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Sequential Fermentation with Non-*Saccharomyces* Yeasts Improves the Chemical and Sensory Characteristics of Albariño and Lado Wines

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Abstract: The application of non-*Saccharomyces* yeast in mixed fermentations with *Saccharomyces cerevisiae* is a useful tool to enhance wine quality. In this study, *Metschnikowia fructicola* Mf278 and *Pichia kluyveri* Pk1 were used in sequential fermentations with *S. cerevisiae* XG3 to ferment grape musts from Albariño and Lado. The development of fermentations was monitored by daily measurements of density and temperature, and sampling at the beginning, tumultuous, and final stages for microbiological control. The basic chemical parameters of wine were determined using the OIV official methodology, whereas the fermentative aroma compounds were quantified by GC–MS. *M. fructicola* Mf278 and *P. kluyveri* Pk1 were the predominant yeasts at the initial stages of sequential fermentations but, after the addition of *S. cerevisiae* XG3, they rapidly declined. A codominance of different *S. cerevisiae* strains was observed at the middle and final stages of fermentation. At the chemical level, Mf278 lowered the volatile acidity and increased the glycerol content of wines. Moreover, *M.* Mf278 and Pk1 increased the content of fermentative esters and fatty acids of wines. These compounds contribute fruity and floral notes to the wines that stood out over wines made only with *S. cerevisiae*, and were better valued at the sensory level.

Keywords: *Metschnikowia fructicola*; *Pichia kluyveri*; sequential fermentation; *Saccharomyces cerevisiae*; wine; chemical composition; fermentative aroma; sensory analysis; Albariño; Lado

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1. Introduction

The potential of non-*Saccharomyces* yeasts in winemaking have long been recognised [1,2]. Thus, in recent decades, these wine yeasts have evolved from being considered spoilage-causing microorganisms to becoming desirable tools for improving wine complexity [3]. There are several technological properties of non-*Saccharomyces* yeasts that have been related to their positive modulation of wine chemical and sensory properties of wines, including an increase in wine acidity and glycerol content, reduction of acetic acid and ethanol content, and the production of secondary metabolites, and secretion of enzymes that enhance wine aroma [4]. Furthermore, it is worth mentioning that the ability to reduce the alcohol content and/or increase the acidity of wines makes non-*Saccharomyces* yeasts a good biological alternative to face the consequences of climate change on wines [5,6]. Nowadays the wide availability of non-*Saccharomyces* yeast at the commercial level confirms their relevant role as starters in the wine industry [7,8]. A broad range of strains of the

species *Torulaspora delbrueckii*, *Lachancea thermotolerans*, and *Metschnikowia pulcherrima* are available as active dry yeasts (ADY), but many other species can be found at different formats such as, active frozen yeast, compressed yeast, cream yeast, encapsulated yeasts, or immobilised yeasts [8].

Metschnikowia spp. are some of the most studied non-*Saccharomyces* yeasts due to their widespread occurrence in grapes and early stages of fermentation, as well as being part of the resident yeast population in wineries (for a review see [9,10]). *Metschnikowia* is characterised by a poor fermentative power and low ethanol tolerance; therefore, it must be used in combination with other yeast like *Saccharomyces cerevisiae* to complete fermentation. Furthermore, *Metschnikowia* is a high producer of extracellular hydrolytic enzymes that release varietal aromas and color precursors enhancing wine quality [9,10]. The improvement in wine complexity is attributed to an increase in the content of volatile compounds such as esters, terpenes, and thiols [11–18]. In the context of climate change, the ability of *Metschnikowia* to reduce the alcohol content is of special interest for wines from warm regions [6,19,20]. Finally, the antimicrobial properties of *M. pulcherrima* against spoilage yeasts and fungi add an extra benefit of *Metschnikowia* as natural bioprotection of wines [21,22]. The production of pulcherrimin, a reddish-brown pigment that chelates iron, is responsible for this antagonism [23]. Recent studies have exploited the bioprotective activity of *Metschnikowia* and its potential as an aroma enhancer for wine diversification [24,25].

The application of *Pichia kluyveri* in winemaking is not as common as that of other non-*Saccharomyces* yeasts. However, the data available on this species support its oenological potential, mainly due to its contribution to the aroma profile and glycerol content of wines [26]. *P. kluyveri* is a biofilm-forming yeast, with very low- or non-fermentative ability; therefore, it is used in mixed fermentations (co- or sequential) with a fermentative yeast such as *S. cerevisiae*. Under these conditions, its oxidative metabolism is responsible for the reduction of ethanol in wines [19]. In addition, the impact of *P. kluyveri* on wine aroma has been associated with the production of compounds such as thiols, fruity esters, and higher alcohol acetates [11,17,27–29]. At the sensorial level, these volatiles positively impact the overall impression of wines and impart fruity and floral notes [11,29].

Beyond the benefits of a given species, it is known that the complex interaction of native non-*Saccharomyces* in spontaneous fermentations imparts a regional character to the wines [30,31]. For this reason, the isolation and selection of yeasts specific to a given region is of great interest in order to differentiate its wines. In this sense, previous studies carried out at Estación de Viticultura e Enoloxía de Galicia (Evega) allowed the isolation and identification of indigenous wine yeasts from different areas in Galicia (NW Spain) [32,33]. These yeasts are maintained at the yeast collection of Evega. A previous survey on the oenological traits of non-*Saccharomyces* yeasts from the Evega collection highlighted the positive oenological characteristics of some *Metschnikowia* spp., *P. kluyveri*, and *L. thermotolerans* strains [34]. Of those, *Metschnikowia fructicola* Mf278 produced Treixadura wines with enhanced aroma and sensory profiles [35].

In this study, two non-*Saccharomyces* yeasts, *M. fructicola* Mf278 and *P. kluyveri* Pk1, were applied in sequential fermentations with *S. cerevisiae* XG3 to ferment grape must from Albariño and Lado, two white grapevine varieties traditional from Galicia. Albariño is a prestigious white aromatic variety cultivated in several zones of Galicia, but also in North of Portugal. The growth of Lado is restricted to the Ribeiro Protected Denomination of Origin (DOP Ribeiro) [36]. This study aims to evaluate the fermentation dynamics, yeast implantation, and the influence of Mf278 and Pk1 on the chemical and sensory profiles of wines. To our knowledge, this is the first time that native non-*Saccharomyces* yeasts have been used to elaborate wines from Lado and Albariño. *M. fructicola* Mf278 or *P. kluyveri*

Pk1 enhanced the wine content of esters, resulting in more complex wines that achieved a better score at the sensory level.

2. Materials and Methods

2.1. Grape Must, Yeast Strains and Inoculum Preparation

Grapes from *Vitis vinifera* Lado and *V. vinífera* Albariño were harvested in Granxa D'Outeiro vineyard and processed in the winery facilities of this farm to obtain the must as described in [37]. Briefly, grapes were destemmed, crushed, and sulphited (24 mg SO_2/Kg grape). Then, the paste was cooled, pressed in a pneumatic press to recover the must and sulphited again (6 mg SO_2/Kg grape). The must obtained was transferred to a stainless-steel tank for a static settling. Lysis VC (2 g/100 L) and Phylia Cys (20 g/100 L)(PHYLIA® CYS (Oenofrance, Magenta, France) enzymes were added to favour settling. Finally, the clean musts from Lado and Albariño were transported to the experimental winery of Evega for fermentation. The basic characteristics of must from each variety are summarised in Tables 1 and 2. In addition, the yeast-assimilable nitrogen (NFA) was 249 mg/L and 154 mg/L for Albariño and Lado musts, respectively.

Table 1. Chemical characteristics of Albariño must and wines obtained by sequential fermentations, XG3 monoculture, and spontaneous fermentation.

Parameter/Albariño Wines	Grape Must	Pk + XG3	Mf + XG3	XG3	ESP
Probable degree/Alcohol content (%*v*/*v*)	13.4	12.9 ± 0.7	12.5 ± 0.6	12.7 ± 0.4	13.5
ºBrix/Glucose + Fructose (g/L) *	22.9	1.6 ± 0.6 ^{a}	0.2 ± 0.0 ^{a}	3.3 ± 1.4 ^{b}	0.3
Total acidity (g tartaric acid/L)	5.9	5.5 ± 0.1	5.6 ± 0.1	5.7 ± 0.1	5.8
Volatile acidity (g acetic acid/L) *		0.35 ± 0.06 ^{b}	0.28 ± 0.01 ^{a}	0.39 ± 0.06 ^{b}	0.27
pH (-)	3.47	3.52 ± 0.02	3.49 ± 0.01	3.52 ± 0.01	3.52
Malic acid (g/L)	3.6	2.5 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	2.6
Tartaric acid (g/L)	3.5	2.5 ± 0.1	2.6 ± 0.1	2.4 ± 0.1	2.4
Glycerol (g/L) *		5.7 ± 0.2 ^{a}	6.6 ± 0.3 ^{b}	6.2 ± 0.1 ^{a}	5.9
Total SO_2 (mg/L)	70	84 ± 3	66 ± 2	84 ± 5	75

Pk + XG3, Mf + XG3, and XG3 data are the mean values of three replicate fermentations ± SD. * Different superscript letters in the same row indicate significant differences according to Tukey's test ($p < 0.05$).

The non-*Saccharomyces* yeast strains used in this study were *M. fructicola* Mf278 and *P. kluyveri* Pk1. *M. fructicola* Mf278 belongs to the yeast culture collection maintained at Evega; its oenological potential has been previously proved [34,35]. *P. kluyveri* Pk1 was isolated from Granxa D'Outeiro musts in a 2022 campaign; preliminary studies at the laboratory scale revealed promising results that made it suitable for application in the winery. Yeast strains were maintained in YPD medium (yeast extract 1% *w*/*v*, peptone 2% *w*/*v*, glucose 2% *w*/*v*, and agar 2% *w*/*v* for solid media) at 4 °C. Inocula for fermentations were prepared as indicated in Blanco et al. [38]. Briefly, a pre-inoculum of each strain was transferred to a 2 L Erlenmeyer flask containing 1 L of YPD and incubated at 28 °C, 150 rpm, for 24 h in a SANYO orbital incubator. The cells were recovered by centrifugation, washed with sterile water and re-suspended in 100 mL of saline solution. The inocula concentration was calculated by serial dilutions and spread on YPD plates in duplicate. Plates were incubated at 28 °C and those containing between 20 and 200 colonies were used for viable yeast counting. *Saccharomyces cerevisiae* XG3 was available in Evega as active dry yeast (ADY) [39].

2.2. Fermentations

The must from each variety was homogeneously distributed in 5 L stainless steel tanks, which were inoculated (in triplicate) with the following yeasts:

- 3X—*M. fructicola* Mf278 + *S. cerevisiae* XG3 (sequential) (Code: Mf);
- 3X—*P. kluyveri* Pk1 + *S. cerevisiae* XG3 (sequential) (Code: Pk);
- 3X—*S. cerevisiae* XG3 (monoculture) (Code: XG3);
- 1X—No yeast addition (Code: ESP).

S. cerevisiae XG3 was added after rehydration as follows: 40 g/hL of yeast was rehydrated in 10 times its weight of warm water (35–40 °C) for 20 min by gently stirring, acclimated gradually by mixing with grape juice (1/2 its volume), and inoculated into the must. For sequential fermentations, the non-*Saccharomyces* yeast strain was first inoculated at 1×10^8 cells/mL. Once the fermentation had started and the density had decreased 5 g/L (day 3), the second inoculum, *S. cerevisiae* XG3, was added (dose 40 g/hL). A spontaneous fermentation (ESP) with indigenous yeast from the must was also performed. Fermentations were carried out at 17 °C in a cold room. Fermentation evolution was monitored by daily measurement of density (using a hydrometer) and temperature. In addition, samples were taken from must and at different stages of fermentation (Fi-initial, Ft-middle, and Ff-final fermentation) for microbiological control including count of viable yeasts and inoculum implantation. Lado fermentations were supplemented at a density of 1070 g/L with OptiFlore®O (20 g/hL) (Lamothe-Abiet, Bordeaux, France) to ensure the nutritional needs of the yeasts and optimise their fermentative activity. When the fermentations finished, the wines were racked to a new tank and sulphited (25 mg/L of free SO_2). Wine samples for chemical analyses were taken at the end of fermentation. In addition, after cold stabilisation for two months, the wines were bottled and stored in the winery until sensory analysis.

2.3. Microbiological Control

The samples from must and at initial, middle, and final fermentation were collected in sterile tubes. In the laboratory, they were adequately diluted in in 2% *w*/*v* buffered peptone water and spread on WL Nutrient Agar (Scharlau Microbiology, Barcelona, Spain) [40]. This medium allows the differentiation of the species used in this study based on their colony morphology. The plates were incubated at 28 °C until visible colonies appeared; those containing between 20 and 200 colonies were used for viable yeast count. Then, a representative number of colonies (20–25 per sample) were selected randomly and isolated on YEPD (yeast extract 1% *w*/*v*, peptone 2% *w*/*v*, glucose 2% *w*/*v*, and agar 2% *w*/*v* for solid media) for further characterisation. These isolates were replicated on Lysine media (Oxoid, Thermo Fisher Scientific, Madrid, Spain) to distinguish between *Saccharomyces* and non-*Saccharomyces* yeasts. The absence of growth on this media confirmed which isolates were *Saccharomyces*.

S. cerevisiae isolates were characterised at the strain level by analysis of mitochondrial DNA restriction profiles (mtDNA-RFLPs). Total DNA from each isolate was obtained as described by Querol et al. [41] and digested with the restriction endonuclease Fast digest *Hinf* I (Thermo Fisher Scientific, Madrid, Spain). The restriction fragments were separated by electrophoresis on a 0.8% (*w*/*v*) agarose gel in 1X Tris-Borate-EDTA (TBE) buffer containing Red SafeTM nucleic acid staining solution (iNtRON Biotechnology, Inc.; supplied by Celta Ingenieros, A Coruña, Spain). DNA pattern bands were visualised under UV light and documented using a Molecular Imager® Gel DocTM XR+ imaging system (BIO-RAD, Madrid, Spain).

The identity of non-*Saccharomyces* yeasts was confirmed by PCR amplification of the 5.8S rRNA gene and the two internal (non-coding) ITS1 and ITS2 spacers using the ITS1 and ITS4 primers [42]. The PCR products were digested with the restriction enzymes FastDigest *Hinf* I, *Bsu* RI, and *Hha* I (Thermo Fisher Scientific, Madrid, Spain); then, the fragments obtained were separated in a 3% agarose gel in 1X TBE buffer and the DNA

pattern bands were visualised under UV light and documented as indicated previously. The pattern bands were confirmed by sequencing of D1/D2 region of 26S rDNA gene as indicated in Castrillo et al. [33].

2.4. Chemical Analysis of Must and Wines

Chemical characteristics of musts including ºBrix, probable alcoholic grade, total acidity, pH, yeast-assimilable nitrogen (YAN), and total SO_2 were determined using the official methodology [43].

Basic parameters of wines (alcohol content; glucose + fructose; titratable and volatile acidity; pH; tartaric, malic, and lactic acids; and glycerol) were determined by Fourier transformed infrared spectrometry using a WineScan FT120 analyser (FOSS Electric, Barcelona, Spain), previously calibrated according to the official methods for wine analysis [43]. In addition, the free and total sulphur dioxide (SO_2) in the wines were also quantified using the OIV methods [43]. Volatile compounds of wines were quantified by gas chromatography–flame ionization detection (GC–MS) according to the protocol described by Lopez et al. [44].

2.5. Sensory Evaluation

The wines were evaluated at the sensory level by a tasting panel with experience in Galician wines. The panel consisted of nine judges, six males and three females aged between 34 and 56 years old. A descriptive score card including 21 descriptors and global wine quality was used [35]. The selected descriptors were specifically chosen for Galician white wines and were scored from 0 (absent) to 9 (most intense). The tasting sessions were held in March at the tasting room of Evega. Wine samples were coded and presented in the same order to the panel in clear tulip-shaped glasses. One wine sample per trial (a blend of the three repetitions) was used for sensory analysis.

The relative intensity (I), relative frequency (F), and geometric mean (GM) of the different descriptors were calculated for each wine. Relative intensity (I) was calculated as the sum of the intensities given by the panel for a descriptor, divided by the maximum possible intensity for this descriptor. F is the number of times that the descriptor was mentioned divided by the maximum number of times that it could be mentioned. GM was calculated as the square root of the product between I and F. Furthermore, the tasters were asked for their personal preference among the evaluated wines for each grape variety.

2.6. Statistical Analysis

The differences in wine basic parameters and volatile compounds, 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 carried out to separate the wines according to their volatile composition considering the main fermentative compounds, and previous standardisation of the data. The PCA was performed using PAST Version 4.17 (2023).

3. Results

M. fructicola Mf278 and *P. kluyveri* Pk1 were used in sequential fermentations with *S. cerevisiae* XG3 to ferment musts from Albariño and Lado grapevine varieties. Control fermentations with XG3 as monoculture and a spontaneous process were also carried out.

3.1. Fermentation Dynamics and Microbiological Control

Figure 1 shows the fermentation curves of Albariño and Lado musts fermented with different yeast strains. The dynamics of Albariño fermentations was similar in all assays, although in those inoculated with the non-*Saccharomyces* yeasts, the rapid decrease in the density was observed one day later (day 6) (Figure 1a). Control fermentation with

S. cerevisiae XG3 and the spontaneous process presented a fast reduction in density from day 4 until day 10, after which the fermentation speed slowed down until the end. The evolution of Lado fermentations was more irregular than that of the Albariño, with a slowdown towards day 7 (Figure 1b).

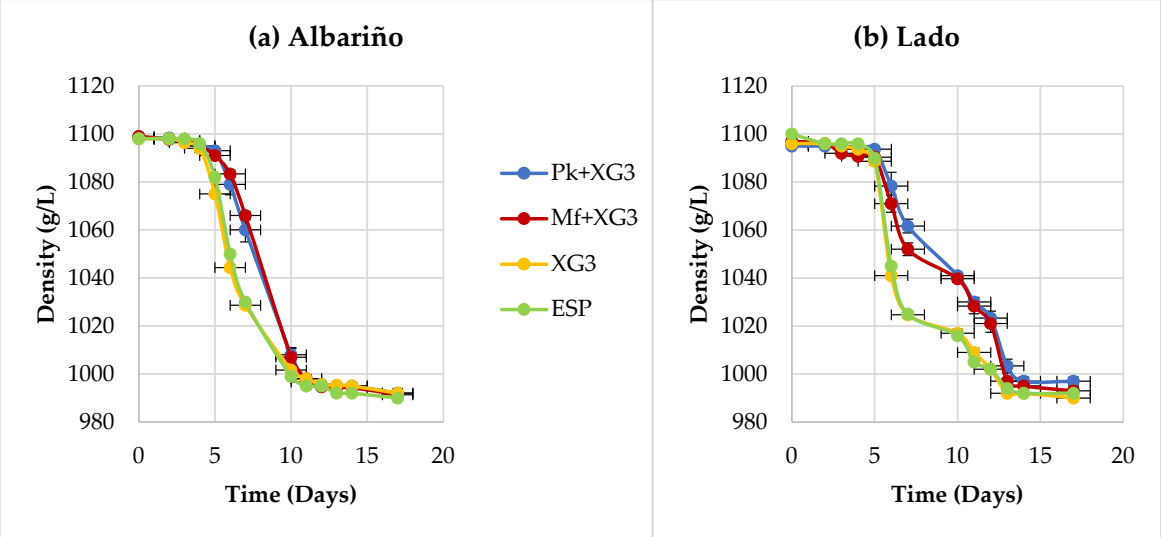


Figure 1. Fermentation curves of (**a**) Albariño and (**b**) Lado grape musts fermented with different yeasts. Pk + XG3—sequential fermentation of *P. kluyveri* Pk1 + *S. cerevisiae* XG3; Mf + XG3—sequential fermentation of *M. fructicola* Mf278 + *S. cerevisiae* XG3; XG3—control fermentation with only *S. cerevisiae* XG3; ESP—spontaneous fermentation. Data are the mean values of three repetitions ± SD.

At the microbiological level, musts from Albariño and Lado contained 9.2×10^2 and 3.3×10^2 of viable yeast/mL, respectively (Figure S1). After inoculation of yeasts, the population in Albariño fermentations reached about 2.0×10^7 viable yeast/mL at the beginning of fermentation, increased up to 1.0×10^8 viable yeast/mL during the tumultuous stage, and fell to 4.0×10^7 viable yeast/mL at the end of fermentation (Figure S1a). The same behaviour was observed in the Lado fermentations with XG3 and the spontaneous process; however, the amount of yeast remained stable at the end in all sequential fermentations of this variety (Figure S1b).

At a qualitative level, the colonies morphology on WL nutrient agar indicated that *P. kluyveri* Pk1 y *M. fructicola* Mf278 were the dominant yeasts at the beginning of fermentations in which they were inoculated. Their identity was confirmed by genetic techniques as indicated in the methodology. However, after the sequential addition of *S. cerevisiae* XG3 (the second inoculum), the populations of Pk1 and Mf278 strains decreased quickly, with their presence being very low or zero at tumultuous fermentation. At this stage and at the end of fermentation, *S. cerevisiae* was the dominant yeast, as well as it was the main species at the beginning of control and spontaneous fermentations. The analysis of *S. cerevisiae* yeasts at the strain level by means of mtDNA-RFLPs allowed the identification of 28 different profiles (named as P1, P2, … P28). The implantation control in Albariño fermentations showed that out of a total of 14 strains, *S. cerevisiae* P2, P8, and P9 were the most abundant yeasts; they appeared in co-dominance in ALB-Mf, ALB-Pk, and ALB-ESP fermentations (Figure 2a). Control fermentations of Albariño, inoculated with *S. cerevisiae* XG3, were dominated by this strain (80% of abundance); however, XG3 was not able to overgrow the natural yeasts in sequential fermentations.

The diversity of *S. cerevisiae* strains in Lado fermentations was higher than in the Albariño ones. The genetic analysis revealed up to 24 different mtDNA-RFLPs profiles of

which only 10 reached abundances higher than 5% in at least 1 fermentation. Strains P1, P2, and P3 were the dominant yeasts in sequential fermentation; they appeared in codominance with frequencies > 10% (Figure 2b). The abundance of the remaining strains was lower than 10%. Spontaneous fermentation was controlled by strains P2 and P3, whereas in XG3 fermentations, this strain was the dominant yeast (frequency of 60%), as expected. Compared to Albariño fermentations, XG3 was found at frequencies of 30% and 20% in sequential fermentations of Lado with Mf and Pk, respectively (Figure 2b).

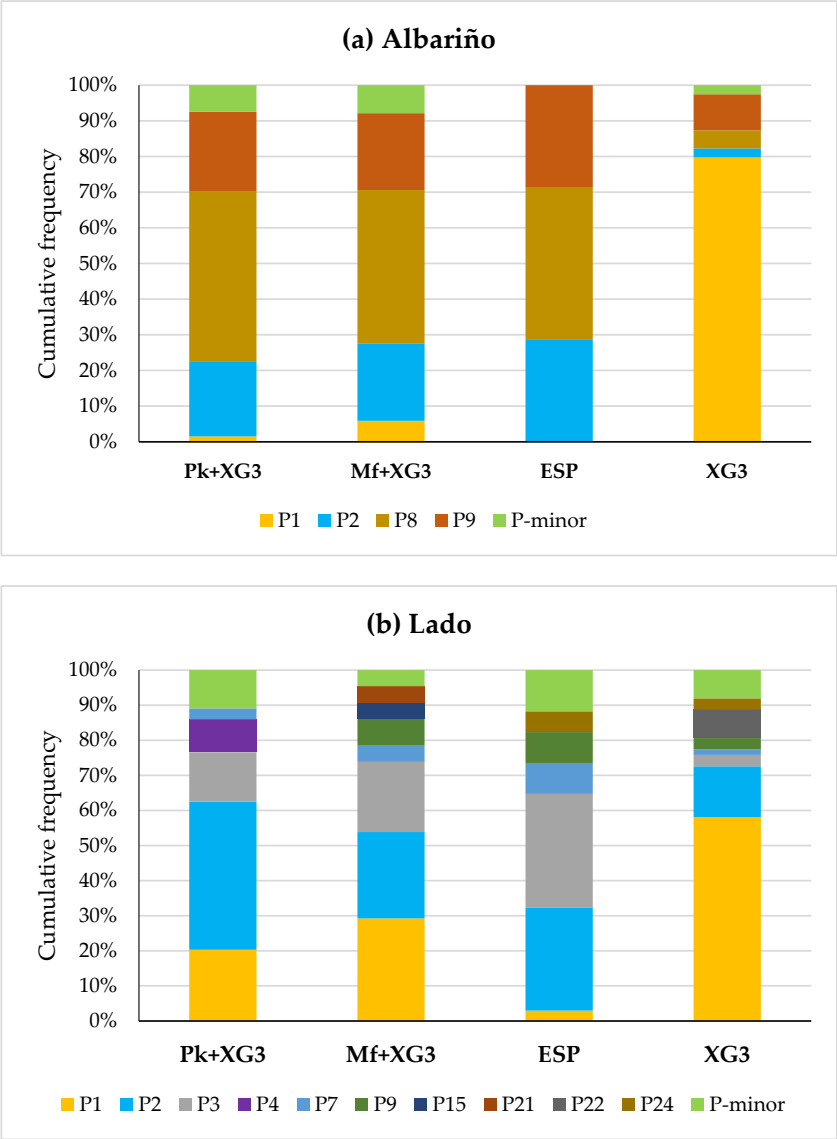


Figure 2. Cumulative frequency of *Saccharomyces cerevisiae* strains isolated from (**a**) Albariño and (**b**) Lado fermentations. Pk + XG3—sequential fermentation of *P. kluyveri* Pk1 + *S. cerevisiae* XG3; Mf + XG3—sequential fermentation of *M. fructicola* Mf278 + *S. cerevisiae* XG3; XG3—control fermentation with only *S. cerevisiae* XG3; ESP—spontaneous fermentation. P1, P2, … P24 are different *S. cerevisiae* strains; P1 = XG3; P-minor: sum of abundance percentage of strains found at frequency < 5%. For fermentations MF + XG3 and Pk + XG3, *S. cerevisiae* isolates from middle and end fermentation were analysed; for XG3 and spontaneous fermentation, *S. cerevisiae* isolates from the initial, middle, and the end of fermentation were analysed.

3.2. Chemical Composition of Albariño and Lado Wines

Table 1 summarises the chemical composition of Albariño wines elaborated with different yeasts. The results evidenced significant differences among wines for residual

sugars, volatile acidity, and glycerol. The content of glucose + fructose was higher in the wines obtained with XG3 than in wines from sequential fermentations. In addition, wines from sequential fermentation with Mf + XG3 had a significantly lower value of volatile acidity and higher content of glycerol than the remaining wines. These fermentations also yielded wines with the lowest content of alcohol, although the differences were not significant.

Similarly, Lado wines obtained by sequential fermentation with Mf + XG3 also had the lowest volatile acidity and the highest concentration of glycerol compared to sequential fermentations with Pk + XG3 or XG3 as monoculture (Table 2). However, with this grapevine variety, XG3 was able to complete fermentations, but the wines from the sequential combination of Pk + XG3 presented residues of unfermented sugars.

Table 2. Chemical characteristics of Lado must and wines obtained by sequential fermentations, XG3 monoculture, and spontaneous fermentation.

Parameter/Lado Wines	Grape Must	Pk + XG3	Mf + XG3	XG3	ESP
Probable degree/Alcohol content (%*v*/*v*)	13.3	12.9 ± 0.4	13.3 ± 0.6	13.1 ± 0.6	13.4
ºBrix/Glucose + Fructose (g/L) *	22.8	7.9 ± 2.6 ^{b}	2.7 ± 0.8 ^{a}	0.8 ± 0.3 ^{a}	3.2
Total acidity (g tartaric acid/L)	6.0	6.1 ± 0.2	6.1 ± 0.1	6.2 ± 0.0	6.3
Volatile acidity (g acetic acid/L) *		0.46 ± 0.03 ^{b}	0.39 ± 0.02 ^{a}	0.45 ± 0.01 ^{ab}	0.34
pH (-)	3.47	3.44 ± 0.02	3.46 ± 0.00	3.45 ± 0.00	3.43
Malic acid (g/L)	3.6	2.4 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	2.6
Tartaric acid (g/L)	5	2.6 ± 0.1	2.7 ± 0.1	2.7 ± 0.1	2.7
Glycerol (g/L) *		5.7 ± 0.2 ^{a}	6.7 ± 0.1 ^{b}	5.7 ± 0.2 ^{a}	5.6
Total SO_2 (mg/L)	46	44 ± 1	43 ± 1	38 ± 2	46

Pk + XG3, Mf + XG3, and XG3 data are the mean values of three replicate fermentations ± SD. * Different superscript letters in the same row indicate significant differences according to Tukey's test ($p < 0.05$).

The results of fermentative aromas evidenced differences among wines according to the yeast used for fermentation. In general, the volatile composition of Albariño and Lado wines showed significant differences for most ethyl esters and fatty acids, as well as for 2-phenylethyl acetate but not for higher alcohols (Tables S1 and S2). Albariño wines obtained with XG3 had a higher content of 1-hexanol than wines obtained by sequential fermentation with *P. kluyveri* Pk1 and *M. fructicola* Mf278. However, the latter had higher contents of esters, fatty acids, and 2-phenylethyl acetate than wines elaborated with *S. cerevisiae* XG3 or by spontaneous fermentation (Table S1). Moreover, between non-*Saccharomyces* yeasts, the content of the above volatile families was higher in Pk1 wines than in Mf278 wines for Albariño. Similarly, the volatile composition of Lado wines also evidenced the impact of sequential fermentations. In this case, Pk1 wines reached a significantly higher content of ethyl hexanoate, ethyl octanoate and ethyl decanoate, and hexanoic acid than wines elaborated with Mf278 or *S. cerevisiae* alone (Table S2). In addition, both non-*Saccharomyces* yeasts produced Lado wines with a higher content of acetates, ethyl butyrate, butyric acid, and octanoic acid than *S. cerevisiae* as monoculture.

The PCA based on the volatile composition of wines allowed their separation in the biplot according to the inoculated yeast. With both varieties, the wines obtained with *S. cerevisiae* were clearly separated on the left side of PC1, from wines obtained by sequential fermentation (Figure 3) due to their higher content of hexanol and 2-phenylethanol. For Albariño wines, the first two principal components, PC1 and PC2, explained 91% of the variance (Figure 3a). Albariño wines obtained with Pk1 were grouped in the second quadrant due to their highest content of 2-phenylethyl acetate, esters, and acids. Mf278 Albariño wines were plotted in the third quadrant, characterised by lower contents of 2-phenylethyl acetate, esters, and acids than Pk1 wines. This PCA also allowed the separation

of wines obtained by ESP fermentation from those made with XG3. Regarding Lado wines, the first two principal components explained 89.5% of the variance (Figure 3b). The wines obtained with XG3 and by spontaneous fermentations were located in the fourth quadrant, negative part of PC1 and PC2, due to their higher content of hexanol and 2-phenylethanol and lower concentrations of esters, acids, and acetates than wines obtained by sequential fermentations. The latter were plotted in the positive part of PC1, with wines from Mf278 located in the second quadrant and Pk1 wines in the third quadrant characterised by the highest content of esters and hexanoic acid.

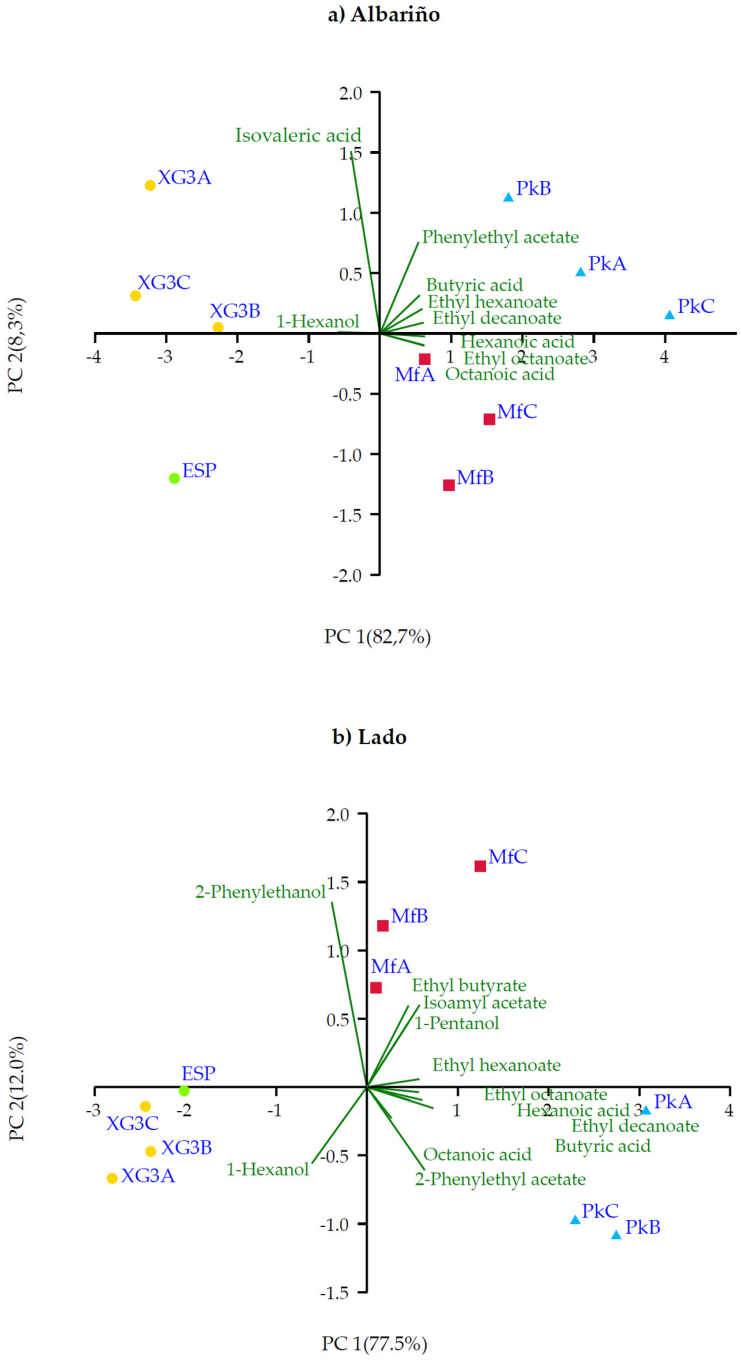


Figure 3. Principal component analysis of (**a**) Albariño and (**b**) Lado wines considering the volatile compounds with significant differences among assays. PkA, PkB y, and PkC—sequential fermentation of *P. kluyveri* Pk1 + *S. cerevisiae* XG3; MfA, MfB, and MfC—sequential fermentation of *M. fructicola* Mf278 + *S. cerevisiae* XG3; XG3A, XG3B, and XG3C—control fermentation with only *S. cerevisiae* XG3; ESP—spontaneous fermentation.

3.3. Sensorial Characterization of Albariño and Lado Wines

The evaluation of the sensory characteristics of Albariño and Lado wines highlighted the wines obtained by sequential fermentation in both cases (Figure 4). These wines obtained the highest punctuation at global level and also for aroma descriptors such as floral or different type of fruits (Figure 4a,b). For instance, Albariño Pk wines achieved the highest global score, and also fruity descriptors including stone, pome, and tropical fruits. The Albariño wine obtained with XG3 stood out for their honey notes whereas the ESP one was by its notes to stone fruits. Finally, the Albariño Mf wines obtained the best mouth feel. In contrast, with Lado variety the wines obtained using Mf278 reached the highest score for all significant descriptors except for stone fruits, which was again higher in ESP fermentation. Accordingly, Albariño Pk1 and Lado Mf278 were the wines preferred by the tasters (Figure 4c).

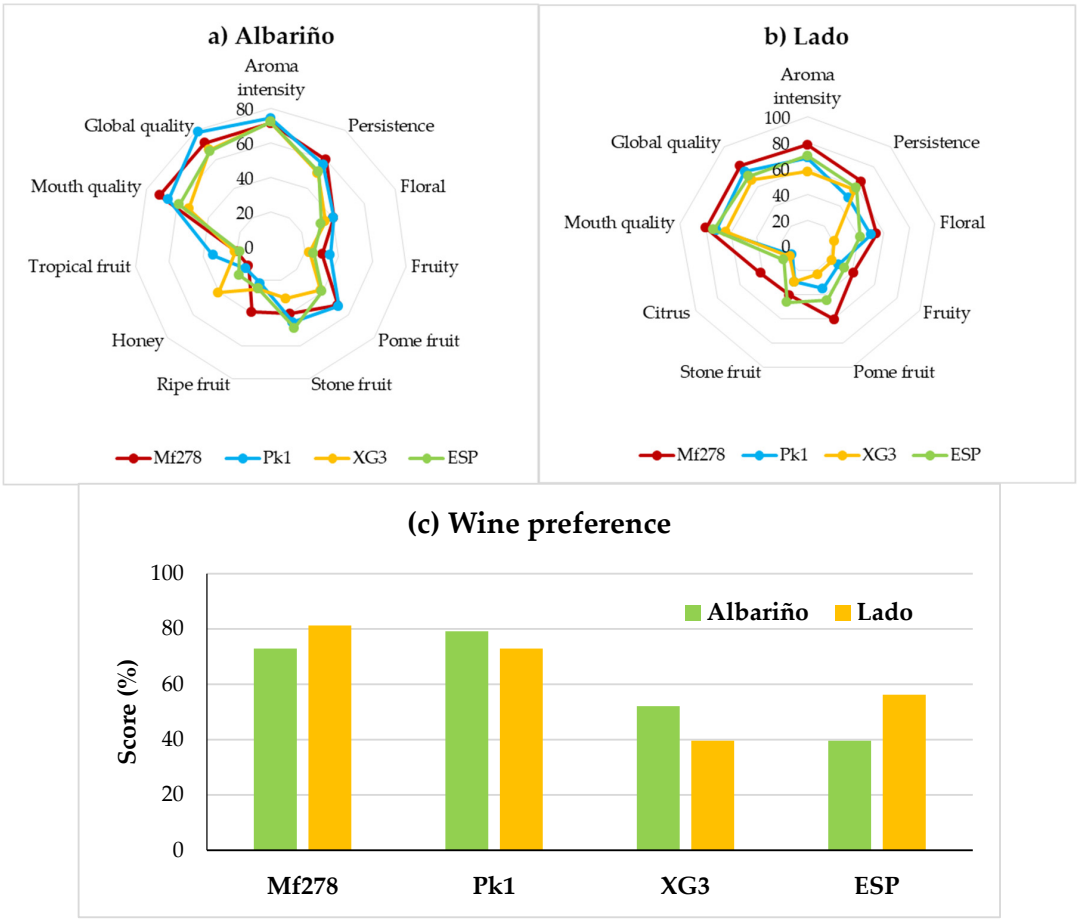


Figure 4. Sensory profiles of (**a**) Albariño and (**b**) Lado wines obtained with different yeasts (only those descriptors with MG > 30% in at least one of the wines are included). (**c**) Score of testers' preferences for wines.

4. Discussion

Wine non-*Saccharomyces* yeasts are considered a useful biological tool to improve wine complexity. Their impact on key wine parameters such as ethanol and glycerol contents, total and volatile acidity, or aroma composition is well documented [2,3]. In addition, in the last decade, differences among microbial populations of grape musts according to the geographical area support the existence of a microbial terroir [45]. Moreover, the contribution of the native yeasts during spontaneous fermentation was related to the regional character of wines from a particular region, which have a great acceptance among

consumers [30,31]. In this context, this study takes advantage of the varietal and microbial resources from Granxa D'Outeiro and Evega [35,37,39]. Thus, we have evaluated the effect of two native non-*Saccharomyces* yeasts in sequential fermentation with *S. cerevisiae* XG3, on the organoleptic properties of wines from Albariño and Lado, two traditional white grapevine varieties grown in Galicia (NW Spain).

The species used in this study, *Metschnikowia* spp. and *P. kluyveri* have been reported as poor fermentative yeasts and they slow down the fermentation [10,26]. As expected, the evolution of our fermentations showed a lag phase of about 3–4 days in all cases; but, once started, the decrease in the density was slower in sequential fermentations compared to control ones. Despite this, the duration of the fermentation process was similar for all the trials. For non-*Saccharomyces* assays, when the completion of fermentation takes longer, it is considered that the advantages of mixed fermentations compensate the slower fermentation. Regarding dynamics of yeast population, the amount of yeast increased until tumultuous stage decreasing towards the end, except for sequential fermentations with Lado, in which the number of viable yeasts was maintained at the end (Figure S1a,b). The delay was probably due to the very low amount of yeast in the musts.

The microbiological control evidenced that *M. fructicola* Mf278 and *P. kluyveri* Pk1 were the predominant yeasts at the beginning of their, respectively, sequential fermentations (Figure 2) with both grape varieties; but their population rapidly declined after *S. cerevisiae* inoculation. Our results are in agreement with previous works reporting the population kinetics for *M. pulcherrima* in sequential fermentations [11,13,15,34]. The decline of *Metschnikovia* was attributed to its low tolerance to ethanol [11]; although its longer survival as monoculture or depending on the timing of *S. cerevisiae* inoculation suggests that other mechanisms, besides ethanol stress, are involved in cell death [46,47]. Certainly, many other factors such as oxygen and nutrient availability, addition of sulphur dioxide, or microbial interactions, including the production of antimicrobial peptides or death mediated by cell-to-cell contact mechanisms, could be responsible for non-*Saccharomyces* yeast survival during fermentation [48,49]. Wang et al. [50] found that some metabolites produced by *S. cerevisiae* affected the viability of the non-*Saccharomyces* yeasts, but this inhibition was species- and strain-dependent. Similar findings were reported in sequential fermentation with *P. kluyveri* strains [11,17]; moreover, the dominance of *S. cerevisiae* over *P. kluyveri* decreased in sequential inoculation compared to co-fermentation [29]. These authors had previously identified a cell–cell contact-mediated inhibition of cell viability between *P. kluyveri* and *S. cerevisiae* [51].

Regarding *S. cerevisiae* XG3 implantation, this strain successfully dominated fermentations when added to Albariño and Lado musts as monoculture; however, it was not able to outgrow wild yeasts in the sequential fermentations. XG3 is an autochthonous strain of *S. cerevisiae* isolated from spontaneous fermentations at Evega. Its enological potential has been proven on a pilot scale with Treixadura, Godello, and Albariño, the three main white grapevine varieties cultivated in Galicia [52,53]. This strain has also been used as a second inoculum in sequential fermentation of Treixadura with non-*Saccharomyces* yeasts, being the responsible for fermentation completion [35]. Recently, XG3 was produced as ADY and has been applied at the industrial level to elaborate Treixadura wines [39]. None of these previous studies reported problems with the implantation of XG3, either as a fresh culture or as ADY. Its failure to control sequential fermentations of Lado and Albariño could be related to the timing of inoculation, which was intentionally retarded to favour the contribution of *M. fructicola* Mf278 and *P. kluyveri* Pk1 to the wine characteristics. Instead, a co-dominance of different strains of *S. cerevisiae* was observed in both sequential and spontaneous fermentations, with dominant strains being variety-dependent (Figure 2). Thus, whereas strains P2, P8, and P9 were the predominant *S. cerevisiae* yeasts in Albariño

fermentations, strains P1, P2, and P3 were the most abundant ones in Lado fermentations. This result is not surprising since we have previously observed that must characteristics influence the diversity of yeast strains during fermentation [54]. In this sense, the total number of strains found in Albariño fermentations (14 strains) was lower than in Lado fermentations (24 strains). Supporting these findings, the diversity of *S. cerevisiae* strains was higher in Lado than in Albariño spontaneous fermentations in the winery of Granxa D'Outeiro [37], from which the grape must used in this study comes. Furthermore, the interaction among non-*Saccharomyces* yeasts and *S. cerevisiae* strains has also been reported [51] and is known to be strain-dependent. Despite its trouble in controlling fermentations, XG3 reached frequencies above 20% in the Lado fermentations, so its contribution to the wine characteristics should be considered. In contrast, its presence in the Albariño fermentations was very low.

In winemaking, the yeasts involved in fermentation convert sugars into ethanol and carbon dioxide, but also into a wide range of secondary metabolites that define the chemical and sensory characteristics of the wine [55]. The contribution of non-*Saccharomyces* yeasts to the chemical composition of wines makes them a valuable tool to enhance wine complexity and to address the consequences of climate change [2,3,5,12]. The enological potential of *Metschnikowia* and *Pichia* species have been reviewed recently. Both yeasts can lower ethanol and volatile acidity of wines, and increase the content of glycerol, esters, and volatile thiols, among other properties [9,10,26]. We did not obtain a significant reduction in alcohol in the wines from sequential fermentations with the strains *M. fructicola* Mf278 and *P. kluyveri* Pk1 in any case; however, Mf278 slightly lowered the ethanol content in Treixadura wines [35]. In contrast, Hranilovich et al. [46] reported the ability of *M. pulcherrima* to decrease ethanol between 0.6% and 1.2% (*v*/*v*) depending on the inoculation timely. Likewise, other studies reported reductions up to 1.0% (*v*/*v*) of alcohol for sequential fermentations of *M. pulcherrima* and *S. cerevisiae* compared to *S. cerevisiae* controls [11–13,20]. Similarly, sequential fermentations with *P. kluyveri* yielded wines with lower ethanol content than their respective controls [11,17]. The oxidative character of these yeasts, as well as their fermentative limitations, are responsible for the ethanol reduction. In addition, they redirect their metabolism toward the production of other compounds of oenological interest such as glycerol, acids, or volatile esters [46]. Accordingly, Mf278 wines had a significantly higher content of glycerol than the remaining wines in agreement with data in the references mentioned above. Glycerol has been associated with wine attributes such oiliness, persistence, and sweetness [56], perceived as positive by consumers. Although *P. kluyveri* can increase the glycerol concentration [26], our results indicated that the strain Pk1 in sequential fermentations did not produce more glycerol than *S. cerevisiae* XG3. Finally, it should be noted that *M. fructicola* Mf278 significantly reduced the volatile acidity of Lado and Albariño wines while *P. kluyveri* Pk1 and *S. cerevisiae* XG3 showed similar values for this parameter. Several studies confirmed that *M. pulcherrima* decreased the content of acetic acid in wines, although the production of equal or even higher values of volatile acidity have also been reported [10]. In line with our results, recent studies that investigated the potential of *Metschnikowia* spp. as biocontrol agents evidenced that they also lowered the production of acetic acid [24,25].

Beyond the influence of *Metschnikowia* and *Pichia* species on the basic chemical parameters of wines, the oenological interest of these yeasts focuses on their production of varietal and fermentative aromas that improve the complexity of wines [2,3]. The positive contribution of *Metschnikovia* to wine fermentative aromas is often related to a higher production of esters and certain fatty acid than *S. cerevisiae;* however, the results about its influence on the higher alcohol content are not conclusive [9,10]. Accordingly, Albariño wines obtained by sequential fermentation with *P. kluyveri* Pk1 and *M. fructicola* Mf278 were clearly separated

by PCA from those obtained with *S. cerevisiae* (Figure 3a) because they had a significant higher content of 2-phenylethyl acetate and most ethyl esters and fatty acids than wines made with *S. cerevisiae*. The highest concentrations of these volatiles were achieved with Pk1 (located in the second quadrant), whereas Mf278 wines appeared in the third quadrant. Similar findings were reported for Riesling wines, especially for 2-phenylethyl acetate and ethyl octanoate, among other compounds [11,17]. Moreover, the increase in some esters, acetates, and/or fatty acids by sequential fermentations with *Metschnikowia* spp. has widely been proved for different types of wines and varieties including sweet "passito" wine [24]; Anglianico [16]; Verdichio [25]; Malvar [20]; Solaris [14]; and Chardonnay, Shiraz, Merlot, and Cabernet Sauvignon [12,13,18]. In Evega, *M. fructicola* Mf278 had been already used to improve the aroma profile of Treixadura wines, resulting in wines with good acceptance at the sensory level [35]. In contrast, Hranilovich et al. [46] found that *M. pulcherrima* increased production of acetate esters and higher alcohols, but not other volatiles. The influence of *P. kluyveri* on the aromatic profile of wines has not been studied as extensively as that of *Metschnikowia*, even though both species have the ability to release volatile thiols that enhance varietal aromas [15,27].

The aroma profile of Lado wines was also significantly modulated by sequential fermentations. These wines were also clearly separated by the PCA (Figure 3b). Unlike Albariño, the content of acetates was significantly increased with Pk1 in Lado wines, especially the concentration of 2-phenylethyl acetate, a compound that imparts pleasant floral notes to wines [57]. In agreement with our results, Delač Salopek et al. [58] reported that wines from *P. kluyveri* stood out by their content of ethyl octanoate and acetate esters. Similarly, an increase in the 2-phenethyl acetate levels of Gewürztraminer wines co-fermented with *P. kluyveri* was described [28]. Recently, the ability of *P. kluyveri* to survive and increase the level of higher alcohol acetates of wines in mixed fermentation was confirmed [29].

The overall quality of wines is positively influence by ethyl and acetate esters which impart fruity and floral notes [59,60]. On the contrary, the contribution of fatty acids to wine is associated with unpleasant aromas such as rancid, pungent, or cheese-like [59], but they are very important for the aromatic equilibrium in wine. Accordingly, at the sensory level, the Albariño and Lado wines obtained by sequential fermentation were the best valued at the global level. They also achieved the highest scores for floral and fruit aroma descriptors which are related to their higher content of esters (Figure 4). The results evidenced that wine preferences depended on the variety. Thus, with Albariño, the tasters choose Pk1 wines due to their global impression and fruity aromas; however, Albariño Mf278 wines obtained the best score for mouth feel descriptor. This result could be related to their higher content of glycerol, which contributed sweetness and oiliness perceived as positive by tasters [55,56]. In contrast, among Lado wines, the most appreciated was the one obtained with Mf278. This wine achieved the best punctuation for most descriptors, although at the chemical level, the wines obtained with Pk1 presented the highest content of some ethyl esters and acetates. In agreement with our results, a positive contribution of *P. kluyveri* and *M. pulcherrima* to the overall impression and descriptors such as pear or flowery was reported in Riesling wines [11]. These findings highlight the importance of making a good choice of yeast inoculum according to the characteristics of the grape variety. For instance, those yeasts that enhance the final fruity ester concentration are recommended to ferment neutral varieties, but they are not adequate for those varieties rich in varietal aromas because they can mask those aromas [57]. Albariño is a prestigious white aromatic variety [61,62], widely used to elaborate quality wines in Galicia. Lado is a minority variety from DOP Ribeiro [36] with an aroma profile that has been recently reported [63]. Furthermore, a chemical characterisation of Lado and Albariño wines from the Granxa D'Outeiro winery (DOP Ribeiro) evidenced their differentiation in terms of aroma [37].

This study has shown that when Albariño and Lado were sequentially fermented with *M. fructicola* Mf278 or *P. kluyveri* and *S. cerevisiae*, their fermentative aromas were further differentiated. Therefore, the use of traditional grapevine varieties joined to the application of native non-*Saccharomyces* species combined with *S. cerevisiae* allowed wine diversification. This approach also benefited the expression of the regional character of wines, which is highly demanded by consumers.

5. Conclusions

The results of this study evidenced that the sequential inoculation with *M. fructicola* Mf278 or *P. kluyveri* Pk1 and *S. cerevisiae* XG3 modulates the chemical and sensory profile of wines from the Albariño and Lado varieties. The sequential fermentations enhance the production of desirable compounds such as acetate and ethyl esters compared to *S. cerevisiae* monocultures. These compounds contributed floral and fruity notes, resulting in more complex wines that were preferred by the tasters at a sensory level. In addition, Mf278 reduces the volatile acidity of wines and increases their glycerol content, improving the wine's mouth feel. Therefore, we recommend the application of both non-*Saccharomyces* strains to enhance the complexity of wines in Galicia. In this sense, future research will focus on optimising the protocols for their use with different traditional varieties of this region.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation11020073/s1, Figure S1: Yeast population dynamics in (a) Albariño and (b) Lado fermentations; Table S1: Volatile composition (μg/L) of Albariño wines obtained with different yeasts; Table S2: Volatile composition (μg/L) of Lado wines obtained with different yeasts.

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