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Research Article

Protist feeding patterns and growth rate are related to their predatory impacts on soil bacterial communities

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One sentence summary: *In vitro* determination of traits, including growth rate and feeding patterns on a selection of bacterial isolates, has the potential to predict the species-specific predatory impact of protists on soil bacterial community.
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Abstract

Predatory protists are major consumers of soil micro-organisms. By selectively feeding on their prey, they can shape soil microbiome composition and functions. While different protists are known to show diverging impacts, it remains impossible to predict *a priori* the effect of a given species. Various protist traits including phylogenetic distance, growth rate and volume have been previously linked to the predatory impact of protists. Closely related protists, however, also showed distinct prey choices which could mirror specificity in their dietary niche. We, therefore, aimed to estimate the dietary niche breadth and overlap of eight protist isolates on 20 bacterial species in plate assays. To assess the informative value of previously suggested and newly proposed (feeding-related) protist traits, we related them to the impacts of predation of each protist on a protist-free soil bacterial community in a soil microcosm via 16S rRNA gene amplicon sequencing. We could demonstrate that each protist showed a distinct feeding pattern *in vitro*. Further, the assayed protist feeding patterns and growth rates correlated well with the observed predatory impacts on the structure of soil bacterial communities. We thus conclude that *in vitro* screening has the potential to inform on the specific predatory impact of selected protists.

Keywords: dietary niche, *in vitro* assay, microbes, microcosm, predation, soil

Introduction

The soil microbiome is a cornerstone of terrestrial ecosystem functioning. In addition to the essential roles of symbiotic and root-associated bacteria and fungi on soil functioning and plant performance (Lugtenberg and Kamilova 2009, Hassani *et al.* 2018), predatory soil protists are increasingly being recognized for their roles in nutrient turnover (Clarholm 1985, Bjørnlund *et al.* 2012, Rønn *et al.* 2012) and as drivers of bacterial community structure (Bonkowski *et al.* 2000, Rønn *et al.* 2002, Gao *et al.* 2019). Predatory protists represent the most dominant protistan functional group in soils (Oliverio *et al.* 2020, Singer *et al.* 2021, Xiong *et al.* 2021). Predation pressure is typically shared unequally on different bacteria as a result of a combination of differential prey defense strategies and predator traits (Singh 1942, Gao *et al.* 2019). Several bacteria have, for instance, evolved different strategies to escape or repel predators (Matz and Kjelleberg 2005) such as the production of antimicrobial compounds (Mazzola *et al.* 2009, Jousset and Bonkowski 2010). Predatory protists can also discriminate between different preys based on traits including cell surface properties and volatile organic compounds (Jousset 2012, Schulz-Bohm *et al.* 2017). Our knowledge is, however, mostly based on a few model species. With the growing awareness that even closely related species may differ in their impact on microbial communities comes the question of how to predict the functional role of the hundreds of protist species coexisting in a single gram of soil (Geisen *et al.* 2018, Gao *et al.* 2019). Indeed, predatory protists typically exhibit a large variety of morphologies and life strategies (e.g. slow or fast grower) that can influence their realized impact on the prey community composition (Jiang and Morin 2005, Ryberg *et al.* 2012).

Various protist traits including phylogenetic distance, morphology such as cell volume and flexibility, and physiological traits such as the growth rate have been studied in relation to the predatory impact of protist on their prey community composition (Glücksman *et al.* 2010, Gao 2020). From these studies, however, it was observed that closely related protists with similar traits could show taxon-specific impact on their prey community. Such distinct impacts likely mirror specificity in the dietary niche of different protist taxon. Therefore, the identification of additional traits more directly related to protist feeding such as dietary niche breadth and overlap, is required to allow better prediction of the predatory impact of protist species. In theory, protists with low prey preferences and thus relatively large dietary niche breadth would feed more or less equally on all bacteria, allowing stochastic processes to dominate bacterial community assembly. On the contrary, protists with clear prey preferences, thus narrower dietary niche breadth, would feed mostly on a subset of preferred prey, leading to more deterministic shifts in the community (Ryberg *et al.* 2012, Filip *et al.* 2014, Johnston *et al.* 2016). Protist species are likely to present different feeding preferences that

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can be linked to relatively narrow to broad dietary niche breadth and impose relatively strong or weak predatory impact on their prey community, accordingly. In addition, predatory impacts of soil protists on bacterial community may be manifest as broad shifts in community structure (Gao 2020, Asiloglu *et al.* 2021) or may only enrich/deplete some species without significantly modifying the whole prey community structure (Asiloglu *et al.* 2020). The relation between protist species-specific feeding patterns as could be assessed in simplified laboratory settings has, however, never been linked to complex predation-induced shifts in soil bacterial communities.

In order to study the informative value of *in vitro* assessed properties in relation to realized predatory impact of protists, we selected eight well-characterized protist isolates spanning several major phylogenetic lineages (Amorphea, Excavates and TSAR; lineages according to Burki *et al.* (2020) and morpho-groups (amoeboid and flagellates), while presenting a similar feeding style (i.e. mostly grasping attached bacteria). We examined their ability to grow on twenty rhizobacterial isolates with well-characterized traits related to plant growth-promoting potential, biocontrol potential (i.e. production of antimicrobial compounds), and environmental stressors, and representing a wide phylogenetic range including Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. We investigated the feeding preferences of the selected protists based on their achieved density on each bacterial strain at two different time points, 3 and 5 days after inoculation, to consider different protist growth stages. We used the emerging protist feeding patterns to estimate the dietary niche breadth of each protist isolate (coefficient of variation of the achieved densities on each bacterium) and to compare protist feeding patterns across our isolates (pair-wise Euclidean distance between the achieved densities on each bacterium). In order to assess the informative power of these feeding-related traits, we analyzed the predatory impact of each protist isolate on a soil bacterial community using a soil microcosm setup and linked these impacts to the *in vitro* feeding patterns. We finally evaluated the importance of different traits for both the predators (phylogenetic distance, volume, and growth rate) and prey (direct-plant growth promotion potential, biocontrol potential, nutrient utilization efficiency and stress-tolerance) in relation to the protist feeding patterns, and the importance of the protist traits in relation to the predatory impacts on the soil bacterial community. Our main hypotheses were that (i) each protist isolate has a distinct feeding pattern as measured by higher achieved densities on a specific set of bacteria, (ii) protists with a relatively narrow dietary niche breadth would have a stronger impact on the prey community structure compared to protists with broader niche breadth, (iii) protists with similar feeding patterns would exert similar impacts on the soil-borne bacterial prey community, and (iv) predator traits related to protist size and prey traits related to biocontrol potential would be the most informative predictors of the observed patterns.

Materials and methods

Plate assay for investigating protist feeding behavior

We used a liquid plate assay to examine the feeding behavior of eight protist isolates (Ecology and Biodiversity collection, Utrecht University, The Netherlands; Table S1) on monocultures of twenty bacterial strains (19 isolates from the rhizosphere of tomato seedlings and *Escherichia coli* OP50; Table S2).

The selected protists were isolated from a range of environments (clay soil, sandy soil and growth substrate) and included two *Acanthamoeba* spp. (isolates C13D2 and C2D2), two *Vannella* spp (isolates P33 and P147), *Naegleria clarki* (isolate P145-4), heterolobosean isolate S18D10 and two *Cercomonas* spp. (isolates C5D3 and S24D2). The protist traits (phylogenetic distance, volume and growth rate) used in this study were derived from previous work by Gao with an adjustment regarding the volume calculation (Gao 2020; see also Tables S1 and S3); in brief, the phylogenetic distance was calculated based on the nearly complete 18S, the volume was estimated assuming a cone shape for the flagellates *Cercomonas* spp. ($\frac{1}{3} * \pi * (\frac{width}{2})^2 * length$), flat fan shape for the amoebae *Acanthamoeba* spp. and *Vannella* spp. (*i.e.*: $length * \frac{width}{2}$) or semi-cylindrical shape for the amoebae *Naegleria clarki* and heterolobosean isolate S18D10 (*i.e.*: $\{length * \pi * (\frac{width}{2})^2\}/2$), and the growth rate was estimated from the daily quantification of individuals over 8 to 11 days by fitting a growth model (grofit::gcFitModel; the mean density and its standard error can be found for each protist and each day in Table S3). The isolate S18D10 could only be related to an uncultured heterolobosean and was identified to have the closest affinity with *Stachyamoeba* ATCC 50 324 (see also Fig. S1).

E. coli OP50 was routinely used as food source for the propagation of the protist cultures

The bacterial strains used were isolated and described by Hu and colleagues (2020; see an overview in Table S2). In brief, the bacteria were isolated from the rhizosphere of tomato plants (*Lycopersicon esculentum*, cultivar 'Jiangsu') in the seedling stage. For each bacterial strain, functional traits were measured to assess their plant growth-promoting potential (production of the auxin indole-3-acetic acid (IAA) and siderophore), biocontrol potential (inhibition of the plant pathogen *Ralstonia solanacearum QL-Rs1115*) and resistance to three environmental stressors (low resource availability, high salinity, and oxidative stress).

Growth conditions and preparation of protist isolates

Once a month, protist cultures were propagated using *E. coli* OP50 as the sole prey (*ca* 10^8 cells mL^{-1}) in Page's Amoeba Saline, a diluted phosphate buffer often used to grow protists (Page 1976); hereafter referred to as PAS) at 15 °C in the dark.

To obtain an active population for co-cultures, we prepared protist cultures as follows: stock protist cultures were washed three times by centrifugation at 800 *g* for 5 min (Heraeus Megafuge 40 Centrifuge, Thermo Fisher Scientific, Langenselbold, Germany) to remove excess nutrients and enrich protists. Because protists do not form a visible pellet, we only discarded 75% of the volume before resuspending the cells in the same volume of PAS. Washed cultures were then amended with *E. coli* OP50 at a density of *ca* 10^8 cells mL^{-1} to support protist growth. Protist cultures were incubated at 15 °C in the dark for 3 or 5 days. The incubation duration was adapted to each protist isolate with the aim of enabling excystation and growth while avoiding new encystation.

To initiate protist-bacterial co-cultures, the obtained active populations of protists were washed as described above, counted and adjusted to 10^3 active individuals mL^{-1}. The numbers of active and encysted individuals were counted on a monitor linked to an inverted microscope (Nikon Eclipse TS 100, Tokyo, Japan) equipped with a DS Camera Control unit DS-L3 with DS-Fi2 camera head (relay lens: 0.7x) using the 20x objective (final magnification on the monitor: 275x). The average count over five screens per well was used to estimate protist density per cm^2. Despite our efforts, the population of *Vannella* sp. P147, *Acanthamoeba* sp. C2D2, *Naegleria* sp. P145-4 and heterolobosean isolate S18D10 were a mixture

of active and encysted individuals (1 : 1; Table S4). Since the washing procedure does not allow for a complete elimination of *E. coli*, we plated a 10-fold dilution series of the washed protist solution on King's B nutrient medium to estimate the level of bacterial load (Table S4).

Growth conditions and preparation of bacterial isolates

Prior to dual culture experiments, the bacteria were grown from a frozen glycerol stocks (−80°C) on 10% TSA (3 g of Bacto Tryptic Soy broth, 15 g of agar for 1 L; BD, New Jersey, USA), with one colony serving to initiate a new culture in liquid TSB (30 g of Bacto Tryptic Soy broth for 1 L) and incubated at 28 °C, 120 r/m, 14–15 h. Bacterial cells were washed three times by centrifugation (9500 *g*, 2 min; Heraeus Megafuge 40 Centrifuge, Thermo Fisher Scientific, Langenselbold, Germany) and resuspended in 0.9% NaCl. Pellets were eventually resuspended in PAS. Based on previously measured calibration curves relating OD_{600} to CFUs, we adjusted the optical density of each bacterial solution to 10^8 cells mL^{-1} (OD_{600} of 0.5 for bacterial isolates 6, 57, and 81 and OD_{600} of 0.2 for the others; SPECTROstar Nano, BMG Labtech, Ortenberg, Germany). The realized bacterial densities were assessed by plating 20 μL of the dilutions 10^3 to 10^6 on 10% TSA, in four replicates. The plates were incubated at 28°C until colonies became visible (24/48 h) for CFU determination. The obtained densities ranged from as low as 1.83×10^6 to 2.98×10^8 CFUs mL^{-1} (Table S5). Because the CFU assay failed for bacterial isolate 14, we used the theoretical value of 8.67×10^8 cells mL^{-1} based on our calibration curve for this strain.

Setup and monitoring of protist cultures

Pure cultures and co-cultures (one bacterial isolate or/and one protist isolate) were prepared in Clear Polystyrene 96-Well Microplates with flat bottom (Corning® 3370, Corning Incorporated, New York, USA; see Table S6 for the volume distribution of each well). We used one plate per protist with each combination set up in triplicate and the location randomized to avoid potential edge effects. Plates were sealed with Parafilm® M tape (Bemis Company Incorporated, Neenah, USA) and incubated in the dark at 20°C for 5 days. We used PAS without additional nutrients so as to limit bacterial growth and activity.

Protist density was determined at days 1, 3, and 5 post inoculation; day 0 corresponds to the day of inoculation. To facilitate protist enumerations across the large number of wells examined, we used pre-defined categories to estimate the number of individuals on the monitor linked to the inverted microscope (Nikon Eclipse TS 100) using the 20x objective (final magnification on the monitor: 275x) instead of direct counts. We used seven different categories (Table S7). To enable the distinction between protist population at relatively low density (i.e. 0–100 individuals per screen), we used four categories starting with the category 0 for the absence of any protists, category 1 for 1 to 10 individuals, category 2 for 11 to 50 individuals and category 3 for 51 to 100 individuals. Larger intervals were used for the three higher categories: category 4 corresponded to 101–400 individuals, category 5 to 401–800 individuals and category 6 to more than 800 individuals per screen. We assigned a category for 3 screens per well (middle line right, middle line center, middle line left) to take potential heterogenous distribution of the protists in the well into consideration. We then averaged the values to obtain a single category value per well and hereby estimated the number of individuals per cm^2 (Table S8).

We measured the OD_{600} over time (at days 0, 1, 3, and 5) with a plate reader (SPECTROstar Nano, BMG Labtech, Ortenberg, Germany) as an indicator of bacterial density (Dalgaard *et al.* 1994). However, the relation between protist growth and the optical density was not necessarily negative and could thus not be used as an indicator of consumption. We, therefore, did not use these data for further analysis.

Determining protist feeding patterns based on in vitro plate assays

Because the initial bacterial densities ranged from as low as 1.83×10^6 to 2.98×10^8 CFUs mL^{-1}, we first investigated the potential impact of the initial bacterial density on the protist achieved density at day 3 and 5 by performing correlation analyses (stats::cor.test).

Second, we investigated the general growth patterns of the protists on all bacteria by plotting their temporal growth. We then visualized protist densities at days 3 and 5 post inoculation on each bacterial food source by deploying the R function gplots::heatmap.2 (normalized per protist with the scale argument set on rows; Warnes *et al.* 2020) and allowing the function to create clusters and draw a dendrogram based on the Euclidean distances for the protist (y-axis). We forced the x-axis to display the bacteria grouped per phylum to help visual comparison between days 3 and 5.

We then calculated the coefficients of variation (CV) at days 3 and 5 post inoculation for each protist as an estimate of dietary niche breadth. The CV is obtained by dividing the standard deviation by the mean of the population, thus providing a standardized measure of the variability. The CV has a value of 0 when the protist isolate reached the same density on each bacterial strain and has an increasingly positive value as the protist isolate reaches very different densities on each bacterial strain; the higher the value, the narrower the dietary niche breadth. To adress similarities between protist feeding patterns, we used the pair-wise Euclidean distance between the achieved densities of each protist across the 20 tested bacterial strains.

Determining the realized predatory impact on soil bacterial communities

We related the obtained descriptive feeding pattern of protists to their impact on a prey community by using data from a soil microcosm experiment that investigated a total of 20 protist isolates for their impacts on soil bacterial community composition (Gao 2020). The eight protists used in the plate assays described above were among the 20 strains used in this previously described experiment. Here, we provide a general description of this microcosm study. Additional details can be found at Gao (2020). In brief, protist-free soil bacterial communities were created using a microbial extraction from a natural sandy soil (Botanische Tuinen, de Uithof, Utrecht, The Netherlands). This protist-free suspension was reinoculated back into sterilized soil. After 2 h of incubation, the protist treatments were added by inoculating 400 μL of each protist suspension (10^4 individuals mL^{-1}) or PAS (control), in 5 replicates. After 10 days, the soil was sampled and DNA was extracted using the DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. Bacterial 16S rRNA gene amplicon sequencing was carried out as follows: a two-steps PCR protocol with the 515F–806R primer-pair targeting the V4 region of the 16S rRNA gene ((Caporaso *et al.* 2011) modified with linker sequences according to the 16S Metagenomic Sequencing Library Preparation protocol (Amplicon primers), purification of the PCR products according to the 16S Metagenomic Sequencing Library Preparation protocol (PCR clean-up) and ampli-

con sequencing (Illumina Inc., San Diego, USA) using a 250-bp V2 paired-end protocol on a MiSeq sequencer (Utrecht Sequencing Facility, The Netherlands). DNA reads were processed using USEARCH, and sequences were identified and clustered into Operational taxonomic units (OTUs) at a 97% sequence similarity level with QIIME 1 (Caporaso *et al.* 2010) using the UCLUST algorithm (Edgar 2010). Taxonomic assignment was obtained by alignment against the SILVA 16S reference database, version 128 (Glöckner *et al.* 2017). Rarefaction was performed to 12 000 reads per sample; at this threshold, seven samples had to be removed.

Data analyses

All statistical analyses were performed with the free software R version 4.0.3 (R Core Team 2020). Unless stated otherwise, results from statistical tests were considered significative for P values < 0.05.

We first investigated the temporal similarity of the emerging protist feeding patterns at days 3 and 5 post inoculation via a Mantel test (vegan::mantel). We then performed an ANOVA on the achieved protist densities on each bacterial isolate to identify significant differences compared to the control (no bacterial cells added; stats::lm and base::summary); our control well allowed us to estimate the growth that each protist could achieve by growing on the *E. coli* cells transferred alongside at the start of the experiment. Due to the relative low number of replicates (triplicates), the use of transformation and/or use of other methods allowing to control for the heteroscedasticity such as nlme::gls (with weights = varIdent(form = ~1 | bacteria)) (Zuur *et al.* 2009) did not seem meaningful; the results of our ANOVA are, thus, informative but should be taken with some caution.

We analyzed the predatory impact of each protist isolate on soil bacterial communities by merging the soil microcosm sequencing data per treatment (phyloseq::merge_samples) and calculating the dissimilarity/distance matrices of the community composition between treatments. We used the Bray-Curtis dissimilarity (vegan::vegdist) and the phylogenetic aware distances weighted UniFrac (phyloseq::distance) and compared them by performing a correlation analysis (stats::cor.test) and a Mantel test (vegan::mantel). We ran a permutational multivariate analysis of variance using both distance matrices (PERMANOVA; vegan::adonis) to assess the predatory impact of protists. In case of a significant result, we performed pair-wise PERMANOVA for each treatment and the control, correcting the P value for multiple testing using the Benjamini and Hochberg procedure (Noble 2009); stats::p.adjust with method = "BH'). We visualized the overall changes in the bacterial community composition is based on a Principal Coordinate Analysis (PCoA) on the Bray-Curtis dissimilarity between samples. We further identified bacterial taxa that increased or decreased in relative abundance upon the inoculation of protists, plotting the log two-fold changes for each treatment compared to the control (DEseq2, (Love *et al.* 2014)); we used an alpha of 0.01, instead of the default value of 0.1, to obtain a meaningful visualization without too many OTUs and as an attempt to control for the reported high false discovery rate (Hawinkel *et al.* 2019). We considered the distance of each treatment relative to the control to adress the magnitude of predatory impact of each protist isolate on the prey community structure and the pair-wise distances between treatments (vegan::vegdist) to investigate similarities in predatory impact between isolates.

We used two analyses to examine the relation between the coefficient of variation (used as proxy for dietary niche breadth) and the magnitude of predatory impact in the soil microcosm. First, we tested the correlation between the coefficient of variation and the distance of each treatment relative to control (stats::cor.test). Second, we calculated the nearest taxon index (NTI; iCAMP::NTI.p; (Ning *et al.* 2020)) for each treatment to examine the prevalence of either phylogenetic clustering (NTI > 2) or overdispersion (NTI < -2) (Zhou and Ning 2017). Phylogenetic clustering is mostly expected when deterministic processes dominate, and overdispersion is expected when stochastic processes dominate (Zhou and Ning 2017). We plotted the obtained NTI values and tested if they were significantly different compared to the non-protist control using an ANOVA (stats::lm and base::summary). We then tested the correlation between the coefficients of variation and the NTI values (stats::cor.test). We visualized with linear regressions (stats: lm) both relations, CV against distance of each treatment relative to control and CV against NTI.

We tested the relations between protist feeding pattern similarities (pair-wise Euclidean distances of the feeding patterns at days 3 and 5 of the plate assay), protist traits (CV, phylogenetic distance, volume and growth rate) and the protist predatory impacts (distance of the bacterial community composition of the soil microcosms relative to control and pair-wise distances between treatments; Bray-Curtis dissimilarity and weighted UniFrac) with linear models and correlation analyses (stats::lm, base::summary and stats::cor.test). We visualized these relations with a principal component analysis (stats::prcomp() and ggbiplot::ggbiplot(), (Vincent 2011)).

In addition, we investigated the correlation between bacterial traits (as measured by Hu and colleagues (Hu *et al.* 2020)) and the protist feeding patterns of our plate assay (stats::cor.test). While bacterial traits such as plant-growth promotion potential are unlikely to dictate the protist feeding patterns, if they are consistently correlated with low protist density, this function could be selected in the soil community when exposed to protist predation.

Results

In vitro protist feeding patterns and relation with predator and prey traits

Each of our protist isolates grew to some extent on all 20 bacterial isolates until day 3 with an increase ranging from about 3.3 to 5.5 x from the day 1, after which they generally decreased by day 5 to about 2.5 to 4.6x the initial population (Fig. S2). Because we only observed a positive correlation between *Acanthamoeba* sp. C2D2 and the initial bacterial densities at day 5 (Table S9), we could consider that the differences in initial bacterial densities did not had an important impact on the protist growth. We distinguished distinct feeding patterns for each protist inferred from significantly higher or lower achieved densities on bacteria compared to the control (no added bacterial cells; Fig. 1 and Fig. S3). The two *Cercomonas* spp. displayed similar feeding patterns that were distinct from all other protists (see also clusters of the y axis in Fig. 1). The protist feeding patterns were similar at days 3 and 5 according to the Mantel test on Euclidean distances between the achieved densities of each protist on the 20 bacteria ($r = 0.965$, significance = 0.001). The coefficient of variation (CV), used as estimate for dietary niche breadth of each protist, ranged from 0.43 to 0.94 (Table S10).

We found a positive correlation between the Euclidean distance and protist growth rate for both days and with the corresponding coefficient of variation at day 5 (Table 1). Our linear models further supported the importance of growth rate to explain the Euclidean distance at days 3 and 5 ($t_{(51)} = 9.495$, $P < 0.001$ and $t_{(51)} = 7.045$, $P <$

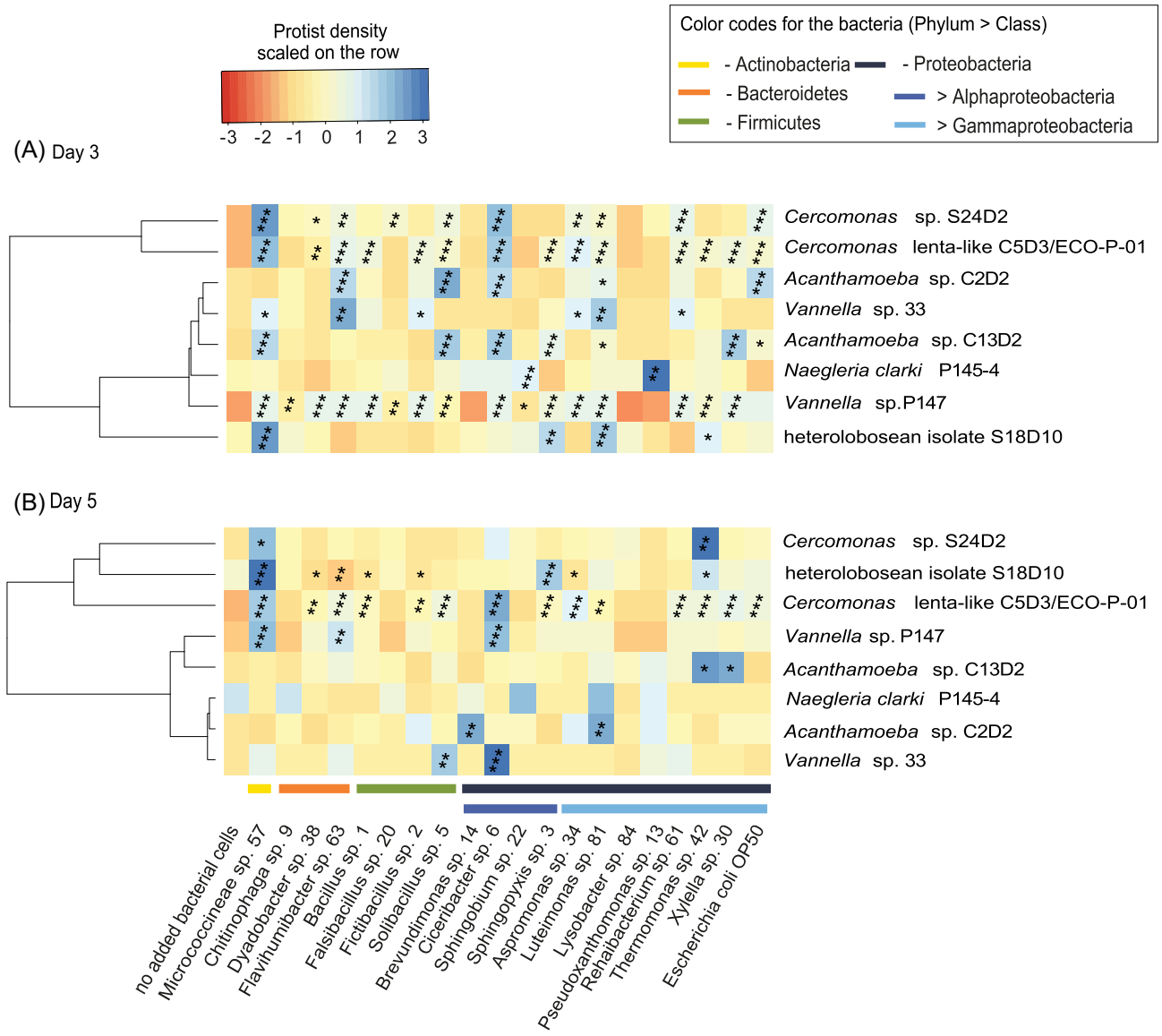

Figure 1. Heatmap of the protist population density on each bacterial isolate representing the feeding patterns at days 3 (panel **A**) and 5 (panel **B**) after inoculation. The protist density has been scaled per row to facilitate comparison; each co-culture was set in triplicates (N = 3). The y-axis is ordered according to similarities between protist feeding patterns (Euclidean distance of the achieved protist density on each bacterium). The x-axis is fixed with the bacteria grouped per phylum. Orange colors correspond to negative values, i.e. lower density compared to average population density, per protist, and blue colors correspond to positive values, i.e. higher density compared to average population density, per protist. Asterisks highlight significantly higher/lower protist densities compared to the control with no added bacterial cells: '*', '**', '***' indicate $P < 0.05$, $P < 001$ and $P < 0.001$ respectively.

0.001, respectively) and also indicated a significant contribution of phylogenetic distance and the volume in explaining the observed feeding patterns at day 3 ($t_{(51)} = 3.277$, $P = 0.002$ and $t_{(51)} = -2.69$, $P = 0.013$, respectively; Table S11). On the prey side, however, we did not find any clear and significant correlations between bacterial traits and the observed protist feeding patterns (Fig. S4).

Predatory impacts of the selected protists on the soil bacterial community

As found for protist feeding patterns, each protist had a significant and distinct impact on its prey community in the soil microcosm experiment (Table 2, Fig. 2 and Figs S5 to S8 for the log two-fold changes of bacterial OTUs for each protist compared to the control). In general, we found that OTUs related to Proteobacteria tended to decrease in relative abundance upon protist inoculation compared to the control, while most OTUs related to Actinobacteria showed relative increases (Figs S5–S8).

Relation between protist traits and the magnitude of the predatory impact on the soil-borne prey community

We did not observe any significant correlation between protist dietary niche breadth and the amplitude of the predatory impact on the prey community (Fig. 3 and Table S12 for the statistical results of the linear models). The coefficients of variation, used as a proxy for the dietary niche breadth for each protist, did not correlate with the impact on the prey community as measured by the Bray-Curtis dissimilarity for each protist relative to the control (for day 3: cor = 0.017, $P = 0.96$ and day 5: cor = 0.516, $P = 0.191$; Table 3 and Table S13 for the weighted UniFrac). In addition, the co-

Table 1. Relation between the pair-wise protist feeding patterns (Euclidean distance of the achieved densities on the bacteria in the plate assay) and pair-wise protist traits including the coefficient of variation (indicator of the dietary niche breadth), the phylogenetic distance, volume and growth rate. The Euclidean distance and coefficient of variation were obtained at two different sampling time points, day 3 and 5, and are indicated as such. Significant results ($P < 0.05$) are highlighted in bold.

	Correlation coefficient	T test	P value
Pair-wise Euclidean distance at day 3			
Coefficient of variation at day 3	−0.205	$t_{(54)} = -1.543$	0.129
Phylogenetic distance	0.056	$t_{(54)} = 0.413$	0.684
Volume	−0.144	$t_{(54)} = -0.742$	0.465
Growth rate	**0.794**	**$t_{(54)} = 9.603$**	**< 0.001**
Pair-wise Euclidean distance at day 5			
Coefficient of variation at day 5	**0.264**	**$t_{(54)} = 2.013$**	**0.049**
Phylogenetic distance	−0.030	$t_{(54)} = -0.219$	0.828
Volume	−0.186	$t_{(54)} = -0.965$	0.343
Growth rate	**0.710**	**$t_{(54)} = 7.406$**	**< 0.001**

Table 2. Predatory impact of each protist compared to the non protist control given as the PERMANOVA results on the Bray–Curtis dissimilarity to Control for each treatment. The *P* value adjustment was performed with the Benjamini and Hochberg correction.

Treatment	F statistic	R^2	P value	Adjusted P value
C13D2	$F_{(1,8)} = 4.599$	0.365	0.008	0.013
C2D2	$F_{(1,8)} = 3.581$	0.309	0.011	0.013
P147	$F_{(1,8)} = 3.125$	0.281	0.009	0.013
P33	$F_{(1,8)} = 3.353$	0.295	0.014	0.014
P145-4	$F_{(1,8)} = 3.516$	0.305	0.010	0.013
S18D10	$F_{(1,8)} = 4.977$	0.384	0.009	0.013
C5D3	$F_{(1,7)} = 7.186$	0.507	0.007	0.013
S24D2	$F_{(1,8)} = 10.74$	0.573	0.010	0.013

Table 3. Correlation between the magnitude of predatory impact (Bray–Curtis dissimilarity relative to the control) and protist traits (coefficient of variation as proxy for the dietary niche breadth, volume and growth rate). The significant result ($P < 0.05$) is highlighted in bold.

Protist trait	Correlation coefficient	Adjusted R^2	P value
Coefficient of Variation (Day 3)	0.017	−0.166	0.96
Coefficient of Variation (Day 5)	0.516	0.144	0.191
Volume	−0.346	−0.026	0.401
Growth Rate	**0.898**	**0.774**	**< 0.01**

efficients of variation were not correlated with the nearest taxon index values (NTI; cor = 0.312, $P = 0.45$ for day 3 and cor = -0.313, $P = 0.45$ for day 5). All the NTI values were higher than 2, indicating phylogenetic clustering (Zhou and Ning 2017). We observed significantly higher NTI values for the bacterial communities exposed to the heterolobosean isolate S18D10 treatment compared to the no protist control, and significantly lower values for the ones exposed to the *Cercomonas* sp. S24D2 treatment (Fig. S9 and Table S14 for the NTI values obtained for each replicate).

From the other protist traits considered, only the protist growth rate was positively correlated with the magnitude of predatory impact on the soil bacterial community composition (Table 3 and S13).

Relation between protist feeding patterns, protist traits and predatory impacts on the soil bacterial community

We found that protists with similar feeding patterns also had a more similar impact on the soil bacterial community. Indeed, the pair-wise Euclidean distance of achieved density on the selected bacteria (i.e. protist feeding patterns) was positively correlated with the pair-wise distances of the soil bacterial community composition (*i.e.* protist predatory impact) (cor = 0.714; Table 4). From the other protist traits investigated, only growth rate was positively correlated with the pair-wise Bray–Curtis dissimilarity (cor = 0.723; Table 4).

Our ordination approach further highlighted the relevant correlations (Fig. 4): growth rate, feeding pattern (pair-wise Euclidean distance at day 3) and predatory impact on the soil-borne bacterial community (Bray-Curtis dissimilarity) all clustered together to explain most of the variation (51.1%) along the first principal component. Phylogenetic distance and volume correlated to explain 31.2% of the variation expressed along the second principal component. Note that due to the observed positive correlation between the Euclidean distance at days 3 and 5, only the day 3 is given as a measure for the feeding pattern in Table 4 and Fig. 4. Similarly, we only present the statistical results using the pair-wise Bray–Curtis dissimilarity. The results of the correlation anal-

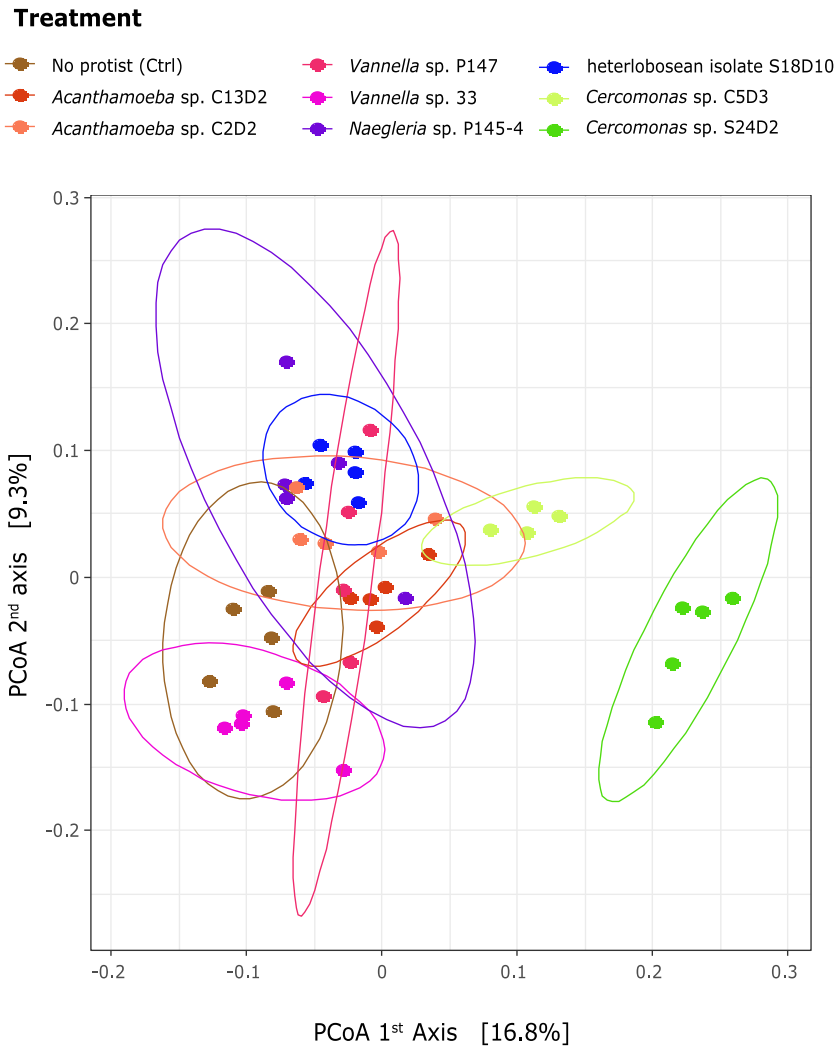

Figure 2. Visualization of the effect of each protist treatment on the bacterial community composition in a soil microcosm. The visualization is based on a Principal Coordinate Analysis (PCoA) on the Bray–Curtis dissimilarity between samples.

Table 4. Correlation between the pair-wise Bray–Curtis dissimilarity and the pair-wise Euclidean distances of protist traits. Not that due to the high positive correlation between the pair-wise Euclidean distance at days 3 and 5, only the result for day 3 is given here below under 'Feeding pattern'. Significant results ($P < 0.05$) are highlighted in bold.

Protist trait	Correlation coefficient	Adjusted R^2	*P* value
Feeding pattern	**0.714**	**0.502**	**< 0.01**
Phylogenetic distance	− 0.124	− 0.003	0.364
Volume	− 0.136	− 0.019	0.49
Growth Rate	**0.723**	**0.513**	**< 0.01**

ysis using the weighted UniFrac were generally similar and can be found in Table S13.

Discussion

We found that despite the relatively broad dietary niche breadth displayed by our selected protists, each of them had a distinct feeding pattern that remained similar for both days 3 and 5 after inoculation. We further found that these *in vitro* feeding patterns and protist growth rate could be related to predatory impacts on the soil-borne bacterial community structure.

In line with our first hypothesis, while all protists could feed to some extent on all 20 bacterial isolates, distinct feeding patterns emerged for each protist depending on the respective protist-bacterium combination. This result is in agreement with previous work showing distinct growth abilities of protist species on various bacteria, due, for instance, to adaptations to feed on or avoid toxin producing bacteria (Jousset *et al.* 2009, Pedersen *et al.* 2011). In addition, distinct feeding patterns between predatory protists were also reported with differential feeding on only 20% of the selected bacterial strains (Singh 1942).

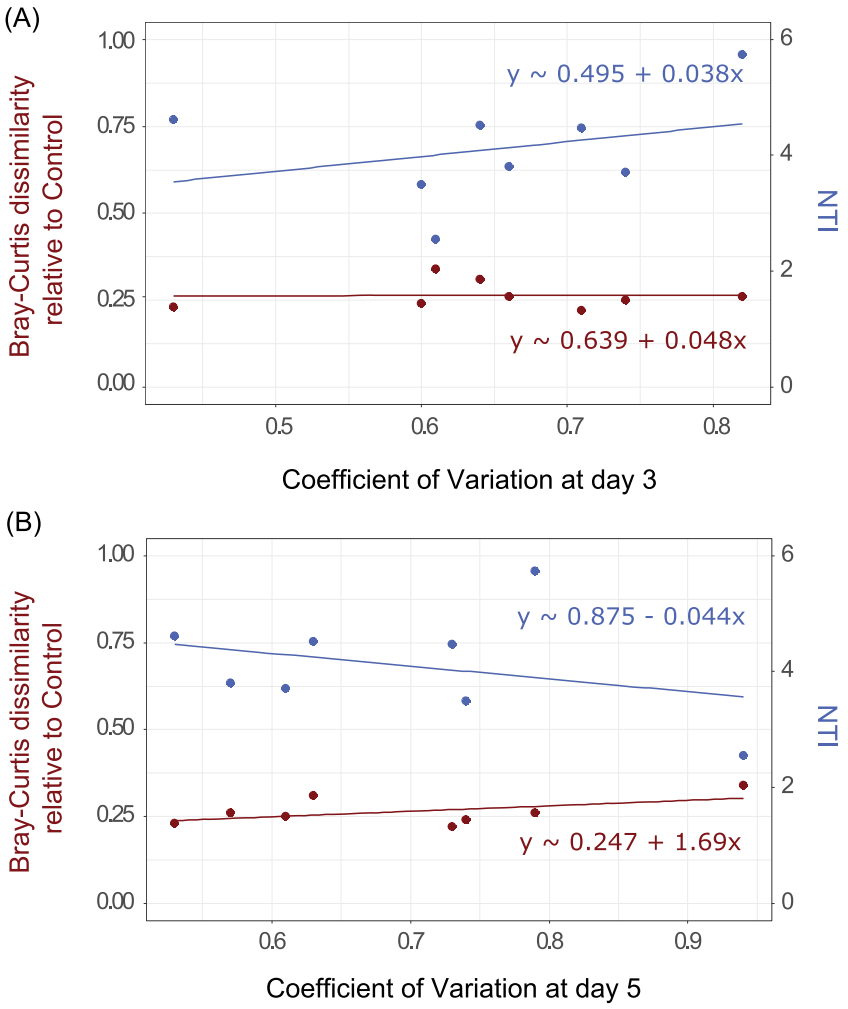

Figure 3. Relation between the dietary niche breadth (coefficient of variation at days 3 and 5, **A** and **B** panel respectively) and the magnitude of the predatory impact on the bacterial prey community (Bray–Curtis dissimilarity of each treatment relative to control and NTI). The y-axis on the left gives the values for the Bray–Curtis to control dissimilarity (dark-brown dot and line) and the y-axis on the right gives the values for the NTI (blue dots and line). Coefficient estimates are given next to the line. Statistical results of the linear model analysis are given in Table S10.

Similarly, each predatory protist exerted a distinct impact on its prey community composition from relatively similar to the control bacterial population for the treatment with *Vannella* sp. P33, for example, to clearly distinct with *Cercomonas* sp. S24D2. Proteobacterial taxa tended to show a reduced relative abundance when communities were subjected to protist predation, which is in line with previous studies suggesting that particularly alpha- and beta-Proteobacteria are sensitive to protist predation (Murase *et al.* 2006, Rosenberg *et al.* 2009). Actinobacteria generally increased in relative abundance in response to protists, which was also in agreement with previous observations (Ekelund *et al.* 2009, Rosenberg *et al.* 2009). Although some trends were found at these higher taxonomic levels, we observed a range of specific responses, both positive and negative, of some OTUs and genera to the presence of specific protist isolates (Fig. S5-S8). Such fine-scale differences between protist impacts on bacterial communities is not surprising given the fact that strong differences in palatability have been observed between isolates of the same bacterial genus such as *Pseudomonas* (Pedersen *et al.* 2011, Amacker *et al.* 2020).

Contrary to our second hypothesis, we did not find any correlation between dietary niche breath and the magnitude of the predatory impact on the prey community composition. This result might reflect the rather generalist feeding nature of the selected protists. Alternatively, niche breath as measured in our pairwise assays may not accurately reflect the extent of realized feeding preference in a complex environment with a highly heterogeneous structure (Erktan *et al.* 2020, Xiong *et al.* 2021). Intriguingly, while heterolobosean isolate S18D10 and *Cercomonas* sp. S24D2 were both reported with the highest coefficient of variation at day 5, suggesting a narrow dietary niche breadth and potentially strong prey selection (Filip *et al.* 2014, Johnston *et al.* 2016), the NTI values observed within bacterial communities exposed to these protists were, respectively, significantly higher and lower compared to the control. This suggests phylogenetic clustering and a higher importance of deterministic processes for the communities exposed to heterolobosean isolate S18D10, but a lower importance of these processes for communities exposed to *Cercomonas* sp. S24D2 (Kembel 2009, Stegen *et al.* 2012). This apparent contradiction could be due to the preferred type of prey. While specialist predators are usually linked to deterministic processes (Chase *et al.* 2009, Ryberg *et al.* 2012), if the predator preferentially feeds on competitively superior prey, this could lower competition between prey species and lead to a stronger influence of stochastic processes in community assembly (Chase 2003, Jiang *et al.* 2011, Johnston *et al.* 2016). Note that we, here, primarily performed our NTI analyses in an exploratory manner and thus only superficially

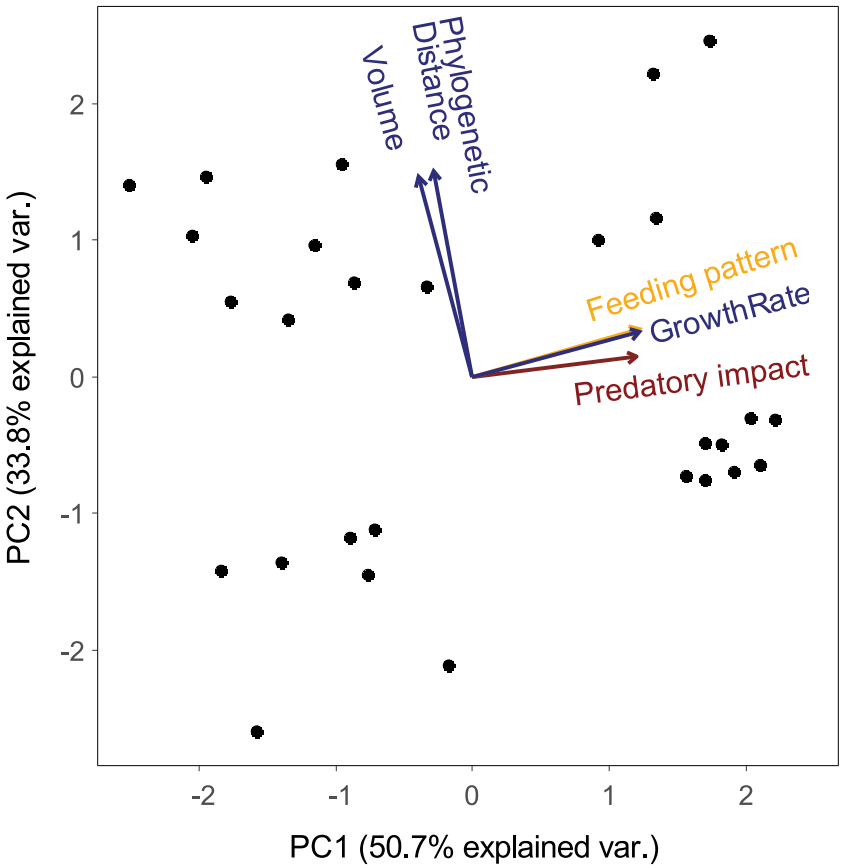

Figure 4. *Principal Component Analysis using the pair-wise Euclidean distances of protist traits (Volume, Phylogenetic distance, and Growth rate; blue color), pair-wise Euclidean distances between protist feeding pattern (plate assay, at day 3, yellow color) and the Bray-Curtis dissimilarity between bacterial community composition (soil microcosm, red-brown color). Each dot represents a protist pair such as P33-S24D2, P33-P147, or S24D2-C5D3; there is a total of 28 pairs.*

address the potential relation between protist feeding patterns and the magnitude of deterministic processes in community assembly.

In line with our third hypothesis, the pair-wise Euclidean distance of the feeding patterns was positively correlated with the pair-wise Bray-Curtis dissimilarity of the predated bacterial community composition. Our feeding assays are, thus, capable of providing characterizations of feeding behavior that have bearing on realized predatory impacts in more complex systems. Similarly, in another study, bacterial biocontrol potential obtained from various controlled assays was found to be a powerful indicator of bacterial performance in a field experience (Agaras *et al.* 2020). Protist feeding assays might thus be used to predict predatory impact of specific protists thereby providing information on how to steer bacterial communities as previously suggested (Gao *et al.* 2019).

From all the protist traits, only growth rate was significantly related to feeding patterns and predatory impacts. In contrast to previous studies (Glücksman *et al.* 2010, Gao 2020), we did not find any relation with protist volume. It should, however, be noted that we only had one isolate in our collection, *Naegleria clarki* P145-4, that was markedly larger (921 μm^3) than the rest (104–455 μm^3). Our experimental collection may therefore not have adequately covered cell volume as a trait variable to make robust conclusions. Furthermore, we did not observe any clear correlation between protist growth and the examined bacterial traits. While the production of antimicrobial compounds has been reported as an efficient defense mechanism against protist predation (Jousset *et al.* 2006, 2009), the absence of additional nutrients in our co-cultures may explain the apparent lack of efficient bacterial defense. Indeed, the production of antimicrobial compounds can be metabolically costly, often requiring sufficient nutrients and a relatively high population density (Haas and Défago 2005). The differences in the protist feeding patterns may also have been regulated by other parameters such as cell size, cell membrane composition and nutritional quality (Boenigk *et al.* 2001, Matz and Kjelleberg 2005).

Thus, laboratory assays have proven useful for examining protist feeding preferences, but we should also keep in mind that they also have their limitations with respect to how well they address *in vivo* feeding behaviors (Montagnes *et al.* 2008). Although we utilized a relatively broad range of species, our selection and standardized assays relying on pairwise interactions cannot provide a full representation of the breadth of species and interactions encountered in the complex soil environment. Nonetheless, such approaches can be highly informative when trying to identify the dietary niche of selected protist species of interest (Montagnes *et al.* 2008, Devictor *et al.* 2010).

Conclusions

Our results indicate that *in vitro* screenings have the potential to instruct on the specific predatory impact of selected protists, with especially feeding patterns and growth rates as informative traits. Our study should be seen as a first step that could be used as a basis for the development of high throughput and standardized plate assays to test the prey adequacy of different species for dif-

ferent protists. The development of such assays could be used to test a bigger set of prey and conditions and would increase our ability to. investigate the multitrophic nature of the microbiome. Such knowledge is typically necessary to develop systematic strategies to steer the soil microbiome by inoculation of specific protist species so to support functions of interest.

Supplementary data

Supplementary data are available at *FEMSEC* online.

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Conflicts of interest statement. No conflict of interest. The authors declare that they have no conflict of interest.

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