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Wine Microbial Consortium: Seasonal Sources and Vectors Linking Vineyard and Winery Environments

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Abstract: Winemaking involves a wide diversity of microorganisms with different roles in the process. The wine microbial consortium (WMC) includes yeasts, lactic acid bacteria and acetic acid bacteria with different implications regarding wine quality. Despite this technological importance, their origin, prevalence, and routes of dissemination from the environment into the winery have not yet been fully unraveled. Therefore, this study aimed to evaluate the WMC diversity and incidence associated with vineyard environments to understand how wine microorganisms overwinter and enter the winery during harvest. Soils, tree and vine barks, insects, vine leaves, grapes, grape musts, and winery equipment were sampled along four seasons. The isolation protocol included: (a) culture-dependent microbial recovery; (b) phenotypical screening to select fermenting yeasts, lactic acid, and acetic acid bacteria; and (c) molecular identification. The results showed that during all seasons, only 11.4% of the 1424 isolates presumably belonged to the WMC. The increase in WMC recovery along the year was mostly due to an increase in the number of sampled sources. Acetic acid bacteria (*Acetobacter* spp., *Gluconobacter* spp., *Gluconoacetobacter* spp.) were mostly recovered from soils during winter while spoilage lactic acid bacteria (*Leuconostoc mesenteroides* and *Lactobacillus kunkeii*) were only recovered from insects during véraison and harvest. The fermenting yeast *Saccharomyces cerevisiae* was only isolated from fermented juice and winery equipment. The spoilage yeast *Zygosaccharomyces bailii* was only recovered from fermented juice. The single species bridging both vineyard and winery environments was the yeast *Hanseniaspora uvarum*, isolated from insects, rot grapes and grape juice during harvest. Therefore, this species appears to be the best surrogate to study the dissemination of the WMC from vineyard into the winery. Moreover, the obtained results do not evidence the hypothesis of a perennial *terroir*-dependent WMC given the scarcity of their constituents in the vineyard environment along the year and the importance of insect dissemination.

Keywords: wine microbial consortium; microbial *terroir*; routes of dissemination; vineyard; grapes; insects; winery; *Hanseniaspora uvarum*

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1. Introduction

Wine production involves a wide diversity of microorganisms with different functions in the process [1]. The so-called wine microbial consortium (WMC) includes various species of yeasts, lactic acid bacteria, and acetic acid bacteria, associated with beneficial or detrimental activities [2]. The main fermenting species are the yeast *Saccharomyces cerevisiae* and the lactic acid bacteria *Oenococcus oeni*, responsible for sugar and malic acid conversions to ethanol and lactic acid, respectively. Non-*Saccharomyces* species are attracting increasing interest as alternative or complementary agents in the process [3,4]. Similarly, lactic acid bacteria other than *O. oeni* have been proposed as starters for malolactic fermentation [5,6]. While yeasts and lactic acid bacteria comprise some spoiling species, all acetic acid bacteria are regarded as harmful to grape or wine quality [7,8].

The dissemination of the WMC in natural and vineyard environments has been thoroughly reviewed [1,2,9,10]. In relation to grapes and wine, the recent focus has been put on evidencing the existence of microbial communities dependent on biogeographical boundaries [11–13], encompassing the concept of *terroir*, associated with the highly reputed features of wine typicity and distinctiveness [14]. The inclusion of a microbial component in the *terroir* concept has been proposed [11,15], but not without critical limitations [16]. Nevertheless, the concept of a "microbial terroir" has gained popularity among researchers as a way of demonstrating the contribution of vineyard microbial assemblages to the valued regional wine distinctiveness [10,17,18]. To attain this achievement, a high diversity of sources (e.g., soil, bark, leaves, flowers) has been sampled in attempt to bridge the vineyard and winery environments, but the results have not yet clarified this issue [19–21]. Indeed, the development of high-throughput metagenomic approaches has provided an increased breadth to microbial ecology studies [22,23]. Still, several reports have failed to evidence the presence of the WMC outside winery environments [20,21]. These powerful techniques mostly detect Operational Taxonomic Units (OTUs) of environmental fungal and bacterial higher taxa, barely evidencing species with a direct influence on wine quality. Thus, the rarity of OTUs of WMC members in environmental samples does not provide support for a perennial community of vineyard microorganisms directly related to wine quality [16]. Moreover, the dispersal of WMC from soils to musts or wines has not yet been demonstrated [13].

Therefore, this study aimed to evaluate the microbial diversity associated with surrounding vineyard environments throughout the year to track possible sources and vectors of the WMC into winery fermentations. The methodology followed a culture-dependent approach given the lack of sensitivity of high-throughput techniques for detecting minor but significant constituents of the vineyard microbiome.

2. Materials and Methods

2.1. Sampling and Microbial Recovery

2.1.1. Samples Collection

Samples were collected during winter (February), spring (May), véraison (July), and harvest (September), from different locations on the campus of the Instituto Superior de Agronomia (38°42′27.95″ N, 9°10′56.34″ W, elevation 51 m), Lisbon, Portugal, as shown in Figure 1. The two analyzed vineyard plots were managed according to conventional practices, using copper oxychloride plus micronized sulfur, metiram plus pyraclostrobin, mancozeb plus pyriofenone, and mancozeb plus metalaxyl-M. Weeds were mechanically controlled and by one spray of carfentrazone. The samples were collected from these two vineyards, in arboreal areas close to each vineyard and from the forest area far from the vineyards, as follows:

(a) Soil: five soil samples (30 g) were taken, four from the vineyard areas and one from a forest area far from the vineyard areas (Figure 1A). Sampling occurred during the four annual periods mentioned above.

(b) Tree and vine bark: five samples were collected from five trees (one sample per tree) close to the vineyard area indicated in Figure 1B, including a wild olive (*Olea europaea var. sylvestris* Mill.), a cork oak (*Quercus suber* L.), a cypress (*Cupressus sempervirens* L.), a Japanese cheese wood (*Pittosporum tobira* Ait.), and from a cypress far from the vineyard area (Figure 1B). Tree samples were taken during the four annual periods mentioned above. The bark samples of two vines (one sample per vine) located in the two different plots were collected during the spring, véraison, and harvest seasons.

(c) Insects: five yellow adhesive chromotropic traps (20 cm × 25 cm, Biosani, Portugal) were set for six days on the places indicated in Figure 1C, including four vines (*Vitis vinifera* L.) and on a cypress far from the vineyard area. Insects (five specimens in each trap) were randomly collected during the four periods mentioned above and were subjected to a simplified identification at Order level [24], considering the Orders Heteroptera and Homoptera as Hemiptera.

(d) Vine leaves: five vine leaves were collected from the places indicated in Figure 1D during the spring, véraison, and harvest seasons.
(e) Grapes: five healthy berries were collected in sterile plastic bags from the locations indicated in Figure 1D at véraison and harvest seasons. Five damaged berries were only collected at harvest time.
(f) Winery: the winery and its equipment were analyzed during véraison and harvest. Five samples from the walls, vats, press, crusher, and cold-stabilizer (one sample each) were collected with cotton swabs.
(g) Grape juices: one sample each of 250 mL red and white grape musts in the winery before starter addition were obtained during the harvest season and analyzed after processing.

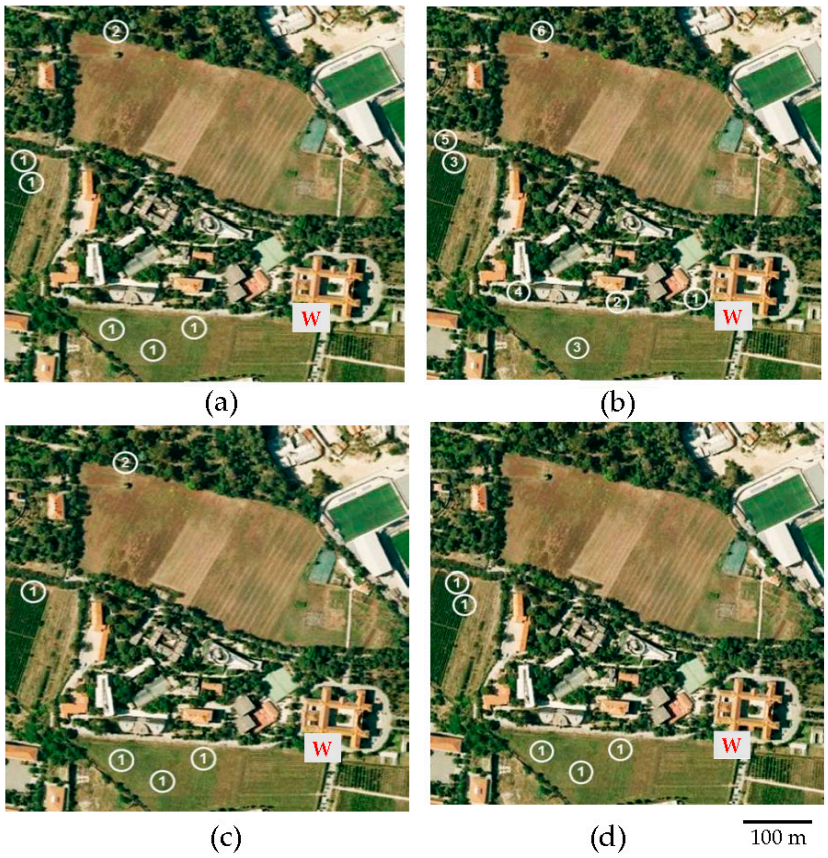

Figure 1. Sites of sampling in the environment at different distance from the winery building (W): (**a**) soils in vineyards ①, soils in forest zone ②; (**b**) tree bark, cork oak ①, cypresses ② ⑥, vines ③, Japanese cheesewood ④, wild olive tree ⑤; (**c**) insects, vineyards ①, forest zone ②; (**d**) vineyard leaves and grapes ①.

2.1.2. Sample Suspension

Soil (30 g), tree bark (10 g), vine bark (10 g), leaves (10 g), and single berry samples were aseptically transferred to 250 mL of peptone water (10 g/L peptone (Biokar Diagnostics, Vienna, Austria), 5 g/L sodium chloride (Merck, Darmstadt, Germany)). Insects were transferred to 10 mL peptone water, and cotton swabs were suspended in 5 mL peptone water. All suspensions were ultra-sonicated for 10 min in a water-bath and stored for 24 h at 25 °C. Then, 100 µL were spread onto different selective media.

Grape juices after winery crushing were analyzed: (a) by direct inoculation (100 µL); (b) after a 24 h auto-enrichment at 25 °C, and (c) after ten days of spontaneous fermentation at 25 °C (only for white juices). After the incubation, 100 µL of each grape must was spread onto the different culture media.

2.1.3. Selective and Differential Media

Yeast isolates were recovered from Malt Yeast Peptone (MYP) agar medium (7 g/L malt extract (Biokar Diagnostics, Vienna, Austria), 0.5 g/L yeast extract (Biokar Diagnostics, Vienna, Austria), 2.5 g/L soy peptone (Biokar Diagnostics, Vienna, Austria), agar 20 g/L (Dário Correia, Odivelas, Portugal)) and Non-*Saccharomyces* Medium (NSM) (20 g/L yeast extract, 10 g/L glucose (Copam, São João da Talha Portugal), 10 g/L tryptone (Biokar), 22 g/L agar, pH 5) [2]. Both media were autoclaved (120 °C, 20 min). Then two antibiotics (0.08 g/L biphenyl (Sigma-Aldrich, St. Louis, MO, USA) and 0.1 g/L chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA)) were added to inhibit the growth of filamentous fungi and bacteria, respectively. The NSM medium was supplemented with 0.1% cycloheximide (Sigma-Aldrich) to inhibit *S. cerevisiae*. The selective and differential media *Zygosaccharomyces* Differential Medium (ZDM) [25] and *Dekkera/Brettanomyces* Differential Medium (DBDM medium) [26] were used to recover *Z. bailii* and *D. bruxellensis*, respectively. Cultures inoculated in MYP, NSM, and ZDM media were incubated at 25 °C for five days, while cultures inoculated in DBDM were incubated at 25 °C for thirteen days.

Lactic acid bacteria were recovered using MRS (De Man, Rogosa, and Sharpe) medium (Biokar, Noack Group, Vienna, Austria) supplemented with 0.1 g/L L-cysteine (Merck, Darmstadt, Germany) and 0.08 g/L biphenyl after autoclavation. For acetic acid bacteria, Glucose Yeast Extract Carbonate (GYC) medium (5 g/L glucose, 1 g/L yeast extract, 3 g/L calcium carbonate, 20 g/L agar) was used, supplemented with 0.08 g/L biphenyl after autoclaving. Cultures inoculated in MRS and GYC media were incubated at 30 °C for two to five days.

2.2. Microbial Screening and Identification

2.2.1. Isolate Purification, Maintenance and Phenotypic Tests

The number of colonies with different dominant morphologies was 3 to 5 in each plate. The purification of each selected colony was performed by streaking onto MYP, MRS, and GYC agar plates without antibiotics, for yeasts, lactic acid bacteria, and acetic acid bacteria, respectively. The pure colonies were transferred to the respective media added with 70% (*v*/*v*) glycerol and preserved at −20 °C. Revival of cryopreserved cells was performed on agar plates of the respective culture medium.

After microscopic observation (400×), the urease test was applied to select ascomycetous yeasts, as described by Kurtzman and Fell [27]. The basidiomycete *Rhodosporidium toruloides* ISA 1854 was used as a negative control, and the ascomycete *Saccharomyces cerevisiae* ISA 1000 as control of a positive response. Yeast isolates showing urease-negative activities were selected for further identification. Preliminary screening for lactic acid and acetic acid bacteria was performed by applying Gram staining, catalase, and oxidase tests on pure bacterial colonies.

2.2.2. DNA Extraction and PCR Amplification

Yeasts

Yeast isolates were grown in NSM medium for 48 h at 25 °C prior to DNA extraction. Then, one colony was streaked into a tube containing 200 µL of sterilized Milli-Q water and heated at 96 °C for 10 min for cell lysis. PCR was performed as described in White et al. [28] in a mixture containing 0.4 µM of each primer ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS4 (5′ CCTCCGCTTATTGATATGC 3′) (STAB VIDA, Caparica, Portugal), 1 mM of $MgCl_2$ (NZYtech, Lisbon, Portugal), 0.1 U of PCR mix (NzyTaq 2× colorless Master Mix) and 1 µL of DNA.

PCR amplifications were performed in a thermocycler (Piko Thermal Cycler, Thermo Scientific, Waltham, MA, USA) as follows: initial denaturing at 95 °C for 5 min and then 35 PCR cycles of the following program denaturing at 94 °C for 1 min, annealing at 50 °C for 2 min, and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. DNA from *S. cerevisiae* ISA 1000 and *Lactobacillus plantarum* (now designated as *Lactiplantibacillus plantarum*) ISA 3960 were used as positive and negative controls, respectively. PCR products

were run through 1.5% agarose (Seakem LE Agarose, Lonza, Basel, Switzerland) gels in 0.5× TBE (Tris-borate-EDTA) buffer, with 0.25× of GelRed (Biotium Inc., Fremont, CA, USA). The gel was visualized under UV light in GelDoc (BioRad, Hercules, CA, USA). A 5000-bp DNA ladder marker (NZYtech, Lisbon, Portugal) served as a size standard.

Bacteria

Bacteria DNA extraction was performed using GeneJET Genomic DNA Purification Kit (Thermo Scientific, Waltham, MA, USA). The PCR for DNA amplification of lactic acid bacteria that were grown in MRS was performed in a mixture containing 0.4 µM of primer PA (5′ AGAGTTTGATCCTGGCTCAG 3′, STAB VIDA) and PH (5′ AAGGAGGTGATCCAGCCGCA 3′, STAB VIDA), as described by Edwards et al. [29]. PCR amplifications were performed in a thermocycler (Piko Thermal Cycler) as follows: initial denaturing at 94 °C for 5 min, and then 30 PCR cycles of the following program denaturing at 94 °C for 30 s, annealing at 49 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. DNA from *L. plantarum* ISA 3960 was used as positive control and *S. cerevisiae* ISA 1000 as negative control.

The PCR for DNA amplification of acetic bacteria that were grown in GYC medium was performed in a mixture containing 0.4 µM of primer Ac1 (5′ GCTGGCGGCATGCTTAACACAT 3′, STAB VIDA) and Ac3 (5′ AACCACATGCTCCACCGCTTG 3′, STAB VIDA) as described in Poblet et al. [30]. To both PCR mixtures, 1 mM of $MgCl_2$ (NZYtech), 0.1 U of PCR mix (NzyTaq 2× Colourless Master Mix) and 1 µL of DNA were added. PCR amplifications were performed in a thermocycler (Piko Thermal Cycler) as follows: initial denaturing at 94 °C for 5 min, and then 35 PCR cycles of the following program denaturing at 94 °C for 1 min, annealing at 64 °C for 2 min, and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. DNA from *Acetobacter cerevisiae* ISA 4409 and *Escherichia coli* ISA 3967 were used as positive and negative control, respectively.

All PCR products were run through 1.5% agarose (Seakem LE Agarose) gels in 0.5× TBE (Tris-borate-EDTA) buffer, with 0.25× of GelRed (Biotium Inc.). Then, the gel was visualized under UV light in GelDoc (BioRad). A 5000-bp DNA ladder marker (NZYtech) served as a size standard. Only the PCR products corresponding to presumptive lactic acid and acetic acid bacteria were sequenced (STAB VIDA). A BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 1 March 2020)) was performed for the DNA sequences obtained. The valid identifications required at least 98% identity.

3. Results and Discussion

3.1. Seasonal Isolation of Yeasts and Bacteria

A total of 1424 yeast and bacterial isolates were recovered from 192 samples during four seasons related to vine growing, grape ripening and wine production (Table 1).

In winter (February), 240 microorganisms were isolated from soil (38%), tree bark (34%), and insects (28%). Bacteria were the main recovered microorganisms (72.5%), comprising 34% of the isolates from the soil, 20% from insects and 18% from tree bark. A total of 27.5% of the recovered yeasts were distributed in soil (4%), tree bark (16%), and insects (8%). In tree bark, the relatively higher yeast incidence may be due to the presence of tree exudates, rich in sugar, facilitating their development [31].

During spring (May), 316 microorganisms were isolated, with 42% being from insects, 26% from tree bark, 19% from soils, and 13% from vine leaves. Like in the winter season, bacteria were the main microbial group (67%) isolated from most of the analyzed reservoirs (soil, trees, and vine leaves). However, the percentage of yeasts isolated from insects was similar to the percentage of bacteria isolated (both represented 21% of the isolates), reflecting a relative increase from winter. This may be due to the beginning of flowering, when the number of insects bearing yeasts increases [32].

At véraison (July), 422 microorganisms were isolated, with insects being the reservoir that most contributed to the isolations (31%), comprising 20% of bacteria and 11% of yeasts. Soil represented 18% of the isolates, followed by trees (17%), grapes (16%), vine

leaves (10%), and the winery and its equipment (9%). As observed in the previous season, bacteria were the main microorganism (67%) isolated from soil, trees, insects, healthy grapes. The winery equipment only showed bacterial isolates. However, the bacteria and yeast percentages in the vine leaves were the same (5%).

Table 1. Number of samples and microbial isolates during all seasons.

Season	Reservoir	Samples	Isolates	Bacteria	Yeasts	Presumptive Lactic Acid Bacteria	Presumptive Acetic Acid Bacteria	Ascomycetous Yeasts
Winter	Soil	5	91	82	9	0	21	0
	Bark	5	82	44	38	0	6	5
	Insects	25	67	48	19	0	6	5
	Total	35	240	174	66	0	33	10
	Total/sample	-	6.9	5.0	1.9	0	0.9	0.3
Spring	Soil	5	60	60	0	1	0	0
	Tree and vine bark	7	82	60	22	3	0	0
	Insects	25	133	67	66	0	0	0
	Vine leaves	5	41	25	16	4	0	12
	Total	42	316	212	104	8	0	12
	Total/sample	-	7.5	5.0	2.5	0.2	0	0.3
Véraison	Soil	5	75	54	21	2	9	0
	Tree and vine bark	7	71	46	25	2	10	3
	Insects	25	130	84	46	13	2	10
	Vine leaves	5	42	21	21	1	1	0
	Healthy grapes	5	67	38	29	0	3	0
	Winery equipment	5	37	37	0	0	0	0
	Total	52	422	280	142	18	25	13
	Total/sample	-	8.1	5.4	2.7	0.4	0.5	0.3
Harvest	Soil	5	45	45	0	4	5	0
	Tree and vine bark	7	76	58	18	3	4	1
	Insects	25	84	71	13	25	6	4
	Vine leaves	5	44	31	13	5	0	0
	Healthy grapes	5	54	36	18	6	1	0
	Damaged grapes	5	45	27	18	0	0	13
	Winery equipment	5	11	9	2	0	0	2
	Must/enriched must	5	74	18	58	9	5	58
	Fermented must	1	13	0	11	0	0	11
	Total	63	446	295	151	52	21	89
	Total/sample	-	7.1	4.7	2.4	0.8	0.3	1.4
	Annual total	192	1424	961	463	78	79	114
	Total/sample	-	7.4	5	2.4	0.4	0.4	0.6

At harvest (September), 446 microorganisms were isolated, 19% from insects, 17% from trees, 12% from healthy grapes, 20% from the grape must (fermented or not), 10% from soils, 10% from damaged grapes, 10% from vine leaves, and 3% from the winery equipment. Bacteria were the main isolated microorganism (66%). In the grape must, bacteria were in a lower percentage (4%) than yeasts (13%). Yeasts (3%) were the most frequent microbial group isolated from fermented grape musts, as was expected due to the low pH values, scarce oxygen availability and depletion of certain nutrients and high levels of ethanol and organic acids [33,34].

The insects trapped during the year belonged mostly to the Orders Diptera, Hemiptera, Lepidoptera, Coleoptera and Hymenoptera, without any specific association to bacterial or yeast isolates (results not shown).

The microbial isolates were subjected to a preliminary phenotyping screening to reduce the number of strains to be identified. Ascomycetous yeasts were only dominant in juice samples, while basidiomycetes represented 89% of all yeasts recovered from outside the winery (Table 1).

The presumptive acetic acid bacteria (Gram-negative, oxidase-negative and catalase-positive or -negative) were further subjected to a nested PCR with primers AcA and AcB applied to the PCR products previously obtained with the primers Ac1 and Ac3

(results not shown). Presumptive acetic acid bacteria produced a restriction band of 550 bp and were selected for sequencing. The DNA of presumptive lactic acid bacteria (Gram-positive, catalase-negative, oxidase-negative) was amplified with primers PA and PH and the resulting band consisted of approximately 1800 bp. The number of DNA sequences consistent with acetic acid and lactic acid bacteria is shown in Table 1. Presumptive lactic acid and acetic acid bacteria in the environment were only represented by 11% of the bacterial isolates during winter, spring, véraison and harvest (Table 1). From these results, a low incidence of the WMC was apparent in the environment and the increase in the number of isolates along the year was mostly due to an increase in the number of sampled sources. Moreover, there was a marked difference in the global microbial assemblage between the vineyard environment and the winery, mostly due to the dominance of ascomycetous yeasts in the winery, as described in the next section.

3.2. Microbial Identification

3.2.1. Yeast Species

During winter, four samples from tree bark and four samples from insects harbored ascomycetous yeasts (Table 2). The DNA sequencing results revealed that the bark isolates belong to *Debaryomyces hansenii* and *D. robertesiae*. The latter species was also isolated from insects. Throughout spring, the ascomycetous identified yeasts were only collected from insects, comprising *Candida cellae*, *C. parapsilopsis*, *Metschnikowia* spp. and *Metschnikowia chrysoperlae* (Table 2). At véraison, the ascomycetous yeasts belonged to the genera *Debaryomyces* spp., *Metschnikowia* spp., and *Candida* spp., *D. hansenii* and *D. fabryi* were isolated from the bark of a cypress located close to the vineyard. The strains isolated from insects belonged to the species *C. albicans*, *C. hawaiiana*, *C. apicola* and *M. rekaufii* (Table 2). There were no yeasts recovered from the winery before harvest, possibly because the winery equipment was new, which is in agreement with other observations [35].

Table 2. Number of samples with ascomycetous species recovered from vineyard environment samples throughout the year.

Season	Species	Insects Far from Vineyard	Insects in the Vineyard	Tree Bark Close to Vineyard	Vine Bark	Damaged White Grapes
Winter	*Debaryomyces hansenii*			3		
	D. robertsiae		4	1		
Spring	*Candida cellae*		4			
	C. parapsilopsis	1	3			
	Metschnikowia spp.	1				
	M. chrysoperlae	3				
Véraison	*C. albicans*		2			
	C. apicola		1			
	C. hawaiiana		2			
	D. fabryi			1		
	D. hansenii			1		
	Metschnikowia spp.		1			
	M. reukaufii		1			
Harvest	*C. hawaiiana*				1	
	C. infanticola		1			
	Hanseniaspora optuntiae		1			
	H. uvarum		1			2
	Pichia ciferrii					1
	P. guilliermondii					1

During harvest, the ascomycetous yeasts isolated from the vine bark belonged to *C. hawaiiana* and those recovered from insects belonged to *H. uvarum*, *H. optuntiae* and *C. infanticola* (Table 2). The ascomycetous yeasts were found in damaged white grapes (*Pichia ciferrii*, *P. guilliermondii* and *H. uvarum*), while red grapes only showed basidiomycetous yeasts. This is in accordance with the observations of Barata et al. [36], who also isolated *H. uvarum* and *P. guilliermondii* from damaged grapes. Chandra et al. [37] also detected *P.*

guilliermondii in soils underneath fruit trees. Regarding the sources, insects were responsible for the majority of isolations throughout the year.

In the winery, yeasts from non-enriched must belonged to the genera *Metschnikowia* spp. (*M. pulcherrima*), *Candida* spp. (*C. zemplinina*, *C. diversa*, *C. helenica*), *Issatchenkia* spp. (*I. terricola*), *Pichia* spp. (*P. fermentans*), and *Hanseniaspora* spp. (*H. uvarum*) (Table 3). Most of these species and *P. manshurica* were found in the enriched must. These results reflect the expected prevalence of yeast species long known as grape juice contaminants [38,39]. The species *S. cerevisiae*, *Z. bailii*, and *P. occidentalis* were only isolated from the fermented white grape must which reflected the expected selection during wine fermentation [34,35].

Table 3. Number of samples with ascomycetous species recovered from the winery.

Season	Species	Non-Enriched Must	Enriched Must	Fermented White Juice	Winery Equipment
Harvest	*Candida boidinii*	1	1		
	C. diversa	2			
	C. hellenica	1			
	C. zemplinina	1	1		
	Hanseniaspora uvarum	1	2		
	Issatchenkia terricola	2	1		
	Lachancea thermotolerans	1	1		
	Metschnikowia spp.	1			
	M. pulcherrima	1	1		1
	P. fermentans	1	1		
	P. kluyveri	1	1		
	P. manshurica		2		
	P. occidentalis			1	
	Saccharomyces cerevisiae			1	1
	Zygosaccharomyces bailii			1	

The species *H. uvarum* was the most frequently isolated, with more than one isolate per sample (results not shown) as reported by Barata et al. [1], and the single species that was simultaneously recovered from insects, grapes in the vineyard and juices in the winery.

3.2.2. Acetic Acid Bacterial Species

In winter, according to DNA sequencing results, 23 samples harbored members of the Acetobacteracea family (Table 4). The most representative genera were *Gluconobacter* and *Gluconoacetobacter* including *G. oxydans* and *G. asukensis*, recovered from all samples of vineyard soils. The species *Gluconobacter oxydans*, *Gluconacetobacter liquefaciens* and *Acetobacter tropicalis* have been associated with wine [40–42]. Acetic acid bacteria were not recovered during spring and véraison. During harvest, the DNA sequencing results revealed that only four samples from the vineyard soil harbored *G. cerinus*, *G. oxydans* and *Acidiphilium* spp. (Table 4).

Table 4. Number of samples with acetic acid bacteria isolates recovered from vineyard environment samples throughout the year.

Season	Species	Vineyard Soil	Soil Far from Vinery	Tree Bark Close to Vineyard	Tree Far from Winery
Winter	*Acetobacter tropicalis*	1			
	Acidisphaera rubrifaciens	1			
	Gluconobacter oxydans	5		1	1
	Gluconacetobacter asukensis	5	1		
	Gl. liquefaciens	1			
	Granulibacter bethesdensis	1			
	Rhodopila globiformis	1			
	Roseomonas cervicalis			1	
Harvest	*Acidiphilium* spp.	1			
	G. cerinus	1			
	G. oxydans	2			

The environmental species identified mainly belonged to the Enterobacteriacea family (*Raoultella* spp., *Klebsiella* spp., *Enterobacter* spp., *Escherichia* spp., *Cronobacter* spp., *Kluyvera* spp., and *Serratia* spp.) and Pseudomonadaceae (*Pseudomonas* spp.) (Table S1).

3.2.3. Lactic Acid Bacterial Species

Lactic acid presumptive bacteria were not recovered during winter. In the next season, samples harbored *Lactococcus* spp. and *Enterococcus* spp. (Table 5), with *Lactococcus lactis* being the single species from vine leaves. This species was also isolated during véraison from insects in the vineyard. The other species isolated during this season in insects belonged to *Enterococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp. and *Vagococcus* spp. One species, *L. kunkeii*, is regarded as a wine spoilage bacterium, and was also found in insects (bees) [43].

Table 5. Number of samples from environmental and winery samples harboring lactic acid bacteria.

Season	Species	Soil Vineyard	Insects in the Vineyard	Insects Far from Vineyard	Tree Bark Close to Vineyard	Vine Leaf	Sound Berry	White Grape Must
Spring	*Enterococcus* sp.				1			
	E. faecalis			1				
	E. faecium	1						
	Lactococcus lactis		1			2		
Véraison	*E. casseliflavus*				1			
	E. faecalis		1					
	E. gallinarum				1			
	Lactobacillus kunkeei		1					
	La. lactis		2					
	Leuconostoc lactis		1	1				
	Le. mesenteroides		1					
	Vagococcus carniphilus		1					
	V. teuberi		1					
Harvest	*Enterococcus* spp.					1		
	E. absuriae		1					
	E. faecalis		5			1		
	E. faecium	1	1		2	1		
	E. gallinarum	1			1			
	E. hirae		1					
	E. lactis	1	1			1		
	E. mundtii		1					
	E. pallens						1	
	E. raffini						1	
	La. lactis	1	2	1				2
	Le. mesenteroides		1					
	Le. pseudomesenteroides		1					
	Fructobacillus tropaeoli						1	

During harvest, the isolates belonged to four genera: *Enterococcus*, *Lactococcus*, *Leuconostoc*, and *Fructobacillus* spp. (Table 5). Lactic acid bacteria belonging to the WMC (*Leuconostoc mesenteroides*) were only isolated from insects, corroborating the findings of Bae et al. [44] and Nisiotou et al. [45], who did not recover lactic acid bacteria belonging to the WMC in healthy and damaged grapes. Similarly, no lactic acid bacteria related to wine could be isolated from the enriched and non-enriched musts, probably due to dominance by yeasts [46]. Among environmental lactic acid bacteria, it is interesting to find that the relatively frequent E. faecalis has also been reported as contaminating insects (*Drosophila melanogaster*) [47]. Indeed, as previously observed with yeasts, insects were the source providing the highest number of isolations.

The environmental species outside the lactic acid bacteria only belonged to the genus *Bacillus* spp. (*B. cereus*, *B. thurigiensis*, *B. megaterium*, *B. nealsonii*, *B. subtilis*) (Table S2).

3.3. Origin and Dissemination of the WMC throughout the Year

The results presented in this study show that the incidence of culturable populations of the WMC in vineyard environments was rather low, thus explaining why metagenomic

techniques barely evidence their presence. For example, Chou et al. [20] reported that the most abundant yeast OTUs belong to *Sporobolomyces*, *Aureobasidium* and *Rhodosporidium* spp., which have no ability to ferment wine [48]. Likewise, bacterial OTUs may include representatives of the families Sphingomonadaceae, Cytophagaceae, Rubrobacteraceae, Acidobacteriaceae (in vine trunk samples), and Bacillaceae, Enterobacteriaceae, Paenibacillaceae, and Oxalobacteraceae (in grape samples) [21]. This taxonomic delimitation at the family level is too high to confirm the presence of bacterial species with significance to winemaking. The family Lactobacillaceae, encompassing environmental and fermenting species, was not among those prevalent [21], contrary to the results presented in this study. Therefore, the metagenomic approaches do not seem appropriate to evidence the existence of vineyard microbial assemblages directly related to wine quality. However, these fungal and bacterial communities may have a role in plant health. Zarraonaindia et al. [49] reported the existence of a grape "core" OTU bacterial phylogeny that was independent of region, climate, or sampling method. This "core" assemblage included soil and plant bacterial taxa related to disease suppression, plant growth and productivity [49]. Liu et al. [50] also described a "core" microbiome for fungal taxa throughout the year. Moreover, considering that the vine plants and microbes may be regarded as holobionts [51], the balance of all microbial communities is relevant to plant and grape health, thus indirectly affecting wine quality. Indeed, grape health is the main factor affecting wine quality [48], as influenced by obligate parasites (e.g., fungal agents of powdery and downy mildews), saprophytic fungi (e.g., *Botrytis cinerea*) or saprophytic yeasts belonging to the WMC [1]. However, these phytopathogenic agents have the ability to overcome natural holobiont microbial defenses [51]. Surprisingly, these agents, with striking effects on wine quality, appear to be overlooked in recent microbial biogeography reviews [10,13,52].

The presence of WMC yeast species in must samples from wineries determined by metagenomic approaches indicates the existence of a nonrandom "microbial *terroir*" [15]. However, as shown in Table 6, must species were seldom coincident with vineyard samples and were entirely different from species recovered from fermented juices. Therefore, a clear division between the microbial communities of vineyards and winery environments is apparent. As remarked by Alexandre [16], whatever the origin of the grapes, yeast species present in the cellar colonize musts that will ferment together and displace the isolates from the vineyard. Accordingly, the results obtained in the present study do not support the view that grape yeast biodiversity with direct influence on wine quality may be correlated with specific *terroirs*.

Table 6. Origin and dissemination of the Wine Microbial Consortium.

Technological Significance	Species	Reservoir	Season
Innocuous yeasts	*Candida parapsilopsis*	Insects	Spring
	C. apicola	Insects	Véraison
	C. diversa	Grape must	Harvest
	C. zemplinina	Grape must	Harvest
	Debaryomyces hansenii	Tree bark	Winter, véraison
	Hanseniaspora uvarum	Insects, damaged grapes, grape must	Harvest
	Issatchenkia terricola	Grape must	Harvest
	Lachancea thermotolerans	Grape must	Harvest
	Metschnikowia pulcherrima	Grape must, winery	Harvest
	Pichia fermentans	Grape must	Harvest
	P. guilliermondii	Damaged grapes	Harvest
	P. kluyveri	Grape must	Harvest
	P. occidentalis	Fermented must	Harvest
Fermenting yeasts	*Saccharomyces cerevisiae*	Fermented must, winery	Harvest
Spoilage yeasts	*Zygosaccharomyces bailii*	Fermented must	Harvest
Acetic acid bacteria	*Acetobacter tropicalis*	Soil	Winter
	Gluconobacter oxydans	Soil, tree bark	Winter, harvest
	G. cerinus	Soil	Harvest
	G. liquefaciens	Soil	Winter
Spoilage lactic acid bacteria	*Lactobacillus kunkeei*	Insects	Véraison
	Leuconostoc mesenteroides	Insects	Véraison, harvest

From an ecological perspective, the likely difference in available carbon sources explains this rupture between vineyard and winery environments. Certainly, Chandra et al. [37] demonstrated the higher incidence of WMC saprophytic yeast species in soils under fruit trees or oak forests probably benefiting from the sugar released by decaying fruits or foliage. Accordingly, in vineyard soils, where grapes are not left to decay, WMC yeasts were very rare [35,37], explaining their weak incidence when metagenomic techniques are used [53] and rotten grapes are not sampled [49]. On the contrary, the absence of *S. cerevisiae* in vine bark was not in accordance with other reports [35,54–56]. This contradiction may be explained by the use, in these studies, of an enrichment step essential to recovery of *S. cerevisiae* present in very low numbers. Another explanation may be the uneven and rare presence of yeast populations in vine barks, which may be overcome when a large number of samples is analyzed [54–56]. Indeed, Nadai et al. [55] did not find *S. cerevisiae* in young vines, in opposition to those that were 8 years old.

Overall, damaged grapes harboring saprophytic microorganisms disseminated by insects constitute the link between vineyard and winery environments [57]. In this study, *H. uvarum* was the single species recovered from vineyard, grape must and insects, in accordance with its reported high frequency of isolation in grapes and at the onset of fermentation [1]. Therefore, this species appears to be the most suitable for microbial source tracking, under an approach similar to that used with foodborne pathogens [58]. Another candidate as an indicator for tracking microbial dissemination that justifies further study would be the lactic acid bacteria *Lactococcus lactis*, recovered from soils, insects and grape must (see Table 5). However, the significance of *Lactococcus* spp. for wine quality is not yet well established [5,44,59,60], seeming to possess relatively little resistance to ethanol [61,62].

4. Conclusions

The results presented in this work showed that the species associated with the WMC are scarce in vineyards and surrounding environments during all seasons. Acetic acid bacteria were mostly recovered from soils during winter. Lactic acid bacteria associated with wine were seldom isolated while their environmental counterparts were mostly recovered from insects throughout spring and véraison. The main bacterial species responsible for malolactic fermentation, *O. oeni*, was not detected during this study.

Regarding yeasts, their diversity was different between vineyard and winery samples, demonstrating the sharp distinction between both microbial assemblages. The agent of wine fermentation, *S. cerevisiae*, was only detected in the fermented grape must. Therefore, the results obtained did not support the existence of a vineyard microbial community that could be related to the *terroir* and influence wine distinctiveness. Furthermore, insects were vehicles of WMC and, due to their natural mode of dispersion, cannot be framed under the boundaries of a precise location, which is the main feature of the *terroir* concept.

Further work is necessary to link ephemeral (e.g., grapes, flowers) with perennial sources (e.g., soil, bark) and the wine microbiota. Given the rarity of fermenting yeasts in these locations, the analytical approach should use an enrichment step to recover the chosen microbial surrogate. *H. uvarum* was the most frequent wine-related microorganism linking vineyard and winery samples during harvest. Therefore, this yeast species appears to be the best candidate for studying routes of dissemination that further require molecular typing at the strain level.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fermentation8070324/s1: Table S1. DNA sequencing results of bacteria not belonging to the acetic acid bacteria groups during winter, véraison and harvest, and Table S2. DNA sequencing results of bacteria not belonging to the lactic acid bacteria group during spring, véraison and harvest.

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