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ORIGINAL ARTICLE

Soil microbiota respond to green manure in organic vineyards

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Keywords
bacteria, biodynamic vineyard, fungi, green manure, microbial community structure, microbial diversity, organic vineyard, soil microbiology, soil vineyard.

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Abstract

Aims: The aim of this work was to investigate the effects of biodynamic management with and without the addition of green manure, in comparison with organic management, on the microbiota in vineyards soil.
Methods and Results: High throughput sequencing was used to compare the taxonomic structure of the soil bacterial and fungal communities from vineyards managed with different methods (organic, biodynamic or biodynamic with green manure). Our results showed that microbial communities associated with biodynamic and organic farming systems were very similar, while green manure was the greatest source of soil microbial biodiversity and significantly changed microbial richness and community composition compared with other soils. Green manure also significantly enriched bacterial taxa involved in the soil nitrogen cycle (e.g. *Microvirga* sp., *Pontibacter* sp. and *Nitrospira* sp.).
Conclusions: Our results showed that the diversity and composition of the microbial communities associated with biodynamic and organic farming systems were similar, indicating that the use of biodynamic preparations 500 and 501 did not cause any significant detectable changes to the soil microbial community in the short term, while the effects of green manure were significant in soil microbiota.
Significance and Impact of the Study: The microbiological richness and structure of soil are used as a sensitive indicator of soil quality. The extension of organic/biodynamic farming, associated with green manure application, could contribute to increase the abundance of functional groups of biological and agronomical relevance and maintaining microbial biodiversity in vineyard soils.

Introduction

Since there are vineyards dating back to pre-Roman times in Europe, the grapevine offers a unique opportunity to study extremely long-term monocultures (Schlegel 1973). The physical and chemical properties of soil generally have a major effect in shaping the microbial population of vineyard soil (Corneo *et al.* 2013). Compared with other agro-ecosystems, vineyard soils receive lower nitrogen fertilizer input and are subjected to relatively infrequent tilling and fewer herbicide applications, which may be reflected in the composition of microbial communities (Steenwerth *et al.* 2008). However, in recent years, grape-growing areas have been subjected to cropping intensification. Thus, traditional vineyards planted along the contours of hills on small terraces have often been abandoned and replaced by larger plots on low to moderate slopes, with chemical fertilization and weed control, which increases grape production, but also intensifies soil degradation (Lopez-Pineiro *et al.* 2011). Indeed, in terms of biochemical properties, vineyard soils are usually highly degraded (Miguens *et al.* 2007). Long-term use of certain inorganic pesticides, in particular copper-based fungicides, has resulted in increased concentrations of heavy metals in the soil, affecting the environmental compartments of soil (Komarek *et al.* 2010). Many

studies have demonstrated that conventional agricultural practices can significantly impact microbial communities in soil ecosystems (Villanueva-Rey *et al.* 2014; Hartmann *et al.* 2015). Although the grapevine is an important crop worldwide and preserving the biological quality of soil is mandatory for sustainable agriculture, knowledge about soil microbiological processes in vineyards is generally limited (Probst *et al.* 2008).

The addition of organic substances is of vital importance for soil quality and health (Baldi *et al.* 2010). Organic fertilizers can be a good solution for maintaining soil health, since the release of nutrients in soil is slower than for chemical fertilizers and often better matches plant needs in the growing season. Of the different organic fertilizers, green manure consists of growing specific crops in the inter-rows of the vineyard and then ploughing them into the terrain to improve soil quality. Green manure crops usually include grass mixtures and legume plants, such as vetch, clover, barley and others. Multiple benefits are produced by green manure. The physical structure of soil is improved, because green manure tends to reduce soil erosion and leaching (Ingels *et al.* 2005). This practice can also help to provide ecological niches supporting predators/parasitoids, improving pest control in the vineyard (Irvin *et al.* 2014). Green manure influences the grapevine plant and its fruit by enhancing the organoleptic characteristics of the grapes (Rotaru *et al.* 2011). Moreover, the organic carbon available for soil micro-organisms is significantly increased by green manure, which also enhances the activity of numerous soil enzymes, especially those involved in the N cycle (Okur *et al.* 2016). Although the advantages of using green manure have been recognized, little is known about the possible modifications that it could cause in soil microbial communities.

Organic agriculture is codified by the Council of the European Union (EC 834/2007) as a management that uses living organisms and mechanical production methods; excludes the use of genetically modified organisms (GMO) and the products derived from them; reduces the external inputs, that are limited to natural or naturally derived substances; and bans the use of chemically synthesized inputs. Biodynamic agriculture can be regarded as a pioneer version of organic agriculture (Kirchmann 1994), and it is currently a certification and labelling system included in the organic production. The principles of biodynamic agriculture were established in Germany by Rudolf Steiner in the 1920s. It adopts a holistic approach to the exploitation of natural resources, taking into consideration the sustainability of different elements, such as the crops themselves, animal life preservation or the maintenance of high-quality soil, in order to recover, preserve or improve ecological harmony. The biodynamic approach in farm practices reflects the metaphysical concepts proposed by its founder. It uses a set of specific compost preparations to be applied to crops to aid fertilization and the application of other homoeopathic treatments based on infusions or plant extracts (Lotter 2003), whose effect did not received scientific validation so far (Chalker-Scott 2013). For example, preparations 500 and 501 consist in cow manure and quartz powder, respectively, placed in a cow horn and buried, and then used in the field after a period of maturation.

Knowledge of how soil management affects soil microbial species richness and abundance is important because microbial diversity and stability determine the soil's ability to react to external changes, the impact and degradation (Munoz *et al.* 2007). Beneficial microbial processes are essential for crop production as they determine the soil's ability to supply nutrients to the plant; they retain nutrients in the profile, contribute to the formation of soil structure, suppress plant pathogens and contribute to soil humus formation (Ingels *et al.* 2005).

The main goal of this study was to investigate the effects of biodynamic viticulture on the microbial community structure and diversity of vineyard soils, with or without the addition of green manure, using high throughput sequencing, in comparison to organic management.

Materials and methods

Sampling site and vineyard management

The study site was located at an experimental site (1·0 ha) in the Trentino-South Tyrol region in northern Italy (San Michele all'Adige, 46·19 N, 11·14 E). The experiment was repeated in two vineyards, according a "split plot" design, with the vineyards (Field 1 and Field 2) as first factor and vineyard management (organic (O), biodynamic (BD) or biodynamic with green manure (BDGM)) as second factor, starting from the autumn of 2011. The vineyards were both planted with Cabernet franc variety (clones 214, 331 and 327) on SO4 rootstock in 2002, and the Guyot vine training system was adopted (2·0 m × 1·0 m). In the O plots, pneumatic leaf removal and mechanical hedging were adopted for canopy management, while in the BD and BDGM plots, pneumatic leaf removal was substituted with manual removal of lateral shoots, and instead of hedging, the shoots were rolled onto the last couple of wires in the vegetative wall. Chemical fertilizers were not applied to any of the plots, while all plots were treated with copper (4·6 kg ha^{-1}) and sulphur (43 kg ha^{-1}) to control fungal disease. In the BD and BDGM plots, biodynamic preparations 500 (100 g ha^{-1}) and 501 (4 g ha^{-1}) were used to stimulate

humus formation, while no fertilizers were applied in the O. The inter-rows in the BDGM plots were sown with cover crops seed mixture for green manure (181 Kg ha^{-1}, Table 1) on 5 October 2011, to limit physical degradation by compaction and dissolution. Green manure plants (dry weight of 0·58 Kg m^{-2}) were mown on 15 May 2012 and incorporated into the soil. Specifically, in the BDGM plots, a chisel plough set at 50 cm was used before cover crop seeding, followed by a rotating harrow. Mechanical weed control was performed during inter row mowing in all the plots.

Soil sampling and processing

Soil sampling was carried out on 2 October 2012. Three sampling points were chosen along two grapevine rows (at the two ends and a central point) in each field and for each type of vineyard management. A total of 36 samples (3 replicates × 2 rows × 2 fields × 3 vineyard managements) were collected.

For each sampling point, three-soil cores (Ø 5 cm, depth 19 cm) were collected from the topsoil and transferred into sterile bags, after removing the first 5 cm of the soil layer (mostly humus). The soil samples were sieved separately to a <2-mm particle size, and an equal amount of soil from each sampling point was transferred into a 50-ml sterile falcon tube (Sarstedt, Germany), lyophilized and stored at −80°C for metagenomic analysis.

Physical and chemical analysis was carried out on the remaining soil, after pooling by field, vineyard management and row (n = 12). The analyses were carried out following the official methods for soil chemical analysis (DM 11/05/92 and DM 13/09/99). The following parameters were measured: total sand (2·0–0·050 mm), silt (0·050–0·002 mm) and clay (<0·002 mm) were determined by measuring the volumetric mass of the water–soil suspension and the distribution of the elementary particles by wet sieving and hydrometer; total soil organic matter (SOM) and total nitrogen content (N) were determined by elemental analysis using the Dumas method; carbon/nitrogen (C/N) ratio, calculated from total C and N. The pH was measured in 1:2·5 soil:water suspension; total $CaCO_3$ by gas-volumetric determination of CO_2 after HCl treatment (ISO 10693) and active limestone using the Drouineau method; Mg, K, exchangeable cations by extraction with ammonium acetate 1 M at pH 7; P using the Olsen method; total Cu, Fe, Mn, Zn, Pb and Cd were quantified in aqua regia.

Table 1 Composition of the cover crop seed mixture sown on 5 October 2011 for green manure and average productivity of the cover crops and natural grass measured on 15 May 2012 as dry weight

Cover crops	% Weight
Vicia sativa	11·0
Pisum sativum	22·1
Vicia faba	55·2
Secale cereale	11·1
Brassica napus	0·6

DNA extraction, amplification and pyrosequencing

Total genomic DNA was extracted from 0·5 g of lyophilized soil using a FastDNA® Spin kit (MP Biomedicals, France), following the manufacturer's instructions, and quantified using a NanoDrop 8 000 spectrophotometer. For bacterial identification, the V1–V3 hypervariable region of 16S rRNA was PCR amplified using the primer set 27f (Weisburg *et al.* 1991) and 518r (Muyzer *et al.* 1993). At the 5′ end, the forward primer carried the 454-adaptor A with a specific Roche-10 nt multiplex identifier (MID) for each soil sample. Each sample was amplified in triplicate in a 25 μl reaction, following the amplification protocol by Nicola *et al.* (2017). The 18S rRNA–5·8S rRNA internal spacer (ITS) of fungal rRNA was amplified using the primer pair ITS1F (Gardes and Bruns 1993)-ITS2 (White *et al.*, 1990). One-way amplicon sequencing was carried out as in Nicola *et al.* (2017). Three independent PCR reactions (technical replicates) were performed for each sample and pooled together. All the PCR products were then analysed with gel electrophoresis and cleaned using an AMPure XP beads kit (Beckman Coulter, Brea, CA). Two final and distinct libraries (16S and ITS) were constructed from the 36 PCR products. Pyrosequencing was performed on a GS FLX+ system (Roche, Mannheim, Germany) using XL+chemistry, following the manufacturer's instructions.

16S rRNA gene and ITS sequence processing

Pyrosequence quality was checked in PRINSEQ (Schmieder and Edwards 2011) and flowgrams were filtered and denoised using FlowClus (Gaspar and Thomas 2015). Denoised microbial reads were processed using Metaxa2 v2·1·3 (Bengtsson-Palme *et al.* 2015) to target the extraction and to verify the 16S rRNA variable regions. Similarly, but for fungal reads, ITSx v1·0·11 (Bengtsson-Palme *et al.* 2013) was used to target the ITS1. USEARCH v7 (Edgar 2013) was used to de-replicate, sort and cluster the extracted regions with 97% pairwise sequence identity. Chimeras were removed by adopting both *de novo* and reference based methods as features of the above-mentioned tool. The RDP classifier train set n.15 (2015/09) was used as a reference database for microbial chimeras, whereas the UNITE reference sequences version n.

7·0 (2016/01) were chosen for fungal ITS chimera detection in UCHIME (Edgar *et al.* 2011). Taxonomy assignment was performed by employing naive Bayesian RDP classifier v2·10 (Wang *et al.* 2007) in QIIME (Caporaso *et al.* 2010) with a minimum confidence of 0·6 against the SILVA database, release 123 (2016/05) (Quast *et al.* 2013) and the UNITE database, version n. 7·1 (2016/08) (Abarenkov *et al.* 2010) for 16S rRNA-based and ITS-based sequences, respectively. Sequence data were made available in the NCBI SRA database under BioProject number PRJNA381189.

16S rRNA gene-based microbial and ITS-based fungal community analysis and statistics

OTU-based analysis was carried out in QIIME to calculate richness and diversity after multiple rarefaction (5 870 and 2 348 read depth for bacteria and fungi, respectively). The OTUs observed were counted and the diversity within each individual sample was estimated using Simpson's diversity index. Richness and diversity values were separately analysed in R, fitting all the factors in generalized linear models (GLMs) assuming a Gamma distribution and validated via graphical representation of residuals vs. fitted values. The statistical significance of the GLMs was inferred by adopting the chi-square test, and *post hoc* pairwise comparisons were calculated using Tukey's HSD test in the multcomp R package (Hothorn *et al.* 2008). Microbial and fungal richness and diversity values were graphically represented as box plots using the ggplot2 R package.

Multivariate analysis of community structure and diversity was performed according to the recommendations of Anderson and Willis (2003): (i) unconstrained ordination offered by Principal Coordinate Analysis (PCoA) (data not shown); (ii) constrained multidimensional scaling using Constrained Analysis of Principal Coordinates (CAP) as re-implemented in the vegan R package (Oksanen *et al.* 2017); (iii) permutation test to assess the significance of the constraints and permutational multivariate analysis of variance (PERMANOVA); (iv) identification and correlation of OTUs responsible for shaping the diversity structure. The effects of rare species were downweighted by applying Hellinger transformation to the rarefied OTU tables.

In more detail, the differences between bacterial communities were investigated using the Bray–Curtis dissimilarity distance and the ordination methods applied to the same distance matrices. All the ordination analyses were computed and CAP plotted in phyloseq (points 1 and 2). The significance of the treatment grouping factor used as a constraint in CAP was assessed via the permutation test in the vegan R package. The null hypothesis of no differences between *a priori* defined groups was investigated using the PERMANOVA approach, implemented in vegan as the ADONIS function and applied to the Bray–Curtis dissimilarity distances.

Permutational pairwise comparisons between the treatments were carried out with the RVAideMemoire R package (Hervé 2017) and *P*-values were FDR-adjusted (point 3). Indicator OTU analysis was applied for calculation of differential OTU abundance in treatments using the indicspecies R package (De Caceres and Legendre 2009) and *P*-values were FDR-adjusted. Procrustes analysis (Lisboa *et al.* 2014) was then applied to CAP ordinations to correlate bacterial and fungal beta-diversity in response to different farming practices (point 4). Differential OTU abundance for treatments at genus level was assessed via permutation ANOVA (RVAideMemoire R package) for both the bacterial and fungal dataset. Significantly different genera (FDR-adjusted *P*-values) were then shown as bar plots (mean ± SD of number of reads), and for each genus, the pairwise permutation *t*-test was applied to all treatment combinations.

Results

The physical and chemical analysis of soil (Table 2) did not reveal statistically significant differences between the types of management.

Pyrosequencing yielded a total of 401 824 raw pyrotags reads for bacteria and 305 990 reads for fungi. After quality filtering and chimera removal, a total of 314 910 16S rRNA sequences and 164 227 ITS sequences remained for community analysis, corresponding to an average ± SD of 8 997 ± 1 726 reads and 4 562 ± 1 367 reads per sample for bacteria and fungi, respectively. A total of 4 809 bacterial OTUs and 633 fungal OTUs were detected (Figs. S1 and S2).

The most abundant bacterial phyla, in all soil samples, were Actinobacteria (31·71%), Proteobacteria (21·96%), Acidobacteria (12·78%) and Gemmatimonadetes (8·29%). A total of 32 phyla, 116 classes, 255 orders, 505 families and 850 genera were detected. As regards genera, the most abundant in vineyard soil were *Gaiella* sp. (5·66%), *Bacillus* sp. (1·99%), *Arthrobacter* sp. (1·74%) and *Nitrospira* sp. (1·26%) for bacteria. The fungal communities were instead dominated by Ascomycota (77%), Basidiomycota (16%) and Zygomycota (7%). Overall, a total of six phyla, 22 classes, 61 orders, 124 families and 220 genera were found in the soil samples. The Ascomycota mostly consisted of Sordariomycetes, followed in decreasing order of relative abundance by Dothideomycetes and Eurotiomycetes. More than 37% of the Sordariomycetes reads belonged to the Hypocreales order, and within this order Nectriaceae were the most abundant family.

Table 2 Physical-chemical analysis of soil samples, divided according to the management system applied (average ± SD). O, samples from organically managed soil; BD, samples from biodynamically managed soil; BDGM, samples from biodynamically managed soil with the addition of green manure as fertilizer

Parameter	O	BD	BDGM
pH	7·97 ± 0·02	7·97 ± 0·01	7·97 ± 0·09
Total limestone (g kg^{-1} $CaCO_3$)	367·50 ± 9·19	365·50 ± 6·36	371·00 ± 1·41
Active limestone (g kg^{-1})	13·50 ± 0·71	13·00 ± 0·00	12·50 ± 0·71
Organic substance (g kg^{-1})	24·00 ± 1·41	22·50 ± 2·12	26·00 ± 8·49
N (g kg^{-1})	1·10 ± 0·00	1·10 ± 0·00	1·40 ± 0·42
C/N	12·51 ± 0·83	11·82 ± 0·75	11·00 ± 0·01
P_2O5 (mg kg^{-1})	59·50 ± 0·71	54·00 ± 11·31	61·00 ± 21·21
K_2O (mg kg^{-1})	229·00 ± 15·56	233·00 ± 2·83	214·00 ± 21·21
MgO (mg kg^{-1})	417·00 ± 43·84	446·00 ± 0·00	466·50 ± 57·28
CSC (meq per 100 g)	14·40 ± 0·71	15·05 ± 0·92	15·80 ± 1·84
Cu DTPA (mg kg^{-1})	27·65 ± 4·31	27·90 ± 7·35	32·85 ± 14·50
Fe DTPA (mg kg^{-1})	10·70 ± 0·00	11·13 ± 1·80	11·00 ± 0·71
Mn DTPA (mg kg^{-1})	10·85 ± 1·06	10·13 ± 0·66	11·05 ± 1·34
Zn DTPA (mg kg^{-1})	4·65 ± 0·72	4·89 ± 1·04	5·71 ± 2·57
Pb DTPA (mg kg^{-1})	21·42 ± 2·81	26·48 ± 10·77	23·45 ± 7·12
Cd DTPA (mg kg^{-1})	0·10 ± 0·00	0·10 ± 0·01	0·10 ± 0·01
Sand (g kg^{-1})	295·50 ± 7·78	263·50 ± 12·02	285·00 ± 9·90
Silt (g kg^{-1})	534·50 ± 6·36	556·50 ± 26·16	550·00 ± 11·31
Clay (g kg^{-1})	170·00 ± 14·14	180·00 ± 14·14	165·00 ± 21·21

Clonostachys sp. (13·29%), *Coprinellus* sp. (8·13%), *Exophiala* sp. (4·15%) and *Fusarium* sp. (4·08%) were the most abundant genera of fungi.

The Glomeromycota phylum, an important soil microbial group that forms one of the most common types of symbiosis (arbuscular mycorrhizal fungi; AMF), presented a low abundance (0·19%) in all soils and the management systems did not influence its diversity. Three classes —Archaeosporales, Glomerales and Paraglomerales—represented this phylum. The Glomeraceae family was more abundant compared with the Ambisporaceae and Paraglomeraceae families. The *Glomus* and *Funneliformis* genera were common with all the management systems, while the genus *Septoglomus* was present in O and BD soils and was almost absent in BDGM soils.

The alpha (within-sample) diversity (observed OTUs) found in bacterial communities in biodynamic soils with green manure (BDGM) was significantly higher than that in organic (O) and biodynamic (BD) soils (Fig. 1a; Table S1). Moreover, the bacterial richness in Field 1 was significantly greater than in Field 2, and the same trend was observed in fungal communities (Fig. 1b). However, the different types of soil management did not influence fungal alpha-diversity. When looking at Simpson's diversity values, no significant differences were found between grouping factors ($P > 0·05$).

When beta (between-sample) diversity was analysed using PERMANOVA, both fungal and bacterial communities were significantly different according to the type of soil management, the field of origin and the interaction between the two ($P < 0·001$, Table S2). With permutational pairwise comparisons, it was ascertained that the microbiome of BDGM soils was significantly different from those in O and BD soils ($P = 0·045$, $P = 0·019$, respectively). Building on these results, constrained analysis of principal coordinates (CAP) was performed on bacteria and fungi (Fig. 2), using the factors that appeared to be significant in PERMANOVA as constraints. The samples were divided according to the type of soil management (BDGM vs O and BD) and the field of origin, both for bacteria and fungi. Procrustes correlation testing for CAP analysis was performed and a correlation of 0·51 (m12 = 0·74) with a significance $P < 0·001$ was found, meaning that bacterial and fungal diversity reacted in a similar way to soil management. Bacterial and fungal indicator OTUs significant for soil management were identified. In BDGM and BD soils, the bacterial indicator species were mainly genera associated with the soil nitrogen cycle, such as *Nitrospira* sp., *Pontibacter* sp. and *Frankia* sp. (Table 3). The fungal indicator species were instead mainly saprobic fungi in each type of soil management (Table 4). *Exophiala* sp., a black yeast often associated with soil enriched with organic waste, was the indicator species in O soils. In BD soils, *Mortierella* sp., *Mortierella Antarctica, Humicola nigrescens* and the antagonistic fungus *Acremonium persicinum* were the indicator OTUs. However, BDGM soils contained the biocontrol agent and plant growth promoting *Cladorrhinum* sp.,

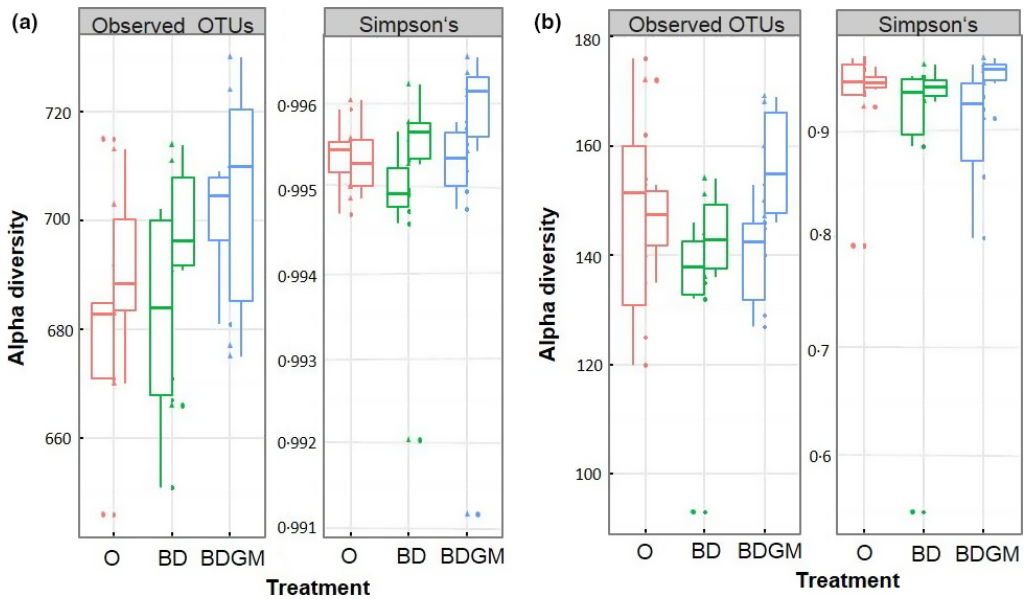

Figure 1 Box plots representing observed OTUs and Simpson indices of bacterial (a) and fungal (b) communities in vineyard soils managed with different sustainable approaches. O, samples from organically managed soil; BD, samples from biodynamically managed soil; BDGM, samples from biodynamically managed soil with the addition of green manure; (●) soil samples from Field 1; (▲) soil samples from Field 2. [Colour figure can be viewed at wileyonlinelibrary.com]

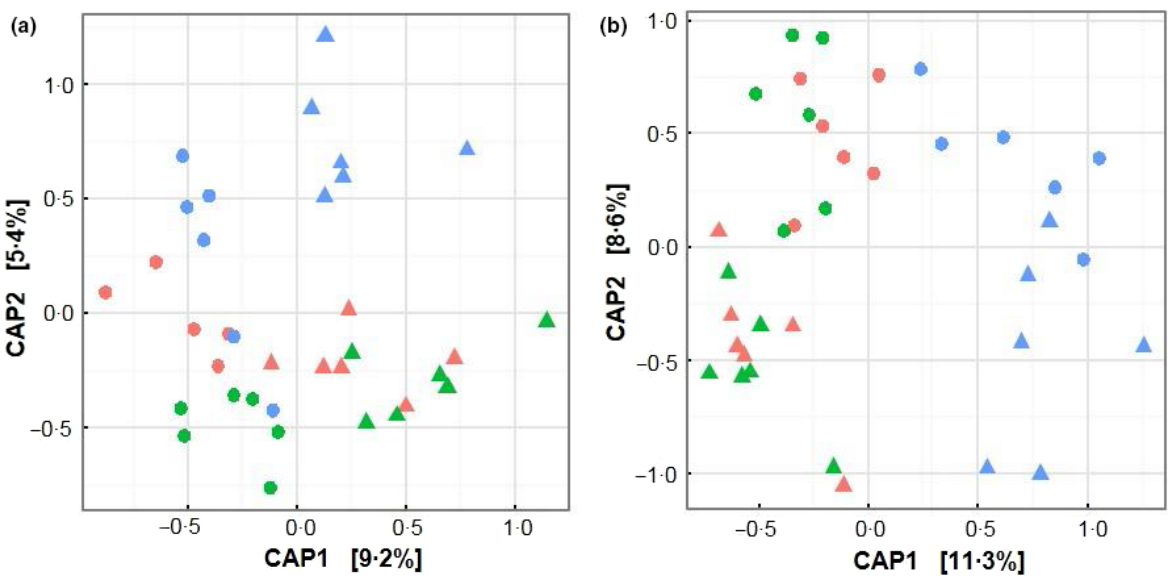

Figure 2 Constrained analysis of principal coordinates (CAP), based on the Bray–Curtis dissimilarity distance of 454 sequencing bacterial (a) and fungal (b) data for soil samples from vineyard soils managed with different sustainable approaches. O, samples from organically managed soil; BD, samples from biodynamically managed soil; BDGM, samples from biodynamically managed soil with the addition of green manure; (●) soil samples from Field 1, (▲) soil samples from Field 2. [Colour figure can be viewed at wileyonlinelibrary.com]

Capnobotryella sp., a black-pigmented fungi, *Cystofilobasidium capitatum*, a pectinolytic yeast and *Exophiala* sp.

As regards the differences in OTU abundance in O soil and BD and BDGM soil, calculated by permutation ANOVA, we found two nitrogen-fixing bacterial genera (*Microvirga* sp. and *Pontibacter* sp.) to be significantly more abundant in BDGM soils, together with *Actinoplanes* sp., which has an important role both in the degradation of fallen leaves and as an antagonist of oomycetes like *Pythium* and *Phytophthora* (Fig. 3,

Table 3 Bacterial indicator OTUs for the different types of vineyard soil management obtained with the indicspecies R package (*P*-values corrected using FDR). O, samples from organically managed soil; BDGM, samples from biodynamically managed soil with the addition of green manure as fertilizer

Soil management	Bacterial OTUs	*P*-values
O	*Lactobacillus sp.*	0·0496
BDGM	*Nitrospira* sp.	0·0037
	Catelliglobosispora sp.	0·0037
	Planosporangium sp.	0·0396
	Paenibacillus sp.	0·0496
	Pontibacter sp.	0·0496

Table 4 Fungal indicator OTUs for the different types of vineyard soil management obtained with the indicspecies R package (*P*-values corrected using FDR). O, samples from organically managed soil; BD, samples from biodynamically managed soil; BDGM, samples from biodynamically managed soil with the addition of green manure as fertilizer

Soil management	Fungal OTUs	*P*-values
O	*Exophiala* sp. 1	0·0440
BD	*Mortierella antarctica*	0·0069
	Acremonium persicinum	0·0092
	Mortierella sp. 04M 158	0·0069
	Humicola nigrescens	0·0166
BDGM	*Exophiala* sp. 2	0·0104
	Capnobotryella sp. MA 4775	0·0173
	Cystofilobasidium capitatum	0·0270
	Cladorrhinum sp.	0·0303

Table S3). However, the genus *Terrimonas*, involved in S cycling in soil, was significantly more abundant in O soil than in BD and BDGM soil. In fungal analysis, the genera *Cladorrhinum*, *Cystofilobasidium* and *Myrmecridium* and the psychrophilic basidiomycetous yeast *Mrakiella* sp. were significantly more abundant in BDGM soils (Fig. 4, Table S4). In addition, the genera *Colletotrichum, Gibberella* and *Leptosphaeria,* which include pathogenic species of plants, were abundant where green manure was applied. In contrast, *Clonostachys* sp. and *Pyrenochaeta* sp., associated with biocontrol and plant pathogens respectively, were more abundant in O and BD samples than in BDGM samples.

Discussion

Scientific studies on biodynamic management in vineyards and its effect on soil microbiota are rare (Burns *et al.* 2016), since most works tend to concentrate on the effects on plants or grapes. Recent work on biodynamic viticulture has affirmed that in terms of grape health, the microbiological and chemical characteristics in these vineyards were comparable or better to those in vineyards cultivated using conventional methods (Guzzon *et al.* 2016).

There are several theories regarding the way in which the biodynamic preparations may interact with crops and may include hormonal stimulation, enhancing crop growth, especially at root level (Villanueva-Rey *et al.* 2014). As regards the effects of biodynamic preparations, according to Chalker-Scott (2013), the addition of these products did not affect the yield of the crops analysed, and other authors have also stated that biodynamic preparations had little influence on plant biotic parameters (Doring *et al.* 2015; Baskar and Shanmugham 2016).

We used the high-resolution power of 454-pyrosequencing to investigate soil microbial biodiversity in sustainably managed vineyards, specifically studying the short-term effects of two types of farming management (O, BD) and green manure application (BD, BDGM) on the diversity, richness and composition of soil microbial communities. In our study, no difference in alpha or beta diversity was noticed between O and BD soil samples. This is in agreement with previous studies on biodynamic management, which have indicated similar behaviour for organic and biodynamic farming systems in terms of microbial soil composition and diversity. In fact, Carpenter-Boggs *et al.* (2000) found that organically and biodynamically managed soils had a similar microbial composition, but they were more biotically active than soils that did not receive organic fertilization. Moreover, organic management enhanced soil biological activity, but additional use of biodynamic preparations did not significantly affect the soil biotic parameters tested. In this study, we have to consider the short-term exposure of the soil microbiota to the different vineyard managements. According to Hartmann *et al.* (2015), the short-term effect of agricultural management has shown a fewer impact than the long-term effects, mainly on bacterial community and the spatiotemporal variability was important to reveal the shift of the structure of the soil microbiota.

Green manure application had a major impact on soil micro-organisms. Our results showed the crucial importance of green manure for soil microbiota, since it promoted higher bacterial richness and significant changes in the microbial communities found in BDGM soils. Bacteria and fungi responded in a similar way to green manure application. These results are in accordance with Ingels *et al.* (2005), who analysed microbial communities using phospholipid fatty acid (PLFA) analysis, showing that biodynamic management associated with green manure application increased the taxonomic and phylogenetic richness, diversity and heterogeneity of soil microbiota compared with other farming systems. Furthermore,

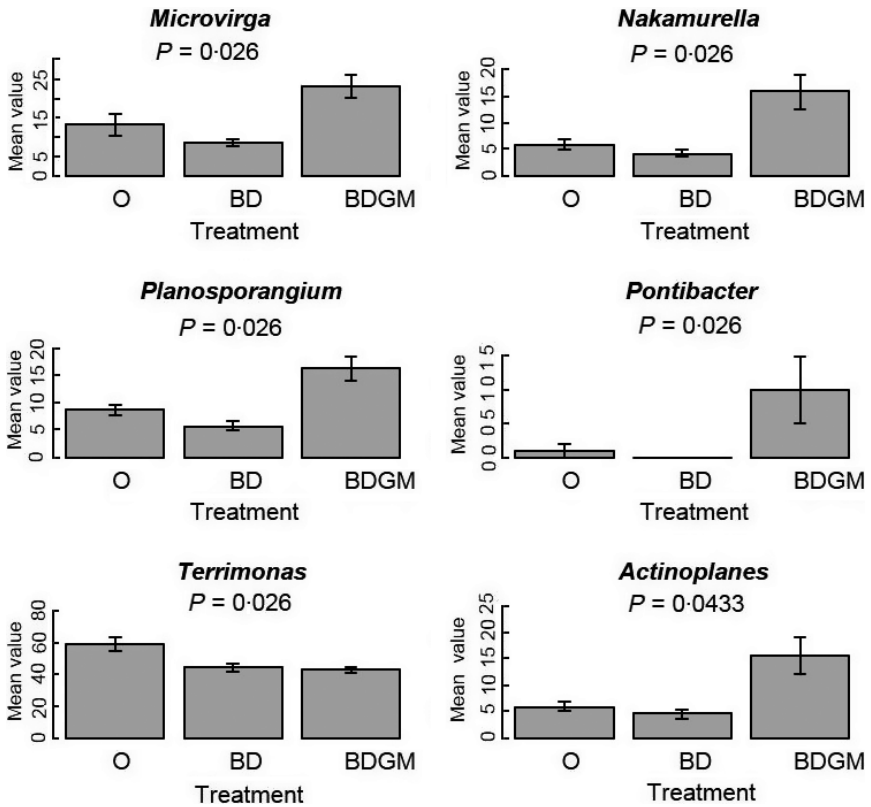

Figure 3 Bar plots of the different bacterial OTU abundance at genus level for different types of vineyard soil management obtained via permutation ANOVA. Only significantly different genera (*P*-values corrected using FDR) are shown (mean ± SD of number of reads). O, samples from organically managed soil, BD, samples from biodynamically managed soil, BDGM, samples from biodynamically managed soil with the addition of green manure.

Wittwer *et al.* (2017) highlighted other benefits of cover crops, such as providing various ecological services to agro-ecosystems, protection against soil erosion, reduction of nutrient losses, improvement of soil and water quality, and to some extent, a reduction in weeds and pests.

Moreover, the addition of green manure significantly enriched the population of bacteria potentially active in the soil nutrient cycle, such as *Microvirga* sp., *Pontibacter* sp. and *Actinoplanes* sp. *Microvirga* sp. is a nitrogen-fixing bacterium that is often found in symbiosis in the root nodules of legumes (Ardley *et al.* 2012; Reeve *et al.* 2014). *Pontibacter* sp. is a Gram-negative genus isolated from different environments, such as different kinds of soil, muddy water and marine water (Srinivasan *et al.* 2014), and some strains carry out nitrogen-fixing activity in soil (Xu *et al.* 2014). However, *Actinoplanes* sp. is often found in leaf litter (Nurkanto *et al.* 2016) and may have an important role in the degradation of fallen leaves and organic matter (Hop *et al.* 2011), in addition to exercising antagonistic activities against several soil-borne pathogens, such as *Pythium* spp. and *Phytophthora megasperma* (Filonow and Lockwood 1985; El-Tarabily *et al.* 2010). Green manure also increased the presence of some fungal OTUs, such as the genus *Cladorrhinum*, a fungal group of prime importance for agriculture and livestock, since some species have biocontrol potential or have been shown to promote plant growth and produce phytases (Carmaran *et al.* 2015), and the cold-adapted heterobasidiomycetous genus *Cystofilobasidium*, which can utilize D-glucuronate and inositol as sole carbon sources and the assimilation of nitrate as sole nitrogen source (Linkind *et al.* 2009). Other increased fungal OTUs in BDGM were *Myrmecridium,* a fungal genus whose members are either saprobes or plant endophytes (Peintner *et al.* 2016) and the psychrophilic basidiomycetous yeast *Mrakiella*. The genera *Colletotrichum*, *Gibberella* and *Leptosphaeria*, which include important phytopathogens of many economically significant plants cultivated around the world, were also more abundant where green manure was applied.

As regards bacterial composition, the two most abundant bacterial phyla in these soil samples, Actinobacteria and Proteobacteria, are copiotrophs in soil, and they are

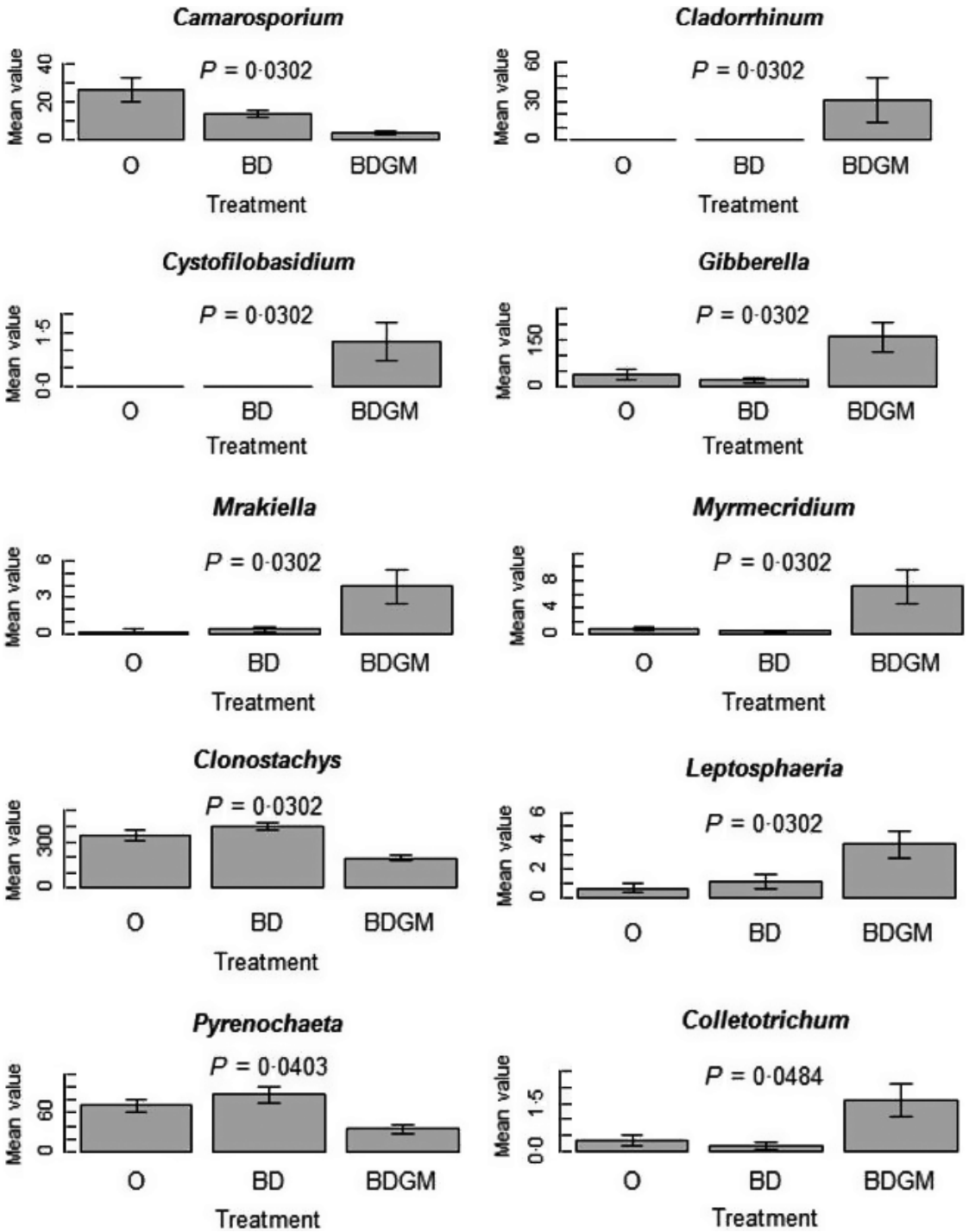

Figure 4 Bar plots of the different fungal OTU abundance at genus level for different types of vineyard soil management obtained via permutation ANOVA. Only significantly different genera (*P*-values corrected using FDR) are shown (mean ± SD of number of reads). O, samples from organically managed soil, BD, samples from biodynamically managed soil, BDGM, samples from biodynamically managed soil with the addition of green manure.

plentiful in conditions of high nutrient availability, exhibiting high growth rates (Fierer *et al.* 2007). The third phylum in order of abundance was Acidobacteria, which instead comprises oligotrophic soil bacteria (Fierer *et al.* 2007; Schimel and Schaeffer 2012). As regards fungi, OTUs belonging to the phylum Ascomycota were

dominant in all types of soil management, which is common in cultivated soil (Sugiyama *et al.* 2010; Franke-Whittle *et al.* 2015; Abujabhah *et al.* 2016), and were followed by those of Basidiomycota, Zygomycota and Chytridiomycota. A similar trend was observed by Orgiazzi *et al.* (2012) when analysing ITS fragments from different soil types with 454 pyrosequencing.

In this work, almost all the microbial genera were found indiscriminately in each of the management systems at both sites. However, differences in abundance could be detected and some OTUs could be associated with specific types of soil management as their indicator OTUs. In O soils, only two indicator OTUs were found, the bacterium *Lactobacillus* sp. and the fungus *Exophiala* sp. *Lactobacillus* sp. often grows on grape skin (Bae *et al.* 2006; Nisiotou *et al.* 2015), but it can also be isolated from soil and it shows antifungal activity against several fungi, among which *Fusarium* spp. (Baffoni *et al.* 2015; Gajbhiye and Kapadnis 2016). According to Franke-Whittle *et al.* (2015), the genus *Exophiala* includes black yeasts that had negative correlations with apple plant growth and that were significant due to the high abundance in the soil. Black yeasts is a *terminus technicus* describing a heterogeneous group of fungi that have in common melanized cell walls and the formation of cells by yeast-like budding (Sterflinger 2006). While no bacteria were significantly associated with this treatment, four common saprotrophic soil fungi were indicator OTUs in BD soils. These were *Mortierella* sp. and *Mortierella antarctica,* which occur mainly in the soil of different ecosystems, including terrestrial habitats of Antarctica (Adams *et al.* 2006); *Humicola nigrescens,* a thermophilic mould capable of efficiently degrading organic materials by secreting thermostable enzymes (Singh *et al.* 2016); and *Acremonium persicinum*, an endophytic fungus of the grapevine with antagonistic activity against both the asexual and sexual spores of *Plasmopara viticola* (Burruano *et al.* 2016). *Acremonium persicinum* also hydrolyses cellulose and produces cephalosporin C, which is a major precursor of semisynthetic cephalosporin antibiotics used to treat a wide range of bacterial infections (Sarookhani and Moazzami 2007).

As regards the bacterial indicator species of BDGM soils, there were three bacterial genera involved in the soil nitrogen cycle (*Nitrospira* sp., *Paenibacillus* sp. and the aforementioned nitrogen-fixing *Pontibacter* sp.). *Nitrospira* sp. belongs to nitrite-oxidizing bacteria (NOB) (Hayatsu *et al.* 2008) and is widely distributed in many habitats, including soil, oceans, freshwater and wastewater treatment plants (Koch *et al.* 2015). In soil, it is often associated with an increased supply of nitrogen from mineral fertilization (Zhou *et al.* 2015). Another nitrogen-fixing bacterium is *Paenibacillus* sp., which is also considered a plant growth promoter due to its production of IAA (indole-3-acetic acid), and it also has biocontrol potential against grapevine pathogens such as *Botrytis cinerea* and *Neofusicoccum parvum* (Grady *et al.* 2016; Haidar *et al.* 2016). As regards fungi, four black yeasts (*Cladorrhinum* sp., *Capnobotryella* sp., *Cystofilobasidium capitatum* and *Exophiala*) were fungal indicator OTUs in BDGM soils. Most of these genera are found as saprobes colonizing inert surfaces, or in hydrocarbon- or heavy-metal-polluted habitats, and several are potential human pathogens (Seyedmousavi *et al.* 2014). Currently, little information about the ecophysiology of other detected indicator OTUs (*Catelliglobosispora* sp., *Planosporangium* sp. and *Capnobotryella* sp.) is available in order to deduce any putative ecological role in the soil system. Two different techniques that are commonly used to investigate the differences in microbial communities (indicator species and OTU identified by permutational ANOVA) detected different groups of OTUs. Both techniques individuated *Planosporangium* sp. and *Pontibacter* sp. as significant bacterial OTUs in BDGM soils, while no correspondences were found in fungal OTUs. These discrepancies in methods indicate how difficult can be a univocal characterization of microbial communities, since different valid statistical tools can give different answers, depending on the algorithms applied. For this reason, we decided to maintain and analyse both results, in exploiting two different techniques of analysis.

Arbuscular mycorrhizal fungi occur in the roots of most plants and are an ecologically important component of the soil microbiome. Analysis of the OTUs belonging to Glomeromycota showed a low level of AMF relative abundance. Also Hartmann *et al.* (2015) have observed only few Glomeromycota associated to different organic and conventional management systems using 454 pyrosequencing analysis. According to Orgiazzi *et al.* (2012), ectomycorrhizal phylotypes are numerous in natural sites covered by trees, but they are almost completely lacking in anthropogenic and grass-covered sites. Ciccolini *et al.* (2016), on studying the community of AMF with 454 pyrosequencing, reported a low level of AMF richness in intense cropping systems. However, we should also consider the limited coverage by the primers used in this work to be partly responsible for the few Glomeromycota observed (Stockinger *et al.* 2010). The genus *Glomus* was most abundant and present in all soils, in accordance with other studies, which have found this genus to be the most abundant AMF in the grapevine (Schreiner and Mihara 2009).

Soil is a nonrenewable resource and most vineyard soils are considered to be highly degraded in terms of loss of organic carbon, as a result of a decrease in nutrient content, an accumulation of metals and organic

pollutants (Coll *et al.* 2011). The effect of agricultural management systems on soil micro-organisms is generally studied with plants undergoing rotation, but less is known about soils used for perennial plants, such as the grapevine. This is one of the few works comparing the microbial communities of soil in organic and biodynamic vineyards using 454 pyrosequencing. Overall, our results showed that the diversity and composition of the microbial communities associated with biodynamic and organic farming systems were similar, indicating that the use of biodynamic preparations 500 and 501 did not cause any significant detectable changes to the soil microbial community in the short term, while the effects of green manure were significant in soil microbiota. The increase in soil microbial diversity associated with the use of green manure could have possible benefits for plant nutrition, considering that in organic farming systems mineralization of organic matter depends on soil micro-organism activity. The incorporation of green manure was shown to increase the diversity of micro-organisms in soil, particularly the abundance of specific bacteria and fungi. Evidence of increased nitrogen-fixing and nitrite-oxidizing bacteria populations in soil as a response to the use of green manure suggests that they can potentially be adopted to increase nitrogen availability. An extension of organic/biodynamic farming associated with green manure application could contribute to maintaining higher microbial biodiversity in vineyard soil and consequently positively influence overall soil quality.

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Conflict of Interest

The authors declare that they have no competing interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Rarefaction curves indicating the observed number of operational taxonomic units (OTUs) related to the number of sequences retrieved in each of the different soil vineyard managements with 16S primer set. O, samples from organically managed soil; BD, samples from biodynamically managed soil; BDGM, samples from biodynamically managed soil with the addition of green manure. The solid line and the dashed line represent, respectively, samples from Fields 1 and 2.

Figure S2. Rarefaction curves indicating the observed number of operational taxonomic units (OTUs) related to the number of sequences retrieved in each of the different soil vineyard managements with ITS primer set. O, samples from organically managed soil; BD, samples from biodynamically managed soil; BDGM, samples from biodynamically managed soil with the addition of green manure. The solid line and the dashed line represent, respectively, samples from Field 1 and 2.

Table S1. Alpha-diversity analysis for bacteria and fungi calculated with a generalized linear model (GLM) and Tukey contrasts as post hoc analysis.

Table S2. Beta-diversity analysis for bacteria and fungi calculated with PERMANOVA on Bray–Curtis dissimilarities and permutational pairwise comparisons.

Table S3. Differentially abundant bacterial genera calculated with permutational ANOVA.

Table S4. Differentially abundant fungal genera calculated with permutational ANOVA.