An investigation of fermentation and maturation temperatures on the chemical and sensory characteristics of the starter-culture based marula fruit wines

by

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DISSERTATION

Submitted in fulfilment of the requirements for the degree of

Master of Science

in

Microbiology

in the

FACULTY OF SCIENCE AND AGRICULTURE

(School of Molecular and Life Sciences)

at the

UNIVERSITY OF LIMPOPO

Supervisor: Prof KLM Moganedi

2024

DECLARATION

I declare that the dissertation hereby submitted to the University of Limpopo, for the degree of Master of Science in Microbiology has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

Signature: Date: 28/03/2024

DEDICATION

This work is dedicated to my entire family, especially my wife Irene Rampyeshe for the endless support and encouragement she gave me throughout the study period.

To myself for never giving up through all the difficult times and challenges I was faced with during the study.

ACKNOWLEDGEMENTS

I will express my heartfelt gratitude and appreciation to the following persons and

Institutions for their significant contributions to the successful completion of this study.

My hardworking and supportive Supervisor, Prof KLM Moganedi for giving me the opportunity and recruiting me into her incredible research team. I appreciate her for the guidance and support she gave me. She was very patient with me and allowed me the space to learn.

Marula research group, for the workmanship we had throughout the project and how we pushed each other.

Ms Baloyi Sibongile from the Pharmacy department, thank you for all the assistance.

Mrs Manape Lekganyane, for her assistance with all the machinery and equipment at the Biotechnology unit.

University of Limpopo, for allowing me to study and payment of my study fees.

Department of Biochemistry, Microbiology and Biotechnology, especially senior staff members who believed in my ability and encouraged me to register.

All the students and colleagues, especially those who took part in the sensory evaluation of the wines.

Last but not least, I will acknowledge my family (my mother, brothers, sister, kids and my beautiful wife for the endless support and love they always gave me).

LIST OF ABBREVIATIONS

AAB – Acetic acid bacteria

Anova – Analysis of variance

ARC – Agricultural Research Council

ATCC – American Type Culture Collection

BJCB - Beer Judge Certification Program

CAF – Central Analytical Facility

CFU – Colony forming units

DNA – Deoxyribonucleic acid

DPPH – 2,2, diphenyl-1-picrylhydrazyl

EC50 – Half maximal effective concentration

FDA – Food and Drug Administration

GC-FID – Gas Chromatography-Flame Ionization Detector

HCL – Hydrochloric acid

HPLC – High Performance Liquid Chromatography

ITS – Internal transcribed spacer

LAB – Lactic acid bacteria

MRS – De Man Rogosa and Sharpe

$NaNO_2$ – Sodium nitrite

NaOAc – Sodium acetate

NaOH – Sodium hydroxide

PCR – Polymerase chain reaction

rRNA – Ribosomal ribonucleic acid

rpm – Revolution per minutes

RID – Refractive index detector

SRM – Standard Reference Method

TA – Titratable acidity

TREC – Turfloop Research Ethic Committee

TSS – Total soluble solids

WLD – Wallerstein differential media

WHO – World Health Organisation

WLN – Wallerstein nutrient agar

YPD – Yeast extract peptone dextrose

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ABSTRACT

Temperature is an important factor that affects the growth and activities of wine microorganisms, which then influences the chemical profile and consequently the organoleptic characteristics of the wine. The aim of the study was to evaluate the influence of varying temperatures for fermentation, maturation and storage conditions on the chemical, nutritional and sensory characteristics of starter-culture-based marula fruit wines. Clarified sweetened marula fruit juices were fermented at 15 °C and 25 °C, and one portion from each was matured and aged at the same temperatures. Another portion from each fermented wine was matured and aged at 4 °C, whereas the reference wine was fermented at 25 °C using the clarified unsweetened marula fruit juice, and matured and stored at 15 °C and 4 °C. The microorganisms isolated from bottled aging wines were identified with the 16S rRNA region. Organic chemicals such as sugars, alcohols, glycerol, pH, acids, esters, tannins, and antioxidants were determined using conventional chemical analysis method, spectroscopy and chromatographic methods. Selected nutrients such as minerals, proteins, cholesterol, crude fats and vitamin C were determined as well. All the wines were subjected to panel testing. The microbial species that were identified in the aging marula wines at different temperatures include *Saccharomyces cerevisiae*, *Lactobacillus brevis*, *Acetobacter lovaniensis*, *Acetobacter ghanensis*, *Raoultella terrigena*, *Raoutella ornithinolytica* and *Bacillus* species. The naturally occurring citric acid which influences titratable and total acidity in wines was detected at higher concentration, while lactic and acetic acids were the least produced. An increase in higher alcohols and spicy esters was observed in the wines that were produced at 25 °C, whereas the level of fruity esters increased in wines that were stored at 4 °C. The wines preserved and improved some of the essential nutrients such as proteins and minerals, whereas vitamin C levels dropped in the wines that were produced at elevated temperatures. The wine that was fermented at 15 °C, matured and stored at 4 °C was the most appreciated as compared to wines that were produced at elevated temperatures. These findings asserted the development of good flavours in wines produced at lower temperatures, as is the case with marula fruit wine wherein the complex intensive flavour profile and good balance can be attained at lower production temperatures.

CHAPTER 1.
INTRODUCTION

1.1. Background of the study

The marula fruit wine is traditionally known as marula beer in African communities. The wine is produced from the juice of ripe marula fruits borne by the Marula tree (*Sclerocarya birrea* subsp. *caffra*) (Hiwilepo van-Hal *et al*., 2013; Shackleton and Shackleton, 2004). The fruits ripen between January and March each year (Sinthumule and Mzamani, 2019), hence the marula fruit wine is produced during this season only. The wine is shared among relatives, neighbours and community leaders during social gatherings (Simatende, 2016; Nyanga, 2012) in the communities, however, it is mainly produced for sale.

Marula fruit wine is produced through the spontaneous fermentation process which has been used for decades and it is still being used in some wineries to produce commercial wines (Oberoi *et al*., 2011; Peng *et al*., 2014). It is an uncontrolled process that involves diverse native microbial species, and this can conversely affect the quality of the wine by potentially causing spoilage and undesirable flavours (Fundira *et al*., 2002; Giraffa and Neviani, 2001). Consequently, most wines produced through spontaneous fermentation are inconsistent from batch to batch in chemical qualities, nutritional qualities and sensory attributes (Combina *et al*., 2005; Dlamini and Dube, 2008).

Traditional marula fruit wine has an average alcohol content of 5% or less as compared to other fruit wines such as grape wines, which contain an alcohol content of between 6 and 14% (Hiwilepo-van Hal, 2013; Mokgolodi *et al*., 2011). This becomes a challenge since wine styles with higher alcohol content than 5% have gained better consumer acceptance and preference (Stockley and Johnson, 2017). The low alcohol content in marula fruit wine is attributed to naturally low sugar content in the fruit juice (Shackleton and Shackleton, 2002) and the 1:1 dilution of marula fruit juice before fermentation (Maluleke *et al*., 2023).

The chemical and sensory attributes of the wine can be influenced by a myriad of factors, some of which include fermenting microorganisms and temperature (Nwonwu, 2006; Fundira *et al*., 2002). Several native microbes with notable contributions to the organoleptic characteristics of marula fruit wine have been previously reported (Belda *et al*., 2015; Fleet, 2003; Maluleke *et al*., 2023; Phiri *et al*., 2022; Torija *et al*., 2001). Hence, selected native yeasts and bacteria such as *Saccharomyces cerevisiae*, non-*Saccharomyces* yeasts and lactic acid bacteria are commonly used to inoculate the must to produce wines (Torija *et al*., 2001).

Summarily, the undesirable aspects of traditional marula fruit wine such as quick spoilage, inconsistent quality and low alcohol content can be mitigated using starter culture, better fermentation design and employing controlled conditions for the fermentation of marula fruit juice. Inoculum-based fermentation has the advantages such as guaranteed production of certain desirable characteristics in wine production (Fleet, 2003; Miah *et al*., 2017). Furthermore, wine alcohol concentration can be increased by adding sugars such as sucrose, dextrose, fructose and glucose to the must through the process called chaptalisation (Liu *et al*., 2018; Schmitt and Christmann, 2016). The use of fermentation systems designed with adequate arrangement for aeration, agitation, temperature and pH control, and proper drain to remove the waste of cultured microorganisms together with fermented products can also contribute to the quality of the wine (Liu *et al.,* 2018).

The purpose of this study was to evaluate the influence of varying temperatures on the chemical, nutritional and sensory characteristics of starter-culture-based marula fruit wines following the fermentation at 15°C and 25°C, maturation and storage at 4 °C, 15 °C and 25 °C. It is important to study and understand the influence of adding sugars, and use of starter cultures such as endogenous non-*Saccharomyces* and *Saccharomyces cerevisiae* yeasts at the different fermenting temperature on the organoleptic characteristic of the marula fruit wine. This application can help to produce improved marula fruit wine in terms of flavours, aromatic components and longevity.

1.2. Aim and objectives

1.2.1. Aim

The study aimed to evaluate the influence of varying temperatures for fermentation, maturation and storage conditions on the chemical, nutritional and sensory characteristics of starter-culture-based marula fruit wines.

1.2.2. Objectives

Marula fruit wines were produced through spontaneous fermentation for reference wines and controlled fermentation at 15 °C and 25 °C for experimental wines, maturation and storage at 25 °C, 15 °C and 4 °C and were compared for the:

- total fermentable sugars (Sucrose, glucose and fructose)
- chemical characteristics (Organic acidity, volatile acidity, pH, esters, aldehydes, alcohol, antioxidant activity, and tannin content)
- nutritional content (Mineral content, crude proteins, vitamin C, fat content and cholesterol)
- sensory characteristics (aroma, texture, taste and colour)

1.3. Research question

What effect does varying the production temperature and using starter culture in the fermentation of marula fruit juice have on the chemical characteristics, nutritional content and sensory characteristics of the marula fruit wine?

1.4. Ethical clearance

Ethical clearance for the study was obtained from Turfloop Research Ethics Committee (TREC) (Appendix A). All volunteers partaking in sensory evaluation of marula wine were from the University and surrounding communities. The project was explained to all volunteers by the principal investigator and the volunteers signed an informed consent form (Appendix B).

CHAPTER 2.

LITERATURE REVIEW

2.1. The Marula tree

The marula tree (*Sclerocarya birrea* sub-species *caffra*) (Fig. 2.1. A) is a deciduous tree that belongs to the *Anacardiaceae* family, and it is considered a South African botanical treasure (Legodi *et al*., 2022; Mashau *et al*., 2022). Although in general, the marula tree is medium-sized, it can still grow up to 18 meters in height under favourable conditions (Hiwilepo-van Hal *et al*., 2013; Mashau *et al*., 2022). The tree is commonly found in many African countries, stretching as far north of Ethiopia, while in South Africa it is widely found in the Limpopo, Mpumalanga, KwaZulu-Natal and Eastern Cape provinces (Fig. 2.1. B) (Molelekoa *et al*., 2018; Phiri *et al*., 2022). All the tree parts, including the stem, leaves, barks and fruits are used for similar and different purposes across the regions.

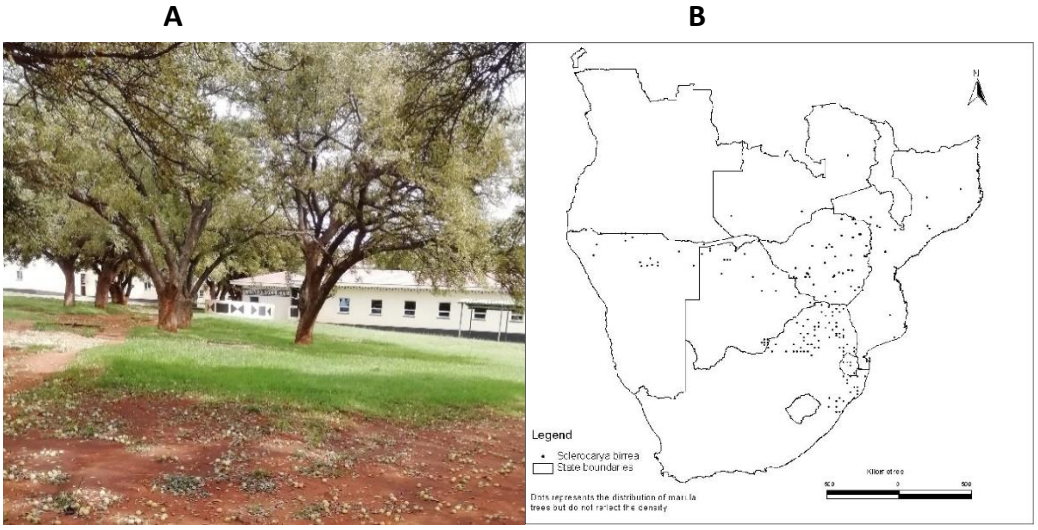
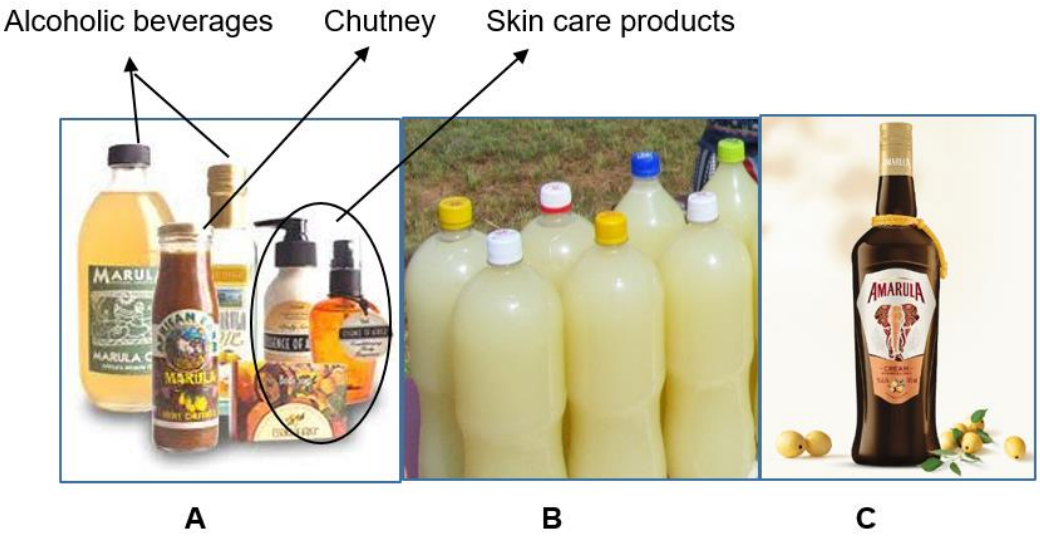


Figure 2.1. The marula tree (A) (Image source: Author) and the distribution of the marula tree in African countries (B) (Image source: Phyto Trade Africa, 2005).

The marula tree bears fruits between January and March each year. The fruits fall to the ground while still green and ripen to a yellow colour (den Adel, 2002) while already on the ground. The fruits are the most valuable part of the tree; they contain average vitamin C content of 403 mg/100 g, bioactive compounds such as phenolic compounds, minerals and other important nutrients as compared to other tropical fruits

(Hiwilepo-van Hal, 2013; Legodi *et al*., 2022; Mashau *et al*., 2022). The ripe fruits are processed into marula fruit wine and other products such as jam, juice, skin care products and chutney (Figs. 2.2) (Mokgolodi *et al*., 2011; Ntshangase and Gadaga, 2015; van Wyk, 2011). The local marula fruit wine brewers have gained experience, through many fruiting seasons, about the desirable fruit quality such as juice acidity and sweetness to produce marula fruit wine and there are specific trees that are mostly targeted for fruit harvest (Shackleton and Shackleton, 2002).



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2.2. The marula fruit wine production

2.2.1. Marula fruit wine

Marula fruit wine is traditionally produced from spontaneous fermentation of diluted marula fruit juice and it is commonly known as marula beer (Hiwilepo-van Hal, 2013). As with grape wines, marula fruit wine has variants that emanate naturally from differences in fruit origin, yield, juice content and the concentrations of available sugars (Shackleton and Shackleton, 2002). The variations in fruit characteristics are due to local environmental factors such as rainfall and temperature, but more specifically due to the environmental factors before fruit harvest (Moganedi *et al*., 2006). The production of marula fruit wine is part of traditional family art and traditional knowledge

which is passed and transferred from generation to generation through the practice (Maluleke *et al*., 2023). There are two types of marula fruit wines traditionally produced, namely, the sweet low-alcohol and higher-alcohol marula fruit wines. To differentiate the two wines, sweet wine is fermented for less than two days, and high-alcohol bitter wine is fermented for more than four days (Maluleke *et al*., 2023). The low alcohol sweet marula wine is served to young generations, and the sour high alcohol wine is served to elderly people (Shackleton and Shackleton, 2004). The marula fruit wine is known for its cloudy and hazy colour, which is sometimes less appreciated by consumers (Fig. 2.3) (Hiwilepo-van Hal, 2013).

Figure 2.3. Traditional marula fruit wine (Source: htch.unesco.org/en/photo).

2.2.2. Extraction and processing of marula juice

Sharp objects have been used to puncture the tough leathery skin of the marula fruit and the juice is squeezed out manually (den Adel, 2002). The kernels are pressed in a separate container, sometimes with the addition of water (den Adel, 2002; Mkwezalamba *et al*., 2015). In modern days, the skin is punctured using forks and pulp-extracting machines (Figs. 2.4). The extraction of juice from marula fruits is a labour-intensive process due to the low juice content of the fruits as the flesh is attached to the central pit and skin (Shackleton and Shackleton, 2002). It generally takes more than four (4) hours to produce at least 50 L of juice, hence this becomes the reason for diluting the juice with equal amounts of water before fermentation just to increase the volume of the wine produced (Shackleton and Shackleton, 2002). Inadvertently, the addition of water dilutes fermentable sugars, including other organic

and inorganic substances that support the process of fermentation for the production of high alcohol content wine.

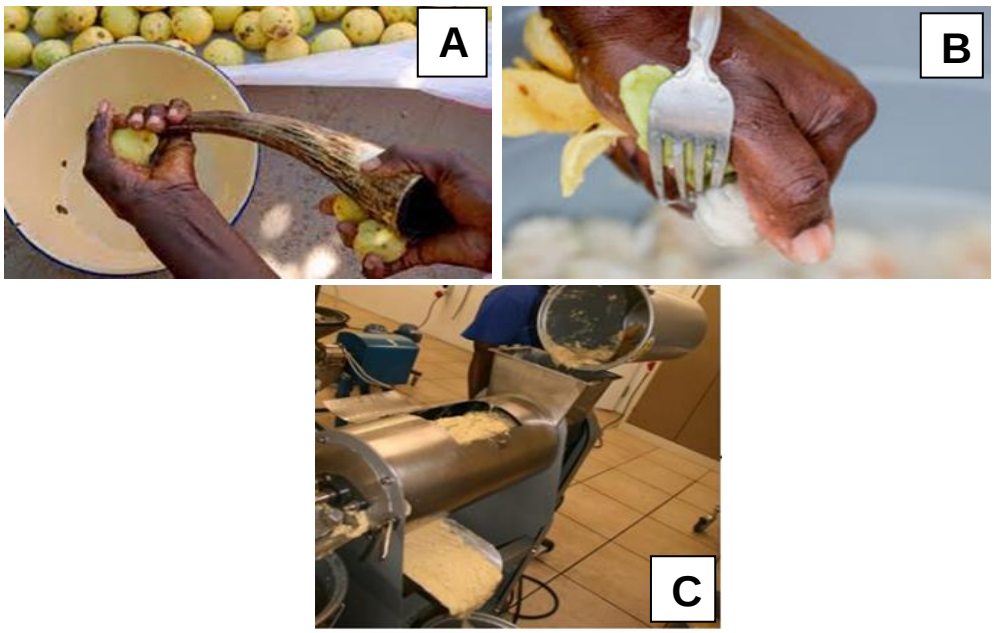

Figure 2.4. Extraction of juice using horn (A) (vambo-marula-fruits-as-socio-economic.html), extraction using a fork (B) (Image by author), extraction using marula fruit juice extraction pulper (C) (Ndlovu, 2016).

2.2.3. Marula juice fermentation process

Marula fruit juice can support the growth of a wide number of microorganisms (du Plessis *et al*., 2002). Traditionally, the wine is produced by allowing the juice to ferment naturally by native microorganisms such as yeast, fungi, acetic acid bacteria, lactic acid bacteria and other bacteria present on the marula fruits. These consume the sugars and other fermentable compounds present in the juice to support their growth (Rainieri and Pretorius, 2000). Ntshangase and Gadaga (2015) reported that the marula juice is left to ferment naturally overnight at ambient temperature and it becomes ready for consumption the following day. This is the case for a sweet low alcoholic marula fruit wine. However, on average it takes 4 – 7 days for the completion of the fermentation process for the production of higher alcohol marula fruit wine (Hiwilepo-van Hal, 2013; Mokgolodi *et al*., 2011).

Marula fruit wine is produced with an average alcohol content of 5% or less as compared to other fruit wines like grape wines which produce wines with alcohol levels

of 6 – 14 % alcohol by volume. The low alcohol content in marula fruit wines is attributed to the dilution of the juice before fermentation. There are attempts through ongoing research studies to produce high-alcohol marula fruit wines using specific starter-culture microorganisms and improved wine production methods (Fundira *et al*., 2002).

2.3. Challenges and possible mitigation strategies for the production of the marula fruit wine

2.3.1. Challenges

Marula fruit wine production has the potential for industrial enterprising, however, the large-scale production of a consistent marula fruit wine is yet still to be explored and such a challenge was reported previously by Fundira *et al*. (2002). Stability and consistency are the two key elements in the production of a commercial product through biological processes (Bartowsky, 2005; Ghosh *et al*., 2015; Simatende, 2016). It is worth noting that the production of a stable and consistent marula fruit wine has not been achieved, especially using the spontaneous fermentation process. This could be attributed to geographical differences that impact the characteristics of the fruit and the autochthonous microbiota that will contribute to wine fermentation in a natural fermentation process.

Wine spoilage and off-flavours with the current traditional marula fruit wine pose a challenge as well. This could emanate from microbial activities and biochemical reactions that occur during fermentation. Some undesirable spoilage microorganisms such as acetic acid bacteria have been reported in traditional marula fruit wine (Legodi *et al*., 2022) and these contribute to the undesirable off-flavours or high levels of acetic acids observed in the traditional marula fruit wine (Phiri *et al*., 2022).

The marula juice is naturally white in colour and cloudy upon extraction and this consequently produces a hazy wine. Hiwilepo-van Hal *et al*. (2013) reported that the haziness is caused by microorganisms that remain suspended in the solution and undissolved organic substances such as proteins, and these contribute to less desirable marula fruit wine, especially for industrial enterprises. In another study,

Lekganyane (2021, unpublished study) reported browning in marula fruit wine produced through pitching and control of bacteria present in the fermenting marula juice. Browning is a common occurrence that has been reported in other fruit wines, including white wines produced from grapes (Styger *et al*., 2011), and the exposure of wine to oxygen contributes to its browning (Fernandes *et al*., 2017).

The alcohol content of marula fruit wine is another matter that warrants consideration. The low alcohol percentages, below 6% (ABV) as found in traditional marula fruit wine, can be less desirable in the wine market since wines with high alcohol have gained consumer acceptance already (Stockley and Johnson, 2017). Furthermore, alcohol contributes to wine taste and feel, hence it can amplify the sensory experience of bitterness or sweetness in wine (Ramoba *et al*., 2022).

2.3.2. Possible mitigation strategy

Spoilage, inconsistency and low alcohol content can be averted through the use of starter culture, better fermentation design and the use of controlled conditions in wine production. Several studies have reported on the influence of *Saccharomyces* strains and other autochthonous microbes from grapes such as non-*Saccharomyces* yeast and *Oenococcus oeni* during grape wine fermentation. Such species have been shown to positively influence the chemical composition of the wine, thus contributing to the organoleptic properties of the wines (Bartowsky, 2005; Fleet, 2003). Autochthonous yeast and bacteria starter cultures can be used in the making of organic wines, owing to their ability to adapt to the fruit must (Berbegal *et al*., 2017). The strategy of studying and using autochthonous microbes from marula fruit as a starter culture for the production of marula fruit wine can positively modify the chemical composition of the wine, consequently contributing to the quality of the marula fruit wine.

Numerous studies have reported some spoilage microbes such as non-*Saccharomyces* species and acetic acid bacteria (AAB) to produce excessive undesirable metabolites such as acetic acid, ethyl acetate and aldehyde resulting in undesirable wines (Comitini *et al.,* 2011). Some studies have indicated that such microbes can contribute positively to wine production if selected for specific wines with optimal oenological properties (Comitini *et al.,* 2011). Using *Saccharomyces* species

as inoculum at the beginning of wine fermentation can lead to the suppression of other spoilage microbes since *Saccharomyces* species are known to develop antagonistic interactions with other microbes (Mas *et al*., 2016). Spoilage microbes can also be removed by pre-treatment methods such as pasteurisation before starter culture inoculation (Ramoba *et al*., 2022).

During wine production, juice clarification can be an important step in removing solids known to impact wine quality (Bowyer, 2008a). Apart from using expensive filtration systems, cold pretreatment can speed up microbial sedimentation anremove other haze-forming protein particles in the juice prior to fermentation (Bowyer, 2008b; Pocock *et al*., 2008). Furthermore, cold maturation at 2 to 4 °C can help to settle out wine particles that remain in the wine during the racking process (Palmer, 2006). Wine oxidation is reported to be the most contributing factor to wine browning (Fernandes *et al*., 2017). Limiting oxygen exposure during racking can assist in reducing wine browning.

Marula fruit wine production commonly produces wine with low alcohol content. The alcohol concentration of wine can be increased by adding sugars such as sucrose, dextrose, fructose and glucose to the must through the process called chaptalisation (Liu *et al*., 2018; Schmitt and Christmann, 2016). Overall, the use of an optimised fermentation process with controlled fermentation conditions such as aeration, temperature, pH and yeast cropping can significantly contribute to the quality of the wine (Liu *et al.,* 2018).

2.4. Traditional uses and importance of marula fruit wine

2.4.1. Socio-cultural importance of marula fruit wine

Marula fruit wine plays a supporting role in the development of different cultures (Shackleton and Shackleton, 2002). It has been integrated into customs and cultural activities in South Saharan countries including South Africa (Ntshangase and Gadaga, 2015). It was previously reported that traditional leaders and elderly members of the community strongly opposed the trading of marula fruit and wine because it was considered taboo (Shackleton and Shackleton, 2002). Notably, the shared production and consumption of marula fruit wine within communities is promoting social networks

among neighbours. The marula fruit wine is also a sacred drink for appeasing the ancestors and acknowledging the beginning of the marula season (Simatende, 2016). These social celebrations reinforce mutual bonds and maintain social networks within the communities (Phiri *et al*., 2022).

Marula beer as traditionally known as “mokhope” by Northern Sotho people. It is culturally considered a spiritual beverage offered to the ancestors using a clay pot at the ceremony where the community traditionalist and traditional spirits will gather under a selected marula tree (Shackleton and Shackleton, 2002). The Tonga rural people used to celebrate the start of marula season by pouring the offering of fresh juice and marula beer over the tombs of their late leaders regarded as ancestors (Shackleton and Shackleton, 2002). Hence most tribes believe that the marula trees were given to the people by the spirits and ancestors (den Adel, 2002; Shackleton and Shackleton, 2002).

2.4.2. Economic importance of marula fruit wine

Apart from aiding social cohesion and cultural activities, marula fruit wine has become an important product that contributes to the livelihood of African communities. Most rural communities in African countries live in poverty and with a high unemployment rate. Unemployed women produce and trade in marula fruit wine to generate income to support their families. Wynberg *et al*. (2002) reported that approximately 150 – 350 L of marula fruit wine is consumed locally by rural households in South Africa. Vuk’uzenzele (2012) newspaper article indicated that the women from the Thohoyandou region, Limpopo province in South Africa have earned an income to support their families by selling 20 L of marula beer for as much as R100 and 2 L for R10. In another report, Shokane *et al*. (2016) stated that 10 000 L of marula wine is produced and earned the brewers a net income of ~R1 500.00 where 2 L was sold for R20 while 750 mL went for R5.00. Marula fruit wine is also revered by community members for its aphrodisiac properties, although there is no empirical evidence to support this. The importance of marula fruit wine in the local markets cannot be underestimated and its potential for the global market cannot be over-emphasised.

2.5. Properties of traditional marula fruit wine

2.5.1. Microbiological contribution

Yeast and bacteria are the common microorganisms that ferment fruit juices into wines. The dynamics of the involvement of yeast and bacteria in wine production have been studied since the 1970s (García *et al*., 2016). Generally, yeast/ fungi, AAB and LAB can grow well in fruit juices (Maluleke *et al*., 2023), however, yeasts are of specific importance in alcohol fermentation. Yeasts can be divided into two categories in wine-making processes: fermenting and non-fermenting yeasts.

2.5.1.1. Non-fermenting yeasts

Non-fermenting yeasts, also referred to as non-*Saccharomyces* yeasts, were traditionally classified as spoilage yeasts in the winemaking process since they were mostly isolated from spoiled wines (Comitini *et al*., 2011; Mestre Furlani *et al*., 2017; Varela *et al*., 2016). The following yeasts have been reported at the early stages of marula fruit juice fermentation, namely, *Hanseniaspora* (*Kloeckera)* sp., *Pichia guilliermondii*, *Hanseniaspora guilliermondii*, *Meyerozyma caribbica, Hanseniaspora uvarum* and *Rhodotorula mucilaginosa* (Phiri *et al*., 2022). It is characteristic of non-*Saccharomyces* yeasts to dominate in the early stages of wine fermentation (Legodi *et al*., 2022; Maluleke *et al*., 2023). They contribute to flavour development of the wine, thus contributing to the complexity and distinctive characteristic of the wine that enhance quality (Comitini *et al*., 2011; García *et al*., 2016). Most of these strains are sensitive to ethanol and thus die soon after the commencement of alcoholic fermentation due to the rising ethanol concentration and depletion of oxygen and other nutrients (Mendoza *et al*., 2009; Nissen and Arneborg, 2003). Nevertheless, the primary wine flavours emerge from the type of fruit used, however, the contribution of some distinctive types of flavours from fatty acid esters, higher alcohols, glycerol, acetaldehyde, acetic acid and succinic acid as from the metabolic activity of non-*Saccharomyces* during active growth is important (García *et al*., 2016).

2.5.1.2. Fermenting yeasts

The most well-studied yeast strain associated with alcoholic fermentation is *Saccharomyces cerevisiae.* Unsurprisingly, *Saccharomyces cerevisiae* species is the dominant fermenting yeast that has been reported in traditional marula fruit wine

(Fundira, *et al*., 2002; Maluleke *et al*., 2023; Phiri *et al*., 2022). The ethanol-tolerant fermenting yeast, *Saccharomyces cerevisiae*, emerges and becomes highly active as ethanol concentration reaches 3 – 4% (v/v) (Fleet and Head, 1993). This has been demonstrated in marula fruit wine as well, wherein S*. cerevisiae* appeared from day 2 of fermentation (Maluleke *et al*., 2023; Phiri *et al*., 2022). *Saccharomyces cerevisiae* strains used in winemaking processes ferment fruit juice at a high rate, producing wines with high ethanol concentrations and small amounts of non-fermentable sugars (Setati *et al*., 2015). The shortcoming of using *Saccharomyces cerevisiae* as a solely selected yeast for large-scale fermentation is that it produces wines with average quality and does not enhance the aromatic traits that characterise the good quality wines (Morgan, 2016; Tristezza *et al*., 2016).

2.5.1.3. Lactic acid bacteria

Lactic acid bacteria (LAB) were isolated from the marula fruit wine as well. These were dominated by *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus buchneri*, *Lactobacillus parabuchneri*, *Lactobacillus nagelii*, *Lactobacillus kefiri*, *Lactobacillus lactis* and *Lactobacillus brevis (*Maluleke *et al.,* 2023*;* Phiri *et al.,* 2022). These species grow on sugars, acids and other metabolites at low oxygen concentrations throughout the fermentation process (Axelsson, 2004). Because *Lactobacillaceae* species are either homofermenters or heterofermenters, species such as *Lactobacillus brevis* cannot survive wines with different acids and alcohols such as ethanol and propanol (Maluleke *et al*., 2023). Lactic acid bacteria are important for malolactic fermentation (Fugelsang and Edwards, 2007), which results in a less tarty wine due to the conversion of a tart-tasting malic acid to a soft-tasting lactic acid in fruits juices that are rich in malic acid like the grape juice (Bartowsky, 2005). However, LAB is undesirable in other wine-making processes due to possible off-flavours resulting from malolactic fermentation which will make the wine unpalatable (Sumby *et al*., 2013).

2.5.1.4. Acetic acid bacteria

Acetic acid bacteria (AAB) are aerobic species that naturally inhabit fruits. This group of *Acetobacteraceae* family oxidises the sugar and alcohol produced by *Saccharomyces* species into acetic acid, which then accumulates in the wine

(Bartowsky, 2005). AABs are used in food industries for the production of vinegar and other acetic acid-fermented food and beverages (Legodi *et al*., 2022). In the presence of oxygen during wine production, acetic acid bacteria rapidly oxidise sugar and alcohol into carbon dioxide, water and acetic acid. *Gluconobacter kondonii* and *Gluconobacter albidus* are the most dominant acetic acid bacteria found in the marula fruit wine appearing at the early stage of the fermentation process while *Acetobacter aceti* was isolated in the late fermentation stage (Phiri *et al*., 2022).

Acetic acid can contribute negatively or positively toward the wine quality, depending on its concentration in the wine. Volatile acids, which are derived from acids of the acetic series present in wine, are required at low concentration sensory thresholds ranging between 200 – 700 mg/L (Lambrechts and Pretorius, 2000; Swiegers *et al*., 2005). Excessive production of volatile acids in wines can result in spoiled wine with an unpleasant vinegar taste and aroma.

2.5.1.5. Other contributing microorganisms

The *Enterobacteriaceae* species such as *Klebsiella oxytoca*, *Raoultella omithinolytica* and *Enterobacter cloacae* were previously isolated from marula fruit wine (Maluleke *et al*., 2023). These strains are pathogenic and could have been introduced by handling during fruit collection or they could be emanating from the soil that has received animal contamination (Bokulich *et al*., 2013; Maluleke *et al*., 2023). *Bacillus* species such as *B. subtilis* is another microorganism that dominates all stages of marula fruit wine production (Maluleke *et al*., 2023). The *Bacillus* species are normally found in soil, water, air and decomposing plant residue and sometimes get into wines through poor handling practices during wine production (Maluleke *et al*., 2023; Ramoba *et al*., 2022). Although *B. subtilis* can dominate the entire process of marula fruit wine production, it remains not biologically active under most conditions and it is not considered pathogenic or toxigenic to humans, animals or plants (Ramoba *et al*., 2022).

2.5.2. Chemical composition and influence on marula wine

The chemical composition of marula fruit wine differs from one area to the other due to different environmental factors such as climate and type of soil (Hillman *et al*., 2008; Moganedi *et al*., 2011). Organic acid, inorganic acid, volatile acids and other

fermentation by-products are known to contribute to the wine flavour profile, i.e., the organoleptic character of the wine (Swiegers *et al*., 2005; Torija *et al*., 2003). Ripe marula fruit naturally contains organic acids such as ascorbic acid, citric acid, malic and tartaric acid, with ascorbic acid and citric acid being the most abundant (Hiwilepo-van Hal *et al*., 2013). These acids either decrease or increase during the fermentation process due to microbial metabolic reactions. A few of the acids detected in the marula fruit wine include acetic acid, butyric acid, lactic acid and succinic acid (Fundira *et al*., 2002; Nyanga, 2012).

During the fermentation process, volatile organic acids, aldehydes, alcohols, esters and other wine constituents are moderately produced by yeast activity at different fermentation conditions (Steger and Lambrechts, 2000). Out of all the compounds produced during fermentation, ethyl esters are the most dominant of the esters, ethanol dominant for alcohols and acetates dominant for high alcohols (Fundira *et a*l., 2002). Acetic acid, which is the dominant volatile, can be produced by spoilage acetic acid bacteria or yeast as well during the fermentation process (Morrissey *et al*., 2004; Silva *et al*., 2004).

2.5.3. Nutritional content

Marula fruit wine contains important micronutrients and macronutrients (Hiwilepo-van Hal *et al*., 2014). Several studies have reported on the nutritional content of marula fruit and pulps. However, few studies report on the nutritional composition of marula fruit wine (Hiwilepo-van Hal *et al*., 2013; Shackleton and Shackleton, 2002). Generally, there is limited literature reporting on the properties and health benefits of different fruit wines as compared to grape wines (Velić *et al*., 2018). Nutritionally, marula fruit and pulp are high in different minerals such as K, Ca, Mg and P and nutrients such as amino acids, proteins, fats and vitamin C (Bille *et al*., 2013; Shackleton and Shackleton, 2002).

Marula fruit also contains low amounts of fats and proteins (Fundira *et al*., 2002). Unsaturated and saturated fatty acids contribute largely to the nutritional and sensory aspects of wines. The unsaturated fatty acids content of marula juice includes 70% oleic acid and 8% linoleic acid of the total fat component (Mariod and Abdelwahab,

2012). These by-products can positively or adversely affect the organoleptic properties of the wines by contributing desirable flavours or off-flavour to the wine. Similar to other African indigenous fruits, marula pulp contains different carbohydrates and phytochemical compounds such as terpenoids, total phenols, saponins, flavonoid, tannins and cardiac glycoside which are essential for human health (Hiwilepo-van Hal *et al*., 2014: van Wyk, 2011).

Developed and developing countries are faced with the challenges of food insecurity and malnutrition. The good nutritional content of marula fruit wine makes it a good source of minerals and other health-benefiting substances. A high number of alcoholics experience deficiencies in proteins and vitamins, hence they are considered malnourished (Lieber, 2003). Consuming high nutritive alcohol beverages such as marula fruit wine can help to improve their level of nutrients intake.

2.6. Important factors in wine production

2.6.1. Quality of starting material

Many studies in the literature demonstrated the feasibility of using grapes to produce wine. The fermentation process and the association between the quality of the grapes juice and the characteristics of the resulting grape wine are well understood and documented as compared to other tropical fruits such as marula fruit, which has the potential to produce a good quality wine (Saranraj and Ray, 2019). Grape juice has a good chemical balance and high sugar concentration, which assist in the fermentation process to produce high-quality wines without the addition of sugars, acids, enzymes or other nutrients (Fleet, 2003; Pretorius and Hoj, 2005).

Sugar is an important substrate for the fermentation of fruits juice into alcohol, apart from other substrates such as proteins, fats, macronutrients and micronutrients which can be converted into wine by-products by microorganisms (Nissen and Arneborg, 2003; Torija *et al*., 2003). Factors such as post-harvest processing method, fruit size and local climate can affect the juice yield, chemical composition and nutritional content which all have a direct influence on the quality of the wine produced (Moganedi *et al*., 2011), hence selections of good quality fruit is a very important step in wine production (Maria *et al*., 2017). The fruit should be slightly overripe with high sugar

(sucrose, glucose and fructose) content and low acidity to avoid cutting down the acidity with added water.

Fruit ripening is an overly complex process to track since the process is continuous. The fruit develops different characteristics such as aromas, flavours, tannins, sugars and acids at different stages of ripening (Andersa, 2020; Watson, 2003). This makes it important to track and monitor the process of fruit ripening (Maria *et al*., 2017). The complexity of the aroma and flavours in the fruit is affected by an increase in sugar content, a decline in acidity level, a rise in pH and an increase in the size of the fruit (Watson, 2003). Tebeila (2022) reported on the use of the total soluble solids and titratable acidity (TSS/TA) ratio to measure the degree of ripeness of marula fruit for wine production wherein a lower value indicates a good balance of the acidity and sugar content for a sweet, good-flavoured marula fruit for the production of quality marula fruit wine.

2.6.2. Wine production process

Wine production is a natural biological process involving the use of microorganisms that release hydrolytic enzymes which convert fruit substrates such as carbohydrates (simple and complex sugars) and other amino acids into alcohol, mainly ethanol (ethyl alcohol), organic/inorganic acid, carbon dioxide and water. There are mainly three processes of wine production, namely, fermentation, maturation and storage processes, with fermentation remaining at the heart of the wine production process. Although the fermentation process is considered the heart of the winemaking process, it remains a fact that the other production processes will impact the quality of the final wine product.

2.6.2.1. Fermentation process

Traditionally, wines (including marula fruit wine) were produced following a natural process using native microorganisms of the fruit juice with less human intervention (Achi, 2005; Legodi *et al*., 2022; Torija *et al*., 2001). Hence, starter cultures nowadays can be used as monoculture or a mixed culture to start up the fermentation process (Fundira *et al.,* 2002). In general, yeast and bacteria dominate the fermentation process during spontaneous fermentation. The non-*Saccharomyces* strains dominate

the early stages while *Saccharomyces* species dominate the late stage of the fermentation process for the production of flavour components and alcohol. Hence, more alcohol concentration is produced during the fermentation process.

Alcohol production during the fermentation process is sometimes followed by lactic acid production by LAB during the process called secondary fermentation (malolactic fermentation). This type of fermentation can occur naturally by the fruit native bacteria or be encouraged by inoculating the desired lactic acid bacteria.

2.6.2.2. Maturation process

Wines can taste flat or flabby immediately after the fermentation process due to low acidity. During this period, the wine is subjected to processes such as malolactic fermentation, and clarification and stabilisation process. These processes are mostly driven by microorganisms that are still active following the fermentation process. It is important to note that there are wines produced for early drinking such as most white wine, thus requiring the minimum of maturation, as compared to red wines that require a long period of maturation process (Fernandes *et al*., 2017; Reeves, 2019). Chemical reactions that ensue during the wine maturation process contribute to the changes in wine composition (Torija *et al*., 2001). The wine colour is the most noticeable change during this process, where white wine changes colour to gold (Reeves, 2019), which further turns to brown if the wine is stored for a longer time, while red wine turns purple to violet tints colour. The change is normally caused by the oxidation process due to wine exposure to oxygen (Legodi *et al*., 2022, Ramoba *et al*., 2022).

2.6.2.3. Wine storage process

Wine storage (aging) takes place after maturation or secondary fermentation processes. Just like the maturation process, storage can improve or develop new wine flavours (Swiegers *et al*., 2005). Wine maturation and aging can be mistaken to be the same process. Maturation refers to the changes in the wine after the fermentation process before bottling, while aging relates to the changes in the wine over time following bottling (Fernandes *et al*., 2017). During this process, the primary chemical compounds from the fruits react with the secondary and tertiary chemical compounds that are produced during wine pre-fermentation and post-fermentation to form the resultant wine flavour components (Swiegers *et al*., 2005; Styger *et al*., 2011).

The improper maturation and storage process can lead to changes in other key chemical and nutritional compounds in the wine thus affecting flavour development. Different wines have different optimal maturation and storage temperatures which minimise risk to the physical, chemical and flavour compounds that the wine can portray. Such conditions play an important role in the development of good-quality wines and their potential for long-term storage.

2.6.3. Effect of temperature on the wine production process

Temperature is one of the major factors affecting the rate at which fermentation takes place (Fundira *et al*., 2002; Guzel-Seydim *et al*., 2005; Jahan *et al*., 2011). Low or higher temperatures affect the growth rate of fermenting microorganisms differently and lead to the production of chemical compounds at different thresholds which affect the organoleptic characteristics of the wines (Janan *et al*., 2011). The regulation of temperature in the winemaking process can slow down or accelerate microbial fermentation patterns and ultimately chemical changes (Molina *et al.,* 2007). To achieve a stable fermentation temperature, a stable cooling system is required since the fermentation process produces heat naturally as a result of chemical reactions taking place (Fundira *et al*., 2002). Desirable fermentation temperatures for red and white grape wine production are well-studied and documented (Reynolds *et al*., 2001). Determining the optimal fermentation condition for wine production is important for the development of a consistent quality wine.

2.6.3.1. Contribution of low temperature

The optimal fermentation temperature for white grape wines is at a range between 15 °C – 20 °C (Gardner, 2002). A low temperature during the fermentation period assists in preserving colour, aroma and fruit flavours of the wine due to a slower fermentation rate (Beltran *et al*., 2008; Gardner, 2002; Torija *et al*., 2003) and discourages the growth of spoilage bacteria and yeasts. Nevertheless, it is important to note that low fermenting temperature can also slow down the development of flavour-rich phenolic compounds such as flavonoid and tannins in wine (Gardner, 2002) and secondly reduce the viability of yeasts and other fermenting microorganisms thus resulting with changes in lipids membrane which is important in the desirable slow fermentation process (Redon *et al*., 2011). Experimenting with parameters of fermentation

temperature can be beneficial for marula fruit wine production, particularly when fermenting at lower temperatures for aromatically delicate varieties which are not fully studied.

2.6.3.2. Contribution of high temperature

The optimal fermentation temperature for red wine ranges between 20 °C and 30 °C (Fundira *et al.*, 2002; Nyanga, 2012), which is a favourable temperature range to enhance the extraction of colour compounds, phenolics and tannins from grape skins (Fundira *et al*., 2002; Gardner, 2002; Reynolds *et al*., 2001). Good temperature control is required because higher temperatures above 30 °C result in the development of off-flavours from undesirable yeast end products (Bartowsky and Henschke, 2004; Reynolds *et al*., 2001) due to the production of sulphur derived compounds such as hydrogen sulphide and disulphides (Beltran *et al*., 2007).

2.7. Wine quality and stability factors

Wines of good quality are known as those that express intense flavours that last on the palate after swallowing and a lingering finish (Buckley, 2018), while moderate or low-quality wines consist of flavour that disappears immediately after swallowing (Peng *et al*., 2014). Maturation and aging can result in the appearance of new secondary compounds termed “bottle bouquet” which are considered to be a quality improvement of fruitiness obtained in young wines (Gonen *et al*., 2021). Hence, two indicators of wine quality and stability that can be affected by maturation and aging methods are complexity and balance (Peng *et al*., 2014), although there are other factors such as wine tepidity and intensity which can affect the quality of wine as well.

2.7.1. Wine complexity

Wine complexity refers to the number of flavour and aromatic compounds produced or enhanced during the aging stage. High-quality wines are stored for a longer time, often releasing complex flavours (more than two) as compared to low-quality wines which lack complex flavour components (D’Alessandro and Pecotish, 2013). Wine complexity can be actual or objective due to its physical and chemical makeup (Parr *et al*., 2011). Wang and Spence (2018) reported that complexity can be viewed in multiple ways, such as the number of wine components perceived, the temporal

evolution of flavours in the mouth (or in the bottle), or holistic integrated perception whose elements may not be indivisible. However, it is not clear what exactly wine complexity refers to in wine tasting, which makes it more of a subjective rather than an objective parameter in the determination of wine quality.

2.7.2. Wine balance

For a wine to be considered balanced, it should have flavour compounds produced at detection thresholds (Parr, 2008). The flavour components such as acidity, tannins, sweetness, alcohol and fruitiness of the wine should be balanced with the taste (D'Alessandro and Pecotish, 2013). However, maturation and storage conditions can damage or enhance one or more of these flavour components, ultimately affecting the quality and stability of the wine.

2.8. Sensory properties of fermented wines

2.8.1. Sensory parameters

The method used for the production of wine determines the quality of the end product (Cabrita *et al*., 2007; Sánchez-Palomo *et al*., 2018). Wine appearance, aroma and flavour components are generally the three elements of sensory parameters that are evaluated and perceived during wine tasting. For that reason, wine characteristics such as taste, aroma, colour and mouth feel are particularly important for market appeal (Deliza *et al*., 2005).

When evaluating wine flavour stimuli, it is important to test the five basic tastes, namely, sweet, sour, salty, bitter and astringency. In addition, aroma is one of the important factors influencing the sensory properties of fermented wines and can be perceived differently in different wines (Sánchez-Palomo *et al*., 2018; Swiegers *et al*., 2005). The aromatic compounds such as esters and high alcohols are the result of biological, biochemical and technological occurrences during the fermentation process (Cabrita *et al*., 2007). These influence other factors mentioned previously such as wine quality and stability, which can directly or indirectly affect the flavour component of the wine. Technological factors and oenological techniques such as the type of equipment used, together with the type of production method used play a significant role in the

types of wine produced in terms of flavours and aroma (Cabrita *et al*., 2007; Swiegers *et al*., 2005).

2.8.2. Type of wine sensory method

Conducting wine sensory evaluation is crucial in determining how the final product can be perceived by consumers (Lesschaeve, 2007). Sensory evaluation methods are pertinent to address key questions regarding wine testing (Chira *et al*., 2011). Methods such as wine appreciation and wine evaluation (Fig. 2.5) (Rani and Dharaiya, 2011) can be used to achieve sensory descriptiveness of the wines.

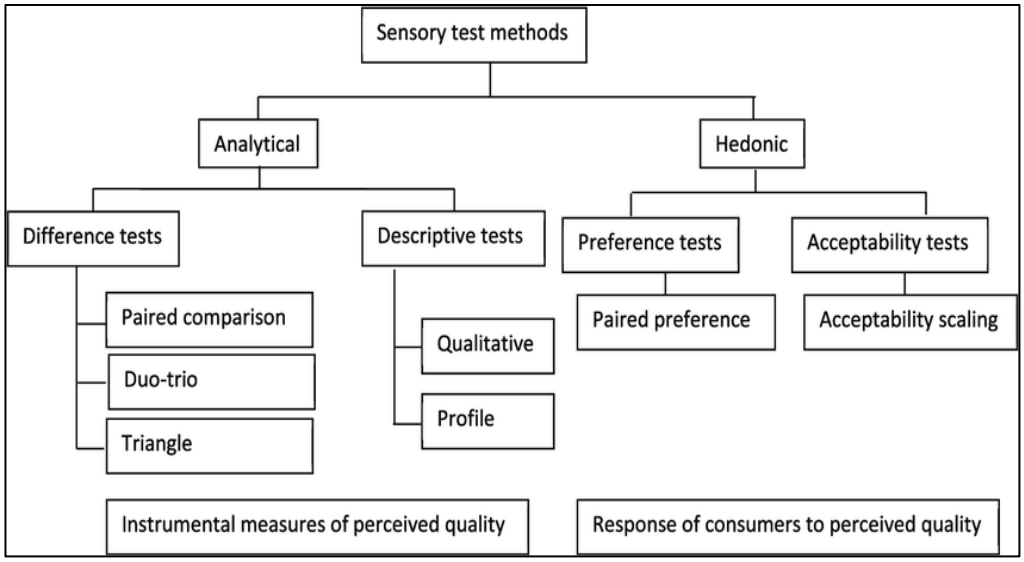

Figure 2.5. The common sensory testing methods (source: Rani and Dharaiya, 2011).

2.8.2.1. Wine appreciation method

A semi-trained panel is used to evaluate the wine sample, usually with full disclosure of the varietal, region of origin, and type of fruits used (Rani and Dharaiya, 2011). This is the wine tasting approach that is mostly used in the wine industry. It consists of both technical and hedonic testing parameters, using some principles borrowed from sensory methodology whereby five senses are used to assess the sensory attributes of the product. These include colour, appearance, size, taste and texture (Chira *et al*., 2011; Lesschaeve, 2007). Little attention is paid to the psychological and physiological biases that can occur in wine tasting, which can affect appreciation (Parr, 2008; Parr *et al*., 2011). The reliability of this wine tasting method can be influenced by several factors such as panellist geographical home area, age group, socioeconomic

background and other regional factors. It is prudent to appreciate that the action of judging wines is naturally subjective; hence the issue of methods reliability can be a subject for statistical analysis.

2.8.2.2. Wine evaluation method

The wine evaluation method uses a trained panel that uses scientific knowledge and principles to evoke, measure, analyse and interpret reactions to those characteristics of wines as perceived by the senses of colour, smell, taste and mouthfeel (Rani and Dharaiya, 2011). There is a minimal disclosure of information on the tasting objectives before or during the testing and this eliminates any biases during sensory experience. Although a trained panellist uses a scientific method to evaluate the wines, issues such as the panellist's level of qualification, and the physical and psycho-emotional state of the panellist can still bring subjectivity to the wine evaluation process (Parr *et al*., 2011).

2.9. The purpose of this study

Traditionally, marula fruit wine production follows a natural fermentation process of the marula fruit juice using indigenous microorganisms with less human intervention (Achi, 2005; Fleet and Heard, 1993; Torija *et al*., 2001). Due to this spontaneous process, there is no control over the fermenting and contributing microbiota and this can conversely affect the quality of the wine by potentially causing spoilage and undesirable flavours (Fundira *et al.,* 2002; Kunze, 2004). Consequently, most wines produced through spontaneous fermentation are inconsistent from batch to batch in chemical qualities, nutritional qualities and sensory attributes (Combina *et al*., 2005). This is the case with traditional marula wine and stability and consistency are key for the production of a commercial product. In addition, the traditional marula wine has a lower alcohol concentration, below 5%, and lacks clarity as well. Hence the purpose of this study was to improve the fermentation, maturation and storage potential of the starter-culture based marula fruit wine by evaluating the chemical, nutritional and sensory characteristic when it is produced at different temperatures as compared to the traditional produced marula fruit wine.

CHAPTER 3.

DETERMINATION OF BACTERIA AND YEASTS DURING THE AGING PROCESS OF MARULA FRUIT WINE

3.1. Introduction

Wine production is a complex biological process where microorganisms have an influential role (Torija *et al*., 2003). Microorganisms involved in wine production release hydrolytic enzymes which hydrolyse raw material substrates such as sugars and amino acids into biochemical compounds attributing to distinctive characteristic of the wines (Fundira *et al*., 2002; Phiri *et al*., 2022). Wine characteristics can somewhat be influenced by a group of native microorganisms originating from the type of fruits used (Legodi *et al*., 2022). Elsewhere different microorganisms such as *Saccharomyces* yeast, non-*Saccharomyces* yeast, and sometimes LAB can be used as starter culture or co-starter culture to begin the fermentation process (Swiegers *et al*., 2005). Furthermore, microorganisms that survive wine fermentation or maturation can easily be transferred into storage bottles and further influence chemical makeup of the wine during the aging process (Renouf *et al*., 2007).

Previous studies have investigated and characterised resident marula fruit wine microorganisms responsible for spontaneous fermentation and their chemical attributes to wine aroma and flavours (Maluleke *et al*., 2023; Molelekoa *et al*., 2018; Phiri *et al*., 2022). The wine harbours different microorganisms that belong to different families such as *Lactobacillaceae*, *Acetobacteriaceae* and *Enterobacteriaceae* (Maluleke *et al*., 2023; Mpofu *et al*., 2008; Phiri *et al*., 2022). The most dominant species are yeast strains *Saccharomyces cerevisiae* (Legodi *et al*., 2022; Molelekoa *et al*., 2018). The non-*Saccharomyces yeasts* generally appear at the early stages of fermentation and disappear at the late stages of fermentation due to an increase in alcohol concentration and depletion of oxygen or other nutrients (Mendoza *et al*., 2009; Nissen and Arneborg, 2003). Nevertheless, many studies in wine production have focused on microorganisms involved in the wine fermentation stage, hence there are few studies which have investigated the microorganisms found in the final product (bottle-aged wines) (Nisiotou and Gibson, 2005; Renouf *et al*., 2007). Thus, there is a

dearth of knowledge on the microorganisms involved during the marula fruit wine aging process.

Although microorganisms found in the aging bottle can attribute positively to the aroma and flavours development of wines (Nisiotou and Gibson, 2005), their presence can also contribute negatively by producing turbid or hazy wines as a result of the accumulation of growing microbes, which sometimes produce polysaccharides that add to wine haziness (Torija *et al*., 2003). Furthermore, these microorganisms can negatively impact wine aroma and flavour characteristics by producing undesired biochemical compounds and undesired carbon dioxide gas in wines (Renouf *et al*., 2007), resulting in the over-carbonation of the wines. This chapter was aimed at revealing the microorganisms in aging marula fruit wine in bottles stored at different temperatures. This knowledge can give a better understanding of how to handle the aging process of marula fruit wine.

3.2. Materials and methods

3.2.1. Collection and preparation of marula juice

Green marula fruits were collected in mid-February from the University of Limpopo grounds (23º53'11.4"S29º44'16.4"E) using cardboard boxes. The boxes were transported to the Microbiology laboratory, where the fruits were left to ripen at room temperature. The fruits were then washed with 70% ethanol and rinsed with tap water prior to extraction to remove soil, other debris and microorganisms from the skin. Juice extraction was carried out under sterile conditions using forks to puncture the skin while the juice together with nuts were collected into the sterilised plastic buckets. The nuts were separated from the juice and the juice volume was measured. Thereafter, the juice was kept in cold storage (4 °C) until use. The juice was then pasteurised in a boiling kettle at 75 °C for 20 minutes. The juice was augmented with white sugar syrup to a specific gravity of 1.067.

3.2.2. Selection and preparation of starter culture

The starter culture isolates used in this study were obtained from previous marula wine studies at the University of Limpopo, Department of Biochemistry, Microbiology and Biotechnology. The choice of starter cultures was based on the results obtained by

Phiri *et al*. (2022) and Maluleke *et al*. (2023*)*, during their studies on traditional marula wines. The identity of the strains is withheld as part of foreground intellectual property and confidentiality and the potential for commercialisation of the resultant product. To prepare the inoculum, the selected organisms were inoculated from stock culture into two 100 mL of sterilised yeast extract peptone (YPD) broth for each yeast isolate. The flasks were incubated in a shaker for 24 hours at 37 °C. Each 100 mL of the active yeast cultures was transferred to separate 1000 mL flasks containing 600 mL sterile marula fruit juice to acclimatise the yeast cells for fermentation conditions. The flasks were incubated at 37 °C for 24 hours.

3.2.3. Marula juice fermentation

Fermentation was performed in two different plastic bucket fermenters with airlocks. Each contained 16 L of pasteurised sweetened marula fruit juice and these were referred to as the experimental wines. One other bucket contained seven litres of unsweetened marula fruit juice that were not inoculated, and this served as the reference wine. Each 100 mL fresh slurry of non-fermenting and fermenting yeast cultures at 105 CFU/mL were sequentially inoculated to each of the 16 L juice fermenters at day 0 and when the fermenting juice reached a specific gravity of 1.050, respectively. One of the two fermenters was placed at 15 °C and the other at 25 °C. Fermentation ensued until sugar content declined to 1.030 specific gravity in wines. The reference wine bucket was placed at ambient temperature for spontaneous fermentation to take place. About 100 mL volumes of the samples were collected at three days intervals for 25 °C and 15 °C experimental wines respectively, while reference wine was collected in two days intervals. Sampling was performed in experimental duplicates. At the end of fermentation, equal volumes of wines were racked from each 16 L bucket into two sterile buckets for each wine, without disturbing the sediments. One bucket from each fermenting temperature was placed at 4°C and the other bucket was put at the same fermenting temperature (15 °C and 25 °C) for maturation. Maturing wines were sampled in 7 days intervals for five weeks. The reference wine was racked into a new bucket and matured at 15 °C for three weeks. After maturation, all wines were transferred into 440 mL brown bottles without disturbing sediments and aged using similar maturation conditions for experimental wines, while reference wine was aged at 15 °C and 4 °C only. Sampling was done for

six months in 30 days intervals. It is important to state that all the samples were collected without disturbing or mixing the wines at all production stages. Traditionally, marula fruit wine does not go through the process of maturation, however it can be stored under the soil to prolong shelf life. It is believed that the temperature is below 25 °C under the soil, hence reference wine at matured at 15 °C. The temperature of 15 °C and 25 °C are commonly used for grape with maturation, but they were never used for marula wine production.

The different wines were labelled by their production temperatures as follows:

15 – 4 °C refers to a wine that was fermented at 15 °C, matured and stored at 4 °C,

25 – 4 °C refers to a wine that was fermented at 25 °C, matured and stored at 4 °C,

25 °C refers to a wine that was fermented at 25 °C, matured and stored at 25 °C and

15 °C refers to a wine that was fermented at 15 °C, matured and stored at 15 °C.

RW 15 °C refers to a reference wine fermented at 25 °C, matured and stored at 15 °C.

RW 15 – 4 °C refers to a reference wine fermented at 25 °C, matured at 15 °C and stored at 4° C.

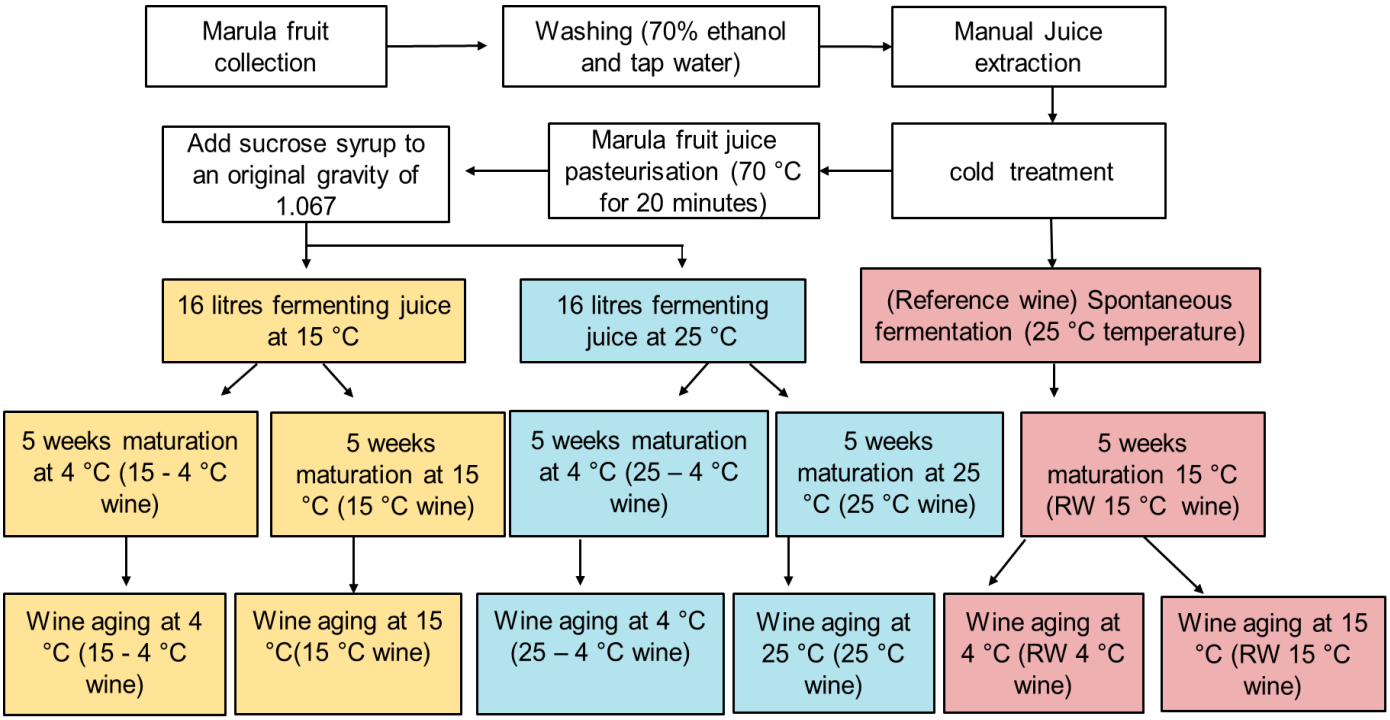

Figure 3.1. Processes for the production of marula fruit wine.

3.2.4. Sample collection and processing

Sterile 2 mL microfuge tubes were used to collect 1.5 mL of wine samples from the 100 mL of the sample collected from aging bottles at different intervals. The tubes were centrifuged at 15000 rpm for 10 minutes using a Sorvall RC 6+ centrifuge machine (Thermo Fischer Scientific). The supernatant was transferred into a new 1.5 mL tube, while the pellet was re-suspended in 50% glycerol solution in 0.85% (w/v) saline solution. The glycerol stock cultures were stored at −80 °C and the supernatant was stored at −20 °C for future use in microbial and chemical analyses, respectively.

3.2.5. Isolation of yeast and bacteria

Ten times serial dilutions from stock cultures were prepared in triplicates and 100 µL from each tube was transferred and spread on Wallerstein Laboratory Nutrient agar (WLN), Wallerstein Differential agar (WLD), Yeast Extract Peptone Dextrose medium agar (YPD) and De Man Rogosa and Sharpe agar (MRS). The cultures on WLD, WLN and YPD media were incubated aerobically at 30 °C for 42 to 72 hours, and cultures on MRS agar were incubated anaerobically at 30 °C for 72 to 94 hours. After incubation, morphologically different colonies were Gram stained for characterisation of cellular morphology. At least 10% of each colony morphology by dominance was sub-cultured on the same media using four-way streaking techniques for purification. Pure colonies were selected randomly looking at morphologies, shape, size and colour for differentiation.

3.2.6. Identification of yeast and bacterial isolates

The pure cultures were sent to Inqaba Biotechnical Industries (Genomics) in Pretoria, South Africa for identification. Genomic DNA was extracted from the isolated cultures using the Quick-DNATM Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The 16S target region was amplified using 16S-27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 16S-1492R (5'-CGGTTACCTTGTTACGACTT-3') primers. Polymerase chain reaction was completed by mixing NEB One*Taq* 2X master mix with standard buffer, genomic DNA (10 – 30 ng/µL), forward primer (10 µM), reverse primer (10 µM), and nuclease free water. The DNA was pre-denatured at 94°C for 5 minutes, denatured at 94 °C for 30 seconds,

annealed at 50 °C for 30 seconds and elongated at 68°C for 1 minute. The PCR was allowed to run for 35 cycles and thereafter held at 68 °C for 10 minutes. The integrity of the PCR amplicons was checked on a 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd), stained with EZ-vision® Bluelight DNA dye. The NEB Fast Ladder was used as a size standard. The fragments were enzymatically purified using the NEB ExoSAP procedure. The amplicons were purified for sequencing (Zymo Research, ZR-96 DNA Sequencing Clean-up KitTM), and sequenced in the forward and reverse direction (Nimagen, BrilliantDyeTM Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) using the ABI 3730xl Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Johannesburg, South Africa).

FinchTV (https://finchtv.software.informer.com/1.4/) was used to view the raw chromatogram files. CLC Bio Main workbench (digitalinsights.qiagen.com) was used to assemble the forward and reverse sequencing reads to form a consensus sequence for each sample. BLASTn analysis (with default parameters) (Altschul *et al*., 1997) was performed on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine if a sequence in the database matches the query sequence above a certain threshold (99% query coverage; 99% identity).

3.3. Results

Various bacteria and the yeast *S. cerevisiae* were isolated from bottled marula fruit wines that were aged at different temperatures. It is noteworthy to state that the isolates from the different marula fruit wines were not representative of all the microbiota present in the wine. Only suspended microorganisms were sampled, i.e., the aging wines were not mixed for homogeneity prior to sampling. Table 3.1 depicts the yeasts and bacterial strains that were isolated from the bottled marula fruit wines that were stored at 4 °C, 15 °C and 25 °C and compared together with the microorganisms isolated from wines in previously published grape wine studies.

Table 3.1. Microorganisms isolated and identified from marula fruit bottled wines that were stored at different temperatures from the current study alongside microorganisms identified at aging phase from previous studies on grape wines.

Microbial type	Wine aging temperature	Experimental wine from this study	Reference wines from this study	Previous studies on grape wine (references a – f)
Yeast	15 °C	*Saccharomyces cerevisiae*		*Saccharomyces cerevisiae* *Brettanomyces. bruxellensis* *Dekkera bruxellensis* *Hanseniaspora uvarum* *Brettanomyces* *Rhodotorula* sp. *Zygosaccharomyces bailii* *Pichia* sp. *Candida* sp.
	15 – 4 °C	*Saccharomyces cerevisiae*		
	25 °C	*Saccharomyces cerevisiae*		
	25 – 4 °C	*Saccharomyces cerevisiae*		
Bacteria	15 °C	*Lactobacillus brevis* *Paenibacillus glucanolyticus* *Bacillus amyloliquefaciens* *Bacillus velezensis* *Bacillus subtilis*	*Bacillus amyloliquefaciens* *Bacillus velezensis* *Acetobacter lovaniensis* *Acetobacter ghanensis* *Bacillus licheniformis* *Bacillus paralicheniformis* *Bacillus haynesi*	*Oenococcus oeni* *Pediococcus parvulus* *Gluconobacter oxydans* *Lactobacillus plantarum* *Pichia membranifaciens* *Zygosaccharomyces bailii* *Komagataeibacter* sp. *Streptomyces bacillaris*
	15 – 4 °C	*Bacillus amyloliquefaciens* *Bacillus velezensis*	*Bacillus siamensis* *Bacillus subtilis* *Bacillus amyloliquefaciens* *Acetobacter ghanensis* *Bacillus licheniformis* *Bacillus paralicheniformis* *Bacillus haynesi*	
	25 °C	*Raoultella terrigena* *Raoutella ornithinolytica* *Acetobacter lovaniensis* *Bacillus amyloliquefaciens*		
	25 – 4 °C	*Bacillus amyloliquefaciens*		

[a]Bartowsky *et al*. (2003); [b]Nisiotou and Gibson (2005); [c]Renouf *et al*. (2007); [d]Ubeda and Briones (1999); [e]Andorra *et al*. (2010); [f]Kioroglou *et al*. (2020); 15 – 4 °C refers to wine that was fermented at 15 °C, matured and stored at 4 °C; 25 – 4 °C refers to wine that was fermented at 25 °C, matured and stored at 4 °C; 25 °C refers to wine that was fermented at 25 °C, matured and stored at 25 °C and 15 °C refers to wine that was fermented at 15 °C, matured and stored at 15 °C.

A total of 14 microbial species were isolated from the marula fruit wines (Table 3.1), which were sampled without prior mixing for homogeneity. The fermenting yeast, *Saccharomyces cerevisiae*, was detected in all the selected aging wines that were stored at 4 °C, 15 °C and 25 °C, but not in the reference wines. Similarly, *Saccharomyces cerevisiae* was the most observed yeast species at the early stages of aging and soon disappeared towards the late stage of aging in grape wines (Table 3.1) in comparison to other non-fermenting yeast species such as *Pichia*, *Candida*, *Zygosaccharomyces*, *Rhodotorula* which were also observed at the early to mid-stages of aging.

The acetic acid bacterium *Acetobacter lovaniensis* appeared in experimental and reference wines that were stored at 25 °C and 15 °C, while *Acetobacter ghanensis* only appeared in the reference wines that were stored at 15 °C and 15 – 4 °C (Table 3.1). The acetic acid bacterial species such as *Gluconobacter*, *Acetobacter* and *Oenococcus* have been detected in grape wines (Table 3.1).

Bacillus species dominated the marula fruit wines, both the experimental and reference wines that were stored at 15 °C and 4 °C. This is contrary to the bacteria detected in grape wines (Table 3.1). Of the Lactic acid bacteria detected, *Lactobacillus brevis* was observed in the experimental wine that was stored at 15 °C. *Raoultella terrigena and Raoutella ornithinolytica*, which are considered pathogenic, were detected from the experimental wine that was stored at 25 °C.

3.4. Discussion and conclusion

Marula fruit wine is a traditional beverage produced through spontaneous fermentation of diluted marula fruit juice and this is promoted by several resident microorganisms on the marula fruit (Legodi *et al*., 2022). Native microorganisms such as yeasts and bacteria consume the sugars and other fermentable compounds present to support their growth (Rainieri and Pretorius, 2000), as a result they produce chemical compounds that contribute to the distinguished characteristics of wines (Fundira *et al*., 2002). Although fermentation of marula fruit juice is not fully discovered like other wines such as grape wine (Legodi *et al*., 2022), there is new evidence showing that microorganisms such as fermenting yeasts, non-fermenting yeasts, lactic acid bacteria

and acetic acid bacteria are responsible for spontaneous fermentation of the traditional marula fruit wine from the start to the end (Phiri *et al*., 2022). Even though the interactions between microorganisms during marula fruit wine fermentation are not well understood, the profiling of specific microorganisms involved during fermentation is growing. Notably, there is a dearth of information on microorganisms that are involved during bottled aging of marula fruit wine as compared to few studies reported on other wines. Thus, this part of the study sought to determine microorganisms which survived the bottle aging of marula fruit wine. Different microorganisms such as yeasts and bacteria were isolated and identified.

It is especially important to note that the species reported in this study were not the only surviving microorganisms in the bottles. The sampling technique, which was carried without mixing the aging wines in the bottles only allowed collection of suspended microorganisms. Furthermore, random colonies were selected for purification and identification.

Saccharomyces cerevisiae was the only dominant yeast species detected from all the aging marula fruit wine. It has been reported elsewhere that resident yeasts acclimatise faster than non-autochthonous starter culture yeasts (Ramírez-Castrillón *et al*., 2017) and could possibly adapt to the maturation and aging conditions. Generally, yeast species have the ability to utilise wine lees (the residue that forms at the bottom of wines, after fermentation and during storage stage) for their growth (Rainieri and Pretorius, 2000). Notably, *S. cerevisiae* was present at the earliest bottling month and became less detected as marula fruit wine aging progressed. The gradually depleting nutrients and absence of oxygen in aging wines are known to contribute to the disappearance of *S. cerevisiae* (Andorra *et al*., 2010; Renouf *et al*., 2007). The other yeast species that were previously reported to survive the bottle aging process include *Candida* sp., *Pichia* sp., *B. bruxellensis*, *Hanseniaspora uvarum*, *Dekkera bruxellensis*, *Rhodotorula* sp. *and Zygosaccharomyces (*Nisiotou and Gibson, 2005; Renouf *et al*., 2007; Ubeda and Briones, 1999), with *B. bruxellensis* and *Pediococcus. parvulus* reported to be the most long surviving dominant species because of their abilities to survive harsh conditions. However, these were not observed in the marula fruit wine in this study.

Importantly, the non-*Saccharomyces* yeasts were not detected in the bottled marula fruit wines, although some species such as *Hanseniaspora guilliermondi*, *Hanseniaspora uvarum, Pichia guilliermondi* and *Pichia fermentans* were reported to dominate the early stages of marula juice fermentation (Maluleke *et al*., 2023). Their absence is important since these non-fermenting yeasts in (aging) wines present a great concern for wine spoilage (Kioroglou *et al*., 2020). However, pasteurisation, as performed in this study and rising levels of alcohol during fermentation suppress the growth of these non-fermenting yeasts.

Lactobacillus brevis was the only lactic acid producing bacteria observed in the marula fruit wine in this study. *L. brevis* species can survive late fermentation stages due to its ability to convert residual hexose sugars into a mixture of lactic acid, ethanol, acetic acid and CO_2 through the hexose fermentation process (Fugelsang and Edwards, 2007). Wines that are fermented at lower temperatures (≤ 15 °C) have residual sugars (Beltran *et al*., 2007) which can support microbial growth. This phenomenon was observed with the marula fruit wine that was fermented and aged at 15 °C. Unlike most other lactic acid bacteria, *L. brevis* can tolerate acidic and high alcohol media, and can use some of organic acids as nutrients for growth (Bartowsky and Borneman, 2011), which gives it an advantage to thrive during the aging conditions. Sometimes lactic acid bacteria can utilise yeast autolysate such as proteins, vitamins, fiber and micronutrients to support their growth (Sumby *et al*., 2013). Previous studies reported the dominance of Lactic acid bacteria such as *Lactobacillus nagelii*, *L. buchneri, L. kefiri, L. parabuchneri* and *L. plantarum* in the early stages of marula fruit wine fermentation process (Maluleke *et al*., 2023; Phiri *et al*., 2022). However, due to their inability to tolerate high ethanol concentration, they disappear as fermentation continues. Renouf *et al*. (2007) noted that *Lactobacillus plantarum* and *Oenococcus oeni* are the common lactic acid bacterial species that are mostly found in aging wine bottles. During wine production, LAB species convert L-malic acid to L-lactic acid, and this becomes a concern in wine production when it is produced at concentrations above the recommended threshold of 1000 – 3000 mg/L (Chidi *et al*., 2018).

Other interesting bacteria in wine production are the acetic acid bacteria. These were not detected in the experimental marula fruit wines in this study, however, *Acetobacter lovaniensis* and *Acetobacter ghanensis* were detected in the 15 °C and 15 – 4 °C

bottled reference wines. The species such as *Gluconobacter oxydans* and *Acetobacter pasteurianus* were previously reported in marula fruit wines (Phiri *et al*., 2022). Acetic acid bacteria are undesirable in wine production due to their capability to consume ethanol as a carbon source and produce acetic acid, which deteriorates wine quality when detected at above 200 – 700 mg/L sensory threshold (Bartowsky, 2005).

Bacillus species were the most detected microbes in all storage conditions of the aging marula fruit wine. Most species of the genus *Bacillus* are present in soil and inner tissues of plants (endophytes), and they are thus transferred into wines through fruit processing for wine production (Ramoba *et al*., 2022). *Bacillus amyloliquefaciens*, *B. paralicheniformis*, *B. haynesi*, *B. velezensis*, *B. siamensis*, *B. subtilis* and *B. licheniformis* were the only identified *Bacillus* species in the study. Maluleke *et al*. (2023) and Phiri *et al*. (2022) detected the same species throughout the marula fruit wine fermentation process. This makes it possible for these species to be transferred into the maturation and storage vessels. Although, several *Bacillus* spp. produce an acidic and bitter wine which affects the wine quality and ultimately results in the spoilage of the wine (Romero-Garcia *et al*., 2009), these genera have some benefits in wine production. These include the ability to produce a wide range of enzymes such as polygalacturonase, pectin esterase and nearly all starch-hydrolysing enzymes involved in smoothening and liquefying the fermenting juice and wines (Tebeila, 2022).

It is a concern that two pathogenic microorganisms *Raoultella terrigena* and *Raoutella ornithinolytica* were detected in the marula fruit wines in this study. These two opportunistic pathogens are rare in wines but were not the only enteric species identified in the marula fruit wine. Maluleke *et al*. (2023) reported pathogenic species such as *Klebsiella oxytoca*, *Raoultella omithinolytica* and *Enterobacter cloacae* at the fermentation stage in marula fruit wine. Although these reported pathogens are not familiar like pathogenic bacteria such as *Escherichia coli*, *Salmonella* spp. and *Listeria monocytogenes*, it become a concern when they are detected in the aging wines due to their virulence (Lekhniuk *et al*., 2021). Noting that these species are not commonly present on surfaces, their presence in the wine can be a result of manual handling during fruit harvesting and processing.

Microorganisms are important in wine production, specifically for driving the fermentation process and the development of unique wine characteristics emanating from their diverse metabolic products. Different microorganisms that include yeasts, LAB, AAB and other bacteria were detected in the aging marula fruit wine. The aging process naturally entails chemical reactions that are important for flavour development in wines, however, the present microorganisms can as well produce secondary metabolites that contribute to the distinguished wine flavours. Inadvertently, spoilage microbiota were present in the marula fruit wines and these should be eliminated due to their undesirable effect on the quality of the wine. Such microorganisms can be eliminated through wine filtration and centrifugation method. It is essential that microorganisms that survive the maturation and aging conditions should be identified and characterised to maintain the good character of the wines.

CHAPTER 4

DETERMINATION OF THE CHEMICAL PARAMETERS OF MARULA FRUIT WINES

4.1. Introduction

The contributing and fermenting yeast or bacteria convert carbohydrates and other substrates into an interminable number of chemical compounds that contribute to flavour and aromatic characteristics of the wines during the fermentation of marula fruit wine (Fundira *et al*., 2002; Molelekoa *et al*., 2018). Apart from being flavour compounds, fermentation by-products influence the pH, acidity, colour and clarity of the wine (Loureiro and Malfeito-Ferreira, 2003). Although some of the primary chemical compounds of the fruits such as organic acid can be detected in wines, microorganisms are generally the major contributors to the modifying fruit wine chemical complexity (Chen *et al.*, 2013), with yeast being the most prominent contributor (Maluleke *et al*., 2023). The naturally occurring chemicals in wine react with the post-fermentation microbial by-products to form secondary and tertiary volatile compounds that contribute to wine taste and aroma (Torija *et al*., 2003).

Secondary metabolites such as higher alcohols (fusel alcohols) and esters are volatile compounds that are produced in wines and are affected by different production conditions such as temperature (Legodi *et al*., 2022). Naturally, the fermentation temperature affects the metabolic activity of fermenting and contributing microorganisms (Molina *et al*., 2007). This influences the rate of fermentation and consequently the production of metabolites including alcohols and esters. Most esters are preserved at lower production temperatures of 15 °C to 20 °C and lost at higher temperatures such as above 20 °C to 30 °C (Fundira *et al*., 2002; Reynolds *et al*., 2001). Notably, higher temperatures are generally less desirable in the production of alcoholic beverages due to the development of high amounts of unpleasant chemical compounds such as *iso*-amyl alcohol, hydrogen sulphide and disulphides (Beltran *et al*., 2007) and loss of important aromatic acetate esters such as *iso*-amyl acetate (Molina *et al*., 2007). Higher alcohols give off a sharp smell or taste, which is often considered unpleasant at above threshold concentration of 300 mg/mL (Fundira *et al*.,

2002) as compared to high concentrations of ethanol which improves wine bitterness or sweetness, thus contributing to wine taste and mouthfeel (Fleet, 2008; Pretorius and Bauer, 2002).

Wine production temperatures can influence the development of flavour-rich phenolic compounds such as tannins and ascorbic acids in wines (Gardner, 2002). These have not been reported in marula fruit wine (Tebeila, 2022) even though the marula fruit pulp and the juice are reported to harbour a large portion of total phenolic compounds (Legodi *et al*., 2022; Tebeila, 2022). Whilst there is a lack of knowledge on the production of high-alcohol marula fruit wine using a starter-culture-based process, there are only a few studies that have documented the chemical components during marula fruit wine production (Fundira *et al*., 2002; Viljoen *et al*., 2008). The starter culture based-marula fruit wines were produced from a sweetened marula fruit juice in this study and were analysed for chemical components that are important in the assessment of the quality of wines.

4.2. Materials and methods

4.2.1. Marula collection

Collection of marula fruits, juice production, selection of starter culture and juice fermentation were performed as outlined in chapter 3, section 3.2.1 to 3.2.3.

4.2.2. Sample collection

It is noteworthy to state that the wines were not mixed for homogeneity prior to sampling. About 100 mL volumes of the samples were collected in two separate sterile 50 mL tubes at day intervals during the fermentation period, weekly intervals during maturation, and monthly intervals during storage. The tubes were stored at – 80 °C prior to analysis.

The different wines were labelled by their production temperatures as follows:
15 – 4 °C refers to a wine that was fermented at 15 °C, matured and stored at 4 °C,
25 – 4 °C refers to a wine that was fermented at 25 °C, matured and stored at 4 °C,
25 °C refers to a wine that was fermented at 25 °C, matured and stored at 25 °C
and

15 °C refers to a wine that was fermented at 15 °C, matured and stored at 15 °C.
RW 15 °C refers to a reference wine fermented at 25 °C, matured and stored at 15 °C.
RW 15 – 4 °C refers to a reference wine fermented at 25 °C, matured at 15 °C and stored at 4° C.

4.2.3. Determination of volatile acid, glycerol, organic acid, sugars and pH

Chemical profiling of the marula fruit juice and wines were determined. Wine samples from both the reference wine and starter culture-based fermentations were sent for analysis to Agricultural Research Council (ARC) Infruitec-Nietvoorbij laboratory in Stellenbosch, in the Western Cape Province, South Africa. The samples were analysed using ALPHA II Wine analyser (Bruker Optics, Germany). The acids that were determined include tartaric acid, acetic acid, malic acid, citric acid, lactic acid, total acid and glycerol content**.** Sample pH, specific sugar (glucose and fructose) and the total sugar were also determined.

4.2.4. Wine titratable acidity

The titration method was used to determine total acidity that was measured as tartaric acid and volatile acidity measured as acetic acid of the samples. A volume of 10 mL of the wine sample was mixed with 10 mL of deionised water in a 50 mL beaker. At least 3 drops of 1% phenolphthalein indicator were added to the mixture and 0.1 N NaOH was slowly titrated into the mixture sample until a change in colour to pale pink was observed. The volume of NaOH used was measured and used to calculate the wine acidity using the formulae below:

Tartaric acid (%) = $\frac{ml\ of\ alkali \times normality\ of\ alkali \times 7.5}{weight\ of\ sample\ (gm)}$

Acetic acid (%) = $\frac{ml\ alkali\ x\ normality\ of\ alkali\ x\ 6.0}{eight\ of\ sample\ (gm)}$

4.2.5. Determination of esters and higher alcohols

Wine samples were analysed at the Central Analytical Facilities (CAF), Stellenbosch University, South Africa. A volume of 5 mL of sample was transferred into a sample tube, followed by the addition of 100 µL of 175 ppm 4-methyl-2-pentanol ($C_6H_{14}O$) (internal standard), 1.5 mL diethyl ether and 3 mL of 20% NaCl solution. The sample

tube was mixed thoroughly with a vortex and sonicated for 30 minutes. Liquid-liquid extraction (LLE) was performed, followed by centrifugation using a Sorvall RC 6+ centrifuge machine (Thermo Fischer Scientific) at 3000 rpm for 1 minute. The diethyl ether layer (top layer) was transferred into a 2 mL vial with 1 mL Na_2SO_4 and then transferred again into a vial insert (positioned inside a 2 mL vial).

Separation was performed on a gas chromatography (GC) instrument (6890N, Agilent Technologies) coupled to flame ionisation detector (FID). Separation of the major volatiles was performed on a polar ZB-FFAP (60 m, 0.32 mm ID, 0.50 µm film thickness) capillary column (Phenomenex). Hydrogen was used as the carrier gas at a flow rate of 1.9 mL/min. The injector temperature was maintained at 250 °C. One microliter of the sample was injected in a 7:1 split ratio. The oven temperature was programmed as follows: 40 °C for 17 minutes and ramped to 240 °C at a rate of 8 °C/min for 6 minutes. The pure compounds (Sigma-Aldrich) of fusel alcohol, straight chain alcohols (2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol) and higher (fusel) alcohols (1-propanol, n-butanol, hexanol and 1-pentanol), acetate esters (isoamyl acetate, hexyl acetate, 2-phenylethyl acetate) and ethyl esters (ethyl acetate, ethyl butyrate, ethyl lactase, ethyl hexanoate, ethyl octanoate, ethyl decanoate, and diethyl succinate) were used as standards to aid in the identification of the compounds present in the marula fruit juice and wine.

4.2.6. Determination of tannins content

The tannin content was determined using the Folin-Ciocalteu method described by Tambe and Bhambar (2014). A total amount of 20 µL of each sample was added into a test tube containing a mixture of 1.5 mL distilled water, 0.1 mL Folin-Ciocalteu reagent and 2 mL 35% Na_2CO_3. The blank was prepared in the same manner as the test solutions without adding any wine sample. All the test tubes were placed at 25 °C in a water bath and shaken gently for 30 minutes. The absorbance of the samples was measured against the blank at 725 nm using a Genesys 10S UV-Vis spectrophotometer (Thermo Fisher Scientific). The experiment was conducted in duplicate and independently repeated.

4.2.7. Determination of antioxidant activity

Free radical scavenging activity of the marula juice and wine samples was quantified using the 2.2 diphenyl-1-picrylhydrazyl (DPPH) method described by Leong and Shui (2002). The known antioxidant, L- ascorbic acid was prepared at different concentrations of 0.625 – 10 mg/mL up to 1 mL with methanol. The wine samples were prepared in the same manner as the L-ascorbic acid. An equal amount of 1 mL of 0.2 mmol/L DPPH-methanol solution was added to each solution and mixed thoroughly. The solution was kept in the dark for 30 minutes. A control mixture was prepared by mixing 1 mL of 0, 2 mmol/L DPPH-methanol solution and 1 mL deionised water. After incubation, the solution was analysed on the UV/Vis spectrophotometer (Genesys 10S, UV-VIS) at the wavelength of 517 nm. The experiment was performed in duplicates. The obtained absorbance was used to calculate the sample's EC50 concentration by using the concentration extrapolated from the derivation of the best-fit line.

4.2.8. Statistical analysis

The differences in the chemical profiles during maturation and storage were analysed using inferential statistics, one-way ANOVA with multiple comparisons using an independent t-test method to compare the mean values of the chemical compounds of different wine production temperatures. The chemical compounds in the wines were compared in the grouping of weekly intervals that ranged from the first week to the fifth week, and monthly intervals that ranged from the first month to the sixth month. The unpaired experimental design and the parametric test was used following analysis for normality. The significant difference of the chemical content produced in all the wines was determined at different levels, namely $p \leq 0.05$ and $p \leq 0.0001$. This was from sufficient to most conclusive evidence that supported the test hypothesis. The test hypothesis states that the temperature influences the chemical composition of the marula fruit wine. Mean values with standard deviations were reported and the experiment was performed in duplicates.

4.3. Results

The selected chemical characteristics of the two starter-culture based and spontaneous marula fruit wines were analysed. The experimental starter culture-

based wines were fermented at 15 °C and 25 °C, and the spontaneously fermented wine (reference wine) was produced at 25 °C. The wines were matured and stored at 15 °C, 25 °C and 4 °C.

It is noteworthy to state that due to the research design; statistical analysis was not used to determine the influence of fermentation temperatures on chemical parameters during the fermentation stage, considering that the sampling period was not the same for the wine that was fermented at 15 °C and 25 °C as illustrated in chapter 3, section 3.2.3. Furthermore, the wines were not mixed for homogeneity prior to sampling based on the method for wine production.

4.3.1. Concentrations of total sugar, fructose and glucose in marula fruit wines

Figure 4.1 represents the utilisation of glucose and fructose during fermentation, maturation and storage conditions at different temperatures for starter-culture-based wines (Fig. 4.1 A) and reference wines (Fig. 4.1 B). The utilisation of both glucose and fructose have a direct influence on the total sugar. The sweetened marula fruit juice used for experimental wines contained more fructose at 62.9 g/L than glucose at 46.6 g/L at day zero (Fig. 4.1 A), whereas the unsweetened marula fruit juice used for the reference wine contained more glucose that was detected at 28 g/L as compared to fructose which was at 18.8 g/L (Fig. 4.1 B).

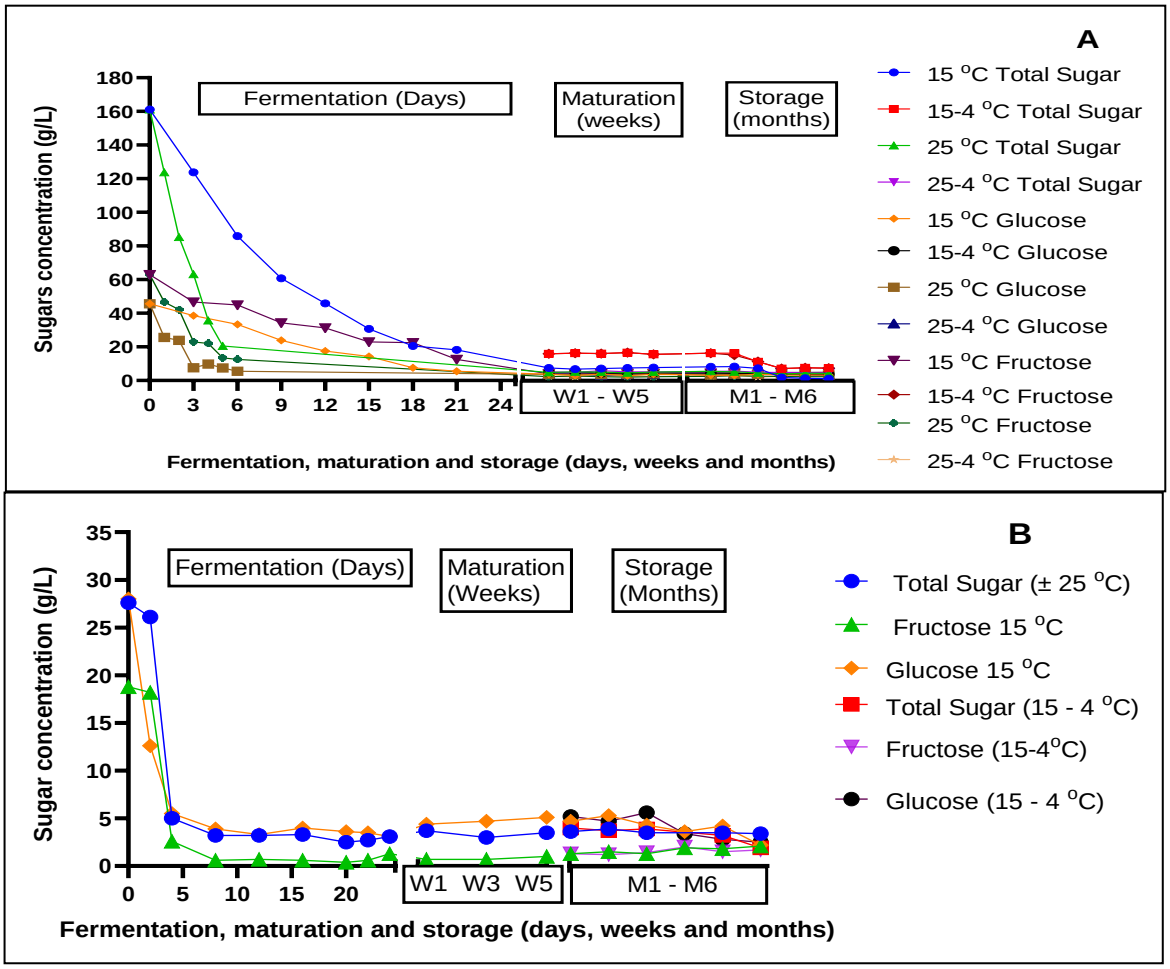

Figure 4.1. Total sugar, fructose and glucose concentrations of the experimental wines (A) and reference wines (B). W refers to time in weeks and M refers to months.

Both glucose and fructose were consumed rapidly in the wine that was fermented at 25 °C and the reference wine, with all their sugars depleted within 5 days of fermentation as compared to the wine that was fermented at 15 °C which took 21 days (Figs. 4.1 A and 4.1 B), hence there was a rapid decrease in total sugar concentration in 25 °C wines during the fermentation stage. The final sugar gravity of 1.030 was targeted to mark the end of fermentation. A 0.1-fold difference in total sugars was noted between the wines that were fermented at 15 °C and 25 °C at the end of fermentation.

The marula wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C) had a higher total residual sugar as compared to its counterpart wines during maturation and storage, while the residual sugar was mostly contributed by a high concentration of fructose sugar (Fig. 4.1 A). As a result, maturation and storage

temperatures revealed a significant difference in residual glucose sugar ($p < 0.01$) and in residual fructose sugars ($p < 0.0001$), of the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C) as compared to wine that was fermented at 25 °C, matured and stored 4 °C (25 – 4 °C). Similar observations were made with the wine that was fermented at 15 °C, matured and stored at 15 °C (15 °C wine) when compared to the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C), while no significant difference ($p > 0,05$) for residual sugar concentration was observed between the wine that was fermented, matured and store at 25 °C (25 °C wine) and that which was fermented at 25 °C, matured and stored at 4 °C (25 – 4 °C wine). The residual glucose concentration ranged from 3.1 to 3.7 g/L and 3.1 to 4 g/L for 25 °C wine and 25 – 4 °C wine respectively, whereas the residual fructose concentration ranged from 2.2 to 2.8 g/L and 2 to 3.2 g/L for 25 °C wine and 25 – 4 °C wine respectively. On the contrary, the residual glucose concentration ranged from 3.2 to 3.9 g/L and 3.6 to 4.8 g/L for 15 °C wine and 15 – 4 °C wine respectively, whereas the residual fructose sugar ranged from 4.1 to 4.9 g/L and 7.3 to 16.6 g/L for 15 °C wine and 15 – 4 °C wine respectively.

4.3.2. Total alcohol concentration

The concentration of total alcohol for all the wines was quantified during fermentation, maturation and storage conditions using chromatographic analysis (Fig. 4.2).

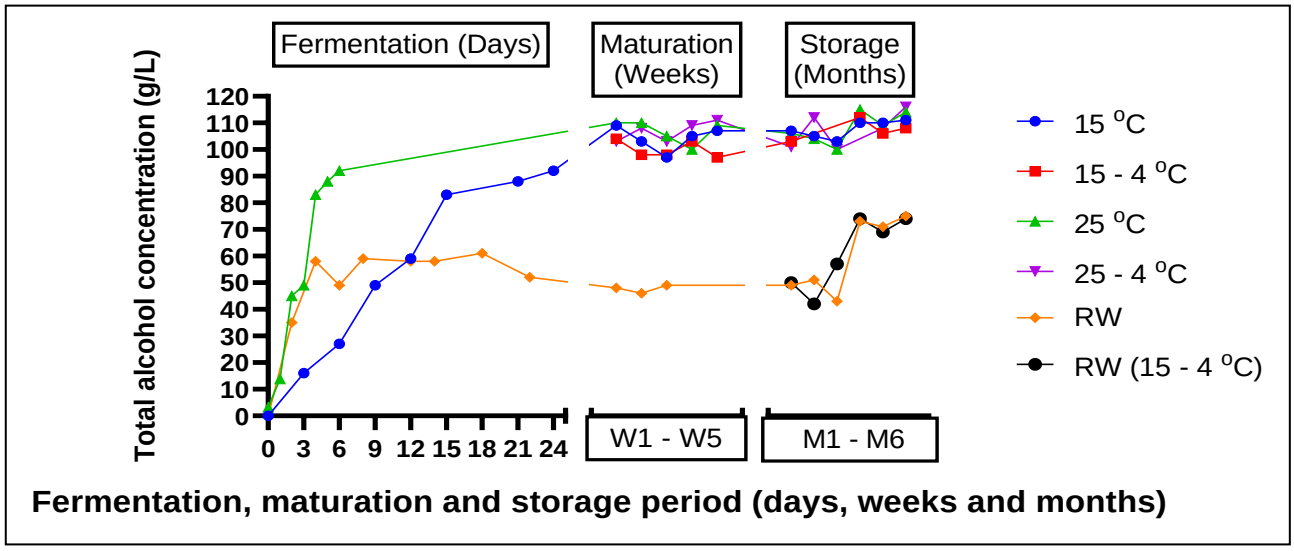

Figure 4.2. Concentration of total alcohol in marula fruit wines produced at different temperatures, RW refers to reference wine, W refers to weeks and M refers to months.

The total alcohol concentration increased steadily in the marula fruit wine that was fermented at 15 °C as compared to a sharp increase in the wine that was fermented at 25 °C (Fig. 4.2). The increase was inversely proportional to the decrease in sugars concentration observed in Fig. 4.1 A. It is worth noting that fermentation for both 15 °C and 25 °C was terminated once the alcohol concentration reached 92 g/L. A 1.14-fold increase in total alcohol for the wines that were matured and stored at 4 °C (15 – 4 °C and 25 – 4 °C) and 1.2 fold increase for the wines that were fermented, matured and stored at 15 °C and 25 °C (15 °C and 25 °C wines) from fermentation phase to maturation phase was observed, showing a slightly higher alcohol content in higher storage temperatures. Interestingly, a 1.06-fold total alcohol difference ($p = 0.012$) was observed between the two wines that were matured and stored at a lower temperature of 4 °C (15 – 4 °C and 25 – 4 °C wines). Even though the total alcohol concentration fluctuate during maturation and storage conditions, the highest total alcohol of 116 g/L was observed from the wine that was fermented at 25 °C, matured and stored at 4 °C (25 – 4 °C wine) in the sixth month of storage, compared to 112 g/L total alcohol which was obtained from the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine) on the fourth months of storage. The total alcohol content for the reference wine increased sharply during the first three days of fermentation and fluctuated between the concentrations of 48 – 61 g/L during the last days of fermentation, maturation and the first three months of storage stage. A sudden increase of total alcohol to 75 g/L during the last three months of storage was unexpectedly observed in the reference wines (Fig 4.2).

Briefly, the pattern of alcohol accumulation and sugar consumption was similar in the reference wine and the wine that was fermented at 25 °C, except for the levels as a factor of the different starting sugar concentrations that expectedly produced different alcohol concentrations in the two wines.

4.3.3. Glycerol content in marula fruit wines

The concentration of glycerol for all the wines was quantified during fermentation, maturation and storage conditions using chromatographic analysis (Fig. 4.3).

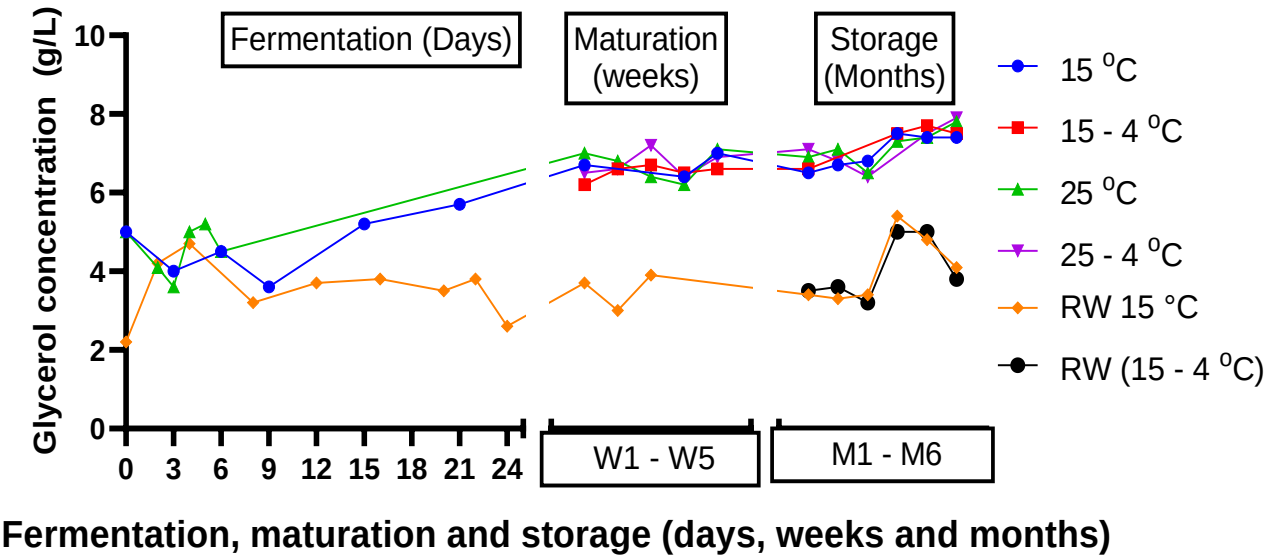

Figure 4.3. Glycerol concentration of wines produced at different temperatures, RW refers to reference wines, W refers to (weeks) and M refers to months.

Glycerol concentration fluctuated between 2.1 – 5.7 g/L for the wine that was fermented at 15 °C and 3.6 – 5.2 g/L for the wine that was fermented at 25 °C, while reference wine fluctuated between 2.6 – 4.7 g/L during fermentation stage (Fig. 4.3). There was a slight increase in glycerol concentration during maturation and storage phase for all the experimental wines which ranged between 5.9 and 7.9 g/L, whereas reference wines were between 3.2 – 5.4 g/L (Fig. 4.3). The reference wine had a low glycerol content comparatively, which was 3.04-fold lower than the experimental wines at the start of the maturation phase. There was a slight 0.8-fold and 1.9-fold increase in glycerol concentration after three months of storage for experimental and reference wines respectively, while there was a 2.5-fold decrease in the concentration of glycerol on the last two days of storage condition in reference wines. A sporadic fluctuation was observed in the reference wine at the end of the storage period.

4.3.4. Concentrations of organic acids

The different organic acid compounds were investigated in the wines during the fermentation, maturation and storage process. Figure 4.4 depicts the levels of total acid, titratable acid and tartaric acid in the wines, whereas citric acid, malic acid, lactic acid and acetic acid are illustrated in figure 4.5.

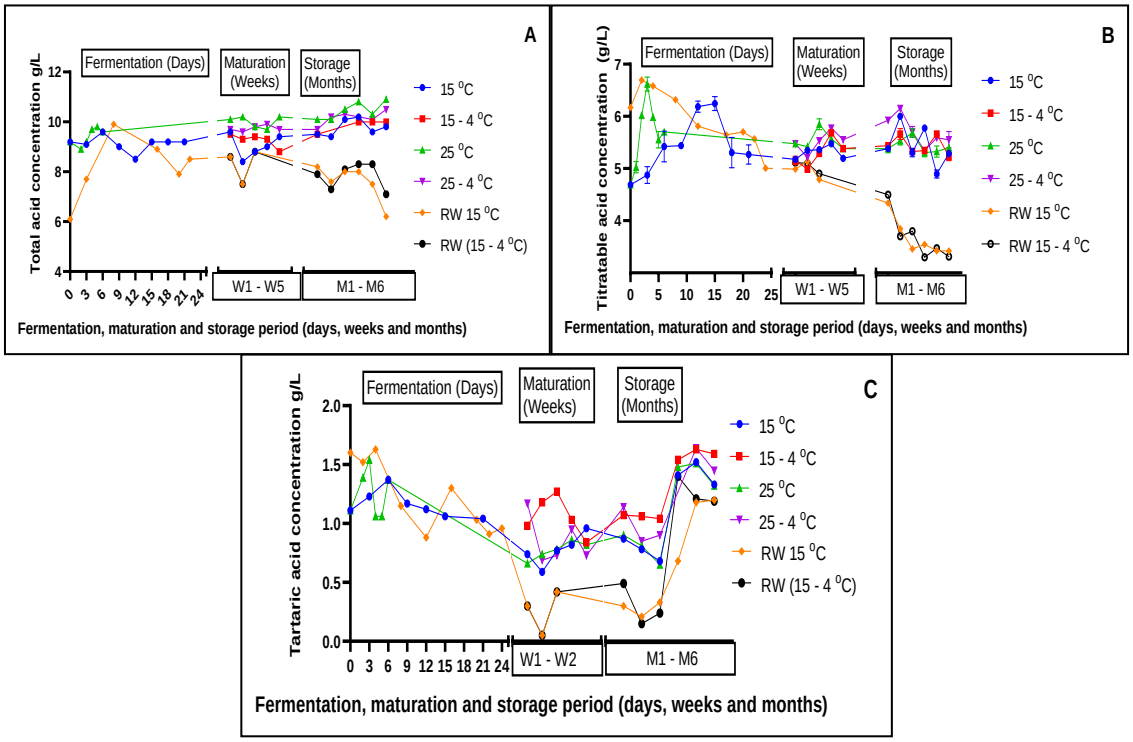

Figure 4.4. Acidity levels in the wines produced at different temperatures. A is total acidity, B is titratable acidity, and C is the level of tartaric acid. RW refers to reference wine, W refers to weeks and M refers to months.

The total acids concentration fluctuated between 9.2 and 9.6 g/L for the wine that was fermented, matured and stored at 15 °C (15 °C wine) and 9.2 – 9.8 g/L for the wine that was fermented, matured and stored at 25 °C (25 °C wine) during fermentation phase (Fig. 4.4 A). The total acid for all the test wines remained constant throughout the maturation and storage stage, except for reference wines (RW 15 °C and RW 15 – 4 °C) whose total acid level dropped to 6.2 g/L and 7.1 g/L, respectively (Fig. 4.4 A). However, the total acid content increased by 1-fold at the maturation and storage stages for the wines that were fermented at 25 °C (25 °C and 25 – 4 °C wines), whereby it ranged from 9.7 to 10.2 and 10.1 to 10.9 g/L respectively (Fig. 4.4 A). A

similar trend was observed during the maturation and storage stages in the wines that were fermented at 15 °C (15 °C and 15 – 4 °C wines), whereby they ranged from 9.6 to 10.2 g/L for both wines (Fig 4.4 A). Consequently, a significant difference ($p < 0.01$) in total acid content between the wines that were fermented, matured and stored at 25 °C (25 °C wines) and the wines that was fermented, matured and stored at 15 °C (15 °C wines) during maturation and storage stages was observed.

Generally, the reference wine and 25 °C wine, both fermented at a higher temperature, displayed a quick rise in titratable acidity which peaked in the second and third days of fermentation respectively, in contrast to a 15 °C wine which was fermented at a lower temperature and had a steady increase in titratable acidity over a 15 days fermentation period. Except for the reference wine that was matured and stored at 15 °C (RW 15 °C), all the other wines which were mostly matured and stored at 4 °C retained a constant amount of titratable acid ranging between 5.1 g/L and 5.9 g/L (Fig. 4.4 B). Maturation and storage stage temperature did not significantly affect the amount of the total titratable acid ($p > 0.05$) produced by all the experimental wines.

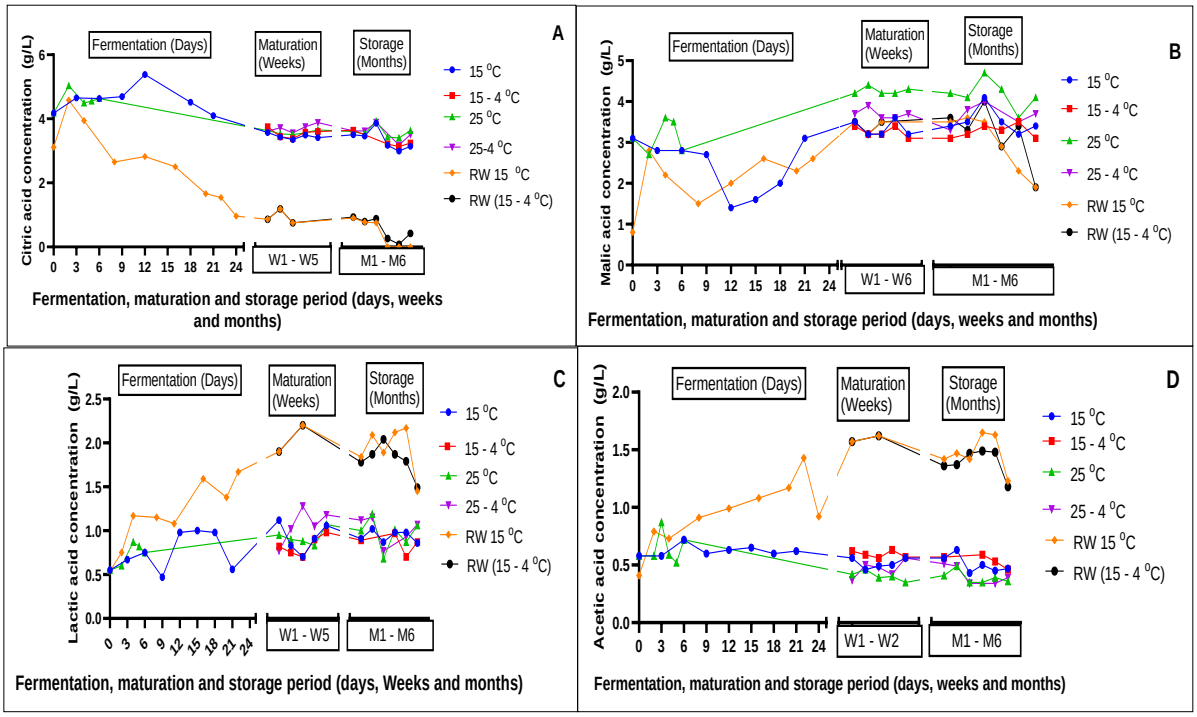

Figure 4.5. Acidity levels, malic acid (A), lactic acid (B), citric acid (C) and acetic acid (D) in the wines produced at different temperatures, RW refers to (reference wine), W refers to weeks and M refers to months.

The naturally occurring tartaric acids ranged between 0.5 – 2 g/L in both experimental wines, while it decreased during fermentation, but stabilised during maturation and early storage stage (Fig. 4.4 C). A steep increase in tartaric acid concentrations was observed on the last three months of storage condition (Fig. 4.4 C), and a similar trend was observed with the reference wine (Fig. 4.4 C).

Citric acid was the most dominant detected titratable acid in all the wines (Fig. 4.5 A) and has a similar accumulation pattern at the titratable acids content (Fig. 4.4. B). The highest citric acid concentration (7,73 g/L) was detected in 15 °C fermenting wine on day 15, whereas 25 °C fermenting wine produced the highest citric acid concentration of 6.1 g/L on day 3 (Fig. 4.5 A). The citric acid content in all the experimental wines decreased and stabilised within a range of 2.99 – 3.9 g/L during maturation and storage, contrary to the steady decrease throughout the production stages observed for the reference wine (Fig. 4.5 A). There was a 0.53 g/L difference ($p > 0.05$) in citric acid concentration obtained at the end of fermentation for 15 °C wine and 25 °C wine insinuating that the length of fermentation did not have any effect on the total citric acid concentration when comparing a 21-day long fermentation period in the 15 °C with the 6 days long period for 25 °C wine.

The concentration of malic acid stabilised and remain constant in all the experimental wines throughout maturation and storage stages, and this ranged from 3.1 to 4.7 g/L. The highest malic acid concentration was detected in the wine that was fermented, matured and stored at 25 °C (25 °C wine) (Fig. 4.5 B), consequently, there was 0.92 fold difference in malic acid concentration ($p < 0.0001$) between the wine that was fermented, matured and stored at 25 °C (25 °C wine) and the wine that was fermented, matured and stored at 15 °C (15 °C wine) during the maturation phase. A similar difference was observed between the wine that was fermented at 25 °C, matured and stored at 4 °C (25 – 4 °C wine) and the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine).

The lactic and acetic acids were the lowest produced acids in the experimental wines, and they ranged between 0.4 – 1.5 g/L and 0.35 – 0.8 g/L respectively, while both acids were highly detected in the reference wines with a range between 0.5 – 2.2 g/L and 0.4 – 1.6 g/L respectively (Figs. 4.5 C and D). The level of lactic acid was not

influenced ($p > 0.05$) by different maturation and storage temperatures, while the acetic acid concentration in the wine that was fermented at 15 °C, matured and stored at 4 ° C (15 – 4 °C wine) was 0.1-fold higher ($p < 0.01$) than other wines during maturation and storage.

4.3.4.1. Contribution of individual organic acid toward total acidity and total titratable acids

The total average amount in percentages of individual organic acids which include malic acid, citric acid, tartaric acid, lactic acid and acetic acid accumulated during the wine production was calculated and determined how they individually contribute to the total acidity and total titratable acidity.

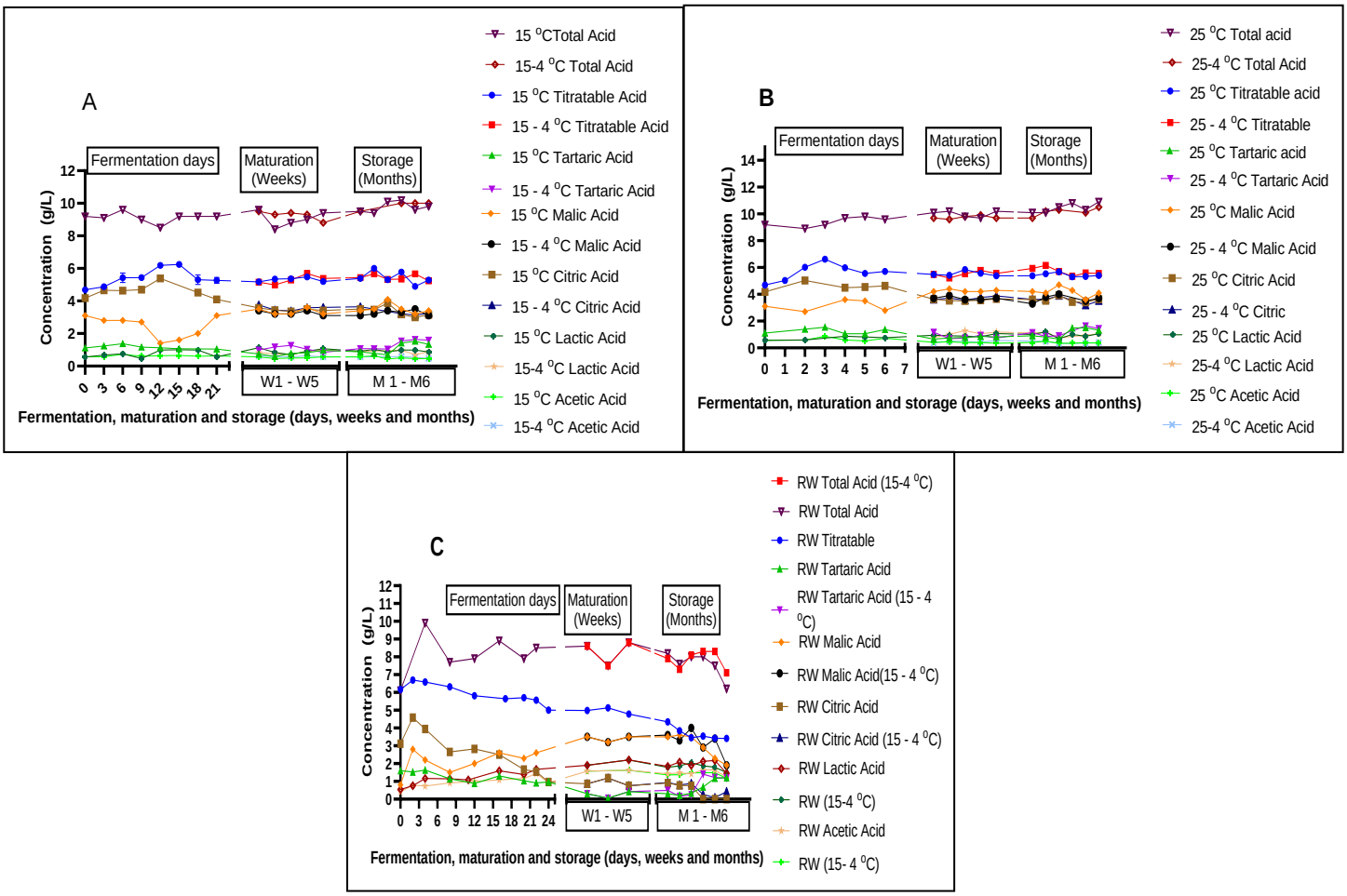

Figure 4.6. Accumulation of individual acids in 15°C produced wines (A), 25 °C produced wines (B) and reference wines (C) toward the total acidity and titratable acidity.

Titratable acid which is usually reported in units of tartaric acid, malic acid, or citric acid contributed to 50% of total acids in non-fermented marula fruit juice (Fig. 4.6), while volatile lactic acid and acetic acid contribute 6.5% each to volatile acid. At the end of fermentation there was 8% increase in titratable acid to 58% which was mostly contributed by citric and malic acid (Fig. 4.6). Marula fruit juice notably contained 37.65% citric acid, 32.23% malic acid, and 12.01% tartaric acid and these reduced to 35.02%, 32.10%, and 11.03% respectively after fermentation in the wine that was fermented at 15 °C (15 °C wine). However citric acid and tartaric acid increased to 39.04% and 13.08% of total acid respectively after fermentation in the wine that was fermented at 25 °C (25 °C wine), whereas malic acid decrease to 27.25% of total acid contribution. Acetic acid and lactic acid remained constant in the wine that was fermented at 15 °C, while they increased to 7.2% and 8.0% respectively in the wine that was fermented at 25 °C. During maturation and storage phase of the wines that were fermented at 15 °C (15 °C wine and 15 – 4 °C wine), citric acid, malic acid, and acetic acid remained the same between the ranges of 32.51% to 35.23%, 32.20% to 34.41% and 5.01% to 6.40% respectively as contribution to total acidy, while tartaric acid increase by 3% on the last month of storage. Similar trend was observed with malic acid and tartaric acid in the wines that were matured and stored at 25 °C (25 °C wine and 25 – 4 °C wine), while tartaric acid increased to a range between 34.34% – 40.05% of total acid contribution. Acetic acid decreased by half percentage, while lactic acid steadily increased to 10% at the end of maturation and storage phase for all the wines that were fermented at 25 °C (25 °C wine and 25 – 4 °C wine).

4.3.5. pH levels in the wines

The graph below (Fig. 4.7) illustrates the change in pH level in all the wines that were produced at different temperatures. In general, the pH of all the wines increased during the fermentation stage, stabilised during the maturation stage and decreased during the late storage stage.

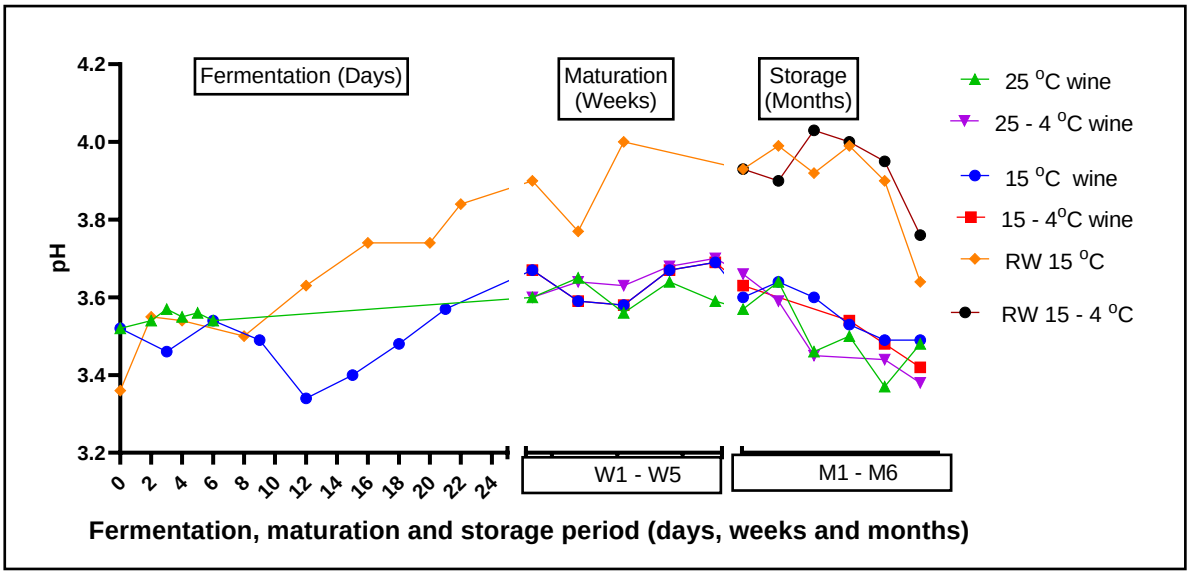

Figure 4.7. The pH values of the wines produced at different temperatures, RW refers to reference wine, W refers to weeks and M refers to months.

The pH for the experimental wines ranged between 3.2 and 3.6, while the reference wine ranged between 3.2 and 4 at the fermentation stage (Fig. 4.7). The pH stabilised during maturation and a decline by 0.1-fold was observed throughout the storage stage for the experimental wines and only in the last three months of storage for the reference wine. However, there was no significant difference ($p > 0.05$) in pH values at maturation and storage conditions between all the experimental wines, while the reference wines' pH remained higher throughout the wine production stages.

4.3.6. Fusel alcohols concentration

The different fusel alcohols were investigated in the wines during different stages of wine production. The production of isoamyl alcohol, isobutanol, 2-phenylethanol and n-butanol in the wines is illustrated in figure 4.8, while the levels of n-propanol, n-pentanol and hexanol are depicted in figure 4.9. Isoamyl alcohol (Fig. 4.8 A) was the most abundant fusel alcohol in all the wines but the concentration was high in wines fermented at 25 °C, while hexanol (Fig. 4.9 D) was the least detected in all the wines.

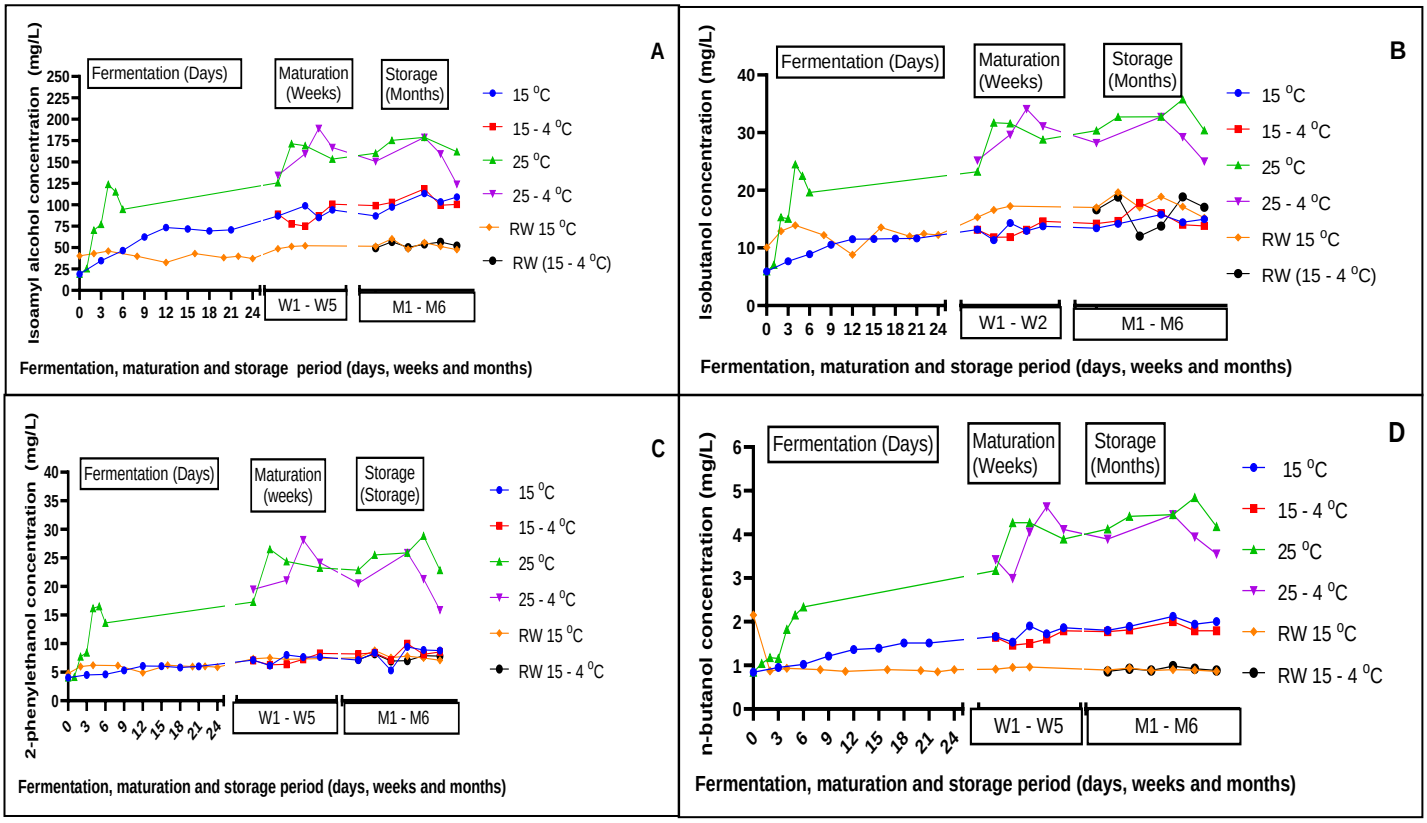

Figure 4.8. Isoamyl alcohol (A), isobutanol (B), 2-phenylethanol (C) and n-butanol high alcohols in the wines produced at different temperatures, RW refers to the reference wine, W refers to weeks and M refers to months.

Isoamyl alcohol, isobutanol, 2-phenylethanol and n-butanol trends were similar in the experimental wines. The concentrations of these alcohols increased throughout fermentation, maturation and storage stages and decreased in the last two months of the storage stage for the wine that was fermented, matured and stored at 25 °C (25 °C wine), while they remained constant throughout all production stages for the reference wines (Fig. 4.8 A – D). On the contrary, the n-butanol concentration of the reference wine decreased from 2.15 to 0.87 mg/L during the fermentation stage and then remained constant throughout (Fig. 4.8 D).

A significant difference ($p = 0.0003$ and $p < 0.0001$) in the concentrations of isoamyl alcohol, isobutanol and 2-phenylethanol was observed between the wine that was fermented, matured and stored at 15 °C (15 °C wine) and the wine that was fermented, matured and stored at 25 °C (25 °C wine) during maturation and storage respectively. Only storage temperature had a significant influence ($p = 0,016$) in n-propanol

concentration between the wine that was fermented, matured and stored at 15 °C (15 °C wine) and the wine that was fermented, matured and stored at 25 °C (25 °C wine).

Isoamyl alcohol was the highest detected fusel alcohol in all the wines and was detected in the wines that were fermented at 25 °C (25 °C and 25 – 4 °C) in the range from 20 to 210 mg/L as compared to 20 to 120 mg/L and 25 to 50 mg/L ranges detected in 15 °C produced wines (15 °C and 15 – 4 °C wines) and the reference wines (RW 15 °C and RW 15 – 4 °C wines) respectively (Fig. 4.8 A). The concentration of isobutanol ranged from 4 to 40 mg/L and 4 to 10 mg/L for the wines which were fermented at 25 °C (25 °C and 25 – 4 °C wines) and 15 °C (15 °C and 15 – 4 °C wines) respectively (Fig. 4.8 B). 2-Phenylethanol ranged in concentration from 3 to 10 mg/L for the wines that were fermented at 15 °C (15 °C and 15 – 4 °C wines) and the reference wines (RW 15 °C and RW 15 – 4 °C wines), whereas the concentration in the wines that were fermented at 25 °C (25 °C and 25 – 4 °C wines) ranged from 3 to 30 mg/L (Fig. 4.8 C). The least detected of the four fusel alcohols was n-butanol, which ranged in concentration from 0.5 to 5 mg/L in the wines which were fermented at 25 °C (25 °C and 25 – 4 °C wines), 0.1 to 2 mg/L in the wines which were fermented at 15 °C (15 °C and 15 – 4 °C wines), and the concentration in the reference wines (RW 15 °C and RW 15 – 4 °C) ranged from 0.1 to 1 mg/L (Fig. 4.8 D).

The concentration of n-propanol (Fig. 4.9 A) increased throughout fermentation, maturation and storage stages and decreased in the last two months of the storage stage, while the occurrence and levels of n-pentanol and hexanol varied throughout, with no clear observable trend (Fig. 4.9 B and C). Notably, the concentration of fusel alcohols in the reference wines dropped at the start of the fermentation process (Fig. 4.9 A – C).

There was no apparent association between the concentration of n–propanol and the different temperatures used in wine production. There was a significant difference ($p < 0.05$) in the concentration of n-pentanol observed between the wine that was fermented, matured and stored at 15 °C (15 °C wine) and the wine that was fermented, matured and stored at 25 °C (25 °C wine) during maturation stage. A similar observation was made between the wine that was fermented at 25 °C, matured and

stored at 4 °C (25 – 4 °C wine) and the wine that was fermented at 15 °C, matured and stored 4 °C (15 – 4 °C wine). There was a definite significance difference between the wines produced at 25 °C (25 °C wine) and 15 °C (15 °C wine) ($p < 0.0001$).

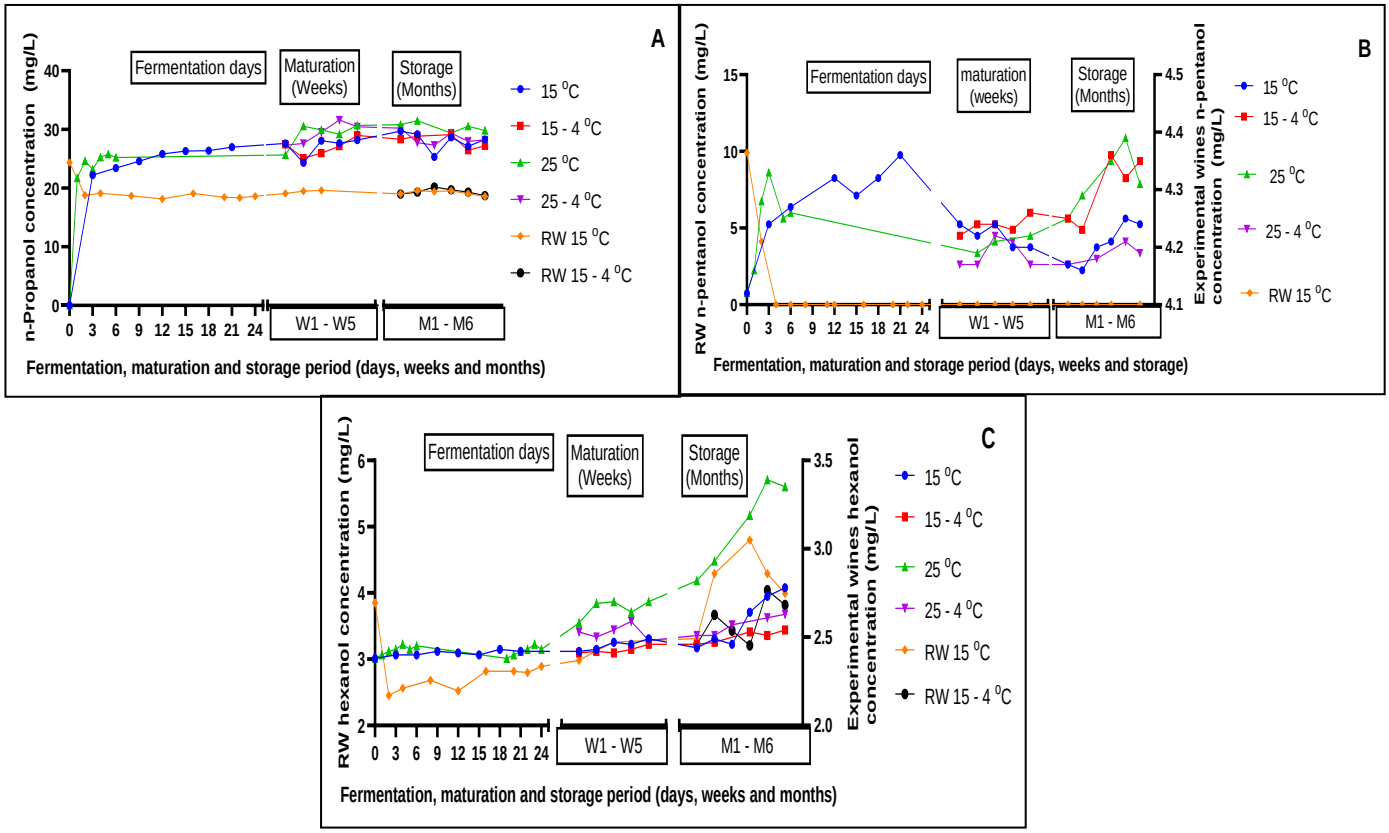

Figure 4.9. n-Propanol (A), n-pentanol (B) and hexanol (C) in the wines that were produced at different temperatures, RW refers to reference wine, W refers to weeks and M refers to months.

The concentration of n-propanol ranged from 20 to 30 mg/L for the experimental wines, and 15 to 20 mg/L in the reference wines (Fig. 4.9 A). The concentration of n-pentanol in the experimental wine ranged from 3.5 to 5 mg/L, while it decreased from 9.9 mg/L and completely depleted within four days of fermentation in the reference wine (Fig. 4.9 B) and the highest n-pentanol concentration of 4.4 mg/L was observed in the 15 °C wine during the fermentation period. Hexanol was the least detected higher alcohol in all the wines and its concentration ranged from 0.5 to 5 mg/L (Fig. 4.9 C).

4.3.7. Concentration of esters

The different ester and acetate compounds were investigated in marula fruit wines that were produced at different temperatures (Fig. 4.10). Ethyl acetate (Fig. 4.10 A) was the most abundant ester in all the wines.

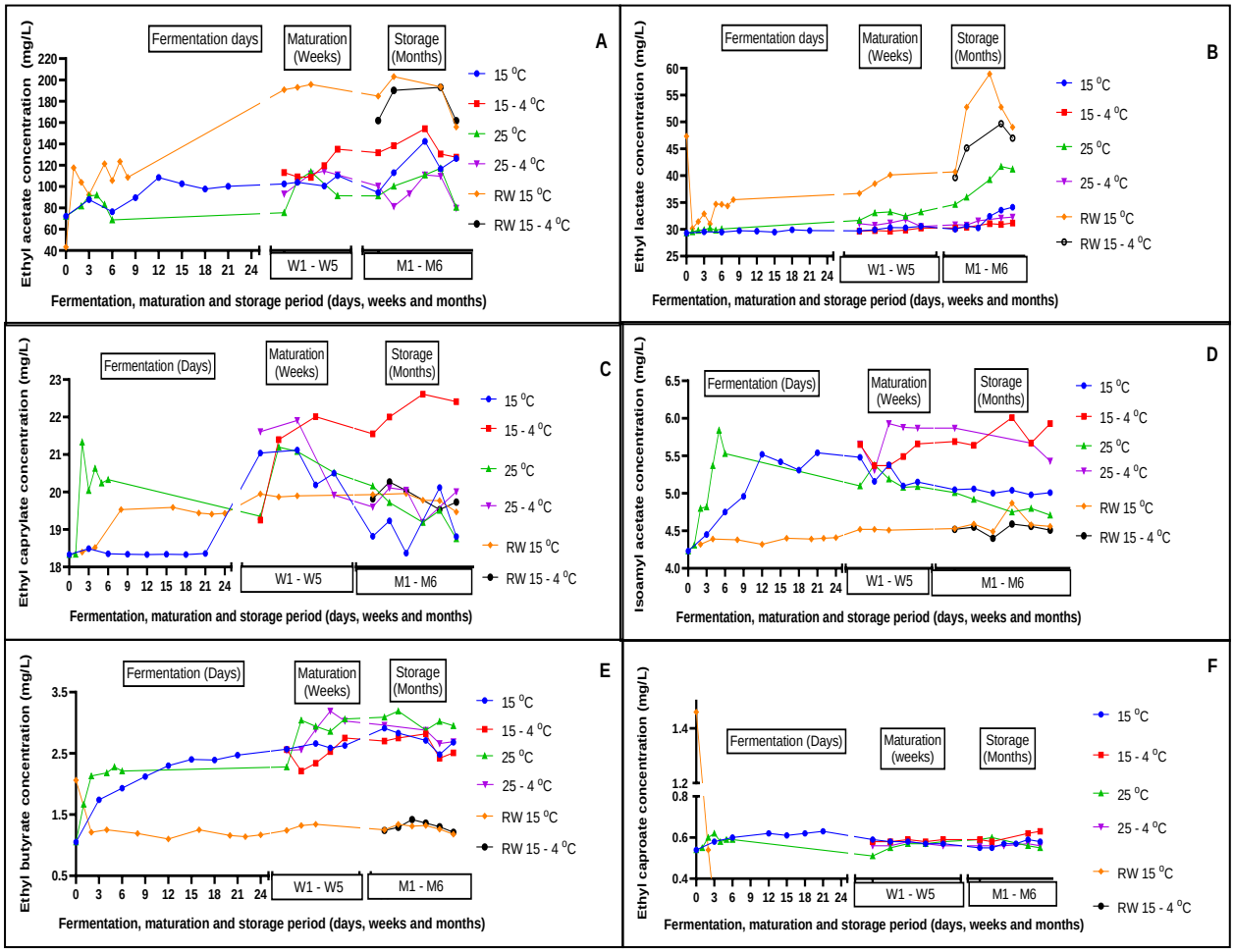

Figure 4.10. Ethyl acetate (A), ethyl lactate (B), ethyl caprylate (C), isoamyl acetate (D), ethyl butyrate (E) and ethyl caproate (F) esters in wines produced at different temperatures, RW refers to (reference wine), W refers to weeks and M refers to months.

Ethyl acetate was the most dominant ester amongst the selected esters analysed at a concentration range from 90 to 205 mg/L for the reference wines (RW 15 °C and RW 15 – 4 °C) (Fig. 4.10 A), and 60 to 145 mg/L in the experimental wines with the highest concentration detected in the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine) (Fig. 4.10 A). The spicy ester, ethyl lactate was the second-

highest ester in concentration. The highest concentration was detected in the reference wine, in the range of 30 to 60 mg/L, followed by the wine that was fermented, matured and stored at 25 °C (25 °C wine) at a concentration range of 30 to 45 mg/L. The wines that were fermented at lower temperature and the wines that were stored at 4 °C (15 °C wine, 15 – 4 °C wine and 25 – 4 °C wine) had a concentration range of 30 to 35 mg/L (Fig. 4.10. B). The concentrations of fruity esters such as ethyl caprylate and isoamyl acetate were detected throughout the production phases at a concentration range of 18 to 23 mg/L and 4 to 6.5 mg/L respectively (Fig. 4.10 D and C). Ethyl butyrate was detected at a lower concentration range of 1 to 3.5 mg/L for the experimental wines, whereas it ranged from 1.1 to 2 mg/L in the reference wines (Fig. 4.10 E). The least concentration of all the esters was ethyl caproate which ranged from 0.5 to 0.7 mg/L in the experimental wines, while it decreased from 1.46 mg/L and completely depleted on the fourth day of fermentation in reference wine (Fig. 4.10 F).

The concentration of ethyl acetate detected in the wines that were fermented at 15 °C (15 °C and 15 – 4 °C wines) was 1.21 folder higher than the wines that were fermented at 25 °C (25 °C and 25 – 4 °C wines). There was significant difference ($p < 0.001$) in the concentration of ethyl acetate observed between the wine that was fermented at 25 °C, matured and stored at 4 °C (25 – 4 °C wine) and the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine) during the maturation and storage stage. The concentration of ethyl lactate detected in the wine that was fermented, matured and stored at 25 °C (25 °C wine) was 1.2-fold higher ($p < 0.001$) than in the wine that was fermented, matured and stored at 15 °C (15 °C wine), and the wine that was fermented at 25 °C, matured and stored at 4 °C (15 – 4 °C wine) during the maturation and storage stage.

A higher concentration of ethyl caprylate and isoamyl acetate were detected in the wines that were stored at 4 °C ($p < 0.0001$) as compared to the wines that were stored at 15 °C and 25 °C. The concentration of ethyl butyrate observed in the wine that was fermented, matured and stored at 25 °C (25 °C) was higher ($p < 0.01$) than that observed in the wine that was fermented, matured and stored at 15 °C (15 °C wine) and the wine that was fermented at 25 °C, matured and stored at 4 °C (25 – 4 °C wine).There was a significant difference ($p < 0.001$) in ethyl caproate concentration observed between the wine that was fermented, matured and stored at 15 °C (15 °C

wine) and the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine) during maturation stage. Whereas similar results were observed during storage stage between the wine that was fermented at 25 °C, matured and stored at 4 °C (25 – 4 °C wine) and the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine).

4.4. Tannin content

The concentration of tannin compounds was quantified using spectrophotometric analysis, in all the wines during fermentation, maturation and storage stage (Fig. 4.11).

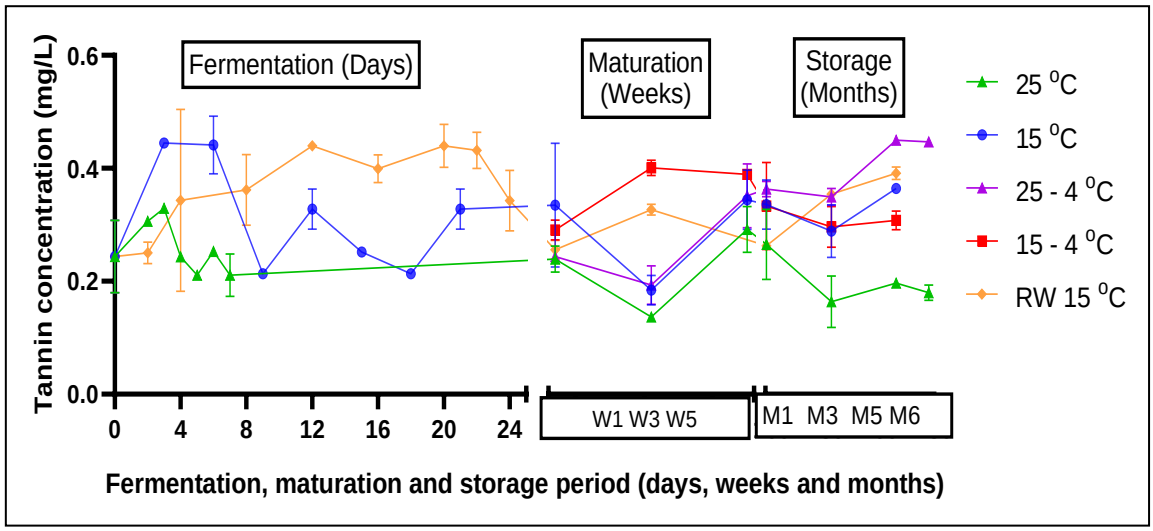

Figure 4.11. Tannin concentration in wines produced at different temperatures, RW refers to reference wine, W refers to weeks and M refers to months.

The concentration of the tannins ranged between 0.1 and 0.5 mg/L for all the wines. The levels in the experimental wines fluctuated throughout the wine production period, while it increased steadily in the reference wines during the fermentation period and decreased on the last three days of fermentation (Fig. 4.11). However, the reference wines had a slightly higher tannin concentration of 0.343 mg/L at the end of fermentation as compared to 0.328 mg/L and 0.252 mg/L detected in wines fermented at 15 °C and 25 °C respectively (Fig. 4.11). The concentrations of the tannin content fluctuated during maturation and storage stage in all the wines, and a significant difference ($p = 0,009$) in tannin concentration between the wine that was fermented, matured and stored at 15 °C (15 °C wine) and the wine that was fermented, matured and stored at 25 °C (25 °C wine) was observed. A similar observation was made between the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C

wine) and the wine that was fermented at 25 °C, matured and stored at 4 °C (25 – 4 °C wine).

4.5. EC50: free radical scavenging activity

The free radical scavenging activity of marula fruit juice and wines was analysed (Table 4.1). The lower EC50 values represent good scavenging activity, and the reverse hold for high EC50 values. All the samples were above 200% EC50 value which is less effective than the 56±13% EC50 value for ascorbic acid.

Table 4.1. Antioxidant activity of wines produced at different temperatures.

Antioxidant activity EC50 (%)							
wine production stage	**Different wines and production conditions**						
	25 °C	**15 °C**	**25 – 4 °C**	**15 – 4 °C**	**RW (15 °C)**	**RW (15 – 4 °C)**	**Positive control**
Ascorbic acid							**56 ± 13%**
Day 0 (juice)	373 ± 26	373 ± 26	373 ± 26	373 ± 26	500 ± 53		
Last day of fermentation	321 ± 93	234 ± 07			327 ± 63		
Maturation week 1	352 ± 13	446 ± 12	379 ± 07	528 ± 01	348 ± 52		
Maturation week 3	486 ± 13	438 ± 14	532 ± 02	667 ± 48	479 ± 08		
Maturation week 5	394 ± 03	408 ± 03	404 ± 18	422 ± 01	526 ± 229		
Storage month 1	463 ± 07	368 ± 20	366 ± 34	460 ± 75	378 ± 11	245 ± 04	
Storage month 3	514 ± 200	387 ± 38	351 ± 13	539 ± 42	426 ± 15	245 ± 01	
Storage months 6	397 ± 22	520 ± 121	430 ± 15	508 ± 52	388 ± 46	262 ± 11	

The lowest concentrations are highlighted for all wines.

The EC50 antioxidant activity, which is the effective concentration at which 50% of DPPH free radicals are scavenged, was analysed for all the wines. The antioxidant activity increased by 1.16-fold, 1.59-fold and 1.52-fold during the fermentation period in the wines that were fermented at 25 °C, 15 °C and the reference wine respectively (Table 4.1). However, the activity decreased and fluctuated in both the experimental wines and the reference wines that were stored at 15 °C during maturation and storage phases, except for the reference wine that was stored at 4 °C (RW 15 – 4 °C) which showed an increase in activity (Table 4.1). There is a general loss in antioxidant activity during the maturation and storage phases (Table. 4.1). Nevertheless, the differences in antioxidant activity

observed during maturation and storage phase between the wines that were fermented at 15 °C and 25 °C was significant (p = 0.004), so was the difference (p = 0,023) between the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine) and wine that was fermented at 25 °C, matured and stored at 4 °C (25 – 4 °C wine).

4.6. Discussion and conclusion

Marula fruit wine is typically produced from fermented marula fruit juice, where yeast converts fermentable sugars into alcohol, carbon dioxide and other important chemical compounds (Phiri *et al*., 2022), which are important to the quality of the wine produced (Maluleke *et al*., 2023). This study evaluated the influence of production temperature on the chemical composition of marula fruit wine that was produced by fermenting sweetened marula fruit juice with native marula fruit yeasts at different fermentation temperatures, maturing and stored at varying temperatures. The intent was to ultimately determine the influence of temperatures on the quality of the marula fruit wine. Quality was primarily inferred from the types of chemical metabolites and profile throughout the production period and consequently the organoleptic characteristics and the storage potential of the wine.

The rate of yeast and bacterial growth and their metabolic activities are related to temperature. Low temperatures generally slow down the metabolic activities and subsequently the growth rate of the yeast and bacteria (Beltran *et al*., 2008; Lambrechts and Pretorius 2000; Torija *et al*., 2003). The reverse also holds true. *Saccharomyces cerevisiae* is generally a glucophillic yeast, whereas the lactic acid bacteria such as *Lactobacillus brevis* and other *Lactobacillus* species identified in the traditional marula wine (Maluleke *et al*., 2023; Phiri *et al*, 2022) are reported to be fructophillic. These microbes are commonly present and dominate the fermentation stage of marula fruit wine and will lead to differential utilisation of the simple fermentable sugars in the fermenting matrix to produce alcohol and other metabolites.

Alcohol and glycerol, the major yeast by-products (Torija *et al*., 2003), were rapidly produced in 25 °C fermented wine. This was three times faster than in the 15 °C fermented wine and a factor of the increased growth rate of the fermenting yeast due to an elevated incubation temperature. The highest total alcohol concentration of 112 g/L (11.20 % ABV) and 116 g/L (11.60 % ABV) achieved from 15 °C and 25 °C wines, respectively, is in concurrence with 11% and 13.80% (ABV) total alcohol achieved from 15 °C and 30 °C wines reported by Fundira *et al*. (2002) during the production of marula fruit wine. Furthermore, the concentration of the total alcohol in the experimental wines was higher than the 7% ABV concentration of total alcohol that

was detected in the reference wines. High concentration of total alcohol can improve wine bitterness or sweetness which contributes to wine taste and mouthfeel (Ramoba *et al*., 2022). The glycerol detected in this study was within the general recommended concentration that ranges from 4 g/L to 10 g/L in dry wines, depending on the type of fruit used and area origin (Gawel *et al*., 2007). Supplementing the natural marula fruit juice with sugar in this study improved the total alcohol and glycerol concentrations in the experimental wines as compared to the unsweetened reference wine. The alcohol content of traditional marula wine is less than 7% ABV (Fundira *et al*., 2002; Maluleke *et al*., 2023; Phiri *et al*., 2022) due to the naturally low sugar content in the marula fruit juice.

Citric acid, malic acid and tartaric acids are reportedly the dominant natural organic acids in wines. These acids contribute to the highest proportion of acidity in the wine (Maluleke *et al*., 2023), with citric acid being the most abundant titratable acid. The high levels of citric acid observed in this study were higher than the recommended concentration of 0.5 to 1 g/L in wines (Chidi *et al*., 2018; Legodi *et al*., 2022). Some microbes, including fermenting microbes, metabolise citric acid as an important mediator in the tricarboxylic acid cycle (Legodi *et al*., 2022) and this could explain the decline in citric acid during the maturation and storage stage. Malic and tartaric acids detected in this study were within the wine recommended concentration ranges of 2 to 6.5 g/L and 5 to 10 g/L, respectively. Although tartaric acid cannot be metabolised during the winemaking process (Loureiro and Malfeito-Ferreira, 2003), it can be lost through physicochemical mechanisms, such as accumulation of ethanol or neutralisation by reacting with cations such as potassium, calcium and sodium, resulting in a drop of wine acidity and a rise in pH levels (Loureiro and Malfeito-Ferreira, 2003). A decrease in tartaric acid and an increase in wine pH during the fermentation stage were thus noted in this study. Wines that were matured and stored at 4° C accumulated more tartaric acid, suggesting that it is more preserved in cold temperatures.

The volatile acetic and lactic acids, which are metabolites of fermenting yeasts and bacteria, were the lowest detected organic acids in this study, with a high presence in the reference wines. The lactic acid and acetic acid bacteria were detected in high

numbers in the reference wines, and these are the high producers of lactic acid and acetic acid in wines. Traditional marula wine has a high presence of LAB and AAB, and this is accompanied by high levels of acetic acid at the latter stages of fermentation (Maluleke *et al*., 2023; Phiri *at al*., 2022), which inadvertently spoils the wine. The amount of organic acids does not specifically inform on the pH of the wine although organic acids do influence the acidity of the wine. Notably, the pH of the marula wines was between 3.2 and 3.6 which was within the general recommended limit of pH 2.9 to pH 4 in wines (Torija *et al*., 2003). Many wine producers prefer a pH between 3.0 and 3.5 to improve wine stability (Bartowsky, 2005).

Higher alcohols have a low effect on wine sensory properties due to the unpleasant taste and aroma when present in high amounts. Higher alcohols, also referred to as fusel alcohols, naturally have a strong pungent smell and taste (Fundira *et al*., 2002). Isobutanol, isoamyl alcohol, 2-phenylethanol, 1-propanol, n-butanol, hexanol and 1-pentanol were identified as the major higher alcohols in this study, congruent with the previous observations in marula fruit and marula fruit wines studies (Fundira *et al*., 2002; Legodi *et al*., 2022; Phiri *et al*., 2022). The wines that were fermented, matured and stored at elevated temperatures comparatively had higher concentrations of the higher alcohols. Molina *et al*. (2008) reported that the high temperature in wine production leads to the high production of higher alcohols because of the high metabolic activity of fermenting microbes. Isoamyl alcohol was the most dominant higher alcohol detected that was produced at the concentration within the recommended wine range of 12 – 311 mg/L, while hexane and n-pentanol were the least detected high alcohols below 4 mg/L. The same observations were reported by Fundira *et al*. (2002) and Samoticha *et al*. (2019), although there are differences in the type of fruits and yeast strains used in wine production between this study and their studies.

Eight different esters such as ethyl acetate, ethyl lactate, ethyl caprylate, isoamyl acetate, ethyl butyrate, ethyl caproate, diethyl succinate and ethyl decanoate were detected in this study. These are common esters found in wines. Diethyl succinate and ethyl caprate were not detected, which could suggest that there were less sufficient succinic and capric acids to start the esterification process with available ethanol. However, both esters were detected at low concentrations in marula fruit wine

in the study by Fundira *et al*. (2002). Ethyl acetate was the most abundant ester in both 15 °C and 25 °C wines in this study, and it is reported to be the most prominent ester in other studies (Fundira *et al*., 2002; Lee and Trinch, 2019). However, it was detected in higher amounts in the reference wine and this could be contributed by acetic acid produced by the acetic acid bacteria which naturally produce high ethyl acetate under low oxygen conditions (Lee and Trinch, 2019). Nevertheless, ethyl acetate was detected within the wine recommended concentrations of 50 -150 mg/L which is below the threshold for spoilage from an unpleasant pungent tang. Lower temperatures preserve most fruity character esters, whereas elevated temperatures are reported to contribute to the loss of banana and strawberry fruity character esters such as isoamyl acetate, ethyl caproate and ethyl caprylate (Scrimgeour *et al*., 2015; Styger *et al*., 2011). These esters were detected in higher amounts in the wines matured and stored at 4 °C (15 – 4 °C and 25 – 4 °C wines). It is worth noting that other spicy character esters such as ethyl butyrate and ethyl lactate were mostly detected in wines that were fermented at 25 °C (25 °C and 25 – 4 °C wines). Ethyl butyrate was detected at levels above the wine-recommended concentration of 0.02 mg/L in all wines (Lambrechts and Pretorius, 2000), while ethyl lactate was detected below the recommended concentration of 150 mg/L (Swiegers *et al*., 2005).

The tannin concentration fluctuated throughout the winemaking process in all the marula fruit wines. Yeast and bacteria reportedly can produce and remove tannins in marula fruit wines (Tebeila, 2022). Heat-stable microbes such as *Bacillus* and *Lactobacillus* species can produce enzymes such as tannin acyl hydrolase (tannase) and phenolic acid decarboxylase which hydrolyse tannins and other phenolic compounds into gallic acid and volatile phenols (Tebeila, 2022), thus resulting in unpredictable changes in tannin concentration in wines. These two bacterial species were detected in the bottle-aging marula fruit wines in this study.

The marula fruit wines displayed low levels of antioxidant activity. Microorganisms can produce antioxidant compounds such as ascorbic and gallic acids (Rodríguez *et al*., 2009) or contribute to their degradation (Leornard *et al*., 2021). *Lactobacillus* species, which were observed in the bottle-aging phase in this study, can contribute to the conversion and depolymerisation of phenolic compounds (Smit *et al*., 2003), thereby reducing the antioxidant scavenging ability of the wine. Interestingly, the same

microbes can release enzymes such as tannase that facilitate the antioxidant compounds extraction in wines (Rodríguez *et al*., 2009). Generally, the antioxidant activity fluctuated throughout the wine production stages in this study, a similar trend was also observed by Tebeila (2022).

It was apparent that temperature influenced the rate of fermentation via the increased or reduced metabolic activities of the microorganisms. This affected the fermentation by-products which in turn influenced the biochemical changes during the wine production process. The maturation and storage temperatures have a direct influence on the production of complex secondary and tertiary compounds. As such, higher alcohols were mostly detected at elevated temperatures while fruity esters were mostly detected at lower temperatures. However, to improve the understanding of how temperature influences the chemical composition of the marula fruit wine, studies on differential expression of genetic elements under varying temperature conditions in native marula fermenting microbiota will be beneficial.

CHAPTER 5

DETERMINATION OF THE NUTRITIONAL PROPERTIES OF MARULA FRUIT WINE

5.1. Introduction

Fruit wines generally refers to fermented alcoholic beverages produced from fruits other than grapes such as marula fruit wine. The wines may inadvertently vary in nutritional composition from grape wines due to the nature of the fruits used. The nutritional content of marula fruit and pulp has been studied (Dlamini and Dube, 2008; Hiwilepo-van Hal *et al*., 2013; Tebeila, 2022) and reported to contain important micronutrients and macronutrients such as ascorbic acid, minerals, carbohydrates, crude fats and protein content (Legodi, *et al*., 2022; Mashau *et al*., 2022). The nutrient profile of marula fruits notably varies according to their place of origin and is influenced by several factors such as climate and harvesting time (Hiwilepo-van Hal *et al*., 2014; Moganedi *et al*., 2011; Tebeila, 2022).

Marula fruit pulp contains vitamin C (ascorbic acid) that ranges from 62 to 400 mg/100 g in content, and this is higher than other tropical fruits such as lemons, grapes, oranges, apples, lemon and papaya amongst many others (Hillman, *et al*., 2008; Hiwilepo-van Hal *et al*., 2012; Legodi *et al*., 2022). Although vitamin C is the most studied compound in marula pulp (Tebeila, 2022), other nutrients such as minerals, lipids (12.32 g/100 mL), proteins (0.31 – 9.93 g/100 mL) and crude fibre (0.70 – 7.03 g/100 mL) were also reported to be present at lower concentrations in marula juice (Hassan *et al*., 2010; Hiwilepo-van Hal *et al*., 2013; Legodi *et al*., 2022). Minerals such as calcium (Ca), magnesium (Mg), potassium (K) and phosphorus (P) are the most abundant minerals in marula juice, whereas sodium (Na), iron (Fe), copper (Cu) and zinc (Zn) are present in smaller quantities (Hiwilepo-van Hal *et al*., 2012; Petje, 2009). Interestingly, marula fruit wine was reported to contain 0.3 – 111 µg/L of proteins, 0.2 – 1.4 g/L of crude fats, 0.6 – 99 mg/L of potassium, 4.3 – 14.7 mg/L of calcium, 2.61 – 17.9 mg/L of phosphorus, 4.15 – 11.2 of sodium, 5.5 – 25.5 of magnesium, 0.15 – 2.7 of zinc and 0.08 – 0.52 of iron (Tebeila, 2022).

Although marula wine is an age-old traditional beverage, it has not been widely and comprehensively studied. There is scanty information about its nutritional composition, although only a few studies reported on its vitamin C and sugar contents (Fundira *et al*., 2002; Hiwilepo-van Hal *et al*., 2012) besides the recent study by Tebeila (2022). It is expected that the nutritional composition of marula fruit juice will be preserved in the wine as has been reported for other wines produced from fruits such as cranberry, sweet cherry, pomegranate and blueberry (Dey *et al*., 2009). Fermentation can influence the nutritional composition of wines while fermenting temperature affects the activities of microorganisms which directly contribute the nutrients such as vitamins and trace elements (Keef *et al*., 2014) or change the level of the nutritional composition of wine by freeing other complex nutrients from the fermentation substrate (Day and Morawicki, 2018; Pranoto *et al*., 2013).

Knowledge of the nutritive quality of marula fruit and consequently the juice, which will infer the retention of these nutrients in the wine will support the importance of the wine as a good health drink that contains essential nutrients. This chapter reports on the nutrient composition of the marula fruit juice and marula fruit wine when the fruit juice was subjected to different fermentation, maturation and storage temperatures.

5.2. Materials and methods

5.2.1. Marula collection

Collection of marula fruit, juice production, selection of starter culture and juice fermentation were performed as outlined in chapter 3, sections 3.2.1 to 3.2.3.

5.2.2. Sample collection

Sample collection was performed as outlined in chapter 4, section 4.2.2.
The different wines were labelled by their production temperatures as follows:
15 – 4 °C refers to a wine that was fermented at 15 °C, matured and stored at 4 °C,
25 – 4 °C refers to a wine that was fermented at 25 °C, matured and stored at 4 °C,
25 °C refers to a wine that was fermented at 25 °C, matured and stored at 25 °C and
15 °C refers to a wine that was fermented at 15 °C, matured and stored at 15 °C.
RW 15 °C refers to a reference wine fermented at 25 °C, matured and stored at 15 °C.

RW 15 – 4 °C refers to a reference wine fermented at 25 °C, matured at 15 °C and stored at 4°

5.2.3. Determination of the mineral composition of marula fruit wine

Mineral analysis was performed at the Limpopo Agro-food Technology Station (LATS) at the University of Limpopo, South Africa. A volume of 5 mL wine samples was mixed with 7.5 mL nitric acid and 1.5 mL hydrogen peroxide in a digestion vessel. The solutions were mixed by carefully shaking the vessels and digesting the samples in a microwave mineral digester for 15 minutes at 203 °C. The digestion vessels were cooled in the laminar flow for 10 minutes at 25 °C. The contents of the vessels were transferred to 50 mL centrifuge tubes and made to a volume of 50 mL with deionised water. Shimadzu Inductively Coupled Plasma Emission Spectroscopy (ICPE-9000) was used to determine the mineral composition (Poitevin, 2012) in the wine samples. Three independent measurements were taken for each sample and expressed in mg/L.

5.2.4. Determination of protein concentration of marula fruit wine

The protein content of wine was determined using the BCA kit (Sigma-Aldrich, South Africa). The wine samples were analysed according to the manufacturer's instructions. The protein standard solution was prepared at concentrations of 0.5 – 30 µg/mL and absorbance were read at 562 nm using Promega Glomax multi detection system. The protein standard curve was used to determine the unknown protein concentrations of the wine samples.

5.2.5. Determination of crude fats concentration in marula fruit wine

The chloroform-methanol solvent extraction method (Folch *et al*., 1957) was used to extract crude fats from the wine samples. A volume of 3 mL wine sample was mixed with 3.7 mL chloroform and 7.5 mL absolute methanol in 50 mL centrifuge tubes. The tubes were incubated in a shaking incubator at 25 °C for 1 hour while shaking at the speed of 100 rpm. After incubation, 3.75 mL of chloroform and absolute methanol were added and left to stand for 5 minutes in the fume cabinet to allow separation of the layers. The top layer was removed, and the remaining bottom layer was mixed with 3.68 mL of chloroform: methanol: water (3: 48: 47 v/v) solution. The mixture was

allowed to stand in the fume cabinet for 2 to 5 minutes to allow formation of layers. The top layer was discarded, and the process was repeated. The bottom layer was transferred into a pre-weighed 10 mL glass vial. The vial was placed under a fan to evaporate the solvent and weighed again after drying. The formula below was used to calculate crude fat concentration.

$$\%\ crude\ fat = \frac{(W2 - W1)x100}{S}$$

W1 is the weight of an empty vial, W2 is the weight of a vial with extracted fat and S is the weight of the sample.

5.2.6. Determination of cholesterol concentration in marula fruit wine

Cholesterol concentration in the wine samples was determined using Liebermann-Burchard (LB) reagent method derived from Kim and Goldberg (1969). The Liebermann-Burchard reagent was prepared by adding 220 mL of cold acetic anhydride and 200 mL of glacial acetic acid (room temperature) in a 500 mL amber glass bottle fitted with a polyseal cap. The two chemicals were mixed by inversion and 30 mL of cold concentrated sulfuric acid was added. The reagent was stored in cold water and used immediately. Cholesterol standard was prepared by dissolving 200 g cholesterol powder into 100 mL glacial acetic acid and stored in an amber glass bottle with a polyseal cap. One millilitre of LB reagent was added to three tubes labelled U, S and B. Precisely, 100 µL of wine sample was added to the tube labelled U, while 100 µL standard solution was added to the tube labelled S and tube B was used as blank with LB reagent only. All the tubes were incubated at 30 °C for 30 minutes and absorbance was read at 625 nm using a spectrophotometer (Genesys 10S, UV-VIS). The concentration of the wine cholesterol was calculated using this formula:

$$Cu\ (wine\ concentration\ = Cs\ (standard\ concentration)X\ Au\frac{wine\ Absorbance}{As(standard\ Absorbance)}$$

5.2.7. Determination of vitamin C concentration in marula fruit wine

Vitamin C content of the wines was determined by using the spectrophotometric method of de Lima and Guilherme (2019). Six millilitres of 5% ice cold trichloroacetic acid (TCA) was added to a test tube, followed by 1 mL of the wine sample. The solution

was mixed thoroughly and centrifuged at 1500 rpm for 5 minutes using a Sorvall RC 6+ centrifuge machine (Thermo Fischer Scientific). About 0.6 mL supernatant was collected into a new tube and 200 µL of 2, 4 dinitrophenylhydrazine-thiorea-copper sulphate (DTC) [20:1:1] was added. The mixture was incubated at 70 °C for 30 minutes. After incubation, 1 mL of 65% sulphuric acid was added and the tubes were kept in the dark for 15 minutes. The blank sample was prepared using the same procedure, without the wine sample. The absorbance was read at 520 nm using a spectrophotometer (Genesys 10S, UV-VIS). Vitamin C standards of different concentrations (5, 10, 15, 20, 25 µg/mL) were used to determine the unknown vitamin C concentrations of the wine samples.

5.2.8. Statistical analysis

The differences in the nutritional content during maturation and storage were analysed using inferential statistics. One-way ANOVA with multiple comparisons using the independent t-test method was used to compare the mean values of the chemical compounds of different wine production temperatures. The nutritional compounds in the wines were compared during fermentation period in day interval, maturation period in weekly interval and storage period in monthly interval. The unpaired experimental design and parametric test was used. The significant difference in chemical content produced in all the wines was determined at different levels, namely $p \leq 0.05$ to $p \leq 0.0001$. This was from sufficient to most conclusive evidence that supported the test hypothesis. Test hypotheses state that the temperature influences the nutritional composition of the marula fruit wine. Mean values with standard deviations were reported and the experiments were performed in duplicates.

5.3. RESULTS

The characteristics of the selected nutritional content of two starter culture-based wines and spontaneous marula fruit wine were analysed. The wines were fermented at 15 °C, 25 °C and ambient temperature respectively, while the wines were matured and stored at 15 °C, 25 °C and 4 °C.

Mineral content

The concentrations of different minerals in marula fruit juice and marula fruit wine at the start and the end of fermentation, maturation and storage stages were determined using spectroscopy (Table 5.1). Generally, the processing of the juice led to some loss of the minerals when reference wines were compared with pasteurised sweetened wines (Table 5.1). It is worth mentioning that non-homogeneity during sampling in all the wines could have contributed to the fluctuation of all the minerals through the production stages (Table 5.1).

Table 5.1. Mineral content from marula fruit wines fermented, matured and stored at different temperatures. The area shaded in orange colour indicate the highest obtained concentration of each mineral.

Production Condition	mineral content					
	Fermentation stage		Maturation stage		Storage stage	
	Start	End	Start	End	Start	End
Potassium (K) mg/L						
15 °C	58,4± 0,0	53,7± 0,6	54,5± 0,1	31,1± 0,3	50,5± 0,1	20,6± 0,9
25 °C	58,4± 0,0	16,2± 0,4	29,3± 0,1	98,8± 0,5	44,9± 0,2	88,1± 0,7
15 – 4 °C			82,8± 0,6	29,0± 0,2	52,9± 0,1	55,6± 0,2
25 – 4 °C			68,2± 0,7	70,7± 0,5	77,3± 0,5	98,8± 0,4

RW 15 °C	63,1± 0,9	48,9± 0,2	55,4± 0,6	50,6± 0,4	77,6± 0,5	106± 0,0
RW (15 – 4 °C)			55,4± 0,6	50,6± 0,4	98,6± 0,2	57,0± 0,5
Sodium (Na) mg/L						
15 °C	12,2± 0,2	10,9± 0,2	4,3± 0,2	13,5± 0,2	11,6± 0,1	3,6± 0,0
25 °C	12,2± 0,2	4,0± 0,2	13,4± 0,2	8,2± 0,2	10,0± 0,3	10,6± 0,2
15 – 4 °C			11,6± 0,1	13,8± 0,1	11,0± 0,1	11,8± 0,1
25 – 4 °C			11,4± 0,0	12,6± 0,1	13,9± 0,1	9,9± 0,2
RW 15 °C	9,0± 0,2	15,1± 0,0	9,4± 0,2	10,2± 0,1	6,0± 0,3	12,6± 0,1
RW (15 – 4 °C)			9,4± 0,2	10,2± 0,1	13,6± 0,2	11,1± 0,5
Phosphorus (P) mg/L						
15 °C	0,8± 0,0	1,7± 0,2	3,0± 0,1	1,0± 0,1	0,4± 0,2	3,6± 0,1
25 °C	0,8± 0,0	2,5± 0,4	0,8± 0,0	4,9± 0,0	0,9± 0,1	3,2± 0,1
15 – 4 °C			2,6± 0,1	1,3± 0,1	0,9± 0,1	1,3± 0,1
25 – 4 °C			2,6± 0,0	3,0± 0,1	3,7± 0,1	3,4± 0,1
RW 15 °C	3,6± 0,1	1,6± 0,1	0,9± 0,3	3,4± 0,1	3,7± 0,1	4,3± 0,1

RW (15 – 4 °C)			0,9± 0,3	4,1± 0,2	4,0± 0,0	0,4± 0,4
Calcium (Ca) mg/L						
15 °C	0,8± 0,0	1,1± 0,0	2,0± 0,0	0,5± 0,0	0,6± 0,0	3,4± 0,0
25 °C	0,8± 0,0	3,3± 0,0	0,4± 0,0	3,3± 0,0	1,3± 0,0	2,1± 0,0
15 – 4 °C			1,6± 0,0	0,5± 0,0	0,7± 0,0	0,9± 0,0
25 – 4 °C			1,7± 0,0	1,4± 0,0	1,5± 0,0	4,6± 0,0
RW 15 °C	2,0± 0,0	1,2± 0,0	0,5± 0,0	2,6± 0,0	1,9± 0,0	2,0± 0,0
Magnesium (Mg) mg/L						
15 °C	1,1± 0,0	1,5± 0,0	2,9± 0,0	0,7± 0,0	1,0± 0,0	4,6± 0,1
25 °C	1,1± 0,0	4,0± 0,1	0,6± 0,0	4,1± 0,0	1,3± 0,0	2,8± 0,0
15 – 4 °C			2,0± 0,0	0,7± 0,0	1,0± 0,0	1,1± 0,0
25 – 4 °C			2,0± 0,0	1,9± 0,0	1,9± 0,0	3,3± 0,0
RW 15 °C	2,7± 0,0	1,1± 0,0	1,8± 0,0	1,6± 0,0	3,5± 0,0	2,6± 0,0
RW (15 – 4 °C)			1,8± 0,0	1,6± 0,0	2,3± 0,0	1,1± 0,0
Iron (Fe) mg/L						

15 °C	0,1± 0,0	0,0± 0,0	0,2± 0,0	0,0± 0,0	0,0± 0,0	0,5± 0,0
25 °C	0,1± 0,0	0,5± 0,0	0,3± 0,0	0,1± 0,0	0,0± 0,0	0,1± 0,0
15 – 4 °C			0,0± 0,0	0,0± 0,0	0,0± 0,0	0,0± 0,0
25 – 4 °C			0,01± 0,0	0,0± 0,0	0,02± 0,01	0,1± 0,0
RW 15 °C	0,2± 0,0		0,0± 0,0	0,0± 0,0	0,2± 0,0	0,02± 0,01
RW (15 – 4 °C)			0,0± 0,0	0,0± 0,0	0,03± 0,03	0,0± 0,0
Zinc (Zn) mg/L						
15 °C	0,002±0	0,05± 0,0	0,3± 0,0	0,02± 0,0	0,0± 0,0	0,2± 0,0
25 °C	0,002±0	0,2± 0,0	0,02± 0,0	0,4± 0,0	0,02± 0,0	0,2± 0,0
15 – 4 °C			0,1± 0,0	0,1± 0,0	0,0± 0,0	0,0± 0,0
25 – 4 °C			0,1± 0,0	0,2± 0,0	0,2± 0,0	0,2± 0,0
RW 15 °C	0,3± 0,0	0,3± 0,0	0,2± 0,0	0,1± 0,0	0,1± 0,0	0,1± 0,0
RW (15 – 4 °C)			0,2± 0,0	0,0± 0,0	0,1± 0,0	0,0± 0,0

Potassium, sodium, phosphorus, calcium, and magnesium were the most abundant minerals in all the wines, with potassium and sodium being present in higher amounts than the other minerals in the range from 16 to 110 mg/L and 4 to 15.5 mg/L, respectively.

Phosphorus, calcium, and magnesium ranged between 0.4 and 5 mg/L (Table 5.1). However, iron and zinc were the lowest detected minerals in the range from 0.01 to 0.5 mg/L and 0.002 to 0.4 mg/L respectively, whereas manganese was not detected in all the wines (Table 5.1). There is no apparent trend on the influence of production temperatures on the wine mineral composition since all minerals fluctuated throughout the production stage. It is noteworthy to mention that the wine samples were not collected from the homogeneous matrix since the fermenting wines were not mixed prior to sampling. In general, there was a recovery and loss of all minerals during the maturation and storage process, either at the start or end of the process. All production temperatures did not have significant influence ($P > 0.05$) in the amount of all minerals detected in different wines.

5.3.2. Protein content

Generally, all the experimental wines contained high concentrations of proteins as compared to the lower amount contained in reference wines (Fig. 5.1).

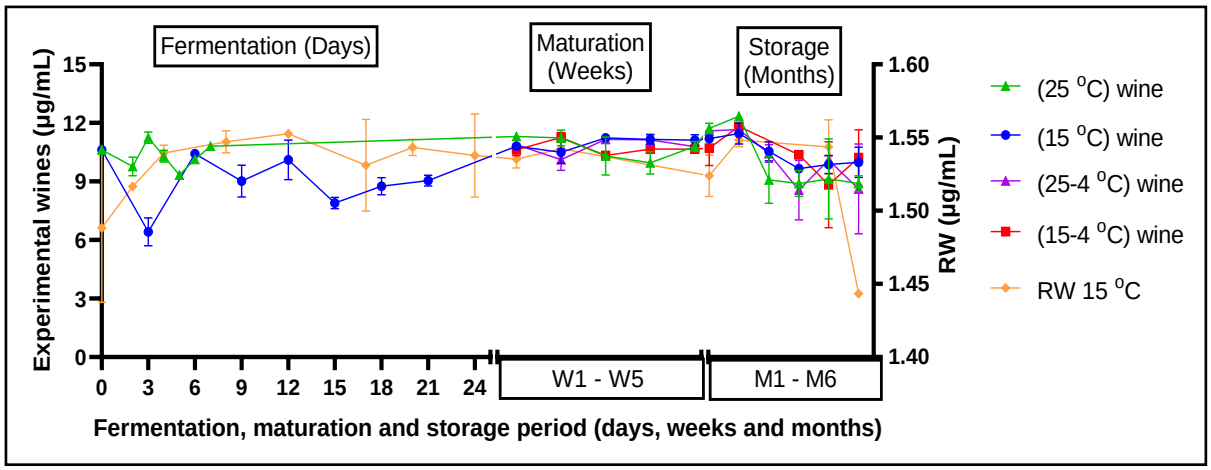

Figure 5.1. Protein concentrations in wines produced at different temperatures, RW refers to reference wine, W refers to weeks and M refers to months.

The experimental wines displayed higher protein concentrations in the range from 6 to 11.2 µg/mL for 15 °C wines, 8.8 to 12.2 µg/mL for 25 °C wines, and the reference wine ranged from 1.40 to 1.60 µg/mL (Fig. 5.1). The wine that was fermented at a lower temperature of 15 °C showed non-significant ($p > 0.05$) low protein content throughout the fermentation period when compared to 25 °C wine, which had a faster fermentation rate (Fig. 5.1). The starting protein content was 10.6 µg/mL for the wine that was fermented at 15 °C and 25 °C (15 °C wine and 25 °C wines), while it was 1.48 µg/mL for the reference wine (Fig. 5.1), considering that the difference between the experimental and reference wines was 9.12 µg/mL. The protein content at the end of fermentation was 10.8 µg/mL for 25 °C wine at day 6 and 9.0 µg/mL for 15 °C wine at day 21 (Fig. 5.1). The protein content remained constant during maturation phase, in the range from 9.9 µg/mL to 11.2 µg/mL for all the experimental wines. A gradual decline was observed in protein content in all the wines during the bottle storage phase to levels of 9.7 µg/mL for wine fermented, matured and stored at 15 °C (15 °C wine), 8.8 µg/mL for wine fermented 15 °C, matured and stored at 4 ° C (15 – 4 °C wine), 8.9 µg/mL for wine fermented, matured and stored at 25 °C (25 °C wine) and 8.6 for wine fermented 25 °C, matured and stored at 4 °C (25 – 4 °C) wine. The protein content in the reference wine was consistently low throughout the different production phases.

There was no significant difference ($p > 0.05$) in protein concentrations for all the wines during the maturation and storage stages.

5.3.3. Wine crude fats

The crude fats concentration for all the wines fluctuated between the ranges of 0 to 1.2 g/L throughout the wine production stages, except for the experimental wine that was fermented, matured and stored at 25 °C which had a crude fat content above 2 g/L during the fermentation stage (Fig. 5.2).

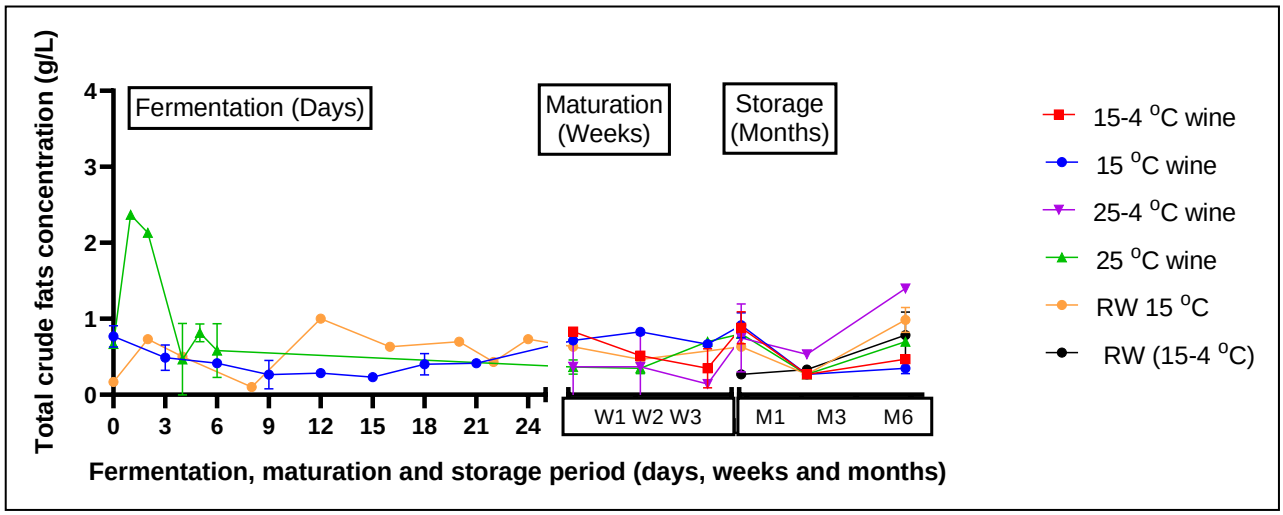

Figure 5.2. Crude fats concentrations in wines produced at different temperatures, RW refers to reference wine, W refers to weeks and M refers to months.

Experimental wines initially contained crude fats at the concentration of 0.77 g/L, whereby the content in 25 °C wine increased by 3-fold to the highest concentration of 2.95 g/L on day 2 (Fig. 5.2). It then reduced further to 0.5 g/L on day 4 and it remained constant in the range of 0 to 1 g/L for 6 months throughout maturation and storage stages. On the contrary, the 15 °C wine gradually decreased by 0.8-fold to the lowest concentration of 0.23 g/L on day 15 (Fig. 5.2), whence it fluctuated throughout the production stage within a range of 0 to 1 g/L (Fig. 5.2). Temperature had no significant effect ($p > 0.05$) on the concentration of crude fats detected between experimental wines during maturation and storage conditions.

5.3.4. Wine cholesterol concentration

Generally, all the experimental wines contained low concentrations of cholesterol as compared to the high amount contained in the reference wines (Fig. 5.3).

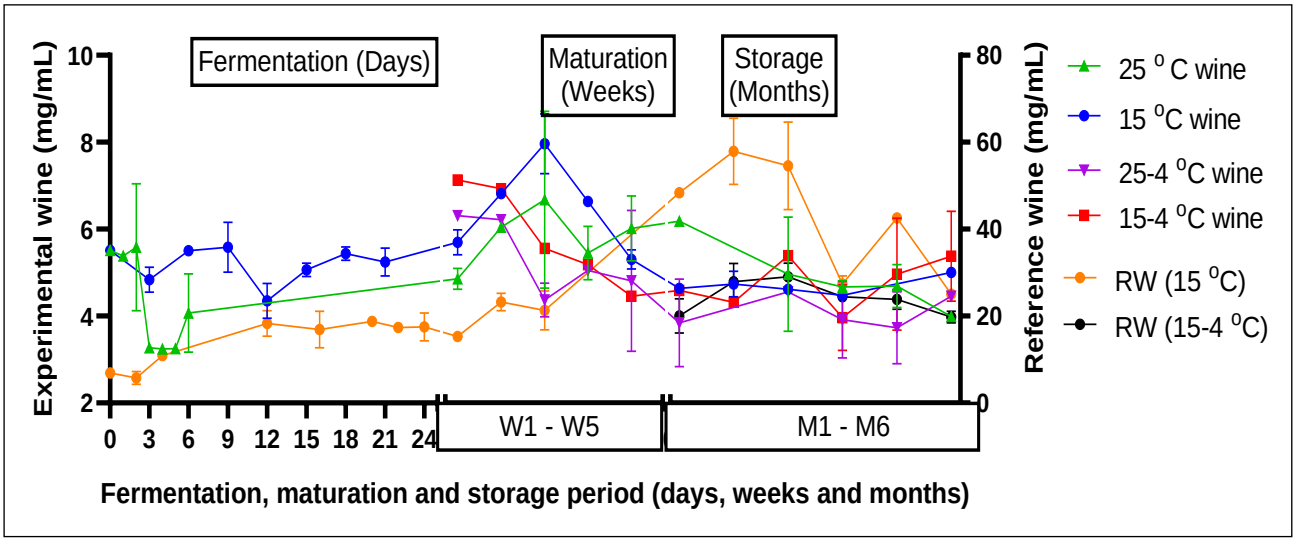

Figure 5.3. Cholesterol concentrations in wines produced at different temperatures, RW refers to reference wine, W refers to weeks and M refers to months.

The reference wines comparatively showed a higher cholesterol content throughout the production stages, with a gradual 7-fold increase from 6.87 mg/mL in the juice to 48.39 mg/L at the beginning of the storage phase (Fig. 5.3), with a further sporadic rise during the storage stage. Comparatively, cholesterol levels in the juice state of the experimental wines was 5.51 mg/mL and decreased steadily to 4.07 mg/mL for the wines that were fermented at 25 °C and 5.24 mg/mL for the 15 °C at the end of fermentation (Fig. 5.3). A non-significant difference ($p > 0.05$) in cholesterol content to a range between 4.46 mg/mL and 6.02 mg/mL was observed amongst the experimental wines at the end of maturation phase. The highest cholesterol concentration of 6.2 mg/mL was detected in the wine that was fermented, matured and stored at 25 °C (25 °C wine), and the lowest cholesterol concentration of 4.46 mg/mL was detected in the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine) (Fig. 5.3). A non-significant drop ($p > 0.05$) in cholesterol content to a range between 3.99 mg/mL and 6.19 mg/mL for wines fermented at 25 °C and 4.48 mg/mL and 5.38 mg/mL for wines fermented at 15 °C was observed for all the experimental wines during bottle storage phase (Fig. 5.3), while a non-significant difference that ranges between 4.45 mg/mL and 4.55 mg/mL was observed for wine that was fermented at 25 °C, matured and stored at 4 °C (15 – 4 °C wine). Generally, the cholesterol concentration in all the wines was not affected by the production temperature at different stages of wine production.

5.3.5. Wine vitamin C concentration

Analysis of the vitamin C concentration of marula wine was performed on both the experimental and reference wines at all the stages of wine production (Fig. 5.4). The concentration of vitamin C was quantified using spectrophotometric analysis.

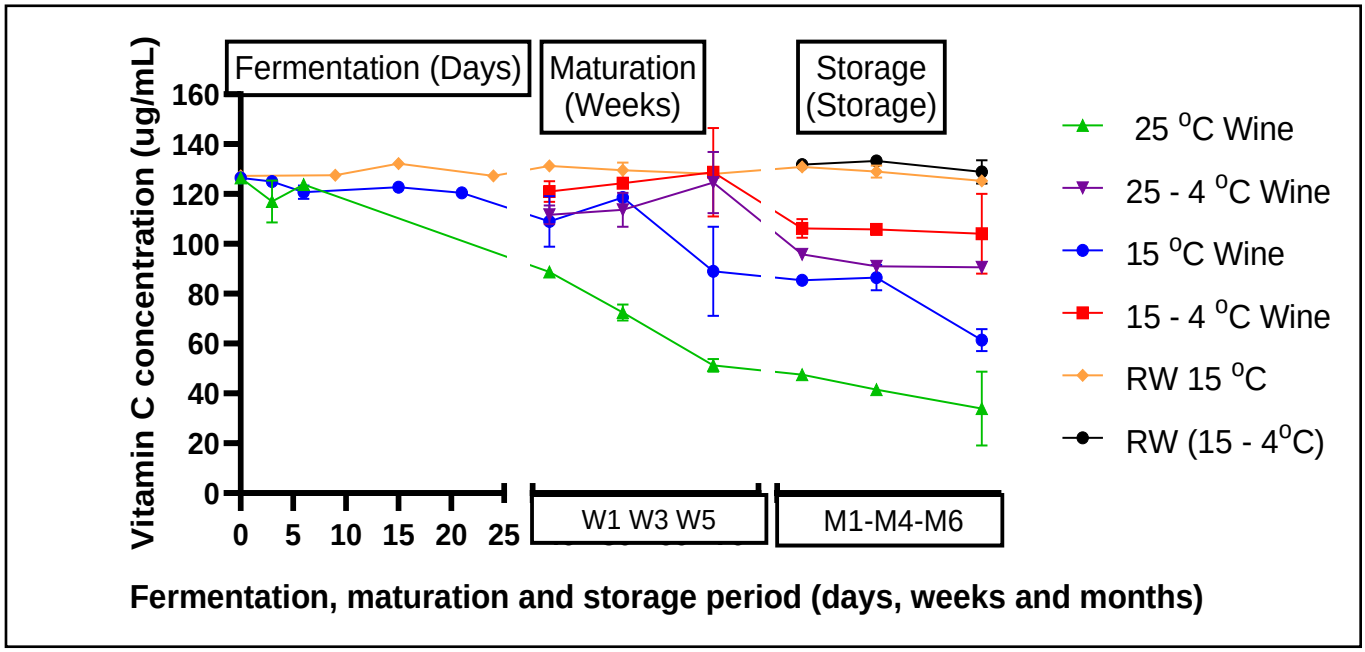

Figure 5.4. Vitamin C concentrations in wines produced at different temperatures, RW refers to (reference wine), W refers to weeks and M refers to months

Vitamin C was notably preserved in the marula fruit wines that were matured and stored at colder temperatures (4 °C). The wines that matured and were stored at higher temperatures (15 °C and 25 °C wines) lost the vitamin C content faster comparatively, more so for the 25 °C wine. A different pattern was observed with the reference wines (RW 15 °C and RW 15 – 4 °C) which were neither pasteurised nor sweetened, their vitamin C content remained constant throughout the different production stages.

5.4. Discussion and conclusion

Wine is an enjoyable drink, and it is reported to have numerous health benefits, including prevention of heart diseases, circulatory diseases and obesity while promoting greater endurance and quality of human life (Wurz, 2019). Marula fruit wine is a beverage that is produced through alcoholic fermentation of ripe marula fruit juice

and is revered by community elders as a potent nutritious drink (Pretorius, 2006). The marula fruit juice has good nutritive value due to the minerals, proteins, carbohydrates, and high amounts of vitamin C it contains (Mashau *et al*., 2022; Tebeila, 2022). The purpose of this study was to evaluate the nutritional composition of marula fruit wines produced at different fermentation, maturation and storage temperatures from a clarified pasteurised marula fruit juice. It is noteworthy to mention that the traditional marula fruit wine is produced from a water-diluted, non-clarified and non-pasteurised marula fruit juice, i.e., a myriad of microorganisms contributes to the spontaneous fermentation of marula fruit juice.

The trace minerals are essential for human health; hence they are required in significant amounts (Marina *et al*., 2013). Minerals such as potassium, sodium, phosphorus, calcium, magnesium, iron and zinc were detected in the marula fruit wine in this study. Wine microorganisms are known to improve the bioavailability of minerals through the production of organic acids or the reduction of the anti-nutritional components (Gharibzahedi and Jafari, 2017; Makokha *et al*., 2002). Interestingly, the microorganisms also use the available or produced minerals during wine production for their growth (Marina *et al*., 2013; Samtiya *et al*., 2021; Tebeila, 2022). Some LAB such as *L. fermentum, L. plantarum, L. casei,* and *L. bulgaricus* are known to enhance the bioavailability of nutrients by reducing the phytic acid content (Marina *et al*., 2013; Samtiya *et al*., 2021). The LAB strains, such as *Lactobacillus nagelii*, *L. buchneri, L. kefiri,* and *L. parabuchneri*, were previously reported in the fermenting marula fruit wine by Maluleke *et al*. (2023) and Phiri *et al*. (2022), whereas *L*. *brevis* and *S. cerevisiae* were detected in the bottled marula fruit wine in this study.

Potassium, sodium, phosphorus, calcium, and magnesium were the most abundant, whereas essential trace elements such as iron and zinc were detected in smaller quantities. A similar observation was reported by Hiwilepo van-Hal *et al*. (2013) and Tebeila (2022) in marula juice and marula fruit wine, respectively. Notably, the concentrations of potassium and sodium detected in this study were 1.5 times higher than the amounts reported by Tebeila (2022). Of importance was that the mineral content of the marula fruit wines was either retained or increased during the maturation and storage for all the wines in different temperatures. Although all the detected minerals were below the WHO recommended dietary allowance per day, their

presence can supplement the much-needed minerals and mitigate the mineral deficiency concerns among the vulnerable populations that consume alcohol without following or having a proper diet, particularly in the rural areas (MacKeown *et al*., 2007). Wine production temperature did not have any influence ($p > 0.05$) on the mineral compositions between the test wines, and there was no noticeable trend in mineral content of the wines.

Marula juice was reported to contain proteins in smaller quantities, in the range from 0.31 – 9.93 g/100 mL (Legodi *et al.,* 2022). The initial protein concentration in marula fruit juice in this study was 10.6 µg/mL (0.0011 g/100 mL) and 1.5 µg/mL (0.0002 g/100 mL) for the experimental wines and reference wine, respectively. Several authors reported that heat treatment of the juice at 60 °C to 75 °C to be the most effective way of pre-treating the juice and retaining high levels of nutrients including the protein content (Li *et al*., 2018; Zhou *et al*., 2015). A 6. 09-fold increase in protein content was observed following pre-treatment of marula fruit juice at 75 °C for 20 minutes.

The protein content in marula fruit wines ranged from 6 to 12.2 µg/mL in both 15 °C and 25 °C wines was lower than 29.8 to 107.1 mg/L and 30.9 mg/mL to 141 mg/mL in grape wine reported by Zoecklein and Gump (2022) and Mesquita *et al*. (2015), respectively. Literature has reported on factors that contribute to protein content and stability in wines. These include yeast and bacteria which can transfer proteins to the wine through autolysis or reduce the protein content by hydrolysing must proteins using exocellular protease enzymes as a response to induced stress (Bowyer, 2008; Tafolo, 2021). Polysaccharide or polymeric tannins can interact with proteins to form protein–tannin complexes which stabilise the soluble protein in the wine (Pocock *et al*., 2008), while the decrease in wines protein content during bottling stage can result from the activity of microorganisms such as lactic acid bacteria (LAB) which express peptidolytic and proteolytic activity (Tafolo, 2021).

Although there were noticeable moderate changes in protein content for all the experimental wines throughout the wine production phases, different production temperatures did not have a significant ($p > 0.05$) effect on protein content. Once more, the low protein concentrations detected in marula fruit wines was mainly contributed

by the fruit juice rather than other contributing factors such as temperature considering the WHO-recommended dietary protein allowance ranging from 34 to 56 g/day.

The fat content detected in all wines was lower than the WHO vitamin C recommended dietary allowance range from 35 – 78 g/day for adults of 19 or older (World Health Organisation, 2010). Fats are part of lipids composition mainly from fruit tissues and yeast cell walls, which importantly influence the sensory properties of wines and can also serve as major yeast nutrients (Phan *et al*., 2022; Tesnière, 2019). The highest crude fats concentration in marula fruit wine of 2.95 g/L was detected in the 25 °C wine during fermentation, followed by 15 °C wine at 0.91 g/L, while that of the reference wines was at 0.73 g/L. Tebeila (2022) reported a range of 0.2 – 1.4 g/L crude fats in different marula fruit juices. The crude fat content in grape wines was comparatively similar at 0.083% (0.83 g/L) lipids concentration in a grape wine that was fermented at the higher temperature of 26 °C and 0.075% (0.75 g/L) at the lowest fermentation temperature (16 °C) (Phan *et al*., 2022). Higher crude fats concentration in wines is commonly a characteristic of high fermentation temperature wherein the fruit tissues and the yeast cell wall breakdown due to rapid fermentation (Sacchi *et al*., 2005). Furthermore, yeast cell autolysis can contribute to the increase in total lipid content, total fatty acids, triacylglycerols and phospholipid content which consequently lead to an increase in crude fats (Phan *et al*., 2022; Tebeila, 2022).

The total cholesterol detected in food products and beverages is referred to as dietary cholesterol (Berger *et al*., 2015). The amount of cholesterol detected in this study ranged between 4 – 8 mg/mL (4×10^6 – 8×10^6 ng/mL) in experimental wines, which is higher than the concentration limit of 300 ng/per day cholesterol as stipulated by World Health Organisation (2003). The highest cholesterol concentration was observed in wines that were produced at 15 °C as compared to those at 25 °C, while cholesterol in the reference wine ranged between 0 – 80 mg/mL (0 – 8×10^7 ng/mL). This was comparatively higher than desired. A factor such as excessive oxygen consumption by yeast cells is known to contribute to high levels of cholesterol in wines (Fornairon-Bonnefond *et al*., 2002) and this could have occurred during racking. Nevertheless, there was no significant difference in cholesterol concentration detected in the wines produced at different temperatures. Generally, there is insufficient literature that reports on the influence of wine production temperature on cholesterol

content. Fats and carbohydrates have a high influence on blood cholesterol level as compared to the amount of cholesterol consumed from food, however the cholesterol concentrations detected in wines can be a concern for most people who consume wines.

Vitamin C is a bioactive compound associated with antioxidant activity and it contributes to human nutrition because of the free radical scavenging activity it possesses (Hiwilepo van-Hal *et al*., 2013; Tebeila, 2022). In this study, the initial vitamin C concentration in marula fruit juice was 126 µg/mL (12.6 mg/100mL), which was 14-fold lower than 172 ± 8 mg/100 mL reported by Hiwilepo van-Hal *et al*. (2013). Generally, heat treatment of the juice and extraction methods used lead to loss in vitamin C (Dlamini and Dube, 2008; Hiwilepo van-Hal *et al*., 2013). The vitamin C concentration detected in marula fruit wine was within the range of other fruit wines such as grapes and rose fruit (Cendrowski *et al*., 2021). The wine that is produced at an elevated temperature loses vitamin C faster as compared to a lower temperature, i.e., by inference, Hiwilepo van-Hal *et al*. (2013) reported that marula wine produced at a lower temperature retained more ascorbic acid (vitamin C) comparatively. However, high vitamin C was retained in the reference wine that was produced at 25 °C. Based on these contrasting outcomes, it is thus difficult to draw a conclusion on the influence of wine production temperature on vitamin C content. Factors such as low pH, phenolic compounds and oxygen concentration level can contribute to the decrease in vitamin C levels (Cendrowski *et al*., 2021; Dlamini and Dube, 2008; Varo *et al*., 2022). The vitamin C content detected in all wines was lower than the WHO vitamin C recommended dietary allowance range of 45 – 75 mg/day for adults of 19 or older (World Health Organisation, 2010).

Marula fruit wine contains a wider range of healthy nutrients. Variations in winemaking fermentation, maturation and storage procedures resulted in clear differences in nutritional composition in the marula fruit wines. Marula fruit wines contain fewer mineral content compared to grape wines and other fruit wines (Table 5.2), although a comprehensive study is required. The knowledge generated in this chapter will contribute to the area of wine nutrition, as well as highlighting the importance of wine production conditions

Table 5.2. Comparative analysis of the nutritional information of marula fruit wines alongside other fruit wines in literature and RDA of essential nutrients.

Wines	**Production Temperature**	**K (mg/L)**	**Na (mg/L)**	**P (mg/L)**	**Ca (mg/L)**	**Mg (mg/L)**	**Fe (mg/L)**	**Zn (mg/L)**
Marula fruit wine	15 °C	20.6 – 38.4	4.3 – 13.5	0.4 – 3.6	0.5 – 3.4	0.7 – 4.6	0.0 – 0.5	0.0 – 0.3
	25 °C	29.3 – 58.4	4.0 – 13.4	0.8 – 4.9	0.4 – 3.3	0.6 – 4.1	0.0 – 0.5	0.0 – 0.4
	15 – 4 °C	29.1 – 82.8	11.0 – 13.8	0.9 – 2.6	0.5 – 1.6	0.7 – 2.0	0.0	0.0 – 0.1
	25 – 4 °C	70.7 – 98.8	9.9 – 13.9	2.6 – 3.7	1.4 – 4.6	1.9 – 3.3	0.0 – 0.1	0.1 – 0.2
	RW 15 °C	48.9 – 106.0	6.0 – 15.1	0.9 – 4.3	0.5 – 2.6	1.1 – 3.5	0.0 – 0.2	0.1 – 0.3
	RW 15 – 4 °C	50.6 – 98.0	9.4 – 13.6	0.4 – 4.1		1.1 – 2.3	0.0 – 0.03	0.0 – 0.2
Previous studies on blackberry, cherry, apple and grape wines (references a – h)								
Blackberry wine	18 – 20 °C	564 – 2014	12 – 213	32 – 119	86 – 457	706 – 381	0.08 – 8.4	0.25 – 6.65
Cherry wine	N/F	834 – 1373	380 – 1650	54 – 179	69 - 84	50 - 72	1.2 – 2.1	0.1 – 0.3
Apple wine	N/F	233 – 955	1.68 – 38	68	69 – 84	13 - 38	0.08 – 0.5	0.04 – 0.2
Grape wine	N/F	265 – 3056	310	0.3 – 47.3	7 – 124	7 - 718	0.06– 23.7	8.9
	RDA(WHO)	4700 mg	2300 – 2400 mg	4700 mg	1000 – 1200 mg	300 – 350 mg	6 – 8.1 mg	8 – 11 mg
Wines	**Production Temperature**	**Protein (µg/mL)**	**Crude fats (g/L)**	**Cholesterol (mg/mL)**	**Vitamin C (µg/mL)**			
Marula fruit wine	15 °C	6.0 – 11.2	0.2 – 0.9	4.3 – 7.9	61.5 – 126.5			
	25 °C	8.8 – 12.2	0.4 -2.9	4.0 – 6.7	33.9 – 126.5			
	15 – 4 °C	8.8 – 11.8	0.3 – 0.8	4.0 – 7.1	104.3 – 128.7			
	25 – 4 °C	9.6 – 11.6	0.4 – 1.4	3.7 – 6.3	90.5 – 124.6			
	RW 15 °C	1.4 – 1.6	0.1 – 1.0	5.8 – 57.9	125.2 – 132.2			
	RW 15 – 4 °C		0.2 – 0.8	5.8 – 29.1	126.1 – 133.3			
Previous studies on melon, rose fruit, and grape wines (references a – h)								
Melon wine		2100	2.7	Not determined	±13 000			
Rose fruit		0.1 - 110	0	Not determined	123 – 1550			
Grape wine		48.5 – 3600	1.32 – 18.02	Not determined	12 - 169			
	RDA(WHO)	3400 – 5600 µg	78 g	0.0003 mg	4500 - 7000 mg			

[a]Velić *et al*. (2018); [b]Pantelić *et al*. (2014); [c]Pohl (2009); [d]Amidžić Klarić *et al*. (2016); [e]Alpeza *et al*. (2014); [f]Mendes-Ferreira *et al*, (2004); [g]Cendrowski *et al*. (2021)

CHAPTER 6.

SENSORY ANALYSIS OF MARULA FRUIT WINE PRODUCED AT DIFFERENT TEMPERATURES

6.1. Introduction

The chemical composition of wine contributes significantly to its organoleptic properties which consequently inform the sensory quality of the wine (Swiegers *et al*., 2005). Different wine characters emanate from the reactions between the primary chemical compounds extracted from the fruits, and secondary and tertiary chemical compounds that are produced in pre-fermentation and post-fermentation processes (Styger *et al*., 2011; Swiegers *et al*., 2005). Flavour compounds such as volatile organic acids, aldehydes, alcohols and esters are responsible for the quality and acceptability of the wine and are assessed through sensory analysis by tasting panellists (Leo and Nollet, 2004). The sensory evaluation is thus important to indicate how the final product can be perceived by consumers. The tasting panel is commonly composed of 12 or more trained or semi-trained individuals of different ages and gender (Kocher and Nikhanj, 2019), which is sufficiently larger to neutralise or buffer individual idiosyncrasies because individual tasters truthfully perceive taste and smell of wine differently (Jackson, 2000; Stone and Sidel, 2004; Zoecklein and Gump, 2022). Most researchers use descriptive sensory analysis to determine if their research experiment data from wine production have made any significant difference in the final product (Lawless and Heymann, 2010; Murray *et al*., 2001), hence descriptive sensory analysis is the most used sensory testing method to profile the products on all the perceived sensory characteristics to characterise wine flavours, appearance, texture, aromas and overall impression of the product (Lawless and Heymann, 2010).

Sensory analysis should give guidance on how testing rooms should be arranged and how samples should be served to the panellists, hence proper arrangements assist to avoid situations where people show prejudice for or against other samples (Pecore *et al*., 2006). Panellists are normally placed individually to allow them to give their judgement on the samples (Hodgoson *et al*., 2008), while sitting in groups can influence the tester to reflect the opinions of people around them rather than their view

(Hodgoson *et al*., 2008; Lawless and Heymann, 2010). Coded labels can also assist to ensure that panellists judge their samples based on their own sensory experiences.

Generally, trained panellists receive training on the full background of the beverage products being tested and the method of evaluation (Stone and Sidel, 2004), hence they can identify small differences in the group of tasted products as compared to semi-trained panellists who are usually used to complete hedonic tests where they are asked to indicate their preferences (Lawless and Heymann, 2010). It is unlikely that the semi-trained panellists can differentiate the mature differences between the products. Consequently, the use of a trained panellist over a semi-trained panellist can guarantee sensory acuity, reliability and cost efficiency (Moskowitz, 1996)

A five (5) point sensory analyst hedonic scale used in this study is one of the tested methods for capturing liking data (Stone and Sidel, 2004). A mean liking score of 5 usually is an indicator of highly acceptable sensory quality, any score achieved could be used confidently as a good and bad illustration of target quality. This study investigated the effect of fermentation, maturation and storage temperatures on sensory attributes of marula fruit wines.

6.2. Materials and methods

6.2.1. Collection of marula

Collection of marula fruit, juice production, selection of starter culture and juice fermentation were performed as outlined in chapter 3, sections 3.2.1 to 3.2.3.

6.2.2. Sample collection

Sample collection was performed as outlined in chapter 4, section 4.2.2.

6.2.3. Sensory analysis of wine

Wine samples from marula fruit wine bottles stored at 4 °C and 15 °C and 25 °C were taken within a six-month study period and analysed using the 5-point hedonic scale (Piggot, 1994) for sensory characteristics (taste, aroma, colour, mouth feel and overall impression). The wine colour was evaluated using Standard Reference Method (SRM) colour chart (Appendix C) (http://www.highwoodsbrewing.com/srm-color.php).

Sensory evaluation was performed at the University of Limpopo, Department of Biochemistry, Microbiology and Biotechnology using a semi-trained panel of 11 adult volunteers of different ages (18 years and above) and gender (7 females and 4 males). The panel was composed of alcohol and non-alcohol consumers.

The panellist underwent in-house training on the guidelines and requirements of the 5-point hedonic scale system and how to score the wines depending on liking. The panellists were trained on the technique to follow when handling the samples and palate-cleansing in between sample tasting. Fresh sliced carrots and cucumber and potable still water were provided for palate cleansing. Clear and transparent glasses were used to collect at least 10 mL of the wine samples. Each wine was given a colour code and distributed to each of the panellist members. All panellists were not given information about the wines, hence blind tasting was conducted. The panellists assessed each wine sample on a scale of 1 to 5 for attributes such as taste, smell, colour, mouth feel and overall impression (Appendix D). The study received ethical clearance for wine tasting (Appendix A).

6.2.4. Statistical analysis

The sensory descriptors' hedonic scores and SRM scores during storage were analysed using inferential statistics, and one-way ANOVA with multiple comparisons using an independent t-test method was used to compare the mean values of the sensory descriptors of different wine production temperatures. The sensory properties of the wines were compared using bottled wines during storage conditions on monthly intervals for six months. The unpaired experimental design and the parametric test were used. The significant difference in sensory descriptors in all the wines was determined at different levels, namely $p \leq 0.05$ to $p \leq 0.0001$. This was from sufficient to most conclusive evidence that supported the test hypotheses. Test hypotheses states that the temperature influences the chemical composition of the marula fruit wine. Mean values with standard deviations were reported and the experiment was performed in duplicates.

6.3. Results

The sensory analysis of the two-starter culture-based wines and spontaneous marula fruit wine was performed by a tasting panel on the basis of taste, aroma, colour, mouth feel and overall impression. The wines were fermented at 15 °C, 25 °C temperature, and were matured and stored at 15 °C, 25 °C and 4 °C. The hedonic score 1 meant extremely disliked and score 5 meant extremely liked, whereas (SRM) scale 1 means lighter colour (pale yellow) and 40 means extremely darker colour.

The different wines were labelled by their production temperatures as follows:

- 15 – 4 °C refers to a wine that was fermented at 15 °C, matured and stored at 4 °C,
- 25 – 4 °C refers to a wine that was fermented at 25 °C, matured and stored at 4 °C,
- 25 °C refers to a wine that was fermented at 25 °C, matured and stored at 25 °C,
- 15 °C refers to a wine that was fermented at 15 °C, matured and stored at 15 °C,
- RW 15 °C refers to a reference wine that was fermented at 25 °C, matured and stored at 15 °C and

  RW 15 – 4 °C refers to a reference wine that was fermented at 25 °C, matured at 15 °C and stored at 4° C.

6.3.1. Sensory descriptors

Figures 6.1 – 6.3 represent the sensory descriptive average score which includes taste, smell, texture, overall impression and colour as reported by all the panellists who have evaluated the different wines on monthly intervals during the storage phase.

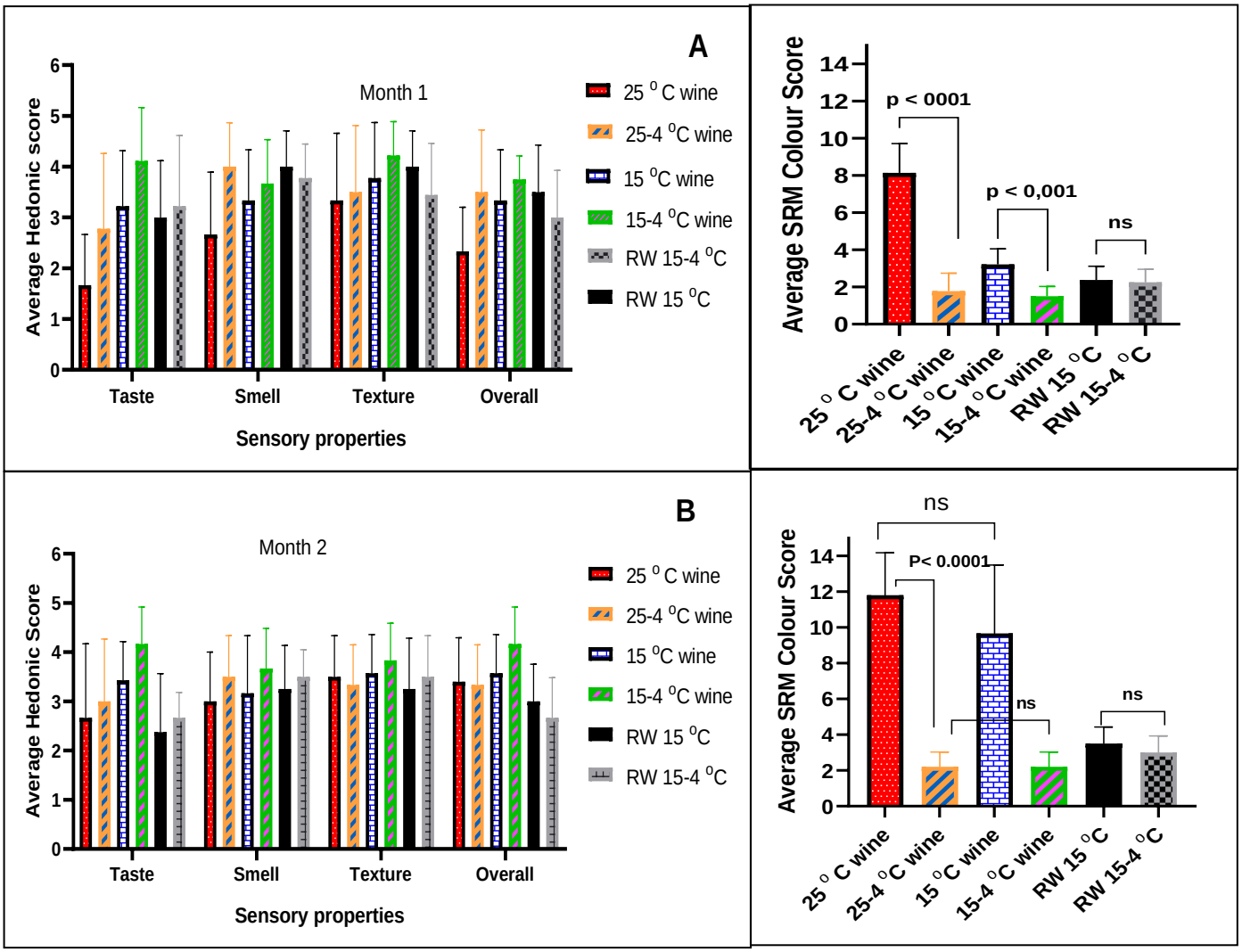

Figure 6.1. Sensory properties of marula fruit wines in month 1 (A) and month 2 (B) of storage at different temperatures, RW refers to reference wine, ns refers to no significant difference and p refers to significant difference.

Texture and smell both received above-average scores for all the wines for the first two months of bottle storage. The wines that were fermented, matured and stored at 25 °C (25 °C wine) and the one stored at 4 °C (25 – 4 °C wine) were liked the least in terms of taste, texture and overall impression, although the colour of 25 °C wine, which was amber to brown, was the most appreciated. A significant difference ($p < 0.0001$) was observed in the taste of the wine that was fermented, matured and stored at 15 °C (15 °C wine) and the wine that was fermented, matured and stored at 25 °C (25 °C wine), and similarly ($p = 0.0002$) between the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine) and the wine that was fermented at 25 °C, matured and stored at 4 °C (25 – 4 °C). On the first month of sensory evaluation,

the wine that was fermented, matured and stored at 25 °C (25 °C wine) had an amber to brown colour at an average of 8.8 SRM score. The wine that was fermented, matured and stored at 15 °C (15 °C wine) received an average score of 3.3 for its gold colour. The wines that were stored 4 °C had pale yellow colour, and they averaged between a range of 1.7 to 1.9 SRM score (Fig. 6.1 A). In the second month, the SRM colour of the wine that was fermented, matured and stored at 25 °C (25 °C wine) increased from 8.8 to 12.7, while that of the wine that was fermented, matured and stored at 15 °C (15 °C wine) increased from to 3.1 to 9.7. There was no difference ($p = 0.309$) between these two wines, while a significant difference ($p < 0.05$) was observed in the colour change of the wines that were stored at 4 °C (Fig. 6.1 B). There was a general improvement in the likeness of all the wines as storage progressed to month 2, with wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C) still dominating in terms of appreciation.

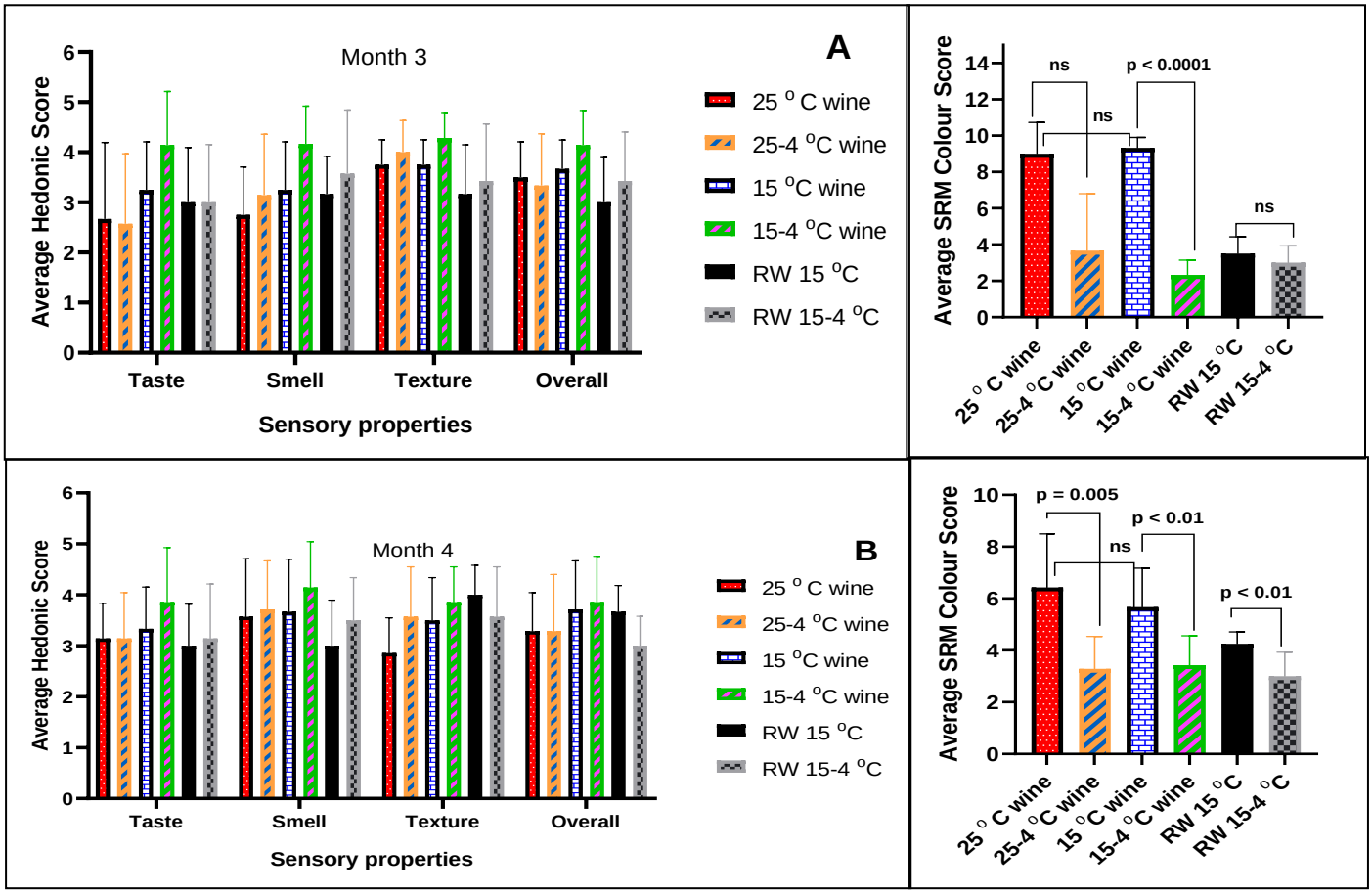

Figure 6.2. Sensory properties for month three (A) and month four (B) wines produced at different temperatures, RW refers to reference wine, ns refers to no significant difference and p refers to significant difference.

Taste improved to an above average score of 3 for the wines produced at 25 °C (25 °C wine and 25 – 4 °C wine) (Fig. 6.2 B) in the third month of storage. Although there was a noticeable decrease in SRM colour score of both the wine that were fermented, matured and stored at 25° C and the wine that was fermented, matured and stored at 15° C on the third month of storage (Fig. 6.1 A), both the wine remained amber to brown in colour (Fig. 6.2 B). However, the SRM colour score of the reference wine that was stored at 15 °C (RW 15 °C wine) and 4 °C (RW 15 – 4 °C wine) received average scores of above 4 and 3.5, respectively. Regardless of the observed changes, the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine) remained the most liked. During the third month of wine storage, production temperature showed an influence on wine taste ($p < 0.05$) for the wine that was fermented, matured and stored at 15 °C (15 °C wine) and the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine). A similar effect ($p < 0.05$) was observed between the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine) and the wine that was fermented at 25 °C, matured and stored at 4 °C (25 – 4 °C wine). Furthermore, there was a significant difference ($p < 0.0001$) in colour SRM score between the wine that was fermented, matured and stored at 15 °C (15 °C wine) and the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine), while a difference was notable ($p = 0.03$) between the wines that were fermented, matured and stored at 25 °C (25 °C) and the wine that was fermented at 25 °C, matured and stored at 4 °C (25 – 4 °C).

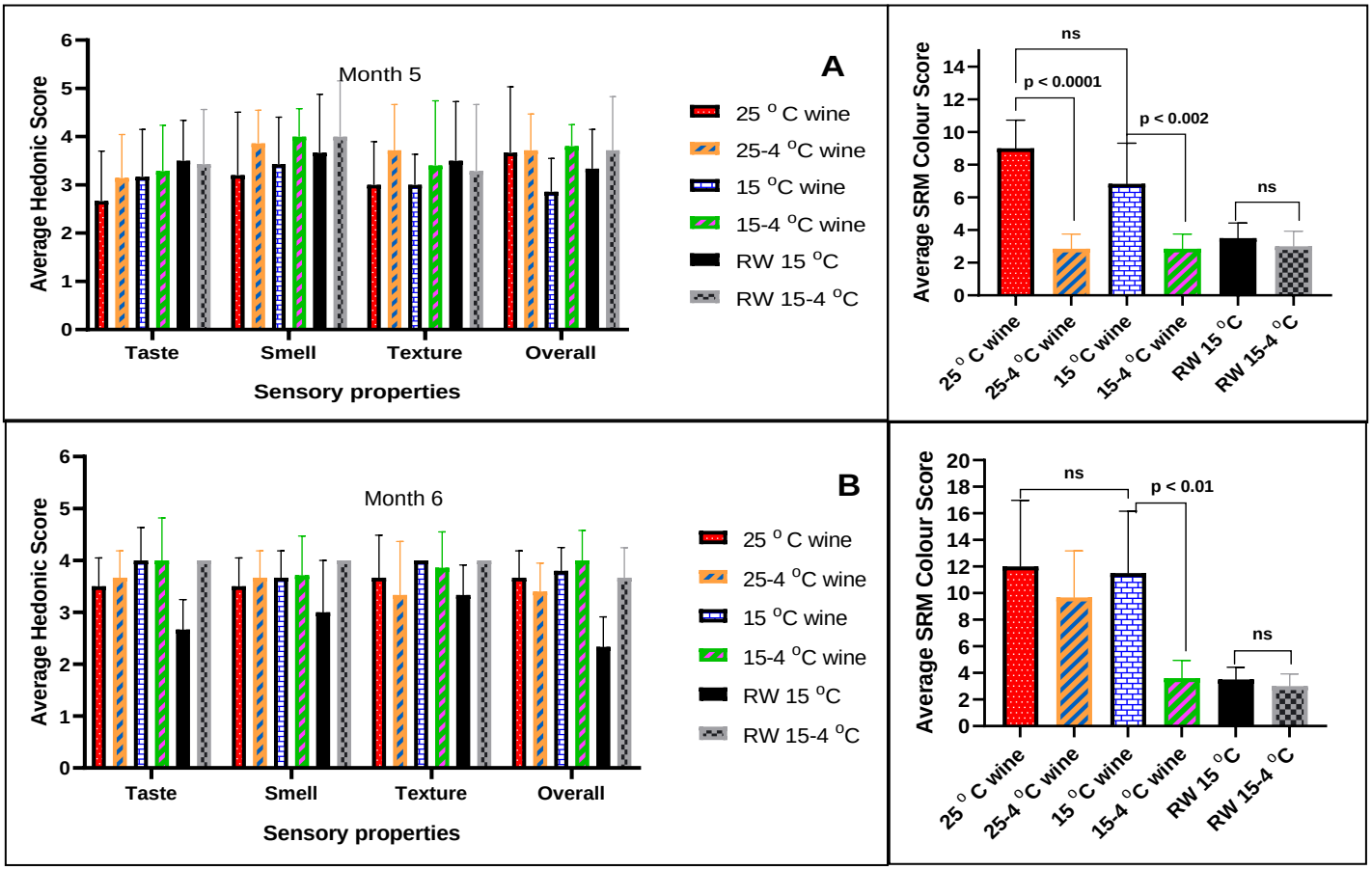

Figure 6.3. Sensory properties for month five (A) and month six (B) wines produced at different temperatures, RW refers to reference wine, ns refers to no significant difference and p refers to significant difference.

The taste of all the wines improved at the sixth month of storage based on the feedback from the panellists, except for the reference wine that was stored at 15 °C (RW 15 °C) (Fig. 6.3 A). However, the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine) remained the most liked wine for taste. Furthermore, there was a general improvement in other parameters such as smell, texture and overall impression for the wine that was fermented, matured and stored at 15 °C (15 °C wine) and the reference wine that was stored at 4 °C (RW 15 - 4 °C) (Fig. 6.3 A). Interestingly, the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine) and the wine that was fermented, matured and stored at 15 °C (15 °C wine) remained the most liked for all the sensory parameters (Fig. 6.3 B). There was a significant change ($p < 0.001$) in SRM colour scored for the wine that was fermented, matured and stored at 15 °C (15 °C wine) and the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine) wherein the wine changed from a gold to an amber colour (Fig. 6.3 B), while the colour of reference wines remain unchanged.

In general, the wines that were matured and stored at 4 °C (15 – 4 °C, 25 – 4 °C and RW 15 – 4 °C wines) were liked slightly more from the first month of storage as compared to their counterparts. This could imply the influence of maturation and storage of lower temperatures on sensory parameters.

6.3.2. Percentage score of panellists who would buy the wine

The data below (Table. 6.1) represent the percentage scores of panellists who would buy the marula fruit wines if it was on the market as compared to those who would not buy. Eleven panellists participated in this evaluation.

Table 6.1. Percentage number of the panellists (n = 11) who would buy the different marula fruit wines.

Wines	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6	Average
	Percentages (%)						
15 °C	56	71	68	71	71	75	69
25 °C	22	43	33	57	17	75	41
15 – 4 °C	78	71	86	86	80	80	80
25 – 4 °C	56	43	40	57	43	25	44
RW 15°C	20	25	29	25	71	50	37
RW 15 – 4 °C	60	29	29	71	71	16	46

The wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine) was the most preferred product by the panellists, followed by the wine that was fermented, matured and stored at 15°C (15 °C wine) (Table 6.1). It is noteworthy that both wines (15 °C and 15 – 4 °C wines) were consistently preferred from the first to the sixth months of storage, with 15 – 4 °C wine being the most liked. The wine that was fermented at 25 °C, matured and stored at 4 °C (25 – 4 °C wine) followed in the third place in the liking scores, while reference wine that was matured at 15 °C and stored at 4 °C (RW 15 – 4 °C wine) was highly appreciated on the fourth and fifth months (Table 6.1). A wine that was fermented, matured and stored at 25 °C (25 °C wine) and reference wine that was matured and stored at 15 °C (RW 15 °C wine) were the lowest appreciated marula fruit wines with the average percentage respectively (Table 6.1).

6.3.3. Common descriptions of marula fruit wines

Table 6.2 represents the average perceptions of individual panellists in terms of descriptions pertaining to appearance, aroma and taste of the marula fruit wines. Generally, the wine that was fermented at 15 °C, matured and stored at 4 °C (15 - 4 °C) was the most liked for its yellow to gold colour, fruity aroma and sweet taste, whereas the wine that was fermented at 25 °C, matured and stored at 25 °C (25 °C) was the least liked for the amber to brown colour, spicy and pungent aroma and bitter, spicy taste.

Table 6.2. Common descriptions of bottled marula fruit wines by panellists. The numbers in parenthesis refer to a number of panellists who scored a particular response.

Wine type	Appearance (respondents)			Aroma (respondents)			Taste (respondents)		
	1 – 2 months	**3 – 4 months**	**5 – 6 months**	**1 – 2 months**	**3 – 4 months**	**5 – 6 months**	**1 – 2 months**	**3 – 4 months**	**5 – 6 months**
15 °C	Yellow to gold (6) Amber (2)	Yellow to gold (1) Amber to brown (6)	Amber to brown (9)	Fruity (2) Pungent (1) Citrus (1) Spicy (2)	Fruity (3) Pungent (1) Citrus (1) Spicy (1)	Fruity (5) Pungent (3) Citrus (2) Spicy (1)	Sweet (3) Sour (6) Bitter (4) Acidic (3)	Sour (2) Bitter (1)	Sweet (5) Sour (4) Bitter (3) Acidic (3)
25 °C	Yellow to gold (3) Amber (4)	Amber to brown (6)	Amber to brown (8)	Fruity (1) Pungent (2) Citrus (1) Spicy (6)	Fruity (2) Pungent (4) Spicy (3)	Pungent (5) Citrus (1) Spicy (6)	Sour (4) Bitter (7) Acidic (2)	Sweet (3) Sour (2) Bitter (4) Acidic (2)	Sweet (1) Sour (4) Bitter (3)
15 – 4 °C	Pale yellow (2) Yellow to gold (4)	Pale yellow (2) Yellow to gold (6)	Yellow to gold (9)	Fruity (1) Pungent (1) Spicy (4)	Fruity (6) Pungent (1) Spicy (5)	Fruity (4) Pungent (2) Citrus (2) Spicy (4)	Sweet (4) Sour (3) Bitter (2) Acidic (1)	Sweet (7) Sour (4) Bitter (6) Acidic (2)	Sweet (4) Sour (6) Acidic (2)
25 – 4 °C	Pale yellow (1) Yellow to gold (7)	Pale yellow (2) Yellow to gold (6)	Yellow to gold (8) Amber to brown (2)	Fruity (7) Pungent (3) Citrus (2) Spicy (2)	Fruity (3) Pungent (8) Citrus (1) Spicy (6)	Fruity (4) Pungent (4) Citrus (1) Spicy (6)	Sour (5) Bitter (5)	Sweet (3) Sour (2) Bitter (5) Acidic (1)	Sweet (3) Sour (1) Bitter (5)
RW 15 °C	Pale yellow (2) Yellow to gold (4)	Pale yellow (2) Yellow to gold (5)	Yellow to gold (5) Amber to brown (2)	Fruity (2) Pungent (2) Spicy (1)	Fruity (3) Spicy (3)	Fruity (1) Pungent (2) Spicy (1)	Sweet (2) Sour (1) Bitter (1) Acidic (2)	Sweet (2) Sour (3) Bitter (2) Acidic (1)	Sweet (1) Sour (2) Bitter (1)
RW 15 – 4 °C	Pale yellow (1) Yellow to gold (4)	Pale yellow (2) Yellow to gold (5)	Yellow to gold (3) Amber to brown (4)	Fruity (2) Pungent (1)	Fruity (2) Pungent (1) Citrus (1) Spicy (1)	Fruity (3) Citrus (1) Spicy (1)	Sour (2) Bitter (2) Acidic (2)	Sweet (2) Sour (4) Bitter (2) Acidic (1)	Sweet (1) Sour (4)

In general, the wine that was fermented, matured and stored at 15 °C (15 °C wine) and that which was fermented, matured and stored at 25 °C (25 °C wine) were perceived to have a yellow to gold and amber colour, respectively in the first two months of storage, which turned into amber – brown and brown colour, respectively as storage progressed (Table 6.2). Their counterpart wines, i.e., both the experimental wines that were matured and stored at 4 °C (15 – 4 °C wine and 25 – 4 °C wine), and the reference wines (RW 15 °C and RW 15 – 4 °C) were perceived to have a pale yellow colour in the first two months of storage and a yellow to gold colour in the third to sixth months of storage by the majority of the panellists (Table 6.2). Furthermore, the wine that was fermented, matured and stored at 25 °C (25 °C wine) was perceived to be less fruity with a strong (pungent) unpleasant aroma, and a sour/ bitter taste, while the majority of the panellists have perceived high spicy aromatic character in this wine throughout the storage phase (Table 6.2). In contrast, the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine) was perceived to a have highly fruity aroma and sweet test on the palate, with less strong unpleasant and spicy aroma (Table 6.2). Although the wine that was fermented at 25 °C, matured and stored at 4 °C (25 – 4 °C) was perceived to have high fruity aromatic character, it was still perceived to have a strong (pungent) unpleasant and spicy aroma, highly sour and bitter taste (Table 6.2). The reference wine that was matured and stored at 4 °C (RW 15 – 4 °C wine) lost its bitterness and acidity, while it gained the fruity and sweet character on the last month of storage (Table 6.2). Whereas the reference wine that was matured and stored at 15 (RW 15 °C wine) retain the spicy and pungent aroma, with bitter and acidity flavours at the end of storage (Table 6.2).

6.4. Discussion and conclusion

Aroma and taste are the most important factors contributing to consumer's attraction to wines or food products in general (Cordente *et al*., 2021), while wine colour and clarity can also have a greater influence (Marić and Firšt-Bača, 2003). Sensory descriptive analysis of the bottled marula fruit wines was performed during a six-month storage period on a monthly interval that is assessing the effect of storage temperature on the sensory attributes of the wines. Marula fruit wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine) was the most liked for taste overall, and at least 80% of the panellists (n =11) stated that they will buy it. This wine was highly

rated for its fruity smell and sweet-to-sour flavours on the palate. The sweet taste is not surprising since it had high residual fructose (7.3 – 16.6 g/L) and glucose (3.6 – 4.8 g/L) following primary fermentation as reported in chapter 4. Its fruity character could be attributed to the esters such as isoamyl acetate, ethyl caprylate and ethyl butyrate that were retained throughout wine storage at levels between 5 to 23 mg/L, 19 to 23 mg/L and 1.5 to 2.6 mg/L, respectively as reported in chapter 4 of this study. These esters are known to impart fruitiness to wines (Scrimgeour *et al*., 2015). Isoamyl acetate gives off the banana, fruits or sweet character and was detected at the threshold above the wine-recommended concentration range of 0.1 to 3.5 mg/L and sensory threshold of 0.03 mg/L (Swiegers *et al*., 2005). Similarly, both ethyl caprylate and ethyl butyrate were detected at levels above the wine-recommended concentration ranges of 0.05 to 3.8 mg/L and 0.02 to 0.06 mg/L, respectively (Lambrechts and Pretorius, 2000, Legodi *et al*., 2022). Ethyl caprylate contributes the pineapple, pear, ripe banana or sweetness characters in wines, whereas ethyl butyrate gives off sour fruit, strawberry, fruity or apple characters (Lambrechts and Pretorius, 2000). Based on the residual sugar content, the wine is considered a semi-dry wine. Semi-dry wines may have 5 to 12 g/L of residual sugar, or up to 18 g/L if the residual sugar content does not exceed the acidity level by more than 10 g (Cook *et al*., 1998).

The wine that was fermented, matured and stored at 15 °C (15 °C wine) did not retain its fruity character, similar to the wine that was fermented at 25°C, matured and stored at 4 °C (25 – 4 °C wine), however, it was the second liked amongst the tasting panellists. Amongst the eleven (11) panellists, seven were women whom the majority (5) do not consume alcoholic beverages. Non-alcohol beverages drinkers often prefer sweeter than bitter or sour beverages (Stockley and Johnson, 2017) like the wine that was fermented, matured and stored at 25 °C (25 °C wine) and the wine that was fermented at 25 °C, matured and stored at 4 °C (25 – 4 °C wine).

The wines that were fermented at higher temperatures (25 °C and 25 – 4 °C wines) were less appreciated by most panellists and were labelled as having a pungent, spicy aroma and bitter taste throughout the storage phase. The spicy aroma was likened to atchar (spicy pickled mango) smell. Fusel alcohols such as isoamyl alcohol, isobutanol and n-propanol which were detected in these wines at levels such as 100 to 200 mg/L, 20 to 40 mg/L and 20 to 30 mg/L, respectively (Chapter 4) reportedly impart an

unpleasant aroma when present at thresholds above 30 mg/L, 50 mg/L and 70 mg/L, respectively (Chidi *et al*., 2018; Lambrechts and Pretorius, 2000; Legodi *et al*., 2022). Furthermore, ethyl acetate which gives off a nail polish smell at a threshold above 10 mg/L was detected at high concentrations in both wines fermented at high temperatures (25 °C and 25 – 4 °C wines) (Chapter 4). Esters such as ethyl butyrate and ethyl lactate are generally detected in wines produced at elevated temperatures above 20 °C (Cordente *et al*., 2021), similarly these were detected in 25 °C and 25 – 4 °C wines at levels between 1.5 to 3.5 mg/L and 30 to 45 mg/L, respectively (Chapter 4). Ethyl butyrate and ethyl contribute to the spicy aroma in wines at a threshold above 0.02 mg/L and 150 mg/L (Swiegers *et al*., 2005). Nevertheless, these wines that were produced at higher temperature (25 °C wine and 25 – 4 °C wine) were appreciated for their sweet to caramel smell.

A wine with a high amount of alcohol (11% to 13 % ABV), balanced level of organic acid (1 g/L to 2 g/L) and higher concentration (< 200 mg/L) of fruity esters is considered to have more flavours (Chidi *et al*., 2018; Fundira *et al*., 2002; Phiri *et al*., 2022). Although some of the important common wine organic acids such as oxalic acid and succinic acid were not analysed in this study, the other organic acid such as tartaric acid, acetic acid and lactic acid were detected below 2 g/L in all the experimental wines (15 °C wine, 15 – 4 °C wine, 25 °C wine and 25 – 4 °C wine) as reported in chapter 4.Notably, the analysed fruity esters such as isoamyl acetate, ethyl caproate and ethyl caprylate were detected at levels above 200 mg/L. These wines obtained the final alcohol content of between 11.2% and 11.6% alcohol by volume (ABV), hence they should be considered to have more flavours. However, the wines that were produced at a lower temperature (15 °C wine and 15 – 4 °C wine) were appreciated more than the wines that were produced at an elevated temperature (25 °C wine and 25 – 4 °C wine). This could be attributed to the high amount of fusel alcohols detected in the wines produced at an elevated temperature as result of high microbial metabolic reaction (Reynolds *et al*., 2001).

Such characteristics were not exhibited by the reference wines; alcohol content was in the range of 4.8% to 7.5% ABV, organic acid content was between 3.4 g/L and 4.3 g/L and the esters that were detected include levels of between 225 mg/L to 270 mg/L, (Chapter 4). The reference wines were highly appreciated for preserving the marula

fruit aroma as reported by panellists, although not appreciated by most in the first three months of storage. They were reported to have a flat sour taste. A wine with a flat taste is deemed lifeless as it is perceived to lack vibrant fruit flavours or either it is weak and tasteless, and sometimes it is perceived as vinegary (Torija *et al*., 2003). There was an improvement in the sensory descriptive scores for taste, smell, texture and overall impression for the reference wines in the last three months of bottle storage. Ethanol can improve wine bitterness and enhance wine sweetness (Pretorius, 2006; Torija *et al*., 2003), while glycerol can improve the texture of the wine, body and mouthfeel due to its viscosity (Gawel *et al*., 2007). The improved sensorial character of the two reference wines (RW 15 °C and RW 15 – 4 °C) could be attributed to the increase in both total alcohol and glycerol concentration in both reference wines (Chapter 4).

It was also noticeable from the SRM colour score that all the wines changed colour as the storage process progressed, mainly the colour intensified with time. The wine that was fermented, matured and stored at 15 °C (15 °C wine) and that which was fermented, matured and stored at 25 °C (25 °C wine) finished with a dark brown colour, while the one that was fermented at 25 °C, matured and stored at 4 °C (25 – 4 °C wine) finished with an amber colour, whereas the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine), together with the reference wines (RW 15 °C and RW 15 – 4 °C wines) finished with a yellow to gold colour. Oxidation resulting from high amounts of oxygen dissolved in wines during wine racking and bottling period (Cendrowski *et al*., 2021) is often associated with colour changes in wines. Oxidation increases wine browning and fast degradation of phenolic compounds such as vitamin C (Echave *et al*., 2021; Scrimgeour *et al*., 2015). A sharp decrease in vitamin C content was observed in both brown-coloured wines stored at 15 °C and 25 °C wines, whereas yellow to gold-coloured wines that were stored at 4 °C retained most of their vitamin C content (Chapter 5). A good balance of temperature is important because lower storage temperatures can slow down the aging process while elevated temperatures above 20 °C can accelerate oxidative reactions. It is worth noting that wine oxidation can also contribute to off-flavours as a result of overproduction of chemical compounds such as acetaldehydes (He *et al*., 2013; Sioumis *et al*., 2005). Hence, the wine that was fermented, matured and stored at 25 °C was less liked amongst the other experimental wines.

Wines that were fermented at a lower temperature (15 °C) and stored at a lower temperature (4 °C) tended to preserve, retain and have improved organoleptic attributes, whereas the wines that were fermented and stored at an elevated temperature (25 °C) lost their sensory characteristic as storage progressed. Browning can be the biggest threat in wine production, and it must be investigated further during marula fruit wine production.

CHAPTER 7
GENERAL DISCUSSION AND CONCLUSION

7.1. General discussion

Marula fruit wine is one of the traditional African indigenous beverages with the potential for industrial commercialisation. The wine is produced by a natural fermentation process using indigenous microorganisms from the marula fruit juice with less human intervention (Fundira *et al*., 2002; Legodi *et al*., 2022; Maluleke *et al*., 2023). The wines that are produced through natural fermentation processes are inconsistent from batch to batch in their chemical qualities, nutritional qualities and sensory attributes (Combina *et al*., 2005). Most wineries use the controlled selected temperatures and selective starter cultures to influence the rate of wine fermentation by enhancing the microbial activity during wine production process (Achi, 2005; Guzel-Seydim *et al*., 2005). Consequently, this affects the chemical and sensory attributes of the wines (Arroy-Lopez *et al*., 2009). This study sought to evaluate the influence of varying temperatures for fermentation, maturation and storage conditions on the chemical, nutritional and sensory characteristics of starter-culture-based marula fruit wines.

Although the marula fruit juice was pasteurised prior fermentation, different microorganisms including *Saccharomyces cerevisiae* yeast, the bacteria *Lactobacillus brevis*, *Acetobacter lovaniensis*, *Acetobacter ghanensis*, *Raoultella terrigena*, *Raoutella ornithinolytica* and other *Bacillus* species survived and were detected in bottled aging wines. The type of microorganisms presents in the different stages of wine development more often enlighten on the possible chemical reactions that contribute to flavour development in wines. The microbial activity can either improve or demean chemical and nutritional composition in the wine thus affecting flavour development. Amongst the detected microbes, there were opportunistic pathogens such as *Raoultella terrigena* and *Raoutella ornithinolytica* which are rare in wines. It is a concern when such pathogens are detected in the wines owing to their virulence activity (Lekhniuk *et al*., 2021). Good hygienic practices prior to marula juice extraction can prevent pathogen contamination considering that they could have been introduced by handling during fruit collection or they could be emanating from the soil that has

received animal contamination (Bokulich *et al*., 2013). The same holds for *Bacillus* species which were dominating during marula fruit wine aging process, although they remain less biologically active under wine production condition (Ramoba *et al*., 2022; Vaz *et al*., 2012).

Nevertheless, the important fermenting microorganisms such as *Saccharomyces* yeasts, non-*Saccharomyces* yeast, lactic acid bacteria and acetic acid bacteria are important for the quality of the wine in terms of flavour development based on the metabolites they produced under wine fermentative and maturing conditions (Beltran *et al*., 2007; Torija *et al*., 2003). Phiri *et al*. (2022) and Maluleke *et al*., (2023) specifically reported on the fermenting microorganisms and broad microbial profiles of marula wines respectively, and these studies alluded to the emergence and evolution of desirable and undesirable microorganisms during marula wine production. *Saccharomyces Cerevisiae* yeast strain was isolated and identified as the important fermenting microorganisms during spontaneous marula fruit wine production (Maluleke *et al*., 2023; Phiri *et al*., 2022), while *Lactobacillus*, *Acetobacter,* and *Gluconobacter* species were also considered important owing to their ability to produce required lactic acid and acetic acid concentrations which confer balance flavors to the wines (Legodi *et al*., 2022; Maluleke *et al*., 2023). The detection of these important marula fruit wine species in the current study will contribute to production of good quality wines.

Marula fruit wine contains organic compounds such as organic acids, sugars, alcohols and esters (Fundira *et al*., 2002; Legodi *et al*., 2022; Mashau *et al*., 2022) and as confirmed in the current study. These various organic compounds were detected at different concentrations throughout the marula fruit wine production process. Basically, microorganisms convert carbon sources such as sugars and amino acids in the marula juice to bioactive primary, secondary and tertiary by-products, whose production is influenced by temperature that affects the growth rate and metabolic reaction in fermenting microorganisms (Swiegers *et al*., 2005; Torija *et al*., 2003). Using elevated temperature (25 °C) for the production of marula fruit wine resulted in rapid fermentation as compared to lower production temperatures (15 °C). The concentrations of volatile compounds such as fusel alcohols and esters increased as fermentation progressed and remain stable during maturation and storage conditions.

The higher concentrations of fusel alcohols, pungent esters and spicy esters were mostly detected in the wines that were matured and stored at elevated temperatures of 25 °C, mainly the wine that was fermented at 25 °C, matured and stored at 25 °C (25 °C wine), whereas the fruity esters were mostly detected in the wines that were matured and stored at lower temperatures of 4 °C, mainly the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine).

Several studies have reported that elevated temperature of 25 °C and above accelerates and enriches wines with secondary metabolites such as tannins and antioxidant compounds (Liu *et al*, 2018; Rodríguez *et al*., 2009). However, there was no specific trend and significant difference between the tannins and antioxidant activity reported in this study. Bacteria belonging to the Bacillus and *Lactobacillus* species detected in the bottled wines can contribute to the conversion and depolymerisation of phenolic compounds (Smit *et al*., 2003), thus resulting in unpredictable changes in tannin and antioxidant concentration in wines.

There is little to no information on the effect of wine production temperatures on nutrient content in the marula fruit wine. Hiwilepo-van Hal *et al*. (2013) reported that there is several contradicting literature reporting on how different factors affect wine nutrient content. However, this study illustrated that there is no clear pattern that demonstrates the effect of different wine production temperatures on the nutritional composition of marula fruit wine. Levels of the selected nutrients fluctuated throughout fermentation, maturation and aging stages with no apparent trend. Vitamin C decreased in the marula fruit wines that were stored at 15 °C and 25 °C, while it was retained in the wines that were stored at 4 °C. In another observation, vitamin C was retained in the reference wines that were fermented, matured and stored at 25 °C. This denotes that temperature was not entirely responsible for the decrease in vitamin C. Although it was not proven in this study, factors such as low pH, microbial bioactive activity and oxygen concentration level can contribute to the decrease in vitamin C levels (Cendrowski *et al*., 2021).

The marula fruit wines that were fermented at an elevated temperature of 25 °C were perceived to have a hash-pungent, spicy aroma and a bitterer to sour taste by wine-tasting panellists. High amount of fusel alcohols (higher alcohols) and pungent and

spicy esters such as isoamyl alcohol, ethyl acetate, ethyl butyrate and ethyl lactate were detected in those marula fruit wines, and these metabolites are attributed to such organoleptic characters. On the contrary, the wine that was fermented at 15 °C, matured and stored at 4 °C (15 – 4 °C wine) was perceived to have fruity and a balance of sweetness to sour flavours and this resulted from the high amount of fruity esters such as isoamyl acetate, ethyl caproate and ethyl caprylate and residual total sugars fructose which ranged between 7.3 and 16.6 g/L and glucose ranged between 3.6 and 4.8 g/L that were detected in the aging wine. Consequently, this wine was highly appreciated by at least 80% out of 11 panellists who evaluated the wine. This wine also retained its yellow to gold colour compared to the wines produced at elevated temperature that turned into brown colour which could have resulted from oxidation.

Different wine production temperature had influence on the final wine characteristics. Although there is overwhelming evidence from this study and reported literature that lower fermentation, maturation and storage temperature can produce wines with a lingering fruity aroma and naturally sweet flavour as compared to elevated temperature produced wines with more spicy and pungent chemical composition, there is still a need to understand how the changes in nutritional and phenolic composition in marula fruit wine affect the sensory parameters. It was evident from this study that some essential nutrients such as proteins were preserved while others such as minerals were improved in quantity in all the marula wines irrespective of the production temperature used. This is important for the high nutritive quality of marula fruit wine, which has been an old age sentiment held in the African communities where the traditional marula fruit wine is produced.

The vulnerability of the aging wine to oxidation which results in browning should be investigated further to find organic means of preventing it. Furthermore, hygiene practices that can prevent contamination of the wine with human health threatening pathogens should be explored. This will be in addition to pasteurisation which was used in this study.

The primary purpose of this study was to investigate temperature conditions that are good for production of a good quality, stable marula fruit wine. A low fermentation temperature (15 °C) and low maturation and storage temperature (4 °C) can be

considered for the establishment of commercial conditions for producing high quality marula fruit wine in industry. These conditions allowed for slower fermentation rate and more time for microbial interactions with nutrients and consequently slower transformation and development of flavours in the maturing and aging wine.

CHAPTER 8

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Appendix A: Ethical certificate

University of Limpopo
Research Administration and Development
Private Bag X1106, Sovenga, 0727, South Africa
Tel: (015) 268 3935, Fax: (015) 268 2306, Email:Anastasia.Ngobe@ul.ac.za

Dear Mr/Ms **L Ramoba**

RE-APPLICATION FOR ETHICS APPROVAL

Title: An Investigation of Fermentation and Maturation Temperatures on the Chemical and Sensory Characteristics of the Starter-Culture Based Marula Fruit Wines.

Researcher: L Ramoba

Degree: Masters of Science in Microbiology.

THIS IS TO CONFIRM THAT YOUR PROPOSAL SERVED AT TREC.

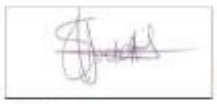
The proposal requires some corrections/amendments to be able to satisfy the requirements for ethical clearance.

1. There should be made mention of measures that will be put in place in case the wine causes adverse reactions for some tasters (e.g. those who may have hidden allergies).
2. A sample informed consent form should be part of the proposal for the tasting panel, as these are human subjects.
3. The researcher should clarify if permission is required at UL the laboratory, If so a permission letter for use of UL's laboratory and staff for tasting is necessary.

NOTE:

i. **This is not a Clearance Certificate and thus cannot allow you to proceed with data collection.**

ii. **You are expected to send the corrected proposal for the finalisation of your ethical clearance.**



AJ Ngobe

Appendix B: Consent Form

PROJECT TITLE: AN INVESTIGATION OF FERMENTATION AND MATURATION TEMPERATURES ON THE CHEMICAL AND SENSORY CHARACTERISTICS OF THE STARTER-CULTURE BASED MARULA FRUIT WINES

PROJECT LEADER/SUPERVISOR:

PROJECT RESEARCHER:

PROJECT DESCRIPTION:

The focus of the study is to produce a long shelf life, high alcohol marula wine using different fermentation temperatures to improve the shelf-life and quality for commercialisation. Participants will be required to provide feedback and opinion on sensorial properties such as the taste, aroma, texture and colour of marula wine in order to evaluate the quality and palatability of the wine. Marula fruit juice obtained from savoury sources and commercial white sugar will be used in the preparation of marula wine. The cultures will be those that were previously isolated from the traditional marula wine.

I, hereby voluntarily consent to participate in the following project:

Investigation of fermentation and maturation temperatures on the chemical and sensory characteristics of the starter-culture based marula fruit wines

I realise that:

1. My participation in this study is voluntary and I can withdraw my participation at any stage.
2. The study entails production of marula wine and the wine contains alcohol.
3. Marula wine is produced from marula fruit juice and sugar. Cultures isolated from traditional marula wines will be used for the starter culture-based wine.
4. The protocol followed does not include any material that may bring any harm to me.
5. The research project and potential outcomes and benefits have been explained to me.

6. An explanation has been provided for the possible discomfort for participating in the study with regard to consumption of a study beverage.

7. The researchers will inform me of any new information that may arise during the course of the study so that I can make an informed decision on whether to continue or discontinue with my participation.

8. All the records assisted with my participation will be accessible to the researchers involved in this study.

9. I can address any question and/or discomfort that I may experience regarding the study with the researchers.

10. Exclusion principles applicable to the study have been explained to me by the researchers.

11. I will advise the research team of any medical issue that arises during the cause of the study.

12. Such medical conditions will be discussed in confidence and medical intervention may be sought.

13. I will not hold the University of Limpopo and any persons involved in this study liable for any harm or any cause whatsoever that may occur to me during the course of this research.

_____ _____

SIGNATURE OF STUDY PARTICIPANT Date

_____ _____

SIGNATURE OF WITNESS Date

_____ _____

SIGNATURE OF RESEARCHER Date

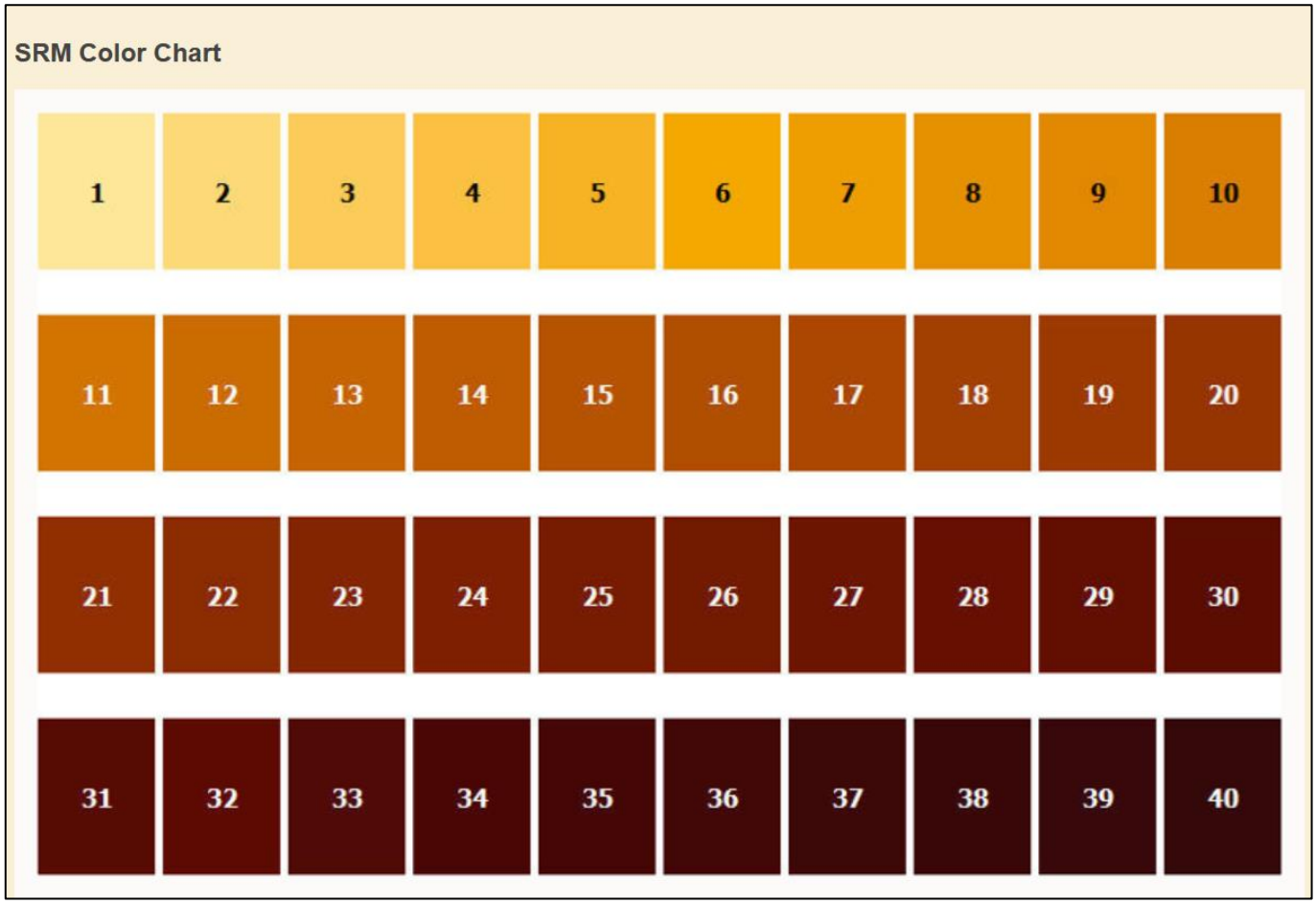
_____ _____

SIGNATURE OF PROJECT LEADER Date

THE STUDY PARTICIPANT

SIGNED AT_____ THIS ____ DAY OF _____ 20__

Appendix C: SRM Colour Cart



0 – 1 pale yellow

1 – 2 pale straw

2 – 3 Straw

3 – 4 pale gold

5 – 6 deep gold

6 – 9 pale amber

9 – 12 medium amber

15 – 12 Medium amber

15 – 18 amber brown

18 – 20 brown

Appendix D: Sensory Evaluation of marula fruit wine

08 July 2022

Do you normally consume alcohol?	**Yes**	**No**

Identification_____ **Colour code_____**

Gender

Female		**Male**		**Other**	

Age group

Under 21		**21-30**		**31-40**	
41-50		**51-60**		**Over 60**	

Sample number						
		1: extremely dislike/ go se rate kudu	2: Dislike/ go se rate	3: Neutral/ magareng	4: Like/ rata	5: extremely like/ go se rate kudu
	Taste/tatso					
	Colour/mmala (SRM number)					
	Smell/monkgo					
	Texture/					
	Overall impression/ kgahlego					
Would you buy it? /O ka e reka?					Yes/ Ee	No/ Aowa

You have been given samples to evaluate. Please taste the samples in order presented. Indicate how much you like or dislike the sample by rating it on a scale of 1-dislike extremely to 5-like extremely. Please eat a carrot in between the tasting.

Comments:

……
……
……