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USE OF NATIVE NON-*SACCHAROMYCES* STRAIN: A NEW STRATEGY IN D.O. "VINOS DE MADRID" (SPAIN) WINES ELABORATION

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ABSTRACT: *The use of native yeasts can be considered a good strategy for enhanced regional identity of wines, and the use of controlled multistarter fermentations to improve special and specific characteristics of wine may be an interesting approach. In this work, the application of native non-Saccharomyces and Saccharomyces strains from D.O. "Vinos de Madrid" in fermentations with Malvar grapes, an autochthonous white variety from Madrid, has been used to select new biotechnological processes which perform wine elaboration. Torulaspora delbrueckii CLI 918, Schizosaccharomyces pombe CLI 1085, Candida stellata CLI 920, Metschnikowia pulcherrima CLI 457, Lachancea thermotolerans 9-6C in pure cultures or mixed and in sequential combination with the Saccharomyces cerevisiae CLI 889 strain have been studied. In general, sequential inoculation has been highlighted by its contribution to higher complexity and quality in the wines produced. The results have special relevance because the implementation of these non-Saccharomyces in winemaking can be used to promote different oenological, aromatic and sensorial properties in wines from D.O. "Vinos de Madrid" according to the requirements of winemakers.*

KEYWORDS: *Native Yeast; Multistarter Fermentation; Malvar Wine; Aroma; Sensorial Analysis*

INTRODUCTION

Grape juice fermentation into wine involves a sequential succession of yeasts. Initially, species of non-*Saccharomyces* genera as *Hanseniaspora* (*Kloeckera*), *Rhodotorula*, *Issatchenkia*, *Debaryomyces*, *Zygosaccharomyces*, *Pichia*, *Torulaspora*, *Schizosaccharomyces*, *Candida*, *Metschnikowia* and *Cryptococcus* are found at low levels in fresh must (Kunkee and Bisson, 1991). Traditionally, the use of non-*Saccharomyces* in the wine elaboration has not been usual due to preceding investigations which showed that several species produce high levels of undesirable compounds that affect wine quality such as acetoin, ethyl acetate, acetic acid and acetaldehyde (Van Kerken, 1963; Cominiti *et al*., 2011). Unfortunately, this exclusion of non-*Saccharomyces* yeasts from the fermentation process may result in a loss of complexity and wines lacking in particular characteristics.

In recent years, a re-evaluation of the role of non-*Saccharomyces* yeasts in winemaking (Esteve-Zarzoso *et al*., 1998) has resulted in several studies that have looked at the use of controlled mixed fermentations as a biotechnological tool in order to improve wine complexity (Clemente-Jimenez *et al*., 2005; Sadoudi *et al*., 2012). The use of controlled fermentations of

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non-*Saccharomyces* yeasts together with *S. cerevisiae* can be suggested as a useful tool for wine production, which allows for the reproduction of microbiological and technical aspects that occur in spontaneous fermentation, as well as an increase in the wine aroma complexity owing to a more complex synthesis of aromatic compounds (Sun *et al*., 2014). This practice has also been reported as being able to increase some desirable metabolites, such as some acetate esters (Rojas *et al*., 2003) and glycerol (Contreras *et al*., 2014). Moreover, some non-*Saccharomyces* yeasts have been reported as being able to release more polysaccharides than *S. cerevisiae* strains (Giovani *et al*., 2012).

The application of non-*Saccharomyces* yeasts in wine elaboration has changed the standardized way to produce wine, improving the quality of the final product. On the other hand, the use of native yeasts is being favoured. These native *Saccharomyces* and non-*Saccharomyces* strains are isolated from different winegrowing regions with typical attributes that could be representative of a certain region (Tello *et al*., 2012) and are better adapted to specific environmental conditions and substrates (Esteve-Zarzoso *et al*., 2000).

The Denomination of Origin "Vinos de Madrid" (with a total extension of 8,390 ha) is located in the centre of Spain and is relatively new, created in 1990. Winemakers of this region base their production on the cultivation of the wine varieties Airén, Malvar, Garnacha and Tempranillo (*Vitis vinifera* L. cv.) to elaborate new styles of wine more competitive in the market (Gil *et al*., 2006).

The aim of this work was the oenological characterization of five non-*Saccharomyces* autochthonous yeast species under several co-culture conditions in combination with selected yeast of *S. cerevisiae* to improve the organoleptic properties of the regional Malvar wines.

MATERIALS AND METHODS

Yeast strains

The non-*Saccharomyces* strains used in this study were selected based on their biotechnological potential (Arroyo *et al*., 2010; Cordero-Bueso *et al*., 2013). *T. delbrueckii* CLI 918, *S. pombe* CLI 1085, *C. stellata* CLI 920, *M. pulcherrima* CLI 457 belong to the yeast collection of IMIDRA Institute and *L. thermotolerans* 9-6C was isolated from spontaneous fermentations of the autochthonous grape variety Malvar (Cordero-Bueso *et al*., 2011). *S. cerevisiae* CLI 889 strain was selected and characterized in our laboratories based on some established and desirable oenological criteria (Arroyo, 2000; Cordero-Bueso *et al*., 2016). All yeast strains used were previously isolated on the Madrid winegrowing region.

Cryogenically preserved strains (-80 °C) were cultured and maintained on YPD plates (1% yeast extract, 2% peptone, 2% glucose, and 20% agar (Conda Laboratories, Madrid, Spain), w/v) and stored at 4 °C. Lysine agar (0.25% L-Lysine monohydrochloride (Sigma-Aldrich, St. Louis, MO, USA), 1.17% yeast carbon base (Difco, Detroit, MI, USA) and 2% agar, w/v) was the medium used in screening as an initial rough discrimination between *Saccharomyces* and non-*Saccharomyces* strains (Fowell, 1965).

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Must preparation

Fresh Malvar grape (*Vitis vinifera* L. cv.) must (vintage 2010), clarified by pectolytic enzymes (Enozym Altair, Agrovin, Spain) (1 g/hL) at 4 °C, was stored at -20 °C until needed. The must was initially adjusted to establish values of pH 3.4, the concentration of sugars was 21.5 °B and total acidity (expressed as g L^{-1} of tartaric acid) was 4.28 g L^{-1}. Musts were supplemented with nitrogen by adding ammonium sulphate up to a level of 250 mgN L^{-1} to avoid slow, sluggish or stuck fermentations because of inadequate amounts of assimilable nitrogen (Bisson, 1999). Yeast assimilable nitrogen (YAN) was determined in must by the formol titration method (Gump *et al*., 2002).

At the time of use, the must was thawed and pasteurized to fluent vapour in an autoclave at 80 °C, 15 min, three times on three consecutive days to avoid caramelization of the sugars. The effectiveness of this treatment was verified by YPD plating.

Laboratory-scale fermentations

Triplicate experiments were carried out in sterile flasks with 1 L of pasteurized Malvar must with constant agitation (150 rpm) in an 18 °C temperature controlled room. The flasks were plugged with a Müller valve filled with 96 % H_2SO_4 (Panreac) to allow the release of CO_2 while preserving the sterile conditions (Vaughnan-Martini and Martini, 1999). Inoculum was performed by growing of yeasts in a YPD liquid medium at 28 °C for 48 h.

The trials were carried out with must of the Malvar variety in two different stages. First, the non-*Saccharomyces* strains, *T. delbrueckii*, *S. pombe* and *C. stellata* were tested against the control *S. cerevisiae* CLI 889 (p-Sc1). In a second step, *M. pulcherrima* and *L. thermotolerans* were assayed with the same strain as control (p-Sc2).

The trials were divided into pure, mixed and sequential cultures. Pure cultures were inoculated with 10^6 cells mL^{-1} of each yeast strain. Mixed fermentation trials were simultaneously inoculated with the same amount (10^6 cells mL^{-1}) of non-*Saccharomyces* cultures and *S. cerevisiae* strain. Sequential fermentation trials were inoculated with 10^6 cells mL^{-1} of the non-*Saccharomyces* cultures at first and the addition of the *S. cerevisiae* strain (10^6 cells mL^{-1}) took place when the wine contained about 5 % alcohol (v/v), estimated according to the weight loss as CO_2 released (g L^{-1}) (Jackson, 2008).

The fermentation progress was monitored by automatic weight using the software OPCEx3 (Resolvica, Inc.) every 24 h until a constant weight was reached. After fermentation, the wines were stored at 4 °C to encourage yeast settling before the analysis.

Microbial and analytical determinations of vinification

Samples were collected daily over the fermentation and diluted appropriately before plating. Non-*Saccharomyces* cells were counted using lysine agar. Total yeast cells were counted in YPD plates. The number of *S. cerevisiae* was given as the difference between the total plate count using YPD and the plate count using lysine agar.

Oenological parameters measured were: alcohol degree, pH, volatile acidity, total acidity, reducing sugars, glycerol, malic acid and lactic acid. They were measured by Fourier transform infrared spectroscopy in the laboratories of Liec Agroalimentaria S.L. (Manzanares, Spain), an

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accredited laboratory for physico-chemical analysis in wines to conform to UNE-EN ISO/IEC 17025:2005 rules.

Quantification of major volatile compounds was carried out in a GC Agilent 6850 with a FID detector equipped with a column DB-Wax (60 m x 0.32 mm x 0.5 µm film thickness) from J&W Scientific (Folsom, CA, USA). The volatile compounds were analysed according to Gil *et al*. (2006) and Balboa-Lagunero *et al*. (2013).

Sensory analysis

The final wines were tested by a trained panel of seven expert judges (four women and three men) from the IMIDRA Institute. This panel had been previously trained in the laboratory in the recognition of wine aromas. Wines from pure, mixed and sequential cultures were compared by triangle tests (ISO 4120:2007) to assess whether aroma differences existed between the different types of cultures. Sensory descriptive analysis was used to describe and quantify attributes of the wines on the basis of a scale from 1 (low intensity) to 10 (high intensity). The final score was obtained as the mean of the wine evaluations with their respective standard deviation (Arroyo *et al*., 2009; Balboa-Lagunero *et al*., 2013).

Statistical treatment

Analysis of variance (ANOVA) was applied on oenological parameters and volatile compounds in pure, mixed and sequential cultures. Tukey HSD post-hoc tests were applied to establish the significance of differences between means ($\alpha = 0.05$). In order to identify the most influential volatile compounds in the differentiation between the different types of cultures, the 32 studied volatile compounds were submitted to discriminant function analysis (DFA). The data were analysed with SPSS Statistics 21.0 Software for Windows (SPSS Inc., Chicago, IL).

RESULTS

In the following sections, the main results of the wine fermentations conducted using *S. cerevisiae* strain CLI 889 and each non-*Saccharomyces* yeasts mentioned above are summarised. To improve the results, one control of *S. cerevisiae* was performed for each lot of must used. In relation to volatile composition, the contribution of each compound to the entire aroma of wines can be estimated by its odour activity value (OAV). Even this value does not take into account the depressive or synergic odour interactions between the different molecules present in wines, OAV can be considered as a first approximation to the potential contribution of each compound to the overall aroma (Ferreira *et al*., 2002; Moyano *et al*., 2002). OAVs of 32 volatile compounds were calculated (see Supplementary Material).

Trials with *Torulaspora delbrueckii* CLI 918 and *S. cerevisiae* CLI 889

Fermentation kinetics of trials of the *T. delbrueckii* and *S. cerevisiae* strains are shown in Figure 1A. Mixed culture (m-Td/Sc) presented a similar fermentation rate in comparison with pure culture of *S. cerevisiae* (p-Sc1, considered as control). When *S. cerevisiae* inoculum (Sc in graphics) was added in sequential inoculation (s-Td/Sc), the CO_2 released was increased considerably. These co-cultures with *T. delbrueckii* and *S. cerevisiae* were characterized by an amount of residual sugar lower to 3 g L^{-1} (Table 1), which were therefore considered as dry

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wines. However, pure culture of *T. delbrueckii* (p-Td) did not consume the total quantity of sugars ending with 33.70 g L^{-1} and induced a slight increase of volatile acidity (0.60 g L^{-1}). This lower fermentative capacity of *T. delbrueckii* pure culture can also be observed in the smaller CO_2 released (Figure 1A). By contrast, the volatile acidity of co-cultures was not significantly different from the control; it was even lower in mixed culture (Table 1). Glycerol concentrations in wines fermented with pure *S. cerevisiae* culture and mixed culture of *T. delbrueckii*/*S. cerevisiae* did not differ significantly. On the contrary, pure *T. delbrueckii* and sequential fermentations produced more glycerol (7.14 and 5.28 g L^{-1}, respectively). Also, it should be noted the ethanol lowering in sequential culture of *T. delbrueckii*/*S. cerevisiae* (Table 1).

Fermentations with the presence of both yeasts species showed an increase of β-phenylethyl alcohol respect to the control, the higher production was notable in the sequential culture (Table 2). Pure culture of this non-*Saccharomyces* strain was clearly defined by its higher concentration of 1-hexanol and ethyl isobutyrate. However, ethyl isobutyrate was only detected on pure fermentation of *T. delbrueckii*. The sequential elaboration with *T. delbrueckii*/*S. cerevisiae* produced larger contents of 2-phenylethyl acetate and ethyl hexanoate (Table 2). Regarding acid composition of the final products, it is possible to observe a reduction on the compounds analyzed when *T. delbrueckii* was inoculated in a sequential way (Table 2).

The significant differences identified in the aroma composition of pure and sequential cultures of *T. delbrueckii* with respect to the control (p-Sc1), were also present in the sensorial analysis (Figure 2A). All tasters were able to distinguish the wines from pure and sequential cultures of *T. delbrueckii* from the control wine by triangle tests (data not shown). Thus, this result indicates the existence of sensory differences between these wines, higher than those observed in the analytical profile. All panellists expressed their preference for the sequential culture. They valued pure and sequential cultures of *T. delbrueckii* as the best ones due to their fruity and flowery aroma, higher aroma intensity and overall quality. The fruity and flowery aroma in these wines is related to a larger concentration in some volatile compounds such as β-phenylethyl alcohol, ethyl isovalerate, ethyl hexanoate, 2-phenylethyl acetate in sequential culture and ethyl isobutyrate in pure culture (Table 2); OAVs of these compounds were higher than 1 therefore they should be considered to have active contribution on the global aroma (see Supplementary Material). For mixed culture of *T. delbrueckii*/*S. cerevisiae*, tasters differentiated this type of inoculation regarding the control with a 5 % significance level, they highlighted the sensory similarity between control and mixed culture. This appreciation of tasters was corroborated by results included in Table 1 and Table 2, where few significant differences between them were observed.

Trials with *S. pombe* CLI 1085 and *S. cerevisiae* CLI 889

As expected, the strain *S. pombe* CLI 1085 presented lower fermentative capacity in pure culture (p-Sp) than the control of *S. cerevisiae* (Figure 1B), however this behaviour is also observed in sequential inoculation (s-Sp/Sc). It can be observed that mixed culture of *S. pombe*/*S. cerevisiae* (m-Sp/Sc) was the best combination to consume almost all sugars (Table 1). In pure and sequential cultures with *S. pombe*, it was reached just over 10 % of ethanol content. This strain of *S. pombe* can metabolize the malic acid, this capacity can be seen using pure and sequential cultures, by contrast, 0.95 g L^{-1} and 0.81 g L^{-1} of malic acid were observed in the control (p-Sc1) and in mixed culture, respectively. It should also be highlighted that the

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volatile acidity was lower in pure and sequential inoculations with this non-*Saccharomyce*s strain (0.33 and 0.38 g L^{-1} respectively), but the fermentation was not finished in both cases.

Significant differences in aroma composition were higher between pure and sequential cultures of *S. pombe* and the control (Table 2). The concentration of volatile compounds related with flowery descriptors as β-phenylethyl alcohol and 2-phenylehyl acetate was higher in cultures elaborated with *S. pombe* strain. In addition, undesirable acid compounds as hexanoic, octanoic and decanoic acids associated with butter and cheese aromas were also elevated in cultures inoculated with *S. pombe*. By contrast, mixed and sequential fermentations showed a reduction on the content of acetoine, also related to butter aroma. In general, the tasters noted that the cultures made with *S. pombe* CLI 1085 strain did not improve the organoleptic properties of Malvar wines (Figure 2B) in comparison with Malvar wines elaborated with an autochthonous *S. cerevisiae* strain. They showed a slight preference for sequential culture indicating their higher fruity aroma, however this fermentation showed a high sugars content (more than 40 g L^{-1}). On the other hand, the panellists were able to differentiate between all types of inoculation in this trial with a 0.1 % significance level by triangle tests.

Trials with *C. stellata* CLI 920 and *S. cerevisiae* CLI 889

Figure 1C shows the fermentation kinetic of *C. stellata* and *S. cerevisiae* cultures, low fermentative capacity of cultures elaborated with *C. stellata* strain can be observed in comparison with the control of *S. cerevisiae* (p-Sc). The addition of *S. cerevisiae* (day 9) produced an important increase in the fermentation rate in sequential fermentation, but still this culture finished with 25.21 g L^{-1} of reducing sugars (Table 1). *C. stellata* CLI 920 strain was the least ethanol tolerant yeast species in this study (Table 1), the pure culture of *C. stellata* (p-Cs) produced 3.40 % (v/v) of ethanol and this fermentation finished with 174.18 g L^{-1} of reducing sugars. In mixed (m-Cs/Sc) culture of *C. stellata*/*S. cerevisiae*, ethanol production was higher than the control (Table 1). This strain of non-*Saccharomyces* showed a high capacity to produce glycerol; the presence of this yeast species on fermentations produce a significant increase of glycerol on final wine, independently of the sugar left on the final product (Table 1).

Moreover, it is possible to observe a significant difference ($p < 0.05$) of volatile composition in mixed and sequential cultures of *C. stellata*/*S. cerevisiae* to the rest, they are characterized by increased esters concentration such as ethyl hexanoate, ethyl butyrate, ethyl octanoate and isoamyl acetate (Table 2). Medium-chain fatty acids such as hexanoic, octanoic and decanoic acids were higher in mixed and sequential cultures with *C. stellata*.

It was also easy for the tasters differentiate pure and co-cultures elaborated with *C. stellata* CLI 920 strain. In spite of the high sugar content left on final product, the pure inoculation of *C. stellata* was the most appreciated by tasters (Figure 2C) for its pleasant sweet flavour and a high concentration in pleasant volatile compounds such as β-phenylethyl alcohol (roses), isoamyl acetate and ethyl hexanoate that are important contributors to the pleasant fruity note of wine (Gil *et al*., 1996).

Trials with *M. pulcherrima* CLI 457 and *S. cerevisiae* CLI 889

As it was showed by *C. stellata* fermentation, the ability to conduct the fermentation of this yeast strain is low, as shown the four different curves (figure 1D). In these trials, *M.*

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pulcherrima CLI 457 strain, in pure and sequential cultures produced wines with low volatile acidity, 0.27 g L^{-1} and 0.50 g L^{-1} respectively, values less than the control p-Sc2 (0.63 g L^{-1}) (Table 1). Besides, the pure use of this *M. pulcherrima* strain was characterized by a higher level of reducing sugars (152.93 g L^{-1}) coinciding with a higher reduction of wine total acidity and a marked increase in pH value. The results have shown a significant decrease of volatile acidity and an increase of glycerol, most clearly evident in pure and sequential cultures of *M. pulcherrima* CLI 457 strain. This strain of *M. pulcherrima* and *C. stellata* CLI 920 are the largest glycerol producers compared to the other strains studied. A rise of acid lactic concentration in pure and co-cultures with this non-*Saccharomyces* strain (Table 1) can also be seen. However, as happened for *C. stellata*, the fermentation was not finished in any case.

M. pulcherrima in mixed culture and the control of *S. cerevisiae* have not presented significant sensorial differences by the tasters, the significance level was higher than 5% through triangle tests. In case of the use of *M. pulcherrima* in Malvar must fermentations, there was no clear preference on sensorial analysis, three of the seven panellists preferred the control, two of them chose the sequential culture and other two chose the mixed culture (Figure 2D). However, the tasters noted in these co-cultures ripe fruit, banana, sweet aromas associated with ethyl isovalerate and isoamyl acetate compounds and a slightly anise-like aroma probably due to ethyl hexanoate (Guth, 1997; Ferreira *et al*., 2000) (Table 2). The tasters also noted the sweet character of the wine elaborated in pure culture of *M. pulcherrima* due to the high sugar concentration of the samples analyzed.

Trials with *L. thermotolerans* 9-6C and *S. cerevisiae* CLI 889

Previous pure cultures of non-*Saccharomyces* strains showed the lowest values of CO_2 released in comparison with other trials, however *L. thermotolerans* pure culture (p-Lt) presented a fermentation kinetics over the sequential culture of *L. thermotolerans*/*S. cerevisiae* (s-Lt/Sc) (Figure 1E). In sequential cultures with other non-*Saccharomyces* tested, it was noted when the *S. cerevisiae* inoculum was added (Sc in graphics) the CO_2 released was increased considerably, however in the sequential culture with *L. thermotolerans* and *S. cerevisiae* this increase in CO_2 released after adding *S. cerevisiae* (day 5) is not so obvious (Figure 1E). In these fermentations, it is possible to observe a two different patterns of CO_2 releasing, one including *S. cerevisiae* pure culture and mixed fermentation, and the other fermentation pattern that includes *L. thermotolerans* pure culture and the sequential fermentation (Figure 1E), as in the case of *S. pombe* fermentations. Biomass evolution in sequential culture of *L. thermotolerans* had also not presented an increase in cells per millilitre values after *S. cerevisiae* inoculation (data not shown).

In Table 1, the ethanol production is seen to be similar in the mixed culture of *L. thermotolerans*/*S. cerevisiae* (m-Lt/Sc) and the control (p-Sc2), while wines from the fermentations of pure (p-Lt) and sequential (s-Lt/Sc) cultures of *L. thermotolerans* lower levels of ethanol were seen (7.19% and 7.29% (v/v), respectively), showing both fermentations a sugar content about 70 g L^{-1}. Besides, a significant increase in total acidity and lactic acid in pure and sequential cultures of *L. thermotolerans* can be observed, which coincided with a consequent reduction of pH and glycerol content. The values of volatile acidity were higher in pure (0.78 g L^{-1}) and sequential (0.77 g L^{-1}) with this non-*Saccharomyces* respect the control (p-Sc2).

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Pure and sequential cultures of *L. thermotolerans* presented significant differences ($p < 0.05$) in its aromatic composition with respect to the control (Table 2). These cultures were noted for their relation with higher alcohols, isobutanol and isoamyl alcohol and β-phenylethyl alcohol that produces a rose aroma; also, esters as ethyl isovalerate and ethyl lactate were found in their aromatic composition (Table 2). Ethyl lactate was the most abundant ester with values of 27.35 and 58.59 mg L^{-1} (pure and sequential cultures of *L. thermotolerans*, respectively), though their OAV values are lower than 1 (see Supplementary Material).

L. thermotolerans 9-6C strain in pure and sequential cultures had similar organoleptic characteristics according to taster conclusions, the significance level was higher than 5% through triangle tests. Besides, the preference of cultures was divided between mixed and sequential cultures of *L. thermotolerans*/*S. cerevisiae*. They highlighted a high acidity flavour, higher aroma intensity and ripe fruit (pear) and floral aroma in pure and sequential cultures with *L. thermotolerans*; in mixed culture, the wine was valued by its freshness, citric aroma and full-bodied due to its lower acidity character (Figure 2E).

Discriminant Function Analysis (DFA)

When stepwise discriminant function analysis was applied to the 32 aromatic compounds analysed for each trial, six discriminant functions were obtained; the first two accounted for 55.9% and 26.4% of the total variance, respectively, so the total variance explained by these two functions was 82.3% (Figure 3). The two discriminant functions allowed us to correctly classify 100% of studied samples. Also, 100% of samples were correctly classified in cross-validation where each case is classified by functions obtained from the rest of the cases. The variables that contributed most to discriminant model were hexanoic acid and isoamyl acetate which negatively correlated with discriminant function 1 and positively correlated with the same function were (Z)-3-hexen-1-ol, isoamyl alcohol, octanoic acid, ethyl isobutyrate and metionol (Table 3). In relation with discriminant function 2, hexanoic acid and ethyl isovalerate were most negatively correlated with this function and positively correlated were octanoic acid, 1-hexanol, β-phenylethyl alcohol, ethyl lactate and 2-phenylethyl acetate (Table 3). Figure 3 shows the distribution in the discriminant space of the different type of cultures with *S. cerevisiae* and non-*Saccharomyces* strains classified by trial. It is necessary to emphasize the clear classification respect on the aromatic composition of wines obtained depending on the non-*Saccharomyces* employed in the fermentation process. *T. delbrueckii* and *C. stellata* trials showed the highest differences in its aromatic composition with respect to their control (Sc2) (Figure 3).

DISCUSSION

Over the last few years, there has been an increase in the use of native yeasts for the control of the fermentation process. Native strains are well known to have adapted to all conditions associated with a wine production area, which allow them to have good fermentation abilities (Tello *et al*., 2012; Tristezza *et al*., 2012; García *et al*., 2016). Moreover, the use of these autochthonous yeasts contributes to obtain wines with specific local characteristics and styles, avoiding the apparent problem of deficit of sensorial diversity among industrial wines (Ugliano, 2009). Also, there has been increasing interest in mimicking the indigenous

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fermentation, but in a controlled way, thus obtaining wines with higher flavour and aroma complexity.

It is widely known that fermentation kinetics could be influenced by non-*Saccharomyces* wine yeasts in fermentations with starter cultures of *S. cerevisiae* (Heard and Fleet, 1988; Ciani *et al*., 2006). In this work, the impact on fermentation kinetics by multistarter cultures has been shown. The expected presence of these yeasts strains during the early stages of fermentation was enough to produce a real impact on the final wines. This presence of non-*Saccharomyces* strains could have been encouraged by the temperature of fermentation since it was reported that the tolerance of non-*Saccharomyces* to ethanol is favoured at 10-20 °C (Fleet and Heard, 1989; Erten, 2002). Furthermore, in a previous study about different fermentation stresses (osmotic pressure, pH and ethanol), the non-*Saccharomyces* and *S. cerevisiae* strains studied showed a good tolerance to those stresses inherent to fermentation (García *et al*., 2016).

In agreement with previous publications, the use of *T. delbrueckii* has been linked to reduced amounts to volatile acidity compared to the control of *S. cerevisiae* strains (Ciani and Maccarelli, 1998; Renault *et al*., 2009). In this work, the ethanol lowering in sequential culture of *T. delbrueckii*/*S. cerevisiae* could be related to Crabtree-positive metabolism of *T. delbrueckii* species described by several authors (Alves-Araújo *et al*., 2007; Bely *et al*., 2008). And contrary to previous findings (Bely *et al*., 2008; Loira *et al*., 2014), this strain of *T. delbrueckii* increased the glycerol content in pure and sequential elaborations. Also, it should be noted that this *T. delbrueckii* CLI 918 strain has been described earlier as a strain with potential interest for its contribution to the aromatic wine profile adding flowery and fruity notes and its use was considered interesting in mixed starter cultures with *S. cerevisiae* (Cordero-Bueso *et al*., 2013).

It is well known that *S. pombe* can metabolise the malic acid (Snow, 1978; Benito *et al*., 2014). The strain *S. pombe* CLI 1085 was able to metabolise malic acid in pure and sequential cultures and the reduction of volatile acidity in the same cultures differs from other investigations where *S. pombe* strains produced a high volatile acidity and could be associated with aroma defects (Tristezza *et al*., 2010; Benito *et al*., 2012).

The use of *C. stellata* as a starter culture to increase glycerol level in wines was suggested by Maurizio Ciani and Picciotti (1995). This yeast specie is known as a high glycerol producer achieving values up to 14 g L^{-1} (Ciani and Ferraro, 1998; Ciani and Maccarelli, 1998). In contrast, *S. cerevisiae* produced between 4 and 10.4 g L^{-1} of glycerol (Radler and Schütz, 1981; Prior *et al*., 2000). Glycerol contributes to the mouthfeel and complexity of wine flavour at lower levels (Prior *et al*., 2000) and concentrations over 5.2 g L^{-1} can produce a sweet taste (Noble, 1984). The mixed culture of *C. stellata* CLI 920 and *S. cerevisiae* CLI 889 presented higher ethanol content than the control and these results are different from the previously published on co-cultures of *C. stellata* employed to reduce the final ethanol content (Ferraro *et al*., 2000; Canonico *et al*., 2016). Mixed and sequential cultures of *C. stellata* CLI 920 and *S. cerevisiae* CLI 889 were characterized by increased esters concentration. Some researchers have indicated that medium-chain fatty acids as hexanoic, octanoic and decanoic acids at concentration of 4 to 10 mg L^{-1} impart mild and pleasant aroma to wine; however, at levels beyond 20 mg L^{-1}, their impact on wine becomes negative (Shinohara, 1985; Jiang and Zhang, 2010). In the present work, *C. stellata* mixed and sequential cultures produced high concentrations of hexanoic, octanoic and decanoic acids that might have a positive impact on the aroma of these wines since their levels were far below 20 mg L^{-1}.

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Some authors have reported that *M. pulcherrima* in monoculture is a low producer of volatile acidity (Cominiti *et al*., 2011; Sadoudi *et al*., 2012). This behaviour was maintained by *M. pulcherrima* CLI 457 strain in pure and sequential culture. In a previous study, Jolly *et al*. (2003) did not detect any relevant changes in chemical composition and fermentative kinetics between *M. pulcherrima* in association with *S. cerevisiae* cultures compared to pure cultures of *S. cerevisiae*; however, *M. pulcherrima* CLI 457 showed a significant decrease of volatile acidity and an increase of glycerol and lactic acid, most clearly evident in pure and sequential cultures with this non-*Saccharomyces*. Also, one *M. pulcherrima* strain commercially available for winemaking has been studied due to its influence on the wine flavour profile (González-Royo *et al*., 2015).

The ability of *L. thermotolerans* to act as an acidifying agent (lactic acid producer) is being considered as a tool to acidify low-acid musts (Kapsopoulou *et al*., 2007; Benito *et al*., 2015). In terms of fermentation kinetics in sequential culture with *L. thermotolerans* 9-6C and *S. cerevisiae* CLI 889, the fermentation was not dominated by *S. cerevisiae*; this behaviour could be owed to the enhanced competition of this non-*Saccharomyces* yeast in the delayed inoculation of *S. cerevisiae* (Kunkee and Bisson, 1993; Mendoza *et al*., 2007), which will probably be produced by competition for nitrogen, as has been previously noticed (Ciani *et al*., 2006) or probably due to an excessive acidity into the wine produced by *L. thermotolerans* previous to *S. cerevisiae* inoculation. Some studies (Gobbi *et al*., 2013; Benito *et al*., 2015) showed that the volatile acidity production in pure and co-cultures with *L. thermotolerans* was significantly reduced while the amount of glycerol can be increased in the same cultures. In our results, the behaviour of *L. thermotolerans* 9-6C strain was completely different in pure and sequential cultures, glycerol decreased in these two cultures and the volatile acidity was increased although these values are high, do not exceed 1 g L^{-1} (Erasmus *et al*., 2004) that could have a negative impact on organoleptic quality of wine. By contrast, our results were coincident with what other authors had also reported on *L. thermotolerans* capacity of β-phenylethyl alcohol production in fermentations with *S. cerevisiae* (Kapsopoulou *et al*., 2007; Gobbi *et al*., 2013). Even though ethyl lactate was the most abundant ester in pure and sequential cultures of *L. thermotolerans*, the real contribution of ethyl lactate and diethyl succinate to the D.O. "Vinos de Madrid" white wines had been previously considered as insignificant (Gil *et al*., 2006; Santos *et al*., 2004).

The influence of non-*Saccharomyces* yeasts on the volatile profile of wines has been widely researched. The significant increment of β-phenylethyl alcohol in the co-cultures of *T. delbrueckii*, *C. stellata* and *L. thermotolerans* seems to be related to the metabolic activity of these non-*Saccharomyces*. This volatile compound contributes with a pleasant floral aroma (rose) in the wine (Swiegers *et al*., 2005) and also is produced by others non-*Saccharomyces* in mixed culture (Andorrà *et al*., 2010). It is worth noting that there have been produced a large amount of higher alcohols in pure and sequential cultures of *T. delbrueckii*, *C. stellata* and *L. thermotolerans* in comparison with their respective controls (Table 3). These compounds can have a positive or negative impact on the flavour and aroma of a wine depending on its final concentration (Beltran *et al*., 2005). Higher alcohols concentrations below 300 g L^{-1} add a desirable level of complexity to wine, while concentrations above 400 mg L^{-1} can have a detrimental effect (Rapp and Versini, 1995).

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CONCLUSION

In summary, the non-*Saccharomyces* strains studied can be used to elaborate wines with different organoleptic requirements. *T. delbrueckii* CLI 918 could be used to produce wines with lower ethanol content and higher fruity and floral aroma. *S. pombe* CLI 1085, though not provide special sensorial characteristics to Malvar wines, could be employed to consume malic acid aimed at achieving the microbiological stabilization and to produce wines with low volatile acidity. *C. stellata* CLI 920 was distinguished for its higher aroma complexity with high concentration in desirable aroma compounds and higher glycerol content, pure culture with this strain could be considered for sweet wine elaboration. *M. pulcherrima* CLI 457 contributed to lower volatile acidity and increase glycerol content, also produces ripe fruit aroma. *L. thermotolerans* 9-6C can be useful for increasing the acidity of wines produced from low acidity musts due to its ability to produce lactic acid.

There is special mention in the results obtained about the best moment to inoculate *S. cerevisiae*. Depending on the strain of non-*Saccharomyces* used, we can conclude that is better to inoculate on sequential way or mixed at the beginning of fermentation. It is possible to observe two different types of fermentation kinetics regarding mixed and *S. cerevisiae* pure fermentations. For mixed fermentation, it seems that there are not significant differences on both CO_2 releasing curves for the species *T. delbrueckii*, *S. pombe* and *L. thermotolerans*, while *M. pulcherrima* exhibited a slight difference, but for *C. stellata* we can observe the highest difference. This fact could be explained because the interaction between both yeast species, there are some cases in which the presence of the non-*Saccharomyces* yeast strains affects the fermentation behaviour of *Saccharomyces*; however, for other yeasts species this interaction does not affect significantly. This interaction has been described (Wang *et al*., 2015; Wang *et al*., 2016) as the result of different consequences, as the production of some metabolites and the change in the concentration of certain metabolites on the media. However, the different behaviour can be explained also, because not all the non-*Saccharomyces* yeast species and all the different strains of the same species showed the same behaviour (Wang *et al*., 2016).

However, when comparing the sequential fermentation with the pure fermentation of non-*Saccharomyces* strains, we can also observe two different types of fermentation kinetics. For yeast species *T. delbrueckii*, *C. stellata* and *M. pulcherrima* an increase on the CO_2 release is observed after *S. cerevisiae* inoculation, while for *S. pombe* or *L. thermotolerans* there are no significant differences among both curves. This fact cannot be explained by the presence of metabolites produced by *S. cerevisiae*, because this fact is observed after *S. cerevisiae* inoculation. In this case, this different behaviour could be explained due to the different uptake of nitrogen compounds (as well-known limiting factor in a wine fermentation) in the media (Andorrà *et al*., 2012). It seems that *L. thermotolerans* and *S. pombe* exhibited higher nutritional requirements than the other species, because when *S. cerevisiae* is inoculated, there is not as much of an increase of the CO_2 release as was expected.

The results of the present study show that sequential cultures of all trials had organoleptic properties closer to pure cultures of non-*Saccharomyces* tested than the control. This type of inoculation in each trial maintained the organoleptic characteristics of its non-*Saccharomyces* pure culture, moreover a higher aroma complexity and higher floral and fruity aroma have been detected by the sensorial panel. Although it should be noted that almost all of sequential

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cultures, except for the *T. delbrueckii* sequential culture, were not able to consume all sugars present in must.

In conclusion, the use of the inoculation of different yeast species on a wine fermentation can improve the sensory properties of Malvar wines. However, depending of the non-*Saccharomyces* yeast strain inoculated, it may be better to use sequential or mixed fermentation. Although the highest differences with respect to the control were observed for sequential inoculation, these differences could be result of stuck or sluggish fermentation. To prevent this behaviour an earlier inoculation of *Saccharomyces* should be done for sequential inoculation. However, for mixed fermentation, in all cases the fermentations were finished, showing the effect of the presence of the non-*Saccharomyces* yeast species on the sensory analysis of the final wines obtained.

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APPENDIX

FIGURE CAPTIONS

Figure 1. Fermentation kinetics of pure (p), mixed (m) and sequential (s) cultures in Malvar must at 18 ^{0}C. A) Cultures with *T. delbrueckii* (Td) and *S. cerevisiae* (Sc). B) Cultures with *S. pombe* (Sp) and *S. cerevisiae* (Sc); C) Cultures with *C. stellata* (Cs) and *S. cerevisiae* (Sc). D) Cultures with *M. pulcherrima* (Mp) and *S. cerevisiae* (Sc). E) Cultures with *L. thermotolerans* (Lt) and *S. cerevisiae* (Sc). Values are the means from triplicate fermentations.

Figure 2. Cobweb graph of mean sensory scores of wines made with five native strains of non-*Saccharomyces* and the control strain of *S. cerevisiae*. A) p-Sc, pure culture of *S. cerevisiae*; p-Td, pure culture of *T. delbrueckii*; m-Td/Sc, mixed culture of *T. delbrueckii* and *S. cerevisiae*; s-Td/Sc, sequential culture of *T. delbrueckii* and *S. cerevisiae*. B) p-Sc, pure culture of *S. cerevisiae*; p-Sp, pure culture of *S. pombe*; m-Sp/Sc, mixed culture of *S. pombe* and *S. cerevisiae*; s-Sp/Sc, sequential culture of *S. pombe* and *S. cerevisiae*. C) p-Sc, pure culture of *S. cerevisiae*; p-Cs, pure culture of *C. stellata*; m-Cs/Sc, mixed culture of *C. stellata* and *S. cerevisiae*; s-Cs/Sc, sequential culture of *C. stellata* and *S. cerevisiae*. D) p-Sc, pure culture of *S. cerevisiae*; p-Mp, pure culture of *M. pulcherrima*; m-Mp/Sc, mixed culture of *M. pulcherrima* and *S. cerevisiae*; s-Mp/Sc, sequential culture of *M. pulcherrima* and *S. cerevisiae*. E) p-Sc, pure culture of *S. cerevisiae*; p-Lt, pure culture of *L. thermotolerans*; m-Lt/Sc, mixed culture of *L. thermotolerans* and *S. cerevisiae*; s-Lt/Sc, sequential culture of *L. thermotolerans* and *S. cerevisiae*.

Figure 3. Application of discriminant analysis to the data expressing as concentration (mg L^{-1}) of volatile compounds of the seven different trials [Sc1, pure *S. cerevisiae* culture considered as a control in *T. delbrueckii*, *S. pombe* and *C. stellata* trials; Td, different cultures (pure, mixed, sequential) elaborated with *T. delbrueckii* CLI 918 strain; Sp, different cultures (pure, mixed, sequential) elaborated with *S. pombe* CLI 1085 strain; Cs, different cultures (pure, mixed, sequential) elaborated with *C. stellata* CLI 920 strain; Sc2, pure *S. cerevisiae* culture considered as a control in *M. pulcherrima* and *L. thermotolerans* trials; Mp, different cultures (pure, mixed, sequential) elaborated with *M. pulcherrima* CLI 457 strain; Lt, different cultures (pure, mixed, sequential) elaborated with *L. thermotolerans* 9-6C strain].

TABLE CAPTIONS

Table 1. Principal oenological parameters at the end of the different inoculations. Values are means ± standard deviations of triplicate fermentations. * Means statistically different from the respective control, $p < 0.05$. [a] Abbreviations related with the type of culture employed and the yeast strains are explained in Figure 2. [b] *S. cerevisiae* pure culture p-Sc1 was taken as the control in *T. delbrueckii* □ *S. cerevisiae*, *S. pombe* □ *S. cerevisiae*, *C. stellata* □ *S. cerevisiae* trials; *S. cerevisiae* pure culture p-Sc2 was taken as control in *M. pulcherrima* □ *S. cerevisiae* and *L. thermotolerans* □ *S. cerevisiae* trials.

Table 2. Volatile compounds (mg L^{-1}), ODE (odour description) and OTV (odour threshold value, mg L^{-1}, Balboa-Lagunero *et al*. 2013) of pure (p), mixed (m) and sequential (s) cultures made with *Saccharomyces* and non-*Saccharomyces* strains. Values

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are the mean ± SD of triplicate fermentations. *Means statistically different from the respective control, $p < 0.05$. Abbreviations related with the type of culture employed and the yeast strains are explained in Figure 2. [a] *S. cerevisiae* pure culture p-Sc1 was taken as the control in *T. delbrueckii* □ *S. cerevisiae*, *S. pombe* □ *S. cerevisiae*, *C. stellata* □ *S. cerevisiae* trials; *S. cerevisiae* pure culture p-Sc2 was taken as control in *M. pulcherrima* □ *S. cerevisiae* and *L. thermotolerans* □ *S. cerevisiae* trials.

Table 3. Standardized coefficients of the discriminant functions. [a] Explained variance proportion (%).

SUPPLEMENTARY MATERIAL

Table S1. ODE (odour description), OTV (odour threshold value, mg L^{-1}, Balboa-Lagunero *et al*. 2013) and OAV (odour activity values; OAV = *x*/OTH, where *x* is the concentration mean value of each volatile compound) of pure (p), mixed (m) and sequential (s) cultures made with *S. cerevisiae* and non-*Saccharomyces* strains (Td, *T. delbrueckii*; Sp, *S. pombe*; Cs, *C. stellata*; Mp, *M. pulcherrima*; Lt, *L. thermotolerans*). [a] *S. cerevisiae* pure culture p-Sc1 was taken as the control in *T. delbrueckii* □ *S. cerevisiae*, *S. pombe* □ *S. cerevisiae*, *C. stellata* □ *S. cerevisiae* trials; *S. cerevisiae* pure culture p-Sc2 was taken as control in *M. pulcherrima* □ *S. cerevisiae* and *L. thermotolerans* □ *S. cerevisiae* trials.

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Figure 1

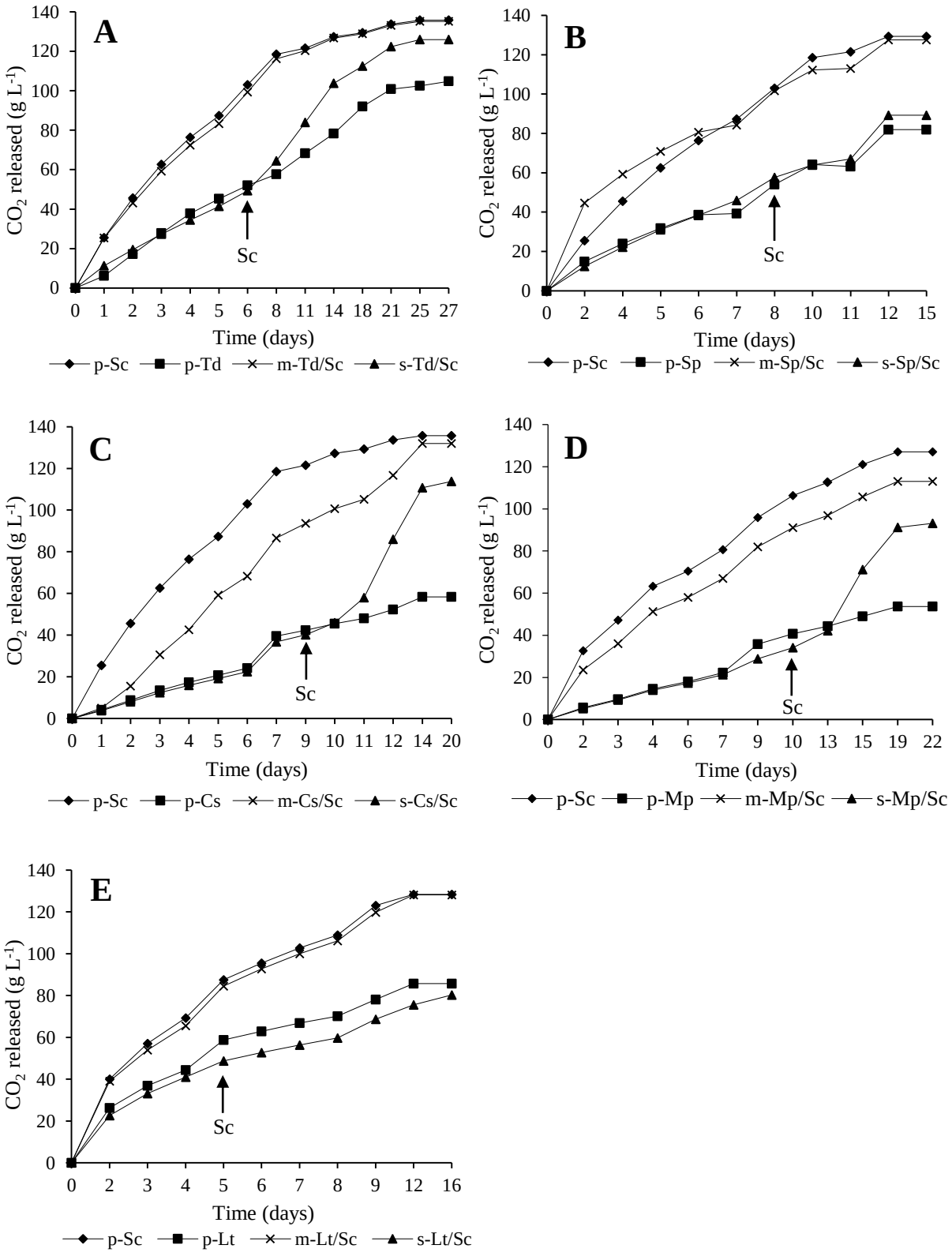

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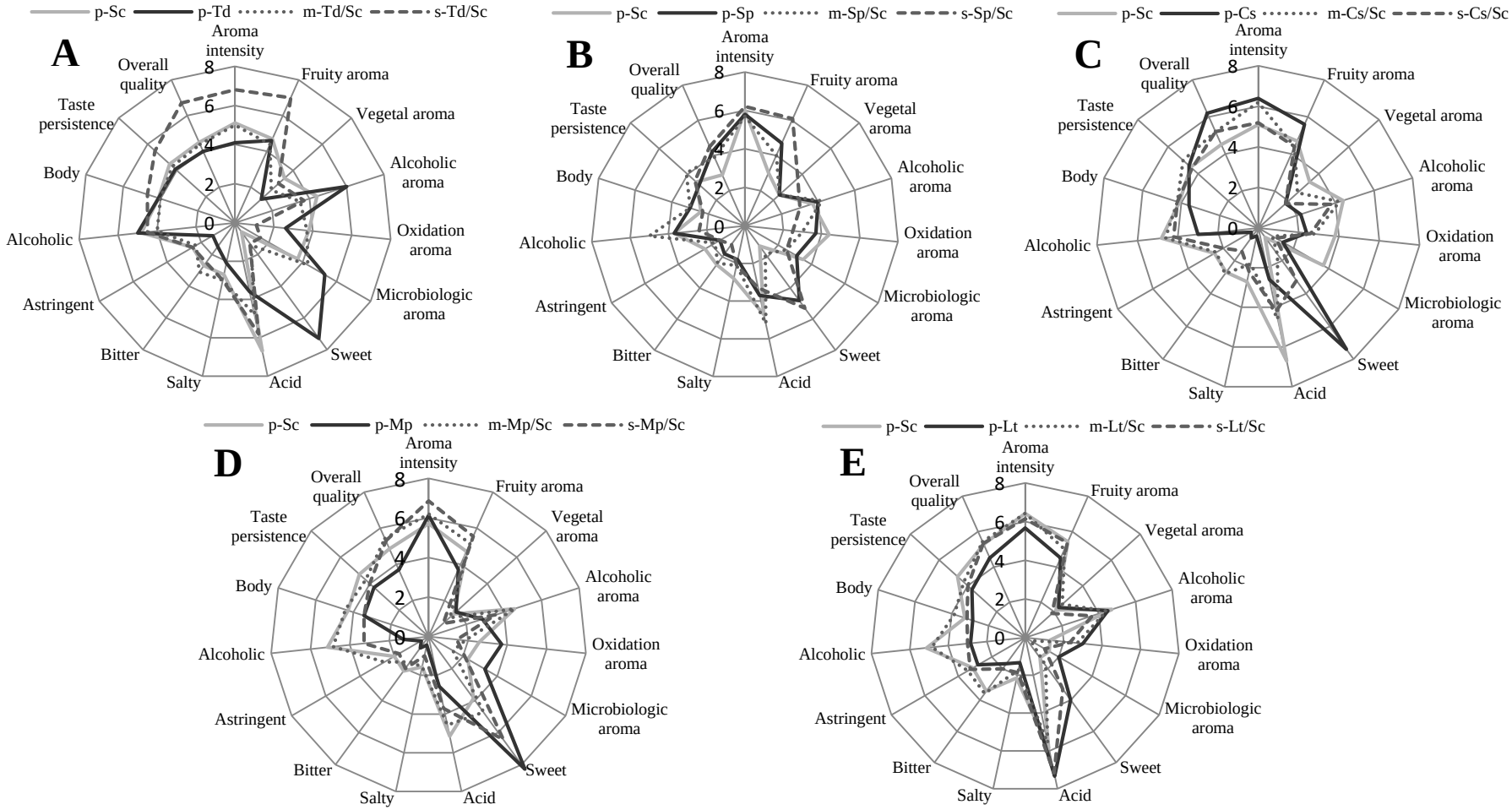
Figure 2



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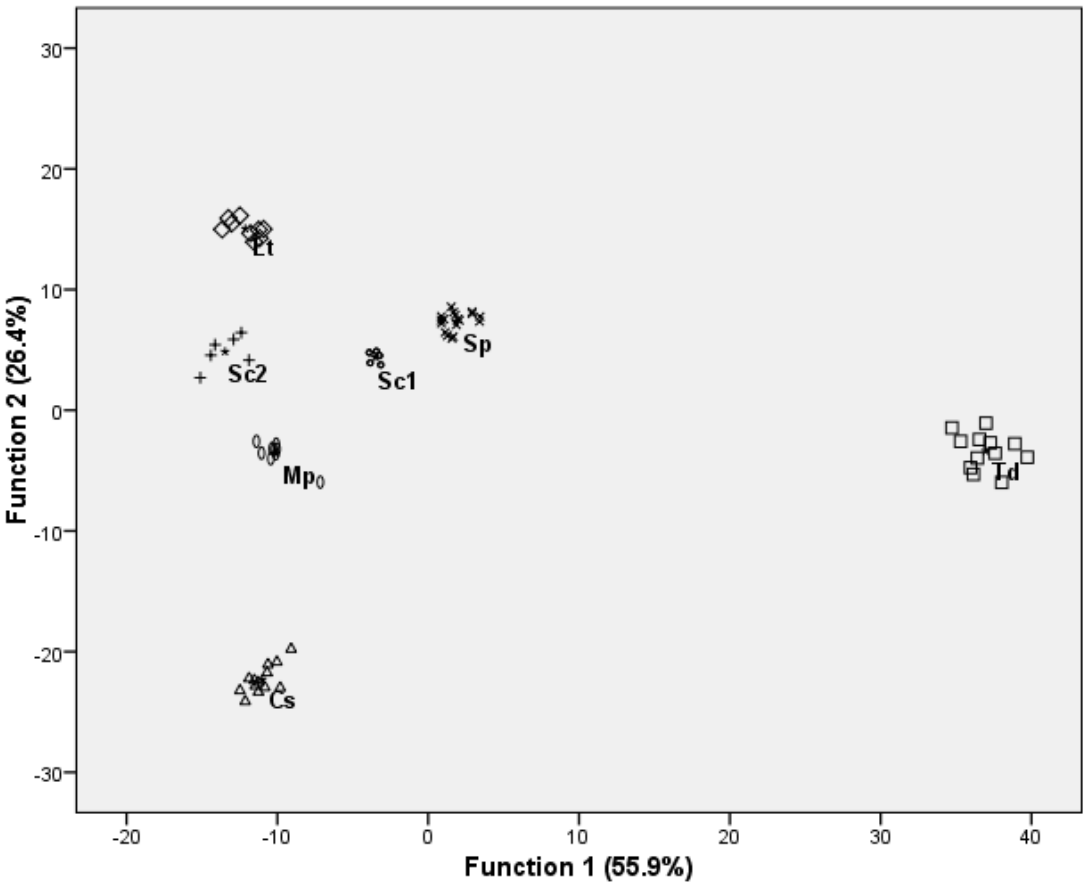

Figure 3

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Table 1

Trial	Type of culture[a]	Ethanol	pH	Volatile acidity	Total acidity	Reducing sugars	Glycerol	Malic acid	Lactic acid
		%, v/v		g/L acetic acid	g/L tartaric acid	g/L	g/L	g/L	g/L
T. delbrueckii - S. cerevisiae	p-Sc1[b]	13.13 ± 0.06	3.30 ± 0.00	0.50 ± 0.01	5.60 ± 0.01	<1.50	3.12 ± 0.14	0.95 ± 0.03	<0.50
	p-Td	10.88 ± 0.19*	3.34 ± 0.03	0.60 ± 0.01*	6.75 ± 0.15*	33.70 ± 2.98*	7.14 ± 0.21*	1.05 ± 0.01	<0.50
	m-Td/Sc	13.03 ± 0.06	3.30 ± 0.01	0.46 ± 0.06	5.46 ± 0.06	<1.50	2.87 ± 0.05	0.90 ± 0.01	<0.50
	s-Td/Sc	12.80 ± 0.00*	3.30 ± 0.00	0.51 ± 0.01	6.06 ± 0.06*	1.70 ± 0.05	5.28 ± 0.20*	0.93 ± 0.06	<0.50
S. pombe - S. cerevisiae	p-Sc1	13.13 ± 0.06	3.30 ± 0.00	0.50 ± 0.01	5.60 ± 0.01	<1.50	3.12 ± 0.14	0.95 ± 0.03	<0.50
	p-Sp	10.21 ± 0.74*	2.98 ± 0.01*	0.33 ± 0.05*	7.08 ± 0.12*	52.93 ± 4.20*	4.45 ± 0.13	<0.20*	0.64 ± 0.07*
	m-Sp/Sc	13.70 ± 0.10	3.20 ± 0.00*	0.73 ± 0.05*	6.56 ± 0.05*	2.43 ± 0.66	5.45 ± 0.17*	0.81 ± 0.03	<0.50
	s-Sp/Sc	10.88 ± 0.94*	3.01 ± 0.01*	0.38 ± 0.05	6.95 ± 0.02*	42.35 ± 6.48*	5.22 ± 0.35*	<0.20*	0.57 ± 0.08*
C. stellata - S. cerevisiae	p-Sc1	13.13 ± 0.06	3.30 ± 0.00	0.50 ± 0.01	5.60 ± 0.01	<1.50	3.12 ± 0.14	0.95 ± 0.03	<0.50
	p-Cs	3.40 ± 0.05*	3.71 ± 0.02*	0.63 ± 0.01*	4.79 ± 0.01*	174.18 ± 0.71*	9.17 ± 0.25*	<0.20*	0.91 ± 0.04*
	m-Cs/Sc	13.70 ± 0.41*	3.37 ± 0.03*	0.70 ± 0.01*	4.93 ± 0.06*	9.66 ± 0.70	5.72 ± 0.12*	0.87 ± 0.09	<0.50
	s-Cs/Sc	13.23 ± 0.23	3.27 ± 0.02	0.55 ± 0.07	4.85 ± 0.06*	25.21 ± 5.19*	6.91 ± 0.68*	0.71 ± 0.15	0.59 ± 0.02*
M. pulcherrima - S. cerevisiae	p-Sc2[b]	12.90 ± 0.04	3.10 ± 0.00	0.63 ± 0.00	6.43 ± 0.00	<1.50	5.15 ± 0.07	0.91 ± 0.01	<0.50
	p-Mp	4.12 ± 0.71*	3.44 ± 0.02*	0.27 ± 0.02*	5.24 ± 0.22*	152.93 ± 13.85*	9.66 ± 0.29*	<0.20*	1.08 ± 0.10*
	m-Mp/Sc	11.31 ± 0.77	3.10 ± 0.02	0.83 ± 0.00*	6.23 ± 0.04	45.19 ± 12.04*	6.02 ± 0.19	0.94 ± 0.06	0.73 ± 0.09*
	s-Mp/Sc	10.66 ± 2.21	3.12 ± 0.01	0.51 ± 0.03*	5.98 ± 0.53	56.50 ± 2.36*	7.40 ± 0.52*	0.85 ± 0.20	0.74 ± 0.15*
	p-Sc2	12.90 ± 0.04	3.10 ± 0.00	0.63 ± 0.00	6.43 ± 0.00	<1.50	5.15 ± 0.07	0.91 ± 0.01	<0.50

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	p-Lt	7.19 ± 0.26*	2.43 ± 0.07*	0.78 ± 0.01*	16.24 ± 0.77*	77.15 ± 4.75*	0.93 ± 0.14*	<0.20*	8.32 ± 0.62*
L. thermotolerans - S. cerevisiae	m-Lt/Sc	12.63 ± 0.05	3.10 ± 0.00	0.46 ± 0.05	6.46 ± 0.11	<1.50	4.75 ± 0.07*	0.61 ± 0.02*	0.56 ± 0.04
	s-Lt/Sc	7.29 ± 0.13*	2.47 ± 0.02*	0.77 ± 0.01*	15.80 ± 0.33*	73.05 ± 2.37*	0.92 ± 0.19*	<0.20*	7.99 ± 0.29*

Table 2

Table 2 (Continued)

C. stellata - S. cerevisiae			***S. cerevisiae***	***M. pulcherrima - S. cerevisiae***			***L. thermotolerans - S. cerevisiae***		
p-Cs	m-Cs/Sc	s-Cs/Sc	p-Sc2[a]	p-Mp	m-Mp/Sc	s-Mp/Sc	p-Lt	m-Lt/Sc	s-Lt/Sc
3.71 ± 0.40	6.71 ± 0.67*	8.75 ± 1.06*	6.27 ± 0.22	1.69 ± 0.09	2.10 ± 0.02	2.51 ± 0.57	12.18 ± 0.68	13.53 ± 0.76	15.61 ± 0.80*
0.01 ± 0.00*	0.73 ± 0.13*	0.85 ± 0.20*	0.32 ± 0.00	0.05 ± 0.00	0.50 ± 0.09	0.15 ± 0.01	0.10 ± 0.03	0.26 ± 0.01	0.09 ± 0.02
101.64 ± 1.02*	26.23 ± 0.90	92.94 ± 0.94*	13.40 ± 3.46	43.33 ± 1.12*	10.47 ± 0.25	40.24 ± 0.98*	49.02 ± 0.86*	16.05 ± 0.14	54.90 ± 0.76*
52.52 ± 1.04*	130.41 ± 1.57*	166.18 ± 0.85*	103.90 ± 4.30	27.26 ± 0.87*	60.48 ± 1.02	81.40 ± 1.12	167.97 ± 0.75*	125.95 ± 0.99	187.05 ± 0.98*
0.13 ± 0.00	0.18 ± 0.09*	0.11 ± 0.01	0.03 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.06 ± 0.01*	0.04 ± 0.01	0.06 ± 0.01*

Table 3

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Compound	ODE		OTV	*S. cerevisiae*	*T. delbrueckii - S. cerevisiae*			*S. pombe - S. cerevisiae*		/Sc	s-Sp/Sc
0.63 ± 0.02*	0.85 ± 0.02	0.38 ± 0.03*	0.23 ± 0.09	0.05 ± 0.01	0.07 ± 0.01	0.09 ± 0.00	0.98 ± 0.20*	0.45 ± 0.07	0.22 ± 0.09	0.00	2.00 ± 0.00
0.63 ± 0.06	0.70 ± 0.12	0.51 ± 0.11	0.20 ± 0.02	0.02 ± 0.00	0.10 ± 0.02	0.10 ± 0.02	0.21 ± 0.01	0.21 ± 0.02	0.52 ± 0.06*	± *	0.33 ± 0.02
0.04 ± 0.00	0.15 ± 0.01	0.14 ± 0.03	0.07 ± 0.02	0.52 ± 0.10*	0.25 ± 0.06*	0.18 ± 0.03	0.06 ± 0.02	0.10 ± 0.03	0.08 ± 0.02	± 2	25.27 ± 2.10
22.86 ± 1.30*	14.20 ± 0.45	18.18 ± 0.45*	15.23 ± 3.02	10.50 ± 0.19	9.55 ± 0.85	8.81 ± 0.90	21.28 ± 0.88	13.51 ± 0.87	41.22 ± 0.32*	5 ± 1	70.44 ± 0.07
182.16 ± 4.02*	180.16 ± 3.96	287.96 ± 3.68*	139.67 ± 11.14	83.40 ± 2.29*	83.58 ± 2.32*	133.51 ± 3.63	251.87 ± 3.44*	170.10 ± 2.90	299.74 ± 3.06*	0.01 ± *	0.11 ± 0.01 0.20 ± 0.01*
0.22 ± 0.03	0.59 ± 0.06*	0.47 ± 0.10*	0.29 ± 0.02	0.06 ± 0.00*	0.16 ± 0.03	0.19 ± 0.00	0.07 ± 0.00*	0.35 ± 0.05	0.07 ± 0.01*	± *	0.36 ± 0.07
0.05 ± 0.00*	0.35 ± 0.02*	0.33 ± 0.05*	0.29 ± 0.03	0.08 ± 0.01	0.30 ± 0.03	0.35 ± 0.06	0.33 ± 0.05	0.26 ± 0.04	0.68 ± 0.08*	0.02	0.05 ± 0.01
0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.14 ± 0.02*	0.00 ± 0.00	0.27 ± 0.01*	4 ± *	9.53 ± 0.96
2.17 ± 0.00*	2.31 ± 0.25*	2.28 ± 0.20*	1.56 ± 0.12	0.18 ± 0.01*	0.76 ± 0.05*	0.94 ± 0.08	0.37 ± 0.03*	2.10 ± 0.23	0.44 ± 0.01*	4 ± 7	108.29 ± 3.25
0.56 ± 0.01	1.12 ± 0.11*	0.82 ± 0.08*	0.42 ± 0.06	0.02 ± 0.00*	0.57 ± 0.10	0.53 ± 0.01	0.05 ± 0.01*	0.54 ± 0.11	0.13 ± 0.05*	0.04	0.31 ± 0.08*
0.01 ± 0.00*	0.17 ± 0.01	0.21 ± 0.04*	0.14 ± 0.00	0.00 ± 0.00*	0.19 ± 0.00	0.15 ± 0.00	0.00 ± 0.00*	0.11 ± 0.02	0.06 ± 0.02		
0.06 ± 0.01	0.14 ± 0.02*	0.14 ± 0.01*	0.05 ± 0.01	0.04 ± 0.00	0.06 ± 0.01	0.05 ± 0.00	0.15 ± 0.02*	0.04 ± 0.00	0.13 ± 0.00*		
0.10 ± 0.01*	0.41 ± 0.03	0.43 ± 0.12	0.28 ± 0.11	0.11 ± 0.04	0.16 ± 0.03	0.06 ± 0.01	0.01 ± 0.00	0.12 ± 0.02	0.02 ± 0.00		
0.04 ± 0.00*	0.38 ± 0.06	0.01 ± 0.00*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00		
0.09 ± 0.02	1.96 ± 0.13*	1.17 ± 0.13*	0.58 ± 0.10	0.07 ± 0.02*	0.31 ± 0.06*	0.20 ± 0.09*	0.03 ± 0.00*	0.61 ± 0.05	0.03 ± 0.00*		
0.27 ± 0.03*	1.47 ± 0.11*	1.14 ± 0.07*	1.09 ± 0.35	3.73 ± 0.45	2.62 ± 0.57	0.52 ± 0.08	27.35 ± 0.48*	3.58 ± 0.45	58.59 ± 0.88*		
3.55 ± 0.10*	8.90 ± 0.80*	6.99 ± 0.80*	4.70 ± 0.88	4.28 ± 0.53	5.15 ± 0.88	2.98 ± 0.33	28.50 ± 0.61*	7.71 ± 0.97	60.44 ± 1.05*		
0.22 ± 0.06*	0.86 ± 0.07	0.43 ± 0.05	2.73 ± 0.51	1.95 ± 0.16	3.50 ± 0.06	2.42 ± 0.30	16.57 ± 0.45*	2.31 ± 0.11	35.22 ± 0.54*		

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Ethyl isovalerate	Fruity, sweet,		0.003	0.24 ± 0.03	0.60 ± 0.03*	0.22 ± 0.01	0.40 ± 0.04	0.22 ± 0.04	0.29 ±		0.22 ± 0.05
]	0.10 ± 0.00	0.25 ± 0.01	0.18 ± 0.01	0.37 ± 0.09	0.28 ± 0.08	0.41 ± 0.06	0.43 ± 0.12	0.13 ± 0.01*	0.32 ± 0.04	0.16 ± 0.06	0.00	0.00 ± 0.00
]	0.02 ± 0.00*	0.11 ± 0.01*	0.21 ± 0.00*	1.54 ± 0.10	0.07 ± 0.02*	2.35 ± 0.10	1.07 ± 0.11	1.82 ± 0.32	1.46 ± 0.09	4.68 ± 0.08*	0.12	0.30 ± 0.07*
]	0.31 ± 0.01*	4.64 ± 0.12*	3.96 ± 0.43*	3.69 ± 0.14	0.19 ± 0.00*	3.53 ± 0.30	4.37 ± 0.11	0.43 ± 0.07*	4.78 ± 0.24*	0.71 ± 0.10*	0.10	0.35 ± 0.08
]	0.09 ± 0.02*	4.65 ± 0.61*	4.33 ± 0.47*	5.89 ± 0.57	0.01 ± 0.00*	4.42 ± 0.22	5.86 ± 0.34	0.23 ± 0.01*	8.08 ± 0.32	0.44 ± 0.09*	0.01	0.09 ± 0.02
]	0.01 ± 0.00*	1.50 ± 0.15*	0.71 ± 0.03	0.06 ± 0.05	0.00 ± 0.00	0.02 ± 0.02	0.02 ± 0.00	0.03 ± 0.00	0.10 ± 0.02	0.13 ± 0.01	0.01	0.03 ± 0.00
]	0.75 ± 0.09*	11.91 ± 0.97*	9.82 ± 0.96*	14.29 ± 1.46	2.49 ± 0.26*	14.24 ± 0.76	14.19 ± 0.98	19.22 ± 0.86	17.04 ± 0.82	44.34 ± 0.88*	0.14 ± *	2.12 ± 0.56* 0.22 ± 0.02*
]	0.06 ± 0.02*	0.13 ± 0.01*	0.22 ± 0.02*	0.06 ± 0.01	0.11 ± 0.03	0.06 ± 0.01	0.09 ± 0.01	0.12 ± 0.02	0.13 ± 0.05*	0.06 ± 0.02	0.18	0.53 ± 0.03
]	0.04 ± 0.01	0.22 ± 0.01*	0.21 ± 0.08*	0.45 ± 0.01	0.00 ± 0.00*	0.39 ± 0.09	0.00 ± 0.00*	0.62 ± 0.07	0.02 ± 0.00*	0.82 ± 0.15*	± *	0.65 ± 0.06*
]	0.00 ± 0.00	0.09 ± 0.00*	0.11 ± 0.04*	0.03 ± 0.00	0.00 ± 0.00*	0.02 ± 0.00	0.01 ± 0.00	0.00 ± 0.00*	0.03 ± 0.00	0.02 ± 0.00	0.78	4.84 ± 0.97*
]	0.08 ± 0.02*	0.13 ± 0.00*	0.12 ± 0.01*	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	0.05	0.91 ± 0.11
]	3.38 ± 0.14*	1.52 ± 0.21	1.61 ± 0.08	1.04 ± 0.41	2.01 ± 0.42	1.27 ± 0.20	0.78 ± 0.04	2.00 ± 0.23	1.32 ± 0.13	1.03 ± 0.01	0.04	0.07 ± 0.00*
]	3.57 ± 0.19*	2.09 ± 0.23	2.28 ± 0.21	1.62 ± 0.43	2.12 ± 0.45	1.75 ± 0.30	0.88 ± 0.05	2.76 ± 0.32	1.54 ± 0.19	1.96 ± 0.18	0.02	0.15 ± 0.01*
]	1.11 ± 0.01	1.72 ± 0.14	1.66 ± 0.15	5.78 ± 0.15	5.44 ± 0.32	5.06 ± 0.13	3.68 ± 0.25	10.33 ± 0.85*	6.32 ± 0.28	14.34 ± 0.56*	0.40	3.79 ± 0.27*
Octanoic acid	Sweet, cheesy		0.5	2.11 ± 0.57	1.15 ± 0.13	2.22 ± 0.68	1.11 ± 0.03	1.14 ± 0.26*	3.07 ± 0.50*		3.32 ± 0.60*	
Decanoic acid	Rancid, fatty		1	0.56 ± 0.05	0.22 ± 0.03	0.73 ± 0.10	0.55 ± 0.09	0.18 ± 0.00*	0.94 ± 0.10*		0.48 ± 0.05	
Σ Acids				7.28 ± 1.05	3.46 ± 0.31*	7.90 ± 1.02	4.66 ± 0.21*	10.52 ± 1.06*	8.53 ± 1.01		8.72 ± 0.97	
Diacetyle	Butter		0.1	0.00 ± 0.00	0.12 ± 0.02*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00		0.00 ± 0.00	
Furfural	Bread, toasty, candy		15	0.02 ± 0.01	0.01 ± 0.00	0.04 ± 0.00	0.13 ± 0.02	0.05 ± 0.01	0.01 ± 0.00		0.01 ± 0.00	
Benzaldehyde	Sweet, candy, wood		5	0.03 ± 0.00	0.04 ± 0.01	0.05 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.04 ± 0.00		0.02 ± 0.00	

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Phenylacetaldehyde	Roses	1	0.00 ± 0.00	0.04 ± 0.00*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Acetoine	Butter	150	1.11 ± 0.41	1.37 ± 0.15	0.64 ± 0.07	2.15 ± 0.09	59.00 ± 2.09*	2.03 ± 0.42	6.81 ± 1.21*
Σ Aldehydes/Ketones			1.16 ± 0.42	1.57 ± 0.18	0.72 ± 0.08	2.30 ± 0.11*	59.06 ± 2.10*	2.07 ± 0.42*	6.84 ± 1.21*
γ-Butyrolactone	Coconut	35	1.18 ± 0.15	0.20 ± 0.02	1.05 ± 0.03	0.45 ± 0.01	1.69 ± 0.05	0.24 ± 0.08	0.29 ± 0.03

Compound	Function	
	1 (55.9)[a]	2 (26.4)[a]
Ethyl Isobutyrate	3.589	−1.470
Diacetyle	−0.738	−0.861
Ethyl Isovalerate	−1.882	−2.636
1-propanol	−0.046	0.644
Isobutanol	0.879	−1.798
Isoamyl acetate	3.235	−2.730
1-butanol	2.067	1.201
Isoamyl alcohol	4.692	1.412
Ethyl hexanoate	1.999	−1.550
Hexyl acetate	1.196	−0.521
Acetoin	1.74	0.513
Ethyl lactate	−0.531	2.552
1-hexanol	2.85	3.836
(Z)-3-hexen-1-ol	5.728	−1.581
Ethyl octanoate	−0.539	0.023
Furfural	−1.771	−1.630
Ethyl-3-	−0.986	−0.068
γ-butyrolactone	−0.353	0.855
Isovaleric acid	0.142	0.014
Metionol	2.151	−2.242

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2-phenylethyl acetate	?0.435	2.373
Hexanoic acid	?5.197	?5.135
Benzyl alcohol	?0.623	?0.321
β-phenylethyl alcohol	?0.893	2.739
Octanoic acid	3.737	9.873
Decanoic acid	?0.838	?1.386

Supplementary Material: Table S1

Compound	**ODE**	**OTV**	**OAV *S. cerevisiae***	**OAV *T. delbrueckii* - *S. cerevisiae***			**OAV *S. pombe* - *S. cerevisiae***			**OAV *C. stellata* - *S. cerevisiae***		
			p-Sc1[a]	p-Td	m-Td/Sc	s-Td/Sc	p-Sp	m-Sp/Sc	s-Sp/Sc	p-Cs	m-Cs/Sc	s-Cs/Sc
1-Propanol	Alcohol, ripe fruit	9	0.35	0.40	0.41	0.22	0.30	0.22	0.22	0.41	0.75	0.95
1-Butanol	Soap, fatty, diesel	150	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.01
Isobutanol	Bitter, fusel, alcohol	40	0.41	0.74	0.39	0.82	0.84	0.35	0.63	2.54	0.66	2.30
Isoamyl alcohol	Harsh, bitter	30	2.98	4.66	3.88	7.73	2.30	3.55	2.35	1.75	4.41	5.52
(Z)-3-Hexen-1-ol	Lemon, fresh	0.4	0.26	0.75	1.42	0.49	0.30	0.31	0.27	0.33	0.45	0.27
1-Hexanol	Green grass, fresh	8	0.11	0.25	0.11	0.10	0.09	0.07	0.03	0.08	0.11	0.05
Metionol	Garlic	1	0.41	1.86	0.57	2.03	0.72	0.82	0.36	0.63	0.70	0.51
Benzyl alcohol	Pleasant, soft	200	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
β-Phenylethyl alcohol	Flowery, roses	14	0.76	2.07	0.91	1.65	0.97	1.81	0.68	1.63	1.01	1.30
Ethyl butyrate	Fruity, sweet, apple	0.02	7.33	9.20	5.40	4.61	4.32	10.01	15.73	10.91	29.76	25.05
Ethyl isovalerate	Fruity, sweet, banana	0.003	78.43	199.93	72.98	81.97	73.25	98.05	73.23	15.04	118.63	109.34
Ethyl isobutyrate	Fruity, pineapple	0.015	0.00	182.71	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

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Isoamyl acetate	Banana, sweet, fruity	0.03	20.04	8.02	13.13	14.18	8.25	24.43	10.03	72.26	77.21	76.54
Ethyl hexanoate	Pineapple, apple	0.014	28.03	15.27	23.91	41.39	14.66	23.75	24.74	39.66	79.72	58.28
Ethyl-3-hydroxybutyrate	Fruity	20	0.00	0.00	0.01	0.00	0.00	0.01	0.00	0.00	0.01	0.01
Hexyl acetate	Fruity, green, pear	1	0.04	0.11	0.06	0.12	0.04	0.03	0.03	0.06	0.14	0.13
2-Phenylethyl acetate	Flowery, lilac	0.25	3.31	0.49	3.43	3.94	6.83	4.99	8.49	0.41	1.66	1.72
Diethyl succinate	Camphor	100	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ethyl octanoate	Fresh, flowery, pineapple	0.58	0.77	0.35	1.52	0.58	0.89	1.09	0.92	0.16	3.39	1.96
Ethyl lactate	Sour milk	154	0.02	0.00	0.02	0.00	0.01	0.01	0.00	0.00	0.01	0.01
Isobutyric acid	Rancid, butter, cheese	0.05	18.24	4.62	18.16	11.09	107.43	14.15	18.24	4.41	17.24	8.42
Butyric acid	Butter, cheese, stinky	0.173	1.68	0.56	1.14	0.31	3.44	0.79	0.39	0.58	1.45	1.02
Isovaleric acid	Cheese	0.033	16.40	0.37	21.78	2.98	7.21	14.25	4.53	0.70	1.69	6.36
Hexanoic acid	Cheese	0.42	6.84	4.14	7.46	5.44	7.14	7.64	9.03	0.74	11.06	9.46
Octanoic acid	Sweet, cheesy	0.5	4.22	2.27	4.44	2.22	2.29	6.14	6.65	0.18	9.27	8.76
Decanoic acid	Rancid, fatty	1	0.56	0.23	0.73	0.55	0.18	0.94	0.48	0.01	1.50	0.73
Diacetyle	Butter	0.1	0.00	1.19	0.00	0.00	0.00	0.00	0.00	0.62	1.37	2.23
Furfural	Bread, toasty, candy	15	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.01
Benzaldehyde	Sweet, candy, wood	5	0.01	0.01	0.01	0.00	0.00	0.01	0.00	0.00	0.02	0.02
Phenylacetaldehyde	Roses	1	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.08	0.13	0.12
Acetoine	Butter	150	0.01	0.01	0.00	0.01	0.39	0.01	0.05	0.02	0.01	0.01
γ-Butyrolactone	Coconut	35	0.03	0.00	0.03	0.01	0.05	0.01	0.01	0.03	0.05	0.05

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Table S1 (Continued)

Compound	ODE	OTV	OAV *S. cerevisiae*	OAV *M. pulcherrima - S. cerevisiae*			OAV *L. thermotolerans - S. cerevisiae*		
			p-Sc2[a]	p-Mp	m-Mp/Sc	s-Mp/Sc	p-Lt	m-Lt/Sc	s-Lt/Sc
1-Propanol	Alcohol, ripe fruit	9	0.70	0.19	0.23	0.28	1.35	1.50	1.76
1-Butanol	Soap, fatty, diesel	150	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Isobutanol	Bitter, fusel, alcohol	40	0.34	1.08	0.26	1.00	1.23	0.40	1.37
Isoamyl alcohol	Harsh, bitter	30	3.46	0.91	2.03	2.71	5.60	4.20	6.26
(Z)-3-Hexen-1-ol	Lemon, fresh	0.4	0.08	0.06	0.06	0.07	0.15	0.10	0.15
1-Hexanol	Green grass, fresh	8	0.03	0.01	0.01	0.01	0.12	0.06	0.03
Metionol	Garlic	1	0.20	0.04	0.10	0.10	0.21	0.21	0.53
Benzyl alcohol	Pleasant, soft	200	0.00	0.00	0.00	0.00	0.00	0.00	0.00
β-Phenylethyl alcohol	Flowery, roses	14	1.09	0.75	0.68	0.63	1.52	0.96	2.92
Ethyl butyrate	Fruity, sweet, apple	0.02	14.41	2.80	8.11	9.54	3.48	17.73	3.73
Ethyl isovalerate	Fruity, sweet, banana	0.003	97.15	27.03	100.07	117.78	109.28	85.38	226.32
Ethyl isobutyrate	Fruity, pineapple	0.015	0.00	1.87	0.00	0.00	9.10	0.00	17.86
Isoamyl acetate	Banana, sweet, fruity	0.03	52.14	5.97	25.18	31.14	12.43	69.86	14.66
Ethyl hexanoate	Pineapple, apple	0.014	30.02	1.34	40.72	37.41	3.92	38.51	9.00
Ethyl-3-hydroxybutyrate	Fruity	20	0.01	0.00	0.01	0.01	0.00	0.01	0.00
Hexyl acetate	Fruity, green, pear	1	0.05	0.04	0.06	0.05	0.15	0.04	0.13
2-Phenylethyl acetate	Flowery, lilac	0.25	1.12	0.43	0.65	0.25	0.04	0.48	0.09

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Diethyl succinate	Camphor	100	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ethyl octanoate	Fresh, flowery, pineapple	0.58	1.00	0.11	0.54	0.33	0.05	1.05	0.05
Ethyl lactate	Sour milk	154	0.01	0.02	0.02	0.00	0.18	0.02	0.38
Isobutyric acid	Rancid, butter, cheese	0.05	54.69	39.05	69.97	48.77	331.40	46.16	705.15
Butyric acid	Butter, cheese, stinky	0.173	2.15	1.60	2.37	2.46	0.73	1.83	0.98
Isovaleric acid	Cheese	0.033	46.80	2.00	71.10	31.96	55.05	44.33	142.47
Hexanoic acid	Cheese	0.42	8.79	0.46	8.42	10.37	1.03	11.38	1.79
Octanoic acid	Sweet, cheesy	0.5	11.78	0.01	8.79	11.75	0.47	16.16	0.89
Decanoic acid	Rancid, fatty	1	0.06	0.00	0.02	0.02	0.04	0.10	0.13
Diacetyle	Butter	0.1	0.64	1.10	0.63	0.85	1.19	1.29	0.57
Furfural	Bread, toasty, candy	15	0.03	0.00	0.03	0.00	0.04	0.00	0.05
Benzaldehyde	Sweet, candy, wood	5	0.01	0.00	0.00	0.00	0.00	0.01	0.00
Phenylacetaldehyde	Roses	1	0.02	0.01	0.01	0.00	0.02	0.03	0.04
Acetoine	Butter	150	0.01	0.01	0.01	0.01	0.01	0.01	0.01
γ-Butyrolactone	Coconut	35	0.17	0.16	0.14	0.10	0.30	0.18	0.41

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