Received: 23 February 2021 | Accepted: 30 June 2021 | Published: 20 July 2021
DOI:10.20870/oeno-one.2021.55.3.4626

OENO One
VINE AND WINE OPEN ACCESS JOURNAL

Grapevine decline is associated with difference in soil microbial composition and activity

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Associate editor: Luigi Bavaresco

ABSTRACT

Grapevine decline is a top concern in viticulture worldwide and is often associated with many biotic and abiotic factors. Grape trunk diseases and viruses are some of the most frequently identified causes of vine dieback. However, a decline is sometimes observed when no mineral deficiency or excess, or pathogenic causes can be identified. Soil enzymatic and microbial activities are relevant bio-indicators since they are known to influence vine health. Grapevine associated microbiota, linked to vine fitness, is known to be influenced by soil microbiota coming from the microbial pool inhabiting the vineyard. This work describes the microbial diversity and activity of four different vineyard plots of the Bordeaux region, selected due to the presence of localised declining areas unexplained yet by disease symptoms. Soils were sampled in declining areas and areas within the same plot showing no decline symptoms, during autumn and spring periods. Significant differences in enzymatic activities, microbial biomass and activity were found among soils even if those soils presented quite similar physicochemical characteristics that could not explain these observed declines. The results of enzymatic assays distinguished patterns in autumn and spring periods with an overall greater enzymatic activity in soils from non-declining areas. This work suggests that soils displaying decline symptoms present a dysbiosis in functionality and diversity which is linked to vine health.

KEYWORDS

enzymatic activities, grapevine decline, microbial diversity, terroir, vineyard soil

Supplementary data can be downloaded through: https://oeno-one.eu/article/view/4626

INTRODUCTION

Aside from its economic value, viticulture in France plays a significant historical and cultural role due to differing agricultural practices depending on pedoclimatic conditions and geographical traditions. Certain French wine production areas, and more global terroirs, such as Val de Loire and Saint-Emilion, are even "World heritage UNESCO" sites (Rochard, 2016). Obviously, the terroir, which is defined as a region linked to a specific ecosystem with a distinct quality of grapes and therefore wines, is shaped by several factors. It is well-known that core parameters of terroir such as climate, soil, plant material and human practices influence vineyard productivity and berry quality (van Leeuwen *et al.*, 2010; Rotaru *et al.*, 2010). Facing the increased challenges posed by climate change, viticulture needs new tools to adapt to these environmental constraints that perpetuate the issue of vineyard decline observed for several decades (Marín *et al*., 2020; Mosedale *et al*., 2015; Reineke and Thiéry, 2016).

Vineyard decline, defined as a vine multiannual yield diminution or its premature, brutal or progressive death, is afflicting viticulture worldwide (Riou *et al*., 2016). It is a high concern in French viticulture with more than 4.6 hl/ha yield loss estimated in 2014, encompassing around 10 % of grapevine plantation at the national scale (BIPE, 2015). The causes of this decline are complex. Grapevine decline is often linked to disease symptoms that can be due to bacteria (Hopkins and Purcell, 2002), fungi and oomycetes (Mondello *et al*., 2018), viruses (Maree *et al*., 2013), pests (Reineke and Thiéry, 2016) and even genetic susceptibility of the rootstock (Renault-Spilmont *et al.*, 2007).

Currently, the best understood pathologies associated with grapevine decline are the grapevine trunk diseases (GTD), which include Eutypa dieback, Esca disease complex and *Botryosphaeria* dieback; these are the most predominant ones caused by specific fungi with well-documented rot symptoms (Bertsch *et al*., 2013). Besides GTDs, viruses such as Grapevine Fanleaf Virus (GFV) and Arabis Mosaic Virus (ArMV) are also known to cause specific symptoms affecting mainly the scion (Martelli, 2017). This type of decline is known to be linked with the soil microbiological status. For instance, Nerva *et al*. (2019) recently showed a link between bulk microbiome composition in vineyard soil and Esca severity by comparing symptomatic and asymptomatic vines, suggesting that bulk soil is the source of GTD inoculum.

However, in many cases, no pathologic causes can be identified in declining vineyards. These declines could be caused by numerous abiotic factors such as climate (e.g., water stress, light exposure and heat stress), viticultural practices, soil quality and/or the use of pesticides.

Physicochemical and biological parameters interact in a delicate balance that may easily flip into vineyard decline. It is assumed that environmental abiotic factors such as climate (Sosnowski *et al*., 2007) and soil features (Lecomte *et al*., 2011) are, most of the time, linked to pathogen predisposition to provoke decline issues. At the microscopic scale, soil microbiota have a broad range of interactions with host-plant, from pathogenic to commensal or beneficial effects that can be observed at a macroscopic scale (Newton *et al.*, 2010). Moreover, soil-inhabiting microbes shape grapevine associated microbiota (Martins *et al*., 2013) and are fully considered as determinant factors for wine quality (Zarraonaindia *et al*., 2015). These micro-organism dynamics play key roles in host plant health and productivity through several direct and indirect processes with for instance plant immune response triggering (Chisholm *et al.*, 2006), carbon (Schimel and Schaeffer, 2012), nitrogen (Mooshammer *et al*., 2014) and phosphorus (Richardson and Simpson, 2011) cycling. Soil microbiota composition in terms of genetic diversity is currently under investigation by the international grapevine scientific community since metagenomics-based tools are more affordable. A strong effort is being made to describe the microbial soil community depending on geographic location, soil physicochemical composition and other parameters such as cultural practices (Berlanas *et al*., 2019; Burns *et al*., 2015; Canfora *et al*., 2018; Coller *et al*., 2019).

Notwithstanding, to our knowledge, only Bacci *et al.* (2018) investigated the relationship between soil microbiome as a biological indicator and a plant health status, the common reed *Phragmites australis*, when subjected to decline without any known causes. Several hypotheses can be offered to explain the unknown decline, but the quality and microbiological balance of the soil may be a coherent biological indicator. One can hypothesise that soil displaying decline features could either have a global downsizing of its microbial diversity and activity or either having a dysbiosis specific to its beneficial microorganisms such as Plant-

TABLE 1. Characteristics of the 4 studied plots and the GPS coordinates of the soils with symptomatic (S) and asymptomatic (AS) vines.

Terroir	Graves				Médoc			
Plot	1		2		3		4	
Plantation year	2011		2008		1990		1963	
Scion/rootstock combinations	CS/RGM		CS/RGM		Merlot/3309C		CS/Kober 5BB	
Inter-row distance (m)	1.1		1.1		1.5		1.3	
Inter-plant distance (m)	0.90		0.90		1		1	
Vine status	S	AS	S	AS	S	AS	S	AS
GPS coordinates	44°45'14.0"N 0°33'31.4"W	44°45'14.1"N 0°33'32.0"W	44°45'13.0"N 0°33'24.8"W	44°45'14.3"N 0°33'25.4"W	45°09'25.5"N 0°48'19.7"W	45°09'25.4"N 0°48'18.8"W	45°09'31.3"N 0°46'19.1"W	45°09'29.9"N 0°46'16.9"W

CS stands for Cabernet-Sauvignon and RGM for Riparia Gloire Montpellier.

Growth Promoting Rhizobacteria (PGPR) or Arbuscular Mycorrhiza Fungi (AMF) which can be reduced or even absent. In grapevine decline problematics, some studies have focused on the restoration of soil microbial diversity and pathogens suppressiveness by adding cover crops which stimulates beneficial microorganisms activity (Richards *et al*., 2020; Vukicevich *et al*., 2018). Nevertheless, the profiles of symptomatic and asymptomatic soil featuring unexplained grapevine decline have yet to be studied within the same plot.

In this context, this work aims to investigate soils displaying decline features with symptoms that were not associated with explainable pathologic causes. To this end, four vineyards from two different terroirs of Bordeaux were chosen to dig out the physicochemical, microbiological and enzymatic differences in bulk soil profile between a declining area and a non-declining one within the same vineyard during autumn and spring.

MATERIALS AND METHODS

1. Studied sites

The Bordeaux wine region is in southwestern France, 20 to 150 km from the Atlantic Ocean coasts, between 44.5° and 45.5°N. The predominant climate is sub-humid temperate with cool nights and a low risk of extreme temperatures (Tonietto and Carbonneau, 2004). Four plots, namely 1, 2, 3 and 4, were selected in two different Appellations with distinct terroir, namely Médoc (north of Bordeaux) and Graves (east of Bordeaux). Each of these plots presented an area displaying decline notable features (e.g. higher percentage of dying plants, smaller vigour of the scions, loss of yield, smaller berries...), compared to the rest of the plot where grapevines showed "normal" growth and yield. The declining areas were annotated with S (for Symptomatic), whereas other areas in the same plot presenting "normal" vines were annotated AS (for ASymptomatic). Each S and AS area was delimited with four rows and 20 plants per row. The two major viruses, Arabis Mosaic Virus (ArMV) and Grapevine Fan Leaf Virus (GFLV) (Boscia *et al*., 1997) were assessed by the ELISA method. DAS-ELISA was carried out with crude plant extracts from leaves samples. GFLV and ArMV were detected using the reagents provided by Bioreba AG (Reinach, Switzerland). Substrate hydrolysis was recorded at 405 nm with a Dynex MRX II microplate reader. The vigour of the vine was measured by weighing the winter-pruned wood of 28 plants spread on four rows within the AS and S areas for each plot. The four plots were all located on sandy soils and according to the World Reference Base for Soil Resources vineyards in Graves (Villenave d'Ornon) are on superior Pleistocene and Holocene sediments whereas Médoc's (Saint Julien) plots are located on inferior Pleistocene sediments (WRBSR, 2015). GPS coordinates, ages of vines and combinations of rootstocks with scions are presented in Table 1.

2. Soil sampling

Bulk soils (10–30 cm of depth) from inter-row vineyards were sampled at those eight different sites. Sampling was performed in November 2018

and in April 2019 using an auger (10 cm × 25 cm) for three subsamples with 1 meter of distance between each that were afterwards pooled. For physicochemical analysis, three aliquot portions were made from this pool whereas five aliquot portions were made for the enzymatic, molecular and microbiological analysis. Regarding Eco-Plates® assays, upper layer soils (5–10 cm) were sampled during the two periods (autumn and spring) and used at their fresh state 24 hours after their sampling.

3. Physicochemical analysis of soils

Five hundred grams from the three subsamples described above were dried at 40 °C for 72 hours, sieved at 2 mm, homogenised and sent to INRAE LAS (62000, Arras, France) to perform physicochemical analysis encompassing granulometry, pH, nutrients and major trace elements contents listed in Table 2. According to Proffitt and Campbell-Clause (2012), the physicochemical parameters measured were not affiliated to mineral deficiencies that could explain the decline observed. At the texture level, the studied soils were all considered as "sand" soils which was defined by the USDA classification. Regarding the pH, all the studied soils were considered as moderate to slightly acid but are among the same rank within plots between S and AS soils.

4. DNA extraction

After sampling, 5 grams of soil from the 5 subsamples described above during autumn and spring were lyophilised for 48 hours using Christ Alpha® 1-4 (Bioblock Scientific) and stored at –80 °C before DNA extraction. DNA was extracted from 250 mg of the lyophilised soils using the DNeasy PowerSoil Pro kit (Qiagen) using the manufacturer recommendations with an additional C5 washing step. DNA samples were quantified on a Qubit® 3.0 fluorometer (Thermo Fisher Scientific) using the Qubit™ dsDNA HS Assay Kit, and their quality was checked with a NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific). DNA was then stored at –20 °C until further use.

5. Quantitative PCR amplification of bacterial and archaeal 16S and fungal 18S rRNA genes

Analyses of qPCR were performed on the DNA extracted from the soil samples using three primers pairs to quantify bacterial and archaeal 16S rRNA genes as well as the fungal 18S rRNA genes (Supplementary Table S1).

Bacterial and archaeal 16S qPCR reactions were monitored in 20 µL mixture consisting of 10 µL of GoTaq® qPCR Master Mix (Promega), 1 and 0.6 µL of each primer (10 µM) for bacterial and archaeal quantification, respectively, and 1 ng of extracted DNA. Cycling conditions were starting with an initial denaturation at 95 °C for 10′ followed by 40 cycles of denaturation at 95 °C for 15″, annealing temperature for 30″ at 60 °C and elongation at 60 °C for 30". Melt curves were obtained at 65 °C by increasing 0.5 °C / 5" until 95 °C. Fungal 18S qPCR reactions were performed in the same conditions except that the annealing temperature was at 50 °C. Each sample was quantified in three replicates in Hard-Shell® 96-Well PCR plates sealed with Microseal® « B » film (Bio-Rad) using the CFX96™ Real-Time PCR Detection System and the CFX™ Manager software, version 3.1 (Bio-Rad Laboratories, France). The software algorithm calculates the efficiency (E) and threshold cycle (CT) based on the kinetics within each reaction. The efficiencies of the qPCR were 85 % to 99 % ($R^2 > 0.99$). The initial template concentration N (gene copy numbers per qPCR reaction volume) was then calculated with the following equation: $N = (1 + E)^{CT}$.

6. Standard curves and absolute quantification of target genes

This qPCR approach based on universal bacterial, archaeal and fungal subunit rRNA genes amplification were followed by absolute abundance quantification using standard curves. To draw those curves, PCR was performed in a T100™ Thermal Cycler (Bio-Rad) on 1 ng of DNA extracted from plot 1 in 20 µL mixture consisted of 10 µL of GoTaq® qPCR Master Mix (Promega), 1 and 0.6 µL of each primer (10 µM) for bacterial and archaeal quantification, respectively. Similar cycling conditions were initial denaturation at 95 °C for 10′ followed by 30 cycles of denaturation at 95 °C for 30″, annealing temperature for 30″ at 50 °C and 60 °C, for 16S and 18S genes respectively, and elongation at 72 °C for 30", finished by a final elongation step at 72 °C for 3′. Obtained amplicons were then sub-cloned using the pGEM®-T easy vector system (Promega) and sequenced to confirm the identity of the amplified fragments. Calibration curves (log gene copy number per reaction volume versus log N) were obtained using serial dilutions of standard from 2×10^8 to 2×10^3 copies of the pGEM-T vector containing the corresponding sequence. The numbers of copies of the qPCR

TABLE 2. Physicochemical characteristics of the inter-row soils from the 4 studied plots with (S) and without (AS) decline symptoms.

Terroir	Graves				Médoc			
Plot	1		2		3		4	
Vine status	S	AS	S	AS	S	AS	S	AS
Basic soil properties								
Sand (%)	84	82	89	88	90	88	95	87
Silt (%)	10	10	7	7	5	7	2	7
Clay (%)	7	8	4	5	5	5	3	6
pH (water)	6.65	6.86	5.79	5.75	6.05	6.78	7.09	6.2
pH (KCl)	5.93	6.01	4.64	4.62	5.07	5.96	6.37	5.13
Organic carbon (%)	0.41	0.56	0.24	0.53	0.43	0.46	0.23	1.39
Total nitrogen (%)	0.03	0.05	0.02	0.04	0.03	0.03	0.01	0.08
Organic matter (%)	0.71	0.97	0.41	0.91	0.73	0.79	0.39	2.40
C/N	11.8	12.2	14.1	12	15.2	13.1	16.4	16.9
Micro/macronutrients								
Phosphorus ($mg.kg^{-1}$)	35	30	17	65	38	13	45	81
CEC ($cmol^{+}.kg^{-1}$)	3.1	3.9	1.3	2.5	2.5	3.3	1.8	5.5
Ca ($cmol^{+}.kg^{-1}$)	2.8	3.5	0.5	1.3	1.7	2.9	1.4	3.4
Mg ($cmol^{+}.kg^{-1}$)	0.4	0.4	0.5	0.2	0.2	0.3	0.3	0.7
K ($cmol^{+}.kg^{-1}$)	3.8	2.6	2.9	4.5	4.7	3.3	5.3	3.1
Na ($cmol^{+}.kg^{-1}$)	0.05	0.04	0.02	0.03	0.02	0.03	0.01	0.03
NO_{3-} ($mg.kg^{-1}$)	9.8	5.2	0.6	5.8	2.7	2.1	0.7	6.9
NH_3-N ($mg.kg^{-1}$)	2.2	2.7	1.7	1.9	2	1.8	1.5	2.3
Trace elements								
Cu ($mg.kg^{-1}$)	18.5	22.3	3.24	5.38	53	56.8	19.9	37.8
Fe ($mg.kg^{-1}$)	117	137	65	195	142	96	27	133
Mn ($mg.kg^{-1}$)	23.4	10.2	3.3	6.6	5.3	6.1	3.4	3.7
Zn ($mg.kg^{-1}$)	3.6	5.6	2.6	1.6	4.7	2.8	5	6.6

Data shown are the values obtained after pooling 3 subsamples.

standards were calculated by assuming average molecular masses of 660 Da for 1 bp of double-stranded DNA.

Copies per nanogram = $\frac{n \times mw}{Na \times 10^{-9}}$

where n is the length of the standard in base pairs, mw is the molecular weight per bp or nucleotide, and Na is the Avogadro constant (6.02×10^{23} molecules per mol).

7. Enzymatic assays

As explained above, the following enzymatic assays were done with fresh, homogenised and sieved soil sampled from each site, coming from five subsamples. One gram of soil for each site was dried and weighted for the final calculation of enzymatic activities.

7.1. Alkaline phosphatase

Colorimetric estimation of the *p*-nitrophenol released by soil phosphatase activity when incubated with basic buffered sodium *p*-nitrophenyl phosphate solution and toluene was used to determine alkaline phosphatase as described by Tabatabai (1994), excepted the filtration step which was replaced by 8000 *g* centrifugation. Assays were performed with 1 gram of homogenised and sieved (2 mm) fresh soil.

7.2. β-glucosidase

Herein, the procedures are similar to those of phosphodiesterase activity (see above) and are based on colorimetric estimation of the *p*-nitrophenol released by soil β-glucosidase

activity when incubated, as described by Tabatabai (1994) with a centrifugation step at 8000 *g* replacing the filtration.

7.3. Arylamidase

Arylamidase activity was detected using Acosta-Martínez and Tabatabai (2000) colorimetric assay, based on 2-naphtylamine released from 1 g of sieved (< 2 mm) fresh soil when incubated with L-Leucine β-naphthylamine.

8. Potential metabolic diversity (PMD) of cultivable bacteria

PMD, represented by functional richness (R) and global metabolic activity (AWCD) were assessed with Biolog Eco-Plates™ system (Biolog Inc., CA) using Calbrix *et al*. (2005) preparation. Those plates are containing 96 wells filled with 31 different carbon sources, plus a control well. Briefly, fresh soil from the 10 cm of the upper layer from 3 biological replicates were pooled, sieved at 2 mm and homogenised prior to suspending 5 grams of fresh soil into 50 mL of 0.85 % NaCl. Suspensions were shaken for 10 minutes at 300 rpm and rested for 10 minutes under ambient temperature. Supernatants were diluted with ultrapure sterile water 1:100 and the 31 Eco-Plate wells were filled with 120 µL of this diluted supernatant, incubated at 20 °C in the dark and subsequently, their absorbance at 590 nm was measured every 24 hours for 4 days. Each Eco-Plate was subdivided into three replicates for each tested soil and the absorbance value of each carbon source was corrected by subtracting the absorbance value of the well containing only water. Negative values were set to zero. Global microbial metabolic activity in each replicate was expressed as the Average Well Colour Development (AWCD). Microbial richness functionality R was calculated as the number of utilised substrates (> higher AWCD mean among the tested soils at 96 h) and Shannon evenness index (SEI) was calculated according to Zak *et al*. (1994) (Supplementary Table S2). Area Under AWCD Curve (AUC), which gives better insights for curve dynamics, was calculated with the trapezoidal method for each soil using "caTools" packaging.

9. Cultivable bacteria and fungi colonies quantification

Quantification of cultivable bacterial population from the eight soils was done on R2A medium (0.5 % yeast extract, 0.5 % proteose peptone, 0.5 % casamino acids, 0.5 % glucose, 0.5 % soluble starch, 0.3% sodium pyruvate, 0.3 % H_2KO_4P, 0.05 % $MgCl_2$, pH 7) amended with 25 mg/L of nystatin to inhibit yeasts and fungi growth. Sterile Petri dishes filled with R_2A medium were plated with the same soil suspensions used above for PMD which were tenfold serial diluted. They were then incubated at 25 °C and Unities Forming Colonies (UFC) were numerated 4 days after plating. Additionally, the cultivable fungal population were quantified on Potato Dextrose Agar (BioKar) amended with 500 µg mL^{-1} of gentamicin and 50 µg mL^{-1} of chloramphenicol to inhibit bacterial growth. Incubation was done at 25 °C and UFC were numerated 7 days after plating.

10. Statistical analysis

All statistical analyses were performed using the R software version 4.0.2 (R Core Team, 2020) and RStudio version 1.3.1056. Histograms and principal component analysis (PCA) were made using *ggplot2*, *ggthemes* and *FactoMineR* packages.

Normality and homogeneity of variances were checked by the Shapiro–Wilk and the Leven tests, respectively (Zar, 1999).

ANalysis Of VAriance (ANOVA) with soil status factor (AS or S) was performed for enzymatic activities, microbial biomass and genes quantities. When significant effects were detected, multiple comparisons of means were done with pairwise t-tests ($\alpha = 0.05$). Residuals were prior checked for their independency, normality and variance homogeneity with the Durbin Watson, Shapiro–Wilk and Bartlett tests, respectively. When assumptions for parametric tests were not respected, a multiple pairwise comparison with the Wilcoxon test was performed after a Kruskal–Wallis test using the *multcomp* package. Bonferroni correction was applied for pairwise comparison. Two-way ANOVA with seasonal (autumn or spring) and terroir factors were performed on molecular biomasses.

RESULTS

1. Declining areas display higher mortality rate and weaker vigour of plants, which are not associated with the presence of viruses

To quantify the decline empirically observed by the winegrowers within the S area in each plot, the percentages of missing vines and young plants, which were recently planted to replace dead plants, as well as the pruning weight of the old vines were

TABLE 3. Characterization of the observed decline in the 4 studied plots by comparing the areas with symptomatic (S) and asymptomatic (AS) vines.

Terroir	Graves				Médoc			
Plot	1		2		3		4	
Decline features	S	AS	S	AS	S	AS	S	AS
Missing vines (%)	0	0	0	0	35	2	0	5
Young plants (%)	65	1	57	1	13	5	38	14
GFLV/ArMV (%)*	0	0	0	0	0	12.5	12.5	100
Number of pruned woods per vine	7.6 ± 0.4	9.6 ± 0.4	8.0 ± 0.3	9.7 ± 0.5	4.4 ± 0.4	7.9 ± 0.4	6.6 ± 0.3	10 ± 0.5
	**		*		***		***	
Pruning weight per vine (g)	197 ± 98	361 ± 120	139 ± 69	307 ± 185	82 ± 46	183 ± 84	104 ± 99	289 ± 113
	***		**		**		***	
Vigour [b]	25 ± 9	38 ± 10	14 ± 6	33 ± 20	19 ± 9	23 ± 8	15 ± 11.	29 ± 8
	***		***		*		***	

Means ± SE (n = 28) are represented. Missing vines correspond to dead plants that were not replaced and young plants are grafted plants that have been recently planted (less than 5 years) to replace the dead ones. For pruning means, asterisks represent significant differences between S and AS soils with $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

[a] The presence of GFLV and/or ArMV viruses has been tested using ELISA tests in eight plants within each area. Data are presented as the percentage of positive samples.

[b]Vigour was calculated as the number of pruned woods divided by the pruning weight.

assessed in symptomatic and asymptomatic areas (Table 3).

Although the mortality of the vines in each plot is higher in S areas compared to AS ones with a higher number of missing plants and/or a higher number of young plants.

Significant differences were detected among the soils regarding the number of pruned woods (ANOVA: $F(7,216) = 19.21$, $P < 0.001$) and the average pruning weight (Kruskal–Wallis: $\chi^2 = 116.35$, ddl = 7, $P < 0.001$) per vine, and lower levels were observed for vines growing in S areas compared to those growing in AS areas, for all the studied plots. Even if no visual disease symptoms could be associated with the observed decline when comparing the two areas within each plot, the presence of the main viruses responsible for "court noué" was checked. GLFV and ArMV were detected in the AS area of plot 3 and in the S and AS areas of plot 4. Thus, the presence of the viruses appeared to be not correlated with the observed decline of vines.

2. Soils from declining areas contain fewer bacteria and archaea DNA than well growing areas

Molecular analyses revealed that the quantity of total DNA extracted per g of dry soil was significantly higher (Kruskal–Wallis: $\chi^2 = 37.49$, ddl = 7, $P < 0.001$) in each AS area compared to its corresponding S area in all plots, whatever the seasonal period (Figure 1 and Supplementary Figure S1). Molecular biomass of bacteria (Kruskal–Wallis: $\chi^2 = 34.016$, ddl = 7, $P < 0.001$) and archaea (Kruskal–Wallis: $\chi^2 = 37.496$, ddl = 7, $P < 0.001$) followed the same pattern except for bacterial 16S detection in plot 3 where no significant difference has been detected ($P = 0.931$). Interestingly, no significant difference was detected for fungal 18S gene between S and AS areas in spring samples, except for plot 4 (Kruskal–Wallis: $\chi^2 = 34.943$, ddl = 7, $P < 0.001$), while higher signals were detected in S soils compared to AS ones within three plots during autumn. It appeared that a higher quantity of DNA was extracted during spring compared to autumn with an increase in S of 5, 85, 70 and 426 % as well as in AS of 24, 258, 137 and 63 % for plots 1, 2, 3 and 4, respectively. To this extent, more bacterial and archaeal 16S genes were found in spring samples compared to autumn ones, especially for plots 1 and 2 located in Graves. For instance, in these plots, in AS soils it took a rise of 1000 and 258 % in 16S archaeal genes and 470 and 259 % in 16S bacterial genes. The seasonal effect was not significant on the number of 18S gene copies (ANOVA: $F(1,76) = 0.23$, $P = 0.63$),

which always remain lower than the number of bacterial, and even the archaeal, 16S genes.

3. Asymptomatic soils displayed more cultivable bacteria and fewer fungi, with lower microbial activity, compared to symptomatic soils

Differences in molecular biomass found between S and AS soils concerning the bacterial and fungal level were confirmed with cultivable approaches. Significant differences were detected among the soils regarding the level of bacterial cultivable populations (ANOVA: $F(7,16) = 33.28$, $P < 0.001$) and a higher level was observed in AS soils compared to S soils, except plot 4 where no significant difference was detected ($P = 0.100$) (Table 4). Unlike the fungi (Kruskal–Wallis: $\chi^2 = 22.273$, ddl = 7, $P = 0.002$), the level of cultivable population was significantly higher in S soils compared to AS soils. The cultivable population of bacteria and fungi were also assessed during autumn which corroborate, as with the spring measurement, with higher and lower population levels of bacteria and fungi, respectively, in AS compared to S soils (Supplementary Table S3). A seasonal effect was observed with a higher number of bacteria and fungi found during spring compared to samplings made in autumn.

Microbial activities during spring sampling represented by AWCD from Biolog Eco-plates™ system were significantly (Kruskal–Wallis: $\chi^2 = 37.496$, ddl = 7, $P < 0.001$) more important in S soils compared to AS soils at the endpoint (96 hours after incubation), except plot 3 where no significant difference was detected ($P = 0.799$) (Figure 2).

The AUC was neither significantly different for plot 3 ($P = 0.8$) (Table 4). Shannon's evenness was not significantly different among the soils (Kruskal–Wallis: $\chi^2 = 20.44$, ddl = 7, $P = 0.0569$), however, the richness functionality R was significantly more important in S soils compared to AS soils (ANOVA: $F(3,8) = 82.83$, $P < 0.001$), except plot 3 where no significant difference was detected ($P = 0.12$). Interestingly, the microbial activity measured by Eco-Plates™ were inverted during the autumn season, with significantly more

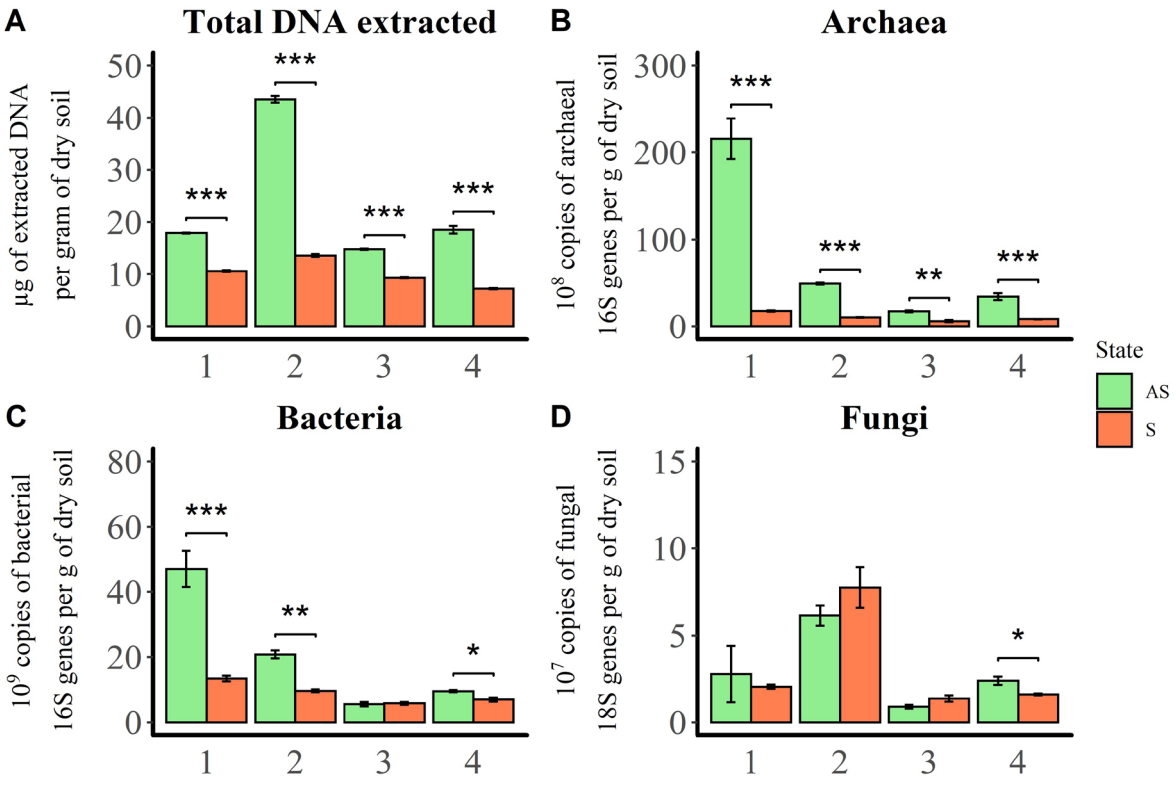

FIGURE 1. Quantifications of (A) total DNA, (B) archaeal 16S rRNA genes, (C) bacterial 16S rRNA genes and (D) fungal 18S rRNA genes in asymptomatic (AS = green) and symptomatic (S = orange) soils, among plots 1, 2, 3 and 4 during spring.

Bars represent means ± SE (n = 5). Significant differences were detected through pairwise t or Wilcoxon tests after an ANOVA or Kruskal test, where $\alpha = 0.05$, corrected with the Bonferroni method. Asterisks represent significant differences between S and AS soils with $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

important values in AWCD (ANOVA: $F(3,8) = 164.4$, $P < 0.001$) and richness R (ANOVA: $F(3,8) = 82.83$, $P < 0.001$) for the AS soils compared to S ones (Supplementary Figure S2; Supplementary Table S3).

The molecular (ANOVA: $F(7, 32) = 11.02$, $P < 0.001$) and cultivable (ANOVA: $F(7, 16) = 94.58$, $P < 0.001$) (B / F) ratios were significantly higher in AS soils compared to the S ones for plots 1, 2 and 4, except for plot 3 where the B/F ratio was significantly lower in the AS soil (Table 5).

4. An overall higher enzymatic activity was detected in asymptomatic soils

Soils within the 4 studied plots showed significant differences in enzymatic analysis (Figure 4). Significantly higher activity in AS soils compared to S soils was observed in arylamidase, β-glucosidase and alkaline phosphatase during

TABLE 4. Cultivable population levels of bacteria and fungi, and Eco-Plates measurements (Area Under Curve (AUC), Shannon's evenness (E) and richness (R) functionality at 96 hours post-incubation) within the 4 studied plots with (S) and without (AS) decline symptoms during spring.

Terroir	Graves				Médoc			
Plot	1		2		3		4	
Status	S	AS	S	AS	S	AS	S	AS
Bacterial counts (log (CFU / g of soil))	7.6 ± 0.03	7.9 ± 0.03	7.6 ± 0.04	7.8 ± 0.04	7.5 ± 0.03	7.7 ± 0.03	7.7 ± 0.01	7.7 ± 0.03
	**		**		**		ns	
Fungal counts (log (CFU / g of soil))	7.3 ± 0.04	6.1 ± 0.04	7.2 ± 0.02	6.9 ± 0.03	6.5 ± 0.04	6.2 ± 0.15	7.5 ± 0.06	7.0 ± 0.15
	***		***		***		***	
AUC	7.6 ± 0.19	6.4 ± 0.13	9.0 ± 0.26	8.0 ± 0.13	8.4 ± 0.21	8.3 ± 0.07	8.5 ± 0.37	7.9 ± 0.3
	***		***		ns		***	
E	0.995 ± 0.002	0.991 ± 0.000	0.998 ± 0.001	0.991 ± 0.004	0.986 ± 0.001	0.992 ± 0.001	0.993 ± 0.001	0.999 ± 0.001
	ns		ns		ns		ns	
R	23.3 ± 0.6	13.3 ± 2.1	27.7 ± 0.6	19.7 ± 0.6	20.7 ± 1.1	22 ± 1	27.3 ± 0.6	25
	***		***		ns		*	

Means Figure SE are presented with (n = 5) for bacterial and fungal counts, whereas (n = 3) for Eco-Plates measurements. Asterisks represent significant differences between S and AS soils with $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

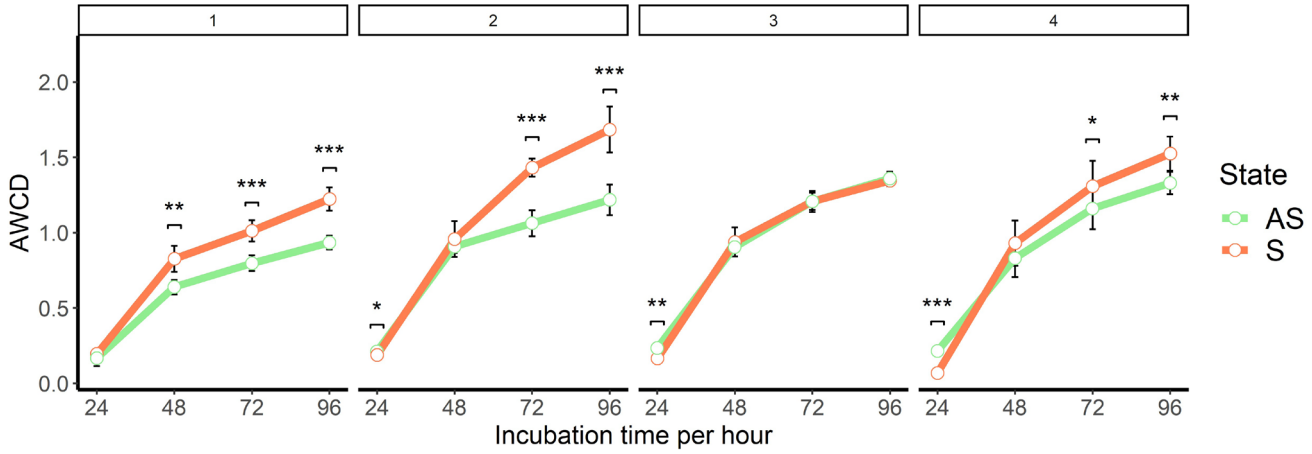

FIGURE 2. Eco-Plates™ measurements displaying microbial activities represented by Average Well Colour Development (AWCD) of metabolised substrates in Eco-Plates based on 96-h incubation (n = 3) in symptomatic (S = red) and in asymptomatic (AS = green) soils of decline among for plots 1, 2, 3 and 4 during spring.

Points on the curves represent means ± SE (n = 3). Asterisks represent significant differences between S and AS soils with $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

TABLE 5. Ratios between 16S bacterial and 18S fungal genes, and between cultivable bacterial and fungal CFUs from the soils within the 4 studied plots with (S) and without (AS) decline symptoms during spring.

Terroir	Graves				Médoc			
Plots	1		2		3		4	
Soil status	S	AS	S	AS	S	AS	S	AS
(B / F) molecular	657 ± 52	1754 ± 100	135 ± 45	345 ± 46	661 ± 110	473 ± 106	440 ± 114	413 ± 79
		***		***		*		ns
(B / F) cultivable	108 ± 17	1911 ± 44	121 ± 14	436 ± 12	1009 ± 295	756 ± 42	72 ± 10	265 ± 98
		***		***		*		**

Means ± SE are represented (n = 5). Asterisks represent significant differences between S and AS soils with $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

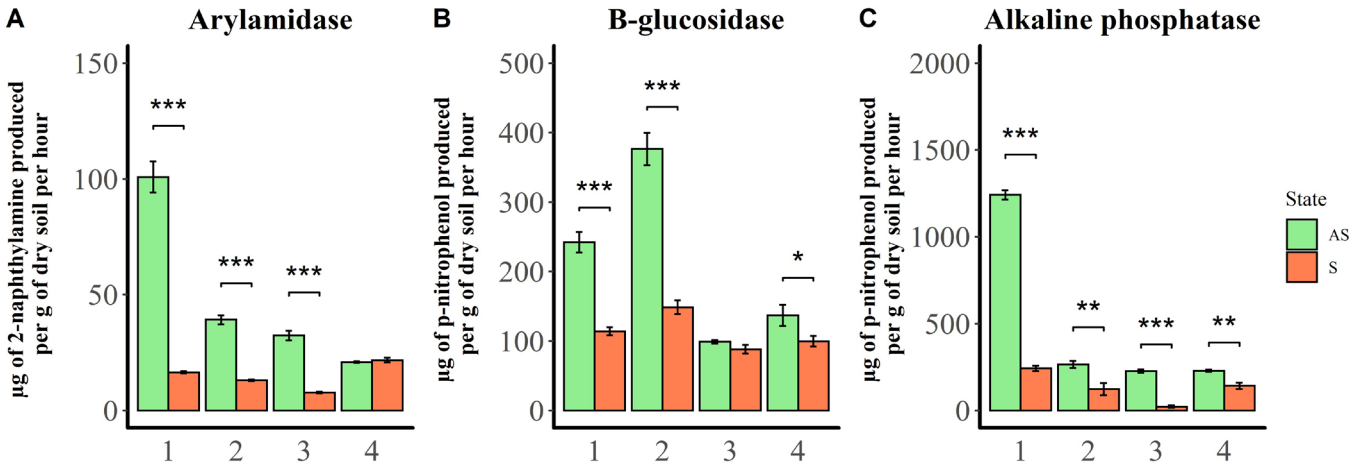

FIGURE 3. Enzymatic activities in asymptomatic (AS = green) and in symptomatic (S = orange) soils among plots 1, 2, 3 and 4. Soils were assessed for the activity of (A) arylamidase l, (B) β-glucosidase and (C) alkaline phosphatase during spring.

Bars represent means ± SE (n = 5). Significance differences corrected with the Bonferroni method were detected through pairwise t or Wilcoxon tests after an ANOVA or Kruskal test. To facilitate the graph reading, usual letters were replaced with annotations: $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

the spring period, except in plot 4 and plot 3 for the arylamidase ($P = 0.967$) and β-glucosidase ($P = 0.339$), respectively. The enzymatic activity was also recorded during autumn but only for plots 1 and 2. Alkaline phosphatase activity increased in spring compared to the samples made in autumn among the soils 1 AS, 1 S, 2 AS and 2 AS with an uprise of 357, 608, 564 and 504 %, respectively (Supplementary Figure S3). Unlike the β-glucosidase where the activity was more important during autumn than spring with an increase of 84, 73, 41 and 6 % for plots 1, 2, 3 and 4, respectively.

5. Linking microbial profiles and enzymes activities

To visualise the similarities and differences between the profiles of the 8 studied soils, a PCA was performed considering all the enzymatic, molecular and microbial values (Figure 4).

The first two dimensions (Dim) accounted for 47.6 % of the variance. Dim1 axis accounted for 24 % of the total variance and was positively correlated with the vigour of the vines, DNA, fungal 18S genes, bacterial and archaeal 16S genes, arylamidase and alkaline phosphatase activities, cultivable fungi and bacteria, with Eco-Plates™ measurements while β-glucosidase activity was negatively correlated. Dim2 axis accounted for 23 % of the total variance and was correlated with the vigour of the vines, DNA, bacterial and archaeal 16S genes, alkaline phosphatase, arylamidase and β-glucosidase activities while cultivable bacteria and fungi, Eco-Plates™ measurements and fungal 18S genes were negatively correlated. The Graves and autumn samples were mainly found on the

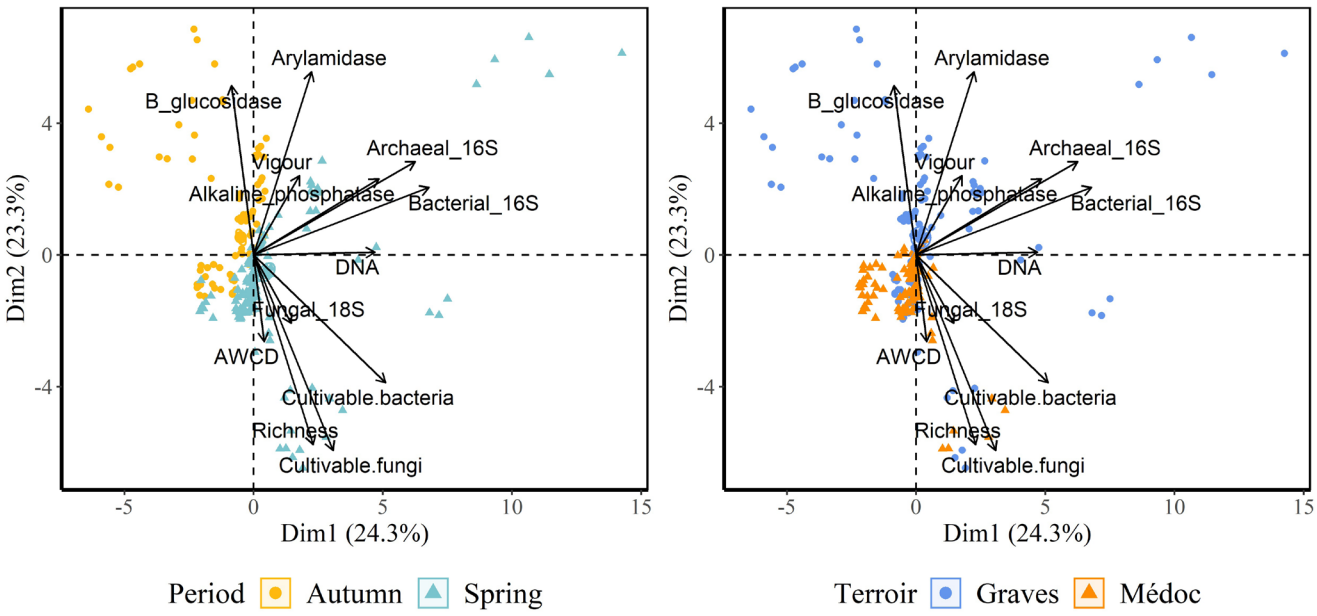

FIGURE 4. Ordination biplot analysis of principal component analysis (PCA) for enzymatic (arylamidase, B-glucosidase, acid and alkaline phosphatases), molecular (total DNA, archaeal and bacterial 16S and fungal 18S) and microbial (Eco-Plates measurements represented by AWCD and richness, cultivable bacteria and fungi) variables among the 4 plots displaying (A) season and (B) terroir.
The size of the arrows indicates the contribution strength of the variables. Standard error ellipses show 95 % confidence areas.

positive side of Dim2 whereas Médoc and spring were mainly found on its negative side.

To understand the variables that could explain the unexplained dieback, PCAs were performed on each vineyard plot considering all the enzymatic, molecular and microbial values (Figure 5).

The first two dimensions (Dim) accounted for 95 %, 93 %, 68 % and 84 % of the variance in plots 1, 2, 3 and 4, respectively. The asymptomatic feature is generically explained by enzymatic activities recorded for arylamidase, β-glucosidase and phosphatase, by the level of cultivable bacteria and by the total DNA extracted coupled with the number of 16S copies of archaeal and bacterial genes. On the other hand, symptomatic features are explained by the number of cultivable fungi for plots 1, 2 and 4 and by the number of 18S fungal genes for plots 2 and 3. Eco-Plates™ variables were not presented in these PCAs since they did not have a clear pattern that could explain the soil status during spring.

DISCUSSION

The decline of vineyards, which has been accelerating over the past few decades, is increasingly worrying stakeholders in the wine industry. Among the main factors that can play a role in these declines, global warming can influence the progression of certain diseases by altering the functioning and microbiome diversity (Dubey *et al.*, 2019). It is well-known that soil microorganisms provide many ecosystem services, such as solubilizing and mineralizing insoluble soil phosphorus or increasing nitrogen available for plants. To better understand the causes of vine decline unexplained by disease symptoms, we investigated the physicochemical, enzymatic and microbial profiles with declining areas and compared them with those of asymptomatic areas within the same vineyard, in four plots from two Bordeaux's appellations.

1. Soil abiotic parameters may not explain the observed decline

Altogether, the physicochemical, enzymatic and microbial components determine the soil ecosystemic processes. These processes are correlated with soil functions which influence vine growth and grape quality (Riches *et al.*, 2013; van Leeuwen *et al.*, 2018). Within these plots, soil with decline features (S) was compared with soil sampled in an area with well-growing and asymptomatic vines (AS). In the eight investigated soils, none of the physicochemical parameters measured in Table 2 could explain the decline

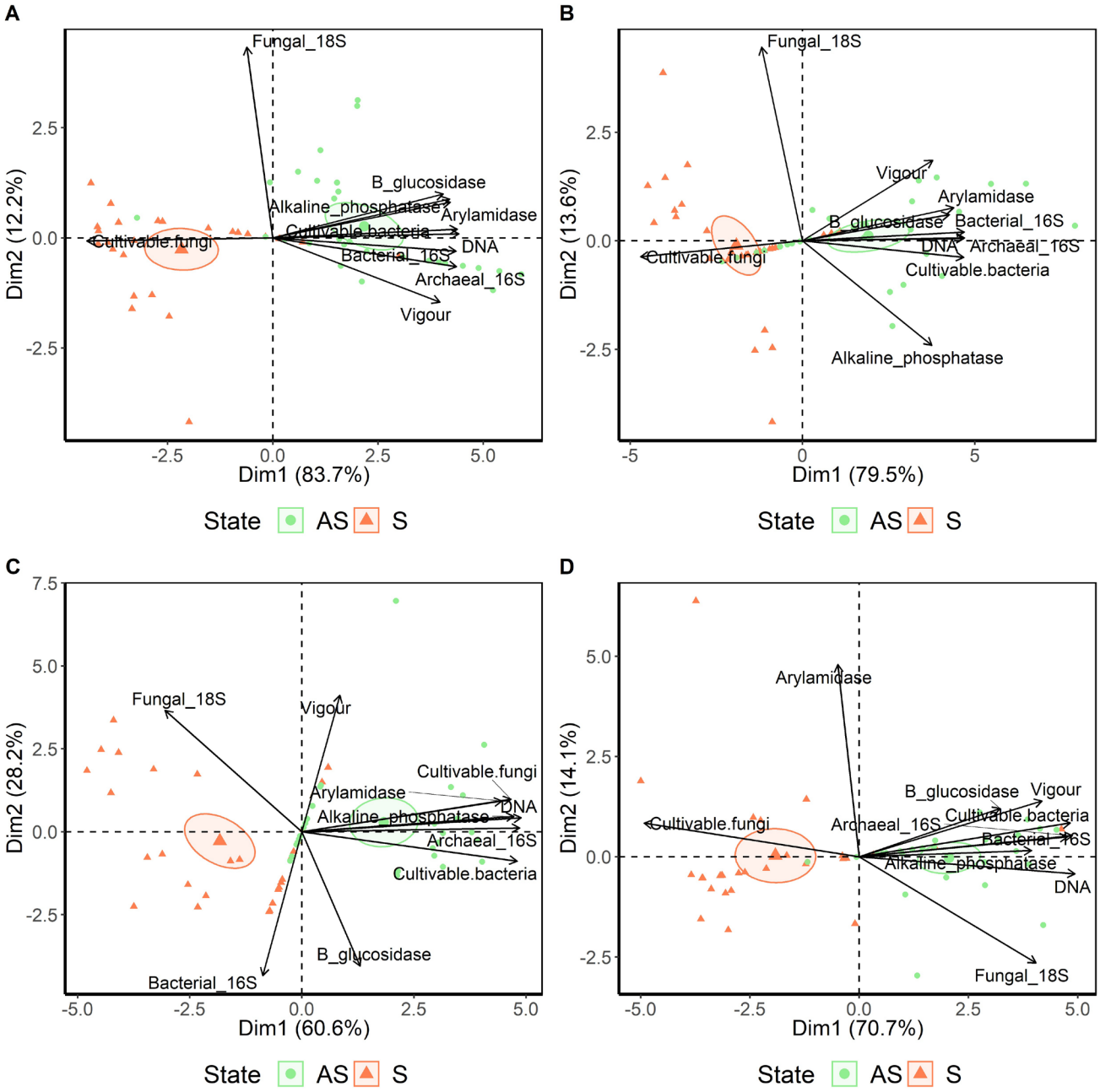

FIGURE 5. Ordination biplot analysis of principal component analysis (PCA) for enzymatic (arylamidase, B-glucosidase, acid and alkaline phosphatases), molecular (total DNA, archaeal and bacterial 16S and fungal 18S) and microbial (cultivable bacteria and fungi) variables among the plots (A) 1, (B) 2, (C) 3 and (D) 4 displaying symptomatic (orange) and asymptomatic (green) features during spring.

The size of the arrows indicates the contribution strength of the variables. Standard error ellipses show 95 % confidence areas.

observed in S soils compared to corresponding AS soils. Indeed, no symptoms could be associated with a lack or excess of trace elements as described in Proffitt and Campbell-Clause (2012) and their toxicity thresholds usually observed in vineyards were above the levels observed in the top 30 cm of the sampled soils. For instance, copper salts (also known as Bordeaux mixture ($Ca(OH)_2$+$CuSO_4$)) have been traditionally used intensively in vineyards since the 19th century to prevent damages provoked by mildew pathogens, which lead to extreme concentrations in top-soils exceeding 500 $mg.kg^{-1}$ of Cu (Brun *et al.*, 2001). Herein the highest value of Cu (56.8 $mg.kg^{-1}$) was found in AS soil from plot 3 and is not considered to have a potentially negative effect on vine growth. Although, it is well known that soil physicochemical parameters are important drivers of the microbial communities (Plassart *et al.*, 2019). Dequiedt *et al.*, (2011) hierarchised

these factors with fine texture and CEC as the top drivers, followed by organic C and N contents and by soil pH. In our study, the AS and S soils among the four plots had a similar fine soil structure. CEC and total C and N contents differed between AS and S soils for only half of the plots (Table 2). This might explain the differences observed in 16S gene copies for archaea (Figure 1B) and bacteria (Figure 1C) found between AS and S soils for these plots, but not for plots 1 and 3. However, only the upper topsoils were analysed with a unique measurement. To unravel the belowground interactions with the physicochemical features of the soil and the vine roots, a deeper analysis should be made.

2. Enzymatic and microbial profiles are relevant biological indicators of the observed decline

Dequiedt *et al*. (2011) observed, through a French survey, that vineyard soils displayed the lowest microbial biomass compared to other land uses. In our study, we obtained higher means in both autumn (Figure S1A) and spring (Figure 1A) periods (1.5 and 3 times more, respectively) compared to Dequiedt *et al*. (2011), which may be due to differences in the DNA extraction methods used.

We observed significant differences between S and AS soils in the four vineyard plots, suggesting that the soils with declining vines have a reduced amount of microbial biomass compared to the soils with non-declining vines. The archaeal and bacterial amounts of specific 16S genes during autumn (Supplementary Figure S1.B&C) and spring periods (Figure 1.B&C) follow the same trend as total DNA. This is less clear for the 18S fungal gene quantity which is significantly more abundant in S soils compared to AS soils during the autumn period for at least three plots (supplementary Figure S1.D) but does not seem to follow this trend during the spring period (Figure 1.D). One explanation of these dissimilarities in the number of 16S gene copies between S and AS soils is that bacterial communities are more sensitive than fungal communities to alteration of nutrient availability (Liang *et al*., 2019).

Alkaline phosphatase in soils is known to be produced exclusively by microorganisms and not by plants (Dick *et al*., 1983). Its activity has been reported to be linked with the level of bacteria under P limiting conditions (Fraser *et al*., 2015). In our study, alkaline phosphatase activity was highly positively correlated with archaeal and 16S genes (Figure 4) and seems to highlight the difference in soil quality between S and AS soils (Figure 3C). Highly positive correlations were also found for arylamidase, with archaeal and bacterial 16S genes, which is considered as a key indicator of soil quality and are primary products of microorganisms (Dodor and Tabatabai, 2002). To a lesser extent, the β-glucosidase activity is more correlated with the fungal 18S gene than with the archaeal or bacterial 16S genes. The level of cultivable fungi appeared to be also correlated with the Eco-Plates measurements such as amino acids, carbohydrates, carboxylic acids and polymers consumptions. Copies of 18S genes and the level of cultivable fungi are correlated and seem to have a strong impact on soil enzymatic activity. Miguéns *et al*. (2007) deciphered the critical level of degradation of vineyard soils and our β-glucosidase and phosphatase activities values were in the same range as in their study which suggests that vineyards display soils with poor enzymatic activities. Among the fungi, the AMF or AM fungal spores are known to be highly influenced by soil conditions and reveal the status of the soil (José *et al*., 2021; Mahmoudi *et al*., 2021). High-throughput sequencing using 18S or ITS specific primers is commonly used for the description of AMF diversity in vineyards (Berruti *et al*., 2017) and might be a promising perspective to evaluate the health status in vineyard soil. In our case, PCAs highlighted, vineyard plot by vineyard plot, that the observed variables could explain the differences between symptomatic and asymptomatic soils for spring samples (Figure 5). Symptomatic features in vineyards were generically explained by the number of cultivable fungi and the number of 18S fungal genes whereas the asymptomatic feature was explained by enzymatic activities, by the level of cultivable bacteria and by the number of 16S copies of archaeal and bacterial genes. These results suggest a dysbiosis in the microbial communities coupled and may be linked to a dysregulation of the ecosystemic processes.

Eco-Plates™ are quite controversial in their interpretation because, like the level of population of cultivable microorganisms, it may be biased because of fast-growing microorganisms which alter the substrate consumption within the wells (Verschuere *et al*., 1997). In vineyard soils, this technology has been used to investigate cover crop (Capó-Bauçà *et al*., 2019) and chemical input (Aballay *et al*., 2017) effects on the physiological profiles of telluric microorganisms. In our case, this system remains interesting to use since we compare similar textures of soil during different

seasons. It appeared that the levels of AWCD (Figure 2), AUC and R (Table 4) measured were significantly higher in S soils compared to AS soils during the spring season whereas this pattern seems inverted during autumn with higher microbial activities in AS soils compared to the S ones. It is hard to explain this inverted tendency, though one of the explanations would be the soil amendment made between the autumn and spring periods that are levelling up the enzymatic and microbial activities in a more important way in the S soils compared to the AS soils due to the higher level of fungi. Indeed, it has been suggested that organic matter derived from fungal metabolic processes may be more chemically resistant and thus increasing the stable carbon storage (Liu *et al*., 2011; Martin & Haider, 1979). This tool remains interesting to compare vineyard physiological profiles, even though the Eco-Plates from Biolog system has yet to be proven as a deep-analysis soil quality indicator as fast-growing microorganisms alter the substrate consumption.

3. Microbial enzymatic activities and molecular biomasses evolve with the season and depend on the terroir

The tendency for higher enzymatic activities, in AS soils compared to S soils, observed during the autumn period (Figure S3) was similar to the observations made during the spring period (Figure 4). Although, phosphatase activities were quite reduced during the autumn compared to the spring period which corroborates the results found in Zuccarini *et al*. (2020), suggesting that warming increases some enzymatic production in soils, but not for β-glucosidase in our case. The higher level of β-glucosidase during autumn might be due to leaf-fall, pruned woods and cover crops degradation that are known to produce organic matter (Mcgourty and Reganold, 2005). This change in enzymatic activities comes up with an alteration in the cultivable level of microbial populations. As far as organic matter is concerned, the amount found in vineyards is considered to be poor compared to other woody perennial crops (Midwood *et al*., 2020). The potential of organic matter, provided by cover crops, is known to increase microbial activity and therefore the soil quality (Belmonte *et al*., 2018; Steenwerth and Belina, 2008; Winter *et al*., 2018). Herein we observed an increase in both bacterial and fungal CFU per gram of dry soil (Table 3; Supplementary Table S3), which is quite different from Corneo *et al*. (2013) results as they had shown no such seasonal effect on cultivable bacteria and fungi over two years of samplings. Measurements made with the Eco-Plates highlighted the microbial dynamics through specific substrates consumption which seems to be more important during spring than autumn (Figure 2; Supplementary Table S3). It may not be surprising as a higher level of bacteria and fungi in cultivable and molecular approaches have been found during spring sampling. Indeed, Hernandez and Menéndez (2019) showed a change in fungal diversity with seasonal fluctuation. Bacterial/fungal (*B*/*F*) gene and cultivable ratios can be used as indicators of soil quality of vineyards but are hardly comparable between studies since different methods to measure biomass were applied (Zehetner *et al*., 2015; Holland *et al*., 2013). Herein, (*B*/*F*) ratios based on copies number of 16S and 18S genes, indicate that bacteria are more abundant than fungi in all soils and globally more abundant in spring than in autumn (Table 5; Supplementary Table S4). In the Graves plots, the (*B*/*F*) ratio is lower in S soils compared to AS soils in both periods of sampling. Interestingly, the (*B*/*F*) ratios based on the cultivable approach are 3460, 45764, 7881 and 4782 % higher in spring compared to autumn samples for 1 S, 1 AS, 2 S and 2 AS soils, respectively. These observations clearly show an increase in the level of cultivable bacteria during spring, suggesting that the richness in bacteria is lowered during autumn whereas cultivable fungi are more stable with the season. This effect was noticed in Pietikäinen *et al*. (2005), with a different approach, that fungi are more adapted to low temperature compared to bacteria, which could drastically affect the (*B*/*F*) ratios that we obtained.

However, the season is assumed to cause less effect on the soil microbiota than the localization (Corneo *et al*., 2013; Siles and Margesin, 2016). Our study demonstrated that both season and terroir strongly impact the variables observed among the soils (Figure 4; Supplementary Table S5). The 18S fungal gene was neither significantly impacted by the terroir (ANOVA: $F(1,76) = 3.645$, $P = 0.32$) and the period of sampling (ANOVA: $F(1,76) = 0.84$, $P = 0.63$), underscoring the idea that fungi were more adapted to low temperature, whereas archaea and bacteria were significantly impacted by both the season and the geographical location.

CONCLUSION

Investigations among four vineyards in the Bordeaux French region that were subjected to unexplained decline revealed a dysbiosis in their

microbial diversity and enzymatic activities. The level of cultivable bacteria coupled to the number of 16S bacterial gene copies were significantly more important in the asymptomatic soils compared to symptomatic ones, while the level of cultivable fungi was higher in the soils subjected to decline. Enzymes involved in N, C and P cycling were significantly more present in the asymptomatic soils, suggesting a decrease in the ecosystemic processes in the area experiencing a decline. The dysregulation of the ecosystemic processes coupled to the microbial dysbiosis observed in studied vineyards in decline is linked to the soil status and therefore the grapevine fitness.

Acknowledgements: This research was supported by FranceAgrimer/CNIV funded as part of the program 'Plan National Dépérissement du Vignoble' within the project Vitirhizobiome. The authors would like to thank the owners of the studied vineyards for their help, as well as Vitinnov for their network and vineyard investigation. The authors would also like to thank Steve Upton for English language editing and review. The authors declare no conflict of interest.

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