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Microbiota Diversity During Grape Drying and Spontaneous Fermentations of Vin Santo

Damiano Barbato [1], Viola Galli [1,*], Silvia Mangani [1], Eleonora Mari [2], Giacomo Buscioni [1], Lisa Granchi [2] and Simona Guerrini [2]

[1] FoodMicroTeam s.r.l., Academic Spin-Off of the University of Florence, Via Santo Spirito, 14-50125 Florence, Italy
[2] Department of Agriculture, Food, Environment and Forestry (DAGRI), Via San Bonaventura, 13-50145 Florence, Italy
* Correspondence: viola@foodmicroteam.it

Abstract: Vin Santo is a *passito* wine produced mainly in Tuscany. In the traditional production of Vin Santo, fermentation occurs naturally. Only a few reports have explored the microbial ecology of Vin Santo. Therefore, the present study aimed to investigate the microbial ecology and its impact on the fermentative kinetics in traditional processes of Vin Santo carried out in two different Tuscan wineries. Despite the different systems used for drying the grapes, both wineries showed similar microbial ecology. Non-*Saccharomyces* yeasts were the dominant microbial population during grape drying in different succession, even though in the end, the dominant species (at different percentages) in both were *Metschnikowia pulcherrima*, *Kloeckera apiculata*, and *Starmerella bacillaris*. The spontaneous fermentations were instead both dominated by *Saccharomyces cerevisiae*, however in different concentration throughout the process, leading to a different ethanol content—12% (*v*/*v*) and 10.8% (*v*/*v*) in winery A and B, respectively. In both wineries, acetic bacteria and moulds did not grow. Considering the intraspecific biodiversity of *S. cerevisiae* populations, the vinifications of both wineries displayed very similar biodiversity indices. No single strain of *S. cerevisiae* dominated the entire fermentation process. The analysis identified 30 distinct genetic patterns in the fermentations of winery A and 23 in the fermentations of winery B. The work provided an insight into the microbial communities and their metabolomic interactions during Vin Santo production which could improve the management and control of the process.

Keywords: *passito* wine; Vin Santo; spontaneous fermentation; microbial biodiversity; *Saccharomyces cerevisiae*; non-*Saccharomyces* yeasts

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1. Introduction

Wines made from dried grapes, known as *passito* wines, have been a century-long tradition in many countries [1]. One example is Vin Santo, a typical *passito* wine produced not only in Tuscany but also in central and northern Italy, as well as on the Greek island of Santorini [2]. The production of Vin Santo involves several phases: harvesting and selecting the grapes, drying them (*appassimento*) in shaded, ventilated rooms or under fully controlled environmental conditions, pressing the healthy grapes, and filling barrels to carry out alcoholic fermentation and ageing for 2–4, or even more, years. The barrels, known as "*caratelli*", are traditional wooden vessels, typically made from chestnut, oak, or cherry, with a capacity ranging from 50 to 200 L, which are kept in the cellar or "*vinsantaia*", a traditional ventilated room. As a result, they are exposed to seasonal temperature variations [2,3].

Although Vin Santo is a niche and traditional product, its production has increased over the years, which is in line with the general increase in demand for *passito* wines [2]. The EU regulates Italian Vin Santo as a Quality Wine Produced in Specific Regions under the specific Protected Designation of Origin (PDO) [4,5]. Due to its lack of standardization, Vin Santo's characteristics can vary significantly depending on the winemaking process. A crucial aspect in the Vin Santo production is the high probability of having stuck or slow fermentations because of the high sugar content (over 300 g/L) associated with high concentrations of organic acids, polyphenols, metal ions such as copper, and by-products from the Maillard reaction [6,7]. In this context, few scientific studies have explored the microbiota of Vin Santo production and its effects on its chemical properties [2,8]. Unlike other *passito* wines [1], the traditional production of Vin Santo usually does not involve starter yeasts; instead, fermentation occurs naturally with the yeasts present on the grapes and in the cellar. The dried grape juice can be enriched with 5–10% of the sediment, known as the "*madre*", collected from the barrels at the end of the ageing of the previous Vin Santo vintage. This sediment appeared to have no direct role as a microbial starter in Vin Santo production, not providing metabolically active *Saccharomyces cerevisiae* populations, but only yeasts belonging to the genus *Zygosaccharomyces* after 3 years of ageing. However, the presence of lipids and other nutritional factors in the sediment seems to have an indirect role in promoting yeast growth and persistence [3]. Grapes are a primary source of microorganisms, and many studies have shown that spontaneous wine fermentation starts with the dominant yeast species occurring on grapes at harvest time and/or during the drying [9–12]. Despite this, little information is available on the microbiota during spontaneous Vin Santo fermentations, even if some recent studies have been conducted on other *passito* wines. The most abundant species found on dried Nebbiolo grapes for 'Sforzato di Valtellina' production were *Hanseniaspora uvarum*, followed by *Metschnikowia* spp. and *Starmerella bacillaris* [13]. As reported by de Filippis et al. [1], the microbial community of *Falanghina* sweet *passito* wine at the beginning of the fermentation was mainly represented by *Aureobasidium*, *Cladosporium*, *Sclerotinia*, while *Candida*, *Debaryomyces*, *Hanseniaspora*, *Metschnikowia*, *Pichia*, *Saccharomyces* and *Zygosaccharomyces* showed a very low incidence. *Hanseniaspora vineae* and/or *Hanseniaspora uvarum* grew after almost 30 days of fermentation, while *S. cerevisiae* occurred in all the phases but became dominant only in the last stages of alcoholic fermentation. A wide diversity of autochthonous yeasts of oenological relevance was also found during spontaneous fermentations in Tostado sweet wine from Galicia, such as *Lachancea thermotolerans*, *Starmerella bacillaris*, *Hanseniaspora uvarum*, *Debaryomyces hansenii*, *Torulaspora delbrueckii*, *Pichia* spp., and *Saccharomyces cerevisiae*, the latter characterized by 19 different dominant strains [14]. Although the above-reported investigations provided insight into the primary yeast indigenous species involved in the production of *passito* wines, there is little information available regarding the microbial dynamics and their subsequent influence on the chemical characteristics during the fermentation and ageing of this kind of wine except for some studies conducted on the aromatic profiles [1,5,8,15]. As reported in the literature, these indigenous yeasts are often inadequate or too stressed by the high osmotic conditions of the grape must to effectively carry out the fermentation process, resulting in low-quality sweet wines with high residual sugar content, low alcohol content, and pronounced "solvent/chemical" notes [1,13,16]. For these reasons, some authors suggest the selection of indigenous yeasts, preferring mixed cultures consisting of *S. cerevisiae* and non-*Saccharomyces* yeasts to improve *passito* wine quality by modulating specific wine attributes and completing the fermentation process without the risk of stuck fermentation [13]. However, to effectively manage Vin Santo production with or without the employment of yeast starters, it is essential to have a deep understanding of the microbial ecology of the process, from grape drying to barrel ageing. To our knowledge,

only a few reports on this topic have been published. Therefore, the present study aimed to investigate the microbiota and, in particular, the yeasts and their impact on the fermentative kinetics in traditional processes of Vin Santo carried out in two different Tuscan wineries.

2. Materials and Methods

2.1. Winemaking

The winemaking process is reported in Figure S1. From each vineyard of two wineries in the Chianti Classico region (A and B), 500 kg of grapes from both the Trebbiano and Malvasia del Chianti varieties were handpicked. The grapes were dried after harvesting in a well-ventilated room (known as *fruttaio*). The clarified must from winery A was immediately transferred to oak wooden barrels of two different capacities (50 and 100 L), filled to 80% of their volume, sealed tightly, and left to ferment spontaneously. On the contrary, in winery B, the clarified must was transferred to the oak wooden barrels of two different capacities (50 and 100 L), after ten days of fermentation in demijohns.

2.2. Microbial Analysis

Wine microorganisms were quantified as reported by Guerrini et al. [17]. Yeasts were quantified on WL Nutrient Agar medium (Thermo Fisher Scientific, Waltham, MA, USA) and Lysine Agar (Oxoid Ltd., Basingstoke, Hampshire, UK), both integrated with sodium propionate (VWR International S.r.l., Milan, Italy) (2 g/L) and streptomycin (VWR International S.r.l., Milan, Italy) (30 mg/L) to inhibit mould and bacteria growth, respectively. The plates were incubated for 48–72 h at 28 °C in aerobic conditions. Lactic acid bacteria were quantified on MRS Agar medium (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 50 mg/L of pimaricin as a yeast inhibitor, and incubated for 5 days at 30 °C under anaerobic conditions. Acetic acid bacteria were quantified on a Lafon-Lafourcade medium supplemented with pimaricin (50 mg/L) as a yeast inhibitor and incubated for three days at 30 °C in aerobic conditions. *Brettanomyces bruxellensis* was quantified on DBDM medium (*Dekkera*/*Brettanomyces* Differential Medium) containing 6.7 g/L 80 YNB (Yeast Nitrogen Base, Difco), ethanol (6% *v*/*v*), 10 mg/L cycloheximide, 100 mg/L p-coumaric acid, 22 mg/L bromocresol green and 20 g/L agar at 28 °C for 5 days in aerobiosis. Finally, moulds were counted on MYPG agar (malt extract 5 g/L, yeast extract 3 g/L, meat extract 5 g/L, glucose 10 g/L and 20 g/L agar) after incubation at 30 °C for 24–48 h. Two samples from each sampling point were subjected to microbial analyses.

2.3. Microbial Identification and Typing of Yeast Strains

After purification by successive streaking on Petri plates, yeast isolates were identified by PCR–RFLP as described by Granchi et al. [18]. The 5.8S rRNA gene and the two ribosomal internal transcribed spacers (ITS) were amplified using the primer pair ITS1/ITS4 and the amplicons were digested with the restriction enzyme *Hae*III (Life Technologies Italia, Monza, Italy). To confirm the identification obtained by PCR–RFLP, the D1-D2 rDNA region products were purified using Nucleo Spin Extract II (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and sent to BMR Genomics (Padua, Italy) for sequencing.

Bacteria were identified by Amplified Ribosomal DNA Restriction Analysis (ARDRA). DNA was extracted, and the 16S rDNA gene was amplified using the primers FD1 (5′ CAACAGAGTTTGATCCTGGCTCAG 3′) and RD1 (5′ GCTTAAGGAGGTGATCCAGCC 3′). The amplicons were purified using Nucleo Spin Extract II (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and sent to BMR Genomics (Padua, Italy) for sequencing.

All the sequences in FASTA format obtained for yeasts and bacteria were compared with those contained in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/

(accessed on 14 March 2024 and 28 February 2025)) using the basic BLAST search tools (version 2.8.1).

Saccharomyces cerevisiae isolates were also characterized at strain level by inter-δ PCR typing with δ12/δ21 primer pair (Thermo Fisher Diagnostics S.p.A., Rodano, Milan, Italy) as reported by Legras and Karst [19]. The genomic patterns were submitted for pairwise comparison using the Dice coefficient and cluster analysis with the unweighted pair group method (UPGMA) by Gel Compare 4.0 software (Applied Math, Kortrijk, Belgium). *S. cerevisiae* diversity was quantified by using the two indices "H" and "e", as proposed by Shannon and Weaver [20].

Non-*Saccharomyces* isolates were characterized at strain level by Randomly Amplified Polymorphic DNA (RAPD) analysis by using the primer M13 (50-GAGGGTGGCGGTTCT-30), while the species was determined by D1/D2 26S rRNA gene sequencing analysis as reported by Mari et al. [21]. All PCR reactions included both negative (DNA-free) and positive controls. Relative frequencies of isolation used to represent yeast species density according to the isolation source were calculated as the number of isolates belonging to each species divided by the total number of isolates and expressed in percentage.

2.4. Chemical Analysis

Glucose, fructose, ethanol, glycerol, 2,3-butanediol, acetic, lactic and succinic acid contents in must and wine were determined by High Performance Liquid Chromatography (HPLC), according to Guerrini et al. [17] utilizing a Rezex ROA-Organic Acid H+ (8%) column (8-µm particle, 300 × 7.8 mm; Phenomenex, Torrance, CA, USA) and a ProStar 210 chromatograph equipped with a DAD at 210 nm and a Refractive Index Detector, in series (Varian Inc., Palo Alto, CA, USA). Malic acid, free amino nitrogen (FAN), NH_3, and Cu^{2+} concentrations were determined enzymatically through Hyperlab (Steroglass, San Martino in Campo, Perugia, Italy), an automatic multi-parametric analyser.

2.5. Phenolic Compounds and Ochratoxin Content Determination

Phenolic compound determination was carried out according to Guerrini et al. [17]. Samples were filtered (0.45 µm) and injected into the HPLC-UV/FLD Jasco series 4000 (Jasco, Japan Spectroscopic co, Hachioji City, Japan) equipped with a pump PU-4180, an autosampler AS-4050, a photodiode array detector MD-4010, and a column oven CO4060 and a reversed-phase column NovaPak C18 (4-µm particle, 300 × 3.9 mm; Waters, Milford, MA, USA), thermostated at 25 °C. The eluent and gradient conditions were those reported by Guerrini et al. [17]. Phenolic compounds were detected by scanning from 210 to 600. nm Hydroxybenzoic and hydroxycinnamic acids, flavonols, flavan-3-ols, stilbenes. Hydroxybenzoic acids and hydroxycinnamic acids were quantified at 280 nm; stilbenes were quantified at 280 nm; flavonols and flavan-3-ols were quantified at 360 nm.

Ochratoxin content of grapes was determined according to Sultan et al. [22]. Grapes were diluted 5 times and homogenized with methanol and water (30:70 v/v) and filtered using Whatman filter paper. The filtrate was diluted with phosphate-buffered saline (PBS) containing 0.01% Tween, loaded and passed through an Ochratoxin Immunoaffinity Columns Milford (VICAM, Waters Corporation, MA, USA) following the manufacturer's instructions. The HPLC system used for OTA analysis was Agilent InfinityLab Poroshell 120 EC-C18 (3.0 × 150 mm 2.7 micron) (Agilent, Berks, UK). The mobile phase was AcN:water:acetic acid (57:41:2, $v/v/v$) using a flow rate of 1 mL min^{-1}. Detection was performed with a fluorescence detector using λ_{ex} 333 nm and λ_{em} 460 nm (excitation and emission wavelengths, respectively). For all experiments, the injection volume was set at 20 µL.

2.6. Statistical Analysis

Data of analytical determinations, performed in duplicate, were analyzed according to ANOVA followed by Tukey's Test. Differences were reported at a significance level of $p < 0.05$ or $p < 0.01$. The statistical analyses were performed by Statistica 7.0 software package (Statsoft GmbH, Hamburg, Germany) or by Graph Pad Prism 8.

3. Results

3.1. Microbiota of Grapes During Drying

The microbiota of grapes during the drying is reported in Figure 1, showing the main microbial groups of oenological interest. In winery A, the grapes were dried by hanging, while in winery B, they were placed on racks. Regardless of the winery and sampling time, non-*Saccharomyces* yeasts were the predominant microbial population. *Saccharomyces cerevisiae* was present in all grape samples at cell concentrations ranging from 10^2 to 10^4 CFU/g. At the end of the drying process, significant levels of moulds, primarily belonging to the *Aspergillus niger* species, were observed in the grape samples from both wineries; nevertheless, the ochratoxin content was less than 0.11 µg/Kg. Acetic acid bacteria were found at higher levels than lactic acid bacteria but at below 10^5 CFU/g. Identification of non-*Saccharomyces* yeast species was conducted at all sampling points, and the results were reported as isolation frequency in Figure 2. At harvest, the yeast-like fungus *Aureobasidium* occurred at a frequency of 56% and 75% on the grapes of winery A and winery B, respectively. During the drying process, yeasts belonging to the *Metschnikowia pulcherrima* and *Starmerella bacillaris* species exhibited a frequency of 80% on grapes from winery A, while on grapes from winery B, *Starmerella apicola*, *Starmerella bacillaris*, and *Kloeckera apiculata* became the dominant yeast species.

3.2. Microbiota and Chemicals Kinetics During Spontaneous Fermentations

By the end of the drying process, the sugar content of the grapes was concentrated at 70% and 60% in wineries A and B, respectively. The chemical composition of the grape must from winery A was: glucose 187 g/L, fructose 199 g/L, malic acid 2.90 g/L, tartaric acid 4.42 g/L, lactic acid < 0.1 g/L, glycerol 0.26 g/L, free amino nitrogen (FAN) 48 mg/L, NH_3 12 mg/L, K^+ 2074 mg/L, Cu^{2+} 3.8 mg/L, pH 3.84. The chemical composition of the grape must from winery B was: glucose 175 g/L, fructose 192 g/L, malic acid 1.84 g/L, tartaric acid 4.49 g/L, lactic acid < 0.1 g/L, glycerol 2.3 g/L, free amino nitrogen (FAN) 86 mg/L, NH_3 27 mg/L, K^+ 1904 mg/L, Cu^{2+} 3.6 mg/L, pH 3.67.

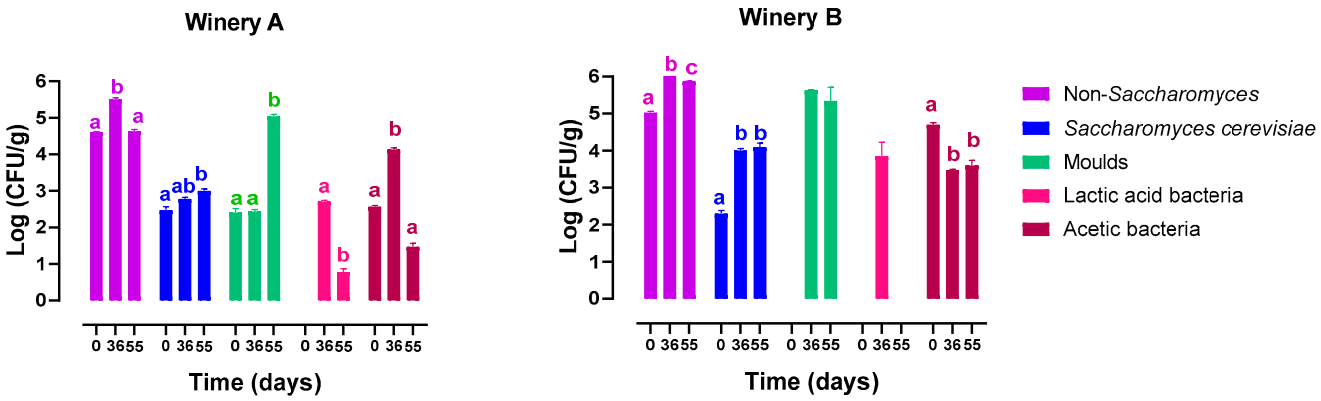


Figure 1. Yeasts, moulds, and bacteria concentrations during grape drying in winery A (hung grapes) and winery B (grapes placed on racks). Different letters indicate statistically significant differences (ANOVA and Tukey's test, $p < 0.05$).

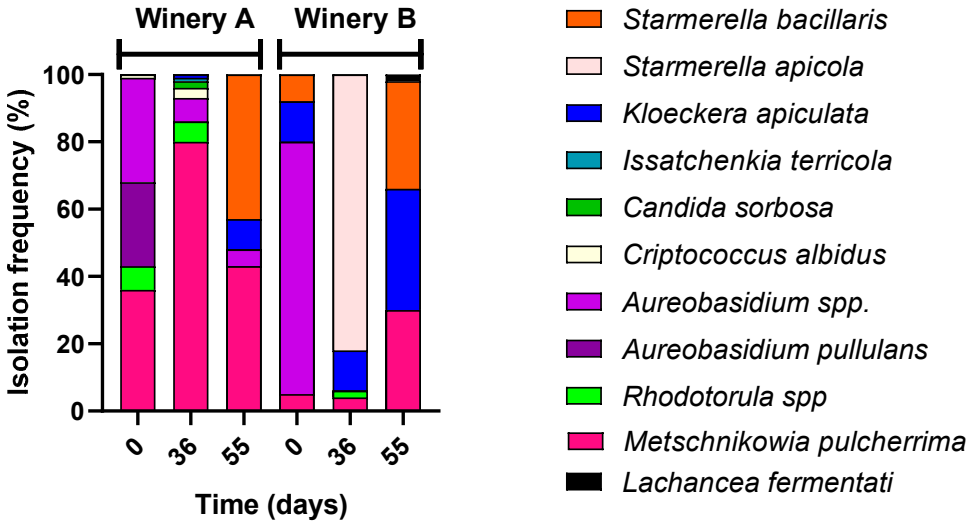


Figure 2. Isolation frequencies expressed as percentages of the non-*Saccharomyces* yeasts found on the grapes during the drying process in the two wineries.

The grape must from winery A was promptly divided between two barrels of different capacities (50 and 100 L) and left to ferment naturally. Meanwhile, the grape must from winery B was initially fermented spontaneously in demijohns for two weeks until the ethanol concentration reached approximately 2.0% (*w*/*v*) and then transferred to two oak wooden barrels of the same capacity as those in winery A. The fermentation process in all four barrels was regularly monitored using microbiological and chemical analyses. The fermentation time course is reported in Figure 3. Both vinifications dominated by *Saccharomyces cerevisiae*, however with different kinetics.

In winery A, in both barrels, *S. cerevisiae* takes over during the first 15 days of fermentation, maintaining concentrations of 10^7 CFU/mL during the first 100 days of fermentation and then decreasing to 10^5 CFU/mL after 200 days. The population of non-*Saccharomyces* increased to approximately 10^6 CFU/mL in the first 15 days of fermentation, decreased below 10^4 CFU/mL in the following 35 days, and remained at this concentration for about three months. Finally, this population grew again up to without reaching 10^5 CFU/mL. Lactic acid bacteria, acetic bacteria, and moulds did not show growth, while *Brettanomyces bruxellensis* was always below the detection limit. After 228 days, the ethanol content was 12% (*v*/*v*) and fructose and glucose concentrations were 134 and 54 g/L, respectively; the acetic acid reached a concentration of 1.4 g/L, lower than the maximum acceptable limit by the Vin Santo production rules (1.8 g/L). Finally, the glycerol content was around 8 g/L, while succinic acid production stopped at 0.7 g/L after 150 days of fermentation.

Regarding winery B, the kinetics of *S. cerevisiae* were quite similar in the two barrels. In both barrels, *S. cerevisiae* never reached 10^7 CFU/mL, and after 150 days, this yeast population entered the death phase, dropping below the detection limit after 228 days. Unlike winery A, the non-*Saccharomyces* yeasts exhibited a progressive loss of cell viability, with a higher rate in the 100 L barrel compared to the 50 L barrel. After 228 days of fermentation, the ethanol content was 10.8% (*v*/*v*) in both barrels, while the residual sugars were 169 g/L in the 100 L barrel and 195 g/L in the 50 L barrel, respectively. Conversely, the 100 L barrel had higher glycerol concentrations than the 50 L barrel (10 g/L versus 9 g/L), but both had identical and elevated levels of acetic acid (2 g/L). Both vinifications recorded low concentrations of succinic acid (less than 0.2 g/L). Lactic acid bacteria were present at 10^5 CFU/mL in both barrels, and the dominant species across all samples was *O. oeni*. In the 50 L barrel, *O. oeni* demonstrated significantly higher concentrations, resulting in complete malic acid degradation within 150 days. In contrast, the vinification in the 100 L

barrel did not complete malolactic fermentation, with malic acid remaining at 0.83 g/L after 228 days. Finally, as seen in winery A, acetic bacteria and moulds did not grow, while *Brettanomyces bruxellensis* was below 10 CFU/mL.

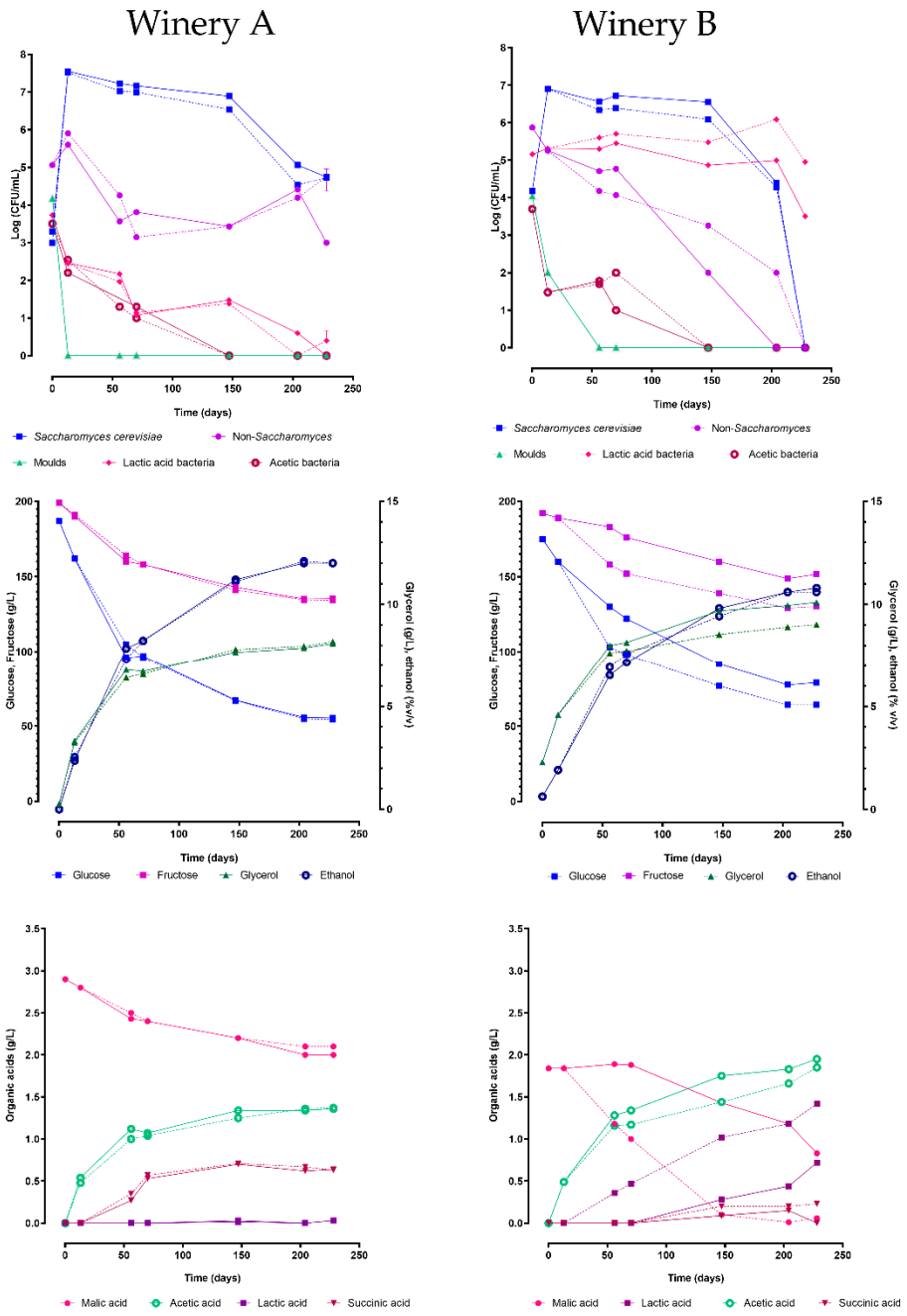


Figure 3. Microbial populations and chemical evolution during alcoholic fermentations carried out in wineries A and B using oak wooden barrels of different capacities (100 L continuous line, 50 L dashed line).

3.3. Biodiversity of Non-Saccharomyces Yeasts During Fermentation

Identification of non-*Saccharomyces* populations was conducted at all sampling points, and the results of non-*Saccharomyces* yeast species were reported as isolation frequency in Figure 4. The non-*Saccharomyces* yeast species in the two wine fermentations conducted in winery A were similar. *M. pulcherrima* was the dominant yeast in the first three sampling points. On the 70th day, the dominant yeast species became *Zygosaccharomyces bisporus*, *Torulaspora delbrueckii*, *Hanseniaspora osmophila*, and *Pichia manshurica* in both fermentations.

Finally, *Zygosaccharomyces* spp. dominated the remaining two sampling points. In contrast, different non-*Saccharomyces* species were found after the first two sample points in barrels in winery B. Indeed, between 56 and 147 days of fermentation, *Lachancea fermentati*, *Candida vini*, *Zygosaccharomyces bailii*, and *Torulaspora delbrueckii* were the dominant species in the 100 L barrel while in the 50 L barrel, *Starmerella bacillaris*, *Hanseniaspora osmophila*, *Pichia manshurica*, and *Zygosaccharomyces bisporus* dominated. Ultimately, the non-*Saccharomyces* yeasts present in the fermentations of the two cellars were characterized by different dominant species. However, this difference does not explain the higher mortality that occurred in both fermentations of cellar B.

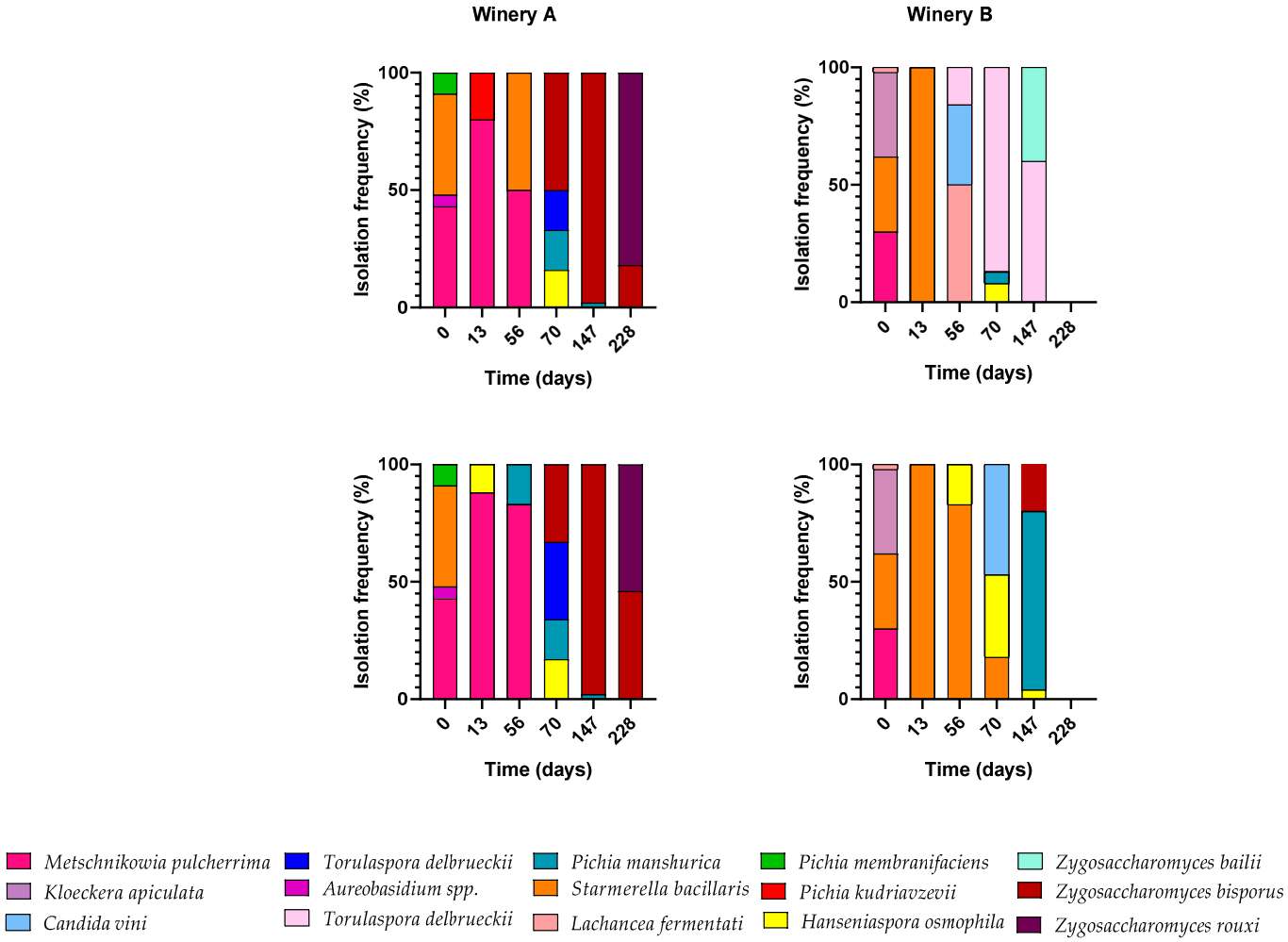


Figure 4. Isolation frequency expressed as a percentage of the non-*Saccharomyces* yeasts found during fermentations in the two wineries (in 100 L barrel at the **top** and 50 L barrel at the **bottom**).

Therefore, to understand the possible reasons for the non-*Saccharomyces* mortality, some compounds considered responsible for stuck or slow fermentations in passito wines, such as polyphenols and copper, were quantified after 230 days of fermentation (Table 1). Gallic acid, protocatechuic acid, caftaric acid and caffeic acid were at higher concentrations in the wines produced by winery B than those of winery A. As for the copper concentration, after 230 days of fermentation, the wines from Winery A had a significantly higher content than those from Winery B, while the initial Cu^{2+} content of the musts was the same (3.8 and 3.6 mg/L). This could be due to an over-intake of Cu^{2+} by the yeast populations of the wines obtained in winery B, contributing to cell death.

Table 1. Polyphenols and copper concentrations quantified after 230 days of fermentation (mean ± SEM) and absorbance at 420 nm; different letters mean significant differences between column (ANOVA and Tukey test, $p < 0.05$).

	Winery A-100 L	Winery A-50 L	Winery B-100 L	Winery B-50 L
Polyphenols (mg/L)				
gallic acid	15.3 ± 0.8 a	20.4 ± 1.0 a	36.3 ± 1.8 b	35.1 ± 0.2 b
protocatechuic acid	13.4 ± 0.7 a	15.2 ± 0.8 a	25.1 ± 0.3 b	21.7 ± 1 b
caftaric acid	7.9 ± 0.4 a	8.2 ± 0.4 a	17.1 ± 0.9 b	18.3 ± 0.9 b
catechin	12.7 ± 0.6 a	12.8 ± 0.6 a	14.2 ± 0.7 a	8.7 ± 0.4 c
vanillic acid	16.8 ± 0.8 a	19.0 ± 0.9 a	25.2 ± 1.26 b	17.0 ± 0.8 a
caffeic acid	4.6 ± 02 a	4.5 ± 02 a	17.2 ± 09 b	18.4 ± 09 b
Total polyphenols index (TPI)	31.19 ± 0.20 ab	32.48 ± 0.10 a	37.62 ± 0.35 c	29.73 ± 0.44 b
Cu^{2+} (mg/L)	2.13 ± 0.10 a	1.35 ± 0.07 b	0.53 ± 0.03 c	0.37 ± 0.02 c
Degree of browning (Abs 420)	0.1032 ± 0.0030 a	0.1072 ± 0.0040 a	0.2370 ± 0.0035 b	0.1804 ± 0.0025 c

The Maillard reaction could also be responsible for the higher mortality of yeasts in the wines of Winery B. Effectively, the degree of browning, which is an indirect measure of the Maillard reaction, showed absorbance values approximately double in winery B than in winery A (Table 1).

3.4. Intraspecific Biodiversity of Saccharomyces cerevisiae

The intraspecific biodiversity of *S. cerevisiae* was evaluated throughout the fermentation process to point out the number of strains present and their distribution over time. This is particularly interesting from an ecological perspective, given the extended duration of the fermentation process and the highly selective environment of Vin Santo. Therefore, 200 *S. cerevisiae* isolates from each vinification were analyzed to detect genetically different strains using the PCR amplification of the inter-δ regions. The analysis identified 30 distinct genetic patterns in the fermentations of winery A and 23 in the fermentations of winery B. The isolation frequencies of the indigenous *S. cerevisiae* strains, calculated for each winemaking and each sampling point, are reported in Figure 5. None of the strain showed significant dominance during the 200 days of fermentation. The profiles shared by the two vinifications of winery A and present in at least one sampling point at concentrations greater than 30% were three (RisI, III, and VIII), while in winery B, they were two (OmII and OmV). As the *S. cerevisiae* population declined in both wineries, previously undetected strains were discovered, reaching isolation frequencies of around 10%. This phenomenon was most evident in the 100 L barrel fermentation of winery A and the 50 L barrel of winery B.

To compare the genetic diversity levels of the *S. cerevisiae* populations isolated from the four vinifications, the values of "H" and "e" indices were calculated (Table 2). According to Shannon's index "H", estimating the richness of *S. cerevisiae* strains found in the four barrels, a similar level was observed for all the vinifications oscillating between 2.2 and 2.6; indeed, no strain emerged as predominant over 200 days of fermentation in the four vinifications. Accordingly, the evenness index "e", which ranges between 0 and 1 and increases as the number of isolates exhibiting the same inter-δ patterns decreases, assumed values between 0.5 and 0.6 in all the vinifications. Finally, all the inter-δ patterns corresponding to the different *S. cerevisiae* strains were analyzed using UPGMA clustering analysis, and the resulting dendrogram is reported in Figure 6. The *S. cerevisiae* strains, at 30% similarity, were grouped into four clusters in both wineries. The clustering of the strains in both cellars were not related to the time of isolation. In particular, the strains isolated at the first sampling point were distributed in different clusters.

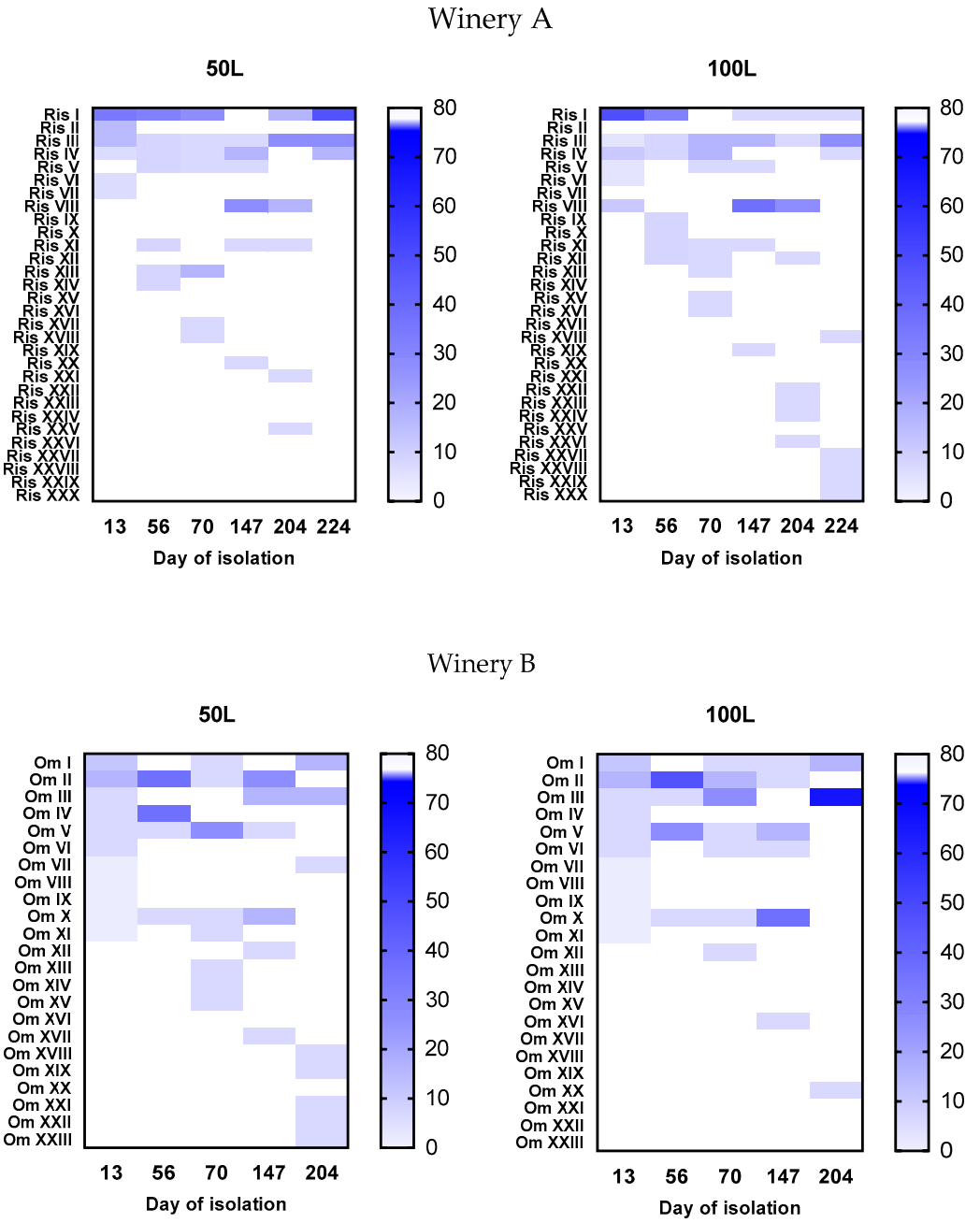


Figure 5. Heat map of the isolation frequencies of the *S. cerevisiae* inter-δ patterns found during fermentations carried out in the two wineries.

Table 2. Biodiversity indices calculated according to Shannon–Weaver for the *S. cerevisiae* population obtained from the four vinifications.

	H Index	E Index
Winery A 100 L	2.28	0.55
Winery A 50 L	2.26	0.55
Winery B 100 L	2.17	0.53
Winery B 50 L	2.65	0.65

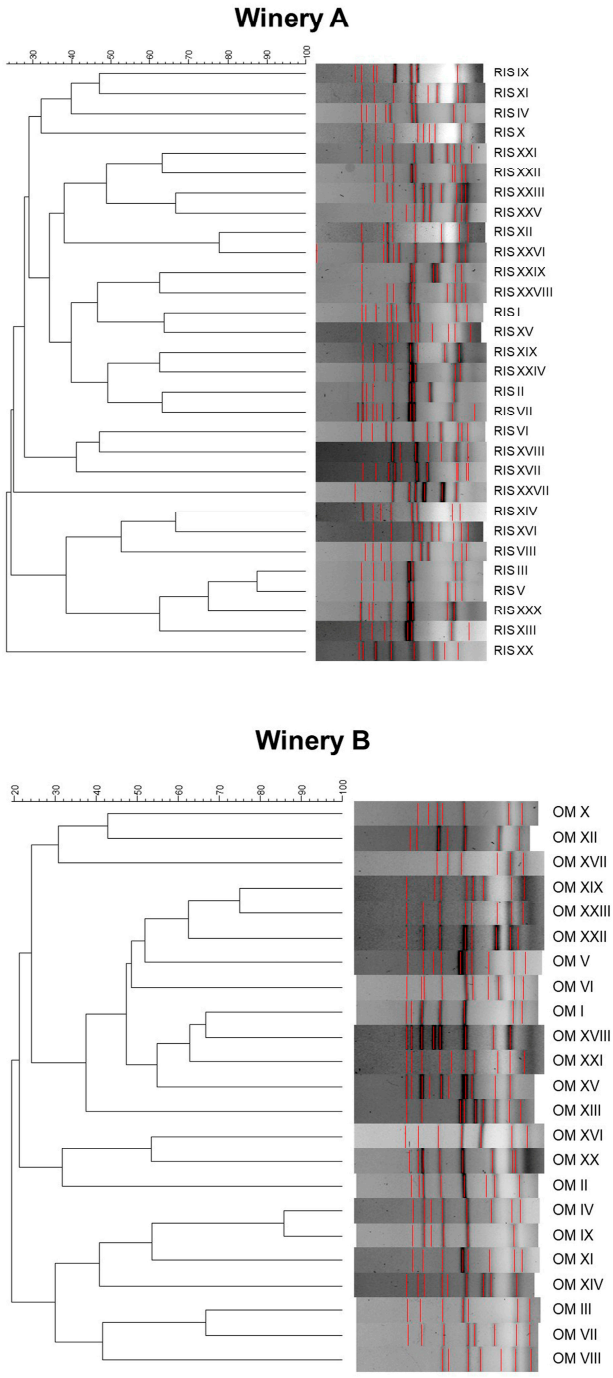


Figure 6. UPGMA dendrogram based on the Dice coefficient of the inter δ-patterns of the indigenous *S. cerevisiae* strains isolated from fermentations carried out in the two wineries.

To further investigate the clonal relationship between genotypes found at the beginning and end of the fermentation process, the inter-δ patterns of the strains present on the thirteenth day and after over 200 days of fermentation were compared using the neighbour-joining method Saitou [23]. The results are reported in Figure 7, where the strains collected at the beginning of fermentation are in green, while the strains collected after more than 200 days of fermentation are in red. The strains present at the end of the fermentation did not show significant similarities with those present at the beginning, except the Ris XXX strain which showed substantial similarities with the Ris III and Ris V strains.

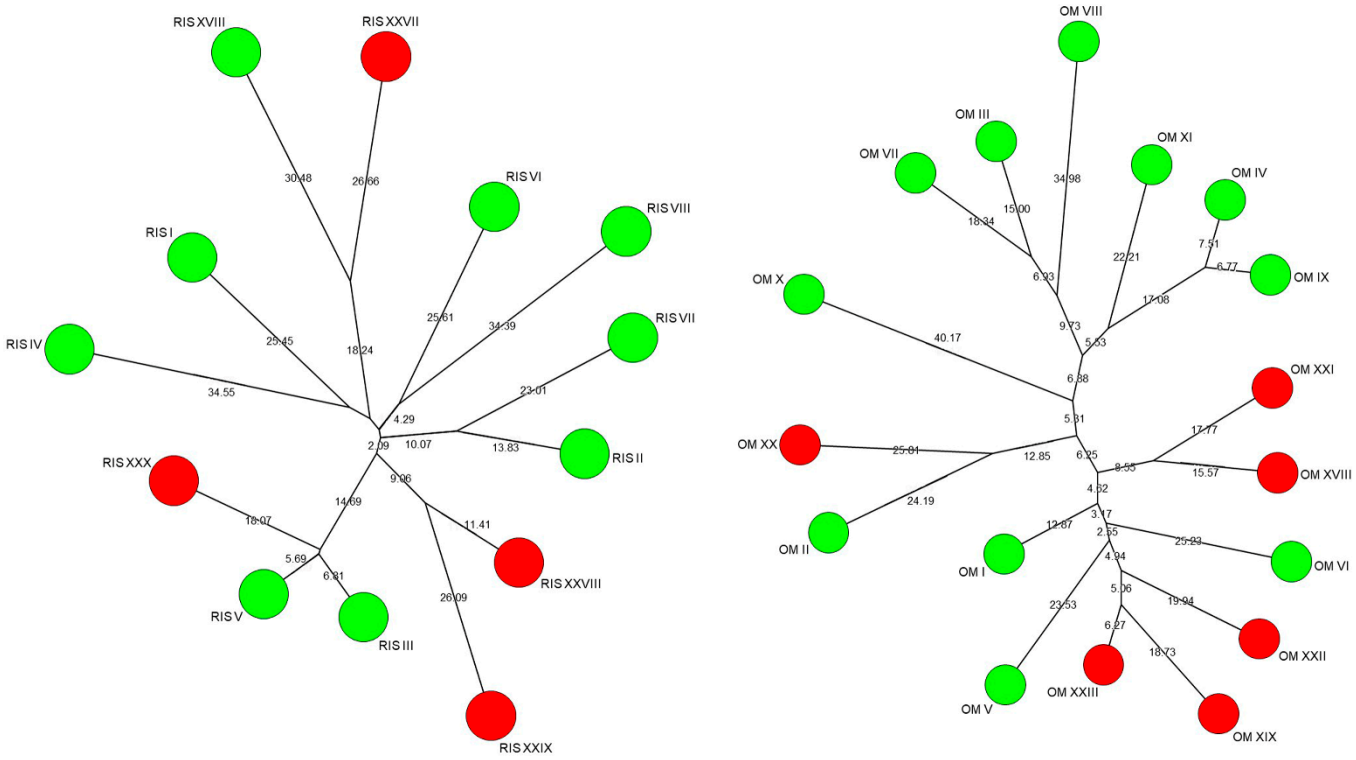


Figure 7. Neighbour-joining consensus tree based on the Dice coefficient of the inter δ-patterns of the *S. cerevisiae* strains present on the thirteenth day (green) and after over 200 days of fermentation (red) in the vinifications carried out in the two wineries (at **left** winery A and **right** winery B).

4. Discussion

The microbial ecology of the traditional process of Vin Santo production was investigated in two wineries in Tuscany. Despite the different systems used for drying the grapes (grapes hung or placed on racks), both wineries showed similar microbial ecology. Non-*Saccharomyces* yeasts were the dominant microbial population, even though the moulds reached comparable concentrations at the end of the drying process. The succession of non-*Saccharomyces* species throughout grape drying differentiated between the two wineries, even though in the end, the dominant species in both were *M. pulcherrima*, *K. apiculata,* and *S. bacillaris,* but at different percentages. Other authors found these yeast species on dried grapes used in Vin Santo and other *passito* wine production [2,24]. Unlike what is generally found in grapes at harvest [12], significant concentrations (10^3–10^4 CFU/g) of *S. cerevisiae* were present from the beginning of the drying phase, this might be due to a *S. cerevisiae* contamination of the drying rooms (*fruttaio*). Regarding the bacterial population, acetic bacteria were most present at the end of the grape-drying process.

Despite the similar microbiological characteristics of the grapes at the end of drying, the fermentation trends in the two wineries were very different. Unlike the winery A vinifications, the winery B vinifications showed low ethanol content and excessive acetic acid content (>1.8 g/L). The low ethanol content in winery B could be due to the non-*Saccharomyces* population in the first two weeks, mainly composed of *S. bacillaris*. This species produces high glycerol and low ethanol concentrations [25]. Being able to tolerate high ethanol concentrations, *S. bacillaris* can compete with *S. cerevisiae*, compromising its fermentation performance. Thanks to these characteristics, some authors have proposed *S. bacillaris* as a starter in co-inoculation with *S. cerevisiae* to reduce the ethanol content of the wines [26]. The high persistence of non-*Saccharomyces* observed in the fermentations of Vin Santo has also been found in other *passito* wines, demonstrating a significant impact of this yeast population on the volatile component and aromatic profile of this type of

wine [1]. Excessive acetic acid content could be due to a non-optimal physiological state of *S. cerevisiae* [27] and/or to the growth of a bacterial population mainly composed of *O. oeni*, an heterolactic lactic acid bacteria, in the presence of high concentrations of hexose sugars. Finally, the yeast population mortality highlighted in the fermentations of winery B starting from the 200th day could be due to the more significant presence in these wines of some polyphenols that have demonstrated certain toxicity for wine microorganisms, although the minimum inhibition concentrations reported in vitro for these compounds were always much higher than those found in the wines analyzed [27–30]. Further studies would be necessary to understand the possible synergistic roles between these compounds and other parameters of *passito* wines compromising the yeast's physiological state, such as ethanol, high sugar concentration, or copper. As reported in scientific literature, concentrations of copper lower than 20 mg/L, as in the case of the winery A and B starting musts, did not affect the cell growth of *S. cerevisiae,* but its fermentation performance can be inhibited to a certain extent [31]. In the wines of winery B, yeast populations could have absorbed copper, compromising the fermentation performance of *S. cerevisiae* yeasts or, otherwise, determining the mortality of non-*Saccharomyces* yeasts.

Considering the intraspecific biodiversity of *S. cerevisiae* populations, the vinifications of both wineries displayed very similar biodiversity indices. No single strain of *S. cerevisiae* dominated the entire fermentation process, which is often observed in spontaneous wine fermentations using non-dried grapes [32]. This lack of dominance could be a typical characteristic of *passito* wines, attributed to the lengthy duration of alcoholic fermentation. Additionally, the clonal relationship between the genotypes present at the beginning and end of the fermentation process was not evident. This suggests that the number of indigenous strains does not increase over time through recombination among the strains present in the initial stages. Instead, different strains seem to attain dominance at various points during fermentation, likely based on their fitness advantages in a culture medium that changes gradually over time. This phenomenon complicates the task of determining the best moment to isolate yeasts for selection as starter strains. It appears that in the case of *passito* wines, a more effective strategy might be to use different yeasts in co-inoculation, as proposed by de Philippis et al. [1]. In this article, the authors proposed the co-inoculation of *S. cerevisiae* with osmotolerant non-*Saccharomyces* yeasts such as *Candida zemplinina* or *Z. bailii*. Recent studies have shown that non-*Saccharomyces* yeasts can modulate and enrich the sensory properties of wines by playing a role in releasing volatile compounds from non-volatile precursors [33,34]. Therefore, a non-*Saccharomyces* starter could contribute favourably to the aromatic bouquet. However, it is important to investigate the metabolic compatibility between strains to avoid competition that can lead to incomplete fermentations. One metabolic capacity of yeasts that could be important for selecting a starter for *passito* wines is the ability to produce succinic acid. Succinic acid production by non-*Saccharomyces* yeast in co-inoculated fermentations with *S. cerevisiae* strains could inhibit *O. oeni* growth [35]. Inhibiting the development of this microbial population could help reduce the concentration of acetic acid, which is often too high in *passito* wines.

5. Conclusions

This study explored the microbial ecology and its impact on the fermentative kinetics in traditional processes of Vin Santo of two wineries. The microbial ecology were similar; during the grape drying non-*Saccharomyces* yeasts were the dominant microbial population, while during the spontaneous fermentation *Saccharomyces cerevisiae* became dominant the fermentation trends in the two wineries were very different. Considering the intraspecific biodiversity of *S. cerevisiae* populations, the vinifications of both wineries

displayed very similar biodiversity indices. No single strain of *S. cerevisiae* dominated the entire fermentation process. The study highlighted the importance of understanding and characterizing microbial communities and their metabolomic interactions, in order to improve the management and control of the process.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation11060310/s1. Figure S1: Winemaking process of Vin Santo.

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