Article

Investigation by High-Throughput Sequencing Methods of Microbiota Dynamics in Spontaneous Fermentation of Abruzzo (South Italy) Wines

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Abstract: Spontaneous wine fermentation is a meaningful topic that cannot be disregarded among winemakers and consumers due to the peculiarity of the organoleptic profile that it confers to the wine. Nevertheless, in this process the activity of indigenous microorganisms might be a threat. We studied the evolution of the spontaneous fermentation process in a traditional Italian winery in order to understand the origin of spoilage microorganisms, and to characterize the peculiarity of the microbiota associated with spontaneous fermentation. Six Trebbiano and Montepulciano wine production chains were monitored by plate counts made by OIV methods and by Illumina MiSeq technique. Despite some compositional deficiencies, all grape musts were characterized by a highly concentrated microbial population. Non-*Saccharomyces* yeasts revealed an unexpected tolerance to ethanol, which has contributed to the evolution of alcoholic fermentation. Lactic bacteria were detectable from the very first steps of the winemaking process, with a prevalence of *Leuconostoc* spp. which is nowadays, rarely isolated in wine. The combination between culture-dependent and high-throughput sequencing (HTS) approaches allowed to estimate microbial diversity and growth dynamics in wine fermentations of different grape varieties and under different treatments; these results could be used by winemakers as a starting point to drive a more mindful, accurate and, controlled fermentation process and to set up the most suitable environmental conditions to enhance wine singularities.

Keywords: spontaneous fermentations; non-*Saccharomyces* yeast; *Leuconostoc*; Illumina MiSeq; Montepulciano

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1. Introduction

The expression "spontaneous fermentation" indicates the production of wine without the employment of selected microbial starter cultures; therefore, entrusting the evolution of alcoholic and malolactic fermentations at the activity of the complex microbiota, including bacteria, yeasts, and other fungi, that populate grapes and winery. This choice might sound archaic, but it is justified by wine producers with the worry of the "standardization of wines", using strains of yeast and bacteria which are not related to the specific wine-environment. Despite the lack of scientific evidence that support this opinion, nowadays there are several examples of wines produced by "spontaneous fermentations" recognized as "high-quality wines" from wine critics and consumers. Therefore, this argument deserves an in-depth study, based on a rigorous scientific method.

The key to obtaining high-quality wines using indigenous microorganisms is to lead a correct driving of microbial community succession during winemaking, acting on the technological variables (temperature, grapes crushing, duration of maceration, design of fermentation vats, etc.), and exploiting the changes in the environment induced by fermentation (alcohol accumulation, nutrient consumption, etc.) [1–4]. Yeast is the main microbial community involved in winemaking, and a large part of the wine character is

due at the balance of the activity of non-*Saccharomyces* and *Saccharomyces* spp. populations. Non-*Saccharomyces* yeasts, are usually found on the raw materials or during the early stages of winemaking; their sudden disappearance form the winemaking scenario is due to the drastic change of status induced by grape crushing, and at the increase of ethanol content of medium [5]. Nevertheless, the contribution of these yeasts on the wine profile is not negligible in terms of organoleptic active compounds produced, but also pose a potential risk for wine quality [6]. *Saccharomyces* yeasts begin to be dominant in the later stages of spontaneous fermentation due to their fermentative vigor and ethanol resistance. The largest part of the sensory qualities of wines depends on the activity of these yeasts, in terms of valorization of grape-native aromas and production of volatile molecules from sugars and aminoacidic metabolisms [7,8]. Lactic acid bacteria (LAB) have been poorly considered for a long time by the wine industry, relegating their activity to the malic acid consumption [9]. Today, their potentiality in the contribution in the organoleptic profile of wines is disclosed [9] and different approaches at malolactic fermentation are proposed [10]. In spontaneous fermentation, it is common to observe the presence of both lactic and acetic bacteria, already in the early stages of winemaking, due to the reduced use of SO_2 and other bacteriostatic compounds [11,12]. The coexistence of yeast and bacteria influences their metabolisms and, is believed to give wines unique features, representing potentially one of the main factors that differentiates wines made by spontaneous fermentation from those obtained by employing selected microbial cultures. Since knowledge about the role of microbiota in oenology is acknowledged, the comprehension of the bacterial and yeast population diversity and growth dynamics during a spontaneous fermentation process is extremely important to understand. The role of each species in the process is vital in order to recognize the early risk of wine spoilage or to allow the selection of autochthonous microorganisms to manufacture wines with territorially unique characteristics [13–15]. Despite the increasing interest observed in recent years among scientists for the investigation of microbiota, there are still several unresolved issues that deserve further study. The first studies on the microbial diversity and dynamics in wine fermentation, based on culture-dependent methods, appear today non-sufficient because they could give misleading information, altered by the selection power of the synthetic media used for microbial growth [16]. In recent years, high-throughput sequencing (HTS) culture-independent techniques have been rapidly developed and used to analyze DNA extracted from samples collected from vineyards, grapes, and during wine fermentations [17–19]. These studies are a gateway to a new level of comprehension of the microbial dynamic that occurs during winemaking, highlighting the identities and features of the microbial communities typical of each single winery. One of the factors that alter most of the profile of a winery's microbiota is the introduction in the last three decades of XX centuries of microbial starters, together with the purchase of grapes/wines outside the winery. These practices are widely used on the current oenological market and can partly explain how difficult it is to identify an original microbial profile of a single winery, with respect to other traditional food production plants.

In Italy, thanks to the ancient winemaking tradition, it is still possible to find wineries that have been producing wine for many decades exclusively using their own grapes and "archaic" oenological techniques. These wineries represent an interesting reservoir of biodiversity that deserves to be explored in depth. This study analyses the bacterial and fungi population diversity and dynamics during the spontaneous fermentation of Montepulciano and Trebbiano wines produced in the Abruzzi region of Italy, from a strongly traditional winery that maintains a preindustrial productive process and obtains highly recognized wines. For this purpose, samples collected at different stages of the spontaneous fermentation in the winery were analyzed using both culture-dependent and culture-independent (HTS) methods furnishing a comprehensive image of how the microbiota evolves through wine production.

2. Materials and Methods

2.1. Alcoholic Fermentation and Sampling

Grapes of Trebbiano (white grape) and Montepulciano (red grape) were harvested in 2018 in six vintage vineyards (two of Trebbiano and four of Montepulciano) located in Abruzzo region, a wine producing area of central Italy. All vineyards are more than 15 years old and were cultured by the Az. Ag. Emidio Pepe (Tornato Nuovo, TE, 42°49′47.2″ N, 13°46′32.5″ E), using the traditional "Tendone" training system, according to an organic protocol. Grapes were hand harvested and transported to the winery; no more than two hours occurred between harvesting and crushing. Trebbiano grapes were pressed with feet in traditional wooden vats with a 1,000 kg grape capacity, without removing the grape stalks. The must was collected in cement vats with a capacity of 500 L. $K_2S_2O_5$ salt (0.05 g/L, Enartis, I) was added into the must after crushing. Montepulciano grapes were manually de-stemmed, not crushed, and fermented in a cement vat with a capacity of 500 L. Grape skins were removed after 10 days of contact with fermenting grape must. In both cases malolactic fermentation took place in the same vats of alcoholic fermentation, the wines were racked for the first time after the complete degradation of the malic acid. Both fermentations were allowed to proceed without yeast inoculation and were considered finished when the must density was below 1000 g/L. Samplings of 50 mL were performed on day 1 (must just after crushing), 3, 6, 9, 15, and 30 (wine) during the fermentation. The samples were immediately analyzed for microbial counts, and then stored at −80 °C before DNA extraction. At each sampling point, traditional microbial cultivation and DNA extraction were performed for each sample.

2.2. Chemical Analysis of Must and Wine

The main measured chemical parameters of grape must and wine were: total sugars (TS), total acidity (TA, expressed as g/L of tartaric acid), pH, ethanol, acetic acid, malic acid, and yeast assimilable nitrogen (YAN) determined by OIV methods [20] or by an automatic analyzer based on the Fourier transform infrared spectroscopy using a WineScan FT120 interferometer (Foss Electric, Hillerød, Denmark). Sugar consumption during alcoholic fermentation was monitored daily by measuring the density (g/cm^3) of the fermenting grape must, by a manual glass densitometer (Polsinelli Enologia, Isola del Liri, FR, Italy).

2.3. Yeast and Bacteria Cultivation

All samples of grapes (fermenting grape, must, and wines) were subjected to microbiological determination, according to the OIV standards [21]. Each sample was serially diluted (1:10 to 1:10^6) in peptone water (1 g/L of mycological peptone) and spread-plated onto WL agar in triplicate cultured at 25 °C for four days in order to count total yeast (TY) and acetic acid bacteria. Non-*Saccharomyces* yeasts (NSY) were counted onto lysine agar medium after 4 days of incubation at 25 °C. Lactic acid bacteria (LAB) population was determined onto MRS agar medium, after two days of incubation at 30 °C under anaerobiosis conditions. All culture media and anaerobic systems were purchased from Oxoid (Thermo Fisher Scientific, Rodano, MI, Italy).

2.4. DNA Extraction

DNA extraction from samples was carried out by centrifugation of two mL of homogenized samples (Eppendorf 5804 centrifuge, Eppendorf, D) for 30 min. at 14,000 rpm, maintaining a temperature of 4 ± 2 °C; the obtained pellet was dissolved in 2 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Small amounts of PVP (Polyvinylpyrrolidone) and 1.1% of ß-mercaptoethanol were added and, after 15 sec. of mixing by Vortex (IKA, San Diego, CA, USA), the samples were incubated at 60 ± 1 °C for 60 min. to eliminate tannins and polyphenols residue from grapes. After incubation, each sample was centrifuged for 15 min at 14,000 g at 4 °C and the pellet was dissolved in a 300 µL TE buffer. All chemicals were purchased at Sigma Aldrich (MS). DNA extraction was then carried

out with the FastDNA Spin Kit for Soil (MP biomedicals, Irvine, CA, USA) following the manufacturer's instructions.

2.5. Miseq Library Preparation and Illumina Sequencing

Amplicon library preparation, quality, and quantification of pooled libraries were performed at the sequencing platform, Fondazione Edmund Mach (FEM, San Michele a/Adige, Italy). Briefly, for each sample, a 464-nucleotide sequence of the V3-V4 region [22,23], of the 16S rRNA gene (*Escherichia coli* positions 341 to 805) and ITS1F (5′- GTTTCCGTAGGTGAACCTGC -3′), and ITS4 (5′- TCCTCCGCTTATTGATATGC -3′) specific for the ITS1-5.8S yeast region [24] were amplified for bacteria and yeasts. Unique barcodes were attached before the forward primers to facilitate the pooling and subsequent differentiation of samples. To prevent preferential sequencing of the smaller amplicons, the amplicons were cleaned using the Agencourt AMPure kit (Beckman coulter) according to the manufacturer's instructions; subsequently, DNA concentrations of the amplicons were determined using the Quant-iT PicoGreen dsDNA kit (Invitrogen) following the manufacturer's instructions. In order to ensure the absence of primer dimers and to assay their purity, the generated amplicon libraries quality was evaluated by a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using the High Sensitivity DNA Kit (Agilent). Following the quantitation, cleaned amplicons were mixed and combined in equimolar ratios. Pair-end sequencing using the Illumina MiSeq system (Illumina, San Diego, CA, USA) was carried out at CIBIO (Center of Integrative Biology)–University of Trento (Trento, Italy).

2.6. Illumina Data Analysis and Sequences Identification by QIIME2

Raw paired-end FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp/blob/master/idemp.cpp, accessed on 29 November 2022) and imported into Quantitative Insights into Microbial Ecology (Qiime2, version 2018.2). Sequences were quality filtered, trimmed, de-noised, and merged using DADA2 [25]. Chimeric sequences were identified and removed via the consensus method in DADA2. Representative bacterial sequences were aligned with MAFFT and used for phylogenetic reconstruction in FastTree using plugins alignment and phylogeny [26,27]. Alpha and beta diversity metrics were calculated using the core-diversity plugin within QIIME2 and emperor [28]. For bacteria, taxonomic, and compositional analyses were conducted using plugins feature-classifier (https://github.com/qiime2/q2-feature-classifier.git, accessed on 29 November 2022). A pre-trained Naive Bayes classifier based on the Greengenes 13_8 99% Operational Taxonomic Units (OTUs) database, which had been previously trimmed to the V4 region of 16S rDNA bound by the 341F/805R primer pair, was applied to paired-end sequence reads to generate taxonomy tables. For fungi, sequences were classified to the species-level using a 97 or 99% threshold dynamic classifier created using UNITE software version 7.2 [29]. The data generated by MiSeq Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under Ac. Number PRJNA833930.

Statistical Analysis

A normality test (Shapiro-Wilk W) was performed, as well as a nonparametric test (Kruskal–Wallis) analyzing the day of collection as independent variables and the microbial plate counts as dependent variables. All the tests on plate counts were performed using the STATISTICA data analysis software system, version 9.1 (StatSoft, Inc., CA, Tulsa, OK, USA). Differences in diversity indices (OTUs number and Shannon diversity index) of different samples were tested by Kruskal- Walli's test by a plug-in implemented in QIIME2. The overall structural changes of the bacterial community were visualized by principal coordinates analysis (PCoA) based on both Weighted Unifrac and Bray-Curtis distance matrices. The statistical significance was assessed via the non-parametric PERMANOVA (permutational multivariate analysis of variance) by means of plug-in implemented in QIIME2.

3. Results and Discussion

3.1. Chemical Composition of the Grape Must and Microbial Evolution of Fermentations

Table 1 reports the main oenological parameters of grape musts involved in this work. According to the physiology of the two grape varieties employed and their need to maintain good balance between sugar content and acidity, grapes were harvested from the 10th of September to the 9th of October.

Table 1. Main chemical parameters of grape must and wine of Trebbiano and Montepulciano involved in this work. Total acidity is expressed as g/L of tartaric acid, YAN means the nitrogen promptly assimilable from yeast. N.d.: not detected.

Grape Must						
Grape variety	Total Sugars g/L	Total Acidity g/L	Malic acid g/L	pH	YAN mg/L	
Trebbiano	232 ± 7	5.9 ± 0.7	2.1 ± 0.3	3.5 ± 0.1	54 ± 1	
Montepulciano	232 ± 9	5.7 ± 0.6	1.7 ± 0.6	3.5 ± 0.1	56 ± 4	
Wine						
Grape variety	Total Sugars g/L	Total Acidity g/L	Malic acid g/L	pH	Acetic acid g/L	Ethanol (%)
Trebbiano	1.8 ± 0.8	4.8 ± 0.7	N.d.	3.8 ± 0.1	0.4 ± 0.1	13.8 ± 0.3
Montepulciano	2.4 ± 1.1	4.8 ± 0.5	N.d.	3.8 ± 0.2	0.6 ± 0.1	13.7 ± 0.6

The values of the main parameters of the obtained wines were similar, 13.8–13.7% of ethanol (*v*/*v*), 1.8–2.4 g/L of residual sugars, 4.8 g/L of total acidity, and 0.990–0.993 g/cm^3 of density (Figure 1) in Trebbiano and Montepulciano wines, respectively. These values were consistent with previous alcoholic fermentations of Trebbiano d'Abruzzo obtained through commercial starter fermentation [30], and Montepulciano d'Abruzzo obtained through autochthonous starter fermentation [31] proceeding without presenting stuck or sluggish fermentations. The Trebbiano fermentation was a little more in starting (Figure 1) and finished 5 days after the Montepulciano fermentation. The end of fermentation (density below 0.995 g/cm^3 in Figure 1) was reached on the 17th and 24th day for Montepulciano and Trebbiano, respectively.

The long time required to end the fermentation was probably due to the dominance of non-*Saccharomyces* in the yeast population during fermentation (Table 2 and Figure 2B) that are known to be slower than *Saccharomyces* cerevisiae in grape wine fermentation. Strains belonging to non-*Saccharomyces* can grow much better than *S. cerevisiae* in poor nitrogen environments [32] and this ability could confer these species a great competitive advantage for starting a fermentation process, in particular in our samples, whose yeast available nitrogen (YAN) was always very low (average value under 100 mg/L, Table 1). YAN of the grape must therefore, is a key factor for the evolution of the alcoholic fermentation and the development of the yeasts. Many factors can influence the YAN on grapes and, consequently, on must, such as environmental conditions and cultural practices [33]. On the other hand, it is noteworthy that despite these unfavorable conditions, the non-*Saccharomyces* yeasts make an efficient alcoholic fermentation. All wines resulted dry, with a sugars average content under 3.0 g/L (Table 1), suggesting that non-*Saccharomyces* yeasts could be useful in vinification as they are less demanding in terms of nutritional factors.

The decrease of temperature has probably prolonged the alcoholic fermentation time to 16 days for Trebbiano (final temperature of 21.7 °C), and 18 days for Montepulciano (final temperature of 24.0 °C). (Figure 1). The total sugar content decreased rapidly from 0 to 5 days, then gradually decreased. Replicated fermentation processes of both Trebbiano and Montepulciano musts were performed without yeast inoculation (Figure 1). Despite the absence of inoculum of selected yeast culture, the maximum fermentation rate was reached after 4 days in the Montepulciano fermentation V_{max} of 4.8×10^{-4} (g/cm^3)/h) and after 5 days in the Trebbiano fermentation (V_{max} of 3.8×10^{-4} (g/cm^3)/h).

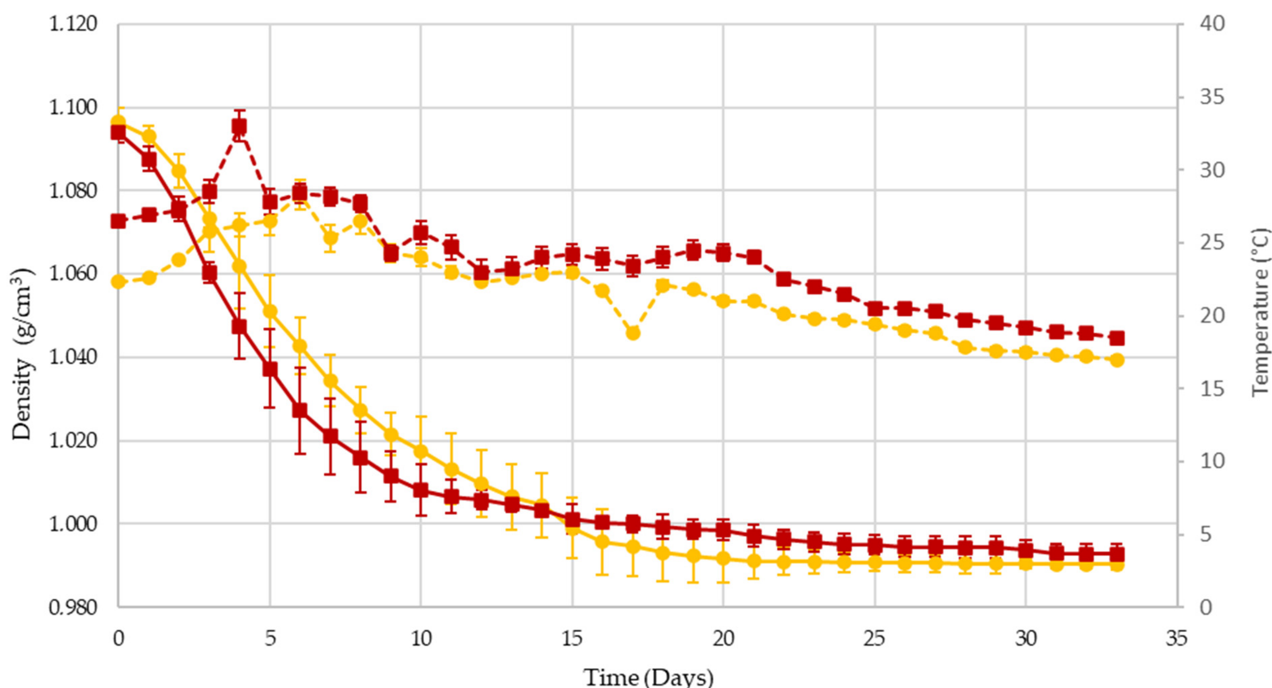

Figure 1. Evolution of spontaneous wine fermentations of Trebbiano (yellow dots) and Montepulciano (bordeaux squares) grapes measured as density (full line; g/cm^3) and temperature (dashed line; °C). Error bars indicated the standard deviation of the replicates ($n = 3$).

Table 2. Microbiological characteristics of samples collected in six different points of fermentation in two wines (Trebbiano and Montepulciano) of Abruzzo. Abbreviation: TY, Total Yeast; NSY, Non-*Saccharomyces* yeast; LAB, lactic acid bacteria, N.d., not detected. Plate counts are expressed as the mean ± SD ($n = 3$). For each column, microbial count, and values with a, b, and c superscripts are significantly different ($p < 0.05$).

Must	TY	NSY	LAB
		(Log CFU/g)	
Trebbiano			
Day 1	5.6 ± 0.6^{a}	5.0 ± 0.4^{a}	N.d.
Day 3	7.4 ± 0.5^{b}	6.5 ± 0.2^{ab}	N.d.
Day 6	7.7 ± 0.6^{b}	6.4 ± 0.6^{ab}	2.5 ± 0.3^{a}
Day 9	7.4 ± 0.8^{b}	6.3 ± 0.4^{ab}	5.8 ± 0.2^{b}
Day 15	7.5 ± 0.5^{b}	5.7 ± 0.2^{a}	6.5 ± 0.6^{b}
Day 30	4.9 ± 0.5^{a}	4.9 ± 0.6^{a}	4.6 ± 0.4^{c}
Montepulciano			
Day 1	7.0 ± 0.8^{ab}	7.1 ± 1.0^{a}	5.7 ± 0.1^{b}
Day 3	7.9 ± 0.2^{b}	6.8 ± 1.5^{a}	6.9 ± 0.5^{c}
Day 6	7.0 ± 0.5^{ab}	6.2 ± 2.0^{a}	5.8 ± 0.2^{b}
Day 9	6.6 ± 1.0^{a}	6.6 ± 1.0^{a}	4.5 ± 1.0^{a}
Day 15	7.3 ± 0.4^{ab}	6.3 ± 1.1^{a}	5.9 ± 0.5^{b}
Day 30	7.6 ± 0.3^{b}	6.9 ± 1.3^{a}	6.8 ± 0.5^{c}

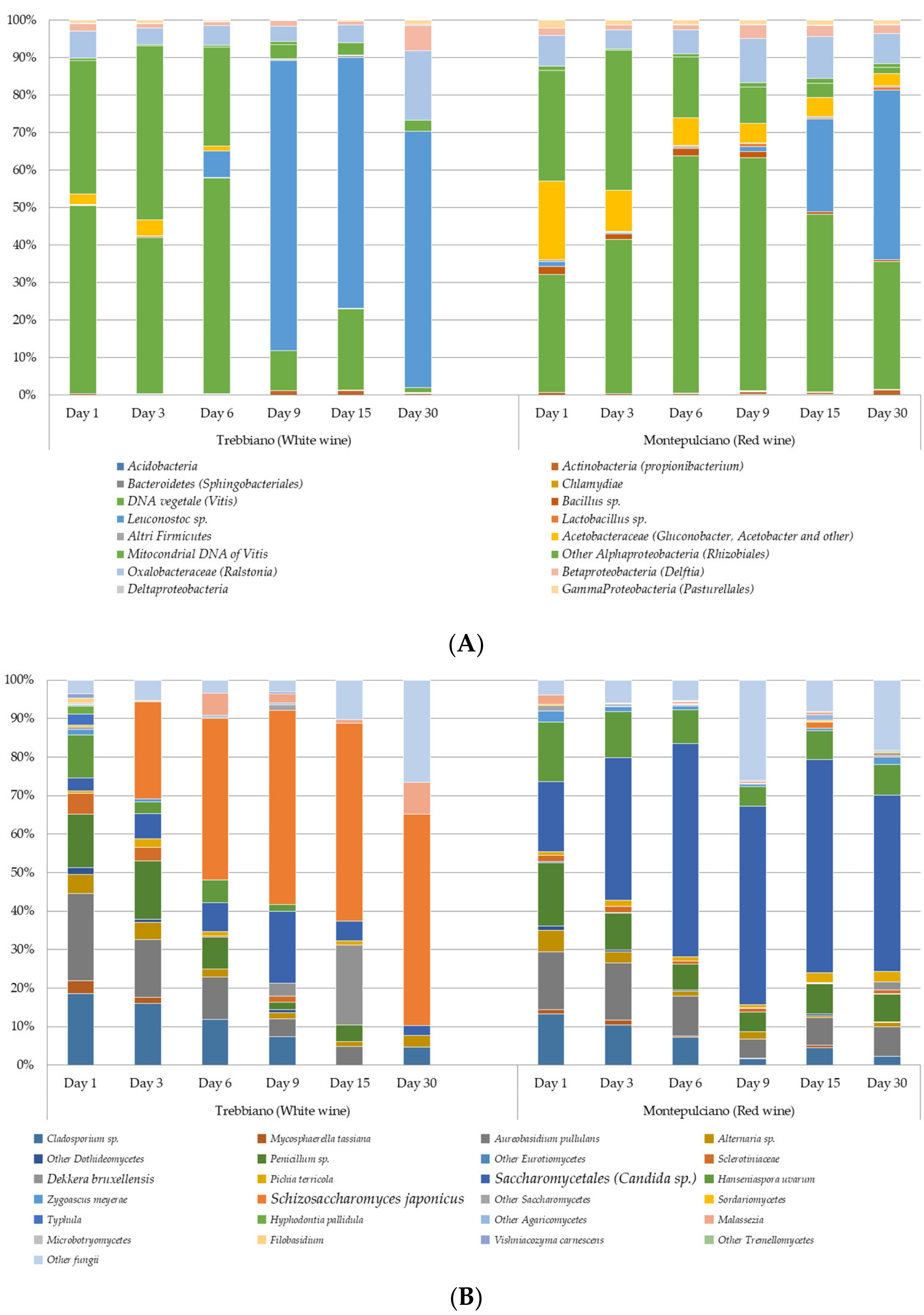

Figure 2. Main Taxa groups (species level or above) of bacterial (**A**) and fungi sequences (**B**) from different wines (Trebbiano and Montepulciano) using Illumina MiSeq. Each bar is a pool of two (Trebbiano) or four (Montepulciano) samples collected on six different days of the fermentation. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

Table 2 shows the microbial concentrations measured by plate counts during fermentation. One day before grape crushing the total yeast (TY) population was 5.6 $\pm$ 0.6 and 7.0 $\pm$ 0.8 Log CFU/g onto Trebbiano and Montepulciano grapes, respectively. TY

population reached the higher amount at D6 and D3 in Trebbiano and Montepulciano fermentation, respectively (Table 2), without a well-defined trend. The sub-population of non-*Saccharomyces* yeast (NSY) in Trebbiano, showed the same trend as TY, with the highest concentration reached at D3. In Montepulciano fermentation, NYS mean values were always in the range between 7.1 and 6.2 Log CFU/g without significant differences in time. In both Trebbiano and Montepulciano fermentations, NSY population was always over 5.0 Log CFU/g. This trend is peculiar and represents a clear difference between spontaneous fermentations and the ones inoculated with selected strains of *S. cerevisiae*. Figure 3 shows the correlation between the yeast's microbial counts and the fermentation rate observed in the six time points of sampling. Both for TY and NSY populations, an almost linear correlation was found, with R^2 of 0.9003 and 9.599 for TY and NSY, respectively. No correlation was found in the case of *Saccharomyces* yeast (R^2 = 0.2786, Figure 3). This evidence would indicate a poor relevance of *Saccharomyces cerevisiae* in driving the alcoholic fermentation in wines obtained from a spontaneous fermentation process.

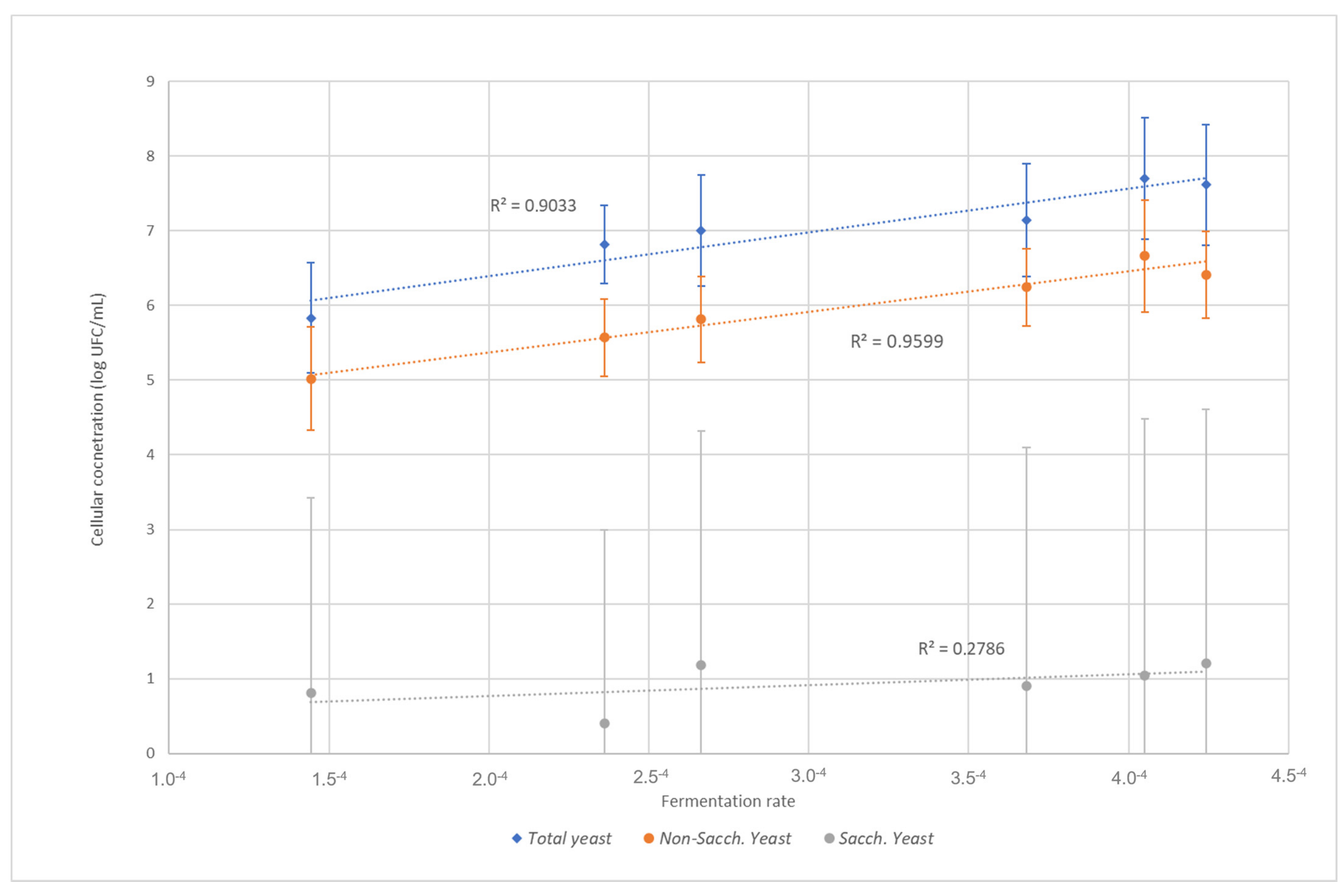

Figure 3. Correlation between the alcoholic fermentation rate and yeast cellular concentration in six points of observation (mean data, *n* = 6).

Lactic acid bacteria (LAB) are the third microbial group of oenological interest that was quantified during all winemaking processes considered in this work. The appearance of LAB was generally premature already before the half of the alcoholic fermentation (Table 2). In Trebbiano samples, LAB appeared at D6 with a concentration of 2.5 Log CFU/g, reaching 6.5 Log CFU/g at D15, a value sufficient to start malolactic fermentation. In Montepulciano samples, LAB resulted detectable at D1, with a concentration of 5.7 log CFU/g and increasing over the 6 Log units already at the D3. Acetic acid bacteria were not counted onto WL agar, probably for the inhibition exerted by the highly concentrated

yeast population. Their detection was determined by a non-cultural technique, as seen in the next paragraph.

We speculated that two main reasons could lead to the LAB early appearance in winemaking: the first one is the absence of grape crushing, a procedure commonly known as "carbonic maceration" that stimulates the growth of aerobic facultative microorganisms due to difficulties of aeration of the whole mass of grapes as already observed by the same authors [11]; the second one is the high pH (pH value starts from 3.50 in both winemaking, Table 1). Some authors [34] reported that when the pH was over 3.50, the low selective pressure exerted by acidity on microorganisms induced the LAB activation of sugars consumptions and the early occurrence of LAB in grape must.

3.2. Microbial Community Dynamic during Montepulciano and Trebbiano Fermentation

MiSeq Illumina was used to identify the succession of the microbial community during the Montepulciano and Trebbiano winemaking. From the 36 samples, the bacterial DNA was always successfully amplified and 1,406,119 reads from the V3–V4 regions of 16SrRNA gene were obtained; except for three Trebbiano and three Montepulciano samples, the yeast DNA was successfully amplified and 931,339 ITS reads were obtained.

Alpha diversity indices, including Observed OTUs number and Shannon diversity index were used to assess the richness and the diversity of microbial communities (Table 3).

Table 3. Observed OTUs (Obs. OTUs) and Shannon diversity index (Shannon div. ind.) for bacteria and yeast in different wines and at different days of the fermentation progression. Results are shown as mean ± SD. For a given wine and day of fermentation, bacteria and fungi Observed OTUs and Shannon diversity index values with ^{a} and ^{b} superscripts are significantly different ($p < 0.05$). When superscripts were not presented, no significant difference was found.

	Obs. OTUs	**Shannon div. ind.**
	Bacteria	
Wine		
Trebbiano	88 ± 50	5.6 ± 0.6
Montepulciano	85 ± 26	5.7 ± 0.6
Stage of Fermentation		
Day 1	93 ± 38	5.8 ± 0.6
Day 3	107 ± 63	5.9 ± 0.4
Day 6	81 ± 80	5.6 ± 0.2
Day 9	87 ± 90	5.6 ± 0.3
Day 15	69 ± 33	5.3 ± 0.9
Day 30	78 ± 31	5.6 ± 0.7
	Fungii	
Wine		
Trebbiano	23 ± 11	2.7 ± 1.2
Montepulciano	27 ± 12	3.0 ± 0.9
Stage of Fermentation		
Day 1	39 ± 12 ^{a}	4.0 ± 0.2 ^{a}
Day 3	29 ± 80 ^{a}	3.5 ± 0.4 ^{a}
Day 6	27 ± 8 ^{a}	2.9 ± 0.6 ^{a}
Day 9	21 ± 4 ^{ab}	2.6 ± 0.7 ^{ab}
Day 15	15 ± 4 ^{b}	2.0 ± 0.9 ^{b}
Day 30	12 ± 10 ^{b}	1.8 ± 0.8 ^{b}

Results showed no significant difference for both bacteria and yeast alpha diversity indices between Trebbiano and Montepulciano; on the contrary, the yeast microbial community showed a significant decreasing trend of diversity from Day 6 to the end of

fermentation. The distribution of the bacterial taxa with relative abundance above 0.1% is presented in Figure 2A. Thirteen taxa had been detected, and the composition of bacteria in the two fermentation methods was very similar without bacterial taxa to distinguish with their presence the two fermentation processes. In brief, the bacterial communities involved in both Trebbiano and Montepulciano fermentations consisted predominantly of *Cyanobacteria, Leuconostoc, Acetobacteraceae* and *Oxalobacteraceae*. Reads belonging to *Cyanobacteria* phylum are probably coming from the amplification and sequencing of *V. vinifera* chloroplasts that originated from *Cyanobacteria* [35]. Most of these were classified as having an "absent/unknown effect" on wine fermentation [36]. The relative abundance values of *Acetobacteraceae* decreased from 2.7% to 0% and from 20.9% to 3.1% during Trebbiano and Montepulciano fermentations, respectively. This result was not surprising because some *Acetobacteraceae* genera belonging to grapes, for example, *Gluconobacter,* have been described as poorly resistant to alcohol and, thus, normally declining during alcoholic fermentation [37,38]. The relative abundance values of *Leuconostoc* were very high at the end of Trebbiano and Montepulciano fermentations and increased from 0 to 68.3% and from 1.3% to 45.1%, respectively, which was expected, as they have been long related with wine fermentations [39]. In particular, *Leuconostoc* species were defined as core microorganisms in spontaneous fermentations and were closely related to a variety of volatile compounds, suggesting that *Leuconostoc* indigenous species can contribute to the formation and increase of the aroma complexity of wine [40]. *Oxalobacteraceae* presence was constant along the fermentation showing a slight increasing trend in Trebbiano (from 7.1 to 18.5%) and no difference in Montepulciano fermentations. *Oxalobacteraceae* family was already detected during alcoholic fermentation and its presence is mostly considered environmental, and not related with the oenological process [41]. We could detect other minor bacterial taxonomic groups during the fermentations (Figure 2A), some of them, such as *Actinobacteria* and *Bacteroidetes* phyla, were detected until the end of the alcoholic fermentation although their role remains unknown [18].

The distribution of the fungi taxa with a relative abundance above 0.1% is presented in Figure 2B. Nineteen taxa had been detected, and the yeast composition in the two fermentation methods was similar. Only two taxa were able to distinguish the Trebbiano from Montepulciano fermentation: *Typhula* and *Hyphodontia pallidula*, which were present only during Trebbiano fermentation at Day 1 (2.9 and 2.1%, respectively) and never detected in Montepulciano. The predominant fungi taxa were *Cladosporium, Aureobasidium pullulans* and *Penicillium* that decreased during the alcoholic fermentation.

Both *Cladosporium* and *Aureobasidium pullulans* are the most abundant species in grape surface, fresh grape juice, and the very early stages of fermentation [42]. *Aureobasidium pullulans* was reported to produce a great diversity of extracellular enzymes, such as pectinases, cellulases, xylanases, glycosidases, and protease [43–45].

The eukaryotic taxa dominating throughout fermentation were *Candida* in Montepulciano, *Schizosaccharomyces japonicus* in Trebbiano, and *Hanseniaspora uvarum* in both fermentations. The change in the relative abundance of *Schizosaccharomyces japonicus* and *Candida* was very similar in both fermentation processes: they increased in the first six days and then stabilized until the end of fermentation constituting about half of the detected fungi. The relative abundance of *Hanseniaspora uvarum* decreased from 11.2–15.6% on Day 1 to 0–8.0% at the end of the Trebbiano and Montepulciano fermentations, respectively (Day 30 in Figure 2B). *Candida* and *Hanseniaspora* genera have been frequently isolated on spontaneous fermentations [46] and these results agreed with previous studies on spontaneous fermentations, describing the abundance of *Hanseniaspora* at the beginning of the fermentation and *Candida* until the end of the alcoholic fermentation [47].

To the best of our knowledge, this is the first time *Schizosaccharomyces japonicus* is found to lead a spontaneous grape wine fermentation. This species has been already described to be isolated from wines showing high levels of acetic acid, sulfidric acid, acetaldehyde, acetoin, and ethyl acetate, but it was never found as dominating the fermentation. Its

presence was related to hot climate regions and showed a special ability to develop in the presence of high temperature (up to 37 °C), sugar content, and osmotic pressure [48].

3.3. β-Diversity of the Bacterial and Fungal Community

The permanova statistical analyses (Table 4) indicated that the differences between Trebbiano and Montepulciano wines were significant only for fungi communities (*p*-value of 0.086 and 0.001 for bacteria and fungi communities, respectively); this result suggests a certain homogeneity of the wines' fermentation in the bacterial pool but strong effects due to the fungi community.

Table 4. Permanova analysis (999 permutations) results for bacteria and fungi communities based on Weighted Unifrac and Bray-Curtis distances, respectively. Abbreviation D: day. Significance levels: * $p < 0.05$; ** $p < 0.01$.

Main Effects	**Pseudo-*F***	***p*-Value**
Bacteria		
Wine	2.815	0.086
Fermentation day	6.374	0.001 **
Pairwise Comparisons for Fermentation Day	Pseudo-*F*	*p*-Value
D1 vs. D3	1.182	0.325
D1 vs. D6	8.295	0.003 **
D1 vs. D9	4.756	0.004 **
D1 vs. D15	6.684	0.003 **
D1 vs. D30	16.570	0.004 **
D3 vs. D6	17.302	0.001 **
D3 vs. D9	7.030	0.003 **
D3 vs. D15	9.373	0.002 **
D3 vs. D30	23.741	0.003 **
D6 vs. D9	2.999	0.061
D6 vs. D15	5.523	0.020 *
D6 vs. D30	17.341	0.007 **
D9 vs. D15	0.311	0.556
D9 vs. D30	2.207	0.181
D15 vs. D30	0.742	0.423
Fungii		
Wine	12.875	0.001 **
Fermentation day	1.659	0.072
Pairwise Comparisons for Fermentation Day	Pseudo-F	*p*-Value
D1 vs. D3	1.085	0.360
D1 vs. D6	3.195	0.010 **
D1 vs. D9	4.865	0.004 **
D1 vs. D15	3.423	0.015 *
D1 vs. D30	3.360	0.015 *
D3 vs. D6	1.222	0.371
D3 vs. D9	2.247	0.080
D3 vs. D15	1.800	0.112
D3 vs. D30	1.892	0.082
D6 vs. D9	0.340	0.659
D6 vs. D15	0.437	0.527
D6 vs. D30	0.674	0.489
D9 vs. D15	0.334	0.717
D9 vs. D30	0.425	0.728
D15 vs. D30	0.712	0.251

The fungi Bray-Curtis PCoA (Figure 4B, total variation explained: 76.45%) was confirming this result showing differences between the two wines Trebbiano (squares) and

Montepulciano (circles) whatever the day of sampling was; probably this difference is due to the clear presence of different yeast species leading the fermentation in all the stages: *Schizosaccharomyces japonicus* and *Candida* in Trebbiano and Montepulciano, respectively (Figure 2B). This result was also in agreement with TY and NSY counts that in both wines were showing no peculiar trend related with the time of sampling.

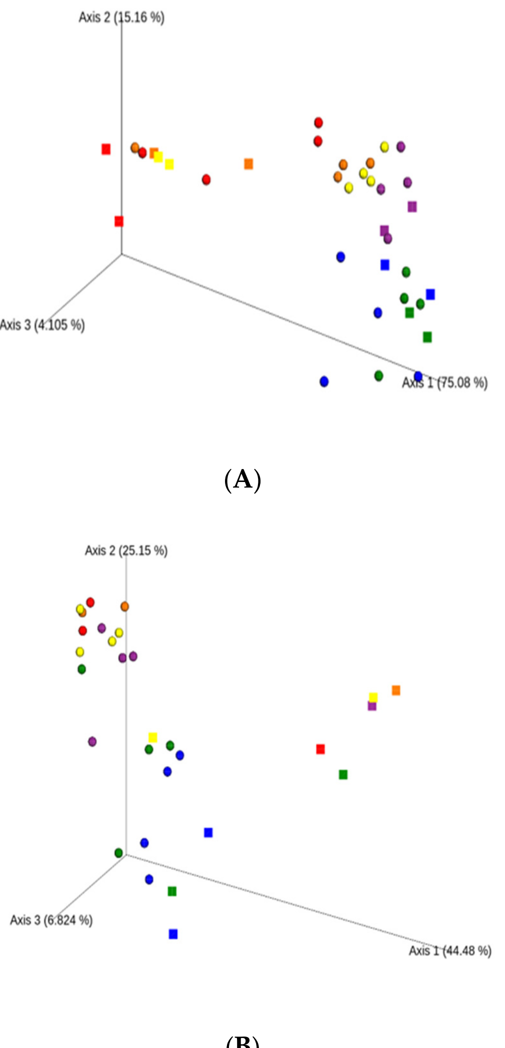

Figure 4. Principal coordinate analysis of Weighted UniFrac distances for bacterial community (**A**) and Bray-Curtis distances for fungi community (**B**) in the fermentation processes. Symbols represent the wine: Trebbiano (squares) and Montepulciano (circles). Different colors represent the different sampling time: Day 1 (green). Day 3 (blue). Day 6 (purple). Day 9 (yellow). Day 15 (orange) and the end of fermentation Day 30 (red). For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

By contrast, bacterial communities were phylogenetically dissimilar between successional phases (D1, D3, D6, D9, D15 and D30). Permanova showed that differences between microbial communities in time were significantly different (p-value = 0.001) only for bacteria (Table 4), and not for fungi community and a distinct succession of bacterial communities occurred during the fermentation processes (Figure 4A). Comparing each other, we could recognize three significantly different stages in bacteria development during the process: D1 and D3 ($p < 0.01$ with all the other time points), D6 and stage D9 until the end. The bacterial Weighted UniFrac PCoA (Figure 4A, total variation explained: 94.35%) confirmed this picture, emphasizing the similarities of the bacterial communities

in three fermentation stages: D1-D3 (green and blue, respectively), D6 (purple) and from D9 until the end (yellow, orange, and red, respectively). It is notable that although the succession of bacterial communities occurred with differences in the days of fermentation, the overall bacterial communities were similar whatever the wine of sampling was (circles and squares). This suggests that there is a similar bacterial phylogenetic turnover pattern in both Trebbiano and Montepulciano processes, alongside a comparable progression. In fact, in both fermentations, *Leuconostoc* species were found in high percentage (Figure 2A) and its presence was more closely related to the time of sampling than to wine.

4. Conclusions

The traditional microbial cultivation method and both culture-dependent and HTS methods were used to study the yeast and bacteria population diversity and dynamics during the spontaneous fermentation of Trebbiano and Montepulciano. The persistence of *Acetobacteraceae* species during Montepulciano alcoholic fermentations suggests that the acetic acid bacteria population is present/active during the spontaneous Montepulciano wine fermentation and are even more abundant than previously believed. We also found that the rising of *Leuconostoc* species during both the Trebbiano and the Montepulciano fermentation is in agreement with previous HTS studies on spontaneous fermentations. Non-*Saccharomyces* yeasts were always co-dominant in all the stages of fermentation, in particular *Schizosaccharomyces japonicus* and *Candida* that imposed themselves after three days in the Trebbiano and Montepulciano fermentation, and accomplished it successfully. Furthermore, the dominance of *Schizosaccharomyces japonicus* is different to what previous HTS studies have described for spontaneous grape wine fermentation. The results of the HTS analysis were more complex and data-rich than those of the culture-dependent method.

The combination of culture-dependent methods and HTS approaches is useful for estimating microbial diversity and population dynamics. Our data suggest that HTS techniques are useful for assessing microbial changes in wine fermentations of different grape varieties or under different treatments and this information could be used by winemakers to drive accurate fermentation processes and to set up the most suitable environmental conditions to enhance wine characteristics.

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