CHARACTERISATION OF THE MICROORGANISMS AND DETERMINATION OF THE CHEMICAL CONSTITUENTS OF MARULA BREWS DURING FERMENTATION

BY

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DECLARATION

I, Evelyn Maluleke, declare that the dissertation titled “CHARACTERISATION OF THE MICROORGANISMS AND DETERMINATION OF THE CHEMICAL CONSTITUENTS OF MARULA BREWS DURING FERMENTATION”, which I hereby submit for the Masters in Microbiology at the University of Limpopo, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: _____ Date: _____

DEDICATION

I dedicate this dissertation to my late mother TSHILIDZI EUNICE MAVHETHA. I shall never forget the love, prayers, support and courage she afforded me. I wish she was here to share this moment. RIP MY SOLDIER.

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List of abbreviations

AAC- Acetic acid bacteria

DNA- Deoxyribonucleic acid

GC- Gas chromatography

HPLC- High performance liquid chromatography

LAB- Lactic acid bacteria

PCR- Polymerase reaction chain

WLD- Wallenstein differential media

WLN- Wallerstein nutrient agar

MLF- Malo lactic fermentation

NGS- Next generation sequencing

MALDI-TOF- Matrix assisted laser desorption-Time of flight

rRNA- ribosome nucleic acid

ITS- Internal transcribed spacer

MRS- De Man, Rogosa and Sharpe agar

YPD- yeast extract peptone dextrose

rpm- Revolutions per minute

ABSTRACT

Marula wine plays a fundamental role in the livelihoods of rural communities where it enhances social cohesion and also provides a reasonable income to the primary traders who often have no alternative source of income. Spontaneous fermentation will inevitably include microbes that produce undesirable metabolites, which lead to the spoilage and short shelf life of the wine. The aim of this study was to profile the microbial and chemical changes during fermentation of marula wine. Marula wines were collected from three areas in the Limpopo province namely: University of Limpopo, The Oaks village and Makhushane village. The bacterial species *Gluconobacter oxydans*, *Acetobacter pasteurianus, Lactobacillus brevis, Lactobacillus nagelii, Lactobacillus parabuchneri* and *Lactobacillus plantarum* species and yeast species *Hanseniaspora guilliermondii, Pichia guilliermondii, Saccharomyces cerevisiae, Rhodotorula mucilaginosa* and *Meyerozyma caribbica* were identified in marula wines at varying stages of fermentation. Non-fermenting yeast species such as *H. guilliermondii* together with lactic acid bacteria such as *L. brevis* and *L. plantarum* and the Enterobacteriaceae dominated the early stages of fermentation, whereas *S. cerevisiae* and Acetic acid bacteria dominated the late stages of fermentation. Chemical profiling of the marula juice and wine, which was achieved using both high performance liquid chromatography (HPLC) and gas chromatography (GC), revealed sucrose as the most abundant sugar in the marula juice with a range of 60.43 mg/mL to 73.20 mg/mL. Volatile organic compounds such as ethanol, ethyl acetate and isobutanol were observed during the fermentation process with none to very little of the volatile compounds detected in marula juice. Ethyl-acetate was the most abundant compound whereas ethanol concentration was observed to be high during the late stages of fermentation at a range of 1.16 g/L to 12.63 g/L. Common microbiota from different marula wines showed low intraspecific diversity indicating that the microorganisms responsible for the spontaneous fermentation are the same throughout the different areas that were selected for this study. The outcomes of the study provide empirical data to develop a wine with a long shelf life.

CHAPTER 1: INTRODUCTION

1.1 Background to the study

Traditionally, home brewed alcoholic beverages have become central elements to the social activities and celebrations in the African communities, i.e., the beverages are an integral part of the day-to-day social gatherings amongst elder in the African communities. There are different types of traditional alcoholic beverages made in African countries; some of these include marula wine, banana wine and sorghum beer. In ancient times, these brews were mainly made for important social and cultural gatherings such as weddings, funerals and rituals depending on the ethnic groups. However, currently most of the traditional brews have entered the local economy due to dire financial situations in many rural villages. Marula wine is the interest of this study. The production of marula wine is exceptionally mainstream in many rural and sub-urban communities in South Africa, particularly in Limpopo province where marula trees and fruits are abundant. Marula wine is currently being produced as an enterprise (Shale *et al*., 2014) and has an existing market in the young and old within African communities across the African continent.

Marula wine is produced from the juice of the marula tree fruits. The juice is traditionally fermented to produce either a high alcoholic drink or a low alcoholic beverage due to the difference in the duration of fermentation. The marula brewing process follows spontaneous fermentation which uses the microorganisms present on the fruits skin to initiate and carry out the fermentation process. An uncontrolled environment is the major limitation of spontaneous fermentation as this results in product of inconsistent quality and generally a short shelf life. The short shelf life is the key limiting factor for mass production of marula wine. The wine does not keep well for a long time due to the off flavours that make it unpalatable. This presents a potential loss of income for the traders who rely on the sale of the wine for daily living during the marula fruiting season. The development of cost effective strategies to avert spoilage of the wine will improve its shelf life, thereby increasing the economic value of wine. There is little information on the identity of the microorganisms and chemical compounds that are involved and produced respectively during the fermentation process of the marula

wine. This is augmented by the subtle differences in the quality and organoleptic properties of the marula wines from different areas with different climatic conditions such as rain and temperature furthermore, the marula fruit quality (Moganedi *et al*., 2011). Knowledge of the microorganisms and the types of compounds that are produced during marula brew fermentation will provide the basis for understanding the dynamics of the processes entailed in the production of marula wine. Hence, the purpose of this study was to isolate and characterise the microorganisms that form the marula wine flora and to further determine the chemical compositions which contribute to the distinctive characteristics of the marula wine.

1.2 The Aim and Objectives of the study

The aim of this study was to profile and characterise the microbiota associated with the fermentation of marula wine and to determine the chemical composition of marula wine during fermentation.

The aim was achieved through the following objectives using marula wines and fruits from different localities in the Limpopo province:

i. Isolation and identification of the bacterial inhabitants of marula fruits used in the production of marula wine.
ii. Isolation and identification of bacterial and yeast microbiota present in the wine during fermentation.
iii. Determination of the chemical composition of the marula juice and marula wines during fermentation.
iv. Molecular typing and characterisation of common microbiota isolated from different marula wines.

CHAPTER 2: LITERATURE REVIEW

2.1. Marula tree and its uses

2.1.1. The Marula tree

Sclerocarya birrea (A. Rich.) Hochst. subsp. *caffra* (Sond.) Kokwaro, is a valuable indigenous tree of Africa. It is found throughout the southern African region, in warm frost-free areas. In South Africa, the marula tree is found growing wildly in the Limpopo, Mpumalanga, KwaZulu-Natal and Eastern Cape Provinces (Komane *et al*., 2015). The most valuable parts of the tree are the fruit, nuts, bark, leaves and stem. The tree is dioecious and deciduous (Due *et al*., 2012). In southern Africa, flowering occurs between September and November and fruiting occurs during January to May (Mkwezalamba *et al.,* 2015). The fruits stay green on the tree and ripen on the ground where they attain a rich yellow colour. The fruit is rich in ascorbic acid and contains sugars (sucrose, glucose, and fructose), phenolic compounds, dietary fibre, minerals such as Na, Ca, Mg, Fe, Zn, Mn, K and sesquiterpene hydrocarbons (Ngemakwe *et al.*, 2017). The sesquiterpene hydrocarbons are terpenes found in plants and have bacteriostatic properties (Mariod and Abdelwahab, 2012). The fruit contains a hard brown seed that encloses a soft white kernel which is rich in oil and proteins. The oil is composed of oleic, palmitic, myristic and stearic acids and the proteins have a predominance of glutamic acid and arginine (Komane *et al*., 2015).

2.1.2. Uses of Marula tree

Marula tree has for decades played an integral part in the lives, food security and spirituality of indigenous communities in southern Africa. Marula has a wide variety of uses. Female trees bear thick leathery skinned fruits with fibrous, juicy, sticky flesh. The fruit is sweet-sour in taste, and is eaten by humans, domestic animals and birds because of its delicious pulp and tasty nuts. The ripe fruit has an average vitamin C content of 403 mg/100 g, compared to other fruits like grapes (38 mg/100 g), oranges (50 mg/100 g) and strawberries (59 mg/100 g) (Hiwilepo-van Hal, 2013).

Fruits are eaten fresh or squeezed to make fresh juice, which can be fermented into an alcoholic drink known in Xitsonga as vukanyi and morula or mokgope in Sepedi, or processed into jam, jelly and chutney (Wynberg *et al*., 2003). The kernels are used as

a food source and a condiment while the oil is used for cooking and as a preservative (Hiwilepo-van Hal, 2013). The bark is mainly used for medicinal purposes such as in treating diarrhoea, fever and malaria (Burlando *et al.,* 2010; Gouwakinnou *et al*., 2011; Street and Prinsloo, 2012; Do *et al*., 2013), while the wood is used for firewood and carvings such as spoons, bowls and plates as well as decorative animal artefacts (den Adel, 2002).

The marula fruit and leaves serve as food for cattle and wildlife. The leaves are nutritious and contribute to a healthy diet for livestock. Marula leaves serve as fodder for livestock during extended drought periods when there is no grass. The marula tree gives excellent shade in garden parks and roads as well (Komane *et al.,* 2015).

2.1.3. Marula products, their socio-economic and cultural values

Women usually carry out production and sales of marula products. Marula products are sold in public spaces such as along the main roads, in their homestead and in places with high human traffic. The income generated from selling marula products tends to be highly variable and is different from one area to another. Marula wine is sold at a price range of R20 to R30/ 2 L bottle and R100 for 20 L, 750 mL is sold at a low price of R5. The marula fruits are also sold for different purposes like for feeding goats and to industry such as for the production of Amarula cream liqueur (Shackleton, 2009). The average income earned from selling the marula fruits is R18.16 for 80 Kg, the price varied significantly in relation to the amounts sold (from R9 to R1016). Marula fruit jam, made from marula fruits or the skin of the marula fruits, was sold at a low annual gross income of R54.61 in 2002. Marula kernels are also sold for different purposes, such as for the extraction of essential oil for cosmetic production. A 200 mL cup of kernels were sold at a price ranging from R2.00 to R5.00 and an 80 Kg bag was sold at prices ranging from R20.00 to R50.00 (Shackleton and Shackleton, 2004).

As marula tree and its products, mainly marula wine, are commonly produced and used in cultural festivities in African communities, the many marula products have entered the mainstream local market due to the socio-economic status of many African households. Trading in marula products requires little or no initial investment in terms of material input because the fruits are available in abundance and the marula tree grows wildly.

2.2. Marula wine

2.2.1. Preparation of marula wine

The fruit characteristics such as size, tree yield, juice and sugar content differ between trees and between localities due to a potential relationship with rainfall or temperature (Moganedi *et al.*, 2011) specifically from the year prior to fruit production. Women use these characteristics to mark the trees with good fruit quality. The phenotypic characteristics of a tree are determined by the environmental factors such as climate changes (Moganedi *et al*., 2011). A big, mature Marula tree can produce 21,000 to 91,000 fruits annually (Mkwezalamba *et al.*, 2015). The traditional juice extraction method is labour-intensive; it takes 3 to 4 hours on average to produce sufficient juice to make 20 – 50 L of wine. Marula juice yield is dependent on the marula size, since large fruits have more juice compared to small ones. It is generally estimated that an 80 kg sack of marula fruits makes 25 L of wine. There is minimum monetary cost involved in the wine preparation as only water is added (Shackleton, 2002).

The preparation of the marula wine is fundamentally a traditional family art, passed on from one generation to another. Marula juice can be fermented to produce a wine, which is further distilled into a spirit called Thothotho by the Bapedi. The marula wine, with an average alcohol content of 5%, is produced by women generally from impoverished households and sold within their communities. This trade is practised in different parts of Africa such as in South Africa (mainly in the Limpopo province), Botswana, Swaziland and Namibia (Shackleton, 2002). Marula wine is traditionally prepared by spontaneous fermentation of a mash obtained from the fruits. The mash is fermented to give two types of drinks namely; a low alcoholic and high alcoholic beverage wherein the lower alcoholic drink is fermented for less than two days while the higher-alcoholic drink is fermented for 4 to 5 days (Hiwilepo-van Hal, 2013). Spontaneous fermentation refers to a process wherein a starter microbial culture is not used. The fruit microflora facilitates the fermentation process, which are usually introduced by fruit flies (Dlamini and Dube, 2008).

2.2.2. The social importance of marula wine

Marula wine is important in African culture. The consumption of the wine is both a social and a cultural activity. First fruits are widely celebrated at national and local levels to give thanks to the ancestors and to mark the beginning of the season of abundance (Simatende, 2016). The ceremonies usually involve the ritual slaughter of a goat or bull under a specifically selected marula tree; the first marula wine is shared between friends, neighbours and relatives at a household level. These neighbourhood gatherings reinforce mutual bonds and obligations, and these are instrumental in building and maintaining social networks within the communities, i.e., the sharing of marula wine builds mutual friendship and support structures within the community (Shackleton, 2002).

2.2.3. Quality and stability of marula wine

The storage temperature influences the shelf life of marula wine. It is short during warm to hot days, about 2 to 4 days. The shelf life of the marula wine is conventionally extended by the addition of the freshly prepared marula juice on a daily basis or by the storage of the prepared brew underground in a tightly closed container (Shackleton and Shackleton, 2004). A shelf life of a product is defined as the time during which the product remains safe, retains the sensory, chemical, physical and microbiological characteristics when stored under certain or recommended conditions (Kilcast and Subramaniam, 2000). Storage temperature plays a vital role in the shelf life of a product since fluctuations in temperature can lead to changes in chemical and microbial composition, the latter may result in the occurrence of unwanted and spoilage microorganisms. Generally, traditional fermented foods vary in quality due to the types of raw materials and equipment used. Examples of these products include mahewu, fermented milk (amasi), umcombotsi and banana beer. The storage practices differ for different products and from one area to another. Some areas prefer the use of plastic containers while others use calabashes. Environmental conditions contribute to the gradual selection of specific microorganisms that are responsible for the perceived flavour (Gadaga *et al*., 1999).

2.3. Microbiota associated with marula wine

The microorganisms that are responsible for the fermentation of marula juice have been poorly studied. They are understood to include wild yeasts and bacteria from fermentations equipment passed from previous fermentation and from the microflora of the marula fruits. Different handling and preparation methods of the brews are more likely to result in marula wines of varying quality due to contribution by different types of microorganisms (Mpofu *et al*., 2008). The most commonly occurring groups of microorganisms in most spontaneously fermented products such as grape wine and mageu (Fermented maize) are the wild yeast, lactic acid bacteria (LAB) and acetic acid bacteria with each group playing a specific role in the quality of the final product (Katongole, 2008).

2.3.1. Lactic acid bacteria

Lactic acid bacteria (LAB) are defined as a group of Gram positive, non-spore forming cocci or rods and obligate aerobes that produce lactic acid as the major end product during fermentation of sugars such as glucose and fructose (Leroy and De Vuyst *et al*., 2004). LAB are subdivided into homo-fermentative and hetero-fermentative groups. Homo-fermentative LAB mostly produce lactic acid only from sugars while hetero-fermentative LAB produce lactic acid, acetic acid and alcohol (Holzapfel and Wood, 1995). The LAB strains improve the shelf life and nutritional quality of the fermented product and beverages and produce organic acids which account for the palatability of the fermented foods (Mokoena *et al*., 2016) and some have antimicrobial properties (Chelule *et al*., 2010, Mokoena *et al*., 2016).

2.3.2. Acetic acid bacteria

Acetic acid bacteria (AAB) refers to a group of microorganisms that belong to the Acetobactereaceae family. These bacteria produce high concentrations of acetic acid from ethanol (Guillamón and Mas, 2017). They are widespread in nature and have been isolated from flowers, fruits, herbs and cereals (Sengun and Karabiyikli, 2011). Industrially, AAB are predominantly used for vinegar production and are also present in wine, ciders and beer (Bhat *et al*., 2014). AAB are classified into the following 12 genera *Acetobacter, Gluconacetobacter, Gluconobacter, Asaia, Acidomonas,*

Granulibacter, Ameyamaea, Neoasaia, Kozakia, Saccharibacter, Swaminathania and *Tanticharoenia*. The involvement of AAB in production of beverages such as wines is ussually detrimental (Sengun and Karabiyikli, 2011) since the conversion of alcohol via acetaldehyde to acetic acid contributes to spoilage of the wine. AAB are considered spoilage microorganisms due to their major metabolites that results in undesirable sensory characteristics (Bartowsky and Henschke, 2008). Wine spoiled by AAB is characterised by a vinegary sourness on the palate and a reduced fruity character and such wines have a low commercial value (Bartowsky and Henschke, 2008). Grapes and grape wines are subjected to spoilage by AAB at many different stages of production. Physical damage to the fruit can lead to contamination by the AAB. Most of the wines become spoiled by AAB during maturation or storage when exposed to air and this spoilage is commonly attributed commonly to *Acetobacter, Gluconobacter* and *Gluconacetobacter* spp. (Bartowsky and Henschke, 2008).

2.3.2. Yeast

Yeasts are unicellular, eukaryotic and aerobic organisms that grow in various niches such as plants, algae, seawater, soil and some can be found on the skin and the intestinal tract of animals (Glazer and Nikaido, 2007). The diversity and the composition of the yeast population significantly contribute to the sensory characteristics of wine, i.e., yeasts influence the chemical composition of wine during fermentation and hence contribute greatly to the flavour of the resulting wine. The growth of each wine yeast species is characterised by a specific metabolic activity, which determines the concentration of flavour compounds in the final product (Romano *et al*., 2003). Yeasts generally contribute positively towards the wine flavour by converting carbohydrates (sugars) to ethanol and other organic compounds that give off the flavour and aroma components. Yeasts are often present in processed food with high sugar content and low pH and produce secondary metabolites such as acids, ester, aldehydes, ketones and sulphur compounds (Fleet, 2003).

The most commonly occurring yeast strain associated with marula wine is *Saccharomyces cerevisiae* (Mpofu *et al*., 2008). Other yeast species include *Pichia anomala, Pichia guilliermondii, Candida tropicalis, and Candida intermedia* (Mpofu *et*

al., 2008). During ancient times, wine would be fermented spontaneously by endogenous microflora known as wild yeasts and these are the yeasts that are naturally occurring on the fruits or the raw material to be used for fermentation. Wild yeasts include *Hanseniaspora* and *Debaryomyces*. *Kloeckera, Metschnikowia pulcherrima*, *Candida pulcherrima* and less frequent isolates are *Pichia membranefaciens, Hansenula anomala, Candida stellata, Cryptococcus* spp*. and Rhodotorula* spp*.* Wild yeasts are responsible for spontaneous fermentation of different wild fruit juice, and these yeasts are primarily responsible for alcoholic fermentation. Thus, the different yeast species developed during fermentation and their dynamics and frequency of appearance determine the taste and flavour characteristics of the final products (Byarugaba-Bazirake, 2008).

2.4. Chemical composition of marula fruit and wine

The chemical composition of marula wine is defined by various factors such as the quality of the fruit used and the fermentation conditions. Numerous chemicals that are found on the fruit skin are subsequently transferred to the brew during the processing. The moisture content of marula fruit is estimated at 85% with more than 2% of the crude fibre. The common sugars found in marula fruit and wine include sucrose, fructose and glucose (Hiwilepo-van Hal, 2013). The most commonly occurring organic acids are ascorbic acid, citric acid, and malic acid (Nyanga *et al.,* 2013). Marula fruit contain significant amount of minerals such as calcium and magnesium, with potassium being dominant. The amino acid of marula fruit varies with climatic conditions with the predominant being asparagine. (Fundira, 2001). Volatile compounds are present on the marula fruits skin, marula juice and wine and these include esters, alcohols, lactones, carbonyl, acetals, phenols, acids and sulphur-containing compounds (Fundira, 2001). Esters and hydrocarbons are the most dominant volatiles by the fruit. Volatiles of the fruit pulp and the whole fruits (skin volatiles) constitute of heptadecene, benzyl 4-methylpentanoate, benzyl butyrate, (*Z*)-13-octadecenal and cyclo-pentadecane. The major alcohol is (*Z*)-3-decen-1-ol and 6-dodecen-1-ol, while the major aldehyde is 11-hexadecanal (Viljoen *et al.*, 2008).

2.5. Production of a stable fruit wine

The making of African traditional fermented foods and beverages generally depends on spontaneous fermentation, which is an uncontrolled process. Since the fermentation is uncontrolled, the product quality becomes inconsistent between different batches. It is of major significance to monitor the microbial dynamics throughout the traditional fermentation processes in order to deliver a high-quality and safe product for consumers (Lv *et al*., 2015). Optimisation of the production processes results in consistent and high quality fermented products. The fermentation process can be improved to a superior controlled process that involves the use of starter cultures. A starter culture is a material that contains large number of different microorganisms or one strain that is added to accelerate the fermentation process. The use of starter cultures can be one of the approaches that could be used to regulate fermentation and to ensure stability in quality (Lv *et al*., 2015). Another process for production of a stable wine is called back slopping which is applied during the traditional fermentation. It involves the use of materials from a preceding batch to initiate the new batch (Corsetti *et al.,* 2012). Through this practice, the initial phase of the fermentation process and the risk of fermentation failure are reduced. Continuous use of back-slopping results in selection of the best-adapted strains which may possess features that are desirable for use as starter cultures (Solieri and Giudici, 2008).

2.6. The development of organoleptic characteristics of wine

Different compounds with different aromatic properties influence the organoleptic characteristics of a wine. These include the flavour compounds that originate from the raw material, compounds formed during extraction of the juice, compounds that are produced by yeasts and bacteria during the fermentation process (alcoholic and malolactic fermentation) and compounds that form during storage (Byarugaba-Bazirake, 2008). Fermentation products usually dominate volatiles identified in wines, since these compounds are present in the highest concentrations. Therefore, conversion of fruit sugars to alcohol and other end products by specific yeast populations may yield wines with diverse organoleptic quality (Zoecklein, 2012). The various yeast species and strains that become established during the overall fermentative process metabolise juice constituents, principally the sugars, to a wide range of volatile and non-volatile end-products, which ultimately influence and

determine the types and concentrations of many by-products that contribute to the aroma and flavour characteristics of the wine (Duarte *et al*., 2010).

Volatiles compounds are divided into higher alcohols, esters, carbonyl compounds and sulphur containing compounds (Kobayashi *et al*., 2008). Volatiles influence the wine aroma, colour and flavour both individually and synergistic or in an antagonistic manner. Some volatiles contribute much more to the flavour. Higher alcohols contribute more on the aroma and flavour of the wine with a strong and pungent smell and taste. The presence of ethanol in a wine directly contributes to the flavour of the wine by giving rise to a warming character. Alcohols with three or more carbon units, ethyl esters (mainly ethyl acetate), and acetaldehyde are the main agents responsible for the flavour of alcoholic beverages and their quantities determine the quality of the wine (Reboredo-Rodríguez *et al.,* 2015). Ethyl acetate has a major effect on the organoleptic characteristics of wine since its presence gives a pleasant aroma with fruity properties, however at a high concentration of above 150 mg/L it turns vinegary and contributes to the spoilage of the beverage (Dragone *et al*., 2009). Acetaldehyde is a carbonyl compound produced by yeasts during alcoholic fermentation, it is highly volatile and it gives a bitter taste to the wine at a high concentration (Rapp and Mandery, 1986). Different flavour compounds are present depending on the type of the wine and the storage conditions. Esters have been found to be responsible for the fruit flavour amongst wines (Swiegers *et al.,* 2005). The aroma of the marula fruit and the wine is attributed to four types of esters, which include the ethyl acetate, isoamyl acetate, ethyl caproate and caprylate and two alcohols, isobutyl, isoamyl and acetaldehyde. Acids constitute a significant group of aroma compounds that impart fruity, cheesy and fatty odours to wines and they contribute to the bitterness stringency and rancidity of wine (Shale *et al.,* 2014). Concentrations of volatile compounds are dependent on the temperature at which the microorganisms are grown. It has been found that more compounds are produced in wines produced at lower temperatures, although this dependent on the strain (Torija *et al.,* 2003). Berger (2012) has revealed that the aroma of most grape wines is mainly composed of higher alcohols and esters formed through the fermentation process, which provide the fruity, clean and fresh herb flavours. Most of the dominating volatiles are those that are produced by yeast metabolism. It has also been reported that alcohols are quantitatively the largest group of volatile compounds in Zalema wines, in accordance with previously published

results, that indicate that alcohols represent 80–90% of the aromatic content of wines. At concentrations above 400 mg/L, they are regarded as negative quality factors that can spoil the wine (Gomez-Miguez *et al.,* 2007).

2.7. Ideal microorganisms for wine fermentation

Fermentation is regarded as the key to food safety and assurance in Africa since it is used at the household level to prepare and preserve food. Common African raw materials that include tropical fruits, milk, sorghum, maize and oil seeds are fermented to produce various African products such as sorghum, sorghum porridges and granules, sorghum breads and flakes, alcoholic beverages, dairy products, fish and meat products, and flavours and substitutes. Methods of their preparations vary from one country to another (Chelule *et al*., 2010). Wine is desired globally as an alcoholic beverage in different forms, namely, a dessert wine, as dry or sweet, still or sparkling and natural or fortified form. Fermentation of wine can occur spontaneously either by native yeasts and bacteria or by inoculation with selected yeasts and bacterial strains (Bisson, 2004). There are different strains of both yeast and bacteria that are regarded as the ideal microorganisms for wine fermentation and these are those that bring the desirable characteristics of wines. Ideal microorganisms for wine fermentation are the Lactic acid bacteria, and yeast and a few strains of acetic acid bacteria (Bartowsky and Henschke, 2008). Although lactic acid bacteria (LAB) and acetic acid bacteria, are prominent in the spoilage of some fruits and fruit products, certain species of LAB can have positive contribution in the production of wines (Nyanga *et al*., 2007). LAB such as *Lactobacillus, Lactococcus, Streptococcus and Leuconostoc* species are considered as the ideal microorganisms for fermentation since they increase the palatability of food and improve the quality by increasing the availability of proteins and vitamins. In addition, LAB confer preservative and detoxifying effects in food. LAB fermented foods boost the immune system and strengthen the body in the fight against pathogenic bacterial infections (Lonvaud-Funel, 1999). The ideal yeasts for winemaking are those that fall under the genus *Saccharomyces* since they are tolerant to ethanol and can ferment the sugar in the juice efficiently and produce good quality wine, for example *Saccharomyces cerevisiae* (Rainieri and Pretorius, 2000).

2.8. Health benefits and effects of fermented foods

Several studies have reported on the different health benefits of fermented foods and beverages. Fermented foods and beverages have probiotic, immunoactive, and anticholesterolemic effects (Shrikhande, 2000). Fermented foods promote the function of the human digestive system in a positive manner (Parvez, 2006). Lactic acid bacteria contained in the fermented products lower the cholesterol level (Pereira and Gibson, 2002). Microorganisms that are involved in the fermentation process such as *Lactobacillus casei and Lactobacillus gasseri* have the ability to bind mutagens and inhibit mutagenic nitrosamines (Rafter, 1995). Some lactic acid bacteria present in fermented foods were found to play a crucial role in the immune system of the host, *L. casei* was found to improve the function of the peritoneal macrophages and increase the production of immunoglobulin A (Shah, 2007).

CHAPTER 3: MICROBIOLOGICAL PROFILING

Determination of bacterial and yeast microbiota of the Marula fruits skin and Marula wines during fermentation

3.1 Introduction

Marula wine is produced by the natural fermentation of marula fruit juice by the Natural microflora i.e., yeasts and bacteria that inhabit the fruits (Nyanga *et al*., 2007). Fruits contain high levels of sugars amongst other nutrients, and their water activity can support the growth of different microorganisms. The various factors such as rainfall, temperature, soil type, fruit maturity, damage due to animal, insects and fungi, mechanical damage, application of fungicides, and insecticides affect the type and load of microbial flora present on the marula fruits (Rawat, 2015). Natural microflora are mainly yeasts and bacteria that are introduced by *Drosophila* or fruit flies that are present on ripe fruits or visit the fruits during the maturing period. The microorganisms on the marula fruit skin are believed to be transferred into the marula juice during the extraction and these initiate the spontaneous fermentation process for marula wine.

Marula fruits fall to the ground while still green and ripen on the ground. Inadvertently, the microbiota on the marula fruit will emanate from the soil. The diverse microbial flora on fruit surfaces play diverse roles during the spontaneous fermentation process (De Vuyst and Weckx, 2016); those that contribute positively to fermentation or harbour spoilage characteristics. The coliforms, lactic acid bacteria (LAB), acetic acid bacteria, yeasts and moulds have been shown to be present during fermentation of marula (Gadaga *et al.,* 1999). The microbial interaction during the fermentation process plays an important role in the quality and goodness of the final product. Yeasts are primarily responsible for alcoholic fermentation. Malolactic fermentation (MLF) follows alcoholic fermentation and is conducted by the LAB (Langer, 2016). These bacteria are important in winemaking and can have a positive or negative effect on the wine quality. Malolactic fermentation in wine is a secondary fermentation that usually occurs at the end of alcoholic fermentation by yeasts, although it sometimes occurs earlier. It entails wine de-acidification in which the dicarboxylic L-malic acid (malate) is converted to the monocarboxylic L-lactic acid (lactate) and carbon dioxide (Liu *et al*., 2017). Benefits of MLF include the lowering of acidity in high acid wines, enhancement

of sensory characteristics and enhanced stability of the LAB (Bloem *et al.,* 2007). The undesirable effects include the excessive reduction in acidity of high pH wines leading to risk of spoilage, production of undesirable flavours, colour changes, and formation of amines (Dharmadhikari, 2002).

The role of acetic acid bacteria in the winemaking process is well known to be associated with the spoilage of wine by acetification of the ethanol produced by yeasts. Growth of these bacteria on fruits and in the juice influence wine composition and possibly affect the growth of yeasts during alcoholic fermentation and lactic acid bacteria during malolactic fermentation (Bartowsky and Henschke, 2008).

Other studies have shown that different communities of microorganisms exist on the surface of fruits (grapes and marula). These microorganisms are generally divided into 3 groups. Group 1: species without fermentation ability (such as *Burkholderia*) Group 2: species with some fermentation ability (*Lactobacillus, Pichia and Candida* which could act during the first stages of winemaking and group 3 were defined as the species that are the main fermentation agents such as *Saccharomyces cerevisiae* and *Oenococcus oeni* (Renouf *et al*., 2007).

It is important to know the type and evolution of the microorganisms involved in the fermentation of marula wine in order to achieve large-scale production without compromising the typical traditional properties and prevent spoilage during storage and maturation. There are molecular techniques that are transforming the study of food microbial ecology and next-generation sequencing (NGS) is one of the current techniques used. NGS achieves parallel sequencing of heterogeneous DNA fragments (Mayo *et al.,* 2014). For the purposes of microbial community observation, these fragments consist of short segments amplified using universal PCR primers targeting known marker genes, principally prokaryotic 16S rRNA and fungal ITS genes. MALDI-TOF biotyper is also considered as one of the reliable and accurate technique used in identifying microbial species (Ferreira *et al.,* 2011). Hence, this study has used both high-throughput technologies to discern the types and dynamics of the microbiota during fermentation of marula wine.

This study sought to investigate the different microorganisms present on the marula fruit surface and those that are present and responsible for marula juice fermentation.

3.2 Materials and methods

3.2.1. Collection of ripe marula fruits and wine samples

Ripe marula fruits were collected from three locations in the Limpopo province namely, the University of Limpopo, Makhushane village (Mopani district, Ba-Phalaborwa municipality) and The Oaks village (Mopani district, Maruleng municipality) during March 2016 and February 2017. Marula trees growing in the wild and the University grounds are a source of marula fruits for marula brewers in the surrounding communities of Mankweng, Mentz, Mamotintane and Ga-Makanye. From the grounds of the University of Limpopo, only ripe yellow fruits that fell to the ground around the marula trees were collected. The three locations were selected randomly without preference over the other.

3.2.2. Isolation of the marula fruit skin microflora

Four to six yellow and green marula fruits were weighed and separately immersed in 100 ml of 0.85% saline solution and incubated with agitation at room temperature for 24 hours. The saline solution was centrifuged, and the pellet was dissolved in sterile distilled water and mixed with absolute glycerol in 1:1 ratio. These served as the stock cultures. One hundred microliters of 10^{-2} and 10^{-3} dilutions of the stock culture were spread-plated onto different selective media. Yeast extract peptone dextrose (YPD) and Wallerstein Nutrient Agar (WLN) were used for yeast isolation and the media were incubated at 30℃. de Man Rogosa and Sharpe (MRS) and Wallerstein differential agar (WLD) were incubated anaerobically at 30℃ for isolation of LAB whereas WLD was incubated aerobically at 35℃ for isolation of AAB. Following incubation, different colonies based on morphology were observed, enumerated and 10% of each type was sub-cultured for purification. The pure cultures were then identified by the use of MALDI TOF biotyper.

3.2.3. Preparation of marula wine

Ripe marula fruits that were collected from the grounds of the University of Limpopo were used in the preparation of the marula wine, and the wine was labelled the LAB.

Clean utensils and tap water were used and the generic recipe was followed. The deskinning and squeezing of the juice were performed with sanitised hands. The fruits were peeled with a fork; the juice was squeezed into a plastic container and mashed together with the marula kernels. The kernels were then separated from the mash and the resulting volume determined. Equivalent volume of water was added to the thick fruit mash and mixed thoroughly with a wooden spoon. The mixture was covered and allowed to ferment at ambient temperature. After a day of fermentation, a very thick layer of pulp on top of the clarified liquid was removed by scooping with a clean plastic spoon. The pulp was continuously removed each day thereafter until there was no further formation of the pulp and the mixture was set for fermentation to continue. The other three wines used in this study were collected from the brewer's homes. Two wines collected from the Oaks were labelled as MST and SKB wines, the wine collected from the Makhushane village was labelled as the MLT wine. All the wines used in this current study were produced following the same procedure.

3.2.4. Sampling of the marula wine

Collection of marula wine samples was performed while fermentation was ensuing. A 50 mL volume of each of the marula wines was sampled using a sterile pipette at 2 day intervals from the point of collection from the different households. The LAB and MLT wines were collected at day 0 of fermentation whereas the MST wine was collected on day 3 and the SKB wine was at day 14 of fermentation process. Sampling was followed by centrifugation at 14000 rpm. In addition, the supernatant was stored in 2 mL micro-centrifuge tubes while the pellet was mixed with sterile glycerol at a ratio of 1:1 then stored at -80 ℃ until microbial isolation was performed.

3.2.5. Isolation of yeasts from the marula wine

Tenfold serial dilutions of the glycerol stock culture suspension were prepared with 0.85% sterile saline solution and the spread plate method was used for cultivation of yeasts on WLN agar, YPD medium and YPD medium supplemented with 5% ethanol, for isolation of ethanol tolerant yeasts. The cultures were incubated aerobically at 30 ℃ for 42 to 72 hours. After incubation different colonies by morphology were

observed, enumerated and 10% of each morphology was sub-cultured on the same media.

3.2.6. Isolation of Lactic acid and Acetic acid bacteria from marula wine

Selective cultivation for isolation of LAB and AAB was performed as described under **section 3.2.2.**

3.2.7. Molecular identification of the bacterial and yeast isolates

Identification of bacteria and yeasts present on the marula fruits and marula wine was achieved with Next Generation Sequencing-Miseq (NGS) and MALDI-TOF Biotyper technique.

3.2.8. Identification of microflora using MALDI-TOF Biotyping technology

A colony of an actively growing bacterial culture was suspended in 300 µL of sterile deionised water in a microcentrifuge tube. This was followed by the addition of 900 µL of absolute ethanol. The mixture was mixed thoroughly and centrifuged at 13000 rpm for 2 minutes. The supernatant was decanted into a beaker and the remaining contents were centrifuged again. The residual ethanol was removed without disturbing the pellet. The pellet was allowed to dry at room temperature. An aliquot of 5 µL of 70% formic acid was added to the pellet and mixed well by vortexing. An equal volume of absolute acetonitrile (Sigma) was added and mixed by vortexing. The mixture was centrifuged for 2 minutes at 13000 rpm such that all the material was collected neatly in a pellet. A small volume of the supernatant (1µL) was spotted onto the MALDI target plate and allowed to dry at room temperature. The spots were overlaid with 1µL of α-cyano-4-hydroxycinnamic acid solution (Sigma) and allowed to dry at room temperature. The samples were then subjected to the MALDI TOF (Bruker) for biotyping. Biotyping experiment was conducted according to the manufacturer instruction. A good identification was signified by a score of 1.700 and above (Bruker Guide to MALDI Sample Preparation, 2015).

3.2.9 Identification of the bacterial and yeast microflora by NGS technology

a) Isolation of DNA

Purified DNA from 1.5 mL of the crude cultures of marula wine samples, collected at different intervals during fermentation, was prepared with the DNeasy® PowerSoil® Kit (QIAGEN) following the manufacturer's instructions. The DNA was stored at –20 ℃ until further analysis.

b) Amplification of the 16S rDNA and ITS region

The primers ITS3 and ITS4 that target the conserved regions of 18S, 5.8S and 28S rDNA gene were used for amplification of yeast DNA and 16S rDNA primers which were used for the bacteria. PCR amplification was performed with a volume of 25 µL which contained 20.5 µL of PCR master mix and 4.5 µL of the DNA template. The PCR master mix was made by combining 7.5 µL of molecular water, 5 µL of 5x buffer, 5 µL of each of the primers, 0.5 µL of 2.5 units/µL HotStart HiFidelity DNA polymerase (QIAGEN). A negative control was prepared by replacing DNA template with molecular grade water. Amplification was carried out as follows: an initial denaturation at 95℃ for 5 minutes; 30 cycles of denaturation at 94℃ for 15 seconds, annealing at 55℃ for 1 minutes, and extension at 72 ℃ for 30 seconds and a final extension at 72℃ for 10 minutes. The GeneAmp PCR system 9600 thermal cycler was used for amplification. Gel electrophoresis was performed in a 1.5% agarose gel and 1x TAE buffer at 80 volts for 30 minutes to check the presence and integrity of the amplicons. A 100-bp DNA ladder was used for sizing of the bands. The gel was viewed under UV light.

c) First clean-up of amplicons

The microtitre plate containing amplicons was centrifuged at 1000 x g at 20℃ for 1 minute to collect condensation and the seal was carefully removed. A multichannel pipette was used to transfer the amplicons to the MIDI plate. A 0.15 X AMPure XP beads (QIAGEN) were vortexed for 30 seconds to evenly spread them. Using a multichannel pipette, 20 µL of AMPure XP beads was added to each of the wells

containing the PCR amplicons. The MIDI plate was sealed and shaken for 2 minutes at 1800 rpm. The plate was incubated at room temperature for 5 minutes without shaking and thereafter it was placed on a magnetic stand for 2 minutes for the supernatant to clear. The supernatant was then removed with a microchannel pipette and discarded. The beads were washed again for 30 seconds with 200 µL freshly prepared 80% ethanol and the supernatant was carefully removed and discarded. A second ethanol wash was performed as mentioned above and the beads were allowed to dry at room temperature for 10 minutes. The plate was removed from the magnetic stand and 52.5 µL of 10 mM Tris (pH 8.5) was added to each well. The plate was then sealed and shaken at 1800 rpm for 2 minutes and this was followed by incubation at room temperature for 2 minutes. With the supernatant cleared for 2 minutes on the magnetic stand, 50 µL of the supernatant was transferred to a new 96-well microtitre plate. The plate was sealed and stored at -15 ℃ until further processing.

d) Index PCR

Using a multichannel pipette, 2.5 µL of the purified PCR amplicons was transferred to a new 96-well plate. The PCR reaction mixture was prepared as indicated in Table 3.1.

Table 3.1: Index PCR components

Component	**Quantity (µL)**
DNA	2.5
Nextera XT Index Primer 1 (N7xx)	2.5
Nextera XT Index Primer 2 (S5xx) (QIAGEN)	2.5
2x KAPA HiFi HotStart ReadyMix	12.5
PCR Grade water	5
	Total 25 µL

The contents were gently mixed and the plate was sealed and centrifuged at 1000 x g for 1 minute. Amplification was performed as follows: an initial denaturation at 95℃ for

3 minutes; 8 cycles of denaturation at 95℃ for 30 seconds, annealing at 55℃ for 30 seconds, and extension at 72℃ for 30 seconds and a final extension at 72℃ for 5 minutes and a final hold at 4 ℃. GeneAmp PCR system 9600 thermal cycler was used for amplification. Gel electrophoresis was performed as outlined in section 3.2.8.2 (b).

e) Second clean-Up of amplicons

Amplicons on a PCR plate were centrifuged at 280 x g at 20 ℃ for 1 minute to collect condensation and the seal was carefully removed. A multichannel pipette was used to transfer the amplicons plate to the MIDI plate. The AMPure XP beads were used for the clean-up of amplicons as described in the first Clean-Up of amplicons

f) Library quantification, normalization, and pooling of the amplicons for NGS MiSeq Illumina

Dilutions of the concentrated amplicons obtained after the second clean-up (final library) were performed using 10 mM Tris pH 8.5 to 4 nM. A volume of 5 µL of diluted DNA was aliquoted from each library and mix aliquots for pooling libraries with unique indices. All the libraries were pooled for one MiSeq run.
The sequences generated by the NGS MiSeq Illumina were trimmed, aligned then analysed with a use of a software called QIIME. Identification of the yeasts and bacteria were done up to the family level.

3.3 Results

3.3.1 Bacterial inhabitants of marula fruits used in the production of marula wine

Ripe marula fruits collected from three different areas in the Limpopo province were used to investigate the inhabiting and contributing bacteria in marula juice fermentation. MALDI-TOF Biotyper technique was used to identify the isolates. The bacterial isolates identified to be those that belong to the family Enterobacteriaceae and these were identified as *Klebsiella oxytoca, Raoultella omithinolytica* and *Enterobacter cloacae*. The observation was similar across all the three areas (Table

3.2). The fruits collected from the Oaks indicated a wide range of Enterobacteriaceae species comparatively. Interestingly only one fermenting *Lactobacillus* species was identified from the fruits collected at the University of Limpopo and this was identified as *Lactococcus lactis*.

Table 3.2: Bacterial isolates from marula fruit skin.

University of Limpopo (by dominance)	Makhushane village (by dominance)	The Oaks (by dominance)
Ripe fruits	Ripe fruits	Ripe fruits
Klebsiella oxytoca *Lactobacillus lactis* *Bacillus. subtilis* *Raoultella ornithinolytica* *Enterobacter cloacae* *Enterobacter aerogenes* *Plesiomonas shigelloides* *Mahura spinosa*	*K.oxytoca* *Klebsiella pneumoniae* *B. subtilis* *R. omithinolytica* *E. cloacae* *E. aerogenes* *Pseudomonas putida*	*K.oxytoca* *K. pneumoniae* *B. subtilis* *R. omithinolytica* *E. cloacae* *E. aerogenes* *Enterobacter ludwigi* *Citrobacter koseri* *Escherichia coli* *Enterobacter radiniatants* *Weissella viridenscens* *Novosphingobium resinovorum*

3.3.2 Bacterial microbiota present in the marula wine during fermentation.

Four marula wine samples obtained from the University of Limpopo (LAB), The Oaks (MST and SKB) and Makhushane village (MLT) were used to study the microbial composition of marula wine. This was done in order to understand the microbial

evolution during the fermentation of marula juice. Different microorganisms that belonged to different families such as Lactobacillaceae, Acetobacteriaceae and Enterobacteriaceae were isolated from all the wines that were investigated in this study (Figures 3.1, 3.3 and 3.5). The occurrence of the Lactobacillaceae and Acetobacteraceae was observed throughout the fermentation period. Bacteria belonging to the Enterobacteriaceae family were present during the early stages of fermentation, however as the fermentation progressed their count dropped drastically (Figures 3.1 and 3.3). Both fermenting and non-fermenting bacterial species were observed at different stages of fermentation. Different LAB species such as *L. plantarum, L. brevis, L. nagelii, L. kefiri* and *L. parabuchneri* (Figures 3.2, 3.4, 3.6 and 3.7) were observed at varying fermentation stages and this data can be supported by the results in figures 3.1, 3.3 and 3.5 which indicated that LAB microorganisms were present throughout the fermentation period. The AAB species were observed in high abundance at the late stages of fermentation such as from days 8 to 16, the main AAB species identified in this study were *Gluconobacter oxydans* and *Acetobacter pasteurianus*.

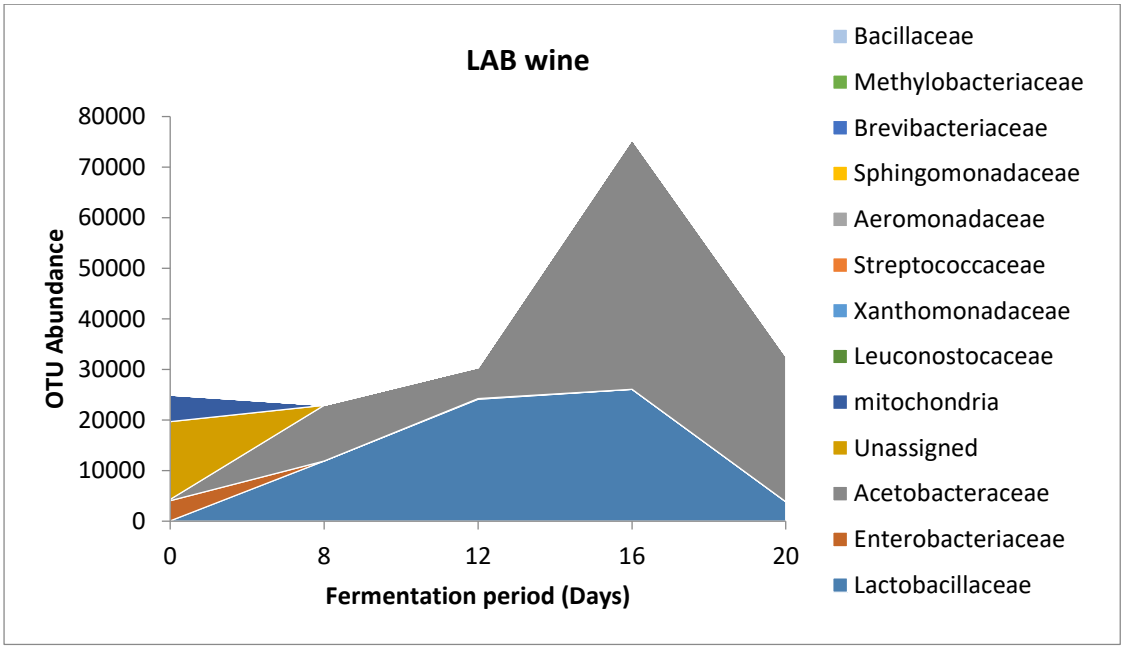

Figure 3.1: Profile of the bacterial families observed in the LAB wine.

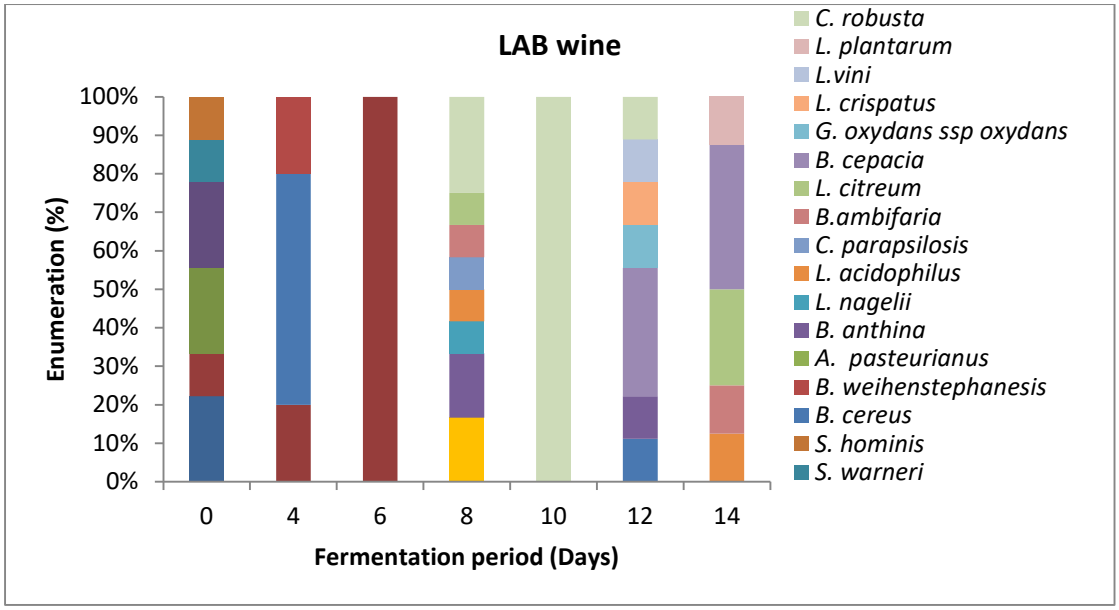

Figure 3.2: Profile of the microbial species observed in the LAB wine.

Figure 3.1 represents the microbiota at family level whereas Figure 3.2 shows the dominant microbiota by 10% of the total number of colonies obtained at different sampling period. The bacterial species shown in figure 3.2 were identified to be those that belonged to the families revealed in figure 3.1.

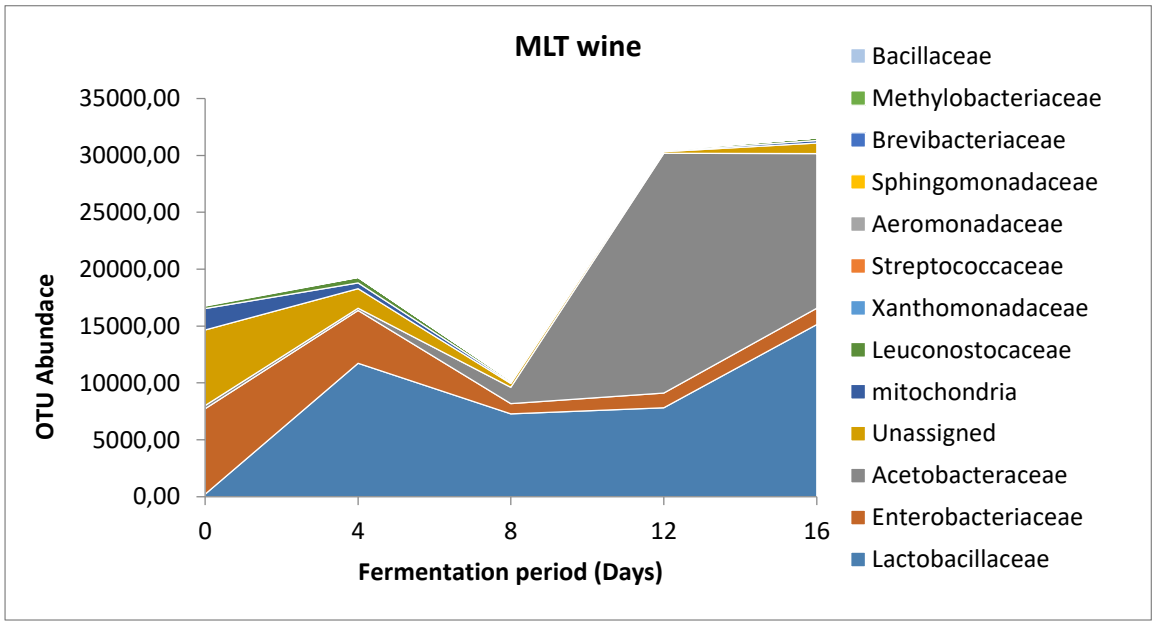

Figure 3.3: Profile of the bacterial families observed in the MLT wine.

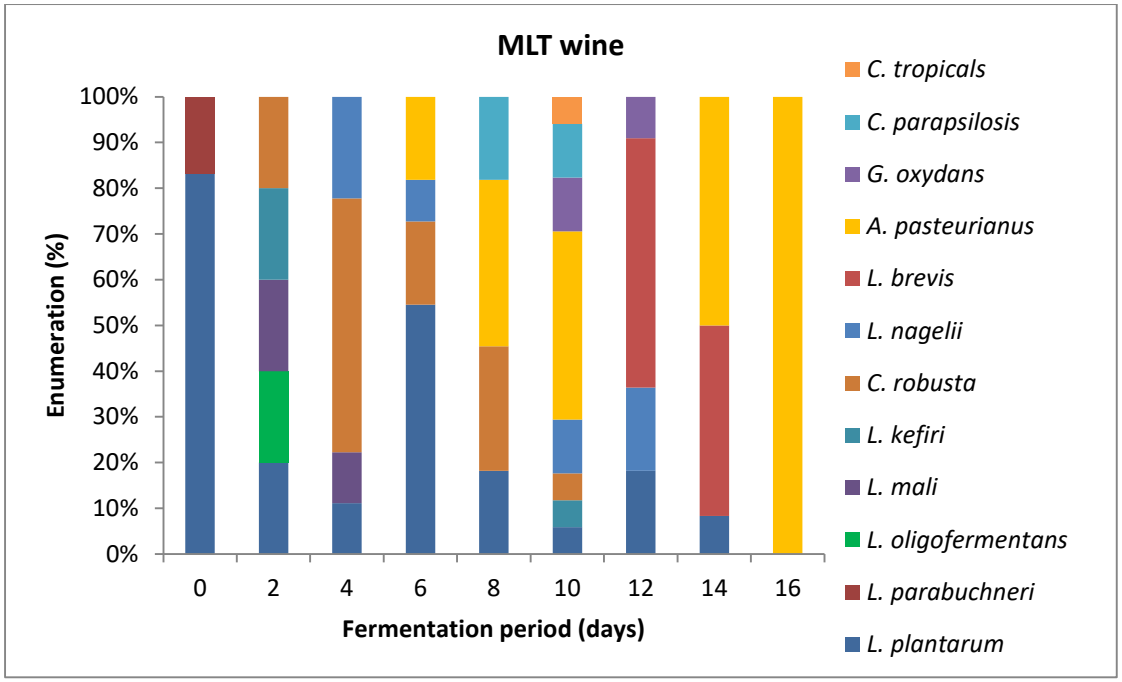

Figure 3.4: Profile of the microbial species observed in the MLT wine.

The NGS data clearly revealed the different families present in the marula juices and wines. The species such as the *L. plantarum, L. mali, L. kefiri, G. oxydans, A. pasteurianus, L. brevis* and *E. cloacae* that were identified from the marula juices (Days 0 in figures 3.2, 3.4.and 3.6) and the marula wines are those that belong to the families such as the Enterobactereaceae, Lactobacillaceae and Acetobacteriaceae (Figure 3.1, 3.3 and 3.5). These were found to be the most dominant. On the other hand, the species that were identified from the marula fruit skin (Table 3.2) belonged to the family Enterobacteriaceae, Bacillaceae and only one species belonged to the family Lactobacillaceae. The sequencing and biotyping assays revealed most of the microorganisms that were not detected on the marula fruit surface such as those belonging to the LAB and AAB groups.

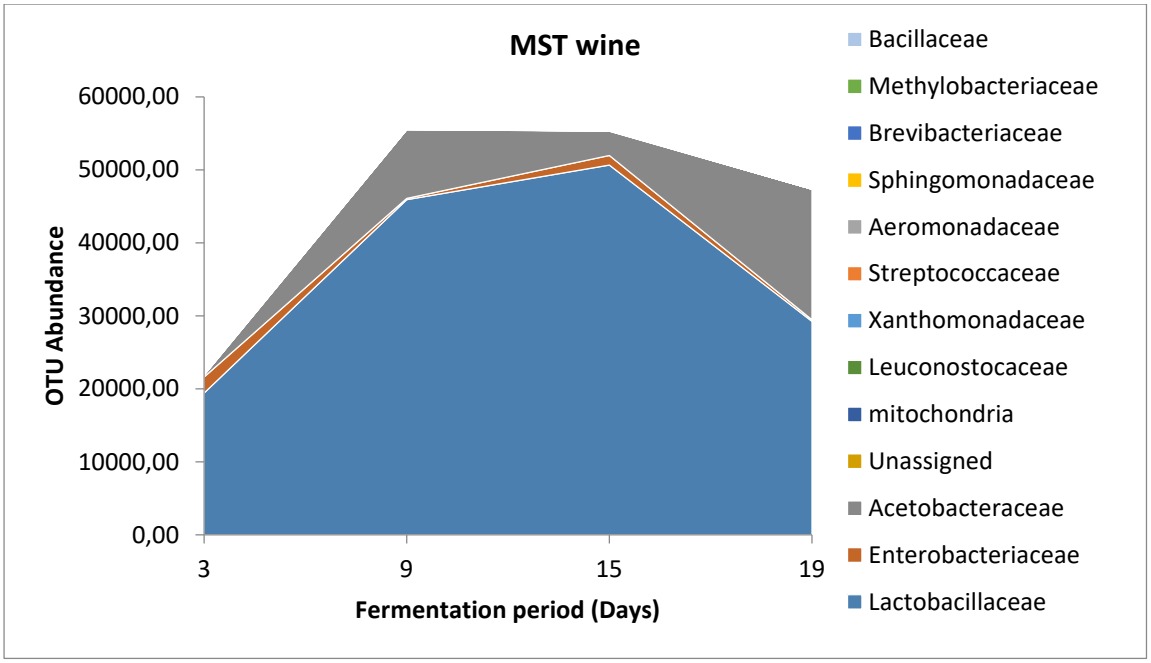

Figure 3.5: Profile of the bacterial families observed in the MST wine.

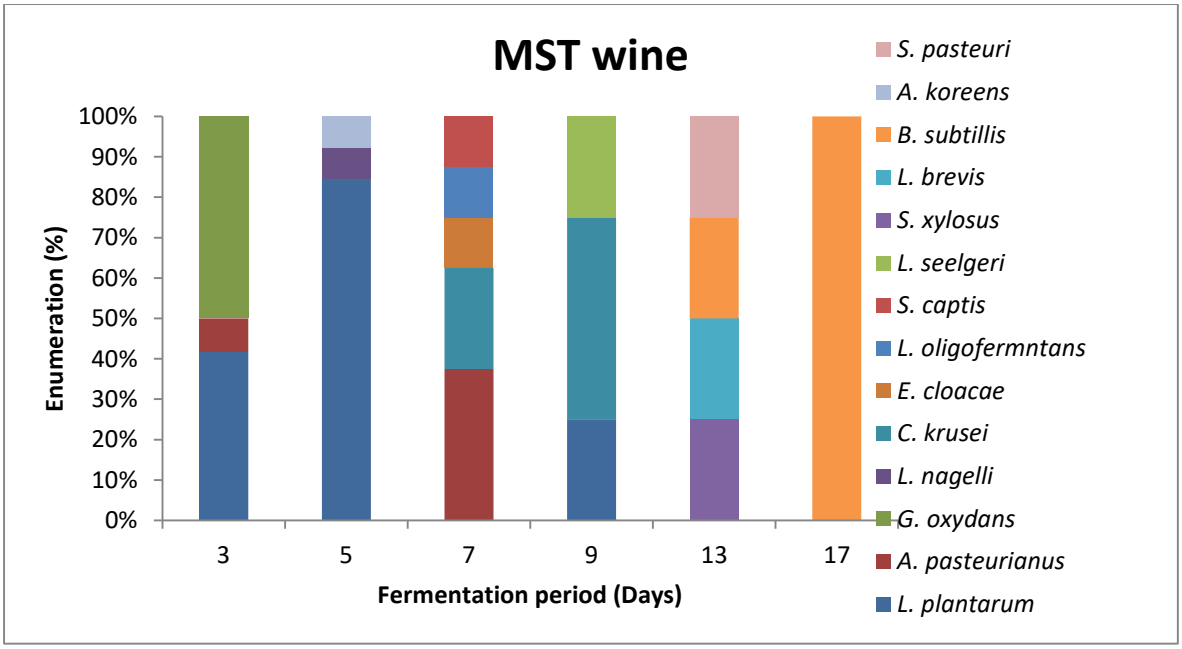

Figure 3.6: Profile of the microbial species observed in the MST wine.

Figure 3.5 represents the microbiota at family level whereas Figure 3.6 shows the corresponding dominant bacteria by 10% of the total number of colonies obtained. Notwithstanding that the juice data was missing due to sampling that started at day 3

of fermentation period, *E. cloacae* was the only bacterium present on the ripe marula fruits skin (Table 3.2) and in the wine.

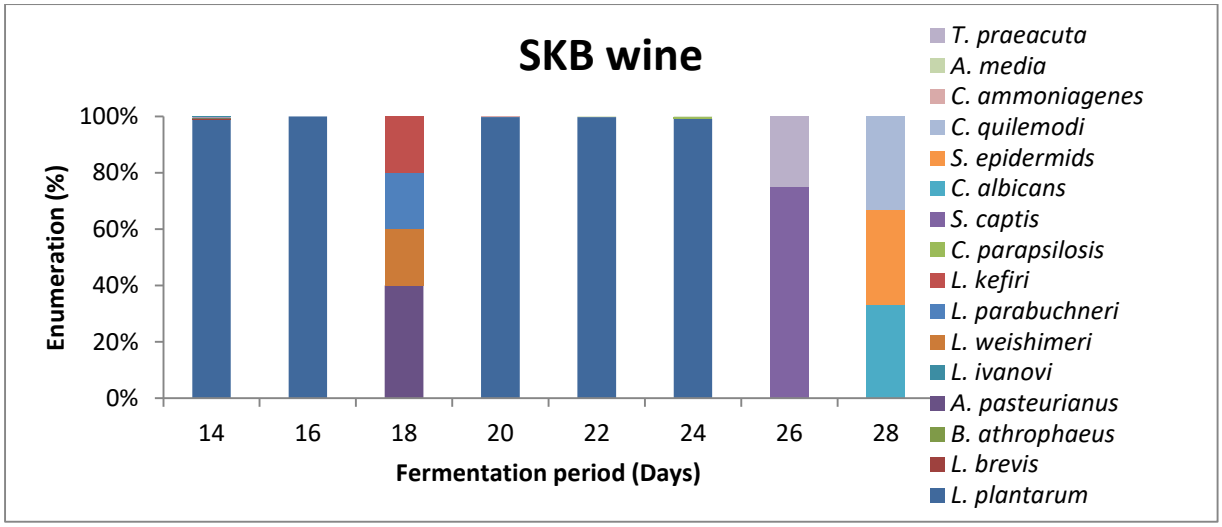

Figure 3.7: Profile of the bacterial species observed in the SKB wine.

3.3.3 Yeast microbiota present in the wine during fermentation

Both fermenting and non-fermenting yeasts were observed at different fermentation stages. Only six different yeast species were identified in the LAB wine, five in the MLT wine, four in the MST wine and two in the SKB wine. *Saccharomyces cerevisiae* was present in all the wines, *Meyerozyma caribbica* was present in three wines namely LAB, MST and SKB wines. The presence of *Pichia guilliermondii* was observed in the LAB and MLT wine at the early stages of fermentation (day 0 to 4). *Hanseniaspora guilliermondii* and *Rhodotorula mucilaginosa* were observed in all the wines except the SKB wine. Non-fermenting yeasts were present in the marula juice (day 0) and at the early stages of fermentation (Days 2 and 4) with *H. guilliermondii* and *M. caribbica* as the dominant isolates (Figures 3.9, 3.11, 3.13 and 3.15). The NGS data revealed the presence of the Saccharomycetaceae yeasts throughout the fermentation period (Figures 3.8, 3.10, 3.12 and 3.14). The presence of various families such as Botryosphaeriaceae and Amphisphaeriaceae were also observed during the early stages of fermentation.

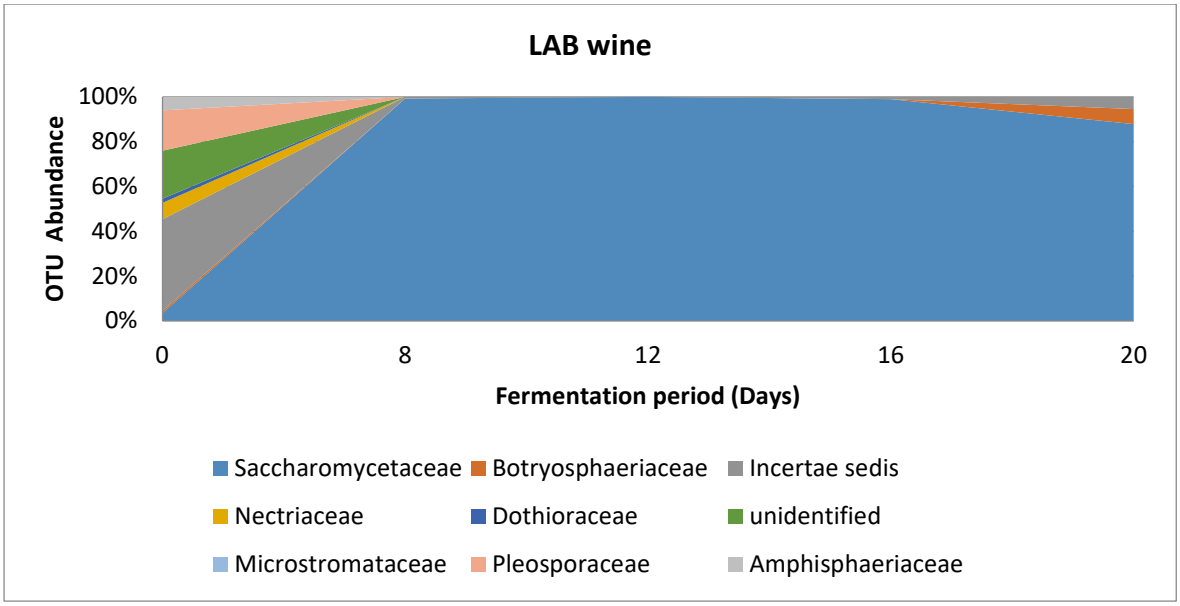

Figure 3.8: Profile of the yeasts families observed in the LAB wine.

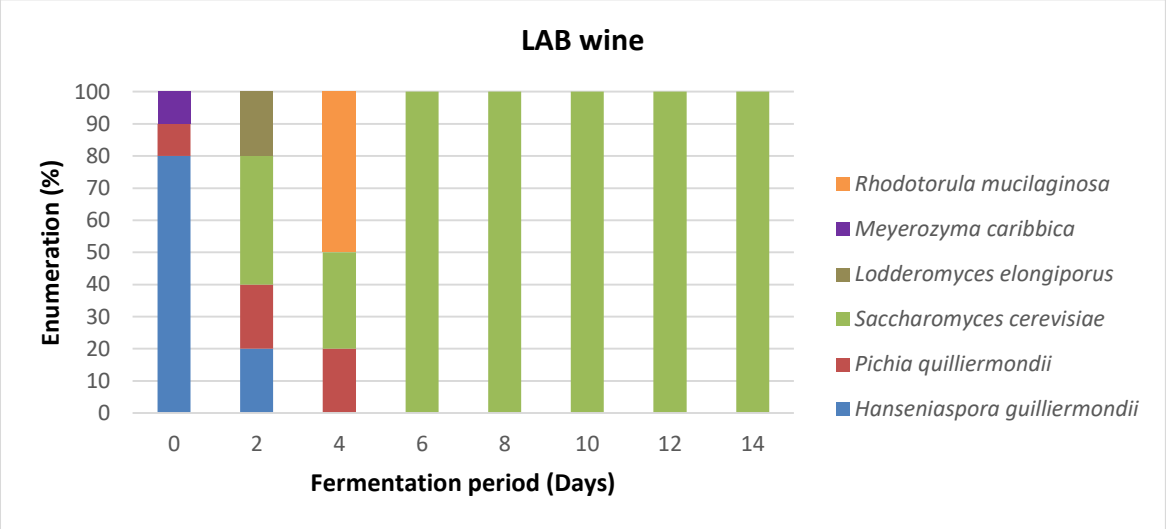

Figure 3.9: Yeasts species from the LAB wine.

Figures 3.8 and 3.9 indicate the families and species that are present in the marula wine, respectively. Various families were observed at the beginning of fermentation such as the Botryosphaeriaceae and Amphisphaeriaceae (Figure 3.8), however, only the Saccharomycetaceae yeasts were present from day 6 and this correlates with the dominance of *Saccharomyces cerevisiae* in figure 3.9.

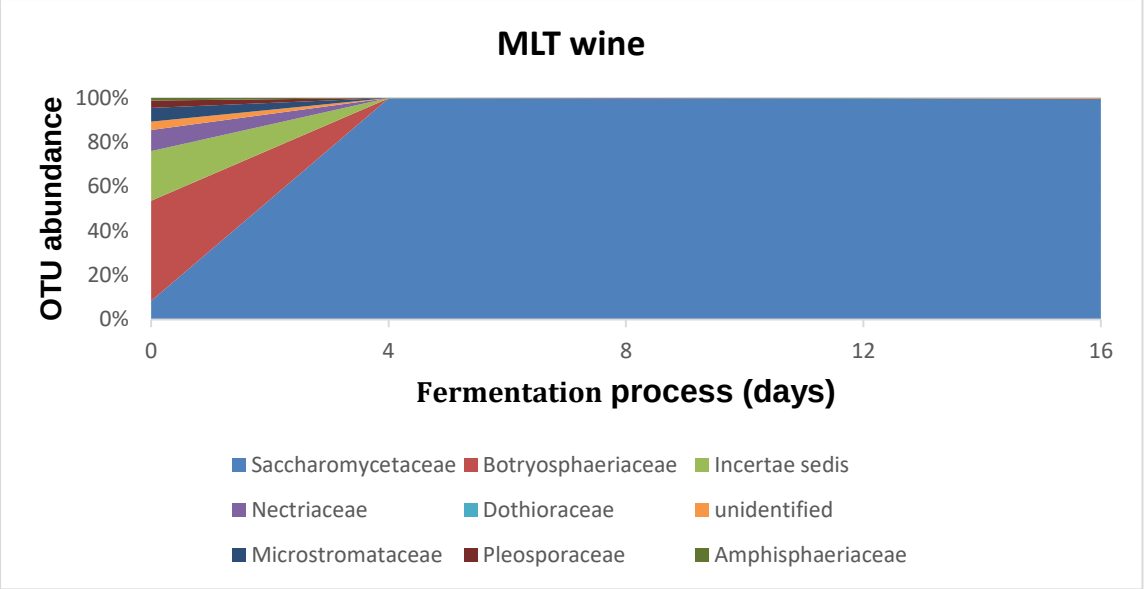

Figure 3.10: Profile of the yeast families observed in the MLT wine

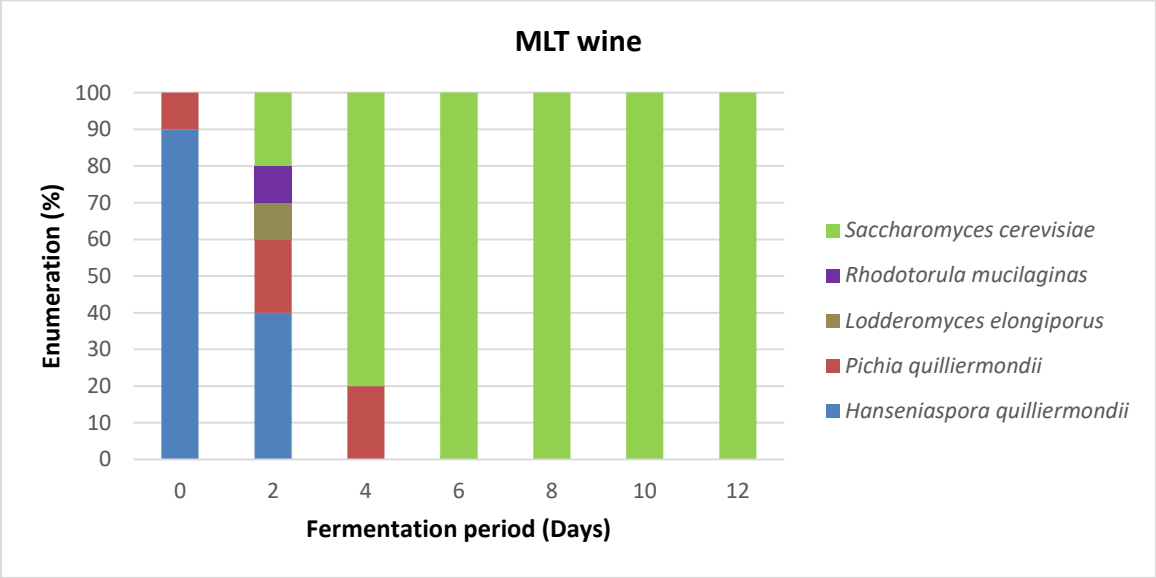

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Figure 3.11: Yeasts species from the MLT wine.

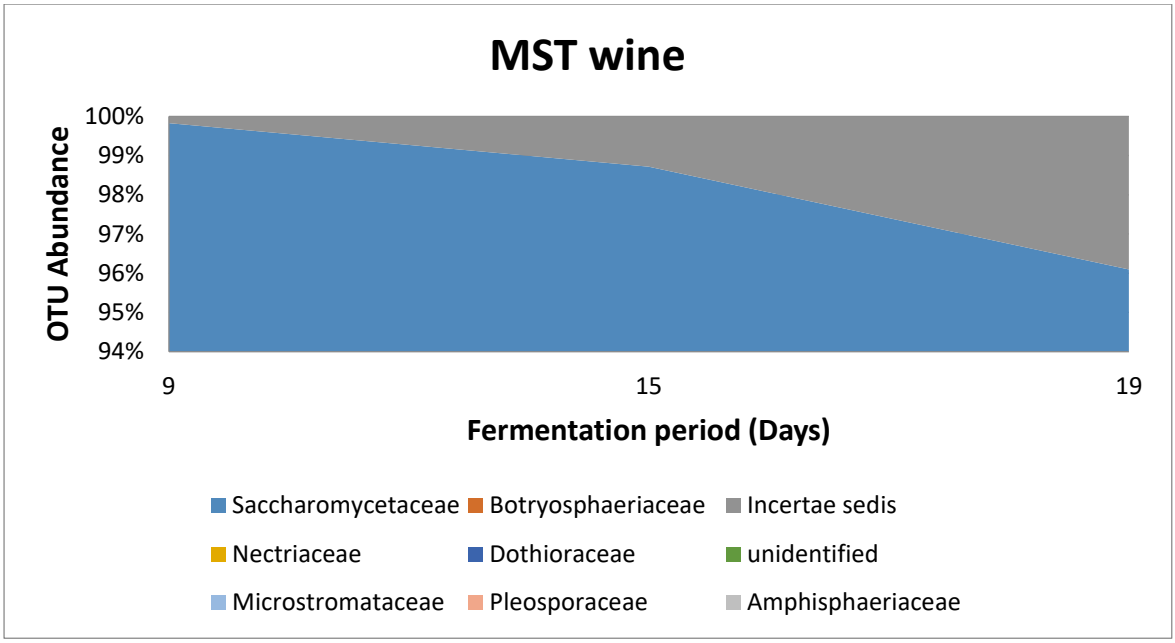

Figure 3.12: Profile of the yeasts families observed in the MST wine.

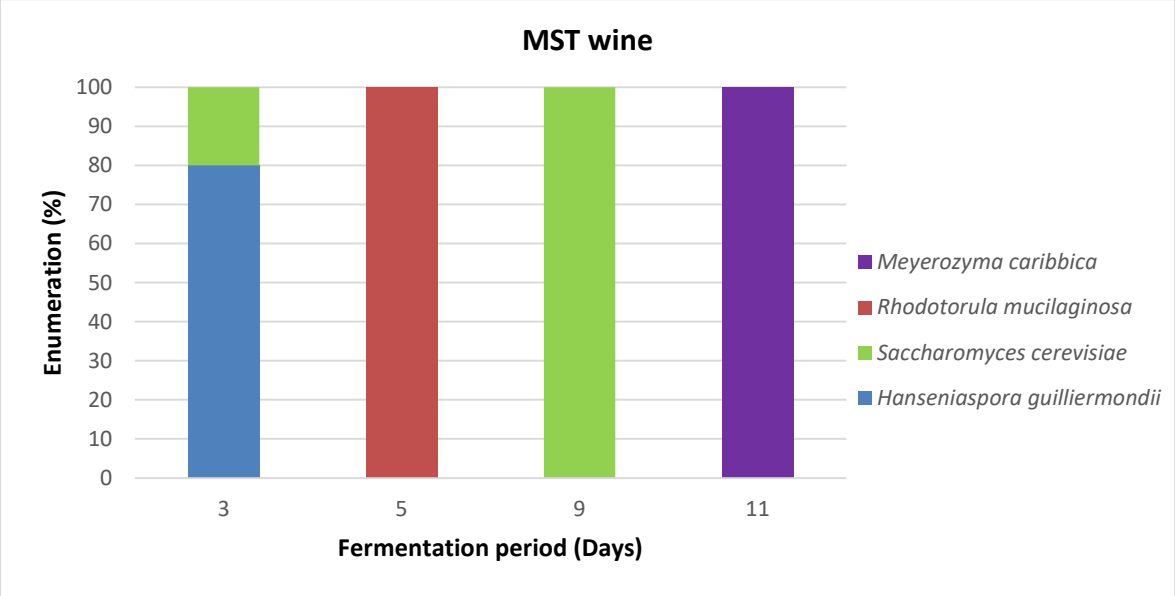

Figure 3.13: Yeasts species from the MST wine.

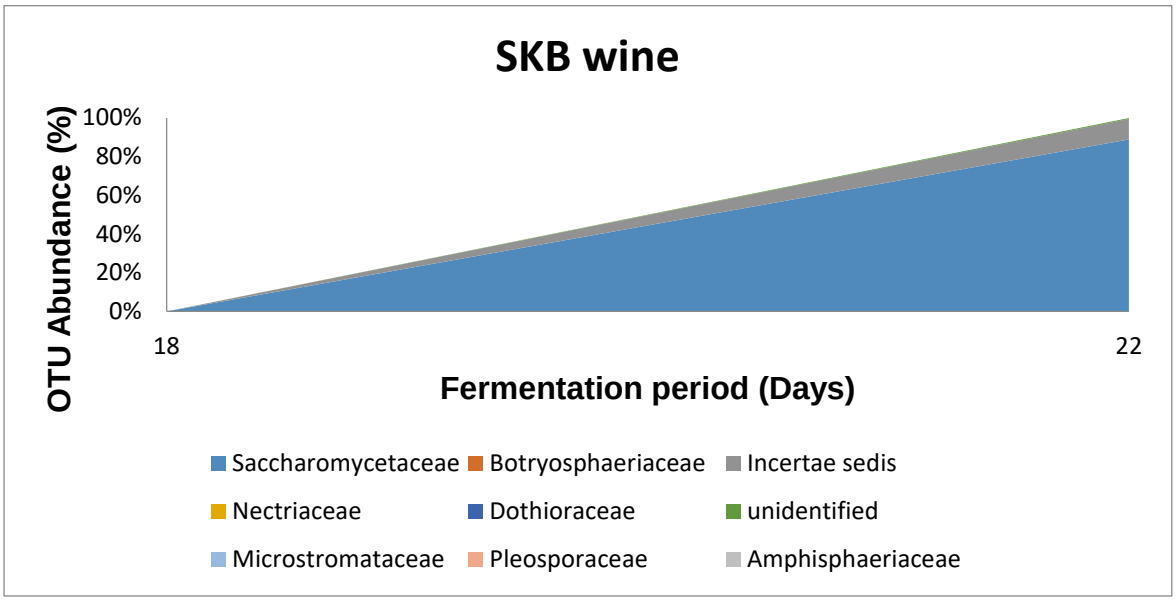

Figure 3.14: Profile of the yeasts families observed in the SKB wine.

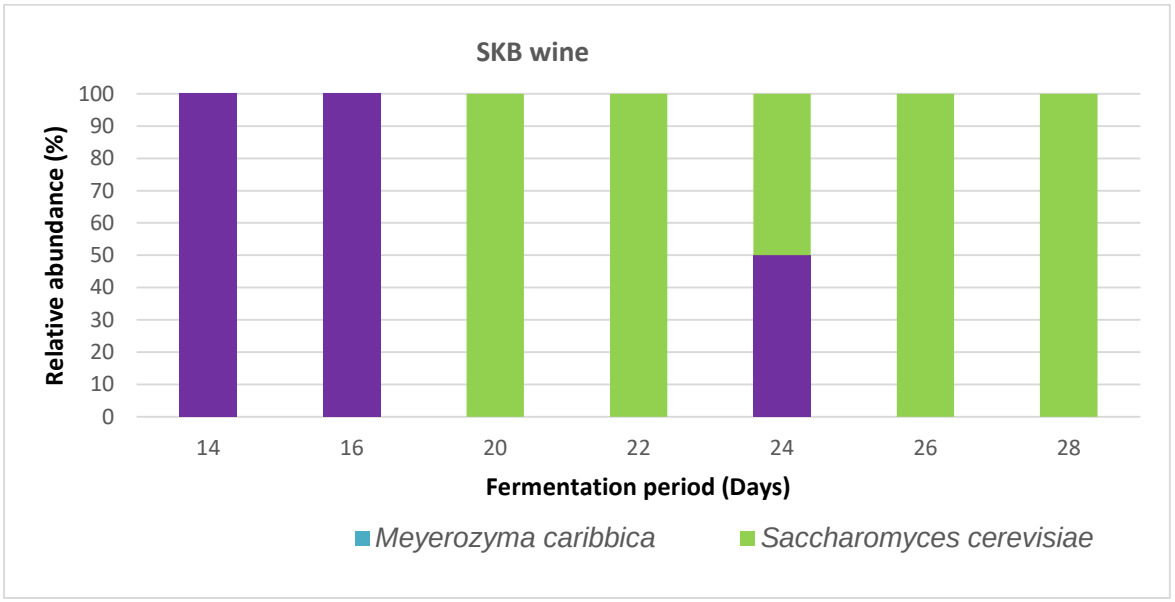

Figure 3.15: Yeasts species from the SKB wine.

Figure 3.10, 3,12 and 3.14 indicate the families of the yeasts obtained from the Oaks village and Makhushane village. The MLT wine (Figure 3.10) showed diverse families whereas the MST and SKB wine only showed the presence of Amphisphaeriaceae and Saccharomycetaceae yeasts. *Saccharomyces cerevisiae* was observed as the dominating yeast strain in all the wines (Figure 3.9,3.11 and 3.13).

3.4 Discussion

This study evaluated the evolution of bacteria and yeasts in four marula wines obtained from different localities with the aim of achieving a profile of the contributing microorganisms to the characteristic taste and aroma of a typical marula wine. The microbial analysis of the ripe marula fruit skins revealed the presence of diverse microorganisms that are known to be associated with soil, animals and humans. These included species such as *B. subtillis*, *E. ludwigi, E. aerogenes, E. radiniatants, K. oxytoca, K. pneumoniae*. Fermenting microorganisms such as *L. lactis* were also isolated. The near-absence of LAB organisms on the marula fruit surface was also observed and reported by Bokulich and colleagues (2013). Microorganisms such as *B. subtilis* isolated from these fruits surface commonly contribute towards breaking down of carbohydrates during the early days of fermentation (Mukherjee *et al.,* 2008). *L. lactis* isolated from the ripe marula fruits is commonly known for malolactic fermentation. This bacterium is mostly associated with plant surfaces, animal skin and hair and it is present in the gastrointestinal tracts of animals (Bauer and Dicks, 2017). Enteric bacteria were previously reported to dominate the surface of fresh fruits such as grapes and vegetables like lettuce (Leff and Fierer, 2013) as was the case in this study. The Enterobacteriaceae species identified in the ripe marula fruits such as *Klebsiella oxytoca, Raoultella omithinolytica* and *Enterobacter cloacae* were pathogenic and their presence on the ripe marula fruits could be attributed to handling during collection or they could be emanating from the soil that has received animal contamination. Feng and colleagues (2015) reported that fruits are exposed to a wide variety of pathogens such as bacteria, viruses, fungi and nematodes. The bacterial species isolated and identified from the ripe marula fruits were observed to be similar irrespective of the area at which the marula fruits were collected. The results obtained in this study were similar to those obtained by Bokulich and colleagues (2013) who observed the presence of Enterobacteraceae and *Bacillus* species through many seasons. They argued that the different microbial species that are associated with fruits surface could possibly be supporting the reproducibility and regionality of wine sensory characteristics (Bokulich *et al.,* 2013).

The wines were sampled at different intervals during the fermentation period for the analysis of bacteria and yeasts profiles and their evolution. The microbiological profiling of the marula wines revealed dominance by the LAB, AAB, Enterobacteria and *Saccharomyces* yeast during the fermentation period. Microorganisms isolated and identified from different marula wines showed high diversity in the microbial community of the marula wine. A progression of LAB and AAB with both the non-fermenting and fermenting yeasts was observed throughout the fermentation. The occurrence of the Lactobacillaceae microorganisms was observed throughout the fermentation process. The Lactobacillaceae species are either homofermenters (*L. mali*) or heterofermenters (*L. brevis*) and this enables them to survive different conditions such as the presence of different acids and alcohols such as ethanol and propanol (Costantini *et al.,* 2009). The occurrence of Acetobacteraceae was observed from early stages of fermentation but at a very low level. However, their increase during the late stages of fermentation is attributed to their ability to survive sugary, acidic and alcoholic environments (Kersters *et al.,* 2006). Similar report was given by Nielsen *et al.* (2007) and the work was based on the traditional fermentation of Ghanaian cocoa.

The presence of LAB and AAB during the spontaneous fermentation period was previously reported by Dlamini and Dube (2008). Bacterial species such as *L. plantarum, L. paracasei, L. brevis, L. curvatis*, *L. sharpie* and *L. rauturi* were identified. *L. rauturi* are commonly known for their participation in the malolactic fermentation and were present in marula wine. The presence of *L. plantarum* was reported as the most active species in the grape wines (Berbegal *et al.,* 2016). The occurrence of *L. plantarum* was also reported in palm wines and it was reported to be responsible for the early acidification of the wine (Amoa-awua *et al.,* 2007). The acetic acid bacteria such as *A. pasteurianus* and *G. oxydans* dominated the late stages of fermentation (from day 6). *A. pasteurianus* grows best at pHs between 5.5-6.3 and temperatures of 25-30℃ (Wang *et al.,* 2015). It is one of the most common organisms responsible for spoilage during storage and ageing because of its ability to metabolise ethanol. Similarly, the obligate aerobe *G. oxydans* utilises alcohol as its primary substrate to produce acetic acid and its continuous presence in wines was reported by Joyeux and colleague (1984).

The Enterobacteriaceae were only detectable during the early stages of fermentation. This group of microorganisms does not survive in acidic and alcoholic environments. The presence of these microorganisms could be an indication of contamination of the wine during the harvesting/handling and processing of the marula fruits and wine. Schutte (2013) obtained similar results which indicated that hygienic standards during traditional fermentation is often poor and therefore, microbial contaminants are possible.

Non-fermenting yeasts such as *Hanseniaspora guilliermondii* and *Meyerozyma caribbica* were only observed during the early stages of fermentation. The non-fermenting yeasts have low tolerance for ethanol and they disappeared from mid to late stage of fermentation at which ethanol production increases in the wine. Expectedly, and similar to a report by Torija and colleague (2001), *S. cerevisiae* was present throughout the fermentation period. *Saccharomyces* is responsible for alcoholic fermentation and grows optimally at slightly acidic environment of pH5 which supports alcoholic fermentation. This lowering of the acidity is attributed to the metabolic activity of the bacteria which produce organic acids under fermentative conditions. Anaerobic conditions promote fermentation and in turn give rise to a selective pressure which inhibits growth of microorganisms that are incapable of fermentative metabolism such as fungi (Pretorius, 2000).

The practice of spontaneous fermentation in this study highlights the importance of indigenous microbial species present on the marula fruit skin, juice and wine. The microbial ecosystem of marula fruits and wine can be used by winemakers and oenologists as a decisive factor to influence wine aroma and consumer's preferences.

CHAPTER 4: CHEMICAL PROFILING

Determination of the chemical composition of Marula juice and wines during fermentation

4.1 Introduction

Alcoholic beverages are made from various types of fruits, and marula fruits have long been used to produce both alcoholic and non-alcoholic beverages for the local market. Depending on the fruit used, there are parameters that are important to keep the end product acceptable to the consumers, these parameters includes the colour, aroma, texture and distinctive taste (Hough and Garitta, 2012). The aroma and flavour profile of wine are the result of an almost infinite number of variations in the different types of chemical compounds present in the brew. The factors that are mostly responsible for the aromatic profile in the wines are the volatile compounds, which are produced either by the fruit plant as a feature of its breed or by yeasts during the alcoholic fermentation. Aroma, which is due to a complex mixture of volatile compounds, is one of the most imperative characteristics for defining wine quality (Berenguer *et al.,* 2016) and these volatile compounds are predominantly formed during the alcoholic fermentation. The typical aroma and flavour compounds present in common wines such as grape wine include isoamyl alcohol, isobutanol and acetic acid, acetaldehyde, *n*-propanol and ethyl acetate. The aroma and flavour compounds originate from the fruits, compounds formed during extraction of juice, compounds produced by yeasts and bacteria during alcoholic and malolactic fermentation and from compounds that appear during the maturation process (van Antwerpen, 2012). Yeast is generally the major contributor for modifying aroma, flavour, mouth-feel, colour and chemical complexity of fruits wines (Chen *et al.,* 2013).

Nutrient composition of the fruits also contributes to the sensory quality of the resulting wine as it affects the viability of fermenting microorganisms and thus reduce the sensorial quality of the final wine. Availability of nutrients in fruits is variable and depends on natural factors such as vineyard characteristics, climatic conditions, fruit maturity, and microbial strains (Fugelsang and Edwards, 2006). The volatile

compounds produced by wine microorganisms (mainly yeasts) in wines include higher alcohols (fusel, marzipan and floral aromas), medium- and long-chain volatile acids (fatty, cheesy and sweaty aromas), acetate esters and ethyl esters (fruity and floral aromas) and aldehydes (buttery, fruity and nutty aromas) (Styger *et al*., 2011).

This part of the study sought to analyse the different chemical compounds that are present in the marula juice and those produced during fermentation. Chemical composition analysis is done in order to assess wine quality and characteristics in relation to different fermentation periods and the metabolites being produced such as the acids and alcohols.

4.2 Materials and methods

4.2.1 Wine sampling

Using a sterile tube, 50 mL of the wine was collected at each interval and centrifuged at 13000 rpm for 5 minutes. The liquid portion was transferred to a new sterile 50 mL centrifuge tube, which was closed tightly and stored at -20℃.

a. HPLC analysis of sugars

The quantities of sucrose, glucose and fructose in the marula wines were determined with high performance liquid chromatography (HPLC). The column used was a Rezex RHM monosaccharide H^+ (300 x 8 mm), and de-ionised water was the mobile phase. The column was operated at a temperature of 85℃ and a flow rate of 0.6 mL/min. A sample volume of 20 µL was used. A refractive index detector was used to detect the separated components. Prepared standards of sucrose (0.390 mg/mL to 400 mg/mL), fructose (0.390 mg/mL to 400 mg/mL) and glucose (0.390 mg/mL to 400 mg/mL) were used to calculate the concentrations of the sugars.

b. Determination of sugar levels and total alcohol content of LAB wine and MLT wine by refractometer

The sugar content was determined by measuring the ˚Brix of the fermenting wine using a refractometer while the alcohol percentage was calculated from the values of original gravity and final gravity using the following formulas: $ABW = 76.08\frac{(OG-FG)}{(1.775-OG)}$ and $ABV = ABW(\frac{FG}{0.794})$.

c. Determination of volatile organic compounds in the LAB and MLT juice and wine by GC

MLT and LAB wines were monitored and sampled from day 0 of fermentation whereas MST and SKB wine were collected at days 3 and 14 of fermentation, therefore MLT and LAB wines were selected for further volatile organic compounds analysis. The analysis of volatile organic compounds was performed with Gas chromatography (GC), (Shimadzu model) with Nukol™ Capillary GC column (30 mm x 0.25 mm) at a flow rate of 1.29 mL/min. Nitrogen gas (carrier gas) was used as the mobile phase using the flow index detector (FID) 2 at a flow rate of 16 mL/min. A sample volume of 1µL was injected into the GC using AOC-20i auto-injector. Volatile organic compounds concentration was quantified by liquid chromatography (LC) solution software version which served to integrate peaks at different retention times based on the calibration curve construction from standard solutions. Compounds such as ethyl acetate, isovaleric acid, hexanoic acid, n-butanol, iso-butanol, ethyl isobutyrate, isobutrylacetate, isoamyl lactate, isoamyl-alcohol, ethyl lactate, ethyl caprylate, 1-octen-3-ol, propanoic acid, isobutyric acid and 2-phynethyl-acetate were used as standards to identify the unknown compounds. Standard concentrations ranged between 0.125 mg/L – 1000 mg/L.

d. Determination of ethanol content of the four marula wines by GC

Ethanol content was determined by GC-2010 Plus Shimadzu. A ZBWAX PLUS column was used with a maximum temperature of 260 ℃. Hellium was used as the carrier gas at a flow rate of 53.74 mL/min at a pressure of 100.0 kPa. A volume of 1µL of the sample was injected to the GC using s split syringe AOC-20i + s at 250 ℃. The ethanol

concentrations were determined by using known ethanol standards (0.625 g/L - 20 g/L).

4.3. Results

A report on the findings of the chemical composition of different marula juice and wines at different fermentation periods is provided.

Marula juice contained more sucrose sugar than fructose and glucose. Furthermore, the juice, which is denoted by day 0 in the representations, generally had little to no volatile compounds. However, different volatiles were observed during the fermentation period with variations at the different stages. The dominating and common compounds in all the wines included ethanol, ethyl acetate and n-butanol.

4.3.1 Sucrose, fructose and glucose content

Sucrose was found to be the most abundant sugar amongst the three sugars analysed in this study (Figures 4.1 and 4.2).

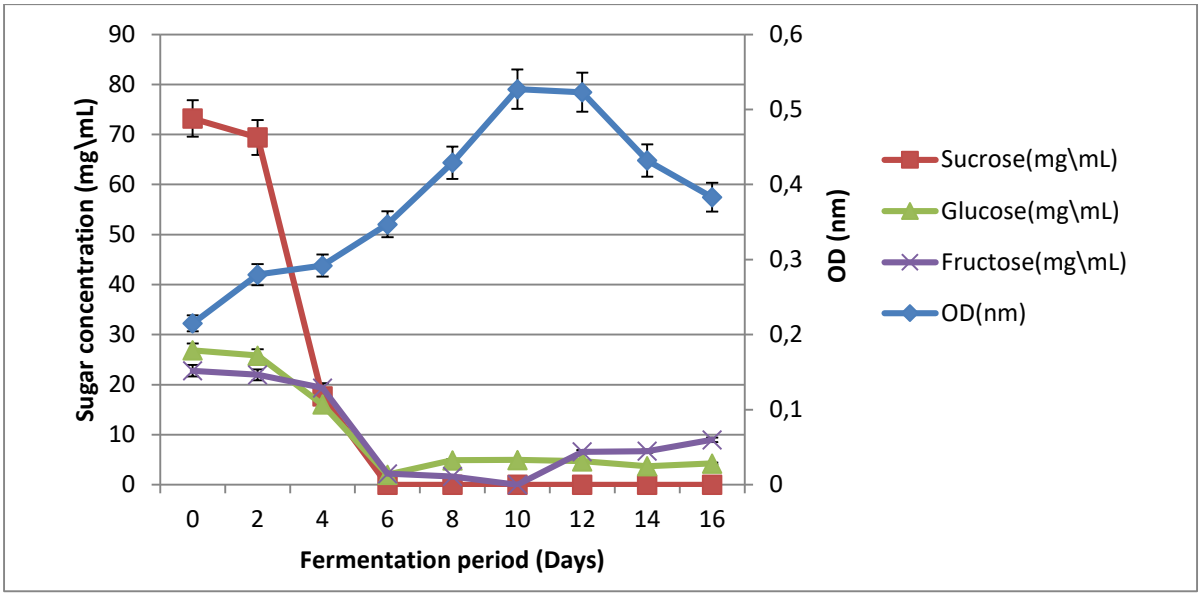

Figure 4.1: Profile of sugars and the microbial load in the LAB wine.

For the LAB and MLT marula wines LAB and MLT, a high sucrose level was observed at day 0 and the level decreased with a corresponding increase in microbial load as fermentation progressed (Figures. 4.1 and 4.2). The concentrations of sucrose, fructose and glucose in the marula juice (Day 0) were 73.20 mg/mL, 22.78 mg/mL and

26.87 mg/mL, respectively (Figure 4.1). The juice and wine from the University of Limpopo (LAB) were observed to have high content of sugars followed by the MLT juice (Day 0) and wine (Figure 4.2).

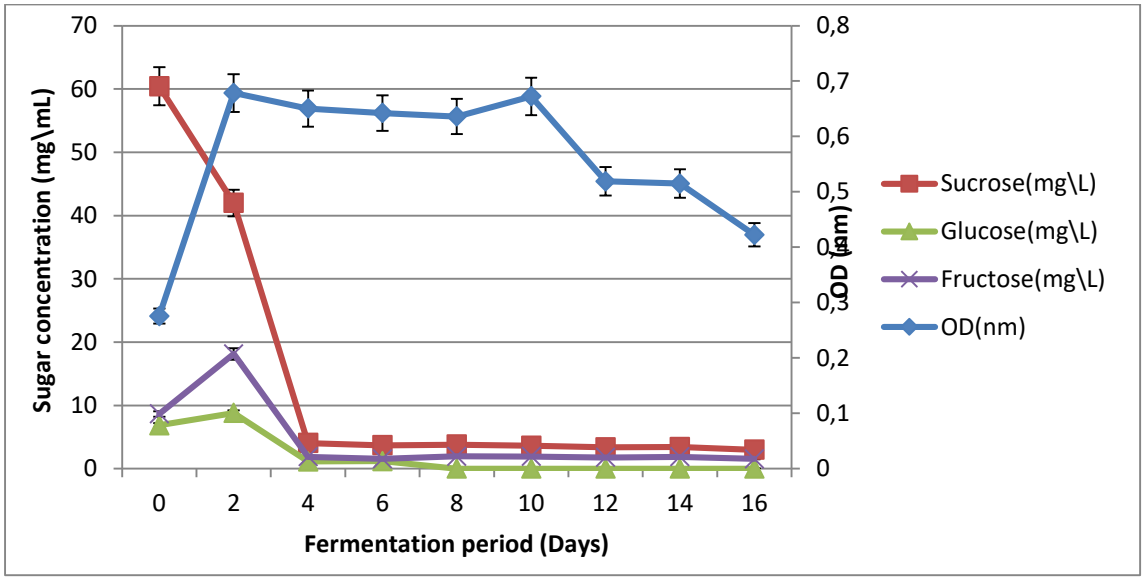

Figure 4.2: Profile of sugars and the microbial load in the MLT wine.

The levels of sugars declined drastically during the first four days and levelled off at the concentrations of 7320 mg/mL and 60.43 mg/mL for sucrose, 26.87 mg/mL and 8.80 mg/mL for glucose and 22.78 mg/mL and 8.66 mg/mL for fructose for LAB and MLT wines respectively. Evidently, the glucose levels were lower than the levels of fructose during the mid to latter stages of fermentation as depicted in figures 4.1 to 4.3. In contrast, the microbial load for the LAB wine increased gradually in 10 days by 2.7 fold when compared to the same level of increase with the MLT which was achieved in 2 days.

The profiles of sugars for MST wine (Figure 4.3) were similar to those of LAB and MLT wines and the gradual decline in fructose level was apparent when compared to sharp decrease in glucose levels. However, the SKB wine revealed complete utilisation of glucose and fructose (Figure 4.4) when the wine was stored and allowed to continue to ferment.

The levels of total sugars in the MLT and LAB wines, as depicted in figure 4.5, were similar to those of sucrose, glucose and fructose (Figures 4.1 and 4.2). Furthermore, better usage of the sugars coincided with a better yield in alcohol content.

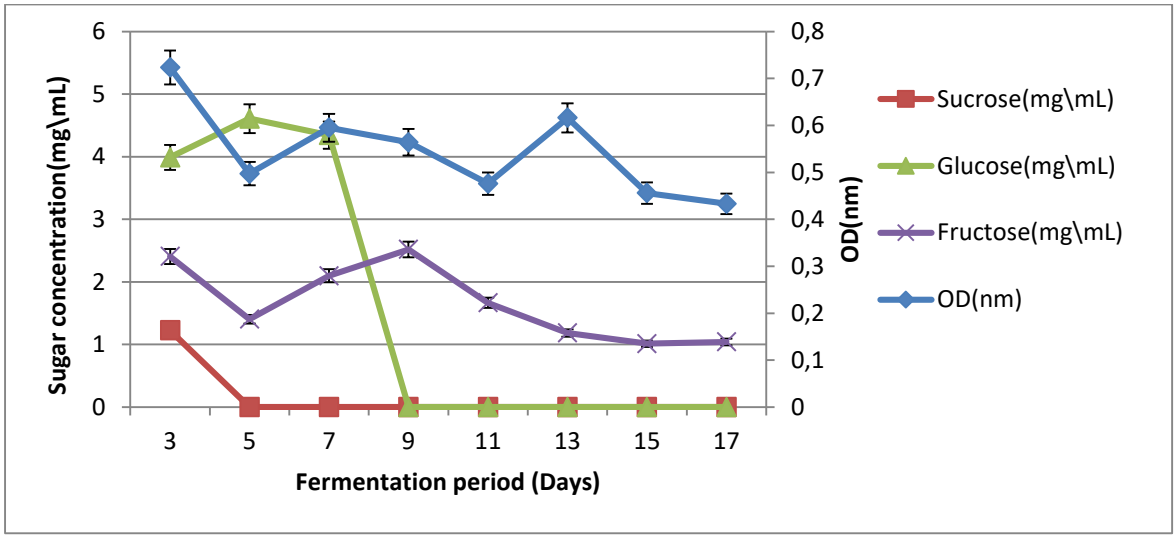

Figure 4.3: Profile of sugars and the microbial load in the MST wine.

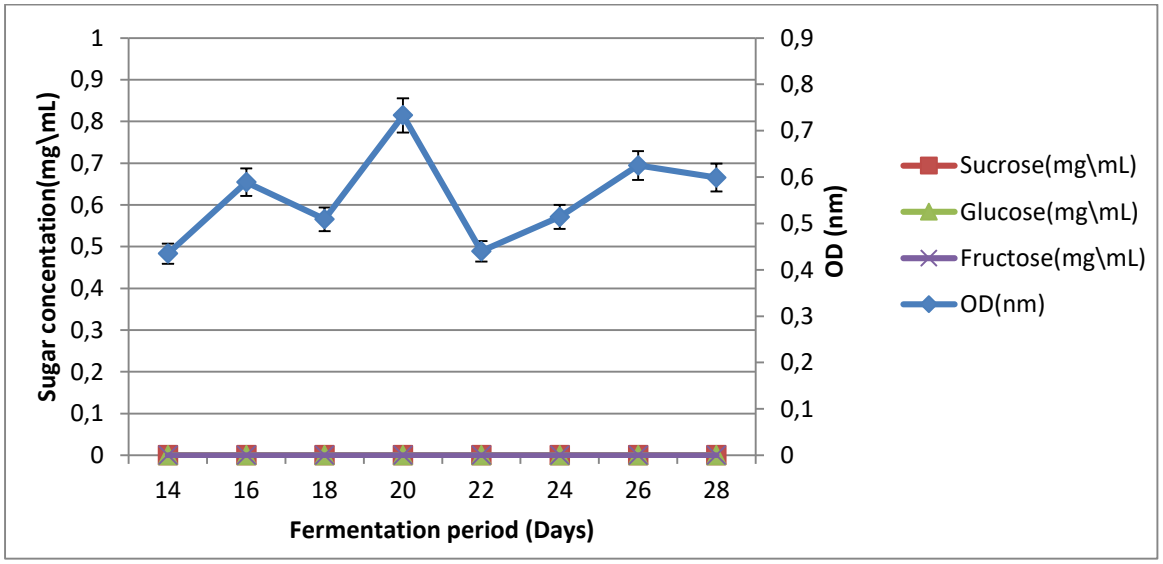

Figure 4.4: Profile of sugars and the microbial load in the SKB wine.

4.3.2 Sugar levels and total alcohol content of LAB wine and MLT wine

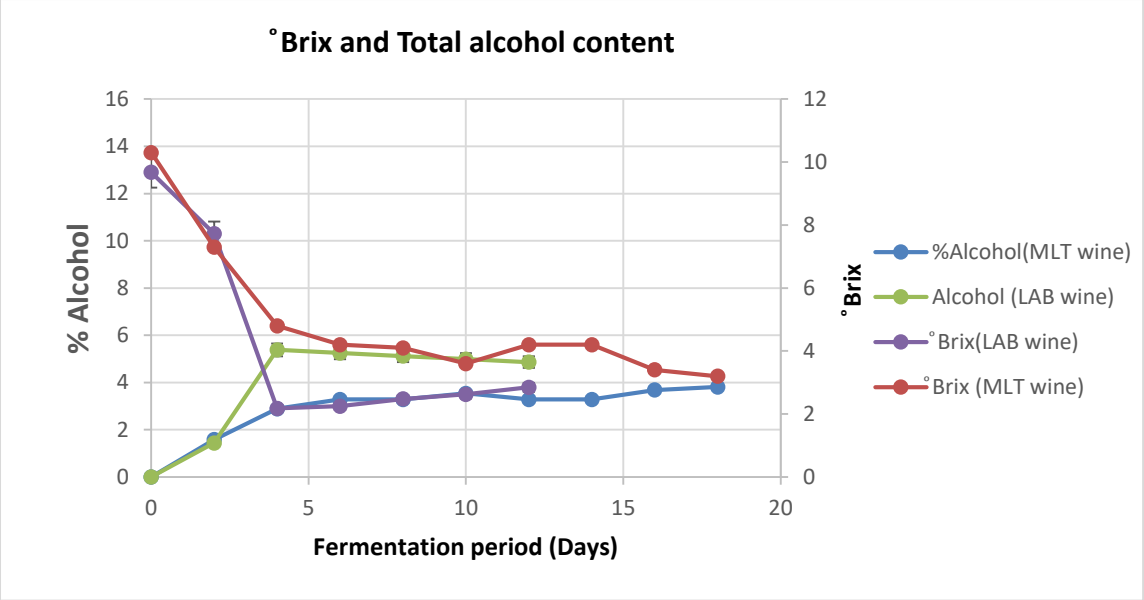

Figure 4.5: Changes in total alcohol levels during marula fruit juice fermentation in LAB wine and MLT wine.

4.3.3 Volatile organic compounds analysis

Volatile organic compounds are metabolites that are produced during fermentation and are considered vital to wine quality and aroma. It is thus of great importance to understand the type of compounds that are present in the juice and that are produced during fermentation as well.

MLT and LAB wines only were analysed in order to trace the profiles from the juice state of the wines since the MST and SKB wines were sampled at days 3 and 14 respectively. Ethyl-acetate was the abundant compound in both the MLT and LAB wines and its concentration increased as the fermentation period increased (Figures 4.6 and 4.7). Interestingly, none to very little of the volatile compounds were detected in the marula juice (Day 0).

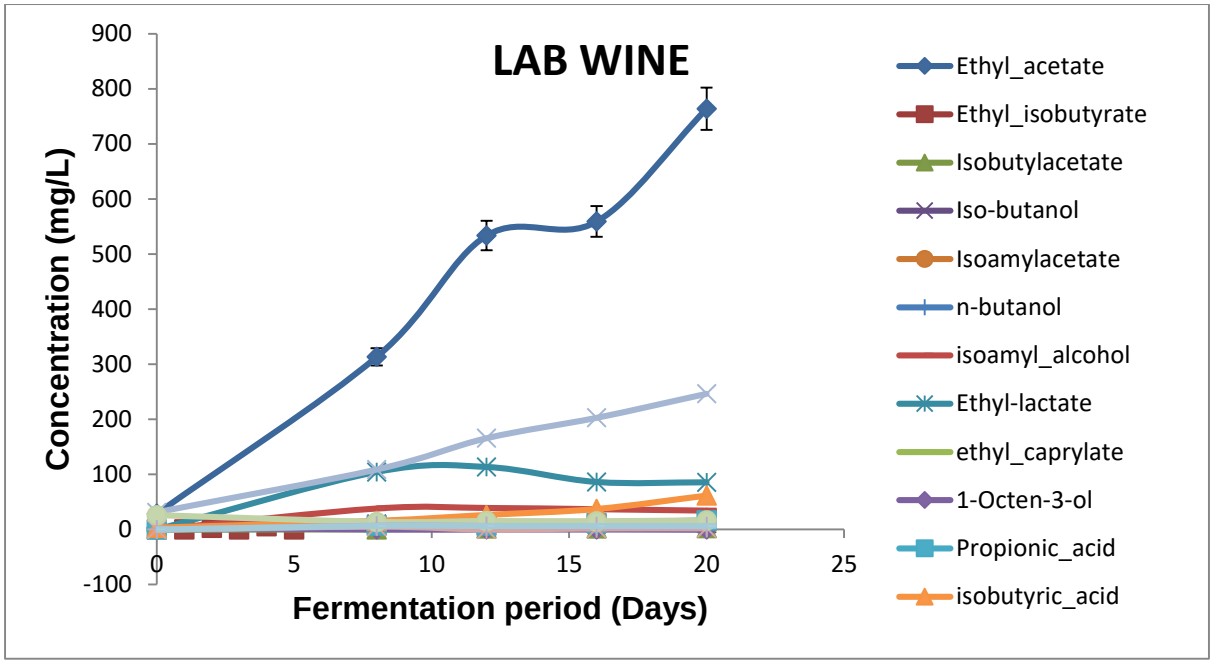

Figure 4.6: Volatile organic compounds of LAB wine.

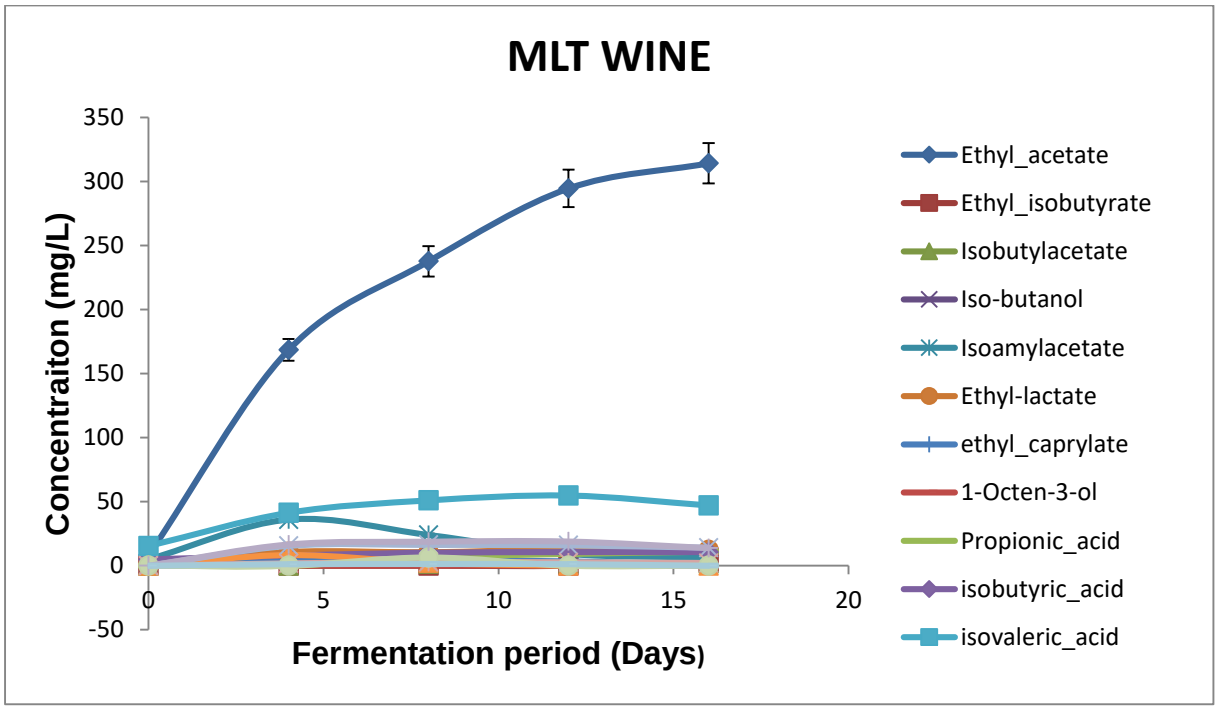

Figure 4.7: Volatile organic compounds of MLT wine.

Ethyl-acetate was found to be the most abundant compound in both the MLT and LAB wines followed by isovaleric acid. The production of these compounds increased with the progression of fermentation process. Higher alcohols such as n-butanol and

isobutanol were low in amount throughout the fermentation period. There was no apparent similarity in the pattern of volatiles detected between the two wines.

4.3.4 Ethanol content of the four marula wines

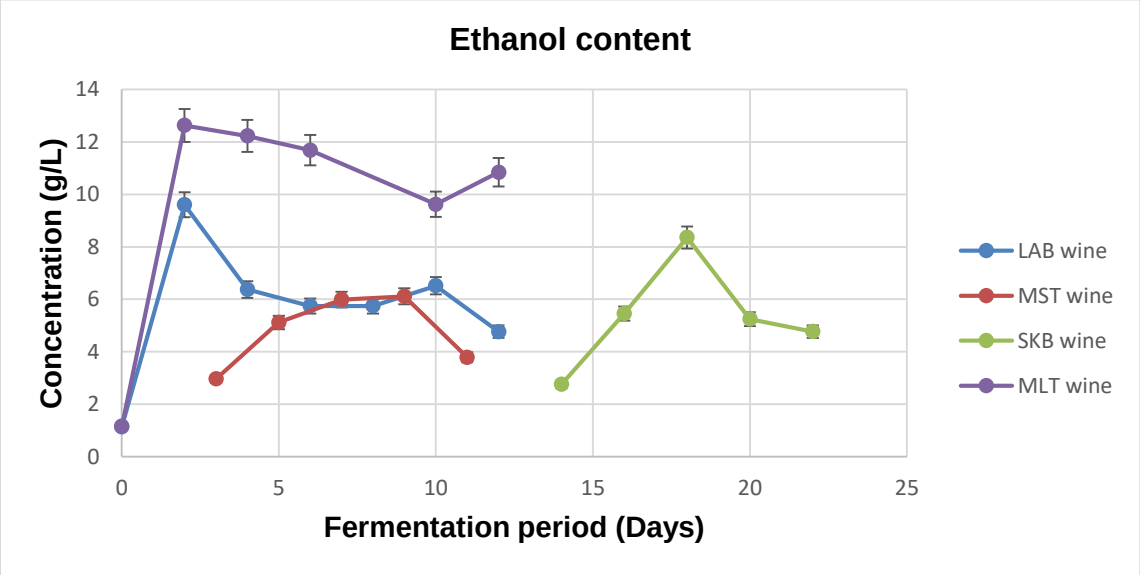

Figure 4.8: Changes in ethanol concentration (g/L) of various marula juices and wines at different fermentation period.

The levels of ethanol were higher in the initial stages of fermentation for LAB and MLT wines and declined gradually thereafter. This pattern coincided with the gradual and proportional rise in ethyl acetate levels observed in figures 4.6 and 4.7. Furthermore, the sharp increases in ethanol levels in all the wines in figure 4 was shown to correspond to the increase in microbial loads (Figures 4.1-4.4) in the same fermentation periods.

4.4 Discussion

This study evaluated the changes in chemical profiles of marula wines during fermentation. Marula juice and wines were profiled for the concentrations of sucrose, glucose and fructose, total sugar, total alcohol, ethanol and other vital volatile organic compounds. Only the LAB and MLT wines were selected for the volatile organic compounds since these wines were sampled from the juice state that was represented

by day 0. The pattern of these compounds was found to be similar between the different areas, with slight differences in the concentrations. These slight variations could be attributed to the differences in the quality of marula fruits in different localities (Moganedi *et al.,* 2011), the starting concentrations of sugars and the type of contributing microbiota in the wines. It was apparent that the microbiota utilised the sugars for growth and the clear absence of volatile compounds in the marula juice signify that these metabolites were produced during active growth of the microorganisms present in the wines. Furthermore, the differences in the types of microorganisms involved in fermentation of marula, as reported in Chapter 3, did not have a noticeable effect on the properties of the resulting wines. This could be explained by the dominance of LAB, AAB and *Saccharomyces cerevisiae*, which replaced the variable microbiota. The differences were in the levels of these microbiota as observed in this chapter and this influenced the concentrations of the chemicals tested.

Fruits accumulate sugars as they grow on the trees through the translocation of sucrose molecules that are produced by photosynthesis from the leaves. During ripening of the marula fruits, the disaccharide sucrose is broken down into its monosaccharaides glucose and fructose (Seymour.*et al.,* 2012). The microorganisms carrying out the fermentation showed to have a slightly higher preference for glucose than for fructose during wine fermentation process as depicted by a sharp decline in glucose levels than fructose. Glucose is a simple substrate that is metabolised by constitutive enzymes and its presence represses utilisation of other catabolites by the catabolite repression mechanism (Ruiz *et al.,* 2010). Marula juice generally contains equal or very similar amounts of glucose and fructose with a high amount of sucrose. The presence of fructose as a residual sugar in wine has a much stronger effect on the final sweetness of wine because it is approximately twice the sweetness of glucose (Mocke, 2013).

The alcoholic content obtained in this study was found to be higher than those of other traditionally fermented beverages, such as sorghum beer which was has a low alcohol content of 2.4 %. The marula wine in Zimbabwe was also previously reported to have a low alcohol content of 2% after 4 days of fermentation (Dlamini, and Dube, 2008). For any fruit juice that is fermented, the alcohol percentage reached is dependent on

the levels of fermentable sugars in the juice, and on the characteristics of the yeasts present. The sugar concentrations obtained in this study were found to be higher compared to those obtained by Phiri (2018). Both the mono-saccharides are co-fermented by microbes such as *S. cerevisiae*, *L. brevis and P. plantarum* amongst others. These bacteria produce numerous compounds such as ethanol, ethyl acetate, isovaleric acid, isobutanol and other vital volatile organic compounds. Volatile compounds of wines are dominated by the compounds that are formed during the fermentation process, some of these compounds such as ethyl acetate were present in high concentrations. This is evident in marula juice fermentation. The majority of volatile organic compounds were detectable as marula juice fermentation progressed and none to very little were detected in the juice. During the early stages of alcohol fermentation of the fruit juice, a number of odorous esters are formed. The aroma of wine is determined by a wide variety of chemical substances. Acetate esters, such as ethyl acetate, hexyl acetate, isoamyl acetate and 2-phenylethyl acetate were reported as the important flavour compounds in wine and other grape-derived alcoholic beverages (Rojas *et al*., 2001). Ethyl acetate is largely responsible for the altered sensory properties typical of souring of the wine. Other detected esters such as isoamyl acetate and ethyl calpryate contribute to wine odour and are especially important for a pleasant fruity note of the wine (Plata *et al.,* 2003). Ethyl calpryate is a volatile ethyl ester found in wine and produced during fermentation by yeasts. This compound is formed by the reaction of ethanol with fatty acid. Ethyl calpryate typically has a pleasant sweet aroma.

Higher alcohols contribute more to the aroma and flavour of the wine with a strong and pungent smell and taste. Higher alcohols that were detected in this study include ethanol, iso-butanol and isobutyl alcohol among others. Ethyl acetate is an ester of ethanol and acetic acid. It is formed by the condensation of the ethanol with acetic acid and this reaction is catalysed by an acid. Esterification reaction occurs at room temperature and the thawing and re-freezing of samples could lead to such reactions occurring. Air causes oxidation of alcohol to acetic acid which catalyses esterification (Lohitharn and Shanks, 2009). The observed decline in ethanol level in the marula wines is attributed to the esterification of ethanol to produce ethyl acetate, which proportionally increased in concentration.

Aroma is enhanced by the formation of organic acids such as propanoic acid, butaryic acid and isovaleric acid. These impart fruity, cheesy and fatty odours to wines and they also contribute to the bitterness, stringency and rancidity of wine (Shale *et al.,* 2014). Propanoic acid, butaryic acid and isovaleric acid were detected at varying amounts throughout the fermentation period in this study, with dominance by isovaleric acid. Ethanol production by *S. cerevisiae* is considered to be a major factor that controls the growth of non-*Saccharomyces* species during fermentation. The yeasts such as *Hanseniaspora, Candida, Pichia* were only observed in the juice and day 1 of fermentation. Generally, the species of *Hanseniaspora, Candida, Pichia* survive in the absence of ethanol due to lack of tolerance for ethanol (Di Maro *et al.,* 2007) and this explains their decline as the fermentation progresses. Ethanol directly contributes to the flavour of the wine by giving rise to a warming character. The results obtained in this current study have demonstrated the different volatile organic compounds present in the marula juice and wine. This data can be used to guide the marula wine makers in controlling the fermentation process. Chemical analyses provide essential information for marula wine characterisation and their link with sensory perception.

CHAPTER 5: MOLECULAR TYPING

Molecular typing and characterisation of common microbiota from different marula wines

5.1. Introduction

Fermented foods and beverages play an important role in contributing to the livelihoods of Africans through enhanced food security and income generation. As a result, there have been investigations to understand the microbial communities that contribute to the fermentation of different beverages (Kergourlay *et al.,* 2015; Barata *et al*., 2012; Fleet, 2007). The isolation, enumeration and identification of LAB yeasts to genus, species and strain levels is fundamental to understanding their occurrence and significance in foods and beverages.

Molecular methods are nowadays superior to traditional, culture-based detection methods for profiling of microbial communities due to their high throughput rate, sensitivity, specificity and better reproducibility as most culture media favour the growth of specific microorganisms, leading to an inaccurate perspective of microbial communities (Kelleher *et al*., 2015). Molecular techniques are robust and inaccuracies are limited due to the stability and consistency of the nucleic acid molecules under different environmental conditions. Multi locus sequence typing (MLST) is a nucleotide sequence based approach used for the unambiguous characterisation of bacterial isolates and other organisms (Maiden *et al*., 2013). It provides a convenient, precise, and highly discriminating typing system which is based on multiple genes and can be used to cluster same strains or distinguish between same species based on the multigenic characteristics of the organisms. MLST of fermenting microorganisms has been applied successfully to *Lactobacillus* and Acetic acid bacteria (Calmin *et al*., 2008). MLST is highly discriminatory; the accumulation of nucleotide changes in housekeeping genes is a relatively slow process and the allelic profile of a bacterial isolate is sufficiently stable over time for molecular typing. The relatedness of microorganisms or isolates can be displayed on a phylogenetic tree wherein isolates that have identical or very similar allelic profiles cluster together from a common ancestor. MLST directly measures the DNA sequence variations in a set of housekeeping genes and characterizes strains by their unique allelic profiles.

The organisms that were used for this part of the study were the LAB and the fermenting yeast *Saccharomyces cerevisiae.* These two groups are commonly known to play a major role in the marula wine fermentation, from carrying out the fermentation to the production of the important volatile compounds. The aim of this chapter was to evaluate the phylogenetic relationships of the common microbiota from different marula wines. This was done in order to understand the diversity and evolution of microorganisms during wine fermentation which is essential for controlling its production.

5.2 Materials and methods

5.2.1 Microbial isolates and culture method

Lactobacillus and yeast strains used in this study are listed in Table 5.1. All the strains were previously isolated in this study during the fermentation process of various marula wines obtained in the Limpopo province and were identified with MALDI-TOF. The yeasts were grown aerobically on YPD and WL nutrient media. The cultures were incubated aerobically at 30℃ for 42 to 72 hours. The *Lactobacillus* species were grown anaerobically on WL differential media at 30℃ for 24 to 72 hours.

Table 5.1: Bacterial and yeasts strains that were used for the molecular typing comparison by the MLST technique.

Strain ID	Strain code	Strain origin (as origin of the wine)
L. plantarum	ELP1	LAB
L. plantarum	ELP9	LAB
L. plantarum	ELP8	LAB
L. plantarum	ELP7	MLT
L. plantarum	ALP4	MOSHIRA*
L. plantarum	ALP5	DENILTON*
L. brevis	ALB1	MOSHIRA*
L. brevis	ALB2	DENILTON*
L. brevis	ELB3	LAB
L. brevis	ELB4	LAB

L. brevis	ELB5	LAB
L. brevis	ELB6	LAB
L. brevis	ELB7	LAB
L. brevis	ELB9	LAB
L. buchneri	ALBU1	MOSHIRA*
L. buchneri	ALBU2	MOSHIRA*
L. buchneri	ALBU3	DENILTON*
L. buchneri	ALBU4	DENILTON*
L. buchneri	ALBU5	MOSHIRA*
L. buchneri	ALBU6	MOSHIRA*
Saccharomyces cerevisiae	EL0DG4	LAB
Saccharomyces cerevisiae	MWRS5	LAB
Saccharomyces cerevisiae	MGS1	LAB
Saccharomyces cerevisiae	EL2DS1	LAB
Saccharomyces cerevisiae	ES20DG	MLT
Saccharomyces cerevisiae	ES22DG	SKB
Saccharomyces cerevisiae	MCS5	LAB
Saccharomyces cerevisiae	MLG1	LAB
Saccharomyces cerevisiae	MRW1	LAB
Saccharomyces cerevisiae	MRWR1	LAB
Saccharomyces cerevisiae	MWRS4	LAB
Saccharomyces cerevisiae	EM6RW1	MLT
Saccharomyces cerevisiae	MRW3	LAB

Keys: * strain was previously isolated and identified by Phiri (2018).

Pure colonies of the identified cultures were sent to Inqaba Biotechnical Industries (Pty) Ltd (Inqaba) for molecular characterization. Six genes namely: *clpx, groel, mure, phes, pyrg and ucrc* were used for LAB MLST analysis and MLST gene regions (ATF1, ITS, NUP116, RPN2, STE50, YBL081W) were used for the yeasts analysis. All processing of the cultures and DNA and the construction of phylogenetic trees were done remotely at Inqaba.

Table 5.2: PCR components and conditions for amplification of bacteria and yeast DNA

EconoTaq PLUS 2X Master Mix	10 µl
gDNA (10-30 ng/µl)	1 µl
F primer (10 µM)	1 µl
R primer (10 µM)	1 µl
Nuclease free water	7 µl
Total volume	20 µl

In short, sequence trimming and alignments were performed with ClustalX algorithm of the MEGA software. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model. The tree with the highest log likelihood (-5541.54) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 20 nucleotide sequences. Evolutionary analyses were conducted in MEGA7 (Ki *et al.,* 2011).

5.3 Results

A selected number of LAB and yeast isolates that were common in all the different marula wines were characterised for strain typing and phylogenetic relationship.

All the selected bacteria formed tight clusters within their respective species, i.e., all the *L. buchneri* isolates clustered together at a bootstrap confidence of 96. So were the *L brevis* which showed a close relationship with *L. plantarum*. Interestingly, the *L. plantarum* strains (ALP strains) which were obtained from a previous study, grouped closely but separately from the strains isolated in this study. A similar relationship to other *L. brevis* strains isolated in this study was observed with the ALB2 strain.

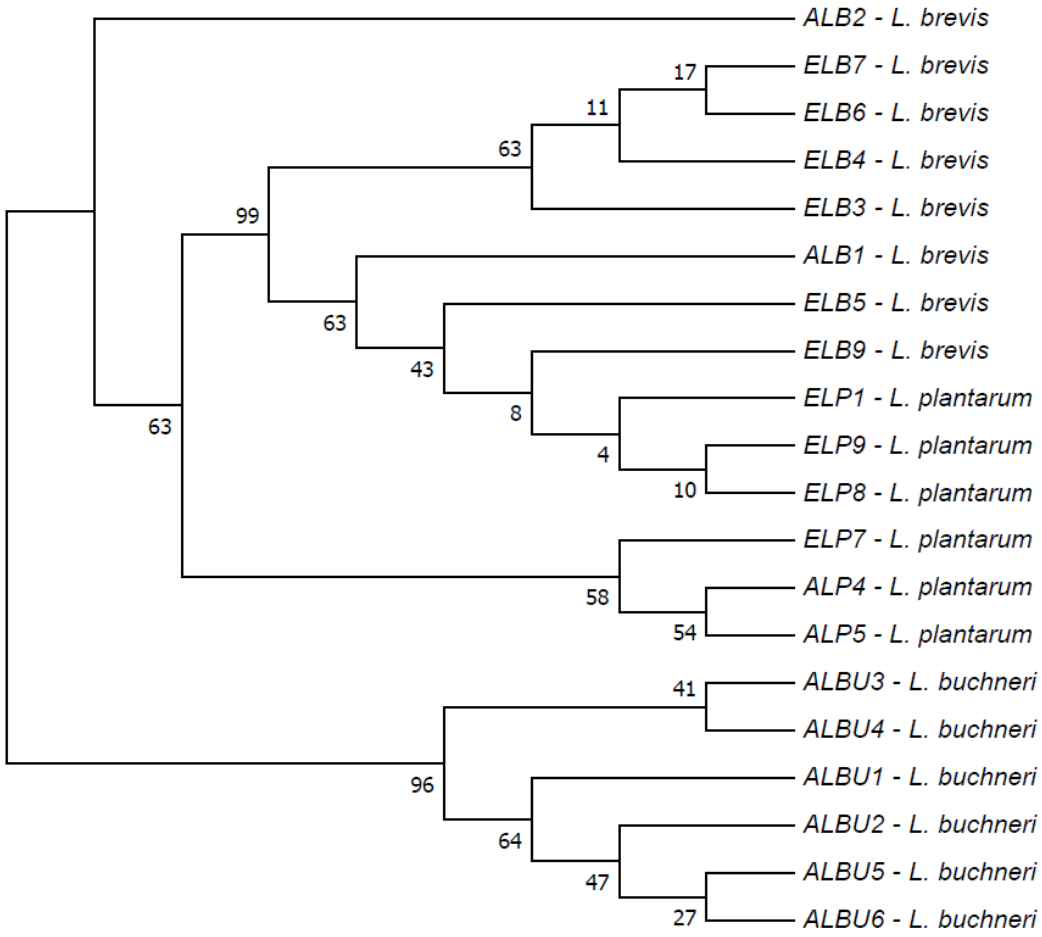

Figure 5.1: An unrooted phylogenetic tree for *Lactobacillus* isolates of marula wines. A total of 1000 bootstrap replicates were applied. Percentage likelihood is shown at node branches.

The *Saccharomyces cerevisiae* strains, contrary to the relationships observed with the LAB strains, showed varying levels of genetic relatedness (figure 5.2). The yeasts clustered loosely by the type of wine they were isolated from, such as those from the LAB wine as shown in figure 5.2. The MLT *S. cerevisiae* isolates ES20DG and EM6RW1 were noticeably separated from ES20DG and branched with an MLT strain ES22DG and a LAB strain MRW3 (Figure 5.2).

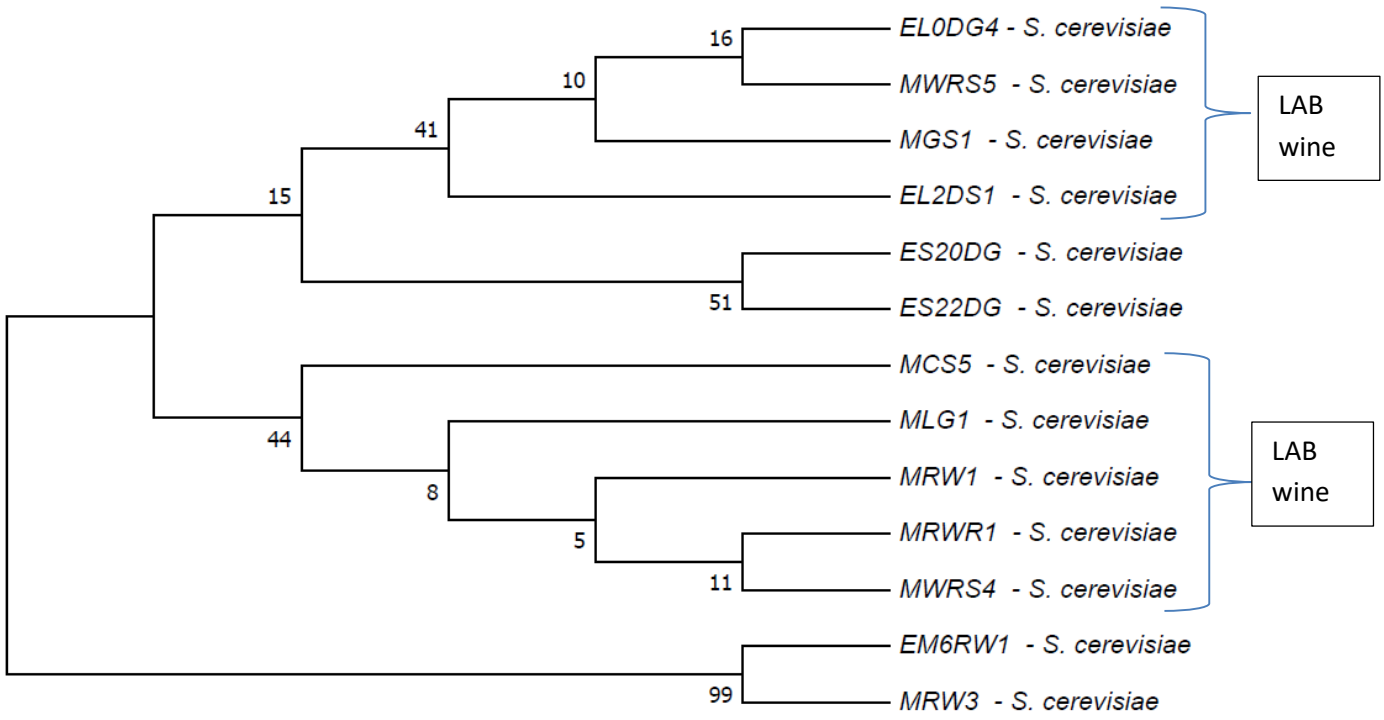

Figure 5.2: An unrooted phylogenetic tree for *Saccharomyces* yeast isolates of marula wines. A total of 1000 bootstrap replicates were applied. Percentage likelihood is shown at node branches.

Common microbiota from the different marula wines showed low level of relatedness. Low genetic diversity was observed amongst all the bacteria (Figure 5.1) tested in this study. The same trend was observed with the yeast strains (Figure 5.2).

5.4. Discussion

Phylogenetic studies are important when studying the ecology and evolution of microbes. This was applied to the fermenting microbiota present in the marula wine in order to study any strain variations that may be present between isolates from different localities.

The findings in this study revealed the LAB and *S. cerevisiae* strains that contributed to the fermentation of the marula wines were related, irrespective of the wine's origin. This implies that the geographical distribution did not place excessive evolutionary pressure on these microbes.

Strain typing is important when studying the diversity of microorganisms that partake in the production of an important commercial product because a consistent organoleptic properties and a long shelf life of the product are required. By knowing the marula wine fermenting strain type, the product shelf life can be extended without compromising its organoleptic properties.

It was interesting to observe the variability in the microorganisms isolated from the marula juices that were obtained from different localities. These findings were presented in Chapter 3 of this study wherein most of the LAB strains and *S. cerevisiae* were not detectable in the marula juice. This did not translate into an equal microbial diversity during the fermentation, however, a convergence of same strains was observed across the isolates from different wines. Noting that the wines were produced by spontaneous fermentation, this infers habitation of marula fruits by similar types of fermenting microbiota. This level of relatedness observed amongst LAB strains and *S. cerevisiae* gives an opportunity to apply these strains in the production of a consistent organoleptic properties and a long lasting wine.

CHAPTER 6
GENERAL DISCUSSION AND CONCLUSION

Fermentation remains one of the preferred methods for food preparation which has health, cultural and economic significance and it is used throughout the world. Wine is an age old fermentation product that is produced from complex interactions between yeasts and bacteria. Marula wine is one such product that is produced through spontaneous fermentation of marula juice. Marula wine is an important traditional African wine that is nowadays produced for commercial benefit. The limitation to its mass production is the short shelf life wherein it attains a bitter unpalatable taste after 2 weeks of production.

Marula fruit cultivation provides the foundation for wine flavour, however, microorganisms, especially yeasts, influence the subtlety and individuality of the flavour response. The microorganisms on the marula fruits surface initiate the fermentation process. The surface of healthy ripen marula fruit had a predominance of Enterobacteriaceae, Bacillaceae, Lactobacillaceae and *Rhodotorula* species, depending on the stage of maturity. Alcoholic fermentation of marula juice is characterised by the successional growth of various bacteria and yeast species and strains. Through yeast-bacterial interactions, this ecology can determine progression of fermentation and the potential growth of spoilage bacteria in the final product.

Bacteria and yeast have been identified at different stages of fermentation and these correspond to the different organic chemicals that have been observed. Microorganisms such as *L. plantarum, L. brevis*, *P. guilliermondii, H. guilliermondii* and *Meyerozyma caribbica* dominated the earlier stages of fermentation. At this stage, sugars were consumed hurriedly, with a steep incline in microbial load in the fermenting wine. It is noteworthy that volatile organic compounds were undetectable in the marula fruit juice. These were produced as fermentation progressed, and differences were detected with changes in the bacterial and yeast profiles. That is, the beginning of fermentation was marked by the presence of yeast *Pichia* and *Hanseniaspora* species and *Lactobacillus* species amongst other bacterial species. The primary contributions of *Pichia* and *Hanseniaspora* species at the early stage of wine production were to initiate the spontaneous fermentation and are believed to influence the wine composition as well as the development of *Saccharomyces* (Torija

et al., 2001). The yeasts were immediately replaced by *Saccharomyces cerevisiae* for alcoholic fermentation. The production of organic acids such as propionic acid and isobutyric acid by bacteria including *Lactobacillus*, and alcohol by *S. cerevisiae* displaced *Pichia* and *Hanseniaspora* yeasts because of their low tolerance for acids and alcohol. On the contrary, *S. cerevisiae* thrives in higher acidic environments.

Production of marula wine at household level takes place in semi-anaerobic conditions. The handling of the marula wine during brewing introduces air into the mixture, and it commonly happens after a week of fermentation when scoops of wine are collected for consumption. The presence of oxygen during fermentation commonly leads to growth of aerobic bacteria. This phenomenon led to the rise of acetic acid bacteria which metabolised alcohol and produced acetic acids. Acetic acid gives off a rancid taste and smell to the wine and these properties were attributed to the spoilage of marula wine. The knowledge of the contributing bacteria and the corresponding chemicals enabled identification of potential spoilage bacteria. This information is pertinent when the shelf life of marula wine is of interest. Interestingly, the phylogenetic study showed high intraspecific similarity between clusters of the *L. buchneri*, *L. brevis* and the yeast *S. cerevisiae*. Similarly, this knowledge will benefit the production of a marula wine of a consistent quality.

CONCLUSION

The microbial relationship commences in the fruits and continues throughout the fermentation and storage processes. The diverse microorganisms present interact throughout the winemaking process, the interactions modulate the hygienic and sensorial properties of the wine. Their significance in contributing to the quality and efficiency of wine production warrants greater recognition. Good hygienic design and maintenance of equipment, with hygiene and health education of food handlers are essential in the control of Enterobacteriaceae contamination especially in order to prolong the shelf-life of the fermented product. The data obtained in this study provided empirical information to develop a long-lasting wine. The results also help to identify the stages at which researchers can control the growth of unwanted microbes.

CHAPTER 7

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