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Evaluation of Autochthonous Non-*Saccharomyces* Yeasts by Sequential Fermentation for Wine Differentiation in Galicia (NW Spain)

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Abstract: Non-*Saccharomyces* yeasts constitute a useful tool in winemaking because they secrete hydrolytic enzymes and produce metabolites that enhance wine quality; in addition, their ability to reduce alcohol content and/or to increase acidity can help to mitigate the effects of climatic change on wines. The purpose of this study was to evaluate the oenological traits of non-*Saccharomyces* yeast strains autochthonous from Galicia (NW Spain). To do that, we carried out sequential fermentation using 13 different species from the yeast collection of Estación de Viticultura e Enoloxía de Galicia (Evega) and *Saccharomyces cerevisiae* EC1118. The fermentation kinetics and yeast implantation were monitored using conventional methods and genetic techniques, respectively. The basic chemical parameters of wine were determined using the OIV official methodology, and the fermentative aroma compounds were determined by GC–FID. The results evidenced the limited fermentative power of these yeasts and the differences in their survival after the addition of *S. cerevisiae* to complete fermentation. Some strains reduced the alcohol and/or increased the total acidity of the wine. The positive effect on sensory wine properties as well as the production of desirable volatile compounds were confirmed for *Metschnikowia* spp. (Mf278 and Mp176), *Lachancea thermotolerans* Lt93, and *Pichia kluyveri* Pkl88. These strains could be used for wine diversification in Galicia.

Keywords: non-*Saccharomyces* yeasts; sequential fermentation; yeast implantation; wine acidity; alcohol reduction; wine aroma; *Metschnikowia* spp.; *Lachancea thermotolerans*; *Pichia kluyveri*

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1. Introduction

The fermentation of grape juice to obtain wine is a complex biochemical and microbiological process in which yeasts play an essential role. It is well known that, during spontaneous fermentations, a succession of yeast species takes place. Aerobic or low fermentative species (e.g., *Pichia*, *Candida*, or *Hanseniaspora*) and other fermentative yeasts, such as *Metschnikowia*, *Lachancea*, *Torulaspora*, or *Zygosaccharomyces*, are present in the early stages. As fermentation progresses, these species are gradually substituted by *Saccharomyces cerevisiae*, which has higher fermentative power and is more tolerant to ethanol [1–3].

For a long time, non-*Saccharomyces* species were associated with problems in wine fermentation because they are poor fermenters and some can produce undesirable metabolites [4]. However, in the last two decades, several studies have evidenced the positive contribution of these yeasts in improving wine quality and complexity; therefore, their role in winemaking has been reconsidered [5–7]. The interesting features of some non-*Saccharomyces* yeasts include an increase in glycerol content and total acidity, a reduction in acetic acid and ethanol content, the secretion of enzymes, and the production of secondary metabolites that enhance the wine aroma profile, or the biocontrol of spoilage microorganisms [4,8].

Nowadays, the benefits of non-*Saccharomyces* species in winemaking are well recognized and documented, especially for certain species such as *Torulaspora delbrueckii*, *Lachancea thermotolerans*, or the *Metschnikowia* genus [9–11]. Due to the fact of their relevance in winemaking, different strains of these and other yeast species are available commercially. Roudil et al. [12] reported the existence of at least 26 commercial starter cultures that offer interesting properties that can improve wine quality. Most non-*Saccharomyces* yeasts show limited fermentative power, so they are normally applied synergistically with *S. cerevisiae* as mixed starters to ensure the completion of fermentation. This practice takes advantage of the positive contribution of non-*Saccharomyces* yeasts to obtaining wines with distinctive properties compared with those elaborated with only *S. cerevisiae*.

The effects of climate change on grapes and wine can be seen in several regions of the world. The increase in temperature leads to an increase in the sugar concentration and a reduction of acidity in musts at harvest; therefore, the wines have higher alcohol content and lower acidity. In this sense, the ability of non-*Saccharomyces* yeasts to reduce the alcohol content and/or to increase the acidity of wines without affecting their sensory properties offers an interesting biological tool with which to mitigate the effects of climate change in winemaking [13–15]. Moreover, most of these strains enhance the chemical and sensory profiles of wines [13,16–19].

Although spontaneous fermentation is dominated by *Saccharomyces cerevisiae*, the complexity of the resulting wines is higher than in those obtained with only *S. cerevisiae* (for a review, see [8]). In addition, these wines are associated with regional characteristics that have been attributed to the contribution of local yeasts during the early stages of fermentation. Therefore, despite the availably of commercial cultures, the oenological potential of autochthonous non-*Saccharomyces* strains in preserving the regional character of wines from a given region is worth evaluating. For this purpose, we studied several non-*Saccharomyces* yeast strains obtained from the yeast culture collection maintained at Estación de Viticultura e Enoloxía de Galicia (Evega-Agacal). This work presents the results of the fermentative ability and yeast survival in the sequential fermentations of several non-*Saccharomyces* strains and their influence on wine chemical composition. The results highlight the positive influence of *Metschnikowia fructicola* Mf278, *Metschnikowia pulcherrima* Mp176, *Lachancea thermotolerans* Lt93, and *Pichia kluyveri* Pkl88 on the chemical and sensory characteristics of wines. Therefore, these autochthonous strains could be used as starters in sequential fermentations to obtain wines with singular properties.

2. Materials and Methods

2.1. Yeast Strains and Culture Media

All yeast strains used in this study came from the yeast culture collection of Evega. Table 1 includes the name of the species, strain number, and codes of the 14 yeast strains evaluated. These strains were selected according to their differential fermentative ability in a preliminary study that included 60 strains belonging to 11 genera of wine yeasts (data not shown). In addition, the commercial yeast *Saccharomyces cerevisiae* EC1118 (Lallemand, Madrid, Spain) was used as a control. Pure yeast cultures were grown on YPD media (yeast extract 1% *w*/*v*, peptone 2% *w*/*v*, glucose 2% *w*/*v*, and agar 2% *w*/*v* for solid media) at 28 °C and kept at 4 °C.

2.2. Sequential Fermentations

Fermentations were carried out using thawed grape juice (obtained from a mix of traditional white grape cultivars from Galicia) supplemented with concentrated juice up to 26.7 °Brix (269.5 g/L sugars, probable alcohol concentration 16.3% *v*/*v*, and total acidity 5.0 g tart/L). The must was pasteurized and distributed in 1 L bottles with 900 mL of must. Yeast inocula were prepared by growing each strain in 250 mL Erlenmeyer flasks containing 100 mL of pasteurized must and incubated at 28 °C and at 150 rpm for 24 h in a SANYO orbital incubator. Then, 1×10^7 cel/mL were added into the must in triplicate and allowed to ferment at 18 °C in a cold room. The evolution of alcoholic fermentation was followed by

daily °Brix measurement. When the fermentation began and a reduction in °Brix (0.6–2.0 depending on the yeast strain) was observed, the second inoculum—*S. cerevisiae* EC1118—was added at a concentration similar to the first. A control with this yeast strain as a monoculture was also performed. To help fermentation, 0.35 g/L of Nutrient Vit Blanc (Lallemand, Madrid, Spain) was added when the second yeast had already been inoculated in all fermentations (day 8). In addition, the wines were also evaluated at the sensory level by 5 members of the staff with experience in wine sensory analysis. The yeast's contribution to the wine's aroma was scored on a scale from 0 to 5, where 0 indicated a negative effect and 5 indicated a positive effect. When the fermentations ended (°Brix repeated for 3 days), the wines were centrifuged, sulfited (25 mg/L of free SO_2), and stored until further chemical analysis.

Table 1. Yeast strains used in this study.

Yeast Species	Strain	Code
Hanseniaspora vineae	129	Hv129
Hanseniaspora uvarum	95	Hu95
Starmerella bacillaris	474	Sb474
Metschnikowia fructicola	278	Mf278
Lachancea thermotolerans	93	Lt93
Torulaspora delbrueckii	315	Td315
Metschnikowiapulcherrima	176	Mp176
Pichia kudriavzevii	158	Pk158
Pichia kluyveri	88	Pkl88
Kluyveromyces dobzhanskii	231	Kd231
Zygosaccharomycesbailii	314	Zba314
Zygosaccharomycesparabailii	181	Zp181
Candidaapicola	31	Ca31
Saccharomyces cerevisiae	EC1118	EC1118

2.3. Microbiological Control

The evolution of the yeast population during fermentation and the interaction among strains were evaluated by taking samples for microbiological control at the beginning (Fi), exponential (Ft), and final (Ff) stages of fermentation. The samples were used to determine the changes in yeast population at quantitative and qualitative levels during the vinification process. The samples were serial diluted in 2% *w*/*v* buffered peptone water, and the adequate dilutions (−4, −5, and −6) were spread on a WL Nutrient Agar medium (Scharlau Microbiology, Barcelona, Spain) in duplicate [20]. The plates were incubated at 28 °C until visible colonies appeared; then, those containing between 20 and 200 colonies were used to count the total viable cells in the sample. The result was expressed as log of colony forming units per milliliter (Log CFU/mL). In addition, this medium allowed us to distinguish among certain wine yeasts based on their colony morphotype. Based on their aspect and frequency, a representative number of colonies (10–20 for each sample) from each sample was selected randomly and isolated on YPD for further characterization at the genetic level. Thus, the yeast identity was confirmed by the PCR amplification of the 5.8S rRNA gene and the two internal (non-coding) ITS1 and ITS2 spacers using the ITS1 and ITS4 primers [21].

When necessary, yeast isolates were grown on a lysine medium (Thermo Scientific™ Oxoid™, Madrid, Spain) to distinguish between *Saccharomyces* and non-*Saccharomyces*, since the former are unable to grow on this medium. Finally, *S. cerevisiae* isolates were characterized at the strain level via analysis of the mitochondrial DNA restriction profiles (mtDNA-RFLPs). The total yeast DNA was obtained, as described by Querol et al. [22], and digested with the restriction endonuclease Fast digest *HinfI* (ThermoFisher Scientific, Madrid, Spain). The restriction fragments were separated by gel electrophoresis on a 0.8% (*w*/*v*) agarose gel in 1X TBE. After staining with ethidium bromide (0.5 µg/mL), the DNA

pattern bands were visualized under UV light and documented using a Molecular Imager® Gel Doc™ XR+ imaging system (BIO-RAD, Madrid, Spain).

2.4. Chemical Analysis

The must parameters, including °Brix, sugar content, and total acidity, were determined using the official methodology [23]. The basic parameters of wines (alcohol content; reducing sugars; pH; titratable and volatile acidity; tartaric, malic, and lactic acids) were determined by Fourier transform infrared spectrometry (FTIR) using a Wine Scan FT120 analyzer (FOSS Electric, Barcelona, Spain) calibrated according to OIV [23]. The ethanol yield (g/g) was calculated as ethanol production (g/L) per sugar consumption (g/L). In addition, the free and total sulfur dioxides were also quantified using the OIV methods.

The volatile compounds of wine were quantified by gas chromatography–flame ionization detection (GC–FID) according to the protocol described by Ortega et al. [24]. All determinations were carried out in duplicate. In addition, the odor activity value (OAV) for each volatile compound was calculated as the ratio between its concentration and its perception threshold; compounds with an OAV > 1 were considered contributors to wine aroma.

2.5. Statistical Analysis

The differences in chemical composition of the wines, considering the yeast strain as a factor, were determined by one-way ANOVA. The Tukey HSD test was used to separate means. These analyses were carried out using SPSS18.0 for Windows.

Principal component analysis (PCA) was used to separate the wines according to their volatile composition considering compounds with an OAV > 1. Previously, the data were standardized using the function $f = \frac{(x-mean)}{standard\ deviation}$ to guarantee their equity in those variables or factors with different values in different units. The PCA was performed using PAST Version 3.26 (2019).

3. Results and Discussion

The recognition of non-*Saccharomyces* yeast's benefits has promoted several studies about the characterization and selection of strains from different species with desirable properties for winemaking [25–28].

Preliminary assays carried out in Evega on the fermentative ability of 60 strains from several yeast species evidenced their limitations in complete fermentation. Thus, when used as monoculture, *Metschnikowia* spp., *H. uvarum,* and *Candida* spp. produced wines with 6.5% of ethanol containing >150 g/L of sugars, whereas wines obtained with *L. thermotolerans*, *K. dobzhanskii*, *T. delbrueckii*, *Starm. bacillaris*, and *Zygosaccharomyces* spp. reached a 12% alcohol content, but they still had sugars to consume (data not shown). Similar differences in the sugar consumption and ethanol yields for wine yeasts have been reported previously [25,29]. Based on these results, a representative strain of each species was chosen, and its potential contribution to the chemical composition of wines was evaluated in sequential fermentations with *S. cerevisiae* EC1118 (a commercial strain) to ensure complete fermentation.

3.1. Kinetics of Sequential Fermentations and Yeast Population Dynamics

The evolution of sequential fermentations with different non-*Saccharomyces* strains showed variations among them, especially in the initial stages (Figure 1). As expected, the control fermentation, with a single inoculum of *S. cerevisiae* EC1118, began to ferment within the first 2 days and showed a higher fermentation speed than sequential vinifications. Some non-*Saccharomyces* yeasts, such as Td315, Sb474, and Hv129, also started fermentation 3 days after inoculation, whereas the remaining strains presented a lag phase of 6–8 days. Despite their slow beginning, after the addition of the second inoculum (EC1118), their fermentation speed was similar to that of the control. However, with Lt93, Td315, Kd231, Zba314, and Zp181, the activity slowed down towards the end, making it difficult to

consume all of the sugars. These results confirmed the widely reported poor or slower fermentative activity of non-*Saccharomyces* species and the need to add a *S. cerevisiae* yeast to successfully complete fermentation [25,27,30,31]. In addition, strain interactions in mixed fermentations and their nutrient requirements determine fermentation completion. The long fermentation times required in our study, even for EC1118 as single inoculum, were probably due to the high sugar concentration of the juice and the controlled low temperature compared with the data previously reported for this strain [31,32].

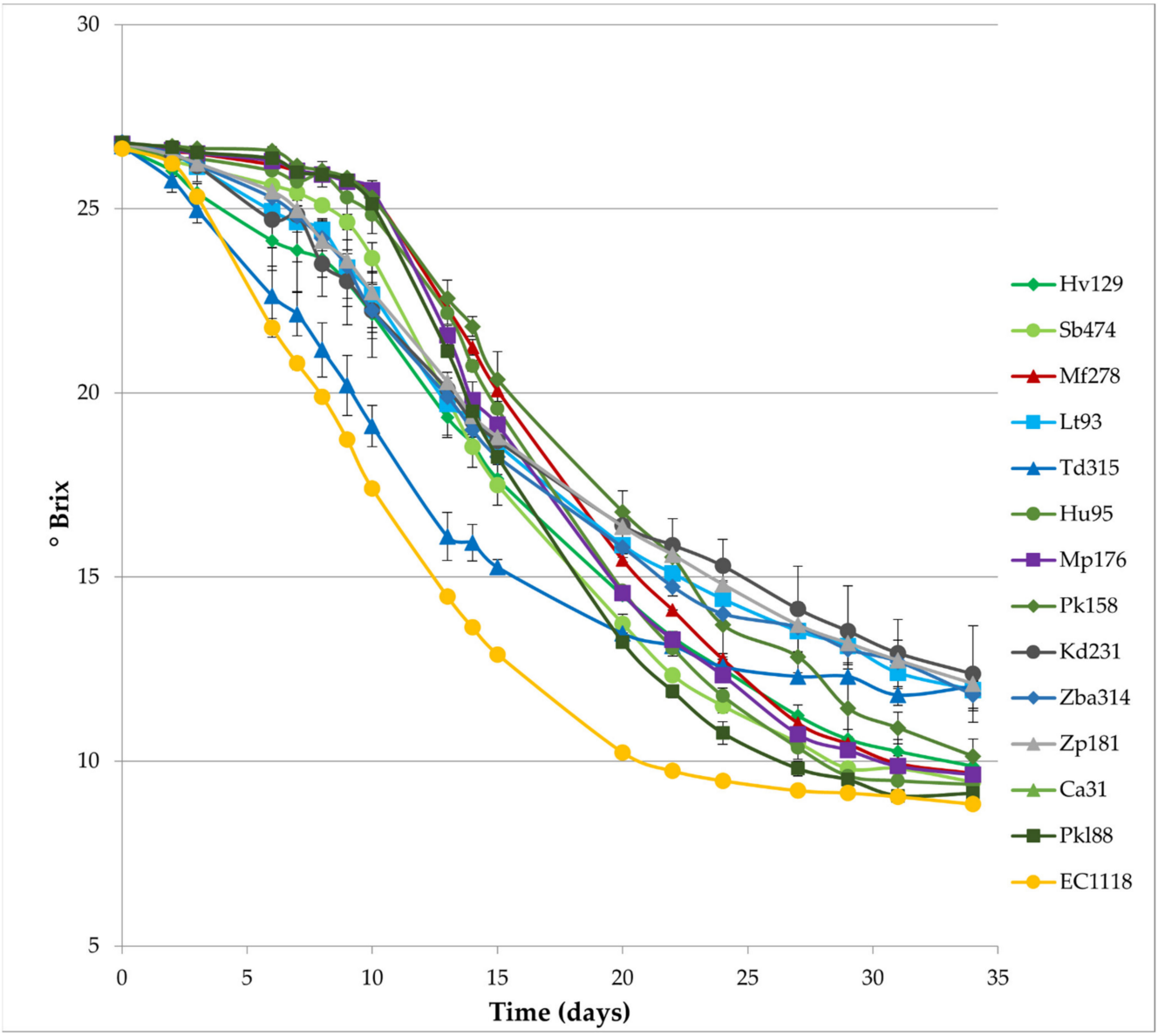


Figure 1. Kinetics of sequential fermentations inoculated with a non-*Saccharomyces* strain + *S. cerevisiae* EC1118. The data are mean values of three replicate fermentations ± SD.

Regarding the evolution of the yeast population at the quantitative level, the total number of yeast at the initial stage of fermentation (Fi) ranged between 7.50 and 8.30 Log CFU/mL (Table 2). The population increased during the exponential fermentation (Ft) (7.61–9.06 Log CFU/mL) in all processes except in the Lt93 assays. At the end, the number of yeasts decreased in all fermentations (5.02–7.74 Log CFU/mL), although with Pkl88, the value was similar to that at the beginning of fermentation. The differences in yeast counts were significant among fermentations only during the exponential phase, with

Sb474 reaching the highest values and Lt93 reaching the lowest ones (Table 2). Considering all fermentation phases, Sb474 showed the highest number of yeasts followed by Kd231; whereas Hv129 and Zp181, Lt93, and Td315 rendered the lowest counts at the initial (Fi), exponential (Ft), or final (Ff) stages, respectively.

Table 2. Heat map of the number of viable yeasts at different stages of sequential fermentation with non-*Saccharomyces* yeasts + *S. cerevisiae* EC1118 (Sc) expressed as log of colony forming units per milliliter (Log CFU/mL). The darkest green indicates the highest number of yeasts, and red indicates the lowest.

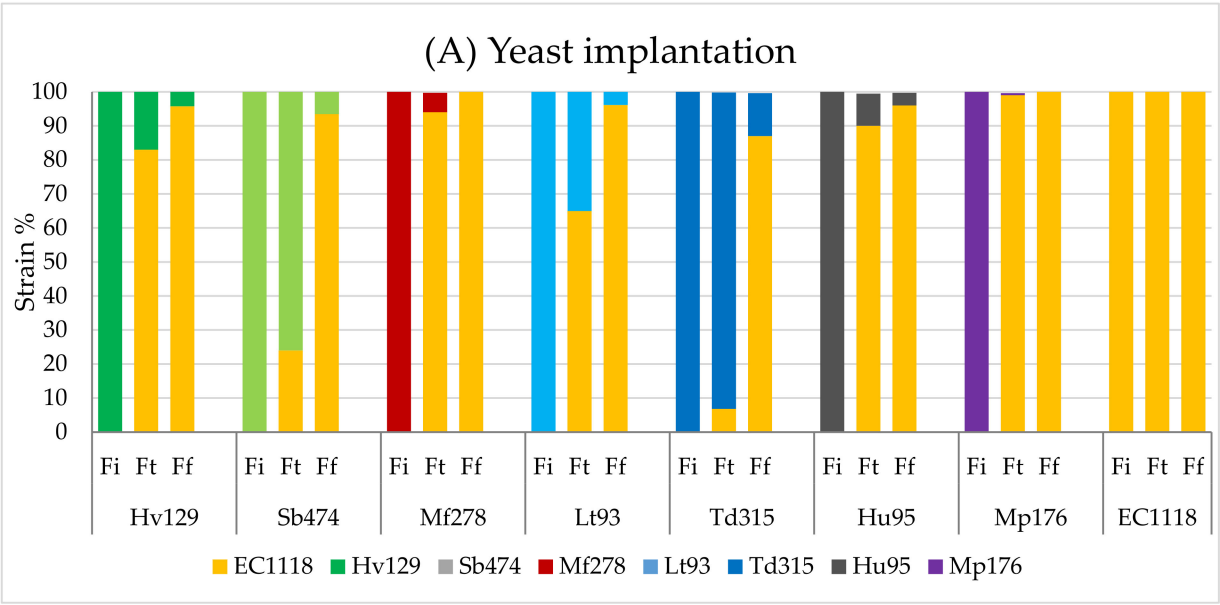
Fermentation	Fi	Ft *	Ff
Hv129 + Sc	7.50	8.19 ^{a}	6.44
Sb474 + Sc	8.26	9.06 ^{b}	7.74
Mf278 + Sc	8.02	8.71 ^{ab}	6.96
Lt93 + Sc	7.91	7.61 ^{a}	6.68
Td315 + Sc	7.96	8.76 ^{ab}	5.02
Hu95 + Sc	7.66	8.78 ^{ab}	6.48
Mp176 + Sc	7.98	8.68 ^{ab}	6.75
Pk158 + Sc	7.83	8.80 ^{ab}	7.49
Kd231 + Sc	8.30	8.97 ^{ab}	7.32
Zba314 + Sc	7.73	8.49 ^{ab}	5.97
Zp181 + Sc	7.51	8.36 ^{ab}	6.39
Ca31 + Sc	7.74	8.90 ^{ab}	6.74
Pkl88 + Sc	7.60	8.57 ^{ab}	7.73
EC1118 (Control-Sc)	7.86	8.38 ^{ab}	6.60

The data are mean values of three replicate fermentations. * Different superscript letters in the Ft column indicate significant differences according to Tukey's test ($p < 0.05$) among fermentations.

3.2. Yeast Implantation in Sequential Fermentations

The proportions of non-*Saccharomyces* and *S. cerevisiae* strains at different stages of mixed fermentations were assessed via colony morphology on WL plates and confirmed by genetic techniques. The results varied among fermentations (Figure 2). At the initial stages, all fermentations were 100% dominated by the first inoculum, as expected, since the must was pasteurized. However, after the sequential addition of *S. cerevisiae*, the proportion of non-*Saccharomyces* was strain dependent. A first group of yeasts, including Td315, Sb474, and Kd321, remained as dominant yeasts during tumultuous fermentation, but their population decreased towards the end. Other species, such as Lt93, Pk158, and *Zygosaccharomyces* spp., were also at important proportions at the middle stages (22–35%) and either decreased or were not isolated in the final stage. The *Hanseniaspora* species survived during all fermentation stages but at frequencies lower than 20% at the tumultuous stage and at less than 5% at the end. Finally, a third group comprised *Metschnikowia* spp., Ca31, and Pkl88, species in which viability decreased seriously after the addition of *S. cerevisiae*; they were isolated at low frequencies at the middle stages (>6%) and were absent at the end.

The loss of viability of the non-*Saccharomyces* yeasts in mixed fermentations was related to several factors, especially the presence of ethanol or anaerobic conditions, which increase as fermentation progresses [2]. Accordingly, several studies revealed that the survival of certain non-*Saccharomyces* increased with oxygen availability [30,33,34]. Recently, cell-to-cell contact mechanisms and the secretion of antimicrobial peptides by *S. cerevisiae* have also been reported as possible causes of inhibition of non-*Saccharomyces* yeasts [35,36]. In addition, the proportion and timing of inoculated strains influence their dynamics during fermentation [32,37].



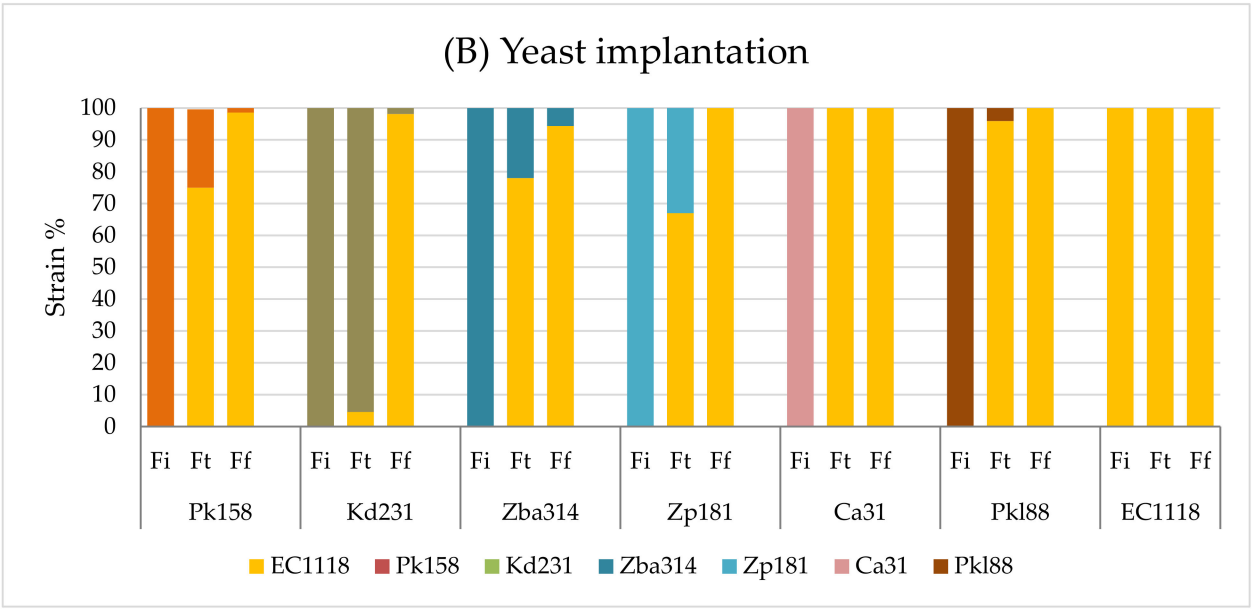


Figure 2. The implantation ability of different non-*Saccharomyces* yeast species at the initial (Fi), middle (Ft), and final stages (Ff) of sequential fermentations of (**A**) Hv129, Sb474, Mf278, Lt93, Td315, Hu95, and Mp176; (**B**) Pk158, Kd231, Zba314, Zp181, Ca31, and Pkl88; and the control EC1118.

In this study, *T. delbrueckii* Td315, *Starm. bacillaris* Sb474, and *K. dobzhanskii* Kd231 were still the dominant yeasts at high proportions after the addition of *S. cerevisiae* (93%, 76%, and 95%, respectively), although their proportions decreased at the end. The prevalence of *T. delbrueckii* in sequential fermentations at the tumultuous and later stages was recently reported in multi-starter fermentations [38], and it had already been found at pilot-scale fermentations [39,40], especially when a killer *T. delbrueckii* strain was used [41,42], or in the production of wines with reduced sulfites [43]. However, other authors observed a reduction in *T. delbrueckii* and other non-*Saccharomyces* from two to four days after the second inoculation [44] in laboratory fermentations. The alcohol tolerance of this species could explain its prevalence until the late fermentation stages [10]. Similarly, the pop-

ulation of *Starm. bacillaris* remains at high frequencies a few days after the sequential inoculation of *S. cerevisiae*, but its populations declined toward the end of fermentation, as previously found [45,46]; the survival time was strain dependent and strongly influenced by the timing of the second inoculum. In addition, the prevalence of *Starm. bacillaris* in mixed fermentations has been related to oxygen availability and cell-to-cell contact mechanisms [34,36]. Moreover, these studies support our findings of a large population of *Starm. bacillaris* during all stages of fermentation (Table 2). *K. dobzhanskii* Kd321 fermentations also had high yeast population levels at the beginning and middle stages. This species has been isolated from damaged grapes [8], but their fermentative potential has not yet been evaluated.

The survival of species, such as *L. thermotolerans* Lt93, *P. kudriavzevii* Pk158, and *Zygosaccharomyces* spp., was lower than the previous yeasts, but they were still isolated at important proportions at the middle stages (22–35%) of fermentation. Several studies have shown that the population of *L. thermotolerans* decreases as soon as the *S. cerevisiae* strain is inoculated (for a review, see [9]) and remains at low levels or is not isolated in the final stages. Further studies in our laboratory evidenced that Lt93 was unable to impose itself in fermentations with white grape varieties, but it successfully dominated red grape fermentation, remaining at high proportions even at the end [47]. Recently, the evaluation of the nitrogen treatment showed that the addition of diammonium phosphate (DAP) resulted in higher cell counts for *L. thermotolerans* and a slower decline after the inoculation of *S cerevisiae* [48]; however, this metabolic trait could be strain dependent [49]. Little information is available about the enological traits of *P. kudriavzevii*, but the data confirmed that, in mixed fermentations, its population rapidly decreased due to the fact of its weak ethanol tolerance [50]. Regarding *Zygosaccharomyces*, Zhu et al. [51] reported a fast decrease in viability in sequential fermentations after 8 days; however, in multi-starter fermentations, the maximum growth was reached in the late fermentation stage, and it was maintained rather stably until the end of fermentation [38].

The *Hanseniaspora* species survived during all stages of fermentation but at frequencies lower than 20% at the tumultuous stage and at less than 5% at the end. Despite the poor fermentation ability of *Hanseniaspora* species, the strains tested here persisted until the late stages of fermentation. Lleixà et al. [52] described slower fermentation kinetics and the persistence of *H. vineae* as a monoculture. In contrast, sequential fermentations using *H. vineae* under standard winemaking conditions evidenced the slower fermentation speed, but its presence was only 1% after day ten [53]. Similarly, a fast decline in the *H. uvarum* population after the addition of *S. cerevisiae* was reported [54,55].

Finally, the *Metschnikowia* spp., Ca31, and Pkl88 viabilities decreased seriously after the addition of *S. cerevisiae*, being found at only approximately 5% at the middle stages and being absent at the end. *Metschnikowia* is resistant to *S. cerevisiae* killer toxins and no antagonistic interactions exist between these species, so the rapid decrease was related to its low ethanol resistance and to the lack of oxygen [11,56]. Regarding *Pichia kluyveri*, some studies reported a fast decline after the addition of *S. cerevisiae* [44,57]; however, when the proportion of *P. kluyveri* to *S. cerevisiae* was 9:1 in a co-inoculation, its persistence was approximately 9 days [58]. In our study, under sequential inoculation, *P. kluyveri* was still present after 15 days of fermentation, although at a low proportion. As with previous yeast species, the results highlight the importance of the inocula ratio and the binomial selection of strains [59].

C. apicola is an osmotolerant yeast that has been isolated in grape must with high sugar content, and it has been barely explored in winemaking [60]. These authors remarked on the ability of some *C. apicola* and strains to grow in 14% *v/v* ethanol; in contrast, Ca31 was already absent at middle fermentation.

3.3. Chemical Characteristics of Wine

The presence of different yeasts during fermentation influences the wine's chemical characteristics. Table 3 summarizes the basic chemical composition of the wines obtained

in this study. The results evidenced the effect of yeast in almost all of the parameters determined. *T. delbrueckii* Td315 reduced the alcohol content by up to 2.7% *v*/*v* compared with the control wines. Wines obtained with other yeast species also decreased the alcoholic degree but to a lesser extent, for instance, 1.1% *v*/*v* in the case of *L. thermotolerans* or *Z. bailii*, 1.3% *v/v* with *K. dobzhanskii*, and 1.5% *v*/*v* with *Z. parabailii*. Importantly, we note that all of these wines contained more than 5 g/L of residual sugars, indicating that these yeasts were unable to complete fermentation. In addition, these strains showed lower ethanol yields than the control. However, certain strains of *T. delbrueckii* and *L. thermotolerans* presented the ability to reduce alcohol and achieved sugar completion [9,10]. The result obtained here could be explained by the extreme sugar conditions in this assay. Further studies carried out at the micro-vinification scale showed that Td315 was unable to complete Treixadura fermentations [61]. Similarly, Velázquez et al. [41] also reported the presence of residual sugars using a killer strain of *T. delbrueckii,* which confirmed that the properties of this species are strain dependent. Concerning *L. thermotolerans*, we recently found that Lt93 reduced the alcohol content of red wine by 0.8%, leaving <4 g/L of residual sugars [47]. Other authors reported lower reductions in alcohol content but a complete depletion of sugars [31,44]. Surprisingly, *Starm. bacillaris* and *Metschnikowia* spp. did not decrease the alcohol content in wine under the conditions of this study. However, both species are widely recognized as a useful biological tool for alcohol reduction [18,45,62,63]. Actually, strains Sb474 and Mf278 did reduce the ethanol in Treixadura wines in micro-vinification assays in the experimental winery of Evega [61].

Table 3. Basic chemical characteristics of wines obtained by sequential fermentation with non-*Saccharomyces* strains + *S. cerevisiae* EC1118 (Sc).

Fermentation	Ethanol (% *v*/*v*)	Glucose + Fructose (g/L)	Ethanol Yield (g/g)	Total Acidity (g Tartaric/L)	Volatile Acidity (g Acetic/L)	Glycerol (g/L)	Sensory Evaluation *
Hv129 + Sc	16.9 ± 0.12 ^{a}	6.8 ± 0.4 ^{bc}	0.52 ± 0.01 ^{ab}	6.4 ± 0.4 ^{abc}	0.61 ± 0.13 ^{abc}	6.7 ± 0.8 ^{fg}	3
Sb474 + Sc	16.9 ± 0.03 ^{a}	3.4 ± 0.2 ^{c}	0.52 ± 0.01 ^{ab}	7.5 ± 0.1 ^{ab}	0.79 ± 0.01 ^{ab}	**11.1 ± 0.5** ^{a}	1−
Mf278 + Sc	16.8 ± 0.05 ^{ab}	3.8 ± 0.4 ^{bc}	0.52 ± 0.01 ^{abc}	7.2 ± 0.0 ^{abc}	0.66 ± 0.01 ^{abc}	8.7 ± 0.2 ^{bcd}	**5+**
Lt93 + Sc	**15.8 ± 0.74** ^{bc}	17.0 ± 11.4 ^{bc}	**0.49 ± 0.02** ^{abc}	**8.8 ± 0.3** ^{abc}	0.62 ± 0.01 ^{abc}	7.2 ± 0.3 ^{cdefg}	**5+**
Td315 + Sc	**14.2 ± 0.21** ^{d}	42.2 ± 4.7 ^{a}	**0.44 ± 0.01** ^{d}	6.9 ± 0.3 ^{gh}	0.47 ± 0.09 ^{c}	6.3 ± 0.5 ^{g}	3
Hu95 + Sc	16.8 ± 0.04 ^{ab}	5.5 ± 0.5 ^{bc}	0.51 ± 0.01 ^{abc}	7.8 ± 0.1 ^{cde}	0.63 ± 0.07 ^{abc}	7.1 ± 0.3 ^{efg}	3
Mp176 + Sc	16.9 ± 0.06 ^{a}	3.2 ± 0.8 ^{c}	0.53 ± 0.00 ^{a}	7.2 ± 0.1 ^{fg}	0.58 ± 0.01 ^{bc}	8.8 ± 0.2 ^{bc}	**5+**
Pk158 + Sc	16.8 ± 0.12 ^{ab}	4.5 ± 1.7 ^{bc}	0.53 ± 0.00 ^{ab}	**8.2 ± 0.2** ^{abc}	0.80 ± 0.14 ^{ab}	7.2 ± 0.1 ^{defg}	3
Kd231 + Sc	**15.6 ± 1.05** ^{c}	18.4 ± 14.6 ^{bc}	**0.48 ± 0.03** ^{bc}	**8.5 ± 0.4** ^{ab}	0.46 ± 0.08 ^{c}	9.7 ± 0.7 ^{ab}	0−
Zba314 + Sc	**15.8 ± 0.41** ^{bc}	15.2 ± 6.7 ^{bc}	**0.49 ± 0.01** ^{abc}	**8.4 ± 0.1** ^{bcd}	0.59 ± 0.14 ^{bc}	8.4 ± 1.1 ^{bcde}	1−
Zp181 + Sc	**15.4 ± 0.30** ^{c}	20.3 ± 4.4 ^{b}	**0.48 ± 0.01** ^{cd}	**8.2 ± 0.2** ^{bcd}	0.68 ± 0.05 ^{abc}	8.2 ± 0.8 ^{bcdef}	2−
Ca31 + Sc	16.9 ± 0.05 ^{a}	2.9 ± 0.3 ^{c}	0.52 ± 0.01 ^{ab}	7.7 ± 0.1 ^{cdef}	0.67 ± 0.03 ^{abc}	7.8 ± 0.2 ^{cdefg}	3
Pkl88 + Sc	16.8 ± 0.04 ^{ab}	4.5 ± 0.2 ^{bc}	0.52 ± 0.01 ^{abc}	7.7 ± 0.1 ^{cdef}	0.82 ± 0.03 ^{a}	7.1 ± 0.4 ^{efg}	**5+**
EC1118 (Sc control)	16.9 ± 0.04 ^{ab}	2.2 ± 0.3 ^{c}	0.53 ± 0.00 ^{ab}	7.9 ± 0.0 ^{bcd}	0.56 ± 0.02 ^{c}	6.4 ± 0.2 ^{g}	3

The data are the mean values of three replicate fermentations ± SD. The initial sugar concentration of the must was 269.5 g/L. The ethanol yield (g/g) was calculated as ethanol production (g/L) per sugar consumption (g/L). Different superscript letters in the same column indicate significant differences according to Tukey's test ($p < 0.05$). * Sensory evaluation: 4 and 5−, positive (+); 3−, no effect; 1 and 2−, negative (−).

The total acidity of wines ranged between 8.8 and 6.4 g/L. The yeast *L. thermotolerans* Lt93 increased the acidity by 0.9 g/L with respect to the control (Table 3) as reported by [37]. Moreover, the wines obtained with this yeast contained lactic acid (1.63 ± 0.32 g/L), whereas in the remaining species, the content of this metabolite was <0.2 g/L (data not shown). This result confirmed that *L. thermotolerans* is a lactic acid producer, as previously described [10,64,65], although this property is strongly variable depending on the strain and fermentation conditions [31]. For instance, Gobbi et al. [37] found 6.38 g/L at the winery scale; similarly, Lt93 produced 7.1 g/L of lactic acid in red wine fermentation [47]. The wines obtained with *K. dobzhanskii*, *P. kudriavzevii*, and *Zygosaccharomyces*

also reached higher values of acidity than the control wine. By contrast, *T. delbrueckii* and *H. vineae* reduced the wine acidity by 1.5 and 1.0 g/L, respectively, compared with the control wine. Similar results were obtained by sequential fermentation with other strains of *T. delbrueckii* [39,41,42]; however, *Hanseniaspora* species have been associated with an increase in wine acidity in the literature [55]. Regarding volatile acidity, the values ranged between 0.46 and 0.82 g/L for wines elaborated with *K. dobzhanskii* Kd231 and *P. kluyveri* Pkl88, respectively.

The content of glycerol has a special relevance at the sensorial level because this compound contributes positively to the mouthfeel, sweetness, and complexity of wine. As generally reported for non-*Saccharomyces* yeasts [3], all yeast strains evaluated here, except Td315, produced higher levels of glycerol in sequential fermentations than *S. cerevisiae* as a monoculture (Table 3). The increment was particularly notorious with Sb474 as well as with *K. dobzhanskii* Kd231 and *Metschnikowia* spp. The increment in glycerol content by non-*Saccharomyces* was attributed to different factors including their ability to redirect sugar consumption for the production of alternative compounds such as glycerol [66].

Finally, at the sensory level, the wines obtained with *Metschnikowia* spp., *L. thermotolerans*, and *P. kluyveri* achieved the best scores, being positively evaluated by all tasters. Several factors influence the sensorial perception of wine including alcohol, acidity, glycerol content, and aroma profile and the interaction and balance among these components [67]. The high alcohol content of wines (>16.8) could have conditioned the evaluation in this case.

3.4. Wine Aroma Composition

Yeasts involved in fermentation modulate the wine aroma by converting the grape components into ethanol and by releasing numerous minor but sensorially important metabolites [7,67]. Particularly, higher alcohols, esters, and volatile fatty acid provide the greatest contribution to secondary aroma. The biosynthesis of these compounds is species- and strain dependent, pointing out the importance of a smart strain selection of strains of biotechnological interest [7].

Accordingly, the yeast strains/species used in this study influenced the fermentative, volatile composition of wines. A total of 26 volatiles, including alcohols, esters, volatile organic acids, and other compounds, were determined. The concentration of the main chemical families and individual compounds are shown in Tables 4 and S1, respectively. The statistical analysis of the results evidenced significant differences among wines for all parameters (Table S1). Moreover, the concentration of 14 compounds were above their OAV.

Higher alcohols are the largest group of volatile compounds. At optimal levels (<300 mg/L), they contribute to the wine aroma complexity and fruity characters, whereas at high concentrations, they impart a pungent smell and taste [67]. The content of higher alcohols ranged between 183.328 and 591.286 mg/L in wines from Zp181 and Kd231, respectively. Only with Kd231 was the concentration over the limits considered negative for wine quality. Seven of the yeasts evaluated produced lower concentrations of these volatiles in wines than *S. cerevisiae*, especially *Zygosaccharomyces, Starm. bacillaris*, and *H. vineae*. Similarly, lower contents of higher alcohols compared with control fermentations with *S. cerevisiae* have been reported in sequential fermentations with *P. kluyveri*, *Kluyveromyces thermotolerans*, and *M. pulcherrima* [57] and with *H. vineae* and *H. uvarum* [52–54]. However, no differences in these volatiles were obtained in mixed fermentations for *Starm. bacillaris* [34,46,68]. On the contrary, another group of yeasts including *Metschnikowia* spp. (Mf278 and Mp176), *T. delbrueckii* Td315, *L. thermotolerans* Lt93, *P. kudriavzevii* Pk158, and *K. dobzhanskii* Kd231 presented a higher content of these alcohols than the control (Table 3). The differences were mainly due to the concentration of isoamyl alcohol, which was especially significant (>2 times) with Kd231; these wines also contained a higher amount of isobutanol (Table S1). These findings agree with those reported for these species by other authors [26,33,39,43,61,62]. By contrast, confirming the great strain variability among

non-*Saccharomyces*, several studied have reported that *L. thermotolerans* and *T. delbrueckii* produced fewer higher alcohols than *S. cerevisiae* [37,41,57,64,69].

Table 4. Heat map of the concentrations (mg/L) of some volatile compound families in the wines obtained by sequential fermentation with non-*Saccharomyces* yeasts + *S. cerevisiae* EC1118 and with *S. cerevisiae* as a monoculture. The darkest green indicates the highest content, and white indicates the lowest content of each compound.

Fermentation	Higher Alcohols	Esters	C3–C5 Fatty Acids	C6–C10 Fatty Acids
Hv129 + Sc	195.824	0.442	3.309	1.640
Sb474 + Sc	185.974	0.347	4.465	2.654
Mf278 + Sc	260.457	0.942	2.015	5.411
Lt93 + Sc	246.885	0.415	2.810	1.565
Td315 + Sc	272.385	0.173	4.156	0.598
Hu95 + Sc	212.950	0.770	2.584	4.342
Mp176 + Sc	235.175	0.792	2.990	4.599
Pk158 + Sc	233.540	0.977	3.969	4.521
Kd231 + Sc	591.286	0.355	6.028	0.976
Zba314 + Sc	191.697	0.303	5.657	0.863
Zp181 + Sc	183.328	0.408	4.613	1.013
Ca31 + Sc	212.528	0.773	5.256	4.196
Pkl88 + Sc	209.564	1.746	3.869	5.312
EC1118 (Sc control)	229.637	0.806	3.453	3.794

The data are the mean values of three repetitions. The differences among wines were significant at $p < 0.001$ according to Tukey's test.

Esters are qualitatively an important group of volatile compounds because they contribute pleasant fruity and floral notes to wine [67]. The main ester in wine is ethyl acetate, which imparts a spoilage character at levels of 150–200 mg/L [70]. In this study, most non-*Saccharomyces* species produced wines containing higher amounts of this compound than *S. cerevisiae*, with Hu95 reaching the highest concentration (Table S1). *Hanseniaspora* species have been reported as high producers of ethyl acetate, a fact that limits its use as a starter, although it can be controlled under sequential fermentations [7,27]. In contrast, the Td315, Kd231, and Lt93 wines presented lower amounts of ethyl acetate than the control wines. Regarding minor esters, their content was improved by *Pichia* and *Metschnikowia* spp. compared with *S. cerevisiae*, with differences especially significant in Pkl88 wines (Table 4). The values ranged between 0.173 and 1.746 mg/L, obtained with Td315 and Pkl88, respectively. Considering individual ethyl esters and isoamyl acetate, Pkl88 wines achieved the highest concentration among them all. Mf278 wines also had higher contents of ethyl hexanoate and ethyl octanoate than the control wines (Table S1). The high production of esters by *P. kluyveri* have been related to the presence of enzymatic activities [59,71,72], although Dutraive et al. [44] did not confirm this ability in wines fermented with a commercial *P. kluyveri* strain. Similarly, the application of *Metschnikowia* spp. strains in mixed cultures usually increases the total concentration of esters in wine [31,62]. In this study, the wines obtained with Mf278 had a content of esters higher than *S. cerevisiae*, whereas no differences were found in the case of Mp176. Despite their ester content, this species contributed positively to wine sensory attributes, as did *L. thermotolerans* Lt93. The latter, similar to the remaining yeasts evaluated, showed a lower content of esters than the control wine as reported in the literature for several non-*Saccharomyces* [37,39,52,64,73]. However, in disagreement with our results, some strains of *Starm. bacillaris* produced higher concentrations of esters than the control fermentations [46,68]. Similarly, several studies revealed that *L. thermotolerans* had a positive impact on wine esters [9]. The Lt93 wines were also highlighted by their content of ethyl lactate (67.773 mg/L), 10-fold more compared with the remining wines, as expected due to the high lactic acid production of this species [31,37]. In the case of *T. delbrueckii*, the data regarding ester production are also contradictory, confirming its strain dependence [10]. The effect of *Hanseniaspora* is less

known, but this species has been associated with an increase in ester content [54]. Likewise, *Z. bailii* was proposed as a potential yeast to increase ethyl esters and, therefore, the aroma complexity of wine [74].

Volatile fatty acids in wines are linked to unpleasant aromas such as cheese and rancid notes; however, they are relevant compounds because they contribute to the equilibrium and complexity of wine as precursors for ethyl esters biosynthesis [70,75]. Consequently, the concentration of C6–C10 fatty acids followed the same trend as esters as expected (Table 4). However, the C3–C5 fatty acids showed an opposite trend. In addition, the sensory results of wines evidenced that the wines with the best scores were those in which the concentration of esters and C6–C10 fatty acids were the highest such as the Pkl88, Mf278, or Mp176 wines. Shinohara [76] already stated that concentrations of 4–10 mg/L of C6–C10 fatty acids provide a mild and pleasant aroma. As an exception, the wine from Lt93 was also well appreciated despite its moderate content of these desirable compounds. The relation between volatile acids and ethyl esters content was observed in numerous mixed fermentations involving different non-*Saccharomyces* yeasts [39,46,52,68].

The concentration of other volatile compounds also differed among the wines elaborated with different strains (Table S1), indicating the importance of yeast species as a tool in modulating the aroma profile of wines. The differences allowed for the separation of wines by principal component analysis (PCA) based on their chemical composition (Figure 3). The PCA, including volatile compounds with an OAV > 1 and the sum of the main chemical families, explained 58.90% of the total variance. Principal component 1 (PC 1) explained 41.71% of the variance, and the second principal component (PC 2) explained 17.19%. Thus, the wines obtained with *Pichia* and *Metschnikowia* spp. (Pkl88, Pk158, Mf278, and Mp76) were located in the first quadrant (the positive part of PC 1 and PC 2) (Figure 3a) of the biplot characterized by a higher content of ethyl esters and long-chain fatty acids (Figure 3b); Ca31 wines were also plotted in this quadrant. In the third quadrant, the wines from Td315, the *Zygosaccharomyces* spp. strains, Hv129, and Lt93, which are associated with higher alcohol contents and short-chain fatty acids, appeared. Sb474 wines were plotted in the negative part of PC 2, whereas Hu95 wines were located in the second quadrant due to the fact of its high content of ethyl acetate. In the same area, the control wines appeared but were closer to the first quadrant. The Kd231 wines were clearly separated from the remaining wines in the fourth quadrant due to the fact of their higher content of alcohols.

To conclude, the results of this study highlighted the enological potential of non-*Saccharomyces* yeast autochthonous from Galicia. The strains evaluated belong to different species, and our findings agree with those previously reported by other authors; however, in some cases, the behavior of a given species was strain dependent, and the *S. cerevisiae* strain chosen as the secondary inoculum also determined the activity of non-*Saccharomyces* yeast. Therefore, future research will focus on the optimization of fermentation conditions for each species/strain, on the selection of appropriate *S. cerevisiae* strains to enhance their effect, and the evaluation of non-*Saccharomyces* yeasts as a tool to differentiate wines from local varieties grown in Galicia.

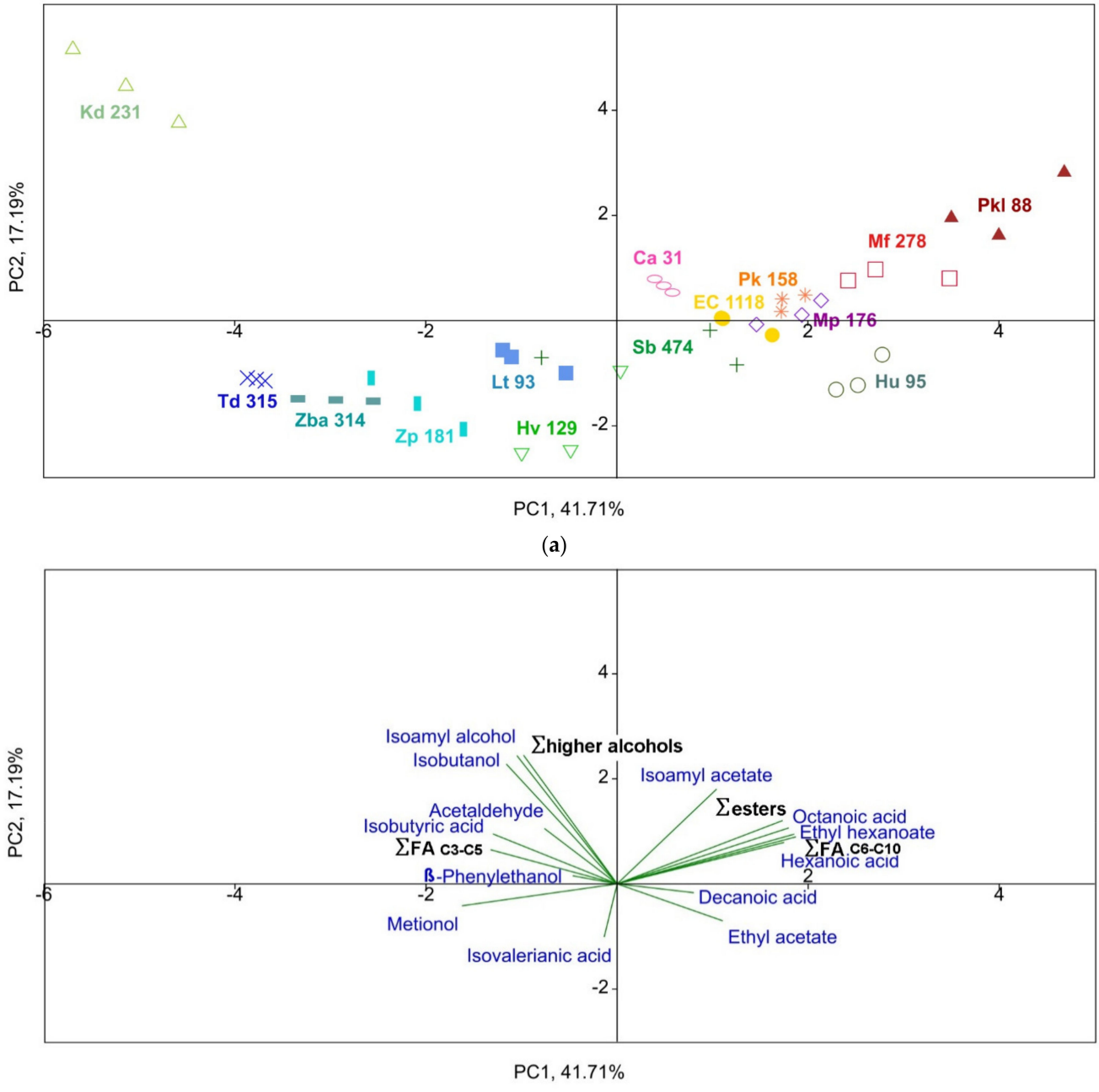


Figure 3. Principal component analysis of wines obtained by sequential fermentation with non-*Saccharomyces* yeasts + *S. cerevisiae* EC1118 and with *S. cerevisiae* as a monoculture based on their volatile composition: (**a**) yeast strain map and (**b**) biplot of the first two components (PC) for compounds with OAV > 1.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fermentation7030183/s1. Table S1: Heat map of the concentration of volatile compounds (mg/L) in the wines obtained by sequential fermentation with non-*Saccharomyces* yeasts + *S. cerevisiae* EC1118 and with *S. cerevisiae* as a monoculture, and significance according to one-way ANOVA.

Author Contributions: P.B. and D.C. conceived and designed the experiments; P.B. and M.J.G. performed the fermentations and carried out the microbial and sensorial analysis; M.J.L. and E.S. carried out the chemical analysis of wines; P.B. and D.C. analyzed the data and wrote and critically revised the manuscript before submission; P.B. administered the project; and P.B. acquired the funding. All authors have read and agreed to the published version of the manuscript.

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