applied sciences

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Use of *Wickerhamomyces anomalus* Strains from Biologically Aged Wines to Improve the Sensorial Profile of Young White Wines

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Abstract: Non-*Saccharomyces* yeasts play a significant role in winemaking, offering unique benefits and contributing to wine complexity and varied and desirable aromatic profiles. This work focuses on the sensory improvement of Pedro Ximénez white wines using selected strains of *Wickerhamomyces anomalus* isolated from biologically aged wines. Chemical and microbiological analyses confirmed the implantation of *W. anomalus*; these yeast strains appear to displace indigenous non-*Saccharomyces* species in the must and produce large amounts of ethyl acetate and lower ethanol content. Wines made with *W. anomalus* strains were judged negatively by the tasting panel due to a nail polish/varnish odor and a strong, bitter taste; however, when these wines were blended with wine normally produced by spontaneous fermentation, the judges rated them positively, highlighting fruity aromas not detected under other conditions. These results conclude that *W. anomalus* strains isolated from biologically aged wines could be useful for modulating the sensory profile of white wines. Moreover, their use in combination with other yeasts or in immobilized form could improve the results obtained and avoid the blending process. The high yield of ethyl acetate produced by these strains could be of interest as an alternative to current methods of producing this compound, including the use of these wines for the production of quality vinegar.

Keywords: *Wickerhamomyces anomalus*; ethyl acetate; alcoholic fermentation; pedro ximénez must

Academic Editors: Nataša Hulak, Gianluigi Mauriello and Katleen Raes

Received: 22 December 2024
Revised: 31 January 2025
Accepted: 31 January 2025
Published: 3 February 2025

Citation: Carbonero-Pacheco, J.; García-Jiménez, Á.; García-García, J.C.; Santos-Dueñas, I.M.; García-Martínez, T.; Moreno, J.; Moreno-García, J.; Mauricio, J.C. Use of *Wickerhamomyces anomalus* Strains from Biologically Aged Wines to Improve the Sensorial Profile of Young White Wines. *Appl. Sci.* **2025**, *15*, 1546. https://doi.org/10.3390/app15031546

1. Introduction

Winemaking is a process that depends on alcoholic fermentation, carried out mainly by yeasts, which transform the grape must into wine. Several species are involved in this process, although *Saccharomyces cerevisiae* has been the main one for many years because of its fermentative capacity and its resistance to high concentrations of ethanol and sulfur dioxide [1,2]. This allows this species to consume all the fermentable sugars in the must and complete the alcoholic fermentation, which, together with the selection of specific *S.*

cerevisiae strains, allows winemakers to achieve control and homogeneity in the industrial must fermentation process [1]. The remaining species are known as non-*Saccharomyces* and have been considered spoilage yeasts for decades. Non-*Saccharomyces* usually appear in the early stages of alcoholic fermentation, are controlled with antimicrobial agents such as sulfur dioxide, and tend to disappear because of the high ethanol concentration produced during alcoholic fermentation [3].

In recent years, non-*Saccharomyces* yeasts have gained popularity among winemakers and the scientific community because of their metabolism, particularly their enzymatic potential, which allows wines with a more complex sensory profile to be obtained through controlled inoculation of these yeasts. Its industrial use is mostly for winemaking in the active dry yeast (ADY) format, being Christian Hansen, Lallemand, and Enartis, the companies with the largest supply of non-*Saccharomyces* yeast products [4]. Some species are *Hanseniaspora uvarum*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima, Pichia kluyveri,* or *Torulaspora delbrueckii,* which are generally used with *S. cerevisiae* to improve wine aroma, lactic acid production, antifungal activity or to reduce the ethanol content of wine or other products obtained by alcoholic fermentation such as beer, mead, or cider [1,5–8]. Sequential fermentation or co-inoculation with *S. cerevisiae* are the most common strategies in winemaking because of the lower fermentative power of non-*Saccharomyces,* which are usually unable to consume all the available sugar in grape must [5,6]

Wickerhamomyces anomalus is a non-*Saccharomyces* yeast found in numerous environments, such as grapes, vineyards, tree exudates, and insects [1,2]. It is associated with esters production, mainly ethyl acetate, which could contribute to floral and fruity notes in wine, and by its ability to tolerate extreme environmental conditions such as oxidative and osmotic stress [9,10]. It is reported that *W. anomalus* is a good producer of relevant enzymes, such as β-glucosidases, and its impact in winemaking has been tested in Airen, Cabernet Sauvignon, or Verdejo must varieties, among others, increasing the complexity of the final wines [1,2,11]; however, it is a yeast with a variable ethanol tolerance range, depending on the strain, which may affect its viability under certain conditions [2].

Recently, *W. anomalus* strains have been isolated from biologically aged wines in the Jerez–Xèrés–Sherry and Montilla–Moriles wine-growing regions [12,13]. This environment is characterized by low availability of nitrogen sources and fermentable carbon and a high ethanol concentration above 15% (*v*/*v*). Although the impact of *W. anomalus* in biological aging has not been explored yet, the strains isolated in Jerez–Xèrés–Sherry show high tolerance to ethanol (up to 16%, *v*/*v*) [14]. This feature could allow them to complete an alcoholic fermentation of grape must on their own, which could be of potential interest in the enological field.

In the Montilla–Moriles protected designation of origin (P.D.O.) region, Pedro Ximénez is the most common grape variety and is characterized as a low-aromatic variety, usually used in the production of biological aged wines [15,16]. This low aromatic profile makes it possible to highlight the aromas produced by the aerobic metabolism of the flor yeasts present during biological aging, such as the nutty and almond notes [17]; however, in the case of the young white wines also produced in this P.D.O., the world market is looking for fruity, floral, and fresh aromas, where the use of non-*Saccharomyces* yeasts such as *W. anomalus* in the alcoholic fermentation could positively contribute to enhancing these desired attributes [18].

The aim of this work is to obtain a distinctive white wine from Pedro Ximénez unfiltered grape must, with fruity aromas, using autochthonous *W. anomalus* strains isolated from biologically aged wines of Montilla–Moriles P.D.O. region.

2. Materials and Methods

The workflow for the Materials and Methods section is detailed in Figure 1.

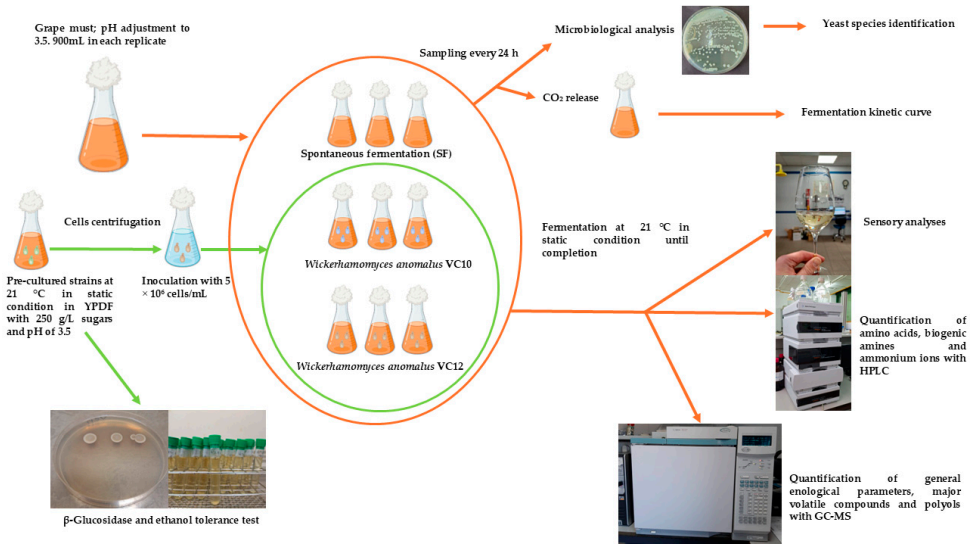

Figure 1. Workflow of material and methods/ experimental design. YPDF: Synthetic medium with 10 g/L yeast extract, 20 g/L peptone, 100 g/L dextrose, and 100 g/L fructose. HPLC: High-Performance Liquid Chromatography. GC-MS: Gas Chromatography–Mass Spectrometry. Created with BioRender.com.

2.1. Microorganisms, Media, and Growth Conditions

The non-*Saccharomyces* yeast strains used in this work were *W. anomalus* VC10 and VC12, isolated from yeast biofilms in biologically aged wines (Yeast Collection of the University of Córdoba, Microbiology Department, Córdoba, Spain) [13]. These strains were previously selected for β-Glucosidase and ethanol tolerance.

Yeast strains were inoculated into 250 mL of YPDF broth (10 g/L yeast extract, 20 g/L peptone, 100 g/L dextrose, and 100 g/L fructose), adjusted to pH 3.5 by the addition of tartaric acid (Sigma-Aldrich; St. Louis, MO, USA) and incubated in static for 5 days at 21 °C to obtain enough cells concentration and to pre-acclimatize the strains to the must conditions.

2.2. β-Glucosidase and Ethanol Tolerance Test

A total of 100 µL of the YPDF broth of each strain was inoculated per triplicate on plates containing β-Glucosidase detection medium (5 g/L arbutine (Sigma-Aldrich; St. Louis, MO, USA), 1 g/L yeast extract, 20 g/L agar and 0.2% of a 1% (*w*/*v*) iron chloride solution). Plates were covered with Parafilm and incubated for 15 days at 28 °C. Dark-black cultures were considered positive [19].

To evaluate the ethanol tolerance of the selected strains, a series of sterile plastic tubes of 15 mL containing 10 mL of a broth containing (3 g/L yeast extract, 5 g/L peptone, and 10 g/L dextrose) and wine ethanol (Alcoholes del Sur, Córdoba, Spain), to obtain a final concentration ranging between 0 and 19%, *v*/*v* of ethanol, were inoculated with 100 µL of the YPDF broth of each strain per triplicate. The tubes were sealed with a hermetic cap and incubated for 15 days at 28 °C. Turbidity or biofilm formation was considered as tolerance to that concentration, and CO_2 production was assessed by shaking or by the detachment of the cap during the incubation.

2.3. Fermentation Conditions

The grape must of Pedro Ximénez grape variety came from the Pérez Barquero SA winery, harvested in 2023. The must had a pH value of 3.9, 200 g/L of sugar, a SO_2

concentration of 61 mg/L, and 4.86 g of tartaric acid/L of titratable acidity after adjusting the pH to 3.5 by adding tartaric acid.

Three conditions were established to assess the impact of *W. anomalus* strains on the alcoholic fermentation of Pedro Ximénez must. Each condition was comprised of three biological replicates, with 900 mL of grape must in a sterile Erlenmeyer flask (nine in total). One of them was carried out following the spontaneous fermentation (SF) of the indigenous yeast present in the must as control. The other two conditions were carried out by adding the selected *W. anomalus* strains previously pre-cultured in the YPDF broth of the *2.1.* section. The strains (VC10 and VC12) were inoculated separately with an initial yeast population of 5×10^6 cells/mL and incubated at 21 °C and 70% of Relative Humidity (RH) under static conditions until alcoholic fermentation ends (weight loss due to CO_2 release less than 1 g/day). During the alcoholic fermentation process, every 24 h, each Erlenmeyer flask was measured to register the mass loss evolution because of CO_2 release, and 5 mL were obtained for subsequent analysis.

2.4. Measurement of Enological Parameters

Chemical analyses were conducted following the International Organisation of Vine and Wine (OIV) recommendations and protocols [20]. These analyses included measurements of ethanol content, pH, titratable acidity, free and total sulfur dioxide (SO_2), and volatile acidity. A Crison GLP 21 + pH meter was employed to determine pH. An Alcolyzer 3001 alcohometer (Anton Paar; Graz, Austria) was used to analyze the ethanol content. The quantification of acetic acid and glycerol content (g/L) was performed with the Y15 chemical analyzer using an absorbance of 500 nm (Biosystems; Barcelona, Spain).

2.5. Quantification of Major Aroma Compounds and Polyols

Gas chromatography using an Agilent 6890 GC (Santa Clara, CA, USA) was employed to analyze the major volatile compounds (concentration >10 mg/L) in wine. These compounds are evaporative under room temperature and play a significant role in the organoleptic characteristics of wine. The chromatograph features a flame ionization detector (FID) and a "CP-Wax 57 CB" column specifically prepared for this analysis. The column dimensions are 60 m × 0.25 μm × 0.2 μm. For each replicate, 0.7 μL of sample is injected, and the total elution time for the major volatiles and polyols being quantified is 80 min [21]. Before sample injection into the chromatograph, 10 mL of wine sample was treated by adding 1 mL of 1.018 g/L 4-methyl-2-pentanol (CAS 108-11-2) as an internal standard in a 14% (*v*/*v*) ethanol solution and 0.2 g of solid calcium carbonate. The prepared mixture underwent sonication in an ultrasonic bath for 30 s and was centrifuged at 5000 rpm for 10 min at 2 °C to remove tartaric acid from the wine. The obtained supernatant from this process was then injected for analysis. The absolute quantification of methanol, higher alcohols (1-propanol, isobutanol, isoamyl alcohol, and 2-phenylethanol), 1,1-diethoxyethane, acetaldehyde, acetoin, ethyl acetate, ethyl lactate, diethyl succinate and the polyols glycerol, and 2,3-butanodiol (levo and meso forms) was performed by a calibration table built with the standard solutions from Thermo Fisher Scientific; Waltham, MA, USA, Merck; Darmstadt, Germany, and Sigma-Aldrich; St. Louis, MO, USA, containing a known concentration of the compounds and subjected to the same treatment as the samples.

2.6. Microbiological Analysis

Samples obtained during alcoholic fermentation and prior to the inoculation of *W. anomalus* strains were cultured in WL (OXOID CM 0501; Hampshire, UK) agar medium (50 g/L dextrose, 4 g/L yeast extract, 5 g/L tryptone, 0.022 g/L bromocresol green, and 20 g/L agar). Bromocresol green gives a blue–green appearance to the medium and acts as a

pH indicator, turning yellow upon acid production by microorganisms. The plates were incubated at 28 °C for 72 h; subsequently, the plates were incubated at 10 °C for 120 h to allow colonies full growth and facilitate colony isolation. Each sample was cultivated by duplicate. Ten random colonies of each WL agar plate were seeded on lysine agar (OXOID CM 0191B; Hampshire, UK) to determine if the colony belonged to non-*Saccharomyces* or *Saccharomyces* group.

Non-*Saccharomyces* yeasts were identified by Matrix-Assisted Laser Desorption/Ionization Time of Flight MALDI-TOF Mass Spectrometry as described in Carbonero-Pacheco [13].

2.7. Quantification of Amino Acids, Biogenic Amines and Ammonium Ions

An adapted derivatization method using diethyl ethoxymethylenemalonate (DEEMM) (Sigma-Aldrich; St. Louis, MO, USA) was employed to analyze amino acids, biogenic amines, and ammonium ions, following the protocol described by Gómez-Alonso et al. [21]. The derivatization process involved combining 0.250 mL of sample without any pretreatment with 0.750 mL of methanol (Labscan; Dublin, Ireland), 1.75 mL of borate buffer 1 M (pH = 9), 0.020 mL of L-2-aminoadipic acid (Sigma-Aldrich; St. Louis, MO, USA) 1 g/L as internal standard, and 0.003 mL of DEEMM in tube over 30 min in an ultrasound bath and heated at 70 °C for 2 h. The HPLC analysis was conducted using an Agilent HPLC 1260 Infinity model (Palo Alto; Santa Clara, CA, USA). Separation was achieved on an ACE C18-HL column (250 mm × 4.6 mm, μm particle size) maintained at 16 °C. The method employed a binary gradient of mobile phases A and B, as described by Gómez-Alonso et al. [22]. Compound detection was performed using a photodiode array detector set to monitor at 280 nm.

2.8. Sensory Analyses

Blind sensory analysis was carried out for each fermentation condition (SF, VC10, and VC12), and two blended wines were obtained by different percentages of each condition. The first one with 90-5-5% and the second one with 80-10-10% of the SF, VC10, and VC12 wines, respectively. The tasting panel was composed of 12 judges, all trained tasters. Wine samples of 25–30 mL were presented at 12–15 °C in clean and clear glasses according to the requirements of the ISO 3591 standards [20]. The samples were randomized and served without indicating each fermentation condition, ensuring blind tasting. The panel evaluated wines on a scale from 0 to 10, with 10 representing the highest level of intensity and 0 representing absence or minimal intensity. To ensure proper conditions and stability, all wine samples were kept at 4 °C for a week prior to the analysis.

2.9. Statistical Analyses

Data presented in Tables and Figures are the average values of a minimum of three biological replicates, each analyzed in triplicate for every studied condition. Statistical analysis was performed using multiple comparison analysis (MCA) for each chemical parameter using the Bonferroni's test at a confidence level of 95% (i.e., a = 0.05 significance level to identify those variables showing significant differences in the wine samples. MCA categorizes samples with significant differences into homogeneous groups (HG). Averages with different HG show statistically significant differences at the 95.0% confidence level. To differentiate between averages, Fisher's least significant difference (LSD) procedure was conducted. Principal Component Analysis (PCA) was employed to reduce the dimensionality of these data and identify the most significant variables in the samples. The results were visualized using a biplot, which graphically represents both the samples and variables. Groups were statistically analyzed to determine the fermentation condition (SF, VC10, and VC12).

3. Results and Discussion

3.1. β-Glucosidase and Ethanol Tolerance Test

Both *W. anomalus* strains showed β-Glucosidase activity in the detection medium; regarding the ethanol tolerance, the VC10 strain was able to grow up to a concentration of 8% (*v*/*v*), with biofilm formation up to 6% (*v*/*v*), whereas the VC12 strain, was able to grow up to a 9% (*v*/*v*) and present biofilm formation up to 5% (*v*/*v*). Strains exhibit different fermentative power depending on the ethanol concentration; VC10 was able to pop up the cap of the tube because of CO_2 production and overpressure up to 6% (*v*/*v*), while VC12 was only capable until an ethanol concentration of 4% (*v*/*v*).

β-Glucosidase activity in *W. anomalus* has been tested in other works, and it is related to the release of terpenes, which has a positive influence on wine; however, both strains tested in this work and isolated in biologically aged wines of the Montilla–Moriles P.D.O. region are less tolerant to ethanol than those isolated in a similar environment in the Jerez–Xèrés–Sherry D.O. region [14]. *W. anomalus* tolerance to ethanol seems to be closely linked to the particular strain tested; some authors establish a 9% (*v*/*v*) ethanol tolerance where, at higher concentrations, the yeast metabolism is considerably reduced, while other studies confirm an ethanol tolerance of 12% (*v*/*v*) [1,23].

The ethanol tolerance test shows that CO_2 production in *W. anomalus* is linked with the % (*v*/*v*) of ethanol in the medium, being higher when the ethanol concentration is low. Studying the fermentative power and ethanol tolerance of a non-*Saccharomyces* yeast is of great importance to evaluate its theoretical activity period during alcoholic fermentation. Ethanol stress in *W. anomalus* is related to amino acid consumption such as arginine, aspartate, or glutamate; however, further studies with the VC10 and VC12 strains are needed to conclude their real tolerance and the possibility of using these strains as monoculture or in co-culture with other yeast species [23].

3.2. Alcoholic Fermentation Rates

Figure 2 shows the mass loss due to the development of CO_2 release in the three fermentation conditions.

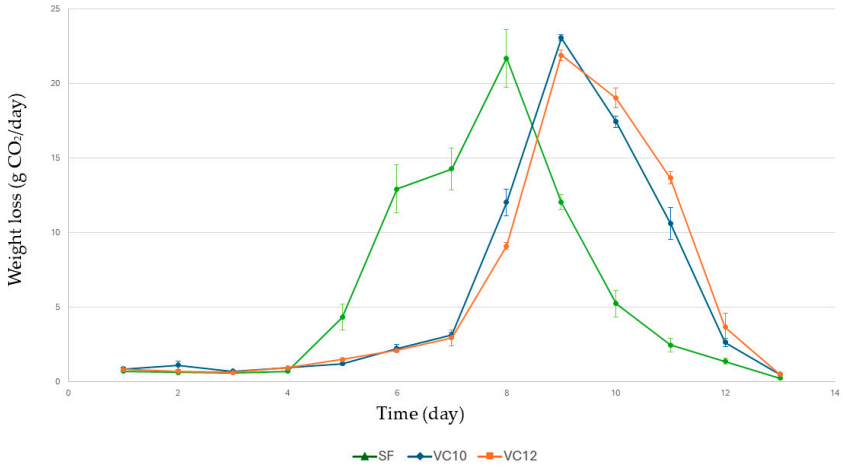

Figure 2. Fermentation kinetics by the development of CO_2 production and release during alcoholic fermentation in the three tested conditions. Spontaneous fermentation (SF): green triangle, *W. anomalus* strain VC10: blue rhombus, *W. anomalus* strain VC12: orange square. The error bars represent the standard deviation over averages from three biological replicates.

The highest fermentation rate in the SF condition was reported on day 8 (21.70 ± 1.93 gCO_2/ day), while in VC10 and VC12 occurred on day 9 (23.07 ± 0.20 and 21.90 ± 0.37 gCO_2/day, respectively). Kinetic curves obtained during the alcoholic fermentations differed between the SF used as control and the ones carried with selected yeast strains, where the peak of CO_2 release was a day later. This could be related to *W. anomalus* inoculation at the beginning of the alcoholic fermentation, which allowed the selected yeast to dominate at the beginning and in the intermediate phase of the alcoholic fermentation. In fact, *W. anomalus* is known to have a lower fermentative power than other yeast, such as *S. cerevisiae*, *T. delbrueckii*, or *Hanseniaspora osmophila* [2,24].

3.3. Microbiological Analysis

Identification of isolated yeast colonies reveals the presence of three non-*Saccharomyces* species, *T. delbrueckii*, *L. thermotolerans*, and *Hanseniaspora opuntiae*, before the start of alcoholic fermentation, detecting 4 × 10^3, 1.1 × 10^3, and 2.8 × 10^2 colony-forming units (CFU)/mL of each species, respectively. Figure 3 shows the relative abundance of the detected yeast during the fermentation process.

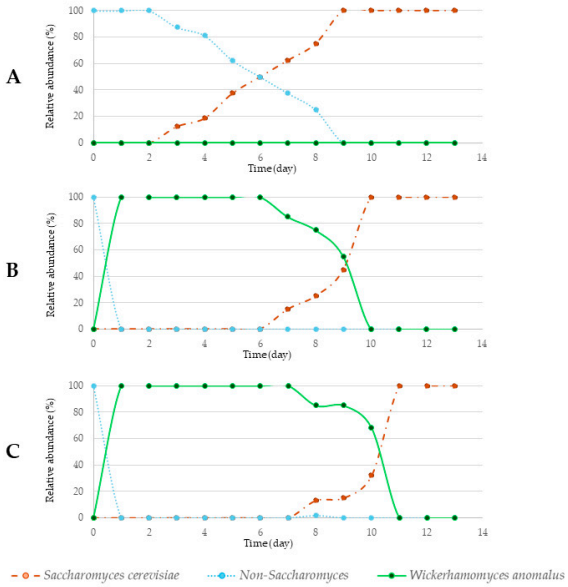

Figure 3. Relative abundance of *S. cerevisiae* (dark brown dashed line), non-*Saccharomyces* yeasts, excluding *W. anomalus* (blue dots) and *W. anomalus* (green line) during the alcoholic fermentation of grape must in each condition (**A**): Spontaneous fermentation (SF), (**B**): *W. anomalus* strain VC10, (**C**): *W. anomalus* strain VC12.

In SF condition, non-*Saccharomyces* yeast appeared from the beginning of the alcoholic fermentation until the eighth day, *T. delbrueckii* the most common yeast species, with 75% of isolates on the first day and *L. thermotolerans* and *H. opuntiae* counting the 20% and 5%, respectively; however, its presences decrease as the fermentation process progresses with no detection of *H. opuntiae* after the second day and with no isolation of *L. thermotolerans* after the sixth day. Last, *T. delbrueckii* was isolated until the eighth day, after which only *S. cerevisiae* was found in the SF condition.

In the VC10 and VC12 conditions, where the *W. anomalus* strains were inoculated at the beginning of the alcoholic fermentation, only this non-*Saccharomyces* species was isolated, with the exception of *T. delbrueckii*, on the eighth day (1.8% of relative abundance), Figure 3. *W. anomalus* was only isolated in the VC10 and VC12 conditions; this yeast species was not found either in the grape must before alcoholic fermentation or during the

sampling in the SF condition, indicating correct implantation of the inoculated strains in the grape must.

S. cerevisiae yeast dominates the alcoholic fermentation at the final stage of the three conditions, being the most abundant species from the sixth day in SF. In the VC10 and VC12 conditions, *S. cerevisiae* was isolated from the eighth day, displacing *W. anomalus* as the most abundant yeast on days 10 and 11, respectively. The emergence of *S. cerevisiae* matches with the highest fermentation rates (gCO_2/day) in all conditions, which highlights the higher fermentation power of this species. This occurrence is common when non-*Saccharomyces* yeasts, such as *M. pulcherrima* or *Pichi kluyveri*, are used in alcoholic fermentation to increase wine complexity because of their lower fermentation power, with expectations such as *L. thermotolerans*, which is capable of surviving when *S. cerevisiae* dominates the fermentation process [5,7,18]. Microbiological analysis and fermentation kinetics data indicate that both VC10 and VC12 *W. anomalus* strains influence the indigenous microbiota of the grape must, displacing non-*Saccharomyces* yeasts and slowing down the emergence and dominance of *S. cerevisiae*. These results match with the studied in Cabernet Sauvignon grape must, where the alcoholic fermentations with *W. anomalus* strains were slower than those carried out with *S. cerevisiae* [2]. In fact, *W. anomalus* has less fermenting power than the other yeast identified in this work; however, his relative abundance does not decrease on the seventh day, being the most common yeast in the VC10 and VC12 conditions until days 9 and 10, respectively. This dominance may be related not only to the initial inoculum of 5×10^6 cells/mL but also to the killer toxin and ethyl acetate production [25–27]. *W. anomalus* killer toxins production has been mainly tested against spoil yeast such as the genus Brettanomyces; however, the antimicrobial activity of this species has also been reported against other non-*Saccharomyces* species during the early stages of grape fermentation and *S. cerevisiae* [1]. *W. anomalus* ethyl acetate production is mediated by an alcohol acetyltransferase called Eat1, which allows this species to produce large amounts of this ester, with strains capable of producing more than 0.55 g/L in natural grape musts and 17 g/L in synthetic controlled media [28–30]. High ethyl acetate producers *W. anomalus* and *H. uvarum* strains have been employed to inhibit *Penicillium roquefortii* filamentous fungus and the oomicetous *Phytophthora nicotianae* [25,31].

Microorganisms inhibition by specific non-*Saccharomyces* yeasts such as *M. pulcherrima* or *L. thermotolerans* during alcoholic fermentation is of significant interest due to their production of pulcherrimin and lactic acid, respectively [5,6]. Further experiments are needed to elucidate the reason that causes the inhibition of non-*Saccharomyces* yeasts in the VC10 and VC12 conditions, although the high ethyl acetate and the killer toxins production by this *W. anomalus* strains seem to be the principal factor.

3.4. Enological Parameters and Major Aroma Compounds and Polyols

Of the nineteen variables analyzed, thirteen were measured by GC-FID and six according to OIV methods (Table 1). A total of fourteen variables have a known odor threshold (OT), and from those, six have exceeded the threshold in SF and VC12, while seven exceeded this threshold in the VC10 condition. The multiple comparison analysis identified six of nineteen variables with three different HG and fourteen of nineteen with at least two different HG; this indicates that the use of the proposed fermentation strategies has a notorious impact on the final wines.

Ethanol, 1-propanol, isoamyl alcohol, and titratable acidity were lower in the fermentation conditions where *W. anomalus* was inoculated than in SF; however, 2,3-butanediol, diethyl succinate, and volatile acidity were higher. A lower ethanol content is usual when non-*Saccharomyces* yeast, such as *M. pulcherrima* and *L. thermotolerans*, are employed alongside *S. cerevisiae* during alcoholic fermentations [16,18,32]; however, with *W. anomalus*, the results vary according to the strain inoculated and the must fermented. In this study,

ethanol content in the wines fermented with *W. anomalus* strains decreased by about 0.4% (*v*/*v*) in comparison with the one obtained by spontaneous fermentation; however, other authors obtained an increase in ethanol concentration with the co-fermentation of grape must between *S. cerevisiae* and *W. anomalus* in Cabernet Sauvignon while no significant differences were observed between verdejo wines fermented with *S. cerevisiae* and co-fermented with *W. anomalus* and *S. cerevisiae* [2,33,34]. Although similar wines have been obtained in the VC10 and VC12 conditions, both *W. anomalus* strains produced different profiles, as can be seen in Figure 4. This PCA summarizes the results obtained when the oenological parameters and major volatile compounds and polyols were analyzed, accounting for 73.24% of the total sample variance. The biplot shows that the three samples are different from each other, being SF more influenced by the alcohols, with the exception of 2-phenylethanol, while ethyl acetate and 2,3-butanediol have more impact in VC10 and VC12 fermentation conditions.

In addition to the lower higher alcohols contents, wines fermented with the selected strains of *W. anomalus* have a higher ethyl acetate content (Table 1). This ester production is mediated by the Eat1 enzyme located in the yeast mitochondria, which uses ethanol and acetyl-coenzyme A as substrates to produce this compound [35]. Other authors which have employed *W. anomalus* strains, highlight the ability of this species to obtain higher yields of ethyl acetate in alcoholic fermentation than those derived from the use of *S. cerevisiae*. For example, the *W. anomalus* P01A017 and Pi09 strains reached an ethyl acetate concentration of 399 and 552.09 mg/L, respectively, in grape must alcoholic fermentations, while the Disva-2 strain was able to produce 792.9 mg/L in YPD medium [26,29,34]. According to available information, the strains tested in this study reach the highest ethyl acetate concentration published using grape must as substrate; however, using sorghum as substrate, *W. anomalus* strains GZ3 and Y3604 yield 2760 and 2990 mg/L of ethyl acetate, respectively [36,37]. These strains were tested to improve the sensory profile of baijiu alcoholic beverages, where an application of VC10 and VC12 strains could be of interest. This high production of ethyl acetate could act as an inhibitor to other yeast species and explain the result obtained in the microbiological analysis. Ethyl acetate is highly demanded as a chemical solvent and is applied in the synthesis of biodiesels, paints, or adhesives, among others; however, nowadays, its production depends on petrochemical resources, which has a negative impact on the environment [28]. For this reason, the *W. anomalus* strains studied in this work could be useful as a sustainable alternative for ethyl acetate production.

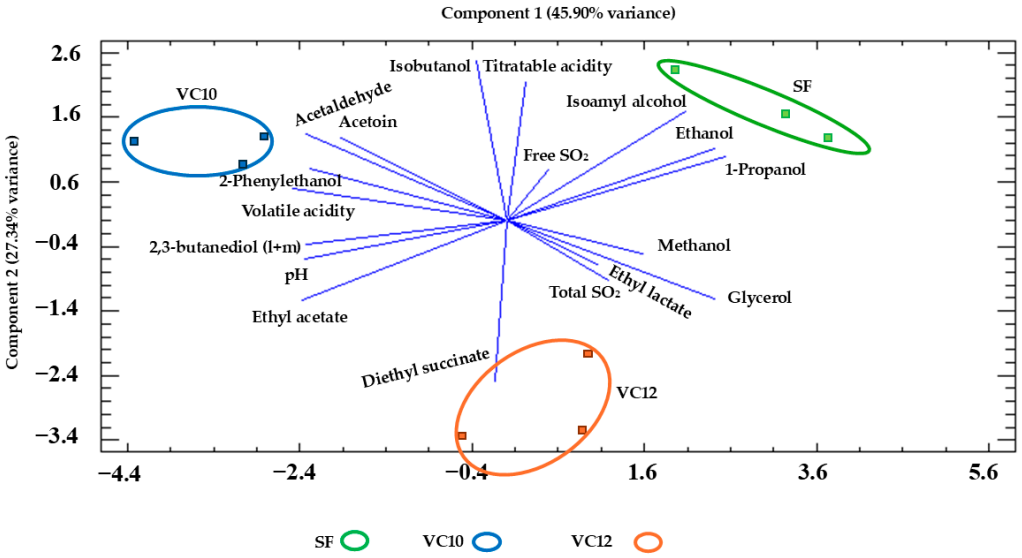

Figure 4. Principal Component Analysis (PCA) of wines obtained with different fermentation strategies. The analysis was carried out with the oenological parameters, major volatile compounds and polyols studied. SF: Spontaneous fermentation. VC10: Fermentation with VC10 *W. anomalus* strain. VC12: Fermentation with VC12 *W. anomalus* strain.

Table 1. Concentration of metabolites detected in the fermented musts. CAS: identification number assigned by the Chemical Abstracts Service. OT: odor threshold. SF: Wine obtained from spontaneous fermentation with indigenous microbiota. VC10: Wine obtained from fermentation with *W. anomalus* VC10 strain. VC12: Wine obtained from fermentation with *W. anomalus* VC12 strain. GC–FID: gas chromatography–flame ionization detector; OIV: International Organisation of Vine and Wine; ±: Standard deviation; abc: homogeneous group among groups of sampling. The different letters indicate homogeneous groups that significantly differ statistically in the parameters between wines ($p < 0.05$, F-test). OT was obtained from Carbonero-Pacheco et al. [32].

Compound	Method of detection	CAS	OT (mg/L)	Odor/flavor description	SF	VC10	VC12
Acetaldehyde (mg/L)	GC–FID	75-07-0	10	Over-ripe apple	59.46 ± 1.26 ^{b}	72.48 ± 1.44 ^{c}	56.93 ± 0.58 ^{a}
Ethyl acetate (mg/L)		141-78-6	7.5	Pineapple, varnish, balsamic	45.34 ± 0.16 ^{a}	1159.35 ± 1.37 ^{c}	998.73 ± 7.51 ^{b}
1,1-Diethoxyethane (mg/L)		105-57-7	1	Refreshing, pleasant, fruity-green	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Methanol (mg/L)		67-56-1	668	Chemical, medicinal	58.63 ± 6.55 ^{a}	50.71 ± 7.07 ^{a}	59.15 ± 1.16 ^{a}
1-Propanol (mg/L)		71-23-8	830	Ripe fruit, alcohol	66.56 ± 0.14 ^{c}	43.04 ± 0.19 ^{a}	49.34 ± 3.05 ^{b}
Isobutanol (mg/L)		78-83-1	40	Alcohol, wine, nail polish	37.73 ± 0.09 ^{b}	37.95 ± 0.02 ^{b}	33.77 ± 0.99 ^{a}
Isoamyl alcohol (mg/L)		123-51-3	30	Alcohol, nail polish	211.23 ± 0.07 ^{b}	185.8 ± 0.14 ^{a}	183.52 ± 5.61 ^{a}
Acetoin (mg/L)		53584-56-8	30	Buttery, creamy	24.97 ± 4.03 ^{ab}	32.32 ± 2.28 ^{b}	21.85 ± 4.46 ^{a}
Ethyl lactate (mg/L)		97-64-3	7.5	Strawberry, raspberry, buttery	15.05 ± 0.20 ^{a}	14.62 ± 0.20 ^{a}	14.99 ± 0.66 ^{a}
2,3-butanediol (l + m) (mg/L)		24347-58-8	668	Buttery, creamy	193.82 ± 18.47 ^{a}	288.10 ± 3.51 ^{b}	239.05 ± 70.87 ^{ab}
Diethyl succinate (mg/L)		123-25-1	100	Over-ripe, lavender	5.84 ± 0.23 ^{a}	6.52 ± 0.12 ^{b}	10.31 ± 0.32 ^{c}
2-Phenylethanol (mg/L)		60-12-8	10	Floral	17.58 ± 0.89 ^{a}	19.65 ± 0.94 ^{b}	17.80 ± 0.65 ^{a}
Glycerol (mg/L)		56-81-5	-	Confers body and smoothness and a sweet taste	4260 ± 90.00 ^{b}	3980 ± 50.00 ^{a}	4300 ± 10.00 ^{b}
Ethanol (% *v*/*v*)	According to OIV	64-17-5	10	Alcoholic	10.77 ± 0.03 ^{c}	10.32 ± 0.04 ^{a}	10.40 ± 0.04 ^{b}
pH		-	-	-	3.06 ± 0.01 ^{a}	3.11 ± 0.02 ^{c}	3.09 ± 0.01 ^{b}
Volatile acidity (g /L)		64-19-7	200	Vinegar	0.09 ± 0.02 ^{a}	0.14 ± 0.01 ^{b}	0.10 ± 0.02 ^{a}
Titratable acidity (g/L)		-	-	-	6.61 ± 0.04 ^{b}	6.54 ± 0.14 ^{ab}	6.31 ± 0.13 ^{a}
Free SO_2 (mg/L)		-	-	-	8.66 ± 1.15 ^{a}	8.66 ± 1.52 ^{a}	8.00 ± 1.00 ^{a}
Total SO_2 (mg/L)		-	-	-	37.66 ± 2.51 ^{a}	37.00 ± 1.73 ^{a}	38.66 ± 1.52 ^{a}

3.5. Nitrogen Compounds

Table 2 shows the composition of nitrogen compounds at different points of the alcoholic fermentation conducted in this work. A decrease in all compound concentrations could be observed throughout the process in the three studied conditions. On day 4, significant differences were found in three of the twenty-one nitrogen compounds analyzed (L-isoleucine, L-leucine, and L-phenylalanine). This could indicate that the non-*Saccharomyces* yeasts found in SF have similar nitrogen source requirements to *W. anomalus* in VC10 and VC12. One of the differences is found in L-phenylalanine, whose concentration is slightly lower in the VC10 and VC12 conditions and could be related to his use as a precursor of 2-phenylethanol in the Erlich pathway by this yeast [38]. On day 8, significant differences are accounted for in eight compounds; however, the most noticeable differences are observed in L-arginine and L-ornithine, which have higher concentrations in the VC10 and VC12 conditions than in SF. This matches with the highest fermentation rate in SF (Figure 2) and the dominance of *S. cerevisiae* over alcoholic fermentation in this condition (Figure 3). In fact, L-arginine is one of the most abundant amino acids in grape must, and its consumption by *S. cerevisiae* is related to ethanol stress tolerance [39]. L-ornithine is known as an intermediate of L-arginine biosynthesis by *S. cerevisiae,* and it is likely transformed into L-arginine because of the high demand for nitrogen by yeast cells during tumultuous fermentation [40]. At the end of the alcoholic fermentation, low concentrations of nitrogen sources were found, the most abundant being γ-aminobutyric acid (GABA), L-proline, and the biogenic amine putrescine. GABA is known to be employed as a nitrogen source by *S. cerevisiae* during alcoholic fermentation when nitrogen is scarce, and it is produced with the GABA shunt pathway from glutamate [41,42]. It is hypothesized that the yeasts in the final stage of alcoholic fermentation are producing this nitrogen source because of the absence of amino acids. L-proline concentration decreases in all conditions until the eighth day, at which time *S. cerevisiae* becomes the dominant species in the fermented musts. In fact, *S. cerevisiae* can not use L-proline during alcoholic fermentation, which would explain the detection of this amino acid at the final stages of the process [43]; however, non-*Saccharomyces* yeast such as *L. thermotolerans* and *T. delbrueckii* are capable of metabolizing that amino acid, which could explain the L-proline decrease in the SF condition [44]. A similar decrease is observed in the VC10 and VC12 conditions, where *W. anomalus* is the dominant species until the ninth day; no published data are available regarding the consumption of L-proline by *W. anomalus*, but it is hypothesized that this yeast is able to metabolize it in the same way as other non-*Saccharomyces*.

Table 2. Concentration of nitrogen compounds detected in the fermented musts. CAS: identification number assigned by the Chemical Abstracts Service. Initial Must: Must before starting the fermentation SF: Wine obtained from spontaneous fermentation with indigenous microbiota. VC10: Wine obtained from fermentation with *W. anomalus* VC10 strain. VC12: Wine obtained from fermentation with *W. anomalus* VC12 strain. -4: nitrogen compounds detected on the fourth day of fermentation. -8: nitrogen compounds detected on the eighth day of fermentation. -F: nitrogen compounds detected at the end of alcoholic fermentation. ±: Standard deviation; abcdef: homogeneous group among groups of sampling., N.D.: not detected. The different letters indicate homogeneous groups that significantly differ statistically in the parameters between wines ($p < 0.05$, F-test).

Compound (mg/L)	CAS	Initial Must	SF-4	VC10-4	VC12-4	SF-8	VC10-8	VC12-8	SF-F	VC10-F	VC12-F
L-aspartic acid	56-84-8	N.D.	16.53 ± 2.31 ^{b}	18.14 ± 3.74 ^{b}	15.95 ± 3.30 ^{b}	N.D.	N.D.	2.06 ± 0.2 ^{a}	N.D.	N.D.	N.D.
L-glutamic acid	56-86-0	N.D.	42.81 ± 9.90 ^{b}	43.47 ± 13.12 ^{b}	37.02 ± 9.77 ^{b}	10.07 ± 0.22 ^{a}	N.D.	N.D.	N.D.	N.D.	N.D.
L-glutamine	56-85-9	N.D.	3.24 ± 0.65 ^{a}	3.24 ± 0.83 ^{a}	3.15 ± 0.20 ^{a}	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
L-histidine	71-00-1	118.42 ± 31.55 ^{b}	47.39 ± 4.38 ^{a}	43.73 ± 8.22 ^{a}	40.01 ± 6.37 ^{a}	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
glycine	56-40-6	8.65 ± 2.48 ^{a}	15.78 ± 2.52 ^{b}	14.50 ± 1.92 ^{b}	14.58 ± 2.16 ^{b}	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
L-threonine	72-19-5	28.14 ± 12.54 ^{a}	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
L-citrulline	372-75-8	11.82 ± 0.43 ^{a}	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
L-arginine	74-79-3	1847 ± 112.44 ^{e}	468.30 ± 22.79 ^{d}	443.61 ± 79.91 ^{d}	412.77 ± 63.00 ^{d}	6.79 ± 0.58 ^{a}	216.33 ± 35.77 ^{b}	305.22 ± 9.01 ^{c}	N.D.	N.D.	N.D.
L-alanine	56-41-7	49.09 ± 0.98 ^{c}	35.11 ± 7.02 ^{b}	41.10 ± 9.82 ^{bc}	39.04 ± 8.73 ^{bc}	2.03 ± 0.34 ^{a}	2.97 ± 0.40 ^{a}	2.26 ± 0.34 ^{a}	2.94 ± 2.32 ^{a}	N.D.	N.D.
γ-Aminobutyric acid	56-12-2	411.00 ± 7.78 ^{d}	20.19 ± 5.37 ^{b}	18.56 ± 5.08 ^{b}	18.81 ± 7.09 ^{b}	N.D.	13.07 ± 0.87 ^{ab}	16.08 ± 4.49 ^{ab}	9.27 ± 1.03 ^{a}	31.54 ± 1.95 ^{c}	32.71 ± 2.10 ^{c}
L-α-amino-n-Butyric acid	2835-81-6	35.15 ± 7.32 ^{c}	42.35 ± 9.50 ^{c}	40.10 ± 8.29 ^{c}	39.25 ± 10.97 ^{c}	N.D.	2.95 ± 5.12 ^{ab}	10.5 ± 0.16 ^{b}	N.D.	N.D.	N.D.
L-proline	147-85-3	70.05 ± 3.39 ^{d}	31.76 ± 3.55 ^{c}	31.80 ± 3.63 ^{c}	31.77 ± 4.74 ^{c}	15.33 ± 0.39 ^{b}	12.44 ± 3.64 ^{ab}	12.59 ± 1.50 ^{a}	15.54 ± 0.60 ^{b}	13.16 ± 0.06 ^{ab}	13.31 ± 0.69 ^{ab}
Ammonium chloride	12125-02-9	N.D.	19.51 ± 5.47 ^{a}	19.68 ± 3.64 ^{a}	20.64 ± 5.43 ^{a}	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
L-valine	72-18-4	6.15 ± 0.17 ^{a}	30.76 ± 4.31 ^{b}	28.88 ± 4.08 ^{b}	28.24 ± 4.74 ^{b}	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
L-isoleucine	73-32-5	N.D.	8.17 ± 1.48 ^{b}	6.85 ± 0.72 ^{a}	6.95 ± 1.18 ^{a}	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
L-tryptophan	73-22-3	N.D.	12.25 ± 1.52 ^{a}	11.73 ± 2.06 ^{a}	11.05 ± 1.76 ^{a}	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
L-leucine	61-90-5	N.D.	9.34 ± 1.82 ^{b}	7.40 ± 1.09 ^{a}	7.44 ± 1.17 ^{a}	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
L-phenylalanine	63-91-2	6.44 ± 0.99 ^{a}	12.06 ± 1.70 ^{c}	8.7 ± 1.62 ^{b}	8.62 ± 2.36 ^{b}	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
L-ornithine	70-26-8	223.75 ± 6.74 ^{f}	63.53 ± 3.69 ^{bcd}	62.27 ± 12.78 ^{bc}	56.48 ± 9.21 ^{b}	6.67 ± 1.51 ^{a}	72.67 ± 1.81 ^{e}	67.33 ± 4.22 ^{de}	4.04 ± 1.02 ^{a}	N.D.	N.D.
L-lysine	56-87-1	121.33 ± 2.48 ^{d}	11.35 ± 7.01 ^{c}	11.35 ± 2.53 ^{c}	13.20 ± 1.29 ^{c}	N.D.	4.67 ± 1.01 ^{b}	4.33 ± 2.13 ^{ab}	N.D.	N.D.	N.D.
putrescine	110-60-1	75.36 ± 2.82 ^{c}	17.59 ± 0.82 ^{b}	17.06 ± 3.00 ^{b}	15.84 ± 2.53 ^{ab}	16.29 ± 0.56 ^{ab}	14.90 ± 0.40 ^{ab}	15.48 ± 0.64 ^{ab}	15.33 ± 0.55 ^{ab}	14.04 ± 0.28 ^{a}	14.16 ± 0.61 ^{a}

3.6. Sensory Analyses

The chemical differences collected in Table 1, and previously discussed, were perceived by the tasting panel, which was not capable of distinguishing between wines from the VC10 and VC12 conditions because of the high ethyl acetate content. The blendings between wines of different fermentation strategies were displayed because of the differences in ethyl acetate content between them. This ester is considered to impart negative sensorial properties when its concentration in wine exceeds 200 mg/L [1].

The tasting panel noted distinct characteristics among the wines presented (Figure 5); they first noticed a nail polish aroma and a strong olfactory intensity and flavor in the VC10 and VC12 conditions. These wines were qualified as unbalanced and defective for most of the tasting panel components. The SF condition achieved the highest score in the herbaceous and vegetable smell, considered a balanced wine by the tasting panel, but most of the judges scored it as mediocre. The blended wines (SF + 10% of VC10/VC12 and SF + 20% of VC10/VC12 (Section 2.8)) (Figure 5) showed a more diverse sensory profile than SF and VC10/VC12, finding floral, herbaceous, vegetables and fruity aromas, being the last one the most outstanding for the tasting panel. While SF + 10% was positively evaluated by most of the components, reaching the highest score in the general gustatory and olfactory score, in SF + 20%, most of the judges rated the wine as unbalanced, highlighting a polish smell.

Although blending was positively evaluated, the use of different inoculation techniques, such as yeast immobilization or sequential fermentation, could be useful to reduce the ethyl acetate production by *W. anomalus* to desirable values and should be tested in further studies [32,45].

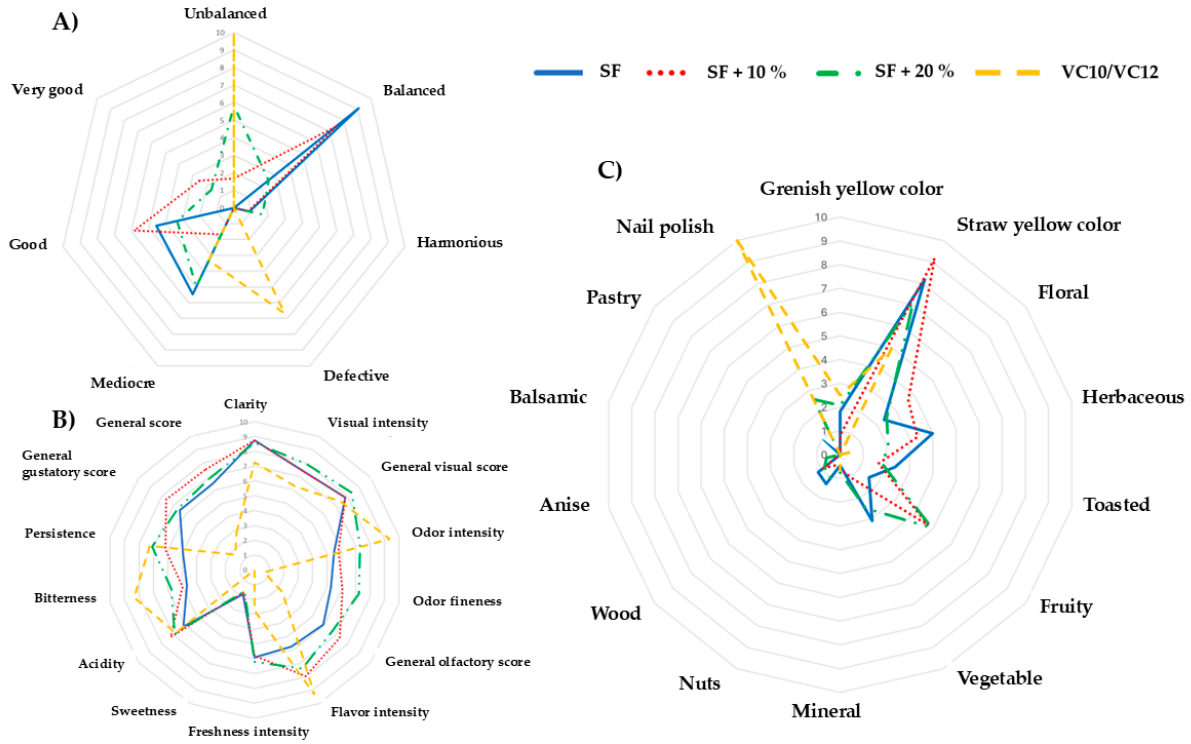

Figure 5. Sensory profile plot in wines produced with different fermentation strategies; SF: Spontaneous fermentation; VC10/VC12: Fermentation with *W. anomalus* strains; SF +10%: Blending of SF (90%), VC10 (5%) and VC12 (5%); SF +20%: Blending of SF (80%), VC10 (10%) and VC12 (10%). Values represent averages from the evaluation made by the tasting panel (n = 12). General scores are represented in (**A**), and specific attributes are represented in (**B**) and (**C**).

4. Conclusions

While non-*Saccharomyces* yeasts offer many benefits, winemakers must carefully select strains and manage their use. Improper use can lead to fermentation issues, high levels of acetic acid and ethyl acetate, or lack of reproducibility; however, when used correctly, these yeasts can significantly contribute to wine quality and help winemakers achieve desired wine styles. The *W. anomalus* strains isolated from biologically aged wines from Montilla–Moriles P.D.O. showed β-G*l*ucosidase activity and were able to grow in an ethanol concentration of up to 9% (*v*/*v*). Both strains were successfully applied in the alcoholic fermentation process and were useful in enhancing the sensorial profile of white wines; however, the high ethyl acetate concentration produced by the strains employed in this work makes their use as monoculture unsuitable in grape must fermentation for winemaking, although its use could be of interest in other processes such as the production of baijiu alcoholic beverage or microorganisms inhibition.

The blend with less aromatic wines has been positively assessed by the tasting panel; however, further studies exploring the volatilome of these strains through platforms such as SBSE-TD-GC-MS (Stir Bar Sorptive Extraction–Thermal Desorption–Gas Chromatography–Mass Spectrometry) and the application of other techniques, such as sequential fermentations along with other yeast species or yeast immobilization could improve the results obtained, increase wine complexity and avoid the overproduction of some compounds which could make the wine unbalanced. In conclusion, once these technique improvements are studied and implemented, the proposed methodology and the use of these strains could be considered to scale up.

Author Contributions: J.C.-P.: investigation, methodology, formal analysis, statistics, writing (original draft); Á.G.-J.: methodology, formal analysis; J.C.G.-G.: methodology, formal analysis; I.M.S.-D.: supervision, methodology, visualization, project administration. T.G.-M.: data curation, review, resources; J.M.: methodology, supervision; J.M.-G.: review, resources; J.C.M.: conceptualization, formal analysis, resources, project administration, writing (review and editing). All authors have read and agreed to the published version of the manuscript.

Funding: This study was co-funded by the Spanish Ministry of Science, Innovation and Universities (MICIU/European Union FEDER), Ref. PID2021-127766OB-I00 (JCM), by a CONTRACT ART. 83 OTRI associated with the CDTI competitive call with Pérez Barquero company, Ref. 12022188 (JCM) and by the "Ayudas para la Explotación de Resultados de investigación de la Modalidad II del X Plan Propio Galileo" (JM-G) granted by "Oficina de Transferencia de Resultados de Investigación" (OTRI).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

Acknowledgments: The authors thank the Pérez Barquero Winery, PAUDIRE company, the staff of the Central Service for Research Support (SCAI) at the University of Córdoba (Spain) for its contribution to yeast identification by MALDI Biotyper and CDTI.

Conflicts of Interest: The authors declare no conflicts of interest.

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