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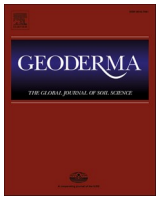

Unraveling the importance of top-down predation on bacterial diversity at the soil aggregate level

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ABSTRACT

The soil microbiome is dynamically structured at the local soil aggregate level by a combination of bottom-up and top-down processes. The soil microbiome is structured at the local soil aggregate scale by a dynamic interplay of bottom-up and top-down processes, yet less attention has been given to the latter (e.g., predation). We aimed to identify distinct groups of predators (protists and nematodes) and prey (bacteria) to determine the effect of predation on microbial gene abundances associated with carbon and nitrogen metabolisms. We partitioned soil aggregate size at three distinct levels to distinguish potential differences in predator–prey microbe interactions that take place at the soil micro-structure level. Our results revealed that the bacterial diversity and the abundance of protists were significantly higher in microaggregates than in macroaggregates. Correlation analysis, structural equation modeling, and co-occurrence networks suggested that predation by protists and nematodes impacted the diversity (Shannon index) and stability (average variation degree) of soil bacterial community, with a more pronounced effect on the bacterial community in soil macroaggregates than in smaller microaggregates. Compared to microaggregates, the higher frequency of predation within macroaggregates was found to promote faster microbiome turnover with direct implications for the abundance of genes involved in carbon and nitrogen metabolisms. Lastly, we also studied the importance of predation as a mechanism promoting bacterial diversity using field and microcosm studies, with a specific focus on the dominant bacterivorous nematode *Protorhabditis*. We addressed the influence of top-down processes on soil microbiome diversity and modulation of the genetic potential of genes involved in carbon and nitrogen metabolisms. Our study provides evidence for the importance of predation impacting the diversity and stability of soil bacterial communities. In addition, it shows that predation alters the abundance of microbial genes associated with carbon and nitrogen metabolisms at the soil aggregate level.

1. Introduction

Understanding the importance of bottom-up and top-down processes modulating biodiversity and ecosystem functioning has long been a central theme in ecology. Increasing evidence suggests that habitat structure influences top-down processes with a more pronounced effect on lower trophic levels than on high trophic levels, thus resulting in direct impacts on ecosystem stability and functionality (Soliveres et al., 2016; Barnes et al., 2020). We consider soils to have a complex hierarchical physical structure composed of microaggregates and macroaggregates, which provide different microhabitats for the top-down regulation of microfaunal grazers on microorganisms (Lehmann and Kleber, 2015; Rillig et al., 2017; Erktan et al., 2020). Soil spatial heterogeneity is characterized by the differences in microhabitat conditions, such as organic matter composition, pore-space networks, water and oxygen availability, and predation pressure (Davinic et al., 2012; Smith et al., 2014). Soil aggregates are dynamically formed through the chemical interactions of organic (transient, temporary, or persistent)

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binding agents leading to microaggregates (20–250 μm) and macroaggregates (>250 μm) (Tisdall and Oades, 1982). Soil macroaggregates often contain fresh and easily decomposable soil organic carbon (SOC), which accelerates microbial metabolisms associated with carbon and nitrogen transformations (Bailey et al., 2013). On the contrary, microaggregates are more often characterized by containing high concentrations of relatively stable and recalcitrant SOC. As such, these are often associated with lower levels of microbial activity (i.e., arylsulfatase and acid phosphatase) compared with macroaggregates (Davinic et al., 2012; Gupta and Germida, 2015). Therefore, the soil aggregate structure determines the spatial stratification of microbial communities and the mechanisms controlling them (Vos et al., 2013; Jiang et al., 2017; Bach et al., 2018).

Trophic interactions between protists, nematodes, and the soil microbiome are fundamental mechanisms associated with patterns of microbial community assembly and turnover (Rønn et al., 2012). Predation by phagotrophic protists and by bacterivorous and omnivorous-predatory nematodes are essential for the maintenance of the diversity of soil bacterial communities (Chesson and Kuang, 2008; Geisen, 2016). Most importantly, the access of bacterivorous and omnivorous-predatory nematodes to their prey is constrained by the soil pore volume that defines the local distribution of space within the soil matrix. For instance, macroaggregates with large pore sizes of > 100 μm can host large-bodied protists and nematodes (Rønn et al., 2001), which is shown to generate stronger predation pressure on prey (i.e., microbial taxa) when compared to the occurrence of this trophic interaction within microaggregates (Briar et al., 2011).

Soil microorganisms are key players in the dynamics of soil carbon (C) and nitrogen (N) cycling, both of which directly affect ecosystem functioning and productivity (Bardgett and van der Putten, 2014). Understanding the mechanisms that influence microbial diversity is of fundamental ecological importance for predicting and harnessing microbiome stability and functionality. In particular, higher bacterial diversity and species interactions have been reported to positively influence C and N metabolisms within the soil microbiome (Delgado-Baquerizo et al., 2016). While most of our knowledge linking biodiversity with ecosystem functioning has been derived from studies focusing on single trophic levels, embracing a food web approach that explicitly incorporates trophic interactions is shedding new light on how complex and dynamic multi-trophic level interactions affect community properties (Barnes et al., 2018). Multi-trophic interactions have been increasingly considered as an important biotic mechanism mediating microbial community assembly with impacts on soil respiration rates, carbon metabolism, and net N mineralization (Guan et al., 2018; Liu et al., 2019). Of particular importance, the extent to which multi-trophic interactions affect bacterial community structure and turnover is still unclear, with direct implications for C and N transformation (Hines and Gessner, 2012; Guerra et al., 2020).

Here, we studied the occurrence of microbe-microfauna predatory interactions in three soil aggregate sizes and determined the role of predation as a top-down process in structuring the bacterial community diversity and causing variation among genes associated with C and N cycling dynamics. To do so, we profiled the bacterial, protist, and nematode communities in three soil aggregate sizes under four manure treatments, and explored the microbial genes associated with C and N metabolisms using metagenomics. Using these combined observations, we focus on testing the following hypotheses: (1) the diversity and composition of the bacterial community and the composition of predators (protists and nematodes) vary across soil aggregate sizes and manure treatments; (2) predation by protists and nematodes results in an increase of the diversity and stability of the bacterial community; and (3) predation exerts an impact on the relative abundance of microbial genes associated with C and N metabolisms.

2. Materials and methods

2.1. Description of the long-term field experiment

The long-term fertilization experiment was conducted at the Yingtan National Agroecosystem Field Experiment Station (The Chinese Academy of Sciences) (28°15′20″N, 116°55′30″E) in Jiangxi Province, China. The experimental site is located at a typical subtropical climate with a mean annual temperature of 17.6 °C and precipitation of 1,795 mm. The soil is classified as Ferric Acrisol according to the classification system of the Food and Agricultural Organization (FAO). The long-term field experiment followed a completely randomized design with four manure treatments (three replicates), including (1) control - no manure amendment (M0), (2) low amount of manure amendment (i.e., 150 kg N ha^{-1} y^{-1}) (M1, 15,528 kg of manure ha^{-1} y^{-1}), (3) high amount of manure amendment (i.e., 600 kg N ha^{-1} y^{-1}) (M2, 62,112 kg of manure ha^{-1} y^{-1}), and (4) high amount of manure amendment (i.e., 600 kg N ha^{-1} y^{-1}) (62,112 kg of manure ha^{-1} y^{-1}) with lime application (i.e., 3,000 kg $Ca(OH)_2$ ha^{-1} $3y^{-1}$) (M3). This experimental system was set up in 2002, containing 12 concrete plots of 2 m length × 2 m width. On average, manure amendments consisted of pig manure that contained 386.5 g kg^{-1} of total carbon, 32.2 g kg^{-1} of total nitrogen (measured on a dry matter basis), and 70% of water content. All the plots were annually planted with corn as a monoculture from April to July and remained bare without planting between August and March. Corn grain in each plot was harvested by hand, i.e., manual picking and shucking. No management practice has been performed at these plots except regular manual weed removal. Weeding carefully by hand results in the complete removal of the weeds (including their roots), causes minimum soil disturbance, and eliminates the potential negative effects of herbicides on the bacterial, protist, and nematode communities.

2.2. Soil sampling and determination of soil physicochemical properties

Soil samples used for the evaluation of bacterial, protist, and nematode communities were collected from each plot after the harvest in late July 2014. We collected samples at a depth of 0–15 cm from five cores (5 cm diameter) using a zigzag sampling procedure, and then reduced the soil volume by the quartering method to ensure a total of 1 kg of soil samples. All collected samples were placed in sterile plastic bags and immediately transported on ice to the laboratory (<24 h). Fresh field soils were passed through a 4 mm sieve to remove rocks and plant debris, and then homogenized for aggregate fractionation. The soils were sieved at field moisture (<10%) and manually fractionated through a series of two sieves (i.e., 2000 μm and 250 μm) to obtain three aggregate sizes: large macroaggregates (>2000 μm; LA), small macroaggregates (250–2000 μm; SA) and microaggregates (<250 μm; MA). In brief, the fresh soil was placed on a 2000 μm sieve, and then horizontally sieved for 2 min. The material passing through a 2000 μm sieve was transferred to a 250 μm sieve for further fractionation. Each aggregate fraction was subdivided into two subsamples (150 g each) and subjected to the determination of soil chemical properties and the analysis of the bacterial, fungal, protistan, and nematode communities.

Soil pH was measured by a glass electrode with water:soil ratio of 2.5:1 (v/w). Soil organic carbon was determined by wet digestion using the potassium dichromate method (Nelson and Sommers, 1996). Total nitrogen and available nitrogen were determined using the micro-Kjeldahl method and the alkaline hydrolysis diffusion method, respectively (Bremner, 1996; Mulvaney, 1996). Ammonium and nitrate were extracted with 2 M KCl and detected on a continuous flow analyzer (Skalar, Breda, Netherlands). Total phosphorus was digested with HF − $HClO_4$ and available phosphorus was extracted with sodium bicarbonate, respectively, and determined using the molybdenum-blue method (Olsen et al., 1954; O'Halloran and Cade-Menun, 2007). Total potassium was digested with HF − $HClO_4$ and available potassium was extracted with ammonium acetate, respectively, and quantified using an atomic

absorption spectrophotometer (Kanehiro and Sherman, 1965). The results of soil edaphic properties are shown in detail in our previous studies (Jiang et al., 2017).

2.3. Gene target amplicon sequencing and processing

Total soil DNA in each sample was extracted from 0.5 g of initial material using the PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA), following the manufacturer's instructions. The extracted DNA samples were quantified using an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). To profile the bacterial and protist communities, we used the primer sets 515F/907R (Kuczynski et al., 2012) and TAReuk454FWD1/TAReukREV3 (Stoeck et al., 2010), targeting the V4 − V5 region of the 16S *rRNA* gene and the V4 region of 18S *rRNA* gene, respectively. Polymerase chain reaction (PCR) mixtures (20 μl) consisted of 2 μl of 10 × reaction buffer, 0.25 μl of each primer (10 μM), 2 μl of 2.5 mM dNTPs, 10 ng template DNA, and 0.4 μl FastPfu Polymerase. PCR conditions were as follows: 3 min of initial denaturation at 95 °C, followed by 40 cycles of 95 °C for 30 s, 60 °C for 5 s, and at 72 °C for 34 s, with a final extension of 72 °C for 10 min. An equal amount of PCR products from each sample was pooled into a single tube and subjected to paired-end 2 × 300 bp sequencing. Sequencing of the 16S rRNA and 18S rRNA amplicons was performed on an Illumina MiSeq platform (Illumina, San Diego, CA, USA).

Raw sequences were processed using the Quantitative Insights into Microbial Ecology (QIIME, version 2) pipeline (Bolyen et al., 2019). Illumina sequences that fully matched the barcodes were quality trimmed, demultiplexed, and further subjected to processing. The assembled reads were processed using chimera detection in UCHIME (Edgar et al., 2011) and clustered into operational taxonomic units (OTUs) at 97% of nucleotide identity (Li and Godzik, 2006). Taxonomical assignments were performed against the Silva database (version 138) for bacteria (Li and Godzik, 2006), and the Protist Ribosomal Reference database (PR2, v4.12) for protists (Guillou et al., 2013). We removed sequences belonging to Streptophyta, Rhodophyta, Metazoa, and Fungi for the protist OTU table. Obtained OTU tables of each organismal type were rarified at an equal depth before statistical analysis for bacterial (47,384 reads) and protist (6,080 reads) communities, respectively. We further assigned the protistan OTUs into different functional groups according to their nutrient-uptake mode, including phagotrophs, phototrophs, parasites, and saprotrophs (Xiong et al., 2018).

2.4. Metagenome sequencing and analysis

We subjected a total of 36 soil samples (4 manure treatments × 3 replicates × 3 aggregate fractions) to shotgun metagenomics. For each sample, 1 μg of purified soil DNA was used to generate sequencing libraries using the Nextera XT DNA library prep kit following the manufacturer's protocols. Metagenome sequencing was carried out on an Illumina HiSeq 4000 platform with a 150-bp read length to a targeted data size of 10 Gb. Raw reads were trimmed using the software Sickle to remove reads with average quality scores below Q20 Phred score and sequence length < 50 bp. Open reading frames (ORFs) were detected using MetaGeneMark (version 3.26) (Besemer and Borodovsky, 1999). Functional annotation was performed by comparing the quality-filtered reads against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa and Goto, 2000) using DIAMOND (version 0.9.29) (Buchfink et al., 2015). The number of each ORF was calculated as the number of base pairs mapped onto the corresponding scaffold region divided by the length of the ORFs. Each gene abundance was computed as the average score values of matched ORFs. Gene abundances were summed up to a total of 4,150 KEGG Orthology (KO) genes that were KEGG defined functional units. We focused our annotation on KEGG pathways involved in carbon and nitrogen transformations. In KEGG, carbon metabolism is involved in six distinct pathways, as follows: carbohydrate metabolism, citrate cycle (TCA cycle), glycolysis, galactose metabolism, pentose phosphate pathway, and pyruvate metabolism. Nitrogen metabolism involves distinct transformations, including nitrogen fixation, nitrification, denitrification, anaerobic ammonium oxidation, nitrogen assimilation, and ammonification.

2.5. Determination of nematode types and abundance

Nematodes were extracted from 100 g of each subsample using a modified Baermann funnel method (Barker, 1985). Four functional groups of nematodes, including bacterivores, fungivores, plant parasites, and omnivores-predators, were identified based on known feeding habits, stoma, and esophageal morphology (Yeates et al., 1993, Bongers and Bongers, 1998). Nematode populations were counted and expressed as nematode numbers per 100 g of dry soil. The number of nematodes was counted using a stereoscopic microscope at 40 × magnification. In addition, at least 100 individuals within each sample were identified to the genus level to determine the nematode community, using an inverted compound microscope at 400 × or 1000 × magnification. The result of nematode assemblages across manure application and aggregate fractions is shown in detail in our previous study (Jiang et al., 2017). The predation pressure of nematodes on bacteria was calculated as the ratio between the number of nematodes and bacterial biomass (Mathisen et al., 2016).

2.6. Microcosm experiment

The predation effect of nematodes on the bacterial diversity was performed in a microcosm experiment as previously described (Jiang et al., 2017). Briefly, soils from each plot were sterilized by acute gamma irradiation at 40 kGy (Buchan et al., 2012). Bacterial suspensions of fresh soils were prepared by passing through 1.0 μm pore-size Millipore filters (Millipore, Bedford, MA, USA) that eliminate nematodes and other small eukaryotes. The most dominant bacterivorous nematode (*Protorhabditis*) isolated from the field experiment was cultivated in a nematode growth medium at 28 °C by feeding on *Escherichia coli* OP50. The nematodes were washed five times with sterile distilled water to minimize the effects of *E. coli* contamination in the experiment. For the microcosms, 50, 150, 500, and 600 *Protorhabditis* individuals were added into 100 g of soil per pot based on their density obtained from the M0, M1, M2, and M3 treatments, respectively. The microcosms were incubated in the dark at 28 °C, and soil moisture was maintained at 25% (*w/w*). A nematode-free control was set up in triplicate to test for potential nematode contamination. Soils were sampled 30 days after inoculation, and then separated into three aggregate fractions later subjected to nematode analysis, as well as the bacterial community assessments.

2.7. Quantification of bacteria in soil aggregates and inside nematodes

Bacterial abundance in soil aggregates was analyzed based on the 16S rRNA gene by quantitative PCR on a CFX96 Optical Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA, USA), using the same primer set described above. Standard curves for the bacterial quantification were obtained using a dilution series (10^{-2} to 10^{-8}) of plasmid DNA containing the 16S rRNA gene fragment from pure cultures by cloning. PCR was performed in mixtures (20 μl) containing 10 μl 2 × SYBR Premix Ex Taq (TaKaRa), 0.2 μM of each primer, and 1 μl DNA template (1–10 ng). Quantitative PCR was run starting with the initial denaturation step at 95 °C for 3 min, followed by 40 cycles (with plate-reading) of 30 s at 95 °C and 45 s at 60 °C, then with a final melt curve step from 72 to 95 °C. The efficiencies of the PCR amplifications ranged from 92.8 − 105.7% with correlation coefficients $R^2 > 0.99$.

The most dominant bacterivorous nematode (*Protorhabditis*) can be identified based on its distinctive morphological characteristics. This specific nematode species was gently lifted to the water surface using the picking tool, and then collected and transferred to a 10 mM sterile

phosphate buffer (pH 7.0) under a dissecting microscope. These harvested bacterivores were introduced into 2% sodium hypochlorite solution for 30 s to avoid microbial interference on the body surface and then washed five times with sterile distilled water. To detect the predation of bacterivores on the bacterial community, 30 *Protorhabditis* individuals were collected and transferred into a 1.5-ml tube for DNA extraction under sterile conditions. Total DNA was extracted from nematodes using a DNeasy blood & tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The bacterial amplicon read abundances inside the bacterivorous nematodes collected from each subsample were determined as amplicon numbers of the 16S rRNA gene by quantitative PCR. No amplification was obtained in the final wash water, thus indicating the successful surface sterilization of the bacterivorous nematodes.

2.8. Statistical analysis

Two-way analysis of variance (ANOVA) was performed to test the influences of manure treatments and soil aggregates on the bacterial community, protist, and nematode communities, and the genetic potential of genes involved in carbon and nitrogen metabolism using Tukey's honestly significant difference (HSD) test in SPSS 23.0 (SPSS, Chicago, IL, USA). Permutational multivariate analysis of variance (PERMANOVA) was conducted to estimate the impacts of manure treatments and aggregate fractions on the compositions of the bacterial, protist, and nematode communities using the 'adonis' function in the 'vegan' package in R statistical software (version 4.0.5) (Anderson, 2001; R Core Team, 2020). The stability of soil bacterial community composition was evaluated by average variation degree (AVD) (Xun et al., 2021). This metric was calculated using the deviation degree from the mean of the normally distributed OTU relative abundances. The variation degree for each OTU was calculated using the following equation ($|a_i| = \frac{|x_i - \bar{x}_i|}{\delta_i}$), in which a_i is the variation degree for an OTU, x_i is the rarefied abundance of the OTU in one sample, $\bar{x}_i$ is the average rarefied abundance of the OTU in one sample group, and δ_i is the standard deviation of the rarefied abundance of the OTU in one sample group. The AVD values were calculated using the following equation ($\mathrm{AVD} = \frac{\sum_{i=1}^{n} \frac{|x_i - \bar{x}_i|}{\delta_i}}{k \times n}$), in which k is the number of samples in one sample group, n is the number of OTUs in each sample group. The lowest average variation degree value indicates the highest community stability.

Correlations between bacterial taxa, protists, and nematodes were run using Spearman's rank correlation test. We conducted network analysis to evaluate the influence of soil aggregate size on the network structure of bacteria-protist-nematode correlations. Co-occurrence networks in three aggregate fractions were obtained by calculating all possible pairwise Spearman's rank correlations between pairs of taxa. The OTUs detected in>75% of the soil samples at the same fraction were kept in the correlation matrix. A valid co-occurrence was considered statistically robust when the correlation coefficient (r) was > 0.8 or < -0.8 and the P-value was < 0.01. The P-values < 0.01 were adjusted using the Benjamini-Hochberg procedure to reduce false positives (Benjamini and Hochberg, 1995). Co-occurrence patterns were calculated and visualized using Cytoscape (Lopes et al., 2010). Random forest modeling was conducted using the randomForest package (Liaw and Wiener, 2002), and the model significance and predictor importance were determined using the A3 (version 1.0.0) (Fortmannroe, 2015) and rfPermute (version 2.1.81) (Archer, 2016) packages in R statistical software (version 4.0.5) (R Core Team, 2020). Structural equation modeling (SEM) was used to model the direct and indirect effects of abiotic and biotic factors on microbial genes associated with carbon and nitrogen metabolisms. Soil properties included pH, soil organic carbon (SOC), and total nitrogen (TN). The bacterial community included biomass, diversity (Shannon index), composition (the first principal coordinates), and stability (average variation degree). A path indicated the partial correlation coefficient and could be interpreted as the magnitude of the relationships between two parameters. Latent variables were used to integrate the effects of multiple conceptually related observed variables into a single-composite effect, aiding the interpretation of model results. All interactions and elements of the model were retained in the final model. SEM was conducted by the robust maximum likelihood approach using Analysis of Moment Structures (AMOS) 20.0 (Byrne, 2001). The SEM fitness was examined based on a non-significant chi-square test ($P > 0.05$), the goodness-of-fit index, and the root mean square error of approximation (Hooper et al., 2008).

3. Results

3.1. The impact of soil aggregates on bacterial diversity

Our results indicated that the Shannon index and Chaol richness of bacterial diversity significantly varied across manure treatments ($P < 0.001$) and aggregate fractions ($P < 0.01$). At the phylum/class/order levels, the MA fraction had a significantly ($P < 0.05$) higher diversity of Acidobacteria (order Acidobacteriales), Actinobacteria (orders Corynebacteriales, Frankiales, Micromonosporales, Propionibacteriales, Streptomycetales, and Streptosporangiales), Alphaproteobacteria (orders Rhizobiales, Rhodospirillales, and Sphingomonadales), Bacteroidetes (class Sphingobacteriia), Chloroflexi (orders Chloroflexales, Ktedonobacterales, and Thermogemmatisporales), Gammaproteobacteria (orders Pseudomonadales and Xanthomonadales), and Firmicutes (order Bacillales) compared with the SA and LA fractions (Fig. 1). The values of average variation degree of the bacterial community suggest a significantly ($P < 0.05$) greater stability in larger aggregate sizes (i.e., MA > SA > LA, values of 0.30 > 0.26 > 0.23, respectively) (Fig. 2A).

3.2. The impact of soil aggregates on protist and nematode community

Data on nematode communities across the manure treatments and aggregate sizes have been previously reported (see Jiang et al., 2017). The soil treatment with manure exerted a significant influence on the protist community, based on Bray-Curtis dissimilarity (PERMANOVA, $F = 26.2$, $R^2 = 0.68$, $P < 0.001$). These differences were also found to be significant for protists at different aggregate sizes ($F = 3.1$, $R^2 = 0.10$, $P = 0.008$). Overall, the protist community was dominated by five phylotypes, including Amoebozoa (42.4% ± 4.7%), Archaeplastida (25.8% ± 6.7%), Rhizaria (15.0% ± 1.2%), Stramenopiles (5.9% ± 1.0%), and Alveolata (5.3% ± 0.8%) (Fig. S1A). Together, the phagotrophic (79.5%) and phototrophic (18.1%) protists represented 97.6% of the functionally assigned protists (Fig. S1B − C). The average abundance of phagotrophs in the MA fraction (84.4%) significantly ($P < 0.01$) exceeded that in the SA (76.3%) and LA (77.9%) fractions. Similarly, the dominant phagotrophic taxa (i.e., Nolandida, *Flamella*, Mycetozoa-Myxogastrea, Euglyphida, and Colpodida) have similar abundances at different soil aggregate sizes (Fig. S1D − I).

3.3. The importance of top-down predation on soil bacterial community in soil aggregates

The inferred predation pressure of bacterivores and omnivores-predators on the bacterial community was estimated by the predator/bacteria ratio. This ratio was shown to be significantly ($P < 0.05$) higher in the LA fraction (25.7) than in the MA (14.5) fraction (Fig. 2B). The amplicon numbers of bacterial community inside *Protorhabditis* were significantly ($P < 0.05$) higher in the LA fraction (269.2 ± 42.4 copies per nematode) than in the MA fraction (204.9 ± 30.9 copies per nematode) (Fig. S2). Phagotrophic protists (i.e., Nolandida, Euglyphida, and Colpodida) ($r = 0.560$–0.721, $P < 0.001$ and $r = 0.690$–0.736, $P < 0.001$), as well as the bacterivorous (*Protorhabditis* and *Cephalobus*) ($r = 0.467$–0.734, $P < 0.001$ and $r = 0.605$–0.702, $P < 0.001$) and

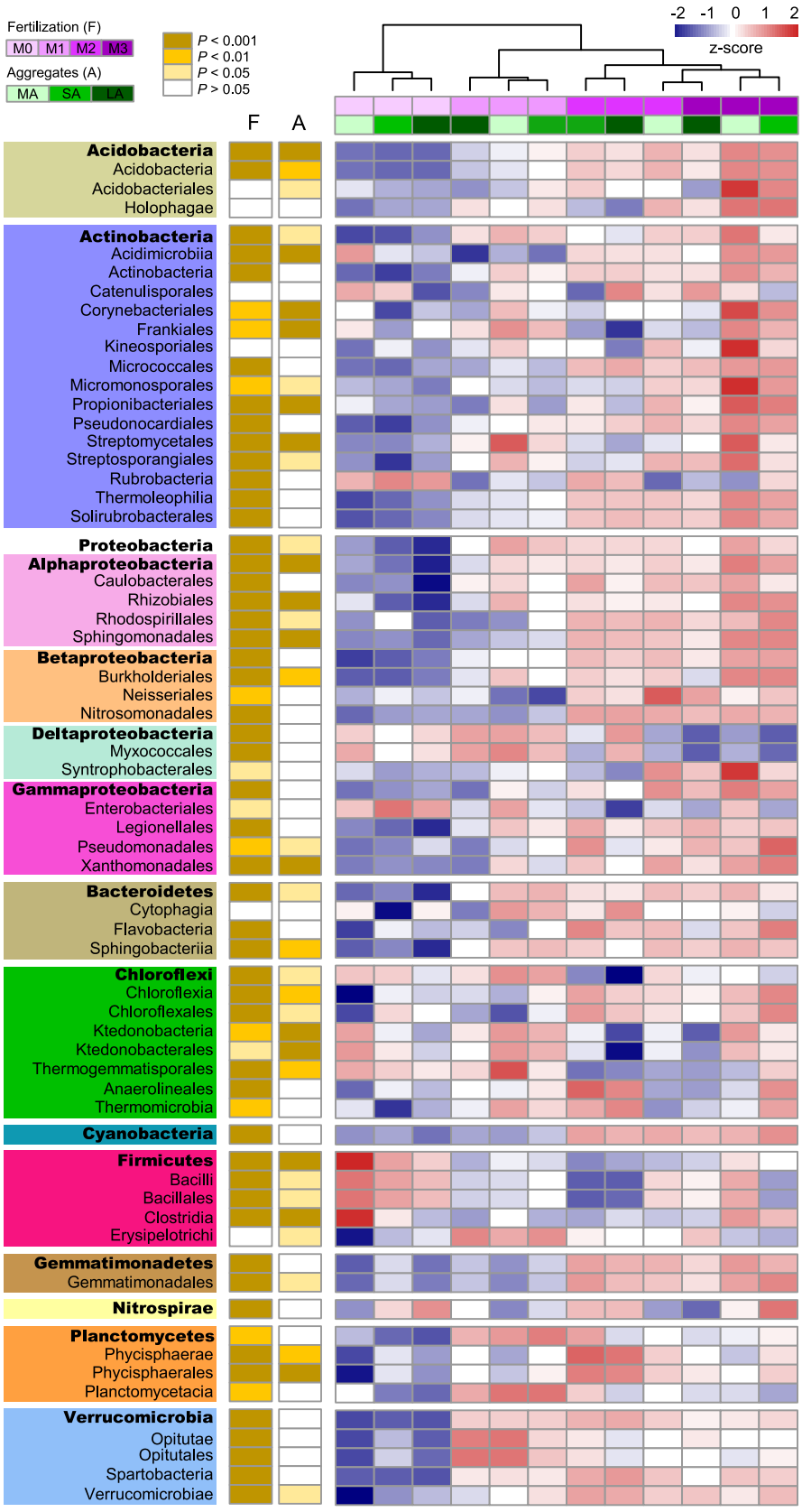

Fig. 1. Normalized diversity of total bacterial community reads across manure treatments and aggregate fractions. Heatmap displaying the Shannon index of dominant bacterial phyla/classes/orders across manure treatments (M0, M1, M2, and M3) and aggregate fractions (MA, SA, and LA). The dendrogram was based on hierarchical clustering using Pearson correlation coefficients for each pairwise comparison. The color scale is based on the normalized values of Shannon index of bacterial taxa using the z-score standardization method. MA, microaggregates; SA, small macroaggregates; LA, large macroaggregates; M0, no manure; M1, low manure; M2, high manure; M3, high manure and lime.

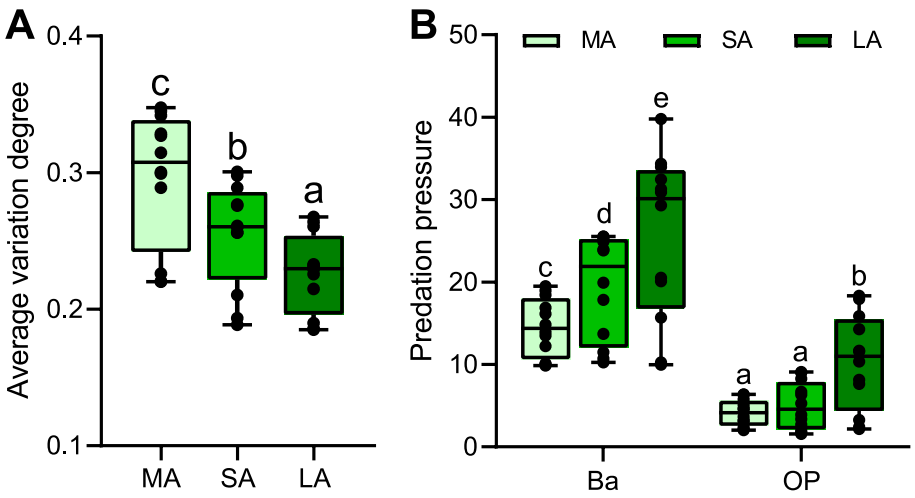

Fig. 2. Average variation degree ('stability') of total bacterial community (A) and inferred predation pressure of nematodes on bacterial taxa (B) across distinct aggregate fractions. Low average variation degree values indicate high stability. Predation pressure of bacterivorous (Ba) and omnivorous-predatory (OP) nematodes on the bacterial community was calculated as the ratio between the density of bacterivorous or omnivorous-predator nematodes and the bacterial biomass. Lines in boxes represent median, top and bottom of boxes represent first and third quartiles, whiskers represent 1.5 interquartile range, and dots represent single observations. Different lowercase letters indicate significant differences based on Tukey's HSD tests ($P < 0.05$). MA, microaggregates; SA, small macroaggregates; LA, large macroaggregates.

omnivorous nematodes (*Aporcelaimus* and *Mesodorylaimus*) (r = 0.490–0.509, $P < 0.001$ and r = 0.394–0.547, $P < 0.05$) were significantly correlated with the diversity of the bacterial community (Fig. S3). Furthermore, dominant groups within the bacterial community, phagotrophic protists, and bacterivores and omnivores were significantly associated with the diversity of major bacterial taxa, including Alphaproteobacteria (r = 0.441–0.808, $P < 0.01$), Betaproteobacteria (r = 0.529–0.794, $P < 0.001$), Bacteroidetes (r = 0.450–0.642, $P < 0.01$), Cyanobacteria (r = 0.502–0.891, $P < 0.01$), Gammaproteobacteria (r = 0.401–0.661, $P < 0.05$), Gemmatimonadetes (r = 0.480–0.809, $P < 0.01$), and Verrucomicrobia (r = 0.571–0.718, $P < 0.001$).

Co-occurrence network analysis further revealed potential patterns of associations between predators (protists/nematodes) and prey (bacterial taxa). Overall, the bacteria-protists-nematodes networks were found to vary greatly when accessing these communities across different soil aggregate sizes (Fig. 3). We found a greater number of correlations between groups of bacteria-protists-nematodes in larger aggregate sizes, ranging from 352 nodes and 19,433 correlations in the MA fraction to 455 nodes and 36,678 correlations in the LA fraction. Compared to the MA and SA fractions, protists and nematodes were strongly correlated with bacterial taxa belonging to the Alphaproteobacteria, Betaproteobacteria, Bacteroidetes, and Gammaproteobacteria in the networks in the LA fraction (Fig. 3A – C). In contrast, protists and bacterivorous nematodes showed significant (albeit weaker) relationships with Betaproteobacteria and Gemmatimonadetes in the bacterial community in the MA fraction.

3.4. The functional potential of soil microbiomes

Metagenomic analyses revealed that microbial genes involved in carbon and nitrogen metabolisms significantly differed – in terms of relative abundances – across manure treatments ($P < 0.001$), and soil aggregate fractions ($P < 0.001$) (Fig. 4). Overall, the relative abundance of genes associated with carbon and nitrogen metabolisms were significantly ($P < 0.001$) higher under high manure treatments (M2 and M3 treatments) compared to the M0 treatment (Fig. S4A – G). Specifically, the high manure treatments displayed significantly ($P < 0.001$) higher relative abundances of genes encoding citrate cycle (genes *icd*, *korA-oorA*, *sdhABCD-frdABCD*, *fumAB*, *acnA*, and *sucC* – 15.3% to 143.9%), pyruvate metabolism (genes *porG* and *pdhCD* – 46.8% to 60.5%), glycolysis (genes *gapA*, *gap2*, *pfkA*, *pyk*, *pgk*, and *fbaA* – 28.9% to 356.3%), galactose metabolism (genes *galEM* and *pmm-pgm* – 30.0% to 214.9.3%), pentose phosphate pathway (genes *talAB*, *prsA*, and *rbsK* – 12.1% to 65.6%), carbohydrate metabolism (genes *adh*, *yiaY*, and *melZ*

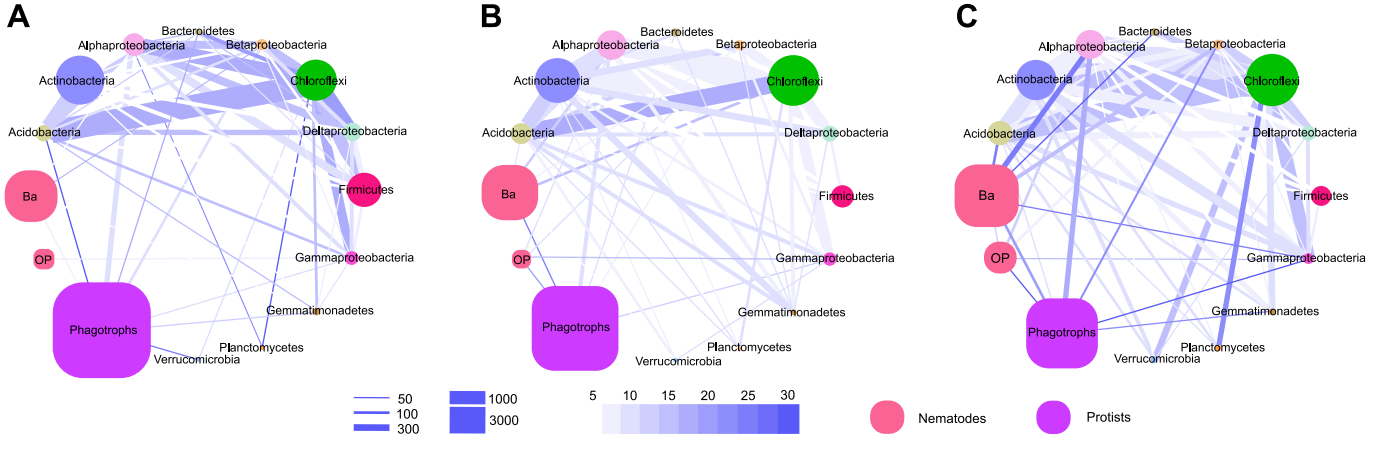

Fig. 3. Network co-occurrence analysis. Networks were built to depict correlations across protists, nematodes and bacterial dominant phyla in the MA (A), SA (B), and LA (C) fractions. Protists include phagotrophs, and nematodes include bacterivores (Ba) and omnivores – predators (OP). Spearman's rank correlations of the relative abundances of all individual species combinations were calculated between pairs of groups. The proportion of significant correlations was divided by the total number of possible correlations to the obtained significant correlations between two groups of species. Line width is proportional to the absolute number of correlations > 0.8. Line color and transparency are proportional to the strength of correlation coefficients, as indicated in the legend. The size of the nodes (circles and squares) is proportional to the relative abundance of taxa in the bacterial, protist, or nematode communities. MA, microaggregates; SA, small macroaggregates; LA, large macroaggregates.

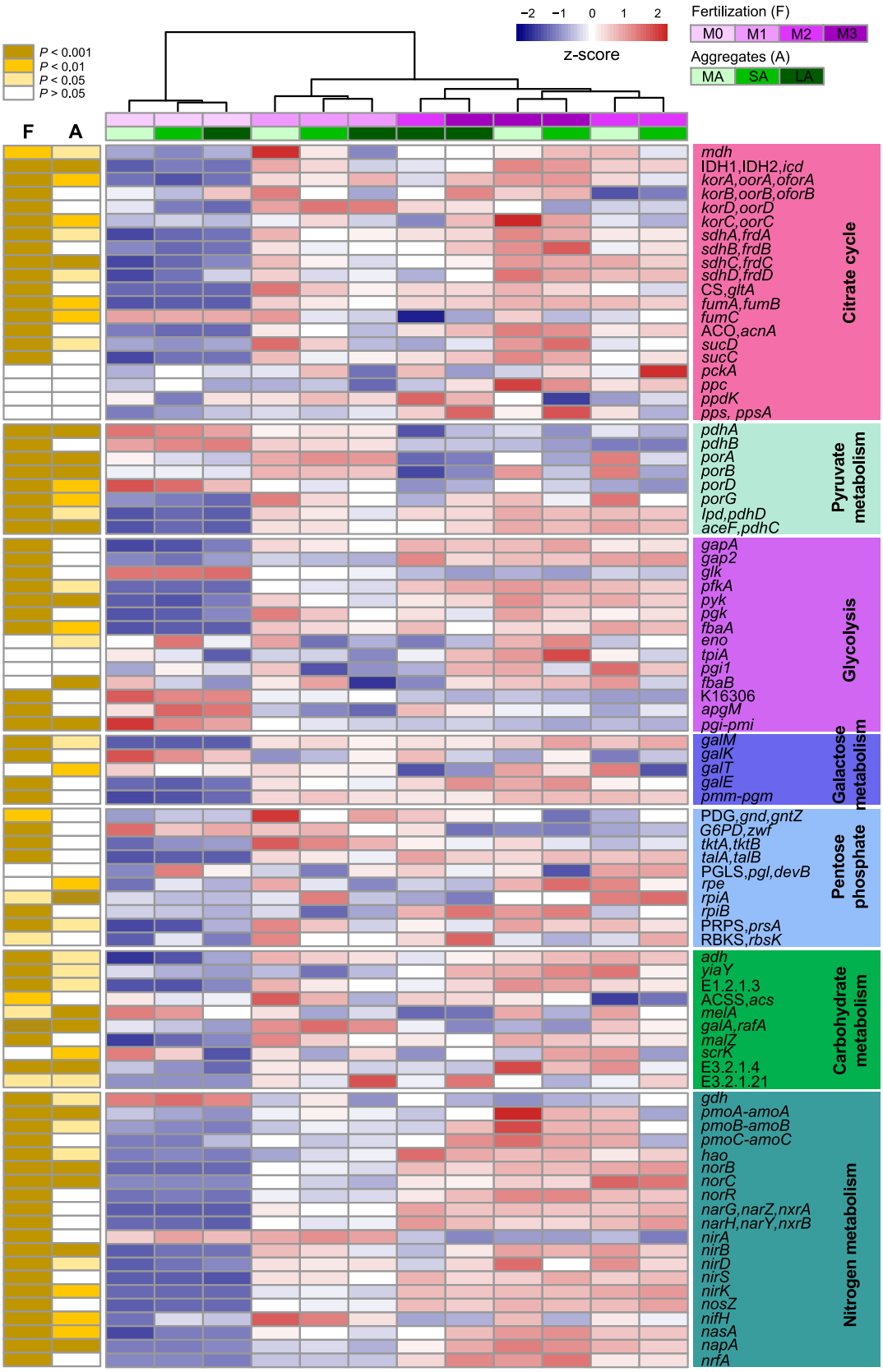

Fig. 4. Heatmap displaying the relative distribution of representative KO genes matching KEGG pathways. Color scale represents row normalized values. The dendrogram was based on hierarchical clustering using Pearson correlation coefficients for each pairwise comparison. MA, microaggregates; SA, small macroaggregates; LA, large macroaggregates; M0, no manure; M1, low manure; M2, high manure; M3, high manure and lime.

– 13.5% to 58.4%), and nitrogen metabolism (genes *amoAC-pmoAC*, *hao*, *norBCR*, *narGHYZ-nxrAB*, *nirBDSK*, *nosZ*, *nasA*, *napA*, and *nrfA* – 50.7% to 1766.7%). However, the relative abundance of genes encoding *pdhAB* and *porD* (pyruvate metabolism), *glk* and *pgi-pmi* (glycolysis), *zwf* (pentose phosphate pathway), *melA* (carbon metabolism), and *gdh* and *nirA* (nitrogen metabolism) followed the opposite trend.

The relative abundances of dominant pathway modules related to carbon metabolism and associated encoding genes were significantly ($P < 0.001$) higher in the MA fraction than in the LA fraction, as observed for the citrate cycle (genes *mdh*, *icd*, *korAC-oorAC*, *oforA*, *sdhACD-frdACD*, *fumABC*, and *sucD*) ($P < 0.05$), pyruvate metabolism (genes *pdhA*, *porABDG*, and *pdhCD*) ($P < 0.05$), glycolysis (genes *pyk*, *fbaAB*, *eno*, and *pgi-pmi*) ($P < 0.01$), galactose metabolism (genes *galM* and *galT*) ($P < 0.01$), pentose phosphate pathway (genes *rpe*, *rpiA*, and *prsA*) ($P < 0.05$), Calvin cycle (genes *rbcS*, *prkB*, and *fbp*) ($P < 0.05$), and carbohydrate metabolism (genes *adh*, *yiaY*, *melA*, *galA-rafA*, and *scrK*) ($P < 0.05$). Similarly, for the nitrogen metabolism, a significantly higher gene relative abundance was observed ($P < 0.001$) in the MA fraction compared to the LA fraction (Fig. S4H). This was evident by differences ($P < 0.05$) in the relative abundances of the genes *amoAB-pmoAB*, *hao*, *norBC*, *nirBDK*, *nifH*, *nasA*, and *napA*.

3.5. Integrating soil edaphic and biotic variables

Random forest modeling indicated that soil pH (6.9%, $P < 0.05$), soil organic carbon (7.3%, $P < 0.05$), total phosphorus (7.1%, $P < 0.05$), and total nitrogen (5.3%, $P < 0.05$) were the best edaphic variables explaining the variations in genetic potential of carbon and nitrogen metabolisms (Fig. S5). As for biotic variables, the biomass, diversity, and stability of the bacterial community were found to significantly ($P < 0.05$) correlate with the variations in genetic potential of carbon and nitrogen metabolisms, respectively. Furthermore, predation by bacterivores and omnivores-predators contributed significantly to changes in the genetic potential of microbiomes involved in carbon and nitrogen metabolisms (6.9% and 6.3%, $P < 0.05$). A similar influence was also found for the predation by phagotrophic protists (7.4%, $P < 0.05$) (Fig. S5).

At the aggregate level, SEM indicated that the potential impact of predation by nematodes on the biomass, diversity, and stability of the bacterial community was higher in the LA fraction ($r = 0.60$, $P < 0.001$) than in the SA ($r = 0.22$, $P > 0.05$) and MA ($r = -0.20$, $P > 0.05$) fractions (Fig. 5). In addition, predation by phagotrophic protists displayed more pronounced effects on the bacterial community in the MA fraction ($r = 0.78$, $P < 0.001$) than in the SA ($r = 0.58$, $P < 0.001$) and LA fractions ($r = 0.49$, $P < 0.01$). Our analysis revealed the predation by phagotrophic protists had a potential direct ($P < 0.001$) effect on increasing the genetic potential of genes involved in carbon and nitrogen metabolism by enhancing the absolute abundance of bacterial communities in the MA fraction (Fig. 5). In contrast, feeding by protists and nematodes in the LA fraction improved carbon and nitrogen metabolism via increasing bacterial abundances ($P < 0.001$), diversity ($P < 0.001$), and community stability ($P < 0.001$).

3.6. Microcosm validation experiment

After 30 days of incubation with the inoculated predator *Protorhabditis*, we found significant ($P < 0.05$) variation in the diversity and stability of bacterial community across the soil treatments (Fig. S6). The treatment amended with *Protorhabditis* resulted in significant increases in the diversity (Shannon index) of the bacterial community (ca. 5.2%) and decreases in the community stability (average variation degree, ca. 7.0%) in the LA fraction. Predation by *Protorhabditis* in the LA fraction had significantly ($P < 0.05$) positive effects on the diversity of the dominant bacterial groups compared to the SA and MA fractions, including Alphaproteobacteria ($\log_2$ fold change (FC) = 0.10–0.16, $P < 0.05$), Betaproteobacteria ($\log_2$FC = 0.10–0.19, $P < 0.01$), Bacteroidetes ($\log_2$FC = 0.18–0.46, $P < 0.01$), Cyanobacteria ($\log_2$FC = 0.12, $P < 0.05$), Gammaproteobacteria ($\log_2$FC = 0.28–0.52, $P < 0.01$), and Gemmatimonadetes ($\log_2$FC = 0.19–0.20, $P < 0.01$) (Fig. S7).

4. Discussion

The main goal of this study was to determine the extent to which top-down predation influences the bacterial diversity across fertilization

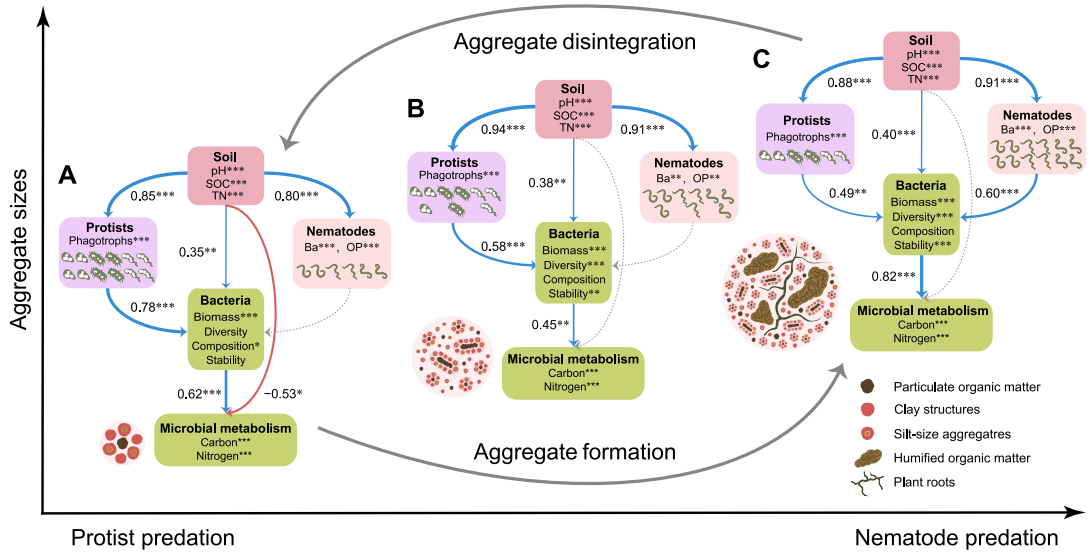

Fig. 5. Conceptual diagram displaying the bacteria-protist-nematode relationships, and how these biotic interactions affect microbial genes associated with carbon and nitrogen metabolisms across soil aggregate sizes. The different models display the direct and indirect relationships across soil properties, protists, nematodes, and total bacterial community metrics potentially influencing carbon and nitrogen metabolism. The structural equation modeling was built based on data acquired for the microaggregates (A), small macroaggregates (B), and large macroaggregates (C). Blue lines indicate positive correlations, and red lines indicate negative correlations. Protists include phagotrophs, and nematodes include bacterivores (Ba) and omnivores – predators (OP). The width of the arrows indicates the strength of significant standardized path coefficients. Paths with no significant coefficients are shown as gray lines. *$P < 0.05$, **$P < 0.01$, **$P < 0.001$.

treatments and soil aggregate sizes. We found the manure treatment to significantly impact soil bacterial, protist, and nematode communities. In fact, such impacts have been long acknowledged in agricultural systems, mostly by manure amendments shifting soil structure, chemistry, and biology (Liu et al., 2016; Xiong et al., 2018; Lupatini et al., 2019). However, we greatly expand on these previous findings by investigating the extent to which the diversity of the distinct bacterial community varies across three soil aggregate sizes, and how bacteria-protist-nematode interactions affect microbial genes associated with soil carbon and nitrogen transformations at different levels of soil aggregation.

4.1. Diversity of bacterial communities varies across soil aggregate sizes

Studies focusing on understanding taxonomical and functional differences in soil microbiomes have largely varied in the scale of sample collection and analysis. At a micro-scale, microbial populations are not equally distributed in the soil matrix but are rather located in patches within and outside soil aggregates (Rillig et al., 2017). Our results support the first hypothesis by showing that the structure and diversity of the bacterial community differ across distinct soil aggregate fractions and the implemented manure treatments. The abiotic (i.e., physico-chemical) variation in soil aggregates can impact bacterial community diversity. In brief, we found that the MA fraction containing higher amounts of nutrients likely supports a more taxonomically diverse bacterial community than the LA fraction, these include greater diversity levels of Acidobacteria, Actinobacteria, Alphaproteobacteria, Bacteroidetes, Chloroflexi, and Gammaproteobacteria. This result corroborates the idea that SOC constitutes an important resource for higher microbial population sizes and the diversity of local communities (Erktan et al., 2020). Our findings of higher diversities of Sphingomonadales and Sphingobacteriia taxa in microaggregates can likely be associated with the composition of available labile carbon driving niche differences across species. For example, the distinct concentrations of complex organic molecules (e.g., phenols and alkyls) across soil aggregates have been shown to select for different bacterial populations (Davinic et al., 2012). Moreover, Bach et al. (2018) suggest that the taxa Sphingomonadales, Sphingobacteriales and Acidobacteriales play an important role in microaggregate formation through active metabolisms involved in organic matter transformation. As such, our findings emphasize the need to better understand the importance of the diversity of these taxa in soil carbon sequestration at the aggregate level. However, it is worth noting that when soil aggregates are homogenized and sieved, the macroaggregates (LA fraction) are broken down into the microaggregates (MA fraction) and lose bacterial diversity.

4.2. Bacterivores predation promotes bacterial diversity in soil aggregates

Although recent findings have highlighted the importance of abiotic edaphic properties (soil physical and chemical characteristics) shaping trophic interactions in soils, the combined influence of soil aggregate sizes with the assembly and dynamics of distinct microbial populations have been rarely integrated into microbial food web ecology. In our study, the abundance of nematodes and protists as well as different trophic groups were unevenly distributed across different aggregate size fractions. Microbial predators, such as protists and nematodes, essentially depend on existing pore spaces as microhabitats to survive and access bacterial prey. Nematodes are known to move through the water-filled pores in the soil matrix with a neck diameter of 30–90 μm, while phagotrophic protists can often transit across smaller pores with a neck size > 5 μm (Rønn et al., 2001; Feng and Balkcom, 2017). In the case of bacterial prey, soil bacterial cells can inhabit small pores within microaggregates (<250 μm) with diameters of 0.8–3 μm, which can, in most cases, avoid predation by larger protists and nematodes (Wright et al., 1995). We showed that both manure treatments and soil aggregate sizes were related to variations in microbial predator–prey interactions. The predation by phagotrophic protists, and bacterivorous and omnivorous-predatory nematodes was found to directly affect the bacterial diversity. This aligns with the rising importance of top-down pressure on structuring the soil microbiome with implications on their functioning (Thakur and Geisen, 2019; Lucas et al., 2020).

Our data from the field and microcosm experiments corroborate findings in demonstrating that the occurrence of predators resulted in higher levels of diversity and stability of the bacterial community across the three soil aggregate sizes. This represents a top-down mechanism impacting bacterial diversity in soils. Besides, it is likely that the type and abundance of predators – associated with their feeding modes, prey preferences, and efficiency – can differentially impact the bacterial community. In this sense, it is worth noting that the ingestion of bacterial taxa by protists and nematodes depends not only on their habitat occupancy, but also on the size of their filtration apparatus, and their preference for distinct bacterial species (Foster and Dormaar, 1991; Jousset, 2011; Giometto et al., 2013). The more pronounced positive effect of nematode predation in macroaggregates might be a consequence of their higher density relative to microaggregates. The soil amendment with manure has been previously reported to increase the proportion of relatively large pores > 100 μm within the LA fraction (Jiang et al., 2017), which likely provides optimal conditions for the successful survival of large-sized protists and nematodes. The higher abundance of bacterivores in the LA fraction is associated with greater top-down predation pressure and higher consumption of bacteria. As such, predation by protists and nematodes contributes to community turnover, which alleviates competitive interactions between bacterial (prey) taxa, thus promoting species diversity and enhancing overall community stability.

Correlational associations between bacteria, protists, and nematodes in co-occurrence networks imply potential direct or indirect trophic interactions. The strong effects of protists and nematodes on bacterial prey may derive from (i) their shared active space in the soil and (ii) their joint activity in performing essential metabolisms. The higher number of both positive and negative correlations in macroaggregates suggests stronger interrelationships between bacteria, protists, and nematodes. It is tempting to speculate that these associations reflect potential trophic interactions structuring the diversity and stability of the bacterial community. Positive correlations between bacteria, protists, and nematodes suggest that the colonization and growth of the bacterial community may increase under predation by protists and nematodes. This positive effect on bacterial populations is partly associated with the recycling of organic molecules and minerals locked up in senescent bacterial tissue, with direct implications for essential carbon cycling (Geisen et al., 2021). Moreover, the greater number of nodes and higher connectivity in networks have been proposed to be indirectly associated with community stability (Coyte et al., 2015; de Vries et al., 2018). Therefore, our results suggest that the bacterial community in macroaggregates has greater stability than those in microaggregates. However, it should be noted that the association of bacteria and bacterivores is bidirectional rather than unidirectional. This implies that variation in bacterial populations directly affects the population size of bacterivores and vice-versa, in a predator–prey dynamic manner.

4.3. Predation by protists and nematodes increases the genetic potential of microbial genes involved in carbon and nitrogen metabolism

Based on KEGG annotations, we found that the pathways of carbon and nitrogen metabolisms significantly varied across manure treatments and soil aggregates. The manure amendment is expected to strengthen the microbial metabolism of distinct carbon sources, such as carbohydrates, amino acids, and saccharides. Most of the carbohydrate-active enzymes detected under manure treatments were related to essential catabolic functions associated with SOM transformation. For example, the relative abundance of microbial metabolic pathways, such as citrate cycle, pyruvate metabolism, glycolysis, galactose metabolism, pentose phosphate pathway, and carbohydrate metabolism, have been reported

to be associated with the degradation of complex dissolved organic matter (i.e., carbohydrates and glycoconjugates) (Cantarel et al., 2008; Li et al., 2018) and release of oligosaccharides into the environment (Berlemont and Martiny, 2016). These oligosaccharides are utilized in the central carbohydrate metabolic pathways, which not only produce energy-yielding products but also produce precursor metabolites for other pathways (Noor et al., 2010).

Our results also revealed the importance of top-down predation indirectly influencing the relative abundance of genes involved in carbon and nitrogen metabolisms. This indirect effect occurs because predation is an essential mechanism associated with bacterial community turnover, which in this case, was shown to vary across distinct soil aggregate sizes, as also shown elsewhere (Chesson and Kuang, 2008; Rillig et al., 2017). Besides, predation pressure caused by the high abundance of bacterivores can stimulate a bacterial-dominated C and N turnover pathway by facilitating a positive-feedback loop of bacterial diversity and activity (Wang et al., 2018). Furthermore, selective feeding of bacterivores on active bacteria can suppress the metabolic quotient (the rate of soil microbial respiration per unit of microbial biomass), therefore enhancing SOC accumulation in macroaggregates (Martin and Sprunger, 2021). In addition, a higher degree of connectivity and stability within the networks in macroaggregates was suggested to support greater levels of microbial functioning. This occurs as an outcome of a higher degree of species interactions that allow for more efficient metabolic transformations in complex soil systems (Delgado-Baquerizo et al., 2020). Thus, assessing the structure and function of nematode and protist communities across aggregate sizes can provide valuable insights that advance our understanding of how trophic interactions dynamically affect C and N transformations at the soil aggregate level.

5. Conclusion

We showed that the bacterial community varied across distinct soil aggregate sizes partly due to variations in trophic predator–prey interactions. This trophic interaction also resulted in a different distribution of microbial genes associated with C and N metabolisms in soil. We provided multiple lines of evidence that protists and nematodes exerted a positive influence on specific bacterial populations, thus promoting community diversity and stability. Furthermore, we found that this top-down regulation of protists and nematodes improved the genes associated with C and N metabolisms. We showed that the macroaggregates provide more opportunities for predation that result in a more dynamic food web with potential cascading effects on soil C and N cycling. Collectively, this study partitions the variation in trophic interactions by taking into account fine-scale soil aggregate sizes, and highlights trophic interactions as an important ecological process with potential micro-scale implications for ecosystem functioning.

Declaration of Competing Interest

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

Data availability

All sequencing reads of the bacterial and protist communities have been deposited in the NCBI Sequence Read Archive under accession numbers SRP090422 and PRJNA906708, respectively. The raw data of metagenomics derived gene catalogs are publicly available under the accession number SRP312216 (Bioproject PRJNA717057).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.geoderma.2023.116658.

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