Improvement of the alcohol content and an investigation of fermentation and maturation temperatures on the chemical and sensory characteristics of unpasteurised starter culture-based marula fruit wine

by

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DEDICATION

I dedicate this Master of Science work to myself, Ramatsobane Phangisile Robyn Mapheto for falling several times and crawling until I was able to complete this degree. It was tough but at the end I was tougher.

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.

Mapheto, RPR (Ms) **Date**

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ABSTRACT

Marula fruit wine is an alcoholic beverage that is produced through fermentation of marula fruit juice. The traditional marula fruit wine has a low alcohol content and spoils quickly. This study investigated the production of a high alcohol wine through starter culture-based fermentation of unpasteurised marula fruit juice. The influence of varying fermentation and maturation temperatures (15 °C and 25 °C) on chemical and sensory properties were explored. Selected chemicals, nutritional content and sensory characteristics were evaluated. The fermentation rate at 25 °C surpassed that at 15 °C, with wines reaching 1.0 °Brix in 10 days and 30 days, respectively. Alcohol content increased gradually and reached 12% as sugar got utilised. A pH of 3.44 – 3.88 deterred spoilage microorganisms yet *Bacillus* species, commonly found in soil and associated with winemaking, were observed in the wines at 15 °C and 25 °C post fermentation. Evaluation of organic acids revealed citric acid dominance (3.28 – 5.70 g/L), along with malic acid (2 – 6 g/L) and low levels of acetic acid (< 2.1 g/L), contributing to desirable sensory characteristics. While temperature variations did not significantly influence higher alcohols, levels at 25 °C were higher than at 15 °C. Mineral concentrations fell below dietary recommendations, with notable potassium and sodium quantities. Protein content ranged from 7.5 to 11 µg/mL. The antioxidant, vitamin C, ranged from 96.6 – 128.7 µg/mL in the 25 – 4 °C wine during the storage phase. The interactions of the chemicals and yeasts contributed to the sensorial characters of the wine. The wine fermented and stored at 15 °C, i.e., 15 – 15 °C and 15 – 4 °C demonstrated to be the most appreciated for taste. This could be attributed to the residual sugar content and higher alcohols for its sweetness and fruity taste. The findings demonstrated that varying temperature did not influence most of the selected nutritional content. Additionally, 25 °C should be used to initiate fermentation, 15 °C for maturation and finally 4 °C for aging to balance the rate of fermentation progression and the production of good wine character. Despite a general understanding of the impact of temperature on flavour development and chemical contribution, the full extent of its influence remains a subject of ongoing exploration.

CHAPTER 1: INTRODUCTION

1.1 Background of the study

Marula wine is an indigenous alcoholic beverage produced usually in the African communities where marula fruit trees are abundant. The beverage has cultural and socio-economic importance to the African people (Francis and Nwonwu, 2006; Petje, 2009). Lately, the traditional wine is mostly produced for trading due to low production costs which results in better profit margins (Maepa and Kgoete, pers. com, IK holders).

The traditional wine is produced from diluted marula fruit juice through spontaneous fermentation, and it is commonly consumed while still at primary fermentation (Torija et al., 2001). The dilution of the juice, which is made to merely increase the volume of the fermentation matrix, leads to a low alcohol content as determined in various studies (Dlamini and Dube, 2008; Maluleke et al., 2023; Phiri et al., 2022). Furthermore, the dilution of the juice may potentially lessen the development of flavour attributes of the wine which is also influenced by the fermenting temperature. The other challenge is that natural fermentation does not yield a product of consistent quality from one production batch to another (Combina et al., 2005) mainly due to variations in the contributing and fermenting microbiota. Notwithstanding these challenges, traditional marula wine has great potential to be developed into a commercial wine for the mainstream market (Nyanga, 2012). However, this would require a product that will not deteriorate in quality during storage, and which is reproducible.

Starter culture-based fermentation of a refined juice in controlled conditions could produce marula fruit wine with improved stability, shelf life and consistency. Autochthonous fermenting yeasts strains from marula wines (Maluleke et al., 2023; Phiri et al., 2022) will be used as inoculum. Autochthonous isolates have the advantage of being well adapted to the fermenting matrix. The production of certain wine characteristics are greatly influenced by the temperature at which they are fermented (Torija et al., 2003).

This study sought to improve the alcohol content of marula wine through the use of undiluted marula fruit juice which has been organically clarified and augmented with sugar syrup to increase its sugar content as well as to investigate chemical and sensory changes during different stages of the production of this wine at different temperatures. Compounds related to fresh and fruity flavours in wines are found at varying growth stages at different temperatures (Molina et al*.,* 2007). The effect of the temperature on enhancing compounds that produce aroma is still not fully understood (Beltran et al*.,* 2006). Consequently, the development of flavour compounds is due to the bioconversions of compounds such as sugars and amino acids during the fermentation process which is driven by microorganisms. Thus, suitable temperature is important to balance the rate of fermentation progression and the production of good wine character. These will be generated by the outcomes of this research project as changes occurring in the wine will be observed at various stages of the fermentation process i.e., primary fermentation, maturation and storage.

1.2 Aim

The aim of the study was to produce a higher alcohol wine through starter culture-based fermentation of unpasteurised marula juice and evaluate the effect of varying fermentation and maturation temperatures on the chemical and sensory properties of the marula fruit wine

1.3 Objectives

Starter-culture based marula fruit wines that were produced at 15°C and 25°C were compared to spontaneously fermenting wine for the:

i. presence and utilisation of fermentable sugars (i.e., glucose and fructose)
ii. chemical characteristics such as organic acidity, volatile acidity, pH, esters, aldehydes, alcohol, antioxidant activity and tannin content
iii. nutritional content such as mineral content, crude protein, vitamin C, fat content and cholesterol
iv. sensory characteristics such as colour, aroma, texture, taste and general impression

CHAPTER 2: LITERATURE REVIEW

2.1 The marula tree

The marula tree (*Sclerocarya birrea*) is one of the most recognised indigenous trees belonging to the Anacardiaceae family. This family comprises more than 600 species and 73 genera (Tapiwa, 2019). The genus *Sclerocarya* has two species that originate in Africa, namely; *Sclerocarya birrea* and *Sclerocarya giletti* (Murye and Pelser, 2018). *Sclerocarya birrea* is further divided into three subspecies, namely, *birrea*, *caffra* and *multifoliolata* (Shackleton et al., 2001).

Morula, as referred to by the Bapedi people, is medium to large in size and has a greyish wide large trunk. The tree height ranges from 7 – 12 m (Seloana et al., 2017) and can double in size up to 18 m when growth conditions are favourable (Mashau et al., 2022). The tree has green leaves which have a watery-latex consistency and produce a strong, resinous smell when they are crushed. The leaves are also compound and divided with a terminal leaflet (Do et al., 2020). The female tree produces numerous amounts of fruits, about 500 Kg of fruits can be produced by one tree in one season (Figure 2.1).

Picture by author

Figure 2.1: Marula tree and marula fruits collected off the ground.

Marula trees grow well in warm and frost-free regions in a wide range of altitudes that vary from sea level to 1800 m above sea level (Sinthumule and Mzamani, 2019). Temperatures from as low as 10 °C are favourable for the growth of marula trees in high altitudes whereas they can still grow in temperatures of up to 40 °C in low-lying areas. Marula tree occurs naturally in various types of woodland, on sandy soil or occasionally sandy loam. It is dioecious, and separately produces male and female flowers (Sinthumule and Mashau, 2019). However, there are instances where trees that produce male flowers can produce female flowers in few amounts (Sinthumule and Mzamani, 2019).

The marula tree is widely distributed and is found in 29 countries in Africa such as Ethiopia, Eritrea, Sudan, Kenya, Uganda, Tanzania, Central Africa Republic, Democratic Republic of Congo and South Africa. In South Africa it is generally found in KwaZulu-Natal, Mpumalanga, Gauteng and Limpopo provinces, although with widespread abundance in the Limpopo province.

2.2 Marula as remedy and other household uses

Humans often need trees to provide oxygen, shade and assistance in absorbing carbon dioxide from the air. The marula tree provides all those attributes and more. The tree components such as the stem, bark, roots, leaves and fruits are used for medicinal purposes (Mashau et al., 2022). The parts can be used individually or infused together to cure a variety of human diseases including infertility, schistosomiasis, epilepsy, diabetes mellitus, fever, diarrhoea and dysentery as well as stomach-aches, headaches, toothaches, backaches, and other physical symptoms (Tapiwa, 2019). In short, the bark is squashed, mixed with cold water to produce a pulp which is then used to treat dysentery and diarrhoea (Fajinmi et al., 2017).

The marula tree roots are used to treat heavy menstruation, bilharzia, coughs, weakness, sore eyes and heart pains when in decoction, infusion or steam form (Shackleton et al., 2002). The leaves' essence is used to treat conditions such as inflammation, bites from crawling animals like spiders and burns (Shackleton et al., 2002). Additionally, the edible *Imbrasia belina* commonly known as mopane worms

are collected from the marula leaves (Mashau et al., 2022). These marula leaves can further be used to treat heartburn when chewed in their raw state. The leaves are also used in making compost and animal feed collectively with the branches and stem. The stem of the tree is further used to provide wood which some use for fire and some process it into timber to make wood products like wooden spoons amongst many others.

2.3 Marula as a sacred tree

The Bapedi people of South Africa treat the marula tree with some respect in honour of its lineage because they believe that the spirits of their ancestors gave it to them. (Sinthumule and Mzamani, 2019). For this reason, marula tree is often the only tree left standing in a ploughed field. It is regarded as a sacred tree among many communities in Africa. The tree is protected in communal lands under the authority of local chiefs. It serves as a meeting place in many localities and is often the ritual centre of kraals and villages.

The Venda people believe that the bark can be used to tell the gender of an unborn child. Women who are expecting consume the bark infusions from the male tree if they desire a son or the female tree if they want a daughter. If a child of the opposite gender is born, he or she is said to be special as they have defied the spirits (Rasethe and Potgieter, 2021). In the Zulu culture the marula tree is labelled the ‘Marriage tree’ as they believe that those who get married beneath the tree will have a happy long-lasting marriage.

Traditional healers take a bath in a marula bark decoction in order to strengthen themselves before treating infectious ailments while sangomas (spiritual healers) use marula stones as a customary element in bone-throwing rites because it is thought to have spiritual significance (Murye, 2017).

2.4 The marula fruit

The individual fruits which are almost the size of a plum or a golf ball vary in shape; from round to oval depending on the tree. The fruits are green when still on the tree (Figure 2.2) and, in most circumstances, remain unripe. They fall and ripen on the ground into a yellow colour. The skin is thick and leathery, and the inside flesh is fibrous and moist. The fibrous moist material is the source of most of the juice and it is called the mesocarp (Dlamini and Dube, 2008). The fruit is 3 – 4 cm in diameter and weighs 15 – 25 g (Mariod and Abdelwahab, 2012).

The fruits can be eaten fresh by removing the peel and sucking out the juice. Otherwise, the peel can be removed followed by crushing the fruit to make what is called “boloilane” in Sepedi and sucking the contents of the fibrous juicy part together with the contents of the kernel (Mahlatji, LE pers.com). The skin can be ashed to make coffee (Mashau et al., 2022) or used to feed animals such as goats, sheep and cattle (Mahlatji, LE pers.com). The nuts obtained from crushing the kernel (seed) can be roasted for food (Mokgolodi et al., 2011) or used to soften animal leather for the production of traditional clothing made out of animal skin (Mahlatji, LE pers.com). The smooth side of the crushed kernels (seeds) (Fig. 2.2) are used together with mud to make decorations in the homesteads.

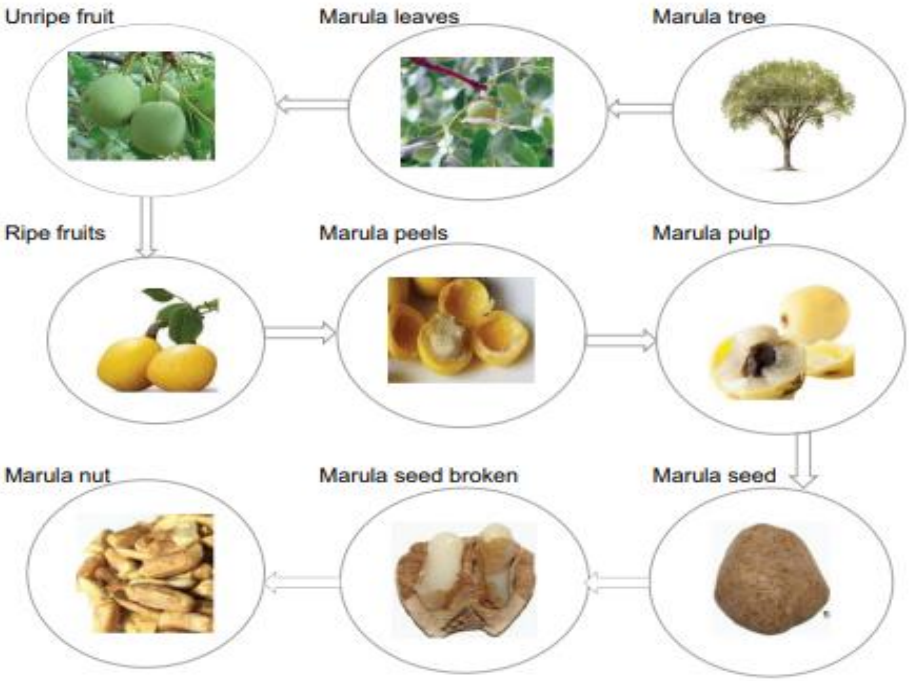

Figure 2.2: The marula fruits. Source: Mashau et al. (2022).

The African people are creative in using marula fruits to make food. In some instances, the fruit juice and its skin are used to make jam, jelly, wine, liqueur flavouring and marula juice which is known as leshong (Mahlatji, pers.com) in some parts of Bapedi communities (Hiwilepo-Van Hal et al., 2014). In other instances, the marula juice is used as a replacement for water to cook porridge, which can be eaten as a snack on its own due to its enhanced marula flavour or it is added to fresh milk to make sour milk due to its sweet-sour taste.

2.5 Marula fruit wine

Marula fruit wine is an alcoholic beverage produced from marula fruit juice in different parts of southern Africa such as Zimbabwe, Namibia and South Africa. In South Africa, Limpopo Province it is produced in Phalaborwa, Mankweng and Sekhukhune amongst many others. The fruit wine is often referred to as marula beer, morula in Sepedi, mukumbi in Tshivenda and vukanyi in Tsonga (Maluleke et al., 2023). The peels of the fruit are removed with a fork to expose the fleshy juicy part of the fruit which is then manually squeezed to extract the juice. The kernels of the fruits which would still have a little bit of the juice, are mashed in water in a separate bucket to extract more juice before they are discarded. Traditionally, less or equal amount of water is added to the juice to increase the volume of the wine. The diluted juice will then be left to ferment at room temperature following spontaneous fermentation. The natural presence of yeasts on the skin of fruits explains the immediate presence and dominance of yeasts in the fermenting matrix (Motlhanka et al., 2018). Fermentation is carried out until pulp formation stops, which is usually after 2 – 3 days. Thereafter, the beverage is ready to be consumed.

Naturally fermented marula wine often has off flavours which can be attributed to the presence and activity of bacteria and yeasts such as *Brettanomyces bruxellensis* during fermentation (Makopa et al., 2023). Fruit flies that frequently visit the marula fruits as they ripen, are most likely responsible for introducing the bacteria involved in the traditional fermentation of marula. The bacteria could include acetic acid bacteria that is known to contribute to wine spoilage by acetification of the ethanol produced by yeasts, which would ultimately result in off flavours like a vinegar-like flavour. An inoculum that consists of desirable fermenting microorganisms can be used to mitigate the effect of spoilage microbiota and to produce a wine of consistent quality.

2.6 Socio-economic benefits of marula fruit wine

The marula fruit wine is produced in many rural parts across Africa and has many benefits. In rural South Africa it is deemed to have “difepammele” to generalise all the health related and nutritional benefits that are not precisely known. Research on the marula fruit wine has and will provide scientific knowledge on the kinds of nutritive and health benefits (difepammele) the beverage contains.

Many rural households depend on the income earned from sales of marula wine. The wine is sold in the homesteads or by street vendors (Figure 2.3), particularly by women as they are typically the ones who prepare this wine. Marula fruit wine is packaged and sold mostly in 2 L plastic bottles which costs R20 – R30 per bottle. The 20 L and 750 mL cost R100 and R5 respectively (Maluleke et al., 2023). On average R18.16 is made from selling 80 Kg of marula fruits while a 200 mL cup of kernels costs R2 to R5 and an 80 Kg bag costs between R20 and R50 (Shackleton and Shackleton, 2004). Development and large-scale production of a long lasting, consistent marula fruit wine would potentially increase the value of the marula tree and the marula fruit juice for economic benefits for the marula wine brewing community. This would in essence create jobs for fruit harvest, distribution and packaging of the product and consequently improve the livelihood of the rural African communities.

Picture by author

Figure 2.3: Marula fruit wine being sold in Motetema, Limpopo province.

2.7 Nutritional value of marula fruits and wine

Good nutritive index is an important aspect in the food and beverage industry. Proper nutrition can prevent the onset of many diseases, especially those linked to oxidative stress including cancer and cardiovascular diseases (Sulmont-Rosse et al., 2019). The critical area of scientific advancement in recent years stands for expanding knowledge of foods with health-beneficial properties. Antioxidants are a class of chemicals that are found naturally in plant matter, animal tissues and microbes (Lorenzo et al., 2018). Marula fruits are rich in these antioxidants (Mashau et al., 2022). These antioxidants have received the most research attention because they protect the body against free radicals (Comert and Gokmen, 2018).

The marula fruit juice comprises of sucrose, glucose and fructose as the main sugars (Phiri et al., 2022). The sugar content generally ranges between 7 and 14 °Brix i.e., 7.18 – 14.77% in marula juice (Legodi et al., 2022). The amount of sugar in marula juice is comparable to other fruit juices used in making wine such as mango. However, the sugar content in some fruit wines such as palm wine is higher than that of marula fruit wine i.e., 10 – 12%. Marula fruits contain other essential nutrients that are important in human diet such as vitamin C, minerals and proteins. Vitamin C content in marula fruit range from 62 mg/100 g to more than 2,100 mg/ 100 g (Hiwilepo-van Hal, 2014). Even at its lowest, vitamin C content of marula fruit is still comparable to that of oranges (Hillman et al., 2008), which are particularly high in vitamin C. Minerals such as iron, potassium, sodium, manganese, calcium, magnesium and zinc have been reported in marula fruits (Ngemakwe et al., 2017) and the high levels in the kernels is understandable wherein phosphorus (808 mg/100 g), magnesium (462 mg/100 g), zinc (5.19 mg/100 g), iron (4.87 mg/100 g) and copper (2.81 mg/100 g) documented for their respective amounts in weight (Tebeila, 2022). The energy value of the marula fruits per 100 g of dry weight ranges from 2699 to 2703 KJ (Tebeila, 2022).

Wine preserves the nutrients found in the fruits when compared to distilled alcoholic drinks such as brandy and whisky (Joshi et al., 2017). During the wine-making process, the bioactive components included in the raw materials are released (Nanni et al., 2021) becoming available for consumption. Hence, it is expected that the nutritive composition of the wine is similar to that of the fruit juice.

2.8 Challenges and opportunities in marula fruit wine production

Marula fruit wine is only produced during the marula fruit season, i.e., between February and April, primarily due to the availability of the fruits. The quick ripening of the marula fruits (Ngemakwe et al., 2017) and quick spoilage of the fruit juice and the wine restrict the production and presence of the wine to only during the fruiting season. These challenges are limiting to the potential production of marula wine in large scale for better profit margin. In addition, the fermenting process for traditional marula fruit wine is uncontrolled, hence various microorganisms with different nature, form and metabolic properties that are introduced by fruit flies and through human handling are involved during wine fermentation. This infers a combination of desirable and undesirable fermenting and contributing microorganisms and the consequence is a wine of varying quality (Sharma et al., 2020) and stability between batches. Furthermore, with spontaneous fermentation, the formation of a product of high quality may not be guaranteed because of a reduced fermentation progression rate or the development of unwanted metabolites due to growth of undesirable flora (Singh et al., 2017). Not all secondary metabolites contribute positive traits to the alcoholic beverages (Motlhanka et al., 2018). The secondary metabolites formed during oxidation of the fruit acids and alcohols after the completion of fermentation affect the quality of the resulting wine and this impact negatively on the consistency required in *en masse* production of wine.

The importance of the physical conditions such as temperature and oxygen level during fermentation should not be underestimated since wine fermentation is an anaerobic process, however, some oxygen is required at the start of the process to allow good level of yeast growth before fermentation ensues. Inadvertently, the variance in environmental conditions leads to variable microorganisms in different batches and consequently the taste of the fruits and ultimately the wine may differ (Legodi et al., 2022). Based on their experience with marula fruit wine sales, winemakers are forced to make only a certain amount of wine where they are certain that it will sell before the wine starts to spoil. Marula fruit wine is then produced periodically and not in large volumes.

Some marula fruit winemakers use backslopping to produce the wine. Backslopping

is a fermentation technique in which a small quantity of the previously fermented matrix is used as the starter for the next fermentation step. Backslopping is commonly applied in the production of sauerkraut, sourdough and koumiss (Kim et al., 2018), however, the microbial ecology remains poorly understood and this technique works to a limited extent. Although this technique is advantageous in providing the starter culture and producing wine of some consistency, undesirable compounds that may be less toxic when in small quantities will accumulate and might reach toxic levels in subsequent brews (Motlhanka et al., 2018) thus affecting the safety of the home brew.

There are a few steps that can be considered to mitigate the challenges outlined above for production of a good quality marula fruit wine. The use of a defined starter culture, pasteurisation of the juice prior to wine fermentation and using desirable, with good control of the fermentation temperature. These are important factors although optimisation of the process is not limited to these. The aspect of selection and use of starter culture in wine production is discussed comprehensively in the subsection below. Pasteurisation, on the other hand, uses heat from 95 – 98 °C to inactivate microorganisms (Panchal et al., 2018), with the desire to remove the unwanted microorganisms (Lindsay et al., 2021). Notably, pasteurisation does not kill all microorganisms. However, if combined with a higher dosage of the starter culture, then the autochthonous microorganisms will be outcompeted and in this way the end product will presumably be the result of the activity of the starter culture (Oberholster et al., 2015).

Another manner of improving the quality of the marula fruit wine could include maturation and aging processes. The temperature, among other environmental conditions, is pertinent to the occurrence of the bioconversions that take place during these phases in wine making. The current traditional method terminates marula fruit wine production in the primary fermentation stage, which is also the point of consumption. These processes include clarification through settling of particulate matter (Oberholster et al., 2015) and a variety of physical, chemical and biological processes that often preserve or enhance the sensory attributes of the wine. Interestingly, most of these changes happen naturally but winemakers generally speed up the processes. Importantly, excessive intervention might distort the wine's natural qualities (Legras et al., 2016).

2.9 Microorganisms associated with wine production

Microorganisms help drive fermentation, both endogenous and exogenous microorganisms are involved in the process of fruit wine fermentation (Morata, 2018). The endogenous cultures are contributed by fruits and winery surfaces while the exogenous are the selected starter cultures. The quality of the end-product wine depends on the contributions made by all microorganisms present in the whole system including bacteria and yeast (Matei and Kosseva, 2017). Traditional wine fermentation follows spontaneous fermentation process and uses the wild microflora present on the surface of fruit-skins and in other instances yeasts available in wineries, which are indigenous are also used.

Lactic acid bacteria (LAB) are generally present in the first steps of winemaking, which is in the must and the start of fermentation. These belong to different species and are generally homofermentative. The most abundant belong to the species *Lactobacillus plantarum, Lactobacillus hilgardii*, *Leuconostoc mesenteroides*, and *Pediococcus* species (Garcia et al., 2020), while to a lesser extent, *Oenococcus oeni* and *Lactobacillus brevis* have also been reported present at the start of fermentation (Guerin et al., 2020). These species have been previously used and shown to lower alcohols, increase fruity and floral attributes as well as to help in driving fermentation (Morata et al., 2019).

These LAB are Gram-positive rod or spherical-shaped. They are distinguished by not sporulating, being catalase-negative and being able to tolerate acid (Bartowsky, 2019). All LAB are anaerobes, however unlike most anaerobes, they grow while exposed to oxygen (Balmaseda et al., 2018). The LAB ability to resist the increased acidity caused by the formation of organic acids allows them to survive better as compared to other bacteria in a natural fermentation. These microbes can be found in soil, water, dairy, meat, fermented meals made from grains, animal gastrointestinal tracts, and plant components (Yu et al., 2020). Their versatility and metabolic variety enable them to live in various environments.

The LAB are also found in marula fruits (Maluleke et al., 2023), they ferment glucose primarily to lactic acid, carbon dioxide and ethanol. Hence, they are found at the initial stages of fermentation where sugar is still abundant. The malolactic fermentation is initiated by the LAB to begin the wine-making process. Yeast cells are employed to initiate the alcoholic fermentation process in most fruit wines following the malolactic fermentation. L-malic acid (dicarboxylic acid) is mostly converted into lactic acid as part of the malolactic fermentation process (monocarboxylic acid) (Mendes Ferreira and Mendes-Faia., 2020). The presence of malolactic and malic enzymes causes this alteration. They generate growth-inhibiting substances and significant volumes of lactic acid, which enhance the flavour and texture of fermented foods and prevent spoilage microorganisms from thriving in the fermented product (Virdis et al., 2021). It is widely acknowledged that non-*Saccharomyces* yeasts predominate in the initial stages of fruit wine fermentation. Later, as the ethanol level rises, *Saccharomyces* becomes the dominating species. This succession of microorganisms is a result of microbial interactions, competition for nutrients and other intrinsic growth factors, and tolerance to environmental constraints such as excessive acidity (Johansen et al., 2019). As a consequence, microorganisms showing a selective advantage emerge in a given period as the dominant populations during fermentation.

Acetic acid bacteria (AAB) come from the family Acetobacteriaceae, and they are also referred to as the vinegar bacteria (Lynch et al., 2019). Acetic acid bacteria are typically thought of as obligate aerobes (Malimas et al., 2017), and even minimal exposure to oxygen, such as those that happen during racking and transferring procedures, are sufficient to promote significant growth of these bacteria (Gomes et al., 2018). *Acetobacter* and *Gluconobacter* are the two genera of AAB that are significant to the wine industry. *Gluconobacter* prefer sugar and seem to be sensitive of high ethanol concentrations and are typically isolated from grapes and must which is most likely the case with marula fruits. On the contrary, *Acetobacter* species are more tolerant of ethanol and may survive fermentation to negatively impact the quality of wines (Lynch et al., 2019). Acetic acid bacteria frequently cause the undesirable vinegary deterioration of wines by producing acetaldehyde and acetic acid from oxidation of ethanol (Gomes et al., 2018).

Bacteria involved in alcoholic fermentation are mostly LAB and AAB. However, there is a bacterium *Zymomonas mobilis* which is also able to carry out alcoholic fermentation (Nassir et al., 2021). *Z. mobilis* is a naturally occurring ethanol producing microorganism that possesses many positive attributes for an industrial biocatalyst, including high specific productivity, high alcohol tolerance, a wide pH range for production (pH 3.5 – 7.5), and the status of being generally considered as safe (Yang et al., 2016).

2.10 A wine starter culture

A starter culture is defined as one or more strains of one or more species of desirable bacteria or yeast or a combination of both that is used to inoculate a raw or pasteurised product to start a fermentation process in food or beverage production (Yadav et al., 2019). In essence, starter cultures are those microorganisms that initiate and carry out the desired fermentation. They may also be described as preparations of live microorganisms or their resting forms (Krieger-Weber, 2017), whose metabolic activity has desired effects in the fermentation substrate (Sottil et al., 2019).

There are different types of starter cultures which can be grouped into bacteria, yeasts and moulds. Furthermore, these starter cultures can be grouped into categories based on composition of microflora, growth temperature, type of products, flavour production and type of fermentation (Thunell and Sandine, 2017). With regards to the composition of the microflora, the starter culture can be a single strain where only a single organism is used, a paired compatible strain where two strains of cultures with complementary activity at known proportion are used, mixed strain which uses more than two organisms which may have different characteristics with unknown proportion and a multiple mixed strain wherein more than two strains in known proportion are used (Parente et al., 2017).

The starter medium often consists of a culture medium, such as grains, seeds, or nutrient solution that has been well adapted by the microorganisms utilised for the fermentation. The type of starter culture used depends on the desired product (Krieger-Weber, 2017) and its behaviour may be predicted because it would have been studied and its concentration and volume would be known which will help in predicting what would happen when used in a certain volume of fermenting medium

(Krieger-Weber, 2017).

2.11 Use of a starter culture in wine production

Starter cultures are employed as a tool to control fermentation because in many cases the behaviour of the starter culture can be predicted based on the type of starter culture used (Pietrafesa et al., 2020). For alcoholic fermentation, yeast starter cultures are used to turn carbohydrates into ethanol and other metabolites. *Saccharomyces* yeasts are frequently utilised in the production of wines. These yeasts are favoured for their high fermentative capacity, quick fermentation rates, minimal risk of fermentation spoiling and the ability to withstand alcohol (Albergaria and Arneborg, 2016). However, *Saccharomyces* yeasts have limited sensory qualities and this has then led to the interest in developing new yeast strains to diversify flavours and the final alcohol content as well (Liu et al., 2021).

Non-*Saccharomyces* yeasts which are also known as non-conventional yeasts are of great interest to the alcoholic beverage industry because of their contribution to aroma complexity and higher yields of desired chemicals (Maicas, 2020). These non-*Saccharomyces* yeasts such as *Torulaspora delbrueckii, Lachancea thermotolerans*, and *Metschnikowia pulcherrima* have previously been considered as contaminants due to the production of undesirable metabolites by many of the species currently known (Maicas, 2020). Nonetheless, a fresh look at these yeast strains has revealed production of metabolites that positively impact wine quality. Hence, *Saccharomyces* and non-*Saccharomyces* mixed fermentations have recently been suggested as a possible method to obtain a wine with more desired attributes (Minnaar et al., 2019). Essentially, the *Saccharomyces* yeast will contribute the alcohol content while flavour attributes will be provided by the non-*Saccharomyces* yeasts.

2.12 Determinants of wine quality

Wines that have just been fermented lose their yeasty and fizzy qualities as they age, while also losing turbidity and developing better microbiological and colour stability in red wines (Tao et al., 2014). The wine's fresh fruity fragrance gradually diminishes

over time, usually after bottling. The results of aging are quite desired if they are connected to the emergence of a favoured aged scent and a smoother mouthfeel.

Wine quality can be described as the extent to which a wine is considered to have desirable attributes. This is a subjective concept since it depends on what a wine consumer finds to be pleasant and thus makes it difficult to identify. Different preferences are borne from different sensations when it comes to wine parameters. Characteristics such as a wine balance, intensity of flavour and finish, complexity, as well as typicity are used in determining the quality of wine (Belda et al., 2017). High quality wines are well balanced (Petropoulos et al., 2017) i.e., acidity, tannins, sugar/sweetness, fruitiness and alcohol levels do not overpower each other (Reynolds, 2022). Wines that take longer to reach maturity are well balanced. On one hand, rich flavours are associated with high quality wines and flavours of quality wines are complex and numerous such that these are intense and can be tasted one after the other for some time after one has swallowed. Flavours that disappear immediately after swallowing indicate a low-quality wine, i.e., a wine with a finish that does not linger and has one or two flavours is considered to be a low-quality wine (Petropoulos et al., 2017).

The aroma of the wine has a great impact on its quality as well and is thought to be the best indicator of wine quality (Gutiérrez-Escobar et al., 2021). The aroma is brought by elements such as the content of volatile molecules, their interactions, the chemical and physical effects of non-volatile wine matrix components (Ferreira et al., 2022) as well as individual perceptions of the scent. Importantly, the aroma perception is divided into primary, secondary and tertiary elements (Wimalasiri et al., 2022). The aroma perceived at primary level occurs naturally in the fruit. In grape wines primary aroma will be extracted in grape varieties during the production process. Secondary aroma of the wine comes from compounds released by the yeast during fermentation, while some aroma only develops later during aging due to oxidation and this is regarded as tertiary aroma perception (Petronilho et al., 2020).

The most efficient method used by well trained and experienced wine judges to assess wines is the sensory evaluation method where parameters such as taste, colour, smell and texture are evaluated. However, the development of an objective quality grading

method would be of great importance in the wine industry (Marangon and Kallithraka, 2021).

2.13 Aroma compounds in wine

Aroma compounds that have been identified in wine were classified into different chemical families and there are several chemical families of aroma molecules. Higher alcohols and esters are the two most significant groups of volatile compounds in wine. There are a variety of other compounds including carbonyls, acids, terpenes, norisoprenoids, sulphur compounds and methoxypyrazines (MPs) (Perestrelo et al., 2020). Each family of aroma molecules, as well as the complex odourless matrix in which these compounds are dissolved, differs significantly between various wine types (Wimalasiri et al., 2022), with various dominant odours in each case, giving each wine a distinct typicity (Perestrelo et al., 2020).

Aroma is made possible by different kinds of chemicals that form and some are suppressed during wine production. These include terpenes such as linalool, geraniol which give citrusy and rose petal flavours (Liu et al., 2021), aldehydes such as hexanol and hexenal which give rise to scents associated with freshly cut grass (Perez-Jimenez et al., 2020). The reactions between alcohols and acids give rise to several esters such as isoamyl, ethyl, octyl and butyl acetate. Esters are associated with pleasurable aromas, such as fruity or floral aromas and improve the quality of wines made from neutral grape varieties with low varietal aroma characteristics. However, other esters such as ethyl acetate are considered to be very undesirable when they dominate the aroma of wine (Prusova et al., 2022). Moreover, ketones such as diacetyl as well as lactones add a melted butter aroma in wine and the released aroma is described as spicy, nutty and toasty respectively (Perez-Jimenez et al., 2020).

Undesirable compounds such as 4-ethylphenol and 4-ethylguaiacol that are produced by *Brettanomyces* yeasts add smell that is related to band aids as well as clove and bacon respectively. Sulphur compounds in wine add unpleasant notes as well such that dimethyl disulfide is perceived as producing corned corn and cabbage notes (Prusova et al., 2022).

2.14 Purpose of study

Wine quality is influenced by a number of interrelated elements, with fermentation environment being majorly important in wine production. Colour and clarity, aroma, flavour are some of the important factors in determining wine quality. Temperature is important for the development of colour (Sadras et al., 2013), hence fermentation, maturation and ageing temperatures should be optimised to achieve the desired colour. On the other hand, aroma and flavour are enhanced by utilisation of lower temperatures and sugar concentration that exceeds 24 ºBrix. Temperature may directly affect the aromatic quality of white wine, favouring either fermentative or varietal scent (Dias et al., 2012).

Traditional marula fruit wine is made through spontaneous fermentation, which results in batch variances due to environmental variables (Motlhanka et al., 2018) that could lead to variations in contributing and fermenting autochthonous microbiota. In the wine industry, starter cultures are frequently used, solely for the purpose of guaranteeing that a certain product's quality remains constant (Barata et al., 2012). The traditional marula fruit wine is produced only through primary fermentation and has low alcohol levels in the range of 3 – 5% (Dlamini and Dube, 2008; Hiwilepo van Hal et al., 2013; Maluleke et al., 2023; Phiri et al., 2022). Commercial wines have alcohol levels ranging from 12 – 15%. The dilution of the juice to increase its volume prior to fermentation consequently produces marula fruit wine with low alcohol content from the low amount of fermentable sugars. Supplementing the sugar content of the fermenting material is a common practice in the wine industry across the world, especially with non-fruit wines such as rice wine called sake (Mateus et al., 2020) or fruits of low sugar content such as kiwi fruit and melon wines (Fundira, 2001). The addition of sugar leads to an increase in the biomass of fermenting yeasts, which in turn proportionally increases their metabolites, including alcohol.

This study sought to improve the alcohol content of marula fruit wine through the use of undiluted marula fruit juice which has been organically clarified and augmented with syrup to increase its sugar content. Autochthonous fermenting yeasts strains from marula fruit wines (Maluleke et al., 2023; Phiri et al., 2022) were used as inoculum. It is worth mentioning that autochthonous isolates have the advantage of being well

adapted to the fermenting matrix.

The production and development of some of the important chemical characteristics of wine are also greatly influenced by the temperature at which the wine is fermented (Torija et al., 2003). In addition, compounds related to fresh and fruity flavours in wines are produced in different growth stages of fermenting microorganisms and with the fermenting temperature still being a major factor (Molina et al., 2007*)*. Although commonly known that the development of flavour compounds is due to the bioconversions of chemicals during the fermentation process and this being attributed to microbial activity to a certain extent, the full effect of temperature on the enhancement of aroma compounds in wine is still not fully understood (Beltran et al., 2006). Thus, suitable temperature is important to balance the rate of fermentation progression and the production of good wine character.

CHAPTER 3: MICROBIAL ANALYSIS

Microbial dynamics during fermentation and storage of marula fruit wine

3.1 Introduction

Marula fruit wine is an alcoholic beverage that is produced through fermentation of marula fruit juice. The process of fermentation entails the breakdown of large organic molecules into simpler ones, mainly through the action of microorganisms. Carbohydrates, mainly in the form of sugars, are converted to alcohols or acids with the aid of enzymes and in minimal oxygen (Canilha et al., 2012). The biochemical changes that take place during fermentation are a natural method of enhancing the nutritive value of food products. Expectedly, different active microorganisms that are involved in the fermentation process contribute different metabolites from the bioconversion of substances, mainly the complex organic molecules. For instance, enzymes produced by yeasts commonly catalyse the transformation of proteins into peptides and amino acids while converting sugars and starches into alcohol (Sharma et al., 2020).

In alcoholic fermentation, numerous microorganisms co-exist and may interact directly or indirectly by modifying their common environment to produce an alcoholic beverage such as wine (Johansen et al., 2019). Alcoholic fermentation, such as in marula fruit wine production can be spontaneous or starter-culture based. Notably, spontaneous fermentation, which occurs naturally, has various limitations such as lack of control of microorganisms involved in the fermentation process that could lead to off-flavours, little or no reproducibility of the process and consequently variations in the product (Virdis et al., 2021).

Microorganisms that are involved in wine production have been explored in various studies at fermentation and maturation level. *Saccharomyces cerevisiae* species has been the mainstay in wine fermentation. *S. cerevisiae* can tolerate fermentation stresses, can increase the complexity of the wine and can survive well and out compete the wild yeasts in the fermentation medium (Krieger-Weber, 2017). On the contrary, the non-*Saccharomyces* yeasts strains seldom carry out alcoholic

fermentation but can be beneficial for the development of good wine attributes. Non-*Saccharomyces* yeasts have slow fermentation progression rate, but they positively contribute towards the development of flavours as well as sensory attributes of the wine (Berbegal et al., 2018).

The non-*Saccharomyces* are mostly used together with *Saccharomyces* species because they cannot tolerate high levels of alcohol. The use of both these yeast strains can prevent stuck fermentation as well as nutrients depletion (Blanco et al., 2021). An example of important non-*Saccharomyces* yeasts include members of the *Hanseniaspora* species*. H. uvarum* is one of the most well-known non-*Saccharomyces* yeasts in fermentation processes (Lee et al., 2020). However, it has some limitations in the production of wine because it produces ethyl acetate and acetic acid (Tristezza et al., 2016). Another species, *H. vineae,* has shown to have better attributes as it can withstand high levels of ethanol and contribute desirable attributes such as floral characters (Zhang et al., 2022).

Some studies in our research laboratory have investigated microorganisms that play a role in the production of traditional marula fruit wine (Maluleke et al., 2023; Phiri et al., 2022), whereas others used autochthonous microbes in starter culture-based marula fruit wine productions (Lekganyane, MA pers. com; Molautsi, PM pers. com; Ramafalo, DM pers. com). Improvement of the quality and stability of marula fruit wine, through the use of specific desirable yeasts, was investigated in this study and knowledge of the microorganisms that remain active post-fermentation of marula fruit wine was of interest for the quality and sensorial character of the resulting wine.

3.2 Methodology

3.2.1 Marula fruit collection

Marula fruits were collected into cardboard boxes from the grounds of University of Limpopo, 23.8888° S, 29.7386° E. Ripe and unripe fruits were collected for immediate and later use respectively. The unripe fruits were kept in boxes used for collection in the laboratory at room temperature.

3.2.2 Marula fruit juice extraction

The ripe marula fruits that were kept in the laboratory were washed with tap water. The skin was removed using a clean metal fork and the juice was aseptically squeezed out manually into a sterilised plastic bucket. The kernels were transferred into the same bucket containing the juice for further extraction. The kernels were then mashed with a wooden spoon to extract more juice from the fleshy part of the fruit and were discarded thereafter. The thick pulp that formed and floated as a top layer was removed with sterilised cups and the juice was sieved with a plastic colander and a mesh strainer that were wiped with 70% ethanol to ensure to remove the fibrous material.

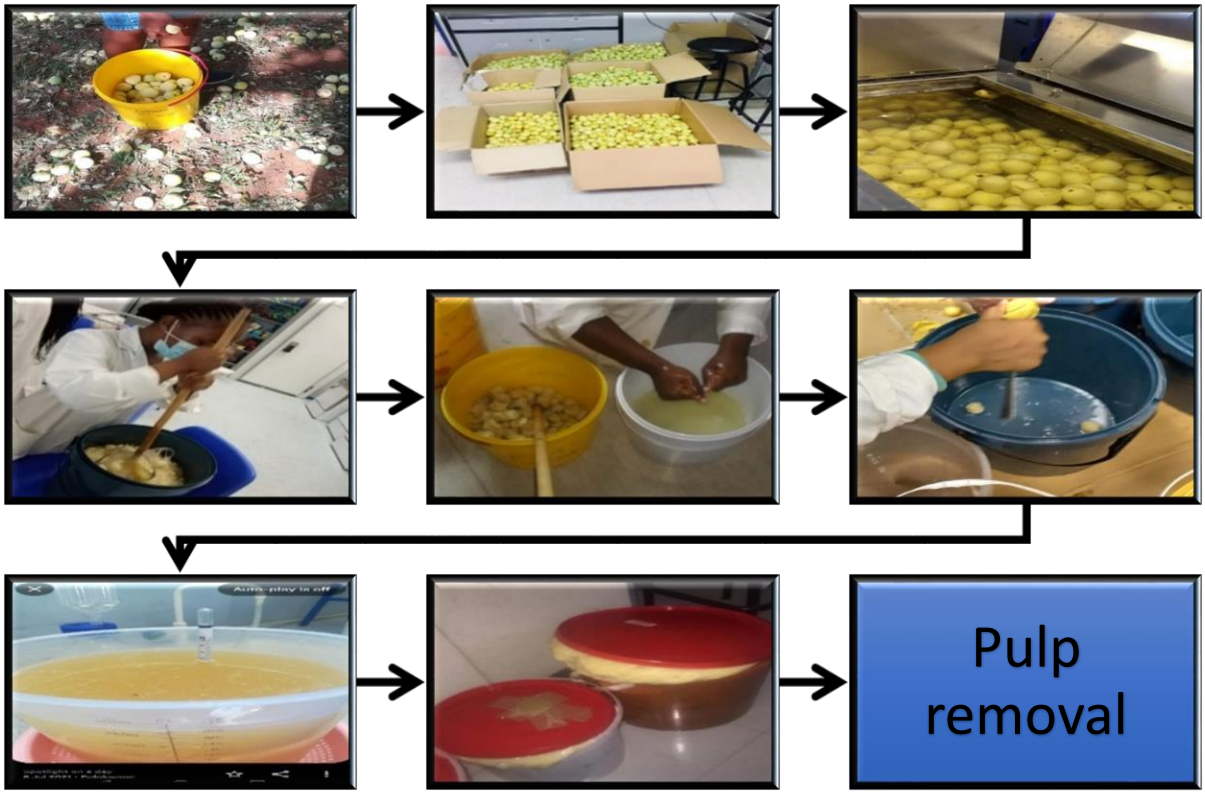

Figure 3.1: Extraction process of juice from marula fruits.

3.2.3 Marula fruit wine preparation

Two yeast cultures which were isolated from marula wine in previous studies were prepared. The identity of the yeast strains and the precise method that was used are

withheld due to the protection under the intellectual property principles (IP policy - University of Limpopo, 2014; WIPO, 2021). These isolates were resuscitated in 200 mL of Yeast Potato Dextrose (YPD) broth for 48 hours at 30 °C in a shaker incubator, followed by inoculating 100 mL of each of the starter cultures into 500 mL of marula juice, and further incubation at 30 °C in a shaker incubator for 48 hours.

The yeast isolates were checked for purity by preparing a wet mount and by Gram staining. Viable counts were performed to determine the pitching rate. Culture media (Sigma-Aldrich) such as YPD agar, Wallerstein Differential (WLD) and Wallerstein Nutrient (WLN) agar plates were prepared as per manufacturer's instructions. A 10-fold dilution factor was used to serially dilute the starter cultures in sterile 0.85% saline solution. Thereafter, 0.1 mL of the diluted cultures were spread onto the different agar plates in duplicates. The number of cells to begin fermentation were 10^6 CFU/mL for each fermenting vessel, excluding the reference wine.

3.2.4 Marula fruit wine production

The prepared marula juice was thoroughly mixed by stirring for homogeneity. A hydrometer was used to check the sugar content of the juice. The 6 L of the juice was kept aside and served as the reference wine. Sugar syrup was added to the juice up to the specific gravity of 1.066 °Brix. The juice was then separated into two different fermenting vessels each containing 16 L of the juice. The 6 L of juice that was kept aside was transferred into its own fermenting vessel and was used as the reference wine with no inoculum. The fermenting vessels were each labelled 25 °C, 15 °C according to the fermenting temperature. The reference wine was fermented at 25 °C. The wines were each racked into different sterile buckets carefully without mixing to avoid transferring lees into the new buckets. The wines were matured at 25 °C,15 °C and 4 °C where each fermentation temperature was divided into two equal volumes i.e., 25 °C wine was divided into equal volumes for maturation at 25 °C and 4 °C and the same for the 15 °C wine. The reference wine was matured at 15 °C. Less lees formation guided whether to continue or stop the racking process. The wines were sampled every 7 days for 5 weeks for the 25°C wine and 3 weeks for the 15 °C wine and reference wine into 50 mL centrifuge tubes and stored at -20 °C for later

use. Sampling was performed every 30 days for a period of 6 months during the storage phase. Two milliliters of the marula fruit wine was aseptically transferred into microcentrifuge tubes at each sampling interval. The wine sample was centrifuged at 10000 rpm for 5 minutes. The supernatant was discarded, and the pellet was washed twice with 0.85% saline solution. The pellet was then stored in 50% glycerol stock solution at -20 °C. Additionally, 10x serial dilutions were prepared from each stock for microbial plating. Thereafter 0.1 mL was plated on different growth media, i.e., Wallerstein Differential media (WLD), Wallerstein Laboratory Nutrient (WLN), Yeast extract peptone dextrose (YPD) and De Man, Rogosa and Sharpe (MRS). The different media were prepared and incubated according to the manufacturer's instructions. Different colonies were sub-cultured on the same media for purification which was ascertained by wet mounts and Gram staining. The colonies with the same morphology were grouped together and only one was selected and sub-cultured and purified. The purified cultures were sent to Inqaba biotechnical industries for identification using 16S sequencing.

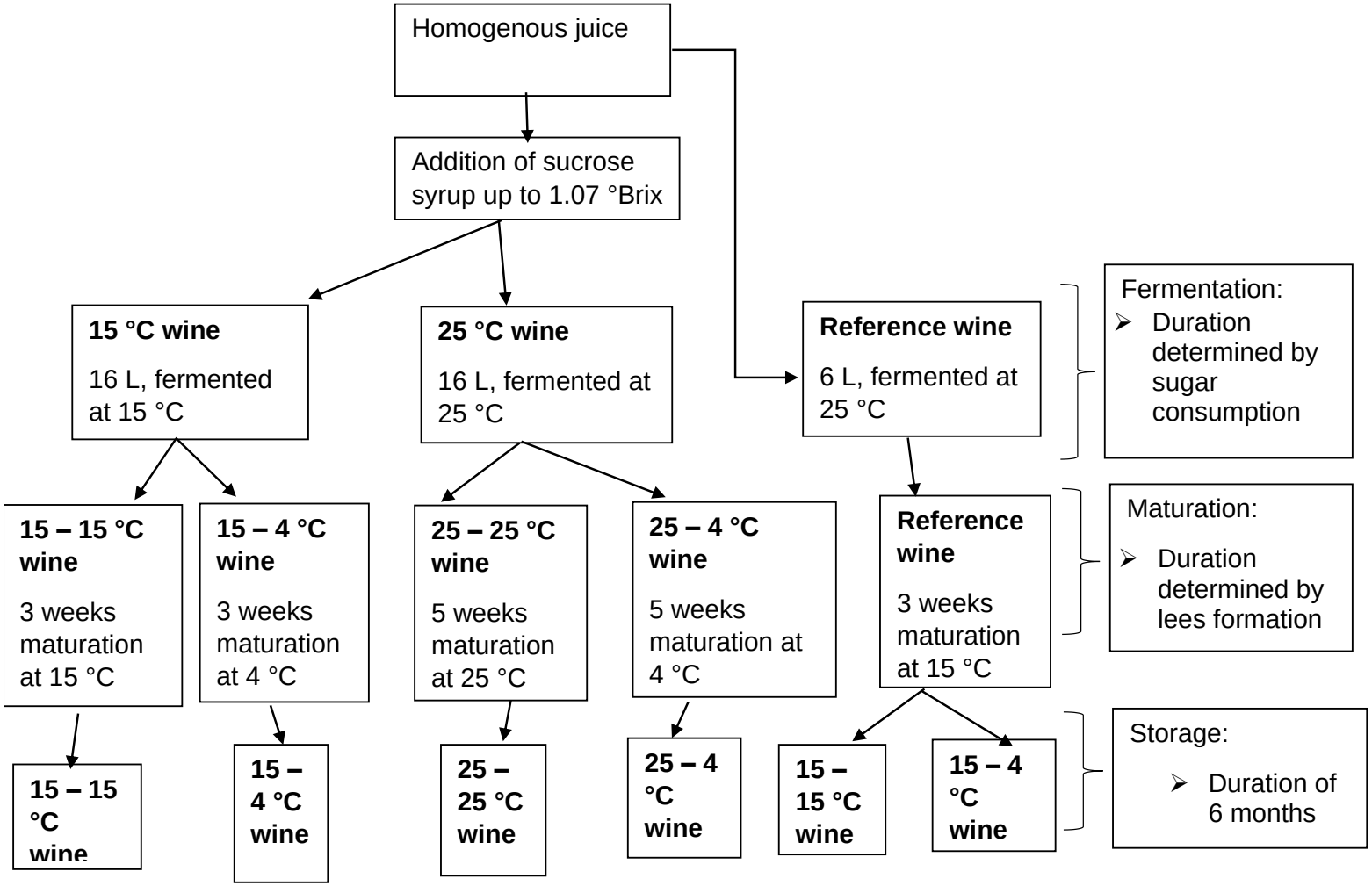

Figure 3.2: Experimental design of the production process

3.3 Results

Comparative analysis was performed between the microorganisms isolated only during the storage phase (bottled wine) i.e., excluding the fermentation and the maturation phase in this study and the microbes that were detected in the primary fermentation period in the previous studies by Maluleke et al. (2023) and Phiri et al. (2022). Notably, the marula wines in the previous studies were produced through spontaneous fermentation, contrary to the current study where a starter culture was used.

The previous studies detected bacterial genera such as *Lacticaseibacillus, Gluconobacter, Lactiplantibacillus, Klebsiella, Lentilactobacillus* and *Acetobacter* from primary fermentation of marula fruit wine (Table 3.1). *Bacillus* emerged to be the dominant genus amidst other various bacterial species detected during the storage phase of marula fruit wine produced through spontaneous fermentation (reference wine) in the current study. Interestingly, this was observed only at the lower storage temperatures of 15 °C and 4 °C. A few *Acetobacter* species were detected as well in the reference wine. On the contrary, *Bacillus* species were detected in all the study storage temperatures for the starter culture-based marula fruit wine. Generally, the bacteria that were observed in the fermentation phase in the previous studies were not detectable except for the acetic acid bacterial species such as *Acetobacter lovaniensis* and *Acetobacter ghanensis. Bacillus* species were not reported in the fermentation phase in the previous studies.

Table 3.1: Microorganisms isolated during fermentation and storage phase of marula fruit wine production.

	Isolates during fermentation phase (Maluleke et al., 2023; Phiri et al., 2022)	**Isolates during storage phase (Current study)**	
		Test marula fruit wine	***Reference marula fruit wine***
Yeasts	*Rhodotorula mucilaginosa* *Meyerozyma caribbica* *Lodderomyces elongiporus* *Saccharomyces cerevisiae* *Pichia guilliermondii* *Hanseniaspora guilliermondii* *Issatchenkia terricola*	15 °C	
		Saccharomyces cerevisiae	
		25 °C	
		Saccharomyces cerevisiae	
		15 – 4 °C	
		Saccharomyces cerevisiae	
		25 – 4 °C	
		Saccharomyces cerevisiae	
Bacteria	*Lacticaseibacillus paracasei* *Acetobacter aceti* *Acetobacter pasteurianus* *Gluconobacter nepheli* *Gluconobacter oxydans* *Asaia bogorensis* *Lactiplantibacillus fabifermentans* *Klebsiella oxytoca* *Lentilactobacillus buchneri* *Lactobacillus brevis* *Lactobacillus plantarum*	15 °C	
		Bacillus subtilis *Paenibacillus glucanolyticus* *Lactobacillus brevis*	*Bacillus amyloliquefaciens* *Bacillus velezensis* *Acetobacter lovaniensis* *Acetobacter ghanensis* *Bacillus licheniformis* *Bacillus paralicheniformis* *Bacillus haynesi*
		25 °C	
		Bacillus velezensis *Bacillus amyloliquefaciens* *Bacillus subtilis*	

	Lactobacillus fermentum	15 – 4 °C	
		Bacillus subtilis *Bacillus amyloliquefaciens* *Bacillus velezensis*	*Bacillus siamensis* *Bacillus subtilis* *Bacillus amyloliquefais* *Acetobacter ghanensis* *Bacillus licheniformis* *Bacillus paralicheniformis* *Bacillus haynesi*
		25 – 4 °C	
		Providencia vermicola *Providencia rettgeri* *Bacillus subtilis*	

Yeast species were not detected in the reference wine (Table 3.1). *Saccharomyces cerevisiae* was detected in all the test wines i.e, in the starter culture-based marula fruit wines stored at different temperatures. However previous studies detected other yeast genera such as *Rhodotorula, Meyerozyma, Lodderomyces, Pichia, Hanseniaspora* and *Issatchenkia* from primary fermentation of marula fruit wine (Table 3.1).

3.4 Discussion

Traditional marula fruit wine is produced from diluted marula fruit juice through spontaneous fermentation. Spontaneous fermentation involves various microorganisms, mostly yeasts and bacteria, with different nature, form and metabolic properties that may be introduced by fruit flies and through human handling (Mashau

et al., 2022). The aim of this chapter was to investigate microbial dynamics during fermentation and storage of marula fruit wine that was produced using starter culture and subjected to varying temperatures. It is also worth noting that the method of sub-culturing as described in the method could have affected the microorganisms detected at storage phase. Only suspended microorganisms could be detected because, by protocol, the wine could not be mixed to homogeneity prior to sample collections. This infers that active microorganisms present in lower numbers may be missed and sedimentation as well could be a plausible reason for missing some the active microorganisms if present.

Maluleke et al. (2023) and Phiri et al. (2022) performed basic studies that comprehensively investigated the microbial populations of both yeasts and bacteria in traditional marula wines obtained from various communities in the Limpopo province. The reference wine in this study was produced following the manner of production of the traditional marula wine as produced in the communities, except that autochthonous yeasts were used as a starter-culture.

The presence of microorganisms in the post-fermentative stage of wines may lead to wine spoilage. *Saccharomyces* is regarded as the spoilage organism if it occurs in wines with residual sugars (semisweet wines) wherein it causes re-fermentation (du Toit and Pretorius, 2000). The fructose residual sugar was at a range of 4.2 – 5.2 g/L for the 15 – 15 °C wine and 15 – 4 °C wine, and 3.2 – 4.2 g/L for glucose. The residual sugars for 25 – 25 °C and 25 – 4 °C wines were 0.9 – 2.4 g/L for fructose and 3.5 – 4.2 g/L for glucose. *S. cerevisiae* was observed in all the experimental ferments excluding the reference wine in the current study. *S. cerevisiae* can survive aerobically and anaerobically depending on the sugars it is utilising (Salari and Salari, 2017). Moreover, *S. cerevisiae* can survive in the presence of high amounts of ethanol. However, marula fruit juice does not have high amounts of sugar (Legodi et al., 2022), and consequently the alcohol content in the traditional marula fruit wine is generally low 5% (Maluleke et al., 2023).

Alcoholic beverages may be spoiled by undesirable strains of *S. cerevisiae*; some strains can produce sulphurous compounds (Garcia et al., 2016). During wine fermentation, indigenous *S. cerevisiae* strains may produce undesirable

characteristics such as haziness which is a common spoilage feature in alcoholic beverages however it was not observed in this study. *S. cerevisiae* killer strains can also prevent the growth of inoculated species (Malfeito-Ferreira, 2011). *S. cerevisiae* can spoil wine by producing volatile phenols contributing to off flavours in the wine. The volatile phenols are produced as a result of decarboxylation of free hydroxycinnamic acids in the clarification process such as those in grape wine (Malfeito-Ferreira, 2011).

Previous studies on marula wines documented bacterial genera such as *Lacticaseibacillus, Acetobacter, Gluconobacter, Asaia, Lactiplantibacillus, Klebsiella, Lentilactobacillus* and *Lactobacillus* that were detected during fermentation. Interestingly, various species of *Bacillus* were detected at different temperatures in stored wines in the current study, more so in the reference wine than the study wines. *Bacillus* species such as *B. amyloliquefaciens, B. velezensis* and *B. haynesi* are found in the soil and can grow aerobically or are facultative aerobes as in the case of marula fruits. Although some like *B. amyloliquefaciens* grow optimally at temperatures of 37 – 40 °C, they can tolerate acidic conditions (Plabon et al., 2021) which is why they still thrive at temperatures not favourable for their growth. *B. velezensis* has been previously reported in marula wine by Maluleke et al. (2023) and Phiri et al. (2022). Furthermore, *B. velezensis* has also been characterised to have antimicrobial compounds (Modikwe et al., 2021).

B. licheniformis and *B. paralicheniformis* are both soil bacteria and *B. licheniformis* is a common feature in wines (Zhang et al., 2022). Although it is a mesophilic microbe, it has been able to survive at lower temperatures of 4°C and 15 °C because of its spore forming character (Muras et al., 2021). This microorganism is further described as having positive attributes for sensorial character such as high esters, thus improving aroma of the wine (Nigris et al., 2018; Zhao et al., 2022). Similarly, *B. velezensis, B. amyloliquefaciens and B. subtilis,* which were observed in the experimental ferments of 15 – 4 °C and 25 – 25 °C wines, are common in winemaking (Bartowsky and Henschke, 2008).

There seem to be no difference in the types of microbiota detected in different temperatures of stored marula fruit wines. Random colonies obtained from stored wines using dominant morphologies were selected and identified. This could explain the obvious differences in the *Bacillus* species types at different temperatures. *Bacillus* species were detected in other wines such as cactus pear wine (Ramoba et al., 2022) and grape wine (Du Toit and Pretorius, 2000; von Cosmos et al., 2017). They can be avoided or reduced by improving the sanitation process. The usage of sulphur dioxide has also been commonly practiced to avoid microbial spoilage (Konig and Frohlich, 2009). However, there is a need for research on how to avoid microbial spoilage using organic means since compounds derived from sulphur dioxide have been reported to cause some allergies (Santos et al., 2012). Generally, *Bacillus* species in wines are regarded as contaminants resulting from the sanitation process and contributed by the fruit fly *Drosophila melanogaster*. This can be problematic when these species are found in large quantities as they can be poisonous, however when in small concentrations, their growth does not affect the sensorial characteristics of the wine (Alarcon-Mendez and Boulton, 2001). Knowledge of the actual loads of these bacterial species is important to denote their influence on quality of the resulting wine and to design control strategies.

Of particular interest are the acetic acid bacteria. *Acetobacter* species were detected only in the stored reference marula wine in the current study*. Acetobacter* species are common in grapes (Lynch et al., 2019) which could be the case with marula fruits as well. The acetic acid bacteria emerge and dominate in the latter stages of fermentation in marula wines (Maluleke et al., 2023; Phiri et al., 2022) and they can survive for a long period in wines (Konig and Frohlich, 2009), which explains their presence at the storage phase. Though common in wines, they are undesirable since they produce products such as ethyl acetate and acetic acid (Konig and Frohlich, 2009) which contribute adversely to the quality of the wine.

In this study *S. cerevisiae* yeasts were the common dominant species found in the aging wine. There are other microorganisms which have been reported in other wines different from the ones observed in the current study. However, previous studies on marula wine by Maluleke et al. (2023) and Phiri et al. (2022) detected *Pichia* and

Hanseniaspora spp. Their absence in the current study infers that these species did not survive the primary fermentation stage. The study by Phiri et al. (2022) clearly demonstrated the disappearance of *Hanseniaspora* sp. at the early to mid-fermentation stage of marula fermentation during emergence and dominance of *S. cerevisiae* at the start of alcohol production. The *Pichia* sp. could have not survived the fermentation stage because of not being able to fully utilise the sugar i.e., glucose since most species cannot utilise fructose and also being overpowered by the alcoholic content in the wines (Vicente et al., 2023). Notably, the detection of *Bacillus* species in all the study wines requires further improvement on the sanitation process.

CHAPTER 4: CHEMICAL ANALYSIS

Characterisation of the chemical profile during the different phases of marula fruit wine

4.1 INTRODUCTION

Marula fruit wine is a traditional alcoholic beverage that is produced from marula fruit juice, and it is common in many southern African countries including South Africa, Zimbabwe and Namibia. The beverage is predominantly produced in Limpopo province in South Africa, in localities such as Phalaborwa, Mankweng, and Sekhukhune. The fruit wine is also known as marula beer, mukumbi in Tshivenda, vukanyi in Tsonga, and morula in Sepedi.

There are several factors that contribute to the chemical makeup of wines. While it is common knowledge that chemical components of the fruits naturally contributes to the chemical profile of the wine, of importance are the metabolites that are produced during fermentation through a variety of chemical and physiological reactions (Robinsons et al., 2014). There are also naturally occurring chemicals in wine which react with the post-fermentation microbial products to form compounds that contribute to wine taste and aroma (Torija et al., 2003).

Wine is mostly constituted by a variety of chemical components such as organic acids, esters, aldehydes, alcohols, antioxidants and tannins. Organic acids such as acetic acid, citric acid, lactic acid, malic acid, glutamic acid and their salts and common in fruit wines (Reynolds et al., 2022). Some of these organic acids develop, transform and diminish during different phases of wine production, and are important for the quality and stability of the wine and have a strong impact on its taste and aroma (Rolbes et al., 2019).

Many chemicals play a role in the aroma of the wine. The most desired fruity and floral aroma are contributed by the esters, which are categorised into two major groups that are acetate esters and ethyl esters (Mbuyane, 2017). Ethyl acetate, isoamyl acetate, isobutyl acetate, phenylethyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl

decanoate are some examples of these esters.

Temperature has a particular effect on the production and transformation of chemicals in wines. The wine industry commonly produces wines at a temperature range of 10 – 25 °C and store these at 4 – 5 °C (Chidi et al., 2018). Foremost, temperature affect the rate of growth of the fermenting microorganisms which contribute new chemicals or transform existing chemicals in the fermenting matrix. Higher alcohols are compounds which generally contribute towards flavour in wines (Legodi et al., 2022). Higher alcohols which are usually found in wines include isobutanol, isoamyl alcohol, propanol as well as butanol. Temperature at a range of 5 – 20 °C results the concentration of these higher alcohols at high concentrations (Kucharczyk and Tuszyński, 2018).

Studies have extensively reported on the chemical changes that take place during fermentation and maturation in marula fruit wine (Maluleke et al., 2023; Phiri et al., 2022; Tebeila, 2022). However, the chemical changes that take place after maturation i.e., during ageing have not been reported in marula fruit wine, which is part of the aim of this chapter.

4.2 Methodology

4.2.1 Marula fruit collection and juice extraction

Marula fruits were collected into boxes from the grounds of University of Limpopo, 23.8888° S, 29.7386° E. Ripe and unripe fruits were collected for immediate and later use respectively. The unripe fruits were kept in the boxes used for collection at room temperature. Juice extraction was performed as outlined in chapter 3, section 3.2.2, marula fruit wine preparation and production in section 3.2.3 and 3.2.4 respectively.

4.2.2 Determination of fermentable sugars, acidity and alcohol content

The assays for fermentable sugars, organic acids, volatile acidity, pH were outsourced to Agricultural Research Council-Wine technologies Stellenbosch. Total sugars, fructose, glucose, acetic acid, citric acid, lactic acid, malic acid, tartaric acid, total acids

pH and alcohol were determined with Fourier transformed-infrared spectroscopy attenuated total reflection (FT-IR ATR technology).

4.2.3 Determination of titratable acidity

Ten millilitres of the wine sample were transferred into a 50 mL clean beaker, followed by the addition of 10 mL of distilled water. Thereafter 3 drops of 1% phenolphthalein were added into the beaker containing the wine sample and the distilled water. The contents of the beaker were then slowly titrated with 0.1 N sodium hydroxide until a pink colour was observed. The titratable acidity was then calculated using the volume of sodium hydroxide used to titrate as indicated below. It is noteworthy to state that the sum of titratable acidity and volatile acidity make the total acidity (Phiri et al., 2022).

$$\text{Total acid (\%)} = \frac{ml\ of\ alkali \times normality\ of\ alkali \times 7.5}{weight\ of\ sample\ (g)}$$

$$\text{Volatile acid (\%)} = \frac{ml\ alkali\ x\ normality\ of\ alkali\ x\ 6.0}{weight\ of\ sample\ (g)}$$

4.2.4 Determination of antioxidant activity

The 2.2-diphenyl-1picrylhydrazyl (DPPH) free radical scavenging assay was used to determine antioxidant activity (Leong and Shui, 2002). DPPH at a concentration of 0.2 mmol/L was prepared in methanol. Thereafter, 0.5 mL of distilled water was added into 5 test tubes then 0.5 mL of each wine sample was serially diluted into the 5 test tubes followed by the addition of 1 mL of DPPH into each test tube. The test tubes were then incubated in the dark for 30 minutes. The test samples were measured using a Thermoscientific UV/VIS spectrophotometer at a wavelength of 517 nm. The blank was prepared similarly to the test samples except without the addition of the wine sample. Ascorbic acid was used as a standard with concentrations ranging from 15.625 – 250 µg/mL.

4.2.5 Determination of total tannin content

Tannin content was determined following the Folin-Ciocalteu (Folin-C) reagent method (Tambe and Bhambar, 2014). A volume of 5 mL of the wine sample was dried with a fan for 4 days and then the dried wine sample was weighed. The dried wine sample was then reconstituted to 10 mg/mL with distilled water. A volume of 3.8 mL of distilled water was transferred into a test tube followed by the addition of 50 µL of the 10 mg/mL of the wine sample and lastly 0.25 mL of Folin-C was added. The solution was mixed thoroughly and 0.5 mL of 35% sodium carbonate was added. Thereafter, 5.4 mL of distilled water was added to make up the volume to 10 mL. The contents of the test tube were then mixed thoroughly and incubated for 30 minutes in the dark. The same procedure was followed for preparation of the blank without the addition of the wine sample. Microtitre plate reader (Glomax) was used to measure the absorbance at 725 nm. Gallic acid was used as a standard with concentrations ranging from 0.0625 – 1 mg/mL. Milligram gallic acid equivalence/gram of wine sample (mg GAE/g) was used to express the tannin content which was calculated using the gallic acid standard curve. The experiment was performed in duplicates.

4.2.6 Statistical analysis

GraphPad prism 9.0 was used for statistical analysis where the built-in one-way Analysis of Variance (ANOVA) was used to analyse the difference between the means of the different wine samples and Tukey's test was used to determine the significance among the different means. A p value less than 0.05 was deemed significant.

4.3 Results

The following results indicate the changes in selected chemical characteristics of starter culture-based marula fruit wine as well as the reference wine from the different production phases which are fermentation, maturation and the storage phase. The experimental marula fruit wines were fermented at 25 °C and 15 °C respectively. The reference wine was fermented at 25 °C. The wines were then matured and stored at their respective temperatures used for fermentation and at 4 °C, excluding the

reference wine. The 25 °C wine was labelled 25 – 25 °C °C to represent a wine fermented, matured, and stored at 25 °C whereas 25 – 4 °C °C represents a wine fermented at 25 °C but matured and stored at 4 °C. This is the same description for the 15 °C wine. The reference wine was fermented at room temperature, matured at 15 °C labelled as 15 – 15 °C °C and stored at 15 °C and 4 °C which are labelled as 15 – 15 °C °C and 15 – 4 °C °C respectively.

4.3.1 Total sugars, glucose and fructose concentrations

Total sugars are broken down into several residual sugars. The experimental wines (Figure 4.1 A) started off with more total sugar than the reference wine (Figure 4.1 B). The total sugar starts off as 85 g/L for the experimental and 28 g/L for the reference wine. The residual sugars that were detected from the breakdown were fructose and glucose.

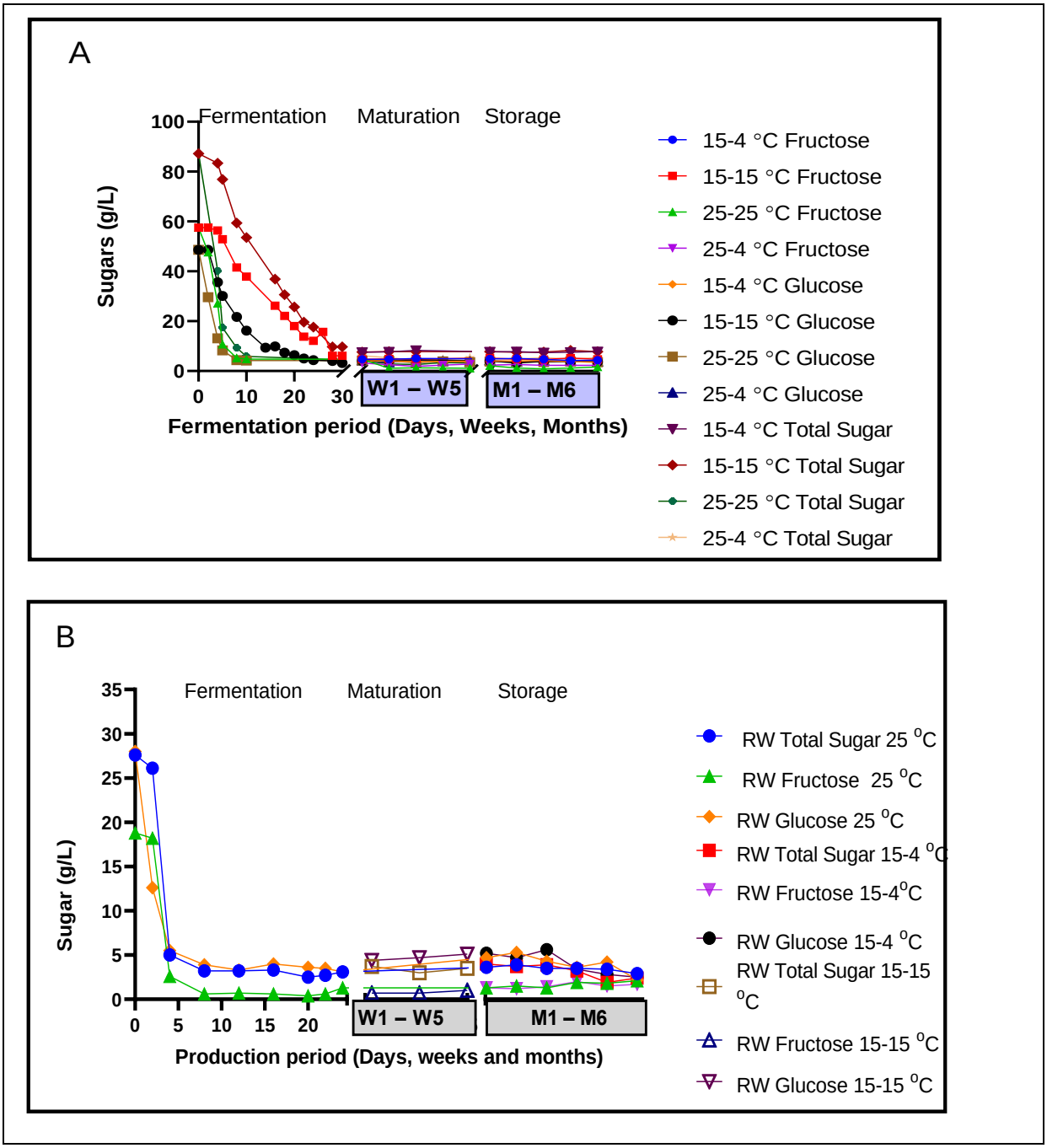

Figure 4.1: Sugar content in the different phases of the production of marula fruit wine. (A) represents the different experimental wines, (B) represents the reference wine. W refers to production period in weeks and M refers to months. RW refers to the reference wine.

Sugar consumption occurred gradually in the fermentation temperature of 15 – 15 °C wine as compared to 25 – 25 °C wine where a rapid decline was observed (Fig. 4.1 A). Glucose was consumed faster than fructose during fermentation in all the

experimental wines (Fig. 4.1 A) except for the reference wines (Fig. 4.1 B). The fermentation rate of the wine fermented at a higher temperature was higher comparatively, i.e., the experimental wines that fermented at 25 °C reached 1.0 ° Brix faster at 10 days when compared to wines that were fermented at 15 °C which took 30 days to reach the same gravity. There total sugar content was significantly different between the 25 – 25 °C and the 15 – 15 °C wines ($p = 0.0498$).

4.3.2 Alcohol concentration

Generally, the alcohol content of all the wines, including the reference wine, increased sharply in the first 2 days, albeit to different levels (Fig. 4.2). The experimental wine that fermented at a lower temperature, i.e., 15 – 15 °C wine, accumulated alcohol at a slower pace comparatively. This is proportional to the rate of sugar consumption observed in figure 4.1.

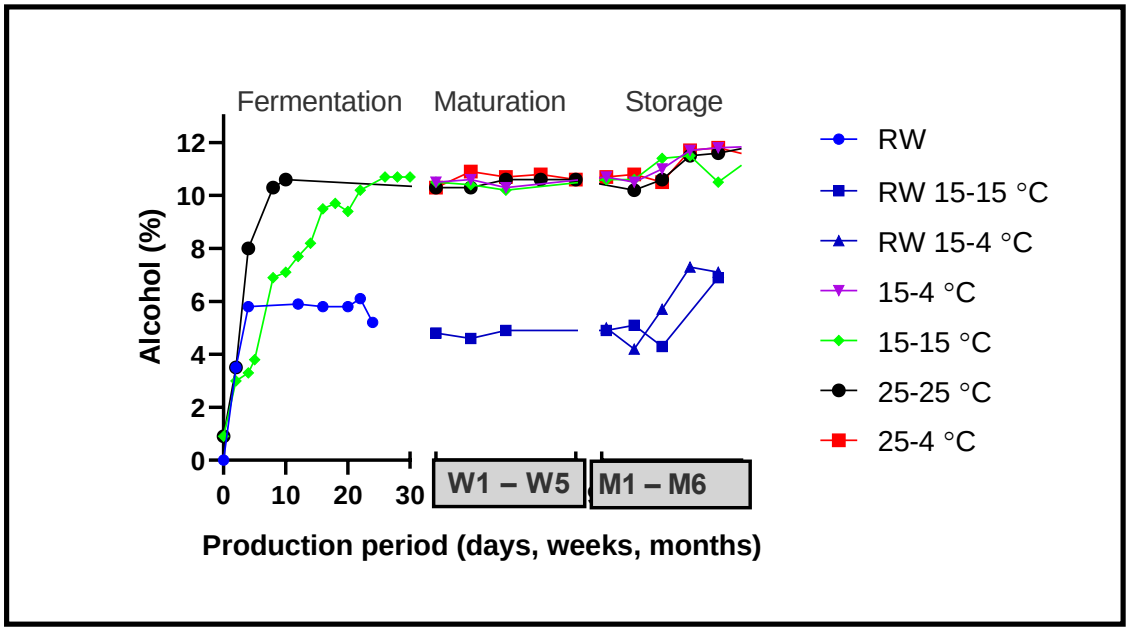

Figure 4.2: Alcohol levels in different phases of the marula fruit wine. W refers to production period in weeks and M refers to months. RW refers to the reference wine.

Alcohol content remained constant in all the wines during the maturation phase and increased non-significantly ($p = 0.0799$) during storage phase from 4.9 to 7.4% for the reference wines and 10.3 to 12% for 25 – 25 ° C wine, 10.3 to 11.9% for 25 – 4 °C

wine (p = 0.7643). There was a non-significant difference (p = 0.999) in alcohol content detected during maturation at 10.3 – 10.8% and storage phase at 10.2 – 12% as a factor of the temperature used for the 15 – 15 °C and 15 – 4 °C wines.

4.3.3 Glycerol concentration

The glycerol range was between 2 – 4.7 g/L in the fermentation phase (Figure 4.3) and ranged from 3.7 – 3.9 g/L in the maturation and storage phase for the experimental wine. The glycerol content increased gradually for both 15 – 15 °C wine and 25 – 25 °C wine during the fermentation and maturation phases. It significantly decreased during the storage phase between the 25 – 25 °C and 25 – 4 °C wines (p = 0.004) as well as with the 15 – 15 °C and 15 – 4 °C wines (p = 0.0364).

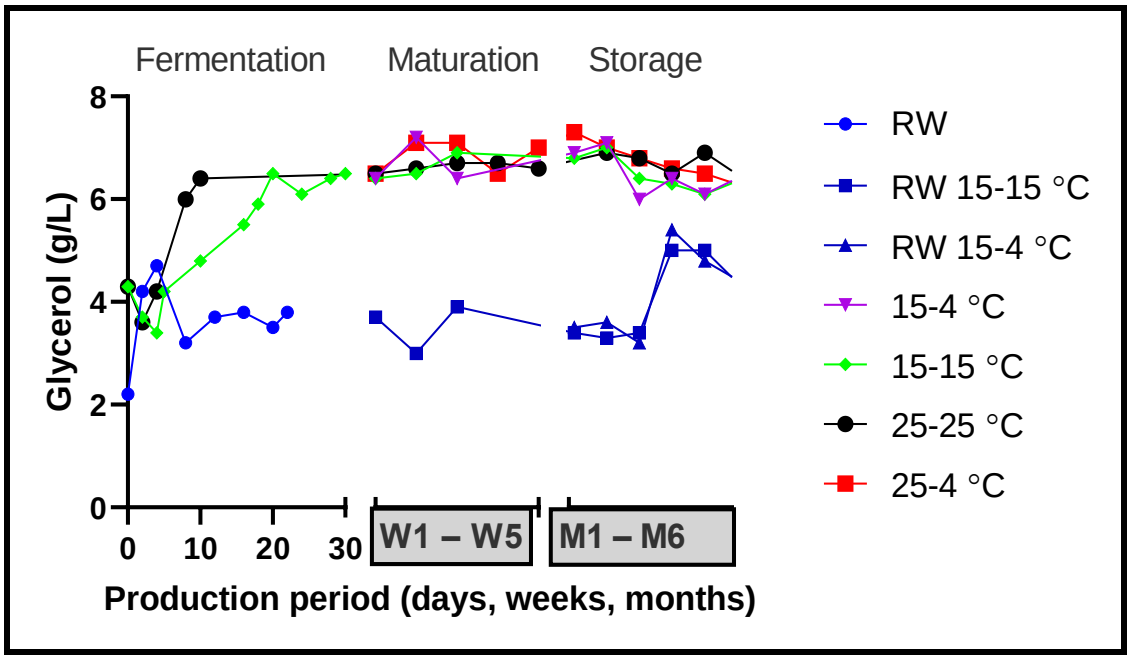

Figure 4.3: Glycerol levels in different phases of the marula fruit wine. W refers to production period in weeks and M refers to months. RW refers to the reference wine.

The glycerol content was between 2 – 4.2 g/L in the fermentation and maturation phase for RW 15 – 15 °C wine and RW 15 – 4 °C wine. It increased sharply in the first few days of fermentation and decreased for RW wine. The glycerol content then

followed a fluctuating pattern (3 – 3.9 g/L) during the maturation phase for the RW. Temperature did not affect the glycerol content since both temperature variations i.e., RW 15 – 15 °C and RW 15 – 4 °C wines followed the same pattern during the storage phase with the content ranging from 3.4 – 5 g/L ($p = 0.5918$) during the storage phase. There was a significant difference between RW 15 – 15 °C and 15 – 15 °C wines ($p=0.0364$) in glycerol content during maturation and storage phase.

4.3.4 Titratable organic acids concentrations

The combination of primary acids, total acidity (Fig. 4.4 A) ranged at 9.6 – 12 g/L in the experimental wines. Titratable acids (Fig. 4.4 E) contributed the most to the total acidity at the range of 5.82 – 6.7 g/L that was at about two-thirds of the total acid contents in the marula fruit wines. In turn, citric acid (Fig. 4.4 C) was the dominating organic acid at 3.28 – 5.70 g/L, which was more than half the titratable acid content. It decreased significantly between RW 15 – 15 °C and 15 – 15 °C wines ($p = 0.0168$) with a percentage difference of 62.68% in the storage phase. There was also a significant decrease in citric acid content between RW 15 – 4 °C and 15 – 4 °C wines in the storage phase ($p = 0.0042$) with a percentage difference of 79.71%. Titratable acidity content (Fig. 4.4 E) followed a rather stable trend in the different production phases for the experimental wines. The malic acid content (Fig. 4.4 D) ranged at 0.98 – 4.3 g/L. Although there was an increase in the malic acid content in the maturation phase, the variance was non-significant.

Tartaric acid (Fig. 4.4 B) was the least dominant, within a range of 0.3 – 1.8 g/L. Interestingly, a sharp decrease in tartaric acid content was observed in the reference wine in the maturation phase i.e., from 1.63 g/L at the end of fermentation to 0.30 g/L in the beginning of the maturation phase. Notably, the reference wine had the lowest primary acid content which contributed to the low total acid content in the reference wine.

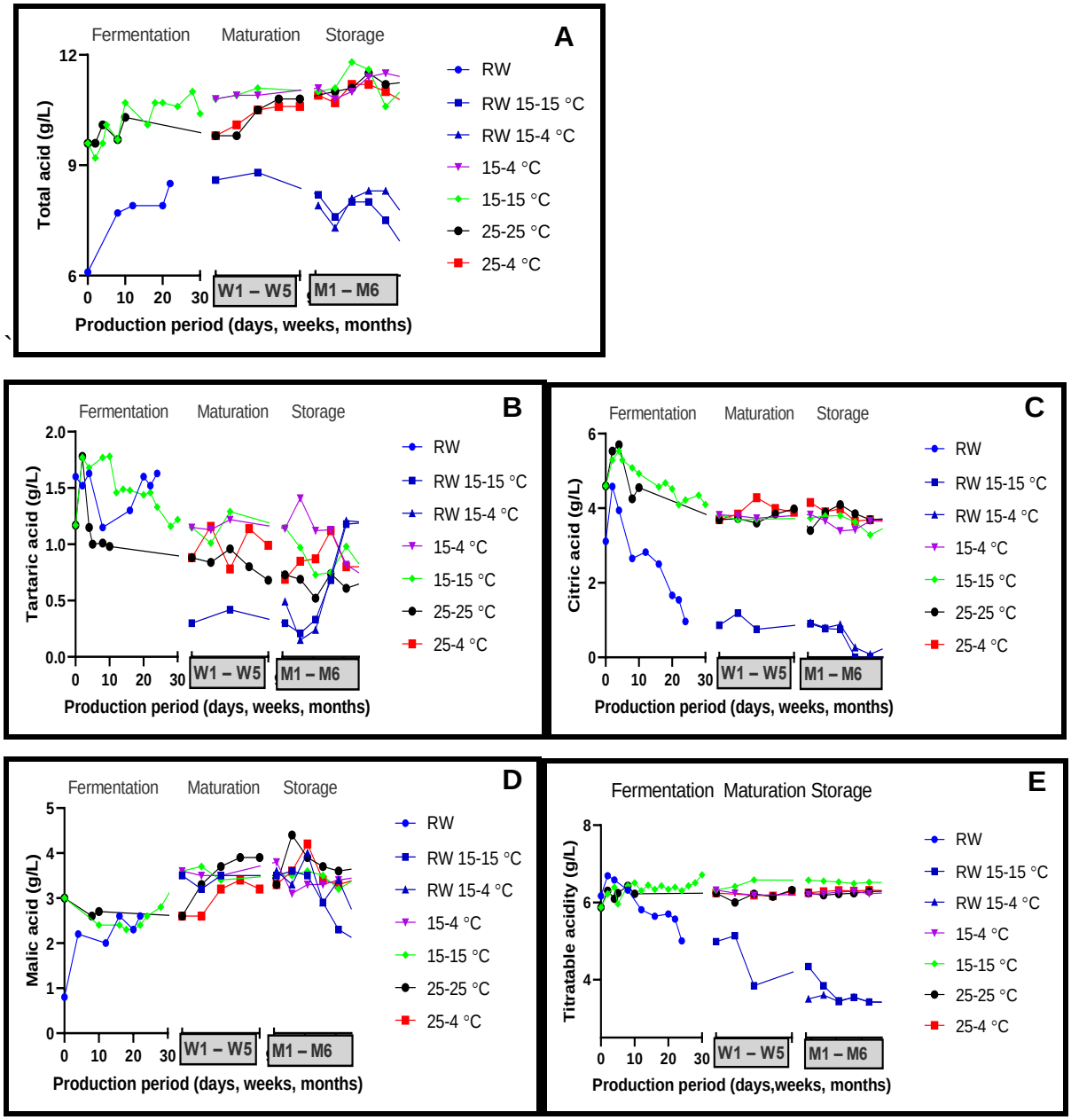

Figure 4.4: Acid content in the various stages of marula fruit wine production. The combination of primary acids (A) total acidity, and primary acids (B) tartaric acid, (C) citric acid, (D) malic acid and (E) titratable acidity. W refers to production period in weeks and M refers to months. RW refers to the reference wine.

4.3.4 Volatile acids concentrations

The volatile acidity component of the marula fruit wines, as represented by lactic and acetic acids in this study, was increasing with progression in fermentation and maturation and storage periods (Fig. 4.5). Lactic acid content increased sharply from 0.59 g/L at the start of fermentation to 2.12 g/L at the end of storage for 15 – 15 °C wine, 2.08 g/L for 15 – 4 °C wine, 2.01 g/L for 25 – 25 °C wine and 1.81 g/L for 25 – 4 °C wine. Notably, the reference wine had the higher content in all the three phases where the concentration ranged from 0.54 g/L during fermentation to 2.2 g/L in the storage phase. The 15 – 15 °C wine had the least lactic acid content throughout. There was a non-significant difference in lactic acid content between RW 15 – 15 °C and 15 – 15 °C wine ($p = 0.6255$) also with RW 15 – 15 °C wine and RW 15 – 4 °C wine ($p = 0.8391$).

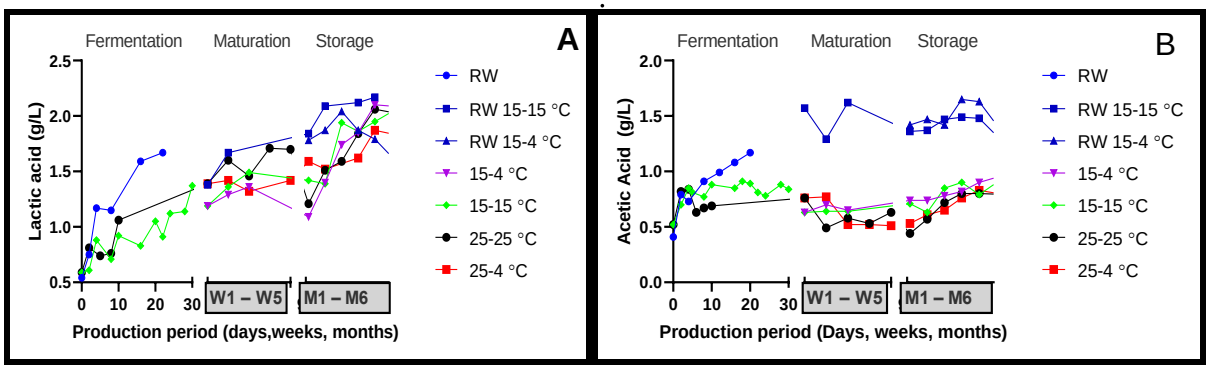

Figure 4.5: Lactic acid (A) and Acetic acid (B) contents during the distinct stages of marula fruit wine production. W refers to production period in weeks and M refers to months. RW refers to the reference wine.

Acetic acid content decreased from 0.84 g/L in the 15 – 15 °C wine at the end of fermentation and 0.69 g/L in 25 – 25 °C to 0.64 g/L at the end of maturation for 15 – 15 °C, 0.65 g/L for 15 – 4 °C wine, 0.63 g/L for 25 – 25 °C wine and 0.51 g/L for 25 – 4 °C wine. Similar to lactic acid content, the reference wine had the highest acetic content in all three phases where the concentration ranged from 0.41 g/L during fermentation to 1.18 g/L and 1.23 g/L for RW 15 – 15 °C and RW 15 – 4°C wines in the storage phase, respectively. There was a significant difference in acetic acid content between RW 15 – 4 °C wine and 15 – 4 °C wine ($p < 0.001$) in the storage

phase with a percentage difference of 1.89%. Whereas a non-significant difference (p = 0.9669) in acetic acid content was observed between RW 15 – 15 °C wine and 15 – 15 °C wine in the maturation phase.

Of the 3 – 6 g/L of volatile acidity in the wines as deducted from the titratable acid content (Fig. 4.4 E) in the total acid content (Fig. 4.4 A), lactic acid contributed about twice the amount more than acetic acid content and together they constituted about half the volatile acidity of the wines.

4.3.5 pH content

pH in the wines was in the range between 3.4 – 3.88 (Fig. 4.6). However, the reference wine had a pH of 3.36 – 4.03 which was higher than the experimental wines in all the different phases.

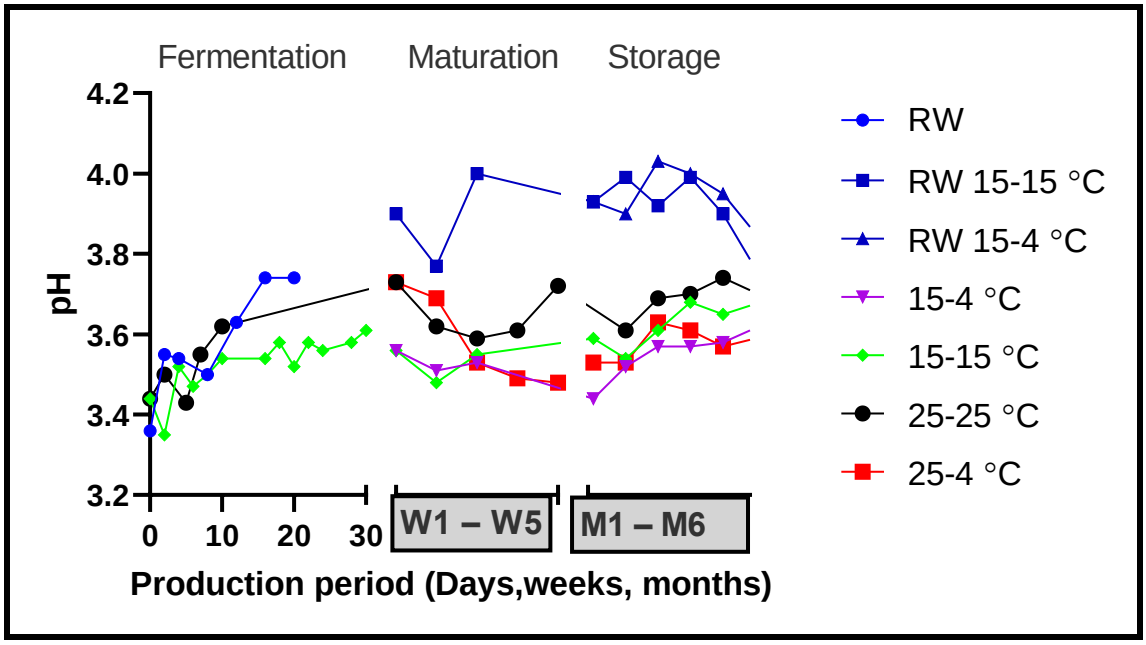

Figure 4.6: pH content during the various stages of marula fruit wine production. W refers to production period in weeks and M refers to months. RW refers to the reference wine.

The experimental wines 15 – 15 °C, 25 – 25 °C had pH values of 3.4 – 3.6 in the fermentation phase. However, at the end of the fermentation phase the 25 – 25 °C had a pH of 3.62 which was higher than pH of 3.52 in the 15 – 15 °C wine. Temperature did not significantly influence the pH in maturation and storage phase between the 25 – 25 °C wine and 25 – 4 °C ($p = 0.6985$). A similar trend for the 15 °C wine indicated that it was not influenced by the temperature as it entered maturation and storage phase. There was a non-significant difference in pH content between RW 15 – 15 °C wine and 15 – 15 °C wine ($p = 0.4431$). Additionally, the pH content between RW 15 – 4 °C wine and 15 – 4 °C wine was not significant ($p=0.4558$) (Fig. 4.6).

4.3.6 Higher alcohol concentrations

Isoamyl alcohol was the dominating higher (fusel) alcohol of all the tested higher alcohols and the lowest in content was n-butanol (Figs. 4.7B and 4.8A). The experimental wines that were produced at a higher temperature, namely 25 –25 °C, 15 – 15 °C have higher content of higher alcohols comparatively except for n-hexanol (Fig. 4.7 and 4.8). Production of all the tested higher alcohols, i.e., 2-phenylethanol, isoamyl alcohol, isobutanol, n-butanol, n-propanol plateaued during maturation and then remained constant for RW 15 – 15 °C wine and RW 15 – 4 °C wine and only isobutanol and n-butanol for 15 – 15 °C wine and 15 – 4 °C wine. Fluctuations were observed in the first three months of storage with an increase of higher alcohols such as 2-phenylethanol, isoamyl alcohol and isobutanol for 25 – 25 °C wine and 25 – 4 °C wine and only isoamyl alcohol for 15 – 15 °C and 15 – 4 °C wines. The increase was non-significant for all these above-mentioned higher alcohols during storage phase, except for n-hexanol which was gradually increasing from fermentation through maturation and in the storage phase as well.

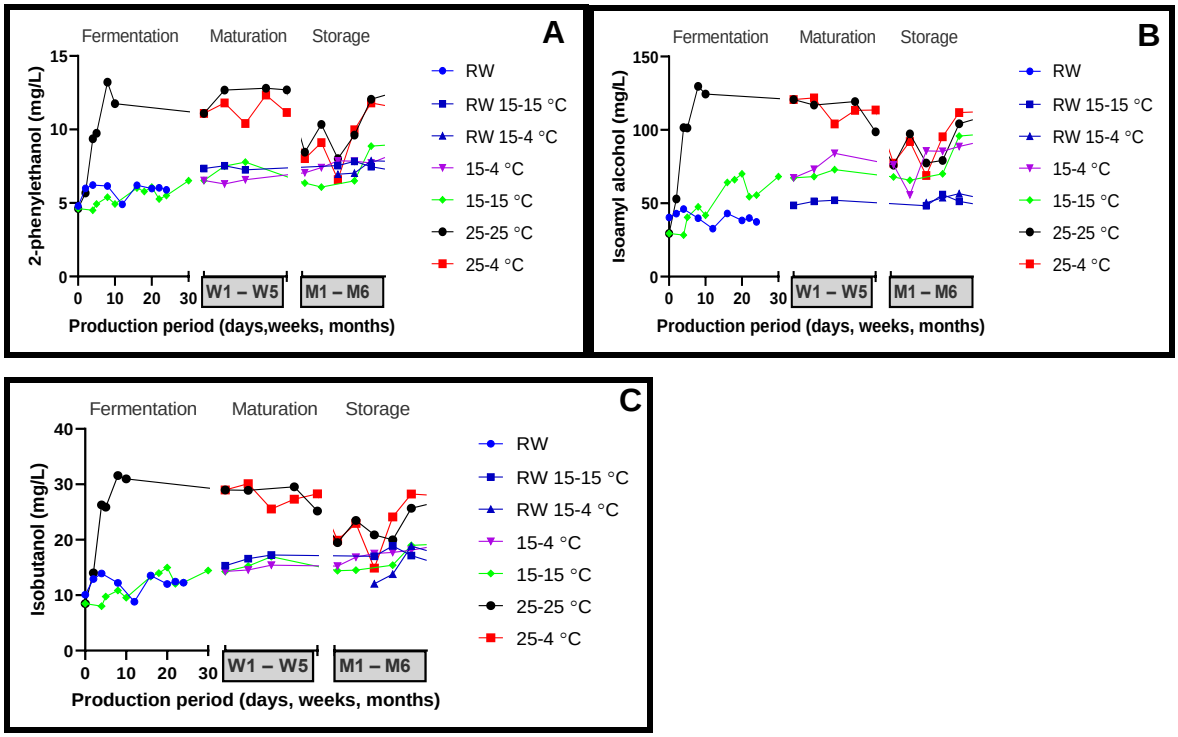

Figure 4.7: Higher alcohols produced in the different phases of marula fruit wine. (A) 2-phenylethanol, (B) isoamyl alcohol and (C) isobutanol. W refers to production period in weeks and M refers to months. RW refers to the reference wine.

The 2-phenylethanol content was higher than all the wines in the fermentation and maturation phase at ranges of 4.62 – 13.22 mg/L and 11.10 – 12.70 mg/L for the 25 – 25 °C wine respectively. (Fig. 4.7 A). The increase in 2-phenylethanol content in the storage phase between the following wines were not significant, namely, RW 15 – 15 °C and 15 – 15 °C ($p = 0.7530$), RW 15 – 4 °C and 15 – 4 °C ($p = 0.4243$), 15 – 15 °C and 15 – 4 °C ($p = 0.5000$) as well as 25 – 25 °C and 25 – 4 °C ($p = 0.0667$). This trend was similarly observed in the isoamyl alcohol content at ranges of 29.48 mg/L at the beginning of fermentation to 120.64 mg/L during the maturation phase and 110.74 mg/L and 112.83 mg/L at the end of storage for 25 – 25 °C wine and 25 – 4 °C wine. The differences were nonetheless not significant between the wines RW 15 – 15 °C and 15 – 15 °C ($p = 0.1511$), RW 15 – 4 °C and 15 – 4 °C ($p = 0.1483$), 15 – 15 °C and 15 – 4 °C ($p = 0.9330$) as well as 25 – 25 °C and 25 – 4 °C ($p = 0.8836$) (Fig. 4.7 B). Additionally, the isobutanol content (Fig. 4.7 C) followed the same trend, the lowest concentration were at a range of 8.47 mg/L at fermentation to a high concentration of 19.28 mg/L at the end of storage phase in RW, 15 – 15 °C and 15 – 4 °C wines.

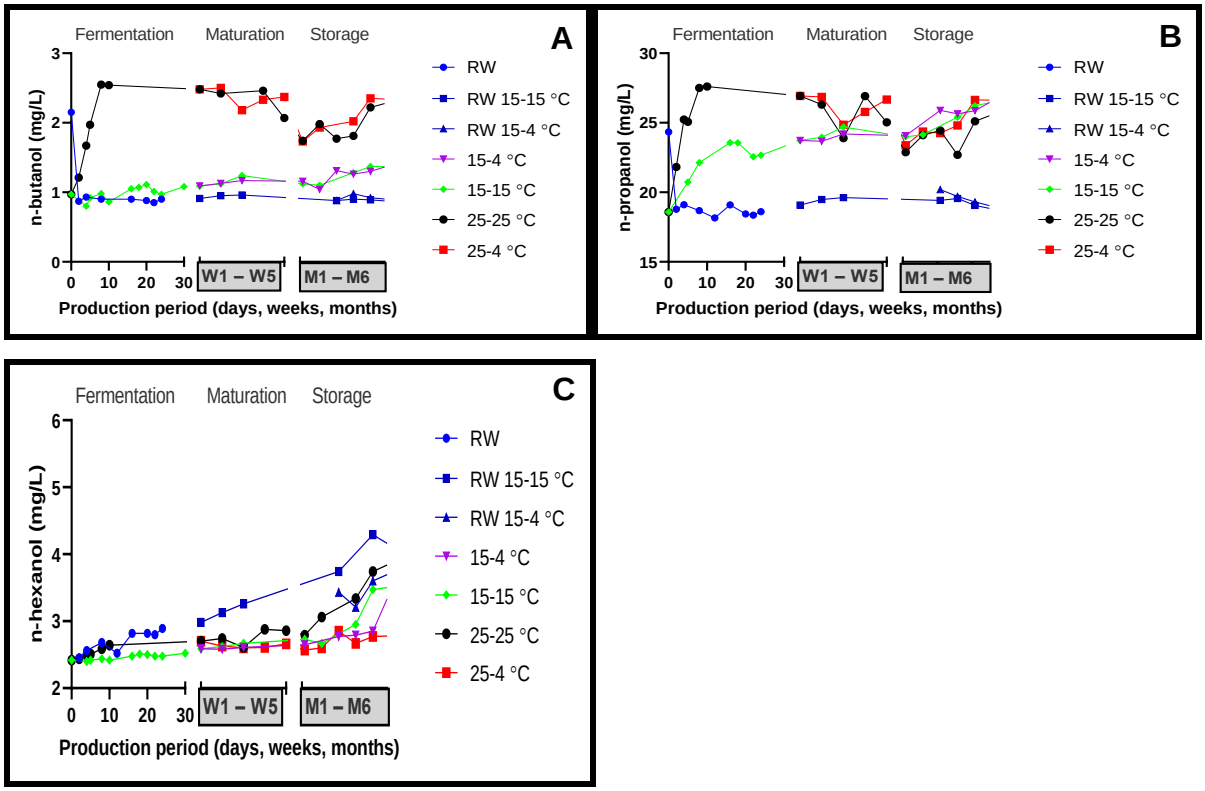

Figure 4.8: Higher alcohols produced in the different phases of marula fruit wine. (A) is n–butanol, (B) is n–propanol and (C) is hexanol. W refers to production period in weeks and M refers to months. RW refers to the reference wine.

Hexanol content gradually increased for all the wines including the reference wine. The hexanol content increased at a range of 2.42 mg/L in fermentation to 3.97 mg/L at the end of the storage phase. Hexanol content was the highest in the reference wine with the concentration at 4.3 mg/L at the end of the storage phase. There was a non-significant difference between the wines RW 15 – 15 °C and 15 – 15 °C ($p = 0.0504$), RW 15 – 4 °C and 15 – 4 °C ($p = 0.3489$), 15 – 15 °C and 15 – 4 °C ($p = 0.1542$) as well as 25 – 25 °C and 25 – 4 °C ($p = 0.1542$) in the storage phase (Fig. 4.8 A). The butanol and propanol contents followed a similar trend, with butanol at a range of 0.98 mg/L at fermentation to 1.45 mg/L at storage phase for the reference wine and the 15 – 15 °C and 15 – 4 °C wines. The butanol content increased non-significantly ($p = 0.0923$) at a range of 0.97 mg/L to 2.55 mg/L at the end of the storage phase between 25 – 25 °C and 25 – 4 °C wines. The propanol content (Fig. 4.8 B) range for the RW wine was low in all the three phases of wine production at 18.6 mg/L – 19.5 mg/L from

fermentation to the storage phase and the increase in propanol content between 25 – 25 °C and 25 – 4 °C wines was non-significant (p = 0.0859) from maturation into storage phase.

4.3.7 Esters concentrations

Levels of ethyl acetate and ethyl lactate were congruent with observed levels of acetic and lactic acid (Fig. 4.5). The range of ethyl acetate content (Fig. 4.9 A) was 80 – 190 mg/L.

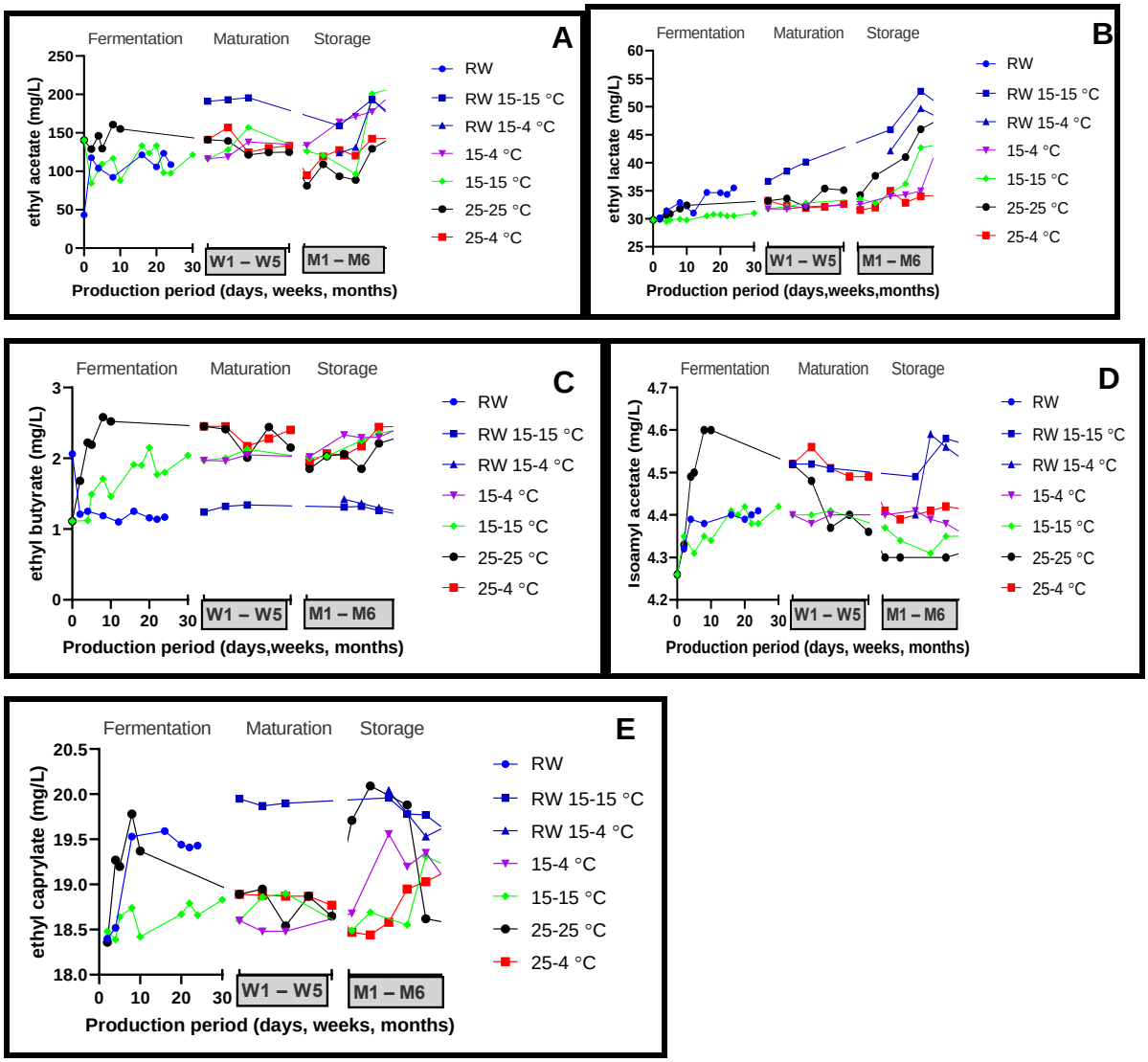

Figure 4.9: Esters produced in the different phases of marula fruit wine. (A) ethyl acetate, (B) ethyl lactate, (C) ethyl butyrate, (D) isoamyl acetate and (E) ethyl caprylate. W refers to production period in weeks and M refers to months. RW refers to the reference wine.

There was a gradual increase in ethyl lactate at a range of 30 – 54 mg/L, and this was significant from maturation into the storage phase between 15 – 15 °C and 15 – 4 °C wines as well as 25 – 25 °C and 25 – 4 °C wines ($p < 0.0001$), with a percentage difference of 2.25% and 1.68% respectively. Other esters were detected at low levels. Ethyl butyrate was at a range of 1.18 – 2.63 mg/L. Isoamyl acetate was detected at a range of 4.26 – 4.60 mg/L, with a significant difference between 15 – 15 °C and 15 – 4 °C wines in the storage phase and 25 – 25 °C and 25 – 4 °C wines ($p < 0.0001$) with a percentage difference of 4.5%. Ethyl caprylate was at a range of 18.4 – 20.2 mg/L with a non-significant decrease between the wines in the storage phase.

4.3.8 Total tannin concentration

The levels of tannins in the marula fruit wine were generally low. There was no apparent trend in tannin contents in the experimental and reference wines (Fig. 4.10). They fluctuated between 0.1 – 0.56 mg GAE/g during fermentation, maturation and storage phase. Varying the temperature did not have any effect on tannin content in the different phases.

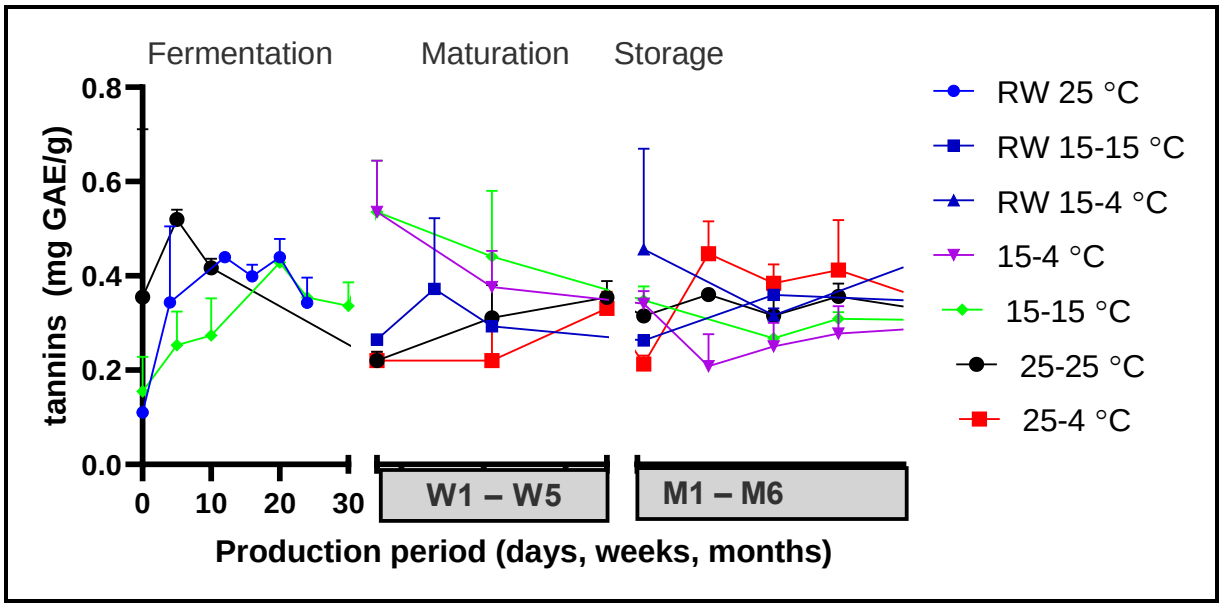

Figure 4.10: Tannin content in the different phases of marula fruit wine production. W refers to production period in weeks and M refers to months. RW refers to the reference wine.

There was a non-significant difference between the RW 15 – 15 °C and 15 – 15 °C wines ($p = 0.8497$), RW 15 – 4 °C and 15 – 4 °C wines ($p = 0.6036$), 15 – 15 °C and 15 – 4 °C wines ($p = 0.2431$) as well as 25 – 25 °C and 25 – 4 °C wines ($p = 0.5582$) from maturation into the storage phase.

4.3.9 Antioxidant activity concentration

The antioxidant activity of the marula fruit wines was low across the different stages of production for all the different wines produced at different fermentation, maturation and storage temperatures when compared to the L-Ascorbic acid (Fig. 4.11). There was no apparent trend for the antioxidant activity in the fermentation phase for the 15 – 15 °C wine. The 25 – 25 °C wine improved with time in the fermentation phase from 659.63 – 445.71 µg/mL. Additionally, temperature did not impact the antioxidant activity in the maturation and storage phase since there was a fluctuation in the EC50 concentration in all the different wines. However, it is worth noting that the antioxidant activity in the 15 – 15 °C wine at the maturation phase had a steady pattern at a range of 300 – 400 µg/mL.

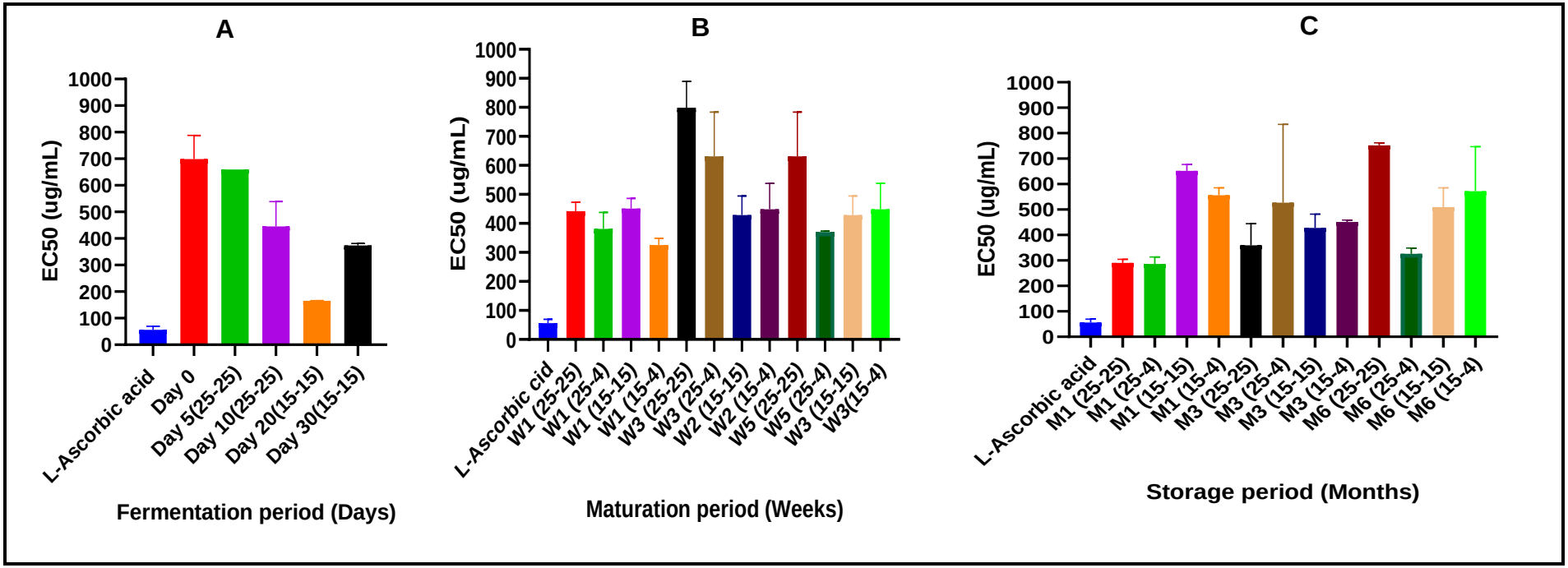

Figure 4.11: The antioxidant activity of marula fruit wine during different production phases. (A) the fermentation phase, (B) the maturation phase and (C) the storage phase. W refers to production period in weeks and M refers to months.

4.4 Discussion

The aim of this chapter was to analyse the presence of fermentable sugars i.e., total sugars, glucose and fructose as well as to characterise organic acidity, volatile acidity, pH, esters, alcoholic content, antioxidant activity and tannin content throughout the different phases of the production period of the marula fruit wine. The production of wine involves the conversion of sugars into alcohol using yeasts. The fermentable sugars in this study were glucose and fructose. The high fermentation temperature of 25 °C led to faster sugar consumption compared to 15 °C which is common in winemaking processes (Mateus et al., 2020). It was observed that glucose was consumed faster than fructose in both temperature variations i.e., 25 – 25 °C wine and 15 – 15 °C wine. This may be due to the fact that the yeasts that drove fermentation had a higher affinity for glucose as compared to fructose. Marula fruit wines had a higher alcohol content at 10.5% during fermentation phase. This is common for commercial wines which is within the desirable range of most commercial wines at 10 – 15%.

The glycerol content is one of the important compounds that contribute to sensorial properties, specifically texture of the wine. The glycerol content was within the recommended range of 4 – 12 g/L (Belda et al., 2015; Mbuyane, 2017) for the experimental wines, albeit low in the reference wine fermented at ambient temperature. Studies reveal that glycerol is mostly produced by non-*Saccharomyces* yeasts (Belda et al., 2015). Additionally, the production of glycerol in non-*Saccharomyces* yeasts were 10 – 14 g/L which was higher than 4 – 10 g/L in a study by (Jolly, 2006). Furthermore, Mbuyane (2017) alluded that non-*Saccharomyces* yeasts produced glycerol, unlike *S. cerevisiae* where the glycerol produced was a result of the sugar already present in the grape. However, the concentrations were desirable since higher glycerol content are associated with contributing to high levels of acetic acid content through redox reactions for osmotic balance that is further maintained through the production of acetic acid (Mbuyane, 2017) which can lead to undesirable off flavours in the wine, compromising its quality. Studies have revealed that *Saccharomyces* strains under oenological conditions were found to be able to consume half the amount of acetic acid subjected to the microorganisms.

Various organic acids, crucial for enhancing the stability of the wine, were identified at diverse concentrations. These primary acids, coupled with the pH levels, significantly influence the overall quality of the wine. Notably, citric acid emerged as a dominant acid among these organic acids, surpassing the conventional concentration range of 0.5 – 1 g/L (Chidi et al., 2018). The high concentration of citric acid, ranging from 3.28 to 5.70 g/L, can be attributed to the inherently rich citric acid content in the marula fruit itself, as elucidated by Legodi et al. (2022). Beyond its impact on acidity, this substantial concentration of citric acid is believed to impart positive effects on the wine. Maintaining adequate acidity is crucial as low acidity in wines can trigger reactions leading to detrimental outcomes such as browning (Chidi et al., 2018). Moreover, during the storage phase of the experimental wines, the elevated citric acid levels potentially played a pivotal role in inhibiting the proliferation of various microorganisms as alluded by Eliuz (2020) for its antimicrobial properties. This preventive action aligns with the findings of Tebeila (2022), which could explain the limited presence of microorganisms detected in the storage phase (Chapter 3 in this study).

Temperature exhibited no discernible impact on the concentration of tartaric acid; rather, the concentrations remained consistently below the typical wine range of 4.5 – 10 g/L, hovering at relatively modest levels between 0.3 – 1.8 g/L. Notably, during the storage phase, there was a non-significant increase in tartaric acid, implying that the increase might be attributed to reactions with other metabolites rather than originating directly from the fruit itself. This observation raises concerns about potential undesirable flavours should the wines undergo aging beyond a six-month period. Evaluation of wine during bottle storage indicates degradation of compounds and browning index (Ferreira et al., 2013).

The acidity profile of the wines is further shaped by the pH, which fell within the desirable range of 3.44 – 3.88. This aligns with the common pH range of 3.1 – 3.9 reported by Legodi et al. (2022), as higher pH levels can potentially encourage the proliferation of undesirable microorganisms. Additionally, the observed pH range in this study correlates with an overall elevated total acidity, a factor intricately linked to pH. Lactic acid and acetic acid concentrations were well within the established wine norms, ranging from 1 – 3 g/L and holding a threshold of 2.1 g/L, respectively. Notably, there was no discernible conversion of malic acid into lactic acid, contributing to the

observed low lactic acid concentration. The absence of spoilage microorganisms associated with a vinegary taste in the study coincided with the recorded low acetic acid concentration, further underscoring the overall quality and stability of the wine.

Higher alcohols emerged as significant constituents, with their concentrations influenced by fermentation temperature. Notably, 2-phenylethanol, isoamyl alcohol, isobutanol, 1-propanol, n-butanol, and hexanol were identified among these alcohols. While the temperature variance did not yield a statistically significant difference, the 25 – 25 °C wine exhibited higher concentrations of these alcohols compared to the 15 – 15 °C wine. Isoamyl alcohol, a prevalent component in white wines, particularly stood out, remaining below the established threshold of 300 mg/L (Carpena et al., 2020). These higher alcohols, in conjunction with esters, played a role in contributing to the desirable aroma of the wine.

A noteworthy observation was the significant difference in ethyl lactate and isoamyl acetate concentrations during the maturation phase of the 15 – 15 °C wine. The presence of esters associated with spicy notes, such as ethyl butyrate, coincided with flavour attributes noted in the 25 – 25 °C wine. Esters that impart fruity notes such as isoamyl acetate and ethyl caprylate were particularly pronounced in the 15 – 15 °C and 15 – 4 °C wines, while the 25 – 25 °C wine exhibited more prominent spicy notes. Additionally, the lower temperatures employed in the 15 – 15 °C and 15 – 4 °C wines were found to preserve desirable sensorial characteristics, described as fresh and fruity, an observation previously reported by Molina et al. (2007).

The tannin level typically depends on the tannin concentration in the fruit itself. The marula fruit does not have a high concentration of tannin content hence the low concentration in the wine. The tannin concentration remained constant at a range of 0.1 – 0.56 mg GAE/g which were similar to tannin levels reported by Gebremedhin et al. (2017) in cactus fruit wine. Additionally, the tannin content in Macedonian wines was also low at a concentration range of 0.51 – 1.30 mmol TE/L. Furthermore, tannins in red wine help to stabilise the colour (Versari et al., 2013) which is quite understandable when it is in lower quantities in white wine like marula fruit wine.

The antioxidant activity in marula fruit wine exhibited no distinct trend; however, a

notable observation was made when compared to L-Ascorbic, as the minimal effective concentration (EC50) decreased. This phenomenon is a common occurrence in wines and is often attributed to the chemical reactions occurring during fermentation. Furthermore, it is established that higher temperatures contribute to a greater loss of antioxidant activity, as highlighted by Balogun et al. (2017). This aligns with the observed steady range of 300 – 400 µg/mL during the maturation phase in the 15 – 15 °C wine. The reduction in antioxidant activity, notably in terms of vitamin C, was further elucidated in Chapter 5 of this study. The loss in vitamin C content is also observed in cashew and apple wine. Furthermore, white wines are expected to contain low antioxidant activity because of the low content of polyphenols which contribute to the low antioxidant activity. In red wine the range was 4.11 – 11.73 mmol TE/L which was higher than 0.51 – 1.18 mmol/TE/L in white wines (Mitsrevska et al., 2020).

Elevated fermentation temperatures at 25 °C accelerated the consumption of glucose over fructose in the wine production process. Glycerol levels remained within acceptable ranges, positively influencing the overall sensory characteristics. The wines exhibited satisfactory pH, acidity, and levels of lactic and acetic acids. Isoamyl alcohol dominated the higher alcohol content, a parameter intricately regulated by temperature. Fluctuations in ester profiles due to temperature variations were identified, contributing to the wine's aromatic complexity. Tannin levels were relatively low, and antioxidant activity displayed variations, indicating a temperature-dependent influence. This underscores that the chemical transformations post-maturation was not solely attributed to yeasts and fruits but also exhibited a noticeable influence from storage temperature to a certain extent.

CHAPTER 5: NUTRITIONAL ANALYSIS

Evaluation of nutritional properties of marula fruit wine and the influence of production temperature on nutrient composition

5.1 Introduction

Proper nutrition plays an important role in the development of many diseases. One of the most important aspect of current scientific advancements is the expansion of knowledge regarding foods with health-promoting benefits (Rolbes et al., 2019). Liquid foods, like drinks, are sources of both fluid and nutrients that replenish and provide energy for day-to-day activities. Some of the locally produced liquid foods are non-alcoholic marula fruit juice as well as the alcoholic marula fruit wine (Maluleke et al., 2023). Traditional marula wine is revered by the African communities for being more nourishing and a potent drink than inducing a drunken state.

Compared to distilled alcoholic beverages like brandy and whiskey, wine commonly retains the nutrients contained in the fruits (Joshi et al., 2017). The bioactive ingredients included in the starting material are released and become accessible during the process of producing the wine (Trolio et al., 2021). Hence, it is expected that the nutritive composition of the wine is similar to that of the fruit juice. Generally, wine is consumed for pleasure and flavour, it is frequently depicted as being bad for human health, regardless of consumption levels. Marula fruits contain essential nutrients that are important in human diet such as vitamin C, minerals and proteins. The vitamin C content in marula fruit, at its lowest, is equivalent to that of oranges (Dlamini and Dube, 2008). It is in the range of 62 mg/100 g – 2100 mg/100 g. Ascorbic acid levels in marula wine range from 96 mg/100 g to 159 mg/100 g, and the wine has an 8.4% sugar content (Tebeila, 2022). Minerals like iron, sodium, manganese, potassium, calcium, magnesium and zinc have been reported in marula fruits (Ngemakwe et al., 2017). Magnesium, calcium, iron, copper and potassium were detected at levels of 10.5 mg/100 g, 6.2 mg/100 g, 0.10 mg/100 g, 0.04 mg/100 g and 548 mg/100 g respectively. There are trace amounts of zinc and sodium in the marula

fruit juice (Hiwilepo-van Hal et al., 2013). Moreover, marula wine has alcohol levels of 2 – 5.5%.

A lot of literature has expanded on the many benefits of wines that are already commercially available such as the grape wine. However, limited information is reported for the marula fruit wine despite the marula fruit wine being a centuries-old traditional drink. Tebeila (2022) investigated the nutritional properties of marula fruit wine during fermentation. However, there is limited information available regarding its nutritional content post fermentation i.e., the maturation and storage phase. This was the purpose of this chapter, more importantly how production temperature affected production and the presence of the selected nutrients.

5.2 Methodology

Marula fruits were collected into boxes from the grounds of University of Limpopo, 23.8888° S, 29.7386° E. Ripe and unripe fruits were collected for immediate and later use respectively. The unripe fruits were kept in the boxes used for collection at room temperature. Juice extraction was performed as outlined in chapter 3, section 3.2.2, marula fruit wine preparation and production in section 3.2.3 and 3.2.4 respectively. Statistical analysis was performed as outlined in chapter 4 section 4.2.6.

5.2.1 Determination of mineral composition in marula fruit wine

Five millilitres of the wine sample were placed in a digestion vessel, and 7.5 mL of nitric acid and 1.5 mL of hydrogen peroxide were added. Following a careful shake of the digestion vessel, the solution was mixed, and the samples were digested for 15 minutes in a microwave mineral digester. The digestive tubes were then allowed to cool for ten minutes in a laminar flow. To avoid contact with the acid, the vessels were subsequently opened in a laminar flow, making sure that the opening faced the laminar flow. After the digesting process was completed, the contents were transferred into 50 mL centrifuge tubes and filled to a capacity of 50 mL with distilled water. Thereafter, the contents were run following the Shimadzu Inductively Coupled Plasma Emission Spectroscopy (ICPE-9000) protocol

5.2.2. Determination of protein concentration of marula fruit wine

The Bicinchoninic acid (BCA) kit (Sigma-Aldrich, South Africa) was used to determine the amount of protein in wine. Analysis of the wine samples were determined as per manufacturer's instructions. The Promega Glomax multi-detection system was used to read the protein standard solution at 562 nm at concentrations of 0.5 to 30 g/mL. Unknown protein concentrations of wine samples were determined using a standard curve.

5.2.3 Determination of total fats

The method described by Folch et al. (1957) was used to analyse crude fats. Firstly, a chloroform-methanol-distilled water solution in the ratio 3:48:47 was prepared were 30mL, 480 mL and 470 mL of chloroform-methanol-distilled water were each transferred into a 1000 mL sterile bottle. Following that, 3 mL of the wine samples, 3.75 mL of chloroform, and 7.5 mL of methanol were added to 50 mL centrifuge tubes. In a shaker incubator set to 25 °C, the tube was shaken for an hour at 100 rpm. Thereafter, 3.75 mL of chloroform and 3.75 mL of distilled water were then added. After removing the supernatant, 3.68 mL of the chloroform-methanol-distilled water solution was added to remove the residual layer. The washing off was repeated. The remaining solution was then transferred to weighed glass vials. The contents of the vials were then evaporated with a fan overnight. Thereafter, the dry sample in the vial was measured on a weighing scale. Total crude fat was then determined using the formula below:

W2-W1/S X 100

W1 = Weight of empty vial (g)
W2 = Weight of vial with extracted fat (g)
S = Weight of sample (mL)

5.2.4. Determination of cholesterol concentration in marula fruit wine

The Liebermann-Burchard (LB) reagent method derived from Kim and Goldberg (1969) was used for cholesterol determination. A 500 mL amber glass bottle with a polyseal cover was filled with 220 mL of cold acetic anhydride and 200 mL of room temperature glacial acetic acid to prepare the Liebermann-Burchard reagent. Following the mixing of the two chemicals by inversion, 30 mL of cold, concentrated sulfuric acid were added. An amber glass bottle with a polyseal cap held the cholesterol standard, which was made by dissolving 200 g of cholesterol powder in 100 mL of glacial acetic acid. Thereafter, 1 mL of LB reagent was added into three tubes labelled U, S and B. Equal volumes of wine sample and the standard solution were added to the labelled tubes i.e., 0.169 mL was added to tubes labelled U and S respectively. Tube B was used as blank with 1 mL of 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:

C_U (wine concentration) = C_S (standard concentration) X A_u (wine Absorbance) / A_s (standard Absorbance)

5.2.5. Determination of vitamin C concentrations in marula fruit wine

The spectrophotometric method described by Lima and Portari (2019) was used to determine vitamin C concentrations. One millilitre of the wine sample was added together with 6 mL of 5% ice cold trichloroacetic acid (TCA). The solution was mixed thoroughly by vortexing and then centrifuged at 1500 rpm for 5 minutes at 4 °C. A new tube was then filled with 0.5 mL of the supernatant and 200 µL of 2, 4 dinitrophenylhydrazine-thiourea-copper sulphate (DTC) in a 20:1:1 ratio. The mixture was incubated at 70 °C for 30 minutes in a water bath. After the incubation period, 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. All tubes were read at 520 nm using a spectrophotometer (Genesys 10S, UV-VIS). Vitamin C standards of different concentrations, namely, 5, 10, 15, 20 and 25 µg/mL were used to determine the unknown vitamin C concentrations of the wine samples.

5.3 Results

The following results indicate the changes in selected nutritional characteristics of starter culture-based marula fruit wine as well as the reference wine from the different production phases which are fermentation, maturation and the storage phase. The experimental marula fruit wines were fermented at 25 °C and 15 °C respectively. The wines were then matured and stored at their respective temperatures used for fermentation and at 4 °C, excluding the reference wine. The 25 °C wine was labelled 25 – 25 °C to represent a wine fermented, matured, and stored at 25 °C whereas 25 – 4 °C represents a wine fermented at 25 °C but matured and stored at 4 °C. This is the same description for the 15 °C wine. The reference wine that was fermented at room temperature, matured at 15 °C was labelled as 15 – 15 °C and stored at 15 °C and 4 °C which are labelled as RW 15 – 15 °C and RW 15 – 4 °C respectively.

5.3.1 Mineral concentrations

Iron and zinc were the least detected minerals in all the wines (Table 5.1). Temperature variation had no effect on the mineral concentrations for all the experimental wines, i.e., 15 – 15 °C, 15 – 4 °C, 25 – 25 °C and 25 – 4 °C. Notably, the reference wine had more mineral concentrations comparatively. RW 15 – 4 °C wine had average mineral concentrations of 92 mg/L and 10.37 mg/L; and RW 15 – 15 °C wine had 79.37 mg/L and 13.37 mg/L for potassium and sodium respectively. Although there was variance between the different mineral concentrations in the different temperatures, these differences of the minerals were non-significant ($p > 0.05$).

Table 5.1: Mineral concentrations during marula fruit wine production.

Sample	Cu mg/L	Fe mg/L	K mg/L	Mg mg/L	Na mg/L	P mg/L	Zn mg/L	Ca mg/L
D0 25 – 25 °C	0,16	0,11	58,40	1,07	12,70	0,81	0,00	0,82
D2 25 – 25 °C	0,16	0,00	38,40	1,01	13,00	2,02	0,05	0,70
D5 25 – 25 °C	0,15	0,00	33,70	0,69	13,50	1,00	0,01	0,49
D10 25 – 25 °C	0,19	0,00	91,70	1,92	12,80	3,21	0,08	1,59
Average	0,17	0,03	55,55	1,17	13,00	1,76	0,04	0,90
W1 25 – 4 °C	0,20	0,08	48,70	1,39	15,80	6,82	0,36	1,20
W3 25 – 4 °C	0,19	0,00	46,50	1,08	13,50	1,92	0,10	1,39
W5 25 – 4 °C	0,15	0,00	38,80	0,75	13,40	1,31	0,00	0,51
Average	0,18	0,03	44,67	1,07	14,23	3,35	0,15	1,03
W1 25 – 25 °C	0,16	0,00	65,10	1,23	12,60	2,01	0,03	1,01
W3 25 – 25 °C	0,17	0,00	34,30	0,67	13,10	0,87	0,00	0,44
W5 25 – 25 °C	0,17	0,00	45,30	1,05	12,80	1,74	0,05	0,69
Average	0,17	0,00	48,23	0,98	12,83	1,54	0,03	0,71
M1 25 – 25 °C	0,14	0,00	30,80	0,63	13,20	0,72	0,00	0,37
M3 25 – 25 °C	0,17	0,00	57,60	1,11	13,60	1,91	0,03	0,82
M6 25 – 25 °C	0,00	0,00	41,10	1,04	4,05	8,51	0,37	1,96

Sample	Cu mg/L	Fe mg/L	K mg/L	Mg mg/L	Na mg/L	P mg/L	Zn mg/L	Ca mg/L
Average	0,10	0,00	43,17	0,93	10,28	3,71	0,13	1,05
M1 25 – 4 °C	0,15	0,00	57,30	1,02	12,10	1,46	0,00	0,77
M3 25 – 4 °C	0,16	0,00	32,30	0,68	13,80	1,23	0,03	0,48
M6 25 – 4 °C	0,00	0,00	46,10	1,27	3,55	6,30	0,24	2,20
Average	0,10	0,00	45,23	0,99	9,82	3,00	0,09	1,15
D0 15 – 15 °C	0,16	0,11	58,40	1,07	12,70	0,81	0,00	0,82
D2 15 – 15 °C	0,16	0,00	65,80	1,21	12,00	1,43	0,00	0,81
D5 15 – 15 °C	0,16	0,00	34,60	0,74	14,00	1,61	0,06	0,52
D10 15 – 15 °C	0,24	0,67	30,90	1,53	12,50	3,41	0,26	1,12
D18 15 – 15 °C	0,14	0,00	6,27	0,21	14,90	2,07	0,14	0,21
D30 15 – 15 °C	0,10	0,00	63,30	0,92	9,47	0,00	0,00	0,57
Average	0,16	0,13	43,21	0,95	12,60	1,56	0,08	0,68
W1 15 – 4 °C	0,15	0,00	37,50	0,76	13,60	1,20	0,01	0,52
W2 15 – 4 °C	0,15	0,00	58,90	1,13	13,10	2,20	0,03	0,93
W3 15 – 4 °C	0,38	0,37	45,70	5,21	3,12	4,43	0,31	3,81
Average	0,23	0,12	47,37	2,37	9,94	2,61	0,12	1,75
W1 15 – 15 °C	0,15	0,00	31,20	0,64	13,20	0,83	0,00	0,45

Sample	Cu mg/L	Fe mg/L	K mg/L	Mg mg/L	Na mg/L	P mg/L	Zn mg/L	Ca mg/L
W2 15 – 15 °C	0,19	0,00	68,60	1,89	10,40	1,70	0,00	1,46
W3 15 – 15 °C	0,14	0,00	56,20	1,01	11,40	0,57	0,00	0,71
Average	0,16	0,00	52,00	1,18	11,67	1,03	0,00	0,87
M1 15 – 4 °C	0,15	0,00	27,00	0,57	13,10	0,62	0,00	0,36
M2 15 – 4 °C	0,16	0,00	37,30	0,78	13,50	1,33	0,04	0,51
M5 15 – 4 °C	0,00	0,28	71,90	3,88	0,66	6,58	0,48	5,74
M6 15 – 4 °C	0,00	0,31	40,60	1,24	5,68	9,11	1,03	4,96
Average	0,08	0,15	44,20	1,62	8,24	4,41	0,39	2,89
M1 15 – 15 °C	0,16	0,00	31,30	0,68	13,70	1,24	0,03	0,45
M2 15 – 15 °C	0,33	0,30	98,30	5,62	6,49	4,85	0,32	4,14
M5 15 – 15 °C	0,00	0,19	41,60	1,19	5,38	8,62	0,50	3,71
M6 15 – 15 °C	0,00	0,00	0,00	0,00	0,75	0,00	0,00	2,11
Average	0,12	0,12	42,80	1,87	6,58	3,68	0,21	2,60
RW D0	0,44	0,54	24,70	4,62	3,60	3,89	0,29	3,58
RW D2	0,28	0,18	63,10	2,65	8,96	3,61	0,29	1,98
RW D4	0,16	0,00	63,00	1,23	12,10	1,31	0,00	0,98
RW D8	0,29	0,18	50,40	2,65	4,96	2,91	0,27	1,88
RW D12	0,30	0,25	86,50	3,76	7,16	4,15	0,37	2,80
RW D16	0,18	0,00	50,30	1,29	11,20	1,54	0,02	0,95
RW D20	0,23	0,10	128,00	3,10	13,30	5,34	0,21	2,68

Sample	Cu mg/L	Fe mg/L	K mg/L	Mg mg/L	Na mg/L	P mg/L	Zn mg/L	Ca mg/L
RW D24	0,19	0,00	48,90	1,06	15,10	2,98	0,16	0,82
Average	0,26	0,16	64,36	2,55	9,55	3,22	0,20	1,96
RW W1	0,21	0,00	55,30	1,81	9,38	1,63	0,06	1,18
RW W2	0,15	0,00	37,40	0,72	13,30	0,86	0,00	0,47
RW W3	0,20	0,00	50,60	1,56	10,20	1,61	0,07	0,99
Average	0,19	0,00	47,77	1,36	10,96	1,37	0,04	0,88
RW M1 15 – 15 °C	0,29	0,20	77,60	3,46	6,01	3,43	0,29	2,57
RW M2 15 – 15 °C	0,22	0,04	92,40	2,47	12,30	3,66	0,16	1,93
RW M3 15 – 15 °C	0,22	0,02	106,00	2,62	12,70	4,27	0,14	2,04
Average	0,24	0,09	92,00	2,85	10,34	3,79	0,20	2,18
RW M1 15 – 4 °C	0,22	0,05	98,60	2,31	13,60	4,09	0,18	1,85
RW M2 15 – 4 °C	0,15	0,00	57,00	1,11	11,10	0,40	0,00	0,76
RW M3 15 – 4 °C	0,20	0,03	82,50	1,70	15,40	4,05	0,20	1,43
Average	0.19	0.03	79.37	1.17	13.37	2.85	0.13	1.35

D refers to days, W refers to weeks, M refers to months and RW refers to reference wine.

5.3.2 Protein content

The protein content (Fig. 5.1) below showed a generally steady pattern of protein concentration. The starting protein content in the experimental wines i.e., 15 – 15 °C and 25 – 25 °C was 10.5 µg/mL and remained constant during maturation. There was no significant difference between protein contents during maturation of the wines at different temperatures ($p > 0.05$).

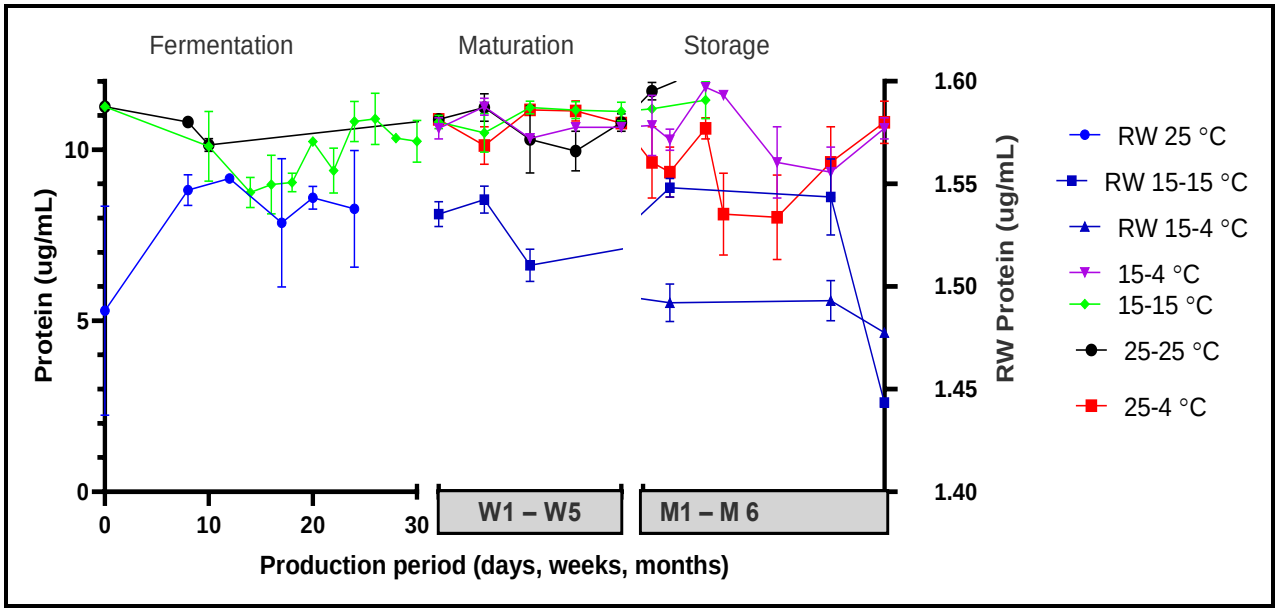

Figure 5.1: Protein content in the different phases of the production of marula fruit wine. W refers to production period in weeks and M refers to months. RW refers to the reference wine.

The protein content in the reference wine was notably lower than the experimental wines at ranges of 1.48 – 1.50 µg/mL and 1.51 – 1.55 µg/mL for RW 15 – 4 °C and RW 15 – 15 °C respectively (Fig. 5.1). Additionally, there was a significant difference between the protein content of the 15 – 15 °C wine and RW 15 – 15 °C wine during maturation and storage, the percentage difference was 76.31% and 78.31% respectively. During the storage phase, there was a significant difference between protein content of 15 – 4 °C wine and RW 15 – 4 °C wine ($p = 0.0006$) with a percentage difference of 76.04%.

5.3.3 Fat content

The original fat concentration (Fig. 5.2) was 1.4 g/L for the experimental wines, and it remained constant in the 15 – 15 °C wine, while it decreased sharply in the 25 – 25 °C wine during the fermentation phase but gradually increased during maturation.

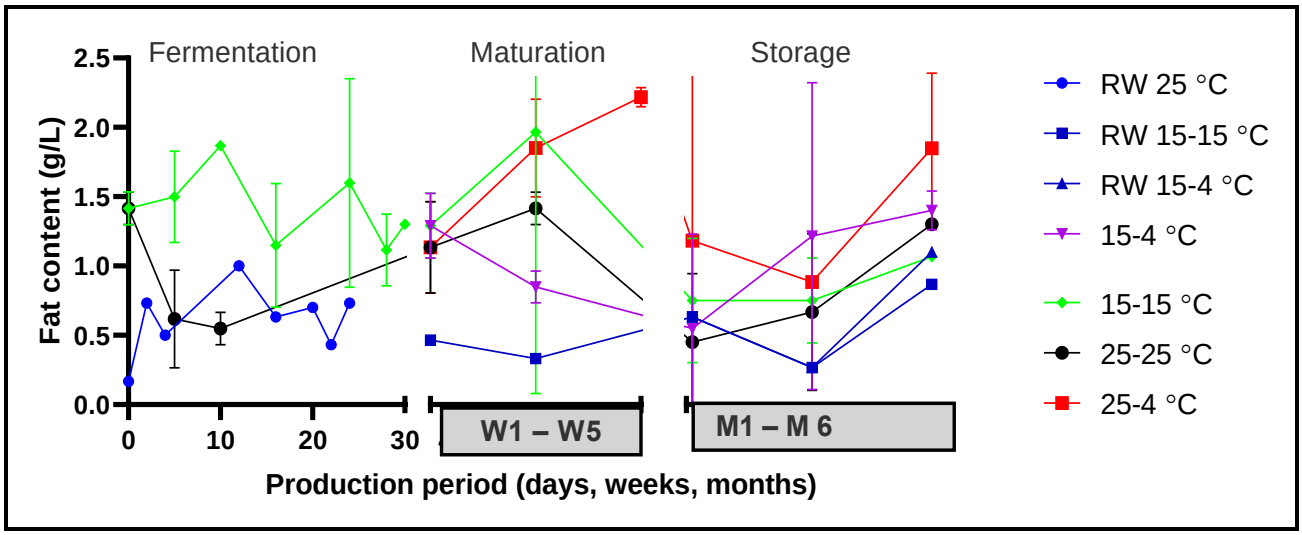

Figure 5.2: Fat content in the different phases of the production of marula fruit wine. W refers to production period in weeks and M refers to months. RW refers to the reference wine.

The fat content steadily increased during the fermentation phase for RW 25 °C. The range of fat content during maturation phase for 15 – 15 °C wine was at 1.5 g/L – 2.0 g/L and 1.0 g/L – 1.5 g/L for 15 – 4 °C wine (Fig. 5.2). The fat concentrations ranged at 1.3 g/L – 1.5 g/L and 0.9 g/L – 2.3 g/L for 25 – 25 °C and 25 – 4 °C, respectively. The fat concentration for both experimental temperature variations increased mid maturation and then decreased non-significantly in the storage phase. There was a significant difference between the 15 – 15 °C wine and RW 15 – 15 °C wine ($p = 0.0001$) and the percentage difference was 23.35% during the storage phase. It is important to state that the samples were not taken from a homogenous mixture.

5.3.4 Cholesterol content

The cholesterol content (Fig. 5.3) below started off at 4.5 mg/mL and the cholesterol concentration in the 15 – 15 °C wine gradually increased in the fermentation phase which is the opposite for the 25 – 25 °C wine. However, the cholesterol content decreased for the 15 – 15 °C wine and 15 – 4 °C wine in the maturation phase and increased for the 25 – 25 °C wine and the 25 – 4 °C wine.

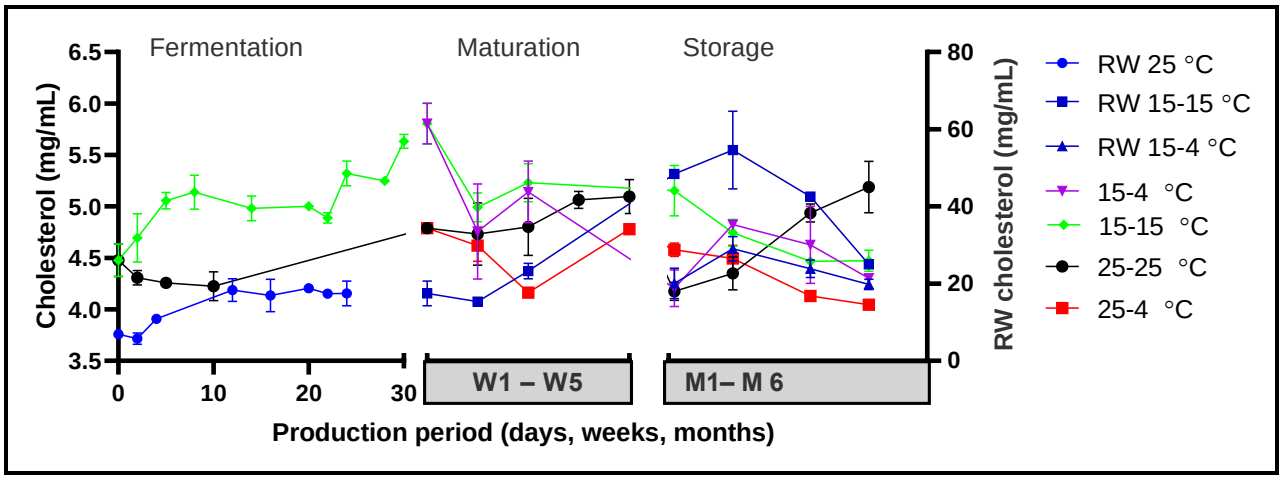

Figure 5.3: Cholesterol content in the different phases of the production of marula fruit wine. W refers to production period in weeks and M refers to months. RW refers to the reference wine.

Cholesterol concentration of the reference wine was notably higher than the experimental wines at ranges of 15.2 – 24.6 mg/mL in the maturation phase. The cholesterol content of RW 15 – 15 °C wine in the storage phase varied significantly with RW 15 – 4 °C wine ($p < 0.05$) with a percentage difference of 5.01%. This was also a similar trend in the 25 – 25 °C wine and the 25 – 4 °C wine ($p < 0.05$) wherein they differed with a percentage difference of 4.3%. A significant difference was also observed between the 15 – 15 °C wine and RW 15 – 15 °C wine in the maturation phase and the storage phase at $p = 0.05$ and $p = 0.0015$, respectively. The percentage difference between 15 – 15 °C wine and RW 15 – 15 °C wine in the maturation and storage phase were 70.68% and 63.29% respectively. The difference in cholesterol content between the 15 – 4 °C wine and 15 – 4 °C wine was significant at $p = 0.0006$ and the percentage difference was 64.30%.

5.3.5 Vitamin C concentration

The vitamin C concentration (Fig. 5.4) had a relatively steady pattern for all the wines in the three phases of production. However, vitamin C concentration in the reference

wine (RW 15 – 15 °C) was the highest in the maturation phase at a range of 124 – 133 µg/mL.

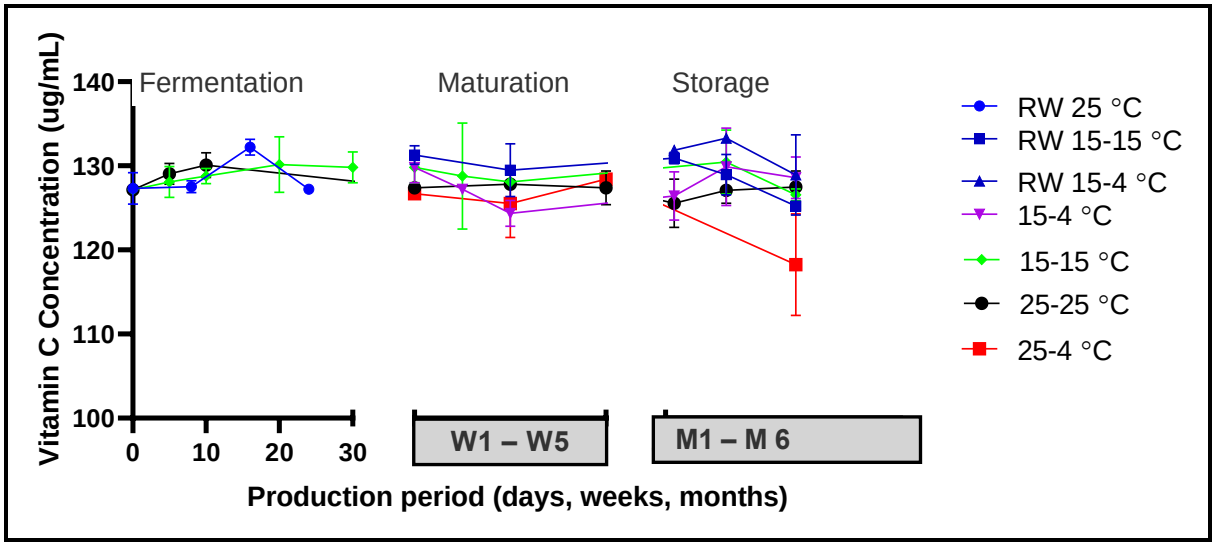

Figure 5.4: Vitamin C concentration in the different phases of the production of marula fruit wine. W refers to production period in weeks and M refers to months. RW refers to the reference wine.

Vitamin C concentration in the 25 – 4 °C wine decreased and was the lowest among the wines in the storage phase at a range of 96.6 – 128.7 µg/mL. The vitamin C concentration in the 15 – 15 °C wine and the 15 – 4 °C experimental wines were slightly above the 25 – 25 °C and the 25 – 4 °C experimental wines. There was no significant difference in vitamin C concentration among the wines produced at different temperatures for the different phases ($p > 0.05$).

5.4 Discussion

Wine is usually arguably portrayed as being harmful to human health regardless of the consumption levels, it is generally drunk for flavour and enjoyment. Other vital nutrients that are crucial to a person's diet can be found in marula fruits which are sometimes passed to the marula fruit wine. When consumed in moderation, wine can be a part of a balanced diet and may provide some possible health advantages. Yet, consuming too much wine can have a number of negative health effects, and the total

effect on a person's health will largely rely on their unique body tolerance to wine consumption levels.

A wide variety of minerals are vital elements for human health because they help in bodily functions such as maintaining nerves and muscles, functioning as cofactors for biochemical pathways as well as acid-base regulation (Gharibzahedi and Jafari, 2017). Marula wines provide macronutrients such as calcium, magnesium, potassium sodium as well as phosphorous and micronutrients such as, copper, iron, and zinc which were observed in this study.

The mineral content in wine is affected by several factors including the environmental factors such as the composition of the soil where the tree grows, how the fruits are processed, clarifying methods as well as the storage conditions (Durgutti et al., 2020). The required dietary allowance (RDA) of calcium, copper, iron, magnesium, phosphorous, potassium, sodium and zinc are 2500 mg/L, 10000 mg/L, 45 mg/L, 350 mg/L, 4000 mg/L, 1500 mg/L and 40 mg/L respectively (WHO, 2004). The concentration of these minerals in the marula fruit wines were lower than the required dietary allowance levels and this is similar to observations by Canuti et al. (2019) in Italian and Californian wines while in contrast to a study by Cox et al. (1977) wherein sodium and potassium were detected in greater quantities. Minerals such as copper, iron and zinc are good for yeast nutrition (Durgutti et al., 2020) and these are consumed during the course of wine production. In addition, white wines are reported to generally have lower mineral content than red wines because of the fermentation technique used (Diaz et al., 2013).

Proteins in wine play several significant functions. They form complexes with substances like tannins and help to stabilise the wine (Cosme et al., 2020). Proteins in wines commonly range from 15 – 700 mg/L (Cosme et al., 2020; Ferreira et al., 2001). The marula fruit wines had lower protein content, and this is important because higher amount of proteins in wine can lead to undesirable sensorial characteristics such as turbidity (Jaeckels et al*.,* 2016) and this was not observed in this study. In grape wine, proteins usually come from the fruit, and can also come from the yeast which facilitates the fermentation process (Ferreira et al., 2001). Starter cultures were

used in this study, yeast autolysis (Durguti et al., 2020) from the experimental wines could explain the difference in the protein content from the experimental and reference wine since no starter culture was used in the reference wine.

Fat content is usually quite low in wine, the highest fat concentration detected in this study was 2.21 g/L in 25 – 4 °C wine and the lowest concentration was in RW wine with a concentration of 0.3 g/L. These values are below the RDA for total fat which is 250 – 350 g/L (Aranceta et al., 2012). The amount of fat concentration is mostly a result of lipolytic bacteria in the wine. The lipolytic bacteria helps in using the triglycerides present in the wine. *L. plantarum* is one of the lipolytic bacteria reported in marula fruit wine (Tebeila, 2022). In this study, microorganisms were isolated based on dominance which could have affected identifying some of the lipolytic bacteria in this study.

The marula fruit has high vitamin C content, and this nutrient can be transferred to marula fruit wine (Balogun et al., 2017). Although the depletion of vitamin C in this study was non-significant, some variance was observed, and the experimental wine at the higher temperatures had the least vitamin C content. Lower fermentation temperature of 15 °C seem to preserve vitamin C better than 25 °C where the loss was significant. Chemical reactions during fermentation as well as exposure of wine to oxygen are attributed to the loss of vit. C (Akinwale et al., 2001; Balogun et al., 2017; Dlamini and Dube, 2008). Legodi (2022) also alluded that the method used to determine the vitamin C content could also contribute to the variance.

It is common in wines to experience loss of nutrients during the wine production process like in marula fruit wine (Tebeila, 2022). The reduction in nutrients in the 25 – 25 °C wine was more prominent when compared to the 15 – 15 °C wine. The wine produced at 15–15 °C compared favourably with other wines and this lower temperature could be important in nutrient preservation in wine production.

CHAPTER 6: SENSORY EVALUATION

Sensory evaluation of marula fruit wines during the storage phase and influence of production temperature on the sensory attributes

6.1 Introduction

Sensory evaluation is one of the key factors in product development in the food industry. It helps in determining the quality of the product, in this case marula fruit wine. Wine quality can be described to the extent at which wine is considered to have desirable attributes. This is a subjective concept since it depends on what a wine consumer finds to be pleasant and thus makes it difficult to identify. Different preferences are borne from different sensations when it comes to wine parameters.

Characteristics such as wine balance, intensity of flavour and finish, complexity, as well as typicity (Belda et al., 2017) are used in determining the quality of wine. High quality wines are well balanced (Petropoulos et al., 2017) i.e., acidity, tannins, sweetness, fruitiness and alcohol levels do not overpower each other (Reynolds, 2022). Wines that take longer to reach maturity are often well balanced. On one hand, rich flavours are associated with high quality wines, and flavours of quality wines are complex and numerous such that these are intense and can be tasted one after the other for some time after one has swallowed. Flavours that disappear immediately after swallowing indicate a low-quality wine, i.e., a wine with a finish that does not linger and has one or two flavours is considered to be a low-quality wine (Petropoulos et al., 2017).

The aroma of the wine has a great impact on its quality, and it is regarded as the best indicator of wine quality (Reynolds, 2022). The aroma is brought by elements such as the content of volatile molecules, their interactions, the chemical and physical effects of non-volatile wine matrix components (Ferreira et al., 2014) as well as individual perceptions of the scent. Importantly, the aroma perception is divided into primary, secondary and tertiary elements (Belda et al., 2017). The aroma perceived at primary level occurs naturally in the fruit. In grape wines, primary aroma will be extracted in

grape varieties during the production process. Secondary aroma of the wine comes from compounds released by the yeast during fermentation, while some aroma only develops later during aging due to oxidation and this is regarded as tertiary aroma perception (Petronilho et al., 2020).

The most efficient method used by well trained and experienced wine judges to assess wines is the sensory evaluation method where parameters such as taste, colour, smell and texture are evaluated. However, the development of an objective quality grading method would be of great importance in the wine industry (Marangon and Kallithraka, 2021).

White wines are not commonly subjected to aging, since they are far less resistant to oxidation, except some sparkling white wines which are fermented in the barrel and few dry whites (Kanavouras et al., 2020). Marula fruit wine in this research study is a dry white wine. This chapter of the study evaluated the sensory profiles of the lab produced marula fruit wines in terms of taste, smell, texture, colour as well as the overall impression and how storage temperature affects these parameters.

6.2 Methodology

Marula fruit collection, juice extraction was performed as outlined in chapter 3, section 3.2.1, 3.2.2 respectively. Marula fruit wine preparation and production in section 3.2.3 and 3.2.4 respectively. Statistical analysis was performed as outlined in chapter 4 section 4.2.6.

6.2.1 Sensory evaluation method

A semi-trained panel consisting of University of Limpopo staff and students who have confirmed to be above 18 years of age, not on any chronic medication and not pregnant voluntarily took part in the evaluation of the marula fruit wine. The study received ethical clearance (Appendix A) and all participants signed a consent form. The marula fruit wine samples were evaluated using the 5-point hedonic scale (Piggot, 1988) (Appendix B). Ten millilitres of each of the wine samples were evaluated each month for a period of 6 months through blind tasting by using codes to label and

differentiate the wine samples. Sliced carrots and cucumbers were used as palate cleansers between the tasting of different samples. The marula fruit wine samples were evaluated for taste, colour using the standard reference method (SRM) colour chart (Appendix C). Smell, texture and the overall impression were assessed on a scale of 1 – 5 where 1 represented extremely dislike and 5 represented extremely like.

6.3 Results

The following results indicate sensory evaluation of marula fruit wines which were stored in amber bottles for a period of 6 months. The marula fruit wine was evaluated for colour, aroma, texture, taste and general impression. The experimental wines are 25 – 4 °C, 15 – 4 °C, 15 – 15 °C, 25 – 25 °C and the reference wines are RW 15 – 15 °C and RW 15 – 4 °C.

The panellists have appreciated all the wines during the first three months of production excluding RW 15 – 15 °C wine which had an average score with a range of 2.2 – 2.5 for taste, 2.5 – 3 for smell, 2.7 – 3.3 for texture and 3 – 3.2 for the overall impression (Fig. 6.1). The 25 – 4 °C wine taste scores gradually decreased as the storage period progressed, a similar trend was observed for this wine with its overall impression. Although the smell was appreciated with a score of 3 – 3.6.

The texture remained constant during the first two months and dropped in the third month (Fig. 6.1). The 15 – 4 °C wine had no apparent trend for taste, but its smell remained constant for the first two months and was disliked in the third month of storage scoring 2.5, the texture was appreciated at a range of 3 – 3.4. The 25 – 25 °C wine taste score did not have an apparent trend, the smell was disliked in the first month but improved as the storage period progressed, the average score ranged at 2.4 – 3.4. The 15– 15 °C wine was liked for its taste and smell with both ranging at 3.3 – 3.6 and the texture ranged at 3.3 – 3.6, overall, it was perceived as a good wine scoring at 2.5 – 3.4 for overall impression. The 15 – 4 °C wine also scored well above 2.5 for all its sensory parameters.

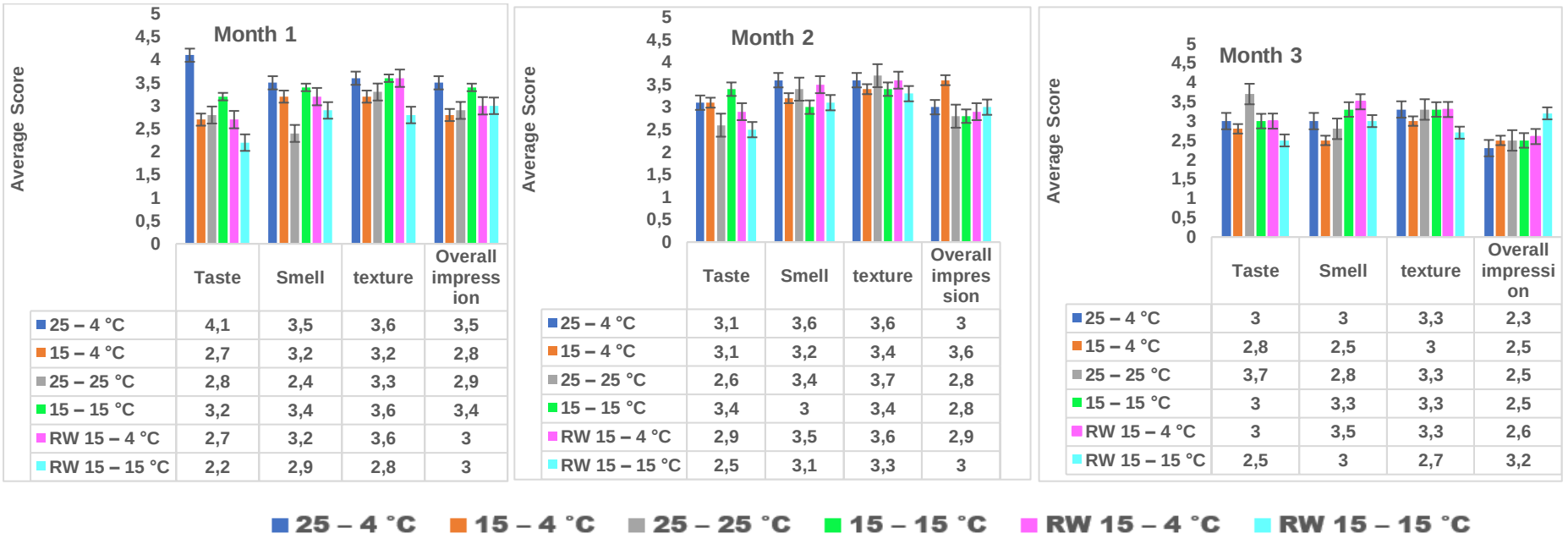

Figure 6.1: Sensory evaluation of marula fruit wine during the first 3 months of the storage period. RW refers to reference wine.

The 15 – 4 °C wine scored at ranges of 2.7 – 3.1, 2.5 – 3.2, 3 – 3.4 and 2.8 – 3.6 for taste, smell, texture and overall impression respectively. Notably, the reference wine RW 15 – 4 °C had a lower score for taste, the score range was 2.7 – 3. The smell was liked with a range of 3.2 – 3.5 and texture 3.3 – 3.6, however the overall impression was lower compared to the other parameters with an average score range of 2.6 – 3. There was no significant difference between the smell ($p = 0.0909$) and taste ($p = 0.2600$) within the ranges. However, for texture there was a significant difference ($p = 0.0435$), the percentage difference was 1.59%.

All the marula fruit wines generally had a good average score during the fourth, fifth and sixth month of the storage period (Fig. 6.2). All the average scores were from 2.5 for all the sensory parameters evaluated. The 15 – 15 °C wine and the 15 – 4 °C wine had the

same score range for taste which was 3.2 – 4, however the RW 15 – 4 °C wine had the highest score range for taste which was 3.4 – 4. Interestingly, the 15 – 15 °C wine also had a high score range for the smell parameter which was at a range of 3.7 – 4.2 and the 25 – 25 °C wine also scored at range reaching closer to 5 which was 2.6 – 4.3. Generally, an improvement in the appreciation of taste and smell for the marula fruit wine, including the reference wine, that were fermented at a lower temperature of 15 °C was observed.

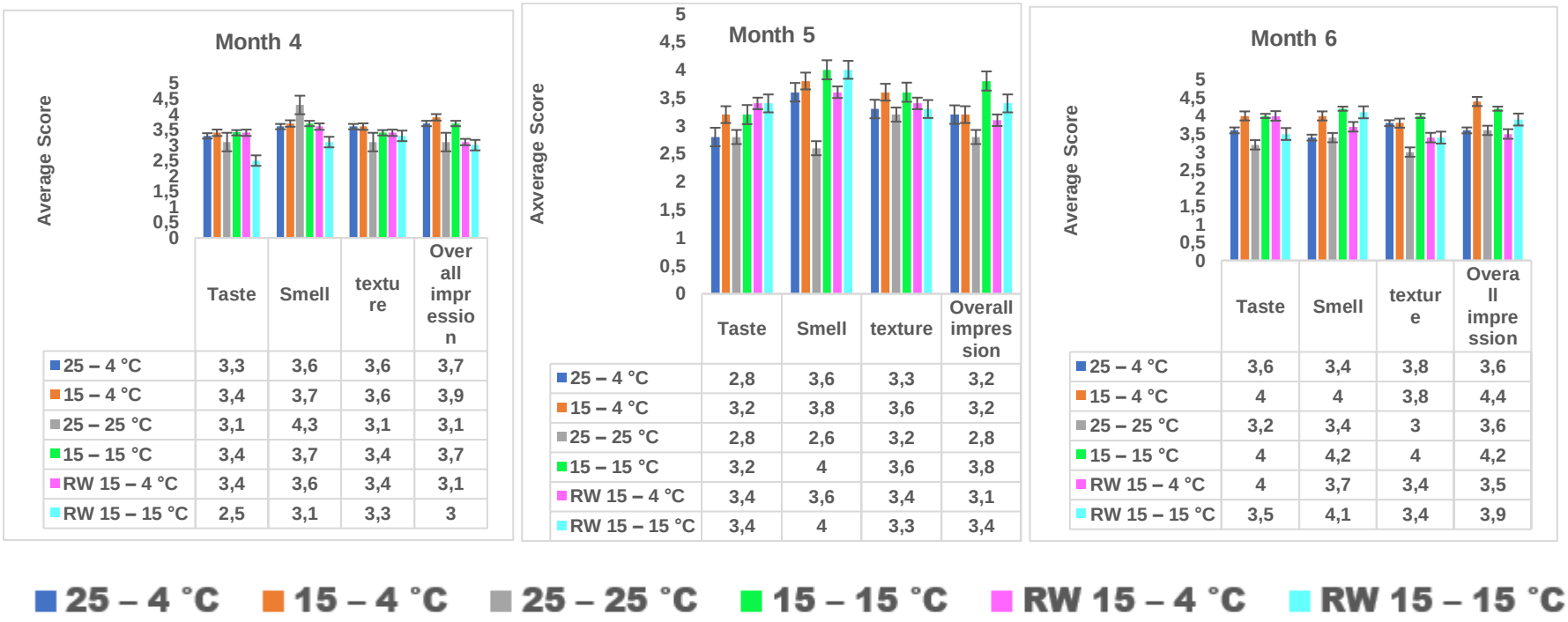

Figure 6.2: Sensory evaluation of marula fruit wine at the second month of the storage period.

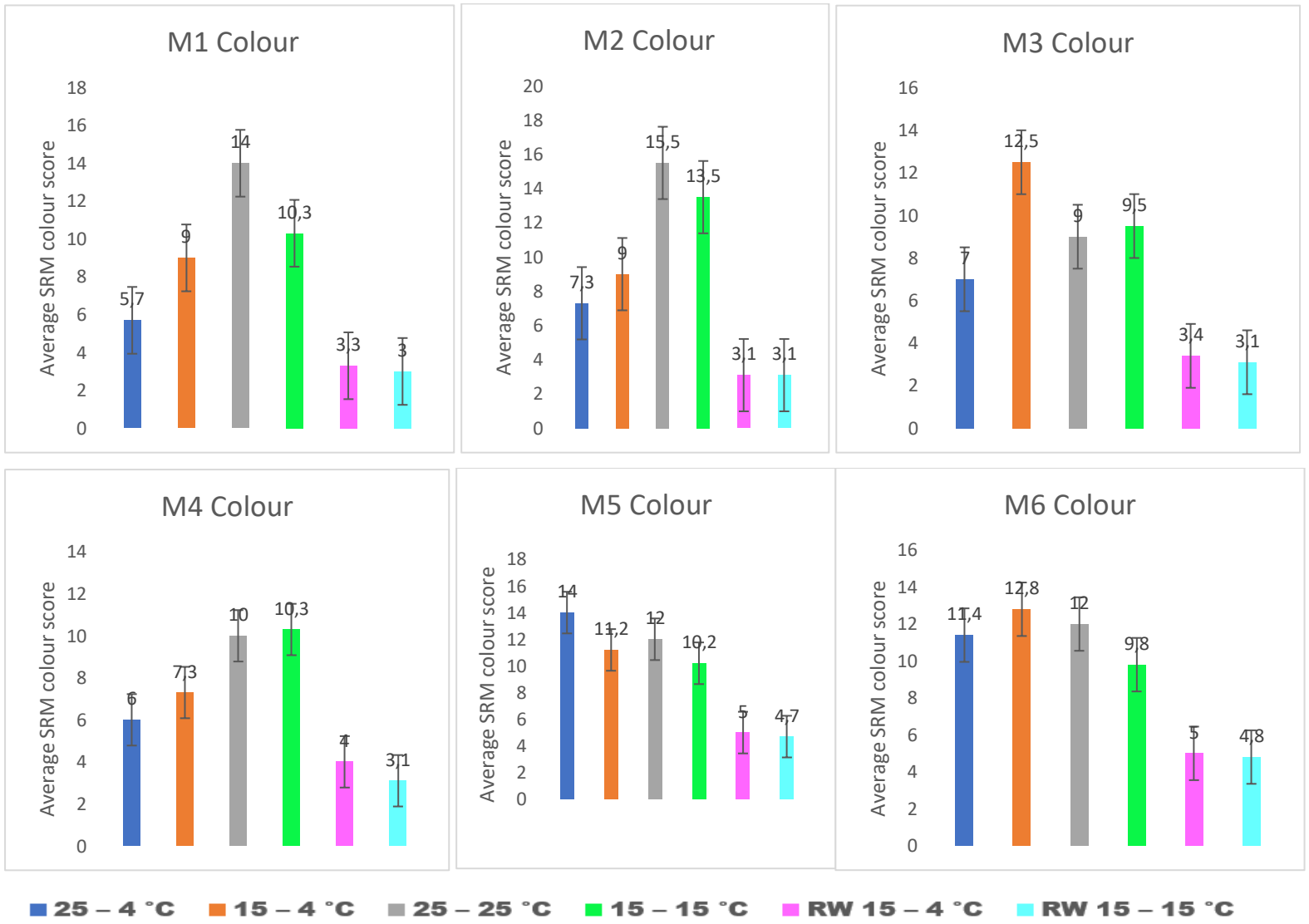

Figure 6.3: The average colour scores of marula fruit wines during the storage phase using the standard reference method (SRM) chart. M represents month.

Additionally, the 15 – 15 °C marula fruit wine also had the highest score with the texture parameter at 3.4 – 4. Overall, the 15 – 15 °C and the 15 – 4 °C wines were the most appreciated wines each scoring at a range of 3.7 – 4.2 and 3.2 – 4.4 respectively (Fig. 6.2). It is worth noting that both these wines i.e., 15 – 15 °C and the 15 – 4 °C had a good score for taste which shows that the taste parameter has a great effect on the panellists' perceptions. There was no significant difference among the ranges in the taste ($p = 0.04236$) and smell ($p = 0.6920$) parameter. However, a significant difference ($p > 0.05$) among the texture ranges was observed at a percentage difference of 8.12%.

The experimental marula fruit wines lost the original yellow-gold colour as aging progressed except for the 25 – 4 °C marula fruit wine which retained the yellow-gold colour for the first four months (Fig. 6.3). There was no apparent pattern observed for the loss of colour due to temperature effect amongst the experimental wines. Interestingly the reference wines in both temperature variations RW 15 – 15 °C, RW 15 – 4 °C retained the hue of the original marula fruit juice even though the SRM average scores were gradually increasing (within the colour range) for both wines at ranges of 3 – 4.8 and 3.1 – 5 respectively.

The perceptions of the panellists regarding the wines were more similar with slight variations throughout the six months storage period (Table 6.1). Interestingly, the two reference wines at different temperature variations did not have distinct tastes.

The experimental wines stored at cooler temperatures i.e., 25 – 4 °C wine and 15 – 4 °C wine were perceived to be more acidic in the first three months of storage and the acidity lingered for the 15 – 4 °C wine and became more distinct in the sixth month. The high level of acidity at months 4 and 6 was observed during this period (Chapter 4, Fig. 4.4 A). The panel described the 25 – 25 °C wine tasted bitter. The tasting panel further noted some spicy notes which were likened to atchar-like (pickled mango) taste. However, the hedonic score was at an average of 2.5 – 2.9 for this wine which indicated that the panel liked the spicy notes within the wine.

Table 6.1: Sensory evaluation comments by marula fruit wine panellists.

Marula fruit wine samples	Common panel comments					
	M1	**M2**	**M3**	**M4**	**M5**	**M6**
25 – 4 °C	Sweet	Acidic	Acidic	Spicy	Spicy	Bitter
15 – 4 °C	Fruity	Sweet	Bitter	Acidic	Fruity	Acidic
15 – 15 °C	Bitter	Salty	Fruity	Acidic	Fruity	Fruity
25 – 25 °C	Bitter	Bitter	Spicy	Spicy	Bitter	Spicy
RW 15 – 15 °C	Flat	No taste	No taste	Tasteless	Flat	Flat
RW 15 – 4 °C	Flat	No taste	No taste	Tasteless	Flat	Flat

RW represents reference wine, M represents month.

The reference wines at 15 – 15 °C and 15 – 4 °C have been described as having no taste, however, the average panel score contradicts this since the score was at a range of 2.2 – 2.5 and 2.7 – 3 for the first 3 months respectively. The panel score indicated that the reference wines were also liked from month 4 – month 6 with a range of 2.5 – 3.5 for 15 – 15 °C wine and 3.4 – 4 for the 15 – 4 °C wine. The panel further described the reference wine as unpleasant water. However, the colour remained closer to that of marula fruit juice i.e., yellow from the earlier months of storage and only diminished to a partly gold colour on month six.

6.4 Discussion

Sensory evaluation of wines involves using senses to understand what is in a glass of wine by tasting, observing colour, smelling, and analysing the texture of the wine and finally determining if the quality of the wine appeals to one's liking. The sensory quality of a wine is the result of several different components interacting in a complex way. For grape wine, the kind of grape variety utilised is crucial since various grapes have unique flavour profiles (Gonzaga et al., 2020). Wine has distinct mineral, earthy, and climatic qualities which is the combination of the soil, climate of the vineyard. Warmer

climates produce richer, fruitier wines, while cooler climates give wines with more acidity and subtler flavours (Ashenfelter and Storchmann, 2014).

There were differences in the chemical compounds that were tested for in the wines including the organic acids and alcohols that are important for the formation of sensory characters. This explains the range of sensory profiles that were perceived. Although the actual perception of the wine depends on an individual. The panellist's perception on the taste has some objectivity in the different temperature variations since the acidity perceived by the panellists coincides with the acidity levels of the wine stored at 15 – 4 °C of as observed in this study (Chapter 4).

Microorganisms such as *S. cerevisiae* which is key in driving fermentation, plays a role in the aroma profile, they produce esters such as ethyl acetate, ethyl lactate and isoamyl acetate among others. The production of these esters in the study coincides with what the panellists perceived. Some of the esters such as isoamyl acetate give off flavours when present in high concentration i.e., above 7.5 mg/L (Mas et al., 2022). However, most of the bitter and spicy notes could be attributed by the high alcoholic content. King et al. (2013) alluded that the primary component of alcohol in wine, ethanol, is known to affect the sensory characteristics of wine by adding bitterness, altering the sweetness by masking the perception of esters and giving the wine a metallic taste, which was similar to the observation in this study.

The other sensory parameter which plays a major role in sensory evaluation is the smell which is often explained as the aroma. Bottle aging could have also influenced the aroma perceived by the panel. Echave et al. (2021) alluded that bottle aging may play a role in diminishing the fruity aroma and turn bright colours like red into a dark brown colour which could be the same with what is observed in these aged marula fruit wines. Nonetheless, bottle aging does not only have detrimental effects, but it may also result in an enhanced flavour profile (Tao et al., 2014).

The presence of dissolved oxygen, total phenols, and amino acids, among other factors, may have contributed to the browning of the wines in addition to the pre-treatment (Yang et al., 2020). It is worth noting that when white wines are stored at low temperatures, they may turn brown (Castellanos et al., 2021). Browning can cause wine to develop permanent flaws that lower the finished product's quality (Yang et al., 2020). Although it is common for white wines to lose their colour and have a brown colour when subjected to aging (Kanavouras et al., 2020). Further investigation is necessary since the reference wine did not diminish in colour. It is also worth noting that the storage conditions of the wines were not only exclusively used for wine.

The wine fermented and stored at 15 °C i.e., 15 – 15 °C and 15 – 4 °C wines demonstrated to be the most appreciated for taste, scored at 3.2 – 4. This could be attributed to the sweetness and fruity taste due to higher sugar content (Fig. 4.1 (A) and higher alcohols (Fig. 4.8) (Chapter 4). Although the higher alcohols were observably higher in the 25 – 25 °C and 25 – 4 °C wines. This demonstrated that a balance between the sensory factors such as sweetness, alcohol content, bitterness, higher alcohols and esters produces a good wine.

The aroma was well appreciated in the 15 – 15 °C wine and 25 – 25 °C wine. This could be attributed to ester compounds responsible for aroma such as isoamyl acetate and higher alcohols like hexanol (Chapter 4, Fig. 4.8 and Fig. 4.9) in this study. Overall, the most liked wine was the one fermented at 15 °C and stored at 4 °C i.e., 15 – 4 °C. This then suggests that for a better wine, all these temperatures should be incorporated into the winemaking process since these temperature conditions have distinct advantages towards the final characteristics of wine. To initiate fermentation, 25 °C should be used, 15 °C for maturation and finally 4 °C should be used for aging to balance the rate of fermentation progression and the production of good wine character.

CHAPTER 7: GENERAL DISCUSSION AND RECOMMENDATION

7.1 Overall discussion for the study

The aim of the study was to produce a high alcohol wine, about 10% through a starter-culture based fermentation of unpasteurised marula juice and evaluate the effect of varying fermentation and maturation temperatures on the chemical and sensory properties of marula fruit wines. Higher fermentation temperature increased the rate of fermentation, i.e., the experimental wines that fermented at 25 °C reached 1.0 ° Brix faster at 10 days when compared to the wines that were fermented at 15 °C which took 30 days to reach the same gravity. Increased levels of glucose and fructose content were especially observed in wines that were fermented at 15 °C and stored at 4 °C, which led to a rise in sweetness.

The use of starter culture-based fermentation with well adapted yeasts proved beneficial for production of a good quality and consistency wine. The utilisation of starter culture and varying fermentation temperatures, acknowledging potential limitations in microbial detection due to sub-culturing and not homogenising the wine before sample collection has aided on more knowledge on the effect of temperature in the marula fruit wine in the storage phase.

This study built on previous studies by Maluleke et al. (2023) and Phiri et al. (2022) that extensively investigated microbial populations in traditional marula wines at different stages of primary fermentation. The occurrence of microorganisms in post-fermentative stages poses a risk of wine spoilage, with *Saccharomyces* recognised as a spoilage organism, particularly in wines with residual sugars (Garcia et al., 2016). Variations in residual sugar levels were noted alongside the presence of *S. cerevisiae*, known to thrive in low-sugar environments. *S. cerevisiae* has the potential to spoil alcoholic beverages by generating sulphurous compounds and volatile phenols, as noted by Malfeito-Ferreira (2011), although these undesirable outcomes were not observed in the current study.

Bacillus species, typically associated with soil and winemaking processes, were identified at different temperatures (15 °C and 25 °C). Meanwhile, Acetic acid bacteria,

known for their adverse impact on wine quality, were only detected in the stored reference marula wine. This underscores the significance of employing a starter culture over natural fermentation, emphasising the importance of controlling the microbial environment to ensure the desired quality and characteristics of the wine. The dominance of *S. cerevisiae* yeasts in aging wine were consistent with previous studies.

In addition to the influence on microbial activity and consequently the rate of fermentation, temperature variation showed a significant influence on the chemical properties and flavour compounds in wine as well. It is thus important to carefully balance the fermentation process in order to produce high-quality wine. The effect of temperature variation was observed as well during exploration of the nutritional aspects of marula fruit wine. Favourable nutrient preservation in marula fruit wine was observed at lower temperatures, particularly at 15–15 °C. For instance, the high vitamin C content in marula fruit showed non-significant depletion during wine production and it was better preserved in lower fermentation temperatures. While the mineral concentrations fell below recommended dietary levels, they contributed to the overall nutritional content. Protein content in the wines was lower, preventing undesirable characteristics such as haze appearance albeit important for good nutrition.

Fruity esters such as isoamyl acetate, ethyl caproate, and ethyl caprylate exhibited greater prominence at lower temperatures, specifically 15 °C, in contrast to the higher temperature of 25 °C. The elevated alcohol content was identified as a contributing factor to bitter and spicy notes. Aroma, recognised as a significant sensorial parameter, was susceptible to potential alterations through bottle aging. Results from sensory evaluation highlighted that wines fermented and stored at 15 °C and 4 °C garnered the highest appreciation for taste, with a good balance between sweetness, fruity notes, and other sensory factors. Meanwhile, the aroma was particularly favoured in wines fermented at both 15 °C and 25 °C. The strategic incorporation of different temperature conditions in the winemaking process emerges as a key determinant for achieving optimal results in terms of flavour, aroma, and overall sensory appeal.

The sensory assessment of the wines encompassed a comprehensive analysis of taste, colour, aroma, texture, and the overall impression. Remarkably, different temperature variations were discerned for imparting distinctive flavour profiles to the wines. Specifically, wines fermented at higher temperatures (25 °C) exhibited a richness in flavour accompanied by spicy notes, while those produced at lower temperatures (15 °C) yielded fruitier wines with heightened acidity. The interplay of chemical compounds, including organic acids and alcohols, played a pivotal role in shaping the sensory characteristics. Microorganisms such as *S. cerevisiae*, contributed to aroma through the production of esters. Significantly, the higher fermentation temperatures were associated with an increased higher alcohol content, impacting the overall wine aroma. Furthermore, the concentrations of esters demonstrated variability based on both fermentation and storage temperatures, emphasising the complex relationship between temperature conditions and the sensory attributes of the wines.

7.2 Conclusion of the study

The study evaluated the effect of different fermentation and maturation temperatures on both chemical and sensory qualities and effectively produced a marula fruit wine with 12% alcohol content, in comparison with the reference traditional wine that had 7% alcohol content. The findings demonstrated the importance of temperature in determining the rate of fermentation, flavour profiles, and general quality of marula fruit wines. The addition of starter cultures proved to be an important component, as it increased the alcohol level and maintained consistency during the fermentation process, which reduced challenges associated with spontaneous fermentation.

7.3 Recommendation for marula wine production

It is apparent from the findings in this study that a variety of temperatures should be incorporated in the winemaking process. The following wine production conditions are proposed from the observation in this study: 25 °C should be used for primary fermentation; 15 °C should be used for maturation of the wine; and 4 °C should be

used for wine aging. Despite a general understanding of the impact of temperature on flavour development and chemical contribution, the full extent of its influence remains a subject of ongoing exploration.

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Appendix A: TREC Ethics clearance certificate

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TURFLOOP RESEARCH ETHICS COMMITTEE

ETHICS CLEARANCE CERTIFICATE

MEETING: **19 April 2022**

PROJECT NUMBER: **TREC/70/2022: PG**

PROJECT:

Title:	Improvement Of The Alcohol Content And An Investigation Of Fermentation And Maturation Temperatures On The Chemical And Sensory Characteristics Of Unpasteurised Starter Culture-Based Marula Fruit Wine
Researcher:	RPR Mapheto
Supervisor:	Prof. KLM Moganedi
Co-Supervisor/s:	N/A
School:	Molecular and Life Sciences
Degree:	Master of Science in Microbiology

PROF D MAPOSA
CHAIRPERSON: TURFLOOP RESEARCH ETHICS COMMITTEE

The Turfloop Research Ethics Committee (TREC) is registered with the National Health Research Ethics Council, Registration Number: **REC-0310111-031**

Note:

i) **This Ethics Clearance Certificate will be valid for one (1) year, as from the abovementioned date. Application for annual renewal (or annual review) need to be received by TREC one month before lapse of this period.**

ii) **Should any departure be contemplated from the research procedure as approved, the researcher(s) must re-submit the protocol to the committee, together with the Application for Amendment form.**

iii) **PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES.**

Finding solutions for Africa

Appendix B: 5 point hedonic scale

Sensory Evaluation of Marula Wine

☐ **Drinker** ☐ **Non-Drinker**

Identification_____ **Colour code**_____

Gender

Female		**Male**		**Other**	

Age group

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

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.

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

Comments

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Appendix C: SRM Colour Scale