Journal of Agriculture and Food Research 18 (2024) 101420

Contents lists available at ScienceDirect

Journal of Agriculture and Food Research

journal homepage: www.sciencedirect.com/journal/journal-of-agriculture-and-food-research

A descriptive study of the microbial populations following biocontrol against powdery mildew in vineyard

L. González-Arenzana *, P. Santamaría, A.R. Gutiérrez, R. Escribano-Viana, I. López-Alfaro

ICVV, Instituto de Ciencias de la Vid y del Vino (Gobierno de La Rioja, Universidad de La Rioja, CSIC). Finca La Grajera, Ctra. Burgos km 6, 26007, Logroño, La Rioja, Spain

ARTICLE INFO

Keywords:
Biocontrol
Powdery mildew
Massive sequencing diversity

ABSTRACT

This study provides a detailed analysis of the bacterial and fungal communities in Biocontrol-treated Tempranillo grape samples, as well as in wines after alcoholic fermentation (AF) and malolactic fermentation (MLF) using the Next Generation Sequencing. Results showed that the bacterial grape community was represented by the Phyla Cyanobacteria and Proteobacteria. The acetic acid bacteria were more abundant and diverse in control wines, while the Family Enterobacteriaceae was significant in wines from Biocontrol-treated grapes. After MLF, the bacteria in both control and Biocontrol-treated samples were represented by *Oenococcus oeni*. Results regarding the fungal community, demonstrated that A*erobasidium pullulans* had high representation, and the genus *Botrytis* accounted for half of the detected OTUs in both types of samples· The genus *Saccharomyces* was predominant in control and Biocontrol-treated grapes. The alpha diversity of the bacterial and fungal communities in control grapes and wines after AF was lower than in Biocontrol samples, and the ecosystem showed no signs of risk or threat of loss of diversity. More similar studies during more years are necessary for stablishing this preliminary result as definitive and for ensuring the safety of the biocontrol in the vineyard.

1. Introduction

*Erysiphe (Erys.) necator (*formerly *Uncinula necator*), the causative agent of grapevine powdery mildew, is a widespread disease that can reduce the yield and quality of grapes, and compromise wine quality [1].

This fungus withstands the winter in sclerotia form till the spring season when it produces asci and ascospores that cause the primary infection, moreover, it produces conidia that triggers the secondary spread of the infection. Thus, *Erys. necator* might infect different parts of the grapevine (shoots, leaves, inflorescences or berries) what entails important economic losses for viticulture and oenological industries [2,3].

The most representative effect of the infection is the appearance of a grey or white pow on both sides of the leaf corresponding with the conidia development and the presence of dark green or brown dots in vine shoots. When the infection becomes important the branches can get dry very quickly. During the berry's development, the fungal conidia coat them with a grey dust that usually causes the chapping and even the breakage of the grapes. Consequently, the yield is importantly reduced after the infection and, besides this, the disease makes easier the affectation by other microorganisms such as fungi or bacteria that may spoilt the final wine quality [2].

There are some studies that quantified the current economic loss such as Scott [1] that talked about $76M per year only in Australia, and Moine et al. [4] who cited $239M in California the cost of managing the powdery mildew disease only in 2015.

The traditional way of managing the *Erys. necator* presence is the application of sulfur-based products, sometimes combined with other chemical substances like copper at several times during the vegetative state of the vines [4,5]. These intensive chemical treatments do not represent an ecofriendly and sustainable strategy due to the environmental risks and to the emerging resistances of the treated fungi. In fact, the European Commission is limiting the chemical products applied in viticulture, the number of applications per year and even the quantity of active substance applied [6].

Due to these foreseeable limitations in the application of chemical fungicides, biological treatments are being implemented. Thus, biological control of *Ery. necator* has been carried out in wheat and marigold with the application of different microorganisms such as *Trichoderma* [7,8]and in vineyard [9] with *Brevibacillus* [10] and *Bacillus subtilis* [11],

* Corresponding author. ICVV, Instituto de Ciencias de la Vid y el Vino (Universidad de La Rioja, Gobierno de La Rioja, CSIC). Finca La Grajera, Ctra LO-20- salida 13, 26071, Logroño, Spain.

E-mail addresses: lucia.gonzalez@icvv.es, lucia.gonzalez@unirioja.es (L. González-Arenzana).

https://doi.org/10.1016/j.jafr.2024.101420
Received 16 July 2024; Received in revised form 6 September 2024; Accepted 12 September 2024
Available online 14 September 2024
2666-1543/© 2024 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

showing variable efficacies according to different factors such as application dose. None of these studies have looked in depth at the impact of these applications on the microbiota of the crop, nor of the fruit, but have focused on the effectiveness of the treatment against infection.

Nowadays, the biological control of the powdery mildew disease or of *Erys. necator* was possible after the industrial exploitation of *Bacillus (B.) pumilus* strain QST2808. This strain produces an antifungal amino sugar compound that disrupts the cell metabolism and destroys cell walls. Moreover, it creates a zone of inhibition on plant surfaces, which prevents fungi from establishing a foothold in the plant, and a physical barrier between the plant leaf and the fungal spores. This strain can also colonize the spores acting as a fungicide. Like several other *B. pumilus* strains, this strain can stimulate the plant's own resistance system by inducing systemic acquired resistance. Since 2013, the European Food Safety Authority (EFSA) has been studying the risk assessment of the active substance of this strain approving their application of farms, or vineyards, and it has also reviewed the legality of the residues concluding that it is a safety strategy of biocontrol for organisms and environments ([12,13]; European Food Safety Authority, 2013). These characteristics make *B. pumilus* strain QST 2808 particularly effective for the control of fungal diseases in plants.

The microorganisms involved in the fermentative stages of winemaking are mainly yeast and bacteria that proceed partly from grapes, so that they could be present in the vineyard. There are several genera and species of yeasts that could be present in the grape surface, but the most important one is *Saccharomyces* (*S.*) *cerevisiae* because it leads the alcoholic fermentation (AF). The bacteria population present in an enological environment can be described as Gram-negative aerobic species, mainly acetic acid bacteria (AAB) or environmental bacteria (EB), and Gram-positive bacteria represented by the lactic acid bacteria (LAB). Regarding bacteria, the AAB and some of the LAB are spoilage microorganisms, the EB role in winemaking is still unknown and among the LAB, *Oenococcus* (*O.*) *oeni* is the main agent of FML [14]. Every treatment applied to the leaves, root, branches, grapes, etc. might disrupt the microbial ecosystem of grapes what could change the natural development of alcoholic and malolactic fermentation even altering the final wine quality [15]. According to previous research about biocontrol, there is no evidence of negative affectation of this type of ecofriendly fungi management in the wine quality, but any of those studies have been focused on the use of *B. pumilus* QST 2808 against powdery mildew [16].

Currently, the study of the microbial populations involved in grape or wine should not be limited to culture dependent techniques because it is well known that natural and wild populations, even of pathogenic microorganisms, could stay in viable but not culturable forms [17]. This means that a great percentage of the viable, alive and totally active microorganisms are not able to grow in culture media so that results of culture dependent technique might be biased from reality [18–21].

This study was planned with one goal that was the description of the alterations of the microbial populations of yeasts and bacteria using culture independent methods after the treatment of a vineyard with *B. pumilus* QST2808.

2. Materials and methods

2.1. Vineyard treatments

To minimize biases from climatic or agronomic conditions, treatments were applied within the same vineyard This study focuses on the typical profile of vine cultivation (*Vitis vinifera* L.) within the Rioja Qualified Designation of Origin (D.O.Ca. Rioja), specifically the Tempranillo variety, planted in 1984 and grown as free-standing vines. The vines were spaced 1.35 × 2.55 m apart, resulting in a plant density of 2905 plants per Ha. The vineyard's soil was managed through tillage.

Two treatments were applied five times after fruit set. The control treatment involved applying 4 kg/ha of Elosal GD (Bayer Crop Bioscience S.L.), a product containing 80 % sulfur, which is the traditional treatment used in the vineyard. The other treatment was a biological fungicide (Sonata®, Bayer Crop Bioscience S.L.), containing 14.35 g of *Bacillus pumilus* strain QST 2808 per liter of the commercial product, applied at a dose of 5 L/ha. Treatments were administered using an automatic knapsack sprayer.

A randomized block design was used for the experiment, with four repetitions per treatment placed at the same row and an average of 33 plants per replicate. Each replicate received the same agronomic management prior to the treatments and was harvested and vinified separately at the optimal time. The vineyard showed no signs of Powdery mildew before the treatments.

2.2. Winemaking conditions

Once a probable alcoholic strength of 13 % was reached, each replicate was harvested, destemmed and crushed and vinified separately in 100 L tanks in the ICVV experimental cellar. The tanks were sulphited to a concentration of 50 mg SO_2/L and kept at 25 °C during spontaneous alcoholic fermentation (AF). Daily, they were punched down and the decrease in density was determined as a measure of sugar consumption. When they reached a density of approximately 990, they were pressed and allowed to finish AF (reducing sugars <2 g/L). The wines were then racked into smaller tanks (50 L) for spontaneous malolactic fermentation (MLF) to begin. MLF was controlled by periodic measurement of malic acid (enzymatic method) until its depletion.

2.3. Sampling and microbiological analysis

The microbiological study of samples was performed at three different times. The first sampling was carried out on freshly harvested grapes, before reaching the winery as Escribano-Viana et al. [16] described. The second sampling was with wines at the end of spontaneous AF, the third one with wine when the spontaneous MLF was depleted.

The microbiological features of samples were analyzed with culture independent methods that allowed the detection of every microorganisms (culturable and non-culturable). For this purpose, the technique employed was the Next Generation sequencing (NGS) or massive sequencing.

Samples from the biomass harvested from grapes in PT, musts and wines were frozen at −80 °C in a volume of 10 mL. Then, the DNA was directly extracted from those samples following the protocol described by González-Arenzana et al. [22]. The DNAs collected from the four replicates of each sample were mixed in a unique sample for performing the massive sequencing focused on Fungi and Bacteria Kingdoms at three different samplings. The Control and Biocontrol-treated samples were completely analyzed by the Foundation for the Promotion of Health and Biomedical Research of Valencia Region (FISABIO) (Valencia, Spain) with NGS or massive sequencing. Every sample passed the quality control and then continued for the library construction.

Yeast community was studied with the Intergenic Transcribed Spacers (ITS) of fungal organisms that were amplified according to the work of Toju et al. [23]. In the case of bacteria, 16 S rDNA gene amplicons were obtained following the 16 S rDNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Cod. 15,044,223 Rev. A). The gene-specific sequences used in this protocol target the 16 S rDNA gene V3 and V4 region and the primers were selected from Klindworth et al. [24]. After ITS and 16 S rDNA amplifications, the multiplexing step was performed using Nextera XT Index Kit. A bioanalyzer was used to verify the size of amplicons and when size was around 500 b b libraries were sequenced using a 2 × 300pb paired-end run (MiSeq Reagent kit v3 on a MiSeq Sequencer according to manufacturer's instructions (Illumina). Quality assessments were performed using prinseq-lite program [25]. R1 and R2 from Illumina sequencing

were joined using the FLASH program [26].

Data were obtained using an ad-hoc pipeline written in RStatistics environment (R Core Team, 2012), making use of several Open-Source libraries such as gdata, vegan, etc. Then, the sequence data were analyzed using qiime 2 pipeline [27].

Denoising, paired ends joining, and chimera depletion was performed starting from paired ends data using DADA2 pipeline [28]. The taxonomic annotation was performed through taxonomic affiliations assigned using the Naive Bayesian classifier integrated in quiime2 plugins. Database used for ITS taxonomic assignation was the KRONA viewer report have been generated using Krona hierarchical browser [29]while for SILVA taxonomic assignation the SILVA_release_132 was used [30]. Tables of OTUs (Operational Taxonomic Units) abundance (%) in each sample were provided by FISABIO.

2.4. Statistical analysis of the alpha diversity parameters

The Alpha diversity of samples was studied with the Simpson index that measures the possibility that two randomly chosen individuals belong to different species [31] and with the Shannon-Weaver index (H) that considers the number of individuals as well as the number of species [32,33]. Those indexes were calculated using the software PAST (V. 4.17) [34]. In addition, statistical analysis of these numerical diversity indices was carried out between control and treated samples at each sampling time, using Student's *t*-test with this software.

3. Results and discussion

Grape weight, pH and probable alcoholic strength followed the expected pattern of the ripening process, increasing until the time of harvest and reaching the usual values for Tempranillo grapes of the D.O. Ca. Rioja appellation of origin (data not shown). Powdery mildew disease was not noticed in situ.

3.1. Microbial characterization of grape surfaces in control and biocontrol-treated samples

Massive sequencing data of OTUs abundance in grape samples are shown in Table 1. Bacteria were mainly represented in control and Biocontrol-treated samples by the Order Chloroplast that belongs to the Phylum Cyanobacteria with 77.4 % and 74.5 %, respectively (Fig. 1). These are photosynthetic bacteria so that they are easily accessible in open environments like vineyards but there were no other studies that found this type of bacteria in the grape's biofilm.

With around the 22.6 % in control grapes and 25.45 % in Biocontrol-treated ones, the Phylum Proteobacteria. Belonging to this Phylum, the Class Alphaproteobacteria was found. It is characterised for being oligotrophic, what means that they do not need very high quantity of nutrients to survives. This Class was represented by the Orders Acetobacterales and Rickettsiales (Fig. 1). The first one included the Family Acetobacteraceae that was more representative into the Biocontrol-treated grapes than in control ones. This Family included two genera. Into the genus *Gluconobacter (G.),* and with similar percentages of abundance, the species *G. cerinus* was found with very similar abundance in both samples. This species is an AAB typically found in grapes at the first stages of AF [35]. Moreover, with lower percentages (minor than 0.5 % in control grapes, and 1.91 % in Biocontrol-treated grapes) the species *Komagataeibacter (K.) intermedius,* an AAB usually linked with the vinegar production, was identified [36]. Other Order of the Class Alphaproteobacteria, Rickettsiales, was represented by the Family Mitochondria and had in control grapes percentages of abundance of 13.8 % and in Biocontrol-treated grapes 12.3 %. The Order Rickettsiales has been reported as a dangerous human pathogen [37] like some of the AAB found in those grape surfaces that have been thought to cause human diseases [38]. The Class Gammaproteobacteria was represented by two Orders, Betaproteobacteriales and Orbales, similarly represented in the two samples (Fig. 1). The Family Burkholderiaceae consisted of the genus *Ralstonia (R.)* and the species *R. picketti*, with percentages of 3.17 % and 2.14 % in control and Biocontrol-treated samples,

Table 1
Colour scale of percentage of identified OTUs, with more than 0.5 % abundance, in control and treated grape samples, with ITS and 16s rDNA massive sequencing. Green dark colour means the highest abundance and white colour the lowest one.

Kingdom	Phylum	Class	Order	Family	Genus	*Species*	Control Grapes	Sonata Grapes
Bacteria	Cyanobacteria	Oxyphotobacteria	Chloroplast	-	-	-	77.4	74.5
	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Gluconobacter*	*G. cerinus*	1.15	1.72
					Komagataeibacter	-	0.63	2.60
						K. intermedius	<0.5	1.91
						Gluconacetobacter sp.	<0.5	0.76
			Rickettsiales	Mitochondria	-	-	13.8	12.3
		Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	*Ralstonia*	*R. pickettii*	3.17	2.14
			Orbales	Orbaceae	-	-	<0.5	0.6
						Bacteria OTUS < 0.5%	3.85	3.45
Fungi	Ascomycota	Dothideomycetes	Capnodiales	-	-	-	<0.5	0.54
				Cladosporiaceae	*Cladosporium*	-	0.71	0.64
			Dothideales	Aureobasidiaceae	*Aureobasidium*	*Au. pullulans*	14.1	18.7
			Pleosporales	Pleosporaceae	*Alternaria*	-	0.75	1.03
		Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*	-	0.90	0.77
		Leotiomycetes	Erysiphales	Erysiphaceae	*Erysiphe*	*Erys. necator*	<0.5	1.13
			Helotiales	Sclerotiniaceae	*Botrytis*	-	43.7	54.7
		Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Saccharomyces*	-	1.49	<0.5
				-	-	-	26.9	4.58
				Saccharomycodaceae	*Hanseniaspora*	*H. uvarum*	6.69	7.41
	Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	*Rhodotorula*	*Rh. graminis*	1.03	2.43
					Sporobolomyces	*Spo. roseus*	0.90	0.85
		Tremellomycetes	Filobasidiales	Filobasidiaceae	*Filobasidium*	*Fi. magnum*	1.15	2.23
						Fungi OTUs <0.5 %	1.76	2.32

- OTU no identified at that level

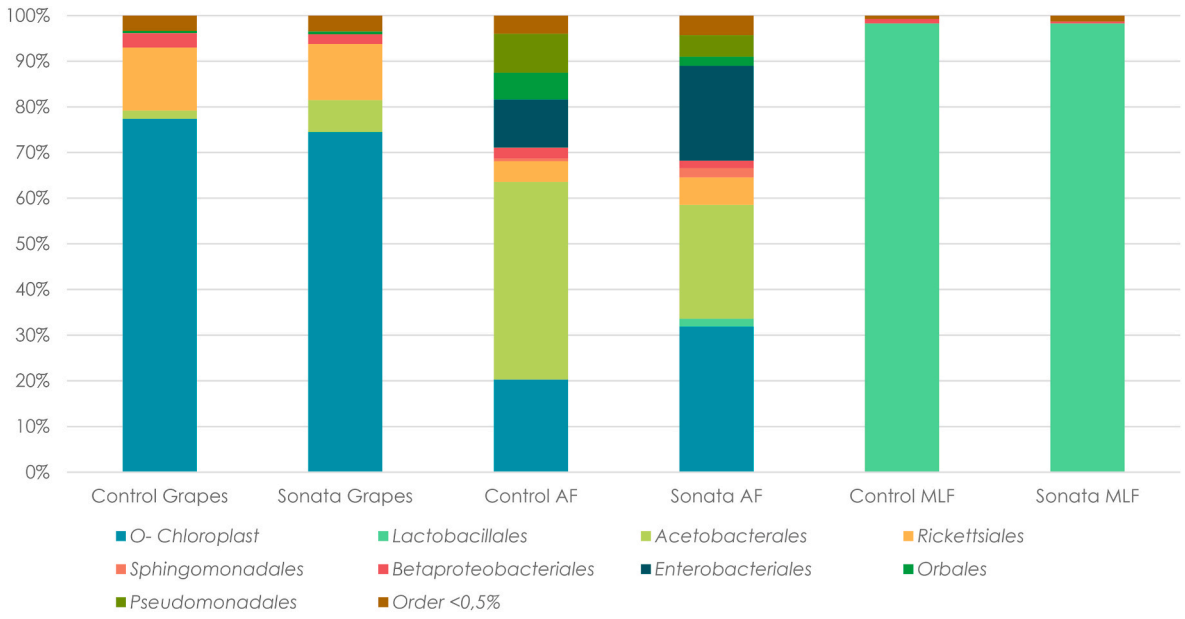

Fig. 1. Percentages of bacterial Orders identified in Control and Sonata treated samples of grapes, wines after the alcoholic fermentation (AF) and after the malolactic fermentation (MLF).

respectively. This species is considered dangerous in medical environments because of their ability to form biofilms in several surfaces [39] so that it may be easy find it in grape biofilm. Into this same Class, the Family Orbaceae was found but with very low percentages (Biocontrol-treated grapes with 0.6 %) thus without a very important role in this vineyard microbiome.

Finally, the percentages of bacteria OTUs identified with percentages of abundance minor than 0.5 % were similar between samples, 3.85 % in control grapes and 3.45 % in grapes Biocontrol-treated with *B. pumilus* strain QST 2808. Although these percentages were not excessively high, only three OTUs were identify at species level in the bacterial community of grapes. Apparently, the identification at genus level was more successful than at species level using NGS.

Related to OTUs identified with ITS massive sequencing (Table 1), two Phyla were described: Ascomycota and Basidiomycota. The Phylum Ascomycota was represented by four Classes, between them the Dothideomycetes, that included the Order Capnodiales. This Order had low percentages of abundances (Fig. 2), in fact the genus *Cladosporium* was the 0.71 % and the 0.64 % of control and Biocontrol-treated grape fungal communities. Inside this same Class, the Order Dothideales, represented by the Family Aureobasidiaceae, the genus *Aureobasidium (Au.)* and the species *Au. pullulans* reached high representation percentages in both types of samples, being the 14.1 % of fungi in control grapes and the 18.7 % in Biocontrol-treated ones (Fig. 2). Usually linked to winemaking environment, this species has been traditionally related to grape microbial communities [40] and it has been also determined as important in similar studies [41] The Order Pleosporales was the last included in this Class (Fig. 2). It was represented by the Family Pleosporaceae and by the genus *Alternaria* but with low percentages of abundance (0.75 % and 1.03 % in control and Biocontrol-treated grapes, respectively). The Class Eurotiomycetes was represented by the genus *Penicillium,* a usual fungus from natural environments, with 0.90 % in control grapes and with 0.77 % in Biocontrol-treated ones. The Class Leotiomycetes was represented by the Order Erysiphales (Fig. 2) with the genus *Eryshipe*

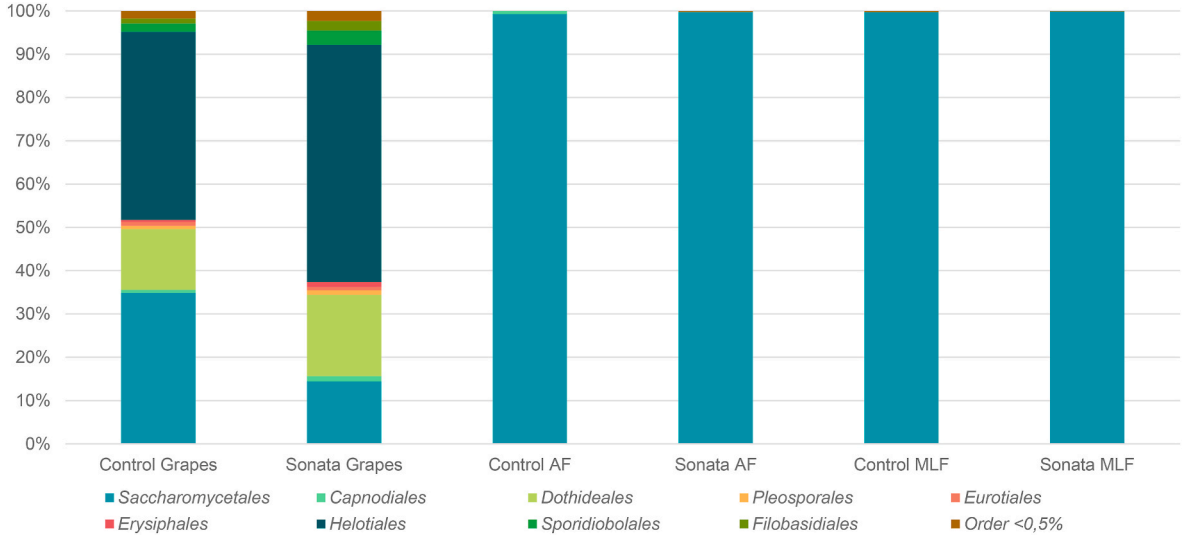

Fig. 2. Percentages of Fungi Orders identified in Control and Sonata treated samples of grapes, wines after the alcoholic fermentation (AF) and after the malolactic fermentation (MLF).

and the species *Erys. necator* that caused the powdery mildew [42]. This fungus was in low percentages of abundance but, curiously, it was slightly higher in Biocontrol-treated grapes (1.03 %) than in control grapes (<0.5 %), but the infection was not visually perceived in the harvested grapes. Belonging to this same Class, the Family Helotiales, represented by the genus *Botrytis*, was the most important one in this study [43]. *Botrytis* was the 43.7 % of the fungal community of control grape samples and the 54.7 % of Biocontrol-treated grape samples.

The Class Saccharomycetales and the Order Saccharomycetales was represented by three families, and it was more important in control grapes than in Biotreated ones, what could mean sensitivity of this yeast to the biofungicide effect of *B. pumilus QST 2808*. The Family Saccharomycetaceae was represented by the genus *Saccharomyces* in very low percentages, being in control grapes lower than 1.49 % and in Biocontrol-treated grapes minor than 0.5 %. The species *S. cerevisiae* is the most important agent of AF and the NGS was not able to find it in grapes. Eventually, the Family Saccharomycodaceae was represented by the species *Hanseniaspora (H.) uvarum* that as *Au. Pullulans, Erys. Necator*

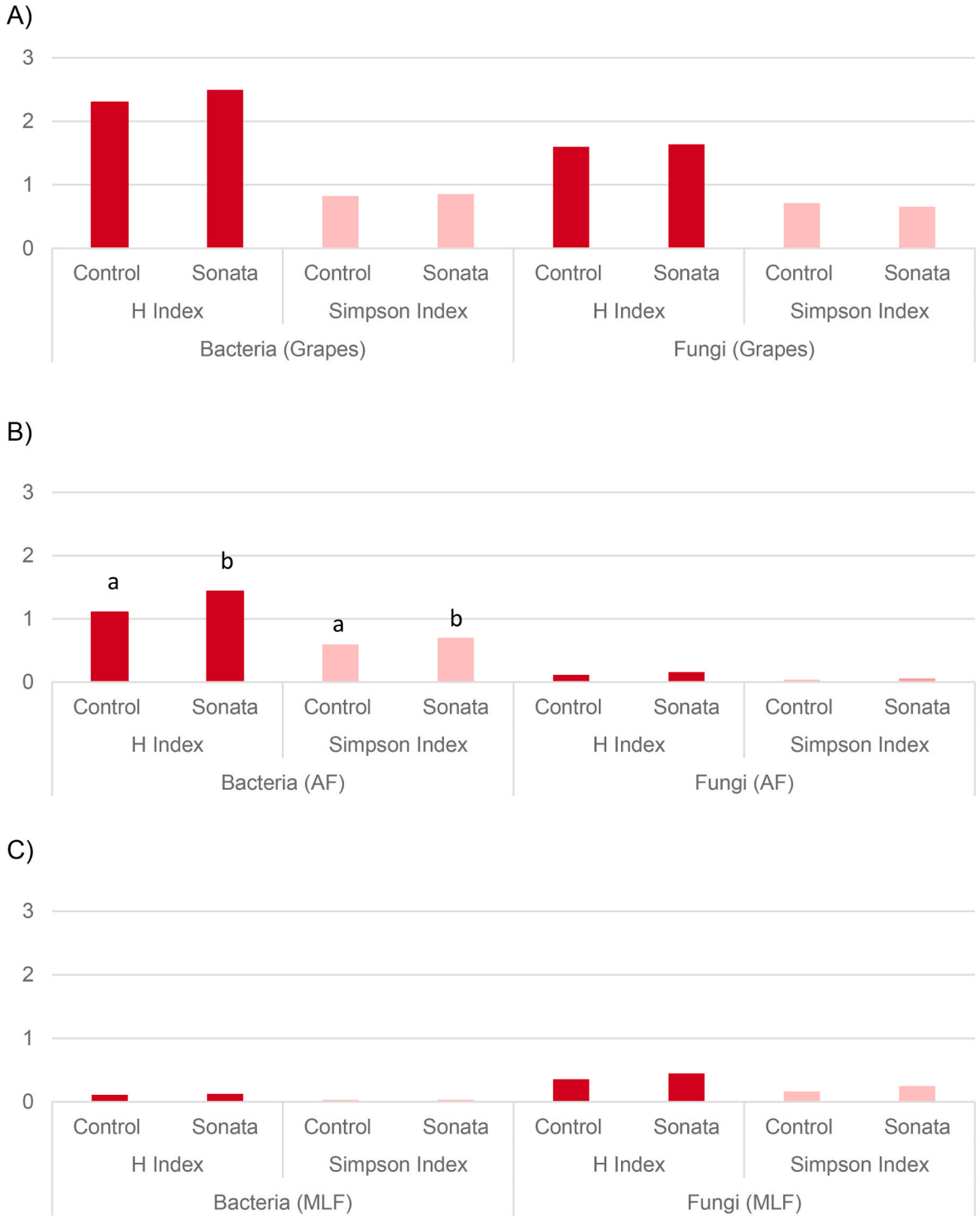

Fig. 3. Alpha diversity parameters (H Index and Simpson index) of Control and Sonata treated samples: A) grapes, B) alcoholic fermentation (AF) and C) malolactic fermentation (MLF). Different letters mean significant differences between control and treated samples for each index ($p < 0.05$).

or even the genus *Botrytis* are thought to be important in early stages of vinification and of course on grape biofilm.

The Phylum Basidiomycota was represented by the Order Sporidiobolales (Fig. 2). This Family included the genus *Rhodotorula (Rh.)* with the species *Rh. graminis* and *Sporobolomyces (Spo.) roseus* was found with percentages of abundance minor than 1 %. Finally, in control and Biocontrol-treated grape samples the 1.15 % and the 2.23 %, respectively, of the fungi community was identified as the species *Filobasidium (Fi.) magnum* that belongs to the Class Tremellomycetes.

The percentage of fungi OTU with abundances minor than 0.5 was of 1.76 % in control grapes and 2.32 % in Biocontrol-treated ones. This minority OTUs was lower than the described for bacteria at the same stage.

The Alpha diversity parameters of grape samples are shown in Fig. 3A). In the context of Alpha diversity, the H-index often refers to the Shannon Diversity Index, denoted as H. This index is a measure of species diversity within a community, considering both species richness or the number of different species and species evenness that is how evenly the individuals are distributed among the species [33]. The higher the value of H, the higher the diversity of species in a particular community. The lower the value of H, the lower the diversity. Regarding The Simpson's Diversity Index, it is a measure used to quantify also the diversity of a biological community. The value for Simpson's Diversity Index ranges between 0 and 1 and the higher the value, the lower the diversity [31].

The H index of bacterial community was a little bit lower in control grapes than in Biocontrol-treated grapes, but Simpson index was practically the same. This tendency was also observed in Fungi community, thought diversity parameters were under the described for bacteria. In any case the differences were statistically significant. In this study, the fungi community of grape biofilm was less rich and diverse than bacteria. In contrast, a slight increase of richness and diversity was observed in bacteria after the treatment with the biocontrol agent *B. pumilus* strain QST 2808. Similar results were observed in studies that tested effect of one biocontrol agent against *Botrytis* [16]. Generally, when an ecosystem is endangered, the Alpha diversity parameters are thought to suffer a reduction so that grape biofilm after Biocontrol-treated treatment may not be threatened or disturbed, in addition no evidence of genetic material of *B. pumilus* was found at grape biofilms.

Other authors have established that the grapes and the early fermentative stages are usually the most diverse stages in microbiological terms related to bacteria population [44] and also to yeast or fungi population [45]. There are also several studies that recognises the detection of bacteria and yeasts, with NGS methods, that are not usually found in winemaking [44–47].

3.2. Microbial characterization of wines after alcoholic fermentation from control and biocontrol-treated samples

Overall, the spontaneous AF of control and Biocontrol-treated samples occurred without complications and had similar durations (data not shown).

Massive sequencing data of OTUs abundance in wine samples from control and Biocontrol-treated grapes after their AF are shown in Table 2. Bacterial community of Biocontrol-treated wines were highly represented by the Order Chloroplast with 31.9 % while in the control wines this Orden meant the 20.3 %. Curiously, this Orden of photosynthetic bacteria was important the grape surface and in the spontaneous AF of both types of samples (Fig. 1).

Inside the Bacteria Kingdom the Phylum Firmicutes was represented

Table 2
Colour scale of percentage of identified OTUs, with more than 0.5 % abundance, in control and treated wine samples after the alcoholic fermentation (AF) with ITS and 16s rDNA massive sequencing. Green dark colour means the highest abundance and white colour the lowest one.

Kingdom	Phylum	Class	Order	Family	Genus	Species	Control AF	Sonata AF
Bacteria	Cyanobacteria	Oxyphotobacteria	Chloroplast	-	-	-	20.3	31.9
	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Lactococcus*	-	<0.5	1.71
	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Acetobacter*	-	1.14	6.81
					Gluconobacter	-	0.65	1.65
						G. oxydans	0.67	3.68
						G. cerinus	33.7	12.8
					Komagataeibacter	-	3.75	<0.5
						K. intermedius	3.33	<0.5
			Rickettsiales	Anaplasmataceae	*Wolbachia*	-	1.01	<0.5
				Mitochondria	*Rhynchosporium*	*Rhy. agropyri*	<0.5	0.56
					-	-	3.54	5.44
			Sphingomonadales	Sphingomonadaceae	*Sphingomonas*	-	0.61	2.00
		Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	*Comamonas*	*Comamonas sp.*	0.93	<0.5
					Massilia	-	1.46	1.67
			Enterobacteriales	Enterobacteriaceae	-	-	1.25	1.60
					Erwinia	-	0.66	1.67
					Pantoea	-	8.63	8.61
					Serratia	-	<0.5	8.91
			Orbales	Orbaceae	-	-	5.85	1.98
			Pseudomonadales	Moraxellaceae	*Acinetobacter*	-	0.60	<0.5
				Pseudomonadaceae	*Pseudomonas*	*Ps. graminis*	<0.5	0.74
						-	7.94	4.01
						Bacteria OTUS <0.5%	3.93	4.29
Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Saccharomyces*	-	98.2	97.1
				-	-	-	1.15	1.98
				Saccharomycodaceae	*Hanseniaspora*	*H. uvarum*	<0.5	0.7
						Fungi OTUs < 0.5%	0.69	0.24

- OTU not lidentified at that level.

by the genus *Lactococcus* of the Class Bacilli, into the Order Lactobacillales and the Family Streptococcaceae. This genus was found in percentages of abundance minor than 0.5 % in control samples and with low percentages in Biocontrol-treated wine samples (1.71 %). This Family is usually very well represented in the winemaking environments because it holds important genera and species involved in the MLF, as for instance *Lactobacillus* or *Oenococcus* that in this study were not detected at this stage. In the case of the genus *Lactococcus*, although is not directly an agent of this secondary fermentative stage is usually found in later stages of AF [15]. This Order was not found in the grape surface (Fig. 1).

The Phylum Proteobacteria accounted for 74.6 % and 62.1 % of the bacterial community in the control and Biocontrol-treated wines, with higher percentages of abundance than those observed in the grape sampling. Belonging to this Phylum, the two taxonomic Classes found in the grape biofilms were also detected in the wines after the AF.

First, the Class Gammaproteobacteria was represented by the Order Acetobacterales (Fig. 1) that was more representative in the wines from control grapes, than in the wines from Biocontrol treatment. The Family Acetobacteraceae inside this Order included three genera. The genus *Acetobacter* was the 1.14 % of the bacteria in control wines and the 6.81 % of the bacteria of Biocontrol-treated wines. This genus is directly linked to the acetification of wines, consequently, its high presence in wines may be dangerous if it develops the metabolic acetification of ethanol [48]. As it was described for grapes, the genus *Gluconobacter* was also found, although in wines two species were identified; the species *G. oxydans* was the 0.67 % of the bacteria community of control wines, but again it was the 3.68 % of Biocontrol-treated wines and the species *G. cerinus*, also detected in grape biofilms, reached in both cases high percentages of abundances, especially in control wines samples in which their presence was the 33.7 % of the bacteria community. In addition, the species *K. intermedius* was again identified but with lower percentages (minor than 0.5 % in Biocontrol-treated wines, and 3.33 % in control ones). With those data, it might be thought that in control and Biocontrol-treated samples, the AAB were highly represented, being more important and diverse in control wines with 43.3 % of representation than in Biocontrol-treated wines with 24.9 %. The presence of Botrytis in the grape surface is usually associated with increased AAB populations what could explain the higher presence of these spoilage bacteria in wines, although they were not predominant in grapes. The Order Rickettsiales of the Class Alphaproteobacteria was found not only in grapes but also in wines (Fig. 1). In these wines, this Order was represented by the genus *Wolbachia* of the Family Anaplasmataceae (1.01 % in control wines and lower than 0.5 % in Biocontrol-treated wines), by the species *Rhynchosporium (Rhy.) agropyri,* (0.56 % in Biocontrol-treated wines and minor than 0.5 % in control wines), and by the Family Mitochondria (3.54 % and in Biocontrol-treated wines with 5.44 %). As it was explained for grapes, the importance of the Order Rickettsiales is its pathogeny ability in humans but in the alcoholic matrix of wines its clinical or oenological role is still unknown. At this stage, the genus *Sphingomonas* of the Order Sphingomonadales (Fig. 1) that is taxonomically included in the Class Alphaproteobacteria was identified. This genus is widely found in nature but can also cause infection because of its opportunistic character [49].

The Class Gammaproteobacteria was represented by four Orders and five Families. The Family Burkholderiaceae, also detected in grapes, consisted of the genus *Comamonas* with percentages of 0.93 % and minor than 0.5 % in control and Biocontrol-treated samples and of the genus *Massilia* with 1.47 % and 1.67 %, respectively. Curiously, those environmental bacteria usually found in open ecological niches, like soils, are ubiquitous gram-negative aerobic and motile bacteria that can go through AF [50]. The Order Enterobacteriales (Fig. 1), with the Family Enterobacteriaceae, was the 10.5 % of the bacteria community of control wines and the 20.8 % of wines whose grapes were Biocontrol-treated with *B. pumilus* strain QST 2808. This Family contains gram-negative facultative anaerobic usually named “enteric bacteria”. In control wine the genera *Erwinia*, *Pantoea* and *Serratia* were detected, being *Pantoea* the 8.63 % of the bacteria community. These genera are ubiquitous bacterium that has been used for soil remediation and for other industrial processes and that are considered important host’s promotor in plants and animals [51,52]. Probably the anaerobic conditions of the AF allowed its presence in wines. In Biocontrol-treated wines, these three genera were found but, in this case, *Pantoea* was the 8.61 % and *Serratia* the 8.91 %. Into this same Class, but in the Order Orbales (Fig. 1), the Family Orbaceae was again found, as in grapes, but with higher percentages (control wines 5.85 % and Biocontrol-treated wine with 1.98 %). In these wines, the Order Pseudomonadales (Fig. 1) was represented by the genus *Pseudomonas (Ps.)*. This Order is composed by gram-negative, motile, aerobic bacillus. The representing species of the genus in this study was *Ps. graminis* that is thought to be useful in biocontrol of food pathogens, so that its presence may be important in wines, but they have scarce percentages of abundances (minor than 0.5 % in control wines and 0.74 % in Biocontrol-treated wines) [53].

Eventually, the percentages of bacterial OTUs identified with percentages of abundance minor than 0.5 % was 3.93 % in control wines and 4.29 % in wines from grapes Biocontrol-treated with *B. pumilus* strain QST 2808; these results were similar in grape and wine stages. As Table 2 shows most of the identification at species level was not possible, therefore, similarly to grape biofilms it seems that identification by NGS is more successful and accurate at genus level.

Regarding the OTUs identified with ITS massive sequencing (Table 2), everyone belonged to the Phylum Ascomycota, to the Class Saccharomycetes and to the Order Saccharomycetales (Fig. 2) in the two types of samples. The Family Saccharomycetaceae was represented by the genus *Saccharomyces* in control wines with a 98.2 % of abundance and in Biocontrol-treated wines with a 97.1 %. The predominance of this genus was expected because it contains the main species for the AF, *S. cerevisiae*, that was not found by NGS. There was a 1.15 % in control wines, and a 1.98 % in Biocontrol-treated wines, of OTUs identified inside this same Order but impossible to locate within the classification at Family level. Eventually, the Family Saccharomycodaceae was represented by the genus *Hanseniaspora (H.)* and by the species *H. uvarum*. This was scarcely represented by minus than 0.5 % in control wines and 0.7 % in Biocontrol-treated wines.

The percentage of fungi OTUs with abundances below 0.5 was 0.69 % in control wines and 0.24 % in Biocontrol-treated wines. Minority OTUs were rather low compared to the results described for bacteria and due to the predominance of some species of the genus *Saccharomyces*, probably the species *S. cerevisiae* which was not identified at species level by NGS despite its high relative abundance.

The Alpha diversity parameters of wine samples are shown in Fig. 3B). The H index and the Simpson index of bacterial and fungi community were lower in control wines than in Biocontrol-treated wines, but the differences were statistically significant only in the bacteria community. This tendency was more evident in bacteria than in fungi although diversity indexes were minor in wine samples than the observed in grape biofilms. Higher diversity indexes mean higher diversity, so that, at this stage after AF, the biofungicide treatment had a beneficial impact on terms of diversity of the bacteria community.

3.3. Microbial characterization of wines after malolactic fermentation from control and biocontrol-treated samples

Overall, the spontaneous MLF of wines elaborated with control and Biocontrol-treated samples occurred without complications and had similar durations (data not shown).

Massive sequencing data of OTUs abundance in wine samples after MLF are shown in Table 3. Bacteria were mainly represented in control and Biocontrol-treated samples after MLF by the species *Oenococcus (O.) oeni*, that belong to the Order Lactobacillales (Fig. 1), with percentages of 98.3 % in both. It was detected by NGS only at this sampling stage. The other species detected was *R. picketti* with 0.93 % of abundance in

Table 3
Colour scale of percentage of identified OTUs, with more than 0.5 % abundance, in control and treated wine samples after malolactic fermentation (MLF) with ITS and 16s rDNA massive sequencing. Green dark colour means the highest abundance and white colour the lowest one.

Kingdom	Phylum	Class	Order	Family	Genus	Species	Control MLF	Sonata MLF
Bacteria	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	*Oenococcus*	*O. oeni*	98.3	98.3
	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	*Ralstonia*	*R. pickettii*	0.93	< 0.5
						Bacteria OTUS <0.5%	0.74	1.32
Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Saccharomyces*	-	91.1	85.6
				-	-	-	7.21	13.5
				Saccharomycodaceae	*Hanseniaspora*	*H. uvarum*	1.45	0.70
						Fungi OTUs <0.5 %	0.27	0.11

- OTU no identified at that level.

control samples and minor than 0.5 in Biocontrol-treated samples. This species was also detected in grape biofilms but is not very usual find it in wines at the final stage of the winemaking process.

Finally, the percentages of bacterial OTUs identified with percentages of abundance minor than 0.5 % were 0.74 % in control wines after MLF and 1.32 % in Biocontrol-treated wines what was considerably lower than the observed in the previous samplings and what was probably due to the predominance of *O. oeni* species. Moreover, the identification at species level of bacteria was successful only at this fermentative stage.

Related to OTUs identified with ITS massive sequencing (Table 3), results were like the described in grape biofilms, but with different percentages. In this sampling after MLF, the Order Saccharomycetales (Fig. 2) was represented by the genus *Saccharomyces* in control wines after MLF with a 91.1 % of abundance and in Biocontrol-treated wines with an 85.6 %. These results were expectable because after the AF *Saccharomyces* is usually found in great percentages. There was a 7.21 % in control wines after MLF, and a 13.5 % in Biocontrol-treated samples, of OTUs identified inside this same Order considered *incertae sedis*. Again, as it was described for all that sampling stages, biofilm the Family Saccharomycodaceae was represented by *H. uvarum* with abundances of 1.45 % in control wines after their MLF and 0.7 % in Biocontrol-treated ones.

The percentage of fungi OTU with abundances minor than 0.5 was of 0.27 % in control samples and 0.11 % in Biocontrol-treated ones. Overall, this minority OTUs was very similar to the described for bacteria of FML stage and probably due to the predominance of bacteria and yeasts Phyla.

The Alpha diversity indices of control and Biocontrol-treated samples after the MLF are shown in Fig. 3C). Diversity of bacteria community was similar between control ad Biocontrol-treated MLF. Only at this stage, diversity of bacteria was minor than fungi diversity, due to the prevalence of *O. oeni*. Fungal diversity of control wines was slightly lower than the observed in wines from grapes Biocontrol-treated with the biofungicide *B. pumilus* strain QST 2808. It was also slightly higher than in wines after AF. The Alpha diversity of both samples was not statistically different so that it would be consider that both treatments did not endanger the microbial ecosystems of winemaking.

There is a lot of research on the most efficient way to apply biocontrol treatments in vineyards [10,54,55] but there are few publications on the impact of this type of treatment on the development of winemaking and on the effects of microbial populations. Escribano-Viana et al. [16,56] found that biocontrol treatment against *B. cinerea* with *Bacillus subtilis QST713* had no negative consequences on grape and wine quality and fermentation processes. The same author explored the implications of this biocontrol on the microbial population and described that the biofungicide had no noticeable impact on the wine microbiota, but some *Bacillus* strains were still present at the end of AF, demonstrating their resistance to the winemaking environment [16, 56]. In contrast, in the present study, *B. pumilus QST 2808* was not observed at any sampling stage, which undoubtedly facilitates its application in the vineyard.

4. Conclusion

Taking into consideration the exposed results, it could be concluded that the Bacteria were mainly represented in control and Biocontrol-treated grape samples by the Order Chloroplast that belongs to the Phylum Cyanobacteria and by the Phylum Proteobacteria, being bold the Family Mitochondria. The bacterial community of wines after the alcoholic fermentation of control and Biocontrol-treated grapes were also mainly represented by these two Phylum, but the Phylum Proteobacteria was identified with higher percentages of abundance that the described in the grape biofilms. Moreover, the acetic acid bacteria were highly represented, being more important and diverse in control wines with 43.3 % of representation than in Biocontrol-treated wines with 24.9 %. The Family Enterobacteriaceae was important in wines whose grapes were Biocontrol-treated with *B. pumilus* strain QST 2808, but their oenological implications are still unknown. The bacteria were represented in control and Biocontrol-treated samples after MLF by *O. oeni,* as it was expected.

Among the Fungi population, the presence of Powdery mildew was determined by NGS in Bio-treated control grapes but with very low percentages. Curiously in these grapes treated with *B. pumilus* it was also found an important presence of *Botrytis*. *Au. pullulans* reached high representation percentages in both types of wine samples while the species *Erys. necator* was in low percentages of abundance. In wines, the genus *Saccharomyces* in control and in Biocontrol-treated samples was identified as the majority one, but the technique NGS did not enable the detection of the species *S. cerevisiae* that is the most important agent of AF.

In general, most of the NGS identifications were more accurate at the genus level and the genetic material of the microorganism applied at the vineyard as biocontrol strategy was not found in the grape biofilms.

The Alpha diversity of the bacterial and fungal community of control grapes and wines after AF was lower than that of samples treated with Biocontrol, but wines after MLF showed similar diversity parameters. Generally, a threatened ecosystem tends to show poor diversity, so it can be concluded that the biocontrol of *B. pumilus* strain QST 2808 did not cause ecological damage or loss of biodiversity in that oenological environment.

Similar studies, over more than one vintage, are necessary to corroborate that these results are maintained over time, and that the microbial ecosystem remains stable after this biological treatment, causing no major effects on the achievement of the winemaking stages.

CRediT authorship contribution statement

L. González-Arenzana: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **P. Santamaría:** Writing – review & editing, Visualization, Validation, Supervision, Data

curation. **A.R. Gutiérrez:** Resources, Data curation, Conceptualization. **R. Escribano-Viana:** Methodology, Investigation, Formal analysis, Conceptualization. **I. López-Alfaro:** Supervision, Resources, Methodology, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Government of La Rioja supported this work under Grant of R. Escribano-Viana BOR nº 34, March 13th, 2015. We also thank the Bayer Crop Bioscience S.L. because of the funding and of the application of those fungicides to the grapevine.

References

[1] E.S. Scott, Grapevine powdery mildew: from fundamental plant pathology to new and future technologies, Australas. Plant Pathol. 50 (1) (2021) 1–6, https://doi.org/10.1007/s13313-020-00761-1.

[2] M. Fernández-González, F.J. Rodríguez-Rajo, O. Escuredo, M.J. Aira, Optimization of integrated pest management for powdery mildew (Unincula necator) control in a vineyard based on a combination of phenological, meteorological and aerobiological data, J. Agric. Sci. 151 (5) (2013) 648–658, https://doi.org/10.1017/S0021859612000743.

[3] H.K. Khiavi, H. Shikhlinskiy, A.B. Ahari, M. Akrami, Study on the biology and epidemiology of *Uncinula necator* the causal agent of grape powdery mildew disease, Life Sci. J. 9 (3) (2012) 1787–1792.

[4] A. Moine, M. Pugliese, M. Monchiero, I. Gribaudo, M.L. Gullino, C. Pagliarani, G. Gambino, Effects of fungicide application on physiological and molecular responses of grapevine (*Vitis vinifera* L.): a comparison between copper and sulfur fungicides applied alone and in combination with novel fungicides, Pest Manag. Sci. 79 (11) (2023) 4569–4588, https://doi.org/10.1002/ps.7660.

[5] S. Möth, M. Redl, S. Winter, F. Hüttner, S. Steinkellner, Efficiency of inorganic fungicides against the formation of Erysiphe necator chasmothecia in vineyards, Pest Manag. Sci. 79 (9) (2023) 3080–3089, https://doi.org/10.1002/ps.7487.

[6] Commission Regulation, Commission Regulation (EC) No 606/2009 of 10 July 2009 laying down certain detailed rules for implementing Council Regulation (EC) No 479/2008 as regards the categories of grapevine products, oenological practices and the applicable restrictions, Off. J. Eur. Union (2009) 1–59, *no. 606/20*(Nº 479/2008).

[7] H.F.A. Ahmed, G.A. Abdel-Wahed, A.M. Mohamed, R.S. Taha, M.F. Seleiman, N. Khan, M.M. Moussa, Using biocontrol agents and sodium nitrophenolate to control powdery mildew and improve the growth and productivity of marigold (*Calendula officinalis* L.), Not. Bot. Horti Agrobot. Cluj-Napoca 52 (1) (2024), https://doi.org/10.15835/nbha52113589.

[8] K.R. Choudaker, V.K. Singh, A.S. Kashyap, A.V. Patel, K.K. Sameriya, D. Yadav, N. Manzar, D. Kamil, L. Prasad, M.S. Saharan, Evaluating the efficacy of microbial antagonists in inducing resistance, promoting growth, and providing biological control against powdery mildew in wheat, Front. Microbiol. 15 (2024), https://doi.org/10.3389/fmicb.2024.1419547.

[9] M.Z. Németh, D. Seress, T. Nonomura, Fungi parasitizing powdery mildew fungi: ampelomyces strains as biocontrol agents against powdery mildews, Agronomy 13 (8) (2023), https://doi.org/10.3390/agronomy13081991.

[10] M. Avan, R. Kotan, E.M. Albastawisi, N. Eftekhari, Biological control of grapevine powdery mildew disease by using *Brevibacillus brevis* strain CP-1, Erwerbsobstbau 65 (6) (2023) 2125–2133, https://doi.org/10.1007/s10341-023-00962-0.

[11] P. Crisp, T.J. Wicks, M. Lorimer, E.S. Scott, An evaluation of biological and abiotic controls for grapevine powdery mildew, 1. Greenhouse studies, Aust. J. Grape Wine Res. 12 (3) (2006) 192–202, https://doi.org/10.1111/j.1755-0238.2006.tb00059.x.

[12] M. Anastassiadou, G. Bernasconi, A. Brancato, L. Carrasco Cabrera, L. Greco, S. Jarrah, A. Kazocina, R. Leuschner, J.O. Magrans, I. Miron, B. Vagenende, A. Verani, Review of the existing maximum residue levels for *Bacillus pumilus QST 2808* according to Article 12 of Regulation (EC) No 396/2005, EFSA J. 18 (5) (2020), https://doi.org/10.2903/j.efsa.2020.6115.

[13] Conclusion, On the peer review of the pesticide risk assessment of the active substance *Bacillus pumilus QST 2808*, EFSA J. 11 (8) (2013), https://doi.org/10.2903/j.efsa.2013.3346.

[14] L. González-Arenzana, P. Santamaría, A.R.A.R. Gutiérrez, R. López, I. López-Alfaro, Lactic acid bacteria communities in must, alcoholic and malolactic Tempranillo wine fermentations, by culture-dependent and culture-independent methods, Eur. Food Res. Technol. 243 (1) (2017) 41–48, https://doi.org/10.1007/s00217-016-2720-2.

[15] L. González-Arenzana, J. Portu, R. López, P. Garijo, T. Garde-Cerdán, I. López-Alfaro, Phenylalanine and urea foliar application: effect on grape and must microbiota, Int. J. Food Microbiol. 245 (2017) 88–97, https://doi.org/10.1016/j.ijfoodmicro.2017.01.017.

[16] R. Escribano-Viana, J. Portu, P. Garijo, A.R. Gutiérrez, P. Santamaría, I. López-Alfaro, R. López, L. González-Arenzana, Evaluating a preventive biological control agent applied on grapevines against *Botrytis cinerea* and its influence on winemaking, J. Sci. Food Agric. 98 (12) (2018) 4517–4526, https://doi.org/10.1002/jsfa.8977.

[17] A. Costantini, E. Vaudano, L. Pulcini, L. Boatti, E. Gamalero, E. Garcia-Moruno, Yeast biodiversity in vineyard during grape ripening: comparison between culture dependent and NGS analysis, Processes 10 (5) (2022), https://doi.org/10.3390/pr10050901.

[18] I. Cools, E. D'Haese, M. Uyttendaele, E. Storms, H.J. Nelis, J. Debevere, Solid phase cytometry as a tool to detect viable but non-culturable cells of *Campylobacter jejuni*, J. Microbiol. Methods 63 (2) (2005) 107–114, https://doi.org/10.1016/j.mimet.2005.02.023.

[19] N. Marouani-Gadri, O. Firmesse, D. Chassaing, D. Sandris-Nielsen, N. Arneborg, B. Carpentier, Potential of Escherichia coli O157:H7 to persist and form viable but non-culturable cells on a food-contact surface subjected to cycles of soiling and chemical treatment, Int. J. Food Microbiol. 144 (1) (2010) 96–103, https://doi.org/10.1016/j.ijfoodmicro.2010.09.002.

[20] J.T. Trevors, Viable but non-culturable (VBNC) bacteria: gene expression in planktonic and biofilm cells, J. Microbiol. Methods 86 (2) (2011) 266–273, https://doi.org/10.1016/j.mimet.2011.04.018.

[21] Z. Wang, Y. Chao, Y. Deng, M. Piao, T. Chen, J. Xu, R. Zhang, J. Zhao, Y. Deng, Formation of viable, but putatively non-culturable (VPNC) cells of beer-spoilage lactobacilli growing in biofilms, LWT 133 (2020) 109964, https://doi.org/10.1016/j.lwt.2020.109964.

[22] L. González-Arenzana, R. López, P. Santamaría, I. López-Alfaro, Dynamics of lactic acid bacteria populations in Rioja wines by PCR-DGGE, comparison with culture-dependent methods, Appl. Microbiol. Biotechnol. 97 (15) (2013) 6931–6941, https://doi.org/10.1007/s00253-013-4974-y.

[23] H. Toju, A.S. Tanabe, S. Yamamoto, H. Sato, High-coverage ITS primers for the DNA-based identification of ascomycetes and basidiomycetes in environmental samples, PLoS One 7 (7) (2012), https://doi.org/10.1371/journal.pone.0040863.

[24] A. Klindworth, E. Pruesse, T. Schweer, J. Peplies, C. Quast, M. Horn, F.O. Glöckner, Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies, Nucleic Acids Res. 41 (1) (2012), https://doi.org/10.1093/nar/gks808 e1–e1.

[25] R. Schmieder, R. Edwards, Quality control and preprocessing of metagenomic datasets, Bioinformatics 27 (6) (2011) 863–864, https://doi.org/10.1093/bioinformatics/btr026.

[26] T. Magoč, S.L. Salzberg, FLASH: fast length adjustment of short reads to improve genome assemblies, Bioinformatics 27 (21) (2011) 2957–2963, https://doi.org/10.1093/bioinformatics/btr507.

[27] J.G. Caporaso, J. Kuczynski, J. Stombaugh, K. Bittinger, F.D. Bushman, E. K. Costello, N. Fierer, A.G. Peña, J.K. Goodrich, J.I. Gordon, G.A. Huttley, S. T. Kelley, D. Knights, J.E. Koenig, R.E. Ley, C.A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, R. Knight, QIIME allows analysis of high-throughput community sequencing data, Nat. Methods 7 (2010) 335.

[28] B.J. Callahan, P.J. McMurdie, M.J. Rosen, A.W. Han, A.J.A. Johnson, S.P. Holmes, DADA2: high-resolution sample inference from Illumina amplicon data, Nat. Methods 13 (7) (2016) 581–583, https://doi.org/10.1038/nmeth.3869.

[29] B.D. Ondov, N.H. Bergman, A.M. Phillippy, Interactive metagenomic visualization in a Web browser, BMC Bioinf. 12 (1) (2011) 385, https://doi.org/10.1186/1471-2105-12-385.

[30] C. Quast, E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, F. O. Glöckner, The SILVA ribosomal RNA gene database project: improved data processing and web-based tools, Nucleic Acids Res. 41 (D1) (2012) D590–D596, https://doi.org/10.1093/nar/gks1219.

[31] P.J. Fedor, I.F. Spellerberg, Reference module in earth systems and environmental sciences, in: Reference Module in Earth Systems and Environmental Sciences, Elsevier, 2013, https://doi.org/10.1016/B978-0-12-409548-9.00602-3.

[32] R. Death, Encyclopedia of ecology, in: Encyclopedia of Ecology, Elsevier, 2008, https://doi.org/10.1016/B978-008045405-4.00117-8.

[33] M. Karydis, G. Tsirtsis, Ecological indices: a biometric approach for assessing eutrophication levels in the marine environment, Sci. Total Environ. 186 (3) (1996) 209–219, https://doi.org/10.1016/0048-9697(96)05114-5.

[34] Ø. Hammer, A.T. H D, D. R P, PAST: paleontological statistics software package for education and data analysis, Palaeontol. Electron. 4 (1) (2001) 1–9, 2001.

[35] E. Mateo, M.J. Torija, A. Mas, E.J. Bartowsky, Acetic acid bacteria isolated from grapes of South Australian vineyards, Int. J. Food Microbiol. 178 (0) (2014) 98–106, https://doi.org/10.1016/j.ijfoodmicro.2014.03.010.

[36] V. Milanović, A. Osimani, C. Garofalo, F. De Filippis, D. Ercolini, F. Cardinali, M. Taccari, L. Aquilanti, F. Clementi, Profiling white wine seed vinegar bacterial diversity through viable counting, metagenomic sequencing and PCR-DGGE, Int. J. Food Microbiol. 286 (2018) 66–74, https://doi.org/10.1016/j.ijfoodmicro.2018.07.022.

[37] M. Rizzo, P. Mansueto, M. Affronti, C. Sferrazza, S. Bucchieri, N. Napoli, G. Cusumano, V. Cannone, F. Catello, G.B. Rini, G. Di Fede, An update on rickettsiosis, Acta Med. Mediterr. 19 (3) (2003) 145–148.

[38] C. Alauzet, C. Teyssier, E. Jumas-Bilak, A. Gouby, R. Chiron, C. Rabaud, F.-P. Counil, A. Lozniewski, H. Marchandin, *Gluconobacter* as well as *asaia* species,

newly emerging opportunistic human pathogens among acetic acid bacteria, J. Clin. Microbiol. 48 (2010) 3935–3942, https://doi.org/10.1128/JCM.00767-10.
[39] M. K Thet, M.L.F. Pelobello, M. Das, M.M. Alhaji, V.H. Chong, M.A.M. Khalil, T. Chinniah, J. Tan, Outbreak of nonfermentative Gram-negative bacteria (*Ralstonia pickettii* and *Stenotrophomonas maltophilia)* in a hemodialysis center, Hemodial. Int. 23 (3) (2019) E83–E89, https://doi.org/10.1111/hdi.12722.
[40] A. Barata, M. Malfeito-Ferreira, V. Loureiro, The microbial ecology of wine grape berries, Int. J. Food Microbiol. 153 (3) (2012) 243–259, https://doi.org/10.1016/j.ijfoodmicro.2011.11.025.
[41] K. Rantsiou, S. Giacosa, M. Pugliese, V. Englezos, I. Ferrocino, S. Río Segade, M. Monchiero, I. Gribaudo, G. Gambino, M.L. Gullino, M.L. Gullino, L. Rolle, Impact of chemical and alternative fungicides applied to grapevine cv nebbiolo on microbial ecology and chemical-physical grape characteristics at harvest, Front. Plant Sci. 11 (2020), https://doi.org/10.3389/fpls.2020.00700.
[42] W. Qiu, A. Feechan, I. Dry, Current understanding of grapevine defense mechanisms against the biotrophic fungus (Erysiphe necator), the causal agent of powdery mildew disease, Hortic. Res. 2 (2015), https://doi.org/10.1038/hortres.2015.20.
[43] J.M. Cantoral, I.G. Collado, R. González, J.M. Cantoral, I.G. Collado, Filamentous fungi (*Botrytis cinerea*), in: Molecular Wine Microbiology, Elsevier, 2011, pp. 257–277, https://doi.org/10.1016/B978-0-12-375021-1.10010-4.
[44] R. Guzzon, M. Malacarne, R. Larcher, E. Franciosi, A. Toffanin, The impact of grape processing and carbonic maceration on the microbiota of early stages of winemaking, J. Appl. Microbiol. 128 (1) (2020) 209–224, https://doi.org/10.1111/jam.14462.
[45] M. Böhmer, D. Smoľak, K. Ženišová, Z. Čaplová, D. Pangallo, A. Puškárová, M. Bučková, T. Cabicarová, J. Budiš, K. Šoltýs, T. Kuchta, T. Szemes, Comparison of microbial diversity during two different wine fermentation processes, FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 367 (18) (2020), https://doi.org/10.1093/femsle/fnaa150.
[46] K.V. Kiselev, N.N. Nityagovsky, O.A. Aleynova, A method of DNA extraction from plants for metagenomic analysis based on the example of grape *Vitis amurensis* rupr, Appl. Biochem. Microbiol. 59 (3) (2023) 361–367, https://doi.org/10.1134/S0003683823030110.
[47] D. Liu, P. Zhang, D. Chen, K. Howell, From the vineyard to the winery: how microbial ecology drives regional distinctiveness of wine, Front. Microbiol. 10 (2019), https://doi.org/10.3389/fmicb.2019.02679.
[48] M.J. Torija, E. Mateo, J.M. Guillamón, A. Mas, Identification and quantification of acetic acid bacteria in wine and vinegar by TaqMan–MGB probes, Food Microbiol. 27 (2) (2010) 257–265, https://doi.org/10.1016/j.fm.2009.10.001.
[49] D.F. Bavaro, M.F. Mariani, E.D. Stea, L. Gesualdo, G. Angarano, S. Carbonara, *Sphingomonas paucimobilis* outbreak in a dialysis room: case report and literature review of an emerging healthcare associated infection, Am. J. Infect. Control (2020), https://doi.org/10.1016/j.ajic.2020.01.018.
[50] Y. Su, Z. He, Y. Yang, S. Jia, M. Yu, X. Chen, A. Shen, Linking soil microbial community dynamics to straw-carbon distribution in soil organic carbon, Sci. Rep. 10 (1) (2020), https://doi.org/10.1038/s41598-020-62198-2.
[51] P.A.D. Grimont, F. Grimont, The genus Serratia, Annu. Rev. Microbiol. 32 (1) (1978) 221–248, https://doi.org/10.1146/annurev.mi.32.100178.001253.
[52] A.M. Walterson, J. Stavrinides, Pantoea: insights into a highly versatile and diverse genus within the Enterobacteriaceae, FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev. 39 (6) (2015) 968–984, https://doi.org/10.1093/femsre/fuv027.
[53] C. Collazo, M. Abadias, I. Aguiló-Aguayo, I. Alegre, E. Chenoll, I. Viñas, Studies on the biocontrol mechanisms of *Pseudomonas graminis* strain CPA-7 against food-borne pathogens in vitro and on fresh-cut melon, LWT - Food Sci. Technol. (Lebensmittel-Wissenschaft -Technol.) 85 (2017) 301–308, https://doi.org/10.1016/j.lwt.2017.02.029.
[54] D. Brulé, M.-C. Héloir, T. Roudaire, J. Villette, S. Bonnet, Y. Pascal, B. Darblade, P. Crozier, P. Hugueney, V. Coma, V. Coma, B. Poinssot, Increasing vineyard sustainability: innovating a targeted chitosan-derived biocontrol solution to induce grapevine resistance against downy and powdery mildews, Front. Plant Sci. 15 (2024), https://doi.org/10.3389/fpls.2024.1360254.
[55] L.G. Vidal, E.P. Armengol, C.G. Bella, J.C. Galimany, C.D. Gustems, E.B. Sebastian, X.E. Vidal, Biocontrol of grapevine diseases, BIO. Web. Conf. 56 (2023), https://doi.org/10.1051/bioconf/20235601014.
[56] R. Escribano-Viana, I. López-Alfaro, R. López, P. Santamaría, A.R. Gutiérrez, L. González-Arenzana, Impact of chemical and biological fungicides applied to grapevine on grape biofilm, must, and wine microbial diversity, Front. Microbiol. 9 (FEB) (2018), https://doi.org/10.3389/fmicb.2018.00059.