Exploring the phenotypic diversity of oenological traits in *Kluyveromyces marxianus* strains

by

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Declaration

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Summary

A wide range of yeast species occur in the wine environment. While *Saccharomyces cerevisiae* dominates alcoholic fermentations, other species may also confer positive organoleptic attributes. Although more frequently isolated from the dairy, cocoa and tequila environments and highly studied for its biotechnological potential, *Kluyveromyces marxianus* has been occasionally reported in the wine environment. Recent reports have demonstrated that strain IWBT Y885, isolated from South African grape must, displays promising properties for winemaking which includes its high pectinase activity and positive contributions to aroma production.

Like other yeast species, a large degree of intraspecific variability has been shown to occur within the species. This study aimed to investigate and compare five different *K. marxianus* strains (L01 to L05) isolated from diverse substrates (dairy, beer, distillery) with strain IWBT Y885, with the underlying goal of interrogating whether the relevant winemaking characteristics previously reported in the latter strain are unique or common amongst *K. marxianus* strains. Along with these strains, the newly produced fusant BF2020 was also investigated. This fusant was generated as a hybrid product of a wine strain of *S. cerevisiae* and *K. marxianus* IWBT Y885 and displays intermediate fermentative capabilities.

Firstly, the morphological traits of the strains investigated were compared. Various differences could be identified with only a select few displaying similar morphologies to IWBT Y885. Subsequently, various oenological traits were investigated in synthetic grape juice. These included fermentation performance, fermentation metabolites, hydrogen sulfide and pectinase production. Variations between strain IWBT Y885 and strains L01 through L05 were found for the majority of traits investigated, regardless of their source of isolation. Following Cluster analysis, three strain groupings emerged. The first group comprised strains L01, L05, Y885 and BF2020 and the second group strains L02 and L04. Strain L03 appeared to show the most variation from the other strains and clustered separately.

Thereafter, fermentations were completed in Chenin blanc as well as Cabernet Sauvignon grape musts under conditions simulating those of industrial settings. Using cluster analysis of the major volatiles produced, clear separations could be observed between all sequential Chenin blanc *K. marxianus* fermentations and the *S. cerevisiae* fermentations. Similar groupings previously observed in synthetic grape juice emerged between the *K. marxianus* strains. However, these chemical differences did not translate into clear sensory differences. It is hypothesized that potential differences in yeast-derived aroma compounds may have been overshadowed by more dominant sensory attributes (fresh green, chalky, bitter, fizzy) listed by the sensory panel.

This study confirmed that strain variation is a common occurrence within *K. marxianus*. In a winemaking context, it leads to the production of varying concentrations of aroma compounds. Subsequently, strain IWBT Y885 stands out with regards to various oenological factors when investigated in synthetic grape juice, however real grape juice comparisons require further investigations.

Opsomming

'n Groot verskeidenheid van gis-spesies kom voor in die wyn-ongewing. Terwyl *Saccharomyces cerevisiae* alkoholiese fermentasie domineer, is dit ook waar dat ander spesies 'n positiewe invloed op die organoleptiese eienskappe van wyn kan uitoefen. Alhoewel *K. marxianus* meestal in suiwel, kakao en tequila omgewings gevind word, word hierdie gis ook gevind in verskeie wyn-omgewings. Onlangse studies demonstreer dat die ras IWBT Y885, wat van Suid-Afrikaanse druiwesap geisoleer was, baie belowende eienskappe vir die wynbedryf inhou. Hierdie sluit in hoë pektinase aktiwiteite sowel as 'n positiewe bydra tot die aroma produksie.

Soos met ander gis-spesies, is daar 'n groot hoeveelheid van intraspesifieke verskille tussen *K. marxianus* rasse. Die hoofdoel van hierdie studie was om vyf verskillende *K. marxianus* rasse (L01 tot L05), wat van verskeie omgewings geisoleer was (suiwel, bier, distilleerdery), met die ras IWBT Y885 te vergelyk. Hierdie vergelyking fokus spesifiek op die relevante wynmaak-einskappe wat reeds in Y885 gevind word. Saam met hierdie rasse was die nuut-gemaakde "fusant" gis BF2020 ingesluit. BF2020 was gemaak as 'n produk van die wyn-gis *S. cerevisiae* en *K. marxianus* Y885.

Eerstens was die morfologiese eienskappe van die rasse ondersoek en vergelyk. Talle verskille was gevind tussen die giste met net sommige giste wat dieselfde morfologiese eienskappe gewys het as IWBT Y885. Daarna was verskeie wynkundige eienskappe ondersoek in kuns druiwesap. Hierdie het bestaan uit fermentasie krag, fermentasie metaboliese produkte, sowel as H_2S en pektinase produksie. Verskille tussen Y885 en die rasse L01 tot L05 was gevind in die meederheid van eienskappe wat ondersoek is. Hierdie verskille het voorgekom ten spyte van die oorsprong van die giste. Nadat 'n cluster analiese gedoen was, het drie groeperings verskyn. Die eerste groep het uit rasse L01, L05, Y885 en BF2020 bestaan en die tweede groep uit L02 en L04. L03 was die mees verskillend van die ander rasse en het die derde groep gemaak.

Na die eksperimente in kuns druiwesap was fermentasies gedoen met Chenin blanc en Cabernet Sauvignon druiwesap. Deur cluster analiese te gebruik vir die vlugtige stowwe het duidelike verskille geblyk tussen die Chenin blanc fermentasies met *K. marxianus* en dié met *S. cerevisiae*. Soortgelyke groeperings het verskyn tussen die *K. marxianus* rasse soos wat gesien was in die kuns druiwesap fermentasies. Ten spyte van die chemiese verskille tussen die wyne, was geen duidelike sensoriese verskille gevind nie. Die hipotese is dat die moonlike verskille wat verskyn het, nie opgetel was nie as gevolg van die meer dominante sensoriese eienskappe wat in die wyn gevind was (vars groen, kalkerig, bitter, bruisend).

Hierdie studie het bevestig dat verskille tussen *K. marxianus* rasse 'n algemene voorkoms is. Spesifiek in die wynbedryf laat dit toe dat wyne met verskillende chemiese komposisies gemaak kan word. Die ras IWBT

Y885 het duidelik uitgestaan met verwysing na verskeie wynkundige eienskappe in kuns druiwesap, maar hierdie eienskappe benodig verdere ondersoek met verband na regte druiwesap eksperimente.

Biographical sketch

Barend Erasmus was born in the Western Cape, South Africa on the 8th of April 1996 and matriculated from Brackenfell High School in 2014. He completed his BSc in Molecular Biology and Biotechnology at Stellenbosch University in 2017 and decided to enter the Wine industry by pursuing an Honours degree in Wine Biotechnology in 2018. Upon completion of his Wine Biotechnology degree at Stellenbosch University, Barend worked as a cellar assistant at Rust en Vrede Wine estate and traveled to Germany to gain work experience abroad. In 2020 Barend commenced with his Master's degree in Wine Biotechnology at Stellenbosch University.

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Preface

This thesis is presented as a compilation of 5 chapters. Each chapter is introduced separately and is written according to the style of the journal *Food Microbiology* to which Chapter 3 was submitted for publication.

Chapter 1 **General Introduction and project aims**

Chapter 2 **Literature review**
Kluyveromyces marxianus and its use in the food industry

Chapter 3 **First research chapter**
Preliminary comparison of oenological traits amongst *Kluyveromyces marxianus* strains under laboratory conditions

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Chapter 1 – General introduction and project aims

1.1 Introduction

In the past few years, the yeast *Kluyveromyces marxianus* has attracted great interest in both the biotechnology as well as food and beverage industry. Indeed, many of these industries make use of its ability to produce various hydrolytic enzymes such as pectinases and inulinases (Amaya-Delgado et al., 2013; Cruz-Guerrero et al., 1995; Merín and Morata de Ambrosini, 2015; Rollero et al., 2018b; Serrat et al., 2002). The latter property along with its ability to grow on a variety of substances and waste products as well as its relatively high ethanol tolerance has made it the focus of many studies (Fonseca et al., 2008; Hart, 2017; Serrat et al., 2002; Yuan et al., 2012; Zoppellari and Bardi, 2013). While only occasionally isolated from the wine environment, its potential for this industry has recently been reported. Indeed, the high pectinase production of *K. marxianus* has been shown to increase the volume of free-run wine in Shiraz (Rollero et al., 2018b). This free-run wine is often regarded as to be of a more desirable quality compared to pressed wine. Wines made using *K. marxianus* have also been shown to exhibit higher phenylethyl acetate and sometimes phenylethanol concentrations (Rollero et al., 2018b; Labuschagne, 2020). These wines being chemically and sensorially distinguishable from wines made with the commercially used *S. cerevisiae* alone (Labuschagne, 2020). Nevertheless, limited literature is available on *K. marxianus* in association with wine and over the past 20 years, only five primary studies investigated the impact of *K. marxianus* on wine aroma compounds (Barone et al., 2021; Kourkoutas et al., 2004; Plata et al., 2003; Rollero et al., 2018b; Vigentini et al., 2016). One of these extended into a series of follow-up studies that included strain IWBT Y885, a strain isolated from South African grape juice at the South African Grape and Wine Research Institute (Labuschagne et al., 2021; Rollero et al., 2021, 2019, 2018a, 2018b; Williams et al., 2019), but the authors never compared their results with other strains. Yet, like many other yeast species**,** *K. marxianus* has been shown to display a large degree of variation between strains. These include variations in their pectinase production and activity as well as ethanol yield (Blanco et al., 1997; Lane et al., 2011). These phenotypical differences likely stem from the many genetic differences reported between strains (Ortiz-Merino et al., 2018). For instance, variable colony morphologies have been linked to the strain's ploidy (Granek and Magwene, 2010). It has yet to be investigated whether this strain heterogeneity extends to the oenological properties described for strain IWBT Y885.

1.2 Project aims

Despite previous studies highlighting *K. marxianus* IWBT Y885's potential, few other *K. marxianus* strains have been investigated for their oenological characteristics. This study therefore aimed to investigate and compare various *K. marxianus* strains regarding oenological characteristics of interest such as pectinase activity, H_2S production, fermentation performance and metabolites produced. The strains investigated

were L01 (originally isolated from dairy), L02 (Dairy), L03 (Dairy), L04 (Baking), L05 (Distillery), five strains belonging to the Lallemand yeast culture collection, as well as the newly produced fusant BF2020 to strain IWBT Y885. As the processes involved in making wine consist of numerous variables, the primary comparisons were completed in synthetic grape juice (SGM) to make use of a more controlled medium and environment. The use of monoculture SGM fermentations also allowed the strains to be characterized holistically without the influence from other yeasts impacting the results. Thereafter, fermentations were completed in both Chenin blanc and Cabernet Sauvignon, sequentially inoculated with *S. cerevisiae,* to compare the strains under conditions closer to an industrial setting.

The main objectives of this study were as follows:

1. Characterize and compare *K. marxianus* strains under laboratory conditions.
   1.1. Compare colony and cell morphology as well as the production of pectinase and hydrogen sulfide.
   1.2. Investigate and compare fermentation performance and production of major oenologically-relevant metabolites in synthetic grape juice.
2. Compare Chenin blanc and Cabernet Sauvignon wines produced using *K. marxianus* with regard to their chemical and sensory profiles.

1.3 References

Amaya-Delgado, L., Herrera-López, E.J., Arrizon, J., Arellano-Plaza, M., Gschaedler, A., 2013. Performance evaluation of *Pichia kluyveri, Kluyveromyces marxianus* and *Saccharomyces cerevisiae* in industrial tequila fermentation. World J. Microbiol. Biotechnol. 29, 875–881. https://doi.org/10.1007/s11274-012-1242-8

Barone, E., Ponticello, G., Giaramida, P., Squadrito, M., Fasciana, T., Gandolfo, V., Ardizzone, F., Monteleone, M., Corona, O., Francesca, N., Oliva, D., 2021. Use of *Kluyveromyces marxianus* to Increase free monoterpenes and aliphatic esters in white wines. Fermentation 7, 79. https://doi.org/10.3390/fermentation7020079

Blanco, P., Sieiro, C., Díaz, A., Reboredo, N.M., Villa, T.G., 1997. Grape juice biodegradation by polygalacturonases from *Saccharomyces cerevisiae*. Int. Biodeterior. Biodegradation 40, 115–118. https://doi.org/10.1016/S0964-8305(97)00055-3

Fonseca, G.G., Heinzle, E., Wittmann, C., Gombert, A.K., 2008. The yeast *Kluyveromyces marxianus* and its biotechnological potential. Appl. Microbiol. Biotechnol. 79, 339–354. https://doi.org/10.1007/s00253-008-1458-6

Granek, J.A., Magwene, P.M., 2010. Environmental and genetic determinants of colony morphology in yeast. PLoS Genet. 6, 1000823. https://doi.org/10.1371/journal.pgen.1000823

Hart, D., 2017. Fermenting solutions to new world problems: Bioremediation of blue agave and cocoa pod wastes (Thesis).

Kourkoutas, Y., McErlean, C., Kanellaki, M., Hack, C.J., Marchant, R., Banat, I.M., Koutinas, A.A., 2004. High-Temperature wine making using the thermotolerant yeast strain *Kluyveromyces marxianus* IMB3. Appl. Biochem. Biotechnol. - Part A Enzym. Eng. Biotechnol. 112, 25–35. https://doi.org/10.1385/ABAB:112:1:25

Labuschagne, P.W.J., 2020. Impact of exogenous thiamine on *Kluyveromyces marxianus* under oenological conditions (Thesis).

Labuschagne, P.W.J., Rollero, S., Divol, B., 2021. Comparative uptake of exogenous thiamine and subsequent metabolic footprint in *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* under simulated oenological conditions. Int. J. Food Microbiol. 184, 109206. https://doi.org/10.1016/j.ijfoodmicro.2021.109206

Lane, M.M., Burke, N., Karreman, R., Wolfe, K.H., O'Byrne, C.P., Morrissey, J.P., 2011. Physiological and metabolic diversity in the yeast *Kluyveromyces marxianus*. Antonie Van Leeuwenhoek 100, 507–519. https://doi.org/10.1007/s10482-011-9606-x

Ortiz-Merino, R.A., Varela, J.A., Coughlan, A.Y., Hoshida, H., da Silveira, W.B., Wilde, C., Kuijpers, N.G.A., Geertman, J.-M., Wolfe, K.H., Morrissey, J.P., 2018. Ploidy Variation in *Kluyveromyces marxianus* separates dairy and non-dairy isolates. Front. Genet. 9, 94. https://doi.org/10.3389/fgene.2018.00094

Plata, C., Millán, C., Mauricio, J.C., Ortega, J.M., 2003. Formation of ethyl acetate and isoamyl acetate by various species of wine yeasts. Food Microbiol. 20, 217–224. https://doi.org/10.1016/S0740-0020(02)00101-6

Rollero, S., Bloem, A., Brand, J., Ortiz-Julien, A., Camarasa, C., Divol, B., 2021. Nitrogen metabolism in three non-conventional wine yeast species: A tool to modulate wine aroma profiles. Food Microbiol. 94, 103650. https://doi.org/10.1016/j.fm.2020.103650

Rollero, S., Bloem, A., Ortiz-Julien, A., Bauer, F.F., Camarasa, C., Divol, B., 2019. A comparison of the nitrogen metabolic networks of Kluyveromyces marxianus and Saccharomyces cerevisiae. Environ. Microbiol. 21, 4076–4091. https://doi.org/10.1111/1462-2920.14756

Rollero, S., Bloem, A., Ortiz-Julien, A., Camarasa, C., Divol, B., 2018a. Fermentation performances and aroma production of non-conventional wine yeasts are influenced by nitrogen preferences. FEMS Yeast Res. 18, 1–11. https://doi.org/10.1093/femsyr/foy055

Rollero, S., Zietsman, A.J.J., Buffetto, F., Schückel, J., Ortiz-Julien, A., Divol, B., 2018b. *Kluyveromyces marxianus* secretes a pectinase in Shiraz grape must that impacts technological properties and aroma profile of wine. J. Agric. Food Chem. 66, 11739–11747. https://doi.org/10.1021/acs.jafc.8b03977

Serrat, M., Bermúdez, R.C., Villa, T.G., 2002. Production, purification, and characterization of a polygalacturonase from a new strain of *Kluyveromyces marxianus* isolated from coffee wet-processing wastewater. Appl. Biochem. Biotechnol. 97, 193–208. https://doi.org/10.1385/ABAB:97:3:193

Vigentini, I., Maghradze, D., Petrozziello, M., Bonello, F., Mezzapelle, V., Valdetara, F., Failla, O., Foschino, R., 2016. Indigenous georgian wine-associated yeasts and grape cultivars to edit the wine quality in a precision oenology perspective. Front. Microbiol. 7, 1–13. https://doi.org/10.3389/fmicb.2016.00352

Williams, D.L., Schückel, J., Vivier, M.A., Buffetto, F., Zietsman, A.J.J., 2019. Grape pomace fermentation and cell wall degradation by *Kluyveromyces marxianus* Y885. Biochem. Eng. J. 150. https://doi.org/10.1016/j.bej.2019.107282

Yuan, W.J., Chang, B.L., Ren, J.G., Liu, J.P., Bai, F.W., Li, Y.Y., 2012. Consolidated bioprocessing strategy for ethanol production from Jerusalem artichoke tubers by *Kluyveromyces marxianus* under high gravity conditions. J. Appl. Microbiol. 112, 38–44. https://doi.org/10.1111/j.1365-2672.2011.05171.x

Zoppellari, F., Bardi, L., 2013. Production of bioethanol from effluents of the dairy industry by *Kluyveromyces marxianus*. N. Biotechnol. 30, 607–613. https://doi.org/10.1016/j.nbt.2012.11.017

Chapter 2 - *Kluyveromyces marxianus* and its use in the food industry

2.1 Introduction

Originally isolated from grapes by Louis Marx in 1888 (Lodder and Kreger-Van Rij, 1953), the yeast *Kluyveromyces marxianus* (named at first *Saccharomyces marxianus*) has since been identified in a large variety of environments. These include soil, dairy (cheese production), beer, wine, tequila (production and waste), dough, coffee (production), coffee (waste), chocolate (production) and cocoa (waste) (Divol and Setati, 2015; Fleet and Mian, 1987; Ho et al., 2014; Inokuma et al., 2015; Lachance, 1995; Nanadoum et al., 2005; Ortiz-Merino et al., 2018; Schwan et al., 2012; Serrat et al., 2002; Tofalo et al., 2014).

Although described early on by E.C. Hansen in 1888 (Lodder and Kreger-Van Rij, 1953), its biotechnological potential has only been acknowledged recently (Fonseca et al., 2008). Indeed, *K. marxianus* is known for its ability to produce various industrially relevant enzymes and for its ability to adapt to a variety of substrates and environmental conditions (Cruz-Guerrero et al., 1995; Rollero et al., 2018c). For instance, its ability to produce hydrolytic enzymes that function efficiently at both high and low temperatures (i.e. 55°C and 4°C) has made it a versatile microorganism that can be used in various industries (Merín and Morata de Ambrosini, 2015; Rollero et al., 2018c; Serrat et al., 2002).

K. marxianus is most frequently isolated from dairy products such as cheese or milk (Fleet and Mian, 1987; Fröhlich-Wyder et al., 2019; Tofalo et al., 2014), most likely because of its ability to metabolize lactose (Ortiz-Merino et al., 2018). It is also frequently isolated from grape environments alongside *Saccharomyces cerevisiae* (Kántor et al., 2015). The latter yeast species is a commonly used bioagent in the food industry and it plays a dominant role in the alcoholic beverage industries, driving alcoholic fermentation and producing a vast array of flavour compounds. Although *K. marxianus* is not a fermenter as strong as *S. cerevisiae*, its ability to produce inulinases, pectinases and metabolize lactose makes it a valuable yeast for these industries. Other industrially relevant traits are largely strain-dependent in *K. marxianus.* This is especially prominent when considering the extracellular hydrolytic enzymes produced. Indeed, both pectinase production and activity have been shown to vary greatly between *K. marxianus* strains (Blanco et al., 1997). This provides an opportunity to find new strains that exhibit enhanced enzyme production or activity, or to engineer new strains using alternative methods such as conventional breeding and hybridisation (Piemolini-Barreto et al., 2014).

Similarly, strain variation occurs regarding ethanol yield (Lane et al., 2011). This large strain variation is likely the reason why *K. marxianus* can be isolated from a large variety of environments. This offers

numerous opportunities for its utilisation in various food and beverage industries due to its ability to survive on various carbon sources and to produce various relevant metabolites.

Although the potential benefits of using *K. marxianus* in the dairy and other biotechnological industries have been reviewed (Fleet and Mian, 1987; Fonseca et al., 2008; Lane and Morrissey, 2010), research has shown that these could extend to other fermentation industries. This review aims to summarise and critically assess the vast body of research published in this field with a particular focus on *K. marxianus*' production of pectinase and inulinase and its overall relevance for various industries connected to the production of wine, grape juice, cocoa, coffee, tequila, bread, dairy products and beer (Figure 2.1).

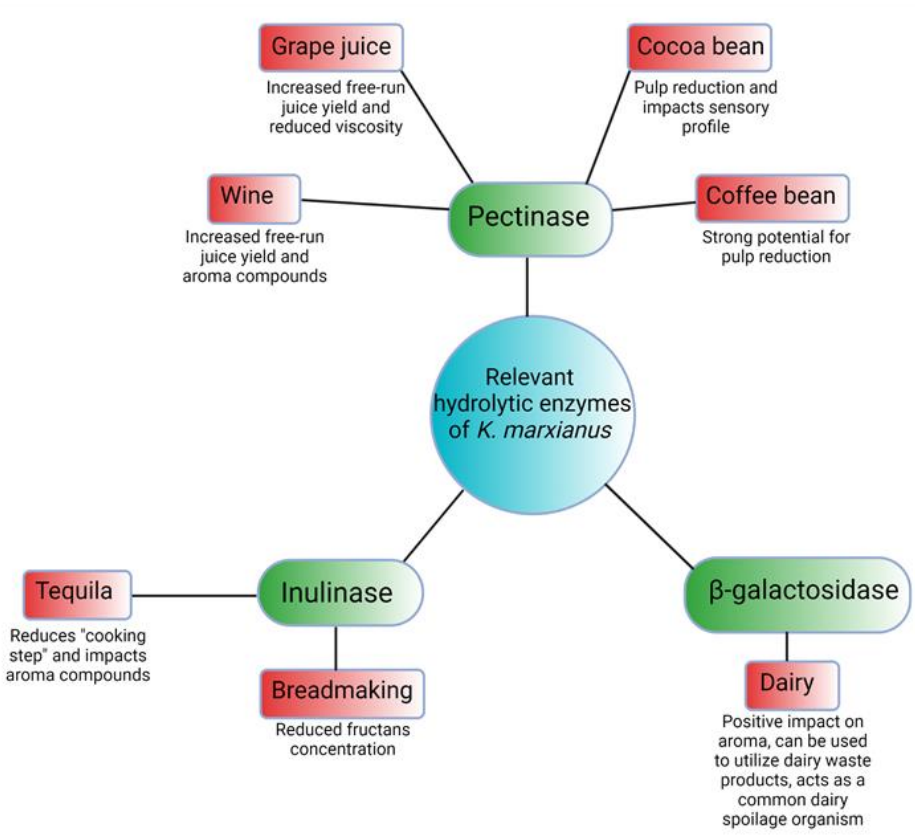

Fig. 2.1. *Relevant branches of the food and beverage industries that benefit and could benefit from the hydrolytic enzymes produced by* K. marxianus*. Created with BioRender.com*

2.2 *K. marxianus'* pectinase and inulinase: two enzymes of high biotechnological relevance

2.2.1 *K. marxianus'* pectinase: an efficient cell wall breaker for the food industries

2.2.1.1 The utilisation of pectinases in the wine and grape juice industry

2.2.1.1.1 Pectin in wine

Pectin is a complex polysaccharide commonly found in plants. In grapes, pectin can be found in large quantities in the middle lamella between plant cells as well in the space between the cellulose-xyloglucan

cell wall scaffold (Ortega-Regules et al., 2008). During fruit ripening, pectin is partially degraded by pectinase enzymes allowing the softening of the fruits. These enzymes include polygalacturonases, which hydrolyse pectic substances by breaking glycosidic alpha (1-4) bonds between galacturonic acid units of the polymer (Ganga et al., 2001). During winemaking, the further breakdown of grape berry cell wall pectin is of great importance. Indeed, it allows the release of various phenolic and aroma compounds as well as the increase in the yield of free-run juice (Álvarez et al., 2006; Gil-Muñoz et al., 2009). Most strains of the main wine yeast *S. cerevisiae* do not produce any or only insignificant amounts of pectinase (Divol and van Rensburg, 2007; Louw et al., 2010).

In order to overcome this issue, the addition of external enzymes of fungal origin during winemaking has been investigated and progressively implemented since the 1950's (Cruess et al., 1951; Reed, 1951) until it became a common step of the process. The timing and enzymes added can vary between the clarification, maceration stage and during fermentation itself, depending on the expected specific goal. These enzymes are typically added in the form of enzyme cocktails to limit the costs associated with purification.

Although their use is now common and broadly beneficial, this process is not without risks. Commercial pectolytic enzymes are majorly obtained from fungal cultures of *Aspergillus.* The supernatant from which the enzymes are extracted contains various enzymes. One of these enzymes that often occurs in commercial enzyme mixtures is pectin methyl esterase, which can ultimately increase the methanol content of the wine through the release of methyl residues into the wine (Revilla and González-SanJosé, 1998). Because of its toxicity (Hodson et al., 2017), the maximum concentration of methanol in wines is strictly regulated (Garg et al., 2016; International Organisation of Vine and Wine, 2004) and operations leading to an increase should therefore be avoided. Non-pectolytic enzymes may also be found such as cinnamoyl esterase. This enzyme has often been cited as responsible for the production of negative phenolic off-flavours (Dugelay et al., 1993), because their activity results in the release of hydroxycinnamic acids, which may be further broken down into volatile phenols by the spoilage yeast *Brettanomyces bruxellensis*. These negative characteristics have been addressed by some commercial enzyme producers however, the removal of these unwanted enzymes generally adds to the cost of the enzyme cocktail.

2.2.1.1.2 *K. marxianus'* pectinase: an enzyme with promising properties for the wine industry

Although a large number of non-*Saccharomyces* yeasts that have been studied, few have been found to display pectinase activity (Blanco et al., 1999; Charoenchai et al., 1997). Among those are *Filobasidium capsuligenum, Rhodotorula dairenensis, Cryptococcus saitoi* and *K. marxianus.* Winemaking conditions expose yeasts and their extracellular enzymes produced to harsh environments. Indeed, typical winemaking conditions consist of high sugar or ethanol concentrations, presence of SO_2, low pH (roughly

between 3 and 4) and cold temperatures. Of the yeasts mentioned above, *K. marxianus* is able to produce pectinases that are still sufficiently active during these conditions (Rollero et al., 2018c).

Pectinase activity by *K. marxianus* was first reported in 1951 (Luh and Phaff, 1951). At the time, *K. marxianus* was classified as *Saccharomyces fragilis.* Since its discovery, it has been studied and characterized numerous times. Reports of the characterisation of this enzyme often differed from each other with some degree as the optimal pH range was found to vary between 3.5 to 5 and temperatures between 40°C and 50°C (Espinoza et al., 1992; Luh and Phaff, 1951; Pereira et al., 1999; Schwan et al., 1997; Schwan and Rose, 1994). These variations can likely be attributed to the large strain variation occurring between *K. marxianus* strains. The use of this enzyme for winemaking was first described by Sieiro et al. (2014). These authors found that the enzyme was able to function under white wine winemaking conditions and at temperatures of 18°C. Confirming this study and providing more information, a study by Rollero et al. (2018c) showed that this enzyme was able to function efficiently under red wine making conditions with an initial cold prefermentative maceration step. This indicated that the enzyme was active at a pH of 3.7 as well as both temperatures of 4°C and 25°C.

The specific method of action of the pectinase produced by *K. marxianus* IWBT Y885 was investigated and found to differ from that of commercial pectinase cocktails. The endopolygalacturonase produced by strain Y885 was found to target specifically pectin polymers found mainly in the middle lamella due to this strains enzyme only being able to hydrolyse α-1,4 glycosidic bonds between nonmethylated galacturonic acid residues (Rollero et al., 2018c). This was confirmed by Mansoldo et al. (2019) who also found similar results using *K. marxianus* CCT3172. This differs from the commercial enzyme cocktails which contain various pectinases that subsequently have multiple targets for hydrolysis. This stronger hydrolytic effect of the enzyme cocktails leads to higher levels of GalA monomers occurring than in treatments where strain Y885 are inoculated. It has been hypothesized that the increased levels of GalA monomers released due to the commercial cocktail used could cause pectin-polyphenol complexes which would aggregate and precipitate out of the wine. This removal of phenolic compounds in treatments where the commercial enzyme cocktails are used could then potentially lead to decreased wine quality compared to those inoculated with strain Y885 (Rollero et al., 2018c).

2.2.1.1.3 Impact of *K. marxianus* pectinase extracts on wine quality

The possibility of using a *K. marxianus* endopolygalacturonase extract was investigated by Sieiro et al. (2014) in order to replace the use of commercial enzyme cocktails. Enzymes were added during fermentation of Albariño grape juice, a white grape varietal. A significant increase in the total aroma fraction, consisting of bound glycosides and free volatiles, was observed for all of the treatments where enzymes were added. The greatest of these being for the treatment with the addition of a 300 U/L of *K.*

marxianus enzyme extract, one unit of activity being defined as the amount of enzyme that releases 1 µmol of galacturonic acid or equivalent in reducing power per minute at 37°C. Of the seven compounds shown to have increased significantly in the 300 U/L *K. marxianus* treatment, only five increased to the extent that the difference could be picked up sensorially. These compounds were α-pinene, linalool, citronellol, β-ionone and eugenol. This increase was especially prominent for β-ionone being 3.6 times higher than in the untreated wines. β-ionone is associated with floral, fruity and violet aroma descriptors, many of which are sought after in various wine styles.

Only the wines treated with the *K. marxianus* enzyme extract exhibited eugenol concentrations above the detection threshold of the human nose. Eugenol is associated with spices, cloves and smoke character (Eder et al., 2012). It is often found in high concentrations when wine is exposed to oak, with the degree of toasting of the oak influencing these concentrations (Bozalongo et al., 2007; Eder et al., 2012). Indeed, toasting of the oak aids in the thermal degradation of the lignin in the wood, which leads to the release of compounds such as eugenol and vanillin (Siegel, 1956; Varanasi et al., 2013). As with wood, grape cell walls also contain lignin (Gao et al., 2019). This lignin is released as the grape cell wall degrades, with this process being enhanced with the aid of pectinase enzymes. Lignin degradation occurs during the winemaking process (Puech, 1987; Setzer, 2011). This is likely why eugenol levels were observed to increase in the study done by Sieiro et al. (2014) as the lignin released was degraded into compounds such as eugenol.

Lignin in grape cell walls can be found in higher concentrations in unripe grapes (Gao et al., 2019). It is possible that one of the reasons why eugenol was found in such high concentrations is that the grapes used were less ripe when picked as the wine made was aimed to be drunk as a “young” wine. Linalool was found to be significantly increased after the addition of *K. marxianus* enzyme extract. This compound is often associated with floral, rose and coriander aromas (Marais, 1983). This increase is significant as linalool can often occur in very low concentrations when grapes are less ripe (Marais, 1983). Therefore, methods to increase compounds such as linalool are sought after.

The study by Sieiro et al. (2014) was performed on a white grape varietal and although extensive, it does not cover all winemaking conditions. Piemolini-Barreto et al. (2014) performed a similar study using a red grape varietal, Cabernet Sauvignon. Juice was fermented on the skins and commercial enzymes or the enzyme extract was added at the start of fermentation. The addition of the *K. marxianus* enzyme extract did not impact the fermentation rate. This contrasts what was found by Sieiro et al. (2014) who showed a significant increase in fermentation rate, of 10 days quicker, with the addition of the *K. marxianus* enzyme extract. Sieiro et al. (2014) attributed the increase in fermentation rate to the increased levels of carbon and nitrogen sources due to the pectin breakdown, however this increase in nutrients would also have occurred in the study performed by Piemolini-Barreto et al. (2014). Unfortunately, the analysis of the grape

juice before fermentation is not provided and therefore nutrient levels are unknown. However, it is possible that the nutrient levels in the Cabernet Sauvignon grape juice were high enough already that an increase in nutrients due to the pectin breakdown would not have had any effect. This saturation effect has been shown previously (Carrau et al., 2008). Indeed, the latter authors found that for *S. cerevisiae* strain M522, no major difference in fermentation rate occurred when the yeast assimilable nitrogen (YAN) was above 75 mg/L. It should also be mentioned that the two studies used different *S. cerevisiae* strains. This is another factor that could influence fermentation rate as different strains may have different nitrogen requirements. Indeed, Carrau et al. (2008) observed that *S. cerevisiae* M522 displayed better fermentation performance than strain KU1 at a YAN concentration of 125 mg/L compared to 75 mg/L.

Similar to previous studies, Piemolini-Barreto et al. (2014) also found a general increase in quality of wine after the addition of the *K. marxianus* enzyme extract. These authors focused largely on the impact of the *K. marxianus* enzyme extract on the phenolic compounds in the wine. One of the factors closely linked to phenolic composition in wines is its colour. Colour and wine clarity are two very important aspects as these are often linked to wine quality and age. Phenolic composition, anthocyanins and proanthocyanins, greatly impact wine colour and its stability. Piemolini-Barreto et al. (2014) showed that the use of *K. marxianus* enzyme extract resulted in an increase of the total anthocyanin concentration by 12% more than the commercial enzyme used and the total polyphenol concentration by 13% more. Along with this, greater wine clarity was observed with these wines, which was attributed to the greater reduction of pectin compounds which can increase the turbidity of wine.

Although multiple studies have shown that *K. marxianus* enzyme extracts can improve certain aspects of the wine, it is important to not deteriorate others at the same time. Piemolini-Barreto et al. (2014) showed that despite increasing the total anthocyanins and polyphenols, many other characteristics were unaffected. Glycerol concentrations, residual sugar and ethanol concentrations, all very important components of wine were similar between the control wines and the wines treated with the *K. marxianus* enzyme extract.

2.2.1.1.4 Impact of *K. marxianus* pectinase extracts on table grape juice quality

Like the wine industry, commercial enzyme cocktails are used to reduce pectinases present in table grape juice. Pectin proves especially troublesome in the table grape juice industry as it increases viscosity, which in turn greatly hinders the clarification and filtration steps.

Piemolini-Barreto et al. (2015) investigated the use of a *K. marxianus* enzyme extract on Ives (*Vitis labrusca*) grape juice, compared to a commercial enzyme treatment. Like in previous studies, an overall increase in extraction yield, decrease in viscosity and increase in clarification of grape juice was found when using the

K. marxianus enzyme extract. However, contrary to the previous studies, these changes were surpassed by the use of the commercial enzyme. Piemolini-Barreto et al. (2015) attributed better performance of the commercial enzyme to the fact that it contained multiple different enzymes, all being able to enhance grape juice characteristics such as clarity and viscosity. It is true that commercial enzymes typically consist of mixtures of different types of pectinases. However, under winemaking conditions, the use of the *K. marxianus* extract still appeared to perform better than that of the commercial enzymes. This difference between the different grape and wine studies is potentially due to differences between the commercial enzyme cocktails used and ratios of enzymes within the commercial products, which can vary. Differences have also been shown to occur regarding the pectin content of wine and table grapes as well as being influenced by ripeness and seasonal variations (Silacci and Morrison, 1990). These pectin differences likely impacted the experiments by Piemolini-Barreto et al. (2015). The specific *K. marxianus* strain IWBT Y885 is able to produce pectinases that are active under high sugar concentrations (Rollero et al., 2018c), while other pectinases such as those produced by *Aspergillus niger* have displayed high inhibitory effects when exposed to high glucose concentrations (Maldonado and Strasser De Saad, 1998). The *K. marxianus* strain used by Piemolini-Barreto et al. (2015) should therefore be investigated regarding glucose inhibitory effects on the pectinases produced. Strain variations could potentially occur regarding this characteristic and would further explain the differences recorded between grape juice and fermenting grape juice studies.

2.2.1.1.5 Inoculating *K. marxianus* during winemaking

Most of the studies mentioned above utilized *K. marxianus* enzyme extracts. However, the use of non-*Saccharomyces* yeasts for sequential fermentations has become an increasingly common practice in the past few years. Rollero et al. (2018c) investigated the impact of performing sequential fermentations using *K. marxianus* and *S. cerevisiae* on pectinase activity during fermentation. The specific strain of *K. marxianus* used was IWBT Y885 which had been isolated from grape juice and displays strong pectinase activity (Rollero et al., 2018c).

The inoculation of two different yeasts during fermentation did not impact the fermentation duration as both wines inoculated with *K. marxianus* as well as those without finished fermentation after around seven days. This contradicts literature which often suggests that the sequential fermentation of an initial non-*Saccharomyces* yeast and secondly a *S. cerevisiae* yeast can lead to an increase in the duration of the fermentation (Puertas et al., 2017; Taillandier et al., 2014).

Overall, Rollero et al. (2018c) found that the inoculation of *K. marxianus* IWBT Y885 increased the free-run juice recovered and the authors attributed this increase to the high production of pectinase by *K. marxianus* IWBT Y885. Increasing the free-run juice is often desired as this juice fraction is typically of better quality than press juice (Darias-Martín et al., 2004; Yokotsuka, 1990). Methanol production was also

found to increase when juice was inoculated with *K. marxianus* IWBT Y885. This increase in methanol was indirectly attributed to the depolymerizing activity of the endopolygalacturonase enzyme. Indeed, the less polymerized pectin macro-structures provide increased access to methoxy groups which can then contribute to the de-esterifying activity (which hydrolyse the methoxy groups) of indigenous microorganisms present (Rollero et al., 2018c). Although high amounts of methanol can be a negative attribute, the methanol levels in the wines were still below the legal limit of 400 mg/L for red wine.

One of the possible benefits of using *K. marxianus* enzyme extracts over the yeast itself is that the amount of enzyme added can be controlled to a better extent. This is potentially why a larger impact was observed by Sieiro et al. (2014) than Rollero et al. (2018c) as the enzyme concentrations added were higher than what the yeast would naturally produce under winemaking conditions, however this still needs to be investigated experimentally. Another concern for using the yeast itself is linked to the yeast's ability to grow and produce pectinase under the cold temperatures during winemaking practices such as prefermentative cold maceration. However, Rollero et al. (2018c) showed that the IWBT Y885 strain was actively growing and secreting enzymes at 4°C (Raimondi et al., 2013). Unlike the addition of commercial enzymes, the use of pectinase-producing yeasts allows for the continuous production of the enzymes throughout fermentation (Williams et al., 2019). This proves advantageous as it allows for increased duration of the presence of pectinase activity.

Utilizing either the *K. marxianus* enzyme extract or the yeast itself has benefits regarding wine quality (Table 2.1). However, more research needs to be conducted before either of these can be used in industry such as the production of large-scale enzyme extracts and the large-scale production of dried *K. marxianus* yeast.

2.2.1.2 Pectin in coffee and cocoa beans

Coffee and chocolate are produced from coffee and cocoa beans, respectively. These productions involve various processes before a coffee or cocoa bean is ready to be used. Coffee beans originate from coffee cherries, which consist of the coffee beans surrounded by various layers such as a mucilage layer, pulp and skin (Figure 2.2). For the beans to be roasted and used to make coffee the surrounding layers must be removed. One of the most commonly used methods is the wet method. A major part of this method is fermenting the beans that are still surrounded by varying quantities of pulp and mucilage, consisting mainly of pectin. Yeasts that are able to produce pectinases are crucial for assisting with the degradation of this pulp and mucilage during fermentation resulting in the naked coffee bean (Garcia et al., 1991; Gonzalez-Rios et al., 2007; Mussatto et al., 2011). In a similar process, cocoa beans, also surrounded by a pulp layer, are recovered (Ho et al., 2014).

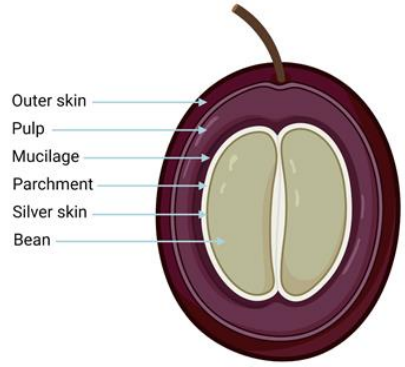

Fig. 2.2. *Diagram representing the composition of a coffee cherry. Created with BioRender.com*

2.2.1.2.1 Enhancing cocoa bean fermentations using *K. marxianus*

The fermentation of cocoa pods facilitates the removal of the pulp (Figure 2.3) and impacts the overall quality of the chocolate produced. Yeasts have been shown to influence various characteristics during fermentation such as the shell content, ethanol produced, higher alcohols, esters, presence of pyrazines, colour of beans and acidity of chocolate produced (Ho et al., 2014). Overall, the latter authors showed that chocolate produced from cocoa beans fermented with bacteria only and no yeasts lacked typical flavours and characteristics associated with chocolate.

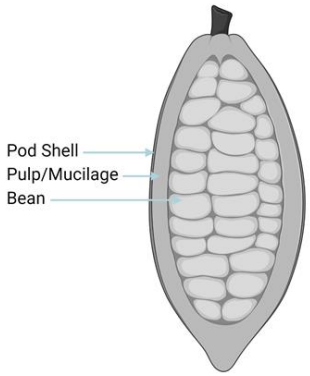

Fig. 2.3. *Diagram representing the composition of a cocoa pod. Created with BioRender.com*

K. marxianus has been previously isolated from cocoa bean fermentations for many years (Ravelomanana et al., 1986). The presence of *K. marxianus* varies to a large extent as traditionally, the fermentations are allowed to occur spontaneously. When the indigenous yeasts present lack the pectinase enzymes or strong

fermentation capabilities, it often leads to increased acidity in the final product, which decreases its quality and economical value. The addition of pectinase enzymes has been utilized in the past, however this is often unaffordable for large scale operations. It has therefore been suggested to inoculate yeasts that are able to produce pectinases, one such yeast being *K. marxianus* (Leal et al., 2008). The latter study revealed that using a hybrid *K. marxianus* strain with increased pectinase activity led to increased pulp reduction, reduced titratable acidity and enhanced sensory properties of the chocolate made from the fermented beans. This was further confirmed in a study by Crafack et al. (2013) who showed that the inoculation of yeasts influences the flavour profile of the final chocolate product to a greater extent than previously suspected. Although the inoculation of yeasts during cocoa bean fermentation has become a more researched topic in recent years, it is still not frequently used in industry. More research is still required regarding various aspects such as the inoculation concentration to have enough yeasts producing pectinases while not inhibiting the indigenous yeast diversity completely.

2.2.1.2.2 Potential to use *K. marxianus* inoculation during coffee bean fermentation

As with cocoa beans, microorganisms play a crucial role in the wet process of coffee bean recovery. In industry, this process is often allowed to occur spontaneously utilizing indigenous yeasts present or by creating starter cultures using previous fermentations. Although this does lead to an increased diversity of yeasts which ultimately increase the complexity of flavours produced by the yeasts, it increases the risk of poor fermentation and pulp removal. It has been suggested to rather use starter cultures consisting of selected yeasts (Bressani et al., 2018). Despite this recommendation, few selected yeasts are available on the market. Various studies have been conducted on finding pectinase-producing yeasts that could be used in this manner (Antier et al., 1993; Haile and Kang, 2019; Masoud and Jespersen, 2006; Oumer and Abate, 2017).

Although *K. marxianus* has been isolated from both natural coffee bean fermentations as well as the wastewater originating from these, very few studies have investigated the potential for using *K. marxianus* during coffee bean fermentations.

In a study by Serrat et al. (2002), a polygalacturonase produced by *K. marxianus,* isolated from coffee fermentation wastewater, was characterized. The enzyme produced was found to have an optimal pH of 4.5 and temperature of 55°C, similar to what had been previously reported. The ability to produce large amounts of pectinase and to ferment at a high rate makes *K. marxianus* a promising candidate for use as a coffee starter culture. It should be noted that this yeast could potentially struggle in the early phase of coffee bean fermentation. This is due to the initial pH of the fermentations generally starting more alkaline, around pH 6, and then becoming more acidic as the fermentation progresses (Velmourougane, 2013). As it

is suggested that increased diversity of yeasts often leads to increased flavour profiles, the use of multiple yeasts in a starter culture could prove beneficial.

2.2.2 *K. marxianus'* inulinase: a potential candidate to help improve current production methods

As described above, *K. marxianus*'s pectinase activity has been extensively researched (Table 2.1). Similarly, its ability to produce inulinase has been investigated. Inulin is a polyfructan that is non digestible by humans. It is found in plants such as wheat and acts as carbohydrate storage unit. The breakdown of inulin by *K. marxianus* has been reported since the 1900's with the production of inulinase by this yeast species being discovered in 1960 by Snyder & Phaff (1960). *K. marxianus*' inulinase was later characterized by Bajpai & Margaritis (1987) who found that inulinases produced by *K. marxianus* performed optimally at a pH between 5 and 6 and temperature between 50°C and 55°C. SO_4^{2-} and Fe^{3+} were found to be strong inhibitors of the enzyme (Cruz-Guerrero et al., 1995; Singh et al., 2007a; Vijayaraghavan et al., 2009).

Inulinase enzymes break down inulin by splitting the terminal fructosyl unit and releasing fructose monomers along with a small amount of glucose. Using enzymes is generally preferred over the chemical hydrolysis of inulin as this can lead to the production of unwanted by-products such as hydroxymethyl furfural and fructose dianhydride. Chemical hydrolysis can also impact product colour which requires downstream processing that can decrease product yield (Singh et al., 2007b).

2.2.2.1 Reducing costs and saving time in tequila production

Tequila production consists of the fermentation and distillation of juice made from *Agave tequilana* (blue agave). The part of the plant that is utilized contains large amounts of fructans such as inulin. In order for microorganisms to be able to ferment the juice, the fructans firstly need to be hydrolysed into simple sugars. Traditionally, this is done through a "cooking" process utilizing steam. However, this process is not 100% effective and often chemical treatments, such as acid hydrolysis, are used. These chemical treatments often have negative consequences. Acidic hydrolysis risks the release of excess salts which inhibit fermentation and increases the rate at which equipment corrodes. Alternatively, commercial enzyme cocktails containing fructanases have recently become available. Utilizing these enzyme cocktails can lead to effectively hydrolysing more than 90 % of fructans present. Although enzyme production can be costly, using enzyme cocktails still proves more economical than utilizing other chemical treatments reducing the cost of producing tequila (Ávila-Fernández et al., 2009; Waleckx et al., 2011).

Current research on new enzyme extracts for commercial use focuses on *A. niger* and *S. cerevisiae.* No research can be found on the use of enzyme extracts produced by *K. marxianus.* As previously mentioned, *K. marxianus* produces inulinase (Vijayaraghavan et al., 2009). Although the inulinase produced by *K.*

marxianus has not shown to be very different to those produced by *A. niger,* inulinase extracts produced by *K. marxianus* possess promising properties for the Tequila industry (Jain et al., 2012; Sirisansaneeyakul et al., 2007).

Recent research focuses on utilizing the yeast *K. marxianus* itself instead of an enzyme extract. *K. marxianus* has been isolated from spontaneous tequila fermentations and is thought to occur commonly (Lachance, 1995). Like other alcoholic beverage industries such as wine and beer, the yeasts present during fermentation have been shown to impact the quality and aromatic compounds present in the final product. *K. marxianus* is a promising yeast to be used in the tequila industry. Indeed, López-Alvarez et al. (2012) found that the *K. marxianus* strain UMPe-1 was able to produce the same level of ethanol as that of a commercially used strain as well as producing greater and different volatile compounds that positively impacted the organoleptic properties of the tequila. This has since been confirmed in another study by Amaya-Delgado et al. (2013) who found that *K. marxianus* produced significantly more esters.

Building on the previous work, Flores et al. (2013) demonstrated that by fermenting with *K. marxianus* strain OFF1, the initial "cooking" and chemical steps could be reduced or skipped.

Although *K. marxianus* has potential to be used in the tequila industry for fermentations, the use of spontaneous fermentations still has many benefits. Indeed, yeast diversity increases the diversity of aroma compounds produced (Lopez et al., 2014). It would therefore be beneficial to inoculate *K. marxianus* along with other yeasts to increase yeast biodiversity. However, this becomes problematic depending on the yeasts used. *S. cerevisiae,* a yeast commonly found and used in tequila fermentations was investigated as an addition to *K. marxianus* fermentations. In most cases, *S. cerevisiae* quickly dominated the fermentations and induced *K. marxianus* loss of viability (Lopez et al., 2014). Despite the latter authors' findings, it can be hypothesized that the loss in viability may not influence the final effect as the main impact of *K. marxianus* is to break down the fructans present in initial steps of tequila production, which would occur early in the fermentation process. A loss in viability in the later stages would therefore not be such a problem. The exact yeast-yeast interaction mechanisms are still not fully understood and more research is needed to better comprehend and manage multi-species consortia.

2.2.2.2 Reducing fructans during breadmaking

Bread can be made in various ways and is consumed in the majority of the world. The most common substrate used to make bread is wheat. This cereal contains a large quantity of fructans which are classified as fermentable oligo-, di- and monosaccharides and polyols (FODMAPs). It has been found that the intake of FODMAPs such as fructans can cause bloating, nausea, and disturbed bowel habits in people suffering from irritable bowel syndrome (IBS) (Goldstein et al., 2000; Truswell et al., 1988). IBS is a chronic functional

gastrointestinal disorder found in populations across the world. Diets low in FODMAPS have shown to reduce symptoms related to IBS by 70% (Struyf et al., 2017).

During the fermentation process of breadmaking, *S. cerevisiae* produces invertase enzymes (Verspreet et al., 2013), which hydrolyse the fructans present into fructose and glucose, but only 50% to 80% of the fructans is hydrolysed. The remaining fructans are often still above the limits of what people with IBS are recommended to consume.

Fructans found in wheat are often of the inulin type. This has generated the speculation that a fermenting yeast that produces inulinase could potentially reduce the levels of fructans present in bread.

Struyf et al. (2017) investigated the effect of using *K. marxianus,* an inulinase-producing yeast with strong fermentative characteristics, on the reduction of fructans during breadmaking (Table 2.1). These authors found that when using either a monoculture of *K. marxianus* or a coculture of both *K. marxianus* and *S. cerevisiae*, the levels of fructans were reduced by more than 90% compared to the 56% of the *S. cerevisiae* monoculture. This decrease was even greater when cocultures were used and the dosage of each yeast was doubled. The authors speculated that the double dosage would have increased the amount of enzyme present to degrade the fructans. Using multiple types of yeast and increasing the amount of yeasts added are not always viable options in industry as both of these add to the cost of production. It is therefore promising that the use of *K. marxianus* monoculture was able to reduce the levels of fructans to below that which would affect people with IBS. Nevertheless, using a coculture of *S. cerevisiae* and *K. marxianus* might prove a better suited option. Indeed, although *K. marxianus* was able to reduce the fructans concentrations, the resulting fructose concentrations increased to a great extent. This is problematic as fructose is a FODMAP which can have the same impact on people with IBS as fructans. This increase in fructose was not observed when both *K. marxianus* and *S. cerevisiae* were used in coculture. This was attributed to the stronger fermentation capability of *S. cerevisiae* being able to degrade fructose at a faster rate and to a greater extent than *K. marxianus* in monoculture (Struyf et al., 2017).

Furthermore, an important aspect of breadmaking is the CO_2 production, which allows the bread to gain volume. Due to the decreased fructose and glucose consumption as well as the inability of *K. marxianus* to consume maltose, the monoculture *K. marxianus* treatment resulted in lower quality bread volume compared to that of the *S. cerevisiae* treatment. However, the usage of both *K. marxianus* and *S. cerevisiae* produced similar results to the monoculture *S. cerevisiae* treatment. A slight decrease was observed during the fermentation after the fructose and glucose had been consumed as the remaining maltose could only be consumed by the *S. cerevisiae* present in the coculture, however this slight decrease in fermentation rate did not make a significant difference to the bread volume (Struyf et al., 2017).

In a follow-up study, the authors investigated whether the CO_2 production of monoculture *K. marxianus* in breadmaking could be improved with the addition of a glucose releasing amyloglucosidase (Struyf et al., 2018). This enzyme would aid in the breakdown of maltose which *K. marxianus* is unable to ferment. Indeed, it successfully aided in the breakdown of maltose and subsequent in the release of glucose, which was subsequently consumed by *K. marxianus*, thereby resulting in increased CO_2 production. Nevertheless, the end glucose concentrations were still relatively high. This is positive as high glucose concentrations can aid with the proper uptake of the FODMAP fructose by people with IBS (Gouyon et al., 2003). Although this increase in glucose can aid people with IBS, the increased glucose and fructose concentrations can lead to sweeter tasting bread which could potentially be perceived as negative by consumers. Similarly, drastic changes in taste or smell can deter customers from trying new products. It is known that *S. cerevisiae* and *K. marxianus* produce different enzymes which leads to different metabolites (Struyf et al., 2018). These different metabolites often lead to variations in aroma and flavour profiles. Despite this, the organoleptic differences between bread made with either of the above-mentioned strains was not great enough to allow consumers to sensorially differentiate between bread made with the two different yeasts (Struyf et al., 2018).

2.3 Beyond hydrolytic enzymes: the overall relevance of *K. marxianus* in various food industries

2.3.1 The frequent occurrence and use of *K. marxianus* in the dairy industry

As mentioned above, the ability to use lactose as a carbon source as well as to grow at both colder and warmer temperatures has led to *K. marxianus* to be frequently isolated from dairy environments. This isolation and association with the dairy industry has been frequently investigated in the past and has been extensively reviewed (Fonseca et al., 2008; Lane & Morrissey, 2010; Varela et al., 2017). In summary of these articles, the association of *K. marxianus* with the dairy industry can be split into two main sections: (1) its prevalence in the production of cheese and utilization of cheese waste and (2) its impact on dairy spoilage (Table 2.1).

K. marxianus is often used in the production of blue, bloomy rind as well as short-ripened cheeses. It has the ability to metabolize lactose and galactose, due to its lactose permease Lac12 and β-galactosidase Lac4. *K. marxianus* also confers various positive aroma and flavour attributes. Cheeses made in association with *K. marxianus* are often associated with acidic, cidery, alcoholic, fermented and fruity flavours. Due to the production of CO_2 by *K. marxianus* during cheese production, *K. marxianus* also aids in the formation of “open texture” characteristics in cheeses. Similarly, downstream effects such as the deacidification of the cheese by *K. marxianus* aids in producing favourable conditions for the growth of necessary ripening bacteria (Fröhlich-Wyder et al., 2019).

The production of cheese generates large amounts of waste, specifically cheese whey. This by-product has been extensively studied with emphasis on recycling and/or reducing the cheese whey generated, ultimately reducing pollution produced. Cheese whey consists of, amongst other nutrients and proteins, lactose which can be metabolized by *K. marxianus.* This has led to two major branches of reuse of cheese whey. Firstly, whey can be used as a carbon source of large-scale growth of *K. marxianus*. The biomass produced could then be used in animal feed and other food processing industries. Secondly, the fermentation of the cheese whey by *K. marxianus* could be used in the subsequent production of ethanol (Fonseca et al., 2008; Lane and Morrissey, 2010; Varela et al., 2017).

Despite being a yeast of great potential in the dairy industry, *K. marxianus* is also one of the most frequently found spoilage organisms. Reasons for this prevalence being the same as to why it is used to such great success in dairy production: its ability to survive in cold temperatures as well as to grow on lactose leads to it being able to grow in dairy products despite refrigeration. Moreover, *K. marxianus* possesses the ability to produce lipolytic and proteolytic enzymes that hydrolyze milk fat and proteins found in dairy products. Similarly, its positive attribute of producing the “open texture” characteristics in many cheeses is only positive when occurring in the correct cheese. Various other cheeses that consumers do not expect to contain this “open texture” characteristics are regarded as spoiled when *K. marxianus* contamination occurs during the production steps (Fleet and Mian, 1987; Fröhlich-Wyder et al., 2019; Mayoral et al., 2005).

2.3.2 *K. marxianus* in the beer industry and its health benefits

As discussed in this review, *K. marxianus* is either utilized or has the potential to be utilized in numerous different food industries (Table 2.1). One of these industries being the alcohol industry. *S. cerevisiae* has been used as a starter culture across the alcoholic beverage industry for many decades. Nevertheless, research on using non-*Saccharomyces* yeasts has increased drastically in recent years with a large majority of this research focusing on the wine industry, but *K. marxianus* has only received minimal attention. As shown with tequila production, its potential to be used in the alcohol industry should not be overlooked.

Currently, little research has been conducted on the utilization of *K. marxianus* in the traditional beer industry. However, it has seen some interest in less traditional beer industries. Indeed, *K. marxianus* is to be largely responsible for the initial fermentation during the production of bili bili, a traditional sorghum beer of Chad (Nanadoum et al., 2005). Bili bili is consumed on a much smaller scale than other commercial beers and this is likely why little research exists on the use and role of different yeasts during its fermentation process. Similarly, a new increasingly popular form of beer in China called milk beer also lacks research. Milk beer was developed in China as a healthier alcoholic drink and consists of a primary step involving lactic acid bacteria as well as a fermentation step by yeasts. Currently, majority of the yeasts used in

industry are *S. cerevisiae* strains which have caused similar flavour profiles across many of the milk beers. A study by Wang et al. (2020) showed the potential to use *K. marxianus* in order to alleviate this problem. Milk beer was successfully produced using *K. marxianus* strain FXJ1 showing increased ester production compared to industrial *S. cerevisiae* strains. The increased ester production led to more fruity and floral milk beer.

Although not currently investigated, it is suggested that the use of *K. marxianus* could have potential health benefits when producing milk beer. Indeed, lactose can be found in milk beer in concentrations high enough to negatively impact people suffering from lactose intolerance. As reported above, *K. marxianus* possesses the ability to metabolize lactose. It has therefore been suggested than using *K. marxianus* during the milk beer fermentation step could reduce the lactose present in the final product. Nevertheless, the extent to which it would be decreased and the subsequent impact on the milk beer still requires more research.

2.3.3 *K. marxianus* stands out amongst non-*Saccharomyces* yeasts in the wine industry

As discussed in section 2.1, both the use of *K. marxianus* pectinase extracts or the inoculation of the yeast itself show great potential to confer positive characteristics when used in wine (Table 2.1). Among these, various properties with regards to inoculating *K. marxianus* into wine have been investigated.

The use of non-*Saccharomyces* yeasts in the wine industry has increased to a large extent over recent years. One large constraint that non-*Saccharomyces* yeasts face is their poor ethanol tolerance compared to commercially used *S. cerevisiae* strains. This results in incomplete fermentations when only using non-*Saccharomyces* yeasts. *K. marxianus* IWBT Y885 shows potential having a higher ethanol tolerance, and is able to ferment to a greater degree compared to most other non-*Saccharomyces* yeasts (Rollero et al., 2018b). However, it should be mentioned that this potential was shown in synthetic grape juice with larger headspaces than would be seen in industrial practices. Indeed, the fermentation performance decreased under real grape juice conditions with larger volumes and reduced headspaces, most probably as a result of a lower oxygen availability (Labuschagne, 2020). It has been suggested that the fermentation performance of *K. marxianus* could be enhanced if fermentations were to be supplemented with specific nutrients such as thiamine (Labuschagne et al., 2021). Nevertheless, *K. marxianus* still lacks the ability to ferment at rates close to those of the commercially used *S. cerevisiae* strains. This makes using *K. marxianus* as a sole yeast in industry unviable as the slow rate of fermentation will likely lead to indigenous *S. cerevisiae* growth to occur and dominate fermentations. This can prove potentially problematic and lead to stuck fermentations if nutrient competition occurs without nutrient supplementation (Rollero et al., 2021; 2018a).

Although *K. marxianus* cannot currently be inoculated as sole starter culture to ferment high sugar grape musts to dryness, its use in mixed culture fermentations such as those suggested in section 2.1 remains of great potential. Analysis of the volatile compounds produced during sequential fermentations showed increased production of phenylethanol and phenylethyl acetate in the wines where *K. marxianus* IWBT Y885 was inoculated (Rollero et al., 2018b, 2018c). These compounds have been shown to be associated with various sought-after aroma descriptors such as fruity, banana and blackcurrant (Fang & Qian, 2005).

Table 2.1 | Impact and use of *K. marxianus* in various food and beverage industries

Food industry	Wine	Grape juice	Cocoa beans	Coffee beans	Tequila	Bread	Dairy products	Beer
Current or potential use in industry	Pectinase enzyme extracts: • Increase in free run. • Increase in positive aroma compounds. • Increase release of nitrogen sources. • Controlled aspect regarding type of pectinase and concentration. • Increase in anthocyanin and polyphenol concentrations.	Pectinase enzyme extracts: • Increase in extraction yield. • Decrease in viscosity. • Increase in clarification.	Inoculation of *K. marxianus:* • Increased pulp reduction. • Reduced titratable acidity. • Improved sensory aspects during chocolate production.	Not currently used however strong potential to be used for pulp reduction.	Inoculation of *K. marxianus:* • Increase in positive aroma compounds. • Able to produce similar levels of ethanol compared to commercially used strains. • Production of inulinases can reduce or skip the required "cooking" step in tequila production.	Inoculation of *K. marxianus:* • Production of inulinases can reduce levels of fructans present in bread reducing levels of potentially negative FODMAPs. • Able to produce sufficient levels of CO_2 required during breadmaking when used in coculture or when amyloglucosidase is added in monoculture. • Despite positive decreases in FODMAPs consumers were unable to distinguish sensorial differences between bread produced by *K. marxianus* and *S. cerevisiae.*	Inoculation of *K. marxianus:* • Increase in positive aroma compounds when used in cheese production. • CO_2 production aids in formation of perforation in cheeses. • Deacidification by *K. marxianus* aids in creating favourable conditions for ripening bacteria to grow. • *K. marxianus* biomass can be produced from cheese whey (cheese waste) for use in animal feed and downstream food processing. • *K. marxianus*	Inoculation of *K. marxianus*: • Frequently used in less traditional beers such as bili bili. • Potential to be used in milk beer and increase the diversity of flavours. • Potential to reduce presence of potentially negative lactose found in milk beers.

		can be used to ferment cheese whey to produce high yielding ethanol.
Inoculation of *K. marxianus:* • Increase in free run. • Produced and active under cold temperatures such as during prefermentative cold maceration. • Continues production of enzyme throughout fermentation. • Increase in positive aroma compounds.		Spoilage by *K. marxianus:* • Spoilage of dairy is commonly caused by *K. marxianus.* • High spoilage rates are due to its ability to grow in cold temperatures and to use lactose as a carbon source. • Perforations produced by *K. marxianus* required in certain cheeses can be considered spoilage when occurring in others where consumers do not expect it.

2.4 Future outlooks

K. marxianus has great potential to be used in various food and beverage industries. Research regarding its use as a starter culture is becoming more frequent and spanning across a range of food industries. Although promising results have shown that it can be used to create enzyme extracts or to be inoculated directly into various mediums, research is still required to fill in various gaps regarding its increased methanol production and poor ability to ferment with other yeasts.

The large amount of strain variation found between *K. marxianus* strains is likely key to it being such a versatile organism for many unrelated industries. This variation has been demonstrated both genotypically and phenotypical. It highlights the adaptive response that *K. marxianus* displays resulting from inhabiting different environments. It is therefore of interest for future studies to investigate the extent of intraspecific variation between *K. marxianus* strains as well as emerging strains such as IWBT Y885.

2.5 References

Álvarez, I., Aleixandre, J.L., García, M.J., Lizama, V., 2006. Impact of prefermentative maceration on the phenolic and volatile compounds in Monastrell red wines. Anal. Chim. Acta 563, 109–115. https://doi.org/10.1016/j.aca.2005.10.068

Amaya-Delgado, L., Herrera-López, E.J., Arrizon, J., Arellano-Plaza, M., Gschaedler, A., 2013. Performance evaluation of *Pichia kluyveri, Kluyveromyces marxianus* and *Saccharomyces cerevisiae* in industrial tequila fermentation. World J. Microbiol. Biotechnol. 29, 875–881. https://doi.org/10.1007/s11274-012-1242-8

Antier, P., Minjares, A., Roussos, S., Raimbault, M., Viniegra-Gonzalez, G., 1993. Pectinase-hyperproducing mutants of *Aspergillus niger* C28B25 for solid-state fermentation of coffee pulp. Enzyme Microb. Technol. 15, 254–260. https://doi.org/10.1016/0141-0229(93)90146-S

Ávila-Fernández, A., Rendón-Poujol, X., Olvera, C., González, F., Capella, S., Peña-Álvarez, A., López-Munguía, A., 2009. Enzymatic hydrolysis of fructans in the tequila production process. J. Agric. Food Chem. 57, 5578–5585. https://doi.org/10.1021/jf900691r

Bajpai, P., Margaritis, A., 1987. Characterization of molecular-sieve-bound inulinase. J. Ferment. Technol. 65, 239–242. https://doi.org/10.1016/0385-6380(87)90172-5

Blanco, P., Sieiro, C., Díaz, A., Reboredo, N.M., Villa, T.G., 1997. Grape juice biodegradation by polygalacturonases from *Saccharomyces cerevisiae*. Int. Biodeterior. Biodegradation 40, 115–118. https://doi.org/10.1016/S0964-8305(97)00055-3

Blanco, P., Sieiro, C., Villa, T.G., 1999. Production of pectic enzymes in yeasts. FEMS Microbiol. Lett. 175, 1–9. https://doi.org/10.1016/S0378-1097(99)00090-7

Bozalongo, R., Carrillo, J.D., Torroba, M.Á.F., Tena, M.T., 2007. Analysis of French and American oak chips with different toasting degrees by headspace solid-phase microextraction-gas chromatography–mass spectrometry. J. Chromatogr. A 1173, 10–17. https://doi.org/10.1016/j.chroma.2007.09.079

Bressani, A.P.P., Martinez, S.J., Evangelista, S.R., Dias, D.R., Schwan, R.F., 2018. Characteristics of fermented coffee inoculated with yeast starter cultures using different inoculation methods. LWT 92, 212–219.

https://doi.org/10.1016/j.lwt.2018.02.029

Carrau, F.M., Medina, K., Farina, L., Boido, E., Henschke, P.A., Dellacassa, E., 2008. Production of fermentation aroma compounds by *Saccharomyces cerevisiae* wine yeasts: effects of yeast assimilable nitrogen on two model strains. FEMS Yeast Res. 8, 1196–1207. https://doi.org/10.1111/j.1567-1364.2008.00412.x

Charoenchai, C., FLeet, G.H., Henschke, P.., Todd, B.E.N.., 1997. Screening of non- *Saccharomyces* wine yeasts for the presence of extracellular hydrolytic enzymes. Aust. J. Grape Wine Res. 3, 2–8. https://doi.org/10.1111/j.1755-0238.1997.tb00109.x

Crafack, M., Mikkelsen, M.B., Saerens, S., Knudsen, M., Blennow, A., Lowor, S., Takrama, J., Swiegers, J.H., Petersen, G.B., Heimdal, H., Nielsen, D.S., 2013. Influencing cocoa flavour using *Pichia kluyveri* and *Kluyveromyces marxianus* in a defined mixed starter culture for cocoa fermentation. Int. J. Food Microbiol. 167, 103–116. https://doi.org/10.1016/j.ijfoodmicro.2013.06.024

Cruess, W.., O'Neal, R., Chong, G., Uchimoto, D., 1951. The Effect of Pectic Enzymes in Wine Making. Am. J. Enol. Vitic. 2, 59–75.

Cruz-Guerrero, A., Garcia-Peña, I., Barzana, E., Garcia-Garibay, M., Gomez-Ruiz, L., 1995. *Kluyveromyces marxianus* CDBB-L-278: A wild inulinase hyperproducing strain. J. Ferment. Bioeng. 80, 159–163. https://doi.org/10.1016/0922-338X(95)93212-3

Darias-Martín, J., Díaz-González, D., Díaz-Romero, C., 2004. Influence of two pressing processes on the quality of must in white wine production. J. Food Eng. 63, 335–340. https://doi.org/10.1016/j.jfoodeng.2003.08.005

Data on wine production and methanol limits. https://www.oiv.int/public/medias/644/oeno-19-2004-en.pdf (accessed on July 25, 2021), International Organisation of Vine and Wine, 2004. Certified in conformity Mainz, 10.

Divol, B., Setati, M., 2015. Secretion of hydrolytic enzymes by non- *Saccharomyces* yeasts – a relevant trait for winemaking? Winel. Mag. 1–7.

Divol, B., van Rensburg, P., 2007. PGU1 gene natural deletion is responsible for the absence of endo-polygalacturonase activity in some wine strains of *Saccharomyces cerevisiae*. FEMS Yeast Res. 7, 1328–1339. https://doi.org/10.1111/j.1567-1364.2007.00284.x

Dugelay, I., Gunata, Z., Sapis, J.C., Baumes, R., Bayonove, C., 1993. Role of cinnamoyl esterase activities from enzyme preparations on the formation of volatile phenols during winemaking. J. Agric. Food Chem. 41, 2092–2096. https://doi.org/10.1021/jf00035a051

Eder, R., Návojská, J., Brandes, W., Nauer, S., Frančáková, H., 2012. Influence of different oak chips on aroma compounds in wine. J. Microbiol. 1, 957–971.

Espinoza, P., Bárzana, E., García-Garibay, M., Gómez-Ruiz, L., 1992. Evaluation of *Kluyveromyces marxianus* for the production of lactase simultaneously to pectinase or inulinase. Biotechnol. Lett. 14, 1053–1058. https://doi.org/10.1007/BF01021058

Fleet, G.H., Mian, M.A., 1987. The occurrence and growth of yeasts in dairy products. Int. J. Food Microbiol. 4, 145–155. https://doi.org/10.1016/0168-1605(87)90021-3

Flores, J.A., Gschaedler, A., Amaya-Delgado, L., Herrera-López, E.J., Arellano, M., Arrizon, J., 2013. Simultaneous saccharification and fermentation of agave tequilana fructans by *Kluyveromyces marxianus* yeasts for bioethanol and tequila production. Bioresour. Technol. 146, 267–273. https://doi.org/10.1016/j.biortech.2013.07.078

Fonseca, G.G., Heinzle, E., Wittmann, C., Gombert, A.K., 2008. The yeast *Kluyveromyces marxianus* and its biotechnological potential. Appl. Microbiol. Biotechnol. 79, 339–354. https://doi.org/10.1007/s00253-008-1458-

Fröhlich-Wyder, M.-T., Arias-Roth, E., Jakob, E., 2019. Cheese yeasts. Yeast 36, 129–141. https://doi.org/10.1002/yea.3368

Ganga, A., Piñaga, F., Querol, A., Vallés, S., Ramón, D., 2001. Cell-wall degrading enzymes in the release of grape aroma precursors. Food Sci. Technol. Int. 7, 83–87. https://doi.org/10.1106/7CAF-U2DW-BBH2-VUQV

Gao, Y., Zietsman, A.J.J., Vivier, M.A., Moore, J.P., 2019. Deconstructing wine grape cell walls with enzymes during winemaking: New insights from glycan microarray technology. Molecules 24, 165. https://doi.org/10.3390/molecules24010165

Garcia, R., Arriola, D., Arriola, Mc., Porres, E., Rolz, C., 1991. Characterization of coffee pectin. LWT -Food Sci. Technol. 24, 125—129.

Garg, G., Singh, A., Kaur, A., Singh, R., Kaur, J., Mahajan, R., 2016. Microbial pectinases: an ecofriendly tool of nature for industries. 3 Biotech 6, 47. https://doi.org/10.1007/s13205-016-0371-4

Gil-Muñoz, R., Moreno-Pérez, A., Vila-López, R., Fernández-Fernández, J.I., Martínez-Cutillas, A., Gómez-Plaza, E., 2009. Influence of low temperature prefermentative techniques on chromatic and phenolic characteristics of Syrah and Cabernet Sauvignon wines. Eur. Food Res. Technol. 228, 777–788. https://doi.org/10.1007/s00217-008-0989-5

Goldstein, R., Braverman, D., Stankiewicz, H., 2000. Carbohydrate malabsorption and the effect of dietary restriction on symptoms of irritable bowel syndrome and functional bowel complaints. Isr. Med. Assoc. J. 2, 583–7.

Gonzalez-Rios, O., Suarez-Quiroz, M.L., Boulanger, R., Barel, M., Guyot, B., Guiraud, J.-P., Schorr-Galindo, S., 2007. Impact of "ecological" post-harvest processing on coffee aroma: II. Roasted coffee. J. Food Compos. Anal. 20, 297–307. https://doi.org/10.1016/j.jfca.2006.12.004

Gouyon, F., Caillaud, L., Carrière, V., Klein, C., Dalet, V., Citadelle, D., Kellett, G.L., Thorens, B., Leturque, A., Brot-Laroche, E., 2003. Simple-sugar meals target GLUT2 at enterocyte apical membranes to improve sugar absorption: a study in GLUT2-null mice. J. Physiol. 552, 823–832. https://doi.org/10.1113/jphysiol.2003.049247

Haile, M., Kang, W.H., 2019. Isolation, identification, and characterization of pectinolytic yeasts for starter culture in coffee fermentation. Microorganisms 7, 401. https://doi.org/10.3390/microorganisms7100401

Ho, V.T.T., Zhao, J., Fleet, G., 2014. Yeasts are essential for cocoa bean fermentation. Int. J. Food Microbiol. 174, 72–87. https://doi.org/10.1016/j.ijfoodmicro.2013.12.014

Hodson, G., Wilkes, E., Azevedo, S., Battaglene, T., 2017. Methanol in wine. BIO Web Conf. 9, 02028. https://doi.org/10.1051/bioconf/20170902028

Inokuma, K., Ishii, J., Hara, K.Y., Mochizuki, M., Hasunuma, T., Kondo, A., 2015. Complete genome sequence of *Kluyveromyces marxianus* NBRC1777, a non-conventional thermotolerant yeast. Genome Announc. 3, 2–3. https://doi.org/10.1128/genomeA.00389-15

Jain, S.C., Jain, P.C., Kango, N., 2012. Production of inulinase from *Kluyveromyces marxianus* using Dahlia tuber extract. Brazilian J. Microbiol. 43, 62–69. https://doi.org/10.1590/S1517-83822012000100007

Kántor, A., Kačániová, M., Kluz, M., 2015. Natural microflora of wine grape berries. J. Microbiol. Biotechnol. Food Sci. 04, 32–36. https://doi.org/10.15414/jmbfs.2015.4.special1.32-36

Labuschagne, P.W.J., 2020. Impact of exogenous thiamine on *Kluyveromyces marxianus* under oenological conditions (Thesis).

Labuschagne, P.W.J., Rollero, S., Divol, B., 2021. Comparative uptake of exogenous thiamine and subsequent metabolic footprint in *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* under simulated oenological conditions. Int. J. Food Microbiol. 184, 109206. https://doi.org/10.1016/j.ijfoodmicro.2021.109206

Lachance, M.-A., 1995. Yeast communities in a natural tequila fermentation. Antonie Van Leeuwenhoek 68, 151–160. https://doi.org/10.1007/BF00873100

Lane, M.M., Burke, N., Karreman, R., Wolfe, K.H., O'Byrne, C.P., Morrissey, J.P., 2011. Physiological and metabolic diversity in the yeast *Kluyveromyces marxianus*. Antonie Van Leeuwenhoek 100, 507–519. https://doi.org/10.1007/s10482-011-9606-x

Lane, M.M., Morrissey, J.P., 2010. *Kluyveromyces marxianus*: A yeast emerging from its sister's shadow. Fungal Biol. Rev. 24, 17–26. https://doi.org/10.1016/j.fbr.2010.01.001

Leal, G.A., Gomes, L.H., Efraim, P., de Almeida Tavares, F.C., Figueira, A., 2008. Fermentation of cacao (Theobroma cacao L.) seeds with a hybrid *Kluyveromyces marxianus* strain improved product quality attributes. FEMS Yeast Res. 8, 788–798. https://doi.org/10.1111/j.1567-1364.2008.00405.x

Lodder, J., Kreger-Van Rij, N.J.W., 1953. The Yeasts: A Taxonomic Study. Science (80-.). 117, 237–237.

López-Alvarez, A., Díaz-Pérez, A.L., Sosa-Aguirre, C., Macías-Rodríguez, L., Campos-García, J., 2012. Ethanol yield and volatile compound content in fermentation of agave must by *Kluyveromyces marxianus* UMPe-1 comparing with *Saccharomyces cerevisiae* baker's yeast used in tequila production. J. Biosci. Bioeng. 113, 614–618. https://doi.org/10.1016/j.jbiosc.2011.12.015

Lopez, C.L.F., Beaufort, S., Brandam, C., Taillandier, P., 2014. Interactions between *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* in tequila must type medium fermentation. World J. Microbiol. Biotechnol. 30, 2223–2229. https://doi.org/10.1007/s11274-014-1643-y

Louw, C., Young, P.R., van Rensburg, P., Divol, B., 2010. Epigenetic regulation of PGU1 transcription in *Saccharomyces* cerevisiae. FEMS Yeast Res. 10, 158–167. https://doi.org/10.1111/j.1567-1364.2009.00599.x

Luh, B.S., Phaff, H.J., 1951. Studies on polygalacturonase of certain yeasts. Arch. Biochem. Biophys. 33, 212–227. https://doi.org/10.1016/0003-9861(51)90100-2

Maldonado, M.C., Strasser De Saad, A.M., 1998. Production of pectinesterase and polygalacturonase by *Aspergillus niger* in submerged and solid state systems. J. Ind. Microbiol. Biotechnol. 20, 34–38. https://doi.org/10.1038/sj.jim.2900470

Mansoldo, F.R.P., Neves Junior, A., Cardoso, V.D.S., Rosa, M.D.S.S., Vermelho, A.B., 2019. Evaluation of *Kluyveromyces marxianus* endo -polygalacturonase activity through ATR-FTIR. Analyst 144, 4111–4120. https://doi.org/10.1039/C9AN00265K

Marais, J., 1983. Terpenes in the aroma of grapes and wines: A Review. South African J. Enol. Vitic. 4, 49–58. https://doi.org/10.21548/4-2-2370

Masoud, W., Jespersen, L., 2006. Pectin degrading enzymes in yeasts involved in fermentation of Coffea arabica in East Africa. Int. J. Food Microbiol. 110, 291–296. https://doi.org/10.1016/j.ijfoodmicro.2006.04.030

Mayoral, M.B., Martín, R., Sanz, A., Hernández, P.E., González, I., García, T., 2005. Detection of *Kluyveromyces marxianus* and other spoilage yeasts in yoghurt using a PCR-culture technique. Int. J. Food Microbiol. 105, 27–34. https://doi.org/10.1016/j.ijfoodmicro.2005.06.006

Merín, M.G., Morata de Ambrosini, V.I., 2015. Highly cold-active pectinases under wine-like conditions from non-*Saccharomyces* yeasts for enzymatic production during winemaking. Lett. Appl. Microbiol. 60, 467–474. https://doi.org/10.1111/lam.12390

Mussatto, S.I., Machado, E.M.S., Martins, S., Teixeira, J.A., 2011. Production, composition, and application of coffee and its industrial residues. Food Bioprocess Technol. 4, 661–672. https://doi.org/10.1007/s11947-011-0565-z

Nanadoum, M., Mbailao, M.H.V.N., Claude, G., Jacques, P., 2005. Identification and typing of the yeast strains isolated from bili bili, a traditional sorghum beer of Chad. African J. Biotechnol. 4, 646–656. https://doi.org/10.5897/AJB2005.000-3117

Ortega-Regules, A., Ros-García, J.M., Bautista-Ortín, A.B., López-Roca, J.M., Gómez-Plaza, E., 2008. Differences in morphology and composition of skin and pulp cell walls from grapes (Vitis vinifera L.): technological implications. Eur. Food Res. Technol. 227, 223–231. https://doi.org/10.1007/s00217-007-0714-9

Ortiz-Merino, R.A., Varela, J.A., Coughlan, A.Y., Hoshida, H., da Silveira, W.B., Wilde, C., Kuijpers, N.G.A., Geertman, J.-M., Wolfe, K.H., Morrissey, J.P., 2018. Ploidy variation in *Kluyveromyces marxianus* separates dairy and non-dairy isolates. Front. Genet. 9, 94. https://doi.org/10.3389/fgene.2018.00094

Oumer, O.J., Abate, D., 2017. Characterization of pectinase from *Bacillus subtilis* strain Btk 27 and its potential application in removal of mucilage from coffee beans. Enzyme Res. 2017, 1–7. https://doi.org/10.1155/2017/7686904

Pereira, M., Schwan, R., Teixeira, J., 1999. Isolation, screening, and characterisation of flocculating and pectinase producing *Kluyveromyces* strains. Food Technol. Biotechnol.

Piemolini-Barreto, L.T., Antônio, R.V., Echeverrigaray, S., 2015. Comparison of a pectinolytic extract of *Kluyveromyces marxianus* and a commercial enzyme preparation in the production of Ives (Vitis labrusca) grape juice. World J. Microbiol. Biotechnol. 31, 755–762. https://doi.org/10.1007/s11274-015-1828-z

Piemolini-Barreto, L.T., Zacaria, J., Delamare, A.P.L., Antonio, R.V., Echeverrigaray, S., 2014. Variation in phenolic compounds, anthocyanins, and color in red wine treated with enzymatic extract of *Kluyveromyces marxianus*. World J. Microbiol. Biotechnol. 30, 1541–1547. https://doi.org/10.1007/s11274-013-1577-9

Puech, J.L., 1987. Extraction of Phenolic Compounds from oak wood in model solutions and evolution of aromatic aldehydes in wines aged in oak barrels. Am. J. Enol. Vitic. 38, 236–238.

Puertas, B., Jiménez, M.J., Cantos-Villar, E., Cantoral, J.M., Rodríguez, M.E., 2017. Use of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* in semi-industrial sequential inoculation to improve quality of Palomino and Chardonnay wines in warm climates. J. Appl. Microbiol. 122, 733–746. https://doi.org/10.1111/jam.13375

Raimondi, S., Zanni, E., Amaretti, A., Palleschi, C., Uccelletti, D., Rossi, M., 2013. Thermal adaptability of *Kluyveromyces marxianus* in recombinant protein production. Microb. Cell Fact. 12, 34. https://doi.org/10.1186/1475-2859-12-34

Ravelomanana, R., Guiraud, J.P., Galzy, P., 1986. Isolation of a pectin-utilizing yeast from cocoa beans. Syst. Appl. Microbiol. 8, 230–233. https://doi.org/10.1016/S0723-2020(86)80083-2

Reed, G., 1951. A review of the action of pectic enzymes and a discussion of their commercial production. Am. J. Enol. Vitic. 2, 54–58.

Revilla, I., González-SanJosé, M.L., 1998. Methanol release during fermentation of red grapes treated with pectolytic enzymes. Food Chem. 63, 307–312. https://doi.org/10.1016/S0308-8146(98)00049-1

Rollero, S., Bloem, A., Brand, J., Ortiz-Julien, A., Camarasa, C., Divol, B., 2021. Nitrogen metabolism in three non-conventional wine yeast species: A tool to modulate wine aroma profiles. Food Microbiol. 94, 103650. https://doi.org/10.1016/j.fm.2020.103650

Rollero, S., Bloem, A., Ortiz-Julien, A., Camarasa, C., Divol, B., 2018a. Fermentation performances and aroma production of non-conventional wine yeasts are influenced by nitrogen preferences. FEMS Yeast Res. 18, 1–11.

https://doi.org/10.1093/femsyr/foy055

Rollero, S., Bloem, A., Ortiz-Julien, A., Camarasa, C., Divol, B., 2018b. Altered fermentation performances, growth, and metabolic footprints reveal competition for nutrients between yeast species inoculated in synthetic grape juice-like medium. Front. Microbiol. 9, 1–12. https://doi.org/10.3389/fmicb.2018.00196

Rollero, S., Zietsman, A.J.J., Buffetto, F., Schückel, J., Ortiz-Julien, A., Divol, B., 2018c. *Kluyveromyces marxianus* secretes a pectinase in Shiraz grape must that impacts technological properties and aroma profile of wine. J. Agric. Food Chem. 66, 11739–11747. https://doi.org/10.1021/acs.jafc.8b03977

Schwan, R., Silva, C., Batista, L., 2012. Coffee fermentation, in: Handbook of plant-based fermented food and beverage technology, Second Edition. CRC Press, pp. 677–690. https://doi.org/10.1201/b12055-49

Schwan, R.F., Cooper, R.M., Wheals, A.E., 1997. Endopolygalacturonase secretion by *Kluyveromyces marxianus* and other cocoa pulp-degrading yeasts. Enzyme Microb. Technol. 21, 234–244. https://doi.org/10.1016/S0141-0229(96)00261-X

Schwan, R.F., Rose, A.H., 1994. Polygalacturonase production by *Kluyveromyces marxianus*: effect of medium composition. J. Appl. Bacteriol. 76, 62–67. https://doi.org/10.1111/j.1365-2672.1994.tb04416.x

Serrat, M., Bermúdez, R.C., Villa, T.G., 2002. Production, purification, and characterization of a Polygalacturonase from a new Strain of *Kluyveromyces marxianus* isolated from coffee wet-processing wastewater. Appl. Biochem. Biotechnol. 97, 193–208. https://doi.org/10.1385/ABAB:97:3:193

Setzer, W.N., 2011. Lignin-derived oak phenolics: a theoretical examination of additional potential health benefits of red wine. J. Mol. Model. 17, 1841–1845. https://doi.org/10.1007/s00894-010-0893-3

Siegel, S.M., 1956. The Biosynthesis of Lignin: Evidence for the participation of celluloses as sites for oxidative polymerization of Eugenol. J. Am. Chem. Soc. 78, 1753–1755. https://doi.org/10.1021/ja01589a076

Sieiro, C., Villa, T.G., da Silva, A.F., García-Fraga, B., Vilanova, M., 2014. Albariño wine aroma enhancement through the use of a recombinant polygalacturonase from *Kluyveromyces marxianus*. Food Chem. 145, 179–185. https://doi.org/10.1016/j.foodchem.2013.08.050

Silacci, M.W., Morrison, J.C., 1990. Changes in pectin content of Cabernet Sauvignon grape berries during maturation. Am. J. Enol. Vitic. 41, 111–115.

Singh, R.S., Dhaliwal, R., Puri, M., 2007a. Partial purification and characterization of exoinulinase from *Kluyveromyces marxianus* YS-1 for preparation of high -fructose syrup. J. Microbiol. Biotechnol. 17, 733–738.

Singh, R.S., Dhaliwal, R., Puri, M., 2007b. Production of high fructose syrup from Asparagus inulin using immobilized exoinulinase from *Kluyveromyces marxianus* YS-1. J. Ind. Microbiol. Biotechnol. 34, 649–655. https://doi.org/10.1007/s10295-007-0237-1

Sirisansaneeyakul, S., Worawuthiyanan, N., Vanichsriratana, W., Srinophakun, P., Chisti, Y., 2007. Production of fructose from inulin using mixed inulinases from *Aspergillus niger* and *Candida guilliermondii*. World J. Microbiol. Biotechnol. 23, 543–552. https://doi.org/10.1007/s11274-006-9258-6

Snyder, H.E., Phaff, H.J., 1960. Studies on a beta-fructosidase (inulinase) produced by *Saccharomyces fragilis*. Antonie Van Leeuwenhoek 26, 433–452. https://doi.org/10.1007/BF02539031

Struyf, N., Laurent, J., Verspreet, J., Verstrepen, K.J., Courtin, C.M., 2017. *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* cocultures allow reduction of fermentable Oligo-, Di-, and Monosaccharides and polyols levels in whole wheat bread. J. Agric. Food Chem. 65, 8704–8713. https://doi.org/10.1021/acs.jafc.7b02793

Struyf, N., Vandewiele, H., Herrera-Malaver, B., Verspreet, J., Verstrepen, K.J., Courtin, C.M., 2018. *Kluyveromyces*

marxianus yeast enables the production of low FODMAP whole wheat breads. Food Microbiol. 76, 135–145. https://doi.org/10.1016/j.fm.2018.04.014

Taillandier, P., Lai, Q.P., Julien-Ortiz, A., Brandam, C., 2014. Interactions between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* in wine fermentation: Influence of inoculation and nitrogen content. World J. Microbiol. Biotechnol. 30, 1959–1967. https://doi.org/10.1007/s11274-014-1618-z

Tofalo, R., Fasoli, G., Schirone, M., Perpetuini, G., Pepe, A., Corsetti, A., Suzzi, G., 2014. The predominance, biodiversity and biotechnological properties of *Kluyveromyces marxianus* in the production of Pecorino di Farindola cheese. Int. J. Food Microbiol. 187, 41–49. https://doi.org/10.1016/j.ijfoodmicro.2014.06.029

Truswell, A.S., Seach, J.M., Thorburn, A.W., 1988. Incomplete absorption of pure fructose in healthy subjects and the facilitating effect of glucose. Am. J. Clin. Nutr. 48, 1424–1430. https://doi.org/10.1093/ajcn/48.6.1424

Varanasi, P., Singh, P., Auer, M., Adams, P.D., Simmons, B.A., Singh, S., 2013. Survey of renewable chemicals produced from lignocellulosic biomass during ionic liquid pretreatment. Biotechnol. Biofuels 6, 14. https://doi.org/10.1186/1754-6834-6-14

Varela, J.A., Gethins, L., Stanton, C., Ross, P., Morrissey, J.P., 2017. Applications of *Kluyveromyces marxianus* in Biotechnology, in: Yeast Diversity in Human Welfare. Springer Singapore, Singapore, pp. 439–453. https://doi.org/10.1007/978-981-10-2621-8_17

Velmourougane, K., 2013. Impact of natural fermentation on physicochemical, microbiological and cup quality characteristics of Arabica and Robusta coffee. Proc. Natl. Acad. Sci. India Sect. B Biol. Sci. 83, 233–239. https://doi.org/10.1007/s40011-012-0130-1

Verspreet, J., Hemdane, S., Dornez, E., Cuyvers, S., Delcour, J.A., Courtin, C.M., 2013. Maximizing the concentrations of wheat grain fructans in bread by exploring strategies to prevent their yeast (*Saccharomyces cerevisiae*)-mediated degradation. J. Agric. Food Chem. 61, 1397–1404. https://doi.org/10.1021/jf3050846

Vijayaraghavan, K., Yamini, D., Ambika, V., Sravya Sowdamini, N., 2009. Trends in inulinase production – a review. Crit. Rev. Biotechnol. 29, 67–77. https://doi.org/10.1080/07388550802685389

Waleckx, E., Mateos-Diaz, J.C., Gschaedler, A., Colonna-Ceccaldi, B., Brin, N., García-Quezada, G., Villanueva-Rodríguez, S., Monsan, P., 2011. Use of inulinases to improve fermentable carbohydrate recovery during tequila production. Food Chem. 124, 1533–1542. https://doi.org/10.1016/j.foodchem.2010.08.007

Wang, L., Gao, E., Hu, M., He, Q., He, Y., Zheng, X., 2020. Comparative analysis of the fermentation performance of high-quality milk beer strains (*Kluyveromyces marxianus*) and optimisation of medium formula for high-density fermentation. Int. J. Dairy Technol. 73, 552–562. https://doi.org/10.1111/1471-0307.12670

Williams, D.L., Schückel, J., Vivier, M.A., Buffetto, F., Zietsman, A.J.J., 2019. Grape pomace fermentation and cell wall degradation by *Kluyveromyces marxianus* Y885. Biochem. Eng. J. 150. https://doi.org/10.1016/j.bej.2019.107282

Yokotsuka, K., 1990. Effect of press design and pressing pressures on grape juice components. J. Ferment. Bioeng. 70, 15–21. https://doi.org/10.1016/0922-338X(90)90023-P

Chapter 3 - Preliminary comparison of oenological traits amongst *Kluyveromyces marxianus* strains under laboratory conditions

3.1 Introduction

Kluyveromyces marxianus is a yeast commonly associated with the dairy industry, but it is also frequently isolated from various other habitats. Its remarkable properties make it a relevant bioagent for an array of industrial applications, including the production of various foods and beverages, but also that of biofuel and enzymes (Fleet and Mian, 1987; Ho et al., 2014; Lachance, 1995; Nanadoum et al., 2005; Ortiz-Merino et al., 2018; Schwan et al., 2012; Serrat et al., 2002; Tofalo et al., 2014). Recently, its potential use for the production of wine has been suggested (Labuschagne et al., 2021; Rollero et al., 2018b, 2018c). Indeed, this yeast species occasionally occurs in vineyards and winemaking environments along with various other so-called non-*Saccharomyces* yeasts (Kántor et al., 2017, 2015; Longo et al., 1991), but its relevance for this industry was only recently reported.

Once decried, the use of non-*Saccharomyces* yeasts in the wine industry is now becoming a common practice following extensive research. Indeed, their positive contribution to various aspects such as the production of favourable flavour and aroma compounds, improvement of mouthfeel, wine colour and production of various oenologically relevant hydrolytic enzymes has been abundantly described in literature (Fonseca et al., 2008; Lane and Morrissey, 2010; Varela et al., 2017). With regard to *K. marxianus* specifically, its high production of phenylethyl acetate and endo-polygalacturonase active under oenological conditions (Rollero et al., 2018a, 2018b, 2018c) make it a yeast with good potential for winemaking. Nevertheless, the authors of the latter two studies only investigated a particular strain isolated from a South African vineyard. While this isolate displayed relatively good fermentation performance (Rollero et al., 2018b), *K. marxianus* is generally described as a poor fermenter in literature because of its Crabtree negative character (Fonseca et al., 2008; Lane and Morrissey, 2010). Furthermore, intraspecific genetic and phenotypic diversity within the *K. marxianus* species has been documented (Cruz-Guerrero et al., 1995; Lane et al., 2011; Ortiz-Merino et al., 2018; Perpetuini et al., 2019; Tofalo et al., 2014), but these investigations did not include oenologically relevant traits.

This chapter aimed to investigate the phenotypic diversity between strains of various origins for oenologically relevant features, including hydrogen sulphide and pectinase production as well as fermentation performance and production of major aroma compounds in a synthetic grape juice medium. A protoplast fusant recently generated from *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* (Unpublished results) was also included in this study.

3.2 Materials and methods

3.2.1 Yeast strains and preculture conditions

The yeasts investigated in this study were *K. marxianus* L01, L02, L03, L04, L05 (Lallemand Inc., Montréal, QC, Canada) as well as strains IWBT Y885 and BF2020 (yeast culture collection of the South African Grape and Wine Research Institute, Stellenbosch University, South Africa). The latter is the product of *K. marxianus* Y885 and *S. cerevisiae* Lalvin QA23™(Lallemand). L01 through L05 were selected as they have been isolated from a variety of habitats as reported in a previous study (Ortiz-Merino et al., 2018). Strain QA23 also served as a non-*K. marxianus* control for fermentation performance and polygalacturonase activity experiments. *Saccharomyces bayanus* Vivace (Renaissance Yeast, Vancouver, BC, Canada) and *S. cerevisiae* WH314 (NRRL Y-567) were used as controls for H_2S production experiments.

Cryopreserved yeasts were thawed at room temperature and streaked onto Yeast Peptone Dextrose (YPD) agar (BioLab, Merck, South Africa). The plates were then incubated at 30°C until colonies reached a usable size. Starter cultures were prepared for each yeast by inoculating a single colony into 5 mL YPD broth (BioLab, Merck, South Africa). The cultures were incubated overnight on a rotating test tube wheel at 30°C. For precultures, 500 µL starter culture was inoculated into 50 mL YPD for all yeasts except strain L04. For the latter, synthetic grape juice medium (SGM) (See section 3.2.3 for composition) was used as a preculture medium instead of YPD to prevent flocculation. Precultures were then incubated on a rotating table (orbital agitation of 125 rpm) at 30°C for 12 h to allow cells to reach exponential growth phase before being inoculated into the final media.

3.2.2 Morphological examination

Precultures were prepared as described above and yeasts were plated onto YPD agar. Plates were then incubated at 30°C. An optical microscope AxioscopeA1 (Zeiss, Oberkochen, Germany) was used along with an N-Achroplan, 100x/1.25 oil Ph3, ∞/0.17 objective (Zeiss) to obtain images of the cells collected from colonies and from liquid cultures in YPD and synthetic grape juice-like medium (SGM) after 0, 4, 8, 12 and 24 h growth.

3.2.3 Fermentation medium and conditions

SGM was used for all fermentations. The medium consisted of 230 g/L total sugar (115 g/L glucose, 115 g/L fructose) and 300 mg/L Yeast Assimilable Nitrogen (YAN). The detailed composition of the medium can be found in supplementary material Tables S1 to S5. Precultures were prepared as described above and after 12 h, yeasts were inoculated at 1 x 10^6 cells/mL into 100 mL SGM, aliquoted into 250-mL Erlenmeyer flasks equipped with a fermentation cap and bubbler. Fermentations occurred at 25°C on a rotating table (orbital

agitation of 125 rpm). All fermentations were performed in triplicate. Fermentation progress was monitored via daily weight loss measurements. Lag phase was determined as the time taken to for each individual strain to release 3 g/L total CO_2 (Zimmer et al., 2014). The maximum rate of fermentation (Vmax) was determined as the maximum rate of CO_2 released. 50 mL samples were taken at the end of fermentation for chemical analysis. The samples were spun down at 9244 *g* at a temperature of 17°C for 5 min using a Herolab HiCen SR centrifuge (Wiesloch, Germany) equipped with a Sorvall™ SLA600TC rotor. The supernatant was filtered through a 0.22-µm cellulose acetate syringe filter (Sterlitech) into a new sample tube and both the pellet and supernatant were frozen separately at -20°C until further use.

Yeast biomass produced during fermentation was determined from pellets retrieved at the end of fermentation. The pellets were frozen at -80°C overnight. Pellets were then freeze-dried over 48 h to remove all moisture using a Martin-Christ BETA 2 – 8 LDplus Freeze dryer. Pellet masses were then determined.

3.2.4 Determination of sugars, glycerol, malic and citric acids by enzymatic assays

At the end of the fermentations (or after 480 h for those that did not finish earlier), glucose, fructose, glycerol, malic acid and citric acid concentrations were determined by enzymatic analyses using an Arena 20XT (Thermo Fisher Scientific, Waltham, MA). The enzymatic assay kits used were Enzytec Liquid D-Glucose (Id-No: E8140, Roche, R-Biopharm) for glucose; Thermo Fisher D-Fructose (Product code 984302) for fructose; Enzytec Fluid Glycerol (Id-No: E5360, Roche, R-Biopharm) for glycerol; Enzytec Liquid L-Malic acid (Id-No: E8280, Roche, R-Biopharm) for malic acid and Roche Yellow line Citric acid (Id-No: 10139076035, Roche, R-Biopharm) for citric acid.

3.2.5 Major volatiles quantification

Major volatiles were quantified at Stellenbosch University's Central Analytical Facility using gas chromatography-mass spectrophotometry (GC-MS). Samples were prepared as follows. 100 µL of 175 ppm 4-methyl-2-pentanol (internal standard), 1.5 mL diethyl ether and 3 mL of 20% sodium chloride (NaCl) solution were added to 5 mL sample. The sample tube was then vortexed, sonicated for 30 min and liquid-liquid extraction (LLE) was performed. Samples were then centrifuged at 1841 *g* for 1 min. The diethyl ether layer (top) was transferred into a new vial with sodium sulphate and then transferred again into 2 mL vial. 1 µL was injected with a 5:1 split ratio onto the GC-MS instrument. Separation was performed on a gas chromatograph (6890N, Agilent technologies network) coupled to an Agilent technologies inert XL EI/CI Mass Selective Detector (MSD) (5975B, Agilent technologies Inc., Palo Alto, CA). The GC-MS system was coupled to a CTC Analytics PAL autosampler. Separation of the major wine volatiles was performed on a ZB-FFAP (60 m, 0.32 mm ID, 0.50 µm film thickness) capillary column (Phenomenex, Torrance, California,

United States of America). Helium was used as the carrier gas at a flow rate of 1.9 mL/min. The injector temperature was maintained at 240°C. The oven temperature was programmed as follows: 35°C for 17 minutes and ramped at a rate of 12°C/min until 240°C and held for 5 minutes. The MSD was operated in a full scan mode and the source and quad temperatures were maintained at 230°C and 150°C, respectively. The transfer line temperature was maintained at 250°C. The mass spectrometer was operated under electron impact (EI) mode at ionization energy of 70eV, scanning from 35 to 650m/z.

3.2.6 Ethanol quantification

Samples were filtered through a 0.22-µm cellulose acetate syringe filter (Sterlitech) and diluted accordingly. High Performance Liquid Chromatography was performed using a Dionex UltiMate 3000 HPLC to determine ethanol concentrations. An ERC Refracto Max520 RI detector along with a 250 x 7.8 mm with guard cartridge were used. The Biorad HPX-87H column (Bio-Rad, Hercules, CA, USA) temperature was 65°C along with a mobile phase and flow rate of 0.005 M H_2SO_4 at 0.6 mL/min.

3.2.7 Oenologically relevant characteristics

3.2.7.1 Polygalacturonase activity

SGM fermentations were performed as described previously with minor modifications. The medium volume increased to 200 mL and fermentation temperature to 30°C. A 1 mL sample was taken after 120 h to evaluate pectinase activity.

Pectinase activity after 120 h was estimated by using a polygalacturonase plate assay. Polygalacturonase activity (PGA) plates were prepared by adjusting 0.68% potassium phosphate to pH 3.5 and adding 1.25% polygalacturonic acid sodium salt from citrus fruit (Sigma-Aldrich) and 2% Difco Agar (BD Biosciences, Franklin Lakes, NJ, USA) (Porter et al., 2019). 10 µL filtered samples were spotted onto the PGA plates. The plates were then incubated for 24 h at 30°C. Following this the plates were flooded with 6N HCl and put on a shaker (The Belly Dancer, Stovall Life Science Incorporation, Greensboro, NC, USA) for 20 min. The HCl was discarded and pectinase activity was visualized by the formation of clear halos (Porter et al., 2019).

3.2.7.2 Hydrogen sulphide production

Hydrogen sulphide production was investigated using Bismuth Sulphite Glucose Glycine Yeast (BiGGY) Agar (Sigma Aldrich, Saint-Louis, MO, USA). Yeasts were precultured as previously described and 16 µL preculture of each yeast was spotted onto BiGGY agar plates. This was done using 2 sets of precultures and both in triplicate. Along with *S. cerevisiae* QA23, *K. marxianus* Y885, L01 through L05 and BF2020, yeasts *S. bayanus* Vivace and *S. cerevisiae* WH314 were spotted. Vivace acted as a low H_2S producing control and WH314 as a high H_2S producing control (Porter et al., 2019). Plates were incubated at 25°C for 5 days and

colony colour was inspected. White to light coloured colonies produced low amounts of H_2S and dark brown to black colonies produced high amounts of H_2S.

In order to quantitatively assess the colour differences between strains, the program ImageJ-win64 was used. The grayscale values were determined across the diameters of the various colonies, with high values depicting light colonies (i.e. low H_2S producing strains). A grayscale value was produced for every pixel across the colonies diameter which was then used to determine the average grayscale value or darkness of the colony. The average grayscale value was then determined between all replicates across the two sets with a standard deviation for the 6 replicates. In order to visualize the data more easily, the grayscale value of WH314 was taken as a maximum 100 % and Vivace as a minimum 0 % as these two controls represented the two extremes of the experiment.

3.2.8 Statistical analyses

XLSTAT version 2020.1.1 was used for principal component analyses (PCA) of major volatiles produced as well as One-way ANOVA with Tukey's multiple comparison test of means and Cluster analysis using Ward's method.

3.3 Results and discussion

3.3.1 Intraspecific comparison of colony and cell morphology

Yeast morphology was investigated by allowing yeasts to grow on YPD agar plates and visualizing colony formation. The two control yeasts *S. cerevisiae* QA23 and *K. marxianus* Y885 both displayed phenotypes similar to those previously described in literature for the corresponding species (Perpetuini et al., 2019; van der Walt and Yarrow, 1984). BF2020, the protoplast fusant of QA23 and Y885, displayed the same colony morphology as QA23 (Table 3.1).

Table 3.1 | Yeast morphology when grown on YPD plates and in YPD broth and SGM

Yeast strain	Colony morphology	Cell morphology (100 x magnification from colonies)	Cell morphology (100 x magnification in YPD broth and SGM) at inoculation	Changes occurring in cell morphology after specified number of hours
S. cerevisiae QA23	White-cream colour, smooth, slightly raised, glossy and round	Round	Round	N/A
K. marxianus IWBT Y885	Similar to QA23, slightly glossier	Round to oval	Round to oval	N/A
BF2020	Same as QA23	Mostly round or oval. A few	Mostly round or oval (no irregularly	N/A

		irregularly shapped cells occur.	shaped cells)	
K. marxianus L01	White, cream colour, shrivelled, raised, varies between round and irregular shaped	A few round and oval cells with the majority displaying filamentous growth	A few round and oval cells with the majority displaying filamentous growth	After 12 h, filamentous cells have disappeared. Mostly round or oval
K. marxianus L02	Similar to QA23, more raised with serrated edges	Round	Round with small groupings of pseudohyphal growth	N/A
K. marxianus L03	Same as Y885	Round to oval	Round to oval	N/A
K. marxianus L04	Similar to QA23 with ring patterns on colonies	Round to oval	Round to oval	Round to oval with large cluster of cells indicating potential flocculation occurring after 8 h in YPD. No flocculation observed in SGM.
K. marxianus L05	White, cream colour, colonies have ring patterns, shrivelled round, raised	Round to oval with a few irregular cells	Round to oval	N/A

The colonies of *K. marxianus* strains L01 through L05 initially appeared similar to *K. marxianus* Y885. However, after 48 h, differences became visible for strains L01, L02, L04 and L05 (Table 3.1), whereas strain L03 maintained similarity to Y885. While the colonies of Y885 remained smooth over time, those produced by strain L01 became shrivelled or displayed a filamentous type of growth over time. This filamentous growth was confirmed when viewing cells taken from the colonies under 100x bright field microscopy as cells appeared as long chained branches (filamentous/pseudohyphal growth).

Cell morphology was also investigated after yeasts were grown in YPD broth and SGM. Strain L01 displayed dimorphic growth with filamentous/pseudohyphal growth reverting fully to a yeast-like morphology after 12 h.

Filamentous/pseudohyphal growth has been previously documented in *S. cerevisiae* as well as *K. marxianus* (Casalone et al., 2005; Ceccato-Antonini and Sudbery, 2004; O'Shea and Walsh, 2000; Reis et al., 2013). An extensive study showed that the growth conditions can significantly influence the cell and colony morphology of dimorphic yeasts strains (O'Shea and Walsh, 2000). The latter authors showed that depending on the growth conditions, *K. marxianus* NRRLy2415 can display various morphologies from

yeast, elongated yeast, true double yeast, double elongated yeast, filament, double filament to pseudohyphal morphology. The authors described double yeast as budding yeast or budding elongated yeast containing a visible constriction at the mother-bud connection (O'Shea and Walsh, 1996). The type of morphology displayed by dimorphic *S. cerevisiae* strains has been correlated to stress factors such as nitrogen or carbon starvation (Ceccato-Antonini and Sudbery, 2004). When starved of nutrients, dimorphic yeasts adopt a filamentous/ pseudohyphal form as it allows the yeasts to move in various directions in order to search for the lacking nutrients. However, the cell morphology of dimorphic *K. marxianus* strains has been linked to colony growth rate (O'Shea and Walsh, 2000). According to the latter study, colonies growing at an optimal growth rate formed yeast-like cells (i.e. large, round to oval shaped cells), which allowed the localization of cells and led to raised colonies, whereas colonies growing at lower rates displayed filamentous/pseudohyphal growth. This could be linked to the nutrient starvation reported for *S. cerevisiae* as when nutrients are higher and growth rates are optimal the round cells of *K. marxianus* allows cells to be localized to the area with high nutrients. However, when nutrients are lacking and growth rates are suboptimal, *K. marxianus* produces filaments or pseudohyphae to search for nutrients.

YPD is a nutrients-rich medium and as such, it allowed strain L01 to display very fast growth and high biomass formation, thereby depleting the nutrients in region of the colonies. This likely led to filamentous/pseudohyphal growth in order for the yeast to try to reach more nutrients in its immediate vicinity. This was then alleviated when the cells were inoculated into YPD broth and SGM as liquid media allowed for renewed access to nutrients.

Strain L02 produced colonies of very similar morphology to strain Y885 but displayed serrated edges. Colony cells as well as cells grown in YPD broth and SGM over the 24 h time period all displayed the same cell morphology being round to oval shaped. However, minor pseudohyphal growth was observed throughout the 24 h time period in both YPD broth and SGM. This pseudohyphal growth suggests that strain L02 is also a dimorphic yeast and that the YPD broth and SGM contain the level of nutrients required to keep the cells in the round to oval cell morphology.

Strain L04 and L05 both showed differing colony morphologies compared to strain Y885 as well as strains L01 through L03 (Table 3.1). Indeed, these two strains displayed white colonies with concentric circle patterns. These patterns have previously been described by Granek & Magwene (2010) who investigated the differing impacts of environmental and genetic factors on colony morphology. The latter authors found that amongst the various factors impacting colony morphology, ploidy appeared to play a role. Diploid and triploid yeast colonies more frequently displayed round smooth colonies when compared haploid strains. Although this was not specifically investigated in the current study, this can also be observed when considering strains L01 through L05 and Y885. Strain L02 is a diploid yeast and L03 is triploid (Ortiz-Merino

et al., 2018). These two strains, along with the diploid Y885 (Unpublished results), displayed smooth colonies whereas the haploid strains L04 and L05 displayed complex colony morphology in the form of circular patterns. The only strain not to follow this pattern was the diploid strain L01 which displayed complex colony morphology despite not being haploid. However, other factors such as nutrients availability and other factors such as presence of ethanol and pH conditions could also play a role, thereby potentially reducing the effect that ploidy may have (Granek and Magwene, 2010).

Despite strains L04 and L05 displaying similar colony morphologies, those of their cells differed when grown in liquid media (Table 3.1). Initially, L04 and L05's cell morphology was round to oval and only individual cells were observed. However, after 8 h in YPD, strain L04 produced large clumps of cells which was hypothesized to be flocculation. This phenomenon was not observed when cells were grown in SGM over the 24 h period. This indicates that the flocculation is stimulated by something present or lacking in the YPD broth. This agrees with previous studies suggesting that yeast morphologies are dependent on various factors such as medium composition and can often change if nutrients are lacking or conditions evolve such as increase in ethanol or pH (Ceccato-Antonini and Sudbery, 2004; Granek and Magwene, 2010).

Strain variation has been shown to occur in various yeast species such as *Brettanomyces bruxellensis* (Lewis et al., 2010; Nicaud, 2012). The latter yeast displays varying forms of cell morphology, in connection with nutrient deficiency and stress such as the presence of SO_2 (Louw et al., 2016). Similarly, variation has been shown for *K. marxianus.* Indeed, among 13 different *K. marxianus* strains, large amounts of diversity in responses were observed with regard to thermal, osmotic and cell wall stress related changes (Lane et al., 2011). Similarly, diversity in biofilm formation amongst several *K. marxianus* strains was suggested to result from variations in the genomes of the *K. marxianus* strains (Perpetuini et al., 2019). Differences were not only found at the level of various single nucleotide polymorphisms (SNPs) but also in ploidy levels, varying from haploid to triploid (Ortiz-Merino et al., 2018). This morphology study confirmed that strain variations occur, including between the different *K. marxianus* strains investigated.

3.3.2 Polygalacturonase activity

Pectinase activity is of great interest in the winemaking industry as the breakdown of grape berry cell wall pectin allows the release of various phenolic and aroma compounds as well as the increase in the volume of free-run juice (Álvarez et al., 2006; Eschstruth and Divol, 2011; Gil-Muñoz et al., 2009; Rollero et al., 2018c).

Table 3.2 | Polygalacturonase activity represented by halo formation on PGA plates

Yeast	QA23	Y885	BF2020	L01	L02	L03	L04	L05
Halo diameter	0 ± 0^{E}	2 ± 0^{B}	2 ± 0^{B}	$1.9\pm 2.22E\text{-}16^{C}$	2 ± 0^{B}	1.8 ± 0^{D}	1.77 ± 0.05^{D}	2.33 ± 0.05^{A}

(cm)	

Mean values ± standard deviation. Strains sharing the same letters are not significantly different at a p<0.05 threshold.

Very little variation could be recorded between the *K. marxianus* strains as well as BF2020 (Table 3.2, Supplementary Material Figure S1), with strain Y885 producing a similar size halo as reported in literature (Williams et al., 2019). Conversely, no degradation halo was visible for *S. cerevisiae* QA23, as previously reported (Divol and van Rensburg, 2007; Fernández-González et al., 2004; Louw et al., 2010; Rollero et al., 2018c). The current study therefore confirms previous reports that *K. marxianus* strains produces high levels of pectinase active at wine pH (Serrat et al., 2002; Sieiro et al., 2009). Nevertheless, this production should be confirmed for strains BF2020 and L01 through L05 under actual winemaking conditions since it has been reported to be dependent on environmental conditions such as temperature (Serrat et al., 2011).

3.3.3 H_2S production

Hydrogen sulphide production (H_2S) by the various *K. marxianus* strains was assessed. The experiment was repeated twice with each set having three plate replicates to account for possible variations linked to colony growth. All replicates of a given strain displayed very little variation with regard to time taken to grow.

As when grown on YPD, colony morphology between yeasts varied to a considerable degree (see supplementary material Figure S2). A large degree of variation could also be observed for grayscale values between the strains (Figure 3.1). Three main groups emerged. Strains Y885, QA23 and BF2020 grouped together with strain L01 as being the lightest and therefore the lowest H_2S producing strains. The intermediate group comprised strains L01, L02, L04 and L05, with strain L01 statistically belonging to both the low and intermediate producer groups. Finally, strain L03 being the strongest producer of H_2S formed a group on its own. Origin of isolation did not seem to play a role in the strain's H_2S production since the three dairy strains, L01, L02 and L03 produced different amounts. Surprisingly, *S. cerevisiae* QA23, a commonly used wine yeast which is known for its low H_2S production during winemaking (Lalvin QA23 technical data sheet), did not produce colonies as white as the low H_2S producing control Vivace. Nevertheless, a previous study showed that results generated through the use of BiGGY agar cannot always be directly translated to what would occur in liquid grape juice (Kumar et al., 2010). Despite this, the use of BiGGY agar does still allow for strain variation to be investigated regarding H_2S production.

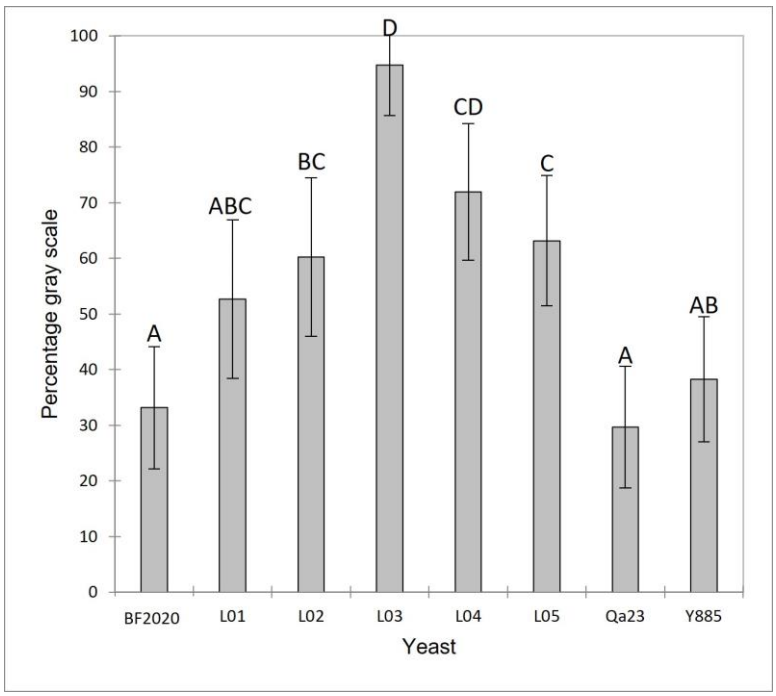

Fig. 3.1. *H_2S production of various strains with higher grayscale percentage indicating higher H_2S production. Strains sharing the same letter are not significantly different at a $p<0.05$ threshold.*

3.3.4 Synthetic grape juice fermentations

3.3.4.1 Fermentation kinetics and performance

Fermentation performance is often a key factor in industry as the length and rate of fermentation influence many costs involved. These factors have also frequently been shown to vary between various non-*Saccharomyces* yeasts (Rollero et al., 2018b), with this study investigating the intraspecific variation between *K. marxianus* strains. The fermentation kinetics and performance of all the yeast strains investigated in this study in SGM are displayed in Figure 3.2 and Table 3.3.

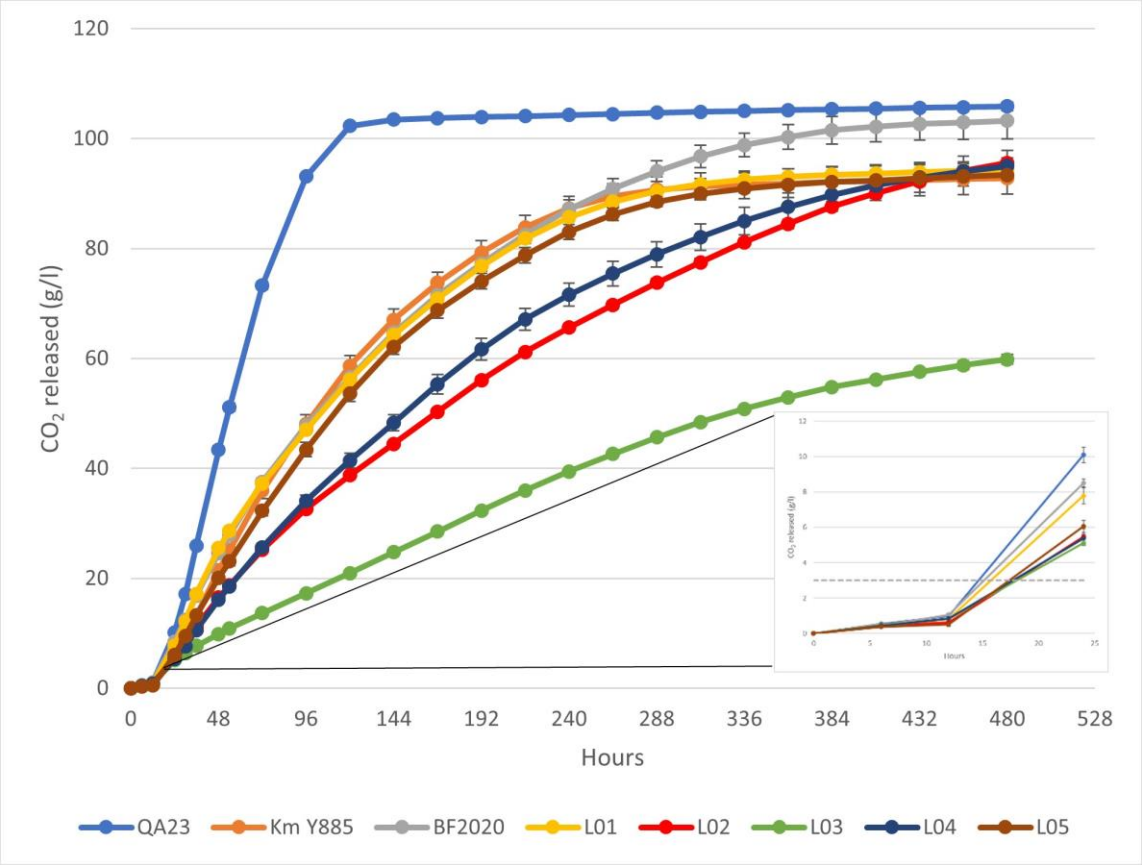

Fig. 3.2. *Fermentation kinetics of 6 strains of* K. marxianus*, one strain of* S. cerevisiae *and one* K. marxianus *x* S. cerevisiae *fusant. A zoomed-in version of the first 24 h is presented (Full size graph can be found in supplementary data Figure S3). The grey dashed line corresponds to 3 g/L released total CO_2 (the threshold considered in this study as exit point of lag phase).*

As expected, *S. cerevisiae* QA23 displayed the fastest kinetics. The *K. marxianus* strains could be split into three groupings. While strains Y885, L01, L05 were the fastest fermenters, strain L03 was the slowest. Strains L02 and L04 displayed intermediate fermentation kinetics. Although they exhibited different fermentation kinetics, all *K. marxianus* strains released similar amounts of CO_2 (approximately 94.2 g/L) with the exception of L03, which only released 59.8 g/L after 480 h. At that time, the fermentations were terminated as most fermentations had already stopped. Very little variation was observed when comparing lag phase times between the different *K. marxianus* strains (Table 3.3), with only strains BF2020 and L01 displaying significantly shorter lag phases. Overall, L04 and L02 had a similar lag phase, times to reach stationary phase, Vmax and consumed more sugar than Y885 (Figure 3.3). Strains L01 and L05 consumed similar amounts of sugar and both performed better than strains L03, L02, and L04. However, strain L01 still outperformed strain L05 with regard to time taken to exit lag phase and to reach stationary phase as well as Vmax. All *K. marxianus* strains as well as BF2020 consumed significantly more glucose than fructose (Figure 3.3), thereby highlighting their glucophilic character. The control *S. cerevisiae* QA23 and BF2020 both consumed the most sugars and subsequently produced the most CO_2. *K. marxianus* strains Y885, L01,

L02, L04 and L05 all consumed similar amounts of total sugars and produced very similar concentrations of CO_2 with strain L03 being the only clear outlier.

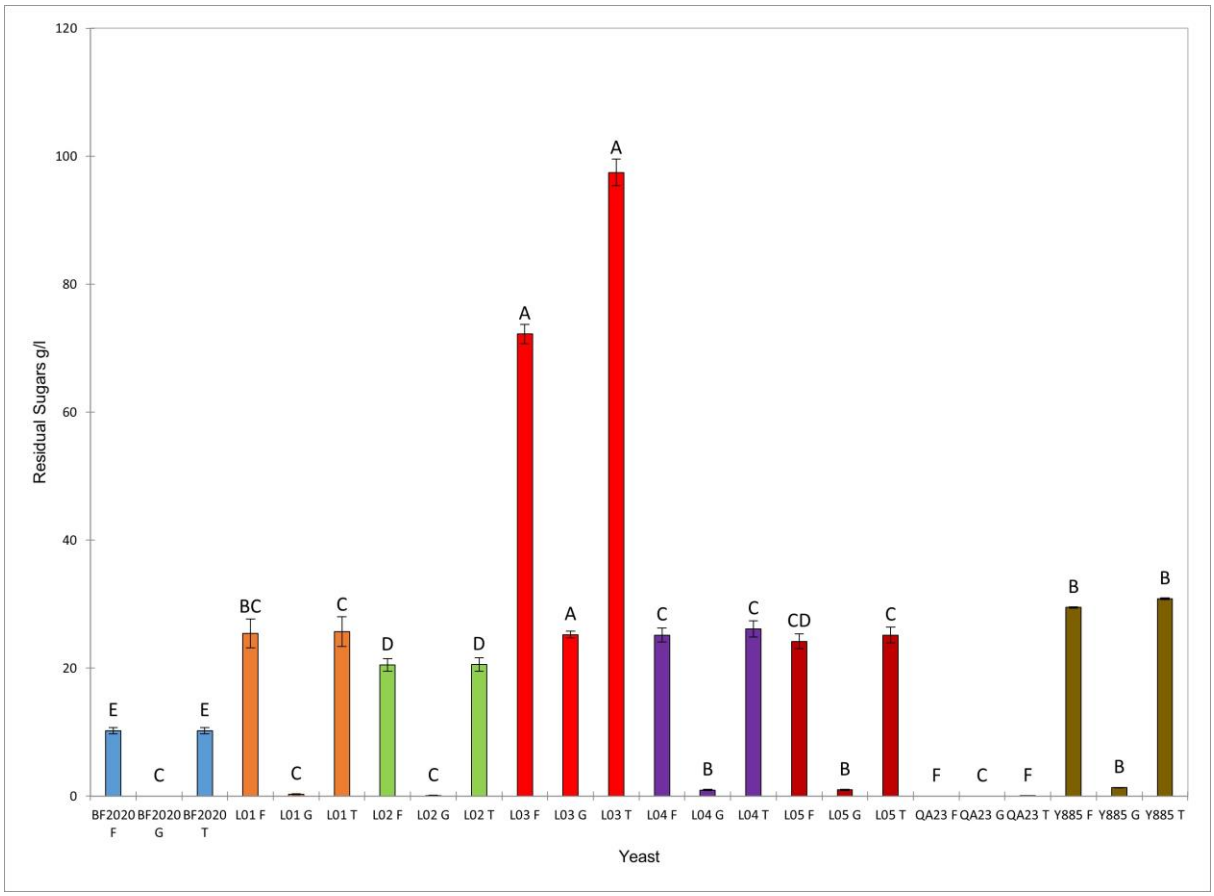

Fig. 3.3. *Comparison of total and average residual sugars at end of fermentation (starting sugar 230 g/L and 115 g/L glucose/fructose respectively). Total sugars (T), Fructose (F), glucose (G). Strain comparisons between sugar groups (i.e. total sugars, fructose or glucose) sharing the same letter are not significantly different at a p<0.05 threshold.*

The fermentation kinetics of Y885 and BF2020 were perfectly identical until 240 h, after which Y885 progressively stopped fermenting, while BF2020 continued. At the end of fermentation (i.e. when sugar consumption stopped), BF2020 had released approximately 103 g/L CO_2 and Y885 92 g/L CO_2. It had a reduced lag phase compared to Y885, an increased Vmax and consumed 20.6 g/L more sugar. Overall, this represents a considerable increase in fermentation performance as shown before (Unpublished results). As shown in Figure 3.3, increased sugar consumption can be seen for glucose as well as an approximately 3-fold decrease in residual fructose for BF2020 compared to Y885. It has been shown that fructose can negatively impact the ethanol tolerance of *S. cerevisiae* strains as well as *K. marxianus* 4D3 (De la Torre-González et al., 2016). Similarly, Berthels et al. (2004) showed that higher ethanol concentrations directly reduce the consumption of glucose and more significantly fructose of *S. cerevisiae* strains. This increase in fructose consumption is likely related to BF2020 possessing the increased ethanol tolerance and fructose consumption abilities of its parental strain QA23.

Table 3.3 | Comparison of fermentation performance factors between the different yeast strains investigated

Yeast	QA23	Km Y885	BF2020	L01	L02	L03	L04	L05
Lag phase (h)	14.66± 0.16^{B}	17.94± 0.65^{A}	15.14± 0.20^{B}	15.78± 0.41^{B}	17.89± 0.16^{A}	18.07± 0.15^{A}	18.07± 0.15^{A}	17.71± 0.27^{A}
Time to reach stationary phase (h)	144± 0^{E}	320± 11.31^{D}	432± 19.60^{B}	360± 19.60^{C}	480± 0^{A}	480± 0^{A}	480± 0^{A}	384± 0^{C}
Vmax (g/L/h)	3.80E-01± 1.71E-02^{A}	2.00E-01± 1.71E-02^{CD}	3.10E-01± 7.23E-03^{B}	2.91E-01± 1.40E-02^{B}	2.03E-01± 6.01E-03^{CD}	1.77E-01± 4.25E-03^{D}	1.89E-01± 8.74E-03^{D}	2.31E-01± 1.12E-02^{C}

Mean values ± standard deviation. Strains sharing the same letters are not significantly different at a p<0.05 threshold.

3.3.4.2 Primary metabolite production

The production of primary metabolites, such as those mentioned in Table 3.4, are frequently investigated with regards to wine yeasts. The production of these metabolites often varies between yeasts and are of great oenological importance.

Table 3.4 | Comparison of fermentation products between the different yeast strains investigated

Yeast	QA23	Km Y885	BF2020	L01	L02	L03	L04	L05
% Ethanol	13.54± 0.11^{A}	11.77± 0.13^{D}	12.87± 0.08^{B}	12.05± 0.22^{CD}	12.52± 0.16^{BC}	7.82± 0.01^{E}	12.06± 0.13^{CD}	12.09± 0.17^{CD}
Molar biomass yield	1.60E-04± 1.88E-07^{A}	1.26E-04± 1.59E-06^{D}	1.12E-04± 2.62E-06^{E}	1.38E-04± 1.24E-06^{C}	1.48E-04± 1.60E-06^{B}	N/A	1.38E-04± 1.26E-06^{C}	1.30E-04± 3.80E-06^{D}
Glycerol (g/L)	4.91± 0.15^{DE}	6.07± 0.11^{B}	5.17± 0.01^{D}	5.55± 0.11^{C}	4.85± 0.01^{E}	3.09± 0.05^{F}	5.90± 0.09^{B}	6.91± 0.08^{A}
Acetic acid (g/L)	1.13± 8.52E-02^{C}	1.58± 5.89E-02^{A}	1.48± 5.42E-02^{AB}	1.17± 8.92E-02^{C}	1.20± 7.58E-02^{C}	1.31± 9.37E-03^{B}	1.67± 1.68E-02^{A}	1.54± 4.21E-02^{AB}
Molar acetic acid yield	2.81E-02± 2.11E-03^{E}	4.53E-02± 1.69E-03^{B}	3.83E-02± 1.40E-03^{CD}	3.28E-02± 2.49E-03DE	3.26E-02± 2.06E-03DE	5.61E-02± 4.03E-04^{A}	4.68E-02± 4.69E-04^{B}	4.30E-02± 1.17E-03^{BC}

Mean values ± standard deviation. Strains sharing the same letter are not significantly different at a p<0.05 threshold. Molar yields are measured as the mol/L biomass or mol/L acetic acid produced/ mol/L sugar consumed.

As expected, ethanol concentrations at the end of fermentation (Table 3.4) revealed the exact inverse trends as those shown in Figure 3.3 for total sugars consumed. However, despite this variation in ethanol production, all strains produced a similar molar yield with a mean value of 3.47 ± 1.92E-02 mol/L Ethanol/mol/L sugar consumed. This indicates that all strains are equally proficient at producing ethanol, but other factors such as ethanol tolerance limited the final amount that the yeasts were able to produce.

At the end of fermentation, the molar biomass yield by the different yeast strains differed significantly (Table 3.4). That of *K. marxianus* strain L03 is not shown because the large quantities of residual sugars caramelized during the freeze-drying process (Supplementary Material Figure S4) and the quantification of

biomass could not be accurately recorded. *S. cerevisiae* QA23 had a significantly higher molar biomass yield than all other yeasts investigated. This trend has been previously reported (Rollero et al., 2018b). Indeed, these authors found *S. cerevisiae* EC1118 reached higher cell counts than *K. marxianus* Y885. BF2020 appeared as a clear outlier with the lowest molar biomass yield of all strains tested. Almost all the other *K. marxianus* strains had higher molar biomass yields (intermediate between QA23 and Y885). Unlike the ethanol yield, which was similar between all strains, the molar biomass yield varied greatly. Subsequently, this indicates that large variations occur in metabolic fluxes between the yeasts. As the ethanol yields are similar, it is likely that these differences would result in the increased production of various other compounds produced during fermentation such as major volatiles.

All *K. marxianus* strains except L02 and L03 exhibited a significantly higher glycerol production than the commercially used *S. cerevisiae* QA23 (Table 3.4). A large amount of variation can nevertheless be observed between the *K. marxianus* strains. Glycerol plays an important biological role in the functioning of the cells. Indeed, the production of glycerol is linked to both the recycling of NADH during fermentation as well as osmoprotection (Shen et al., 1999). Therefore, these variations in glycerol production suggest differences in redox balance management and/or osmoprotection requirements.

In synthetic grape juice fermentations, it was found that all *K. marxianus* strains, other than strains L01 and L02, produced significantly more acetic acid than QA23 (Table 3.4). Despite this, QA23 also produced significantly high concentrations of acetic acid. Acetic acid production can partly be linked to the production of glycerol during fermentation. Indeed, an over-production of glycerol leads to redox imbalance due to a production of NAD^+. Acetic acid is then produced in order to regenerate NADH (Chidi et al., 2018). As discussed previously, all *K. marxianus* strains other than L03 produced either similar or significantly more glycerol than QA23, this likely explains why these strains also produced more acetic acid. This increase in acetic acid production is seen to even greater extents in strain L03. This extreme increase suggests that strain L03 is suffering from redox imbalances with an over production of NADH, which in turn is likely to be connected to its overall poor fermentation performances.

Fermentations occurred in semi-anaerobic conditions as fermentation caps were fitted to prevent the entry of oxygen into the flasks. However, the flasks were not sparged to remove the initially present oxygen. This along with a large headspace to allow for better mixing likely allowed for increased dissolved oxygen concentrations in the SGM. Increased dissolved oxygen typically leads to increased biomass production (Merico et al., 2009). This leads to increased glycerol production to regenerate NAD^+ which in turn increases acetic acid production in order to maintain redox balance. This likely explains the relatively high acetic acid production observed for all strains including QA23, compared to what is typically observed in an industrial grape juice fermentation setup.

When looking at the general trends in Table 3.4, various groupings occur. Both strains Y885 and L05 displayed an increase in glycerol with a corresponding decrease in biomass production. Strain Y885 displayed the same increase for acetic acid with a corresponding decrease in biomass production. In contrast, strains QA23 and L02 displayed a decrease in glycerol, acetic acid and corresponding increase in biomass production, respectively. For strains QA23, Y885, L02 and L05 glycerol and acetic acid appeared to be linked as the production either both increased or decreased together with the respective increase or decrease in biomass. L03 was the only exception displaying a clear decrease in glycerol and increase in acetic acid production. No clear pattern could be seen for BF2020, L01 and L04. This highlights the variation between strains with regards to primary carbon metabolism, again with strain L03 appearing the most different from the various *K. marxianus* strains. Interestingly, strain L02 grouped with the *S. cerevisiae* strain QA23 despite appearing to be very different with regards to other characteristics.

3.3.4.3 Major volatiles

The major volatiles produced were quantified at the end of fermentation (480 h). The respective molar yields of each compound were determined by normalizing the data to the concentration of total sugars consumed during fermentation. These yields are shown in Table 3.5 with the shades of colours representing higher (green) to lower (red) production and in Figure 3.4 in a PCA plot.

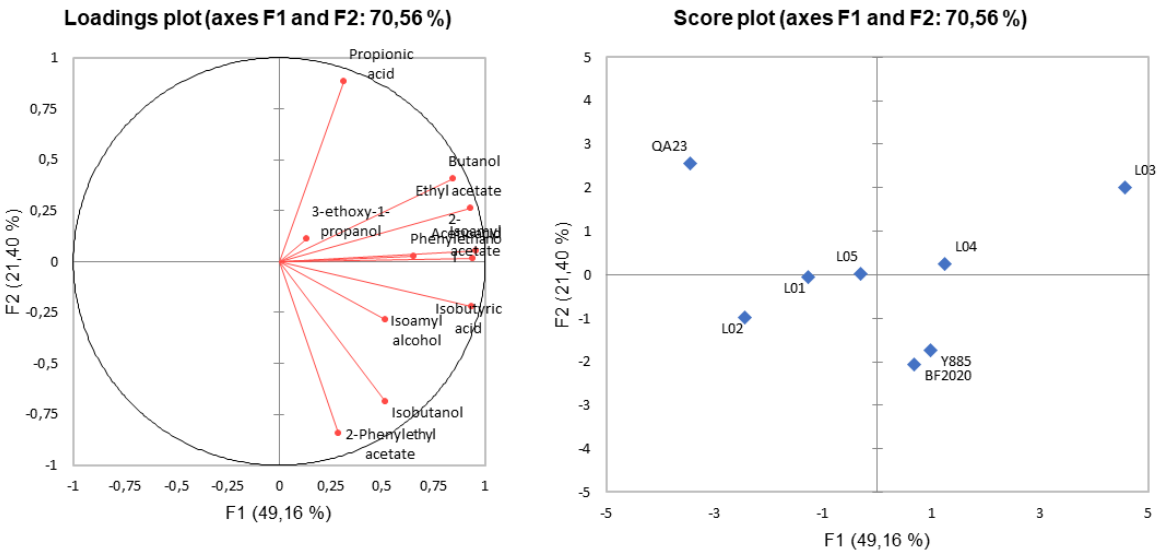

Fig. 3.4. *Principal component analysis (PCA) of molar major volatile production at the end of alcoholic fermentation (480h).*

All *K. marxianus* strains produced significantly more of most volatile compounds quantified than QA23. In particular, they produced more phenylethanol and isobutanol (but not butanol), and more esters (namely isoamyl acetate, ethyl acetate, 2-phenylethyl acetate). Significant differences were nevertheless noted between strains of *K. marxianus*. Indeed, while strain L02 produced low yields of most compounds (and

even the lowest of all strains for some compounds), L03 behaved in an opposite fashion. Interestingly, while strain Y885 produced low yields of most compounds, it produced the highest yields of 2-phenylethyl acetate, followed closely by BF2020. Overall, the latter strain behaved similarly to Y885.

As shown in previous studies, Y885 produced more phenylethanol and isobutanol compared to *S. cerevisiae*. This was supported by gene expression analysis (Rollero et al., 2021, 2019). Comparatively, a large amount of variation can be observed between Y885 and strains L01 through L05. This suggests that the gene expression between these strains subsequently varies as well. Rollero et al. (2019) suggested that the *ARO8/9* gene expression could be increased for phenylethanol production by Y885, which would promote the transamination of aromatic amino acids into α-ketoacids instead of the direct incorporation into biomass. This is further supported as both Y885 and BF2020 were two of the lowest biomass-producing and high phenylethanol producing strains. It should be mentioned this trend did not apply to L01. Similarly, both Y885 and the likely genetically similar BF2020 produced significantly more isoamyl alcohol and 2-phenylethyl acetate compared to other *K. marxianus* strains and QA23, thereby indicating more variation in gene expression and metabolic flux.

Isoamyl acetate, ethyl acetate and 2-phenyl acetate are all important esters that are found in wine. These esters among others play a crucial role in influencing wine flavour and aromas. Their synthesis has been shown to be closely linked to lipid and acetyl-CoA metabolism (Thurston et al., 1982). Isoamyl acetate (pear), ethyl acetate (fruity at concentrations lower than 50 mg/L) and 2-phenyl acetate (honey, fruity, flowery) are associated with pleasant aromas such as those mentioned above. The production of these esters by *K. marxianus* strains have been shown in previous studies, subsequently the use of *K. marxianus* in winemaking could lead to wines with increased fruity or floral characteristics (Gethins et al., 2015; Rapp and Mandery, 1986; Rollero et al., 2018b). Despite higher concentrations of the above-mentioned esters being positive, high concentrations can also lead to negative aromas. This can be seen specifically with ethyl acetate which when in very high concentrations (more than 150 mg/L) can be perceived as a solvent-like aroma (Lilly et al., 2000; Swiegers and Pretorius, 2005).

Isobutanol was also found to be produced significantly more by all *K. marxianus* strains. This higher alcohol is synthesized by the catabolism of both sugars and amino acids such as leucine and valine. Isobutanol, along with other higher alcohols, is generally known to contribute to the solvent or alcoholic aroma found in some wines, however this has been shown to vary greatly between different types of wines.

K. marxianus strain L02 showed a large amount of variation producing significantly less propionic acid, 3-ethoxy-1-propanol, 2-Phenylethanol, isoamyl alcohol but more isobutanol. With both the increased and decreased production of various higher alcohols, it is difficult to predict the impact that strain L02 would potentially have on wine aroma or flavour.

3-ethoxy-1-propanol has been stated to have a chemical or organic solvent odour, however when found in red wine, this compound is generally associated with blackcurrant aroma (Tao and Zhang, 2010). Although found in very low concentrations in fermentations using strain L02, strain L04 was found to produce 2-fold more 3-ethoxy-1-propanol compared to all other strains including QA23. 3-ethoxy-1-propanol has been shown to be produced by *Torulaspora delbrueckii* in real grape juice and therefore investigating the production of 3-ethoxy-1-propanol using strain L04 in real red grape juice could provide significant insight into the production of this compound by non-*Saccharomyces* yeasts (Loira et al., 2014).

Of the various *K. marxianus* strains, strain L03 showed the greatest variation. Strain L03 produced more isoamyl acetate, propionic acid, ethyl acetate, butanol, isobutyric acid, phenylethanol and acetic acid. Despite the production of fruity esters isoamyl acetate and ethyl acetate, the concentrations they are produced at could likely lead to solvent like aromas. Similarly, the increased production of higher alcohols and organic acids are also likely to result in wines with chemical, alcoholic or vinegar aromas.

Table 3.5 | Comparison of molar major volatile yield between the different yeast strains investigated

Yeast	Isoamyl acetate	Propionic acid	3-ethoxy-1-propanol	Ethyl acetate	Butanol	Isobutyric acid	2-Phenylethyl acetate	2-Phenylethanol	Isoamyl alcohol	Isobutanol
QA23	1.44E-05± 3.09E-07^{E}	8.31E-05± 9.69E-07^{A}	5.03E-05± 1.17E-06^{B}	4.93E-04± 2.92E-05^{E}	1.45E-05± 7.03E-07^{BC}	2.16E-05± 5.23E-07^{E}	2.34E-06± 2.89E-07^{F}	1.48E-04± 3.96E-06^{C}	1.74E-03± 3.58E-05^{CD}	4.42E-04± 2.20E-05^{E}
Y885	2.04E-05± 8.43E-07^{CD}	5.65E-05± 1.73E-06^{BC}	4.45E-05± 6.71E-07^{C}	1.87E-03± 5.37E-05^{BCD}	1.63E-05± 4.67E-07^{B}	1.05E-04± 3.01E-06^{C}	8.49E-05± 1.01E-06^{A}	2.43E-04± 6.73E-06^{A}	2.13E-03± 1.52E-05^{AB}	1.36E-03± 1.52E-05^{BC}
BF2020	2.15E-05± 1.05E-06^{C}	5.68E-05± 1.50E-06^{BC}	3.98E-05± 6.54E-07^{D}	1.99E-03± 1.43E-04^{BC}	1.45E-05± 1.26E-06^{BC}	1.40E-04± 3.82E-06^{B}	6.70E-05± 3.72E-06^{B}	1.92E-04± 8.14E-06^{B}	2.23E-03± 8.96E-05^{A}	1.58E-03± 5.70E-05^{A}
L01	1.86E-05± 2.28E-07^{D}	5.74E-05± 2.58E-06^{BC}	2.96E-05± 1.29E-06^{EF}	1.35E-03± 9.85E-05^{CD}	1.39E-05± 2.84E-07^{C}	7.81E-05± 2.80E-06^{D}	1.75E-05± 1.15E-06^{E}	2.62E-04± 6.24E-06^{A}	1.71E-03± 4.33E-05^{D}	1.19E-03± 3.70E-05^{CD}
L02	1.85E-05± 4.97E-07^{D}	5.06E-05± 2.86E-06^{C}	2.61E-05± 6.00E-07^{F}	1.25E-03± 2.38E-05^{D}	1.28E-05± 3.95E-07^{C}	7.51E-05± 1.92E-06^{D}	3.00E-05± 2.50E-06^{D}	1.20E-04± 5.68E-06^{D}	1.30E-03± 5.81E-05^{E}	1.54E-03± 4.98E-05^{AB}
L03	2.80E-05± 6.51E-07^{A}	8.47E-05± 7.50E-06^{A}	3.14E-05± 1.71E-06^{E}	5.69E-03± 3.65E-04^{A}	2.14E-05± 7.81E-07^{A}	1.69E-04± 1.43E-05^{A}	1.60E-05± 1.15E-06^{E}	2.69E-04± 1.25E-05^{A}	1.88E-03± 1.22E-05^{CD}	1.38E-03± 9.00E-05^{B}
L04	2.49E-05± 9.53E-07^{B}	7.48E-05± 1.85E-07^{A}	1.03E-04± 1.03E-06^{A}	2.44E-03± 4.19E-04^{B}	1.49E-05± 4.52E-07^{BC}	1.35E-04± 1.17E-06^{B}	3.98E-05± 1.17E-06^{C}	1.74E-04± 5.70E-06^{BC}	1.83E-03± 6.19E-05^{CD}	1.38E-03± 7.68E-05^{BC}
L05	2.18E-05± 2.36E-07^{C}	6.15E-05± 1.37E-06^{B}	2.70E-05± 9.03E-07^{F}	1.96E-03± 5.41E-05^{BC}	1.42E-05± 2.34E-07^{BC}	8.84E-05± 2.00E-06^{D}	3.13E-05± 7.88E-07^{D}	2.01E-04± 1.23E-05^{B}	1.94E-03± 9.84E-05^{BC}	1.00E-03± 4.97E-05^{D}

Molar yield is represented as a spectrum from red to green with green indicating higher yield and red lower yield. Strains sharing the same letter are not significantly different at a p<0.05 threshold. Molar yields are measured as the mol/L major volatile produced/ mol/L sugar consumed.

The PCA in Figure 3.4 was generated to visualise the overall strain variation regarding major volatile production. As expected from the data presented in Table 3.5, *S. cerevisiae* QA23 is largely separated from the *K. marxianus* strains and BF2020 both along the x- and y-axis. The various *K. marxianus* strains broadly separate along the x-axis with strain L03 being the most distant. This separation of L03 is due to the increased butanol, isoamyl alcohol, isobutyric acid, acetic acid, ethyl acetate and 2-phenyl ethanol production. Both the separation of QA23 and L03 correspond with these yeasts appearing as outliers in Table 3.5. As in Table 3.5, similarities between strain Y885 and BF2020 can be seen with these two strains grouping closely together. This close grouping is likely due to their close genetic relatedness, standing apart from other *K. marxianus* strains, in particular because of their increased 2- phenylethyl acetate production. Defined media, such as synthetic grape juice medium, often underrepresent the complexity of compounds found in environments such as wine. Therefore, it is suggested that increased strain variation could be seen in these more complex environments.

3.3.5 Strain comparison summary

The PCA analysis in section 3.4.3 was produced using solely the molar major volatile data. Therefore, in order to summarize the comparison of the strains a more holistic approach was taken incorporating the data from molar major volatiles, glycerol production, pectinase activity, H_2S production and fermentation performance in a cluster analysis (Figure 5).

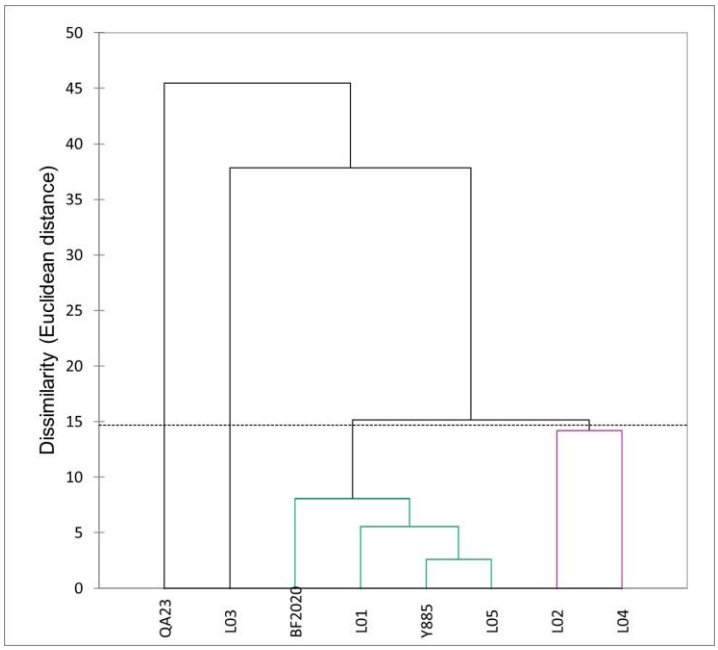

Fig. 3.5. *Dendrogram comparing the dissimilarity between the* K. marxianus *strains and BF2020 investigated in this study. The horizontal line indicating the separation of clusters.*

4 clusters are apparent from the dendrogram seen in Figure 5 namely cluster 1 (QA23), cluster 2 (L03), cluster 3 (L01, L05, Y885 and BF2020) and cluster 4 (L02 and L04). These clusters or groupings, with the

exception of BF2020, are the same as what was seen for the fermentation kinetics in Figure 3.2. As discussed previously, strain L03 appeared the least similar to other *K. marxianus* strains. The clustering of L02 and L04 is largely due to the very similar fermentation performance (Table 3.3), as the molar major volatiles production between the two strains varied to a large degree (Figure 3.4). Source of isolation appeared not to play a role as the three dairy strains L01, L02 and L03 appeared in different clusters from one another.

3.4 Conclusion

As in previous studies, variation between *K. marxianus* strains could be observed over numerous traits, focusing here mostly on those of oenological interest. All strains other than L05 displayed significant variation compared to Y885 with more than half the characteristics investigated. These variations also extended to different degrees with major differences being observed for 2-phenyl acetate, isobutyric acid, 3-ethoxy-1-propanol production, fermentation performance and biomass produced.

Included in this comparative study was the product of protoplast fusion of *K. marxianus* Y885 and *S. cerevisiae* QA23. This fusant, BF2020, displayed improved fermentation performance compared to the parental strain Y885, but retained similarities with Y885, especially with regards to the production of major volatile compounds.

The various strains investigated in this study were isolated from four different sources: wine, dairy, baking and distillery. Surprisingly, despite having the same source of isolation, strains L01, L02 and L03 appeared significantly different from one another for the majority of characteristics ranging from colony morphology to production of major volatiles. Conversely, strains isolated from different environments such as L01 (Dairy), L05 (Distillery) and Y885 (Wine) appeared very similar with numerous characteristics. This chapter has shown that great strain variation occurs amongst *K. marxianus* strains, with regards to oenologically relevant traits, and that this variation occurs regardless of the source of isolation.

3.5 Supplementary material

Table S1 | Synthetic grape juice medium composition

Synthetic grape juice medium composition		Per litre
Carbon sources	Glucose	120 g
	Fructose	120 g
Acids	Potassium L-tartrate	2.5 g
	L-malic acid	3.0 g
	Citric acid	0.2 g
Salts	Potassium hydrogen phosphate (K_2HPO_4)	1.14 g

	Magnesium sulphate heptahydrate ($MgSO_4$, $7H_2O$)	1,23 g
	Calcium chloride dehydrate ($CaCl_2$, $2H_2O$)	0,44 g
Trace elements	Stock solution X1000	1 mL
Vitamins	Stock solution X100	10 mL
Anaerobic factors	Stock solution	2 mL
Nitrogen sources	Stock solution	9.6 mL

Table S2 | Trace element stock solution composition (X1000)

Trace elements stock composition	Per litre
Manganese(III) chloride tetrahydrate ($MnCl_2$, $4H_2O$)	0.2 g
Zinc chloride ($ZnCl_2$)	0.135 g
Iron(III) chloride ($FeCl_2$)	0.03 g
Copper(III) chloride ($CuCl_2$)	0.015 g
Boric acid (H_3BO_3)	0.005 g
Cobalt(II) nitrate hexahydrate ($Co(NO_3)2$, $6H_2O$)	0.030 g
Sodium molybdate dehydrate ($NaMoO_4$, $2H_2O$)	0.025 g
Potassium iodate (KlO_3)	0.01g

Table S3 | Vitamin stock solution composition (X100)

Vitamin stock composition	Per litre
Myo-inositol	10 g
Pyridoxine hydrochloride	0.2 g
Nicotinic acid	0.2 g
Calcium pentothenate	0.1 g
Thiamin hydrochloride	0.05 g
PABA K	0.02 g
Riboflavin	0.02 g
Biotin	0.0125 g
Folic acid	0.02 g

Table S4 | Amino acid stock solution composition

Amino acid stock composition	Per litre
Tyrosine	1.83 g
Tryptophane	17.93 g
Isoleucine	3.27 g

Aspartic acid	4.45 g
Glutamic acid	12.04 g
Arginine	37.34 g
Leucine	4.84 g
Threonine	7.59 g
Glycine	1.83 g
Asparagine	5.31 g
Glutamine	50.52 g
Alanine	14.52 g
Valine	4.45 g
Methionine	3.14 g
Phenylalanine	3.80 g
Serine	7.85 g
Histidine	3.27 g
Lysine	1.70 g
Cysteine	1.31 g
Proline	61.26 g
NH_4Cl	46 g

Table S5 | Amino Anaerobic factors stock solution composition

Anaerobic factors stock composition	Per litre
Ergosterol	100 mg
Tween 80	5 mL
Complete with absolute ethanol to 20 mL	

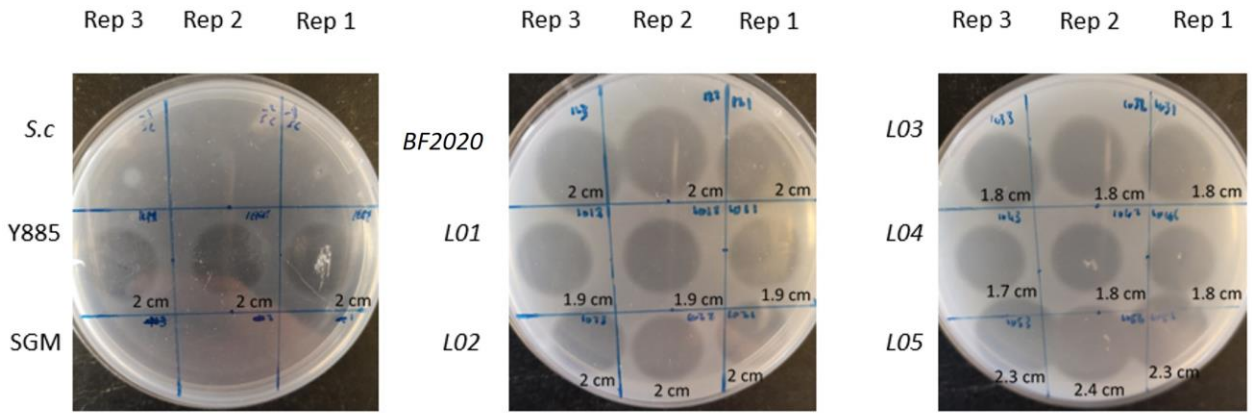

Fig. S1. *Polygalacturonase activity represented by halo formation on PGA plates. Distance indicated in cm is the relative halo diameter.*

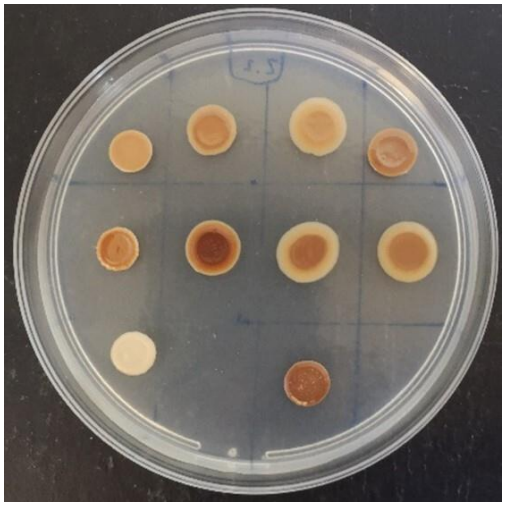

Fig. S2. *Comparison of H_2S production using BiGGY agar. Top left to right:* S. cerevisiae *QA23;* K. marxianus *Y885; BF2020;* K. marxianus *L01. Middle left to right:* K. marxianus *L02; L03; L04; L05. Bottom left to right:* S. bayanus *Vivace (low H_2S producing control);* S. cerevisiae *WH314 (high H_2S producing control).*

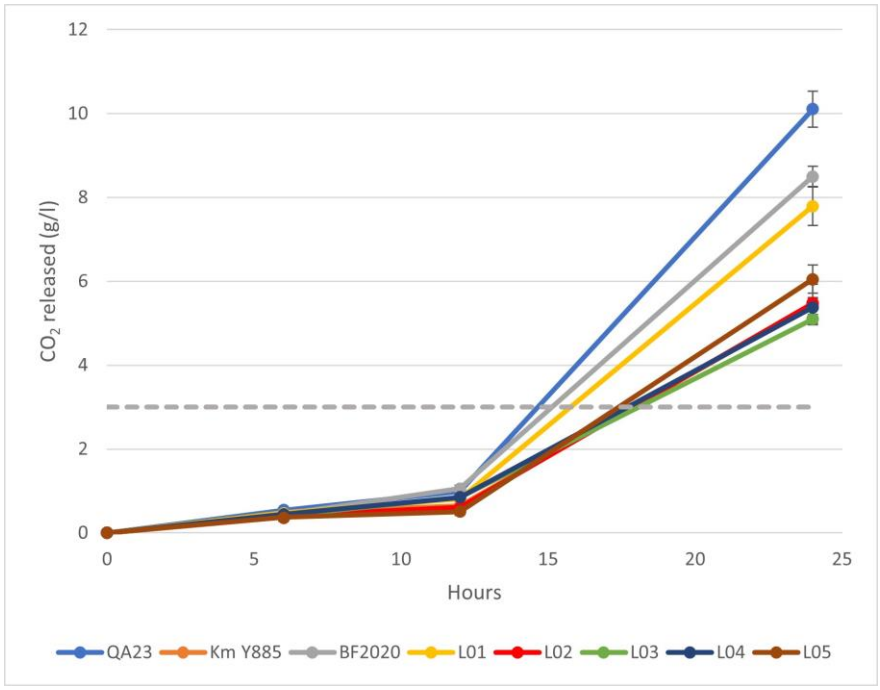

Fig. S3. *Fermentation kinetics, after 24 h, of 6 strains of* K. marxianus*, one strain of* S. cerevisiae *and one* K. marxianus *x* S. cerevisiae *fusant. The grey, dashed line corresponds to 3 g/L released total CO_2 (the exit of lag phase).*

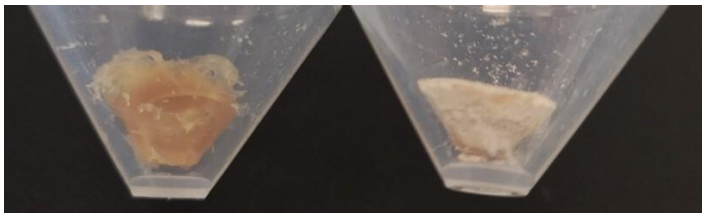

Fig. S4. *Pellets retrieved after centrifugation from 50 mL samples taken at end of fermentation. Strain* K. marxianus *L03 (left),* K. marxianus *Y885 (right).*

Chapter 4 – Impact of *Kluyveromyces marxianus* strains in mixed culture Chenin blanc and Cabernet Sauvignon wine production

4.1 Introduction

One of the main aims of this study was to compare the IWBT strain Y885, with regard to its oenological characteristics, to a number of other *Kluyveromyces marxianus* strains isolated from non-winemaking environments. In Chapter 3, strain Y885 was compared to strains L01 through L05 and BF2020 regarding fermentation performance, metabolites produced and a few other oenological parameters. However, these parameters were only investigated in laboratory conditions, making use of synthetic grape must (SGM) and monoculture fermentations in Erlenmeyer flasks. While these provided valuable information, a number of limitations prevent laboratory conditions to be a true reflection of a winemaking environment. As a consequence of the relatively slow fermentation rate of non-*Saccharomyces* yeasts, all SGM fermentations were performed while shaking to increase fermentation rate. To allow for thorough shaking and mixing, a large headspace (68.25 % of the flask volume) was left above the synthetic medium. This headspace, which is minimised in industrial winemaking conditions, resulted in increased initial oxygen concentrations that likely impacted *K. marxianus'* metabolism. Similarly, all SGM fermentations occurred at 25°C to allow for fermentations to be completed within a practical time frame. Despite 25°C being used in industrial fermentations, this temperature is not representative of all temperatures used in the wine industry, since white varieties are typically fermented below 20°C. Finally, while monoculture fermentations were performed in SGM to test the individual strains' performances and attributes, sequential fermentations with *S. cerevisiae* (i.e. a common practice when using other commercial non-*Saccharomyces* yeasts) would be required in industry, because *K. marxianus* is unable to ferment to dryness (i.e. consume all sugars) on its own (Chapter 3). The use of monocultures in SGM did therefore not allow to assess the impact of yeast interactions such as nutrient competition, which must be considered. In order to investigate how these strains would respond to real winemaking environments, both Chenin blanc and Cabernet Sauvignon wines were made using the strains mentioned above.

S. cerevisiae Lalvin QA23 was utilized for all sequential inoculations. Due to having shown increased fermentation performance (Chapter 3) BF2020 was used in two treatments for Chenin blanc fermentations, one monoculture treatment and one which was sequentially inoculated with QA23. The grape juices were not sterilized, in accordance with industry practices. PCR-ARISA was used to monitor population dynamics and presence of the inoculated strains. Chemical and sensory analyses were performed at the end of fermentation to determine the impact of the various strains on wine properties.

4.2 Materials and methods

4.2.1 Yeast strains and preculture conditions

As in Chapter 3, the yeasts investigated were *K. marxianus* L01, L02, L03, L04, L05 (Lallemand Inc., Montréal, QC, Canada) (Ortiz-Merino et al., 2018) as well as strains IWBT Y885 and BF2020 (yeast culture collection of the South African Grape and Wine Research Institute, Stellenbosch University, South Africa). Strain Lalvin QA23™ (Lallemand Inc.) also served as a non-*K. marxianus* control for all real grape juice experiments.

Cryopreserved yeasts Y885, L01 through L05 and BF2020 were thawed at room temperature and streaked onto Yeast Peptone Dextrose (YPD) agar (BioLab, Merck, South Africa). The plates were then incubated at 30°C until colonies reached a usable size. Starter cultures were prepared for each yeast by inoculating a single colony into 10 mL YPD broth (BioLab, Merck, South Africa). The cultures were incubated overnight on a rotating test tube wheel at 30°C. For precultures, 5 mL starter culture was inoculated into 500 mL YPD for all yeasts. Precultures were then incubated on a rotating table (orbital agitation of 125 rpm) at 30°C for 12 h to allow cells to reach exponential growth phase. These precultures were then used to inoculate all real grape juice fermentations at an initial cell density of 1×10^6 cells/mL. Prior to the inoculation of all yeasts an acclimatisation step was performed. After the yeasts were collected and washed with distilled water to remove YPD residue, the yeasts were resuspended in 20 mL distilled water at room temperature. Either Chenin blanc or Cabernet Sauvignon grape juice, depending on the experiment, was then added to reduce the yeast suspension temperature by 5°C. This process was repeated until the yeast suspension was approximately within 10°C of the grape juice temperature. Following this, the yeast suspensions were added to the respective fermentation vessels.

Where QA23 was inoculated either for a control or for sequential fermentations, dried QA23 yeasts were used and inoculated according to the recommended method as described by the Lalvin specification sheet for QA23.

4.2.2 Chenin blanc fermentation procedure

Chenin blanc grapes were harvested at Wolwedans vineyards (Stellenbosch, South Africa) on the 16th of February 2021. The grapes were crushed destemmed and processed at the Stellenbosch University Department for Viticulture and Oenology experimental cellar. The grapes were then pressed at 1.2 bar with the addition of dried ice (CO_2) pellets into the juice collection tray to reduce oxidation. 4 ml/hl Rapidase Clear (Oenobrands SAS, Montferrier-sur-Lez, France) was added along with 30 ppm SO_2. The juice was then cold settled for 48 h at 4°C and analysed for the following: Yeast Assimilable Nitrogen (240.42 mg/L); Malic acid (4.63 g/L); Glucose (115.71 g/L); Fructose (118.06 g/L); pH 3.48 and a Total acidity (7.62 g/L). The juice was split into 4-L bottles and inoculated with strains QA23, Y885, L01, L02, L03, L04, L05 and two sets of

BF2020. The first set of BF2020 was labelled Seq, as this set was later sequentially inoculated with QA23. The second set of BF2020 was labelled Noseq, as this set was only inoculated in monoculture throughout fermentation. 40 g/L Fermaid K^+ (Lallemand) was added to both the QA23 as well as BF2020 Noseq treatments. All treatments were then moved to 15°C. Fermentation kinetics were monitored using weight loss. Fermentations proceeded very slowly and therefore all treatments were moved to 22°C after 3 days. Due to the very low initial rate of fermentation the sequential inoculation of *S. cerevisiae* was delayed providing the *K. marxianus* and BF2020 strains increased potential to contribute sufficiently to the wine profile. After 134 h, samples were taken for ARISA analysis. Following this QA23 was sequentially inoculated into treatments Y885, L01, L02, L03, L04, L05 and BF2020 Seq. At the same time 40 g/L Fermaid K^+ was added to these treatments. Once fermentations had finished samples were taken for ARISA. 50 ppm SO_2 was added, and all treatments were moved to -4°C. After 2 weeks, the wines were racked and bottled for further analysis. All treatments were performed in triplicate.

4.2.3 Cabernet Sauvignon fermentation procedure

Cabernet Sauvignon grapes were harvested at Bellevue vineyards on the 26th of March 2021. The grapes were crushed destemmed and processed at the Stellenbosch University Department for Viticulture and Oenology experimental cellar. The crushed grapes and juice were analysed for the following: YAN (160 mg/L) Malic acid (4.41 g/L); Glucose (118.48 g/L); Fructose (129.59 g/L); pH 3.28 and a Total acidity (7.84 g/L). The crushed berries and juice were split into 10 l buckets (7 kg per bucket). 40 mg/L DAP along with 20 ppm SO_2 was added to each bucket. Strains Y885, L01, L02, L03, L04, L05 and two sets of BF2020 were then inoculated at 1 x 10^6 cells/mL as described above. The first set of BF2020 was labelled A and the second B. Sets A and B acted as two duplicate treatment sets in order to test the repeatability of using the newly produced fusant strain. All treatments were incubated at 4°C for 4 days for prefermentative cold maceration (PCM). Thereafter, all treatments were transferred to a 25°C room and samples were taken immediately for ARISA. All treatments were then inoculated with QA23. 20 g/L Fermaid K^+ was added to all treatments at the end of PCM and again after 67% of the sugars had been consumed. Fermentation kinetics were monitored using weight loss. At the end of alcoholic fermentation, the must and grape skins were transferred to a bladder press. The free-run wine was collected and measured as soon as the flux started to drip. The skins were then pressed and the volume of pressed wine was measured. The free-run and press wines were combined in 4-L bottles for each individual treatment and incubated at 20°C after inoculation with *Oenococcus oeni* Lalvin VP41 (Lallemand) to induce malolactic fermentation. At the end of malolactic fermentation (i.e. malic acid concentration below 0.3 g/L), all treatments were moved to -4°C and 50 ppm SO_2 was added. After 2 weeks, the wines were racked and bottled. All treatments were performed in triplicate.

4.2.4 Assessment of yeast species diversity through PCR-ARISA

Samples taken during fermentation were thawed and washed using distilled water. Initially, DNA extractions were performed for both Chenin blanc and Cabernet Sauvignon fermentations using the rapid isolation of yeast chromosomal DNA method from Hoffman (1997). However, after multiple attempts and troubleshooting including the addition of a Polyvinylpyrrolidone (PVP) wash step, no DNA could be extracted. Subsequently, DNA was successfully extracted using ZymoBIOMICS DNA Miniprep Kits (Zymo Research, Irvine, CA, USA) following the protocol provided. DNA was stored at -20°C until used for PCR-ARISA.

The ITS1-5.8S rRNA-ITS2 DNA region was amplified using the carboxy-fluorescein labelled ITS1 primer (5'-6-FAM- TCC GTA GGT GAA CCT GC GG-3') and ITS4 (5'- TCC TTC GCT TAT TGA TAT GC-3') in a 25 µL reaction. The reaction consisted of DNA, 1U Ex Taq DNA polymerase (TaKaRa Bio Inc.), 1 × Taq buffer, 0.25 µM of each primer, 400 µM dNTP mix and 1 mM $MgCl_2$. The PCR reactions were performed in a MiniAmp Thermal Cycler model A37028 (Thermo Fisher Scientific) under the following set conditions: Initial denaturation stage of 3 min at 94°C, 40 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec and an extension at 72°C for 45 sec, final extension stage at 72°C for 10 min (Bagheri et al., 2017). The PCR products were separated using capillary electrophoresis on an ABI 3010x Genetic Analyzer (Applied Biosystems) with a ROX 1.1 labelled size standard (75-1121 base pairs) at Stellenbosch University's Central Analytical Facility. Thermo Fisher Scientific peak scanner was used to identify the peaks generated and to determine the relative abundance of the yeast populations.

4.2.5 Determination of sugars and malic acid by enzymatic assays

Glucose, fructose and malic acid concentrations were initially determined by enzymatic analyses using an Arena 20XT (Thermo Fisher Scientific, Waltham, MA). The enzymatic assay kits used were Enzytec Liquid D-Glucose (Id-No: E8140, Roche, R-Biopharm) for glucose; Thermo Fisher D-Fructose (Product code 984302) for fructose and Enzytec Liquid L-Malic acid (Id-No: E8280, Roche, R-Biopharm) for malic acid.

4.2.6 Determination of sugars and ethanol concentrations by ALPHA spectroscopy

During the later stages of this study the enzymatic assays were unavailable and therefore glucose, fructose and ethanol concentrations were subsequently determined using an Alpha-P Mid Infrared spectrometer (Bruker Optics, Ettlingen, Germany) equipped with a wine universal attenuated total reflectance (ATR) sampling accessory. All control and selections were made using OPUS wine wizard software (OPUS for Microsoft, Bruker Optics, Ettlingen, Germany).

4.2.7 Quantification of major volatiles

Major volatiles were quantified at Stellenbosch University's Central Analytical Facility using gas chromatography-Flame ionization detection (GC-FID). Samples were prepared as follows. 100 µL of 175 ppm 4-methyl-2-pentanol (internal standard), 1.5 mL diethyl ether and 3 mL of 20% sodium chloride (NaCl) solution were added to 5 mL sample. The sample tube was then vortexed, sonicated for 30 min and liquid-liquid extraction (LLE) was performed. Samples were then centrifuged at 1841 *g* for 1 min. The diethyl ether layer (top) was transferred into a new vial with sodium sulphate and then transferred again into 2 mL vial. 1 µL was injected with a 5:1 split ratio onto the GC-FID instrument. Separation was performed on a gas chromatograph (6890N, Agilent technologies network) coupled to an Agilent technologies inert XL EI/CI Mass Selective Detector (MSD) (5975B, Agilent technologies Inc., Palo Alto, CA). The GC-FID system was coupled to a CTC Analytics PAL autosampler. Separation of the major wine volatiles was performed on a ZB-FFAP (60 m, 0.32 mm ID, 0.50 µm film thickness) capillary column (Phenomenex, Torrance, California, United States of America). Helium was used as the carrier gas at a flow rate of 1.9 mL/min. The injector temperature was maintained at 240°C. The oven temperature was programmed as follows: 35°C for 17 minutes and ramped at a rate of 12°C/min until 240°C and held for 5 minutes. The MSD was operated in a full scan mode and the source and quad temperatures were maintained at 230°C and 150°C, respectively. The transfer line temperature was maintained at 250°C. The mass spectrometer was operated under electron impact (EI) mode at ionization energy of 70eV, scanning from 35 to 650m/z (Louw et al., 2009).

4.2.8 Sensory flash profile tests

A trained group of 10 tasters, whom all had previous professional experience with wine sensory evaluations, were gathered as the panellists for the following sensory tests. The sensory tests conducted were adapted from the flash profile technique originally described by Dairou and Sieffermann (2002). 50 mL of the various Chenin blanc wine treatments were aliquoted into bottles which were distributed to panellists. Due to Covid-19 regulations, all sensory evaluations occurred at the individual panellists' homes. The Compusense sensory data capturing software package was used to capture the panellists' responses. All treatments were given randomized codes and the tasting order was randomised across the panellists. The panellists were instructed to perform 2 sessions with 5 to 10 min intermissions in-between. During the first session all panellists were to smell and taste all wines and provide descriptive attributes. During the intermission all attributes were pooled and provided to the panellists. During the second session the panellists were asked to rank each wine from least to most intense for the attributes chosen in the first session. This process was repeated for each of the 3 replicates for each treatment with every replicate being tasted on a different day. For further accuracy, each replicate was tasted in duplicate with 10-min breaks in between. All data were pooled and the rank sum of each attribute for each treatment across all panellists was calculated. This rank sums were then used to generate a PCA in order to visualize similarities or differences between treatments. Ethical clearance for all sensory tests was obtained from the

Stellenbosch University's Research and Ethics Committee (REC): Humanities department under the reference number REC-2021-21710.

4.2.9 Statistical analyses

XLSTAT version 2020.1.1 was used for principal component analyses (PCA) of major volatiles produced as well as One-way ANOVA with Tukey's multiple comparison test of means and Cluster analysis using Ward's method.

4.3 Results and discussion

4.3.1 Chenin blanc fermentations and population dynamics

Chenin blanc fermentations were initially started at 15°C. However, after 3 days, no to very little fermentation activity was observed. The fermentation vessels were then transferred to a 22°C room for the remainder of fermentation. This slow start was unexpected as both *S. cerevisiae* and *K. marxianus* are known to ferment at this temperature (Barone et al., 2021). It is however possible that the temperature acclimatisation step during the yeast inoculation may not have been long enough, thereby causing the delay in fermentation. Yeast rehydration nutrients are often used in the rehydration step to aid with inoculation. As all *K. marxianus* strains used were colonies precultured in YPD no rehydration nutrients were used. Being precultured in YPD, a nutrient rich and well-suited medium for the yeasts, the shock of inoculation into the high sugar, low temperature grape juice was likely worsened. In future experiments, it would be of interest to add a form of the yeast rehydration buffer to increase the yeasts' ability to adapt to the medium change. Excluding treatments QA23 and BF2020 Noseq, all treatments consisted of an initial inoculation of a *K. marxianus* strain followed by the sequential inoculation of QA23 after 134 h. Little variation could be seen for the fermentation kinetics of the sequential fermentations (Figure 4.1). Fermentations ended with an average alcohol concentration of 12.78 % ± 0.71 %. Slight variations occurred with regards to the measured CO_2 weight loss at fermentation end points however these can be attributed to experimental errors as all treatments were found to have an average residual sugar of 1.31 g/L ± 1.70 g/L at the end of fermentation.

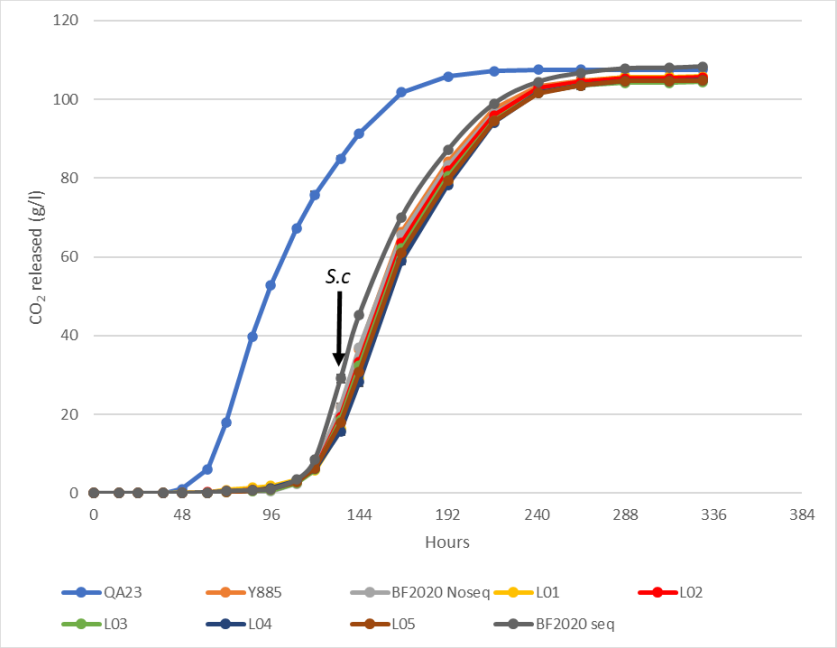

Fig. 4.1. *Chenin blanc fermentation kinetics of 6 strains of* K. marxianus*, one strain of* S. cerevisiae *(QA23) and two* K. marxianus *x* S. cerevisiae *fusants (BF2020). QA23 was sequentially inoculated after 134h (indicated by arrow) for all treatments except BF2020 Noseq. Average standard deviation for all fermentations was less than 0,33 g/L CO_2 released and, although included, are therefore not visible on graph.*

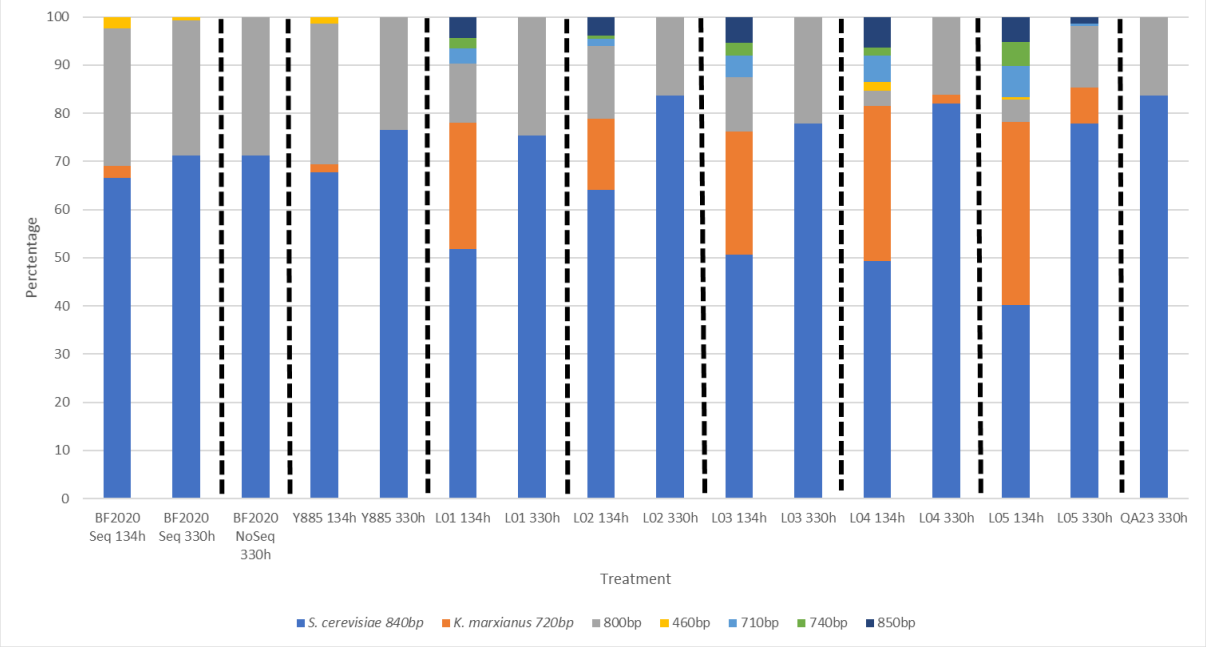

Fig. 4.2. *Chenin blanc population dynamics immediately before the sequential inoculation of QA23 as well as at the end of fermentation.*

Samples were taken for all sequential fermentations just before QA23 was inoculated as well as at the end of fermentation for PCR-ARISA analysis in order to determine population dynamics at these points (Figure 4.2). Initially, a rapid DNA extraction method was used to extract DNA for the PCR-ARISA. However, after multiple attempts DNA could not be successfully extracted from the yeast samples taken. This was attempted for both Chenin blanc and Cabernet Sauvignon samples with the addition of multiple wash steps

including washing with Polyvinylpyrrolidone (PVP) to reduce potential interference from polyphenolic compounds. Finally, DNA was successfully extracted and utilised for PCR-ARISA with the aid of ZymoBIOMICS DNA Miniprep Kits. As the DNA miniprep kits include more extensive wash and purification steps, it is likely that the complex grape juice matrixes contained compounds that were interfering with the rapid DNA extraction method.

After analysing the population dynamics in Figure 4.2, it is clear that *S. cerevisiae* had already become dominant in all fermentations after 134 hours, even though QA23 had not been inoculated yet. Despite this, the presence of the initially inoculated *K. marxianus* or fusant strains could still be observed. The extent of this presence varied to a large degree with a much greater presence for strains L01 through L05 (27.38 % ± 7.77 %) compared to strain Y885 and BF2020 (2.13 % ± 0.40 %) after 134 h. By the end of fermentation, most treatments no longer had any presence of *K. marxianus* yeasts, the only exceptions being strains L04 and L05, which appeared to have survived, albeit at a low proportion (4.64 % ± 2.79 %).

The significant proportion of an unknown yeast species, revealed by the presence of a PCR amplicon of 800 bp should be noted. Unfortunately, the PCR-ARISA process used did not allow for the identification of the yeast without further tests. It was expected to find other yeasts present throughout the fermentation as the fermentation conditions and grape juice were not sterile, but the extended presence of the 800 bp yeast, which accounted for approximately 20,83 % ± 5,30 % of the yeast population at the end of fermentation, was surprising. This yeast may have contributed to the sensory and chemical profiles of the wines.

As *S. cerevisiae* dominated the fermentation from a reasonably early stage, it is unsurprising that the fermentation kinetics presented in Figure 4.1 showed little variation between sequential treatments. Despite BF2020 having shown increased fermentation performance in Chapter 3, it was outcompeted by indigenous *S. cerevisiae* in these real grape must fermentations. Unfortunately, no sample was taken during fermentation, so it remains unclear at which stage of fermentation BF2020 became no longer detectable.

4.3.2 Major volatiles produced during Chenin blanc fermentations

The concentrations of major volatile compounds were measured after the wine had been bottled and aged for 4 months. The results are presented in Table S6 and S7 and compiled in the form of a PCA (Figure 4.3).

Despite the high presence of *S. cerevisiae* early on during fermentation, a clear separation between the QA23 treatment and those inoculated with *K. marxianus* or BF2020 occurred along both the x- and y-axis. Treatment L01 also greatly separated from the various *K. marxianus* treatments along the y-axis (Figure 4.3).

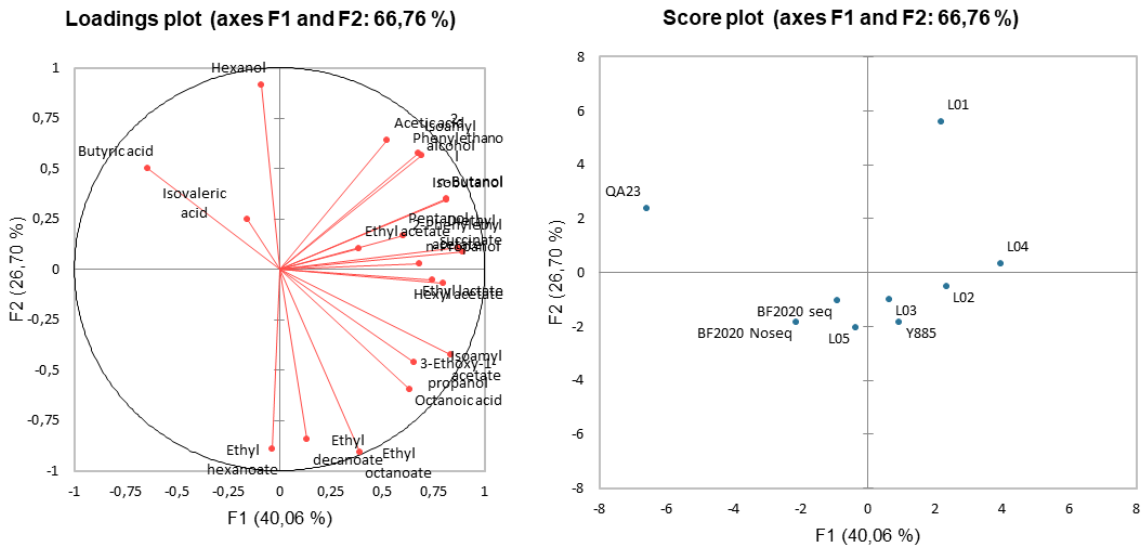

Fig. 4.3. *Principal component analysis (PCA) of molar major volatile production of Chenin blanc wines.*

Using the PCA data generated from Figure 4.3, a cluster analysis was performed in order to establish the degree of similarity between treatments (Figure 4.4). Although not the exact same, similar trends emerged between the results shown in Figure 4.4 and those generated when using synthetic grape juice (Figure 3.5). As in the synthetic grape juice experiments, treatments Y885, BF2020 and L05 grouped together as well as treatments L02 and L04 grouping together. Surprisingly, amongst the *K. marxianus* strains, treatment L01, formerly closely grouping with treatments BF2020, Y885 and L05, was now the least related. Conversely, the former least related treatment L03 now grouped closely with L02 and L04. BF2020 Noseq and BF2020 seq grouped closely together. As seen in Figure 4.2, *S. cerevisiae* dominated at the end of fermentation despite not being inoculated in the BF2020 Noseq treatment. With BF2020 Noseq and BF2020 seq both grouping closely it is likely that potential cross contamination occurred and that the *S. cerevisiae* strain(s) present at the end of both of these treatments was/were the same. This would explain why major volatiles produced were similar. However, as both treatments grouped away from the monoculture *S. cerevisiae* treatment a clear impact from the BF2020 strains can still be inferred. It is therefore also possible that they grouped closely because of the impact of the BF2020 strains on major volatiles produced and not because of the impact of *S. cerevisiae.*

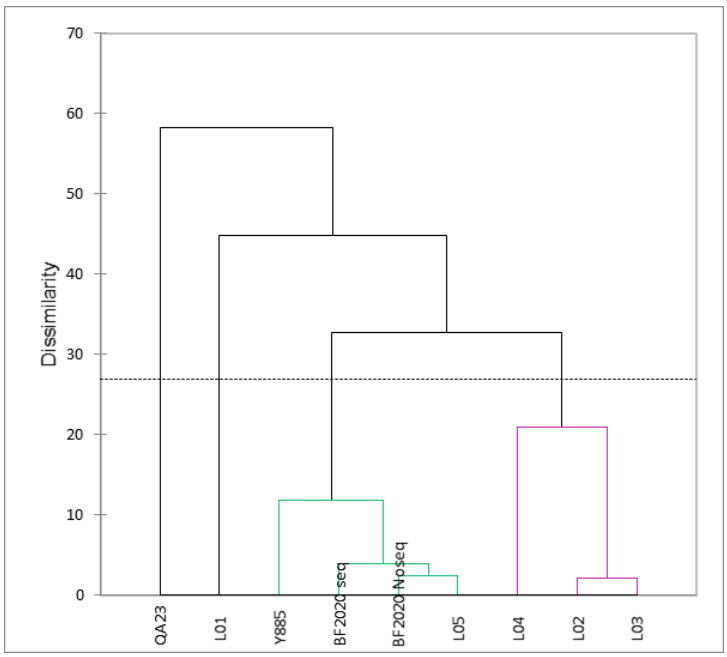

Fig. 4.4. *Dendrogram comparing the dissimilarity between the* K. marxianus *strains, BF2020 and QA23 investigated in this study. The horizontal line indicating the separation of clusters.*

One of the major drivers for the separation of the *K. marxianus* treatments and QA23 is the high production 2-phenylethyl acetate by *K. marxianus* strains (Table S6 and S7). All *K. marxianus* and BF2020 treatments produced more than 2-fold 2-phenylethyl acetate compared to QA23 (Figure 4.5). This high production of 2-phenylethyl acetate has been shown to be characteristic of *K. marxianus* in Chapter 3 as well as previous studies (Rollero et al., 2018a, 2018b, 2018c).

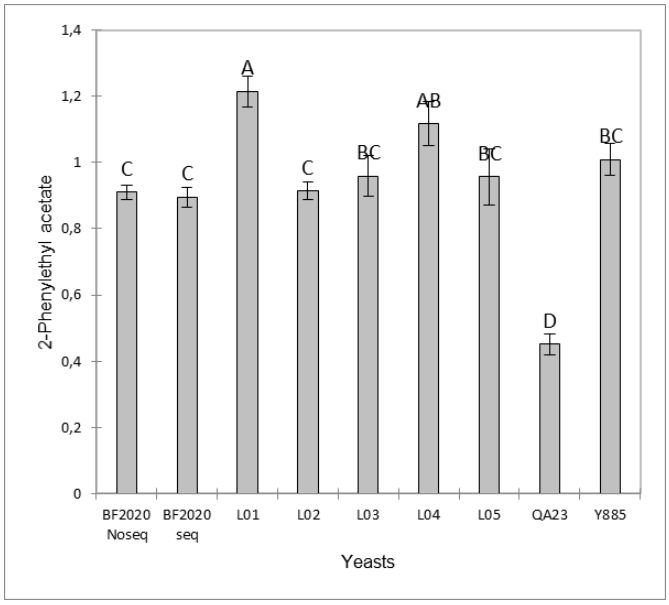

Fig. 4.5. *2-phenylethyl acetate production (mg/L) of Chenin blanc* wines *of various strains. Strains sharing the same letter are not significantly different at a p<0.05 threshold.*

Figure 4.5 shows that the highest 2-phenylethyl acetate concentrations were found in the L01 treatment. This varies considerably from the synthetic grape juice experiments (Chapter 3) where L01 was found to be one of the lowest 2-phenylethyl acetate producers. As discussed previously, various limitations exist in

creating a realistic industrial winemaking environment when using synthetic grape juice. It is possible that the interactions with the dominant *S. cerevisiae,* decrease in oxygen concentration and/or the composition of the must (especially the concentration of phenylalanine, i.e. the main metabolic precursor of 2-phenylethyl acetate) all impacted the production of 2-phenylethyl acetate. This highlights the already known fact that despite providing a good overall idea of what would happen in real grape juice, the results produced from synthetic grape juice studies can often vary to real grape juice.

4.3.3 Cabernet Sauvignon fermentations and population dynamics

Cabernet Sauvignon fermentations consisted of an initial 4-day prefermentative cold maceration (PCM) step at 4°C after which the fermentation proceeded at 25°C. All *K. marxianus* and BF2020 treatments were inoculated at the start of PCM. QA23 was sequentially inoculated at the end of PCM. The BF2020 strain was inoculated into two different treatments termed A and B respectively to test repeatability using this yeast.

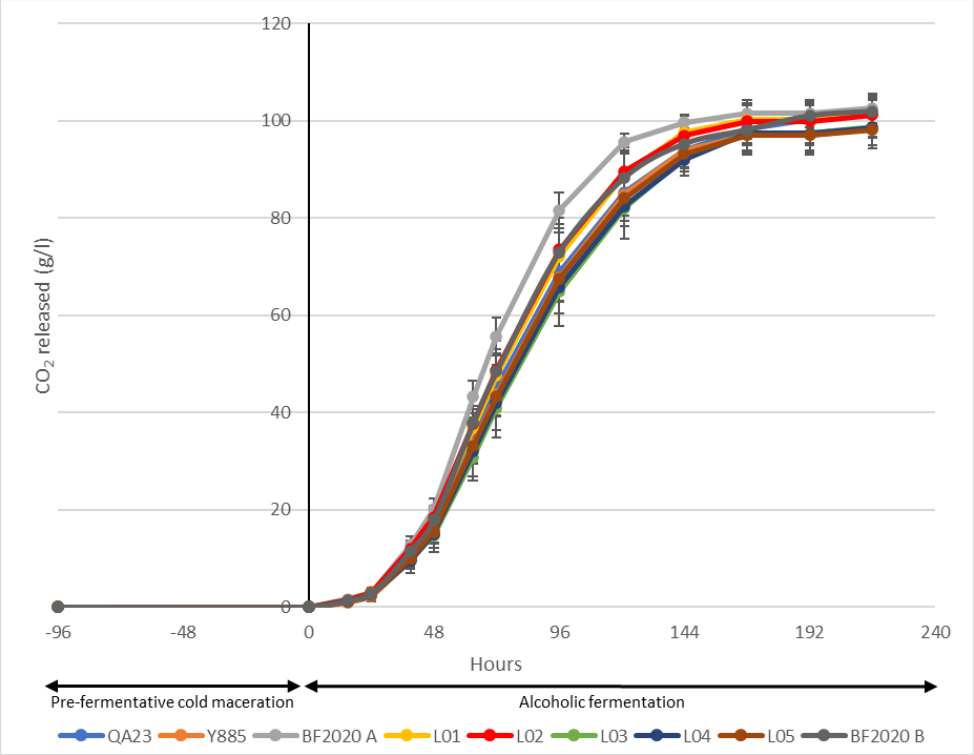

Fig. 4.6. *Cabernet Sauvignon fermentation kinetics of 6 strains of* K. marxianus*, one strain of* S. cerevisiae *and* two K. marxianus *x* S. cerevisiae *fusants (BF2020). QA23 was sequentially inoculated after the completion of PCM at time indicated as time 0h.*

As with the Chenin blanc fermentations the fermentation kinetics appeared to be very similar to one another with only treatment BF2020 A fermenting slightly faster than the others (Figure 4.6). Slight variations occurred with regard to the fermentation end points, however these can be attributed to experimental errors as all treatments were found to have an average residual sugar of 1.46 g/L ± 1.59 g/L at the end of fermentation. Malolactic fermentation was completed in all treatments with the final malic acid concentrations being below 0.3 g/L.

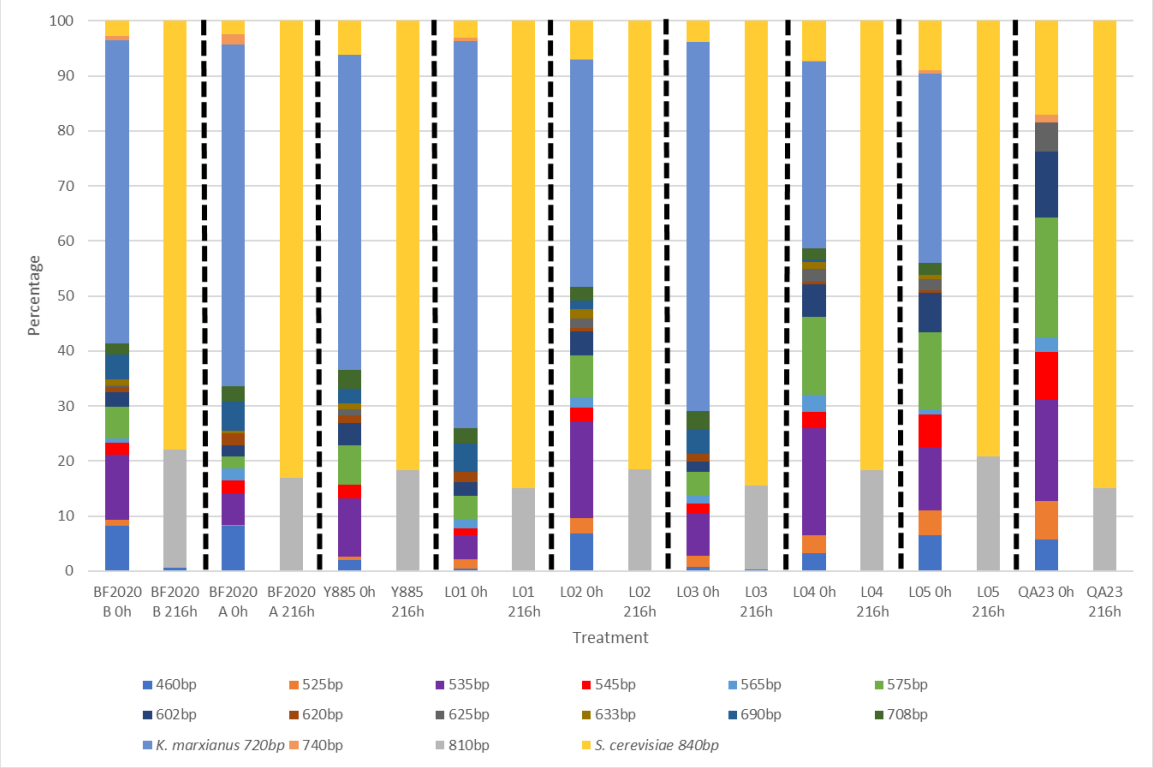

Fig. 4.7. *Cabernet Sauvignon population dynamics immediately before the sequential inoculation of QA23 as well as at the end of fermentation.*

Using PCR-ARISA, yeast diversity was explored at the end of PCM and at the end of fermentation. A large diversity of indigenous yeasts was observed at the end of PCM, although the different strains of *K. marxianus* were clearly dominant (Figure 4.7). Indigenous *S. cerevisiae* could be detected, albeit in very low proportion (less than 10%). Despite this high initial presence, no *K. marxianus* presence could be detected for any of the treatments at the end of alcoholic fermentation. A much greater diversity of indigenous yeasts was seen for the Cabernet Sauvignon fermentations compared to the Chenin blanc fermentations. This can likely be attributed to the cold temperature of PCM which delayed the start of fermentation and extended the survival of non-*Saccharomyces* yeasts.

4.3.4 Major volatiles produced during Cabernet Sauvignon fermentations

As with Chenin blanc the major volatiles of the Cabernet Sauvignon wines were quantified 4 months after bottling (Table S8 and S9). This data was then compiled in the form of a PCA (Figure 4.8). The metabolic footprint and overall impact of the *K. marxianus* and BF2020 strains during the Cabernet Sauvignon fermentations appeared to be limited compared to the Chenin blanc fermentations. Indeed, the treatments did not group as clearly as in the Chenin blanc fermentations (Figure 4.8). QA23 and L01 again separated from the treatments Y885, BF2020 B and L01 through L04 along the x-axis. However, this time, QA23 grouped closely to L02 and L03 along the y-axis. The two inoculation repeats BF2020 A and BF2020 B

appeared very similar and did not separate along the y-axis however showing separation along the x-axis. Although intended to be repeats the ARISA data from Figure 4.7 shows a 7 % difference in BF2020 A and B populations between the two treatment repeats. This difference could potentially explain the separation seen along the x-axis.

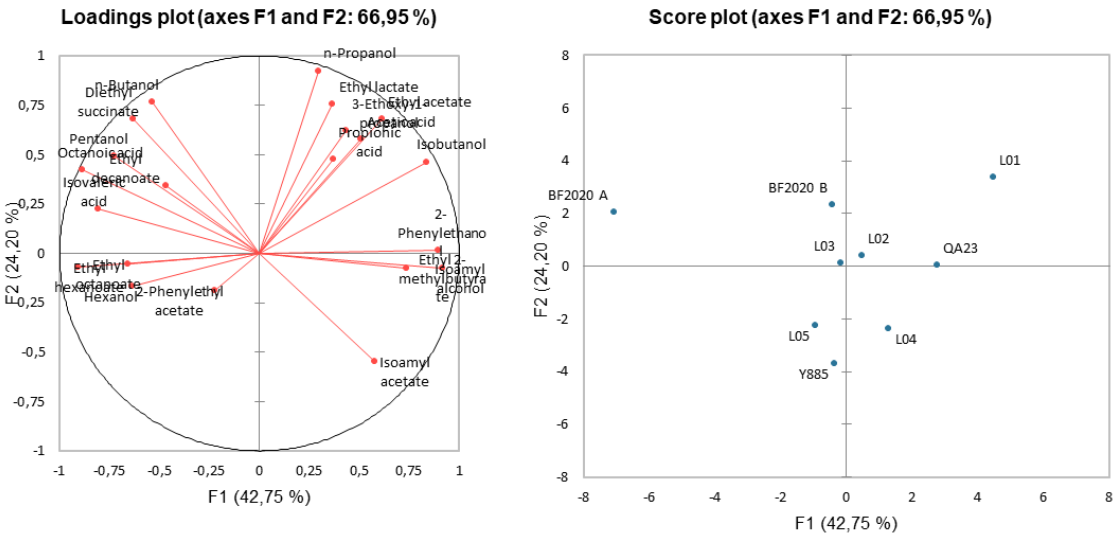

Fig. 4.8. *2- Principal component analysis (PCA) of molar major volatile production at the of Cabernet Sauvignon wines.*

Although certain strains did show separation in Figure 4.8, the significance is not very high. After the major volatiles were compared to determine statistically significant variations, only 6 out of 21 compounds showed statistically significant differences between treatments (Table S8 and S9). This indicates that the *K. marxianus* and BF2020 strains did not impact the fermentations to a large extent. This is further supported by the 2-phenylethyl acetate production (Table S9). The difference between 2-phenylethyl acetate produced by the QA23 and *K. marxianus* treatments varied to a much lesser extent than expected (Figure 4.9). These final concentrations produced were also significantly lower than those produced in the Chenin blanc fermentations indicating that the *K. marxianus* strains likely contributed very little to the major volatiles produced. This could be attributed to the different prefermentation operation, but also possibly to the matrices themselves. In particular, it would have been of interest to quantify phenylalanine concentration, since it has been directly linked to 2-phenylethylacetate production (Rollero et al., 2019).

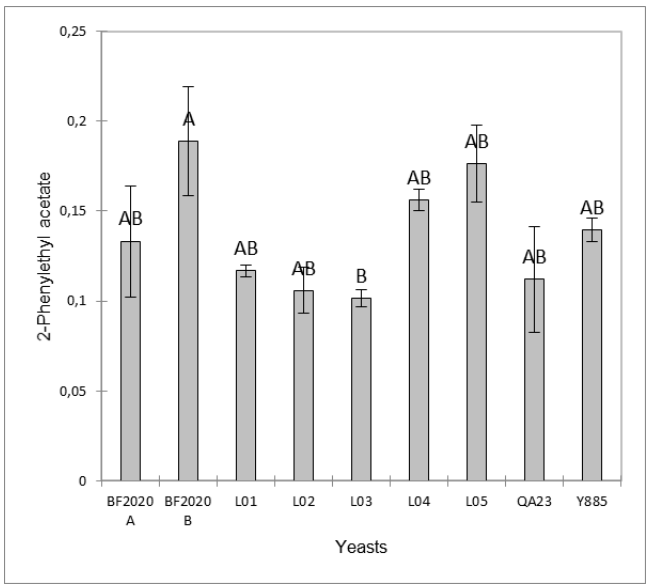

Fig. 4.9. *2-phenylethyl acetate production (mg/L) of Cabernet Sauvignon wines of various strains. Strains sharing the same letter are not significantly different at a p<0.05 threshold.*

4.3.5 Impact on Cabernet Sauvignon free-run wine volume

Once the Cabernet Sauvignon fermentations were completed, the free-run wine volume was measured for all treatments. In order to compare treatments to one another, the free-run to press wine ratio was determined. No significant differences was noticed between any of the treatments including that of the QA23 treatments (Figure 4.10). This outcome differs from that reported by Rollero et al. (2018c) who found the inoculation of *K. marxianus* Y885 along with a PCM treatment led to a great increase in free run juice. The experimental parameters between the study by Rollero et al. (2018c) and the current study were largely the same with the only exception being the use of Shiraz grapes by Rollero et al. (2018c) and Cabernet Sauvignon in the current study.

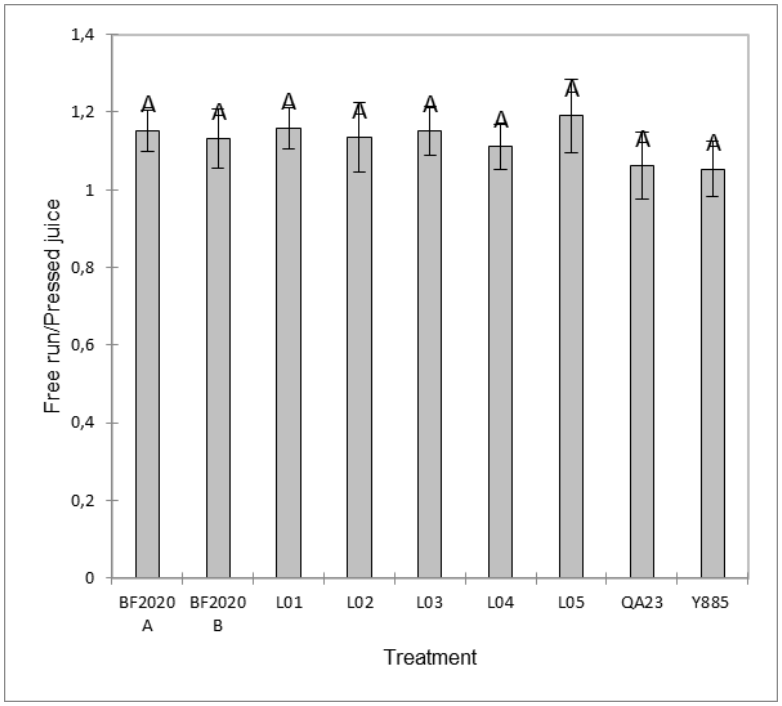

Fig. 4.10. *Free run ratio at the end of Cabernet Sauvignon alcoholic fermentations of various strains. Strains sharing the same letter are not significantly different at a p<0.05 threshold.*

The difference in grape cultivars can likely explain the difference in impact by *K. marxianus* on free-run ratio. In a study by Apolinar-Valiente et al. (2015), it was suggested that the lower content of uronic acids in the skins of Shiraz grapes compared to Cabernet Sauvignon grapes would indicate a lower amount of pectin in Shiraz. Although not investigated in the current study, a lower pectin concentration in Shiraz may have contributed to the differences seen between the current study and that by Rollero et al. (2018c). Indeed, a large difference in pectin concentration in Cabernet Sauvignon might require increased concentrations of pectinases in order to observe the same effects as seen with Shiraz grapes. Furthermore, the degree of methylation has been shown to vary to a large extent between different grape cultivars (González-Centeno et al., 2010; Ortega-Regules et al., 2008). The latter studies both confirmed that Cabernet Sauvignon grapes had higher degree of methylation than Shiraz grapes. Increased degrees of methylation have been found to directly lead to a decrease in hydrolysis by endopolygalacturonase produced by *Fusarium moniliforme* (Bonnin et al., 2002). Although differences may occur between endopolygalacturonases produced by *K. marxianus* and *F. moniliforme*, it is likely that the same decrease in hydrolysis may occur and explain the differences observed between the current study and that by Rollero et al. (2018c). This is further substantiated by the fact that pectinases produced by *K. marxianus* specifically target α-1,4 glycosidic bonds between non-methylated galacturonic acid residues (Rollero et al., 2018c). This cultivar difference was noted by the latter authors. They found that although still having an effect, much less of an impact was seen on the free-run ratio of Merlot wines than in Shiraz (Unpublished data). As with Cabernet Sauvignon, González-Centeno et al. (2010) also found Merlot to have a higher degree of methylation than Shiraz substantiating the above hypothesis. However, it should be mentioned that contrary to this, Ortega-

Regules et al. (2008) found Merlot to have a lesser degree of methylation than Shiraz. These differences could potentially be due to the ripeness of the grapes investigated between the two studies.

As no significant differences were observed in the free-run wine ratios, it is possible that the expected contribution of PCM in the release of aroma precursors was also limited. This could potentially explain why the QA23 treatment did not separate as well from the *K. marxianus* treatments as previously described in Shiraz grape juice by Rollero et al. (2018c). It would therefore be of interest to repeat the current study using Shiraz grapes to have a better comparison to previous literature and assess *K. marxianus*' contribution in different cultivars. For future studies, it would be beneficial to monitor the pectinase activity throughout or at least during the initial phase of fermentation. However, it should be mentioned that although confirming whether the yeasts produce pectinase enzymes this will not confirm whether the enzymes actively contribute to the breakdown of pectin. It would therefore be of interest to also measure the GalA monosaccharides released as a result of pectin breakdown as performed by Rollero et al. (2018c).

4.3.6 Sensory analysis

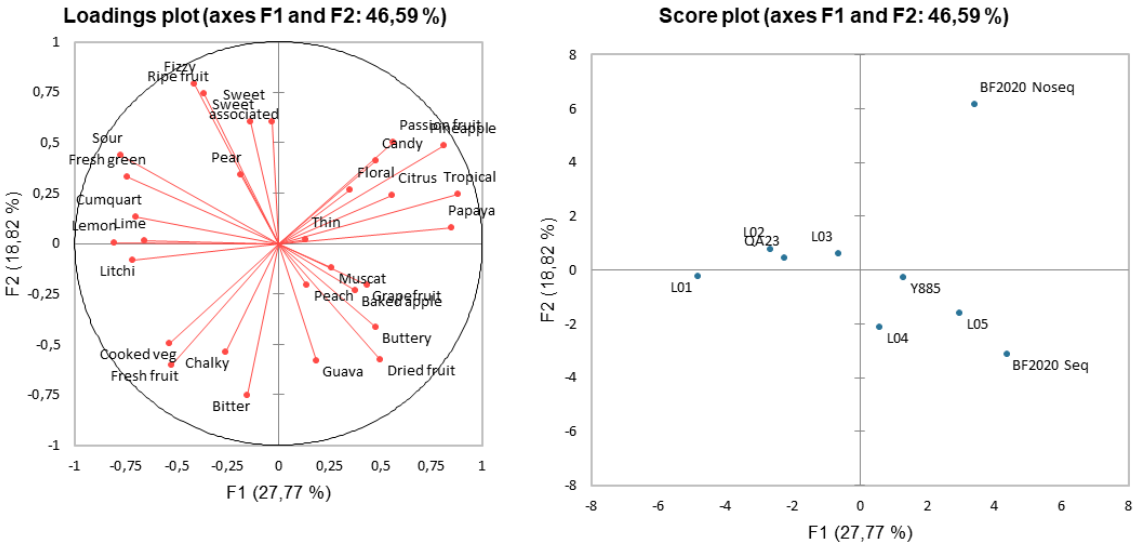

Fig. 4.11. *2- Principal component analysis (PCA) of sensory data generated for Chenin blanc wines.*

A sensory evaluation was performed on the Chenin blanc wines in order to determine whether the chemical differences reported between treatments would result in wines with different sensory profiles. Using the rank sum data, generated by a trained panel, the PCA in Figure 4.11 was generated. As the results from the chemical analysis of Cabernet Sauvignon wines did not result in significant differences between treatments it was decided not to perform sensory analysis on these wines.

A number of treatments grouped relatively closely with the QA23 treatment, indicating little sensory impact from strains L01, L02 and L03. Despite this, separation can be observed for strains Y885, BF2020 Seq, L04 and L05 along the x-axis. Similarly, treatment BF2020 separated along both the x- and y-axis.

Although this separation would suggest clear sensory differences, the overall explained variance was fairly low (46.59%). Secondly, a number of high contributors to the already low explained variance were factors likely not impacted by the major volatiles quantified. Indeed, these descriptors included fresh green, chalky, sour, bitter and fizzy. As these attributes appeared to be very prominent in the wines produced, it is likely that they overshadowed potential differences, seen with the major volatiles PCA (Figure 4.3), that might have been detected otherwise.

It can therefore be concluded that despite the potential still existing for the various yeasts used to impact the sensory profiles, no clear differentiation could be seen in the current study between wines produced with QA23 and those produced using the *K. marxianus* or BF2020 strains.

4.4 Conclusion

Using synthetic grape juice is often the first step in wine microbiology studies. While it allowed us to compare *K. marxianus* strains, it underestimated the complexity of the grape juice matrix and ignored that of yeast interactions. Subsequently, results obtained in synthetic grape juice frequently do not fully reflect those obtained when using real grape must. The current study clearly confirmed this statement. Indeed, although differences could be identified between *K. marxianus* strains using real grape juice, they were more limited than those observed in synthetic grape juice. Nevertheless, clear differentiations could be seen between the QA23 and *K. marxianus* treatments regarding major volatiles produced for Chenin blanc wines, but these did not translate into differences in the sensory profiles of these wines.

This study has also shown that despite certain strains displaying weaker fermentation performance in synthetic grape juice, these strains could be used to effectively produce wines with the sequential inoculation of *S. cerevisiae*. Similarly, strains that displayed high H_2S production in chapter 3 did not result in wines with negative off-flavours using real grape juice. Although the sensory data generated in the current study did not provide conclusive results, it can still be stated that the use of the *K. marxianus* and BF2020 strains impacted the chemical composition of the wines produced, with a larger impact seen for Chenin blanc wines. The lesser impact of *K. marxianus* strains on the Cabernet Sauvignon wines can be attributed to a number of factors. Although the cultivar differences between Cabernet Sauvignon and Shiraz grapes as well as the high presence of indigenous yeasts likely all played a role, the experimental procedure itself could also have had an impact. Despite *K. marxianus* being able to survive and produce pectinases during the prefermentative cold maceration step, the impact of the yeasts on metabolites produced during this step was likely limited. Following the immediate sequential inoculation of *S. cerevisiae* once moved to 25°C, the *K. marxianus* strains were likely to have been outcompeted by *S. cerevisiae* early on. In order to induce a greater impact from the *K. marxianus* strains, the inoculation of *S. cerevisiae* could potentially have been delayed.

4.5 Supplementary material

Table S6 | Comparison of major volatiles of Chenin blanc wines (mg/L)

Yeast	Ethyl acetate	n-Propanol	Isobutanol	Isoamyl acetate	n-Butanol	Isoamyl alcohol	Ethyl hexanoate	Pentanol	Hexyl acetate	Ethyl lactate	Hexanol
QA23	8.49E+01± 3.05E+00^{ABC}	5.07E+01± 4.27E+00^{BC}	2.69E+01± 5.96E-01^{D}	3.89E+00± 9.43E-02^{D}	1.32E+00± 8.06E-02^{B}	2.20E+02± 4.31E-01^{B}	8.49E-01± 1.63E-02^{B}	5.91E-01± 7.36E-03^{A}	2.22E-01± 5.15E-03^{B}	1.38E+01± 4.90E-01^{B}	1.03E+00± 2.10E-02^{AB}
Y885	8.88E+01± 4.91E+00^{ABC}	6.72E+01± 5.69E+00^{AB}	3.59E+01± 1.84E+00^{ABC}	5.91E+00± 2.78E-01^{A}	1.63E+00± 2.30E-01^{AB}	2.37E+02± 5.32E+00^{AB}	1.03E+00± 7.40E-02^{A}	5.98E-01± 6.01E-03^{A}	2.82E-01± 1.30E-02^{AB}	1.51E+01± 5.96E-01^{B}	8.96E-01± 1.32E-02^{C}
BF2020 seq	8.15E+01± 3.85E+00^{BC}	6.12E+01± 5.80E+00^{ABC}	3.26E+01± 2.98E+00^{CD}	5.21E+00± 7.53E-02^{ABC}	1.41E+00± 7.90E-02^{AB}	2.30E+02± 1.14E+01^{AB}	9.51E-01± 4.73E-02^{AB}	5.93E-01± 9.25E-03^{A}	2.53E-01± 9.34E-03^{B}	1.61E+01± 5.64E-01^{B}	9.39E-01± 1.19E-02^{C}
BF2020 Noseq	7.61E+01± 3.99E+00^{C}	4.76E+01± 3.71E+00^{C}	3.06E+01± 1.52E+00^{CD}	4.69E+00± 5.47E-02^{C}	1.35E+00± 8.09E-02^{B}	2.21E+02± 6.61E+00^{B}	1.04E+00± 6.94E-03^{A}	5.89E-01± 5.99E-03^{A}	2.50E-01± 6.08E-03^{B}	1.65E+01± 3.17E-01^{B}	9.24E-01± 1.78E-02^{C}
L01	8.57E+01± 5.40E+00^{ABC}	6.35E+01± 9.44E+00^{ABC}	4.17E+01± 2.63E+00^{A}	4.95E+00± 2.46E-01^{BC}	1.73E+00± 9.84E-02^{A}	2.55E+02± 8.92E+00^{A}	7.91E-01± 5.44E-02^{B}	6.00E-01± 1.13E-02^{A}	2.63E-01± 1.37E-02^{B}	1.65E+01± 9.39E-01^{B}	1.12E+00± 4.05E-02^{A}
L02	9.24E+01± 1.76E+00^{AB}	7.21E+01± 5.61E+00^{A}	3.96E+01± 2.02E+00^{AB}	5.64E+00± 1.18E-01^{AB}	1.63E+00± 8.30E-02^{AB}	2.35E+02± 2.26E+00^{AB}	9.35E-01± 2.96E-02^{AV}	5.98E-01± 2.92E-03^{A}	2.93E-01± 4.16E-03^{AB}	1.84E+01± 5.85E-01^{AB}	9.77E-01± 1.70E-02^{BC}
L03	9.61E+01± 3.43E+00^{A}	6.39E+01± 1.41E+00^{ABC}	3.01E+01± 1.14E+00^{CD}	5.67E+00± 3.52E-	1.62E+00± 3.16E-	2.21E+02± 6.65E+00^{B}	9.06E-01± 8.83E-	5.90E-01± 4.65E-	2.84E-01± 3.04E-	1.69E+01± 3.29E-01^{B}	9.43E-01± 2.67E-

				01^{AB}	02^{AB}		02^{AB}	03^{A}	02^{AB}		02^{BC}
L04	8.62E+01± 5.47E+00^{ABC}	5.95E+01± 4.23E+00^{ABC}	3.68E+01± 2.10E+00^{ABC}	5.66E+00± 3.22E-01^{AB}	1.62E+00± 6.42E-02^{AB}	2.40E+02± 1.16E+01^{AB}	8.98E-01± 4.01E-02^{AB}	6.41E-01± 7.17E-02^{A}	3.71E-01± 6.80E-02^{A}	2.28E+01± 3.98E+00^{A}	9.41E-01± 3.73E-02^{C}
L05	7.98E+01± 3.45E+00^{BC}	5.59E+01± 1.59E+00^{ABC}	3.37E+01± 1.68E+00^{BCD}	5.16E+00± 1.46E-01^{ABC}	1.36E+00± 2.16E-02^{B}	2.31E+02± 2.07E+00^{AB}	9.62E-01± 2.59E-02^{AB}	5.91E-01± 5.34E-03^{A}	2.44E-01± 2.07E-02^{B}	1.67E+01± 5.26E-01^{B}	9.77E-01± 2.55E-02^{BC}

2 *Strains sharing the same letter are not significantly different at a p<0.05 threshold.*

Table S7 | Comparison of major volatiles of Chenin blanc wines continued (mg/L)

Yeast	3-Ethoxy-1-propanol	Ethyl octanoate	Acetic acid	Ethyl decanoate	Butyric acid	Diethyl succinate	Isovaleric acid	2-Phenylethyl acetate	2-Phenylethanol	Octanoic acid
QA23	2.17E+00± 2.21E-01^{B}	6.17E-01± 2.85E-02^{A}	2.64E+02± 3.79E+01^{B}	1.50E-01± 1.49E-02^{AB}	3.13E+00± 1.43E-01^{A}	2.41E-01± 7.06E-02^{B}	2.16E+00± 6.77E-02^{AB}	4.51E-01± 3.05E-02^{D}	2.98E+01± 1.32E-01^{C}	6.73E+00± 2.00E+00^{B}
Y885	2.93E+00± 2.42E-01^{AB}	7.94E-01± 6.87E-02^{A}	2.57E+02± 5.88E+00^{B}	1.80E-01± 2.07E-02^{AB}	2.68E+00± 6.57E-02^{B}	3.14E-01± 1.15E-02^{AB}	2.24E+00± 1.57E-02^{A}	1.01E+00± 4.97E-02^{BC}	3.39E+01± 7.14E-01^{B}	1.23E+01± 3.33E-01^{A}
BF2020 seq	2.83E+00± 3.36E-01^{AB}	7.46E-01± 6.69E-02^{A}	2.67E+02± 1.21E+01^{B}	1.73E-01± 1.94E-02^{AB}	2.64E+00± 6.81E-02^{B}	3.21E-01± 1.55E-02^{AB}	2.25E+00± 4.73E-02^{A}	8.96E-01± 3.00E-02^{C}	3.35E+01± 2.06E+00^{BC}	1.22E+01± 4.26E-01^{A}
BF2020 Noseq	2.60E+00± 1.58E-01^{B}	7.64E-01± 1.10E-02^{A}	2.88E+02± 8.69E+00^{AB}	1.73E-01± 3.83E-03^{AB}	2.66E+00± 4.71E-02^{B}	3.24E-01± 1.11E-02^{AB}	2.08E+00± 1.81E-02^{BCD}	9.11E-01± 2.12E-02^{C}	3.20E+01± 1.07E+00^{BC}	1.24E+01± 5.38E-01^{A}
L01	2.47E+00± 3.58E-01^{B}	6.19E-01± 6.44E-02^{A}	3.30E+02± 1.27E+01^{A}	1.37E-01± 8.42E-03^{B}	2.75E+00± 6.19E-02^{B}	3.98E-01± 1.09E-02^{A}	2.21E+00± 5.03E-02^{AB}	1.22E+00± 4.72E-02^{A}	4.18E+01± 1.56E+00^{A}	1.07E+01± 4.93E-01^{A}

L02	3.65E+00± 1.98E-01^{A}	7.76E-01± 6.06E-02^{A}	2.96E+02± 1.12E+01^{AB}	1.80E-01± 1.25E-02^{AB}	2.81E+00± 2.44E-02^{B}	3.54E-01± 2.57E-02^{AB}	1.98E+00± 2.93E-02^{CD}	9.15E-01± 2.64E-02^{C}	3.41E+01± 4.50E-01^{B}	1.17E+01± 1.40E-01^{A}
L03	2.92E+00± 2.13E-01^{AB}	7.68E-01± 1.13E-01^{A}	2.93E+02± 6.05E+00^{AB}	1.73E-01± 2.62E-02^{AB}	2.62E+00± 2.33E-02^{B}	3.50E-01± 3.51E-02^{AB}	1.96E+00± 3.70E-02^{D}	9.59E-01± 6.22E-02^{BC}	3.29E+01± 7.94E-01^{BC}	1.14E+01± 6.01E-01^{A}
L04	2.94E+00± 2.37E-01^{AB}	7.61E-01± 1.36E-02^{A}	2.95E+02± 1.93E+01^{AB}	1.69E-01± 9.99E-03^{AB}	2.62E+00± 2.70E-02^{B}	4.52E-01± 8.80E-02^{A}	2.12E+00± 4.38E-02^{ABC}	1.12E+00± 6.64E-02^{AB}	3.60E+01± 1.54E+00^{B}	1.14E+01± 4.92E-01^{A}
L05	2.81E+00± 1.38E-01^{AB}	8.19E-01± 4.11E-02^{A}	2.65E+02± 6.82E+00^{B}	2.12E-01± 3.47E-02^{A}	2.58E+00± 4.25E-02^{B}	3.76E-01± 1.13E-02^{AB}	2.13E+00± 4.26E-02^{AB}	9.57E-01± 8.57E-02^{BC}	3.49E+01± 5.37E-01^{B}	1.22E+01± 1.01E-01^{A}

Strains sharing the same letter are not significantly different at a p<0.05 threshold.

Table S8 | Comparison of major volatiles of Cabernet Sauvignon wines (mg/L)

Yeast	Ethyl acetate	n-Propanol	Ethyl 2-methylbutyrate	Isobutanol	Isoamyl acetate	n-Butanol	Isoamyl alcohol	Ethyl hexanoate	Pentanol	Ethyl lactate	Hexanol
QA23	6.44E+01± 8.27E+00^{A}	5.42E+01± 5.60E+00^{A}	2.06E+00± 2.46E-01^{A}	4.57E+01± 3.96E+00^{AB}	5.67E-01± 1.63E-02^{A}	2.77E+00± 2.51E-01^{A}	3.96E+02± 1.23E+01^{A}	3.21E-01± 1.47E-02^{A}	6.70E-01± 6.62E-03^{AB}	3.40E+01± 2.25E+00^{A}	2.74E+00± 1.78E-01^{A}
Y885	5.38E+01± 7.47E+00^{A}	4.76E+01± 7.34E+00^{A}	1.73E+00± 7.03E-02^{AB}	4.12E+01± 2.15E+00^{B}	5.78E-01± 1.95E-01^{A}	2.57E+00± 1.42E-01^{A}	3.62E+02± 1.18E+01^{AB}	3.39E-01± 1.77E-02^{A}	6.50E-01± 1.85E-02^{AB}	3.20E+01± 5.03E+00^{A}	2.79E+00± 1.70E-01^{A}
BF2020 A	5.52E+01± 6.22E+00^{A}	5.41E+01± 6.63E+00^{A}	1.58E+00± 1.44E-01^{B}	3.79E+01± 4.54E+00^{B}	4.32E-01± 1.26E-01^{A}	3.05E+00± 5.69E-01^{A}	3.12E+02± 3.84E+01^{B}	3.93E-01± 5.98E-02^{A}	8.78E-01± 1.35E-01^{A}	3.33E+01± 6.94E+00^{A}	2.92E+00± 6.08E-01^{A}
BF2020 B	5.85E+01±	5.59E+01±	2.00E+00±	4.60E+01±	5.37E-01±	2.83E+00±	3.69E+02±	3.61E-01±	6.92E-01±	3.52E+01±	2.94E+00±

	1.05E+01^{A}	1.36E+00^{A}	1.16E-01^{AB}	2.83E+00^{AB}	7.07E-02^{A}	2.56E-01^{A}	2.46E+01^{AB}	4.80E-03^{A}	1.69E-02^{AB}	4.71E+00^{A}	4.98E-01^{A}
L01	7.30E+01± 1.17E+01^{A}	6.10E+01± 4.91E+00^{A}	1.96E+00± 1.56E-01^{AB}	5.44E+01± 3.12E+00^{A}	5.41E-01± 6.30E-02^{A}	2.75E+00± 1.89E-01^{A}	3.84E+02± 1.25E+01^{A}	3.08E-01± 1.36E-03^{A}	6.85E-01± 2.29E-02^{AB}	3.76E+01± 2.98E+00^{A}	2.64E+00± 1.08E-01^{A}
L02	5.66E+01± 5.19E+00^{A}	5.58E+01± 3.51E+00^{A}	1.99E+00± 7.28E-03^{AB}	4.78E+01± 6.50E-01^{AB}	5.32E-01± 4.65E-02^{A}	2.82E+00± 1.61E-01^{A}	3.81E+02± 6.55E+00^{A}	3.34E-01± 4.40E-03^{A}	7.70E-01± 1.39E-01^{AB}	3.59E+01± 3.73E+00^{A}	2.85E+00± 1.55E-01^{A}
L03	5.78E+01± 5.99E+00^{A}	5.55E+01± 1.51E+00^{A}	2.00E+00± 3.45E-02^{AB}	4.59E+01± 2.29E+00^{AB}	6.41E-01± 6.67E-02^{A}	2.78E+00± 1.37E-01^{A}	3.67E+02± 1.49E+00^{AB}	3.61E-01± 2.16E-02^{A}	6.62E-01± 3.29E-03^{AB}	3.21E+01± 3.60E+00^{A}	2.94E+00± 2.69E-01^{A}
L04	5.34E+01± 7.10E+00^{A}	4.93E+01± 2.06E+00^{A}	2.18E+00± 9.35E-02^{A}	4.49E+01± 9.83E-01^{AB}	6.08E-01± 5.61E-02^{A}	2.54E+00± 1.35E-01^{A}	3.65E+02± 1.57E+01^{AB}	3.49E-01± 1.20E-02^{A}	6.40E-01± 1.74E-02^{B}	3.12E+01± 5.81E+00^{A}	2.76E+00± 2.34E-01^{A}
L05	5.07E+01± 3.77E+00^{A}	4.97E+01± 3.12E+00^{A}	1.92E+00± 5.20E-02^{AB}	4.25E+01± 2.72E+00^{B}	5.80E-01± 6.70E-02^{A}	2.60E+00± 3.70E-02^{A}	3.72E+02± 4.16E+00^{AB}	3.71E-01± 1.13E-02^{A}	6.71E-01± 2.67E-02^{AB}	3.32E+01± 6.34E+00^{A}	3.07E+00± 1.39E-01^{A}

Strains sharing the same letter are not significantly different at a p<0.05 threshold.

Table S9 | Comparison of major volatiles of Cabernet Sauvignon wines continued (mg/L)

Yeast	3-Ethoxy-1-propanol	Ethyl octanoate	Acetic acid	Propionic acid	Ethyl decanoate	Diethyl succinate	Isovaleric acid	2-Phenylethyl acetate	2-Phenylethanol	Octanoic acid
QA23	4.92E+00± 4.20E-01^{A}	1.05E-01± 2.77E-03^{A}	3.77E+02± 1.50E+01^{A}	1.00E+01± 1.07E+00^{A}	2.46E-01± 1.18E-01^{A}	4.23E-01± 4.56E-02^{A}	5.66E+00± 5.79E-01^{A}	1.12E-01± 6.58E-03^{AB}	8.85E+01± 2.55E+00^{A}	5.98E+00± 6.32E-02^{A}
Y885	4.58E+00±	1.16E-01±	3.46E+02±	9.09E+00±	2.73E-01±	4.47E-01±	5.08E+00±	1.39E-01±	7.90E+01±	5.95E+00±

	9.22E-01^{A}	6.70E-04^{A}	3.08E+01^{A}	8.01E-01^{A}	5.83E-02^{A}	7.86E-02^{A}	1.89E-01^{A}	2.96E-02^{AB}	2.22E+00^{AB}	2.34E-02^{A}
BF2020 A	4.70E+00± 9.33E-01^{A}	1.22E-01± 3.80E-02^{A}	3.60E+02± 6.30E+01^{A}	9.23E+00± 8.71E-01^{A}	3.53E-01± 2.61E-01^{A}	8.79E-01± 0.00E+00^{A}	6.52E+00± 2.18E+00^{A}	1.33E-01± 6.58E-03^{AB}	6.72E+01± 1.19E+01^{B}	6.56E+00± 1.08E+00^{A}
BF2020 B	5.03E+00± 5.41E-02^{A}	1.32E-01± 5.52E-03^{A}	4.08E+02± 1.02E+01^{A}	1.12E+01± 1.62E+00^{A}	4.02E-01± 6.91E-02^{A}	7.48E-01± 3.19E-02^{A}	5.54E+00± 6.74E-01^{A}	1.89E-01± 2.96E-02^{A}	8.24E+01± 6.55E+00^{AB}	6.14E+00± 6.79E-02^{A}
L01	4.94E+00± 1.62E-01^{A}	9.32E-02± 2.32E-03^{A}	4.11E+02± 2.41E+01^{A}	1.05E+01± 2.04E+00^{A}	2.79E-01± 7.72E-02^{A}	6.44E-01± 1.65E-02^{A}	4.96E+00± 2.32E-01^{A}	1.17E-01± 3.08E-02^{AB}	8.56E+01± 2.09E+00^{A}	5.91E+00± 3.46E-01^{A}
L02	4.68E+00± 1.61E-01^{A}	1.07E-01± 1.30E-02^{A}	3.68E+02± 3.60E+01^{A}	8.93E+00± 4.96E-01^{A}	3.57E-01± 3.34E-02^{A}	5.34E-01± 1.45E-01^{A}	5.14E+00± 2.43E-01^{A}	1.27E-01± 3.02E-02^{AB}	8.43E+01± 1.46E+00^{AB}	6.10E+00± 2.45E-01^{A}
L03	4.91E+00± 1.52E-01^{A}	1.25E-01± 1.63E-02^{A}	3.79E+02± 5.30E+00^{A}	1.02E+01± 1.34E+00^{A}	3.72E-01± 5.18E-02^{A}	6.84E-01± 1.28E-01^{A}	5.45E+00± 4.03E-01^{A}	1.02E-01± 3.54E-03^{B}	7.95E+01± 2.16E+00^{AB}	6.09E+00± 2.40E-01^{A}
L04	4.84E+00± 4.36E-01^{A}	1.03E-01± 6.00E-03^{A}	3.85E+02± 2.01E+01^{A}	9.69E+00± 1.18E+00^{A}	3.24E-01± 4.37E-02^{A}	5.03E-01± 1.09E-01^{A}	5.36E+00± 3.16E-01^{A}	1.56E-01± 1.28E-02^{AB}	7.91E+01± 5.19E+00^{AB}	5.88E+00± 4.88E-02^{A}
L05	4.63E+00± 3.39E-01^{A}	1.22E-01± 1.33E-02^{A}	3.83E+02± 5.34E+01^{A}	9.94E+00± 1.04E+00^{A}	3.04E-01± 4.87E-02^{A}	5.56E-01± 6.34E-02^{A}	5.56E+00± 2.60E-01^{A}	1.76E-01± 4.68E-03^{AB}	8.19E+01± 2.30E-01^{AB}	6.01E+00± 1.73E-01^{A}

4 *Strains sharing the same letter are not significantly different at a p<0.05 threshold*

4.6 References

Álvarez, I., Aleixandre, J.L., García, M.J., Lizama, V., 2006. Impact of prefermentative maceration on the phenolic and volatile compounds in Monastrell red wines. Anal. Chim. Acta 563, 109–115. https://doi.org/10.1016/j.aca.2005.10.068

Apolinar-Valiente, R., Romero-Cascales, I., Gómez-Plaza, E., López-Roca, J.M., Ros-García, J.M., 2015. Cell wall compounds of red grapes skins and their grape marcs from three different winemaking techniques. Food Chem. 187, 89–97. https://doi.org/10.1016/j.foodchem.2015.04.042

Bagheri, B., Bauer, F.F., Setati, M.E., 2017. The impact of *Saccharomyces cerevisiae* on a wine yeast consortium in natural and inoculated fermentations. Front. Microbiol. 8, 1–13. https://doi.org/10.3389/fmicb.2017.01988

Barone, E., Ponticello, G., Giaramida, P., Squadrito, M., Fasciana, T., Gandolfo, V., Ardizzone, F., Monteleone, M., Corona, O., Francesca, N., Oliva, D., 2021. Use of *Kluyveromyces marxianus* to increase free monoterpenes and aliphatic esters in white wines. Fermentation 7, 79. https://doi.org/10.3390/fermentation7020079

Berthels, N., Corderootero, R., Bauer, F., Thevelein, J., Pretorius, I., 2004. Discrepancy in glucose and fructose utilisation during fermentation by wine yeast strains. FEMS Yeast Res. 4, 683–689. https://doi.org/10.1016/j.femsyr.2004.02.005

Bonnin, E., Le Goff, A., Körner, R., Vigouroux, J., Roepstorff, P., Thibault, J.-F., 2002. Hydrolysis of pectins with different degrees and patterns of methylation by the endopolygalacturonase of Fusarium moniliforme. Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol. 1596, 83–94. https://doi.org/10.1016/S0167-4838(02)00207-8

Casalone, E., Barberio, C., Cappellini, L., Polsinelli, M., 2005. Characterization of *Saccharomyces cerevisiae* natural populations for pseudohyphal growth and colony morphology. Res. Microbiol. 156, 191–200. https://doi.org/10.1016/j.resmic.2004.09.008

Ceccato-Antonini, S.R., Sudbery, P.E., 2004. Filamentous growth in *Saccharomyces cerevisiae*. Brazilian J. Microbiol. 35, 173–181. https://doi.org/10.1590/S1517-83822004000200001

Chidi, B.S., Bauer, F.F., Rossouw, D., 2018. Organic acid metabolism and the impact of fermentation practices on wine acidity - A review. South African J. Enol. Vitic. 39, 315–329. https://doi.org/10.21548/39-2-3172

Cruz-Guerrero, A., Garcia-Peña, I., Barzana, E., Garcia-Garibay, M., Gomez-Ruiz, L., 1995. *Kluyveromyces marxianus* CDBB-L-278: A wild inulinase hyperproducing strain. J. Ferment. Bioeng. 80, 159–163. https://doi.org/10.1016/0922-338X(95)93212-3

Dairou, V., Sieffermann, J.-M., 2002. A comparison of 14 jams characterized by conventional profile and a quick original method, the flash profile. J. Food Sci. 67, 826–834. https://doi.org/10.1111/j.1365-2621.2002.tb10685.x

De la Torre-González, F.J., Narváez-Zapata, J.A., López-y-López, V.E., Larralde-Corona, C.P., 2016. Ethanol tolerance is decreased by fructose in *Saccharomyces* and non-*Saccharomyces* yeasts. LWT 67, 1–7. https://doi.org/10.1016/j.lwt.2015.11.024

Divol, B., van Rensburg, P., 2007. *PGU1* gene natural deletion is responsible for the absence of endo-polygalacturonase activity in some wine strains of *Saccharomyces cerevisiae*. FEMS Yeast Res. 7, 1328–1339. https://doi.org/10.1111/j.1567-1364.2007.00284.x

Eschstruth, A., Divol, B., 2011. Comparative characterization of endo-polygalacturonase (Pgu1) from *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* under winemaking conditions. Appl. Microbiol. Biotechnol. 91, 623–634. https://doi.org/10.1007/s00253-011-3238-y

Fernández-González, M., Úbeda, J.F., Vasudevan, T.G., Otero, R.R.C., Briones, A.I., 2004. Evaluation of polygalacturonase activity in *Saccharomyces cerevisiae* wine strains. FEMS Microbiol. Lett. 237, 261–266. https://doi.org/10.1016/j.femsle.2004.06.042

Fleet, G.H., Mian, M.A., 1987. The occurrence and growth of yeasts in dairy products. Int. J. Food Microbiol. 4, 145–155. https://doi.org/10.1016/0168-1605(87)90021-3

Fonseca, G.G., Heinzle, E., Wittmann, C., Gombert, A.K., 2008. The yeast *Kluyveromyces marxianus* and its biotechnological potential. Appl. Microbiol. Biotechnol. 79, 339–354. https://doi.org/10.1007/s00253-008-1458-6

Gethins, L., Guneser, O., Demirkol, A., Rea, M.C., Stanton, C., Ross, R.P., Yuceer, Y., Morrissey, J.P., 2015. Influence of carbon and nitrogen source on production of volatile fragrance and flavour metabolites by the yeast *Kluyveromyces marxianus*. Yeast 32, 67–76. https://doi.org/10.1002/yea.3047

Gil-Muñoz, R., Moreno-Pérez, A., Vila-López, R., Fernández-Fernández, J.I., Martínez-Cutillas, A., Gómez-Plaza, E., 2009. Influence of low temperature prefermentative techniques on chromatic and phenolic characteristics of Syrah and Cabernet Sauvignon wines. Eur. Food Res. Technol. 228, 777–788. https://doi.org/10.1007/s00217-008-0989-5

González-Centeno, M.R., Rosselló, C., Simal, S., Garau, M.C., López, F., Femenia, A., 2010. Physico-chemical properties of cell wall materials obtained from ten grape varieties and their byproducts: grape pomaces and stems. LWT - Food Sci. Technol. 43, 1580–1586. https://doi.org/10.1016/j.lwt.2010.06.024

Granek, J.A., Magwene, P.M., 2010. Environmental and genetic determinants of colony morphology in yeast. PLoS Genet. 6, 1000823. https://doi.org/10.1371/journal.pgen.1000823

Ho, V.T.T., Zhao, J., Fleet, G., 2014. Yeasts are essential for cocoa bean fermentation. Int. J. Food Microbiol. 174, 72–87. https://doi.org/10.1016/j.ijfoodmicro.2013.12.014

Hoffman, C.S., 1997. Preparation of yeast DNA, RNA, and proteins. Curr. Protoc. Mol. Biol. 39, 11–14. https://doi.org/10.1002/0471142727.mb1311s39

Kántor, A., Kačániová, M., Kluz, M., 2015. Natural microflora of wine grape berries. J. Microbiol. Biotechnol. Food Sci. 04, 32–36. https://doi.org/10.15414/jmbfs.2015.4.special1.32-36

Kántor, A., Mareček, J., Ivanišová, E., Terentjeva, M., Kačániová, M., 2017. Microorganisms of grape berries. Proc. Latv. Acad. Sci. Sect. B Nat. Exact, Appl. Sci. 71, 502–508. https://doi.org/10.1515/prolas-2017-0087

Kumar, G.R., Ramakrishnan, V., Bisson, L.F., 2010. Survey of hydrogen sulphide production by wine yeasts. Am. J. Enol. Vitic. 61, 365–371.

Labuschagne, P.W.J., Rollero, S., Divol, B., 2021. Comparative uptake of exogenous thiamine and subsequent metabolic footprint in *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* under simulated oenological conditions. Int. J. Food Microbiol. 184, 109206. https://doi.org/10.1016/j.ijfoodmicro.2021.109206

Lachance, M.-A., 1995. Yeast communities in a natural tequila fermentation. Antonie Van Leeuwenhoek 68, 151–160. https://doi.org/10.1007/BF00873100

Lane, M.M., Burke, N., Karreman, R., Wolfe, K.H., O'Byrne, C.P., Morrissey, J.P., 2011. Physiological and metabolic diversity in the yeast *Kluyveromyces marxianus*. Antonie Van Leeuwenhoek 100, 507–519. https://doi.org/10.1007/s10482-011-9606-x

Lane, M.M., Morrissey, J.P., 2010. *Kluyveromyces marxianus*: A yeast emerging from its sister's shadow. Fungal Biol. Rev. 24, 17–26. https://doi.org/10.1016/j.fbr.2010.01.001

Lewis, J.A., Elkon, I.M., McGee, M.A., Higbee, A.J., Gasch, A.P., 2010. Exploiting natural variation in *Saccharomyces cerevisiae* to identify genes for increased ethanol resistance. Genetics 186, 1197–1205. https://doi.org/10.1534/genetics.110.121871

Lilly, M., Lambrechts, M.G., Pretorius, I.S., 2000. Effect of increased yeast alcohol acetyltransferase activity on flavor

profiles of wine and distillates. Appl. Environ. Microbiol. 66, 744–753. https://doi.org/10.1128/AEM.66.2.744-753.2000

Loira, I., Vejarano, R., Bañuelos, M.A., Morata, A., Tesfaye, W., Uthurry, C., Villa, A., Cintora, I., Suárez-Lepe, J.A., 2014. Influence of sequential fermentation with *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* on wine quality. LWT - Food Sci. Technol. 59, 915–922. https://doi.org/10.1016/j.lwt.2014.06.019

Longo, E., Cansado, J., Agrelo, D., Villa, T.G., 1991. Effect of climatic conditions on yeast diversity in grape musts from Northwest Spain. Am. J. Enol. Vitic. 42, 141–144.

Louw, C., Young, P.R., van Rensburg, P., Divol, B., 2010. Epigenetic regulation of PGU1 transcription in *Saccharomyces cerevisiae.* FEMS Yeast Res. 10, 158–167. https://doi.org/10.1111/j.1567-1364.2009.00599.x

Louw, L., Roux, K., Tredoux, A., Tomic, O., Naes, T., Nieuwoudt, H.H., Van Rensburg, P., 2009. Characterization of selected South African young cultivar wines using FTMIR Spectroscopy, Gas chromatography, and multivariate data analysis. J. Agric. Food Chem. 57, 2623–2632. https://doi.org/10.1021/jf8037456

Louw, M., du Toit, M., Alexandre, H., Divol, B., 2016. Comparative morphological characteristics of three *Brettanomyces bruxellensis* wine strains in the presence/absence of sulfur dioxide. Int. J. Food Microbiol. 238, 79–88. https://doi.org/10.1016/j.ijfoodmicro.2016.08.040

Merico, A., Galafassi, S., Piškur, J., Compagno, C., 2009. The oxygen level determines the fermentation pattern in *Kluyveromyces lactis*. FEMS Yeast Res. 9, 749–756. https://doi.org/10.1111/j.1567-1364.2009.00528.x

Nanadoum, M., Mbailao, M.H.V.N., Claude, G., Jacques, P., 2005. Identification and typing of the yeast strains isolated from bili bili, a traditional sorghum beer of Chad. African J. Biotechnol. 4, 646–656. https://doi.org/10.5897/AJB2005.000-3117

Nicaud, J.-M., 2012. Yarrowia lipolytica. Yeast 29, 409–418. https://doi.org/10.1002/yea.2921

O'Shea, D.G., Walsh, P.K., 2000. The effect of culture conditions on the morphology of the dimorphic yeast *Kluyveromyces marxianus* var. marxianus NRRLy2415: a study incorporating image analysis. Appl. Microbiol. Biotechnol. 53, 316–322. https://doi.org/10.1007/s002530050027

O'Shea, D.G., Walsh, P.K., 1996. Morphological characterization of the dimorphic yeast *Kluyveromyces marxianus* var. marxianus NRRLy2415 by semi-automated image analysis. Biotechnol. Bioeng. 51, 679–690. https://doi.org/10.1002/(SICI)1097-0290(19960920)51:6<679::AID-BIT6>3.0.CO;2-E

Ortega-Regules, A., Ros-García, J.M., Bautista-Ortín, A.B., López-Roca, J.M., Gómez-Plaza, E., 2008. Differences in morphology and composition of skin and pulp cell walls from grapes (Vitis vinifera L.): technological implications. Eur. Food Res. Technol. 227, 223–231. https://doi.org/10.1007/s00217-007-0714-9

Ortiz-Merino, R.A., Varela, J.A., Coughlan, A.Y., Hoshida, H., da Silveira, W.B., Wilde, C., Kuijpers, N.G.A., Geertman, J.-M., Wolfe, K.H., Morrissey, J.P., 2018. Ploidy Variation in *Kluyveromyces marxianus* Separates Dairy and Non-dairy Isolates. Front. Genet. 9, 94. https://doi.org/10.3389/fgene.2018.00094

Perpetuini, G., Tittarelli, F., Suzzi, G., Tofalo, R., 2019. Cell wall surface properties of *Kluyveromyces marxianus* strains from dairy-products. Front. Microbiol. 10, 1–8. https://doi.org/10.3389/fmicb.2019.00079

Porter, T.J., Divol, B., Setati, M.E., 2019. Investigating the biochemical and fermentation attributes of *Lachancea* species and strains: Deciphering the potential contribution to wine chemical composition. Int. J. Food Microbiol. 290, 273–287. https://doi.org/10.1016/j.ijfoodmicro.2018.10.025

Rapp, A., Mandery, H., 1986. Wine aroma. Experientia 42, 873–884. https://doi.org/10.1007/BF01941764

Reis, V.R., Bassi, A.P.G., Silva, J.C.G. da, Ceccato-Antonini, S.R., 2013. Characteristics of *Saccharomyces cerevisiae* yeasts exhibiting rough colonies and pseudohyphal morphology with respect to alcoholic fermentation. Brazilian

J. Microbiol. 44, 1121–1131. https://doi.org/10.1590/S1517-83822014005000020

Rollero, S., Bloem, A., Brand, J., Ortiz-Julien, A., Camarasa, C., Divol, B., 2021. Nitrogen metabolism in three non-conventional wine yeast species: A tool to modulate wine aroma profiles. Food Microbiol. 94, 103650. https://doi.org/10.1016/j.fm.2020.103650

Rollero, S., Bloem, A., Ortiz-Julien, A., Bauer, F.F., Camarasa, C., Divol, B., 2019. A comparison of the nitrogen metabolic networks of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*. Environ. Microbiol. 21, 4076–4091. https://doi.org/10.1111/1462-2920.14756

Rollero, S., Bloem, A., Ortiz-Julien, A., Camarasa, C., Divol, B., 2018a. Fermentation performances and aroma production of non-conventional wine yeasts are influenced by nitrogen preferences. FEMS Yeast Res. 18, 1–11. https://doi.org/10.1093/femsyr/foy055

Rollero, S., Bloem, A., Ortiz-Julien, A., Camarasa, C., Divol, B., 2018b. Altered fermentation performances, growth, and metabolic footprints reveal competition for nutrients between yeast species inoculated in synthetic grape juice-like medium. Front. Microbiol. 9, 1–12. https://doi.org/10.3389/fmicb.2018.00196

Rollero, S., Zietsman, A.J.J., Buffetto, F., Schückel, J., Ortiz-Julien, A., Divol, B., 2018c. *Kluyveromyces marxianus* secretes a pectinase in shiraz grape must that Impacts technological properties and aroma profile of wine. J. Agric. Food Chem. 66, 11739–11747. https://doi.org/10.1021/acs.jafc.8b03977

Schwan, R., Silva, C., Batista, L., 2012. Coffee Fermentation, in: Handbook of plant-based fermented food and beverage technology, Second Edition. CRC Press, pp. 677–690. https://doi.org/10.1201/b12055-49

Serrat, M., Bermúdez, R.C., Villa, T.G., 2002. Production, purification, and characterization of a polygalacturonase from a new strain of *Kluyveromyces marxianus* Isolated from coffee wet-processing wastewater. Appl. Biochem. Biotechnol. 97, 193–208. https://doi.org/10.1385/ABAB:97:3:193

Serrat, M., Rodríguez, O., Camacho, M., Vallejo, J.A., Ageitos, J.M., Villa, T.G., 2011. Influence of nutritional and environmental factors on ethanol and endopolygalacturonase co-production by *Kluyveromyces marxianus* CCEBI 2011. Int. Microbiol. 14, 41–49. https://doi.org/10.2436/20.1501.01.134

Shen, B., Hohmann, S., Jensen, R.G., Bohnert, a H., 1999. Roles of sugar alcohols in osmotic stress adaptation. Replacement of glycerol by mannitol and sorbitol in yeast. Plant Physiol. 121, 45–52. https://doi.org/10.1104/pp.121.1.45

Sieiro, C., Sestelo, A.B.F., Villa, T.G., 2009. Cloning, characterization, and functional analysis of the EPG1-2 Gene: A new allele coding for an endopolygalacturonase in *Kluyveromyces marxianus*. J. Agric. Food Chem. 57, 8921–8926. https://doi.org/10.1021/jf900352q

Swiegers, J.H., Pretorius, I.S., 2005. Yeast modulation of wine flavor, in: Advances in Applied Microbiology. pp. 131–175. https://doi.org/10.1016/S0065-2164(05)57005-9

Tao, Y., Zhang, L., 2010. Intensity prediction of typical aroma characters of cabernet sauvignon wine in Changli County (China). LWT - Food Sci. Technol. 43, 1550–1556. https://doi.org/10.1016/j.lwt.2010.06.003

Thurston, P.A., Quain, D.E., Tubb, R.S., 1982. Lipid metabolism and the regulation of volatile ester synthesis in *Saccharomyces cerevisiae*. J. Inst. Brew. 88, 90–94. https://doi.org/10.1002/j.2050-0416.1982.tb04078.x

Tofalo, R., Fasoli, G., Schirone, M., Perpetuini, G., Pepe, A., Corsetti, A., Suzzi, G., 2014. The predominance, biodiversity and biotechnological properties of *Kluyveromyces marxianus* in the production of Pecorino di Farindola cheese. Int. J. Food Microbiol. 187, 41–49. https://doi.org/10.1016/j.ijfoodmicro.2014.06.029

van der Walt, J.P., Yarrow, D., 1984. Methods for the isolation, maintenance, classification and identification of yeasts, in: The Yeasts. Elsevier, pp. 45–104. https://doi.org/10.1016/B978-0-444-80421-1.50009-7

Varela, J.A., Gethins, L., Stanton, C., Ross, P., Morrissey, J.P., 2017. Applications of *Kluyveromyces marxianus* in biotechnology, in: Yeast diversity in human welfare. Springer Singapore, Singapore, pp. 439–453. https://doi.org/10.1007/978-981-10-2621-8_17

Williams, D.L., Schückel, J., Vivier, M.A., Buffetto, F., Zietsman, A.J.J., 2019. Grape pomace fermentation and cell wall degradation by *Kluyveromyces marxianus* Y885. Biochem. Eng. J. 150, 107282. https://doi.org/10.1016/j.bej.2019.107282

Zimmer, A., Durand, C., Loira, N., Durrens, P., Sherman, D.J., Marullo, P., 2014. QTL dissection of lag phase in wine fermentation reveals a new translocation responsible for *Saccharomyces cerevisiae* adaptation to sulfite. PLoS One 9, 37–39. https://doi.org/10.1371/journal.pone.0086298

Chapter 5 – General discussion and conclusions

5.1 General discussion

Finding new and improved yeasts is a constant endeavour in the biotechnology and food industries. This often aims to improve the yeasts ability to survive in harsh industrial conditions (such as wine related temperatures and ethanol tolerance), improve their abilities to produce specific metabolites of interest (industrially relevant enzymes) or to perform specific functions more effectively (e.g. ferment or produce biomass). The availability of new yeasts is often dependent on native strain variation and the ability to generate improved yeasts through methods such as hybridisation or genetic modification. In this study, six indigenous *K. marxianus* strains isolated from wine, dairy, baking and distillery environments were investigated for their oenological attributes. One fusant recently obtained by protoplast fusion at the South African Grape and Wine Research Institute (unpublished) was also added to the study. Phenotypical similarities and differences were observed in all strains, regardless of their original source of isolation, as summarised in Table 5.1 where the different strains are compared to IWBT Y885, the only strain of the study originating from the broader wine environment.

Table 5.1 | General comparison of strain Y885 with the other *K. marxianus* strains investigated in this study under laboratory conditions

Characteristic investigated	BF2020	L01	L02	L03	L04	L05
Polygalacturonase activity	-	^	-	^	^	v
H_2S production	-	-	-	^	^	^
Fermentation kinetics	v	-	^	^	^	^
Lag phase	v	v	-	-	-	-
Time taken to reach stationary phase	^	^	^	^	^	^
Vmax	v	v	-	-	-	-
Total sugars consumed	v	v	v	^	v	v
Average ethanol production		-			-	-
Molar ethanol yield	-	-	-	-	-	-
Molar biomass yield						-
Glycerol production	^	^	^	^	^	v
Molar major volatile production						
Isoamyl acetate	-	-	-			-
Propionic acid	-	-	-			-
3-ethoxy-1-propanol						
Ethyl acetate	-	-	-		-	-
Butanol	-				-	-
Isobutyric acid						
2-Phenylethyl acetate						
2-Phenylethanol		-		-		
Isoamyl alcohol	-					-
Isobutanol		-	-	-	-	
Acetic acid					-	-

Total characteristics where strains are similar	8/22	9/22	9/22	5/22	8/22	11/22
Characteristics where Y885 is more suited for oenologically relevant conditions	2/8	3/8	3/8	6/8	5/8	3/8
Characteristics where Y885 is less suited for oenologically relevant conditions	4/8	3/8	1/8	0/8	1/8	3/8

Statistical variation between strains with grey (-) strains represented as being statistically similar, green (^) strains represented as statistically less suited than Y885 and red (ᵛ) strains statistically more suited than Y885. For ethanol, biomass and major volatile production no colours were allocated as producing more or less cannot definitively be said to be more or less suited for use in the wine industry. Grey strains are not significantly different at a $p<0.05$ threshold.

Of the characteristics investigated, large degrees of variation can be seen when comparing strains BF2020 and L01 through L05 to strain Y885 (Table 5.1). Other than strain L05, all strains displayed significant differences in more than half of the characteristics investigated. This also extends to BF2020 which is likely to share a large amount of genetic material with its parental strain Y885. Despite strain L05 displaying the most similarities and grouping together during the cluster analysis (Figure 3.5), strain L05 and Y885 still located relatively far apart on the PCA plot in Figure 3.4. This could be explained by the fact that when variations occurred in the production of major volatiles, these often occurred to large extents with for example strain Y885 producing more than 2.5 times more 2-phenylethyl acetate than strain L05, thereby confirming the high production of 2-phenylethyl acetate by Y885 reported in previous studies (Rollero et al., 2018b, 2018c). Overall, strain L03 displayed the most differences compared to strain Y885 with only 5 similar parameters between strains, despite having the most similar morphological characteristics when grown on and in YPD as well as SGM, indicating that morphological characteristics are poor indications of strain variation with regards to oenological characteristics.

One of the underlying aims of this study was to investigate whether Y885, isolated from a wine environment, displayed increased potential to be used in the wine industry over strains isolated from non-wine environments. Despite the variations that were found between the strains, all strains still displayed conserved relevant characteristics such as the production of pectinase enzymes, increased glycerol and various major volatiles production. Nevertheless, strain Y885 broadly appeared more suited than strains L02, L03 and L04 for winemaking when investigated under laboratory conditions.

BF2020 was previously generated in an attempt to create a yeast strain that is similar to *K. marxianus* strain Y885, however possessing increased fermentation performance (unpublished results). Despite being significantly more similar to Y885 than QA23, this study confirmed that BF2020 is more suited for oenologically relevant conditions than its parental strain Y885 in 4 characteristics under laboratory conditions. Interestingly, despite BF2020's increased fermentation performance, major volatile production appeared significantly similar between the two strains, thereby confirming their close genetic relatedness. However, despite many of compounds investigated having an impact on wine aroma, their production is

dependent on several factors such as ethanol concentration, fermentation temperature, grape must pH, aeration, level of solids, grape variety, maturity, and skin contact time. Due to this the impact of these strains on during real grape juice fermentations was investigated.

The major volatile data generated from Chenin blanc and Cabernet Sauvignon wines produced using strains Y885, L01 through L05, BF2020 and QA23 yielded contrasting results. While clear separations could be observed regarding *K. marxianus* treatments and the QA23 treatments for Chenin blanc wines, these were much more limited in the Cabernet Sauvignon wines. The lack of impact on Cabernet Sauvignon wines was attributed to the large presence of indigenous yeasts, high degree of methylation found in Cabernet Sauvignon grapes and rapid increase in *S. cerevisiae* populations (González-Centeno et al., 2010; Ortega-Regules et al., 2008). Unfortunately, the different chemical profiles of the Chenin blanc wines did not translate into clear sensory differences.

Despite not yielding a clear sensory difference, the real grape juice experiments still delivered valuable results. Two of the main positive aspects of using strain Y885 are that it displays high pectinase activity and produces high amounts of 2-phenylethyl acetate suggested to contribute floral and fruity aromas (Gethins et al., 2015; Rapp and Mandery, 1986). The SGM experiments showed that pectinase activity between the *K. marxianus* strains did not vary to a large extent and despite Y885 producing the most 2-phenylethyl acetate in SGM experiments, it was not the highest producer in Chenin blanc fermentations. However, all *K. marxianus* treatments still showed increased 2-phenylethyl acetate production compared to the control *S. cerevisiae* treatment. It was also noted that certain negative aspects identified under laboratory conditions such as high H_2S production by some strains and the generally high acetic acid production did not occur in real grape juice experiments. From this study, it can be concluded that regarding pectinase activity and the production of 2-phenylethyl acetate in Chenin blanc wines, strain Y885 did not stand out amongst the strains investigated.

5.2 Limitations and future research prospects

The fact that the genomes of strains L01 through L05 have been sequenced (Ortiz-Merino et al., 2018) was one of the reasons that guided their selection for this study, to allow for a potential follow-up study in which phenotypic differences could be investigated at a genomic level. It would be of interest to sequence the genome of Y885 (currently underway at the time of writing) in order to perform a comparative genetic study. Similarly, investigating the genetic differences between the newly produced strain BF2020 and Y885 (also underway at the time of writing) could provide insights into the differences observed in fermentation performance.

As evidenced in the current study, working with real grape juice can often be challenging and requires careful planning. It would therefore be of interest to repeat the above real grape juice experiments using methods that would enhance the survival of *K. marxianus*. Indeed, despite seeing an impact on the major volatiles produced for Chenin blanc wines, *K. marxianus'* contribution towards the wines' sensory profiles remained limited, probably as a result of its low survival rate after 134 h. Similarly, a longer survival of the *K. marxianus* strains investigated for Cabernet Sauvignon could limit the impact of the indigenous populations. The commercially recommended inoculation concentration is typically 1×10^7 cells/mL for non-*Saccharomyces* yeasts, however due to practical constrains, the current study used 1×10^6 cells/mL, as performed in previous studies (Rollero et al., 2018c). It would therefore be of benefit to increase the inoculation concentration of the yeasts used. Similarly, determining the best inoculation method when preculturing in YPD could allow for a more successful inoculation process. Despite the success reported by Rollero et al. (2018c), delaying the sequential inoculation of *S. cerevisiae* for experiments including a PCM step could enhance the survival of *K. marxianus* by decreasing early competition with *S. cerevisiae.* Furthermore, *K. marxianus* has been shown to have a higher requirement for thiamine in order to ensure effective fermentation performance (Labuschagne et al., 2021). Determining the grape juice thiamine concentration and subsequent supplementation of thiamine could therefore be considered to induce a longer survival of *K. marxianus.*

Although the limited impact of *K. marxianus* on Cabernet Sauvignon wines partially attributed to be due to a low presence of *K. marxianus* during fermentation, the choice of grape cultivar likely also played a crucial role. The contrasting results generated in the current study and those obtained when using Shiraz grapes (Rollero et al., 2018c) was in part hypothesized to be due to cultivar differences in pectin concentration and/or structure. It would therefore be of interest to repeat the current study using multiple cultivars to assess the extent of cultivar impacts on the traits investigated. Linked to differences between grapes and initial grape juice composition would be the nitrogen concentrations. It would be of interest to quantify specifically the phenylalanine concentrations at the start of fermentation in future studies as this has been shown to be directly linked to the production of phenylethanol and 2-phenylethyl acetate and could likely influence the impact *K. marxianus* would have on the sensory profile (Rollero et al., 2019).

As this study focused on the impact of a specific yeast on the sensory profile and chemical composition of wines, it was important to determine the relative population dynamics to be able to determine whether indigenous yeasts may have impacted the study. In order to confirm the presence of the strains investigated at the time of sequential inoculation and the end of fermentation PCR-ARISA was utilized. Although this provided a fair overview of the yeast diversity, increasing the number of sampling points would provide a better understanding of the population dynamics and potentially yeast interactions throughout fermentations. As mentioned in Chapter 4, a large diversity was observed regarding the indigenous yeasts present for both Chenin blanc and Cabernet Sauvignon fermentations. It would be of

interest to identify a number of these yeasts, specifically those that appeared to have a high presence at the end of fermentation such as the “800bp” yeast in Chenin blanc fermentations. Prior to ARISA, the DNA required was unsuccessfully extracted using rapid DNA extraction methods and ultimately required a commercial DNA extraction and purification kit to be successful. In order to increase the number of sampling points for ARISA, it would be of interest to determine exactly why the rapid DNA extraction methods does not work and how to improve this to allow for extractions without utilizing costly commercial kits.

Despite the presence of various indigenous yeasts, a clear impact was seen by *K. marxianus* on the chemical composition of Chenin blanc wines. Due to the large amount of samples, a flash profile sensory approach was used. One of the most important limitations regarding the sensory evaluations was the overshadowing of attributes such as green, chalky and fizzy. The aromas and factors that were aimed to be investigated and impacted by *K. marxianus* were not focused on by the sensory panelists. This likely happened as the sensory tests used allowed the panelists to use their own descriptors and were not given any specifications regarding the attributes they were to compare. This overshadowing of lesser impacted attributes means that if no correlation was found, it would be either because there was no correlation or because the differences that did occur were not focused on. It would therefore be of benefit for future similar studies to consider guiding the sensory panel with regard to what attributes should be focused on chemical data produced. Alternatively, a more thorough descriptive analysis could be performed. However, this would require more time and subsequently be more costly. As in flash profile sensory tests, trained panelists would be used, however instead of ranking the wines against each other, each wine would be investigated individually. In this case, the panelists would be presented with a list of attributes and be asked to determine the intensity of the attribute in the wine such as detectable, weak, moderate, strong, very strong or strongest imaginable (Ristic et al., 2007). This would allow for a more controlled sensory analysis without the influence of overshadowing aroma attributes such as seen in the current study.

Finally, a major limiting factor in the current study was the number of yeasts compared. Although strains isolated from dairy, baking, distillery, and wine environments were compared, it mostly consisted of one strain from each source (except for dairy). It would be of interest to confirm the findings in this study using more strains as well as specifically to compare strain Y885 to *K. marxianus* strains that are also isolated from winemaking environments.

5.3 References

Gethins, L., Guneser, O., Demirkol, A., Rea, M.C., Stanton, C., Ross, R.P., Yuceer, Y., Morrissey, J.P., 2015. Influence of carbon and nitrogen source on production of volatile fragrance and flavour metabolites by the yeast *Kluyveromyces marxianus*. Yeast 32, 67–76. https://doi.org/10.1002/yea.3047

González-Centeno, M.R., Rosselló, C., Simal, S., Garau, M.C., López, F., Femenia, A., 2010. Physico-chemical properties of cell wall materials obtained from ten grape varieties and their byproducts: grape pomaces and stems. LWT -

Food Sci. Technol. 43, 1580–1586. https://doi.org/10.1016/j.lwt.2010.06.024

Labuschagne, P.W.J., Rollero, S., Divol, B., 2021. Comparative uptake of exogenous thiamine and subsequent metabolic footprint in *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* under simulated oenological conditions. Int. J. Food Microbiol. 184, 109206. https://doi.org/10.1016/j.ijfoodmicro.2021.109206

Ortega-Regules, A., Ros-García, J.M., Bautista-Ortín, A.B., López-Roca, J.M., Gómez-Plaza, E., 2008. Differences in morphology and composition of skin and pulp cell walls from grapes (Vitis vinifera L.): technological implications. Eur. Food Res. Technol. 227, 223–231. https://doi.org/10.1007/s00217-007-0714-9

Ortiz-Merino, R.A., Varela, J.A., Coughlan, A.Y., Hoshida, H., da Silveira, W.B., Wilde, C., Kuijpers, N.G.A., Geertman, J.-M., Wolfe, K.H., Morrissey, J.P., 2018. Ploidy variation in *Kluyveromyces marxianus* separates dairy and non-dairy isolates. Front. Genet. 9, 94. https://doi.org/10.3389/fgene.2018.00094

Rapp, A., Mandery, H., 1986. Wine aroma. Experientia 42, 873–884. https://doi.org/10.1007/BF01941764

Ristic, R., Downey, M.O., Iland, P.G., Bindon, K., Francis, I.L., Herderich, M., Robinson, S.P., 2007. Exclusion of sunlight from Shiraz grapes alters wine colour, tannin and sensory properties. Aust. J. Grape Wine Res. 13, 53–65. https://doi.org/10.1111/j.1755-0238.2007.tb00235.x

Rollero, S., Bloem, A., Ortiz-Julien, A., Bauer, F.F., Camarasa, C., Divol, B., 2019. A comparison of the nitrogen metabolic networks of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*. Environ. Microbiol. 21, 4076–4091. https://doi.org/10.1111/1462-2920.14756

Rollero, S., Bloem, A., Ortiz-Julien, A., Camarasa, C., Divol, B., 2018a. Altered fermentation performances, growth, and metabolic footprints reveal competition for nutrients between yeast species inoculated in synthetic grape juice-like medium. Front. Microbiol. 9, 1–12. https://doi.org/10.3389/fmicb.2018.00196

Rollero, S., Zietsman, A.J.J., Buffetto, F., Schückel, J., Ortiz-Julien, A., Divol, B., 2018b. *Kluyveromyces marxianus* secretes a pectinase in Shiraz grape must that impacts technological properties and aroma profile of wine. J. Agric. Food Chem. 66, 11739–11747. https://doi.org/10.1021/acs.jafc.8b03977