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INNOVATIVE MICROBIAL STRATEGIES FOR MODULATING THE AROMATIC PROFILE OF WINES AND PROTECTION AGAINST ALTERATION PROCESSES

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Summary

CHAPTER 1

General introduction

1.1 Trend of oenological microbiology

For thousands of years, wine and fermented alcoholic beverages have been produced without understanding the biological, biochemical and chemical processes involved. Only nowadays we know that a complex microbial consortium is involved in the conversion of grape juice/must into wine, with yeasts dominating the alcoholic fermentation. The main bioprocess during alcoholic fermentation is the conversion of carbohydrates into ethanol. However, a large variety of primary and secondary yeast metabolites are responsible for greatly impacting wine quality (Di Maro et al., 2007; Petruzzi et al., 2015).

Malolactic fermentation one of the most important stages of wine production, is carried out by acid lactic bacteria. This so-called secondary wine fermentation involves several lactic acid bacteria species, including *Lactobacillus*, *Pediococcus*, and *Oenococcus* (Lonvaud-Funel, 1999). *Oenococcus oeni* is the most adaptable to wine conditions (low pH and high ethanol concentration) and is the most common bacteria species detected in wines during malolactic fermentation.

Lactic acid bacteria are commonly used in food biotechnology, and effective management of these microbiological processes necessitates a better understanding of bacterial behaviour and metabolism. Currently, only a few commercial starter cultures of this species have been demonstrated to successfully perform malolactic fermentation, and more research into novel starter cultures with defined technological and flavouring skills is necessary (Alegría et al., 2004).

Several studies have shown that the microbial population originally present in the must is very diverse in terms of eukaryotic microbes, and that *Saccharomyces cerevisiae* is not the dominant species of the microbial population during the early stages of spontaneous alcoholic fermentation (Di Maro et al., 2007; Andorrà et al., 2008; Mendoza et al., 2010). Indeed, yeasts of oenological significance that do not belong to the *Saccharomyces* genus can be found in considerable numbers. As a result, this diverse group of microorganisms is commonly referred to as "non-*Saccharomyces*." The earliest steps of alcoholic fermentation are driven by non-*Saccharomyces* species, followed by the dominance of *S. cerevisiae*, which is in responsible for the core and closing phases of alcoholic fermentation. Only *Saccharomyces* species (*S. cerevisiae* or, less frequently, *Saccharomyces uvarum*) can complete alcoholic fermentation.

These starter microorganisms can assist winemakers in achieving a variety of benefits, including the completion of alcoholic fermentation and malolactic fermentation, the reduction of time required for fermentation/production (with a corresponding reduction in production costs), and the control of negative effects caused by indigenous microorganisms. Furthermore, microbial starters may enable the development of specialised products (e.g. sparkling wines) (Vogel et al., 2011; Garofalo et al.,

2016). As a result, commercial starter cultures enabled winemakers to manufacture stable and sustainable products, primarily by utilising the large diversity of genotypically and technologically characterised *S. cerevisiae* strains (Bartowsky et al., 2005; Mateo et al., 2001).

In other words, this biotechnological approach has transformed the way wine is produced, reducing the spread of spontaneous indigenous wine while guiding and accelerating microbial-based bioprocesses of interest in oenology. Along with continuing interest in the selection of new and suitable *S. cerevisiae* and *O. oeni* strains, as well as the design of new microbial combinations, an increasing emphasis has been placed in the last decade on the selection of non-*Saccharomyces* species/strains for the formulation of new starter cultures capable of driving alcoholic fermentation. According to several studies, several species have been shown to have relevant qualities in the creation of aromatic compounds as well as natural bio-protection of wine against spoilage yeasts and bacteria. Some of the consequences of using selected non-*Saccharomyces* species include: the production of mannoproteins, the reduction of volatile acidity, the reduction of alcohol content, the production of esters, terpenes, and thiols, the production of polysaccharides, the stabilisation of colour, and the consumption of malic acid (Benito 2008; Wang et al., 2016). Less recent research has investigated into mixed and sequential fermentations of non-*Saccharomyces* and *Saccharomyces* to see how they affect the volatile component of wine (Zironi et al., 1993). Although the study of non-*Saccharomyces* is not a new issue, further research on species other than those commonly employed in oenology is required.

1.2 High sugar matrix as a source of yeasts

The primary biocatalysts of alcoholic fermentation are yeasts of the species *S. cerevisiae*, which are frequently utilised as starters in a variety of fermentation processes (such as wine, beer and bread). In particular, *S. cerevisiae* becomes the dominating species in the alcoholic fermentation of grape musts as the ethanol concentration rises.

As a result, naturally occurring *S. cerevisiae* is typically isolated by spontaneous must fermentations, which in the past has led some to believe that *S. cerevisiae* is commonly found on grapes.

According to Taylor et al. (2014), the fungal population on mature grapes in vineyards is primarily made up of non-*Saccharomyces* species and contains less than 0.00005% *Saccharomyces* spp. For these reasons, it appears that the vineyard is not the main source of this yeast.

Numerous studies have documented the spread of several yeast species, including *S. cerevisiae*, in other naturally occurring settings such other fruits, honey, insects, oak fluxes, and other broad-leaved trees. These results support the notion that the vineyard is a "transient" environment where the presence of *S. cerevisiae* is primarily related to grape berry ripening. When fruit is not accessible,

other habitats could function as a refuge.

1.2.1 Sources of yeasts from wine

Today, the standard method of wine production includes the use of commercial *S. cerevisiae* strains as starters to guarantee the reproducibility of fermentation through the vintages, helping to produce wines that are more harmonious. Due to the decreased diversity in the microbial populations participating in fermentation, this oenological practise has led to a certain reduction and flattening of the wine sensory features. The end consequence is that wine traits are similar around the world.

Since native yeasts from particular wine regions are likely better adapted to various climatic conditions, there is growing interest in the isolation and selection of these strains. The creation of unique odours depends primarily on native microorganism.

In terms of technical and metabolic features, the *S. cerevisiae* yeast strains isolated by Capece et al. (2019) from the must of Primitivo di Manduria grapes displayed great variability. Due to their ability to compete with the innately existing spontaneous microflora, these native yeast strains appear to be more adapted to the environmental conditions of grape must. Comparing wine made with commercial starter to wine made with indigenous inoculation starters, this characteristic ensures that the fermentative process is carried out by indigenous inoculated starter, providing wine with desired characteristics and the distinctive flavour of Primitivo di Manduria. Similar observations have been made with strains isolated from Nero d'Avola (Capece et al. 2010) and Uva di Troia (Petruzzi et al. 2017) grape musts, demonstrating the ability of native strains to exalt wine peculiarities and ensure the preservation of the typical sensory proprieties of the wine of any given region.

1.2.2 Other sources of yeasts

S. cerevisiae yeast strains are not exclusive to the viticulture and oenology fields, as already mentioned. The fermentation of bread, beer, apple cider, and other alcoholic beverages really employs a variety of strains.

Other than grape musts, researchers have isolated *S. cerevisiae* yeast strains from sugar matrices.

According to Resende et al. (2018), *S. cerevisiae* is the main agent responsible for the alcoholic fermentation of Chicha (also known as indigenous Andean beer), the oldest and most well-known beverage in several South American countries. In order to demonstrate the potential of these yeast strains to metabolise a variety of uncommon carbon sources, such as sorbose, glucosamine, ribose, arabinose, ramnose, melibiose, starch and cellobiose, the authors isolated many yeast strains from fruit and/or cereals, such as maize or rice (the main ingredients for producing this beverage).

S. cerevisiae has also been isolated from honey (Carvalho et al., 2005), along with additional yeast

species such *Metschnikowia* spp. (Seijo et al., 2011), *Zygosaccharomyces rouxii*, *Zygosaccharomyces mellis*, *Saccharomyces mellis*, and *Saccharomyces rosei*. These can modify the ratio of sucrose, glucose, and fructose, lowering the sugar concentration and activating fermentations process.

1.3 Yeasts with fermentative and non-fermentative activity

1.3.1 Non-Saccharomyces *yeast*

Despite grape must contain naturally complex and diverse microbial community, the alcoholic fermentation process is mainly conducted by *S. cerevisiae* with either inoculated of indigenous strains.

Inoculation with active dry yeasts of *S. cerevisiae* is a common practice since the 20th century, to assure prompt and reliable fermentations and wines with a consistent and predictable quality.

Many additional yeast genera and species, however, can survive the fermentation process due to their tolerance to ethanol.

Due to their low fermentative ability and excessive production of off flavours like acetaldehyde, acetic acid, acetoin of ethyl acetate, or undesirable volatile phenols (i.e. from *Brettanomyces* species) (Esteves et al. 2019), these species are commonly referred to as "non-*Saccharomyces* yeasts" and have secondary relevance during the fermentation of grape must to wine

Numerous studies have revealed that these undesirable chemical compounds are species- and strain-dependent, and since the 1980s, it has been established that some non-*Saccharomyces* species have favourable effects, primarily enhancing the complexity, texture, and flavour integration of wines during spontaneous fermentations (Gschaedler, 2017).

In fact, they are able to offer distinctive flavour complexity or mouthfeel while expressing terroir-associated qualities (Binati et al. 2019), which helps satisfy consumer desire for new wine types. Non-*Saccharomyces* yeasts could also have positive impacts to wines characteristics.

Typically, non-*Saccharomyces* yeasts (primarily from the genera *Hanseniaspora*/*Kloeckera*, *Candida*, *Pichia*, *Zygosaccharomyces*, *Schizosaccharomyces*, *Torulaspora*, *Kluyveromyces*, and *Metschnikowia*) dominate the initial stages of alcoholic fermentation before being replaced by *S. cerevisiae*, which completes the process (Jolly et al. 2014; Gschaedler, 2017).

1.3.2 Non-Saccharomyces *without fermentative activity*

The largest number of these strains often originates from the surface of grape berries, cellar equipment and winery environment, expiring quickly once the fermentation starts. At first, it was thought that these strains expired as a result of the increased concentration of ethanol or added SO_2. Recent research has revealed that the cause is more intricate and undoubtedly strain-specific: many non-

Saccharomyces species, in fact, can persist and survive at significant levels even at the late stage of fermentation process (Andorra et al. 2011; David et al. 2014; Wang et al. 2014; Albertin et al. 2017; Gschaedler 2017). The *Brettanomyces* genus is well recognised among non-*Saccharomyces* yeasts since it degrades wines all over the world. Five species now make up the Pichiaceae family-related genus *Brettanomyces*: *B. custersianus*, *B. naardenesis*, *B. nanus*, *B. anomalus*, and *B. bruxellensis*. They can be categorised as facultative anaerobic yeasts and Crabtree-positive yeasts, like *S. cerevisiae*, and can survive in both aerobic and anaerobic environments.

In addition to beer, sherries, dairy products, sourdough, cider, kombucha, olives, tequila, and tamarind, *Brettanomyces* yeasts have also been discovered in grape berries, wine, and wine-making equipment.

B. bruxellensis often is present in lower quantities throughout fermentation than other yeasts, but with time and during malolactic fermentation, it may rise and take over, negatively altering the sensory characteristics of wine (Renouf et al., 2006).

According to Chatonnet et al. (1992), *B. bruxellensis* may indeed synthesize 4-vinylphenol and 4-ethylphenol from p-coumaric acid, as well as 4-vinylguaiacol and 4-ethylguaiacol from ferulic acid, which provide characteristics like animal leather, barnyard, horse sweat, and animal smells to wine. Additionally, this yeast may create biogenic amines such cadaverine, hexylamine, phenylethylamine, putrescine, and spermidine, according to Agnolucci et al. (2009).

The typical antimicrobial agent used in winemaking to successfully combat *B. bruxellensis* in grape must and wine is sulphur dioxide. When wine is destined for ageing in oak barrels, eliminating *B. bruxellensis* becomes even more crucial. According to Malfeito-Ferreira et al. (2004), residual yeast cells in wine could enter wood pores and cracks and create favourable ecological niches that can contaminate and damage ageing wine.

1.3.2 Non-Saccharomyces *with fermentative activity*

During the pre-fermentative stage, three major genera (*Hanseniaspora* spp., *Candida* spp., and *Metschnikowia* spp.) predominate: *Hanseniaspora uvarum* has been described as a major non-*Saccharomyces* apiculate yeast during the early stages of fermentation; *Starmerella bacillaris* has been isolated from grape must regardless of wine-producing region or grape variety; *Metschnikowia pulcherrima* has been reported as a high population in grape musts (Albertin et al. 2017). Furthermore, they may have an immediate impact on wine fermentation by creating flavour or indirectly by influencing the growth and metabolism of *S. cerevisiae*. *M. pulcherrima* and *S. bacillaris*, for example, may increase 2-phenylethyl alcohol synthesis, which is associated with pleasant fragrances at moderate concentrations (Clemente-Jimenez et al. 2004; Andorra et al. 2010),

despite *H. uvarum* is widely reported to create fruity aromas (Matraxia et al 2021).

Several strains of non-*Saccharomyces* species, primarily *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *M. pulcherrima*, and *Pichia kluyveri*, are now available as dry or frozen active yeast. These species are used in winemaking with objectives such as: i) increasing the varietal aroma fraction of wines (Ruiz et al. 2018); ii) controlling wine acidity properties (Gobbi et al. 2018); iii) improving colour extraction and mouthfeel properties (Belda et al 2016); iv) reducing ethanol content (Binati et al. 2020); v) improving foaming properties of sparkling wines (Medina-Trujillo et al. 2017). *T. delbrueckii* is one of the most studied non-*Saccharomyces* species in wine: its intense ß-glucosidase activity produces a variety of aromatic chemical compounds, including norisoprenoids, terpenols, lactones, and higher alcohols. *M. pulcherrima* produces extracellular enzymes such as ß-glucosidases, which hydrolyze the glycosylated aroma precursors present in grape juice, and it also produces more 3-sulfanilesan-1-olo than *S. cerevisiae* (Chasseriaud et al. 2018). Non-*Saccharomyces* have poor fermentative abilities and are unable to dominate numerically throughout the fermentation because to their low ethanol tolerance. Considering the metabolic influence of these yeasts during the early stages of fermentation is sufficient to cause considerable changes in the volatile profile of the wine, they are appropriate for inoculation as a co-starter with *S. cerevisiae* (Binati et al., 2020). The strains employed in mixed culture and the inoculation procedures will determine the wine quality. For mixed fermentation, two approaches have been investigated. Co-inoculation involves adding *Saccharomyces* and non-*Saccharomyces* strains to wine at the same time, but concentrations may differ. Sequential inoculation involves adding non-*Saccharomyces* species first, followed by *S. cerevisiae*, so the first strain can ferment on their own for a set amount of time before *S. cerevisiae* takes over the fermentation (Pandilla et al. 2016; Binati et al 2020).

Both procedures aim to replicate the natural process of spontaneous fermentation, increasing the complexity of the wine (Whitener et al. 2016).

1.4 Novel inoculum strategy in wine production

The notion of controlled fermentations for alcoholic beverages, in particular in wine, was established in the early 1980s, with a selected yeast inoculation in the must to be fermented. For many decades, *S. cerevisiae* was the only yeast species recommended for inoculation of wine fermentations. However, beginning in the 1990s, there was an increase in interest in non-*Saccharomyces* yeasts, and within three decades, non-*Saccharomyces* went from being an unwanted yeast associated with damaged wines to microorganisms that may enhance their aromatic profile (Jolly et al., 2014; González et al., 2006).

Non-*Saccharomyces* yeasts decrease ethanol content, increase glycerol production, decrease acidity,

and stabilise colour through enzymatic reactions (Benito et al., 2019; Ciani et al., 2016; Comitini et al., 2011; Gamero et al., 2016; Morata et al., 2019; Varela, 2016). There are numerous schemes for the application of these yeasts in wine fermentation, in which co-cultures of the non-*Saccharomyces* yeast of interest with *Saccharomyces* yeasts have been used to provide complete fermentation.

Several unconventional yeast strains have been explored in laboratory co-fermentations for wine production. This process could be carried out using various strategies, including: (a) growing each yeast strain separately and combining the different cultures with *S. cerevisiae* at a certain point to end the fermentation; (b) inoculating the non-conventional yeast first, and then adding *S. cerevisiae* later to finish the fermentation; or (c) inoculating strains with equal amounts or in different ratios, with cell numbers varying by several orders of magnitude (Morales et al., 2019)

1.5 Yeast nutrition and yeast derivates

1.5.1. Yeast nutrition

Yeasts can use a wide variety of nitrogen-containing substances in their natural environments. Based on the nature of the nitrogen compound and the species that uses it, assimilation of these compounds can occur in a variety of ways and degrees, generally optimising their development and metabolic activity (Barbosa et al., 2012). The assimilable nitrogen in grape juice is constituted by ammonium and amino acids in similar proportions (Henschke & Jiranek, 1993). The effect of carbon and nitrogen delivery on yeast fermentative fragrance is well recognised, since various research, particularly in wine production, have concentrated on this issue. The principal fruity or floral aroma-active chemicals in wine are due to yeast activity during AF, and their synthesis can be greatly influenced by winemaking practises. Many production aid tools, such as enzymes, clay minerals, organic acids, antioxidants, and yeast nutrients, are available on the oenological market and can promote the easy process of alcoholic fermentation and, in general, the quality of the final wine (Claus et al., 2018; Kemp et al., 2015). The yeast will encounter diverse working conditions depending on the content of the matrix to be fermented, with the fermentation environment varying in terms of pH, acidity, accessible sugars, assimilable nitrogen, vitamins, mineral salts, and the presence of inhibitory substances.

1.5.2. Yeast derivates

Yeast derivative products (YDPs) have been widely used in the winemaking process for fermentation management and wine stabilisation for numerous years. These products were used to provide assimilable nitrogen, stimulate yeast and lactic bacteria growth, and prevent stuck fermentations, but

also to increase wine colloidal stability (Angeles Pozo-Bayón et al., 2009, Comuzzo et al., 2011, Morata et al., 2018). YDPs have recently been widely utilised to either improve the technological process or safeguard the chromatic and sensory qualities of wines (Andjar-Ortiz et al., 2014, Charpentier and Feuillat, 1992, Feuillat and Charpentier, 1982, Lubbers et al., 1994, Pozo-Bayón et al., 2009).

Inactivated yeast is defined as "killed yeast that has lost its fermenting capacity and has not been extracted or added" (JOCE, n°C5, January 8, 1975). In the oenological context, the general technique for creating inactivated yeasts consists in inactivating a *Saccharomyces* yeast cream by heat and/or pH change; yeast cells may have undergone natural autolysis under the action of endogenous enzymes (Resolution OIV-OENO 459-2013). Although cell integrity is not preserved because cell wall membranes have been destroyed, inactivated yeast still has yeast cell content. If autolysis has occurred, the cellular contents are more soluble and have a lower molecular weight. Yeasts that have been treated in this way are often spray-dried. If autolysis occurs, it must be modest in order to meet OIV standards, which require an insoluble fraction larger than or equal to 60% w/w of dry matter (Resolution OIV-OENO 459-2013).

Yeast autolysate is a concentrated hydrolysate produced by autolysis of yeast biomass, which may be supplemented with heat treatments and/or pH changes. The self-digestion of proteins and other cellular elements by enzymes found in yeast cells is referred to as autolysis (Resolution OIV-OENO 496-2013). The autolysate has not been extracted and comprises both soluble and insoluble cellular components. Before autolysis, yeast is diluted with water to a specific solids content (Alexandre, 2011). Salt can be added to the resultant slurry to aid in cell membrane rupture (plasmolysis). During the autolysis process, yeast components (proteins, nucleic acids, lipids, cell wall polysaccharides) are solubilized and hydrolyzed. Cells are subsequently heated (30 to 60 °C) to promote further cell disintegration (Alexandre and Guilloux-Benatier, 2006; Alexandre, 2011). Although yeast autolysate is highly soluble in water, the soluble portion of dry matter present in the autolysate, whether dry or liquid, must be less than 80% (Resolution OIV-OENO 496-2013). Yeast autolysates are employed as nutrients during alcoholic fermentation as well as for rehydrating active dry yeasts.

Recently, among yeast-derived products OIV (resolution OIV-OENO 533-2017) approved wine treatment employing inactivated yeasts with guaranteed glutathione levels to minimise the oxidation of specific varietal aromatic components disclosed by yeast metabolism, particularly thiols. Angeles Pozo-Bayón et al. (2009) evaluated the oenological applications of these yeast derived compounds.

The phenolic compounds most susceptible to chemical oxidation in wine include caffeic acid and its esters, catechin, epicatechin, and gallic acid (Li et al., 2008). It has been demonstrated that enough

glutathione could inhibit oxidative coloration by delaying the formation of carboxymethine-bridged (+)-catechin dimers formed in the model wine system (Sonni et al., 2011a). Sonni et al. (2011b) showed that this delay was due to the ability of glutathione to form addition products with carbonyl compounds, such as glyoxylic acid. However, our understanding of these agents' mechanisms of action on wine organoleptic features is typically empirical, and these mechanisms are not well known.

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CHAPTER 2

Study of the oenological aptitudes of yeasts isolated from high sugar matrix (manna and honey by-products) as starters and co-starters for winemaking process.

ABSTRACT

The group of non-*Saccharomyces* yeasts has recently become of great interest in oenology. This is due to their metabolic and enzymatic characteristics. In fact, their use in co-inoculation with *S. cerevisiae* can determine a qualitative improvement of the wines from a sensory point of view. It can also give a greater complexity to the final product.

Several studies have shown that high sugar matrices, such as manna and honey, are very rich in microbial biodiversity. These ecological niches represent extremely selective conditions in terms of osmotic pressure and can be a source of yeasts with useful properties for use in oenology.

In the present study, a group of yeasts isolated from manna and honey by-products in previous research work were subjected to a genotypic identification at the species level by sequencing the D1/D2 variable domains of the 26-sRNA gene and the ITS region of the 5.8S gene. A differentiation of strains was carried out by RAPD-PCR. All the strains were subjected to *in vitro* screening (H_2S production, β-glucosidase activity, osmotic pressure, and resistance to different concentrations of ethanol, copper and potassium metabisulphite). The technological definition of each strain was then carried out. A micro-vinification experiment was then carried out using the strains with the best technological characteristics (*L. thermotolerans*, *Starmerella lactis-condensi*, *C. oleophila*). These strains were sequentially inoculated with a control strain of *S. cerevisiae*. There were also two fermentation trials with a single culture of *S. cerevisiae* isolated from manna and a control fermentation using *S. cerevisiae* control strain. Alcoholic fermentation was carried out at 20 °C. Microbiological and physico-chemical parameters were measured during alcoholic fermentation.

L. thermotolerans showed high tolerance to ethanol and increased glycerol production, whereas *S. lactis-condensi* reduced volatile acidity. As far as the species are concerned, *C. oleophila* showed a low metabolic activity with a low effect on the chemical parameters. A strain of *S. cerevisiae* from manna showed similar behaviour to the control strain. This strain is a promising starter for winemaking. The results obtained can be evaluated for the selection of new starter and co-starters for using in the vinification of regional wines.

2.1 INTRODUCTION

Conventional wine fermentation practice includes the use of commercial strains of *S. cerevisiae* as starters to ensure reproducibility of the fermentation and contribute to the production of more balanced wines. This oenological practice has led to a certain reduction and flattening of the sensory characteristics of the final product, due to a decrease in the diversity of the microbial populations involved in the fermentation process. Inoculation with *S. cerevisiae* active dry yeast has been used since the 20th century to ensure fast and reliable fermentations and wines of consistent and predictable quality. However, there are many other species and genera of yeast that can persist during the fermentation process. These, generally called "non-*Saccharomyces* yeasts", are a group of species of secondary importance in must fermentation, even considered spoilage organisms (Binati et al., 2020) due to their low fermentative capacity and overproduction of off flavours such as acetaldehyde, acetic acid, ethyl acetate acetoin (Esteves et al., 2019) or unwanted volatile phenols like *Brettanomyces* spp. (Ruiz et al., 2019). Many studies showed that these negative traits are strain dependent. Since the 1980s, the positive effect of certain non-*Saccharomyces* has been demonstrated. This is mainly due to the improvement of wine complexity, texture, and flavour integration in spontaneous fermentations (Gschaedler, 2017).

The positive effects and characteristics imparted to wines by non-*Saccharomyces* yeasts are in line with consumer demand for new wine styles (Rollero et al., 2018); in fact, they can impart unique aromatic complexity or mouthfeel, while expressing characteristics associated with terroir (Binati et al., 2019). In general, non-*Saccharomyces* yeasts (mainly belonging to the genera *Hanseniaspora*, *Candida*, *Meyerozyma*, *Zygosaccharomyces*, *Schizosaccharomyces*, *Torulaspora*, *Kluyveromyces* and *Metschnikowia*) are dominant in the early stages of alcoholic fermentation and are then replaced by *S. cerevisiae*, which finishes the fermentation process (Jolly et al., 2014; Gschaedler, 2017). Typically, most of these strains originate directly from grape berry surfaces, cellar equipment surfaces or the cellar environment and die shortly after the onset of fermentation. It was initially thought that the death of these strains was due to the increasing concentration of ethanol and the addition of SO_2. More recent research has shown that the reason is more complex and is certainly strain dependent. In fact, even in the late stages of fermentation, many non-*Saccharomyces* species can persist and survive at significant levels. (Zott et al., 2008; Andorra et al., 2011; David et al., 2014; Wang et al., 2014; Albertin et al., 2017; Gschaedler, 2017).

Three main genera (*Hanseniaspora* spp., *Candida* spp. and *Metschnikowia* spp.) dominate during the pre-fermentative phase: *H. uvarum* was described as one of the main non-*Saccharomyces* yeasts during the initial phase of the fermentation process, *Starmerella bacillaris* was isolated from grape must irrespective of the wine production region or grape variety under consideration, and

Metschnikowia spp. was reported as a high population in grape must. (Albertin et al., 2017). They could also have an impact on wine fermentation, either directly through flavour production or indirectly through modulation of *S. cerevisiae* growth and metabolism. Indeed, *Metschnikowia pulcherrima* and *Starmerella bacillaris* can increase 2-phenylethyl alcohol production, associated with pleasant flavours at moderate concentrations (Clemente-Jimenez et al., 2004; Andorra et al., 2010), while *H. uvarum* often produces acetate and fruity esters (Viana et al., 2008; Matraxia et al., 2021). Several strains of non-*Saccharomyces* species, mainly *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima* and *Pichia kluyveri*, are now available as dry or frozen active yeasts. These species will be used in the production of wine with specific objectives such as (i) increasing the varietal aromatic content of wines (Ruiz et al., 2018); (ii) control of wine acidity characteristics (Gobbi et al., 2013); (iii) improvement of colour extraction and mouthfeel characteristics (Belda et al., 2016); (iv) reduction of ethanol content (Binati et al., 2020); (v) improvement of sparkling wine effervescence characteristics (Medina-Trujillo et al., 2017). Non-*Saccharomyces* generally have a low fermentation performance and are unable to dominate the total fermentation numerically due to their low tolerance to ethanol.

The metabolic impact of these yeasts during the early stages of fermentation is sufficient to induce significant changes in the volatile profile of the wine, making them suitable for inoculation as co-starters with *S. cerevisiae* (Pandilla et al., 2016; Binati et al., 2020).

Several studies suggest that matrices with high sugar content are rich in *Saccharomyces* and non-*Saccharomyces* yeasts, which are potentially applicable in the field of oenology and fermented beverages. Matraxia et al. (2021) used *H. uvarum*, isolated from honey by-products in beer fermentation, in co-inoculation with *S. cerevisiae*. Alfonzo et al. (2021) successfully applied *S. cerevisiae* strains isolated from honey in winemaking and found great differences compared to *S. cerevisiae* isolated from grapes.

The microbial communities that characterise a specific food matrix are generally a major contributor to its composition and properties for food applications. In a study by Guarcello et al. (2019), the culturable microbial ecology of Sicilian manna ash was analysed to obtain new information on the hygienic quality, shelf-life and potential applications of this traditional food, with the aim of characterising the culturable microorganisms associated with the different products obtained during manna processing.

Gaglio et al. (2017) also investigated the microbial biodiversity of honey by-products used to produce "*Spiritu re fascitrari*", finding a niche rich in *Saccharomyces* and non-*Saccharomyces* yeasts. For this reason, the aim of the present study was: i) the identification of a group of yeasts isolated from manna and honey; ii) verify their potential use in oenology, through specific tests of resistance,

osmotolerance and enzymatic activity; iii) employ same strain, with specific activities, as starters and co-starters *in vitro* fermentation.

2.2 MATERIALS AND METHODS

2.2.1 Isolates origins, DNA extraction and species identification

The yeasts employed in this research work belongs to the collection of the Department of Agricultural, Food and Forest Sciences (SAAF; University of Palermo, Italy), they were isolated from manna and honey by-products.

Yeast isolates were identified by molecular techniques. DNA was extracted using the Quick-DNA Microprep Kit (Zymo research) according to the instructions of the manufacturer. To make an initial discrimination of yeast, all isolates were analysed by restriction fragment length polymorphism (RFLP) of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8 S rRNA gene. DNA amplification was performed with the ITS1/ITS4 primer pair in accordance with Esteve-Zarzoso et al. (1999). The resulting amplicons were then digested with CfoI, HaeIII and HinfI (MBI Fermentas, St. Leon-Rot, Germany) at 37 °C for 8 h. ITS amplicons and the corresponding restriction fragments were analysed on an agarose gel using 1.5% and 3% (w/v) agarose in 1 × TBE (89 mM Tris-borate, 2 mM EDTA pH 8) buffer, stained with SYBR safe DNA gel stain (Invitrogen, Milan, Italy), visualised by UV transillumination and captured on Gel Doc 1000 video gel documentation system (Bio-Rad, Richmond, CA). The standard DNA ladders used were 1 kb Plus and 50 pb (Invitrogen). At least one isolate per group was further processed by 26S rRNA gene D1/D2 region sequencing (Gaglio et al., 2017). The data were compared with the sequence published in the GenBank database by means of the BLAST alignment tool (http://blast.ncbi.nlm.nih.gov/).

2.2.2 Strain typing

The intraspecific characterisation of the isolates belonging to the *S. cerevisiae* strains was carried out by Interdelta analysis with primers delta 12 and delta 21 (Legras and Karst, 2003). The intraspecific characterisation of the isolates belonging to the non-*Saccharomyces* strains was carried out by different RAPD-PCR assays with primers M13 (Francesca et al., 2014) and XD5 (Di Maro et al., 2007). PCR products were analysed and visualised as described by Settanni et al. (2012).

2.2.3 Technological screening

All strains were tested for technological characteristics, H_2S production, β-glucosidase activity, osmotolerance and resistance to ethanol, potassium metabisulphite and copper. In addition, growth tests on lysine were conducted. The ability to produce H_2S was tested using a qualitative method performed on Bismuth Sulfite Glucose Glycerin Yeast extract (BiGGY) agar (Oxoid, Milan, Italy) (Jiranek et al.1995). H_2S was estimated by colony blackening after 3 days of incubation at 28 °C. A

four-level scale was used: -, no growth; +, growth and low H_2S production; P, growth and medium H_2S production; PP, growth and high H_2S production. Only strains with low H_2S production were subjected to additional tests. The β-glucosidase activity was evaluated as described by Rosi et al. (1994) on agar plates containing arbutin as substrate. Strains with this activity hydrolyse the substrate, and dark brown colour develops in the agar.

The resistance tests were performed in modified YPD medium containing different doses of each stress agent and according to the selection criteria for non-*Saccharomyces* yeasts described by Mestre Furlani et al. (2017). Accordingly, the following concentrations were used 4, 8 or 12% (v/v) of ethanol; 220, 270 and 320 g/L of glucose to test osmotolerance; 150 and 200 mg/L of sulphur dioxide (SO_2) by addition of potassium metabisulphite ($K_2S_2O_5$); 2.5, 5 and 10 mM of copper, supplied as copper sulphate.

2.2.4 Growth kinetics on single source of sugar

The strains were also evaluated for their ability to grow in the presence of single sugars matrix using the procedure described by Kurtzman et al. (2011) with the following modifications: the tests were performed in rimless tubes (16 x 180 mm), each containing 10 mL (yeast extract, 3 g/L, triptone, 5 g/L; glucose or fructose, 200 g/L) and inoculated with the pure strain cultures as reported by Hall et al. (2014).

Growth of pure strain cultures in synthetic media was assessed by measuring optical density (OD) at 600 nm in a 96-well microtitre plate. Measurement was performed every 24 h for 4 days using ScanReady microplate photometer P-800 (Life Real Biotechnology Co., Ltd, Hangzhou, China). The temperature of the incubation was set at 25 °C. A blank measurement was subtracted from each OD reading. All analyses were performed in triplicate. Total growth of the strains was calculated as the integrated area underlying the curve up to 4 days ad described by Hall et al., (2014).

2.2.5 Fermentation of grape must

The strains with low H_2S production, high resistance to ethanol and potassium metabisulphite and the ability to grow rapidly on glucose and fructose substrates were evaluated for their ability to ferment a grape must.

The grapes cv. Traminer were harvested during the 2021 vintage. All the microvinifications were carried out in the Department of Agricultural, Food and Forestry Sciences (SAAF; University of Palermo, Italy). The grapes were harvested, destemmed and crushed by hand. The must obtained was divided into twenty-one batches (1 litre each) and pasteurised at 72°C for 15 seconds. The yeasts were inoculated in liquid concentrated form [approximately 6.0 log (CFU)/g], from TR1 to TR4 trials were inoculated with different strains of non-*Saccharomyces*, each belonging to the species: *L.*

thermotolerans (two strains), *S. lactis-condensi*, *C. oleophila*. While experiments TR5 and TR6 were inoculated with two different strains of *S. cerevisiae* from manna, TRC was inoculated with a commercial strain of *S. cerevisiae* (EC1118). The experimental design is shown in Figure 3. After 4 days, each experiment from TR1 to TR4 was inoculated with the commercial strain of *S. cerevisiae*. The alcoholic fermentation of all experiments was carried out at 20°C for 30 days.

At the end of the alcoholic fermentation, potassium metabisulphite (8 g/hL) was added to all experiments. Samples were taken at different stages of vinification: at the inoculum of non-*Saccharomyces* strains, after 3 days of AF, after the *S. cerevisiae* inoculum, after 8 days of AF and at the end of AF. All samples were analysed within 24 h of collection. All analyses were performed in triplicate. To allow the removal of CO_2, the flasks were sealed with a Müller valve (Ciani & Rosini 1987) and the weight loss was monitored until it fell below 0.01 g per day (end of fermentation).

2.2.6 Microbiological and oenological parameters

All samples collected during AF were analysed for yeast populations. Musts samples were diluted in Ringer's solution (Sigma-Aldrich, Milan, Italy) and analysed in triplicate for presumptive *Saccharomyces* spp. yeasts on Wallerstein Laboratory (WL) nutrient agar (Pallmann et al., 2001), non-*Saccharomyces* were counted on lysine agar (Martin et al., 2018). All media and supplements were purchased from Oxoid (Thermofisher, Milan, Italy).

The wines obtained were analysed by means of WineScan (FOSS, Hillerød, Denmark) to determine volatile acidity (VA), reducing sugars, ethanol, glycerol, malic acid and lactic acid. The instrument was calibrated according to the EEC 2676 standard procedure (European Commission, 1990; Sannino et al., 2013). pH was determined according to the OIV-MA-AS313-15 method (OIV, 2020a) and total acidity (TA) according to the method described in OIV-MA-AS313-01 (OIV, 2020b). All chemical analyses were performed in triplicate.

2.2.7 Statistical analysis

The ANOVA test was used to identify significant differences between the chemical parameters determined during the winemaking process (residual sugar, ethanol, glycerol, malic acid, lactic acid and volatile acidity, total acidity and microbial load of presumptive *Saccharomyces* and non-*Saccharomyces*). The post-hoc Tukey's method was used for pairwise comparison of all data. Statistical significance was set at $P < 0.05$ (Mazzei et al., 2010).

An exploratory multivariate approach using Principal Component Analysis (PCA) was used to investigate the relationships between the data obtained at the end of the AF (ethanol, residual sugar, glycerol, malic acid, lactic acid, pH, total acidity and volatile acidity) from the different treatments (Alfonzo et al., 2021).

2.3 RESULTS AND DISCUSSION

2.3.1 Isolation, identification and strain typing of yeasts.

A total of 80 isolated yeast from manna and honey by-product, stored in the strain collection of the SAAF department, were subjected to genotypic characterization. The restriction analysis of ITS1-5.8S-ITS2 separated the isolates into eight groups (Table 1).

Table 1. Molecular identification of yeast species isolated from manna and honey samples.

Species	Restriction profile	5.8S-ITS PCR (bp)	Size of restriction fragment [a]			Number of isolates
			*Cfo*I	*Hae*III	*Hinf*I	
Candida aaseri	I	680	n.c.	550	285	1
Debaryomyces hansenii	II	660	295 + 280	400 + 120 + 75	315 + 315	1
Starmerella lactis-condensi	III	500	175 + 100	300	180 + 170	2
Citeromyces matritensis	IV	700	330 + 210	450 + 200 + 80	390 + 320	4
Lachancea thermotolerans	V	680	320 + 275	300 + 210 +80	345	57
Meyerozyma guillermondii	VI	600	310 + 260	400 + 125 + 80	320	5
Starmerella magnoliae	VII	400	180 + 175	280 + 190	220	1
Candida oleophila	VIII	620	300 + 280	430 + 140 + 80	315	1
Zygosaccharomyces bisporus	IX	800	300 + 270	700 + 100	390 + 230 +150	2
Zygosaccharomyces bailii	X	790	320 + 270 + 95 + 95	690 + 90	340 + 225 + 160 + 55	2
Saccharomyces cerevisiae	XI	850	370 + 330	310 + 240 + 175 + 130	370 + 360 + 120	4

[a] Values refer to the number of base pairs per fragment.

Nine groups were preliminary identified at species level by comparison of the restriction profiles with those reported in literature (Esteve-Zarzoso et al., 1999; Francesca et al., 2014; Sannino et al., 2013). Specifically, the isolates were identified as *Candida aaseri* (group I), *Debaryomyces hansenii* (group II), *S. lactis-condensi* (group III), *Citeromyces matritensis* (group IV), *L. thermotolerans*, (group V), *Meyerozyma guillermondii* (group VI), *Starmerella magnoliae* (group VII), *Candida oleophila* (VIII), *Zygosaccharomyces bisporus* (group IX), *Zygosaccharomyces bailii* (group X) and *Saccharomyces cerevisiae* (group XI). The genotypic identification of yeasts was completed by pairwise alignment of D1/D2 sequence that successful confirmed the species identification (Table 1). With regards to genera/species distribution among samples, most isolates belonged to the *L. thermotolerans* group (57 isolates). The species *S. cerevisiae*, *M. guillermondii* and *C. matritensis* were also isolated from several samples. The following isolated were further investigated at strain level by RAPD-PCR. Molecular analysis revealed 34 different strains (data not shown).

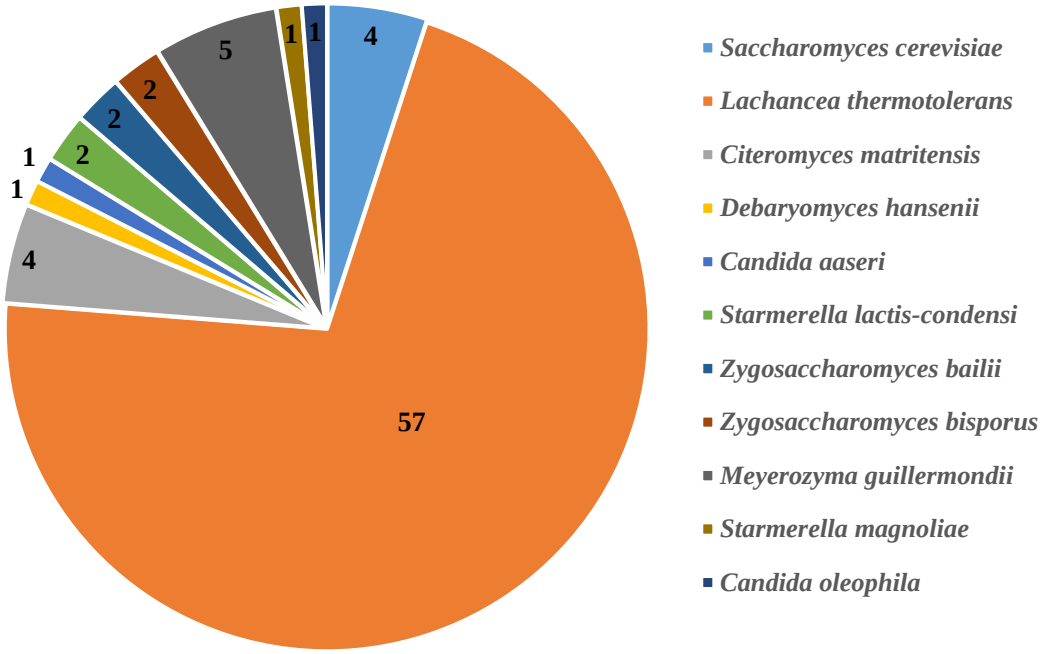

Figure 1. Number of isolates among yeast species

2.3.2 Technological characteristics of yeast strains

Result of technological screening are reported in Table 2. Several inter-specific differences were found. All strains were examined for their ability to produce H_2S, with the exception of those from the genus *Zygosaccharomyces* spp., which are typical wine-altering microorganisms (Alonso et al., 2015), were tested for their production of H_2S. However, only the strains belonging to the species *C. matritensis*, *C. aaseri* and *M. guillermondii* showed high H_2S production and were therefore excluded from the subsequent resistance tests. In ethanol resistance tests, strains belonging to the species *L. thermotolerans* and *S. lactis-condensi* were resistant to 8% (v/v) ethanol, while strains belonging to the species *C. aaseri*, *S. magnoliae* and *C. oleophila* were resistant to 4% (v/v) ethanol, whereas *S. cerevisiae* was resistant to 12% (v/v) ethanol. Moreover, all strains showed growth in presence of 200 mg/L of potassium metabisulphite. With regard to copper resistance, high variability was found among strains of the species *L. thermotolerans*, while only strains of the species *D. hansenii*, *S. magnoliae* and *C. oleophila* resisted the highest copper concentrations (10 mM).

Regarding β-glucosidase activity, only strains YS209 (*C. oleophila*) and MN114 (*D. hansenii*) showed positive result.

Table 2. Technological screening of yeast strains.

Strain (Species)	H_2S [a]	Ethanol resistance [b]			MBSK resistance [c]		Osmotic resistance [d]			Copper Resistance [e]			β-glucosidase activity
		4%	8%	12%	150 mg/L	200 mg/L	220 g/L	270 g/L	320 g/L	2,5 mM	5 mM	10 mM	
MN113 (*S. cerevisiae*)	+	+	+	+	+	+	+	+	+	+	+	+/-	-
YS210 (*S. cerevisiae*)	+	+	+	+/-	+	+	+	+	+	+	+	+/-	-
MN28 (*L. thermotolerans*)	-	+	+	-	+	+	+	+	+	+	+	+/-	-
MN136 (*L. thermotolerans*)	-	+	+	-	+	+	+	+	+	+	+	+/-	-
MN93 (*L. thermotolerans*)	-	+	+	-	+	+	+	+	+	+	+/-	-	-
MN400 (*L. thermotolerans*)	-	+	+	-	+	+	+	+	+	+	+	+/-	-
MNF104 (*L. thermotolerans*)	-	+	+	-	+	+	+	+	+	+	+	+/-	-
MNF105 (*L. thermotolerans*)	-	+	+	-	+	+	+	+	+	+	+	+/-	-
YS186 (*L. thermotolerans*)	-	+	+	-	+	+	+	+	+	+	+	+/-	-
YS1 (*L. thermotolerans*)	-	+	+	-	+	+	+	+	+	+	+	+/-	-
YS42 (*L. thermotolerans*)	-	+	+	-	+	+	+	+	+	+	+	+/-	-
YS45 (*L. thermotolerans*)	-	+	+	-	+	+	+	+	+	+	+	+/-	-
YS55 (*L. thermotolerans*)	-	+	+	-	+	+	+	+	+	+	+	-	-
XV11 (*L. thermotolerans*)	-	+	+	-	+	+	+	+	+	+	+	-	-
XV22 (*L. thermotolerans*)	-	+	+	-	+	+	+	+	+	+	+/-	-	-
XV34 (*L. thermotolerans*)	-	+	+	-	+	+	+	+	+	+	+/-	-	-
XV47 (*L. thermotolerans*)	-	+	+	-	+	+	+	+	+	+	+	-	-
MN114 (*D. hansenii*)	-	+	-	-	+	+	+/-	+/-	+/-	+	+	+	+
MN412 (*S. lactis-condensi*)	-	+	+	-	+	+	+	+	+	+	+/-	-	-
MN417 (*S. lactis-condensi*)	-	+	+	-	+	+	+	+	+	+	+/-	+/-	-
MN117 (*C. aaseri*)	P	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MNF138 (*C. matritensis*)	PP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MNF308 (*C. matritensis*)	PP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MNF289 (*C. matritensis*)	PP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
YS82 (*C. matritensis*)	PP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
YS292 (*S. magnoliae*)	-	+	-	-	+	+	+/-	+/-	+/-	+	+	+	-
YS209 (*C. oleophila*)	-	+	-	-	+	+	+/-	+/-	+/-	+	+	+	+
YS246 (*M. guillermondii*)	PP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
YS300 (*M. guillermondii*)	P	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
YS247 (*M. guillermondii*)	P	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
YS271 (*M. guillermondii*)	P	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a] H_2S Production: -, no growth; +, growth and low H_2S production; P, growth and medium H_2S production; PP, growth and high H_2S production.
[b] growth on YPD supplied with different ethanol percentages [4, 8 and 12 % (v/v)].
[c] growth on YPD supplied with different concentrations of potassium metabisulphite (150 and 200 mg/L).
[d] growth on YPD supplied with different glucose concentrations (220, 270 and 320 g/L).
[d] growth on YPD supplied with different copper concentrations (2.5, 5 and 10 mM).
[e] β-glucosidase activity: +, growth; -, no growth; on medium containing arbutin.

From the previous technological tests, all 22 strains were selected. Their growth kinetics on fructose and glucose media were further investigated (Fig. 2).

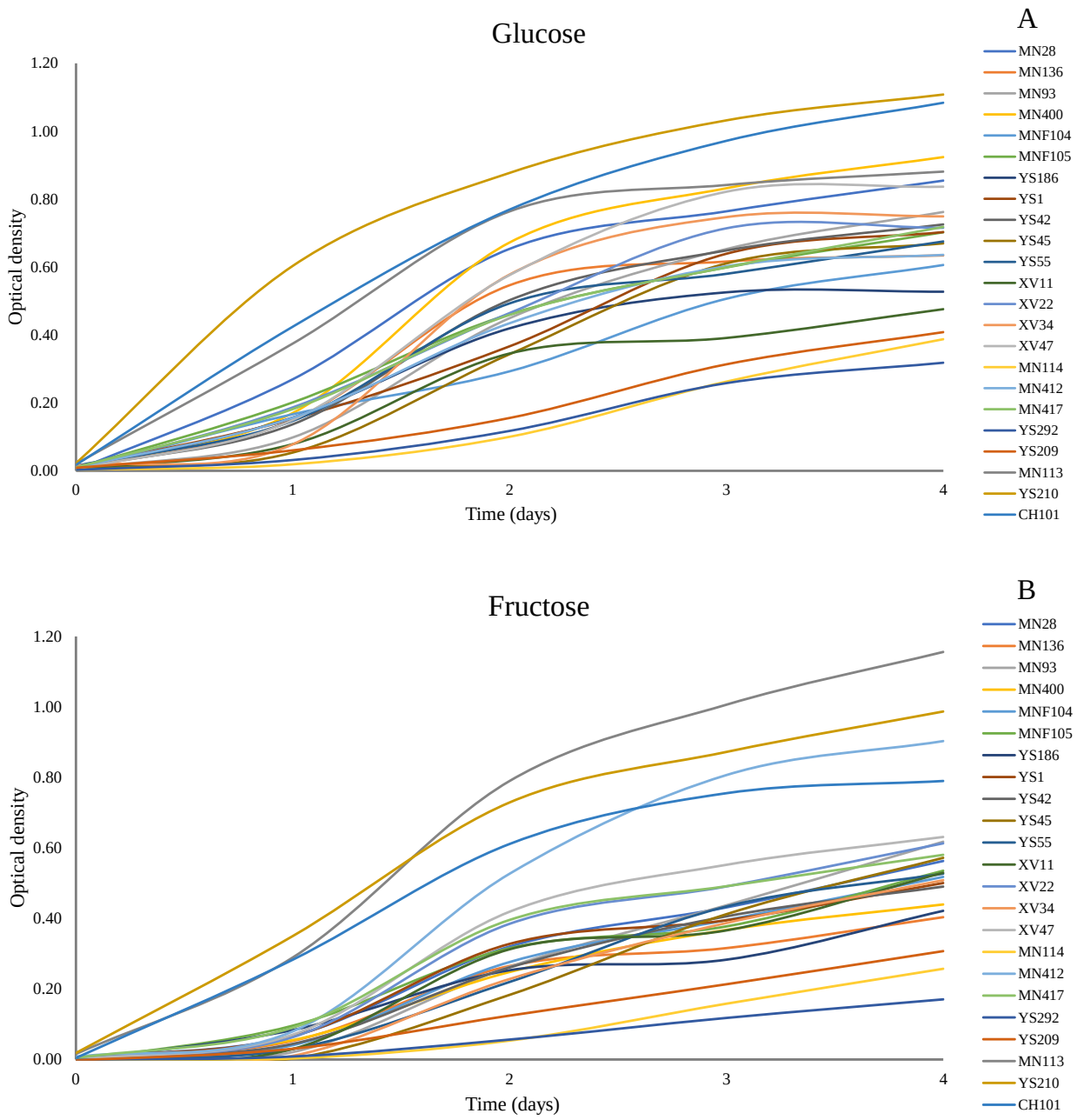

Figure 2. Growth of different strains in single sugar matrix of glucose (A) and fructose (B). The growth was measured by OD values at 600 nm in triplicate.
Values of standard deviations ranged between 0 and 0.16 and are not showed for a better graphical visualization of figures.

After 24 h of incubation, the highest level of growth on glucose was found for *S. cerevisiae* YS210 strain, which showed the highest OD until day 4 (1.10). On the other hand, the highest OD value at the 4th day of fructose fermentation (1.15) was observed for *S. cerevisiae* MN113. Among non-*Saccharomyces*, the best growth values were recorded for strain MN400 *L. thermotolerans* with 0.92 after 4 days of glucose fermentation. Regarding fructose fermentation, strain MN412 *S. lactis-condensi* showed OD values (0.90) higher than control strain CH101 *S. cerevisiae* (0.79) after 4 days incubation. This character could be related to a fructophilic activity of the strain MN412. In both

glucose and fructose media, the worst growth values were recorded for strain MN117 *C. aaseri*. Total growth values are shown in the table. Regarding the total growth of the strains on glucose, among the non-*Saccharomyces* strains, MN400 (*L. thermotolerans*) showed the highest value (2.14). On the other hand, the non-*Saccharomyces* strain with the highest growth on fructose (1.87) was MN412 (*S. lactis-condensi*). Also, on fructose, *S. cerevisiae* strains isolated from manna showed higher growth than CH101 strain, which was used as a control.

Table 3. Total growth on synthetic medium containing a single source of sugars (glucose and fructose).

Area under the growth curve		
Strain	**Glucose**	**Fructose**
MN28	2.11	1.10
MN136	1.63	0.84
MN93	1.58	1.03
MN400	2.14	0.89
MNF104	1.27	0.97
MNF105	1.61	1.06
YS186	1.37	0.83
YS1	1.51	1.04
YS42	1.65	0.96
YS45	1.34	0.88
YS55	1.56	0.95
XV11	1.05	0.97
XV22	1.72	1.25
XV34	1.78	0.88
XV47	1.97	1.36
MN114	0.58	0.34
MN412	1.51	1.87
MN417	1.60	1.27
YS292	0.57	0.27
YS209	0.73	0.52
MN113	2.42	2.66
YS210	3.07	2.44
CH101 (Control)	2.71	2.04

Result indicates the total growth calculated as the integrated area underlying the curve up to four days.

2.3.3 Micro-fermentation

The two *L. thermotolerans* strains (MN400 and XV47) with high copper resistance and the best growth dynamics on glucose and fructose, respectively, were selected for the TR1 and TR2 trials. The strain MN412 (*S. lactis-condensi*) with high growth on fructose and YS209 (*C. oleophila*) with high β-glucosidase activity were used as co-starters for the TR3 and TR4 experiments. In two fermentation experiments (TR5 and TR6), the two *S. cerevisiae* strains MN113 and YS210 were used as starters.

In the microfermentations, the weight lost (CO_2 emitted) was monitored daily for 30 days (end of AF).

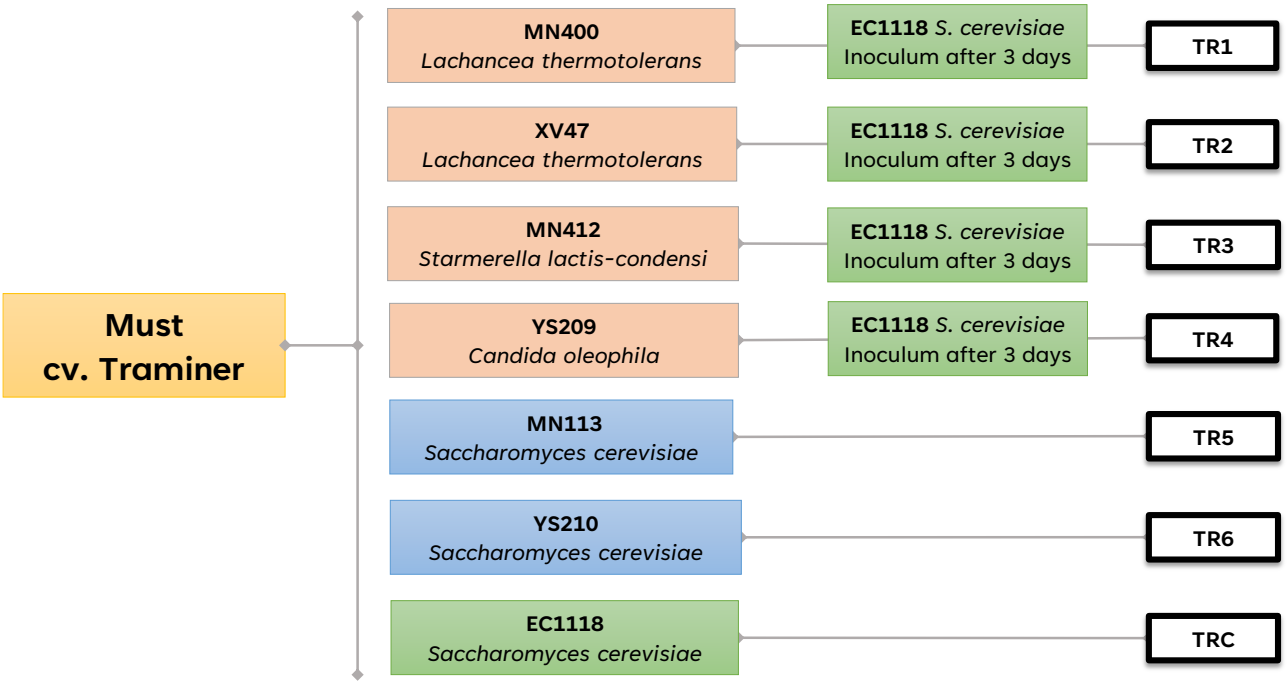

Figure 3. Experimental plan of micro-vinification. After 3 days of alcoholic fermentation the trials with non-*Saccharomyces* have been inoculated with control strain of *S. cerevisiae*.

The results of the fermentation kinetics (Fig. 4) showed that 3 days after inoculation, the non-*Saccharomyces* species with the greatest weight loss were *S. lactis-condensi* (TR3) and the two *L. thermotolerans* strains (TR1 and TR2). The other strains showed very low, almost negligible, fermentation activity. *S. cerevisiae* strain MN113 showed a similar trend to the control. Regarding the percentage of weight lost, the TR1 and TR5 trials lost more than the TRC control. At the end of the process, TR2 and TR6 showed the lowest weight loss, probably because fermentation stopped.

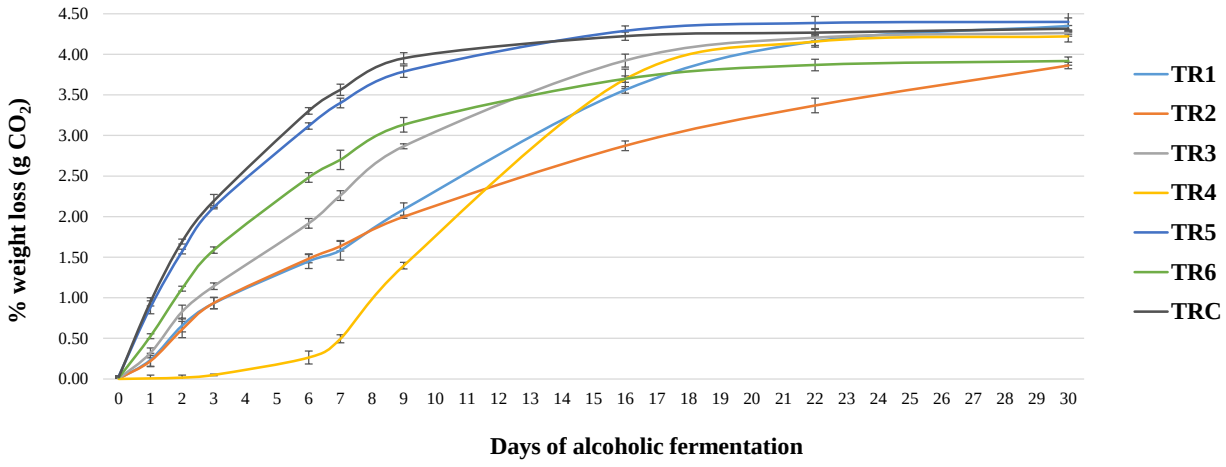

Figure 4. Weight loss during the microvinifications.

2.3.4 Microbiological counts

Microbial yeast counts during fermentation are shown in Table 4. Microbiological monitoring for presumptive counts of *Saccharomyces* and non-*Saccharomyces* was performed at the inoculation of the starter/co-starter (T0), at day 3 (inoculum of *S. cerevisiae* in non-*Saccharomyces* trials), at day 8 and at the end of the AF. The inoculation of the non-*Saccharomyces* was in the range of 5.9 to 6.2 log (CFU/mL), whereas the *Saccharomyces* (MN113, YS210 and control) were inoculated at a concentration of around 6.5-6.8 log (CFU/mL). After 3 days of alcoholic fermentation for all tests, yeast population growth was observed with values of 6.4 and 7.2 log (CFU/mL). On the 3th day, *Saccharomyces* was inoculated at a concentration of 6.5 log (CFU/mL) for each of the experiments.

Table 4. Monitoring of yeast populations during experimental micro-fermentation.

Samples	Microbial loads							
	TR1	**TR2**	**TR3**	**TR4**	**TR5**	**TR6**	**TRC**	**S.S.**
***Saccharomyces* spp.**								
T0	n.d.	n.d.	n.d.	n.d.	6.49±0.15^{a}	6.69±0.21^{a}	6.79±0.23^{a}	n.s.
T3 ^{a}	6.53±0.12^{b}	6.53±0.12^{b}	6.53±0.12^{b}	6.53±0.12^{b}	7.44±0.24^{a}	7.27±0.18^{a}	7.36±0.17^{a}	***
T8	7.18±0.20^{b}	7.54±0.32^{b}	7.18±0.21^{b}	7.26±0.37^{a}	7.35±0.22^{a}	7.24±0.23^{a}	7.26±0.24^{a}	***
End of AF	6.67±0.33^{a}	6.64±0.12^{a}	6.36±0.15^{a}	6.32±0.16^{a}	6.40±0.16^{a}	6.15±0.16^{a}	6.23±0.21^{a}	n.s.
Non-*Saccharomyces* spp.								
T0	4.13±0.27^{a}	4.49±0.30^{a}	4.78±0.11^{a}	4.54±0.10^{a}	n.d.	n.d.	n.d.	n.s.
T3	7.20±0.32^{ab}	6.41±0.37^{b}	7.23±0.10^{a}	7.11±0.13^{ab}	n.d.	n.d.	n.d.	*
T8	6.65±0.28^{a}	6.54±0.19^{a}	6.48±0.22^{a}	< 2.00	n.d.	n.d.	n.d.	n.s.
End of AF	4.25±0.20^{a}	4.20±0.10^{a}	4.30±0.14^{a}	< 2.00	n.d.	n.d.	n.d.	n.s.

^{a} inoculum of *S. cerevisiae* EC1118 in trials TR1, TR2, TR3 and TR4.

Results indicate average values ± standard deviation of three plate counts. Log CFU/mL for must and wine samples.

Abbreviations: T0, must after yeast inoculum; T3, 3 days of alcoholic fermentation; T8, 8 days of alcoholic fermentation; AF, alcoholic fermentation.

S.S., statistical significance; n.d., not determined. P value: *, P < 0.05; **, P < 0.01; ***, P < 0.001; n.s., not significant.

After 8 days of alcoholic fermentation, theses inoculated with *L. thermotolerans* and *S. lactis-condensi* had non-*Saccharomyces* counts one logarithmic cycle higher than presumptive *Saccharomyces*. In TR4 the non-*Saccharomyces* were below the detection limit after 8 days of AF due to their low resistance to ethanol. The same trend was found by Binati et al. (2020) fermenting three different non-*Saccharomyces* species combined with *S. cerevisiae*. At the end of the alcoholic fermentation, TR1, TR2 and TR3 showed non-*Saccharomyces* counts of 4.2-4.3 log (CFU/ml), while *Saccharomyces* counts were about 6.1-6.7 log (CFU/ml). Several authors agree that co-inoculating *S. cerevisiae* and non-*Saccharomyces* yeast species can lead to death or loss of variability of the non-*Saccharomyces* once *S. cerevisiae* dominates the fermentation and becomes stress resistant to the inhibiting ethanol.

In addition, secretion of inhibitory substances has been reported as a possible cause of inhibition in non-*Saccharomyces* yeasts (Englezos et al., 2019). Therefore, they suggest that sequential inoculation (non-*Saccharomyces* followed by *S. cerevisiae*) is a better technique than mixed culture, allowing a higher expression of the metabolism of non-*Saccharomyces* yeast (Loira et al., 2014). Trials inoculated with single culture MN113 *S. cerevisiae* (TR5) isolated from manna had similar trends to control trials (TRC) inoculated with grape yeast. Alfonzo et al. (2021) used *S. cerevisiae* isolated from honey by-products in wine production and found a comparable microbiological behaviour to that of *S. cerevisiae* isolated from grapes.

2.3.5 Physical-chemical analysis

The influence of manna yeasts (*Saccharomyces* and non-*Saccharomyces*) on the chemical composition of the wines was evaluated even by quantifying the main analytical components at the end of alcoholic fermentation. The results of the chemical analyses are summarised in Table 5. Regarding glycerol content, the highest values were found in the trials inoculated with *L. thermotolerans* (7.1-7.4 g/L) compared to the other batches inoculated with non-*Saccharomyces*, the same trend in terms of glycerol increase was found by Hranilovic et al. (2018). For the pure *S. cerevisiae* inoculated theses, the TR3 test showed the lowest level of volatile acidity (0.18 g/L) compared to the TRC control (0.38 g/L acetic acid). Except for TR3 and TR6, no significant differences were found between the theses in terms of VA, whose values were below 0.80 g/L, the threshold above which wine quality is compromised (Capozzi et al., 2015).

Table 5. Chemical parameters determined during the micro-vinification process.

Parameters	Musts	Micro-vinification							
		End of alcoholic fermentation							
		TR1	**TR2**	**TR3**	**TR4**	**TR5**	**TR6**	**TRC**	**S.S.**
Residual sugars $^{\beta}$	221.83±2.26	0.24±0.05^{c}	14.10±1.16^{b}	0.15±0.04^{c}	0.26±0.09^{c}	0.28±0.05^{c}	23.0±1.50^{a}	0.24±0.04^{c}	***
Ethanol $^{\gamma}$	n.d.	11.46±0.05^{a}	10.79±0.05^{b}	11.48±0.05^{a}	11.35±0.05^{a}	11.47±0.05^{a}	10.36±0.05^{c}	11.50±0.05^{a}	***
Malic acid $^{\beta}$	1.71±0.15	1.47±0.04^{a}	1.64±0.02^{a}	1.57±0.06^{a}	1.59±0.04^{a}	1.66±0.07^{a}	1.64±0.08^{a}	1.66±0.06^{a}	n.s.
Lactic acid $^{\beta}$	n.d.	0.69±0.04^{b}	1.63±0.05^{a}	0.02±0.04^{c}	0.03±0.05^{c}	0.06±0.02^{c}	0.02±0.02^{c}	0.03±0.01^{c}	***
Glycerol $^{\beta}$	n.d.	7.40±0.15^{a}	7.10±0.10^{a}	6.20±0.17^{b}	5.30±0.11^{c}	4.80±0.10^{c}	5.00±0.12^{c}	5.10±0.14^{c}	***
pH	3.63±0.01	3.46±0.02^{a}	3.52±0.03^{b}	3.42±0.02^{a}	3.49±0.02^{a}	3.45±0.02^{a}	3.53±0.02^{b}	3.45±0.01^{a}	**
VA $^{\beta}$	n.d.	0.36±0.02^{b}	0.57±0.03^{a}	0.18±0.02^{c}	0.28±0.03^{bc}	0.30±0.04^{bc}	0.66±0.05^{a}	0.38±0.02^{b}	***
TA $^{\beta}$	5.10±0.02	4.52±0.05^{a}	4.69±0.06^{a}	4.58±0.10^{a}	4.61±0.06^{a}	4.42±0.12^{a}	4.73±0.08^{a}	4.52±0.07^{a}	n.s.

Result indicates mean value ± standard deviation of three determinations from two replicates. Data within a line followed by the same letter are not significantly different according to Tukey's test.
$^{\beta}$, expressed in g/L; $^{\gamma}$, expressed in % (v/v).
Abbreviations: VA, volatile acidity (acetic acid g/L); TA total titratable acidity (tartaric acid g/L); n.d, not detected.
S.S., statistical significance; n.d., not determined. P value: *, P < 0.05; **, P < 0.01; ***, P < 0.001; n.s., not significant.

At the end of the alcoholic fermentation, many of the wines obtained had a low residual sugar content (< 0.5 g/L), which is a common characteristic of dry wines (Malfeito-Ferreira et al., 2019). This confirms the completion of fermentation by yeasts. On the other hand, TR2 and TR6 did not complete alcoholic fermentation, resulting in high residual sugar levels (>14 g/L). Except for TR2 and TR4 the other trials showed ethanol content values comparable to that of the TRC control thesis (11.50% v/v). A PCA was performed on the main chemical data of the wines obtained to better compare and visualise the technological variability introduced by the strains used (Fig. 5).

Principal components 1 and 2 (PC1 and PC2) accounted for 85.07% of the variance. There was a positive correlation with ethanol content for the strains located in the first and fourth quadrants (TR1, TR3, TR4, TR5 and TRC). Theirs ability to perform fermentation and produce wines with low residual sugar content could make this group of strains interesting. In the third quadrant, the TR6 strain has a positive correlation with residual sugar content, malic acid and volatile acidity, while in the second quadrant, the TR2 strains have a positive correlation with glycerol and lactic acid content, total acidity and pH.

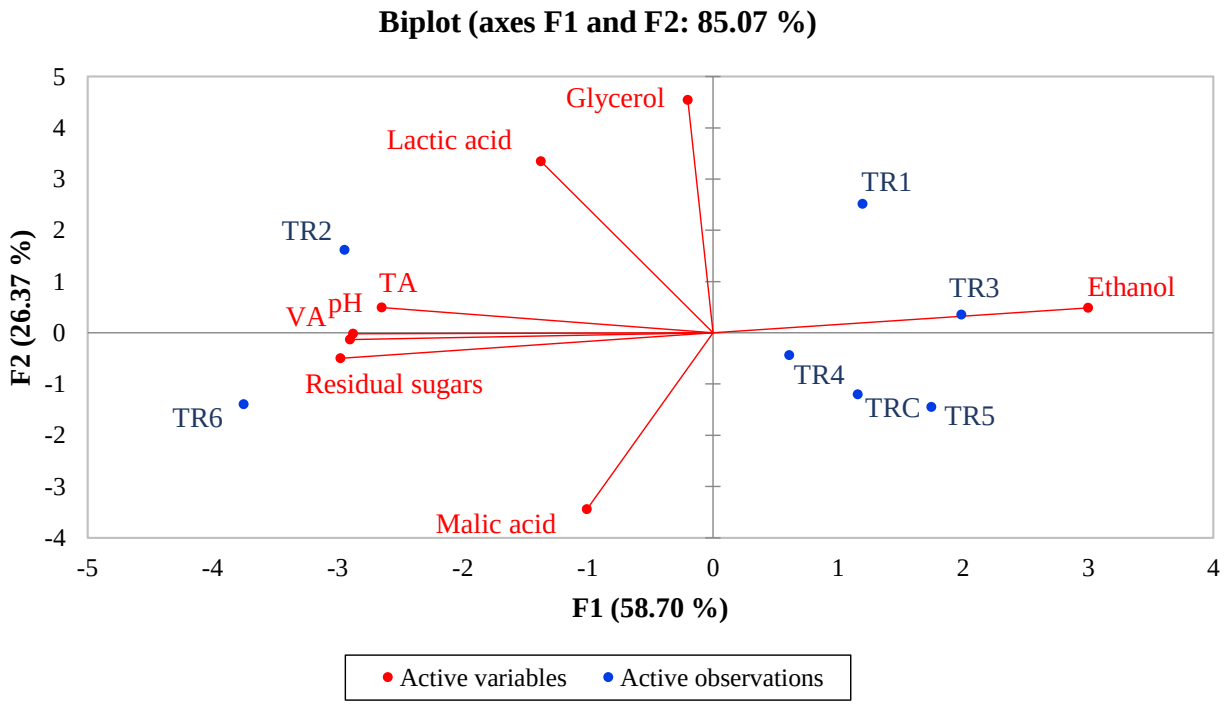

Figure 5. Biplot of principal component analysis of the main oenological parameters at the end of alcoholic fermentation.

Some strains could be further evaluated for sequential fermentation trials with *S. cerevisiae* in large-scale winemaking, considering the non-*Saccharomyces* yeasts with good oenological properties. In particular, MN400 (*L. thermotolerans*), MN412 (*S. lactis-condensi*), YS209 (*C. oleophila*) were the most interesting non-*Saccharomyces* strains. Among the *S. cerevisiae* strains tested, only MN113

showed similar behaviour to the control strain, thus proving to be a potential starter for wine fermentation in single culture. The effect of the strains on the volatile organic compound component will be further investigated in future research work.

2.4 CONCLUSIONS

This study used culture-dependent and molecular methods to assess yeast diversity in high-sugar matrices such as manna and honey, focusing on yeasts as a starting point for a study verifying their potential use in oenology. A high diversity of non-*Saccharomyces* and *Saccharomyces* yeasts was found in manna and honey by-products. An intraspecific grouping of the isolates was carried out based on genotypic and phenotypic clusters. In order to verify their application in the oenological field as starters and co-starters, an extensive technological characterisation was carried out. In particular, strains with a limited production of H_2S and those with a higher tolerance to ethanol, to osmotic stress, to sulphur and to copper have been selected.

As most of the characteristics analysed are species and strain dependent. The results highlight the importance of characterising many isolates for the selection of new starters and co-starters to be used in monoculture or mixed fermentations with the aim of improving wine quality and provide wines with unique characteristics. Further research is needed to evaluate the contribution to the volatile organic component of the selected strains in sequential inoculation with *S. cerevisiae.*

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CHAPTER 3

Impact of two new non-conventional yeasts (*Starmerella lactis-condensi* and *Candida oleophila*), isolated from sugar-rich substrates, on Frappato wine aroma

ABSTRACT

The interest of non-*Saccharomyces* yeasts in wine fermentation increased constantly in last years. This study reports for the first time the enological potential of two strains *Starmerella lactis-condensi* MN412 and *Candida oleophila* YS209. In an innovative way, these strains were used in winemaking to improve floral and fruity aroma of Frappato red wine, which has not been explored. The enological performances of the two non-*Saccharomyces* strains were compared to a wine strain of *Starmerella bacillaris*, namely Cz3, previously characterized in winemaking conditions. In these three cases, the non-*Saccharomyces* strain was sequentially inoculated with *S. cerevisiae* wine strain NF213, used as control. The *S. lactis-condensi* MN412 was isolated from Sicilian manna, a sugar-rich matrix, extracted from *Fraxinus angustifolia* trees (Oleaceae). The strain *C. oleophila* YS209 was isolated from honey by-products. Microbiological counts showed the ability of MN412 and YS209 to maintain high counts up to 6 days of alcoholic fermentation. Regarding chemical parameters, Cz3 showed the highest glycerol production. Analysis of VOCs revealed that the trials with non-*Saccharomyces* yeasts were characterized by a higher concentration of esters that contributed positively to the fruity aroma of the wines. The sensory analysis confirmed that the use of MN412 and YS209 impacted positively the final wines in terms of fruity and floral intensity, respectively, while did not generate sensory defects. In conclusion, non-conventional yeasts represent strategy to improve floral-fruity freshness of wine aroma and sugar-rich matrices such as manna ash and honey might represent novel ecological niches as source of potential oenological yeast.

3.1 INTRODUCTION

Non-*Saccharomyces* yeasts *constitute the largest microbial* group present on grape berries (Borren and Tian, 2021). Generally, they play an important role in the first days of fermentation when the levels of ethanol are quite low (Benito et al., 2019a). During the alcoholic fermentation, the composition of non-*Saccharomyces* yeast populations changes in relation to the evolution of ethanol concentration with the species sensitive replaced by those moderately resistant to the increasing levels of ethanol (Zhao et al., 2021), with *Saccharomyces* yeasts being predominant in the last stages of alcoholic fermentation (Mateus et al., 2020). Due to the key role played by yeasts on the sensory traits of wines (Romani et al., 2020; Varela, 2016), in the last decade, studies on oenological microbiology have focused also on the impact of non-*Saccharomyces* yeasts (Benito et al., 2019b).

Among the different non-*Saccharomyces* species, *Candida* and *Starmerella* species have recently been successfully used in mixed fermentation with *S. cerevisiae* to reduce ethanol content (Englezos et al., 2016a), increase glycerol concentration (Giaramida et al., 2013) and generating pleasant esters in wine (Englezos et al., 2016b). Currently, most of the *Candida* and *Starmerella* species used in winemaking are derived from oenological sources, mainly grapes and must (Di Maio et al., 2012). Recent studies proved that matrices with a high sugar content (e.g. honey by-products) are rich in microorganism, in particular *Saccharomyces* and non-*Saccharomyces* yeasts (Gaglio et al., 2017; Sinacori et al., 2014). Consequently, some of these strains present in these matrices have shown good aptitude for use in fermentation processes (Francesca et al., 2022). Moreover, Prestianni et al. (2022) applied *Saccharomyces cerevisiae* and *Hanseniaspora uvarum* isolate from honey by-product to improve and stabilize the quality of mead. Alfonzo et al. (2021) also tested the suitability of *S. cerevisiae* strains from non-oenological sources in winemaking and evidenced consistent differences with *S. cerevisiae* of grape origin.

A previous study conducted by Guarcello et al. (2019) analysed the cultivable microbial ecology of Sicilian manna ash, a sugar-rich matrix, and isolated several non-*Saccharomyces* yeasts, including *S. lactis-condensi* strains.

S. lactis-condensi were isolated from different oenological sources such as *Vitis labrusca* grapes (Čadež et al., 2020), botrytized Tokaj Essence wines (Csoma et al., 2021). Battistelli et al. (2021) have found a high presence of *S. lactis-condensi* investigating the cultivable microbiota of "mothers" of *Vino cotto*. Recently, Csoma et al. (2023) clarified the fructosophilic role of the dominant species *S. lactis-condensi* in Essences, a typical sweet wine from the Tokaj wine region in Hungary.

Franco et al. (2021) isolated *C. oleophila* in spontaneous fermentations of grape musts, tested its fermentative capabilities, and conducted sequential fermentation with *S. cerevisiae* in laboratory bioreactors. The same authors found high acetic acid production by *C. oleophila* but did not

investigate the impact of this yeast on the composition of volatile organic compounds (VOC) or the sensory profile of wines. Other authors, Lachance et al. (2011) found the ability of *C. oleophila* to metabolize glucose at various levels, and Aplin et al. (2019) described *C. oleophila* under laboratory winemaking conditions finding high acetic acid production. Therefore, to date, *C. oleophila* has been not used as starter or co-starter in any winemaking process.

Sicily is among the main Italian regions active in the production of red and rosé wines. In 2020, about 2 million hL/year of red wines were produced in Sicily (ISTAT, 2020). Among red grapes, Frappato is an autochthonous cultivar mainly cultivated within the provinces of Ragusa and Trapani with a total surface of about 750 ha for the production of Controlled and Guaranteed Denomination of Origin wine "Cerasuolo di Vittoria" (Asciuto and Bacarella, 2008). Frappato wines are characterised by a light ruby red color, brilliant, vinous, fruity and floral notes (Leder, 2020), but very little is known about the evolution of physicochemical, microbiological and aromas parameters of these wines. Frappato wines are commonly produced with commercial strains of *S. cerevisiae*, the species that ensures fermentation reproducibility and wine balancing.

However, many other yeast species with secondary importance during fermentation persist for the entire process. The positive effect of non-*Saccharomyces* yeasts in developing high taste-olfactory complexities has been highlighted (Fazio et al., 2023). This aspect well encounters the current consumer demand for novel wine styles (Comitini et al., 2023).

To our knowledge, however, no previous work has evaluated the effect of *S. lactis-condensi* and *C. oleophila* strains in sequential inoculation with *S. cerevisiae* during wine fermentation and investigated for their capability to improve aroma. Both *S. lactis-condensi* and *C. oleophila* strains have been isolated from novel ecological niches, such as manna ash and honey by-products with high sugar content.

Based on the above considerations, the present study aimed to: (i) to evaluate two non-conventional yeast strains (*S. lactis-condensi* MN412 and *C. oleophila* YS209) isolated from "natural environments" (manna and honey) for their potential in Frappato winemaking; (ii) to deepen our knowledge on VOCs composition of Frappato red wine.

3.2 MATERIAL AND METHODS

3.2.1 Strain preparation, experimental plan and sample collection

Non-*Saccharomyces* strains *S. lactis-condensi* MN412 isolated from manna (Guarcello et al., 2019), *C. oleophila* YS209 isolated from honey by-product, and *S. cerevisiae* NF213 isolated from grape must (Settanni et al., 2012) belong to the oenological yeast collection of the Department of Agricultural, Food and Forestry Sciences (SAAF; University of Palermo, Italy). All strains were reactivated from -80°C stock in Yeast Peptone Dextrose (YPD) at 28°C for 48 h and were reproduced

in a concentrated liquid suspension by Bionova srl (Villanova sull'Arda, Piacenza, Italy). *St. bacillaris* Cz3 is a strain of oenological origin (Di Maio et al., 2012) deposited in the yeast collection of the Sicilian Regional Institute of Wine and Oil (IRVO, Palermo, Italy) and marketed by Bioagro srl (Thiene, Vicenza, Italy). Grape of "Frappato" cultivar were donated by the winery "Caruso & Minini srl" located in Marsala (Italy).

The experimental plan of the present study (Fig. 1) consisted of four treatments: N1, sequential inoculum of *S. lactis-condensi* MN412/*S. cerevisiae* NF213; N2, sequential inoculum of *C. oleophila* YS209/*S. cerevisiae* NF213; N3, sequential inoculum of *St. bacillaris* Cz3/*S. cerevisiae* NF213; N4, single inoculum of *S. cerevisiae* NF213. In trials N1-N3, *S. cerevisiae* NF213 was inoculated 72 h after the addition of non-*Saccharomyces* strains.

All vinification were conducted at Department SAAF of University of Palermo, Italy and samples were collected at different stages of vinification: after grape pressing, after yeast inoculation, during alcoholic fermentation at day 1, 2, 3, 6, and at the end of fermentation (14 days). All analyses were performed in triplicate.

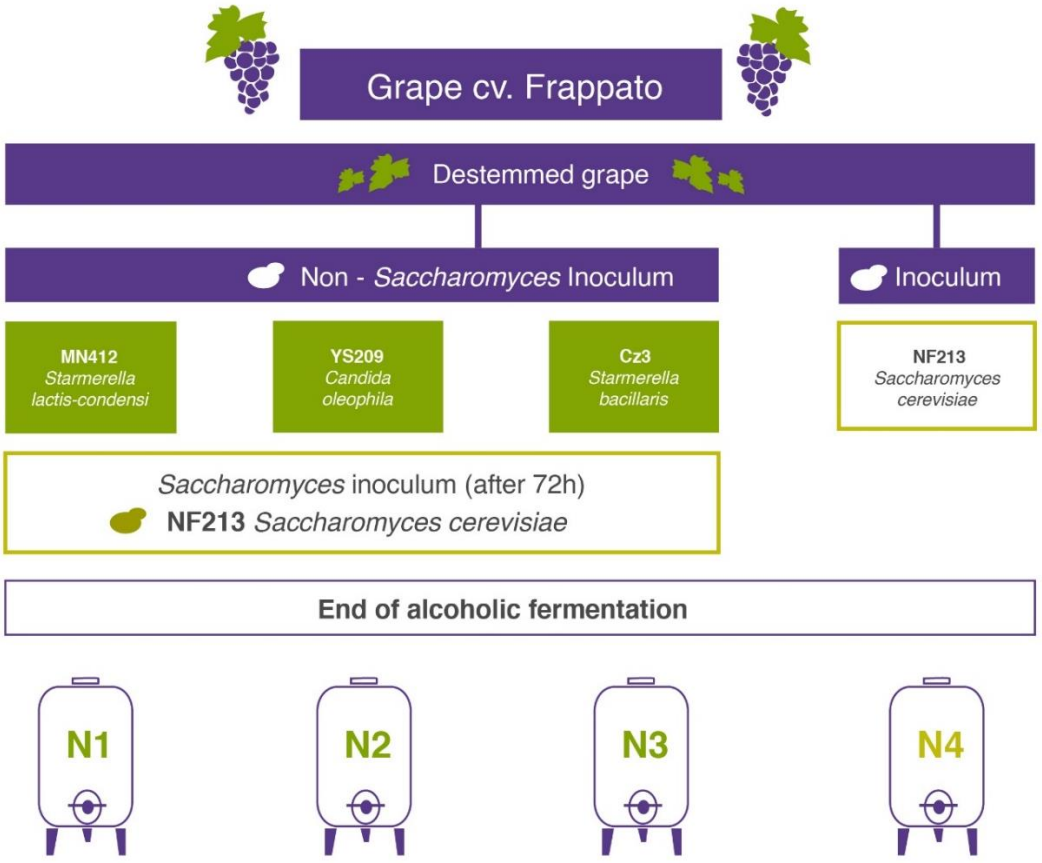

Figure 1. Experimental design of Frappato wines vinified with different non-*Saccharomyces* yeast strain.

3.2.2 Winemaking

Grapes were stemmer-crushed and supplemented with 2 g/hL of potassium metabisulphite (Chimica Noto s.r.l., Partinico, Italy). Bulk grape must was used to fill three test tanks (250 L each) for a total of 12 vats. Before yeast inoculation, 20 g/hL of diammonium phosphate (Chimica Noto SRL) and 20 g/hL of Fermaid E^{TM} (Lallemand, Castel D'Azzano, Italy) were added to each vat. All strains in concentrated liquid suspension [approx. 7.00×10^{10} colony-forming units (CFU)/g] were inoculated (20 mL/hL) according to the experimental plan; the alcoholic fermentation was conducted at 22 °C. At the end of alcoholic fermentation, 5 g/hL of potassium metabisulphite was added. The wines were aged in steel tanks 18 °C for two months. At bottling, free sulfur dioxide was adjusted to an approximate concentration of 30 mg/L. Bottled wines were kept at 15 °C. The winemaking process followed an oenological protocol used extensively by several wine companies. The process was performed at experimental wine cellar of University of Palermo based in Palermo city, Sicily (Italy).

3.2.3 Microbiological analysis

All samples collected during alcoholic fermentation were analysed for yeast colonies forming units, using various selective/differential culture media. Ten milliliters of each must sample were diluted in 90 mL of Ringer's solution (Sigma-Aldrich, Milan, Italy) and plated on Wallerstein Laboratory (WL) nutrient agar (incubated at 28 ºC for 72h) for *Saccharomyces* yeast quantification, and on lysine agar (incubated at 28 ºC for 5 days) for non-*Saccharomyces* (Di Maio et al., 2011). All media and supplements were purchased from Oxoid (Basingstoke, UK).

3.2.4 Yeast isolation, molecular identification and strain typing.

The dominance of the three non-*Saccharomyces* strains selected for this study was verified after three days of alcoholic fermentation, while that of *S. cerevisiae* was investigated at the end of alcoholic fermentation. At least five colonies of each yeast group with different morphology were selected from the respective culture media using the morphological criteria described by Cavazza et al. (1992) and Pallmann et al. (2001). All isolates were purified by successive sub-cultures on YPD agar (Lai et al., 2022) and their purity was verified by light microscopy (Carl Zeiss LTd, Berkochen, Germany). Three isolates with the same morphology from a given sample were then subjected to genetic characterization.

Genomic DNA for PCR assays was extracted by InstaGene Matrix kit (Bio-Rad, Hercules, CA, USA) following the protocol provided by the manufacturer. Yeasts differentiation was performed by RFLP using the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene (Esteve-Zarzoso et al., 1999). One isolate per group was further analysed by sequencing the D1/D2 region of the 26S rRNA gene to confirm the preliminary identification obtained by RFLP analysis as indicated by Alfonzo et al. (2020). DNA sequencing reactions were performed at AGRIVET

(University of Palermo, Italy). Sequence identity was determined by BlastN search against the NCBI non-redundant sequence database (http://www.ncbi.nlm.nih.gov). Sequences were manually corrected using Chromas 2.6.2. (Technelysium Pty Ltd., Australia).

The dominance of *S. cerevisiae* NF213 was confirmed by comparing the interdelta profile of the isolates from the highest cell dilution of musts with that of the pure strain. Interdelta analysis was conducted as described by Legras and Karst (2003). The persistence of non-*Saccharomyces* was carried out by comparing randomly amplified polymorphic DNA(RAPD)-PCR patterns of the isolates with those of the pure strains. RAPD-PCR was performed with primers M13 (Francesca et al., 2014) and XD5 (Di Maro et al., 2007). PCR products were visualised and compared as reported by Alfonzo et al. (2021).

3.2.5 Physicochemical analysis of musts and wines

The concentration of glucose, fructose, ethanol, glycerol, ammoniacal nitrogen, alpha-amino nitrogen, malic acid, lactic acid, and acetic acid were evaluated by means of the enzymatic analyser iCubio iMagic M9 (Shenzhen iCubio Biomedical Technology Co. Ltd., Shenzhen, China) as described by Matraxia et al. (2021). Samples were centrifuged (9000 rpm, 10 min) and analysed following the manufacturer's protocol. All reagents were purchased from R-Biopharm AG (Darmstadt, Germany). The values of pH were determined by OIV-MA-AS313-15 method (OIV, 2020a), total acidity was determined by the methodology described by OIV-MA-AS313-01 (OIV, 2020b), and free and total sulfur dioxide were measured in accordance with the methods described by OIV-MA-AS323-04B (OIV, 2020c). All chemical analyses were carried out in triplicate.

3.2.6 Analysis of VOCs in wine samples

5.2.6.1 Liquid-liquid extraction

Volatile compound composition of wine samples right after the end of alcoholic fermentation was determined with the following protocol: wine samples (10 mL) from all trials were mixed with MS SupraSolv® dichloromethane (5 mL) in a 50-mL conical flask, stirred at room temperature for 30 min, and centrifuged at 4000 rpm for 10 min by low Speed Centrifuge (ScanSpeed 416) with Swing Rotor (LaboGene ApS Industrivej 6–8, Vassingerød, DK- 3540 Lynge, Denmark); the aqueous phase was removed, added with anhydrous sodium sulphate (1 g), and centrifuged at 4000 rpm for 5 min; dichloromethane layer was removed, and dried under N_2 gas to 0.2 mL.

5.2.6.2 Identification and quantification of VOCs by GC-MS

Gas chromatographic analyses were performed with Agilent 7000C GC system, fitted with a fused silica Agilent DB-5MS capillary column (30 m × 0.25 mm i.d.; 0.25 μm film thickness), coupled to

an Agilent triple quadrupole Mass Selective Detector MSD 5973; ionization voltage 70 eV; electron multiplier energy 2000 V; transfer line temperature, 295 ◦C. Solvent Delay: 3.5 min. Helium was the carrier gas (1 mL/min).

The temperature was initially maintained at 40 °C for 1 min, gradually increased to 250 °C at a rate of 3 °C/min for 30 min, and finally maintained at 250 °C at 10 °C/min. One microliter of each sample was injected at 250 °C automatically and in the splitless mode: transfer line temperature, 295 °C. The individual peaks were analysed using the GC MS Solution package, Version 2.72. Identification of compounds was carried out using Adams, NIST 11, Wiley 9 and FFNSC 2 mass spectral database (Adams, 2007; Nist, 2008). These identifications were also confirmed by other published mass spectra and linear retention indices (LRI). LRI were calculated using a series of n-alkanes (C8-C40). Quantification was carried out using the three calibration lines. For compounds belonging to other classes than the standards, similarity was used for quantification. A dilution factor was used for the reported data.

3.2.7 Sensory analysis

Sensory evaluation of experimental wines was performed by quantitative descriptive analysis. Fourteen judges (8 men and 6 women, ranging from 26 to 45 years old) were recruited from University of Palermo. All judges had experience in winemaking and participated in previous studies as members of panels judging wines. Besides, they were subjected to preliminary tests to determine their sensory performances on basic tastes and aromas of wines. Sensory analysis of wine was conducted as described by Jackson (2016). The 14 panellists compared the four experimental wines during different sessions. They consensually generated 16 sensory descriptive attributes regarding appearance (colour), odour (intensity, complexity, floral, fruity, spicy, balsamic, and overall odour quality), flavour, taste (intensity, persistence, sour, salty and smoothness, overall taste quality), and overall quality. The panellists also generated a consensual descriptive ballot (Biasoto et al., 2014; Jackson, 2016) and the descriptors were associated to a 9 cm unstructured scale (1 = extremely low, 5 = moderate intensity, 9 = extremely high). The four wine samples were evaluated in distinct tasting sessions carried out on successive days. Overall, each judge evaluated each of the four wines with two repetitions. A given bottle represented a single technical repeat.

3.2.8 Statistical analysis

ANOVA test was applied to identify significant differences among physicochemical parameters (pH, total acidity, acetic acid, residual sugars, glucose, fructose, alpha-amino nitrogen, ammoniacal nitrogen, ethanol, glycerol, malic acid, lactic acid, free and total SO_2), levels of *Saccharomyces* and non-*Saccharomyces* and sensory analysis. The post-hoc Tukey's method was applied for pairwise

comparison of all data. Statistical significance was attributed to $P < 0.05$ (Mazzei et al., 2010). A heat map clustered analysis (HMCA), based on hierarchical dendrogram with heat map plot, was generated from VOCs and the individual content values of data matrix are graphically represented as colours (Martorana et al., 2017). The relative values of VOCs concentration were depicted by colour intensity from yellow (lowest quantity) to red (highest quantity). Heat map analysis was performed using the autoscaled data using ascendant hierarchical clustering based on Ward's method and Euclidian distance (Gaglio et al., 2017) to show the similarities between VOCs and wine obtained with different yeast starter strains and nutrition regimes.

Sensory Product Characterization Analysis (SPCA) was applied in order to determine the sensory differences of the wines produced by means of an analytical method based on the attributes describing each trial. For each session, the score was evaluated considering product, judge and session effect. A histogram chart of different colours was created for each wine. Blue is associated with coefficients that show a significant positive value and the red color with coefficients showing a significant negative value. Differences between trials were represented graphically with a sensory profile plot.

Statistical data processing and graphic construction were performed with the XLStat software version 2019.2.2 (Addinsoft, New York, USA) for Excel.

3.3 RESULT AND DISCUSSION

3.3.1 Kinetics of yeast populations during fermentation

The growth of yeasts during the alcoholic fermentation is graphically shown in Fig. 2. The levels of non-*Saccharomyces* and *Saccharomyces* populations of Frappato must, at the beginning of monitoring, were 5.3 Log CFU/mL and < 2.0 Log CFU/mL, respectively. Cell density of non-*Saccharomyces* increased at 6.0-7.3 Log CFU/mL just after inoculation; these densities are considered adequate to influence the sensory characteristics of wines (Du Plessis et al., 2017). After 3 d, the trials N1-N3 were inoculated with *S. cerevisiae* NF213 until 7.3 to 8.3 Log CFU/mL. After further 3 d, all trials showed a decrease of non-*Saccharomyces*, a trend already registered by Binati et al. (2020), who followed a sequential inoculum of *S. bacillaris* and *S. cerevisiae.* Specifically, values lower than 2-3 Log CFU/mL were registered for trial N1, N2 and N3, after 6 d from the beginning of fermentation. The decrease of non-*Saccharomyces* populations in sequential inoculum with *S. cerevisiae* is determined by several events, mainly increased ethanol concentrations, secretion of inhibitory substances, and competition phenomena (Wang et al., 2016). According to Binati et al. (2020), at the end of alcoholic fermentation (14 d), non-*Saccharomyces* populations were at levels lower than the detection limit. On the contrary, *Saccharomyces* were in the range 7.9-8.6 Log CFU/mL for all trials.

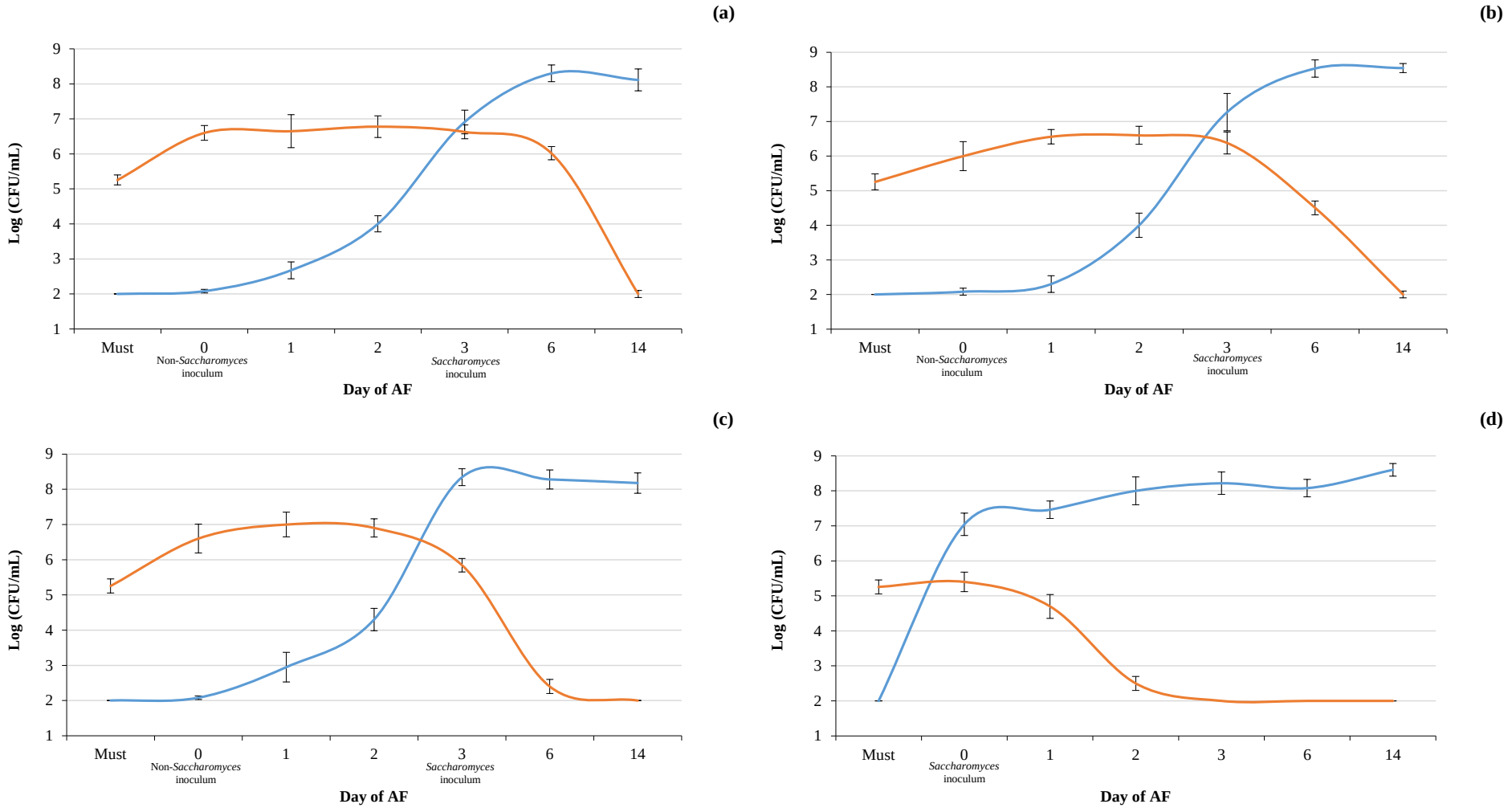

Figure 2. Evolution of yeast populations of presumptive *Saccharomyces cerevisiae* and non-*Saccharomyces* populations during alcoholic fermentation: **(a)** sequential inoculum *Starmerella lactis-condensi* MN412/*Saccharomyces cerevisiae* NF213 (N1); **(b)** sequential inoculum *Candida oleophila* YS209/*Saccharomyces cerevisiae* NF213 (N2); **(c)** sequential inoculum *Starmerella bacillaris* Cz3/*Saccharomyces cerevisiae* NF213 (N3); **(d)** single inoculum *Saccharomyces cerevisiae* NF213 (N4; control). Legend: —, presumptive *Saccharomyces*; —, non-*Saccharomyces*.

3.3.2 Dominance of inoculated yeasts

A total of 1003 colonies that had grown on WL were isolated, sequentially re-propagated on WL and checked for their colony colour, colony topography and microscopic observations (Cavazza et al., 1992; Pallmann et al. 2001); 748 colonies were classified as *Saccharomyces*. The analysis of 5.8S-ITS amplicons confirmed that all these isolates shared a 5.8S-ITS region of 880 bp typical of *S. cerevisiae* and the profile of the restriction fragments obtained with C*fo*I, *Hae*III and *Hinf*I confirmed that these isolates were *S. cerevisiae*. (Guillamón et al., 1998).

The other unclassified 255 isolates were assigned to the non-*Saccharomyces* yeast group.

Eighty-nine isolates were characterized by an ITS amplicon of 480 bp and were presumptively identified and *S. lactis-condensi*. In fact, the same ITS amplicon sizes were found by Solieri et al. (2006), who worked on *S. lactis-condensi*. Eighty-two isolates showed ITS amplicons of 630 and were considered presumptive *C. oleophila* (n=82) while 67 were allocated to the species *S. bacillaris* (n=67) based on the 430 bp amplicon (Gordún Quiles et al., 2018; Wang et al., 2019). The remaining isolates (n=17) showed an ITS amplicon between 750 (n=11) and 760 (n=6) bp with a colony morphology on WL agar similar to that of yeasts of the genus *Hanseniaspora*, which are very common in sicilian Frappato musts (Romancino et al., 2008). RFLP profiles of non-*Saccharomyces* species confirmed what observed by other authors who identified yeasts (Esteve-Zarzoso et al., 1999; de Llanos Frutos et al., 2004; Solieri et al., 2006; Wang et al., 2019).

Interdelta analysis confirmed the presence of three different strains of *S. cerevisiae*. The different interdelta profiles also indicated the presence of indigenous grape *S. cerevisiae* (Aponte et al., 2020). The direct comparison of the interdelta profiles showed that *S. cerevisiae* NF213 was the strain most frequently isolated (>96%). RAPD pattern comparison indicated that each non-*Saccharomyces* inoculated strains showed a dominance percentage higher than 90%. Yeast genotypic identification was completed by pairwise alignment of D1/D2 sequence of the 30% isolates with those of type strains (*C. oleophila* CBS2219^T, *S. cerevisiae* CBS 1171^T, *S. lactis-condensi* CBS 52^T and *S. bacillaris* CBS9494^T); D1/D2 sequence from the strains Cz3, MN412, NF213 and YS209, and used in this study showed 100% homology with type strains.

3.3.3 Chemical monitoring

The results of the chemical analyses are summarized in Table 1. The initial sugar content of Frappato grape must of this study was 231.83 g/L (114.18 g/L glucose and 117.65 g/L fructose), total titratable acidity (TTA) of 8.11 g/L tartaric acid, 2.13 g/L malic acid and pH 3.15.

After 72 h, the majority of chemical parameters showed significant differences among trials, while pH and concentrations of malic acid, lactic acid, free and total SO_2 were quite comparable.

Table 1.

Chemical parameters determined during the winemaking process.

Parameters	Musts	Vinification									
		3 d of alcoholic fermentation					End of alcoholic fermentation				
		N1	N2	N3	N4	S.S.	N1	N2	N3	N4	S.S.
Ammoniacal nitrogen$^{\alpha}$	249.17±0.12	210.98±0.14^{c}	218.45±0.09^{a}	215.75±0.13^{b}	89.22±0.18^{d}	***	48.59±0.14^{d}	88.13±0.09^{a}	73.84±0.17^{b}	51.26±0.18^{c}	***
Alpha-amino nitrogen$^{\alpha}$	192.94±0.15	192.48±0.12^{b}	192.51±0.11^{b}	207.92± 0.19^{a}	59.61±0.04^{c}	***	97.86±0.12^{d}	105.68±0.11^{b}	102.58±0.11^{c}	116.47±0.04^{a}	***
Residual sugars$^{\beta}$	231.83±0.26	174.90±0.12^{a}	181.73±0.20^{b}	172.31±0.15^{b}	90.01±0.26^{c}	***	0.14±0.02^{a}	0.07±0.01^{b}	0.07±0.03^{b}	0.12±0.02^{ab}	*
Glucose$^{\beta}$	114.18±0.10	112.49±0.08^{a}	109.55±0.06^{b}	110.86±0.10^{b}	29.32±0.05^{c}	***	0.03±0.01^{a}	0.02±0.00^{a}	0.07±0.03^{a}	0.03±0.01^{a}	n.s.
Fructose$^{\beta}$	117.65±0.15	62.41±0.21^{a}	72.18±0.12^{a}	61.45±0.08^{b}	60.69±0.14^{c}	***	0.11±0.02^{a}	0.05±0.02^{ab}	0.00±0.00^{b}	0.09±0.04^{a}	**
Acetic acid$^{\beta}$	0.02±0.02	0.06±0.02^{b}	0.04±0.03^{b}	0.21±0.04^{a}	0.09±0.02^{b}	**	0.31±0.02^{a}	0.28±0.04^{a}	0.31±0.06^{a}	0.26±0.01^{a}	n.s.
Malic acid$^{\beta}$	2.13±0.03	2.08±0.02^{a}	2.02±0.03^{a}	2.11±0.06^{a}	2.10±0.02^{a}	n.s.	1.90±0.02^{b}	1.87±0.03^{b}	1.99±0.04^{a}	1.91±0.02^{b}	*
Lactic acid$^{\beta}$	0.04±0.01	0.02±0.01^{a}	0.04±0.01^{a}	0.05±0.02^{a}	0.03±0.01^{a}	n.s.	0.06±0.02^{a}	0.07±0.01^{a}	0.07±0.02^{a}	0.06±0.02^{a}	n.s.
Glycerol$^{\beta}$	0.35±0.01	0.75±0.14^{c}	0.70±0.17^{c}	2.76±0.05^{b}	6.68±0.11^{a}	***	7.90±0.16^{b}	8.26±0.10^{b}	10.31±0.17^{a}	8.29±0.14^{b}	***
Ethanol$^{\gamma}$	0.01±0.01	2.87±0.02^{c}	2.99±0.01^{b}	2.22±0.06^{d}	7.13±0.01^{a}	***	11.70±0.06^{bc}	11.78±0.03^{b}	11.65±0.02^{c}	11.99±0.03^{a}	***
pH	3.15±0.01	3.16±0.02^{a}	3.15±0.01^{a}	3.17±0.00^{a}	3.15±0.01^{a}	n.s.	3.13±0.02^{a}	3.14±0.01^{a}	3.14±0.00^{a}	3.16±0.01^{a}	n.s.
Total titratable acidity$^{\delta}$	8.11±0.09	6.82±0.12^{b}	6.84±0.10^{b}	6.88±0.10^{b}	7.17±0.10^{a}	*	6.35±0.10^{b}	6.30±0.10^{b}	6.35±0.10^{b}	6.70±0.10^{a}	**
Free-SO_2^{α}	8.00±0.00	8.00±0.50^{a}	8.50±0.50^{a}	8.00±0.00^{a}	8.75±0.50^{a}	n.s.	17.00±0.50^{a}	16.50±0.50^{a}	17.50±1.00^{a}	18.00±0.50^{a}	n.s.
Total-SO_2^{α}	9.50±0.50	10.00±1.50^{a}	10.50±1.00^{a}	10.00±0.50^{a}	12.00±1.00^{a}	n.s.	32.00±1.50^{a}	28.00±1.00^{b}	30.00±1.00^{ab}	32.00±1.00^{a}	*

Parameters	Vinification				
	Bottling				
	N1	N2	N3	N4	S.S.
Ammoniacal nitrogen$^{\alpha}$	n.d.	n.d.	n.d.	n.d.	n.d.
Alpha-amino nitrogen$^{\alpha}$	n.d.	n.d.	n.d.	n.d.	n.d.
Residual sugars$^{\beta}$	0.04±0.03^{a}	0.02±0.01^{a}	0.03±0.01^{a}	0.05±0.02^{a}	n.s.
Glucose$^{\beta}$	0.00±0.00^{b}	0.00±0.00^{b}	0.03±0.01^{a}	0.02±0.01^{ab}	**
Fructose$^{\beta}$	0.04±0.03^{a}	0.02±0.01^{a}	0.00±0.00^{a}	0.03±0.01^{a}	n.s.
Acetic acid$^{\beta}$	0.33±0.04^{a}	0.29±0.04^{a}	0.35±0.03^{a}	0.28±0.01^{a}	n.s.
Malic acid$^{\beta}$	1.84±01^{c}	1.83±0.01^{c}	1.91±0.01^{a}	1.88±0.01^{b}	***
Lactic acid$^{\beta}$	0.06±0.02^{a}	0.08±0.01^{a}	0.07±0.02^{a}	0.07±0.03^{a}	n.s.
Glycerol$^{\beta}$	7.67±0.07^{c}	8.02±0.11^{b}	10.13±0.08^{a}	8.00±0.07^{b}	***
Ethanol$^{\gamma}$	11.75±0.05^{b}	11.74±0.03^{b}	11.68±0.03^{b}	11.94±0.02^{a}	***
pH	3.18±0.02^{a}	3.16±0.01^{a}	3.19±0.00^{a}	3.20±0.01^{a}	n.s.
Total titratable acidity$^{\delta}$	6.20±0.10^{a}	6.20±0.10^{a}	6.30±0.10^{a}	6.40±0.10^{a}	n.s.
Free-SO_2^{α}	31.00±1.50^{a}	30.50±1.00^{a}	29.50±1.50^{a}	30.50±1.00^{a}	n.s.
Total-SO_2^{α}	55.00±1.00^{a}	49.00±1.50^{a}	50.00±0.50^{a}	51.00±1.00^{a}	n.s.

Result indicates mean value ± standard deviation of three determinations from three replicates. Data within a line followed by the same letter are not significantly different according to Tukey's test.
Symbols: $^{\alpha}$, mg/L; $^{\beta}$, expressed in g/L; $^{\gamma}$, % v/v; $^{\delta}$, tartaric acid g/L.
Abbreviations: S.S., statistical significance; P value: *, P < 0.05; **, P < 0.01; ***, P < 0.001; n.s., not significant; n.d., not determined.

The trials inoculated with *S. lactis-condensi* MN412 (N1) showed the lowest values of residual sugars (174.90 g/L). The trials N1 – N3 showed a higher consumption of fructose rather than glucose, compared to the trial N2. Fructose preference is a common characteristic of certain *Candida* strains (Englezos et al., 2019; Magyar and Tóth, 2011). The highest concentrations of ethanol and glycerol were registered for trial N4 [7.13 % (v/v) and 6.68 g/L, respectively]. Among the sequential inoculation trials carried out, N2 was the trial showing the highest ethanol concentration [2.99 % (v/v)], while trial N3 showed the highest glycerol concentration (2.76 g/L). No decrease in the production of ethanol was found in the trial inoculated with *S. bacillaris* (N3), as also determined by Giaramida et al. (2013).

All the fermentations were completed in two weeks and the wines obtained were characterised by a residual sugar content of less than 1 g/L. *S. lactis-condensi* (N1) and *C. oleophila* (N2) did not cause any change in the oenological parameters in terms of acetic acid content. Also, no statistically significant differences were observed between trials in terms of glucose concentration, acetic acid, lactic acid and pH values.

Trial N2 inoculated with *C. oleophila*, contrary to what reported by Franco et al. (2021), produced little acetic acid (0.28 g/L). Aplin et al. (2019) tried to select *C. oleophila* as a co-starter, but the strain produced acetic acid higher than 0.8 g/L, for this reason it was discarded and never applied in vinification. To our knowledge, the present work is the first report on application of *C. oleophila* in wine fermentation under real winemaking condition, since the previous authors used strain of *C. oleophila* only in bioreactor and/or *in vitro* investigation (Aplin et al., 2019).

Significant differences were found for TTA values, which were lower for the trials inoculated with non-*Saccharomyces* (6.30-6.35 g/L tartaric acid). At the end of fermentation, ethanol concentrations ranged between 11.65 and 11.99% (v/v). The highest values in ethanol were observed in the control trial N4 and the use of non-*Saccharomyces* in sequential inoculation with *S. cerevisiae* can promote the reduction of ethanol content of wines. In this study, differences in ethanol content ranged from 0.21-0.34 % (v/v). Benavides et al. (2022) who tested different sequential inoculum combinations to lower ethanol content in wines observed similar results.

Malic acid concentration decreased slightly from the beginning of monitoring (2.13 g/L) to the end of fermentation for all trials (1.87-1.99 g/L). The negligible decrease of malic acid concentration observed during the alcoholic fermentation could be due to *Saccharomyces* spp. strains, which can degrade malic acid initially, present in must from 3% to 45% (Saayman and Viljoen-Bloom, 2006). Lactic acid was present in trace amount for all trials. Regarding glycerol, a much higher content than in the other trials was found in trial N3 inoculated with *S. bacillaris* (10.31 g/L), a similar increase was found by Giaramida et al. (2013). This compound influences wine sensory properties, especially

in red wines where it positively contributes to smoothness, sweetness, and complexity (Comitini et al., 2011). The increase in glycerol content of wines produced with *C. oleophila* and *S. bacillaris* is a common phenomenon (Englezos et al., 2018; Franco et al., 2021). At bottling, chemical parameters changed insignificantly. For the first time, *C. oleophila* has been used in a grape must obtain bottled wine according to commercial protocols. To our knowledge, *S. lactis-condensi* strains have only recently been selected for oenological applications and have shown greater efficiency in fructose utilisation and tolerance to sugar, alcohol and sulphur content compared to *S. bacillaris* (Csoma et al., 2023).

3.3.4 Volatile organic compounds of wines

The VOCs of wines are listed in Table 2. Quantitative differences were found among trials. The 29 identified compounds were grouped into alcohols, ethers, aldehydes, carboxylic acids, esters and other compounds. The heat map reports the differences between the aromatic profiles and VOC amounts (Fig. 3).

Alcohols are dominant wine VOCs resulting from yeast fermentation (Kotseridis and Baumes, 2000). The compounds mainly detected in this study were phenylethyl alcohol and 1-penthanol with values varying from 2.08 ppm (N2) to 40.63 ppm (N4) and from 47.94 ppm (N4) to 67.16 ppm (N2), respectively. These higher alcohols are responsible for floral notes (Cordente et al., 2021). Trial N3 inoculated with *S. bacillaris* distinguished from the others for the high concentrations of 3-hexenol and 2-butanol, both related to fusel note (Juan et al., 2012; Furdíková et al., 2014). Among ethers, 3-Ethoxy-1-propanol, a compound that gives a fruity aroma (Velázquez et al., 2015), was the only compound detected and the concentrations varied from 0.30 ppm (N2) to 1.15 ppm (N3).

Due to their rancid and cheesy smells (Călugăr et al., 2020), carboxylic acids are undesirable in wines and the experimental wines obtained in this study were characterized by very low concentrations (< 0.06 ppm).

Esters compounds are released during fermentation and directly influence the aromatic complexity of wines (Tempère et al., 2018). Within this class, ethyl acetate was significantly higher in wines processed with the sequential inoculum (N1-N2-N3) than single culture of *S. cerevisiae* (N4).

Ethyl acetate, which is also associated with the development of fruity flavours (Renault et al., 2015). Englezos et al. (2019) found a similar increase in ethyl acetate content during mixed fermentation of *S. bacillaris*/S. *cerevisiae*.

Among the treatments, N1 and N2 were distinguished from the others by higher levels of ethyl octanoate, 0.90 ppm and 1.15 ppm respectively.

Table 2.

Volatile organic compounds detected in the four Frappato wines (all values in ppm).

LRI	Compounds[1] (Common name)	Aroma description[2]	N1[3]	N2[3]	N3[3]	N4[3]	S.S.[4]
\	**Σ Alcohols**						
625	2-Methyl-2-butanol	Plastic, solvent, fly spray	0.71±0.02^{b}	1.07±0.03^{a}	0.76±0.01^{b}	0.61±0.02^{c}	***
664	2-Butanol	Alcoholic	0.00±0.00^{b}	0.00±0.00^{b}	8.28±0.07^{a}	0.00±0.00^{b}	***
760	1-Pentanol	Fusel	50.84±1.14^{bc}	67.16±1.23^{a}	51.71±1.35^{b}	47.94±1.21^{c}	***
796	2.3-Butanediol[6]	Buttery, creamy	0.17±0.01^{a}	tr	tr	tr	***
796	2.3-Butanediol[6]	Buttery, creamy	tr	0.00±0.00	tr	0.03±0.01	n.s.[5]
829	3-Methyl-1-pentanol	Fusel	0.10±0.01^{a}	tr	0.00±0.00^{b}	0.11±0.01^{a}	***
857	3-Hexenol	Grass, moss	0.06±0.01^{b}	0.00±0.00^{c}	0.15±0.02^{a}	0.05±0.01^{b}	***
872	1-Hexanol	Green	2.33±0.04^{b}	2.25±0.03^{c}	3.93±0.02^{a}	1.30±0.02^{d}	***
1039	Benzyl alcohol	Sweet, flower	0.00±0.00^{b}	0.17±0.01^{a}	0.00±0.00^{b}	0.00±0.00^{b}	***
1108	Phenylethyl alcohol	Floral, rose	28.33±1.34^{b}	2.08±0.02^{d}	14.18±0.54^{c}	40.63±1.76^{a}	***
1442	p-Thyrosol	Sweet, floral, fruity	0.28±0.02^{b}	0.44±0.03^{a}	0.06±0.01^{c}	tr	***
1502	2.4-Di-tert-butylphenol	Unknown	1.11±0.08^{b}	4.54±0.05^{a}	0.89±0.07^{c}	0.59±0.03^{d}	***
	Σ Ethers						
816	3-Ethoxy-1-propanol	Fruit	0.86±0.02^{b}	0.30±0.01^{d}	1.15±0.04^{a}	0.55±0.03^{c}	***
	Σ Aldehydes						
960	Benzaldehyde	Bitter almond, nutty, smoky	0.00±0.00^{b}	0.00±0.00^{b}	0.06±0.01^{a}	0.00±0.00^{b}	***
	Σ Carboxylic acids						
875	3-Methyl-butanoic acid	Cheese, rancid	0.00±0.00^{b}	0.03±0.01^{a}	0.00±0.00^{b}	0.00±0.00^{b}	*
976	Hexanoic acid	Mild, fatty	0.00±0.00^{b}	0.03±0.01^{a}	tr	tr	*
---	4-Ethoxy-4-oxobutanoic acid	Unknown	0.06±0.01^{a}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	***
	Σ Esters						
613	Ethyl acetate	Ethereal, fruity	10.57±0.23^{b}	13.91±0.21^{a}	13.95±0.18^{a}	4.56±0.10^{c}	***
713	Propyl acetate	Pear	0.03±0.01^{a}	0.04±0.01^{a}	0.00±0.00^{b}	0.00±0.00^{b}	***
800	Ethyl butanoate	Apple	0.19±0.01^{b}	0.25±0.02^{a}	0.13±0.01^{c}	0.14±0.01^{c}	***
876	Isopentyl acetate	Banana, fruity tropical	0.30±0.02^{a}	0.00±0.00^{c}	0.00±0.00^{c}	0.20±0.01^{b}	***
879	2-Methylbutyl acetate	Fruity	0.04±0.01^{a}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	***
937	Ethyl 3-hydroxybutyrate	Fruity, grape, green	0.19±0.02^{a}	0.02±0.01^{c}	0.13±0.01^{b}	0.16±0.02^{ab}	***
999	Ethyl Hexanoate	Sweet fruity, pineapple, green apple	0.77±0.04^{b}	0.74±0.03^{bc}	1.07±0.05^{a}	0.66±0.04^{c}	***
1153	Diethyl butanedioate (Diethyl succinate)	Fruit	0.82±0.06^{a}	0.47±0.05^{c}	0.68±0.03^{b}	0.82±0.01^{a}	***
1188	Ethyl octanoate (Ethyl caprylate)	Fruity, pear	0.90±0.04^{b}	1.15±0.06^{a}	0.69±0.02^{c}	0.57±0.03^{d}	***
1296	Ethyl nonanoate	Fruity, fatty	0.18±0.02^{b}	0.24±0.01^{a}	tr	0.05±0.01^{c}	***
	Σ Others						
1245	1.3-Di-tert-butylbenzene	Unknown	0.90±0.02^{c}	3.80±0.04^{a}	1.57±0.03^{b}	0.91±0.04^{c}	***
-----	Tryptophan	Unknown	0.06±0.01^{c}	1.05±0.07^{a}	0.49±0.02^{b}	0.00±0.00^{c}	***

[1]Compounds are classified in order of retention time; [2]Aroma descriptions are reported in the online database of Good Scents Company Information (http://www.thegoodscentscompany.com/), Flavornet (http://www.flavornet.org/) and LRI & Odour Database (http://www.odour.org.uk/);[3] Relative amounts, expressed as ppb; [4] statistical significances; [5] not significative; [6] stereoisomers not identified.

Abbreviations: tr: trace amount < 0.01 ppm.

Data within a line followed by the same letter are not significantly different according to Tukey's test.

P value: ***, P < 0.001

For the first time the impact on VOCs by *C. oleophila* (N1) was studied. Previously, Franco et al. (2021) used this species as a co-starter *in vitro* winemaking experiment, analysing only the basic chemical-physical parameters. This is the first paper to report a study of VOCs associated with *C. oleophila* for oenological use as a co-starter. Its previous use has been in agriculture as a biocontrol agent (Raspor et al., 2010). The growing interest in non-*Saccharomyces* strains with must bio-protective action (Naselli et al., 2023), offers interesting insights into the selection of new co-starter strains. Further investigations will be necessary to verify the possible bio-protective action of the strain YS209 *C. oleophila.* Matraxia et al. (2021) applying non-*Saccharomyces* strains isolated and selected from honey by-products, also found an increase in the ester content of beers. To our knowledge, strains of *S. lactis-condensi* have only recently been selected for oenological applications (Csoma et al., 2023).

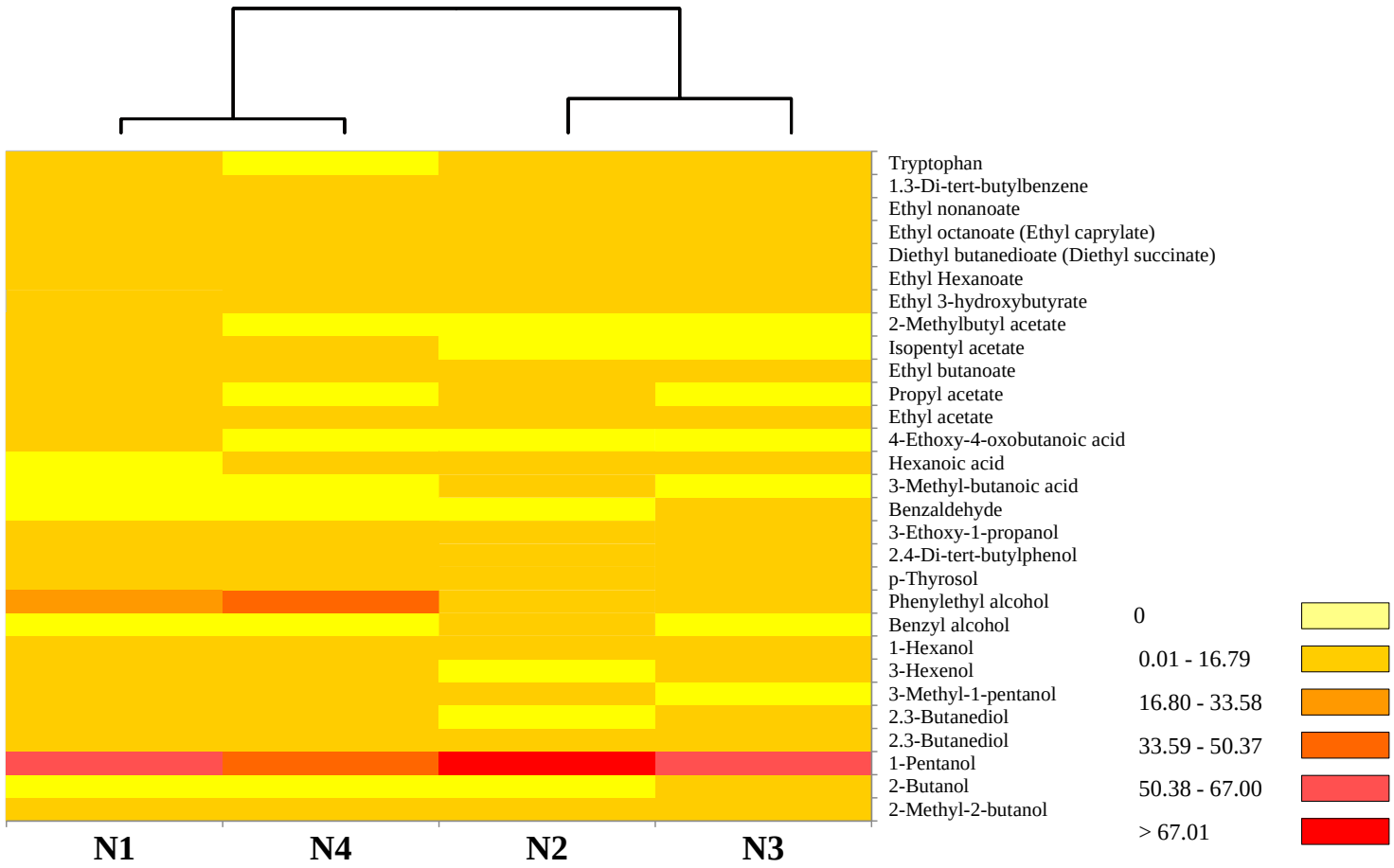

Figure 3. Distribution of volatile organic compounds among wines. The heat map plot depicts the relative concentration of each VOCs. Frappato must fermented by: N1, sequential inoculum *Starmerella lactis-condensi* MN412/ *Saccharomyces cerevisiae* NF213; N2, sequential inoculum *Candida oleophila* YS209/ *Saccharomyces cerevisiae* NF213; N3, sequential inoculum *Starmerella bacillaris* Cz3/ *Saccharomyces cerevisiae* NF213; N4, single inoculum *Saccharomyces cerevisiae* NF213.

3.3.5 Sensory evaluation

Among the 15 attributes that defined the sensory profile of each wine, SPCA indicated that the highest discriminating power was represented by odour overall quality, flavour overall quality and overall quality, while the lowest discriminating power was shown by colour. The definition of the sensory characteristics of each wine, expressed in model coefficients for each product-descriptor combination is shown in Fig. 4.

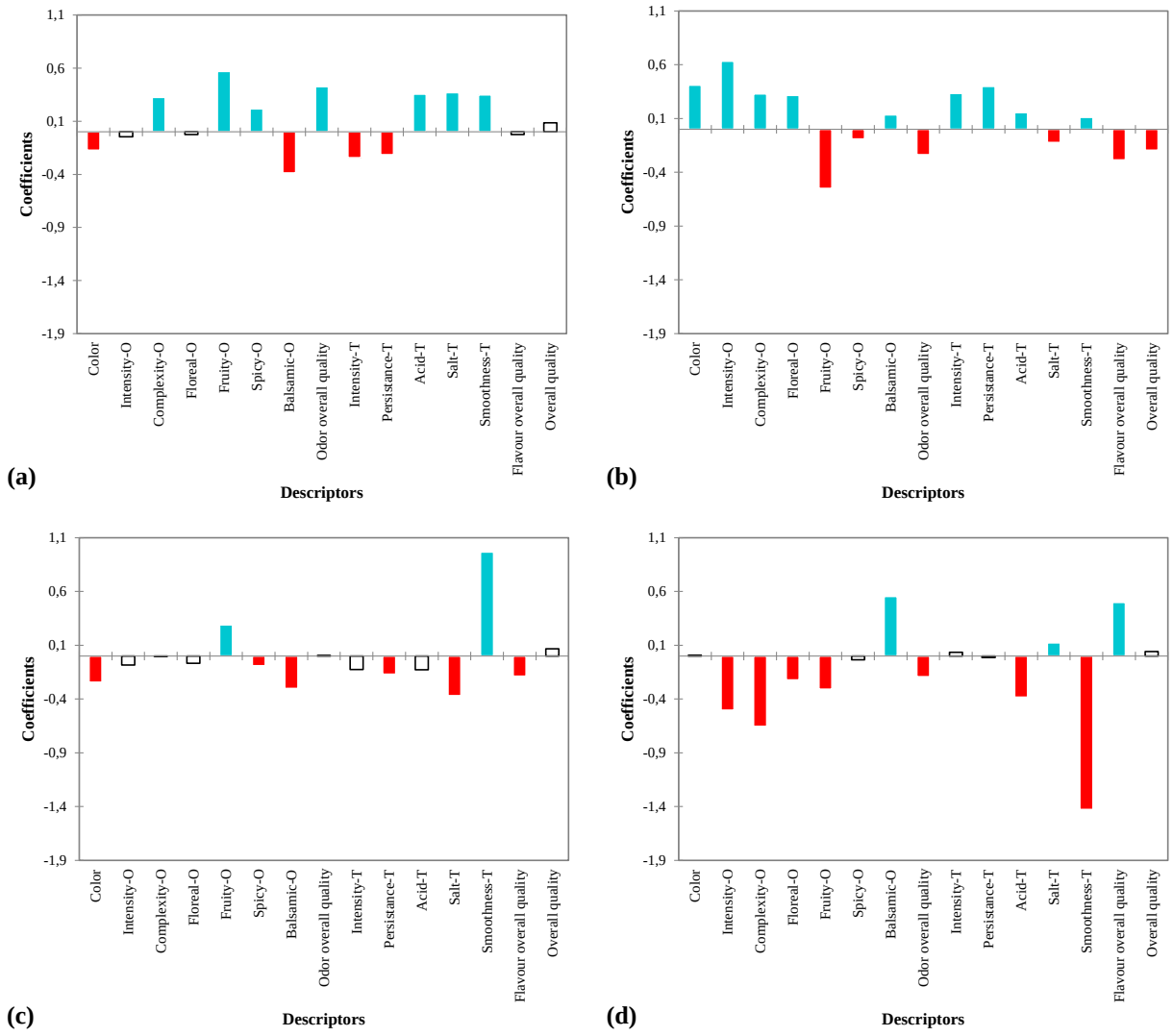

Figure 4. Sensory profiles of Frappato wines obtained with sequential inoculation of: (a) N1, sequential inoculum *Starmerella lactis-condensi* MN412/ *Saccharomyces cerevisiae* NF213; (b) N2, sequential inoculum *Candida oleophila* YS209/ *Saccharomyces cerevisiae* NF213; (c) sequential inoculum *Starmerella bacillaris* Cz3/ *Saccharomyces cerevisiae* NF213; (d) N4, single inoculum *Saccharomyces cerevisiae* NF213. The blue colour is associated to coefficients that have a significant positive value and the red colour is associated to coefficients that have a significant negative value.

Trials N1 and N2 showed a number of attributes with significant positive effect of 7 and 9, respectively. Treatment N3, showed 6 attributes with significant negative effect. The coefficients defining the complexity, fruity and spicy odours of wine from trial N1 produced with sequential inoculation of *S. lactis-condensi* showed the highest coefficients when compared to all other trials.

The wine from trial N3 was characterized by a strong smoothness on the palate (Fig. 4c). The high smoothness values could be related to the amount of glycerol of wine (Ciani and Ferraro, 1998). The activity of *S. bacillaris* before addition of *S. cerevisiae* was sufficient to increase the glycerol until values defining for the smoothness of this wine, a similar behaviour was found by Giaramida et al. (2013). The final wine of the control trial (N4) was characterised by balsamic odours, low intensity and complexity odour, and the taste was neither acidic nor smooth (Fig. 4d). However, none of the wines analysed showed off-odour.

In order to better evaluate the differences among Frappato wines, the data of the sensory analysis performed were illustrated in the sensory profile graph (Fig. 5).

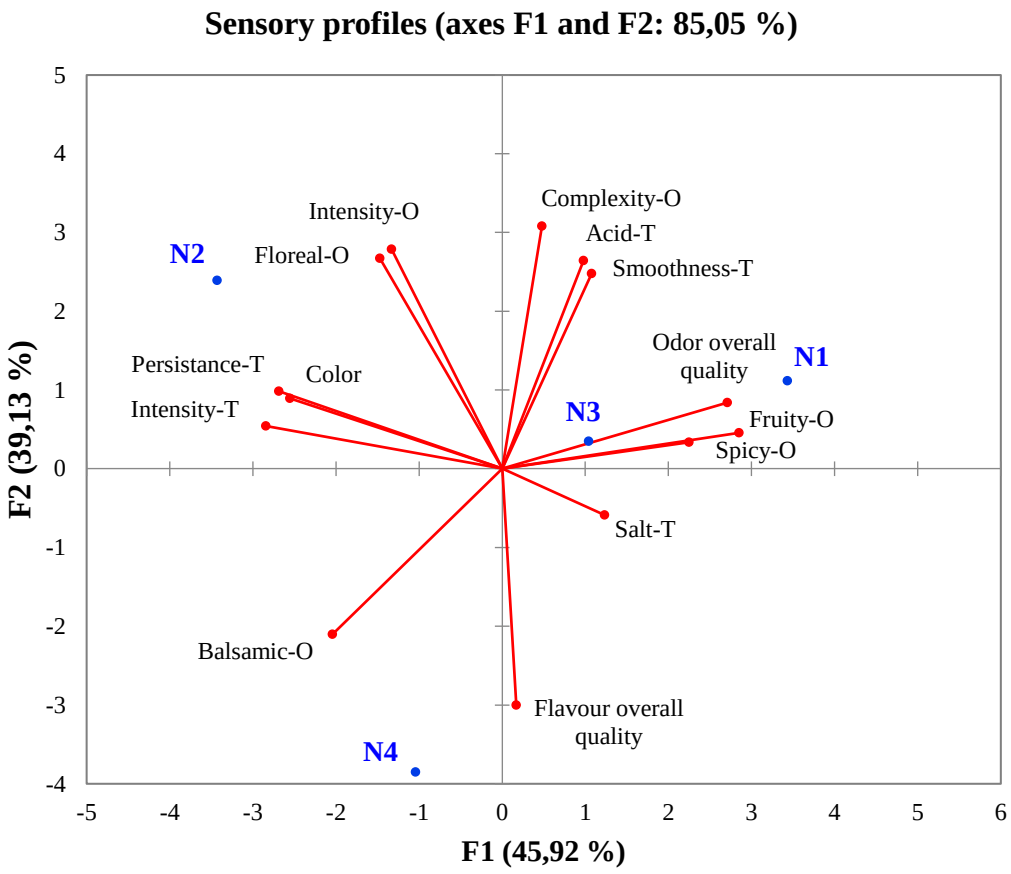

Figure 5. Sensory profiles graph showing the distribution of different Frappato wine in relation to the taste and odour attributes. Codes: N1, *sequential inoculum Starmerella lactis-condensi* MN412/ *Saccharomyces cerevisiae* NF213; N2, sequential inoculum *Candida oleophila* YS209/ *Saccharomyces cerevisiae* NF213; N3, sequential inoculum *Starmerella bacillaris* Cz3/ *Saccharomyces cerevisiae* NF213; N4, single inoculum *Saccharomyces cerevisiae* NF213.

The biplot graph correlates the attributes of wines variables that explained 85.05% of the total variability as function of factor 1 (45.92%) and 2 (39.13%). This graph reveals a clear grouping of the wines into 3 clusters. In the first quadrant, the wines from the trial N1 and N3 were correlated with the attributes of taste (acid and smoothness), odour (complexity, fruity and spicy) and odour overall quality. In the third quadrant, trial N4 wine was associated with balsamic odour attribute. In the last quadrant, trial N2 wine was strongly associated with colour, intensity, and persistence of taste, also for floreal and intensity odour. In all trials, Frappato wines showed different sensory profiles. The wine from trial N1 and N2 wine produced with *S. lactis-condensi* and *C. oleophila* were of considerable interest and showed high scores for most of the descriptors of sensory evaluation.

3.4 CONCLUSION

In this research, for the first time, non-*Saccharomyces* yeast isolated from manna and honey by-products were applied to winemaking process. The effect of *S. lactis-condensi* and *C. oleophila* in sequential inoculation with *S. cerevisiae* on the oenological parameters of Frappato wines and the VOCs composition was evaluated in comparison with the non-*Saccharomyces* control strain *S. bacillaris*, previously used in industrial winemaking. Very low production of acetic acid by *C. oleophila* were found in contrast to other author reports.

An increment of glycerol content was registered in wine produced with mixed culture of *S. bacillaris*/*S. cerevisiae* that increased the final smoothness of wines. VOC profiles confirmed wine differences especially for esters that, due to fruity aroma, contributed to the peculiar definition of the resulting wines, mainly by *C. oleophila.* The sensory analysis confirmed that the use of *S. lactis-condensi* and *C. oleophila* influenced positively the final wines in terms of fruity and floral intensity while did not generate sensory defects.

This work reports scientific data on the role of two novel non-conventional yeast species, *S. lactis-condensi* and *C. oleophila*, as potential co-starters to modulate the aromatic and organoleptic profile of wines. Novel information has been also showed on VOCs composition of a Frappato red wine cultivated in southern of Italy.

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CHAPTER 4

Effects of different yeast strains, nutrients and glutathione-rich inactivated yeast addition on the aroma characteristics of Catarratto wines

ABSTRACT

Catarratto is one of the most common non-aromatic white grape varieties cultivated in Sicily (Southern Italy). In order to improve the aromatic expression of Catarratto wines a trial was undertaken to investigate the effect of yeast strain, nutrition and reduced glutathione. Variables included two *Saccharomyces cerevisiae* strains, an oenological strain (GR1) and one isolated from honey by-products (SPF52), three different nutrition regimes (Stimula Sauvignon Blanc™ (SS), Stimula Chardonnay™ (SC) and classic nutrition practice), and a specific inactivated yeast rich in reduced glutathione to prevent oxidative processes [Glutastar™ (GIY)] ensuing in ten treatments (T1-T10).

Microbiological and chemical parameters demonstrated the aptitude of strain SPF52 to successfully conduct alcoholic fermentation. During fermentation, the *Saccharomyces* yeast populations ranged from 7-8 logarithmic CFU/mL. All wines had a final ethanol content ranging between 12.91 and 13.85 % (v/v). The dominance of the two starter strains over native yeast populations was higher than 97% as estimated by Interdelta analysis. The addition of nutrients SS or SC increased the aromatic complexity of the wines as reflected by volatile organic compounds (VOCs) composition and sensory profiles. In particular, 32 VOCs were identified; alcohols (62.46-81.1 %), thiols (0.27-0.87 %), ethers (0.09-0.16%), aldehydes (0-1.21%), ketones (0-2.28 %), carboxylic acids (4.21-12.32 %), esters (0-10.85%), lactones (0.9-1.49 %) and other compounds (0.77-6.9 %). Sensory analysis demonstrated a significant impact on wine aroma in relation to yeast starter strain used, the type of nutrition (SS, SC or classic nutrition) and the presence/absence of GIY. The wines produced with GR1 yeast strain and SS (T2), SPF52 with SC (T9) both in presence of GIY showed higher overall quality. Trials T2 and T9 showed the highest scores for 13 and 18 attributes, respectively. The different nutrition, addition of GIY and the yeast starter strains diversified and enhanced sensory expression of Catarratto wines.

4.1 INTRODUCTION

Sicily is the largest Italian wine region accounting for about 17.5% of the overall Italian wine production (Fracassetti et al., 2018). In this region, approximately 100,000 hectares of cultivated land are vineyards. Furthermore, Sicily has an ancient wine tradition and contributes to make Italy one of the three leading European countries for wine production. Among the white grape varieties, Catarratto is the most cultivated grape cultivar in Sicily (Carimi et al., 2010) and the second most cultivated in Italy (Robinson et al., 2013). Catarratto wines have a moderate alcohol by volume and a significant total acidity with variable pH values (Sannino et al., 2013). These parameters are variable according to the altimetry of the viticultural areas, in particular in hilly zones Catarratto wines show high values of total acidity, malic acid and low pH. Wines produced with this grape variety have a sufficient olfactory intensity, particularly characterized by descriptors of orange blossom and citrus fruits (Leder, 2020). From a gustatory point of view, Catarratto wines are commonly sapid with a long finish (Sannino et al., 2013). However, there is limited knowledge on Catarratto wine aroma, physicochemical and microbiological characteristics (Fracassetti et al., 2018; Sannino et al., 2013).

Aroma is one of the principal wine attributes influencing wine consumer preferences (Mouret et al., 2015). The majority of fruity/floral aroma compounds are produced by yeast during alcoholic fermentation (AF) and their synthesis can be significantly influenced by oenological practices such as clarification, aeration, nutrient addition and fermentation temperature (Hernandez-Orte et al., 2006; Torrea et al., 2011). Moreover, the aromatic profile of wine is also influenced by the *Saccharomyces cerevisiae* strain used as a starter to conduct AF (Lambrechets and Pretorius, 2000). Indigenous yeast represents an important resource in winemaking; numerous *S. cerevisiae* strains isolated from grape berries and spontaneously fermented musts are being used in winemaking (Cappello et al., 2004). In order to expand the choice of *S. cerevisiae* strains able to enrich the aromatic complexity of wines, their isolation from natural matrices not related with winemaking is becoming a common practice; some studies regarding the ecology of *S. cerevisiae* demonstrated that this species is present in natural sugar matrices such as manna (Guarcello et al., 2019), honey (Carvalho et al., 2005), honey by-products (Gaglio et al., 2017), fruits (Lee et al., 2011), and nectar (Dandu and Dhabe, 2011); *S. cerevisiae* isolated from honey have a high fermentative capacity and can be used for alcoholic mead production (Pereira et al., 2009). Several studies have evaluated the performance of oenological *S. cerevisiae* strains in mead production (Pereira et al., 2013; Pereira et al., 2014; Sottil et al., 2019), however, none have focused on using *S. cerevisiae* strains isolated from honey for winemaking.

Nitrogen is important for an efficient fermentation and the synthesis of various yeast-derived volatile compounds (Barbosa et al., 2012). Grape juice/must contain assimilable nitrogen in different forms, inorganic (ammonium) and organic (amino acids and peptides), which are assimilated differently by

yeast (Ayestaran et al., 1995). Yeast nutrition management during fermentation is important for the wine aroma profile (Molina et al., 2009), and is commonly supplemented with diammonium phosphate, or yeast derivate nutrients to prevent problems related to nitrogen deficiency, such as slow/stuck fermentations and H_2S production (Vilanova et al., 2007).

To prevent loss of aroma, wine must be protected against oxidation at the earliest stages of the winemaking process and can be achieved via the addition of natural antioxidant compounds, such as glutathione (L-g-glutamyl-L-cysteinyl-glycine) (Kritzinger et al., 2013). Glutathione is a tripeptide, which contains three constitutive amino acids, glutamate, cysteine and glycine, formed from the natural metabolism of yeast. In wine, glutathione can be present as a reduced (GSH) or oxidized form (GSSG). Glutathione is important in wine in its reduced form because it can scavenge orthoquinones responsible for browning and aroma loss due to oxidation mechanisms (Lavigne et al., 2007). It is well known that GSH is a more potent antioxidant than ascorbic acid (Cojocaru and Antoce, 2016). The International Organisation of Vine and Wine (OIV) has recently adopted and incorporated a monograph (OIV-OENO 603-2018) on inactivated yeasts with guaranteed glutathione levels into the international oenological codex.

In order to better investigate of the effect of the nutritional management of yeasts during AF and the use of antioxidant compounds on wine aroma composition, in the present research, two yeast strains isolated from different ecological niches (grape and honey), two yeast nutritional managements and the addition of glutathione-rich inactivated yeast on the aroma composition and sensory quality of Catarratto wine were evaluated.

4.2 MATERIALS AND METHODS

4.2.1 Experimental design and sample collection

The experimental plan consisted of three variables: (i) addition of Glutastar™ inactivated yeast (GIY) as antioxidant; (ii) addition of Stimula Sauvignon Blanc™ (SS) and Stimula Chardonnay™ (SC) as yeast nutrient supplementation; and (iii) the inoculation of two yeast starters strains (GR1 and SPF52), conducted in duplicate (Fig. 1).

GIY is an inactivated yeast with a guaranteed glutathione level and also rich in other nucleophilic peptides (Bahut et al., 2020). SS and SC are organic nutrients, consisting of yeast autolysates formulated to provide optimal levels of amino acids, sterols, vitamins and minerals to promote the aromatic metabolism of yeasts; SS contains pantothenate, thiamine, folic acid, zinc and manganese and is formulated to improve volatile thiols, while SC contains biotin, vitamin B6, magnesium and zinc and is formulated to optimize the biosynthesis of volatile esters. GIY, SS and SC were provided by Lallemand Inc. (Castel D'Azzano, Verona, Italy).

Saccharomyces cerevisiae yeast strain GR1 and SPF52 belong to the oenological yeast collection of the Department of Agricultural Food and Forestry Sciences (SAAF) (University of Palermo. Italy). The strain GR1 was isolated from grapes (Francesca et al., 2010) and is used in industrial winemaking, while the strain SPF52 was isolated from fermented honey by-products (Gaglio et al., 2017) and selected for its high performance to ferment grape must.

Grapes of the "Catarratto bianco lucido" cultivar were harvested from a vineyard located in San Giuseppe Jato (37°59'20'' N; 13°11'34'' E, Palermo, Sicily, Italy) in the 2019 vintage. Wine production was conducted at "Cantina Sperimentale G. Dalmasso" of the Istituto Regionale del Vino e dell'Olio (IRVO) winery located in Marsala (Trapani, Sicily, Italy), Di Bella Vini s.r.l. winery and Azienda Agricola Buonivini (San Giuseppe Jato, Palermo, Italy).

Samples were collected during grape harvest, from clarified bulk must, just after yeast starter inoculation, during AF (day 3, 6, 12 and 18), ageing in steel vat (1, 3 and 5 months) and at bottling. All samples were transported at 4 °C in a portable fridge and subjected to analysis within 24 h from collection.

4.2.2 Winemaking process and monitoring

The grapes were manually harvested, and stemmer-crushed. Potassium metabisulphite (5g/hL) was added to the bulk must and clarified into stain less-steel tank by cold settling for 24 h in presence of pectolytic enzymes (4 g/hL). The clarified bulk must was divided into twenty steel vats (2.5 hL each); each treatment consisted of two 2.5 hl tanks, for a total of 10 experimental treatments (T1 to T10; Fig. 1).

Prior to yeast inoculation, GIY (40 g/hL) was added to treatments T2, T4, T7 and T9 ; nutrient SS (40 g/hL) was added to treatments T1, T2, T6 and T7 ; nutrient SC (40 g/hL) was added to treatments T3, T4, T8 and T9 . Yeasts were inoculated in liquid concentrated form (approx. 7.00×10^{12} colony-forming units (CFU)/g) at 20g/hL, T1 to T5 and T6 to T10 with *S. cerevisiae* strains GR1 and SPF52, respectively.

Treatments 5 and 10 were controls (control-A and control-B, respectively), with no addition of GIY, SS and SC, but received an addition of diammonium phosphate (15 g/hL; Chimica Noto s.r.l., Partinico, Italy). The AF was conducted at 18 °C. At the end of AF, the wines were settled, racked off lees, and transferred into stainless-steel tanks at 15° C, and topped with nitrogen to avoid oxidation up to bottling stage.

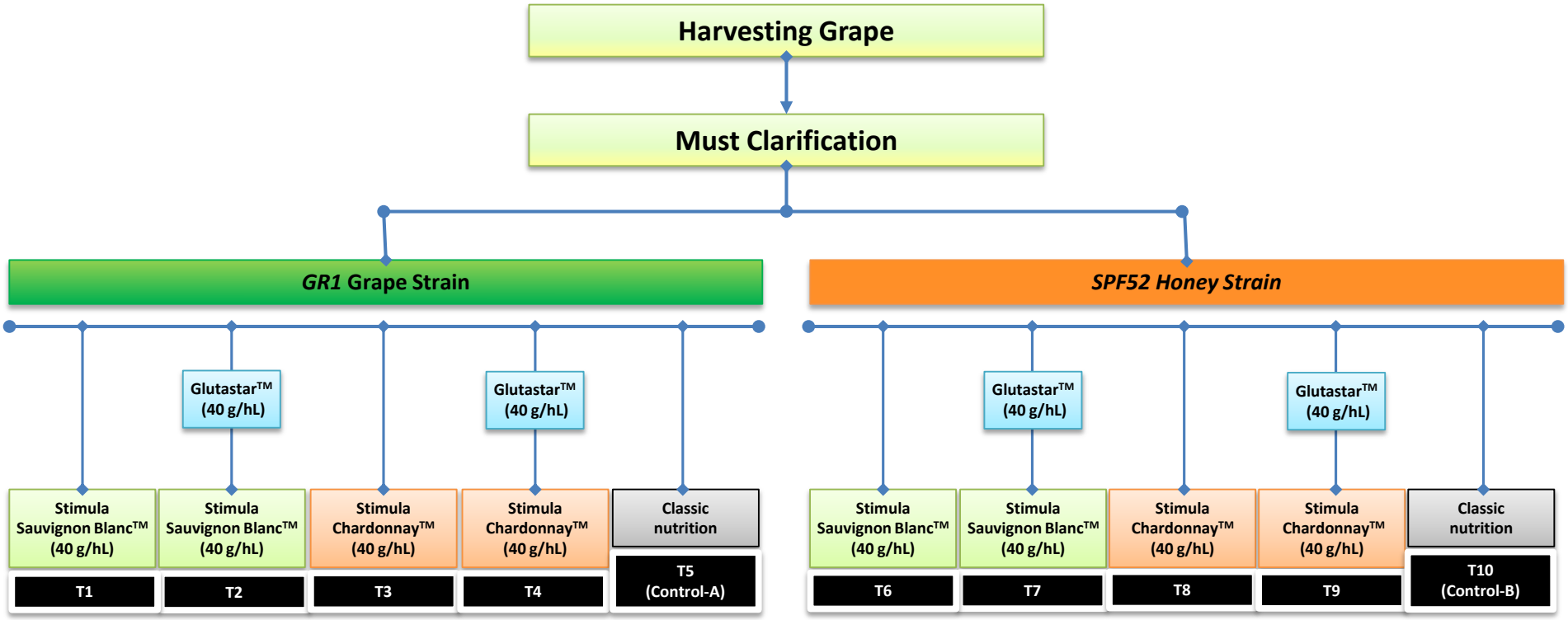

Figure 1. Experimental design of Catarratto wines vinified with different yeast strains, nutrient regime and addition of an antioxidant.

4.2.3 Microbiological analysis

All samples collected during wine production were analysed for yeast and bacteria populations. Must samples were diluted in Ringer's solution (Sigma-Aldrich, Milan, Italy) and analysed in duplicate for total yeasts (TY) on Wallerstein Laboratory (WL) nutrient agar (Pallmann et al., 2001), mesophilic rod lactic acid bacteria (LAB) on de Man–Rogosa–Sharpe agar (Capozzi et al., 2012), coccus LAB on glucose M17 agar (Francesca et al., 2014), acidophilic LAB on medium for *Leuconostoc oenos* agar (Caspritz and Radler, 1983) and acetic acid bacteria (AAB) on Kneifel agar medium (OIV, 2010). All media and supplements were purchased from Oxoid (Thermofisher, Milan, Italy).

4.2.4 Yeast isolation and genotypic identification

Yeasts were isolated from WL medium with at least five colonies per morphology randomly selected from the agar plates. The isolates were purified by successive sub-culturing on WL and their purity was verified under an optical microscope (Carl Zeiss Ltd.). Three isolates (from each sample) with the same morphology were then subjected to genetic characterisation (Cavazza et al., 1992).

Genomic DNA for PCR assays was extracted (Alfonzo et al. 2021) and yeast differentiation was by RFLP using the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene (Settanni et al. 2012). One isolate per group was further analysed by sequencing the D1/D2 region of the 26S rRNA gene to confirm the preliminary identification obtained by RFLP analysis (Alfonzo et al. 2020a). DNA sequencing reactions were performed at AGRIVET (University of Palermo, Italy). The sequence identity was determined by BlastN search against the NCBI non-redundant sequence database (http://www.ncbi.nlm.nih.gov). Sequences were manually corrected using Chromas 2.6.2. (Technelysium Pty Ltd, Australia).

4.2.5 Strain typing of S. cerevisiae *isolates*

In order to verify the dominance of GR1 and SPF52 strains during AF, all isolates at the highest cell concentration were characterized by Interdelta analysis. Genetic diversity within *Saccharomyces* isolates was assessed by Interdelta analysis (Legras and Karst, 2003). Interdelta patterns were analysed using the GelCompar II software (v. 6.5. Applied-Maths, Sin Marten Latem, Belgium) and similarities among patterns were assessed; profiles showing more than 95% of similarity were considered identical.

4.2.6 Physicochemical analysis

4.2.6.1 Wine composition

Enzymatic assays for glucose, fructose, ethanol, glycerol, ammoniacal nitrogen, alpha-amino nitrogen and acetic acid were conducted on a iCubio iMagic M9 (Shenzhen iCubio Biomedical

Technology Co. Ltd. Shenzhen, China) as described by Barbaccia et al. (2021). The reagents were purchased from R-Biopharm AG (Darmstadt, Germany).

Residual sugars were determined with a WineScan (FOSS, Hillerød, Denmark) calibrated following UNI CEI EN ISO/IEC 17025, 2018.

The pH was determined by OIV-MA-AS313-15 method (OIV, 2020a), total acidity was determined by the methodology described by OIV-MA-AS313-01 (OIV, 2020b), and free and total sulphur dioxide were measured in accordance with the methods described by OIV-MA-AS323-04B (OIV, 2020c). All chemical analyses were carried out in triplicate.

4.2.6.2 Oenological-Chemical parameters

Wine samples were analysed for total extract, total phenols, total acidity and buffering power as described by CEE, 2676/90, ash alkalinity following the methodology of Usseglio-Tomasset (1995), flavans reactive to p-dimethylamino cinnamaldehyde (Di Stefano et al., 1989), absorbance at 420 nm by spectrophotometer (UV-1601-Shimadsu) and the oxidation test (POM test) as described by Müller-Späth (1992). All analyses were carried out in duplicate.

4.2.6.3 Volatile organic compounds

Volatile compound composition was determined following the protocol described by Reddy and Dillon (2015). Wine samples (10 mL) from all trials were mixed with MS SupraSolv® dichloromethane (10 mL) in a 100-mL conical flask, stirred at room temperature for 30 min, and centrifuged at 3000 rpm for 10 min by Low Speed Centrifuge (ScanSpeed 416) with Swing Rotor (LaboGene ApS Industrivej 6-8, Vassingerød, DK-3540 Lynge, Denmark). The aqueous phase was removed and was added anhydrous sodium sulphate (1 g) before centrifuging at 3000 rpm for 10 min. The dichloromethane layer was removed and dried under N_2 gas to 1 mL.

Gas chromatographic analyses were performed in two different GC-MS apparatus with two different columns. The first one was an Agilent 7000C GC system, fitted with a fused silica Agilent DB-5MS capillary column (30 m × 0.25 mm i.d.; 0.25 μm film thickness), coupled to an Agilent triple quadrupole Mass Selective Detector MSD 5973; ionization voltage 70 eV; electron multiplier energy 2000 V; transfer line temperature, 295 ˚C. Solvent Delay: 5 min. Helium was the carrier gas (1 mL/min). The second apparatus was a Shimadzu QP 2010 plus equipped with an AOC-20i autoinjector (Shimadzu, Kyoto, Japan) and with a Supelcowax 10 capillary column (30 m x 0.25 mm i.d.; 0.25 μm film thickness); ionization voltage 70 eV; transfer line temperature, 280 ˚C. Helium was the carrier gas (1 mL/min). For both columns, the temperature was initially kept at 40 ˚C for 5 min. Then gradually increased to 250 ˚C at 2 ˚C/min rate. Held for 15 min and finally raised to 270 ˚C at

10 ˚C/min. One µL of sample was injected at 250 ˚C automatically and in the splitless mode; transfer line temperature, 295 ˚C.

The individual peaks were analysed using the GC MS Solution package, Version 2.72. Identification of compounds was carried out using Adams, NIST 11, Wiley 9 and FFNSC 2 mass spectral database. These identifications were also confirmed by other published mass spectra and linear retention indices (LRI). The LRI were calculated using a series of *n*-alkanes (C8-C40). In addition, some of the compounds were confirmed by comparison of mass spectra and retention times with standard compounds available at the Department STEBICEF – University of Palermo.

4.2.7 Sensory evaluation

Sensory evaluation of experimental wines consisted of two steps: (*i*) sensory acceptance tests performed by consumers and (*ii*) quantitative descriptive analyses carried out by panellists to define aroma and sensory profiles. The sensory assessments were performed as described by Alfonzo et al. (2020b).

4.2.7.1. Acceptance test

Samples of experimental wines were evaluated for their overall acceptability (Biasoto et al., 2014; Villanueva and Da Silva, 2009). A total of 87 consumers were recruited from the University of Palermo; lecturers, researchers, technicians and graduate students were invited to take part by filling in a recruitment form, and from a group of 25 habitual consumers of white wine 13 women and 12 men whose ages ranged from 21 to 42 years were selected. The selection criterion of the subjects was the consumption of at least one glass of white wine per week with no experience on wine sensory analysis.

All the consumers evaluated the overall acceptability of the 10 wine samples using a hybrid hedonic scale of 10 cm which included three points: dislike extremely (0), neither like or dislike (5) and like extremely (10). The ten wine samples were evaluated in two separate tasting sessions and carried out over two successive days. The effects of the presentation order and first-order carry-over of the samples were controlled using the crossover design (Wakeling and MacFie 1995).

4.2.7.2. Quantitative descriptive analyses

Sixteen judges (9 women and 7 men, ranging from 23 to 41 years old) were recruited from Oenologist Associations: National Organization of Wine Taster (ONAV, Italy), Italian Sommelier Association (AIS, Italy) and University of Palermo. All had experience in winemaking and participated in previous studies as sensory judges.

The judges were subjected to preliminary tests to determine their sensory performances on basic tastes and the aromas associated with wines. The sensory profiles (ISO 13299, 2016) of the Catarratto wines

were constructed using two selected panels (ISO/CD 8586, 2019) each of eleven judges trained over several sessions.

The sensory analysis of wine was conducted following the methodology by Jackson (2016).

The 16 panellists compared the ten experimental wines during different sessions. They consensually generated 50 sensory descriptive attributes regarding appearance, odour, flavour, taste, overall quality, and finish over several sessions. The set of attributes were: appearance (yellow colour, green reflexes); odour (intensity, persistence, floral, orange flowers, fruity, peach, apricot, plum, green apple, citrus fruit, grapefruit, tropical fruit, pineapple, banana, tamarind, small fruit, strawberry, liquorice, caramel, honey, wax, bread crust, box tree and cat pee); gustatory taste (sweet, sour, salty and bitter); mouth-feel (body or balance); flavour (intensity, persistence, floral, fruity, citrus fruit, tropical fruit, caramel, honey, box tree and cat pee), overall quality (overall quality, odour, taste, mouth-feel and flavour) and finish (after-smell and after-taste).

The panellists were also trained for the identification of wine off-odors and off-flavour: microbial (mouldy, corky, yeasty, buttery and cheesy); pungent (vinegary, alcoholic, sulfur); putrid (rancid, rotten egg, rubbery); petroleum (fusel, plastic, solvent), other (Issa-Issa et al. 2020; Jackson, 2016).

The panellists also generated a consensual descriptive ballot for the wines in which the descriptors were associated with a 9 cm unstructured scale anchored at the left and right extremes with the terms “none/weak” and “strong”, respectively (Biasoto et al., 2014; Jackson, 2016).

The ten wine samples were evaluated in distinct tasting sessions carried out on successive days. Overall, each judge evaluated each of the ten wines with three repetitions. For each repetition, a different wine bottle was opened. To control the contrast effect amongst the samples an incomplete balanced block design was used (Cochran and Cox 1957).

4.2.8 Statistical and explorative multivariate analyses

ANOVA test was applied to identify significant differences among chemical parameters determined during the winemaking process (pH, total acidity, volatile acidity, residual sugars, glucose, fructose, alpha amine nitrogen, ammoniacal nitrogen, ethanol, glycerol, malic acid and lactic acid), microbiological analysis (*Saccharomyces* and non-*Saccharomyces* microbial counts), oenological chemical parameters (total extract, total phenols, p-DACA flavans, absorbance, oxidation n test, buffering power and ash alkalinity) and sensory analysis (acceptance test and quantitative descriptive analyses). The post-hoc Tukey’s method was applied for pairwise comparison of all data. Statistical significance was attributed to $P < 0.05$ (Mazzei et al., 2010).

An explorative multivariate approach was employed to investigate relationships among data obtained during AF (ammoniacal nitrogen, alpha-amino nitrogen, ethanol, fructose, glucose, glycerol, malic acid, pH, residual sugars, total acidity and volatile acidity) from the different treatments.

The agglomerative hierarchical clustering (AHC) and principal component analysis (PCA) of data were performed to investigate relationships among treatments.

To graphically represent the VOCs concentrations, a heat map clustered analysis (HMCA), based on hierarchical dendrogram with heat map plot, was employed to show the individual content values contained in the data matrix as colours (Martorana et al., 2017). The relative values of VOCs concentration were depicted by colour intensity from yellow (lowest quantity) to red (highest quantity). Heat map analysis of the volatile levels was performed using the autoscaled data (Gaglio et al., 2017). The heat map was generated using ascendant hierarchical clustering based on Ward's method and Euclidian distance to show the similarities between VOCs and wine obtained with different yeast starter strains and nutrition regimes.

Multiple factor analysis (MFA) was performed on the data matrix consisted of 10 rows (trials) × 50 columns (50 attributes for sensory analysis) to explore the correlation between variables and different treatments, as well as discrimination among the treatments. Agglomerative hierarchical cluster analysis (AHCA) was also performed on the same data matrixes MFA to explore the variations and similarities of the treatments in relation to the sensory analysis.

Statistical data processing and graphic construction were performed with the XLStat software version 2020.3.1 (Addinsoft, New York, USA) for Excel.

4.3 RESULTS AND DISCUSSION

4.3.1 Dynamics of Saccharomyces *spp. and non-*Saccharomyces *populations*

The levels of *Saccharomyces* and non-*Saccharomyces* yeast populations are extremely important to understand the selective effect of the different nutrients used in the different treatments with starter yeasts in Catarratto wines. Adequate yeast nutrition contributes to improve the quality factors that can affect the value of wine (Bell and Henschke, 2005).

The yeast populations during fermentation are shown in Fig. 2. Presumptive *Saccharomyces* (PS) and non-*Saccharomyces* (NS) yeast populations were 4.1 Log CFU/mL (Fig. 2a) and 3.7 Log CFU/mL (F respectively, in the Catarratto must. These concentrations are comparable with those reported in other studies (Scacco et al., 2012). *Saccharomyces cerevisiae* strains (GR1 and SPF52) were inoculated between 7.1 and 7.7 Log CFU/mL, and the initial ratio of PS/NS were between 2 and 2.5. Initial PS values were slightly higher than those reported by Scacco et al. (2012), where PS levels in Catarratto ranged between 5.8-6.3 Log CFU/mL with an initial *Saccharomyces*/non-*Saccharomyces* ratio between 1.7 and 4.6. After 3 days of AF, an increase of PS population up to 7.6 - 8.0 Log CFU/mL was observed for all treatments, whereas NS yeasts showed values in the range of 2.1-3.9 Log CFU/mL. Maximum PS levels were similar to those reported in the literature (7.9-8.1 Log CFU/mL), in fact, Scacco et al. (2012) in fermenting Catarratto musts observed the maximum concentration

from 4 to 9 days after the start of AF. At the 6th day of AF, PS concentrations were observed at 7 Log CFU/mL and this trend was also observed until the 12th day. In this case, SS and SC showed typical growth kinetics for both starter yeasts. In contrast, NS populations were undetectable from the 6th day of AF onwards. This would be attributed to the increase in ethanol content, competition with *Saccharomyces* yeast and the reduction in growth factors (Morata and Loira, 2019). By the end of AF, a reduction of PS levels was observed for all treatments. At the end of AF, PS concentrations were between 6.6-7.0 Log CFU/mL, and continued to decline during aging in steel until bottling to 2.0 Log CFU/mL.

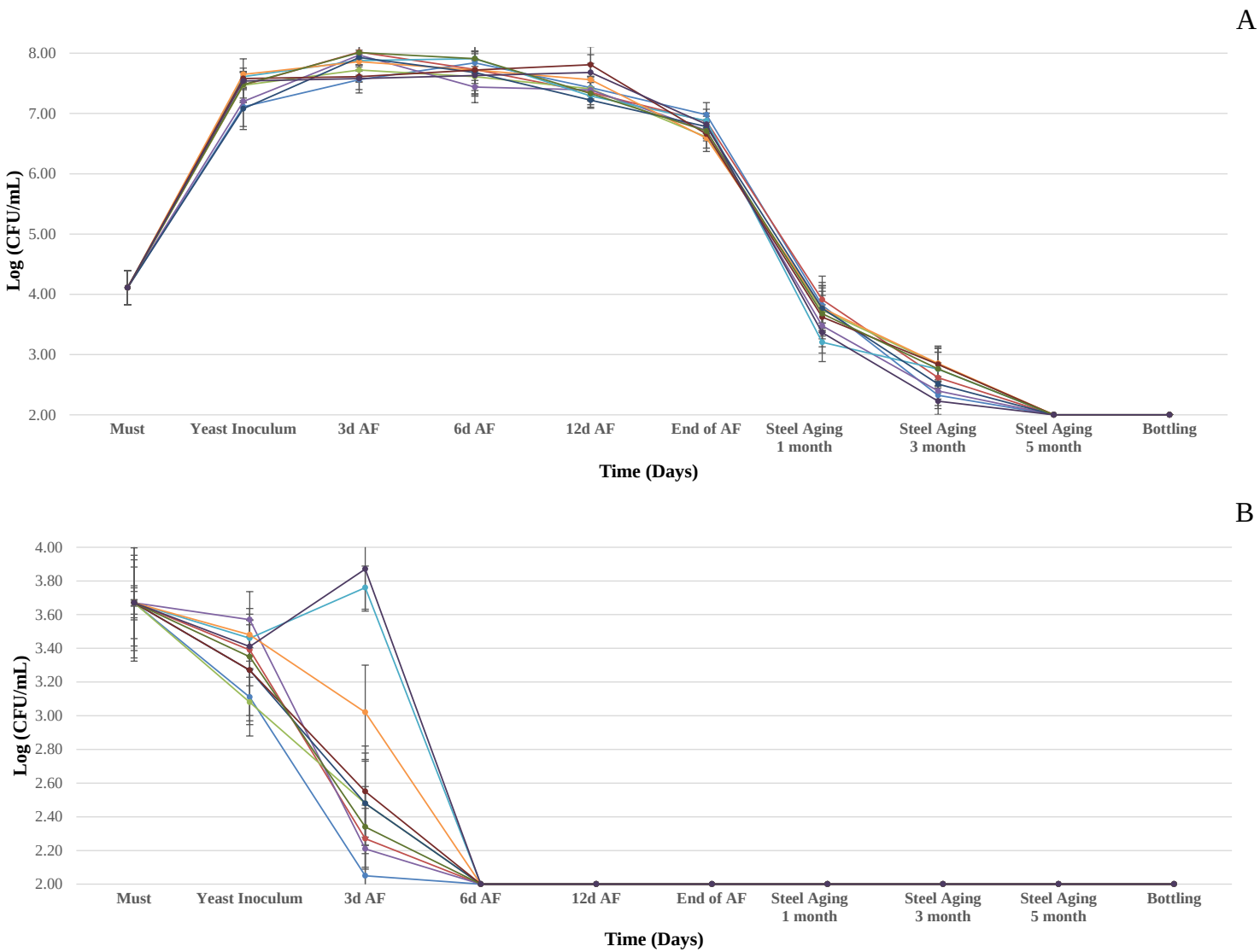

Figure 2. Yeast population (Log CFU/mL) evolution during alcoholic fermentation and wine storage: (A) Presumptive *Saccharomyces*; (B) non-*Saccharomyces*. Legends: ●, T1; ●, T2; ●, T3; ●, T4; ●, T5 (Control-A); ●, T6; ●, T7; ●, T8; ●, T9; ●, T10 (Control-B).

4.3.2 Molecular analysis of yeast

A total of 3084 yeast colonies from WL media were isolated, purified to homogeneity and separated on the basis of WL colony morphology and 2767 isolates shared the morphological characteristics of *Saccharomyces*. Furthermore, analysis of 5.8S-ITS amplicons showed that all these isolates had the typical *Saccharomyces* spp. 5.8S-ITS region of 800 - 900 bp (White et al., 1990). The other isolates were assigned into the non-*Saccharomyces* yeast group, since their ITS amplicon sizes were different from 800-900 bp. All PS were further examined by restriction analysis of 5.8S-ITS region and directly identified as *S. cerevisiae* by comparing their restriction bands with those available in literature (Cordero-Bueso et al., 2011a, b; Esteve-Zarzoso et al., 1999). For each RFLP group, one isolate was subjected to sequencing of D1/D2 domain the 26S rRNA gene that successful confirmed the species identification. Interdelta profiles indicated that 22 different *S. cerevisiae* strains were isolated at the highest cell densities from the ten treatments. The direct comparison of the Interdelta profiles showed that *S. cerevisiae* GR1 and SPF52 were the strains most frequently (>97%) isolated, and thus demonstrated the dominance of the starter strains GR1 and SPF52 during AF. This is consistent with observations in the literature where the same approach to monitor the persistence and evaluate the dominance of the inoculated strains was used (Alfonzo et al., 2020b; Xufre et al., 2011).

4.3.3 Alcoholic fermentation

The conversion of grape sugars to alcohol by yeast is of course fundamental to winemaking and through their metabolism the production, various aromatic compounds the final wine quality and nuances are achieved (Swiegers et al., 2005). Consequently, nutrients are key compounds both to support yeast growth and to ensure a regular and complete fermentation (Lambrechts and Pretorius, 2000).

The main wine composition parameter are shown in Table S1. The final wine compositions are in agreement with that predicted from the initial grape must composition. The residual sugar varied between the treatments with *S. cerevisiae* SPF52 having slightly more than GR1. Final ethanol content was variable in the different treatments (13.64-14.02 % (v/v)). Considering the initial sugar content of the must of Catarratto grapes (221.50 g/L) and the final ethanol content, these values are similar to those reported in the literature by Fracassetti et al. (2018) who predicted the use of the commercial strain of *S. cerevisiae* 20 CRU611 on musts with similar chemical characteristics. Glycerol produced by GR1 and SPF52 yeast strains ranged between 5.98 and 6.68 g/L: at these concentrations, glycerol contributes to the viscosity and softness of the wine, with a positive effect on its taste (Noble and Bursick, 1984). No statistical significance was found for total acidity in all treatments (5.36 to 5.40 g/L) and was slightly lower than that described by Fracassetti et al. (2018) and Scacco et al. (2012) who reported values more than 6 g/L of tartaric acid. The volatile acidity was variable between the

treatments, but at the end of AF, values of 0.31 g/L were found for all the wines. This concentration is mainly due to the acetic acid produced by the yeast during AF, and the values were equivalent to those observed by other studies in Catarratto wines produced in Sicily (Fracassetti et al., 2018; Sannino et al., 2013; Scacco et al., 2012). Slight differences were observed for malic acid content (1.26 and 1.59 g/L). Lactic acid was not detected in any treatment. The free SO_2 and total SO_2 values ranged between 30-32 mg/L and 80-85 mg/L, respectively.

The effect of different strains of *S. cerevisiae* strains (GR1 and SPF52), chemical parameters (ammoniacal nitrogen, alpha-amino nitrogen, ethanol, fructose, glucose, glycerol, malic acid, residual sugars, total and volatile acidity), nutrient strategy (SS and SC) and the presence/absence of antioxidant compounds (Glutastar^{TM}) in the final wines was evaluated by a multivariate statistical analysis approach (Fig. 3).

Agglomerative hierarchical clustering (AHC) classified the trials in accordance with their mutual dissimilarity and relationships (Fig. 3a). This analysis classified the trials using ten variables selected on the basis of the results from chemical monitoring. All treatments were clearly separated into three clusters with a dissimilarity of 15%. The most numerous clusters was cluster 3 which included six treatments (T2, T3, T4, T5, T6 and T7). Whereas cluster 2 and cluster 1 were represented by the T8-T10 and T1-T9 trials, respectively. Cluster 1 was the most heterogeneous and this is confirmed by the Within-class variable. The variables that greatly impacted trial clusterization were different for each cluster. The trend of pH, total acidity, ethanol and malic acid during AF generated cluster 1. Glycerol was the variable that most discriminated against cluster 2, while cluster 3 was obtained by volatile acidity, residual sugars, glucose, fructose and α-amino nitrogen.

The biplot illustrated in Fig. 3b highlights the distribution of the different treatments in relation to the same chemical parameters used for AHC analysis. Treatments T8 and T10 clustered into one group that was statistically correlated with α-amino nitrogen, malic acid and volatile acidity. Treatments T3 and T6 were associated with fructose, glucose and residual sugars. On the other quadrant of biplot, T2, T4, T5 and T7 grouping was driven by ammonium nitrogen and glycerol. Finally, treatments T1 and T9 were related with ethanol, pH and total acidity.

This behaviour is in agreement with several authors who observed that the use of different yeast strains, subjected to different nutritional regimes, can lead to variations in chemical parameters during the AF of must (Julien et al., 2001; Sablayrolles, 2009).

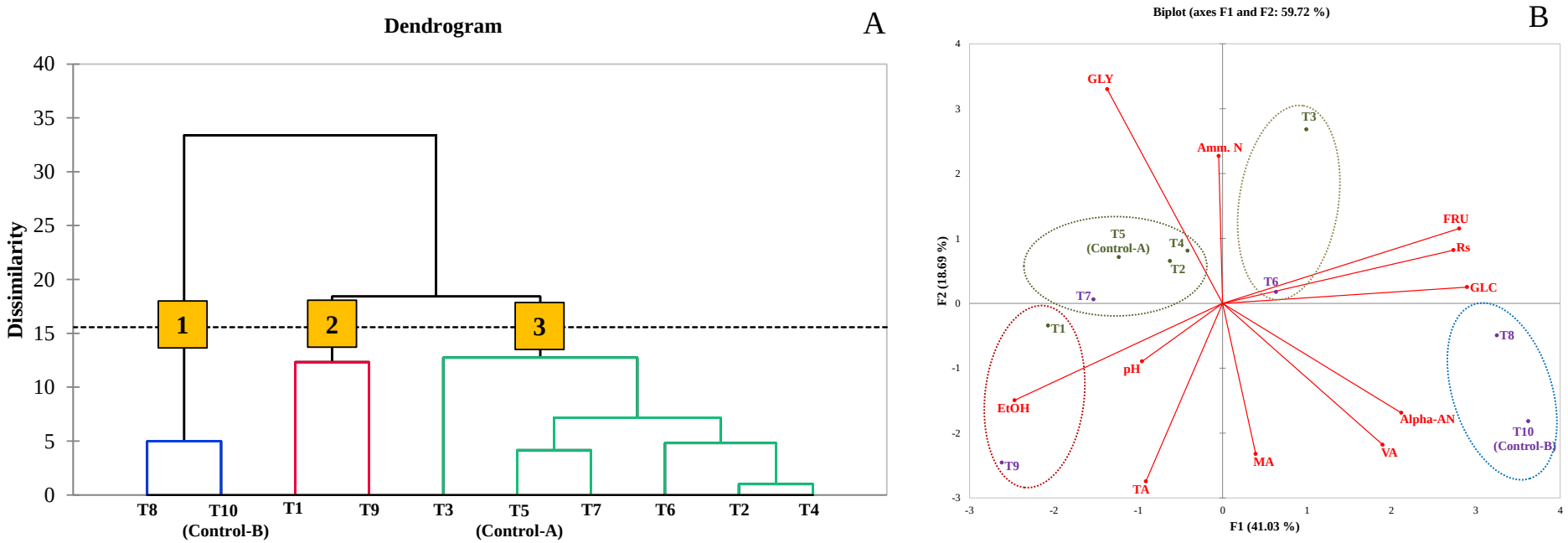

Figure 3. Agglomerative hierarchical clustering (A) and biplot (B) using chemical parameters detected during alcoholic fermentation. Abbreviations: Amm. N, Alpha-AN, alpha-amino nitrogen; EtOH, ethanol; FRU, fructose; GLC, glucose; GLY, glycerol; MA, malic acid; Rs, residual sugars; TA, total acidity; VA, volatile acidity.

4.3.4 Oenological-Chemical parameters

Results from oenological-chemical analysis of wines are shown in Table 1. Treatment wines T1 and T2, produced with GR1 yeast strain, SS and GIY additions, showed the lowest values of wine susceptibility to oxidation by POM test. A high value from POM test characterises the wine potential in preserving the wine phenolics and oxidation potential (Comuzzo et al., 2006).

Table 1. Phenols and oxidation indicators of experimental wines.

Treatment	Total extract (g/L)	Total phenols (mg/L catechins)	*p*-DACA flavans (mg/L catechins)	Absorbance (420nm)	Oxidation n test (POM)	Buffering power (meq/L)	Ash Alkalinity (meq/L)
T1	18.30±0.13^{bc}	103.21±1.66^{a}	21.65±0.17^{b}	0.073±0.02^{a}	6.49±0.04^{b}	34.48±0.59^{b}	13.83±0.25^{b}
T2	18.00±0.31^{bc}	102.26±1.60^{a}	23.63±0.32^{a}	0.074±0.01^{a}	9.09±0.13^{a}	37.04±0.17^{a}	15.61±0.19^{a}
T3	18.10±0.05^{bc}	101.21±0.63^{a}	17.23±0.05^{d}	0.074±0.00^{a}	2.59±0.04^{d}	32.26±0.58^{cd}	12.66±0.08^{c}
T4	18.10±0.09^{bc}	100.54±1.00^{a}	19.22±0.34^{c}	0.074±0.01^{a}	4.81±0.03^{c}	33.33±0.57^{bc}	13.46±0.07^{b}
T5 (Control-A)	16.80±0.16^{d}	102.54±1.40^{a}	19.18±0.14^{c}	0.075±0.01^{a}	4.76±0.06^{c}	29.41±0.57^{fg}	10.82±0.05^{e}
T6	18.20±0.14^{bc}	86.72±0.39^{cd}	1.91±0.03^{f}	0.101±0.02^{a}	0.00±0.00^{e}	31.25±0.09^{de}	12.65±0.10^{c}
T7	19.00±0.10^{a}	88.48±0.26^{bc}	2.81±0.04^{e}	0.091±0.02^{a}	0.00±0.00^{e}	31.25±0.37^{de}	12.65±0.06^{c}
T8	18.40±0.15^{b}	84.72±0.64^{d}	1.47±0.02^{f}	0.100±0.01^{a}	0.00±0.00^{e}	30.30±0.18^{ef}	12.08±0.05^{d}
T9	19.10±0.13^{a}	83.39±1.73^{d}	1.55±0.02^{f}	0.085±0.00^{a}	0.00±0.00^{e}	33.33±0.03^{bc}	13.41±0.23^{b}
T10 (Control-B)	17.80±0.38^{c}	91.48±1.02^{b}	3.10±0.03^{e}	0.105±0.01^{a}	0.00±0.00^{e}	28.57±0.10^{g}	10.36±0.19^{f}
Statistical significance	**	**	**	n.s.	**	**	**

Result indicate mean value ± standard deviation of two determinations.
Data within a column followed by the same letter are not significantly different according to Tukey's test.
P value: **, P < 0.01; n.s., not significant.

In this case, the highest values found in the POM test for T2 (9.09) demonstrates how this typology of wine is able to preserve a determined phenolic component (Voce et al., 2020). In fact, the total polyphenols content was higher in wines made with the GR1 strain (100.54-103.21 mg/L catechins), with and/or without the addition of SS, SC and GIY compared to those obtained with the SPF52 strain (83.39-91.48 mg/ L catechins). In this case, total polyphenol content was found to be nondependent on the presence/absence of glutathione, and the type of nutrition in T1-T5 treatments, whereas variations occurred in T6-T10 treatments fermented with SPF52. Most likely, as suggested by Grieco et al. (2019), the differences could be of microbiological nature. In fact, during the vinification process specific yeast strains are able to produce polysaccharides capable of establishing stable complexes with polyphenols (Brandolini et al., 2007). In addition, the p-DACA flavans content was higher in treatments T1-T5 made with GR1 yeast strain, and the highest values were observed in T1 (21.64 mg/L catechins) and T2 (23.63 mg/L catechins). The content of catechins is also an important quality parameter, to verify the level of oxidation of wine and the influence on color (Katalinić et al., 2004).

Treatments T1 and T2, also stood out from the other experimental wines for their high content in buffering power corresponding to a higher amount of salified acids This might suggest a long

gustatory perception and minerality/acidity taste perceived for these wines (Blouin and Peynaud, 2005). Based on this observation, the use of *S. cerevisiae* GR1 strain and/or GIY (mainly trials 3 and 4) did not reach the same values of trials T1 and T2, as well as with control thesis (T5; classic nutrition) the values obtained were the lowest compared to those observed in the trials T1-T4.

The experimental production obtained by strain SPF52 (T6-T10) showed a similar oenological characteristics and significant differences were found with respect to trials conducted with strain GR1. The oxidation POM-test showed no impact of GIY in these wines; there was no protection of oxidations of the phenolic compounds. Consequently, SPF52 strain treatment wines had lower levels of p-Daca flavans than GR1 wines, thus resulting in an increase of 420 nm optical density.

The highest content of total dry extract was in wines T7 and T9 (19.00 g/L and 19.10 g/L, respectively); all other treatments ranged from 16.80-18.40 g/L. These values are comparable with Catarratto wine studies (20.6-22.1 g/L) (Scacco et al., 2012).

Ash alkalinity was lower in the control wines (T5 and T10), with the highest value in T2 wine (15.61 meq/L). There was no correlation between the yeast strain, nutritional scheme nor the absence or presence of glutathione-rich inactivated yeast.

4.3.5 Volatile organic compound composition

The composition of VOCs of the ten samples is reported in Table 2. More quantitative than qualitative differences were observed in the composition of the ten wines. Thirty-two compounds of different chemical classes (alcohols, thiols, ethers, aldehydes, ketones, carboxylic acids, esters, lactones) were identified, representing more than 90% of total volatile wine components. The esters were classified into different chemical structure families ethyl esters of fatty acids (EEFAs), higher alcohol acetates (HAAs), ethyl esters of branched acids (EEBAs), and miscellaneous esters (MEs) (Puertas et al. 2018).

Alcohols were the most abundant compounds (52.44-80.60%), then carboxylic acids (4.21-12.32%), EEBAs (1.04-10.35%) and HAAs (1.46-6.56%). The most abundant alcohol in all samples (Fig. 4) is 3-methyl-1-butanol (isoamyl alcohol). Both yeast strains, GR1 (grape) and SPF52 (honey), both nutrients (SS and SC) and the presence of the antioxidant GIY, promoted the production of 3-methyl-1-butanol compared to controls. Furthermore, the presence of antioxidants (GIY) significantly increased the amount of 3-methyl-1-butanol in T2, T4 and T7, while T9 displayed only a small increase.

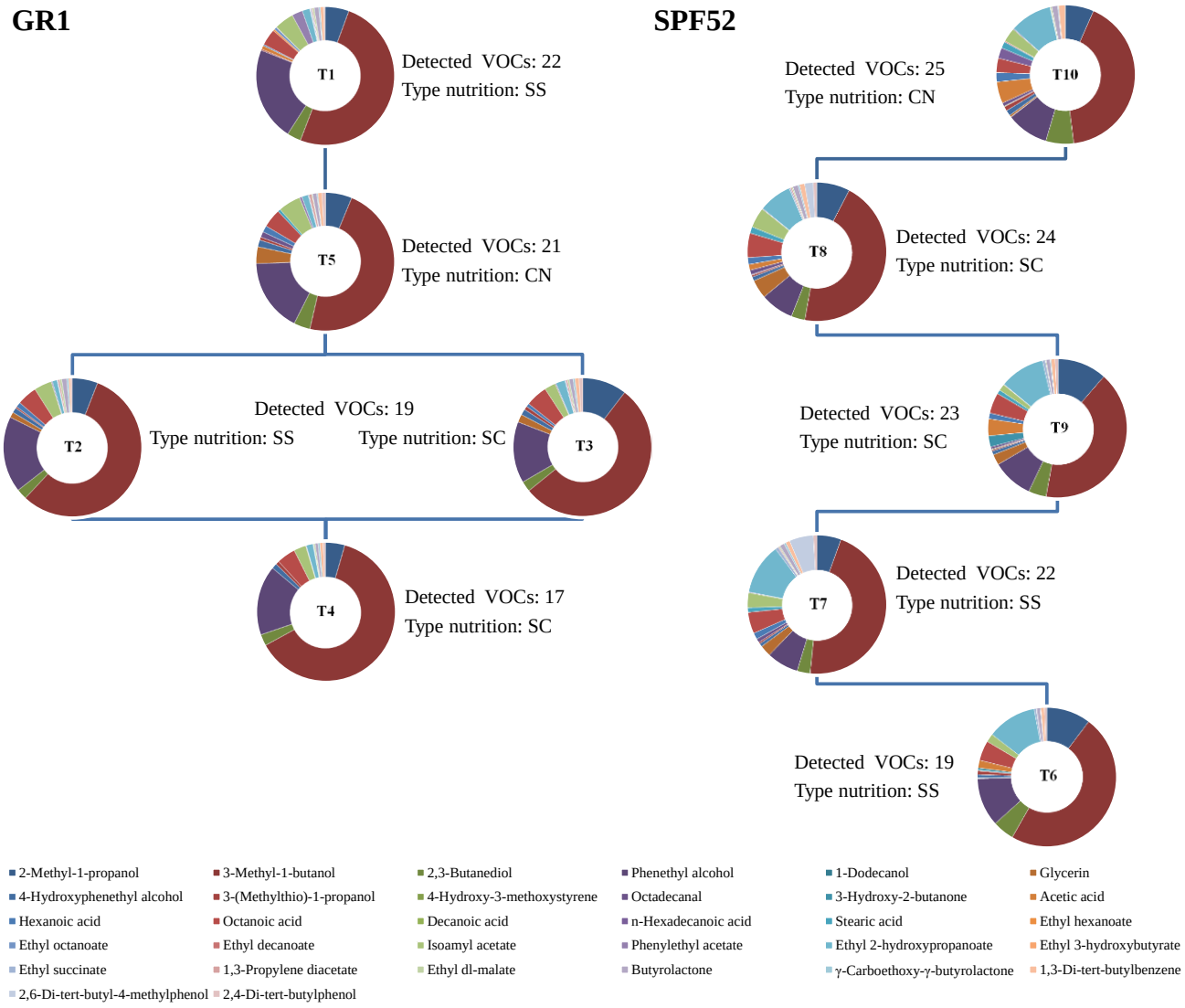

Figure 4. Distribution of wines in relation to the number of volatile organic compounds (VOCs).

Esters influence wine aroma, not only directly but also via complex synergistic interactions. The fermentative strategy immensely affects the total ester content (Puertas et al., 2018). The total amount of esters generated by the honey strain SPF52 was higher than the GR1 grape strain. The most abundant class of esters was EEBAs, with ethyl 2-hydroxypropanoate as the unique compound of this family. It is present in higher quantities in the wines made with SPF52 honey strain, and could be responsible for the caramel and/or honey aroma noted in the sensory analysis of these wines.

Table 2. Volatile organic compounds (VOCs) of wines obtained from treatments T1-T10.

LRI[a]	LRI[b]	Ident.[c]	Compounds	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
			Σ Alcohols										
1076		1. 2	2-Methyl-1-propanol	5.17	5.55	9.66	4.30	5.81	9.22	5.16	7.11	10.15	5.75
1190	736	1. 2	3-Methyl-1-butanol	46.36	52.17	50.18	59.87	43.86	43.06	42.49	41.62	36.99	35.34
1515	808	1. 2	2.3-Butanediol isomer	4.89	3.90	3.72	4.31	5.63	7.23	4.69	5.04	6.11	8.46
1553	913	1. 2	2.3-Butanediol isomer	1.13	0.71	0.82	0.81	1.43	1.74	1.01	0.99	1.34	2.85
1867	1122	1. 2	Phenethyl alcohol	20.34	16.57	13.39	15.62	15.72	10.17	6.93	7.25	8.50	8.50
1939	1387	1. 2	1-Dodecanol	0.00	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.00	0.07
2304	1103	1. 2. 3	Glycerin	0.00	1.19	1.74	0.00	3.60	0.00	2.43	4.07	2.21	0.38
2976	1444	1. 2	4-Hydroxyphenethyl alcohol	0.00	1.09	1.28	1.19	1.49	0.69	0.70	0.90	0.74	1.11
3427	3041	1. 2. 3	Cholesterol	t	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

			Σ Thiols										
1677	988	1. 2	3-(Methylthio)-1-propanol	0.27	0.35	0.80	0.79	0.64	0.80	0.36	0.40	0.29	0.87
			Σ Ethers										
2171	1321	1. 2	4-Hydroxy-3-methoxys Tyrene	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.09	0.16	0.14
			Σ Aldehydes										
2323	2010	1. 2	Octadecanal	0.00	0.00	0.00	0.00	1.21	0.00	0.82	0.99	0.48	0.70
			Σ Ketones										
1259	-	1. 2	3-Hydroxy-2-butanone	0.00	0.00	0.00	0.00	0.00	0.51	0.00	0.00	2.28	0.00
			Σ Carboxylic Acids										
1454	-	1. 2. 3	Acetic acid	0.82	0.00	0.00	0.00	0.00	1.61	0.00	1.26	3.46	4.27
1847	1028	1. 2	Hexanoic acid	0.28	1.08	0.72	0.00	1.40	0.00	1.39	1.44	1.17	1.83
2046	1206	1. 2	Octanoic acid	3.60	4.36	4.75	4.21	4.06	4.20	4.54	5.19	4.20	2.83
2283	1382	1. 2	Decanoic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2900	1387	1. 2	*n*-Hexadecanoic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.09
3123	2383	1. 2	Stearic acid	0.00	0.00	0.00	0.00	0.59	0.00	0.99	1.37	0.84	1.30
			Σ EEFAs										
1213	996	1. 2	Ethyl hexanoate	0.37	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1427	1194	1. 2	Ethyl octanoate	0.57	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1611	1400	1. 2	Ethyl decanoate	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.21
			Σ HAAs										
1111	873	1. 2	Isoamyl acetate	4.23	3.84	2.41	2.73	4.89	1.76	3.16	4.31	1.35	2.80
1778	1260	1. 2	Phenylethyl acetate	2.33	0.24	0.22	0.14	0.55	0.00	0.16	0.16	0.11	0.18
			Σ EEBAs										
1319	814	1. 2	Ethyl 2-hydroxypropanoate	1.66	1.04	1.99	1.50	1.39	10.35	10.85	7.03	9.26	8.43
1486	936	1. 2	Ethyl 3-hydroxybutyrate	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
			Σ MEs										
1645	1181	1. 2	Ethyl succinate	0.26	0.17	0.22	0.14	0.22	0.43	0.65	0.30	0.55	0.13
1702	1108	1. 2	1.3-Propylene diacetate	0.23	0.39	0.34	0.00	0.57	0.00	0.22	0.29	0.00	0.00
2012	1272	1. 2	Ethyl dl-malate	0.37	0.47	0.38	0.32	0.19	0.07	0.24	0.26	0.18	0.27
			Σ Lactones										
1589	913	1. 2	Butyrolactone	0.99	1.08	0.83	0.65	0.91	0.75	0.97	1.13	0.79	1.17
2191	1305	1. 2	*γ*-Carboethoxy-*γ*-butyrolactone	0.34	0.41	0.41	0.43	0.26	0.15	0.42	0.34	0.22	0.19
			Others										
1399	1254	1. 2	1.3-Di-tert-butylbenzene	0.73	0.41	0.91	0.71	0.90	0.72	0.91	1.10	0.83	1.34
1874	1508	1. 2	2.6-Di-tert-butyl-4-methylphenol	0.00	0.00	0.00	0.00	0.00	0.00	5.19	1.84	0.00	0.00
2270	1525	1. 2	2.4-Di-tert-butylphenol	0.29	0.36	0.83	0.51	0.78	0.51	0.80	0.75	0.66	1.19
			Total compounds	**95.39**	**95.38**	**95.60**	**98.23**	**96.10**	**96.47**	**95.08**	**95.23**	**92.87**	**92.40**

[a] LRI: Supercowax10 column; [b] LRI: DB5-MS column; [c] Ident.: 1= retention index identical to bibliography; 2= identification based on comparison of MS; 3= retention time identical to authentic compounds; t: trace amount < 0.05%. EEFAs: ethyl esters of fatty acids; HAAs: higher alcohol acetates; EEBAs: ethyl esters of branched acids; MEs: miscellanea esters.

Among the various treatments, only the T8 showed lower amounts of ethyl 2-hydroxypropanoate than control. The addition of GIY (T7 and T9) favours the formation of 2-hydroxypropanoate. It is clearly the opposite for the must fermented with GR1 grape strain: in this case, the absence of antioxidants stimulates a greater production of EEBAs. Regarding the acetates deriving from long chain alcohols (HAAs), GR1 strain produced higher amounts (2.63 - 6.56%) than the SPF52 strain (1.46 - 4.47%). Differences can also be noted regarding the impact of nutrients and antioxidant on the final composition of the wine. In fact, SS in absence of GIY, produced higher concentrations of HAAs; while a lower amount of the latter was present when the yeast was treated with SC. SPF52 honey strain, in the presence of SC and in the absence of GIY, favoured a higher content of HAAs in wine samples than those treated with GIY; in turn, the presence of antioxidants seemed to favour the production of HAAs with SS compared to T6. As reported by Renault et al. (2015), the EEFAs, compounds which contribute to fruity aromas, are present in minimal quantities in all samples (0.21 - 1.02%) and reflected in the sensory analysis.

The most abundant carboxylic acid in all wines was octanoic acid, except in T10 where it was acetic acid. GR1 strain produced wines, had minimal (T1) acetic acid.

Other volatile compounds identified in all samples were 2.4-DTBP and the corresponding aromatic compound without phenolic group. In T7 and T8 wines, 4-methyl-2.6-DTBP was also present. These bioactive secondary metabolites, produced by various groups of organisms, are reported in the literature (Zhao et al., 2019).

4.3.5.1 Statistical multivariate analysis of VOCs composition

The graphical representation of VOCs analysis is shown in Fig. 5. The double hierarchical dendrogram combined with heat map plot showed that all additions (yeast, nutrient and GIY) significantly affected VOCs composition of the wines.

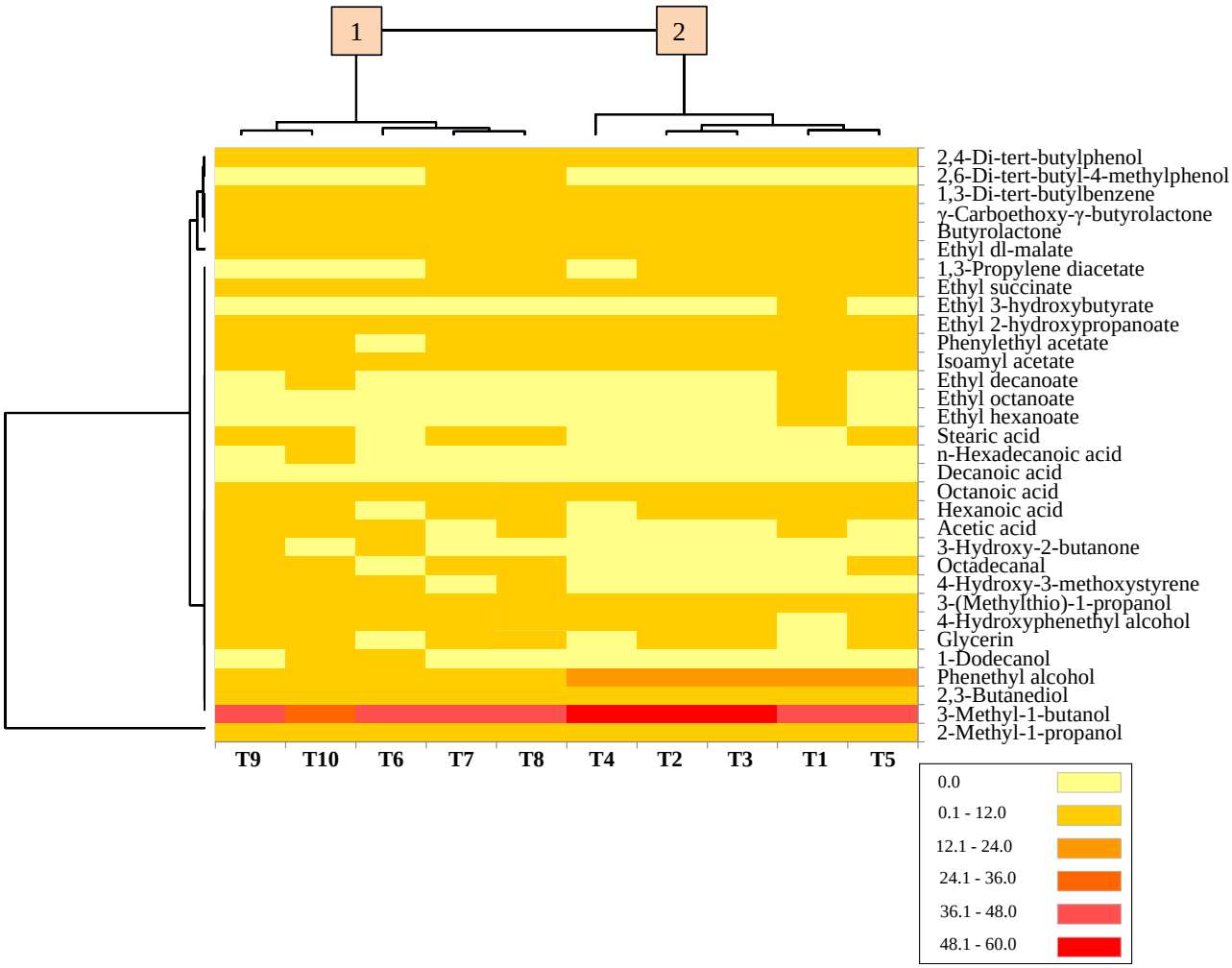

Figure 5. Distribution of the volatile organic compounds from wines expressed as relative peak areas (peak area of each compound/total area) × l00. The hierarchical dendrogram is based on the values of volatile organic compounds (VOCs). The heat map plot depicts the relative percentage of each compound within each wine.

There are two distinct VOCs clusters, with the most important being the high quantity of ethyl esters of branched acids (EEBAs 7.04–10.85%) and a lower presence of alcohols (62.46–72.26%) in the

T6-T10 group compared to the T1-T5 group (EEBAs 1.04-1.74%; alcohols 77.54–86.10%). Control A and control B are characterised by the lower quantity of alcohols in the two groups (77.54% in control A and 62.46% in control B).

Interestingly, the T1-T5 wines were grouped into one mega-cluster with the discriminator as yeast strain, GR1 grape strain. T4 showed the highest dissimilarity values, in contrast to T1 and T5, T2 and T3 with similar VOCs composition. T4 exhibited the highest alcohols content (86.10 %) and the lowest carboxylic acids content (4.21 %).

The second cluster (T6-T10) are the experimental wines produced with SPF52 honey strain. This cluster has three subclasses: T9 and T10, T7 and T8, and T6 which represented a separate subclass. It could be proposed that the SPF52 honey strain produces very different VOCs depending on the nutrition strategies (SS and SC) and antioxidant (GIY) addition. Furthermore, GR1 grape strain significantly affected wine composition independently by nutrition strategies and antioxidant addition.

4.3.6 Sensory analysis

The highest overall acceptability scores were found for T1 (2.71) based on wine consumers' response (Fig. 6a), whereas T2 (3.33) and T9 (3.48) were rated highest by wine expert (Fig. 6b). It is worthy of note that wines T2 and T9 had very high overall acceptability scores from both wine consumers and wine experts. From the sensory acceptance test, wines produced with strain GR1, SS and GIY (T2) and strain SPF52, SC and GIY (T8) were the most appreciated. Probably, the aromatic behaviour of the strains and the type of nutrition in conjunction with antioxidants guaranteed a higher acceptability of the wine. None of the ten wines were judged as "unpleasant/unacceptable".

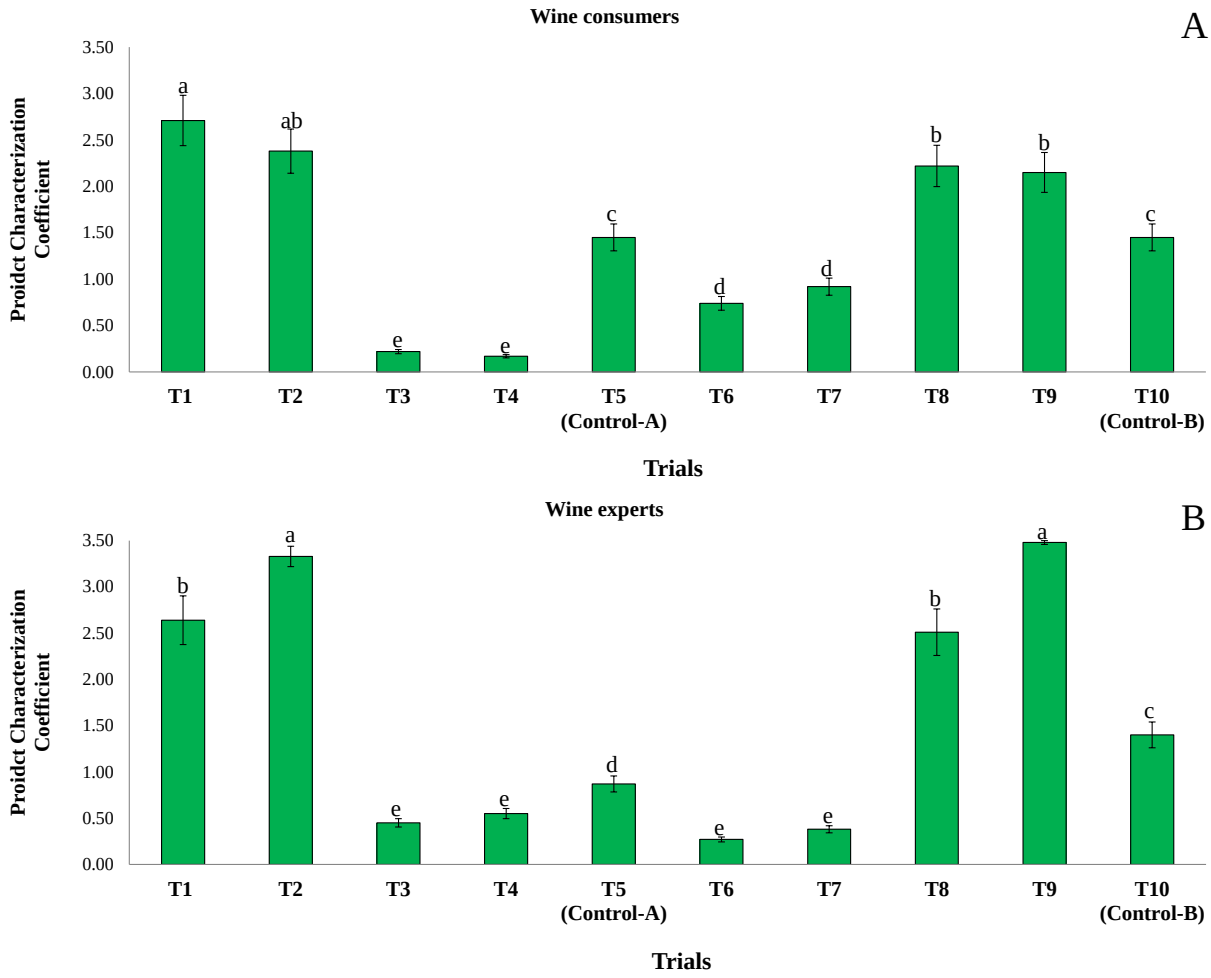

Figure 6. Sensory analysis-based product characterization for overall acceptability of wines (T 1-10): (A) wine consumers; (B) wine experts.

The quantitative sensory analysis results are reported in Table 3. All the wines mainly showed differences related to the yeast strain used (GR1 or SPF52). This phenomenon has been extensively studied by numerous authors and especially with indigenous *S. cerevisiae* strains where it is possible to differentiate the wines by sensory characteristics (Liu et al., 2016; Puertas et al., 2018). A significant impact on the sensory characterisation of wines produced by the same yeast strain, and also by the nutrition type (SS, SC or classic nutrition) and the presence/absence of GIY. Differences in wine appearance were variable in the treatments with variations in yellow colour (6.7-7.39), and green reflexes (3.01-3.89). Treatments T2 and T9 showed the highest scores for 13 attributes (odour: intensity, floral, orange flowers, green apple, citrus fruit and grapefruit; taste: salty and bitter; mouth-feel: balance; flavour: citrus fruit; overall quality: taste; finish: after-smell and after-taste) and 18 attributes (odour: intensity, persistency, fruity, tropical fruit, caramel, honey and bread crust; taste: sweet; mouth-feel: body; flavour: persistency, tropical fruit, caramel and honey; overall quality: odour, mouth-feel and flavour; finish: after-smell and after-taste), respectively.

Table 3. Sensory attributes of the experimental Catarratto wines.

Attributes	Trial										SEM	Statistical significance	
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10		Judges	Wine
Appearance													
Yellow color	6.85^{f}	6.70^{h}	6.85^{f}	6.80^{g}	6.78^{g}	7.39^{a}	7.20^{d}	7.28^{c}	7.15^{e}	7.35^{b}	0.02	*	*
Green reflexes	3.36^{e}	3.46^{d}	3.83^{b}	3.89^{a}	3.74^{c}	3.12^{fg}	3.01^{h}	3.09^{g}	3.19^{f}	3.18^{f}	0.03	*	*
Odor													
Intensity	7.15^{d}	8.30^{a}	7.80^{b}	5.25^{f}	5.28^{f}	6.25^{e}	7.56^{c}	7.22^{d}	8.19^{a}	7.35^{d}	0.09	*	*
Persistency	7.38^{d}	8.10^{b}	6.82^{f}	5.01^{h}	4.10^{i}	5.98^{g}	7.65^{c}	7.11^{e}	8.64^{a}	7.68^{c}	0.11	**	***
Floral	6.88^{b}	7.30^{a}	2.15^{f}	3.25^{e}	3.25^{e}	6.29^{c}	6.10^{c}	2.20^{f}	3.20^{e}	5.38^{d}	0.16	***	***
Orange flowers	7.20^{b}	7.70^{a}	1.00^{d}	1.00^{d}	1.00^{d}	1.00^{d}	2.90^{c}	1.00^{d}	1.00^{d}	1.00^{d}	0.21	**	***
Fruity	6.70^{d}	6.20^{e}	7.70^{b}	4.25^{f}	4.50^{f}	6.71^{d}	3.10^{g}	7.30^{c}	8.02^{a}	2.50^{h}	0.16	*	**
Peach	1.00^{e}	1.00^{e}	7.12^{a}	3.85^{d}	1.00^{e}	6.65^{b}	1.00^{e}	1.00^{e}	5.25^{c}	1.00^{e}	0.20	***	***
Apricot	1.00^{d}	1.00^{d}	7.08^{a}	1.00^{d}	1.00^{d}	6.41^{b}	1.00^{d}	1.00^{d}	4.25^{c}	1.00^{d}	0.20	***	***
Plum	1.00^{c}	1.00^{c}	1.00^{c}	1.00^{c}	1.00^{c}	1.00^{c}	1.00^{c}	6.82^{a}	5.58^{b}	1.00^{c}	0.17	***	***
Green apple	1.00^{b}	3.20^{a}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	0.05	*	**
Citrus fruits	6.20^{b}	7.90^{a}	3.54^{c}	3.25^{c}	1.00^{d}	1.00^{d}	1.00^{d}	1.00^{d}	1.00^{d}	1.00^{d}	0.20	**	***
Grapefruit	4.35^{b}	7.70^{a}	2.65^{c}	1.00^{d}	1.00^{d}	1.00^{d}	1.00^{d}	1.00^{d}	1.00^{d}	1.00^{d}	0.18	**	**
Tropical fruits	1.00^{c}	1.00^{c}	8.30^{a}	1.00^{c}	5.25^{b}	1.00^{c}	1.00^{c}	8.12^{a}	7.98^{a}	1.00^{c}	0.27	***	***
Pineapple	1.00^{c}	1.00^{c}	7.77^{a}	1.00^{c}	4.00^{b}	1.00^{c}	1.00^{c}	1.00^{c}	1.00^{c}	1.00^{c}	0.18	**	**
Banana	1.00^{c}	1.00^{c}	7.62^{a}	1.00^{c}	4.80^{b}	1.00^{c}	1.00^{c}	1.00^{c}	1.00^{c}	1.00^{c}	0.18	**	***
Tamarind	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	7.75^{a}	1.00^{b}	1.00^{b}	0.17	*	**
Small fruits	3.50^{c}	3.98^{b}	4.20^{b}	4.01^{b}	1.00^{d}	5.12^{a}	4.12^{b}	1.00^{d}	1.00^{d}	1.00^{d}	0.13	**	***
Strawberry	1.00^{b}	1.00^{b}	6.75^{a}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	0.14	**	*
Licorice	6.50^{a}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	0.03	*	*
Anice	6.87^{a}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	0.02	*	*
Caramel	1.00^{d}	1.00^{d}	1.00^{d}	1.00^{d}	1.00^{d}	6.10^{c}	7.20^{b}	7.35^{ab}	7.52^{a}	7.12^{b}	0.14	**	***
Honey	1.00^{d}	1.00^{d}	1.00^{d}	1.00^{d}	1.00^{d}	5.87^{c}	7.75^{a}	7.85^{a}	7.86^{a}	7.01^{b}	0.15	**	***
Wax	1.00^{d}	1.00^{d}	1.00^{d}	1.00d	1.00^{d}	6.15^{c}	6.98^{b}	6.18^{c}	6.58^{bc}	7.45^{a}	0.25	***	***
Bread crust	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	7.10^{a}	1.00^{b}	6.98^{a}	1.00^{b}	0.27	***	***
Box tree	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	7.12^{a}	0.15	**	**
Cat pee	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	8.70^{a}	0.19	**	**
Taste													
Sweet	2.40^{g}	2.54^{f}	2.97^{c}	2.58^{f}	2.34^{g}	2.98^{c}	2.72^{e}	3.48^{b}	3.59^{a}	2.91^{d}	0.03	*	*
Sour	7.86^{a}	7.42^{b}	6.65^{d}	6.75^{d}	6.12^{e}	5.15^{g}	6.80^{d}	5.38^{f}	5.37^{f}	6.98^{c}	0.07	**	*
Salty	6.28^{c}	7.10^{a}	5.50^{f}	5.15^{g}	4.45^{h}	5.25^{g}	6.50^{b}	5.70^{e}	5.85^{d}	6.58^{b}	0.06	*	*
Bitter	2.15^{b}	2.38^{a}	1.80^{e}	2.05^{c}	1.92^{d}	1.38^{f}	1.22^{g}	1.10^{h}	1.25^{g}	1.38^{f}	0.04	*	*
Mouthfeel													
Body	7.15^{d}	7.88^{b}	6.82^{e}	6.15^{g}	6.35^{f}	6.31^{f}	7.51^{c}	7.80^{b}	8.42^{a}	6.75^{e}	0.06	*	*
Balance	6.80^{c}	8.32^{a}	6.25^{e}	5.98^{f}	5.00^{g}	6.17^{e}	6.89^{c}	6.50^{d}	7.49^{b}	6.20^{e}	0.07	*	*
Flavour													
Intensity	6.93^{c}	7.50^{b}	6.26^{d}	5.14^{f}	5.25^{f}	5.71^{e}	7.45^{b}	7.80^{a}	7.85^{a}	7.10^{c}	0.08	*	**
Persistency	6.82^{e}	8.00^{b}	5.44^{f}	5.68^{f}	4.82^{g}	5.58^{f}	7.15^{d}	7.70^{c}	8.78^{a}	6.87^{e}	0.10	**	**
Floral	6.12^{a}	5.10^{b}	2.25^{h}	3.25^{f}	2.50^{h}	3.15^{f}	4.12^{d}	3.52^{e}	4.82^{c}	2.20^{h}	0.10	**	**
Fruity	6.25^{d}	6.90^{c}	7.62^{a}	4.08^{f}	5.12^{e}	5.12^{e}	1.96^{h}	6.92^{c}	7.25^{b}	2.80^{g}	0.15	**	**
Citrus fruits	6.65^{b}	7.20^{a}	1.60^{c}	1.00^{d}	1.00^{d}	1.00^{d}	1.00^{d}	1.00^{d}	1.00^{d}	1.00^{d}	0.20	**	***
Tropical fruits	2.25^{d}	2.98^{c}	8.10^{a}	1.00^{e}	4.62^{b}	1.00^{e}	1.00^{e}	8.12^{a}	7.87^{a}	1.00^{e}	0.25	***	***
Caramel	1.00^{d}	1.00^{d}	1.00^{d}	1.00^{d}	1.00^{d}	3.85^{c}	6.25^{b}	7.10^{a}	7.02^{a}	4.25^{c}	0.21	***	***
Honey	1.00^{d}	1.00^{d}	1.00^{d}	1.00^{d}	1.00^{d}	6.27^{b}	7.64^{a}	7.85^{a}	7.35^{a}	3.88^{c}	0.25	***	***
Box tree	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	7.15^{a}	0.15	**	**
Cat pee	1.00^{c}	1.00^{c}	1.00^{c}	1.00^{c}	3.02^{b}	1.00^{c}	1.00^{c}	1.00^{c}	1.00^{c}	8.75^{a}	0.19	**	***
Overall quality	7.58^{b}	8.38^{a}	6.89^{c}	6.10^{d}	4.32^{e}	6.27^{d}	7.70^{b}	7.50^{b}	8.57^{a}	2.20^{f}	0.16	*	**
Odor	7.68^{c}	8.40^{b}	7.03^{e}	5.15^{g}	4.10^{h}	6.17^{f}	7.50^{cd}	7.20^{de}	8.86^{a}	1.50^{i}	0.18	**	**
Taste	7.25^{c}	7.89^{a}	6.92^{e}	6.12^{f}	5.30^{h}	5.78^{g}	7.10^{d}	7.01^{de}	7.54^{b}	6.12^{f}	0.07	*	**
Mouthfeel	7.10^{cd}	7.55^{b}	6.50^{e}	5.89^{g}	5.90^{g}	6.37^{f}	6.97^{d}	7.20^{c}	8.32^{a}	5.80^{g}	0.06	*	**
Flavour	7.41^{c}	8.20^{b}	6.80^{e}	6.32^{f}	4.39^{g}	6.32^{f}	7.22^{cd}	6.98^{de}	8.81^{a}	1.93^{h}	0.16	**	***
Finish													
After-smell	7.31^{b}	8.00^{a}	6.80^{c}	6.21^{d}	4.10^{e}	6.15^{d}	7.11^{bc}	6.80^{c}	8.15^{a}	1.59^{f}	0.16	**	***
After-taste	7.11^{b}	8.10^{a}	6.30^{c}	6.32^{c}	5.10^{d}	6.11^{c}	7.35^{b}	7.10^{b}	7.96^{a}	1.38^{e}	0.15	**	***

Result indicate mean value.
Data within a line followed by the same letter are not significantly different according to Tukey's test.
P value: ***, P < 0.001; **, P < 0.01; * P < 0.05

These results confirmed the results of the wine experts reported for the sensory acceptance test. Consequently, the combination of the GR1 yeast strain with SS and GIY produced wines with a high overall quality (8.38). On the other hand, the combination of SPF52, SC and GIY obtained wines with

overall quality values of 8.57. The use of different “Stimula” (specific nutrient), in combination with the yeast strain, were able to enhance particular aromas. Torrea and Henschke (2004) observed how the impact of three different concentrations of yeast assimilable nitrogen (YAN) on Chardonnay must can influence the composition of the descriptors of the final wine. Evidently, the different nutrition and addition of GIY in relation to the yeast strain resulted in different sensory expressions. Just as different yeast autolysates are able to influence the perception of wine aroma (Comuzzo et al., 2006). It is interesting to note that in T1 and T2 wines, aromas of orange flowers were perceived (7.2 and 7.7, respectively) which were absent in wines produced using SC (T3 and T4) and classic nutrition (T5). On the other hand, the peach aroma attribute was detected in trials involving the addition of SC (T3 and T4) compared to those involving the use of SS (T1-T2) or classical nutrition (T5). The wines produced with SPF52, and nutrient SC (T8-T9) showed the presence of tropical fruit (8.12 and 7.98, respectively) and plum (6.82 and 5.58, respectively) aromas while no such aromas were detected for the wines produced with SS (T6-T7) and classic nutrition (T10). Wines T6 and T7, made with yeast SPF52, aromas of small fruits were perceived when the nutrition regime included SS, such aromas were not detected in wines produced with SPF52 and SC (T8-T9) and those with classic nutrition (T10).

In some cases, the experimental wines produced aromas recognised exclusively in one treatment: green apple in T2, tamarind in T8, strawberry in T3, liquorice and aniseed in T1. Only in T10 wine, the attributes of box tree and cat pee were detected as odour and taste. The analysis of aromas revealed the presence of the caramel, honey and wax descriptors in the wines inoculated with SPF52 with variable scores in the T6-T10 wines, regardless of the nutritional scheme and the presence/absence of GIY. These attributes were not perceptible for wines produced with the GR1 strain. This tendency was also observed in the wine flavour (caramel and honey). The attributes describing the taste of the wines differed between the wines. High ratings were observed in T9 for sweet, T1 for sour, T2 for salty and bitter. Mouthfeel also showed statistically significant differences in almost all wines. The attribute body showed highest rating in T9 (8.42), whereas in T2 the highest rating of balance was found (8.32). For all wines, off-aromas and off-flavours were not detected. The addition of GIY in some cases increased flavour in terms of intensity and persistence, which was greater than in wines without antioxidants. In fact, treatment with GIY resulted in obtaining the improved wines (T2 and T9) with more complexity of sensory profiles. Badea et al. (2017) demonstrated that doses of 40 mg/L of glutathione added in musts before AF helped to protect varietal aromas of wines and result in wines with sensory profiles highly appreciated by consumers.

4.3.6.1 Multiple factor analysis of sensory scores

Multiple factor analysis (MFA) was used determine if there are any correlations between the winemaking variables in the sensory data. This led to the identification of four factors with eigen values higher than 1, indicating that the total number of variables (50) for the 10 wines could be grouped into only four factors which explained 86.46% of the total variance. The association between the variables and the MFA factor is indicated by the contribution and cos^2 value. Interestingly, specific aroma, taste and flavour descriptors were attributable to different factors. The aromas (orange flowers, citrus fruit, grapefruit, caramel, honey and wax), taste (sweet, sour, bitter) and flavours (intensity, persistency, floral, fruity, citrus fruit, caramel and honey) were associated with F1, whereas the aromas (fruity, plum, box tree and cat pee), flavours (box tree and cat pee) and overall quality (odour, taste, mouth-feel and flavour) were associated with F2, and the aromas (tropical fruit, pineapple and banana), taste (salty), mouth-feel (body and balance) and flavour (tropical fruit) were associated with F3. The aromas (intensity, floral, peach, apricot and strawberry) and finish (after-smell and after-taste) a were associated to F4. As shown in Fig. 7a,b, the two-dimension model of MFA of variables explained 55% of the total variance, with F1 and F2 accounting for 32.48 and 22.52%, respectively. The variables loading plot of MFA (Fig. 7a) showed that 18 variables were located in the first quadrant, twelve in the second quadrant, twelve in the third quadrant and 8 in the fourth quadrant. Fig. 7b shows that the trials were grouped into three clusters. However, both MFA observation plot (Fig. 7b) and AHCA dendrogram (Fig. 7c) showed that the T5 (Control-A) grouped with wines T1, T2, T3, and T4. Interestingly, the wine T10 (Control-B) did not cluster with the group of wines made with yeast strain SPF2. Indeed, the wines T6, T7, T8 and T9 represented a different cluster. Within each cluster, wine pairs T1-T2, T3-T4, T6-T7 and T8-T9 showed low dissimilarity. This attribute-by-attribute comparison was also plotted from the MFA, showing the degree of similarity in sorting between the groups for each attribute. The groups of variables had different influences in each trial, as indicated in Fig. 8. The shorter the arm, the more similarly the groups sorted that attribute.

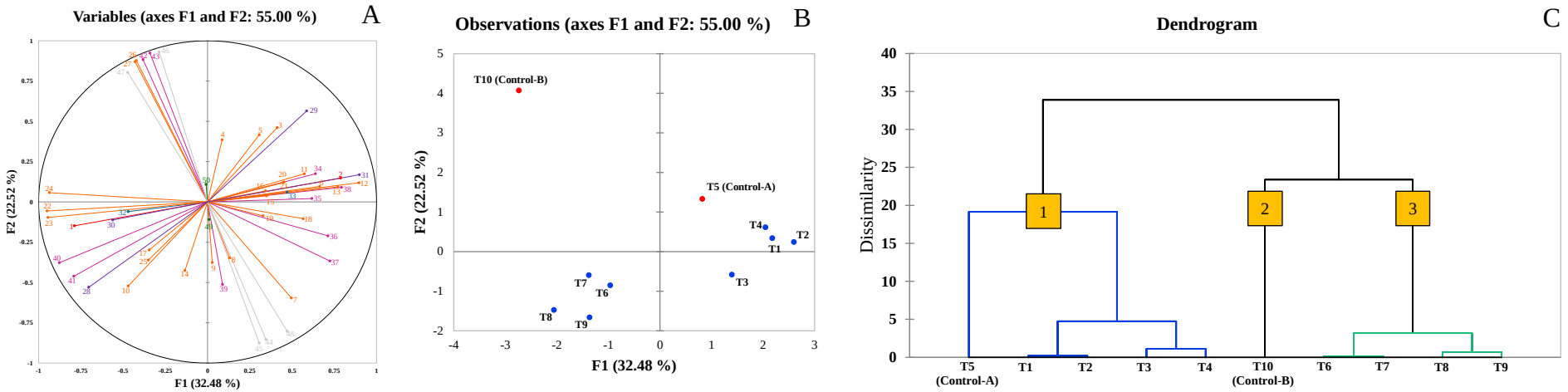

Figure 7. Correlations of the sensory analysis and discrimination among different wines:

(A) Variable loading plot of MFA: ■ appearance (1, yellow color; 2, green reflexes), ■ odor (3, intensity; 4, persistence; 5, floral; 6, orange flowers; 7, fruity; 8, peach; 9, apricot; 10, plum; 11, green apple; 12, citrus fruit; 13, grapefruit; 14, tropical fruit; 15, pineapple; 16, banana; 17, tamarind; 18, small fruit; 19, strawberry; 20, licoric; 21, anice; 22, caramel; 23, honey; 24, wax; 25, bread crust; 26, box tree; 27 cat pee), ■ taste (28, sweet; 29, sour; 30, salty; 31, bitter), ■ mouth-feel (32, body; 33, balance), ■ flavour (34, intensity; 35, persistence; 36, floral; 37, fruity; 38, citrus fruit; 39, tropical fruit; 40, caramel; 41, honey; 42, box tree; 43, cat pee, ■ overall quality (44, overall quality; 45, odor; 46, taste; 47, mouth-feel; 48, flavour), ■ finish (49, after-smell; 50, after-taste); (B) sample scores of MFA analysis; (C) AHC dendrogram of trials based on their dissimilarity.

4.4 CONCLUSIONS

In this study, different experimental protocols were evaluated to obtain different aromatic expressions for a Catarratto cultivar classified as non-aromatic. The *S. cerevisiae* SPF52 strain, isolated from sugary matrices different from grape must, was proven to be suitable for wine production. The addition of nutrients Stimula Sauvignon Blanc™ or Stimula Chardonnay™ before the inoculation of starter yeasts allowed to increase the aromatic complexity of the final wines, as confirmed by VOCs and sensorial analysis. Finally, the addition of GSH-enriched inactivated yeast Glutastar™ was useful to prevent the chemical oxidation of musts and wines and to generate the highest aromatic intensity. The study focused on the aromatic evaluation of wines bottled after 5 months of stain-less steel tank aging. Further studies are needed to investigate the aromatic evolution of wines during the ageing in bottle.

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Table S1. Chemical parameters determined during the winemaking process

Parameters	Must	Yeast Inoculum	Vinification										
			3 days of alcoholic fermentation										
			Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10	S.S.
pH	3.27±0.01^{a}	3.32±0.02^{a}	3.09±0.01^{d}	3.08±0.01^{de}	3.10±0.00^{cd}	3.07±0.01^{def}	3.03±0.01^{efg}	3.01±0.02^{g}	3.15±0.01^{bc}	3.12±0.03^{cd}	3.02±0.01^{fg}	3.18±0.03^{b}	***
TA	5.53±0.01^{a}	5.53±0.03^{a}	5.51±0.03^{a}	5.52±0.04^{a}	5.52±0.04^{a}	5.52±0.01^{a}	5.51±0.06^{a}	5.52±0.04^{a}	5.51±0.01^{a}	5.52±0.04^{a}	5.51±0.04^{a}	5.52±0.05^{a}	n.s.
VA	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	n.s.
RS	221.50±0.26^{a}	208.20±0.11^{b}	153.79±0.14^{d}	137.44±0.12^{h}	171.68±0.16^{c}	140.02±0.20^{g}	150.50±0.27^{e}	120.73±0.31^{j}	125.20±0.26^{i}	144.94±0.25^{f}	116.08±0.24^{k}	139.76±0.26^{g}	***
Glucose	105.00±0.10^{a}	87.4±0.09^{b}	57.16±0.08^{f}	51.28±0.08^{i}	63.71±0.07^{c}	54.64±0.06^{g}	59.24±0.06^{e}	49.16±0.05^{k}	54.19±0.05^{h}	49.84±0.09^{j}	51.19±0.11^{i}	60.59±0.15^{d}	***
Fructose	99.00±0.15^{a}	74.5±0.13^{b}	68.19±0.17^{f}	57.94±0.21^{i}	65.83±0.33^{h}	69.73±0.12^{e}	68.03±0.11^{f}	53.89±0.21^{j}	67.36±0.14^{g}	71.72±0.36^{c}	46.75±0.19^{k}	70.72±0.24^{d}	***
Ethanol	0.54±0.03^{g}	0.99±0.04^{g}	2.86±0.07^{e}	3.31±0.10^{cd}	2.16±0.11^{f}	2.96±0.05^{de}	2.93±0.02^{de}	4.54±0.11^{b}	4.96±0.20^{a}	3.56±0.19^{c}	4.75±0.31^{ab}	3.31±0.06^{cd}	***
Glycerol	0.91±0.04^{b}	0.98±0.10^{b}	1.98±0.14^{a}	2.02±0.16^{a}	2.02±0.17^{a}	1.98±0.13^{a}	1.99±0.09^{a}	2.02±0.11^{a}	2.02±0.14^{a}	1.99±0.13^{a}	1.99±0.12^{a}	1.98±0.08^{a}	***
Malic acid$^{\Psi}$	1.90±0.03^{a}	1.77±0.02^{b}	1.75±0.05^{bcd}	1.66±0.02^{de}	1.76±0.04^{bc}	1.64±0.03^{e}	1.62±0.04^{e}	1.62±0.04^{e}	1.59±0.02^{e}	1.67±0.03^{cde}	1.64±0.01^{e}	1.64±0.04^{e}	***
Lactic acid$^{\Psi}$	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	n.s.
Amm. N	84.30±0.12^{a}	83.27±0.19^{b}	67.98±0.18^{f}	72.61±0.14^{c}	67.46±0.21^{g}	68.66±0.09^{e}	66.19±0.16^{i}	66.75±0.12^{h}	68.17±0.18^{f}	66.61±0.31^{h}	68.32±0.15^{ef}	70.43±0.11^{d}	***
Alpha-AN	33.46±0.15^{a}	33.25±0.11^{b}	25.19±0.07^{j}	29.06±0.12^{f}	27.43±0.07^{h}	31.25±0.11^{c}	29.76±0.09^{e}	26.58±0.08^{i}	23.03±0.04^{k}	30.98±0.10^{d}	21.88±0.07^{l}	27.69±0.03^{g}	***

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. Result indicates mean value ± standard deviation of three determinations from two replicates. Data within a line followed by the same letter are not significantly different according to Tukey's test.$^{\Psi}$, expressed in g/L.

Parameters	Vinification										
	6 days of alcoholic fermentation										
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10	S.S.
pH	3.07±0.03^{e}	3.11±0.01^{de}	3.10±0.02^{de}	3.13±0.00^{cde}	3.15±0.00^{bcd}	3.21±0.03^{a}	3.18±0.02^{abc}	3.19±0.01^{ab}	3.22±0.02^{a}	3.17±0.01^{abc}	***
TA	5.47±0.02^{a}	5.47±0.04^{a}	5.45±0.06^{a}	5.46±0.05^{a}	5.46±0.03^{a}	5.45±0.01^{a}	5.46±0.07^{a}	5.45±0.04^{a}	5.47±0.02^{a}	5.46±0.03^{a}	n.s.
VA	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	n.s.
Rs	67.89±0.03^{g}	75.55±0.11^{e}	85.03±0.28^{c}	74.88±0.35^{f}	82.02±0.30^{d}	75.04±0.09^{ef}	60.00±0.10^{h}	94.24±0.12^{b}	57.79±0.18^{i}	97.34±0.17^{a}	***
Glucose	26.60±0.11^{g}	30.56±0.11^{f}	33.66±0.19^{c}	31.66±0.41^{e}	24.85±0.38^{h}	32.42±0.02^{d}	27.16±0.15^{g}	34.41±0.27^{b}	21.94±0.14^{i}	37.45±0.30^{a}	***
Fructose	30.69±0.12^{f}	37.60±0.36^{d}	45.13±0.05^{a}	33.39±0.09^{e}	25.67±0.15^{h}	37.09±0.20^{d}	29.02±0.12^{g}	44.56±0.16^{b}	22.15±0.17^{i}	41.44±0.14^{c}	***
Ethanol	8.00±0.05^{d}	7.95±0.08^{d}	7.47±0.11^{e}	7.98±0.09^{d}	8.87±0.04^{b}	8.33±0.06^{c}	9.00±0.09^{ab}	6.57±0.07^{f}	9.19±0.19^{a}	6.76±0.02^{f}	***
Glycerol	5.05±0.01^{ab}	5.31±0.11^{a}	5.30±0.12^{a}	5.18±0.23^{a}	5.29±0.08^{a}	5.03±0.05^{ab}	5.19±0.04^{a}	5.05±0.06^{ab}	5.16±0.08^{a}	4.82±0.12^{b}	**
Malic acid	1.65±0.14^{a}	1.57±0.07^{a}	1.62±0.11^{a}	1.52±0.10^{a}	1.52±0.06^{a}	1.58±0.04^{a}	1.49±0.06^{a}	1.58±0.03^{a}	1.58±0.00^{a}	1.57±0.07^{a}	n.s.
Lactic acid	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	n.s.
Amm. N	67.50±0.04^{b}	70.63±0.17^{a}	66.66±0.19^{d}	67.27±0.08^{bc}	63.68±0.14^{h}	64.62±0.35^{g}	65.50±0.24^{f}	66.10±0.03^{e}	67.24±0.11^{bc}	67.03±0.08^{c}	***
Alpha-AN	15.66±0.09^{h}	18.94±0.05^{e}	16.87±0.11^{g}	17.75±0.04^{f}	14.95±0.09^{i}	21.57±0.18^{b}	15.74±0.13^{h}	22.64±0.06^{a}	20.56±0.05^{d}	21.13±0.06^{c}	***

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Abbreviations: TA, total titratable acidity (tartaric acid g/L); VA, volatile acidity (acetic acid g/L); RS, reducing sugar (g/L); Amm. N, ammoniacal nitrogen (mg/L); Alpha-AN, alpha-amino nitrogen (mg/L); S.S., statistical significance; n.d., not determined.
P value: *, P < 0.05; **, P < 0.01; ***, P < 0.001; n.s., not significant.

Parameters	Vinification 12 days of alcoholic fermentation										
	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**	**Trial 6**	**Trial 7**	**Trial 8**	**Trial 9**	**Trial 10**	**S.S.**
pH	3.16±0.01^{e}	3.19±0.01^{d}	3.17±0.00^{de}	3.23±0.02^{c}	3.31±0.00^{a}	3.30±0.00^{a}	3.26±0.00^{b}	3.26±0.00^{b}	3.29±0.00^{a}	3.24±0.01^{bc}	***
TA	5.43±0.05^{a}	5.42±0.04^{a}	5.42±0.03^{a}	5.43±0.06^{a}	5.41±0.04^{a}	5.41±0.05^{a}	5.42±0.03^{a}	5.42±0.11^{a}	5.43±0.04^{a}	5.43±0.09^{a}	n.s.
VA	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	n.s.
Rs	9.16±0.01^{j}	19.93±0.05^{g}	20.39±0.11^{f}	17.65±0.17^{h}	15.40±0.08^{i}	34.90±0.04^{c}	23.14±0.06^{e}	46.69±0.18^{b}	27.33±0.15^{d}	48.66±0.16^{a}	***
Glucose	3.15±0.03^{i}	6.24±0.05^{h}	7.60±0.08^{f}	6.87±0.07^{g}	1.58±0.02^{j}	14.21±0.06^{c}	8.49±0.08^{e}	20.83±0.11^{a}	10.17±0.09^{d}	19.62±0.04^{b}	***
Fructose	3.87±0.04^{g}	9.26±0.09^{e}	10.20±0.10^{c}	7.13±0.07^{f}	1.98±0.02^{h}	18.26±0.11^{b}	9.72±0.14^{d}	18.45±0.06^{ab}	10.11±0.11^{c}	18.54±0.04^{a}	***
Ethanol	12.48±0.03^{ab}	12.21±0.05^{bcd}	12.10±0.06^{cd}	12.33±0.08^{abc}	12.60±0.15^{a}	11.37±0.04^{f}	11.97±0.07^{de}	10.60±0.11^{g}	11.76±0.10^{e}	10.44±0.14^{g}	***
Glycerol	5.65±0.07^{bc}	5.79±0.11^{abc}	5.89±0.03^{ab}	5.98±0.08^{a}	5.17±0.07^{d}	5.96±0.04^{ab}	5.58±0.05^{c}	5.14±0.21^{d}	5.18±0.10^{d}	5.24±0.10^{d}	***
Malic acid	1.54±0.02^{ab}	1.51±0.05^{ab}	1.60±0.06^{a}	1.49±0.03^{ab}	1.48±0.00^{ab}	1.56±0.06^{ab}	1.47±0.03^{ab}	1.46±0.02^{b}	1.48±0.05^{ab}	1.51±0.03^{ab}	*
Lactic acid	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	n.s.
Amm. N	66.59±0.12^{b}	67.48±0.17^{a}	65.98±0.17^{c}	64.52±0.04^{d}	61.42±0.10^{f}	64.44±0.07^{d}	48.19±0.11^{i}	49.43±0.23^{h}	61.81±0.11^{e}	56.11±0.19^{g}	***
Alpha-AN	12.75±0.05^{i}	16.70±0.10^{f}	16.05±0.00^{g}	16.82±0.12^{f}	18.12±0.06^{e}	20.82±0.04^{c}	15.24±0.16^{h}	21.81±0.17^{a}	20.53±0.31^{d}	21.09±0.04^{b}	***

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Parameters	Vinification End of alcoholic fermentation (18 days of alcoholic fermentation)										
	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**	**Trial 6**	**Trial 7**	**Trial 8**	**Trial 9**	**Trial 10**	**S.S.**
pH	3.08±0.00^{d}	3.04±0.00^{e}	3.07±0.01^{de}	3.08±0.00^{d}	3.16±0.01^{c}	3.48±0.02^{a}	3.43±0.00^{b}	3.45±0.00^{ab}	3.48±0.02^{a}	3.43±0.01^{b}	***
TA	5.41±0.02^{a}	5.41±0.06^{a}	5.40±0.05^{a}	5.40±0.05^{a}	5.40±0.04^{a}	5.41±0.06^{a}	5.41±0.05^{a}	5.42±0.05^{a}	5.43±0.06^{a}	5.42±0.04^{a}	n.s.
VA	0.31±0.02^{a}	0.31±0.02^{a}	0.31±0.02^{a}	0.31±0.02^{a}	0.31±0.03^{a}	0.31±0.00^{a}	0.31±0.01^{a}	0.31±0.00^{a}	0.31±0.00^{a}	0.31±0.01^{a}	n.s.
Rs	1.64±0.01^{f}	1.72±0.04^{f}	1.96±0.06^{e}	1.71±0.05^{f}	1.94±0.04^{e}	4.80±0.10^{c}	2.90±0.05^{d}	3.00±0.03^{d}	5.20±0.06^{b}	5.90±0.06^{a}	***
Glucose	0.73±0.00^{de}	0.62±0.02^{ef}	0.88±0.09^{c}	0.55±0.04^{f}	0.64±0.03^{ef}	1.20±0.02^{b}	0.80±0.01^{cd}	1.60±0.00^{a}	1.10±0.00^{b}	1.60±0.04^{a}	***
Fructose	0.78±0.01^{e}	0.72±0.00^{e}	1.01±0.02^{d}	0.68±0.03^{e}	0.98±0.04^{d}	3.20±0.07^{a}	1.70±0.06^{c}	1.80±0.03^{c}	2.10±0.02^{b}	3.20±0.03^{a}	***
Ethanol	12.91±0.06^{f}	13.00±0.05^{f}	13.30±0.04^{e}	13.08±0.03^{f}	13.49±0.04^{cde}	13.71±0.05^{ab}	13.69±0.06^{abc}	13.85±0.08^{a}	13.52±0.12^{bcd}	13.47±0.12^{de}	***
Glycerol	6.08±0.05^{d}	6.64±0.06^{ab}	6.66±0.05^{a}	6.61±0.07^{ab}	5.88±0.08^{de}	6.35±0.04^{c}	6.51±0.06^{abc}	5.78±0.06^{ef}	6.46±0.08^{bc}	5.67±0.06^{f}	***
Malic acid	1.54±0.02^{ab}	1.51±0.01^{ab}	1.60±0.10^{a}	1.49±0.02^{ab}	1.48±0.03^{b}	1.56±0.04^{ab}	1.47±0.03^{b}	1.34±0.03^{c}	1.27±0.00^{c}	1.26±0.00^{c}	***
Lactic acid	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	n.s.
Amm. N	25.46±0.06^{i}	26.56±0.02^{f}	26.13±0.04^{h}	25.59±0.09^{i}	27.22±0.04^{e}	35.99±0.11^{c}	26.34±0.07^{g}	30.17±0.08^{d}	38.07±0.05^{a}	36.27±0.09^{b}	***
Alpha-AN	12.34±0.07^{h}	14.20±0.02^{f}	13.24±0.01^{g}	14.27±0.00^{f}	12.12±0.03^{i}	17.93±0.13^{b}	14.52±0.12^{e}	16.91±0.10^{c}	20.14±0.09^{a}	16.28±0.10^{d}	***

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Parameters	Vinification										
	1 month of steel aging										
	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**	**Trial 6**	**Trial 7**	**Trial 8**	**Trial 9**	**Trial 10**	**S.S.**
pH	3.24±0.00^{f}	3.31±0.01^{e}	3.30±0.00^{e}	3.36±0.01^{cd}	3.34±0.00^{de}	3.44±0.03^{a}	3.39±0.02^{bc}	3.40±0.02^{abc}	3.42±0.00^{ab}	3.39±0.01^{bc}	***
TA	5.40±0.03^{a}	5.40±0.06^{a}	5.39±0.04^{a}	5.40±0.00^{a}	5.39±0.01^{a}	5.39±0.02^{a}	5.39±0.03^{a}	5.40±0.03^{a}	5.41±0.02^{a}	5.41±0.02^{a}	n.s.
VA	0.33±0.02^{a}	0.31±0.01^{a}	0.30±0.03^{a}	0.35±0.05^{a}	0.37±0.06^{a}	0.37±0.04^{a}	0.42±0.04^{a}	0.35±0.00^{a}	0.42±0.06^{a}	0.40±0.04^{a}	n.s.
Rs	0.90±0.02^{g}	1.10±0.02^{f}	1.30±0.03^{e}	1.10±0.01^{f}	0.80±0.04^{g}	3.50±0.05^{c}	2.40±0.04^{d}	2.50±0.04^{d}	4.30±0.03^{b}	5.20±0.05^{a}	***
Glucose	0.23±0.00^{d}	0.11±0.01^{ef}	0.14±0.00^{e}	0.10±0.00^{f}	0.00±0.00^{g}	0.50±0.02^{c}	0.20±0.01^{d}	0.10±0.00^{f}	1.00±0.01^{b}	1.30±0.02^{a}	***
Fructose	0.41±0.04^{d}	0.29±0.02^{de}	0.22±0.02^{ef}	0.21±0.01^{ef}	0.10±0.00^{f}	0.75±0.06^{b}	0.60±0.03^{c}	0.40±0.04^{d}	0.87±0.09^{b}	1.25±0.02^{a}	***
Ethanol	13.65±0.04^{def}	13.84±0.03^{bcd}	13.96±0.05^{abc}	13.99±0.04^{ab}	14.04±0.02^{a}	13.74±0.04^{de}	13.71±0.11^{de}	13.81±0.09^{cd}	13.63±0.05^{ef}	13.51±0.07^{f}	***
Glycerol	6.55±0.04^{ab}	6.69±0.03^{a}	5.98±0.04^{d}	6.68±0.06^{a}	5.95±0.06^{d}	6.24±0.00^{c}	6.56±0.04^{ab}	5.75±0.05^{e}	6.43±0.06^{b}	5.91±0.08^{d}	***
Malic acid	1.52±0.02^{ab}	1.49±0.03^{ab}	1.58±0.05^{a}	1.47±0.04^{ab}	1.46±0.03^{b}	1.54±0.02^{ab}	1.46±0.07^{b}	1.32±0.05^{c}	1.26±0.02^{c}	1.26±0.00^{c}	***
Lactic acid	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	n.s.
Amm. N	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
Alpha-AN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	

Parameters	Vinification										
	3 month of steel aging										
	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**	**Trial 6**	**Trial 7**	**Trial 8**	**Trial 9**	**Trial 10**	**S.S.**
pH	3.23±0.01^{f}	3.30±0.00^{e}	3.31±0.00^{e}	3.35±0.01^{cd}	3.33±0.01^{de}	3.44±0.02^{a}	3.38±0.02^{bc}	3.38±0.02^{bc}	3.41±0.01^{ab}	3.40±0.00^{b}	***
TA	5.39±0.05^{a}	5.38±0.06^{a}	5.39±0.04^{a}	5.36±0.05^{a}	5.37±0.05^{a}	5.38±0.05^{a}	5.38±0.06^{a}	5.39±0.05^{a}	5.40±0.06^{a}	5.40±0.04^{a}	n.s.
VA	0.20±0.02^{b}	0.33±0.03^{a}	0.28±0.02^{ab}	0.35±0.04^{a}	0.35±0.04^{a}	0.35±0.04^{a}	0.37±0.06^{a}	0.35±0.04^{a}	0.36±0.02^{a}	0.38±0.04^{a}	*
Rs	0.67±0.01^{e}	0.88±0.08^{d}	0.77±0.07^{de}	0.65±0.05^{e}	0.42±0.02^{f}	1.18±0.08^{c}	1.23±0.03^{c}	1.14±0.04^{c}	1.45±0.05^{b}	1.78±0.08^{a}	***
Glucose	0.11±0.01^{ab}	0.02±0.02^{b}	0.03±0.03^{b}	0.01±0.01^{b}	0.00±0.00^{b}	0.09±0.05^{ab}	0.02±0.01^{b}	0.00±0.00^{b}	0.21±0.11^{a}	0.18±0.06^{a}	***
Fructose	0.17±0.03^{a}	0.10±0.02^{ab}	0.06±0.03^{ab}	0.08±0.08^{ab}	0.02±0.01^{b}	0.12±0.02^{ab}	0.09±0.03^{ab}	0.05±0.03^{ab}	0.10±0.04^{ab}	0.17±0.06^{a}	*
Ethanol	13.63±0.09^{bc}	13.81±0.05^{abc}	13.94±0.14^{ab}	13.97±0.15^{ab}	14.02±0.02^{a}	13.72±0.00^{abc}	13.72±0.14^{abc}	13.80±0.15^{abc}	13.64±0.11^{bc}	13.50±0.13^{c}	*
Glycerol	6.54±0.08^{ab}	6.68±0.09^{a}	6.00±0.05^{de}	6.67±0.07^{a}	5.97±0.06^{ef}	6.22±0.04^{cd}	6.57±0.08^{ab}	5.77±0.07^{f}	6.44±0.08^{bc}	5.90±0.10^{ef}	***
Malic acid	1.51±0.09^{ab}	1.50±0.09^{abc}	1.59±0.08^{a}	1.46±0.07^{abc}	1.44±0.05^{abc}	1.54±0.07^{ab}	1.45±0.08^{abc}	1.32±0.09^{bc}	1.26±0.11^{c}	1.26±0.05^{c}	**
Lactic acid	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	n.s.
Amm. N	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
Alpha-AN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	

Parameters	Vinification Bottling Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10	S.S.
pH	3.22±0.00^{e}	3.28±0.01^{d}	3.31±0.02^{cd}	3.35±0.03^{bc}	3.35±0.02^{bc}	3.40±0.01^{a}	3.37±0.02^{ab}	3.39±0.00^{ab}	3.40±0.01^{a}	3.40±0.01^{a}	***
TA	5.36±0.02^{a}	5.35±0.00^{a}	5.36±0.02^{a}	5.35±0.03^{a}	5.35±0.01^{a}	5.35±0.11^{a}	5.33±0.07^{a}	5.34±0.08^{a}	5.37±0.10^{a}	5.36±0.12^{a}	n.s.
VA	0.26±0.01^{a}	0.30±0.01^{a}	0.30±0.00^{a}	0.33±0.02^{a}	0.35±0.01^{a}	0.36±0.00^{a}	0.39±0.04^{a}	0.34±0.01^{a}	0.40±0.12^{a}	0.39±0.10^{a}	n.s.
Rs 1	0.21±0.05^{abcd}	0.38±0.13^{ab}	0.30±0.04^{abc}	0.18±0.00^{cd}	0.07±0.01^{d}	0.41±0.12^{a}	0.21±0.02^{bcd}	0.18±0.00^{cd}	0.26±0.08^{abcd}	0.20±0.03^{bcd}	***
Glucose	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	n.s.
Fructose	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	n.s.
Ethanol 2	13.65±0.19^{bcd}	13.82±0.14^{abc}	13.95±0.10^{ab}	13.97±0.11^{ab}	14.02±0.10^{a}	13.73±0.05^{abcd}	13.72±0.06^{bcd}	13.80±0.03^{abc}	13.64±0.02^{cd}	13.50±0.06^{d}	**
Glycerol 3	6.50±0.07^{abc}	6.69±0.08^{a}	5.99±0.08^{d}	6.69±0.07^{a}	5.96±0.06^{d}	6.27±0.06^{c}	6.58±0.05^{ab}	5.77±0.03^{d}	6.45±0.17^{bc}	5.91±0.00^{d}	**
Malic acid	1.50±0.03^{bc}	1.48±0.02^{bcd}	1.58±0.01^{a}	1.45±0.00^{cd}	1.43±0.01^{d}	1.52±0.03^{b}	1.45±0.02^{cd}	1.30±0.03^{e}	1.25±0.01^{ef}	1.24±0.01^{f}	**
Lactic acid	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	n.s.
Amm. N	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
Alpha-AN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	

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Parameters	Vinification 5 month of steel aging Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10	S.S.
pH	3.25±0.01^{e}	3.27±0.02^{de}	3.30±0.00^{cd}	3.37±0.02^{b}	3.32±0.01^{c}	3.42±0.02^{a}	3.38±0.01^{ab}	3.39±0.01^{ab}	3.40±0.01^{ab}	3.38±0.01^{ab}	***
TA	5.38±0.03^{a}	5.36±0.05^{a}	5.37±0.04^{a}	5.35±0.02^{a}	5.37±0.04^{a}	5.36±0.04^{a}	5.36±0.06^{a}	5.36±0.06^{a}	5.39±0.02^{a}	5.38±0.04^{a}	n.s.
VA	0.22±0.02^{b}	0.32±0.05^{ab}	0.29±0.04^{ab}	0.34±0.03^{a}	0.36±0.04^{a}	0.34±0.03^{a}	0.38±0.02^{a}	0.35±0.05^{a}	0.38±0.03^{a}	0.37±0.02^{a}	**
Rs	0.22±0.11^{bcd}	0.43±0.08^{abc}	0.32±0.03^{bc}	0.20±0.02^{cd}	0.08±0.01^{d}	0.62±0.02^{a}	0.34±0.17^{bc}	0.28±0.06^{bcd}	0.31±0.08^{bcd}	0.45±0.02^{ab}	***
Glucose	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	n.s.
Fructose	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	n.s.
Ethanol	13.64±0.15^{bcd}	13.83±0.10^{abc}	13.95±0.11^{ab}	13.98±0.12^{ab}	14.01±0.11^{a}	13.72±0.11^{abcd}	13.73±0.08^{abcd}	13.79±0.06^{abcd}	13.62±0.00^{cd}	13.50±0.14^{d}	**
Glycerol	6.51±0.07^{abc}	6.69±0.07^{a}	6.01±0.06^{d}	6.66±0.07^{a}	5.98±0.06^{d}	6.31±0.06^{c}	6.55±0.07^{ab}	5.76±0.06^{e}	6.42±0.06^{bc}	5.88±0.06^{de}	**
Malic acid	1.51±0.05^{a}	1.48±0.06^{a}	1.58±0.11^{a}	1.45±0.05^{ab}	1.44±0.00^{ab}	1.54±0.03^{a}	1.45±0.05^{ab}	1.31±0.02^{bc}	1.25±0.03^{c}	1.26±0.03^{c}	*
Lactic acid	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	n.s.
Amm. N	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
Alpha-AN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	

CHAPTER 5

Improving the Aromatic Profiles of Catarratto Wines: Impact of *Metschnikowia pulcherrima* and Glutathione-Rich Inactivated Yeasts

ABSTRACT

Catarratto is one of the most widely cultivated grape varieties in Sicily. It is an indigenous non-aromatic white grape variety. Despite its widespread use in winemaking, knowledge of the aroma and chemical and microbiological properties of Catarratto wines is quite limited. The influence of *Metschnikowia pulcherrima* combined with *Saccharomyces cerevisiae* on the aromatic expression of Catarratto wines was investigated with and without the addition of glutathione-rich inactivated yeast. The substance is a natural specific inactivated yeast with a guaranteed glutathione level used to limit oxidative processes. The aromatic profiles of the final wines were determined through analysis of the volatile organic compounds using a solid phase microextraction technique that identified 26 aromatic compounds. The addition of *M. pulcherrima* in combination with the natural antioxidant undoubtedly increased the aromatic complexity of the wines. Dodecanal was exclusively detected in the wines processed with glutathione-rich inactivated yeasts. Furthermore, the presence of this natural antioxidant increased the concentration of six esters above the perception threshold. Sensory analysis was also performed with a panel of trained judges who confirmed the aromatic differences among the wines. These results suggest the suitability of glutathione-rich inactivated yeasts for determining the oxidative stability of Catarratto wines, thus preserving its aromatic compounds and colour.

5.1 INTRODUCTION

Identifying new strategies for the aromatic enhancement of wine produced from non-aromatic grape varieties is one of the major objectives of oenological microbiology research. During alcoholic fermentation (AF), yeast metabolic activity and winemaking techniques determine the biosynthesis of several products that influence wine aroma (Izquierdo Cañas et al., 2014; Alfonzo et al., 2021). The application of non-conventional yeasts isolated during the fermentation of traditional fermented beverages represents an alternative for producing a variety of alcoholic beverages (Varela et al., 2016), including wine.

Raw materials with a high sugar content if subjected to spontaneous fermentation can provide potential starters with interesting traits. Wild *Saccharomyces* and non-*Saccharomyces* spp. may generate flavour profiles with desirable characteristics to be applied at industrial level (Ciani and Comitini, 2011). Several authors have isolated strains of *Saccharomyces cerevisiae* from high-sugar-containing matrices such as manna, honey and honey by-products (Francesca et al., 2022; Guarcello et al., 2019) that were successfully applied in experimental Catarratto cultivar winemaking (Alfonzo et al., 2021).

Recently, the controlled inoculation of selected non-*Saccharomyces* and *S. cerevisiae* strains has permitted the production of higher quality wines. The current trend is to exploit non-*Saccharomyces*/*Saccharomyces* sequential inoculation to achieve a positive impact in terms of aroma (Lappa et al., 2020). The result is closely related to the species of the multi-starter cultures involved in the sequential inoculum (Lappa et al., 2020).

The final aroma of wines can be modulated not only by *Saccharomyces* but also by non-conventional yeasts. The metabolic impact of non-*Saccharomyces* yeasts during the early stages of fermentation is sufficient to trigger significant changes to the wine's volatile profile; they are suitable for inoculation as co-starters with strains of *S. cerevisiae* (Rossouw and Bauer, 2016).

Fermentation processes using mixed strains with the sequential addition of non-*Saccharomyces* and *S. cerevisiae* strains tend to reproduce what happens naturally during spontaneous wine fermentation concerning population dynamics (Gonzalez and Morales, 2022). Indeed, the levels of non-*Saccharomyces* yeast populations are reduced over time, leaving space for *S. cerevisiae* to dominate and conclude the AF (Fleet, 2003).

The use of non-*Saccharomyces* yeasts has improved the primary aromas of wines, as the production of specific enzymes enables the precursors present in the must to release volatile molecules. Their activity also affects secondary aromas through the production of volatile organic compounds (mainly alcohols and esters) that can influence typical aromatic expressions such as fruity notes (Gonzalez and Morales, 2022).

The impact determined by sequential inoculation can influence various aspects of wine characteristics. In recent oenological studies, strains belonging to the species *L. thermotolerans* and *Starmerella bacillaris* in sequential inoculation with *S. cerevisiae* achieved wines with a significant amount of lactic acid and glycerol, respectively, while strains of *Torulospora delbrueckii* and *Hanseniaspora uvarum* in mixed cultures and in sequential inoculation with *S. cerevisiae*, on the other hand, seemed to influence the composition in terms of higher alcohol and ester contents (Lappa et al., 2020).

Among the non-*Saccharomyces* yeasts, *Metschnikowia pulcherrima* is one of the species most abundant in the initial phase of AF of grape musts. In mixed cultures with *S. cerevisiae*, *M. pulcherrima* rapidly declines due to its low resistance to the ethanol produced by *S. cerevisiae* (Wang et al., 2016). Some strains of *M. pulcherrima* are known to synthesize fruity esters and can increase the concentrations of terpenes or thiols generally masked by higher alcohols (Benito et al., 2015). Non-aromatic grape varieties lack varietal aromatic precursors (terpenes or thiols) and the presence of fruity aromas (pineapple) due to the fact that an increased ethyl octanoate content determines a positive sensorial impact. Some thiol precursors such as 4-methyl-4-sulfanylpentan-2-one, as well as those produced by *S. cerevisiae*, can be synthesized by *M. pulcherrima* at much higher concentrations, thus significantly influencing the characteristics of wine (Ruiz et al., 2018). Recently, *M. pulcherrima* was successfully used in a sequential inoculation with *S. cerevisiae* for a reduction in ethanol content in Merlot wines (Aplin et al., 2021) and to improve the aromatic complexity in Shiraz and Cabernet Sauvignon wines (Varela et al., 2021).

A solution aimed at limiting the loss of aromaticity in white wines is represented by the addition of a natural antioxidant such as glutathione at the beginning of the vinification process (Kritzinger et al., 2013). The application of glutathione during winemaking has positive effects on the colour and aroma stability of white wines (Badea and Antoce, 2015). The glutathione content naturally present in musts is relatively low, and its quantities are closely related to the reactions that characterise the fermentation process as well as the metabolic activities of the yeasts (Aplin et al., 2021). Its use in oenology provides considerable advantages as its antioxidant activity is capable of limiting browning in white grape must as it inhibits polyphenol polymerisation and severely limits the production of compounds such as sotolone that give wine a fenugreek or curry odour (Binati et al., 2022). Glutathione's degree of protection also extends to the aromatic molecules in wines, especially the esters, volatile thiols and terpenes produced by yeasts during alcoholic fermentation that are present in greater quantities when glutathione is added to the must (Lyu et al., 2021). Some sulphite-free wines are produced by exploiting the antioxidant activity of glutathione in place of potassium

metabisulphite, meeting the needs of consumers who are more sensitive to the negative health effects of sulphur dioxide (Ferrer-Gallego et al., 2017).

This study focused on the potential of non-*Saccharomyces* and *Saccharomyces* yeasts for the aromatic improvement of wine produced from non-aromatic grape varieties such as Catarratto. We evaluated the sequential inoculation of a commercial non-*Saccharomyces* yeast strain (*M. pulcherrima*) and *S. cerevisiae* SPF52 isolate from honey by-products to simulate what would occur during spontaneous fermentation. Secondly, we assessed the ability of exogenous glutathione addition during fermentation in the form of inactivated yeast to influence the technological and aromatic properties of wine.

The aims of this research were to investigate: (i) the impacts of *M. pulcherrima* associated with *S. cerevisiae*; (ii) the effect of an antioxidant compound on the aroma and sensory profiles of Catarratto wine; and (iii) the volatile organic compound composition of Catarratto white wine.

5.2 MATERIALS AND METHODS

5.2.1 Experimental Drawing and Sampling

The experimental design (Fig. 1) was composed of four treatments: T1, sequential inoculum of FLAVIA® MP346/*S. cerevisiae* SPF52; T2, the use of Glutastar™ to the bulk must and sequential inoculum with FLAVIA® MP346/ *S. cerevisiae* SPF52; C1, single inoculum of *S. cerevisiae* SPF52; C2, the addition of Glutastar™ and fermentation by *S. cerevisiae* SPF52.

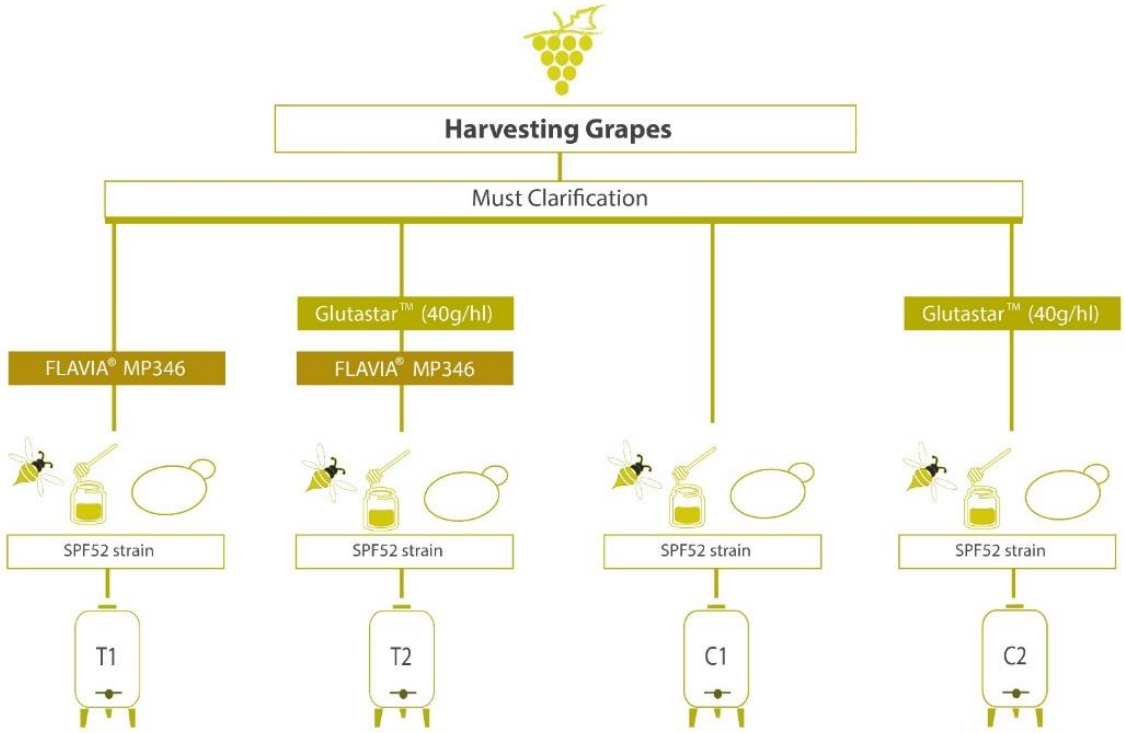

Figure 1. Experimental plan of wines obtained from Catarratto grape must.

FLAVIA® MP346 is a pure culture of *M. pulcherrima* selected by the Universidad de Santiago de Chile (USACH) for its specific capacity to release enzymes with arabinofuranosidase activity (Ganga et al., 2013). Glutathione-rich inactivated yeast (GIY) is an inactivated yeast mass with a guaranteed glutathione level (Bahut et al., 2020). GIY and FLAVIA® MP346 were provided by Lallemand Inc. (Castel D'Azzano, Verona, Italy). The *S. cerevisiae* SPF52 strain used in this study belonged to the yeasts collection of the Department of Agricultural, Food and Forestry Sciences (SAAF; University of Palermo, Italy); it was isolated from fermented honey by-products (Gaglio et al., 2017) and selected for its high performance in fermenting Catarratto grape must (Alfonzo et al., 2021).

Samples were collected from clarified bulk must just after the inoculum of *M. pulcherrima* MP346, after the inoculation of *S. cerevisiae* SPF52, during AF (day 3, 6, 12 and 18), during ageing in a steel tank (1, 3 and 5 months) and at bottling. All samples were transported at 4 °C into a portable fridge and subjected to analysis within 24 h after collection.

5.2.2 Winemaking

After hand harvesting, grapes were stemmer-crushed and treated with 2 g/hL of potassium metabisulphite (Chimica Noto s.r.l., Partinico, Italy). Clarification of the must was carried out at 4 °C for one day by using pectolytic enzymes [Lallzyme® C-Max (Lallemand Inc. Italia, Castel D'Azzano, Verona, Italy); dosage: 4 g/hL].

T1 and T2 were inoculated with FLAVIA® MP346 at 25 g/hL when the clarified must had reached a temperature of 16 °C. The strain *S. cerevisiae* SPF52 was used in a liquid concentrated form [about 7.00×10^{10} colony-forming units (CFU)/g].

After 24 h, T1 and T2 were inoculated with *S. cerevisiae* SPF52 (20 g/hL), while the controls, C1 and C2, were inoculated immediately with the SPF52 strain at the same dose. Before the inoculum of the starter yeast, GIY (40 g/hL) was added to treatments T2 and C2. The organic nutrient Stimula Chardonnay™ (SC; Lallemand Inc. Italia, Castel D'Azzano, Verona, Italy) was added to all tanks (40 g/hL) prior to *S. cerevisiae* yeast inoculation. The use of Stimula Chardonnay™ with *S. cerevisiae* SPF52 was chosen because of the results obtained by previous vinifications on Catarratto wines (Alfonzo et al., 2021). The fermentation was carried out at 18 °C in 12 steel tanks with a volume of 2.5 hL each. At the end of AF, the wines were cold-settled, their yeast lees were racked off and they were transferred into stainless-steel tanks at 15 °C and topped with nitrogen to avoid oxidation until bottling. During ageing, malolactic fermentation was prevented by keeping the free SO_2 values above 35 mg/L until bottling. Tartaric stability was ensured through the addition of 8 g/hL of metatartaric acid (Chimica Noto s.r.l., Partinico, Italy). Each treatment was performed in triplicate.

5.2.3 Monitoring Yeast Populations

During the AF, all must samples were microbiologically analysed to determine the total yeast concentration (TY) using the protocol described by Pallmann et al. (2001). *Saccharomyces* and non-*Saccharomyces* yeasts colonies were distinguished as reported by Valera et al. (2016). The analyses were conducted in triplicate.

5.2.4 Yeast Collection and Genotypic Characterization

Yeasts were isolated from WL medium, purified on the same medium and then subjected to morphological analysis, as reported by Pallmann et al. (2001), and genotypic characterisation.

Genomic DNA for PCR assays was prepared from yeast isolates after growth in YPD broth media at 25 °C for 48 h. Cells were harvested, and DNA was extracted using the InstaGene Matrix kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. According to Sinacori et al. (2014), yeasts were discriminated by RFLP of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene. Species-level identification of each group was confirmed by sequencing the D1/D2 region of the 26S rRNA gene following the procedure described by Guarcello et al. (2019). DNA sequencing reactions were performed at AGRIVET (University of Palermo, Italy). Sequences were manually corrected using Chromas 2.6.2. (Technelysium Pty Ltd., Australia). Nucleotide sequences were compared to GenBank sequences through BLASTn searches.

5.2.5 Dominance of S. cerevisiae *and M. pulcherrima isolates*

The dominance of the inoculated *S. cerevisiae* and *M. pulcherrima* was verified as reported by Legras and Karts (2003), and Barbosa et al. (2012). Fingerprinting profiles were analysed as reported by Alfonzo et al. (2020).

5.2.6 Must and Wine Analysis

5.2.6.1 Chemical Properties

Chemical properties such as sugars (glucose and fructose, g/L) and residual sugars (g/L), yeast-assimilable nitrogen (ammoniacal nitrogen and alpha-amino nitrogen, g/L), organic acids (malic acid, lactic acid and acetic acid, g/L), glycerol (g/L) and ethanol (% v/v) were quantified during and at the end of the AF using the methods described by Prestianni et al. (2022).

The pH values were measured using a pH 70 Vio FOOD pH meter (XS Instruments, Carpi, Italy), and total acidity (g/L of tartaric acid) was detected through the procedure proposed by OIV-MA-AS313-01 (2020). Free and total sulphur dioxide was determined in accordance with Alfonzo et al. (2021).

The analysis of the chemical composition of wines analysed included ash alkalinity, buffering power, total extract, total phenols, flavans reactive to 4-(dimethylamino)cinnamaldehyd, oxidation tests, total phenols and extracts were performed as reported by Alfonzo et al. (2021).

5.2.6.2 Volatile Organic Compounds

All reagents were of analytical grade. Ethyl benzoate was purchased from Sigma-Aldrich (82024 Taufkirchen, Germany). n-Alkane standards (C8 to C40) were purchased from Aldrich Chemical Co. (St. Louis, MO, USA).

An automatic SPME holder (Supelco®, Bellefonte, PA, USA) was used for evaluation of VOC profiles. A fiber 50/30 µm divinylbenzene (DVB)/carbowax (CAR)/polydimethylsiloxane (PDMS) of 1 cm length was used for fractionation of volatile compounds from the headspace (HS) of the conditioned wines. Prior to its use, the fiber was conditioned for 1.5 h at 250 °C in the inlet of the gas chromatograph according to Supelco® Co. Analysis of wine aroma was performed following a slightly modified method proposed by Sagratini et al. (2012). For extraction, each aliquot (10 mL) of the wine samples and 2.2 g of NaCl were placed into a 20 mL vial (75.5 × 22.5 mm) (Supelco, Bellefonte, PA, USA). The samples were equilibrated at 35 °C for 15 min, stirring at 600 rpm. The SPME fiber was exposed to the wine samples for 30 min in the headspace of the sample kept at 35 °C. The flavour compounds were desorbed for 5 min from the fiber to the column through a splitless injector at 250 °C. The SPME fibres were cleaned to prevent cross-contamination by inserting the fibre into the auxiliary injection port at 250 °C for 30 min and were then re-used. All samples were prepared and analysed in triplicates in standard 20 mL volume headspace vials.

Semi-quantification of volatile compounds was performed using an Agilent 7000C GC system fitted with a fused silica apolar DB-5MS capillary column (30 m × 0.25 mm i.d.; 0.25 µm film thickness) (Santa Clara, CA, USA) coupled to an Agilent triple quadrupole Mass Selective Detector MSD 5973. The ionization voltage was 70 eV, the electron multiplier energy was 2000 V and the transfer line temperature was 270 °C. The solvent delay was 0 min. Helium was the carrier gas (1 mL/min). The temperature programme was from 35 °C (0 min) to 270 °C at 8 °C min^{-1}, from 270 °C (2 min) to 300 °C at 15 °C min^{-1} and then 300 °C for 5 min. Volatile compounds were injected at 250 °C automatically in the splitless mode. Linear retention indices were calculated using *n*-alkanes as reference compounds. For the analysis of alkane solutions (C_8-C_{40}) (Sigma-Aldrich, USA), the injector mode was set in the 10:1 split mode. The individual peaks were analysed using the GC-MSolution package, version 2.72. Identification of compounds was carried out using the Adams, NIST 08, Wiley 9 and FFNSC 2 mass spectral databases.

For each volatile organic compound identified, the odour activity value (OAV) as described by Butkhup et al. (2011) was calculated in order to assess which VOCs contributed significantly to the odour series characterising each wine.

5.2.7 Sensory Analysis

A total of 15 judges (7 women and 8 men, ranging from 25 to 46 years old) with previous experience in wine tasting participated in the evaluation of the sensory profile of the wines carried out as described by Jackson (2017). The judges were subjected to preliminary tests to determine their sensory performances in terms of their basic taste and the aromas associated with the wines. The sensory profiles of the wines obtained from Catarratto grapes were constructed using two selected panels each of ten judges trained over several sessions. The fifteen panellists compared the four experimental wines during different sessions. They consensually generated 36 sensory descriptive attributes for appearance, odour, flavour, taste, overall quality and finish in several sessions. The set of attributes were: appearance (green reflexes and yellow colour); odour (banana, citrus, fatty, floral, fruity, grape, green almond, intensity, pear, persistence, pineapple and sweet fruit); taste (bitter, salty, sour and sweet); mouthfeel (body or balance); flavour (banana-like, cherry pit, citrus, fruity, intensity, mandarin orange, persistence, pineapple, sweet apple and sweet fruit), overall quality (flavour, mouth-feel, odour and taste) and finish (after-smell and after-taste). The different descriptors were quantified using a 9-point intensity scale as reported by Alfonzo et al. (2020).
The sensory test was carried out following the procedures described by Alfonzo et al. (2021).

5.2.8 Statistical Analysis

In order to determine statistically significant differences between the properties monitoring during the AF (chemical and technological data) and in the final wines (sensory analysis and VOCs composition), the ANOVA test was applied. Tukey's test was used for multiple mean comparisons (statistical significance: $p < 0.05$).
Multiple factor analysis (MFA) was carried out in order to distinguish the different treatments from the data acquired during the sensory analysis following the methodology reported by Alfonzo et al. (2021). Agglomerative hierarchical clustering (AHC) was performed to group the trials according to their dissimilarity, as measured by Euclidean distances and Ward's method.
In order to assess the existing correlation between the aromas detected during the sensory analysis and the VOCs with an odour activity value >1, a principal component analysis (PCA) was performed using the XLstat software version 2019.2.2 (Addinsoft, New York, NY, USA) for Excel.

5.3 RESULTS AND DISCUSSION

5.3.1 Microbial Growth Dynamic

The concentrations of yeasts (presumptive *Saccharomyces* (PS), non-*Saccharomyces* (NS) and presumptive *Metschnikowia* (PM)) during the alcoholic fermentation (AF) are shown in Figure 2. The PS and NS levels in the Catarratto must were around four logarithmic cycles (Figure 2a, b), while no isolates attributable to the genus *Metschnikowia* were detected (Figure 2c). Catarratto musts are

usually poor for the presence of indigenous *Metschnikowia* spp., although in musts from Sicilian Catarratto grapes, *M. pulcherrima* has been isolated at percentages ranging from 0.2 to 1.1% (Romancino et al., 2008).

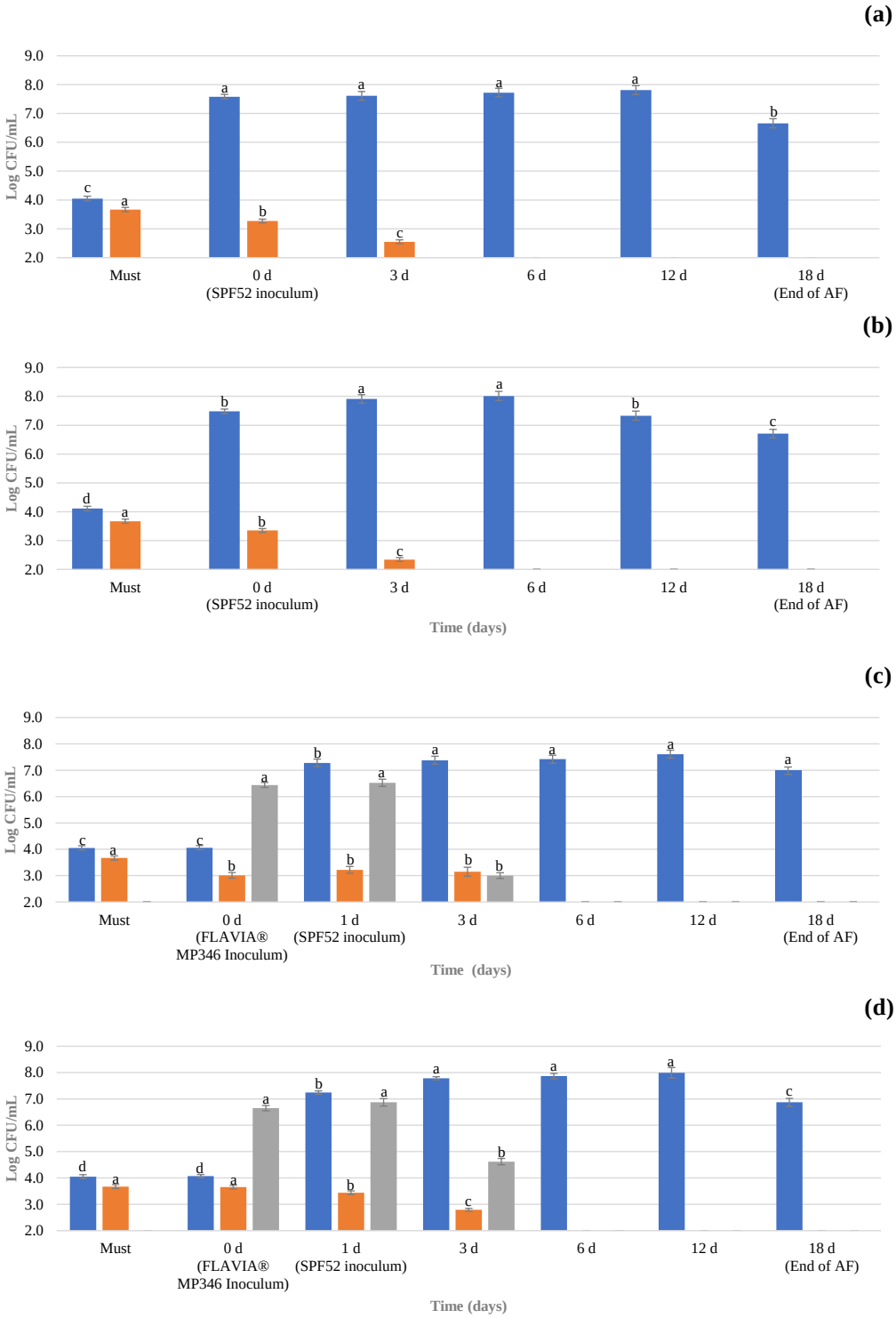

Figure 2. Microbiological concentration (Log CFU/mL) of samples during alcoholic fermentation: (**a**) C1, single inoculum with *S. cerevisiae* SPF52; (**b**) C2, glutathione-rich inactivated yeasts and single inoculum with *S. cerevisiae* SPF52; (**c**) T1, sequential inoculum with *M. pulcherrima* MP346/*S. cerevisiae* SPF52; (**d**) T2, glutathione-rich inactivated yeasts and sequential inoculum with *M. pulcherrima* MP346/*S. cerevisiae* SPF52. For each microbiological group, different letters indicate statistically significant values determined by using

Tukey's test ($p \leq 0.05$). Legends: ■, Presumptive *Saccharomyces*; ■, non-*Saccharomyces* (except *Metschnikowia* spp.); ■, Presumptive *Metschnikowia* spp.

The *M. pulcherrima* MP346 inoculum concentration in T1 and T2 was close to 6.5 Log CFU/mL. The concentration of PS after the SPF52 inoculum ranged from 7.3 (T1) to 7.6 (C1) Log CFU/mL in all treatments. On day 3 of AF, PS showed an increase to 7.4–8.0 Log CFU/mL for all trials. The NS populations were lower and in the range of 2.3–3.2 Log CFU/mL. The reduction in the NS yeast populations during AF is a known phenomenon attributable to several causes such as metabolite production by *S. cerevisiae*, nutrient limitation and low resistance to ethanol (Wang et al., 2016). The PM levels were 3.0 Log CFU/mL for T1 and 4.6 Log CFU/mL for T2 after 3 days of AF and were lower than the limit of detection in the C1 and C2 samples. Indeed, the lower microbial load of the PM populations observed at 3 days of AF in T1 and T2 compared with C1 and C2 could be due to the lower ethanol concentration detected in T1 and T2 (Table S1). At day 6 of AF, when the ethanol reached concentrations above 6% v/v, the PS values reached levels in the range of 7.0–8.0 Log CFU/mL, whereas both NS and PM were undetectable in any trials. The absence of *M. pulcherrima* in trials inoculated with the commercial preparation FLAVIA® MP346 (T1 and T2) could be due to the above-mentioned factors. Some authors have recorded a significant decrease in the concentration *of M. pulcherrima* after 9 days of AF when sequential inoculation with *S. cerevisiae* occurred (Ruiz et al 2018).

From the 12th day until the end of AF (18 d), the PS populations decreased slightly from 7.3–8.0 to 6.7–7.0 Log CFU/mL in all treatments. The microbiological count values for *S. cerevisiae* were found by Scacco et al. (2012) on Sicilian Catarratto musts inoculated with selected starter strains of the same species.

5.3.2 Molecular Analysis

In relation to the macro- and microscopic characteristics, 949 colonies were analysed; from these, 592 isolates showed the typical characteristics of yeasts belonging to the *Saccharomyces* genus. The amplicon size of the 5.8S-ITS region was around 850 bp and confirmed the presumptive species identity of *S. cerevisiae* for all isolates. The other isolates (n = 357) were assigned to the NS yeast group.

A total of 233 isolates were morphologically identified as *Metschnikowia* spp. and showed an ITS amplicon between 380 and 400 bp. The ITS amplicon sizes were equivalent to those reported in the literature for *M. pulcherrima*. The isolates of the PS group (n = 592) and PM (n = 233) were characterised by RFLP analysis of the 5.8S-ITS region.

The PS RFLP profiles were similar to those indicated by Granchi et al. (1999). Consequently, the PS group represented putative *S. cerevisiae*. The sizes of the RFLP profiles of the PM were equivalent to those described in the literature for the species *M. pulcherrima* (Granchi et al.,1999).

The different profiles may have been caused by the presence of native *S. cerevisiae*, although less representative, being present among the isolates obtained. Indeed, the PS count values detected before SPF52 inoculation (4.1 Log CF/mL) clearly explain the presence of eight additional Interdelta profiles. The Interdelta profile of *S. cerevisiae* SPF52 was the most frequently (>93%) isolated. The strain typing of *M. pulcherrima* was carried out by RAPD-PCR. The results from these analyses showed that all the 233 isolates represented a unique strain.

The genotypic identification of the yeasts was completed by pairwise alignment of the D1/D2 sequence with the type of strain of each species (*S. cerevisiae* CBS 1171^T and *M. pulcherrima* CBS 5833^T). A comparison of the sequences of the D1/D2 region of the two reference strains showed a 100% similarity to the sequences of the type strains of each species, confirming the identification obtained by the RFLP analysis.

5.3.3 Kinetics of the Main Oenological Properties

The fermentations carried out in the presence of *S. cerevisiae* SPF52 as the only inoculated strain (C1 and C2) and the corresponding trials with *M. pulcherrima* (T1 and T2) were able to conclude the AF as determined by the complete consumption of sugars.

The trends of the principal oenological data during AF are shown in Table S1. The fermentation was concluded in 18 days on average.

After 3 d of AF, differences in pH, TA and the concentrations of sugars, ethanol, ammonia nitrogen and alpha amine nitrogen were observed among the trials. The highest differences in the sugar, glycerol and ethanol contents were registered at day 6 of AF. Specifically, C2 showed the lowest values in residual sugars (58.79 g/L); glucose was 25.80 g/L and fructose was 32.99 g/L, and consequently, it showed the highest values of ethanol (8.44\% *v*/*v*). The glycerol contents observed in T1, T2 and C2 were similar (5.19–5.28 g/L), whereas the lowest values were found in C1 (5.06 g/L). This trend was observed until the 12th day of AF.

At the end of AF, the glucose concentrations ranged from 1.10 (T1 and T2) to 1.62 g/L (C1), whereas the fructose concentration was slightly higher and within the range of 1.39–2.60 g/L. No differences were observed for TA, whereas VA's values ranged from 0.27 (T1) to 0.31 (C1 and C2) g/L acetic acid. The pH values varied between treatments, where T1 and T2 had slightly lower values (3.41 and 3.43, respectively) when compared to both the control trials C1 (3.47) and C2 (3.51). The ethanol concentrations ranged between 11.35 and 11.43% (*v*/*v*); the comparison between the T1 and T2 and the C1 and C2 treatments showed no significant differences. In contrast, Contreras et al. (2015)

reported that some strains of *M. pulcherrima* are able to decrease the amount of ethanol by as much as 1% (*v*/*v*) during fermentation. An analysis of the ethanol production during AF a revealed lower ethanol production in the T1 and T2 trials after 3 d of AF. After AF, differences in the ethanol concentration between the different trials were not statistically significant. This phenomenon could be attributable to the presence of *M. pulcherrima* up to the 3rd day of AF (3.0–4.6 Log CFU/mL).

The malic acid levels decreased in all the treatments from an initial concentration of 1.90 g/L in the must to 1.28–1.50 g/L at the end of AF. Contrary to the reports of Ruiz et al. (2018), no decreases were recorded in T1 and T2 compared to C1 and C2, although these authors showed that in fermentations conducted with *M. pulcherrima*/*S. cerevisiae*, a decrease in the malic acid content of 0.2 g/L occurred in the wines. Lactic acid was absent in all the trials. The highest concentration of glycerol was found in C2 (6.57 g/L), and lower values (>5 g/L) were detected in the other wines. In this case, the sequential inoculum with *M. pulcherrima*/*S. cerevisiae* did not produce an increase in the glycerol concentration, in contrast to what has been observed in white wines made with the Verdejo variety (Ruiz et al., 2018).

During the five months of ageing in stainless steel tanks, there were no substantial changes in the monitored chemical properties (Table S2). There was a decrease in residual sugars, glucose and fructose, and all the other properties remained constant or showed minimal variations.

5.3.4 Oenological Data Analysis

The values of the physico-chemical properties of the wines are reported in Table 1.

The free and total SO_2 values were variable in the different wines. In particular, the highest free SO_2 values were observed in T1 and C2 (29 mg/L), while the highest total SO_2 value was observed in T1 (128 g/L).

Table 1. Oenological properties of four Catarratto wines.

Sample	SO_2 Free (mg/L)	SO_2 Total (mg/L)	Total Extract (g/L)	Total Phenols (mg/L Catechins)	p-DACA Flavans (mg/L Catechins)	Absorbance (420 nm)	Oxidation Test (%)	Buffer Power (meq/L)	Ash Alkalinity (meq/L)
T1	29.00 ± 1.00^{a}	128.00 ± 0.00^{a}	18.80 ± 0.09^{b}	92.74 ± 0.84^{a}	19.80 ± 0.08^{a}	0.079 ± 0.000^{c}	5.74 ± 0.09^{a}	31.25 ± 0.08^{b}	12.28 ± 0.05^{c}
T2	22.00 ± 0.00^{b}	115.00 ± 2.00^{b}	19.10 ± 0.07^{a}	93.47 ± 0.39^{a}	10.23 ± 0.11^{b}	0.080 ± 0.003^{c}	1.12 ± 0.03^{b}	31.25 ± 0.17^{b}	12.58 ± 0.07^{b}
C1	16.00 ± 2.00^{c}	109.00 ± 1.00^{c}	18.50 ± 0.08^{c}	84.38 ± 1.13^{c}	1.63 ± 0.08^{d}	0.101 ± 0.001^{a}	0.00 ± 0.00^{c}	31.32 ± 0.12^{b}	12.11 ± 0.07^{d}
C2	29.00 ± 1.00^{a}	105.00 ± 1.00^{d}	19.00 ± 0.11^{ab}	83.36 ± 0.67^{b}	3.12 ± 0.05^{c}	0.093 ± 0.002^{b}	0.00 ± 0.00^{c}	32.34 ± 0.13^{a}	13.43 ± 0.02^{a}
S.S.	***	***	***	***	***	***	***	***	***

Result indicates mean value ± standard deviation of three determinations. Abbreviations: S.S., statistical significance; T1, sequential inoculum with *M. pulcherrima* MP346/ *S. cerevisiae* SPF52; T2, glutathione-rich inactivated yeasts and sequential inoculum with *M. pulcherrima* MP346/ *S. cerevisiae* SPF52; C1, single inoculum with *S. cerevisiae* SPF52; C2, glutathione-rich inactivated yeasts and single inoculum with *S. cerevisiae* SPF52. Data in the same column followed by the same letter are not significantly different according to Tukey's test. *p* value: ***, $p < 0.001$.

The total extract was higher than the minimum legal values, which for white wines are fixed at >14 g/L (MIPAAF, 2017). In this study, all the wines exceeded this threshold; the values were in the range of 18.50–19.10 g/L for C1 and T2, respectively, which were comparable to the results described in Scacco et al. (2012) on Sicilian Catarratto wines.

The T1 and T2 trials retained a greater susceptibility to undergo oxidation than the C1 and C2 controls, which was independent of the use or non-use of GIY with oxidation test values of 5.74 and 1.12% (T1 and T2) and 0% (C1 and C2). The presence of *M. pulcherrima* therefore appeared to exert a bio-protective action by predicting oxidations at the pre-inoculation of *S. cerevisiae*. The decrease in polyphenols was not due to the synthesis of polysaccharides by *M. pulcherrima* but to its bioprotective and inhibiting action against grape tyrosinases. In fact, in the pre-fermentative stage in the C1 and C2 controls, the absence of *M. pulcherrima* favoured a significant increase in the optical density at 420 nm. At the same time, in the same controls there would have been a significant decrease in the total polyphenols resulting from the decrease in the phenolic class of the ortho-diphenols detected by means of the p-DACA reagent. The total polyphenol content was independent of the presence/absence of GIY. The null POM test values observed in the controls C1 and C2 may be due to a series of oxidation reactions of polyphenolic compounds that not even the addition of GIY in T2 was able to limit. The colonisation of the must by *M. pulcherrima* in the pre-fermentation phase probably led to a reduction in oxidative activities (Cinquanta et al., 2019).

Regarding buffering power, there were negligible variations, and only the wine C2 reached statistically significant values compared to the other trials. The highest buffering power value was in C2 (32.34 meq/L), which was comparable to those reported in the literature in Sicilian Catarratto wines (Cinquanta et al., 2019). This was similar for ash alkalinity, where C2 had the highest value (13.43 meq/L); the wine values were within the range of 11–17 meq/L, which were similar to those reported in the literature for white wines (Corona, 2010).

5.3.5 Volatile Organic Compound Composition

The samples showed differences mainly at the quantitative level. Twenty-six compounds were detected, and they were grouped into several classes: alcohols, ethers, aldehydes, ethyl esters of fatty acids (EEFAs), higher alcohol acetates (HAAs), ethyl esters of branched acids (EEBAs), miscellaneous esters (MEs) and other compounds. For clarity, the classification of esters was reported as described by Alfonzo et al. (2021). The most-concentrated compounds in all the samples were EEFAs (2318.98–1401.74 ppb) followed by MEs (233.83–98.84 ppb) and alcohols (36.48–18.84 ppb).

The must inoculated with *M. pulcherrima* MP346 produced less alcohols than the controls. 3-methyl-1-butanol and phenylethyl alcohol were the compounds detected in the highest quantity in C2. A

similar condition was observed in Riesling wines fermented by sequential inoculation with *M. pulcherrima*/*S. cerevisiae* (Benito et al., 2015).

The compound most commonly detected in the aldehyde class was dodecanal. In the wines produced in the absence of GYI, it reached a maximum concentration in C1 (11.06 ppb). Aldehydes, particularly decanal and dodecanal if they are present in high concentrations, can result in the appearance of an unpleasant "green" odour in wines (Liu et al., 2016).

Esters directly and indirectly influence wine aroma by means of highly varied interactions. The fermentation process applied significantly influences the quality and quantity of esters [43,44]. The wine samples inoculated with *M. pulcherrima* MP346 showed a higher content of esters (2318.98 ppb in T1 and 2056.15 ppb in T2) than the controls (1401.74 ppb in C1 and 1848.45 ppb in C2). Among the esters, the most representative was ethyl decanoate, which was produced in amounts over 1000 ppb in the wines inoculated with *M. pulcherrima* MP346. Indeed, in Riesling musts inoculated with the same commercial strain of *M. pulcherrima*, the quantities detected were half of those present in the Catarratto musts (Benito et al., 2015; Mislata et al., 2021).

The ethyl decanoate content reported by Benito et al. (2015) and Mislata et al. (2021) does not appear to have been impacted by the presence of *M. pulcherrima* MP346. However, in the Catarratto wines in this study, the levels of ethyl decanoate were significantly higher in the fermented wines with sequential inoculum.

A different situation was observed for ethyl octanoate, where the second EEFA was detected in greater quantities. Higher levels of ethyl octanoate were found in the experimental wines C1 and T1 without the addition of GIY. The effect of the glutathione-enriched inactivated yeast on ethyl octanoate was unclear, although these highly volatile hydrophobic esters exhibit significant variations in wines containing yeast-derivative products (Rigou et al., 2021). Among the 2-phenylethyl esters, two opposite situations were found for 2-phenylethyl hexanoate, which was detected only in C1 and C2, while 2-phenylethyl acetate was present exclusively in T1 and T2.

The determination of VOCs in the different wines is reported in Table 2.

However, the 2-phenylethyl acetate concentrations were lower than those determined for Riesling wines produced using *M. pulcherrima* MP346. Most likely, the strain of *S. cerevisiae* used as the starter for AF significantly influenced the levels of this ester (Xu et al., 2022).

Among the twenty-six VOCs, only seven compounds showed an OAV greater than 1 (Table 2), i.e., one aldehyde (dodecanal) and six esters (ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl 9-decenoate, 3-methyl-1-butanol acetate and methyl benzoate). Esters represent a group of compounds of considerable importance that are formed during AF through yeast metabolism and have a strong impact on the aromatic profile of wine (Tempère et al., 2018).

Table 2. Volatile organic compounds detected in the four Catarratto wines (all values in ppb).

t_R (min.s) [1]	LRI [1]	Compounds [2]	Aroma Description [3]	Odour Threshold [3]	C1[4] (OAV)	C2[4] (OAV)	T1[4] (OAV)	T2[4] (OAV)	S.S.[5]
		Σ Alcohols			33.00±1.32^{b}	36.48±1.46^{a}	18.84±0.75 ^{c}	19.19±0.76^{c}	***
10.55	758	3-methyl-1-butanol	Fusel	40000 [7]	26.65±1.07^{b} (<1)	29.59±1.18^{a} (<1)	12.82±0.51^{d} (<1)	15.59±0.62^{c} (<1)	***
36.49	1110	Phenylethyl alcohol	Floral, rose	125000 [8]	6.35±0.25^{b} (<1)	6.89±0.28^{a} (<1)	6.02±0.24^{b} (<1)	3.60±0.14^{c} (<1)	***
		Σ Ethers			4.75±0.19^{a}	4.16±0.17^{b}	4.24±0.17^{b}	2.53±0.10^{c}	***
32.14	1042	Ethyl benzyl ether	Tropical fruit, pineapple	unknown	4.75±0.19^{a} (n.d.[6])	4.16±0.17^{b} (n.d.[6])	4.24±0.17^{b} (n.d.[6])	2.53±0.10^{c} (n.d.[6])	***
		Σ Aldehydes			17.37±0.69^{a}	4.91±0.20^{c}	11.85±0.47^{b}	2.73±0.11^{d}	***
24.89	958	Benzaldehyde	Bitter almond, cherry	1500 [9]	6.31±0.25^{a} (<1)	4.91±0.20^{b} (<1)	3.60±0.14^{c} (<1)	2.73±0.11^{d} (<1)	***
37.08	1203	Decanal	Floral, orange peel citrus	0.1 [10]	tr (n.d.[6])	tr (n.d.[6])	tr (n.d.[6])	tr (n.d.[6])	n.d.[6]
56.38	1411	Dodecanal	Citrus, floral	2 [11]	11.06±0.44^{a} (5.53)	0.00±0.00^{c} (<1)	8.25±0.33^{b} (4.13)	0.00±0.00^{c} (<1)	***
		Σ EEFAs			1401.74±56.08^{d}	1848.45±73.94^{c}	2318.98±92.76^{a}	2056.15±82.25^{b}	***
27.64	989	Ethyl hexanoate	Sweet fruity, pineapple, green apple	5 [11]	33.79±1.35^{b} (6.76)	48.86±1.95^{a} (9.77)	27.85±1.11^{c} (5.57)	32.14±1.29^{b} (6.42)	***
37.44	1208	Ethyl octanoate	Fruity, pear	2 [11]	901.19±36.05^{a} (450.60)	730.52±29.22^{b} (365.26)	837.67±33.51^{a} (418.84)	596.78±23.87^{c} (298.39)	***
51.00	1379	Ethyl decanoate	Fruity, grape	200 [11]	273.88±10.96^{c} (1.37)	928.14±37.13^{b} (4.64)	1253.71±50.15^{a} (6.27)	1236.22±49.45^{a} (6.18)	***
54.98	1391	Ethyl 9-decenoate	Fruity, fatty	100 [15]	184.44±7.38^{ab} (1.84)	137.73±5.51^{c} (1.38)	199.75±7.99^{a} (2.00)	178.82±7.15^{b} (1.79)	***
67.44	1599	Ethyl dodecanoate	Sweet, waxy, floral	2000 [11]	8.44±0.34^{b} (<1)	3.20±0.13^{c} (<1)	0.00±0.00^{d} (<1)	12.19±0.49^{a} (<1)	***
		Σ HAAs			15.10±0.60^{b}	19.09±0.76^{a}	6.25±0.25^{d}	9.71±0.39^{c}	***
18.59	882	3-methyl-1-butanol acetate	Sweet fruity, banana	0.75 [8]	15.10±0.60^{b} (20.13)	19.09±0.76^{a} (25.45)	6.25±0.25^{d} (8.33)	9.71±0.39^{c} (12.95)	***
		Σ EEBAs			12.94±0.52^{b}	8.02±0.32^{c}	0.00±0.00^{d}	14.56±0.58^{a}	***
58.69	1447	Isopentyl octanoate	Fruity, pineapple, coconut	125 [14]	12.94±0.52^{b} (<1)	8.02±0.32^{c} (<1)	0.00±0.00^{d} (<1)	14.56±0.58^{a} (<1)	***
		Σ MEs			233.83±9.35^{a}	106.27±4.26^{bc}	118.12±4.74^{b}	98.84±3.96^{c}	***
6.80	611	Ethyl acetate	Ethereal, fruity	7500 [11]	65.36±2.61^{a} (<1)	9.10±0.36^{d} (<1)	33.72±1.35^{c} (<1)	38.29±1.53^{b} (<1)	***
34.79	1089	Methyl benzoate	Green almond	10 [12]	36.94±1.48^{a} (3.69)	25.00±1.00^{b} (2.50)	24.22±0.97^{b} (2.42)	14.93±0.60^{c} (1.49)	***
46.19	1268	2-phenylethyl hexanoate	Sweet, honey, floral	94 [13]	10.28±0.41^{a} (<1)	5.03±0.20^{b} (<1)	0.00±0.00^{c} (<1)	0.00±0.00^{c} (<1)	***
46.24	1542	2-phenylethyl acetate	Rose	250 [11]	0.00±0.00^{c} (<1)	0.00±0.00^{c} (<1)	3.69±0.15^{b} (<1)	5.45±0.22^{a} (<1)	***
		Σ Others			121.25±4.85^{a}	67.62±2.70^{b}	56.49±2.27^{c}	40.17±1.61^{d}	***
7.50	634	Tetrahydrofuran	Butter, caramel	unknown	40.89±1.64^{a} (n.d.[6])	35.68±1.43^{b} (n.d.[6])	26.44±1.06^{c} (n.d.[6])	23.34±0.93^{c} (n.d.[6])	***
18.14	876	1,3-dimethylbenzene	Plastic odour	unknown	12.08±0.48^{a} (n.d.[6])	8.03±0.32^{b} (n.d.[6])	4.14±0.17^{c} (n.d.[6])	2.89±0.12^{d} (n.d.[6])	***
29.59	1023	*o*-cymene	Herb	unknown	15.37±0.61^{a} (n.d.[6])	9.97±0.40^{b} (n.d.[6])	5.41±0.22^{c} (n.d.[6])	3.67±0.15^{d} (n.d.[6])	***
34.04	1097	1-butenyl benzene	unknown	unknown	2.81±0.11^{a} (n.d.[6])	2.05±0.08^{b} (n.d.[6])	1.40±0.06^{c} (n.d.[6])	0.76±0.03^{d} (n.d.[6])	***
44.34	1232	Benzothiazole	Sulfury, rubbery, vegetable	unknown	16.45±0.66^{a} (n.d.[6])	0.00±0.00^{b} (n.d.[6])	0.00±0.00^{b} (n.d.[6])	0.00±0.00^{b} (n.d.[6])	***
50.79	1302	6-ethyltetralin (isomer)	unknown	unknown	6.85±0.27 (n.d.[6])	3.10±0.12 (n.d.[6])	3.44±0.14 (n.d.[6])	tr (n.d.[6])	n.d.[6]
51.29	1311	6-ethyltetralin (isomer)	unknown	unknown	7.66±0.31 (n.d.[6])	0.00±0.00 (n.d.[6])	2.97±0.12 (n.d.[6])	tr (n.d.[6])	n.d.[6]
54.53	1368	2-ethenyl-naphtalene	unknown	unknown	11.50±0.46 ^{a} (n.d. [6])	6.36±0.25^{c} (n.d.[6])	10.83±0.43^{a} (n.d.[6])	9.51±0.38^{b} (n.d.[6])	***
59.64	1485	2,6-di-tert-butylquinone	unknown	unknown	7.64±0.31 (n.d. [6])	2.43±0.10 (n.d.[6])	1.86±0.07 (n.d.[6])	tr (n.d.[6])	n.d.[6]

[1] Linear retention index obtained through the modulated chromatogram reported for DB-5 MS apolar column;
[2] compounds are classified in order of retention time;
[3] Odor thresholds reported in the literature (http://www.thegoodscentscompany.com/; http://www.flavornet.org; http://www.odour.org.uk; Vilanova and Martinez, 2007);
[4] Relative amounts expressed as ppb with respect to calibration curve of ethyl benzoate;
[5] statistical significance;
[6] not determinable. Abbreviations: EEFAs: ethyl esters of fatty acids; HAAs: higher alcohol acetates; EEBAs: ethyl esters of branched acids; MEs: miscellaneous esters; OAV, odour activity value; tr: trace amount < 0.05%; T1, sequential inoculum with *M. pulcherrima* MP346/ *S. cerevisiae* SPF52; T2, glutathione-rich inactivated yeasts and sequential inoculum with *M. pulcherrima* MP346/ *S. cerevisiae* SPF52; C1, single inoculum with *S. cerevisiae* SPF52; C2, glutathione-rich inactivated yeasts and single inoculum with *S. cerevisiae* SPF52. Data in the same line followed by the same letter are not significantly different according to Tukey's test. *p* value: ***, $p < 0.001$.
[7] American Industrial Hygiene Association, 1989; [8] Darici et al., 2014; [9] Ferreira et al., 2004; [10] Duan et al., 2015; [11] Pino et al., 2010; [12] Tao and Zhang, 2010; [13] Zhang et al., 2020; [14] Zhang et al., 2013; [15] Xu et al 2022.

5.3.6 Sensory Analysis

The data from the sensory evaluation are shown in Table 3. The trials revealed some differences correlated with the presence/absence of *M. pulcherrima* MP346 and GIY.

The wines showed variability in terms of the attributes that defined appearance. The yellow colour values were in the range of 7.15–7.29, whereas, the green reflexes ranged between 3.63–4.04. The yellow colour values observed were higher than those shown by Scacco et al. (2012), while the ratings associated with the green reflections attribute were similar.

Table 3. Sensory score for experimental Catarratto wines.

Attributes	Trial				SEM	Statistical Significance	
	C1	C2	T1	T2		Judges	Wine
Appearance							
Yellow colour	7.28 [a]	7.15 [a]	7.21 [a]	7.29 [a]	0.01	n.s.	n.s.
Green reflexes	4.04 [a]	3.63 [b]	3.74 [b]	3.68 [b]	0.02	***	***
Odour							
Banana	3.63 [b]	3.94 [a]	2.79 [d]	3.15 [c]	0.07	***	***
Citrus	2.40 [a]	1.00 [c]	1.74 [b]	1.00 [c]	0.09	***	***
Fatty	1.35 [b]	1.22 [c]	1.62 [a]	1.32 [b]	0.02	***	***
Floral	2.53 [a]	1.00 [c]	1.97 [b]	1.00 [c]	0.10	***	***
Fruity	8.54 [c]	8.02 [d]	8.88 [a]	8.68 [b]	0.05	***	***
Grape	2.97 [c]	2.99 [c]	4.17 [a]	3.43 [b]	0.07	***	***
Green almond	7.67 [a]	6.84 [b]	6.77 [b]	5.71 [c]	0.11	***	***
Intensity	6.68 [c]	7.19 [b]	8.26 [a]	7.40 [b]	0.09	***	***
Pear	5.14 [b]	5.44 [a]	4.76 [d]	4.91 [c]	0.04	***	***
Persistence	7.11 [d]	8.64 [b]	8.12 [c]	8.97 [a]	0.10	***	***
Pineapple	3.62 [a]	3.63 [a]	2.96 [c]	3.44 [b]	0.04	***	***
Sweet fruit	7.25 [b]	7.57 [a]	5.75 [d]	6.59 [c]	0.10	***	***
Taste							
Sweet	3.48 [a]	3.59 [a]	2.78 [b]	2.68 [b]	0.06	***	***
Sour	5.38 [b]	5.37 [b]	8.11 [a]	8.24 [a]	0.21	***	***
Salty	5.70 [c]	5.85 [c]	7.99 [b]	8.39 [a]	0.18	***	***
Bitter	1.10 [c]	1.25 [b]	1.20 [b]	1.42 [a]	0.02	***	***
Mouthfeel							
Body	7.80 [c]	8.42 [b]	8.55 [b]	8.97 [a]	0.06	***	***
Balance	6.50 [d]	7.49 [c]	8.10 [b]	8.65 [a]	0.12	***	***
Flavour							
Banana-like	2.47 [b]	2.75 [a]	1.93 [d]	2.22 [c]	0.07	***	***
Cherry pit	3.67 [a]	3.84 [a]	3.77 [a]	2.70 [b]	0.07	***	***
Citrus	3.92 [a]	1.00 [b]	3.58 [a]	1.00 [b]	0.21	***	***
Fruity	6.15 [c]	6.26 [c]	7.79 [a]	6.80 [b]	0.10	***	***
Intensity	7.80 [c]	7.85 [c]	8.12 [b]	8.56 [a]	0.04	***	***
Mandarin orange	1.74 [a]	1.00 [c]	1.40 [b]	1.00 [c]	0.05	***	***
Persistence	7.70 [c]	8.78 [a]	7.97 [b]	8.94 [a]	0.08	***	***
Pineapple	7.11 [a]	6.89 [b]	6.86 [b]	6.14 [c]	0.05	***	***
Sweet apple	2.51 [c]	2.66 [c]	3.89 [a]	3.54 [b]	0.09	***	***
Sweet fruit	7.12 [b]	7.56 [a]	5.75 [d]	6.58 [c]	0.10	***	***
Overall quality	7.50 [d]	8.57 [b]	8.25 [c]	8.80 [a]	0.07	***	***
Flavour	6.98 [c]	8.81 [a]	8.11 [b]	8.91 [a]	0.11	***	***
Mouthfeel	7.20 [c]	8.32 [a]	7.88 [b]	7.97 [b]	0.06	***	***
Odour	7.20 [c]	8.86 [a]	8.01 [b]	8.74 [a]	0.10	***	***
Taste	7.01 [d]	7.54 [c]	7.82 [b]	8.11 [a]	0.06	***	***
Finish							
After-smell	6.80 [c]	8.15 [b]	8.21 [b]	8.50 [a]	0.10	***	***
After-taste	7.10 [c]	7.96 [b]	8.22 [b]	8.71 [a]	0.09	***	***

Results indicate mean value of three replicate sessions. Abbreviation: SEM, standard error of the mean; T1, sequential inoculum with *M. pulcherrima* MP346/ *S. cerevisiae* SPF52; T2, glutathione-rich inactivated yeasts and sequential inoculum with *M. pulcherrima* MP346/ *S. cerevisiae* SPF52; C1, single inoculum with *S. cerevisiae* SPF52; C2, glutathione-rich inactivated yeasts and single inoculum with *S. cerevisiae* SPF52. Data in the same line followed by the same letter are not significantly different according to Tukey's test. *p* value: ***, $p < 0.001$; n.s., not significant.

The T2 sample displayed a high score for 13 descriptors. The *M. pulcherrima* MP346 and GIY wine (T2) had the highest overall quality score (8.80). With regards to the odour attributes, the T1 and T2 wines showed the highest values for intensity and persistence, respectively. In addition, the T1 wine showed high scores for grape, fruity and fatty odours, the C1 wine showed high scores for citrus, floral, green almond and pineapple odours and the C2 wine was characterised by the presence of odours associated with banana, pear, pineapple and sweet fruit. The T2 wine was characterised by odour attributes with intermediate scores. In wines to which GIY was added (C2 and T2), citrus and floral odours were not perceived. Nevertheless, banana, citrus, floral, fruity and pear aromas were present in the Catarratto wines reported by Scacco et al. (2012) but at lower levels.

The descriptors associated with taste enabled discrimination of the wines. T1 and T2 showed high scores for sour flavours, whereas salty flavours showed high values in T2. In terms of mouthfeel, the T2 wine achieved high values for the body and balance attributes. No unpleasant odours or flavours were revealed for all the wines. The GIY increased the flavour intensity and persistence, confirming. the results described by Alfonzo et al. (2021). Indeed, the treatment with GIY in combination with *M. pulcherrima* MP346 significantly improved the aromatic complexity of the T2 wine.

The T2 wine showed high intensity and persistence scores for flavours. The sensory descriptors with high flavour values were pineapple (C1), sweet fruit (C2) and fruity (T1 and T2). The T2 wine also excelled compared to the other wines for after-smell (8.50) and after-taste (8.71).

Correlations of the sensory analyses were examined by MFA. The number of sensory attributes (thirty-six variables) for the four wines made it possible to define two factors with an Eigen > 1 that represented a total variance of 89.64%. The correlation between the variables and the MFA factor was expressed by the value of the contribution and cos^2. The incidence of the factors F1 (56.41%) and F2 (33.23%) on the total variance discriminated the different wines. Examining the loading plot (Fig. 3), eight variables were located in both quadrants I and IV, ten were located in quadrant II and eleven were located in quadrant III.

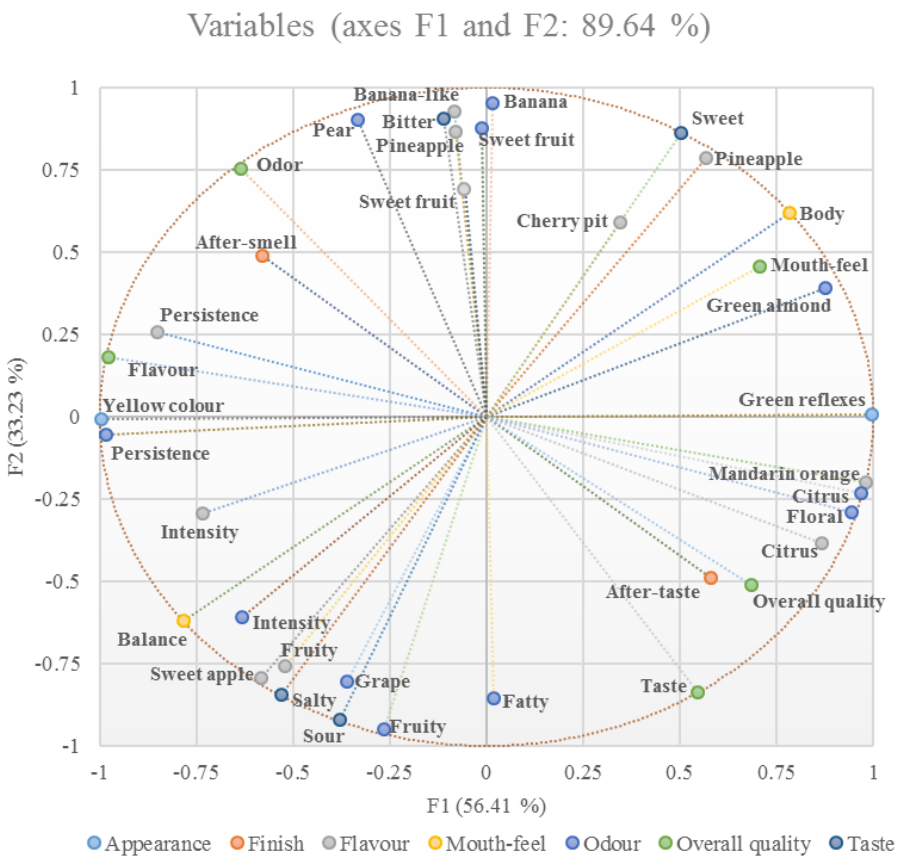

Figure 3. Variable loading plot of MFA.

Figure 4 reveals that the wines were clustered into three groups. In Figure 4a (MFA) and Figure 4b (AHCA), it is possible to observe how T1 and T2 represented a unique cluster. Interestingly, trial C1 did not cluster with trial C2. Indeed, the C1 and C2 trials represented a different cluster.

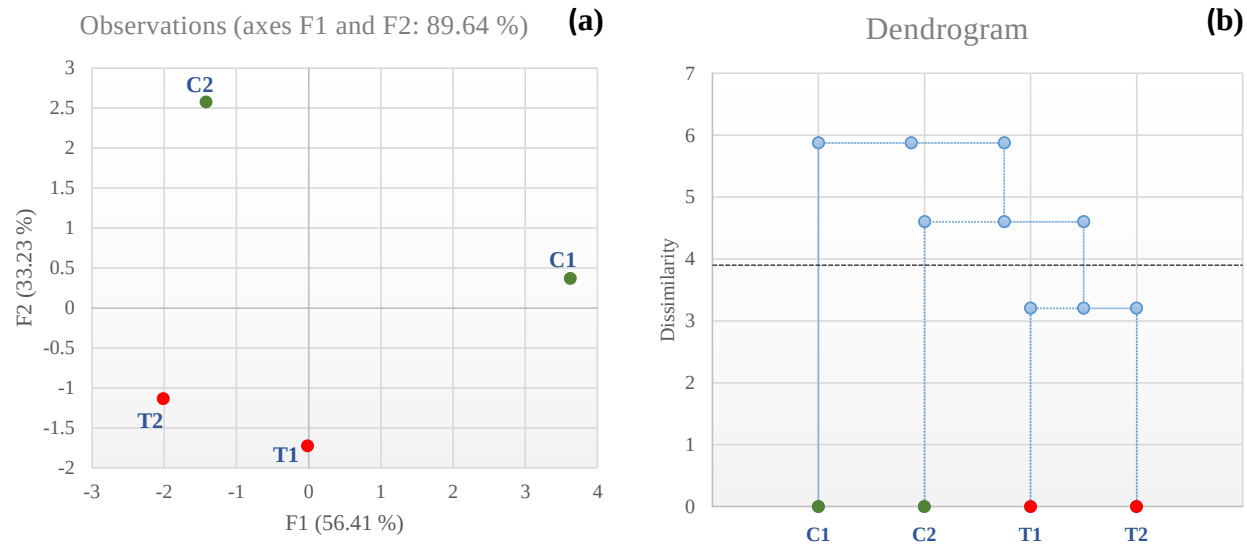

Figure 4. Multiple factor analysis applied to sensory analysis of Catarratto wines: (**a**) sample score; (**b**) agglomerative hierarchical clustering (AHC) dendrogram. Abbreviations: T1, sequential inoculum with *M. pulcherrima* MP346/ *S. cerevisiae* SPF52; T2, glutathione-rich inactivated yeasts and

sequential inoculum with *M. pulcherrima* MP346/*S. cerevisiae* SPF52; C1, single inoculum with *S. cerevisiae* SPF52; C2, glutathione-rich inactivated yeasts and single inoculum with *S. cerevisiae* SPF52.

5.3.7 Sensory Profiles Associated with Volatile Organic Compounds

A PCA was used to evaluate the correlation between VOCs and aroma attributes. According to Figure 5, the F1 factor contributed 66.11% of the total variance, whereas the F2 factor explained 28.60% of the total variance. Each wine, as can be seen from the biplot graph, was separate from the others.

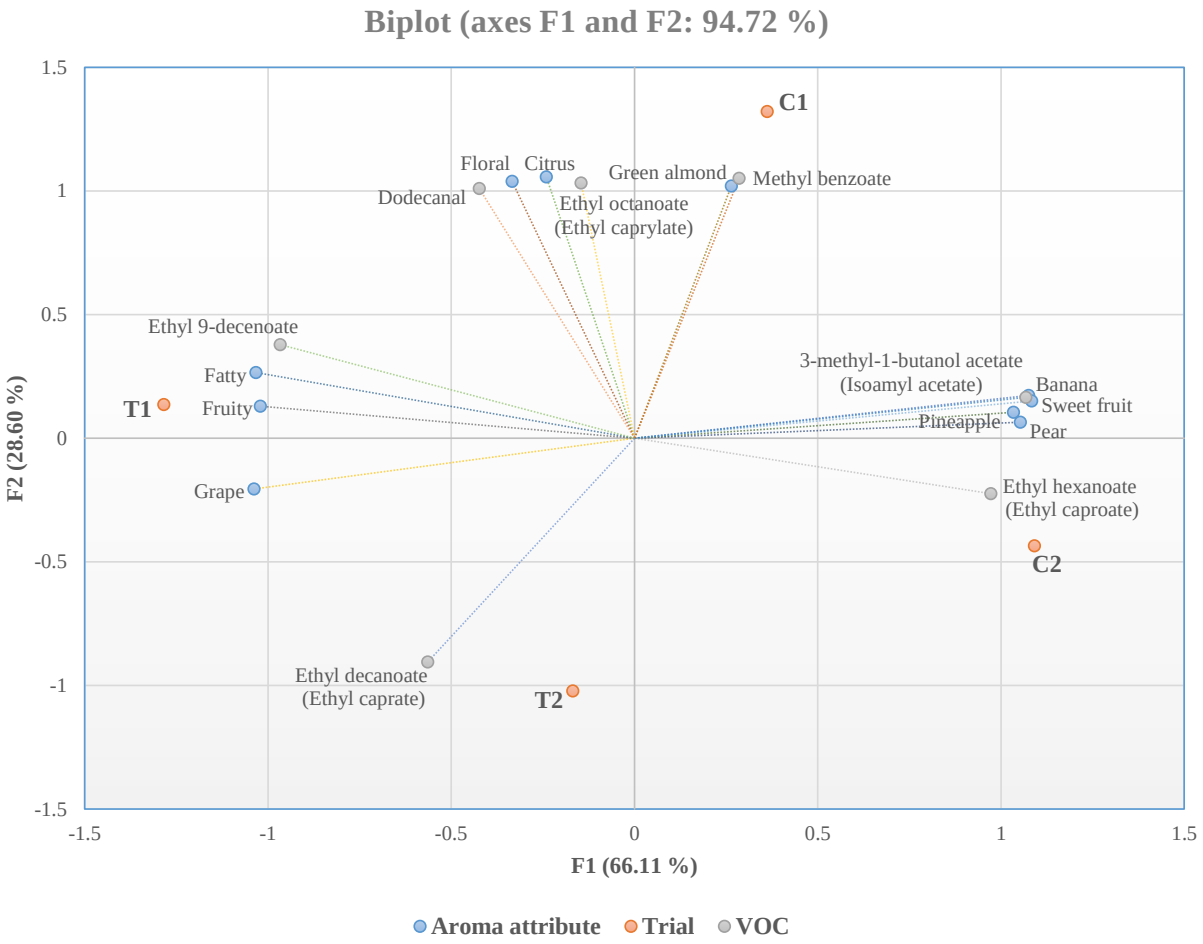

Figure 5. Principal component analysis (PCA) biplot for VOCs and aroma attributes. Abbreviations: T1, sequential inoculum with *M. pulcherrima* MP346/ *S. cerevisiae* SPF52; T2, glutathione-rich inactivated yeasts and sequential inoculum with *M. pulcherrima* MP346/ *S. cerevisiae* SPF52; C1, single inoculum with *S. cerevisiae* SPF52; C2, glutathione-rich inactivated yeasts and single inoculum with *S. cerevisiae* SPF52.

The C1 wine was associated with methyl benzoate, which produced green almond aromas (Burdock, 2017). A sensory analysis confirmed this attribute, and the highest scores were achieved in this trial. Ethyl 9-decenoate was the compound closely correlated with the T1 wine. This ester produces fruity and fatty odours (Ribéreau-Gayon et al., 2006), which were also detected in the sensory analysis, with the scores of fruity being higher than fatty. The grape aroma emitted by ethyl decanoate (Vázquez-

Pateiro et al., 2020) represented the T2 wine. The highest sensory analysis attributes detected in the T2 wine were fruity and sweet fruit, and the grape aroma showed modest values. However, fruity and grape aromas are also associated with the presence of ethyl decanoate (Fang and Qian, 2005). Finally, the C2 wine was closely associated with four odour descriptors (pineapple, sweet fruit, banana and pear). Only 3-methyl-1-butanol acetate and ethyl hexanoate were above the odour threshold and were responsible for the odours detected in the C2 wine by sensory analysis (Burdock, 2017).

The imperfect correlation between the highest OAV values of VOCs and the sensory analysis might be attributable to the synergistic interaction of odour molecules (high OAVs with low OAVs) with each other. As a result, the odours related to specific compounds were absent or very slightly perceived during the sensory analysis.

5.4 CONCLUSIONS

In this research, four treatments were examined in order to investigate the effect of *M. pulcherrima* and an antioxidant on the aroma and sensory profile of Catarratto wines. The use of *S. cerevisiae* SPF52 from a non-winemaking origin confirmed that yeasts from honey and its derivatives can potentially be used as starter strains in oenology. The combined use of *M. pulcherrima* MP346 and GIY had a positive impact on the taste–olfactory complexity of the wines. These differences were also confirmed by a sensory analysis. The VOC profiles generated by the wines obtained in the presence/absence of *M. pulcherrima* MP346 were correlated to the addition of GIY from the point of view of the quantity–intensity effect.

Dodecanal was only detected in the wines without GIY, whereas six esters had an OAV > 1 and actively contributed to the aroma definition of the different wines. Among the esters, ethyl decanoate was the most abundant in the wines inoculated with *M. pulcherrima* MP346, regardless of the presence/absence of GIY. However, the differences in the VOC profiles enabled the wines produced with the different winemaking protocols to be distinguished.

The modulation of the aromatic profile of each wine was also confirmed by a sensory analysis, which made it possible to differentiate the wines into three groups. The presence of *M. pulcherrima* MP346 and the absence of GIY did not allow the T1 and T2 wines to be discriminated from a sensory profile, while these differences were greater in the C1 and C2 wines, where the only variable was represented by the addition of GIY.

Further studies are needed to evaluate the antioxidant effects of the specific inactive yeast with a guaranteed glutathione content at different times during the pre-fermentation stage (on the crushed-stemmed and drained must during the pressing stage) of Catarratto grapes.

The use of *S. cerevisiae* of a non-oenological origin, *M. pulcherrima* in the pre-fermentation stage and specific inactivated yeast with a high antioxidant power resulted in a better preservation of

aromatic the compounds and colour, increasing the positive impact on the oxidative stability of the wines.

5.5 REFERENCES

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Table S1. Chemical parameters determined during alcoholic fermentation time (days): 3, 6, 12, and 18.

→ continued

Parameters	Must	Vinification									
		3 days of alcoholic fermentation				S.S.	6 days of alcoholic fermentation				S.S.
		T1	T2	C1	C2		T1	T2	C1	C2	
pH	3.30±0.02	3.15±0.01a	3.10±0.01b	3.14±0.01a	3.06±0.02c	***	3.17±0.02bc	3.16±0.01c	3.21±0.01b	3.27±0.02a	**
TA	5.53±0.09	5.48±0.06b	5.56±0.00ab	5.62±0.04a	5.55±0.02ab	*	5.44±0.10a	5.45±0.05a	5.50±0.06a	5.55±0.07a	n.s.
VA	0.00±0.00	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	n.s.	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	n.s.
Rs	223.99±0.92	172.87±1.20a	171.12±1.93a	146.26±0.24b	117.20±0.35c	***	112.89±0.36a	83.19±0.72c	95.75±0.85b	58.79±0.32d	***
Glu	113.05±0.55	79.97±0.19a	78.32±1.16b	60.38±0.21c	61.93±0.24c	***	64.35±0.24a	41.62±0.20b	44.43±0.15c	25.80±0.11d	***
Fru	110.91±0.36	92.90±1.01a	92.80±0.77a	85.88±0.03b	55.27±0.10c	***	48.54±0.12b	41.57±0.52c	51.32±0.70a	32.99±0.21d	***
Eth	0.01±0.00	2.66±0.01c	2.67±0.01c	3.93±0.09b	5.40±0.08a	***	5.69±0.08d	7.16±0.12b	6.57±0.11c	8.44±0.03a	***
Gly	0.92±0.00	2.00±0.03a	2.00±0.01a	2.02±0.02a	2.01±0.01a	n.s.	5.28±0.01a	5.27±0.04a	5.06±0.04b	5.19±0.07a	**
MA	1.90±0.03	1.65±0.02a	1.66±0.02a	1.69±0.02a	1.66±0.03a	n.s.	1.59±0.02a	1.60±0.02a	1.60±0.02a	1.60±0.03a	n.s.
LA	0.00±0.00	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	n.s.	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	n.s.
Amm. N	85.42±1.01	161.57±0.31ab	160.86±0.11a	159.51±0.77a	157.27±0.11b	*	146.23±0.28c	147.39±0.31bc	150.96±0.34a	149.91±1.22ab	*
Alpha-AN	33.90±0.39	78.16±0.09c	70.21±0.21d	80.32±0.32b	81.63±0.35a	***	73.44±0.11c	71.39±0.41d	75.97±0.41b	78.66±0.16a	***

Parameters	Vinification									
	12 days of alcoholic fermentation				S.S.	End of alcoholic fermentation (18 days)				S.S.
	T1	T2	C1	C2		T1	T2	C1	C2	
pH	3.24±0.01c	3.21±0.01c	3.31±0.00b	3.35±0.02a	***	3.41±0.02b	3.43±0.01b	3.47±0.00a	3.51±0.01a	***
TA	5.48±0.03a	5.45±0.07a	5.45±0.07a	5.48±0.08a	n.s.	5.41±0.09a	5.40±0.09a	5.42±0.02a	5.50±0.08a	n.s.
VA	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	n.s.	0.27±0.00c	0.29±0.00b	0.31±0.00a	0.31±0.00a	***
Rs	49.51±0.34a	28.80±0.12c	47.80±0.20b	27.60±0.30c	***	3.70±0.04a	2.60±0.05d	3.01±0.03c	3.24±0.03b	***
Glu	21.43±0.25a	12.07±0.09b	21.09±0.06a	10.26±0.12c	***	1.10±0.01b	1.10±0.01b	1.62±0.01a	1.11±0.01b	***
Fru	28.08±0.09a	16.73±0.03d	26.71±0.14b	17.34±0.18c	***	2.60±0.03a	1.50±0.04c	1.39±0.02d	2.13±0.02b	***
Eth	9.00±0.11b	10.11±0.06a	9.03±0.03b	10.09±0.08a	***	11.35±0.15a	11.43±0.13a	11.36±0.08a	11.35±0.07a	n.s.
Gly	5.34±0.02b	5.92±0.09a	5.20±0.03b	5.22±0.05b	***	5.58±0.04c	5.31±0.05d	5.85±0.08b	6.57±0.12a	***
MA	1.55±0.01	1.58±0.02	1.47±0.01	1.50±0.01	***	1.49±0.02a	1.50±0.02a	1.36±0.01b	1.28±0.02c	***
LA	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	n.s.	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	n.s.
Amm. N	119.88±0.43a	112.28±0.10b	110.38±0.44c	109.22±0.37c	***	49.35±0.27a	48.78±0.17a	45.61±0.21b	44.41±0.13c	***
Alpha-AN	67.32±0.08c	68.74±0.18b	63.84±0.13d	69.79±0.15a	***	55.21±0.08b	53.19±0.06c	56.81±0.11a	57.32±0.25a	***

Result indicate mean value ± standard deviation of three determinations. Data in the same line followed by the same letter are not significantly different according to Tukey's test.

Abbreviations: TA, total titratable acidity (tartaric acid g/l); VA, volatile acidity (acetic acid g/L); RS, reducing sugar (g/L); Glu, glucose (g/L); Fru, fructose (g/L); Eth, ethanol (% v/v); Gly, glycerol (g/L); MA, malic acid (g/L); LA, lactic acid (g/L); Amm. N, ammoniacal nitrogen (mg/L); Alpha-AN, alpha-amino nitrogen (mg/ L); n.d., not determined. P value: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant.

Table S2. Chemical parameters determined during steel aging time (month): 1, 3 and 5.

Parameters	Vinification									
	1 month of steel aging				S.S.	3 month of steel aging				S.S.
	T1	T2	C1	C2		T1	T2	C1	C2	
pH	3.39±0.01b	3.39±0.02b	3.41±0.00b	3.46±0.01a	***	3.40±0.01b	3.39±0.01b	3.43±0.00a	3.44±0.02a	*
TA	5.40±0.01a	5.40±0.08a	5.49±0.03a	5.45±0.10a	n.s.	5.38±0.09a	5.39±0.02a	5.45±0.04a	5.46±0.10a	n.s.
VA	0.30±0.00c	0.30±0.00c	0.35±0.01b	0.43±0.00a	***	0.33±0.00b	0.34±0.01b	0.36±0.00a	0.36±0.00a	*
Rs	3.00±0.02b	2.90±0.01c	2.51±0.00d	3.36±0.02a	***	1.25±0.02b	1.19±0.02c	1.15±0.03c	1.47±0.01a	***
Glu	1.62±0.02a	0.47±0.00c	0.43±0.00d	1.47±0.02b	***	0.31±0.01b	0.30±0.00b	0.35±0.02a	0.21±0.01c	***
Fru	1.38±0.00d	2.43±0.01a	2.08±0.00b	1.89±0.00c	***	0.94±0.01b	0.89±0.02c	0.80±0.01b	1.26±0.00a	***
Eth	11.35±0.08a	11.43±0.13a	11.36±0.08a	11.35±0.07a	n.s.	11.35±0.07a	11.43±0.10a	11.36±0.00a	11.35±0.09a	n.s.
Gly	5.68±0.08bc	5.61±0.07c	5.82±0.03b	6.48±0.08a	***	5.67±0.06bc	5.60±0.09c	5.82±0.00b	6.49±0.08a	***
MA	1.35±0.02a	1.33±0.02a	1.32±0.03ab	1.27±0.01b	**	1.34±0.01a	1.32±0.01a	1.33±0.01a	1.27±0.00b	*
LA	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	n.s.	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	n.s.
Amm. N	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Alpha-AN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Parameters	Vinification				
	5 month of steel aging				S.S.
	T1	T2	C1	C2	
pH	3.38±0.01b	3.38±0.02b	3.41±0.00a	3.43±0.00a	***
TA	5.35±0.06a	5.38±0.05a	5.38±0.10a	5.42±0.05a	n.s.
VA	0.34±0.00c	0.36±0.01b	0.35±0.00b	0.38±0.00a	***
Rs	0.61±0.00a	0.50±0.03b	0.28±0.01d	0.31±0.01c	***
Glu	0.20±0.00a	0.20±0.01a	0.12±0.01b	0.15±0.01b	***
Fru	0.41±0.00a	0.30±0.02b	0.16±0.00c	0.16±0.00c	***
Eth	11.35±0.17a	11.43±0.02a	11.36±0.06a	11.35±0.08a	n.s.
Gly	5.64±0.08bc	5.59±0.09c	5.80±0.01b	6.47±0.06a	***
MA	1.32±0.02a	1.31±0.02ab	1.30±0.00ab	1.27±0.02b	**
LA	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	n.s.
Amm. N	n.d.	n.d.	n.d.	n.d.	n.d.
Alpha-AN	n.d.	n.d.	n.d.	n.d.	n.d.

Result indicate mean value ± standard deviation of three determinations. Data in the same line followed by the same letter are not significantly different according to Tukey's test. Abbreviations: TA, total titratable acidity (tartaric acid g/l); VA, volatile acidity (acetic acid g/L); RS, reducing sugar (g/L); Glu, glucose (g/L); Fru, fructose (g/L); Eth, ethanol (% v/v); Gly, glycerol (g/L); MA, malic acid (g/L); LA, lactic acid (g/L); Amm. N, ammoniacal nitrogen (mg/L); Alpha-AN, alpha-amino nitrogen (mg/ L); n.d., not determined. P value: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant.

CHAPTER 6

General conclusions

This PhD thesis aims to represent a new approach to modulate the chemical and organoleptic quality of wines. The approach taken explored various aspects of wine microbiology, with a focus on the use of new yeasts, *Saccharomyces* and non-*Saccharomyces* from non-conventional matrices.

For the first time, culture-dependent and molecular methods were used to assess the diversity of yeasts in high-sugar matrices such as manna and honey. The results showed that certain strains belonging to the species *S. lactis-condensi* and *C. oleophila* proved to have aptitude for use as co-starter.

In a second study, for the first time, non-*Saccharomyces* yeast isolated from manna and honey by-products and selected in previous study, were applied to winemaking process. The effect of *S. lactis-condensi* and *C. oleophila* in sequential inoculation with *S. cerevisiae* on the oenological parameters of Frappato wines. The VOCs composition of wines obtained was evaluated in comparison with the non-*Saccharomyces* control strain *S. bacillaris*, previously used in industrial winemaking. VOC profiles confirmed wine differences especially for esters that, due to fruity aroma, contributed to the peculiar definition of the resulting wines, mainly by *C. oleophila*. The sensory analysis confirmed that the use of *S. lactis-condensi* and *C. oleophila* influenced positively the final wines in terms of fruity and floral intensity while did not generate sensory defects.

This work reports scientific data on the role of two novel non-conventional yeast species, *S. lactis-condensi* and *C. oleophila*, as potential co-starters to modulate the aromatic and organoleptic profile of wines.

In a third study, different experimental protocols were evaluated to obtain different aromatic expressions for a Catarratto cultivar classified as non-aromatic. The *S. cerevisiae* SPF52 strain, isolated from sugary matrices different from grape must, resulted to be suitable for wine production. Also, the impact of nutrients Stimula Sauvignon Blanc™ or Stimula Chardonnay™ before the inoculation of starter yeasts allowed to increase the aromatic complexity of the final wines. The addition of glutathione-rich inactivated yeast (GIY) Glutastar™ was useful to prevent the chemical oxidation of musts and wines and to generate the highest aromatic intensity.

Finally, four different treatments were examined to investigate the effect of *M. pulcherrima* and an antioxidant on the aroma and sensory profile of Catarratto wines. The combined use of *M. pulcherrima* MP346 and GIY Glutastar™ had a positive impact on the taste-olfactory complexity of the wines. These differences were also confirmed by a sensory analysis. The VOC profiles generated by the wines obtained in the presence/absence of *M. pulcherrima* MP346 were correlated to the addition of GIY from the point of view of the quantity-intensity effect. Among the esters, ethyl decanoate was the most abundant in the wines inoculated with *M. pulcherrima* MP346, regardless of the presence/absence of GIY. The modulation of the aromatic profile of each wine was also confirmed by a sensory analysis. The use of *S. cerevisiae* of a non-oenological origin, *M. pulcherrima* in the pre-

fermentation stage and specific inactivated yeast with a high antioxidant power resulted in a better preservation of aromatic the compounds and colour, increasing the positive impact on the oxidative stability of Catarratto wines.

The thesis demonstrated how the utilisation of non-conventional yeasts and yeast-derived nutrients can be a promising technique for modulate the quality of wine. Following in the footsteps of the wine industry, where the use of selected microbial cultures is a common practise, the introduction of innovative yeasts can lead to a better structuring of the aromatic profiles of fermented beverages, as well as changing the inoculation methods of the selected strains. The study of microbial ecology of high sugar matrices can be a valuable source of microorganisms that can be used to characterise and differentiate wine in an increasingly demanding consumer market. This technique not only increase consumers' desire for novelty but can also the profitability of the wine sector.

CHAPTER 7

List of publications

Effects of different yeast strains, nutrients and glutathione-rich inactivated yeast addition on the aroma characteristics of Catarratto wines

Alfonzo A.[a], **Prestianni R.**[a], Gaglio R.[a], Matraxia M.[a], Maggio A.[b], Naselli V.[a], Craparo V.[a], Badalamenti N.[b], Bruno M.[b], Vagnoli P.[c], Settanni L.[a], Moschetti G.[a]

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International Journal of Food Microbiology Vol. 360, 16 December 2021, Article number 109325

ABSTRACT

Catarratto is one of the most common non-aromatic white grape varieties cultivated in Sicily (Southern Italy). In order to improve the aromatic expression of Catarratto wines a trial was undertaken to investigate the effect of yeast strain, nutrition and reduced glutathione. Variables included two *Saccharomyces cerevisiae* strains, an oenological strain (GR1) and one isolated from honey by-products (SPF52), three different nutrition regimes (Stimula Sauvignon Blanc™ (SS), Stimula Chardonnay™ (SC) and classic nutrition practice), and a specific inactivated yeast rich in reduced glutathione to prevent oxidative processes [Glutastar™ (GIY)] ensuing in ten treatments (T1-T10). Microbiological and chemical parameters demonstrated the aptitude of strain SPF52 to successfully conduct alcoholic fermentation. During fermentation, the Saccharomyces yeast populations ranged from 7 to 8 logarithmic CFU/mL. All wines had a final ethanol content ranging between 12.91 and 13.85% (v/v). The dominance of the two starter strains over native yeast populations was higher than 97% as estimated by interdelta analysis. The addition of nutrients SS or SC increased the aromatic complexity of the wines as reflected by volatile organic compounds (VOCs) composition and sensory profiles. In particular, 32 VOCs were identified; alcohols (62.46–81.1%), thiols (0.27–0.87%), ethers (0.09–0.16%), aldehydes (0–1.21%), ketones (0–2.28%), carboxylic acids (4.21–12.32%), esters (0–10.85%), lactones (0.9–1.49%) and other compounds (0.77–6.9%). Sensory analysis demonstrated a significant impact on wine aroma in relation to yeast starter strain used, the type of nutrition (SS, SC or classic nutrition) and the presence/absence of GIY. The wines produced with GR1 yeast strain and SS (T2), SPF52 with SC (T9) both in presence of GIY showed higher overall quality. Trials T2 and T9 showed the highest scores for 13 and 18 attributes, respectively. The different nutrition, addition of GIY and the yeast starter strains diversified and enhanced sensory expression of Catarratto wines.

Improving the Aromatic Profiles of Catarratto Wines: Impact of *Metschnikowia pulcherrima* and Glutathione-Rich Inactivated Yeasts

Naselli, V.[a], **Prestianni, R.**[a], Badalamenti, N.[b], Matraxia, M.[a], Maggio, A.[b], Alfonzo, A.[a], Gaglio, R.[a], Vagnoli, P.[c], Settanni, L.[a], Bruno, M.[b], Moschetti, G.[a], Francesca, N.[a]

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Antioxidants, Vol. 12, February 2023, Article number 439

ABSTRACT

Catarratto is one of the most widely cultivated grape varieties in Sicily. It is an indigenous non-aromatic white grape variety. Despite its widespread use in winemaking, knowledge of the aroma and chemical and microbiological properties of Catarratto wines is quite limited. The influence of *Metschnikowia pulcherrima* combined with *Saccharomyces cerevisiae* on the aromatic expression of Catarratto wines was investigated with and without the addition of glutathione-rich inactivated yeast. The substance is a natural specific inactivated yeast with a guaranteed glutathione level used to limit oxidative processes. The aromatic profiles of the final wines were determined through analysis of the volatile organic compounds using a solid phase microextraction technique that identified 26 aromatic compounds. The addition of *M. pulcherrima* in combination with the natural antioxidant undoubtedly increased the aromatic complexity of the wines. Dodecanal was exclusively detected in the wines processed with glutathione-rich inactivated yeasts. Furthermore, the presence of this natural antioxidant increased the concentration of six esters above the perception threshold. Sensory analysis was also performed with a panel of trained judges who confirmed the aromatic differences among the wines. These results suggest the suitability of glutathione-rich inactivated yeasts for determining the oxidative stability of Catarratto wines, thus preserving its aromatic compounds and colour.

Impact of two new non-conventional yeasts (*Starmerella lactis-condensi* and *Candida oleophila*), isolated from sugar-rich substrates, on Frappato wine aroma

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Food Bioscience (under review)

ABSTRACT

The interest of non-*Saccharomyces* yeasts in wine fermentation increased constantly in last years. This study reports for the first time the enological potential of two strains *Starmerella lactis-condensi* MN412 and *Candida oleophila* YS209. In an innovative way, these strains were used in winemaking to improve floral and fruity aroma of Frappato red wine, which has not been explored. The oenological performances of the two non-*Saccharomyces* strains were compared to a wine strain of *Starmerella bacillaris*, namely Cz3, previously characterized in winemaking conditions. In these three cases, the non-*Saccharomyces* strain was sequentially inoculated with *S. cerevisiae* wine strain NF213, used as control. The *St. lactis-condensi* MN412 was isolated from Sicilian manna, a sugar-rich matrix, extracted from *Fraxinus angustifolia* trees (Oleaceae). The strain *C. oleophila* YS209 was isolated from honey by-products. Microbiological counts showed the ability of MN412 and YS209 to maintain high counts up to 6 days of alcoholic fermentation. Regarding chemical parameters, Cz3 showed the highest glycerol production. Analysis of VOCs revealed that the trials with non-*Saccharomyces* yeasts were characterized by a higher concentration of esters that contributed positively to the fruity aroma of the wines. The sensory analysis confirmed that the use of MN412 and YS209 impacted positively the final wines in terms of fruity and floral intensity, respectively, while did not generate sensory defects. In conclusion, non-conventional yeasts represent strategy to improve floral-fruity freshness of wine aroma and sugar-rich matrices such as manna ash and honey might represent novel ecological niches as source of potential oenological yeast.

Technological screening and application of *Saccharomyces cerevisiae* strains isolated from fermented honey by-products for the sensory improvement of *Spiritu re fascitrari*, a typical Sicilian distilled beverage

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Food Microbiology Vol. 104, June 2022, Article number 103968

ABSTRACT

"*Spiritu re fascitrari*" is a Sicilian alcoholic beverage obtained through distillation of a decoction of spontaneously fermented honey by-products (FHP). The production process often leads to sensorial defects due to the unstable alcoholic fermentation. The objective of this work was to select *Saccharomyces cerevisiae* strains from FHP to be used as starter in decoction fermentation. Based on chemical, microbiological and technological data, from a total of 91 strains three *S. cerevisiae* were selected for further testing to produce FHP at laboratory scale level. After FHP distillation, the analysis of volatile organic compounds showed a complex mixture of sensory active molecules, mainly alcohols and aldehydes. Among the alcohols, 3-methyl-1-butanol, 2-methyl-1-butanol, phenylethyl alcohol, hexadecanol and octadecanol were found at the highest concentrations. Among the carboxylic acids, acetic acid was mainly detected in the spontaneously fermented samples. FHP fermented with the three selected strains were not characterized by the presence of off-odours or off-flavours. The results obtained in this work demonstrate that the selected *S. cerevisiae* strains are promising starters to stabilize the production of distilled alcoholic beverages produced from honey by-products.

Use of sequentially inoculation of *Saccharomyces cerevisiae* and *Hanseniaspora uvarum* strains isolated from honey by-products to improve and stabilize the quality of mead produced in Sicily

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Food Microbiology, Vol. 107, May 2022, Article number 104064

ABSTRACT

Mead is a beverage produced by alcoholic fermentation of honey-must. The starter yeasts that are commonly used for the alcoholic fermentation of honey-must are oenological *Saccharomyces cerevisiae* strains. The objective of the present work was, for the first time, to apply yeasts of honey by-products origin to evaluate the influences the taste-olfactory attributes of mead. For this purpose, three experimental productions were set up, which included: (i) single inoculation of *S. cerevisiae*; (ii) single inoculation of *Hanseniaspora uvarum*; (iii) sequential inoculation of *H. uvarum*/*S. cerevisiae*. Two control trials were performed, using a commercial strain of *S. cerevisiae* of oenological origin and a spontaneous fermentation. The results of the chemical parameters showed differences between the trials in terms of residual sugars, acetic acid, glycerol, ethanol and volatile organic compounds. Sensorial analysis also showed a high heterogeneity among trials. The attributes of sweetness, honey and floral were found in mead fermented with *H. uvarum*, whereas all meads obtained with *S. cerevisiae* were dry, balanced and without off-odors and off-flavours. The results obtained showed that the controlled application of conventional and non-conventional yeast strains isolated from honey by-products origin could be a promising approach to improve the quality of meads.

Non-conventional yeasts from fermented honey by-products: Focus on *Hanseniaspora uvarum* strains for craft beer production

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Food Microbiology Vol. 99, October 2021, Article number 103806

ABSTRACT

The increasing interest in novel beer productions focused on non-*Saccharomyces* yeasts in order to pursue their potential in generating ground-breaking sensory profiles. Traditional fermented beverages represent an important source of yeast strains which could express interesting features during brewing. A total of 404 yeasts were isolated from fermented honey by-products and identified as *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii* and *Hanseniaspora uvarum*. Five *H. uvarum* strains were screened for their brewing capability. Interestingly, *H. uvarum* strains showed growth in presence of ethanol and hop and a more rapid growth than the control strain *S. cerevisiae* US-05. Even though all strains showed a very low fermentation power, their concentrations ranged between 7 and 8 Log cycles during fermentation. The statistical analyses showed significant differences among the strains and underlined the ability of YGA2 and YGA34 to grow rapidly in presence of ethanol and hop. The strain YGA34 showed the best technological properties and was selected for beer production. Its presence in mixed- and sequential-culture fermentations with US-05 did not influence attenuation and ethanol concentration but had a significant impact on glycerol and acetic acid concentrations, with a higher sensory complexity and intensity, representing promising co-starters during craft beer production.

Influence of indigenous *Hanseniaspora uvarum* and *Saccharomyces cerevisiae* from sugar-rich substrates on the aromatic composition of loquat beer.

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International Journal of Food Microbiology, Vol. 379, March 2022, Article number 109868

ABSTRACT

The demand for unique and exclusive food products and beverages is constantly on the increase. One of the products that mostly evolved to encounter market dynamics in the last decade is craft beer. For a long time, craft breweries have included fruit in beer production to enrich flavour and aroma profile of different beer styles. In this study, for the first time, the use of *Saccharomyces* and non-*Saccharomyces* yeast strains isolated from high-sugar matrices (manna and fermented honey by-products) were investigated to diversify fruit craft beer production, in order to improve the fermentation process and highlight the complexity of aroma profiles generated during alcoholic fermentation. Two yeast strains, *Hanseniaspora uvarum* YGA34 and *Saccharomyces cerevisiae* MN113, were tested as co-starters and starters for their beer production capacity. Commercial yeast strain US-05 was used as control. Loquat juice was added at the end of primary alcoholic fermentation in all trials. Interestingly, *S. cerevisiae* MN113 consumed sugars faster than control strain *S. cerevisiae* US-05, including maltose, even in the case of sequential inoculation. This strain showed an excellent ability to consume rapidly sugars present. All strains showed their concentrations ranged between 5 and 8 Log cycles during fermentation. The absence of off-odours and the improvement of aromatic perception were observed in experimental trials involving the use of *S. cerevisiae* MN113 as a monoculture and in sequential combination with *H. uvarum* YGA34. Esters and alcohols were the most abundant compounds emitted from the beers. The beers produced with sequential inoculation of *H. uvarum* YGA34 and *S. cerevisiae* MN113 or US-05 are characterised by a higher ester and lower alcohol concentration. These two unconventional yeast strains from high sugar matrices showed great technological properties, representing promising co-starters and starters during craft fruit beer production.

A novel microbiological approach to impact the aromatic composition of sour loquat beer

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Food Bioscience Vol. 55, August 2023, Article number 103011.

ABSTRACT

The growing interest in novel beer development determined the exploitation of unconventional yeasts isolated from novel ecological niches to generate unexplored sensory profiles. In recent years, there is an increasing interest in generating beers brewed with the addition of fruits. For the first time, *Lachancea thermotolerans* MNF105 and *Saccharomyces cerevisiae* MN113 isolated from manna, were tested as starter cultures to process loquat beer to improve the sensory profile. Innovatively, the yeast species *L. thermotolerans* was investigated for the production of sour fruit beer. Sour fruit beers produced with *L. thermotolerans* MNF105 were more balanced than the respective control, especially in terms of perceived acidity during sensory analysis. This could be due to the lower lactic acid production (0.49 g/L) compared to the respective control (1.74 g/L). The overall organoleptic investigation showed a preference for *S. cerevisiae* MN113 (TF1) isolated from manna. Experimental trials conducted with the selected strains demonstrated the absence of off-odour and off-flavour and improved aroma perception. Aldehydes and alcohols were the most abundant compounds emitted from the beers. *S. cerevisiae* MN113 and *L. thermotolerans* MNF105, manna related yeasts, showed great technological properties, representing promising starters for the production of fruit beer and sour fruit beer.

Application of technological protocols on an industrial scale to improve Seville-style table olive production in Italy and Spain

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A B S T R A C T

Improving the fermentation performance of starter strains used in the fermentation of table olives is a biotechnological solution of current interest to improve the quality characteristics of the final product. The aim of this study was to evaluate the use of *Lactiplantibacillus pentosus* OM13 as a starter culture for the fermentation of Seville-type table olives in two different production areas: Italy and Spain. The starter strain *L. pentosus OM13* was inoculated into two different table olive varieties: Nocellara del Belice in Italy and Manzanilla in Spain.

Lactiplantibacillus plantarum Vege-Start 60 was used as a commercial control, while an additional control production was carried out by spontaneous fermentation. The industrial productions consisted of three different protocols, differing in the type of nutrient and the presence/absence of acclimatisation of the starter strain. All trials were subjected to microbiological monitoring, evaluation of acidification dynamics and sensory analysis of the final product. After 90 days, the pH reached values below 5 in the different treatments. The LAB reached microbial loads varying between 6.5 and 8.7 log CFU/mL throughout the monitoring period. The microbial populations of spoilage and/or potential pathogenic microorganisms were variable depending on the microbial group monitored. However, after 12 days of fermentation, Enterobacteriaceae showed values below the detection limit. In contrast, a fluctuating trend was observed for yeasts, Pseudomonadaceae and Staphylococcaceae.

Sensory analyses showed variable differences depending on the technological protocol used. Table olives obtained with *L. pentosus* OM13 in the presence of nutrient, activator and acclimatisation period achieved higher overall acceptability values compared to the other trials. The use of adjuvants (nutrients and activators) is a strategy used in the production of table olives fermented with *L. pentosus* OM13 to improve the sensory characteristics of table olives.

Publications on national journals

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