA Recovery Kit), and sequenced in the forward and reverse directions on the ABI PRISM$^{TM}$ 3500xl Genetic Analyser. Purified sequencing products (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit$^{TM}$) were analysed using CLC Main Workbench 7 followed by a BLAST search (NCBI) (Altschul et al 1997).

### *7.2.4. Statistical analysis*

The experiments were done two times and each measurement was done in duplicate. The arithmetic means and standard deviations were calculated.

## 7.3. Results and Discussion

### *7.3.1. Growth of Enterococcus* spp. *on MacConkey agar*

The average *Enterococcus* counts of *emasi*, Swazi naturally fermented milk ranged from not detectable to 8.58 ± 0. 07 log cfu/ml (Table 7.1.), while those in *emahewu*, ranged from not detectable to 6.72 ± 0.10 log cfu/ml (Table 7.2.).

The presence of *Enterococcus* ssp. in fermented products is attributed to poor general hygienic practices (GHP) such as sanitation programmes, personal hygiene and preparation processes (Trivedi and Karpišková 2008). In addition, *Enterococcus* ssp. are know to be pschrotropic in nature, have high heat resistance and generally adapt well to different substrates and growth conditions. *Enterococcus* ssp. has been reported to increase in number during cooling. Other sources of *Enterococcus* ssp. into the products may have been due to use of partially cleaned preparation containers and utensils. These products are prepared at homestead mostly by women who have had no form of training in hygiene. The women who prepare *emasi* and *emahewu* are involved in other household tasks such as child care and cleaning the surroundings. During interchange of tasks there is no guarantee that good hygiene practices is observed (Mortarjemi and Nout (1996). This may have led to contamination of the products.

**Table 7.1.**

The viable *Enterococcus* spp. counts of *emasi*, Swazi naturally fermented milk.

| **Sample code** | **Viable *Enterococcus* spp. counts (log cfu/ml)** |
|---|---|
| MOT-emasi | 7.91 ± 0.06 |
| Nt-emasi-1 | 8.58 ± 0.07 |
| Nt-emasi-2 | 8.43 ± 0.09 |
| L-emasi | 4.13 ± 0.04 |
| M-emasi | Not detectable |
| Mb-emasi 1 | 7.19 ± 0.10 |
| Mb-emasi 2 | 6.48 ± 0.10 |
| Mb-emasi 3 | 7.26 ± 0.07 |
| Mb-emasi 4 | 5.42 ± 0.03 |
| Mb-emasi 5 | Not detectable |
| Mb-emasi 6 | Not detectable |
| Mb-emasi 7 | 6.49 ± 0.11 |
| Mb-emasi 8 | 6.45 ± 0.04 |
| Mb-emasi 9 | 5.60 ± 0.03 |

**Table 7.2.**

The viable *Enterococcus* spp. counts of *emahewu*, a Swazi non-alcoholic fermented beverage.

| **Sample code** | **Viable *Enterococcus* spp. counts (log cfu/ml)** |
|---|---|
| L-emah-1 | 6.66 ± 0.20 |
| L-emah-2 | 6.72 ± 0.10 |
| L-emah | 6.59 ± 0.08 |
| Lemah-3 | 4.43 ± 0.07 |
| Nt-emah | 5.69 ± 0.20 |
| Z4-emah-1 | 6.36 ± 0.10 |
| L-emah-20 | Not detectable |
| L-emah-21 | 4.22 ± 0.18 |
| L-emah-22 | 4.56 ± 0.30 |
| L-emah-23 | 3.65 ± 0.10 |
| L-emah-24 | 4.43 ± 0.07 |
| L-emah-25 | 4.26 ± 0.05 |
| L-emah-26 | 5.17 ± 0.09 |
| L-emah-27 | 4.67 ± 0.30 |
| Ez-emah | 4.15 ± 0.21 |
| Ez-emah-2 | 5.41 ± 0.07 |
| Mv-emah | 4.69 ± 0.12 |
| M-emah | Not detectable |
| Fonteyn-emah | 4.65 ± 0.13 |
| Fonteyn-emah-2 | 5.08 ± 0.06 |
| Mangw-emah | Not detectable |
| Mangw-emah-2 | 4.85 ± 0.14 |
| Mangw-emah-3 | 4.88 ± 0.16 |
| Nkoy-emah | 5.38 ± 0.20 |

The findings of the current study are similar to Dobranić et al (2016) where it was observed that total viable counts; psychrophilic bacteria, lactic acid bacteria, staphylococci, *Escherichia coli*, enterococci, enterobacteria and listeria spp. were isolated in milk originating from healthy cows and antibiotic-treated cows. The log CFU/ml for enterococci in milk from healthy cows ranged from <1 – 3.6 and in milk from treated cows ranged from <1 – 5.

### *7.3.2. Identification of viable Enterococcus spp. by sequencing the 16S rDNA*

The isolates were identified to species level by sequencing the 16S rDNA (Table 7.3.). The isolates were obtained from colonies that had deep red centre and a translucent halo around them. Five isolates from *emasi* were *Enterococcus durans, Enterococcus hirae, and Enterococcus lactis*. One isolate from *emasi* was *Enterococcus durans and Enterococcus lactis*.

**Table 7.3**.

The name and code for the isolates.

| Name | Code |
|---|---|
| *Enterococcus durans, Enterococcus hirae, Enterococcus lactis* | EM1 (emasi) |
| *Enterococcus durans, Enterococcus lactis* | EM2 (emasi) |
| *Enterococcus durans, Enterococcus hirae, Enterococcus lactis* | EM3 (emasi) |
| *Enterococcus durans, Enterococcus hirae, Enterococcus lactis* | EM4 (emasi) |
| *Enterococcus durans, Enterococcus hirae, Enterococcus lactis* | EM5 (emasi) |
| *Enterococcus durans, Enterococcus hirae, Enterococcus lactis* | EM6 (emasi) |

The medium MacConkey agar (Oxoid) is a selective medium used for enumeration of gram negative bacteria (MacConkey'agar 2016). The gram negative bacteria include indicators of poor hygiene such as coliforms. The group coliforms include at least four genera such as *Escherichia*, *Klebsiella*, *Citrobacter* and *Enterobacter* (Mclandsborough 2005). The differential selectivity of MacConkey agar is due to medium ingredients such as bile salts, crystal violet dye, lactose and neutral pH red indicator. However, bile salts inhibit most gram positive bacteria, except *Enterococcus* and some *Staphylococcus* spp (Vlab.amarita.edu. 2016). In the current study none of the most common members of coliforms genera were found, but *Enterococcus* spp. The *Enterococcus* spp were able to grow on MacConkey agar (Oxoid) because the bile salts in the medium although inhibit from most gram positive bacteria does not inhibit growth of *Enterococcus* spp. The presence of *Enterococcus* spp in milk and milk products have been reported in some studies. Collison and Jonnnes (1999) observed that due to improper sanitary conditions during the handling and processing of milk sold in Gaborone (Botswana), *Enterococcus* spp were present in milk. The enterococci counts

of in milk ranged from 0 to 1.0 x$10^4$ cfu/ml (0 to 4.0 log cfu/ml). The presence of enterococci in milk was associated with the ability of enterococci to tolerate unfavourable conditions such as moderate heat treatment (survive at 63.5$^o$C for 30 min), low temperature and post contamination after processing (Chajęcka-Wierzchowska et al. 2017; Collison and Jonnnes 1999; Trivedi and Karpišková 2008). During the traditional preparation of Swazi *emasi*, raw milk is used. The fermentation process occurs naturally under room temperature. This therefore, is a conducive condition for the enterococci to survive as they can tolerate low pH and can utilise different substrates for growth during harsh growth conditions. The commonest strains isolated from Botswana milk by percentage were *Enterococcus faecalis* (72%), *Enterococcus faecium* (14%), *Enterococcus durans* (9%) and *Enterococcus lactis* (5%). The presence of enterococci compromises the quality of the product because some enterococci are opportunistic pathogens in immunocompromised hosts (Chajęcka-Wierzchowska et al (2017). Besides, some enterococci have been reported to posses intrinsic resistance to a number of antimicrobial agents. Some are known to acquire resistance to antimicrobials.

Trivedi and Karpišková (2008) also reported the presence of *Enterococcus faecium* as the main strains in several dairy products in their study in Zagreb, Croatia. The commonest strains isolated from dairy products by percentage were *Enterococcus faecalis* (53.3%), *Enterococcus faecium* (26.7%), *Enterococcus casseliflavus* (8%), *Enterococcus durans* (4%), *Enterococcus mundtii* (4%), *Enterococcus raffinosus* (2.7%), and *Enterococcus malodoratus* (1.3%). The presence of enterococci in these dairy products was attributed to poor hygiene starting from milking stage up to processing stage. The other reason is their psychotropic in behaviour, heat resistance and their ability to utilise different substrates for growth under

unfavourable conditions. Their presence in dairy food is undesirable because enterococci can decarboxylase tyrosine leading to production of tyramine in fermented foods. The of presence tyramine and histamine causes illnesses such as blood pressure, headache, urticarial, nausea and vomiting in sensitive people (Trivedi and Karpišková 2008).

Similarly Oguntoyinbo and Okueso (2013) reported the presence of enterococci in Nigerian traditional fermented dairy foods, *nunu* and *wara*. The average viable counts from *nunu* were 4.17 log cfu/g and 4.55 log cfu/g for *wara*. The common strains of enterococci identified were *Enterococcus faecium* (84%) and *Enterococcus faecalis* (16%). With respect to safety of *nunu* and *wara* four (13.3%) of the strains were found to be sensitive to chloramphenicol (30 µg) but 33.7% of the total strains were resistant to 5 µg of vancomycin. The antibiotic resistance profile is necessary to ensure that the strains that are resistant to some antibiotics do not transfer drug resistant genes to some pathogens. Futhermore, *Enterococcus* spp. were also isolated and identified from powdered infant formula and follow-on formulas (TaeMi and JongHyun 2012). It was observed that > 90% of *Entorococcus* in powdered infant formula and follow-on formulas were identified as *Enterococcus faecium, Enterococcus casseliflavus and Enterococcus faecalis* (TaeMi and JongHyun 2012). The safety of the products was evaluated by looking at the susceptibility of *Enterococcus* spp. to some antibiotics. All enterococci were susceptible to ampicillin, penicillin, tetracycline and vancomycin. The enterococci isolates were susceptible to erythromycin, ripampin and streptomycin.

Dobranić et al (2016) investigated if drinking raw milk posse as potential risk due to the presence of foodborne pathogens. They found that enterococci were present in the raw milk and the common strains were *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus durans*. The average bacterial counts for Enterococci was <1-3.6 log cfu/ml (Health cows) and <1-5.0 log cfu/ml (Treated cows). The Enterococci counts in treated cows were higher than in health cows this may to to the fact that some Enterococci strains had developed resistance to some antibiotics.

### *7.3.3. Conclusions*

The viable microorganisms on MacConkey agar (Oxoid) were identified as *Enterococcus durans, Enterococcus hirae, Enterococcus lactis* (from *emasi*), *Lactobacillus plantarum* and *Lactococcus lactis* (from *emahewu*). The Swazi traditional fermented foods maybe safe for consumption since the strains recovered are known to be non pathogenicproducts.

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# CHAPTER 8: GENERAL DISCUSSION

## 8.1. Summary of study investigations and findings

The purpose of the present study was to document the preparation methods of *emahewu* (maize based non-alcoholic beverage), *emasi* (fermented milk), *umcombotsi* (alcoholic beverage) and *buganumarula* (fermented fruit mashes wine) at household level in Swaziland. The study further focused on the enumeration, isolation and identification of lactic acid bacteria (LAB) (by sequencing 16SrDNA) in *emasi* and *emahewu which are popular* Swazi traditional fermented foods. LAB were enumerated, isolated and identified, from samples that were collected from households in Hhohho region of Swaziland. The probiotic properties of LAB in Swazi traditional fermented foods focusing on *emahewu* were evaluated in terms of several parameters, including growth under low pH, bile tolerance, susceptibility to antibiotics and antimicrobial activity of LAB. The safety of *emasi* and *emahewu* to consumers was investigated.

The detailed fermentation steps were documented for *umcombotsi*, *emahewu*, *buganu* and *emasi*. With reference to preparation procedures and practices, all respondents indicated that they had prepared different fermented foods at one time or another. The most regularly prepared and readily available traditional fermented foods were *umcombotsi* (alcoholic beverage), *emahewu* (non-alcoholic beverage), *buganu* (*marula* wine), and *emasi* (spontaneously fermented milk). Both gender (men and women) responded that they prepared *umcombotsi*, and only women stated that they prepared *emahewu*, *buganu*, and *emasi*. *Umcombotsi* was essentially prepared for sale, while *buganu*, *emahewu* and *emasi* were for sale as well as household consumption. *Umcombotsi* was generally prepared by mixing maize

meal, un-milled sorghum malt (*magayiwe*), and brown sugar (3 kg) in water (20 L). The initial stage involved the cooking the mixture to gelatinise the starch. This was followed by fermentation at ambient temperature (25-30$^{o}$C) for about 72 h. Preparation of *Umcombotsi* takes about 4 to 5 days. *Emahewu* was prepared by mixing maize meal (1 kg) with water (ca. 5L) and then cooking to make a soft porridge. The cooled porridge was left to ferment at room temperature (25-35$^{o}$C). Some women who prepared *Emahewu* reported adding sugar or a pilled potato to aid the fermentation process. *Emasi* was prepared by letting raw milk to naturally ferment at room temperature in either metal or plastic containers (buckets) for 2 to 3 days. *Buganu* was prepared from marula fruit (*amaganu*) juice and pulp mixed with water (ca. 10 L) and sugar (ca. 2 kg). The mixture was allowed to ferment at ambient temperature for about 3 days, sieved and then served. *Umcombotsi*, *emahewu*, *buganu* and *emasi* were the fermented foods commonly prepared at household level in the Hhohho region, Swaziland. The main ingredient used for preparing *umcombotsi* and *emahewu* was maize meal. Un-milled sorghum malt was also added during preparation of *umcombotsi*. The methods of preparation of *umcombotsi* were in many ways similar to the traditional preparation of *Sesotho joala* (Traditional fermented beer from Lesotho) reported by Gadaga et al (2013). Other traditional fermented beers similar to *umcombotsi* are *doro*, a sorghum beer from Zimbabwe, (Gadaga et al 1999; Benhura and Chingombe 1989) and *Umqombothi* fermented sorghum beer in South Africa (Novellie 1966; Katongele 2008). The preparation of Swazi *emahewu* is similar to *mahewu/mageu* (South Africa sorghum or millet malt beverage (Katongele 2008). The preparation of Swazi *emasi* was similar to that practiced in many Southern African countries in preparing *amasi*, *sethemi*, *mafi*, or *madila* (Gadaga et al 1999; Beukes et al 2001; Kebede et al 2007; Gadaga et al 2013; Ohiokpehai and Jagow 1998).

The home-made *emasi* was found to have an average pH of 4.68 and TA of 0.89%. The LAB counts were 8.25 log cfu/mL. Similarly, *Emahewu*, a maize based non-alcoholic beverage had pH of about 3.62 and TA of 0.43%. This product had LAB counts of 8.10 log CFU/mL.

The LAB counts in all two products were consistent with observations for similar African fermented foods. The LAB from *emasi* and *emahewu* were identified through profiling using Gram stain, catalase reaction, sugar assimilation tests using API 50 CH test strips and through sequencing the 16S rDNA. Nine morphologically different isolates were identified from *emasi*, while sixteen (16) were identified from *emahewu*. It was found that *Lactococcus lactis* subsp. *lactis* and *Leuconostoc mesenteroides* were the common strains in *emasi*. *Lactobacillus plantarum, Lactobacillus paracasei* ssp. *paracasei* and *Lactobacillus brevis* were also detected. In *emahewu, Lactobacillus plantarum* were the most common strains, followed by *Leuconostoc mesenteroides* ssp. *mesenteroides, Lb. fermentum* and *Lb. brevis, Wessella confusa, Lactobacillus acidophilus* and *Lactococcus lactis*. It was concluded that the microflora found in Swazi traditional fermented foods was similar to that found in fermented South African milk where the common genera were *Leuconostoc, Lactococcus* and *Lactobacillus* (Buekes et al 2001). The strains in *emahewu* compares well with other similar products like *ting* (South African spontaneously fermented sorghum non-alcoholic beverage) where the main strains were *Lactobacillus plantarum, Lactococcus lactis, Lactobacillus fermentum, Lactobacillus rhamnsosus, Weissella cibaria*, and *Enterococcus faecalis* (Madoroba et al 2011). The carbohydrate metabolism of LAB is this study compares well LAB isolated from Ethiopian naturally fermented buttermilk and from Sudanese fermented Camel's milk (Negussie et al (2016). These LAB were able to utilise carbohydrates namely

galactose, maltose, glucose, fructose, mannose, lactose, trehalose, meliobiose, xylitol and sorbose.

Sixteen LAB isolates belonging to *Leuconostoc lactis, Lactobacillus plantarum, Lb. acidophilus, Weissella confusa, Lactococcus lactis* and *Leuconostoc pseudomesenteroides* were tested for ability to grow at low pH, in presence of bile, antibiotics, and for their antimicrobial properties. All the strains were able to survive at pH 3. *Leuconostoc lactis, Lb. plantarum, Lb. acidophilus, L. lactis, Weissella confusa* and *Leuconostoc pseudomesenteroides* grew in the presence of 0.3, 0.5, and 1.0% (w/v) oxgall bile. All isolates were sensitivity to ampicillin, amoxicillin and tetracycline. However, *Lb. acidophilus* was resistant to ampicillin, and a strain of *Lb. plantarum* (M9) was moderately susceptible to tetracycline. All isolates showed resistance to streptomycin, ciprofloxacin and nalidixic acid, while *Weissella confusa* and *Lb. acidophilus* were moderately susceptible to ciprofloxacin. Neutral supernatant from each of the actively growing cultures of the isolates were inhibitory to *Salmonella typhimurium* ATCC 13311 and *Escherichia coli* ATCC 25922. Similarly, Mathara et al (2008) reported that *Lb. plantarum* tested survived at pH 2 for 2 h exposure. Finding of the current study are similar to those reported by Jose et al (2015), Mathara et al (2008) and Turchi et al (2013) where it was found out that *Lb. plantarum* and *Lb. brevis* isolates tolerated effects of 0.3% (w/v) bile. With respect to susceptibility to antibiotics the results in the current are to conclusions drawn by Jose et al (2015), Mathara et al (2008) and Owusu-Kwateng et al (2015). Lactic acid bacteria have been reported to possess antimicrobial activity against pathogens (Yu et al 2013).

The viable counts of *Enterococcus* spp. colonies (from colonies with deep red centre and a translucent halo around them on MacConkey agar - Oxoid) for *emasi*, ranged from not detectable to 8.58 ± 3.10 log cfu/ml and not detectable to 6.72 ± 0.10 log cfu/ml from *emahewu*. Five isolates from *emasi* were identified as *Enterococcus durans, Enterococcus hirae, and Enterococcus lactis*. One isolate from *emasi* was *Enterococcus durans and Enterococcus lactis*. Despite concerns raised by earlier observations on coliforms counts, the current research suggest that the traditional fermented Swazi foods maybe safe for consumption since the strains recovered are known to be non pathogenic. The findings of the current study are similar to other studies in milk based fermented products. Enterococci have been reported in raw milk and fermented milk products (Collison and Jonnnes 1999; Chajęcka-Wierzchowska et al 2017). Therefore, the fermented foods are safe to consumers, but the presence of enterococci in *emasi* may pose a health risk to immunocomprimised consumers.

## 8.2. The significance of the findings to food and nutrition security

Food insecurity is a major challenge at household level in Swaziland. Most household cannot afford to buy commercial food products regularly to meet the daily requirements. Household who cannot afford to buy commercial food product, as a result they purchase and consume poor quality foods. On the other hand Swazi traditionally fermented foods are easily produced at homesteads. These foods are relatively cheap to buy. The traditionally fermented Swazi foods play different roles at household that border on nutritional security. *Emasi* in Swaziland provide some much needed proteins in Swazi diets (Masarirambi et al 2009). *Emasi* contains about 2 ppm diacetyl, a range of volatile and non-volatile organic compounds

such as lactic acid, acetaldehyde, acetic acid, succinic acid, citric acid, pyruvic acid and ethanol that makes the product nutritionally acceptable (Mutukumira et al 2008).

The people in Swazi homestead are known to take two to three meals per day (Kgaphola and Viljoen 2004) and fermented Swazi foods make part of the portions eaten daily in homestead. And amongst the common taken traditionally fermented foods is *emahewu* and *emasi*. Other commonly taken fermented foods is the fermented porridges (*incwancwa*). The alcoholic beverage (*umcombotsi*) and fermented fruit mashes *(buganu*/*marula* wine) are consumed at homestead and also sold as source of income. The traditional fermented Swazi foods major play an important role in the diet and socio-economic activities of the population in Swaziland. Therefore play major role in food security at household in Swaziland. The study showed that among the strains tested, *Lb. acidophilus Lb. plantarum* as probiotics had properties could be beneficial to health of consumers. The fermented foods were safe to consumers. Figure 8.1 shows how the four pillars of food security were integrated in the study in achieving Food and Nutritional Security.

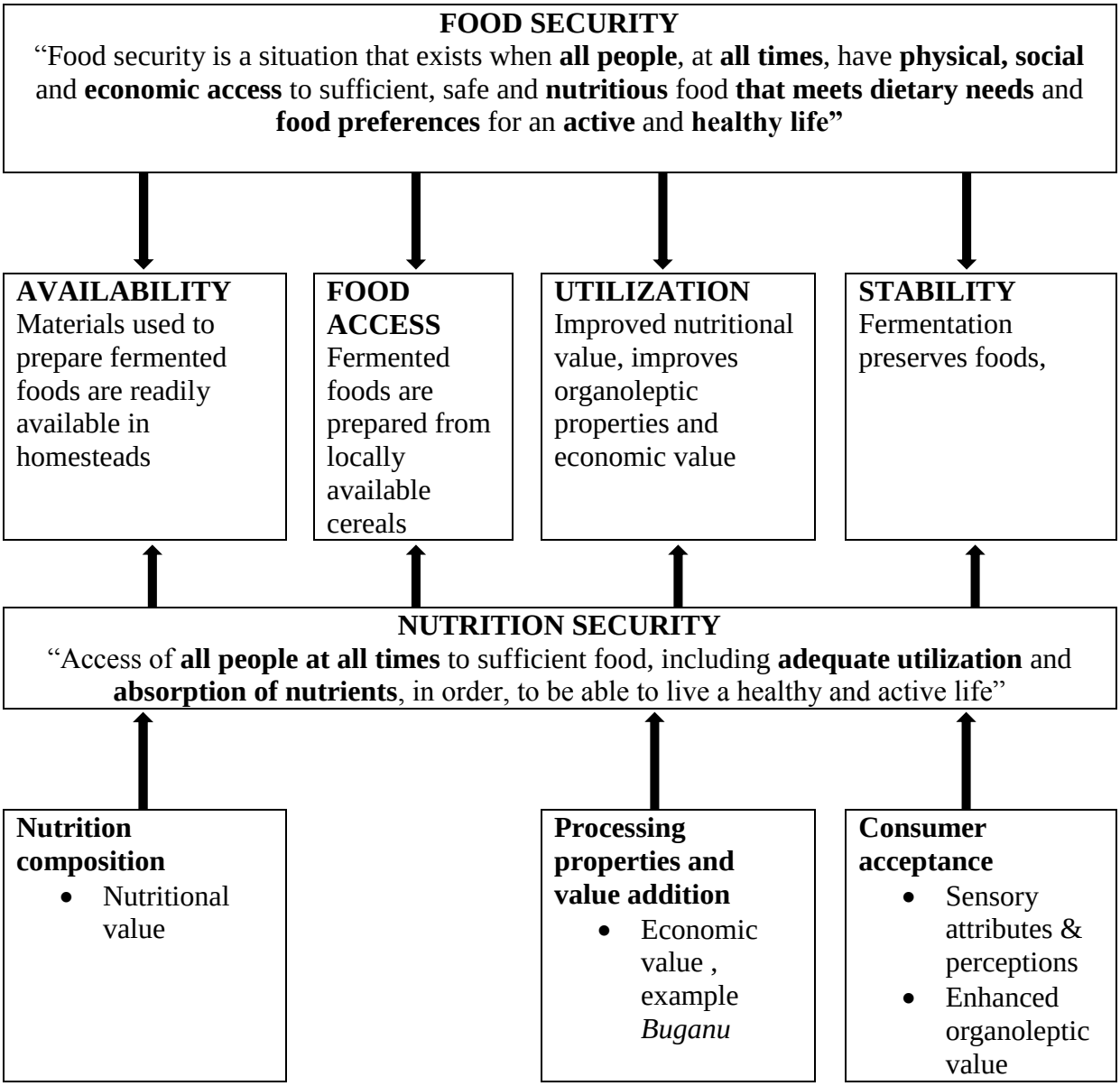


**Figure 8.1.** Significance of study findings to food and nutrition security.

## 8.3. The major strengths and weaknesses of study methodology

Documentation of preparation methods of Swazi traditional fermented foods was based on scientifically accepted methods. The methods used during the enumeration, isolation and identification of LAB (by sequencing 16SrDNA) were scientifically accepted methods. The probiotic properties of LAB in Swazi traditional fermented foods focussing on *emahewu* were studied using scientifically accepted methods. The safety of *emasi* and *emahewu* were conducted by growing *Enterococcus* spp on MacConkey agar. The identification of *Enterococcus* spp (by sequencing 16SrDNA) was based on scientifically accepted methods. The methods for safety of *emasi* and *emahewu* may need improvement. There is need to conduct IMViC tests on isolated pure colonies from MacConkey agar before identification by sequencing 16SrDNA. The IMViC tests refer to Indole test, Methyl Red (MR) test, Voges-Proskauer (VP) test and Citrate test.

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# CHAPTER 9: CONCLUSIONS AND RECOMMENDATIONS

## 9.1. Conclusions

In the current study, it was found out that *Umcombotsi*, *emahewu*, *buganu* and *emasi* were the fermented foods commonly prepared at household level in the Hhohho region. The fermented foods were prepared for own consumption as well as for sale. The main ingredient used for preparing *umcombotsi* and *emahewu* was maize meal. Un-milled sorghum malt (*magayiwe* or *mnandi*) was also added during preparation of *umcombotsi*. However, typically no malt was added during the preparation of *emahewu*.

The findings of this study highlight that *Emasi* (spontaneously fermented milk) and *emahewu* (non-alcoholic beverage) traditional fermented foods of Swaziland are fermented largely by LAB. *Leuconostoc mesenteroides* and *Lactococcus lactis* subsp. *lactis* were typical strains in *emasi*, while the *Lactobacillus* genus especially *Lb. plantarum* was typical in *emahewu*. *Emasi* and *emahewu* help in enhancing dietary diversity and in Swaziland they are popular foods taken by children and adults.

This study futher indicates that LAB from fermented Swazi foods in particular *emahewu* are able to tolerate low pH (3) exposure for 3 h. The LAB isolates exhibited tolerance to different concentration of bile salts (0.3, 0.5 and 1%) for a period not less than 4 h. Characteristically, most LAB isolates were found sensitive to antibiotics that inhibit bacterial cell wall synthesis (ampicillin and amoxicillin) and resistant to antibiotics that inhibit the synthesis of nucleic acids (ciprofloxacin and nalidixic acid). The different LAB isolates tested in the current study showed different responses (some were resistant and others were susceptible) to antibiotics

that inhibit the synthesis of proteins (tetracycline and streptomycin). The LAB have some antimicrobial activity against *S. typhimurium* ATCC 13311 and *E. coli* ATCC 25922. From the properties studied, it was concluded that LAB have significant probiotic properties that could be beneficial to the health of consumers.

With regard to the safety of Swazi traditional fermented foods (*emasi* and *emahewu*) *Enterococcus durans, Enterococcus hirae, Enterococcus lactis* (from *emasi*), *Lactobacillus plantarum* and *Lactococcus lactis* (from *emahewu*) were the only identified genera from colonies on MacConkey agar (Oxoid). These presumptive coliform colonies appeared deep red at centre and with a translucent halo around them. None of these isolates were identified as coliforms (*Escherichia*, *Klebsiella*, *Citrobacter* and *Enterobacter*) from *emasi* and *emahewu*. Therefore, the Swazi traditional fermented foods maybe safe consumption since the strains recovered are known to be non pathogenic.

### 9.2. Recommendations

There is still need to conduct futher studies on evaluation of probiotic potential of LAB. Such study should cover areas like testing the antimicrobial activities of LAB on a number of common pathogens (*Listeria monocytogenes, Staphilococcus* aures) that consumers may get through taking fermented foods. Study on amylase activities, haemolytic activity and adhesion capacity to caco-2 cell line of LAB. The enterocci found in *emasi* should further be characterised on their susceptibility to antibiotics to confirm the enterococcal safety. This is inline with approaches conduct on *Enterococcus* spp by TaeMi and JongHyun (2012). The outlined recommendation can help to further conduct some studies in developing starter

cultures that can be used in fermented foods in Swaziland. This would lead to studies in commercialising the fermented food products in Swaziland by using LAB starter cultures whose probiotics potential would have be known.

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## APPENDICES

**Appendix 1.** Publications from this work

1. Simatende, P., Gadaga, T.H., Nkambule, S.J. and Siwela, M. 2015. Methods of preparation of Swazi traditional fermented foods. J Ethnic Foods. 2: 119-125. DOI: http://dx.doi.org/10.1016/j.jef.2015.08.008
2. Simatende, P., Gadaga, T.H. and Siwela, M. 2016. The microbial and biochemical aspects of Swazi traditional fermented foods and identification of lactic acid bacteria in Swazi foods focusing on *emasi* and emahewu foods. J Food Sci Res. (Under review).
3. Simatende, P., Gadaga, T.H. and Siwela, M. 2016. Evaluation of probiotic properties of lactic acid bacteria strains isolated from *Emahewu*, a Swazi fermented food. J Food Sci Biotechnol. (Under review).
4. Simatende, P., Gadaga, T.H. and Siwela, M. 2016. The safety to consumers of traditionally fermented Swazi foods focusing on *emasi* and *emahewu*. Food Contr. (Under review).

**Appendix 2.** Semi-structured questionnaire on the preparation of fermented foods in Swaziland.

**UNIVERSITY OF SWAZILAND**

**DEPARTMENT OF ENVIRONMENTAL HEALTH SCIENCE**

## METHODS OF PREPARATION OF TRADITIONAL FERMENTED FOODS IN SWAZILAND

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**Purpose of the study**

This study aims at collecting information on the methods of preparation of traditionally fermented *emasi* (fermented milk), *emahewu* (non-alcoholic sorghum or maize meal beverage), *incwancwa* (sour porridge) and *umcombotsi* (sorghum meal/maize meal beer). This questionnaire will not take more than 30 minutes to complete.

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1. Date of interview:

2. Place of interview:

3. Gender of the person preparing the fermented food:

   a. Male ☐
   b. Female ☐

4. Marital status:

   Married ☐ Single ☐ Divorced ☐ Separated ☐

5. Type(s)/Name(s) of fermented food prepared

   …….

   …….

   …….

   …….

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6. Ingredients used for each product:

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7. Equipment used for each product:

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8. The preparation flow chart. (Please show all the steps, the time taken for each step, temperatures and quantities where possible)

9. How often do you prepare the food(s)?

a. Once a week ☐
b. Once every month ☐
c. When the ingredients are available ☐
d. During special occasions ☐
e. Other (specify) ☐ …….

10. Who consumes the food(s) in the family?

a. The whole family ☐
b. Infants (weaning) ☐
c. School going children ☐
d. The adults ☐

11. Is any category of people **prohibited** from consuming any of the fermented products?

a. Yes ☐

b. No ☐

12. If Yes, please state which one and why.

13. Do you prepare the food for sale?

a. Yes ☐

b. No ☐

14. If Yes, is this your major source of income?

a. Yes ☐

b. No ☐

15. If, No, what is your other source of income?

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**THANK YOU FOR TAKING TIME TO PARTICIPATE IN THIS INTERVIEW.**

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