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EDITED BY
David Johnston-Monje,
University of Valle, Colombia

REVIEWED BY
Oluwadara Pelumi Omotayo,
North-West University, South Africa
Marketa Sagova-Mareckova,
Crop Research Institute (CRI), Czechia

*CORRESPONDENCE
Expedito Olimi
expedito.olimi@tugraz.at

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Deciphering the microbial composition of biodynamic preparations and their effects on the apple rhizosphere microbiome

Expedito Olimi[1]*, Samuel Bickel[1], Wisnu Adi Wicaksono[1], Peter Kusstatscher[1], Robert Matzer[4], Tomislav Cernava[1] and Gabriele Berg[1,2,3]*

[1]Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria, [2]Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB), Potsdam, Germany, [3]Institute for Biochemistry and Biology, University of Potsdam, Potsdam, Germany, [4]Independent consultant for soil management (BODENmanagement.net), Graz, Austria

Soil microbial communities are crucial for plant growth and are already depleted by anthropogenic activities. The application of microbial transplants provides a strategy to restore beneficial soil traits, but less is known about the microbiota of traditional inoculants used in biodynamic agriculture. In this study, we used amplicon sequencing and quantitative PCR to decipher microbial communities of composts, biodynamic manures, and plant preparations from Austria and France. In addition, we investigated the effect of extracts derived from biodynamic manure and compost on the rhizosphere microbiome of apple trees. Microbiota abundance, composition, and diversity of biodynamic manures, plant preparations, and composts were distinct. Microbial abundances ranged between 10^{10}-10^{11} (bacterial 16S rRNA genes) and 10^{9}-10^{11} (fungal ITS genes). The bacterial diversity was significantly higher in biodynamic manures compared to compost without discernible differences in abundance. Fungal diversity was not significantly different while abundance was increased in biodynamic manures. The microbial communities of biodynamic manures and plant preparations were specific for each production site, but all contain potentially plant-beneficial bacterial genera. When applied in apple orchards, biodynamic preparations (extracts) had the non-significant effect of reducing bacterial and fungal abundance in apple rhizosphere (4 months post-application), while increasing fungal and lowering bacterial Shannon diversity. One to four months after inoculation, individual

taxa indicated differential abundance. We observed the reduction of the pathogenic fungus *Alternaria*, and the enrichment of potentially beneficial bacterial genera such as *Pseudomonas*. Our study paves way for the science-based adaptation of empirically developed biodynamic formulations under different farming practices to restore the vitality of agricultural soils.

KEYWORDS

biodynamic farming, compost microbiome, biodynamic manures, biodynamic preparations, rhizosphere microbiome, 16S rRNA/ITS amplicon sequencing, organic soil amendments

Introduction

The soil microbiome plays an essential role in crop production, but is a strongly influenced component in the Anthropocene (1, 2). Therefore, it is critical to identify threats that cause microbiome disturbances, as well as to find sustainable restoration strategies for affected microbiomes (2). Microbial diversity is crucial for soil and plant health (3) and for human health as well, which can be measured in taxonomic and functional diversity as well as by total gene count within the microbiota (4). However, enriching microbial diversity in the soil is a challenge, which can be managed directly by applying (i) microbiome transplants, (ii) microbes with beneficial properties, (iii) microbiota-active metabolites, or indirectly by changing environmental conditions in a way that microbiomes also shift their structure and function from dysbiosis into a healthy state (1, 5). The use of compost in field fertilization, a classical microbiome transplant, can be traced back to the advent of human civilization, marked by the agricultural revolution (6). Numerous benefits for enhancing soil ecosystem functions, soil quality, and plant health, are attributed to composting in arable and natural ecosystems (7–9). However, many extensive farming systems from the past have been replaced by what is nowadays known as intense, conventional agriculture. It is characterized by high-yield cultivars, which need high inputs of synthetic fertilizer, and pesticides and commonly result in reduced soil vitality, emerging and multi-resistant soil-borne pathogens and an overall yield decline, which prompts a re-evaluation of agricultural practices (10–12).

An ongoing evaluation of current agricultural practices has shifted attention towards alternative and environmentally sustainable systems, including biodynamic farming to restore the "lost soil microbiome" (13–15). Furthermore, the globally increasing demand for foods and beverages produced under organic management practices (16–18) motivates a deeper investigation of microbial constituents that are present in the utilized organic amendments. Biodynamic (BD) farming was first conceptualized by the Austrian philosopher Rudolf Steiner (1861-1925) (19) and is considered the first systematic form of ecological or organic farming (15, 19, 20). Although organic and BD farming are related, the latter considers specific practices which are intended to influence the biological metaphysical aspects including natural rhythms concerning the sun and moon, weather, and seasons of the farm (10, 15, 21). A special feature of BD agriculture is the use of specific BD manure and fermented plant preparations (20). These products are applied as solid manure or liquid extracts in form of field sprays (i.e., soil or foliar application) to enhance soil quality and stimulate plant health (22–24). Recent studies highlighted the role of BD preparations, especially in enhancing soil diversity (15, 18, 25), and have also found application in viticulture (17), especially in enhancing winegrape quality (26). In addition, long-term field trials indicated differences in the soil microbiome between BD, integrated, and organic farming systems (27, 28) even under desert farming conditions (29).

BD farming is rooted in anthroposophy, which views humans as the primary link between the cycles of the earth and the cosmos, with humans bridging a gap between the spiritual and material worlds (17). Special natural biodynamic preparations (dubbed "Steiner preparations") were formulated and utilized in this system to replace synthetic products (17). The procedure to obtain BD formulations involves fermenting cow manure in cow horns to create the so called biodynamic extracts (compost teas), which are then applied according to Steiner (30). While the reason for fermentation in cow horns may still be unknown, preparations from this method have been proven to have special qualities including soil fertility improvement and enhancing plant physiological responses to light radiation (25). Moreover, (26) found a substantial impact of BD preparations in the enhancement of wine quality, winegrape canopy and chemistry, despite contradicting results regarding effects on soil quality. Yet, the microbiome of BD formulations (manures and plant preparations), the differences between BD manures and non-biodynamic composts as well as their impact on the rhizosphere microbiota remain unexplored.

Therefore, we analysed bacterial and fungal communities of BD formulations (manures and plant preparations) and composts (standard-, horse-, and apple composts). Extracts from the BD manures and composts are commonly applied as field sprays; thus, we also investigated the microbiome of liquid extracts and their precursor materials. The two analysed extracts were applied in differently managed (organic and integrated) apple orchards and samples were taken at bloom (one month), and post-harvest (four months) to investigate the temporal variability in the rhizosphere microbiome. The study was based on the hypotheses that: (i) BD manures and composts have a different microbiome; (ii) the microbiome of BD manures is affected by the amendment with biodynamic plant preparations; (iii) the microbiome of BD manures differs between regions and the year of production; (iv) the microbiomes of extracts obtained from BD manure and compost are different from the precursor materials, and (v) the microbiome of the apple rhizosphere is affected by the application of extracts.

Material and methods

Description of the biodynamic and compost formulations

We sampled four BD manure products and six biodynamic plant preparations for amplicon sequence analysis. BD horn manures were obtained from Demeter (Vienna, Austria) and BioDynamie Services sarl (Château, France), while all plant preparations were obtained from France. In the BD farming community, the plant preparations are referred to as P-502, P-503, P-504, P-505, P-506, and P-507. In the production process, BD manure (500) is prepared from cow manure that is fermented in cow horns and buried in the soil at a depth of 50 cm for six months (i.e., during autumn and winter). After the horns are recovered from the soil, the retrieved horn manure (500) can be amended with six different plant preparations (P-502 to P-507) and referred to as 500P.

The BD formulations used in this study originated from two different regions of production: France (FR-500/FR-500P) and Austria (AT-500/AT-500P). Apart from differences in the region of origin, we included BD products from different production years (Table 1). According to the producers, BD preparations were stored in ceramic pots and placed in a double-walled wooden box covered with a wooden lid. The boxes were kept in cellars exclusively dedicated for storage of preparations at constant environmental conditions. We analysed BD manure samples without preparations (AT/FR-500) produced in the years 2019, 2020, and 2021 and BD manures with preparations (AT/FR-500P) from the years 2012, 2016, and 2020 (Table 1). The BD manure FR-500P produced in 2016 was amended with four plant preparations coded F, T, S, and V, according to the producer in France.

For comparison between biodynamic manures and compost, three composts (standard-, apple, and horse compost) were included in the analysis (Table 1). Briefly, the different composts were constituted as follows: (i) Standard compost (70% shrub cuttings, 5% soil, and 25% organic waste); (ii) Horse compost (60% shrub cuttings, 5% soil, and 35% horse manure with straw); and (iii) Apple compost (75% shrub cuttings, 5% soil, and 20% pressed organic apple fruits).

Field treatments and experimental design

As a common practice in BD farming, sprays for field application are extracted from BD formulations. Similarly, compost can be applied in liquid form as "compost tea"; it is prepared by mixing liquid extracts from compost with molasses and stone dust. We applied two extracts from the BD manure (AT-500P) and compost (standard compost) in two apple orchards, which were maintained following organic and integrated management practices. The organic and integrated orchards were located at 47.1289°N, 15.7807°E and 47.2125°N, 15.8569°E, respectively. Apple trees were grown for approximately ten years in each orchard. Treatments were performed at the beginning of spring (May 2021). The BD preparations were applied by sprinkling the extracts in the vicinity of the plant roots, using the "Demeter" recommended application rate of 1:10 (i.e. liquid extract: water) liters per acre. For each orchard, a randomized plot design of 4 plots per treatment and 8-10 trees per plot was chosen. To account for spatial variations, plots were randomly distributed across six neighbouring rows. Rhizosphere samples were randomly taken from each plot using an auger of 5 cm diameter. Ten soil cores from each plot were pooled to comprise a biological replicate. Rhizosphere (soil associated with roots) sampling was performed one- and four- months after treatment and corresponded with the spring and autumn seasons. Samples were kept cooled and transported within six hours to the laboratory at the Institute of Environmental Biotechnology (Graz University of Technology, Graz, Austria).

Sample processing and deoxyribonucleic acid extraction

For compost, BD manures, and BD plant preparations, four grams of sample were stored at -70°C until DNA was extracted. Before DNA extraction from liquid extracts ("compost tea"), the extracts from compost and BD manure were centrifuged at 16000g for 16 minutes, and the microbial pellet was frozen at -70°C until DNA extraction. For orchard samples, approx. four

TABLE 1 Overview of the studied composts (i.e., apple-, horse-, and standard compost) and biodynamic manures (with/without plant preparations).

Sample ID	Formulation*	Year	Sample type	Region#	Description
C1	Compost tea[A]	2021	Extract		Field spray extract (from C12)
C2	Compost tea	2021	Extract		Extract from C12, but same as C1
C3	Compost tea	2021	Extract		Extract from C13
C4	FR-500P	2012	Solid	FR	500P- Horn manure preparation -2012
C5	FR-500	2019	Solid	FR	500-Horn manure-2019
C6	FR-500P	2016	Solid	FR	500P- Horn manure preparation-2016(F)
C7	FR-500P	2016	Solid	FR	500P- Horn manure preparation-2016(T)
C8	FR-500P	2016	Solid	FR	500P- Horn manure preparation-2016(S)
C9	FR-500P	2016	Solid	FR	500P- Horn manure preparation-2016(V)
C10	FR-500P	2020	Solid	FR	500- Horn manure-2020
C11	FR-500P	2020	Solid	FR	500P- Horn manure preparation-2020
C12	Standard compost		Solid	AT	
C13	Apple compost		Solid	AT	
C14	Horse compost		Solid	AT	
D1	AT-500	2020	Solid	AT	500- Horn manure
D2	AT-500	2021	Solid	AT	500- Horn manure
D3	AT-500P	2020	Solid	AT	500P- Horn manure Preparation
D4	AT-500P[A]	2021	Extract	AT	Field spray extract (from D3)
D5	AT-500P	2021	Extract	AT	Field spray extract (from D3)
P1	P-502		Solid	FR	*Achillea millefolium* (Achillee; P-502)
P2	P-503		Solid	FR	*Matricaria rectutita* (Camomille; P-503)
P3	P-504		Solid	FR	*Urtica dioica* (Ortie (P-504)
P4	P-505		Solid	FR	*Quercus robur* (Ecorce DeChene; P-505)
P5	P-506		Solid	FR	*Taraxacum dens-leonis* (Pissenlit; P-506)
P6	P-507		Liquid	FR	*Valeriana officinalis* (Valeriane; P-507)

#Acronyms FR, France, AT, Austria indicate regions of manure and preparation production.
*The superscript A indicates extracts which were used in field treatments.
BD plant preparations (P502-507) and their respective plant species from which they are derived were also included.

grams of rhizosphere soil were stored at -70°C. For all samples, total microbial genomic DNA was extracted using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Inc.; Norcross-Georgia, United States) following the manufacturer's instructions. DNA was quality checked using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA) and stored at -20°C before performing PCR and amplicon sequencing.

Real-time quantitative PCR of total bacterial and fungal abundance

The primer pairs Unibac-II-515f/Unibac-II-806r for bacteria as previously used by (29) and ITS1f/ITS2r for fungi (31) were used for real-time qPCR quantifications to determine the copy numbers of 16S rRNA and ITS genes in the different samples. Reactions were performed in a total volume of 10 µL in a reaction mix composed of 5 µL of KAPA SYBR Green (Bio-Rad, Hercules, CA, U.S.A.), 0.5 µL of each primer (10 µM), 3 µL of PCR grade water and 1 µL template DNA (samples were diluted 1:10 in PCR grade water). Amplifications were performed in triplicates for each sample using a Rotor-GeneTM 6000 series thermal cycler (Corbett Research, Sydney, Australia) with the following program settings: initial denaturation (95°C,5 min) followed by 35 cycles of denaturation (95°C,10 s); annealing (54°C, 15 s); extension (72°C, 10 s); then melt down from 72 to 96°C. Serial dilutions of standards containing defined copy numbers were generated according to (29) and used to calculate gene copy numbers in different samples. As standards, known concentrations of 16S rRNA gene fragments from a *Bacillus* sp., and the ITS region from a *Penicillium* sp. were used.

Amplicon library preparation

The extracted DNA was further used for amplicon library preparation based on the hypervariable V4 region of the bacterial 16S rRNA gene and the ITS1 regions of fungal DNA.

We employed one-step PCR using the primer pair 515F (5'-GTGCCAGCMGCCGCGGTAA-3'), and 806R (5'-GGACTACHVGGGTWTCTAAT-3') for the bacterial library preparation (32). Both forward and reverse primers contained sample-specific barcodes to facilitate multiplexed sequencing. For each PCR, 1 µL of extracted DNA was used as a template in a 30 µL reaction. Peptide nucleic acid (PNA) PCR clamps were used to block the amplification of plant plastid and mitochondrial 16S rRNA genes during the PCR amplification of the bacterial community (33, 34). The reaction mixture contained 6 µL (5xTaq &GO, PCR pre-mix, MP Biomedicals), 0.6 µL (10 µM 515F/806R) primers, 0.45 µL (50 µM mPNA and pPNA), and 20.9 µL of PCR grade water. All reactions were performed in triplicates in a thermocycler (Bio-metra GmbH, Jena, Germany). The PCR program included an initial denaturation (96°C, 5 min), followed by 30 cycles (94°C for 60 s, 78°C PNA step for 5 s, 54°C for 1 min, 74°C for 60 s), followed by 74°C for 10 min and then a cool down to 10°C.

For the fungal community, we used the primer pair ITS1f (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2r (5'-GCTGCGTTCTTCATCGATGC-3') (31, 35) for library preparation. All amplifications were performed in triplicates. One µL of extracted DNA was used in each 30 µL reaction. The reaction mixture contained 6 µL (5xTaq &GO, PCR pre-mix, MP Biomedicals), 0.6 µL (10 µM ITS1F/ITS2R) primers, 21.8 µL of PCR grade water. The PCR program included the first step of initial denaturation (96°C, 5 min), followed by 30 cycles (denaturation: 96°C for 60 s, annealing: 58°C for 60 s, and extension: 74°C for 60 s), followed by 74°C for 10 min (final extension) and a cool-down step to 10°C. Successful PCR amplifications at the correct amplicon size were confirmed by gel electrophoresis. PCR amplicons were purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) following the manufacturer's instructions. Purified PCR amplicons were quantified using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA) and pooled in equimolar concentrations. Paired-end Illumina MiSeq 2x 250 sequencing of the amplicon library was performed at Eurofin (Berlin, Germany). All raw reads obtained from the sequencing company were deposited at the European Nucleotide Archive (ENA) under study accession number PRJEB53400.

Bioinformatic pipeline

Paired-end reads from the sequencing facility were quality-checked and demultiplexed using Cutadapt (36). Demultiplexed reads were analysed using QIIME2 version 2021.11.0 (37). Primer sequences were removed, and the DADA2 algorithm in the QIIME2 environment was employed to quality filter, denoise, and remove chimeric sequences (38); thus generating amplicon sequence variants (ASVs), and a feature table of microbial counts. Taxonomic assignment of denoised reads was performed using the VSEARCH (39) tool in QIIME2 by comparing the reads against reference databases: SILVA132 for 16S rRNA (40, 41) and UNITE v7 (42) for ITS sequence reads.

Statistical analysis

All data were analysed with R statistical software (version 4.0.3) using RStudio (Version 1.1.423) (43), supplemented by the web-based microbiome analyst tools (44). The obtained microbial ASV tables and taxonomic assignment were processed using phyloseq (45) and vegan 2.5.7 (46) packages.

For alpha and beta diversity analyses, the statistics were performed on datasets rarefied to minimum sampling depths of 1000 and 700 reads per sample for 16S and ITS analyses, respectively. The sample-specific rarefaction curves for bacterial and fungal communities were visualized (Figure S1). One sample of a biodynamic plant preparation (P-507) was excluded from the analysis due to insufficient replicates (one sample was retained).

Microbial alpha diversity was calculated using the Shannon diversity index (H'). The non-parametric Kruskal-Wallis test (47), followed by Dunn's test (corrected for multiple comparisons using the Bonferroni method) was performed on Shannon diversity index and abundance data to test for differences between the samples (e.g., BD manures vs. composts, extracts vs. precursor materials); and treatment comparisons (extract from standard compost and AT-500P) post-inoculation in the different orchards. Also, the effect of treatment on the rhizosphere microbiome at different sampling times was tested. Permutational analysis of variance (PERMANOVA, 999 permutations) based on Bray-Curtis dissimilarity was used to test for differences in community structure between sample groups. For significant factors (and combinations), pairwise comparisons were performed using the 'pairwise.adonis2' function in vegan (46) with p-values adjusted using the Bonferroni method. The differences in microbial community structure between the sample groups were visualized using dendrograms (generated by complete clustering of Bray-Curtis distances), and non-metric multidimensional scaling (NMDS).

Microbial taxonomic composition based on the percentage relative abundance of top20 taxa at class, order, and family levels was represented using stacked bar plots. Linear discriminant analysis Effect Size (LEfSE) (48) implemented in microbiome analyst (44) was used to identify the taxa underlying the observed microbiome differences between the composts and biodynamic preparations, as well as revealing treatment effects in the different orchards and sampling times. The common microbiome (defined by a prevalence of 50%, and detection threshold of 0.001% relative abundance) was used to obtain unique and shared ASVs associated

with the different samples. The list of ASVs was visualized using a Venn diagram generated by the systemPipeR package (49) in R studio.

We used the SourceTracker R script (50) to estimate the proportion of microbiota in the apple rhizosphere that potentially originated from the field sprays extracted from compost and BD manures after soil treatment. SourceTracker estimates the proportion of microbiota that originates from a set of source and sink environments as previously described (50). In our case, the extracts from BD manure (AT-500P) and standard compost which were used for orchard treatment were considered as the source environment and the rhizosphere soil as the sink environment. By considering control soil as the microbial source, we also tracked microbiota in the treated rhizosphere soil which was not of extract origin, but likely originated from the soil.

Results

The biodynamic manures and composts were distinct in microbiome structure and composition

Amplicon sequencing of bacterial and fungal communities yielded 2,213,856 and 4,534,963 high-quality reads, assigned to 9,387 bacterial and 2,742 fungal ASVs, respectively. Generally, we observed significant differences in microbiome structures between BD manures (AT-500/P or FR-500/P) and composts (i.e. apple-, horse-, standard composts), especially for the bacterial community (Figure 1). Moreover, a separation based on the country of origin of BD manures was observed (Figures 1A, B). Significant differences (P=0.008) in Shannon diversity between compost and BD manures were observed for bacterial communities (Table S3A), while no significant differences (P=0.19) were observed for the fungal community (Table S4A). Microbial abundance was significantly different for bacterial (P=0.04; Table S1A) and fungal communities (P=0.009; Table S2A).

The bacterial abundance (16S rRNA gene fragments per gram soil) was higher in BD manure than in compost and ranged as follows and ranged as follows: (BD manure: 2.52×10^{11} to 4.17×10^{11} vs compost: $8.89.16\times10^{10}$ to 1.46×10^{11}). Meanwhile, the fungal community (ITS region copies per gram soil) were: (BD manure: 1.34×10^{9} to 1.18×10^{11} vs compost: 8.89×10^{10} to 1.46×10^{11}). However, a significant difference ($P<0.05$) was observed only for the fungal abundance between BD manure (AT-500) and horse compost (Figure S2B), while no significant differences ($P>0.05$) were observed for the bacterial community (Figure S2A).

The bacterial Shannon index was higher in BD manures than in composts and ranged as follows: BD manure (5.28-5.63)

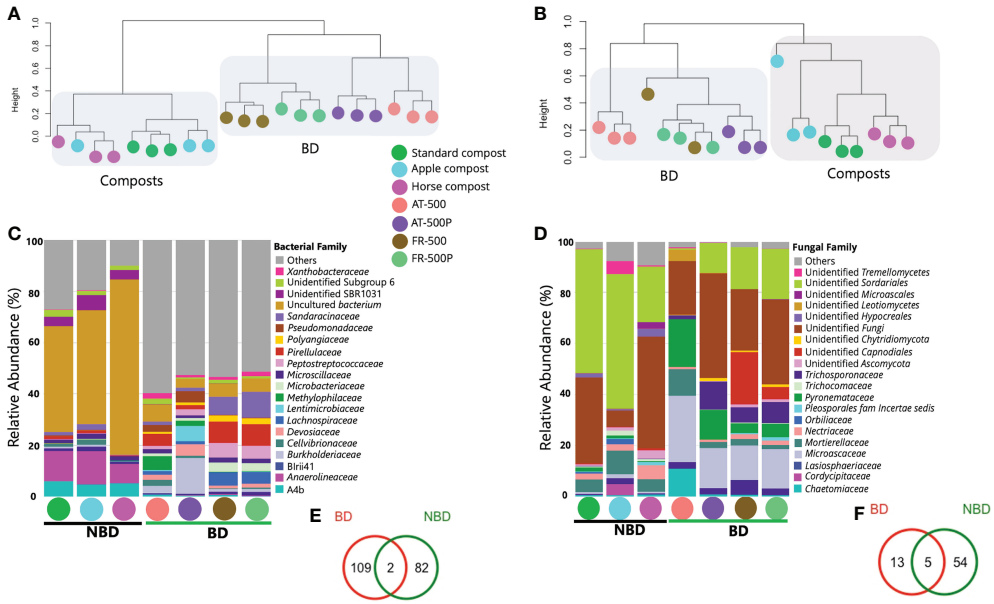

FIGURE 1
Microbial community structure and composition of BD manures and composts. Coloured circles represent the different BD manures (500/P) and non-biodynamic (NBD) composts. Figures **(A, B)** are dendrogram representation of the bacterial and fungal community obtained by complete clustering of the Bray Curtis distance matrix. **(C, D)** are stacked bar plots showing the bacterial, and fungal taxonomic composition in the different BD and compost samples; while **(E, F)** represent shared and unique ASVs between BD manures and composts for bacterial and fungal communities, respectively. The common microbiome was defined as ASVs with prevalence >50% and relative abundance >0.001%. AT and FR indicate countries Austria and France, respectively.

versus compost (1.81-3.54). Significantly higher bacterial diversity (P=0.02) in BD manures (AT-500 and FR-500) compared with horse compost was observed (Figure S2C). In contrast, the fungal Shannon diversity ranged between (2.63-3.13) for BD manures as compared to compost (2.41-2.60), and significant differences (P>0.05) in Shannon diversity were observed for the fungal community (Figure S2D).

PERMANOVA between BD manures and composts revealed significant differences in community compositions between these organic formulations for bacteria (R^2 = 0.85, P=0.001) and fungi (R^2 = 0.85, P=0.001). In addition, BD manures amended with plant preparations (AT/FR-500P) could be distinguished from those without preparations (AT/FR-500), especially for the bacterial community (Figures 1A, B).

Comparing higher-level taxonomy of BD manures and composts revealed differences in microbial composition between samples. Dominant phyla included *Chloroflexi* (average percentage abundance: 35.0%), *Proteobacteria* (29.5%), and *Firmicutes* (8.9%). The phylum *Chloroflexi* was dominant in composts (standard-, apple-, and horse), with abundance (69.8%, 72.4%, and 87.7%, respectively). Meanwhile, *Proteobacteria*, *Firmicutes*, and *Actinobacteria* were highest in BD manures with relative abundances of 43.7%, 14.8%, and 9.5%, respectively. For the fungal community, the phyla *Ascomycota* (59.7%), *Basidiomycota* (5.7%), and *Mortierellomycota* (5.1%) were predominant. We observed differences among compost microbiomes, especially for the bacterial community. The bacterial family *Anaerolineaceae* was present only in composts; however, with a dominance of uncultured bacteria (Figure 1C). Meanwhile, BD manures were seen to contain members of families *Pseudomonadaceae*, *Peptostreptococcaceae*, *Microbacteriaceae*, *Lentimicrobiaceae*, *Lachnospiraceae*, *Devosiaceae*, and *Burkholderiaceae* (Figure 1C). The fungal families including unidentified *Microascales*, unidentified *Hypocreales*, and *Cladosporiaceae* were observed in composts (Figure 1D). *Trichosporonaceae*, *Microascaceae*, *Lasiosphaeriaceae*, *Mortierellaceae*, and *Chaetomiaceae* were observed in BD manures, while the fungal family *Cordycipitacea* was only found in the apple compost (Figure 1D).

Moreover, bacterial genera including *Bacillus*, *Devosia*, and *Pseudomonas* were mainly associated with BD manures (AT-500/P and FR-500/P). The fungal genera *Lecanicillium*, and *Arthrobotrys* were mainly present in composts, while *Trichosporon, Scutellinia*, and *Sporobolomyces*, were associated with BD manures.

Analysis of the common microbiome analysis revealed that 109, and 82 bacterial ASVs were unique to BD manures and composts, respectively, while only two ASVs were shared (Figure 1E). Some bacterial genera in the common microbiome of BD manure were *Paenibacillus*, *Mesorhizobium*, *Mycobacterium*, *Cellulomonas*, and *Clostridium*. On the other hand, 13 and 54 unique fungal ASVs were detected in BD manures and compost, while five ASVs were shared between both microbiomes (Figure 1F). The shared fungal ASVs included unidentified *Capnodiales* and unidentified *Microascacea.*

Biodynamic manures varied in structure and composition between the different countries of production, but shared a common microbiome

We compared BD manures that differed in production year and region of production (Austria or France). Differences (P<0.05) in fungal diversity were observed between FR-500P [2012] and AT-500 [2021] as shown (Figure S3D), while the fungal abundance was substantially higher in AT-500 [2020/2021] as compared to FR-500P [2016: S, T, and V] (Figure S3B). No significant differences (P>0.05) in bacterial abundance and diversity were observed among the biodynamic manures, for the two regions and different years of production (Figures S3A, C).

In contrast to microbial diversity and abundance, we observed substantial differences in microbial composition between BD manures based on the country of production as follows: bacterial community (BD manure: R^2 = 0.78, P=0.001, country: R^2 = 0.24, P=0.001), and fungal community (BD manure: R^2 = 0.78, P=0.001, country: R^2 = 0.26, P=0.001). These differences were also shown by NMDS in which clear separation of samples according to the region and BD manure type was observed (Figure S4).

The dominant bacterial classes were *Gammaproteobacteria* (19.3 average percentage relative abundance), *Clostridia* (14.5%) and *Alphaproteobacteria* (12.1%). Predominant fungal classes included *Sordariomycetes* (47.9%), *Tremellomycetes* (6.7%), *Pezizomycetes* (5.9%) and *Dothideomycetes* (5.2%). Differences between regions were visible, especially for the bacterial community. For instance, the order *Actinomarinales* was only found in BD manures produced in France (Figure S3C). Meanwhile, *Sphingobacteriales* were observed exclusively in Austrian BD manure samples (Figure S4C). The fungal community between the two regions was separated by the presence of orders *Capnodiales*, *Ascosphaerales*, and *Pleosporales* were associated with the BD manures produced in France. Independent of the region of production, BD manures were found to contain bacterial families such as *Bacillaceae*, *Burkholderiaceae*, *Clostridiaceae*, *Flavobacteriaceae*, and *Pseudomonadaceae*.

The assessment of the common microbiome of BD manures from the two countries revealed that 30 bacterial and 13 fungal ASVs were shared. Most bacterial ASVs were unique (120 and 123 for Austria and France, respectively). In contrast, a total of 16 and 11 fungal ASVs were unique for Austria and France (Figures S4E, F). Some of the shared bacterial ASVs included *Devosia, Azotobacter, Acidibacter, Clostridium, Romboutsia, Cellulomonas, Sorangium, Solirubrobacter*, and *Turicibacter.* The shared fungal genera were *Trichosporon*, *Mortierella*,

unidentified *Microascaceae*, unidentified *Lasiosphaeriaceae* and unidentified *Sordariales*.

Common microbiome of plant preparations and preparation-amended BD manures

To establish whether BD plant preparations altered the microbiome of BD manures (FR/AT-500P), the community structure and diversity of BD preparations (manure and plant preparations) were analysed. Significant differences in microbiome composition were seen between amended BD manures and the different plant preparations (bacterial: R^2 = 0.83, P=0.001; fungal community: R^2 = 0.94, P=0.001. NMDS visualization showed a clear separation between BD manures and plant preparations. Microbiome separation between plant preparations was observed for bacterial and fungal communities, respectively (Figures S5A, C).

There was a high microbiota load both in BD plant preparations and manures (bacterial: 8.12×10^{10} to 2.65×10^{11}), and fungal (9.70×10^{9} to 1.34×10^{10}). A significant difference (P<0.05) was observed for the fungal abundance between FR-500 and P-505 (Figure S6B), and no differences (P>0.05) were observed for the bacterial community (Figure S6A). On the other hand, the Shannon diversity index of BD plant preparations and amended manure (i.e. FR-500P) ranged between 4.38-5.28 and 2.20-3.18, both for bacterial and fungal communities, respectively. Significant differences (Kruskal-Wallis, P<0.05) in bacterial diversity were observed between BD manure (FR-500P) and plant preparation (P-505), as well as between P-504 with P-505 and P-506 (Figure S5C). The fungal diversity was substantially higher (P<0.05) in preparations (P-505 and P-506) as compared to P-503 (Figure S6D).

The bacterial order *Bacillales* was present both in BD manures and plant preparations, except in preparation P-505, and was highest in P-503 and P-504 with average relative abundances of 11.8% and 36.0%, respectively. The bacterial orders *Rhizobiales*, *Pseudomonadales*, and *Flavobacteriales* were observed in both plant preparations and BD manures (Figure 5B). Meanwhile, *Bacteroidales was* especially present in AT-500/P and plant preparations (P-503 and P-505). For the fungal community, BD manures were distinguished from plant preparations by orders *Capnodiales*, *Hypocreales*, *Ascosphaerales*, *Eurotiales* and Unidentified fungi. The fungal orders *Microascales*, and *Pezizales* were associated with BD manures and plant preparations (Figure S4D).

A comparison between preparation amended and non-amended manures revealed that 120 and 155 bacterial ASVs were shared between (AT-500 and AT-500P), as well as (FR-500 and FR-500P), respectively (Figures S7B, D). Similarly, for the fungal community 25 and 23 ASVs were shared (Figures S8B, D). There was a common microbiome shared between BD manures and plant preparations, both for bacterial (Figures S7A, C) and fungal communities (Figures S8A, C), respectively. More unique than shared members of the bacterial (Figures S7A, C) and fungal (Figures S8A, C) ASVs were observed for plant preparations and BD manures. The shared part of the common microbiome between BD manures and plant preparations was composed of bacterial genera *Romboutsia*, *Azotobacter*, *Pedomicrobium*, and *Bacillus*. Meanwhile, shared fungal genera were *Fusarium*, *Arthrographis*, Unidentified *Microascaceae*, Unidentified *Chaetomiaceae*, and Unidentified *Nectriaceae*.

LEfSe analysis identified the bacterial and fungal genera explaining the differences between BD manures (FR-500P) and plant preparations (Figures 2A, B, respectively). The BD plant preparations (P-502, P-503, P-504, P-505, and P-506) were differentiated by the bacterial genera *Luteibacter*, *Oceanobacillus*, *Virgibacillus*, *Mycobacterium*, and *Staphylococcus* (Figure 2A). *Lentimicrobium*, *Romboutsia*, and *Devosia* were associated with BD manures (AT/FR-500P). However, the genus *Bacillus* was shared between BD manures (AT/FR-500P) and plant preparations. Meanwhile, fungal genera *Scutellinia*, *Trichosporon*, and *Sporobolomyces* were mainly associated with BD manures, while *Fusarium*, *Ascosphaera*, *Pseudallescheria*, *Arthrographis*, *Chaetomium*, *Cyberlindnera*, and *Scedosporium* were associated with BD plant preparations (Figure 2B).

Extracts and precursor materials of BD manure and standard compost contained a common microbiome

The microbial diversity and abundance comparison between extracts and precursor materials of BD manure (AT-500P) and standard compost revealed that extracts generally contained a higher microbial diversity than precursor materials, except for AT-500P (Figures S9C, D); and vice versa for microbial abundance (Figures S9A, B). Significantly higher (t-test: P<0.05) microbial abundance was observed in the precursor of BD manure (AT-500P) in comparison to the respective extract, both for bacterial and fungal communities (Figures S9A, B). No significant differences (P>0.05) in microbial abundance were observed between the precursor and extract of standard compost (Figures S9A, B). The bacterial diversity in the standard compost extract was significantly lower (t-test, P=0.004) than in the extract derived from the compost materials (Figure S9C). Contrarily, the fungal diversity in extract derived from standard compost and BD manure (AT-500P) was significantly higher (t-test, P<0.05) in comparison to precursor material (Figure S9D).

The microbial community structure between extract and precursor materials of BD manure (AT-500P) and standard compost was distinct. This was confirmed by PERMANOVA analysis in which we observed significant differences between the

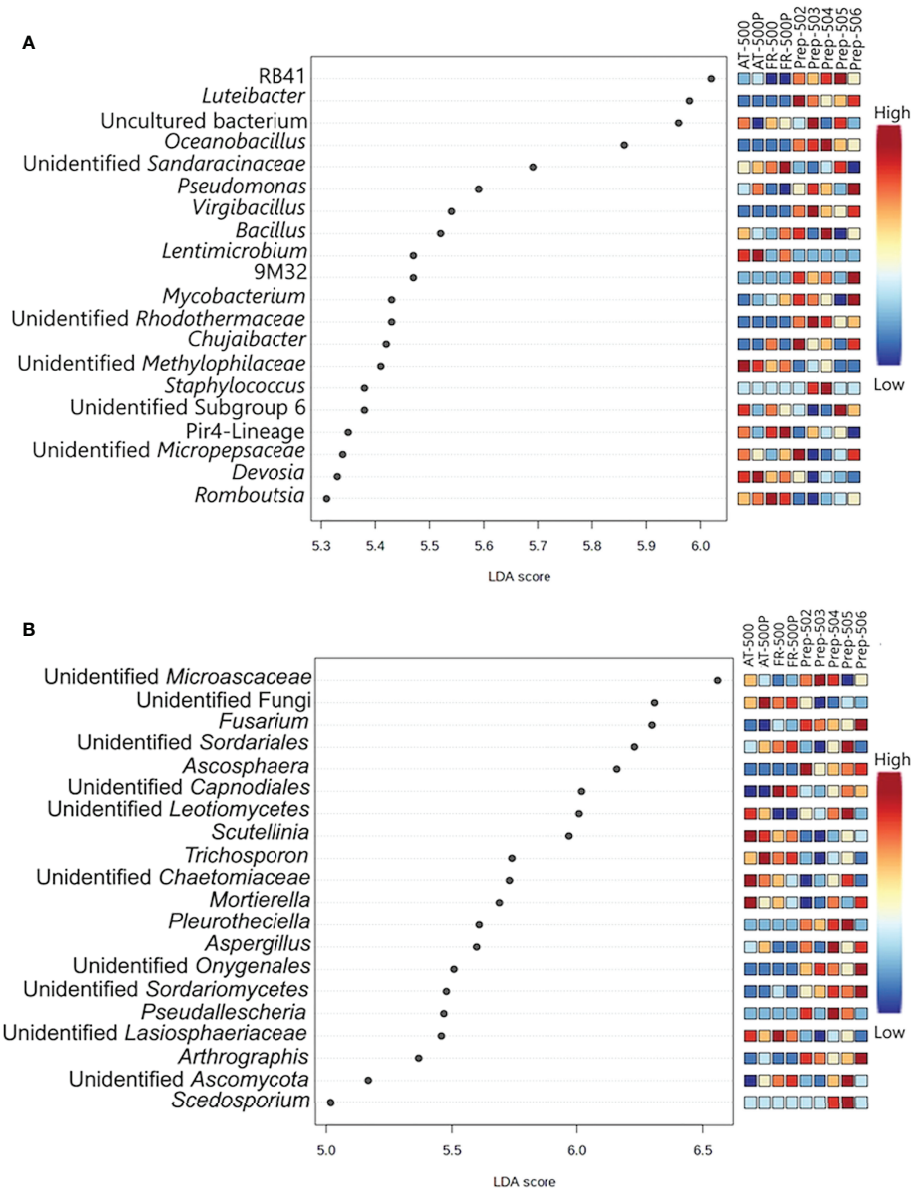

FIGURE 2
Dot plot representation of differentially abundant bacterial **(A)** and fungal **(B)** genera between BD manures and BD plant preparations. Plant preparations are shown as P-502, P-503, P-504, P-505, and P-506; while biodynamic manures were represented as AT -500/P and FR-500/P. Acronym P- represent preparation, while AT and FR represent the countries Austria and France where the BD manures were produced. The differentially abundant genera with LDA scores between 5 and 7 and were based on FDR adjusted P-value. The coloured bar represents the relative abundances of the differentially abundant genera, with the lowest indicated as blue and highest as red.

extracts and their derivative materials (i.e., bacteria: R^2 = 0.65, P=0.001 and fungi: R^2 = 0.72, P=0.001). In addition, pairwise community comparison revealed significant differences (P<0.05) in community structure between precursor materials (standard compost and AT-500P) and their respective extracts, both for bacterial and fungal communities (Tables S7 and S8, respectively).

The extracts and precursor materials of standard compost were distinct in microbial composition. BD manure and their respective extract displayed similar microbiome compositions (Figures 3A, B). The bacterial classes *Anaerolineae* (61.6%), and *Chloroflexia* (8.2%) predominated in standard compost, while *Gammaproteobacteria* (52.3%) and *Bacilli* (34.0%) were mainly present in the extract of standard compost (Figure 3A). The BD manure (AT-500P) and extract were mainly associated with the bacterial classes *Gammaproteobacteria* (precursor: 30.8% and extract: 20.6%), *Bacteroidia* (18.0% and 11.3%), *Alphaproteobacteria* (11.8% and

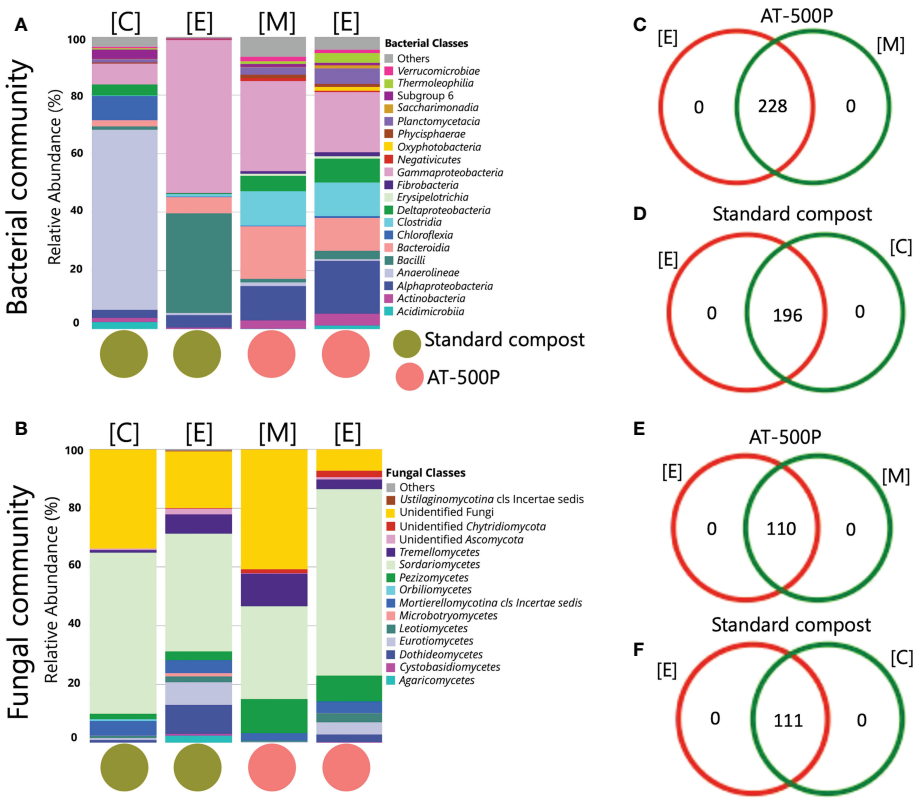

FIGURE 3
Comparison of microbial structure composition between AT-500P and standard compost as well as their respective extract. Panels **(A, B)** are stacked barplot representations of the bacterial and fungal community composition in precursors and extracts of compost and BD manure. Panels **(C, D)** are Venn diagrams representing the common bacterial ASVs shared between extract and precursor materials of compost and BD manure, while **(E, F)** represent the same for the fungal community. The acronyms [E], [C], and [M] represent extract, compost, and BD manure, respectively.

18.1%), *Clostridia* (11.8% and 11.5%)), and *Deltaproteobacteria* (5.2% and 8.2%). For the fungal community, extracts of standard compost and BD manure as compared to the precursor materials contained a higher abundance of *Dothideomycetes* (10.0% versus 1.2%, and 2.6% versus 0.1%, respectively), *Eurotiomycetes* (7.7% versus 1.2%, and 4.1% versus 0.3%), as well as *Leotiomycetes* (2.0% versus 0.7%, and 3.1% versus 0.5%) (Figure 3B).

Interestingly, BD manure (AT-500P) and the associated extract shared 228 and 110 ASVs of the common microbiome, both for bacterial and fungal communities, respectively (Figures 3C, E). Conversely, standard compost and extract were found to share 196 and 111 ASVs (Figures 3D, F). Differential abundance analysis revealed the enrichment of bacterial genera like *Bacillus*, *Pseudomonas, Paenibacillus*, *Flavobacterium*, and *Azotobacter* in the extract of standard compost as compared to precursor materials (Figure S10A). Meanwhile, in extract (AT-500P) as compared to precursor material, an enrichment of bacterial genera including *Roseobacter* and *Polynucleobacter* was observed, while *Lentimicrobium, Devosia*, and *Simplicispira* were enriched in AT-500P (precursors) Figure S10B.

For the fungal community, genus-level differences between extracts and precursor materials of standard compost and BD manure (AT-500P) were marked by the enrichment of fungal genera *Fusarium*, *Trichosporon* and *Aspergillus* in the extract as compared to standard compost and AT-500P precursors (Figures S10C, D). In addition, *Penicillium*, and *Scutellinia* comprised the extracts of standard compost (Figure S10C), while *Arthrographis* was enriched in AT-500P extract (Figure S10D).

Extracts have a time-dependent effect on the apple rhizosphere microbiome under organic and integrated management systems

The microbial structure and composition, as well as diversity and abundance analyses, were performed with rhizosphere samples from two apple orchards managed using organic and integrated management systems. Samples were obtained at two

sampling times (spring and autumn) from both orchards. There were no significant differences (P>0.05) in microbial abundance between treatments and controls, both for organic and integrated orchards at one- and four-months post-inoculation (Figure S11). Generally, after four months, bacterial diversity was lower in treatments in comparison to controls. In contrast, the fungal diversity was higher in treatments as compared to controls (Figure S12). At four months post-inoculation, the bacterial diversity was significantly lower (P<0.05) in standard compost (extract treatment) compared to the control in the organic orchard, as well as between AT-500P and the control for the integrated orchard (Figure S12B). Furthermore, the fungal diversity was significantly higher in the AT-500P treatment compared to the control in the integrated orchard four-month post-inoculation (Figure S12D).

For beta diversity, significant microbial structure differences between orchard types and sampling time were observed: orchard type (bacterial: R^2 = 0.14, P=0.001; fungal: R^2 = 0.30, P=0.001) and sampling time (bacterial: R^2 = 0.06, P=0.001; fungal: R^2 = 0.06, P=0.004). An overall effect of treatment on the microbiome was observed in the organic orchard, both after one month (PERMANOVA; bacterial: R^2 = 0.32, P=0.02; fungal: R^2 = 0.26, P=0.001) and four months (bacterial: R^2 = 0.27, P=0.002; fungal: R^2 = 0.23, P=0.05) as shown (Tables S7, S8 for the bacterial and fungal community, respectively). However, in the integrated orchard, a significant effect of treatment on bacterial community structure was observed four months after treatment (R^2 = 0.32, P=0.002), while no significant effect (P>0.05) was seen for the fungal community (Tables S7, S8). Pairwise significant differences (P<0.05) between treatment and control were evidenced for the fungal community at one-month post-inoculation (Table S8), and for the bacterial community in both orchards at four months post-inoculation (Table S7). The microbial community clustering by NMDS was shown for different treatments and orchards, at one month (Figures S13A, C), and four months (Figures S14A, C) post inoculation, both for bacterial and fungal community, respectively.

The apple rhizosphere of the two orchards was dominated by the bacterial phyla *Proteobacteria* (Organic: Integrated; 26.1%: 25.8%), *Firmicutes* (30.0%: 3.4%), *Actinobacteria* (18.8%: 26.9%), *Chloroflexi* (13.1%: 15.5%), and *Acidobacteria* (17.2%; 10.4%). On the other hand, the fungal community was predominated by *Ascomycota* (70.1%: 68.1%), *Mortierellomycota* (21.8%: 21.8%), and *Basidiomycota* (6.5%: 8.3%). For both orchards, the bacterial composition was similar at one-month post-inoculation, but a higher average relative abundance of less abundant taxa categorized into ("others") was seen in rhizosphere soil treated with extract of BD manure (AT-500P) and standard compost as compared to the control (Figure S13B). For the fungal community, we observed that families such as *Microascaceae*, and *Chaetomiaceae* were higher in treatments as compared to the control (Figure S13D).

At four months post-inoculation, the family-level taxonomic composition was similar, but differences were observed in the less abundant taxa ("others"), whose abundance was high in treatments as compared to the control (Figures S14B, D). In contrast, a high abundance of the family *Chaetomiaceae* was observed in treatments as compared to controls, both for organic and integrated orchards (Figure S14D).

All microbial genera which were responsible for significant pairwise differences in community composition between treatments and controls were further explored. At one-month post-inoculation, enrichment in the control of the fungal genera *Alternaria*, *Thelonectria*, and *Solicoccozyma* was observed in the organic orchard (Figure 4A). Meanwhile, the genus *Chaetomium* was highly abundant in the rhizosphere which was treated with the extract from BD manure (AT-500P). At four months post-inoculation, bacterial genera including *Bacillus*, *Bradyrhizobium*, *Pseudolabrys*, and unidentified *Burkholderiacea* were enriched in the controls, compared to the treatments, while *Chthniobacter* and *Candidatus Xiphinemebacter* were enriched in the rhizosphere after treatments in the organic orchard (Figure 4B). For the integrated orchard, the bacterial genera *Pseudomonas*, *Mycobacterium*, and *Candidatus Udeobacter* were enriched in the rhizosphere that had been treated with AT-500P (extract), while *Candidatus Xiphinemebacter*, and uncultured *Pirellulaceae* were enriched in rhizosphere treated with the extract from standard compost (Figure 4B).

Application of extracts from standard compost and BD introduces new microbes into the apple rhizosphere, especially in the integrated apple orchard

SourceTracker was employed to reveal the portion of the rhizosphere microbiome, which was potentially introduced by the treatments that included extracts from standard compost and AT-500P for organic and integrated orchards (Figure 5) at two sampling times. We observed time-dependent differences in the proportion of the rhizosphere microbiome that was introduced by extracts. A higher proportion of the microbiota at one month post-inoculation, than at four months post-inoculation could be traced back to the treatments, especially for the integrated orchard.

We observed that treatments introduced a higher proportion of bacteria in integrated than in the organic orchard, especially at one-month post-inoculation, and the average percentage proportions ranged as follows: organic (1%-2%) and integrated management (1%-20%). However, a high proportion of bacteria (10% to 60%), and fungi (0%- 20%) from the extracts (i.e. labelled as "unknown") could not be traced in the rhizosphere (Figure 5). For the fungal community, both in the organic and integrated orchard, the extract from standard compost as compared to AT-500P was found to potentially introduce more fungi into the rhizosphere: organic (AT-500P: 0%-2% vs.

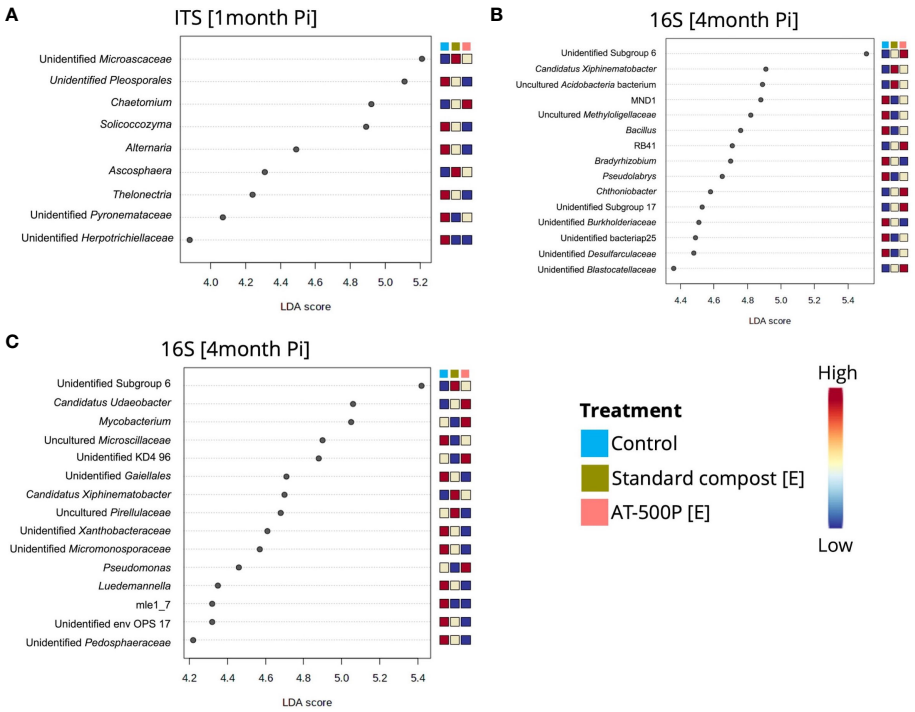

FIGURE 4
Dot plot representation of differentially abundant genera in treatments which showed significant pairwise differences to the control for the different orchards and sampling times. **(A)** shows differentially abundant fungal genera at one-month post inoculation in organic orchard, while **(B, C)** represent significant differentially abundant bacterial genera between treatment and control in organic and integrated orchard at four-months post inoculation, respectively. The significant ($P<0.05$) differentially abundant genera were obtained by LEfSe, and the genera with high LDA scores (3 to 6) were shown. The [E] in the legend represents extract, and the coloured scale bar represents relative abundances, with the lowest indicated as blue, and highest as red.

standard compost: 0%-3%), and integrated management (0%-1% vs. 0%-2%). Considering control soil as the microbial source, we observed that a high proportion of the microbiome (bacteria: 40% to 90%, and fungi: 80% to 95%) was shared with the treated rhizosphere soil.

Discussion

Biodynamic manures, composts, and plant preparations carried distinct microbiomes. We observed a higher microbial diversity in BD manures than in BD plant preparations and composts (apple-, horse-, and standard composts). While the impact of type and quantity of organic material on compost microbiomes was previously reported (51), our study reveals for the first time specific microbiome differences between BD manures and composts. These variations could be attributed to various factors such as the type of starting material and the composting technology used (52). Moreover, the role of physical and chemical parameters such as oxygen, temperature, moisture content, pH, and nutrient availability shape the compost microbiome (53).

Specific to BD farming, the fermentation of animal waste and the subsequent amendment with plant preparations form a critical part of BD products (20). This study revealed that plant preparations and amended BD manures contain a distinct microbiome. In addition, the microbiome varied among the different plant preparations. Such variation among preparations might be attributed to plant materials from which they are derived. Moreover, the effect of the plant genotype on the microbiome has been reviewed elsewhere (54, 55). Our study provides a link between the plant microbiome and the microbiome of plant-derived organic amendments. We propose that the specific procedures used in making BD manures might have contributed significantly to the uniqueness of BD products. This is apparent from the clear differences between composts and their BD counterparts (Figures 1A, B for bacterial and fungal communities, respectively). However, a common microbiome comprised of *Paenibacillus*, *Cellulomonas*, and *Clostridium* was shared between all BD manures and composts. These taxa play essential roles as proficient degraders of plant biomass (56). The presence of members under the family *Clostridiaceae* could be attributed to the use of cow manure in BD preparations. *Clostridium* is associated

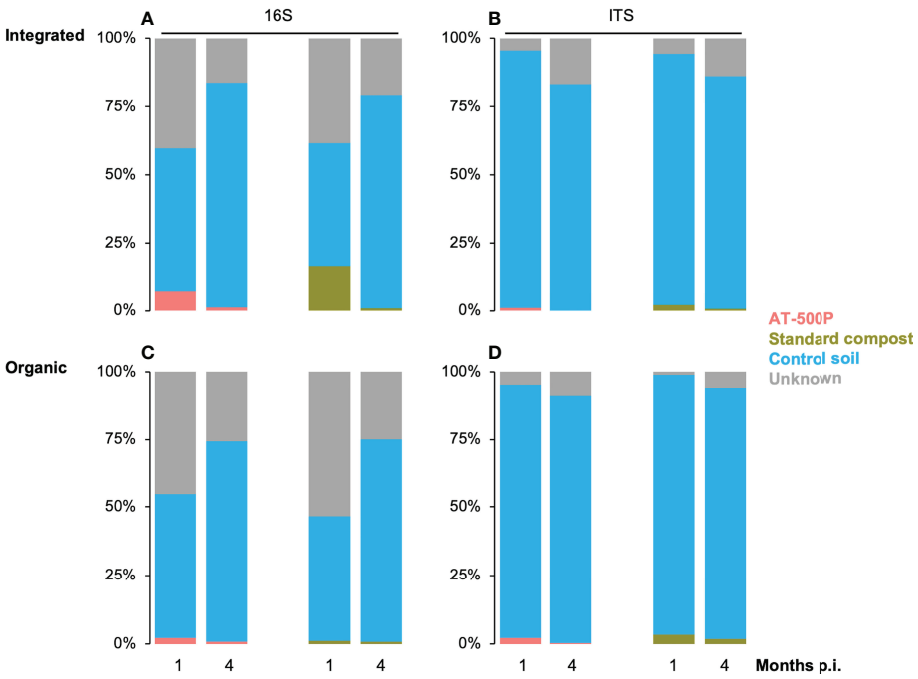

FIGURE 5
Tracking the microbiome transfers from standard compost and BD manure (AT-500P) extracts (i.e. microbiome source) into the treated rhizosphere soil under different management systems and sampling times. Panel **(A)** and **(B)** shows the proportion of microbiota tracked from treatment into rhizosphere in the integrated orchard for bacterial and fungal community, respectively, while **(C)** and **(D)** represent the same for the organic orchard. The proportion of the microbiome in the treated rhizosphere which was shared with the untreated soil (i.e. control soil) is shown both for organic and integrated orchards. The unknown part of the microbiome includes the percentage proportion of microbiota in treatments which could not be traced in the rhizosphere. Coloured stacked bars represent proportions of microbiome in treated rhizosphere (i.e. AT-500P and standard compost extracts), shared microbiome between control and treated rhizosphere soil, as well as the unknown microbiome (i.e. the unassigned part of treatment microbiome). The x-axis represents time in months post inoculation. The y-axis shows the proportion of the community originating from the different sources.

with severe bacillary hemoglobinuria in cattle and sheep, and it was suggested that its incidence can be reduced by the annual vaccination of herds (57). *Clostridia* in traditional BD preparations could fulfil similar functions. Additionally, Nitrogen fixation by *Clostridia* (58, 59), underpins the potential effect of organic amendments in promoting beneficial soil microbial traits.

Biodynamic manures amended with preparations contained a high abundance of *Actinobacteria*, a phylum known for its active role in the composting process (60, 61). Evidence linking *Actinobacteria* in a consortium of *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* to the breakdown of lignocellulose has recently been revealed (56). Potential plant-beneficial bacterial families like *Pseudomonadaceae, Burkholderiaceae*, and *Bacillaceae* (62) were observed to dominate the BD formulations and plant preparations. This suggests a potential contribution of the microbiome associated with BD manure and plant preparations in farming systems.

Interestingly, a common microbiome of the bacterial genus *Azotobacter*, and the fungal genera *Fusarium* and *Arthrographis* was observed between BD manures and plant preparations and further emphasizes the potential soil enrichment with beneficial bacteria. The genus *Fusarium* mainly contains plant pathogenic species (63), and was observed to be mainly associated with BD plant preparations. However, non-pathogenic and beneficial *Fusarium* species are well known (64) supporting the idea that intraspecific microbial diversity can supress pathogenicity (3). The fungal family *Ascosphaeracea* was observed in some of the BD plant preparations and has previously been found associated with the microbiome of bees (65). We suggest that this could have originated from the flowers of the plants used as starting materials in the production of BD plant preparations.

Independent of the production year (storage effect), the microbiome of BD manures was distinct between the regions of production (Austria and France). We suggest that various factors including plant and animal raw materials, as well as product handling in the two regions, have contributed significantly to the observed microbiome differences. Our study extends this insight to explain the likely cause of the region-specific difference observed in BD manures. Furthermore, the microbiome consistency across years suggests that BD manures can be reproduced in a production line. The specific procedures to

make BD preparations have been practised for decades by the BD farming communities and were also standardized (10, 15, 20), which provides an explanation for the reproducibility.

The use of liquid extracts from BD manures as field sprays is a common practice in BD farming (15). While the biochemical characteristics of such extracts were already deciphered (66, 67), our study serves as a primer to further understand the microbiome of BD extracts. The microbiomes of extracts and precursor materials of composts and BD manures were distinct but shared a common microbiome. We likely attribute the shared microbiome to the material from which the extracts are derived; also the procedure that materials are subjected during the fermentation. Moreover, the common microbiome of compost, mainly composed of *Firmicutes* and *Actinobacteria* was recently revealed (68). Bacterial genera including *Pseudomonas*, *Paenibacillus*, *Azotobacter*, and unidentified *Sphingomonadaceae* were associated with standard compost extract Microbially enhanced compost extracts were previously found to be essential in improving plant growth and nutrition (69). Thus, their microbiome composition could potentially explain the effects attributed to extract application on plant health.

Several fungal genera such as *Fusarium*, *Aspergillus Penicillium*, *Mortierella*, and *Lecanicillium* were observed in extracts of the different compost and BD manures. Interestingly, *Lecanicillium* has been found to have entomopathogenic properties, which led to its use as a biocontrol agent against aphids (70), as well as phytoparasitic nematodes and fungi (71, 72). The fungal genus *Penicillium* is diverse and widely distributed in the soil environment (73–75). It can exert positive effects through the production of secondary products (76), as well as play critical roles in plant growth and defence (77–79). High microbial diversity was found in extracts, as compared to the precursor standard compost and BD manure. This could be attributed to additives such as molasses and stone dust which were used to make the extracts. In addition, water as a solvent used in the extract production process may have contributed to its microbiome. Recent studies often found high microbial diversity in water microbiomes (80–82).

Overall, a positive impact of BD manure and compost amendments on soil fungal diversity was found, especially at four months post-inoculation, both for organic and integrated orchards. Enrichment effects of BD formulations on the soil microbiome were reported in a forgoing study (27), and further confirmed by the current study. Previous studies indicated that the use of BD manures and associated extracts in viticulture affects soil biodiversity and ecosystem functions (13, 18, 83, 84), and plays important roles in improving soil quality (23); as well as their potential role in biodynamic viticulture including the enhancement of wine quality (17, 25, 26). In the present study, treatments increased *Acidobacteria*, both in organic and integrated orchards. *Acidobacteria* is a rather underrepresented phylum, yet ubiquitous in the soil environment (85, 86), and involved in various processes in biogeochemical cycles, decomposition of biopolymers, exopolysaccharide secretion, and plant growth promotion (85, 87, 88). The fungal genus *Alternaria* was found to be decreased in treatments as compared to the untreated rhizosphere at one-month post-inoculation in the organic orchard. Thus, we suggest that one potential role of BD treatments is the reduction of plant pathogenic fungi, which also includes *Alternaria*. This genus is ubiquitous with saprophytic, endophytic, and pathogenic species which are commonly found associated with various plants, including fruits (89–91). Moreover, some *Alternaria* species were linked with allergies in humans (92, 93). Interestingly, strains of *Fusarium* and *Alternaria* have recently been found to be strongly associated with seeds of numerous crop plants, where they are vertically transmitted to the root and rhizosphere (94). On the other hand, the bacteria genera *Pseudomonas* and *Mycobacterium* were found to be enriched in the apple rhizosphere of the integrated orchard which had been treated with extracts from BD manure. Thus, in the context of BD farming, our findings provide insights into the potential role of BD products especially in increasing the abundance of beneficial bacterial genera. Moreover, the determination of various soil quality indicators suggests larger response in the BD system, than in conventional management systems (95, 96).

Our attempt to track the microbiome from extracts to the rhizosphere revealed that a specific proportion of the extract microbiome was transferable. In contrast to a previous study by (27), we observed that a higher proportion of the bacterial and fungal community was transferred to the rhizosphere in the integrated management orchard when compared the organic orchard. This implies that the use of BD extracts differently affects local soil microbiomes. This might be due to competition with indigenous microorganisms that are present in these soils. However, microbial signatures of organic amendment origin can survive in the soil microbial biomass (97, 98). Interestingly, even with the low applied rates for BD preparations (i.e. 1L:10L; extract: water) as shown by Koepf etal. (99) and the international "Demeter" guidelines for biodynamic farming (https://demeter.net/), we observed a significant effect of treatment on the microbial community structure and diversity, particularly an increase in fungal diversity during autumn for the integrated orchard. Thus, we suggest that an increase in the frequency of treatment application could more effectively modulate the local soil microbiome. Moreover, the application of small quantities (i.e. ≥ 5%) of suppressive soil was found to induce suppressiveness to disease conducive soils (100, 101). The low proportion of microbes transferred to the apple rhizosphere of the organic as compared to the integrated management orchard could be associated with high competition from the resident

microbiota. Limitations for the establishment of bioinoculant strains in soil environments were previously described (102, 103), and may include competition, as well as limitation in colonization due to different soil physico-chemical parameters.

Conclusion

The findings of this study provide first insights into the microbiome of biodynamic manures and their applied liquid extracts. Overall, our findings provide knowledge on approaches to enhance microbial diversity in agricultural soils, especially in intensively managed fields. Moreover, the in-depth assessment of biodynamic products in orchards subjected to different field management systems provides valuable information related to their contribution to plant rhizosphere microbiomes. Interestingly, the low standardization of compost amendments criticized in the past (104),
can be an advantage in future agriculture, which needs more diversification at all levels. We detected potentially beneficial microbes originating from biodynamic treatments that may be established in different farming systems upon their introduction; although, consistent application will be needed to provide expected benefits in the long term.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

GB, PK, EO, and RM designed the study. EO, PK, and RM performed the experiment and took the samples. EO performed library preparation and other laboratory work. EO, PK and WW analysed the data and interpreted it together with TC, SB, and GB. EO, SB, and PK wrote the manuscript. All authors read, corrected, and approved the final version.

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Conflict of interest

Author RM was employed by the company BODENmanagement.net.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsoil.2022.1020869/full#supplementary-material

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