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USE OF NON-CONVENTIONAL YEASTS ISOLATED FROM SUGAR-RICH SUBSTRATES FOR MODULATING THE AROMATIC PROFILE OF FRUIT AND SOUR BEERS

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CHAPTER 1

General introduction

1.1 Brewing history

Beer is one of the oldest alcoholic beverages and it is consumed all over the world. It is produced through the alcoholic fermentation of beer wort, made mainly from barley malt, or other cereals as well as wheat, wheat malt, oats, rice, corn and millet. All these raw materials provide maltose and glucose (Briggs et al., 2004). The conversion of sugars into ethanol and carbon dioxide is a process that occurs through the action of yeast, which performs alcoholic fermentation. The two yeast strains, *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* (also known as *Saccharomyces carlsbergensis*), are the primary species that are able to perform this process (Legras et al., 2007).

For a long time, craft breweries have included fruit and spices in beer production to enrich flavour and aroma profile of different beer styles. To this purpose, generally the products added are typical of a given area strongly link the final beers to the production/transformation area (Glover, 2001; Protz, 1995). The origins of beer can be traced back to a period of considerable antiquity. The earliest evidence of the preparation of a beer-like drink is attributed to the Sumerians, the inhabitants of the fertile strip of land between the Tigris and the Euphrates, and dates back to approximately 6,000 years ago (Katz and Maytag, 1991). Nevertheless, the methods employed for the consumption of alcohol varied considerably. For example, Mesopotamia was the first region to witness the emergence of the brewer profession. The workers engaged in the preparation of the beverage were compensated with beer. At that time, a variety of beer types were produced, including dark, light, red, strong, sweet, and aromatic beers. It is estimated that there were approximately twenty types of beer available on the market in Babylon, the richest city in Mesopotamia. The beer also had a religious and ritual significance. Indeed, it was drunk during funerals to celebrate the virtue of the deceased and was offered to the divinity to guarantee a peaceful rest to the dead. In ancient Egypt, beer held a similar significance. It was consumed by the subjects of the pharaohs from an early age and was also used as a food and medicine (Arnold, 1911). Until the Middle Ages, the brewing process was exclusively the domain of women. Gradually, this exclusive right was superseded as beer production was initiated in monasteries. This craft was adopted by monks (primarily Belgian and Dutch) to perpetuate the

association between beer and religion. Indeed, the first Babylonian women to produce beer were in fact the temple's priestesses. Over time, the practice of brewing became exclusively the domain of men. In due course, the monks began to produce in excess of their immediate requirements, and thus began to sell their surplus. Unfortunately, the rulers of the time perceived the potential financial gain that could be derived from the beer trade and sought to impede the monks, who were exempt from taxation, from engaging in such a lucrative enterprise (Meussdoerffer, 2009). Over time, "Grut", a mixture of juniper berries, blackthorn, oak bark, wormwood, anise, rosemary and other herbs that were later deemed dangerous, was first used in beer production and is now replaced by hops (Hornsey, 2003). In 1516, the Duke of Bavaria issued a decree stipulating that only barley (including barley malt), hops, and pure water could be used for beer production. The use of hops imparted a flavour to the beverage that was similar to that of beer as it is known today. However, the use of yeast was still unknown, and fermentation was considered to be a random process. It was not until the sixteenth century that brewers were able to successfully control barley fermentation, leading to improvements in terms of both quality and quantity.

1.2 Beer production technology

The current beer production process has been achieved thanks to a series of changes made over time, involving the following steps:

1. Malting: Malt is obtained primarily from barley, although it can also be derived from other cereals. Nevertheless, barley has consistently been the most prevalent cereal utilized in the production of beer for a number of reasons. These include its high starch content, favorable organoleptic qualities, and relatively low-fat content (Giardini and Baldoni, 2000). The malting process involves:

   - The steeping process involves the cleaning of barley or other cereals, which are then placed in maceration tanks where they are hydrated and oxygenated. This facilitates germination.

- Germination is the phase during which the embryonic development of the barley grain takes place. Following this, the bud begins to feed on the starchy substances of the endosperm, which results in the rupture of the grain and the formation of the enzymes essential for the production of beer. This process is described by Bamforth (1993). In this manner, barley is transformed into malt.

- In the initial drying phase, the temperature is maintained at 50-60 °C in order to reduce the humidity of the seed. Subsequently, the temperature is elevated to approximately 80-90 °C, contingent on the desired type of malt. The type of malt employed and the manner in which it is processed are of paramount importance for the quality of the final product. The purposes of this phase are manifold: stop germination, reduce the humidity of green malt to increase its shelf life, make the malt crumblier, provide the desired aroma and color to the malt (Bertinotti and Faraggi, 2015).

2. Mashing: Milled malt is combined with hot water in order to extract cereal components (principally starch) and to facilitate the activity of enzymes. During this phase, the starch is converted into fermentable sugar by the action of enzymes.

3. Wort boiling: During this phase, hops are added, imparting the final flavour of the beverage. Hops contain essential oils that contribute to the hoppy, floral, and spicy aromas characteristic of beer. It is important to note that many of these compounds are volatile and can therefore be lost by evaporation during boiling. To circumvent this issue, the brewer may opt to add some of the hops at either the midpoint or the conclusion of the boiling process. Furthermore, during this phase, the beer must undergo browning due to the reactions that occur between the reducing sugars and the primary amines (Baxter and Hughes, 2001).

4. Fermentation: Following boiling, the wort is cooled and then subjected to fermentation. The process of beer fermentation represents a pivotal stage in the production of a quality final product. The various types of beer are distinguished by a number of factors, including the characteristics of the fermentation process, the types of yeasts used, the times, temperatures

and processing methods employed. The objective of wort fermentation is to ensure the consistent metabolization of wort constituents into ethanol, carbon dioxide and other fermentation products (Carlson, 1987). This enables the production of beer with satisfactory quality and stability. In the context of beer fermentation, yeast cultures are used that can be safely reintroduced into subsequent beers (Speers, 2016). It is important to note that brewing is the only fermentation process that recycles yeast cultures from one fermentation to the next. Fermentation processes, such as those employed in the production of alcohol for human consumption and in the manufacture of industrial alcohol, as well as in the production of wine, sake and cider, utilise their yeast culture on a single occasion. The management of the yeast culture between fermentations represents a crucial aspect of the brewing process. It is of the utmost importance to meticulously safeguard the quality of the cultured yeast, as it will be used to initiate the subsequent wort fermentation. Consequently, the quality of the resulting beer will be significantly influenced by the quality of the yeast used. Over the years, considerable efforts have been made to study the biochemistry and genetics/molecular biology of brewer's yeast (along with industrial strains in general). Two distinct types of fermentation may be identified, namely top and bottom fermentation. In the first method, beer is fermented by yeast belonging to the *S. cerevisiae* species, operating at a temperature between 18-24 °C. This process takes approximately two weeks to complete. This process encompasses, for example, English ale beers, Trappist, Irish stout and British Indian pale ale (IPA). Beer fermentation can also take place using different yeasts and temperature, by adding yeast from the *S. pastorianus* species to the must. These act at a temperature between 7-9 °C and require at least 5 or 6 weeks for complete maturation. Once fermentation is complete, the remains of the yeasts settle on the bottom. An example are the "lager" beers (Briggs et al., 2004).

5. Maturation: Subsequently, the beer is transferred to special tanks where it will remain for a period of four to six weeks, during which time it will undergo maturation. In this phase, all

components are refined and stabilised, a process which results in a natural clarification and the acquisition of the beer's definitive flavour. Subsequently, the majority of beers undergo further filtration to eliminate any residual yeast and other components that might impart a cloudy appearance. At this juncture, the beer is nearly ready for packaging and consumption (Fix, 1989).

Figure 1. Flow-chart of beer production technology
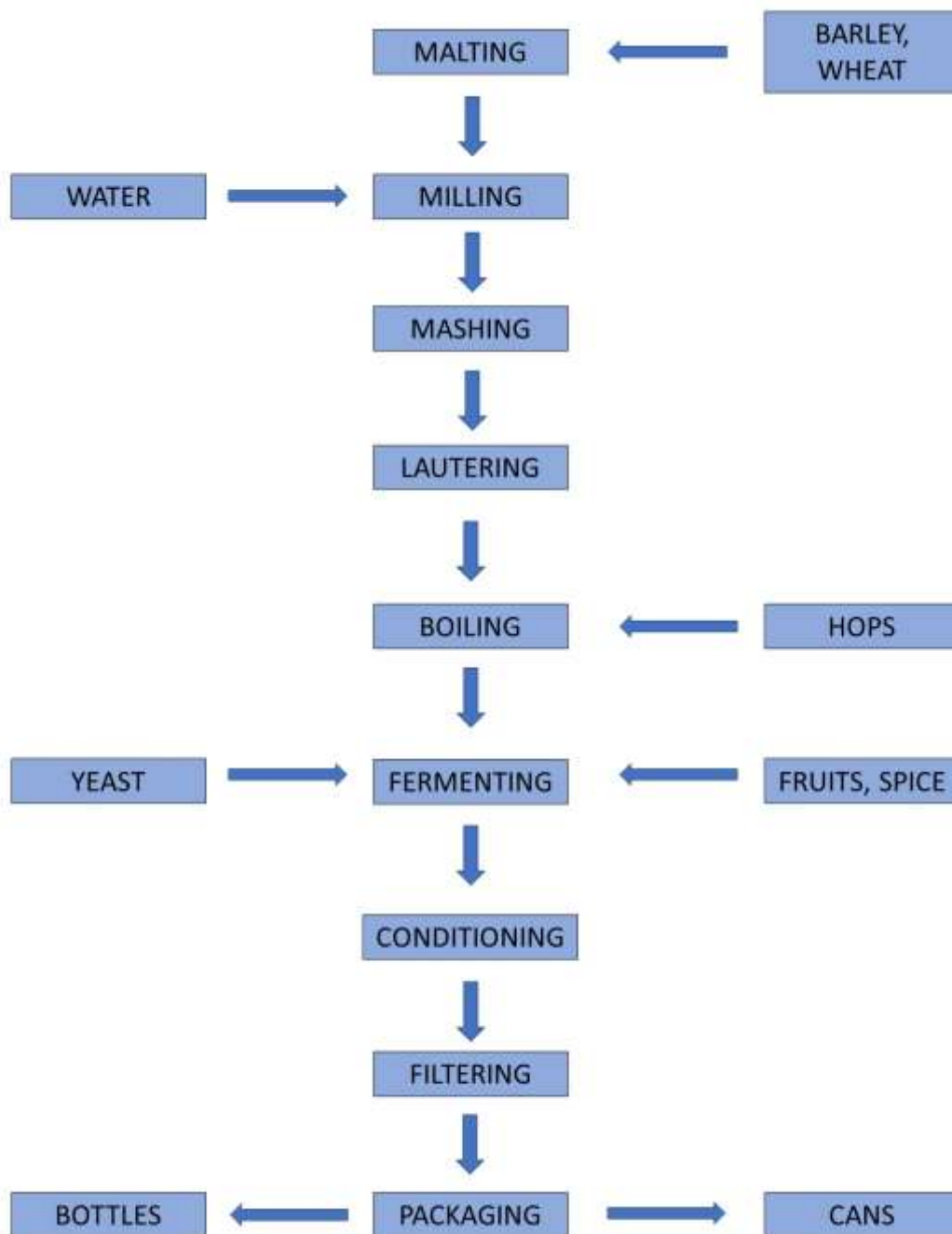
1.3 Fermentative microorganism: *Saccharomyces* and non-*Saccharomyces* yeasts

The conversion of sugar into an alcoholic beverage is dependent upon the fermentation process, which is carried out by a multitude of microorganisms, including yeasts (*Saccharomyces* and non-*Saccharomyces*) and bacteria.

1.3.1 *Saccharomyces* yeasts

Over time, numerous beer styles have emerged, each with its distinctive character and flavour, shaped by the cultural and geographical context of its country of origin (Protz et al., 1995; Glover et al., 2001). Despite the numerous changes that have occurred within the beverage industry, the role of yeast has remained consistent. Since the earliest records of beer production by humankind, numerous modifications have been implemented that have culminated in the modern beer-brewing process. Two types of brewing yeast were originally classified based on flocculation behaviour: top fermenting (ale and weizen yeast) (Jentsch et al., 2007) and bottom fermenting (lager yeast). The behaviour of these yeasts is sufficiently distinct that the two main classes of beer types (ales and lagers) are based on the two yeast types. Ale yeast exhibits greater genetic diversity than lager yeast, and, similar to weiss yeasts, ferments at higher temperatures (18–24 °C). In contrast, lager yeast is more conserved and ferments at lower temperatures (8–14 °C). The yeasts are of fundamental importance, not only because they determine the main characteristics of fermented products through the synthesis of primary metabolites such as ethanol and carbon dioxide, but also because they contribute to defining part of the quality characteristics, influencing above all the olfactory sensory aspect. An example of this is the production of Weizen beers, which are produced with *S. cerevisiae* in particular (Jentsch et al., 2007).

The genus *Saccharomyces*, which belongs to the fungal kingdom, currently contains some of the most important species for the food and beverage industry. A total of nine species have been identified within this genus. The genus *Saccharomyces* currently comprises nine species: *S. cerevisiae*, *Saccharomyces paradoxus*, *Saccharomyces mikatae*, *Saccharomyces kudriavzevii*, *Saccharomyces*

arboricola, *Saccharomyces eubayanus*, *Saccharomyces uvarum*, *S. pastorianus* and *Saccharomyces bayanus* (Stewart et al., 2016; de Melo Pereira et al. 2010).

The *Saccharomyces sensu stricto* species complex (Vaughan-Martini & Martini, 1998) contains some of the most important species for the food industry. These include *S. cerevisiae* (Meyen ex EC Hansen), which is responsible for wine, bread, ale and weiss beer and sake fermentations; or *S. bayanus* (wine and cider fermentations) and *S. pastorianus* (EC Hansen), which is responsible for lager beer fermentation (Rainieri et al., 2006). This species can be found in a wide variety of environments and matrices. Although its importance is often associated with the food sector, *S. cerevisiae* is the main player in the alcoholic fermentation of beverages, precisely due to its high capacity to metabolise and convert sugar into ethanol (Cavalieri et al., 2003). The fermentation of beer conducted with *S. cerevisiae* is designated as "top fermentation," a term derived from the position of the yeast on the surface of the wort. The fermentation temperature varies according to the single strain, ranging between 14 and 32 °C. The fermentation process typically takes between 10 and 15 days to complete (Krescankova et al., 2015). Beer fermentation with *S. cerevisiae* is called "top fermentation", due to the position of the yeast on the surface of the wort. Depending on the individual strain, the fermentation temperature varies between 18 and 24 °C and it takes 10 to 15 days to complete fermentation (Krescankova et al., 2015).

The yeasts grow in a common yeast medium such as yeast-peptone-dextrose extract (YPD) *S. cerevisiae* is globular in shape and white in colour, while on WL Nutrient Agar medium, the colonies are large, light green in colour with a grey border, smooth and shiny surface, buttery consistency with a full margin. The optimum growth temperature is 30 °C, under facultative anaerobic conditions (Dimmer et al., 2002).

The commercial strains are subjected to technological screening for oenological parameters, including fermentation vigour, power and purity, sedimentation capacity, and aromatic compounds production (Bauer and Pretorius, 2000). However, their use may not align with the sugars beer-must composition, potentially leading to stuck fermentations and unfavourable sensorial characteristics

(Ferreira et al., 2010). Another characteristic of this microorganism is its ability to generate hundreds of volatile compounds, whose abundance, both qualitative and quantitative, depends on the strain used. These differences are implicit in the natural metabolic biodiversity present within the species. In the context of wine fermentation, this natural variability has been exploited for decades by offering different selected strains of *S. cerevisiae* with different metabolic and physiological characteristics, generally in the form of active dry yeast (Vaudano, 2014).

In contrast, *S. pastorianus* is a bottom-fermenting yeast of great importance to the brewing industry (Mosher et al., 2021). With the advent of methodologies based on molecular biology, gene sequencing of ale and lager yeast strains has shown that *S. pastorianus* is an interspecies hybrid between *S. cerevisiae* and *S. eubayanus*, with homologous relationships between them and also with *S. bayanus*, a yeast species used in wine fermentation and identified as a wild yeast in beer fermentations (Libkind et al., 2011). This "lager yeast" is capable of fermentation at higher temperatures, although it differs from *S. cerevisiae* in that it remains active at lower temperatures, up to 5 °C. Moreover, *S. pastorianus* is capable of hydrolysing melibiose into more fermentable monosaccharides than *S. cerevisiae*. This results in a greater utilisation of the available sugars, which in turn leads to a drier, crisper beer. It has a distinctive genetic profile that is optimal for fermenting sugars, resulting in the production of highly palatable beers (Monerawela et al., 2017). This yeast is employed in the production of lager-type beers, which require considerably lower temperatures for fermentation than those required for other industrial fermentation processes. *Saccharomyces pastorianus* is capable of operating at temperatures between 5-15 °C. It differs from other strains in that it does not impart the final fruity or floral aromas and flavours (Gibson and Liti, 2015). The strains involved in lager fermentation are cryotolerant and exhibit a high capacity for fermenting maltose and maltotriose (capacities inherited from the species of origin or due to hybridization). They tend to settle after flocculation and to sink to the bottom of the fermentation vessel (Vidgren and Londesborough, 2011).

1.3.2 Non-*Saccharomyces* yeasts

Historically, non-*Saccharomyces* yeast species have been viewed negatively in brewing processes, as and especially some of them could present problems associated with beer safety, turbidity, filterability, off-flavours, acidity and other changes in flavour profile (Miguel et al., 2022; Varela, 2016). However, the correct use and selection of non-*Saccharomyces* yeasts in the brewing process can lead to the acquisition of favourable properties. In recent times, non-*Saccharomyces* yeasts have been subjected to investigation with a view to elucidating their distinctive metabolic and enzymatic pathways. The reasons for this interest are due to the criticisms that are increasingly being directed at fermentations that are guided by the massive initial inoculations of *S. cerevisiae* commercial strains. Many studies have been conducted on these yeasts over the years, and it has been discovered that they can play an important role in defining beer aroma and improving organoleptic characteristics (Van Rijswijck et al., 2017). During spontaneous fermentation, the development and succession of different species and strains could result in greater complexity and distinctive sensorial characteristics in the final product (Vaudano et al., 2014).

Yeasts responsible for spontaneous fermentation, the so-called non-*Saccharomyces* yeasts, are also referred to as "indigenous" or "wild" yeasts, in order to distinguish them from the added exogenous yeast cultures (Varela, 2016). Several non-*Saccharomyces* yeast species are found during spontaneous fermentations of certain beer styles (Belgian Lambic beer and American coolship ales), including *Meyerozyma guilliermondii*, *Debaryomyces* spp., *Pichia* spp., *Wickerhamomyces anomalus*, *Brettanomyces anomalus*, *Brettanomyces custersii, Brettanomyces bruxellensis*, *Candida krusei, Cryptococcus keutzingii* and *Rhodotorula mucilaginosa* (Bokulich and Mills, 2012; Spitaels et al., 2014). The metabolic processes of these yeasts result in the formation of hundreds of active aromatic compounds, which impart the distinctive aroma and flavour to fermented beverages. In fact, it is generally accepted that indigenous yeasts are unable to complete alcoholic fermentation due to their low alcohol tolerance and the excessive production of secondary compounds. The impact of yeast selection on the metabolites that contribute to the characteristic flavour of beer, such as acetate

and ethyl esters and higher alcohols, is widely recognised (Pires et al., 2014). This has led researchers to investigate the use of non-*Saccharomyces* strains in beer production.

The main reasons for using non-conventional yeasts in brewing are bioflavouring, acidification, development of enzymes that release aromatic precursors, production of low-calorie and low-alcohol beers, production of probiotic or fortified beers (Francesca et al., 2023; Holt et al., 2018; Puligundla et al., 2021; Toh et al., 2020). Although the positive characteristics of non-*Saccharomyces* yeast are a recent area of research, their potential in the fermented beverage industry, particularly in brewing, is becoming clearer.

Numerous species, including *Candida oleophila* and *Starmerella lactis-condensi* (Francesca et al., 2024), *Candida zemplinina* (Di Maio et al., 2013), *Metschnikowia pulcherrima* (Canonico et al., 2019; Morata et al., 2019; Naselli et al., 2023), *Torulaspora delbrueckii* (Simonin et al., 2018), *Kluyveromyces marxianus* (Barone et al., 2021), *Pichia kluyveri* (Hu et al., 2021), *Lachancea thermotolerans* (Vaquero et al., 2021) have been employed in oenology in the last decade to pursue several objectives, like microbial acidification, pre-fermentation bio-protection, increased glycerol, reduced ethanol and in general improve the overall quality of wines. Instead, several non-*Saccharomyces* yeasts isolated from different matrices, including *Zygosaccharomyces*, *Pichia*, *Saccharomycodes*, *Torulaspora*, *Lachanchea* and *Hanseniaspora*, have been studied for applications in brewing (Sannino et al., 2019).

For instance, Canonico et al., (2016) found that the presence of *T. delbrueckii* resulted in beers with a low alcohol content while displaying a particularly analytical and aromatic profile. Similarly, Callejo et al. (2019) applied yeasts for beer production and found that the species *T. delbrueckii* and *Saccharomycodes ludwigii*, followed by *L. thermotolerans*, are capable of producing beers with low ethanol content and characteristic aroma profiles or *Schizosaccharomyces pombe* can increase both ethanol and acetaldehyde content.

Instead, Matraxia et al. (2021) found that *Hanseniaspora uvarum* effectively improved glycerol and acetic acid concentrations. This strain also exhibited higher sensory complexity and intensity and

grew rapidly in the presence of ethanol and hops. Additionally, Pirrone et al. (2022) demonstrated that the beers produced with sequential inoculation of *H. uvarum* and *S. cerevisiae* are characterised by a higher ester and lower alcohol concentration. Francesca et al. (2023) demonstrated that the yeast strain *L. thermotolerans* MNF105 had lower lactic acid production, which contributed to the flavour balance of beer. Additionally, it was capable of producing ethyl lactate, a compound commonly produced by this species. The ability of certain yeast species and strains to ferment sugars selectively allows for their sequential use with *S. cerevisiae*, resulting in improved flavour and reduced sweetness of beers due to residual sugars. Non-*Saccharomyces* yeasts have a low tolerance to ethanol and are not always able to consume the main sugars of the beer wort. Therefore, they are typically used in co-fermentation with *Saccharomyces* species to complete fermentation (Cubillos et al., 2019). Thus, the mixed or sequential inoculation of non-*Saccharomyces* and Saccharomyces yeasts is a promising strategy for producing innovative flavours in beer (Holt et al., 2018).

1.4 Fermentative cultures inoculum strategy

In recent years, the brewing industry has seen an increase in production, not only in terms of volume, but also in terms of new beer styles and products (Aquilani et al., 2015). Drinkers around the world are increasingly seeking innovative, tasty, and complex craft beers (Betancur et al., 2020).

To achieve new beer styles, numerous studies have been conducted on the main materials of beer, such as malt (Gugino et al., 2023; Zdaniewicz et al., 2020a), hops (Paguet et al., 2024), and other ingredients such as fruit (Pirrone et al., 2022). Another alternative for creating new beers with distinctive flavours is the use of non-conventional yeasts. To achieve this goal, the strategy of increasing microbial and fermentative complexity seems promising. Based on the established success of Belgian Lambic and in general of mixed fermentation beers, several developments are going on in this direction. The ability of certain yeast species and strains to selectively ferment sugars allows them to be used in sequence with *S. cerevisiae*, resulting in improved flavour and increased attenuation of beers. In the wine sector, numerous studies have shown that the use of several yeast

strains, either simultaneously or sequentially, is a widespread and well-established strategy for increasing the complexity and quality value of wine production. In fact, this subject has been studied for several years in Italy, as shown by the study by Zironi et al. (1993). In brewing, with a few years delay compared to the wine sector, the same practices are spreading to obtain innovative productions with non-conventional fermentations, using non-*Saccharomyces* strains. The interactions between different microorganisms and their compatibility and competition during fermentation need to be carefully considered when using multiple species. The use of different micro-organisms may occur at different stages of the process. To enhance bio-flavouring, Holt et al. (2018) screened 17 non-conventional yeast species, inoculating them 48 h before the use of a commercial strain of *S. cerevisiae*. Bourbon-Melo et al. (2021) evaluated *Hanseniaspora guilliermondii* and *Hanseniaspora opuntiae* to determine their potential for beer bio-flavouring: both species were used in dual inoculums with *S. cerevisiae* and the mixed-cultures, while did not influence attenuation and ethanol concentration of beer, exerted a significant impact on the volatile composition.

Canonico et al. (2016) used the *T. delbrueckii* DiSVA 254 strain in co-inoculation with a commercial *S. cerevisiae* US-05 starter strain at different ratios (1:1, 10:1, 20:1). They showed that the non-Saccharomyces strain influenced the analytical and aromatic profile of the beer when the inoculation ratio exceeded 10:1 (*T. delbrueckii/S. cerevisiae*). Under these mixed fermentation conditions, they observed an increased consumption of Yeast Assimilable Nitrogen (YAN), probably related to the synthesis of aromatic compounds. The presence of more than one inoculated strain from different species can be improved by modulating parameters like temperature. In any case, the presence of a secondary strain affects the growth of the principal one, as reported by Gobbi et al. (2013). Instead, Matraxia et al. (2021) found that *H. uvarum* applied at different inoculum ratios with *S. cerevisiae* US-05 (sequential inoculum) effectively improved glycerol and acetic acid concentrations. In conclusion, as shown by the above studies, there does not seem to be one way of inoculating a culture that absolutely guarantees better results than others. Rather, the choice between a sequential inoculum

and a co-inoculum must be evaluated on a case-by-case basis, depending on the type of matrix to be fermented, the conditions of pH, sugars, YAN, temperatures and compatibility of the strains used.

1.5 Novel brewing technologies and ingredient

1.5.1 Fruit beer

Beer market is worldwide dominated by traditional beer types, but there is an increasing interest in generating beers brewed with the addition of fruits (Patraşcu et al., 2018). Although the incorporation of fruit inevitably increases the cost of beer production, it satisfies consumer needs for new taste, smell and visual stimuli. Moreover, it is particularly appreciated by female consumers (Yang et al., 2022). Oliver and Colicchio (2011) has defined fruit beers as beers with fruit flavouring, rather than fruit-based alcoholic beverages. This definition distinguishes alcoholic beverages produced from pressed fruit, such as grape wines and apple ciders, which are not grain-based, from grain-based beers with added fruit and from beers mixed with other beverages, such as lemon juice or lemonade. In the case of wines and ciders, the carbohydrates present in the fruit juices (must) serve as the primary source of energy for the microorganisms involved in the fermentation process, resulting in the production of a beverage that contains minimal residual carbohydrates (Apolinar-Valiente et al., 2021). In contrast, fruit beer production is based on the fermentation of wort, an extract of the cereals used, followed by the addition of fruit or fruit juices during the brewing process. The addition of whole fruit to beer is a traditional practice in Belgium, whereby cherry lambic (Kriek) or raspberry lambic (Framboise) is produced by the addition of sour cherries (*Prunus cerasus* L.) or raspberries (*Rubus idaeus* L.) to fermenting lambic in casks. In the case of cherry lambic, 150–400 g/L of intact sour cherries are added to wooden casks filled with a blend of old and young lambic beer. The sugar present in the fruits initiates a secondary fermentation. During the fermentation and maturation of fruit beers, a range of flavours and bioactive compounds are extracted from the fruits, including carotenoids and polyphenols, which are the most abundant. Consequently, the addition of fruit during

the fermentation process may contribute to an enhancement of the content of bioactive compounds in beer (De Keersmaecker, 1996; Glover, 2001; Protz, 1995; Spitaels et al., 2014).

This type of beverage became popular due to its rich fruity flavor and refreshing properties due also to a pleasant acidity (Gorzelany et al., 2022; Martìnez et al., 2017; Zapata et al., 2019). Almost all kinds of fruits can be added to beer, such as drupes (peaches, mangoes, etc.), kernel fruits (pears, apples, etc.), and berries (strawberries, blueberries, etc.) (Marin et al., 2021). At the same time, the consumption of tropical fruits is also becoming popular worldwide due to their nutritional and health properties (Aquilani et al., 2014). Many of these fruits, such as banana, passion fruit, annona, mango, and loquat, are being studied for their use in brewing, with the aim of increasing the amount of ethanol by adding sugars, for enrichment in terms of volatile organic compounds and, in some cases, to improve the final acidity (Carvalho et al., 2009; De Melo et al., 2017; Gasiński et al., 2020; Pirrone et al., 2022; Santos et al., 2021). Fruit beers possess a distinctive flavour and taste profile, and have undergone a period of rapid development in recent years. It is also important to consider the impact of fruit on the fundamental physico-chemical parameters of beer. Furthermore, the addition of fruit can improve the anti-oxidative properties of beer, increase the content of volatile compounds and offer consumers a new sensory experience. Although considerable progress has been made in this field, it is still in its infancy. There is still much to be done to address the shortcomings and to fully explore the potential of fruit beers.

1.5.2 Sour beer

Sour beer is a very diverse genre of beer that defies any specific definition based on production process, raw material or geographical origin. A common denominator of sour beers is a higher concentration of organic acids, resulting in a lower pH (pH 3.0 to 3.9) compared to 'normal' beers. Sour beers are intentionally designed to be acid and can also be fermented with wild microorganisms or fruit, barrel-aged, or blended with younger beers (Bossaert et al., 2019; Tonsmeire, 2014). There are different microbiological approaches for brewing sour beers, through spontaneous fermentation

or through inoculum of acidifying bacteria, such as acetic acid or lactic acid bacteria or yeasts of the genus *Brettanomyces* or *Lachancea* (Dysvik et al., 2020).

Osburn et al. (2018) introduced the concept of 'primary souring', which focuses on using different yeast species, including strains of *L. thermotolerans*, to brew sour beer without bacteria, resulting in a significant reduction in the duration and variability of the transformation process. This species is capable of heterolactic fermentation of sugar into lactic acid, ethanol, CO_2, and at the same time, producing pleasant aromatic and flavour compounds (Domizio et al., 2016; Osburn et al., 2018).

Recently, researches have conducted studies on *L. thermotolerans* for brewing application. Postigo et al. (2023) determined the fermentation capacity of 10 yeast strains of *L. thermotolerans* and showed that beer fermented with strain CLI 1232 had a balanced acidity with a fruity flavour profile and honey notes, whereas strain 1-8B had a balanced acidity but less fruity and citrus flavour than strain CLI 1232. Canonico et al. (2019) confirmed that *L. thermotolerans* strains can lower the pH of the inoculation medium due to the formation of significant amounts of lactic acid, as well as they consistently produce ethyl butyrate and ethyl acetate. However, Zdaniewicz et al. (2020) demonstrated that certain strains of *L. thermotolerans* have poor lactic acid production and have a slight effect on pH lowering, although they generated higher amounts of ethyl lactate than *S. cerevisiae*. In conclusion, this strain is perfectly suited for the production of sour or slightly sour beer, as it has excellent characteristics in terms of attenuation, flocculation, acidification and bio-aromatisation. Although many of these characteristics are strain-related, it is always useful to know the characteristics of the strain used.

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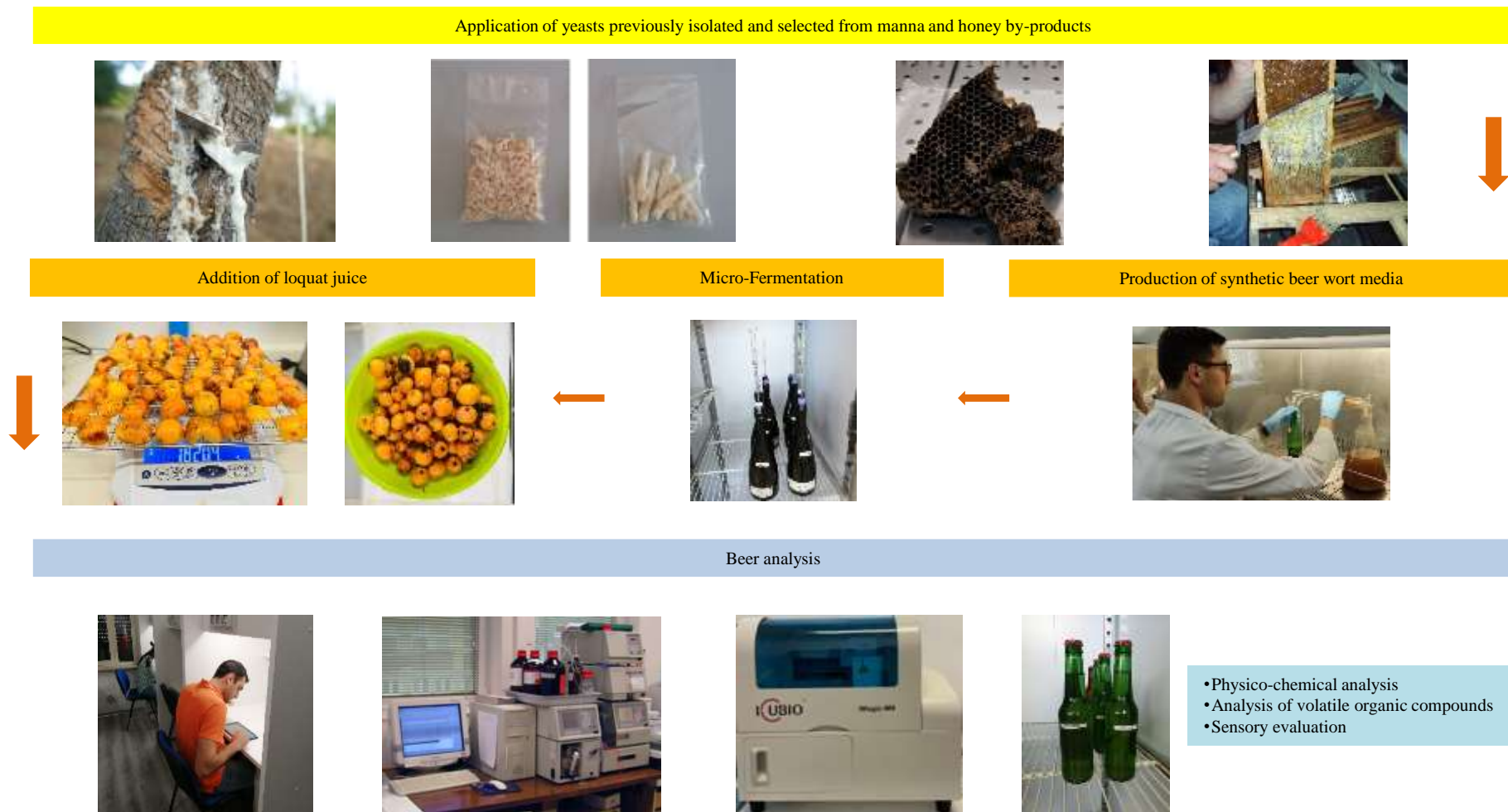
CHAPTER 2

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

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 starter during craft fruit beer production.

1   **Fig. 1.** Graphical abstract of experimental research

Application of yeasts previously isolated and selected from manna and honey by-products

Addition of loquat juice | Micro-Fermentation | Production of synthetic beer wort media

Beer analysis

• Physico-chemical analysis
• Analysis of volatile organic compounds
• Sensory evaluation

2

3

2.1 Introduction

Beer is one of the oldest alcoholic beverages and it is consumed all over the world. It is produced through the alcoholic fermentation of beer wort, made mainly from barley malt, or other cereals as well as wheat, wheat malt, oats, rice, corn and millet. All these raw materials provide maltose and glucose (Briggs et al., 2004). For a long time, craft breweries have included fruit and spices in beer production to enrich flavour and aroma profile of different beer styles. To this purpose, generally the products added are typical of a given area strongly link the final beers to the production/transformation area (Glover, 2001; Protz, 1995). Various fruits such as cherries and raspberries have been used for centuries as brewing adjunct and flavouring agents in Belgium; as a matter of fact, cherry lambic (Kriek) and raspberry lambic (Framboise) are considered traditional beer styles (De Keersmaecker, 1996; Spitaels et al., 2014).

In the last years, tropical fruits such as mango, passion fruit and banana were tested in brewing and were found to play several positive roles especially in terms of increased ethanol production (Carvalho et al., 2009; Gasiński et al., 2020) and enrichment in terms of volatile and aromatic organic compounds (De Melo et al., 2017). Loquat, one of the most popular tropical fruit commonly cultivated in Sicily, is generally locally used as raw material in brewing. Sicilian loquats ecotypes express high quality characteristics particularly appreciated by consumers; in particular, they were perceived as tastier than imported varieties (Testa et al., 2020). Thus, local Sicilian loquat cultivars show very interesting properties from the sensory perspective (Farina et al., 2016).

Beer is characterised by volatile and semi-volatile compounds such as alcohols, esters, acids, terpenoids, C13-norisoprenoids, and volatile phenols (Witrick et al., 2017). Fermenting yeasts, mainly non-conventional yeast strains play a crucial role (Van Rijswijck et al., 2017) for defining beer aroma. Very recently, research in this field focused on the selection of yeasts isolated from alternative matrixes in order to intensify the aromatic characteristics of the beers (Colomer et al., 2019; Larroque et al., 2021). To this purpose, recent studies have been conducted on the isolation of yeast strains from sugar rich sources, like manna ash (Guarcello et al., 2019) or highly alcoholic

beverages carried out by fermented honey by-products (Matraxia et al., 2021). A novel approach for the bio-aromatisation of fermented beverages is also represented by the application of unconventional yeasts, both *S. cerevisiae* and non-*Saccharomyces* strains, isolated from unstudied raw matrices, like manna ash and honey, which might be characterised by an intensive production of enzymes, mainly hydrolases, such as glucosidase that can release aroma precursors or aroma-active substances (Rodriguez et al., 2007, 2010; Ruiz et al., 2018; Sadineni et al., 2012). These fermentation products are volatile compounds that can contribute to enrich flavour and aroma profiles of beers (Pires et al., 2014).

Among non-*Saccharomyces* yeasts with potential in brewing, Saccharomycotina genera including *Pichia*, *Saccharomycodes*, *Zygosaccharomyces*, *Hanseniaspora* and *Torulaspora* are being studied to be used as starter cultures (Sannino et al., 2019). *Hanseniaspora* was found to fulfil this duty very well; *Hanseniaspora uvarum* showed a significant impact on glycerol and acetic acid concentrations, with a higher sensory complexity and intensity, and the ability to grow rapidly in presence of ethanol and hop (Matraxia et al., 2021). Bourbon-Melo et al. (2021) evaluated *Hanseniaspora guilliermondii* and *Hanseniaspora opuntiae* to determine their potential for beer bio-flavouring: both species were used in mixed inocula with *Saccharomyces cerevisiae* and the mixed-cultures, while did not influence attenuation and ethanol concentration of beer, exerted a significant impact on the volatile composition. Non-*Saccharomyces* yeasts alone do not possess the capacity to perform an optimal alcoholic fermentation and, for this reason, they are usually used in co-fermentation with *Saccharomyces* species (Cubillos et al., 2019). Thus, mixed or sequentially inoculation of non-*Saccharomyces* and *Saccharomyces* yeasts represents a promising strategy for enhancing desirable flavours in beer (Holt et al., 2018). Furthermore, the use of novel strains of *S. cerevisiae* isolated from unstudied matrices, such manna ash, might represent novel biotechnological approach to improve beer aroma. Based on the above reasons, the present study aimed to: (i) evaluate the effect of a novel *S. cerevisiae* strain isolated from manna ash as potential starter cultures in fruit brewing; (ii) evaluate

the sequential inoculum of *H. uvarum* and *S. cerevisiae* yeast strains; (iii) improve the organoleptic quality of loquat craft beer.

2.2 Materials and methods

2.2.1 Yeast strains and media

Yeast strains used in this research are *S. cerevisiae* MN113 and *H. uvarum* YGA34. All strains belong to microbial culture collection of the Department of Agricultural, Food and Forest Sciences (SAAF; University of Palermo, Italy). *S. cerevisiae* MN113 was previously isolated from manna ash (Guarcello et al., 2019). *Hanseniaspora uvarum* YGA34 was isolated from honey by-products and evaluated for its performances in monoculture and dual sequential inoculum with *S. cerevisiae* (Matraxia et al., 2021). The commercial strain *S. cerevisiae* US-05 (Fermentis, Lesaffre, France) was used as control. Yeasts from the microbial colture collection were revived from cryogenic storage and streaked onto Yeast Peptone Dextrose Agar (YPDA) plates and incubated for 2 days at 30 °C before being moved to room temperature storage until propagation. Wort media (WM) used for fermentation assays was prepared according to Larroque et al. (2021). All media and chemical components of WM were purchased from Oxoid (Rodano, Italy).

2.2.2 Experimental drawing and sampling

Experimental beers were produced at a laboratory-scale level (0.75 L sterilised batch) using the monocultures of *S. cerevisiae* MN113 and US-05 (the last strain used for control production) and two different sequential inoculums of *H. uvarum* YGA34 with both *S. cerevisiae* strains, as visible in Fig. 2.

Loquat juice used as raw material for beer production was squeezed from fruits of the white-fleshed local cultivar "Claudia" (*Eriobotrya japonica* Lindl) harvested at complete maturity using skin color as harvest index from an orchard located in Palermo (38°4'38.65"N, 13°22'21.95"E). Fruits were

collected randomly in plastic bags and transported by a portable ice box to the laboratory of SAAF Department where they underwent a washing procedure including a step of 2 min in chlorinate water solution (0.2%, v/v) followed by a rinse in cold (1–2 ◦C) water to remove chlorine (Alfonzo et al., 2018). The fruits were then dried under laminar hood to avoid further surface contamination and then manually peeled and cut longitudinally. Loquat juice was extracted by means of the RoboDiet Compact juice extractor (De Longhi, Treviso, Italy). The juice was subjected to pH and sugar (°Bx, degrees Brix) by a pH meter Mod.70 XS/50010162 (Cheimika, Pellezzano, Italy) and penetrometer TR5325 (Turoni, Forlì, Italy), respectively. The juice was then filtered by Navaris filter bags (Berlin, Germany) to eliminate suspended particles and immediately frozen at -20 °C (Tarantino et al., 2021). Loquat juice (20% v/v) was added to all trials at the end of the alcoholic fermentation (day 10) as reported by Gasiński et al. (2020).

Specifically, four experimental trials were inoculated following this scheme: T1, inoculated with *S. cerevisiae* US-05, used as control; T2, inoculated with *S. cerevisiae* MN113; T3, inoculated sequentially with YGA34 strain and after 48 h with MN113 strain; T4, inoculated sequentially with YGA34 strain and after 48 h with US-05 strain.

Each yeast strain was inoculated at a cell density of approximately 2.0×10^6 cells/mL (Holt et al., 2018). Samples were taken at different phases of beer production: uninoculated must, must just after yeast inoculum, just after the second strain addition in case of sequential inoculums (day 2), at the end of primary alcoholic fermentation (day 9), after addition of loquat juice (day 10) and at the end of the secondary alcoholic fermentation (day 22). All experimental fermentation trials were performed in triplicate.

Fig. 2. Experimental plan of loquat beer production
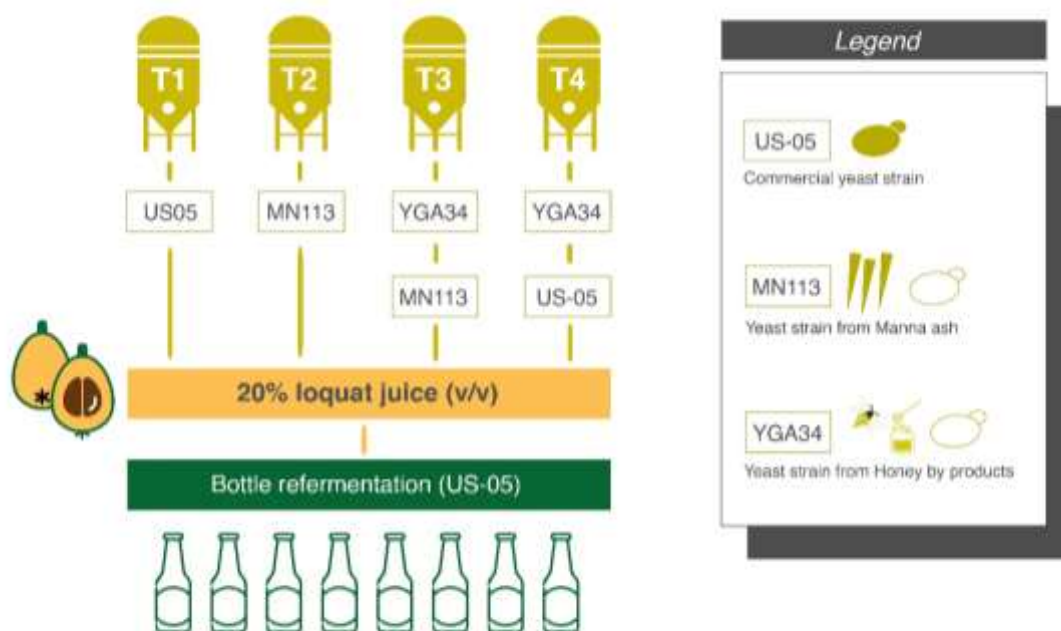
2.2.3 *Monitoring of wort fermentation*

The fermentation was monitored daily by measuring weight loss up to day 22. According to Ciani and Maccarelli (1998), fermentation rate (FR) was calculated as the amount of carbon dioxide produced after 3 days of fermentation [CO_2 released (g/L)]. Alcoholic fermentation was completed when no residual sugar changes were detected for two successive days. Samples were collected at different stages of beer production: uninoculated must, must just after yeast inoculum, just after the second strain addition in case of sequential inoculums (day 2), at the end of primary alcoholic fermentation (day 9), after addition of loquat juice (day 10) and at the end of the secondary alcoholic fermentation (day 22). All samples were immediately subjected to microbiological investigation carried out by plate counts.

Cell densities of *Saccharomyces* populations were evaluated on Wallerstein Laboratory (WL) nutrient agar, while those of non-*Saccharomyces* on Lysine Agar (LA) medium applying the conditions

described by Di Maio et al. (2011) and Iris et al. (2020). After growth, all colonies were classified as presumptive *Saccharomyces* and *Hanseniaspora* according to their morphological characteristics on agar plates and after microscopic investigation (Cavazza et al., 1992). All analyses were performed in triplicates.0.

2.2.4 Physicochemical analysis

The measurement of pH of the several samples collected was conducted with a pH meter Mod.70 XS/50010162 (Cheimika, Pellezzano, Italy) while Brix degrees (°Bx) were determined with a refractometer DBR Salt (Zetalab srl, Padova, Italy). The determination of acetic acid, fructose, glucose, glycerol, malic acid, maltose, sucrose and tartaric acid, was performed as reported by Matraxia et al. (2021). All reagents and standards were purchased from R-Biopharm AG (Darmstadt, Germany). All samples were diluted until the optimal concentration indicated by the apparatus calibration curve was reached.

2.2.5   Determination of volatile organic compounds

All reagents were of analytical grade. Ultra-pure water was used to prepare standard and diluted solutions. Individual standard of each compound: ethyl caprylate, ethyl caproate (standard for the ester fraction), benzaldehyde (standard for the aromatic fraction), and 1-hexanol (standard for the alcoholic fraction) were purchased from Sigma-Aldrich (82024 Taufkirchen, Germany). *n*-Alkane standards (C_8 to C_{40}) were purchased from Aldrich Chemical Co. (St. Louis, Mo., USA). Standard solutions containing a mixture of the four standards were prepared at five different concentrations. Stock solutions were obtained by dilution commercial standards in hydroalcoholic solutions containing ethanol (5.0-5.2%) in pure water at pH 3.2-3.3, partially simulating the beer composition. An automatic SPME holder (Supelco®, Bellefonte, PA, USA) was used for the evaluation of VOCs profiles. A 50/30 μm divinylbenzene (DVB)/carbowax (CAR)/polydimethylsiloxane (PDMS) fiber of 1 cm length was used for fractionation of volatile compounds from the headspace (HS) of

conditioned beers. 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 beer aroma was performed following the method proposed by Thompson-Witrick et al. (2015). For extraction, each aliquot (10 mL) of beer sample was placed into a 20 mL vial (75.5×22.5 mm) (Supelco, Bellefonte, PA, USA). The samples were equilibrated at 40 °C for 30 min. The SPME fiber was exposed to the beers for 30 min in the headspace of the sample kept at 40 °C. The flavour compounds were desorbed for 8 min from the fiber to the column through a splitless injector at 250 °C. All samples were prepared and analysed in triplicates in standard 20 mL-volume headspace vials.

Quantification of volatile compounds was performed using an Agilent 7000C GC system, fitted with a fused silica DB-5 MS 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; ionization voltage 70 eV; electron multiplier energy 2000 V; transfer line temperature, 270 °C. Solvent delay: 0 min. Helium was the carrier gas (1 mL/min). The GC temperature was an initial temperature of 40 °C for the first 2 min, then from 40 °C to 240 °C, increasing 7 °C min^{-1}, and held for 10 min. Volatile compounds were injected at 250 °C automatically with the splitless mode. Linear retention indices were calculated using n-alkanes as reference compounds. For the analysis of alkane solution (C_8-C_{40}) (Sigma-Aldrich, USA), the injector mode was set in 10:1 split mode. The individual peaks were analysed using the GC-MSolution package, Version 2.72. Identification of compounds was performed with the Adams (Adams, 2007), NIST 11, Wiley 9 and FFNSC 2 mass spectral database.

2.2.6 *Sensory analysis*

Sensory analysis of the fruit beers produced in this study was conducted as reported by Larroque et al. (2021). Fourteen judges (ranging from 26 to 47 years old) were recruited from University of Palermo and trained for fruit beer evaluation. All panellists had experience in brewing and participated in previous studies as sensory judges. Judges were trained through preliminary sessions to calibrate sensory attributes that describe the aromas associated with fruit beers. Loquat beers were

served to tasters in individual test booths with standardised lighting to exclude the perception of beer colour on flavour perception. Samples were labelled with randomized numerical codes and served (100 mL at 16 °C) in standard ISO type tasting glasses topped with watch glass. The descriptors used for fruit beer attribute evaluation were: odour (intensity, complexity, fruity, citrus, loquat, floral, spicy, herbaceous/vegetal, earthy, honey/caramel, toasted, wheat/cereal, sulphury, acetic, winey, oxidized/aged and lactic). The sensory attributes were assessed using an unstructured nine-point scale anchored at the left end with "absent" and at the right end with "high" (Gugino et al., 2024; Jackson et al., 2016).

2.2.7   Statistical and explorative multivariate analyses

ANOVA test was applied to determine statistical significance between chemical parameters detected during laboratory-scale fruit beer productions (D-glucose, D-fructose, sucrose, maltose, acetic acid, glycerol, tartaric acid and malic acid), microbiological analysis (total yeast counts and weight loss), VOCs and sensory analysis (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). The differentiation of the VOC profiles generated by each experimental beer was represented through heat map clustered analysis (HMCA) as reported by Martorana et al. (2017). The VOC concentration is graphically represented by a colour change from yellow to red in relation to the low or high levels determined (Gaglio et al., 2017). The chemical and sensory parameters that characterised the different experimental beers were differentiated by agglomerative hierarchical clustering (AHC). Multivariate statistical analysis was elaborated using XLStat software version 2019.2.2 (Addinsoft, New York, USA) for Excel.

2.3 Results and discussion

2.3.1 Wort fermentation

The kinetics of wort weight loss as CO_2 production was daily monitored for 22 d (end of the fermentation process) and is graphically reported in Fig. 3.

Fig. 3. Fermentation kinetics of synthetic wort beer inoculated with different yeast strains expressed as the concentration of CO_2 released (g/L). Abbreviations: T1, *S. cerevisiae* US-05; T2, *S. cerevisiae* MN113; T3, sequential inoculum with *H. uvarum* YGA34 and *S. cerevisiae* MN113; T4, sequential inoculum with *H. uvarum* YGA34 and *S. cerevisiae* US-05.
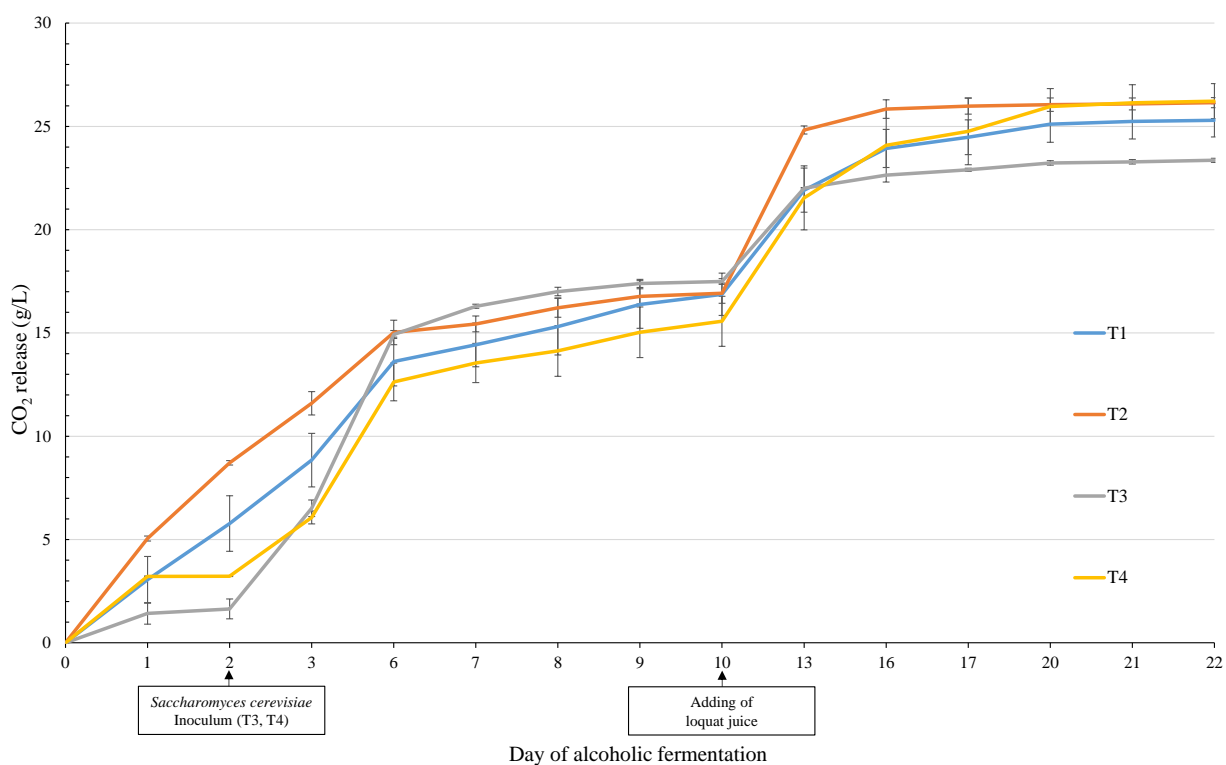
After 72 hours of fermentation, trial T2 showed the highest value of FR (8.71 g/L). Interestingly, the strain *S. cerevisiae* MN113 showed better performances than commercial control *S. cerevisiae* strain US-05, for this parameter. The lowest FR values were registered in presence of *H. uvarum* (1.64 and 3.23 g/L for T3 and T4, respectively). These results of FR showed a very low ability of *H. uvarum* strains to ferment beer wort. The low FR does not represent a limitation for beer production; the genus *Hanseniaspora* spp. is characterized by very low fermentation power and/or vigour, but it is able to improve the aroma complexity of fermented beverages such as wine and beer (Martin et al., 2018, Matraxia et al., 2021).

After the sequential inoculum of the two *S. cerevisiae* strains, in trials T3 and T4 showed an increasing of FR. At the 10th day of AF (addition of the loquat juice), greatest weight loss was measured for trial

T3 (17.49 g/L). Even in the case of the trial with sequential inoculum (trials T3 and T4), after 48 hours from the inoculum of the *Saccharomyces*, the *S. cerevisiae* MN113 strain showed better performance of FR than *S. cerevisiae* commercial strain US-05. Accordingly, with Bourbon-Melo et al. (2021), sequential inoculation of *Saccharomyces* with *Hanseniaspora* spp. significantly accelerated fermentation rates. At the end of the alcoholic fermentation process, the highest value of FR was found for the sequential fermentation of *H. uvarum* YGA34 with the control strain *S. cerevisiae* US-05 (26.23 g/L). Sequential fermentation with control strain presented the highest fermentation rate and was comparable to *Saccharomyces* in single culture.

2.3.2 Population dynamics of yeasts

The evolution of yeast populations during the alcoholic fermentation is reported in Fig. 4.

Fig. 4. Monitoring of yeast concentrations during AF. Beer fermented by: (a) US-05 [T1]; (b) MN113 [T2]; (c) sequential inoculum with YGA34 and MN113 [T3]; (d) sequential inoculum with YGA34 and US-05 [T4]. Different superscript letters indicate significant differences on microbial concentrations were performed at each sampling time according to Tukey's test for $P < 0.05$. Abbreviations: WL, Wallerstein nutrient agar for yeasts; LA, Lysine Agar for non-*Saccharomyces* group.
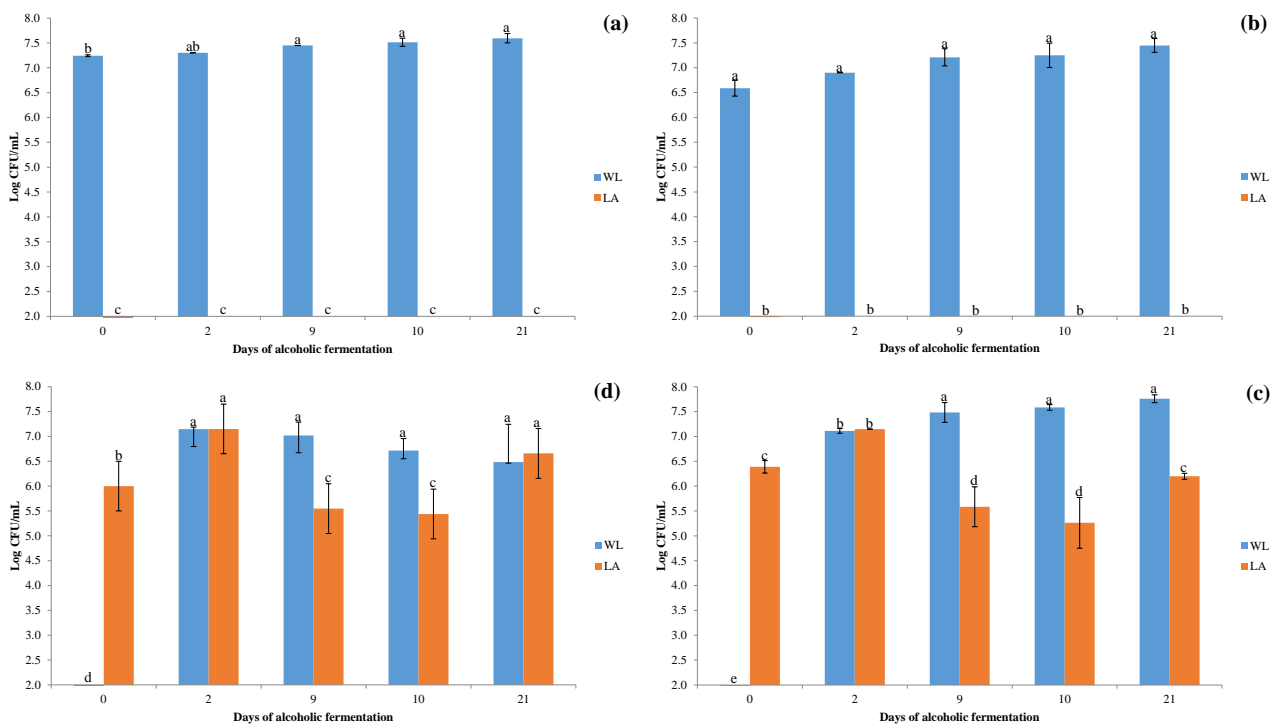
Total yeast count of must before starter inoculums and loquat juice, both on WL Nutrient Agar and LA, was below the detection limit. After inoculation, yeast cell densities ranged between 6.00 and 7.24 Log CFU/mL. The persistence of the strains inoculated was morphologically examined by means of colony shape and cellular morphology to recognize typical members of *Hanseniaspora* and *Saccharomyces* genera (Cadez et al., 2014; Jindamorakot et al., 2009; Kurtzman et al., 2011). Starter yeasts increased of about 0.5 Log cycles after 1 d for all trials and these results follow the general dynamics of yeast growth in fermenting must-beer (Capece et al., 2021). At day 2, the trials T3 (Fig. 4c) and T4 (Fig. 4d) were then inoculated with 7.11 and 7.15 Log CFU/mL of *S. cerevisiae* MN113 and commercial control strain US-05, respectively. At the next sampling point (day 9 of AF), these trials showed a decrease of presumptive *Hanseniaspora* spp. populations, levels were 5.59 and 5.55 Log CFU/mL, while those of *S. cerevisiae* were still high (7.48 and 7.02 Log CFU/mL for T3 and T4, respectively). At the same time, the levels of *S. cerevisiae* in T1 (Fig. 4a) and T2 (Fig. 4b) were 7.21 and 7.45 Log CFU/mL, respectively. The decrease of *Hanseniaspora* spp. population is due to the nature of the interaction with *S. cerevisiae* (Tristezza et al., 2016), probably due to competition for the same nutrients or the presence of metabolites produced by *S. cerevisiae* that obstruct the growth of *H. uvarum* (Domizio et al., 2011; Wang et al., 2015). After loquat juice addition (day 10 of fermentation), the microbial levels increased for all trials. In particular, *H. uvarum* in trial T4 showed a remarkable increment of its population starting from day 10 up to end of the process. As stated by Mateus et al. (2020), *H. uvarum* has a marginal preference for fructose. At the end of the alcoholic fermentation, the highest cell counts were registered for *S. cerevisiae* MN113 in trial T3 (7.76 Log CFU/mL), showed higher values respect sequential fermentation with control strain *S. cerevisiae* US-05 in trial T4 (7.59 Log CFU/mL). Growth dynamics of yeasts during the alcoholic fermentation of fruit beer were comparable to those reported by (De Melo et al., 2017).

2.3.3 Physicochemical analysis

The physicochemical values of juice and must are reported in Table 1. The initial wort had a pH value

of 3.00 and 9.70 °Bx, whereas the loquat juice had a pH of 3.75 and 11 °Bx.

Table 1. Conventional chemical parameters identified in beer wort and *Eriobotrya japonica* juice.

	Wort	Loquat juice
D-fructose (g/L)	2.00 ± 0.20	37.08 ± 0.60
D-glucose (g/L)	39.63 ± 0.28	30.66 ±0.40
Maltose (g/L)	20.77± 0.05	n.d.
D-sucrose (g/L)	24± 0.21	36.05 ± 0.20
Acetic acid (g/L)	0.04 ± 0.08	0.04 ± 0.04
L-Malic Acid (g/L)	0.20 ± 0.01	12.81 ± 0.11
Glicerol (g/L)	0.06 ± 0.01	n.d.
Tartaric acid (g/L)	0.09 ± 0.01	0.87 ± 0.12

About the different trials, the pH registered at the end of alcoholic fermentation were between 3.28

and 3.55. The analyses on physicochemical parameters highlighted several differences among strains

in terms of glucose, fructose, sucrose, maltose, glycerol, acetic acid, malic acid, and tartaric acid

concentration evolution (Table 2). Except for T2 and T3, all experimental trials reached a final sugar

concentration below 2.3 g/L after 22 days of fermentation. In the first two days, trials T3 and T4,

inoculated with *H. uvarum*, partially fermented glucose, fructose and sucrose, but it cannot show

fermentation of maltose, confirming the inability of this species to consume it (Madrera et al., 2021;

Matraxia et al., 2021). For this genus, no genes for maltose assimilation have been identified in the

publicly available genomes of *Hanseniaspora* spp. (Steenwyk et al, 2019; Cadez et al., 2019). Instead,

this species is characterized by the ability to assimilate glucose and fructose rapidly (Pretorius, 2000)

and for its high production of β-glycosidase (Arèvalo Villena et al., 2005).

Interestingly, *S. cerevisiae* MN113 in T2 consumed more sugars than the control strain *S. cerevisiae*

US-05 in T1, including maltose, after 2 days of fermentation. This strain showed an excellent ability

to consume sugars present rapidly. After the sequential inoculum the values of maltose decreased. In

fact, to the next sampling day, MN113 together with YGA34 (T3) consumed sugars faster than

YGA34 with US-05 (T4). This confirmed that MN113 even in the case of sequential inoculation has demonstrated an excellent ability to rapidly ferment sugars. At the addition of the juice the concentrations of glucose, sucrose and fructose increased, but at the end of alcoholic fermentation they were completely fermented. The kinetic of sugars consumption by *S. cerevisiae* correspond according to which glucose and fructose are consumed first and then maltose (Tan et al., 2021; Tronchoni et al., 2009). The values of glycerol, malic acid, acetic acid and tartaric acid, produced during fermentation, are reported in Table 2.

Table 2. Conventional chemical parameters monitored in samples beer during the alcoholic fermentation of beer.

	T1	T2	T3	T4	S.S.
D-fructose (g/L)					
2d	0.00 ±0.00[a]	0.00 ±0.00[a]	0.00 ±0.00[a]	0.00 ±0.00[a]	N.S.
9d	0.00 ±0.00[a]	0.00 ±0.00[a]	0.00 ±0.00[a]	0.00 ±0.00[a]	N.S.
10d (+Fr)	6.18 ± 1.22[a]	6.18 ± 1.22[a]	6.18 ± 1.22[a]	6.18 ± 1.22[a]	N.S.
End AF	0.00 ±0.00[a]	0.00 ±0.00[a]	0.00 ±0.00[a]	0.00 ±0.00[a]	N.S.
D-glucose (g/L)					
2d	8.49 ± 0.77[a]	0.35 ± 0.25[b]	14.03 ± 2.91[a]	13.94 ± 2.75[a]	**
9d	3.66 ± 0.04[a]	0.28 ± 0.27[b]	3.65 ± 0.06[a]	3.65 ± 0.09[a]	***
10d (+Fr)	5.65 ± 0.43[a]	2.03 ± 1.77[b]	3.08 ± 0.80[a]	5.29 ± 0.29[a]	*
End AF	0.06 ± 0.01[a]	0.04 ± 0.03[a]	0.04 ± 0.04[a]	0.03 ± 0.01[a]	N.S.
Maltose (g/L)					
2d	15.55 ± 0.54[a]	11.47 ± 1.25[b]	18.98 ± 1.36[a]	18.85 ± 1.81[a]	***
9d	7.14 ± 0.92[b]	3.33 ± 0.15[c]	2.55 ± 0.18[c]	14.21 ± 1.41[a]	***
10d (+Fr)	4.87 ± 0.29[b]	1.64 ± 0.14[c]	2.6 ± 0.19[c]	9.75 ± 0.54[a]	***
End AF	0.37 ± 0.09[b]	1.98 ± 0.23[a]	1.90 ± 0.21[a]	0.94 ± 0.24[b]	***
D-sucrose (g/L)					
2d	6.18 ± 0.45[c]	0.61 ± 0.08[d]	13.88 ± 1.25[b]	16.60 ± 1.05[a]	***
9d	0.36 ± 0.06[a]	0.38 ± 0.10[a]	0.36 ± 0.05[a]	0.39 ± 0.15[a]	N.S.
10d (+Fr)	3.88 ± 0.13[a]	1.06 ± 0.15[c]	2.96 ± 0.05[b]	4.32 ± 0.24[a]	***
End AF	0.37 ± 0.04[b]	0.34 ± 0.09[b]	0.36 ± 0.13[b]	0.36 ± 0.04[b]	N.S.
Acetic acid (g/L)					
2d	0.00 ±0.00[a]	0.09 ± 0.01[a]	0.13 ± 0.13[a]	0.10 ± 0.02[a]	N.S.
9d	0.05 ± 0.04[a]	0.12 ± 0.02[a]	0.13 ± 0.05[a]	0.10 ± 0.01[a]	N.S.
10d (+Fr)	0.00 ±0.00[a]	0.12 ± 0.06[a]	0.13 ± 0.04[a]	0.12 ± 0.02[a]	*
End AF	0.00 ±0.00[a]	0.14 ± 0.02[a]	0.15 ± 0.02[a]	0.12 ± 0.12[a]	N.S.
L-Malic Acid (g/L)					
2d	0.03 ± 0.00[b]	0.04 ± 0.01[a]	0.02 ± 0.00[b]	0.01 ± 0.00[c]	*
9d	0.01 ± 0.01[a]	0.02 ± 0.00[a]	0.01 ± 0.00[a]	0.01 ± 0.01[a]	N.S.
10d (+Fr)	1.61 ± 0.10[a]	1.54 ± 0.37[a]	1.55 ± 0.12[a]	1.55 ± 0.21[a]	N.S.
End AF	1.16 ± 0.03[b]	1.56 ± 0.03[a]	1.36 ± 0.04[ab]	1.39 ±0.21[ab]	*
Glicerol (g/L)					
2d	0.54 ± 0.02[a]	0.51± 0.01[a]	0.59 ± 0.05[a]	0.56 ± 0.08[a]	N.S.
9d	0.55 ± 0.00[a]	0.54± 0.00[a]	0.56 ± 0.09[a]	0.55 ± 0.04[a]	N.S.
10d (+Fr)	0.52 ± 0.01[a]	0.51± 0.02[a]	0.55 ± 0.07[a]	0.54 ± 0.01[a]	N.S.
End AF	0.51 ± 0.01[b]	0.53± 0.02[b]	0.65 ± 0.04[a]	0.57 ± 0.02[b]	*
Tartaric acid (g/L)					

2d	0.15±0.01[b]	0.17±0.02[b]	0.20±0.05[b]	0.18±0.01[a]	***
9d	0.22±0.10[a]	0.26±0.05[a]	0.20±0.11[a]	0.14±0.02[a]	N.S.
10d (+Fr)	0.25±0.03[a]	0.25±0.02[a]	0.26±0.00[a]	0.27±0.06[a]	N.S.
End AF	0.33±0.02[a]	0.32±0.03[a]	0.37±0.00[a]	0.32±0.05[a]	N.S.

Values are expressed as average of three measurements.
Abbreviations: S.S., statistical significance.
Beer fermented by: US-05 (T1), MN113 (T2), sequential inoculum with YGA34 and MN113 (T3), sequential inoculum with YGA34 and US-05 (T4). Data within a line followed by the same letter are not significantly different according to Tukey's test.
Symbols: ***, P < 0.001; **, P < 0.01; * P < 0.05; N.S., not significant.

Acetic acid concentrations were variable among trials and the values measured at the end of AF ranged from 0.05-0.14 g/L. *H. uvarum* YGA34 showed acetic acid values higher than control trial already after the first 48 hours. The acid contents at the end of the secondary alcoholic fermentation were comparable to those reported by Matraxia et al. 2021 and Viana et al., 2021. Glycerol content showed the highest value in trial T3 (0.65 g/L). Regarding this parameter, the trials showing the highest amounts was that involving the sequential inoculation. Malic and tartaric acid showed an increase with the addition of loquat juice, a fruit with a high amount of these compounds (Toker et al. 2013), and no significant differences were found among trials.

2.3.4 Volatile organic compound composition

As a first step, the loquat juice was analysed. The volatile composition of juice was mainly represented by aldehydes (46.65%), alcohols (28.38%), terpenoids (11.35%), and esters (9.86%) (Table 3) were also compared.

Table 3. Volatile compounds identified in *Eriobotrya japonica* juice using HS-SPME/GC-MS.

No.	LRI[a]	Compounds[b]	Content (%)[c]
		Σ Alcohols	28.38
1	885	(*E*)-2-Hexen-1-ol	28.38
		Σ Aldehydes	46.65
2	799	Hexanal	14.04
3	812	Furfural	0.93
4	856	(*E*)-2-Hexanal	16.51
5	949	Benzaldehyde	1.14
6	1005	Octanal	2.70
7	1102	Nonanal	7.19
8	1204	Decanal	4.14
		Σ Esters	9.86
9	605	Ethyl acetate	3.23
10	776	Methyl 2-methylbutanoate	3.86
11	839	Ethyl 2-methylbutanoate	0.76
12	910	Methyl hexanoate (Methyl caproate)	0.79

13	1194	Ethyl octanoate (Ethyl caprylate)		1.22			
		Σ Terpenoids		**11.35**			
14	931	*α*-Pinene		0.60			
15	947	Camphene		1.89			
16	1003	*α*-Terpinene		0.89			
17	1023	*p*-Cymene		1.56			
18	1031	Limonene		2.22			
19	1219	*β*-Cyclocitral		1.76			
20	1288	Thymol		1.41			
21	1385	*β*-Damascenone		1.02			
		Σ Others		**3.76**			
22	772	Toluene		1.61			
23	893	Styrene		2.15			

[a] LRI: DB-5MS column; [b] Compounds are classified in order of linear retention time of non-polar column; [c] Values are expressed as a percentage content.

Beers fermented with *S. cerevisiae* monocultures, US-05 (T1) and MN113 (T2), respectively, had a similar flavour profile (Table 4). In the composition of both products only two classes of volatile organic compounds have been found, alcohols and esters. Alcohols were quantitatively more abundant than esters, 124.85 ppm in T1 and 109.37 ppm in T2, and even if represented by a smaller number of molecules. The most abundant alcohol in both samples was isoamyl alcohol, 93.59 ppm in T2, and 89.12 ppm in T1, followed by phenethyl alcohol (14.28 ppm and 13.04 ppm, in T2 and T1, respectively). The class of esters was present by 22 different compounds, among which the most abundant was ethyl caprate in T2 and T1 (6.36 ppm and 6.62 ppm, respectively) followed in order of abundance by ethyl caprate in T2 (4.84 ppm). In T1, the other esters were present with a concentration lower than 1 ppm. The beers in which YGA34 strain of *H. uvarum* was used with *S. cerevisiae* MN113 and US-05 in sequence (T3 and T4), are characterized by a higher concentration of esters (19.07 ppm in T3 compared to 16.02 ppm in T2, and 10.92 ppm in T4 compared to 9.13 ppm in T1), and a lower concentration of alcohols (93.23 ppm in T3 compared to 109.37 ppm in T2, and 75.92 in T4 compared to 124.85 ppm in T1).

Table 4. Volatile compound concentrations (ppm) in samples of beer. Compounds detected by HS-SPME/GC-MS.

LRI[1]	Ident.[2]	Compounds	T1[3]	T2[3]	T3[3]	T4[3]	S.S.
		Σ Alcohols	**124.85±1.75[a]**	**109.37±1.43[b]**	**93.23±1.17[c]**	**75.92±1.14[d]**	***
621	1, 2	2-Methyl-1-propanol	21.42±0.27[a]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	***
736	1, 2	3-Methyl-1-butanol (Isoamyl alcohol)	89.12±1.11[b]	93.59±1.17[a]	81.38±1.02[c]	58.89±0.74[d]	***
870	1, 2, 3	1-Hexanol	1.27±0.04[b]	1.50±0.02[a]	0.90±0.01[c]	1.51±0.02[a]	***
1122	1, 2	Phenethyl alcohol	13.04±0.33[c]	14.28±0.24[b]	10.95±0.14[d]	15.52±0.38[a]	***
		Σ Esters	**9.13±0.28[d]**	**16.02±0.42[b]**	**19.07±0.42[a]**	**10.92±0.34[c]**	***
605	1, 2, 3	Ethyl acetate	0.39±0.01[d]	0.83±0.02[c]	3.60±0.04[b]	5.91±0.25[a]	***
708	1, 2	Ethyl propanoate	0.11±0.00[c]	0.13±0.00[b]	0.10±0.00[c]	0.28±0.01[a]	***
750	1, 2	Ethyl isobutanoate	0.04±0.00[b]	0.36±0.01[a]	0.05±0.00[b]	0.04±0.00[b]	***

771	1, 2	Isobutyl acetate (2-Methylpropyl acetate)	0.07 ± 0.02^a	0.00 ± 0.00^b	0.00 ± 0.00^b	0.08 ± 0.01^a	**
787	1, 2	Ethyl 2-hydroxypropionate (Ethyl lactate)	0.06 ± 0.00^c	0.40 ± 0.01^a	0.18 ± 0.00^b	0.07 ± 0.00^c	***
799	1, 2	Ethyl butanoate	0.12 ± 0.00^b	0.69 ± 0.02^a	0.12 ± 0.00^b	0.08 ± 0.00^c	***
849	1, 2	Ethyl 3-methylbutanoate	0.03 ± 0.01^a	0.05 ± 0.02^a	0.03 ± 0.01^a	0.02 ± 0.00^a	N.S.
856	1, 2	Ethyl 2-methylbutanoate	0.03 ± 0.00^{bc}	0.06 ± 0.01^a	0.05 ± 0.01^{ab}	0.03 ± 0.00^c	*
876	1, 2	3-Methyl-1-butyl acetate (Isoamyl acetate)	0.78 ± 0.03^c	0.95 ± 0.02^b	2.53 ± 0.06^a	2.57 ± 0.04^a	***
1004	1, 2, 3	Ethyl hexanoate (Ethyl caproate)	0.25 ± 0.00^b	0.29 ± 0.01^a	0.24 ± 0.00^b	0.21 ± 0.00^c	***
1032	1, 2	3-Methylbutyl 2-hydroxypropanoate (Isoamyl lactate)	0.00 ± 0.00^b	0.00 ± 0.00^b	0.07 ± 0.00^a	0.00 ± 0.00^b	***
1194	1, 2, 3	Ethyl octanoate (Ethyl caprylate)	6.62 ± 0.24^a	6.36 ± 0.19^a	6.39 ± 0.18^a	1.00 ± 0.04^b	***
1247	1, 2	3-Methylbutyl hexanoate (Isopentyl hexanoate)	0.05 ± 0.01^a	0.04 ± 0.01^a	0.00 ± 0.00^c	0.02 ± 0.00^b	**
1255	1, 2	Phenylethyl acetate	0.03 ± 0.00^c	0.05 ± 0.01^b	0.06 ± 0.00^b	0.08 ± 0.01^a	**
1294	1, 2	Ethyl nonanoate	0.14 ± 0.01^a	0.11 ± 0.00^b	0.14 ± 0.00^a	0.11 ± 0.01^b	**
1298	1, 2	Ethyl (E)-4-decenoate	0.06 ± 0.00^c	0.38 ± 0.01^a	0.34 ± 0.01^b	0.03 ± 0.00^d	***
1335	1, 2	2-Methylpropyl octanoate (Isobutyl octanoate)	0.02 ± 0.00^b	0.03 ± 0.00^b	0.05 ± 0.01^a	0.00 ± 0.00^c	**
1400	1, 2	Ethyl decanoate (Ethyl caprate)	0.10 ± 0.00^b	4.84 ± 0.12^a	4.79 ± 0.13^a	0.17 ± 0.00^b	***
1453	1, 2	2-Methylbutyl octanoate	0.08 ± 0.01^a	0.09 ± 0.00^a	0.10 ± 0.02^a	0.04 ± 0.00^b	**
1576	1, 2	Ethyl dodecanoate (Ethyl laurate)	0.11 ± 0.00^d	0.24 ± 0.01^a	0.15 ± 0.00^b	0.13 ± 0.00^c	***
1635	1, 2	3-Methylbutyl decanoate (Isopentyl decanoate)	0.01 ± 0.00^b	0.04 ± 0.01^a	0.04 ± 0.01^a	0.02 ± 0.00^b	*
1993	1, 2	Ethyl hexadecanoate (Ethyl palmitate)	0.03 ± 0.00^b	0.08 ± 0.01^a	0.04 ± 0.00^b	0.03 ± 0.00^b	***
		Σ Others	$\mathbf{0.28\pm0.01^b}$	$\mathbf{0.20\pm0.00^c}$	$\mathbf{0.09\pm0.00^d}$	$\mathbf{1.08\pm0.04^a}$	***
893	1, 2	Styrene	0.28 ± 0.01^b	0.20 ± 0.00^c	0.09 ± 0.00^d	1.08 ± 0.04^a	***

[1] LRI: DB5 column; [2] Ident.: 1= retention index identical to bibliography; 2= identification based on comparison of MS; 3= retention time identical to authentic compounds. Compounds are classified in order of linear retention time of non-polar column; [3] Values are expressed in mg L^{-1}, averaged over three samples each analysed in triplicate. Data within a line followed by the same letter are not significantly different according to Tukey's test. Symbols: ***, P < 0.001; **, P < 0.01; * P < 0.05; N.S., not significant.

As previously reported, (Sun et al., 2020) the C_6-aldehydes, that derive from the lipoxygenase (LDX) pathway, were the most abundant volatile aldehyde component. (E)-2-Hexenal (16.51%) and hexanal (14.4 %), with a green-type flavour, were the most important aldehyde in loquat fruit and, such as acetaldehyde, may play important roles in fresh loquat aroma. A previously analysis of free volatile compounds of 'Claudia' loquat cultivar (Planeta et al., 2021) reported a predominance of compounds belonging to the class of acids, probably obtained from a natural oxidation, prevented in our case. Aldehydes and terpenoids were totally absent in the aroma of the final products. Furthermore, alcohols are represented exclusively by (E)-2-hexen-1-ol, that contributes to loquats aromatic character. This compound has a green-type odour and a green-type flavour, but it was not present in the alcohols of the aroma of beers. Among the esters, ethyl acetate, ethyl 2-methylbutanoate and ethyl octanoate (syn. ethyl caprylate) were present in both loquat juice and beer, as well as styrene.

2.3.5 Sensory analysis

The results of the quantitative sensory analysis are reported in Fig. 5. All experimental loquat beers showed consistent differences highlighting the impact due to the starter yeasts (US-05 or MN113) and the presence/absence of *H. uvarum*. As far as the hop aroma is concerned, since hop were not

present in the initial substrate, it is generally described as floral, citrusy, green and spicy, making the hop aroma an easily recognisable descriptor for panelists. Thus, certain combinations of components (Eyres and Dufour, 2008) and/or yeast metabolites can also generate an aroma described as hop aroma. None of the experimental beers showed off-odours. The single culture of control strain US-05 was clearly discriminated from MN113 *S. cerevisiae* strains and YGA34 *H. uvarum* strains.

Fig. 5. Sensory analysis performed on odor of beers: spider plot of average scores for aroma determined by judges during tasting sessions. Beer fermented by: US-05 (T1); MN113 (T2); sequential inoculum with YGA34 and MN113 (T3); sequential inoculum with YGA34 and US-05 (T4). Symbols: ***, P < 0.001; **, P < 0.01; * P < 0.05.
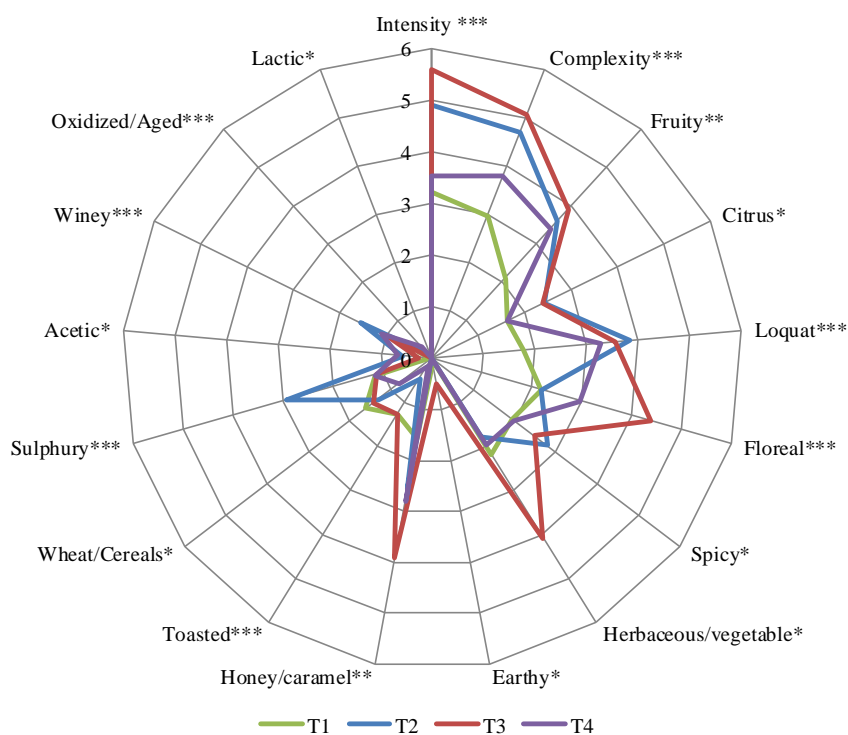
The trial T2 presented the highest scores for five parameters (citrus, loquat, spicy, winey and sulphury), while T3 for seven parameters (intensity, complexity, fruity, floreal, herbaceous/vegetable, earthy and honey/caramel), evidencing a completely different perception of the two beers obtained from these trials. However, in T2 beer fruity, floreal and honey/caramel attributes were clearly recognized. T1 showed the highest scores for wheat/cereals. All panelists recognised the loquat aroma.

Control yeast strain (T1) showed the lowest values for the most all attributes and it was easily recognised by the other yeast strains. Trial T3, sequentially inoculated with YGA34 *H. uvarum* and MN113 *S. cerevisiae* selected strains revealed the highest scores for the most all attributes.

These results demonstrate that non-conventional yeast strains, both *S. cerevisiae* and non-*Saccharomyces*, can increase flavour complexity under sequential culture processes, which agree with some studies. Many researches in fermented beverages have shown that the perception of aroma compounds depends on several factors, called the 'matrix effect', as well as antagonistic or synergistic interactions (Cortese et al., 2020; Holt et al., 2018)

2.3.6   Statistical and explorative multivariate analyses

The graphical representation of VOCs analysis is shown in Fig. 6.

Fig. 6. Distribution of volatile organic compounds among beers. The heat map plot depicts the relative concentration of each VOCs. Beer fermented by: US-05 (T1); MN113 (T2); sequential inoculum with YGA34 and MN113 (T3); sequential inoculum with YGA34 and US-05 (T4).

Fig. 7. Dendrogram of strains resulting from AHC based on values of chemical and sensory aspect of beer experimental productions Dissimilarity is calculated by Euclidean distance. Agglomeration is calculated by Ward's method. Beer fermented by: US-05 (T1); MN113 (T2); sequential inoculum with YGA34 and MN113 (T3); sequential inoculum with YGA34 and US-05 (T4).
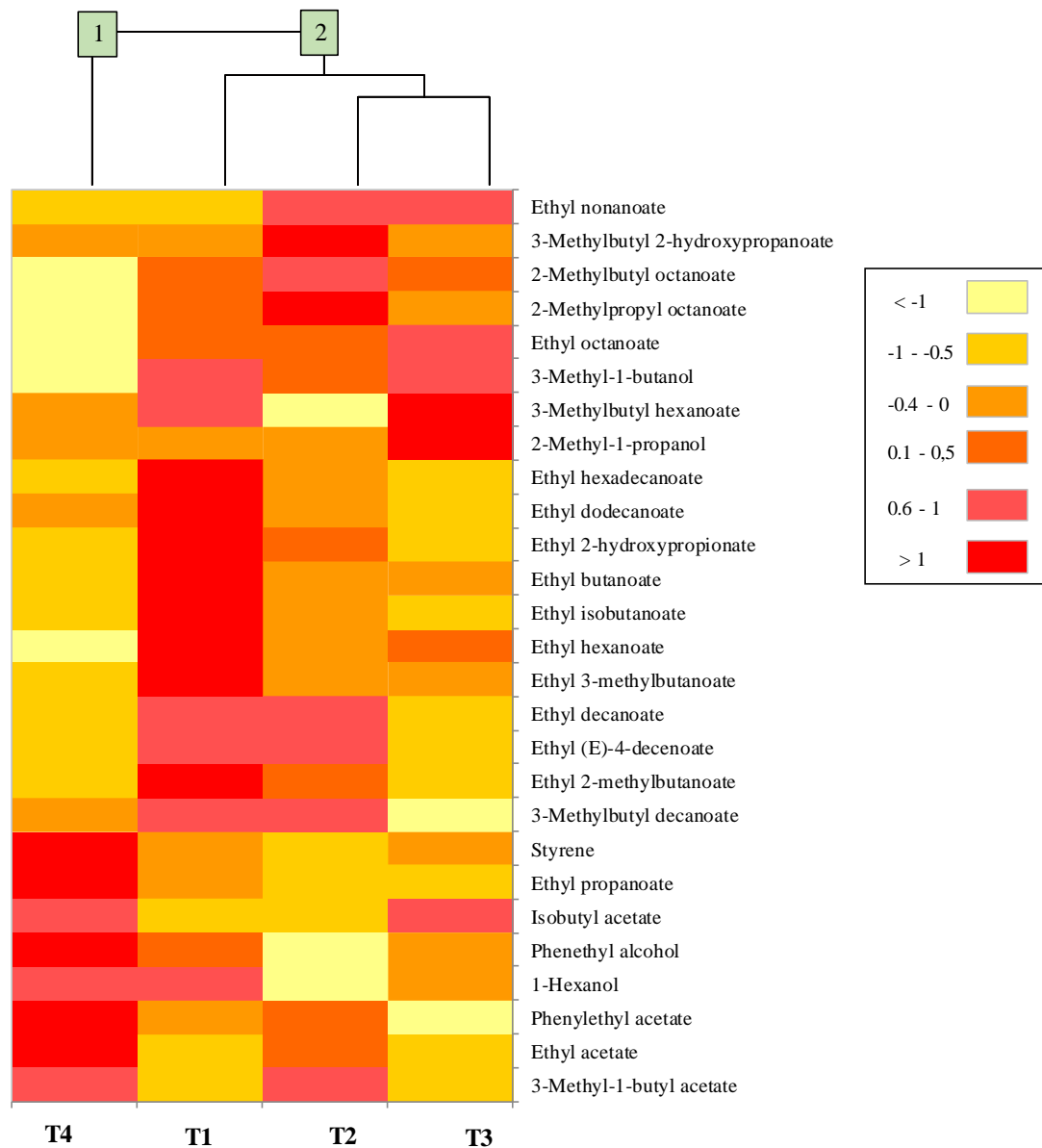
The hierarchical dendrogram combined with heat map plot showed that the inoculation mode and the different strains significantly affected VOCs emitted from beers. The concentrations of the VOCs among trials determined the grouping of trial T1, T2 and T3 in one main cluster.

AHC classified the trials in accordance to their mutual dissimilarity and relationship (Fig. 7).

Dendrogram
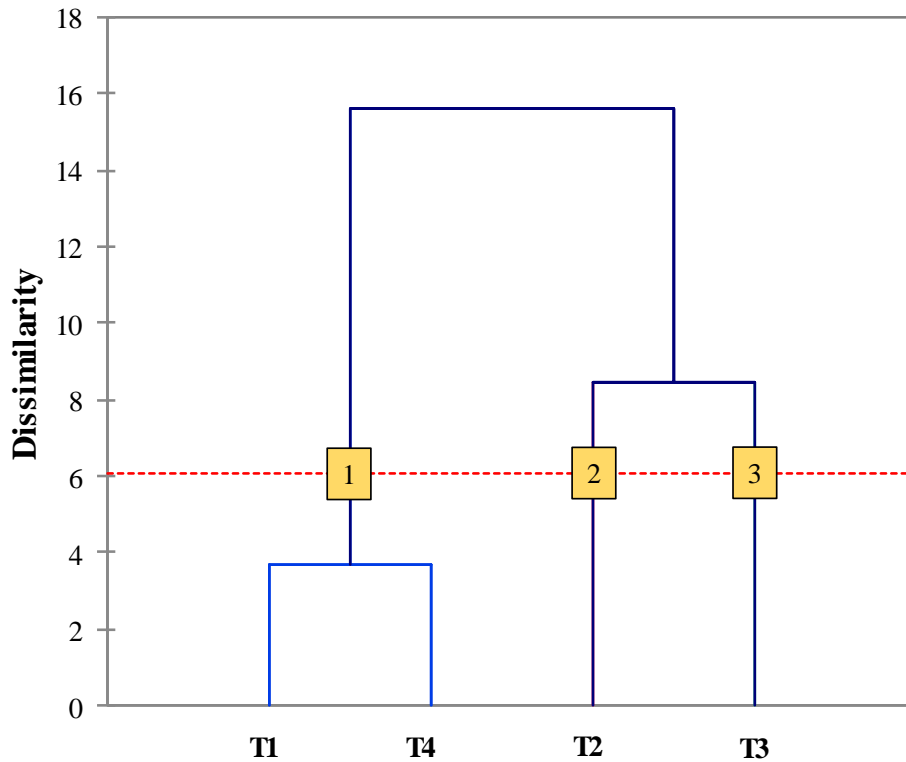
This analysis classified the trials using twenty-five variables selected on the basis of the results from chemical parameters and sensory attributes. All experimental fruit beers were clearly separated into three clusters considering a dissimilarity of 10%. Trials which involved the use of MN113 (T2) and MN113 with YGA34 (T3) were separately grouped, and trials T1 and T4 in one cluster. The variables that greatly impacted trial clusterization resulted maltose, intensity and honey/caramel.

2.4 Conclusions

The information presented in this work help to better understand the sensory brewing potential of unconventional yeasts. The yeast strains, belonging to the genera *S. cerevisiae* and *H. uvarum* previously isolated from manna ash and honey by-products, were screened and evaluated for their performance in fruit beer application as starter or co-starter cultures. For the first time yeast strains of different genera isolated from high sugar matrices were tested in combination to evaluate their effect on physico-chemical and sensory properties of Sicilian loquat beer. The different inoculum combinations among the selected strains showed better results in several aspects than the fermentation

conducted by the commercial control *S. cerevisiae* US-05. 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. However, the higher levels of maltose detected in beers fermented with *S. cerevisiae* MN113 might provide the beverage with a sweeter taste and more palatable to consumers. 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. Regarding VOCs, esters and alcohols were the most abundant compounds emitted from the beers. The beers produced with sequential inoculation of YGA34 *H. uvarum* and *S. cerevisiae* MN113 or US-05 (T3 and T4) are characterised by a higher ester and lower alcohol concentration. This work enriches the very limited scientific knowledge on the role of unconventional yeast from high sugar matrices as potential starter and co-starter for fruit beer production.

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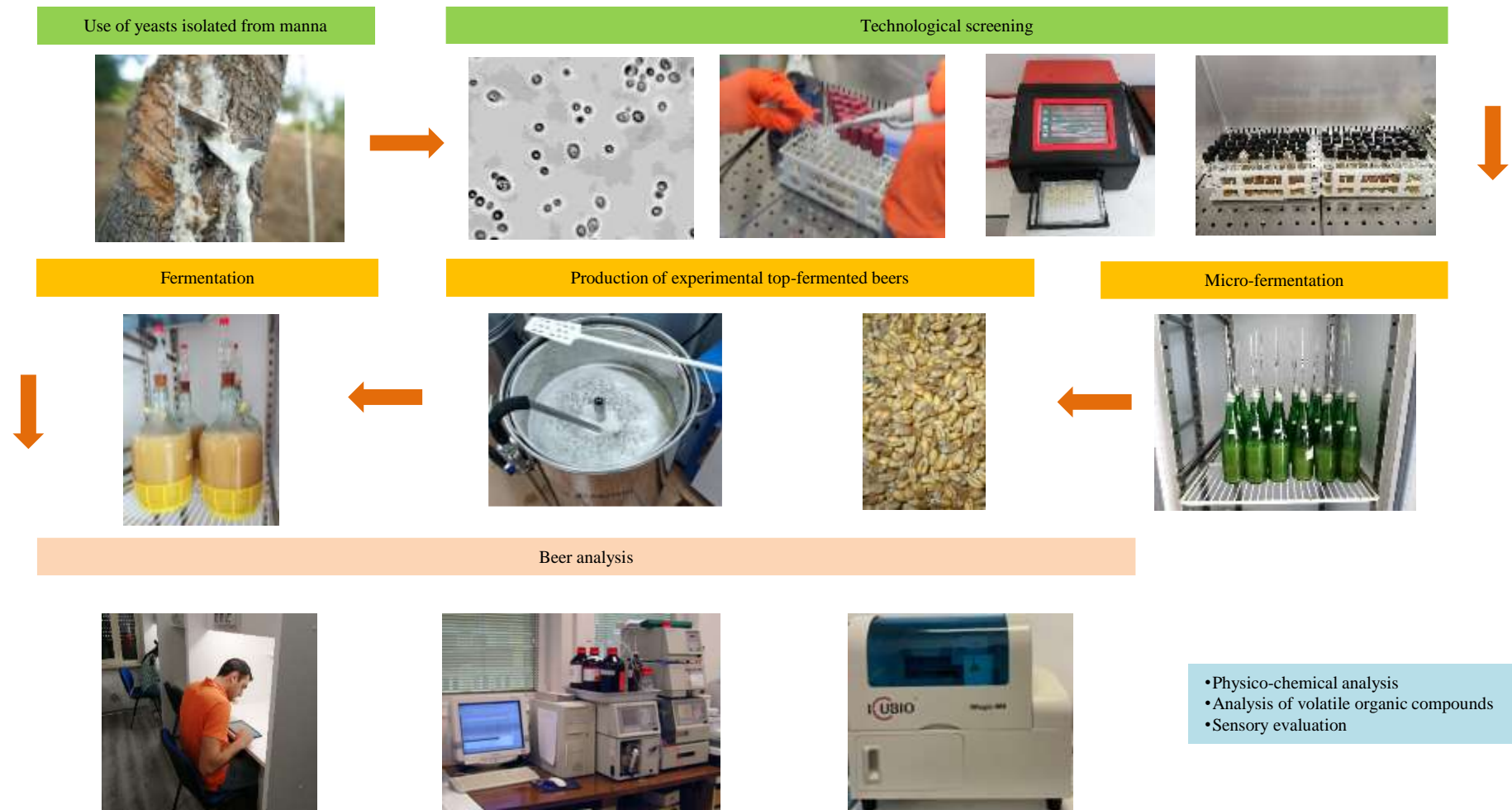
CHAPTER 3

Exploring the diversity of native *Lachancea thermotolerans* strains isolated by sugary extracts from manna ash to modulate the flavour of sour beers

ABSTRACT

The craft beer industry is increasingly interested in sour beers. A new approach, called "primary souring", employs different yeast species, including *Lachancea thermotolerans*, to generate sour beer without the involvement of bacteria. In this study, fifteen strains of *L. thermotolerans* were isolated by sugary extracts from manna ash and evaluated for their ability to produce sour beers with a relatively reduced amount of lactic acid. In particular, five strains exhibited notable resistance to ethanol, sugar and hops, as well as comparable lactic acid production (ranging from 0.33 to 0.45 g/L). Experimental beers produced using MNF105 (T1) were perceived as the most "fruity". Consequently, elevated levels of ethyl decanoate (165.30 mg/L), ethyl hexanoate (29.4 mg/l), ethyl octanoate (29.7 mg/l) and ethyl acetate (36.1 mg/l) were found in T1 beer, exceeding the perception threshold. The limited production of lactic acid by this strain is a desirable characteristic for brewing, particularly for the creation of sour beers with a balanced acid taste and a higher appreciation rating, as demonstrated by sensory analysis. The yeast *L. thermotolerans* MNF105, which is related to manna, has excellent technological properties and is a promising starter for beer production with the ability to light bio-acidify and modulate flavour.

1   **Fig. 1.** Graphical abstract of experimental research

2

3

3.1 Introduction

In recent years, the brewing industry has seen an increase in production, not only in terms of volume, but also in terms of new beer styles and products (Aquilani et al., 2015). Drinkers around the world are increasingly seeking innovative, tasty, and complex craft beers (Betancur et al., 2020). To achieve new beer styles, numerous studies have been conducted on the main materials of beer, such as malt (Gugino et al., 2023; Zdaniewicz et al., 2020a), hops (Paguet et al., 2024), and other ingredients such as fruit (Pirrone et al., 2022). Another alternative for creating new beers with distinctive flavours is the use of non-conventional yeasts. In fact, many researches are focused on selecting, characterizing, and applying non-conventional yeasts (Burini et al., 2022; Larroque et al., 2021; Sampaolesi et al., 2023; Simões et al., 2023).

Moreover, sour beer is a very diverse genre of beer that defies any specific definition based on production process, raw material or geographical origin. A common denominator of sour beers is a higher concentration of organic acids, resulting in a lower pH (pH 3.0 to 3.9) compared to 'normal' beers. Sour beers are intentionally designed to be acid and can also be fermented with wild microorganisms or fruit, barrel-aged, or blended with younger beers (Bossaert et al., 2019; Tonsmeire, 2014). There are different microbiological approaches for brewing sour beers, through spontaneous fermentation or through inoculum of acidifying bacteria, such as acetic acid or lactic acid bacteria or yeasts of the genus *Brettanomyces* or *Lachanchea* (Dysvik et al., 2020).

Osburn et al. (2018) introduced the concept of 'primary souring', which focuses on using different yeast species, including strains of *Lachancea thermotolerans*, to brew sour beer without bacteria, resulting in a significant reduction in the duration and variability of the transformation process. This species is capable of heterolactic fermentation of sugar into lactic acid, ethanol, CO_2, and at the same time, producing pleasant aromatic and flavour compounds (Domizio et al., 2016; Osburn et al., 2018). Using lactic acid bacteria in breweries is widely acknowledged to result in significant contamination risks, along with escalating sanitisation expenses (Maifreni et al., 2015).

29 Recently, researches have conducted studies on *L. thermotolerans* for brewing application. Postigo et

30 al. (2023) determined the fermentation capacity of 10 yeast strains of *L. thermotolerans* and showed

31 that beer fermented with strain CLI 1232 had a balanced acidity with a fruity flavour profile and

32 honey notes, whereas strain 1-8B had a balanced acidity but less fruity and citrus flavour than strain

33 CLI 1232. Canonico et al. (2019) confirmed that *L. thermotolerans* strains can lower the pH of the

34 inoculation medium due to the formation of significant amounts of lactic acid, as well as they

35 consistently produce ethyl butyrate and ethyl acetate. However, Zdaniewicz et al. (2020b)

36 demonstrated that certain strains of *L. thermotolerans* have poor lactic acid production and have a

37 slight effect on pH lowering, although they generated higher amounts of ethyl lactate than *S.*

38 *cerevisiae.*

39 Some authors (Guarcello et al., 2019; Matraxia et al., 2021; Sinacori et al., 2014) have recently

40 conducted researches on the selection of yeasts from high sugar matrices as honey, honey by-products

41 and manna. Manna is a sugary product obtained from the solidification of the processed sap that

42 emerges from incisions made in the stem and main branches of certain species of the genus *Fraxinus*

43 sp. during the summer season (Schicchi et al., 2007; Yücedag and Sen, 2008). Manna hosts

44 microorganisms that are osmophilic, meaning they can survive in a viable form under intense stress

45 conditions caused by osmotic pressure due to the high sugar content. The most abundant yeast species

46 found in manna is *L. thermotolerans* (Guarcello et al., 2019). Yeast strains isolated from this matrix,

47 both *Saccharomyces* and non-*Saccharomyces*, have great technological properties and can improve

48 the flavour profile of beers produced (Francesca et al., 2023; Pirrone et al., 2022).

49 The research aimed to achieve the following objectives: (i) characterizing *L. thermotolerans* strains

50 isolated from manna for their brewing properties; (ii) selecting the yeast strain that has demonstrated

51 the best effective fermentation performance in the production of innovative craft beer; and (iii)

52 evaluating the effect of the best strain during beer fermentation and the sensory quality of bottled sour

53 beer.

54

3.2 Materials and methods

3.2.1 Yeast strains and media

The yeasts used in this study, were previously isolated from Manna and identified in a precedent work by Guarcello et al. (2019). These strains were stored in glycerol stocks at -80 °C at the microbial collection of Department of Agricultural, Food and Forest Sciences (SAAF; University of Palermo, Italy). The strains were recovered and grown on YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose] at 30 °C for 2 days. All media components were purchased from Thermo Fischer (Milan, Italy). The investigation focuses on fifteen different strains (MN28, MN136, MN93, MN400, MNF104, MNF105, YS186, YS1, YS42, YS45, YS55, XV11, XV22, XV34, and XV47) belonging to the *L. thermotolerans* species. Commercial yeast strains *L. thermotolerans* Philly Sour and *S. cerevisiae* US-05 (both sourced from Lallemand Inc., Montreal, Canada) were utilized as controls in this study.

3.2.2 Technological screening of Lachancea thermotolerans *strains*

3.2.2.1 Hydrogen sulphide production

The production of hydrogen sulphide (H_2S) was evaluated by culturing the strains onto bismuth sulphite agar (Biggy Agar), Wilson-Blair medium (Merck, Darmstadt, Germany; Jiranek et al., 1995). The results were evaluated by measuring the degree of colony blackening after 3 days of incubation at 28 °C, using a five-level scale: 0 = white, 1 = beige, 2 = light brown, 3 = brown, 4 = dark brown, 5 = black (Matraxia et al., 2021). The positive control was represented by *S. cerevisiae* GR1 (SAAF Department collection), which exhibited a brown colouration (3 = brown).

3.2.2.2 Sugar and ethanol tolerance assays

To evaluate ethanol tolerance, dilutions of pure exponential cultures were transferred onto tubes containing liquid YPD media supplemented with 6, 8 and 10% (v/v) ethanol. All samples were incubated at 28 °C for 3 days. Sugar stress tolerance assays were obtained using the same procedure as described by Binati et al. (2019). Stress conditions were tested in YPD medium containing different

81 doses of stress agent. YPD medium was added (1%) to a previously grown culture and incubated

82 overnight at 27 °C with agitation to reach the initial stationary phase. Accordingly, the following

83 glucose concentrations were used: 220, 270 and 320 g/L. YPD medium without stress agent was used

84 as a negative control. A commercial *S. cerevisiae* US-05 was used as a positive control strain. All

85 analyses were carried out in triplicate.

86 3.2.2.3 Glucose, fructose, and maltose assimilation test

87 The ability of the strains to grow in the presence of different sugars was assessed using the procedure

88 illustrated by Kurtzman et al. (2011) with the following modifications: tests were conducted in rimless

89 tubes (16 × 180 mm), each one containing 10 mL of Yeast extract – Malt extract medium (YM

90 composition: yeast extract, 3 g/L, triptone, 5 g/L; glucose, fructose or maltose, 200 g/L) and

91 inoculated with pure strain cultures as reported by Hall et al. (2014). Growth was assessed by visual

92 inspection (Kurtzman et al., 2011). The growth of the pure strain cultures in wort was further

93 investigated by optical density (OD) measurement at 600 nm wavelength into a 96-well microtitre

94 plate (Michel et al., 2016). The measurement was performed at 24 h intervals for 4 d using the

95 ScanReady Microplate photometer P-800 (Life Real Biotechnology Co., Ltd, Hangzhou, China).

96 Incubation temperature was set at 25 °C. Blank measurement was subtracted from each OD reading.

97 The variables describing the growth curves were represented by the sum of the values of the subtended

98 areas of the curves measured daily until the end of the experiment. A commercial *L. thermotolerans*

99 (Philly sour) was used as a control strain, and a same medium without inoculum was used as a

100 negative control. All analyses were performed in triplicates in two independent experiments.

101 3.2.2.4 Cross resistance to hop and ethanol

102 The five strains of *L. thermotolerans* that showed the best technological performances, i.e. low H_2S

103 production, ethanol resistance, sugar stress tolerance, and excellent sugar assimilation ability, were

104 evaluated for their ability to hop resistance, flocculate, and ferment beer wort. The tolerance of *L.*

105 *thermotolerans* strains to hop was evaluated applying the procedure illustrated by Matraxia et al.

106 (2021). Growth was assessed by visual inspection (Kurtzman et al., 2011; Michel et al., 2016). A

commercial *S. cerevisiae* (US-05) was used as a control strain, and the same medium without inoculum was used as a negative control. All analyses were performed in triplicates.

3.2.2.5 Flocculation assay

The flocculation assay was carried out as previously described by Tofalo et al. (2014). Flocculation was also evaluated using the Helm's assay. In this assay, flocculation type and sedimentation volume were measured and evaluated in a calcium sulphate solution buffered at pH 4.5 according to Casey et al. (1994). A commercial *S. cerevisiae* (US-05) was used as a control strain, and the same medium without inoculum was used as a negative control. All analyses were executed in triplicates.

3.2.2.6 Microfermentation: monitoring of weight loss, strain concentration and physicochemical analysis

To ensure standardized conditions for all trials, the wort fermentation medium was prepared as described by Matraxia et al. (2021). Aliquots of 200 mL of wort were placed in 300 mL flasks, sealed with a Müller valve to allow CO_2 produced during fermentation to leave the system and autoclaved at 110 °C for 15 min. After sterilization, the extracted wort was allowed to cool to 18 °C and then inoculated with each yeast strain. Fermentation was performed at 18 °C under static conditions and was monitored daily by measuring weight loss until day 12. To facilitate CO_2 removal, the flasks were occluded with a Müller valve (Ciani & Rosini 1987) and the weight loss was monitored until a daily decrease below 0.01 g (end of fermentation process). According to Ciani and Maccarelli (1998), the fermentation rate was determined as the daily production of CO_2 after 3 days and at the end of alcoholic fermentation, and fermentation vigour as the maximum amount of ethanol produced (as % v/v).

A commercial yeast of *L. thermotolerans* (Philly sour) was used as a positive control, and a wort without inoculum was used as a negative control. The cell density of *L. thermotolerans* strains was monitored at different stages of the fermentation process: at the first inoculum (D_0), at the 3rd day (D_3), at the 6th day (D_6), at the 9th day (D_9), at the 12th day (D_{12}), and at the end of alcoholic fermentation (D_{15}). The samples were serially diluted in Ringer's solution (Sigma-Aldrich, Milan,

Italy) and spread-plated (0.1 mL) into Wallerstein Laboratory (WL) nutrient agar to evaluate *Saccharomyces* populations and Lysine Agar medium (LA) for non-*Saccharomyces* (Di Maio et al., 2011; Iris et al., 2020). Lysine Agar is a medium that does not allow the growth of *S. cerevisiae* (Lin, 1975), and was used for the evaluation of theses with *L. thermotolerans* strains. All different colonies were identified as putative *Saccharomyces* and *Lachancea* after microscopic examination (Cavazza et al., 1992). Total yeast counts were determined by incubating the samples at 28 °C for 48–72 h. At the end of fermentation, the beers were analysed for pH, residual sugar, lactic acid, acetic acid, and glycerol content using standard methods. The pH of the several samples collected was conducted using a pH meter Mod.70 XS/50010162 (Cheimika, Pellezzano, Italy). Brix degrees (°Bx) of the wort were determined with a refractometer DBR Salt (Zetalab srl, Padova, Italy). The determination of lactic acid, acetic acid, and glycerol was performed through the enzymatic analyser iCubio iMagic M9 (Shenzhen iCubio Biomedical Technology Co. Ltd. Shenzhen, China) following the procedure reported by Matraxia et al. (2021). All reagents and standards were purchased from R-Biopharm AG (Darmstadt, Germany). All analyses were performed in triplicates.

3.2.3 Experimental design and sample collection for beer production

The role of the inoculum during fermentation was followed on experimental top-fermented beers produced at a medium-scale level (5 L batch) using an all-in-one microbrewing plant Klarstein mod. 10031629 (Chal-Tec GmbH Berlin, Germany). For beer production, 4 kg wheat malt and 4 kg pilsen malt were used (BestMalz, Heidelberg, Germany). The malts were ground through a roller mill placed at 1.20 mm. The malts were then added to 30 L of water with 8 g of calcium sulfate ($CaSO_4$) and 8 g of calcium chloride ($CaCl_2$) for pH correction (Marconi et al., 2016). The mash was carried out according to the procedure of Francesca et al. (2023). Subsequently, the grains were rinsed with 16 L of water previously heated to 78 °C during the lautering phase, yielding in a total wort volume of 35 L. After filtration, the liquid portion of the wort was boiled and hops (Mandarina Bavaria - pellets, 40 g, 9.7 % w/w α-acids) were added at the beginning of this stage. At the end of this process, the

total volume was 33 L. The wort was then clarified with a whirlpool involving a 10-min recirculation and a 10-minute rest. Finally, the wort was cooled to the yeast inoculation temperature of 21 °C using a stainless-steel wort cooling coil. Three experimental trials were inoculated as follows: T1 with *L. thermotolerans* MNF105, T2 with *L. thermotolerans* Philly sour and T3 with *S. cerevisiae* US-05. Therefore, to better evaluate the behaviour of the native strain, it was compared with a commercial strain of *L. thermotolerans* and one of *S. cerevisiae*.

Yeast strain was inoculated at a cell density of approximately 2.0×10^6 cells/mL (Holt et al., 2018). Fermentation was conducted at 20 °C in glass fermenters with hermetic closure and compensation valve. Samples were collected at different stages of beer production, including uninoculated must, after the inoculum of yeast strains, at 3, 6, 9 and 12 d. At the end of the fermentation process, the beer was transferred into 0.33 L bottles. At the end of the fermentation process, the beer was transferred into 0.33 L bottles by adding 6 g/L of dextrose. The bottles were conditioned at 20 °C for 25 days (Francesca et al., 2023). At the end of this process, the beers were subjected to volatile organic compounds and sensory analysis. All fermentation experiments were carried out in triplicate.

3.2.4 Microbiological counts and dominance of the inoculated strains and determination of physicochemical parameters

Microbiological counts and determination of physicochemical parameters were performed as described at paragraph 2.2.6. For each treatment and sampling point, isolates were obtained from the highest dilutions of the respective culture medium (Lysine Agar for non-*Saccharomyces* and WL agar for presumptive *Saccharomyces*) in order to verify the dominance of *L. thermotolerans* MNF105, Philly Sour and *S. cerevisiae* US-05. Five yeast colonies with the same macroscopic morphology were purified to obtain axenic colonies. These colonies were then observed under a light microscope to determine their cell morphology. Yeasts with a cell morphology similar to that of the genus *Lachancea* (Lachance and Kurtzman, 2011) and *Saccharomyces* (Vopálenská et al., 2005) were subjected to DNA extraction. The identification of the species *L. thermotholerans* and *S. cerevisiae*

was confirmed through Restriction Fragment Length Polymorphism (RFLP) analysis, in accordance

with the methodology described by Esteve-Zarzoso et al. (1999), utilising the restriction enzymes

C*fo*I, H*inf*, and H*ae*III. The strain typing of *L. thermotholerans* strains was carried out by DNA

(RAPD)-PCR analysis using the primer M13 (Binati et al., 2019), while for *S. cerevisiae*, interdelta

analysis (Legras and Karst, 2003) was used. Amplification, visualisation of bands and result analysis

were conducted using Gelcompar II software, version 6.5 (Applied-Maths, Sint-Martens-Latem,

Belgium), in accordance with the methodology described by Alfonzo et al. (2021). The comparison

of the polymorphic and interdelta profiles of the isolates from the different trials and the inoculated

strains (*L. thermotholerans* MNF105, Philly Sour and *S. cerevisiae* US-05) enabled the percentage of

dominance to be determined.

BeerFoss™ FT Go (FOSS Italia srl, Padova, Italy) was used to measure alcohol (% vol), density

(FG), real extract (°P), energy (kcal/100g), apparent extract (°P), original extract (°P), specific gravity

(°P) and real attenuation (%) of the final beers (Gugino et al., 2024; Francesca et al., 2023).

3.2.5 Analysis of Volatile organic compounds (VOCs) of beer samples

3.2.5.1 *Standard solutions*

Limonene (Fisher Scientific S.L.C, 28108 – Alcobendas-Madrid) was used as standard for calibration

line. Standard solutions were prepared at five different concentrations (31.2 mg/L, 62.5 mg/L, 125

mg/L, 169 mg/L and 250 mg/L)

3.2.5.2 SPME analysis

Beer samples (10 mL) were placed in a 20 mL SPME glass vial (Gerstel, 75.5 × 22.5 mm) together

with 1 g of sodium chloride. The column temperature was initially kept constant at 40 °C for 2 min

(during splitless injection), subsequently, increasing the temperature by 4 °C/min was set to 60 °C, at

which it was kept constant for 2 min. Increasing the temperature by 2 °C/min., it was raised to 90 °C,

from 190 °C to 230 °C, increasing by 5 °C/min and finally left at 230 °C for 15 min. The analyses in

the fiber were automatically injected at 250 °C with the splitless mode. The mass spectrometer was

set in MS mode to acquire all mass-to-charge ratios from 35 to 450 amu (0.1 amu). 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. Quantification was carried out using limonene calibration line.

3.2.5.3 Identification and quantification of VOCs by GC-MS

Gas chromatographic analyses were performed using 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: 3.5 min. Helium was the carrier gas (1 mL/min). The odour activity value (OAV) was calculated for each VOC detected, following the approach proposed by Butkhup et al. (2011), to determine the VOCs that contributed significantly to the odour series characterising each beer.

3.2.6 Sensory analysis

Fifteen judges (10 men and 5 women) aged between 26 and 46 were selected from the University of Palermo to evaluate the beer produced. All panelists had experience in brewing and participated in previous studies as sensory judges. The beer evaluation procedure was carried out as described by Francesca et al. (2023) with some modifications to the descriptors as follows: odour (intensity, complexity, fruity, floral, hoppy, wheat/cereal, honey/caramel, acetic, oxidized/aged, sulphury, alcohol and DMS) and taste (intensity, complexity, sweet, bitter, acid, astringent, fruity, spicy, hoppy, sapidity, wheat/cereal, burnt/cooked, alcohol, body, DMS and oxidized/aged) and overall acceptance. The sensory attributes were assessed using an unstructured nine-point scale anchored at the left end with "absent" and at the right end with "high" (Gugino et al., 2024; Jackson et al., 2016). The average of the three assessments was used to obtain the final scores.

3.2.7 Statistical analysis

The results of physicochemical parameters of micro-fermentation and fermentation were statistically processed by analysis of variance (ANOVA), and homogeneous groups were identified by Tukey's test (statistical significance: $P < 0.05$). Sensory product characterization was performed to differentiate between the different treatments based on data collected during sensory analysis, conducted following the methodology outlined by Alfonzo et al. (2023). The objective of this study is to evaluate the correlation between the aromas identified in the VOCs with an odour activity value exceeding 1 and the sensory analysis (only aroma), a principal component analysis (PCA) was performed using the XLstat software version 2019.2.2 (Addinsoft, New York, NY, USA) for Excel.

3.3 Results and Discussion

3.3.1 Technological characteristics of Lachancea thermotolerans yeast strains for beer production

3.3.1.1 H_2S production, alcohol resistance and sugar stress tolerance

The results of technological screening are reported in Table 1. All strains were found to be devoid of H_2S production, confirming the findings of Porter et al. (2019). The evaluation of H_2S production revealed no strain variability. However, these results are in contrast with those of Comitini et al. (2011), who demonstrated that some strains belonging to the genus *Lachancea* can high amounts of this compound (3 to 5 on a scale of 0 to 5). It is important to note that this trait is not dependent on the genus or species, but on the strain. All strains of *L. thermotolerans* showed growth on lysine agar (25 °C). Additionally, the strains exhibited tolerance to ethanol. In our study, all strains of *L. thermotolerans* showed the ability to resist alcohol at 6 % and 8 % (v/v), and five strains showed high resistance at 10% (v/v). Moreover, *L. thermotolerans* spp. is known to be more resistant to ethanol than other non-*Saccharomyces* species. However, this characteristic depends on the strain, with some strains showing greater resistance than others. For example, some strains of *L. thermotolerans* have been shown to be more resistant than other species of this genus, including *Lachanchea lanzarotensis* and *Lachanchea fermentati* (Porter et al., 2019a; Porter et al., 2019b). Similarly, it has been shown to

be more resistant than other species such as *H. uvarum*, *Metschnikowia pulcherrima* or *Starmerella Bacillaris* (Aponte & Blaiotta, 2016). The *L. thermotolerans* strains chosen for this investigation may be used as starters to produce beer with up to 10% (v/v) ethanol. Regarding sugar stress tolerance, all isolates were able to grow at the three concentrations tested and showed no difference in growth at the increased osmotic pressure caused by high glucose concentrations. This contrasts with Binati et al. (2019), who showed a decrease in yeast culture growth with increasing sugar content. This may be related to strain and sampling matrix, as yeast strains isolated from manna, a high sugar matrix, may be more resistant to osmotic pressure (Guarcello et al., 2019).

Table 1. Technological characteristics of *Lachancea thermotolerans* strains for beer production

	Production of H$_2$S[a]	Growth on LA[b]	Ethanol resistance			Sugar test tolerance		
			6%	8%	10%	220 g/L	270 g/L	320 g/L
MN28	-	+	+	+	+	+	+	+
MN136	-	+	+	+	-	+	+	+
MN93	-	+	+	+	+	+	+	+
MN400	-	+	+	+	+	+	+	+
MNF 104	-	+	+	+	-	+	+	+
MNF 105	-	+	+	+	+	+	+	+
YS186	-	+	+	+	-	+	+	+
YS1	-	+	+	+	-	+	+	+
YS42	-	+	+	+	-	+	+	+
YS45	-	+	+	+	-	+	+	+
YS55	-	+	+	+	-	+	+	+
XV11	-	+	+	+	-	+	+	+
XV22	-	+	+	+	-	+	+	+
XV34	-	+	+	+	-	+	+	+
XV47	-	+	+	+	+	+	+	+
PC[c]	+/-	-	+	+	+	+	+	+
NC[d]	-	-	-	-	-	-	-	-

Symbols: +, positive growth; −, no growth; +/- , weak growth.
Abbreviations: [a] H$_2$S, Hydrogen sulphide; [b] LA, lysine agar, [c] CP, positive control, [d] NC, negative control.

All strains were found to be devoid of H$_2$S production, confirming the findings of Porter et al. (2019). The evaluation of H$_2$S production revealed no strain variability. However, these results are in contrast with those of Comitini et al. (2011), who demonstrated that some strains belonging to the genus *Lachancea* can high amounts of this compound (3 to 5 on a scale of 0 to 5). It is important to note

that this trait is not dependent on the genus or species, but on the strain. All strains of *L. thermotolerans* showed growth on lysine agar (25 °C). Additionally, the strains exhibited tolerance to ethanol. In our study, all strains of *L. thermotolerans* showed the ability to resist alcohol at 6 % and 8 % (v/v), and five strains showed high resistance at 10% (v/v). Moreover, *L. thermotolerans* spp. is known to be more resistant to ethanol than other non-*Saccharomyces* species. However, this characteristic depends on the strain, with some strains showing greater resistance than others. For example, some strains of *L. thermotolerans* have been shown to be more resistant than other species of this genus, including *Lachanchea lanzarotensis* and *Lachanchea fermentati* (Porter et al., 2019a; Porter et al., 2019b). Similarly, it has been shown to be more resistant than other species such as *H. uvarum*, *Metschnikowia pulcherrima* or *Starmerella Bacillaris* (Aponte & Blaiotta, 2016). The *L. thermotolerans* strains chosen for this investigation may be used as starters to produce beer with up to 10% (v/v) ethanol. Regarding sugar stress tolerance, all isolates were able to grow at the three concentrations tested and showed no difference in growth at the increased osmotic pressure caused by high glucose concentrations. This contrasts with Binati et al. (2019), who showed a decrease in yeast culture growth with increasing sugar content. This may be related to strain and sampling matrix, as yeast strains isolated from manna, a high sugar matrix, may be more resistant to osmotic pressure (Guarcello et al., 2019).

3.3.1.2 Glucose, fructose and maltose assimilation test

The strains were tested for their ability to assimilate fructose, glucose, and maltose during the fermentation of the main sugar in the wort (Fig. 2a, 2b, 2c). The results showed that all 15 isolates grew in the presence of single sugar as the sole carbon source, but some of them showed more growth than others.

Figure 2. Fructose, glucose and maltose (a, b and c) consumption monitored during the alcoholic fermentation of the wort, inoculated with the different yeast strains, over 4 days of sperimentation (Values are expressed as average of three measurements ± standard deviation).
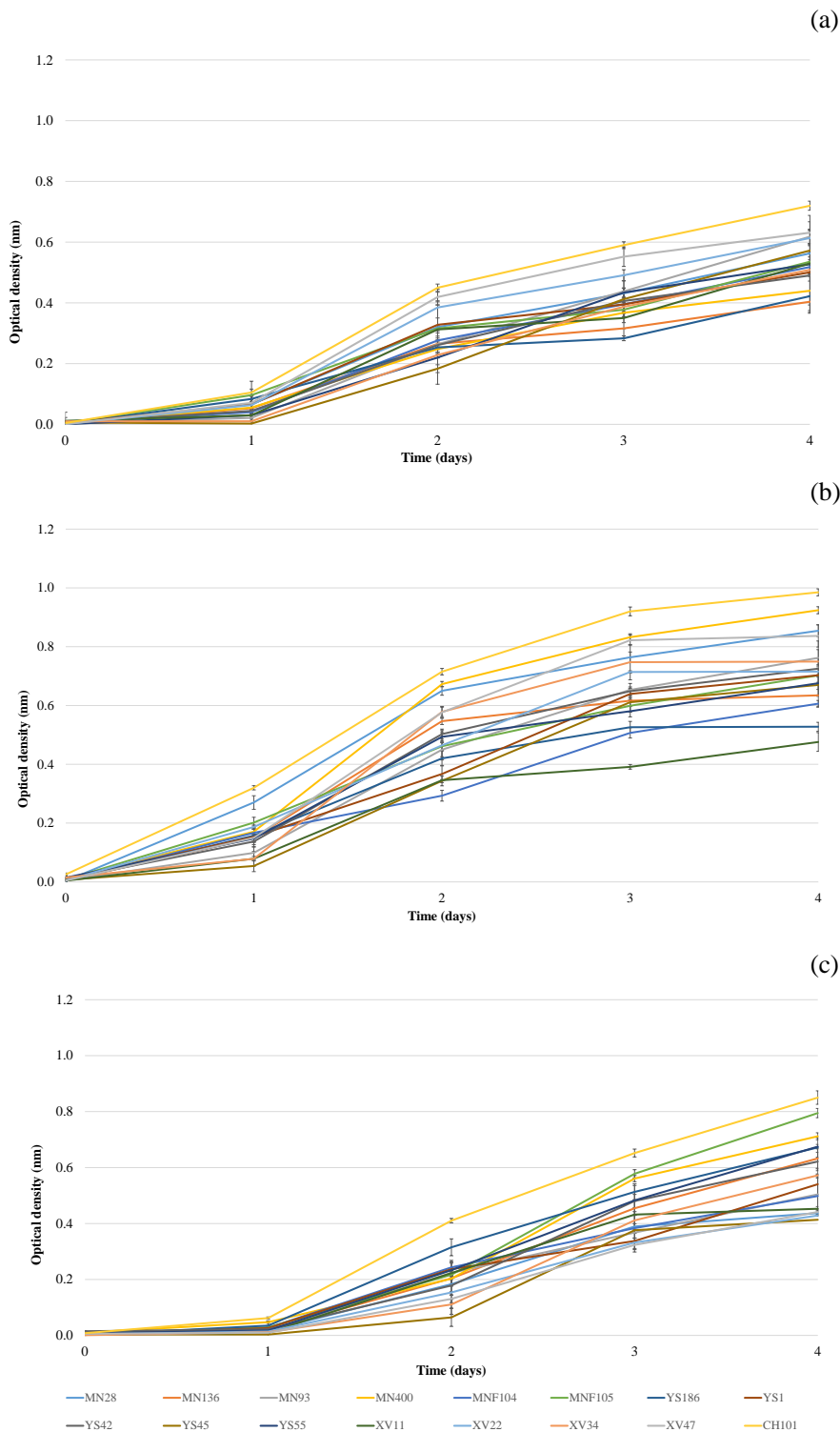
Strain XV47 showed the highest total growth during fermentation, in terms of fructose consumption (Fig. 2a), followed by strain XV22. However, in terms of glucose consumption (Fig. 2b), the highest total growth was achieved by strain MN400, while the second-best strain was MN28. As

70

309 demonstrated, this species is characterized by the ability to ferment sugars such as glucose and

310 fructose, confirming numerous studies (Cominiti et al., 2011; Toh et al., 2020; Zdaniewicz et al.,

311 2020b). After fructose and glucose uptake, yeast assimilates maltose, the most abundant fermentable

312 sugar in brewing wort (Boulton & Quain, 2006). *L. thermotolerans* is known to be able to consume

313 maltose, but not all strains are able to do so (Domizio et al., 2016; Toh et al., 2020; Postigo et al.,

314 2022). In this case, all strains were able to consume maltose, with the highest total growth regarding

315 maltose consumption found for strain MNF105 (Fig. 2c), followed by strain MN400. These results

316 suggest that the strains selected for this research can be used as starter strains for brewing applications,

317 with some of these strains being particularly suited to beer production.

318 3.3.1.3 Cross resistance to hop and ethanol

319 The study evaluated the growth of yeasts in the presence of iso-α-acid and in the cross presence of

320 iso-α-acid and ethanol to select the most resistant strains (Table 2).

321 **Table 2.** Cross resistance to hop and ethanol of the different yeast strains

Strain code	Resistance to hop				Cross resistance				Flocculation assay[a]	Sedimentation volume[b]
	0 IBU	25 IBU	50 IBU	90 IBU	0 IBU/5% ethanol	25 IBU/5% ethanol	50 IBU/5% ethanol	90 IBU/5% ethanol		
MN28	+	+	+	+	+	+	+	+	0	0.75
MN93	+	+	+	+	+	+	+	+	0	0.65
MN400	+	+	+	+	+	+	+	+	0	0.55
MNF105	+	+	+	+	+	+	+	+	0	0.45
XV47	+	+	+	+	+	+	+	+	0	0.60
PC	+	+	+	+	+	+	+	+	0	0.75
NC	-	-	-	-	-	-	-	-	0	0

322 Symbols: +, positive growth; −, no growth; +/- , weak growth.
323 Abbreviations: IBU, International Bitterness Unit; PC, positive control; NC, negative control;
324 [a] Flocculation degree after 22 days of incubation;
325 [b] Mean sedimentation volume (mL) expressed according to Helm's Assay.
326

327 All strains were able to grow in liquid medium containing 0, 25, 50, and 90 IBU. In terms of cross-

328 resistance to ethanol and hops, all strains showed vigorous growth in the presence of all the limiting

329 conditions present (Table 2). Accordingly, to Domizio et al. (2017) demonstrated that this species is

330 not affected by IBU levels, at least up to 60 IBU.

331

3.3.1.4 Flocculation assay

The mean sedimentation volumes ranged from 0.45 to 0.75 mL as measured by the Helm's assay. All

the yeasts exhibited type II flocculation with an ascending interface near the bottom of the tubes,

typical of non-flocculating yeasts, as described by Casey et al. (1994). The flocculation capacity of

brewer's yeast is directly related to the yield, clarity and filtration performance of green beer

(Govender, Kroppenstedt and Bauer, 2011; Varela et al., 2020). Furthermore, low flocculation can

result in higher attenuation (Panteloglou, Smart and Cook, 2012). Consequently, yeasts with low

flocculation tend to remain in suspension, resulting in a cloudy appearance of the finished beer.

Nevertheless, beer turbidity may also be a desired quality in unfiltered or naturally turbid beers, such

as wheat beer (Kahle, Zarnkow and Jacob, 2021).

3.3.1.5 Microfermentation

The strains were evaluated for their ability to grow under technological conditions simulating beer

production. The results of the fermentation kinetics of wort weight loss as a function of CO_2

production (Fig. 3) showed that the MNF105 strain lost slightly more weight than the control strain

Philly Sour. After 3 days of alcoholic fermentation, strain MN28 showed the greatest weight loss

(2.80 g). From day 3 to day 15, the greatest weight loss was measured for strain MN93 (10.39 g). At

the end of the alcoholic fermentation process, the greatest weight losses, ranging from 10.80 g to

11.15 g, were observed for strains MN28 and MNF105, respectively. Yeast strain MN28 showed a

higher value for fermentation rate (2.80 v/v), while MNF105 showed a higher value for fermentation

vigour (4.61 g of CO_2/day). In general, both strains exhibited lower values than the control strain.

These results contrast withthose obtained by Zdaniewicz et al. (2020b), who reported a high

fermentation intensity in the first days of the process.

360

361   The growth kinetics of the different inoculated strains are shown in Fig. 4. The microbiological counts

362   of the wort without inoculation were below the detection limit. After inoculation, yeast cell densities

363   varied between 6.4 and 7.0 Log CFU/mL, which increased just after 1 d for all trials. Starter yeasts

364   increased by about 1 Log cycles after 3 days for all trials. At the next sampling point (day 6 of alcoholic

365   fermentation), trials MN93, MN400, and XV47 started to decrease the presumed populations of

366   *Lachancea* spp., while the other trials continued to increase. Instead, on day 9, all trials started to

367   decrease except trial MN28, which started from the next sampling point. The presumptive *L.*

368   *thermotolerans* strains in the different trials showed similar dynamics, with an increase in the first few

369   days with a peak between 7.7 and 7.2 (Log CFU/mL), followed by a subsequent decrease. The trial

370   inoculated with the MNF105 strain showed higher values than the control strain. At the end of the

371   alcoholic fermentation, the highest cell counts were registered for the MNF105 trial (7.7 Log

372   CFU/mL). The yeast growth dynamics during the alcoholic fermentation of beer were like to those

373   described by (Domizio et al., 2016).

374

Fig. 4. Evolution of *L. thermotolerans* concentrations during alcoholic fermentation. Synthetic beer wort fermented by: MN28, MN93, MN400, MNF105, XV47 and *L. thermotolerans* Philly Sour (TC) (Values are expressed as average of three measurements ± standard deviation).
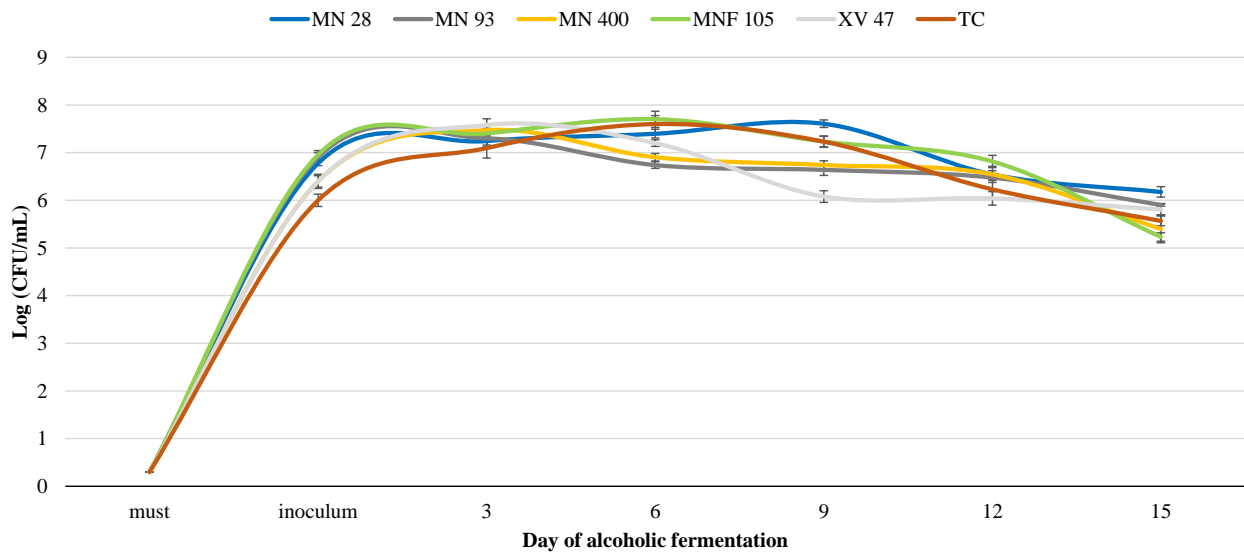
The physicochemical parameters of the final beers are presented in Table 3. Yeast strain MNF105 proved to be more capable to produce lactic acid than the other strains. In terms of final density, MNF105 was also the strain with the closest values to the control strain and these results are particularly encouraging. The analysis of the physicochemical parameters and the evolution of the glycerol, lactic acid, and acetic acid concentrations showed several differences between the strains (Table 3). The strain XV47 produced the highest amount of acetic acid (0.32 g/L). In contrast, MNF105 exhibited the highest glycerol production (2.17 g/L), exceeding the control (2.10 g/L). This strain demonstrated a higher lactic acid production (0.45 g/L) than the other strains, yet remained below the control (2.55 g/L).

388   **Table 3.** Physicochemical analysis the different trials of microfermentation samples and main fermentation properties.

	MN28	MN93	MN400	MNF105	XV47	TC	S.S.
Fermentation vigour as ethanol % (v/v)	2.80 ± 0.10^{ab}	2.48 ± 0.17^{b}	2.54 ± 0.13^{ab}	2.55 ± 0.14^{ab}	2.50 ± 0.07^{b}	2.85 ± 0.11^{a}	**
Fermentation rate[a] (g of CO_2/day)	4.52 ± 0.14^{ab}	4.34 ± 0.12^{b}	$4.53 \pm 0.11a^{b}$	4.61 ± 0.08^{ab}	4.40 ± 0.13^{ab}	4.76 ± 0.20^{a}	*
pH	3.85 ± 0.10^{a}	3.89 ± 0.11^{a}	3.79 ± 0.08^{a}	3.75 ± 0.11^{a}	3.78 ± 0.15^{a}	3.60 ± 0.05^{a}	N.S.
Density (FG)	1.017 ± 0.007^{a}	1.019 ± 0.008^{a}	1.017 ± 0.003^{a}	1.016 ± 0.009^{a}	1.018 ± 0.007^{a}	1.015 ± 0.006^{a}	N.S.
Acetic acid (g/L)	0.11 ± 0.01^{bc}	0.07 ± 0.00^{c}	0.12 ± 0.00^{bc}	0.18 ± 0.01^{b}	0.32 ± 0.05^{a}	0.17 ± 0.01^{b}	***
Lactic acid (g/L)	0.33 ± 0.03^{b}	0.34 ± 0.03^{b}	0.39 ± 0.04^{b}	0.45 ± 0.07^{b}	0.41 ± 0.05^{b}	2.55 ± 0.12^{a}	***
Glycerol (g/L)	1.85 ± 0.12^{ab}	1.80 ± 0.13^{b}	2.04 ± 0.08^{ab}	2.17 ± 0.07^{a}	1.97 ± 0.07^{ab}	2.10 ± 0.12^{a}	**

389   [a] Time period of 3 days.
390   Values are expressed as average of three measurements ± standard deviation.
391   Abbreviations: N.S., not significant.
392   Data in the same line followed by the same letter are not significantly different according to Tukey's test. Symbols: ***, $P < 0.001$; **, $P < 0.01$; * $P < 0.05$.

3.3.2 Beer production

3.3.2.1 Yeast growth during fermentation

The evolution of the concentrations of yeasts during the alcoholic fermentation of beer wort fermentation is shown in Fig. 5. On both WL and LA medium, the uninoculated wort's microorganism concentrations were below the detection limit. The inoculation rate showed a slight difference between trials, 6.2 Log CFU/mL for T1 and 6.1 and 6.5 Log CFU/mL for T2 and T3, respectively. These yeast strains showed a similar fermentation trend, which followed general fermentation dynamics of beer wort (Toh et al., 2020; Francesca et al., 2023). For both tests, the microbial load values increased until day 6 and then started to decrease. This phenomenon could be imputable to several factors, including the lack of appropriate nutrients (Domizio et al., 2016; Michel et al., 2016). All strains remained viable until the end of fermentation with high values. In fact, on the last day of fermentation (day 12), the microbial load values of the trials were 6.1 Log CFU/mL (trial T1) and 5.9 Log CFU/mL (control trial T3). The trial with the highest score was T3 on day 6 with a score of 7.2 Log CFU/mL, which was higher than the other trials. Thus, the new strain MNF105 in the T1 trial demonstrated a fermentation growth curve comparable to that of both the T2 and T3 control strains. A total of 225 yeast isolates were collected. Specifically, 75 yeast isolates were obtained from each trial (T1, T2 and T3). DNA analysis (RAPD-PCR) demonstrated that all *L. thermotolerans* strains exhibited an identical RAPD profile to the inoculated strains (*L. thermotolerans* MNF105 and Philly Sour) in the respective trials (T1 and T2). In the T3 treatment, the interdelta profiles of the *S. cerevisiae* were comparable to those of the inoculated *S. cerevisiae* strain US-05.
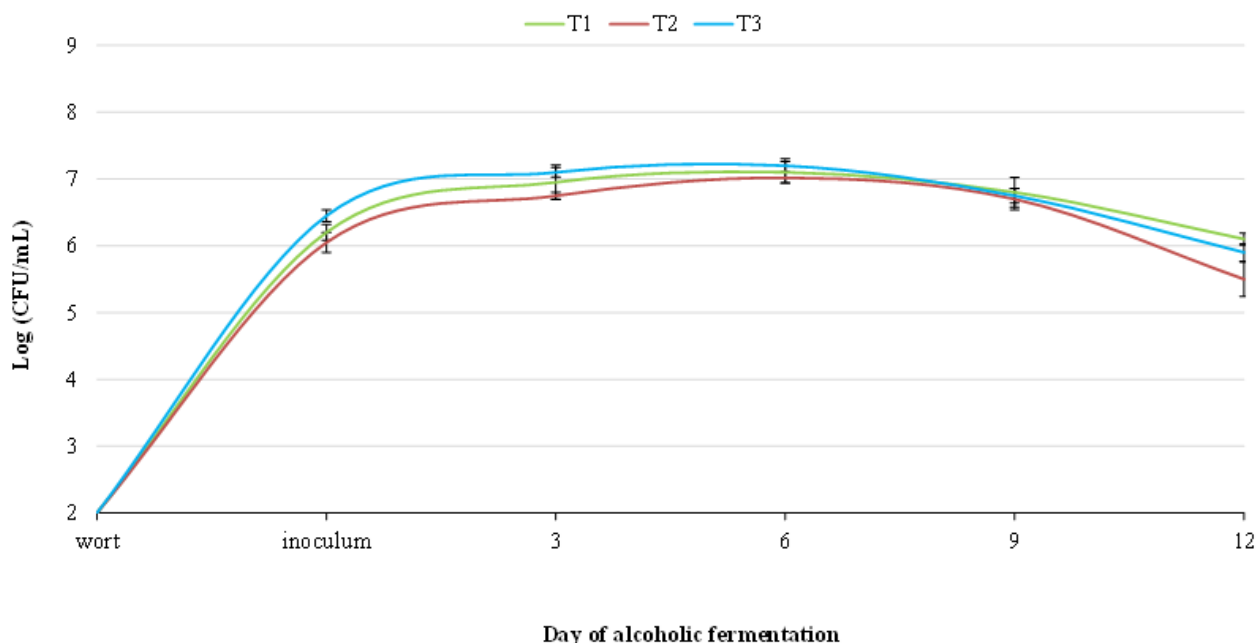
418 **Fig. 5**. Evolution of yeasts concentrations during wort fermentation under real conditions. Beer
419 fermented by: *L. thermotolerans* MNF105 (T1); *L. thermotolerans* Philly Sour (T2), *S. cerevisiae*
420 US-05 (T3).

421

422

423 3.3.2.2 Physicochemical analysis obtained during fermentation

424 The wort had a pH of 5.70 and 12.20 °Bx. The sugar composition was: 4.60 g/L of fructose, 7.50 g/L

425 of glucose, 37.50 g/L of maltose, and 15.95 g/L of sucrose. The data recorded by the FOSS analysis

426 is reported in Table 4.

427 **Table 4.** Physicochemical parameters identified in the beers by FOSS.
428

	T1	T2	T3	S.S.
Alcohol % (v/v)	4.07 ± 0.11^c	4.33 ± 0.09^b	4.86 ± 0.08^a	***
Density (FG)	1016 ± 1.20^a	1014 ± 1.00^a	1010 ± 1.15^a	**
Real extract (°P)	5.45 ± 0.12^a	5.04 ± 0.09^b	4.65 ± 0.10^c	***
Energy (kcal/100g)	50.34 ± 0.15^a	45.04 ± 0.13^b	44 ± 0.11^c	**
Apparent extract (°P)	4.08 ± 0.11^a	3.57 ± 0.12^b	2.91 ± 0.09^c	**
Original extract (°P)	11.67 ± 0.09^b	11.70 ± 0.12^b	12.20 ± 0.10^a	***
Real attenuation (%)	52.9 ± 0.24^c	56.4 ± 0.36^b	62.80 ± 0.42^a	***
pH	3.87 ± 0.12^b	3.56 ± 0.10^c	4.12 ± 0.17^a	***

429 Values are expressed as average of three measurements ± standard deviation
430 Abbreviations: S.S., statistical significance; N.S., not significant.
431 Beer fermented by: *L. thermotolerans* MNF105 (T1); *L. thermotolerans* Philly Sour (T2), *S. cerevisiae* US-05 (T3). Data
432 in the same line followed by the same letter are not significantly different according to Tukey's test. Symbols: ***, P <
433 0.001; **, P < 0.01; * P < 0.05.

The *L. thermotolerans* strain MNF105 was able to reduce the pH from 5.70 to 3.87. Furthermore, the beers produced with this strain showed a slightly higher pH than that showed by the commercial control *L. thermotolerans* (T2) but lower than that determined by the *S. cerevisiae* strain used (T3), which reached a final value of 3.56 and 4.12, respectively. Our results are consistent with those of Domizio et al. (2016); indeed, *L. thermotolerans* 101 was able to produce beers with a final pH of 3.77. In contrast, Zdaniewicz et al. (2020b) showed that the strain MN477031 only marginally influences the drop of pH compared to *S. cerevisiae*. In terms of sugar consumption, the results of the new strain were comparable to those of the commercial control *L. thermotolerans* (T2), with a final density of 1.016 compared to 1.014. However, none of them equalled the outcomes of the *S. cerevisiae* strain in the T3 trial. There is a correlation between ethanol production, residual sugar amount, and attenuation, with consistent differences between the trials inoculated with *L. thermotolerans* (T1 and T2) and those inoculated with *S. cerevisiae* (T3). However, the results among the strains for the main sugar, acids and glycerol strains were encouraging (Table 5).

Table 5. Monitoring of the chemical composition of samples during the alcoholic fermentation of beer.

	T1	T2	T3	S.S.
D-fructose (g/L)				
3d	0.22 ± 0.01^a	0.15 ± 0.02^b	0.12 ± 0.02^b	***
6d	0.00 ± 0.00^a	0.00 ± 0.00^a	0.02 ± 0.00^a	N.S.
9d	0.00 ± 0.00^a	0.00 ± 0.00^a	0.02 ± 0.00^a	N.S.
End AF	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	N.S.
D-glucose (g/L)				
3d	0.58 ± 0.04^a	0.47 ± 0.05^b	0.15 ± 0.01^c	***
6d	0.48 ± 0.06^a	0.09 ± 0.01^b	0.04 ± 0.00^b	***
9d	0.04 ± 0.01^a	0.03 ± 0.00^a	0.02 ± 0.01^a	N.S.
End AF	0.00 ± 0.00^a	0.01 ± 0.00^a	0.00 ± 0.00^a	N.S.
Maltose (g/L)				
3d	25.85 ± 0.36^a	23.58 ± 0.28^b	7.74 ± 0.15^c	***
6d	16.88 ± 0.48^a	15.58 ± 0.26^b	3.38 ± 0.05^c	***
9d	7.89 ± 0.16^a	6.58 ± 0.18^b	1.45 ± 0.07^c	***
End AF	6.47 ± 0.18^a	5.49 ± 0.09^b	0.95 ± 0.15^c	***
D-sucrose (g/L)				
3d	3.24 ± 0.09^a	2.04 ± 0.11^b	0.35 ± 0.03^c	***
6d	0.15 ± 0.01^a	0.14 ± 0.02^a	0.18 ± 0.02^a	N.S.
9d	0.09 ± 0.01^b	0.11 ± 0.01^b	0.17 ± 0.02^a	***
End AF	0.03 ± 0.00^a	0.01 ± 0.00^a	0.02 ± 0.00^a	N.S.
Lactic acid (g/L)				
3d	0.07 ± 0.01^a	0.07 ± 0.00^a	0.05 ± 0.01^a	*

	T1	T2	T3	S.S.
6d	0.41 ± 0.06^b	1.49 ± 0.12^a	0.09 ± 0.00^c	***
9d	0.45 ± 0.06^b	1.56 ± 0.09^a	0.12 ± 0.01^c	***
End AF	0.47 ± 0.07^b	1.62 ± 0.09^a	0.08 ± 0.00^c	***
Acetic acid (g/L)				
3d	0.03 ± 0.00^b	0.14 ± 0.01^a	0.01 ± 0.00^c	***
6d	0.06 ± 0.01^b	0.15 ± 0.02^a	0.02 ± 0.00^c	***
9d	0.15 ± 0.02^a	0.17 ± 0.02^a	0.04 ± 0.01^b	***
End AF	0.10 ± 0.01^b	0.13 ± 0.01^a	0.04 ± 0.01^c	***
L-Malic Acid (g/L)				
3d	0.13 ± 0.01^{ab}	0.1 ± 0.01^b	0.16 ± 0.02^a	**
6d	0.15 ± 0.03^{ab}	0.11 ± 0.01^b	0.18 ± 0.01^a	*
9d	0.17 ± 0.01^b	0.14 ± 0.01^b	0.22 ± 0.02^b	***
End AF	0.18 ± 0.02^{ab}	0.15 ± 0.03^b	0.22 ± 0.03^a	N.S.
Glycerol (g/L)				
3d	2.57 ± 0.10^b	3.11 ± 0.08^a	2.96 ± 0.05^a	***
6d	3.14 ± 0.17^a	3.16 ± 0.09^a	3.04 ± 0.15^a	N.S.
9d	3.20 ± 0.22^a	3.28 ± 0.08^a	3.08 ± 0.09^a	N.S.
End AF	3.47 ± 0.09^a	3.32 ± 0.11^{ab}	3.16 ± 0.04^b	*
Tartaric acid (g/L)				
3d	0.15 ± 0.07^a	0.16 ± 0.03^a	0.18 ± 0.05^a	N.S.
6d	0.16 ± 0.01^b	0.17 ± 0.01^b	0.22 ± 0.03^a	*
9d	0.15 ± 0.03^a	0.17 ± 0.04^a	0.21 ± 0.02^a	N.S.
End AF	0.18 ± 0.01^b	0.20 ± 0.01^b	0.24 ± 0.02^a	**

449 Values are expressed as average of three measurements ± standard deviations.
450 Abbreviations: S.S., statistical significance; AF, alcoholic fermentation; N.S., not significant.
451 Beer fermented by: *L. thermotolerans* MNF105 (T1); *L. thermotolerans* Philly Sour (T2), *S.*
452 *cerevisiae* US-05 (T3). Data in the same line followed by the same letter are not significantly
453 different according to Tukey's test. Symbols: ***, $P < 0.001$; **, $P < 0.01$; * $P < 0.05$.

454 Consequently, trial T1 (6.00 g/L) demonstrated a higher final sugar content than trials T2 and T3,

455 with values of 5.51 g/L and 0.98 g/L, respectively. In terms of sugar consumption kinetics, the control

456 strain *S. cerevisiae* US-05 (T3) demonstrated superior results. The MNF105 strain demonstrated

457 comparable behaviour to the Philly Sour strain in the T2 trial, both of which exhibited a slower rate

458 of sugar consumption than *S. cerevisiae*. Finally, MNF105 exhibited a lower rate of sugar

459 consumption than the other trials. After 3 days of fermentation, the strains have almost consumed all

460 the sugars except maltose. Subsequently, maltose was the only sugar remaining even after the

461 completion of alcoholic fermentation. In contrast, Toh et al. (2020) showed that yeast strain of *L.*

462 *thermotolerans* Concerto consumed all sugars present in the wort, including maltose. Our results

463 show that *L. thermotolerans* could assimilate maltose, but this capacity is strain-dependent and overall

464 lower than that of *S. cerevisiae* (Callejo et al., 2019). The two strains of *L. thermotolerans* produced

465 higher levels of lactic acid than the *S. cerevisiae* strain, confirming the acidifying capacity of this

species. However, differences were found between the two strains, with the novel strain producing 0.47 g/L at the end of alcoholic fermentation compared to 1.62 g/L for the control strain. These results are significantly higher than those demonstrated by Domizio et al. (2016) and Zdaniewicz et al. (2020b) and particularly encouraging as the low lactic acid content makes this strain interesting to produce low-sour beers, which, unlike sour beers, appeal to a wider range of consumers. The results of the trials indicated that the values for acetic acid, malic acid and tartaric acid were notably low. Regarding glycerol, the concentrations registered in our study (3.16 – 3.47 g/L) are higher than the range of values reported by Domizio et al. (2016) and Viana et al. (2021), but lower to those reported by Zdaniewicz et al. (2020b).

3.3.2.3 Determination of volatile organic compounds

The experimental beers contained a diverse range of aromatic organic compounds, as shown in Table 6. The composition of VOCs in the final beer showed great complexity, characterised by five classes: alcohols, esters, carboxylic acids, terpenes and others. The use of the novel MNF105 (T1) strain for beer fermentation resulted in the highest concentration of VOCs, with a higher amount of esters compared to the other control trials. The ester class was the most abundant, with a particularly high concentration in T1 (324.5 mg/L) followed by T3 inoculated with *S. cerevisiae* US-05 (259.7 mg/L). The ester class was characterised by twenty-one different compounds. Of these, T1 trial yielded particularly interesting results, demonstrating higher values for eleven compounds. The presence of esters is responsible for the fruity flavour of fermented beverages, and their concentration depends on the enzyme activity of the yeast strains (Pires et al., 2014). The main ester compound identified in this study was ethyl decanoate, which provided rich fruity and floral properties (Liu et al., 2022). The T1 trial yielded highly encouraging results, with a value of 165.3 mg/L compared to 12.1 mg/L and 93.0 mg/L for the control strains T2 and T3, respectively. This compound has been detected in stout, lager and wheat beers (Ruvalcaba et al., 2019; De Flaviis et al., 2022).

490   **Table 6.** Volatile organic compound detected in beer samples. Compounds detected by SPME (all values in mg/L).

491

RT	Compounds	Aroma Description	Odour Threshold	T1 (OAV)	T2 (OAV)	T3 (OAV)	S.S.
	∑Alcohols			100.0 ± 3.2^c	127.0 ± 4.1^b	216.9 ± 8.5^a	***
5.748	2-Methyl-1-propanol	Alcoholic	100^1	0.0 ± 0.0^b (<1)	0.0 ± 0.0^b (<1)	32.4 ± 1.6^a (<1)	***
8.402	1-Pentanol	Alcoholic, iodoform-like	80^1	45.1 ± 1.6^c (<1)	66.3 ± 2.3^b (<1)	122.5 ± 4.6^a (1.53)	***
8.502	2-Methyl-1-butanol	Alcoholic, banana, vinous	65^1	19.8 ± 0.7^a (<1)	19.3 ± 0.8^a (<1)	0.0 ± 0.0^b (<1)	***
19.549	Phenethyl alcohol	Rose, perfumy	125^1	35.1 ± 1.0^c (<1)	41.4 ± 1.1^b (<1)	62.0 ± 2.4^a (<1)	***
	∑Esters			324.5 ± 11.0^a	110.1 ± 5.0^c	259.7 ± 9.3^b	***
5.403	Ethyl acetate	Fruity, sweet	30^1	36.1 ± 0.8^a (1.20)	20.6 ± 0.7^c (<1)	29.1 ± 0.8^b (<1)	***
9.048	Pentyl lactate	Acetic acid, vinegar, milky	100^2	0.0 ± 0.0^b (<1)	0.0 ± 0.0^b (<1)	3.6 ± 0.1^a (<1)	***
9.497	Isobutyl acetate	Banana, sweet, fruity	1.6^2	0.0 ± 0.0^b (<1)	0.0 ± 0.0^b (<1)	3.7 ± 0.1^a (2.31)	***
10.347	Ethyl butanoate	Papaya, apple, perfumed	0.40^1	0.0 ± 0.0^b (<1)	0.0 ± 0.0^b (<1)	3.6 ± 0.2^a (9.00)	***
12.701	Isoamyl acetate	Fruity, banana, pear	0.50^2	4.3 ± 0.3^a (8.60)	4.9 ± 0.2^a (9.80)	28.0 ± 0.8^a (56.00)	***
13.800	Isoamyl isobutyrate	Green, rummy, cocoa	0.03^2	2.0 ± 0.1^a (66.66)	1.7 ± 0.1^b (56.66)	0.0 ± 0.0^c (<1)	***
16.200	Ethyl hexanoate	Apple, fruity, aniseed	0.17^1	29.4 ± 1.8^a (172.94)	18.0 ± 1.2^b (105.88)	7.5 ± 0.4^c (44.11)	***
16.700	Isoamyl butanoate	Fruity, melon, berry	unknown	6.3 ± 0.2^b (n.d.)	7.0 ± 0.2^a (n.d.)	0.0 ± 0.0^c (n.d.)	***
17.050	Methyl-3-ethyl-2-pentanoate	unknown	unknown	2.2 ± 0.1^a (n.d.)	2.1 ± 0.1^a (n.d.)	0.0 ± 0.0^b (n.d.)	***
18.799	Ethyl heptanoate	Fruity, perfumed, fatty	0.40^2	7.8 ± 0.2^a (19.50)	5.8 ± 0.2^b (14.50)	5.8 ± 0.3^b (14.50)	***
18.949	Linalyl butanoate	Citrus, bergamot, berry	unknown	10.2 ± 0.3^a (n.d.)	9.2 ± 0.3^b (n.d.)	5.3 ± 0.2^c (n.d.)	***
21.198	Ethyl octanoate	Apple, sweet, fruity	0.37^1	29.7 ± 1.3^a (99.00)	16.0 ± 0.8^b (43.24)	9.52 ± 0.7^c (25.72)	***
22.394	Isoamyl caproate	Perfumed, tropical fruits	0.90^2	0.0 ± 0.0^b (<1)	0.0 ± 0.0^b (<1)	5.1 ± 0.4^a (5.66)	***
22.644	Phenylethyl acetate	Roses, honey, apple, flowery	3.80^2	0.0 ± 0.0^b (<1)	0.0 ± 0.0^b (<1)	24.0 ± 1.2^a (6.31)	***
23.498	Ethyl nonanoate	Fruity, fatty acids, sweet	1.20^2	9.9 ± 0.2^b (8.25)	7.0 ± 0.3^c (5.83)	9.5 ± 0.2^a (7.91)	***
24.148	Methyl geraniate	Floral, rose-fatty	unknown	3.5 ± 0.1^a (n.d.)	2.6 ± 0.2^b (n.d.)	3.2 ± 0.1^a (n.d.)	**
25.547	Ethyl-trans-4-decenoate	Green, pineapple, pear	unknown	13.0 ± 0.6^a (n.d.)	1.2 ± 0.1^b (n.d.)	0.0 ± 0.0^c (n.d.)	***
25.647	Ethyl decanoate	Caprylic, fruity, apple	0.57^2	165.3 ± 4.8^a (290.00)	12.1 ± 0.7^c (21.22)	93.0 ± 2.4^b (163.15)	***
26.593	Isoamyl caprylate	Fruity, orange, pear, melon	2.00^2	0.0 ± 0.0^b (<1)	0.0 ± 0.0^b (<1)	8.9 ± 0.8^a (4.45)	***
29.596	Ethyl laurate	Caprylic, estery	2.00^2	4.8 ± 0.2^b (2.40)	1.9 ± 0.1^c (<1)	14.2 ± 0.5^a (7.10)	***
36.290	Ethyl palmitate	Fatty acids, fruity, sweet	1.50^2	0.0 ± 0.0^b (<1)	0.0 ± 0.0^b (<1)	2.8 ± 0.1^b (1.86)	***
	∑Carboxylic acids			0.0 ± 0.0^b	0.0 ± 0.0^b	13.2 ± 0.6^a	***
20.944	Octanoic acid	Caprylic	15.00^1	0.0 ± 0.0^b (<1)	0.0 ± 0.0^b (<1)	13.2 ± 0.6^a (<1)	***
	∑Terpenes			4.80 ± 0.4^b	7.00 ± 0.5^a	0.0 ± 0.0^c	***

16.000	β-Myrcene	herbs, resinous, spicy	0.20^2	1.8 ± 0.2^b (9.00)	4.5 ± 0.4^a (22.50)	0.0 ± 0.0^c (<1)	***
17.149	Limonene	Citrus, fruity	0.1^1	3.0 ± 0.2^a (30.00)	2.5 ± 0.1^b (25.00)	0.0 ± 0.0^c (<1)	***
	∑Other			16.20 ± 0.8^a	14.10 ± 0.5^b	6.0 ± 0.3^c	***
5.703	3-Methyl butanolide	unknown	unknown	10.8 ± 0.5^a (n.d.)	9.5 ± 0.2^b (n.d.)	0.0 ± 0.0^c (n.d.)	***
8.102	Glycerol formal	viscosity	10000^2	1.2 ± 0.1^a (<1)	0.7 ± 0.1^b (<1)	0.0 ± 0.0^c (<1)	***
13.201	Styrene	balsamic	20^2	4.2 ± 0.2^b (<1)	3.9 ± 0.2^b (<1)	6.0 ± 0.3^a (<1)	***

492   Values are expressed as average of three measurements ± standard deviation.
493   Concentrations are calculated using limonene as the standard for the calibration line.
494   Compounds in each class are ordered according to their retention time.
495   Odour threshold as reported in literature.
496   Abbreviations: S.S., statistical significance; RT, retention time using a non-polar DB-5MS column; OAV, odour activity value; n.d., not determinable; N.S., not significant.
497   Beer fermented by: *L. thermotolerans* MNF105 (T1); *L. thermotolerans* Philly Sour (T2), *S. cerevisiae* US-05 (T3). Data in the same line followed by the same
498   letter are not significantly different according to Tukey's test. Symbols: ***, P < 0.001.
499   [1]Maarse, H. (2017). Volatile compounds in foods and beverages. Routledge.
500   [2]Zunkel, M., Gastl, M., Schoenberger, C., Sedin, D., Becker, T. (2011). Beer flavor database. In ASBC 74th Annual Meeting. Ft. Myers.

The use of the novel MNF105 (T1) strain for beer fermentation resulted in the highest concentration of VOCs, with a higher amount of esters compared to the other control trials.

The ester class was the most abundant, with a particularly high concentration in T1 (324.5 mg/L) followed by T3 inoculated with *S. cerevisiae* US-05 (259.7 mg/L). The ester class was characterised by twenty-one different compounds. Of these, T1 trial yielded particularly interesting results, demonstrating higher values for eleven compounds. The presence of esters is responsible for the fruity flavour of fermented beverages, and their concentration depends on the enzyme activity of the yeast strains (Pires et al., 2014). The main ester compound identified in this study was ethyl decanoate, which provided rich fruity and floral properties (Liu et al., 2022). The T1 trial yielded highly encouraging results, with a value of 165.3 mg/L compared to 12.1 mg/L and 93.0 mg/L for the control strains T2 and T3, respectively. This compound has been detected in stout, lager and wheat beers (Ruvalcaba et al., 2019; De Flaviis et al., 2022). The other most abundant esters were ethyl hexanoate (29.4 mg/L in T1 followed by 18.0 in T2), ethyl acetate (36.1 mg/L in T1 followed by 29.1 mg/L in T3), and ethyl octanoate (29.7 in T1 followed by 16.0 mg/L in T2). These compounds are secondary metabolites of yeasts and contribute to the aroma of beer. It should be noted that the main active esters in beer are phenylethyl acetate (flowers, rose, honey), isoamyl acetate (fruit, banana), ethyl acetate (fruit, sweet), and ethyl hexanoate and ethyl octanoate (sour apple) (Verstrepen et al., 2003). These compounds were also found in other studies where they brewed beers with certain strains of *L. thermotolerans* (Gobbi et al., 2013; Postigo et al., 2023). Ethyl acetate was also noted in barley beers brewed with a yeast strain belonging to the *Lachancea* genus (Galaz & Franco, 2023), and in sorghum beers fermented with *S. cerevisiae* (Tokpohozin et al., 2019). However, it has also been detected in fruit lambic beers, which are spontaneously fermented and usually associated with *Brettanomyces* activity (Bongaerts et al., 2021). Regarding alcohols, the results of the two trials inoculated with *L. thermotolerans* are comparable, with the T2 control trial (127.0 mg/L) showing a higher value than T1 trial (100.0 mg/L). The trial T3 inoculated with *S. cerevisiae* exhibited greater amounts of alcohols (216.9 mg/L) in contrast to the other two trials. The class of alcohols is characterised by alcoholic,

floral or solvent aromas (Eβlinger, 2009). Compounds belonging to the terpenoid class, such as limonene and β-myrcene, have been found in beers and can be attributed to hops. Limonene is the second most abundant terpenoid in nature and is an important aroma compound associated with hops that gives beer a citrus or pine flavour (Ramírez and Viveros, 2021; Jiang et al., 2023). This compound is mainly found in lemons and other citrus fruits. However, it is generally found in more than 300 plants (Jongedijk et al., 2016). β-myrcene is also a compound derived from hops (Brendel et al., 2020). Brendel et al. (2020) detected its presence in beer made with different variety of hops. Among the thirty-one VOCs, seventeen compounds showed an odour activity value greater than one, specifically one alcohol (1-Pentanol), fifteen esters (ethyl acetate, isobutyl acetate, isoamyl acetate, isoamyl isobutyrate, ethyl hexanoate, ethyl heptanoate, ethyl octanoate, isoamyl caproate, phenylethyl acetate, ethyl nonanoate, ethyl decanoate, isoamyl caprylate, ethyl laurate, and ethyl palmitate) and two terpenes (β-myrcene and limonene). Esters are the principal and significant group of compounds generated by yeasts metabolism during alcoholic fermentation. These compounds exert a considerable influence on the beer's ultimate flavour profile (Holt et al., 2019).

3.3.3 Sensory analysis

The resulting beers were evaluated by sensory analysis to determine the sensory suitability of the strain tested. Fig. 6 shows histograms of the sensory analysis results for aroma and taste. The differences between the experimental beers were significant, and the novel yeast had a different effect on the sensory characteristics of the final products. No off-flavours or off-odours were reported by the panellists. Consequently, these descriptors have not been included in Fig. 6.

Fig. 6. Sensory profiles of experimental beers: (a) *L. thermotolerans* MNF105 (T1) (aroma); (b) *L. thermotolerans* Philly Sour (T2) (aroma); (c) *S. cerevisiae* US-05 (T3) (aroma); (d) *L. thermotolerans* MNF105 (T1) (taste); (e) *L. thermotolerans* Philly Sour (T3) (taste); (f) *S. cerevisiae* US-05 (T3) (taste). Blue-colored histograms are associated with coefficients that have a significantly positive value, while red-colored histograms are associated with coefficients that have a significantly negative value.
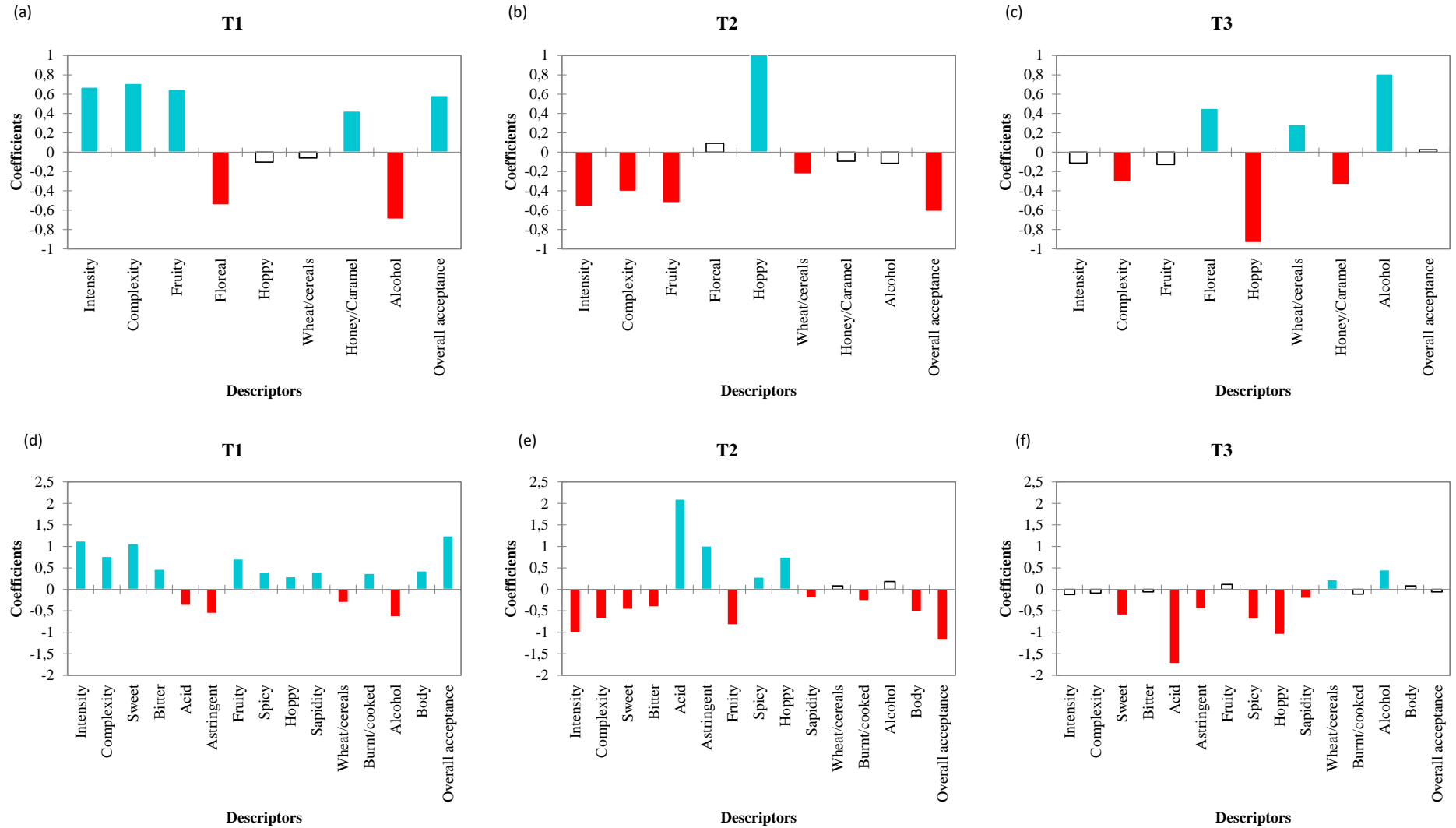
These results agree with those obtained by Francesca et al. (2023), who tested the strain MNF105 during fruit beer production and was shown to enhance the aromatic characteristics. The sensory characterisation of the beers defined coefficients for 9 aroma descriptors and 15 flavour descriptors. These coefficients were quantified by how much the calculated value was significantly above or below the overall average. The three trials were characterized by different descriptors above the mean values. According to many studies, the use of *L. thermotolerans* increases the perceived acidity of beers due to its ability to produce lactic acid determining a drop of pH (Osburn et al., 2018; Peces-Pérez, et al., 2022; Postigo et al., 2023; Romero-Rodríguez et al., 2023).

The overall organoleptic study showed a preference for T1 beer inoculated with strain MNF105, which presented fruity notes and a balanced acidity. Instead, it showed different descriptors above the mean values, five for aromas (intensity, complexity, fruity, honey/caramel and overall acceptance) and eleven for flavours (intensity, complexity, sweet, bitter, fruity, spicy, hoppy, sapidity, burnt/cooked, body and overall acceptance). Beers brewed with commercial *L. thermotolerans* (T2) were too acidic and therefore unbalanced, while those brewed with *S. cerevisiae* (T3) were drier and with a higher alcohol content. These results confirm the research of Postigo et al. (2023), who showed that the yeast strain *L. thermotolerans* CLI 1232 has a balanced acidity with a fruity flavour profile. Furthermore, these analyses showed that the new strain isolated from manna can produce beers with a low-acidity level, which are more balanced and have flavours that could be appreciated by a larger number of consumers.

3.3.4 *Correlation between volatile organic compounds and sensory profiles*

A PCA was conducted to evaluate the relationship between VOCs and sensory profile (aroma attributes). The results (Fig. 7) showed that the F1 factor contributed 65.67% of the total variance, whereas the F2 factor explained 34.33% of the total variance. The biplot graph indicated that each beer was distinct from the others. The T1 beer was associated with ethyl acetate, ethyl decanoate, ethyl heptanoate, ethyl octanoate, and ethyl hexanoate, which produced fruity and sweet aromas

(Verstrepen et al., 2003). These esters exceeded the odour threshold and were also identified in the sensory analysis, with higher fruity and honey/caramel scores than in the other trials. The hop aroma, higher in the T2 beer, is associated with limonene and β-myrcene, which is attributable to hops (Jiang et al., 2023). Instead, the T3 beer was associated with four odour descriptors (wheat/cereals, alcohol and floreal). Phenylethyl acetate is responsible for the floral in T3 beer, as it can produce floral aromas (Verstrepen et al., 2003).

Fig. 7. Principal component analysis (PCA) biplot for VOCs and aroma attributes. Abbreviations: *L. thermotolerans* MNF105 (T1); *L. thermotolerans* Philly Sour (T2); *S. cerevisiae* US-05 (T3).

Biplot (axes F1 and F2: 100.00 %)
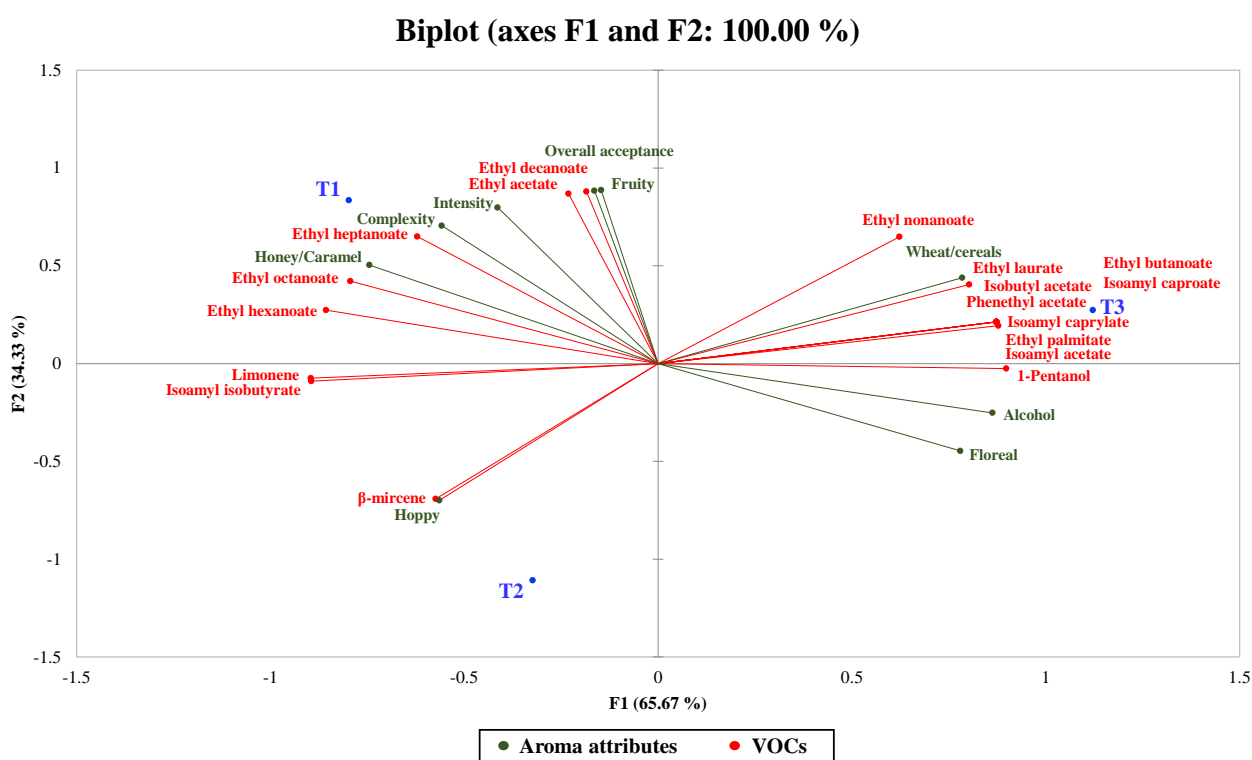
3.4 Conclusion

In conclusion, this study examines *L. thermotolerans* strains that originate from high-sugar matrices, specifically manna ash, for their potential brewing applications. The research enriches our limited scientific knowledge of sugar extracts from manna ash on a microbial level and sheds new light on the potential role of *Lachancea* yeasts as starters for sour beer production. Five strains demonstrated significant resistance to 10 % ethanol and 320 g/L of sugar and interesting weak lactic acid production (between 0.33 g/L and 0.45 g/L). The strain MNF105 has demonstrated excellent fermentation performances and has been chosen for use in brewing conditions. During sensory analysis, beers

produced using MNF105 (T1) were the most preferred and perceived as the most "fruity" with an acid taste lower than control, confirming the aptitude to produce light sour beer. As a results, elevated levels of ethyl decanoate (165.30 mg/L), ethyl hexanoate (29.4 mg/L), ethyl octanoate (29.7 mg/L) and ethyl acetate (36.1 mg/L) were found in T1 beer, exceeding the perception threshold. The use of this strain in the beer production process resulted in improvements in physicochemical parameters, VOCs, and sensory characteristics, indicating the potential of the strain as a brewing starter for a wide range of beer production styles. The limited generation of lactic acid (0.47 g/L) by this strain is a favourable trait for the brewing of beer, especially for creating beers with a reduced amount of lactic acid, thus a well-balanced acid or sour taste and innovative flavours that could be appreciated by a larger number of consumers. Manna sugary exudate remains an innovative source of potential starter yeasts for fermented alcoholic beverage. Further research is required to assess the impact of these strains on wort fermentation in industrial settings and under varying wort compositions.

3.5 References

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CHAPTER 4

A novel microbiological approach to impact the aromatic

composition of sour loquat beer

4   **ABSTRACT**

5   The growing interest in novel beer development determined the exploitation of unconventional yeasts

6   isolated from novel ecological niches to generate unexplored sensory profiles. In recent years, there

7   is an increasing interest in generating beers brewed with the addition of fruits. For the first time,

8   *Lachancea thermotolerans* MNF105 and *Saccharomyces cerevisiae* MN113 isolated from manna,

9   were tested as starter cultures to process loquat beer to improve the sensory profile. Innovatively, the

10   yeast species *L. thermotolerans* was investigated for the production of sour fruit beer. Sour fruit beers

11   produced with *L. thermotolerans* MNF105 were more balanced than the respective control, especially

12   in terms of perceived acidity during sensory analysis. This could be due to the lower lactic acid

13   production (0.49 g/L) compared to the respective control (1.74 g/L). The overall organoleptic

14   investigation showed a preference for *S. cerevisiae* MN113 (TF1) isolated from manna. Experimental

15   trials conducted with the selected strains demonstrated the absence of off-odour and off-flavour and

16   improved aroma perception. Aldehydes and alcohols were the most abundant compounds emitted

17   from the beers. *S. cerevisiae* MN113 and *L. thermotolerans* MNF105 manna related yeasts showed

18   great technological properties, representing promising starters for the production of fruit beer and

19   sour fruit beer.

20

21

22   **Keywords**: Alcoholic fermentation; Loquat beer; *Lachancea thermotolerans*; *Saccharomyces*

23   *cerevisiae*; Volatile organic compounds.

24   **Fig. 1.** Graphical abstract of experimental research
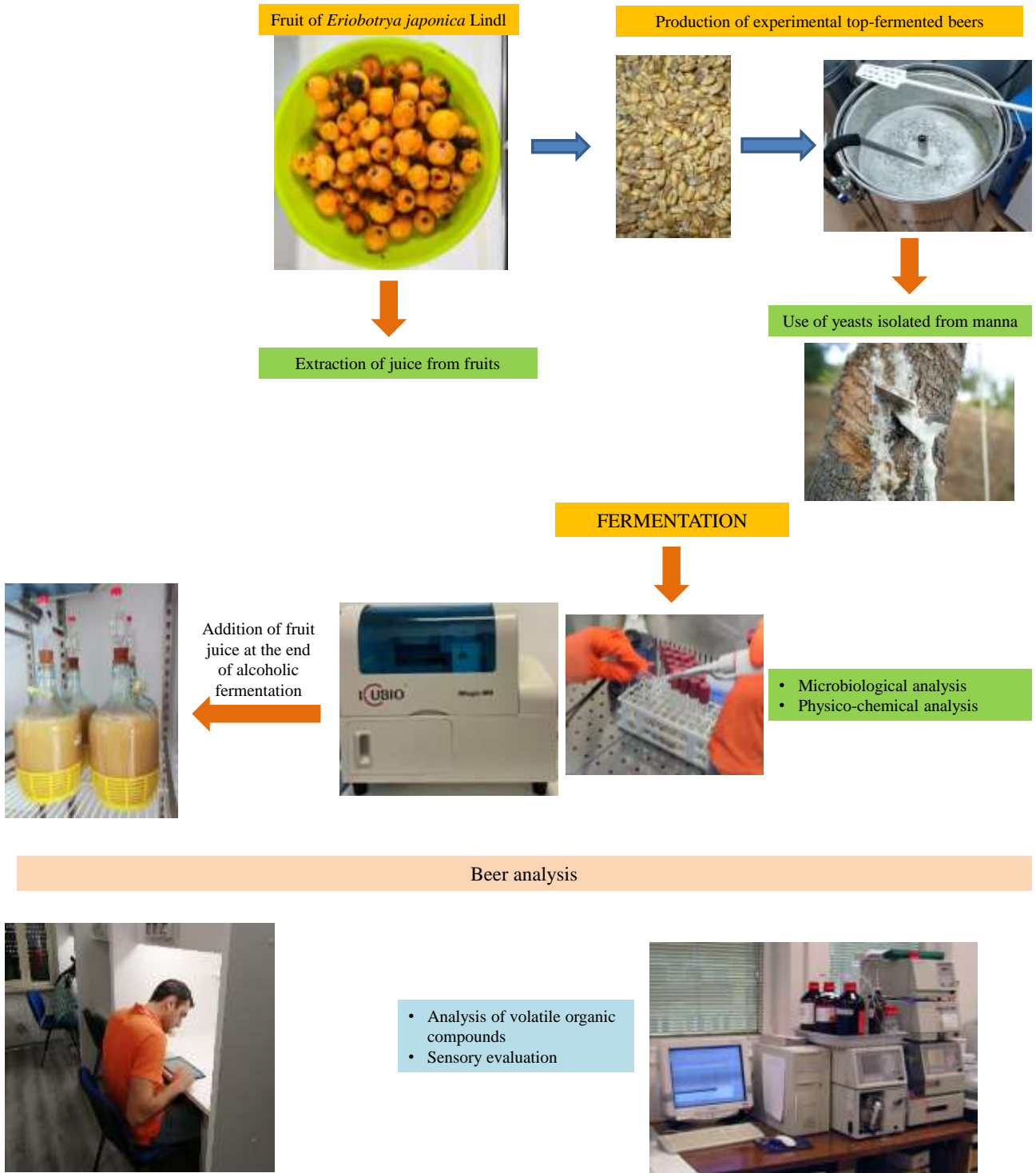
4.1 Introduction

Beer is the oldest and most popular alcoholic beverage in the world. Precisely, it ranks third among beverages, after tea and water (Anderson et al., 2019; Callejo et al., 2020). Recent development has focused on the selection of *Saccharomyces* and non-*Saccharomyces* yeasts from sugar-rich sources, in order to find new yeast strains capable of producing innovative fermented alcoholic beverages, e.g. Guarcello et al. (2019) provided a survey on the ecological niches associated with the highly sugary source represented by manna, while Matraxia et al. (2021) investigated yeast composition of a highly alcoholic beverages (Spiritu re fascitrari) obtained from the fermentation of honey by-products or Sinacori et al. (2014) deepened the knowledge of the microbial community of southern Italian honeys. In particular, manna is a sugary substance produced from the solidification of processed sap of various *Fraxinus* sp. (Schicchi et al., 2007; Yücedag & Sen, 2008). As being a highly sugar containing source, manna hosts osmophilic microorganisms, in particular that are able to survive in a viable form under the extremely stressing conditions generated by the osmotic pressure (Guarcello et al., 2019). The study conducted by Guarcello et al. (2019) resulted in the isolation of several yeast species and *Lachancea thermotolerans* showed characteristics useful to act as starters or co-starters in food applications such as sour beer production. *Lachancea* and other non-*Saccharomyces* yeasts including *Pichia*, *Saccharomycodes*, *Zygosaccharomyces*, *Hanseniaspora*, and *Torulaspora* are being evaluated for their possible use as starter cultures in brewing (Sannino et al., 2019). Domizio et al. (2016) registered a lactic acid production by *L. thermotolerans* allowing the production of sour beer without the deliberate addition of bacteria with a consistent shortening of the transformation process and positively affecting the taste and aroma. Canonico et al. (2019) confirmed the notable decrease of pH of the medium inoculated with pure cultures of *L. thermotolerans* due to the generation of large amounts of lactic acid and registered also a defined production of ethylbutyrate and ethylacetate. On the other hand, Zdaniewicz et al., (2020) showed that some strains of *L. thermotolerans* possess a limited lactic acid production capacity with a marginal influence on pH drop, but observed a higher production of ethyl lactate in comparison to *S. cerevisiae*.

Beer market is worldwide dominated by traditional beer types, but there is an increasing interest in generating beers brewed with the addition of fruits (Patraşcu et al., 2018). Several traditional beer processes are being implemented by the addition of fruits to produce novel sour fruit beers, e.g. the typical Belgian lambic beer, brewed with a blend of barley malt and unmalted wheat id added with "Kriek" cherries or "Framboise" raspberries, and subjected to spontaneous fermentation (De Keersmaecker, 1996; Glover, 2001; Protz, 1995; Spitaels et al., 2014). This type of beverage became popular due to its rich fruity flavor and refreshing properties due also to a pleasant acidity (Gorzelany et al., 2022; Martìnez et al., 2017; Zapata et al., 2019). At the same time, the consumption of tropical fruits is also becoming popular worldwide due to their nutritional and health properties (Aquilani et al., 2014). Many of these fruits, such as banana, passion fruit, annona, mango, and loquat, are being studied for their use in brewing, with the aim of increasing the amount of ethanol by adding sugars, for enrichment in terms of volatile organic compounds and, in some cases, to improve the final acidity (Carvalho et al., 2009; De Melo et al., 2017; Gasiński et al., 2020; Pirrone et al., 2022; Santos et al., 2021). Many tropical fruits such as mango, avocado and papaya recently spread out from their origin areas to Mediterranean countries (Adiletta et al., 2020; Farina et al., 2020; Migliore et al., 2017) whilst other ones such as loquat have been around for a long time. Loquat (*Eriobotrya japonica* Lindl.) is an evergreen tree native to southeastern China. Today, loquat trees are cultivated in many countries around the world (Badenes et al., 2013). In particular, the species *E. japonica* is well adapted throughout Mediterranean countries (Reig et al., 2014) when Spain is the first country for fruit production (Reig et al., 2011). Italian production of loquat fruit is almost entirely concentrated on the northern coast of Sicily, mainly within Palermo province (Farina et al., 2011). Sicilian loquat is characterized by orange-fleshed and white-fleshed fruits that are rich in nutrients, highly aromatic and with high acidity (Gentile et al., 2016); for this reason, these fruits were considered for brewing purposes (Farina et al., 2016; Pirrone et al., 2022). Furthermore, their acidity makes them of great interest for the production of sour fruit beers. To our knowledge, however, no previous research has assessed the effect of *L. thermotolerans* isolated from a novel ecological niche such as manna ash or

other high sugar matrices to produce sour fruit beer. Based on the above considerations, the present research aimed to: (i) evaluate for the first time the effect of *L. thermotolerans* strain (MNF105) isolated from manna for fruit sour beer production; (ii) improve the knowledge on a *S. cerevisiae* strain (MN113) isolated from manna as a possible starter culture in fruit beer production; (iii) study for the first time loquat fruit addition to produce craft beer; (iv) to deepen our knowledge on microbial ecology of manna ash as novel source of yeast starter.

4.2 Materials and methods

4.2.1 Yeast strains and media

Yeast strains applied in this research were *S. cerevisiae* MN113 and *L. thermotolerans* MNF105. Yeast strains belongs to the collection of the Department of Agricultural, Food and Forest Sciences (SAAF; University of Palermo, Italy); they were isolated from manna (Guarcello et al., 2019) and selected for their high performances during beer wort fermentation. For the control trials, commercial yeast strains *L. thermotolerans* Philly Sour and *S. cerevisiae* US-05 (both from Allemand Inc., Montreal, Canada) were employed. Yeast reactivation from cryogenic storage was carried out as reported by Pirrone et al., (2022). Yeast propagation was then carried out in broth cultures with YPD medium, incubated overnight at 28°C and then re-inoculated in sterile flasks containing YPD, where the cells were left to grow. Media component were procured from Oxoid (Rodano, Italy).

### 4.2.2 Experimental plan

Experimental high fermentation beers were conducted on a medium scale (5 L batch) employing four different inocula to better understand the impact of inoculum during fermentation. The wort for the fermentation trials was produced with a 40-L all-in-one microbrewing plant Klarstein mod. 10031629 (Chal-Tec GmbH Berlin, Germany). 4.5 kg of Pilsen malt and 4.5 kg of wheat malt (BestMalz, Heidelberg, Germany), pre-ground by a double roller mill (Brouwland, Beverlo, Belgium) with roller distance at 1.20 mm, were added to 34 L of water containing $CaSO_4$ (10 g) and $CaCl_2$ (10 g) for pH

correction (Marconi et al., 2016). The mash was performed at different temperature/time combinations: 45 °C for 15 min (acid rest); 52 °C for 15 min (protease step); 62 °C for 30 min (β-amylase); 72 °C for 20 min (α-amylase); and 78 °C for 10 min (mash-out); until the sugars are completely converted (Mayer et al., 2016). The lautering phase was performed by rinsing the grains with 18 L of H_2O heated at 78 °C; the resulting total volume of wort was 41 L. Subsequently, the wort was boiled for 60 min. At the beginning of boiling, 45 g of hops (Mandarina Bavaria - pellets, 9.7 % w/w α-acids) were added. After that, the resulting volume was 37 L. Clarification of the wort was carried out using a whirlpool that included recirculation for 10 min and resting for 10 min (Marconi et al., 2016). The wort was cooled for 20 min in a stainless-steel wort chiller until 21 °C and then prepared for yeast inoculation. Quality parameters of beer wort were: 5.60 pH and 12°Bx (Brix degree). Loquat juice, used to prepare the fruit beers, was extracted from the fruits of the cultivar 'Claudia' of *Eriobotrya japonica* Lindl reaped from a local orchard (37°5'39.54 "N, 13°25'25.85 "E). The fruits were harvested when fully ripe as determined by colorimeter (Minolta, Osaka, Japan). After cutting and pre-washing, subsequent washings were performed as reported by Alfonzo et al., (2018). Subsequently, fruit juice was obtained as reported by Pirrone et., (2022). At the conclusion of the alcoholic fermentation (day 10th), 20% (v/v) of loquat juice was added in all experiments according to Gasiski et al. (2020). The values of pH and the content of sugars of the juice were measured before addition to beer. Four experimental trials were inoculated as reported in Figure 2, as follows: TF1 with *S. cerevisiae* MN113; TF2 with *S. cerevisiae* US-05 (control trial for *Saccharomyces*); TF3 with *L. thermotolerans* MNF105; TF4 with *L. thermotolerans* Philly sour (control trial for *Lachancea*). Trials were inoculated with each yeast strain approximately at 2.0×10^6 cells/mL (Holt et al., 2018). Fermentation took place at 20 °C in glass fermenters (5 L) with hermetic closure equipped with an airlock valve. Samples were collected from uninoculated wort, after the inoculum of each yeast strain, at 3 and 6 days of fermentation, upon completion of primary alcoholic fermentation (day 10), the following day after adding the loquat juice (day 11) and at the conclusion of secondary alcoholic fermentation (day 16). At the end of the fermentation process, the beer was transferred into 0.33 L

135   bottles by adding 6 g/L of dextrose. The bottles were conditioned at 20 °C for 25 days (Callejo et al.,

136   2019). After this period, sensory analysis was conducted on the beers. All fermentation experiments

137   were carried out in triplicate.

138   **Fig. 2.** Experimental plan of loquat beer production
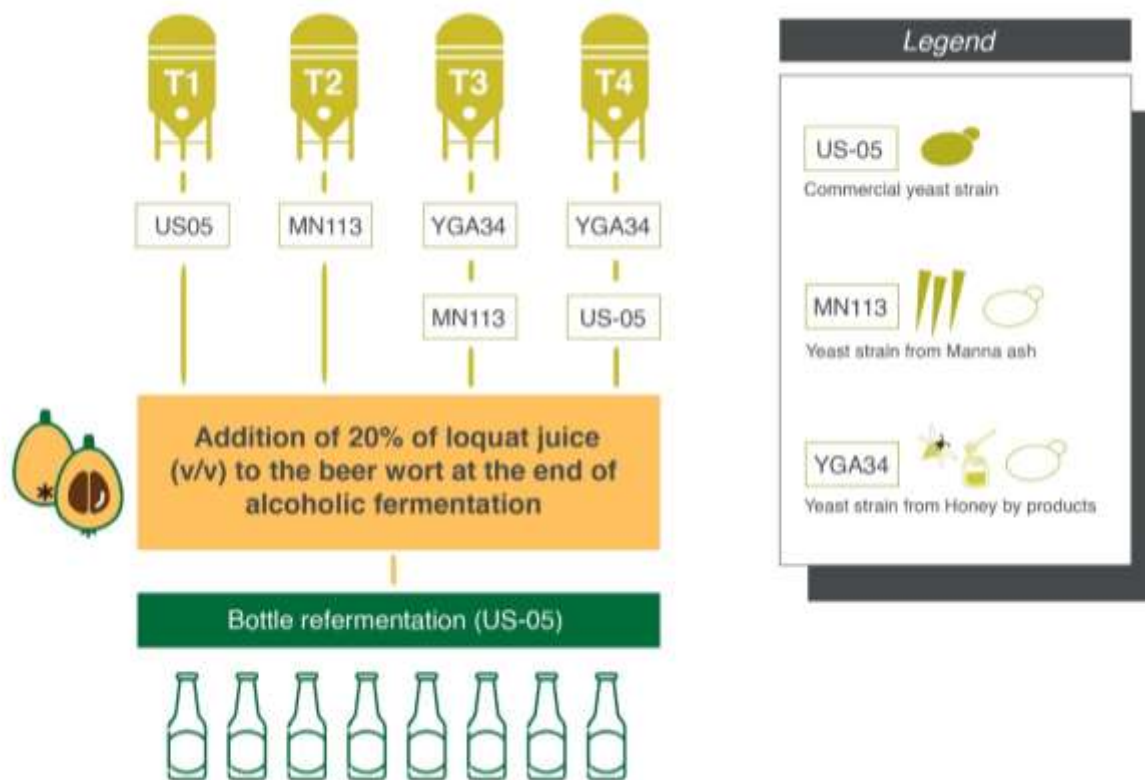
139

140   *4.2.3   Microbiological analyses*

141   Each sample was subjected to microbiological analysis with plate counts. Two different media were

142   used: Wallerstein Laboratory nutrient agar (WL) and Lysine Agar medium (LA) for *Saccharomyces*

143   (Di Maio et al., 2011) and non-*Saccharomyces* (Iris et al., 2020) populations, respectively. Based on

144   their morphological characteristics, the colonies from the two agar media were presumptively

145   identified as *Saccharomyces* and *Lachancea* only after cell morphology determination by microscopic

146   inspection (Cavazza et al., 1992). All analyses were performed in triplicates.

147

4.2.4 Physicochemical analysis

The values of pH were measured with a pHmeter, model number Mod.70 XS/50010162 (Cheimika, Pellezzano, Italy) and the °Bx were estimated with a refractometer, model number DBR Salt (Zetalab srl, Padova, Italy). The determination of acetic acid, lactic acid, tartaric acid, fructose, glucose, glycerol, malic acid, maltose, and sucrose, was carried out as reported by Matraxia et al. (2021). BeerFoss™ FT Go (FOSS Italia srl, Padova, Italy) was used to measure alcohol (% vol), density (FG), real extract (°P), energy (kcal/100g), apparent extract (°P), original extract (°P), specific gravity (°P) and real attenuation (%) of the final beers.

4.2.5 Analysis of volatile organic compounds in beer samples

Analysis of volatile organic compounds in beer samples was carried out as reported by Alfonzo et al., (2021). Quantification was carried out using three calibration lines. For compounds belonging to classes other than standards, similarity was used for quantification. A dilution factor was used for the reported data.

4.2.6 Sensory evaluation

Thirteen judges (aged between 27 and 45, 8 man and 5 women) were selected from the University of Palermo to evaluate fruit beer. All panellist had experience in beer production and acted as beer judges in several beer tasting sessions. The judges received preliminary training to target the sensory characteristics that depict the attributes of the fruit beers. To eliminate the effect of beer colour perception on taste perception, samples were offered to panellists in private tasting cabins with uniform illumination. Samples were appropriately labelled with randomly generated number codes and delivered in standard ISO tasting glasses with a watch glass stopper (100 mL at 16 °C). Sensory evaluations were carried out under blind tasting conditions at the sensory analysis laboratory of SAAF Department – University of Palermo, Italy. Beer sensory evaluations were conducted in accordance with the methodology described by Marconi et al. (2016) and the ISO standards. The attributes

174   evaluated were: visual perception (appearance), odour-based olfactory sensations (through the nostril,

175   orthonasally) and flavour (through the back of the throat, retronasally), oral sensations that are based

176   on taste, mouthfeel and overall quality. All panellists identified 32 sensory descriptive attributes in

177   terms of appearance, odour, flavour, taste and overall quality. The sensory attributes were assessed

178   using an unstructured nine-point scale anchored at the left end with "absent" and at the right end with

179   "high" (Gugino et al., 2024; Jackson et al., 2016). In addition, panellists also visually judged the

180   intensity of the colour using the same scale with the terms "straw yellow" and "amber orange"

181   anchored to the left and right limits (Barry et al., 2018; Jackson, 2016). For evaluating the fruit beer

182   attributes, the following descriptors were used: appearance (color), odour (intensity, complexity,

183   fruity, loquat, floral, hoppy, wheat/cereal, honey/caramel, acetic, oxidized/aged, sulphury, alcohol and

184   DMS) and taste (intensity, complexity, sweet, bitter, acid, astringent, fruity, loquat, spicy, hoppy,

185   sapidity, wheat/cereal, burnt/cooked, alcohol, body, DMS and oxidized/aged). The average of the

186   three assessments was used to obtain the final scores.

187

188   *4.2.7   Statistical and explorative multivariate analyses*

189   ANOVA test was performed to identify significant differences between the chemical parameters

190   determined during the brewing process (lactic acid, acetic acid, tartaric acid, glucose, fructose,

191   sucrose, maltose, glycerol, and malic acid), microbiological analysis (yeast counts), VOCs and

192   sensory analysis (descriptive quantitative analysis). The post-hoc Tukey's method was used to

193   pairwise compare all the data. Statistical significance was attributed to $P \leq 0.05$ (Mazzei et al., 2010).

194   A heat map clustered analysis (HMCA) was used to visualize VOC concentrations, based on a

195   hierarchical dendrogram with a heat map graph, displaying individual content values in the data

196   matrix as colors (Martorana et al., 2017). Color intensity was used to represent the relative VOC

197   concentration values, ranging from yellow (lowest quantity) to red (highest quantity). Heat map

198   analysis of VOC concentration was carried out using the autoscaled data (Gaglio et al., 2017). The

199   heat map was created using ascending hierarchical clustering based on Ward's method, while

200 statistical data analysis and graph construction were conducted with XLStat software version

201 2019.2.2 (Addinsoft, New York, USA) for Excel. The data collected during the alcoholic fermentation

202 (VOC's, sensory and chemical parameters) from the several trials were compared to investigate

203 relationships using an explorative multivariate technique. Agglomerative hierarchical clustering

204 (AHC) was performed to explore the relationships among the trials particularly between sensory and

205 chemical parameter data.

206

207 **4.3 Results and discussion**

208 *4.3.1 Evaluation of population dynamics*

209 Growth kinetics during fermentation are shown in Figure 3.

210 **Fig. 3.** Monitoring of yeast concentrations during alcoholic fermentation. Beer fermented by: *S. cerevisiae*

211 MN113 (TF1); *S. cerevisiae* US-05 (TF2); *L. thermotolerans* MNF105 (TF3); *L. thermotolerans* PHILLY

212 SOUR (TF4).

Day of alcoholic fermentation
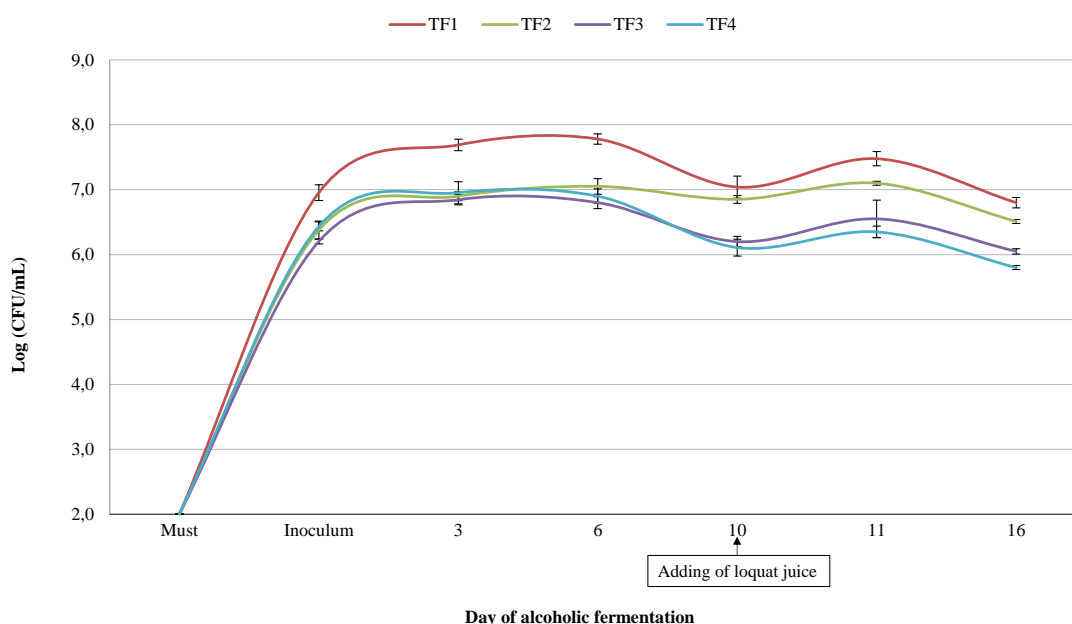
214 Microbial levels in uninoculated wort as well as loquat juice were below the detection limit, both on

215 WL and LA media (data not shown). On the contrary, all inoculated trials showed yeast cell densities

216 varying between 6.2 and 7.0 Log CFU/mL; these values increased just after 1 d. Starter yeasts

217 increased about 0.5 Log cycles their levels after 3 d for all trials. Similar trends were observed for *L.*

218 *thermotolerans* (Fig. 3), which increased significantly over the first 3 d until 6.9 and 7.0 log

219   (CFU/mL) for trials TF3 and TF4, respectively. From the 6th day of fermentation onward the

220   inoculated trials began to decrease the numbers of *Lachancea*. Similarly, the levels of *S. cerevisiae* in

221   trials TF1 and TF2 showed an increase in the first few days and displayed cell densities of 7.8 and

222   7.1 (CFU/mL), respectively, and then began to decrease. Interestingly, trial TF1 trial inoculated with

223   *S. cerevisiae* MN113 showed a more consistent growth than trial TF2 inoculated with the control

224   strain US-05. The results are comparable to the dynamics of yeast growth during fermentation in wort

225   beer (Matraxia et al., 2021; Toh et al., 2020). The decrease of *Lachancea* levels after day 4 can be

226   due to various factors including the decrease in available nutrients as sugar (Domizio et al., 2016;

227   Michel et al., 2016). On day 11th of fermentation, with the addition of loquat juice, yeast cell density

228   increased in all trials. *S. cerevisiae* MN113 in trial TF1 had the greatest cell counts at the conclusion

229   of AF (6.8 Log CFU/mL), while *L. thermotolerans* MNF105 in trial TF3 (6.1 Log CFU/mL)

230   demonstrated higher results than control TF4 (5.8 Log CFU/mL). Yeast growth dynamics occurred in

231   fruit beer, confirmed those observed by De Melo et al. (2017) and Pirrone et al. (2022).

232

233   *4.3.2 Physico-chemical analysis*

234   Physicochemical composition of loquat juice and wort are presented in Table 1.

235   **Table 1.** Conventional chemical parameters identified in beer wort and *Eriobotrya japonica* juice.

Parameters (g/L)	Wort	Loquat juice
D-fructose	4.52 ± 0.22	38.25 ± 0.64
D-glucose	7.56 ± 0.28	31.56 ±0.45
Maltose	36.24 ± 0.05	0.00 ± 0.00
D-sucrose	18.05 ± 0.21	35.15 ± 0.30
Acetic acid	0.00 ± 0.00	0.04 ± 0.04
Lactic acid	0.00 ± 0.00	0.04 ± 0.04
L-Malic Acid	0.10 ± 0.01	12.51 ± 0.15
Glycerol	0.00 ± 0.00	0.00 ± 0.00
Tartaric acid	0.09 ± 0.01	0.95 ± 0.13

236   Loquat juice was characterized by pH 3.70 and 11.20 °Bx (data not shown), whereas the original wort

237   by pH 5.60 and 12 °Bx (data not shown). Instead, Table 2 shows data registered after FOSS analysis.

Table 2. Physicochemical parameters identified in the final fruit beers.

	TF1	TF2	TF3	TF4	S.S.
Alcohol (% vol)	4.35 ± 0.12^b	5.04 ± 0.11^a	4.28 ± 0.15^b	4.21 ± 0.21^b	***
Density (FG)	1012.6 ± 1.1^a	1007.4 ± 1.2^b	1013.4 ± 0.8^a	1013.2 ± 0.5^a	***
Real extract (°P)	5.19 ± 0.12^a	4.13 ± 0.13^b	5.33 ± 0.08^a	5.28 ± 0.07^a	***
Energy (kcal/100g)	44 ± 0.12^a	43 ± 0.13^a	44 ± 0.08^a	43 ± 0.08^a	N.S.
Apparent extract (°P)	3.69 ± 0.12^a	2.35 ± 0.12^b	3.87 ± 0.12^a	3.83 ± 0.12^a	***
Original extract (°P)	11.78 ± 0.08^a	11.8 ± 0.06^b	11.8 ± 0.10^a	11.65 ± 0.08^a	***
Specific gravity (°P)	1014.5 ± 1.10^a	1009.2 ± 0.90^b	1015.2 ± 1.20^a	1015 ± 1.23^a	***
Real attenuation (%)	57.5 ± 1.15^b	66.4 ± 1.82^a	56.4 ± 1.35^b	56.2 ± 1.38^b	***
pH	3.80 ± 0.10^a	3.81 ± 0.08^a	3.65 ± 0.12^{ab}	3.49 ± 0.06^b	*

Values are expressed as average of three measurements.
Abbreviations: S.S., statistical significance.
Beer fermented by: *S. cerevisiae* MN113 (TF1); *S. cerevisiae* US-05 (TF2); *L. thermotolerans* MNF105 (TF3);
L. thermotolerans PHILLY SOUR (TF4). Data in the same line followed by the same letter are not significantly
different according to Tukey's test. Symbols: ***, P < 0.001; **, P < 0.01; * P < 0.05; N.S., not significant.

Ethanol production was not significantly different between the trials, and therefore no differences were shown between *L. thermotolerans* and *S. cerevisiae* for this parameter. Instead, pH values at the end of alcoholic fermentation ranged from 3.44 to 3.81. According to Domizio et al. (2016), *L. thermotolerans* strain 101 was able to reduce pH from 5.60 to 3.77 during wort fermentation. Our isolate *L. thermotolerans* MNF105, that produced noticeably sour beers, reached final pH values from 5,60 to 3.65, while the control strain reached a lower value of 3.49. In this study *L. thermotolerans* determined a pH dropping similar to that observed with *S. cerevisiae*. Trials TF1 and TF3 showed similar alcohol, density and attenuation values, while trial TF2 showed lower density values and consequently higher alcohol concentration and attenuation. However, some differences among strains were registered for physicochemical parameters and for the main sugars, acids and glicerol (Table 3). Regarding sugar content, except trial TF2, which had a final sugar content of 0.57 g/L, the other trials reached higher values between 5.62 and 6.07 g/L after 16 d of fermentation (Table 3). The sugar consumption kinetics showed that the selected control strain US-05 (TF2) was characterized by the best performances, although the behaviour of strain MN113 in trial TF1 was almost comparable. After 3 d of alcoholic fermentation, both strains of *S. cerevisiae* consumed entirely glucose and fructose, but only partially the other sugars. In case of maltose, trial TF1 showed a slower consumption than

what observed for trial TF2. Similar observations were made with *Lachancea* strains and the residual maltose content in all trials were comparable. These results confirm those of Domizio et al. (2016) who stated that *L. thermotolerans* and *S. cerevisiae* strains possess similar maltose utilization capacities. In contrast, Callejo et al. (2019) registered a lower maltose fermentation capacity in *Lachancea* strains when compared to *S. cerevisiae* strain. The kinetic of sugars consumption by *S. cerevisiae* registered in our study followed the general trend of this species, with glucose and fructose used before maltose (Pirrone et al., 2022; Tan et al., 2021). Glucose, fructose and sucrose concentrations increased when loquat juice was added to the wort, and were totally fermented during the alcoholic fermentation.

Table 3. Conventional chemical parameters monitored in samples beer during the alcoholic fermentation.

	TF1	TF2	TF3	TF4	S.S.
D-fructose (g/L)					
3d	0.21 ± 0.04^a	0.12 ± 0.03^a	0.20 ± 0.05^a	0.10 ± 0.02^a	*
6d	0.04 ± 0.03^b	0.15 ± 0.03^a	0.00 ± 0.00^b	0.00 ± 0.00^b	***
10d	0.04 ± 0.03^b	0.19 ± 0.06^a	0.00 ± 0.00^b	0.00 ± 0.00^b	**
11d (+Fr)	5.54 ± 0.25^{ab}	5.26 ± 0.12^b	6.10 ± 0.23^a	5.62 ± 0.18^{ab}	*
End AF	0.01 ± 0.00^b	0.01 ± 0.00^b	0.02 ± 0.01^{ab}	0.04 ± 0.01^a	**
D-glucose (g/L)					
3d	0.27 ± 0.09^{bc}	0.12 ± 0.05^c	0.65 ± 0.10^a	0.44 ± 0.08^{ab}	***
6d	0.04 ± 0.02^a	0.05 ± 0.02^a	0.10 ± 0.02^a	0.08 ± 0.05^a	N.S.
10d	0.03 ± 0.01^a	0.02 ± 0.01^a	0.03 ± 0.00^a	0.04 ± 0.00^a	N.S.
11d (+Fr)	7.38 ± 0.15^a	7.41 ± 0.49^a	7.31 ± 0.38^a	7.22 ± 0.32^a	N.S.
End AF	0.03 ± 0.00^b	0.04 ± 0.01^b	0.05 ± 0.05^a	0.04 ± 0.02^b	**
Maltose (g/L)					
3d	7.40 ± 0.40^b	3.70 ± 0.31^b	26.17 ± 1.75^a	24.00 ± 2.20^a	***
6d	6.95 ± 0.12^b	1.40 ± 0.19^c	17.16 ± 1.15^a	16.02 ± 1.11^a	***
10d	6.84 ± 0.15^a	1.37 ± 0.14^b	8.01 ± 0.89^a	7.66 ± 1.20^a	***
11d (+Fr)	5.84 ± 0.21^a	0.82 ± 0.10^b	5.92 ± 0.19^a	5.79 ± 0.18^a	***
End AF	5.42 ± 0.22^a	0.45 ± 0.05^b	5.82 ± 0.61^a	5.53 ± 0.83^a	***
D-sucrose (g/L)					
3d	0.34 ± 0.09^c	0.24 ± 0.05^c	2.30 ± 0.06^a	2.11 ± 0.04^b	***
6d	0.19 ± 0.03^{ab}	0.22 ± 0.03^a	0.12 ± 0.04^b	0.13 ± 0.02^b	*
10d	0.14 ± 0.10^a	0.20 ± 0.04^a	0.11 ± 0.01^a	0.10 ± 0.05^a	N.S.
11d (+Fr)	5.95 ± 0.40^{ab}	5.46 ± 0.32^{ab}	6.34 ± 0.12^a	5.21 ± 0.41^b	*
End AF	0.16 ± 0.05^a	0.07 ± 0.02^a	0.18 ± 0.09^a	0.13 ± 0.08^a	N.S.
Lactic acid (g/L)					
3d	0.07 ± 0.05^a	0.08 ± 0.02^a	0.07 ± 0.02^a	0.07 ± 0.02^a	N.S.
6d	0.06 ± 0.03^c	0.07 ± 0.01^c	0.51 ± 0.05^b	1.59 ± 0.26^a	***
10d	0.09 ± 0.02^{bc}	0.08 ± 0.04^c	0.61 ± 0.08^b	2.25 ± 0.34^a	***.
11d (+Fr)	0.07 ± 0.04^b	0.09 ± 0.05^b	0.51 ± 0.12^b	2.20 ± 0.43^a	***
End AF	0.04 ± 0.02^b	0.05 ± 0.02^b	0.49 ± 0.08^b	1.74 ± 0.36^a	***
Acetic acid (g/L)					
3d	0.13 ± 0.02^a	0.01 ± 0.00^b	0.02 ± 0.01^b	0.16 ± 0.05^a	**
6d	0.13 ± 0.03^{ab}	0.02 ± 0.00^c	0.05 ± 0.02^{bc}	0.16 ± 0.04^a	**
10d	0.15 ± 0.05^{ab}	0.04 ± 0.01^b	0.14 ± 0.04^{ab}	0.18 ± 0.06^a	*

11d (+Fr)	0.08 ± 0.02^a	0.04 ± 0.01^a	0.09 ± 0.02^a	0.12 ± 0.07^a	N:S.
End AF	0.14 ± 0.02^a	0.03 ± 0.01^b	0.09 ± 0.02^{ab}	0.13 ± 0.05^a	N.S.
L-Malic Acid (g/L)					
3d	0.16 ± 0.08^a	0.17 ± 0.05^a	0.13 ± 0.05^a	0.10 ± 0.02^a	N.S.
6d	0.16 ± 0.05^a	0.18 ± 0.03^a	0.15 ± 0.03^a	0.12 ± 0.03^a	N.S.
10d	0.18 ± 0.05^a	0.20 ± 0.05^a	0.17 ± 0.04^a	0.14 ± 0.04^a	N.S.
11d (+Fr)	2.23 ± 0.19^a	2.41 ± 0.19^a	2.25 ± 0.15^a	2.13 ± 0.14^a	N.S.
End AF	1.56 ± 0.12^a	1.81 ± 0.11^a	1.83 ± 0.15^a	1.80 ± 0.21^a	N.S.
Glicerol (g/L)					
3d	3.18 ± 0.19^a	2.95 ± 0.18^{ab}	2.54 ± 0.20^b	3.23 ± 0.10^a	*
6d	3.20 ± 0.20^a	3.02 ± 0.20^a	3.15 ± 0.12^a	3.34 ± 0.22^a	N.S.
10d	3.38 ± 0.18^a	3.08 ± 0.14^a	3.38 ± 0.15^a	3.52 ± 0.28^a	N.S.
11d (+Fr)	3.28 ± 0.20^a	3.15 ± 0.11^a	3.50 ± 0.10^a	3.48 ± 0.19^a	N.S.
End AF	3.32 ± 0.22^a	3.17 ± 0.09^a	3.54 ± 0.16^a	3.51 ± 0.21^a	N.S.
Tartaric acid (g/L)					
3d	0.15 ± 0.02^a	0.18 ± 0.02^a	0.14 ± 0.01^a	0.16 ± 0.03^a	N.S.
6d	0.15 ± 0.02^a	0.21 ± 0.02^a	0.14 ± 0.03^a	0.16 ± 0.04^a	N.S.
10d	0.13 ± 0.03^a	0.22 ± 0.03^a	0.15 ± 0.04^a	0.17 ± 0.04^a	N.S.
11d (+Fr)	0.32 ± 0.05^a	0.37 ± 0.04^a	0.38 ± 0.09^a	0.34 ± 0.05^a	N.S.
End AF	0.32 ± 0.04^a	0.38 ± 0.09^a	0.38 ± 0.08^a	0.34 ± 0.08^a	N.S.

Values are expressed as average of three measurements.
Abbreviations: S.S., statistical significance.
Beer fermented by: *S. cerevisiae* MN113 (TF1); *S. cerevisiae* US-05 (TF2); *L. thermotolerans* MNF105 (TF3); *L. thermotolerans* PHILLY SOUR (TF4). Data in the same line followed by the same letter are not significantly different according to Tukey's test. Symbols: ***, P < 0.001; **, P < 0.01; * P < 0.05; N.S., not significant.

The concentrations of lactic acid, acetic acid, malic acid and tartaric acid, produced during fermentation, are reported in Table 3. Comparing *L. thermotolerans* MNF105 (TF3 test) with the commercial strain, it produced moderate amounts of lactic acid and had a slight influence on pH (TF4) (0.52 and 2.25 g/L, respectively), but although limited, both *L. thermotolerans* showed an ability not possessed by *S. cerevisiae*. In the present study, in accordance with the work of Domizio et al. (2016), the ability of *L. thermotolerans* to produce lactic acid by acidifying beer wort was highlighted. In contrast, the study conducted by Zdaniewicz et al. (2020) showed that this species does not possess the necessary capacity for beer acidification. However, the limited lactic acid production by *L. thermotolerans* MNF105 strain can be considered a positive trait for the production of fruit beers, as these which are characterized by a high basal acidic taste. At the end of the secondary alcoholic fermentation, the amounts of acids other than lactic acid, were comparable to those previously reported by Pirrone et al. (2022) and Viana et al. (2021), who analyzed beers and fruit beers. Regarding glycerol, the concentrations registered in our study (3.17 – 3.54 g/L) are higher than those reported by Gazinski et al. (2020) and Kawa-Rygielska et al., (2019), where they studied fruit

291 beers. With the addition of loquat juice, the levels of malic and tartaric acid, particularly high in loquat

292 fruits (Toker et al., 2013), increased in all trials. Moreover, all strains consumed malic acid, with *S.*

293 *cerevisiae* strain MN113 consuming the largest amount.

294

295 *4.3.3 Volatile organic compound composition*

296 The volatile organic compound of loquat juice is characterised by esters (1.42 ppm), terpenes (0.65

297 ppm), alcohols (0.55 ppm), ketones (0.17 ppm) and aldehydes (0.13 ppm) (Table 4).

298 **Table 4**. Volatile compounds identified in *Eriobotrya japonica* juice using liquid-liquid/GC-MS.

Compoaut Name	Loquat juice (ppm)
∑**Esters**	**1.42**
Ethyl acetate	0.60
Methyl 2-methylbutanoate	0.02
Methyl cinnamate	0.04
N-Acetyl-L-proline methyl ester	0.07
Decyl decanoate	0.05
Methyl 3,4,5 trimethoxycinnamate	0.64
∑**Alcohols**	**0.55**
2,3-Butanediol	0.02
2-Hexen-1-ol	0.19
3-Hexen-1-ol	0.10
3-Phenyl-2-butanol	0.02
2,4-Di-tert-butylphenol	0.22
∑**Aldehydes**	**0.13**
Hexanal	0.07
3,4-Dimethylbenzaldehyde	0.06
∑**Ketones**	**0.17**
Acetoin	0.08
2-Hydroxy-2-methyl-1-phenyl-1-propanone	0.09
∑**Terpenes**	**0.65**
Carvomenthene	0.16
Limonene	0.19
Isocaqmalendiol	0.06
Squalene	0.24
∑**Other**	**0.18**
Toluene	0.08
m-Xylene	0.10

299 The analyses performed on the juice are similar to those of Pirrone et al. (2022), but ketones were

300 detected in this case. In contrast to Planeta et al. (2021), who analyzed several loquat fruits, no

301 compounds belonging to the acid class were detected, probably due to a decrease in oxidative

302 phenomena. The final beers showed a higher VOC complexity, characterized by seven classes:

alcohols, aldehydes, ketones, carboxylic acids, esters, terpenes and others. The experimental beers

differed for a variety of aroma compounds, as shown in the heat map (Fig. 4), where the relationships

among the beers are based on the concentration of each compound detected. Beers fermented with *S. cerevisiae* MN113 (TF1) showed the highest content of aroma compounds, particularly alcohols (Table 5).

Fig. 4. Distribution of volatile organic compounds among fruit beers. The heat map plot depicts the relative concentration of each VOCs. Beer fermented by: *S. cerevisiae* MN113 (TF1); *S. cerevisiae* US-05 (TF2); *L. thermotolerans* MNF105 (TF3); *L. thermotolerans* PHILLY SOUR (TF4).
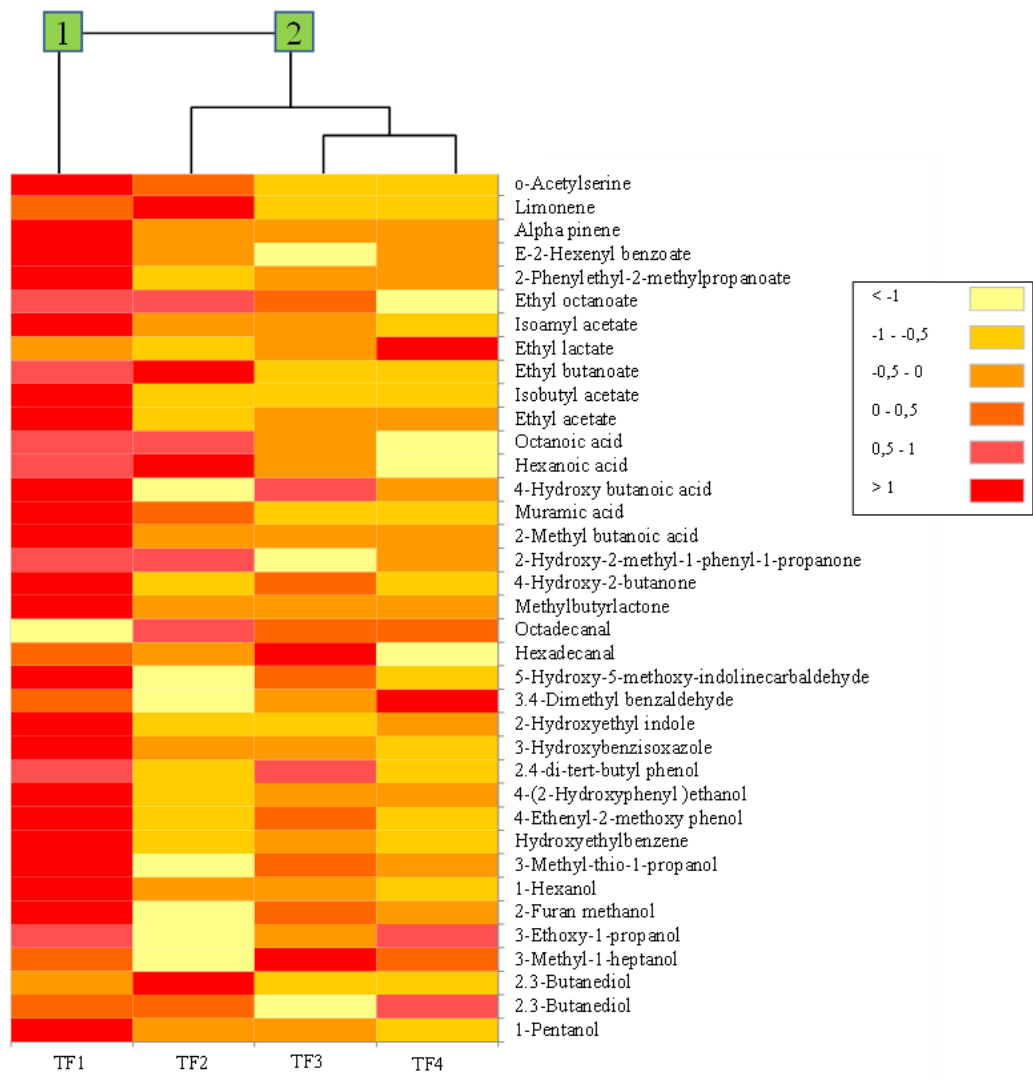
Table 5. Volatile compound concentrations in beer samples. Compounds detected by liquid-liquid/GC-MS.

Compounds	TF1[1]	TF2[1]	TF3[1]	TF4[1]	S.S.
∑Alcohols	**100.63±1.89[a]**	**35.09±1.03[c]**	**40.66±0.95[b]**	**30.99±0.83[d]**	***
1-Pentanol	34.99±0.26[a]	14.98±0.24[b]	14.21±0.12[c]	10.06±0.31[d]	***
2,3-Butanediol	0.81±0.04[a]	0.82±0.06[a]	0.48±0.04[b]	0.88±0.03[a]	***
2,3-Butanediol	0.30±0.02[b]	0.58±0.03[a]	0.20±0.01[c]	0.23±0.01[c]	***
3-Methyl-1-heptanol	0.13±0.01[b]	0.00±0.00[c]	0.20±0.02[a]	0.12±0.01[b]	***
3-Ethoxy-1-propanol	0.14±0.01[a]	0.08±0.01[b]	0.10±0.01[b]	0.13±0.00[a]	***
2-Furan methanol	0.11±0.01[a]	0.00±0.00[c]	0.07±0.01[b]	0.06±0.01[b]	***
1-Hexanol	0.17±0.00[a]	0.11±0.00[b]	0.12±0.02[b]	0.09±0.01[b]	***
3-Methyl-thio-1-propanol	0.24±0.02[a]	0.11±0.01[c]	0.18±0.02[b]	0.16±0.01[b]	***
Hydroxyethylbenzene	46.30±0.88[a]	16.77±0.55[c]	21.70±0.46[b]	13.91±0.15[d]	***
4-Ethenyl-2-methoxy phenol	0.36±0.04[a]	0.11±0.01[c]	0.26±0.02[b]	0.12±0.01[c]	***
4-(2-Hydroxyphenyl) ethanol	0.19±0.01[a]	0.11±0.01[c]	0.13±0.01[bc]	0.14±0.01[b]	***
2,4-di-tert-butyl phenol	0.70±0.04[a]	0.53±0.03[b]	0.71±0.06[a]	0.52±0.02[b]	**
3-Hydroxybenzisoxazole	1.00±0.06[a]	0.40±0.02[b]	0.38±0.03[b]	0.13±0.01[c]	***
2-Hydroxyethyl indole	15.19±0.49[a]	0.49±0.06[d]	1.92±0.12[c]	4.44±0.24[b]	***
∑Aldehydes	**3.40±0.13[a]**	**6.34±0.23[a]**	**3.88±0.17[a]**	**3.44±0.19[a]**	N.S.
3,4-Dimethyl benzaldehyde	0.24±0.01[b]	0.14±0.01[d]	0.20±0.00[c]	0.30±0.02[a]	***
5-Hydroxy-5-methoxy-indolinecarbaldehyde	0.29±0.01[a]	0.14±0.00[c]	0.24±0.01[b]	0.17±0.02[c]	***
Hexadecanal	1.42±0.04[ab]	1.37±0.12[ab]	1.58±0.11[a]	1.12±0.08[b]	*
Octadecanal	1.45±0.07[b]	1.99±0.11[a]	1.86±0.05[a]	1.85±0.07[a]	***
∑Ketones	**0.78±0.04[a]**	**0.41±0.03[b]**	**0.41±0.01[b]**	**0.41±0.02[b]**	***
Methylbutyrlactone	0.04±0.01[a]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	***
4-Hydroxy-2-butanone	0.39±0.02[a]	0.06±0.01[d]	0.23±0.00[b]	0.12±0.00[c]	***
2-Hydroxy-2-methyl-1-phenyl-1-propanone	0.35±0.01[a]	0.35±0.02[a]	0.18±0.01[c]	0.29±0.02[b]	***
∑Carboxylic acids	**1.91±0.12[a]**	**1.76±0.07[a]**	**1.05±0.06[b]**	**0.47±0.03[c]**	***
2-Methyl butanoic acid	0.11±0.01[a]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	***
Muramic acid	0.05±0.00[a]	0.04±0.00[ab]	0.03±0.01[b]	0.03±0.00[b]	*
4-Hydroxy butanoic acid	0.09±0.01[a]	0.00±0.00[d]	0.07±0.00[b]	0.03±0.00[c]	***
Hexanoic acid	0.50±0.04[a]	0.57±0.03[a]	0.31±0.02[b]	0.20±0.01[c]	***
Octanoic acid	1.16±0.06[a]	1.15±0.04[a]	0.64±0.03[b]	0.21±0.02[c]	***
∑Esters	**2.71±0.18[a]**	**1.20±0.08[c]**	**1.69±0.09[b]**	**2.89±0.17[a]**	***
Ethyl acetate	1.27±0.08[a]	0.32±0.02[c]	0.53±0.04[b]	0.49±0.03[b]	***
Isobutyl acetate	0.05±0.01[a]	0.04±0.01[a]	0.04±0.00[a]	0.04±0.00[a]	N.S.
Ethyl butanoate	0.08±0.01[a]	0.11±0.02[a]	0.00±0.00[b]	0.00±0.00[b]	***
Ethyl lactate	0.47±0.03[c]	0.22±0.01[d]	0.69±0.03[b]	2.06±0.12[a]	***
Isoamyl acetate	0.19±0.02[a]	0.09±0.01[b]	0.09±0.00[b]	0.07±0.00[b]	***
Ethyl octanoate	0.24±0.01[a]	0.22±0.00[a]	0.16±0.01[b]	0.00±0.00[c]	***
2-Phenylethyl-2-methylpropanoate	0.20±0.01[a]	0.06±0.00[c]	0.08±0.01[bc]	0.09±0.01[b]	***
E-2-Hexenyl benzoate	0.21±0.01[a]	0.14±0.01[b]	0.10±0.00[c]	0.14±0.01[b]	***
∑Terpenes	**0.14±0.02[a]**	**0.16±0.01[a]**	**0.08±0.01[b]**	**0.06±0.00[b]**	***
Alpha pinene	0.03±0.01[a]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	***
Limonene	0.11±0.01[b]	0.16±0.01[a]	0.08±0.01[c]	0.06±0.00[c]	***
∑Others	**0.09±0.01[a]**	**0.07±0.01[a]**	**0.04±0.00[b]**	**0.03±0.00[b]**	***
o-Acetylserine	0.09±0.01[a]	0.07±0.01[a]	0.04±0.00[b]	0.03±0.00[b]	***

[1]Values are expressed in ppm, averaged over three samples each analysed in triplicate. Data in the same line followed by the same letter are not significantly different according to Tukey's test. Symbols: ***, $P < 0.001$; **, $P < 0.01$; * $P < 0.05$.

Abbreviations: S.S., statistical significance; N.S., not significant.

Alcohols were quantitatively and numerically the most abundant class in all beers, with the highest concentration in TF1 (100.63 ppm) followed by TF3 (40.66 ppm). This class is known for floral, solvent or alcoholic flavors (Eβlinger, 2009). The most abundant alcohol in both fruit beers was hydroxyethylbenzene (46.30 and 21.70 ppm in TF1 and TF, respectively) followed by pentanol (34.99 ppm and 14.98 ppm in TF1 and TF2, respectively). Eight different ester-class compounds are present in the samples; however, their levels are strain-dependent (Pires et al., 2014). The main ester active

in aroma is ethyl acetate, a secondary metabolite of alcoholic fermentation, which is responsible for the fruity aroma (Canonico et al., 2016). Moreover, ethyl lactate was also present, especially in beers from trials TF3 and TF4 (0.69 ppm and 2.06 ppm, respectively), because this compound in generally produced by the species *L. thermotolerans*. Some strains within this yeast species have been shown to produce ethyl lactate and have been found in sour beers (Witrick et al., 2017). In addition, this compound is also detected during wine fermentation (Gobbi et al., 2013). Among the compounds detected, 2,3-butanediol, 2,4-di-tert-butyl phenol, 3,4-dimethyl benzaldehyde, 2-hydroxy-2-methyl-1-phenyl-1-propanone, ethyl acetate and limonene were present in both loquat juice and fruit beer. Specifically, 2,3-butanediol is a molecule produced by *S. cerevisiae* (Song et al., 2019), while 2,4-di-tert-butyl-phenol is produced by yeasts and was found in *Luzhou*-Flavor Liquor (Ding et al., 2015). Instead, 3,4-dimethylbenzaldehyde was found in dry-cured hams, where several yeasts were found, in particular *Debaryomyces* was the dominant species of yeast (Gong et al., 2023). Furthermore, 2-hydroxy-2-methyl-1-phenyl-1-propanone was found in a traditional Chinese liquor (Huangjiu) obtained by fermenting a pool of different species of bacteria, yeasts and fungi (Wang et al., 2022). Instead, ethyl acetate has been found in sorghum beers fermented with *S. cerevisiae* yeast (Tokpohozin et al., 2019). But it has also been found in fruit lambic beers, mainly associated with activity by *Brettanomyces* (Bongaerts et al., 2021). Limonene is found in lemon and other citrus fruits, but in general in more than 300 plants (Jongedijk et al., 2016). Moreover, this compound is the second most distributed terpenoid in nature and can also be associated with hops (Ramírez & Viveros, 2021). Finally, most of these compounds are produced by the metabolism of microorganisms, while only limonene is attributed to fruit and hops.

4.3.4 Sensory evaluation

The data from the sensory evaluation are reported in Figure 5. The differences among loquat experimental beers were significant, thus, yeasts isolated from manna impacted differently from control yeasts beer aroma. All panellists were able to recognize loquat addition and the use of wheat malt in all beers and they did not reveal any defect. In particul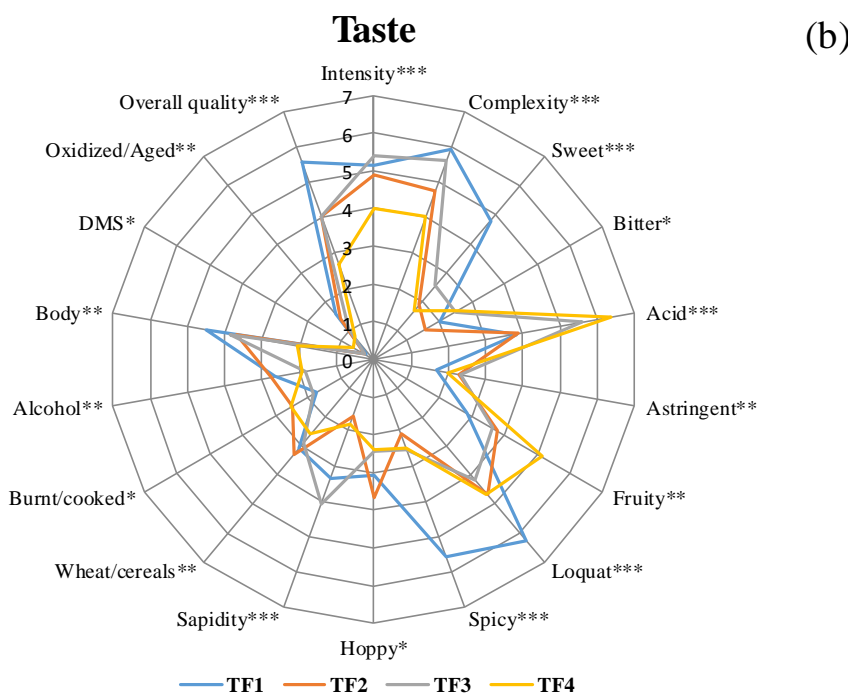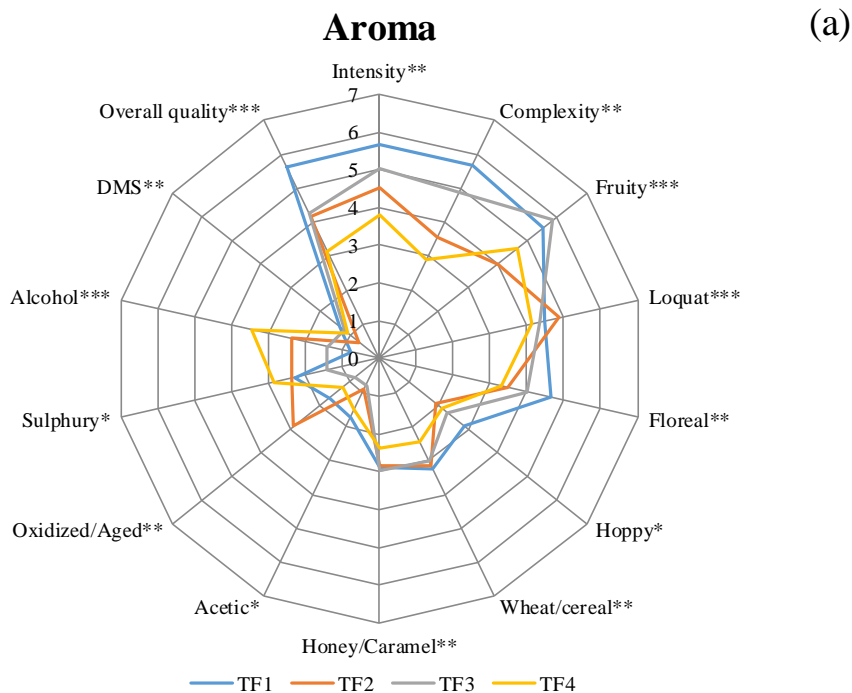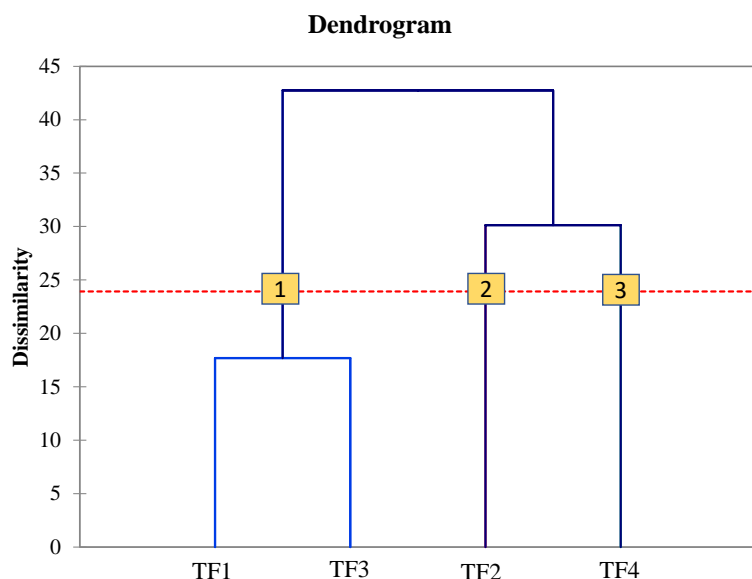ar, trial TF1 beers showed the highest scores for fourteen attributes (aroma: intensity, complexity, floreal, hoppy, wheat/cereal, acetic and overall impression; taste: complexity, sweet, loquat, spicy, body, oxidized/aged and overall impression), while those from trial TF3 only for five (aroma: fruity, honey/caramel; taste: intensity, astringent and sapidity). The beer obtained from trial TF2 were easily recognized from the other beers and they reached a general low acceptance, but the commercial *L. thermotolerans* strain used in trial TF4 determined an excessive acidity and the beers received the worst appreciation. On the other hand, the beers from TF1 and TF3 received fair overall quality values (5.56 and 4.01, respectively) (Fig. 5b). Several studies reported that the use of *L. thermotolerans* increases perceived acidity due to an increase in total acidity of beers (Osburn et al., 2018; Peces-Pérez et al., 2022; Romero-Rodríguez et al., 2022). The overall organoleptic investigation showed a preference for trial TF1 beers, which showed a higher residual sugar content at the end of fermentation with pronounced notes of spice and loquat flavour, followed by those from trial TF3. The differences among loquat experimental beers were significant, thus, yeasts isolated from manna impacted differently from control yeasts beer aroma. All panellists were able to recognize loquat addition and the use of wheat malt in all beers and they did not reveal any defect.

Fig. 5. Sensory analysis performed on beers: spider plots of average scores for aroma (a), taste attributes and overall quality of bottled fruit beers (b), determined by judges during tasting sessions. Beer fermented by: *S. cerevisiae* MN113 (TF1); *S. cerevisiae* US-05 (TF2); *L. thermotolerans* MNF105 (TF3); *L. thermotolerans* PHILLY SOUR (TF4). Symbols: ***, P < 0.001; **, P < 0.01; * P < 0.05.

In particular, trial TF1 beers showed the highest scores for fourteen attributes (aroma: intensity, complexity, floreal, hoppy, wheat/cereal, acetic and overall impression; taste: complexity, sweet, loquat, spicy, body, oxidized/aged and overall impression), while those from trial TF3 only for five (aroma: fruity, honey/caramel; taste: intensity, astringent and sapidity). The beer obtained from trial TF2 were easily recognized from the other beers and they reached a general low acceptance, but the commercial *L. thermotolerans* strain used in trial TF4 determined an excessive acidity and the beers received the worst appreciation. On the other hand, the beers from TF1 and TF3 received fair overall quality values (5.56 and 4.01, respectively) (Fig. 5b). Several studies reported that the use of *L. thermotolerans* increases perceived acidity due to an increase in total acidity of beers (Osburn et al., 2018; Peces-Pérez et al., 2022; Romero-Rodríguez et al., 2022). The overall organoleptic investigation showed a preference for trial TF1 beers, which showed a higher residual sugar content at the end of fermentation with pronounced notes of spice and loquat flavour, followed by those from trial TF3.

4.3.5 Statistical and explorative multivariate analyses

The AHC categorized the evidence according to their mutual dissimilarity and relationship (Fig. 6). This evaluation categorized the trials using forty-two variables chosen based on the outcomes from sensory characteristics and chemical parameters. The different trials of loquat beers were visibly divided into three clusters, considering a dissimilarity of 35%. In one cluster were grouped theses in which manna-isolated strains were used, namely MN113 (TF1) and MNF105 (TF3), while in two other different clusters were grouped theses in which controls were used, namely US-05 (TF2) and MNF105 (TF4). The variables that greatly influenced the clustering were fruity, intensity of color and acid. The graphical representation of the VOC analysis is reported in Fig. 4. The hierarchical dendrogram combined with heat map graph revealed that different strains significantly influence the VOCs released from the trials. The concentrations of the VOCs among loquat beers resulted in a cluster with TF1 trials and a main cluster with a grouping of TF2, TF3 and TF4 trials.

Fig. 6. Dendrogram of strains resulting from AHC based on values of chemical and sensory aspect of beer experimental productions Dissimilarity is calculated by Euclidean distance. Agglomeration is calculated by Ward's method. Beer fermented by: *S. cerevisiae* MN113 (TF1); *S. cerevisiae* US-05 (TF2); *L. thermotolerans* MNF105 (TF3); *L. thermotolerans* PHILLY SOUR (TF4).

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4.4 Conclusion

In this work, yeast strains belonging to the species *L. thermotolerans* and *S. cerevisiae* isolated from sugar-rich matrices, i.e., manna, were tested to assess their effect as starter cultures on the physicochemical and organolectical properties of loquat beer. In particular, the selected strain *L. thermotolerans* MNF105 derived from these matrices was applied for the first time in brewing. From different point of view, fermentation with the chosen strains produced better results compared to that with the corresponding commercial controls. Experimental trials conducted with the selected strains demonstrated the absence of off-odour and off-flavour and improved aroma perception. Instead, these strains have been shown to be able to conduct beer fermentation, producing a good amount of alcohol, and also the ability to produce particular flavors that can modify and enhance the aromatic complexity of fruit beers. Moreover, the modest lactic acid production of the *L. thermotolerans* MNF105 strain is a positive ability for the production of sour fruit beers, as these already have excessive acidity as a base due to the low pH of the fruit. The overall organoleptic investigation showed a preference for *S. cerevisiae* MN113 (TF1), which showed higher residual sugar content at the end of AF, followed by TF3, in which *L. thermotolerans* also isolated from manna was used, which produced a more balanced beer than the commercial control. Aldehydes and alcohols were the most prevalent VOCs in beers. Beers brewed with *S. cerevisiae* MN113 (TF1) are characterized by higher concentrations of alcohols, ketones and carboxylic acids. In particular, ethyl acetate was also higher, a secondary metabolite of alcoholic fermentation responsible for the fruity aroma. Interestingly, the samples inoculated with *Lachancea* strains have a greater content of ethyl lactate, a compound produced by this species. *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.

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CHAPTER 5

Use of non-conventional yeasts (*Candida oleophila*, *Starmerella lactis-condensi*, *Hanseniaspora uvarum* and *Lachancea thermotolerans*) for enhancing the sensory quality of craft beer
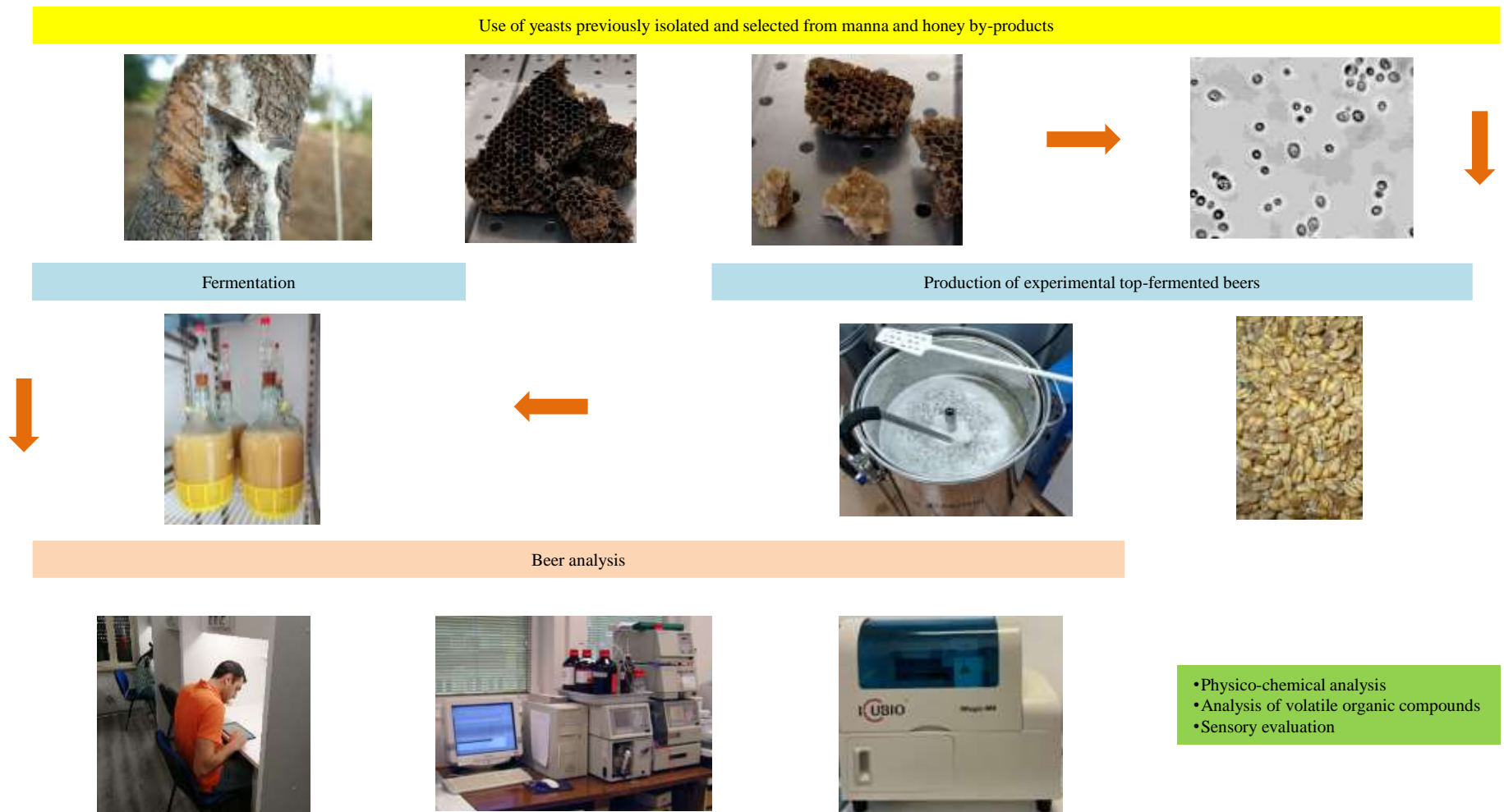
ABSTRACT

Craft beer production has experienced significant growth in recent years. There is a growing interest in using non-conventional yeasts to produce beers with unique flavors. This work investigated the impact of unconventional yeast strains in sequential inoculum with *Saccharomyces cerevisiae* US-05, including *Hanseniaspora uvarum* YGA34 (EP1), *Lachanchea thermotolerans* MNF105 (EP2), *Candida oleophila* YS209 (EP3) and *Starmerella lactis-condensi* MN412 (EP4), as co-starter cultures on the physico-chemical and sensory properties of beer. The control trial was inoculated with *S. cerevisiae* US-05 (TC). The strains were selected from matrices with high sugar content, such as manna and fermented honey by-products. In particular, the strains *C. oleophila* YS209 and *St. lactis-condensi* MN412 were applied for the first time in brewing. All strains showed their concentrations ranged between 5 and 8 Log cycles during fermentation. Furthermore, *L. thermotolerans* MNF105 and *St. lactis-condensi* MN412 had consumed almost all of the fructose, glucose and sucrose prior to inoculation of the commercial strain. *Lachancea thermotolerans* MNF105 consumed slightly more maltose than the other strains, but slightly less than the control strain. Esters were the most detected compounds in the beer samples, with ethyl octanoate being the main compound. The EP2 trial had the highest ester content (237.3 mg/L). Moreover, control trial TC had the highest value of ethyl octanoate at 157.1 mg/L, followed by the EP2 trial at 125.5 mg/L. The other most abundant esters detected were ethyl-trans-4-decenoate (31.4 mg/L in TC followed by 25.9 in EP2), ethyl decanoate (caprate) (38.1 mg/L in EP2 followed by 2.7 mg/L in EP3), and isoamyl acetate (27.0 in EP2 followed by 2.0 mg/L in EP1 and EP3). The overall organoleptic investigation showed a preference for EP2 trial, which showed spicy and fruity flavour. Overall, these unconventional yeast strains from high sugar matrices showed great technological properties, representing promising co-starters and starter during craft beer production.

Keywords: *Candida oleophila*; *Starmerella lactis-condensi; Hanseniaspora uvarum*; *Lachanchea thermotolerans*; Non-*Saccharomyces*; Beer fermentation; volatile organic compounds; Beer aroma.

127

657 **Fig. 1.** Graphical abstract of experimental research

Use of yeasts previously isolated and selected from manna and honey by-products

Fermentation

Production of experimental top-fermented beers

Beer analysis

- Physico-chemical analysis
- Analysis of volatile organic compounds
- Sensory evaluation

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659

5.1 Introduction

Historically, non-*Saccharomyces* yeast species have been viewed negatively in brewing processes, as and especially some of them could present problems associated with beer safety, turbidity, filterability, off-flavours, acidity and other changes in flavour profile (Miguel et al., 2022; Varela, 2016). However, the correct use and selection of non-*Saccharomyces* yeasts in the brewing process can lead to the acquisition of favourable properties. Many studies have been conducted on these yeasts over the years, and it has been discovered that they can play an important role in defining beer aroma and improving organoleptic characteristics (Van Rijswijck, Wolkers–Rooijackers, Abee, & Smid, 2017). The impact of yeast selection on the metabolites that contribute to the characteristic flavour of beer, such as acetate and ethyl esters and higher alcohols, is widely recognised (Pires, Teixeira, Bràanyik, & Vicente, 2014). This has led researchers to investigate the use of non-*Saccharomyces* strains in beer production.

Due to their characteristics, some of these yeasts have been shown to be suitable for the production of low-alcohol beers, functional beers, sour beers and beer bioflavouring (Basso et al. 2016; Holt, Mukherjee, Lievens, Verstrepen, & Thevelein, 2018, Francesca et al., 2023; Toh, Chua, Lu, & Liu, 2020). Although the positive characteristics of non-*Saccharomyces* yeast are a recent area of research, their potential in the fermented beverage industry, particularly in brewing, is becoming clearer.

Recently, research in this field has also focused on selecting yeasts isolated from alternative sources to identify novel yeasts capable of intensifying the aromatic characteristics of beers (Colomer, Funch, & Forster, 2019; Larroque et al., 2021). Indeed, some research has conducted isolations from highly sugary matrices, such as manna ash (Guarcello et al., 2019) or from highly alcoholic beverages obtained from fermented honey by-products (Matraxia et al., 2021).

Several non-*Saccharomyces* yeasts isolated from different matrices, including *Zygosaccharomyces*, *Pichia*, *Saccharomycodes*, *Torulaspora*, *Lachanchea* and *Hanseniaspora*, have been studied for applications in brewing (Sannino, Mezzasoma, Buzzini, & Turchetti, 2019).

For instance, Canonico, Agarbati, Comitini and Ciani (2016) found that the presence of *Torulaspora delbrueckii* resulted in beers with a low alcohol content while displaying a particularly analytical and aromatic profile. Similarly, Callejo et al. (2019) applied yeasts for beer production and found that the species *Torulospora delbrueckii* and *Saccharomycodes ludwigii*, followed by *Lachanchea thermotolerans*, are capable of producing beers with low ethanol content and characteristic aroma profiles or *Schizosaccharomyces pombe* can increase both ethanol and acetaldehyde content.

Instead, Matraxia et al. (2021) found that *Hanseniaspora uvarum* effectively improved glycerol and acetic acid concentrations. This strain also exhibited higher sensory complexity and intensity and grew rapidly in the presence of ethanol and hops. Additionally, Pirrone et al. (2022) demonstrated that the beers produced with sequential inoculation of *Hanseniaspora uvarum* and *Saccharomyces cerevisiae* are characterised by a higher ester and lower alcohol concentration. Francesca et al. (2023) demonstrated that the yeast strain *L. thermotolerans* MNF105 had lower lactic acid production, which contributed to the flavour balance of beer. Additionally, it was capable of producing ethyl lactate, a compound commonly produced by this species.

The ability of certain yeast species and strains to ferment sugars selectively allows for their sequential use with *S. cerevisiae*, resulting in improved flavour and reduced sweetness of beers due to residual sugars. Non-*Saccharomyces* yeasts have a low tolerance to ethanol and are not always able to consume the main sugars of the beer wort. Therefore, they are typically used in co-fermentation with *Saccharomyces* species to complete fermentation (Cubillos, Gibson, Grijalva-Vallejos, Krogerus, & Nikulin, 2019). Thus, the mixed or sequential inoculation of non-*Saccharomyces* and *Saccharomyces* yeasts is a promising strategy for producing innovative flavours in beer (Holt et al., 2018). To our knowledge, there are no articles in the literature on the use of *Candida oleophila* and *Starmerella lactis-condensi* in brewing. This study is the first to report results associated with these two yeasts as co-starters in brewing. Previously, they had only been applied in oenological studies (Francesca et al., 2024).

Based on the above considerations, the present study aimed to: (i) improve the knowledge on the role of non-*Saccharomyces* for beer production; (ii) to evaluate for the first time the effect of *C. oleophila* and *St. lactis-condensi* for beer production; (iii) improve the organoleptic quality of craft beer; and (iv) to enhance our understanding of the ecology of yeasts associated with high sugar matrices.

5.2 Materials and methods

5.2.1 Yeast strains and media

The yeast strains used in this research were *H. uvarum* YGA34, *L. thermotolerans* MNF105, *C. oleophila* YS209, and *St. lactis-condensi* MN412. Yeast strains applied in this study belong to the collection of the Department of Agricultural, Food and Forest Sciences (SAAF; University of Palermo, Italy). They were isolated from manna (Guarcello et al., 2019) and honey by-products (Gaglio et al., 2017) and were selected for their high-performance during beer wort fermentation. The commercial yeast strain *S. cerevisiae* US-05 (from Lallemand Inc., Montreal, Canada) was used for sequential inoculation after 72 h and for the control trial. Yeast reactivation from cryogenic storage was performed following the procedure described by Pirrone et al. (2022). All media and the supplements used were purchased from Oxoid (Thermofisher, Milan, Italy).

5.2.2 Experimental plan

Experimental high fermentation beers were conducted on a medium scale (5 L batch) employing four different inocula to better understand the impact of inoculum during fermentation. The wort for the fermentation trials was produced in double batch with a 40-L all-in-one microbrewing plant Klarstein mod. 10031629 (Chal-Tec GmbH Berlin, Germany).

The recipe for the beer included 5.5 kg of pilsner malt (BestMalz, Heidelberg, Germany), 3 kg of pale ale malt (BestMalz, Heidelberg, Germany), 0.5 kg of wheat malt (BestMalz, Heidelberg, Germany), and 0.1 kg of acidified malt (Mich. Weyermann® GmbH & Co. KG Brennerstraße 17-19, 96052 Bamber) to lower the pH of the mash. The malts were ground using a double roller mill (Brouwland,

736   Beverlo, Belgium) with a roller distance of 1.20 mm. They were then added to 35.5 L of water

737   containing 10 g each of $CaSO_4$ and $CaCl_2$ for pH correction, as reported by Marconi, Rossi, Galgano,

738   Sileoni, & Perretti, et al. (2016). The mashing process was conducted at 65°C for one hour until

739   complete saccharification, which was tested using an iodine solution, following the method described

740   by Mayer et al. (2016). The temperature was subsequently raised to 72°C and maintained for 10

741   minutes. Enzyme deactivation was achieved by heating the mixture to 78°C for 10 minutes, followed

742   by the lautering phase with a recirculation flow of wort over the spent and rinsed grains with 19 L of

743   water heated to 78°C. The resulting wort volume was 46.5 L. The wort was then boiled for 60 minutes.

744   Ten g of Styrian wolf pellets with 12% w/w α-acids were added at the beginning of the boil, followed

745   by another 30 g of the same hops 5 minutes before the end of the boil. The resulting volume was 40

746   L. The wort was clarified using a whirlpool that involved a 10-min recirculation and a 10-min rest,

747   as described by Marconi et al. (2016). After cooling for 20 minutes in a stainless-steel wort chiller,

748   the wort was prepared for yeast inoculation. The beer wort's standard quality parameters were pH 5.2

749   and 14°P (Plato degree). The experiment utilised 80 litres of must that were produced in a double-

750   batch process on the same day.

751   Five experimental trials were inoculated as reported in Fig. 2, as follow: EP1 with *H. uvarum* YGA34

752   and after 72 h with US-05 strain; EP2 with *L. thermotolerans* MNF105 and after 72 h with US-05

753   strain; EP3 with *C. oleophila* YS209 and after 72 h with US-05 strain; EP4 with *St. lactis-condensi*

754   MN412 and after 72 h with US-05 strain; and TC with US-05 strain as control. Yeast strains were

755   inoculated at a density of approximately 2.0×10^6 cells/mL, as reported by Holt et al. (2018). The

756   fermentation process was carried out at a temperature of 20°C in hermetically sealed glass fermenters

757   (5 L) that were equipped with an airlock valve. Samples were collected from uninoculated wort, after

758   the inoculum of each yeast strain, every three days until the end of fermentation (day 3, 6, 9 and 12).

759   After completing the fermentation process, the beer was transferred into 0.5 L bottles and 6 g/L of

760   dextrose was added, as reported by Francesca et al. (2023). Bottle conditioning was subsequently

761   carried out at 20°C for a period of 25 days (Callejo et al., 2019). Following this period, the

762   experimental beers underwent sensory analysis. All fermentation experiments were conducted in

763   triplicate.

764   **Fig. 2.** Experimental plan of beer production
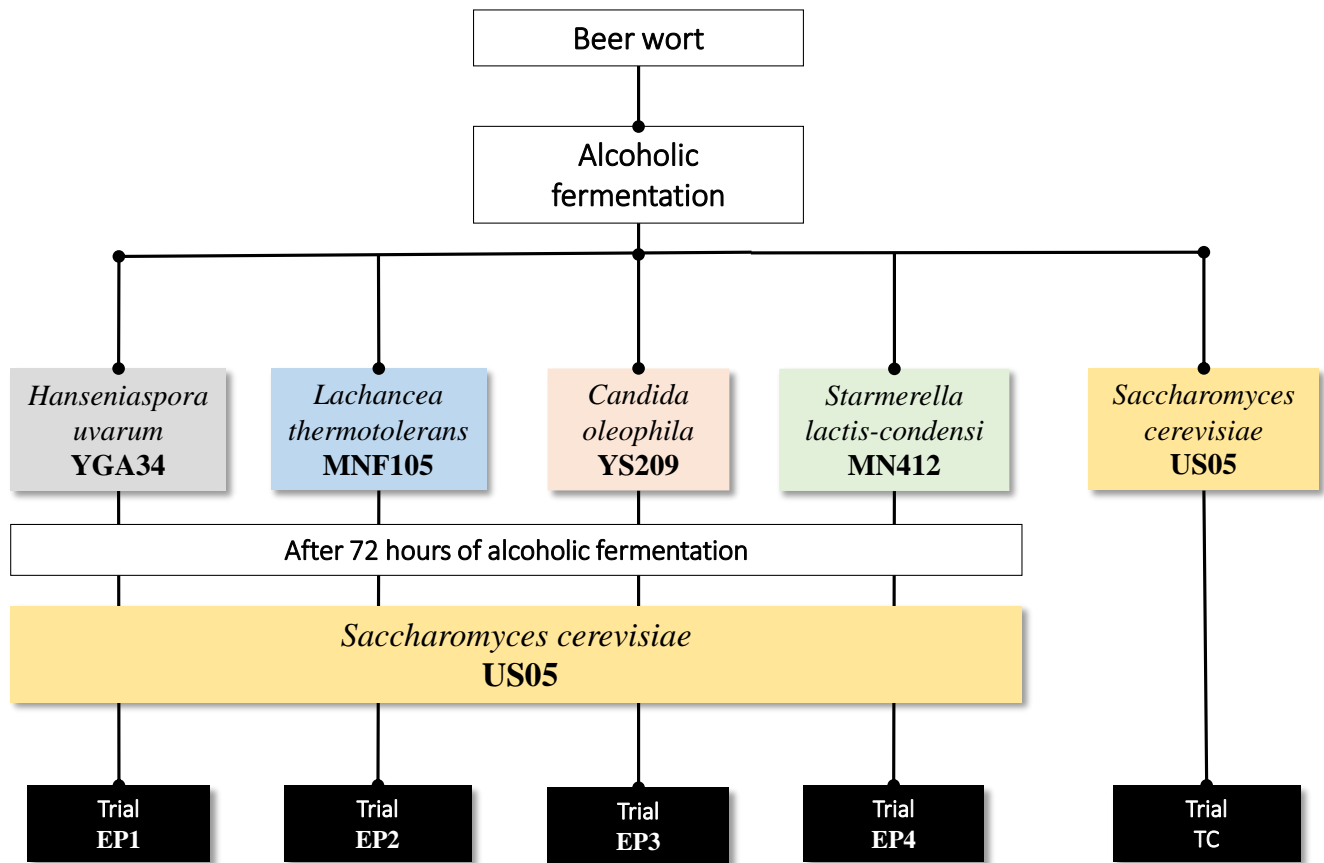
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766   *5.2.3 Microbiological analyses*

767   The samples were subjected to microbiological analysis with plate counts immediately after sampling.

768   The following media were used: Wallerstein Laboratory nutrient agar (WL) for *Saccharomyces*

769   populations (Di Maio, Polizzotto, Planeta, & Oliva, 2011) and Lysine agar (LA) for non-

770   *Saccharomyces* populations (Iris, Antonio, Antonia, & Antonio, 2020). The colonies from the two

771   agar media were presumptively identified based on their morphological characteristics. Identification

772   was confirmed only after determining cell morphology through microscopic inspection (Cavazza,

773   Grando & Zini, 1992). All analyses were performed in triplicates.

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5.2.4. Determination of physicochemical parameters

The values of pH,°Bx, acetic acid, lactic acid, tartaric acid, fructose, glucose, glycerol, malic acid, maltose, sucrose, alcohol (% vol), density (FG), real extract (°P), energy (kcal/100g), apparent extract (°P), original extract (°P) and real attenuation (%), have been carried out as reported by Francesca et al. (2023).

5.2.5. Determination of volatile organic compounds of beer samples

Analysis of volatile organic compounds in beer samples was carried out as reported by Alfonzo et al., (2021). Quantification was carried out using three calibration lines. For compounds belonging to classes other than standards, similarity was used for quantification. A dilution factor was used for the reported data.

5.2.6. Sensory evaluation

Sixteen judges (9 men and 7 women) aged between 28 and 48 were selected from the University of Palermo to evaluate the beer produced. The beer evaluation procedure was conducted following the methodology described by Francesca et al. (2023), with some modifications made to the descriptors as outlined below: odour (intensity, complexity, fruity, floral, hoppy, honey/caramel, wheat/cereal, acetic, spicy, sulfury, alcohol and DMS), taste (intensity, complexity, sweet, bitter, acid, astringent, fruity, spicy, hoppy, sapidity, wheat/cereal, balsamic, alcohol, body, DMS, oxidized/aged) and overall acceptance. The average of the three assessments was used to obtain the final scores.

5.2.7. Statistical and explorative multivariate analyses

An analysis of variance (ANOVA) was carried out to identify any significant differences between the physico-chemical parameters during the brewing process, the VOCs and the sensory analysis of the final beer. The post-hoc Tukey's method was used to perform pairwise comparisons on all the data. Statistical significance was attributed to $P \leq 0.05$ (Mazzei et al., 2010). In order to assess the

correlation between the aromas identified in the VOCs and the sensory analysis, a Principal Component Analysis (PCA) was performed using XLstat software version 2019.2.2 (Addinsoft, New York, NY, USA) for Excel, considering only those aromas with an odour activity value greater than 1.

5.3 Results

5.3.1. *Evaluation of population dynamics*

Growth kinetics during fermentation are shown in Figure 3. Microbial levels in uninoculated wort were below the detection limit, both on WL and LA media. Instead, the inoculum cell densities varied between 5.95 and 6.82 Log CFU/mL. The values increased by approximately 1 Log cycle after 3 days for all trials. Similar trends were observed for the control trial TC. Moreover, on day 3th at the sequential inoculation of the commercial yeast strain *S. cerevisiae* US-05, the range of cell density of different trials for *Saccharomyces* genus varied between 5.95 and 6.82 Log CFU/mL. On the next sampling day, an increase in the number of *Saccharomyces* was observed, except for the control trial TC, which showed a decrease. In addition, the trials showed a decrease in all non-*Saccharomyces*. However, starting from sample day 9th, there was a decrease in both *Saccharomyces* and non-*Saccharomyces*, which persisted until the end of the fermentation process. The highest cell counts were registered at the day 3th for *C. oleophila* YS209 in the trial EP3 (7.54 Log CFU/mL) followed by *L. thermotolerans* MNF105 in trial EP2 (7.36 Log CFU/mL). Instead, *S. cerevisiae* US-05 in EP2 (7.30 Log CFU/mL) and EP4 (7.16 Log CFU/mL) showed more consistent growth than the control trial TC, which grew to values of 7.10 Log.
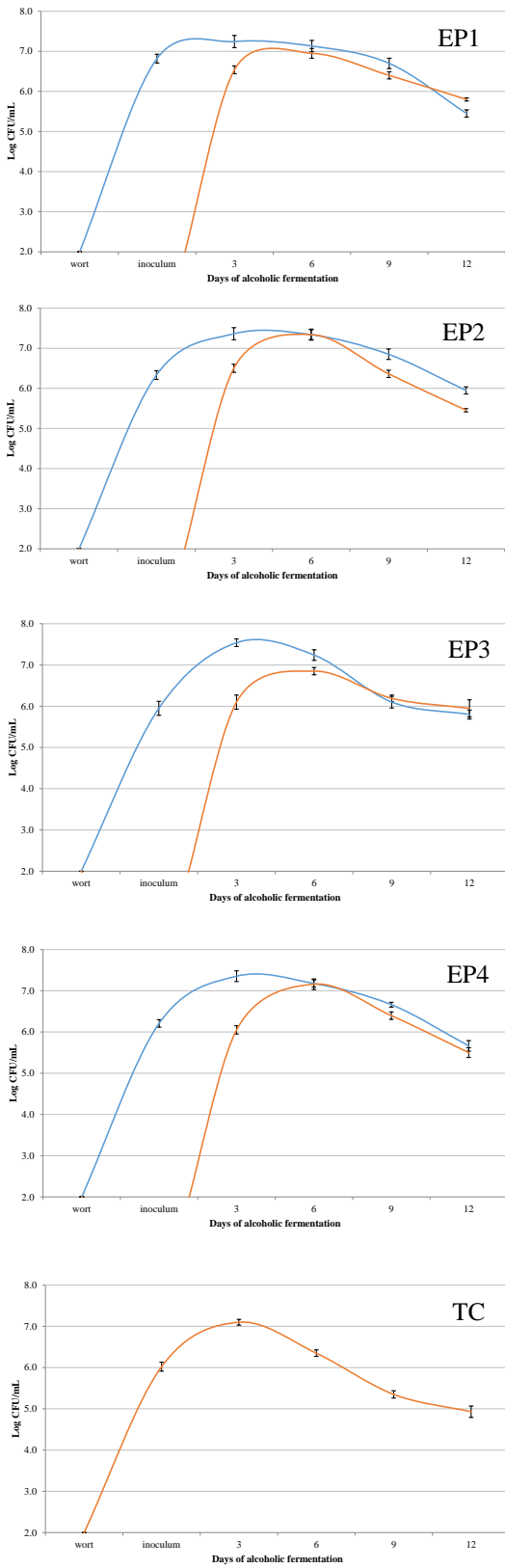
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834 5.3.2 *Physicochemical changes during fermentation*

835 Chemical composition of the wort was: 42.66 g/L maltose, 13.65 g/L sucrose, 12.64 g/L glucose and

836 1.56 g/L fructose. The pH values obtained at the end of alcoholic fermentation for the different trials

837 ranged from 3.58 to 4.10. Chemical parameter analyses showed notable differences among strains

838 regarding the evolution of sugar, glycerol, acetic acid, and lactic acid concentrations (Table 1).

839 **Table 1.** Conventional chemical parameters monitored in samples beer during the alcoholic fermentation of
840 beer.

Parameters	Trials					S.S.
	EP1	EP2	EP3	EP4	TC	
D-fructose (g/L)						
3d	0.31 ± 0.02^a	0.01 ± 0.00^c	0.22 ± 0.01^b	0.32 ± 0.02^a	0.04 ± 0.01^c	***
6d	0.51 ± 0.03^a	0.00 ± 0.00^c	0.07 ± 0.01^b	0.03 ± 0.01^{bc}	0.01 ± 0.01^c	***
9d	0.02 ± 0.00^a	0.00 ± 0.00^b	0.02 ± 0.00^a	0.00 ± 0.00^b	0.03 ± 0.01^a	***
12d	0.00 ± 0.00^a	0.00 ± 0.00^a	0.02 ± 0.00^a	0.01 ± 0.01^a	0.04 ± 0.01^a	N.S.
D-glucose (g/L)						
3d	6.13 ± 0.12^a	0.16 ± 0.02^d	4.81 ± 0.15^b	0.57 ± 0.04^c	0.15 ± 0.02^d	***
6d	2.34 ± 0.11^a	0.08 ± 0.02^b	0.20 ± 0.01^b	0.15 ± 0.01^b	0.07 ± 0.00^b	***
9d	0.09 ± 0.01^b	0.06 ± 0.00^c	0.10 ± 0.01^{ab}	0.06 ± 0.01^c	0.12 ± 0.01^a	***
12d	0.07 ± 0.00^{bc}	0.05 ± 0.00^c	0.10 ± 0.01^{ab}	0.06 ± 0.01^c	0.11 ± 0.02^a	***
Maltose (g/L)						
3d	39.25 ± 0.42^b	28.50 ± 0.28^c	39.71 ± 0.21^b	41.26 ± 0.34^a	26.27 ± 0.19^d	***
6d	25.54 ± 0.18^a	17.50 ± 0.24^d	24.05 ± 0.17^b	23.01 ± 0.31^c	12.54 ± 0.16^e	***
9d	9.20 ± 0.15^b	7.24 ± 0.10^c	10.24 ± 0.21^a	9.24 ± 0.12^b	2.25 ± 0.05^d	***
12d	1.30 ± 0.08^a	0.71 ± 0.04^c	0.92 ± 0.03^b	0.75 ± 0.08^c	0.53 ± 0.03^d	***
D-sucrose (g/L)						
3d	4.38 ± 0.15^a	0.13 ± 0.02^c	3.70 ± 0.14^b	0.26 ± 0.07^c	0.18 ± 0.06^c	***
6d	6.48 ± 0.21^a	0.13 ± 0.03^b	0.20 ± 0.07^b	0.17 ± 0.04^b	0.13 ± 0.03^b	***
9d	0.11 ± 0.03^a	0.10 ± 0.02^a	0.13 ± 0.02^a	0.09 ± 0.01^a	0.14 ± 0.03^a	N.S.
12d	0.09 ± 0.02^a	0.10 ± 0.03^a	0.11 ± 0.01^a	0.09 ± 0.04^a	0.10 ± 0.01^a	N.S.
Lactic acid (g/L)						
3d	0.16 ± 0.02^b	0.69 ± 0.05^a	0.17 ± 0.04^b	0.16 ± 0.04^b	0.16 ± 0.02^b	***
6d	0.15 ± 0.02^b	0.71 ± 0.05^a	0.16 ± 0.01^b	0.15 ± 0.02^b	0.12 ± 0.01^b	***
9d	0.15 ± 0.03^b	0.72 ± 0.04^a	0.16 ± 0.02^b	0.15 ± 0.03^b	0.16 ± 0.02^b	***
12d	0.18 ± 0.04^b	0.70 ± 0.04^a	0.17 ± 0.03^b	0.16 ± 0.05^b	0.15 ± 0.01^b	***
Acetic acid (g/L)						
3d	0.12 ± 0.02^b	0.18 ± 0.03^a	0.05 ± 0.01^c	0.01 ± 0.00^c	0.03 ± 0.01^c	***
6d	0.15 ± 0.01^b	0.23 ± 0.02^a	0.05 ± 0.00^c	0.02 ± 0.01^c	0.03 ± 0.01^c	***
9d	0.15 ± 0.03^b	0.25 ± 0.02^a	0.06 ± 0.01^c	0.01 ± 0.00^c	0.04 ± 0.01^c	***
12d	0.15 ± 0.04^b	0.24 ± 0.03^a	0.07 ± 0.02^{bc}	0.02 ± 0.01^c	0.05 ± 0.02^c	***
Glicerol (g/L)						
3d	0.00 ± 0.00^c	0.71 ± 0.05^b	0.00 ± 0.00^c	0.00 ± 0.00^c	1.93 ± 0.12^a	***
6d	1.83 ± 0.09^{ab}	2.04 ± 0.13^a	1.67 ± 0.08^b	1.05 ± 0.09^c	1.95 ± 0.11^{ab}	***
9d	2.30 ± 0.05^b	2.62 ± 0.04^a	2.15 ± 0.06^b	1.96 ± 0.04^c	2.52 ± 0.09^a	***
12d	2.91 ± 0.11^b	3.04 ± 0.10^b	2.78 ± 0.08^{bc}	3.36 ± 0.09^a	2.55 ± 0.10^c	***

841 Values are expressed as average of three measurements.

848 After 12 days of fermentation, all trials resulted in a final sugar concentration of less than 1.46 g/L.

849 Regarding fructose, glucose, and sucrose, the EP2 and EP4 trials, inoculated with *L. thermotolerans*

850 MNF105 and *St. lactis-condensi* MN412, respectively, consumed almost all of them after three days

851 of fermentation before inoculation of the strain *S. cerevisiae* US-05. After three days, MNF105 strain

852 consumed the most maltose compared to the other strains, but slightly less than the control strain.

853 After the addition of the commercial strain *S. cerevisiae* US-05, all sugars were consumed and their

854 levels decreased in all trials, with levels comparable to the control trial.

855 Furthermore, the EP2 trial inoculated with *L. thermotolerans* MNF105 had a higher lactic acid

856 concentration of 0.70 g/L at the end of alcoholic fermentation compared to the other trials, which had

857 values between 0.15 and 0.18 g/L. In contrast, the EP2 trial showed slightly higher levels of acetic

858 acid compared to the other trials, with values of 0.24 g/L, while the other trials had values ranging

859 from 0.02 to 0.15 g/L. Glycerol contents were similar in all the trials. However, the EP4 trial, which

860 was inoculated with *St. lactis-condensi*, had the highest value of 3.36 g/L.

861 Table 2 shows the data recorded after the FOSS analysis. Ethanol values ranged from 5.80 to 6.32.

862 The lowest value was observed for EP2, while the values determined for the other tests were similar.

863 No differences in density values were found between the trials. However, the highest values for real

864 attenuation were recorded for EP3 (67.70%), EP4 (69.20%) and TC (67.70%), intermediate for EP1

865 (66.20%) and the lowest for EP2 (63.20%).

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Table 2. Conventional chemical parameters monitored in samples beer during the alcoholic fermentation of
beer.

	EP1	EP2	EP3	EP4	TC	S.S.
Alcohol (v/v)	6.06 ± 0.12^a	5.80 ± 0.11^b	6.19 ± 0.15^a	6.32 ± 0.21^a	6.19 ± 0.14^a	***
Density (FG)	1.010 ± 0.09^a	1.012 ± 0.08^a	1.009 ± 0.12^a	1.008 ± 0.09^a	1.009 ± 0.10^a	N.S.
Real extract (°P)	4.59 ± 0.12^b	5.01 ± 0.13^a	4.39 ± 0.08^{bc}	4.18 ± 0.07^c	4.39 ± 0.10^{bc}	***
Energy (kcal/100g)	36.00 ± 0.12^b	41.30 ± 0.13^a	33.35 ± 0.08^c	30.71 ± 0.08^d	33.35 ± 0.09^c	***
Apparent extract (°P)	2.56 ± 0.10^b	3.07 ± 0.11^a	2.31 ± 0.08^{bc}	2.05 ± 0.13^c	2.31 ± 0.07^{bc}	***
Original extract (°P)	13.80 ± 0.08^a	13.78 ± 0.06^a	13.82 ± 0.10^a	13.81 ± 0.08^a	13.80 ± 0.07^a	N.S.
Real attenuation (%)	66.20 ± 1.15^{ab}	63.20 ± 1.82^b	67.70 ± 1.35^a	69.20 ± 1.38^a	67.70 ± 1.25^a	**
pH	3.80 ± 0.10^a	3.81 ± 0.08^a	3.65 ± 0.12^a	3.49 ± 0.06^a	3.82 ± 0.15^a	N.S.

Values are expressed as average of three measurements.
Beer fermented by: *H. uvarum* YGA34 and after 72 h with US-05 strain (EP1); *L. thermotolerans* MNF105
and after 72 h US-05 strain (EP2); *C. oleophila* YS209 and after 72 h US-05 strain (EP3); *St. lactis-condensi*
MN412 and after 72 h with US-05 strain (EP4); US-05 strain as control (TC).
Abbreviations: S.S., statistical significance; N.S., not significant.
Data in the same line followed by the same letter are not significantly different according to Tukey's test.
Symbols: ***, $P < 0.001$; **, $P < 0.01$.

5.3.3. Volatile organic compound composition

As shown in Table 3, the experimental beers contained a large number of different aromatic organic

compounds. The composition of volatile organic compounds (VOCs) in the final beer was complex,

with six classes identified: alcohols, ketones, carboxylic acids, esters, terpenes and others. As a

encourage results, all novel strains applied for beer production produced a high variability of detected

compounds. The novel *L. thermotolerans* MNF105 (EP2) produced the highest concentration of

aromatic compounds, specifically with a greater amount of esters than the other trials.

887 **Table 3.** VOCs detected in beer samples. Compounds detected by SPME (all values in mg/L).

RT (min)	Compounds	Aroma Description	Odour Threshold	EP1 (OAV)	EP2 (OAV)	EP3 (OAV)	EP4 (OAV)	TC (OAV)	S.S.
	∑Alcohols			95.4 ± 2.7^b	89.8 ± 3.0^{bc}	85.2 ± 2.4^c	128.9 ± 3.3^a	66.7 ± 2.4^d	***
3.453	1-Pentanol	Alcoholic, iodoform-like	80^1	47.8 ± 1.6^{ab} (<1)	46.7 ± 1.2^b (<1)	45.0 ± 1.1^b (<1)	51.2 ± 0.8^a (<1)	32.8 ± 0.8^c (<1)	***
14.000	1-Octanol	coconut, walnut, oily	0.04^1	1.7 ± 0.1^a (42.5)	1.7 ± 0.1^a (42.5)	1.5 ± 0.2^{ab} (37.5)	1.2 ± 0.0^b (30.0)	1.6 ± 0.1^a (40.0)	**
15.000	Phenethyl alcohol	Rose, perfumy	125^1	42.2 ± 0.8^b (<1)	38.1 ± 1.6^{bc} (<1)	35.7 ± 0.9^c (<1)	71.8 ± 2.2^a (<1)	28.1 ± 1.1^d (<1)	***
20.649	2-Undecanol	fatty acids, coconut	0.5^2	3.7 ± 0.2^{bc} (7.4)	1.0 ± 0.0^d (2.0)	3.0 ± 0.2^c (6.0)	4.7 ± 0.3^a (9.4)	4.2 ± 0.4^{ab} (8.4)	***
20.799	4-Vinylguaiacol	spicy, cloves, phenolic	0.12^2	0.0 ± 0.0^b (<1)	2.3 ± 0.1^a (19.2)	0.0 ± 0.0^b (<1)	0.0 ± 0.0^b (<1)	0.0 ± 0.0^b (<1)	***
	∑Ketones			0.2 ± 0.0^{ab}	0.2 ± 0.0^{ab}	0.1 ± 0.0^b	0.3 ± 0.1^a	0.1 ± 0.0^b	**
17.150	3-Hydroxy-2-methyl-1-phenyl-1-propanone	unknown	unknown	0.2 ± 0.0^{ab} (n.d.)	0.2 ± 0.0^{ab} (n.d.)	0.1 ± 0.0^b (n.d.)	0.3 ± 0.1^a (n.d.)	0.1 ± 0.0^b (n.d.)	**
	∑Carboxylic acids			0.7 ± 0.1^a	0.0 ± 0.0^c	0.0 ± 0.0^c	0.0 ± 0.0^c	0.3 ± 0.0^b	***
2.354	Acetic acid	acid, acetic, pungent	71^2	0.7 ± 0.1^a (<1)	0.0 ± 0.0^c (<1)	0.0 ± 0.0^c (<1)	0.0 ± 0.0^c (<1)	0.0 ± 0.0^b (<1)	***
19.949	β-hydroxydodecanoic acid	unknown	unknown	0.0 ± 0.0^a (<1)	0.0 ± 0.0^a (<1)	0.0 ± 0.0^a (<1)	0.0 ± 0.0^a (<1)	0.3 ± 0.0^a (<1)	N.S.
	∑Esters			127.4 ± 5.0^c	237.3 ± 8.2^a	105.5 ± 2.6^d	91.2 ± 2.6^d	198.5 ± 5.7^b	***
1.904	Ethyl acetate	Fruity, sweet	30^1	1.0 ± 0.1^b (<1)	3.2 ± 0.2^a (<1)	0.7 ± 0.0^{bc} (<1)	0.5 ± 0.0^c (<1)	0.7 ± 0.1^{bc} (<1)	***
4.303	Isobutyl acetate	Banana, sweet, fruity	1.6^2	0.0 ± 0.0^a (<1)	0.5 ± 0.0^a (<1)	0.0 ± 0.0^a (<1)	0.0 ± 0.0^a (<1)	0.0 ± 0.0^a (<1)	N.S.
5.203	Ethyl butanoate	Papaya, apple, perfumed	0.40^1	0.6 ± 0.0^a (1.5)	0.5 ± 0.1^{ab} (1.2)	0.5 ± 0.0^{ab} (1.2)	0.4 ± 0.0^b (1.0)	0.4 ± 0.0^b (1.0)	N.S.
8.002	Isoamyl acetate	Fruity, banana, pear	0.50^2	2.0 ± 0.2^b (4.0)	27.0 ± 1.0^a (54.0)	2.0 ± 0.1^b (4.0)	0.0 ± 0.0^c (<1)	1.8 ± 0.1^b (3.6)	***
12.151	Ethyl hexanoate (caproate)	Apple, fruity, aniseed	0.17^1	4.2 ± 0.4^b (24.7)	5.6 ± 0.5^a (32.9)	1.9 ± 0.2^c (11.2)	5.5 ± 0.3^a (32.3)	1.8 ± 0.1^c (10.6)	***
12.551	Hexyl acetate	aromatic, perfumed	3.5^2	0.2 ± 0.0^a (<1)	1.2 ± 0.0^a (<1)	0.3 ± 0.0^a (<1)	0.4 ± 0.0^a (<1)	0.3 ± 0.0^a (<1)	N.S.
12.651	2-Methylbutyl isobutanoate	unknown	unknown	0.2 ± 0.0^a (n.d.)	0.0 ± 0.0^a (n.d.)	0.0 ± 0.0^a (n.d.)	0.0 ± 0.0^a (n.d.)	0.4 ± 0.0^a (n.d.)	N.S.
18.199	Ethyl octanoate	Apple, sweet, fruity	0.37^1	103.2 ± 3.4^c (278.9)	125.5 ± 2.5^b (339.1)	91.8 ± 1.8^d (248.1)	77.3 ± 2.1^e (208.9)	157.1 ± 3.2^a (424.2)	***
19.299	Isoamyl hexanoate (caproate)	Apple, fruity, aniseed	0.17^1	0.2 ± 0.0^a (1.2)	0.4 ± 0.0^a (2.3)	0.2 ± 0.0^a (1.2)	0.4 ± 0.0^a (2.3)	0.5 ± 0.0^a (2.9)	N.S.
19.499	2-Phenylethyl hexanoate (caproate)	unknown	unknown	0.9 ± 0.1^b (n.d.)	3.9 ± 0.2^a (n.d.)	0.8 ± 0.1^b (n.d.)	1.1 ± 0.0^b (n.d.)	0.5 ± 0.0^c (n.d.)	***
20.199	Propyl octanoate	unknown	unknown	0.0 ± 0.0^a (n.d.)	0.3 ± 0.0^a (n.d.)	0.0 ± 0.0^a (n.d.)	0.0 ± 0.0^a (n.d.)	0.0 ± 0.0^a (n.d.)	N.S.

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RT	Compound	Odour description	Odour threshold	EP1	EP2	EP3	EP4	TC	S.S.
20.449	Ethyl nonanoate	Fruity, fatty acids, sweet	1.20[2]	1.5 ± 0.2[a] (1.3)	1.1 ± 0.1[bc] (<1)	1.4 ± 0.1[ab] (1.2)	0.9 ± 0.0[c] (<1)	1.1 ± 0.0[bc] (<1)	***
21.698	Butyl octanoate (caprylate)	unknown	unknown	0.4 ± 0.0[a] (n.d.)	0.7 ± 0.0[a] (n.d.)	0.3 ± 0.0[a] (n.d.)	0.3 ± 0.0[a] (n.d.)	0.6 ± 0.0[a] (n.d.)	N.S.
22.698	Ethyl-trans-4-decenoate	Green, pineapple, pear	unknown	10.6 ± 0.6[c] (n.d.)	25.9 ± 1.1[b] (n.d.)	1.6 ± 0.1[d] (n.d.)	1.7 ± 0.1[d] (n.d.)	31.4 ± 2.1[a] (n.d.)	***
22.748	Ethyl decanoate (caprate)	Caprylic, fruity, apple	0.57[2]	0.8 ± 0.0[b] (1.4)	38.6 ± 2.3[a] (67.7)	2.7 ± 0.2[b] (4.7)	1.2 ± 0.1[b] (2.1)	0.0 ± 0.0[b] (<1)	***
23.998	Isoamyl caprylate	spicy, orange, pear	2[2]	1.1 ± 0.0[b] (<1)	1.5 ± 0.1[a] (<1)	0.9 ± 0.0[c] (<1)	1.0 ± 0.0[c] (<1)	1.3 ± 0.1[b] (<1)	***
27.197	Ethyl dodecanoate (laurate)	caprylic, soapy, estery	2[2]	0.5 ± 0.0[bc] (<1)	1.2 ± 0.1[a] (<1)	0.4 ± 0.0[c] (<1)	0.5 ± 0.0[bc] (<1)	0.6 ± 0.0[b] (<1)	***
34.645	Ethyl palmitate	Fatty acids, fruity, sweet	1.50[2]	0.0 ± 0.0[a] (<1)	0.2 ± 0.0[a] (<1)	0.0 ± 0.0[a] (<1)	0.0 ± 0.0[a] (<1)	0.0 ± 0.0[a] (<1)	N.S.
	∑Terpenes			37.5 ± 2.7[b]	34.1 ± 3.8[b]	26.9 ± 1.4[b]	33.9 ± 2.8[b]	162.2 ± 6.7[a]	
10.351	Camphene	unknown	unknown	0.0 ± 0.0[b] (n.d.)	0.0 ± 0.0[b] (n.d.)	0.0 ± 0.0[b] (n.d.)	0.0 ± 0.0[b] (n.d.)	2.1 ± 0.2[a] (n.d.)	***
11.301	β-Myrcene	herbs, resinous, spicy	0.20[2]	0.0 ± 0.0[b] (<1)	0.0 ± 0.0[b] (<1)	0.0 ± 0.0[b] (<1)	0.0 ± 0.0[b] (<1)	6.1 ± 0.3[a] (15.5)	***
11.801	β-Pinene	pine, mint, eucalyptus	unknown	0.0 ± 0.0[b] (n.d.)	0.0 ± 0.0[b] (n.d.)	0.0 ± 0.0[b] (n.d.)	0.0 ± 0.0[b] (n.d.)	8.7 ± 0.5[a] (n.d.)	***
12.851	o-Cymene	unknown	unknown	1.7 ± 0.1[b] (n.d.)	1.3 ± 0.1[bc] (n.d.)	1.0 ± 0.0[c] (n.d.)	1.6 ± 0.1[b] (n.d.)	5.6 ± 0.4[a] (n.d.)	***
12.951	Limonene	Citrus, fruity	0.1[1]	12.3 ± 1.2[a] (123.0)	14.0 ± 2.1[a] (140.0)	7.3 ± 0.5[b] (73.0)	5.2 ± 0.4[b] (52.0)	10.8 ± 0.31[a] (108.0)	***
14.850	p-Cymenene	unknown	unknown	3.1 ± 0.2[a] (n.d.)	2.5 ± 0.2[a] (n.d.)	2.4 ± 0.2[a] (n.d.)	2.7 ± 0.2[a] (n.d.)	8.3 ± 0.6[a] (n.d.)	***
15.200	Linalool	citrus, rosewood-like, aniseed	0.008[2]	20.4 ± 1.2[ab] (2550.0)	16.3 ± 1.4[b] (2037.5)	16.2 ± 0.7[b] (2025.0)	24.4 ± 2.1[a] (3050.0)	21.6 ± 1.6[a] (2700.0)	***
	∑Other			42.1 ± 2.1[b]	33.0 ± 1.8[c]	73.7 ± 2.4[a]	18.1 ± 0.9[d]	29.7 ± 1.7[c]	***
8.402	Styrene	balsamic	20[2]	42.1 ± 2.1[b] (2.1)	33.0 ± 1.8[c] (1.6)	73.7 ± 2.4[a] (3.7)	18.1 ± 0.9[d] (<1)	29.7 ± 1.7[c] (1.5)	***

888 Values are expressed as average of three measurements ± standard deviation.
889 Concentrations are calculated using limonene as the standard for the calibration line.
890 Compounds in each class are ordered according to their retention time.
891 Odour threshold as reported in literature.
892 Abbreviations: S.S., statistical significance; RT, retention time using a non-polar DB-5MS column; OAV, odour activity value; n.d., not determinable;
893 N.S., not significant.
894 Beer fermented by: *H. uvarum* YGA34 and after 72 h with US-05 strain (EP1); *L. thermotolerans* MNF105 and after 72 h US-05 strain (EP2); *C.*
895 *oleophila* YS209 and after 72 h US-05 strain (EP3); *St. lactis-condensi* MN412 and after 72 h with US-05 strain (EP4); US-05 strain as control
896 (TC). Data in the same line followed by the same letter are not significantly different according to Tukey's test. Symbols: ***, $P < 0.001$; **, $P <$
897 0.01; * $P < 0.05$.
898 [1]Maarse, H. (2017). Volatile compounds in foods and beverages. Routledge.
899 [2]Zunkel, M., Gastl, M., Schoenberger, C., Sedin, D., Becker, T. (2011). Beer flavor database. In ASBC 74th Annual Meeting. Ft. Myers.
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In the study, esters were the most abundant class, with a particularly high concentration of 237.2 mg/L in the EP2 trial, followed by TC inoculated with *S. cerevisiae* US-05 with 198.5 mg/L. The ester class consists of eighteen different compounds, seven of which have odour activity values greater than one. These include ethyl butanoate, isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl hexanoate, ethyl nonanoate and ethyl decanoate. In the EP2 trials, twelve compounds showed higher values, but only six of them were above the odour threshold, compared to the control trial TC which showed five. Furthermore, in the EP1 trial inoculated with *H. uvarum* YGA34 and in the EP3 trial inoculated with *C. oleophila* YS209, seven compounds with an odour activity greater than one were detected. These strains exhibited a greater ability to enhance the aromatic complexity and variability of the beers. In this study, the main ester compound identified was ethyl octanoate. The highest value of 157.1 mg/L was observed in the control trial (TC), followed by 125.5 mg/L in the EP2 trial inoculated with *L. thermotolerans* MNF105. The other most abundant esters were ethyl-trans-4-decenoate (31.4 mg/L in TC followed by 25.9 in EP2), ethyl decanoate (caprate) (38.1 mg/L in EP2 followed by 2.7 mg/L in EP3), and isoamyl acetate (27.0 in EP2 followed by 2.0 mg/L in EP1 and EP3). For alcohols, the results of the trials are comparable, with the EP4 trial (128.90 mg/L) inoculated with *St. lactis-condensi* MN412 showing a higher value compared to the other trials. In all trials, values of 1-Octanol and 2-Undecanol were detected with odour activity value greater than one, as described in Table 3. The EP2 trial identified more active compounds than the other trials. Notably, it detected 4-vinylguaiacol (2.30 mg/L), a compound that was absent in the other trials. Seven terpenoid compounds were detected, of which only three had an odour activity value greater than one: limonene, β-myrcene, and linalool. The trial inoculated with the commercial *S. cerevisiae* strain US-05 exhibited higher values of active compounds, specifically β-Myrcene (6.1 mg/L present only in TC), Limonene (14.0 mg/L in EP2 followed by 12.3 mg/L in EP1) and Linalool (24.4 in EP4 followed by 21.6 mg/L in EPC). Styrene exhibited an odour activity value greater than one. Overall, EP3 trial inoculated with *C. oleophila* YS209 exhibited the highest value with 73.7 mg/L.

5.3.4. Sensory evaluation

The results of the quantitative sensory analysis are reported in Fig. 4. The experimental beers exhibited significant differences, indicating that the non-*Saccharomyces* yeast strains used had a positive impact on the organoleptic quality of the beer.

Fig. 4. Sensory analysis performed on beers: spider plots of average scores for aroma (a), taste attributes and overall quality of bottled beers (b), determined by judges during tasting sessions. Beer fermented by: *H. uvarum* YGA34 and after 72 h with US-05 strain (EP1); *L. thermotolerans* MNF105 and after 72 h US-05 strain (EP2); *C. oleophila* YS209 and after 72 h US-05 strain (EP3); *St. lactis-condensi* MN412 and after 72 h with US-05 strain (EP4); US-05 strain as control (TC). Symbols: ***, P < 0.001; **, P < 0.01; * P < 0.05.
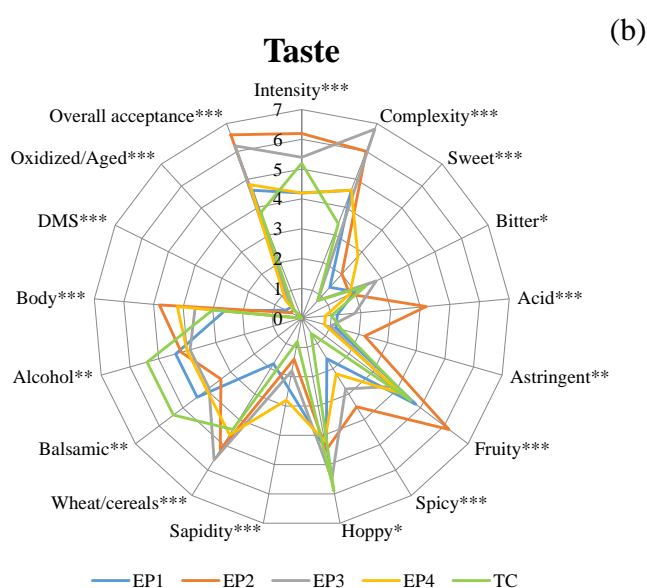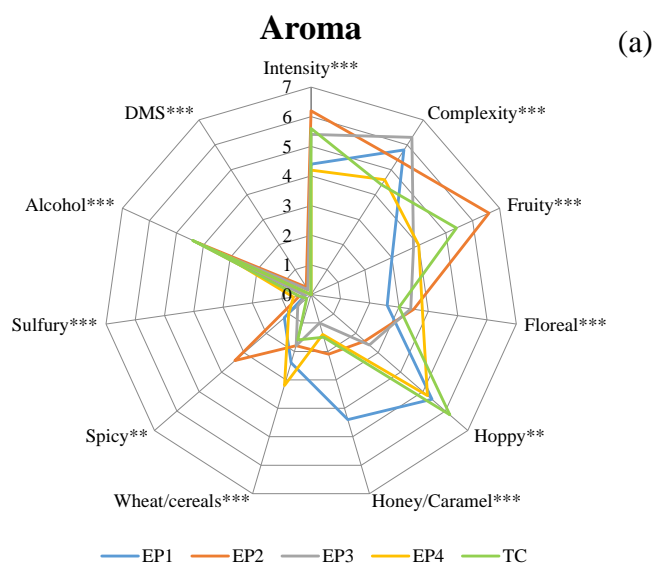
The panellists appreciated all trials, and similar results were found for some parameters. Specifically, all panellists identified the aroma and taste of the hops and malt in all beers and detected no defects. The EP2 and EP3 trials were particularly notable for their overall acceptability (6.6 and 6.2, respectively). The former received higher scores (aroma) for intensity and fruity notes (6.6 and 6.2, respectively), while the latter scored higher for complexity (6.3 points).

Instead, the trial with the highest overall acceptability scores was the EP2 trial, and specifically showed higher scores for 10 attributes (aroma: intensity, spicy and fruity; taste: intensity, acidity, astringency, fruity, spicy, body and overall acceptability). The second trial most appreciated by the panellists was the EP3 trial, which was more complex and specifically showed higher values for 5 attributes (aroma: complexity; taste: complexity, bitter, wheat/cereal and oxidised/aged).

The highest score for the aroma was the parameter "fruity" in EP2 (6.6 points), while the highest score for the flavour was the parameter "complexity" in EP3 (6.8 points). Moreover, trials EP1 and EP4 both demonstrated some relevant parameters. The EP1 trial is notable for its high complexity, while the EP4 trial successfully highlights the hop aroma.

The beers obtained from the different trial were easily distinguishable from the control trial. Indeed, the commercial strain of *S. cerevisiae* US-05 used in the TC trial demonstrated higher values for some parameters such as "fruity", but overall had low complexity and body.

5.3.5 Correlation between VOCs and sensory profiles

A principal component analysis (PCA) was performed to evaluate the correlation between VOCs and sensory attributes related to aroma. The findings, as illustrated in Figure 5, indicate that the F1 factor accounted for 67.74% of the total variance, while the F2 factor explained 37.89% of the total variance. The biplot graph demonstrates that there is diversity among the beers, with the exception of EP1 and EP3, which are similar.

Fig. 5. Principal component analysis (PCA) biplot for VOCs and aroma attributes. Beer fermented by: *H. uvarum* YGA34 and after 72 h with US-05 strain (EP1); *L. thermotolerans* MNF105 and after 72 h US-05 strain (EP2); *C. oleophila* YS209 and after 72 h US-05 strain (EP3); *St. lactis-condensi* MN412 and after 72 h with US-05 strain (EP4); US-05 strain as control (TC).

Biplot (axes F1 and F2: 67.74 %)
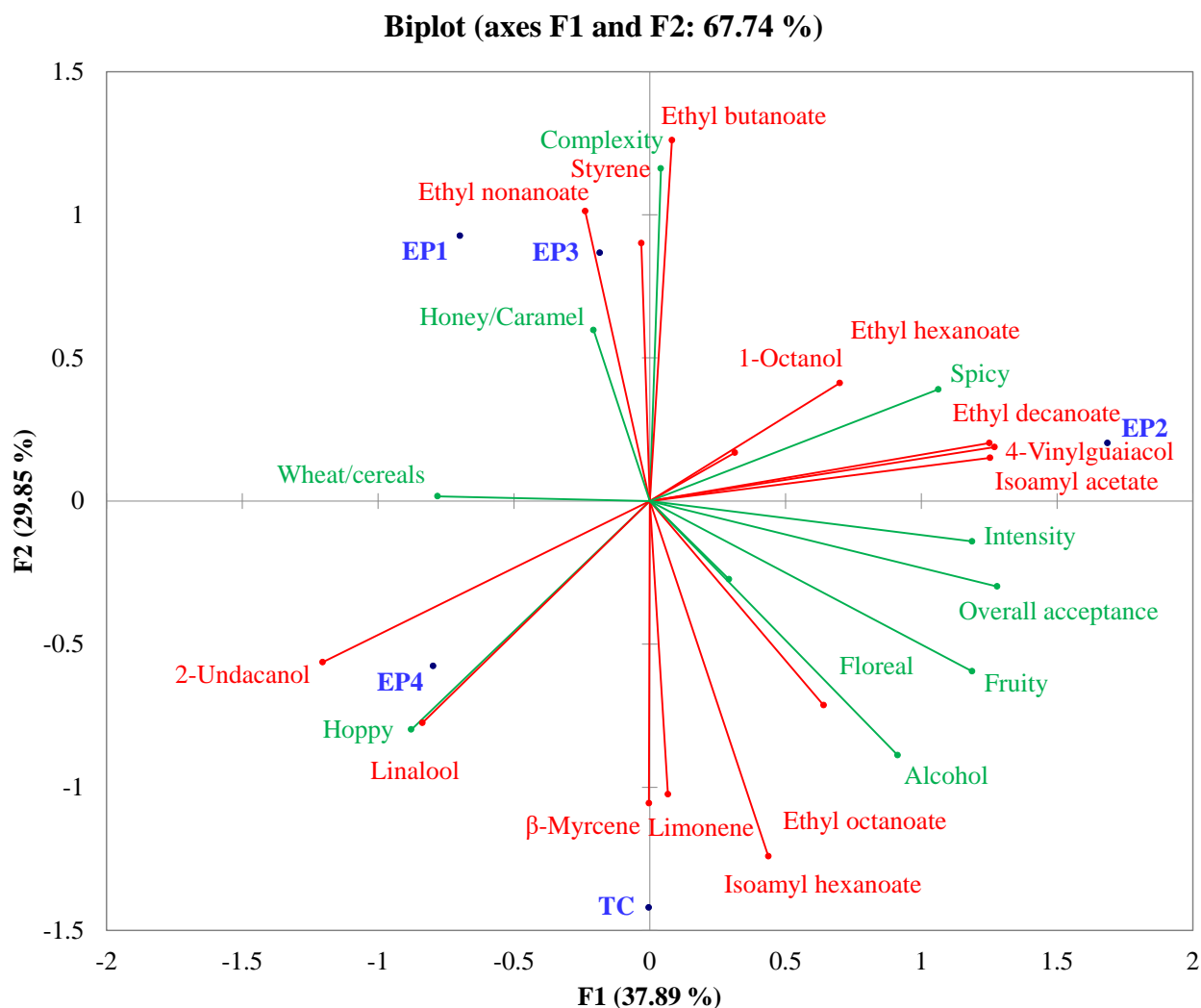
EP2 beer was found to be associated with several esters, including ethyl hexanoate, ethyl decanoate, and isoamyl acetate, as well as octanol, which produced fruity aromas (Verstrepen et al., 2003). This trial was also associated with the production of 4-vinylguaiacol, which gives rise to spicy notes (Vanbeneden, Gils, Delvaux, & Delvaux, 2008). The aroma of hops, which is more pronounced in the EP4 beer, is attributed to Linalool (Jiang et al., 2023). The same applies to the control trial (TC) associated with Limonene and β-myrcene, which are attributable to hops, as well as esters such as Ethyl octanoate and Isoamyl hexanoate, which are responsible for the fruity aroma (Verstrepen et al.,

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2003). Instead, the EP1 and EP3 beer was associated with three odour descriptors (Styrene, Honey/caramel and Ethyl nonanoate). Ethyl nonanoate and styrene are responsible for the Honey/caramel aroma, as they can produce sweet and balsamic aromas, respectively (Meilgaard, 1975).

5.4. Discussion

The results of microbiological analysis follow the general dynamics of yeast growth with sequential inoculation in fermenting wort (Bourbon-Melo et al., 2020; Matraxia et al., 2021). The persistence of the inoculated strains was investigated phenotypically by examining colony shape and cellular morphology to identify typical members of the *Hanseniaspora*, *Candida*, *Starmerella*, *Lachanchea* and *Saccharomyces* genera (Cadez, Pagnocca, Raspor, & Rosa, 2014; Jindamorakot et al., 2009; Chand-Goyal, Eckert, Droby, & Atkinson, 1998; Csoma, Kállai, Czentye, & Sipiczki, 2023; Fell, Statzell-Tallman, & Kurtzman, 2004; Kurtzman, Fell, Boekhout, & Robert, 2011). Immediately after inoculation, the cell density of all non-*Saccharomyces* increased to values above 7 Log CFU/mL, with slight differences between the trials. Previous studies have shown that these values can have an impact on the sensory quality of the final product (Du Plessis et al., 2017). After day 6, the decrease in non-*Saccharomyces* levels may be attributed to a reduction in available nutrients, such as sugars, and an increase in stress agents, such as ethanol (Domizio, House, Joseph, Bisson, & Bamforth, 2016; Matraxia et al., 2021; Francesca et al., 2024). Simultaneously, the reduction in non-*Saccharomyces* population is attributed to the interaction with *S. cerevisiae* (Tristezza et al., 2016). This may be due to competition for nutrients or the presence of metabolites produced by *S. cerevisiae* that inhibit the growth of non-*Saccharomyces* (Domizio et al., 2011; Wang, Mas, & Esteve-Zarzoso 2015). The fermentation dynamics of beer with sequential yeast inoculation were confirmed, consistent with previous observations by Pirrone et al. (2022), Matraxia et al. (2021), and Wu et al. (2024).

Regarding the physicochemical analysis, the presence of different non-*Saccharomyces* strains in trials EP1, EP2, EP3 and EP4 did not affect the attenuation capacity of the commercial *S. cerevisiae* strain

US-05. In fact, these trials showed similar values to those obtained in the control trial (TC). In particular, *H. uvarum* YGA 34 in trial EP1 demonstrated limited fermentation capabilities for the principal sugars in beer wort. This is consistent with the findings of Matraxia et al. (2021), which confirm that this species is unable to consume maltose. These results are also supported by Pirrone et al. (2022). The *St. lactis-condensi* yeast strain MN412 used in the EP4 trial has shown a strong ability to consume glucose and fructose during the initial fermentation period. It should be noted that this strain is not able to consume maltose. Other species within this genus, such as *Starmerella bombicola*, have also shown an inability to consume this sugar (García, Esteve-Zarzoso, Cabellos, & Arroyo, 2018).

In the EP3 trial inoculated with *C. oleophila* YS209 exhibited a similar trend, consuming glucose, fructose, and sucrose in the initial fermentations prior to inoculation with *S. cerevisiae*. Additionally, this strain was unable to consume maltose. Moreover, Francesca et al. (2024) demonstrated that this yeast strain has a preference for fructose over other sugars.

In contrast, among all the non-*Saccharomyces* yeast strains tested in the EP2 trial, only *L. thermotolerans* MNF105 showed a similar trend to the control strain by consuming maltose. Moreover, Domizio et al. (2016) confirmed that certain strains of *L. thermotolerans* and *S. cerevisiae* have comparable maltose utilization capabilities. In contrast, Callejo et al. (2019) found that *Lachancea* strains had a lower capacity for maltose fermentation compared to the *S. cerevisiae* strain. Furthermore, results obtained from the yeast strain *L. thermotolerans* MNF105 are consistent with those reported by Francesca et al. (2023). The sugar consumption kinetics of the *S. cerevisiae* US-05 in the trial TC in our study followed the general pattern of the species, with glucose and fructose being consumed before maltose (Pirrone et al., 2022; Tan et al., 2021).

In terms of the value of lactic acid, *L. thermotolerans* MNF105 in trial EP2 exhibited significantly higher levels of this compound respect other trials, due of the ability of this species to produce it, as demonstrated by various studies during fermentation of beer wort (Domizio et al., 2016; Francesca et

al., 2023). Furthermore, Zdaniewicz, Satora, Pater, and Bogacz, (2020) demonstrated that this species lacks the capacity for beer acidification, indicating that this characteristic is strain-dependent.

The acetic acid values at the end of fermentation are also comparable between the different trials, which is consistent with several studies on beer wort fermentation (Matraxia et al., 2021; Pirrone et al., 2022; Viana et al., 2021). The trials showed similar glycerol contents, with the EP4 trial inoculated with *St. lactis-condensi* MN412 exhibiting the highest value. The glycerol values of the different trials are consistent with those reported by Matraxia et al., (2021) and Viana et al. (2021), where they studied beer. Overall, the alcohol and final attenuation values obtained were higher than those reported by Cirlincione et al. (2023) who produced beers fermented with the commercial *S. cerevisiae* strain US-05. This difference may be due to a higher sugar content in the initial wort.

Instead, esters are mainly responsible for the sweet and fruity aroma in fermented beverages and their concentration is dependent on the enzymatic activity of the yeast strains (Pires et al., 2014). These compounds have a significant impact on the final flavour profile of the beer (Holt, Miks, de Carvalho, Foulquie-Moreno, & Thevelein, 2019). Ethyl octanoate is rich in fruity and sweet properties, especially apple-like flavour (Verstrepen et al., 2003). This compound has been detected in wheat beers produced with Maiorca wheat malt (Gugino et al., 2024). There are over 90 esters that can be produced in beer, but the most significant and commonly detected ones are ethyl octanoate (sour apple), isoamyl acetate (fruit, banana), ethyl acetate (fruit, sweet), and ethyl decanoate (fruit, apple) (Verstrepen et al., 2003; Viejo, Fuentes, Torrico, Godbole, & Dunshea 2019). These compounds are secondary metabolites of yeasts and contribute to define the aroma of beer. Due to the synergy of multiple compounds, even small changes in ester concentrations can have a significant impact on the aroma of beer (Thompson-Witrick et al., 2015).

Ethyl trans-4-decenoate was detected in beers produced with *S. cerevisiae* var. *diastaticus*, *S. cerevisiae* var. *bayanus* and *Brettanomyces claussenii* by Matukas et al. (2022). Moreover, Ocvirk, Mlinarič, and Košir (2018) found ethyl decanoate in wheat beer produced with a condenser placed on top of the fermenter to reduce losses, as well as in different beers produced from the three types

of fermentation (top, bottom, and spontaneous) (Viejo et al., 2019). Isoamyl acetate was observed in samples taken at different stages of the brewing process of lager beer and in traditional sorghum beer (Alves et al., 2020; Attchelouwa et al., 2020).

The class of alcohols is distinguished by alcoholic, floral or solvent aromas (Esslinger, 2009). In particular, Octanol has been detected in beers during a study conducted by Ferreira et al., (2022) on the impact of temperature during beer storage on its chemical profile. Instead, Undecanol has been detected by Praia, Herkenhoff, Broedel, Frohme, & Saad, (2022) in sour beers during a study that proposed to evaluate the probiotic strain *Lacticaseibacillus paracasei* subsp. *paracasei* F19 (F19) with the yeast *S. cerevisiae* US-05. Moreover, Larroque et al. (2021) detected 4-vinylguaiacol in beer brewed using synthetic wort and fermented with *Pichia anomala* BCMO15_2 and *S. cerevisiae* 00/30. Furthermore, 4-vinylguaiacol, was historically categorised as an off-flavour, but is now known to be an essential aroma in some white beers (Vanbeneden et al., 2008).

The terpenes detected are attributed to hops (Brendel, Hofmann & Granvogl, 2020; Dietz, Cook, Huismann, Wilson, & Ford, 2020; Ramírez & Viveros, 2021). Brendel et al. (2020) detected β-myrcene in alcohol-free beer made with various hop varieties. Additionally, Limonene, the second most abundant terpenoid in nature, is an important aroma compound associated with hops that gives beer a citrus or pine flavour (Ramírez and Viveros, 2021; Kemp et al., 2021). Sharp, Qian, Clawson, and Shellhammer (2016) identified limonene in beers hopped with Citra and Simcoe varieties. Instead, Van Holle et al. (2021) detected it in beers brewed with three industrially important hop varieties: Amarillo, Cascade, and Centellian, from different parts of the world. Specifically, one of the most significant components of the overall beer aroma is certainly linalool (Rutnik, Knez Hrnčič, & Jože Košir, 2022; Dietz et al., 2020). Linalool is a significant component of beer aroma and is one of the most desired hop oil compounds. It imparts a pleasant floral aroma. Rutnik et al. (2023) found that this component was more prevalent in beer brewed with Styrian Wolf hops than in beer brewed with other hop varieties.

Furthermore, Styrene is an aromatic compound commonly found in wheat beers, as indicated by several studies in the literature (Schwarz, Stübner, & Methner, 2012; Gugino et al., 2024). It is formed by the decarboxylation of cinnamic acid during boiling or through an enzymatic process during fermentation. Styrene can be found in small amounts in grains, as well as in coffee or dried fruits. This compound has been found in beers brewed with wheat Sicilian grains, such as Maiorca, in varying percentages, as demonstrated by Gugino et al., (2024).

According to many studies, the use of different non-*Saccharomyces* yeasts could improve the flavour profile of fermented beverages, particularly beer (Gutiérrez, Boekhout, Gojkovic, Katz, 2018; Larroque et al., 2021; Pirrone et al., 2022). The overall organoleptic study showed a preference for EP2 beer inoculated with *L. thermotolerans* yeast strain MNF105, which presented intensity and fruity notes. These results are consistent with those obtained by Francesca et al. (2023), who tested the MNF105 strain during fruit beer production and found that it enhanced the beer's aromatic characteristics. Instead, Postigo, Esteban, and Arroyo (2023) confirmed our results, showing that the yeast strain *L. thermotolerans* CLI 1232 has a balanced acidity with a fruity aroma profile. But overall, the other trials were also particularly appreciated by panellists. Moreover, several studies have investigated the yeast *H. uvarum*. Matraxia et al. (2021) showed that this yeast can increase the fruity aroma. Pirrone et al. (2022) also demonstrated that beers produced with sequential inoculation of *H. uvarum* YGA34 and *S. cerevisiae* MN113 or US-05 (T3 and T4) have a higher ester and lower alcohol concentration.

This is the first paper to report a study of *C. oleophila* and *St. lactis condensi* for brewing use as a co-starter. Furthermore, Francesca et al. (2024) demonstrated that the use of *St. lactis condensi* MN412 and *C. oleophila* YS209 had a positive impact on the final wines in terms of fruity and floral intensity, respectively, as confirmed by sensory analysis.

5.5. Conclusion

In this work, we investigated the effects of unconventional yeast strains as co-starter cultures, specifically *H. uvarum*, *L. thermotolerans*, *C. oleophila* and *St. lactis condensi*, on the physico-chemical and sensory properties of beer. The strains were obtained from matrices with high sugar content, such as manna and honey by-products. In particular, the selected strains *C. oleophila* YS209 and *St. lactis condensi* MN412 were applied and studied for the first time in brewing. After 72 hours of fermentation, the commercial yeast strain *S. cerevisiae* US-05 was inoculated. The various selected strains exhibited superior performance in several aspects compared to the fermentation carried out exclusively by the commercial strain *S. cerevisiae* US-05. Furthermore, experimental trials conducted with the selected strains showed the absence of off-odour and off-flavour and greater aromatic complexity. Moreover, *L. thermotolerans* MNF105 and *St. lactis-condensi* MN412 had consumed almost all of the fructose, glucose and sucrose prior to inoculation of the commercial strain. MNF105 consumed slightly more maltose than the other strains, but slightly less than the control strain.

These strains have the ability to enhance the aromatic complexity and variability of the beers. The beers produced are characterised by a higher ester concentration and lower alcohol content. The main ester compound identified in this study was ethyl octanoate. On other hand, the EP2 trial identified more active aromatic compounds than the other trials. The overall organoleptic investigation showed a preference for EP2 trial inoculated with *L. thermotolerans*, which showed spicy and fruity flavour, followed by EP3, inoculated with *C. oleophila* YS209 resulting with high complexity. This work enriches the limited scientific knowledge on the role of non-*Saccharomyces* yeasts as co-starters for beer production. Specifically, for the first time, the yeast species *C. oleophila* and *St. lactis condensi* were investigated for their potential role in beer production.

5.6. References

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CHAPTER 6

General conclusion

This PhD thesis presents a number of approaches to enhancing the overall quality of fermented beverages. The search for fermenting microorganisms that can improve the microbiological, physical, chemical, sensory and organoleptic properties of fermented alcoholic beverages such as wine, beer, mead and cider is considered to be a key point in the development of the industry today. A comprehensive analysis of the microbial ecology of high-sugar matrices enabled the assessment of the potential of different yeast strains.

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. Two yeast strains, *Hanseniaspora uvarum* YGA34 and *Saccharomyces cerevisiae* MN113, were tested as co-starters and starters for their beer production capacity. The beers produced with sequential inoculation of *H. uvarum* YGA34 and *S. cerevisiae* MN113 or US-05 were 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 starter during craft fruit beer production.

In a second study, fifteen strains of *Lachanchea thermotolerans* were isolated by sugary extracts from manna ash and evaluated for their ability to produce sour beers with a relatively reduced amount of lactic acid. After a deep characterization of strains for their brewing properties (ethanol tolerance, hop resistance, cross resistance, flocculation, hydrogen sulphide production, growth kinetics), the best strain was applied for beer production. The experimental beers brewed with *L. thermotolerans* MNF105 were perceived as the most "fruity", which was correlated with the high quantity of esters. This yeast, which is related to manna, has excellent technological properties and is a promising starter for beer production with the ability to light bio-acidify and modulate flavour.

In a third study, *L. thermotolerans* MNF105 and *S. cerevisiae* MN113 isolated from manna, were tested as starter cultures to process loquat beer to improve the sensory profile. Sour fruit beers produced with *L. thermotolerans* MNF105 were more balanced than the respective control, especially in terms of perceived acidity during sensory analysis. Moreover, these strains have been shown to be able to conduct beer fermentation, producing a good amount of alcohol, and also the ability to produce particular flavors that can modify and enhance the aromatic complexity of fruit beers.

A fourth study has investigated the impact of unconventional yeast strains in sequential inoculum with *S. cerevisiae* US-05, including *H. uvarum* YGA34, *L. thermotolerans* MNF105, *Candida oleophila* YS209 and *Starmerella lactis-condensi* MN412, as co-starter cultures on the physico-chemical and sensory properties of beer. For the first time, the strains *C. oleophila* YS209 and *St. lactis-condensi* MN412 were applied in brewing process. After 72 hours of fermentation, the commercial yeast strain *S. cerevisiae* US-05 was inoculated. The selected strains demonstrated

superior performance in several aspects, including the production of VOCs, when compared to the fermentation carried out exclusively by the commercial strain *S. cerevisiae* US-05. Furthermore, experimental trials conducted with the selected strains showed the absence of off-odour and off-flavour and greater aromatic complexity. Indeed, it has been demonstrated that beers brewed with distinct non-*Saccharomyces* yeast strains exhibit enhanced complexity, which can be attributed to the capacity of these strains to generate diverse perceptible aroma compounds. This PhD thesis demonstrated the potential of unconventional yeasts as a strategy for enhancing the quality of fermented beverages. Furthermore, it is evident that the use of innovative yeasts or fruits can lead to an improvement in the flavour and taste profiles of fermented beverages. The study of microbial ecology in territorial and traditional matrices can be an important source of potentially usable microorganisms that, in addition to fruit, can characterise and distinguish fermented beverages in a rapidly expanding market. Therefore, the utilisation of these novel species isolated from unconventional matrices by brewing companies rappresents a valuable opportunity to diversify their product range and produce novel products with distinctive flavours that are highly sought after by consumers. This approach not only fosters consumer's desire for novelty, but can also have a positive impact on the profitability of companies in the sector.

CHAPTER 7

List of publications, conferences and projects

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

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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 starter during craft fruit beer production.

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

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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.

Exploring the diversity of native *Lachancea thermotolerans* strains isolated by sugary extracts from manna ash to modulate the flavour of sour beers

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ABSTRACT

The craft beer industry is becoming increasingly interested in the production of innovative beers. A novel approach, designated as "primary souring," employs diverse yeast species, including *Lachancea thermotolerans*, to produce sour beers. Furthermore, there is a growing interest in utilising unconventional yeasts to produce beers with distinctive flavours. For the first time, yeast strains of *L. thermotolerans*, isolated from sugar extracts of manna ash, were evaluated for their ability to produce and improve the sensory properties of sour beers. In particular, five strains exhibited notable resistance to ethanol, sugar and hops, as well as comparable lactic acid production (ranging from 0.33 to 0.45 g/L). Experimental beers produced using MNF105 (T1) were perceived as the most "fruity". This is the first study to examine the impact of this novel indigenous strain, derived from

unconventional matrixes such as manna, on the organoleptic quality of craft sour beers. Consequently, elevated levels of ethyl decanoate (165.30 mg/L), ethyl hexanoate (29.4 mg/l), ethyl octanoate (29.7 mg/l) and ethyl acetate (36.1 mg/l) were found in T1 beer, exceeding the perception threshold. The ability of this strain to perform light bio-acidification is a valuable feature for the development of new brewing techniques, particularly for the creation of sour beers with balanced acidity and innovative flavours. The yeast *L. thermotolerans* MNF105, which is related to manna, has excellent technological properties and is a promising starter for beer production with the ability to light bio-acidify and modulate flavour.

Use of non-conventional yeasts (*Candida oleophila, Starmerella lactis-condensi, Hanseniaspora uvarum* and *Lachancea thermotolerans*) for enhancing the sensory quality of craft beer

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ABSTRACT

In recent years, craft beer production has grown significantly, sparking interest in using non-conventional yeasts to produce beers with distinctive flavors. This work investigated the impact of unconventional yeast strains, including *Hanseniaspora uvarum* YGA34 (EP1), *Lachanchea thermotolerans* MNF105 (EP2), *Candida oleophila* YS209 (EP3) and *Starmerella lactis-condensi* MN412 (EP4), as co-starter cultures alongside *Saccharomyces cerevisiae* US-05. The control trial was inoculated with *S. cerevisiae* US-05 (TC) alone. For the first time, *C. oleophila* and *St. lactis-condensi* have been applied for beer production and also result have been compared with *H. uvarum* and *L. thermotolerans*. These strains were previously selected from high-sugar matrices like manna

and fermented honey by-products, exhibited logarithmic growth cycles ranging from 5 to 8 during experimental fermentations. Interestingly, *St. lactis-condensi* MN412 and *L. thermotolerans* MNF105 strains efficiently consumed fructose, glucose, and sucrose in the beer must before the commercial strain *S. cerevisiae* US-05 was introduced. *Lachancea thermotolerans* MNF105 consumed more maltose than the other strains, albeit slightly less than the control strain. Among the beer samples, esters were the most prevalent compounds, ranging from 91.2 to 237.3 mg/L. Notably, the EP2 trial exhibited the highest ester content (237.3 mg/L). Specifically, ethyl octanoate was the dominant compound, identified at 125.5 mg/L in the EP2 trial. These unconventional yeast strains exhibited significant differences compared to beers brewed with *S. cerevisiae* alone. Additionally, their application led to an increase in volatile organic compounds. In conclusion, novel yeast strains isolated from high-sugar matrices showed excellent technological properties, making them promising co-starters and starter in innovative craft beer production.

Maiorca wheat malt: A comprehensive analysis of physicochemical properties, volatile compounds, and sensory evaluation in brewing process and final product quality

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ABSTRACT

This study explores the potential of Maiorca wheat malt as an alternative ingredient in beer production, investigating its impact on the brewing process and beer quality at different recipe contents (50 %, 75 %, 100 %). The study encompasses a comprehensive analysis of key malt parameters, revealing Maiorca malt's positive influence on maltose, glucose, filterability, extract, free amino nitrogen, and fermentability. Notably, the malt exhibited heightened levels of α-amylase and β-amylase enzymes compared to conventional commercial malt. Furthermore, the analysis of aroma compounds and subsequent sensory evaluations unveiled a significant correlation between the proportion of Maiorca malt in the formulation and intensified estery, fruity, malty, honey, complemented by a reduction in attributes such as aromatic compounds, phenolic, yeasty, sulfury, oxidized, and solvent-like odors. This research underscores the favorable contribution of Maiorca

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wheat malt to enhancing both the brewing process and final beer quality, highlighting its potential as an innovative ingredient in brewing practices.

Investigating optimal malting regimes for the Sicilian old landrace Perciasacchi durum wheat

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ABSTRACT

This study investigated the malting process of ancient Sicilian durum wheat variety Perciasacchi, focusing on steeping's impact on malt quality. Four malting trials were conducted, with different steeping times (9 and 13hours) and temperatures (15°C and 25°C). The 25°C steeping temperature enhanced the quality parameters of the malt. The malt steeped 25 hours at 25°C exhibiting notable enhancements in extract yield, free amino nitrogen content, enzyme activity, fermentability, sugar content, and reduced beta-glucans and wort viscosity. The malt obtained by steeping at 15°C exhibited inadequate modification, with low enzyme levels and prolonged saccharification times. Microbrewing trials with the malt (P4) produced beer with higher color, turbidity, foam retention, and a complex flavor profile compared to commercial malt beer. This innovative approach highlights the potential of ancient wheat varieties to create high-quality malts with distinctive sensory characteristics, suitable for producing unique beers.

Technological and Organoleptic Parameters of Craft Beer Fortified with Powder of the Culinary–Medicinal Mushroom *Pleurotus eryngii*

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ABSTRACT

Beer is one of the oldest and most popular alcoholic beverages and is currently consumed worldwide. The various components used in the brewing process have a physiological impact on the consumer and current research aims to improve its technological and functional properties through the addition of natural compounds (plants or mushrooms). In this work, the addition of two different amounts (5 and 10 g/L) of *Pleurotus eryngii* var. eryngii in powder form added at different production stages (PRE and POST alcoholic fermentation) showed the improvement in yeast viability during the alcoholic fermentation, increased the alcoholic content, and improved the sensorial profile. Regarding the organoleptic profile in the experimental samples, cocoa/chocolate and mushroom aromas were found and the samples PRE10 and POST5 received the best ratings with respect to all evaluated parameters.

Reduction of Pericarp Browning and Microbial Spoilage on Litchi Fruits in Modified Atmosphere Packaging

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ABSTRACT

The pericarp browning and postharvest microbiological decay of litchi fruit (*Litchi chinensis* Sonn cv Kwai Mai) significantly reduce its commercial potential in the fresh market. In this study, different combinations of modified atmosphere packaging (MAP) were applied at 5 ± 1 °C based on the use of natural gases that are innocuous to human health and an alternative to commercially adopted sulfur dioxide (SO_2) treatment. The results showed that control fruits, after 6 days of storage, begin to show the first symptoms of decay, revealed by the appearance of lesions and microbial infections determined by total mesophilic microorganisms and molds. This is not the case in the MAP-treated fruits and the MAP 3-treated (5% O_2 + 20% CO_2 + 75% N_2) fruits that show the best results. The control fruits, moreover, turned completely brown by the end of the storage period. The MAP 3 treatment was the most effective in preventing browning and the loss of the red pericarp color and vitamin content and in maintaining acceptable SSC/TA levels and flavor. In addition, a microbiological analysis revealed that all the MAP-treated litchi fruits did not harbor undesirable microorganisms during the entire cold storage period. In conclusion, the MAP 3 conditions delayed pericarp browning and maintained the better organoleptic quality of litchi fruits.

Effects of Tray-Drying on the Physicochemical, Microbiological, Proximate, and Sensory Properties of White- and Red-Fleshed Loquat (*Eriobotrya Japonica* Lindl.) Fruit

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ABSTRACT

Loquat fruits, highly valued by consumers for their characteristic aroma and pleasant taste, have a short post-harvest life and are susceptible to mechanical damage, loss of firmness, and initial organoleptic characteristics. The aim of this work was to develop a drying method suitable for storing loquat fruits in polyamide/polyethylene (PA/PE) bags containing two gaseous mixtures (treatments): MAPN2 (100% N2) and MAPP (21% O2 and 0.04% CO2), at room temperature (20 ± 1 °C) for at least 2 months. The effects of these conditions on the physico-chemical, microbiological, proximate, and sensory properties of fruit stored over a 50-day time interval were studied. The results showed that convective tray dehydration treatment at 70° for 12 h had good drying efficiency for loquat slices. In addition, the MAPN2 packaging limited the browning of the slices, keeping the microbial groups below the detection limits, with a clear positive effect on some minerals and vitamins, which were higher in concentration compared to the MAPP-packed samples. From an applicative point of view, the tray drying method for loquat fruits is useful on a small scale but could also be easily industrialized.

Effect of hot air-drying and modified atmosphere packaging on the quality and stability of blood orange slices

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ABSTRACT

The choice of time/temperature combination is critical for ensuring microbiological stability and retaining the characteristic taste of dried blood orange slices. This study found that hot air-drying at 70 ºC for 12 hours preserved shrinkage without altering the longitudinal diameter, though thickness was significantly reduced, especially in samples with passive modified atmosphere packaging (MAP). Increased hardness and masticability were noted due to water removal, with active MAP maintaining high hardness and colour integrity up to D100. Sensory analysis revealed differences in colour intensity and flavour between active and passive MAP-stored slices. HS-SPME chromatography identified key chemical compounds contributing to aroma and flavour, highlighting the complex interplay between temperature, storage conditions, and volatile organic compounds production. The study demonstrates that drying combined with MAP storage enhances organoleptic qualities and nutritional value, offering a method to produce a healthy, tasty, and visually appealing snack.

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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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.

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

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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 *St. lactis-condensi* MN412 was isolated from Sicilian manna, a sugar-rich matrix,

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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.

Protective effect of glutathione-rich specific inactivated yeast in pre-fermentative phase on organic Catarratto grape must

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ABSTRACT

The aim of this research was to explore the possible effects of the use of a glutathione-rich specific inactivated yeast (GIY) on pre-fermentative stages of winemaking process. The research evaluated the single and combined antioxidant effects of GIY, with or without *Metschnikowia pulcherrima*, at different stages of grape pressing process. The antioxidant effect was evaluated by measuring soluble oxygen and colour browning of musts. The impact of different oenological protocols on the aromatic profile of the final wines was assessed through the volatile organic compounds (VOC) and sensory analysis. The inoculation treatment of *M. pulcherrima* during the pre-pressing phase, followed by the addition of GIY during the post-pressing stage, limited both O_2 uptake (1.49 mg/L) and the browning phenomenon (0.093 OD at 420 nm) during the pre-fermentative phase. The protocol involving the addition of GIY during pressing showed the highest concentrations of certain compounds mainly

associated to fruity and floral aromas, such as ethyl octanoate (26.03 mg/L) and ethyl decanoate (26.87 mg/L). The sensory evaluation of the wines revealed the absence of off-odours and off-flavours in all treatments. However, the treatment involving the addition of *M. pulcherrima* and GIY received the greatest scores for the attributes smoothness and colour. In conclusion, the study found that GIY added treatments can be used in winemaking to enhance wine colour and counteract the detrimental effects of oxidation. These findings offer a potential alternative to reduce the use of SO_2 in wine production.

Oenological capabilities of yeasts isolated from high sugar matrices (manna and honey) as potential starters and co-starters for winemaking

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ABSTRACT

Non-*Saccharomyces* yeasts have recently garnered significant interest in oenology. When co-inoculated with *Saccharomyces cerevisiae*, they contribute to enhancing wine quality from a sensory perspective. In the present study, a group of yeasts previously isolated from manna and honey by-products were subjected to a genotypic identification. The D1/D2 variable domains of the sRNA gene and the ITS region of the 5.8S gene were sequenced. Additionally, a differentiation of strains was carried out by RAPD-PCR. All strains underwent in vitro screening. Subsequently, a micro-vinification experiment was conducted, focusing on strains with favourable technological characteristics: *Lachancea thermotolerans*, *Starmerella lactis-condensi*, and *Candida oleophila*).

These strains were sequentially inoculated alongside a control strain of *Saccharomyces cerevisiae*. Technological screening revealed that some strains exhibited limited H_2S production, ethanol tolerance (up to 8% v/v), resistance to potassium metabisulfite (200 mg/L), osmotic stress tolerance (up to 320 g/L of glucose), and copper resistance (on average 5 mM). The findings from this study can guide the selection of new starters and co-starters for regional wine production.

Technological affinity index for interaction between lactic acid bacteria and *Saccharomyces cerevisiae* strains to modulate the fruity and floreal aroma of Catarratto wines

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ABSTRACT

Microbial interactions during the fermentation process influence the sensory characteristics of wines. Alongside alcoholic fermentation, malolactic fermentation also plays a crucial role in determining the aromatic traits of wines. The time (t), rate (m) and volatile organic compounds (VOC) of malolactic fermentation are linked to the interaction between yeast and lactic acid bacteria. The study investigated the interactions between *Lactiplantibacillus plantarum* or *Oenococcus oeni* with *Saccharomyces cerevisiae* by using the Technological Affinity Index (TAIndex). The co-inoculation

of *L. plantarum*/*S. cerevisiae* resulted in a higher TAIndex than the co-inoculation of *O. oeni*/*S. cerevisiae* conditions. A low TAIndex led to increased aromaticity of the wines. The time and rate of malolactic fermentation have a strong impact on the synthesis of VOCs with a high olfactory impact. Therefore, knowledge of the TAIndex could play a decisive role in improving winemaking planning to produce wines with higher fruit and floral perceptions.

The impact of a *Saccharomyces cerevisiae* bio-protective strain during cold static clarification on Catarratto wine.

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ABSTRACT

The study aimed to evaluate the impact of early addition of a *Saccharomyces cerevisiae* HD A54 strain before pressing during winemaking. This approach aimed to reduce the dissolved oxygen in the grape must, thereby preserving the wine characteristics. Two different treatments were settled, Trial A, which was not added with sulphite or other substances during pressing; and Trial B, which was added with a *S. cerevisiae* strain at the pressing stage. Chemical parameters were determined through an enzymatic analyser which indicated a faster fructose consumption compared to glucose in Trial A. Plate counts were carried out to monitor microbial groups during vinification. Both treatments showed regular trends with respect to *Saccharomyces* population. Trial B exhibited oxygen consumption higher than control trial., especially in the early stages of winemaking. This was determined through dissolved O_2 analysis. Furthermore, trial B had lower absorbance values at post-pressing and pre-clarification stages. Both dissolved oxygen and absorbance analyses underscore the

positive impact of the *S. cerevisiae* HD A54 strain in protecting against oxidative processes in the musts at the pre-fermentative stage. The analysis of volatile organic compounds detected 30 different compounds, including alcohols and esters. Trial B had higher alcohol levels, particularly hydroxyethylbenzene (135.31 mg/L vs. 44.23 mg/L in Trial A). Trial A had almost four times higher ethyl acetate concentration than Trial B, which is an indicator of oxidation. Interestingly, trial B showed higher concentrations of 3-methyl-butyl acetate and 2-phenylethyl acetate, molecules correspond to fruity (banana) and floreal (rose) aromas, respectively. Regarding sensory analysis, Trial B received better scores for fruity and floral attributes, as well as overall wine quality.

Enhancing the quality and safety of Nocellara del Belice green table olives produced using the Castelvetrano method

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ABSTRACT

The Castelvetrano method is the most widely used among the various table olive processing styles in Sicily. After debittering, the product is stored at low temperatures to prevent the growth of undesirable microorganisms. In an effort to enhance the production process, yeast isolates underwent genotypic characterization and technological screening. The screening process identified two yeast strains *Candida norvegica* OC10 and *Candida boidinii* LC1, which can grow at low temperatures and tolerate high pH values (up to 10) and salinity [10% (w/v)]. During the monitoring period, the inoculated trials showed limited presence of spoilage/pathogenic microorganisms. Additionally, the yeasts limited oxidative phenomena and softening of the drupes. The organic compounds detected were higher in the inoculated trials than in the control, and cold storage induced aromatic decay, which was less pronounced in the trial inoculated with *C. norvegica*. Sensory analysis revealed that the inoculated trials scored higher in sweetness, hardness and crispness.

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

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ABSTRACT

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.

Co-inoculation approach combining lactic acid bacteria and yeasts to enhance the production of Nocellara del Belice green split table olives

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ABSTRACT

Table olives are a popular fermented food in the Mediterranean region. In southern Italy, split green table olives are traditionally fermented spontaneously. However, this method poses a risk of product spoilage due to undesirable microorganisms. To address this challenge, driven fermentation using selected starter strains offers a solution, ensuring a safer and more predictable production process. Three distinct experimental productions of Nocellara del Belice split table olives were conducted. In the control trial, a commercial strain of *Lactiplantibacillus pentosus* OM13 (referred to as OS3) was inoculated individually. In the OS1 and OS2 trials, *L. pentosus* was coinoculated with *Candida boidinii* LC1 and *Candida norvegica* OC10, previously selected for their bioprotective properties. During the 90-day fermentation process, critical parameters such as pH, salinity and microbial populations were monitored. The olives underwent a comprehensive analysis of colour, pulp texture, volatile organic compounds and sensory traits. During this fermentation, the dominant microorganisms were those deliberately inoculated, mainly lactobacilli and yeasts (> 6 Log CFU/mL). Notably, co-inoculated treatments showed a significant reduction in undesirable microbial populations. The pH values of the brine decreased below 4.5 in all treatments by day 75. Overall, this

strategic approach that involved fermentation and coinoculation ensured a microbiologically safe product and achieved a higher pulp hardness compared to the control trial. The flavour profiles obtained also varied based on the specific inoculum combination, as revealed by sensory analysis, which highlighted significant differences in texture, flavours and global appreciation, without any detectable odours or off-flavours

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- Abstract: Effetti della disidratazione in corrente di aria calda e dell'atmosfera modificata sulla qualità di frutti di mango – Tinebra I., Passafiume, R., Culmone, A., Roppolo, P., Ruggeri, A., Gaglio, R., **Pirrone, A**., Palazzolo, E., Farina, V. - 21-22 giugno 2023 - Torino. XIV Giornate Scientifiche SOI. In Acta Italus Hortus - Numero 28

- Abstract: Effetti della disidratazione convettiva e successiva conservazione in atmosfera modificata su frutti di arancia rossa di Sicilia - Roppolo, P., Passafiume, R., Tinebra, I., Guarino, V., Ruggeri, A., **Pirrone, A**., Gaglio, R., Palazzolo, E., Inglese, P., Farina, V. - 21-22 giugno 2023. Torino. XIV Giornate Scientifiche SOI - In Acta Italus Hortus numero 28

- Abstract and oral presentation: "Use of non-*Saccharomyces* yeasts isolated from new ecological niches (honey by-products and manna ash) for wine and beer production" **Pirrone, A**. - March 14th, 2022 – Palermo & Hybrid (on-line) - FORTHEM Food Science Lab Scientific Workshop

- Poster and abstract: Technological development of new tropical fruit products and beverages. Pirrone, A. 26th Workshop PhDFood2022- 19-21 settembre 2022 – UniASTISS in Asti, Italy

- Poster and abstract: Use of *Hanseniaspora uvarum* and a novel strain of *Saccharomyces cerevisiae* isolated from manna ash to produce loquat beer. **Pirrone, A**., Matraxia, M., Prestianni, R., Tinebra, I., Farina, V, Guzzon, R., Gaglio, R., Alfonzo, A., Naselli, V., Moschetti, G., Francesca, N. Microbial Diversity 2021- Virtual Event, 14-15 December, 2021

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Collaboration to drafting, management and reporting of the following research projects:

- Partnership for Reaserch and Innovative in the Mediterranean Area - Call 2022, Tematic Area 3-Food value chain: Topic 2.3.1-2022 (RIA) Enabling the transition to healthy and sustainable dietary behaviour, title of the project proposal: "Transnational Movement to Support the Sustainable Transition towards a Healthy and Eco-friendly Agri-Food System through the Promotion of MEDIET and its Lifestyle in Modern Society." CUP: B73C23000060001;

- LIVING LAB NEBRODI - Partnership for innovation pursuant to art. 65 of Legislative Decree 50/2016 - Executive design and implementation and management of the work programme related to the development of the Living Lab - Area Interna Nebrodi - Action 1.3.2 of the PO FESR SICILIA 2014-2020: AINEB 50 Intervention Nebrodi Internal

Strategy - Creation of open innovation environments: Living and Fab Lab, CUP: F47H20003430009;

Partnership: EURIS SRL; ITALIACAMP SRL; UNIVERSITY OF THE STUDIES OF PALERMO, DEPARTMENT OF AGRICULTURAL, FOOD AND FORESTRY SCIENCES

Scientific Responsible: Prof. Nicola Francesca

Amount of funding: 846,300.00

Duration: 20/01/23 - 31/12/23

- Agreement for research activities on behalf of third parties between HTS enologia di Luigi Scavone legal seat Marsala (TP) and Dipartimento Scienze Agrarie, Alimentari e Forestali Università di Palermo Cod. Fisc. 80023730825, P. IVA 00605880822. Research title and activities: Agreement for activities in research and development of biotechnologies in viticulture and oenology. Project Code CON-0405.

  Scientific Responsible: Dr. Nicola Francesca

  Partners: HTS oenology of Luigi Scavone University of Palermo-Dip. SAAF

  Activity period: September 2021 - September 2024

  Total funding: 60,000.00 euro.

- Agreement for research activities between HTS enologia di Luigi Scavone legal seat Marsala (TP) and Dipartimento Scienze Agrarie, Alimentari e Forestali Università di Palermo Cod. Fisc. 80023730825, P. IVA 00605880822. Research title and activities: Sustainable Biotechnologies for the Improvement of the Technological and Sensory Quality of Wines. Project Code CON-0404.

  Scientific Responsible: Dr. Nicola Francesca

  Partners: HTS oenology of Luigi Scavone University of Palermo-Dip. SAAF

  Activity period: September 2021 - September 2024

Total funding: 450,000.00 euro

- Agreement for research activities between Azienda agricola G. Milazzo-Terre della Baronia s.r.l. with registered office in Campobello di Licata (AG) and Dipartimento Scienze Agrarie, Alimentari e Forestali Università di Palermo Cod. Fisc. 80023730825, P. IVA 00605880822. Research title and activities: Research and development of biotechnologies in the field of viticulture and oenology - sparkling wines. Project Code CON-0405.

  Scientific Responsible: Dr. Nicola Francesca

  Partners: Azienda agricola G. Milazzo-Terre della Baronia s.r.l and University of Palermo-Dip. SAAF

  Activity period: September 2021 - September 2024

  Total funding: 144,000.00 euro.

- Agreement for research activities between Azienda Geolive Belice srl with registered office in Castelvetrano (TP) and Dipartimento Scienze Agrarie, Alimentari e Forestali Università di Palermo Cod. Fisc. 80023730825, P. IVA 00605880822. Research title and activities: Research and development of biotechnologies in mesa olive growing and agro-food sector. Project code CON-0407.

  Scientific Responsible: Dr. Nicola Francesca

  Partners: Azienda Geolive Belice srl and University of Palermo-Dip. SAAF

  Activity period: September 2021 - September 2024

  Total funding: 270,000.00 euro.

CHAPTER 8

Industrial scale application

The results of the experiments described in Chapter 5 have also been applied at an industrial scale level. In particular, two beers were produced using *Candida oleophila* YS209, isolated from manna, in sequential inoculation and in co-inoculation with *Saccharomyces cerevisiae* US-05. At the same time, Maiorca malt and cereals were used, following the results obtained in the Manuscript "Maiorca wheat malt: A comprehensive analysis of physicochemical properties, volatile compounds, and sensory evaluation in brewing process and final product quality". Specifically, 6000 bottles were produced in a first production and 10000 bottles in a second production at the commercial brewery Epica srl (Sinagra, Messina). This research was partly financed by the research project of the Sicilian Region for the support of inland areas (LIVING LAB NEBRODI - Partnership for Innovation according to art. 65 of Legislative Decree no. 50/2016 - Executive design and implementation and management of the work programme related to the development of the Living Lab - Area Interna Nebrodi - Action 1.3.2 of the PO FESR SICILIA 2014-2020: Intervention AINEB 50 Nebrodi internal strategy - Creation of open innovation environments: Living and Fab Lab, CUP code: F47H20003430009)

Fig. 1. Experimental beers produced