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Biodiversity and Enological Potential of Non-*Saccharomyces* Yeasts from Nemean Vineyards

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Received: 19 March 2018; Accepted: 23 April 2018; Published: 2 May 2018

Abstract: Vineyards in Nemea, the most important viticultural zone in Greece, were surveyed for indigenous non-*Saccharomyces* (NS) yeasts of enological potential. NS populations were isolated from the final stage of alcoholic fermentation and identified by a range of molecular methods. The enological profiles of *Hanseniaspora guilliermondii*, *H. osmophila*, *Lachancea thermotolerans*, *Starmerella bacillaris* and *Torulaspora delbrueckii* strains were evaluated. Significant interspecies variation was observed in fermentation kinetics. *H. osmophila* and *T. delbrueckii* showed the highest capacity for prompt initiation of fermentation, while *S. bacillaris* achieved a higher fermentation rate in the second half of the process. Significant differences were also observed in the chemical parameters of NS strains. *S. bacillaris* SbS42 and *T. delbrueckii* TdS45 were further evaluated in mixed-culture fermentations with *Saccharomyces cerevisiae*. NS strains achieved lower population densities than *S. cerevisiae*. SbS42 exhibited a higher death rate than TdS45. The chemical profiles of different ferments were separated by principal component analysis (PCA). Both NS strains were associated with lower levels of ethanol, when compared to single *S. cerevisiae* inoculation. TdS45 increased the ethyl acetate levels, while SbS42 caused a different production pattern of higher alcohols. This is the first report to explore the enological potential of NS wine yeast populations from Nemea. Based on prominent enological traits identified, the selected *S. bacillaris* and *T. delbrueckii* strains may be further exploited as co-culture starters for improving the quality and enhancing the regional character of local wines.

Keywords: non-*Saccharomyces* yeasts; mixed-culture fermentations; enological traits; yeast genotyping; wine chemical analysis

1. Introduction

The alcoholic fermentation of grape must is principally conducted by *Saccharomyces cerevisiae*, the major wine yeast. However, during spontaneous fermentation several other yeasts naturally found on grape skins, known as non-*Saccharomyces* (NS) yeasts, may contribute significantly to the quality of the wine. Species of *Hanseniaspora*/*Kloeckera* and *Candida* are the most frequently isolated yeasts in freshly crushed grape juice, while a less frequent but occasionally considerable number of isolations belong to the genera *Metschnikowia*, *Pichia*, *Kluyveromyces*/*Lachancea*, *Torulaspora*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Issatchenkia* and *Cryptococcus* [1–7].

Although NS yeasts initiate fermentation, most of them are not detectable at the end of the course, either because they are ethanol-intolerant or incapable of withstanding microbial antagonism [8,9]. However, in various later studies it has been shown that several NS species, such as *I. occidentalis*, *L. thermotolerans*, *M. pulcherrima*, *Starmerella bacillaris* (synonym *C. zemplinina*) and *Z. bailii*, were able to maintain high viable populations (up to 6–7 log CFU/mL) for longer periods than previously thought and were even isolated from the final stages of the alcoholic fermentation [4,7,10,11]. These species may play a crucial role in winemaking, due to the production of important metabolites that shape the flavor and contribute to the style of wines [1,12]. These metabolites include both "volatile" compounds, like aldehydes and esters, and "non-volatile" compounds, like glycerol, acetic acid and lactic acid.

In this context, there is a growing interest in the use of well-selected NS yeasts such as *Torulaspora*, *Candida*, *Metschnikowia* and *Lachancea/Kluyveromyces* species, which when combined with *S. cerevisiae* in mixed-culture starters can improve wine fermentation and final product quality [13]. Several NS strains have been produced commercially as active dry yeasts and novel starter cultures are continuously developing to address specific challenges in modern winemaking [14]. To this end, there is a great need for a thorough description and evaluation of the natural yeast biota associated with different cultivars/viticultural zones in order to identify elite genotypes of enological interest [15]. Well-selected indigenous strains are invaluable in modern winemaking for designing next-generation starter cultures to mimic spontaneous fermentation and thus enhance the typicity and complexity of wines. Through this alternative approach, the potential risks of uncontrolled (uninoculated) fermentations associated with product quality and human health are avoided [16].

In the present study, the culturable yeast communities present at the end of alcoholic fermentation of five spontaneous "Agiorgitiko" ferments originating from different vineyards of Nemea, the major viticultural zone in Greece, were assessed. Strains of *S. bacillaris* and *T. delbrueckii* were further evaluated in mixed-culture fermentations along with *S. cerevisiae*. The respective wine phenotypes were characterised through chemical analysis and compared.

2. Materials and Methods

2.1. Isolation of Non-Saccharomyces (NS) Yeasts

Grape samples belonging to the "Agiorgitiko" grape cultivar (*Vitis vinifera* L.) were collected from five vineyards within the Nemea protected designation of origin (PDO) region. The grapes were crushed with a stomacher and allowed to ferment spontaneously in sterile bottles. Fermentation progress was followed daily by weight determinations. For the isolation of non-*Saccharomyces* yeasts, samples from grape juices were taken at the end of the alcoholic fermentations and plated on lysine medium agar. Ten to 20 colonies were randomly selected from plates and examined microscopically. Isolates were purified by the streak plate technique and stored at −80 °C until further analysis.

2.2. Molecular Identification of Yeasts

The 5.8S-ITS rDNA region of yeasts was amplified by polymerase chain reaction (PCR) as previously described [4]. About 500 ng of the PCR products were subjected to digestion with the restriction endonucleases *Hinf*I, *Hae*III and *Hha*I [17]. *Dde*I and *Dra*I were additionally applied for differentiating species within the genera *Hanseniaspora* and *Candida* [4]. Restriction fragments were loaded on a 3% (*w*/*v*) agarose gel, detected by ethidium bromide staining, and photographed under ultraviolet (UV) light. Sizes of fragments were estimated using a 100-bp molecular size marker. PCR products of the 5.8S-ITS region from representative restriction patterns were purified using the QIAquick PCR purification kit and directly sequenced (Macrogen; http://www.macrogen.com). Nucleotide basic local alignment search tool (BLAST) searches were performed at the National Center for Biotechnology Information (NCBI)/GenBank database.

For molecular typing of non-*Saccharomyces* species the tandem repeat-tRNA (TRtRNA) PCR method [18] using the TtRNASc primer (5′-GCTTCTATGGCCAAGTTG-3′) with 5GAC

(5′-CAGCAGCAGCAGCAG-3′) or ISSR-MB (5′-CTCACAACAACAACAACA-3′); and random amplified polymorphic DNA (RAPD) analysis with the primers RF2 (5′-CGGCCCCTGT-3′) and R5 (5′-AACGCGCAAC-3′) were applied according to Paffetti et al. [19] and Martin et al. [20], respectively.

2.3. Pure-Culture Fermentations

Pure-culture fermentations were conducted in duplicate at 26 °C in 150 mL flasks containing 100 mL of pasteurized (72 °C for 10 min) grape must (pH 3.78; sugars 292 g/L; titratable acidity 3.9 g/L, as tartaric acid; yeast assimilable nitrogen 184 mg/L). Thirty ppm of SO_2 were added to the grape must as potassium metabisulfite. The flasks were closed with silicone stopper supplied with a fermentation lock containing glycerol 50% *v*/*v* to allow only CO_2 to escape. Yeast strains were added at 6 Log CFU/mL. The course of the fermentation was monitored daily by weight loss due to CO_2 exhaust.

2.4. Mixed-Culture Fermentations

Fermentations were performed in triplicate at 25 °C in 1 L flasks with 750 mL of pasteurized (72 °C, 10 min) grape must (sugars 230 g/L; pH 3.44; titratable acidity 5.7 g/L, as tartaric acid; yeast assimilable nitrogen 240 mg/L). Thirty ppm of SO_2 were added to the grape must as potassium metabisulfite. Yeasts were pre-cultured in the same grape must for 12 h at 26 °C with agitation (225 rpm). Yeast inocula were added to the grape must at 6 Log CFU/mL under the following inoculation schemes: simultaneous inoculation of *S. bacillaris* SbS42 (SbCo) or *T. delbrueckii* TdS45 (TdCo) and *S. cerevisiae* ScNM18 and single inoculation of *S. cerevisiae* ScNM18 (SSc). The course of the fermentation was monitored daily by weight loss. For the enumeration of total yeasts, *S. cerevisiae* and non-*Saccharomyces* species, grape must serial dilutions were plated on Wallerstein laboratory nutrient agar (WL), ethanol sulfite agar (ESA) or yeast-peptone-dextrose (YPD) agar, and lysine medium agar (LA), respectively. Plates were incubated at 28 °C for 2–5 days, except for YPD which was incubated at 37 °C.

2.5. Analytical Determinations

Reducing sugars, total and volatile acidity, pH, and total and free SO_2 were determined according to the methods in the Compendium of International Methods of Analysis of Musts and Wines [21]. The yeast-assimilable nitrogen (YAN) was estimated as described previously [22]. Organic acids (citric, tartaric, malic, succinic, lactic, acetic), sugars (glucose, fructose), glycerol and ethanol were determined by high-performance liquid chromatography (HPLC) with refractive index detection according to OIV-MA-AS313-04, slightly modified [18]. Separations were performed on a polystyrene/divinylbenzene Agilent Hi-Plex H column (300 mm × 7.7 mm, particle size 8 μm) operating at 75 °C. The mobile phase was 4 mM H_2SO_4 with a flow rate of 0.4 mL/min. The major volatile compounds (acetaldehyde, ethyl acetate, methanol, 1-propanol, 2-methyl-1-propanol [isobutanol], 3- and 2-methyl-1-butanol) of wine fermentations were determined by gas chromatography (GC) according to EC No 2870/2000 [23].

2.6. Statistical Analysis

Comparison between means was conducted by analysis of variance (ANOVA, $p < 0.05$). Principal component analysis (PCA) was applied to enological characteristics in order to explore relationships between samples and variables using the JMP®, Version 8. SAS Institute Inc., Cary, NC, USA, 1989–2007.

3. Results and Discussion

3.1. Heterogeneity of Non-Saccharomyces Yeasts in Fermented Musts

"Agiorgitiko" is the major Greek red grapevine variety. It is cultivated throughout the country, but its origin and principal area of cultivation is Nemea, in the Peloponnese. Despite the great importance of

the Nemean viticultural zone, the indigenous yeast biota is still unexplored. Here, five "Agiorgitiko" grape must samples (NAG1, NAG2, NAG3, NAG4, and NAG5) were collected from five respective vineyards of the Nemea region and allowed to ferment spontaneously. Non-*Saccharomyces* (NS) yeasts were isolated from the final fermentation stage and species heterogeneity was assessed through restriction enzyme and sequence analyses of the 5.8S-ITS rDNA region. In total, seven yeast populations were identified, i.e., *Hanseniaspora guilliermondii*, *H. osmophila*, *Lachancea thermotolerans*, *Starmerella bacillaris* (synonym *Candida zemplinina*), *Torulaspora delbrueckii*, *Saccharomycodes ludwigii* and *Zygosaccharomyces bailii*. NS species richness ranged among different ferments between the presence of just a single population in sample NAG5 to 4 different species in samples NAG2 and NAG3 (Table 1). *T. delbrueckii*, *L. thermotolerans* and *H. guilliermondii* were the most frequently encountered species followed by *S. bacillaris* and *H. osmophila*. NS species richness declines during fermentation and at the end of the course only a few populations can be recovered [4,24]. Spoilage yeasts, such as *S. ludwigii* and *Z. bailii*, also encountered in the present study, are often isolated from the late fermentation phase [25,26]. Due to their high ethanol and SO_2 tolerance, these species can cause wine spoilage as they can persist during wine storage [27]. However, yeasts of enological value can be also isolated, which may play a significant role in improving wine quality, since they resist the elevated ethanol concentrations accumulating during fermentation [25]. Strains of *L. thermotolerans*, *S. bacillaris* and *T. delbrueckii*, which fall into this category, were isolated and further considered for use in winemaking, since they may exhibit a higher ethanol tolerance than other NS yeasts and withstand microbial antagonism during alcoholic fermentation [4,28].

Table 1. Population frequencies (%) of non-*Saccharomyces* yeast species at the end of fermentation in different ferments.

Yeast Species	Ferment				
	NAG1	**NAG2**	**NAG3**	**NAG4**	**NAG5**
Hanseniaspora guilliermondii	64	25	25		
Hanseniaspora osmophila		37		6	
Lachancea thermotolerans	9	13	25		
Torulaspora delbrueckii		25	25	19	
Saccharomycodes ludwigii	27				
Starmerella bacillaris			25	75	
Zygosaccharomyces bailii					100

TRtRNA-PCR and RAPD analyses were applied for intraspecific genotyping of NS yeast species. Two different strains were identified for each of *L. thermotolerans* and *T. delbrueckii*, whereas all the other species were comprised of a single genotype. In the case of *T. delbrueckii*, genotype discrimination was accomplished only when the TRtRNA-PCR method employing the ISSR primer was applied, suggesting that the genotypes are rather closely related to each other. The *L. thermotolerans* strains were detected in different ferments, while the *T. delbrueckii* strains (TdI and TdII) coexisted in NAG2 ferment. TdI accounted for 75% and TdII for the rest 25% of the total species population.

3.2. Technological Characterization of NS Yeast Strains

Four NS species (*H. guilliermondii*, *H. osmophila*, *T. delbrueckii* and *S. bacillaris*) isolated from the final stage of spontaneously fermented grape musts were subjected to technological characterization to identify strains of enological value. Significant interspecies variation was observed in the fermentation kinetics, while strains within each species showed similar fermentation profiles (Figure 1 and Table 2). *H. guilliermondii* exhibited the lowest fermentation power (max CO_2 = 5.50 ± 0.03 g of CO_2, $p < 0.005$). By contrast, *H. osmophila*, *S. bacillaris* and *T. delbrueckii* strains released much higher amounts of CO_2 (7.81 ± 0.10 g, 7.96 ± 0.05 g and 7.78 ± 0.11 g, respectively) compared to *H. guilliermondii*, suggesting better adaptation to the alcoholic fermentation environment. *H. osmophila* and *T. delbrueckii* appeared

to have the highest capacity for prompt and rapid initiation of fermentation, a characteristic that may facilitate their prevalence against other NS yeasts at the onset of fermentation. *H. osmophila* further showed the highest fermentation rate during the entire course, therefore producing the shortest fermentation duration by 4 days. As opposed to *T. delbrueckii*, *S. bacillaris* showed lower fermentation vigor early during fermentation, but was faster at the final stages, an interesting feature to accelerate the rate in sluggish fermentations.

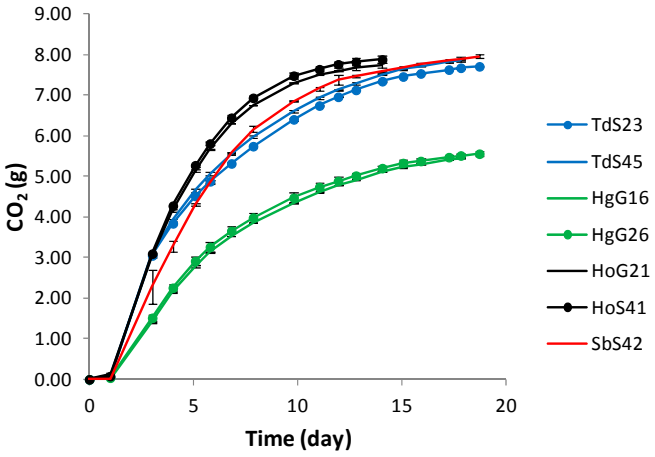

Figure 1. Fermentation kinetics (CO_2 production) of non-*Saccharomyces* yeast isolates in sterile grape must. Hg: *Hanseniaspora guilliermondii*; Ho: *Hanseniaspora osmophila*; Sb: *Starmerella bacillaris*; Td: *Torulaspora delbrueckii*.

Fermentations conducted in the presence of *T. delbrueckii* or *S. bacillaris* showed higher titratable acidity values than those observed in *H. osmophila* or *H. guilliermondii* inoculated fermentations ($p < 0.0075$; Table 2). The pH values were also affected accordingly, with *T. delbrueckii* causing the highest drop. Significant intraspecies variation was observed in *T. delbrueckii* regarding volatile acidity (VA) production. The strain TdS45 produced relatively low levels of VA, while TdS23 produced twice as much ($p < 0.0001$). This difference was also observed in the concentration of acetic acid. Although *T. delbrueckii* is generally considered to be a low VA producer [29], recent studies report significant intraspecies variation in this important enological characteristic [30], suggesting that a thorough examination of *T. delbrueckii* diversity is required to select strains that can assure the production of wines with low VA. The highest VA values were recorded for *H. guilliermondii* (0.73 ± 0.00 mg/L to 0.76 ± 0.01 mg/L) and *S. bacillaris* (0.76 ± 0.01 mg/L). The same species also produced the highest amounts of acetic acid ($p < 0.0001$). Both yeasts are known to produce rather high levels of acetic acid [31,32]. At the same time, increased concentration of glycerol was associated with *S. bacillaris* or *H. guilliermondii* activity, as opposed to *H. osmophila* and *T. delbrueckii* ($p < 0.0001$). Since glycerol improves the oraganoleptic profile of wines, the use of high glycerol-producing yeasts, such as *S. bacillaris*, appear appealing in modern winemaking [13].

H. osmophila and *T. delbrueckii* showed higher preference for glucose than fructose, as also previously recorded [33], with glucose-to-fructose ratios (G/F) of 0.5 and 0.6, respectively. On the other hand, *H. guilliermondii* exhibited a rather fructophilic character (G/F = 1.6). *S. bacillaris* was strongly fructophilic with SbS42 being able to ferment all available fructose but only 20% of glucose. The high affinity of *S. bacillaris* for fructose has been well documented previously [34], and strains of *H. guilliermondii* have also been reported to be fructophilic [35]. The fructophilic nature of certain NS species is of great enological importance as they can act in a complementary way to *S. cerevisiae*, which consumes glucose at faster rate than glucose, in grape must sugar fermentation. Therefore, they can be exploited in the fermentation of high sugar grape musts or in the restart of stuck or sluggish fermentations in which the low G/F ratios disturb sugar depletion.

Table 2. Enological properties of non-*Saccharomyces* yeast strains. Values [1] within the same row followed by a common letter are not significantly different ($p < 0.05$). Hg: *Hanseniaspora guilliermondii*; Ho: *Hanseniaspora osmophila*; Sb: *Starmerella bacillaris*; Td: *Torulaspora delbrueckii*.

Enological Parameter	Yeast Strain						
	TdS45	**TdS23**	**HoS41**	**HoG21**	**HgG26**	**HgG16**	**SbS42**
Fermentation rate (gCO_2/day, time period 3 days)	1.05 ± 0.01^{a}	1.03 ± 0.02^{a}	1.05 ± 0.00^{a}	1.05 ± 0.01^{a}	0.51 ± 0.03^{c}	0.47 ± 0.01^{c}	0.75 ± 0.13^{b}
Fermentation vigour (gCO_2 /100 mL at d 7)	22.34 ± 0.18^{a}	21.63 ± 0.11^{a}	24.93 ± 0.16^{a}	24.39 ± 0.11^{a}	13.62 ± 0.49^{d}	12.98 ± 0.12^{d}	20.25 ± 0.55^{a}
CO_2 production (g/flask)	7.86 ± 0.03^{a}	7.67 ± 0.01^{a}	7.88 ± 0.09^{a}	7.74 ± 0.06^{a}	5.44 ± 0.01^{b}	5.56 ± 0.05^{b}	7.95 ± 0.04^{a}
Total acidity (as tartaric acid g/L)	5.7 ± 0.1^{a}	5.5 ± 0.0^{b}	5.1 ± 0.0^{c}	4.9 ± 0.1^{d}	4.9 ± 0.0^{d}	4.9 ± 0.0^{d}	5.5 ± 0.0^{b}
pH	3.60 ± 0.01^{f}	3.65 ± 0.00^{e}	3.75 ± 0.00^{c}	3.80 ± 0.01^{a}	$3.79 \pm 0.1^{a,b}$	3.78 ± 0.00^{b}	3.70 ± 0.00^{d}
Volatile acidity (as acetic acid g/L)	0.33 ± 0.01^{d}	0.66 ± 0.01^{c}	0.72 ± 0.01^{b}	0.67 ± 0.00^{c}	0.76 ± 0.0^{a}	0.73 ± 0.01^{b}	0.76 ± 0.02^{a}
Malic acid (g/L)	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Lactic acid (g/L)	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Succinic acid (g/L)	1.4 ± 0.0^{a}	1.1 ± 0.0^{b}	0.7 ± 0.0^{c}	0.7 ± 0.0^{c}	0.7 ± 0.0^{c}	0.8 ± 0.0^{c}	0.5 ± 0.0^{d}
Glucose (g/L)	46.4 ± 0.8^{e}	48.2 ± 0.2^{d}	41.8 ± 1.3^{g}	43.2 ± 0.3^{f}	103.4 ± 0.8^{c}	105.4 ± 0.0^{b}	122.6 ± 0.4^{a}
Fructose (g/L)	77.5 ± 0.6^{d}	80.4 ± 0.0^{c}	82.0 ± 1.0^{b}	87.3 ± 0.4^{a}	64.2 ± 0.7^{f}	66.1 ± 0.1^{e}	0.9 ± 0.0^{g}
Glycerol (g/L)	6.0 ± 0.1^{d}	6.8 ± 0.0^{c}	$5.7 \pm 0.4^{d,e}$	5.5 ± 0.0^{e}	8.6 ± 0.1^{b}	8.7 ± 0.0^{b}	14.1 ± 0.1^{a}
Acetic acid (mg/L)	290 ± 5^{f}	628 ± 12^{e}	$664 \pm 6^{c,d}$	661 ± 9^{d}	682 ± 1^{c}	712 ± 9^{b}	853 ± 11^{a}
Ethanol (g/L)	78.8 ± 0.5^{c}	78.5 ± 0.3^{c}	80.8 ± 0.8^{a}	$80.5 \pm 0.4^{a,b}$	55.3 ± 0.7^{d}	53.9 ± 0.6^{e}	$79.3 \pm 0.4^{b,c}$

[1] Mean value ± standard deviation (SD) of duplicate fermentations.

T. delbrueckii strains produced the highest levels of succinic acid among the NS yeasts analyzed ($p < 0.0001$). On the contrary, succinate was undetectable in the microfermentation conducted by *S. bacillaris*, while *H. guilliermondii* and *H. osmophila* produced intermediate amounts. *H. guilliermondii* yielded the lowest levels of ethanol ($p < 0.0001$). The other species produced significantly higher amounts ranging from 78.7 ± 0.2 g/L for *T. delbrueckii* to 80.7 ± 0.2 g/L for *H. osmophila*, which was the highest yield recorded.

Principal component analysis (PCA) was applied to resolve the associations among enological profiles of NS yeasts (Figure 2). The added total variation of PC1 and PC2 was at 82.30% (49.69% and 32.61%, respectively). The different NS species were all located in different quadrants of the PCA plot. By contrast, although positioned on the same quadrant, strains of *T. delbrueckii* formed two distinct groups along the negative scale of PC1. This dissociation could be assigned to the high values of succinic acid that loaded negatively and the low levels of acetic acid and volatile acidity produced by TdS45 that loaded positively on PC1. *S. bacillaris* was by far the most distantly located yeast species, owing to the high values of the G/F ratio and glycerol, both loaded positively on PC1 and PC2.

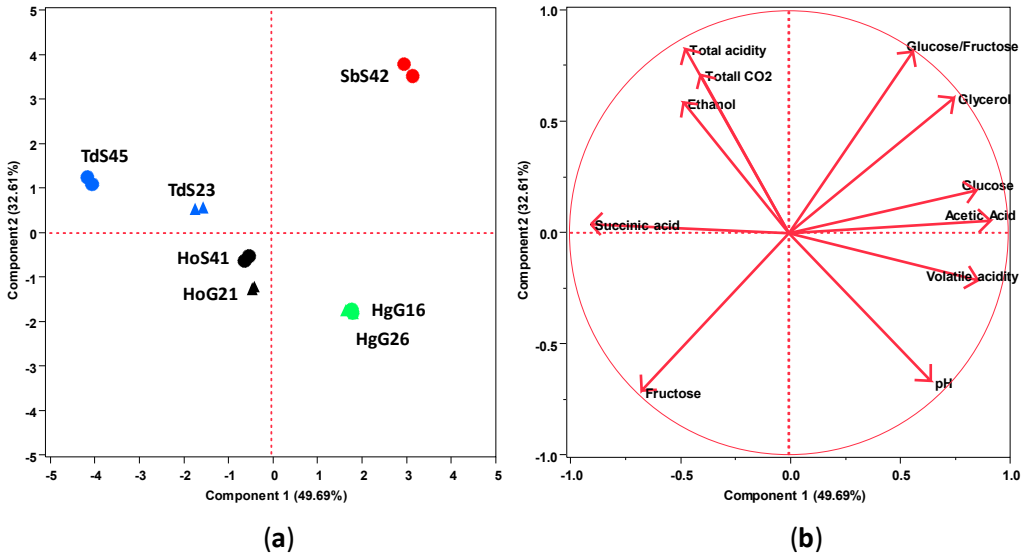

Figure 2. Principal component analysis (PCA) performed on the enological characteristics of non-*Saccharomyces* yeasts. PC1 and PC2 account for 49.69% and 32.61% of the total variation, respectively. (**a**) PCA score plot of chemical profiles; (**b**) PCA loading plot of chemical parameters. Hg: *Hanseniaspora guilliermondii*; Ho: *Hanseniaspora osmophila*; Sb: *Starmerella bacillaris*; Td: *Torulaspora delbrueckii*.

Overall, the NS yeasts analyzed here showed interesting features that could offer technological advances and/or improve wine quality. For instance, *H. osmophila* showed the most vigorous fermentation kinetics, a positive technological feature for a starter culture as it can facilitate its prevalence over other detrimental species/strains. However, high production levels of acetic acid and ethyl acetate restricts its use in wine production [33]. Despite that, a strain of *H. osmophila* has been proposed as a good candidate for development of mixed starters, mainly because of the high production of 2-phenylethyl acetate [31]. Therefore, it seems that this species, although previously considered a spoilage yeast, deserves further investigation. Among *Hanseniaspora* species, *H. guilliermondii* has been also considered for use in winemaking since it is a strong producer of 2-phenylethyl acetate and isoamyl acetate, two important flavour compounds that contribute to the make up of a wine's aroma [31]. The fructophilic character and the elevated production of glycerol by strains HgG16 and HgG26 observed in this study are also important technological characteristics; however, further consideration is required because of the high levels of acetic acid that *H. guilliermondii* strains produce. *T. delbrueckii* was one of the first NS yeast species to be commercially available in 2003 [13] and, since then, several strains have

been released to address specific technological needs [14]. When compared to other NS strains, TdS45 exhibited several positive enological characteristics, such as low production of acetic acid and volatile acidity, higher fermentation rate, and increased total acidity. Therefore, TdS45 was further evaluated in mixed fermentations with *S. cerevisiae*. *S. bacillaris* has been recently proposed as a good candidate starter culture for use in winemaking, mainly because of its strong fructophilic character that could facilitate the fermentation of high sugar musts, as is the case in the Nemea region [34]. Strain SbS42 produced high levels of glycerol and showed high fructophilic behaviour, and it was used in mixed fermentations with *S. cerevisiae*.

3.3. Fermentation Kinetics and Yeast Population Dynamics in Mixed-Culture Fermentations

S. bacillaris strain SbS42 and *T. delbrueckii* strain TdS45 were further evaluated in mixed fermentations with *S. cerevisiae* strain ScNM18 (CoTd and CoSb, respectively). Fermentation with a single inoculation of *S. cerevisiae* ScNM18 (SSc) was also performed as a reference. The CO_2 evolution of different fermentation trials is shown in Figure 3a. Significantly lower levels of CO_2 (p = 0.003) were released by the mixed culture of *S. cerevisiae* with *T. delbrueckii* (101.34 ± 0.34 g) than with *S. bacillaris* (102.59 ± 0.29 g) or *S. cerevisiae* alone (102.25 ± 0.11 g). The duration of the fermentation ranged between 5.2 d for SSc to 6.0 d for CoTd or CoSb. The yeast population dynamics are shown in Figure 3b–d. Strain ScNM18 showed similar kinetics in both single- and mixed-inoculated fermentations. Present data coincide well with previous results where the co-inoculation addition of *S. bacillaris* did not influence the growth kinetics of *S. cerevisiae* in mixed-culture fermentations [36].

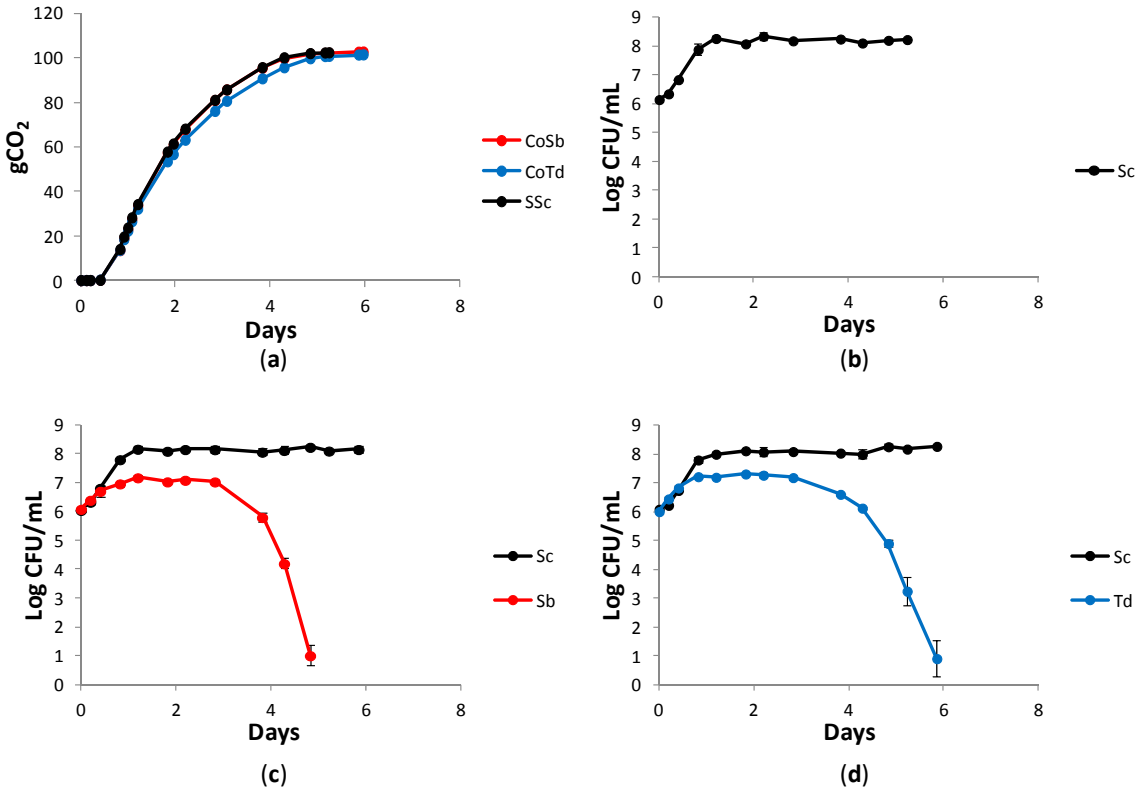

Figure 3. CO_2 production (**a**) and population dynamics (**b**–**d**) in sterile grape must inoculated with *S. cerevisiae* ScNM18 (black line), *S. bacillaris* SbS42 (red line) or *T. delbrueckii* TdS45 (blue line).

In all cases, *S. cerevisiae* reached stationary phase within 30 h of inoculation. At this stage, average counts were at 8.21 ± 0.08 Log CFU/mL, 8.15 ± 0.05 Log CFU/mL and 8.15 ± 0.10 Log CFU/mL for SSc, CoSb and CoTd, respectively, and were retained at these levels until the end of the fermentation course. Both non-*Saccharomyces* yeasts reached stationary phase within 20 h of inoculation. However,

they achieved lower population densities by approximately 1 log CFU/mL as compared to *S. cerevisiae*. In more detail, SbS42 and TdS45 peaked at 7.18 ± 0.10 Log CFU/mL and 7.31 ± 0.00 Log CFU/mL, being 1.06 Log CFU/mL and 0.96 Log CFU/mL lower, respectively, than the maximum populations of *S. cerevisiae*. The higher capacity of *S. cerevisiae* to use and convert available nitrogen into biomass probably confers competitive advantage for its numerical supremacy against NS yeasts [37]. Although NS populations both started to decline at 2.8 d, SbS42 exhibited a higher death rate than TdS45.

3.4. Chemical Profiles of Wines Produced in Mixed-Culture Fermentations

The chemical characteristics and the major volatiles of ferments produced in mixed-culture fermentations are shown in Table 3. PCA was performed to explore the relationships among the chemical profiles of the different ferments. The cumulative variation as composed by the first two components was 61.40% (35.33% and 26.07% for PC1 and PC2, respectively). It was shown that the chemical profiles of the different ferments formed three well-separated, tight clusters on the PCA plot (Figure 4a). SSc and CoSb ferments were placed on opposite quadrants along PC2, mainly due to the high values of malic acid, total acidity, and 2-methyl-1-butanol recorded for SSc that loaded positively on PC1 (Figure 4b), while propanol, isobutanol and acetaldehyde with high values for the CoSb were negatively loaded on PC1. CoTd was separated from both SSc and CoSb along the PC1 axis. The chemical profile of CoTd seemed to equilibrate between positive and negative characteristics loaded on PC1. This dissociation could be attributed to the high glycerol, acetic acid and ethanol values for both SSc and CoSb that were positively loaded on PC2, in contrast to CoTd that was valued negatively on PC2, mainly due to the presence of ethyl acetate.

Table 3. Chemical characteristics and major volatiles of wines produced in mixed-culture fermentations. Data are means ± SD of three replicates. Values with different superscript letters within each row are significantly different, according to Student's *t* test ($p < 0.05$). SSc: *S. cerevisiae* NM18; CoSb: *S. bacillaris* SbS42 and *S. cerevisiae* ScNM18 added simultaneously; CoTd: *T. delbrueckii* TdS45 and *S. cerevisiae* ScNM18 added simultaneously.

Compound	Inoculation Protocol		
	SSc	CoSb	CoTd
Total acidity (as tartaric acid g/L)	8.1 ± 0.1 ^{a}	8.0 ± 0.1 ^{a}	8.1 ± 0.0 ^{b}
pH	3.51 ± 0.01 ^{a,b}	3.53 ± 0.01 ^{a}	3.50 ± 0.02 ^{b}
Volatile acidity (as acetic acid g/L)	0.22 ± 0.02 ^{a}	0.25 ± 0.01 ^{a}	0.23 ± 0.01 ^{a}
Free SO_2 (mg/L)	6 ± 2 ^{a}	6 ± 1 ^{a}	6 ± 1 ^{a}
Total SO_2 (mg/L)	16 ± 1 ^{a}	16 ± 2 ^{a}	15 ± 2 ^{a}
Citric acid (mg/L)	897 ± 16 ^{a}	855 ± 1 ^{b}	843 ± 18 ^{b}
Tartaric acid (g/L)	1.9 ± 0.1 ^{a}	1.7 ± 0.1 ^{b}	1.8 ± 0.0 ^{a,b}
Malic acid (g/L)	2.3 ± 0.1 ^{a}	2.2 ± 0.0 ^{b}	2.2 ± 0.1 ^{b}
Succinic acid (g/L)	1.2 ± 0.0 ^{a}	1.2 ± 0.0 ^{a}	1.2 ± 0.1 ^{a}
Acetic acid (mg/L)	205 ± 5 ^{b}	221 ± 2 ^{a}	178 ± 5 ^{c}
Glucose (g/L)	1.0 ± 0.1 ^{a}	0.9 ± 0.0 ^{a}	0.9 ± 0.0 ^{a}
Glycerol (g/L)	10.4 ± 0.2 ^{a}	10.4 ± 0.1 ^{a}	9.9 ± 0.3 ^{b}
Ethanol (g/L)	113.6 ± 1.6 ^{a}	109.2 ± 0.6 ^{b}	107.0 ± 2.2 ^{b}
Acetaldehyde (mg/L)	5.7 ± 0.9 ^{a}	7.8 ± 1.3 ^{a}	5.9 ± 1.3 ^{a}
Ethyl acetate (mg/L)	31.8 ± 0.4 ^{b}	33.5 ± 0.5 ^{ab}	34.4 ± 1.6 ^{a}
Methanol (mg/L)	38.4 ± 1.3 ^{a}	38.8 ± 0.4 ^{a}	37.3 ± 2.3 ^{a}
Propanol (mg/L)	26.6 ± 0.8 ^{b}	31.5 ± 1.8 ^{a}	28.2 ± 2.1 ^{a,b}
Isobutanol (mg/L)	68.3 ± 0.9 ^{b}	76 ± 0.9 ^{a}	70.8 ± 2.2 ^{b}
2-Methyl-1-butanol (mg/L)	74.9 ± 1.0 ^{a}	70.7 ± 0.2 ^{b}	74.5 ± 2.9 ^{a}
3-Methyl-1-butanol (mg/L)	340.4 ± 5.7 ^{a}	335.1 ± 2.5 ^{a}	341.7 ± 12.6 ^{a}

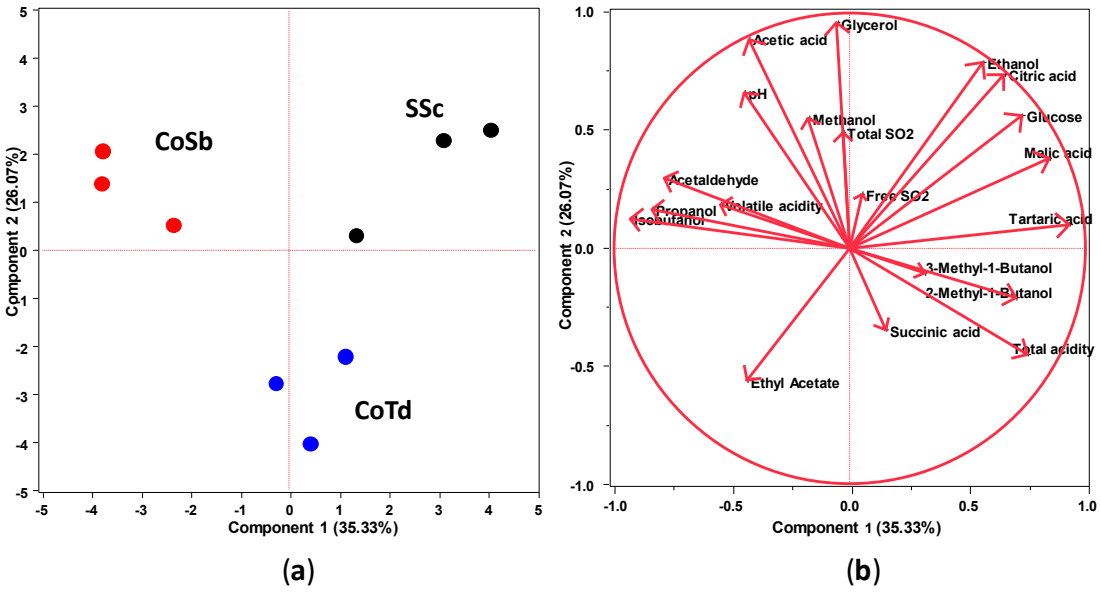

Figure 4. PCA performed on the chemical parameters of ferments produced by single inoculation with *S. cerevisiae* ScNM18 (SSc) or mixed inoculation of ScNM18 with *S. bacillaris* NM11 (CoSb) or *T. delbrueckii* TdS45 (CoTd). PC1 and PC2 account for 35.33% and 26.07% of the total variation, respectively. (**a**) PCA score plot of chemical profiles; (**b**) PCA loading plot of the chemical parameters.

ANOVA was applied to the chemical parameters of Table 3 to reveal differences between fermentation trials. When compared to the SSc inoculation trial, the use of SbS42 significantly increased the levels of acetic acid ($p = 0.0035$), contrary to TdS45 that caused the reverse effect ($p = 0.0003$), as also observed in pure-culture fermentations. Significant differences were observed in the final ethanol concentration, which was lower in the case of mixed fermentations (p values at 0.0157 and 0.0024 for CoSb and CoTd fermentations, respectively) as compared to the SSc inoculation trial. The highest decrease of ethanol by 6.6 g/L was achieved in the CoTd fermentation trial. The TdS45 strain also caused a rather small, albeit statistically significant, increase ($p = 0.0177$) in the ethyl acetate levels compared to the SSc ferment. Finally, the use of *S. bacillaris* caused a different production pattern of higher alcohols as compared to both SSc and CoTd, by increasing the levels of propanol ($p < 0.05$) and isopropanol ($p < 0.005$) and lowering the content of 2-methyl-1-butanol ($p < 0.005$).

The reduction in the ethanol content by NS yeasts can extenuate the rise in grape sugar levels caused by climate change in accordance with the new market trends and needs. The increased production of secondary metabolites by NS yeasts may, at least partially, lower the ethanol yield [38]. *T. delbrueckii* has been shown to cause decreases in ethanol production in the range of 0.15% to 0.5% (v/v) [39–41]. However, no significant ethanol reductions were observed in other studies [42–44]. The above results highlight the existence of high intraspecies variation in *T. delbrueckii* regarding the ethanol yield. *S. bacillaris* has been also shown to significantly reduce ethanol levels in mixed fermentations with *S. cerevisiae*, especially when added at higher counts [32,36].

Present results show that when strains SbS42 and TdS45 are used in mixed fermentations with *S. cerevisiae*, this may provide solutions to emerging problems, such as the increasing ethanol content of wines. Both strains significantly lowered the ethanol concentration in the final wine by 0.6% and 0.8% vol in CoSb and CoTd fermentation trials, respectively. *T. delbrueckii* TdS45 can also be used to improve the sensorial characteristics of wines by lowering the acetic acid levels.

4. Conclusions

The analysis of the NS yeast populations isolated at the last stage of the "Agiorgitiko" grape must alcoholic fermentations revealed seven species of various enological attributes. The fermentations

conducted on a mixed culture of *S. cerevisiae* and *S. bacillaris* SbS42 or *T. delbrueckii* TdS45 resulted in wine products with significantly different chemical profiles to each other and also to ferments driven solely by *S. cerevisiae*. *S. bacillaris* increased the levels of propanol and isopropanol, while lowering the content of 2-methyl-1-butanol. Significantly lower ethanol levels were also produced in mixed-culture fermentations as compared to *S. cerevisiae* alone, corroborating recent studies pointing to the potential use of NS for reducing wine alcohol content [45]. Present results highlight the suitability and value of well-selected *S. bacillaris* and *T. delbrueckii* strains in mixed-culture fermentations to improve the unique character of local wines and bring about diverse products in the wine market.

Author Contributions: G.S. and I.C. performed the experiments and analyzed the data; A.M. supervised and evaluated the HPLC and GC analyses; Y.P. contributed the grape samples; G.B. contributed to data analysis and manuscript preparation; A.N. conceived and designed the experiments, analyzed the data and wrote the paper.

Acknowledgments: This work was partially supported by the European Regional Development Fund (ERDF) and National Resources, under the Operational Program Competitiveness and Entrepreneurship (EPAN II), Action "COOPERATION 2011" (Grant Number 11SYN_2_704).

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; and in the decision to publish the results.

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