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Research Paper

Species-specific predation determines the feeding impacts of six soil protist species on bacterial and eukaryotic prey

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ABSTRACT

Predatory protists play a central role in nutrient cycling and are involved in other ecosystem functions by predating the microbiome. While most soil predatory protist species arguably are bacterivorous, some protist species can prey on eukaryotes. However, studies about soil protist feeding mainly focused on bacteria as prey and rarely tested both bacteria and eukaryotes as potential prey. In this study, we aimed to decipher soil predator–prey interactions of three amoebozoan and three heterolobosean soil protists and potential bacterial (*Escherichia coli*; 0.5–1.5 μm), fungal (*Saccharomyces cerevisiae*; 5–7 μm) and protist (*Plasmodiophora brassicae*; 3–5 μm) prey, either as individual prey or in all their combinations. We related protist performance (relative abundance) and prey consumption (qPCR) to the protist phylogenetic group and volume. We showed that for the six soil protist predators, the most suitable prey was *E. coli*, but some species also grew on *P. brassicae* or *S. cerevisiae*. While protist relative abundances and growth rates depended on prey type in a protist species-specific manner, phylogenetic groups and volume affected prey consumption. Yet we conclude that protist feeding patterns are mainly species-specific and that some known bacterivores might be more generalist than expected, even preying on eukaryotic plant pathogens such as *P. brassicae*.

1. Introduction

Soils host approximately 59 % of all known species on Earth (Anthony et al., 2023). The majority of this soil biodiversity, mainly fungi and bacteria, belongs to the soil microbiome, which performs important ecosystem functions such as controlling the cycling of carbon (C) and nitrogen (N), as well as pathogen suppression (Banerjee and van der Heijden, 2022; Sokol et al., 2022). However, a considerable amount of the nutrients that bacteria and fungi process are kept inside their cells, non-accessible to plants. These nutrients are made available to plants by microbiome predators, particularly predatory protists (Bonkowski, 2004; Geisen et al., 2016).

Predatory protists represent the dominant protist group in soils (Oliverio et al., 2020), whose functional role arguably is predominantly their preying on bacteria (de Ruiter et al., 1995; Gao et al., 2019; Geisen et al., 2018). However, especially in the last years, it has become apparent that the functional importance of predatory protists (subsequently termed protists) in soils might also include fungivory and predation on other eukaryotes (Dumack et al., 2016; Geisen et al., 2016). For example, protists have been shown to prey on plant-pathogenic eukaryotes such as *Plasmodiophora brassicae* (Schwelm et al., 2023) or *Fusarium culmorum* (Geisen et al., 2016). Nevertheless, although protists prey on a broad range of organisms, their predation can be selective (Amacker et al., 2022; Dumack et al., 2019; Glücksman et al., 2010).

Protist feeding might be determined by traits of both predator and prey, most particularly body size (biomass or volume) (Amacker et al., 2022; Geisen et al., 2022; Hu et al., 2023; Pedersen et al., 2011). In fact, the sizes of both predator and prey determine successful predation as many (especially amoeboid) protists engulf the entire prey or use their "mouth" opening in the case of ciliates (Leander, 2020). Although size restrictions might be especially prevalent for eukaryotic prey, due to their larger size compared to bacterial prey, predation of protists on larger eukaryotes such as nematodes has been observed (Geisen et al., 2015). As a result of trait conservatism, variation in feeding by protists has also been suggested to be predicted by phylogenetic relationships (Gao, 2020; Pedersen et al., 2011). However, even closely related protist

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species have been shown to exhibit different feeding preferences (Amacker et al., 2022), mainly driven by predator volume (Glücksman et al., 2010). This suggests that phylogenetically conserved traits are of minor relevance to explain feeding differences, but that more variable traits have a more dominant role. Lastly, prey traits such as nutrient content might also influence protist-prey interactions as shown for marine ciliates (Gruber et al., 2009). In turn, prey selection might be a consequence of the prey's nutrient content and ratios (Flynn et al., 1996).

Historically, most studies investigating protist–prey relationships focused on bacterivory (Amacker et al., 2022; Gao, 2020; Glücksman et al., 2010) and rarely included prey sources belonging to other kingdoms (Estermann et al., 2023), allowing only limited insights into the relevance of protists in soil food webs. Furthermore, no study focused on a combination of prey from different kingdoms (only manipulations of bacterial diversity, e.g., Petchey, 2000; Saleem et al., 2013; Yang et al., 2018) including an increased diversity of prey containing more varied nutrient sources, which might increase predator performance.

In this study, we aim to investigate predator–prey interactions among three Amoebozoa and three Heterolobosea species, which are common soil predatory protists (Geisen et al., 2014), and three potential microbial prey species (a plant pathogenic protist species, *Plasmodiophora brassicae*, 3–5 μm in diameter; a common eukaryotic model species, *Saccharomyces cerevisiae*, 5–7 μm in diameter; and a common prokaryotic model species, *Escherichia* coli, 0.5–1.5 μm in diameter), either as a single prey as well as in all possible combinations. To do so, we calculated protist and prey abundances after three, seven, and nine days. We correlated feeding preferences to body size (calculated as volume) and the phylogenetic group of the predator species to determine potential traits predicting predator–prey links. Our hypotheses were as follows: (1) protist growth (an increase of relative abundance compared to controls) differs depending on both predator and prey identity, with bacteria (*E. coli*) representing a more suitable prey for protist growth than the eukaryotes *P. brassicae* and *S. cerevisiae*; (2) protist volume determines prey suitability, with larger predator size enabling increased predation on larger prey (the two eukaryotic taxa) positively affecting protist abundance, while phylogenetic group has no effect on prey suitability; (3) protist volume determines the amount of prey consumed, with larger predators consuming more than smaller ones, while phylogenetic group has no effect on prey consumption; and (4) increasing prey diversity increases protist growth.

2. Material and methods

2.1. Cultivation

2.1.1. Escherichia coli

The uracil-requiring *Escherichia coli* strain OP50 was bought from Caenorhabditis Genetics Center (CGC; https://cgc.umn.edu/). This specific strain was selected because of its inability to reproduce without an external uracil source, limiting its growth. Prior to inoculation of the protist cultures, OP50 was grown in Lysogeny broth (LB; 1 L demineralized water with 10 g bacterial peptone (OXOID, UK) + 10 g NaCl (Duchefa biochemie, The Netherlands) + 5 g yeast extract (OXOID, UK)) at 37 °C and placed on an orbital shaker (New Brunswick Innova 4335 Refrigerated incubator shaker, USA) at 100 rpm for 16 h. Afterward, the bacterial cultures were washed three times by centrifugation (Multifuge 3 S-R Refrigerated Centrifuge (Heraeus, US)) at 1500 rpm for 5 min, removing the supernatant, and resuspending in 40 mL Neff's Modified Amoebae Saline (NMAS, Page, 1976) before the final resuspension in 50 ml NMAS. Optical density (OD) OD_{600} of the *E. coli* OP50 solution was used to estimate bacterial abundance using a Pharmacia Novaspec II spectrophotometer (Pharmacia Biotech Novaspec II, The Netherlands).

2.1.2. Protists

Six soil protist species of in-house cultures were used: *Cryptodifflugia operculata* (isolate 75), *Acanthamoeba* sp. (isolate M4), and *Vannella* sp. (isolate P147) belonging to the Amoebozoa phylum and *Naegleria clarki* (isolate P2881), *Allovahlkampfia* sp. (isolate P10), and Heterolobosea sp. (isolate 32 M) for the Heterolobosea phylum. The protist species used in the experiment were derived from established cultures and identified by their 18S rRNA gene sequence (*C. operculata* 75, *Acanthamoeba* sp. M4, *Vannella* sp. P147, *N. clarki* P2881, and *Allovahlkampfia* sp. P10) (Amacker et al., 2022; Gao et al., 2022) or isolated from soils (Heterolobosea sp. 32 M) as previously described (Geisen et al., 2014).

For the experiment, precultures were grown in NMAS in six-well plates (Greiner Bio-One B.V., The Netherlands), which were sealed with parafilm and incubated at 15 °C in the dark. After seven and 14 days, the plates were checked for protist growth under an inverted Zeiss Axioskope 2 Plus (Zeiss, Germany) microscope at 10× and 20× magnification (objectives; Leica, Germany). Subsequently, wells containing single protist species were selected, and 15 μL were transferred to each well of a six-well plate (Greiner Bio-One B.V., The Netherlands) with 2 mL of 10 % NB–NMAS + 90 % NMAS.

To obtain sufficient numbers of active protists, the cultures were subcultured weekly for three weeks. 150 μL of protist culture in the six-well plates were transferred into five 60 × 15 mm Petri dishes (Greiner Bio-one, Germany) with 5 mL of 10 % NB-NMAS + 90 % NMAS. Plates were kept in the dark at 15 °C. At the end of the third week, bacteria were minimized from the protist cultures by washing three-fold (centrifugation in 50 ml Falcon tubes at 1200 rpm for 5 min with a Multifuge 3 S-R Refrigerated Centrifuge), discarding the supernatant until 7.5 mL was left to avoid losing protists and refilled with 32.5 mL NMAS. We used an IXplore Standard inverted microscope (Leica, Germany) to calculate the abundance of each of the six protist species. To do so, we added 100 μL of each species to three wells in a 96-well plate (Greiner Bio-One, The Netherlands) and counted the individuals in three different areas of each well at 100× magnification. To standardize the counting for every species, we counted the individuals present in one screen on the top of the well, one on the bottom left and one on the bottom right, creating a triangle. The average of the three screens per well was calculated, and the total amount of protists in the entire well was calculated by extrapolating the number of individuals present in the surface counted (0.00264 cm^2 per screen) to the total well area (0.32 cm^2).

2.1.3. Saccharomyces cerevisiae

Commercial baker's yeast (Dr. Oetker, Bielefeld, Germany) was used as *S. cerevisiae* yeast prey. The yeast was suspended in NMAS and the concentration was determined by manual cell counting with a hemocytometer and adjusted to 1.25 million cells/ml in NMAS prior inoculation of protist cultures.

2.1.4. Plasmodiophora brassicae

Resting spores of *P. brassicae* were isolated from clubroots of *Brassica oleracea*. Therefore, frozen clubroots were washed with tap water and homogenized in a household blender in 500 ml of sterile water. The homogenate was filtered through two layers of 220 mm filter paper (Universal Hygia, The Netherlands) and the filtrate was sedimented by centrifugation. Spores were further purified using a two-step ficoll gradient (32 and 16 %, Ficoll 400 (w/v), Carl Roth GmbH & Co. KG, Germany) centrifugation (Mehrabi et al., 2018) to separate the resting spores from bacteria and soil particles. Spores were collected from the interphase of the 16–32 % ficoll layer, and sedimented by centrifugation (11,800 rpm for 1 min) in a standard microcentrifuge (Eppendorf 5417, Germany). The spores were resuspended in 70 % ethanol by vortexing, incubated for 1 min and the suspension was then diluted with three times the volume of sterile water. Spores were sedimented again by centrifugation followed by three-times washing by centrifugation in sterile water. Resting spore concentration was determined by manual cell counting using a hemocytometer and the concentration was adjusted to 1.25 million spores/ml in NMAS prior inoculation of protist cultures.

Table 1
Morphological measurements, groups, and formulas used to calculate biovolumes of the six studied predatory protist species.

Species	Morph. group	Formula	Length (μm)	Width (μm)	Volume (μm^3)
Cryptodifflugia operculata 75	Sub-cylindrical	VC = hπr^2	23.31	17.48	5593.9
Heterolobosea sp. 32 M	Sub-cylindrical	VC = hπr^2	22.33	11.80	2442.0
Naegleria clarki P2881	Sub-cylindrical	VC = hπr^2	23.47	6.02	668.1
Allovahlkampfia sp. P10	Sub-cylindrical	VC = hπr^2	12.95	7.85	626.8
Vannella sp. P147	Flat/Fan-shaped	VC = hr/2	21.22	12.41	131.7
Acanthamoeba sp. M4	Flat/Fan-shaped	VC = hr/2	17.52	9.62	84.2

2.2. Protist volume measurement

We first measured length and width by inspecting protist cultures under an IXplore Standard inverted microscope (Leica, Germany) and analysed with an Axiocam 712 color camera (Zeiss, Germany) using the software Zeiss Zen 3.7. The software was calibrated for every microscope's objective beforehand. We took pictures and measured the width and length (active individuals) of 10 different individuals per species. Then, we calculated a proxy for volume by dividing the protist into two morphological groups as previously done by Amacker et al. (2022) and Gao (2020), and calculating the volume in consequence (Table 1). For species with mainly cylindrical or sub-cylindrical shape (*Allovahlkampfia* sp. P10, *C. operculata* 75, Heterolobosea sp. 32 M, and *N. clarki* P2881), we used the formula VC = hπr^2 where h = length and r = width/2. For flat or fan-shaped species (*Acanthamoeba* sp. M4 and *Vannella* sp. P147), we used the formula VC = hr/2 where h = length and r = width.

2.3. Experimental design

This *in vitro* experiment was set up to examine predator–prey interaction between three prey species and six soil predatory protists (prey: *E. coli*, *P. brassicae*, and *S. cerevisiae;* predators: *Acanthamoeba* sp. M4, *Allovahlkampfia* sp. P10, *C. operculata* 75, Heterolobosea sp. 32 M, *N. clarki* P2881, and *Vannella* sp. P147). The three prey species possess different nutrient contents. Resting spores of *P. brassicae* are mainly composed of chitin, lipids, and carbohydrates (Bi et al., 2016; Clarholm, 1981), *S. cerevisiae* cell walls are mainly composed of polysaccharides, proteins, and chitin (Cabib et al., 2001), while *E. coli* is mainly composed of polysaccharides, phospholipids and proteins (Schnaitman, 1970). Each predator was cultured with the prey in all the possible combinations, resulting in eight treatments (Fig. 1). The experiment was carried out using six-well plates (Greiner Bio-One B.V., The Netherlands) in which five of the wells were used as replicates per species per treatment, ending up in a total of 55 six-well plates and 275 filled wells (Fig. 1). This approach prevented possible contamination between different prey and predator treatments. Each plate was filled with NMAS and the respective protist and/or prey species. As controls, we used the predatory protist species in the liquid media (NMAS) without prey, and also the respective prey species in NMAS media without the predatory protists.

The concentration of both prey source and protists was normalized to add approximately the same abundance to each well. The initial abundance of predatory protists was 167 individuals per well, except for 95 individuals per well of the by far largest organism, *C. operculata* 75. As food source, and to adjust the concentration of the prey based on prey size, the abundance of *E. coli* was 2.5 million cells/well, for yeast and *P. brassicae* 1.25 million cells/well. In the combined prey treatments, the total amount of prey was the same as for the individual prey treatments. To achieve so, we reduced the abundance of each prey by half when combined with another prey, and to one-third when combined with two other prey. Prey and protist species were inoculated on the same day.

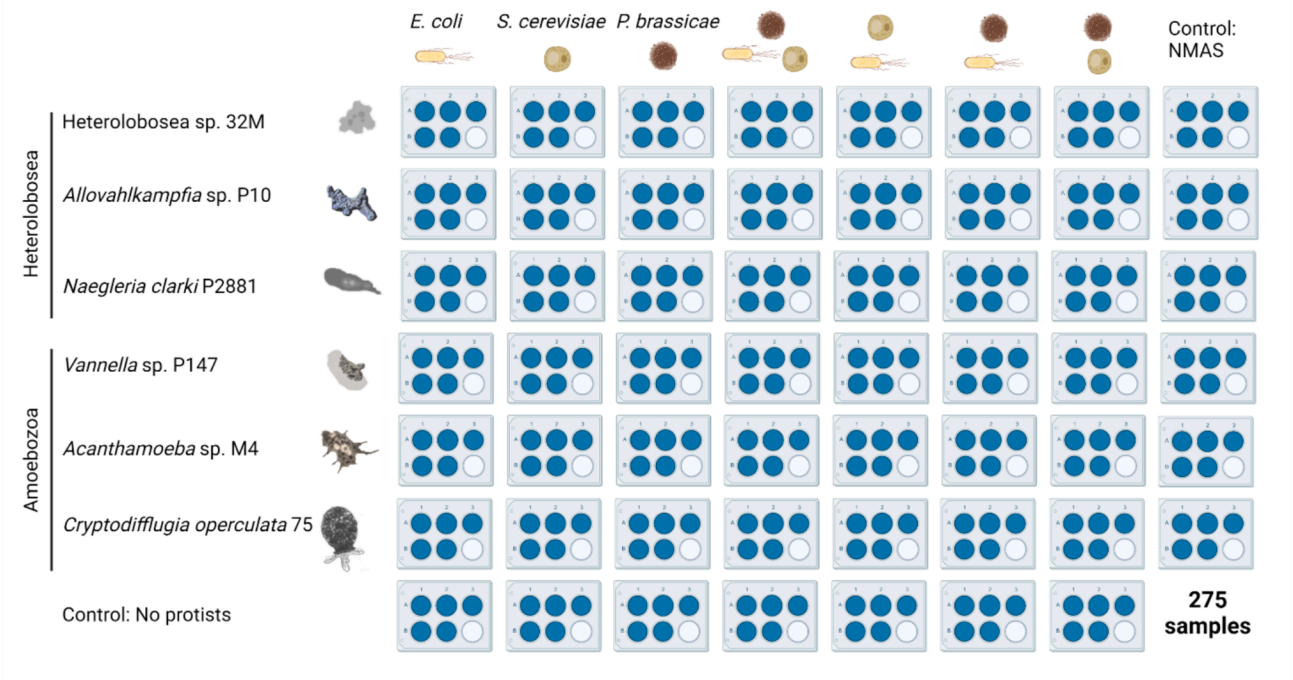

Fig. 1. Graphical representation of the experimental design. The three prey organisms and their combinations are shown on top. The six predatory protist species are depicted on the left. Each six-well plate had five replicates, here coloured in dark blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.4. Regular counting of protists

Both active and inactive (cysts) protist cells were counted after three, seven and nine days post inoculation for each of the 275 wells. However, we only focus on the total abundance as an integration of both protist stages. Therefore, the six-well plates were placed under an Ixplore Standard inverted microscope (Leica, Germany) with a 20× magnification objective and active protists and cysts in three screens were counted as described above. The counting was performed by the same person to reduce biases. We used three approaches to determine protist growth: 1) The total abundance of the different protist species calculated as active cells + inactive cells. 2) Relative abundance in respect to the controls calculated as 'abundance day 9 of the protist with food source/abundance day 9 of the control'. In this way, we obtained the fold-change increase in protist abundance per treatment when compared to the control. A result of 1 would therefore show no difference in abundance with the control. 3) Growth rate was calculated as (ln(end abundance)–ln(initial abundance))/(duration of the experiment in days). We calculated growth rates for different time intervals (0 to 3, 3 to 7, 7 to 9, or 0 to 9 days post-inoculation).

2.5. Determination of prey consumption rate by qPCR

At 3 days post-inoculation and 9 days post-inoculation, 1 ml of each well was collected in a 2 ml centrifuge tube and centrifuged for 3 min at 14,000 rpm on a standard table microcentrifuge (Eppendorf 5417, Germany). Immediately after centrifugation, the top 800 µl were carefully removed and the remaining sample was frozen, lyophilized and resuspended in 25 µl sterile milliQ water (Purelab/Elga, United Kingdom). DNA extraction was performed as previously described. Therefore, an equal volume of lysis buffer containing 0.2 M NaCl (Duchefa Biochemie, The Netherlands), 0.2 M Tris–HCl (pH 8.0; VWR, The Netherlands), 1 % (v/v) β-mercaptoethanol (Sigma, The Netherlands) and 800 µg/ml proteinase-K was added followed by incubation at 65 °C and 750 rpm for 1.5 h and 5 min incubation at 95 °C in a Thermomixer (Eppendorf, Hamburg, Germany). The lysate was then diluted ten times and stored at −20 °C for posterior qPCR analyses. The amount of prey in each sample was estimated by qPCR using specific primers for *E. coli* (401F and 611R, Walker et al., 2017), *S. cerevisiae* (SCDF and SCDR, Chang et al., 2007), and *P. brassicae* (TC1F and RTPbR1a, Cao et al., 2014). The qPCRs conditions for *S. cerevisiae* were as follows: 98 °C for 3 min, followed by 35 cycles of 10 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C and a final step of 10 min at 72 °C. The qPCR conditions for *E. coli* were as follows: 98 °C for 3 min, followed by 35 cycles of 10 s at 95 °C, 20 s at 68 °C, 45 s at 72 °C and a final step of 10 min at 72 °C. The qPCR conditions for *P. brassicae* were as follows: 94 °C for 3 min followed by 40 cycles of 30 s 94 °C, 30 s at 60 °C, 30 s at 72 °C and a final step of 10 min at 72 °C. All qPCRs were performed using the iQTM SYBR® Green Supermix (Bio-Rad, Hercules, USA).

2.6. Statistical analysis

All statistical analyses were performed in R software ver. 4.2.2 (Development Core Team, 2015). Figures were created using the package ggplot2 (Wilkinson, 2011) and finalized using Adobe Illustrator ver. 26.5.3 (https://www.adobe.com/products/illustrator.html). We decided to use relative abundances (fold change with respect to controls) and growth rates instead of total abundances to be able to compare results among species. In this way, we avoided marked differences in total abundances derived from growth rates inherited from individual species' ecology. Prior to the analyses of treatment effects, we checked for the residual's normality of the models by visually inspecting the model assumptions using the function *plot*. Furthermore, a Saphiro–Wilk test was performed to assess the normality by using the nortest package (Gross and Ligges, 2015). We applied a log + 1-transformation in case models of relative abundance data (which included zeros) exhibited violated normality assumptions. We modelled 'Growth rate' or 'Protist relative abundance' in response to fixed factors 'Protist species' and 'Prey treatment', as well as their interaction, and examined their significance using ANOVA. Tukey's HSD post hoc tests (R package multcompView) were performed to detect the underlying drivers of significant main effects on protist abundance. To be consistent with the analyses performed for abundance (total and relative) where we focused on the measurements from the last day, we present the results of growth rates from day 0 to day 9 in the main text and show other time intervals in the Supplementary material.

While model assumptions regarding normality and independence of the data were not violated in these models, we acknowledge that the residuals of this model indicate non-homogeneity of variances, with *Cryptodifflugia operculata* and *Acanthamoeba* sp. exhibiting strongly different variances (see Supplementary Fig. S1). To overcome this problem we also analysed the data with total predator counts as response variable. To this aim, we constructed a generalized linear model with a negative binomial distribution (glm.nb) to correctly model our overdispersed data. This analysis revealed very similar effects of predator species identity and prey treatment on predator abundance. Because our analysis based on relative abundances visually allows for comparisons among predator species, we decided to present the figures based on this model.

To test for the effect of the phylogenetic group on protist abundance, we modelled 'Protist relative abundance' in response to fixed factors 'Protist species' and 'Phylogenetic group'. While 'Protist species' should have ideally been used as a random effect, we only had three species per phylogenetic group, what impeded us to use it as such. In terms of volume effects on protists abundance, we correlated 'Average relative abundance' and 'Protist volume' via the function *cor.test*. The decision to average all data points of relative abundance per protist species and the correlation with protist volume was done to avoid pseudo-replication in our analysis.

To model predator species' effects on prey consumption, we used qPCR-based Cq-values. There is an inverse relation between Cq values and copy numbers of target genes (approximately representing DNA amount of target organisms), with higher Cq-values indicating lower amounts of DNA. In order to test predator identity and prey species effects on prey consumption, we modelled Cq-values from the last time point (day 9) using Cq-value from the first extraction (day 3) as a covariate, in addition to fixed effects 'Protist species' and 'Prey species' and their interaction. We added Cq values from the first day as a covariate to control for potential differences in prey abundance at the time of inoculation. On the other hand, to model phylogenetic group or volume effects on prey consumption, we modelled Cq values from the last time point with 'Cq-value from the first day' and 'Protist species' as a covariate, in addition to fixed effects 'Phylogenetic group' or 'Volume' and 'Prey species and their interaction'.

Lastly, to model prey diversity effects on predator abundances (hypothesis 4) we also used both models: 1) A linear model with 'Protist relative abundance' in response to fixed factors 'Protist species' and 'Prey Diversity' (0, 1, 2, 3), as well as their interaction, and 'Prey Treatment' as a random factor to then examine their significance using ANOVA (*car::Anova*). 2) A generalized linear mixed effects model with a Poisson distribution (*glm*) with 'Protist relative abundance' in response to fixed factors 'Protist species', 'Prey Diversity' and 'Bacteria Presence' as well as their interaction, and used 'Prey Treatment' as random factor. Because our analysis based on relative abundances visually allows for linear regressions, we decided to present the figures based on this model, and leave the ones representing total counts in Supplementary material.

3. Results

3.1. Protist abundance depending on food source

We found that protist population growth (relative abundances) depended on predator–prey species combination (ANOVA, p-value < 0.001; Fig. 2; for total abundances, see Supplementary Fig. S2). On

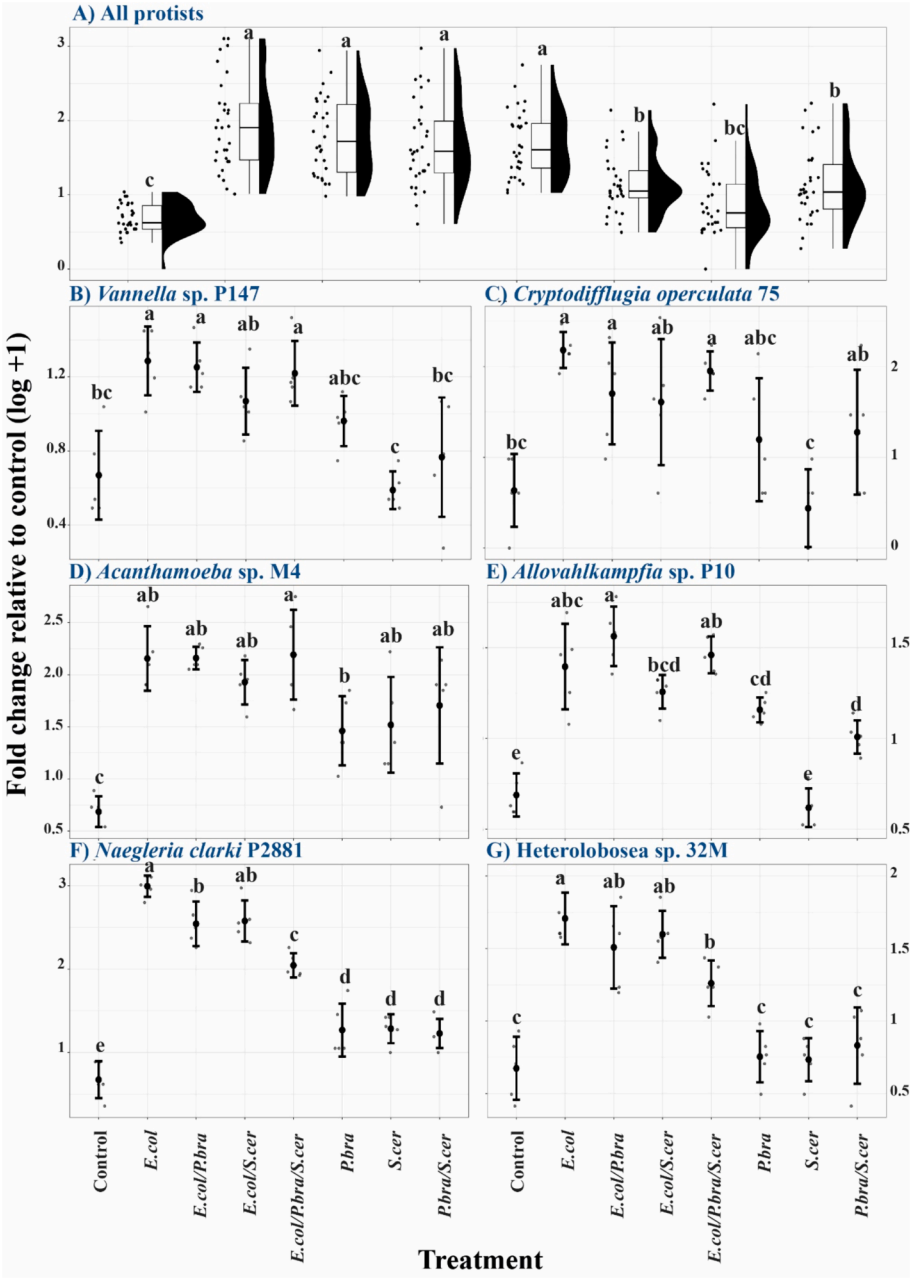

Fig. 2. Protist abundance relative to control (fold change) at 9 days post inoculation for all protist species combined (A) and separately (B–G). (**A**) Here we show the distribution of the data in three complementary ways: through the visualization of the raw data (dots), through a boxplot showing the quartiles, and in a violin plot. The wider the part of the violin plot, the more data points are accumulated in that fraction. The legend of panel A corresponds to the same legend as the one for panels B–G. (**B–G**) Individual species with their scientific name shown above. Letters represent the statistical comparisons obtained through a Tukey HSD Test. If letters are shared, no significant differences were observed. Error bars represent the standard deviation and the thick dot represents the mean. Data shown in this figure were log + 1 transformed to meet the normality of the residuals. *E.col*, *Escherichia coli*; *P.bra*, *Plasmodiophora brassicae*; *S.cer*, *Saccharomyces cerevisiae*.

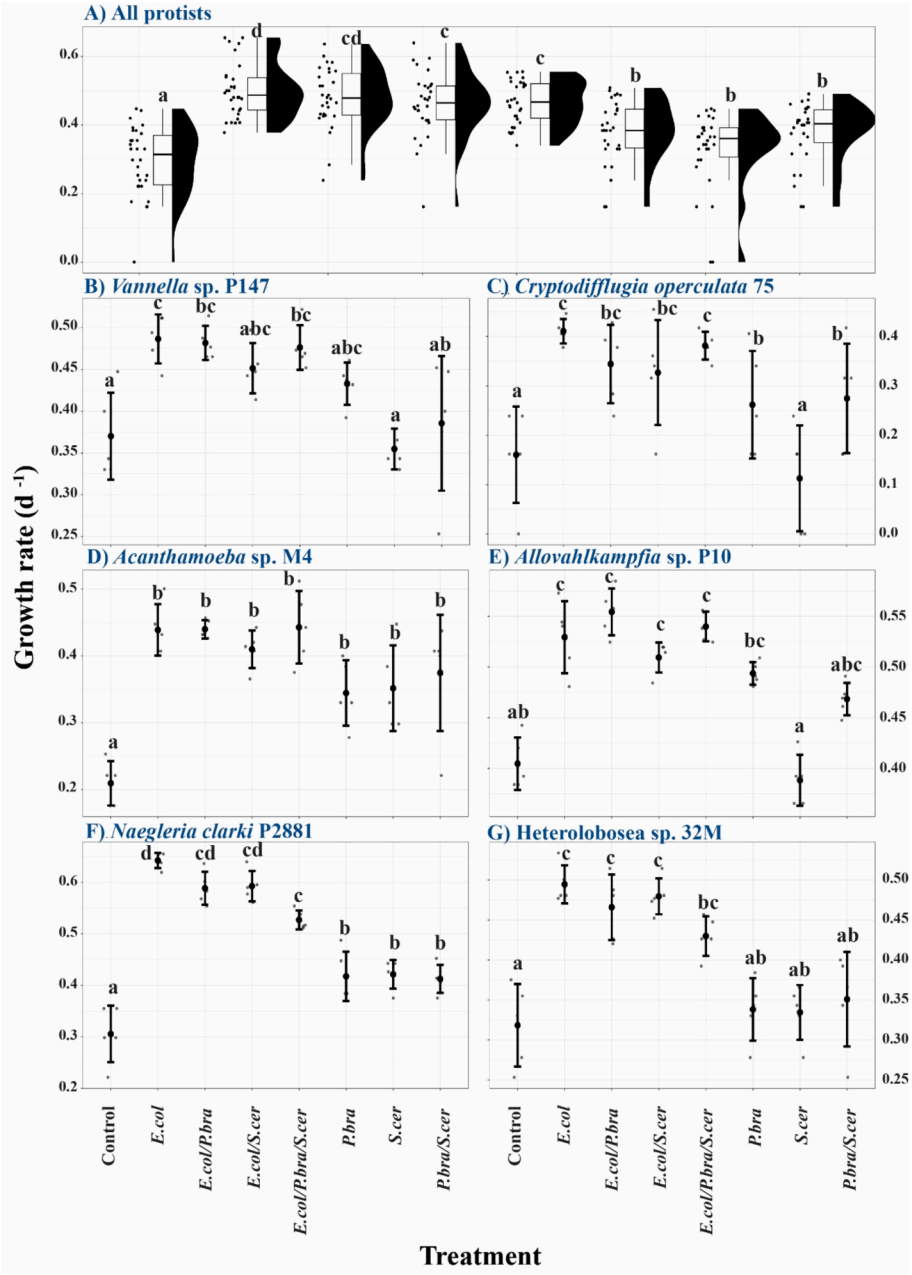

Fig. 3. Growth rates from 0 to 9 days post inoculation for all protist species combined (A) and separately (B–G). (**A**) Here we show the distribution of the data in three complementary ways: through the visualization of the raw data (dots), through a boxplot showing the quartiles, and in a violin plot. The wider the part of the violin plot, the more data points are accumulated in that fraction. The legend (x-axis) of panel A corresponds to the same legend as the one for panels B–G. (**B–G**) Individual species with their scientific name shown above. Letters represent the statistical comparisons obtained through a Tukey HSD Test. If letters are shared, no significant differences were observed. Error bars represent the standard deviation and the thick dot represents the mean. *E.col, Escherichia coli*; *P.bra, Plasmodiophora brassicae*; *S.cer, Saccharomyces cerevisiae*.

average, the highest protist growth was shown with *E. coli* as the single food source (6x compared to controls without prey source; Fig. 2A; Tukey test; p-value < 0.001) and lowest with only *S. cerevisiae* present (no significant difference to controls without prey; Fig. 2A; Tukey test; p-value $= 0.77$). Similarly, when looking at the individual protist species, the highest growth was detected when *E. coli* as prey was present (e.g., 3x increase to control of *N. clarki* P2881 with only *E. coli* as prey; Fig. 2B–G). Compared to the control, Heterolobosea sp. 32 M did not grow without *E. coli* as present prey. For *N. clarki* P2881, the growth appeared to correspond to the total amount of *E. coli* prey in the treatment. Therefore, the highest growth was shown with *E. coli* as sole prey (19.1x), which then was reduced to 12.5x (*E. coli/S. cerevisiae*) and 12.1x (*E.coli/P. brassicae*) and to 6.8x in the *E. coli/P. brassicae/S. cerevisiae* combination (Fig. 2F). *Acanthamoeba* sp. M4 (3.5x; Tukey test; p-value $= 0.028$), *Allovahlkampfia* sp. P10 (2.18x; Tukey test; p-value < 0.001), and *N. clarki* P2881 (2.7x; Tukey test; p-value $= 0.003$) showed population growth in the treatment with *P. brassicae* as the only prey. Additionally, *Acanthamoeba* sp. M4 (4x; Tukey test; p-value $= 0.015$) and *N. clarki* P2881 (2.65x; Tukey test; p-value $= 0.002$) also grew with *S. cerevisiae* as the only prey. We also found that *S. cerevisiae* and *P. brassicae* cells grouped around or even were ingested by *C. operculata* 75 (Supplementary Fig. S3).

Moreover, we found that growth rates from day 0 to day 9 varied depending on predator–prey species combination (ANOVA, p-value < 0.001; Fig. 3; for growth rates of other time intervals, see Supplementary Fig. S4–S6). These growth rates positevely correlated with the relative abundance results observed at the last time point (Pearson correlation test, p-value < 0.001, correlation coefficient of 0.59). Consequently, and to be consistent with qPCR-based results, we decided to use analyses based on relative abundances throughout the manuscript.

3.2. Protist phylogenetic group and volume related to their abundances

Neither protist volume nor the interaction between volume and treatment (Supplementary Fig. S7) affected protist growth when combining all treatments (ANOVAs; p-value > 0.05; Fig. 4).

While the protist growth was not impacted by their phylogenetic group (ANOVA; p-value $= 0.48$; Fig. 5), their growth was impacted differently depending on the treatment (ANOVA; p-value $= 0.02$; Supplementary Fig. S8).

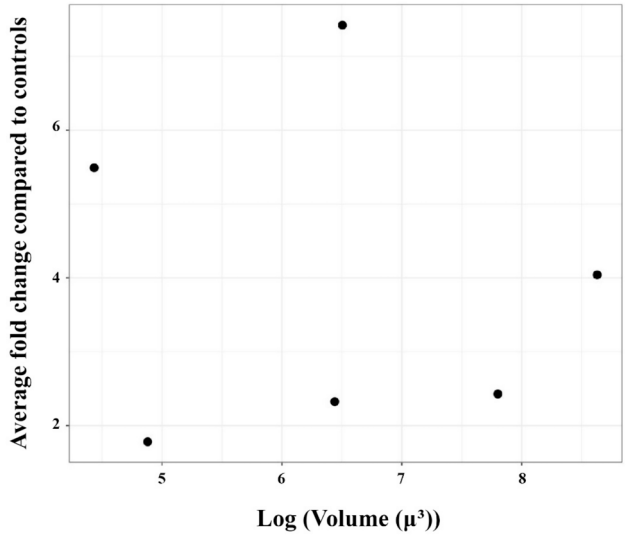

Fig. 4. Scatter plot showing the relationship between average protists abundance (fold change relative to control; y–axis) and protist volume (log-transformed for visualization purposes).

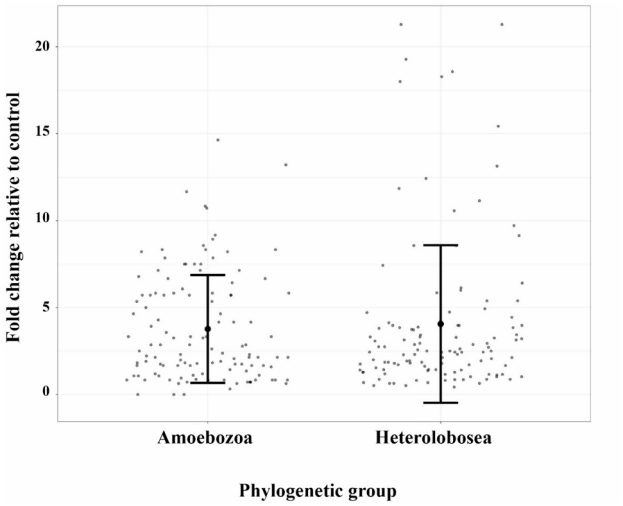

Fig. 5. The relationship between protist abundance folds changes relative to the control (y-axis) and phylogenetic group (x-axis) with all data combined. Small dots represent the raw data, error bars represent the standard deviation and the thicker dot represents the mean.

3.3. Prey consumption depending on phylogenetic group and volume

When testing for the effect of protist species identity on prey consumption, we observed a significant interaction between protist identity and prey type (ANCOVA; p-value < 0.001; Supplementary Fig. 9). When sorting the predator species from smaller to larger volumes, we observed no correlation between volume and prey consumption for any of the prey species (*E. coli*, *P. brassicae*, or *S. cerevisiae* (ANCOVAs; p-value > 0.05; Fig. 6). However, when removing *C. operculata* 75 from the data, we observed a positive linear effect of volume on *E. coli* consumption (ANCOVA; p-value < 0.001; $R^2 = 0.26$; Supplementary Fig. S10). Additionally, the prey consumption was also differently influenced by the interaction between treatment and protist species (ANCOVAs; p-value $= 0.04$; Supplementary Fig. S11).

The phylogenetic group had a significant effect on prey consumption depending on prey type (ANCOVA; p-value < 0.001; Fig. 7) or treatment (ANCOVA; p-value < 0.001; Supplementary Fig. S12). The consumption of *S. cerevisiae* was not different between phylogenetic groups (t-test; p-value > 0.05). But in the case of *P. brassicae*, Amoebozoa showed a higher consumption—as seen by the higher Cq values—compared to Heterolobosea (t-test; p-value $= 0.007$; Cq decrease of 3.4 % from Amoebozoa to Heterolobosea). Similarly, *E. coli* consumption was also affected by the protist phylogenetic group with Amoebozoa consuming less (lower Cq values) than Heterolobosea (t-test; p-value < 0.001; Cq increase of 19 % from Amoebozoa to Heterolobosea).

3.4. Prey diversity effect on protist abundance

An increasing number of prey species positively affected protist growth (ANOVA; p-value $= 8.081e{-}14$; $R^2 = 0.08$; Fig. 8A; for total abundances, see Supplementary Fig. S13). This effect was, in turn, dependant on protist species (ANOVA; p-value $= 0.04$). *Vannella* sp. P147 (ANOVA; p-value $= 0.04$; $R^2 = 0.13$; Fig. 8B), *Acanthamoeba* sp. M4 (ANOVA; p-value < 0.001; $R^2 = 0.33$; Fig. 8D), *Allovahlkampfia* sp. P10 (ANOVA; p-value $= 0.01$; $R^2 = 0.29$; Fig. 8E), and *Naegleria clarki* P2881 (ANOVA; p-value < 0.001; $R^2 = 0.35$; Fig. 8F) increased growth with an increasing number of different potential preys. *Cryptodifflugia operculata* 75 only showed a marginally significant linear trend (ANOVA; p-value $= 0.07$; $R^2 = 0.10$; Fig. 8C), while Heterolobosea sp. 32 M growth was not affected by an increasing number of prey species (ANOVA; p-value > 0.05; Fig. 8G). When considering only treatments containing prey (1–3 species, no controls without prey), a positive linear trend was only

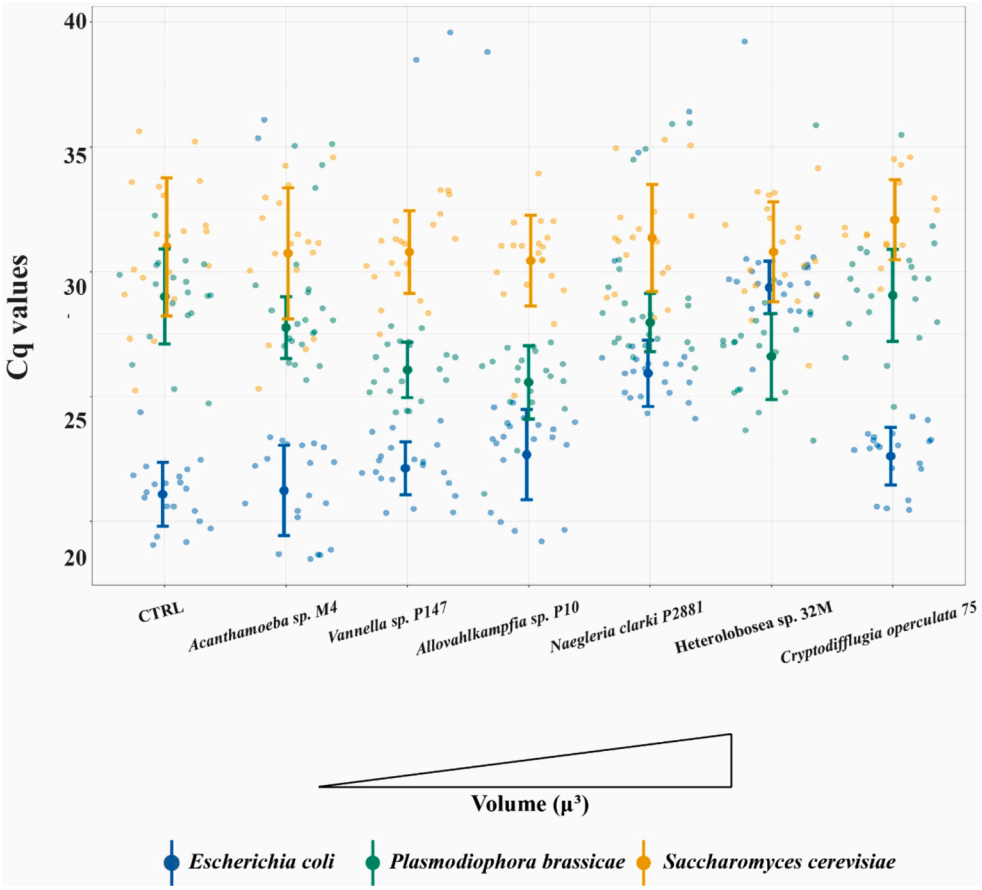

Fig. 6. Scatter plot representing the relationship between protist species identity (x-axis) and the Cq values of the different prey at 9 days post inoculation. While the model was performed using the Cq values at the first time point as a covariant to control for variations in the inoculation, here we only show the Cq values at 9 days post inoculation for visualization purposes. The y-axis representing Cq values has to be interpreted as follows: the higher the Cq value the lower the amount of DNA. The data shown represents all treatments together (for data separated by treatment, see Supplementary Fig. S8). The small dots represent the raw data, the bars represent the standard deviation, and the thicker dot represents the mean. Species are sorted in an increasing volume, from the smallest *Acanthamoeba* sp. M4 to the largest *C. operculata* 75. CTRL, Control (only prey added).

visible for *Acanthamoeba* sp. M4 (ANOVA; *p*-value = 0.07; R^2 = 0.09; Fig. 8D) and *Allovahlkampfia* sp. P10 (ANOVA; *p*-value = 0.04; R^2 = 0.13; Fig. 8E), while no significant relationship was observed for the other protist species. Furthermore, when testing for total counts and adding bacterial presence as variable, we observed a three-way interaction between 'Prey Number', 'Protist species' and 'Bacteria Presence' (ANOVA; *p*-value < 0.001; Supplementary Fig. S13). However, when removing *E. coli* data and focusing only on treatments containing prey (1–2 species, no controls without prey), no protist species significantly grew when comparing treatments with one or two species (Supplementary Fig. S13).

4. Discussion

Our results revealed that the growth of the six soil protist species studied here was affected differently by prey type (*E. coli*, *S. cerevisiae*, and *P. brassicae*) and protist identity. This supports our hypothesis of species-specific feeding preferences among protist species (Amacker et al., 2022; Geisen et al., 2016). Protist population growth was highest with *E. coli* as food source and was lower (or even zero) with *P. brassicae* and *S. cerevisiae.* Despite the recognition that some protist species can feed on different eukaryotic prey (Dumack et al., 2016; Estermann et al., 2023; Geisen et al., 2016), soil protists are considered to be mainly bacterivorous in soils (Clarholm, 1981; Gao et al., 2019; Geisen et al., 2016; Krome et al., 2009; Rønn et al., 2002), which is in line with the fact that the strongest population growth of the six protist species was observed when feeding on *E. coli*. Furthermore, the fact that the six protist species performed better with *E. coli* might also be driven by our cultivation on *E. coli* as a prey source in the laboratory, promoting it as a suitable prey. While having a common suitable prey, protist species identity determined growth depending on prey identity. In fact, the propagation of *Allovahlkampfia* sp. P10, *N. clarki* P2881, and *Acanthamoeba* sp. M4 on *P. brassicae* and *N. clarki* P2881 and *Acanthamoeba* sp. M4 on *S. cerevisiae* confirms previous studies showing that some assumed protist bacterivores might actually be generalist feeders (Amacker et al., 2022; Geisen et al., 2016; Potapov et al., 2022). Although we did not observe a statistically significant growth increase of *C. operculata* with *S. cerevisiae* contrasting previous findings (Estermann et al., 2023; Geisen et al., 2016) or *P. brassicae*, we observed that *C. operculata* interacted and even ingested both prey cells, as also reported previously (Geisen et al., 2016; Schwelm et al., 2023). We can also argue that due to the interaction between *C. operculata* 75 and *P. brassicae/S. cerevisiae* cells (Supplementary Fig. S2), protists may serve as vectors for phoresy rather than as predators of specific organisms, as suggested for other soil organisms such as earthworms or nematodes (Topalović and Geisen, 2023; Williams et al., 2006).

We also showed that both predator volume and phylogenetic group did not affect protist growth in a prey-dependent way, and therefore found no support for our second hypothesis. The fact that phylogenetic group did not determine prey suitability is in line with other studies showing that protist feeding patterns are often species-specific and not driven by phylogenetically conserved traits (Amacker et al., 2022;

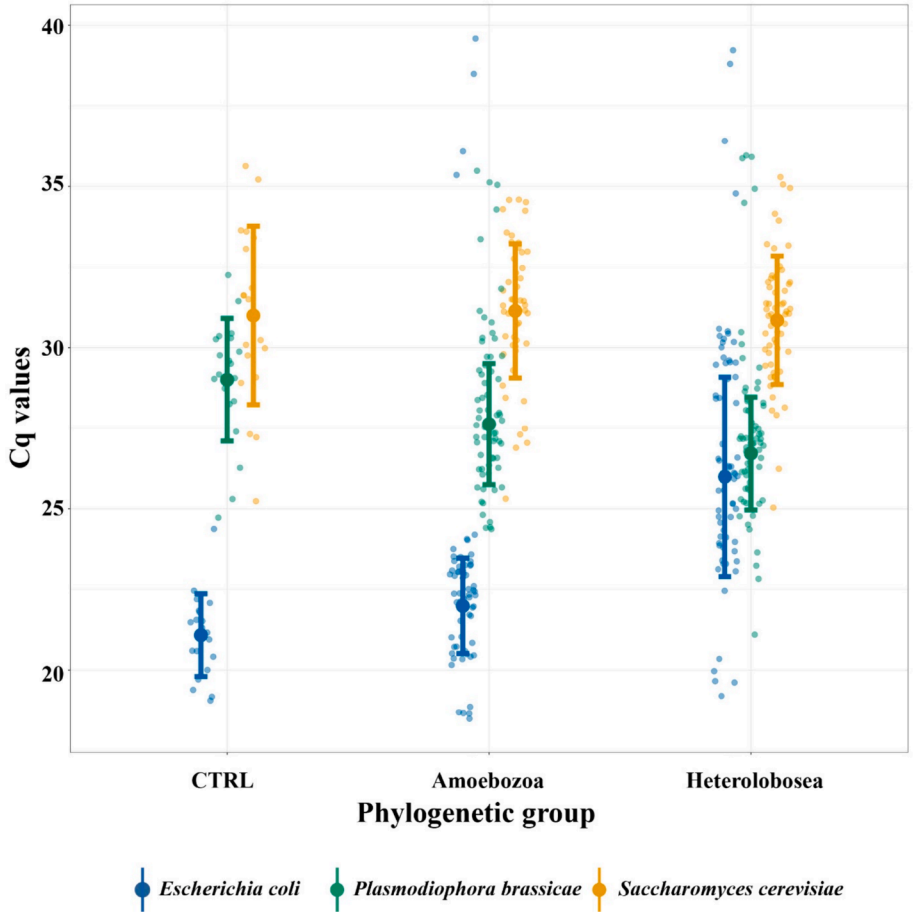

Fig. 7. Scatter plot representing the relationship between the protist phylogenetic group (x-axis) and the Cq values of the different preys at 9 days post-inoculation. The y-axis representing Cq values has to be interpreted as follows: the higher the Cq value the lower the amount of DNA. The small dots represent the raw data, the bars represent the standard deviation, and the thicker dot represents the mean.

Estermann et al., 2023; Guo et al., 2022; Page, 1977). Contrary to our results, protist body size was previously shown to be a key trait determining the feeding preferences and impacts of protists on bacterial communities (Glücksman et al., 2010). The contradiction in our findings, indicating volume as a non-determining trait, compared to earlier studies demonstrating the opposite, could be attributed to differences in prey types utilized. Previous research predominantly concentrated on specific bacteria strains (Amacker et al., 2022; Gao, 2020; Glücksman et al., 2010) whereas our study incorporates prey from three distinct kingdoms with varying sizes. Furthermore, we studied here only a limited number of protist species, making generalizations about body size difficult.

Contrary to our third hypothesis, we expected that prey consumption was not determined by phylogenetic group. However, we observed differences in the prey consumption between both investigated protist phylogenetic groups. This result aligns with those of Glücksman et al. (2010), which showed that closely phylogenetically related protists appear to have similar impacts on bacterial community compositions, potentially suggesting the existence of conserved ecophysiological traits underlying high-taxonomical phylogenetic groups. While an effect of the phylogenetic group on prey consumption was observed, we found no effect of volume on the consumption of any prey. This finding is in contrast with our third hypothesis but aligns with studies showing no effect of protist volume on bacteria consumption (Amacker et al., 2022; Gao, 2020; Glücksman et al., 2010). However, when removing the data from *C. operculata*, we observed a potential influence of volume on *E. coli* consumption, suggesting that other traits like growth rates (*C. operculata* shows a particularly slow growth rate) contribute to differential feeding impacts. Further studies using more protist species are needed to deepen our understanding on the most important traits determining feeding pressure on bacterial communities.

Lastly, we observed that an increase in prey richness positively influenced protist growth. However, this correlation was mainly driven by the presence of *E. coli*, leading to the rejection of our fourth hypothesis. The link between prey richness and protist abundance was then shaped by the likelihood of protists encountering their most suitable prey (*E. coli*) in our experimental setups, rather than a potential positive effect derived from the presence of different nutrient sources (Flynn et al., 1996; Gruber et al., 2009). Therefore, protists reached their highest abundance when all prey species were included, resulting in a 100 % probability of encountering *E. coli* as a potential prey. Notably, the absence of an impact on protist growth concerning prey richness contrasts with the findings of Saleem et al. (2013), where an increased diversity of suitable prey was shown to boost protist cell numbers. In turn, Petchey (2000) showed that an increasing prey diversity did not impact protist abundance but reduced the variation in protist population density. Therefore, to reliably estimate the importance of prey species richness on protist performance future experiments should consider different prey from different phylogenetic groups and different variables to be measured apart from abundances such as population stability.

5. Conclusions

While protist feeding preferences might impact ecosystem functions such as nutrient cycling or services such as pathogen suppression, protist feeding studies are mainly limited to a few species of both protists and

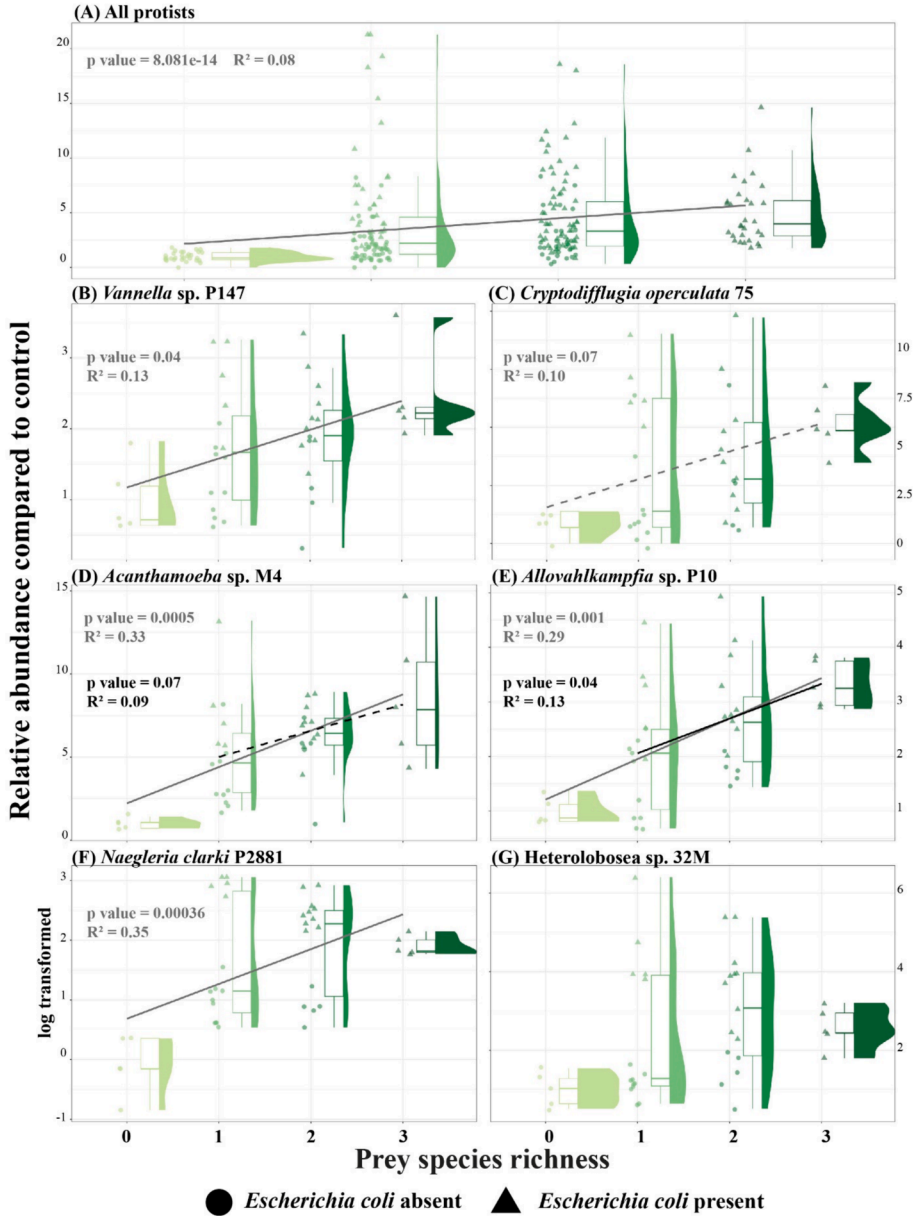

Fig. 8. Combined violin and box plot showing the relationship between an increasing number of preys (x-axis; from 0 species until 3) and the relative abundance of protists compared to control at 9 days post inoculation (y-axis). Here we show the distribution of the data in three complementary ways: through the visualization of the data points (dots), through a boxplot showing the quartiles, and in a violin plot. The wider the part of the violin plot, the more data points are accumulated in that fraction. Panel A represents the totality of the data, combining all protist species. Panels B–G represent one protist species. Lines represent statistically significant linear relationships (continuous lines; p-value < 0.05) or marginally significant ones (dotted lines; p-value between 0.05 and 0.1). Black lines represent the fitted model without the control (0 prey species), and grey lines depict the fitted model considering the control. Dot shapes represent whether *E. coli* was absent (circle) in the treatment or present (triangle).

prey. Here we tested predator–prey links between six soil protist species and three prey types (and all their combinations) and detected that differences in protist growth and prey consumption are mainly species-specific. While *E. coli* was the generally preferred prey, some protist species also thrived on the eukaryotic prey *P. brassicae* and/or *S. cerevisiae* suggesting that these species are more generalist feeders. While we did not observe effects of volume and prey suitability, it is worth noting that our setup lacks the complexity of soil structure, where volume might matter accessing different spatial areas and their associated prey. Additionally, the soil protists used in the experiment achieved their maximum growth rate within the initial three days post-inoculation. This outcome emphasizes the rapid reproduction of many common soil protists in the range of hours in these lab conditions, which should be considered for future feeding experiments.

The fact that three protist species fed and grew on the plant-pathogen *P. brassicae* suggests including protists in more studies to test their potential as future biocontrol agents. Overall, future studies should increase the number of morphologically diverse organisms in more natural soil systems research to investigate other traits that potentially better explain feeding preferences and their potential role in ecosystem functions.

CRediT authorship contribution statement

Alejandro Berlinches de Gea: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Stefan Geisen:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Franka Grootjans:** Writing – review & editing, Methodology, Investigation. **Rutger A. Wilschut:** Writing – review & editing, Formal analysis, Data curation. **Arne Schwelm:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization.

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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejop.2024.126090.

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