MICROBIAL AND CHEMICAL DYNAMICS DURING MARULA FERMENTATION

by

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DECLARATION

I Archie Phiri declare that the dissertation hereby submitted to the University of Limpopo (Turfloop campus) for the degree of Master of Science in Microbiology has not been previously submitted by me for the degree at this or any other University, that is my own work in design and execution, and that all materials contained herein have been duly acknowledged.

Signature: _____ Date: 03/09/2012

DEDICATION

I would like to dedicate this work to the almighty God, my late grandmother and my loving family and friends for the unfailing love and support.

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ABSTRACT

Marula wine is produced through a spontaneous fermentation process that is mediated by the native microbiota present on the fruits and those transferred from the surface of the fermentation utensils, mainly by *Saccharomyces* and non-*Saccharomyces* yeasts. Information regarding the diversity of yeasts and bacteria as well as their dynamics and contribution to the chemical composition of the final product remains obscure. The current study monitored the bacterial and yeast populations and the chemical profile throughout fermentation of the marula wines obtained from different communities in Limpopo province. Furthermore, potential spoilage microorganisms were identified. The microbial analysis revealed that the yeast species *Hanseniaspora guilliermondii* and the *Lactobacillus* species dominated the initial stages of fermentation whereas *Saccharomyces cerevisiae* and *Acetobacter* species dominated the latter stages. The marula wines commonly contained low fructose (1.8 – 2 mg/mL), sucrose (0 – 1.65 mg/mL) and no glucose at the end of fermentation study period. The changes in the microbiota during fermentation of marula wine corresponded with the sugar consumption and the change in the production of the volatiles. The volatile compounds included aldehydes, esters, ethanol, higher alcohols and organic acids such as acetic and formic acids were in high levels at a range of 11 – 87% at the latter stages of fermentation. High levels of acetic acid could be the cause for the off taste and spoilage associated with *Acetobacter aceti* and *Acetobacter pasteuriannus*. These findings are important in the downstream preservation process and subsequent improvement of storage life of the marula wine.

CHAPTER 1

Introduction

The marula tree, known as *Sclerocarya birrea* subsp. *caffra* is a deciduous and dioecious tree which is a member of the southern Africa's indigenous flowering fruit-bearing trees. It belongs to the family known as Anacardiaceae. Its distribution is from Ethiopia to South Africa and it is considered native in several countries such as South Africa, Malawi, Namibia, Nigeria, Botswana, Sudan, Swaziland, Gambia, Kenya, Tanzania, Angola, Uganda, Zambia, Ethiopia and Zimbabwe (Orwa *et al.*, 2009; Chirwa and Akinnifesi, 2008). The African communities use all parts of the tree to produce a wide variety of products, from furniture to cosmetics for sale. The female tree bears a small plum-size fruit called marula with a pale-yellow color from January to late March (Nerd and Mizrahi, 1993; Ojewole *et al.*, 2010). The marula fruit is one of the most commonly used fruits in the African countries (Shackleton and Shackleton, 2005; Shackleton *et al.*, 2009). These fruits are rich in nutritional minerals and carbohydrates and are either eaten fresh or processed into beverages (Moganedi *et al.*, 2007) or jam. In many local communities, the non-alcoholic and alcoholic beverages are prepared for domestic use or for trading within the communities. This provides a reasonable income for the families which have turned it into an enterprise during the marula season (Moganedi *et al.*, 2007). Fermentation of marula wine is mainly mediated by the natural microorganisms associated with the marula fruit, and the wine has a short shelf-life of 2 – 4 days depending on the ambient temperature. Marula wine, known as Vukanyi in Xitsonga and Morula in Sepedi, forms an integral part of the livelihoods as well as social and cultural activities of many communities in the Limpopo province. Marula has recently gained commercial value in southern Africa since its fruits and other products attributes have entered local, regional and international trade (Mokgolodi *et al.*, 2011). The development of marula wine into a global commercial beverage requires that the wine must be stable with consistent quality and must have a long shelf life. Marula wine has a significant economic value because this tree has found good use in social and cultural activities in the communities across the continent of Africa, hence a significant market already exists. With grape wine as a model, marula wine can be improved and developed into a commercial drink which is available throughout the

year. However, there is a need to first find strategies to preserve the marula wine thereby increasing its shelf life. For such a method to be devised, it is necessary to have a clear understanding of the dynamics during the fermentation process. Hence this study generated an inventory of the microbiota present throughout the marula wine fermentation process and has further profiled the chemical dynamics that occur during fermentation.

1.1 Aim and objectives

The aim of the study was to determine the microbiological and chemical changes that occur at different stages during the fermentation process of the marula wines.

The aim was achieved through the following objectives:

i. Determination of the chemical compounds and sugar content from the marula fruits and the wines during fermentation process.
ii. Identification of the microbiota present in the marula fruit juice and wines and their evolution during the fermentation process.
iii. Analysis of the complexity of yeast and bacterial populations during the fermentation period.
iv. Identification of potential spoilage microorganisms in the marula wines.

CHAPTER 2

Literature review

2.1 The marula tree

The genus *Sclerocarya*, is derived from its hard seed (*Sclero* = hard and *carya* = nut) which comprises of four species of which *Sclerocarya birrea* is the most widely distributed (Shackleton *et al.,* 2002). *Sclerocarya birrea* is commonly known as Marula in southern Africa. Three subspecies of *S. birrea* are known, namely, *S. birrea* subsp. *multifoliata*, *S. birrea* subsp. *birrea* and *S. birrea* subsp. *caffra*. *S. birrea* subsp. *caffra* is the most widespread species found in the Sahelian region and the east and south tropical Africa (Shackleton *et al.,* 2003). It occurs in 29 countries from the west to east and north to south countries such as Senegal, Guinea Bissau, Ivory Coast, Mauritania, Mali, Burkina, Ghana, Togo, Benin, Niger, Nigeria, Chad and Sudan, in low Africa; Eritrea, Ethiopia, Uganda, Kenya, and Tanzania, in eastern high Africa; and Angola, southern Congo, Zambia, Malawi, Mozambique, Namibia, Botswana, Zimbabwe, South Africa, Lesotho and Swaziland in southern Africa (Figs. 2.1 and 2.2) (Hall *et al.*, 2000). The different names used for marula tree in different regions include mng'ongo (Swahili), mafula (Tshivenda), morula (Sepedi) and Nkanyi (Tsonga) are also used.

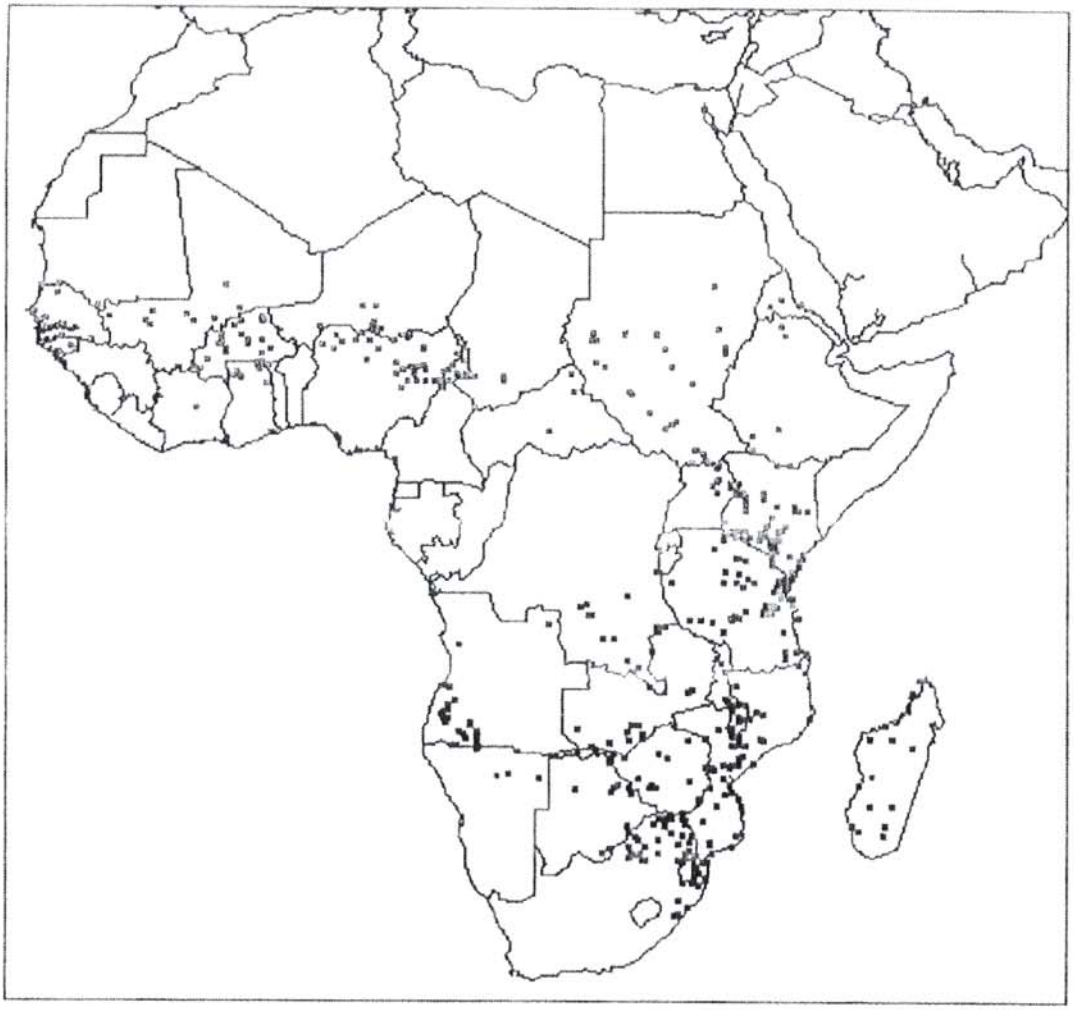

Figure 2.1. Distribution of *Sclerocarya birrea* (marula) (Hall *et al.*, 2000) from Herbaria Royal botanic garden.

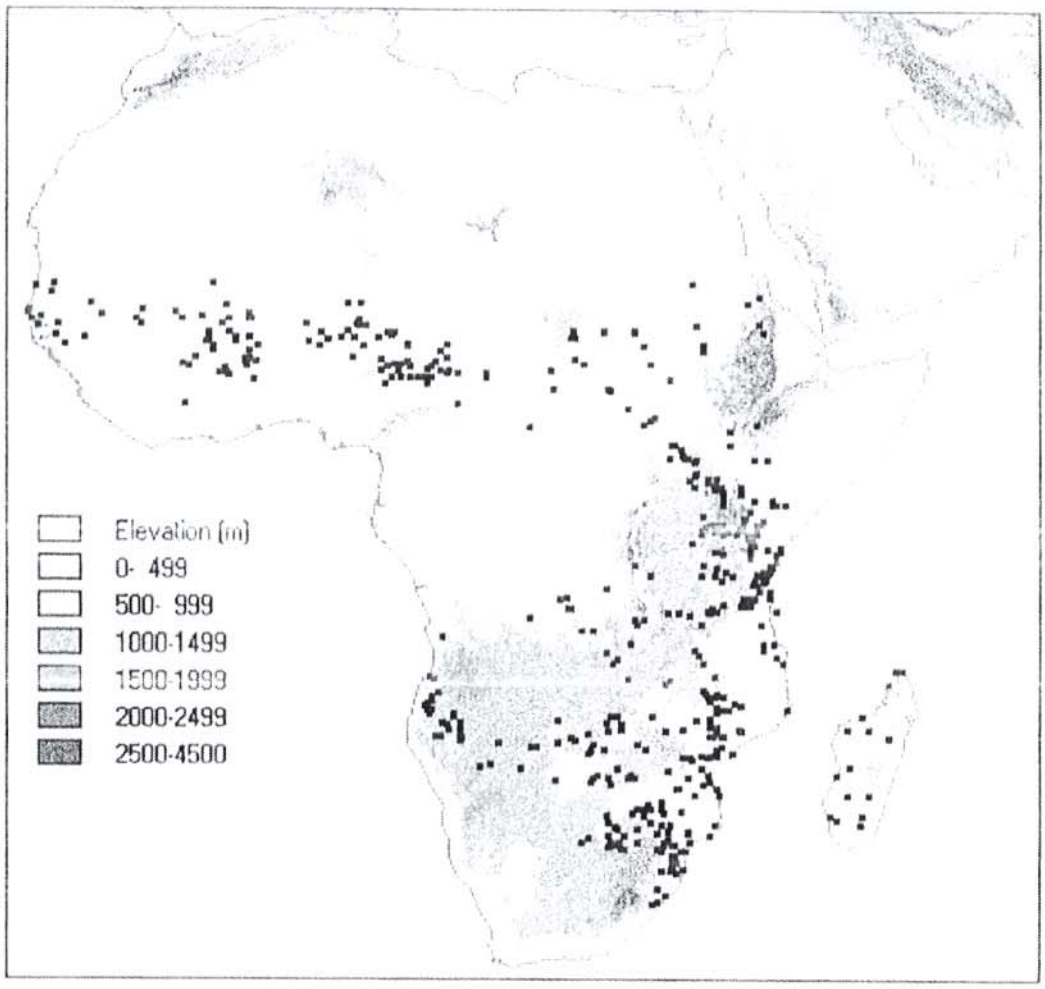

Figure 2.2. Distribution of *Sclerocarya birrea* in relation to elevation (Hall *et al.*, 2000). Adapted from Herbaria: Royal botanic garden.

The marula tree has been considered a desirable species in support of the development of rural communities based on the fruits, wine or nuts, and thus as a valuable species for potential domestication (Shackleton, 2002) due to its wide distribution and usage. It has been regarded as a protected species in South Africa since 1962 (Shackleton *et al.*, 2005).

Marula is one of the most valuable fruit bearing indigenous tree in the African continent. The tree is medium to large sized and grows to a length of up to 18m tall (Fig. 2.3 A). It is single stemmed with dense spreading crown and deciduous foliage. It has grey barks (Fig. 2.3 B) which usually peel off in flat round disks and exposing

underlying angular depressions, which gradually turn to a brown-grey colour (Shone, 1979). It is either cultivated or grows naturally in east, Sahel or southern part of Africa outside the humid forest zone (Orwa *et al.*, 2009). However, it grows wildly in most parts of the African continent. The tree grows well in warm frost-free areas, it is salt-tolerant (du Plessis, 2002), survives well in sandy loam and clay soils and is commonly found in areas receiving annual rainfall of about 200-1370 mm (Hiwilepo-van Hal, 2013). The marula tree leaves are unevenly, pinnately compound consisting of 7-13 pairs of leaflets plus a terminal one alternately arranged and crowded near the ends of branches (Fig. 2.4 A) (Venter and Venter, 2005). In southern Africa, flowering occurs in September-December, and fruiting predominantly occurs during January to March. The female tree has blood red flowers (Fig. 2.4 A), which changes from purple to white producing fruits while the male tree only displays dark red flowers, which turns pink and white when mature.

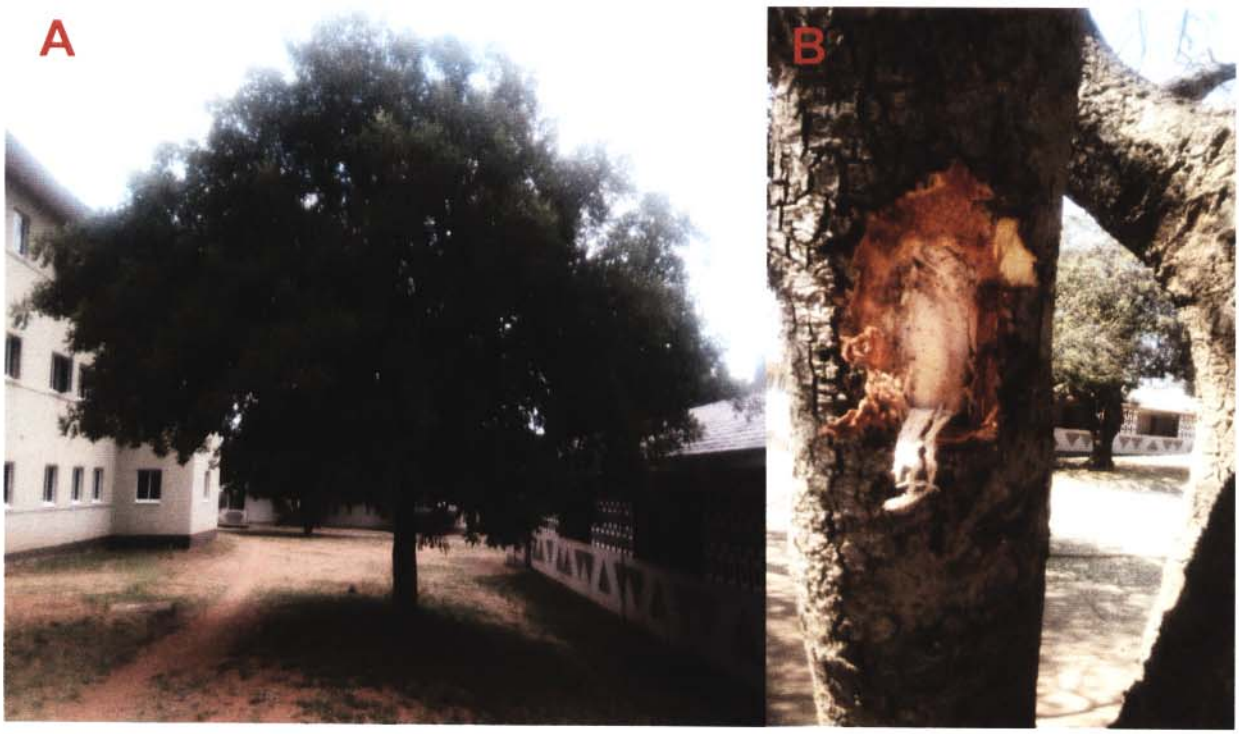

Figure 2.3. The marula tree (A) and its bark (B). Photos taken by Mr A. Phiri.

The fruits are produced in wild trees at an early age of 7 – 10 years and the yield increases as the tree grows older (Shackleton *et al.*, 2002). The size and quality of the fruits vary from tree to tree, with the mass ranging from 10 g to more than 60 g (Botelle *et al.*, 2002; Shackleton, 2003). The fruit is a 30 – 40 mm round oval drupe

with thick leathery soft exocarp which encloses a mucilaginous flesh (Gous *et al.*, 1988). The mucilaginous flesh has a tart, sweet refreshing taste and a slightly turpentine-like aroma and adheres tightly to the single stone (Palmer and Pitman, 1972). The marula fruit has unique flavour and it is nutritive and rich in vitamins. The Mean fruit yield per tree is significantly higher for the trees in the villages (> 17 000 fruits) than the trees in protected areas (< 3 500) despite accounting for differences in tree size (Shackleton *et al.*, 2003). The colour of the fruit turns into yellow after abscission (Fig. 2.4 B).

Figure 2.4. Marula tree leaves (A) and the ripe marula fruits after abscission (B). Photos taken by Mr A. Phiri.

2.2 Traditional uses and importance of marula tree

The interest in the marula tree is vast. This is mainly due to its socio-economic value and its engraved appreciation in the African community. The marula tree is a multipurpose indigenous tree. This tree has for decades played an integral part in the lives, food security, cultural and social cohesiveness of the indigenous communities in the southern Africa. Due to its multiple uses and its significance amongst communities, several African cultures have specific beliefs and hold ceremonies

associated with this species (Walker, 1989). For thousands of years the parts of this indigenous plant have been used for food, beverages, medicine, and other artefacts. Numerous studies have focused on its pharmacological characteristics (Eloff, 2001; Sarkar *et al.*, 2014), nutritional properties (Hiwilepo-van Hal *et al.*, 2012) and its other economic value (Van Wyk, 2011; Vermaak *et al.*, 2011) which constitute the main uses of the marula tree. The marula wine is a worthwhile enterprise for the poor households because it is liked by both rural and urban African people. The brewing and trading of marula wine is mainly driven by the women in the communities. The preparation of the marula wine is cost effective as it requires only water and ripe marula fruits. Hence the profit margin is substantial for these households considering the input investment. Most marula wine traders (41%) reported to be self-employed in 2004, citing the sales of the wine as their most crucial source of income when the fruits are in season (Shackleton, 2004). However, the short shelf life of the wine necessitates frequent brewing. Consequently, for continuous trading, the brewers must prepare fresh beverage every 7 to 8 days and this puts immense pressure on the women who also take care of the households. This problem is exacerbated by the lack of proper cooling storage facilities.

2.2.1 Traditional medicine

Indigenous use of the marula tree for management of health conditions is an age old tradition (Hutchings *et al.*, 1996). Several studies have been performed in evaluation of the medicinal properties of the marula tree and various cultures have long utilised the parts of the tree to manage, control, and treat numerous ailments. Almost all the parts of the plant, but especially the bark and leaves are exploited for medicinal purposes (Hall *et al.*, 2000). In various African countries, powdered stem-bark has been used for the treatment of dysentery and diarrhoea, rheumatism, and insect bites and is also believed to be a cure for malaria and proctitis (Gurib-Fakim *et al.*, 2010). Decoctions, infusions or steam from boiled roots is used to treat heavy menstruation, bilharzia, coughs, weakness, sore eyes, heart pains and also as an antiemetic (Shackleton *et al.,* 2002). The leaves are said to provide a relief from abscesses, burns and spider bites. The marula oil is used as a balm to treat ear, nose and throat infections (Gurib-Fakim *et al.*, 2010). Moreover, both the bark and

leaf extracts contains antidiarrhoeal, antidiabetic, anti-inflammatory, antiseptic, antimicrobial, antiplasmodial, antihypertensive, anticonvulsant and antioxidant properties (Gondwe *et al.*, 2008; Ojewole *et al.*, 2003).

The leaves contain tannins and flavonoids (Gueye, 1973). The gum from the tree is rich in tannin (0.4%), and it is often used in making ink (Watt and Breyer-Bradwijk, 1962; Mariod *et al.*, 2012). Marula fruit has high antioxidants activity of 2960 mg/100g and 1872 mg/100g equivalence to L-ascorbic acid at pH 4.5 and pH 7, respectively (Mdluli and Owusu-Apenten, 2002). Compared to other edible fruits they contain high amount of phenolics (Gil *et al.*, 2002). The marula juice was reported to have an antioxidant capacity of 382 mg/mL equivalent to vitamin C (Borochov-neori *et al.*, 2008). Furthermore, the juice contains high content of polyphenols (2262 ug GAE/g) and flavonoids (202 ug catechin/g) (Ndhlala *et al.*, 2007). Hiwilepo *et al.* (2012) reported a positive correlation between the antioxidant activity and polyphenols. The antioxidant activity was not affected by pasteurisation and only 14% was lost after 4 weeks of storage at 18 °C (Hillman *et al.*, 2008). This gives a clear pharmacological support for the ethnotherapeutic uses of marula in traditional treatment of health challenges.

2.2.2 Artefacts

The wood of the tree was used for furniture, panelling, flooring and carvings (Shackleton *et al.*, 2002). The inner fibre layer of the bark was used to make a strong rope and the wood was carved for drums, yokes, boats and other different products such as spoons, plates, and decorative animal figures such as warthogs and rhinos (Lawes *et al.*, 2004). However the harvesting of the wood from the tree was later banned in 1962 after the tree was officially declared a protected species across the entire country (Orwa *et al.*, 2009).

2.2.3 Food and beverages

The marula tree is valued by the rural communities in the southern Africa for its highly aromatic sweet-sour fruits. Marula fruits are commonly used as a good source of nutrition during the season when there is drought and shortage of subsistence products. Marula is commonly used as a very crucial source of nutrition in affected people (Mojeremane and Tshwenyane, 2004). These fruits are either eaten fresh as a snack or prepared into a variety of food products. These include jam, jelly, chutney and some non-alcoholic and alcoholic beverages (Shackleton *et al.*, 2009). Over 90% of households in the Limpopo province of South Africa who collect marula fruits mainly make an alcoholic beverage, and secondarily for consumption as fresh fruits or process the fruits into juice, and/or process into jam (Shackleton *et al.*, 2009). There is a biased preference of alcoholic over non-alcoholic marula beverages in the rural communities (Maroyi, 2013). The alcohol content in the marula wine is up 5% depending on the period of fermentation (Mokgolodi *et al.*, 2011). The fruit kernels are eaten or used for oil extraction (Mutshinyalo and Tshisevhe, 2003) which can be used for cooking and for production of cosmetics (du Plessis, 2002; Mojeremane and Tshwenyane, 2004). The marula kernels are regarded as a delicacy and are commonly used as diet supplement during the winter season (Taylor *et al.*, 1995; Shackleton *et al.,* 2002). Besides snacking, the nut can also be ground up and mixed with vegetables and meat or for baking traditional breads (Shackleton *et al.,* 2002). The skin of the fruit is often dried and used to replace coffee (Hiwilepo-van Hal, 2013), and the leaves are cooked and eaten as relish (Shackleton *et al.*, 2002). Regardless of the different ethnomedical and commercial uses of the other parts of the tree such as stem-barks, leaves and roots, the fruit still has the highest commercial potential comparatively (Ojewole *et al.*, 2010).

2.2.4 Nutritional value of marula

Marula fruit plays an important role in the nutrition of people in the rural communities who utilise it for food (Hiwilepo-van Hal *et al.*, 2013). The nutrient and chemical composition of marula fruits differs widely depending upon genetic and environmental factors. The marula fruit contains high content of minerals such as

potassium, magnesium and calcium (Shackleton *et al.,* 2010). These minerals and high content of vitamin C have significantly increased the interest in marula fruit utilisation for industrial purposes (Dube *et al.,* 2012). Marula fruit provides about 2 mg of vitamin C per gram of fresh juice, approximately four to five times the level of average fresh orange juice (Mojeremane and Tshwenyane, 2004). The fruit provides energy estimated at about 130 kJ per 100 g of fruit fresh (Shackleton *et al.*, 2003). Vitamin C accounts for about 70% of the total antioxidant activity of the marula fruit, which is 20 – 40 times higher compared to that of common reported edible fruits (Mdluli and Owusu-Apenten, 2002). The pulp of the marula fruit is abundant in citric, malic and tartaric acids and sugars such as sucrose, glucose and fructose (Van Wyk *et al.,* 2002; Hillman *et al.,* 2008). The sugar content is 7 – 14% brix with sucrose as the main sugar while glucose and fructose are present in smaller quantities (Fundira, 2001).

The kernel is rich in oil and protein (Van Wyk *et al.*, 2002; Hillman *et al.*, 2008). Crude fixed oil from the nuts is a valuable source of essential fatty acids (Mariod *et al.,* 2012) and the oil is considered to have a significant commercial potential, and ethnobotanical records suggest that such properties have been acknowledged and exploited by local populations for a long time (Palmer and Pitman, 1972). The oil is currently used in the cosmetic industry as a main ingredient in body lotions. Fatty acids found in the oil includes palmitic acid (12 g 100 g-1 fatty acid), stearic acid (9.2 g 100 g-1 fatty acid), oleic acid (69.9 g 100 g-1 fatty acid), and linoleic acid (7.8 g 100 g-1 fatty acid) (Moyo, 2009). The fatty acid composition is affected by harvesting time. A quantitative increase in the oil content was observed to reach as high as 63.0% at the end of the last harvesting date which is around June (Mariod *et al.,* 2012). In high levels, the bioactive lipids play a vital role in nutritional applications (Mariod *et al.,* 2012).

The marula fruit flesh has an energy value of approximately 130 kJ per 100 g of the fruit flesh (von Teichman, 1983), and the energy value of the kernel is approximately 2,699 – 2,703 kJ per 100 g, which is generally higher than most other commonly consumed nuts, except walnut (Shackleton *et al.,* 2003; Wynberg *et al.,* 2002). Additionally, the marula kernels have high levels of magnesium (467 mg/100 g), phosphorous (836 mg/100 g) and potassium (677 mg/100 g) which contributes to the importance of the nuts in the diets of local rural communities (Wynberg *et al.,* 2002).

The fruit juice contains 0.7 g per 100 g dietary fiber with the ash content of 1 g per 100 g (Borochov-neori *et al.*, 2008). The marula fruit skin has the highest ash content (1 – 4.2 g/100 g) with the pulp having 0.2 g/100 g and the juice being 0.09 g/100 g of the ash content (Taylor, 1985). The ash content from the kernel of marula fruit is 1.7% (Aganga and Mosase, 2001). The reported amounts of the ash content vary a lot and this is suspected to be the result of the different methods used in the analysis and depend on the time and temperature used (Hiwilepo-van-Hal *et al.*, 2013).

2.3 Production of marula wines

The production of marula wine was initially performed at subsistence level; this was eventually turned into an enterprise due to an increased level of poverty and unemployment in the rural areas (Shackleton, 2004). Production of a marula alcoholic beverage on an industrial commercial scale has been achieved in South Africa and a commercial wild fruit cocktail, Amarula, is now available on the market. However, the process of producing a local wine at communal level is different. Many households (74%) in the Limpopo province where marula trees grow wildly produce between 138 L and 311 L of marula fruit wine each season (Simatende *et al.*, 2015). The brewing process of marula wine simply involves the extraction of the juice from ripe fruit and incubation of the water diluted juice to spontaneously ferment over days (Figs. 2.5 A and 2.5 B). The processing of the extracted juice vary amongst brewers, in which different proportion of water is added to the marula juice. This influences the fermentation period wherein more diluted juices take longer to ferment adequately. Subsequently the strength and alcohol content of the resulting wine is affected.

Figure 2.5. Marula wine brewers from Nobody village, Limpopo province (A) rinsing and extraction of marula juice and (B) shows a settling and separation of the pulp into clear juice and the floating upper layer sludge. Photos taken by Mr A. Phiri.

The processing methods of the marula fruit wine are different from one village or country to another and, in few cases the fruits are first subjected to a heat to soften the fruits outer skin before pressing. This heat treatment affects the overall aroma profile of the marula juice and pulp by forming compounds resulting in undesirable off-flavours (Hiwilepo-van-Hal *et al.*, 2013). Lower alcoholic or higher alcoholic beverages can be produced from marula juice depending on the fermentation period. Lower alcoholic drink is produced by fermenting the juice for 2 days or less while the higher alcoholic drink is fermented for 4 – 5 days (Hiwilepo-van Hal *et al.*, 2013).

The use of the fruits of different degrees of ripeness can produce sourness which makes the wine unpalatable. There are no reports on variations due to the odour, flavour and after taste of the juices prepared from different cultivars (Schäfer and McGill, 1986).

There is no addition of a starter culture in traditional fermentation of marula wine. The natural microbiota, mainly yeasts introduced by the Drosophila is responsible for fermentation of the marula juice (Hiwilepo-van Hal *et al.*, 2013). The sugar content and the yeast present in the juice largely contribute to the alcohol content of the fermented marula juice (Dlamini and Dube, 2008).

Spontaneous fermentation is complex and unpredictable in terms of length of fermentation and the quality of the resulting product. Spontaneous fermentation often produces products with a shortened shelf life which are sometimes not safe due to high potential for contamination by pathogens (Nout and Rombouts, 1992). Traditionally, extension of the shelf-life is practised by daily addition of the freshly prepared marula juice or by storage of the prepared marula wine underground in tightly closed containers. In the food industry, thermal treatment is frequently applied as an important step to increase the shelf-life by inhibition of the spoilage caused by certain microorganisms and enzymes. Although a necessary step in food processing, heating can affect the overall aroma profile of the product (Hiwilepo-van Hal *et al.*, 2013) and result in formation of undesirable off-flavours. The development of off-flavours was found in a pasteurised guava puree stored at frozen temperatures, which indicated that the quality is not guaranteed even upon low temperature storage (Augusto *et al.*, 2000).

The literature has mainly reported on the presence of various microorganisms during spontaneous fermentation of wine and some of their contributions to the final product of the fermentation. However there are still gaps to be filled regarding the dynamics and transition of the microbial population as well as the change in the chemicals produced by these microorganisms at different stages of the spontaneous fermentation. Several studies done on marula only provided limited information on the microbial population and the chemical composition which could not contribute to the comprehensive knowledge on the changes that occur during the marula fermentations. Therefore there is need for further investigation on the microbial and chemical dynamics of marula wine at different stages of spontaneous fermentation.

CHAPTER 3

Microbiological analysis of marula wines during fermentation

3.1 Introduction

Numerous studies have been conducted on the microorganisms found on marula fruits, as well as their fermented and unfermented juices. However the development and dynamics of the microorganisms have not been detailed. In spontaneous fermentation the natural microorganisms in the fruits are responsible for the fermentation. Amongst these microorganisms are the yeasts introduced by the fruit flies, *Drosophilia* (Hiwilepo-van Hal *et al.*, 2012). Some of the microbial contaminants are introduced to the wine during and after its processing through unhygienic handling of the fruits (Amusa and Odunbaku, 2009; Lues *et al.*, 2011). These microorganisms play a huge role in the quality and the palatability of the resulting wine. Understanding of the succession and evolution of these microorganisms during the fermentation will be effective in developing rapid efficient methods to eliminate the undesired microorganisms without altering the organoleptic properties and quality of the wine.

The diversity and quantity of the microbial populations are affected by several factors such as the climatic and microclimatic factors which include temperature, UV exposure, rainfall, sunlight and winds (Kowalchuk *et al.*, 2010). The marula juice fermentation involves a mixed and complex interaction of microorganisms introduced onto the surface by fruit flies, *Drosophila* (Dlamini and Dube, 2008). Yeast species such as *Aureobasidium pullulans, Geotrichum capitatum, Trichosporon brassicae, Rhodotorula mucilaginosa, Hansenula anomala* and *Hansenula jadinii* have previously been isolated from the marula fruits (Okagbue and Siwela, 2002), while *Saccharomyces apiculata* was isolated from the marula wine (Dlamini and Dube, 2008). The *Hansenula* species are considered fermentative, and these species may be playing a role in marula fermentation (Dlamini and Dube, 2008).

The yeast community of the marula wines from different localities were characterised using Automated Ribosomal Intergenic Spacer Analysis (ARISA) described by Fisher

and Tripplet (1999). The fingerprinting technique allows profiling of the both the culturable and unculturable microorganisms. ARISA provides estimates of microbial richness and diversity, and has become a very useful tool for comparing community structure between different and multiple samples based on profile patterns (Ni *et al.*, 2007; Torzilli *et al.*, 2006). The microbial communities of the different wines at different stages of fermentation were profiled using genetic fingerprinting techniques.

In this study the culture-based and molecular-based techniques were used to evaluate the microbial communities and identify the fungal and bacterial species associated with marula wine and their dynamics during fermentation.

3.2 Materials and methods

3.2.1 Study area and collection of marula fruits and wines

The study was conducted in Limpopo province, South Africa. Nine liters of fermenting marula wines and fruits were collected from Mentz (S23 54′ 3.54″ E29 46′ 28.02″), Moshira in Sekhukhune (S24 20′ 35.84″ E30 5′ 33.07″), while from Blompoort, Dennilton (S25 10′ 60″ E29 11′ 60″) only the wine was collected. One wine sample (Lab wine) was kept and allowed to ferment in the Microbiology laboratory, University of Limpopo (UL) and this was used for comparison in terms of reduced handling events commonly done when serving the wine.

Fermenting wine samples were collected from the local producers at different stages of fermentation. All the wines were produced by the traders.

3.2.2 Preparation of Lab marula wine

The lab wine was prepared from the ripe marula fruits that were collected from UL campus. The preparation of the wine was done following community brewer's instructions. Approximately 20 kg of ripe marula fruits were collected from the ground (S23 52′ 57.43″; E29 43′ 1.77″) after abscission, washed with tap water and peeled off using a fork to squeeze the juice out. The juice was mixed with an equal part of

tap water and divided into two equal portions. The pulp, pip and juice were put into a clean bucket, tap water was then added to cover the pips and the mixture was thoroughly mixed by kneading until the liquid appeared thicker. The juice was strained into a new container, and combined with the juice. An equal portion of tap water was added, mixed thoroughly and divided into two equal portions. One portion was kept and allowed to ferment in the Microbiology laboratory and the other was transported to the brewer's (Mrs R Maipa) home in Mentz and allowed to spontaneously ferment at room temperature.

Both the fruits and the fermenting wines were transported to the laboratory at UL for further processing. At each sampling point 1.5 mL of marula wine was drawn, transferred into a sterile micro-centrifuge tube and centrifuged at 10 000 rpm for 5 min at room temperature. The pellet was suspended in 50% glycerol water and stored at -80°C until needed.

3.2.3 Sampling of marula wine

The sampling of the fermenting marula wines was carried out as shown on Table 3.1.

Table 3.1. Marula wine sampling.

Day intervals	Lab	Mentz	Moshira-A	Moshira-B	Blompoort
0	x				
1			x		
2	x	x		x	
4	x	x	x		
5				x	x
6		x			
7	x		x		x

8		x		x	
9	x		x		x
10		x		x	
11	x		x		x

3.2.4 Microbial analysis of the marula wines

3.2.4.1 Enumeration and isolation of bacteria and yeast

The yeast population was enumerated by direct plating on Wallerstein Laboratory Nutrient (WLN) agar (Sigma Aldrich, Steinheim, Germany) supplemented with 34 mg/L chloramphenicol (Sigma Aldrich) to suppress bacterial growth and 250 mg/L biphenyl (Riedel-de Haen AG, Seelze, Germany) to inhibit the growth of moulds. Bacteria were isolated on de Man Rogosa and Sharpe (MRS) medium (Biolab, Merk, South Africa) supplemented with 100 mg/L Delvocid (DSM, The Netherlands) to inhibit fungal growth. Ten-fold dilutions of the samples were prepared in saline (0.9% w/v NaCl) by adding 100 µL of the sample into 900 µL of saline and 100 µL of the undiluted and diluted samples were spread-plated for enumeration of the microorganisms. The plates were incubated at 30°C until growth was visible. Colony counts (CFUs) were performed and ten percent of morphologically distinct colonies were randomly picked, sub-cultured and purified through successive streaking on the WLN agar for yeast and MRS agar for bacteria. The pure cultures were then identified by means of the MALDI-TOF Biotyper or rRNA genes sequencing.

3.2.4.2 Molecular identification of the yeast and bacteria isolates

3.2.4.2.1 Biotyping of bacterial isolates

Maldi-TOF Biotyper® (Bruker Daltonik GmbH, Bremen, Germany) was used for identification of the purified bacterial isolates. A single bacterial colony from a 24 hrs culture was thoroughly suspended in 300 µL distilled water and 900 µL of absolute ethanol was added. The cell suspension was vortexed and then centrifuged at 10

000 rpm for 2 min. The supernatant was removed and 50 µL of formic acid was added to the cell pellet before vortexing. Acetonitrile (50 µL) was added, the mixture was vortexed vigorously and this was followed by centrifugation at 10 000 rpm for 2 min. One microliter of the supernatant was spotted onto the MALDI-TOF target plate and allowed to dry. The samples were overlaid with 1 µL of HCCA matrix (α-cyano-4-hydroxycinnamic acid [Vitek MS-CHCA 41107] in 50% acetonitrile and 1.5% trifluoroacetic acid) and dried at room temperature. The samples were applied to a MicroFlex LT mass spectrometer (Bruker Daltonik), and the results were analysed by MALDI Biotyper 3.0 software (Bruker Daltonik) in automatic mode using the manufacturer's settings.

3.2.4.2.2 Amplification and sequencing of the rRNA genes

Pure morphologically distinct bacterial isolates were inoculated into 5 mL of MRS broth and incubated at 30°C for 24 hrs with shaking at 180 rpm on a rotary shaker. Yeast isolates were routinely cultured in YPD broth and incubated at 30°C for 24 hrs with shaking at 180 rpm on a rotary shaker. The DNA was used as template for 16S rDNA amplification and sequencing.

Yeast genomic DNA was extracted from 1mL of the sample following the extraction method described by Sambrook and Rusell (2001), while the bacteria genomic DNA was extracted from the 1 mL of the sample according to the bacterial mini-preparation procedure described by Neumann *et al.* (1992). Yeast ITS-5.86 rRNA region was amplified using primers set ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC) (White *et al.*, 1990). The bacterial 16S rDNA was amplified using the primer set EUbB (27F) (5′-AGAGTTTGATCMTGGCTCAG-3′) and EUbA (1522R) (5′-AAGGAGGTGATCCANCCANCCRCA-3′) (Suzuki and Giovannoni, 1996). The Polymerase chain reaction (PCR) was performed in a 25 µL reaction mixture containing 2.5 µL Ex Taq buffer (10 X buffer, 1 Unit of Ex Taq™ polymerase) (TaKaRa Bio inc., Olsu, Shiga, Japan), 1 µL of 25 mmol/L $MgCl_2$, 4 µL of 2.5 mmol/L DNTPs, 1 µL of a template DNA (100 ng/µL) and 11.3 µL of sterilised de-ionized H_2O.

For yeast 18S rDNA amplification, the PCR conditions consisted of an initial denaturation at 94°C for 3 min followed by 40 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 45 sec, and a final extension at 72°C for 10 min. The amplification of the 16S rDNA of the bacteria was also carried out in the similar PCR condition except that it was for 35 cycles at an annealing temperature of 55°C.

The PCR amplicons were subjected to Sanger's sequencing using the ITS4 primer for yeasts and the EUbA for 16S rDNA sequencing for bacteria. The PCR amplicons of both the yeast and bacteria were purified using the Zymmoclean™ Gel DNA recovery kit (Zymo Research Corporation, Irvine, CA, USA), following the manufacturer's protocol and the purified PCR products were sent to the Central Analytical Facility (CAF), Stellenbosch University for sequencing. The resulting sequences were aligned using Bio-edit (Informer Technologies, Inc) and the taxonomic assignment of individual isolates was performed by comparing the ITS-region and 16S rRNA sequences to the sequences available in GenBank (NCBI) (http://www.ncbi.nlm.nih.gov/pubmed) using the Basic local alignment search tool (BLAST) algorithm (Altschul *et al.*, 1997).

3.2.4.2.3 Crude DNA extraction and ARISA fingerprinting

The yeast communities associated with marula wine were evaluated using PCR and ARISA. The glycerol stock sample of marula wines at varying stages of fermentation was centrifuged at 5630 x g for 10 min to collect microbial biomass. The pellet was re-suspended in TE lysis buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) and the DNA was extracted as described by Hoffman and Winston (1987). The ITS1-5.8S-ITS2 rRNA region was amplified with the FAM-labelled ITS1 primer (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), using the Phire® Plant Direct PCR kit (FINNZYMES OY, Espoo, Finland) under the following conditions: an initial denaturation for 6 min at 98°C, followed by 40 cycles at 98°C for 20 sec, 54°C for 30 sec, 72°C for 1 min, and a final extension of 10 min at 72°C. The ARISA-PCR fragments were separated by capillary electrophoresis on an ABI3010xl Genetic Analyzer (Applied Biosystems, CA, USA) to obtain electropherograms of the different fragment lengths and fluorescent intensities. A

ROX1.1 size standard was used. The ARISA data was analysed using Genemapper 4.1 software (Applied Biosystems).

A threshold of 50 fluorescent units was used to exclude background fluorescence. The software converted fluorescence data into electropherograms, where the peaks represented fragments of different sizes, and the peak areas represented the relative proportion of these fragments. The number of peaks in each electropherogram was interpreted as the OTU richness in the community. The fragment lengths and fluorescence for each sample were aligned using an Excel Macro. Only fragment sizes larger than 0.5% of the total fluorescence and between 300 and 1000 bp in length were considered for analysis. A band size of 3 bp for fragments below 700 bp and 5 bp for fragments above 700 bp was employed to minimise the inaccuracies in the ARISA profiles. All elution points in the electropherograms that did not contain a peak in at least one sample were removed with the use of a custom built Perl program. This process resulted in a matrix in which each row represented a sample and each column represented an OTU (species). Principal component analysis (PCA) of the ARISA profile matrix was performed in STATISTICA software Version 10.

3.3 Results

The current study sought to monitor the microbial population dynamics in marula wine fermentations using wines prepared in different areas of the Limpopo province in South Africa. The marula wine (lab wine) prepared and sampled under controlled hygienic conditions was used as a reference.

3.3.1 Population structure and evolution of the microbiota during fermentation

3.3.1.1 Microbial population of the marula wines

The bacteria and yeast populations showed a similar trend for all the ferments except that there the bacterial population was comparatively higher at the beginning of the fermentation at a range of $2.27 \times 10^3 - 1.57 \times 10^5$ CFU/mL. This trend was

maintained as fermentation progressed until the latter stages where a slight decline was observed (Fig. 3.1 B). The total yeast load for the Lab, Mentz and Blompoort ferments was lower ($2.22 \times 10^4 - 2.31 \times 10^4$ CFU/mL) but the Moshira ferments had a higher yeast load at the early stage of fermentation. With the exception of Moshira B and Blompoort ferments, the yeast load of the ferments increased steadily during the fermentation period but decreased at the late stage when the wines were becoming bitter and not palatable. Interestingly, the Lab wine which was kept under controlled laboratory conditions maintained a low bacterial load throughout and the two Moshira wines both had higher bacterial (1.26×10^5 CFU/mL and 1.57×10^5 CFU/mL) and yeast (1.17×10^6 CFU/mL and 2.50×10^7 CFU/mL) loads at days 1 and 2 and (1.42×10^6CFU/mL and 1.54×10^5 CFU/mL; 1.71×10^5 CFU/mL and 5.63×10^4 CFU/mL) at days 10 and 11 respectively (Fig. 3.1)

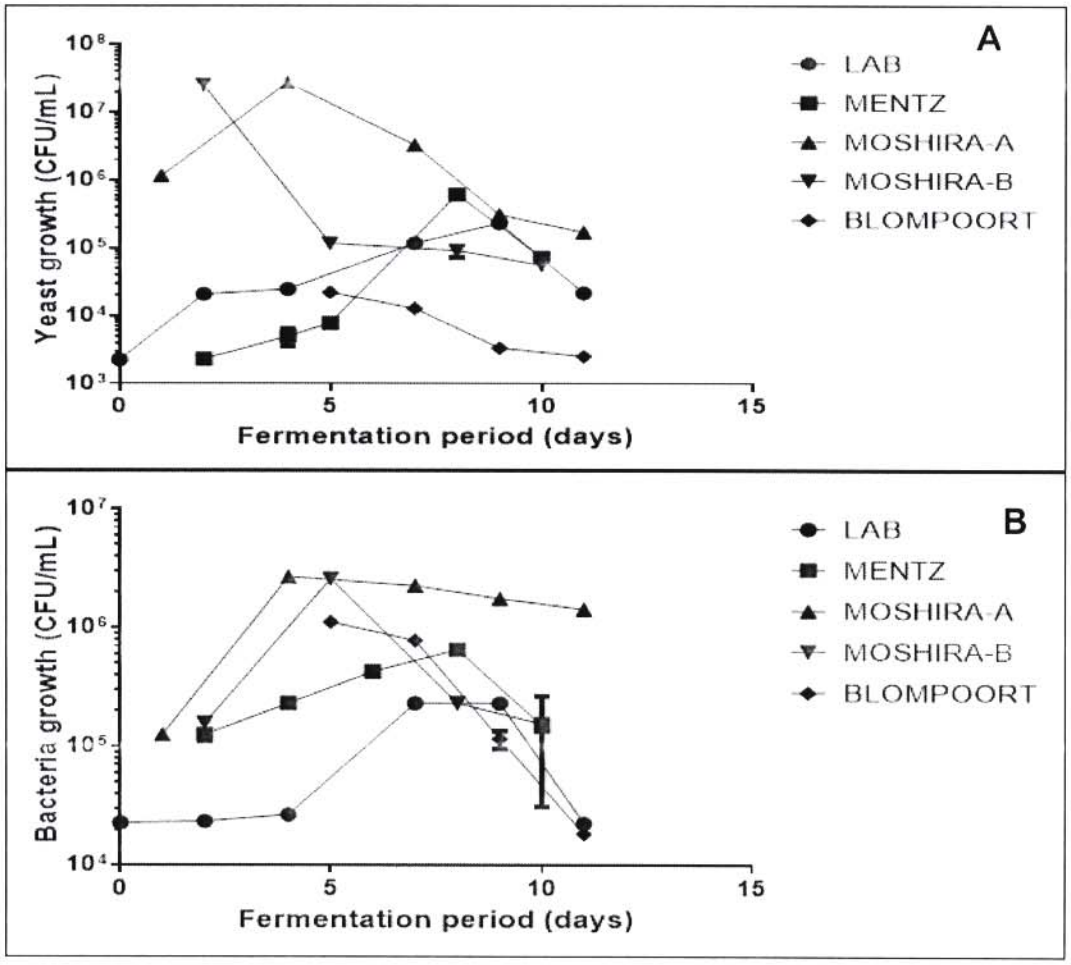

Figure 3.1. Yeast and bacteria numbers during fermentation of marula wines from Lab, Mentz, Moshira-A, Moshira-B, and Blompoort.

3.3.1.2 Yeast dynamics during spontaneous fermentation

Fermenting and non-fermenting yeasts were observed at varying stages of fermentation with clear evidence of replaceability. Only yeast species representing more than 1% of the total population were reported. Four different yeast species were identified in the Mentz, Lab, and both Moshira wines and a total of five species in the Blompoort wine. *Hanseniaspora guilliermondii, Saccharomyces cerevisiae, Issatchenkia terricola* and *Rhodotorula mucilaginosa*, were present in all wines while *Pichia kudriavzevii* was only present in the Blompoort wine. Non-*Saccharomyces* yeasts were dominant during the early stages of fermentation, i.e., days 0 to 4 with *Hanseniaspora guilliermondii* as the dominant isolate (Figs. 3.2 – 3.11). The mid and late stages (days 5 to 11) of fermentation were dominated by *Saccharomyces cerevisiae* in all the wines. *Pichia kudriavzevii* which was only present in the Blompoort wine appeared after 6 days of fermentation at a low abundance of and became undetectable after day 9 (Fig. 3.6).

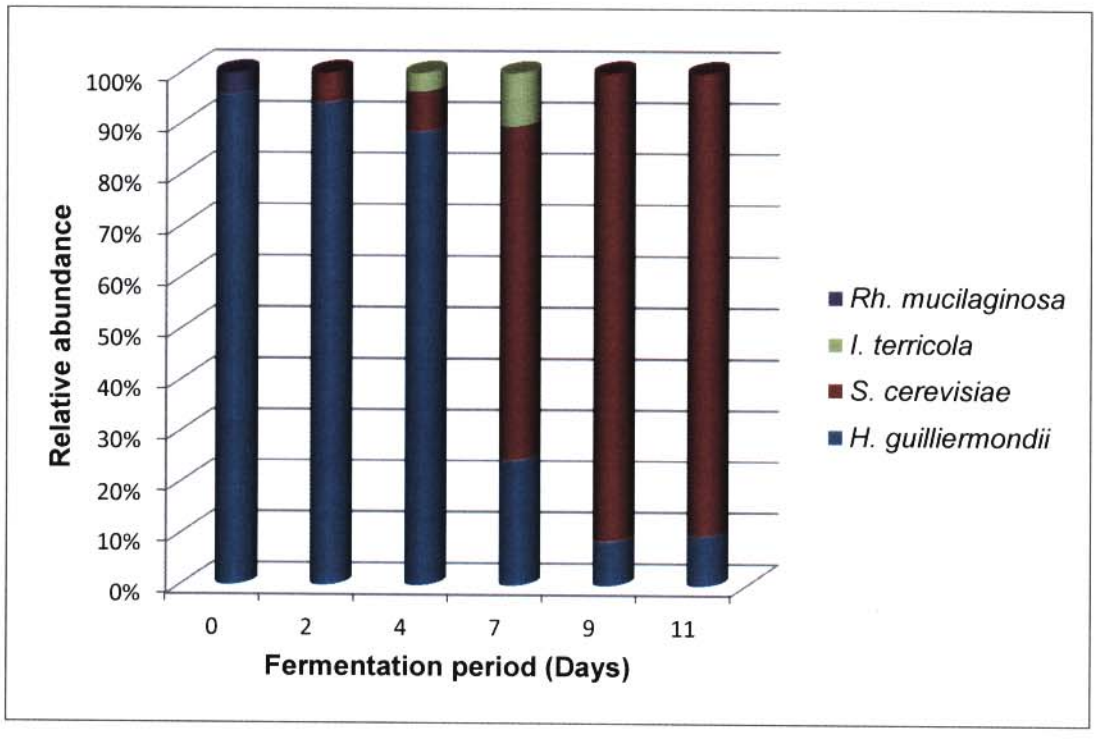

Figure 3.2. Population structure and relative abundance of the yeast species identified in the marula wine from Lab during fermentation.

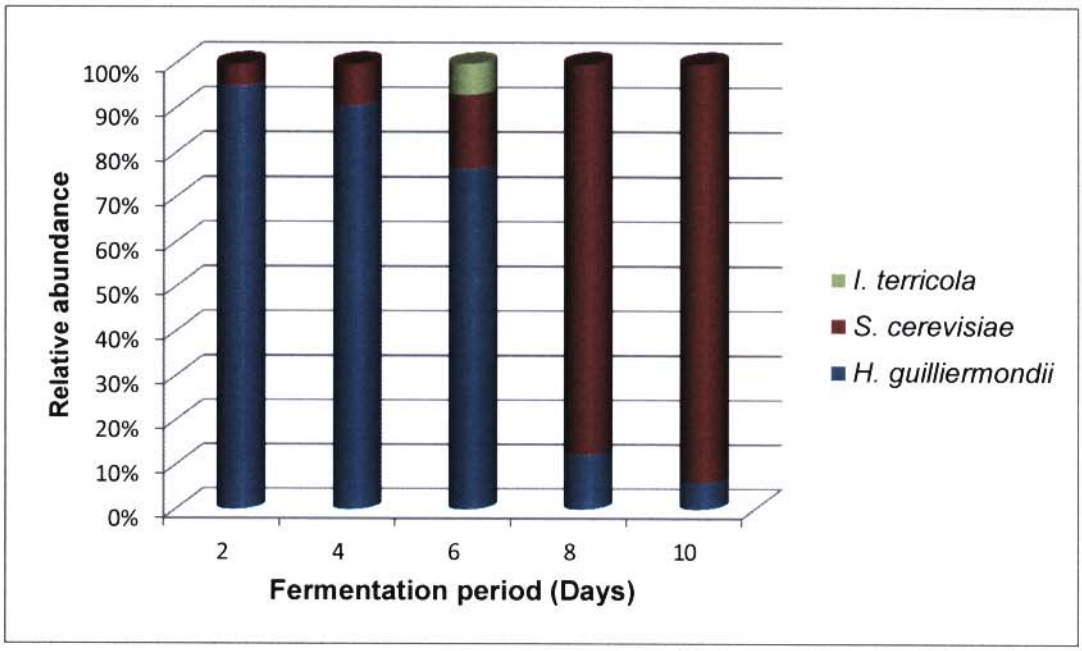

Figure 3.3. Population structure and relative abundance of the yeast species identified in the marula wine from Mentz during fermentation.

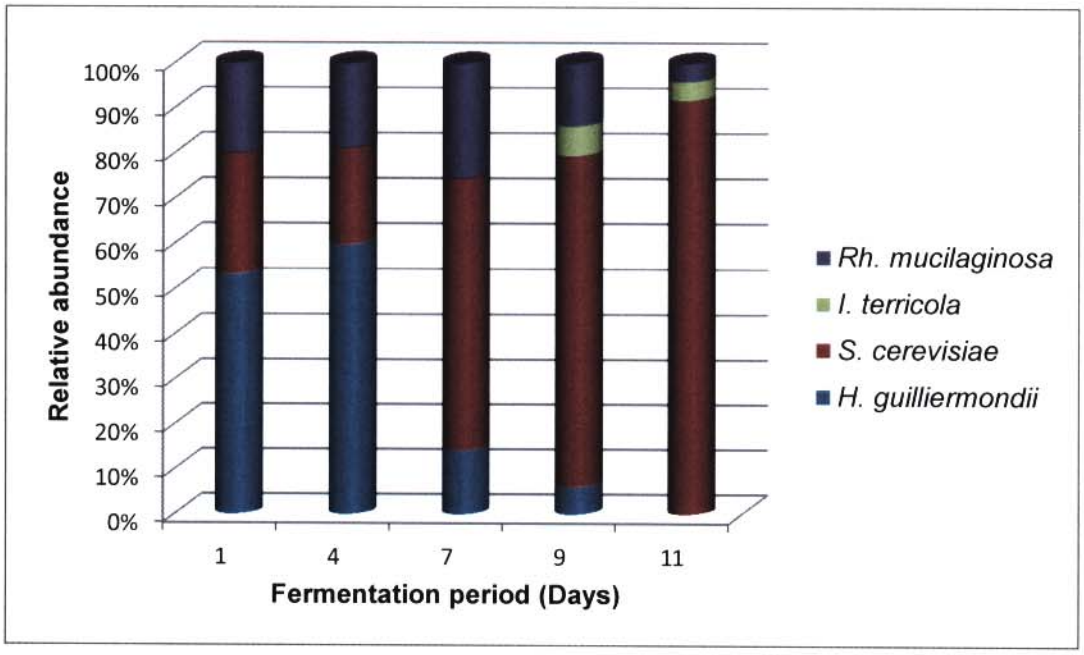

Figure 3.4. Population structure and relative abundance of the yeast species identified in marula wine from Moshira-A during fermentation.

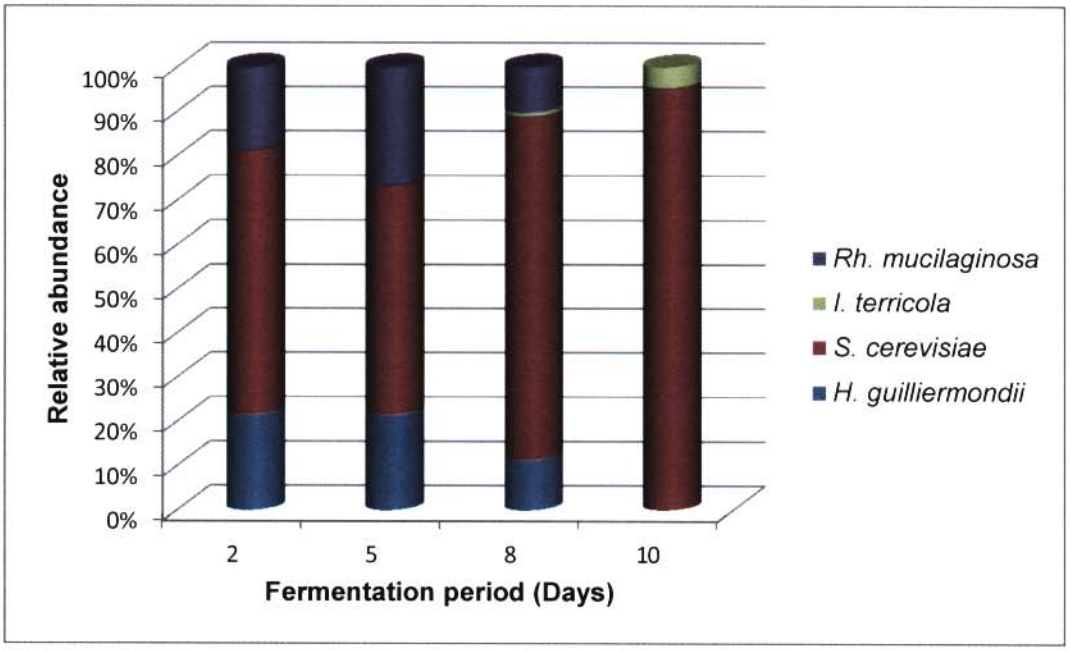

Figure 3.5. Population structure and relative abundance of the yeast species identified in the marula wine from Moshira-B during fermentation.

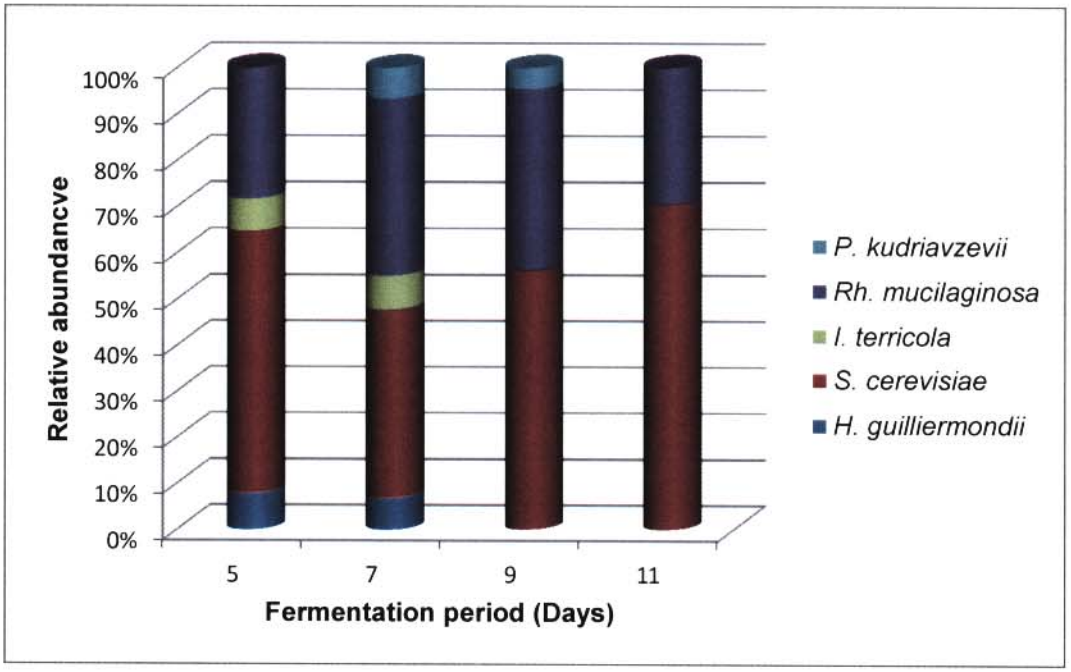

Figure 3.6. Population structure and relative abundance of the yeast species identified in marula wine from Blompoort during fermentation.

3.3.1.3 Bacteria dynamics during spontaneous fermentation of marula wine

The spontaneous fermented marula wines were found to harbour various species of Lactic acid and Acetic acid bacteria. A total of nine different species of *Lactobacilli* were isolated from the marula wines obtained from different localities with *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus buchneri, Lactobacillus parabuchner,* and *Lactobacillus brevis* dominating throughout the fermentation period. Acetic acid bacteria included *Gluconobacter oxydans, Acetobacter pasteuriannus* and *Acetobacter aceti* (Figs. 3.7 – 3.10). Bacterial species including *Asaia bogorensis* and *Lactobacillus fabifermentans* were only observed in the Lab wine in low numbers (Fig. 3.7).

The pattern of bacterial distribution was different from the yeast profile that showed a distinct dominance of non-fermenting yeasts at the early stage of fermentation and was displaced by the fermenting *S. cerevisiae* at the mid stage of fermentation. With the bacterial population, the *Lactobacillus* species were present throughout the fermentation period, with relative total abundance of 30 – 90% in the first 5 days. *L. buchneri* was present throughout in significant amount whereas *L. fabifermentans*, *L. brevis* and *L. paracasei* were mostly detected at the earlier stages of fermentation. Similarly, few Acetic acid bacteria such as *G. nepheli* and *A. bogorensis* were detected in low numbers at the early stages of fermentation and the latter stages were dominated by *A. aceti* and *A. pasteuriannus*.

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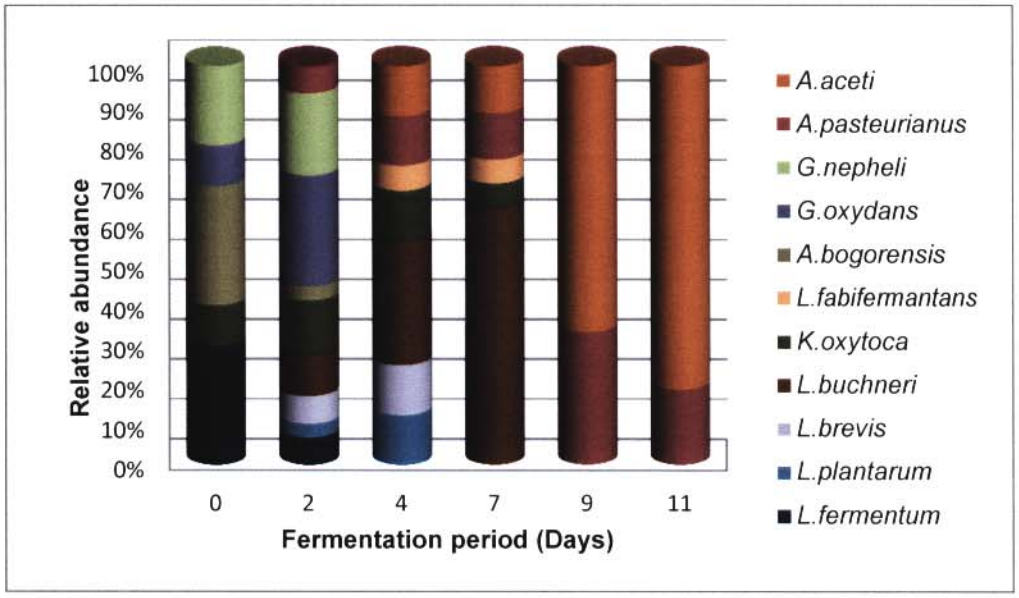

Figure 3.7. Population structure and relative abundance of bacterial species identified in marula wine from Lab during fermentation.

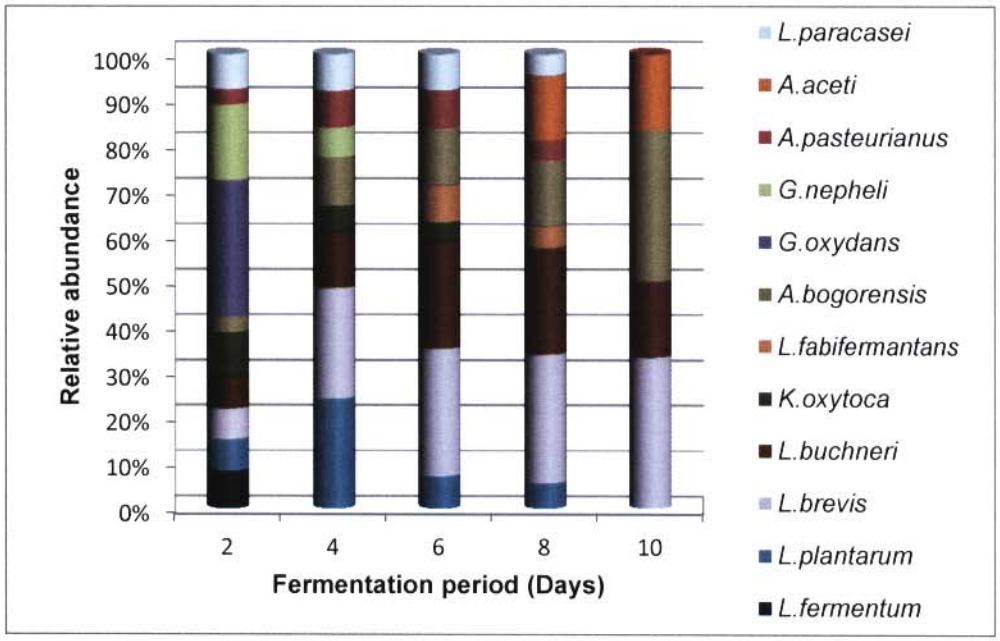

Figure 3.8. Population structure and relative abundance of the bacterial species identified in the marula wine from Mentz during fermentation.

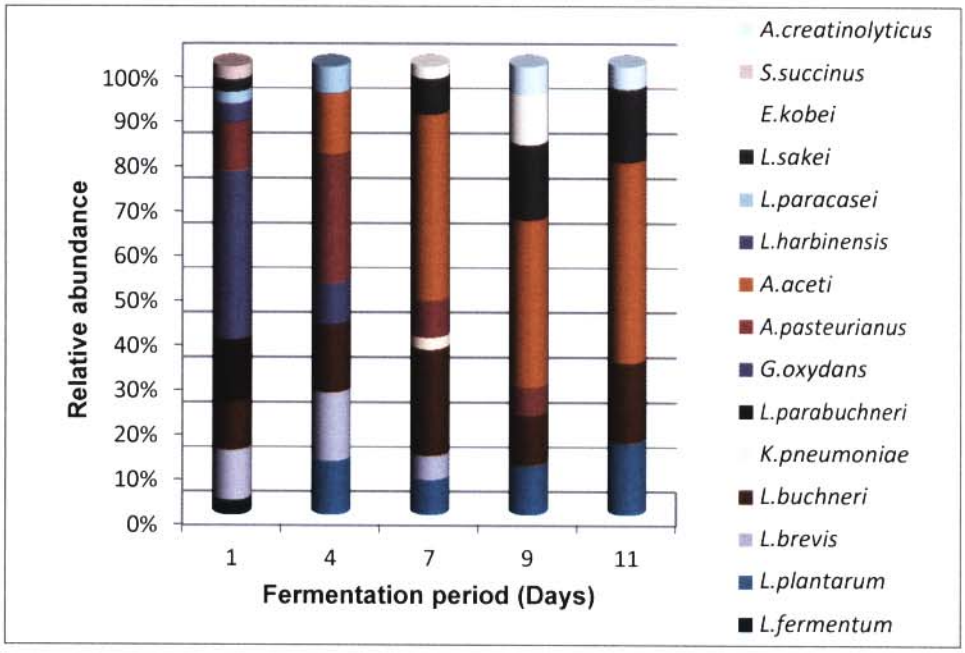

Figure 3.9. Population structure and relative abundance of the bacterial species identified in the marula wine from Moshira-A during fermentation.

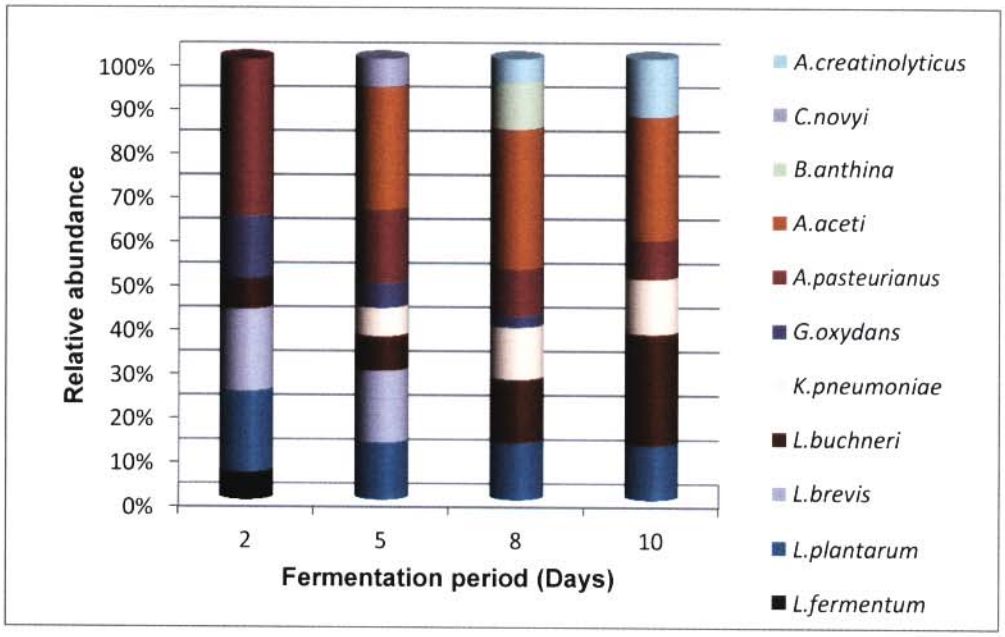

Figure 3.10. Population structure and relative abundance of the bacterial species identified in the marula wine from Moshira-B during fermentation.

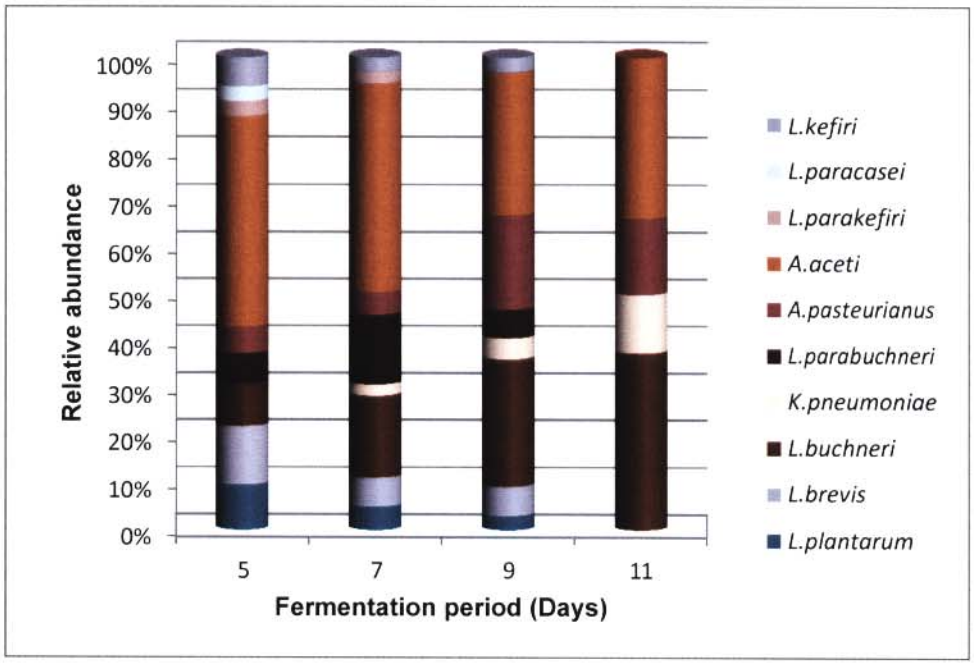

Figure 3.11. Population structure and relative abundance of the bacterial species identified in marula wine from Blompoort.

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3.3.2 Variability of the marula wine associated fungal communities

The diversity and variability of the fungal community structures associated with marula were evaluated using ARISA for 11 consecutive days of spontaneous fermentation. PCA analysis including the most abundant OTUs from each wine was performed to show the variation between the different marula wines. The marula wines were then differentiated according to the ARISA fingerprints as illustrated in figure 3.12. The plot data revealed a variation in the fungal community structures of the marula wines where the first and second principal components explained 34.2% and 15.7% of total variance respectively (Fig. 3.12). The Mentz wine fungal community showed a similar structure to that of the reference Lab wine. The community network of these two wines showed an evidence of highly connected OTUs revealing an overlap of their fungal communities (Fig. 3.12). On the other hand, the fungal community structures of the two Moshira wines showed different structures which were also different to that of the reference Lab wine. The scatter plot showed a different clustering of the fungal communities of the wines. The relative peak heights showed more variability.

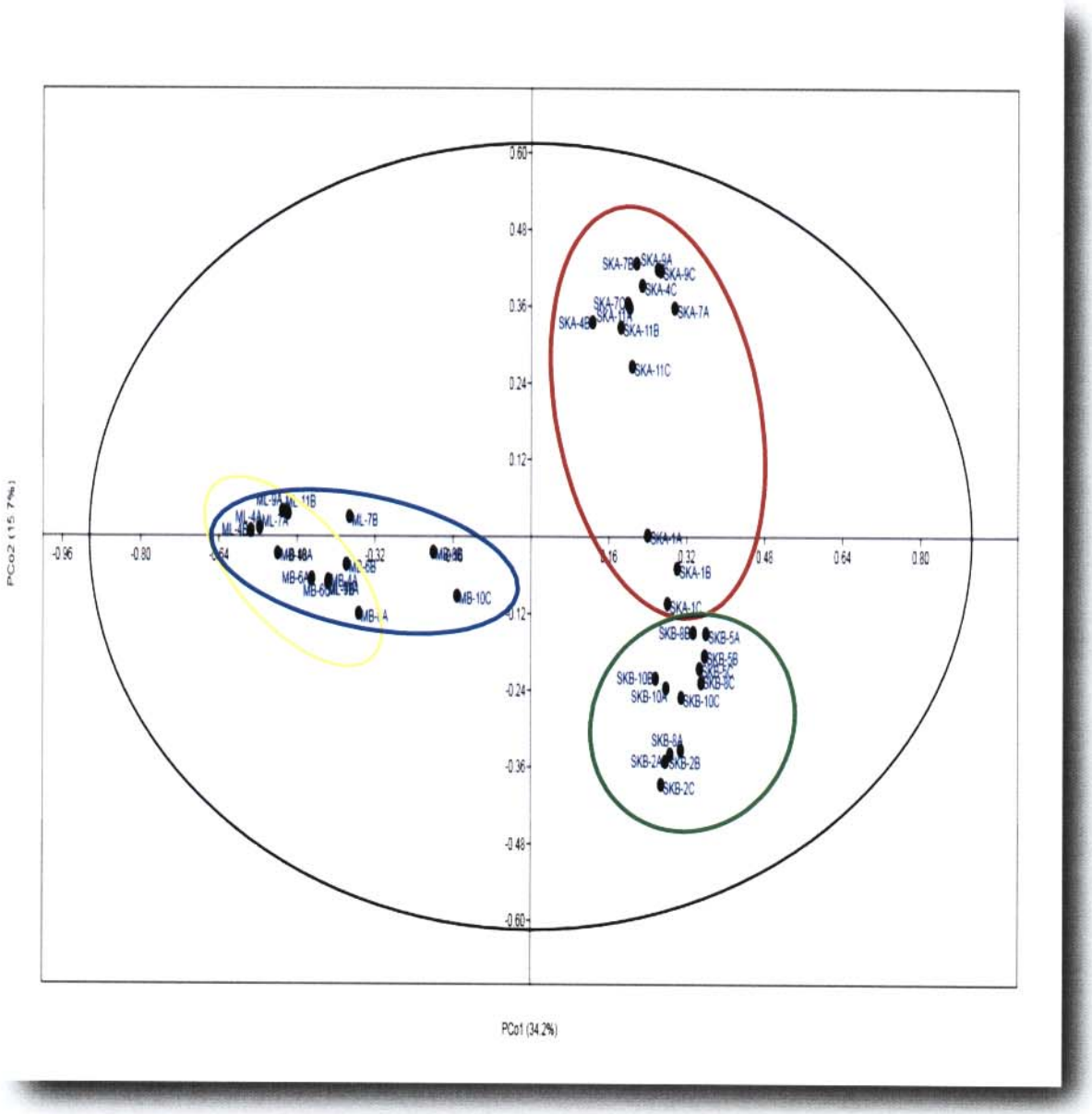

Figure 3.12. Two-dimensional principal component analysis (PCA) on fungal community structures in the marula wine assessed by ITS1-5,8S-ITS2 rRNA gene ARISA profile.

Lab, Mentz, Moshira-A, Moshira-B

3.4 Discussion

The current study evaluated the change in the microbial profiles and also the yeasts diversity of five different wines during fermentation over an eleven day period. This period covered from the juice state to the stage when the wine was bitter and not palatable. The wine sampling was performed at different stages of fermentation for analyses of the bacteria and yeast profiles and their evolution throughout fermentation. This study sought to investigate the contributing microbiota to the characteristic taste and aroma of the marula wine. The microbial analysis revealed Lactic acid bacteria, Acetic acid bacteria and yeasts as the main microorganisms present in the fermentation of the marula wines. A succession of the yeast species of both non-*Saccharomyces* and *Saccharomyces* together with the Lactic acid and Acetic acid bacterial species were observed throughout fermentations. *H. guilliermondii* was the dominant yeast species at the early stage of marula juice fermentation, the period where the sugar content was higher. The reduction of *H. guilliermondii* from day 4 onwards coincided with the appearance of *S. cerevisiae* as more sugars were being metabolised into volatile compounds such as ethanol and some organic acids including acetic acid and formic acid.

The presence of non-fermenting yeast such as *H. gulliermondii* and *I. terricola* in wine was previously reported by Wang and Liu (2013). These yeast species have low tolerance for ethanol (Jolly *et al.*, 2006) and they disappear from mid to late stage of fermentation when ethanol production increases in the wine. Similar pattern of yeast evolution has been reported in the alcoholic fermentation of grape wine and juice where the non-*Saccharomyces* yeast species of *Hanseniaspora*, *Candida*, *Pichia* and *Metschnikowia* initially found in the grape must decreased as *S. cerevisiae* numbers increased (Fleet, 2003). However, these non-*Saccharomyces* yeast species have been noted to re-appear in the latter stages which are dominated by the *S. cerevisiae* although in low numbers while *Rh. mucilaginosa* persisted throughout the fermentation period (Diaz *et al.*, 2013). Interestingly *P. kudriavzevii* was only found in the marula wine from Blompoort. The presence of *P. kudriavzevii* was observed in wines with high residual sugar content (Clemente-Jimenez *et al.*, 2004) which was the case with marula wine from Blompoort in this study. *Pichia kudriavzevii* was observed in the presence of *S. cerevisiae* during fermentation of

grapes (Aponte and Blaiotta, 2016) and in some instances it dominated *S. cerevisiae* at the late stages of fermentation of the grape wine (Wang and Liu, 2013).

The succession of the bacteria observed in this study is similar to that reported by Dlamini and Dube (2008). It is apparent that the fermentation of marula wine is mediated by a complex mixture of yeast and bacterial species. Bacterial species such as the hetero-fermentative *L. fermentum*, *L. plantarum*, *L. paracasei* and *L. brevis* were present in high numbers in marula wines. Acetic acid bacteria such as *G. oxydans* emerged in low numbers at the initial stages of fermentation and it disappeared during the mid to latter stages wherein *A. aceti* dominated the more acidic wine. *Gluconobacter oxydans* is associated with sugar rich environment (Bartowsky and Henschke, 2008) but it has a low tolerance for ethanol (De Ley *et al.*, 1984). *Lactobacillus plantarum* is deemed the most active species in wine fermentation (Berbegal *et al.*, 2016). Amoa-awua *et al.* (2007) also reported in their study of traditional fermented palm wine a presence of some Lactic acid bacteria mainly *L. plantarum* and some acetic acid bacteria of both genera *Acetobacter* and *Gluconobacter*. The presence of *L. plantarum* and *L. mesenteriodes* in the palm wine were reportedly responsible for a rapid acidification of the wine during the first day whilst the acetic acid bacteria were responsible of the acidification at the latter stages (Amoa-awua *et al.*, 2007). In contrast, *L. fermentum* which was also detected in the current study was identified as the main Lactic acid bacteria responsible for the souring of the *burukutu*, a traditional fermented sorghum beer produced in Ghana (Atter *et al.*, 2014).

Generally, spontaneous fermentation of marula wine involves a complex interaction of the yeasts mainly *Saccharomyces* and Lactic acid bacteria and to a lesser extent Acetic acid bacteria initially found on the surface of the fruits (Dlamini and Dube, 2008). The yeast and Lactic acid bacteria have a symbiotic relationship in which the Lactic acid bacteria produce a favourable acidic environment for the proliferation of the yeast genera (Wilson *et al.*, 2012) while the yeast contributes to the growth of the Lactic acid bacteria through production of vitamins and other nutritional factors such as amino acids (Jespersen, 2003).

This study also evaluated the diversity and variability of the yeast communities associated with the Lab, Mentz, and the two Moshira wines. The dynamics of the

yeast populations were monitored during the spontaneous fermentation. The yeast population of the wines was at 10^4 to 10^6 CFU/mL, similar order to magnitude reported in the grape wine (Bagheri *et al.*, 2015). Molecular ecological distributions based on the profiles derived from the ARISA fingerprinting were employed to assess the variability in the yeast community structures between the evaluated samples. The yeast populations from different areas were substantially distinctive. Despite the common yeast population present in the wines, the fungal community of each wine demonstrated distinctive clustered yeast population. The Lab and Mentz marula wines that were produced from the same marula fruit juices showed similarities in the structure of the yeast community which is an evidence of connected OTUs and this is further shown by the overlapping of the community structures between the two wines. The study also showed that there are OTUs distinctive to a certain wine. Despite having marula wines produced from marula fruits obtained from the same area there is a difference in the yeast community compositions to separate the wines from each other. This was observed with the two Moshira wines which were produced from the same marula fruit collection but prepared separately. This infers that there may be several OTUs which are unique to a specific marula wine, thus prevalence of species heterogeneity in the wines. Setati *et al.* (2012) reported a prevalence of significant species heterogeneity between grape juice samples from the same vineyard.

The variability in the yeast composition of the wines in the current study also revealed a phylogenetic divergence over geographic space, where the yeast community structures between marula wines from different areas clustered far apart. The geographical factors such as climatic conditions and the soil differences between villages could result in the variation in the composition of the plant-associated yeast population present in the raw material. The soil structure mainly determines the microbial community associated to the soil and also present in the plant (Corneo *et al.*, 2013). Moreover, the temperature of the soil also affects the microbial behaviour (Lavelle and Spain, 2001), hence the discrepancies in microbial communities (Corneo *et al.*, 2013). This is evident in the current study where the yeast communities in the marula wines within the area showed less percentage of variance than between the areas. Colehour *et al.* (2014) also highlighted the similar findings to this study in their study of the bacterial communities in spontaneous

fermented cassava beers. The bacterial communities in the cassava beer from the households showed correlation in the beer from the same village than between the villages (Colehour *et al.*, 2014). Despite the apparent disparities in the yeast diversity, the data from the study evidently highlighted the similar yeast community profiles between marula wines produced from the marula fruits from the same geographical area despite a slight variability.

CHAPTER 4

Chemical composition of marula juice and wines during fermentation

4.1 Introduction

The composition of the marula juice has an influence on the fermentation product that is, the chemical composition and sensory quality of the wine. During the process of fermentation, microorganisms metabolise the fermentable sugars present in the must and produce varying types of chemicals. These are dependent on the fermenting yeast and contributing bacterial strains and the properties of the raw materials. Yeast strains commonly produce alcohol and, the Lactic acid bacteria convert sugars into lactic acid and Acetic acid bacteria convert the produced ethanol to acetic acid (Dlamini and Dube, 2008). Yeasts such as *S. cerevisiae* convert the sugar from the fruits to ethanol and carbon dioxide and other end products such as organic acids, higher alcohols and esters and, to a lesser extent, aldehydes (Rapp and Versini, 1991). Alcohol contributes to the flavour, giving rise to a warming character and further plays a significant role in the flavour perception of consumers (Campillo *et al.*, 2009; Pinho *et al.*, 2006).

Volatile organic compounds are constituents of many fermented beverages and commonly comprise of esters, alcohols, aldehydes, acids, terpenes, ketones, sulphur compounds, amines and phenols (Cortacero-Ramírez *et al.*, 2003; Lui *et al.*, 2005). However, the volatile component of fermented products varies depending on the microflora involved, as well as the processing conditions such as fermentation, drying, brining or ageing (Leejeerajumnean *et al.*, 2001). The volatile organic compound such as organic acids and other secondary metabolites are produced by microorganisms that are involved in the fermentation process and they contribute to the aroma and taste of the beverage. Moreover, these compounds are considered important in the brewing industry due to their impact on the quality of the beverage and that they enhance consumer acceptance (Guillaume *et al.*, 2009; Lui *et al.*, 2005). Organic acids influence the overall acid-balance of the wine, thus wine with too low acid contents will have a flat taste while high acidic levels results in an excessively sharp acidic or sour taste (Mato *et al.*, 2005). Secondary metabolites,

such as higher alcohols and esters, produced by yeast and bacteria during alcoholic fermentation are the mostly highly volatile compounds and they as well particularly contribute to the aroma of the wine (Styger *et al.*, 2011). Various organic acids present in indigenous beer have several significant roles in the beverage, as they affect the character and organoleptic sensory properties, stability, nutrition, acceptability and quality of the finished product (Aka *et al.*, 2008; Castiňeira *et al.*, 2002; Santalad *et al.*, 2007).

Over the past years, numerous studies have been conducted on chemical composition of various spontaneously fermented indigenous beverages (Atter *et al.*, 2014; Mimura *et al.*, 2014; Shale *et al.*, 2013). However the profiles of the organic volatile compounds and sugars of the unfermented and fermented marula juice at different stages of fermentation have not been studied. Sugars and organic volatile compounds play a significant role in the fermented products in terms of the quality and acceptability of the product. This study sought to profile the chemical dynamics of the marula fresh juice and fermented juice at different stages of fermentation.

4.2 Materials and methods

The supernatants of the marula juice and wines which were collected at different stages of fermentation were centrifuged at 10 000 rpm for 5 min. The resulting supernatant was filtered through 0.22 µm Whatman filter membrane into 1.5 mL HPLC vials.

4.2.1 Determination of sugar content of marula juice and wines

Fermentable sugars, namely, sucrose, glucose and fructose from the marula juice and fermenting wine samples were determined by HPLC using Shimadzu Prominence 20 HPLC system. Samples of 20 µL were injected into a Rezex RHM-monosaccharide H^+ column (300 x 7.8 mm) and eluted using water at a flow rate of 0.6 mL/min. The column temperature was kept at 85°C. The separated components were detected using a Shimadzu RID10A refractive index detector. Data was processed using LC Solutions software. Using the external standard method, the

analytical standards (Sigma-Aldrich) such as sucrose, glucose and fructose at varying concentrations of 0.39 – 100 mg/mL were prepared in distilled water. The standard curves of the sugars were constructed and used to determine the concentrations of the sugars.

4.2.2 Determination of volatile compounds in marula juice and wines

The volatile organic compounds in marula juice and wine samples were analysed by capillary gas chromatography using Shimadzu GC2010Plus gas chromatograph on a Nukol^{TM} capillary column (15 m x 0.53 mm x 0.5 µm). Nitrogen was used as the carrier gas. A sample of 1 µL was injected into the column at an injection temperature of 220°C. The column temperature was kept at 200°C with a flow rate of 1.33 mL/min. The volatile organic compounds were detected using flame ionization detector (FID), with the detector temperature maintained at 220°C. The peaks were processed using GC Solutions software. The pure compounds (Sigma-Aldrich) of organic acid (acetic acid, formic acid, propionic acid, isobutyric acid, butyric acid isovaleric acid, valeric acid isocaproic acid, caproic acid, heptanoic acid, nonanoic acid), alcohols (ethanol, n-butanol, 1-pentanol. 1-propanol, 2-methyl-1-propanol), aldehydes (formaldehyde, acetaldehyde butyraldehyde and isobutyraldehyde) and ester (isopentyl acetate) were used as standards to aid in the identification of the compounds present in the marula juice and wine.

4.2.3 Statistical analysis

The statistical analysis of the results was done using Graphpad Instat version 3 software analysis of variance (ANOVA) with Tukey test for comparison of any significant differences between the means. The significance of the differences was considered at $P < 0.05$.

4.3 Results

This study investigated the chemical profile of different marula wines at the varying stages of fermentation. The unfermented marula fruit juice and fermented wines contained various volatile compounds which are metabolites of microbial activities and other chemical reactions taking place in the wines. The results below represent the chemical dynamics of different marula wines at different stages of fermentation.

4.3.1 Analysis of sugars in marula fruits and juice

The change in the marula wine sugars was determined as a standard equivalent. Sucrose accounted for 70% of the total sugars, with glucose and fructose making up the balance of 30% (Fig. 4.2). The concentrations of sucrose, fructose and glucose in the fresh pulp used for marula wine fermentation were 36.38 mg/mL, 10.54 mg/mL and 9.65 mg/mL, respectively in the Lab ferment.

A high significant difference ($p < 0.0013$) for sucrose, glucose and fructose in the fresh marula fruits from Mentz was observed as compared to the sugar contents of the marula fruits from Moshira ($p < 0.0025$). Sucrose differed significantly ($p < 0.01$) from both glucose and fructose in marula fruits from both areas. However, there was no significant difference ($p > 0.05$) between fructose and glucose in both marula fruit samples.

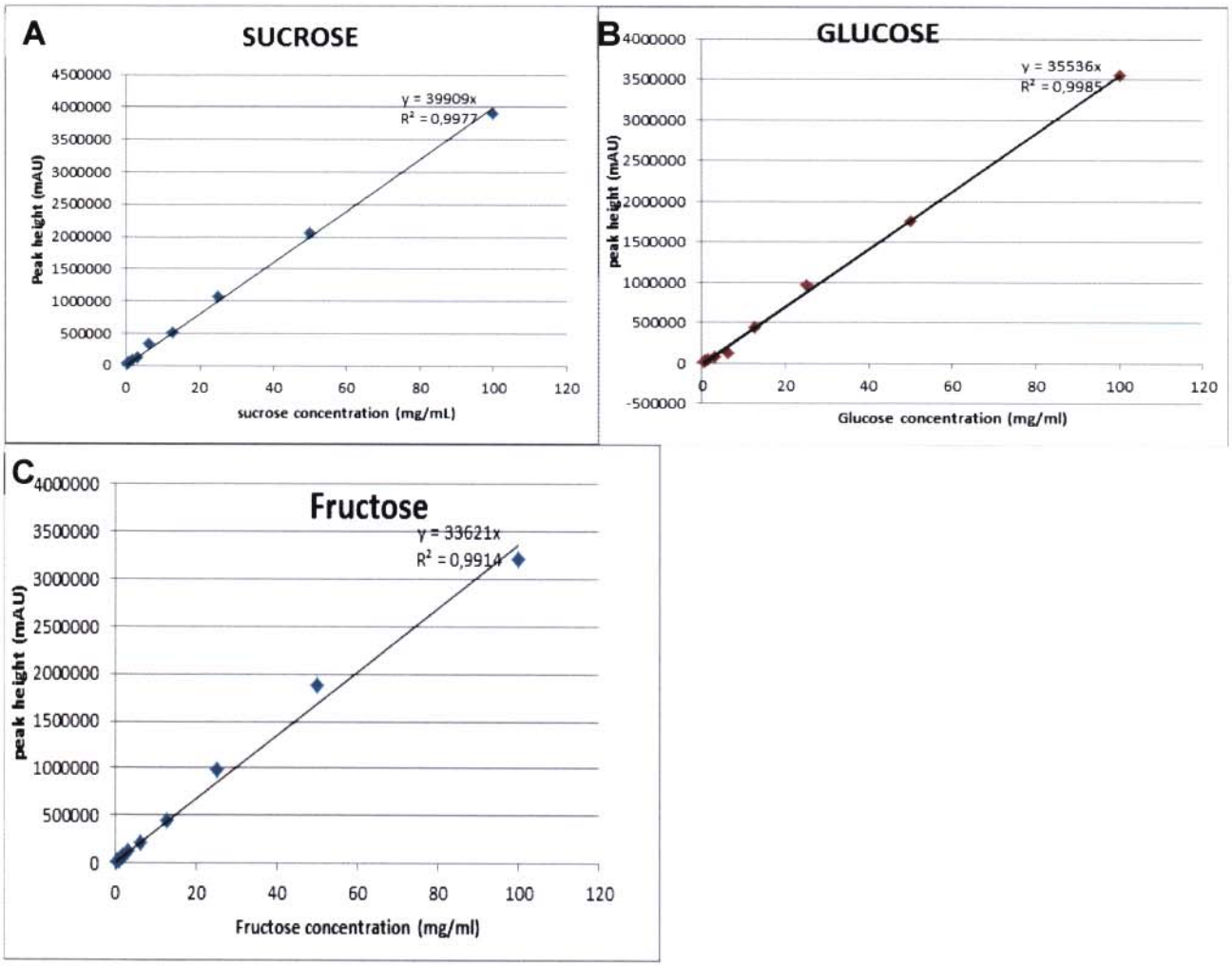

Figure 4.1. Standard curves of sucrose (A), glucose (B) and fructose (C) at concentration range of 0.39 – 100 mg/mL.

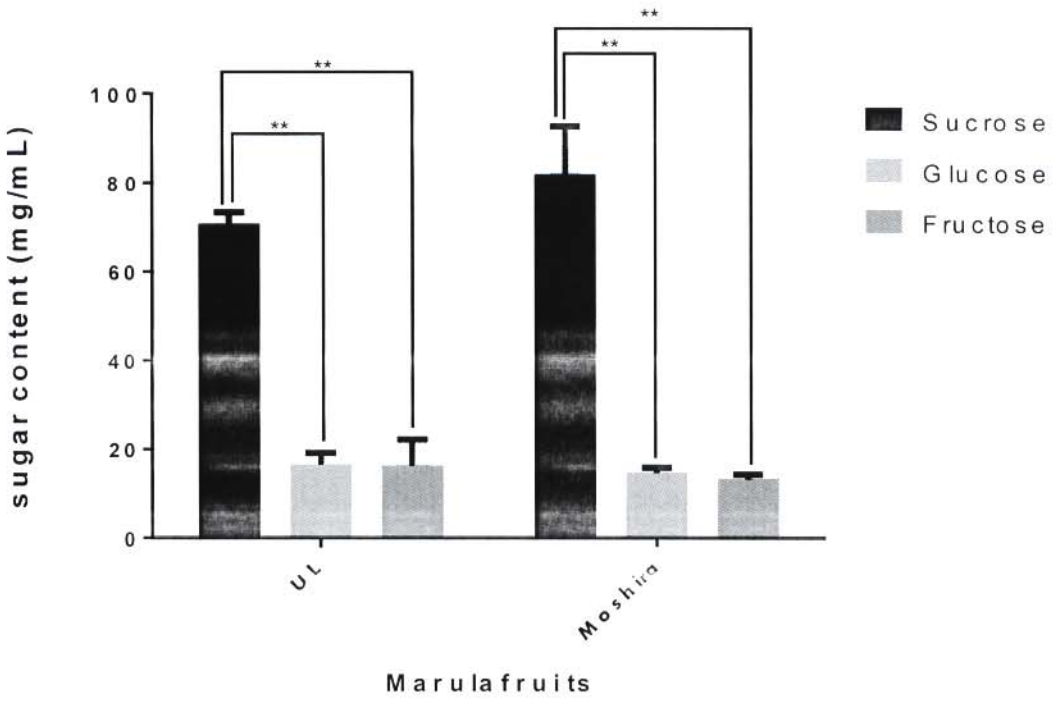

Figure 4.2. Sucrose, glucose and fructose content in marula fruits.

4.3.2 Sugar utilisation during fermentation

There was an abundance of sucrose at the initial stages of fermentation in comparison to glucose and fructose, which increased in amount as the fermentation progressed and expectedly when the amount of sucrose declined. This pattern was observed for all the different marula wines and in addition, the sucrose and glucose got depleted within 7 days of fermentation (Figs. 4.3 – 4.7). Fructose showed a slow decline comparatively for all the wines. The Lab and Mentz fermentations, which come from the same marula juice stock mixture, showed a rapid decline in sucrose with glucose and fructose fluctuating initially before finally declining to zero between day 7 and 10 (Figs. 4.3 and 4.4). The Mentz fermentation proceeded slower than the Lab wine. The two marula fermentations from Moshira showed the same trend for consumption of sucrose and glucose, while fructose levels remained at ≈ 2 mg/mL from day 5 (Figs. 4.5 and 4.6). In contrast, the Blompoort fermentation was sluggish, as evident with the slow consumption of the fructose and glucose which remained at levels above 10 mg/mL after 10 days of fermentation (Fig. 4.7).

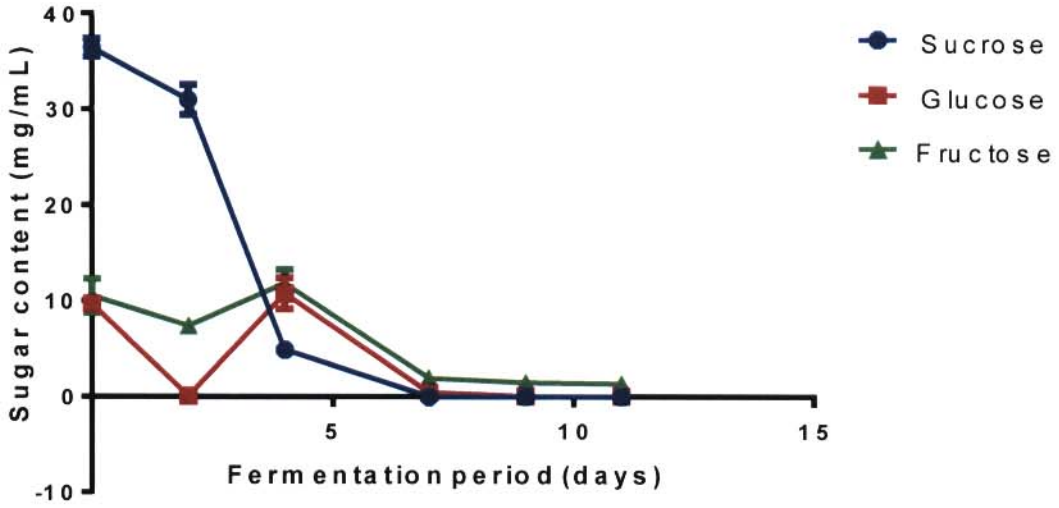

Figure 4.3. Change in the sucrose, glucose and fructose during fermentation of marula wine from Lab.

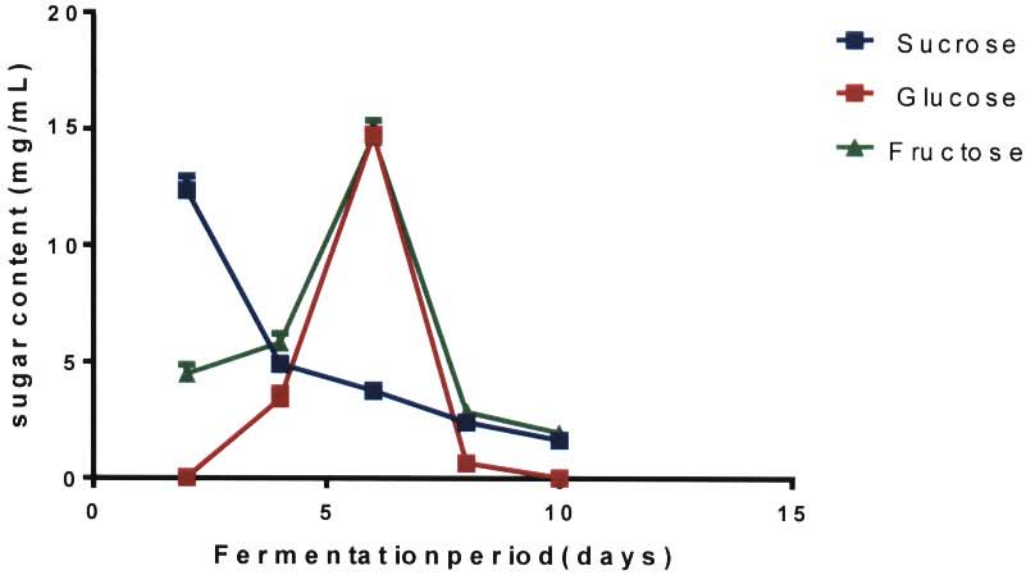

Figure 4.4. Change in the sucrose, glucose and fructose during fermentation of marula wine from Mentz.

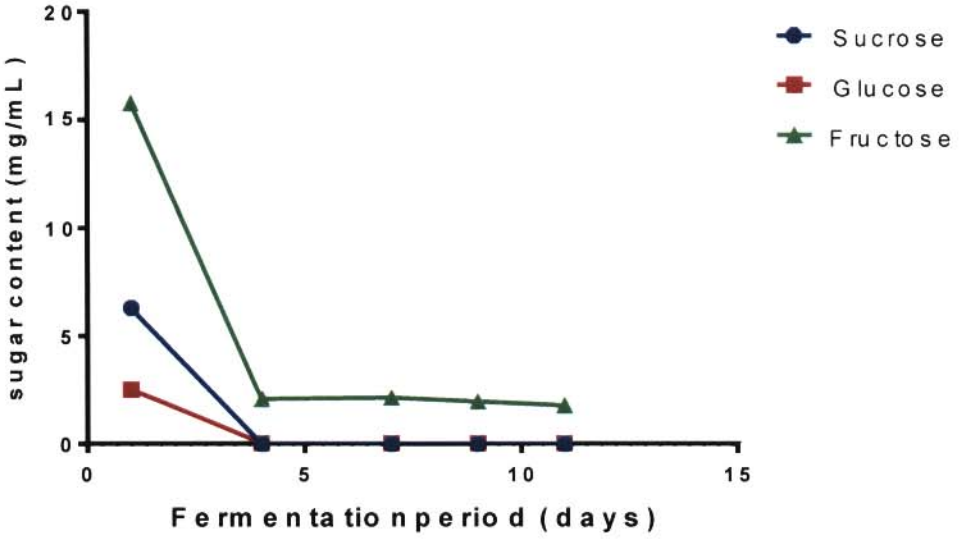

Figure 4.5. Change in the sucrose, glucose and fructose during fermentation of marula wine from Moshira-A.

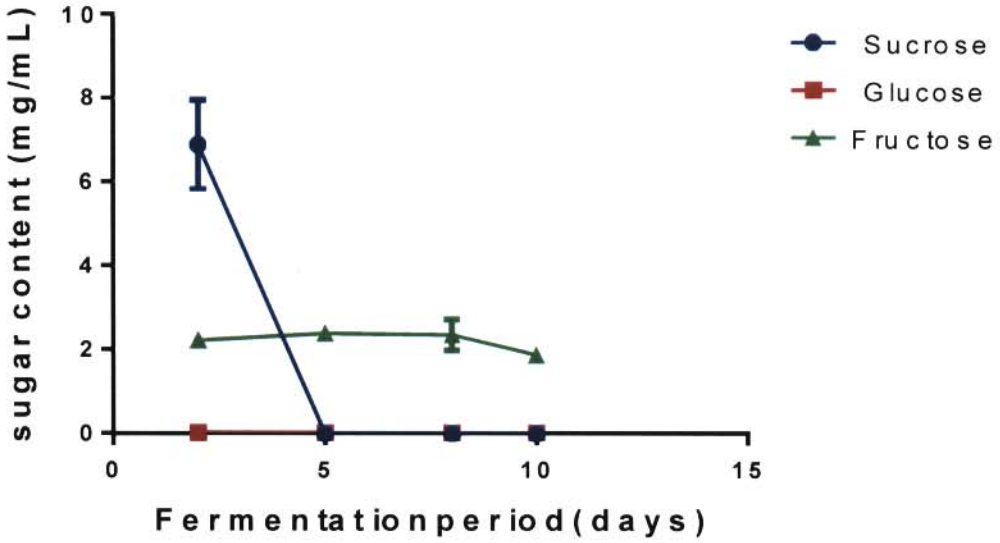

Figure 4.6. Change in the sucrose, glucose and fructose during fermentation of marula wine from Moshira-B.

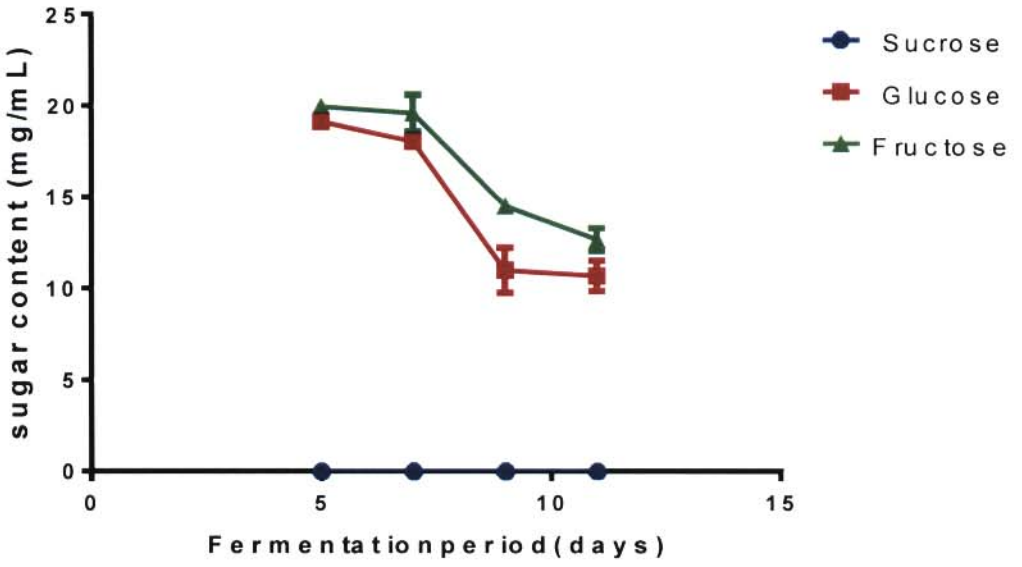

Figure 4.7. Change in the sucrose, glucose and fructose during fermentation of marula wine from Blompoort.

4.3.3 Changes in the composition of volatile compounds during fermentation

The analysis of the volatile compounds associated with marula wine during the fermentation was performed in the different marula wines at varying stages of fermentation. Nineteen volatiles (six alcohols, three aldehydes, one ester and nine organic acids) were identified in all the marula wines from selected areas. The alcohols present in all the wines included 1-propanol, 1-pentanol, 2-methyl-1-propanol, n-butanol and ethanol. Ethanol was the main alcohol in all the wines. The higher fusel alcohols (1-propanol, 1-pentanol, 2 methyl-1-propanol and hexanol) with acetate esters and aldehydes including isopentyl acetate and isobutyraldehyde were present at high concentration during earlier stages of fermentation in marula wines (figs. 4.8 – 4.10). Ethanol levels increased as fermentation time increased and it was the dominant alcohol at an abundance of 90% at the end of fermentation (Fig. 4.8). Among the organic acids tested, acetic acid and formic acid dominated throughout the fermentation (Fig. 4.9). The formic acid levels were higher in the Lab, Mentz and both Moshira wines. Contrary to the other marula wines analysed, the Blompoort marula wine was dominated by acetic acid at the later stages of the fermentation study period.

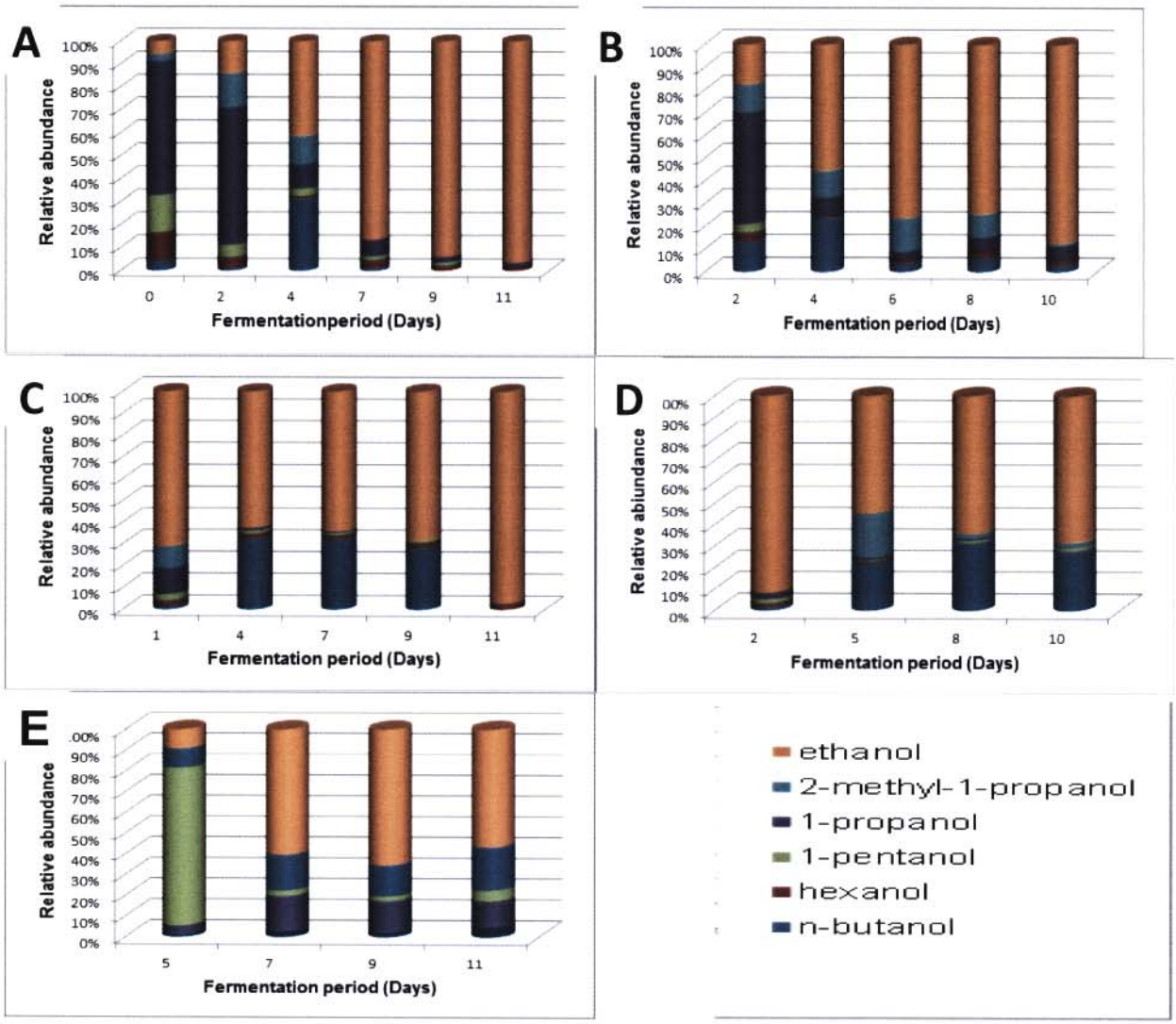

Figure 4.8. Change in the alcohol profile of marula wines from (A) Lab, (B) Mentz, (C) Moshira-A, (D) Moshira-B and (E) Blompoort during fermentation.

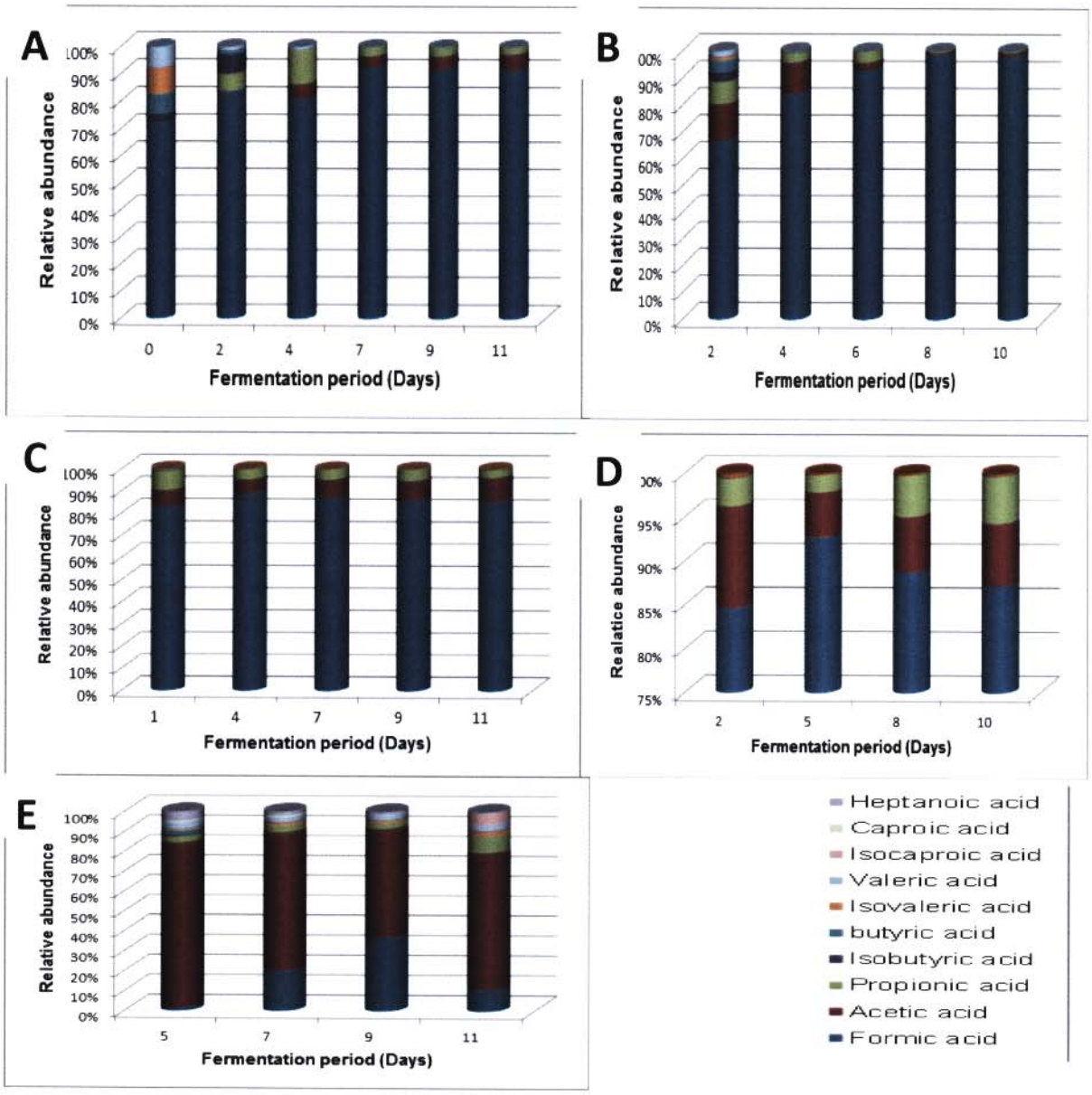

Figure 4.9. Change in the organic acid profiles of marula wines from (A) Lab, (B) Mentz, (C) Moshira-A, (D) Moshira-B and (E) Blompoort during fermentation.

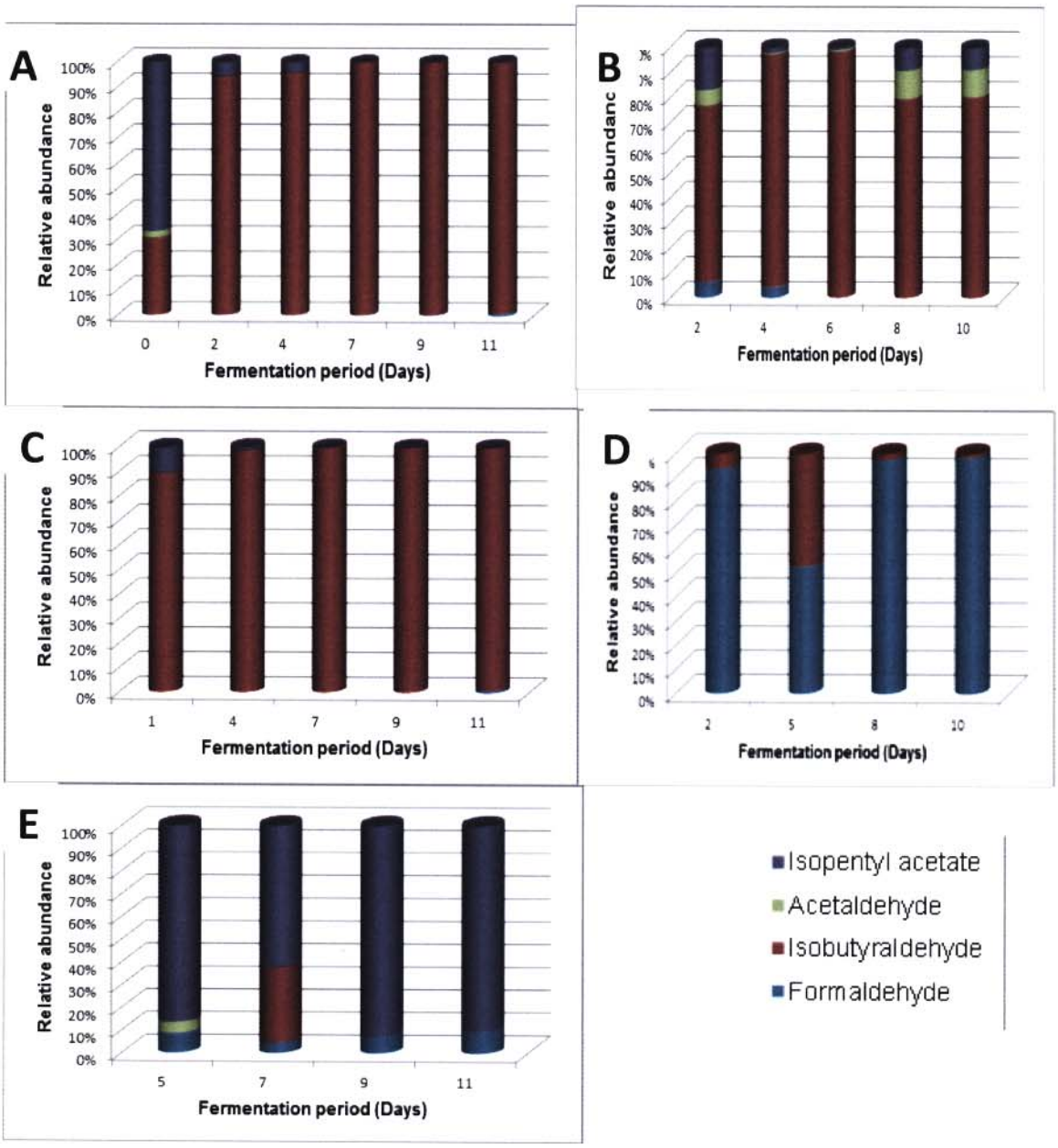

Figure 4.10. Change in the aldehydes and esters profile in the marula wines from (A) Lab, (B) Mentz, (C) Moshira-A, (D) Moshira-B, and (E) Blompoort during fermentation.

4.4 Discussion

The current study evaluated the change in the chemical profiles of five different marula wines during fermentation over an eleven day period. Marula fruits and wines

at different stages of fermentation were profiled for the selected sugars, alcohols, esters and aldehydes. Only ripe marula fruits were analysed for the sugars.

Previous related studies have revealed sucrose to be the main sugar in the marula juice (Fundira, 2001; Weinert *et al.*, 1990). The sugar concentration in the fresh marula fruit observed in this study was significantly higher than those reported for marula fruits harvested in Namibia (47 mg/mL sucrose, 4.9 mg/mL glucose and 22 mg/mL fructose) which was measured in the marula juice extracted from ripe marula fruits. Climatic conditions and amount of rainfall influence the juiciness and sugar content of the marula fruits (Moganedi *et al.*, 2011) and subsequently this will affect the organoleptic characteristics of the resulting wine, noting that the only ingredient for making the wine are the fruit juice and water.

In the production of any alcoholic beverage, the concentration of the sugar is important for the final alcohol content of the resulting brew. Naturally marula juice has high sucrose content, and this study showed that sucrose decreases rapidly and becomes transformed into glucose and fructose at the initial stage of fermentation. The main fermenting yeast species observed in this study was *S. cerevisiae*, which is gluconophilic. At the mid to late stages where this yeast species was dominant glucose was consumed at a faster rate than other sugars since glucose is its preferred carbon (Berthels *et al.*, 2004), hence it is depleted faster than fructose. Evidently, the utilisation of sugars proceeded faster in the presence of fermenting *S. cerevisiae* than in the earlier stages of fermentation which were dominated by the weakly fermentative non-*Saccharomyces* yeasts.

In alcoholic beverages, organic acids play a major role, contributing to flavour, colour, and aromatic properties. The presence of these acids are indicators of a fermentation processes (Rodriguez *et al.*, 2010) and they constitute an important group of volatile compounds that can result in vinegary, cheesy, and fatty odours of the wine's sensory properties as well as bitterness, stringency and rancidity (Pinho *et al.*, 2006). Higher alcohols such as 1-propanol, 1-pentanol, 2-methyl-1-propanol, n-butanol and hexanol were present in high proportions in all the marula wines during early stages of fermentation, the period which was dominated by the non-*Saccharomyces* yeast species. Non-*Saccharomyces* yeast such as *Hanseniaspora guilliermondii* which was detected in the marula wine produce aromatic compounds

such as higher alcohols (fusel alcohols) and some acetate esters through the Erhlich pathway (Gamero *et al.*, 2016). *Hanseniaspora guilliermondii* and *Pichia anomala* are strong producers of acetate esters, 2-phenylethyl acetate and isoamyl acetate in wine (Rojas *et al.*, 2001). Fusel alcohols such as 1-pentanol and 1-hexanol have been reported as the major alcohols in the fresh marula pulp (Pretorius *et al.,* 1985) and these alcohols dominated the early stages of fermentation at 16% for 1-pentanol, 12% for 1-hexanol and propanol at 58%. However, their levels steadily decreased as fermentation progressed. The high levels of fusel alcohols in fermented wine results in off-flavours whereas they contribute to the aroma of the resulting wine when in low amount, this is in combination with their esters. These aroma characteristics can be used as an organoleptic signature of different wines (Belda *et al.*, 2017). The fusel alcohol propanol was also identified as a major higher alcohol in the Korean traditional rice wine together with 2-methyl-1-propanol which resulted in low quality of the wine when in higher levels (Kim *et al.*, 2013).

Expectedly, ethanol was the major alcohol in the marula wines with relative abundance of up to 90% and more at the latter stages of the fermentation wherein *S. cerevisiae* was the dominant yeast. Ethanol is an important volatile compound in wine and contributes to the wine's strong and pungent smell as well as taste (Shale *et al.*, 2013). Similarly, acetic acid dominated the latter stages of fermentation where the Acetic acid bacteria were present in high numbers and this infers that the bitter vinegary taste of unpalatable marula wine at the late stage of wine production is attributable to acetic acid produced by *A. aceti* and *A. pasteuriannus*.

Although different fruits may share most of these aromatic characteristics, the differences arise from the complex mixture of the volatiles which will inadvertently give a distinctive aroma (Tucker, 1993). Aromatic compounds such as isopentyl acetate, isobutyraldehyde, formaldehyde and acetaldehyde are known to be present in small quantities in fermented beverages such as wine where they play an important role in influencing the flavour (Shale *et al.*, 2013). Such esters and aldehydes were noted at the initial stage and to the lesser extent at the later stage of marula fermentation in this study. The non-fermenting yeast species *H. guilliermondii*, *R. mucilaginosa* and *I. terricola* are known to contribute to the aroma profile of wines through production of flavour-active compounds (Romano *et al.*, 2003) and their dominating presence matched the production of desirable flavour

compounds in the early stage of fermentation in marula wines. On the other hand, acetate esters which have been reported to be responsible for the desirable fruity-like character of young grape wines (Marais and Pool, 1980) can be detrimental to the aroma and quality of a wine if present in levels above sensory threshold wherein they give off a vinegar off-flavour (Guth, 1997; Ferreira *et al.*, 2000). This may be responsible for the spoiled characteristics of the marula wine when is kept in storage for two weeks and more.

All these metabolic volatile compounds contribute either positively or negatively to the overall structure, flavour, and aroma the final product depending on their level of production during fermentation. Elevated levels of most of these compounds in the wine are considered undesirable aromatic characteristics thus the wines are described as having off-flavours.

CHAPTER 5

Identification of potential spoilage microorganisms in the marula wine

5.1 Introduction

Wine is an alcoholic drink that is produced from a complex interaction of variety of microbial species which include yeasts, Lactic acid and Acetic acid bacteria and filamentous fungi (Fleet, 1993). The microorganisms harboured in the fermenting medium play a pivotal role in the quality and aroma of the end product of fermentation. However the degree at which these microbial species are present can conversely affect the quality of the product with potential to cause spoilage (Giraffa, 2001). Microbiological spoilage results in the deterioration of the sensory quality and typically presents as off-flavours, odours and visual changes in the final product. These microorganisms affect the quality by causing acidification, hazes, sediments and ropiness of beverages. Metabolism of several sugars and polyols by bacteria can result in wine spoilage. Desirability of a compound in wine is dependent on concentration and wine style (Francis and Newton, 2005). Some genera of Lactic acid bacteria and all species of the Acetic acid bacteria genera and to the lesser extent some yeast are considered spoilage microorganisms because they produce spoilage compounds such as acetic acid, acetaldehyde and ethyl acetate. These spoilage volatile compounds, acetic acid and acetaldehyde are formed from the oxidative metabolism of ethanol by the Acetic acid bacteria (Adachi *et al.*, 1978). Uncontrolled Acetic acid bacteria activities results in a large production of volatile acids in the wine and this turns the wine into vinegar. Spoilage as a result of Acetic acid bacteria is associated with volatility characteristics, a vinegary taste as well as a range of acetic, nutty, sherry-like, solvent or bruised apple aromas which are produced through oxidation of the ethanol to acetaldehyde and acetic acid (Du Toit and Pretorius, 2000; Fleet, 2007). This leads to a reduction in the fruity sensorial characteristics of wine (Bartowsky *et al.*, 2003). Microbiological spoilage that results from the Lactic acid bacteria includes acidification, mannitol taint, diacetyl production, mousiness, acrolein formation, bitterness, tartaric acid degradation, geranium off-odour and biogenic amines formation (Sponholz, 1993). Lactic acid

bacteria change the aroma and the flavour of the resulting wine. The Lactic acid bacteria responsible for spoilage of wines include the species from *Lactobacillus*, *Oenococcus* and *Pediococcus* (Bartowsky, 2008). Wine spoilage by yeast species during fermentation results in high esters content and formation of acetic acid and hydrogen sulphide. Certain yeasts from the genera *Candida*, *Pichia* and *Hansenula* grow as biofilm in the wine if it is exposed to air (Fleet, 2007), and the wine will taste less acidic and more oxidised as a result of high acetaldehydes concentrations (Sponholz, 1993; Fugelsang and Edwards, 2007).

The spoilage of the fermented marula juice is presumably compounded by the unhygienic handling during extraction of the juice from the marula fruits in the households. The lack of knowledge in food handling, poor personal hygiene, poor sanitary and equipment often results in microbial contamination of the traditional beverages during and after processing, including through the addition of contaminants such as battery acids and concoctions only known to the brewers (Amusa and Odunbaku, 2009; Ikalafeng, 2008; Lues *et al.*, 2011).

The spoilage of the marula wine results in a short seasonal availability of this wine thus affects the communities that benefits financially from its trade. It is important to detect and identify the possible potential spoilage microorganisms in the wines in an effort to develop the methods that can be employed to eliminate these microbes. The culture-based and molecular-based methods were used in this study to detect and identify the potential spoilage microorganisms in the marula wines.

5.2 Materials and methods

The wines were processed as described on section 3.2.2 and the pellet-glycerol stocks were cultured for identification of the potential spoilage microorganisms.

5.2.1 Determination of the spoilage microorganisms

The NBB agar and broth base (Sigma-Aldrich) were used for enrichment of the spoilage microorganisms. The microorganisms were first enriched in the NBB®-B broth prior to cultivation on the NBB®-A agar. Five millilitres of the NBB broth was

transferred into the test tubes and then inoculated with 100 µL of each marula wine. The test tubes were incubated at 25 °C in a rotary shaker at a speed of 180 rpm for 5 days. The growth in the liquid media was identified by the change in colour from red to yellow and cloudiness of the liquid media. The NBB®-A agar was inoculated by spread plating 100 µL of the undiluted and serially diluted enriched broth culture. The plates were incubated at 25 °C for a period 5 days until the growth was visible. The isolated microorganisms were separated by distinct colony morphologies and sub-cultured on the fresh cultivation media. The pure cultures were then identified by the MALDI-TOF Biotyper as described in section 3.2.4.2.1.

5.3 Results

5.3.1 Identification of spoilage microorganisms

A variety of Lactic acid and Acetic acid bacteria and one yeast species were observed and identified in this analysis (Table 5.1).

Table 5.1. Possible spoilage microorganisms in the marula wines.

	Lab wine	Mentz	Moshira-A	Moshira-B	Blompoort	Spoilage metabolites
LAB	*L. plantarum* *L. buchneri* *L. brevis* *L. fermentum*	*L. plantarum* *L. buchneri* *L. brevis* *L. fermentum*	*L. plantarum* *L. buchneri* *L. brevis* *L. fermentum*	*L. plantarum* *L. buchneri* *L. brevis* *L. fermentum*	*L. plantarum* *L. buchneri* *L. brevis* *L. fermentum*	Acetic acid (Bartowsky, 2008)
AAB	*A. bogorensis* *G. nepheli* *A. pasteuriannus* *A. aceti*	*A. bogorensis* *G. nepheli* *A. pasteuriannus* *A. aceti*	*A. pasteuriannus* *A. aceti*	*A. pasteuriannus* *A. aceti*	*A. pasteuriannus* *A. aceti*	Acetic acid (Bartowsky, 2008) Acetaldehyde (Francis and Newton, 2005) (Lawrence,

						1988) Formic acid (Sponholz, 1993)
Yeast	Not detected	Not detected	Not detected	Not detected	*P. kudriavzevii*	Acetaldehyde and acetic acid (Fugelsang and Edwards, 2007)

5.4 Discussion

Marula wine is an alcoholic beverage traditionally produced from the spontaneous fermentation of the mash obtained from the marula fruits. Just like any other traditional beverage, marula wine harbours a complex microbial ecosystem which contributes to the broad diversity of tastes, aromas, and textures that are associated with them.

The evaluation of the microorganisms associated with wine spoilage in this study revealed that both bacteria and yeast may be involved in the deterioration of the quality of the marula wine. The present study revealed that Acetic acid bacteria, Lactic acid bacteria and yeast species *P. kudriavzevii* which was only identified in the Blompoort wine may be responsible for marula wine spoilage. Acetic acid is the major volatile acid which results in spoilage of wine and the spoilage by this volatile compound is often referred to as volatile acidity and may contribute to 50% of the volatile acid in wines (Du Toit and Pretorius, 2000). The *Lactobacillus* species mainly affect the wine quality by formation of lactic acid and through the glycerol metabolism, and this results in the bitterness (Bartowsky and Pretorius, 2008), and may also cause an increase in acidity of the wine (Sponholz, 1993; Fugelsang and Edwards, 2007). Acidification by Lactic acid bacteria mainly occurs in wines with residual sugars when there are enough nutrients for the proliferation of these bacteria (Wibowo *et al.*, 1985). In the current study high levels of acetic acid were

observed in the Blompoort wine which was the only wine containing residual sugars at the latter stage of fermentation. Depending on the type of species and growth conditions, sugar utilisation can also lead to formation of ethanol, acetate, formate or succinate (Hammes and Herter, 2009). Amongst the spoilage *Lactobacillus* sp. *L brevis* is regarded as the most common cause of wine spoilage (Back, 2005).

The wine spoilage by the acetic acid bacteria mainly arise through the formation of compounds such as acetic acid, acetaldehyde and ethyl acetate during the wine fermentation process (Bartowsky, 2008). *Acetobacter aceti* and *Acetobacter pasteuriannus* are the main Acetic acid bacteria that cause the spoilage of wine by conversion of ethanol into acetic acid and acetaldehydes which results in the vinegary off-flavour in the wine (Du Toit and Pretorius, 2000; Fleet, 2007). Furthermore yeast species *P. kudriavzevii* identified in the Blompoort fermentation together with other species from the genus *Pichia* are known to cause spoilage of wine through formation of a film layer on the wine and producing acetaldehydes which then gives rise to a pungent sherry-like aroma resembling that of a bruised apple (Kurtzman, 2011; Fugelsang and Edwards, 2007).

Spoilage of wine is commonly influenced by the presence of spoilage microorganisms both yeasts and bacteria which produce undesirable compounds. The isolated acetic acid and lactic acid bacteria, which when in abundance, produce organic acids which lower the pH and produce off flavours that lead to unpalatability of the wine. Therefore good management and control of the growth of the contributing microbiota will inadvertently prevent spoilage of the wine.

CHAPTER 6

General discussion and conclusion

4.1 Discussion

Marula has been well acknowledged as one of the food resources adapted for subsistence and commercial utilisation by the traditional societies where it is native. Marula wine is traditionally produced by spontaneously fermenting the mash obtained from the marula fruits. The fermentation process occurs as the result of the activity of the microorganisms present on the surface of the marula fruits. These natural microorganisms influence the organoleptic properties of the wine through metabolism of several compounds that are naturally found in the marula must. Therefore it is important to know the dynamics of microorganisms present and their role during the spontaneous fermentation. Although yeasts are the main microorganisms responsible for alcoholic fermentation which affects the sensory quality of the wine by producing secondary metabolites (Salvado *et al.*, 2011), bacteria also play an important role in fermentation by producing volatile compounds that contributes to the aroma and flavour of the finished product (Bartowsky, 2008). The current study evaluated the chemical and microbial profiles of five different marula wines during fermentation over an eleven day period. This period covered from the juice state to the stage when the wine was bitter and not palatable. The wine sampling was performed at different stages of fermentation for analyses of the chemical, bacterial and yeast profiles.

The bacterial and yeast populations were monitored and high bacterial load was observed in the initial stages with the two Moshira ferments exhibiting the highest bacterial load and the reference wine with the lowest. Most of the microorganisms isolated in the latter stages in the fermented wine in this study were initially present in the beginning of the fermentations, which suggests that these microorganisms were harboured in the marula fruits. The same pattern of succession in the bacterial and yeast population was maintained in all the wines despite prevalence of some differences in the composition during fermentation. The bacteria population in the spontaneous fermentations of the current study showed a diverse group of Lactic

acid and Acetic acid bacteria throughout the fermentation process. These two groups of bacteria are particularly significant in wine fermentation where they interact with the various yeasts present in the must/juice through their metabolic activities to produce the final alcoholic product. This interaction can either result in a positive or negative effect on the quality of the wine. The participation of the bacteria present in the marula fermentations is common with all the other traditional spontaneous fermented wines. Despite the yeast metabolism predominating the fermentations, the Lactic acid bacteria were present in high numbers than that of yeasts In the spontaneous fermentation of most traditional beverages the Lactic acid bacteria and yeasts commonly occur in association during the process (Jespersen, 2003; Nout, 2003). This association was also observed in the current study where the co-existence of the Lactic acid bacteria and yeasts especially the non-*Saccharomyces* occurred in the unfermented marula pulp and also in the wine, which suggests that the spontaneous fermentation process is achieved through alcoholic and lactic acid fermentation. The Lactic acid bacteria observed in this study have also been reported in spontaneous fermentation of other fruits wines and these bacteria were mainly dominated by *L. paracasei* which is a facultative hetero-fermentative species of the *Lactobacilli* (Laureys and De Vuyst, 2014). *L. paracasei* has been suggested as a potential probiotic candidate (Chiang and Pan, 2012), therefore the marula ecosystem could be a possible source of this novel probiotic species. The Lactic acid bacteria dominated the initial stages whereas the latter stages of alcoholic fermentation were dominated by the acetic acid bacteria. The dominance of Lactic acid bacteria in the beginning of the fermentation in this study is in line with what has been reported in the previous study of marula juice fermentation by Dlamini and Dube (2008). The authors suggested that initial stages of marula fermentation presented favourable conditions for proliferation of the Lactic acid bacteria to pre-dominate the fermentation and metabolise the sugars to produce secondary metabolites. The Acetic acid bacteria such as *A. pasteuriannus* and *A. aceti* as observed in this study dominated the latter stages, which is not surprising because they prefer ethanol as the carbon source (Bartowsky and Henschke, 2008), and they oxidise it to mainly acetic acid which is the main volatile organic acid in wine.

This study elucidated the sugars consumption and metabolites production profiles of the microbiota described above. A variation in the utilisation of the sugars by the

microorganisms during fermentation was exhibited, which could explain the differences in production of a variety of volatile compounds. The variation in the sugar consumption by yeast and the proportion of the production of volatile compounds mainly ethanol further suggests distinctive difference in the strains of yeast species present in the wines. This has been recently demonstrated by Ohimain (2016). The author found that the proportion at which the ethanol is produced was different in fermentations carried out by different strains of fermenting yeast. Various volatiles compounds have different properties therefore affect the flavour and aroma of the product at different degree (Kobayashi *et al.*, 2008 and Silva *et al.*, 2008). These volatiles directly affect the sensorial quality of the product in a positive or negative way as they greatly enhance the favour of the beverage (Riu-Aumatell *et al.*, 2004; Lui *et al.*, 2005). The production of the volatile compounds varies among yeast species (Romano *et al.*, 2003), thus the subtle variations in the type and quality of the volatile compounds in the different marula wines in this study might be the resultant of the different relative abundance of the different yeasts between the localities. The ethanol levels increased linearly with the decrease in the sugars in the fermentations and this also coincided with an increase in the production of the organic acids mainly the acetic acid and formic acid. Ethanol was the main alcohol in the fermented wines during the period that was dominated by *S. cerevisiae*. The effect of the ethanol production in the main fermentation by the *S. cerevisiae* was reflected by the decrease in the population of the low alcohol-tolerant apiculate yeast *H. guilliermondii*. Blompoort fermentation which exhibited slow rate of fermentation and residual sugars showed low levels of ethanol as compared to the other fermentations including that of a reference wine. Huang *et al.* (1996) demonstrated that Lactic acid bacteria can affect the rate of yeast-driven alcoholic fermentation, and can result in production of high amounts of acetic acid in wine with residual sugars (Boulton *et al.*, 1996).

The diverse microbial species of both yeasts and bacteria involved in the fermentation of spontaneous wine produces a variety of volatile compounds through their metabolic pathways and some of this compounds affect the wine quality to a degree that the wine is considered spoiled. The results of these spoilages include mousy taints, bitterness, geranium notes, volatile acidity, slimy-texture and biofilm formation (Bartowsky, 2008). The potential spoilage microorganisms identified in the

present study were the species of Lactic acid bacteria, Acetic acid bacteria and to the lesser extent yeasts. It is noteworthy to indicate that the spoilage is subject to the specific beverage. In terms of marula wine the acidity provided by the LAB and AAB is important to the typical taste of marula during alcoholic fermentation stage. The accumulation of bacteria and the resultant high organic acid levels wherein alcohol is also converted to acetic acid by acetic acid bacteria is undesirable and leads to an unpalatable drink. At this stage, the contributing microbiota are deemed as spoilage organisms. This spoilage microbiota were common in all the wines including the reference wine, however the degree at which the spoilage metabolites were produced in the wines was different. *Acetobacter* species are the main spoilage microorganisms and they produced acetic acid, acetaldehyde and formic acid as the main spoilage metabolites in the wine (Bartowsky, 2008; Francis and Newton, 2005; Sponholz, 1993). Acetaldehydes and acetic acid are formed through oxidation of ethanol (Bartowsky and Pretorius, 2008) and this reduces alcohol content and aroma of the resulting wine.

Overall, through employment of the culture-based and molecular-based techniques in this study, we were able to profile the population structure of the microorganisms associated with marula wine and the dynamics of these microorganisms together with the various compounds produced in the process.

4.2 Conclusion

Traditional wines provide a much needed income for the households that trade in the wine, noting that their production is very low in cost and requires very few raw materials. The knowledge of the bacteria and yeast involved in the fermentation of marula brew is essential for the production of a wine of consistent quality. This study revealed the variations but dominant microbiota and chemicals that are responsible for the taste, aroma and possible spoilage of the resulting marula wine. This knowledge gives courage and confidence for the production of marula wine that has a long shelf life but same organoleptic characteristics as the natural spontaneously fermented marula wine produced currently in the villages.

CHAPTER 7

REFERENCES

Adachi, O., Miyagawa, E., Shinagawa, K., Matsushita, K., Ameyama, M., 1978. Purification and properties of particulate alcohol dehydrogenase from *Acetobacter aceti*. Agric. Biol. Chem. 42, 2331 – 340.

Aganga, A. A., Mosase, K. W., 2001. Tannin content, nutritive value and dry matter digestibility of *Lonchocarpus capassa, Zizyphus mucronata*, *Sclerocarya birrea*, *Kirkia acuminata* and *Rhuslancea* seeds. Anim. Feed Sci. Technol. 91,107 – 113.

Aka, S., Camara, F., Nanga, Y.Z., Loukou, Y.G., Koffi, M.D., 2008. Evaluation of organic acids and sugars contents during the production of ‗Tchapalo', a traditional sorghum beer in Côte d'Ivoire. J. Food Technol. 6 (5), 189 – 195.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25, 3384 – 3402.

Amoa-Awua, W.K., Sampson, E., Tano-Debrah, K., 2007. Growth of yeasts, lactic and acetic acid bacteria in palm wine during tapping and fermentation from felled oil palm (*Elaeis guineensis*) in Ghana. J. Appl. Microbiol. 102, 599 – 606.

Amusa, N.A., Odunbaku, O.A., 2009. Microbiological and nutritional quality of hawked kunun (a sorghum-based non- alcoholic beverage) widely consumed in Nigeria. Pak. J. Nutr. 8, 20 – 25.

Aponte, M., Blaiotta, G., 2016. Potential role of yeast strains isolated from grapes in the production of Taurasi DOCG. Front. Microbiol. 7, 809.

Atter, A., Obiri-Danso, K., Amoa-Awua, W.K., 2014. Microbiological and chemical processes associated with the production of burukutu a traditional fermented beer in Ghana. Int. Food Res. J. 21, 1769 – 1776.

Augusto, F., Valente, A. L. P., Tada, E. S., Rivellino, R. S., 2000. Screening of Brazilian fruit aromas using solid-phase microextraction–gas chromatography–mass spectrometry. J. Chrom A. 873, 117 – 127.

Back, W., 2005. Winery. In: Back, W. (Ed) Colour atlas and handbook of veverage biology. Verlag Hans Carl: Nürnberg, Germany, pp. 113 – 135.

Bagheri, B., Bauer, F.F., Setati, M.E., 2015. The diversity and dynamics of indigenous yeast communities in grape must from vineyards employing different agronomic practices and their influence on wine fermentation. S. Afr. J. Enol. Vitic. 36(2), 243 – 251.

Bartowsky, E.J., 2003. Lysozyme and winemaking. Aust NZ Grapegrow. Winemak. 473a, 101 – 104.

Bartowsky, E.J., Henschke, P.A., 2008. Acetic acid bacteria spoilage of bottled red wine—A review. Int. J. Food Microbiol. 125, 60 – 70.

Bartowsky, E.J., Pretorius, I.S., 2008. Microbial formation and modification of flavour and off-flavour compounds in wine. In Konig, H., Unden, G., Frohlich, J. (Eds) Biology of microorganisms on grapes, in must and wine. Heidelberg, Springer, pp. 211 – 233.

Borochov-Neori, H., Judeinstein, S., Greenberg, A., Fuhrman, B., Attias, J., Volkova, N., Hayek, T., and Aviram, M., 2008. Phenolic antioxidants and antiatherogenic effects of marula (*Sclerocarrya birrea* Subsp. *caffra*) fruit Juice in healthy humans. J. Agric. Food Chem. 56(21), 9884 – 989.

Belda, I., Ruiz, J., Esteban-Fernández, A., Navascués, E., Marquina, D., Santos, A., Moreno-Arribas, M.V., 2017. Microbial contribution to wine aroma and its extended use for wine quality improvement. Molecules. 22, 189.

Berbegal, C., Peña, N., Russo, P., Grieco, F., Pardo, I., Ferrer, S., Spano, G., Capozzi, V., 2016. Technological properties of *Lactobacillus plantarum* strains isolated from grape must fermentation. Food Microbiol. 57, 187 – 194.

Berthels, N., Cordero-Otero, R., Bauer, F., Thevelein, J., Pretorius, I., 2004. Discrepancy in glucose and fructose utilization during fermentation by *Saccharomyces cerevisiae* wine yeast strains. FEMS Yeast Res. 4, 683 – 689.

Berthiller, F., Schuhmacher, R., Buttinger, G., Krska, R., 2005. Rapid simultaneous determination of major type-A and B trichothecenes as well as zearalenone in maize by high performance liquid chromatography tandem mass spectrometry. J. Chrom. A. 1062, 209 – 216.

Bille, P.G., Steppich, G., 2008. Transformation of marula (*Sclerocarya birrea*), monkey orange (*Strychnos cocculoides*) and eembe (*Berchemia discolor*) into food products. Sci. Hortic. 117, 321 – 328.

Botelle, A., du Plessis, P., Pate, K., Laamanen, R., 2002. A survey of marula fruit yields in North-Central Namibia. CRIAA SA-DC, Windhoek, Namibia.

Boulton, R.B., Singleton, T.L., Bisson, L.E., Kunkee, R.E., 1996. Principles and practices of winemaking. Chapman and Hall, New York.

Campillo, N., Peñalver, R., López-García, I., Hernández-Córdoba, M., 2009. Headspace solid-phase micro-extraction for the determination of volatile organic sulphur and selenium compounds in beers, wines, and spirits using gas chromatography and atomic emission detection. J. Chrom. A. 1216, 6735 – 6740.

Castiñeira, A., Peña, R.M., Herrero, C., García-Martín, S., 2002. Analysis of organic acids in wine by capillary electrophoresis with direct UV detection. J. Food Comp. Anal. 15, 319 – 331.

Chirwa, P.W., Akinnifesi, F.K., 2008. Ecology and biology of *Uapaca kirkina Strychnos cocculoides* and *Sclerocarya birrea* in Southern Africa. In F.K. Akinnifesi, R.R.B. Leakey, O.C. Ajayi, G. Sileshi, Z. Tchoundje, P. Matakala, F.R. Kwesiga (Eds.), Indigenous fruit trees in the tropics: Dometication, utilisation and commercialisation. CABI International, London, pp. 322 – 340.

Clemente-Jimenez, J.M., Mingorance-Cazorla, L., Martinez-Rodriguez, S., Las Heras-Vazquez, F.J., Rodriguez-Vico, F., 2004. Molecular characterization and oenological properties of wine yeasts isolated during a spontaneous fermentation of six varieties of grape must. Food Microbiol. 21, 149 – 155.

Colehour, A.M., Meadow, J., Liebert, M.A., Cepon-Robins, T.J., Gildner, T.E., Urlacher, S.S., Bohannan, B.J.M., Snodgrass, J.J., Sugiyama, L.S., 2014. Local domestication of lactic acid bacteria via cassava beer fermentation. PeerJ 2, 479.

Corneo, P.E., Pellegrini, A., Cappellin, L., Roncador, M., Chierici, M., Gessler, C., Pertot, I., 2013. Microbial community structure in vineyard soils across altitudinal gradients and in different seasons. FEMS Microbiol. Ecol. 84, 588 – 602.

Cortacero-Ramírez, S., Hernáinz-Bermúdez, M.D.C., Antonio SeguraCarretero, A., Cruces-Blanco, C., Fernández-Gutirrez, A., 2003. Analysis of beer components by capillary electrophoretic methods. Trends in Anal. Chem. 22(7-8), 441 – 455.

De Ley, J., Gillis, M., Swings, J., 1984. Family VI. Acetobacteraceae, In N.R. Krieg, J.G. Holt (Eds.), Bergey's Manual of Systematic Bacteriology. Krieg, N.R.; Holt, J.G., Williams & Wilkins, Baltimore, pp. 267 – 278.

Díaz, C., Molina, A.M., Nähring, J., Fischer, R., 2013. Characterization and dynamic behaviour of wild yeast during spontaneous fermentation in steel tanks and *amphorae*. Biomed. Res. Int. 2013, 540465.

Diaz-Campillo, M., Urtiz, N., Soto, O., Barrio, E., Rutiaga, M., Paez, J., 2012. Effect of glucose concentration on the rate of fructose consumption in native strains isolated from the fermentation. World J. Microbiol. Biotechnol. 28, 3387 – 3391.

Dimo, T., Rakotonirina, S.V., Tan, P.V., Azay, J., Dongo, E., Kamtchouing, P., Cros, G., 2007. *Sclerocarya birrea* (Anacardiaceae) stem bark methylene chloride/methanol extract on streptozotocin-diabetic rats. J. Ethnopharmacol. 110, 434 – 438.

Dlamini, N.R., Dube, S., 2008. Studies on the physico-chemical, nutritional and microbiological changes during the traditional preparation of marula wine in Gwanda, Zimbabwe. Nutr. Food Sci. 38, 61 – 69.

Dube, S., Shereni, I., Dlamini, N.R., Sibanda, T., 2012. Extending the shelf life of fresh marula (*Sclerocarya birrea*) juice by altering its physico-chemical parameters, In J.C. Jimenez-Lopez (Ed.), Biochemical testing. INTECH open publisher, Croatia, pp. 181 – 196.

du Plessis, P., 2002. Promoting indigenous fruits in Namibia. CRIAA SA-DC. Namibia.

Du Toit,, W.J., Pretorius, I.S., 2002. The occurrence, control and esoteric effect of acetic acid bacteria in winemaking. Ann. Microbiol, 52, 155 – 179.

Eloff, N., 2001. Antibacterial activity of marula (*Sclerocarya birrea* (A.Rich) Hochst. subsp. *caffra* (Sond) Kokwaro)(Anacardiaceae) bark and leaves. J. Ethnopharmacol. 76, 305 – 308.

Ferreira, V., Lopéz, R., Cacho, J.F., 2000. Quantification determination of the odorants of young red wines from different grape varieties. J. Sci. Food. Agric. 80, 1659 – 1667.

Fisher, M.M., Triplett, E.W., 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. Appl. Environ. Microbiol. 65, 4630 – 4636.

Fleet, G.H., Heard, G.M., 1993. In: Fleet, G.H. (Ed). Wine Microbiology and Biotechnology. Harwood Academic Publishers, Chur, pp. 27 – 54.

Fleet, G.H., 2003. Yeast interactions and wine flavour. Int. J. Food Microbiol. 86, 11 – 22.

Fleet, G.H., 2007. Wine. In: Doyle, M.P., Beuchat, L.R. (Eds), Food microbiology fundamentals and frontiers (3rd ed.). ASM Press, Washington DC, pp. 863 – 890.

Francis, I.L., Newton, J.L., 2005. Determining wine aroma from compositional data. Aust. J. Grape Wine Res. 11, 114 – 126.

Fugelsang, K.C., Edwards, C.G., 2007. Wine microbiology. Practical applications and procedures. Springer Science and Business Media, New York, pp. 3 – 179.

Fundira, M., 2001. Optimization of fermentation processes for the production of indigenous fruit wines (Marula). Doctoral dissertation, Stellenbosch, Stellenbosch University.

Gamero, A., Quintilla, R., Groenewald, M., Alkema, W., Boekhout, T., Hazelwood, L., 2016. High-throughput of a large collection of non-conventional yeasts reveals their potential for aroma formation in food fermentation. Food Microbiol. 60, 147 – 159.

Giraffa, G., Neviani, E., 2001. DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems. Int. J. Food Microbiol. 67, 19 – 34.

Gondwe, M., Kamadyaapaa, D.R., Tuftsa, M., Chuturgoonb, A.A., Musabayane, C., 2008. *Sclerocarya birrea* [(A. Rich.) Hochst.] [Anacardiaceae] stem-bark ethanolic extract (SBE) modulates blood glucose, glomerular filtration rate (GFR) and mean arterial blood pressure (MAP) of STZ-induced diabetic rats. Phytomedicine. 15, 699 – 709.

Gous, F., Weinert, I.A.G., van Wyk, P.J., 1988. Selection and processing of marula Fruit (*Sclerocarya birrea* subsp. *Caffra*). LWT - Food Sci. Technol. 21, 259 – 266.

Gueye. S., 1973. Contribution a l'étude pharmacodynamique d'une plante antidiabétique (*Sclerocarya birrea*). Thèse faculté mixte de médecine et de pharmacie du Sénégal.

Guillaume, L.E., Rodrigues, J.E.A., Gil, A.M., Barros, A.S., Esteves, V.I., 2009. Analysis of non-aromatic organic acids in beer by CE and direct detection mode with diode array detection. J. Chrom. A. 70, 1737 – 1742.

Gurib-Fakim, A., Brendler, T., Philips, L.D., Eloff, J.N., 2010. Green gold success stories using southern African plant species, AAMS publishing, Port Louis, Mauritius.

Guth, H., 1997. Quantification and sensory studies of character impact odorants of different white wine varieties. J. Agric. Food Chem. 45, 3027 – 3032.

Hall, J.B., O'brien, E.M., Sinclair, F.L., 2000. *Sclerocarya birrea*: a monograph. School of Agriculture and Forest Sciences Publication Number X, University of Wales, Bangor.

Hammes WP, Hertel C. Genus I. Lactobacillus Beijerink., 2009 In: De Vos P, Garrity GM, Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.H., Whitman,

W.B., (Eds). Bergey's manual of systematic bacteriology (2nd ed.). Berlin: Springer; . 3, 465 – 510.

Hillman, Z., Mizrahi, Y., Beit-Yanni, E., 2008. Evaluation of valuable nutrients in selected genotypes of marula (Sclerocarya birrea ssp. caffra). Scientia Horticulturae, 117, 321 – 328.

Hiwilepo-van Hal, P., Bille, P.G., Verkerk, R., Dekker, M., 2013. The effect of temperature and time on the quality of naturally fermented marula (*Sclerocarya birrea* subsp. *Caffra*) juice. LWT - Food Sci. Technol. 53, 70 – 75.

Hiwilepo-van Hal, P., Bosschaart, C., van Twisk, C., Verkerk, R., Dekker, M., 2012. Kinetics of thermal degradation of vitamin C in marula fruit (*Sclerocarya birrea* subsp. *caffra*) as compared to other selected tropical fruits. LWT - Food Sci. Technol. 49, 188 – 191.

Hoffman, C.S., Winston, F.,1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Genet. 57, 267 – 272.

Holzapfel, W.H., 2002. Appropriate starter culture technologies for small-scale fermentation in developing countries. Int. J. Food Microbiol. 75, 197 – 212.

Huang, Y.C., Edwards, C.G., Peterson, J.C., Haag, K.M.,1996. Relationship between sluggish fermentation and the antagonism of yeast by lactic acid bacteria. Am. J. Enol. Vitic 47, 1 – 10.

Hutchings, A., Scott, A.H., Lewis, G., Cunningham, A., 1996. Zulu Medicinal Plants: An Inventory.

Ikalafeng, B.K., 2008. Microbiota and mycotoxins in traditional beer of the greater Kimberly area and associated brewing and consumption practices. Doctor of Technologiae: Environmental Health. Central University of Technology, Free State, Bloemfontein, South Africa.

Jespersen, L., 2003. Occurrence and taxonomic characteristics of strains of *Saccharomyces cerevisiae* predominant in African indigenous fermented foods and beverages. FEMS Yeast Res. 3, 191 – 200.

Jolly, N.P., Augustyn, O.P.H., Pretorius, I.S., 2003. The effect of non-*Saccharomyces* yeasts on fermentation and wine quality. S. Afr. J. Enol. Vitic. 24, 55-62.

Kim, E., Chang, Y.H., Ko, J.Y., Jeong, Y., 2013. Physiochemical and microbial properties of the Korean rice wine, Makgeolli, supplemented with banana during fermentation. Prev. Nutr. Food Sci. 18(3), 203 – 209.

Kleiman, R., Ashley, D.A., Brown, J.H., 2008. Comparison of two seed oils used in cosmetics, moringa and marula. Ind. Crops. Prod. 28, 361 – 364.

Kobayashi, M., Shimizu, H., Shioya, S., 2008. Beer volatilecompounds and their application to low malt beer fermentation. J. Biosci. Bioeng. 106, 317 – 323.

Komane, B., Vermaak, I., Summers, B., Viljoen, A., 2015. Safety and efficacy of *Sclerocarya birrea* (A.Rich.) Hochst (Marula) oil: A clinical perspective. J. Ethnopharmacol. 176, 327 – 335.

Kowalchuk , G.A., Yergeau, E., Leveau, J.H.J., Sessitsch, A., Bailey, M., 2010. Plant-associated microbial communities. In W.T. Lui, J. Jansson (Eds.), Environmental Molecular Microbiology. Caister Academic Press, New York, pp. 131 – 148.

Kurtzman, C.P., Fell, J.W., Boekhout T., 2011. The yeasts, a taxonomic study (5th ed.). Elsevier.

Lafon-Lafourcade, S., Larue, F., Ribereau-Gayon, P., 1979. Evidence for the existence of survival factors as an explanation for some perculiarities of the yeast growth, especially in grape must of high sugar concentration. Appl. Environ. Microbiol. 38, 1069 – 1073.

Laureys, D., De Vuyst, L., 2014. Microbial species diversity, community dynamics, and metabolite kinetics of water kefir fermentation. Appl. and Environ. Microbiol., 80(8), 2564 – 2572.

Lawes, M.J., Eeley, H.A.C., Shackleton, C.M., Geach, B.G.S., 2004. Indigenous forests and woodlands in South Africa: Policy, people and practice, University of KwaZulu-Natal Press, Scottville, South Africa.

Leakey, R.R.B.,1999. Potential for novel food products from agroforestry trees. Food Chem. 66, 1 – 14.

Leejeerajumnean, A., Duckham, S.C., Owens, J.D., Ames, J.M., 2001. Volatile compounds in *Bacillus* - fermented soybeans. J. Sci. Food. Agric. 81:525 – 529.

Lui, M., Zeng, Z., Xiong, B., 2005. Preparation of novel solid-phase micro-extraction gas chromaytographic analysis of aroma. J. Chrom. A. 1065, 287 – 299.

Marais, J., Pool, H.J.,1980. Effect of storage time and temperature on the volatile composition of dry white table wine. Vitis J. Grapevine Res. 19, 151 – 164.

Mariod, A., Matheus, B., Eichner, K., 2004. Fatty acid, tocopherol and sterol composition as well as oxidative stability of three unusual Sudanese oils. J Food Lipids 11, 179 – 189.

Mariod, A.A., Abdelwahab, S.I., 2012. *Sclerocarya birrea:* Biochemical composition, nutritional and medicinal uses--a review. Nat. Prod. Res. Rev 1, 100 – 120.

Maroyi, A., 2013. Local knowledge and the use of marula Sclerocrrya birrea (A. Rich) Hochst in South-central Zimbabwe. Indian J. Tradit. Knowle, 12(3), 398 – 403.

Mato, S., Suarez-Luque, S., Huidobro, J.F., 2005. ‗A review method of the analytical methods to determine organic acids in grape juices and wines'. Food Res. Internat. 38, 1175 – 1188.

Mdluli, K., Owusu-Apenten, R., 2002. Enzymatic browning in marula fruit 1: Effect of endogenous antioxidants on marula fruit polyphenol oxidase. J. Food Biochem. 27, 67 – 82.

Mimura, N., Isogai, A.,, Iwashita, K., Bamba, T., Fukusaki, E.. 2014. Gas chromatography/mass spectrometry based component profiling and quality prediction for Japanese sake. J. Biosci. Bioeng. 118(4), 406 – 414.

Moganedi, K., Sibara, M., Grobler, P., Goyvaerts, E., 2011. An assessment of genetic diversity among marula populations using the amplified fragment length polymorphism (AFLP) technique. Afr. J. Agric. Res. 6, 790 – 797.

Moganedi, K.L.M., Colpaert, N., Breyne, P., Sibara, M.M., Goyvaerts, E.M.A., 2007. Determination of genetic stability of grafted marula trees using AFLP markers. Sci. Hortic. 111, 293 – 299.

Mojeremane, W., Tshwenyane, S. O., 2004. The resource role of marula (*Sclerocarya birrea*): A multipurpose indigenous fruit tree of Botswana. J. Biol. Sci, 4, 771 – 775.

Mokgolodi, N.C., Ding, Y., Setshogo, M.P., Ma, C., Liu, Y., 2011. The importance of an indigenous tree of Southern African communities with specific reference to its domestication and commercialization: a case of the marula tree. Forest. Stud. China. 13, 36 – 44.

Moyo, M., 2009. Micropropagation and secondary metabolites of *Sclerocarya birrea*. Doctoral dissertation, Pietermaritzburg, University of Kwazulu-Natal.

Mutshinyalo, T., Tshisevhe, J., 2003. *Sclerocarya birrea* (A.Rich.) Hochst. Subsp. *caffra* (Sond.) Kokwaro. Pretoria National Botanical Garden.

Mpofu, A., Kock, J.L.F., Pretorious, E.E., Pohl, C.H., Zvauya, R., 2008. Identification of yeast isolated from mukumbi, a Zimbabwean traditional wine. J. Sus. Dev. Africa. 10(3), 88 – 103.

Ndhlala, A.R., Kasiyamhuru, A., Mupure, C., Chitindingu, K., Benhura, M.A., Muchuweti, M., 2007. Phenolic composition of *Flacourtia indica*, *Opuntia megacantha* and *Sclerocarya birrea*. Food Chem. 103, 82 – 87.

Neumann, B., Pospiech, A., Schairer, H., 1992. Rapid isolation of genomic DNA from gram-negative bacteria. Trends Genet. 8, 332 – 333.

Ni, H.J.,Bao, Q.H., Sun, T.S.,Chen, X.,Zhang, H.P., 2007. Identification and biodiversity of yeasts isolated from Koumiss in Xinjiang of China. Wei Sheng Wu Xue Bao. 47(4), 578 – 82.

Nouts, M.J.R., Rombouts, F.M., 1992. Fermentative preservation of plant foods. J. Appl. Bacteriol. 73, 136 – 147.

Ohimain, E.I., 2016. Methanol contaminaton in traditionally fermented alcoholic beverages: the microbial dimension. Ohimain SpringerPlus 5, 1607.

Ojewole, J. A., 2003. Evaluation of the anti-inflammatory properties of *Sclerocarya birrea* (A. Rich.) Hochst. (family: Anacardiaceae) stem-bark extracts in rats. J Ethnopharmacol. 85, (2-3), 217 – 220.

Ojewole, J.A.O., Mawoza, T., Chiwororo, W.D.H., Owira, P.M.O., 2010. *Sclerocarya birrea* (A. Rich) Hochst. [‗Marula'] (Anacardiaceae): A review of its phytochemistry, pharmacology and toxicology and its ethnomedicinal Use. Phytother. Res. 24, 633 – 639.

Okagbue, R., Siwela, M., 2002. Yeasts and related microorganisms isolated from ripe marula fruits *(Sclerocarya caffra)* in Zimbabwe. S. Afr. J. Sci. 98, 551 – 552.

Palmer, E., Pitman, N., 1972. Trees of southern Africa. Balkema, Cape Town. 226 – 246.

Orwa, C., Mutua, A., Kindt, R., Jamnadass, R., Simons, A., 2009. Agroforestry Database: a tree reference and selection guide version4.0. 1-4. (http://www.worldagroforestry.org/af/treedb/)

Pinho, O., Ferreira, I.M.P.L.V.O., Santos, L.H.M.L.M., 2006. Method optimization by solid-phase microextraction in combination with gas chromatography with mass spectrometry for analysis of beer volatile fraction. J. Chromatogr. A. 1121, 145 – 153.

Pretorius, V., Rohmer, E., Rapp, A., Holtzhausen, L.C., Mandery, H., 1985. Volatile flavour components of marula juice. Z. Lebensmittel. Fors. 181, 458 – 461.

Prins, W.A., Botha, M., Botes, M., de Kwaadsteniet, M., Endo, A., Dicks, L.M.T., 2010. *Lactobacillus plantarum* 24, isolated from the marula fruit (*Sclerocarya birrea*), has probiotic properties and harbours genes encoding the production of three bacteriocins. Curr. Microbiol. 61, 584 – 589.

Rapp, A., Versini, G., 1991. Influence of nitrogen compounds in grapes on aroma compounds of wine. In: RANTZ (Ed). Proceedings of the International symposium on nitrogen in grapes and wines. Davis, CA: Am. J. Enol. Vitic. 156 – 164.

Riu-Aumatell, M., Castellari, M., López-Tamames, E., Galassi, S., Buxaderas, S., 2004. Characterisation of volatile compounds of fruit juice and nectars by HS/SPME and GC/MS. Food Chem. 87, 627 – 637.

Rodriguez, M.E., Infante, J.J., Molina, M., Domínguez, M., Rebordinos, L., Cantoral, J.M., 2010. Genomic characterization and selection of wine yeast to conduct industrial fermentation of a white wine produced in SW Spain winery. J. Appl. Microbiol. 108, 1292 – 1302.

Rojas, V., Gil, J., Pinaga, F., Manzanares, P., 2001. Studies on acetate ester production by non-*Saccharomyces* wine yeast. Int. J. Food Microbiol. 70, 283 – 289.

Romano, P., Fiore, C., Paraggio, M., Caruso, M., Capece, A., 2003. Function of yeast species and strains in wine flavour. Int. J. Food Microbiol. 86, 169 – 180.

Salvadó, Z., Arroyo-López, F. N., Barrio, E., Querol, A., Guillamón, J. M., 2011. Quantifying the individual effects of ethanol and temperature on the fitness advantage of *Saccharomyces cerevisiae*. Food Microbiol. 28, 1155 – 1161.

Sambrook, J., Rusell, D.W., 2001. Rapid isolation of yeast DNA, In J. Sambrook, D.W. Rusell (Eds.), Molecular cloning, a laboratory manual. Cold Harbor Laboratory Press, New York, pp. 631 – 632.

Sambrook, J., Russell, D.W., 2001. Molecular cloning : a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Santalad, A., Teerapornchaisit, P., Burakham, R., Srijaranai, S., 2007. Capillary zone electrophoresis of organic acids in beverages. LWT Food Sci. Technol. 20, 1741 – 1746.

Sarkar, R., Chaudhary, S.K., Sharma, A., Yadav, K.K., Nema, N.K., Sekhoacha, M., Karmakar, S., Braga atsabisa, M.D., Mukherjee, P.K., Sen, T., 2014. Anti-biofilm activity of Marula – A study with the standardized bark extract. J. Ethnopharmacol. 154, 170 – 175.

Schäfer, G., McGill, A. E. J., 1986. Flavour profiling of juice of the marula (*Sclerocarya birrea* subsp. *caffra*) as an Index for cultivar selection. Acta Horticulturae. 194, 215 – 222.

Setati, M.E., Jacobson, D., Andong, U.C., Bauer, F., 2012. The Vineyard Yeast Microbiome, a Mixed Model Microbial Map. PLoS ONE. 7, e52609.

Shackleton, C., Shackleton, S.E., Gambiza, J., Nel, E., Rowntree, K., Urquhart, P., Fabricius, C., Ainslie, A., 2010. Linking ecosystems service and poverty alleviation in the arid and semi-arid lands of southern Africa Nova Publishers, New York.

Shackleton, S., 2004. Livelihood benefits from the local level commercialization of savanna resources : a case study of the new and expanding trade in marula (*Sclerocarya birrea*) beer in Bushbuckridge, South Africa. S. Afr. J. Sci. 100, 651 – 657.

Shackleton, S.E., Shackleton, C.M., Wynberg, R., Sullivan, C.A., Leakey, R.R.B., Mander, M., McHardy, T., Den Adel, S., Botelle, A., Du Plesis, P., Lombard, C., Laird, S., Cunningham, T., O'Regan, DO., 2009. 'Livelihood trade-offs in the commercialisation of multiple-use NTFP: lessons from marula (Sclerocarya birrea subsp. caffra) in southern Africa'. In: Uma Shaanker, R., Hiremath, A.J., Joseph, G., Rai, N.D. (Eds), Non-timber forest products: conservation, management and policy in the tropics, Ashoka Trust for Research in Ecology and the Environment (ATREE), Bangalore, India, pp. 139 – 173.

Shackleton, S.E., Shackleton, C.M., 2005. The contribution of marula fruits and fruit products to rural livelihoods in the Bushbuckridge district South Africa: balancing domestic needs and commercialisation. Forests, Trees and Livelihoods, 15, 3 – 24.

Shackleton, S.E., Shackleton, C.M., Cunningham, T., Lombard, C., Sullivan, C.A., Netshiluvhi, T.R., 2002. Knowledge on *Sclerocarya birrea* subsp. *caffra* with emphasis on its importance as a non-timber forest product in South and southern Africa: A Summary. S. Afr. Forestry J. 194, 27 – 41.

Shackleton, S.E., Shackleton, C.M., Wynberg, R., Sullivan, C.A., Leakey, R.R.B., Mander, M., McHardy, T., Den Adel, S., Botelle, A., du plessis, P., Lombard, C., Laird, S., Cunningham, T., O'Regan, D.O., 2009. Livelihood trade-offs in the commercialisation of multiple-use NTFP: lessons from marula (*Sclerocarya birrea* subsp. *caffra*) in southern Africa, In R.U. Shaanker, A.J. Hiremath, G. Joseph, N.D. Rai (Eds.), *Non-timber forest products: conservation, management and policy in the*

tropics.. Ashoka Trust for Research in Ecology and the Environment (ATREE), Bangalore, India, pp. 139 – 173.

Shackleton, S.E., Wynberg, R.P., Sullivan, C.A., Shackleton, C.M., Leakey, R.R.B., Mander, M., McHardy, T., den Adel, S., Botelle, A., du Plessis, P., Lombard, C., Laird, S.A., Cunningham, A.B., Combrinck, A., O'Regan, D.P., 2003. Marula commercialization for sustainable and equitable livelihoods: Synthesis of a Southern African case study. Winners and Losers:Final technical report, DFID FRP R7795, Appendix 3.5. Centre for Ecology and Hydrology, Walling-ford, UK.

Shale, K., Mukamugema, K.J., Lues, R.J., Venter, P., Mokoena, K.K., 2013. Characterization of selected volatile organic compounds in Rwandan indigenous beer urwagwa by dynamic headspace gas chromatography-mass spectometry. Afr. J. Biotechnol. 12, 2990 – 2996.

Shone, A.K.,1979. Notes on the marula. Dept. Water Aff. For. Bull. 58, 1-89.

Silva, G.A.D., Augusto, F., Poppi, R.J., 2008. Explotary analysis of the volatile profile beers by HS-SPME-GC. Food Chem. 111, 1057 – 1063.

Simatende, P., Gadaga, T.H., Nkambule, S.J., Siwela, M., 2015. Methods of preparation of Swazi traditional fermented foods. J. Eth. Foods. 2, 119 – 125.

Soriano, J.M., Dragacci, S., 2004. Occurance of fumonisins in foods. Food Res. Int. 37, 985 – 1000.

Sponholz, W. R. (1993). Wine spoilage by microorganisms. In: Fleet, G.H (eds) Wine Microbiology & Biotechnology. Switzerland: Harwood Academic Publishers. pp. 395 – 413.

Styge, G., Jacobson, D., Bauer, F.F., 2011. Identifying genes that impact on aroma profiles produced by *Saccharomyces cerevisiae* and the production of higher alcohols. Appl. Microbiol. Biotechnol. 91(3), 713 – 30.

Suzuki, M.T., Giovannoni, S.J., 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. 62, 625 – 630.

Taylor, F. W., 1985. The potential for the commercialisation of indigenous plants in Botswana. In: Wickens, G. E., Goodin, J.R, & Field, D.V (Eds) Plants for arid lands. Presented at International Conference on Economic Plants for Arid Lands. Royal Botanical Gardens, Kew.

Taylor, F.W., Butterworth, K.J., Mateke, M.M., 1995. The importance of indigenous fruit trees in semi-Arid areas of Southern and Eastern Africa, —Paper presented at the African Academy of Sciences second round table discussion on non-wood/timber products —, Pretoria, South Africa.

Torzilli, A.P., Sikaroodi, M., Chalkley, D., Gillevet, P.M., 2006. A comparison of fungal communities from four salt marsh plant using automated ribosomal intergenic spacer analysis (ARISA). Mycologia. 98, 690 – 698.

Tucker, G.A., 1993. Introduction, In G.B. Seymour, J.E. Taylor, G.A. Tucker (Eds.), Biochemistry of fruit ripening. Chapman and Hall, London, pp. 1 – 55.

Van Wyk, B.E., 2011. The potential of South African plants in the development of new food and beverage products. S. Afr. J. Bot. 77, 857 – 868.

Van Wyk, B.E., Van Oudtshoorn, B., Gericke, N., 2002. Medicinal plants of South Africa (2nd ed.). Briza, Pretoria, 234 – 235.

Venter, F., Venter, J., 2005. Making the most of indigenous trees (2nd ed.). Briza, Pretoria, 274.

Vermaak, I., Kamatou, G.P.P., Komane, B., Viljoen, A.M., Beckett, K., 2011. African seed oils of commercial importance — Cosmetic applications. S. Afr. J. Bot. 77, 920 – 933.

Viljoen, A.M., Kamatou, G.P.P., Baser, K.H.C., 2008. Head-space volatiles of marula (*Sclerocarya birrea* subsp. *caffra*). S. Afr. J. Bot. 74, 325 – 326.

Von Teichman, I., 1983. Notes on the distribution, morphology, importance and uses of the indigenous Anacardiaceae. 2. The Importance and Uses of *Sclerocarya birrea* (The Marula). Trees in South Africa. pp. 2 – 7.

Walker, N., 1989. King of foods. Marula economics in the Matabos. African Wildlife. 43, 281 – 285.

Wang, C., Liu, Y., 2013. Dynamic study of yeast species and *Saccharomyces cerevisiae* strains during the spontaneous fermentations of Muscat blac in Jingayang, China. Food Microbiol. 33, 172 – 177.

Watt, J. M., Breyer-Brandwijk, M.G., 1962. The medicinal and poisonous plants of southern and eastern Africa, E.S. Livingstone, Edinburgh.

Weinert, I.A.G., van Wyk, P.J., Holtzhausen, L.C.,1990, -*Marula*ll, in Nagy, S.,Shaw, P.E., Wardowski, W.F. (Eds), Fruits of Tropical and Subtropical Origin: Composition, Properties and Uses. Florida Science Source, Inc., Lake Alfred, FL, pp. 88 – 113.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, In M.A. Innis, D.H. Gelfand, J.J. Sninsky (Eds.), PCR protocols: A guide to methods and applications. Academic Press Incorporated, New York, pp. 315 – 322.

Wibowo, D., Eschenbruch, R., Davis, C.R., Fleet, G.H., Lee, T.H., 1985. Occurrence and growth of lactic acid bacteria in wine: A review. Am. J. Enol. Vitic. 36. 302 – 313.

Wilson, P., David, T., Sam, B., 2012. Microbial and chemical changes occuring during production of traditional Rwandese banana beer urwagwa. J. Ferm. Technol. 1, 104.

Wynberg, R., Cribbins, J., Leakey, R., Lombard, C. Mander M., Shackleton, S., Sullivan, C., 2002. Knowledge on *Sclerocarya birrea* subsp. *caffra* with emphasis on its importance as a non-timber forest product in South and southern Africa: A Summary Part 2: Commercial use, tenure and policy, domestication, intellectual property rights and benefit-sharing. S. Afr. Forestry J., 196, 67 – 78.