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Effect of Non-*Saccharomyces* Species Monocultures on Alcoholic Fermentation Behavior and Aromatic Profile of Assyrtiko Wine

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Abstract: Six wild-type non-*Saccharomyces* strains, belonging to the species *Zygosaccharomyces bailii*, *Priceomyces carsonii*, *Trigonopsis californica*, and *Pichia manshurica*, were evaluated for white wine production using Assyrtiko grapes from Santorini in Greece. Fermentation kinetics, in terms of glucose and fructose consumption and sensory analysis, was first employed to test the enological potential of the yeast strains. Based on their performance, two strains of *Z. bailii* (Zb-A19Y5 and Zb-K29Y2) and one strain of *T. californica* (Tc-A9Y1) selected for further analysis. The selected strains were tested in larger fermentation volumes for sugar consumption, while the produced wines were assessed for classical enological parameters, volatile compounds (GC/MS), and sensory analysis. Tc-A9Y1 could lead to dry wine (1 g/L of residual sugars) with 1.6 vol (12%) less alcohol than the other experimental wines. The wines that were fermented with the strain Zb-K29Y2 exhibited very high concentrations of isoamyl alcohol (604.33 ± 76.8 mg/L), but at the same time, they were characterized by low fruity notes. None of the produced wines presented any off-flavor aromas. Exploiting non-*Saccharomyces* strains with great fermentation capacity, which are able to produce high-quality wines and adapted to global warming conditions, is a new challenge for the wine industry.

Keywords: non-*Saccharomyces* yeast; assyrtiko; winemaking; *Zygosaccharomyces bailii*; *Priceomyces carsonii*; *Trigonopsis californica*

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1. Introduction

Assyrtiko is a Greek grape variety with unique sensorial attributes. This dynamic variety is famous worldwide due to the production of high-acidity wines, with typical varietal aromas, even more so when grown in the volcanic terroir of Santorini Island [1]. Assyrtiko is considered a variety that is well adapted to climate change, characterized by resistance to heat and drought. Recently, a great collection of wine yeast has been collected from Greek wines at the end of the fermentation process, including Assyrtiko wines [2]. The wine market is becoming extremely competitive, so an effort to differentiate and create a signature wine style is vital [3]. The role of microorganisms constitutes an integral modulator of wine quality [4,5]. Modern winemaking seeks and invests in the selection of so-called indigenous, autochthonous, or native local strains. Indigenous strains are believed to be able to maintain the typical sensory properties and enhance the unique properties of a wine, demonstrating improved adaptation to their native environment [6]. Complexity can be added through the inoculation of various species either under co-inoculation or sequential inoculation schemes [7–9].

Non-*Saccharomyces* yeasts are usually found in the early stages of alcoholic fermentation, and the most dominant ones belong to the genera of *Hanseniaspora*, *Candida*, *Torulaspora*, *Pichia*, *Rhodotorula*, and *Zygosaccharomyces* [4,10]. These non-*Saccharomyces* yeasts may play

a positive role in wine quality through the production of secondary metabolites with enhanced sensorial contribution [11–13]. A new application of non-*Saccharomyces* has emerged in terms of bioprotection, aiming for a reduction in sulfite in wines [11]. Furthermore, their ability to increase the release of volatile thiols, particularly during the pre-fermentation stage, when this microbiological sub-population is dominant, was previously noted [12]. In any case, their low fermentation capacity and high sensitivity to alcohol make them inappropriate starters for wine fermentation. Thus, mixed cultures with *S. cerevisiae* strains designed, leading to more or less complex wines based on the yeast strains and winemaking conditions [14–16]. Non-*Saccharomyces* yeast can also be classified as spoilage wine agents. The species that are most at risk of provoking organoleptic deviation is *Brettanomyces bruxellensis*, through the production of active odorous compounds that are described as "horse sweat", "animal", "plastic", etc. Volatile phenols are the main compounds implicated in the spoilage effect, which can also be produced by genera such as *Candida*, *Pichia*, and *Trigonopsis* [17–19]. In more detail, *Trigonopsis* species were shown to be able to grow in the presence of ethanol by expressing heat shock proteins and a DNA damage-related protein and to be able to confer spoilage characteristics on wine [17]. Additionally, *Pichia* species that are recovered from grapes, grape juice, and winery equipment that is in contact with grape juice, but not from wines, produced 4-ethylphenol, an unpleasant taint described as a "phenolic odor" [19].

The present study aims to evaluate the performance of indigenous and rarely isolated non-*Saccharomyces* strains in monoculture fermentation schemes for their enological potential. Namely, the previously isolated species *Zygosaccharomyces bailii*, *Priceomyces carsonii*, *Trigonopsis californica,* and *Pichia manshurica* [2] were tested for their fermentation capacity and their sensorial contribution to Assyrtiko must. All the aforementioned species, based on the bibliography, are often correlated with microbial spoilage and have never before been evaluated for any beneficial potential.

2. Materials and Methods

2.1. Yeast Isolates

Seventeen microbial isolates belonging to 5 different species were previously isolated and identified in wines at the end of spontaneous alcoholic fermentation (AF) [2]. More specifically, *Trigonopsis californica* (1 isolate), *Zygosaccharomyces bailii* (8 isolates), *Priceomyces carsonii* (1 isolate), and *Pichia manshurica* (7 isolates) survived and co-existed with *S. cerevisiae* strains during AF. All the isolates were preserved at −20 °C in YPD broth [(g/L): yeast extract 10, Bacteriological peptone 20, Dextrose 20] with 30% of glycerol added.

2.2. Genotyping by Rep-PCR

Total genomic DNA from the isolates was extracted in previous work and stored at −20 °C [2]. Quantification and quality control of DNA extract were performed by spectrophotometer (Epoch, Biotek, Winooski, VT, USA) at wavelengths of 260, 280, and 230 nm. Prior to determination of the isolated yeast strains, rep-PCR (Repetitive Polymerase Chain Reaction) with GTG5 (5′-GTG GTG GTG GTG GTG-3′) primer was performed.

PCR amplification was conducted in 20 μL final reaction volumes, containing 10 μL of OneTaq-quick load 2× Mastermix with standard buffer (New England Biolands, Hitchin, UK), 10 mM GTG5, and 50 ng of template DNA. The amplification program consisted of 2 min of initial denaturation at 95 °C, 35 cycles of 30 s at 94 °C, 1 min at 45 °C, 4 min at 72 °C, concluding with 5 min at 65 °C. All PCR products were run on a 1.5% (*w*/*v*) agarose gel in 1 × TAE buffer, stained with ethidium bromide (20 min), at 110 V for 140 min and scanned under ultraviolet light (MiniBIS, DNR, Jerusalem, Israel). A 1Kb DNA ladder (Nippon Genetics, Düren, Germany) served as size standard. The resulting fingerprints were digitally captured, converted, normalized, and analyzed using the Dice coefficient with Bionumerics software version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium). Means of the Unweighted Pair Group Method using the Arithmetic Average (UPGMA) clustering algorithm led to the formation of the strain-specific dendrogram.

2.3. Experimental Design and Winemaking Conditions

2.3.1. Fermentation Kinetics of Six Strains

Micro-fermentation trials evaluated the fermentation potential of the yeast strains. Fermentations were carried out in 50 mL of pasteurized (72 °C, 10 min) Assyrtiko must, which was provided by Gaia (Santorini, Greece) winery (vintage 2021), under static conditions. The initial pasteurized grape must (pH = 3.20 ± 0.03, total acidity = 5.77 ± 0.06 g tartaric acid/L, YAN = 609.00 ± 4.76 mgN_2/L, 16.60 ± 0.01 mgSO_2, 5.10 ± 0.10 free SO_2) contained 119.5 ± 2.5 g/L of glucose and 120.1 ± 3.1 g/L of fructose. Precultures were grown in YPD broth at 28 °C for 48 h, and then used to inoculate each fermentation (10^6 cell/mL). The viability of the inoculum was examined by classical microbiological techniques at 0 and 48 h. The pasteurized must was also microbiologically analyzed, and no viable, culturable cells were noticed, ensuring the success of pasteurization. Briefly, 1 mL of the wine or must sample was serially diluted in 9 mL Ringer's solution following decimal dilutions. Then, 0.1 mL of the appropriate dilutions spread in duplicate on YPD agar [(g/L): yeast extract 10, Bacteriological peptone 20, Dextrose 20, Agar 20] substrate to quantify the microbial population. All assessed fermentations duplicated, and each analysis conducted twice.

Fermentation kinetics were monitored by residual sugar (glucose and fructose) determination on a daily basis using Enzytec kit-liquid Glucose-Fructose (R-biopharm, Darmstadt, Germany). Fermentations were carried out at 18 °C in 100 mL Duran under static conditions. The temperature condition was chosen so as to be close to the real winemaking conditions. As previously described, cv. Assyrtiko fermentations are usually performed at 18 °C [20]. The sample was homogenized before every sampling point. Double trials of pure cultures were carried out for each different strain.

2.3.2. Fermentation Kinetics of Three Selected Strains

The strains with higher enological potential were further subjected to bigger laboratory fermentations (1.2 L total volume). The fermentation media and inoculum preparation were the same as described before Section 2.1. Fermentations were also carried out at 18 °C in 1.5 L glass bottles under static conditions. The sample was homogenized before every sampling point. Fermentation kinetics were monitored by residual sugar (glucose and fructose) determination a daily using an automatic enzymatic Analyzer (Hyperlab, Steroglass, San Martino in Campo, Italy). Two trials of pure cultures were carried out for each different strain.

2.4. Chemical Analysis

2.4.1. Classical Enological Parameters

Alcoholic fermentation was monitored daily through the determination of glucose and fructose. AF was considered completed when the total sugar concentration was below 4 g/L. Analysis of glucose/fructose, acetic acid, and malic acid was determined using an automatic enzymatic Analyzer (Hyperlab, Steroglass, San Martino in Campo, Italy). pH was measured using a pHmeter (HI2210, Hanna Instruments, Smithfield, VA, USA). Yeast assimilable nitrogen (YAN) was measured following the formol method [21]. Determination of free and total SO_2 was performed by the iodometric method, and ethanol content and total acidity were determined according to OIV methods [22]. All duplicated fermentations were analyzed twice.

2.4.2. Identification and Quantification of Volatile Compounds

The methodology selected for optimizing the extraction of aromatic compounds from the wine samples, preceding their introduction into the gas chromatography–mass spectrometry (GC-MS) apparatus, involved the implementation of a liquid–liquid extraction technique. This extraction approach was adapted from the protocol established previously [23]. All duplicated fermentations were analyzed twice.

The analytical instruments employed in this study included a gas chromatograph (Shimadzu (Kyoto, Japan), Nexis GC-2030) equipped with an autosampler/injector (Shimadzu

(Kyoto, Japan), AOC 20i Plus). In the case of liquid–liquid extraction (LLE), the injector was maintained at a temperature of 250 °C, operating in split/splitless mode with a 1/100 split ratio. THE MEGA-WAX MS capillary column was utilized, measuring 30 m in length, with an i.d. of 0.25 mm and a film thickness of 0.25 µm. The stationary phase consisted of polyethylene glycol (PEG) (Agilent Technologies, Santa Clara, CA, USA). The injector temperature was maintained at 250 °C. The temperature program for the gas chromatograph commenced at 50 °C for 2.5 min, followed by a ramping rate of 2.5 °C/min to 100 °C, 4 °C/min to 165 °C, and 7 °C/min to 250 °C, with a 2 min hold at the final temperature. Helium served as the carrier gas, with a constant flow rate of 1.5 mL/min.

The mass spectrometer was operated in electron impact mode at 70 eV within the mass range of m/z 50–550 amu, while maintaining the ion source and transfer line at 230 °C. All analyses were conducted in duplicate. In accordance with established protocols, concentrations of volatile compounds were determined utilizing an internal standard (n-undecane). Compound identification was validated when a minimum match factor of 80% was attained by comparing the mass spectra of the compounds to those available in the NIST library.

2.5. Sensory Analysis

The six produced wines from the fermentation of six strains of five species were categorized based on their aromatic profile (odor) using a free sorting task test, as described previously [2]. Free sorting task is an efficient technique for assessing the perception of a set of products by a panel of subjects and is also widespread in wine science [24–26]. Samples were evaluated in individual booths, using covered, ISO glasses (NF V09-110, 1971) containing about 25 mL of liquid, coded with three-digit random numbers. A total of 10 experienced panelists participated in the pilot study. Panelists were not informed about the nature of the samples. All duplicated fermentations were evaluated twice by each panelist (n = 4).

Sensory analysis of the selected strains was performed as described by Dimopoulou et al. [15]. Samples were evaluated by descriptive analysis in individual booths, using covered, ISO glasses (NF V09-110, 1971) containing about 25 mL of liquid, coded with three-digit random numbers [15]. The panel was composed of 10 trained tasters, selected for their experience in assessing aromas in Greek white wines. Panelists were not informed about the nature of the samples, and all duplicated fermentations were evaluated twice by each panelist (n = 4). The intensity of the examined sensory attributes was evaluated using a 10-point scale (1: null; 10: very strong) and scored manually.

2.6. Statistical Analysis

Significant differences were evaluated by One-way Analysis of Variance (ANOVA), which was followed by Tukey's post hoc test ($p < 0.05$). All statistical analyses were performed using Statgraphics Centurion 18 software (Statgraphics Technologies, Inc., The Plains, VA, USA). Encoding free sorting data was the key to categorizing wine samples based on the results of the sensory assessment. For each group, results were encoded in an individual similarity matrix (wines × wines), in which 1 stands for two wines set in the same group and 0 for two wines placed in different groups. These individual matrices were summed across subjects; the resulting co-occurrence matrix represents the global similarity matrix, where larger numbers indicate higher similarity between samples. The assumption underlying this method is that grouped samples are more similar than samples that are sorted into different groups. The resulting co-occurrence matrix was submitted to HCA (ward coefficient) to derive a spatial arrangement of wines with R (3.6.2) software analysis.

3. Results and Discussion

During the spontaneous fermentation process, non-*Saccharomyces* yeasts usually dominate and start the AF, while the conversion of sugars into ethanol is often completed by *S. cerevisiae* strains [27,28]. However, all the 18 non-*Saccharomyces* yeasts that were

investigated in this survey, presented in Table 1, are isolated from spontaneous fermented wines which exhibited no off-odor or off-flavor characteristics, as previously described [2]. The aforementioned species, which have rarely been isolated from the wine environment, are often characterized as spoilage yeasts. More precisely, *T. californica* is characterized by the literature as a contaminant of Californian wines and genetically closely related to *Trigonopsis cantarellii*, *Trigonopsis variabilis*, and *Trigonopsis vinaria* [29,30]. Recent research suggests that *T. californica* is considered a high acetic acid producer and has been isolated from wines at the end of AF and during aging, conferring a "Brett" character to wine [31,32]. Additionally, *P. manshurica* is found in several fermented foods and alcoholic drinks [33–36]. The spoilage capacity of *P. manshurica* regional isolates was evaluated in red wine, and the production of volatile phenols and cadaverine was evidenced [34,35,37].

Table 1. Sample coding, species, and strain differentiation of the isolates. The selected strains are marked in bold.

Isolate ID	Species	Strain	Code ID
A9Υ1	*Trigonopsis californica*	Tc	**Tc-A9Υ1**
K16Y1	*Zygosaccharomyces bailii*	Z1	**Zb-K16Y1**
K16Y1	*Zygosaccharomyces bailii*	Z1	Zb-K16Y1
A19Y5	*Zygosaccharomyces bailii*	Z2	**Zb-A19Y5**
K21Υ7	*Zygosaccharomyces bailii*	Z3	Zb-K21Υ7
K21Υ9	*Priceomyces carsonii*	Pc	**Pc-K21Υ9**
K29Υ2	*Zygosaccharomyces bailii*	Z3	**Zb-K29Υ2**
K29Υ4	*Zygosaccharomyces bailii*	Z3	Zb-K29Υ4
K29Υ5	*Zygosaccharomyces bailii*	Z3	Zb-K29Υ5
K29Υ8	*Zygosaccharomyces bailii*	Z3	Zb-K29Υ8
A30Y5	*Zygosaccharomyces bailii*	Z3	Zb-A30Y5
K33Υ1	*Pichia manshurica*	P1	Pm-K33Υ1
K33Υ2	*Pichia manshurica*	P1	Pm-K33Υ2
K34Υ4	*Pichia manshurica*	P1	Pm-K34Υ4
K34Υ10	*Pichia manshurica*	P1	**Pm-K34Υ10**
K34Υ17	*Pichia manshurica*	P1	Pm-K34Υ17
K34Υ19	*Pichia manshurica*	P1	Pm-K34Υ19
K34Υ20	*Pichia manshurica*	P1	Pm-K34Υ20

However, only a few researchers have investigated their contribution to a wine's microbial environment. For instance, *Z. bailii* stands out as a spoilage yeast, mainly in acidified food products, various non-carbonated fruit drinks, and in wine with fermentative metabolism [38–40]. Despite the higher levels of produced sorbic, benzoic, and especially acetic acid that are produced by *Z. bailii* compared to those produced by *S. cerevisiae*, no other off-flavor observations have been reported to indicate spoilage behavior in wines [40,41]. Finally, according to Cordero-Bueso et al. [42], *P. carsonii* was reported to influence the volatile composition of musts from grape berries and was not able to catabolize all residual sugars on must. *P. carsonii* has also been isolated from oils, fermented sausages, and lambic beer [43–45]. The main objective of the present survey was to examine the performance of these indigenous and rarely isolated non-*Saccharomyces* strains in monocultures' fermentation schemes as potential starter cultures.

Strain differentiation was achieved using repetitive extragenic palindromic PCR (rep-PCR) using the primer GTG5 (Table 1). For isolates from the same species, which were also identified within the same wine samples, a lack of strain diversity was predominantly observed. Consequently, only one strain per species of *T. californica*, *P. carsonii*, and *P. manshurica* was identified. On the contrary, when investigating *Z. bailii*, nine isolates originating from various samples were analyzed, and molecular fingerprinting revealed the presence of three distinct strains. It is noteworthy that Zb-Z3 is detected in both red and white wines from two different wineries. A single representative strain for each species was subsequently selected for further investigation of its enological potential.

3.1. Yeast Growth and Enological Parameters during Fermentation

Fermentation kinetics were monitored for each single monoculture by sugar consumption. Figure 1 shows the glucose and fructose consumption during the AF of pasteurized Assyrtiko must under the different inoculation schemes. The microbiological analysis affirmed the initial inoculum concentration (0 h) and the survival of the inoculated strains at 48 h. The population of the inoculum in all modalities at 48 h ranged from 7.8 ± 0.02 to 8.2 ± 0.05 log CFU/mL. After 20 days of fermentation at 18 °C, two strains (Pc-K21Y9, Pm-K34Y10) could not lead to dry wines. Pc-K21Y9 demonstrated fructophilic behavior, since fructose was catabolized quickly, but it could not catabolize glucose, while the residual sugars were estimated at 24.7 ± 1.4 g/L. The term "fructophilic" refers to the preferential capacity of metabolizing fructose when both fructose and glucose are present in high concentrations in the surrounding environment [46]. Additionally, the strain Pm-K34Y10, after 20 days of AF, could not catabolize a concentration of 20.8 ± 2.2 g of glucose/L and 51.78 ± 3.0 g fructose/L. All fermentations that were performed with *Z. bailii* and *T. californica* strains led to dry wines. More specifically, Zb-A19Y5, Zb-K29Y2, Tc-A9Y1, and Zb-K16Y1 completed alcoholic fermentation at 12, 16, 20, and 16 days, respectively. A recent study investigated 20 indigenous *S. cerevisiae's* fermentative capacity using the same pasteurized Assyrtiko must, under the same laboratory conditions [2]. The majority of these strains had completed alcoholic fermentation after 9 days. Consequently, the investigated non-*Saccharomyces* are characterized by a lower fermentation rate compared to *S. cerevisiae* starters. It is widely recognized that some non-*Saccharomyces* species are capable of surviving and persisting until the completion of fermentation, as they exhibit a heightened tolerance to ethanol [38]. Our results are in line with this observation, since the increment of alcohol did not significantly impact most of the species in this survey. Numerous non-*Saccharomyces* yeasts have been explored for their fermentative capabilities, primarily in co-inoculation with *S. cerevisiae*, given their inability to complete alcoholic fermentation when used as monocultures [37,40,47,48]. The fermentation rate of monocultures of non-*Saccharomyces* species has been proven to be lower than those with monocultures of *S. cerevisiae*, sequential inoculations, or co-cultures of non-*Saccharomyces* species with *S. cerevisiae* strains. Nevertheless, it is worth noting that the successful completion of alcoholic fermentation by monocultures of non-*Saccharomyces* species has already been reported [49,50]. Notably, certain strains of the *Z. bailii* species have been previously identified for their high fermentative efficiency, reaching up to 89% [49].

To provide a holistic and non-verbal evaluation of the resulting wines, a sensory analysis was performed. Based on the aroma bouquet of Assyrtiko wines, the determination of the differences among non-*Saccharomyces* fermentation schemes was achieved. According to the free sorting assessment, wines were grouped according to their perceived similarity by panelists, and sensory descriptors were provided for each group (Figure 2). Two distinct groups are noticed. Firstly, the pink group (Pm-K34Y10, Zb-K16Y1, and Pc-K21Y9) was described by mainly negative attributes (oxidation, H2S, and other off-odor aromas). Additionally, the green group (Zb-K29Y2, Tc-A9Y1, and Zb-A19Y5) was associated with positive attributes such as "fruitiness", "floral", "tropical fruits", and "apple". The results were expected due to the abnormal fermentation of Pm-K34Y10 and Pc-K21Y9 strains. However, Zb-K29Y2 was the only *Z. bailii* strain that exhibited off-flavor aromas. Our findings align with the literature that supports strains, belonging to the genera *Hanseniaspora* and *Zygosaccharomyces*, revealing differences at both the interspecies and intraspecies levels [37].

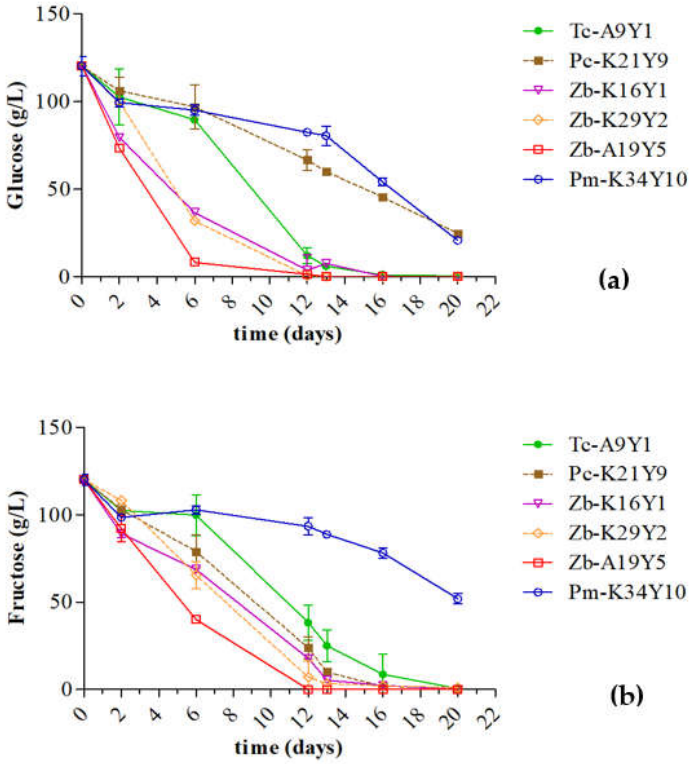

Figure 1. Changes in sugar consumption [(**a**) D-glucose and (**b**) D-fructose] during alcoholic fermentations for the 6 strains. Sample coding: Tc = *Trigonopsis californica*, Zb = *Zygosaccharomyces bailii*, Pc = *Priceomyces carsonii*, Pm = *Pichia manshurica*.

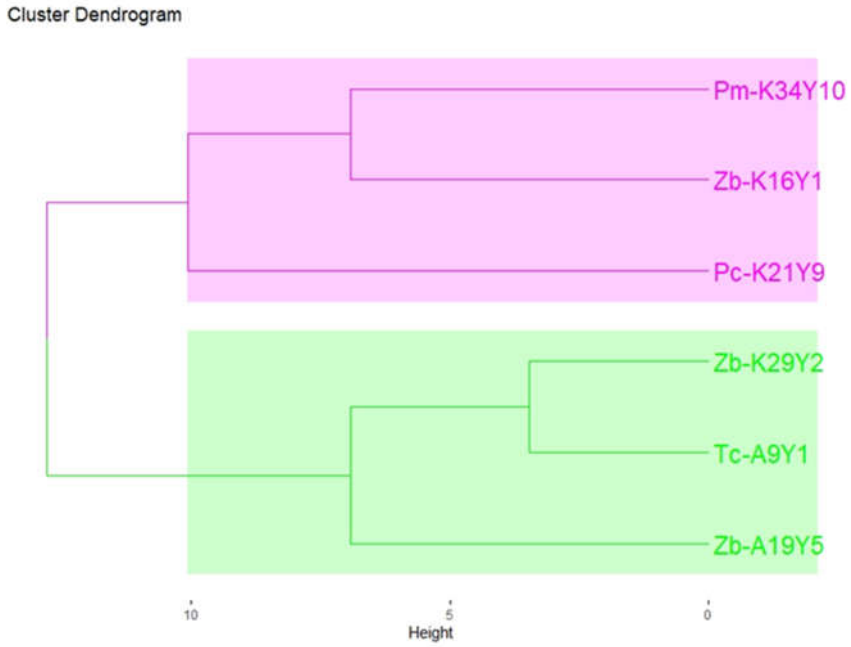

Figure 2. Hierarchical cluster analysis (HCA) of the different aromatic profiles of the 6 produced wines from different non-*Saccharomyces* strains, based on the results of the free sorting task with Ward coefficient. Sample coding: Tc = *Trigonopsis californica*, Zb = *Zygosaccharomyces bailii*, Pc = *Priceomyces carsonii*, Pm = *Pichia manshurica*. The two groups are distinct by colors: Group A = pink, Group B = green.

To further explore the enological potential of the highest performing strains by performing more biochemical analyses, the strains that constituted the green group were subjected to a second laboratory fermentation in a bigger volume (1.2 L) so that a further

analysis could be performed. The microbiological analysis affirmed the initial inoculum concentration (0 h) and the survival of the inoculated strains after 48 h of inoculation. The population of the inoculum in all modalities at 48 h varied between 7.9 ± 0.03 and 8.3 ± 0.04 log CFU/mL. As Figure 3 illustrates, the monocultures of the *Z. bailii* strains (Zb-A19Y5 and Zb-K29Y2) completed alcoholic fermentation within 504 h, while *T. californica* Tc-A9Y1 required 648 h for fermentation completion. Notably, in both fermentations, it is evident that the *T. californica* strain exhibited a lower fermentation rate compared to the *Z. bailii* strains. According to the literature, *Z. bailii* isolates have been noticed to exhibit fructophilic behavior, but it was difficult to conclude whether the observed phenotype could be linked to high sugar adaptation [38,46,51,52].

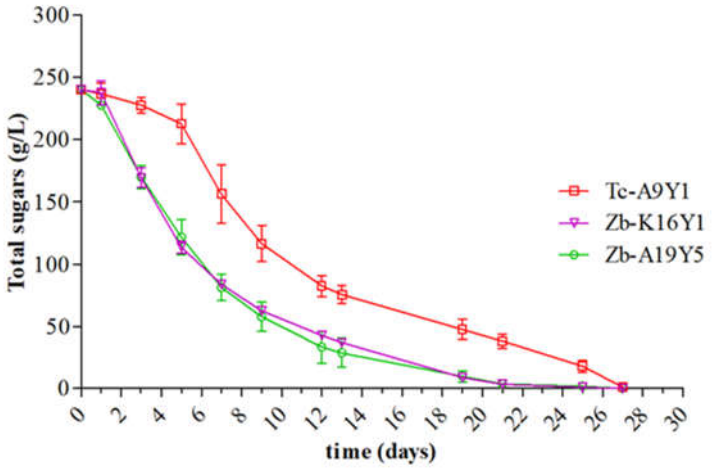

Figure 3. Changes in total sugar consumption [D- glucose and D- fructose] during alcoholic fermentations for the two *Z. bailii* (Zb-K16Y1 and Zb-A19Y5) and one *T. californica* (Tc-A9Y1) strain. Sample coding: Tc = *Trigonopsis californica*, Zb = *Zygosaccharomyces bailii*.

3.2. Chemical Analyses

In Table 2, the standard enological parameters of the must before and after the completion of alcoholic fermentation are presented. The final concentration of total sugars was <4 g/L for each wine sample, 3.85 ± 1.16 g/L for Zb-A19Y5, and 3.65 ± 1.32 g/L for Zb-K29Y2 after 21 days, whereas for Tc-A9Y1, it was 1.00 ± 0.00 g/L after 27 days. According to the chemical analysis results, *Z. bailii* strains, namely, Zb-A19Y5 and Zb-K29Y2, led to the production of wines with a high alcohol content (13.41 ± 0.07%vol–13.11 ± 0.05%vol). In contrast, the *T. californica* (Tc-A9Y1) strain led to a reduced ethanol wine content (11.79 ± 0.04%vol). Moreover, the yield of ethanol that was produced per unit of total sugar consumed ($Y_{EtOH/TS}$, in g/g) was calculated. The ethanol yield of the two *Z. bailii* strains (0.45 and 0.44 g/g) was higher than those of *T. californica* (0.39 g/g). Using the Zb-A19Y5 strain, the ethanol yield was 88% of the maximum theoretical yield (0.51 g/g). The use of strains that lead to low final sugar values and a high alcohol yield is mostly wanted in winemaking. In Assyrtiko must, *S. cerevisiae* strains have also been tested with a higher alcohol yield (0.49–0.50 g/g) [53,54]. An innovative strategy to produce reduced-alcohol wines takes advantage of wine yeasts that do not belong to the *S. cerevisiae* species and that metabolize sugar without generating ethanol or do so with less efficiency [55].

Furthermore, the pH value ranged between 3.12 ± 0.01 and 3.19 ± 0.01, with the highest value observed in the wine made with Tc-A9Y1. The total acidity of the wines ranged between 3.92 ± 0.02 and 4.35 ± 0.08 g tartaric acid/L, and yeast-assimilable nitrogen ranged between 99.40 ± 0.74 and 105.70 ± 3.01 mg N_2/L, with the highest values observed in the wine made with Zb-A19Y5. The total SO_2 in wines made with *Z. bailii* strains showed the same values of 14.10 ± 1.80 mg/L and 16.00 ± 4.51 mg/L as those where *T. californica* was inoculated. Low values of SO_2 indicate the need for further protection of the produced wines from oxidation. Additionally, even if the values of free and total SO_2

are not statistically significant, the higher value of combined SO_2 of Tc-A9Y1 compared to *Z. bailii* strains could be correlated to a lower fermentation rate and possible commitment with the produced acetaldehyde. No statistically significant differences were found between the values of yeast-assimilable nitrogen and sulfur dioxide, whereas L-malic acid was not detected in any of the samples. *Z. bailii* is conventionally acknowledged as a malic acid-consuming yeast and has been suggested for adjusting pH in fermenting musts with an excess of malic acid [46,56]. Regarding the volatile acidity, the values of acetic acid ranged between 0.41 ± 0.02 and 0.47 ± 0.01, with the highest value observed in the wine made with Tc-A9Y1. The levels in wines made with *Z. bailii* strains were significantly different from those made with *T. californica*, but comparable to those obtained from wines inoculated with *S. cerevisiae* strains in Assyrtiko must [57].

Table 2. Mean concentration of enological parameters of Assyrtiko must and wines produced using three different non-*Saccharomyces* strains (Zb-A19Y5, Tc-A9Y1, and Zb-K29Y2). Different letters in each row indicate the significance of One-way Analysis of Variance (ANOVA), which was followed by Tukey's post hoc test ($p < 0.05$).

	Must	Zb-A19Y5	Tc-A9Y1	Zb-K29Y2
Total sugars (g/L)	230 ± 0.03	3.85 ± 1.16 ^{c}	1.00 ± 0.00 ^{a}	3.65 ± 1.32 ^{b}
Ethanol (%vol)		13.41 ± 0.07 ^{c}	11.79 ± 0.04 ^{a}	13.11 ± 0.05 ^{b}
Ethanol Yield ($Y_{EtOH/TS}$, g/g)		0.45	0.39	0.44
pH	3.20 ± 0.03	3.15 ± 0.01 ^{a}	3.19 ± 0.01 ^{b}	3.12 ± 0.01 ^{c}
Total acidity (g tartaric acid/L)	5.77 ± 0.06	4.35 ± 0.08 ^{a}	4.33 ± 0.03 ^{a}	3.92 ± 0.02 ^{b}
Volatile acidity (g acetic acid/L)		0.42 ± 0.03 ^{a}	0.47 ± 0.01 ^{b}	0.41 ± 0.02 ^{a}
YAN (mg N_2/L)	609.00 ± 4.76	105.70 ± 3.01 ^{a}	100.10 ± 4.83 ^{a}	99.40 ± 0.74 ^{a}
Total SO_2 (mg/L)	16.60 ± 0.01	14.10 ± 1.82 ^{a}	16.00 ± 1.51 ^{a}	14.10 ± 1.80 ^{a}
Free SO_2 (mg/L)	5.10 ± 0.10	6.40 ± 0.05 ^{a}	5.80 ± 0.09 ^{a}	5.80 ± 0.07 ^{a}
L-malic acid (g/L)	n.d.	n.d.	n.d.	n.d.

n.d.: not detected.

3.3. Volatile Compound Analyses

More than a thousand aromatic compounds, encompassing higher alcohols, esters, organic acids, and other constituents, have been recognized in wine. While it is likely that not all of these compounds play a role in shaping the wine's aroma, the diverse array of chemical families contributes to the remarkable aromatic complexity of wine. The wine samples were subjected to further analysis of some volatile compounds. These volatiles are potential contributors to the global aroma of wine. It has to be mentioned that Assyrtiko wines are mostly characterized by typical varietal aromas such as earthy, mushroom, and nutty odors, as well as lemon and honey [1]. Therefore, the impact of the inoculated strains on boosting the aromatic profile and typicity of the final product will be very important. Retention time and kovats index of the identified volatile aromatic compounds is reported in Table S1 (Supplementary Materials).

3.3.1. Higher Alcohols

The analysis of volatile alcohols in samples that were fermented with the strains Zb-A19Y5, Tc-A9Y1, and Zb-K29Y2 reveals intriguing insights into the distinct chemical signatures of each sample (Table 3). In general, interspecies differences among *Z. bailii* strains are noticed regarding the production of higher alcohols. In more detail, isoamyl alcohol demonstrates a significant difference, with sample Zb-K29Y2 exhibiting the highest concentration at 604.33 ± 76.80 mg/L. This discrepancy suggests that variations in isoamyl alcohol levels may contribute to the distinction in the flavor profiles of these wines. Isoamyl alcohol is generally described by banana- and fusel-type odors. A previous study demonstrates an amplification of butyric notes with the addition of isoamyl alcohol (3-methylbutan-1-ol) in a fruity model solution [58]. The literature provides evidence that higher alcohols are found in total concentrations ranging from 0.2 to 1.2 g/L in white

wines [59]. While 2-phenylethan-1-ol imparts a rose-like aroma, other higher alcohols are often associated with fusel oil, solvent, or malt scents [60]. Research indicates that higher alcohols may enhance the aromatic complexity of wine or, in some instances, mask certain flavors, contingent upon their concentrations [61]. When present below 300 mg/L, higher alcohols are generally considered contributors to a desirable complexity of wine; however, concentrations exceeding 400 mg/L are deemed to have a negative impact on wine's quality [61]. Phenylethyl alcohol, linked with floral notes, exhibits a pronounced concentration in the samples, ranging from 246.63 ± 15.09 mg/L to 319.92 ± 36.05 mg/L.

Table 3. Higher alcohols (mg/L) of wines produced in pilot scale fermentations (±SD) with different non-*Saccharomyces* strains (Zb-A19Y5, Tc-A9Y1, and Zb-K29Y2). Different letters in each row indicate the significance of One-way Analysis of Variance (ANOVA), which was followed by Tukey's post hoc test ($p < 0.05$).

	Zb-A19Y5	Tc-A9Y1	Zb-K29Y2
1-Butanol, 3-methyl-(Isoamyl alcohol)	376.23 ± 4.42 ^{a}	534.38 ± 39.20 ^{ab}	604.33 ± 76.8 ^{b}
1-Hexanol	7.15 ± 1.64 ^{a}	5.58 ± 1.03 ^{a}	10.21 ± 0.11 ^{a}
1-Hexanol, 2-ethyl-	4.72 ± 2.30 ^{a}	6.59 ± 3.06 ^{a}	n.d.
Phenylethyl Alcohol	246.63 ± 15.09 ^{a}	276.16 ± 2.00 ^{a}	319.92 ± 36.05 ^{a}
Behenic alcohol	2.7 ± 0.13	n.d.	n.d.
3,3,6-Trimethyl-1,4-heptadien-6-ol	2.68 ± 0.20	n.d.	n.d.
Tryptophol	21.21 ± 0.81 ^{b}	20.62 ± 0.80 ^{b}	8.76 ± 0.74 ^{a}
Ethanol, 2-phenoxy-	n.d.	n.d.	0.78 ± 0.09

n.d.: not detected.

Intriguingly, tryptophol, a secondary metabolite, also reveals noteworthy differences among the samples. Wines that were fermented with Zb-A19Y5 and Tc-A9Y1 strains were registered as having a concentration of 21.21 ± 0.81 mg/L and 20.62 ± 0.80 mg/L, respectively, whereas wines that were inoculated with Zb-K29Y1 differed significantly, with 8.76 ± 0.74 mg/L. This divergence underscores the role of tryptophol, which is a compound that is strongly impacted by yeast metabolism. According to previous research, aromatic alcohols such as phenylethanol contribute positively to the organoleptic characteristics of wines [62]. According to previous research, *Z. bailii* was found in Maotai-flavor liquor fermentation and enhanced the production of higher alcohols, which greatly impact the unique aroma [63].

3.3.2. Acids

Concerning the analysis of volatile acids in wine samples, several compounds reveal substantial variations in concentration among the three samples (Table 4). Wines that were inoculated with the Zb-A19Y5 strain showed the highest concentration of propanoic acid and propanoic acid, 2-methyl, which imparts an acidic, cheesy aroma, of octanoic acid, with its characteristic waxy notes, and finally oleic acid, which offers a slightly fatty aroma [64]. Meanwhile, the wine that was fermented with the Tc-A9Y2 yeast strain shows its significant differences in its hexanoic acid concentration, known for its pungent, sweaty odor, and octanoic acid, with its characteristic sweaty notes. Finally, the wine that was inoculated with the Zb-K29Y2 strain has the largest concentration of hexadecanoic acid, a fatty acid with the potential for a subtle wax or candle-like aroma, while pentadecanoic acid can contribute to a creamy and waxy aroma. These variations underscore the intricate and diverse nature of wine's flavor and aroma, reflecting the complex interplay of these compounds in defining the sensory experience. It has been noted that fatty acids can influence the complexity of wine aromas, and this influence is contingent on the concentration of acids in the wine [65], although it is not linked to the overall quality of the wine [66]. For instance, at concentrations ranging from 4 to 10 mg/L, fatty acids may contribute to enhancing the complexity of wine, but the elegance of the wine aroma could be negatively impacted when their concentration exceeds 20 mg/L [67].

Table 4. Volatile acids (mg/L) of wines produced in pilot scale fermentations (±SD) with different non-*Saccharomyces* strains (Zb-A19Y5, Tc-A9Y1, and Zb-K29Y2). Different letters in each row indicate the significance of One-way Analysis of Variance (ANOVA), which was followed by Tukey's post hoc test ($p < 0.05$).

	Zb-A19Y5	Tc-A9Y1	Zb-K29Y2
Propanoic acid	3.89 ± 0.5 ^{a}	2.77 ± 0.42 ^{a}	4.39 ± 0.13 ^{a}
Propanoic acid, 2-methyl-	4.75 ± 0.04 ^{b}	2.34 ± 0.45 ^{a}	2.9 ± 0.41 ^{a}
Butanoic acid	9.84 ± 0.28 ^{a}	9.73 ± 0.12 ^{a}	8.41 ± 0.84 ^{a}
Pentanoic acid, 3-methyl-	21.52 ± 0.23 ^{b}	9.99 ± 1.64 ^{a}	9.54 ± 1.08 ^{a}
Hexanoic acid	115.65 ± 0.56 ^{ab}	67.66 ± 7.62 ^{a}	144.17 ± 26.7 ^{b}
2-Hexenoic acid	1.95 ± 0.05	n.d.	n.d.
Octanoic acid	184.9 ± 3.11 ^{b}	115.68 ± 8 ^{a}	176.29 ± 22.74 ^{ab}
Nonanoic acid	2.89 ± 0.81 ^{a}	1.91 ± 0.11 ^{a}	1.64 ± 0.44 ^{a}
n-Decanoic acid	65.91 ± 4.44 ^{b}	36.64 ± 3.33 ^{a}	47.86 ± 7.01 ^{ab}
Undecylenic acid	7.95 ± 0.84 ^{a}	6.74 ± 2.14 ^{a}	n.d.
Dodecanoic acid	8.68 ± 1.52 ^{a}	5.45 ± 0.38 ^{a}	n.d.
Tetradecanoic acid	9.36 ± 1.84 ^{a}	6.67 ± 0.4 ^{a}	7.27 ± 1.2 ^{a}
Pentadecanoic acid	2.26 ± 0.16 ^{ab}	1.41 ± 0.01 ^{a}	3.79 ± 0.67 ^{b}
n-Hexadecanoic acid	236.21 ± 9.51 ^{b}	206.62 ± 6.86 ^{b}	129.3 ± 24.84 ^{a}
Palmitoleic acid	4.29 ± 0.99 ^{a}	1.62 ± 0.24 ^{a}	4.33 ± 2.18 ^{a}
Octadecanoic acid	135.28 ± 15.71 ^{a}	127 ± 13.5 ^{a}	74.09 ± 13.54 ^{a}
Oleic Acid	24.32 ± 0.16 ^{b}	21.38 ± 2.35 ^{ab}	9.98 ± 4.87 ^{a}
9,12-Octadecadienoic acid (Z,Z)-	2.33 ± 0.44 ^{a}	4.56 ± 0.98 ^{a}	n.d.
Benzoic acid, 4-(methylamino)-	1.12 ± 0.24	n.d.	n.d.
Eicosanoic acid	4.52 ± 0.06 ^{a}	4.59 ± 0.22 ^{a}	n.d.
9-Oxo-1,17-heptadecanedioic acid	50.79 ± 3.48	n.d.	n.d.
Docosanoic acid	n.d.	4 ± 0.14	n.d.
9-Decenoic acid	n.d.	37.19 ± 3.03 ^{a}	30.24 ± 4.89 ^{a}

n.d.: not detected.

3.3.3. Esters

All the produced wines were exposed to volatile ester analysis. The wine samples presented the most evident differences in the concentration of octanoic acid, ethyl ester, and decanoic acid, ethyl ester (Table 5). It is known that esters not only contribute to the fruity aroma of young wines but also serve as important markers for fruity notes in wines of different ages [68,69]. Esters have been observed to influence the fruity aroma of wine, even at subthreshold levels, due to their intricate synergistic effects [68,70,71], highlighting their significant contribution to the overall aroma of wine. The big difference between the number of esters in each sample and the concertation highlights the crucial role of the yeasts [72]. For instance, octanoic acid, ethyl ester, was not detected in sample Zb-A19Y5, and Ethyl 9-decenoate was not detected in Tc-A9Y1, while the other samples exhibited a high concentration. Additionally, the fact that wines from the two *Z. bailii* strains showed statistically significant differences can imply intraspecies variability.

Table 5. Esters (mg/L) of wines produced in pilot scale fermentations (±SD) with different non-*Saccharomyces* strains (Zb-A19Y5, Tc-A9Y1, and Zb-K29Y2). Different letters in each row indicate the significance of One-way Analysis of Variance (ANOVA), which was followed by Tukey's post hoc test ($p < 0.05$).

	Zb-A19Y5	Tc-A9Y1	Zb-K29Y2
Hexanoic acid, ethyl ester	16.92 ± 4.25 ^{a}	28.68 ± 0.68 ^{a}	22.99 ± 3.38 ^{a}
Octanoic acid, ethyl ester	n.d.	57.73 ± 1.97 ^{a}	81.73 ± 12.84 ^{b}
Decanoic acid, ethyl ester	10.77 ± 1.91 ^{a}	24.06 ± 1.34 ^{ab}	52.4 ± 12.39 ^{b}
Butanedioic acid, diethyl ester	0.74 ± 0.12 ^{a}	n.d.	1.88 ± 0.03 ^{b}

Table 5. *Cont.*

	Zb-A19Y5	Tc-A9Y1	Zb-K29Y2
Ethyl 9-decenoate	23.64 ± 1.71 [a]	n.d.	22.59 ± 2.59 [a]
Ethyl hydrogen succinate	15.38 ± 1.34 [a]	18.62 ± 0.34 [a]	19.46 ± 3.67 [a]
1,2-Benzenedicarboxylic acid, bis 2-methylpropyl) ester	3.59 ± 0.42 [a]	4.31 ± 1.07 [a]	2.42 ± 0.7 [a]
Hexanedioic acid, bis 2-ethylhexyl) ester	5.05 ± 2.26 [a]	9.19 ± 0.73 [a]	6.98 ± 0.32 [a]
Eicosanoic acid, isopropyl ester	2.03 ± 1.14 [a]	n.d.	n.d.
Didecyl phthalate	1.48 ± 0.35 [a]	3.16 ± 0.12 [b]	n.d.
Octadecanoic acid, 10-oxo-, methyl ester	47.85 ± 1.67 [c]	39.59 ± 3.02 [b]	1.59 ± 0.48 [a]
Ethyl trans-4-decenoate	n.d.	16.78 ± 0.21	n.d.
Acetic acid, hexyl ester	n.d.	n.d.	5.44 ± 0.26

n.d.: not detected.

3.4. Sensory Impact

The Assyrtiko wines that were produced using the different fermentation schemes described above were all evaluated by a sensory panel of experts. Figure 4 depicts the mean scores of ten sensory characteristics, with a maximum score of 10, of the wines that were inoculated with the different non-*Saccharomyces* pure cultures. The statistical analysis (ANOVA) indicated a significant difference ($p < 0.05$) for the fruitiness, citrus, and aromatic aftertaste of the produced wines.

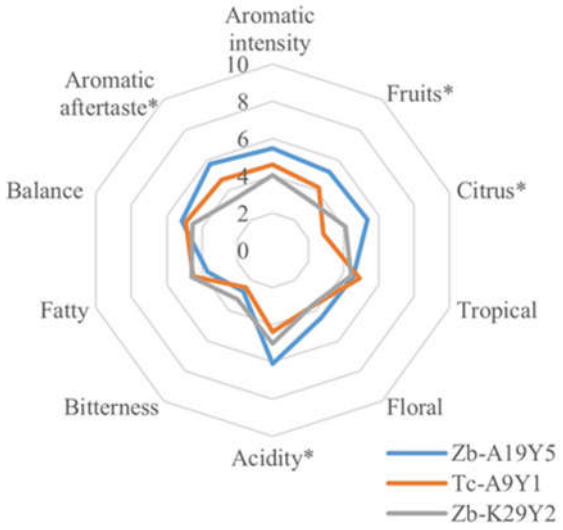

Figure 4. Spider plot of means of the sensory profile of Assyrtiko wines fermented with different non-*Saccharomyces* strains at the end of fermentation process. Sample coding: Tc = *Trigonopsis californica*, Zb = *Zygosaccharomyces bailii.* (*) indicates the existence of statistically significant differences based on one-way ANOVA, $p < 0.05$.

In more detail, the statistical analysis (Tuckey's test) revealed that wines that were inoculated with Zb-A19Y5 and Tc-A9Y1 strains differed the most in terms of fruitiness, citrus, and acidity descriptors. These results are also confirmed by the results of volatile compound analysis. Reversely, wines that were inoculated with Zb-A19Y5 and Zb-K29Y2 strains differed the most in terms of aromatic aftertaste, where the first strain was ranked significantly higher. It is noteworthy that no off-flavor or unpleasant characteristics were noticed by the panelists. Zb-A19Y5 exhibited the highest scores for several attributes, namely, aromatic intensity, fruitiness, citrus, acidity, and aromatic aftertaste. Although the Zb-K29Y2 wines exhibited very high concentrations of isoamyl alcohol (604.33 ± 76.8 mg/L) and differed statistically from the other strains, the fruitiness of these samples was remarkably low. One hypothesis that could explain this phenomenon is the very high concentrations of isoamyl alcohol. It has been underlined, previously, that the presence of isoamyl alcohol alone

led to a significant decrease in the "olfactory threshold" of the fruity reconstitution [58]. Motivated by climate change, as well as health issues, significant research efforts have been directed toward strains of yeasts that are capable of generating wines with lower ethanol levels while maintaining a harmonious sensorial profile [72–75]. According to our results, the *T. californica* strain Tc-A9Y1 seems to have promising enological potential, well adapted to the wine's needs. Moreover, the beneficial attributes of the examined yeasts may be remarkably increased or decreased if they are used as co-cultures with *S. cerevisiae* strains. Additional complementary experiments, designed to investigate the behavior of these yeasts in a must, while considering the presence of other microorganisms, and explore their interactions with *S. cerevisiae*, could be a very interesting endeavor. For instance, *Z. bailii* was successfully proposed as a co-culture with *S. cerevisiae* to improve the production of ethyl esters, which remarkably contributed to the fruitiness and floral wine aroma [76]. Furthermore, mixed starters of *Z. bailii* and *S. cerevisiae* also enhanced the wine taste and body by increasing the production of polysaccharides [37].

4. Conclusions

The current study unravels the enological potential of some rarely isolated non-*Saccharomyces* species that were previously isolated at the end of alcoholic fermentation of spontaneously fermented wines. Strains of *Zygosaccharomyces bailii*, *Priceomyces carsonii*, *Trigonopsis californica*, and *Pichia manshurica* were investigated for their fermentation capabilities and their impact on the sensory attributes of Assyrtiko must from Santorini. *P. carsonii* and *P. manshurica* revealed abnormal fermentations and exhibited an off-odor character. However, *T. californica* and the two *Z. bailii* strains led to dry wines which exhibited an interesting organoleptic profile. The ethanol yields of the two *Z. bailii* strains (0.45 and 0.44 g/g) were higher than those of *T. californica* (0.39 g/g). Our results confirm that some non-*Saccharomyces* yeasts are capable of making a positive contribution to volatile compounds in wine. The wines that were inoculated with Zb-A19Y5 were noted as the most promising regarding the overall enological and organoleptic evaluation. Additionally, the ability of a yeast strain to complete fermentation while the final wines show no off-flavor characteristics and reduce the ethanol concentration is also remarkable. Further investigation of the effect of the proposed strains in different must matrixes and with different inoculation combinations is highly recommended.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app14041522/s1, Table S1: Retention time and kovats index of the identified volatile aromatic compounds.

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