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Insights into the Belowground Biodiversity and Soil Nutrient Status of an Organic Apple Orchard as Affected by Living Mulches

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Abstract: The impact of living mulches established with three officinal plants (*Alchemilla vulgaris*, *Fragaria vesca* and *Mentha x piperita*) on the soil bacterial microbiome and activity, the nematodes population, and the nutrient status of an organic apple orchard was assessed. The composition and diversity of the bacterial communities were differentially modified by living mulches. The activity of the bacterial microbiome associated with *F. vesca* was higher and utilized more C sources in comparison to other treatments. The combined analysis of the core bacterial microbiome and metabolic activity pointed to a potential effect of *F. vesca* on different levels of the soil's trophic network. The living mulches did not affect the overall number of nematodes, but in some cases, they modified the structure of the population: *F. vesca* induced the highest share of bacteria feeders and the lowest number of herbivores and fungal feeders. The living mulches modified the availability of some nutrients and the pH. Multivariate analysis of the whole dataset showed several potential inter-dependencies between the assessed parameters that are worthy of further study. In conclusion, the introduction of multifunctional living mulches based on officinal plants induced changes to the soil's genetic and functional biodiversity and chemical properties. These modifications could deliver ecosystem services particularly relevant to organic apple orchards.



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Keywords: *Alchemilla vulgaris*; *Fragaria vesca*; *Mentha x piperita*; soil bacteria; nematodes

1. Introduction

The preservation of natural resources and improving environmental biodiversity have become urgent goals that must be achieved [1]. These issues have also been promoted through policies at the EU (Green Deal and Farm to Fork strategies) and worldwide (2030 Sustainable Development Goals) levels. In this framework, the concept of agroecology has been pursued as a tool that fosters biodiversity with economic and social sustainability, and new intercropping strategies have been proposed for different cropping systems [2,3]. Besides the expected increase in aboveground biodiversity [4], the effect of intercropping has also been assessed in terms of soil biodiversity, particularly on microbial activities related to nutrient cycling [5,6], but also considering other ecosystem services and their effect from economic and human well-being perspectives [7,8].

Living mulches are a specific declination of intercrops for fruit crops, which were initially developed as a tool for weed control on tree rows [9,10], particularly for organically managed orchards [11,12]. However, recently, they have also been evaluated for their possible exploitation as providers of other ecosystem services related to plant protection and their positive effect on the overall aboveground insect community [13,14]. To foster the adoption of living mulches, which require high technical knowledge for their correct implementation [15], solutions with multifunctional species, including medicinal and aromatic plants, could represent an interesting perspective as providers of additional income to farmers [16] and support biological control of soil-borne pathogens [17].

Nematodes are the most abundant soil metazoan, and their populations are useful biological indicators of soil health that can reveal natural and human-induced changes in soil agro-ecosystems [18]. Several agronomic practices can impact nematode populations (e.g., tillage, fertilization, and plant protection) [19,20]. However, root exudates can also exert diverse effects on several nematode taxa and feeding guilds [21], which are sometimes associated with soil-borne plant syndromes [22].

The soil microbiome is recognized as having a crucial role in soil health and fertility. Microbial communities are involved in direct and indirect interactions with plants, which depend on environmental and agronomic factors [23,24]. However, the soil microbiome is also interconnected with the major nematode trophic groups (bacteria and fungi feeders). A comprehensive understanding of the mechanisms underlying all these interactions will enable the development and implementation of strategies optimizing soil management through the better prediction of the changes induced by agricultural practices, thus fostering the provision of ecosystem services [25].

Considering all these aspects, knowledge of the impact of living mulches on orchard soil biodiversity and the ecosystem services they could provide related to soil nutrient status could further increase the interest of farmers in adopting this practice into fruit cropping systems. The hypothesis of a possible positive modification of the soil biome derived from living mulches was thus assessed in an organic apple orchard. Three medicinal plants (*Alchemilla vulgaris*, *Fragaria vesca*, and *Mentha x piperita*) were compared to natural soil cover for their effect on the functional biodiversity of the soil with regard to bacteria and nematode populations and in relation to its nutrient content.

2. Materials and Methods

2.1. Experimental Site and Management Practices

The trial was conducted on a ten-year-old apple orchard (cv. Gala on M9 rootstock) established on loamy sand soil (sand 78% + silt 14% + clay 4%) with 3.22% soil organic matter and a pH of 6.2. The orchard, belonging to the National Institute of Horticultural Research experimental farm (central Poland, 51°58'0" N, 20°9'0" E), is located in an area characterized by an average annual temperature of 12 °C and an average annual precipitation of 512 mm. The orchard, with trees trained in a spindle form and spaced at 3.5 m × 1.6 m, was managed according to the rules of organic farming, with localized fertilization with dry bovine manure and stillage (total of 12 g N/tree) and drip irrigation.

A Randomized Complete Block Design (RCBD) was laid out with four treatments of understorey soil management, each with three replications, including three living mulching species: *Alchemilla vulgaris*, *Fragaria vesca*, and *Mentha x piperita*. Natural soil cover was considered as the control, and it was managed with three-time mowing during the growing season. Each replication consisted of 20 trees for a total row length of about 30 m. The living mulching species were planted at a rate of 10 plants/m² randomly along the tree row in mid-May 2021. The plots were hand-weeded twice to promote the good establishment of the living mulches during the first growing season and during the experiment.

2.2. Soil Sample Collection

Soil samples for chemical and biological analyses were collected three times during the season with well-established living mulches (May, July, and September 2022). Sampling was performed using Egner's auger (2.5 cm diameter), collecting at least 10 subsamples at 0–20 cm depth within an area of 30 cm radius/distance around the apple tree trunk. The subsamples were pooled and mixed. Visible animals, organic parts (plant residues, roots) and small stones were removed at this stage. The samples were processed immediately or stored at 4 °C for up to one week for soil chemical analysis, nematode biodiversity assessment or microbial biodiversity and activity measurements. Samples for DNA extraction were stored at −80 °C until analysis.

2.3. Soil Chemical Analysis

The soil samples were analyzed in terms of pH, salinity, and the levels of basic nutrients: N-NO₃, N-NH₄, P, and K. After drying at a temp. of 65 °C, the soil was mineralized in concentrated nitric acid in a Candela Mars 5 microwave digestion oven. Nitrogen was determined colorimetrically with a Skalar SanPlus automated flow analyzer [26], and phosphorus and potassium were analyzed with a Perkin Elmer OPTIMA 2000 DV plasma spectrometer [27]. The pH of the soil samples was determined on KCl extract: 10 g of homogenized and air-dried soil was mixed with 25 mL of 1 M KCl, and the measurement was carried out on the solution after 24 h using a pH meter (Fisher Scientific, Warsaw, Poland).

2.4. Soil Microbial Biodiversity and Activity Assessment

Soil microbial biodiversity and activity were determined using the BIOLOG[®] system and EcoPlates, which enabled us to evaluate the microbial activity towards 31 different potential carbon sources. A suspension was obtained from 1 g of soil using sterile distilled water (1:9 *w:v*) and serially diluted three times. The plates were inoculated with 100 µL of the soil suspension per each well and incubated in the dark at 26 °C. After 72 h, the absorbance at 590 nm (OD) and 750 nm was measured. The activity of the microorganisms was estimated on the basis of Average Well Color Development (AWCD) [28], and the activity index was calculated according to the following formula:

$$AWCD = \sum OD_i / 31$$

where OD_i is the optical density of the individual wells. The microbial biodiversity index was estimated using the Shannon–Weaver coefficient (H')

$$H' = -\sum p_i (\ln p_i)$$

where p_i is the level of microbial activity in each well (OD_i) divided by the activity in all of the wells (Σ OD_i) [29]. When calculating the activity levels of the microorganisms for the H' index and the amount of metabolized substrates, the threshold value OD = OD_i − OD_(control well) was used. Substrate richness (S), which is the number of utilized carbon substrates, was calculated using an OD₅₉₀ value of 0.500 as a threshold for positive response. The results were used for comparison and visualization of the potential metabolic profiles of the soil microbial communities of each soil sample.

2.5. Soil Nematode Biodiversity Assessment

Nematodes were extracted from the soil using a modified Baermann method [30]. Briefly, 100 mL of soil was uniformly spread on a 250 µm sieve, moistened with distilled water and left for 24 h at room temperature. The nematodes extracted from the soil were recovered from the water suspension using a 25 µm sieve. Afterward, they were killed by heating at 65 °C for 2 min and preserved in TAF solution (2 mL of triethanolamine, 10 mL of 40% formalin, and distilled water added to a final volume of 100 mL). Fifty randomly selected nematodes from each sample were chosen for permanent glycerin slide preparation and detailed classification to the genus level according to the taxonomical keys [31]. All of the identified nematode genera were assigned to five trophic groups (bacterial feeders, fungal feeders, omnivores, predators, or plant feeders) following Yeates et al. [32] and to colonizer–persister (c–p) classes [33,34]. The observations and photographic documentation were carried out using a Nikon Eclipse 80i microscope equipped with a Nikon DS-Fi1 camera under 400× magnification.

2.6. DNA Extraction and Sequencing

DNA was extracted from a 0.5 g soil sample of each treatment and replicate (in total, three isolations per treatment) using the E.Z.N.A.[®] Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. After the isolation, the DNA was purified using Mag-Bind[®] TotalPure NGS (Omega Bio-Tek, Norcross, GA, USA) according

to the manufacturer's instructions and finally resuspended in 50 µL of water. The quality and concentration of the DNA extracts were evaluated via spectrophotometer analysis (NanoDrop 1000, Thermo Fisher Scientific Inc., Wilmington, DE, USA).

The metagenome analysis of archaea and bacteria was performed based on the hypervariable V3–V4 region of the 16S rRNA gene. The library construction, sequencing, and initial bioinformatic analysis were outsourced to Genomed S.A. (Warsaw, Poland). In short, the 341F and 785R primers were used together with a Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs Inc., Ipswich, MA, USA). The sequencing was performed through the use of paired-end technology (PE) at 2×300 nt with an Illumina v3 kit using a MiSeq instrument (San Diego, CA, USA). The preliminary analysis of the obtained data was carried out with a MiSeq Reporter (MSR) v2.6 software (Illumina, San Diego, CA, USA). It consisted of two steps: the automatic demultiplexing of the samples and generating the fastq files containing the raw reads. The quality control of the reads, together with the analysis of the error profile of individual samples, was performed with the FIGARO tool. Subsequently, pre-processing of the data relied on the removal of the adapter sequences and rejection of the short reads (<30 nt) with the Cutadapt tool [1]. Bioinformatic analysis allowing for the taxonomic classification of the soil-inhabiting microbes based on 16S rRNA reads was carried out with QIIME 2 [2]. The obtained reads were denoised using the DADA2 pipeline [3], and unique sequences of biological origin, i.e., Amplicon Sequence Variants (ASVs), were assigned. In the next step, the taxonomic classification of the ASVs was performed using a self-trained naive Bayes classifier that acquired data from the reference Silva 138 nr_v138 database [4]. The amplicon sequences were deposited in the NCBI's SRA database (BioProject: PRJNA1040432).

2.7. Statistical Analysis

Statistical analysis of the data was performed using R software version 4.1.3 [35]. The Shapiro–Wilk test was used to verify if the data followed a normal distribution, and Levene's test was used to verify the homogeneity of the variances. Thus, the data were analyzed through the use of ANOVA, and the mean differences were tested with Tukey's test at $p \leq 0.05$ with a HSD test function from the “agricolae” package. In the case of a not normal distribution, nonparametric Kruskal–Wallis analysis with Fisher's least significant difference post hoc test was utilized, introducing the Benjamini–Hochberg correction, with significance set to $p \leq 0.05$, using the Kruskal function from the “agricolae” package, while for two-way factorial designs, nonparametric Scheirer–Ray–Hare from the “rcompanion” package was used. PCA was performed using the “prcomp” command from the “stats” package and visualized using “ggplot2” and “ggfortify” packages. Heatmaps were generated using “heatmap.2” command from “gplots” package, and the phylogenetic tree of core microbiome taxa was generated using “ggtree” and “ggtreeExtra” packages. NGS data were analyzed using both “phyloseq” package and STAMP v2.1.3 software [36]. Correlations were calculated and visualized using “stats” and “ggcorrplot” packages, respectively.

3. Results

3.1. Effect of Living Mulches on Soil Nutrient Availability

The majority of the measured soil chemical parameters showed statistically significant differences in their levels between sampling points during the vegetative period, irrespective of the treatment (Supplementary Materials Table S1, Figure 1). Only P levels were independent of the seasonal factor. Spring time samples were the most different from the other two sampling timepoints, which also emerged from the PCA (Figure 1). The observed pH values decreased significantly during the vegetative season (on average, from 6.30 in May to 5.74 in September). Both nitrogen forms showed a concentration peak in summer (46.25 g/L for N-NO₃ and 227.50 g/L for N-NH₄). However, while nitrate levels remained stable till the end of the season (on average 41.08 g/L), the concentration of N-NH₄ decreased significantly, reaching a level comparable to that observed in spring at the autumn sampling time. Similar trends were observed for potassium and calcium levels,

which also showed the highest concentrations during summer, while the concentrations observed in autumn were either comparable (for K) or lower (for Ca) than at the beginning of the vegetative season. Mg concentration followed a different pattern, with a decrease in summer.

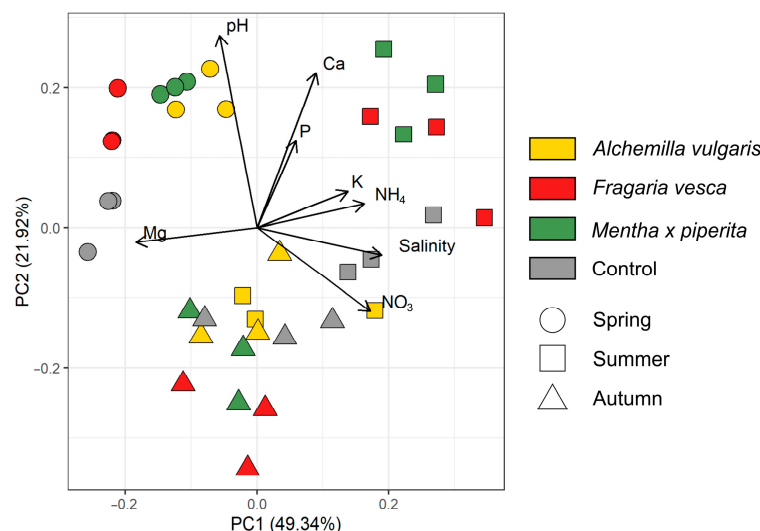


Figure 1. PCA of soil chemical parameters in relation to sampling time and living mulch treatment.

The living mulch species resulted in affecting the concentration of some nutrients and chemical parameters, but not salinity, magnesium, and N-NH₄ levels. *F. vesca* induced an increase in the soil pH and P levels (which also significantly increased in apple leaves, data beyond the scope of this article, manuscript in preparation) in comparison to the control, and lowered N-NO₃ levels. *A. vulgaris* induced a slight increase in N-NO₃ levels and significantly decreased K levels when compared to the control. *M. x piperita* resulted in increased K levels and decreased Ca concentration in soil. It is worth noting that most of the observed parameters showed a significant interaction between timepoint and treatment factors. The observed changes in the soil nutrients only marginally affected the nutrient content of the apple leaves: the N, K, and Mg content in leaves were not affected, while negligible changes occurred for Ca (data beyond the scope of this article, manuscript in preparation). Moreover, no differences in yield were observed between the treatments.

3.2. Impact of Living Mulches on Soil Microbiome Metabolic Activity and Diversity

The data obtained through Biolog EcoPlates analysis were used to calculate the metabolic activity and biodiversity indices as well as assess the metabolic potential of soil microbiomes toward individual compounds or compound classes.

The highest average bacterial activity was observed in autumn (Table 1). *F. vesca* induced a significantly higher activity compared to the control or *A. vulgaris* treatment, and soil from *M. x piperita* had intermediate activity. Substrate richness was not affected by the seasonal factor but was significantly lower in *A. vulgaris* treatment in comparison to the other treatments. No statistically significant differences between treatments or sampling timepoints were observed for the H Index calculated on microbial metabolic potential, but a higher diversity was observed in spring ($p = 0.058$) compared to summer samples or in control compared to other treatments, particularly *A. vulgaris* ($p = 0.064$). Moreover, no interaction between the sampling timepoint and treatment was observed for any of the three parameters.

Five out of thirty-one C sources (four carbohydrates and one polymer) displayed statistically significant differences within at least one sampling timepoint, but only D-Xylose displayed significant differences between treatments across the whole season (Table 2). The soil microbiome associated with *A. vulgaris* showed significantly decreased activity toward this compound during spring in comparison to any other treatment and during summer in comparison to the control and *F. vesca*, and the *M. x piperita* soil microbiome also showed significantly lower activity toward it. On the other hand, the soil microbiome from *F. vesca* displayed stable high activity toward this carbohydrate during the whole season. Considering the overall changes induced by the living mulching species on these C sources, *A. vulgaris* induced decreased activity of the metabolic profile, except for the D-Galactonic- γ -Lactone utilization during spring time. On the contrary, the changes induced by the *F. vesca* microbiome usually increased metabolic activity.

Table 2. Average absorbance at 590 nm for five compounds with significant changes observed in at least one sampling point between soil managed with living mulch species or natural cover. Different letters show statistically significant differences between treatments and sampling points for $p \leq 0.05$.

Class	Carbohydrate	Carbohydrate	Carbohydrate	Carbohydrate	Polymer
Compound	β -Methyl-D-Glucoside	D-Galactonic- γ -Lactone	D-Xylose	D-Cellobiose	Tween 40
Spring					
Control	1.99 cdef	1.07 c	1.25 bcde	1.85 ab	2.06 ab
<i>A. vulgaris</i>	2.38 abcd	1.98 ab	0.38 efg	1.19 ab	2.10 ab
<i>F. vesca</i>	2.60 a	1.26 bc	2.41 ab	1.99 ab	2.02 ab
<i>M. piperita</i>	1.84 def	1.63 abc	1.51 bcd	2.10 ab	2.26 ab
Summer					
Control	2.26 abcde	1.97 ab	2.54 a	2.65 a	2.41 ab
<i>A. vulgaris</i>	0.42 f	1.50 abc	0.19 g	0.99 b	2.10 ab
<i>F. vesca</i>	2.48 abc	2.22 a	2.00 abc	2.43 ab	2.41 ab
<i>M. piperita</i>	1.55 ef	1.42 abc	0.46 defg	1.51 ab	2.46 ab
Autumn					
Control	2.57 ab	1.92 abc	0.41 defg	2.18 ab	1.98 b
<i>A. vulgaris</i>	1.58 abcdef	1.96 ab	1.11 cdef	2.01 ab	2.33 ab
<i>F. vesca</i>	2.26 abcd	1.74 abc	2.50 ab	2.57 ab	2.50 a
<i>M. piperita</i>	2.03 bcdef	1.48 abc	0.30 fg	1.73 ab	2.24 ab

3.3. Impact of Living Mulches on Soil Microbiome Genetic Diversity

To better explore the outcomes from the metabolic analysis, the microbial genetic biodiversity was analyzed by applying 16S rDNA V3V4 amplicon sequencing for the summer sampling timepoint, which was the most variable and interesting from a practical standpoint.

A total of 1,978,037 paired reads were obtained from Illumina MiSeq sequencing, with reads per sample ranging from 115,202 to 203,316. Quality control and merging of the paired reads using the dada2 software package resulted in the retention of, on average, $45.74 \pm 2.31\%$ of the reads per sample (Supplementary Materials Table S2). The rarefaction curves of these data showed that rarefying the library size to the smallest library size provided enough sequencing depth for downstream analyses (Supplementary Materials Figure S1). In the analyzed dataset, 99.88 to 100% of the reads were classified to the Bacteria kingdom, while the others were assigned to Archaea. Thus, in this study, we decided to analyze only the reads assigned to the Bacteria kingdom.

Even though 39 different bacterial phyla were identified, only 11 of them constituted at least 1% of the whole community (Supplementary Materials Table S3). Interestingly, the abundance of all of them was significantly different, but the three analyzed samples for each treatment were clustered together, confirming a consistent impact of the living mulch (Figure 3, left side, colored strip). *M. x piperita* and *F. vesca* bacterial populations clustered together, separate from *A. vulgaris*, and all were clearly separated from the control (Figure 3,

left side clustering). Proteobacteria was the most abundant phylum, especially in the control treatment (33.6%), and it was significantly reduced by about 25% with the introduction of any living mulch, especially *A. vulgaris*. Actinobacteriota, the second most abundant phylum, was most commonly present in the natural covered soil (28.3%) and significantly less abundant in *A. vulgaris* (25.0%), *F. vesca* (20.1%), or *M. x piperita* (20.8%) treatments. Acidobacteriota, the third major phylum, was, on the contrary, less abundant in the control (10.0%) and increased by *M. x piperita* (13.5%), *F. vesca* (14.1%), and *A. vulgaris* (15.3%) treatments. Changes in the abundance of less present phyla (e.g., Bacteroidota, Chloroflexi, and Firmicutes) were also evident, especially for Bacteroidota, which were much more abundant in soil mulched with *F. vesca* and *M. x piperita* than in the other treatments. The other four phyla each accounted for less than 5% of the community. Myxococcota and Planctomycetota were enriched to a similar level (about 2.4%) by all living mulches in comparison to the control (about 1.6%). On the other hand, some specific changes were observed for the different living mulching species: Verrucomicrobiota was more abundant in *F. vesca*, followed by *M. x piperita*, while Patescibacteria was enriched by *M. x piperita*, especially when compared to the control.

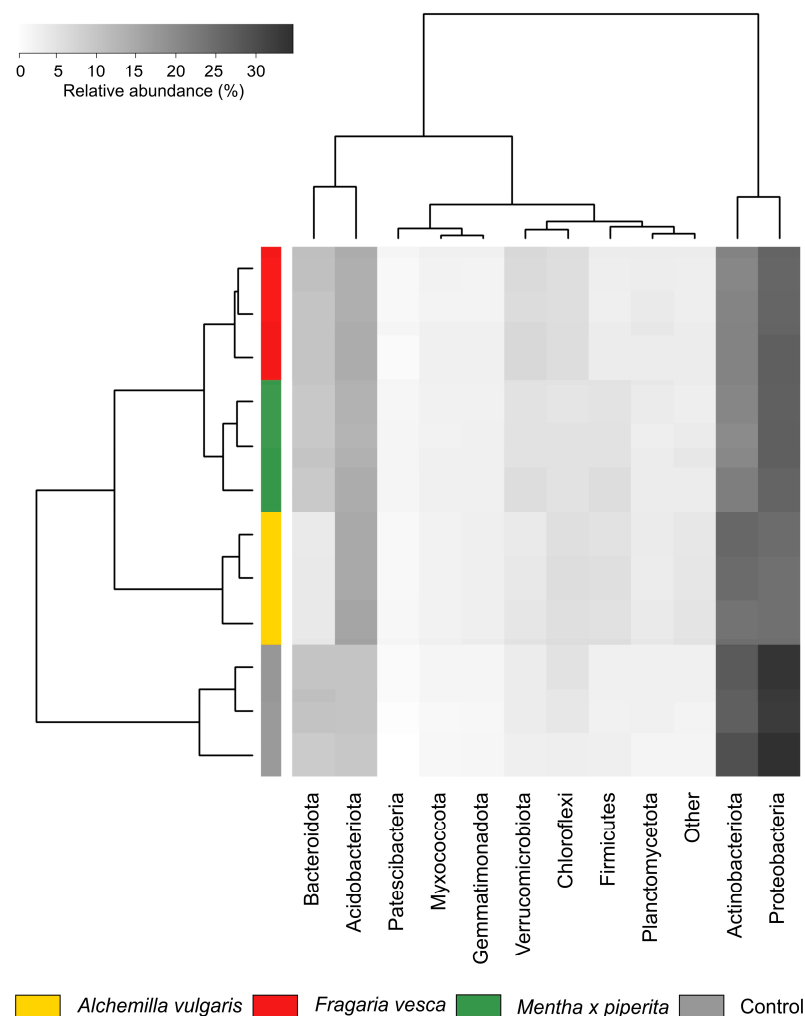


Figure 3. Relative abundance of the most abundant phyla (>1%) across the samples collected from soil managed with different living mulch species or naturally covered (control).

At the genus level, 838 different taxa were identified. Few genera were unique (present in all three replicates for a certain treatment and absent in other treatments/samples) in terms of the different methods of soil management: six genera in the *A. vulgaris* treatment (*Exiguobacterium*, *Corallococcus*, *Sorangium*, *Spirochaeta*, and an unclassified genus

representing Patescibacteria phylum and an unclassified genus representing the *Geminicoccaceae* family), seven in *F. vesca* (*Psychrobacter*, *Buttiauxella*, *Candidatus Glomeribacter*, *Actinomandura*, *Candidatus Lumbricinola*, *S15-21*, and *211ds20*), eight in *M. x piperita* (*Micromonospora*, *Sphingobacterium*, *Oceanobacillus*, *Planococcus*, *Planomicrobium*, LD29, an unclassified representative of the *Microbacteriaceae* family and an unclassified representative of *Solirubrobacterales* order), and five in control (*Conyzicola*, *Dysgonomonas*, *Acidovorax*, *Duganella*, and *Erwinia*).

Filtering the genera to the taxa present in each sample (identified in each library) with at least 0.1% relative abundance and showing statistically significant differences between treatments allowed us to distinguish 82 different bacterial taxa (Figure 4) representing 12 different phyla, mainly belonging to Proteobacteria and Actinobacteria, hereinafter referred to as the core bacterial microbiome.

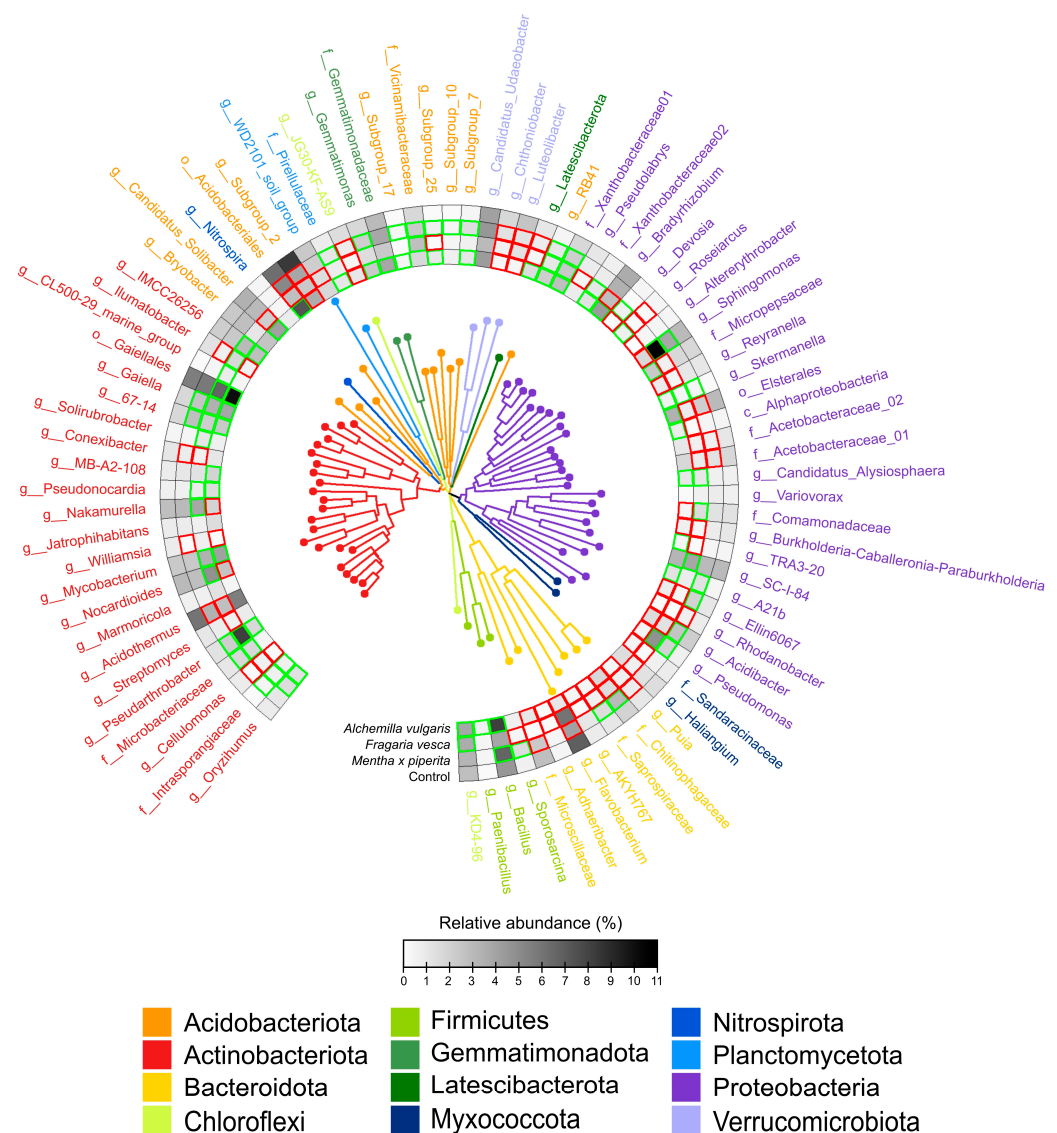


Figure 4. The phylogenetic tree of core microbiome taxa is based on the V3V4 fragment of the most abundant ASV assigned to certain taxa together with the average relative abundance of these taxa in the tested treatments. Tiles with red or green borders represent the significantly lowest or highest, respectively, values of the treatments in comparison to the control. The core microbiome taxa were chosen by filtering the data to the genera present in each replicate of each treatment with at least 0.1% relative abundance and showing significant changes between treatments based on the Kruskal–Wallis test with Benjamini–Hochberg correction ($p \leq 0.05$).

About 92% of the taxa showed changes in abundance in comparison to the control (Figure 4, tiles with red and green border). The *A. vulgaris* treatment was characterized by the largest number of enriched taxa (35), followed by *F. vesca* (27) and *M. x piperita* (25). On the contrary, *F. vesca* treatment showed the largest number of taxa with reduced abundance (37), followed by *A. vulgaris* (27) and *M. x piperita* (19). Only seven taxa (*Skermanella*, RB41, *Gaiella*, 67-14, *Pseudoarthrobacter*, *Xanthobacteraceae01*, and *Intrasporangiaceae*) were always enriched, and only nine (*Microscillaceae*, *Flavobacterium*, *Puia*, *Rhodanobacter*, *Luteolibacter*, *Chtoniobacter*, JG30-KF-AS9, and *Subgroup_2*) were always reduced in each living mulch treatment when compared to the control (details in Figure 4). The overall soil metagenome response of *A. vulgaris* treatment seemed to share more abundance shifts (the same direction of relative frequency changes for individual taxa observed) with *F. vesca* treatment (17) than with *M. x piperita* (10) or between *F. vesca* and *M. x piperita* (12). Interestingly, almost all similarities (9 out of 10) shared only between *A. vulgaris* and *M. x piperita* were defined as enriched taxa abundance in comparison to the control (details in Figure 4). Furthermore, *F. vesca* treatment was characterized by the highest number of taxa uniquely changed by the treatment in comparison to the control. These shifts included abundance enrichment of three taxa (*SC-I-84*, *CL500-29 marine group*, *Solirubrobacter*) and a decrease in four taxa (*Haliangium*, *Pseudolabrys*, *Streptomyces* and *WD2101 soil group*). In the case of *A. vulgaris*, the changes concerned an increased abundance of *Paenibacillus* and MB-A2-108 and a reduced abundance of *Alterythrobacter*, while no specific changes were observed in the *M. x piperita* treatment.

A significant increase in biodiversity (Shannon and Simpson indexes) and, to a lesser extent, community richness (Chao1 index) was observed in the soil covered with living mulches compared to the natural cover (control) (Table 3). According to their overall impact on the soil bacterial diversity, the mulching species could thus be ranked as follows: *M. x piperita* > *F. vesca* > *A. vulgaris*.

Table 3. Alpha diversity indices of bacterial amplicon sequencing data from tree understory soil managed with living mulch species or natural cover of an organic apple orchard. Different letters show statistically significant differences between treatments for $p \leq 0.05$.

Treatment	ChaO1	Shannon	Simpson
<i>A. vulgaris</i>	508.72 ab	5.16 c	0.989 c
<i>F. vesca</i>	513.67 ab	5.28 b	0.991 b
<i>M. piperita</i>	553.71 a	5.36 a	0.992 a
Control	410.33 b	4.98 d	0.986 d

3.4. Impact on Soil Nematodes Abundance and Diversity

Between 370 and 524 nematode individuals per sample were isolated from the soil samples collected during the summer sampling timepoint. The total number of nematodes was not affected by the treatment, although some differences were observed in the relative abundance of bacterivore and fungivore feeding groups (Table 4). *F. vesca* induced an increase in bacterivores compared to the control and *A. vulgaris*. On the other hand, *A. vulgaris* showed a significantly higher share of fungi feeders only in comparison to *F. vesca*, even though the average was also higher in comparison to the control.

Even though the number of identified taxa did not differ significantly between the treatments (on average 13.9), up to 35 different nematode taxa were found (Supplementary Materials Table S4). Ten of them represented bacterivores, eleven were members of the herbivore trophic group, fungivores and predators had five taxa each, and four were from the omnivore group. Only two taxa were always present in any of the analyzed samples: *Rhabditis* (bacterivores) and *Pratylenchus* (herbivores). The following taxa were found in the majority of samples: *Plectus* and *Filenchus* (each in 91.7% of the samples), *Cephalobus* (in 83.3% of the samples), and *Eucephalobus* (75.0%). The other genera that were found in about half of the samples included bacterivores (*Acrobeloides* and *Panagrolaimus*), fungivores (*Aphelenhoides*, *Aphelenchus*, and *Ditylenchus*), and herbivorous genera (*Coslenchus*, *Paraty-*

lenchus, and Tylenchus). The core nematode taxa (found in at least one replicate per each treatment) consisted of 12 genera representing bacterivores, fungivores, and herbivores (Figure 5). Omnivores and predators were present in up to one-third of the analyzed samples and thus were specific to the selected treatments. Certain taxa, particularly of the herbivores group, were identified (with different abundance) only in specific treatments: *Bitylenchus*, *Trichodorus*, *Merlinius*, or *Meloidogyne* in samples from the *A. vulgaris* treatment or *Helicotylenchus* in samples from the *M. x piperita* treatment. Bacteria feeders (*Monhystera*), fungi feeders (*Ditylenchus*), herbivores (*Cephalenchus*), and omnivores (*Microdorylaimus*) were typical of *F. vesca* mulched soil. On the other hand, the control was characterized by the presence of several predators and omnivores like *Nygolaimus*, *Aporcelaimellus*, or *Mesodorylaimus*.

Table 4. Total nematode population and trophic group abundance of tree understory soil managed with living mulch species or natural cover of an organic apple orchard. Means ± SEM. Different letters show statistically significant differences between treatments for $p \leq 0.05$.

Treatment	Total	Bacterivores	Fungivores	Herbivores	Omnivores	Predators	Number of Taxa
<i>A. vulgaris</i>	478.0 ± 8.2 a	48.8 ± 1.0 b	26.3 ± 2.5 a	19.3 ± 2.8 a	3.0 ± 1.8 a	0.9 ± 1.2 a	15.0 ± 2.0 a
<i>F. vesca</i>	412.0 ± 6.2 a	75.5 ± 2.3 a	7.8 ± 2.4 b	9.2 ± 1.9 a	0.7 ± 1.1 a	4.2 ± 1.9 a	14.7 ± 1.2 a
<i>M. piperita</i>	480.0 ± 6.3 a	65.4 ± 3.2 ab	14.8 ± 2.8 ab	15.7 ± 3.3 a	2.5 ± 1.6 a	1.6 ± 1.7 a	11.7 ± 1.2 a
Control	466.0 ± 4.6 a	48.5 ± 3.6 b	12.2 ± 2.6 ab	24.1 ± 2.5 a	4.5 ± 2.1 a	9.1 ± 2.7 a	14.0 ± 2.4 a

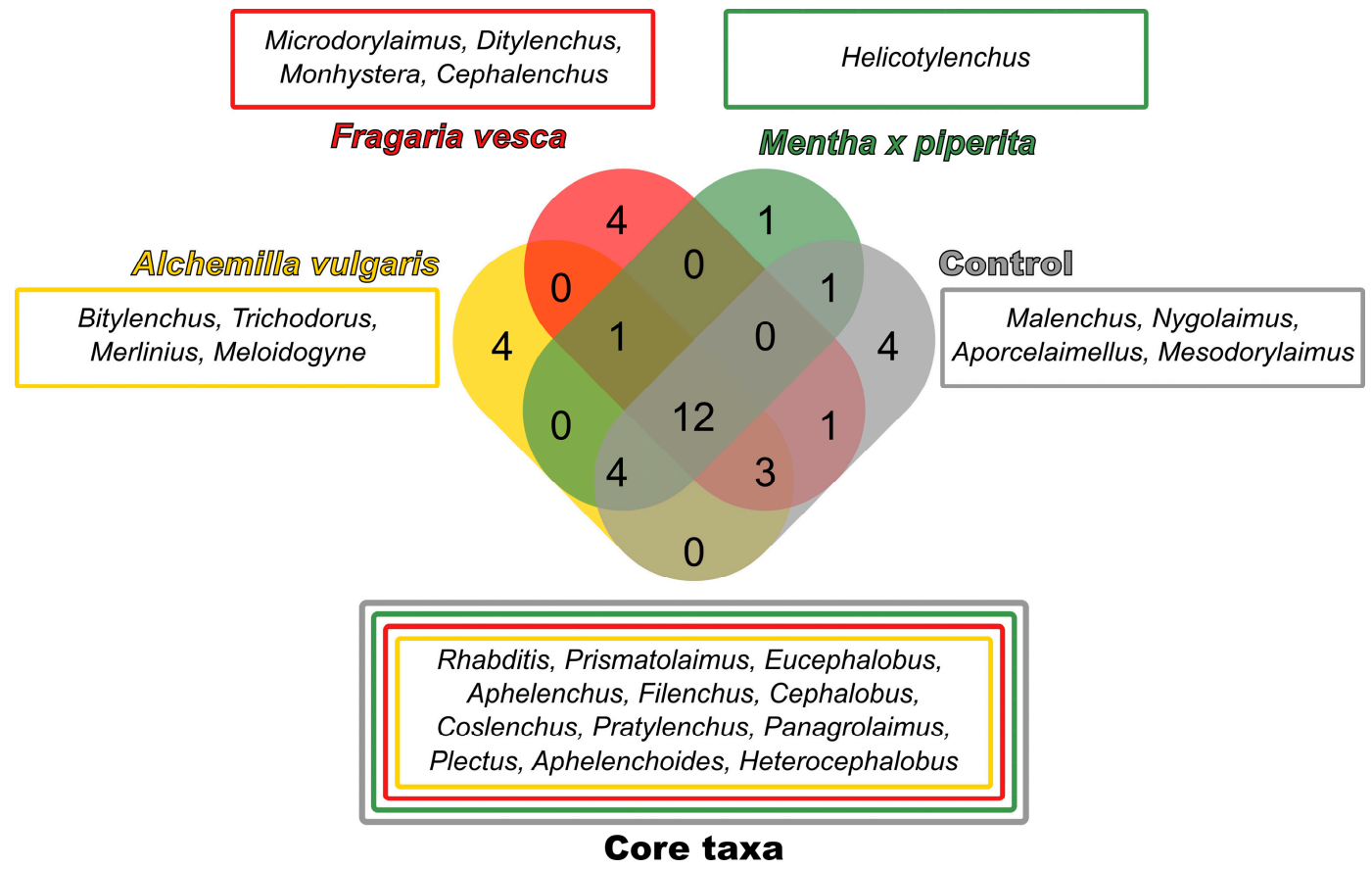


Figure 5. Venn diagrams based on the counts of nematode taxon identified at least once in the samples of soil treated with different living mulches. The taxa specific to each treatment and the core nematode taxa are also listed in boxes.

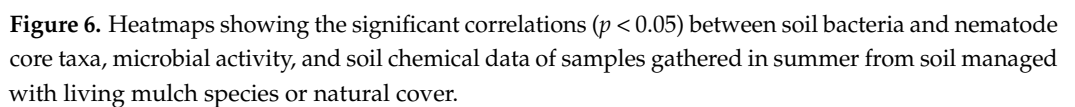
Only nine taxa, belonging to all but the omnivore groups, out of the thirty-five identified taxa, had significant differences in terms of relative abundance (Table 5). Nematode communities extracted from the samples of soil from *A. vulgaris* mulches showed a significantly higher abundance of *Aphelenchoides* and *Bitylenchus* than any other treatment. On the contrary, communities associated with soil from *F. vesca* mulches were enriched in *Cephalenchus* and *Eucephalobus* individuals in comparison to other treatments and had reduced presence of *Paratylenchus* and *Nygolaimus* compared to the control. Mulches of *M. x piperita* had the least effect on the soil nematode community composition in comparison to the control, as these two treatments differed significantly only in *Paratylenchus* and *Nygolaimus* abundance.

Table 5. Nematode taxa with significant differences in relative abundance (%) between samples from tree understory soils managed with living mulch species or natural cover of an organic apple orchard. CEP—*Cephalobus*; EUC—*Eucephalobus*; APH—*Aphelenchoides*; DIP—*Diphtherophora*; DIT—*Ditylenchus*; BIT—*Bitylenchus*; CEH—*Cephalenus*; PAR—*Paratylenchus*; NYG—*Nygolaimus*. Means $\pm$ SEM. Different letters show statistical differences between treatments for $p \leq 0.05$.

Treatment	CEP	EUC	APH	DIP	DIT	BIT	CEH	PAR	NYG
<i>A. vulgaris</i>	9.3 $\pm$ 2.3 a	3 $\pm$ 1.8 b	7.5 $\pm$ 1.4 a	3.3 $\pm$ 1.0 a	0.0 $\pm$ 0.0 b	4.4 $\pm$ 1.4 a	0.0 $\pm$ 0.0 b	3.8 $\pm$ 2.1 ab	0.0 $\pm$ 0.0 b
<i>F. vesca</i>	7.7 $\pm$ 1.2 ab	9.8 $\pm$ 1.3 a	0.7 $\pm$ 1.1 b	0.0 $\pm$ 0.0 b	1.4 $\pm$ 1.1 a	0.0 $\pm$ 0.0 b	1.4 $\pm$ 1.1 a	0.0 $\pm$ 0.0 b	0.0 $\pm$ 0.0 b
<i>M. piperita</i>	3.8 $\pm$ 1.1 ab	3.2 $\pm$ 1.3 b	1.6 $\pm$ 1.2 b	0.7 $\pm$ 1.1 ab	0.0 $\pm$ 0.0 b	0.0 $\pm$ 0.0 b	0.0 $\pm$ 0.0 b	1.5 $\pm$ 1.1 b	0.0 $\pm$ 0.0 b
Control	0.8 $\pm$ 1.2 b	0.7 $\pm$ 1.1 b	0.7 $\pm$ 1.1 b	1.6 $\pm$ 1.2 ab	0.0 $\pm$ 0.0 b	0.0 $\pm$ 0.0 b	0.0 $\pm$ 0.0 b	11.8 $\pm$ 2.1 a	3.8 $\pm$ 1.8 a

3.5. Correlations between Soil Nutrient Content, Microbial Activity and Nematological Biodiversity

To better understand the potential inter-dependency between the soil biodiversity data and microbial activity in relation to its chemical properties, an analysis with data obtained from the summer samples was performed, including the chemical parameters of the soil, indices derived from the Biolog EcoPlate assay, and the relative abundance of the core bacterial microbiome and of the core nematode taxa (consisting of 82 and 12 taxa, respectively). More than 1800 statistically significant relationships were found among the dataset composed of 135 parameters (Supplementary Materials Table S5). These included some easily explained or expected correlations, such as a strong positive or negative correlation between nematode genera belonging to the same or different trophic group or between AWCD and metabolic potential towards several carbohydrates or increasing soil phosphorus levels, along with pH increase. An in-depth analysis of all these interactions was considered beyond the scope of this article. However, an assessment of significant correlations, which is useful in terms of understanding the impact of the introduction of any living mulch species into an orchard environment from a practical (agronomical) perspective, was performed as a case study (Figure 6). Interestingly, *Aphelenchoides*, *Cephalobus*, and *Filenchus* showed the highest number of correlations with all groups of parameters (49, 45, and 34, respectively). *Aphelenchoides* and *Cephalobus* correlated with several bacteria taxa (13 and 6, respectively). On the other hand, *Heterocephalobus*, *Panagrolaimus*, and *Prismatolaimus* were not correlated with any bacterial taxa and with one or a few of the other parameters.



4. Discussion

The introduction of living mulches to officinal plant species in an organic apple orchard was considered a practice that can fulfill several ecosystem services and functions: weed control, enhancing aboveground arthropod populations that support pest control and yield, and providing a secondary cash crop, increasing the potential income of the orchard. The present study showed that this practice can also promote changes in soil biodiversity at different trophic levels (bacteria and nematodes), affecting their composition and activity after only two years following the establishment of the living mulch, which, in turn, can impact nutrient availability.

4.1. Living Mulches Effect on Soil Biodiversity

Living mulches modified the composition of soil bacterial communities when the diversity was assessed via amplicon sequencing data. The bacterial genetic biodiversity of the orchard described at the phylum level, with a clear dominance of three phyla (Proteobacteria, Actinobacteriota, and Acidobacteriota), was similar to that of other studies performed in apple orchards [37,38] regardless of the living mulch treatment applied. The soil bacteria microbiome associated with *F. vesca* was enriched with microorganisms, which are either described as endosymbionts of AMF (e.g., *Candidatus Glomeribacter*) [39] or present in the earthworm gut microbiome (e.g., *Candidatus Lumbricinola*) [40] as well as microorganisms identified in various soil environments, like *Psychrobacter* [41] or *Butti-
auxella* [42,43], suggesting that the use of *F. vesca* as a living mulch could affect the soil environment at different levels of the trophic network. Taxa specific only to *A. vulgaris*, e.g., *Corallococcus*, *Patescibacteria*, or *Geminicoccaceae*, have been rarely explored [44–46]; thus, their importance and functional role in orchard soil environments need further study. Bacteria taxa characteristic to the *M. x piperita* soil microbiome are poorly known; however, some of them, like *Micromonospora*, are known to colonize plant rhizosphere, particularly N-fixing species [47], have already been isolated from mint species [48], allowing us to hypothesize a specific plant–bacteria association or the selection of this taxa by the living mulch species that could be useful in terms of nutrient cycling or plant protection. Indeed, endophytic colonization by this genus was found to improve the growth of *M. x piperita* and to modify the synthesis of (R)-pulegone [49,50], a terpene that shows high control capacity as a volatile against different plant pests [51].

Plant pathogens like *Erwinia* [52] or *Acidovorax* [53] found only in the soil with natural cover (control) may lead to the conclusion that the living mulches may reduce the presence of these pathogens or that the natural cover could favor their occurrence. This result could provide evidence that the increased soil microbial diversity following the introduction of the selected living mulches may also lead to additional specific ecosystem services. Even though no fire blight symptoms were observed during the trial, a deeper analysis in this regard with metagenomic, metatranscriptomic, or even metabolomic approaches could shed light on such an effect.

Considering the metabolic data, it is noteworthy that the activity was higher in the bacterial microbiome associated with *F. vesca* compared to that of the other two living mulch species and natural soil cover. In addition, the bacterial population associated with *A. vulgaris* and *M. x piperita* used a smaller number of compounds as a carbon source compared to *F. vesca*. Such modifications could be related to the process of soil microbiome selection by the living mulch species, which is probably mediated by the specific secondary metabolites or essential oils released by the root systems into the soil environment [54–57]. On the other hand, the bacteria population of all living mulches was similarly active toward compounds reported as constituents of plant root exudates like L-asparagine, L-serine, L-arginine, phenylethylamine, D-malic acid, or 4-hydroxy benzoic acid [58]. Therefore, they may be common to generic root exudates also released by the tested species. However, D-xylose, also commonly found in root exudates [59,60], and D-cellobiose or β -methyl-D-glucoside, were differentially metabolized by the bacteria microbiome of the three living mulch species, with that from *F. vesca* presenting the highest metabolism potential for

D-xylose. The ample potential to exploit different C sources and high metabolism activity expressed by the bacterial microbiome associated with the soil of *F. vesca* could thus be considered a possible beneficial effect of this living mulch on the transformation of soil organic matter, which is particularly useful in organic orchards management. The capacity of the bacterial microbiome to exploit D-cellobiose or β -methyl-D-glucoside as C sources was positively related to the abundance of *Cellulomonas* ($\rho = 0.63$), which is known to degrade these compounds [61], and *Luteolibacter* ($\rho = 0.59$), which acts as a secondary consumer of cellulosic carbon co-occurring with primary degraders [62].

Several studies have shown that soil bacterial composition can be modified via agronomic practices or external drivers [63,64], but in our case, it is difficult to mechanistically explain the observed correlation between diversity and function. Nevertheless, it has also been shown that simple NGS analysis can bias the analyses of the function [65] as it also includes sequences from dead or dormant state cells or extracellular DNA [66], making it feasible to hypothesize that the discrepancy observed in the evaluation of the bacterial biodiversity could be accounted to these biases.

The living mulches did not affect the overall number of nematodes as it was similar to the number seen in natural cover, but in some cases, they modified the structure of the population. The bacteria feeders were the most abundant trophic group, composing between 48.5 and 75.5% of the community, which is common for various terrestrial environments [67]. The limited presence of predatory or omnivorous nematodes representing higher trophic levels is also a common feature of diverse soil environments [68]. However, mulching with *F. vesca* induced the highest share of bacteria feeders and the lowest number of herbivores and fungal feeders, especially mulching with *A. vulgaris*. The observed changes were mainly related to increased *Aphelenchoides* abundance in *A. vulgaris*, although all three living mulch species are known to be hosts of this genus [69]. However, it is worth mentioning that according to the classification implemented in the NINJA tool [70], which is widely used and cited in publications concerning the biological monitoring of nematodes, *Aphelenchoides* and the related *Aphelenchus* and *Ditylenchus* genera are assigned to the fungal feeder group and not to herbivorous species, thus making it possible to differently interpret the observed changes. The reduced abundance of *Paratylenchus* in the living mulch species compared to the natural cover could derive from the observed modification of weed density and biodiversity [11]. *Amaranthus*, *Hypochaeris*, *Polygonum*, *Rumex*, *Stellaria*, and *Trifolium* were the less abundant weed genera in the three living mulch species treatments, which may have reduced the host availability for polyphagous plant-parasitic nematodes like *Paratylenchus* [71]. The absence of soil disturbance in the natural cover could have accounted for the higher presence of predators and omnivores compared to the living mulches [72].

4.2. Living Mulches Impact on Soil Nutrient Elements Availability and Cycling

The modifications of the chemical characteristics of the soil, namely the pH and availability of some nutrients, observed in the soil mulched with the officinal plants could be the effect of both plant growth and microbial activity but also the result of season-dependent changes in plant uptake and microbial activity (also derived from the fungal population). The observed decrease in soil pH with the living mulches could be ascribed to the higher bacterial activity (i.e., respiration) observed with Biolog EcoPlates but also to a higher release of root exudates by the living mulch species compared to natural cover. The balance between microbial activity and plant uptake has been found to shape the availability of ions (e.g., Ca^{2+} , K^{+} , and NO_3^{-}) [73,74], which is also related to electrical conductivity (salinity) and pH as well [75]. The modification of the soil pH could have also affected the availability of P, one of the least mobile macroelements and a limiting factor for plant growth [76,77]. Soil covered by *F. vesca*, characterized by higher bacterial activity and the presence of genera associated with mycorrhizal fungi, which are known for their P solubilization capacity, also showed the highest content of phosphorus. Even though this could be the result of lower P requirements of *F. vesca* in comparison to the other

living mulch species due to smaller plant biomass [12] or other phenological characteristics (e.g., flowering time, length, and intensity), the impact of *F. vesca* in this regard could be considered a specific additional positive effect (ecosystem service) of this species.

The low level of nitrogen forms in spring, which is typical of this period when the intensive vegetative growth of the apple trees occurs [78], was not affected by the high activity of the bacteria population found in the living mulches, which is likely due to the additional uptake of these plants. However, later in the season, the concentrations of nitrogen increased, which may be a result of the increased microbial activity induced by living mulches and the mineralization of organic matter, eventually combined with reduced plant requirements. In the case of *A. vulgaris*, it is worth mentioning that the removal from a grassland of a species close to it (*A. monticola*) strongly affected the bacterial community, resulting in a decreased rate of plant litter decomposition and soil respiration [79].

The K levels of the soil proved to be the parameter that was most dependent on the living mulches and species-specific: *A. vulgaris* reduced the K content of the soil, which could suggest that the long-term cultivation of this species as a living mulch may lead to competition with apple trees for this element. On the other hand, *M. x piperita* and *F. vesca* increased the availability of K compared to natural cover. Several bacteria species (e.g., *Pseudomonas*, *Burkholderia*, *Acidithiobacillus*, *Bacillus*, and *Paenibacillus*) have shown K solubilization capacity from minerals such as mica, illite, muscovite, biotite, and orthoclases [80,81], increasing K availability up to 15% [82]. From our analysis, *Pseudomonas* was significantly enriched by both species in comparison to the control, *Bacillus* was increased in mint and *Paenibacillus* was slightly enriched in mint, while *Burkholderia* was lower in *F. vesca* than in the control. However, the impact could have derived from their activity (protein expression) and not directly related to their abundance. Moreover, our previous study performed within the same experimental orchard showed that these living mulches did not affect the NPK content in leaves or apple tree root development [12], which, together with the results of the current study, suggests no evidence of immediate competition between the tree and the living mulches for soil nutrients resources.

4.3. Impact of Living Mulches on Interactions between Soil Microorganisms

The potential dependencies between the different soil biodiversity, metabolic, and chemical parameters found with the dataset gathered in this study partially confirmed the results of previous studies. A positive correlation between the abundance of *Pratylenchus* and the abundance of *Burkholderia*–*Caballeronia*–*Paraburkholderia* is supported by the results of soybean infestation with *P. brachyurus*, which also increased *Burkholderia* and *Paraburkholderia* abundance in the soil [83]. Interestingly, infestation of the same plant species with a root-knot nematode—*Meloidogyne*—increased the frequency of *Rhodanobacter* [83], a species that is positively correlated with the abundance of *Pratylenchus* in our trial. Most studies have focused on plant parasitic nematode genera typical of vegetable crops, which were not found in our orchard (e.g., *Globodera*, *Meloidogyne*, or *Heterodera*). Nevertheless, some microbial taxa associated with these nematodes, including *Bradyrhizobium* and *Nitrospira* [84–87], also showed positive correlations with other species, like *Coslenchus*, *Filenchus*, or *Aphelenchoides*, which are present in the apple orchard. Interestingly, the correlations discovered in terms of *Filenchus* and *Aphelenchoides* largely overlap and include different microbial and chemical parameters even though they belong to different trophic groups [32]. An interesting bacteria taxon, which could be further explored as a potential novel biological active agent against nematodes, is *Devosia*. It is a plant growth-promoting bacterium [88], which was negatively correlated with the *Meloidogyne* population [83] and, according to our analyses, also with *Filenchus* and *Aphelenchoides* abundance.

The majority of the probable associations between bacteria and nematodes need further clarification and verification as many taxa belonging to the identified core bacterial microbiome of the apple orchard belong to newly known or identified taxa and are frequently present only in metagenomic datasets [89–91]. However, it is worth mentioning that some of them showed similar negative correlations with more than one plant-parasitic

nematode species, raising the possibility of their application in plant protection or as markers of soil health status. Nonetheless, the observed relations could also be the result of the complex functional interactions between the plant and the soil life web [92].

5. Conclusions

The introduction of multifunctional living mulches based on officinal plants induced changes to the soil genetic and functional biodiversity of bacteria and nematode populations, which resulted in modifications to the chemical properties of the soil. These modifications could deliver ecosystem services related to nutrient availability and plant protection, which can directly impact major crops. These services are particularly relevant to organic orchards due to the limitations in using external inputs (both fertilizers and pesticides), making this knowledge suitable to promote the use of such living mulches in practice. However, the changes noted in relation to most of the bacterial taxa identified in the study between the treatments are still limited and based only on the individual characterization of each taxa. A deeper view of their functioning and interactions with other soil-living organisms would be needed to better exploit the soil biodiversity through sustainable practices in soil management.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture14020293/s1>, Figure S1. Rarefaction curves showing observed species richness in soil samples collected from apple orchard with different living mulch species. The violet dotted line represents the sequencing depth used for data analysis; Table S1. Soil chemical parameters during 2022 vegetative season. Different letters show statistically significant differences between treatments for $p \leq 0.05$; Table S2. General sequence information; Table S3. Relative abundance of the main phyla (>1%) across soil microbiome associated with living mulch species or natural cover. Different letters show statistically significant differences between treatments for $p \leq 0.05$; Table S4. Frequency of occurrence (%) of the identified taxa in soil samples assigned to different treatments; Table S5. Spearman correlation matrix of selected nematodes genera abundance, core microbiome abundance, microbial activity and soil chemical properties. Only significant ($p < 0.05$) values are shown.

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