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Yeast Biodiversity in Vineyard during Grape Ripening: Comparison between Culture Dependent and NGS Analysis

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Abstract: In this study, the evolution of the yeast microflora present on the berry surface, during the ripening of Barbera grapes, was monitored. Sampling was performed in three vineyards located in the "Nizza" Barbera d'Asti DOC zone and different methodologies have been employed. A culture-dependent method based on the identification of strains grown on solid media by ARDRA (Amplified Ribosomal DNA Restriction Analysis) and the D1-D2 domain of ribosomal 26S DNA capillary sequencing was coupled to NGS (Next Generation Sequencing) targeting ITS (Internal Transcribed Sequence) amplicons with the Illumina MiSeq platform. By using culture-dependent techniques, the most frequently detected species was the yeast-like fungus *Aureobasidium pullulans*, which was dominant in the culturable fraction. Among yeasts, the presence of oligotrophic basidiomycetes such as *Cryptococcus* spp., *Rhodotorula graminis* and *Sporidiobolus pararoseus* was observed at the beginning of ripening. Afterward, upon approaching the harvest, a succession of oxidative or weakly fermentative copiotrophic species occurs, such as *Saturnispora diversa*, *Issatchenkia terricola*, *Hanseniaspora opuntiae*, *Starmerella bacillaris* and *Hanseniaspora uvarum*. The massive sequencing revealed a larger number of species, respect to the culture-dependent data. Comparing the two different approaches used in this work, it is possible to highlight some similarities since *Aureobasidium*, *Rhodotorula* and *Sporobolomyces* were detected by both methods. On the contrary, genera *Hanseniaspora*, *Issatchenkia* and *Saturnispora* were revealed by culture-dependent methods, but not by NGS, while *Saccharomyces* spp. were identified, with low frequency, only by NGS. The integrated application of NGS sequencing and culture-dependent techniques provides a comprehensive view of mycodiversity in the wine-growing environment, especially for yeasts with low abundance.

Keywords: grape berries; fungi; mycobiota; "Nizza" Barbera; ripening

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1. Introduction

Wine is a product strictly related to the production region. The idea of *terroir* is intended as a set of climatic, pedological and anthropic viticultural factors influencing the characteristics of wine in a typical and unrepeatable way. This concept has been defined as pseudo-scientific ("nebulous" according to Bokulich) [1], but, in recent years, new analytical techniques have revealed the actual territorial differences in even relatively close production areas. The microbiological aspect, usually underestimated when talking about *terroir*, has revealed all its importance thanks to the new high throughput sequencing (HTS) techniques, allowing differences between the viticultural areas in terms of microbial biodiversity to be identified, not related to chance, but typical and characteristics of those areas [1–3]. The microbiological characteristics that are present in the fermenting

must and, consequently, in wine are derived from the microbial population on the grape surface and from the microorganisms present in the soil, which ultimately represents its reservoir [4]. Studies based on culture-dependent techniques, resumed in the review of Barata [5] have revealed that the yeast population on berries is substantially divided into three groups. The first group is represented by oligotrophs, usually Basidiomycetes yeasts with oxidative metabolism, including *Cryptococcus* spp. and *Rhodotorula* spp. that dominate the nutrient-poor grape surfaces. The group is made up of ubiquitous species favored by nutrient-poor environments such as healthy berries. In addition to the aforementioned Basidiomycetes, the yeast-like dimorphic Ascomycete fungus *Aureobasidium pullulans* is very widespread. The second group includes copyotrophs, ascomycete yeasts with oxidative or weakly fermentative metabolism, mainly represented by *Metschnikowia pulcherrima* and by some species of the genus *Candida*, *Hanseniaspora* and *Pichia*. While Basidiomycetes are the predominant yeasts on healthy bunches, *Candida zemplinina* and *Hanseniaspora uvarum* dominate on damaged berries. In general, the apiculate *H. uvarum/Kloeckera apiculata* is the most common species on grape bunches all over the world and the predominant at the beginning of the spontaneous fermentation of the must. The third group involves copyotrophs, strongly fermenting yeasts found on damaged grapes, such as *Zygosaccharomyces*, *Candida* and *Torulaspora*. The proliferation of these yeasts is explained by the high availability of nutrients that results in damaged grapes. Basidiomycetes are the minority in this phase due to the proliferation of Ascomycetes species such as *Pichia* spp., *Zygosaccharomyces* spp., *Zygoascus* spp. and *Torulaspora* spp.

The balance between these groups depends on the availability of nutrients on the grape surface. According to Pretorius [6], the main species on berries are the apiculate yeasts, *Kloeckera* and *Hanseniaspora*, while numerically less significant are *Candida*, *Brettanomyces*, *Cryptococcus*, *Kluyveromyces*, *Pichia* and *Rhodotorula. Saccharomyces* spp., which is dominant during fermentation, is rarely found on grapes [7].

Since the culture-dependent techniques are not able to spot about 90% of the microbial species, they, although useful, have the disadvantage of being limited by the cultivability of the microorganisms [8,9]. The development of alternative techniques such as DGGE (Denaturing Gradient Gel Electrophoresis) and qPCR partially solved this problem and are useful for identifying and counting single microorganisms, but they cannot provide general information on the entire microbial population present in a matrix because of their low sensitivity or specificity.

NGS techniques allow the qualitative–quantitative study of the whole microbial population present in a matrix and have been recently applied in a wide variety of studies concerning different aspects of the viticultural–oenological chain ranging from microbial diversity to vineyard, microbial diversity in fermentation, microbial zoning interactions, and the influence of viticultural practices on microbiota [1,2,10–12]. Despite numerous studies conducted on populations under different conditions, there is a lack of information on what happens during the annual cycle of the vine, in particular during ripening. In general, studies based on culture-dependent techniques highlighted that during ripening, the oligotrophic species were replaced with fermentative copiotrophic species, which become dominant due to a greater presence of nutrients deriving from a micro-cracking that occurs on the berry surface and to the presence of volatile organic compounds [13]. Despite this information, there are a lack of data regarding the evolution of the entire mycobiota, including non-cultivable or rare species, which can only be detected with NGS techniques.

This study was aimed to investigate the evolution of the fungal microbial population during the last phase of maturation with culture-dependent techniques and high throughput sequencing methodologies, on Barbera grapes belonging to the Nizza appellation, one of the finest in Piedmont.

2. Materials and Methods

2.1. Samples Collection and Recovery

The study was conducted on three Barbera vineyards located in Nizza Monferrato (Italy), namely, Mastrandrea (MAS), Gaffoglio (GAF) and Amandola (AMA) Figure 1.

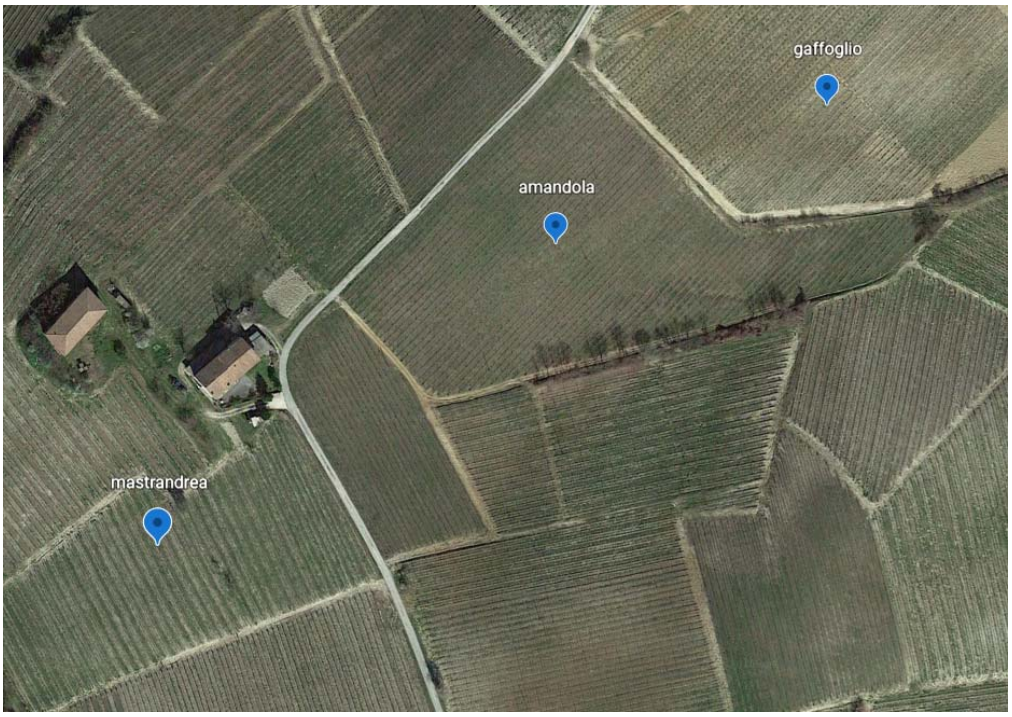

Figure 1. Geolocalization of the vineyards: Mastrandea: 44°47′1.58″ N 8°19′6.55″ E, Gaffoglio 44°47′7.67″ N 8°19′17.66″ E, Amandola: 44°47′5.68″ N 8°19′13.35″ E.

The vineyards were homogeneous in terms of exposure, altitude, pesticide treatments, and with sound grapes. Vineyards were conventionally managed, and the last treatment was done in July. Samples from each vineyard were drawn three times during September 2015 (1 September, 10 September and 21 September), which corresponded to an average °Brix of 22.8, 24 and 26, respectively.

Nine bunches from each vineyard were taken during maturation from the same vines.

To isolate yeasts from grape skin, 27 grape berries, three for each bunch, at the top, in the middle and at the bottom were randomly picked from each sampling, put in a sterile flask with 200 mL of sterile physiologic solution and incubated at 25 °C overnight on a shaker. These washing solutions were pelleted at 5000 rpm for 15 min, and then pellets were divided into two aliquots: one was re-suspended in 3 mL of new physiologic solution and immediately used for isolation with laboratory media; the second was washed twice with Ultrapure water and stored at −20 °C for NGS analysis.

2.2. Specie Identification by Culture-Dependent Methodology

2.2.1. Isolation in Pure Culture

WL (Wallerstein Laboratory) (MERCK, Milan, Italy) agar was used to isolate yeasts from grape skin. To these were added 100 mg/L of ampicillin sodium salt and 400 mg/L of biphenyl (Sigma-Aldrich, Milan, Italy), to prevent the growth of bacteria and molds, respectively. Suspensions were diluted and spread on WL, and the plates were incubated at 25 °C for eight days. Considering plates with 100–200 colonies, single colonies showing different morphologies were sampled, and grown on WL agar for eight days at 25 °C. At least two colonies belonging to different morphologies were isolated. In addition, colonies showing unique morphologies were also isolated. Isolates were grown in YEPG (Yeast Extract 1%, Peptone 1% and Glucose 2%) medium for 8 days at 25 °C and analyzed.

2.2.2. ARDRA Analysis

DNA was extracted according to [14] and amplified using the primers ITS1 and ITS4 as previously described [15]. *Cfo*I, *Hae*III and *Hinf*I were used as restriction enzymes. Data obtained were analyzed with Bionumerics software (Applied Maths, Belgium). Clustering was performed using the option "average of experiments", using ITS amplicons and restriction fragment patterns. Dendrograms were built with the UPGMA method considering 90% of similarity as a grouping cut-off. A cophenetic correlation was applied to determine reliable and unreliable clusters as described by [16].

2.2.3. 26S Sequencing

After grouping, one sample per group was sequenced using the D1-D2 domain as a target, for yeast unequivocal species identification [17].

The PCR products were run by electrophoresis at 80 V for 60 min on a 1.2% (*w*/*v*) agarose gel. Amplicons were purified using Illustra CFX kit (GE Healthcare, Little Chalfont, UK). Sequencing was performed in both directions with primers NL1-NL4 [17] using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Monza, Italy) following the manufacturer's instructions. Products were purified with Illustra Autoseq Kit (GE Healthcare), denatured in formamide, and finally analyzed with an ABI 310 Genetic Analyzer (Applied Biosystem).

Sequences were compared by Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 10 September 2017) and confirmed by alignment with type strain sequences downloaded from the CBS database (http://www.westerdijkinstitute.nl/Collections/Biolomics.aspx?Table=Yeasts%202011, accessed on 10 September 2017) or, when possible, with D1-D2 sequence of the type strains conserved in CREA-VE (Centro di Ricerca Viticoltura ed Enologia) culture collection. All the identified microorganisms were stored at −80 °C.

2.3. NGS Sequencing

Genomic DNA was extracted from each sample using the PowerSoil® DNA Isolation Kit according to the manufacturer's instructions (MO BIO Laboratories, Inc., Carlsbad, CA, USA).

Fungal ITS1 amplification was performed with primers ITS1F_KYO2 and ITS2_KYO2 (amplicon length about 350 bp) [18]. PCR reactions containing about 5 ng of genomic DNA were performed in a total volume of 20 µL (1× KAPA HiFi HotStart ReadyMix, 2.5 mM $MgCl_2$ and 0.3 µM of each primer) in a Bio-Rad thermocycler (CX1000, Bio-Rad, Italy).

PCR products were purified using the Agencourt® AMPure® Kit (Beckman Coulter, CA, USA), quantified using QuantiT PicoGreen® kit (Thermo Scientific, Wilmington, DE, USA) and pooled for sequencing on an Illumina MiSeq platform using the MiSeq reagent kit v2 (500 cycle—2 × 250 bp) to obtain about 70,000 reads for each sample.

Sequencing raw data were processed using a custom bioinformatics pipeline. The taxonomic assignment was performed using the UNITE fungal ITS database (release 7.2) [19] (min. coverage for species identification ≥ 97%).

All raw sequence data in read-pairs format were deposited at the National Centre for Biotechnology Information (NCBI) in Sequence Read Archive (SRA) under BioProject: PRJNA803359.

2.4. Statistical Analysis

Descriptive statistical analysis was conducted with RAM package of R statistical software to obtain: (i) biodiversity indices (Shannon–Wiener Index, Simpson Index, Observed species) and (ii) Beta diversity graph.

3. Results

3.1. Climatic Data

The period before the harvest was warm with a maximum temperature in July of 39 °C, in August 40.1 °C and in September 31.3 °C. August 2015 was the 12th warmest month,

from 1958 to today, with a temperature of about 1 °C higher than the climatic average of the period 1971–2000.

The humidity was constantly very high throughout the year. Rainfall was abundant in March and August with values above 100 mm. In particular, August recorded a precipitating surplus of around 54%, in 6th place among the wettest months of August in the last 58 years. (Source: Arpa.)

3.2. Species Identification and Abundance

After isolation of different morphologies found in all samples, 53 colonies were maintained in pure culture. Because the colonies were isolated on a medium added with ampicillin and biphenyl in the growing media, the identification of bacteria and molds was precluded. These cultures were characterized by ARDRA and clustered. Then, one representative for the group was further analyzed and the domain D1-D2 or ITS1-ITS4 was sequenced. Table 1 shows the frequencies of species found in each vineyard.

Table 1. Relative abundance of species during maturation obtained by plating on WL agar.

Vineyard	Sample	Sampling Time	Species	Frequency of Isolation (%)
Amandola	1	1 September	*Aureobasidium pullulans*	52.6
			Sporidiobolomyces roseus	32.5
			Rhodotorula graminis	14.9
	2	10 September	*Rhodotorula graminis*	55.6
			Aureobasidium pullulans	44.4
	3	20 September	*Aureobasidium pullulans*	92.6
			Candida diversa	4.1
			Issatchenkia terricola	2.2
			Hanseniaspora opuntiae	1.1
Mastrandrea	4	1 September	*Aureobasidium pullulans*	62.5
			Rhodotorula graminis	16.3
			Cryptococcus flavescens	12.8
			Sporidiobolomyces roseus	8.4
	5	10 September	*Aureobasidium pullulans*	100.0
	6	20 September	*Aureobasidium pullulans*	100.0
Gaffoglio	7	1 September	*Aureobasidium pullulans*	56.7
			Rhodotorula graminis	19.3
			Cryptococcus carnescens	12.7
			Hanseniaspora opuntiae	11.3
	8	10 September	*Aureobasidium pullulans*	53.2
			Starmerella bacillaris	21.3
			Hanseniaspora opuntiae	12.8
			Issatchenkia terricola	12.8
	9	20 September	*Hanseniaspora opuntiae*	92.9
			Hanseniaspora uvarum	7.1

In Amandola's vineyard, the population found was 6.04×10^3 CFU/g of grape in the first sampling time, the species identified were: *Aureobasidium pullulans* with 52.6% frequency, *Sporidiobolus pararoseus* with 32.5% and *Rhodotorula graminis* with 15%. In the second sampling time, CFU was 1.44×10^3 CFU/g of grape, and the species composition was 55.6% *Rhodotorula graminis* and 44.4% *Aureobasidium pullulans.* Finally, the species found in the third sampling were 92.6% *Aureobasidium pullulans*, 4.1% *Saturnispora diversa*, 2.2% *Issatchenkia terricola* and 1.1% *Hanseniaspora opuntiae*, in this last sample the population was 5.4×10^3 CFU/g of grape.

In Mastrandrea's vineyard, the first sampling time included *Aureobasidium pullulans*, *Cryptococcus flavescens*, *Sporidiobolus pararoseus* and *Rhodotorula graminis* (62.5%, 12.8%, 8.4%

and 16.3%, respectively). The total cultivable count was 3×10^3 CFU/g of grape. The CFU at the second sampling time was 9.4×10^3 CFU/g of grape and the only species identified was *Aureobasidium pullulans*; this species was also uniquely found at the third sampling time where the plate count resulted in 1.36×10^4 CFU/g of grape.

In Gaffoglio, seven species were identified: at the first time of sampling, having a total population of 6.4×10^3 CFU/g of grape, *Aureobasidium pullulans*, *Rhodotorula graminis*, *Cryptococcus carnescens* and *Hanseniaspora uvarum* were found. At the second one (total CFU/g of grape 1.12×10^3) *Aureobasidium pullulans*, *Starmerella bacillaris*, *Issatchenkia terricola* and *Hanseniaspora opuntiae* were identified. In the third, *Hanseniaspora opuntiae* and *Hanseniaspora uvarum* were present and the total count resulted in 1.0×10^4 CFU/g of grape.

The identified species and their respective frequencies in the three samplings of the Gaffoglio's vineyard can be seen in Table 1. *Aureobasidium pullulans* was identified in the first two samples only, with a respective frequency of 56.7%, and 53.2%. *Rhodotorula graminis* was present in the first sampling time with a frequency of 19.3%. *Hanseniaspora opuntiae* was found in all samples, with a frequency of 11.3% in the first, 12. 8% in the second and 92. 9% in the third. *Cryptococcus carnescens* was detected only at the first sampling time with a frequency of 12.7%. *Issatchenkia terricola* appears at the second sampling time, with a frequency of 12.8%. *Starmerella bacillaris* was identified in the second sampling with a frequency of 21.3%. Finally, *Hanseniaspora uvarum* was found only in the third sample with a frequency of 7.1%.

Biodiversity indexes calculated on these data are shown in Table 2. The Shannon diversity index, changed among samples ranging from 0 to 1.19, evidencing how yeast diversity changes in the same vineyard during the last phase of maturation and also among vineyards. A similar trend was observed for the Simpson index. Data also highlighted a tendency of reducing richness, expressed as the Margalef index, as a function of the sampling time, except in Amandola.

Table 2. Microbial diversity index calculated on species isolated on WL agar plates.

Vineyard	Sample No.	Simpson	Shannon	Margalef
Amandola	1	0.6	0.99	0.35
	2	0.5	0.69	0.23
	3	0.14	0.34	0.54
Mastrandrea	4	0.56	1.06	0.52
	5	0	0	0
	6	0	0	0
Gaffoglio	7	0.62	1.15	0.59
	8	0.64	1.19	0.49
	9	0.13	0.26	0.24

3.3. NGS Analysis

The fungal communities associated with grapevine were investigated twenty days before harvest, ten days before and at the harvest time. The number of used reads ranged from 41,822 to 116,524. Overall, the fungal populations at a *phylum* level were very similar and mainly comprised Ascomycota (62.7%) followed by Basidiomycota [3,20,21]. *Phyla* as *Mucoromicota* and *Chytridiomycota* had an extremely low abundance.

NGS sequencing showed a large predominance of filamentous fungi with *Mycosphaerella* spp. as prevalent species in all samples (Figures 2–4), in particular, in samples 1, 2, 3, 4, 5, and 6 (Amandola Figure 2 and Mastrandrea Figure 3, respectively); although, in Gaffoglio (Figure 4) its percentage was lower. In general, the number of reads corresponding to *Mycospherella* decreased in proximity to the harvest time. Taking into account their frequencies, this genus fungus was followed by *Stemphylium*, *Pseudopithomyces*, *Ramularia*, *Cladosporium* and *Chalastospora*. While *Stemphylium* decreased at the third sampling time in all the three vineyards, the occurrence of the other species did not follow a specific trend.

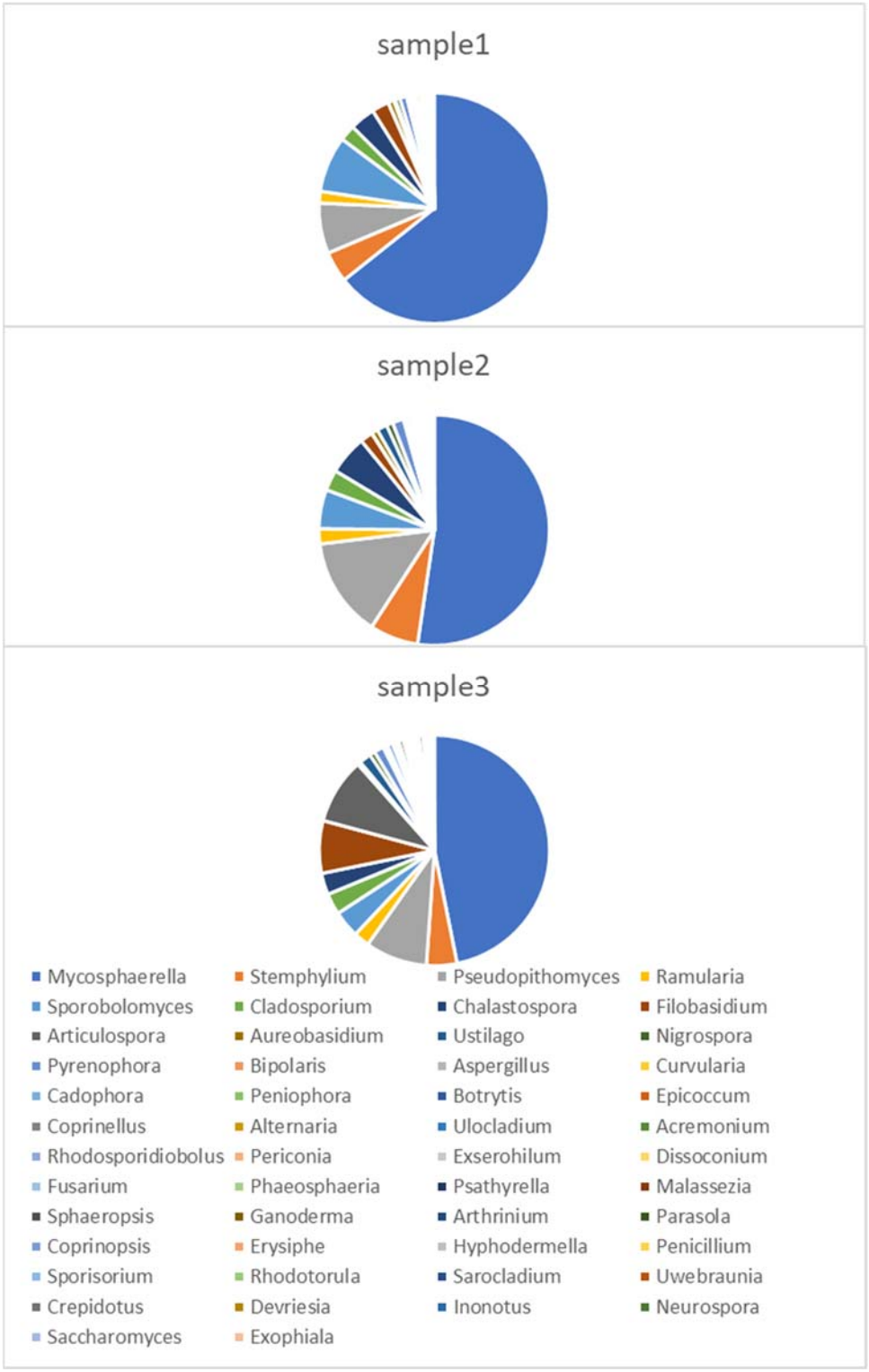

Figure 2. Relative abundance of the main fungi obtained by NGS analysis in Amandola.

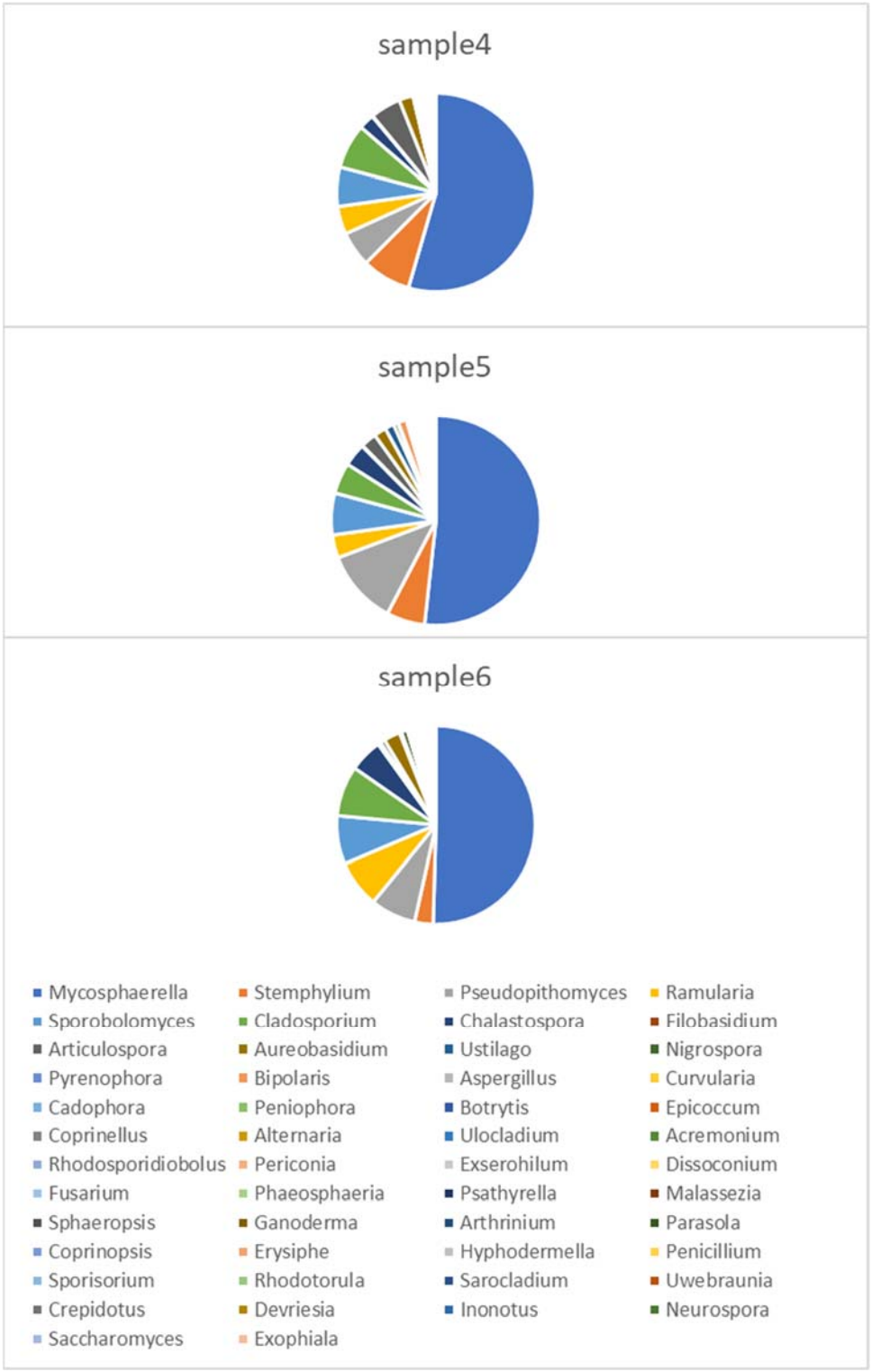

Figure 3. Relative abundance of the main fungi obtained by NGS analysis in Mastrandrea.

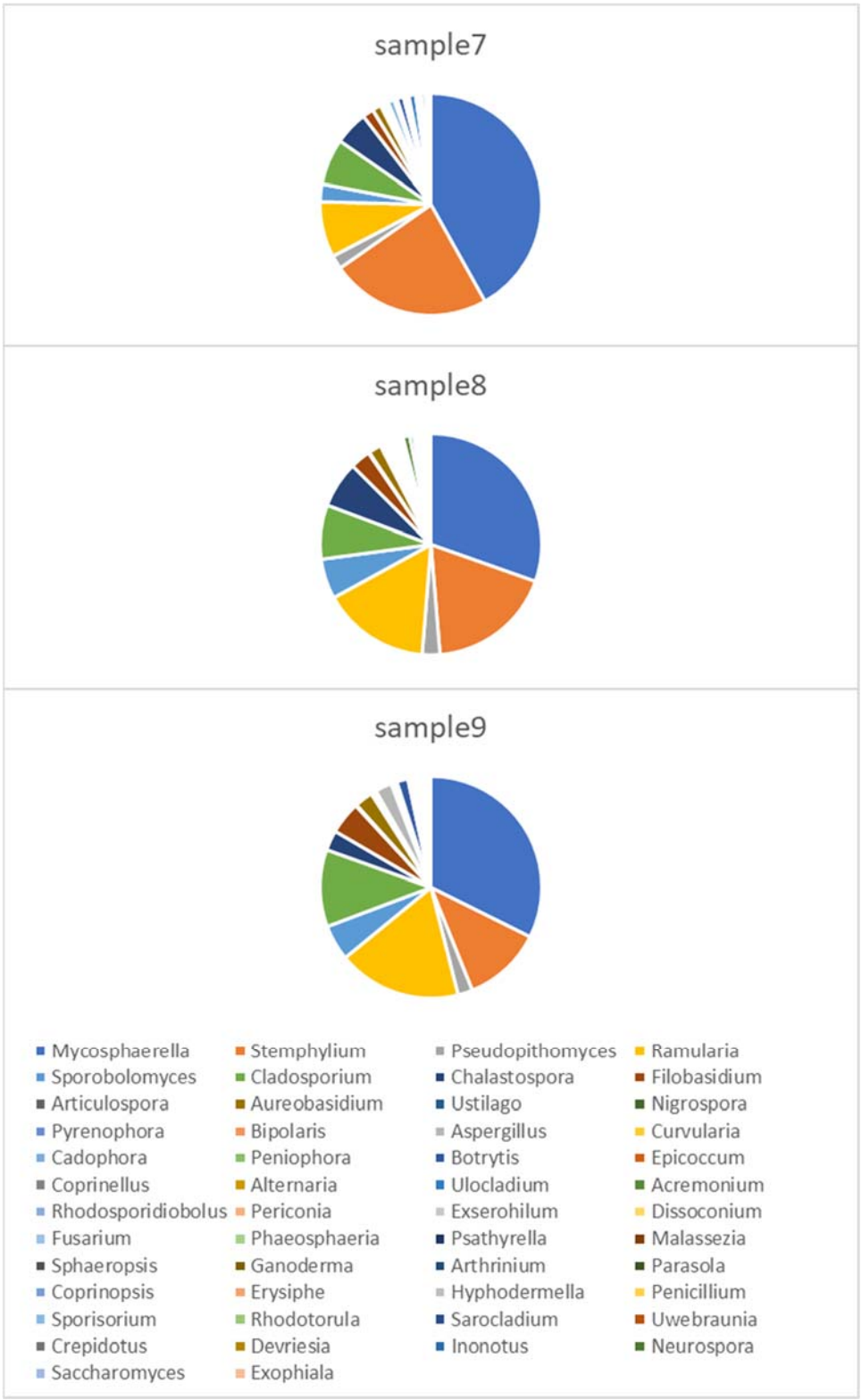

Figure 4. Relative abundance of the main fungi obtained by NGS analysis in Gaffoglio.

Considering the differences among vineyards, it is possible to note that *Pseudophytomyces and Nigrospora* were more abundant in Amandola and Mastrandrea. *Ramularia* was more present in Gaffoglio and less in Amandola. Genera *Ganoderma*, *Coprinellus Curvularia* and *Pyrenophora* had slightly higher percentages in Amandola than in other vineyards.

Sequences closely assigned to yeasts were comprised among 0.82% and 2.97% of total reads with a predominance of *Sporobolomyces* spp., *Filobasidium* spp. (strictly related to *Cryptococcus* genus) and *Aureobasidium pullulans* (Table 3). Considering the percentages, *Sporobolomyces* spp. was less abundant in the Gaffoglio vineyard, *Filobasidium* was less frequent in the Mastrandrea vineyard, while *Aureobasidium* was more abundant in the Mastrandrea vineyard; this is also observable in Figures 2–4.

Table 3. Percentage of sequences assigned to yeasts with NGS analysis.

	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample 6**	**Sample 7**	**Sample 8**	**Sample 9**
Yeast/Yeast-like Genus	**Amandola I**	**Amandola II**	**Amandola III**	**Mastrandrea I**	**Mastrandrea II**	**Mastrandrea III**	**Gaffoglio I**	**Gaffoglio II**	**Gaffoglio III**
Sporobolomyces spp.	2.10%	1.15%	0.81%	1.30%	1.37%	1.59%	0.38%	0.85%	0.61%
Filobasidium spp.	0.64%	0.37%	1.58%	0.02%	0.02%	0.09%	0.24%	0.43%	0.56%
Aureobasidium spp.	0.21%	0.19%	0.10%	0.47%	0.41%	0.54%	0.19%	0.27%	0.31%
Rhodosporidiobolus spp.	0.01%	0.02%	0.01%	0.03%	0.04%	0.01%	0.02%	0.09%	0.06%
Rhodotorula spp.	nd	nd	0.01%	0.01%	0.01%	nd	<0.01%	nd	0.06%
Saccharomyces spp.	0.02%	0.01%	0.01%	0.01%	0.01%	nd	nd	<0.01%	nd
Candida spp.	nd	nd	nd	nd	nd	nd	nd	0.01%	nd
Cryptococcus spp.	nd	nd	nd	nd	nd	nd	<0.01%	nd	nd
Total	2.97%	1.74%	2.50%	1.84%	1.86%	2.23%	0.82%	1.64%	1.60%

NGS was unable to detect *Hanseniaspora*, *Issatchenkia* and *Saturnispora* genera previously revealed by culture-dependent methods. Moreover, *Saccharomyces* spp. was detected, with low frequency, only by NGS, but not in all samples, confirming the rarity of this yeast in the vineyards and on healthy grapes.

Microbial diversity indexes calculated using NGS data are reported in Table 4.

Table 4. Microbial diversity index calculated on NGS data.

Vineyard	**Sample**	**Obs. Species**	**Simpson Index**	**Shannon Index**	**Chao Index**	**ACE Index**
Amandola	1	175.0	0.8	1.8	191.9	191.6
	2	159.0	0.7	1.8	190.2	178.5
	3	214.0	0.7	1.9	181.0	266.1
Mastrandrea	4	168.0	0.7	1.6	227.0	237.9
	5	169.0	0.7	1.7	241.8	209.5
	6	181.0	0.7	1.6	208.0	219.8
Gaffoglio	7	199.0	0.8	1.9	268.3	272.9
	8	157.0	0.7	1.7	198.4	191.1
	9	121.0	0.8	1.7	133.0	141.2

The Simpson index and Shannon index were very similar among samples, while the Chao index, which estimates the richness of species, decreased in all vineyards in the third sampling time. Beta diversity showed the stability of the differences in biodiversity in the three vineyards during grapes maturation (data not shown) and it did not reveal a specific difference due to the time of sampling. Therefore, it is reasonable to suppose that no genera were related to the stage of grape maturity.

4. Discussion

In the last few years, the microbial community on the wine–grape surface has been studied due to its possible effect on wine characteristics, style and quality. All these parameters are affected by various factors, such as geography, climate and viticultural practices (e.g., herbicides, fertilizers, pesticides and fungicides) [20,21].

In this work, attention was also focused on the evolution of the fungal microbial population during the last phase of maturation. To our knowledge, only a few studies have focused on this topic, and a small number of works have used different approaches to compare the results to explore the biodiversity.

In our study, both the culture-dependent method by plating on WL agar and the culture-independent method by NGS analysis targeting the ITS domain were used.

It is important to highlight that the introduction of ampicillin and biphenyl in growing media precluded the identification of bacteria and molds. Differently, sequencing allows the identification of the main part of the eukaryotic microorganisms, where the most frequent species are molds, and yeasts are the minority.

According to Barata et al. [5], the yeast population on healthy grapes fluctuates between 10^2 and 10^4 CFU/g, but there are various publications in the literature reporting higher values. For example, Guerzoni and Marchetti [22] in their work carried out in Italy, found a population ranging from 10^2 to 10^7 CFU/g of grape, or that of Sabate et al. [23] carried out in Spain. This may be due to the presence of some apparently healthy berries, but not completely intact, which induces an increase in the total number of copiotrophic species. Comparing the results of this work with the literature, it is, however, clear that the total count carried out in the three vineyards under study always range between 10^2 and 10^4 CFU/g except at the third sampling of Gaffoglio, near the harvest, at the time of ripening.

Considering the set of samples, it is possible to observe how the diversity of the species that can be cultivated in the laboratory is rather limited. It ranges from a minimum of three species for the Mastrandrea vineyard to a maximum of seven for the Gaffoglio vineyard.

Since these vineyards are adjacent, it is probable that the factors influencing the number of species are not related to climatic variables, such as temperature and rainfall, but rather to local variables such as exposures, parasitic treatments, or particular humidity conditions. In our case, a variable that could have influenced the number of species is exposure; in fact, Mastrandrea has a southeast position and, therefore, is sunnier with a consequent limited number of species. No differences related to the number and type of pesticide treatments were found. It is interesting to note that there is no correlation between the number of species detected and the total number of cells occurring on the grape, neither by observing the data from a temporal point of view during sampling, nor by comparing the values and species detected between the vineyards.

Regarding the species found in this investigation using agar plates, differences were detected among samples. *Aureobasidium pullulans* was the most abundant and widespread species. It is a yeast-like filamentous fungus, also known as "black yeast", ubiquitous with a high degree of polymorphism that lives as a saprophyte on various substrates such as soil, river water, and plant material and is also present in the air. In our work, the species was detected in all samples except in the last sampling time of Gaffoglio's vineyard, while in Mastrandrea's vineyard, *Aureobasidium* represents the only species detected in the second and third sampling times. These data confirm the findings of Alessandria et al. [24] conducted on a greater area and on a greater number of Barbera vineyards, which also includes the reference area of our study. The great environmental diffusion of *A. pullulans* is also recognized in the work of Barata et al. [5], in which this species is the main one on healthy grapes with a percentage ranging between 95 and 100%; the same observation was reported by Bokulich et al. [3].

Together with *A. pullulans*, the basidiomycete *R. graminis* was the only yeast that was detected in all three vineyards. Other Basidiomycetes belonging to the *Cryptococcus* genus were detected: in particular, *C. carnescens* was identified at the first sampling time on Gaffoglio grapes. Consistently, this species was also identified in Vaudano et al. [25] in their study on Grignolino grapes. *Cryptococcus flavescens* was identified in the first sampling of Mastrandrea, while to the best of our knowledge, it does not appear in the literature. However, in the review by Barata et al. [5], a mention was made of the genus *Cryptococcus*.

Yeasts belonging to the genus *Hanseniaspora* have been frequently detected. In particular, *H. opuntiae* was detected in the third sampling time of Amandola and in all samples of Gaffoglio, where, at the third sampling time, it represented the predominant species together with *H. uvarum*. The presence of *Hanseniaspora* is not a novelty, since this apiculate yeast was frequently found on fruits, and it forms part of the grape and fermentation mi-

crobiome [26,27]. Its association with grapes and the first stages of alcoholic fermentation has been reported in most vineyard regions worldwide [28,29].

Issatchenkia terricola was found in two samples (3 and 8) corresponding to the third sampling time in the Amandola vineyard and to the second sampling time in Gaffoglio. This species had also already been found on grapes. Baffi et al. [30], in their study on Brazil vineyards, found that the most frequent species was *Hanseniaspora uvarum*, followed by *Issatchenkia occidentalis*, *I. orientalis* and *I. terricola*. Other species were *Aureobasidium pullulans* and *Sporidiobolus pararoseus*.

S. pararoseus was detected in the first sampling time both in the Amandola and in Mastrandrea vineyards, while in the other sampling time, it was no longer identified. Similarly, *Saturnispora diversa* (synonym *Candida diversa)* was identified in the third sampling time in the Amandola vineyard. This species was found in the study made by Zhang et al. [31] on Cabernet Sauvignon in China, while Gao et al. [32] found this species among those species associated with sour rot-affected grapes.

The Gaffoglio vineyard appears to be the one with the higher biodiversity (Shannon index was 1.15). *Starmerella bacillaris* (synonym of *Candida zemplinina*), frequently associated with grapes and must [33], was identified in the second sampling time. Its presence was previously reported by Vaudano et al. [25] on Grignolino grapes and by Bokulich et al. [3].

Data found in the literature are very similar to ours. Castrillo et al. [34] observed that the predominant yeasts found on grapes collected in Galicia were *Aureobasidium* spp., *Metschnikowia* spp., *Hanseniaspora uvarum*, and *Cryptococcus* spp.; these data agree with ours except for *Metschnikowia.* Moreover, they also found, in a lower amount, *Issatchenkia terricola* and *Starmerella bacillaris*, but their presence was related to a specific region.

Overall, the literature analysis demonstrates several concordances among the different works.

The yeasts identified in this work can also be categorized according to their nutritional needs. In fact, in the results relating to the first sampling time made at the beginning of September, the genera of yeasts identified are all attributable to Basidiomycetes. These yeasts have limited nutritional needs, and are called oligotrophs, namely, *Rhodotorula*, *Sporidiobolus*, *Cryptococcus. A. pullulans*, which is common to all samples, is an Ascomycetes belonging to oligotrophs. The exception is *Hanseniaspora opuntiae*, identified in Gaffoglio in the first sampling time.

In the second sampling time (10 September), in addition to the Basidiomycetes described above, Ascomycete yeasts were also identified, such as *Starmerella* and *Hanseniaspora,* which have an oxidative or weakly fermentative metabolism, therefore needing a more mature fruit as habitat, with greater availability of nutrients.

At the third sampling time, the genera *Issatchenkia*, *Saturnispora* (Candida) and *Hanseniaspora* were found, and they are commonly reported as being associated with grapes and wine environments [5].

In the three vineyards, using culture-dependent methods, it was not possible to detect the species *Saccharomyces cerevisiae* or other species of the same genus. These observations confirm the observations reported by various authors [6,7,35] who affirm that this yeast is very rare in the vineyard and difficult to sample directly from healthy grapes. Mortimer and Polsinelli [7] estimated that only about one in one-thousand grape berries carries *Saccharomyces cerevisiae.*

Consistently with Morgan et al. [10], NGS data revealed more filamentous fungal species than yeast species, especially those associated with the grape berry surface.

The top ten of fungal genera, found in all samples were Mycosphaerella, Stemphylium Pseudophytomyces, Ramularia, Sporobolomyces, Cladosporium, Chalastospora, Filobasidium, Articulospora and Aureobasidium.

Kioroglou et al. [36], in their study on the microbiota of Australian grapes, observed that the most abundant genera across all samples were *Mycosphaerella* and *Aureobasidium*; this last has been frequently isolated and also detected on grape skin in previous studies by culture-independent techniques around the world [3,20,26,37]. The genus *My-*

cosphaerella has been rarely reported and species of *Mycosphaerella* are generally considered plant pathogens, but recently, Dissanayake et al. [38] identified both *Aureobasidium* and *Mycosphaerella* within the endophytic community in stems grapevine.

Aureobasidium pullulans and *Sporidiobolus pararoseus* were detected by Kecskeméti et al. [21] who reported that, on grape berries, they can act as effective antagonists against microorganisms known to have negative effects on wine quality such as *Botrytis cinerea*. *Mycosphaerella*, *Stemphylium* and *Aureobasidium* were also found by Kamilari [12] in their metataxonomic analysis of grape microbiota in Cyprus.

Cladosporium is a common genus of fungi, because of its saprophytic and pathogenic nature, this genus has a wide host range [39], and it is also present on the grape surface as reported by [10,12,37].

Filobasidium species (*F. floriforme* and *Filobasidium* sp.) were detected from leaf and berry samples by Bougreau et al. [40] in their study on Texas vineyards.

Chalastospora ellipsoidea and *Cladosporium delicatulum* were detected by DGGE analysis by Alessandria et al. [24], but they were not found by other authors [10,12].

Ramularia was not found in the previously cited works, but it was found in vine wood by Del Frari et al. [41], while *Articulospora* and *Pseudophytomyces* were not reported in grape microbiota according to our knowledge.

In general, our data agree with the actual literature. Moreover, very recently, Liu and Howell [42] highlighted the occurrence of a core microbiome that is prioritized over space and time, constituted by fungi including fermentative yeasts (*Saccharomyces*, *Debaryomyces*), yeast-like fungi (*Aureobasidium*, *Cryptococcus*, and *Vishniacozyma*), filamentous fungi (*Cladosporium*, *Alternaria*, *Penicillium*, and *Fusarium*) and other genera (*Mycosphaerella*, *Didymella*, *Ramularia*, and *Epicoccum*).

It has been frequently reported that the composition of the microbial community on the grape surface depends on various factors, such as geography climate and agricultural practices [43–46]. Comparing the different samples, since the geography and viticulture practices were similar, in this work no specific differences in microbial population were found, but one aspect that can be evidenced concerns the presence of *Saccharomyces* in some samples.

Regarding the maturation stages, a decrease in the Chao index was observed in all vineyards. Kioroglou et al. [36] studied the microbial population in vineyards of two Australian regions at two stages of maturation and observed that the fungal community composition varied significantly across the distinct vineyards due to diverse altitudes and climate conditions; the region was the factor that had the strongest effect on the sample differentiation by taxa composition while the stage of maturation was not so determinant.

Zhu et al. and Wei et al. [47,48] studied the succession of the fungal microbial community in grapes during different maturation phases. Based on the Shannon index, Wei et al. [48] observed that the microbial alpha diversity of the Cabernet Sauvignon grape decreased during grape development. This result is in agreement with Zhu et al. [47] for the same grape variety.

In contrast, Liu and Howell [42] observed that the fungal diversity of grapes increased significantly during berry development. However, it should be outlined that in those studies, the microbiota was analyzed from the beginning of veraison to the harvest; therefore, the data are not properly comparable with ours.

Innovations in DNA sequencing and, in particular, high-throughput sequencing technologies, together with bio-informatic tools, have considerably improved the study of microbial communities.

However, there are some limits both on culture-dependent identification and on NGS methods. The application of culture-dependent methods is considered weak to support the total biodiversity, since only a small part of microorganisms can be detected.

DNA may not be recovered from all genotypes, in fact less abundant ones could not be detected [49]. NGS analyses are principally targeted on ITS regions [50]. Since the SSU, ITS and LSU all comprise both variable and highly conserved regions, it is not easy to obtain

correct taxonomic assignments to short fragments (100–450 bases) at lower taxonomic levels [51] Correct sequence alignments are difficult to obtain for some fungal taxa; for this reasons, this method is not reliable for species-level identification, which is usually reported at the genus level or even higher taxonomic levels, such as family or order [52]. Another constraint of NGS is that the correspondence of OTU (Operational Taxonomic Unit) with species can be unreliable because some species have genes that are 97% similar, which will result in merged OTUs containing multiple species [49].

Sometimes, it is difficult to understand whether the fungi identified by this technique actually exist in the natural system [53]; therefore, to obtain a good determination in species identification, a combination of both approaches (traditional and culture-independent) should be employed [49,54].

In wine, most of the fungi detected on the grapes are not present. Most of the fungi are sensitive to the SO_2 that is added during crushing grapes. Among the yeast found in this study, the only species that can be present in wine must are *Metschnikowia pulcherrima*, *Issatchenkia* spp., *Candida* spp., *Hanseniaspora* spp. and *Starmerella bacillaris* [55]. These species participate in the initial phase of fermentation, and influence wine flavor and aroma, but with the ethanol increase, they usually die, with the exception of *Starmerella*. In recent years, the attention on non-*Saccharomyces* has increased because of their ability to produce secondary metabolites that improve the organoleptic profile of wine [56]. Grapes are a natural reservoir of yeasts, which can be explored to investigate their possible employment in the production of typical wines.

5. Conclusions

The study of yeast biodiversity is important because it allows us to gain more information about the communities present on the grape surface and their impact. The present study was focused on exploring the microbial diversity in three vineyards of Barbera during the last month of grape maturation by using two different methods.

Comparing the two approaches, it is possible to highlight the similarities since *Aureobasidium*, *Rhodotorula* and *Sporobolomyces* were found with both methods; differences were found for genus *Hanseniaspora*, *Issatchenkia* and *Saturnispora* revealed by culture-dependent methods, but not by NGS, while *Saccharomyces* spp. were detected, with low frequency, only with NGS.

The integrated application of NGS sequencing and culture-dependent techniques provides a comprehensive view of mycodiversity in the wine-growing environment, especially for yeasts with low abundance. NGS allows us to have an almost complete view of the fungal population present on the berries. The culture-dependent methods and the identification of cultivable autochthonous species lead to the selection of yeasts that could be interesting for application purposes and, also, for the maintenance of the typicality of the final product.

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