NEW PERSPECTIVES ON MALOLACTIC FERMENTATION AND ORGANOLEPTIC IMPROVEMENT OF WINES: INFLUENCE OF TORULASPORA DELBRUECKII ON DIFERENT TYPES OF WINEMAKING

Candela Ruiz de Villa Sardón

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ROVIRA i VIRGILI

New perspectives on malolactic fermentation and organoleptic improvement of wines: Influence of *Torulaspora delbrueckii* on different types of winemaking

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CANDELA RUIZ DE VILLA SARDÓN

DOCTORAL THESIS
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New perspectives on malolactic fermentation and organoleptic improvement of wines: Influence of *Torulaspora delbrueckii* on different types of winemaking

Doctoral Thesis

Supervised by Dr. Nicolas Rozès and
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Dr. Nicolas Rozès and Dra. Cristina Reguant Miranda CERTIFY,

that the present study, entitled "New perspectives on malolactic fermentation and organoleptic improvement of wines: Influence of *Torulaspora delbrueckii* on different types of winemaking", presented by Candela Ruiz de Villa for the award of the degree of Doctor, has been carried out under our supervision at the Department of Biochemistry and Biotechnology of Universitat Rovira i Virgili.

This thesis is eligible to apply for the Degree of Doctor with International Mention.

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Doctoral Thesis Supervisors

Dr. Nicolas Rozès

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"El que sabe dónde va,
Va despacio,
Para paladear
El *ir llegando*"

- Gloria Fuertes -

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A mi madre,
a mi abuela María

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INTRODUCTION

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Wine, originating in Georgia in the South Caucasus region, dating to the early Neolithic period (ca. 6000–5000 BC) has evolved into one of the most widely produced and consumed fermented beverages in modern times (McGovern et al., 2017). Italy holds the leading position as the main wine-producing country, closely followed by France, Spain, and the United States. However, countries in the southern hemisphere, such as Chile, Argentina, Australia, and South Africa, are gaining significance in terms of wine production. In 2022, the global wine trade reached a volume of 106 MHL, demonstrating a growing trend in trade value (OIV, 2022).

Fermentation processes have been studied historically since the discovery of alcoholic fermentation (AF) by Antoine Lavoisier (Chambers and Pretorius, 2010). The expansion of the wine industry has helped these studies to develop exponentially. In this context, scientists and winemakers are exploring innovative approaches to enhance wine production. Currently, one of the main research areas focuses on enhancing the fermentation process through the application of biotechnological tools applicable to the different vinification methods. By focusing on achieving better microbiological control, wine production is progressively improving, thereby ensuring the production of high-quality wines.

1. Grape berry and grape must composition

Producing high-quality wine relies on a crucial factor: the cultivation of high-quality grapes. To achieve this goal, favourable climate conditions and meticulous viticulture techniques are essential. The main species of grapevine cultivated in the wine industry is *Vitis vinifera*. However, there is an increasing focus on studying other *Vitis* species, such as American and Asian *Vitis*, for their interesting properties like disease and drought resistance or tolerance, with the aim of genetically enhancing grapevines (Buonassisi et al., 2017). In addition to wine production, grapevines are also commercially cultivated for table grapes and raisins, highlighting the significant importance of grapevine cultivation.

Grapevines are caducifolious plants that perform three essential functions: (i) the development of branches and roots, (ii) the formation of inflorescences and subsequent grape berries growth, and (iii) the storage of reserves for sprouting in the following year, following a period of winter dormancy. Achieving a balance between the distribution of reserves for vegetative growth and

berry development (reproductive cycle) is a key aspect of viticulture. However, the different phases of this cycle vary depending on the grape cultivar. Over the years, an extensive range of grape cultivars has been developed through breeding and mutations. Many of these cultivars originated in Europe and have since spread to different grape production regions, where they have further evolved into various cultivars and sub-cultivars. Therefore, oenologists and viticulturists face the challenge of acquiring in-depth knowledge about the different cultivars, in relation to both viticultural and oenological behaviour. Besides, they have to achieve a good management of cultivars to achieve a harmonious balance among grape components and quality. The morphology and composition of the grapes, and their subsequent relationship to the vinification and fermentation processes will be discussed below.

1.1. Morphology of grape berry

Grape berries are clustered together in bunches and connected to the stem by the pedicel, which has conducting vessel for nutrient transport. The pedicel continues to grow until *véraison*, and as it becomes lignified during grape maturation, it accumulates a high concentration of organic acids and phenolic compounds. The grape berry itself is composed of different tissues (Figure 1), the pericarp, which surrounds the seeds, and the exocarp. Among these tissues, the outermost layer, known as the grape berry skin or exocarp, performs multiple functions. Its waxy layer acts as a hydrophobic barrier, protecting the berry from fungal infections and preventing dehydration and physical damage. As we will discuss later, the exocarp tissue, contains polyphenols and other compounds that contribute to pigmentation, flavour, and aroma (Lecas and Brillouet, 1994). Moreover, this surface is inhabited by a diverse community of microorganisms, including aerobic fungi, yeast, acetic acid bacteria (AAB), and lactic acid bacteria (LAB). This microbiota will arrive to the winery along with grapes during harvest. However, under winemaking conditions, most of these microorganisms do not persist or develop, leaving room only for fermentative microorganisms (Bisson and Walker, 2015). Below the grape berry skin, the pericarp consists of the mesocarp and the endocarp, collectively known as the grape flesh. This important part is characterized by large vacuoles that contain the must: a water-based liquid with a very high density due to the abundance of solutes, primarily sugars, organic acids, nitrogen compounds and phenolic compounds (Keller, 2015). Figure 1 illustrates the distribution of these components in the

different parts of the grape. Finally, the grape seeds are located within the endocarp. The seeds consist of water, carbohydrate compounds, lipids, nitrogenous substances, minerals and tannins.

Furthermore, it is worth nothing to highlight that the composition of the different parts of the grape berry is influenced by the level of maturity and the grape cultivar.

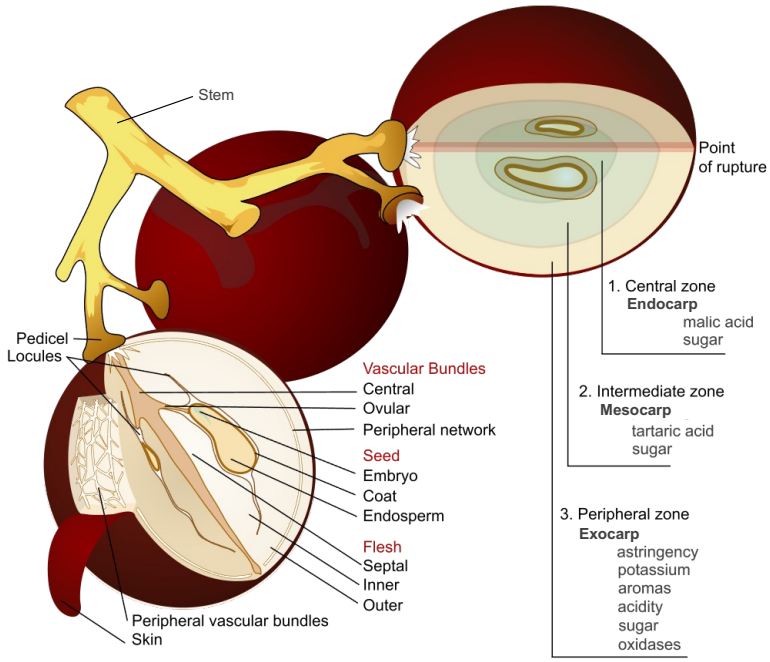

Figure 1. Basic grape berry structure

1.2. Sugars

During the second phase of growth, when ripening starts, grape berries begin to accumulate sugars, specifically fructose and glucose, primarily derived from the photosynthesis process (Figure 2). These sugars are stored in the vacuoles and serve as an essential source for berry development (Hidalgo Togores, 2010). At the optimal point of maturity, grape berries typically contain between 150 to 250 g/L of glucose and fructose, in an almost equal proportion (Ribéreau-Gayon et al., 2006b). However, the concentration of sugars, much like acidity, is also impacted by climate change, resulting in must with higher sugar content that produce wines with higher ethanol content (Mira de Orduña, 2010). Musts with high sugar

content can have significant microbiological consequences, inducing stress responses in yeast. This may result in an increase in the production of undesirable metabolites during fermentation, such as acetic acid, as a stress response mechanism. Moreover, the high osmotic pressure in these musts can lead to stuck of fermentations (García et al., 2016).

Both sugars and acids play a fundamental role in determining the ideal harvesting time, requiring a delicate balance between them. This critical point is known as technological maturity.

1.3. Organic acids

During the initial phase of berry growth, water and organic acids accumulate in grape flesh (Figure 2). The acidity of grape berries and, consequently, grape must, is primarily composed of tartaric acid (L-tartaric acid enantiomer), malic acid (L-malic acid enantiomer) and citric acid in smaller concentration (Hidalgo Fernández-Cano, 2011).

Additionally, due to microorganism metabolism, grapes contain other organic acids in small amounts, such as succinic acid resulting from yeast metabolism and lactic acid produced by LAB in grapes (Volschenk et al., 2006). Each acid contributes different to the overall sensory characteristics, with tartaric and citric acid imparting freshness sensations, while malic acid can be perceived as harsh, although their impact varies with concentration (Vicente et al., 2022).

After *véraison*, the concentration of the main acids in grapes decrease, reaching concentrations of approximately 10 to 3.5 g/L for tartaric acid and 5 to 1 g/L for L-malic acid. However, their concentrations are influenced by climatic conditions, such as precipitation and temperature. These external factors are especially problematic for L-malic acid. Although tartaric acid is generally present in grape must at higher concentrations than L-malic acid, the latter is actually the stronger acid. Consequently, the variation in its concentration can pose challenges for winemakers in certain types of vinification (Volschenk et al., 2006). For instance, some cool Atlantic regions may struggle with high L-malic acid concentrations, which could reach levels above 6 g/L (Vicente et al., 2022) or even up to 15 g/L (Gallander, 1977). Conversely, in warm regions affected by climate change, wine acidity is decreasing, also in terms of malic acid concentration, leading to imbalanced wines.

Beyond sensory effects, acidity and pH values outside acceptable concentrations can cause technical problems in the metabolism of certain microorganisms, thus affecting AF and malolactic fermentation (MLF). In response, winemakers may employ acidification and deacidification techniques to achieve a suitable acidity balance, employing methods ranging from physical to biological approaches (Ferreira and Mendes-Faia, 2020; Vicente et al., 2022).

1.4. Phenolic compounds

Phenolic compounds, also known as polyphenols, are secondary metabolites of the grapevine (Figure 2). These compounds consist of a phenol group, which means at least a benzyl core attached to a hydroxyl group. They are formed through the condensation of erythrose 4-phosphate, an intermediate product of the pentose phosphate cycle, with 2-phosphoenolpyruvate. This biosynthetic process, referred to as the shikimic acid pathway, results in the synthesis of benzoic and cinnamic acids, along with aromatic amino acids such as phenylalanine and tyrosine.

Within these metabolic routes, the enzyme phenylalanine ammonia-lyase plays an important role. This enzyme directs phenylalanine away from protein synthesis channelling it towards the production of trans-cinnamic acid and other phenolic compounds.

Structurally, they can be classified into two main groups: non-flavonoid and flavonoid compounds. Non-flavonoid compounds include phenolic acids, such as hydroxycinnamic and benzoic acids, and stilbenes, such as resveratrol. These compounds are predominantly located in the flesh of grapes, but also present at low concentration in the skin. On the other hand, flavonoid compounds include flavonols, anthocyanins, and tannins, which are mainly found in the skin and seeds, but also in the flesh of grapes (Teixeira et al., 2013). Among these, flavonols responsible for the yellow colour in wine, are present in relatively low concentrations, typically ranging from 10 to 300 mg/kg, depending on the grape variety (Monagas et al., 2005). However, tannins are the phenols with higher concentrations in grape berries and are mainly responsible for the astringency of wine. Tannins are categorized into three groups based on their structure: Hydrolysable tannins (gallotanins and ellagitannins) from oenological additives and/or oak barrels and condensed tannins from the grape. The latter are formed by polymers and oligomers of flavan-3-oles, which can also be found in their

monomeric form. The primary monomeric flavan-3-ols in grapes include catechin, epicatechin, epigallocatechin, and epicatechin gallate (Coombe, 1992; Hidalgo Fernández-Cano, 2011; Monagas et al., 2005).

Furthermore, anthocyanins are essential phenolic compounds responsible for the colour of the grape skin and, consequently, the wine. However, certain grape cultivars known as "red grape flesh" or "*teinturier*" contains anthocyanins in both the flesh and skins (Santiago et al., 2008). In *Vitis vinifera*, five anthocyanins 3-O-glucoside have been identified: cyanidin, delphinidin, peonidin, petunidin, and malvidin, with malvidin-3-O-glucoside being the most abundant. Besides, glucose can also be acylated at the 6-position with acetic acid, p-coumaric acid, and caffeic acid (Hidalgo Fernández-Cano, 2011; Monagas et al., 2005).

The evolution of phenolic compounds during grape berry ripening is a complex process influenced by numerous factors. Typically, their concentration increases as the grapes mature, reaching a peak and subsequently decreasing at the time of ripeness (Ribéreau-Gayon, 2006a). This dynamic evolution significantly impacts the overall quality of the resulting wine. Consequently, one of the key challenges for oenologists is to identify the optimal harvesting time, which is related with both the concentration and extractability of phenolic compounds. To obtain high-quality wines it is necessary to synchronize the technological maturity of the grapes with their phenolic maturity, but this is a difficult task as both maturities are affected by the effects of climate change (Gutiérrez-Gamboa et al., 2021; Jones et al., 2005; Mira de Orduña, 2010).

1.5. Aromatic compounds and aroma precursors

During the final stages of grape maturity, varietal aromatic compounds and aroma precursors are accumulated as secondary metabolites in both the grape skin and flesh (Figure 2). These primary aromas, known as varietal aromas, along with fermentative and aging aromas, play a significant role in shaping the overall aroma profile of the resulting wines. The expression of these aromas is not only dependent on the grape cultivar but also influenced by various factors, including soil, climate conditions, and viticultural practices, specifically water supply.

Certain free aromatic compounds, such as terpenes (including monoterpenoids, sesquiterpenoids, and C13 norisoprenoids), some methoxypyrazines (especially in unripe

grapes), and certain thiols, are present in the grapes. However, the majority of these compounds exist in grapes as odourless precursors, usually glycosylated for terpenes, or cysteinylated or glutathionylated for volatile thiols (Hjelmeland and Ebeler, 2015; Ribéreau Gayon et al., 2006a). During the vinification process, particularly during AF, a cleavage by β-lyase (specific trait of certain strains of *S. cerevisiae*) of these precursors occurs during AF leading to the release of odorant molecules like aromatic aldehydes, esters, and thiols.

Among different grape cultivars, the Muscat family stands out due to its notably high concentration of terpenes, often over the olfactory perception thresholds. Another distinct characteristic of certain cultivars is the presence of methoxypyrazines, green pepper smell, above the perception threshold, notably found in Sauvignon Blanc, Cabernet Sauvignon, Merlot and Cabernet Franc. Sauvignon Blanc is also renowned for its concentration of specific odoriferous volatile thiols, adding to its unique aromatic profile (Ribéreau Gayon et al., 2006a).

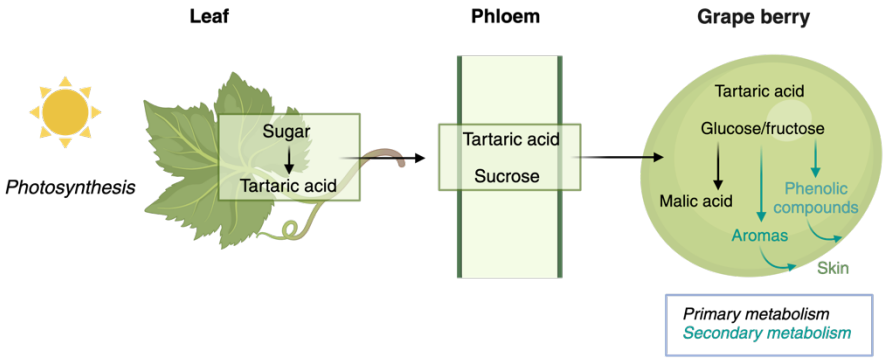

Figure 2. Leaf and grape metabolism.

1.6. Nitrogen composition

The grape nitrogen composition is a crucial factor for the successful development of both AF and MLF in winemaking. Total nitrogen content in grape must consists of various forms, including inorganic nitrogen as ammonium salts, as well as organic forms such as peptides, proteins, and amino acids. Typically, grape must contains a variable total nitrogen concentration ranging from 0.1 to 1.0 g N/L, being the optimal concentration for subsequent fermentations between 140 to 400 mg N/L (Ribéreau Gayon et al., 2006b). However, this

concentration can vary widely, both in terms of the overall amount and the proportion of different nitrogen compounds. Various factors influence the nitrogen content, including grape cultivar, level of maturity, viticultural practices, timing and rate of nitrogen application to the vineyard, soil management techniques, and even the presence of *Botrytis cinerea* infection (Bell and Henschke, 2005).

From an oenological perspective, the most interesting fraction of nitrogen is the Yeast Nitrogen Assimilable (YAN) fraction. It comprises ammonium, certain oligopeptides, and amino acids (excluding proline and hydroxyproline), which are the nitrogen forms that yeast can metabolize during fermentation.

The concentration of ammonia decreases during grape ripening, reaching around 10% of the total nitrogen content when the grape is fully ripened. In contrast, amino acids increase in concentration during ripening and are closely related to the application of nitrogen to the vineyard. Correcting the concentration of ammonium in the must is important when it falls below 25 mg/L, or YAN is below 150 mg/L, to ensure optimal yeast performance, as it is a crucial nutrient for their metabolism. However, it is essential to control the addition of ammonia, usually in the form of diammonium phosphate or diammonium sulphate, as excessive amounts can decrease the concentrations of certain odoriferous compounds, such as higher alcohols, esters, and particularly acetates (Ribéreau Gayon et al., 2006b).

On the other hand, the concentration of assimilable amino acids increases during grape ripening, regardless of nitrogen application in the vineyard. They play a critical role in yeast metabolism and are closely related to wine aroma thus limiting the production of hydrogen sulphide and mercaptans by *S. cerevisiae* are closely linked to wine aroma (Rapp and Versini, 1995).

1.7. Lipids

Grapes contain a lipid fraction primarily located in the seeds, with variable percentages also found in the wax of grape skin and in the flesh. The total lipid content depends on grape cultivars, viticulture practices, and fermentation management, with some examples of great lipid concentration such as the Sauvignon blanc cultivar reaching 2.8 g/L (Tumanov et al., 2015).

Most of the lipid fraction is composed of fatty acids (FAs) in free form or esterified into neutral lipids, phospholipids or glycolipids. Unsaturated fatty acids (UFAs), particularly oleic, linoleic, palmitic and linolenic acids, represent the major components of total lipids in grape berries (Le Fur et al., 1994). Among these, linoleic acid (C18:2) is the most abundant in must, with a content ranging from trace amounts to 280 mg/L (Santos et al., 2011; Tumanov et al., 2015).

In addition to FA, grape must also contains certain sterols, such as phytosterols present on the skin and flesh. The primary component is β-sitosterol, accounting for 85-90% of the sterol fraction, while campesterol and stigmasterol make up 5%. Yeasts incorporate these sterols for growth; however, high concentrations can lead to a stuck fermentation (Luparia et al., 2004).

The importance of lipids lies in their intervention in yeast metabolism (Thurston et al., 1981) as it will be described in the later section "2.1.1 Yeast metabolism", making them significant factors in winemaking.

1.8. Polysaccharides

Polysaccharides present in grapes and grape must arise from the breakdown and solubilization of pectic substances found in both the skin and flesh cell walls. The concentration of these polysaccharides fluctuates as grapes progress through different stages of ripeness. Notably, during the *véraison* period, the previously insoluble protopectins, primarily composed of galacturonic acid, become solubilized, leading to an elevated concentration of soluble pectic substances within the grape must. As the grapes continue to mature, a general reduction in the overall concentration of soluble polysaccharides in the must is commonly observed (Ribéreau-Gayon et al., 2006a).

1.9. Other compounds

Grape must contains trace elements that play a significant role in the fermentation process. Inorganic elements, which serve as essential components for cell metabolism, function as aprotic cofactors in the activities of specific enzymes like oxidoreductases and kinases. They also contribute to the maintenance of pH levels and ion balance in yeasts (Ribéreau-Gayon et al., 2006a) Predominantly, cations such as potassium, sodium, calcium and magnesium are found in the must, together with anions such as chlorates, phosphates and sulfates (Aranda et al., 2011).

Notably, vitamins also play an important role, as their presence within grape must stimulates the growth and metabolic activity of yeasts. Working as growth factors, vitamins act as coenzymes in numerous biochemical reactions (Ribéreau Gayon et al., 2006a). Although must generally contains a range of vitamins, some concentrations may be suboptimal. Therefore, the addition of vitamins can promote growth, especially when grapes have been affected by fungal infections (Aranda et al., 2011).

Among these vitamins, the addition of thiamine to the wort for winemaking purposes is legal in the European Union at a concentration of 0.6 mg thiamine hydrochloride/L according to the International Code of Oenological Practices Issue 2020 (OIV 2020). Thiamine, an essential nutrient for metabolic processes involved in yeast proliferation, is particularly useful in increasing growth and fermentation rates, and decreasing the formation of ketonic acids responsible for lowering free SO_2 in wines (Labuschagne and Divol, 2021).

2. Fermentation process and wine microorganisms

The transformation of grape must into wine involves a complex series of biochemical processes, with two main fermentation processes: AF and MLF. AF consists mainly of the biotransformation of sugars in ethanol and carbon dioxide (CO_2) by yeasts action. Regarding to MLF, process which is not present in all type of wines, the LABs decarboxylate L-malic acid into L-lactic acid and CO_2 by malolactic enzyme. Beyond these main reactions, the compounds present in grape must which have been described previously (*"1. Grape and grape must composition"*), are continuously metabolized by microorganisms, significantly contributing to the overall aroma, colour, and flavour characteristics of the wine.

The microbiota coming from the grape and/or from the winery environment encompass a wide variety of yeast and LAB species, while other microorganisms like filamentous fungi and AAB can also be found within the wine microbiota (Ferreira and Mendes-Faia, 2020). The yeast population in grapes typically ranges from 10^2 to10^4 cell/g, whereas the population of LAB is lower, usually below 10^2 cell/g and the population of AAB is even lower, generally below 10 cell/g. However, these concentrations can vary considerably depending on the ripeness of the grapes, changing climatology and damaged berries, which generally implies an increase in the populations of bacteria and non-*Saccharomyces* (Barata et al., 2012).

The impact of these microorganisms on the winemaking process and the final product can vary depending on several factors, including their concentration, timing of appearance, species and the type of wine being produced. This variability determines whether they have beneficial or undesirable effects on the overall winemaking outcome. In this context, all microorganisms interact and contribute to the overall wine quality.

Throughout this introduction it will be explain the contribution of population dynamics and different yeast and LAB species to the specific metabolic transformations, which ultimately influence the success of winemaking and the quality of the final product.

2.1. Alcoholic fermentation

Yeasts are the main protagonists of AF, the main biotransformation produced during winemaking where glucose and fructose from grape must are metabolized into ethanol, as introduced. AF has been discovered by Antoine Lavoisier, and from that moment the research focused on wine microorganisms have revealed the role of yeast on this complex biochemical process (Jolly et al., 2014). The variety of yeast species changes during the process of vinification depending on several factors which affect population dynamics at species and strain level. First, the microbiota present in grape skin depends on the viticultural practices, the climatological conditions or even the grape cultivar (Bordet et al., 2020). Then, in the fermenting must, yeast species are conditioned by the selective pressure related with stressful factors such as low pH, high acidity, high sugar content which leads to higher alcohol degree, or lack of nitrogen sources (García-Ríos and Guillamón, 2019). Furthermore, some oenological practices as an excess of sulphur dioxide (SO_2), an incorrect nutritional supplementation or problems with the control of temperature, production by the yeast of certain toxic compounds for itself such as medium-chain fatty acids in white vinification could leads to a sluggish fermentation (Bisson, 1999). In this context, yeast of *Saccharomyces* genus, especially *Saccharomyces cerevisiae*, becomes the yeast species which dominates AF due to their excellent fermentative capacity and ethanol resistance (García-Ríos and Guillamón, 2019). However, this yeast species is not present in the grapes surface but in the winery environment and consequently do not appear at the beginning of fermentation (Barata et al., 2012).

Conversely, these first stages of AF are dominated by a group of yeast called non-*Saccharomyces* yeasts, which are increasingly considered in winemaking (Jolly et al., 2014).

Concerning the presence of yeasts in grape must, AF is originally carried out spontaneously by indigenous yeasts, however this practice could lead to deviations in the fermentation process due to lack of microbiological control. During the last decades the trend of inoculating commercial starter cultures have increased with the aim to obtain a more controlled fermentation (Fleet and Heard, 1993). The starter cultures are mainly produced as active dry yeast (ADY), whose production consist of a dehydration of cells, resulting below 8% (w/v) of water which maintain a state of suspended metabolism and is genetically more stable, allowing longer periods of storage (Dupont et al., 2014). However, the problem that this practice of inoculation seems to pose is that the wines could lose their typicity of terroir, unlike spontaneous fermentation. That is why, with the development of biotechnological techniques, another type of inoculation has been proposed, which consist of the selection of autochthonous yeast from grape and winery microbiota, followed by a characterization of the most interesting yeast species and strains, in terms of fermentation dynamics and organoleptic properties achieved. The selected yeasts could be used as starter culture to inoculate grape must getting the advantages of both yeast management, a microbiological controlled AF while preserving the identity of wines.

2.1.1. Yeast metabolism

As it has been widely described, yeasts are the responsible for the biochemical transformations of grape compounds in fermenting must. The main metabolism is the carbon metabolism, which involves the glycolysis, the AF, the glycero-pyruvic pathway and the Krebs cycle or tricarboxylic acid cycle (TCA). Nitrogen metabolism is also important for the survival of the yeast. Derived from these main metabolisms, several aromatic compounds are produced which provide interesting, or sometimes undesirable, characteristics to the wine aroma. These metabolisms and their interactions will be described in the following subsections.

2.1.1.1. Carbon metabolism

Under oenological conditions, yeast degrades glucose/fructose via glycolysis and the end product, pyruvate, is transformed into ethanol via acetaldehyde in the cells' cytosol (Figure 3). During this pathway is releasing energy in the form ATP and NADH. This metabolic pathway from glucose to pyruvate is known as the Embden-Meyerhof-Parnas (EMP) pathway.

First, pyruvate is decarboxylated to acetaldehyde and CO_2 by pyruvate decarboxylase. Then, acetaldehyde is reduced to ethanol by alcohol dehydrogenase, or it can be metabolized to acetate and subsequently to cytosolic acetyl-CoA by acetaldehyde dehydrogenase and acetyl-CoA synthase. Acetyl-CoA is used for the biosynthesis of lipids and amino acids. Acetic acid is the main volatile acid in wine and its production depends on the yeast strain, the fermentation conditions, the health of the grapes and the chemical composition of the grape must; for example, the higher the sugar content, the higher the acetic acid content of the wines will be.

In addition, its level can be increased especially during bacterial alteration (acetic acid impairment and lactic acid disease). In addition, acetate with pyruvate can form citramalic acid in wine up to 300 mg/L apart from citramalate from grape must (Ribérau-Gayon et al., 2006b).

The Crabtree effect, also known as catabolic repression of sugars, is an important concept to understand. *S. cerevisiae* can only metabolize sugars through the fermentation pathway. This effect is particularly pronounced in grape must, where the sugar concentration is high (more than 9 g/L). Even when oxygen is present, *S. cerevisiae* can only ferment the sugars and cannot utilize respiration pathway (Ribéreau-Gayon et.al, 2006b).

Yeasts have the ability to utilize an alternative pathway called glycerol-pyruvic fermentation. In this process, an intermediate of glycolysis is converted to glycerol-3-phosphate by the enzyme glycerol 3-phosphate dehydrogenase. Subsequently, glycerol 3-phosphatase is metabolized to glycerol as the end product of this fermentation pathway. The development of this pathway varies depending on the yeast species and serves as a mechanism to cope with the osmotic pressure present in grape must (García-Ríos and Guillamón, 2019).

Another secondary product of pyruvate-derived fermentation can be produced in the early stages of AF. Yeasts can form 200–300 mg/L of D-lactic acid and only a dozen mg/L of L-lactic

acid (Ribéreau-Gayon et al., 2006b). In some cases, the lactic acid content can increase in wines in the presence of LAB, transforming L-malate into L-lactate by MLF and/or sugar metabolism depending on the presence of D- and/or L-Lactate dehydrogenase of LAB species. Additionally, some non-*Saccharomyces* species and more specifically *Lachancea thermotolerans* can use sugar to produce a higher level of L-lactic acid during AF (Hranilovic et al., 2018).

In another scenario, when oxygen is present, and there is a low sugar concentration (lower than 2 g/L), respiration occurs in the mitochondrion. In the respiration pathway, pyruvate is first oxidized to acetyl-CoA by the pyruvate dehydrogenase complex, producing one molecule of NADH and one molecule of CO_2. This acetyl-CoA is then further oxidized in the Krebs to CO_2 and H_2O through eight enzymatic reactions. Generally, this pathway yields up to 38 molecules of ATP from one molecule of glucose, including the two ATPs produced by glycolysis. Thus, respiration is a more energetically efficient metabolism compared to fermentation.

Although the Krebs cycle does not function as an energy pathway under fermentation conditions, it serves as an essential biosynthetic pathway for certain intermediate metabolites, such as oxaloacetate, citrate, α-ketoglutarate, succinate, fumarate and malate (Waterhouse, 2002), (Figure 3B). Indeed, certain enzymes of the Krebs cycle have minimal activity under these conditions, in particular citrate lyase and succinic dehydrogenase, which implies that this cycle operates along two pathways: the oxidative pathway which allows the accumulation of a-ketoglutarate and the pathway reductive which allows the accumulation of succinate (Ribéreau-Gayon et al., 2006b). In addition, Camarasa et al., (2003) demonstrated that the reductive branch of the TCA pathway was the major pathway for succinate production if aspartate was used as a source of nitrogen and that a surplus of succinate was produced by oxidative decarboxylation of α -oxoglutarate if glutamate was the sole nitrogen source of the growth medium during anaerobic glucose fermentation. Malic acid and citric acid are usually not produced in significant quantities, as their concentrations are already high in the grape must and can be directly incorporated from it. Therefore, after FA there is typically little citrate consumption but 10 to 25% malate can be consumed by *Saccharomyces* species depending on the strain and fermentation conditions (Ribéreau-Gayon et al., 2006b). On the other hand, certain strains of *S. cerevisiae* can produce from 0.5 to 3 g/L of L-malic acid during alcoholic

fermentation (Vion et al., 2023; Yéramian et al., 2007). In the context of climate change, this trait can be interesting for preserving or better biologically acidifying wines.

2.1.1.2. Nitrogen metabolism

As it was described in grape composition YAN involves different forms of nitrogen as ammonium, amino acids and small peptides. The uptake of amino acids and ammonium is regulated by the Nitrogen Catabolic Repression (NCR) of yeast (Magasanik and Kaiser, 2002). Amino acids are taken up by yeast through a system of 19 permeases, whereas ammonium is transported via three permeases that are all active only when ammonium is found in low concentrations (Godard et al., 2007). Although yeasts species can use a wide variety of different nitrogen compounds, they tend to show a preference for some of them (Torija et al., 2003). Studies have examined these preferences in various yeast species, revealing interspecific differences that depend on the nitrogen composition of the medium (Roca-Mesa et al., 2020).

2.1.1.3. Secondary aroma metabolism

From these primary metabolisms, secondary aromatic compounds are produced in wines (Figure 3). In young wines, these aromas, along with the varietal aromas, create complex aromatic profiles that vary depending on the yeast species used.

Continuing with the carbon metabolism, FAs are synthesized from acetyl CoA. These reactions are catalysed by the multienzyme fatty acid synthase complex (FAS) (Tehlivets et al., 2007).

Fatty acids synthesized have significant importance as they play a crucial role in cellular membranes and, consequently, in the response to environmental stresses (Nielsen, 2009). They are classified based on their chain length: short-chain FAs (SCFA) with 3 to 5 carbon atoms, medium-chain FAs (MCFA) with 6 to 12 carbon atoms, and long-chain FAs (LCFA) with more than 12 carbon atoms. SCFA and MCFA have an impact on the volatile profile of wines, while LCFA are responsible for maintaining cell membrane integrity. Additionally, an accumulation of MCFA also influence both AF and MLF performance (Saerens et al., 2008).

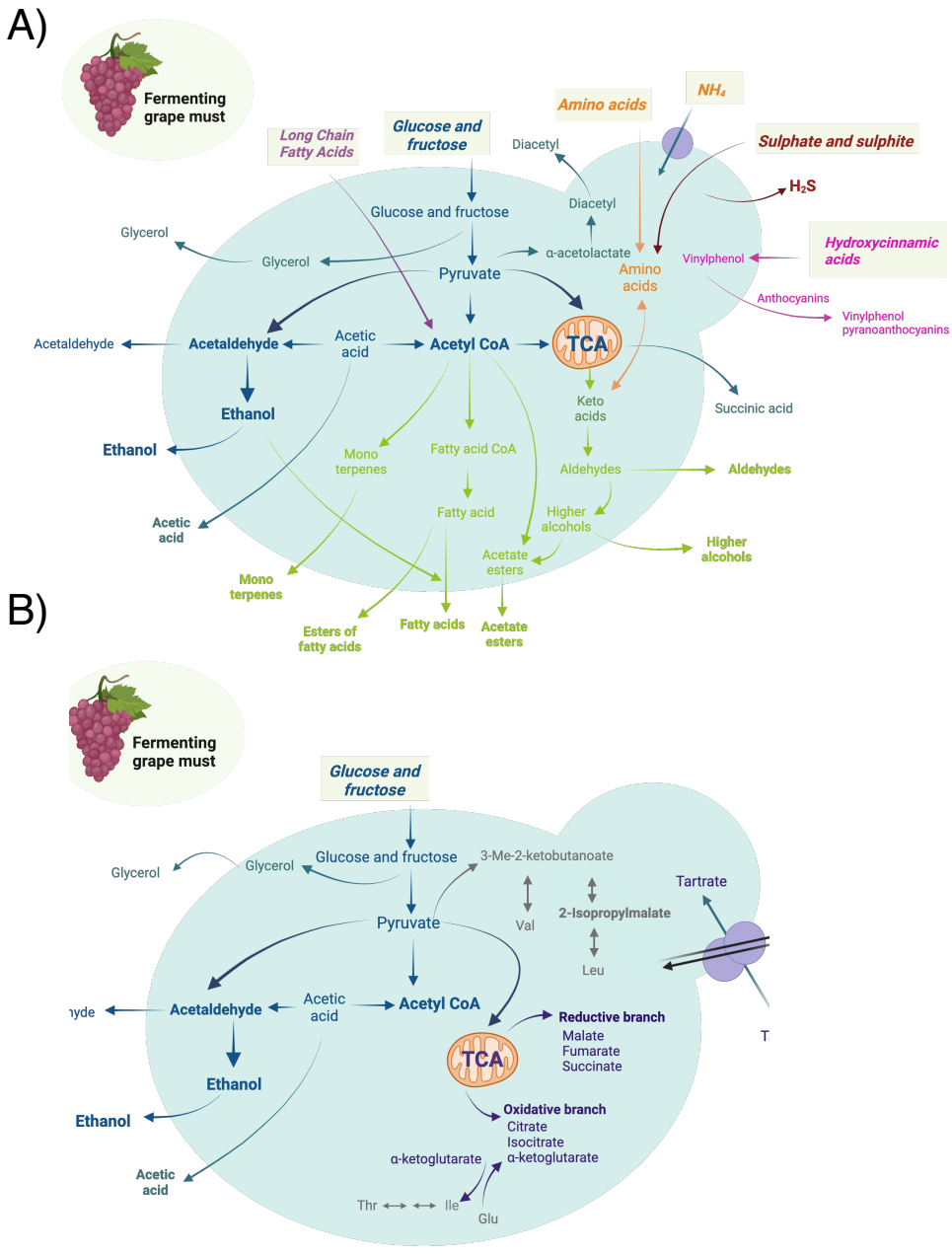

Figure 3. A) General Yeast metabolism during alcoholic fermentation in fermenting grape must**.** Adapted from Dzialo et al. (2017) and Swiegers et al. (2005) and B) Detailed metabolism of glycolysis and TCA.

The presence of LCFA in grape must also plays a role in yeast metabolism. It has been observed that *S. cerevisiae*, the main yeast involved in AF, absorb and incorporate linoleic acid into their membrane lipids, despite their inability to synthesize it (Thurston et al., 1981). This effect appears to enhance yeast viability (Beltran et al., 2008). The utilization of linoleic acid (C18:2) influences the primary carbon metabolism of *S. cerevisiae*, leading to increased glucose consumption and ethanol production under anaerobic conditions. This, in turn, affects the glycolytic pathway, the TCA cycle, and up-regulates amino acid production (Casu et al., 2018). Additionally, even with a supplementation of only 120 mg/L of linoleic acid, its presence affects the production of volatile compounds, such as thiols (Casu et al., 2018, 2016). Furthermore, a high presence of lipids has been linked to increased synthesis of higher alcohols. This effect could be partially attributed to impaired activity of alcohol acetyl transferases in the presence of lipids, which catalyse the conversion of higher alcohols into the corresponding esters (Guittin et al., 2021).

On the other hand, the FAs synthesized can form esters of FAs through esterification. The production of esters of FAs can occur through two different reactions: enzymatic catalysis or chemical esterification at low pH during aging (Sumby et al., 2010). These compounds are formed from an acid group (carboxylic group) and an alcohol group, which can be ethanol or a complex alcohol derived from amino acid metabolism (Saerens et al., 2008). However, this process is highly dependent on oxygen availability (Mason and Dufour, 2000). Ethyl esters of FAs are associated with fruity aroma descriptors, which are typically desired by winemakers in young wines, for instance: ethyl hexanoate has green apple and violet aroma descriptors and the ethyl octanoate have pineapple and pear aroma descriptor (Sumby et al., 2010).

Another important group of fermentative volatile compounds is the fusel or higher alcohols. Depending on their concentration, they can positively contribute to the wine's complexity (below 300 mg/L) or have a negative impact (above 400 mg/L) (Ribéreau-Gayon et al., 2006a). Fusel alcohols are derived from certain amino acids, such as branched-chain amino acids (isoleucine, leucine, and valine), aromatic amino acids (phenylalanine, tyrosine and tryptophan), and the sulphur-containing amino acid (methionine), through the Ehrlich catabolic pathway (Dzialo et al., 2017). The regulation of the Ehrlich pathway is influenced by the yeast species and the availability of carbon and nitrogen compounds (González et al.,

2018; Lacroux et al., 2008). Fusel alcohols are categorized into aliphatic alcohols, such as propanol, isobutyl alcohol or isoamyl alcohol, and aromatic alcohols, such as tyrosol, tryptophol, and 2-phenylethanol (Dzialo et al., 2017).From fusel alcohols, fusel alcohol acetate esters are formed through enzymatic acetylation by acetyltransferase enzymes. Higher alcohol acetates include isobutyl acetate, whose descriptor is apple aroma, ethyl 2-phenylacetate, with rose aroma, and isoamyl acetate, whose descriptor is banana aroma (Sumby et al., 2010).

Aldehydes are also derived of the Ehrlich pathway. Even they are present in lower concentrations have a notable impact on the aromatic profile of wines due to their low sensory threshold values. Especially acetaldehyde which comprise 90% of the aldehydes, even though in high concentrations it is considered a defect (Lambrechts and Pretorius, 2000).

During AF, volatile thiols are produced due to their release by carbon-sulphur lyase enzymes of yeast from their precursors, which are naturally present in grapes as it has been described. These compounds contribute to the aromatic complexity of the wine and are particularly notable for their tropical descriptors, which are especially prominent in certain white grape cultivars.

However, not all volatile compounds produced during yeast metabolism are desirable to winemakers. One of the less favourable by-products is hydrogen sulphide (H_2S), notorious for its unpleasant sulphurous aroma like to rotten eggs. The formation of H_2S is related to the nitrogen metabolism, specifically when yeast reduce exogenous sulphate during the synthesis of sulphur-containing amino acids like cysteine and methionine. There is a complex relationship between the concentration of YAN and the production of H_2S during AF. Adequate YAN levels are crucial to manage and minimize the formation of H_2S, as low YAN concentrations may lead to increased H_2S production (Ugliano et al., 2009). However, strain-dependent variations have been observed in this regard, suggesting that interactions between YAN and the genotype of different yeast strains can influence the amount of H_2S formed during AF (Ugliano et al., 2011).

2.1.2. *Saccharomyces cerevisiae*

As it has been introduced *S. cerevisiae* is the yeast specie best adapted to the restrictive winemaking conditions limiting the growth of competing microorganisms (Pretorius, 2016). The fermentative capacity displayed by *S. cerevisiae* is a result of the regulation via the Crabtree effect, which is not commonly found in most non-*Saccharomyces* yeast species.

Winemaking biotechnology companies have successfully used the wide inter-strain variability of yeast to select and offer starter cultures that are better suited for various types of vinifications (Table 1), (Vejarano and Gil-Calderón, 2021). These offerings include specific *S. cerevisiae* strains tailored for white, rosé, or red wines, as well as strains that exhibit greater resistance to osmotic pressure, making them ideal for second fermentation in sparkling wines. Some starter cultures are indicated as incompatible with the MLF process due to the specific release of specific compounds. This is particularly interesting for winemaking scenarios where MLF is not desired, such as white or sparkling wines in warm climates. Indeed, the differences between various *S. cerevisiae* strains go beyond their technological aspects, such as fermentative capacities and the release of mannoproteins (Masneuf-Pomarède et al., 2010).

These strains also exhibit aromatic metabolic differences, which can significantly influence the aroma profile of the wine they produce (Romano et al., 2022). This offers winemakers an interesting proposition, as they can select specific *S. cerevisiae* strains based on their described aromatic production in terms of composition and intensity. By doing so, winemakers can choose a strain that contributes a distinctive aroma to the wine. Alternatively, they could choose a neutral strain to highlight the aromatic properties of the indigenous microbiota and the grape cultivar, or even the aromatic impact of other species which could be inoculated. This strategic selection of *S. cerevisiae* strains and potential co-inoculations offers winemakers greater control over the sensory characteristics and aromatic complexity of their wines.

Table 1. Starter cultures of *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast available from different wine biotechnological companies. Underlined strains indicate the ones used in experimental work.

Yeast species	Commercial name	Company	Characteristics
Saccharomyces cerevisiae	Lalvin QA23	Lallemand	Increase the varietal complexity. Low nitrogen demand. Good fermentative characteristics. Indicated for red and rosé wines.
	Lalvin CLOS	Lallemand	High tolerance to ethanol and SO_2. Good compatibility with. MLF Indicated for red wines.
	Lalvin ICV K1 Marquée	Lallemand	High production of fermentative esters. Delay MLF. Indicated for red and white wines.
	Lalvin BM4x4	Lallemand	Increase the varietal complexity. Indicated for red wines.
	Viniferm Revelación	Agrovin	Increase of volatile thiols. Indicated for white and rosé wines.
	Zymaflore Klima	Laffort	Reduction of alcohol degree. Increase varietal character.
	DVS JAZZ	Chr. Hansen Holding AS	Good fermentative characteristics even at low temperatures. Good compatibility with MLF.
Torulaspora delbrueckii	Biodiva TD291	Lallemand	Increase ester concentration. Reduce volatile acidity. Recommended for late harvest wines.
	Viniflora Prelude	Chr. Hansen Holding AS	Decrease production of toxic medium-chain fatty acid promoting MLF. High production of mannoproteins. Increase flavour complexity.
	NSA1 Viniferm NSDT	Agrovin	Increase 2-phenyl ethanol and varietal character (high β-lyase activity). High mannoprotein release.
	Zymaflore Alpha	Laffort	Increase of volatile thiols. Low production of volatile acidity, acetoin, acetaldehyde and H_2S.
	EnartisFerm Q TAU FD	Enartis	Increase ester concentration. High resistance to osmotic shock.
	Oenovin *Torulaspora* BIO	Oeno	Increase flavour complexity. Low production of volatile acidity.
Metschnikowia pulcherrima	Flavia	Lallemand	Increase of thiol and terpene aromas. Recommended for white and rosé wines.
	Initia	Lallemand	Bioprotection, recommended for white and rosé wines.
	Guardia	Lallemand	Bioprotection, recommended for red wines.
	Zymaflore Khio^{MP}	Laffort	Fast imposition at low temperatures. Bioprotection in white and rosé wines and grapes during long pre-fermentative phases at low temperature.
Lacchancea thermotolerans	Viniferm NS Chance	Agrovin	Increase of lactic acid concentration. High aromatic complexity and unctuosity, for the high glycerol production.
	Laktia	Lallemand	Increase of lactic acid concentration. High aromatic complexity.
	Concerto	Chr. Hansen Holding AS	Increase of lactic acid concentration. High aromatic complexity. Production of ethyl isobutyrate
Pichia kluyveri	Viniflora FrootZen	Chr. Hansen Holding AS	High increase of volatile thiols.
T. delbrueckii and *M. pulcherrima*	Zymaflore Ëgide^{TDMP}	Laffort	Bioprotection. Neutral organoleptic characteristics.
Kluyveromyces thermotolerans, *T. delbrueckii* and *S.cerevisiae*	Melody	Chr. Hansen Holding AS	Increase of aromatic intensity, especially tropical aromas.

2.1.3. Non-*Saccharomyces*

Aside from *Saccharomyces* yeast, there exists a diverse group of species known as non-*Saccharomyces* yeast. These yeast species are naturally present in both the grapevine and winery environment (Beltran et al., 2002) and take the lead in the initial stages of AF. In the past, non-*Saccharomyces* yeasts were considered undesirable since they were related with wine alterations (Padilla et al., 2016). However, in recent years, wine research has focus to non-*Saccharomyces* yeasts due to interesting abilities, which had caused the development of new starter (Table 1) (Roudil et al., 2019). These yeast species have been selected for their multiple benefits, including the enhancement of mannoproteins (Domizio et al., 2014; Giovani et al., 2012), biocontrol effects (Canonico et al., 2023; Kuchen et al., 2019), acidification (Escott et al., 2022; Vaquero et al., 2022), modulation of the volatile profile (Azzolini et al., 2015; Hu et al., 2018; Liu et al., 2016; Oliveira and Ferreira, 2019; Renault et al., 2015; Velázquez et al., 2015), influence on colour composition (Balmaseda et al., 2021b; Božič et al., 2020; Chen et al., 2018; Minnaar et al., 2018), lower production of volatile acidity (Ciani et al., 2021; Jolly et al., 2014), alcohol reduction (Contreras et al., 2014; Garciá et al., 2020; Zhu et al., 2021), and more recently, interactions with MLF (Balmaseda et al., 2023, 2021c; Ferrando et al., 2020) and protection against browning (Giménez et al., 2023).

Non-*Saccharomyces* yeast species are predominant during AF, especially in spontaneous fermentations without an early inoculation of *S. cerevisiae*. However, as the fermentation progresses, their presence reduces due to their slow growth and poor fermentative activity, with lower tolerance to ethanol, particularly when exposed to SO_2 (Jolly et al., 2014). Additionally, nutrient limitation and competition with *S. cerevisiae* have a repressive effect on their growth (Roca-Mesa et al., 2022).

These non-*Saccharomyces* yeast species can be divided into three subgroups: aerobic non-*Saccharomyces*, such as *Pichia spp.*, *Debaryomyces spp.*, *Rhodotorula spp.*, and *Candida spp.*; apiculate yeasts with low fermentative activity, such as *Hanseniaspora uvarum*, *Hanseniaspora guilliermondii,* or *Hanseniaspora occidentalis*; and species with fermentative metabolism, such as *Torulaspora delbrueckii, Metschnikowia pulcherrima, Zygosaccharomyces bailii, Lachancea thermotolerans* and *Schizosaccharomyces pombe*. It is

important to note that there are other yeasts present in the wine environment that are not considered non-*Saccharomyces* yeast, but as they are spoilage yeasts, such as *Dekkera/Brettanomyces*.

2.1.3.1. *Torulaspora delbrueckii*

One of the most studied non-*Saccharomyces* yeast species is *T. delbrueckii*, formerly known as *Saccharomyces rosei, T. rosei, or T. fermentati*. It shares a close genetic relationship with *S. cerevisiae*, due to its resistance to ethanol and SO_2, and its good fermentative activity, *T. delbrueckii* remains in the final stages of AF (Benito, 2018). *T. delbrueckii* has been the first non-*Saccharomyces* species commercialized as a starter culture, due to its potential applications in various oenological challenges. Currently, a wide range of strains of this yeast are commercially available as ADY (Table 1). Some of the reported benefits of using *T. delbrueckii* include its low acetic acid production, reduction of alcohol production, increase of glycerol production and increase of mannoproteins release. The increment of foamability and foam persistence in sparkling wine production it is another interesting application of this species (González-Royo et al., 2015; Medina-Trujillo et al., 2017). Moreover, the impact of *T. delbrueckii* has been deeply studied on the aromatic composition of wines, particularly in white wines (Azzolini et al., 2015; Velázquez et al., 2015) but also in red wines (Balmaseda et al., 2021b; Ramírez et al., 2016). Besides, for red wines, studies have described its influence on colour composition and phenolic compounds (Balmaseda et al., 2021b; Escribano-Viana et al., 2019; Minnaar et al., 2018). This impact on aroma and colour composition is attributed to specific enzymes that are more developed in some non-*Saccharomyces* yeasts. However, in *T. delbrueckii* only an increase in β-glucosidase (Maturano et al., 2012) and β-lyase activity (Belda et al., 2015) has been studied, varying significatively among different strains. Further research is needed to better understand and characterize the enzymatic behaviour of *T. delbrueckii* and its implications on the organoleptic composition of wine, especially concerning colour.

Interestingly, the potential of this yeast in other types of products is starting to be explored, such as its application in rosé wine (Muñoz-Redondo et al., 2021), botrytized wines (Bely et al., 2008), or even wines fermented from other fruits (Liu et al., 2023; Tocci et al., 2023; Wang et al., 2023)

Finally, another promising application that has been observed recently is its ability to enhance MLF development (Balmaseda et al., 2023), although as we will see in the next sections, this effect depends on several factors.

2.1.3.2. *Metschnikowia pulcherrima*

M. pulcherrima, is another interesting non-*Saccharomyces* yeast species commonly found in the grape ecosystem, however it exhibits low fermentative activity (Comitini et al., 2011). Therefore, it is often used in combination with other yeast strains with higher fermentative capacity. Its presence tends to decrease over time when co-fermenting with *S. cerevisiae* due to its limited tolerance to ethanol, but it shows moderate resistance to SO_2 compared to other non-*Saccharomyces* species (Loira et al., 2020).

Several commercial *M. pulcherrima* strains are also available as ADY due to its interesting oenological applications (Table 1). One notable use is its role as a biocontrol agent (Canonico et al., 2023), as it produces a natural antifungal compound, the pulcherrimin, which also gives it its characteristic colour, red extracellular pigment, when growing on plates (Morata et al., 2019b). Moreover, *M. pulcherrima* is known for expressing a wide range of hydrolytic enzymes, such as pectinolytic, protease, glucanase, lichenase, β-glucosidase, cellulase, xylanase, amylase, sulphite reductase, lipase, and β-lyase activities (Barbosa et al., 2018; Belda et al., 2016a; Canonico et al., 2023; Fernández et al., 2000). These enzymatic activities have a significant impact on the aroma and colour of wines fermented in its presence (Carpena et al., 2021).

Additionally, *M. pulcherrima* has an interesting capacity of reducing alcohol content, making it a potential biotechnological tool for alcohol reduction (Garciá et al., 2020; Hranilovic et al., 2020). Its respiratory catabolism of sugars since it is a Crabtree-negative species under aerobic conditions efficiently reduces ethanol production. Studies on sterilized natural must have reported alcohol reductions of up to 1.6% (v/v), although its effectiveness may vary when interacting with the indigenous microbiota (Contreras et al., 2014).

M. pulcherrima's application has also been studied in other alcoholic beverages such as beer (Postigo et al., 2022) and other fruits fermentations (Kręgiel et al., 2022; Zhang et al., 2022).

Furthermore, it has been investigated for its potential to reduce enzymatic browning, as it efficiently consumes oxygen, thereby reducing its availability for polyphenol oxidases.

2.1.4. Other yeast-derived changes in wine: colour and polysaccharides

In the previous sections, we detailed the main metabolic changes that occur during AF by both *Saccharomyces* and non-*Saccharomyces* yeast, and their implications for wine quality. However, there are other important metabolisms and changes that can also impact wine quality and subsequent processes such as MLF.

The colour composition of wines is modified by yeast though different mechanisms (Tofalo et al., 2021). In the case of red wines, the colour intensity can be reduced due to the adsorption of pigments on the yeast cell wall (Assunção Bicca et al., 2023; Morata et al., 2003). Interestingly, different yeast species exhibit varying effects, possibly due to differences in cell wall composition (Figure 4) or thickness (Božič et al., 2020). On the other hand, wines fermented with certain non-*Saccharomyces* species, such as *T. delbrueckii* or *M. pulcherrima*, have been found to show an increase in anthocyanins concentration (Balmaseda et al., 2021b; Chen et al., 2018; Escribano-Viana et al., 2019; Minnaar et al., 2018). This effect might be associated to pectinolytic enzymes that promote the extraction of anthocyanins from the grape skin (Tofalo et al., 2021), however this effect has only studied in *M. pulcherima* (Belda et al., 2016a). On the contrary, the colour could also decrease, mechanism related with the β-glucosidase activity associated to certain yeast strains (Maturano et al., 2012), This activity can break the polyphenols-sugar bond, exhibiting a decolorizing effect (Manzanares et al., 2000).

Moreover, pyruvic acid and acetaldehyde produced by yeasts can also interact with anthocyanins through cycloaddition reactions forming pyranoanthocyanins such as Vitisin A and Vitisin B respectively (Schwarz et al., 2003), forming more stable compounds. Yeast also metabolizes hydroxycinnamic acids during AF and aging of wine by of the hydroxycinnamate decarboxylase enzyme (HCDC) forming vinylphenols which in red and rosé wines could reacts with anthocyanins forming pyranoanthocyanins which also contributes to wine stabilization (P. Zhang et al., 2021). Differences in HCDC activity have been reported among yeast species (Božič et al., 2020) suggesting varying impacts on the wine colour and stability. However, yeast species with vinylphenol reductase activity, such as *Brettanomyces*, is able to convert

vinyl phenols into ethyl phenols leading to negative impacts on the aroma of wine. In this sense when vinylphenols react with anthocyanins the risk of the formation of ethyl phenols is reduced (Morata et al., 2019b). Understanding how different yeast species influence colour and other sensory characteristics of wines can contribute to the development of wines with desired qualities.

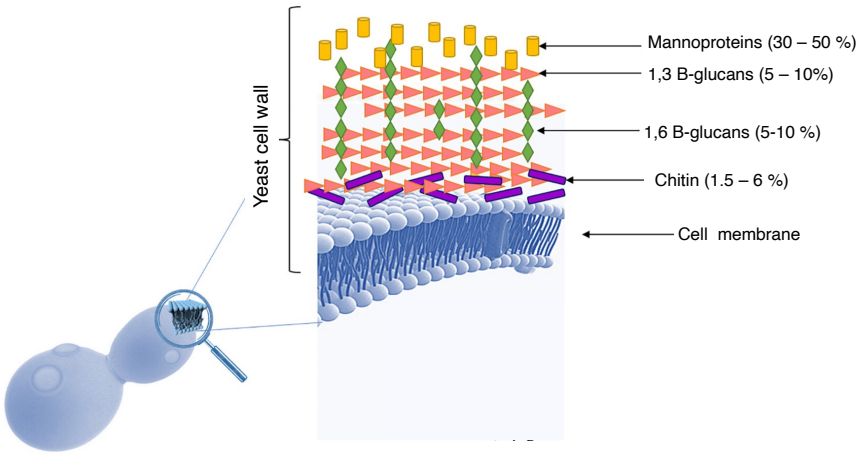

Figure 4. Yeast cell wall composition. Percentage of different compounds indicated in parentheses (% by weight). Adapted from Tofalo et al. (2021).

Polysaccharides are essential compounds in wine that influence both its sensory and physicochemical properties, being their research focus of numerous current studies. Apart from the polysaccharides derived from grapes, yeast -mainly- and bacteria also release polysaccharides, particularly mannoproteins, from their cell walls during their growth and during autolysis. Mannoproteins are located in the outermost layer of the yeast cell wall and, through β-1,6 glucan chains, are connected to the innermost layer, which are composed of β-1,3 glucan chains and chitin. The concentration of released polysaccharides varies depending on the yeast strain and the fermentation conditions. Giovani et al. (2012) described that some non-*Saccharomyces* yeast strains produce higher levels of polysaccharides compared to *S. cerevisiae* strains. Interestingly, these strains were some spoilage yeasts like *Zygosaccharomyces bailii* and *Brettanomyces bruxellensis*, as well as beneficial non-*Saccharomyces* species like *T. delbrueckii* or *S. pombe*. Other authors have

also reported higher presence of mannoproteins in wines with the presence of *T. delbrueckii* (Balmaseda et al., 2022a; Belda et al., 2015).

These macromolecules have numerous positive effects on wine, which is why wines are often aged with their lees - autolyzed yeast and other compounds. First, depending on the polysaccharide structure they interact through different interactions with malvidin-3-O-glucoside modulating the colour of wines (Assunção Bicca et al., 2023). They are also involved in protein and tartaric stability (Dupin et al., 2000; Lubbers et al., 1994). But in general, the most valuated benefit is the reduction of astringency and the increase of body and mouthfeel (Gawel et al., 2016).

2.2. Malolactic fermentation

The other significant metabolic process in winemaking is the malolactic fermentation (MLF). MLF process consist of a decarboxylation process where L-malic acid present in the grape must is converted to L-lactic acid by LAB (Pilone and Kunkee, 1970). This transformation leads to a reduction in acidity and an increase in pH (Liu, 2002; Lonvaud-Funel, 1999). The consequences of MLF on the wine's organoleptic properties include a smoother taste due to the pungent acidity of malic acid and the smooth acidity of lactic acid. Additionally, MLF provides microbial stability, as less substrates are present in the media for contaminant microorganism, and can modify the aromatic profile of wines, due to LAB metabolism (Liu, 2002).

While MLF is not necessary for all types of winemaking, it is very common in red wines and is also performed in complex and high-acidity rosé wines, as well as certain white wines like some Chardonnay wines. However, the occurrence of MLF is influenced by climate change; in warmer areas, L-malic acid concentrations in grapes are decreasing, but in colder wine regions, if any still exist today, higher concentrations of malic acid may require MLF, even in white and sparkling wines.

LABs are present on the grape surface and are maintained during the initial stages of AF (Bae et al., 2006; Barata et al., 2012; Semon et al., 2001). However, their population is not high, and as ethanol concentration increases and SO_2 is added, LAB population decreases, with the exception of *Oenococcus oeni*, a species well-adapted to wine conditions. Managing MLF in

the stressful conditions of wine production can be unpredictable, as it requires a LAB population of 10^6 cells/mL to begin (Lonvaud-Funel, 1999). Due to the high cost of commercial LAB cultures, many wineries opt for spontaneous MLF with indigenous LAB, which may extend the duration of the process. However, specific temperature conditions are needed to reach the appropriate population, which may lead to stuck MLF during winter. Commercial LAB cultures have been developed offering better control and effectiveness of the MLF process (Henick-Kling and Park, 1994; Jussier et al., 2006). Most of these starter cultures consist of *O. oeni* due to its tolerance characteristics, but there are also other species available like *Lactiplantibacillus plantarum* (Table 2).

In the following sections, we will go further into the metabolism of LAB during MLF and the challenging conditions they must manage.

2.2.1. Wine Lactic Acid Bacteria metabolism

The main metabolism of LAB is the fermentation of sugars, which can be classified in homofermentative and heterofermentative LAB. Homofermentative LAB transform the hexoses that they use, especially glucose, into L-lactic acid and/or D-lactic acid or only L-lactic depending on the species, via EMP pathway. While heterofermentative species via pentose phosphate pathway, transform hexoses into lactate, ethanol, acetate and CO_2 (Ribéreau-Gayon et al., 2006b).

The energy obtained from the fermentation of residual sugars is generally sufficient to support the biomass required to initiate the MLF (Ribéreau-Gayon et al., 2006b). This way *O. oeni* and other LAB species can survive due to their ability to rely on L-malic acid as their energy source in the wine. They consume L-malic acid through its decarboxylation process in the cytosol. The L-malic acid enters the cell as monoanionic malate ($HMal^-$) through a permease called MleP. The malate is utilized in the cytosol where is decarboxylated, a proton (H^+) is consumed in the catalysis and an efflux of neutral lactate (HLac) is produced. Then the intracellular pH increases and an electrochemical gradient is generated creating a proton motive force which enables the entrance of H^+ through ATPase for the synthesis of ATP.

The genes responsible for this metabolic pathway are organized in an operon, with the malolactic enzyme gene (*mleA*) and the malate permease gene (*mleP*) being part of it. These genes are preceded by *mleR*, which encodes a transcriptional regulatory protein that controls the expression of the operon.This reaction is the one that define MLF, however LAB are responsible of other transformations during MLF which must be considered.

Citric acid can be metabolized by *O. oeni* and other LAB species. This consumption has been also associated to a mechanism of stress (Olguín et al., 2009). First, citric acid is divided into an oxaloacetate and acetate by the citrate lyase enzyme. The oxaloacetate is then decarboxylated to pyruvate and further transformed into α-acetolactate. Under anaerobic conditions, which are typical during wine fermentation, α-acetolactate is fermented to produce acetoin and 2,3-butanediol as end products. These end products can enhance buttery aromas; however, high concentrations are considered undesirable (Bartowsky and Henschke, 2004).

Moreover, in addition to fermenting residual sugars, LAB also need nitrogen sources, even when wine's nitrogen content is typically low after AF. However, wine microorganisms do not have the ability to metabolize proteins present in wine. *O. oeni*, in particular, prefers peptides as a nitrogen source, which can be found in the wine at levels of around 100 g N/L after AF. LAB have the capacity to break down these peptides and release free amino acids into the wine, which they subsequently consume (Remize et al., 2006). Consequently, they can also utilize the existing free amino acids in the wine. It usually representing about 20 mg N/L (Roca-Mesa et al., 2020).

Nevertheless, the breakdown of amino acids can sometimes lead to the formation of undesirable compounds. For instance, the catabolism of arginine might give rise to the production of the precursors of ethyl carbamate, a carcinogenic compound, or putrescine, a biogenic amine (Gil-Sánchez et al., 2018). Biogenic amines are toxic substances formed through substrate-specific enzymatic decarboxylation of their respective amino acid precursors, such as ornithine, histidine, and tyramine. Their content in wine can vary from traces up to 130 mg/L (Soufleros et al., 1998) although they are generally found at lower levels than in other fermented products (Silla Santos, 1996). Some of the most commonly identified biogenic amines in wine include putrescine, histamine and tyramine however it has been demonstrated that the capacity to produce them is specie and strain dependent.

During MLF, LAB also play a significant role in producing desirable secondary (fermentative) aromas (Cappello et al., 2017). The production of esters is highlighted, however esterase activity in wine LAB (Matthews et al., 2004) has been less extensively studied compared to yeast. The LAB ester production depends on various factors, such as the specific LAB strain and species, as well as the media (Fia et al., 2018; Pozo-Bayón et al., 2005). It has been found to be particularly effective in producing short-chain esters, particularly ethyl fatty acid esters (Cappello et al., 2017; Ugliano et al., 2003).These compounds, when present in appropriate concentrations, enhance the fruity aroma of the wine. Moreover, certain species of wine LAB have the ability to release grape variety-specific aromas by means of their β-glucosidase activity, for instance C13-norisoprenoides and monoterpenes (Grimaldi et al., 2005). However, the extent of this activity varies depending on the specific LAB strains and the terpene substrates involved.

Furthermore, LAB can decarboxylate phenolic acids into 4-vinyl derivatives and then could be reduced to 4-ethyl derivatives, which are associated to off-flavours (Cavin et al., 1993). Indeed, wine colour can also be influenced by the metabolism of LAB (Virdis et al., 2021). Typically, there is a decrease in colour intensity after MLF, a phenomenon attributed to various mechanisms. For example, the metabolism of acetaldehyde and pyruvic acid by LAB can influence the formation of polymeric pigments, thereby impacting colour development (Burns and Osborne, 2013). Furthermore, it has been observed by Devi et al., (2020) that LAB, similar to yeast, are capable of assimilating anthocyanin glucosides, likely through their cell walls. Through their β-glycosidase activity, these LAB can cleave the glycosidic bonds of anthocyanin glucosides and subsequently break down the aglycons into compounds such as phloroglucinol aldehyde and corresponding phenolic acids (Devi et al., 2020).

2.2.2. Wine Lactic Acid Bacteria species: *Oenococcus oeni*

In winemaking, there is a wide variety of LAB species belonging to the phylum *Firmicutes* and families *Lactobacillaceae* and *Leuconostocaceae* (Ribéreau-Gayon et.al, 2006b). Initially, the LAB diversity is high on the grape surface, but it starts to decrease during AF due to their specific nutritional requirements and different stresses (Bae et al., 2006; Terrade and Mira de Orduña, 2009). Nevertheless, some species manage to survive and grow, eventually leading

to the spontaneous occurrence of MLF. The dominant LAB species found in wine is *Oenococcus oeni*, but there are also other species, including the former *Lactobacillus, Pediococcus*, *Leuconostoc,* and *Oenococcus* (Ribéreau-Gayon et.al, 2006b).

Table 2. Starter cultures wine Lactic Acid Bacteria available at different wine biotechnological companies. Underlined strains indicates the ones used in experimental work.

Yeast species	Commercial name	Company	Main characteristics
Oenococcus oeni	Lalvin VP41	Lallemand	High resistance to ethanol, low pH and SO_2. Low production of diacetyl. Indicated for red and white wines.
	Lalvin SILKA	Lallemand	Is not able to produce biogenic amines. Recommended for co-inoculation and sequential inoculation with yeast, and for MLF with wood.
	Beta Co-Inoc	Lallemand	Good synergy with yeast, better results for co-inoculation.
	Viniferm OE 322	Agrovin	Pull of strains. Specific for wines with low pH and high L-malic content. Recommended for white wines.
	Viniferm OE 104	Agrovin	Pull of strains. Adapted to wines with high polyphenolic content. Recommended for red wine.
	Lactoenos 450 PreAc	Laffort	High resistance to ethanol. Low production of diacetyl.
	Lactoenos BERRY Direct	Laffort	High L-malic consumption yield. Low production of diacetyl.
	Viniflora CH11	Chr. Hansen Holding AS	Specific for wines with low pH and for MLF at low temperature. Recommended for white wines.
	Viniflora CH35	Chr. Hansen Holding AS	High resistance to SO_2. High production for diacetyl. Indicated for white wines with MLF and aged in barrel.
Lactiplantibacillus plantarum	ML Prime	Lallemand	Do not produce acetic acid. Rapid consume of L-malic acid. Only for co-inoculated wines.
	NOVA	Chr. Hansen Holding AS	Do not increase pH. Recommended for must with low malic acid content

Among the former *Lactobacillus* genus, the species commonly associated with the wine environment include *Lactiplantibacillus plantarum, Levilactobacillus brevis, Lentilactobacillus buchneri, Lentilactobacillus hilgardii,* and *Fructilactobacillus fructivorans* (Endo et al., 2009). Notably, *Lactiplantibacillus plantarum* is prominent for its high prevalence and occasional involvement in spontaneous MLF. However, this genus can produce undesirable compounds such as biogenic amines, although it depends on the strain (Silla Santos, 1996). On the positive side, it exhibits good esterase activity (Pozo-Bayón et al., 2005) Due to these characteristics, specific *L. plantarum* strains have been selected and are commercially available as starter cultures for initiating MLF in wine (Table 2).

Pediococcus genus is also present in both grape and winery environments. However, it is often associated with spoilage and undesirable effects. Four species, *P. damnosus*, *P. parvulus*, *P. pentosaceus*, and *P. inopinatus*, are known to be associated with wine (Endo et al., 2009). It has been described that this specie together with *Lactobacillus* genus are had higher capacity than *O. oeni* for producing 4-ethyl derivatives (Cavin et al., 1993).

O. oeni, formerly known as *Leuconostoc oenos*, is the main specie of wine, which serves as its primary ecological niche. This species has a high fast-evolving capacity, resulting in a wide variety of strains that contribute to its remarkable adaptability. Among these, the strain PSU-1 (ATCC BAA-331) stands out as the first *O. oeni* genome to be sequenced and has since become a model strain in research on this species (Neeley et al., 2005).

It is present in grape surface and in winery environment (Franquès et al., 2017; Reguant et al., 2005), despite its high nutrient requirements *O. oeni* is able to produce exopolysaccharides and biofilms and colonize winery surfaces (Dimopoulou et al., 2016).

O. oeni exhibits good resistance to low pH levels, high ethanol content and limited nutrient availability, that contribute to its successful establishment in wine. In response to the hostile conditions, *O. oeni* employs diverse stress responses, predominantly related to acidity and ethanol levels (Balmaseda et al., 2022b; Margalef-Català et al., 2017; Olguín et al., 2015). Notably, wine could present different challenges that can negatively affect *O. oeni*, including SO_2 (Reguant et al., 2005), polyphenols (Reguant et al., 2000) and negative interactions with yeast. Nevertheless, certain strains of *O. oeni* have developed specific adaptation to deal with

these unfavourable factors. Furthermore, some *O. oeni* strains have also the capacity to produce biogenic amines (Izquierdo-Cañas et al., 2009), being their low production an important criterion of selection.

In this context, the wine biotechnological companies offer an extensive selection of *O. oeni* starter cultures for MLF, many of which comprise selected strains known for their desirable properties (Table 2). Additionally, there are pools of *O. oeni* strains available, reflecting the rich diversity within this species. However, even when *O. oeni* is intentionally inoculated, it may not always successfully complete the MLF, thus research in improving MLF performance is still necessary.

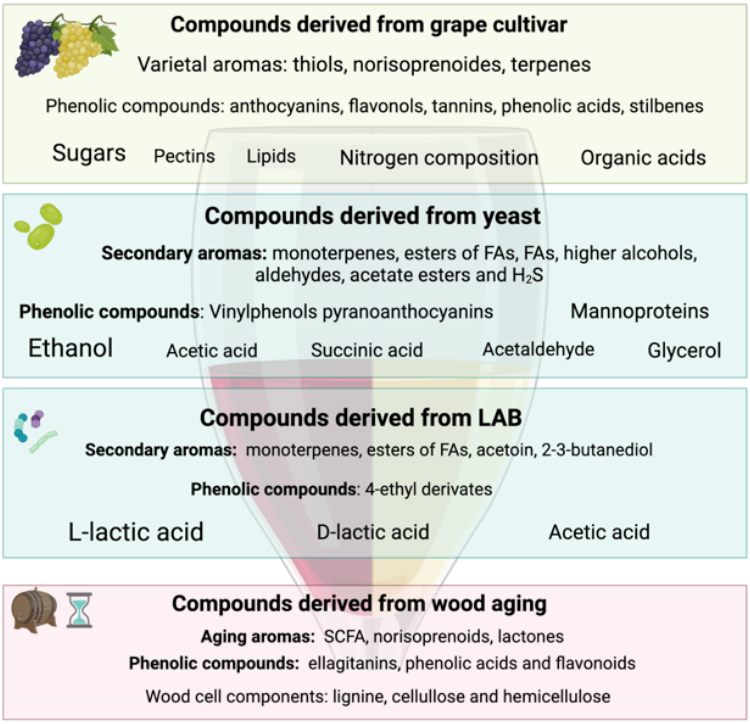

Figure 5. Main wine compounds derived from grape berries, yeast metabolism, Lactic Acid Bacteria (LAB) metabolism and wood aging. FAs correspond to Fatty Acids and SCFA correspond to Short Chain Fatty Acids.

2.3. Microbiological interactions

The microbial ecology that develops in wine involves various interactions between different groups of microorganisms, species, and strains. It is not always possible to enhance or avoid these desirable or undesirable interactions, respectively. However, with the increasing

knowledge in this field and the wide availability of starter cultures, winemakers now have the means to better control these interactions, allowing them to produce wines that best align with the preferences of the oenologist (Englezos et al., 2022). Given the complexity of the wine matrix (Figure 5), which varies significantly between different types of vinifications, grape cultivars, production areas, and more, understanding these interactions requires further study. It can be challenging to attribute the effects of specific compounds, making research in this area essential for the continuous improvement of winemaking processes.

2.3.1. Yeast-yeast interactions

In the context of winemaking, it is essential to examine the interactions between the two yeast groups: *Saccharomyces* and non-*Saccharomyces* yeasts. As mentioned earlier, the dominance of non-*Saccharomyces* is typically followed by the presence of *S. cerevisiae*. However, the dynamics of yeast populations depend on the type of fermentation, whether it is sequential or inoculated.

In the case of inoculated AF, it is common to first combine non-*Saccharomyces* yeasts to take advantage of their properties, and then inoculate *S. cerevisiae* to ensure a successful fermentation process. This combined inoculation, utilizing selected or commercial yeast strains, offers various options that can have different effects on the final wine characteristics and the dynamics of yeast populations (Martín-García et al., 2020; Roullier-Gall et al., 2022; Taillandier et al., 2014; Zhao et al., 2022). These options range from co-inoculation, where both types of yeast are inoculated simultaneously, to sequential inoculation, with varying times of contact for the non-*Saccharomyces* yeasts before inoculating *S. cerevisiae*.

Regarding interactions between yeast species, it is possible to distinguish between mutualism, when one specie benefits from the metabolites of the other, and competence or amensalism, when species compete for the nutrients or produce inhibitory compounds (Figure 6).

In relation to nitrogen competition although some non-*Saccharomyces* yeasts can enrich the medium due to their proteolytic activity (Ciani et al., 2010) in general, their growth causes depletion of essential nutrients for *S. cerevisiae*. This is especially problematic, in combined fermentations with yeasts with high fermentative capacity, such as *T. delbrueckii* and *L. thermotolerans* compromising the imposition of *S. cerevisiae*. Especially in sequential

inoculations, since *S. cerevisiae* has problems to grow and consume sugars, more due to the nutrient limitation of that medium, than to the presence of antimicrobial compounds secreted by non-*Saccharomyces* (Roca-Mesa et al., 2022, Taillandier et al., 2014).

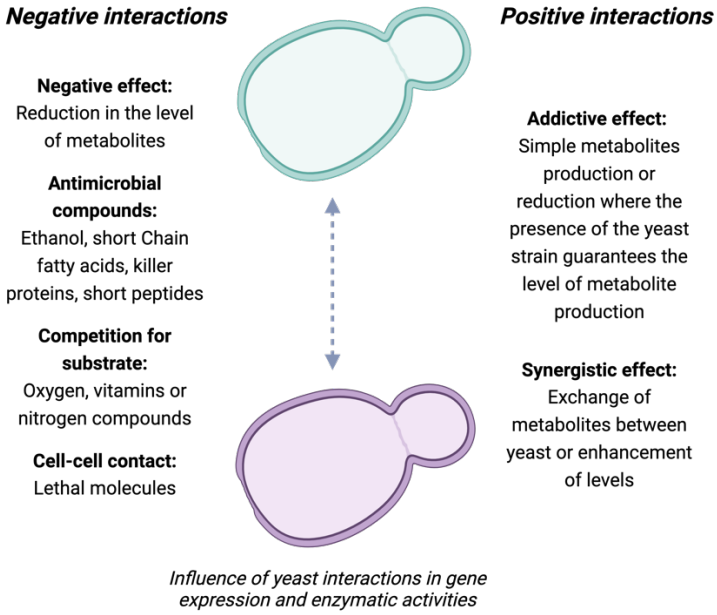

Figure 6. Possible yeast-yeast interactions during alcoholic fermentation (Adapted from Ciani et al., 2010)

Furthermore, some metabolisms are toxic for some yeast species, for instance ethanol which is more toxic for non-*Saccharomyces* (Ciani et al., 2010) since *S. cerevisiae* have a higher resistance. However, the most significant compounds in terms of toxicity are the killer toxins, which are harmful to susceptible yeast species. Various phenotypes can be distinguished among yeast strains: killer strains, which generate killer toxins; sensitive phenotypes, vulnerable to toxins produced by other yeast; and neutral phenotypes, displaying resistance to the toxin without producing it. Interestingly, killer strains may also be susceptible to a different toxin the one they produce (Ribéreau-Gayon et.al, 2006b). The killer phenotype has been widely described in *S. cerevisiae*, but also it has also been described in some strains of *T. delbrueckii* (Ramírez et al., 2016; Velázquez et al., 2015), *H. uvarum* (Mendoza et al., 2019) and *Pichia membranifaciens* (Santos et al., 2009).

During fermentation, yeast cells communicate with each other through the secretion of certain molecules that are recognized by other yeasts, thereby modifying their behaviour and controlling cell density. This phenomenon is known as quorum sensing and plays a significant role in cell-cell contact interactions observed in yeasts during the fermentation process. Studies have investigated cell-cell contact interactions in various yeast species, including *T. delbrueckii* and *S. cerevisiae*. It has been observed that when these species come into contact, it leads to the death of *T. delbrueckii* (Renault et al., 2015). A similar effect was found in the case of *L. thermotolerans*, together with antimicrobial peptides from *S. cerevisiae*. Furthermore, in the case of *Pichia kluyveri and S. cerevisiae*, their cell-cell contact results in a decrease in the population of both species (Hu et al., 2022).

2.3.2. Yeast – *O. oeni* interactions

The interactions between yeast and *O. oeni* have become a topic of great interest. Oenologists and researchers aim to identify the right yeast-LAB combinations to either avoid or enhance the MLF process in different types of wine. To gain a deeper understanding of this context, studying the composition of wine after AF is crucial, as it contains all the stimulating or inhibitory compounds that can influence the MLF (Figure 7). Besides wine composition, studies have shown that the combination of specific strains plays a significant role in determining the impact of these interactions (Alexandre et al., 2004; Balmaseda et al., 2018; du Plessis et al., 2017a; Lafon-Lafourcade et al., 1984). To fully explore this topic, it will be discussing not only *S. cerevisiae* but also non-*Saccharomyces* yeast due to their growing utilization in winemaking. There are recent studies which evaluate the good synergy between some non-*Saccharomyces* yeast and *O. oeni* (Ferrando et al., 2020), being the effect of *T. delbrueckii* the most studied (Balmaseda et al., 2023, 2021c, 2021b).

Regarding stimulatory effects, within the compounds released by yeast that are of more interest for enhancing MLF, are mannoproteins, which are released during the autolytic process of yeast in AF. Mannoproteins have multiple effects, with the main one being that they serve as a nutritional source for *O. oeni* (Jamal et al., 2013) producing a stimulatory growing (Diez et al., 2010). Additionally, they have been associated with detoxification of the media (Lafon-Lafourcade et al., 1984) by reducing MCFA concentration, which are reported

to be toxic for *O. oeni*, as MCFA can disrupt the fluidity of the cell membrane (Edwards and Beelman, 1987). Furthermore, it has been observed that non-*Saccharomyces* yeast, such as *M. pulcherrima* and *T. delbrueckii*, produce higher quantities of mannoproteins than *S. cerevisiae* after AF and during aging (Balmaseda et al., 2021a; Belda et al., 2016b; Ferrando et al., 2020). For instance, Balmaseda et al., (2021a) found that in wines fermented in the presence of *T. delbrueckii* genes related to mannose uptake of certain *O. oeni* strains showed an increased relative expression. This study also revealed that mannose metabolism was more active under stressful conditions in the presence of *T. delbrueckii*.

On the other hand, yeast have also an effect on the nutrient availability for *O. oeni* which have complex nutritional requirements (Terrade and Mira de Orduña, 2009). In this context after AF there are a different composition of nutrients that depends on the media (Arnink and Henick-Kling, 2005) and on the nutritional preferences of the inoculated yeast (Roca-Mesa et al., 2020) For example High fermentative yeast such as *T. delbrueckii*, or *S. cerevisiae* consume high concentration of amino acids producing wines with low amino acids content (Roca-Mesa et al., 2020).

Furthermore, the presence of inhibitory compounds for LAB derived from yeast metabolism can be reduced by certain non-*Saccharomyces* species, which may enhance the performance of MLF. Among these inhibitory compounds are found MCFA, which have been mentioned. Some non-*Saccharomyces* yeast species, such as *H. uvarum* (Hu et al., 2018; Liu et al., 2016), *M. pulcherrima* (Hranilovic et al., 2020), *L. thermotolerans* (Fairbairn et al., 2021), and *T. delbrueckii* (Balmaseda et al., 2021b), have shown lower production compared to *S. cerevisiae*, specially of octanoic and decanoic acids (Fairbairn et al., 2021), potentially reducing their toxic effect. However, its production, apart from being dependent from the nitrogen composition of must, is strain-dependent (Hu et al., 2018).

Ethanol content is another significant factor that can cause stress during MLF (Capucho and San Romão, 1994). However, studies have shown that certain non-*Saccharomyces* yeast can reduce the alcohol degree in wines (Contreras et al., 2014; Garciá et al., 2020; Hranilovic et al., 2020; Quirós et al., 2014; Zhu et al., 2021), which could ultimately enhance the activity of *O. oeni*.

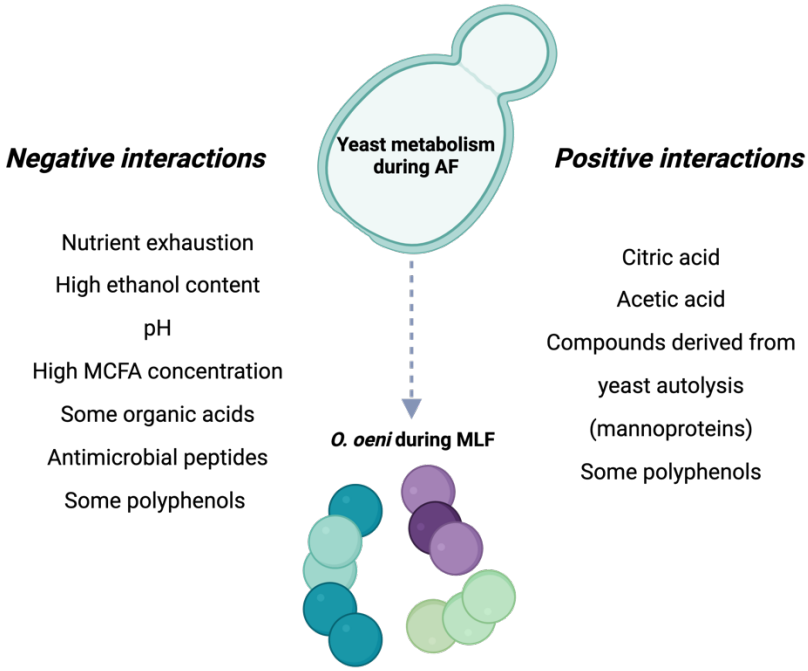

Figure 7. Yeast – *O. oeni* interactions during malolactic fermentation. Adapted from Balmaseda et al. 2018).

Sulphur dioxide (SO_2) is known to be inhibitory to LAB as it leads to a decrease in ATPase activity (Reguant et al., 2005), but the concentration produced by yeast is usually not sufficient to have a strong antibacterial effect.

Low pH is also a key aspect affecting MLF performance by *O. oeni*. Some non-*Saccharomyces* species, like *M. pulcherrima* and *T. delbrueckii*, have been found to modulate pH (Martín-García et al., 2020) potentially increasing it (Balmaseda et al., 2022a; Chen et al., 2018). Conversely, *L. thermotolerans* it is used to acidify the wines and consequently can decrease the pH of wines, affecting MLF. Furthermore, the high lactic acid concentration produced by *L. thermotolerans* can lead to an inhibitory effect, since it can be transported into the interior of the cell, lowering the intracellular pH and causing stress on *O. oeni* (Snyder et al., 2021).

Succinic acid is another compound related to the inhibition of MLF, as it can competitively inhibit the active site of the malolactic enzyme. Torres-Guardado et al. (2022b) described a reduction of malolactic activity with a concentration of 1 g/L of succinic acid. Some non-*Saccharomyces*

species have been found to reduce succinic acid production, although the effect seems to vary depending on the strains used, since (Hranilovic et al., 2020) showed an increase in succinic acid in wines with *M. pulcherrima*, while Martín-García et al., (2020) observed a slight reduction in wines with *M. pulcherrima* and *T. delbrueckii*. In sequential fermentations with *T. delbrueckii* strains some authors did not observe significant changes when comparing with *S. cerevisiae* (Ferrando et al., 2020; Torres-Guardado et al., 2023). However, some authors observed a decrease (Balmaseda et al., 2022a), and others an increase (Contreras et al., 2014).

Phenolic compounds have been described as potentially stressful for *O. oeni*, although the impact depends on their specific structure (Reguant et al., 2000). Interestingly, *T. delbrueckii* has been reported as an enhancer of MLF, even in the presence of high polyphenolic content (Balmaseda et al., 2021b).

Table 3. Challenges and opportunities for future wine microbiology research (Englezos et al., 2022).

Challenges	Opportunities
Further investigation of the interaction mechanisms among wine microorganisms	Full control of the fermentation process and improved management of specific microorganisms using omics-based technologies
Integration of the knowledge of microbial dynamics and their impact on wine	Modulation of the concentration of specific metabolites in wine
Exploration of the potential of omics-based technologies in wine production	Better prediction of the behaviour of microorganisms during fermentation
Production of wines with less SO_2 by using bioprotective microorganisms	Fulfilling consumer demands for wines free of chemical additives considered negative for health
Accessing the impact of low SO_2 addition to microbial interactions	Improved management of SO_2 in wines

Nevertheless, further research is necessary to better understand interactions between microorganism in wine matrix (Table 3). Being particularly interesting the role of yeast – especially non-*Saccharomyces* – in MLF. In addition to strain compatibility, it is important to study how the inoculation strategy or contact time may interact with *O. oeni* activity, and how these interactions could affect other types of vinifications beyond traditional white and red wines. In this context is important to consider all of these interactions to better choose a starter culture and develop new ones.

3. Winemaking techniques

Winemaking is a complex process influenced by multiple factors. In this introduction, we have explored how grape composition, varieties, microbiological dynamics and species interactions contribute to the diverse quality and composition of wines. Therefore, in addition to managing viticulture and fermentations, winemaking practices play a vital role in achieving the desired end product.

Traditional white, red and sparkling wines have historically dominated in terms of production volume worldwide. However, there are other types of vinifications with rich traditions, such as rosé, skin-macerated white wines, high sugar-wines, fortified wines or wines of carbonic maceration, among others, which have received comparatively less research attention. Nevertheless, due to their distinct vinification processes and resulting wines, these vinifications deserve deeper investigation.

Moreover, some *special* vinifications are now gaining popularity in current oenology and an increasing number of wineries are producing them. Therefore, extensive research in these wines is necessary to gain a comprehensive understanding of their unique compositions and how improvements studied in red and white wines can translate to these diverse types of wines. In the following sections we will comment the principal aspects of the different types of vinifications as well as innovative techniques applicated.

3.1. Winemaking process: general steps

In general, the winemaking process follows several key steps, but there are variations among different techniques, which will be explained in the following sections (Figure 8).

Among these processes, various control analyses, corrections with additives, transfers between tanks or are performed. Another crucial point before the processing of the grapes is the time of harvest whether manual or mechanized. Depending on the sanitary state and the technological (sugar/total acidity ratio) or phenolic maturity of the grapes, variants (thermovinification, pre-fermentation maceration, etc.) for the type of vinification can be introduced but the fundamental steps are as follows (Ribéreau-Gayon et al., 2006b):

- Grape selection: Wineries aiming for high-quality wines excellence will carefully choose undamaged grapes either by hand or using a sorting table.

- Destemming: This process consist of the separation of the grapes from the stems, leaves and any wood part. It is done in order to reduce the excessive astringency caused by tannin concentrations of the stem, or any green taste and consequently improve the organoleptic quality.

- Grape crushing: Grapes are crushed to facilitate pressing. This process is performed carefully to avoid seed crushing, which would lead to increased astringency and green flavours.

- Must racking or debourbage: In rosé and white wines, this process involves the precipitation of grape must solids to obtain a clearer must. It is usually performed at low temperatures, and sometimes pectolytic enzymes are used.

- Maceration: During this process, the grape skins are in contact with the fermenting must for varying durations, allowing for the extraction of aromas and phenolic compounds. The duration of maceration can be adjusted to achieve desired flavour profiles and characteristics in the final wine.

- Fermentation process: This includes both alcoholic and malolactic fermentations, as detailed previously on section "2. Wine fermentation".

- Devatting: After maceration, the grape skins are separated from the wine, and the wine is transferred to another tank.

- Pressing: Pomace is pressed to extract the remaining liquid. The type of press, pressure, and number of press cycles influence the final quality, with the first press cycle yielding wine of better quality.

- Aging: Final steps may involve storing wines some time to achieved final characteristics. Within the possibilities wines usually undergo wood aging in barrels, although newer materials (concrete, clay, …) are now used. After aging, the wine goes through additional aging in the bottle.

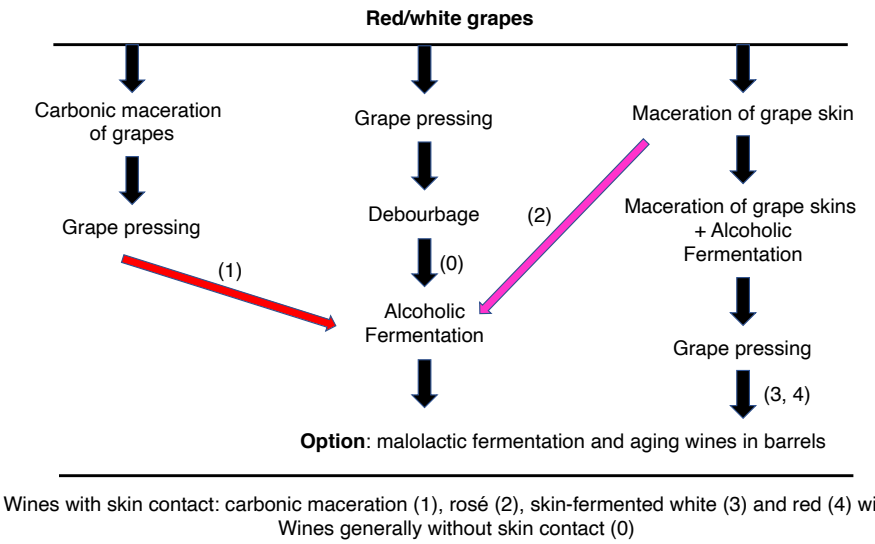

Figure 8. General steps and modifications of winemaking.

3.2. White winemaking

White wines are also well appreciated and involve a wide variety of types. It is worth noting that white wines are not always produced with white grapes, since there are white wines made from red grapes, such as *blanc de noirs* from Champagne, made from Pinot noir grapes. In this case grapes, with low phenolic concentration, are pressed in conditions that prevent anthocyanins extraction (Ribéreau-Gayon et al, 2006b).

White wines are pressed and do not have maceration with the skins as in red wines. However, this do not mean that white wines may not have maceration since before pressing during the pre-fermentation phase sometimes a pre-fermentative maceration in the absence of alcohol is performed in order to extract volatile aromas and precursors from grape skins.

In white wines one of the most important parts is the rigorous control of the pre-fermentative process avoiding the oxidation of grape must. This is of outmost importance in white vinifications due to the low phenolic concentration, as these compounds serve as oxidation barrier in red vinifications being the critical process steps: transfers and grape must movement. Grape must browning could be controlled with SO_2, due to the antioxidant properties of this additive, however, with the increasing trend of reduce SO_2 addition, other

browning control proposals have appeared as using glutathione or bio-protection with *M. pulcherrima* (Giménez et al., 2023).

Regarding MLF is not a common process in white wines. Nevertheless, traditionally, some Chardonnay wines, especially from Burgundy, are famous by their complexity and smoothness, due to an on-lees aging and the performance of MLF, which also provides their characteristic buttery aromas (Semon et al., 2001). Furthermore, the acidity in white wines is very important, but with climate change new problems have emerged such as the imbalance of grape maturity (decreased acidity while having a great concentration of sugars) and the emergence of cooler new production areas which leads to higher acidity. In this context in cool areas the deacidification is need either by microbiological methods such as allowing MLF to happen or the use of *S. pombe* to reduce L-malic acid (Vicente et al., 2022; Vilela et al., 2019) or by physical methods as cryogenic freezing, electrodialysis, organic extraction, and anion-exchange resin (Li et al., 2019). In the same way warm areas are needing of acidification, with the most innovative strategies being the addition of fumaric acid (Gancel et al., 2022) and the use of *L. thermotolerans* (Escott et al., 2022; Vaquero et al., 2022)

Fining is a critical part of white vinification, as this type of wines are more exposed to visual evaluation. In relation to aging new winery trends have include this step in the vinification process, usually with on-lees aging (del Barrio-Galán et al., 2016) and the use of new - ancient - materials as concrete eggs or amphoras to perform this process in (Baiano et al., 2017; Gil i Cortiella et al., 2020; Nevares and Del Alamo-Sanza, 2021).

3.3. Red winemaking

Red wine is produced all over the world and is characterized by the maceration of the grape skin in order to extract colour. Red winemaking process is illustrated in Figure 9.

First, grapes usually are destemmed however, some regions use stems as a natural additive to enhance freshness, complexity, and phenolic structure, ultimately improving chemical stability during aging. This practice has been increasingly adopted in various wine production areas worldwide, although it may not be suitable for all grape cultivars or vintages (Blackford et al., 2021).

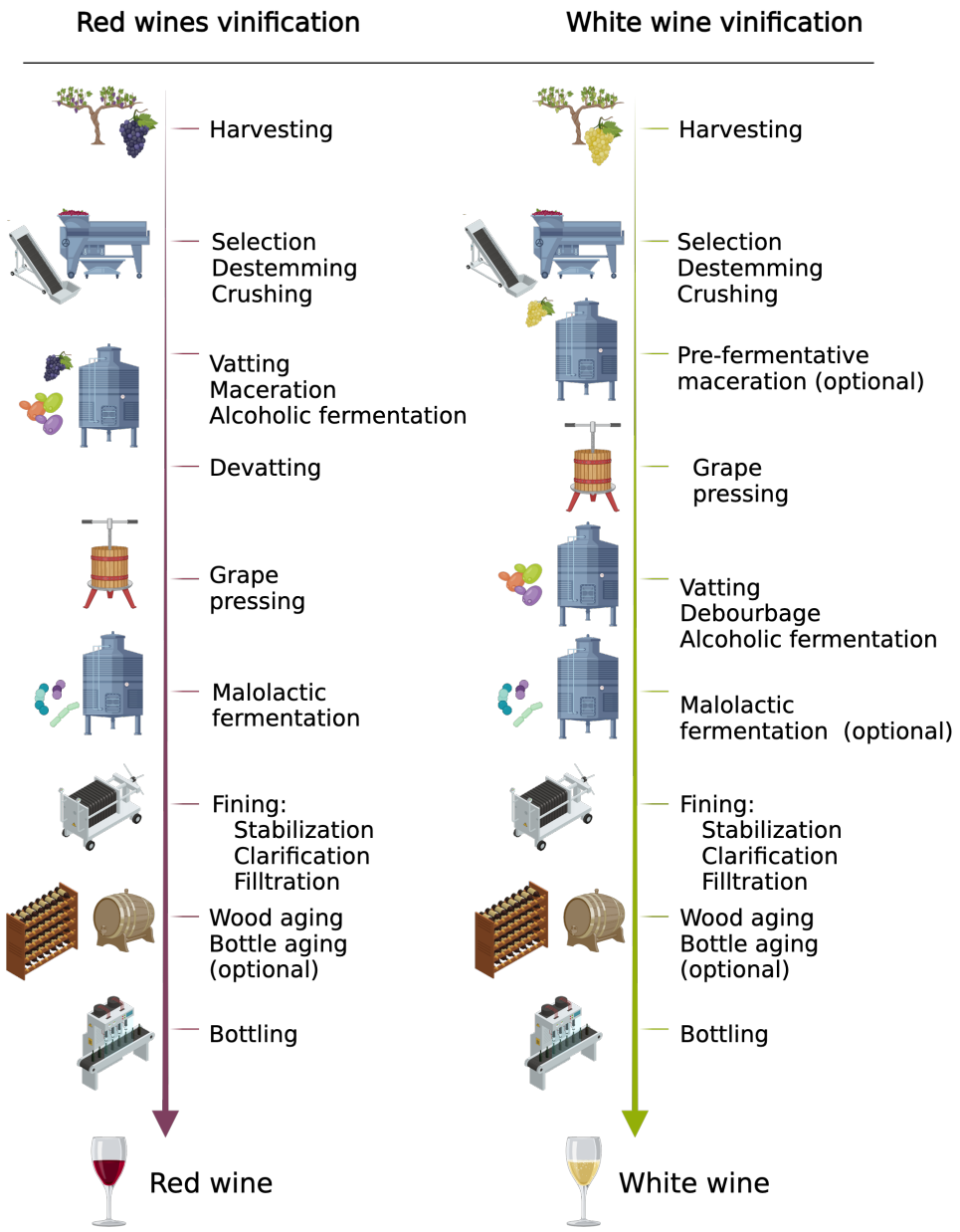

Figure 9. Red and white winemaking.

Following destemming and crushing, the must with pomace is vatted in a tank for AF and maceration. The maceration process involves a concentration-driven diffusion mechanism from grape berry skin into the must (Giacosa et al., 2023). As it has been described grapes must be on the optimum point of maturity to achieve the optimal accumulation of phenolic compounds. Grapes with low anthocyanin and tannin concentrations are used for young red wines, which are fresher and fruitier. In contrast, grapes rich in phenolic compounds are used for more complex wines that normally undergo an aging process. The extraction of phenolic compounds during vinification can be modulated to achieve the desired wine type and aging potential. Oenologists can influence the level of phenols in the wine through various winemaking practices, which not only affect the colour but also the taste of the final product. Techniques such as the presence or absence of stems, time and temperature of maceration, addition of pectolytic enzymes, number of pumping-overs or punch-downs, pressure of the press, aging, or the use of specific microorganisms are used to modulate phenolic extraction, among other techniques (Ribéreau-Gayon et al., 2006b). During winemaking, numerous reactions occur between anthocyanins and tannins.

Climate change has led to an imbalance between technological maturity and phenolic maturity, resulting in wines with poor phenolic composition in warm climates. Several techniques are being studied to improve the phenolic extraction and address this issue, such as pulse electric fields (Ricci et al., 2020), ultrasound (Martínez-Lapuente et al., 2022) and the use of microwaves (Pérez-Porras et al., 2022), among others. However, the most widely adopted solution is pre-fermentation cold maceration, which involves keeping the crushed grapes at low temperatures, delaying AF and achieving greater phenolic extraction (Lasanta et al., 2023).

Once AF is complete, a post-fermentative maceration can be prolonged until the winemaker determines the optimal vatting time based on the desired wine type. After this, the wine is run off the tank, and the pomace is devatted. MLF almost always takes place in red wines, either in stainless steel tanks or sometimes in barrels, to decrease pungent acidity in red wines and obtaining a well-rounded wine. Finally, fining, which includes tartaric stabilization, clarification, and filtration, is an optional step recommended for some types of wines, that would be performed after aging if the latter is performed. This process is performed by using

of enzymes or coagulants, and cold storing to precipitate molecules and avoid these processes to occur in the commercialized bottles.

Regarding aging in red wines, it is a complex process involving many chemical changes during both wood and bottle aging. It is worth noting that new alternatives are emerging such as woods different from oak for barrel aging (del Alamo-Sanza and Nevares, 2018; Martínez-Gil et al., 2022) and other materials such as concrete, clay, etc (Guerrini et al., 2022; Nevares and del Alamo-Sanza, 2021).

3.4. Rosé winemaking

Rosé wines are the wine type less valuated for the medium consumers, however due to the complexity to obtain good quality and organoleptic complex rosé wines it is a very interesting type of vinification. Regarding the grapes used in general are red grapes, however in some vinification areas are authorized due to a traditional use, the blend of red and white grapes. In relation to their production, rosé wines are usually classified by their colour intensity, exiting a wide spectrum, whose elaboration depends on the area, consumer preferences, trends, etc. We are going to distinguish between intense rosé wines and blush rosé wines (Figure 10).

Blush rosé wines, whose name comes from *California blush wines*, are elaborated as a white wine, without presence of grape pomaces during AF. The grapes are directly pressed; however, a pre-fermentative maceration is performed in this case directly in the press cage to extract a bit of colour. These wines are characterized by their pale colour and freshness in aroma and taste (Ribéreau-Gayon et al., 2006b).

Regarding intense rosé wines are produced also similarly to white wines, however in this case the pre-fermentative maceration in tank, usually during a range from 2 to 20 hours, is required to extract more phenolic compounds. However, excessive skin contact may result in excessive colour, astringency and bitterness. These wines are deeper-coloured and have more body and complexity (Ribéreau-Gayon, 2006b).

Figure 10. Macerated and direct pressing rosé winemaking.

As in white wines, in rosé wines it is necessary to keep the aromas and protect the colour, this way the protection against oxidation is quite important. The research interest for this type of wine is growing, being mainly focus on colour protection and improve its aromatic characteristics. New technological (Gutiérrez-Escobar et al., 2023; Labrador Fernández et al.,

2023; Leborgne et al., 2023; Zhang et al., 2023) and microbiological (Del Fresno et al., 2021; Mulero-Cerezo et al., 2023; Puyo et al., 2023) approaches are being tested for the colour protection of must, fermentation, and evolution on bottle.

Decades ago, the MLF in rosé wines was not performed since they were intended to be fresh and fruity wines. However, now with the acidity changes discussed in "3.3 White wines" section and the change of consumer preferences, MLF may be also performed on rosé wines (Dimopoulou et al., 2022). This process makes the rosé wines fuller and soft, which becomes more necessary as the maceration time increases. However, sometimes it is difficult to perform it since it requires a moderate sulfiting which may be a risk for the colour (Ribéreau-Gayon, et al. 2006b).

3.5. Carbonic maceration winemaking

Carbonic maceration wines are technically a red wine vinification, however due to their special characteristics regarding production a different section is specifically dedicated. Areas such as Beaujolais, Rioja, Burgundy or Riceys in Champagne have experimented an increasing interest in this vinification during the last decades. Traditionally, other areas have produced wines with method similar but not equal to carbonic maceration such as Georgia (Ribéreau-Gayon et al., 2006b). For example, Beaujolais Nouveau is the most famous and popular carbonic maceration red wine made from Gamay grapes produced in the Beaujolais region of France, officially released on the third Thursday of November in the year of its production.

Invented by Michel Flanzy in 1934, this specific winemaking process is characterised by vinified the whole grape bunches without crushing or destemming into a closed tank with a carbon dioxide-rich atmosphere (Tesniere and Flanzy, 2011), (Figure 10). The tank is filled with carbon dioxide (CO_2) to create an anaerobic environment in order to enhance the anaerobic fermentative mechanism produced inside the grapes (Tesniere and Flanzy, 2011). During this firs stage absorb variable amounts of CO_2, depending on the harvest temperature. Then the anaerobic metabolism starts which is associated with different intracellular changes in berries. The temperature control is important in this phase, as it influences the intensity of the anaerobic metabolism.

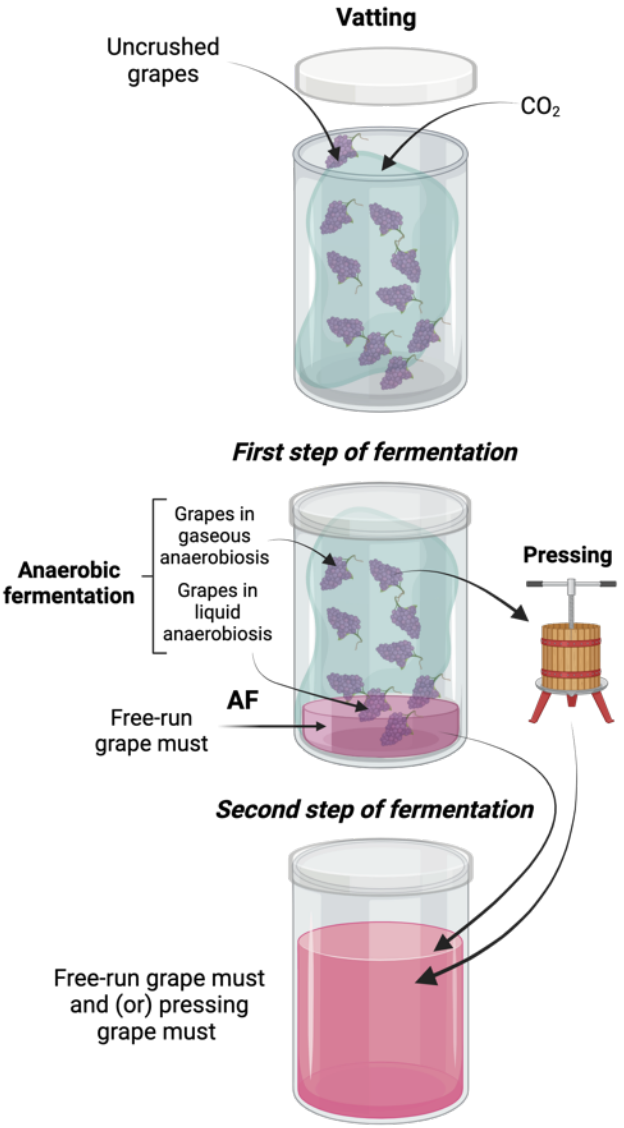

Figure 11. Carbonic maceration process (Adapted from (Tesniere and Flanzy, (2011).

Due to the intracellular swelling caused by the CO_2 production, grape broke down and a fraction of grape must is released and deposited at the bottom of the tank. In this context several reactions are produced since there is three different material phases (Figure 12): liquid (free-run must), solid (grapes) and gaseous (CO_2). During this process small amounts of ethanol are produced variably due to the process temperature (1.5 – 2.0 %, v/v), and other

secondary products such as glycerol and acetaldehyde are accumulated. At the same time, a degradation of L-malic acid is produced as it is catabolized to ethanol or succinic acid. Furthermore, a diffusion of phenolic and aromatic compounds from the skin to the flesh is produced. In addition, another compound very characteristic of the intracellular metabolism of berries under anaerobic fermentation is the formation of isoamyl acetate as a fruity, banana-flavoured ester.

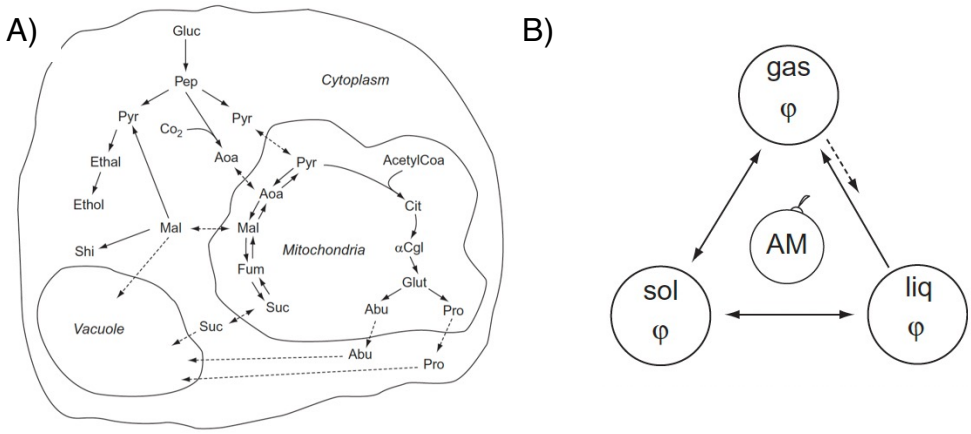

Figure 12. A) Interactions among various phases during the initial phase of fermentation in the carbonic maceration winemaking method. AM represents the anaerobic metabolism of grape berries; gas (gaseous phase); sol (solid phase, grape berries) and liq (liquid phase, must). B) AM within a grape berry cell. Solid arrows indicate the pathways of anaerobic metabolism in grape berries under hypoxia. Dotted arrows depict the exchanges occurring between different cellular compartments (Tesniere and Flanzy, 2011).

Simultaneously, a lot of changes are produced in the free-run must as a consequence of the interaction produced in grape and the microbiological action. Yeasts become to growth and becomes a spontaneous AF. Through diffusion, an exchange of volatile compounds released from the fermentation that is produce to the grapes not immersed in the free-run must.

Afterwards, the process enters its second phase where the grapes are pressed and based on the decision of the oenologist, the pressed wine, with better quality, is vinified with the free-run wine or both fraction by separated (Figure 11). Due to the presence of LAB in the grape surface sometimes the MLF starts spontaneously, in this case is recommended to vinify both fractions separately. This is because the higher sugar content in the free-run grape must together with the presence of LAB, can potentially lead to lactic spoilage and high acetic acid

concentration (Ribéreau-Gayon et al., 2006b; Tesniere and Flanzy, 2011). AF usually last from 2 to 7 days depending on the temperature, nitrogen conditions, etc. Then the MLF takes place, however sometimes due to improper management both fermentations may occur simultaneously. Due to these complex processes and microbiological interactions the resulting wines have very interesting organoleptic characteristics which are very appreciated by consumers. Carbonic maceration wines are characterized by high content of esters and acetates, which increase their fruity aroma, especially with high concentration of isoamyl acetate and ethyl cinnamate (Antalick et al., 2014; González-Arenzana et al., 2020). Concerning colour composition, in comparison to traditional red wines, they have a very different phenolic content, influenced by factors including grape variety, ripeness, vintage, and winemaking conditions (Chinnici et al., 2009; González-Arenzana et al., 2020; Portu et al., 2023).

Nevertheless, a certain disparity exists concerning the aging potential of carbonic maceration wines. While some experts comment that these wines retain their characteristic properties for up to two years of aging, others argue that beyond a year of aging, these wines may not exhibit proper evolution, suggesting that they not evolve as harmonious as the traditional red wine (Ribéreau-Gayon, et al., 2006b).

Despite the limited research on carbonic maceration wines, most of the existing studies focus on studying their colour composition (Castillo-Sánchez et al., 2006; Chinnici et al., 2009; González-Arenzana et al., 2020; Pace et al., 2014; Portu et al., 2023). However, there are also some recent studies which characterize the diverse autochthonous microbial population of these type of wines (González-Arenzana et al., 2020; Gutiérrez et al., 2023, 2022; Guzzon et al., 2020).

3.6. Skin-fermented winemaking

Orange wines or skin-fermented of white grapes, a distinctive and historically significant style of winemaking, have gained considerable attention. As discussed in the "3.2 White Winemaking" section, pre-fermentative maceration is occasionally employed. However, there exists an alternative approach involving the fermentation of white grapes as in red wine vinification, whereby they are fermented in the presence of grape pomace (see Figure 13).

The origin of wine traces back 8000 years to the South Caucasus, which it was also the birthplace of orange wines, situated in the present-day Republic of Georgia. Ancient Georgian winemaking involved fermenting white grape must with skins completed with a later aging ranging from months to years within a distinctive large amphora named "kvevri," with capacities of 2 to 15 litres (Bene and Kállay, 2019; Glonti, 2001). This traditional method still endures in Georgia and was acknowledged in 2013 as part of the UNESCO Representative List of the Intangible Cultural Heritage of Humanity (Zsuzsanna Bene, 2018). Over the last decade, diverse European winemaking regions, including Italy's Friuli–Venezia Giulia Wine Region, Slovenia and Croatia, and more recently Romania, Hungary, Spain, France, Chile, Australia, South Africa, Oregon, and California, have incorporated this technique (Bene and Kállay, 2019). Notably, the OIV has added "White wine with maceration" to its International Code of Oenological Practices, marking it as the eighth special wine category recognized by the OIV (Resolution OIV-ECO 647-2020).

Pre-fermentative skin maceration is a more extensively explored and practiced method. Its aim is to elevate phenolic compounds that contribute to the aging potential of white wines, enhancing aromatic extraction without altering the colour profile or inducing excessive tannin concentration and astringency (Cheynier et al., 1989; Darias-Martín et al., 2000; Falqui and Fernandez, 1996; Ferreira et al., 1995; Marais, 1998). Achieving the appropriate level of aromatic grape ripeness is crucial to prevent the extraction of undesirable herbaceous traits (Sokolowsky et al., 2013). Studies have indicated that conducting this maceration at low temperatures for 15 hours enhances the presence of aromatic fruity esters (Marais, 1998).

Nonetheless, the concept of skin-fermented wines denotes a distinct product and objective. The outcome of skin fermentation is a highly aromatic wine with an amber or orange hue and a fuller-bodied and tannic profile compared to conventional white wines (Jackson, 2008). Despite this, the study of skin-fermented wines and their characteristics remains relatively limited. Current research often centres on its phenolic composition. Bene and Kállay, (2019) observed an elevation in its polyphenolic content, particularly catechin. Sokolowsky et al., (2013) investigated the sensory attributes of wines produced from aromatic grape varieties such as Riesling and Gewurztraminer, demonstrating that it enhances the varietal aromas. The obtained skin-macerated wines had an increase in bitterness and colour in Riesling wines,

while Gewurztraminer wines exhibited significant differences in bitterness, sweetness, sourness, astringency, colour, and certain aromas. Despite the growing popularity of skin-fermented wines, further research is essential for a better understanding about the composition of skin-fermented white wines and to enhance their distinctive attributes.

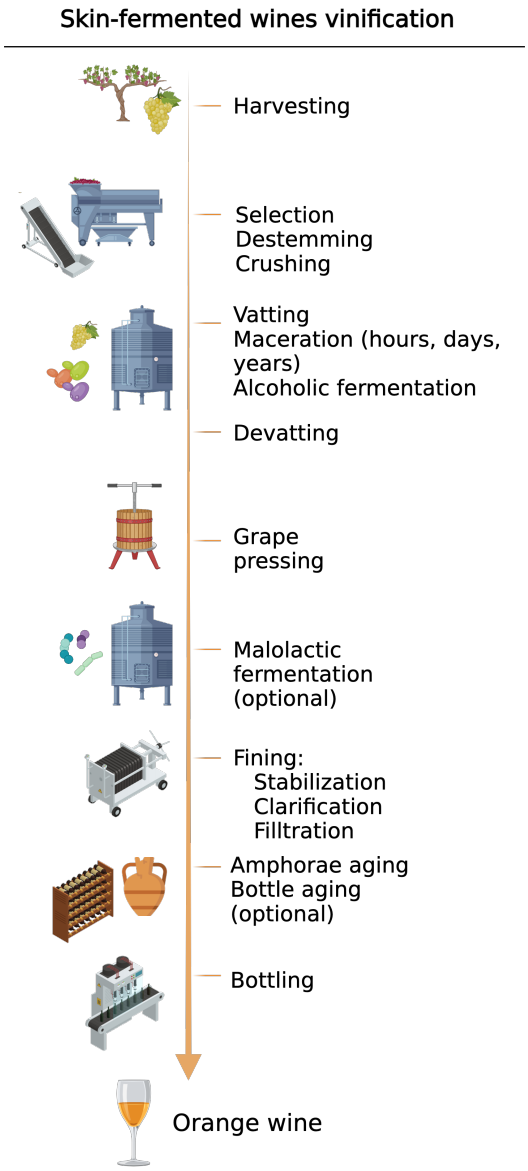

Figure 13. Skin-fermented White wine winemaking.

3.7. Climate change and new oenological practices

Climate change is exerting its impact on global agriculture, and viticulture is certainly not exempt from this challenge. The rising temperatures and scarce precipitation (with periods of too many precipitations) in the conventional wine-producing regions present a complex and significant threat to wine production, affecting both vineyards and the winemaking process in diverse manners. It is essential to underline that these established winemaking regions are undergoing changes, with warmer zones potentially becoming arid in the near future, thus hindering grapevine cultivation. Nonetheless, there are also emerging new wine production regions in cooler climates, primarily in the northern hemisphere's north and the southern hemisphere's south (Droulia and Charalampopoulos, 2022; Verdugo-Vásquez et al., 2023).

The Impacts of climate change are initially found in the vineyards, affecting grape quality and yields, and consequently influencing overall wine characteristics. While the primary challenges arising from climate change in the preceding sections were introduced, we will now delve deeper into these changes and explore some of the current research solutions.

Starting with grapevines, the epicentre of this issue, as elucidated in "1. Grape and Must Composition" section, is grape composition as it is undergoing transformations due to reduced water availability and elevated temperatures, which trigger heat and water stress. These stresses directly affect vine physiology and, consequently, the grape berry composition (Rogiers et al., 2022) The notable alterations include heightened sugar concentrations, diminished acid levels, and the creation of a disparity between phenolic, aromatic and technological maturity, all while impacting overall yield (Mira de Orduña, 2010).

Mitigation strategies from vineyard management are being proposed to address these concerns, encompassing the search for alternative grape cultivars and rootstocks that better suit the changing conditions. Additionally, the implementation of efficient irrigation strategies, delayed pruning, diverse considerations for row orientation, training systems with canopy manipulation, sustainable vineyard floor management (such as cover cropping), and sequential harvesting are being explored (Rogiers et al., 2022; Sun et al., 2023). Furthermore, the search for cooler or higher altitude areas for vineyard expansion has also been proposed (Arias et al., 2022).

Apart from these challenges, climate change leads to other problems directly or indirectly linked to grape cultivation, including smoke taint (Mirabelli-Montan et al., 2021) waterlogging, increased plague and disease prevalence in grapevines, and shifts in soil properties (Rogiers et al., 2022). In response to these multifaceted challenges, the concept of precision viticulture has emerged. This approach involves new technologies like remote sensing, drones, sensor networks and geographic information systems (GIS) to gather and analyse data on vineyard parameters. Then resulting insights, the specific information is then used to make targeted decisions focused on manage a better control over these complex issues in specific field areas or vine zones (Sun et al., 2023).

Upon arrival at the winery, the altered grape berry composition brings forth a range of challenges in AF, MLF and the final quality of the wine. AF and MLF faces a significant concern with the increase of ethanol content, potentially leading to sluggish or stuck fermentations (Gutiérrez-Gamboa et al., 2021). Moreover, the high alcohol levels can adversely affect the wine's sensory attributes, contributing to imbalances alongside decreased acidity (Jones et al., 2005). Consumer trends shown less acceptance for high alcoholic wines as alcohol consumption is experiencing a decreasing trend. Furthermore, wines with high alcohol content face elevated taxes in several countries (Elder et al., 2010) and the consumption of such wines can raise health-related concerns (Saliba et al., 2013) Several strategies have been proposed to address the challenge of high ethanol content, with some focused -on fermentation techniques. An effective approach, as was detailed upon in the "2.1.3. Non-*Saccharomyces* Yeast" section, involves the selection of starter cultures from specific yeast species like *M. pulcherrima* or *T. delbrueckii* due to their lower transformation rate of sugars to ethanol (Contreras et al., 2014; Garciá et al., 2020; Quirós et al., 2014; Zhu et al., 2020).

Another strategy for alcohol content management involves the management of yeast activity through controlled moderate temperatures and restricted nutrient availability, effectively slowing down fermentation and reducing alcohol production (Sam et al., 2021). Scarce nutrient availability generates that yeast metabolism use sugars to produce stress response molecules, decreasing the amount of sugar available for fermentation. An interesting proposal under research is the addition of water to the grape must, to dilute sugars and diminish osmotic pressure, or substitution of must by water making constant the

amount of final wine. of water to the grape must, These processes have been predominantly studied in red wines, achieving ethanol reductions ranging from 0.6% v/v to 5.9% v/v (Gardner et al., 2022; Piccardo et al., 2019; Schelezki et al., 2020; Schelezki, Smith, et al., 2018; Schelezki, Šuklje, et al., 2018; Teng et al., 2020). However, the allowance authorization for water addition varies across countries and regions, with California and Australia permitting it for facilitating AF, while the European Union, South Africa, Chile, and other wine-growing areas typically restrict it unless specific additive dissolution requirements are need (Xynas and Barnes, 2022). Furthermore, technological avenues exist for post-fermentation alcohol reduction, including membrane separation techniques, such as nanofiltration, reverse osmosis and pervaporation, and thermal distillation techniques which involves vacuum distillation and spinning cone column (Sam et al., 2021).

Another significant challenge faced by wines from current warm regions is the decline in acidity. Beyond viticultural interventions, acidity can be increased through the direct addition of approved acids, namely tartaric acid, citric acid, malic acid, lactic acid, and more recently, fumaric acid (Gancel et al., 2022). Moreover, techniques such as ion exchange resins, electro membrane treatments, and biological acidification can be employed to enhance acidity (Vicente et al., 2022).

Biological strategies for acidity management include the use of specific species. Some strains of the *Saccharomyces* genus have also been explored for their propensity to overproduce L-malic or succinic acid (Vicente et al., 2022). *Candida stellata*, which produces more succinic acid, while *Starmerella bacillaris* is able to produce α-ketoglutaric and pyruvic acids (Vicente et al., 2022). Additionally, *L. thermotolerans* produces lactic acid in significant quantities, although its effectiveness is strain-dependent. Among these options, *L. thermotolerans* stands out due to its efficiency and wide commercial availability (Escott et al., 2022; Fairbairn et al., 2021; Vaquero et al., 2022).

However, as previously mentioned in the section "3.2. White winemaking", the migration to cooler regions can lead to an excess of acidity, necessitating de-acidification measures. In such situations, various technological approaches can be employed, including cryogenic freezing, electrodialysis, organic extraction, anion-exchange, reverse osmosis, as well as the addition of calcium carbonate or potassium bicarbonate (Li et al., 2019; Ribéreau-Gayon, et al., 2006b).

Biological deacidification is closely linked to malic acid metabolism, with the most common method being MLF Typically, the LAB species *O. oeni* is employed due to its excellent attributes and adaptability, although some strains of *L. plantarum* are also utilized as starter cultures. While both species exhibit comparable deacidification efficiency, there exist differences in their adaptation. *O. oeni* shows enhanced ethanol tolerance, whereas *L. plantarum* demonstrates great resistance to high osmotic pressure (Liu et al., 2002; Vicente et al., 2022). Additionally, *S. pombe* is under investigation for this purpose, as it metabolizes L-malic acid into ethanol in a process called maloalcoholic fermentation. However, certain strains of *S. pombe* have the drawback of producing excessive amounts of acetic acid (Schoondermark et al., 2022).

Another significant issue arising from climate change is the discordance between technological maturity and phenolic maturity. This challenge entails a situation where grapes attain the correct ripeness in terms of sugar and acid concentrations, yet the phenolic compounds have not reached their optimal levels. If grapes are harvested in this moment, colour and phenolic extraction may be difficult and specific colour characteristics will not be easy to meet. However, if viticulturist waits to fully obtain optimal berry colour characteristics, grape sugars are excessive and sugar to acidity ratio is unbalanced. To address this, the most used techniques are the use of enzymes and the management of skin maceration durations and temperatures for effective polyphenol extraction while preserving other wine properties. However, various alternative approaches have been explored to enhance colour extraction. Among technological strategies, methods such as thermovinification, ultrasound-assisted extraction (UAE), pulsed electric field (PEF), microwaves, and cryomaceration have been employed. However, due to the higher costs associated with these processes, other alternative solutions are being sought. An emerging biotechnological alternative is the utilization of non-*Saccharomyces* yeast species. Some Certain non-*Saccharomyces* strains, owing to their exhibit high enzymatic activities, show promise in extracting which offer potential for enhance more phenolic extractions from grape skins (Balmaseda et al., 2021b; Chen et al., 2018; Escribano-Viana et al., 2019). Although further research is required, these non-*Saccharomyces* species hold potential promise as effective tools in enhancing to improve colour extraction

HYPOTHESIS AND OBJECTIVES

UNIVERSITAT ROVIRA I VIRGILI
NEW PERSPECTIVES ON MALOLACTIC FERMENTATION AND ORGANOLEPTIC IMPROVEMENT OF WINES: INFLUENCE OF TORULASPORA DELBRUECKII ON DIFERENT TYPES OF WINEMAKING
Candela Ruiz de Villa Sardón

The landscape of oenology is constantly evolving, driven by the need to address a variety of challenges, including the impact of climate change and shifting consumer preferences. In response, the integration of biotechnological advancements into the winemaking process has become an increasingly explored area. This research area focuses on the interaction of selected microbial species in relation to two stages of the winemaking process: alcoholic fermentation (AF) and malolactic fermentation (MLF). Particular emphasis is placed on the role of non-*Saccharomyces* yeast species, which have a significant impact on the aromatic profile and overall characteristics of wines. The complex interplay between various yeast strains can lead to complex metabolic interactions and activities, thus shaping the end result of MLF. This complex process is influenced both by climatic variations and by the specific type of winemaking, which makes its performance more or less desirable. In addition, the sensitivity of lactic acid bacteria to changes in the wine matrix adds an additional difficulty. The role of *Torulaspora delbrueckii* has been mainly studied in traditional vinifications, white and red wines, however, recently is investigated its application in other types of beverages such as high-sugar wines, cider or other fruit fermentations. In recent years, it has been described that its presence has the potential to modulate the availability of certain compounds that can facilitate the activity of *O. oeni* during the MLF. Further research is needed for other types of wine matrices, such as special winemaking methodologies, which are increasingly in demand by some consumers.

Among these conceptual changes, non-*Saccharomyces* yeasts have also been proposed as a solution to the challenge of high alcohol levels in wines due to climate change, notably involving sustainable technological interventions, such as the addition of water to the must. However, more research is needed in order to understand how effective are and how affect the organoleptic parameters of the final wine, especially in white wines.

Within this oenological context, the hypothesis of this thesis was that these innovative treatments have a different impact on the performance of both AF and MLF, consequently shaping the organoleptic characteristics of wines post-fermentation. Understanding the dynamics between non-*Saccharomyces* yeast, *S. cerevisiae* and *O. oeni* will help winemakers to make informed decisions about yeast selection and MLF management, ultimately contributing to the production of wines with specific and desired characteristics.

Therefore, the main objective of this study is to further investigate in more detail the impact of T. delbrueckii inoculation on sensory attributes as well as AF and MLF performance in specific types of wine, with the aim of providing more knowledge that can improve the effectiveness of MLF. Additionally, to suggest strategies to mitigate alcohol levels while preserving the different sensory qualities of the wines after AF and MLF. To achieve this main goal, four specific objectives are proposed, which will be developed through three chapters:

- **Objective 1:** characterize the impact of different *T. delbrueckii* and *S. cerevisiae* strain combinations on MLF and the inoculation strategy used. (Chapter 1: 1.1 and 1.2).

- **Objective 2:** formulate a synthetic grape must for conducting proper AF followed by MLF. (Chapter 1: 1.1 and 1.2).

- **Objective 3:** study the influence of *T. delbrueckii* on the sensory attributes and MLF progression in distinct vinification processes using natural grape must: rosé wines, carbonic maceration wines and skin-fermented white wines. (Chapter 2: 2.1, 2.2, and 2.3).

- **Objective 4:** assess the implications of adding pre-fermentation water and inoculating *Metschnikowia pulcherrima* as strategies to mitigate alcohol content on the sensory characteristics, as well as AF and MLF efficiency in white wines. (Chapter 3: 3.1 and 3.2).

RESULTS

UNIVERSITAT ROVIRA I VIRGILI
NEW PERSPECTIVES ON MALOLACTIC FERMENTATION AND ORGANOLEPTIC IMPROVEMENT OF WINES: INFLUENCE OF TORULASPORA DELBRUECKII ON DIFERENT TYPES OF WINEMAKING
Candela Ruiz de Villa Sardón

Chapter 1

Impact on malolactic fermentation of *Torulaspora delbrueckii*: inoculation strategy and strain combination on a new synthetic must

Along **Chapter 1** it is presented a comparative study using different strains of *T. delbrueckii* and *S. cerevisiae* for sequential AF, along with multiple strains of *O. oeni* for subsequent MLF. The main focus of this comparative study was to manage AF to obtain the desired parameters in wine AF and enhance the efficiency of MLF. Notably, previous research by our research group has highlighted the potential improvement of MLF through the use of *T. delbrueckii*. The experiments detailed in this chapter were conducted under controlled and reproducible laboratory conditions. These investigations were carried out using a newly developed synthetic must, which facilitated the performance of both fermentation processes.

In **Section 1.1**, several strains were examined to determine the most suitable *T. delbrueckii* - *S. cerevisiae* combination for AF, as well as the optimal *O. oeni* strain for subsequent MLF. The selected strain combinations from this selection process were subsequently employed in the following chapters, encompassing both synthetic and natural must fermentations. Additionally, the initial must composition was developed in order to carry out both fermentations successfully.

Section 1.2 delves into the study of different time of *T. delbrueckii* contact (2, 4, and 6 days), alongside a co-inoculation of *T. delbrueckii* and *S. cerevisiae*, compared to a sole inoculation of *S. cerevisiae*. This investigation involved the use of various strains identified in the previous characterisation, ultimately leading to the identification of the most effective inoculation strategy for MLF enhancement. Furthermore, the synthetic must was optimised by the addition of β-sitosterol and linoleic acid, resulting in a more efficient fermentation process.

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Section 1.1

Screening of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* strains in relation to their effect on malolactic fermentation

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Abstract

The use of *Torulaspora delbrueckii* in the alcoholic fermentation (AF) of grape must is increasingly studied and used in the wine industry. In addition to the organoleptic improvement of wines, the synergy of this yeast species with the lactic acid bacterium *Oenococcus oeni* is an interesting field of study. In this work, 60 strain combinations were compared: 3 strains of *Saccharomyces cerevisiae* (Sc) and 4 strains of *T. delbrueckii* (Td) in sequential AF, and four strains of *O. oeni* (Oo) in malolactic fermentation (MLF). The objective was to describe the positive or negative relationships of these strains with the aim of finding the combination that ensures better MLF performance. In addition, a new synthetic grape must has been developed that allows the success of AF and subsequent MLF. Under these conditions, the Sc-K1 strain would be unsuitable for carrying out MLF unless there is prior inoculation with Td-Prelude, Td-Viniferm or Td-Zymaflore always with the Oo-VP41 combination. However, from all the trials performed, it appears that the combinations of sequential AF with Td-Prelude and Sc-QA23 or Sc-CLOS, followed by MLF with Oo-VP41, reflected a positive effect of *T. delbrueckii* compared to inoculation of Sc alone, such as a reduction in L-malic consumption time. In conclusion, the obtained results highlight the relevance of strain selection and yeast-LAB strain compatibility in wine fermentations. The study also reveals the positive effect on MLF of some *T. delbrueckii* strains.

Keywords

Non-*Saccharomyces*, *Torulaspora delbrueckii*, *Oenococcus oeni*, synthetic grape must, alcoholic fermentation, malolactic fermentation.

1. Introduction

Winemaking is particularly influenced by microorganisms, which are responsible in a large part for the quality of the final product. Yeasts transform grape must sugars (glucose and fructose) into ethanol, carrying out alcoholic fermentation (AF), which is the main reaction of wine production (Ribéreau-Gayon et al., 2006b).

The other highlighted biochemical transformation is the malolactic fermentation (MLF), which is desired to happen in a wide range of wines, such as red wines and some specific white and *rosé* wines. In this case, lactic acid bacteria (LAB) decarboxylate L-malic acid into L-lactic acid. Apart from deacidification, MLF assures microbiological stability and improves organoleptic characteristics (Chambers and Pretorius, 2010; Paramithiotis et al., 2022). In addition, there are other interesting microbiological reactions such as those involved in secondary aromas, i.e., the production of ethyl esters or higher alcohols during fermentation (Belda et al., 2015; Carpena et al., 2021).

Saccharomyces cerevisiae is the species that mainly conducts AF of grape must due to its good fermentation ability (Ribéreau-Gayon et al 2006b). Nevertheless, there are a large number of yeast species with useful properties that are included as non-*Saccharomyces* yeasts. For many years, they have been considered undesirable microorganisms, but their use has increased (Jolly et al., 2014). Because they are present in grape and winery microbiota, they play an important role in spontaneous fermentation, giving varietal character to wines (Rossouw and Bauer, 2016). Some of the most relevant species are *Hanseniaspora uvarum, Starmerella bacillaris, Lachancea thermotolerans, Metschnikowia pulcherrima, Torulaspora delbrueckii* and *Zygosaccharomyces bailii* (Beltran et al., 2002; Du Plessis et al., 2017a; Fairbairn et al., 2021; Jolly et al., 2014). Most of these non-*Saccharomyces* enhance the aromatic profile of wines thanks to their enzymatic activities that release volatile compounds from their precursors (Belda et al., 2015; Carrau and Henschke, 2021; Padilla et al., 2016; Russo et al., 2020). Furthermore, some species have shown other advantages, such as the capacity to reduce ethanol content (Hranilovic et al., 2020; Zhu et al., 2021). Some of them are available as starter cultures and are also used by oenologists in the first stages of fermentation. *T. delbrueckii*, *M. pulcherrima* and *L. thermotolerans* are the most common ones and distributed as commercial products.

Recent studies show the synergy between non-*Saccharomyces* and *Oenococcus oeni*, the LAB species mainly responsible for MLF (Arnink and Henick-Kling, 2005; Ferrando et al., 2020; Paramithiotis et al., 2022). Specifically, several studies have revealed that wines fermented with *T. delbrueckii* can improve MLF performance and consequently the final product (Balmaseda et al., 2021b). Therefore, *T. delbrueckii* is a species with potential applications, for example, advantageous changes in organoleptic profiles of red wines, increasing their aromatic characteristics, complexity, mouthfeel and colour (Balmaseda et al., 2021b; Ramírez et al., 2016; Zhang et al., 2018).

Despite this improvement in wine quality linked to the use of non-*Saccharomyces* species, it is necessary to check the compatibility between strains of non-*Saccharomyces*, *S. cerevisiae* and *O. oeni*. Sometimes there may be a stimulatory effect between the yeast-bacteria strain combination, but there may also be complex inhibitions specific for each strain blend. Generally, non-*Saccharomyces* yeasts influence LAB development and consequently, the MLF, in a species and strain dependent manner (Alexandre et al., 2004; Du Plessis et al., 2017b; Englezos et al., 2022; Russo et al., 2020; Torres-Guardado et al., 2022a). The interactions between *Saccharomyces*, non-*Saccharomyces* and *O. oeni* are also dependent on the inoculation regimes of yeast and bacteria. Although here we have considered only the most usual way of performing MLF after AF, there is an emerging trend of co-inoculating bacteria with yeasts, and not only *O. oeni* but also other LAB (Bartowsky et al., 2015; Englezos et al., 2022).

The aim of this work was to study the potential suitability or incompatibility of a wide range of yeast-bacteria combinations in a synthetic grape must to perform AF followed by complete MLF to confirm the advantages of *T. delbrueckii*.

2. Materials and methods

2.1. Microorganism strains

Seven yeast strains were used to carry out AF. Three *S. cerevisiae* strains from Lallemand Inc. (Montreal, Canada): Lalvin-QA23 (Sc-QA), Lalvin-CLOS (Sc-CL) and ICV K1 Marquée (Sc-K1). Even though Sc-K1 is not a strain recommended for MLF by the manufacturer (Lallemand Inc.),

it was selected with the aim of observing whether its combination with *T. delbrueckii* improves the MLF performance.

In addition, four *T. delbrueckii* strains were tested: *Biodiva TD291* (Lallemand Inc., Montreal, Canada) (Td-B), Viniflora Prelude (Chr. Hansen Holding AS, Hoersholm, Denmark) (Td-P), NSA1 Viniferm NSDT (Agrovin, Spain) (Td-V) and Zymaflore Alpha (Agrovin S. A, Spain) (Td-Z). Yeast inocula were prepared from active dry yeast (ADY) for both species after rehydration according to the manufacturer's instructions, 37 °C and 30 °C for *Saccharomyces* and *Torulaspora* species respectively.

Regarding *O. oeni* strains, Lalvin VP41 (Lallemand Inc., Montreal Canada) (VP41), Viniflora CH11 (Chr. Hansen Holding AS, Hoersholm, Denmark), 1Pw13 (own collection, CECT 8893) and PSU-1 (ATCC BAA- 331) were used for MLF. LAB strains were preserved in MRS plates. MRS medium was supplemented with 4 g/L D, L-malic acid and 5 g/L fructose at pH 5. Then, isolated colonies from these plates were grown in MRS broth at 27 °C in a 10% CO_2 atmosphere. The inocula (2×10^7 cells/mL) were obtained from a pre-culture in the final phase of exponential growth cultivated in 50 mL of the same MRS medium under the same conditions for 3 days at 28 °C.

2.2. Alcoholic fermentation

A new synthetic must was developed in order to perform MLF after AF. Since studies which work with both, AF and MLF, usually tested fermentations in natural must (Ferrando et al., 2020; Martín-García et al., 2020). AF was performed in triplicate with a new synthetic must (110 g/L glucose, 110 g/L fructose, 5 g/L L-tartaric acid, 2 g/L L-malic acid, 0.5 g/L citric acid, 1.7 g/L yeast nitrogen base w/o amino acids and ammonium sulphate, 50 mg/L ammonium chloride and 1.505 g/L amino acid stock (Table SD1) with a total nitrogen content of 243 mg N per L at pH 3.5. The composition of this must is similar to that of natural must but without either lipid factors or phenolic compounds. Bottles of 500 mL were used for the fermentation of 450 mL at 22 °C with agitation at 120 rpm, using a cap with two valves that allows carbon dioxide to escape and sample extraction.

For the sequential fermentation, the four strains of *T. delbrueckii* were separately inoculated, followed by *S. cerevisiae* inoculation after 48 h. Fermentation with the three *S. cerevisiae* strains were used as a control. So, the number of different AF was four *T. delbrueckii* strains by three *S. cerevisiae*, plus the controls with only *S. cerevisiae* strains, *id est*, a total of 15 wines obtained. Each one was performed in triplicate. The initial population inoculated was 2.5×10^6 cells/mL for both species.

Samples were taken every two days, and the fermentation kinetics were monitored by measuring the density of a centrifuged sample with an electronic densimeter (Densito 30PX Portable Density Metre (Mettler Toledo, Spain)). The end of AF was established when the sugar concentration was lower than 2 g/L using the Miura One Multianalyzer (TDI, Barcelona, Spain).

2.3. Malolactic fermentation

Once AF was finished, wines were centrifuged at 3730 g for 15 min at 4 °C and filtered with 0.22 μm filters (Merck, Germany). Biological triplicates were mixed to avoid differences in MLF. The 15 resulting wines were distributed in 50 mL tubes to perform the MLF. Four strains of *O. oeni* were inoculated in a population of 2×10^7 cells/mL, obtaining a total of 60 combinations of yeast-LAB, fermented in triplicate. Anaerobic and static conditions at 20 °C were used to perform the MLF. Consumption of L-malic acid was monitored by measuring its concentration every day using Miura One Multianalyzer (TDI, Barcelona, Spain). The MLF was considered completed with a concentration of L-malic acid < 0.1 g/L. No supplementation with L-malic acid was performed before MLF. Viable populations were analysed to check the populations and verify the AF and MLF inocula by plating serial dilutions in YPD and MRS media, respectively.

2.4. Analyses of metabolites

Wines after AF and MLF were analysed to calculate the concentration of organic acids and other metabolites. Acetic acid and L-malic acid were quantified with a Miura One Multianalyzer (TDI, Barcelona, Spain). Citric acid, glycerol and ethanol were determined using an Agilent 1100 HPLC (Agilent Technologies, Germany) (Quirós et al., 2014). The wine samples were filtered with 0.22 μm pore filters before injection (Agilent Technologies). The

HPLC had a Hi-Plex H (300 mm x 7.7 mm) column inside a 1260 MCT (Infinity II Multicolumn Thermostat). The column conditions were 60 °C for 30 min, and the mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 mL/min. Finally, the chromatograph was equipped with two detectors, an MWC detector (G1365B multi-wavelength detector) and an RID detector (1260 Infinity II refractive index detector) (Agilent Technologies).

The following analysis was performed only in wines after AF. Succinic acid was analysed by an enzymatic kit with microplates (Megazyme, Wicklow, Ireland). The content of mannoproteins after AF was determined as equivalents of mannose using a D-mannose and D-glucose enzymatic assay kit (Megazyme). Mannoprotein precipitation and acid hydrolysis to release mannose from mannoproteins were performed following the procedure described in Balmaseda et al., (2021a) The variations in acetic, succinic and L-malic acids as well as glycerol and mannoproteins in wines are calculated as follows: [$C_{metabolite\ seq.FA}$ - $C_{metabolite\ control.FA}$/$C_{metabolite\ control.FA}$]* 100, where $C_{metabolite\ seq.FA}$ is the metabolite concentration in the sequential FA and $C_{metabolite\ control.FA}$, the metabolite concentration in the control wine for each sequential combination and *S.cerevisiae* strains.

Finally, the volatile compounds were liquid/liquid extracted with 400 µL of dichloromethane in presence of 2.5 g $(NH_4)_2SO_4$ using 4-methyl-2-pentanol (0.8 g/L) and heptanoic acid (0.7 g/L) as internal standards, following Ortega et al. (2001) modified by (Balmaseda, et al., 2021a). All reagents were analytical grade from Sigma-Aldrich (Barcelona, Spain). The organic phase was extracted and 2 µL was injected in split mode (10:1, 30 mL/min) into a gas chromatograph (Agilent Technologies, Germany) with a FFAP column of 30 m × 0.25 mm × 0.25 µm. All aromatic volatile compounds were identified and quantified by comparison with standards. They included fusel alcohols (1-butanol, isoamyl alcohol, 1-pentanol, cis-3-hexen-1-ol, 2-phenylethanol), their corresponding acetate esters (isobutyl acetate, isoamyl acetate, hexyl acetate, 2-phenyletanol acetate), other alcohols (2-butanol, isopropanol), short-chain fatty acids (propionic, isobutyric, butyric, 3-methyl butanoic and valeric acids), medium-chain-fatty acids (hexanoic, octanoic, decanoic and dodecanoic acids) and their corresponding ethylic esters of C6, C8, C10 and C12.

2.5. Statistical analysis

All alcoholic and malolactic fermentations were performed using three independent biological samples. These data were statistically analysed using ANOVA and Tukey test analyses performed by XLSTAT 2020.2.3 software (Addinsoft, Paris, France). The statistical significance level was considered at *p*-value < 0.05. The heat map was created to examine the correlation between metabolites and samples (conditions). All data used to construct the heat map were centred and reduced. The clusters were constructed from centred and reduced data by using XLSTAT 2020.2.3 software. In addition, multiple linear regressions were performed for each *O. oeni* strain using an optimisation index (OI) as the dependent variable and the mean values of the families of volatile compounds, acetic acid, succinic acid and mannoproteins, as independent variables. Briefly, the OI was calculated from MLF data values, using the means of total MLF time, malic acid consumption rate and percentage of MLF completion. For each parameter, the OI was calculated as follows: the maximum value for a parameter was considered as 1 (x/x), if it had a positive effect on FML performance, or 0 (1 x/x) whether it had a negative effect OI was calculated, based on (Borrull et al., 2016).

3. Results and discussion

3.1. Sequential alcoholic fermentation

3.1.1. Fermentation kinetics

AFs were performed in the new synthetic must at 22 °C. All the strains showed common viable populations for alcoholic fermentation, starting from the desired initial population. Both control and sequential fermentations finished the AF up to a concentration lower than 2 g/L of sugars in a range of 12-25 days (Table SD2). Despite that, the first 48 h of fermentation with *T. delbrueckii* started a slightly faster consumption of sugars than the controls, mainly in Sc-QA23 and Sc-K1 AF (Figure 1). There were some differences between controls: AF with Sc-QA23 lasted 13 days (Figure 1A), AF with Sc-CLOS lasted 13 days (Figure 1C), and AF with Sc-K1 lasted 15 days (Figure 1B). The total time of AF increased in sequential fermentations. According to the literature, the slower kinetics in sequential fermentations may be due to nutritional competition between non-*Saccharomyces* and *S. cerevisiae* (Balmaseda et al., 2018; Belda et al., 2015;

Martín-García et al., 2020; Romano et al., 2022) Additionally, depending on the combination of *T. delbrueckii* and *S. cerevisiae* strains, differences were observed between the four *T. delbrueckii* strains (Figure 1, Table SD2). Td-Prelude was the fastest in almost every combination, especially with Sc-QA23, with a final time of 17 days. However, in the Sc-K1 batch, Td-Biodiva finished one day before Td-Prelude. In addition, of note, the fermentation kinetics of Sc-K1 showed a lower slope and consequently a lower consumption rate of sugars, specifically during sequential fermentation with Td-Zymaflore (Figure 1B).

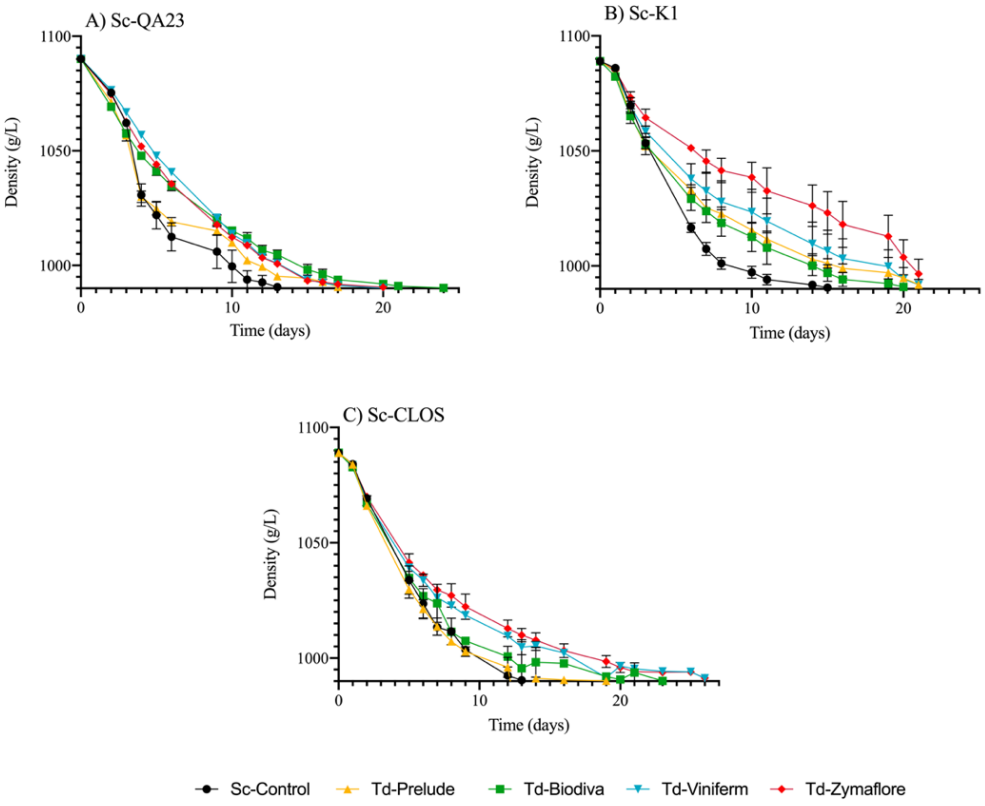

Figure 1. Evolution of alcoholic fermentation kinetics: AF with the different *S. cerevisiae* strains (Sc): A) Sc-QA23, B) Sc-K1, C) Sc-CLOS. Sc-Control, Td-Biodiva, Td-Prelude, Td-Viniferm and Td-Zymaflore correspond to *S. cerevisiae* control fermentations and *T. delbrueckii* (Biodiva, Prelude, Viniferm and Zymaflore) + *S. cerevisiae* sequential fermentations. ● Sc-Control, ■ Td-Biodiva, ▲ Td-Prelude, ▼ Td-Viniferm and ♦ Td-Zymaflore.

Consumption of 50% total sugars occurred in the first three, four and five days depending on the type of yeast inoculation (Table SD2), probably because the speed slows down due to an increase in alcohol concentration and a decrease in nutrients (Ribéreau-Gayon et al., 2006b).

3.1.2. Oenological parameters after AF

Several metabolites were analysed after AF with the aim of describing the obtained wine and observing the potential advantages in MLF. Regarding organic acids, there were no substantial changes between the conditions (Table SD2). However, there were differences in relation to the formation of some products depending on the combination *T. delbrueckii*/*S. cerevisiae* strains (Figure 2). The presence of Td-Biodiva strains induced less acetic acid formation regardless of the strains of *S. cerevisiae* used (Figure 2A). Moreover, in Sc-QA23 wines, a reduction in acetic acid production was observed under these conditions when *S. cerevisiae* was inoculated after two days of growth of the *T. delbrueckii* strains. With the other *S. cerevisiae* strains (K1 and Clos), acetic acid production was dependent on the *T. delbrueckii* strain. The presence of *T. delbrueckii* strains generally induced less acetic acid formation regardless of the strains of *S. cerevisiae* used (Figure 2A). The formation of acetic acid as well as glycerol in wine mainly depends on the initial sugar level due to the adaptation mechanism of the *Saccharomyces* strains to a medium with a high sugar concentration (Ribéreau-Gayon et al., 2006b). Sequential inoculation or co-inoculation of *T. delbrueckii* strains with *Saccharomyces* is generally well known to decrease the acetic acid level of natural grape must (red or white) regardless of fermentation types. As reported by several authors, *T. delbrueckii* can go from 0.05 g/L (Balmaseda et al., 2021c) to 0.51 g/L (Canonico et al., 2019), or even 1.6 g/L in a synthetic medium (Contreras et al., 2015).

Normally, the formations of glycerol and ethanol by *Saccharomyces* come respectively from glyceropyruvic and alcoholic fermentations, which influences the yield of the two products in AF (Ribéreau-Gayon et al 2006b) In our study, we can observe that strains of *T. delbrueckii* had lower glycerol concentrations than those obtained by *S. cerevisiae* monocultures (Figure 2B). Nevertheless, Td-Biodiva seemed to behave differently compared to other *T. delbrueckii* strains in the presence of the Sc-QA23 strain because the glycerol content decreased, whereas it increased in the presence of Td-Viniferm and Td-Zymaflore. Glycerol values were

reasonable (5-8 g/L) (Ribéreau-Gayon et al, 2006b) except for monoculture Sc-K1, which showed an overproduction around 10 g/L. Nevertheless, those differences in glycerol could not be related to ethanol reduction (Table SD2) as reported by (Zhu et al., 2020). However, ethanol values were similar under all conditions, near 12% (v/v) without a significant variation.

When a molecule of glycerol is formed, a molecule of pyruvate cannot be transformed into ethanol after its decarboxylation to ethanal. In AF condition, this pyruvate molecule enters the incomplete citric acid cycle (inactivation of succinodehydrogenase) which leads to the formation of succinic acid (Ribéreau-Gayon et al., 2006b) Regarding this by-product of AF, which is described in the literature as a competitive inhibitor of MLF (Lonvaud-Funel et al., 1988; Caridi and Corte 1977), no significant difference was observed in the final content of the wines (Table SD2). However, depending on the strains used for the sequential inoculation, some variations were observed (Figure 2C). In fact, the greatest increase in succinic acid compared to the control wines was detected for the Sc-K1 strains.

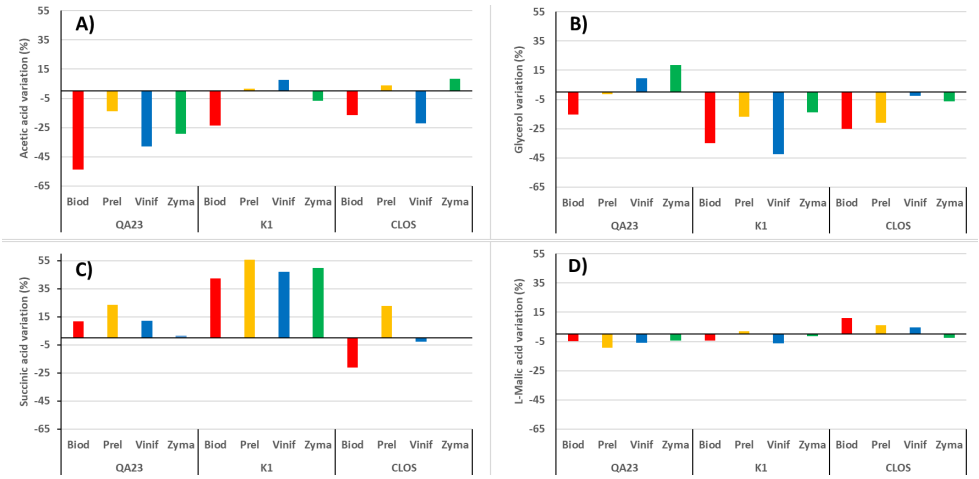

Figure 2. Variation in the production of acetic acid (A), glycerol (B), succinic acid (C) and L-malic acid (D) at the end of alcoholic fermentation by the *T. delbrueckii*/*S. cerevisiae* combination compared to the corresponding *S. cerevisiae*. Biod, Prel, Vinif and Zyma correspond to the *T. delbrueckii* strain: Td-Biodiva, Td-Prelude, Td-Viniferm and Td-Zymaflore, respectively. The values expressed the average of three biological replicates for each condition.

The greatest incidence of the *S. cerevisiae*/*T. delbrueckii* was noted in the variation in malic acid consumption (Figure 2D). The L-malic acid concentration decreased from the initial value of 2 g/L, particularly in Sc-QA23 fermentations, due to the partial consumption by yeast (10-25%) (Ribéreau-Gayon et al., 2006b). Lower L-malic acid consumption was achieved in Sc-K1 fermentations in comparison with Sc-QA23 and Sc-CLOS fermentations. Nevertheless, it was decided to start the MLF with the remaining L-malic acid and not to increase it to the initial concentration, reproducing real winemaking.

Finally, citric acid consumption was generally higher in Td-Prelude fermentations than in other sequential fermentations (Table SD2). Furthermore, this consumption for the Td-Prelude strains was significantly different from that for all *Saccharomyces* strains and Td-Viniferm strains (Figure SD6).

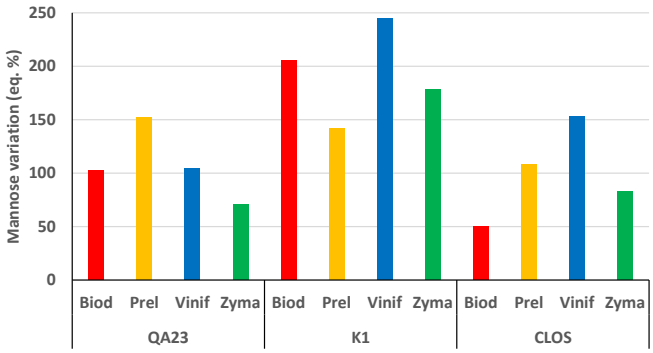

Figure 3. Variation of mannoproteins (equivalent mannose) at the end of alcoholic fermentation according to the *Saccharomyces cerevisiae* strain used in sequential inoculation respect to *S. cerevisiae* strain alone. Sc and Td correspond to wines fermented with *S. cerevisiae* and *T. delbrueckii*, respectively. Biod, Prel, Vinif and Zyma correspond to the *T. delbrueckii* strain: Td-Biodiva, Td-Prelude, Td-Viniferm and Td-Zymaflore, respectively. The values expressed the average of three biological replicates for each condition.

Mannoproteins are released in wine during the autolysis of yeasts (Guilloux-Benatier et al., 1995). A higher quantity of these compounds was related to a better performance of MLF because *O. oeni* is able to hydrolyse them and metabolise some of the resultant products such as mannose (Alexandre et al., 2004; Balmaseda et al., 2021a). Recent studies have shown that non-*Saccharomyces* species increase the release of mannoproteins (González-Royo et al.,

2015). Sequential fermentations with *T. delbrueckii* had significantly higher equivalent mannose values than controls (Figure 3), especially the Td-Prelude and Td-Viniferm strains.

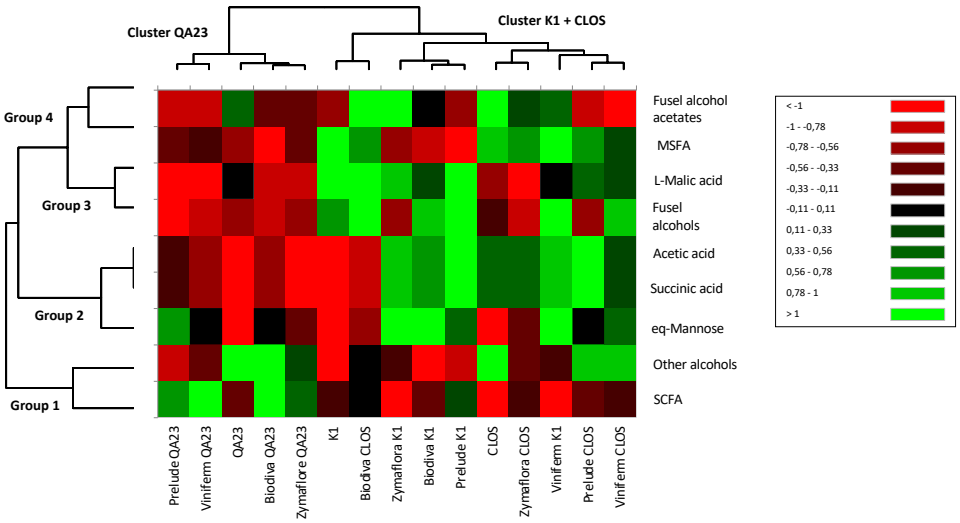

Figure 4. Heat map of the strains used in this work in relation to the products of alcoholic fermentation (Green higher concentrations, red lower concentrations). Group 1: Short-Chain Fatty Acids + Other alcohols; Group 2: Mannoproteins + Succinic acid + Acetic acid; Group 3: Fusel alcohols + L-malic acid and Group 4. Medium-Chain Fatty Acids + Fusel alcohols acetates. QA23, K1 and CLOS correspond to the *S. cerevisae* strains. B, P, V and Z correspond to the *T. delbruekii* strains: Td-Biodiva. Td-Prelude. Td-Viniferm and Td-Zymaflore, respectively. The values expressed the average of three biological replicates for each condition.

Concerning the volatile compounds, there were few significant differences between the conditions (Table SD3). However, a trend regarding medium chain fatty acids (MCFAs) was observed. Wines fermented only with *S. cerevisiae* (control fermentations) showed higher values than wines from sequential AF with *T. delbrueckii*. This trend is clearly higher in the Sc-K1 wines. This reduction of MCFA in fermentations performed by non-*Saccharomyces* has been previously shown in other studies (Balmaseda, et al., 2021b) and could be related to their binding to mannoproteins (Guilloux-Benatier et al., 1995). In contrast, the corresponding ethyl esters of MCFA were more highly produced by some wines fermented in sequential AF. This fact confirmed the modulation of aroma produced by non-*Saccharomyces* (Padilla et al., 2016). This favourable effect was demonstrated for ethyl octanoate, which increased with *T. delbrueckii* (Viana et al., 2008); in this case, Td-Viniferm showed a tendency towards higher

concentrations (Table SD3). Anyway, there is clearly a strain-specificity of non-*Saccharomyces* strains in shaping the aromatic characteristics of wine (Zhang et al., 2021).

All abovementioned differences in metabolic parameters among the different conditions are shown in a heatmap (Figure 4). Fermentation was clustered into two groups according to the *S. cerevisiae* strain used: Sc-QA23 was separated from Sc-K1 and Sc-CLOS. Of note, relevant physiological differences have been found between strains *S. cerevisiae* QA23 and K1; thus, sometimes, Sc-QA23 is considered as *S. cerevisiae bayanus* and Sc-K1 as *S. cerevisiae cerevisiae* (Ruiz-de-Villa et al., 2022). Similarly, Sc-CLOS is considered as *S. cerevisiae* var. *cerevisiae* by the manufacturer.

3.2. Malolactic fermentation

3.2.1. Fermentation kinetics

After AF, all wines were inoculated with the four strains of *O. oeni,* and there were large differences in the behaviour of different combinations (Figure 5). Of note that almost all MLFs reach the end, leaving the L-malic acid concentration below 0.1 g/L, except for some wines. Thus, it is possible to determine that this must is useful for both fermentations. The goal of using a synthetic must for both fermentations is to standardise the experimental conditions, obtaining more reproducibility compared to the natural musts used in other studies (Ferrando et al., 2020; Martín-García et al., 2020). There are very few studies regarding synthetic musts used for the whole process, AF plus MLF. However, some researchers developed a methodology to study the compatibility between yeasts and LAB (Costello et al., 2003).

A heat map (Figure 6) was created to determine the most successful MLFs compared to control wines. However, some of the combinations did not finalize the process, resulting in a stuck MLF. For example, in this study, it was verified that the strain *S. cerevisiae* K1 induces a stop of the MLF. Nevertheless, the positive effect of *T. delbrueckii* becomes clear in this case; thus, the main sequential Sc-K1 improved the final L-malic acid consumption (Figure 6). Indeed, this synergy was significant in the combination of Oo-VP41 with Td-Prelude, Td-Viniferm and Td-Zymaflore, and L-malic acid was completely consumed. In addition, Td-Prelude with Sc-QA23 and Sc-CLOS improved the performance of MLF with the four strains of *O. oeni* in relation to

the control condition and the other *Td* strains. These data are consistent with those reported in the literature describing faster MLF in wines inoculated with *T. delbrueckii* and *S. cerevisiae* in comparison to the wines inoculated only with *S. cerevisiae* (Balmaseda et al., 2021b).

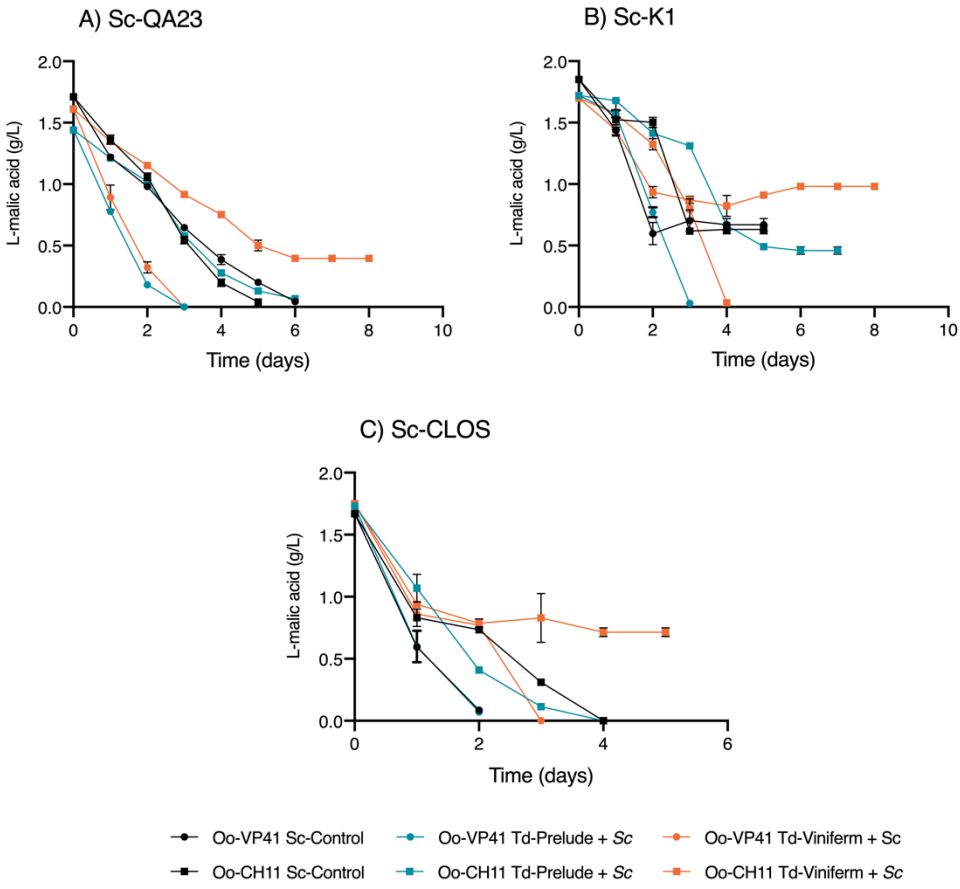

Figure 5. Evolution of malolactic fermentation kinetics in some of the wines. A) Sc-QA23, B) Sc-K1 and C) Sc-CLOS correspond to wines fermented with *S. cerevisiae* QA23, K1 and CLOS in combination with *T. delbrueckii* strains: Td-Prelude and Td-Viniferm which correspond to P and V respectively. Oo-VP41 and Oo-CH11 corresponds to MLF performed with the *Oenococcus oeni* strains VP41 and CH11.

As mentioned above, wines fermented with *T. delbrueckii* showed higher concentrations of equivalent mannose than the control, which may be related to this MLF improvement. The type of mannoproteins consumed is variable for *O. oeni* strains. It has been described that yeast mannoproteins up to 200 mg/L can positively influence the growth of many LAB and

especially commercial mannoproteins of intermediate molecular weight (6-22 kD) activate growth of most of *O. oeni* strains in the presence of ethanol (Diez et al., 2010) In contrast, Sc-K1 fermentations were the ones with the highest mannoprotein values and in general showed negative MLF results. This indicates that there are other inhibiting factors that were affected more in this condition.

MCFAs are one of these metabolites that could negatively influence MLFs, especially decanoic and dodecanoic acids, which have been reported to be inhibitors of *O. oeni* (Capucho and San Romão, 1994; Edwards and Beelman, 1987). MCFA content in wines are significantly different between *Saccharomyces* strains and some variations between *Torulaspora* strains were observed (Table SD3). In some wines, the lower presence of MCFA could be correlated with the improvement of the MLF, as in some of the sequential fermentations.

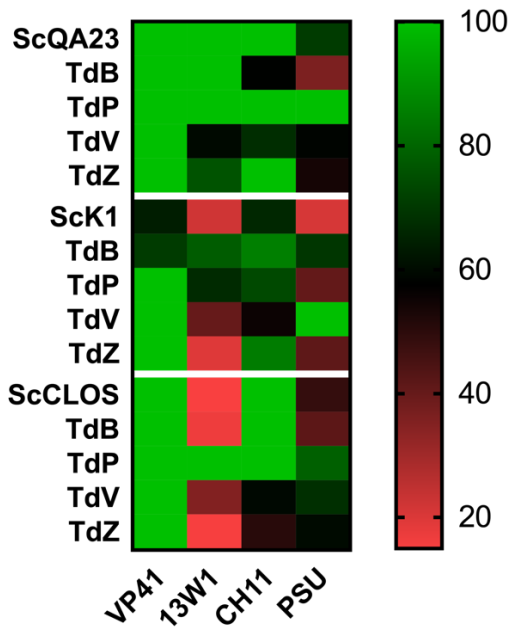

Figure 6. Heat map of final L-malic consumption percentage in malolactic fermentations with all strain combinations (Green finished MLF. red unfinished MLF). Sc and Td correspond to wines fermented with *S. cerevisiae* and *T. delbrueckii*. respectively. B. P. V and Z correspond to the *T. delbrueckii* strain: Td-Biodiva, Td-Prelude, Td-Viniferm and Td-Zymaflore, respectively. VP41, 1Pw13, CH11 and PSU-1.

Table 1. L-malic acid consumption until stop or final of MLF (percentage and days); consumption rate (g/L·day). Mean ± standard deviation (n=3). Sc corresponds to wines fermented with *S. cerevisiae* and Td to wines fermented with a sequential fermentation of *T. delbrueckii* and *S. cerevisiae*. B, P, V and Z correspond to the *T. delbrueckii* strains: Td-Biodiva, Td-Prelude, Td-Viniferm and Td-Zymaflore. respectively. Oo-VP41, Oo-1Pw13, Oo-CH11 and Oo-PSU-1 correspond to different *Oenococcus oeni* strains.

	Final L-malic consumption (%)/ (days)				Consumption rate⁺ (g/L·day)			
Strains	**Oo-VP41**	**Oo-1PW13**	**Oo-CH11**	**Oo-PSU-1**	**Oo-VP41**	**Oo-1PW13**	**Oo-CH11**	**Oo-PSU**
Sc-QA23	100.00 (4)	100.00 (6)	100.00 (8)	70.37 (9)	33±0.01	48±0.03	38±0.01	17±0.00
Td-B	100.00 (4)	100.00 (5)	57.87 (5)	36.20 (4)	36±0.02	33±0.01	16±0.04	10±0.00
Td-P	100.00 (3)	100.00 (4)	100.00 (6)	100.00 (6)	63±0.01	39±0.02	29±0.00	23±0.01
Td-V	100.00 (3)	59.35 (4)	67.24 (6)	58.18 (6)	64±0.02	28±0.01	23±0.00	18±0.10
Td-Z	100.00 (3)	75.85 (5)	100.00 (5)	53.85 (6)	72±0.03	37±0.03	30±0.01	15±0.03
Sc-K1	63.84 (4)	22.04 (4)	66.09 (4)	20.84 (4)	63±0.04	31±0.03	17±0.02	16±0.09
Td-B	70.45 (4)	77.84 (5)	85.53 (5)	69.33 (4)	45±0.06	44±0.00	57±0.06	51±0.00
Td-P	100.00 (3)	66.74 (5)	73.43 (5)	40.46 (5)	47±0.02	19±0.03	14±0.00	25±0.03
Td-V	100.00 (4)	39.90 (6)	55.43 (5)	100.00 (6)	19±0.02	18±0.06	42±0.01	31±0.01
Td-Z	100.00 (3)	19.56 (3)	84.78 (4)	41.39 (4)	50±0.00	30±0.00	42±0.09	38±0.05
Sc-CLOS	100.00 (2)	14.97 (3)	100.00 (4)	48.50 (5)	84±0.04	19±0.03	33±0.02	28±0.09
Td-B	100.00 (3)	16.76 (3)	100.00 (5)	41.62 (5)	80±0.06	20±0.00	41±0.06	27±0.00
Td-P	100.00 (2)	100.00 (3)	100.00 (4)	78.61 (4)	88±0.02	26±0.03	56±0.00	55±0.03
Td-V	100.00 (3)	35.14 (4)	59.14 (4)	67.71 (4)	45±0.02	32±0.06	45±0.01	45±0.01
Td-Z	100.00 (3)	15.31 (3)	50.63 (3)	59.69 (5)	41±0.00	23±0.00	34±0.09	40±0.05

Looking at *O. oeni* strains, Oo-VP41 was the only strain, which assures the total consumption of L-malic acid in almost all combinations. This strain also improved the MLF duration in some cases; for example, MLF in Td-Prelude – Sc-QA23 with Oo-VP41 required three days while that with Oo-CH11 required 6 days (Table 1). In addition, it is important to highlight that this strain had a significantly better L-malic acid consumption rate than the other strains.

Apart from Oo-VP41, the other strain, which obtained favourable results, was Oo-CH11 and Oo-1Pw13, especially for Sc-CLOS wines. On the other hand, MLFs performed with Oo-PSU-1 were mostly incomplete with the lowest L-malic acid consumption rates. This behaviour was different compared to some results of MLF from natural must. For example, (Balmaseda et al., 2021b) observed that in high polyphenolic wines, the strain Oo-PSU-1 showed a better MLF performance than Oo-CH11.The differences found with previous works can be explained by the fact that the behaviour of *O. oeni* strains depend on the specific wine conditions, due to the numerous factors affecting its survival in this harsh environment that can be very variable in wine.

1.1.1. Wine composition after malolactic fermentation

Regarding wine metabolites after MLF, it is worth noting that wines with Oo-VP41 had higher citric acid consumption, which is significantly different from Oo-1Pw13 (Table SD2). Moreover, the glycerol content was higher in Oo-VP41 MLF than in Oo-CH11 and Oo-1Pw13.

The difficulty in interpreting the results of MLFs led us to perform multiple linear regressions for each *O. oeni* strain used in this work. For this, an optimization index (OI) was calculated from three MLF parameters as response variables (Table SD5) and parameters of the chemical composition of wines (SCFA, MCFA, fusel alcohols, fusel alcohol acetates, other alcohols, succinic acid, acetic acid, L-malic acid and mannoproteins) as explanatory variables. From a multiple linear regression calculated according to the best model, we were able to obtain information concerning the behaviour of each LAB strain under our MLF conditions.

As observed in Table 2 and in the results of MLF kinetics, *O. oeni* strains have different behaviours depending on the chemical composition of the wines. The variability of the response variable (OI) was explained by two explanatory variables for each strain, and it was 46, 61.5, 35.2 and 60.9% for the Oo-VP41, Oo-1Pw13, Oo-CH11 and Oo-PSU-1 strains,

respectively. For all analyses of total variance, the *p*-value was less than 0.05. The results in Table 2 also showed that the OI of strain Oo-VP41 was explained by acetic acid and mannoproteins, that of strain Oo-1Pw13 by fusel alcohols and MCFA, that of Oo-CH11 by acetic acid and mannoproteins and that of Oo-PSU by other alcohols and mannoproteins.

Table 2. Multiple linear regression results. R2. Coefficient of regression; Type III sum of squares analysis. variables providing significant information to explain the variability of the Total dependent variable; AcAc, acetic acid; Mps, Mannoproteins; FA, Fusel alcohols; FAA, Fusel alcohols acetates; SCFA, Short-chain of fatty acids; MCFA, Medium-chain of fatty acids; OAl. Other alcohols (2-butanol, isopropanol); Coef. Coefficient.

	Oenococcus oeni strains			
	Oo-VP41	**Oo-1PW13**	**Oo-CH11**	**Oo-PSU-1**
R^2	0.463	0.565	0.436	0.499
Adjusted R^2	0.373	0.492	0.341	0.415
p-value of variance	0.024	0.007	0.003	0.016
Explanatory variables (*p*-value of type III sum of squares analysis)				
First variable	AcAc (0.017)	MCFA (0.163)	AcAc (0.010)	OAl (0.105)
Second variable	Mps (0.012)	FA (0.013)	Mps (0.068)	Mps (0.005)
Equation of the model				
Intercept	1.192	2.36	0.714	1.274
Coef first variable	3.050	-0.099	3.749	0.061
Coef. second variable	-5.275	-0.119	-4.003	5.136

Surprisingly, the strains Oo-VP41 and Oo-CH11 were found to be more accommodating to high acetic acid content (positive correlation, equation coefficient of 3.05 and 3.75 respectively) regardless of yeast species tested in fermentation, but negatively (equation coefficient of -5.3 and -4 respectively) related to mannoprotein level which however increased in sequential AF. Of these two variables, the concentration of mannoproteins most influenced the model obtained by this regression analysis. These wines were obtained by the fermentation of the *S. cerevisiae* K1 strain, which hypothetically could have caused a faster lysis of the *T. delbrueckii* strains during AF due to the production of K1 toxin, increasing the mannoprotein content.

According to Figure 3, this pattern could be explained by the fact that the more mannoproteins there are in the wines, the worse is the malolactic fermentation. Nonetheless, this negative

correlation must be related to other yeast metabolites not studied in this work because in the literature, a high mannoprotein content is reported to be positive for MLF performance (Diez et al., 2010). For example, Rizk et al., (2018) described that *Saccharomyces cerevisiae* Uvaferm BDX produces nine peptides that are able to inhibit MLF in vitro. Thus, the toxins identified in Sc-K1 (Reiter et al., 2005) could have some implications in the inhibition of *O. oeni*. In addition, the manufacturers of Lallemand Inc. do not recommend strain K1 for further MLF (Lallemand Inc., n.d.). Thus, from an industrial point of view, the use of this strain in AF may be an interesting option in some vinifications in which MLF is an undesirable process.

Regarding the performance of the strain Oo-1Pw13 in the same wines, the two explanatory variables are fusel alcohols (FA) and short-chain of fatty acids (MCFA) (Table 2). Both variables are negatively related to OI. This may indicate that the higher are the concentrations of FA and MCFA, the more difficult the MLF will be with this yeast combination. However, there is no description of an inhibition caused by these metabolites; therefore, they would not be the only ones producing this inhibition, as discussed above. In general, and this is confirmed by the results in Table SD3, the fewer higher alcohols there were in the wines, the more that 100% of MLF was observed.

The two explanatory variables of strain Oo-CH11 were acetic acid (AcAc) and mannoproteins (Mps), the first being the most influential statistical variable. Finally, the content of mannoproteins and other alcohols has a positive effect on the performance of the MLF produced by the Oo-PSU strain although the first one was statistically the best (Table 2). Indeed, for this strain, its behaviour in the presence of high levels of mannoproteins in the wines seems to improve, although only 20% (3/15 wines) of the MLFs can go to the end. Finally, based on the Type III sum of squares (Table 2), the following variables bring significant information to explain the variability of the OI for each LAB strains: acetic acid and mannoproteins for Oo-VP41, fusel alcohols for Oo-1PW13, acetic acid for Oo-CH11 and mannoproteins for Oo-PSU-1. Results of the linear regressions, although limited to the conditions of our study, show that for the same chemical composition of the wines, the LAB strains prepared under the same conditions are differently sensitive to the metabolites of the wine. Many factors and metabolites affect the compatibility between yeast and *O. oeni* strains.

As we have seen, not only non-*Saccharomyces* species influence MLF (Balmaseda et al., 2018; Ferrando et al., 2020; Balmaseda et al., 2021d), but *S. cerevisiae* strains can also inhibit or promote *O. oeni* development (Arnink and Henick-Kling, 2005; Comitini and Ciani, 2007; Osborne and Edwards, 2007). Thus, it becomes clear that a correct choice of the right yeast-bacteria starter culture combination is very important for a successful MLF.

2. Conclusion

In this study, fifteen alcoholic fermentations were combined with four strains of *O. oeni,* resulting in a total of 60 combinations. Most of them showed different behaviours depending on the *T. delbrueckii*, *S. cerevisiae* and *O. oeni* strains used. Wines fermented with Sc-K1 are unsuitable for performing MLF, which confirms the manufacturer's recommendation. However, this strain can be recommended for the fermentation of wines in which MLF is not desired. On the other hand, the positive effect of *T. delbrueckii* was highlighted in combinations of AF with Td-Prelude and Sc-QA23 or Sc-CLOS followed by MLF with *O. oeni* VP41. This blend assures the end of MLF as well as a good L-malic acid consumption rate. It is possible to conclude that the performance of MLF under these conditions depends on the strains of yeasts/lactic acid bacteria used. Other studies, such as the optimal moment of presence of *T. delbrueckii* in the must before inoculation with *S. cerevisiae,* will allow to determine the most favourable conditions for the realisation of the MLF. This study also confirms that the sequential use of *Torulaspora* strains during AF induces a low level of acetic acid and MCFA in wines, negative factors for performing MLF. On the other hand, the content of MCFA ethyl esters and fusel alcohol acetates has increased, which makes it possible to obtain more aromatic wines.

Further studies should focus on *Torulaspora*/*Saccharomyces* combinations in order to know the effect of the *Saccharomyces* strain on the lysis of *Torulaspora* along the AF, which could induce a release of nitrogen or another nutrient favourable to the realization of the MLF.

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Supplementary data

Table SD1. Must nitrogen composition. GABA corresponds to ácido gamma-aminobutírico.

	mg/L	mg N/L
Ammonium chloride	50	13.09
Proline	300	36.48
GABA	50	6.79
Glutamine	260	49.81
Arginine	115	36.97
Tryptophan	75	10.28
Alanine	90	14.14
Glutamic acid	210	19.98
Serine	80	10.66
Threonine	60	7.05
Leucine	25	2.76
Aspartic acid	45	4.73
Valine	35	4.18
Phenylalanine	25	2.12
Isoleucine	25	2.67
Histidine	40	10.83
Methionine	10	0.94
Tyrosine	10	0.77
Glycine	10	1.86
Cysteine	15	1.73
Lysine	10	1.92
Asparagine	10	2.12
Citrulline	5	1.20
Total	1555	243.00

Table SD2. Time of consumption of 100% and 50% of sugars in AF (days) and analytical parameters of wines after AF. Mean ± standard deviation (n=3). Different lower-case letters indicate a significant difference between values using the Tukey (HSD) test at *p*-value < 0.05. Sc and Td correspond to wines fermented with *S. cerevisiae* and *T. delbrueckii*. respectively. B. P. V and Z correspond to the *T. delbrueckii* strain: Td-Biodiva. Td-Prelude. Td-Viniferm and Td-Zymaflore. respectively.

	Duration (days)		Wine after AF parameters						
Yeast strains	100%	50%	L-malic acid (g/L)	Succinic acid (g/L)	Acetic acid (g/L)	Citric acid (g/L)	Glycerol (g/L)	EtOH (v/v)	pH
Sc-QA23	13	4	1.71 ± 0.02 ^{abc}	0.47 ± 0.11	0.80 ± 0.05 ^{ab}	0.44 ± 0.06 ^{ab}	7.55 ± 0.56 ^{abc}	12.0 ± 0.23	3.60 ± 0.04 ^{abc}
Td-B	24	5	1.63 ± 0.01 ^{cd}	0.53 ± 0.15	0.37 ± 0.03 ^{e}	0.39 ± 0.09 ^{ab}	6.39 ± 0.29 ^{abc}	12.4 ± 0.48	3.60 ± 0.01 ^{abc}
Td-P	17	5	1.54 ± 0.03 ^{d}	0.58 ± 0.13	0.69 ± 0.08 ^{abcd}	0.34 ± 0.06 ^{ab}	7.44 ± 0.29 ^{abc}	11.6 ± 0.06	3.59 ± 0.01 ^{abc}
Td-V	20	4	1.61 ± 0.03 ^{cd}	0.53 ± 0.17	0.50 ± 0.15 ^{de}	0.48 ± 0.07 ^{ab}	8.27 ± 0.56 ^{abc}	12.2 ± 0.41	3.61 ± 0.01 ^{abc}
Td-Z	20	4	1.56 ± 0.07 ^{cd}	0.48 ± 0.08	0.57 ± 0.08 ^{cde}	0.50 ± 0.02 ^{ab}	8.96 ± 0.56 ^{ab}	12.7 ± 0.44	3.65 ± 0.04 ^{ab}
Sc-K1	15	3	1.85 ± 0.05 ^{a}	0.47 ± 0.11	0.57 ± 0.13 ^{abc}	0.52 ± 0.07 ^{ab}	10.09 ± 0.56 ^{a}	12.3 ± 0.24	3.60 ± 0.02 ^{abc}
Td-B	20	3	1.72 ± 0.05 ^{abc}	0.67 ± 0.04	0.59 ± 0.01 ^{bcde}	0.42 ± 0.08 ^{ab}	6.98 ± 0.9 ^{c}	11.7 ± 0.68	3.60 ± 0.03 ^{abc}
Td-P	21	3	1.88 ± 0.03 ^{a}	0.73 ± 0.02	0.78 ± 0.09 ^{abc}	0.41 ± 0.04 ^{ab}	8.40 ± 0.58 ^{ab}	11.8 ± 0.06	3.59 ± 0.04 ^{abc}
Td-V	21	4	1.70 ± 0.09 ^{abc}	0.69 ± 0.12	0.83 ± 0.05 ^{a}	0.51 ± 0.04 ^{ab}	5.81 ± 0.64 ^{bc}	11.8 ± 0.11	3.63 ± 0.03 ^{abc}
Td-Z	21	5	1.84 ± 0.06 ^{ab}	0.70 ± 0.05	0.72 ± 0.07 ^{abcd}	0.42 ± 0.11 ^{ab}	8.70 ± 1.76 ^{ab}	11.3 ± 0.81	3.55 ± 0.01 ^{c}
Sc-CLOS	13	3	1.67 ± 0.03 ^{bcd}	0.70 ± 0.05	0.71± 0.07 ^{abcd}	0.54 ± 0.03 ^{ab}	8.82 ± 0.99 ^{ab}	12.2 ± 0.47	3.63 ± 0.03 ^{ab}
Td-B	23	3	1.85 ± 0.06 ^{a}	0.51 ± 0.10	0.59 ± 0.07 ^{bcde}	0.57 ± 0.09 ^{a}	6.60 ± 0.44 ^{abc}	12.0 ± 0.46	3.58 ± 0.01 ^{bc}
Td-P	19	3	1.73 ± 0.02 ^{abc}	0.79 ± 0.09	0.73 ± 0.12 ^{abcd}	0.32 ± 0.09 ^{b}	6.95 ± 0.64 ^{abc}	12.3 ± 0.31	3.55 ± 0.01 ^{c}
Td-V	26	4	1.75 ± 0.07 ^{abc}	0.62 ± 0.17	0.55 ± 0.01 ^{cde}	0.48 ± 0.07 ^{ab}	8.59 ± 0.45 ^{ab}	11.6 ± 0.54	3.66 ± 0.02 ^{a}
Td-Z	26	4	1.60 ± 0.06 ^{cd}	0.64 ± 0.03	0.77 ± 0.01 ^{abc}	0.37 ± 0.02 ^{ab}	8.27 ± 0.40 ^{abc}	11.9 ± 0.21	3.64 ± 0.04 ^{ab}

Table SD3. Volatile compounds (mg/L) of wines after AF. Mean ± standard deviation (n=3). Sc and Td correspond to wines fermented with *S. cerevisiae* and *T. delbrueckii*. respectively. B. P. V and Z correspond to the *T. delbrueckii* strain: Td-Biodiva. Td-Prelude. Td-Viniferm and Td-Zymaflore, respectively. Different lower-case indicate a significant difference between Sc-control and Td strains, and uppercase letters between Sc strains using the Tukey (HSD) test at *p*-value < 0.05. SFCA, Short-Chain Fatty Acids (Propionic acid, isobutyric acid, butyric acid, 3-methyl butanoic acid, valeric acid); MCFA, Medium-Chain Fatty Acids (hexanoic acid (C6), octanoic acid (C8), decanoic acid (C10) and dodecanoic acid, C12); Ethyl esters of MCFA (ethyl esters of C6, C8, C10 and C12); Fusel alcohols (1-butanol, isoamyl alcohol,1-pentanol, cis-3-hexen-1-ol); Fusel alcohol acetates (isobutyl acetate, isoamyl acetate, hexyl acetate, 2-phenyletanol acetate) and Other alcohols (2-butanol,isopropanol).

	SCFA	MCFA	Ethyl esters of MCFA	Fusel alcohols	Fusel alcohol acetates	Other alcohols
Sc-QA23	0.82 ± 0.10 ^{aB}	2.23 ±1.06 ^{bcA}	12.77 ± 2.54 ^{aB}	2.99 ± 0.39 ^{bcA}	4.26 ± 0.65 ^{aA}	9.55 ± 0.93 ^{bB}
Td-B	2.33 ± 0.66 ^{cB}	1.44 ± 0.40 ^{aA}	17.29 ± 1.44 ^{abB}	2.63 ± 0.65 ^{cdA}	3.50 ± 1.14 ^{aA}	10.70 ± 0.75 ^{abB}
Td-P	0.96 ± 0.08 ^{bcB}	2.44 ± 0.67 ^{aA}	27.30 ± 3.37 ^{abB}	2.01 ± 0.94 ^{abA}	3.06 ± 0.97 ^{aA}	3.61 ± 0.49 ^{aB}
Td-V	3.20 ± 0.32 ^{cB}	2.87 ± 0.60 ^{cA}	24.86 ± 0.83 ^{abB}	2.87 ± 0.15 ^{dA}	2.99 ± 0.50 ^{aA}	4.98 ± 0.29^{abB}
Td-Z	1.64 ± 0.16 ^{bB}	2.55 ± 0.32^{abA}	27.32 ± 2.89 ^{bB}	3.15 ± 0.57 ^{aA}	3.35 ± 0.38 ^{aA}	6.41 ± 0.72 ^{abB}
Sc-K1	0.97 ± 0.11 ^{aA}	4.58 ± 0.79 ^{bcB}	14.38 ± 1.44 ^{aA}	5.71 ± 0.76 ^{bcC}	3.25 ± 1.06 ^{aA}	2.79 ± 0.21 ^{bA}
Td-B	0.87 ± 0.09 ^{cA}	2.15 ± 0.31 ^{aB}	16.80 ± 0.52 ^{abA}	6.06 ± 0.24 ^{cdC}	3.98 ± 0.76 ^{aA}	1.77 ± 0.12 ^{abA}
Td-P	1.40 ± 0.26 ^{bcA}	1.13 ± 0.12 ^{aB}	19.24 ± 4.59 ^{abA}	6.68 ± 0.56 ^{abC}	3.29 ± 2.36 ^{aA}	3.49 ± 0.38 ^{aA}
Td-V	0.45 ± 0.07 ^{cA}	5.53 ± 0.39 ^{cB}	18.25 ± 5.70 ^{abA}	7.28 ± 1.52^{dC}	4.25 ± 3.08 ^{aA}	5.43 ± 0.27 ^{abA}
Td-Z	0.43 ± 0.02 ^{bA}	2.38 ± 0.07 ^{abB}	21.61 ± 3.56 ^{abB}	3.17 ± 0.59 ^{aB}	5.11 ± 0.43 ^{aA}	5.49 ± 1.59 ^{abA}
Sc-CLOS	0.23 ± 0.07 ^{aA}	4.08 ± 0.34 ^{bcC}	24.44 ± 5.76 ^{aB}	4.13 ± 0.44 ^{bcB}	5.57 ± 1.48 ^{aA}	8.94 ± 0.88 ^{bB}
Td-B	1.15 ± 0.20 ^{cA}	4.07 ± 1.02 ^{aC}	25.33 ± 5.75 ^{abB}	6.51 ± 0.43 ^{cdB}	5.72 ± 0.39 ^{aA}	6.73 ± 2.56 ^{abB}
Td-P	0.96 ± 0.24 ^{bcA}	3.86 ± 0.60 ^{aC}	16.87± 3.64 ^{abB}	3.28 ± 0.94 ^{abB}	2.95 ± 0.17 ^{aA}	8.61 ± 1.62 ^{aB}
Td-V	1.12 ± 0.22 ^{cA}	3.45 ± 0.41 ^{cC}	22.65 ± 1.00 ^{abB}	5.82 ± 0.66 ^{dB}	2.57 ± 0.19 ^{aA}	8.70 ± 2.25 ^{abB}
Td-Z	1.06 ± 0.19 ^{bA}	4.00 ± 0.64 ^{abC}	18.70 ± 2.94 ^{bB}	2.75 ± 0.78 ^{aB}	4.20 ± 1.13 ^{aA}	4.79 ± 0.97 ^{abB}

Table SD4. Analytical parameters of wines after MLF. Mean ± standard deviation (n=3). Different lowercase indicate a significant difference between yeast combination, and uppercase letters between Oo strains using the Tukey (HSD) test at *p*-value < 0.05. Sc, Td and Oo corresponds to species *S. cerevisiae, T. delbrueckii* and *O. oeni*. respectively. B. P. V and Z correspond to the *T. delbrueckii* strain: Td-Biodiva. Td-Prelude. Td-Viniferm and Td-Zymaflore. respectively.

Yeast	*O. oeni* strains	Citric acid	Glycerol	pH
Sc-QA23	Oo-VP41	0.22 ± 0.04 BCDa	6.48 ± 0.79 ^{Ab}	3.77 ± 0.02 ^{Aab}
	Oo-CH11	0.52 ± 0.04 ^{BCDab}	6.61± 0.17 ^{Aa}	3.76 ± 0.05 ^{Aa}
	Oo-1Pw13	0.27 ± 0.06 ^{BCDb}	6.74± 0.34 ^{Aa}	3.79 ± 0.02 ^{Ab}
Td-B + Sc-QA23	Oo-VP41	0.50 ± 0.11 ^{CDa}	8.48 ± 0.21 ^{BCb}	3.72 ± 0.02 ^{Aab}
	Oo-1Pw13	0.38 ± 0.14 ^{CDb}	7.93 ± 0.21 ^{BCa}	3.86 ± 0.01 ^{Ab}
Td-P + Sc-QA23	Oo-1Pw13	0.23 ± 0.02 ^{BCb}	6.90 ± 0.25 ^{Ba}	2.34 ± 0.01 ^{Ab}
	Oo-CH11	0.33 ± 0.05 ^{BCab}	8.43 ± 0.12 ^{Ba}	3.76 ± 0.01 ^{Aa}
	Oo-PSU-1	0.25 ± 0.01 ^{BCab}	8.27 ± 0.65 ^{Ba}	3.76 ± 0.01 ^{Aab}
Td-V + Sc-QA23	Oo-VP41	0.33 ± 0.09 ^{ABa}	7.45 ± 1.19 ^{BCb}	3.77 ± 0.01 ^{Aab}
Td-Z + Sc-QA23	Oo-VP41	0.22 ± 0.03 ^{ABa}	8.32 ± 0.42 ^{BCb}	3.81 ± 0.02 ^{Aab}
Td-P + Sc-K1	Oo-VP41	0.22 ± 0.02 ^{ABa}	9.03 ± 0.14 ^{Ab}	3.81 ± 0.02 ^{Aab}
Td V+ Sc-K1	Oo-VP41	0.24 ± 0.07 ^{Aa}	6.40 ± 0.64 ^{BCb}	3.78 ± 0.02 ^{Aab}
Td Z+ Sc-K1	Oo-VP41	0.09 ± 0.03 ^{Da}	8.91 ± 0.29 ^{Cb}	3.78 ± 0.04 ^{Aab}
Sc CLOS	Oo-VP41	0.47 ± 0.02 ^{BCa}	9.11 ± 0.20 ^{Cb}	3.80 ± 0.04 ^{Aab}
Td B+ Sc-CLOS	Oo-CH11	0.30 ± 0.05 ^{ABab}	9.45 ± 0.15 ^{Aa}	3.77 ± 0.02 ^{Aa}
	Oo-VP41	0.25 ± 0.06 ^{ABa}	6.67 ± 0.56 ^{Ab}	3.81 ± 0.03 ^{Aab}
Td P+ Sc-CLOS	Oo-CH11	0.19 ± 0.02 ^{BCab}	6.20 ± 0.53 ^{Aa}	3.74 ± 0.03 ^{Aa}
	Oo-1Pw13	0.22 ± 0.02B ^{Cab}	6.53 ± 1.08 ^{Aa}	3.72 ± 0.02 ^{Ab}
Td V+ Sc-CLOS	Oo-VP41	0.23 ± 0.04 ^{BCDa}	5.57 ± 0.11 ^{BCb}	3.79 ± 0.07 ^{Aab}
Td Z+ Sc-CLOS	Oo-VP41	0.43 ± 0.03 ^{CDa}	5.32 ± 0.50 ^{BCb}	3.78 ± 0.07 ^{Aab}

Table SD5. Variables used to calculate the multiple linear regression results: Final time of MLF, rate, final L-malic consumption percentage, final time of MLF optimised, rate optimised, final L-malic consumption percentage optimised and optimisation index (OI). Sc, Td and Oo correspond to species *S. cerevisiae*, *T. delbrueckii* and *O. oeni*, respectively. B, P, V and Z correspond to the *T. delbrueckii* strain: Td-Biodiva, Td-Prelude, Td-Viniferm and Td-Zymaflore, respectively.

Yeast	*O.oeni* strains	Final time FML	Rate	%FML	Final time op	Rate op	%FML op	OI
Sc-QA23		4	0.33	100.00	0.50	0.29	1.00	1.79
Td-B		4	0.36	100.00	0.50	0.32	1.00	1.82
Td-P		3	0.63	100.00	0.67	0.56	1.00	2.22
Td-V		3	0.64	100.00	0.67	0.57	1.00	2.24
Td-Z		3	0.72	100.00	0.67	0.64	1.00	2.30
Sc-K1		4	0.63	63.84	0.50	0.55	0.64	1.69
Td-B		4	0.45	70.45	0.50	0.40	0.70	1.61
Td-P	**Oo-VP41**	3	0.47	100.00	0.67	0.42	1.00	2.08
Td-V		4	0.19	100.00	0.50	0.17	1.00	1.67
Td-Z		3	0.56	100.00	0.67	0.50	1.00	2.16
Sc-CLOS		2	1.08	100.00	1.00	0.95	1.00	2.95
Td-B		3	1.02	100.00	0.67	0.90	1.00	2.56
Td-P		2	1.13	100.00	1.00	1.00	1.00	3.00
Td-V		3	0.49	100.00	0.67	0.43	1.00	2.10
Td-Z		3	0.43	100.00	0.67	0.38	1.00	2.04
Sc-QA23		6	0.48	100.00	0.50	0.43	1.00	1.93
Td-B		5	0.34	100.00	0.60	0.30	1.00	1.90
Td-P		4	0.39	100.00	0.75	0.35	1.00	2.10
Td-V		4	0.28	59.36	0.75	0.25	0.59	1.59
Td-Z		5	0.38	75.85	0.60	0.33	0.76	1.69
Sc-K1		4	0.31	22.04	0.75	0.27	0.22	1.24
Td-B		5	0.44	77.84	0.60	0.39	0.78	1.77
Td-P	**Oo-1Pw13**	5	0.19	66.74	0.60	0.17	0.67	1.44
Td-V		6	0.18	39.90	0.50	0.16	0.40	1.05
Td-Z		3	0.26	19.56	1.00	0.23	0.20	1.43
Sc-CLOS		3	0.10	14.97	1.00	0.09	0.15	1.24
Td-B		3	0.12	16.76	1.00	0.10	0.17	1.27
Td-P		3	0.20	100.00	1.00	0.18	1.00	2.18
Td-V		4	0.16	15.31	0.75	0.14	0.15	1.04
Td-Z		3	0.30	35.14	1.00	0.27	0.35	1.62
Sc-QA23		8	0.38	100.00	0.38	0.34	1.00	1.71
Td-B		5	0.16	57.87	0.60	0.14	0.58	1.75
Td-P		6	0.29	100.00	0.50	0.26	1.00	2.26
Td-V		6	0.23	67.24	0.50	0.20	0.67	1.80
Td-Z		5	0.30	100.00	0.60	0.26	1.00	2.46
Sc-K1	**Oo-CH11**	4	0.17	66.09	0.75	0.15	0.66	1.99
Td-B		5	0.57	85.53	0.60	0.50	0.86	2.46
Td-P		5	0.14	73.43	0.60	0.12	0.73	1.88
Td-V		5	0.42	55.43	0.60	0.37	0.55	2.02
Td-Z		4	0.45	84.78	0.75	0.39	0.85	2.59

Sc-CLOS		4	0.31	100.00	0.75	0.28	1.00	3.03
Td-B		5	0.43	100.00	0.60	0.38	1.00	2.58
Td-P	**Oo-CH11**	4	0.66	100.00	0.75	0.58	1.00	3.33
Td-V		4	0.48	59.14	0.75	0.43	0.59	2.37
Td-Z		3	0.32	50.63	1.00	0.29	0.51	2.39
Sc-QA23		9	0.17	70.37	0.44	0.15	0.70	1.85
Td-B		4	0.16	100.00	1.00	0.14	1.00	3.14
Td-P		6	0.29	100.00	0.67	0.26	1.00	2.76
Td-V		6	0.19	58.18	0.67	0.16	0.58	2.13
Td-Z		6	0.15	53.85	0.67	0.13	0.54	2.05
Sc-K1		4	0.16	20.84	1.00	0.14	0.21	2.18
Td-B		4	0.51	69.33	1.00	0.45	0.69	2.97
Td-P	**Oo-PSU-1**	5	0.25	40.46	0.80	0.22	0.40	2.26
Td-V		6	0.31	100.00	0.67	0.28	1.00	2.78
Td-Z		4	0.38	41.39	1.00	0.34	0.41	2.59
Sc-CLOS		5	0.24	48.50	0.80	0.21	0.49	2.21
Td-B		5	0.22	41.62	0.80	0.19	0.42	2.12
Td-P		4	0.63	78.61	1.00	0.56	0.79	3.18
Td-V		4	0.49	67.71	1.00	0.43	0.68	2.94
Td-Z		5	0.41	59.69	0.80	0.36	0.60	2.47

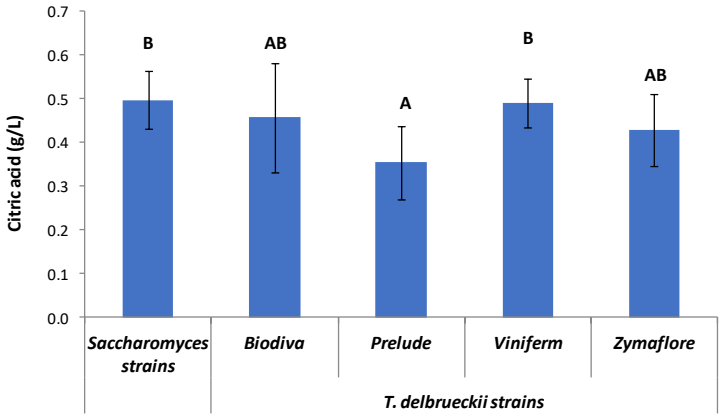

Figure SD6. Citric acid consumption (g/L) after AF. Mean ± standard deviation (n=9). Different uppercase letters indicate a significant difference between values using the Tukey (HSD) test at $p < 0.05$. *Saccharomyces* strains correspond to the citric acid consumption of QA23, CLOS and K1 strains.

UNIVERSITAT ROVIRA I VIRGILI
NEW PERSPECTIVES ON MALOLACTIC FERMENTATION AND ORGANOLEPTIC IMPROVEMENT OF WINES: INFLUENCE OF TORULASPORA DELBRUECKII ON DIFERENT TYPES OF WINEMAKING
Candela Ruiz de Villa Sardón

Section 1.2

Comparative study of inoculation strategies of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* on the performance of alcoholic and malolactic fermentations in an optimized synthetic grape must

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Abstract

Progress in oenological biotechnology now makes it possible to control alcoholic (AF) and malolactic (MLF) fermentation processes for the production of wines. Key factors in controlling these processes and enhancing wine quality include the use of selected strains of non-*Saccharomyces* species, *Saccharomyces cerevisiae*, and *Oenococcus oeni*, as well as the method of inoculation (co-inoculation or sequential) and the timing of inoculation.

In the present work, we investigated the effects of different inoculation strategies of two *Torulaspora delbrueckii* (Td-V and Td-P) strains followed by *S. cerevisiae*. Times (two, four, and six days) and types (co-inoculation and sequential) of inoculation were evaluated on the AF of a synthetic grape must. Furthermore, this synthetic medium was optimized by adding linoleic acid and β-sitosterol to simulate the natural grape must and facilitate reproducible results in potential assays. Subsequently, the wines obtained were inoculated with two strains of *Oenococcus oeni* to carry out MLF. Parameters after AF were analysed to observe the impact of wine composition on the MLF performance. The results showed that the optimization of the must through the addition of linoleic acid and β-sitosterol significantly enhanced MLF performance. This suggests that these lipids can positively impact the metabolism of *O. oeni*, leading to improved MLF efficiency. Furthermore, we observed that a 4-day contact period with *T. delbrueckii* leads to the most efficient MLF process and contributed to the modification of certain AF metabolites, such as the reduction of ethanol and acetic acid, as well as an increase in available nitrogen. The combination of Td-P with Oo-VP41 for 4 or 6 days during MLF showed that it could be the optimal option in terms of efficiency. By evaluating different *T. delbrueckii* inoculation strategies, optimizing the synthetic medium and studying the effects on wine composition, we aimed to gain insights into the relationship between AF conditions and subsequent MLF performance. Through this study, we aim to provide valuable insights for winemakers and researchers in the field of wine production and will contribute to a better understanding of the complex interactions between these species in the fermentation process.

Keywords

Oenococcus oeni; Inoculation time; β-sitosterol; Linoleic acid

1. Introduction

The use of different yeast species in wine alcoholic fermentation (AF) has been an interesting subject of study in recent years (Beltran et al., 2002; Bordet et al., 2020; Englezos et al., 2022). In the past, it was common to inoculate only *Saccharomyces cerevisiae* into the fermentation process. However, in recent years, the use of non-*Saccharomyces* starter cultures and spontaneous fermentation has become more widespread (Bordet et al., 2020; Fazio et al., 2023; Jolly et al., 2014; Roudil et al., 2019). Currently, there are different species, or mixtures of species, available as commercial active dry yeast (ADY) that are used in the wine industry, e.g., *Torulaspora delbrueckii*. *Metschnikowia pulcherrima*, *Lachancea thermotolerans or Pichia kluyveri* (Fazio et al., 2023; Roudil et al., 2019; Viana et al., 2008; Vicente et al., 2021). Their use is an excellent biotechnological solution for better process control and a more stable product apart from other organoleptic improvements (Benito, 2018; Roudil et al., 2019).

Regarding *T. delbrueckii*, it has been demonstrated that, among other benefits, its use improves aromatic complexity (Belda et al., 2017; Carpena et al., 2021; Minnaar et al., 2015), reduces acetic acid production (Bely et al., 2008; Canonico et al., 2019) and improves the red wine colour (Balmaseda et al., 2021b; Du Plessis et al., 2017a; Minnaar et al., 2018). However, this effect depends on the grape variety used; Minnaar et al. (2015) found that *T. delbrueckii* can decrease phenolic compounds in Pinotage wine but decrease in Cabernet franc wines. Furthermore, researchers have been dedicating more attention to understanding the compatibility of non-*Saccharomyces* with malolactic fermentation (MLF) (du Plessis et al., 2017b; Russo et al., 2020; Yılmaz and Gökmen, 2021). Especially, the species *T. delbrueckii* has shown positive impacts enhancing the efficiency and effectiveness of the process (Balmaseda et al., 2021a, 2022; Ferrando et al., 2020). MLF has a great impact on wine characteristics, mainly in overall acidity but also in flavour and aroma, due to the decarboxylation of L-malic acid into L-lactic acid by lactic acid bacteria (LAB), with *Oenococcus oeni* being the main bacteria responsible for this process in winemaking (Arnink and Henick-Kling, 2005; Lonvaud-Funel, 1999).

Yeast-bacteria synergy is strain-dependent (Benito, 2018b); that is, different strains of each species may interact differently and produce different metabolites (Ruiz-de-Villa et al., 2023b).

In addition, the timing of inoculation of non-*Saccharomyces* into the must before the inoculation of *S. cerevisiae* can influence the outcome of the fermentation process (Hranilovic et al., 2020; Snyder et al., 2021; Zhao et al., 2022). During AF, non-*Saccharomyces* species are typically present in the early stages, while *S. cerevisiae* dominates and completes the fermentation (Ribéreau-Gayon et al., 2006b). Industrially, non-*Saccharomyces* can be inoculated as co-inoculation or sequential inoculation with *S. cerevisiae*. Consequently, these strategies can have different effects on the final wine and the subsequent behaviour of *O. oeni* during MLF (Martín-García et al., 2020; Zhao et al., 2022). In order to investigate the microbiological interactions effectively, it is necessary to utilize reproducible media that allow for the analysis of fermentation behaviours under controlled conditions, without relying on the complex composition of natural grape must. However, the composition of designed synthetic grape musts sometimes does not lead to successful MLF outcomes in the corresponding obtained wines. Linoleic acid and β-sitosterol are two of the main lipids found in grape skin, between polyunsaturated fatty acid and phytosterols, respectively (Guittin et al., 2021; Le Fur et al., 1994). Therefore, it has been proposed to improve previously developed synthetic grape must by incorporating these lipids, with the aim of improving MLF assays.

The aim of this work was, firstly, to optimize a synthetic grape must by incorporating linoleic acid and β-sitosterol to achieve reproducible conditions for other possible tests in this context. Secondly, we evaluated the effect of various inoculation strategies and the timing involving different strains of *T. delbrueckii* and *S. cerevisiae* on the outcome of the AF products. Lastly, the third objective was to provide a comprehensive understanding of how wine composition, resulting from different AF conditions, affects the progression and efficiency of MLF with two *O. oeni* strains. According to these three objectives, we sought to study the influence of this optimized grape must on AF and MLF performance.

2. Materials and methods

2.1 Microorganisms and media

The yeast strains used in this study were a commercial *Saccharomyces cerevisiae* Lalvin-QA23 (Sc-QA) from Lallemand Inc. (Montreal, Canada) and two *Torulaspora delbrueckii* strains: Viniflora Prelude (Chr. Hansen Holding AS, Hoersholm, Denmark) (Td-P) and NSA1 Viniferm NSDT (Agrovin, Spain) (Td-V). Two strains of *Oenococcus oeni* were used: VP41 from Lallemand Inc. (Montreal, Canada) and CH11 from Christian Hansen A/S (Hørsholm, Denmark). These strains were chosen considering the results obtained in a previous work by Ruiz de Villa et al. (2023a), which aimed to study the interactions between different strains of *S. cerevisiae*, *T. delbrueckii* and *O. oeni.* In this way, the strains that showed the best results in different parameters, such as AF and MLF kinetics, were selected for this study.

Total yeast population kinetics and inocula were monitored by counting viable cells from YPD plates containing 10 g/L yeast extract (Panreac, Barcelona, Spain), 20 g/L peptone (Panreac), 20 g/L glucose (Panreac) and 20 g/L agar (Panreac). Similarly, the viability of *T. delbrueckii* was determined on a lysine medium (Lysine agar 6.6% (w/v), 2 mL/L potassium lactate 10% (v/v) and 5 mL/L lactic acid (v/v) (Sigma-Aldrich, Barcelona, Spain).

The *O. oeni* strains were precultured in an MRS broth medium (De Man et al., 1960) (Difco Laboratories, Detroit, MI, USA) supplemented with 4 g/L DL-malic acid (Sigma–Aldrich) and 5 g/L D-fructose (Panreac) at a pH of 5. The inocula were incubated at 28 °C in a CO_2 (10%) incubator. The same conditions and counting medium (MRS plates) were used to evaluate the *O. oeni* populations.

2.2 Alcoholic fermentation

Two grape musts were used to perform AF; the first one (Sc-Lip-) was described by Ruiz de Villa et al. (2023a), and the second one (Sc-Lip+) had the same composition but 10 mg/L β-sitosterol (ref 85451, Sigma–Aldrich) and 200 mg/L linoleic acid (Sigma–Aldrich) was added.

Before the inoculation of the synthetic grape must, the yeast strains were rehydrated from ADY according to the manufacturer's indications: 37 °C for 30 min for the *S. cerevisiae* and

30 °C for 30 min for the *T. delbrueckii* strains. The initial population was 2.5×10^6 cells/mL for both yeast species.

AF was carried out in triplicate with the synthetic must without lipids (Sc-Lip-) and in the presence of lipids (Sc-Lip+, chosen as a control throughout the study) in 500 mL bottles containing 450 mL of the must at 22 °C with agitation at 120 rpm in an Innova 42 incubator shaker (New Brunswick Scientific, Madrid, Spain). The closures allowed carbon dioxide to escape and sampling. AF kinetics were monitored by measuring density with an electronic densimeter (Densito 30PX, Mettler-Toledo, Barcelona, Spain). In addition, the population dynamics of the total yeast and *T. delbrueckii* were controlled with YPD and a lysine media, respectively. The AF was considered finished when residual sugars (glucose + fructose) was under 2 g/L.

Four inoculation strategies were tried: a co-inoculation at the same time point of *S. cerevisiae* and *T. delbrueckii* and three sequential inoculations with different time points of *T. delbrueckii* contact followed by the inoculation of *S. cerevisiae*: 2, 4 and 6 days. The different inoculation strategies were tested with both *T. delbrueckii* strains.

2.3 Malolactic fermentation

When AF was finalized, the wines were stabilized for 7 days at 4 °C. Then, they were centrifuged at 1500 x *g* for 20 min at 4 °C and filtered with 0.22 μm filters (Merck, Darmstadt, Germany). To avoid biological differences, the replicates were mixed and then divided into tubes with 50 mL of wine by triplicate during static fermentation at 20 °C. The MLFs were inoculated with one of the two *O. oeni* strains described previously at a population of 2×10^7 cells/mL. The wines were not supplemented with L-malic acid before MLF. The process was monitored by measuring the consumption of L-malic acid enzymatically every day using the Y15 analyser (Biosystems S.A., Barcelona, Spain). The MLFs were considered finished when the L-malic acid concentration was < 0.1 mg/L. Viable populations were determined by plating serial dilutions in an MRS media.

2.4 Calculation of the area under the curve (AUC)

The measurement of the area under the curve (AUC), the decrease in AF density or L-malic consumption in the MLF, was used as an indicator of fermentation performance which allowed us to overcome the total times and fermentation kinetics. The AUCs were calculated by integrating either the decreasing density during the AF or the decreasing L-malic acid consumption during the MLF between two consecutive times. The calculation formula is the sum of consecutive AUCs = Σ [$((d_2+d_1)/2) * (t_2-t_1) + \ldots. + ((d_n+d_{n-1})/2) * (t_n-t_{n-1})$] where d_1, d_2, …d_{n-1}, d_n are the densities of the grape must at times 1, 2, n-1 and n, respectively.

2.5 Chemical analysis

2.5.1 General oenological metabolites

The general parameters of the wines were analysed after the AF. Glycerol, ethanol, and citric acid were determined with high-performance liquid chromatography (HPLC) using an Agilent 1100 HPLC (Agilent Technologies, Waldbronn, Germany) (Zhu et al., 2020). Sample pre-treatment consisted of filtering the samples with 0.22 μm pore filters (Agilent Technologies). The column used was a Hi-Plex H (300 mm x 7.7 mm) column inside a 1260 MCT (Infinity II Multicolumn Thermostat). The mobile phase consisted of 5 mM H_2SO_4 at a flow rate of 0.6 mL/min. The HPLC was equipped with two detectors, an MWC detector (G1365B multiwavelength detector) and a RID detector (1260 Infinity II refractive index detector, Agilent Technologies). Glycerol, ethanol and citric acid concentrations were calculated from external calibration curves of known standards.

Ammonia, α-amino nitrogen (PAN), acetic acid and residual sugars were quantified with Biosystem's enzymatic kits. While succinic acid (Megazyme, Wicklow, Ireland) was analysed by an enzymatic kit using a microplate reader (POLARstar Omega, BMG LABTECH, Ortenberg, Germany).

The extraction of mannose to analyse the number of mannoproteins was performed as described by (Balmaseda et al., 2021a). Quantification was performed with a D-mannose and D-glucose enzymatic assay kit (Megazyme).

2.5.2 Volatile compound analysis

The volatile compounds of the wines obtained after alcoholic fermentation were analysed by liquid/liquid extraction with a methyl tert-butyl ether/hexane mixture (1/1). Briefly, 50 μL of H_3PO_4 (1/3) and 25 μL of internal standards (3-octanol, 1.98 g/L; heptanoic acid, 3.33 g/L and heptadecanoic acid, 1.03 g/L) were added to 5 mL of wine. To extract the volatile compounds, 400 μL of MTBE/hexane was added, and the mixture was stirred for 2 min by vortexing. After that, the wines were centrifuged at 5,200 x *g* for 5 min at room temperature. The organic extract was placed in an insert placed inside the vial to then injected into a GC-FID chromatograph. The chromatographic conditions were as follows: injection volume, 2 μL; injection mode: spitless; inlet and detector temperatures, 250 °C; column: HP-FFAP (30 m x 250 μm 0.25 μm, Agilent). The concentrations of the volatile compounds were calculated from external calibration curves of known standards. The volatile compounds determined were fusel alcohols acetates (isobutyl acetate, isoamyl acetate, 2-phenylethaol acetate), fusel alcohols (FA: isoamyl alcohol, hexanol, *cis*3-hexenol, 2-phenylethanol), ethyl esters of fatty acids (ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl dodecanoate), short-chain fatty acids (SCFA: propanoic, butanoic (butyric acid) and pentanoic (valeric acid)), medium-chain fatty acids (MCFA: hexanoic, octanoic, decanoic acids), and long-chain fatty acids (LCFA: tetradecanoic (myristic acid), hexadecanoic (palmitic acid), octadecanoic (stearic acid), and 9,12-octadecadienoic acid (linoleic acid)).

2.6 Statistical analysis

The fermentation was performed by triplicate biological samples. The statistical software used was XLSTAT version 2021.5.1 (Addinsoft, Paris, France). The data were analysed with two-way ANOVA with a post hoc Tukey test (Honesty Significant Difference) with a confidence interval of 95% and significant results with a p-value < 0.05. Principal component analysis was also performed to describe the effects of the yeast and bacterial strains on the alcoholic and malolactic performance. For this, we used the AUC calculated from the AF and MLF kinetics.

3. Results and discussion

3.1. Alcoholic fermentation

3.1.1. Fermentation kinetics

The synthetic grape must (Sc-Lip+) used in this study was modified from the version previously published (Sc-Lip-) in the work by Ruiz-de-Villa et al. (2023a). To improve the performance of AF and MLF, linoleic acid and β-sitosterol were added. These two components are among the main lipids found in the skin of grapes (Beltran et al., 2008; Guittin et al., 2021; Le Fur et al., 1994). Linoleic acid is the major polyunsaturated fatty acid in grapes, (Liu et al., 2018) described concentrations which varies from trace to 280 mg/L depending on the cultivar and winemaking conditions, for instance the Ugni Blanc cultivar contains approximately 12.7 mg/g dry matter variety (Guittin et al., 2021). β-sitosterol is the main phytosterol of grapes, and according to previous studies, it can be 70% (Le Fur et al., 1994) or 84% (Guittin et al., 2021) of the total phytosterols.

The original synthetic must (Sc-Lip-) and the modified must (Sc-Lip+) were compared to observe possible modifications in the AF and MLF. The duration of the AF was shorter when using the Sc-Lip+ must (Figure 1), finishing the AF in 16 days compared to 18 days for the original synthetic must (Sc-Lip-). The addition of linoleic acid and β-sitosterol to the Sc-Lip+ must appeared to have a positive impact on the alcoholic fermentation performance. In previous studies, it was observed that the addition of linoleic acid and β-sitosterol was observed to increase yeast viability values throughout the AF (Beltran et al., 2008). Since *S. cerevisiae* cannot synthesize unsaturated fatty acids (UFAs) in the absence of molecular oxygen, their presence in grape must is essential for yeast growth (Casu et al., 2016). The incorporation of β-sitosterol can be detected in *S. cerevisiae* biomass, suggesting that it is incorporated by yeast and used for growth (Luparia et al., 2004).

However, it has been reported that the addition of phytosterols, especially β-sitosterol, to fermentative media may lead to stuck fermentation in the absence of oxygen (Luparia et al., 2004). However, in the present study, we found that the addition of β-sitosterol at a concentration of 10 mg/L under our experimental conditions, without entry of oxygen and

agitation, did not result in stuck fermentation, and the AF was completed successfully. These results suggest that the specific concentration of β-sitosterol used in our study may have played a role in the successful performance of the AF.

On the other hand, the use of *T. delbrueckii* in the co-inoculation or sequential inoculation with *S. cerevisiae* impacted the duration of the AF differently (Figure 1). The co-inoculation AF kinetics resulted in shorter fermentation times with respect to the control, particularly when using the Td-P strain (Figure 1A), which took 14 days to complete the fermentation. In comparison, the Td-V strain took 16 days to complete fermentation, the same amount of time as the Sc-Lip+ condition (Figure 1B).

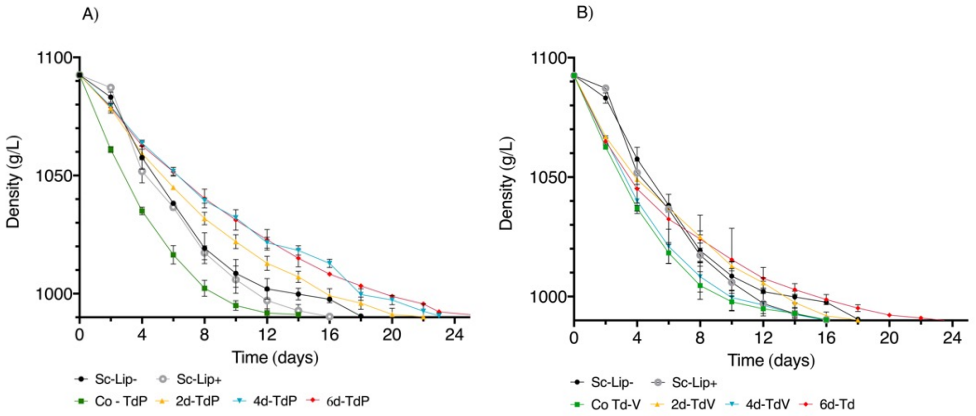

Figure 1. Evolution of the alcoholic fermentation kinetics: Fermentations with A) *T. delbrueckii* Prelude + *S. cerevisiae* QA23 and B) *T. delbrueckii* Viniferm + *S. cerevisiae* QA23. Sc-Lip- and Sc-Lip+ correspond to the *S. cerevisiae* control fermentations with the original synthetic must and the modified synthetic must, respectively; Co-TdP and Co-TdV correspond to the co-inoculated fermentations. 2, 4 and 6 days correspond to the sequential fermentations with *T. delbrueckii* and *S. cerevisiae* inoculated at these times. ● Sc-Lip-, ● Sc-Lip+, ■ Td-Co, ▲ 2d-Td, ▼ 4d-Td and ♦ 6d-Td.

When considering sequential fermentations, the total fermentation time was increased given both species are major competitors for the nitrogenous nutrients within the grape must (Zilelidou and Nisiotou, 2021). Comparing the results in Figures 1A and 1B, Td-V (B) fermentations tended to be shorter in duration than Td-P (A) fermentations. Moreover, the data suggest that the duration in which Td was present during the fermentation process was directly proportional to the duration of the fermentation process. In the case of Td-P, 2, 4 or

6 days of its presence within the must resulted in 22, 23, or 26 days of AF, respectively. Similarly, in the case of Td-V, 2, 4 or 6 days of its presence within the must resulted in 16, 18, or 24 days of AF, respectively. These findings highlight the importance of considering the specific strain of Td and its duration within the AF process.

Regarding the areas under the curve (AUCs) calculated from the density decrease in AF kinetics in Figure 1, there were significant differences that supported the AF kinetics results (Table 1). The AUCs made it possible to indirectly associate the duration of fermentation with the rate of sugar consumption. Among these differences, the co-inoculation conditions had the lowest AUC, followed by the single cultures of *S. cerevisiae*, with a faster AF for Sc-Lip+ than for Sc-Lip-. Moreover, the Td-V strain decreased the AF duration in the sequential inoculation compared to the co-inoculation treatment, while for the Td-P strain, the opposite was observed (Table 1).

3.1.2. Viable yeast population

With regard to the population of the viable cells of the two fermentations (S-lip- and S-lip+), the results showed that the populations of the viable cells of *S. cerevisiae* were higher in the presence of lipids than in their absence (Figure SD1). This trend suggests that the addition of β-sitosterol and linoleic acid was beneficial for the growth and proliferation of the yeast under the conditions of the control fermentations, as has been reported in previous studies (Beltran et al., 2008; Casu et al., 2016).

In Figure 2 is shown the evolution as a percentage of the viable cells of the two yeasts, *Torulaspora* and *Saccharomyces*, determined by the use of a selective solid growth media during the AF. Total population of both yeast species is shown in Figure SD3. Regarding the co-inoculated AF, we observed that during the AF, the strain Td-P had a slightly higher abundance than Td-V (Figure 2 A and B). On the other hand, when comparing the abundance of the species in the co-inoculation with sequential AF, as previously reported (Bordet et al., 2020; Lleixà et al., 2016), *S. cerevisiae* dominated the fermentation from the middle to the end of the process.

Table 1. Analytical parameters of the wines after AF. Mean and standard deviation (SD) (n=3). Different uppercase letters indicate a significant difference between the values of inoculation strategies and must conditions, and lowercase letters indicate a significant difference between the values of the different strains using the Tukey (HSD) test at *p*-value < 0.05. Sc-Lip- and Sc-Lip+ correspond to *S. cerevisiae* control fermentations with original synthetic must and modified synthetic must, respectively; Co-TdP and Co-TdV correspond to the co-inoculated fermentations with *T. delbrueckii* Prelude or Viniferm + *S. cerevisiae*. 2 days, 4 days and 6 days correspond to the sequential fermentations with *T. delbrueckii* (Prelude or Viniferm) and *S. cerevisiae* inoculated at these times.

	AUC	pH	L-malic acid (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (%, v/v)	Eq-Mannose (g/L)	α-amino nitrogen (mg N/L)
Sc-Lip-	18495 ± 54 ^{h}	3.32 ± 0.03 ^{aA}	1.72 ± 0.03 ^{aA}	0.95 ± 0.06 ^{cA}	7.94 ± 0.25 ^{cB}	10.98 ± 0.15 ^{cdB}	0.07 ± 0.03 ^{aA}	5.67 ± 1.15 ^{bA}
Sc-Lip+	16460 ± 31 ^{f}	3.45 ± 0.07 ^{bcA}	1.65 ± 0.03 ^{bA}	0.84 ± 0.05 ^{bcA}	7.67 ± 0.49 ^{cB}	11.66 ± 0.56 ^{dB}	0.08 ± 0.05 ^{aA}	6.67 ± 1.53 ^{bA}
Co-TdP	15269 ± 29 ^{i}	3.49 ± 0.03 ^{abA}	1.53 ± 0.04 ^{aB}	0.37 ± 0.11 ^{aB}	4.37 ± 0.10 ^{aA}	9.49 ± 0.06 ^{aB}	0.20 ± 0.07 ^{bB}	4.00 ± 1.00 ^{aB}
Co-TdV	17281 ± 15 ^{g}	3.35 ± 0.01 ^{abA}	1.61 ± 0.04 ^{aA}	0.45 ± 0.13 ^{aA}	4.69 ± 0.34 ^{aB}	9.13 ± 0.12 ^{aA}	0.16 ± 0.01 ^{bB}	4.00 ± 0.01 ^{aA}
2 days- TdP	22565 ± 23 ^{d}	3.51± 0.01 ^{cA}	1.72 ± 0.04 ^{abB}	0.79 ± 0.04 ^{bB}	5.91 ± 0.42 ^{bA}	11.71 ± 0.29 ^{bcB}	0.14 ± 0.03 ^{abB}	9.33 ± 0.58 ^{bB}
4 days-TdP	23708 ± 24 ^{c}	3.66 ± 0.03 ^{cA}	1.78 ± 0.03 ^{bB}	0.82 ± 0.05 ^{bB}	6.02 ± 0.57 ^{bcA}	11.54 ± 0.50 ^{bcB}	0.17 ± 0.02 ^{bB}	9.33 ± 1.53 ^{bB}
6 days-TdP	26682 ± 42 ^{a}	3.65 ± 0.03 ^{cA}	1.71 ± 0.03 ^{abB}	0.84 ± 0.08 ^{bB}	4.63 ± 0.49 ^{bA}	11.36 ± 0.13 ^{bB}	0.13 ± 0.02 ^{abB}	11.33 ± 1.53 ^{bB}
2 days- TdV	19436 ± 25 ^{e}	3.56 ± 0.02 ^{dA}	1.62 ± 0.03 ^{bA}	0.62 ± 0.08 ^{bA}	5.85 ± 0.26 ^{bB}	10.51 ± 0.08 ^{bcA}	0.10 ± 0.01 ^{abB}	6.33 ± 0.58 ^{bA}
4 days-TdV	17312 ± 52 ^{g}	3.64 ± 0.02 ^{dA}	1.60 ± 0.04 ^{abA}	0.55 ± 0.13 ^{bA}	6.82 ± 0.90 ^{bcB}	10.53 ± 0.12 ^{bcA}	0.18 ± 0.02 ^{bB}	8.33 ± 1.53 ^{bA}
6 days-TdV	24413 ± 100 ^{b}	3.62 ± 0.03 ^{dA}	1.61 ± 0.04 ^{aA}	0.68 ± 0.07 ^{bA}	6.44 ± 0.22 ^{bB}	10.04 ± 0.12 ^{bA}	0.13 ± 0.04 ^{abB}	7.00 ± 1.00 ^{bA}

However, we found that in the co-inoculation, *S. cerevisiae* was more prevalent at the end of the AF process, while in the sequential fermentations, *T. delbrueckii* maintained a higher percentage at the end of the process, particularly in the Td-P strain, which comprised of 40-30% of the total population (Figure 2 C, E and G). Renault et al. (2015) also observed more growth of *T. delbrueckii* in sequential inoculation than in co-inoculation. This higher presence of Td-P during the fermentation may be related to the longer duration of the AF, while in Td-V 2-Days and 4-Days, it was shorter, as in the co-inoculation AF (Figure 1). Regarding the differences between the sequential conditions, it appears that during the fermentation with 4 days of *T. delbrueckii* contact, the population was higher than that at 2 and 6 days (Figure 2 E and F).

Other studies have also observed the impacts of the inoculation strategy on the relative abundance of *T. delbrueckii* and *S. cerevisiae* during AF. For example, testing other strains, Roca-Mesa et al. (2022) found that in a sequential AF at 48 hours, *T. delbrueckii* was the dominant species, with 60% at the end of the fermentation. In the case of co-inoculation, with a 1:1 proportion of the two species, they observed an imposition of *S. cerevisiae,* but *T. delbrueckii* still made up 40% of the total population. Taillandier et al. (2014); Zhu et al. (2021) also reported the dominance of *S. cerevisiae* in co-inoculated fermentations at the end of AF.

Overall, our results and those of other studies suggest that the inoculation strategy can impact the relative abundance of *T. delbrueckii* and *S. cerevisiae* during AF. Additionally, the proportion of *T. delbrueckii* to *S. cerevisiae* appears to be strain dependent.

3.1. General oenological parameters analysed.

The metabolic composition of the wines after the AF changed among the conditions. The original and modified controls showed significant differences in their metabolic compositions. Regarding the pH of the final wines, Sc-Lip+ presented a higher pH than Sc-Lip- (Table 1). On the other hand, L-malic acid had a decreasing trend in the Sc-Lip+ wines. The ethanol content was higher in the wines containing lipids (Lip+) (Table 1). As has been described, ethanol can increase up to 1% vol. with the presence of linoleic acid in the fermentative medium (Liu et al., 2018). Furthermore, there was a decrease in the concentration of acetic acid in the modified synthetic must (Table 1). These results are consistent with previous findings, which

have demonstrated that the presence of linoleic acid and β-sitosterol can lead to a reduction in acetic acid concentrations (Beltran et al., 2008).

The *T. delbrueckii* wines showed significant differences in pH and the concentration of ethanol and α-amino nitrogen (Table 1). The pH of the wines produced under the different fermentation conditions was found to vary significantly (Table 1). The Sc-Lip+ wines and the co-inoculated wines had lower pH values at the end of the AF compared to the sequential fermentation conditions. Also, there were significant differences in pH among the different durations of *T. delbrueckii* contact: the wines fermented with 2 days of *T. delbrueckii* contact had a lower pH than those fermented with 4 or 6 days of contact. Among the *T. delbrueckii* strains, there were no significant differences in pH. The extended presence of *T. delbrueckii* resulted in wines with high pH values. Balmaseda et al. (2022a), Chen et al. (2018) and Martín-García et al. (2020) reported a pH increase of 0.1 or more in sequential wines with *T. delbrueckii* for 4 days with respect to the control with *S. cerevisiae*.

Significant differences in α-amino nitrogen were found at the end of the AF (Table 1). The remaining α-amino nitrogen was lower in the *S. cerevisiae* control wines and in the co-inoculated wines, which was significantly lower compared to the sequential fermentation wines. Among them, there were significant differences in the α-amino nitrogen remaining in the 4-Day and 6-Day *T. delbrueckii* contact conditions. When considering only the Sc-controls and *T. delbrueckii* strains, the Td-P wines displayed significantly higher α-amino nitrogen levels compared to the other wines (9.33 – 11.33 mg/L). Previous studies (Bely et al., 2008; Martín-García et al., 2020) have reported higher concentrations of residual α-amino nitrogen in wines fermented with *T. delbrueckii*. However, the extent of this increase can vary depending on the specific strain used, as they may differ in their ability to release amino acids, their nitrogen requirements (Benito, 2018) or the occurrence of *T. delbrueckii* autolysis.

This suggests that the choice of the *T. delbrueckii* strain and its interaction with the fermenting process can impact the levels of residual α -amino nitrogen in the resulting wine.

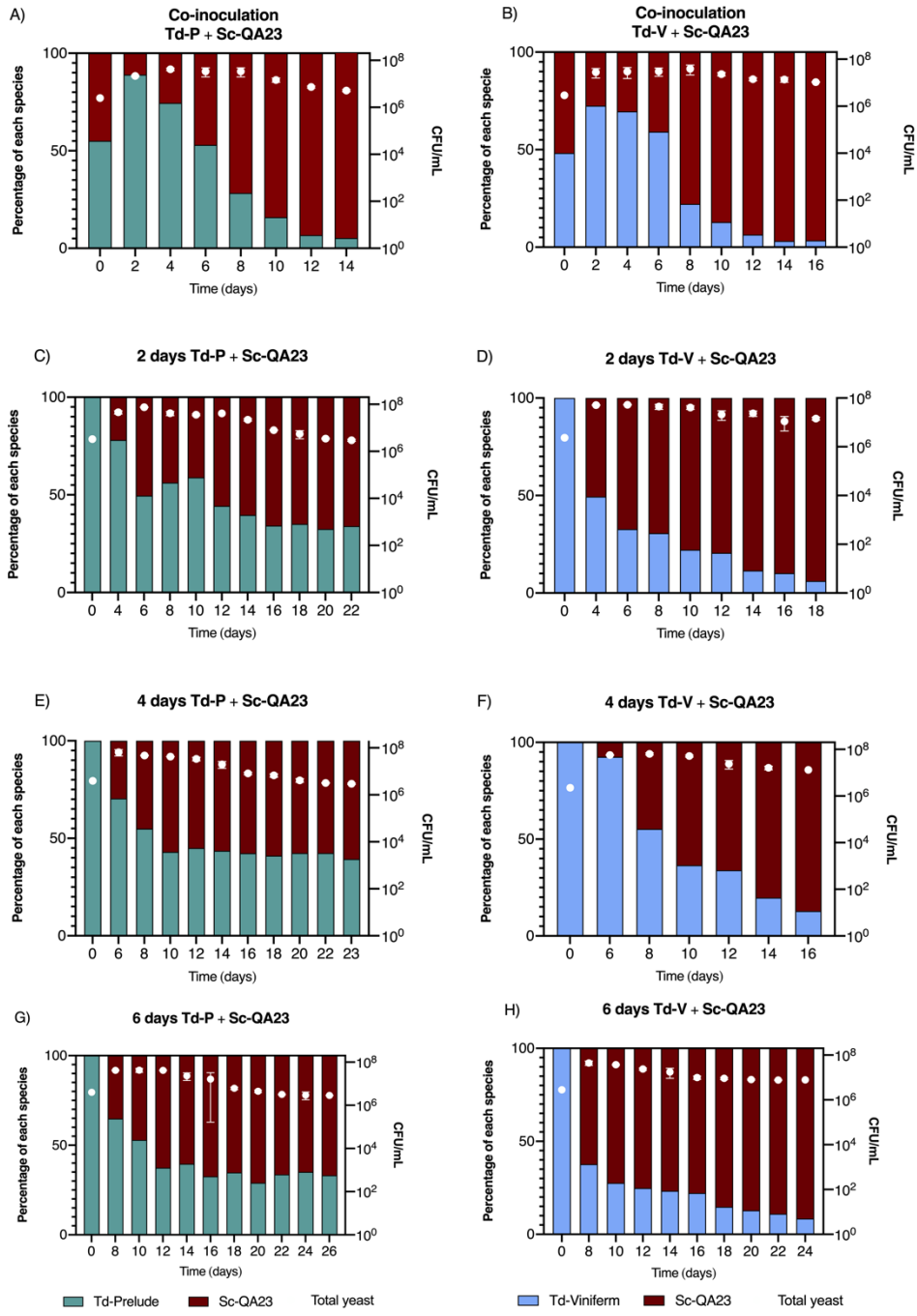

Figure 2. Total yeast viability (CFU/mL) and percentage of each species (*Saccharomyces cerevisiae* and *Torulaspora delbrueckii)* in co-inoculated and sequential fermentations at 2, 4 or 6 days. Sc-QA23, Td-P and Td-V correspond to *S. cerevisiae*, *T. delbrueckii* Prelude and *T. delbrueckii* Viniferm, respectively.

The glycerol concentration exhibited significant differences based on the timing of the inoculation strategy and the strain (Table 1). Literature reported that glycerol increases using *T. delbrueckii being* related to a decrease in ethanol (Balmaseda et al., 2022a; Belda et al., 2015; González-Royo et al., 2015). However, the co-inoculated wines tended to have lower glycerol concentrations compared to sequential and control conditions in spite of the fact they also showed the lowest ethanol concentrations (Table 1). It has been described that the production of glycerol relies on the development of the glycero-pyruvic pathway (Benito, 2018) but in our experimental conditions, some results showed that as described by Zhu et al. (2020, 2021) and Rodrigues et al. (2016) that the reduction of glycerol in non-*Saccharomyces*/*Saccharomyces* sequential fermentations in a synthetic grape must did not necessarily imply that glycerol production was the main route of ethanol reduction in mixed fermentations in culture. Regarding ethanol content between conditions wines fermented only with *S. cerevisiae* (Sc-Lip+ and Lip-) presented the highest values, followed by the sequential fermentations, being the lowest values in 6-days condition (Table 1). The decrease was related not only to the time but also to the strain, and the Td-V wines had a larger reduction in ethanol, from 1 to 1.5% (v/v). In synthetic must, Zhu et al. (2020) described a decrease of 0.29 or 0.47% (v/v) depending on the strain. Other authors have also observed a reduction in alcohol degree in sequential fermentations, despite they had other conditions as natural grape must (Belda et al., 2015) or the presence of other species in the starter culture (Yılmaz and Gökmen, 2021).

L-malic acid consumption by yeast varies between 10 and 25% depending on the species (Ribéreau-Gayon et al., 2006b). The sequential Td-P and control wines consumed a lower amount of L-malic acid, ranging from 0.22 to 0.36 g/L, while the Td-V wines consumed slightly more, from 0.38 to 0.4 g/L. Finally, the co-inoculated wines had the highest consumption of L-malic acid (Table 1).

In addition, the higher decrease in acetic acid levels was found when using the co-inoculation strategy with a final concentration of 0.37 - 0.45 g/L (Table 1). The sequential fermentation conditions also resulted in lower levels of acetic acid compared to the control (Sc-Lip+). Furthermore, the Td-V wines had significantly lower concentrations of acetic acid. Similarly, Taillandier et al. (2014) reported a decrease of 0.3 g/L in synthetic grape must through the

use of *T. delbrueckii* in sequential fermentations for 48 hours. However, there are also variations between different yeast strains. Du Plessis et al. (2017b) also reported in synthetic must with *T. delbrueckii* monoculture fermentations a reduction in volatile acidity with *T. delbrueckii* compared to *S. cerevisiae*. The concentrations of citric and succinic acids did not show significant differences.

Consistent with previous research, the results for mannoproteins determined in eq-mannose showed a trend towards higher mannoprotein values in the wines fermented with *T. delbrueckii* (Belda et al., 2015; Ruiz-de-Villa et al., 2023b). However, there were no significant differences between the Td strains (Table 1).

3.2. Volatile composition

Volatile composition after AF is shown in Table 2. Some authors described a relationship between the presence of UFA in the grape must and the formation of volatile compounds derived from yeast (Sumby et al., 2010). However, any differences were found between Lip- and Lip+ regarding volatile composition, thus the addition of linoleic acid at this concentration did not affect the volatile composition.

The most significant differences between inoculation strategies were found in the content of SCFA and MCFA (Table 2). SCFAs with the sequential fermentations showing remarkably higher concentrations than the co-inoculation and Sc-Lip+, with butyric acid being the main factor responsible for this difference.

Conversely, in MCFA, there was a significant decrease in the levels of octanoic acid and decanoic acid in wines with sequential presence of *T. delbrueckii*, regardless of the inoculation time, compared to the co-inoculated wines. Additionally, an increasing trend was observed in Sc-Lip+ and Lip- wines compared to the sequential wines. Literature also describes a decrease in MCFA content resulting from the presence of *T. delbrueckii* during sequential fermentations of natural grape must (Balmaseda et al., 2018, 2021b).

In terms of ethyl esters of FA and fusel alcohol, no significant differences were found. However, there was an increasing trend observed in sequential wines, particularly in the 6 days-TdV condition, with respect to fusel alcohols. Several authors have also reported an

increase in fusel alcohol content, primarily in wines derived from natural must (Azzolini et al., 2015; Belda et al., 2015; Ruiz-de-Villa et al., 2023b).

3.3. Influence of *T. delbrueckii* inoculation strategy on malolactic fermentation

To perform the MLF, the wines produced from different alcoholic fermentations were inoculated with two *O. oeni* strains, Oo-VP41 and Oo-CH11. Our results showed that with the modified synthetic must (Sc-Lip+), the MLF process was completed one day earlier than the original must (Figure3). In a previous study (Ruiz-de-Villa et al., 2023b), the same synthetic must was used but without the addition of linoleic acid and β-sitosterol, different strains were tested in sequential fermentation and resulted in some cases of MLF failure. However, this study's conditions successfully allowed all wines to complete the MLF process. This suggests that adding β-sitosterol and linoleic acid to synthetic grape must enhances the efficiency of the MLF process. It is hypothesised, as for the yeast viability described above, that LAB can improve the fermentation performance in the presence of linoleic acid and/or β-sitosterol.

Furthermore, this synthetic must proposed has a simpler formulation than the one proposed by Costello et al., (2003) and showed similar or even better results in relation to MLF efficiency (Du Plessis et al., 2017b).

When the two yeast species were co-inoculated, the MLF process was as slow as the Sc-control fermentations (Figure SD4) but lasted longer than the wines produced with sequential fermentation (Figure 3). Martín-García et al. (2020) described a similar effect in the case of co-inoculations with these species. This behaviour could be linked to the lower abundance of *T. delbrueckii* during alcoholic fermentation (AF), where *S. cerevisiae* dominated the fermentation. Thus, affecting the metabolic profiles of the resulting wines, which in this case, as previously described, the co-inoculated wines had the highest concentration of MCFA, compounds toxic to *O. oeni* (Capucho and San Romao, 1994) via the destabilization of their membrane (Sereni et al., 2020). Furthermore, these wines exhibited the lowest concentration of α-amino nitrogen, which is an important nutrient for the growth and metabolism of *O. oeni* (Remize et al., 2006).

Table 2. Volatile compounds of the wines after AF. Σ: Fusel alcohol acetates (isobutyl acetate, isoamyl acetate and 2-phenylethanol acetate), Ethyl esters of FA (ethyl butanoate, ethyl hexanoate, ethyl octanoate and ethyl dodecanoate), Fusel alcohols (isoamyl alcohol, 1-hexanol, cis-3-hexen-1-ol, 2-phenylethanol), SCFA (propionic, butyric and valeric acids), MCFA (octanoic and decanoic acids), LCFA (myristic acid, palmitic acid and stearic acid). Mean and standard deviation (SD) (n=3). Different lowercase letters indicate a significant difference between conditions using the Tukey (HSD) test at *p*-value < 0.05. Sc-Lip- and Sc-Lip+ correspond to *S. cerevisiae* control fermentations with original synthetic must and modified synthetic must, respectively; Co-TdP and Co-TdV correspond to the co-inoculated fermentations with *T. delbrueckii* Prelude or Viniferm + *S. cerevisiae*. 2 days, 4 days and 6 days correspond to the sequential fermentations with *T. delbrueckii* (Prelude or Viniferm) and *S. cerevisiae* inoculated at these times.

	∑ Fusel alcohol acetates	∑ Ethyl esters of FA acetates	∑ Fusel alcohols	∑ SCFA	∑ MCFA	∑LCFA
Sc-Lip-	1.58 ± 0.26 ^{a}	1.09 ± 0.17 ^{a}	334.06 ± 52.71 ^{ab}	2.65 ± 0.56 ^{a}	0.83 ± 0.31 ^{bc}	4.70 ± 1.77 ^{a}
Sc-Lip+	1.67 ± 0.17 ^{a}	0.92 ± 0.18 ^{a}	333.28 ± 26.75 ^{ab}	2.89 ± 0.76 ^{a}	0.72 ± 0.12 ^{bc}	4.24 ± 1.24 ^{a}
Co-TdP	1.69 ± 0.09 ^{a}	1.04 ± 0.25 ^{a}	251.30 ± 70.97 ^{a}	2.68 ± 1.63 ^{a}	1.20 ± 0.39 ^{c}	4.96 ±1.36 ^{a}
Co-TdV	1.52 ± 0.32 ^{a}	0.96 ± 0.03 ^{a}	285.73 ± 68.58 ^{ab}	2.87 ± 1.83 ^{a}	1.20 ± 0.50 ^{c}	3.72 ± 0.01 ^{a}
2 days- TdP	1.02 ± 0.12 ^{a}	1.10 ± 0.09 ^{a}	368.66 ± 24.72 ^{ab}	10.30 ± 0.57 ^{b}	0.31 ± 0.03 ^{b}	4.47 ± 1.22 ^{a}
4 days-TdP	1.33 ± 0.27 ^{a}	0.87 ± 0.13 ^{a}	325.24 ± 20.81 ^{ab}	8.25 ± 1.47 ^{b}	0.56 ± 0.03 ^{ab}	6.47 ± 1.94 ^{a}
6 days-TdP	1.15 ± 0.23 ^{a}	0.66 ± 0.06 ^{a}	354.67 ± 70.75 ^{ab}	10.82 ± 3.50 ^{b}	0.34 ± 0.02 ^{ab}	5.78 ± 1.09 ^{a}
2 days- TdV	1.43 ± 0.10 ^{a}	1.04 ± 0.20 ^{a}	348.99 ± 24.05 ^{ab}	7.23 ± 1.40 ^{ab}	0.33 ± 0.10 ^{ab}	6.86 ± 0.21 ^{a}
4 days-TdV	1.07 ± 0.54 ^{a}	1.07 ± 0.17 ^{a}	364.47 ± 55.31 ^{ab}	8.39 ± 0.44 ^{b}	0.08 ± 0.07 ^{b}	6.33 ± 2.05 ^{a}
6 days-TdV	1.21 ± 0.16 ^{a}	1.02 ± 0.30 ^{a}	411.70 ± 39.59 ^{b}	11.20 ± 0.96 ^{b}	0.09 ± 0.05 ^{b}	7.39 ± 0.36 ^{a}

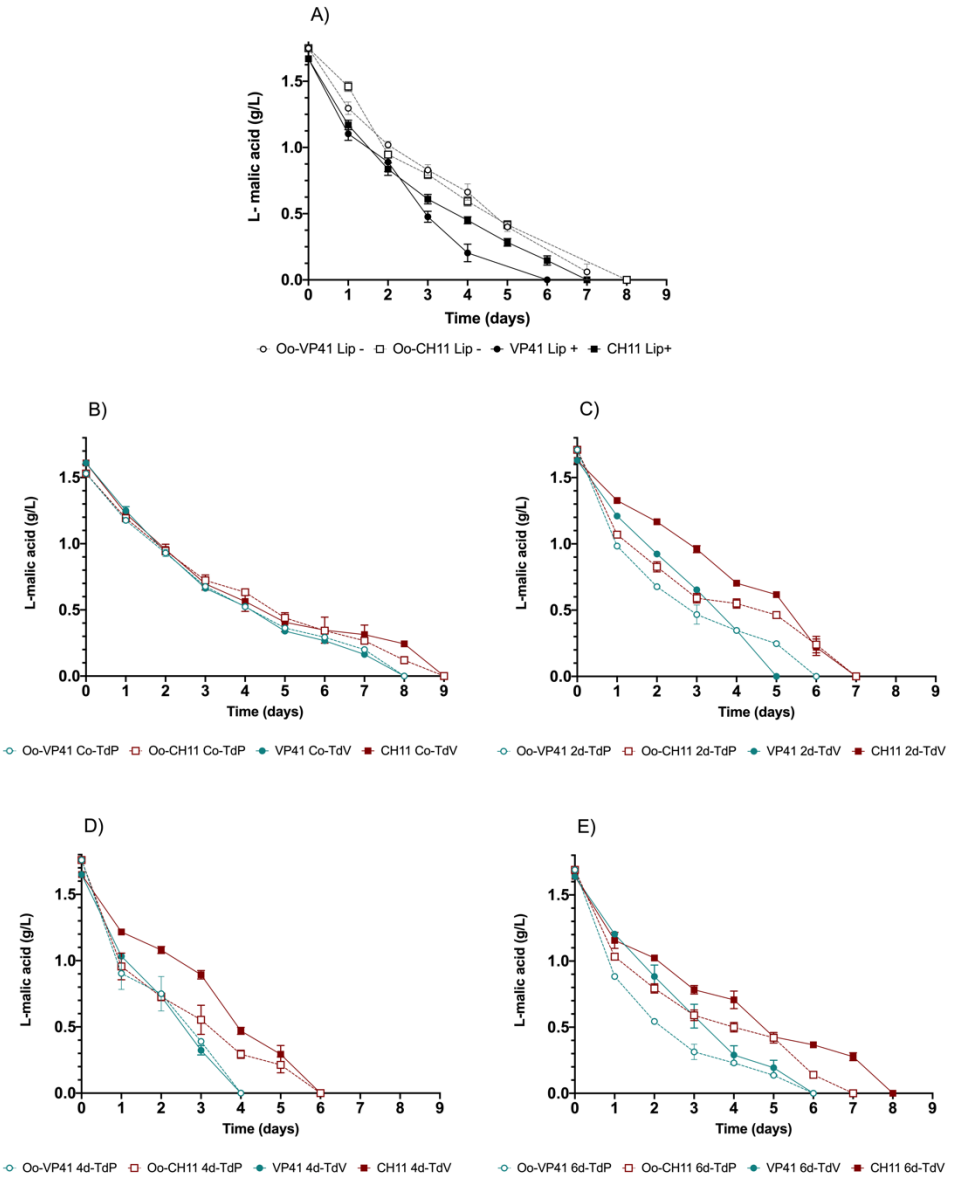

Figure 3. Evolution of malolactic fermentation kinetics: A) Sc-Lip- and Sc-Lip+ correspond to the *S. cerevisiae* control fermentations with the original synthetic must and the modified synthetic must, respectively; B) Co-TdP and Co-TdV correspond to the co-inoculated fermentations with *T. delbrueckii* Prelude or Viniferm + *S. cerevisiae* QA23. C) 2, D) 4 and E) 6 days correspond to the sequential fermentations with *T. delbrueckii* (Prelude or Viniferm) and *S. cerevisiae* inoculated at these times. Oo-VP41 and Oo-CH11 correspond to the two strains of *O. oeni* VP41 and CH11. The values are expressed as the average of three biological replicates for each condition.

The positive impact of *T. delbrueckii* on the MLF process (Balmaseda et al., 2022; Balmaseda, et al., 2021b; Ferrando et al., 2020) can be observed in sequential fermentations, particularly when using the Oo-VP41 strain, which completed the MLF process faster than the Oo-CH11 strain in all the experimental conditions (Figure3), agreeing with previous results (Ruiz-de-Villa et al., 2023b). In the 2-Day condition, it was observed that the time of MLF and L-malic acid consumption rate improved compared to the control condition, especially with the Td-P strain. For the 4-Day conditions, the improvement was even greater, with the Oo-VP41 strain enhancing the MLF performance by three days (with lower AUCs) and the Oo-CH11 strain by two days (Figure 3). Both the Td-P and Td-V strains reduced the L-malic consumption rate compared to the *S. cerevisiae* wines. The positive effects observed under the 4-Day conditions may be attributed to the higher percentage of *T. delbrueckii* during AF, particularly with Td-P. As previously mentioned, the resulting wines presented lower alcohol content and a slightly higher pH, which creates a more favourable environment for the metabolism of LAB. Additionally, the elevated nitrogen composition in the form of mannoproteins and α-amino nitrogen (Table 1) can further promote the performance of MLF, because *O. oeni* can utilize these nutrients as a source of energy and carbon (Alexandre et al., 2004; Balmaseda et al., 2021a; Diez et al., 2010). Additionally to this factor, the decreased concentrations of MCFAs in this condition may have indirectly facilitated the progression and efficiency of MLF by creating an environment more favourable to the activity of the selected *O. oeni* strains, as discussed earlier. Furthermore, these two factors could be related since the presence of higher amount of mannoproteins (Table 1) could be related with an absorption of MCFA detoxifying the media (Lafon-Lafourcade et al., 1984).

Finally, in the 6-Day condition, the MLF performance was improved by two days with the Oo-VP41 strain, specifically with Td-P, but with the Oo-CH11 strain, the MLF performance was slower than the control.

To summarize the findings of this study, a PCA was conducted using the AUC values obtained for both AF and MLF. This analysis allows us to visualize the relationship between AF and MLF, as depicted in Figure 2. Interestingly, when the AUC for AF was higher, the AUC for MLF was lower, indicating an inverse correlation. This suggests that a more

prolonged and gradual AF results in a shorter and faster MLF. This pattern was particularly evident in the Td-P wines, especially after 4 and 6 days of contact with *T. delbrueckii*, which is equivalent to inoculating *S. cerevisiae* at densities of approximately 1060 and 1050 g/L, respectively. Similarly, controlled fermentations conducted without lipids and co-inoculated wines, which exhibited significantly faster AF, demonstrated a slower and more protracted MLF, as previously described.

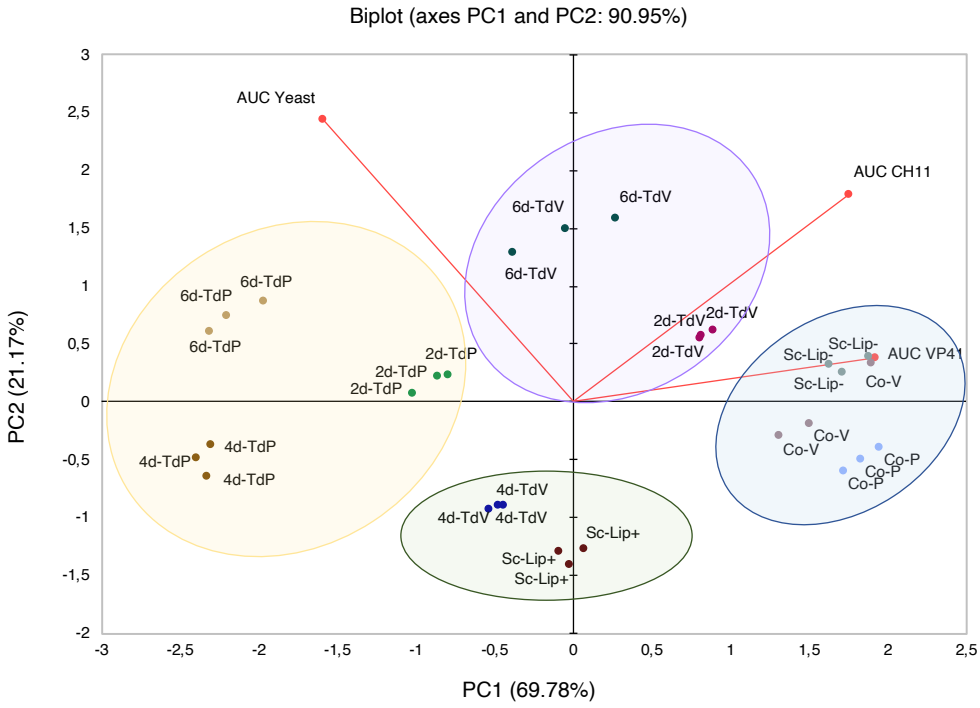

Figure 4. Principal component analysis biplots built from the following variables: AUC of AF data and AUC of MLF data, MLF driven by the *O. oeni* strains VP41and CH11. The observations are Sc-Lip- and Sc-Lip+, which correspond to the S. cerevisiae control fermentations with the original synthetic must and the modified synthetic must, respectively; Co-TdP and Co-TdV correspond to the co-inoculated fermentations with *T. delbrueckii* Prelude or Viniferm + *S. cerevisiae* QA23. 2 days, 4 days and 6 days correspond to the sequential fermentations with *T. delbrueckii* (Prelude or Viniferm) and *S. cerevisiae* inoculated at these times.

4. Conclusions

In summary, in relation to the synthetic fermentative medium, the optimization performed by adding linoleic acid and β-sitosterol was found to be effective in promoting good MLF performance, which suggests that these lipids can improve the metabolism of *O. oeni*. Regarding the co-inoculated wines, where *S. cerevisiae* dominated the AF, the performance of MLF was slower, which can be attributed to a higher concentration of MCFA and a low concentration of α-amino nitrogen.

Whereas sequential fermentation was a better option to obtain an efficient MLF process and improve AF metabolites, such as a reduction in ethanol or acetic acid, especially with Td-V. The 4 days of *T. delbrueckii* contact allowed a higher presence of these species during the AF, which, due to their positive synergy with *O. oeni*, improved the MLF. Even though the Td-V wines showed better oenological parameters, the use of Td-P for 4 or 6 days, when combined in the MLF with Oo-VP41, was the best option in terms of MLF efficiency. In addition, our results showed that there is a relationship between the duration and speed of AF and how it affects MLF. Overall, these findings highlight the importance of considering both the inoculation strategy and the specific strains to obtain a better understanding of the complex interactions between these species throughout the fermentation process.

Acknowledgements

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Supplementary data

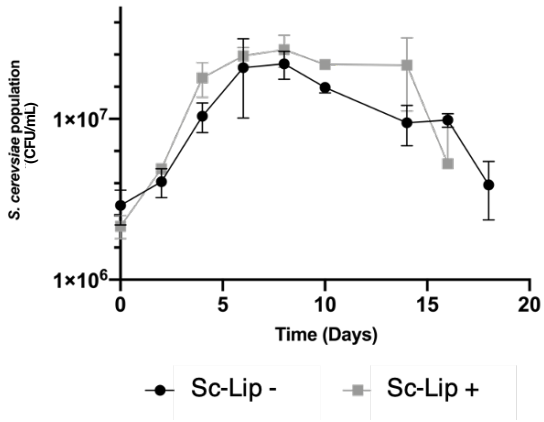

Figure SD1. Total yeast viability (CFU/mL) of the original synthetic must (Sc-Lip-) and the modified must with linoleic acid and β-sitosterol (Sc-Lip+). Mean and standard deviation (SD) (n=3)

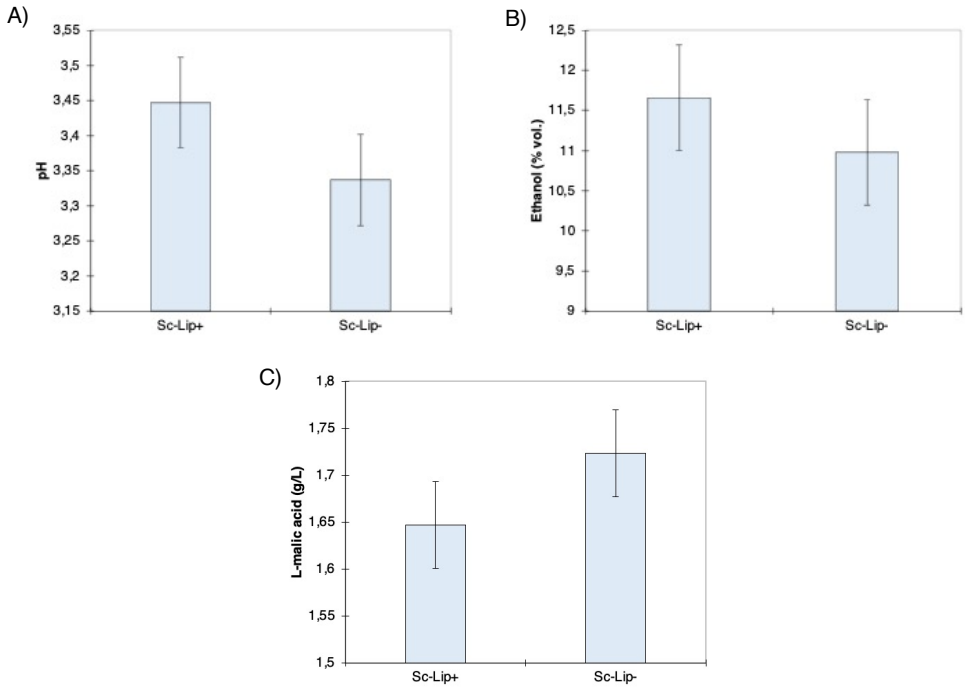

Figure SD2. Final pH (A), ethanol (% vol.) and (B) L-malic acid (g/L) (C) at the end of the alcoholic fermentation of the original synthetic must (Sc-Lip-) and the modified must with linoleic acid and β-sitosterol (Sc-Lip+). Mean and standard deviation (SD) (n=3)

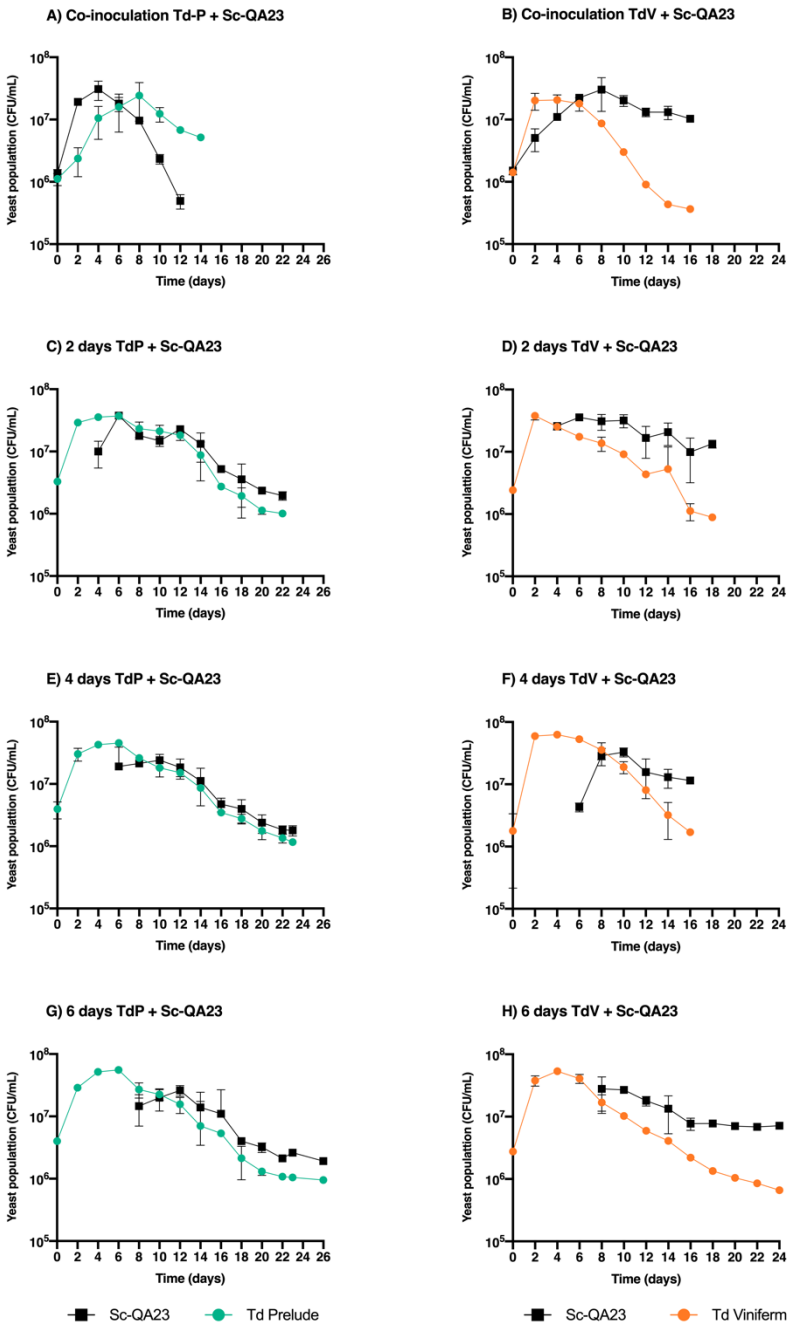

Figure SD3. Yeast viability (CFU/mL) of each species (*Saccharomyces cerevisiae* and *Torulaspora delbrueckii)* in co-inoculated and sequential fermentations at 2, 4 or 6 days. Sc-QA23, Td-P and Td-V correspond to *S. cerevisiae*, *T. delbrueckii* Prelude and *T. delbrueckii* Viniferm, respectively.

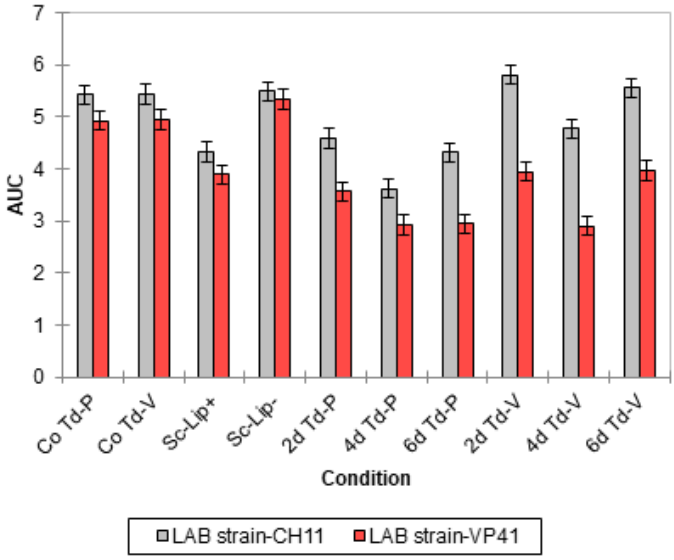

Figure SD4. The area under the curves (AUCs) from the L-malic acid consumption in malolactic fermentation kinetics. Mean and standard deviation (SD) (n=3). Sc-Lip- and Sc-Lip+ correspond to *S. cerevisiae* control fermentations with the original synthetic must and the modified synthetic must, respectively; Co-TdP and Co-TdV correspond to the co-inoculated fermentations with *T. delbrueckii* Prelude or Viniferm + *S. cerevisiae* QA23. 2 days, 4 days and 6 days correspond to the sequential fermentations with *T. delbrueckii* (Prelude or Viniferm) and *S. cerevisiae* inoculated at these times. *O. oeni* strains VP41 and CH11 correspond to *O. oeni* strains Oo-CH11 and Oo-VP41

UNIVERSITAT ROVIRA I VIRGILI
NEW PERSPECTIVES ON MALOLACTIC FERMENTATION AND ORGANOLEPTIC IMPROVEMENT OF WINES: INFLUENCE OF TORULASPORA DELBRUECKII ON DIFERENT TYPES OF WINEMAKING
Candela Ruiz de Villa Sardón

Chapter 2

Torulaspora delbrueckii effect on non-common vinifications

After investigating the modulation of wine composition by *T. delbrueckii* and its impact on MLF under controlled laboratory conditions using synthetic must (discussed in Chapter 1), this phenomenon was further explored under semi-industrial winemaking conditions in **Chapter 2**. In contrast to most previous studies, which have mainly investigated the influence of *T. delbrueckii* on wine composition and MLF in traditional white and red winemaking, our focus was to study alternative vinification approaches.

Firstly, in **Section 2.1** was performed an experiment involving rosé winemaking using Cabernet Sauvignon grape must. Interesting results were obtained in terms of colour attributes. In particular, the inoculation of *T. delbrueckii* appeared to diminish the concentration of anthocyanin while increasing the proportion of pyranoanthocyanins. This is favourable for the production of pale rosé wines, a category in high demand by consumers. Additionally, certain strains demonstrated the ability to enhance the MLF process.

Secondly, in **Section 2.2** the influence of *T. delbrueckii* was studied in wines produced through Carbonic Maceration using Grenache grape must. Different wines were produced regarding its sensory profiles, including increased colour intensity and enhanced concentration of characteristic aromas associated with carbonic maceration, such as isoamyl acetate. Furthermore, an improvement in MLF was achieved, highlighting the good results for spontaneous MLF.

Finally, in **Section 2.3,** the influence of *T. delbrueckii* was assessed in the context of skin-fermented white wines compared to conventional white winemaking, at different fermentation temperatures from Muscat of Alexandria grape must. A significant effect on the organoleptic and metabolomic composition was observed in wines due to the three factors: the presence of skins during AF, the inoculation of *T. delbrueckii* and the temperature of fermentation. Furthermore, *T. delbrueckii* promoted spontaneous MLF in a grape must with a rich biodiversity of LAB.

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Section 2.1

Sequential inoculation of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* in rosé wines improves colour and enhances malolactic fermentation

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Abstract

The production of rosé wines involves colour extraction, depending on the grape variety and maceration time, and malolactic fermentation in some cases. The aim of this study was first to observe the organoleptic modification by sequential inoculation of different strains of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* on Cabernet Sauvignon rosé wines. Then, using different strains of *Oenococcus oeni*, we performed a malolactic fermentation (MLF) on these rosé wines obtained. The presence of *T. delbrueckii* caused significant changes in the colour composition of the wines. Concerning the anthocyanin and pyranoanthocyanins compositions, the formation of the latter and the diminution of the former depended on the strains of *T. delbrueckii* and *S. cerevisiae*. Regarding the performance of MLF, a positive synergy between *T. delbrueckii* and *O. oeni* was observed in wines fermented and MLF was completed more quickly than by the control wines. In conclusion the reduction in total anthocyanins and colour intensity offers an interesting technique for producing rosé wines with a lighter colour.

Keywords

Oenococcus oeni; anthocyanins; malolactic fermentation; pyranoanthocyanins

1. Introduction

The presence of microbiota in grapes and the cellar environment plays a significant role in the final complexity of wine (Belda et al., 2017; Beltran et al., 2002). In recent years, studies have investigated the use of diverse microbiota, especially non-*Saccharomyces* yeasts, in alcoholic fermentation (AF) (Jolly et al., 2014; Padilla et al., 2016). Currently, there is significant interest in using non-*Saccharomyces* yeasts as starter cultures together with *Saccharomyces cerevisiae* because their organoleptic characteristics and technological aspects are beneficial (Vejarano and Gil-Calderón, 2021; Viana et al., 2008). It is essential to discuss *Torulaspora delbrueckii* because of its relevance in biotechnology (Fernandes et al., 2021). Studies have stated that utilizing *T. delbrueckii* can lead to significant changes in the characteristics of the final product, as the production of acetic acid is generally reduced and the ethanol content in wine can be lowered, which may be desirable in certain contexts (Benito, 2018). It has also been reported that sequential inoculation with *T. delbrueckii* and *S. cerevisiae* improves the foam properties of sparkling wine (González-Royo et al., 2015). Furthermore, due to the high enzymatic activity exhibited by some strains of this species, the concentration of some volatile compounds in wine can be increased (Azzolini et al., 2015; Carpena et al., 2021; Renault et al., 2015). For example, a high production of total esters and other volatile compounds has been reported (Balmaseda et al., 2021d; Renault et al., 2015), although this aroma modulation depends on the specific strain and the population of *T. delbrueckii* and *S. cerevisiae* present (Renault et al., 2015).

While most research has focused on aroma modification, *T. delbrueckii* has been shown to exhibit a significant impact on the colour of wine. Studies have demonstrated that *T. delbrueckii* increases anthocyanin extraction during alcoholic fermentation in red wines (Balmaseda, et al., 2021b; Escribano-Viana et al., 2019; Minnaar et al., 2018). All these effects could be interesting in rosé wines. Although the fermentation process for *rosé* wines is similar to that for white wines, achieving the desired colour is crucial for these wines. Different nuances and intensities can be achieved by adjusting the process of skin maceration for red grape varieties; for example, for pale *rosé* wines, shorter maceration times are needed, which can result in low release of aromas (Ribéreau-Gayon et al., 2006b). Consequently, non-*Saccharomyces* yeasts are an attractive option for this type of wine.

In general, *rosé* wines are known for their freshness. However, some of these wines must undergo malolactic fermentation (MLF) to achieve greater complexity. This can be a challenging process with *rosé* wines due to the amount of sulphur dioxide necessary to protect their colour. Fortunately, some studies have shown that *T. delbrueckii* can promote MLF, even in red wines (Balmaseda et al., 2021b). This effect could be particularly useful for *rosé* wines, and several studies have demonstrated the potential of *T. delbrueckii* in this regard (Balmaseda, et al., 2021b; Ruiz-de-Villa et al., 2023b).

The aim of this study was to investigate the potential of *T. delbrueckii* in improving Cabernet Sauvignon *rosé* wines in several ways. Specifically, the use of *T. delbrueckii* was proposed as a method to modulate colour, preserve aroma, and improve MLF. To achieve this goal, various strains of *S. cerevisiae*, *T. delbrueckii*, and *O. oeni* were tested with the aim of identifying the optimal combination to achieve the aforementioned improvements. From an industrial perspective, the results of this study could have significant implications for the production of high-quality rosé wines.

2. Materials and methods

2.1 Microorganism strains and inocula

The following *S. cerevisiae* strains were used in this work: Lalvin-QA23 (ScQ) and ICV K1 Marquée (ScK1) from Lallemand S.A. (Montreal, Canada). For sequential fermentations, the following strains of *T. delbrueckii* were also tested: *Biodiva TD291* (TdB, Lallemand S.A.) and Vinoflora Prelude (TdB, Chr. Hansen Holding AS, Hoersholm, Denmark). The inocula were prepared from dry active yeast as recommended by the manufacturers for 30 min at 37 °C for *S cerevisiae* strains and 30 °C for *T. delbrueckii* strains. Fermentation flasks were inoculated with an initial population of 2×10^6 cells/mL.

Regarding MLF, the following strains of *O. oeni* strains were used: Lalvin VP41 (Oo-VP41, Lallemand S.A.), Viniflora CH11 (Oo-CH11, Chr. Hansen Holding AS), 1Pw13 (Oo-1Pw13, own collection), and PSU-1 (Oo-PSU-1, American Type Culture Collection BAA- 331). These strains were replicated from isolated colonies and grown in MRS broth (Difco Laboratories, Detroit, MI, USA) medium (De Man et al., 1960) modified following the procedure described in

Margalef-Català et al. (2017) at pH 5 supplemented with 4 g/L DL-malic acid (Sigma–Aldrich, Barcelona, Spain) and 5 g/L D-fructose (Panreac, Barcelona, Spain). Then, the inocula were prepared from a preculture in 50 mL of modified MRS until the final phase of exponential growth. The inoculation volume was calculated with growth curves, depending on the strain. The growing conditions were 27 °C in a 10% CO_2 atmosphere. The populations of *O. oeni* were counted by plating on modified MRS plates containing 2% (w/v) agar (Panreac) and supplemented with 100 mL/L of centrifuged tomato juice diluted 1:10 (Aliada, Madrid, Spain), 100 mg/L of nystatin (Panreac) to avoid yeast growth and 25 mg/L of sodium azide (BioSciences, St. Louis MO, USA) to prevent acetic acid bacteria growth.

2.2 Fermentation conditions

Must from a Cabernet Sauvignon grape variety (*Vitis vinifera* L.) was used for all fermentations. Grapes were harvested and processed in the experimental winery *Mas dels Frares* of *Rovira i Virgili* University (41°08'44.1''N 1°11'51.0''E), which belongs to the AOC Tarragona, during the 2022 vintage harvest. The must was clarified at 7 °C after maceration for 2 hours at 25 °C to extract the colour. After that, the clear must was treated for 24 h with 1 mL/L dimethyl dicarbonate (Fisher Scientific, Hampton, USA) to eliminate undesirable microorganisms. No nutrient supplementation in the form of added nitrogen or thiamine was used in the fermentations. Alcoholic fermentation (AF) was performed in 500 mL bottles filled with 450 mL of must at 22 °C. The bottle was closed using a system of two valves, allowing sample extraction to be performed and carbon dioxide to be released. The initial parameters of grape must was as follows: 1,102.4 g/L density, pH 3.6, 48 mg N/L a-amino nitrogen (NOPA) and 17 mg N/L NH_4.

The following groups were utilized in triplicate: control ScQ, control ScK1, sequential TdB+ScQ, sequential TdP+ScQ, sequential TdB+ScK1 and sequential Td-P+ScK1. These sequential fermentations were inoculated first with *T. delbrueckii*, and after 48 h of AF with *S. cerevisiae*, in an initial population of 2 x 10^6 cells/mL, a viable inoculum population was determined by plating a 1:10 serial dilution in YPD agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 17 g/L agar, Panreac).

AF monitoring was performed every day by measuring the density of the centrifuged samples using an electronic densimeter (Densito 30PX Portable Density Metre (Mettler Toledo, Barcelona, Spain). AF was estimated to end when the glucose/fructose concentration was below 2 g/L, analysed enzymatically using a Y15 Enzymatic Autoanalyzer (Biosystems S. A, Barcelona, Spain).

Prior to MLF, wines were stabilized for 4 days at 4 °C. Then, the wines were centrifuged at 3000 x g for 15 minutes at 4 °C and sterilized by filtration with a 0.22 μm membrane (Merck, Germany). With the objective of reducing variability, triplicates were mixed and divided again for each condition. The final concentration of L-malic acid was corrected to obtain an initial concentration of 2 g/L, and the pH was adjusted before L-malic acid was added. At this time, MLF was performed in small volumes of 50 mL at 20 °C in anaerobic and static conditions. Each *O. oeni* strain was inoculated to reach an initial population of 2×10^7 cells/mL. The consumption of L-malic acid was measured daily up to a concentration lower than 0.1 g/L using the Y15 Enzymatic Autoanalyzer (Biosystems).

2.3 Calculating the area under the curve (AUC)

To evaluate the performance of fermentation, the area under the curve (AUC) was measured by analysing the decrease in density during AF and consumption of L-malic acid during MLF. This approach allowed us to assess fermentation performance independent of total fermentation times and kinetics. The AUCs were calculated by integrating the density decrease during AF or L-malic acid consumption during MLF between two consecutive time points. The formula used for the calculation was the sum of consecutive AUCs, which was obtained by summing the areas of consecutive data points as follows: Σ [((d2+d1)/2) * (t2-t1) + …. + ((dn+dn-1)/2) * (tn-tn-1)], where d1, d2, … dn-1, dn represent the densities or L-malic acid values at times 1, 2, n-1, and n, respectively.

2.4 Physico-chemical parameter analysis

Citric acid, glycerol and ethanol were determined by using an Agilent 1100 HPLC (Agilent Technologies, Waldbronn, Germany) according to Quirós et al. (2014). Wine samples were filtered with 0.22 μm pore filters (Merck) before injection. The HPLC was equipped with a Hi-Plex H column (300 mm x 7.7 mm) inside a 1260 MCT (Infinity II Multicolumn

Thermostat) with two detectors, an MWC detector (Multiwavelength detector, Agilent Technologies) and an RID detector (1260 Infinity II refractive index detector, Agilent Technologies).In addition, acetic acid, L-malic acid, pyruvic acid and acetaldehyde were enzymatically measured by a Y15 Enzymatic Autoanalyzer (Biosystems). Succinic acid was also analysed by an enzymatic method with microplates (Megazyme, Wicklow, Ireland) using the POLARstar Omega (BMG LABTECH, Ortenberg, Germany).

To estimate the mannoproteins present, mannoprotein precipitation was first performed with 95% ethanol from *rosé* wines, and then acid hydrolysis at 90 °C was performed, which led to the release of mannose. The released mannose was analysed by following the procedure described in Balmaseda et al. (2021a). Then, the equivalents of mannose were quantified with a D-mannose and D-glucose enzymatic assay kit (Megazyme, Ireland).

Finally, a Crison micro pH 2002 pH meter (Hach Lange Spain, Barcelona, Spain) was used to determine the pH of the wines.

2.5 Volatile composition

The volatile composition of the wines after AF was analysed. The pre-treatment of the samples consisted of a liquid/liquid extraction with a methyl tert-butyl ether/hexane mixture (1/1). The internal standards (Sigma-Aldrich) used were 3-octanol (1.98 g/L), heptanoic acid (3.33 g/L) and heptadecanoic acid (1.03 g/L), which were added to 5 mL of wine. The organic phase was injected into a GC-FID chromatograph (Agilent Technologies). The chromatographic conditions were as follows: injection volume, 2 μL; injection mode, spitless; inlet and detector temperatures, 250 °C; and column, HP-FFAP (30 m x 250 μm 0.25 μm, Agilent). The concentrations of the volatile compounds were calculated from known external standards by calibration curves. The volatile compounds identified were acetates of fusel alcohols (AFA): isobutyl acetate, isoamyl acetate, 2-phenylethaol acetate); fusel alcohols (FA): amyl and isoamyl alcohols, hexanol, *cis*3-hexenol, 2-phenylethanol); ethyl esters of fatty acids (EEFA): ethyl butanoic, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl dodecanoate, ethyl lactate and diethyl succinate); short-chain fatty acids (SCFA): propanoic, butanoic (butyric acid), isobutyric (2-methylpropanoic acid), pentanoic (valeric acid) and isovaleric acids (3-methylbutanoic acid); medium-chain fatty acids (MCFA):

hexanoic, octanoic, decanoic and dodecanoic acids) and long-chain fatty acids (LCFA): tetradecanoic (myristic acid), hexadecanoic (palmitic acid), 9-hexadecenoic (palmitoleic acid), octadecanoic (stearic acid), 9-octadecenoic (oleic acid) and 9,12-octadecadienoic acid (linoleic acid)).

2.6 Colour and anthocyanin analysis

CIELab coordinates were determined as described Ayala et al. (1997). The colorimetric coordinates hue (H*), lightness (L*) and chroma (C*) were calculated with MSCV software (Ayala *et al.*, 2001). The colour intensity (CI) was calculated from the sum of absorbances at 420, 520 and 620 nm and measured in a spectrophotometer using a method described by Glories et al. (1984).

The content of free and acylated anthocyanins as well as pyranoanthocyanins was analysed with an Agilent 1200 series liquid chromatograph (HPLC–diode array detection) using an Agilent Zorbax Eclipse XDB-C18 column (4.6, 250 mmx 5 mm, Agilent Technologies) with the procedure described by (Gil et al., 2012).

2.7 Statistical analysis

To ensure the reliability of the results, all assays were performed in triplicate. The data obtained were subjected to statistical analysis using ANOVA and Tukey's HSD test (honestly significant difference) with XLSTAT 2020.2.3 software (Addinsoft, Paris, France). A *p*-value of less than 0.05 was considered statistically significant. A principal component analysis (PCA) was also carried out to describe the relationship between colour and the content of phenolic compounds in *rosé* wine according to AF conditions.

3. Results and discussion

3.1 Alcoholic fermentation

The yeast combinations used in this study were selected based on a previous investigation (Ruiz-de-Villa et al., 2023b), which aimed to identify the most effective strains to promote malolactic fermentation (MLF). The results showed that TdP+ScQ was the most efficient for MLF performance, followed by TdB and NSA1 Viniferm NSDT (Td-Viniferm, Agrovin, Spain).

As a result, Td-P and TdB were chosen for this study. Two strains of *S. cerevisiae*, ScQ and ScK1, were also used. ScK1 did not perform well in MLF when synthetic grape must was applied, but we wanted to test it with real grape must.

Therefore, six different yeast combinations were tested. Due to the low yeast assimilable nitrogen (YAN) concentration in the must, the alcoholic fermentations lasted for several days (Figure SD1). No nitrogen was added to the fermentation process, and no additional nutrients were introduced to evaluate their effects under less favourable conditions. This approach prevented fermentation from occurring too rapidly; thus, the potential of the yeast combinations could be more comprehensively examined.

The results of this study demonstrate that the AF duration was significantly shorter for *S. cerevisiae* strains compared to sequential fermentations. Specifically, the AF duration for ScQ and ScK1 was 17 and 19 days, respectively, while sequential fermentation with TdP and ScK1 took a maximum of 25 days (Figure SD1). These findings align with previous studies, which indicate that *T. delbrueckii* has a significant nutrient requirement that restricts the subsequent fermentation activity of *S. cerevisiae*; as a result, competition occurs between the two yeast species (Belda et al., 2015; Romano et al., 2003; Ruiz-de-Villa et al., 2023). In Table 1, the areas under the curves (AUCs) are compared for each fermentation condition. The results showed that ScQ achieves a significantly faster fermentation rate than that of ScK1. Additionally, differences were observed with and without *T. delbrueckii*. In general, the sequential fermentations were slower except for TdB with ScK1, as the fermentation rate was faster even though the process lasted longer.

3.2 Physico-chemical parameters after alcoholic fermentation

The wines obtained from different fermentation conditions were analysed, and the levels of general parameters were compared, including the levels of organic acids, ethanol, and glycerol; equivalents of mannose; and pH (Table 1). The results indicate that the levels of citric acid, succinic acid or L-malic acid did not show significant differences among wines under these fermentation conditions. However, the use of TdP resulted in a significant reduction in the levels of acetic acid compared to that of the Sc Control condition (Table 1). Interestingly, a reduction in acetic acid was also observed when TdB was used in

combination with ScQ but not with ScK1. Taillandier et al. (2014) and Ruiz de Villa et al. (2023b) described sequential fermentations with some strains of *T. delbrueckii,* and *S. cerevisiae* produced less acetic acid in synthetic media.

For natural must, an increase in volatile acidity has been reported in red wines, while a reduction has been observed in white wines (Balmaseda, et al., 2021b; Balmaseda, et al., 2021d; Oliveira and Ferreira, 2019). In rosé wines, a slight decrease in volatile acidity has been described (Muñoz-Redondo et al., 2021). These findings suggest that the impact of *T. delbrueckii* on acetic acid in wines depends on the strain and type of vinification, as previously described in the literature.

The ethanol content in wines produced using the different strains of *S. cerevisiae* varied significantly, and ScK1 wines exhibited lower ethanol values than those of ScQ wines. Additionally, the use of TdB in conjunction with ScK1 resulted in a significant decrease in alcohol content. Although the reduction of ethanol has been linked to the use of some *T. delbrueckii* strains (Zhu et al., 2020), the extent of this decrease can vary depending on factors such as the strain, must and winemaking conditions (Balmaseda, et al., 2021d). The glycerol content was found to be significantly higher in wines produced using TdB. This trend may be related to the reduction in ethanol content observed when this strain was used in combination with ScK1, although the development of the glycerol-pyruvic pathway has been described as strain-dependent (Benito, 2018).

For the pH, no significant differences were found (Table 3) with 2 days of *T. delbrueckii* contact. Regarding equivalents of mannose, there were significant differences among wines fermented with *S. cerevisiae* and sequential fermentations, in which the concentration was significantly higher, as previously described (Belda et al., 2015; Ruiz-de-Villa et al., 2023a). In addition, TdP showed the highest concentrations of equivalents of mannose, since this strain is an overproducer of mannoproteins (Benito, 2018).

Table 1. Principal parameters analysed from alcoholic fermentation. ScQ and ScK1 correspond to the pure fermentation with *S. cerevisiae* QA23 and *S. cerevisiae* K1, respectively; TdB+ScQ and TdP+ScQ correspond to sequential fermentations with *S. cerevisiae* QA23 and *T. delbrueckii* Biodiva or *T. delbrueckii* Prelude, respectively; TdB+ScK1 and TdP+ScK1: correspond to sequential fermentations with *S. cerevisiae* K1 and *T. delbrueckii* Biodiva or *T. delbrueckii* Prelude, respectively. AUC, Area Under the Curve. Lowercase letters indicate a significant difference between *S. cerevisiae* strains at *p*-value < 0.05; Capital letters indicate a significant difference between *S. cerevisiae*, *T. delbrueckii* Biodiva and *T. delbrueckii* Prelude at *p*-value < 0.05. Mean ± standard deviation (n=3).

	AUC	Malic acid (g/L)	Citric acid (g/L)	Succinic acid (g/L)	Acetic acid (g/L)	Ethanol (% vol)	Glycerol (g/L)	Eq-Mannose (mg/L)
ScQA23	113.3 ± 1.1 ^{aB}	1.73 ± 0.03 ^{aA}	0.15 ± 0.16 ^{aA}	0.19 ± 0.06 ^{aA}	0.41 ± 0.04 ^{aB}	12.24 ± 0.01 ^{bAB}	9.14 ± 0.14 ^{aA}	89.7 ± 18.5 ^{aA}
TdB+ScQ	107.8 ± 1.8 ^{aA}	1.75 ± 0.02 ^{aA}	0.24 ± 0.06 ^{aA}	0.22 ± 0.05 ^{aA}	0.28 ± 0.04 ^{aB}	12.66 ± 0.19 ^{bA}	11.33 1.06 ^{aB}	217.1 ± 40.6 ^{aB}
TdP+ScQ	104.2 ± 2.4 ^{aB}	1.66 ± 0.05 ^{aA}	0.19 ± 0.03 ^{aA}	0.25 ± 0.1 3^{aA}	0.14 ± 0.05 ^{aA}	13.16 ± 0.17 ^{bB}	9.66 ± 0.77 ^{aA}	224.2 ± 1.4 ^{aC}
ScK1	139.6 ± 0.9 ^{bB}	1.74 ± 0.06 ^{aA}	0.36 ± 0.07 ^{aA}	0.20 ± 0.05 ^{aA}	0.26 ± 0.06 ^{aB}	12.75 ± 0.06 ^{aAB}	9.80 ± 0.28 ^{aA}	85.2 ± 14.9 ^{aA}
TdB+ScK1	137.5 ± 2.9 ^{bA}	1.63 ± 0.03 ^{aA}	0.16 ± 0.06 ^{aA}	0.19 ± 0.06 ^{aA}	0.29 ± 0.08 ^{aB}	11.12 ± 0.58 ^{aA}	10.06 ± 0.53 ^{aB}	173.0 ± 9.2 ^{aB}
TdP+ScK1	148.2 ± 1.2 ^{bB}	1.73 ± 0.04 ^{aA}	0.17 ± 0.11 ^{aA}	0.20 ± 0.04 ^{aA}	0.15 ± 0.07 ^{aA}	12.24 ± 0.47 ^{aB}	9.04 ± 0.44 ^{aA}	245.3 ± 24.9 ^{aC}

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3.3. Volatile composition

Different volatile compounds were detected (Table 2), including fusel alcohols (isoamyl alcohol, 1-hexanol and 2-phenyl ethanol), short-chain fatty acids (SCFAs) (butyric acid, isobutyric acid and valeric acid), medium-chain fatty acids (MCFAs) (octanoic acid and decanoic acid), LCFAs (myristic acid, palmitic acid and stearic acid), fusel alcohol acetates (isobutyl acetate, isoamyl acetate, hexyl acetate, 2-phenyl ethanol acetate), and ethyl esters of FA (ethyl butanoate, ethyl hexanoate and ethyl dodecanoate). It has been shown that *T. delbrueckii* lead to higher concentrations of fusel alcohols, especially TdB; compared to wines fermented only with *S. cerevisiae*, wines produced with *T. delbrueckii* generated significantly higher concentrations of isoamyl alcohol. The high values of fusel alcohols with this species have already been reported in other studies (Azzolini et al., 2015; Belda et al., 2017; Benito, 2018; Muñoz-Redondo et al., 2021; Ruiz-de-Villa et al., 2023b). Moreover, the presence of *T. delbrueckii* led to an increasing trend in the concentrations of 2-phenylethanol. This combination of strains in sequential wines also exhibited significantly higher concentrations of fusel alcohol acetates, even though some authors described a decrease in their concentration with *T. delbrueckii* (Azzolini et al., 2015; Belda et al., 2017). This increase in some volatile compounds could be related to the high enzymatic activity of *T. delbrueckii* (Romano et al., 2003).

However, in regard to MCFAs, a decreasing trend was observed in the presence of *T. delbrueckii*. Wines fermented with *S. cerevisiae*, especially the strain ScQ, generated higher values of MCFAs than those fermented with *T. delbrueckii*, as reported by Balmaseda, et al. (2021b) and Ruiz-de-Villa et al. (2023b).

3.4. Anthocyanins, pyranoanthocyanins and colour parameters

Figure 1 shows the total anthocyanin concentration (1A) and the pyranoanthocyanin concentration (1B) of the different wines after AF determined by HPLC. Those parameters were also analysed in wines after MLF; however, no significant differences of interest were observed (data not shown). Figure 1 also shows the relative proportions, expressed as percentages (%), of different pigments detected in the different samples (1C). As expected, nonacylated anthocyanins were predominant, and malvidine-3-O-glucoside was the main

anthocyanin detected (data not shown). Acylated anthocyanins were also detected, but only the acetylated forms were present in the wines. Among the acylated anthocyanins, malvidin-3-O-acetylglucoside practically monopolizes this category.

These data agree with previous research indicating that acetylated anthocyanins were the predominant type among the acylated anthocyanins found in Cabernet wines (Gil et al., 2012; Gombau et al., 2020). The total anthocyanin concentration (Figure 1A) was significantly higher in control samples fermented with pure cultures of *S. cerevisiae* (ScQ or ScK1) compared to corresponding wines fermented with sequential inoculation of *T. delbrueckii* strains (TdB+ScQ, TdP+ScQ, TdB+ScK1 and TdP+ScK1). Furthermore, it seems that wines fermented only with ScK1 showed higher anthocyanin concentrations than wines fermented only with Sc-Q, although these differences did not reach statistical significance. As previously discussed, all sequential fermentations showed significantly lower anthocyanin concentrations than those of their respective control wines. The concentrations decreased by approximately 50% on average compared to that of the controls; the only exception was TdB+ScK1, which showed a higher reduction of approximately 60%. This reduction in anthocyanin concentration could be attributed to different factors. On the one hand, literature has described that some *T. delbrueckii* strains could have high β-glucosidase activity (Maturano et al., 2012). Consequently, the presence of β-glucosidase activity in these *T. delbrueckii* strains may promote the formation of aglycones from anthocyanins, making these pigments more susceptible to oxidation (Vidana Gamage et al., 2022). It has been observed that the *T. delbrueckii* strains used in this study had a higher β-glucosidase activity than the *S. cerevisiae* strains tested (Figure SD2).

On the other hand, anthocyanins can react with different compounds, such as ethanal, to form flavanol-ethyl-anthocyanin adducts (Es-Safi et al., 1999). Additionally, anthocyanins can react with ethanal, pyruvic acid and vinylphenols through cycloaddition reactions to form pyranoanthocyanins (Bakker and Timberlake, 1997; Schwarz et al., 2003). However, in this case, the last process is not responsible since no differences were found in pyranoanthocyanin concentrations among the different samples. Besides, pyruvic acid and acetaldehyde were analysed after AF but there were no significant differences that could be related to variations in anthocyanin concentrations (Table SD1).

Table 2. Volatile compounds (mg/L) analysed from alcoholic fermentation. Sum of Fusel alcohol acetates (isobutyl acetate, isoamyl acetate and 2-phenylethanol acetate), Sum of Ethyl esters of FA (ethyl butanoate, ethyl hexanoate, ethyl octanoate and ethyl dodecanoate), Sum of Fusel alcohols (isoamyl alcohol, 1-hexanol, *cis*-3-hexen-1-ol, 2-phenylethanol), Sum of SCFA (propionic, butyric and valeric acids), Sum of MCFA (octanoic and decanoic acids), Sum of LCFA (myristic, palmitic and stearic acids). ScQ and ScK1 correspond to the pure fermentation with *S. cerevisiae* QA23 and *S. cerevisiae* K1, respectively; TdB+ScQ and TdP+ScQ correspond to sequential fermentations with *S. cerevisiae* QA23 and *T. delbrueckii* Biodiva or *T. delbrueckii* Prelude, respectively; TdB+ScK1 and TdP+ScK1: correspond to sequential fermentations with *S. cerevisiae* K1 and *T. delbrueckii* Biodiva or *T. delbrueckii* Prelude, respectively. Mean ± standard deviation (n=3). Lowercase letters indicate a significant difference between *S. cerevisiae* strains at *p*-value < 0.05.

	Fusel alcohols acetates	Ethyl esters of FA	Fusel alcohols	SCFA	MCFA	LCFA
ScQ	1.23 ± 0.22^{a}	0.54 ± 0.20^{a}	776 ± 65^{a}	11.5 ± 2.0 ^{bc}	1.18 ± 0.52^{b}	1.96 ± 0.61^{a}
TdB+ScQ	1.90 ± 0.15^{b}	1.03 ± 0.38^{a}	1274 ± 70 ^{c}	12.6 ± 3.1^{c}	0.01 ± 0.01^{a}	2.58 ± 0.80^{ab}
TdP+ScQ	1.09 ± 0.31^{a}	0.55 ± 0.09^{a}	90 ± 29 ^{ab}	17.1 ± 3.8 ^{cd}	0.09 ± 0.03^{a}	3.80 ± 0.34^{b}
ScK1	1.08 ± 0.24^{a}	0.80 ± 0.10^{a}	878 ± 79 ^{ab}	5.7 ± 2.8 ^{ab}	0.45 ± 0.05^{a}	2.46 ± 0.28^{ab}
TdB+ScK1	2.29 ± 0.21^{b}	0.82 ± 0.22^{a}	1275 ± 35 ^{c}	2.1 ± 1.3 ^{a}	0.14 ± 0.02^{a}	3.07 ± 0.89^{ab}
TdP+ScK1	2.03 ± 0.25^{b}	0.79 ± 0.13^{a}	1035 ± 67 ^{b}	20.0 ± 0.6 ^{d}	0.19 ± 0.03^{a}	2.60 ± 0.04^{ab}

Another factor to consider is that the different yeast species or even yeast strains could show different capacities to adsorb pigments, such as anthocyanins (Morata et al., 2003; Tofalo et al., 2021). Moreover, the kinetics of all sequential fermentations were slower than those of pure *S. cerevisiae* fermentations (ScQ and ScK1) (Figure SD1). Thus, it is worth noting that the risk of oxidation is higher when the fermentation is longer since the wines remain unprotected for a longer period without the addition of sulphur dioxide.

Despite our results, some authors reported the opposite effect, as the total anthocyanins increased with sequential inoculation of *T. delbrueckii* in red wine (Balmaseda et al., 2021b; Chen et al., 2018; Escribano-Viana et al., 2019; Minnaar et al., 2018). However, it is important to note that these studies involved the production of red wine, while our study involved the production of *rosé* wine. This distinction leads to significant differences. First, in *rosé* winemaking, the contact time between the grape skins and the juice is relatively short, resulting in a lower overall extraction of anthocyanins. Second, in *rosé* winemaking, yeast inoculation is performed after the maceration process, when the extraction of pigments from the grape skins has already been completed. In contrast, during the production of red wine, yeast inoculation occurs while grape skins are present, and maceration is prolonged for a much longer period. The presence of grape skins during fermentation serves as a source of anthocyanins, contributing to a potentially higher concentration of total anthocyanins in red wines (Gil et al., 2012).

Vitisin A and Vitisin B, as shown in Figure 1B, were detected in all samples, and both are derived from malvidin-3-O-monoglucoside and malvidin-3-O-acetylmonoglucoside (He et al., 2012). However, Vitisin A and Vitisin B concentrations were statistically similar in all wines. Thus, the formation of pyranoanthocyanins was not influenced by the presence of *T. delbrueckii* strains and was also independent of the *S. cerevisiae* strain used. Nevertheless, as shown in Figure 1C, some variations in the relative proportion of pyranoanthocyanins to total pigments (including anthocyanins and pyranoanthocyanins) were detected after AF. In this context, wines produced through sequential fermentation with ScQ (TdB+ScQ and TdP+ScQ) exhibited a higher proportion of pyranoanthocyanins than that of their control.

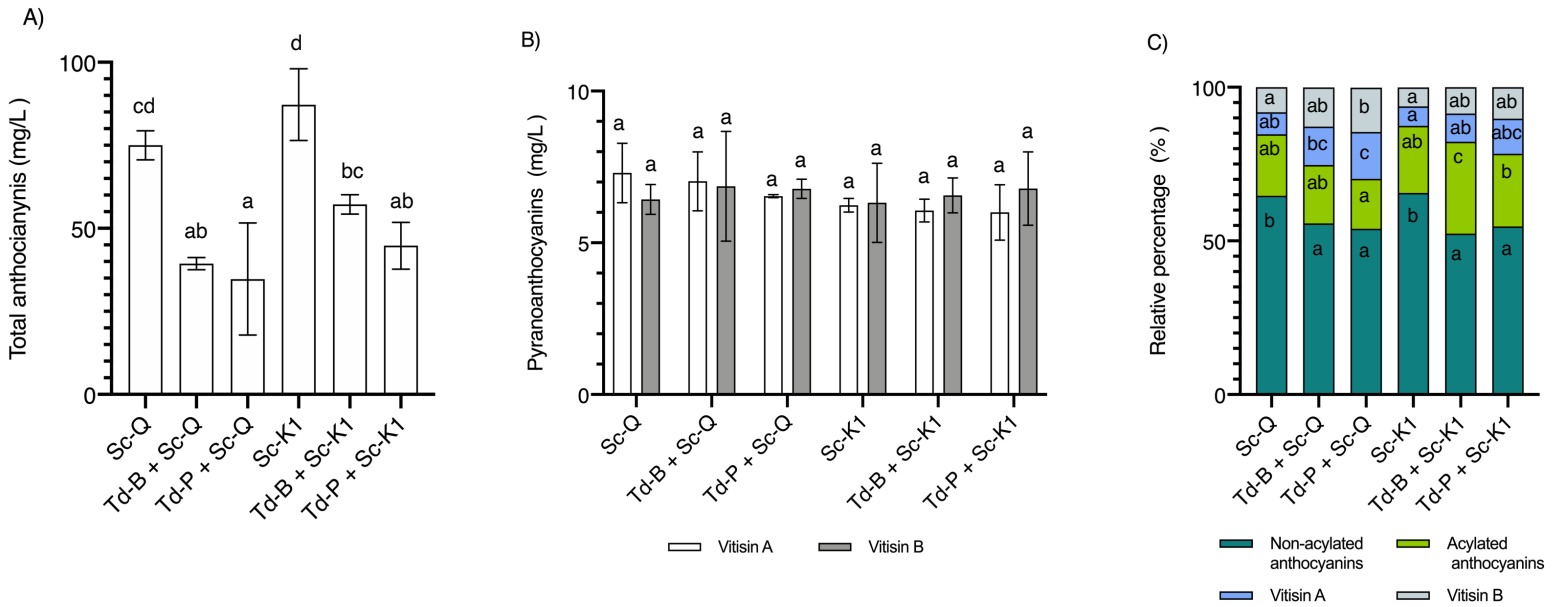

Figure 1. Total anthocyanins and pyranoanthocyanins concentration at the end of the alcoholic fermentation. ScQ and ScK1 correspond to the pure fermentation with *S. cerevisiae* QA23 and *S. cerevisiae* K1, respectively; TdB+ScQ and TdP+ScQ correspond to sequential fermentations with *S. cerevisiae* QA23 and *T. delbrueckii* Biodiva or *T. delbrueckii* Prelude, respectively; TdB+ScK1 and TdP+ScK1 correspond to sequential fermentations with *S. cerevisiae* K1 and *T. delbrueckii* Biodiva or *T. delbrueckii* Prelude, respectively The values are expressed as the mean of three biological replicates for each condition. Different lowercase letters indicate the existence of significant difference between the samples (p-value < 0.05).

This increase in pyranoanthocyanins can be attributed to the lower concentration of anthocyanins in these samples (Figure 1A). A similar trend was observed in samples with sequential fermentation using the ScK1 strain and both *T. delbrueckii* strains, although these differences were not statistically significant. Moreover, the presence of the TdP strain leads to wines with a higher proportion of pyranoanthocyanins than those of wines fermented with the TdB strain.

Comparing the different fermentation conditions, the proportion of acylated anthocyanins was statistically similar in both wines fermented with pure cultures of *S. cerevisiae* (ScQ and ScK1). Regarding wines fermented with the ScK1 strain, the sequential inoculations showed a higher proportion of acylated anthocyanins and a lower proportion of nonacylated anthocyanins compared to that of the ScK1 control wine. Thus, the degradation of anthocyanins observed in TdB+ScK1 and TdP+ScK1 (Figure 1A) was mainly attributed to nonacylated anthocyanidins. However, for sequential fermentation in the presence of ScQ and both *T. delbrueckii* strains, no significant differences were detected in the proportion of acylated anthocyanins. Therefore, the decrease in anthocyanin observed in these samples mainly resulted from the degradation of acylated and nonacylated anthocyanin.

Differences in the relative proportions of pyranoanthocyanins and acylated anthocyanins can lead to changes in wine colour. It has been reported that pyranoanthocyanins contribute more to a yellowish hue than anthocyanins (De Freitas and Mateus, 2011). These derived pigments are also less sensitive to pH changes and less prone to discolouration caused by the action of sulphur dioxide (Fulcrand et al., 1997). On the other hand, acetylated anthocyanins have been described to exhibit a higher bluish hue than that of their corresponding nonacetylated counterparts (De Villiers et al., 2004). Moreover, the structure of acylated anthocyanins is more resistant to nucleophilic attack by water, which results in the formation of hemiketal forms because their structure favours intrapigmentation phenomena (Trouillas et al., 2016; Vidana Gamage et al., 2022). All these phenomena collectively show the potential to significantly modulate the intensity and hue of wine colour.

Table 3 shows the colour parameters of the different wines. Some differences in colour parameters cannot be attributed to the pH since all the samples have similar values. Colour

intensity (CI) was higher in conventional fermentations with ScK1 and ScQ strains. However, in all the wines produced through sequential fermentations, a lower colour intensity was observed, which could be attributed to the lower concentration of anthocyanins detected in these wines. There is limited research on the impact of *T. delbrueckii* on *rosé* wines. In a previous study, the sensory effects in *rosé* wines of *T. delbrueckii* as well as *Metschnikowia pulcherrima* were examined (Muñoz-Redondo et al., 2021). The same strain (Td-Biodiva) was employed and a significant reduction in CI was observed, even though the *S. cerevisiae* strain was different.

Table 3. Colour parameters in wines after alcoholic fermentations. ScQ and ScK1 correspond to the pure fermentation with *S. cerevisiae* QA23 and *S. cerevisiae* K1, respectively; TdB+ScQ and TdP+ScQ correspond to sequential fermentations with *S. cerevisiae* QA23 and *T. delbrueckii* Biodiva or *T. delbrueckii* Prelude, respectively; TdB+ScK1 and TdP+ScK1: correspond to sequential fermentations with *S. cerevisiae* K1 and *T. delbrueckii* Biodiva or *T. delbrueckii* Prelude, respectively. All data is expressed as the mean of three biological replicates ± standard deviation, Different lowercase letters indicate the existence of significant difference between the samples (p-value < 0.05).

	CI	H*	L*	C*	pH
ScQ	3.98 ± 0.01 ^{b}	28 ± 1 ^{ab}	41 ± 1 ^{ab}	67 ± 2 ^{abc}	3.53 ± 0.03
TdB+ScQ	3.41 ± 0.28 ^{a}	37 ± 2 ^{c}	45 ± 3 ^{bc}	73 ± 1 ^{cd}	3.51 ± 0.01
TdP+ScQ	3.46 ± 0.11^{a}	33 ± 2 ^{bc}	45 ± 2 ^{bc}	71 ± 1 ^{cd}	3.49 ± 0.03
ScK1	4.28 ± 0.51^{b}	33 ± 1 ^{bc}	36 ± 4 ^{a}	70 ± 1 ^{bcd}	3.56 ± 0.05
TdB+ScK1	3.14 ± 0.59 ^{a}	24 ± 6 ^{a}	46 ± 4 ^{bc}	67± 2 ^{ab}	3.50 ± 0.03
TdP+ScK1	2.99 ± 0.03 ^{a}	26 ± 6 ^{ab}	48 ± 1 ^{c}	66 ± 2 ^{a}	3.54 ± 0.02

The CIELab coordinates L* (Lightness) and C* (Chroma) provide quantitative information about colour characteristics. In all sequential wines, an increase in L* values were detected in comparison with *S. cerevisiae* control wines, especially for ScK1 (Table 3). This finding aligns with the CI results since L* is usually negatively correlated with CI. Therefore, sequential fermentations showed less intense colour. The C* values of sequential ScK1 wines (TdB+ScK1 and TdP+ScK1) decrease compared to that of the ScK1 control. However, in the ScK1 wines, the differences were more pronounced because the decrease in CI between the control and sequential wines was larger compared to that of the ScQ wines.

When considering the CIELab coordinate H* (hue), which relates to the qualitative aspects of colour, the control samples (ScQ and ScK1) exhibited similar values. Therefore, both tested strains of *S. cerevisiae* did not have any significant effect on the hue of the wines when conventional inoculation was carried out. In contrast, sequential fermentations conducted with the ScQ strain and both *T. delbrueckii* strains (TdB+ScQ and TdP+ScQ) demonstrated significantly higher H* values compared to that of the control (ScQ). Thus, sequential fermentation with ScQ resulted in wines with more pronounced yellowish nuances. For sequential fermentation in the presence of ScK1, the H* values showed a decreasing trend compared to that of their control, indicating a less yellowish hue. One possible explanation is that these sequential fermentations (TdB+ScK1 and TdP+ScK1) did not exhibit statistically significant differences in the proportion of pyranoanthocyanins compared to the control (ScK1) (Figure 1C).

A principal component analysis (PCA) was performed to clarify which factors contribute to the overall colour variation observed in rosé wines under these conditions (Figure 2). The following parameters was used to perform the PCA: colour intensity, L*, C*, H*, total anthocyanins, relative proportion of nonacylated anthocyanins, relative proportion of acylated anthocyanins and relative proportions of pyranoanthocyanins. The first principal component (PC1) explains 52.67% of the variance, while the second (PC2) explains 34.69%; therefore, the combined variance explained by the first two components was 87.36%.

The loading variables presented in Figure 2b indicates the contribution provided by the two components related to their length and direction. The loadings on PC1 are related to CI, anthocyanin total concentration and proportion of nonacylated anthocyanins and are directed towards the positive values (corresponding to the right in Figure 2a), indicating that there was a correlation between these variables. In contrast, the L* coordinate, as it has opposite loadings on PC1, is directed towards the negative values being negatively correlated with the previous variables. This result was expected since the higher the CI of the wines was, the lower the L* value. Loading on PC1 separated samples into two clusters, control (ScQ and ScK1) in the positive values and sequential in the negative values. Therefore, it may be concluded that sequential fermentations, with the presence of *T. delbrueckii* strains, produced wines with lower anthocyanin concentrations and consequently, wines with less intense colours.

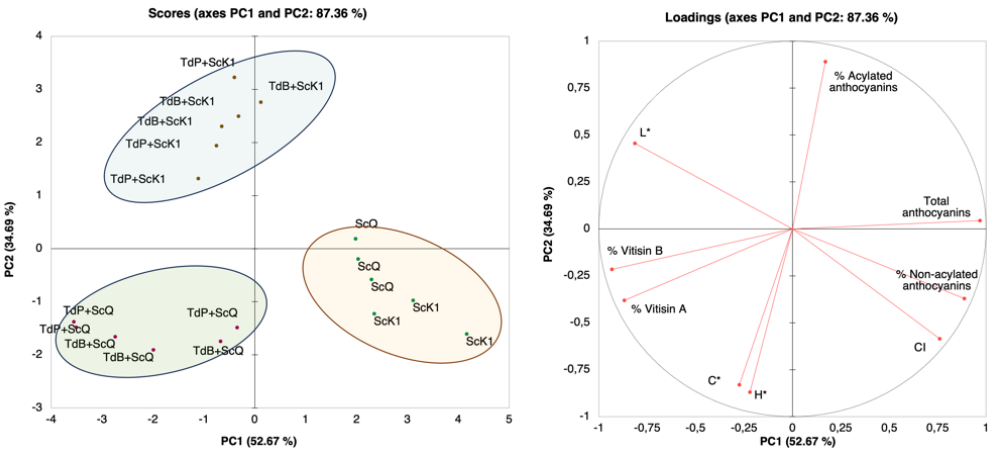

Figure 2. Principal component analysis biplots built from the following loadings: total anthocyanins, acylated anthocyanins, non-acylated anthocyanins, CI, Vitisin A, Vitisin B, C*, L*, H*. The scores are the followings: ScQ and ScK1: correspond to the pure fermentation with *S. cerevisiae* QA23 and *S. cerevisiae* K1, respectively; TdB+ScQ and TdP+ScQ which correspond to sequential fermentations with *S. cerevisiae* QA23 and *T. delbrueckii* Biodiva or *T. delbrueckii* Prelude, respectively; TdB+ScK1 and TdP+ScK1: correspond to sequential fermentations with *S. cerevisiae* K1 and *T. delbrueckii* Biodiva or *T. delbrueckii* Prelude, respectively.

Moreover, the proportion of pyranoanthocyanins (vitisin A and vitisin B) was negatively correlated with the total anthocyanin concentration, as they had opposite loadings on PC1. Thus, according to our results, sequential fermentation with *T. delbrueckii* strains seems to promote the degradation of anthocyanins, which increased the relative proportion of pyranoanthocyanins in the final wines.

The loadings on PC2 explained the H* (hue) and the proportion of acylated anthocyanins. The loadings corresponding to the proportion of acylated anthocyanins are directed towards the positive values of this PC. In contrast, H* loading is directed towards the negative values. Moreover, the loadings corresponding to pyranoanthocyanins (Vitisin A and Vitisin B) correlated positively with H* loadings. Therefore, the H* becomes higher as the proportion of pyranoanthocyanins increases and the proportion of acylated anthocyanins decreases, indicating that the wines were more yellowish.

Thus, according to PC2, the sequential fermentations carried out with ScK1 (TdB+ScK1 and TdP+ScK1) were clustered as a different group from sequential fermentations carried out with

ScQ (TdB+ScQ and TdP+ScQ) and control wines (ScQ and ScK1). This allowed us to separate the different sequential fermentations. In this sense, regardless of the *T. delbrueckii* strain, when sequential fermentation was carried out with ScQ, the produced wines contained a higher proportion of pyranoanthocyanins. However, when sequential fermentation was carried out with ScK1, the produced wines contained a higher proportion of nonacylated anthocyanins. This different pigment proportion led to wines with different H* values. In particular, the sequential fermentation of ScQ led to wines with a more yellowish hue, while the sequential fermentation with ScK1 led to wine with a more bluish hue.

3.5. Malolactic fermentations (MLF)

In terms of the MLF results, interesting differences were observed among the strains (Figure 3). In these conditions for natural *rosé*, fermentation could not be completed by Oo1Pw13 (data not shown); previously, it had been observed that L-malic consumption by this strain was very slow (Balmaseda, et al., 2021b).

As it has been described, although ScK1 is not a *S. cerevisiae* strain recommended for MLF, the presence of *T. delbrueckii* during AF in combination with ScK1 can have a positive effect (Ruiz-de-Villa et al., 2023b). Consequently, in this study the most interesting differences were found in the MLF of the ScK1 set, in which none of the control conditions could complete MLF, as expected. However, wine fermented in presence of *T. delbrueckii* TdP completed MLF with three of the strains, OoVP41 (6 days), OoCH11 (6 days) and OoPSU-1 (7 days), and TdB with OoCH11 (5 days) (Figure 3D).

Considering *O. oeni* strains, confirming previous results (Ruiz-de-Villa et al., 2023b), OoVP41 showed excellent MLF performance. Wines fermented with ScQ finished the MLF when OoVP41 was used, and even for one condition of the ScK1 set, the wine fermented with TdP+ScK1 (Figure 3A and 3D). Regarding ScQ wines, OoPSU-1 MLF managed to consume all L-malic acid only with TdB+ScQ and TdP+ScQ (5 days), contrary to the control condition (Figure 3B). These findings showed the beneficial effect of the synergy between *T. delbrueckii* and *O. oeni* described previously (Balmaseda et al., 2022a; Balmaseda et al., 2021a; Ruiz-de-Villa et al., 2023b). In this case, this improvement could be related to the higher content of mannoproteins (Table 1) in wines due to the presence of *T. delbrueckii* during the AF. In

addition, a lower concentration of MCFAs was observed in sequential wines (Table 2), compounds that have been described as toxic to *O. oeni* (Capucho and San Romão, 1994) because the membrane of *O. oeni* is destabilized (Sereni et al., 2020). Even though the minimum concentrations reported of decanoic and dodecanoic acids with inhibitory effects in *O. oeni* were above 12.5 and 2.5 mg/L, respectively (Capucho and San Romão, 1994), these differences detected among conditions could have had a slight effect.

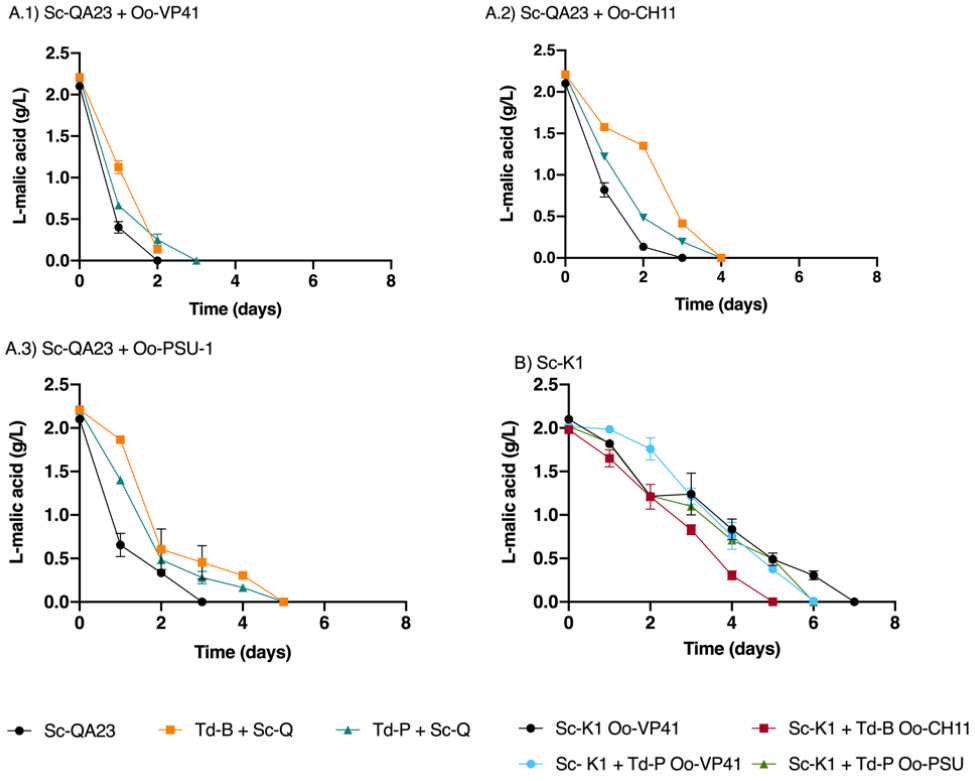

Figure 3. Consume of L-malic acid during MLF. ScQ and ScK1 correspond to the pure fermentation with *S. cerevisiae* QA23 and *S. cerevisiae* K1, respectively; TdB+ScQ and TdP+ScQ correspond to sequential fermentations with *S. cerevisiae* QA23 and *T. delbrueckii* Biodiva or *T. delbrueckii* Prelude, respectively; TdB+ScK1 and TdP+ScK1 correspond to sequential fermentations with *S. cerevisiae* K1 and *T. delbrueckii* Biodiva or *T. delbrueckii* Prelude, respectively. A) Corresponds to ended MLF of ScQ wines with: A.1) OoVP41 strain, A.2) OoCH11 strain and A.3) OoPSU-1 strain. B) Corresponds to ended MLF of ScK1 wines with OoVP41, OoCH11 and OoPSU-1 strains. All data are expressed as the mean of three biological replicates ± standard deviation.

The positive synergy with these two species could have involved a reduction in pH or ethanol helping to ease the stressful environment for *O. oeni*; however, in our study, this behaviour was not observed. The reduction in polyphenolic compounds due to the presence of *T. delbrueckii* (Figure 1) may have exert an effect since certain phenolic compounds are stressful for *O. oeni* (Bech-Terkilsen et al., 2020).

4. Conclusion

In this study, our objective was further examining the oenological potential of using *T. delbrueckii* in sequential inoculation with *S. cerevisiae*, focusing specifically on its application in *rosé* wines. To achieve this objective, both the organoleptic characteristics and MLF performance were examined. Interesting results were obtained regarding the organoleptic profile of the wines. Wines fermented in the presence of *T. delbrueckii* exhibited higher concentrations of fusel alcohols and acetates. However, the most noteworthy results involved the colour composition of the wines. Compared to control conditions, the presence of *T. delbrueckii* resulted in a significant colour change. Furthermore, sequential fermentations resulted in a reduction in anthocyanins and, consequently, a decrease in colour intensity. However, the behaviour varied depending on the *S. cerevisiae* strain used (ScQ or ScK1). It appears that TdB+ScQ and TdP+ScQ wines contained a higher percentage of pyranoanthocyanins than that of TdB+ScK1 and TdP+ScK1 wines. The increase in these pigments is associated with an increase in yellow hues, as indicated by CIELab coordinates. Additionally, the reduction in anthocyanins in sequential ScQ fermentations was attributed to a decrease in acylated and nonacylated anthocyanins, while in sequential ScK1 wines, it was primarily related to nonacylated anthocyanins. For wines fermented with TdB+ScK1 and TdP+ScK1, a higher percentage of acylated anthocyanins was observed, resulting in an increase in bluish hues. The possible reasons for this colour reduction are as follows: the reduction in anthocyanins may be due to the formation of aglycones, the capacity of the two different species to adsorb pigments, or the oxidation resulting from the longer duration of AF in sequential wines.

MLF exhibited significant differences among wines with different AF conditions and among *O. oeni* strains. The best combination was observed in the wine fermented with TdP+ScQ,

followed by MLF performed by OoVP41. Additionally, a positive synergy between *T. delbrueckii* and *O. oeni* was observed in wines fermented with TdP+ScK1, as MLF was completed more quickly than by the control wines, even though ScK1 is not typically recommended for MLF.

In conclusion, the use of *T. delbrueckii* in sequential fermentation with *S. cerevisiae* achieved promising results with rosé wines. Sequential fermentations with ScQ increase the proportion of pyranoanthocyanins, which could enhance colour stability—an essential characteristic for rosé wines. The reduction in total anthocyanins and colour intensity presents an interesting oenological tool for producing rosé wines with a lighter colour, which is currently highly sought after by consumers. Furthermore, the improvement in MLF performance is another valuable aspect of *T. delbrueckii*, although the performance is highly influenced by the specific strain combination.

Acknowledgments

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Supplementary data

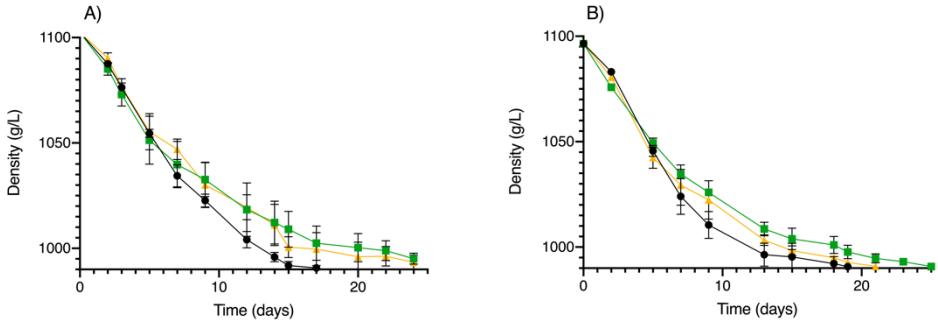

Figure SD 1. Kinetic of alcoholic fermentation. ● Sc, ■ TdB, ▲ TdP. A) Corresponds to AF with ScQ and B) Correspond to the AF with ScK1; TdB and TdP corresponds to the fermentations with *S. cerevisiae* and *T. delbrueckii* Biodiva or *T. delbrueckii* Prelude, respectively. All data is expressed as the arithmetic average of three biological replicates ± standard deviation (n=3).

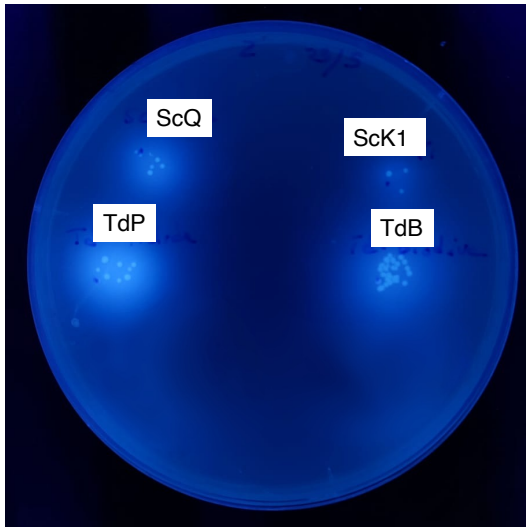

Figure SD 2. β-glucosidase activity of the tested strains. A) Correspond to *S. cerevisiae* ScQ, B) corresponds to S.cerevisiae ScK1, C) Correspond to *T. delbrueckii* TdB and TdP corresponds to the fermentations with *S. cerevisiae* and *T. delbrueckii* Biodiva or *T. delbrueckii* Prelude, respectively.

Table SD1. Pyruvic acid and acetaldehyde after alcoholic fermentation. ScQ and ScK1 correspond to the pure fermentation with *S. cerevisiae* QA23 and *S. cerevisiae* K1, respectively; TdB+ScQ and TdP+ScQ correspond to sequential fermentations with *S. cerevisiae* QA23 and *T. delbrueckii* Biodiva or *T. delbrueckii* Prelude, respectively; TdB+ScK1 and TdP+ScK1: correspond to sequential fermentations with *S. cerevisiae* K1 and *T. delbrueckii* Biodiva or *T. delbrueckii* Prelude, respectively. Lowercase letters indicate a significant difference between *S. cerevisiae* strains at *p*-value < 0.05; Capital letters indicate a significant difference between *S. cerevisiae*, *T. delbrueckii* Biodiva and *T. delbrueckii* Prelude at *p*-value < 0.05. Mean ± standard deviation (n=3).

	Pyruvic acid (mg/L)	Acetaldehyde (mg/L)
ScQA23	101 ± 32.65	34 ±12 ^{aA}
TdB+ScQ	99 ± 59.53	54 ±20 ^{aA}
TdP+ScQ	73 ± 2	37 ±13 ^{aA}
ScK1	43 ± 15	147 ±11 ^{bA}
TdB+ScK1	92 ± 43	113 ± 38 ^{bA}
TdP+ScK1	58 ± 14	79 ± 37 ^{bA}

Section 2.2

Torulaspora delbrueckii improves the organoleptic properties and promotes malolactic fermentation in carbonic maceration wines

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Abstract

This study investigates the impact of inoculating *Torulaspora delbrueckii* (Td) strains during the initial phase of Carbonic Maceration (CM) vinification, aiming to enhance the fermentative process and unique characteristics of CM wines. CM is a winemaking technique where intact grapes are enclosed in tanks filled with carbon dioxide, inducing intracellular fermentation. The study compares the effects of two Td strains on the MLF performance and sensory characteristics of CM wines, using both inoculated and spontaneous MLF strategies. Although general physicochemical parameters remained consistent across conditions, organoleptic attributes showed significant differences due to *T. delbrueckii* presence. *T. delbrueckii* introduction during CM resulted in wines with increased anthocyanin content and a particular volatile profile. Isoamyl acetate, a key aroma in CM wines, was notably elevated, especially in the TdP strain. Sensory evaluations also revealed distinctions, with TdV wines displaying more pronounced aromas of red fruit, banana, and grass. Regarding MLF, *T. delbrueckii* presence notably enhanced performance, particularly in spontaneous MLF cases, accelerating fermentation completion. Inoculating the *Oenococcus oeni* strain OoVP41 also shortened MLF duration. These findings highlight the potential of Td strains to improve MLF efficiency and sensory attributes in CM wines. Employing *T. delbrueckii* strains strategically enables winemakers to optimize MLF and improve sensory profiles, offering a opportunity to produce higher-quality CM wines.

Key words

Oenococcus oeni; maceration; anthocyanins; isoamyl acetate

1. Introduction

Carbonic maceration (CM) vinification is a specific winemaking technique that is characterised by not crushing or destemming the grapes. Instead, the intact grapes are placed in tanks, which are then filled with carbon dioxide (CO_2) to create an anaerobic environment (Tesniere and Flanzy, 2011). Within the grapes, an anaerobic fermentative metabolism initiates, known as intracellular fermentation, causing the berries to undergo physicochemical changes. As a result, the grapes begin to break down, releasing the juice or must, which accumulates at the bottom of the tank and undergoes alcoholic fermentation (AF) through the action of wild yeasts, present in the skin of the grapes (Tesniere and Flanzy, 2011). Furthermore, this process also triggers other metabolic reactions, including the extraction of phenolic compounds from the grape skins, the release of volatile compounds and a partial degradation of malic acid (Tesniere and Flanzy, 2011). This grapes berries would result in a wine with improved organoleptic characteristics, due to intra-berry metabolic pathways. Finally, the second part of the process takes place, where the free-run wine and the press-wine finish the AF, mixed or separated depending on the oenologist decision (Portu et al., 2023; Tesniere and Flanzy, 2011).

CM vinification process offers a wide range of possibilities, as it can be varied at different points, for instance using rotating tanks to homogenise the whole grains with the free-run juice or prolonging the maceration after the AF (Tesniere and Flanzy, 2011). During the first part of the process, there may be a biochemical decarboxylation of L-malic acid in the free-run fermenting must through malolactic fermentation (MLF), depending on the population of lactic acid bacteria (BAL) present in the grape must. Consequently, if MLF has already begun in the first step in the free-run fermenting must, it is advisable to vinify the two wine fractions separately. This separation is important because the higher sugar content in the grape berry can potentially lead to lactic spoilage if both fractions are combined. In this context, AF is usually fast, between 2 or 7 days, and then takes place the MLF, however sometimes due to improper management both fermentations may occur simultaneously (Tesniere and Flanzy, 2011).

The distinctive organoleptic characteristics of CM wines, which have a lot of consumer appreciation, have contributed to the growing interest in studying this winemaking method (Antalick et al., 2014; Etaio et al., 2016; González-Arenzana et al., 2020; Portu et al., 2023).

These wines are known for having a higher content of esters and acetates, which increase their fruity aroma (Antalick et al., 2014; González-Arenzana et al., 2020). Specially, literature highlights the higher concentration of isoamyl acetate and ethyl cinnamate compared to wines produced using traditional fermentation methods (Antalick et al., 2014; Tesniere and Flanzy, 2011). Additionally, in regard to colour characteristics, CM wines have been found to have lower or higher levels of phenolic compounds and colour intensity, it depends on the grape variety, the grape ripeness, the vintage or the winemaking conditions ((Chinnici et al., 2009; González-Arenzana et al., 2020; Portu et al., 2023).

Recent studies have focused on exploring the high diverse autochthonous microbial population present in CM wines, with a large presence of non-*Saccharomyces* in the early stages (Gutiérrez et al., 2022; Guzzon et al., 2020). The utilization of non-*Saccharomyces* commercial yeast presents an interesting prospect to maintain these special characteristics and have a better microbiological control. For instance, the use of the species *Torulaspora delbrueckii,* commercialized as starter culture, has been studied in sequential fermentation with *Saccharomyces cerevisiae* in white wines (Azzolini et al., 2015; Renault et al., 2015) red wines (Balmaseda et al., 2021d; Chen et al., 2018), rosé wines (Muñoz-Redondo et al., 2021; Ruiz-de-Villa, 2024a) or even in botrytized wines (Bely et al., 2008) owing to its impact on the organoleptic properties. The utilization of *T. delbrueckii* in winemaking has been found to have significant effects on the composition of volatile profile. The presence of this species leads to an increase in specific volatile compounds, such as fusel alcohols (Azzolini et al., 2015; Belda et al., 2015; Benito, 2018; Ruiz-de-Villa et al., 2023b). Additionally, the presence of *T. delbrueckii* during fermentative maceration in red wines has been associated with a greater release of phenolic compounds (Balmaseda et al., 2021b; Chen et al., 2018).

Furthermore, several research studies have focussed the potential of *T. delbrueckii* in enhancing the performance of malolactic fermentation (MLF) by the main LAB in wine, *Oenococcus oeni* (Balmaseda et al., 2021d; Ferrando et al., 2020; Martín-García et al., 2020; Ruiz-de-Villa et al., 2023b). In CM context, it has been recently studied how the yeast species inoculation affect to the bacterial population (Gutiérrez et al., 2023). As described above, in the case of CM vinification MLF may occur spontaneously which can significantly impact the wine's flavour profile. Besides, it is important to note that a population of 10^6 CFU/mL and

optimal conditions for LAB are necessary to carry out the MLF. In cases where this does not occur, the inoculation of LAB starter cultures and the use of *T. delbrueckii* could enhance the complete and successful malic acid degradation.

In summary, the objective of this research is to investigate the oenological implications of inoculating *T. delbrueckii* during the initial stage of CM vinification with the aim of improve the fermentative process and distinctive character of these wines. The study compares the effects of two different strains of this species on both the organoleptic characteristics and the performance of inoculated and spontaneous MLF.

2. Materials and methods

2.1. Carbonic maceration and alcoholic fermentation

The study evaluated three yeast species: one strain of *Saccharomyces cerevisiae*, CLOS YSEO (from now on referred as Sc), obtained from Lallemand Inc. (Montreal, Canada), and two strains of *Torulaspora delbrueckii*: Viniflora Prelude (Td-P), obtained from Chr. Hansen Holding AS (Hoersholm, Denmark), and Viniferm NS Td (Td-V), obtained from Agrovin Alcázar de San Juan, Spain). The yeast strains were inoculated from Dry Active Yeast and rehydrated following the manufacturer's instructions. The *S. cerevisiae* strain was rehydrated at 37 °C for 30 minutes, while the *T. delbrueckii* strains were rehydrated at 30 °C for the same duration.

The fermentations were conducted using Grenache grape variety (*Vitis vinifera* L.) supplied by the cellar *Mas dels Frares* of *Rovira i Virgili University*, which belongs to the PDO Tarragona. The grapes were harvested and processed in this experimental winery of the university. Semi-industrial scale fermentations were performed in 15 L food-grade plastic container, maintaining a constant temperature of 22 °C. Initially, uncrushed grape berries, without stems, were placed in the tanks. The Control condition involved no inoculation during carbonic maceration (CM) and was left with the endogenous microbiota. The sequential conditions were inoculated with the two strains of *T. delbrueckii* at an initial concentration of 2×10^6 cells/mL. After yeast inoculation, CO_2 was pumped inside to create an anaerobic environment, and the tanks were hermetically closed. After five days of CM, the grapes were pressed, and the two must fractions (free-run must and press must) were mixed. The resulting fermenting

must from each condition was transferred to another tanks, and *S. cerevisiae* was inoculated in all three conditions at a concentration of 2 x 10^6 cells/mL to finish AF. Thus, three different conditions were tested: Control, TdP, and TdV.

Samples after CM (CM point) and at the final of AF (Final AF point) were stored to analyse them. In addition, final wines were bottled to perform the sensory analysis.

Two media were used to determine inocula and population dynamics by plating a 1:10 serial dilution. For total yeast was used YPD agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 17 g/L agar (Panreac Química SLU, Barcelona, Spain)) and for non-*Saccharomyces* yeasts the selective medium Lysine (Difco Laboratories, Detroit, MI, USA).

An electronic densimeter (Densito 30PX Portable Density Meter (Mettler Toledo, Spain) was used for monitoring AF by measuring density each day. AF was considered finished when reductive sugars were under 2 g/L of residual concentration. Residual glucose and fructose were determined by an enzymatic autoanalyzer Y15 Enzymatic Autoanalyzer (Biosystems S.A, Barcelona, Spain).

2.2. Malolactic fermentation

After completing AF, replicates of each condition were mixed, and the resulting wines were divided into three conditions for MLF. The wines were stored at 4°C for one week to stabilize them. The two strains of *Oenococcus oeni* used for inoculation were Lalvin VP41 (Oo-VP41) Lallemand Inc. and Viniflora CH11 (Oo-CH11) Chr. Hansen Holding AS. These strains were inoculated from commercial lyophilized products and rehydrated following the manufacturer's recommendations. *O. oeni* strains were rehydrated in wine at 20 °C for 15 minutes. Furthermore, a spontaneous MLF was performed (Sp).

As a result, the initial three AF conditions were expanded to a total of nine MLF conditions: Control, TdP, and TdV, each with their respective MLF condition of OoVP41, OoCH11, and Sp, with triplicate samples for each condition.

The MLF inoculations were performed at a population of 2 x 10^7 cell/mL. MLF was carried out in 1 L volumes at a temperature of 20°C under anaerobic and static conditions. The progress

of L-malic acid consumption was monitored daily until it reached a concentration below 0.1 g/L using the Y15 Enzymatic Autoanalyzer (Biosystems S.A, Barcelona, Spain).

The inoculum and populations of LAB were controlled by plating on modified MRS medium (De Man et al., 1960) (Difco Laboratories, Detroit, MI, USA) following the modification described in (Margalef-Català et al., 2017). The medium was adjusted to a pH 5 and supplemented with 4 g/L DL-malic acid (Sigma-Aldrich), 5 g/L D-fructose (Panreac), 100 mL/L of tomato juice (Aliada, Madrid, Spain), 100 mg/L of nystatin (Panreac) to prevent yeast growth, and 25 mg/L of sodium azide (BioSciences, St. Louis MO, USA) to prevent acetic acid bacteria growth. The plates were incubated at 27 °C in a 10% CO_2 atmosphere.

2.3. Area Under the Curve (AUC)

The area under the curve (AUC) was calculated to assess significant differences in the AF performance. This was achieved by analysing the decrease in density during AF and then integrating that values between two consecutive time points. The formula used for the calculation involved summing the areas of consecutive data points as follows: Σ [((d2 + d1)/2) * (t2 - t1) + … + ((dn + dn-1)/2) * (tn - tn-1)], where d1, d2, ..., dn-1, dn represent the at times 1, 2, n-1, and n, respectively.

2.4. Physicochemical analysis

2.4.1. General oenological parameters

The pH of the wines was determined using a Crison micro pH 2002 pH-meter (Hach Lange Spain, L'Hospitalet, Spain). The content of citric acid, glycerol, and ethanol was determined following the procedure described in Quirós et al. (2014) using an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany). Prior to injection, the wine samples were filtered using 0.22 µm pore filters (Merck). The HPLC system was equipped with a Hi-Plex H column (300 mm x 7.7 mm) inside a 1260 MCT (Infinity II Multicolumn Thermostat). Two detectors were coupled: a MWC detector (Multi-wavelength detector, Agilent Technologies) and a RID detector (1260 Infinity II refractive index detector, Agilent Technologies).

The concentrations of acetic acid and L-malic acid were enzymatically determined using the Y15 Enzymatic Autoanalyzer (Biosystems S.A, Barcelona, Spain). Succinic acid was analysed using a manual enzymatic method (Megazyme, Wicklow, Ireland) with the UV-Vis spectrometer POLARstar Omega (BMG LABTECH, Ortenberg, Germany).

To estimate the mannoprotein content, a precipitation with 95% ethanol followed by an acid hydrolysis at 90 °C was performed following the procedure described in Balmaseda et al. (2021a). The resulting mannoproteins were quantified in terms of mannose equivalents using a D-mannose and D-glucose enzymatic assay kit (Megazyme).

2.4.2. Volatile composition

In the present study, the volatile composition of the wines was extracted using a liquid/liquid extraction method, 400 µL of dichloromethane, in the presence of 2.5 g of ammonium sulphate $(NH_4)_2SO_4$), was used. Two internal standards (IS), 4-methyl-2-pentanol (0.8 g/L) and heptanoic acid (0.7 g/L), were employed. The extraction method used was based on the protocol described by (Ortega et al., 2001) with modifications as outlined by (Balmaseda et al., 2021d). After the extraction, the organic phase was collected. Subsequently, 2 µL of the organic phase was injected into a gas chromatograph coupled to a FID detector (Flame Ionization Detector) (Agilent Technologies, Germany) using a FFAP column with dimensions of 30 m × 0.25 mm × 0.25 µm. The injection was performed in split mode with a split ratio of 10:1 and a flow rate of 30 mL/min. The volatile compounds identified were the following: fusel alcohol acetates (isobutyl acetate, isoamyl acetate, 2-phenyletanol acetate), fusel alcohols (2-metil-propanol, 1-propanol, isoamyl alcohol, 1-pentanol, 1-hexanol, *cis*-3-hexen-1-ol, 2-phenylethanol), other alcohols (2-butanol, 1-butanol), ethyl esters of FA (Ethyl butanoate, ethyl octanoate, ethyl decanoate, diethyl butanoate, ethyl dodecanoate, ethyl hexanoate), short-chain fatty acids (SCFA) (propionic, isobutyric, butyric, butyric acids), medium-chain fatty acids (MCFA) (octanoic, decanoic and dodecanoic acids). All reagents were analytical grade from Sigma-Aldrich (Barcelona, Spain).

2.4.3. Colour and anthocyanins analysis

The following colour parameters were determined: CIELab coordinates, Colour Intensity and Total Polyphenolic Index (TPI). CIELab coordinates were determined as describe Ayala et al. (1997). The colorimetric coordinates were calculated with MSCV software (Ayala *et al.*, 2001), including the red-greenness (a*) and yellow-blueness (b*) and their derivate magnitudes: hue (H), lightness (L) and Chroma (C). The Colour Intensity (CI) was calculated from the sum of absorbances at 420, 520 and 620 nm, measured in a spectrophotometer, using the method described by Glories et al. (1984). The content of free anthocyanins, non-acylated and acylated anthocyanins as well as pyranoanthocyanins, was determinate in two points of AF: after MC and after the AF. Samples from final AF were only filtered with a 0.22 μm pore filters (Merck) before injection. However, samples from the final of CM were pre-treated by using PVPP (Sigma-Aldrich) columns in order to eliminate sugars which could interfered in the HPLC measure. Then both batches of samples were analysed by using was analysed with an Agilent 1200 series liquid chromatograph (HPLC–diode array detection) using an Agilent Zorbax Eclipse XDB-C18 column (4.6 x 250 mmx 5 mm, Agilent Technologies) with the procedure described by (Gil et al., 2012).

The total anthocyanin content was determined by spectrophotometry using the method described by Niketic-Aleksic and Hrazdina (1972).

2.4.4. Sensory analysis

After the AF and MLF, sensory analyses were conducted by a trained tasting panel consisting of 12 tasters. First, the panel compared three wines through a blind triangle test to identify any noticeable differences between them. Subsequently, a descriptive test was performed on wines that were found to be significantly different in the triangle test. In the descriptive test, tasters evaluated the intensity of five attributes using a numerical scale (from 0 to 5): acidity, red fruit aroma, banana aroma, grass aroma, and global perception.

2.4.5. Statistical analysis

All the fermentations were performed in triplicate to improve the consistency of the results. Statistical analysis of data was done by using the ANOVA and Tukey test with XLSTAT

2021.2.3 software (Addinsoft, Paris, France). A *p*-value of less than 0.05 was considered statistically significant. A principal component analysis (PCA) was used to describe the volatile composition of wines after MC and AF. Panel check V1.4.2 (Tomic et al., 2010) was used for sensory data.

3. Results and discussion

3.1. Alcoholic fermentation

As it has been described, CM offers several possibilities for conducting the vinification process. The following vinification process was carried out: after undergoing five days of CM under anaerobic conditions, the free-run must and press must were blended to observe and analyse the complete wine produced during CM, comparing samples with and without the presence of *T. delbrueckii*.

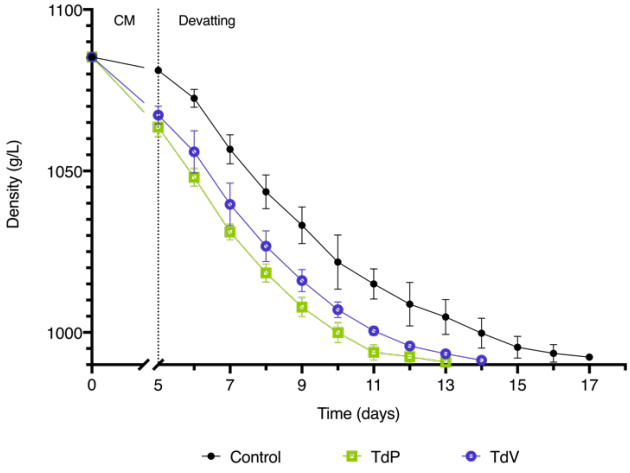

Figure 1. Kinetic of alcoholic fermentation after five days of carbonic maceration. Control corresponds to the pure fermentation with *S. cerevisiae* CLOS; TdP and TdV corresponds to the fermentations with *S. cerevisiae* and CM with *T. delbrueckii* Prelude or *T. delbrueckii* Viniferm, respectively. All data is expressed as the arithmetic average of three biological replicates ± standard deviation (n=3).

Subsequently, various parameters of the resulting wines were analysed at two stages: after CM and AF. The initial parameters in the grape must were as follows: pH 3.3, total acidity 4.85 g/L, α-amino nitrogen 121 mg/L and ammonium 87 mg/L. The initial density was 1085.3 g/L,

and as the yeast started the AF in the free-run must the density gradually decreased. In the Control condition with spontaneous yeasts, the density only decreased to 1081.2 ± 0.4 g/L. However, in the CM condition with *T. delbrueckii*, the decrease was significantly more pronounced. Specifically, for TdP, the density decreased to 1063.6 ± 3.0 g/L, and for TdV, it decreased to 1067.3 ± 2.8 g/L (Figure 1). This reduction in initial density facilitated a shorter duration of AF in wines containing *T. delbrueckii*. The TdP condition lasted for 13 days, while the TdV condition lasted for 14 days. In contrast, the control condition with *S. cerevisiae* alone extended for a duration of 17 days.

The AUC (Area Under the Curve) values were calculated for wines sampled at the end of the CM and sampled at the end of AF (Table 1). Significant differences were observed in the CM samples, where the presence of *T. delbrueckii* resulted in significantly lower AUC values compared to the CM Control, indicating a faster AF during CM. However, after the inoculation of *S. cerevisiae* and the performance of AF, Control wines exhibited higher AUC values than wines with *T. delbrueckii* (TdP and TdV). This indicates that despite *T. delbrueckii* consuming sugars during CM, *S. cerevisiae* alone leads to a faster AF, which aligns with previous studies that have reported *S. cerevisiae* to have faster AF compared to sequential or coinoculated fermentations with non-*Saccharomyces* species due to the competition between species (Balmaseda et al., 2021c; Roca-Mesa et al., 2022).

In terms of yeast populations, it was observed that the presence of non-*Saccharomyces* yeasts, detected in lysine medium, was higher in wines that were inoculated with *T. delbrueckii* as expected. It has been previously reported that when *T. delbrueckii* is used in sequential fermentation with *S. cerevisiae*, there is a high percentage of *T. delbrueckii* observed at the end of AF (Roca-Mesa et al., 2022; Ruiz-de-Villa et al., 2023b).

Few differences were observed in the general oenological parameters analysed. A slight production of ethanol was observed after CM, between 1 and 3%, as stated in literature (Tesniere and Flanzy, 2011). However, in this sampling point, the ethanol content was higher in the musts inoculated with *T. delbrueckii* (TdP and TdV) than in the Control condition. This was predictable since the AF in the free-run must were less advanced in Control condition due to the absence of inoculated yeast, producing only a 1.06% of ethanol. Besides, there

was a decreasing trend in ethanol production after AF in TdP and TdV wines compared to Control. Many authors have stated that ethanol decreases in sequential AF with the use of *T. delbrueckii* (Belda et al., 2015; Contreras et al., 2014; Zhu et al., 2020). However, this decrease depends on the vinification conditions (Benito, 2018); thus, the reduction was not significant in these CM conditions, No significant differences were observed in glycerol, pH, citric acid, or succinic acid among the samples. However, L-malic acid was more consumed in Td wines than in the Control wines after AF. This could be explained as slight consumption of malic acid has been observed in yeast for their metabolism (Ribéreau-Gayon, 2006b). It has been reported that the consume is higher in *T. delbrueckii* fermentations (Balmaseda et al., 2022a; Escribano-Viana et al., 2019; Ruiz-de-Villa et al., 2023b). Variations were found in acetic acid levels, with a noticeable increasing trend in the Control samples at both the CM and AF stages. In sequential AF with *T. delbrueckii* and *S.cerevisiae* has been observed a reduction of acetic acid to a greater or lesser degree depending on the strain and winemaking conditions (Balmaseda et al., 2021b; Bely et al., 2008; Martín-García et al., 2020).

Furthermore, in terms of nitrogen content at the end of CM, wines fermented with *T. delbrueckii* exhibited significantly higher consumption compared to the Control, which can be attributed to a larger yeast population. However, the Control wine after AF showed complete consumption of ammonia and slightly higher consumption of α-amino nitrogen compared to TdP and TdV wines. It is worth noting that after CM, TdP wine displayed higher consumption of α-amino nitrogen compared to TdV wine. Additionally, there was an increase in mannoproteins presence at the end of AF, consistent with previous reports (Balmaseda et al., 2021a; Domizio et al., 2014; Ruiz-de-Villa et al., 2023b), indicating higher nitrogen concentration availability in TdP and TdV wines after AF.

Table 1. Oenological parameters analysed. End of CM corresponds to the sampling after carbonic maceration, before to inoculate *S. cerevisiae*, and End of AF corresponds to the sampling after alcoholic fermentation. Control corresponds to the control fermentation; TdP and TdV corresponds to the fermentations with *T. delbrueckii* Prelude or *T. delbrueckii* Viniferm, respectively. AUC, Area Under the Curve. Different lowercase letters indicate the existence of significant difference between samples at the end of CM (p-value < 0.05). Different uppercase letters indicate the existence of significant difference between samples at the end of AF (p-value < 0.05). All data is expressed as the arithmetic average of three biological replicates ± standard deviation (n=3).

General oenological parameters	End of CM			End of AF		
	CM Control	CM TdP	CM TdV	AF Control	AF TdP	AF TdV
AUCs	5416 ± 1 ^{a}	5372 ± 7 ^{b}	5381 ± 7 ^{b}	11287 ± 49 ^{B}	8119 ± 17 ^{A}	9165 ± 28 ^{A}
Glycerol (g/L)	2.04 ± 0.12 ^{a}	3.43 ± 0.79 ^{a}	3.38 ± 0.55 ^{a}	7.51 ± 0.76 ^{AB}	6.56 ± 0.59 ^{A}	7.55 ± 0.09 ^{B}
Ethanol (% v/v)	1.06 ± 0.04 ^{a}	2.99 ± 0.28 ^{b}	2.84 ± 0.16 ^{b}	11.06 ± 0.21 ^{A}	11.58 ± 0.16 ^{A}	11.70 ± 0.09 ^{A}
Ammonia (mg/L)	68 ± 9 ^{b}	40 ± 11 ^{a}	46 ± 6 ^{a}	n.d	11 ± 4 ^{B}	7 ± 5 ^{AB}
a-amino nitrogen (mg/L)	109 ± 3 ^{b}	60 ± 7 ^{a}	97 ± 7 ^{b}	19 ± 7 ^{A}	27 ± 4 ^{A}	22 ± 3 ^{A}
Succinic acid (g/L)	0.42 ± 0.02 ^{b}	0.27 ± 0.07 ^{a}	0.31 ± 0.10 ^{ab}	0.34 ± 0.04 ^{A}	0.31 ± 0.05 ^{A}	0.29 ± 0.03 ^{A}
Citric acid (g/L)	0.26 ± 0.04 a	0.34 ± 0.10 a	0.28 ±0.04 a	0.23 ±0.02 ^{A}	0.25 ± 0.03 ^{A}	0.25 ± 0.05 ^{A}
Acetic acid (g/L)	0.34 ± 0.07 b	0. ± 0.02 a	0.61 ± 0.06 ab	0.54 ± 0.10 ^{A}	0.47 ± 0.05 ^{A}	0.31 ± 0.06 ^{A}
L-malic acid (g/L)	-	-	-	1.73 ± 0.04 ^{A}	1.64 ± 0.04 ^{B}	1.58 ± 0.04 ^{C}
pH	-	-	-	3.21 ± 0.03 ^{A}	3.25 ± 0.03 ^{A}	3.27 ± 0.04 ^{A}
Eq. mannose (mg/L)	-	-	-	114 ± 13 ^{A}	301 ± 7 ^{B}	311 ± 8 ^{B}

3.2. Volatile composition

In order to examine the impact of both *T. delbrueckii* strains on the volatile composition of wines, a Principal Component Analysis (PCA) was conducted (Figure 2). The identified volatile compounds were categorized into the following families: Ethyl esters, fusel alcohols acetates, fusel alcohols, MCFA, SCFA and other alcohols (Table SD 2).

On one hand Figure 2A shows that PC1 separated the wines in two groups. The samples after CM and Control wines after AF (AF control) are separated in a group, while wines with *T. delbrueckii* after AF (AF TdP and AF TdV) are grouped in another group. On the other hand, PC2 separate wines with *T. delbrueckii* regarding the strain used (AF TdP and AF TdV).

The Control condition with spontaneous yeast during CM did not undergo changes during the completion of AF, with the exception of some compounds. Instead, it remained similar to samples after CM being positively correlated with the other alcohols and MCFA variables, and negatively correlated with the rest (Figure 2). Only few compounds showed a significant increase after the completion of AF in all conditions, such as 1-propanol, 2-methyl propanol, ethyl dodecanoate and 2-phenylethanol (Table SD2). Previous studies have reported that a wide range of aromatic compounds in carbonic maceration wines are formed in the initial stage of the process (CM), which occurs under a CO_2 atmosphere (González-Arenzana et al., 2020; Salinas et al., 1996). Some differences were observed in relation to the presence or not of *T. delbrueckii* after CM phase. Ethyl butanoate, 2-methyl propanol, ethyl dodecanoate and 2-phenyletanol were increased significantly (Table SD2) in CM TdP and CM TdV wines, regardless the strain.

However, as shown in Figure 2, it is evident that several volatile compounds showed an increase only after AF in *T. delbrueckii* conditions (AF TdP and AF TdV), positively correlated with ethyl esters, fusel alcohols, SCFA and fusel alcohol acetates. This implies that the final wines in the presence of *T. delbrueckii* after the entire process have higher concentrations of these volatile families. These compounds include: 1-propanol, 2-methyl-propanol, isoamyl acetate, 2-phenylethanol acetate, ethyl decanoate, ethyl dodecanoate, and isopropanol, volatile compounds characterized by alcohol, wine, banana, roses, grape and leave descriptors, respectively (Sumby et al., 2010) (Table SD2).

The modulation of aroma due to the presence of *T. delbrueckii* showed in this work confirmed previous results in red (Balmaseda et al., 2021b; Belda et al., 2015) and white wines(Azzolini et al., 2015), or even in rosé wine (Muñoz-Redondo et al., 2021; Ruiz-de-Villa et al., 2024a). This effect has been also associated to an increase in esters and fusel alcohols, in spite that this impact depends on the strain and wine. Regarding the results of this study TdP strain increase significatively more than TdV strain the concentrations of 2-phenlyethanol, 2-methyl propanol and isoamyl acetate (Table SD2). Isoamyl acetate is a characteristic aromatic compound found in CM wines (Tesniere and Flanzy, 2011), characterized for its banana descriptor (Sumby et al., 2010). However, after CM the concentration of isoamyl acetate in these wines only reached concentrations of 0.62 mg/L (Control), 0.63 mg/L (TdP) and 0.47 mg/L (TdV) (Table SD2) without significant differences, maybe due to the grape cultivar. However, it is worth noting that TdP and TdV increased significantly this isoamyl concentration in wine after AF, reaching a concentration of 3.37 mg/L and 1.32 mg/L, respectively, compared to the AF Control wine with a concentration of 0.58 mg/L. Previous studies have reported that CM vinification process leads to higher concentrations of this volatile compound compared to traditional vinification methods, with differences between cultivars and types of wines. For instance, in French wines from blend of cultivars Antalick et al., (2014) observed concentrations of 3.20 mg/L in CM wines compared to 0.51 mg/L in traditional young red wines, however González-Arenzana et al., (2020), described concentrations of 2.78 mg/L in CM wines compared to 1.14 mg/L in traditional red wines.

In addition, it worth to highlight that the negative correlation of AF TdP and AF TdV with MCFA was due to a trend on a decrease of this family (Figure 2)., especially associated to decanoic acid. It has been reported that the presence of *T. delbrueckii* during AF leads to a reduce in MCFA. This reduction has been associated with a detoxification due to the presence of more mannoproteins in *T. delbrueckii* wines (Table1)

These findings indicate that the presence of *T. delbrueckii* during CM not only changes the volatile composition during the CM phase but also carries its influence throughout the AF process, especially with TdP.

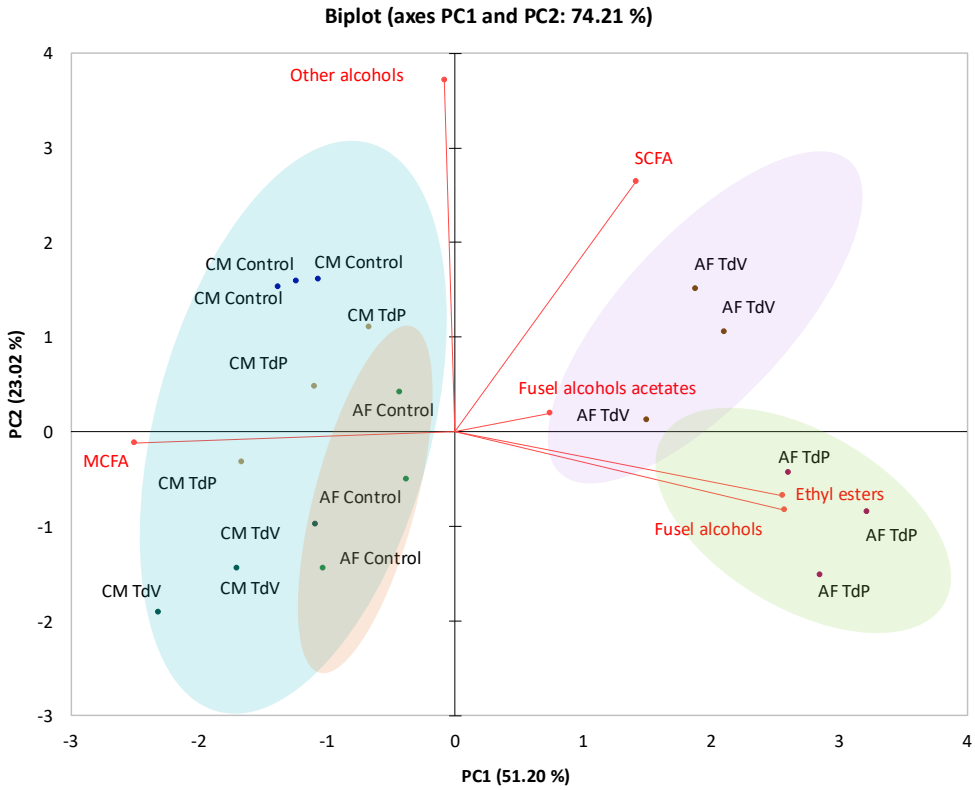

Figure 2. Principal component analysis biplot built from the following variables: Ethyl esters, fusel alcohols acetates, fusel alcohols, MCFAs, SCFAs and other alcohols. The samples are the followings: CM Control: corresponds to control condition at the end of Carbonic maceration; CM TdP and CM TdV which corresponds to conditions inoculated with *T. delbrueckii* Prelude or *T. delbrueckii* Viniferm, respectively, at the end of carbonic maceration; Control: corresponds to control condition at the end of alcoholic fermentation; TdP and TdV: corresponds to conditions inoculated with *T. delbrueckii* Prelude or *T. delbrueckii* Viniferm, respectively, at the end of alcoholic fermentation.

3.3. Colour parameters and anthocyanins composition

In which refers to anthocyanins and colour composition interesting difference were found between conditions. Table 2 presents the results of anthocyanins composition and colour parameters. It is evident that the concentration of anthocyanins is relatively low, which could be attributed to the grape cultivar (Gombau et al., 2020) or maybe to the young age of the Grenache vineyard from which the grapes were harvested. It is noteworthy than anthocyanins content and colour characteristics in CM differs between different studies, some authors described these wines with high phenolic content and greater or similar colour intensity than

conventional wines (González-Arenzana et al., 2020). However traditionally CM wines has been described as wines with less intense colour and less total phenolic compounds and anthocyanins (Portu et al., 2023).

In terms of microbiological treatments, the presence of *T. delbrueckii* during the CM phase significantly increased the concentration of anthocyanins in wines, particularly in TdV wines. This effect could be related with an oxidation of anthocyanins in Control wines due to the fact that the AF took longer to start (Figure 1). Furthermore, the high anthocyanins content of wines fermented in presence of *T. delbrueckii* have been previously documented in traditional red wines with this specific TdV strain (Balmaseda et al., 2021b), as well as with other strains (Chen et al., 2018; Escribano-Viana et al., 2019; Minnaar et al., 2018). This phenomenon may be attributed to the elevated pectolytic activity observed in certain non-*Saccharomyces* yeast, such as *Metschnikowia pulcherrima*, (Belda et al., 2016a) however it has not been proved in *T. delbrueckii*.

After AF the wines produced with TdP and TdV still maintained a higher proportion of free anthocyanins compared to the Control wine (Table 2). However, when comparing the concentration of anthocyanins after CM and after AF, it had a decreasing trend in the presence of *T. delbrueckii*, with a higher reduction observed in TdV wine. This effect was not observed in Control wine after AF. As has been reported previously, an AF with *T. delbrueckii* without the presence of grape skins can lead to a decrease in anthocyanin levels, as observed in rosé wines (Ruiz-de-Villa et al., 2024a). This reduction could be attributed to the formation of aglycones from anthocyanins, which are susceptible to oxidation (Vidana Gamage et al., 2022), or it could be due to the high β-glucosidase activity of certain *T. delbrueckii* strains (Maturano et al., 2012). Additionally, it could be related to the absorption of pigments, which has been shown to vary depending on the yeast species or strain (Morata et al., 2003; Tofalo et al., 2021).

Anthocyanidin-3-O-monoglucosides and other free anthocyanin concentrations, determined by HPLC-DAD, exhibited a similar trend to that reported for total anthocyanins measured using spectrophotometry. This correlation was anticipated, as spectrophotometric analysis includes the detection of other pigments, potentially leading to an overestimation of the total anthocyanin concentration. Conversely, HPLC-DAD methods solely detect free anthocyanins (Rivas-Gonzalo et al., 1992).

In terms of pyranoanthocyanins (Vitisin A and Vitisin B), significant differences were observed. In Control samples at the end of CM were not detect any pyranoanthocyanins, while they were detected in TdP and TdV samples after CM. However, after AF, pyranoanthocyanins were detected in all wines, with a higher concentration in TdP and TdV wines. It was also observed that Td wines after AF, the concentration decreased in comparison to Td samples after CM. It worth noting that in rosé wines it has been observed previously a higher proportion of pyranoanthocyanins in wines fermented with *T. delbrueckii* compared to wines fermented with *S. cerevisiae* (Ruiz-de-Villa et al., 2024a). These pigments derived contribute to improved colour stability in wines, as they are less affected by changes in pH and are less likely to experience discoloration due to the presence of sulphur dioxide (Fulcrand et al., 1997).

At the CM stage, there were no significant differences in CI among the different conditions, although there was a slight decreasing trend observed in the Control CM sample. However, after the completion of AF, Control wine exhibited a significant reduction in CI (Table 2). In this context, it was observed that Control wines exhibited significantly higher L* values and lower C* values compared to Td wines. This suggests that wines fermented with *T. delbrueckii*, regardless of the strain used, had a more intense and vibrant colour than the control samples, which is related to the higher concentration of anthocyanins. Regarding the H* coordinate, Control samples showed higher values than Td wines, indicating a more pronounced yellowish tone. This, along with the lower concentration of anthocyanins observed, is likely associated with an anthocyanin oxidation. Conversely, the Td wines exhibited the opposite trend. In terms of the a* coordinate, Td wines had higher values compared to the Control wines, indicating a stronger red component in both after CM and after AF, which could be also related with the higher concentration of anthocyanins. However, in terms of the b* coordinate, Td wines had lower values after CM, resulting in yellowish hues which agrees with high H* values. Nevertheless, there were no differences in the b* coordinate after AF.

Table 2. Phenolic compounds and colour parameters analysed. End of CM corresponds to the sampling after carbonic maceration, before to inoculate *S. cerevisiae*, and End of AF corresponds to the sampling after alcoholic fermentation. Control corresponds to the control fermentation; TdP and TdV corresponds to the fermentations with *T. delbrueckii* Prelude or *T. delbrueckii* Viniferm, respectively. Different lowercase letters indicate the existence of significant difference between samples at the end of CM (p-value < 0.05). Different uppercase letters indicate the existence of significant difference between samples at the end of AF (p-value < 0.05). All data is expressed as the arithmetic average of three biological replicates.

Phenolic compounds and colour parameters	End of CM			End of AF		
	CM Control	CM TdP	CM TdV	AF Control	AF TdP	AF TdV
Total anthocyanins (mg/L) quantified by spectrophotometry	61 ± 2 ^{a}	106 ± 4 ^{b}	118 ± 3 ^{b}	49 ± 3 ^{a}	114 ± 17 ^{b}	112 ± 7 ^{b}
Free anthocyanins (mg/L) quantified by HPLC	20 ± 7 ^{a}	85 ± 13 ^{bc}	97 ± 23 ^{c}	24 ± 2 ^{a}	69 ± 3 ^{bc}	66 ± 7 ^{c}
Pyranoanthocyanins (mg/L)	n.d	2.43 ± 0.10 ^{d}	2.46 ± 0.12 ^{d}	1.76 ± 0.01 ^{b}	2.08 ± 0.06 ^{c}	2.06 ± 0.03 ^{c}
L*	53 ± 2 ^{bc}	45 ± 1 ^{a}	45 ± 3 ^{a}	59 ± 1 ^{c}	48 ± 2 ^{ab}	48 ± 4 ^{ab}
C*	48 ± 2 ^{b}	51 ± 1 ^{b}	53 ± 5.63 ^{b}	33 ± 3 ^{a}	53 ± 4 ^{b}	52 ± 3 ^{b}
H*	46.2 ± 4.8 ^{c}	22.5 ± 6.4 ^{b}	23.0 ± 1.9 ^{b}	17.9 ± 0.4 ^{ab}	12.6 ± 1.7 ^{a}	11.1 ± 1.6 ^{a}
a*	33 ± 3 ^{a}	47 ± 3 ^{b}	50 ± 4 ^{b}	33 ± 2 ^{a}	49 ± 7 ^{b}	51 ± 3 ^{b}
b*	34.6 ± 2.8 ^{c}	19.3 ± 4.9 ^{b}	21.2 ± 0.1 ^{b}	10.6 ± 0.4 ^{a}	10.8 ± 1.9 ^{a}	9.9 ± 0.8 ^{a}
CI	2.45 ± 0.18 ^{b}	2.79 ± 0.09 ^{b}	2.76 ± 0.13 ^{b}	1.48 ± 0.34 ^{a}	2.55 ± 0.26 ^{b}	2.52 ± 0.23 ^{b}

3.4. Malolactic fermentation

After the end of CM and AF, three MLF strategies were implemented: inoculation with *O. oeni* strains OoVP41 and OoCH11, and spontaneous fermentation. At the end of AF, the initial LAB concentration was low to become a MLF ($2 \cdot 10^3$ CFU/mL) and the consumption of L-malic acid was also small (Table 1), suggesting that MLF did not have commenced yet. The purpose of inoculating the *O. oeni* starter cultures was to observe their potential under competitive pressure of endogenous LAB.

Figure 3 shows the MLF kinetics of the three wine conditions with their respective MLF strategies. The inoculation of OoVP41 resulted in a shorter MLF duration compared to OoCH11 and spontaneous MLF. Wines TdP and TdV, which underwent MLF in the presence of OoVP41, completed MLF two days earlier (8 days) than the control wines with the same MLF starter culture (10 days). Previous studies conducted under laboratory conditions (Ruiz-de-Villa et al., 2023b) have also described OoVP41 as a highly efficient fermentative strain. This study further demonstrates its successful performance under competitive conditions. The effect of *T. delbrueckii* on MLF was also observed with the use of the OoCH11 starter culture. However, in this case, the MLF duration was only reduced by one day (11 days vs. 12 days) without differences between strains.

The greater differences were found in spontaneous MLF, in the case of control wines they took to start the MLF 7 days while TdP took 4 days and TdV only 3 days. Regarding the total time MLF TdP and TdV were also shorter, Td P lasted 15 days and TdV 16 days, notably less time that the control, which lasted 20 days. It has been reported an interesting effect on spontaneous MLF. Furthermore, the most underlining differences were observed in spontaneous MLF. Control wines took 7 days to initiate MLF, whereas TdP took 4 days and TdV only took 3 days. Apart from bring forward the beginning of MLF, the total duration was also shorter in TdP (15 days) and TdV (16 days) compared to the control, which lasted 20 days.

The presence of *T. delbrueckii* has been associated with various factors that contribute to the enhanced efficiency of *O. oeni*. These factors include the reduction of inhibitory compounds such as succinic acid, SO_2, or MCFA, the mitigation of stressful conditions such as low pH or high ethanol content, and the increase of beneficial compounds like mannoproteins.

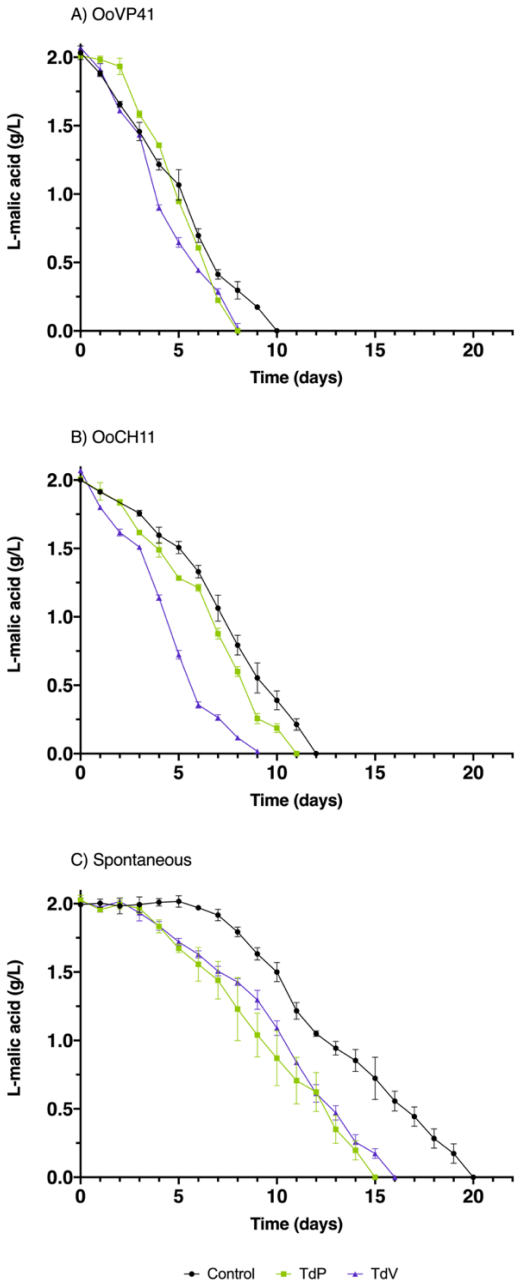

Figure 3. Consumption of L-malic acid during malolactic fermentation. A) corresponds to OoVP41 MLF; B) corresponds to OoCH11 MLF, and C) corresponds to Spontaneous MLF. Control corresponds to control condition only fermented with Sc, TdP and TdV corresponds to the fermentations with *T. delbrueckii* Prelude and Viniferm, respectively. All data is expressed as the arithmetic average of three biological replicates ± standard deviation (n=3).

However, in the present study, no differences were observed in ethanol levels or pH under these conditions. Therefore, the improved performance of MLF may be attributed to the reduction of MCFA (Figure 2), which is known to be toxic to *O. oeni* (Capucho and San Romao, 1994) or the increase in ammonia and mannoproteins (Table 1). Apart of a nutritional intake the increase of mannoproteins could be associated to the reduction of MCFA, since they could absorb these lipids detoxifying the media (Lafon-Lafourcade et al., 1984). In addition, previous research has linked increased mannoprotein levels to improved MLF performance, being observed that the relative expression of certain *O. oeni* genes involved in mannose uptake and other sugars was elevated in *T. delbrueckii* wines (Balmaseda et al., 2021a). These authors also described that the metabolism of mannoproteins is more active under stressful conditions. Therefore, it is possible that in this study, the metabolism of mannoproteins was more activated due to the high concentrations of anthocyanins compared to the Control. Certain phenolic compounds can impose stress on *O. oeni*, but not all of them do. Interestingly, previous studies have reported an improvement in MLF even under higher polyphenolic conditions than the control in traditional red winemaking methods (Balmaseda,et al. 2021b). Thus, it is noteworthy that the inoculation of *T. delbrueckii* in CM wines also promotes MLF, assisting *O. oeni* in adapting to challenging conditions.

3.5. Organoleptic characteristics

To better understand the effects of *T. delbrueckii* treatment during CM on wines, a sensory analysis was conducted after AF and MLF. Initially, a triangle test was performed, comparing Control wines to TdP, Control wines to TdV, and TdP to TdV. The results revealed significant differences between Control and TdP, as well as between Control and TdV. However, the panel of tasters was unable to distinguish between TdP and TdV. Regarding the MLF comparison, the three MLF conditions (Spontaneous, OoVP41, and OoCH11) were evaluated for each AF condition, but the tasters were unable to differentiate between the three MLF conditions in any of the wines. Consequently, a descriptive test was conducted exclusively on wines after AF.

The PCA represented in figure 5A shows a discrimination of the three wines according of the results obtained in the descriptive test. All variables are correlated positively with *T. delbrueckii* strains, especially with TdV.

In figure 5B the same trend in some of the analysed parameters is observed. For instance, TdV exhibited pronounced red fruit aroma, grass aroma, banana aroma, and higher values in overall perception. In the case of TdP, the red fruit and grass aromas were similar to those of the control wines, while the banana aroma and overall perception were improved compared to the Control wine. Among these parameters, the banana aroma was the only one that showed a significant difference between *T. delbrueckii* wines and Control wine (Figure 5B). Thus, it can be concluded that consumers associated a stronger banana aroma with the presence of *T. delbrueckii* during CM. It is noticeable that testers had preference for TdV wines (Verdugo-Vásquez et al., 2023).

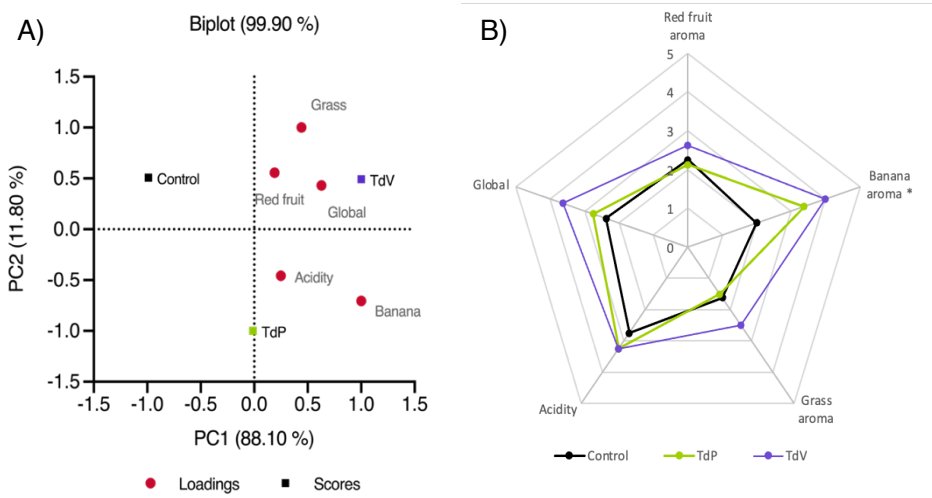

Figure 5. A) Principal component analysis biplots built from the following variables: Red fruit, grass, banana, acidity and global perception. B) Spiderweb diagram for sensory analysis of CM wines after AF. Asterisks (*) indicate attributes that showed significant differences (p-value < 0.05). The samples correspond to wines after alcoholic fermentation. Control corresponds to control condition at the end of alcoholic fermentation; TdP and TdV correspond to conditions inoculated with *T. delbrueckii* Prelude or *T. delbrueckii* Viniferm, respectively.

4. Conclusions

In this study, we investigated the impact of two *T. delbrueckii* strains (TdP and TdV) on the MLF performance and sensory characteristics in carbonic maceration wines. Then tree strategies of MLF were tested: the inoculation of two *O. oeni* strains (OoVP41 and OoCH11) and a spontaneous MLF. While the general physicochemical parameters did not exhibit significant differences between conditions, the organoleptic parameters showed noteworthy changes with the presence of *T. delbrueckii.* When this species was introduced during CM, it resulted in wines with enhanced anthocyanin content and a distinct volatile profile. Notably, *T. delbrueckii* strains contributed to significantly higher levels of the aroma compound isoamyl acetate, a key aroma in carbonic maceration wines. The TdP strain, in particular, led to even higher concentrations of this aroma compared to the TdV strain. A sensory evaluation panel also discerned differences between the treatments, with TdV wines exhibiting more pronounced aromas of red fruit, banana and grass.

Regarding MLF, the presence of T. delbrueckii significantly improved the performance, especially in cases of spontaneous MLF, where the fermentation started earlier and required less time to complete. Moreover, the inoculation of OoVP41 also contributed to a two-day reduction in MLF duration. These findings underscore the potential benefits of utilizing *T. delbrueckii* strains for enhancing MLF efficiency and overall sensory attributes in CM wines. In regions with increased acidity due to climate change, particularly in the new north regions, the inoculation of LAB becomes crucial to ensure complete malic acid degradation and maintain the desired wine quality. This is of particular importance in the context of CM vinification. By strategically employing these strains, winemakers can optimize the MLF process and enrich the sensory profile of carbonic maceration wines, providing an avenue for producing wines of enhanced quality and distinctive characteristics.

Acknowledgements

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Supplementary data

Table SD1. Citric and acetic acid analysed after MLF. Control corresponds to the control fermentation; TdP and TdV corresponds to the fermentations with *T. delbrueckii* Prelude or *T. delbrueckii* Viniferm, respectively. Oo-VP41, OoCH11 and Spontaneous correspond to MLF performed with the strain Vp41, the strain CH11 and endogenous LAB. Different lowercase letters indicate the existence of significant difference between samples at the end of CM ($p < 0.05$). Different uppercase letters indicate the existence of significant difference between samples at the end of AF (p-value < 0.05). All data is expressed as the arithmetic average of three biological.

MLF condition	AF condition	Citric acid	Acetic acid
OoVP41	Control	0.25 ± 0.02	0.50 ± 0.08
	TdP	0.20 ± 0.03	0.51 ± 0.04
	TdV	0.22 ± 0.04	0.38 ± 0.07
OoCH11	Control	0.23 ± 0.03	0.54 ± 0.12
	TdP	0.26 ± 0.03	0.39 ± 0.06
	TdV	0.24 ± 0.06	0.37 ± 0.08
Spontaneous	Control	0.21 ± 0.02	0.63 ± 0.02
	TdP	0.19 ± 0.01	0.57 ± 0.03
	TdV	0.20 ± 0.03	0.48 ± 0.07

Table 2. Total volatile compounds analysed (mg/L). End of CM corresponds to the sampling after carbonic maceration, before to inoculate *S. cerevisiae*, and End of AF corresponds to the sampling after alcoholic fermentation. Control corresponds to the control fermentation; TdP and TdV corresponds to the fermentations with *T. delbrueckii* Prelude or *T. delbrueckii* Viniferm, respectively. All data is expressed as the arithmetic average of three biological replicates.

	End of CM			End of AF		
	CM Control	CM TdP	CM TdV	AF Control	AF P	AF V
Isobutyl acetate	5.01 ± 0.39	4.64 ± 0.76	4.26 ± 0.58	3.83 ± 0.81	2.79 ± 0.30	2.53 ± 0.87
Isoamyl acetate	0.62 ± 0.12	0.63 ± 0.12	0.47 ± 0.06	0.57 ± 0.09	3.37 ± 0.11	1.32 ± 0.12
2-fenylethanol acetate	0.02 ± 0.04	0.33 ± 0.19	0.21 ± 0.14	0.09 ± 0.03	0.27 ± 0.04	0.63 ± 0.23
Σ Fusel alcohols acetates	5.65 ± 0.45	5.61 ± 0.78	4.94 ± 0.78	4.50 ± 0.87	6.43 ± 0.22	4.49 ± 1.16
Ethyl butanoate	0.28 ± 0.02	1.02 ± 0.19	0.63 ± 0.24	0.47 ± 0.19	0.47 ± 0.08	0.53 ± 0.03
Ethyl hexanoate	0.95 ± 0.26	1.38 ± 0.17	0.89 ± 0.14	0.95 ± 0.20	0.87 ± 0.06	n.d
Ethyl octanoate	3.53 ± 0.01	3.62 ± 0.61	3.21 ± 0.58	2.72 ± 1.16	2.21 ± 0.45	1.85 ± 0.29
Ethyl decanoate	0.30 ± 0.01	0.29 ± 0.13	0.14 ± 0.04	0.58 ± 0.23	1.32 ± 0.08	1.51 ± 0.39
Diethyl butanedioate	n.d	n.d	n.d	0.73 ± 0.03	1.60 ± 0.27	0.50 ± 0.01
Ethyl dodecanoate	0.17 ± 0.04	0.66 ± 0.08	0.29 ± 0.03	1.24 ± 0.27	5.11 ± 0.40	4.32 ± 0.44
Σ Esters de FA	5.2 ± 0.3	6.9 ± 0.7	5.2 ± 0.3	6.7 ± 0.9	11.6 ± 0.9	8.2 ± 0.1
2-metil-propanol	12.8 ± 2.1	19.8 ± 3.2	20.9 ± 1.4	16.3 ± 1.9	49.9 ± 4.0	34.0 ± 3.6
1-propanol	1.11 ± 0.25	1.31 ± 0.42	2.53 ± 1.65	6.02 ± 1.47	10.49 ± 1.32	12.26 ± 1.43
Isoamyl alcohol	2.53 ± 0.21	2.38 ± 0.32	1.21 ± 0.59	1.44 ± 1.44	1.90 ± 0.29	1.25 ± 0.38
1-pentanol	0.25 ± 0.04	0.20 ± 0.19	0.37 ± 0.12	0.24 ± 0.01	0.29 ± 0.09	0.31 ± 0.01
1-hexanol	0.72 ± 0.03	0.67 ± 0.09	0.56 ± 0.16	0.32 ± 0.32	0.33 ± 0.04	0.56 ± 0.24
cis-3-hexen-1-ol	0.10 ± 0.01	0.14 ± 0.24	0.44 ± 0.03	0.20 ± 0.20	n.d	n.d
2-pheniletanol	5.67± 0.9	15.9 ± 2.7	9.7 ± 0.3	26.9 ± 5.3	56.4 ± 2.4	44.3 ± 6.0
Σ Fusel alcohols	24.0 ± 2.9	41.8 ± 5.1	36.6 ± 0.2	52.4 ± 0.2	120.2 ± 5.7	92.7 ± 5.5

2-butanol	0.18 ± 0.01	0.32 ± 0.03	0.12 ± 0.12	0.25 ± 0.05	0.43 ± 0.12	0.46 ± 0.17
1-octanol	4.35 ± 0.14	3.51 ± 0.72	1.89 ± 0.46	2.48 ± 0.21	2.34 ± 0.75	3.07 ± 0.35
Σ Other alcohols	4.53 ± 0.14	3.83 ± 0.71	2.01 ± 0.34	2.74 ± 0.16	2.77 ± 0.75	3.53 ± 0.51
Propionic acid	0.88 ±0.03	0.56 ± 0.48	n.d	n.d	n.d	n.d
Isobutyric acid	0.19 ± 0.05	0.23 ± 0.39	0.29 ± 0.29	0.69 ± 0.69	0.78 ± 0.24	1.12 ± 0.37
Butyric acid	0.41 ± 0.07	0.33 ± 0.03	0.45 ± 0.16	0.54 ± 0.09	0.51 ± 0.04	1.14 ± 0.23
Σ SCFA	1.48 ± 0.13	1.12 ± 0.07	0.74 ± 0.13	1.22 ± 0.78	1.29 ± 0.26	2.26 ± 0.33
Octanoic acid	0.06 ± 0.01	0.24 ± 0.05	0.13 ± 0.03	0.15 ± 0.04	0.06 ± 0.01	0.17 ± 0.05
Decanoic acid	2.15 ± 0.13	2.40 ± 0.53	2.37 ± 0.51	1.88 ± 0.35	0.62 ± 0.13	0.87 ± 0.19
Dodecanoic acid	0.05 ± 0.05	0.07 ± 0.03	0.02 ± 0.02	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.01
Σ MCFA	2.26 ± 0.13	2.72 ± 0.55	2.52 ± 0.56	2.07 ± 0.37	0.72 ± 0.14	1.09 ± 0.25

UNIVERSITAT ROVIRA I VIRGILI
NEW PERSPECTIVES ON MALOLACTIC FERMENTATION AND ORGANOLEPTIC IMPROVEMENT OF WINES: INFLUENCE OF TORULASPORA DELBRUECKII ON DIFERENT TYPES OF WINEMAKING
Candela Ruiz de Villa Sardón

Section 2.3

Influence of skin-fermentation and *Torulaspora delbrueckii* inoculation on white wine production: changes in fermentation dynamics and wine composition

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Manuscript in preparation

Abstract

In current white wine production innovative techniques such as extended skin contact and malolactic fermentation (MLF) are garnering attention. In this work it was been proposed the application of *Torulaspora delbrueckii* in skin-fermented white wines using a sequential inoculation strategy alongside *Saccharomyces cerevisiae*. Furthermore, the study also explores the influence of varying vinification temperatures on the dynamics of fermentation. Results reveal interesting variations in the duration and initiation of AF. Furthermore, the investigation of MLF dynamics highlights the crucial role of *T. delbrueckii* and its interaction with *Oenococcus oeni* since a spontaneous MLF occurred in presence of this yeast specie. In addition, significant differences in relation to the metabolome were observed with the influence of all factors: the presence of skins, the presence of *T. delbrueckii* and the temperature. These factors produced significant changes in compounds such as phenolic acids, volatile compounds and the presence of 2-isopropylmalic acid, providing a better understanding of the interaction of factors that define the unique profile of the resulting wines.

Keywords

Oenococcus oeni; *Saccharomyces cerevisiae*; phenolic acids; organic Krebs acids, orange wine

1. Introduction

White wine vinification offers a wide range of possibilities, with evolving trends moving from fresh and youthful styles to aged expressions. Winemakers are increasingly exploring alternative techniques, such as aging in barrels, ceramic amphorae or concrete egg-shaped vessels, to produce value-added products (del Alamo-Sanza and Nevares, 2018; Gil i Cortiella et al., 2020). These methods can impart distinctive characteristics and contribute to the complexity of white wines. Another interesting practice in white wine vinification is the intentional contact of fermenting grape must with the grape skins. While the traditional approach involves only brief skin contact for hours or days, an alternative is to extend the duration of skin contact during the alcoholic fermentation (AF) (Kemp et al., 2021; Lorteau, 2018). This approach similar to the vinification of red wines, leads to the production of orange wines, also known as skin-fermented white wines.

There is a growing consumer appreciation for orange wines (Bene and Kállay, 2019), since this extended skin contact results in a deeper colour and a more textured and tannic profile compared to traditional white wines (Jackson, 2008). Moreover, it allows the expression of varietal components that are concentrated in the grape skins and seeds, emphasizing wine distinctive characteristics (Singleton et al., 1975). Despite these wines becoming widely popular in the last decades and spreading through a lot of winemaking regions, their origin can be traced back 8000 years ago to the Republic of Georgia (Glonti, 2001). The practice involved fermenting white wines with the skins for up to six months in large egg-shaped earthenware vessels called *Qvevri* (Bene and Kállay, 2019; Glonti, 2001). However, currently the regulation of orange wines is limited to four countries: Georgia, the United States of America, South Africa and the Canadian province of Ontario. However, only the latter two countries have established statutory criteria to define these wines (Lorteau, 2018). The production of orange wines offers winemakers the opportunity to experiment with different grape varieties and achieve a distinct flavour profile. Aromatic varieties such as Riesling, Gewurztraminer or Muscat are suitable for producing orange wines due to the high levels of free and glycosidically bound compounds present in their skins (Kemp et al., 2021; Sokolowsky et al., 2013).

Furthermore, white wine vinification is changing in another aspects, for instance, malolactic fermentation (MLF) in white wines has been rarely conducted, primarily limited to certain Chardonnay wines (Semon et al., 2001). However, the current trends towards producing more complex wines and the impact of climate change have led to an increased adoption of MLF in white wine production. As winemaking regions shift towards northern latitudes, they are characterized by cooler climates and higher acidity levels (Gutiérrez-Gamboa et al., 2021). As a result, MLF may become necessary in these regions to achieve desired wine characteristics.

Microorganisms play a crucial role in the production of white wine, and non-*Saccharomyces* yeast strains have gained significant attention for their potential to enhance the sensory characteristics of wines. Non-*Saccharomyces* are particularly used to improve the varietal aromatic profile of wines, which is of great importance in white wines (Canonico et al., 2019; Ciani et al., 2010; Jolly et al., 2014; Oliveira and Ferreira, 2019; Puertas et al., 2017; Roudil et al., 2019).

Among the non-*Saccharomyces* species, *Torulaspora delbrueckii* stands out as an interesting choice. It has been demonstrated to possess the ability to enhance the aroma profile of white wines (Azzolini et al., 2015; Belda et al., 2015; Renault et al., 2015; Velázquez et al., 2015). Furthermore, *T. delbrueckii* has been found to contribute to the improvement of MLF performance carried out by the lactic acid bacteria (LAB) *Oenococcus oeni* (Balmaseda et al., 2023). The use of starters cultures of *T. delbrueckii* in white or orange wines could be an interesting option to preserve and improve the organoleptic typicity of these wines apart from assure a good microbiological control.

Within this context, given the limited literature on skin-fermented or orange wines and MLF in white wines, this research introduces a novel perspective on the utilization of *T. delbrueckii* in Skin-fermented white wines. It proposes a sequential inoculation approach employing *T. delbrueckii* and *S. cerevisiae* to optimize the fermentation performance of both AF and MLF, thereby enhancing the overall organoleptic profile of the wines. As a secondary objective, the vinification process has been evaluated at two distinct temperatures to investigate the resulting changes in oenological characteristics.

2. Materials and methods

2.1. Alcoholic fermentation

The AF conducted in this study were performed with Muscat of Alexandria grape cultivar (*Vitis Vinifera* L.). The grapes were sourced from the experimental winery Mas dels Frares, affiliated with the *Rovira i Virgili* University, located within the Tarragona designation of origin (DO Tarragona). The entire grape process, including harvesting, processing and AF, took place at the winery. The AF was carried out in 10 L food-grade plastic tanks. The initial parameters in the grape must were the following: pH 3.5, total acidity 4.5 g/L, α-amino nitrogen 65 mg/L, and ammonium 30 mg/L.

Two distinct vinification methods were employed. Firstly, half of the grapes underwent pressing, followed by clarification with Lallzyme-C-Max pectinases (Lallemand Inc., Montreal, Canada) at 4 °C and were vinified as a traditional white wine (W). Conversely, the other half of the grapes were vinified as skin-fermented wine/orange wine (S), where fermentation took place with the grape skins during maceration (Table 1).

Regarding the AF temperature, two different conditions were tested: a high temperature of 25 °C and a low temperature of 16 °C. Additionally, two microbiological treatments were implemented: a control condition using *S. cerevisiae* as the sole inoculant, and a sequential condition involving the inoculation of *T. delbrueckii* followed by *S. cerevisiae* after a four-day interval. The yeast strains used in the study were QA23 (Sc, from Lallemand S.A, Montreal, Canada) for the control condition and Prelude Vinoflora (TdP) for the sequential condition (Chr. Hansen Holding AS, Hoersholm, Denmark). Both active dry yeasts were rehydrated following the instructions provided by the manufacturer to achieve an initial population of 2 x 10^6 cells/mL. The *S. cerevisiae* strain was rehydrated at a temperature of 37 °C for 30 minutes, while the *T. delbrueckii* strains were rehydrated at 30 °C for the same duration. Three biological replicates were performed.

Monitoring of AF progress involved daily density measurements using the Densito 30PX Portable Density Meter from Mettler Toledo, Spain. AF was considered complete when the concentration of reductive sugars reached below 2 g/L. To evaluate the inocula and

population dynamics, two different media were employed: YPD agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 17 g/L agar from Panreac Química SLU, Castellar del Vallés, Spain) was used for total yeast assessment, while the selective medium Lysine (BDDifco, Massachusetts, United States) was utilized to evaluate non-*Saccharomyces* yeasts.

Table 1. Conditions tested in the study.

	Temperature	Vinification	Yeast
H-S-Sc	High (25 °C)	Skin-fermented wine	*S. cerevisiae*
H-S-Td	High (25 °C)	Skin-fermented wine	*T. delbrueckii + S. cerevisiae*
L-S-Sc	Low (16 °C)	Skin-fermented wine	*S. cerevisiae*
L-S-Td	Low (16 °C)	Skin-fermented wine	*T. delbrueckii + S. cerevisiae*
H-W-Sc	High (25 °C)	White wine	*S. cerevisiae*
H-W-Td	High (25 °C)	White wine	*T. delbrueckii + S. cerevisiae*
L-W-Sc	Low (16 °C)	White wine	*S. cerevisiae*
L-W-Td	Low (16 °C)	White wine	*T. delbrueckii + S. cerevisiae*

2.2. Malolactic fermentation

Following AF, MLF was carried out in all conditions. The replicates of each condition were combined and stored at a temperature of 4 °C for one week to allow for stabilization. The final concentration of L-malic acid was adjusted to achieve an initial concentration of 2 g/L, and the pH was readjusted to its value prior to the addition of L-malic acid. Subsequently, MLF was performed in 500 mL volumes at a temperature of 20 °C under anaerobic and static conditions. The consumption of L-malic acid was monitored daily until its concentration dropped below 0.1 g/L, when the fermentation was considered finished, using the Y15 Enzymatic Autoanalyzer (Biosystems S.A, Barcelona, Spain).

The conditions were divided into triplicates once again to perform three MLF conditions: two inoculated with *Oenococcus oeni* and one spontaneous MLF. The two *O. oeni* strains used were Lalvin VP41 (Oo-VP41) from Lallemand S.A. and PSU-1 (ATCC BAA-331). Each *O. oeni* strain was inoculated to achieve an initial population of 2×10^7 cells/mL. The strains were pre-cultured in an MRS broth medium (De Man et al., 1960) (from Difco Laboratories, Detroit, MI, USA) supplemented with 4 g/L DL-malic acid (Sigma-Aldrich) and 5 g/L D-fructose (Panreac)

at a pH of 5. The inocula were incubated at 28 °C in a CO_2 (10%) incubator. The inocula and populations of *O. oeni* were monitored by plating on modified MRS medium, as described in (Margalef-Català et al., 2017). The modified MRS medium had in addition 100 mL/L of centrifugated tomato juice (Aliada, Madrid, Spain), 100 mg/L of nystatin (Panreac) to prevent yeast growth, and 25 mg/L of sodium azide (BioSciences, St. Louis MO, USA) to prevent acetic acid bacteria growth. The plates were also incubated at 28 ºC in a 10% CO_2 atmosphere.

2.3. Microorganism identification

2.3.1. Yeast identification

Apart from using YPD and lysine medium to differentiate yeast species the species were also identified based on the amplicon size of the ITS-5.8S rDNA region (Esteve-Zarzoso et al., 1999). Twenty colonies were isolated from must before *T. delbrueckii* or *S. cerevisiae* inoculation, must before inoculating *S. cerevisiae* in sequential AF and wine at the end of AF.

2.3.2. LAB identification and typing of *Oenococcus oeni*

First, ten random colonies for the inoculated MLF wines and 20 for spontaneous MLF wines were isolated. DNA of colonies was extracted with a High Pure PCR Template Preparation Kit (Roche, Barcelona, Spain). LAB isolates were confirmed to be *O. oeni* by the species-specific PCR (Zapparoli et al., 1998). *O. oeni isolated* were typed by the multilocus variable number tandem repeat (VNTR) (Claisse and Lonvaud-Funel, 2014). For the DNA extraction was followed the procedure described in Balmaseda et al. (2021b) based on Claisse and Lonvaud-Funel (2012). Samples were analysed by using capillary electrophoresis by Eurofins Genomics Europe (Edersberg, Germany).

2.3.3. General parameters

The pH of the wines was measured using a Crison micro pH 2002 pH-meter (Hach Lange Spain in L'Hospitalet, Spain). Acetic acid, L-malic acid, L-lactic acid, D-lactic acid, residual glucose and fructose were analysed using the Y15 Enzymatic Autoanalyzer provided by Biosystems S.A in Barcelona (Spain).

To estimate the amount of mannoproteins, the mannoproteins were first precipitated using 95% ethanol. Subsequently, an acid hydrolysis with sulphuric acid 5 M at a temperature of 90 °C was carried out, which resulted in the release of mannose. The released mannose was then analysed following the procedure outlined in (Balmaseda et al., 2021a). The quantification of mannose equivalents was performed using a D-mannose and D-glucose enzymatic assay kit provided by Megazyme.

For the analysis of citric acid, succinic acid, glycerol and ethanol, an Agilent 1100 HPLC system manufactured by Agilent Technologies in Waldbronn, Germany was utilized. The method described by (Quirós et al., 2014) was followed. Before injection, the wine samples were filtered using 0.22 µm pore filters from Merck. The HPLC system consisted of a Hi-Plex H column (300 mm x 7.7 mm) housed within a 1260 MCT (Infinity II Multicolumn Thermostat). The system incorporated two detectors: a MWC detector (Multi-wavelength detector, Agilent Technologies) and a RID detector (1260 Infinity II refractive index detector, Agilent Technologies).

2.4. Volatile composition

The volatile compounds present in the wines after AF were analysed following the procedure outlined in Ruiz de Villa et al. (2024a). In summary, the samples underwent a pre-treatment process involving liquid-liquid extraction using a mixture of methyl tert-butyl ether and hexane (1:1) with 5 mL of wine. For this analysis, 25 µL of three internal standards were used (3-octanol, 1.98 g/L; heptanoic acid, 3.33 g/L and heptadecanoic acid, 1.03 g/L). The organic phase obtained after separation was injected into a gas chromatograph with flame ionization detection (GC-FID). The GC-FID analysis was carried out under the following chromatographic conditions: an injection volume of 2 µL, a spitless injection mode, an inlet temperature of 250 °C, a detector temperature of 250 °C, and a HP-FFAP column (30 m x 250 µm, 0.25 µm, Agilent) was used. The concentrations of the volatile compounds were quantified using known external standards and calibration curves.

The identified volatile compounds were acetates of fusel alcohols such as isobutyl acetate, isoamyl acetate, and 2-phenylethanol acetate; fusel alcohols such as amyl alcohol, isoamyl alcohol, hexanol, cis-3-hexenol, and 2-phenylethanol; ethyl esters of fatty acids (FA) such as ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl dodecanoate, ethyl

lactate, and diethyl succinate; short-chain fatty acids (SCFA) including propanoic acid, butyric acid, isobutyric acid (2-methylpropanoic acid), pentanoic acid (valeric acid), and isovaleric acid (3-methylbutanoic acid); medium-chain fatty acids (MCFA) such as hexanoic acid, octanoic acid, decanoic acid, and dodecanoic acid; and long-chain fatty acids (LCFA) including tetradecanoic acid (myristic acid), palmitic acid, palmitoleic acid, stearic acid, oleic acid, and linoleic acid.

2.5. Metabolomic analysis

Wine metabolite compounds were determined by GC-MS. The methodology will not be explained because it is undergoing the process of publication. However, the metabolites were extracted by an organic solvent and then derivatized to make them volatiles and allow their identification in GC-MS. Identification of metabolites was performed using the NIST17 library and confirmed by an own library (injection of pure standards). For approximately 150 metabolites detected by GC-MS and identified by libraries, we only used metabolites of greater than 70% quality, to a final number of 64 compounds used. The results were normalized by calculating the ratio of the area of each identified metabolite to the area of the internal standard. Three biological replicates were performed.

2.6. Sensory analysis

A trained tasting panel comprising 12 tasters conducted both a blind triangle test and a descriptive sensory analysis. The purpose of the triangle test was to identify any discernible differences between the wines produced using different microbiological treatments: *S. cerevisiae* alone versus the sequential inoculation of *T. delbrueckii* and *S. cerevisiae*. Three wines were compared in a blind manner during this test. Following the triangle test, a descriptive sensory analysis was carried out on the wines that exhibited significant differences according to the results of the triangle test. In this descriptive test, the tasters evaluated the intensity of five specific attributes using a numerical scale ranging from 0 to 5. These attributes included pear, tropical and grass aroma, acidity, bitterness, and global perception. The tasters provided ratings based on their perception of the intensity of each attribute in the wines.

2.7. Statistical analysis

To ensure the consistency of the results, both AF and MLF processes were carried out in biological triplicates. For the statistical analysis of the obtained data, the ANOVA method and the Tukey test were employed. These analyses were performed using XLSTAT 2022.2.3 software (Addinsoft, Paris, France). A *p*-value threshold of less than 0.05 was chosen to determine statistical significance. To process the sensory analysis data, Panel Check software was utilized.

A principal component analysis (PCA) was also performed to describe the relationship between the main metabolites analysed and the wine conditions. The main metabolites used were the following (numbers correspond to numbers in the PCA, Figure 2): 4, Lactic acid; 12, Pyruvic acid; 20, Succinic acid mono ethyl ester; 32, Malic acid 1-ethyl ester; 33, Malic acid 4-ethyl ester; 39, Tyrosol; 40, 2-Isopropylmalic acid; 43, Tartaric acid ethyl ester; Krebs, sum of organic acids in the Krebs's cycle; Phenols, sum of phenolic acids. Also, a 3-way ANOVA was performed to describe the effect of the three factors used in the experimental design and their interaction (yeast, type of vinification and fermentation temperature) on the family of metabolites such as Krebs organic acids, total phenolic acids or ethyl esters of organic acids.

Finally, Analysis of Variance (ANOVA)-Simultaneous Component Analysis (ASCA) was used to decompose the variability sources affecting volatile and metabolomic data. ASCA is a multivariate extension of ANOVA, which decomposes the variation in the data into the main effects and their binary combinations, obtained from a predefined experimental design (Smilde et al., 2005). In this study, three variability factors were considered: yeast species, vinification style, fermentation temperature, and the interactions between them. A permutation test of 10000 iterations was performed in each ASCA model to assess the significance of each factor (a *p*-value under 0.05 means the factor is significant), (Bertinetto et al., 2020).

3. Results and Discussion

3.1. Alcoholic fermentation

The successful completion of the AF was observed across all conditions, as depicted in Figure 1. However, noteworthy variations were observed among the different experimental setups. As expected, sequential fermentations exhibited longer durations compared to the control

group (Ruiz-de-Villa et al., 2023a). This disparity can be attributed to the presence of the two different yeast species, which engage in a competitive relationship for essential nutrients (Roca-Mesa et al., 2020). Interestingly, the difference between sequential and control fermentations became more pronounced in the case of white wine at high temperature H-W-Sc compared to H-W-Td. It was also observed that skin-fermented wines initiated the AF earlier, regardless of the yeast combination or temperature (Figure 1). In contrast, white wines experienced a lag phase during the initial three days of fermentation, indicating a slower start. It could be explained since the presence of skins could enhance the yeast fermentative activity due to the content of lipids present in the grape skin (Le Fur et al., 1994; Santos et al., 2011; Tumanov et al., 2015). It has been described that some fatty acids (FAs) such as linoleic acid, which yeast are able to assimilate (Thurston et al., 1981) increasing their viability (Beltran et al., 2008). Furthermore, sterols, such as β-sitosterol, major phytosterol of grape can be also incorporate by yeast for growth (Luparia et al., 2004).

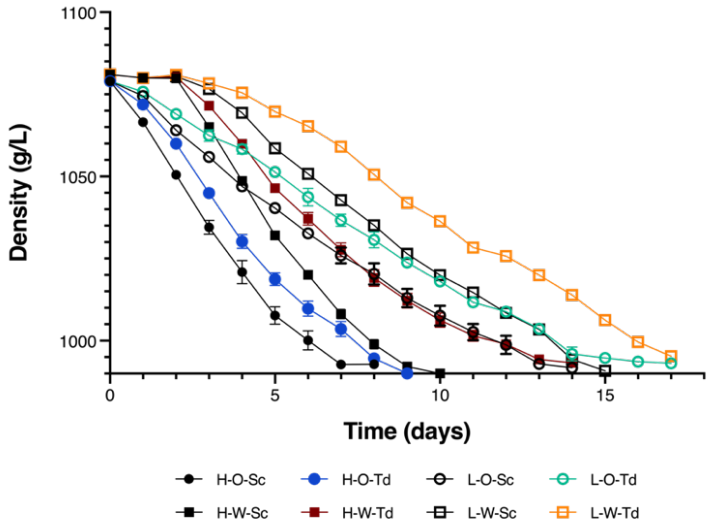


Figure 1. Kinetic of alcoholic fermentation. Wines: 25 °C corresponds to high temperature of fermentation, 16 °C corresponds to low temperature of fermentation, Skin-fermented correspond to wines fermented in presence of grape skins, W correspond to white wines with racking, Sc corresponds to fermentation solely with *S. cerevisiae* and Td corresponds to sequential fermentation with *T. delbrueckii* and *S. cerevisiae*. All data is expressed as the arithmetic average of three biological replicates ± standard deviation (n=3).

Regarding temperature, it was found that fermentations conducted at high temperatures had shorter durations compared to those at lower temperatures. This observation aligns with previous literature, which suggests that 25 °C is the optimal temperature of *S. cerevisiae* (Heard and Fleet, 1988). Consequently, at lower temperatures, fermentation progresses slower.

Moreover, focusing on yeast populations (Fig 1B), grape must had an initial concentration of yeasts of 8.4 x 10^4 CFU/mL (total yeasts). In the case of sequential wines, *T. delbrueckii* was detected at more than 80% before inoculating *S. cerevisiae* (data not shown) and after AF remained a percentage of 30%. It is worth emphasizing that at lower temperatures, the percentage of *T. delbrueckii* present was higher in both orange and white wines, compared to higher temperatures. This indicates that lower temperatures provide a more favourable environment for the persistence of *T. delbrueckii*. On the other hand, studies have shown that *S. cerevisiae* gains an advantage over non-*Saccharomyces* yeast, such as *T. delbrueckii* at higher temperatures (Salvadó et al., 2011). Besides, it is interesting that in all conditions *T. delbrueckii* remained at high population at the end of the AF as it has been previously described (Roca-Mesa et al., 2020, Ruiz-de-Villa et al., 2024a).

3.2. Physicochemical parameters after alcoholic fermentation

3.2.1. General oenological parameters

Various physicochemical parameters were analysed following the completion of AF, as it is shown in Table 2. Notably, a spontaneous MLF was observed under specific conditions (L-S-Td, H-W-Td, and L-W-Td). This is evident from the data presented in Table 2, where L-malic acid consumption and subsequent L-lactic acid production were noted. Consequently, a slight increase in pH was produced. Regarding D-lactic acid, significant variations were found between conditions. Particularly noteworthy is the elevated concentration of D-lactic acid in H-W-Td wine, followed by L-S-Td wine. This behaviour is likely attributed to the heterofermentative metabolism of some LAB species, known to enzymatically synthesize D-lactic acid from sugars (Ribéreau-Gayon et al., 2006b). Additionally, a significant reduction in citric acid was observed in L-S-Td wines, possibly owing also to LAB metabolism, while no corresponding rise in acetic acid levels was detected. These results highlighted the presence of autochthonous LAB in the initial grape must in a population enough to carry on spontaneously MLF.

Glycerol exhibited an increase in skin-fermented wines. An observable increase in glycerol content was found in wines fermented with *T. delbrueckii* (H-S-Td and L-S-Td), being significant at low temperature. Similarly, an upward trend in *T. delbrueckii* wines was seen in white wines. This increase has previously described to the high glycerol-pyruvic pathway activity of *T. delbrueckii* (Belda et al., 2015), although strain-specific effects have also been (Loira et al., 2012). Interestingly, a comparison between skin-fermented and white wines revealed a lower trend in ethanol concentrations for the former. This effect was significant between H-S-Sc and H-W-Sc, and between L-S-Sc and L-W-Sc. Supporting this observation, Bene and Kállay (2019) reported lower values of alcohol in traditional *Qvevri* wine (skin-fermented white wine in an amphora) when comparing with a traditional white wine vinification.

In relation to mannoproteins, a notable increase was detected in wines fermented in the presence of *T. delbrueckii*. The intensified release of mannoproteins and their subsequent concentration in final wines in the context of non-*Saccharomyces*, particularly *T. delbrueckii*, has been previously reported across various wine types (Ruiz-de-Villa et al., 2024a; Balmaseda et al., 2022a; Belda et al., 2015). Additionally, another interesting trend appeared when comparing skin-fermented wines and white wines. Specifically, the L-S-Td condition exhibited a significant mannoprotein concentration, suggesting that the presence of *T. delbrueckii* and pomace during AF at low temperatures may promote the release of mannoproteins. The increase of mannoproteins is one of the factors that could favour the performance of the MLF, as we will see below.

3.2.2. Volatile composition of wines

The volatile composition of wines after AF was analysed for each biological replicate. The most interesting differences were found between the type of vinification: Skin-fermented wines and white wines. Notably, significant variations were observed within volatile families (Table 3). The presence of skins was associated with significantly higher levels of fusel alcohols. In contrast, the traditional white wine vinification process exhibited elevated concentrations of esters of FAs and acetates of fusel alcohols.

Table 2. Oenological parameters analysed. Wines: H corresponds to high temperature of fermentation, L corresponds to low temperature of fermentation, S correspond to wines fermented in presence of grape skins (Skin-fermented wines), W correspond to White wines with racking (White wines), Sc corresponds to fermentation solely with *S. cerevisiae* and Td corresponds to sequential fermentation with *T. delbrueckii* and *S. cerevisiae*. Different lowercase letters indicate the existence of significant difference between samples at the end of alcoholic fermentation (*p*-value < 0.05). All data is expressed as the arithmetic average of three biological replicates ± standard deviation (n=3).

	H-S-Sc	H-S-Td	L-S-Sc	L-S-Td	H-W-Sc	H-W-Td	L-W-Sc	L-W-Td
Citric acid (g/L)	0.34 ± 0.02 ^{ab}	0.24 ± 0.04 ^{a}	0.50 ± 0.09 ^{b}	0.26 ± 0.03 ^{a}	0.29 ± 0.10 ^{a}	0.28 ± 0.03 ^{a}	0.29 ± 0.04 ^{a}	0.23 ± 0.01 ^{a}
Succinic acid (g/L)	0.47 ± 0.06 ^{a}	0.48 ± 0.06 ^{a}	0.51 ± 0.02 ^{a}	0.39 ± 0.03 ^{a}	0.46 ± 0.02 ^{a}	0.50 ± 0.00 ^{a}	0.48 ± 0.03 ^{a}	0.53 ± 0.14 ^{a}
Acetic acid (g/L	0.21 ± 0.01 ^{a}	0.18 ± 0.01 ^{a}	0.44 ± 0.02 ^{c}	0.26 ± 0.04 ^{ab}	0.25 ± 0.06 ^{ab}	0.34 ± 0.01 ^{bc}	0.41± 0.05 ^{c}	0.41 ± 0.08 ^{c}
L-malic acid (g/L)	1.35 ± 0.09 ^{c}	0.95 ± 0.13 ^{b}	1.23 ± 0.11 ^{c}	0.16 ± 0.01 ^{a}	1.25 ± 0.05 ^{c}	n.d	1.25 ± 0.04 ^{c}	0.03 ± 0.02 ^{a}
L-lactic acid (g/L)	n.d	0.34 ± 0.30 ^{ab}	n.d	0.86 ± 0.44 ^{b}	n.d	1.51 ± 0.02 ^{c}	n.d	0.81 ± 0.11 ^{b}
D-lactic acid (g/L)	0.13 ± 0.01 ^{a}	0.13 ± 0.01 ^{a}	0.13 ± 0.05 ^{a}	0.32 ± 0.01 ^{b}	0.07 ± 0.01 ^{a}	0.49 ± 0.01 ^{c}	0.15 ± 0.04 ^{a}	0.09 ± 0.01 ^{a}
Glycerol (g/L)	5.89 ± 0.80 ^{abc}	7.25 ± 0.41 ^{bc}	5.56 ± 0.67 ^{ab}	7.55 ± 0.17 ^{c}	4.93 ± 0.97 ^{a}	5.97 ± 0.86 ^{abc}	4.86 ± 0.46 ^{a}	5.67 ± 0.41 ^{ab}
Ethanol (%v/v)	9.11 ± 0.37 ^{a}	9.43 ± 0.03 ^{ab}	9.71 ± 0.24 ^{abc}	9.77 ± 0.13 ^{bcd}	10.02 ± 0.22 ^{bcd}	9.70 ± 0.09 ^{abc}	10.34 ± 0.33 ^{d}	10.09 ± 0.13 ^{cd}
pH	3.37 ± 0.02 ^{bc}	3.33 ± 0.03 ^{b}	3.19 ± 0.01 ^{a}	3.42 ± 0.01 ^{c}	3.33 ± 0.01 ^{b}	3.40 ± 0.04 ^{c}	3.24 ± 0.04 ^{a}	3.40 ± 0.01 ^{c}
Eq-mannose (mg/L)	143 ± 18 ^{ab}	164 ± 19 ^{bc}	116 ± 8 ^{ab}	211 ± 17 ^{c}	114 ± 41 ^{ab}	117 ± 34 ^{ab}	94 ± 14 ^{a}	110 ± 8 ^{b}

While limited studies have studied the impact of skin presence during AF in white wines, certain authors have also observed an increase in fusel alcohols and a corresponding decrease in several ethyl esters when AF was performed in presence of skins in red grape varieties (Bertrand et al., 1983; Herraiz et al., 1900). Other researchers have explored the effects of pre-fermentative maceration in white wines. For instance, an investigation involving Muscat grape variety revealed that a 23-hour maceration at 18 ºC led to higher levels of both free and glycosylated aroma compounds (Sánchez Palomo et al., 2006). However, under fermentative conditions, the grape and yeast glycoside activity may not be sufficient. As a potential solution, the addition of glycosidic and pectolytic enzymes could enhance the release of varietal volatile compounds in skin-fermented white wines (Cabaroglu et al., 2003). Furthermore, significant differences were found in terms of SCFA and LCFA, which exhibited higher concentrations for skin-fermented wines. This phenomenon may be attributed to the presence of FAs in the skins of the grapes, which as it has been discussed above could potentially contribute to the improved performance of AF observed in these wines. Correspondingly, (Herraiz et al., 1990) also noted an increase in SCFA with the inclusion of grape skins during AF.

Temperature effects became evident in the case of SCFA and MCFA. Notably, SCFA concentrations increased at higher temperatures in both skin-fermented and white wines. Regarding MCFA, it exhibited a reduction in white wines fermented at lower temperatures with *S. cerevisiae* (L-W-Sc). In contrast, within skin-fermented wines, MCFA levels experienced an increase at lower temperatures.

Interestingly, the type of vinification and AF temperature appeared to influence the behaviour of *T. delbrueckii*. In white wines, regardless of temperature, the presence of *T. delbrueckii* notably increased SCFA content (H-W-Td and L-W-Td). However, in skin-fermented wines in presence of with *T. delbrueckii* (H-S-Td) diminish SCFA content at higher temperatures, while did not display any impact at lower temperatures (L-S-Td).

Table 3. Composition of wine in volatile compounds (mg/L). FAs correspond to Fatty Acids, SCFA correspond to Short-Chain Fatty Acids, MCFA correspond to Medium-Chain Fatty Acid and LCFA correspond to Long-Chain Fatty acid. Wines: H corresponds to high temperature of fermentation, L corresponds to low temperature of fermentation, S correspond to wines fermented in presence of grape skins (Skin-fermented wines), W correspond to White wines with racking (White wines), Sc corresponds to fermentation solely with *S. cerevisiae* and Td corresponds to sequential fermentation with *T. delbrueckii* and *S. cerevisiae*. Different lowercase letters indicate the existence of significant difference between samples at the end of alcoholic fermentation (*p*-value < 0.05). All data is expressed as the arithmetic average of three biological replicates ± standard deviation (n=3).

	H-S-Sc	H-S-Td	L-S-Sc	L-S-Td	H-W-Sc	H-W-Td	L-W-Sc	L-W-Td
Fusel alcohol acetates	0.83 ± 0.19 ^{ab}	1.37 ± 0.16 ^{abc}	1.08± 0.62 ^{ab}	0.49 ± 0.08 ^{a}	2.71 ± 0.19 ^{d}	1.80 ± 0.17 ^{bcd}	2.31 ± 0.20 ^{cd}	1.81 ± 0.67 ^{bcd}
Ethyl esters of FAs	2.42 ± 0.41 ^{ab}	1.56 ± 0.06 ^{a}	1.57 ± 0.11 ^{a}	3.25 ± 0.75 ^{bc}	4.36 ± 0.65 ^{c}	2.99 ± 0.15 ^{abc}	4.37 ± 0.23 ^{c}	3.31 ± 1.32 ^{bc}
Fusel alcohols	328 ± 16 ^{abc}	407 ± 13 ^{c}	354 ± 75 ^{bc}	298 ± 12 ^{ab}	264± 25 ^{ab}	264 ± 10 ^{ab}	269 ± 14 ^{ab}	254 ± 32 ^{a}
SCFA	37 ± 1 ^{e}	26 ± 2 ^{d}	18 ± 2 ^{bc}	19 ± 2 ^{c}	67 ± 1 ^{a}	23 ± 3 ^{cd}	6 ± 1 ^{a}	11 ± 3 ^{b}
MCFA	1.12 ± 0.21 ^{a}	0.83 ± 0.07 ^{a}	2.22 ± 0.33 ^{bc}	2.22 ± 0.12 ^{bc}	2.76 ± 0.27 ^{c}	1.48 ± 0.06 ^{ab}	1.03 ± 0.17 ^{a}	1.46 ± 0.88 ^{ab}
LCFA	9.6 ± 1.9 ^{bc}	7.1 ± 0.6 ^{ab}	11.4 ± 1.4 ^{c}	5.8 ± 0.1 ^{a}	8.3 ± 1.5 ^{abc}	6.6 ± 0.8 ^{ab}	7.2 ± 0.2 ^{ab}	5.5 ± 0.9 ^{a}

Notably, skin-fermented wines with *T. delbrueckii* at lower temperatures (L-S-Td) showed elevated esters content, accompanied by reduced LCFA levels, as compared to their respective controls (H-S-Td and L-S-Td). Furthermore, at elevated temperatures, *T. delbrueckii* inoculated wines displayed significantly lower MCFA concentrations, particularly pronounced in H-W-Td and displaying a decreasing trend in H-S-Td. Interestingly, at lower temperatures, wines fermented with *T. delbrueckii* (L-W-Td and L-S-Td) did not exhibit diminished MCFA values. It has been described that sequential fermentations with *T. delbrueckii* and *S. cerevisiae* reduce the concentration of MCFA in the final wine (Balmaseda et al., 2021b; Balmaseda et al., 2021d; Zhang et al., 2018), this reduction seems to depend on the strain and on the conditions (Balmaseda et al., 2023) probably related to the temperature of fermentation.

3.2.3. Metabolomic analysis

In order to better understand the effect that the three factors had in the resulting wines a metabolomic analysis was performed. First a principal component analysis (PCA) was carried out to describe which metabolites could contribute to the overall variation observed in the wines obtained under these conditions (types of vinification and inoculation, and fermentation temperature), (Figure 2). The following parameters were used to perform the PCA: 4, Lactic acid; 12, Pyruvic acid; 20, Succinic acid mono ethyl ester; 32, Malic acid 1-ethyl ester; 33, Malic acid 4-ethyl ester; 39, Tyrosol; 40, 2-Isopropylmalic acid; 43, Tartaric acid ethyl ester; Krebs, sum of organic acids in the Krebs's cycle; Phenols, sum of phenolic acids.

The first principal component (PC1) explains 58.62% of the variance, while the second (PC2) explains 18.23%; therefore, the combined variance explained by the first two components was 76.85%. The loading variables presented in Figure 2B indicates the contribution provided by the two components related to their length and direction. The loadings on PC1 are related decreasing order of contribution, to organic Krebs acids, malic acid 4-ethyl ester, sum of phenolic acids, tyrosol, pyruvate, malic acid 1-ethyl ester and 2-isopropylmalate are directed towards the positive values (corresponding to the right in Figure 2B), indicating that there was a correlation between these variables and the first component.

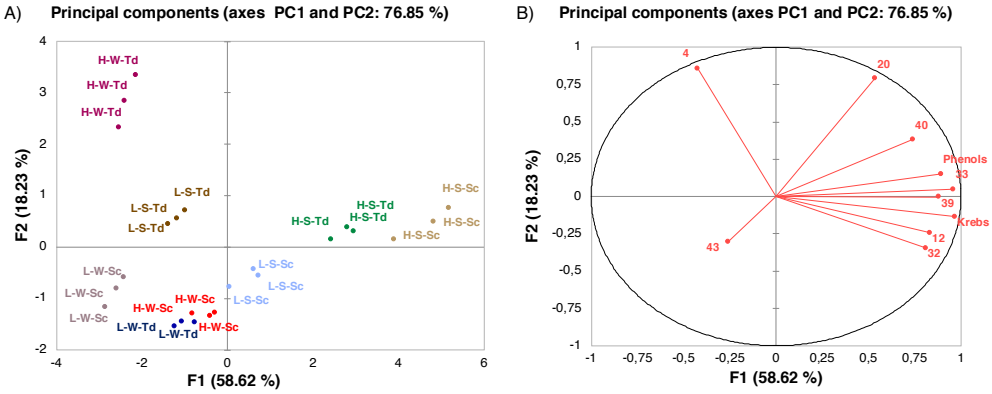


Figure 2. Principal component analysis of the metabolites analysed. The two-dimensional principal subspace for wine data (A) and the variables used (B). Variables: 4, Lactic acid; 12, Pyruvic acid; 20, Succinic acid mono ethyl ester; 32, Malic acid 1-ethyl ester; 33, Malic acid 4-ethyl ester; 39, Tyrosol; 40, 2-Isopropylmalic acid; 43, Tartaric acid ethyl ester; Krebs, sum of organic acids in the Krebs's cycle; Phenols, sum of phenolic acids. Wines: H corresponds to high temperature of fermentation, L corresponds to low temperature of fermentation, S correspond to wines fermented in presence of grape skins (Skin-fermented wines), W correspond to White wines with racking (White wines), Sc corresponds to fermentation solely with *S. cerevisiae* and Td corresponds to sequential fermentation with *T. delbrueckii* and *S. cerevisiae*.

From figure 2A, we can observe that all the Skin-fermented wines were mainly related to a high content of organic Krebs acids and phenolic acids, while the wines obtained by the grape must racking (White wines) were distributed in the part left of figure 2A, indicating some presence of phenolic acids and a lowest content of organic Krebs acids. However, there is an exception for L-S-Td. This result was expected since the phenolic acids were mainly located in the grape skins (Hidalgo Fernández-Cano, 2011). In addition, the skin-fermented wines on the left side of the figure were separated into four clusters, according to the type of inoculation and the fermentation temperature. Thus, a higher temperature (25 °C) leads to a greater expected richness in phenolic acids in the wines but also the wines resulting from a single inoculation were the richest compared to the sequential inoculation for the same temperature. This characteristic is also linked to a higher content of ethyl malate esters, organic Krebs acids, pyruvate and 2-isopropylmalate. The loadings on PC2 explained the lactate and succinate mono ethyl ester contents. Thus, according to PC2, the sequential

fermentations were separated from the rest of the conditions because a spontaneous MLF was detected.

In this context the most interesting compounds were analysed by separated. In figure 3 it is possible to observe the variation between conditions of organic acids in Kreb's cycle, phenolic acids and 2-isopropylmalic acid. Regarding organics acid from Krebs cycle, it was found a significant increase in skin-fermented wines at high temperature, especially only with *S. cerevisiae* (H-S-Sc). However, regarding white wines it was observed a significant decrease in organic acids in *S. cerevisiae* wines at both temperatures but specially at 16 °C. On the contrary, in presence of *T. delbrueckii* organic acids were decreased significantly only at high temperature (H-W-Td).

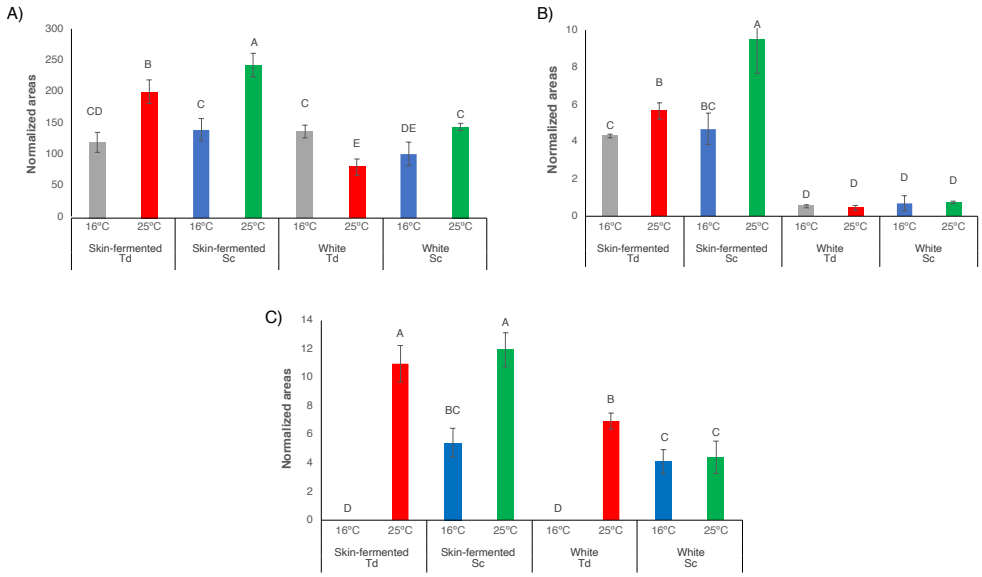


Figure 3. Normalized areas of more highlighted compounds: A) Organic acids from the Krebs Cycle, B) Phenolic compounds and C) 2-isopropylmalic acid. Wines: 25°C corresponds to high temperature of fermentation, 16°C corresponds to low temperature of fermentation, Skin-fermented correspond to wines fermented in presence of grape skins, W correspond to White wines with racking, Sc corresponds to fermentation solely with *S. cerevisiae* and Td corresponds to sequential fermentation with *T. delbrueckii* and *S. cerevisiae*.

In relation to phenolic acids (Figure 3B) it was notably that the abundance in white wines was significantly lower. It was expected since with the skin maceration during AF the phenolic

compounds present in the grape skin were released. The higher content of total phenolic compounds in skin-fermented wines in comparison to traditional white wines have been described (Bene and Kállay, 2019). Furthermore, this increasing effect in these compounds has been also observed in pre-fermentative maceration (Darias-Martín et al., 2000). Furthermore, Ramey et al., (1986) described an increase of hydroxycinnamic acids and in general phenolic compounds associated with pre-fermentative maceration and with higher temperatures. Regarding the presence of *T. delbrueckii*, a notable reduction in hydroxycinnamic acids compared to *S. cerevisiae* was detected, especially in p-coumaric acid, although this effect was higher at lower temperatures. Previously Ngqumba et al., (2017) has reported a reduction in certain phenolic acids (ferulic and gallic acid) in wines produced through monoculture and co-inoculation of *T. delbrueckii* and *S. cerevisiae*. These findings align with the hydroxycinnamate decarboxylase (HCDC) activity observed in *T. delbrueckii* strains (Božič et al., 2020), which has been also observed highly expressed in other non-*Saccharomyces* strains like *Pichia guillermondii* or *Wickerhamomyces anomalus* (Benito et al., 2011; Božič et al., 2020). Notably, the HCDC activity detected in *T. delbrueckii* strains by Božič et al. (2020) is comparatively lower than the average activity observed in *S. cerevisiae*. This suggests a potential synergistic effect occurring from the co-presence of both species. This observation implies that *T. delbrueckii* might metabolize hydroxycinnamic acids into vinylphenols. This enzymatic activity holds particular significance in red wines (Ruiz-de-Villa, et al., 2024b), as vinylphenols can combine with anthocyanins to form highly stable compounds known as vinyl phenolic pyranoanthocyanins (Benito et al., 2011; Schwarz et al., 2003). However, in wines originating from white grapes, this phenomenon could yield a dual impact. On one hand, the reduction of hydroxycinnamic acids might reduce the astringency that provide these compounds (Ferrer-Gallego et al., 2014), which could be beneficial in skin-fermented or white wines. On the other hand, the absence of anthocyanins coupled with the presence of vinylphenols could elevate the risk of 4-ethylphenol formation, particularly when yeast with vinylphenol reductase activity, such as *Brettanomyces*, are present (Zhang et al., 2021). This emphasises the potential need for caution when employing *T. delbrueckii* and other non-*Saccharomyces* strains in vinification processes involving white grapes, especially in scenarios where *Brettanomyces* is a potential concern. Regarding *S. cerevisiae*, a parallel decrease was observed at lower temperatures. As it has been commented, this yeast strain

also possesses HCDC activity (Božič et al., 2020), although with varying levels depending on the specific strain. Consequently, this activity appears to be less pronounced in conditions of higher temperatures (Figure 3B), potentially contributing to the production of wines with elevated astringency but limited vinylphenol availability. This reduction of hydroxycinnamic acids could be related to the metabolism of some LAB species due to the spontaneous MLF detected. However, as will be described in "3.3 Malolactic fermentation" all the strains isolated during the AF and MLF were *O. oeni*, species which is not able to decarboxylate hydroxycinnamic acids (De Las Rivas et al., 2009).

Finally, the identification of 2-isopropylmalic acid (2-IPMA) (Figure 3C) was particularly interesting, due to lack of knowledge about this compound in the wine environment. Along with 3-isopropylmalic acid (3-IPMA), 2-IPMA it has been detected in wine, serving as intermediates in the leucine biosynthesis pathway within yeast. 2-IPMA is generated in the mitochondria of *S. cerevisiae* from isoketovalerate, subsequently being transported to the cytosol where it is isomerized to 3-IPMA. The latter compound then undergoes a two-step conversion to leucine (Dumlao et al., 2008; Marobbio et al., 2008). As a result, yeast cells naturally release these compounds into the surrounding medium, Dumlao et al., (2008) suggested that secretion may occur during amino acid starvation. Regarding the obtained results at elevated temperatures, skin-fermented wines showed significantly higher 2-IPMA levels compared to white wines. This suggests the potential presence of this organic acid within grape skins. This finding is consistent with existing literature, as higher content of 2-IPMA has been reported in red wines compared to white wines (Ricciutelli et al., 2020, 2019). While information about the association of 2-IPMA with grapes is currently lacking, it has been detected in apple skins (Sugimoto et al., 2021). In this case the origin is as intermediate of the citramalate pathway, demonstrated in plants, which contributes to aroma-active ester formation during apple ripening (Sugimoto et al., 2021). Considering the concentration of 2-IPMA in wines fermented in the presence of *T. delbrueckii*, significant differences were observed, suggesting a potential implication on the metabolism of these species. Furthermore, at lower temperatures, concentrations were generally lower across conditions, these also suggested a potential temperature-dependent influence on 2-IPMA concentration. Regarding the properties of 2-IPMA, it has been recognized for its ability to detoxify aluminium for yeast cells (Suzuki et al., 2007). Moreover, this compound has exhibited

antimicrobial effects against certain pathogenic bacteria, including both gram-positive (*L. monocytogenes* and *S. aureus*) and gram-negative (*E. coli*, *S. enterica*, and *Y. enterocolitica*) species (Ricciutelli et al., 2020). In light of these findings, further investigation is needed to better understand the role of this bioactive compound in grapes and wine, as well as its potential bactericidal impact on wine-related microorganisms, including LAB or acetic acid bacteria (AAB).

3.3. Variability sources

An ASCA model was calculated to study the variability in metabolome and volatile data associated to the different factors considered: yeast, types of vinification and fermentation temperature. ASCA results are expressed in terms of % effect, which indicates the contribution of each factor to the matrix variability. The ASCA results for the 62 and 23 identified molecules in metabolomic and volatile analysis, respectively, are summarized in Table 4.

Table 4. ASCA results for the metabolomic and volatile analysis showing the percentage of variance (Effect (%)) for each factor and the *p*-value obtained from the permutation test. * Corresponds to a *p*-value < 0.05, which means that the factor is significant.

Factor	Effect (%)
Yeast	11.51*
Type of vinification	28.16*
Fermentation temperature	13.23*
Yeast x Type of vinification	6.08*
Yeast x Fermentation temperature	7.50*
Type of vinification x Fermentation temperature	9.22*

As can be seen by the ASCA results (Table 4), all the considered factors significatively impact the metabolome and volatile composition of the obtained wines. The "Type of vinification" factor shows the higher contribution to the overall variability of the wines (28.16%), which highlights the great contribution of grape skins in the characteristics of the wine. Although, "Fermentation temperature" and "Yeast" factors are also significative, as could be suspected from the great literature of the use of *T. delbrueckii* (Azzolini et al., 2015; Belda et al., 2015; Puertas et al., 2017; Renault et al., 2015) and the temperature impact on organoleptic wine

characteristics (Beltran et al., 2008). Additionally, the binary combinations of the factors, even though not so high, also significative which means that different factors affect differently depending on the other factor. This would mean that temperature does not impact the same on wines fermented with *S. cerevisiae* than by sequential inoculation using *T. delbrueckii*. Finally, the remain unexplained variability (24.30%) may be due to the inherent variability of biological replicates. These results mean that by modulating the three factors the winemaker is able to obtain different wines.

On the other hand, as ASCA individualize the variability of the data in each of the considered factors, it is possible to attribute the greater variability to a specific factor. In that sense, for "Type of vinification" factor shows the greater influence on the phenolic composition for skin-macerated wines, but also on other compounds such as tyrosol and succinate, fumarate and oxaloacetates (Krebs cycle metabolites) in terms of metabolome, and fusel alcohols (specially 2-phenylethanol) and SCFA for volatile composition. White wines vinifications are more related to acetates and ester concentrations. Regarding, the factor "Fermentation temperature", the high temperature is related to an increase in 2-IPMA concentration, as previously observed, and the low temperature to MCFA and LCFA. Finally, "Yeast" factor is related to *T. delbrueckii* inoculation with many acids from Krebs cycle, as well as 1-hexanol and valeric acid concentrations.

3.4. Malolactic fermentation

After AF the focus was initiating MLF. However, the presence of indigenous LAB was unexpectedly high in must, reaching 4 x 10^4 CFU/mL. In L-S-Td, H-W-Td and L-W-Td wines the LAB population remained high at the end of AF, still at 1 x 10^5 CFU/mL. In the other wines LAB presence was detected but at a lower concentration, around 2 x 10^3 CFU/mL. Consequently, spontaneous MLF occurred in wines fermented in the presence of *T. delbrueckii* (L-S-Td, H-W-Td, and L-W-Td), with the exception of H-S-Td.

This effect corroborates earlier findings observed in different types of vinifications (Ruiz-de-Villa et al., 2024b; Balmaseda et al., 2021b). The synergic relationship between *T. delbrueckii* and *O. oeni* has been identified as a key factor in accelerating the consumption of L-malic acid by the inoculated strains (Ruiz-de-Villa et al., 2024a, Ruiz-de-Villa et al., 2024b, Balmaseda et al., 2023;

Balmaseda et al., 2021b). Furthermore, it has been observed that *T. delbrueckii* also promotes spontaneous MLF in red wines produced using the traditional (Balmaseda et al., 2021b) and carbonic maceration process (Ruiz-de-Villa et al., 2024b). However, in those studies due to the lower LAB population spontaneous MLF took more time to start.

Consequently, in wines where spontaneous MLF did not occur during AF (H-O-Td, H-O-Sc, L-O-Sc, H-W-Sc, and L-W-Sc) and where L-malic acid still remained, MLF was carried out per two different strains and spontaneous fermentation. Figure 4 provides an overview of the MLF kinetics. It is noteworthy that in the H-O-Td wine inoculated with *O. oeni,* both strains exhibited a faster consumption of L-malic acid compared to the other conditions. In the case of spontaneous MLF, L-O-Sc displayed the shortest MLF duration. However, upon comparing H-O-Td with its control equivalent, H-O-Sc, a significant disparity appeared. The consumption of L-malic acid concluded eleven days earlier in H-O-Td. In this instance, MLF lasted only three days, whereas in H-O-Sc, it extended for a period of 17 days before finishing the MLF, although the subsequent consumption of L-malic acid was fast.

The beneficial impact of *T. delbrueckii* has been documented across diverse matrices, including white wines (Balmaseda et al., 2021d). This effect is the result of a combination of factors, where *T. delbrueckii* positively influences the final composition of the wine enhancing the activity of *O. oeni*. This begins with the reduction of certain compounds toxic to *O. oeni*, such as MCFA (Edwards and Beelman, 1987). It is worth noting that the reduction of these compounds depends on the strain of *T. delbrueckii* and the specific winemaking conditions. Notably, only In the H-W-Td conditions, a significant reduction in MCFA compared to *S. cerevisiae* was observed (Table 3).

Conversely, the increased release of mannoproteins into the media by *T. delbrueckii* have the potential to improve the performance of MLF. These mannoproteins can be metabolized by *O. oeni* (Balmaseda et al., 2021a; Jamal et al., 2013), and can contribute to the detoxification of the media from inhibitory compounds (Lafon-Lafourcade et al., 1984), supporting MLF progression. This study has indeed observed an increase in sequential fermentations with *T. delbrueckii* especially at low temperatures of fermentation (Table 2).

Furthermore, hydroxycinnamic acids have been described as inhibitory for *O. oeni*, especially p-coumaric acid (Reguant et al., 2000). In this context, the reduction of certain hydroxycinnamic acids, such as p-coumaric acid identified in skin-fermented wines with sequential AF involving *T. delbrueckii*, could be linked to the acceleration of MLF, in combination with other contributory factors.

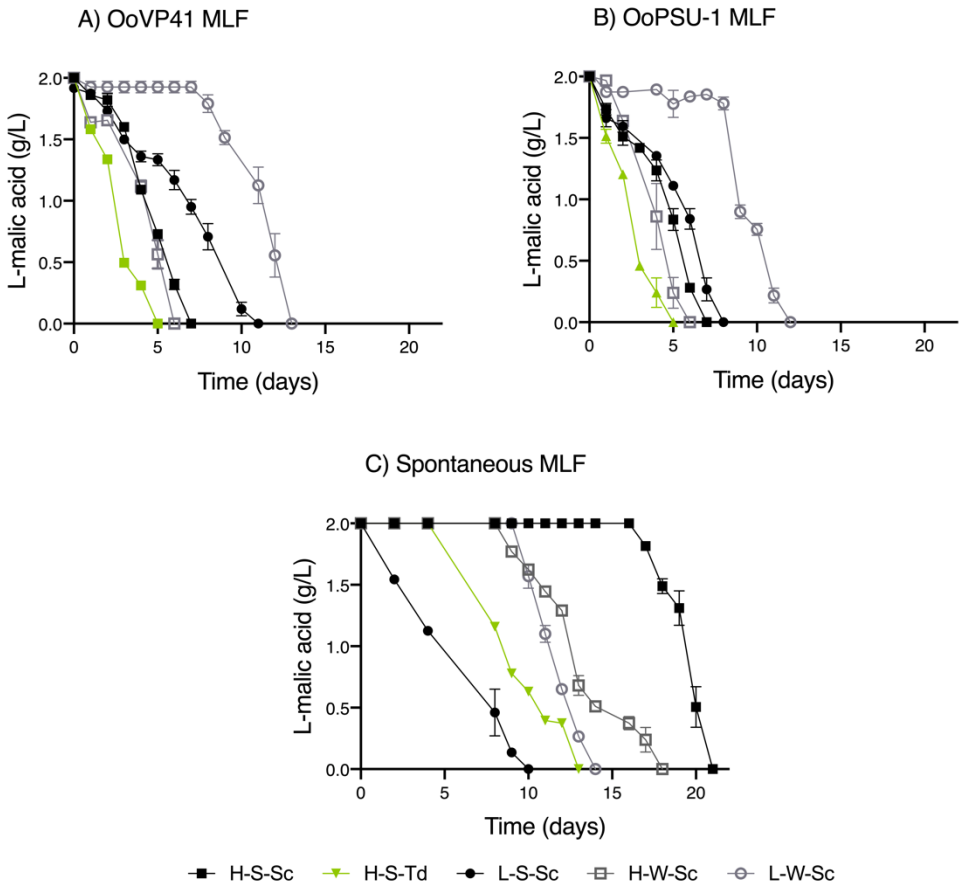


Figure 4. Consumption of L-malic acid during malolactic fermentation. A) corresponds to OoVP41 MLF; B) corresponds to OoPSU-1 MLF, and C) corresponds to spontaneous MLF. Wines: 25 °C corresponds to high temperature of fermentation, 16 °C corresponds to low temperature of fermentation, Skin-fermented corresponds to wines fermented in presence of grape skins, W corresponds to White wines with racking, Sc corresponds to fermentation solely with S. *cerevisiae* and Td corresponds to sequential fermentation with *T. delbrueckii* and *S. cerevisiae*. All data is expressed as the arithmetic average of three biological replicates ± standard deviation (n=3).

3.4.1. Lactic Acid Bacteria identification

Considering the high population of LAB detected, species and strain identification were performed. All isolates were identified as *Oenococcus oeni* using species-specific PCR. Subsequently, 19 different VNTR profiles were identified (Figure 5), which could be associated with different *O. oeni* strains (Claisse and Lonvaud-Funel, 2014). In the L-S-Td, H-W-Td and L-W-Td wines, inoculated strain identification was not possible since these wines underwent spontaneous MLF during AF.

Significant variability in profiles was observed among the different conditions, including variations in the percentage of specific profiles. Two profiles, M1 and M2, were isolated from the must. Particularly, M2 had a high prevalence at various stages of fermentation, persisting after AF and spontaneous MLF. it has been mainly imposed in spontaneous MLF in H-W-Sc and H-W-Td wines, and it appeared at almost 20 - 50% in the other spontaneous MLF cases. Additionally, during the OoVP41 MLF of H-S-Sc wine, this profile shared prevalence with the inoculated strain VP41. This high imposition suggests that this particular strain could be an interesting candidate for further investigation. Another profile with notable presence during MLF was C5. This strain was present at the end of MLF in all inoculated wines with OoVP41, indicating that it might have a good capacity of adaptation to wine conditions and competitive characteristics to colonize this niche.

Regarding the inoculated strains, it was observed that OoVP41 did not exhibit strong imposition during MLF maybe due to the high competence of autochthonous population. It appeared in inoculated wines after MLF at higher percentages at low AF temperature wines, particularly in white wine (L-W-Sc). Moreover, it is noteworthy that a profile (VP41*) identical to that of OoVP41 was found in stages where the strain was not inoculated, indicating that this strain had colonized the winery environment. However, OoPSU-1 demonstrated good prevalence, exceeding 70%, in all inoculated wines. The high imposition of OoPSU-1 has been observed in previous works in red and white wine where it reaches an imposition of the 100% (Balmaseda et al., 2021b, 2021d). The fact that OoVP41 did not dominate MLF may have been related to the fact that the MLF kinetic of

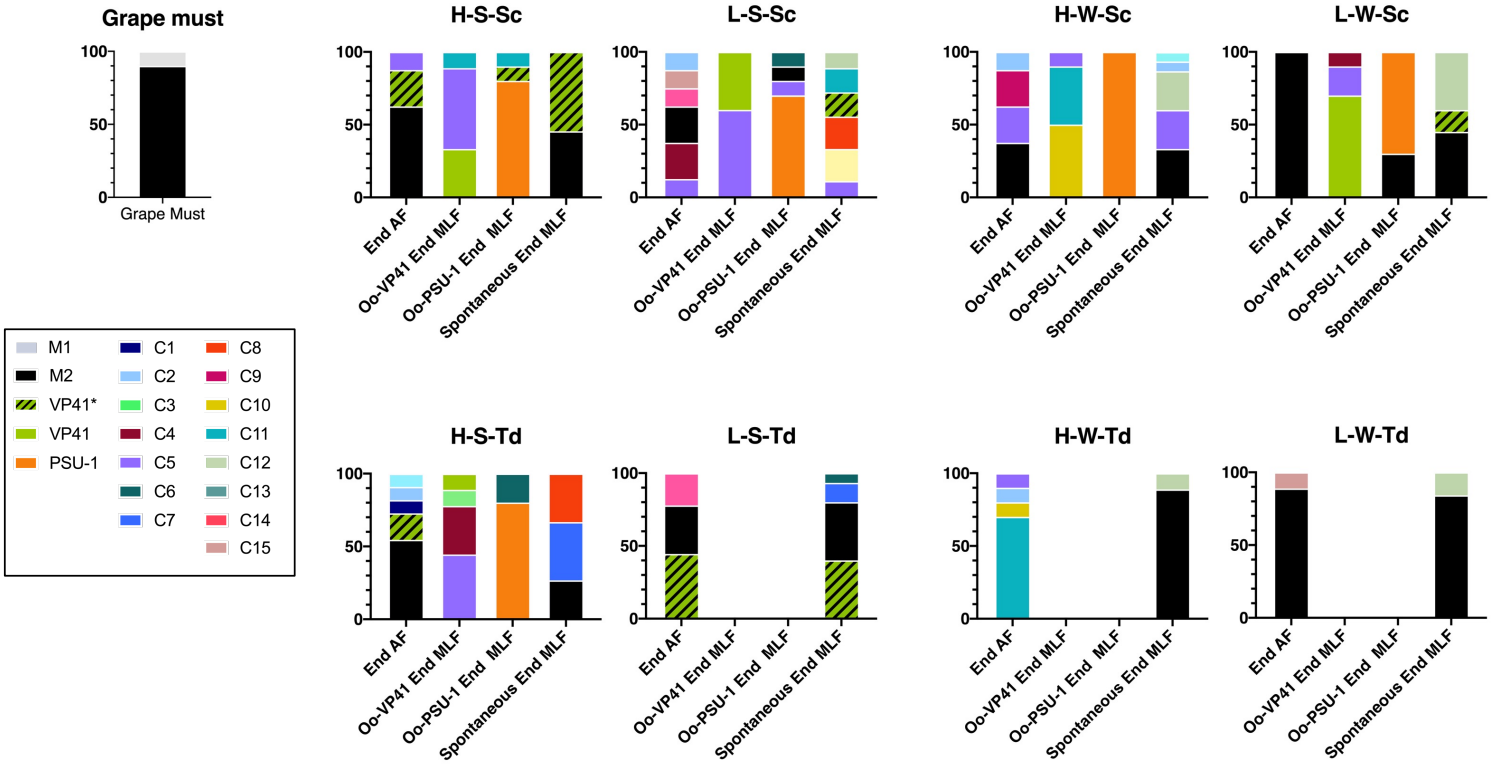
wines inoculated with this strain did not go faster than with OoPSU-1, as has happened in other studies in which there was a notable fermentative difference between them.

3.5. Sensory analysis

Regarding the organoleptic analysis, it focused on the sensory characteristics resulting from the use of *T. delbrueckii*. With this purpose the wines after AF were subjected to a triangular analysis. This analysis compared wines fermented solely with *S. cerevisiae* to wines produced using sequential fermentation with *T. delbrueckii* (H-S-Sc vs H-S-Td, L-S-Sc vs L-S-Td, H-W-Sc vs H-S-Td, L-W-Sc vs L-S-Td) according to the different vinification conditions. Trained tasters, ranging from 12 to 19 participants depending on the tasting session, evaluated the wines. The results showed significant differences in all combinations, indicating that regardless of temperature or the presence of grape skins during AF, the use of *T. delbrueckii* in sequential fermentation had a distinct impact on the organoleptic properties of the wines (data not shown). These differences could be attributed to the spontaneous MLF, however even in the condition without MLF finished the tasters differenced the wine fermented with *T. delbrueckii*.

Subsequently, a descriptive analysis of the wines was conducted, focusing on tropical aroma, pear aroma, terpenic aroma, grass aroma, acidity, bitterness, and overall harmony. These attributes were selected based on the grape cultivar and wine type. As shown by the PCA results in Figure 3A the skin-fermented wines with sequential fermentation were separated from those fermented solely with *S. cerevisiae* based on PC1. H-S-Td wines exhibited positive correlations with pear, tropical, terpenic aromas, and global harmony, while L-S-Td wines showed stronger associations with terpenic and grass aromas.

Figure 5. Percentage of *O. oeni* strains during vinification according to VNTR profiles. M1 and M2 refers to profiles of *O. oeni* identified in grape must, VP41* corresponds to OoVP41 strain identified from the winery environment, VP41 corresponds to OoVP41 inoculated and PSU-1corresponds to OoPSU strain inoculated; from C1- to C15 correspond to profiles of *O. oeni* identified during alcoholic fermentation (AF) and malolactic fermentation (MLF). Wines: 25 °C corresponds to high temperature of fermentation, 16 °C corresponds to low temperature of fermentation, Skin-fermented correspond to wines fermented in presence of grape skins, W correspond to White wines with racking, Sc corresponds to fermentation solely with S. cerevisiae and Td corresponds to sequential fermentation with *T. delbrueckii* and *S. cerevisiae*



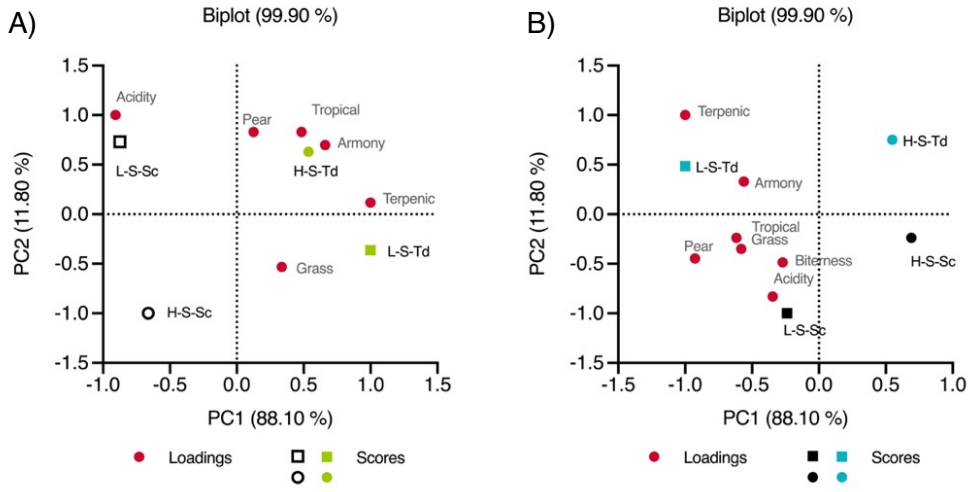


Figure 6. Principal component analysis biplots built from the following variables: Pear, grass, terpenic aromas, bitterness, acidity and harmony. A) Correspond to skin-fermented wines and B) Corresponds to white wines. Wines: 25 °C corresponds to high temperature of fermentation, 16 °C corresponds to low temperature of fermentation, Skin-fermented correspond to wines fermented in presence of grape skins, correspond to white wines with racking, Sc corresponds to fermentation solely with *S. cerevisiae* and Td corresponds to sequential fermentation with *T. delbrueckii* and *S. cerevisiae*.

As has been observed, there is an influence of the factors considered as well as the interaction between them, justifying the absence of significant differences in many cases. In this context although only a limited number of significant variations were found in the analysis of volatile compounds between sequential wines with *T. delbrueckii* and those solely fermented with *S. cerevisiae*, a more intricate difference in aromatic profiles became evident in sensory analysis. This phenomenon can be attributed to an aromatic synergy, wherein the interplay of various compounds contributes to the overall sensory perception. It is important to note that additional aromatic distinctions may arise from varietal terpenes, a component that was beyond the scope of our analytical method.

4. Conclusion

In summary, the performance of AF varied depending on the experimental conditions. Sequential fermentations, driven by competition between yeast species, took longer than the control group. Skin-fermented wines consistently initiated the AF earlier, while white wines

exhibited a lag phase. Fermentations at high temperatures had shorter durations compared to low temperatures, consistent with the optimal temperature range for *S. cerevisiae*. Additionally, the presence of *T. delbrueckii* persisted for more days at lower temperatures, highlighting its adaptability to such conditions. Conversely, *S. cerevisiae* demonstrated a competitive advantage over non-*Saccharomyces* yeast at higher temperatures. These results contribute to our understanding of the complex dynamics of alcoholic fermentation through the influence of fermentation temperature, the type of vinification involved and the interactions between microorganisms, yeasts and lactic acid bacteria present in grape must, on this process.

These findings shed light on the intricate dynamics of MLF and highlight the influence of various factors, including the presence of *T. delbrueckii* and the synergistic relationship with *O. oeni*. The spontaneous occurrence of MLF and the differential kinetics observed among the tested conditions contribute to our understanding of the complex microbial interactions during wine fermentation. Further research in this area is warranted to explore the underlying mechanisms and optimize MLF processes to enhance the quality and sensory characteristics of wines.

Acknowledgements

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Supplementary data

Table SD 1. Citric acid (g/L), acetic acid (g/L) and pH of wines after MLF. Wines: 25 ºC corresponds to high temperature of fermentation, 16 ºC corresponds to low temperature of fermentation, Skin-fermented correspond to wines fermented in presence of grape skins, W correspond to white wines with racking, Sc corresponds to fermentation solely with *S. cerevisiae* and Td corresponds to sequential fermentation with *T. delbrueckii* and *S. cerevisiae*. OoVP41 and OoPSU corresponds to wines inoculated with these strains, respectively and Spontaneous corresponds to spontaneous MLF during AF. All data is expressed as the arithmetic average of three biological replicates ± standard deviation (n=3).

		Citric acid (g/L)	Acetic acid (g/L)	pH
OoVP41	H-S-Sc	0.26 ± 0.01^{a}	0.28 ± 0.04 ^{a}	3.42 ± 0.01
	H-S-Td	0.22 ± 0.04 ^{a}	0.23 ± 0.02 ^{a}	3.42 ± 0.01
	L-S-Sc	0.43 ± 0.08 ^{c}	0.50 ± 0.03 ^{d}	3.44 ± 0.01
	H-W-Sc	0.28 ± 0.07 ^{ab}	0.46 ± 0.04 ^{cd}	3.40 ± 0.01
	L-W-Sc	0.23 ± 0.02 ^{a}	0.45 ± 0.03 ^{cd}	3.40 ± 0.01
OoPSU	H-S-Sc	0.25 ± 0.04 ^{a}	0.27 ± 0.03 ^{a}	3.38 ± 0.01
	H-S-Td	0.23 ± 0.03 ^{a}	0.23 ± 0.05 ^{a}	3.41 ± 0.01
	L-S-Sc	0.40 ± 0.03 ^{bc}	0.48 ± 0.02 $_{cd}$	3.76 ± 0.07
	H-W-Sc	0.25 ± 0.03 ^{a}	0.43 ± 0.02 ^{bcd}	3.73 ± 0.5
	L-W-Sc	0.25 ± 0.04 ^{a}	0.46 ± 0.05 ^{cd}	3.38 ± 0.01
Spontaneous	L-S-Td	0.26 ± 0.03 ^{ab}	0.39 ± 0.01 ^{bc}	3.42 ±0.04
	H-W-Td	0.28 ± 0.09 ^{ab}	0.34 ± 0.02 ^{ab}	3.40 ± 0.01
	L-W-Td	0.23 ± 0.01 ^{a}	0.41 ± 0.08 ^{bcd}	3.40 ± 0.01

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Candela Ruiz de Villa Sardón

Chapter 3

Ethanol reduction with pre-fermentative water addition and Metschnikowia pulcherrima: effect on alcoholic and malolactic fermentation

The modulation of wine composition by *T. delbrueckii* has been extensively discussed throughout previous chapters. However, the field of modern oenology is in a constant state of evolution, and novel approaches are constantly being tried to enhance wine production and to improve the quality of the final product. In the current landscape, the focus is on climate change, an issue of great importance, which is mainly associated with investigations focused on reducing ethanol content. **Chapter 3** involves a study of diverse sustainable strategies for ethanol reduction, specifically delving into two distinct methodologies: the addition of different percentages of water and the sequential inoculation of *M. pulcherrima* and *S. cerevisiae*.

In **Section 3.1**, the impact of these treatments was evaluated both in terms of wine composition and ethanol reduction efficiency. Different behaviours were observed, with a notable dilution effect particularly pronounced in the context of higher water additions. The findings highlight the potential benefits of a moderate water addition, in synergy with the capacity of sensory modulation capacity attributed to *M. pulcherrima*.

In **Section 3.2** the implications of these treatments on the subsequent MLF were analysed. It was observed that ethanol reduction facilitates the process of MLF, as expected. Moreover, the study confirms that *M. pulcherrima*, as well as *T. delbrueckii*, contributes to the enhancement of MLF through distinctive metabolic changes in the wine. Furthermore, aromas change after MLF, however the addition of water was not significant as in wines after AF. This suggest that in a less stressful media, LAB also significantly impact the aromatic composition.

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Candela Ruiz de Villa Sardón

Section 3.1

Physicochemical and organoleptic differences in Chardonnay Chilean wines after ethanol reduction practises: Pre-fermentative water addition or *Metschnikowia pulcherrima*

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Fermentation

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Abstract

Climate change is posing a major challenge to the wine industry, with rising alcohol levels emerging as an issue of concern affecting quality, economics and health. This study explores two methods to reduce alcohol content in Chardonnay wines from Chile. Firstly, 5% and 10% of water was added to grape must. Secondly, the sequential inoculation of *Metschnikowia pulcherrima* with *Saccharomyces cerevisiae* was examined. The main objectives were to assess the efficacy of these treatments in reducing alcohol levels and their impact on organoleptic properties.

Our findings revealed that the presence of *M. pulcherrima* in winery conditions was less effective in reducing ethanol. Nevertheless, wines resulting from this treatment exhibited an interesting composition with distinct sensory profiles. Furthermore, Sc-5%W condition, displayed promising results by reducing ethanol content by 0.47% (v/v), with less significant changes in the sensory profile. Although the Sc-10%W wines showed a more substantial ethanol reduction of 1.73% (v/v), they exhibited a decreasing trend in volatile compounds and polysaccharides, ultimately being perceived as less complex in sensory analysis and not being preferred by consumers. This research contributes to understand how these approaches affect the alcohol content and sensory attributes of white wines is fundamental to the sustainability and ability of the sector to recover from climate challenges.

Keywords

Watering; low alcohol wines; non-*Saccharomyces.*

1. Introduction

In the last decades climate change has had a significant and profound impact on the agricultural industry. Therefore, viticulture and winemaking are being affected by this issue in wine regions around the world (Gutiérrez-Gamboa et al., 2021; Jones et al., 2005; Mira de Orduña, 2010). While climate change is causing challenges and negative effects in traditional wine making regions, it has also resulted in the emergence of new wine-producing areas due to the displacement of climate patterns (Droulia and Charalampopoulos, 2021; Verdugo-Vásquez et al., 2023). Chilean wine regions are also grappling with global warming. Coquimbo, Aconcagua and Central Valley Regions have undergone a change from warm to hot climates (Verdugo-Vásquez et al., 2023). It is worth noting that the Maule region, which provided the grape must for the study, is part of the Central Valley region. The global warming worldwide produces an increase in temperatures and a reduction of water availability due to draught. In the vineyard this leads to increased sugar concentrations in grape berries, reduced the total acidity levels in grapes and a lag between phenolic and technological maturity (Gutiérrez-Gamboa et al., 2021). As a result, the wines produced present organoleptic imbalance with higher alcohol contents and an increased risk of stuck fermentations (Gutiérrez-Gamboa et al., 2021; Jones et al., 2005).

Furthermore, it is important to note that the increase in alcohol content is related by higher taxes, as a result of policy interventions implemented in numerous countries (Elder et al., 2010). Another factor contributing to the reduction in alcohol degree is the growing trend towards adopting healthier lifestyles that involve consuming less alcohol (Saliba et al., 2013).

A wide variety of practices have been proposed to address the issue of increased alcohol content in wines. Many of these approaches focus on viticulture practices, such as modifying irrigation techniques or adjusting pruning management (Gutiérrez-Gamboa et al., 2021). However, there is also growing research into microbiological modulation to reduce alcohol levels in wines. While carbon metabolic pathways in yeast species are generally conserved, variations exist in terms of ethanol yields (Hranilovic et al., 2020; Jolly et al., 2014). Studies have demonstrated that certain non-*Saccharomyces* yeasts can effectively reduce alcohol content through co-fermentation with *Saccharomyces cerevisiae* under aeration conditions

(Jolly et al., 2014; Quirós et al., 2014). Among these yeasts, *Metschnikowia pulcherrima* has been reported as one of the most effective species reducing ethanol levels through its respiratory catabolism of sugars (Hranilovic et al., 2020; Quirós et al., 2014; Zhu et al., 2021). Physical practices have been also proposed as osmotic distillation, reverse osmosis or vacuum distillation (Rolle et al., 2018; Schmidtke et al., 2012). Another oenological practice studied to reduce alcoholic content is the water addition or substitution in grape must. A reduction between 0.6% (v/v) to 5.9% (v/v) has been reported in literature, most of these studies has been performed in red wines (Piccardo et al., 2019; Schelezki et al., 2020b, 2020a; Teng et al., 2020). However, it is worth noting that Gardner et al., (Gardner et al., 2022) conducted a water addition study with the Viognier and Marsanne grape cultivars. Addition of water is not authorized in all wine regions due to varying legislation. For instance, in California state (not below 22° Brix) and Australia (not below 24° Brix), the addition of water has been permitted to facilitate alcoholic fermentation (AF) of must with high sugar content. However, in the European Union, South Africa, and other wine-growing regions, the addition of water is generally prohibited unless necessary for additives (Xynas and Barnes, 2022). In Chile, water addition is authorized in musts with higher levels of soluble solids up to 23.5 °Brix, but it is limited to a maximum of 3.5% of the total water allowed for the addition of additives (Ministry of Agriculture of Chile, 2019).

These findings highlight the potential for exploring alternative practices to mitigate the increase of alcohol content in wines. Furthermore, there is a lack of studies that focus on these practices in white wines, making the research on the Chardonnay cultivar particularly interesting due to its high grape berry maturity (Sadras and Petrie, 2011).

The objective of this study is to compare the effectiveness of reducing the alcohol content of a wine by adding water (5 and 10%) or by using a microbiological treatment, *M. pulcherrima* and *S. cerevisiae* sequential fermentations, under pilot plant like conditions. We performed an assessment of the physicochemical and sensory properties of the wines with the end goal to determine the most effective method of alcohol reduction and understand its impact on the final product.

2. Materials and Methods

2.1. Microorganisms and alcoholic fermentation

Two yeast species were evaluated: *S. cerevisiae* QA23 (Sc) for the control and water addition conditions, and *M. pulcherrima* Level2 Flavia (Mp) both from Lallemand Inc., Canada. The yeasts were inoculated from Dry Active Yeast and rehydrated according to the manufacturer's instructions. *S. cerevisiae* strains were rehydrated at 37 °C for 30 minutes, while *M. pulcherrima* strains were rehydrated at 30 °C for the same duration. *S. cerevisiae* was inoculated at a concentration of 2 x 10^6 cell/mL and *M. pulcherrima* at a concentration of 1 x 10^7 cell/mL.

The fermentations were conducted using Chardonnay natural grape must supplied by Viña Correa Albano from the Maule region, Chile. Pilot plant scale fermentations were performed in 20 L food-grade plastic tanks, maintaining a temperature of 16 °C without agitation. Two different watering conditions were tested by adding distilled water to the grape must: 5% and 10% (which will be referred in text as Sc-5%W and Sc-10%W, respectively). In addition to the watering conditions, a sequential fermentation was carried out. *M. pulcherrima* (Mp+Sc) was initially inoculated, it remained in grape must for 3 days at 22 °C while a manual aeration three times a day. Then *S. cerevisiae* was inoculated and continue the AF at 16 °C without aeration. Therefore, four conditions were studied in triplicate: Sc-Control (*S. cerevisiae* under control conditions), Sc-5%W (*S. cerevisiae* under 5% watering condition), Sc-10%W (*S. cerevisiae* under 10% watering condition), and Mp+Sc (sequential fermentation with *M. pulcherrima* followed by *S. cerevisiae*). To ensure proper nutrition for yeast growth, 48 hours after the inoculation, nutrients (Nutrienvit, Lallemand Inc., Montreal, Canada) were added to the fermentations at a concentration of 150 mg/L following suppliers' indications.

Inocula and population dynamics were determined by plating a 1:10 serial dilution in YPD agar (10 g/L of yeast extract, 20 g/L of peptone, 20 g/L of glucose, 17 g/L of agar, Panreac Química SLU, Castellar del Vallés, Spain). In addition, populations of *M. pulcherrima* and non-*Saccharomyces* were controlled by Wallerstein selective medium (BDDifco, Massachusetts, United States).

The monitoring of AF was performed by measuring density each day with Densito 30PX Portable Density Meter (Mettler Toledo, Spain). Fermentation was considered finished when the density remains stable and the reducing sugars concentration was less than 2 g/L. Reducing sugars were analysed following the official method of the OIV (OIV, 2022). After AF wines were sulphited (10 mg/L $K_2S_2O_5$) and stabilized at 4 °C until. Then were bottled and stored before the sensory analysis.

2.2. Oenological parameters

General oenological parameters analysed after AF were the following: Titratable acidity (expressed as g of equivalent tartaric acid per litter), volatile acidity (expressed as g of equivalent acetic acid per litter), ethanol contents (% v/v) and Total Polyphenols Index (I_{280}). They were determined by the official method of the OIV (2022).

2.3. Analysis of volatile compounds

The volatile compounds in the wine samples were analysed using the procedure described in (Úbeda et al., 2017). Briefly, before the analysis, the wine samples underwent a pre-treatment process to extract the volatile compounds using Headspace Solid Phase Microextraction (HS-SPME). A 2 cm 50/30 µm Carboxen/ Divinylbenzene/ Polydimethylsiloxane (CAR/DVB/PDMS) SPME fibber (Supelco, Bellefonte, PA, USA) was employed. The sample volume used was 7.5 mL where was added 10 µL of 4-methyl-2-pentanol (0.75 mg/L) used as internal standard. Headspace sampling was done by using an autosampler, with the vial incubated at 45 °C for 20 minutes and agitated at 500 rpm. Subsequently, injection was performed using the spitless mode for 3 minutes, with a transfer line temperature of 280 °C. The gas chromatography analysis was conducted using a 7890B Agilent GC system coupled to a quadrupole mass spectrometer Agilent 5977 inert (Agilent Technologies, Palo Alto, CA, USA). A DB Wax capillary column (60 m × 0.25 mm x 0.25 µm film thickness, J & W Scientific, Folsom, CA, USA) was used, and the carrier gas was Helium flowing at a rate of 1 mL/min.

Compound identification was performed by using an MS ChemStation (Agilent Technologies). Data were presented as relative area values. To calculate the relative area, the peak area of

the main ion of each compound was divided by the peak area of the main ion of internal standard, normalizing it.

2.4. Determination of Soluble polysaccharides

The analysis of polysaccharides was conducted following the procedure outlined in (Fanzone et al., 2012). Initially, the polysaccharides were extracted from wine matrix using a precipitation method involving cold acidified ethanol. Then, the determination of polysaccharides was carried out using HRSEC-RID technique.

2.5. Analysis of low-molecular-mass phenolic compounds

Low-molecular-mass phenolic compounds were analysed following the procedure described in (Peña-Neira et al., 2007). Briefly, wine phenolic compounds were extracted three times with 25 mL diethyl ether and three times with 25 mL of ethyl acetate. Then the extracts were evaporated under vacuum and dissolved in 2 mL of methanol/water (1:1, v/v). Samples were analysed by using a HPLC-DAD (Agilent Technologies, Santa Clara, CA, USA). Finally, the identification and quantification were done by comparison of their spectra and retention time with external standards (Sigma Aldrich, Santiago de Chile, Chile).

2.6. Sensory analysis

Three different sensory evaluations were conducted to assess the wines. Each glass contained 50 mL of wine for all the analysis.

The first evaluation was a triangle sensory analysis, where a panel of 30 tasters, consisting of trained experts, compared the treatments with the Sc-Control to determine significant differences in a binomial test. To eliminate visual subjectivity, the wines were served in dark glasses and labelled with random 3-digit codes.

The second evaluation involved a descriptive analysis, aiming to provide a detailed sensory profile of the samples that showed significant differences in the triangular sensory analysis. Transparent glasses were used to evaluate the wines. A panel of 10 trained and expert tasters performed this analysis. The tasters used a 15 cm unstructured scale to rank the intensity of various attributes, including colour intensity, aroma intensity, mouthfeel intensity, compote

aroma, tropical aroma, stone fruit aroma, floral aroma, lactic aroma, acidity, unctuosity, bitterness, and persistence.

Lastly, a consumer preference evaluation was conducted, involving 75 consumers. The purpose was compared the Sc-10%W treatment wine with the Sc-Control wine. Samples were presented following a Latin square design. The panel involves 27 females, 45 males and 3 nonbinaries, with an average age of 24.6 years old. All the consumers were students and staff from the Faculty of Agronomy (University of Chile).

2.7. Statistical analysis

All conditions were performed in triplicate biological samples. The statistical software used was XLSTAT version 2022.5.1 (Addinsoft, Paris, France). The data were analysed with two-way ANOVA with a post hoc Tukey test (Honesty Significant Difference) with a confidence interval of 95% and significant results with a p-value < 0.05. PLS-DA was used to discriminate samples regarding volatile compounds.

Descriptive sensory analyses were conducted using the software Panel Check (V1.4.2 2012), applying an ANOVA test with a significance level of 95% and utilizing the Least Significance Differences (LSD) test for post hoc comparisons. Consumer paired preference analyses were assessed using the binomial distribution and the probability was calculated according to Golden et al. (2010).

3. Results

3.1. Alcoholic fermentation kinetics

There were notable differences in the fermentation time among different conditions in terms of alcoholic fermentation (AF) kinetics considering the time needed to cosume 50% of sugars (T50%) and the maximal consumption rate, as it is shown in Figure 1 and in Table SD1. Usually, non-*Saccharomyces* species tend to prolong the AF process due to nutrient competition (Roca-Mesa et al., 2020). However, in the case of Mp+Sc wines the AF duration was faster compared to the other conditions (Table SD1). This behaviour can be attributed the presence of *S. cerevisiae* and non-*Saccharomyces* yeasts (Figure SD1) in the grape must which started

the fermentation. The sugar consumption was higher than in the other conditions may be due to the temperature change (22 °C) during the three-day contact period of *M. pulcherrima* with the grape must, as well as the manual aeration three times a day. These conditions were implemented to optimize the *M. pulcherrima* respiratory metabolism (Morales et al., 2015) and improve the ethanol reduction effectiveness.

Among the water addition conditions, it is noteworthy that Sc-10%W followed by Sc-5%W exhibited a faster AF compared to Sc-Control, despite the initial lower density resulting from dilution effects.

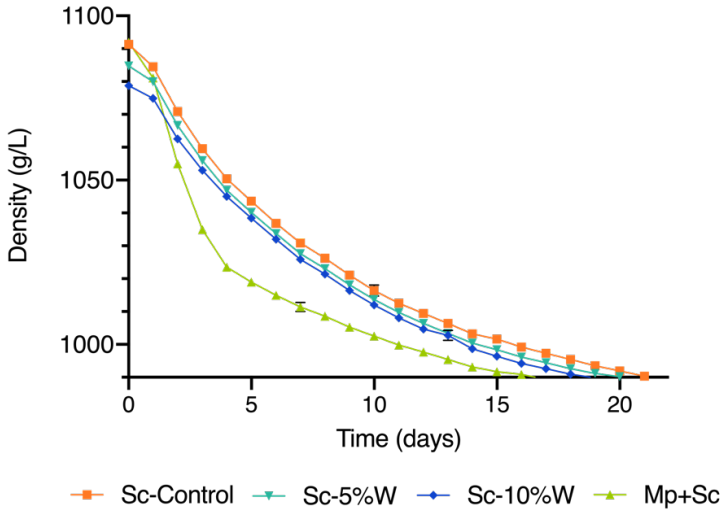


Figure 1. Alcoholic fermentation kinetics of the different experimental conditions. The Sc-Control represents wines fermented solely with *S. cerevisiae*, while Sc-5%W and Sc-10%W indicate wines with a pre-fermentative water addition of 5% and 10%, respectively. The Mp+Sc wines depict sequential fermentation with *M. pulcherrima* and *S. cerevisiae*. Means accompanied by standard deviations (SD) based on three replicates (n = 3).

3.2. Chemical general analysis

In terms of reducing the alcohol content (Table 1), Mp+Sc condition showed a tendency towards ethanol reduction of approximately 0.30% (v/v), although these differences were not statistically significant from Sc-Control. Previous studies conducted on natural white must have reported alcohol reductions of up to 0.99% (v/v) (Contreras et al., 2014), ranging from 0.6%

to 1.2% (v/v) in Chardonnay must (Hranilovic et al., 2020) or 0.84% to 1.25% (v/v) in the case of the Malvar cultivar (Garciá et al., 2020). In Muscat wines Zhu et al. (Zhu et al., 2020) described a reduction up to 0.74% (v/v) using the same strain combination as in the present study. It is important to note all these studies involved the sterilization of grape must. However, in our study, we aimed to replicate real semi-industrial vinification conditions, and therefore, we decided not to sterilize the fermenting must. As a result, there were naturally occurring spontaneous yeast present at the beginning of alcoholic fermentation (Figure SD1). Consequently, the competition between *M. pulcherrima* and the spontaneous yeast (Wang et al., 2016) could explain the relatively low ethanol reduction observed. Additionally, the differences observed between strains in literature (Hranilovic et al., 2020; Zhu et al., 2020) suggested that the compatibility between *M. pulcherrima* and *S. cerevisiae* strains may influence the effectiveness of alcohol reduction.

Table 1. Main oenological parameters of final wines. The Sc-Control represents wines fermented solely with *S. cerevisiae*, while Sc-5%W and Sc-10%W indicate wines with a pre-fermentative water addition of 5% and 10%, respectively. The Mp+Sc wines depict sequential fermentation with *M. pulcherrima* and *S. cerevisiae*. Different lowercase letters indicate the existence of significant difference between the samples (*p*-value < 0.05). Data are expressed as the mean of three experimental replicates ± standard deviation.

	Sc-Control	Sc-5%W	Sc-10%W	Mp+Sc
Ethanol % (vol/vol)	13.93 ± 0.21 ^{c}	13.53 ± 0.15 ^{b}	12.20 ± 0.10 ^{a}	13.67 ± 0.06 ^{bc}
Titratable acidity (g/L de T_2H)	5.1 ± 0.3	4,9± 0.3	5.2 ± 0.3	5.2 ± 0.4
pH	3.21 ± 0.01	3.19 ± 0.01	3.21 ± 0.02 ^{a}	3.19 ± 0.01
Volatile acidity (g/L)	0.69 ± 0.03	0.68 ± 0.03	0.62 ± 0.09	0.64 ± 0.03
Reducing sugars (g/L)	1.8 ± 0.1 ^{ab}	1.8 ± 0.3 ^{ab}	1.9 ± 0.1 ^{b}	1.3 ± 0.4 ^{a}
I_{280}	9.05 ± 0.36 ^{c}	7.25 ± 0.93 ^{ab}	7.71± 0.31 ^{a}	8.95 ± 0.54 ^{bc}

On the other hand, the water addition methods managed a better reduction of ethanol content. Sc-5%W resulted in 0.47% (v/v) and Sc-10%W showed a reduction of 1.73% (v/v), both significantly lower compared to Sc-Control (Table 1). Schelezki et al (Schelezki et al., 2020a) reported a reduction of 1% (v/v) with a similar water addition in Shiraz musts than in our study (11.6% for early harvest and 10.2% (v/v) for late harvest). The addition of water has

been tested in other red wines with other percentages. For instance, an addition of 7.5% of water decreased ethanol content by 0.9% (v/v) in Shiraz wines (Schelezki et al., 2020a), an addition of 14% v/v resulted in a reduction of 2.1% (v/v) also in Shiraz wines (Teng et al., 2020) and 8% of water addition decreased the ethanol content by 1.1% v/v in Tempranillo wines (Piccardo et al., 2019). The results suggest that the effectiveness of ethanol reduction can depend on the fermentation approaches and grapes cultivars (Xynas and Barnes, 2022).

Regarding general chemical parameters as titratable acidity, pH or volatile acidity there were no significant differences among treatments. Regarding titratable acidity, a decrease was anticipated due to the dilution effect caused by the addition of water. However, it is worth noting that the concentration of titratable acidity could be lower in the Sc-Control and Mp+Sc treatments due to tartrate precipitation. Tartaric acid exhibits higher insolubility in ethanol, which means that as the ethanol content increases, the precipitation of tartrates becomes more pronounced compared to treatments with water addition. This way the reduction of acidity by tartaric precipitation in Sc-Control and Mp+Sc could be compensated by dilution effect in treatments with water addition, explaining the lack of statistical differences among treatments.

Nevertheless, I_{280} suffered the dilution effect of grape must being significantly higher in Sc-Control and Mp+Sc than in water treatments (Table 1). This behaviour as expected has been reported previously in water addition studies (Schelezki et al., 2020b).

3.3. Volatile compounds in wines

The relative abundance of the identified 57 volatile compounds was analysed (Table SD 2). As it was shown on table 2 the volatile compounds detected were grouped in following families: fusel alcohol acetate esters (7), ethyl esters (19), other esters (2), Short-Chain Fatty Acids (SCFA) (3) (acetic acid, isobutyric acid and isovaleric acid), Medium-Chain Fatty Acids (MCFA) (3) (octanoic acid, decanoic acid and dodecanoic acid), Alcohols (10), Aldehydes (2), Ketones (2) and Terpenes (8).

The first two latent variables (LV) of PLS-DA (Partial Least Squares-Discriminant Analysis) LV1 and LV2 explained the 58.48% of the total variance of the **Y**-block (classes of treatments) (Figure2A). Thus, LV1 was effective in discriminating between wines treated with Ss-control

and water, and those produced through sequential fermentation (Mp+Sc), which were separated. Most of the volatile compounds were positively correlated with the Mp+Sc treatment (Figure 2B). On the other hand, LV2 helped in differentiating Sc-control wines from Sc-10%W wines, while Sc-5%W wines occupied a middle position between them. In this case some esters, terpenes and MCFA were positively correlated with Sc-Control and Sc-5%W. On the contrary, compounds appear negatively correlated with Sc-10%W.

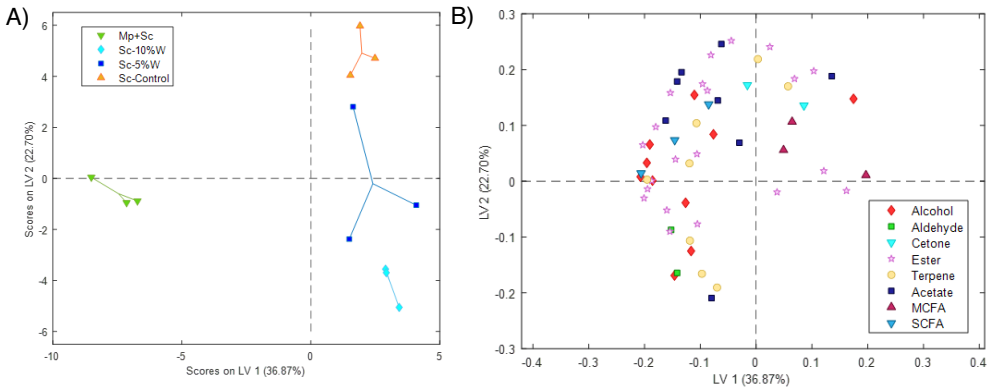


Figure 2. First two latent variables for the PLS-DA model (58.48% of the variance) of the volatile compounds. A) Scores and B) Loadings on the PLS-DA model. The Sc-Control represents wines fermented solely with S. cerevisiae, while Sc-5%W and Sc-10%W indicate wines with a pre-fermentative water addition of 5% and 10%, respectively. The Mp+Sc wines depict sequential fermentation with *M. pulcherrima* and *S. cerevisiae*. Means accompanied by standard deviations (SD) based on three replicates (n=3).

Regarding the relative abundance of volatile families, there were some interesting differences observed (Table 2). The presence of *M. pulcherrima* during AF appeared to increase the overall levels of fusel alcohols, which contributes to a floral aroma. This increase in higher alcohols has been previously reported in white wines fermented with *M. pulcherrima* in sequential culture with *S. cerevisiae* (Carpena et al., 2021; Contreras et al., 2014; Garciá et al., 2020; Hranilovic et al., 2020). Specifically, the presence of 2-phenyletanol in sequential fermentation with non-*Saccharomyces* yeasts has been attributed to *M. pulcherrima* (Padilla et al., 2016). Hranilovic et al. (Hranilovic et al., 2020) proposed that this effect could be linked to a response from sequential inoculation. Wines fermented with a combination of *M. pulcherrima* and *S. cerevisiae* (Mp+Sc) also exhibited an increase in total aldehydes, which contributes to a fruity aroma. Furthermore, certain

terpenes showed increased levels in Mp+Sc wines. This could be associated with the higher activity of β-glucosidase, activity described in *M. pulcherrima* (Comitini et al., 2011). In contrast, the abundance of ethyl esters was reduced in Mp+Sc wines which agrees with Tronchoni et al. (Tronchoni et al., 2018), who described a higher presence of ethyl esters in wines fermented with *S. cerevisiae* monocultures compared with wines than in sequential fermentations with *M. pulcherrima*. However, other studies have reported no variation or an increase in ethyl esters with the presence of *M. pulcherrima* (Contreras et al., 2014; Hranilovic et al., 2020). These findings suggest that the production of ethyl esters associated with *M. pulcherrima* may be influenced by the winemaking conditions. Furthermore, the abundance of MCFA was significantly reduced in Mp+Sc wines in accordance with several reports (Contreras et al., 2014; Hranilovic et al., 2020; Tronchoni et al., 2018). Specifically, Balmaseda et al. (2021d) observed a higher reduction in the white grape variety Macabeo compared to Cabernet sauvignon, due to the differences in the vinification process. This decrease is noteworthy because these compounds are known to be toxic to *Oenococcus oeni* and consequently reduce malolactic activity (Capucho and San Romão, 1994; Edwards and Beelman, 1987).

In the context of pre-fermentative water treatments, it has been observed that the reduction of volatile compounds varies among different families. Notably, total esters have shown a significant decrease when subjected to water treatments. This dilution effect has been previously described in water addition by other researchers (Gardner et al., 2022; Schelezki et al., 2020a). However other volatile families as acids, alcohols or aldehydes had not change in relation to the Sc-control. Moreover, it is worth highlighting that, apart from total terpenes, there are no significant differences observed between the two dilution percentages, Sc-5%W and Sc-10%W. This suggests that the effect of dilution with water prior to the AF on wine volatile compounds is not strongly influenced by the dilution percentage within this range.

Table 2. Volatile compounds expressed as relative abundance. The Sc-Control represents wines fermented solely with *S. cerevisiae*, while Sc-5%W and Sc-10%W indicate wines with a pre-fermentative water addition of 5% and 10%, respectively. The Mp+Sc wines depict sequential fermentation with *M. pulcherrima* and *S. cerevisiae*. Different lowercase letters indicate the existence of significant difference between the samples (p-value < 0.05). Data are expressed as the mean of three experimental replicates ± standard deviation.

	Sc-Control	Sc-5%W	Sc-10%W	Mp+Sc
∑ Acetate esters	22.3 ± 0.7 ^{b}	17.9 ± 0.3 ^{a}	17.4 ± 1.0 ^{a}	21.3 ± 1.5 ^{b}
∑ Ethyl esters	60.0 ± 2.6 ^{c}	44.1 ± 4.4 ^{ab}	40.1 ± 2.8 ^{a}	49.1 ± 2.6 ^{b}
∑ Other esters	1.1 ± 0.17 ^{a}	1.25 ± 0.10 ^{a}	1.04 ± 0.12 ^{a}	1.51 ± 0.05 ^{b}
∑ Total esters	83 ± 3.4 ^{c}	63.1 ± 4.6 ^{a}	58.4 ± 3.9 ^{a}	71.7 ± 2.9 ^{b}
∑ SCFA	0.21 ± 0.02	0.21 ± 0.04	0.16 ± 0.01	0.29 ± 0.06
∑ MCFA	13.6 ± 0.9 ^{b}	13.9 ± 1.9 ^{b}	12.9 ± 1.5 ^{ab}	9.7 ± 0.4 ^{a}
∑ Total acids	14.5 ± 1.1 ^{a}	14.6 ± 1.9 ^{a}	13.5 ± 1.5 ^{a}	10.6 ± 0.5 ^{b}
∑ Alcohols	23.8 ± 0.9 ^{a}	23.4 ± 1.6 ^{a}	23.1 ± 1.0 ^{a}	33.4 ± 3.0 ^{b}
∑ Aldehydes	0.19 ± 0.01 ^{a}	0.19 ± 0.02 ^{a}	0.28 ± 0.02 ^{a}	0.35 ± 0.08 ^{b}
∑ Ketones	0.14 ± 0.03	0.09 ± 0.03	0.10 ± 0.01	0.10 ± 0.01
∑ Terpenes	0.67 ± 0.07 ^{b}	0.59 ± 0.05 ^{ab}	0.64 ± 0.05 ^{a}	0.77 ± 0.07 ^{ab}

3.4. Soluble polysaccharides in wines

The method employed in this study enabled the identification of four distinct fractions containing polysaccharides from grapes and microorganisms (yeasts and bacteria) (Fanzone et al., 2012). Figure 3 illustrates these different fractions detected, each corresponding to a specific molecular weight range: the high-molecular-weight fraction (HMWf) with a number average molecular weight Mn = 158.7 ± 2.4 KDa, the medium-molecular-weight fraction (MMWf) with a Mn = 34.3 ± 0.6 KDa, the low-molecular-weight fraction (LMWf) with a Mn = 16.3 ± 0.6 KDa, and the oligosaccharide fraction (OLIGf) with a Mn = 5.9 ± 0.2 KDa.

In terms of total concentration, significant differences were observed among the Chardonnay wines examined in this study. Sequential fermentation with *M. pulcherrima* resulted in notably

higher concentrations of total polysaccharides. While slight differences were observed for MMWf and OLIGf, the higher increment was found in the HMWf fraction, which exhibited a 20.45% increase in Mp+Sc wines compared to the Sc-Control wine (Table 3). Previous studies have reported that non-*Saccharomyces* yeasts release more polysaccharides during AF, which are essentially mannoproteins (Domizio et al., 2014; García et al., 2017; Giovani et al., 2012). González-Royo et al. (2015) concluded that the presence of *M. pulcherrima* in white wine fermentation leads to an overall increase in total polysaccharides, with the most substantial increase observed in the HMWf fraction (Figure3).

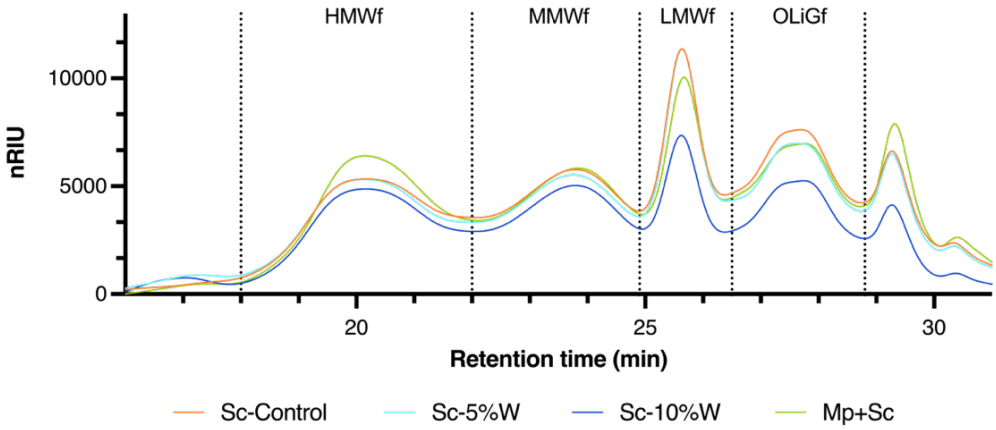


Figure 3. Molecular weight distribution of soluble polysaccharides fractions, by HRSEC-RID. The Sc-Control represents wines fermented solely with *S. cerevisiae*, while Sc-5%W and Sc-10%W indicate wines with a pre-fermentative water addition of 5% and 10%, respectively. The Mp+Sc wines depict sequential fermentation with *M. pulcherrima* and *S. cerevisiae*.

When observing the effects of water addition treatments, it was found that polysaccharides experienced a significant reduction. However, the fractions that exhibited the most significant variations were MMWf, LMWf and OLIGf (Table 3).

Moreover, noteworthy differences were observed between the different percentages of water addition (Figure 3). In the case of MMWf, a non-significant reduction of 4.8% was observed in Sc-5%W wines, while a larger reduction of 10.8% was observed in Sc-10%W wines. In the LMWf fraction, a 35.4% reduction was observed in Sc-5%W, compared to the higher reduction of 41.7% in Sc-10%W wines. Lastly, the OLIGf fraction experienced a 17.3% reduction in Sc-

5%W, whereas a more significant reduction of 32.7% was observed with a 10% water addition (Table 3). Piccardo et al. (2019), Schelezki et al. (2018) and Teng et al. (2020) also described a reduction in the content of polysaccharides in red wines with the addition of water.

Table 3. Polysaccharide fractions (mg/L). The Sc-Control represents wines fermented solely with *S. cerevisiae*, while Sc-5%W and Sc-10%W indicate wines with a pre-fermentative water addition of 5% and 10%, respectively. The Mp+Sc wines depict sequential fermentation with *M. pulcherrima* and *S. cerevisiae*. Different lowercase letters indicate the existence of significant difference between the samples (p-value < 0.05). Data are expressed as the mean of three experimental replicates ± standard deviation.

Fraction (mg/L)	Sc-Control	Sc-5%W	Sc-10%W	Mp+Sc
HMWf	43.3 ± 3.0 ^{a}	44.1 ± 2.4 ^{a}	40.8 ± 4.1 ^{a}	52.2 ± 0.7 ^{b}
MMWf	37.1 ± 1.7 ^{bc}	35.3 ± 0.8 ^{ab}	33.1 ± 3.1 ^{a}	40.3 ± 0.2 ^{c}
LMWf	31.6 ± 1.1 ^{b}	20.4 ± 4.4 ^{a}	18.4 ± 1.9 ^{a}	31.6 ± 5.2 ^{b}
OLIGf	41. 2 ± 1.0 ^{c}	34.1 ± 0.2 ^{b}	27.8 ± 2.5 ^{a}	43.2 ± 3.0 ^{c}
∑ Polysaccharides	153 ± 5 ^{b}	133 ± 6 ^{a}	128 ± 8 ^{a}	167 ± 6 ^{b}

3.5. Low-molecular-mass phenolic compounds in wines

The present study investigated the impact of different treatments on the phenolic composition of wines. Various phenolic compounds were identified, including hydroxybenzoic acids (Gallic acid and protocatechuic acid), hydroxycinnamic acids and derivatives (*trans*-caftaric acid, *trans*-coutaric acid, *cis*-coutaric acid, caffeic acid and hexose ester of *trans* p-coumaric acid), phenolic alcohols (tyrosol), flavanols (catechin, epicatechin and procyanidins) and flavonols (astilbin, quercetin and derivatives, and other flavanols).

The heatmap presented in Figure 4 illustrates the proportions of phenol compounds between different treatments. In the case of Mp+Sc wines, there was a tendency towards increased levels of flavanols, hydroxybenzoic acids and hydroxycinnamic acids. Conversely, a decrease was observed in flavonols and phenolic alcohols, although these differences did not reach statistical significance. However, two notable significant variations were observed. The content of epicatechin increased from 8.30 mg/L in the Sc-Control wine to 9.47 mg/L in the presence of *M. pulcherrima*. On the other hand, quercetin levels decreased from 0.92 mg/L in the Sc-Control wine to 0.61 mg/L in Mp+Sc wines (Table SD3).

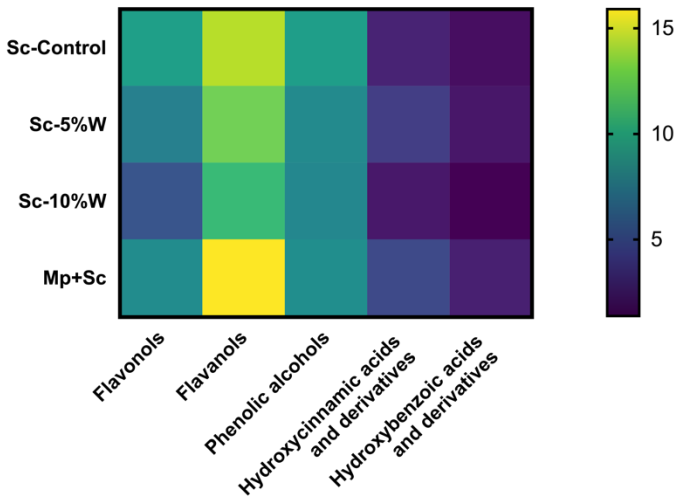


Figure 4. Heat map of low-molecular-mass phenolic compounds families detected in wines. The Sc-Control represents wines fermented solely with *S. cerevisiae*, while Sc-5%W and Sc-10%W indicate wines with a pre-fermentative water addition of 5% and 10%, respectively. The Mp+Sc wines depict sequential fermentation with *M. pulcherrima* and *S. cerevisiae*.

There have been relatively few studies analysing the modification of low-molecular-mass phenolic compounds in white wines with the presence of *M. pulcherrima*. However, it has been described that *M. pulcherrima* can increase polyphenolic content in red wines (Balmaseda et al., 2021b; Escribano-Viana et al., 2019). In addition, Sorrentino et.al (2012) reported an increase in certain phenolic compounds such as epicatechin, catechin and gallic acid, with the use of another species of *Metschnikowia* genera: *M. fruticola*. This increase has been attributed to higher polygalacturonase activity (Belda et al., 2016a) as suggested by some authors, although no statistical differences were observed for wine soluble polysaccharides, which should be also related with enzymatic activity through maturity and AF.

Regarding the Sc-5%W water treatment, there was a general decreasing trend observed in all low-molecular mass compounds, although no significant differences were found among the phenolic compound families (Figure 4). Nevertheless, specific phenols such as epicatechin, quercetin, and certain procyanidins showed reductions (Table SD2). On the contrary Sc-10%W wines exhibited lower values across all families of compounds (Figure 4), indicating a dilution effect like what has been observed in some volatile compounds and polysaccharides. These

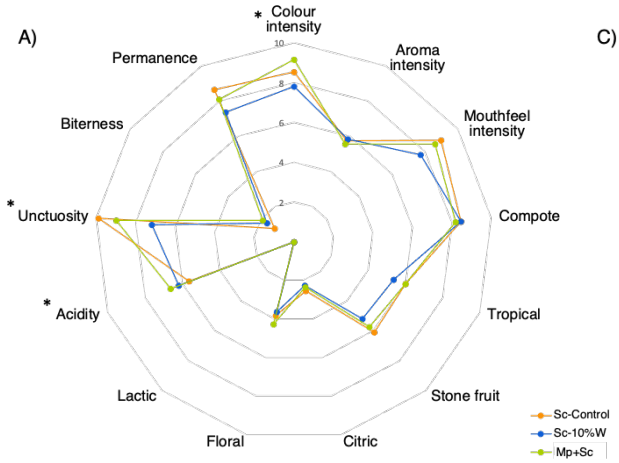
results would agree with the reported effects of water additions to the fermenting must, although this was observed in red wines (Piccardo et al., 2019; Schelezki et al., 2020b; Teng et al., 2020).

3.6. Sensory analysis

A triangular sensory analysis was conducted to assess the differences between the control and the treatments. The results showed that out of 30 tasters, 21 were able to differentiate the Mp+Sc wine from the Sc-Control, 14 were able to differentiate the Sc-10%W wine, and 12 were able to differentiate the Sc-5%W wine from the Sc-Control. Based on these results, only the Mp+Sc wine and Sc-10%W wine were found to be significantly different from the Sc-Control wine, with a significance level of p-value < 0.1. Following the triangular analysis, a descriptive analysis was performed by a professional tasting panel to further evaluate the sensory differences between the significantly different wines. The treatments compared in this analysis were the Sc-Control, Sc-10%W, and Mp+Sc, as depicted in Figure 4.

Figure 5A and 5B indicates that there were only significant differences between colour intensity, acidity and unctuosity. The Sc-10%W wines had significantly less unctuosity and colour intensity suggesting that the addition of water clearly influences the decrease of unctuosity. On the contrary, the acidity perception was not the higher, as could be expected. Mp+Sc wines were rated as the most acidic in comparison to the Sc-Control even the analytical value of total acidity was not different. Regarding aromas, although no significant differences were found between the conditions, there was a decreasing trend in aroma intensity for Sc-10%W wines, which aligns with the reduction in volatile compounds mentioned above. Other researchers studying red wines with added water also reported a decrease in flavour intensity, colour, and structural characteristics, which became more pronounced with higher water addition percentages, however, the tannin levels remained stable (Schelezki et al., 2020a, 2020b).

Substitution methods had fewer effects on the sensory profiles. Piccardo et al. (2019) described wines with addition or substitution water as having more vegetal and acidic characteristics. In red wines, sensory changes may be more pronounced due to the higher complexity compared to white wines.



B)

	Sc-control	Sc-10%W	Mp+Sc
Colour Intensity	ab	b	a
Unctuosity	ab	b	a
Acidity	a	ab	b

Figure 5. A) Spider plot of organoleptic parameters analysed in descriptive analysis. Asterisks (*) indicate significant differences between conditions: * *p*-value < 0.05. B) Letters indicating significant differences (p-*value* < 0.05) between conditions. C) Percentage of preference in the analysis of consumer preference. The Sc-Control represents wines fermented solely with *S. cerevisiae*, while Sc-10%W indicate wines with a pre-fermentative water addition of 10%. The Mp+Sc wines depict sequential fermentation with *M. pulcherrima* and *S. cerevisiae*.

Wines fermented with the presence of *M. pulcherrima*, the tropical and fruity notes were not significantly perceived, which is consistent with the lower ester concentrations compared to the Sc-Control wines. However, the floral notes were still present, possibly due to a high concentration of some fusel alcohols. In addition, it has been described in other sensory analysis that wines fermented in presence of *M. pulcherrima* had oxidation and spirit-like aromas (Tronchoni et al., 2018), which could be attributed to the high levels of isoamyl alcohol.

Finally, to validate the impact of water addition on the wine profile, we conducted a preference test, comparing Sc-Control with Sc-10%W. The results clearly demonstrated a strong preference for the Sc-Control sample without water addition, with a *p*-value of 5.6×10^{-5} (Figure 5C). This preference could be attributed to the higher levels of unctuosity observed in the Sc-Control sample. Interestingly, while a previous study by Niimi et al. 2017), indicated that an increase in body did not influence preference in red wine, our findings suggest that consumers associate unctuosity with quality specifically in white wine.

4. Conclusions

This study aimed to investigate different methods for reducing alcohol content in wines, including the use of *Metschnikowia pulcherrima* in sequential fermentation with *Saccharomyces cerevisiae* and the addition of water (5% and 10%) to the fermentative must. The experiments focused on Chardonnay wines, as there is a lack of research on these technologies in white wines. In order to simulate real conditions, the fermentative must was not sterilized, in order to study the reduction of ethanol and sensory modifications of treatments under competitive pressure of endogenous microorganisms.

Our findings indicated that the presence of *M. pulcherrima* in alcoholic fermentation was less effective in reducing ethanol, likely due to the presence of other yeast species in the must. However, the resulting wines had different compositions, with higher levels of HMWf polysaccharides and a tendency towards increased concentrations of certain phenolic compounds, particularly epicatechin. In terms of volatile compounds, there was an increase in alcohols, which could be linked to heightened floral notes of the wines in sensory analysis. Regarding pre-fermentative water addition, the Sc-5%W condition, which reduced ethanol by 0.47 ± 0.06% (v/v), showed promising results in terms of analytical parameters, with no significant differences observed in low-molecular-weight phenolic compounds. This condition also exhibited a slighter reduction trend in volatile compounds and polysaccharides compared to the Sc-10%W wines, which had an ethanol reduction of 1.73 ± 0.10% (v/v). Wines with a pre-fermentative water addition of 10% were described as less complex in sensory analysis, showing a decreasing trend in all analysed organoleptic parameters.

These results demonstrate that adding high percentages of water leads to a general decrease in the concentration of most wine components, although it can also increase wine production, which may pose a challenge. However, the addition of lower percentages of water, such as 5% or even less, can effectively reduce ethanol content without significantly altering the organoleptic profile of the wines. Further research could explore the combination of low water addition percentages with the use of non-*Saccharomyces* yeast, such as *M. pulcherrima*, to achieve both ethanol reduction and improvements in organoleptic

characteristics. This line of research is important considering the rapid progress of climate change and the limited approval rates for these methods in most wine-producing countries.

Acknowledgments

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Supplementary data

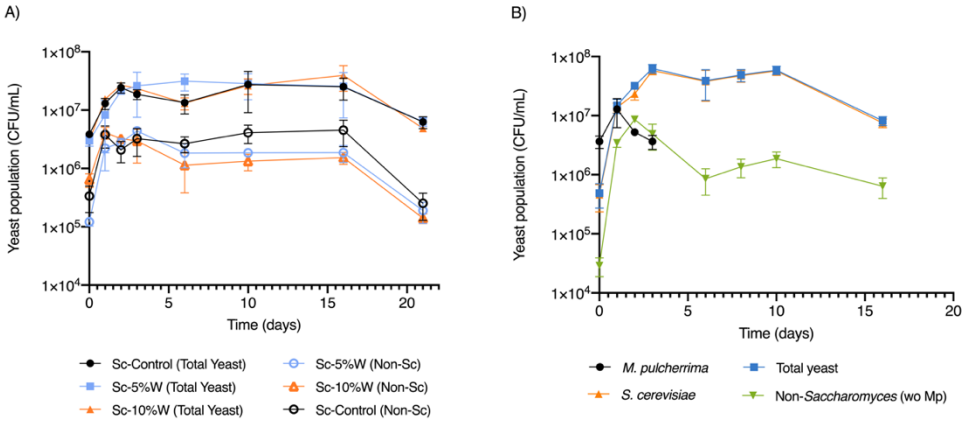

Figure SD1. A) Sc-Control, Sc-5%W and Sc-10%W yeast populations during AF. B) Mp-Sc yeast populations during AF. The Sc-Control represents wines fermented solely with *S. cerevisiae*, while Sc-5%W and Sc-10%W indicate wines with a pre-fermentative water addition of 5% and 10%, respectively. The Mp+Sc wines depict sequential fermentation with *M. pulcherrima* and *S. cerevisiae*. Means accompanied by standard deviations (SD) based on three replicates (n=3).

Table SD1. Maximal Consumption Rate (g/L/day) and days to consume 50% of sugars (T50%). The Sc-Control represents wines fermented solely with *S. cerevisiae*, while Sc-5%W and Sc-10%W indicate wines with a pre-fermentative water addition of 5% and 10%, respectively. The Mp+Sc wines depict sequential fermentation with *M. pulcherrima* and *S. cerevisiae*. Different lowercase letters indicate the existence of significant difference between the samples (*p-value* < 0.05). Data are expressed as the mean of three experimental replicates ± standard deviation.

	Sc-Control	Sc-5%W	Sc-10%W	Mp+Sc
Max. Consumption Rate (g/L/day)	13.6 ± 0.5 ^{b}	13.4 ± 0.3 ^{ab}	12.4 ± 0.1 ^{a}	26.2 ± 0.6 ^{c}
T 50% (days)	5.44 ± 0.02 ^{b}	5.45 ± 0.07 ^{b}	5.69 ± 0.04 ^{c}	3.75 ± 0.04 ^{a}

Table S2. Volatile compounds detected in wines (relative area). The Sc-Control represents wines fermented solely with *S. cerevisiae*, while Sc-5%W and Sc-10%W indicate wines with a pre-fermentative water addition of 5% and 10%, respectively. The Mp+Sc wines depict sequential fermentation with *M. pulcherrima* and *S. cerevisiae*. Data are expressed as the mean of three experimental replicates ± standard deviation.

	Sc-Control	Sc-5%W	Sc-10%W	Mp+Sc
Esters				
Acetate esters				
Ethyl acetate	5.23 ± 0.14	4.10 ± 0.57	4.21 ± 0.22	5.28 ±0.30
Isobutyl acetate	0.31 ± 0.03	0.27 ± 0.03	0.09 ± 0.01	0.37 ± 0.02
Isoamyl acetate	8.01 ± 0.27	5.92 ± 0.24	6.11 ± 0.43	7.33 ± 0.44
Hexyl acetate	3.02 ± 0.20	2.13 ± 0.59	2.39 ± 0.19	1.84 ± 0.11
3-Hexenyl acetate	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
Heptyl acetate	0.05 ± 0.01	0.04 ± 0.02	0.04 ± 0.01	0.05 ± 0.01
2-Phenethyl acetate	5.67 ± 0.42	5.35 ± 0.62	4.46 ± 0.40	6.37 ± 0.65
Ethyl esters				
Ethyl butanoate	0.58 ± 0.02	0.38 ± 0.03	0.41 ± 0.01	0.54 ± 0.02
Ethyl hexanoate	10.47 ± 0.73	7.15 ± 2.17	7.82 ± 0.43	8.26 ± 0.60
Ethyl heptanoate	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ±0.01
Ethyl octanoate	30.22 ± 0.68	20.13 ± 5.85	20.03 ± 2.06	25.91 ± 2.13
Ethyl 7-octenoate	0.11 ± 0.01	0.09 ± 0.02	0.07 ± 0.01	0.10 ± 0.01
Ethyl nonanoate	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
Ethyl decanoate	13.27 ± 2.04	10.58 ± 2.16	8.59 ± 0.59	9.84 ± 2.11
Ethyl 4E-decenoate	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.08 ± 0.01
Ethyl dodecanoate	4.93 ± 1.23	5.19 ± 0.33	2.71 ± 0.24	3.51 ± 0.88
Ethyl tetradecanoate	0.15 ± 0.08	0.15 ± 0.03	0.10 ± 0.01	0.20 ± 0.02
Ethyl hexadecanoate	0.16 ± 0.10	0.25 ± 0.01	0.22 ± 0.01	0.36 ±0.02
Other esters				
Isoamyl octanoate	0.32 ± 0.08	0.42 ± 0.06	0.42 ± 0.05	0.48 ± 0.04
Isoamyl decanoate	0.16 ± 0.04	0.20 ± 0.01	0.17 ± 0.01	0.10 ± 0.02
Isobutyl hexanoate	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.05 ± 0.01
Isobutyl octanoate	0.07 ± 0.02	0.06 ± 0.01	0.05 ± 0.01	0.10 ± 0.01
Butyl 9-decenoate	0.04 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.14 ± 0.01
Methyl octanoate	0.15 ± 0.01	0.13 ± 0.01	0.13 ± 0.03	0.16 ± 0.01
Methyl decanoate	0.12 ± 0.04	0.14 ± 0.02	0.10 ± 0.03	0.08 ± 0.02
Methyl 4-decenoate	0.04 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	0.07 ± 0.01
Methyl undecanoate	0.06 ± 0.01	0.09 ± 0.02	0.04 ± 0.01	0.15 ± 0.03

Alcohols				
Isobutanol	0.50 ± 0.02	1.51 ± 0.20	1.38 ± 0.11	2.35 ± 0.15
Isoamyl alcohol	11.65 ± 0.14	10.98 ± 1.16	11.03 ± 0.70	13.79 ± 1.06
Isohexanol	0.05 ± 0.03	0.04 ± 0.02	0.03 ± 0.01	0.05 ± 0.01
1.3-Methyl-4-penten-1-ol	0.14 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.09 ± 0.01
1-Hexanol	0.66 ± 0.02	0.66 ± 0.07	0.67 ± 0.04	0.71 ± 0.03
1-Heptanol	0.13 ± 0.02	0.12 ± 0.01	0.12 ± 0.02	0.22 ± 0.04
2.3-Butanediol	0.21 ± 0.12	0.11 ± 0.05	0.04 ± 0.02	0.21 ± 0.02
1-Octanol	0.10 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.12 ± 0.01
1-decanol	0.09 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.11 ± 0.01
2 -phenylethanol	10.75 ± 0.86	9.71 ± 1.46	9.50 ± 0.34	15.72 ± 1.75
Acids				
Acetic acid	0.65 ± 0.08	0.46 ± 0.21	0.37 ± 0.02	0.62 ± 0.09
SCFA				
Isobutyric acid	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.09 ± 0.01
Isovaleric acid	0.16 ± 0.02	0.16 ± 0.03	0.12 ± 0.01	0.20 ± 0.01
MCFA				
Hexanoic acid	1.33 ± 0.08	1.29 ± 0.23	1.12 ± 0.12	1.09 ± 0.11
Octanoic acid	7.22 ± 0.50	7.26 ± 0.87	6.75 ± 0.77	6.29 ± 0.61
Decanoic acid	5.08 ± 0.44	5.34 ± 0.84	5.09 ± 0.64	2.26 ± 0.04
Aldehydes				
3-methyl-benzaldehyde	0.19 ± 0.01	0.19 ± 0.02	0.23 ± 0.03	0.29 ± 0.02
4-ethyl-benzaldehyde	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.06 ± 0.01
Ketones				
Isovalerone	0.11 ± 0.01	0.07 ± 0.03	0.09 ± 0.01	0.10 ± 0.01
2-Undecanone	0.03 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
Terpenes				
Mesitylene	0.32 ± 0.13	0.23 ± 0.12	0.27 ± 0.02	0.23 ± 0.06
Benzocyclobutene	0.12 ± 0.02	0.08 ± 0.03	0.08 ± 0.01	0.09 ± 0.01
o-Cymene	0.04 ± 0.02	0.10 ± 0.07	0.07 ± 0.05	0.13 ± 0.01
Durene	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
Linalol	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
Terpinen-4-ol	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.05 ± 0.01
Caryophyllene	0.05 ± 0.02	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01
Nerolidol	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
Viridiflorene	0.03 ± 0.02	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01

Chapter 3.2

Impact of Ethanol Reduction Practices on Malolactic Fermentation: Comparative Analysis of Pre-Fermentative Water Addition and *Metschnikowia pulcherrima* inoculation

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Manuscript in preparation

Abstract

Climate change poses significant challenges to the wine industry, with rising temperatures, sugar content, and declining water availability impacting alcohol levels, acidity, and fermentation processes. Despite various techniques proposed for alcohol reduction, their impact on malolactic fermentation (MLF) remains understudied. Stressful compounds in wine, particularly ethanol, can hinder MLF progression. This study aims to investigate the effects of alcohol reduction strategies on MLF dynamics, focusing on strain-dependent responses of *Oenococcus oeni*. Analysing aromatic differences post-alcoholic fermentation (AF) and their influence on MLF performance, along with changes in aroma composition post-MLF, offers insights into the impact of alcohol reduction practices on final wine composition. Notably, significant shifts in aroma composition were observed post-MLF compared to wines analysed after AF. Surprisingly, the less stressful conditions resulting from water additions led to enhanced aroma production, including acetates esters and fusel alcohols. Water addition conditions exhibited improved *O. oeni* performance in terms of process kinetics and aroma production. Furthermore, the sequential inoculation strategy involving *M. pulcherrima* showed its potential for enhancing MLF.

Key words

Oenococcus oeni; esters; aromatic composition; alcohol reduction; climate change

1. Introduction

Climate change presents an important concern to the wine industry. Increasing temperatures and sugar content, as well as decreasing water availability contribute to increased alcohol levels and reduced acidity, and disparities between technological, aromatic, and phenolic fermentations (Droulia and Charalampopoulos, 2022; Mira de Orduña, 2010). Among these challenges, the increase of alcohol content stands out as the main issue. Various techniques for alcohol reduction have been proposed, encompassing viticultural practices, such as pruning management (Sun et al., 2023), physical practices, such as adding water to grape must (Xynas and Barnes, 2022), and microbiological methods, such as the use of some non-*Saccharomyces* species (Contreras et al., 2014). However, slight attention has been devoted to comprehensively investigating the impact of these alcohol reduction approaches on malolactic fermentation (MLF).

MLF involves the decarboxylation of L-malic acid into L-lactic acid. It is a fundamental process mainly performed in red wines and occasionally in specific white wines, such as certain Chardonnay wines (Semon et al., 2001). This transformation is carried out by lactic acid bacteria (LAB), with *Oenococcus oeni* as the predominant species in the wine environment, due to its remarkable adaptation to wine conditions (high acidity and alcoholic content, scarce nutrient availability, etc,). Nonetheless, in wine there are several stressful compounds for LAB including *O. oeni* which could potentially delay the performance of MLF (Margalef-Català et al., 2017; Olguín et al., 2010; Reguant et al., 2000, 2005). Ethanol is one of the main stress factors and consequently its reduction could ultimately enhance the activity of *O. oeni*.

It is important to note that the methods employed to reduce ethanol levels may sometimes trigger alterations in the resulting composition of the wine after the alcoholic fermentation (AF). These changes could subsequently impact the activity of *O. oeni* and therefore influence the progression of MLF, as well as the sensory qualities of the final wine. This might include adjustments in inhibitory or stimulatory compounds for *O. oeni*. Moreover, the complex metabolic pathways of *O. oeni* exhibit diverse responses to variations in wine composition (Bech-Terkilsen et al., 2020). These responses are strain-dependent and ranges from adaptation responses to stress to the development of secondary metabolisms. For instance, its

β-glucosidase and esterase capacity, which can catalyse hydrolysis and synthesis of esters, is an attribute that could exert a modulating influence on the aromatic profile of wines post MLF. It has been described to be influenced by the composition of wine after AF (Fia et al., 2018).

In light of these considerations, the objective of this short communication is to study the impact of alcohol reduction strategies tested in a preceding study (Ruiz-de-Villa, 2024c) on the subsequent dynamics of MLF. To discern potential strain-dependent effects, the evaluation encompasses two distinct *O. oeni* strains: a commercially available strain and a model strain.

2. Materials and methods

2.1. Alcoholic fermentation

For a comprehensive understanding of the experimental setup and analytical techniques employed during alcoholic fermentation, readers are directed to the previous publication (Ruiz-de-Villa et al., 2024c). Briefly, regarding the wine production, Chardonnay wines were produced from natural grape must supplied by *Viña Correa Albano* from the Maule region, Chile. Four distinct alcoholic fermentation conditions were explored: a control condition (Sc-Control) fermented solely with *S. cerevisiae* (Lalvin QA23, Lallemand Inc., Montreal, Canada); two conditions involving the addition of 5% and 10% water to the grape must (Sc-5%W and Sc-10%W, respectively) and a sequential fermentation involving *M. pulcherrima* (Mp+Sc), with initial inoculation of *M. pulcherrima* (Level2 Flavia, Lallemand Inc) followed by *S. cerevisiae* after two days. *S. cerevisiae* was inoculated at a concentration of 2 x 10^6 cell/mL and *M. pulcherrima* at 1 x 10^7 cell/mL.

2.2. Malolactic fermentation

For this study, replicas of these wines were blended with the addition of 0.8 mg/L of potassium metabisulfite ($K_2S_2O_5$, Panreac, Química SLU, Castellar del Vallés, Spain) and stabilized at 4 °C for one week. Subsequently, the L-malic acid content was adjusted to 2 g/L to ensure a more accurate comparison of malolactic fermentation (MLF) kinetics. Following the L-malic acid correction, the pH was readjusted using concentrated NaOH to match the original levels post-AF for each respective condition. Then, the wines were

divided in triplicates in 3 L food-grade plastic tanks at 20 °C and separately inoculated with two strains of *O. oeni*: Lalvin VP41 (OoV) from Lallemand and PSU-1 ATCC BAA-331 (OoP).

Consequently, the initial four conditions led to the establishment using two *O. oeni* strains of eight distinct MLF conditions, which were performed per triplicate: Control-OoV, 5%W-OoV, 10%W-OoV, Mp+Sc-OoV, Control-OoP, 5%W-OoP, 10%W-OoP, and Mp+Sc-OoP.

The inoculation of MLF conditions was carried out at a concentration of 2 x 10^7 UFC/mL. The *O. oeni* strains were precultured in an MRS broth medium (De Man et al., 1960) (Panreac), supplemented with 4 g/L of DL-malic acid (Sigma–Aldrich, Santiago de Chile, Chile) and 5 g/L of D-fructose (Panreac), maintaining a pH of 5. The *O. oeni* populations were evaluated using MRS plates and both the inocula and plates were cultivated under conditions of 28 °C with a CO_2-enriched environment.

The progression of the process was monitored through daily enzymatic measurements of L-malic acid consumption (Biosystems S.A., Barcelona, Spain). The completion of MLF was determined upon reaching a L-malic acid concentration of < 0.1 mg/L.

2.3. Analysis of volatile compounds

The volatile compounds after MLF were analysed using the procedure described in (Ubeda et al., 2019). A 7.5 mL of wine sample was utilized, to which 10 µL of 4-methyl-2-pentanol (0.75 mg/L, Sigma-Aldrich) was added as an internal standard. To extract the volatile compounds, the wine samples underwent a pre-treatment process using Headspace Solid Phase Microextraction (HS-SPME). A 2 cm 50/30 µm Carboxen/ Divinylbenzene/ Polydimethylsiloxane (CAR/DVB/PDMS) SPME fiber (Supelco, Bellefonte, PA, USA) was employed. The headspace sampling procedure employed an autosampler, with the vial incubated at 45 °C for 20 minutes and agitated at 500 rpm. Subsequently, injection was carried out in spitless mode for 3 minutes, with a transfer line temperature of 280 °C. Volatile compounds were detected by Selective Ion Monitoring (SIM) mode using a 7890B Agilent GC system coupled with a quadrupole mass spectrometer Agilent 5977 inert (Agilent Technologies, Palo Alto, CA, USA) was utilized. A DB Wax capillary column (60 m × 0.25 mm

x 0.25 µm film thickness, J & W Scientific, Folsom, CA, USA) was employed, and Helium was used as the carrier gas at a flow rate of 1 mL/min.

Identification of metabolites was performed using the NIST17 library of MS ChemStation software. (Agilent Technologies). The data were presented as relative area values. To compute the relative area, the peak area of the primary ion of each compound was divided by the peak area of the main ion of the internal standard, thereby normalizing it.

2.4. Sensorial analysis

To find potential organoleptic differences between the two MLF conditions, a triangular sensory analysis was conducted. The panel comprised 30 tasters, including trained experts. Treatments were compared to identify significant differences by using a binomial test. To mitigate any visual subjectivity, the wines were presented in opaque glasses and labelled with randomized 3-digit codes.

2.5. Statistical analysis

Each set of conditions was replicated in triplicate biological samples. The collected data were analysed using a two-way ANOVA, followed by a post hoc Tukey test (Honest Significant Difference) at a 95% confidence interval, with significance determined at a p-value < 0.05. Discrimination of samples based on volatile compounds was performed by using PLS-DA. The statistical software employed for these analyses was XLSTAT version 2022.5.1 (Addinsoft, Paris, France).

3. Results and discussion

3.1. Malolactic fermentation performance

The consume of L-malic was quite different between two tested strains (Figure 1), indicating that there are differences between strains for the same AF conditions. Moreover, since each MLF condition had distinct kinetics, the resulting wines after the different AF conditions changed their compositions, leading to either enhancement or alteration of this process (Table 1).

Table 1. Main parameters after alcoholic fermentation which could have an effect on malolactic fermentation. Wine conditions: Control represents wines fermented solely with *S. cerevisiae*, while 5%W and 10%W indicate wines with a pre-fermentative water addition of 5% and 10%, respectively. The Mp wines corresponds to sequential fermentation with *M. pulcherrima* and *S. cerevisiae*. MCFA corresponds to Medium Chain Fatty Acids expressed as relative abundance. Different letters indicate statistical differences for each parameter (p-value < 0.05).

	Control	5%W	10%W	Mp
Ethanol % (v/v)	13.93 ± 0.21 ^{c}	13.53 ± 0.15 ^{b}	12.20 ± 0.10 ^{a}	13.67 ± 0.06 ^{bc}
pH	3.21 ± 0.01	3.19 ± 0.01	3.21 ± 0.02 ^{a}	3.19 ± 0.01
MCFA	13.62 ± 0.99 ^{b}	13.89 ± 1.92 ^{b}	12.96 ± 1.53 ^{ab}	9.65 ± 0.45 ^{a}
Polysaccharides (mg/L)	153 ± 5 ^{b}	133 ± 6^{a}	128 ± 8 ^{a}	167 ± 6 ^{b}

On one hand, the fermentations inoculated with OoV strain finished under all conditions. However, the total duration of MLF show significant differences between conditions (Figure 1). Notably, the reduction in alcohol content exhibited a clear effect; the condition involving a 10% addition of water (10%W) concluded MLF within four days, marking a considerable reduction in time compared to Control-OoV conditions, which took nine days to complete MLF. This effect can be attributed to the decrease in alcohol content, dropping from 13.93% to 12.20% (v/v) (Table 1). A similar effect was observed in the case of 10%W-OoP and Control-OoP. The former lasted nine days to complete MLF, while the latter faced a stop of MLF at 1.36 g/L of L-malic acid. Ethanol exhibited a toxic effect on *O. oeni* in wine since it interacts with the lipids of the membrane tending to be more fluid (Capucho and San Romão, 1994). Consequently, certain processes are affected, leading to the leakage of intracellular compounds such as ions, which produces internal acidification, which leads to rigidity of the membrane.

The main impact is related with the destabilization of the membrane since the specific changes to cell wall of *O. oeni* in response to ethanol remain relatively unexplored. Previous studies have identified the activation of cell wall-associated genes as a response to ethanol (Margalef-Català et al., 2017). An inhibitory effect of 11.8% (v/v) ethanol at a pH of 3.2 was described by Solieri et al. (2010), and similarly, at the same pH, Knoll et al. (2011) observed stop of MLF at 9.86% and 11.83% (v/v) with two different commercial *O. oeni* strains, respectively. In our conditions, at the same pH but with higher ethanol content we have

observed only two stops of MLF, Control-OoP and 5%W-OoP, suggesting that the different strains have different tolerance to ethanol.

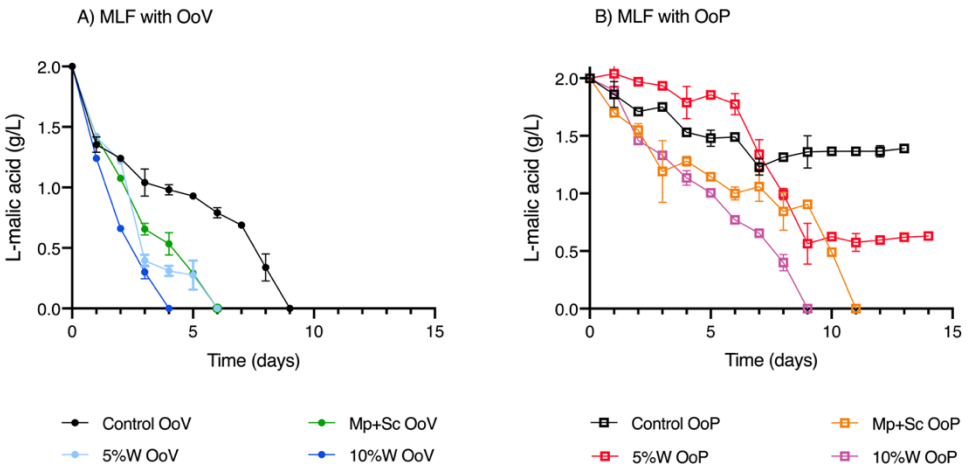

Figure 14. Kinetics of malolactic fermentation (MLF). A) MLF inoculated with OoV and B) MLF inoculated with OoP. Wine conditions: Control represents wines fermented solely with *S. cerevisiae*, while 5%W and 10%W indicate wines with a pre-fermentative water addition of 5% and 10%, respectively. The Mp wines corresponds to sequential fermentation with *M. pulcherrima* and *S. cerevisiae*. Oo corresponds to *Oenococcus oeni* strains inoculated to perform MLF, OoV and OoP. Means ± Standard deviation of three biological replicates.

In the case of the 5%W-OoV and Mp+Sc-OoV conditions, they exhibited a similar pattern, with both concluding MLF in six days (Figure 1A). Regarding 5%W-OoV, the shorter MLF performance in comparison to the Control-OoV could likely be attributed to its lower alcohol content. However, the scenario with Mp+Sc posed an interesting contrast. Although there was a trend towards lower alcohol content (Ruiz-de-Villa, et.al., 2024c) compared to the Control-OoV condition, the difference was not statistically significant. Consequently, alternative factors may be responsible for the enhanced MLF performance. The influence of non-*Saccharomyces* species on MLF has been explored, notably with *T. delbrueckii* (Balmaseda et al., 2021b; Ruiz-de-Villa et al., 2023b), and to a lesser extent, with *M. pulcherrima* (Balmaseda et al., 2021d). This positive effect of non-*Saccharomyces* in MLF is due to their influence in specific compounds implicated in *O. oeni* activity. In this case, a significant reduction in medium-chain fatty acids (MCFA), recognized for their potential toxicity for *O. oeni* (Capucho and San Romão, 1994; Guilloux-Benatier et al., 1998) was

observed, alongside an increase trend in polysaccharides (Table 1). Quantified polysaccharides include polysaccharides of grape must and those from the cell wall released from yeast autolysis. In this context, despite the total was not significant in comparison to the Control (Table 1), the high and medium-molecular-weight fraction (HMWf and MMWf) of those polysaccharides were increased significantly in Mp condition (Ruiz-de-Villa et al., 2024c). This increase could potentially boost MLF performance by detoxifying the environment of compounds such as MCFA (Lafon-Lafourcade et al., 1984). Furthermore, it has been documented that the metabolism of mannoproteins (Balmaseda et al., 2021a; Jamal et al., 2013) could stimulate *O. oeni* growth (Diez et al., 2010). Notably, a substantial increase in mannoprotein release during AF by non-*Saccharomyces* strains has been reported, with a particularly noteworthy correlation between higher mannoprotein content in wines fermented with *T. delbrueckii* and improved MLF performance. The effect becomes even more evident with the same ethanol content (about 13.6 %, v/v) when considering the use of OoP, specifically, in the case of Mp+Sc-OoP, contrasted with 5%W-OoP. While Mp+Sc-OoV completed MLF, 5%W-OoP suffered a stop of MLF at 0.5 g/L of L-malic acid and Control-OoP at 1.36 g/L of L-malic acid. This trend could potentially be attributed to the same metabolites.

3.2. Volatile compounds after MLF

To better understand the impact of inoculating two distinct *O. oeni* strains, an analysis of volatile compounds was undertaken to explain their contribution to aroma attributes.

Initially, a comparison was conducted between the volatile compounds analysed in samples after AF in the previous study (Ruiz-de-Villa, et al. 2024c) and those analysed after MLF. Subsequently, we further examined the variations within MLF conditions by comparing the effects of OoV and OoP inoculation. Both comparative analyses were conducted using the same statistical analysis. First, ANOVA was employed to discern significant differences among conditions in relation to their individual volatile compounds, data shown in supplementary material (Table SD1). Nonetheless, owing to the difficulties in interpreting the data due to its dimensionality, a Principal Component Analysis (PCA) was conducted (data not shown). However, the considerable variability of the samples due to their inherent nature posed challenges in observing inter-group differences and assessing characteristic volatile profiles.

Consequently, a Partial Least Square-Discriminant Analysis (PLS-DA) was subsequently carried out. This aimed to not only discern disparities between volatile compounds following AF and MLF, but also to show the variance attributed to the groups studied, thereby facilitating the assessment of the volatile profiles of each group (Figures 2 and 3).

The comparison between wines after AF and MLF revealed variations in specific volatile compounds among wines (Table SD1). The first two latent variables (LV) of PLS-DA, LV1 and LV2, explain the 47.36% of the total variance of the **Y**-block (classes of treatments) (Figure 2A). Therefore, LV1 effectively distinguished wines post-AF from those post-MLF. It is evident that most volatile compounds exhibited a positive correlation with samples after MLF, indicating an increase subsequent to MLF. A significant enhancement was observed in the total acetates esters, with specific esters of fatty acids displaying the same trend.

Interestingly, it was observed that even in some cases MLF was not completed (Control-OoP and 5%W-OoP) there were changes in volatile composition. Knoll et al., (2011) described that even a partial MLF was conducted, enzymatic changes were produced due to the presence of LAB in the media.

Wine LAB have been demonstrated to elevate ester content through enzymatic activities involving esterases and alcohol acetyltransferases (Matthews et al., 2004; Pozo-Bayón et al., 2005). After MLF, there was a significant increase in ethyl butanoate, associated with apple-like and pineapple aromas, as well as ethyl octanoate, with a concurrent rise in ethyl hexanoate, both linked to fruity aromas (Table SD1). Previous research highlights that *O. oeni* metabolism contributes to the increment of ethyl octanoate and hexyl octanoate (Costello et al., 2013; Lerm et al., 2010).

Sumby et al., (2013)revealed that purified esterases of *O. oeni* exhibit dual activity involving the synthesis and hydrolysis of ethyl butanoate, ethyl hexanoate, and ethyl octanoate. An increase of ethyl esters has been also described in similar conditions, Chardonnay wines with pH 3.2, however these authors observed the increase specifically on acetic acid ethyl esters (Knoll et al., 2011).

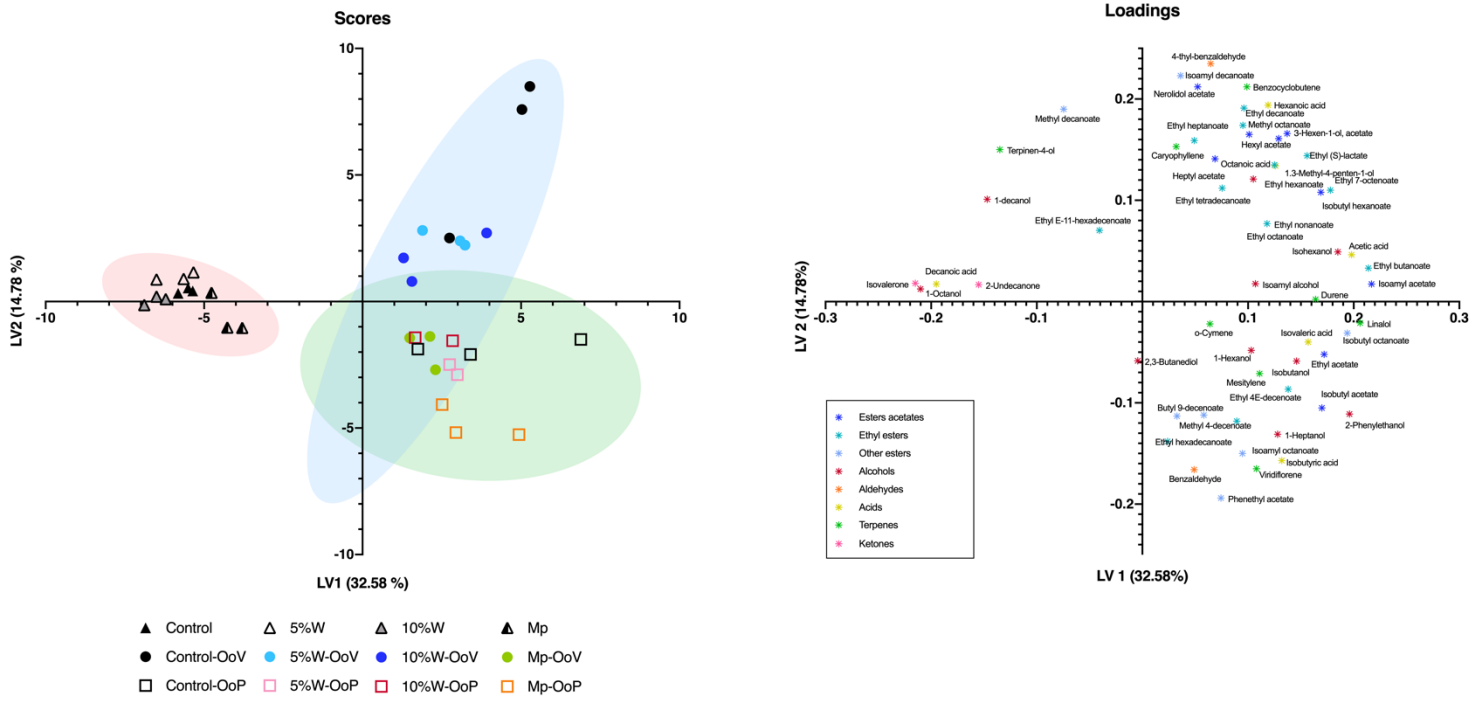

Figure 2. First two latent variables for the PLS-DA model (% of the variance) of the volatile compounds after alcoholic fermentation (red ellipse) and malolactic fermentation. A) Scores: Control represents wines fermented solely with *S. cerevisiae*, while 5%W and 10%W indicate wines with a pre-fermentative water addition of 5% and 10%, respectively. The Mp wines corresponds to sequential fermentation with *M. pulcherrima* and *S. cerevisiae*. Oo corresponds to *Oenococcus oeni* strains inoculated to perform MLF, OoV (Blue ellipse) and OoP (Green ellipse); and B) Loadings on the PLS-DA model.

However, according to the literature, ethyl esters demonstrated a more substantial increase post-MLF than acetate esters (Cappello et al., 2017; Lerm et al., 2010; Liu, 2002) although in this study an increment in some acetates esters has been observed (e.g. ethyl acetate, isobutyl acetate and isoamyl acetate). Nevertheless, some authors have also reported increases in ethyl acetate and isoamyl acetate (Delaquis et al., 2000; Maicas et al., 1999).

Ethyl lactate was detected in samples which have been performed MLF, as expected (Cappello et al., 2017; Delaquis et al., 2000; Maicas et al., 1999), which is associated with fruitiness, milky aromas and increased mouthfeel.

Regarding fusel alcohols, there were observed an increasing trend after MLF, especially on Control and Mp conditions, which have more alcohol content. Specially, there were observed significant differences in 2-phenylethanol, which was higher in samples with MLF, especially in Mp conditions. However, between Control and samples with water treatments (5%W and 10%W), was incremented in the same extend. This aligns with Knoll et al. (2011), who observed higher increment of this compound in wines with lower ethanol content.

Terpenes, however, demonstrated a converse effect, with linalool increasing post-MLF while terpen-4-ol decreased, and in most cases, was undetectable. It is known that *O. oeni* possesses glycosidase activities which positively influence wine aroma by hydrolysing grape-derived aroma precursors like monoterpenes.

Few changes were detected in SCFA and MCFA, however there was detected an increasing trend in octanoic acid and remarkable significant decrease in decanoic acid. The decrease in decanoic acid after MLF has been previously reported by other authors.

Together with the decrease of decanoic acid other compounds were negatively correlated with wines after MLF, such as ketones which were identified after AF but not after MLF.

All these differences together with other trends revealed the different volatile composition of wines after MLF in comparison to AF, as LV1. On the other hand, LV2 revel the differences between AF conditions, even in wines after MLF. Furthermore, in LV2 was observed a separation between wines inoculated with OoV and those inoculated with OoV.

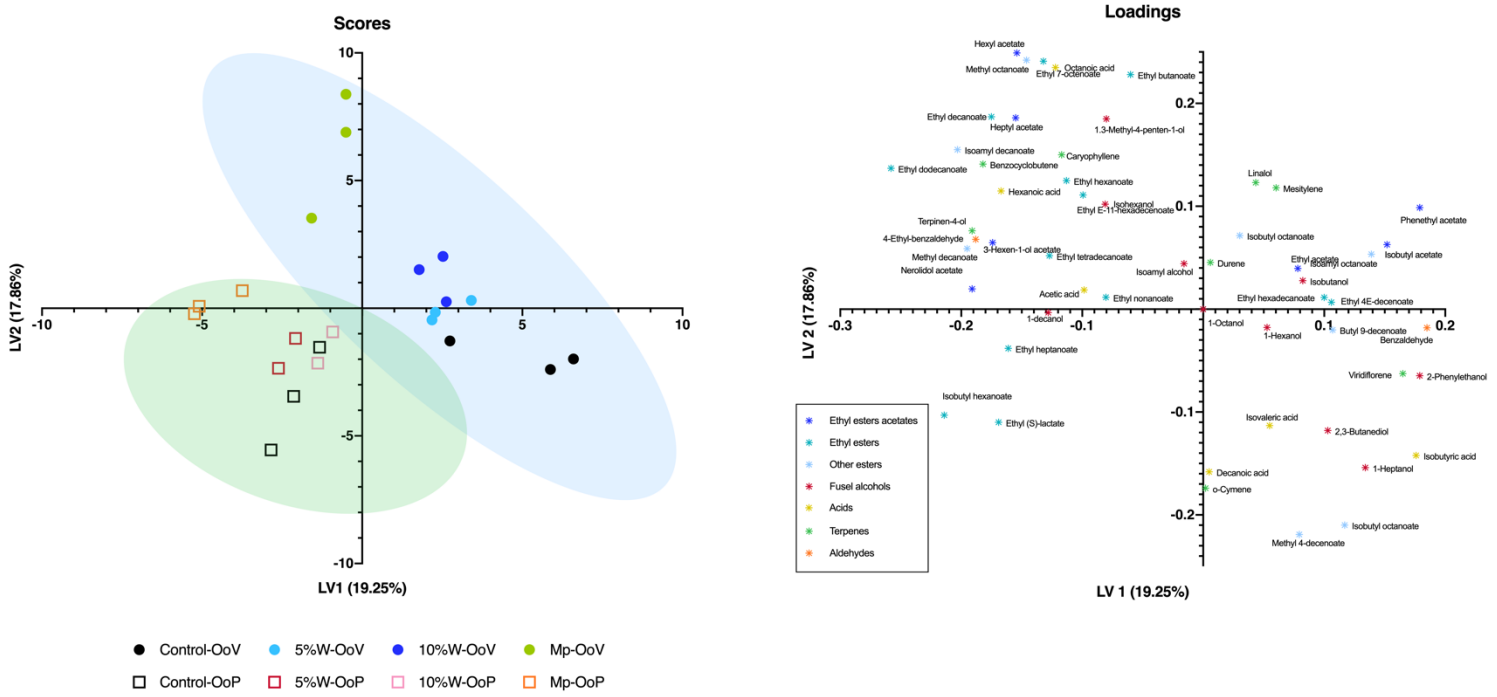

Figure 3. First two latent variables for the PLS-DA model (% of the variance) of the volatile compounds after malolactic fermentation. A) Scores: Control represents wines fermented solely with *S. cerevisiae*, while 5%W and 10%W indicate wines with a pre-fermentative water addition of 5% and 10%, respectively. The Mp wines corresponds to sequential fermentation with *M. pulcherrima* and *S. cerevisiae*. Oo corresponds to *Oenococcus oeni* strains inoculated to perform MLF, OoV (Blue ellipse) and OoP (Green ellipse); and B) Loadings on the PLS-DA model.

The distinct variations observed in together with other trends showed the contrasting volatile composition of wines subsequent to MLF as characterised LV1. Conversely, LV2 shows the differences inherent in AF conditions, even extending to wines post-MLF. Moreover, within LV2, a separation emerges between wines inoculated with OoV and those inoculated with OoP. In this context another statistical study, specifical for OoV and OoP wines was performed. The correspondent PLS-DA is shown in Figure 3. In this case the LV1 and LV2 explain the 37.11%.

It is observed that LVs separated effectively in one direction the wines inoculated with OoV from the wines inoculated with OoP. Furthermore, in the perpendicular direction it is observed that samples maintain the separation observed in wines after AF, MpOoV and MpOoP are clearly separated from the rest. However, in this case, after MLF the dilution effect was not as clearly observed as after AF. This effect could be due to the fact that some compounds were incremented in 5%W and 10%W at the same percentage that Control wines. For instance, total content of ester acetates or ethyl esters, especially with OoV. Notably, this might be attributed to the potential modulation of certain enzymatic activities by ethanol concentration (Cappello et al., 2017)

Regarding the differentiation between OoV and OoP wines, it is s due to the fact that most of the changes in volatile compounds produced during MLF are strain-dependent (Cappello et al., 2017; Costello et al., 2013; Fia et al., 2018; Knoll et al., 2011) such as the ability to hydrolysed or synthesized esters. Isobutyl acetate, isoamyl acetate and ethyl butanoate were significantly increased in OoP wines, while ethyl lactate, methyl decanoate and ethyl dodecanoate were higher in OoV wines. In Figure 3 it is possible to observe that there is a high variability between the correlation of samples. In general, it is observed that OoV wines were more correlated with most of compounds, especially esters of FAs while OoP wines was positively correlated with alcohols, overall. However, despite these statistical differences it worth noting that sensory analysis did not show any significant differences between wines inoculated with both *O. oeni* strains.

5. Conclusion

The aromatic differences between AF conditions and their impact on the performance of MLF, as well as the obtained differences between aroma composition after MLF, allows to understand how the alcohol reduction practices impact wine final composition. It was observed several significant changes in aromatic composition after MLF in comparison with wines analysed after AF. Even though a dilution effect was expected for the water addition conditions, the less stressful media for microorganism significatively impact the composition, enhancing the production of aroma rich compounds, especially fusel alcohols acetates and fusel alcohol. Water addition conditions exhibit better performance for *O. oeni* in terms of kinetics of the process and production of aroma. Additionally, the results of the sequential inoculation strategy using *M. pulcherrima* improve the knowledge regarding the use of this species in MLF performance. In this context, as climate change is a recurring problem in wine making and novel solutions should be taken, more research about the water addition needs to be carried out.

Acknowledgments

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Supplementary data

Table SD1. Volatile compounds expressed as relative abundance detected after malolactic fermentation. Wine conditions: Control represents wines fermented solely with *S. cerevisiae*, while 5%W and 10%W indicate wines with a pre-fermentative water addition of 5% and 10%, respectively. The Mp wines corresponds to sequential fermentation with *M. pulcherrima* and *S. cerevisiae*. Uppercase letters indicate significant differences between AF treatments and lowercase letters indicates significant differences between MLF condition (no MLF, OoV and OoP (p-value < 0.05).

	Control	Control-OoV	Control-OoP	5%W	5%W-OoV	5%W-OoP	10%W	10%W-OoV	10%W-OoP	Mp	Mp-OoV	Mp-OoP
Ethyl acetate	5.23 ± 0.14^{Ba}	8.36 ± 0.57^{Bb}	9.19 ± 1.53^{Bb}	4.43 ± 0.57^{ABa}	7.75 ± 0.43^{ABb}	8.37 ± 0.05^{ABb}	4.21 ± 0.22^{Aa}	7.17 ± 0.86^{Ab}	8.15 ± 0.34^{Ab}	5.28 ± 0.30^{ABa}	8.12 ± 0.41^{ABb}	8.38 ± 0.57^{ABb}
Isobutyl acetate	0.31 ± 0.03^{ABa}	0.59 ± 0.15^{ABb}	0.74 ± 0.21^{ABc}	0.27 ± 0.03^{ABa}	0.50 ± 0.32^{ABb}	0.90 ± 0.12^{ABc}	0.09 ± 0.01^{Aa}	0.29 ± 0.24^{Ab}	0.82 ± 0.09^{Ac}	0.37 ± 0.02^{Ba}	0.72 ± 0.14^{Bb}	0.73 ± 0.15^{Bc}
Ethyl butanoate	0.58 ± 0.02^{Ba}	0.98 ± 0.05^{Bb}	0.89 ± 0.15^{Bb}	0.45 ± 0.13^{ABa}	0.82 ± 0.02^{ABb}	0.87 ± 0.07^{ABb}	0.41 ± 0.01^{Aa}	0.73 ± 0.03^{Ab}	0.86 ± 0.09^{Ab}	0.54 ± 0.01^{Aa}	0.73 ± 0.02^{Ab}	0.80 ± 0.04^{Ab}
Isoamyl acetate	8.01 ± 0.27^{Ba}	14.09 ± 0.59^{Bb}	14.19 ± 2.52^{Bb}	6.59 ± 1.24^{ABa}	13.22 ± 0.60^{ABb}	13.51 ± 0.27^{ABb}	6.11 ± 0.43^{Aa}	11.50 ± 1.39^{Ab}	12.22 ± 0.66^{Ab}	7.33 ± 0.44^{Aa}	9.91 ± 1.85^{Ab}	12.64 ± 0.91^{Ab}
Ethyl hexanoate	10.47 ± 0.73^{Aa}	16.16 ± 1.64^{Aa}	10.75 ± 7.74^{Aa}	7.15 ± 1.19^{Aba}	15.69 ± 1.02^{ABa}	7.73 ± 2.77^{ABa}	7.82 ± 0.43^{Aa}	12.78 ± 1.53^{Aa}	13.69 ± 0.91^{Aa}	8.26 ± 0.60^{Aa}	5.04 ± 0.06^{Aa}	12.10 ± 0.73^{Aa}
Hexyl acetate	3.02 ± 0.20^{Ca}	4.44 ± 0.28^{Cb}	4.13 ± 0.78^{Cb}	2.42 ± 0.59^{Aa}	4.09 ± 0.25^{Ab}	3.30 ± 0.16^{Ab}	2.39 ± 0.19^{Ba}	3.62 ± 0.58^{Bb}	3.35 ± 0.19^{Bb}	1.84 ± 0.11^{Aa}	1.70 ± 0.70^{Ab}	2.37 ± 0.26^{Ab}
3-Hexen-1-ol acetate	0.04 ± 0.01^{Aa}	0.10 ± 0.05^{Ab}	0.06 ± 0.02^{Aa}	0.03 ± 0.01^{Ba}	0.12 ± 0.01^{Bb}	0.04 ± 0.01^{Bca}	0.03 ± 0.01^{Aa}	0.10 ± 0.06^{Ab}	0.07 ± 0.03^{Aa}	0.04 ± 0.01^{Aa}	0.06 ± 0.02^{Ab}	0.07 ± 0.02^{Aa}
Ethyl heptanoate	0.02 ± 0.01^{Aa}	0.08 ± 0.04^{Ac}	0.03 ± 0.01^{Aab}	0.03 ± 0.02^{Aa}	0.03 ± 0.01^{Ac}	0.02 ± 0.01^{Ab}	0.02 ± 0.01^{Aa}	0.03 ± 0.01^{Ac}	0.04 ± 0.01^{Ab}	0.02 ± 0.01^{Aa}	0.06 ± 0.03^{Ac}	0.02 ± 0.01^{Ab}
Isobutyl hexanoate	0.04 ± 0.01^{Aa}	0.29 ± 0.03^{Ab}	0.17 ± 0.02^{Ab}	0.04 ± 0.01^{Aa}	0.29 ± 0.02^{Ab}	0.24 ± 0.01^{Ab}	0.03 ± 0.01^{Aa}	0.30 ± 0.01^{Ab}	0.27 ± 0.01^{Ab}	0.05 ± 0.01^{Aa}	$0.33 \pm 0,06^{Ab}$	$0.06 \pm 0,03^{Ab}$
Heptyl acetate	0.05 ± 0.01^{Aa}	0.06 ± 0.01^{Ac}	0.05 ± 0.01^{Aa}	0.04 ± 0.01^{Aa}	0.06 ± 0.01^{Ac}	0.04 ± 0.01^{Aa}	0.04 ± 0.01^{Aa}	0.05 ± 0.01^{Ac}	0.05 ± 0.01^{Aa}	0.05 ± 0.01^{Aa}	0.03 ± 0.01^{Ac}	0.04 ± 0.01^{Aa}
Methyl octanoate	0.15 ± 0.01^{Ba}	0.23 ± 0.01^{Ba}	0.20 ± 0.04^{Ba}	0.14 ± 0.03^{Aba}	0.21 ± 0.02^{ABa}	0.16 ± 0.01^{ABa}	0.13 ± 0.01^{ABa}	0.21 ± 0.04^{ABa}	0.17 ± 0.01^{ABa}	0.16 ± 0.01^{Aa}	0.07 ± 0.05^{Aa}	0.16 ± 0.02^{Aa}
Ethyl (S)-lactate	n.d	0.06 ± 0.01^{Ab}	0.01 ± 0.01^{Aa}	n.d	0.05 ± 0.02^{Ab}	0.03 ± 0.01^{Aa}	n.d	0.04 ± 0.01^{Ab}	0.03 ± 0.01^{Aa}	n.d	0.06 ± 0.02^{Ab}	0.05 ± 0.02^{Aa}
Ethyl octanoate	30.2 ± 0.6^{Ba}	61.1 ± 9.9^{Bb}	60.1 ± 2.5^{Bb}	20.1 ± 2.8^{Aa}	52.8 ± 2.3^{Ab}	42.4 ± 1.0^{Ab}	20.0 ± 2.1^{Aa}	45.12 ± 7.8^{Ab}	42.0 ± 0.6^{Ab}	25.9 ± 2.1^{Aa}	24.7 ± 8.5^{Ab}	36.6 ± 4.2^{Ab}
Ethyl 7-octenoate	0.11 ± 0.01^{Ba}	0.22 ± 0.01^{Bb}	0.20 ± 0.02^{Bb}	0.09 ± 0.02^{Aa}	0.15 ± 0.01^{Ab}	0.12 ± 0.01^{Ab}	0.07 ± 0.01^{Aa}	0.12 ± 0.02^{Ab}	0.12 ± 0.01^{Ab}	0.10 ± 0.02^{Aa}	$0.08 \pm 0.02b$	0.12 ± 0.01^{Ab}
Ethyl nonanoate	0.03 ± 0.01^{Aa}	0.06 ± 0.01^{Ab}	0.07 ± 0.01^{Aab}	0.03 ± 0.01^{Aa}	0.07 ± 0.01^{Ab}	0.06 ± 0.01^{Aab}	0.03 ± 0.01^{Aa}	0.05 ± 0.01^{Ab}	n.d	0.04 ± 0.01^{Aa}	0.05 ± 0.01^{Ab}	0.04 ± 0.04^{Ab}
Octanoic acid. isobutyl ester	0.07 ± 0.02^{Aa}	0.13 ± 0.04^{Ab}	0.13 ± 0.03^{Ab}	0.06 ± 0.01^{Aa}	0.14 ± 0.01^{Ab}	0.12 ± 0.02^{Ab}	0.05 ± 0.01^{Aa}	0.15 ± 0.03^{Ab}	0.12 ± 0.01^{Ab}	0.10 ± 0.01^{Aa}	0.12 ± 0.03^{Ab}	0.15 ± 0.02^{Ab}
Methyl decanoate	0.09 ± 0.04^{Ba}	0.08 ± 0.04^{Bb}	0.07 ± 0.01^{Ba}	0.14 ± 0.02^{Ba}	0.12 ± 0.01^{Bb}	0.04 ± 0.01^{Ba}	0.10 ± 0.03^{Ba}	0.16 ± 0.05^{Ab}	0.01 ± 0.02^{Aa}	0.06 ± 0.04^{Aa}	n.d	n.d
Ethyl decanoate	13.27 ± 2.04^{Ca}	22.17 ± 8.00^{Cb}	21.15 ± 2.44^{Cb}	10.58 ± 0.54^{BCa}	21.79 ± 0.98^{BCb}	13.33 ± 0.39^{BCb}	8.59 ± 0.59^{Ba}	19.25 ± 3.39^{Bb}	10.13 ± 0.98^{Bb}	7.87 ± 2.11^{Aa}	4.69 ± 1.30^{Ab}	6.93 ± 1.65^{Ab}
Methyl 4-decenoate	0.01 ± 0.02^{Aa}	0.05 ± 0.01^{Aa}	0.05 ± 0.01^{Ab}	0.04 ± 0.01^{Aa}	0.03 ± 0.01^{Aa}	0.08 ± 0.03^{Ab}	0.02 ± 0.01^{Aa}	0.06 ± 0.03^{Aa}	0.05 ± 0.01^{Ab}	0.06 ± 0.02^{Aa}	0.14 ± 0.09^{Aa}	0.04 ± 0.03^{Ab}
Isoamyl octanoate	0.35 ± 0.08^{Aa}	0.34 ± 0.37^{Aab}	0.87 ± 0.08^{Ab}	0.42 ± 0.06^{Aa}	1.08 ± 0.05^{Aab}	0.82 ± 0.01^{Ab}	0.42 ± 0.05^{Aa}	0.37 ± 0.57^{Aab}	0.78 ± 0.08^{Ab}	0.48 ± 0.04^{Aa}	0.63 ± 0.20^{Aab}	0.80 ± 0.14^{Ab}
Ethyl 4E-decenoate	0.04 ± 0.01^{ABa}	6.13 ± 0.49^{ABb}	15.66 ± 2.10^{ABb}	0.04 ± 0.01^{Aa}	0.11 ± 0.02^{Ab}	0.07 ± 0.01^{Ab}	0.03 ± 0.01^{Aa}	12.13 ± 2.03^{ABb}	3.99 ± 1.57^{ABb}	0.08 ± 0.01^{Ba}	7.77 ± 7.00^{ABb}	17.43 ± 1.81^{ABb}
Phenethyl acetate	5.67 ± 0.42^{Aa}	3.30 ± 5.53^{Aa}	9.70 ± 1.27^{Aa}	5.35 ± 0.62^{Aa}	8.56 ± 0.62^{Aa}	8.32 ± 0.37^{Aa}	4.46 ± 0.40^{Aa}	6.85 ± 0.80^{Aa}	6.42 ± 0.22^{Aa}	6.37 ± 0.65^{Ba}	5.06 ± 1.31^{Ba}	9.50 ± 0.55^{Ba}
Ethyl dodecanoate	5.32 ± 1.23^{Cb}	8.30 ± 1.87^{Cb}	3.47 ± 0.36^{Ca}	5.25 ± 0.33^{BCb}	5.57 ± 0.28^{BCb}	1.53 ± 0.10^{BCa}	2.71 ± 0.24^{ABb}	4.07 ± 0.66^{ABb}	2.20 ± 0.43^{ABa}	3.17 ± 0.88^{Ab}	0.82 ± 0.51^{Ab}	1.49 ± 0.50^{Aa}
Isoamyl decanoate	0.16 ± 0.04^{Ba}	0.26 ± 0.08^{Ba}	0.23 ± 0.01^{Ba}	0.20 ± 0.01^{Ba}	0.30 ± 0.01^{Ba}	0.16 ± 0.01^{Ba}	0.17 ± 0.01^{Ba}	0.30 ± 0.03^{Ba}	0.14 ± 0.02^{Ba}	0.10 ± 0.02^{Aa}	0.02 ± 0.04^{Aa}	0.16 ± 0.01^{Aa}

Ethyl (11E) hexadecenoate	0.05 ± 0.01 ^{ABb}	0.07 ± 0.02 ^{ABab}	0.05 ± 0.01 ^{ABa}	0.06 ± 0.01 ^{ABb}	0.07 ± 0.01 ^{Abab}	n.d	0.03 ± 0.01 ^{Ab}	0.05 ± 0.01 ^{Aab}	n.d	0.16 ± 0.06 ^{Bb}	0.02 ± 0.03 ^{Bab}	0.06 ± 0.01 ^{Ba}
Butyl 9-decenoate	0.06 ± 0.03 ^{Aa}	0.08 ± 0.03 ^{Aa}	0.06 ± 0.01 ^{Aa}	0.07 ± 0.01 ^{Aa}	0.11 ± 0.01 ^{Aa}	0.07 ± 0.01 ^{Aa}	0.06 ± 0.01 ^{Aa}	0.12 ± 0.01 ^{Aa}	0.07 ± 0.01 ^{Aa}	0.14 ± 0.01 ^{Aa}	0.11 ± 0.03 ^{Aa}	0.33 ± 0.35 ^{Aa}
Nerolidol. acetate	0.04 ± 0.01 ^{Aa}	0.10 ± 0.08 ^{Aa}	0.04 ± 0.03 ^{Aa}	0.04 ± 0.01 ^{Aa}	0.06 ± 0.01 ^{Aa}	n.d	0.05 ± 0.01 ^{Aa}	0.05 ± 0.01 ^{Aa}	0.05 ± 0.01 ^{Aa}	0.05 ± 0.01 ^{Aa}	0.03 ± 0.01 ^{Aa}	0.04 ± 0.01 ^{Aa}
Ethyl tetradecanoate	0.15 ± 0.08 ^{Aa}	4.31 ± 7.02 ^{Aa}	0.12 ± 0.17 ^{Ab}	0.15 ± 0.03 ^{Aa}	0.33 ± 0.05 ^{Aa}	0.04 ± 0.01 ^{Ab}	0.10 ± 0.01 ^{Aa}	0.07 ± 0.01 ^{Aa}	0.35 ± 0.01 ^{Ab}	0.20 ± 0.02 ^{Aa}	0.24 ± 0.04 ^{Aa}	0.21 ± 0.04 ^{Ab}
Ethyl hexadecanoate	0.21 ± 0.10 ^{Aa}	0.35 ± 0.14 ^{Aab}	0.47 ± 0.41 ^{Ab}	0.25 ± 0.01 ^{Aa}	0.34 ± 0.04 ^{Aab}	0.44 ± 0.02 ^{Ab}	0.22 ± 0.01 ^{Aa}	0.46 ± 0.02 ^{Ab}	0.28 ± 0.05 ^{Ab}	0.36 ± 0.05 ^{Aa}	0.31 ± 0.04 ^{Ab}	0.60 ± 0.12 ^{Ab}
Isobutanol	0.70 ± 0.02 ^{ABa}	2.60 ± 0.14 ^{ABb}	2.61 ± 0.47 ^{ABa}	1.51 ± 0.20 ^{ABa}	1.90 ± 0.96 ^{ABb}	2.45 ± 0.11 ^{ABa}	1.38 ± 0.11 ^{Aa}	2.13 ± 0.49 ^{Ab}	1.14 ± 0.44 ^{Aa}	2.35 ± 0.15 ^{Ba}	2.54 ± 1.09 ^{Bb}	3.09 ± 0.21 ^{Ba}
Isoamyl alcohol	11.65 ± 0.14 ^{Aa}	19.57 ± 0.83 ^{Aa}	6.34 ± 0.88 ^{Ab}	10.98 ± 1.16 ^{Aa}	18.31 ± 1.35 ^{Aa}	19.01 ± 0.36 ^{Ab}	11.03 ± 0.70 ^{Aa}	17.91 ± 2.29 ^{Aa}	19.48 ± 0.50 ^{Ab}	13.79 ± 1.06 ^{Aa}	13.53 ± 1.72 ^{Aa}	22.13 ± 1.29 ^{Ab}
Isohexanol	0.05 ± 0.03 ^{Aa}	0.10 ± 0.04 ^{Ab}	0.11 ± 0.04 ^{Aa}	0.04 ± 0.02 ^{Aa}	0.12 ± 0.03 ^{Ab}	0.11 ± 0.01 ^{Aa}	0.03 ± 0.01 ^{Aa}	0.11 ± 0.01 ^{Ab}	0.11 ± 0.01 ^{Aa}	0.05 ± 0.01 ^{Aa}	0.09 ± 0.01 ^{Ab}	0.08 ± 0.01 ^{Aa}
1.3-Methyl-4-penten-1-ol	0.14 ± 0.01 ^{Ba}	0.21 ± 0.01 ^{Ba}	0.21 ± 0.04 ^{Bb}	0.12 ± 0.01 ^{Aa}	0.15 ± 0.06 ^{Aa}	0.14 ± 0.06 ^{Ab}	0.12 ± 0.01 ^{Aa}	0.13 ± 0.07 ^{Aa}	0.10 ± 0.01 ^{Ab}	0.09 ± 0.01 ^{Aa}	0.10 ± 0.02 ^{Aa}	0.13 ± 0.01 ^{Ab}
1-Hexanol	0.66 ± 0.02 ^{Aa}	1.09 ±0.04 ^{Aab}	0.79 ± 0.63 ^{Ab}	0.66 ± 0.07 ^{Aa}	0.41 ± 0.61 ^{Aab}	1.06 ± 0.01 ^{Ab}	0.67 ± 0.03 ^{Aa}	1.04 ± 0.09 ^{Ab}	1.07 ± 0.03 ^{Ab}	0.71 ± 0.04 ^{Aa}	1.05 ± 0.01 ^{Ab}	1.11 ± 0.05 ^{Ab}
1-Heptanol	0.13 ± 0.02 ^{Aa}	0.23 ± 0.04 ^{Ab}	0.18 ± 0.01 ^{Ab}	0.12 ± 0.01 ^{Aa}	0.19 ± 0.01 ^{Ab}	0.18 ± 0.03 ^{Ab}	0.12 ± 0.02 $^{A a}$	0.18 ± 0.01 ^{Ab}	0.18 ± 0.01 ^{Ab}	0.22 ± 0.06 ^{Ba}	0.32 ± 0.03 ^{Bb}	0.36 ± 0.02 ^{Bb}
2.3-Butanediol	0.21 ± 0.12 ^{ABa}	0.12 ± 0.01 ^{Aba}	0.10 ± 0.04 ^{ABa}	0.11 ± 0.05 ^{ABa}	0.11 ± 0.04 ^{ABa}	0.12 ± 0.02 ^{ABa}	0.04 ± 0.02 ^{Aa}	0.08 ± 0.03 ^{Aa}	0.14 ± 0.01 ^{Aa}	0.21 ± 0.02 ^{Ba}	0.17 ± 0.08 ^{Ba}	0.17 ± 0.03 ^{Ba}
1-octanol	0.10 ± 0.01 ^{ABb}	n.d	n.d	0.08 ± 0.01 ^{Ab}	n.d	n.d	0.08 ± 0.01 ^{Ab}	n.d	n.d	0.12 ± 0.01 ^{Bb}	n.d	n.d
1-decanol	0.09 ± 0.01 ^{Ab}	0.08 ± 0.02 ^{Aa}	0.05 ± 0.02 ^{Aa}	0.10 ± 0.01 ^{Ab}	0.08 ± 0.02 ^{Aa}	0.05 ± 0.23 ^{Aa}	0.10 ± 0.01 ^{Ab}	0.08 ± 0.02 ^{Aa}	0.06 ± 0.01 ^{Aa}	0.11 ± 0.01 ^{Ab}	0.06 ± 0.02 ^{Aa}	0.08 ± 0.01 ^{Aa}
2-Phenylethanol	10.75 ± 0.86 ^{Ba}	19.46 ± 0.98 ^{Bb}	20.41 ± 3.19 ^{Bb}	10.55 ± 1.46 ^{ABa}	17.57 ± 1.34 ^{ABb}	19.04 ± 0.01 ^{ABb}	9.50 ± 0.34 ^{Aa}	16.79 ± 1.50 ^{Ab}	17.42 ± 0.57 ^{Ab}	15.72 ± 1.75 ^{Ca}	22.93 ± 2.32 ^{Cb}	25.62 ±1.30 ^{Cb}
Benzaldehyde	0.19 ± 0.01 ^{Aab}	0.22 ± 0.04 ^{Aa}	0.30 ± 0.03 ^{Ab}	0.19 ± 0.02 ^{Aab}	0.19 ± 0.05 ^{Aa}	0.19 ± 0.01 ^{Ab}	0.23 ± 0.03 ^{Ab}	0.17 ± 0.01 ^{Aa}	0.19 ± 0.02 ^{Ab}	0.29 ± 0.09 ^{Bb}	0.28 ± 0.03 ^{Ba}	0.34 ± 0.02 ^{Bb}
4-ethyl-benzaldehyde	n.d	1.75 ± 1.60 ^{Aa}	n.d	n.d	n.d	n.d	0.04 ± 0.01 ^{Aa}	n.d	n.d	0.06 ± 0.01 ^{Aa}	0.02 ± 0.04 ^{Aa}	n.d
Acetic acid	0.65 ± 0.08 ^{Aa}	1.60 ± 0.54 ^{Ab}	1.14 ± 0.26 ^{Ab}	0.58 ± 0.21 ^{Aa}	1.25 ± 0.29 ^{Ab}	1.47 ± 0.05 ^{Aa}	0.37 ± 0.02 ^{Aa}	1.25 ± 0.13 ^{Ab}	1.34 ± 0.01 ^{Aa}	0.62 ± 0.09 ^{Aa}	1.32 ± 0.33 ^{Ab}	1.27 ± 0.05 ^{Aa}
Isobutyric acid	0.05 ± 0.01 ^{Ba}	0.07 ± 0.01 ^{Bb}	0.08 ± 0.02 ^{Bb}	0.05 ± 0.01 ^{ABa}	0.07 ± 0.01 ^{ABb}	0.08 ± 0.01 ^{ABa}	0.04 ± 0.01 ^{Aa}	0.06 ± 0.01 ^{Ab}	0.07 ± 0.01 ^{Aa}	0.09 ± 0.01 ^{Ca}	0.12 ± 0.01 ^{Cb}	0.12 ± 0.01 ^{Ca}
Isovaleric acid	0.16 ± 0.02 ^{Ba}	0.27 ± 0.05 ^{Bb}	0.24 ± 0.04 ^{Bb}	0.16 ± 0.03 ^{ABa}	0.22 ± 0.01 ^{ABb}	0.20 ± 0.01 ^{ABa}	0.12 ± 0.01 ^{Aa}	0.19 ± 0.02 ^{Ab}	0.18 ± 0.01 ^{Aa}	0.20 ± 0.07 ^{Ca}	0.31 ± 0.07 ^{Cb}	0.32 ± 0.01 ^{Ca}
Hexanoic acid	1.33 ± 0.08 ^{Ba}	4.51 ± 2.94 ^{Bb}	2.14 ± 0.31 ^{Bab}	1.29 ± 0.23 ^{ABa}	1.99 ± 0.12 ^{ABb}	2.15 ± 0.05 ^{ABa}	1.12 ± 0.12 ^{ABa}	1.79 ± 0.15 ^{ABb}	1.85 ± 0.07 ^{ABa}	1.09 ± 0.11 ^{Aa}	1.45 ± 0.15 ^{Ab}	1.60 ± 0.09 ^{Aa}
Octanoic acid	7.22 ± 0.50 ^{Ba}	11.36 ± 0.69 ^{Bb}	10.58 ±1.26 ^{Bb}	7.26 ± 0.87 ^{Ba}	10.88 ± 0.88 ^{Bb}	10.15 ± 0.10 ^{Bb}	6.75 ± 0.77 ^{Ba}	10.08 ± 0.77 ^{Bb}	8.94 ± 0.40 ^{Bb}	6.56 ± 0.61 ^{Aa}	4.53 ± 3.89 ^{Ab}	7.72 ± 0.29 ^{Ab}
Decanoic acid	5.08 ± 0.44 ^{Ab}	0.03 ± 0.04 ^{Aa}	0.04 ± 0.03 ^{Aa}	5.34 ± 0.84 ^{Ab}	0.01 ± 0.01 ^{Aa}	0.17 ± 0.02 ^{Aa}	5.09 ± 0.64 ^{Ab}	0.29 ± 0.04 ^{Aa}	2.17 ± 0.54 ^{Aa}	2.26 ± 0.04 ^{Ab}	2.73 ± 4.24 ^{Aa}	2.17 ±0.55 ^{Aa}
Mesitylene	0.32 ± 0.02 ^{Aa}	0.40 ± 0.43 ^{Ab}	0.71 ± 0.09 ^{Ab}	0.23 ± 0.13 ^{Aa}	0.71 ± 0.11 ^{Ab}	0.89 ± 0.17 ^{Ab}	0.27 ± 0.02 ^{Aa}	0.57 ± 0.08 ^{Ab}	1.00 ± 0.21 ^{Ab}	0.23 ± 0.06 ^{Aa}	0.40 ± 0.25 ^{Ab}	0.36 ± 0.02 ^{Ab}
Benzocyclobutene	0.12 ± 0.02 ^{Ba}	0.27 ± 0.17 ^{Bb}	0.15 ± 0.05 ^{Bab}	0.08 ± 0.03 ^{ABa}	0.15 ±0.01 ^{ABb}	0.11 ± 0.01 ^{Abab}	0.08 ±0.01 ^{ABa}	0.13 ± 0.02 ^{ABb}	0.16 ± 0.04 ^{ABab}	0.09 ± 0.00 ^{Aa}	0.07 ± 0.02 ^{Ab}	0.08 ± 0.02 ^{Aab}
o-Cymene	0.04 ± 0.02 ^{Aa}	0.13 ± 0.05 ^{Aa}	0.11 ± 0.01 ^{Aa}	0.10 ± 0.07 ^{Aa}	0.09 ± 0.05 ^{Aa}	0.12 ±0.04 ^{Aa}	0.07 ±0.05 ^{Aa}	0.10 ± 0.01 ^{Aa}	0.09 ± 0.02 ^{Aa}	0.13 ± 0.01 ^{Aa}	0.19 ± 0.11 ^{Aa}	0.10 ± 0.02 ^{Aa}
Durene	0.03 ± 0.01 ^{Ba}	0.13 ± 0.02 ^{Bb}	0.08 ± 0.03 ^{Bb}	0.01 ± 0.01 ^{Aa}	0.02 ± 0.03 ^{Ab}	0.09 ±0.03 ^{Ab}	0.04 ± 0.01 ^{ABa}	0.10 ± 0.03 ^{ABb}	0.08 ± 0.01 ^{ABb}	0.04 ± 0.03 ^{ABa}	0.09 ± 0.04 ^{ABb}	0.11 ± 0.05 ^{ABb}
Linalol	0.07 ± 0.01 ^{Aa}	0.13 ± 0.03 ^{Ab}	0.12 ± 0.02 ^{Ab}	0.07 ± 0.01 ^{Aa}	0.11 ± 0.01 ^{Ab}	0.12 ±0.01 ^{Ab}	0.08 ± 0.01 ^{Aa}	0.11 ± 0.01 ^{Ab}	0.12 ± 0.03 ^{Ab}	0.08 ± 0.01 ^{Aa}	0.11 ± 0.02 ^{Ab}	0.14 ± 0.01 ^{Ab}
Terpinen-4-ol	0.03 ± 0.01 ^{Ab}	0.05 ± 0.01 ^{Aa}	n.d	0.03 ± 0.03 ^{Ab}	n.d	n.d	0.03 ± 0.01 ^{Ab}	n.d	n.d	0.05 ± 0.01 ^{Ab}	n.d	n.d
Caryophyllene	n.d	0.14 ± 0.07 ^{Ba}	0.09 ± 0.03 ^{Ba}	0.01 ± 0.01 ^{Aa}	n.d	n.d	0.05 ±0.01 ^{ABa}	n.d	n.d	0.06 ± 0.02 ^{ABa}	n.d	n.d
Viridiflorene	0.02 ± 0.01 ^{ABa}	n.d ^{ABab}	0.11 ± 0.04 ^{ABb}	0.01 ± 0.01 ^{Aa}	0.02 ± 0.01 ^{Aab}	0.03 ± 0.01 ^{Ab}	0.01 ± 0.01 ^{ABa}	0.09 ± 0.01 ^{ABb}	0.02 ± 0.01 ^{ABb}	0.04 ± 0.01 ^{Ba}	0.09 ± 0.02 ^{Bb}	0.10 ± 0.01 ^{Bb}
Isovalerone	0.11 ± 0.02 ^{Ab}	n.d ^{Aa}	n.d	0.09 ± 0.03 ^{Ab}	n.d ^{Aa}	n.d	0.09 ± 0.02 ^{Ab}	n.d	n.d	0.10 ± 0.01 ^{Ab}	n.d	n.d
2-Undecanone	0.03 ± 0,01 ^{Ab}	n.d ^{Aa}	n.d ^{Aa}	0.01 ± 0.01 ^{Ab}	n.d ^{Aa}	n.d	0.02 ± 0,01 ^{Ab}	n.d	n.d	0.01 ± 0.01 ^{Ab}	n.d	n.d

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General Discussion

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Winemaking is a constantly evolving process to improve both the technological aspects of production and the final quality of the wine product. The goal of wineries to meet customer preferences and solve new challenges such as climate change requires specialized research focused on the use of new sustainable, microbiological and technological alternatives. In this context the management of AF and MLF have a key role as the main process in winemaking. This management can involve the deliberate inoculation of commercial starter cultures, yeast and lactic acid bacteria, or alternatively, the deliberate selection of indigenous microorganisms. In relation to yeast, non-*Saccharomyces* species stand out for many interesting properties such as aromatic enhancement or the modulation of alcohol content (Contreras et al., 2014; Zhu et al., 2020). Furthermore, recent research has revealed the potential of certain non-*Saccharomyces*, such as *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*, to improve the performance of MLF (Balmaseda et al., 2023d). However, further research is needed to better understand the interactions between microorganisms in different types of winemaking to provide winemakers with informed guidance on effective starter cultures management.

There is a wide variety of starter cultures available from commercial companies, offering a high variability of characteristics (Roudil et al., 2019; Vejarano and Gil-Calderón, 2021) However, the metabolic interactions among them may determine their compatibility, not only between yeast species but also with LAB.

T. delbrueckii metabolism and winemaking conditions influences the chemical wine composition

In **Section 1.1** different strain combination of *T. delbrueckii* (Td-Biodiva, Td-Prelude, Td-Viniferm and Td-Zymaflore) and *S. cerevisiae* (Sc-QA23, Sc-CLOS and Sc-K1) were tested in sequential AF in synthetic must. The resulting wines were then inoculated with several strains of *O. oeni* (Oo-VP41, Oo-CH11, Oo-PSU and Oo-1Pw13) to perform MLF. During this study diverse behaviours have been observed, that affected several aspects of the resulting wines. For instance, the utilization of Sc-K1 alone was incompatible with all strains of *O. oeni*, leading stuck of MLF. On the other hand, certain yeast strain combinations exhibited the potential to reduce time of MLF, such as the sequential AF involving Td-P with Sc-QA23 or Sc-CLOS, followed by MLF utilizing Oo-VP41. Notably, a synthetic must developed specifically for this

study was used in these experiments which can be used in future research. Conventionally, microbiological experiments involving both AF and subsequent MLF are undertaken using natural must or concentrated commercial alternatives. However, the inherent variability in composition between vintages or batches, together with seasonal limitations of availability, hampers the reproducibility of these media. While a synthetic must was previously developed by Costello et al. (2003), it had a complex composition. Thus, this issue was addressed by the development of a simplified synthetic must.

In **Section 1.2**, to optimize AF and MLF performances, the composition of this synthetic must was refined. To this purpose, two lipids commonly found in grape must were added: linoleic acid and β-sitosterol. The inclusion of these lipids appeared to have a positive effect on the overall completion rates. It demonstrated successful completion of the MLF, suggesting that these lipids have the potential to enhance the metabolic processes of *O. oeni*.

Moreover, in **Section 1.2** the most suitable yeast strains Sc-QA23, Td-P and Td-V were tested with the four *O. oeni* strains. In addition, different inoculation strategies of *T. delbrueckii* and *S. cerevisiae* were compared with this strain combination in order to find the better option to enhance the subsequent MLF. Co-inoculation of *T. delbrueckii* and *S. cerevisiae,* despite its short AF, resulted in a longer and slower MLF. Martín-García et al. (2020) described the ineffective effect of co-inoculation of non-*Saccharomyces*. In contrast, a 4-day period of contact with *T. delbrueckii* followed by inoculation with *S. cerevisiae* improved MLF efficiency. This strategy promoted a higher prevalence of *T. delbrueckii* during AF, which appeared to favour its positive synergy with *O. oeni*. In the same context 6 days of *T. delbrueckii* contact resulted in a shorter MLF. Both cases were equivalent to inoculating *S. cerevisiae* at densities of approximately 1060 and 1050 g/L. This improvement was particularly noticeable with the strains Td-P and OoVP41 for MLF.

The effect of *T. delbrueckii* has been studied under cellar conditions showing a high dependence on winemaking conditions and strains. Moreover, most studies have focused on white and red wines. Therefore, the main reason of **Chapter 2** was to explore the effects of the chosen strains across various winemaking contexts, encompassing rosé fermentations, carbonic maceration wines and white wines with and without skin contact. By assessing

physicochemical, volatile, polyphenolic and sensory changes, as well as the impact on MLF dynamics, a comprehensive understanding of the strains was achieved.

Initially, the rosé fermentations in **Section 2.1** were carried out using Cabernet Sauvignon grape must. The focus was to evaluate the behaviour of *T. delbrueckii* strains Td-B and Td-P during sequential AF with *S. cerevisiae* strains Sc-QA23 and Sc-K1, to assess their performance in a natural grape must context. Subsequently, MLF was performed using the four initial strains. Moving on to **Section 2.2**, the study focused on carbonic maceration wines produced from the Red Grenache grape cultivar. *T. delbrueckii* strains Td-P and Td-V were chosen in combination with Sc-CLOS due to their suitability for red wines. In this context, MLF conditions involved the inoculation of commercial strains Oo-VP41 and Oo-CH11, alongside a spontaneous MLF with autochthonous LAB. Lastly, in **Section 2.3**, the focus shifted to white wines elaborated from the Muscat of Alexandria grape cultivar. These wines were produced with the presence of grape skins during AF (skin-fermented white wines) and without skins during AF (white wines). For this study, only the best performing strain of T. *delbrueckii*, Td-P, was used, and then sequentially inoculated with Sc-QA23. Concerning MLF, the strains Oo-VP41 and Oo-PSU were tested.

In the initial two chapters, several physicochemical parameters were analysed and it was possible to observe how yeasts species and strains modulate the wine composition showing differences between grape musts and types of winemaking. For instance, it has been described that the use of *T. delbrueckii* leads to a reduction in ethanol content (Azzolini et al., 2015; Belda et al., 2015; Zhu et al., 2020). Nevertheless, this reduction is not uniform, and its level depends on several factors, including the strain of *T. delbrueckii*, the characteristics of the grape must, and the winemaking process. In the context of these experiments, slight reductions in ethanol content were found. Notably, a significant decrease in alcohol content was observed in the co-inoculation strategy employed within the synthetic must, a scenario explored in **Section 1.2**. Conversely, sequential AF approaches in **Section 1.1** and **1.2** exhibited no significant changes in ethanol content. However, rosé wines demonstrated a notable decrease in ethanol content, particularly pronounced when utilizing the Sc-K1 strain (**Section 2.1**). Conversely, carbonic maceration wines (**Section 2.2**) and white wines (**Section 2.3**) did not show significant changes in ethanol content. Interestingly, in the case of skin-fermented wines there was observed a

decreasing trend when comparing with white wines. In summary, these results suggest that the use of *T. delbrueckii* for the purpose of alcohol reduction depends on many factors. Thus, the combination of this species with other techniques such as those shown in **Chapter 3** could be a better option for the problem of high ethanol content.

Another compound that can pose challenges at elevated concentrations is acetic acid. Existing literature indicates that *T. delbrueckii* plays a role in decreasing the concentration of acetic acid (Bely et al., 2008; Canonico et al., 2019; Contreras et al., 2014). The experimental observations along **Chapter 2** corroborate this reduction. Regarding synthetic must fermentations, the most pronounced reduction in acetic acid was identified within the co-inoculation approach involving *T. delbrueckii* and *S. cerevisiae*. Moving to wines derived from natural musts, a consistent reduction in acetic acid was evident across all vinifications, with the exception of white wines fermented without skin contact.

Another parameter that displays a uniform trend across all fermentation processes is the increased concentration of mannoproteins in wines (**Sections 1.1, 1.2, 2.1, 2.2 and 2.3**), which are compounds released in wine during the autolysis of yeasts. Literature have reported the capacity of some non-*Saccharomyces* yeasts, including *T. delbrueckii* (Balmaseda et al., 2021a; Belda et al., 2015), to rise mannoprotein concentration. These results confirm that mannoproteins increase in presence of *T. delbrueckii* regardless the grape must or the strain, under our experimental conditions. This observation will be particularly interesting since it is a compound related with the improvement of MLF, a topic that will be delved into in subsequent discussions.

Moreover, the volatile composition of the wines exhibited different profiles influenced by the presence of *T. delbrueckii*, which varied depending on fermentation conditions. It is noteworthy, as highlighted in previous studies, that there is a strain-specific effect on the modification of the aromatic profile of the wine (Azzolini et al., 2015; Balmaseda et al., 2021b; Renault et al., 2015).

Regarding synthetic must experiments (**Sections 1.1 and 1.2),** the variations in volatile compounds were relatively limited, potentially attributed to the absence of aromatic precursors of the grape cultivar. In this context, an increasing trend was observed for fusel alcohols and specific ethyl esters of FAs, particularly in the condition involving a 6-day contact with *T. delbrueckii* (**Section 1.2**). Additionally, a significant reduction in medium-chain fatty

acids (MCFA) was detected in the presence of *T. delbrueckii*. This trend of decreasing MCFA was consistently observed across the natural must vinifications, encompassing rosé wines, carbonic maceration wines, skin-fermented white wines, and white wines (**Sections 2.1, 2.2 and 2.3**). However, a noteworthy behaviour was found in white wines, as the reduction in MCFA associated with *T. delbrueckii* presence was only evident at higher temperatures of AF (25 °C), in contrast to a lower AF temperature (16 °C) (**Section 2.3**). The reduction of MCFA have been reported previously (Balmaseda et al., 2021b; Azzolini 2015; Zhang et al 2018) and it is also particularly interesting due to their negative influence on MLF.

Furthermore, the inoculation of *T. delbrueckii* during fermentation led to additional aromatic variations, including a general increase in fusel alcohols, particularly on isoamyl alcohol and 2-phenylethanol (**Sections 2.1, 2.2**). Other interesting changes were detected in the context of carbonic maceration wines, wherein the influence of *T. delbrueckii* appeared to increase several compounds in addition to fusel alcohols. This increase involved specific esters of FAs, such as ethyl decanoate and ethyl dodecanoate, alongside particular acetates of fusel alcohols, like 2-phenylethanol acetate and isoamyl acetate. In this context, the strain Td-P in particular, exhibited higher concentrations of 2-phenylethanol, associated with rose descriptors, and isoamyl acetate, characterized by banana descriptor. Notably, isoamyl acetate is recognized as a distinctive aromatic compound often found in carbonic maceration wines (Tesniere and Flanzy, 2011) which indicates that the use of *T. delbrueckii* would improve the characteristic profile of these wines.

White wines exhibited less observable aromatic effect from *T. delbrueckii*, whereas in skin-fermented white wines, variations were influenced by fermentation temperature. Specifically, at higher temperatures (25 °C), an increase in fusel alcohols was noted, while lower temperatures enhanced levels of ethyl esters of FAs. Beyond the previously mentioned effect on MCFA, there was an effect of *T. delbrueckii* regarding to short-chain fatty acids (SCFA) and long-chain fatty acids (LCFA).

Lastly, in **Section 2.3,** the observed notable variations in volatile compounds were linked to the presence of grape skins during fermentation. Skin-fermented wines exhibited significantly

elevated levels of fusel alcohols, whereas white wines showed higher concentrations of esters of FAs and fusel alcohols acetates.

Focusing on the fermentation of rosé wines, the inoculation of *T. delbrueckii* involved a significant impact on the wine's colour (**Section 2.1**). This influence was translated into a reduction in anthocyanins and colour intensity, with distinct responses observed across different strains. The strains Td-P and Td-B in sequential inoculation with Sc-QA23, contributed to the decrease through the reduction of both acylated and non-acylated anthocyanins. In particular, a higher relative proportion of pyranoanthocyanins (vitisin A and vitisin B) was observed, which are stable compounds that potentially contribute to the colour preservation of rosé wine. These compounds are related to the yellow hues detected in the wines.

Conversely, the decrease in anthocyanins within sequential wines involving Sc-K1 was attributed to a reduction in non-acylated anthocyanins. This differentiation resulted in Sc-K1 wines exhibiting a more blueish tone due to the higher content of acylated anthocyanins. The observed reduction in anthocyanins could potentially come from multiple mechanisms. It could involve the adsorption of pigments onto the yeast cell wall (Morata et al., 2003) or oxidation due to the extended duration of AF in sequential inoculation. Another plausible mechanism involves the formation of aglycones. This process is potentially influenced by the β-glucosidase activity associated with certain *T. delbrueckii* strains (Maturano et al., 2012), as this enzyme breaks the polyphenol-sugar bond, leading to a decolorizing effect (Manzanares et al., 2000).

In carbonic maceration wines there was also observed a clear effect on phenolic composition (**Section 2.2**). After *T. delbrueckii* contact with grapes during the initial stage of carbonic maceration a greater release of phenolic compounds was observed. These could be due to the enzymatic activity of this species, as it has been probed in other species such as *M. pulcherrima* which due to its pectinolytic enzymes (Belda et al., 2016a) that promote the extraction of anthocyanins from the grape skin. However, once the grape skins were retired and the AF was finished the same decreasing trend in anthocyanins was observed in *T. delbrueckii* wines.

Remarkably, higher concentrations of pyranoanthocyanins were evident in carbonic maceration wines inoculated with *T. delbrueckii*. This behaviour could be linked to the augmented proportion of pyranoanthocyanins in sequential Sc-QA23 wines. This increase may be attributed

to the interaction between pyruvic acid (vitisin A) and acetaldehyde (vitisin B), both produced by yeasts, engaging in cycloaddition reactions with anthocyanins to form pyranoanthocyanins (Schwarz et al., 2003). Moreover, hydroxycinnamic acids are metabolized by yeast through the hydroxycinnamate decarboxylase enzyme (HCDC). *T. delbrueckii* strains exhibit this activity (Božič et al., 2020) together with other non-*Saccharomyces* strains (Benito et al., 2011; Božič et al., 2020) Alongside *S. cerevisiae*, which also demonstrates robust HCDC activity, *T. delbrueckii* can metabolize hydroxycinnamic acids into vinylphenols. These compounds can then interact with anthocyanins, resulting in the formation of highly stable compounds known as vinyl phenolic pyranoanthocyanins (Benito et al., 2011; Schwarz et al., 2003).

This observation aligns with the findings from **Section 2.3**, where a significant reduction in hydroxycinnamic acids, particularly p-coumaric acid, was noted in sequential *T. delbrueckii* wines compared to *S. cerevisiae*. However, in the case of white wines, in the absence of anthocyanins, the formation of vinylphenols is a potential risk as it could increase the potential formation of unwanted 4-ethylphenols in the presence of yeasts with vinylphenol reductase activity, such as *Brettanomyces* (Zhang et al., 2021).

Considering these findings, the use of *T. delbrueckii* could help to protect the wine colour via the formation of pyranoanthocyanins. Besides, it would be recommendable if it is desired to obtain rosé wines with low colour intensity and red wines with high colour intensity. Having into account the risk of vinylphenols presence in white wines.

Lastly, comparing skin-fermented wines in comparison to traditional white wines, a notable rise in phenolic acids was observed. (Bene and Kállay, 2019). Together with these results, in the metabolome analysis performed in this chapter were found some other interesting changes such as the presence of 2-isopropylmalic acid (2-IPMA). Interestingly, skin-fermented wines showed significantly higher 2-IPMA abundance compared to white wines, besides *T. delbrueckii* wines showed significant increase, depending on the temperature, suggesting a potential implication on the metabolism of these species. That was particularly interesting since recent investigation have reported an ant bactericidal effect of this compound (Ricciutelli et al., 2020).

Concluding metabolic changes **Section 2.3**, an ANOVA-Simultaneous Component Analysis (ASCA) model was executed to explore how yeast (*T. delbrueckii* and *S. cerevisiae*),

fermentation temperature (16 or 25 °C) and type of fermentation (presence or absence of grape skins) impacted final wine composition. This analytical approach revealed the collective influence of these factors on the matrix variability. Remarkably, the type of vinification was the most influential factor contributing to the overall variance of the wines, followed by fermentation temperature and yeast species, which all of them exhibited statistically significant impact, as expected (Beltran et al. 2008; Renault et al. 2015).

A sensory analysis was carried out on the carbonic maceration wines explored in Chapter 2.2, as well as the skin-fermented and white wines performed in Chapter 2.3. The objective was to detect whether trained tasters were able to differentiate the wines fermented in presence of *T. delbrueckii*. Both sensory analyses were significant. However, the panel of tasters in carbonic maceration wines was unable to distinguish between TdP and TdV. In relation to the white wines, they were significant regardless of temperature or the presence of grape skins during AF.

Alcohol reduction techniques affect wine composition

Having delved into the influence of *T. delbrueckii* inoculation in the selected vinification conditions, the subsequent focus will shift to the discussion of how the alcohol reduction strategies, explored in **Section 3.1**, have impacted the composition of the wines. In **Section 3.1** three different strategies were tested in order to reduce alcoholic degree of Chardonnay Chilean wines. Two of them were physical techniques involving the pre-fermentative addition of distillate water to the grape must, at two percentages, 5% (Sc-5%W) and 10% (Sc-10%W). In addition, a sequential inoculation of *M. pulcherrima* (Mp+sc) was performed followed by *S. cerevisiae* inoculated after 48 hours.

Different behaviours were observed, firstly with regard to the efficiency of ethanol reduction. Specifically, Sc-5%W resulted in a significative ethanol reduction of 0.47% (v/v), while Sc-10%W produced a more pronounced reduction of 1.73% (v/v). It is important to note that research of pre-fermentative water addition strategies has mainly been conducted in the context of red wines, showing different effectiveness depending on fermentation approaches and grape cultivars (Xynas and Barnes, 2022). For instance, Schelezki et al. (2020) reported a reduction of 1% (v/v) through a water addition of 11.6% for early harvest and 10.2% (v/v) for

late harvest in Shiraz musts. In Teng et al. (2020) observed that a 14% v/v addition led to a 2.1% (v/v) reduction in Shiraz wines.

Regarding the Mp+Sc approach, the reduction in alcoholic content was not statistically significant, however it displays a tendency of approximately 0.30% (vol/vol). This observation could potentially be attributed to the presence of other yeast species competing in the grape must. It is important to highlight that the study aimed to replicate real semi-industrial vinification conditions, thus opting not to sterilize the fermenting must. The literature provides a range of reported ethanol reductions, reaching up to 0.99% (v/v), (Contreras et al., 2014) varying from 0.6% to 1.2% (v/v) in Chardonnay must (Hranilovic et al., 2020). The results reported in literature are also dependent on the specific *M. pulcherrima – S. cerevisiae* strain combination, similar to the dynamics observed with *T. delbrueckii*.

The three treatments produced distinct effects on wine composition after the AF. With regards to the water addition treatments, the expected dilution effect was evident, although not across all parameters. In the case of Sc-5%W, an intermediate trend in volatile composition and total polysaccharides was noted between Sc-Control and Sc-10%W. Notably, no significant differences emerged between the two dilution percentages. This suggests that the impact of pre-fermentative dilution on total volatile compounds and polysaccharides was not so influenced by the specific percentage of dilution within this range. Regarding low-molecular-mass phenolic compounds, Sc-5%W showed a general declining trend; however no significant differences were detected among the phenolic compound families. Conversely, Sc-10%W wines exhibited consistently lower values across all compound families. These results are in line with findings observed in red wines (Piccardo et al., 2019; Schelezki et al., 2020a; Teng et al., 2020) utilizing pre-fermentative water additions. The slight differences, combined with the reduction in alcohol content, led to different sensory results, as tasters were able to discern the Sc-10%W condition while not the Sc-5%W condition.

Regarding the Mp+Sc condition, a notable modulation of wines was observed, similar to the observations with *T. delbrueckii*. Main changes involve volatile composition, showing a significant increase in the concentrations of aldehydes and alcohols, particularly 2-phenylethanol. Furthermore, an increasing trend, significant for certain compounds, in the levels of flavanols, hydroxybenzoic acids, and hydroxycinnamic acids. This increase was

attributed mainly to the high polygalacturonase activity described for *M. pulcherrima* (Belda et al., 2016a). Although no statistical differences were noted in the total soluble polysaccharides, the Mp+Sc condition led to an increase in two polysaccharides fractions (HMWf and MMWf). These observed compositional effects were corroborated by sensory analyses, where tasters were able to distinguish wines under this condition from those of Sc-control.

In conclusion, the incorporation of a low percentage of water combined with the inoculation of *M. pulcherrima*, as previously discussed with *T. delbrueckii*, could provide a means of shaping the sensory characteristics of wines while achieving more effective alcohol reduction. This strategy presents a synergistic effect and a promising sustainable solution to address the challenge of rising alcohol content in wines.

How wine composition after AF influenced MLF

The diverse fermentative conditions have been shown to produce substantial influence on various aspects of wine composition. However, these effects do not conclude there; they extend to the subsequent stage in the winemaking process – the MLF.

The process of MLF is not always desired since it depends on the type of wine. However, in regions characterized by cooler climates and elevated grape acid concentrations (Droulia and Charalampopoulos, 2022; Verdugo-Vásquez et al., 2023), MLF becomes necessary. Consequently, there exists a need to examine the behaviour of LAB, particularly *O. oeni*, across diverse wine types.

In this thesis it has been shown how *T. delbrueckii* and *M. pulcherrima* – and other fermentative treatments – modulates the wine composition. Now we will discuss how this modulation, could improve the performance of MLF and consequently the activity of different strains of *O. oeni*.

In **Chapter 1**, as previously discussed, was described that the Td-P strain, followed by Td-B and Td-V, exhibited enhanced MLF. This effect was particularly pronounced when coupled with the *O. oeni* strain Oo-VP41, which consistently demonstrated the best efficiency for MLF across multiple chapters. In **Section 1.1**, the presence of *T. delbrueckii* accelerated MLF, even when combined with the Sc-K1 strain, which is not typically recommended for MLF. This observation was further corroborated in the context of rosé wines explored in **Section 2.1**.

In relation to the inoculation strategy investigated in **Section 1.2**, it was found that the approach involving 4 days of contact followed by 6 days of contact with *T. delbrueckii* presented the most favourable results for MLF enhancement. Conversely, the co-inoculation strategy did not have the same benefits for accelerating the MLF. Martín-García et al. (2020), have reported that co-inoculation of *T. delbrueckii* and *M. pulcherrima* was not effective in improving MLF. Besides **Section 1.2** exposed an interesting correlation: a prolonged AF, resulting from the presence of *T. delbrueckii*, was associated with an improved MLF performance.

In **Section 2.2**, the influence of *T. delbrueckii* was also evident in both inoculated and spontaneous MLF. Wines inoculated with TdP and TdV during carbonic maceration exhibited an earlier completion of MLF compared to the Control condition, particularly when coupled with the inoculation of OoVP41 in MLF. This pattern was maintained in the context of spontaneous MLF for wines initially inoculated with *T. delbrueckii*, resulting in a faster start of MLF compared to Control wines.

A special scenario emerged in the MLF analysis presented in **Section 2.3**. Across this chapter, the employment of *T. delbrueckii* was found to promote MLF during AF due to the elevated LAB concentration already presented in the grape must. This observation aligns with the tendency of this species to encourage spontaneous MLF (Balmaseda et al., 2023, 2018). Consequently, it suggests that in vintages characterized by a high population of LAB, the use of *T. delbrueckii* might not be recommended since it could lead into lactic spoilage.

However, in **Section 2.3** to explore the impact of type of vinification and temperatures on MLF, an inoculation involving OoVP41 and OoPSU, alongside a spontaneous MLF, was conducted to analyse the MLF dynamics and strains imposition. Notably, a pronounced imposition of OoPSU compared to OoVP41 was observed, a phenomenon that has been reported before (Balmaseda et al., 2021b). Moreover, two *O. oeni* strains consistently appeared throughout the fermentation process, with one of them present from the grape must. This fact suggest that characterize these strains could be interesting for further research due to their adaptability to the specific wine conditions and competitive attributes to colonizing this environment.

Lastly, **Section 3.2** delved into the effects of alcohol reduction treatments, revealing that the reduction of ethanol via water addition enhanced MLF, as ethanol is toxic to *O. oeni*.

Additionally, OoVP41 displayed a greater ethanol tolerance compared to OoPSU. This chapter also showed the beneficial impact of *M. pulcherrima* on MLF, an effect that has not been as extensively studied as *T. delbrueckii.*

During this last chapter was also observed how the alcohol reduction impact to the aromatic profile of wines, which was due to the esterase activity of *O. oeni* (Costello et al., 2013; Fia et al., 2018). Surprisingly, it was observed that although a dilution effect was expected in the context of water addition treatments, the less stressful growth environment for *O. oeni* had a substantial impact on volatile composition leading to the enhancement of aroma-rich compounds, notably esters of FAs.

The synergistic interactions between *T. delbrueckii* and *M. pulcherrima*, particularly the former, with *O. oeni* have been subject to laboratory investigations as well as specific winemaking scenarios (Balmaseda et al., 2022a; Balmaseda et al., 2021b, 2021d).The findings from these studies proposed that this effect could be attributed to a range of parameters. Besides, Balmaseda et al., (2022b) reported that non-*Saccharomyces* can contribute to decrease *O. oeni* stress in wine since their presence is related to the lower abundance of the stress indicator protein Hsp20. Some of these parameters align with the beneficial effect showed in our experimental conditions.

It is worth noting that higher pH creates a less stressful environment for *O. oeni* ethanol. While this effect was not consistently observed in wines from natural must, **Section 1.2** indicated a slight increase in pH during the 4-day *T. delbrueckii* contact, which was associated with improved MLF. The pH increase due to the presence of *T. delbrueckii* varies in the literature, but some authors have reported similar findings (Balmaseda et al., 2021d; Martín-García et al., 2020). Ethanol content is another stressful factor for *O. oeni* (Capucho and San Romão, 1994) and its reduction enhance the activity of *O. oeni* as it was observed in **Chapter 3**.

Regarding to the effect of *T. delbrueckii* on nutritional content after AF for *O. oeni,* in **Section 1.2** was showed a higher content of α-amino nitrogen with the condition of 4-day of *T. delbrueckii* contact. While peptides are the preferred nitrogen source in wine for *O. oeni* (Remize et al., 2006), the increased availability of α-amino nitrogen could still contribute to its nutritional intake.

The increase in mannoproteins associated with *T. delbrueckii* presence, a recurring observation across the chapters, is a key factor. Mannoproteins could potentially enhance *O. oeni* growth, as the mannose released from them can serve as a nutritional source for this LAB species (Jamal et al., 2013). Balmaseda et al., (2021a) reported that increased mannoprotein levels linked to *T. delbrueckii* presence led to elevated expression of certain *O. oeni* genes involved in mannose and other sugars uptake. Furthermore, they described that the metabolism of mannoproteins by *O. oeni* becomes more active under stressful conditions, such as those found in **Section 2.2**, where anthocyanin levels were increased in the presence of *T. delbrueckii*. This supports the findings of Balmaseda et al. (2021b), which demonstrated the improvement of MLF with *T. delbrueckii* in high polyphenolic wines.

It is noteworthy that not all families of polyphenols are inhibitory to *O. oeni*. For instance, hydroxycinnamic acids are inhibitory only at high concentrations (Reguant et al., 2000) This observation could be linked to **Section 2.3**, where the presence of *T. delbrueckii* led to a reduction in hydroxycinnamic acids and significantly promoted MLF.

Regarding mannoproteins, these compounds are also related to another class of compounds that can be inhibitory to *O. oeni* – MCFAs (Capucho and San Romão, 1994). Mannoproteins have the ability to detoxify the media by eliminating certain compounds, including MCFAs (Lafon-Lafourcade et al., 1984). As consistently observed in our chapters, wines fermented in the presence of *T. delbrueckii* and *M. pulcherrima* exhibited lower levels of MCFAs, and the higher presence of mannoproteins could contribute to this beneficial effect.

In conclusion, the decision to use *T. delbrueckii* depends on the objectives of the winemaker, the microbiological state of grapes and the specific type of winemaking. While there are both advantages and disadvantages to this inoculation in relation to MLF, it is crucial to carefully consider how these effects align with the desired characteristics of the final product.

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CONCLUSIONS

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The main conclusions obtained from this thesis are:

1. In our experimental conditions, different interactions were observed among *T. delbrueckii*, *S. cerevisiae* and *O. oeni* strains. The most effective combination for enhance MLF was found to be the use of Td-P and Sc-QA23 or Sc-CLOS during AF, followed by MLF with Oo-VP41.

2. Sequential AF involving 4 days of *T. delbrueckii* followed by *S. cerevisiae* inoculation was as the most effective strategy for enhancing MLF. Conversely, co-inoculation of both species led to a prolonged MLF process.

3. A synthetic must supplemented with linoleic acid and β-sitosterol ensures a correct AF and subsequent MLF.

4. Rosé wines, carbonic maceration wines, skin-fermented white wines and traditional white wines showed improved MLF, particularly in the spontaneous MLF scenario, in the presence of *T. delbrueckii*.

5. The inoculation of *T. delbrueckii* have an effect on anthocyanins: decrease their concentration in rosé wines, increase it in carbonic maceration wines and promote the formation of pyranoanthocyanins.

6. The inoculation of *T. delbrueckii* modulates the volatile profile of wines. Specially in carbonic maceration leading to an increase of isoamyl acetate.

7. The presence of skin during AF in white wines resulted in several changes in metabolic composition of wines, since organic acids of the Krebs cycles to phenol compounds and volatile compounds increased.

8. Pre-fermentative water addition in white grape must was effective in reducing alcohol content. However, this reduction could be further complemented and organoleptically improved by inoculating *M. pulcherrima*.

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NEW PERSPECTIVES ON MALOLACTIC FERMENTATION AND ORGANOLEPTIC IMPROVEMENT OF WINES: INFLUENCE OF TORULASPORA DELBRUECKII ON DIFERENT TYPES OF WINEMAKING
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APPENDIX

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Publications derived from this PhD thesis:

- Screening of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* strains in relation to their effect on malolactic fermentation. Ruiz-de-Villa, C., Poblet, M., Cordero-Otero, R., Bordons, A., Reguant, C., and Rozès, N. **Food Microbiology (2023).**
- Comparative study of inoculation strategies of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* on the performance of alcoholic and malolactic fermentations in an optimized synthetic grape must. Ruiz-de-Villa, C., Poblet, M., Bordons, A., Reguant, C., and Rozès, N. **International Journal of Food Microbiology (2023).**
- Sequential inoculation of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* in rosé wines improves colour and enhances malolactic fermentation. Ruiz-de-Villa, C., Gombau, J., Poblet, M., Bordons, Canals J.M., Zamora, F., Reguant, C. and Rozès, N. **Submitted to LWT- Food Science and Technology.**
- *Torulaspora delbrueckii* improves the organoleptic properties and promotes malolactic fermentation in carbonic maceration wines. Ruiz-de-Villa, C., Gombau, J., Poblet, M., Bordons, Canals, J.M., Zamora, F., Reguant, C. and Rozès, N. **Manuscript in preparation.**
- Influence of skin-fermentation and *Torulaspora delbrueckii* inoculation on white wine production: changes in fermentation dynamics and wine composition. Ruiz-de-Villa, C., García-Viñola, V., Schorn-García, D., Poblet, M., Bordons, A., Reguant, C., and Rozès, N. **Manuscript in preparation.**
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Other publications

- Differentiation of *Saccharomyces* species by lipid and metabolome profiles from a single colony. Ruiz-de-Villa, C., Poblet, M., Bordons, A., Reguant, C., and Rozès, N. **Food Microbiology (2022).**
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Contribution to national and international meeting

Oral Communications

Screening del efecto de diferentes cepas de *Saccharomyces cerevisiae* y *Torulaspora delbrueckii* durante la fermentación maloláctica. Ruiz-de-Villa, C., Poblet, M., Cordero-Otero, R., Bordons, A., Reguant, C., and Rozès, N. **14ª Reunión Red Nacional de Bacterias Lácticas**. Online (04/2021)

Beneficios de *Torulaspora delbrueckii* en vinos rosados: impacto en el color y en la fermentación maloláctica. Ruiz-de-Villa, C., Gombau, J., Poblet, M., Bordons, Canals J.M., Zamora, F., Reguant, C. and Rozès, N. **Congreso Jóvenes Investigadores Ciencia, Ingeniería y Tecnología de los Alimentos.** Salamanca, Spain (10/2022).

Poster Communications

Efecto de *Torulaspora delbrueckii* sobre el perfil organoléptico y la fermentación maloláctica en vinos de maceración carbónica. Ruiz-de-Villa, C., Gombau, J., Poblet, M., Bordons, Canals J.M., Zamora, F., Reguant, C. and Rozès, N. **16ª Reunión Red Nacional de Bacterias Lácticas**. Madrid, Spain. (05/2023).

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Appendix

Differentiation of *Saccharomyces* species by lipid and metabolome profiles from a single colony

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Appendix

Abstract

Yeast metabolism depends on growing conditions, which include the chemical composition of the medium, temperature and growth time. Historically, fatty acid profiles have been used to differentiate yeasts growing in liquid media. The present study determined the fatty acids of *Saccharomyces* species in colonies. Using the same method, the effect of that the number of colonies and growth time had on solid media allowed us to determine the metabolomic profiles of the cells. Our results showed that the lipid and metabolomic profiles of the cells evolved as the colony grew. Interestingly, some strains of *Saccharomyces cerevisiae* have been differentiated using the fatty acid profile of a colony; concretely indeed EC1118 and QA23 strains were separated from ICV-K1 and BM4x4. The synthesis of saturated fatty acids was greater than that of unsaturated fatty acids during the first two days of cell growth on a solid medium compared to a liquid medium. Unsaturated fatty acids subsequently became predominant. Finally, this methodology could be useful for carrying out physiological studies in a complete or defined solid growth medium allowing the supplementation of compounds, which inhibit or activate the growth of yeasts.

Keywords

Wine yeast; fatty acids; trehalose; squalene; *Saccharomyces cerevisiae*; *S. uvarum*; *S. kudriavzevii*

1. Introduction

Microbial diversity, both in vineyards and cellars, plays an important role in winemaking. Yeasts and lactic acid bacteria (LAB) are crucial to winemaking, as they are responsible for alcoholic and malolactic fermentations, respectively (Fugelsang and Edwards, 2007). The dominant species in alcoholic fermentation are *Saccharomyces cerevisiae* and the closely related *Saccharomyces bayanus*, due to their efficient fermentative catabolism (Pretorius, 2000). A wide variety of commercial *S. cerevisiae* strains – including *S. bayanus* ones, sometimes mistakenly labelled as *S. cerevisiae* – are used as starter cultures. In the wine industry, it is argued that the use of these starters is associated with a loss of wine typicity (Philip et al., 2021) but there is no clear scientific evidence for its existence. For this reason, it is increasingly common for wineries to isolate and select indigenous *S. cerevisiae* strains that are associated with particular characteristics typical of wines of the area (Tempère et al., 2018). In addition to *S. cerevisiae* and *S. bayanus*, there are other interesting species in the *Saccharomyces* genus, such as *S. uvarum* and *S. kudriavzevii*. These species have been studied for their remarkable properties (Minebois, 2020), including their high production of volatile compounds (Gamero et al., 2013; Masneuf-Pomarède et al., 2010).

Nevertheless, under uncontrolled conditions, other undesirable yeast species may also be found (Pretorius, 2000). For this reason, microbiological control is necessary for avoiding spoilage, for analysing intraspecific variability and for verifying the fermentation strains. Typically, to do this, the microorganisms involved in the process must first be isolated and then identified. Depending on the physiology of the microorganism, specific culture media are used for this isolation. For yeasts, the most widely used media are nutrient rich, the most common of which is yeast extract peptone dextrose agar (YPDA). Selective and differential media, such as a medium with ethanol and sodium metabisulfite, can also inhibit the growth of sensitive microorganisms (Kish et al., 1983).

Several methods have been used to characterize and identify yeasts, from biochemical phenotypic tests to molecular techniques (Pincus et al., 2007). Examples include phenotypic yeast identification methods such as the rapid screening tests, the analytical profile index (API) system and the use of the previously described selective media. Nevertheless, in recent years

these methods have been almost entirely supplanted by more accurate molecular biology techniques. Regardless of their discriminating power (species or strains), examples include DNA–DNA hybridization; karyotyping methods; fingerprinting methods such as interdelta polymorphism fingerprinting; and microsatellites (Querol et al., 1992; Ivey and Phister 2011).

Molecular techniques also include several culture-independent techniques, such as quantitative real-time polymerase chain reaction (q-PCR), and next-generation sequencing (NGS) techniques, which allow genetic polymorphisms to be detected through massive sequencing (Bokulich and Mills, 2012).

However, as suggested by Pincus et al. (2007), rapid conventional identification methods such as chromogenic media and rapid enzymatic methods allow rapid and presumptive detection of the most critical and common opportunists in the health sector. Another interesting example is obtaining the fingerprints of long-chain fatty acids of yeast by gas chromatography (GC) (Pretorius et al., 1999). This technique has been used in some studies since Abel et al. (1963) demonstrated that microorganisms can be classified into genera and species according to their lipid composition, which is determined using GC with a flame ionization detector (FID). Other studies include a rapid method of identifying several species of *Candida* based on the presence or absence of certain fatty acids in their lipid composition (Gunasekaran and Hughes, 1980), the differentiation of wine yeasts – *S. cerevisiae* from other spoilage yeasts – by their composition in total fatty acids (Rozès et al., 1992), and the identification of different spoilage yeasts in a wine bottling plant (Malfeito-Ferreira et al., 1989). The latter study also reported the use of solid media YPDA to standardize growth conditions and minimize any variation in fatty acid composition.

On the other hand, other gas chromatography techniques have also been applied to metabolome analyses for identifying and quantifying extracellular and intracellular metabolites with molecular masses lower than 1000 Da (Villas Bôas et al., 2005). Several authors have focused on determining the exometabolome – i.e. by-products of yeast metabolic activities – during alcoholic fermentation (Skogerson et al., 2009; Pinu et al., 2014a; Minebois et al., 2020). Others have evaluated the effect of growth conditions, such as low temperature. (López-Malo et al., 2013) or during Sauvignon must fermentation (Pinu et al.,

2014b). All the above-mentioned studies were All this previous work was carried out from liquid culture and to our knowledge, the production over time of intracellular metabolites during yeast colony growth has never been investigated. no bibliographic results mention the study of intracellular metabolites of yeast during their growth in solid medium.

The aim of this study was to develop a new gas chromatography-mass spectrometry (GC-MS) method based on the lipid and metabolome profiles of a single picked colony to differentiate species of *Saccharomyces*. The optimization took into account culture media, number of colonies used, and growth time. In addition, this methodology could allow physiological studies to be performed in a complete or defined solid growth medium to assess the yeast response to inhibitor or activator compounds.

2. Materials and methods

2.1. Microorganism strains and culture media

Six *Saccharomyces* strains were used in this study (Table 1). The strains Lalvin QA23® and Lalvin EC 1118® are labelled as *Saccharomyces cerevisiae bayanus* by the manufacturer. Since this denomination is not clear regarding the species, we have chosen to name them as *S. cerevisiae*, following López-Malo et al. (2013).

The inocula were prepared from commercial active dry yeast (ADY) and from liquid frozen cells. The ADY was rehydrated according to the supplier's instructions: 30 min in water at 37 °C. For cells from liquid frozen cultures, the strains were first incubated in YPD liquid (20 g/L of dextrose, 20 g/L of peptone, 10 g/L of yeast extract (Cultimed, Barcelona, Spain)) at 28 °C for two days. Then for all yeasts an overnight preculture in YPD was performed. Next, the yeast cells were spread onto solid media using decimal dilutions for colony counting. Two different media were used, the first was YPDA (YPD with 17 g/L of agar [Cultimed] at pH 4.5), and the second was liquid YPD. YPDA was used throughout the study for all the species and liquid YPD (20 mL) was only used to determine the fatty acid composition of the yeast cells grown statically overnight at 28 °C in an Erlenmeyer flask. The cells were harvested after centrifuging and the fatty acids were extracted and silylated, as explained below in the metabolite-extraction procedure.

Appendix

To study the effect of time and determine the minimal number of yeast colonies, the strain Lalvin QA23 was used as a reference. It was incubated for two, three, and seven days at 28 °C in aerobic conditions on YPDA. In contrast, the different *Saccharomyces* strains were incubated for two days at 28 °C on YPDA (Table 1). One colony (diameter of ca 3 mm) was taken from the corresponding growth media to compare the lipid and metabolome profiles between the strains.

Table 4. *Saccharomyces* strains used in this study.

Species	Strain commercial name or collection number	Source
Saccharomyces cerevisiae [c]	Lalvin QA23®	Lallemand [a]
Saccharomyces cerevisiae [c]	Lalvin EC 1118®	Lallemand
Saccharomyces cerevisiae	ICV K1 *Marquée*	Lallemand
Saccharomyces cerevisiae	Lalvin BM4X4	Lallemand
Saccharomyces kudriavzevii	11825	CECT [b]
Saccharomyces uvarum	1969	CECT
Saccharomyces pastorianus	1940	CECT

[a] Lallemand Inc., Montreal, Canada
[b] CECT, Spanish Type Culture Collection
[c] Labelled as *Saccharomyces cerevisiae bayanus* by the manufacturer

2.2. Estimation of total cell number in the colonies

The total cell number in a colony was estimated using the relationship between the optical density (OD) and the total cell number. The reference strain was also Lalvin QA23 and it was grown on YPDA at 28 °C. Between one and four colonies were randomly picked and immediately resuspended in 400 µL of sterile water. After homogenization, the OD of the decimal cell solution was determined at 600 nm. From the same decimal dilution, the cells were counted using a Neubauer chamber under a light microscope (Leica Microsystems, Wetzlar, Germany).

2.3. Metabolite extraction procedure

The metabolome and lipid extraction procedure was the same for all the strains. Briefly, using a modified version of the procedure described in López-Martínez et al. (2014), the specified numbers of colonies grown on the different media were introduced into Eppendorf tubes containing ca 100 mg of 0.5-mm glass beads (BioSpec Products, Qiagen) and 400 µL of methanol-water (1:1, v/v). As internal standards (IS), 10 µL of ribitol at 1 mg/mL (Sigma-Aldrich, Barcelona, Spain) and 10 µL of α-cholestane at 1 mg/mL (Sigma-Aldrich) were used for the metabolomic and lipidomic approaches, respectively. After shaking vigorously with a vortex mixer (30 sec), the microtube was heated to 90 °C for 5 min. Once cooled, 800 µL of chloroform was added and the tube was then shaken at 120 rpm for 20 min and centrifuged at 10000 rpm for 2 min. The two phases were physically separated from one another. Finally, the aqueous and organic phases were dried in a SC110 speed vacuum system (Savant Instruments, USA) for 4 h.

The dried residues were redissolved and derivatized. The aqueous phase (metabolome) was heated for 30 min at 70 °C in 40 µL of 20 mg/mL methoxyamine hydrochloride in pyridine (Sigma-Aldrich), followed by a 30 min treatment at 70 °C with 40 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, Sigma-Aldrich). For the organic phase (lipid), the samples were only derivatized with 80 µL of MSTFA at 70 °C for 30 min.

2.4. Gas chromatography-mass spectrometry analysis

Both the metabolome and lipid profiles were analyzed using GC-MS, with a 6890N GC system (Agilent Technologies, Germany) equipped with a DB-5HT column (30 m × 0.25 mm × 0.1 µm; Agilent Technologies) and an automatic injector (7683B, Agilent Technologies). Helium was used as the carrier gas at a constant flow of 1.0 mL/min. The compounds were detected with a mass selective detector (MSD, model 5975, Agilent Technologies). The MSD temperatures were 300 °C, 180 °C and 280 °C for the transfer, quadrupole, and source, respectively. The MSD data were acquired in electronic ionization scan mode at 70 eV within the 35 – 650 amu range after a solvent delay of 3 min and then analyzed. using the Agilent MSD Chemstation software (Agilent Technologies). The metabolites were identified using an in-house MS and the NIST 2005 libraries. The relative abundance of each identified

compound was calculated according to the respective chromatographic peak heights corrected in relation to the IS peak height, ribitol and α-cholestane for metabolome and lipid analysis, respectively. The results were expressed as arbitrary units (AU).

4.1. Metabolomic analysis

Two µL of the derivatized cell extract were injected at a split ratio of 20:1 at an injector temperature of 200 °C. The column oven temperature was initially held at 80 °C for 4 min and then increased, first to 200 °C at a rate of 5 °C/min, and then to 300 °C at a rate of 25 °C/min, where it was held for 7 min.

4.2. Lipidomic analysis

Three µL of the derivatized lipid extract were injected at a split ratio of 5:1 at an injector temperature of 300 °C. The column oven temperature was initially held at 90 °C for 1 min and then increased, first to 320 °C at a rate of 15 °C/min, and then to 380 °C at a rate of 4 °C/min, where it was held for 1 min.

4.3. Statistical analysis

Each combination of strains per condition (number of colonies, growth time, strains and solid growth media) was analysed using three independent biological samples. An ANOVA (Tukey honestly significant difference [HSD] test) and principal component analysis (PCA) were performed using the XLSTAT software 2018.7 package (Addinsoft, Paris, France) with a statistical significance level of p-value < 0.05.

5. Results and discussion

The composition of yeast cells, i.e., lipids (fatty acids, squalene and ergosterol), amino acids, sugars, organic acids and other metabolites, depends on the growing conditions, which include, among other factors, the chemical composition of the medium, and whether the conditions are aerobic or anaerobic (Klug, 2014; Malfeito-Ferreira et al., 1989; Manzanares et al., 2011). Therefore, the first objective of this work was to study the response of the *Saccharomyces cerevisiae* Lalvin QA23 strain over time, during its growth on YPD agar medium. In addition, the optimal number of colonies was verified in order to standardize the

parameters of the method and to determine the minimum yeast population necessary to yield a reproducible detection in the GC analysis. Thus, one to four colonies were randomly picked from the same plate after two days and pooled together. The average number of cells according to each colony pool (1 to 4) was significantly different, ranging from 0.75 x 10^7 to 2.23 x 10^7 cells/mL (Table 2). However, the relationship between the number of cells/mL and the colonies was well correlated (R^2 = 0.9822), which allows us to validate the results between the number of colonies and the metabolite profiles. Growth was analysed at two, three and seven days to determine the best and minimum time to obtain well-differentiated metabolome and lipid profiles.

Table 2. Relationship between the number of sampled colonies and the number of cells/mL determined by counting the cells of the strain Lalvin QA23 at two days. Different lower-case letters indicate a significant difference (*p*-value < 0.05) between the number of colonies using the Tukey (HSD) test.

Number of colonies	cells/mL 10^7
1	0.75 ± 0.014 [a]
2	1.06 ± 0.085 [b]
3	1.74 ± 0.092 [c]
4	2.23± 0.014 [d]

5.1. Effect of number of colonies and growth time on the lipid composition of strain Lalvin QA23

The lipids determined were grouped as unsaturated fatty acids (UFAs), saturated fatty acids (SFAs), sterol, and squalene (Figure 1). Palmitoleic (C16:1) and oleic (C18:1) acids were the main UFAs detected. Myristic (C14:0), palmitic (C16:0) and stearic (C18:0) acids were the SFAs identified. The lipid profiles of Lalvin QA23 identified by GC-MS agreed with the fatty acid composition described for *S. cerevisiae* (Klug, 2014); in other words, no polyunsaturated fatty acids were detected. Under the growth conditions used (YPDA), the only medium-chain fatty acids (MCFAs, C6 to C12) detected were capric acid (C10) and lauric acid (C12), regardless of the growth time (Supplementary material 1). These MCFAs are generally found in higher concentrations in hypoxic conditions, such as white winemaking or during the anaerobic growth of *Saccharomyces* and allow the cell to modulate its structural and functional

membrane integrity (Beltran et al., 2008). The total *S. cerevisiae* fatty acids detected in strain Lalvin QA23 were distributed as follows: 50 – 55% oleic acid, 25 – 30% palmitoleic acid, 25 – 30% palmitic acid, 8 – 9% stearic acid, and only 1.8 – 3.4% MCFAs.

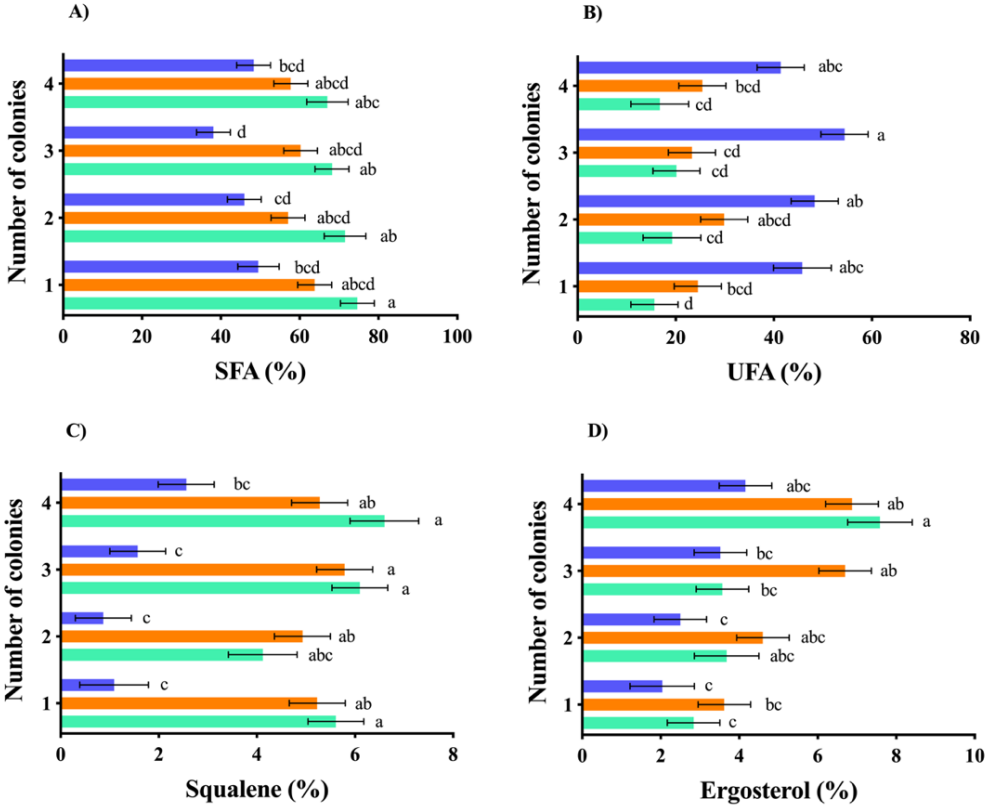

Figure 1. Effect of time (■ 2 days, ■ 3 days, ■ 7 days) on the lipid composition (%) of strain Lalvin QA23 cells according to the number of colonies. Saturated fatty acids (SFA) (A), unsaturated fatty acids (UFA) (B), squalene (C) and ergosterol (D). Mean ± standard deviation (n=3). Different lower-case letters indicate a significant difference between the number of colonies using the Tukey (HSD) test at *p*-value < 0.05.

Interestingly, using our simple extraction procedure (colony resuspended in a two-phase solvent system and heating the cells for 5 min) we detected squalene, the precursor of sterol biosynthesis, and ergosterol, the final product of this biosynthesis in yeast. The detection of ergosterol, the main free sterol present in the membranes of yeast cells (Tuller et al., 1999) was expected. Surprisingly, squalene, which is normally present in low percentage in yeast cells grown under aerobic conditions, has also been detected; no other sterol precursors were detected under our experimental conditions (growth culture and lipid determination).

No significant changes were found in percentages of SFA, UFA, squalene, and ergosterol according to the number of colonies (Figure 1). The major lipids identified were fatty acids, which represented around 87% of total lipids detected, regardless of how many colonies were analysed (Figure 1A and 1B). In comparison, squalene and ergosterol were detected in the 3.3 – 4.8% and 2.8 – 6.2% ranges, respectively (Figure 1C and 1D). Nevertheless, the ergosterol percentage increased significantly from 1 to 4 colonies (Figure 1D), while the percentage of squalene remained constant (Figure 1C). Based on these results, analysing just one colony should be sufficient to provide a statistically consistent lipid profile.

The evolution of yeast lipids as a function of growth time on solid media has not previously been described. However, many studies have evaluated lipid evolution under different fermentation conditions, due to the alteration of the membrane lipids in stressful environments (Bardi et al., 1999; Torija et al., 2003). For example, as fermentation progresses, under anaerobic conditions, yeasts cannot synthesize sterols or long-chain unsaturated fatty acids (Aranda, 2011). The only study that has analysed yeast cell lipids from a colony was performed by Malfeito et al. (1997), using a large 2-cm diameter colony. In our study, we observed that increasing the growing time of colonies on YPDA played an important role in the ratios of total cell lipids (Figure 1). The same pattern was generally observed for all lipids, regardless of the number of colonies. On other hand, the percentage of SFA decreased over time as the number of colonies grew (Figure 1A), while the opposite pattern was observed for the percentage of UFAs: the longer the growth time, the higher the percentage of UFAs (Figure 1B). With respect to the proportion of squalene found in the cells over time, the same pattern was observed as for SFA, in other words, percentages decreased as a function of growth time (Figure 1C). In addition, the percentage of ergosterol increased until the third day in all samples analysed. However, the observed diminution at day 3 for four picked colonies could be due to the saturation of signal detection in our analytical conditions (Figure 1D).

Appendix

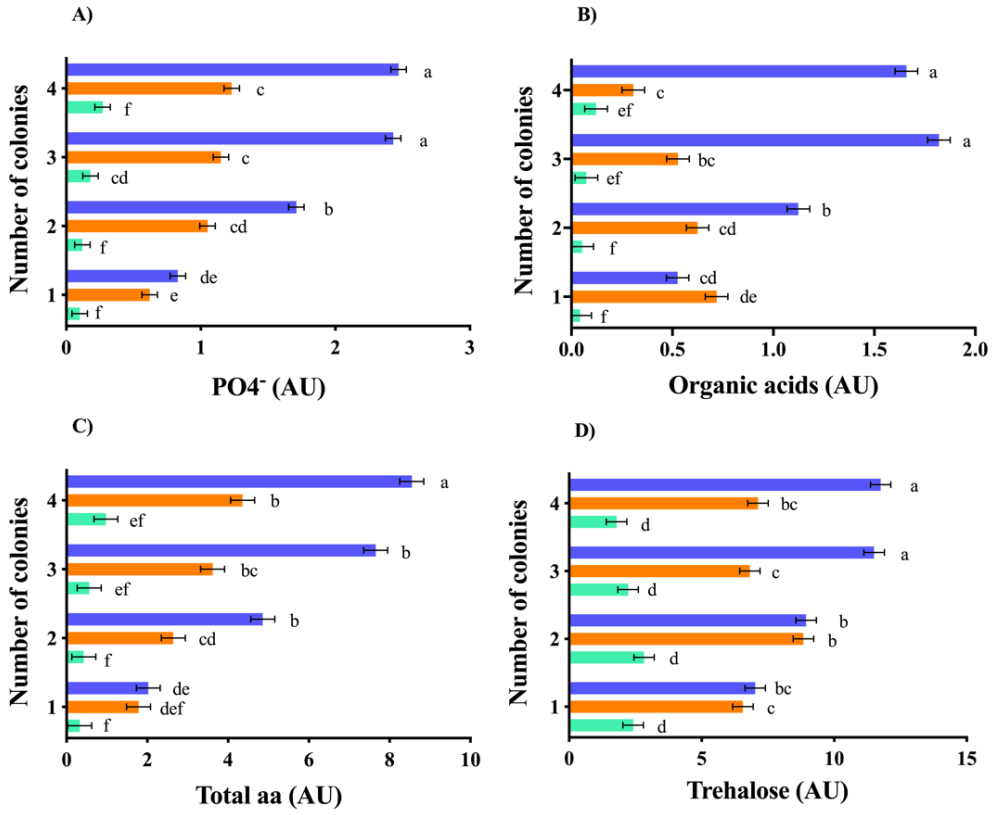

Figure 2. Effect of time (■ 2 days, ■ 3 days, ■ 7 days) on the main metabolomic compounds of strain Lalvin QA23 cells according to the number of colonies. (A), organic acids (B), total amino acids (Total AA) (C), and trehalose (D). Mean ± standard deviation (n=3). Different lower-case letters indicate a significant difference between the number of colonies and time using the Tukey (HSD) test at *p*-value < 0.05. AU, arbitrary units: height of the metabolite normalized with the height of the internal standard. Clusters were used to visualize the different groups; they are not based on statistical intervals/analysis.

5.2. Effect of number of colonies and growth time on the metabolome composition of strain Lalvin QA23

From an average of more than 150 peaks detected in one run, several substances have been identified in the metabolic profiles of strain Lalvin QA23. Among these metabolites we identified amino acids, polyamines, organic acids, sugars (mono- and disaccharides), and polyols (sugar alcohols such as inositol, erythritol, 2,3-butanediol, glycerol, etc.). The main results are shown in Figure 2. The compounds were clustered in four groups: phosphate anions ($PO4^-$); organic acids (succinic, fumaric, malic and citric acids); total amino acids

identified (Total AA), which also included some biogenic amines (putrescine and cadaverine), and trehalose (Supplementary material 1).

To the best of our knowledge, the temporal evolution of the yeast metabolome on plates of solid growth medium has never been studied, and that was one of the objectives of this work. Indeed, just as with lipidomic studies, all metabolomic analysis using GC-MS are currently performed with extra- and intracellular yeast samples growing in different liquid media. Examples of this include the alcoholic fermentation of a defined medium (Minebois et al., 2020), the fermentation of grape juice (Ritcher et al., 2015), and even studies of intracellular metabolic changes during bioethanol fermentation (Chen et al., 2016).

It was observed that all the metabolites followed an upward trend over time. In almost all cases there were significant differences between the cultures at two, three, and seven days. An increasing trend emerges when the significant differences between the number of colonies are considered individually. In all the groups of metabolites, one colony and two colonies are clustered in two different groups that are significantly different from a third group formed by three and four colonies. There was one exception: total AA were separated into four classes according to the number of colonies.

If the statistical results are analysed together, the impact of the number of colonies considered becomes apparent mainly on the seventh day (Figure 2). For $PO4^-$ (Figure 2A), organic acids (Figure 2B), and trehalose (Figure 2D), there were no differences between three and four colonies on the seventh day. As with the lipid profiles, this could be due to a large quantity of these metabolites in the yeast cells during this growth period, inducing a non-linear response of the detector. This would therefore make it necessary to dilute the sample. The same happened between two and three colonies when detecting the amino acids identified. In relation to evolution over time, some differences were also found between the second and third day. However, one colony was an exception among all the clusters; this is more clearly described in Figure 3.

As can be seen in Figure 2A, the content of the phosphate anion ($PO4^-$) derived from phosphoric acid in cells increased with time, but only slightly according to the number of colonies, for the reasons explained above. Inorganic phosphate is an essential nutrient for all

organisms and required for basic vital needs, including the synthesis of nucleic acids, phospholipids, and cellular metabolites. It is taken up from outside the cells through several membrane transport systems and is compartmentalized in yeast vacuoles as free phosphate and polyphosphate (Persson et al., 2003). Sommer (1996) reported that yeast extract contains ca. 1.8% (w/w), suggesting that there is no limitation of extracellular phosphate. In addition, Markham and Byrne (1967, 1968) showed that the limiting concentration of P with 5% (w/v) glucose in the growth medium was 65 μg/mL. This could mean that, under our conditions, the increasing levels of intracellular phosphate detected over time could be due to an increase in the release of phosphate from its storage form in vacuoles and hence its greater availability, or an experimental artifact related to the sampling process.

For the total organic acids (succinic, malic, fumaric and citric acids), a plateau was observed at day 3 regardless of the number of colonies analysed; however, subsequently (on day seven), the percentage was proportional to the cell concentration (Figure 2B). Aside from lactic acid, which is produced from pyruvic acid, the other organic acids are formed in the Krebs cycle. After 2–3 days of growth on YPDA medium, the yeast cells had consumed the sugars, producing CO_2 and generating an anaerobic atmosphere, which could explain the slow formation of these acids. However, after depletion of sugars, the production of these acids increased. These aging stress conditions could influence the increasing concentration of trehalose over time in a similar way (Figure 2D). Indeed, it has been proven that the accumulation of trehalose in yeasts is a response to certain stressful conditions, such as fermentation (Wang et al., 2014).

An interesting result is the increase over time in the levels of some amino acids and amines, such as ornithine and putrescine. In yeast cells, ornithine is decarboxylated to putrescine, which is the main substrate for polyamine biosynthesis (Tabor et al., 1982). It should be noted that ornithine and putrescine, as well as other polyamines not identified in our extract but likely to have been present, appeared from the third day onward, and their increase in time correlated with actively growing yeast (Kay et al., 1980). These metabolites are related to aging cells and as a response to the generation of reactive oxygen species (ROS) (Eisenberg et al., 2009). It must be remembered that the YPD growth medium contains amino acids due to the presence of peptone and yeast extract, and that these may be assimilated by yeast and

not synthesized. To our knowledge, there is no information available on whether these products contained other polyamines, such as cadaverine, spermine, or putrescine.

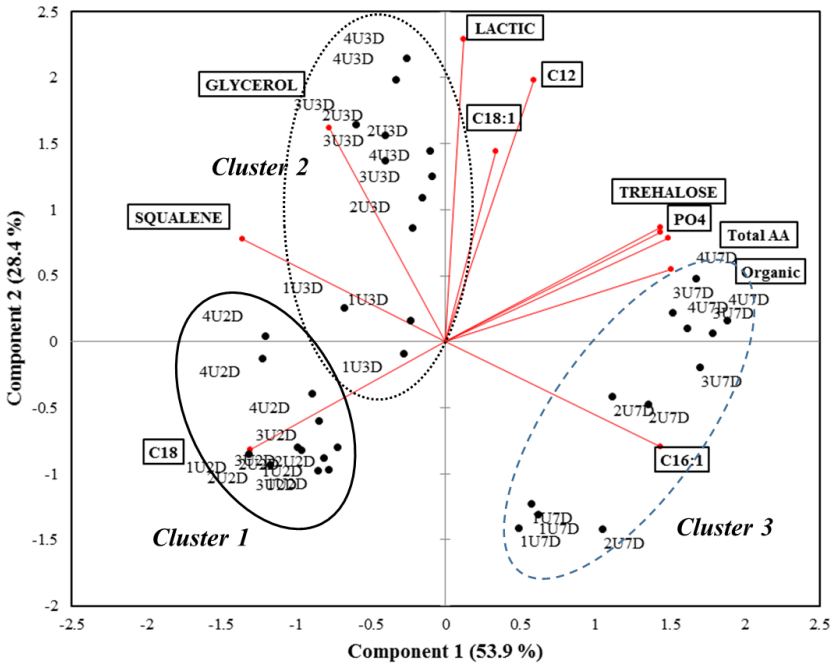

Figure 3. Biplot of principal component analysis (PCA) with varimax rotation of the principal identified metabolites and lipids of the Lalvin QA23 strain. Score plots of factor 1 (55.39%) against factor 2 (15.11%) where the samples were grouped in three clusters: Cluster 1: second day; Cluster 2: third day; and Cluster 3: seventh day. U, corresponds to the number of colonies; D, corresponds to the number of days.

In addition, other metabolites were detected with a high degree of certainty (> 80% confidence), including glycerol and inositol. There was a significant increase in inositol related to the number of days and number of colonies. This increase could arise from two different mechanisms: firstly, because these yeasts produce inositol from glucose, and secondly, because it also has inositol transporters that incorporate it from the medium (Nikawa, 1991). Inositol is an important component of several secondary messenger molecules and phospholipids (Ploier et al., 2014); their rising concentrations could therefore be due to the increase of biomass over time.

In contrast, the concentration of glycerol only presented significant differences for one colony; in fact, the percentage of this increased on the third day and significantly decreased on the

seventh day. This decrease could be due to the increase in other metabolites on day 7 as the metabolite levels were calculated from an area ratio.

5.3. Effect of number of colonies and growth time on the lipid and metabolome composition of strain Lalvin QA23

The lipidomic and metabolomic results of the strain Lalvin QA23 samples were subjected to a principal component analysis (PCA), which used C12, C18, C16:1, C18:1, and squalene for lipid compounds and lactic acid, glycerol, total AA, $PO4^-$, organic acids, and trehalose for metabolomic compounds. The resulting model had two factors that explained 82.3% of the variance. The samples visibly clustered into three groups according to growth day: cluster 1: second day; cluster 2: third day; and cluster 3: seventh day. PC1 described the time-evolution of the major part of the organic acids, total AA, $PO4^-$, trehalose, C16:1, and C18.

Table 3. Main effects of number of colonies and growth time on YPDA and the interaction between them on the lipid composition and main metabolomic compounds of strain Lalvin QA23. Medium-chain fatty acids (MCFA), saturated fatty acids (SFA), unsaturated fatty acids (UFA), Erg/Sq, ergosterol/squalene, (n=3 independent colonies); ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

	Colony effect	Time effect	Colony x Time
MCFA	***	***	***
SFA	ns	***	***
UFA	ns	***	***
UFA/SFA	ns	***	***
Ergosterol	***	ns	***
Squalene	*	***	***
Erg/Sq	ns	***	***
Organic acids	***	***	***
Total AA	***	***	***
Trehalose	***	***	***
PO_4^-	***	***	***

Even though these differences were mostly significant, it was observed that the signal obtained for one colony and two days is sufficient and reproducible. Thus, the analysis is most

efficient the growth time is two days, as this is the shortest growth time necessary. Furthermore, selecting just one colony could avoid possible cross-contamination on the agar plate, which would vary the results. This should also avoid the problem of signal saturation that was observed with three and four colonies in some cases.

Furthermore, similarly to the ANOVA results (Table 3), this component also separated the third cluster according to the number of colonies, due to the increase and decrease in the metabolites and lipids mentioned previously. We should also point out that there was evident separation of one colony sample from the second and third group, located near the first cluster. With respect to the second principal component, it was observed that the main contributors to the separation of clusters were C12, C18:1, glycerol, lactic acid, and to a lesser extent squalene, which allowed a good separation of cluster 2 (colonies from the second day) from the other clusters. Finally, the first group was clearly associated with C18 synthesis.

Table 3 shows that the differences between colonies, growth time, and their interaction were statistically significant in most cases. There were some exceptions with regard to the number of colonies (which had no significant effect on SFAs, UFAs, UFA/SFA and ergosterol/squalene) and the growth time effect in the case of ergosterol. It was remarkable that the effect of the number of colonies on squalene was only significant with a confidence interval of 95%.

5.4. Differentiation of different *Saccharomyces* species with the method developed

Different strains of *S. cerevisiae* (K1, BM4x4, QA23 and EC 1118) were analysed to observe the differences between their lipid profiles and metabolomes. These were characterized under the previously optimized conditions of two days' growth, and one colony on YPDA.

The metabolome results (two days, one colony) obtained for all strains were not sufficiently different to include them in the PCA analysis. However, a variation trend can be observed between the two species. The main differences are reflected in the fatty acid profile. The strains QA23 and EC 1118 (*S. bayanus*) had higher concentrations of SFAs than K1 and BM4x4 (*S. cerevisiae*), particularly C18. On the other hand, the last strains cited had higher percentages of UFAs than the others (results not shown). This variation was even more pronounced when comparing C18:1. Even though these differences were not significant, the

trend can be seen clearly in Figure 4. The four strains were separated by principal component 1 and, the SFA and C14 variables determined this discrimination.

Comparing these strains of *S. cerevisiae* with three other species of *Saccharomyces* (*S. uvarum*, *S. pastorianus*, and *S. kudriavzveii*) reveals significant differences. Principal component 2 separated the *S. cerevisiae* strains from others influenced by variable UFAs (C18:1 and C16:1) (Figure 4). However, *S. uvarum* is separated from *S. pastorianus* and *S. kudriavzveii* by the principal component 1. This species presents higher SFA values, particularly C16, than the others, which had higher UFA concentrations.

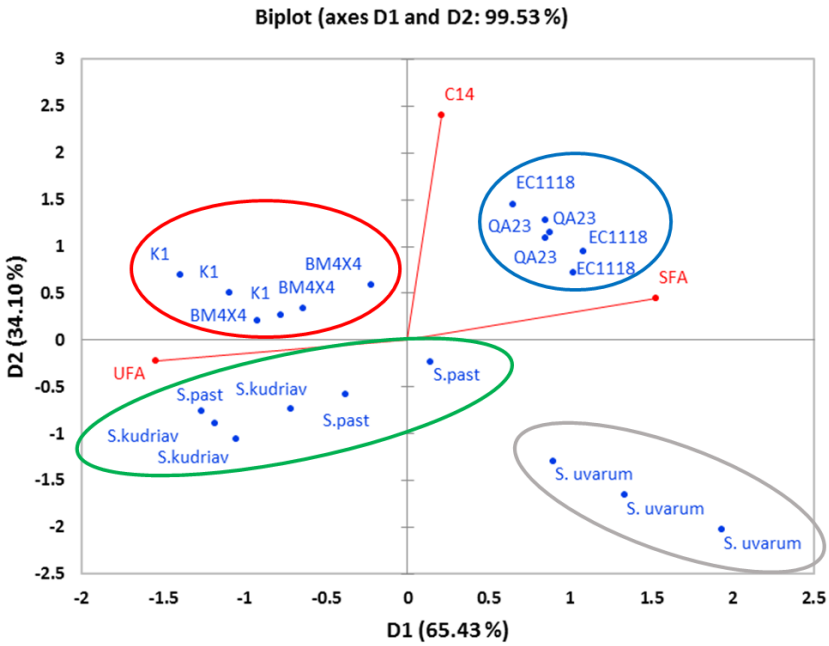

Figure 4. Biplot of the principal component analysis (PCA) with varimax rotation of the main fatty acid composition of the cells of the *Saccharomyces* species. SFA, saturated fatty acids (sum of C14, C16 and C18); UFA, unsaturated fatty acids (sum of C16:1 and C18:1); K1 and BM4X4, *S. cerevisiae* strains (red circle); EC1118 and QA23, *S. cerevisiae* strains (blue circle); *S. kudriavzevii*, *S. pastorianus* (green circle) and *S. uvarum* (gray circle). The ellipses added to the PCA are used as visual aids to identify the different clusters, they are not based on statistical intervals/analysis.

The metabolomic profiles of the seven species, after two days of growth on YPDA picking one colony, were subjected to ANOVA analysis. After two days of growth, few intracellular metabolites were identified, and significant differences were only recorded for lactic acid,

trehalose, and the sum of AA as well as organic acids. For the other metabolites detected, PO4-, glycerol, and inositol, no significant differences were observed. With respect to lactic acid and trehalose synthesis (Figure SD1), *S. kudriavzevii* had the lowest content of intracellular metabolites compared to the other species. This is likely to be related to the weak growth of this species at 28 °C, as its optimum temperature for growth is 24 °C (Arroyo-López et al., 2009). Greater levels of trehalose were accumulated by strain Lalvin QA23. The total AA percentage was higher in *S. cerevisiae* (K1 and BM4x4) and *S. uvarum* than in *S. kudriavzveii*, *S. pastorianus* and in the other *S. cerevisiae* strains, EC1118 and QA23.

5.5. Influence of the physical state of the culture medium on the lipid composition

It was very interesting to note that the state of the growing medium, liquid or solid, made a significant difference to the UFA composition in the cells of all yeast species (Figure 5). The same behaviour was observed for all the other lipids determined (Suplementary material 2). This result shows that, independently of the yeast species analysed, SFAs are synthesized in the first moments of cell growth on a solid growth medium. One might hypothesize that the liquid growth condition is more hypoxic than the solid medium, promoting the synthesis of unsaturated fatty acids, but this is not the case.

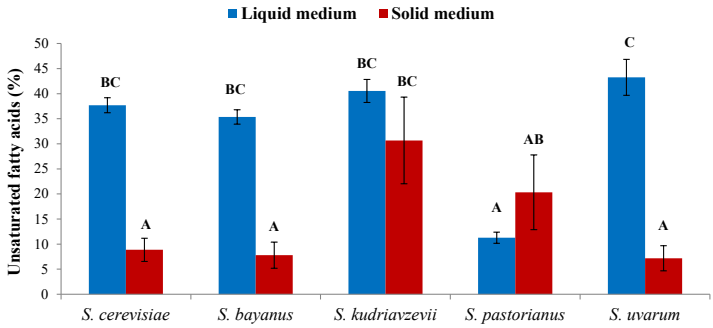

Figure 5. Unsaturated fatty acid composition in yeast cells growing in either liquid or solid YPD. Unsaturated fatty acids (sum of C16:1 and C18:1); *S. cerevisiae* K1 strain; *S. cerevisiae* Lalvin QA23 strain; *S. kudriavzevii* strain, *S. pastorianus,* and *S. uvarum*. Different upper-case letters indicate a significant difference in UFA composition using the Tukey (HSD) test at *p*-value < 0.05.

The same behaviour was observed for *S. cerevisiae* (K1 and Lalvin QA23 strains) and *S. uvarum* but not for *S. kudriavzevii* and *S. pastorianus* (Figure 5). No significant difference was found between the *S. kudriavzevii* results in the two culture conditions: liquid vs solid. However, for *S. pastorianus*, although there are no significant differences, the trend is reversed, with more UFAs being recorded in the solid medium than in the liquid medium.

6. Conclusions

In this study, we used gas chromatography to conduct a lipidic and metabolomic analysis of several strains of different *Saccharomyces* species, from colonies grown on agar plates. The method conditions were optimized by studying differences in terms of number of colonies, growth time, and culture media. There were significant changes over time and in terms of the number of colonies, as in the case of the metabolome, but we did not observe any significant changes on day 2 regardless of the number of colonies. We concluded that one single colony grown for two days is the most efficient and quickest way of producing a good chromatographic signal. To our knowledge, this is the first time it has been observed that when wine yeasts are cultivated on a solid medium there is (i) greater synthesis of C18 than of C18:1, (ii) increasing temporal evolution of intracellular metabolites, and (iii) differentiation between *Saccharomyces* species on the basis of metabolomic and lipid profiles. In this study, certain strains of *S. cerevisiae* were differentiated from each other using the fatty acid profile of a colony. Some manufacturers. Curiously, two of these strains, EC1118 and QA23 have occasionally been named by the manufacturer as *S. cerevisiae bayanus*. Moreover, despite the hybrid origin of *S. bayanus* (*S. cerevisiae* x *S. uvarum* x *S. eubayanus*) (Ono et al., 2020), its different profile from *S. cerevisiae* confirms that there are some physiological differences between these two species. The major advantage of the method developed is that it may be very useful for differentiating those and other yeast species and even other wine microorganisms grown under the same conditions, and for tracking their main metabolomic changes over time.

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Supplementary data

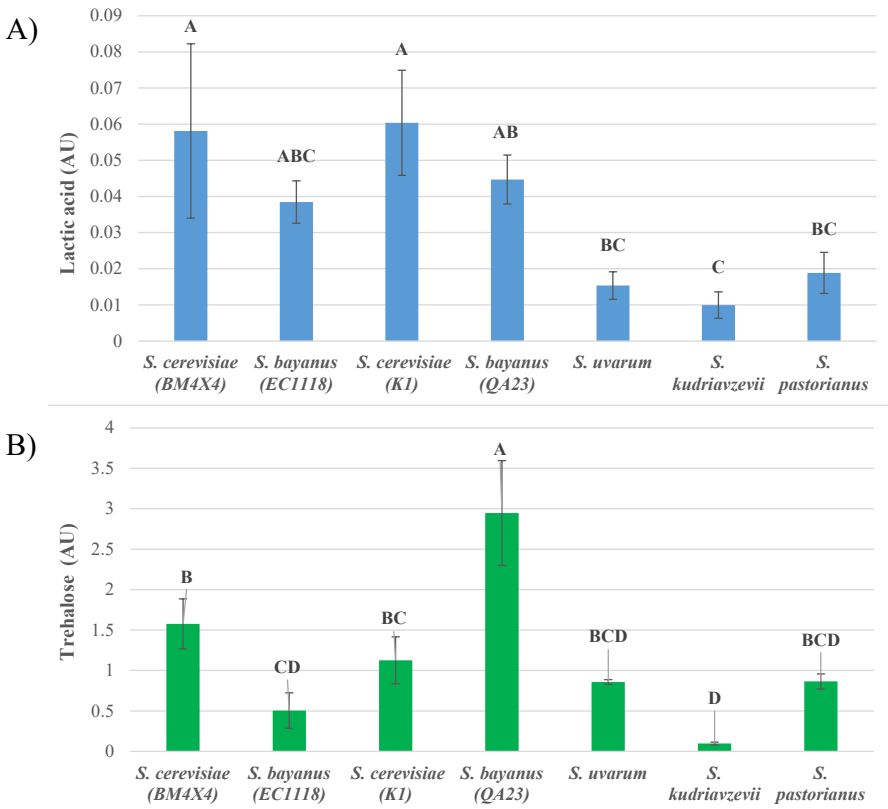

Figure SD 1. Effect of two days of growth on the intracellular content of lactic acid and trehalose for different *Saccharomyces* species by analysis of a single colony. Mean ± standard deviation (n=3). Different upper-case letters indicate a significant difference between *Saccharomyces* strains using the Tukey (HSD) test at *p*-value < 0.05. AU, arbitrary units: height of the metabolite normalized with the height of the internal standard (ribitol).

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