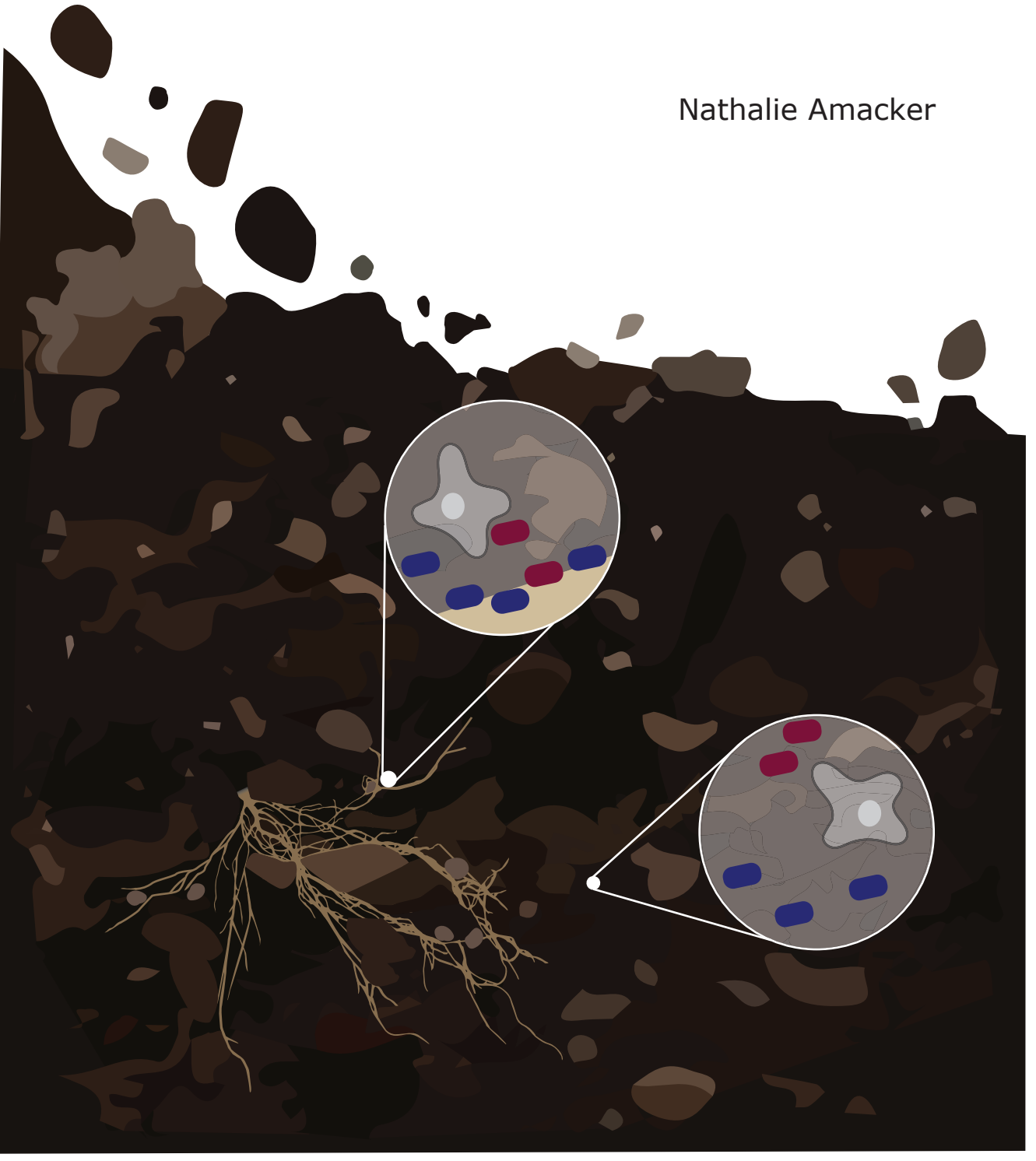
Harnessing the potential of predatory protists to support a beneficial soil microbiome

Nathalie Amacker



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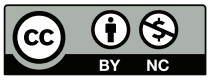

Harnessing the potential of predatory protists to support a beneficial soil microbiome

Het potentieel van roofzuchtige protisten benutten om een gunstig bodemmicrobioom te ondersteunen
(met een samenvatting in het Nederlands)

Exploiter le potentiel des protistes prédateurs pour soutenir un microbiome bénéfique du sol
(avec un résumé en français)

Nutzung des Potenzials räuberischer Protisten zur Unterstützung eines günstigen Bodenmikrobioms
(mit einer Zusammenfassung in deutscher Sprache)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
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rector magnificus, prof.dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

woensdag 22 juni 2022 des middags te 2.15

door

Nathalie Amacker

geboren op 18 december 1990
te Sion, Zwitserland

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Chapter 1

General Introduction

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1. A necessary shift toward sustainable agriculture

The explosion of the global human population during the last century is associated with an increasing demand for food, fuel and fibers (Kopittke *et al.* 2019). At the same time, the intensive exploitation of soils to produce these goods is linked to a variety of environmental costs including land degradation, increased emissions of greenhouse gases, decrease of soil organic matter, loss of above- and below ground biodiversity, and alteration of biogeochemical and hydrological cycles (Balmford and Bond 2005; FAO *et al.* 2020). Especially, the excessive use of fertilizers and pesticides are destroying soils, threatening biodiversity and causing major human health issues (Carson 1962; van Lexmond *et al.* 2015; Foster and Custodio 2019). There is thus an increasingly urgent need to mitigate these effects and move toward sustainable practices so to maintain a viable and favorable habitat for current and future human societies (Foley *et al.* 2011; Funabashi 2018).

2. Plant-microbes interactions

One of the promising approach is to take advantage of the diverse microbial communities inhabiting the soil. Several soil microorganisms deliver important functions to the plants, such as nutrient cycling, hormonal balance optimization and protection against diseases and pests (Bender, Wagg and van der Heijden 2016). This pool of microbes is not only overlooked but has been mismanaged for decades. Agricultural breeding has in many cases diminished the ability of plant cultivars to interact with beneficial microbes (Philippot *et al.* 2013; Rossmann *et al.* 2020) and many agricultural practices tend to lower soil microbial biodiversity (Tsiafouli *et al.* 2015). The inoculation and/or enrichment of specific microorganisms has been seen as a promising approach to restore soil fertility and support yields while using less agrochemicals (Compant *et al.* 2019). Research in this direction has enabled the identification of specific taxa and the associated traits that would lead to improved plant performance. Such plant beneficial micro-organisms include bacteria such as Pseudomonads (Haas and Défago 2005) and *Bacillus* spp. (Saxena *et al.* 2020) as well as fungi such as *Trichoderma* spp. and symbiotic mycorrhizal fungi (Szczałba *et al.* 2019). Various traits associated with biological control of pathogens and pests (*e.g.*, production of antibiotics, resource competition), direct plant growth promotion (*e.g.*, nutrient supply, production of growth hormone) and root colonization have been further linked to a positive effect of the inoculated microorganisms on plant development (Lugtenberg and Kamilova 2009). Inoculating microbial isolates harboring these plant-beneficial traits and/or enhancing them in naturally occurring communities have been increasingly suggested as a way to support plant development (Finkel *et al.* 2017; Wallenstein 2017). While promising in laboratory and well-controlled settings, the application typically faced numerous limitations in term of efficiency and reproducibility in field conditions (Sessitsch, Pfaffenbichler and Mitter 2019).

In addition to bacteria and fungi, it was suggested that the soil protists may be equally or even superior in supporting plant performance by acting as stimulators for the nutrient turnover and regulators of the native microbiome (Gao *et al.* 2019).

3. The protists

Protists are a paraphyletic group referring to all Eukaryotes except plants (Chloroplastida), animals (Metazoa) and Fungi (Figure 1; Burki *et al.* 2020). Soil protists are mostly unicellular and usually display a size range of few micrometers to millimeters (Geisen *et al.* 2017). As to be expected from their broad phylogenetic coverage (Figure 1), protists exhibit a huge variety of morphotypes with the classical division between amoebae, flagellates, ciliates and shell-forming (testate) types (Geisen *et al.* 2018; Burki *et al.* 2020). Including autotrophs, heterotrophs, mixotrophs and parasites, protists exhibit a range of ecological functions from primary producers to decomposers (Geisen *et al.* 2018). In the soil context, the major group is represented by heterotrophic, free-living protists (Figure 1; Oliverio *et al.* 2020; Singer *et al.* 2021; Xiong *et al.* 2021), which were traditionally referred to as "protozoa". Many heterotrophic protists are predators and feed on relatively small prey such as bacteria and yeast by ingesting them via phagocytosis, but others graze and/or attack bigger organisms such as hyphal-forming fungi, nematodes and other micro-eukaryotes (Geisen *et al.* 2015; Geisen 2016).

4. Predatory impacts of protists on the bacterial communities

In their quality of predators, soil protists are mostly known for their role as main consumers of bacteria. As such, protists typically reduce the bacterial biomass (de Ruiter, Neutel, and Moore 1995; Clarholm 2005). Counterintuitively, however, the bacterivorous activity of protists is usually linked with an increase in microbial activity (Saleem and Moe 2014). This discrepancy is generally explained by the increased nutrient turnover that results from protist consumption (Sherr, Sherr, and Berman 1983; Clarholm 1984) as well as the removal of less active, senescent bacterial cells which are more likely to be preyed upon first. The removal of senescent cells lowers competition for space and makes nutrients available for the more active bacterial cells (Bonkowski 2004).

Another crucial characteristic of protist consumption is prey selectivity (Montagnes *et al.* 2008). The protists can sense and discriminate between different prey based on size, shape and chemical properties including specific membrane-bound proteins but also volatiles compounds (VOCs) (Jousset 2012; Schulz-Bohm *et al.* 2017). Such prey discrimination and selectivity cause protists to modify bacterial community composition, favoring a specific subset of species (Bonkowski and Brandt 2002; Rønn *et al.* 2002). Noteworthy, distinct food preferences have been observed for phylogenetically closely related protists with similar morphotypes suggesting that each species has its own food preferences (Glücksman *et al.* 2010).

In response to this predatory pressure, bacteria have evolved different escape and/or defense strategies, including changes in bacterial cell morphology, colony formation, escaping movement and/or production of toxic compounds (Matz and Kjelleberg 2005). Numerous toxic compounds produced by soil Pseudomonads that have long been known

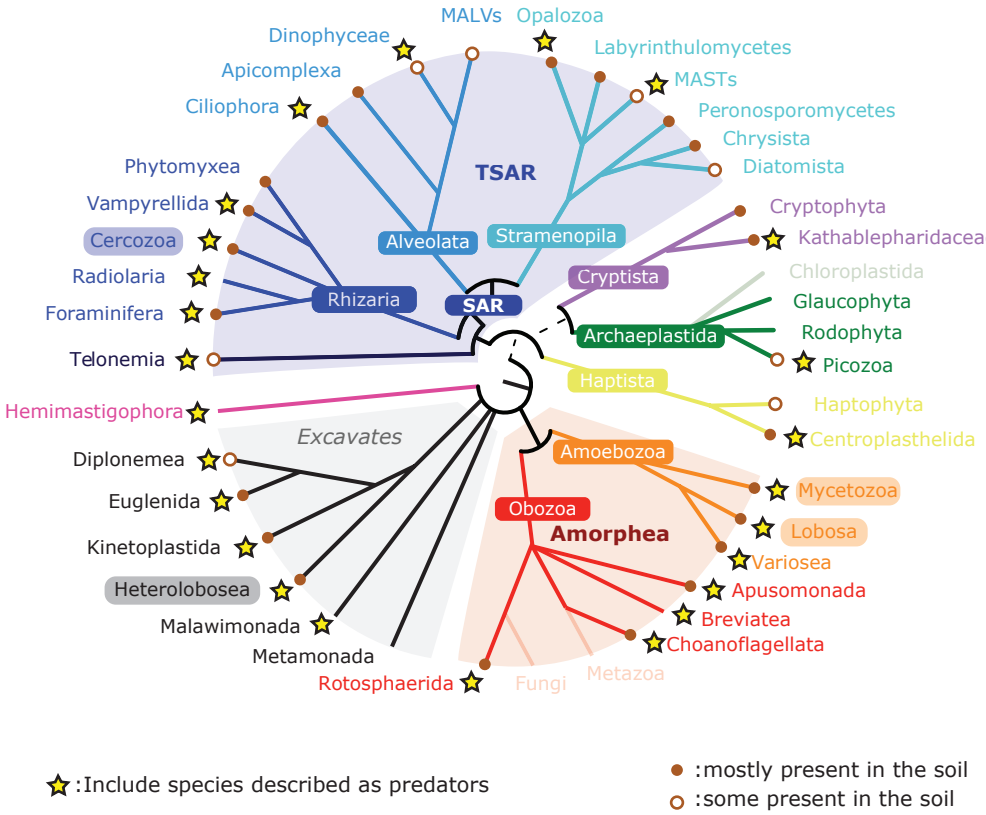

Figure 1:Schematic representation of the eukaryotic diversity based on Burki, Sandin, and Jamy 2021. Stars indicate groups that include predatory protists. Brown dots indicate groups that include terrestrial species based on the literature (see also table S1 for details); the groups were considered as "mostly present" in the soil if they ranked 1st and 2nd compared to freshwater and marine habitat in the study of Singer et al. 2021, and "some present" for the ones ranked 3rd. Note that the supergroup of Excavates is not supported by the most recent phylogenetic analyses (Burki et al. 2020) and is therefore given in italics. Chloroplastida, Metazoa and Fungi are given in transparency as they are not part of the protists. The groups (Cercozoa, Heterolobosea, Mycetozoa and Lobosa) are highlighted because that include protist isolates used in the present thesis.

for their antifungal activity, such as 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide, pyrrolnitrin or phenazines, also help protect bacteria against protist predation (Jousset *et al.* 2006). Depending of the extent of this observed overlap between pathogen inhibition and inhibition of predator protists, the role of predation pressure might be essential in the very appearance and maintenance of such biocontrol traits in the microbial community (Jousset 2012). Soil protists could thus play a valuable role in keeping soils healthy with low (plant-) pathogen densities by promoting biocontrol traits.

5. Protist predation in relation to plant development

By releasing nutrients from the bacterial biomass and modifying their community structure, soil protists have been associated with beneficial effects for plant development (Figure 2). The application of protists has been linked with an increase in shoot biomass (Bonkowski *et al.* 2000; Bonkowski and Clarholm 2012), a more elongated and branched root system (Kreuzer *et al.* 2006; Krome *et al.* 2010), a higher content of nitrogen (Kuikman and Van Veen 1989a; Koller *et al.* 2013) and other elements in the shoot (Bonkowski, Jentschke and Scheu 2001; Herdler *et al.* 2008). Further, protist introduction can enrich plant-beneficial taxa in the bacterial community (Bonkowski 2004; Bonkowski and Clarholm 2012), suggesting that their effect on plant growth is driven by alterations in microbiome functionality. For instance, protist introduction was related to an increased survival and activity of introduced plant-beneficial bacteria such as *Pseudomonas* spp. (Weidner *et al.* 2016) and *Azospirillum* sp. B510 (Asiloglu *et al.* 2020). Based on these numerous beneficial effects for the plants, the inoculation of protists was suggested as a strategy to support plant growth by restoring microbiome functionality (Gao *et al.* 2019).

In addition, most soil protists can produce resistant and long-living resting cysts (Shmakova, Bondarenko and Smirnov 2016) that could be dried. This is of particular relevance for biotechnological applications in order to design formulations with a long shelf life. As mentioned earlier, predatory protists are the most abundant functional groups in soils and they have a particular position in the soil food web, potentially acting as a central hub linking bacterial and fungal population in the soil microcosm (Xiong *et al.* 2018; Zhao *et al.* 2019). With this central position, soil protists are promising candidates to manipulate the soil microbiome to promote functions of interest (Gao *et al.* 2019).

6. Gaps of knowledge

Despite these promising features to support plant development, the application of protists remains a trial and error process due to the unpredictability of their impact. In order to make targeted applications of soil protists but also to understand their ecological importance, we need to understand the extent of their predatory impacts, which is intimately linked to the selective nature of protist feeding (Montagnes *et al.* 2008). Predatory protists are taxonomically diverse and are likely to present an equally high diversity in their predatory impacts and functional role in the ecosystem (Burki, Sandin and Jamy 2021). It is thus essential to consider this vast diversity and work with non-model species while studying protist functional importance (Geisen *et al.* 2017). The amount, rate and quality of nutrients released from the bacterial pool may, for instance, vary depending on the protist species (Davidson *et al.* 2005). Similarly, while we know that protist modifies bacterial community composition, we are still unable to predict these changes (Montagnes *et al.* 2008; Geisen *et al.* 2017) and thus cannot predict their effects on plants. While phylogeny may give indications for distinctions between higher taxonomic levels (Pedersen *et al.* 2011), closely-related protist species induce distinct changes in bacterial communities (Glücksman *et*

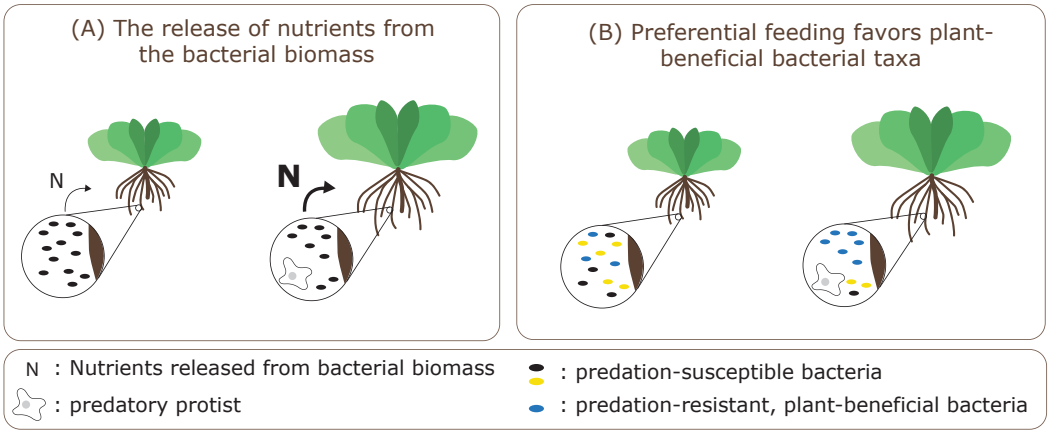

Figure 2: Schematic representation of the two main mechanisms linked to protist beneficial impact on plant development; note that they are not mutually exclusive. The protist predation unlocks nutrients from the bacterial biomass and the nutrients are available for other organisms, including the plant (panel A). The protist feeds preferentially on some bacteria, leading to a change on the bacterial community composition that can be beneficial for the plant (panel B).

al. 2010) making phylogenetic distance a relatively poor predictor. Protist morphological traits, including volume and cell flexibility, could be related to shifts in the prey community (Glücksman *et al.* 2010; Gao 2020), but how exactly these traits relate to the realized predatory impacts of protists remain unclear and does only provide a modest predictive power. The identification of measurable functional traits or properties to relate protist feeding preference to their predatory impact is necessary to screen between isolates, describe their functional role as predators and understand their potential impact on the soil microbiome and on plant development. In addition, most practical aspects such as the type of inoculants (*e.g.*, single or mixed-species) or the appropriate time for inoculation are mostly unknown. Indeed, most research has been done with either the model amoeba *Acanthamoeba castellanii* or unidentified protists, and single inoculation time usually few days before seedling transfer (reviewed in Bonkowski 2004; Gao *et al.* 2019).

7. Objectives and outline of the thesis

The main question of this thesis is:

"Can we harness the potential of predatory protists to support a beneficial soil microbiome?"

With the main hypothesis that taxonomic diversity of predatory protists relate to a similarly high functional diversity and thus impact on the soil ecosystem functioning, my aim was to identify protist taxa and associated traits, as well as application methods, that would lead to plant beneficial outcome. I especially focused on the role of predatory protists in modifying their bacterial prey community structure. I used non-model protist isolates, from different eukaryotic lineages including the Cercozoa, Heterolobosea, Lobosa and Mycetozoa (these groups are highlighted in Figure 1). First, I analyzed which bacterial traits were best correlated with a successful defense against predation by different protists (**Chapter 2**); such traits would allow bacteria to resist predation, improve their chances to establish and could thus be useful to consider when screening for plant-beneficial bacterial taxa. Then, I investigated the prey consumption patterns of different protist isolates in a plate assay and related these *in vitro* patterns to realized predatory impacts in a soil microcosm (**Chapter 3**); *in vitro* feeding patterns could help predict the importance of different protists in the soil by highlighting functional overlaps and unique predatory impacts. Then, I tested the effects of different protists on plant growth via single-species inoculation using lettuce as a model crop (**Chapter 4**). I also investigated the importance of the timing of protist inoculation and of a single- or mixed-species inoculation to support the growth of lettuce in a greenhouse setup (**Chapter 5**). These two chapters, 4 and 5, were meant to help optimizing protist amendment by identifying the best protist candidate (from our collection), inoculation type (single- or mixed-species) and inoculation time, as well as to provide some insights into the potential relevance of changes in the microbial communities in relation to plant properties. Eventually, I synthesized and discussed the results obtained in this thesis, proposing future research directions and examining the promises and challenges linked to protist amendment to support plant development (**Chapter 6**).

In the **chapter 2**, I studied the potential of seven Pseudomonads to inhibit the growth of six predatory protists in a plate assay. Each bacteria had been previously characterized in term of its plant-beneficial potential including biocontrol potential index (BPI, *i.e.*, ability to suppress pathogens), direct plant growth potential (DGPI, *e.g.*, production of phytohormones), and colonization potential index (CPI) (Agaras et al. 2015). I found a significant correlation between biocontrol traits and protist inhibition.

In the **chapter 3**, I investigated the consumption patterns of eight well-characterized protists on twenty bacterial isolates in a plate assay and related these patterns to the protist impact on soil bacterial communities (via 16S rRNA amplicon sequencing) in a soil microcosm. I also studied the potential relations between protist traits such as phylogenetic distance, cell volume, growth rate, and the observed feeding patterns. I found that feeding patterns, growth rate and predatory impacts correlated well together: protists with *in vitro* similar feeding patterns had similar predatory impacts on the prey community in the soil microcosm.

In the **chapter 4**, I tested the effects of six different protists on the performance of lettuce as model crop and on a soil bacterial community in the soil, prior plant transfer, and in the rhizosphere soil at the end of the experiment via 16S rRNA amplicon sequencing. We inoculated the protists three weeks before seedling transfer and let the plant grow for three weeks before destructive harvest to analyze the plant biomass and shoot nutrient content. I found a relatively modest but taxon-specific effect of the protists on the plant shoot-to-root ratio and on the rhizosphere-associated bacterial community structure at the end of the experiment.

In the **chapter 5**, I studied the role of inoculation time for plant-beneficial protist application in a greenhouse experiment with lettuce as model crop. I inoculated either a single-species or three-species mixture solution one week prior seedling transfer, at the same time with seedling transfer and one week after seedling transfer. After 30 days of growth, I harvested the plant for biomass and nutrient content measurements, as well as rhizosphere soil to analyze the bacterial and protistan communities (via 16S and 18S gene amplicon sequencing). I found that an early inoculation increased the aboveground plant biomass the most without distinction between single- or mixed-species and without drastic changes in the rhizosphere microbial community composition.

Finally, in the **chapter 6**, I synthesized and discussed the key elements of this thesis. I discussed the predatory impact of soil protists on the bacterial communities and on plant development. I compared the effects of the same protist taxon (*Cercomonas* sp. S24D2) on the bacterial community in three different experiments (chapter 3, 4 and 5) to examine the context-dependency of its predatory impact. I considered the utility of plate assays to relate protist traits to ecological functions in the soil. Eventually, I discussed some limitations and future perspectives linked to this work.

Supplementary Material

Table S1: Overview of the different groups presented in Figure 1 and associated references for the functional group assignation. Note that we here use the term "predator" instead of "consumer" that was used in Singer et al 2021; similarly we did not distinguish between bacterivore, mycophagous or omnivore, as Xiong et al. 2021 did, but grouped them all under "predator". The groups including the protist isolates used in the present thesis are highlighted in bold.

"supergroup"	Phylum	Functional group	Reference	Code of the isolates
	Telonemia	predator	Singer *et al.* 2021	
Rhizaria	Foraminifera	predator	Singer *et al.* 2021	
Rhizaria	Radiolaria	predator	Singer *et al.* 2021	
Rhizaria	**Cercozoa**	**predator**		**S24D2, C5D3**
Rhizaria	Vampyrellida	predator	Xiong *et al.* 2021	
Rhizaria	Phytomyxea	parasite	Singer *et al.* 2021	
Alveolata	Ciliophora	predator	Singer *et al.* 2021	
Alveolata	Apicomplexa	parasite	Singer *et al.* 2021; Xiong *et al.* 2021	
Alveolata	Dinophyceae	predator, phototroph and parasite	Singer *et al.* 2021	
Alveolata	MALVs	NA		
Stramenopila	Opalozoa	predator	Singer *et al.* 2021	
Stramenopila	Labyrinthulomycetes	parasite, saprotroph	Xiong *et al.* 2021 ; Schärer *et al.* 2007	
Stramenopila	MASTs	predator	Singer *et al.* 2021	
Stramenopila	Peronosporomycetes	parasite, saprotroph	Strullu-Derrien *et al.* 2011	
Stramenopila	Chrysista	NA		
Stramenopila	Diatomista	phototroph	Adl *et al.* 2018	
Cryptista	Chryptophyta	phototroph	Freshwater Algae of North America, 2003	
Cryptista	Kathablepharidaceae	predator	Vørs 1992	
Archeaplastida	Chloroplastida	phototroph	Adl *et al.* 2018	
Archeaplastida	Glaucophyta	phototroph	Adl *et al.* 2018	
Archeaplastida	Rhodophyta	phototroph	Xiong *et al.* 2021	
Archeaplastida	Picozoa	predator	Singer *et al.* 2021	
Haptista	Haptophyta	phototroph	Singer *et al.* 2021	
Haptista	Centroplasthelida	predator	Adl *et al.* 2018	
Amoebozoa	**Mycetozoa**	**predator**	**Xiong *et al.* 2021**	**P1-1**
Amoebozoa	**Lobosa**	**predator**		**C13D2, C2D2, P147, P33**
Amoebozoa	Variosea	predator	Berney *et al.* 2015	

(the table continues in the next page)

"supergroup"	Phylum	Functional group	Reference	Code of the isolates
Obozoa	Apusomonada	predator	Heiss *et al.* 2013	
Obozoa	Breviatea	predator	Adl *et al.* 2018	
Obozoa	Choanoflagellata	predator	Adl et al 2018	
Obozoa	Metazoa	predator		
Obozoa	Fungi	saptrotroph		
Obozoa	Rotosphaerida	predator	Adl *et al.* 2018	
Excavates	Metamonada	parasite	Singer *et al.* 2021; Xiong *et al.* 2021	
Excavates	Malawimonada	predator	Singer *et al.* 2021	
Excavates	Diplonemea	predator	Singer *et al.* 2021	
Excavates	Euglenida	predator	Chan *et al.* 2013	
Excavates	Kinetoplastida	parasite, predator	Adl *et al.* 2018	
Excavates	**Heterolobosea**	**predator**	**Singer *et al.* 2021**	**NL81, P145-4, S18D10, NL10**
Orphan	Hemimastiogophora	predator	Lax *et al.* 2018	

Chapter 2

Biocontrol traits correlate with resistance to predation by protists in soil pseudomonads

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Abstract

Root-colonizing bacteria can support plant growth and help fend off pathogens. It is clear that such bacteria benefit from plant-derived carbon, but it remains ambiguous why they invest in plant-beneficial traits. We suggest that selection via protist predation contributes to recruitment of plant-beneficial traits in rhizosphere bacteria. To this end, we examined the extent to which bacterial traits associated with pathogen inhibition coincide with resistance to protist predation. We investigated the resistance to predation of a collection of *Pseudomonas* spp. against a range of representative soil protists covering three eukaryotic supergroups. We then examined whether patterns of resistance to predation could be explained by functional traits related to plant growth promotion, disease suppression and root colonization success. We observed a strong correlation between resistance to predation and phytopathogen inhibition. In addition, our analysis highlighted an important contribution of lytic enzymes and motility traits to resist predation by protists. We conclude that the widespread occurrence of plant-protective traits in the rhizosphere microbiome may be driven by the evolutionary pressure for resistance against predation by protists. Protists may therefore act as microbiome regulators promoting native bacteria involved in plant protection against diseases.

Keywords: rhizobacteria, PGPR, protozoa, multitrophic interactions, biocontrol

1. Introduction

Plant-associated microorganisms are an essential component of plant growth and health (Berendsen, Pieterse and Bakker 2012; Mendes, Garbeva and Raaijmakers 2013). Plant roots are in particular a hot-spot of plant-microbe interactions, with root-associated microorganisms modulating plant hormonal balance (Lugtenberg and Kamilova 2009; Ravanbakhsh *et al.* 2018) and plant immune responses (van Loon 2007; Zamioudis and Pieterse 2012). Microbe-microbe interactions are also linked to plant health, as plant-associated bacteria are known to be able to protect plants against pathogens by producing inhibitory secondary metabolites or competing for resources (Raaijmakers *et al.* 2009; Gu *et al.* 2020). The participation of microorganisms to plant health can contribute to a natural immunity of soils (Cook *et al.* 1995; Weller *et al.* 2002) and thereby reduce the need for environmentally harmful pesticides (*e.g.*, Haas and Défago 2005; Fravel 2005). Although many root-associated microorganisms have the potential to protect plants, these activities can be highly variable, and we still have little information regarding drivers affecting pathogen-suppressive microbes. In natural systems, plant-associated microorganisms face multiple biotic interactions that constrain their fitness (Finkel *et al.* 2017; Wallenstein 2017; Sessitsch, Pfaffenbichler and Mitter 2019). One particularly strong fitness pressure is that imposed by predation by free-living, phagotrophic protists. These act as key regulators of rhizosphere microbiome assembly through their intense and selective predatory activity (Gao *et al.* 2019).

Protists are a paraphyletic group encompassing most micro-eukaryotes and are present in the soil at densities in the range of 10^4 individuals per gram (Adl *et al.* 2018; Geisen *et al.* 2018). Covering a wide range of sizes, typically from micrometers to few millimeters (Geisen *et al.* 2017), and morphotypes (*e.g.*, testate and naked amoebae, flagellates, ciliates), protists occupy numerous ecological niches within the soil food web (Geisen *et al.* 2018). In line with their high taxonomic and functional diversity, protist species differ widely in their feeding behaviour (Weekers and Drift 1993; Jürgens and Matz 2002). For instance, different morphotypes vary in their ability to physically reach their prey. While flagellates mainly feed by filtering the liquid around them using their flagellum (Boenigk *et al.* 2001), amoebae glide on surfaces, using their pseudopods to reach small cavities (Anderson 2016). Prey selection goes even beyond discrimination based solely on physical accessibility: some protists can further select their bacterial prey based on their size and cell surface biochemistry (John and Davidson 2001; Wootton *et al.* 2006). Thus, predation by protists acts as a selective pressure on bacterial communities, and this predatory pressure depends on the protist species. Interestingly, while closely related protists can in some cases elicit similar changes in the composition of the bacterial communities, in other cases they induce highly disparate modifications (Glücksman *et al.* 2010; Pedersen *et al.* 2011).

In order to escape predation, bacteria have developed a range of defence mechanisms. Common strategies include morphological changes such as filament formation, size shifts

(Güde 1979), but also behavioural and physiological changes such as biofilm formation (Raghupathi *et al.* 2018), enhanced motility (Matz and Jürgens 2003) and/or the production of inhibitory compounds (Matz and Kjelleberg 2005). While these adaptations have been mostly studied and reported in aquatic systems (Pernthaler 2005), they may be of direct relevance for soil and rhizosphere microbiome functioning. From a plant perspective, indeed, some traits conferring resistance to protists also contribute to disease suppression. Indeed, numerous compounds produced by soil pseudomonads that have long been known for their antifungal activity, such as 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide, pyrrolnitrin or phenazines, also help protect bacteria against protist predation (Jousset *et al.* 2006). In addition, the presence of protists can induce the biosynthesis of the lipopeptidic surfactants massetolide and viscosin, which have been primarily investigated for their antimicrobial activities against plant pathogens (Andersen and Winding 2004; Mazzola *et al.* 2009). Further, the exoprotease *AprA* inhibits various bacterivorous protists (Jousset *et al.* 2006) while also contributing to the suppression of plant-parasitic nematodes (Siddiqui, Haas and Heeb 2005). If there is a large degree of overlap between bacterial defence against protist predation and traits conferring plant protection, then introduction of soil protists could promote soil functionality and plant health. Increased selective pressure imposed by predators would thus coincide with increases in plant protective capabilities. A recent study by Asiloglu and colleagues (2020) further supports this idea: they showed that the application of soil protists enhanced the survival of the plant-beneficial bacterium *Azospirillum* sp. B510 in the rhizosphere of rice (*Oryza sativa* L.).

While highly attractive, the proposed link between predation resistance and plant beneficial activity still requires empirical verification to allow for the development of effective and predictable levels of soil function enhancement. In the present study, we therefore sought to (1) investigate the extent to which predatory pressures align with phylogenetic proximity for both predator and prey, and (2) test whether protist-bacteria interactions can be predicted as a function of bacterial traits known for their contribution to plant growth promotion, pathogen suppression and/or root colonization success. We scrutinised the interactions between seven soil *Pseudomonas* spp. described in relation to their plant-beneficial activity (Agaras *et al.* 2015) and six heterotrophic protists. The genus *Pseudomonas* was chosen as a model due to the well-known role of many of its members in plant growth promotion and protection (Haas and Défago 2005; Lugtenberg and Kamilova 2009), thus an extensive available literature and a high interest for application. Protist species were selected to cover three phylogenetic supergroups (Rhizaria, Excavata and Amoebozoa) as well as the morphotype categories of amoebae, amoebo-flagellates and flagellates. The bacterial and protist isolates were cultivated in all pairwise predator-prey combinations, and the growth of both bacteria and protists were recorded and related to the characterised bacterial traits. We hypothesized that bacterial isolates harbouring traits associated with pathogen suppression would be more resistant to protist predation.

2. Materials & methods

2.1 Bacterial and protist isolates

We selected seven bacterial strains from a collection of *Pseudomonas* spp. isolated from Argentinian agricultural soils and previously characterized as described in Agaras *et al.* (2015). The selection comprises *Pseudomonas fluorescens* strain RBBP4, *Pseudomonas donghuensis* strain SVBP6, *Pseudomonas putida* strain SVMP4, *Pseudomonas asplenii* strain RPBP2 and three *Pseudomonas chlororaphis* strain SVBP8, strain SMMP3 and strain SVBP3 (see also Table S1 for an overview and the density at the day of inoculation). Studying *Pseudomonas* spp. has the advantage to build upon an extensive literature that scrutinized its plant-beneficial activity, including both plant promotion and pathogen suppression, thus offering a vast array of available data regarding genetic and physiological traits (Walsh, Morrissey and O'Gara 2001; Haas and Défago 2005; Lugtenberg and Kamilova 2009). The bacterial isolates were chosen to cover a range of plant-beneficial traits linked with plant growth promotion and disease suppression. In a previous greenhouse study, we showed that the plant-beneficial activity of these bacterial isolates was stimulated by the presence of the amoeba *Acanthamoeba castellanii* (Weidner *et al.* 2016) prompting further investigation on predator-prey interactions.

Escherichia coli OP50 was included in the setup to serve as a positive control for the growth of the protists. *E. coli* OP50 is routinely used as food source for our protist cultures. To our knowledge, *E. coli* OP50 does not possess any antagonistic activities against plant pathogens, nor any anti-predation strategies.

The protist isolates were selected to represent some of the main phyla of soil-dwelling free-living protists, while also including some closely related isolates (two Rhizaria, two Excavata, two Amoebozoa), and covering various morphotypes (two flagellates, two amoebo-flagellates, two amoebae; Table 1). Protists were isolated from a range of environments (clay soil, sandy soil and growth substrate) in the Netherlands and grown on *E. coli* OP50. The taxonomic assignment of the protists was obtained by extracting DNA from cultures of each protist isolate. Several pairs of general eukaryotic primers were used to facilitate the recovery of nearly full-length 18S rRNA gene sequences from each strain. Resulting sequences were subjected to BLASTn searches against NCBI GenBank (for more details see Gao 2020, Chap.3).

Table 1: Description of the protist isolates used in the present study. Taxonomic assignment is as described in Gao (2020). The eukaryotic supergroups were assigned according to Adl et al. (2018).

Code	Taxonomic assignment	Eukaryotic supergroup	Mophotype	Origin	Reference
C5D3	*Cercomonas lenta*	Rhizaria	Flagellate	Clay soil	DSM 32401*
S24D2	*Cercomonas* sp.	Rhizaria	Flagellate	Sandy soil	Gao, 2020
P147	*Vannella* sp.	Amoebozoa	Amoeboid	Growth substrate	Gao, 2020
C13D2	*Acanthamoeba* sp.	Amoebozoa	Amoeboid	Clay soil	Gao, 2020
NL81	*Naegleria clarki*	Excavata	Amoebo-flagellate	Growth substrate	Gao *et al. (in prep.)*
P145-4	*Naegleria clarki*	Excavata	Amoebo-flagellate	Growth substrate	Gao, 2020

**The protist isolate Cercomonas lenta C5D3 has been deposited in 2015 by ECOstyle BV at the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures as Cercomonas lenta ECO-P-01 DSM 32401.*

2.2 Growth conditions and preparation of bacterial isolates

All bacterial isolates were kept as frozen glycerol stocks (-80°C). Prior to the experiments, bacteria were grown on King's B plates (KB; King, Ward, and Raney 1954), with one colony serving to initiate a new liquid culture in King's B (28°C, 120 rpm, 14-15 hours). For practical reasons, we worked with a modified KB recipe using potassium dihydrogen phosphate (KH_2PO_4); the pH of the solution was adjusted to 7.0. Bacterial cells were washed three times by centrifugation (9,500 *g*, 2 min) and resuspension in 0.9% NaCl. The pellets were eventually resuspended in Page's Amoeba Saline, a diluted phosphate buffer used to grow protists (Page 1976; hereafter referred to as PAS) and adjusted to an OD_{600} of 1.5. By plating a 10-fold serial dilution of the bacterial suspension, we estimated the cell densities for each isolate (Table S1).

2.3 Growth conditions and preparation of protist isolates

The protist cultures were routinely propagated supplemented with *Escherichia coli* OP50 as sole prey in PAS at 15°C, in the dark; fresh cultures were initiated once a month. *E. coli* OP50 was typically added at a density of ca 10^8 cells mL^{-1}. The protist stock cultures are thus usually a mixture of cyst and active individuals.

To obtain an active population for the co-cultures, we prepared protist culture as follows: stock protist cultures were washed three times by gentle centrifugation at 100 *g* for 10 min to remove spent medium, dead cells and potential contaminations. After centrifugation, the protists are concentrated in the lower part of the tube. Because they do not form any visible pellet, we only discard seventy-five percent 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 duration was adapted to each protist isolate with the aim to enable excystation and growth while avoiding new encystation.

To initiate the co-cultures, the obtained active populations of protists were washed as described in the previous paragraph, counted and adjusted to 10^3 active individuals mL^{-1}; note that despite our procedure the population of *Naegleria* sp. NL81 was already mostly encysted (Table S2). To ensure that the protist inoculation was consistent across all wells, we estimated the protist density before, during, and after the inoculation procedure. The density was estimated by transferring a volume of 10 µL in Clear Polystyrene 96-Well Microplates with flat bottom (Corning® 3370). The cells were enumerated over the full surface created by the drop on a monitor connected to an inverted microscope Nikon Eclipse TS 100 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).

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 number of cells transferred along with the protists (Table S2); these remaining *E. coli* cells represented 1-10% of the total bacterial density in the co-cultures. To examine the potential influence of these residual *E. coli* cells on protist growth, we set up wells without any addition of prey cells. These wells are referred to as "No added cells".

2.4 Setup and monitoring of the cultures

Pure cultures and co-cultures (one bacterial isolate, one protist isolate) were prepared in Clear Polystyrene 96-Well Microplates with flat bottom (Corning® 3370; see table S3 for the volume distribution for each well, supplementary material). Each combination was set up in five replicates. The location of each culture was randomized to take potential edge effects into account. Plates were sealed with Parafilm® and incubated in the dark at 20°C for 5 days. The growth medium (2% King's B, diluted in PAS) was chosen to mimic a low nutrient system. The OD_{600} was measured every day with a plate reader (SPECTROstar Nano, BMG Labtech) as indicator of bacterial density. Measured OD_{600} values were corrected for path length, so that depicted values correspond to a standard light path of 1 cm. Preliminary calibrations revealed that protist present in the wells did not significatively affect the optical density. Before each measurement, plates were briefly shaken (double orbital, 5 seconds at 500 rpm) to homogenize the cultures.

The co-cultures were also set up in triplicate in PAS to investigate the ability of the protist isolates to grow on the bacterial isolates under nutrient-limiting conditions. In case the protist would not grow on the bacteria in PAS, nor in 2% KB, it would indicate that the bacteria represent an inappropriate food source. On the other hand, if the protist does grow on the bacteria in PAS but not in 2%KB, this would indicate an active defense mechanism like the production of antibiotic compounds. An additional scenario might be observed, where the protist cannot grow on the bacterial isolate in PAS but can grow in 2% KB suggesting our system to be bottom-up regulated.

Protist density was estimated in a non-destructive manner after 1, 3 and 5 days of incubation. Encysted and active individuals were enumerated separately on three surface areas (264,000 μm^2 per area), covering two non-central and one central location per well. The average of these three counts was then used to estimate the density per well. Cells were counted on a monitor connected to a Nikon Eclipse TS 100 inverted microscope with a phase contrast. We mainly used the 20x objective (final magnification on the monitor: 275x) but also the 40x objective (final magnification on the monitor: 550x) in cases where it was difficult to differentiate between active cells, cysts and/or cluster of bacterial cells. As all used organisms are living attached to the surface and not in suspension, protist concentration is expressed as individuals cm^{-2}.

We chose to focus our data analysis on the third day after inoculation due to the specific growth pattern of the *Naegleria* spp. on *E. coli* OP50 in 2% KB and in PAS (Fig. S1). The population of both *Naegleria* spp. (P145-4 and NL81) showed an optimum density at day 3 before decreasing markedly at day 5, while the *Cercomonas* spp. (S24D2 and C5D3), the *Acanthamoeba* sp. (C13D2) and the *Vannella* sp. (P147) strains grew following similar patterns on the bacterial isolates at day 3 and 5 after inoculation. Noteworthy, even though *Naegleria* sp. NL81 cultures started with only cysts, excystation occurred rapidly, and the protists followed a similar growth pattern than the other *Naegleria* sp. used in this study (Fig. S1).

2.5 Statistical analyses

All data were analyzed using the version 3.4.3 of the open source statistical software R (R Core Team 2017).

First, we investigated the growth of all protists combined on the different bacterial isolates in 2% KB. Note that we infer growth from density measurements, without incorporating maintenance and death as component of the density due to limitations of our methodology (amount of time points and ability to distinguish between living and dead cells); the same is true for the analysis of the bacteria. Due to zero inflation and overdispersion, we could not use a GLM assuming a Poisson distribution (Zuur *et al.* 2009). We decided to use the zero-inflated model hurdle or two-part for our data (Zuur *et al.* 2009). The hurdle model is comprised of two models: one model fits the abundance of the data, and the other

model is a logistic regression reporting the probability of a non-zero count (presence/absence) (Zuur *et al.* 2009). We used the pscl::hurdle (Zeileis, Kleiber and Jackman 2008) function specifying the count model family to be negative binomial because of the observed overdispersion in our data. Using the base::summary function on the model, we extracted the significance of each explanatory variables (*i.e.*, the bacterial isolates) to explain the observed protist densities.

We also investigated the growth of each protist separately on each bacterial isolate. The generalized and/or two-part models were not suitable for these analyses, potentially due to the lower number of data per group (five data points per group) and the high number of zeros for some groups. To correct for the heteroscedasticity of the data, we used a square root transformation on the data. We ran an ANOVA analysis (stats::lm and base::summary) on the transformed data, using bacterial isolates as explanatory variable for the protist density at day 3 in 2% KB.

We computed a heatmap to show the protist density of each species in co-culture with each bacterial isolate (gplots::heatmap; Warnes *et al.* 2020). The protist density was centered and scaled per row, *i.e.*, per protist isolate, to enable a visual comparison between species. The protist and the bacterial isolates are displayed according to their phylogenetic proximity.

Bacterial phylogenetic analyses were carried out with concatenated partial 16S rRNA, *rpoB* and *oprF* gene sequences (Mulet, Lalucat, and García-Valdés 2010; Agaras *et al.* 2015). For this, we selected 510 nt within the 5' region of the 16S rRNA gene (positions 110–619 in *Pseudomonas protegens* Pf-5, AJ417072) plus 480 nt within the 5' region of the *rpoB* gene (positions 1575–2085 in *P. protegens* Pf-5; NC_004129.6) and 510 nt of the *oprF* gene (positions 263–742 in *P. protegens* Pf-5, NC004129). The corresponding concatenated sequences (1490 bp) of the seven pseudomonads isolates were included in the analysis inferred by the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980). Evolutionary analysis was conducted in the software MEGA7 (Kumar, Stecher and Tamura 2016). All positions containing alignment gaps and missing data were eliminated (All deletion option).

Phylogenetic analyses for protists were carried out using nearly full-length 18S rRNA gene sequences (see details in Gao, 2020). Maximum-Likelihood phylogenetic trees were constructed within SeaView Version 4 (Gouy, Guindon and Gascuel 2010). In order to assess the stability of the clades, phylogenetic analysis was performed based on Bayesian analysis using MrBayes 3.2 (Huelsenbeck and Ronquist 2001). The evolutionary model was conducted under 6 General Time Reversible (GTR) substitution types with gamma-distributed rate variation across sites and a proportion of invariable sites.

We further investigated the effect of protists on the bacterial density using as proxy the OD_{600}. We plotted the treatment mean of bacterial density against the treatment mean of

protist density (log_{10}(active cells cm^{-2} + 1); addition of a one because of the presence of zeros) at day 3 in 2% KB and computed a Spearman rank correlation (stats::cor.test). To further investigate this relationship, we ran an ANOVA analysis (stats::lm and base::summary) using protist isolates as explanatory variable for the bacterial density (OD_{600} values) at day 3 in 2% KB. We ran the analysis separately for each bacterial isolate. We computed a heatmap to show the bacterial density of each isolate exposed to each predator protist (gplots::heatmap; Warnes *et al.* 2020).

We then investigated correlations between the protist density and specific bacterial traits. Most data on bacterial traits were obtained from Agaras *et al.* (2015). The protocol and results of the drop collapse assay to identify biosurfactant production were reported for SVBP6 in Agaras, Iriarte, and Valverde (2018); results for the other bacterial isolates can be found in the supplementary material of the present manuscript (Table S4). We used Spearman rank correlations (stats::cor.test) to analyze the relation between protist density and the bacterial traits with counts or continuous data (*i.e.*, number of inhibited fungi, inhibition of Pythium, HCN production in liquid medium, phospholipase relative activity in egg-yolk agar, exoprotease relative activity in milk agar, production of siderophore, solubilization of inorganic phosphorous, 1-aminocyclopropoane-1-carboxylate (ACC) deaminase activity, production of auxin indole-3-acetic acid (IAA), swimming, swarming and twitching motility). We performed point-biserial correlations (ltm::biserial.cor; Rizopoulos 2006) to study the relation between the protist density and dichotomous data of the bacterial traits (*i.e.*, presence/absence of the genes *phzF* for production of phenazines, *prnD* for pyrrolnitrin, and *pltB* for pyoluteorin, presence/absence of biosurfactant (drop collapse activity), and presence/absence of the N-acylhomoserine lactone (AHL) type of quorum sensing signals). All correlations were combined into one correlation matrix (corrplot::corrplot; Wei and Simko 2017). We computed the statistical significance tests using stats::cor.test specifying the method to be Spearman or Pearson for the point-biserial correlation.

Using the Spearman rank correlation (stats::cor.test), we also investigated the correlation between the combined densities of all protist isolates at day 3, in 2 %KB, and plant-beneficial related indexes proposed by Agaras *et al.* (2015), as well as the correlation between the combined bacterial densities at day 3 in 2 %KB, and the indexes. The results were displayed using corrplot::corrplot. The indexes proposed by Agaras *et al.* (2015) are: the Biocontrol Potential Index (BPI, *e.g.*, antibiotic genes, HCN production, lytic enzymes) and the Direct Growth Promotion Index (DGPI, *e.g.*, P solubilization, IAA, ACC deaminase). The Colonization Potential Index (CPI) was constructed considering motility, quorum sensing, biofilm activities separately from the rest. Each index was computed for every bacterial isolate based on a ratio between its activity value and the highest measurement in the set of tested isolates, normalized with the number of measured activities (see Agaras *et al.* 2015).

3. Results

3.1 Impact of soil pseudomonas on protist performance

Five of the seven pseudomonads significantly inhibited protist growth (Fig. 1 and Table S5). The isolates *P. donghuensis* SVBP6, *P. putida* SVMP4, and *P. chlororaphis* SVBP3 inhibited all six protist isolates, while the other bacterial isolates let at least one protist isolate grow to similar density compared to the positive control *E. coli* OP50 (Fig. 2 and Table S6 for the ANOVA table).

The bacterial ability to inhibit protist growth was only partially mirroring phylogenetic proximity. Indeed, all *P. chlororaphis* strains of the study inhibited the six protist isolates (Fig. 2). However, the anti-predator potential of *P. donghuensis* SVBP6 is much more similar to that of *P. putida* SVMP4, compared to its closely related *P. fluorescens* RBBP4 (Fig. 2).

Similarly, the growth patterns of the different protist isolates were only partially consistent with eukaryotic supergroups (Fig. 2). For instance, the two *Cercomonas* spp. (Rhizaria) grew well on *P. fluorescens* RBBP4, while the Amoebozoa and Excavata showed an average growth or even lower growth (compared to *E. coli* OP50). Similarly, the two *Naegleria* spp. (Excavata), achieved their highest densities on the low density of *E. coli* OP50 given by

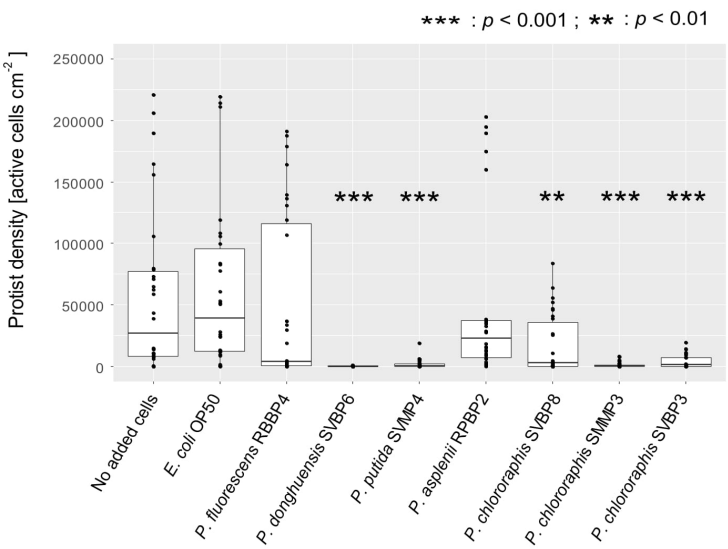


Figure 1: Active protist densities grown on different bacterial isolates (No added cells, E. coli OP50, and Pseudomonas spp.) at day 3, in 2% KB, shown for all protists together. Asterisks indicate significant differences compared to the control (protist grown on the E. coli OP50) reported from the binomial regression part of the hurdle model (see also supplementary Table S5).

the "no added cells" wells. In other cases, species-specificity was observed: for example, *Cercomonas* sp. S24D2 grew well on *P. asplenii* RPBP2, while *Cercomonas lenta* C5D3 did not. Similarly, *Naegleria clarki* NL81 grew well on *P. fluorescens* RBBP4, but *Naegleria clarki* P145-4 did not.

While we mainly focused on the co-cultures in 2% KB when analyzing predator-prey interactions, we briefly report here important and contrasting patterns observed in the nutrient limiting conditions of the PAS setup. In the co-cultures grown in PAS, *P. donghuensis* SVBP6 and two of the *P. chlororaphis* (SVBP3 and SMMP3) did not inhibit any of the protist isolates (see also Table S7 and Fig. S2). In addition, *Naegleria* spp. formed cysts with all bacterial strains when in PAS, but not in the presence of the bacterial isolates *P. donghuensis* SVBP6, *P. putida* SVMP4 and the three *P. chlororaphis* SVBP3, SVBP8 and SMMP3 in 2 %KB.

3.2 Impact of protist isolates on bacterial performance

The bacterial density was in general negatively correlated with protist density (Spearman's rank correlation coefficient: -0.37, p=0.016; Fig. 3).

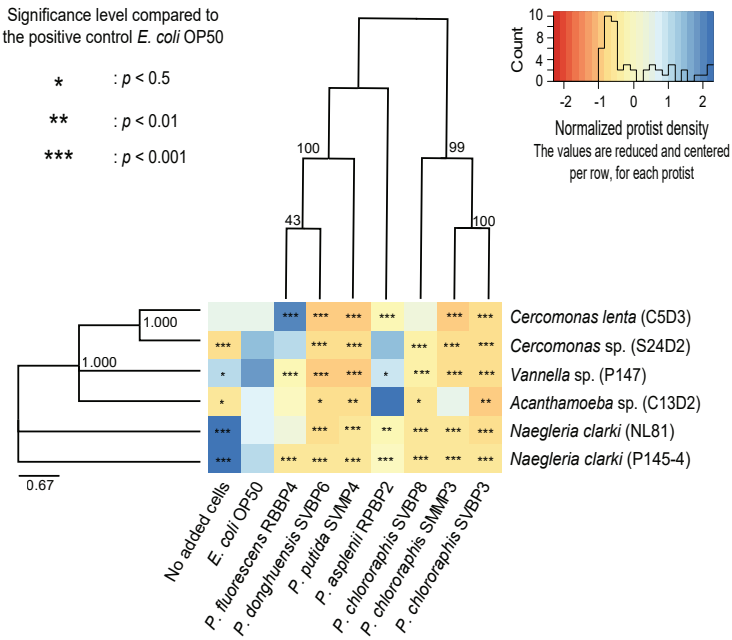


Figure 2: Active protist densities grown on each bacterial strain at day 3, in 2% KB, shown for individual predator-prey co-cultures. The different colors of the heatmap represent the normalized protist density on each bacterial isolate. White (corresponding to a value of 0) indicates the average density per protist isolate (per row). Orange indicates lower density compared to the average of a protist isolate and blue indicates higher density compared to the average of a protist isolate. Asterisks indicate level of significance in protist density grown on the given bacterial isolate relative to growth with E. coli OP50. The protist isolate and bacterial isolates are displayed based on their phylogenetic relatedness. Phylogenetic trees are based on the Maximum-Likelihood Method using the concatenated partial sequences from 16S rRNA, rpoB and oprF genes for the pseudomonads and the 18S rRNA gene for the protist isolates.

With reference to bacterial performance, we observed a clear distinction between those bacterial isolates that could inhibit the protist isolates as opposed to those that could not. Except for *P. chlororaphis* SMMP3, the bacterial isolates able to inhibit all protists were not affected by the presence of the predators. These bacterial isolates achieved similar optical density independent of the protist presence and identity (Table 2 and Fig. S3). The other bacterial isolates (*E. coli* OP50, *P. asplenii* RPBP2, and *P. fluorescens* RBBP4) were all influenced in their density by the presence of at least one protist isolate; we observed both lower and higher means compared to the control treatment (no protist) (Table 2 and Fig. S3).

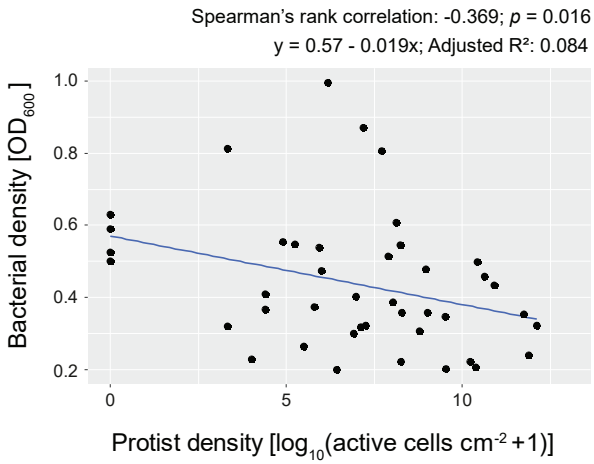


Figure 3: Bacterial density (OD_{600}) plotted against protist density at day 3, in 2% KB. Each point represents the mean of 5 replicates.

Table 2: ANOVA table on bacterial density (expressed as OD_{600}) after 72h incubation in 2% KB, explained by using the protist presence (seven categories: no protist present, or one of the six protist isolates used in the study: Acanthamoeba sp., Vannella sp., Naegleria spp., Cercomonas spp.) as categorical variable. The bacterial isolates found to inhibit protist growth (Fig.1) are highlighted in bold.

Bacteria	**$F_{(6,28)}$**	***p* value**	**adj. R^2**	**significance level**
E. coli OP50	14.65	< 0.001	0.707	***
P. asplenii RPBP2	21.52	< 0.001	0.784	***
P. fluorescens RBBP4	8.57	< 0.001	0.572	***
***P. chlororaphis* SVBP8**	1.15	0.362	0.025	
***P. chlororaphis* SVBP3**	0.65	0.688	-0.065	
***P. chlororaphis* SMMP3**	3.77	0.007	0.328	**
***P. donghuensis* SVBP6**	1.26	0.308	0.043	
***P. putida* SVMP4**	2.18	0.075	0.172	

3.3 Correlation between plant-beneficial traits and resistance to predation

We further investigated the correlation between a suite of bacterial traits related to plant growth and health and protist density. The bacterial traits had been previously measured for each isolate by Agaras and colleagues (2015, 2018).

In general, bacterial traits associated with pathogen suppression showed negative trends with protist growth (Fig. 4). The inhibition of fungal plant pathogens was for instance significantly negatively correlated with the growth of four protist isolates (*Cercomonas* spp. S24D2 and C5D3, and *Naegleria* spp. P145-4 and NL81; Fig. 4 and Table S8 for the statistical tests). The relative exoprotease activity shown in milk agar was further negatively correlated with the density of four assessed protist isolates (Fig. 4, Table S8). Other traits (the inhibition of the oomycete *Pythium ultimum*, production of phospholipase, of biosurfactant, of hydrogen cyanide and of siderophores) showed a negative, yet only marginally significant trend with the growth of all protist isolates (Fig. 4, Table S8). The genetic potential to produce antibiotics (phenazines, pyrolnitrin, pyoluteorin) was only negligibly correlated with protist growth inhibition; we even observed significantly positive correlations between the *pltB*-carrier (for production of pyoluteorin) and the density of *Cercomonas lenta* C5D3 and *Naegleria clarki* NL81 (Fig. 4, Table S8).

We also detected positive trends between protist density and two direct plant growth promotion traits (inorganic phosphorus solubilisation and auxin production). Surprisingly, ACC deaminase was negatively correlated with protist density (Fig. 4, Table S8).

We further mainly observed negative correlations between protist density and bacterial traits related to root colonization. Especially swimming motility was associated with a low density of all protist isolates (Fig. 4; Table S8).

Looking at the general patterns, the total protist density was significantly negatively correlated with the Biocontrol Potential Index (BPI) and the Colonization Potential Index (CPI). In contrast, the bacterial density (all isolates together) was positively correlated with the Biocontrol Potential Index (Fig. 5).

4. Discussion

We examined the extent of the overlap between resistance to predation and traits related to pathogen inhibition typically reported in rhizosphere bacteria. Because resistance to predation can be directly related to bacterial fitness, the observed overlap could explain the prevalence of plant-beneficial bacterial traits in the rhizosphere.

In our study, resistance to predation was found to be correlated with level of investment in plant-protective traits of *Pseudomonas* spp., a prevalent taxon in the rhizosphere. In contrast, predator-prey interactions generally could not be well predicted by the phylogenetic

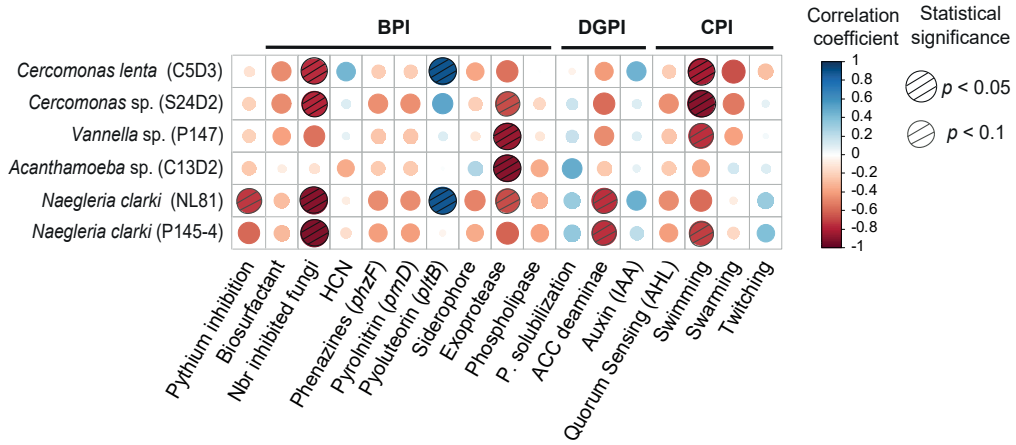


Figure 4: Correlation matrix between protist growth and selected prey bacterial traits. Red and blue dots indicate negative and positive correlations, respectively. Dashed circles are statistically significant. The different bacterial traits are grouped together according to the indexes: Biocontrol Potential Index (BPI), Direct Plant Growth Promotion (DGPI) and Colonization Potential Index (CPI).

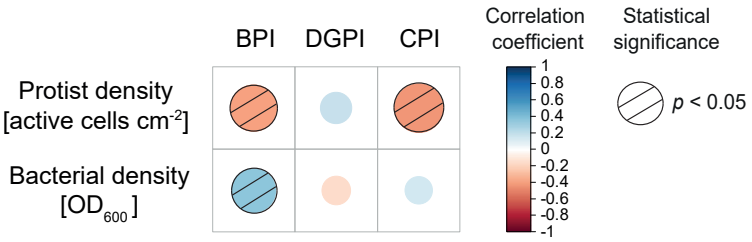


Figure 5: Spearman rank correlation between the combined protist density and the combined bacterial density with the different bacterial indices calculated by Agaras et al. (2015). Red and blue dots indicate negative and positive correlations, respectively. Dashed circles are statistically significant. Biocontrol Potential Index (BPI; e.g., antibiotic genes, HCN production, lytic enzymes), Direct Growth Promotion Index (DGPI; e.g., P solubilization, IAA, ACC deaminase), and Colonization Potential Index (CPI; motility, quorum sensing, biofilm).

relatedness of either the microbial prey or predators. We therefore argue that protists may offer new approaches to support a pathogen-suppressive microbiome.

4.1 Specificity of predator-prey interactions

Bacterial isolates varied in their effect on the protist predators, from no inhibition to strong inhibition of all species tested. The bacterial isolates *Pseudomonas asplenii* RPBP2 and *Pseudomonas fluorescens* RBBP4 supported growth of most of the protist isolates. In contrast, the predator-resistant isolates *Pseudomonas donghuensis* SVBP6, *Pseudomonas putida* SVMP4, *Pseudomonas chlororaphis* SVBP3, SVBP8 and SMMP3 were highly effective

at inhibiting the tested protist isolates. The predator-resistant bacterial isolates seem thus to harbor defense mechanisms effective against a range of organisms, spanning at least three eukaryotic supergroups. Such broad defense suggests nonspecific mechanisms such as production of broad-range antibiotics or extracellular lytic enzymes (Whipps 2001; Raaijmakers, Vlami and de Souza 2002). Interestingly, protist inhibition only occurred in our 2% KB system, and not under nutrient-limiting conditions setup (*i.e.*, PAS). This suggests that the bacterial isolates enable protist growth, but if enough nutrients are available, they reach higher densities and actively defend themselves provided they harbor the necessary genetical toolbox for antipredator activity. This is further supported by the fact that all bacterial isolates reached higher densities in the 2%KB system compared to the PAS system (mean OD_{600} in 2%KB: 0.41, in PAS: 0.14; $t_{(403)}$=-19, p<0.001; data not shown).

Although some closely related predator and prey strains acted similarly in our co-culture assays, phylogenetic proximity was not a strong predictor of protist feeding patterns. Preferential feeding of protists has previously been reported, but the contribution of taxonomy and/or phenotypic traits to this selectivity remain mostly unclear (Montagnes *et al.* 2008). For example, Pedersen *et al.* (2011) observed both a similar response for protists belonging to the same supergroup on secondary metabolite producing *Pseudomonas* spp. as well as a better resistance of the amoeboid taxa. However, Glücksman *et al.* (2010) showed that closely related and morphologically similar protists could have a very different impact on bacterial communities. Thus, even though some patterns can be generalizable to higher taxonomic level, very species-specific interactions occur as well and the prediction of the predatory effect of a protist species remains difficult. We are currently still lacking measurable traits of protists that might help to understand and potentially predict such interactions. In future research, consideration of protist traits such as cell flexibility, growth rate and prey density optimum in addition to the morphotype and taxonomy may help to define predictors of predator-prey interactions.

Surprisingly, no cysts were observed for any of the tested protist isolates when grown in the presence of the inhibitory bacteria. Encystation is a widespread survival mechanism used to help withstanding stressful conditions such as environmental extremes (Shmakova, Bondarenko and Smirnov 2016), and it has been shown to be induced by microbial compounds such as 2,4-diacetylphloroglucinol (DAPG), pyoluteorin (Jousset *et al.* 2006) and putrescine (Song *et al.* 2015). Bacterial compounds can, however, also adversely affect cyst formation and/or viability as reported for *Naegleria americana* with viscosin (Mazzola *et al.* 2009) and putrescine (Song *et al.* 2015). Adverse effects on encystation may therefore explain the absence of cysts in our study.

Bacterial density was negatively correlated with protist density, but, interestingly, the predation-resistant bacterial isolates were in general not influenced by exposure to protists. The isolates *P. donghuensis* SVBP6, *P. putida* SVMP4, SVBP3 and SVBP8 all achieved similar

optical densities independent of the exposure to any predators. Our results are in line with previous work by Pedersen *et al.* (2009) where the predation-resistant *Pseudomonas protegens* CHA0 was not affected by the presence of either the flagellate *Cercomonas longicauda* or the nematode *Caenorhabditis elegans.* The bacterial isolates affected by the presence of predators showed both lower and higher densities compared to the no-protist control, in a species-specific manner (Fig. S3).

Based on the predator-prey interactions studied in the present study, we cannot predict species-specific interactions based only on phylogeny. While all *P. chlororaphis* spp. had a significant adverse effect to all protist isolates, *Acanthamoeba sp.* could still grow on *P. chlororaphis* SMMP3 and *Cercomonas* sp. C5D3 on *P. chlororaphis* SVBP8. In addition, the bacterial isolate *P. donghuensis* SVBP6 shared a much more similar inhibition pattern with *P. putida* SVMP4 than with its closer relative *P. fluorescens* RBBP4.

We speculate that the strong and broad anti-protist activity of the bacterial isolates *P. donghuensis* SVBP6, *P. putida* SVMP4, *P. chlororaphis* SVBP3, SVBP8 and SMMP3 is likely transferable to other predators. We expect that the ability to inhibit a broad range of predators would increase the survival and establishment of inoculated bacteria.

4.2 Bacterial traits correlated to predation resistance

Bacterial traits related to pathogen suppression conferred a broad protection against protists. The ability of bacteria to resist predation can provide a competitive advantage compared to predation-susceptible bacteria. When exposed to predation and in mixture with other bacteria, *Pseudomonas protegens* CHA0 was shown to grow better compared to its isogenic *gacS* deficient mutant (Jousset, Scheu, and Bonkowski 2008) or compared to other *Pseudomonas* spp. described with lower predation resistance (Pedersen *et al.* 2009). Here we link such general resistance to previously reported measurable bacterial traits.

In particular, the ability of the bacterial isolates to inhibit pathogens (fungal pathogens and *Pythium ultimum*) strongly overlapped with their ability to inhibit the protist isolates. The combined inhibition of pathogens and predator protists has been reported in previous studies for various amoebae of the Amoebozoa supergroup (Andersen and Winding 2004; Jousset *et al.* 2010; Novohradská, Ferling, and Hillmann 2017), common soil flagellates from the Rhizaria and Excavata (Pedersen *et al.* 2010) as well as for the ciliate *Tetrahymena pyriformis* (Schlimme *et al.* 1999). Our study further supports this overlap by showing that members of the Amoebozoa, Rhizaria and Excavata are all similarly inhibited by the same set of soil pseudomonads, and by identifying traits strongly correlated with the observed inhibition.

Interestingly, the production of lytic enzymes such as proteases or lipases was a better predictor of general anti-protist activity than antimicrobial compounds. Lytic enzyme production is one of the factors driving the biocontrol activity of fungi (Segers *et al.* 1994;

Bonants *et al.* 1995) and bacteria (Dunne *et al.* 1997; Siddiqui, Haas and Heeb 2005) against pathogen and pests. In a study comparing functional mutants of *Pseudomonas protegens*, Jousset *et al.* (2006) also reported a contribution of the extracellular protease *AprA* to the toxicity against protists. Phospholipase are another group of lytic enzymes known to promote cytolysis of macrophages (Schmiel and Miller 1999). Because macrophages and amoebae share many similarities (Escoll *et al.* 2013; Novohradská, Ferling and Hillmann 2017), phospholipase could contribute to protist inhibition. The present work suggests that exoenzymes contribute to a general protection mechanism against protist predation.

The potential for antibiotic production was, in contrast, only marginally correlated with protist inhibition. For instance, bacteria able to produce biosurfactants, which have previously been proposed to suppress protists (Mazzola *et al.* 2009; Song *et al.* 2015), only had a weak, non-significant effect on *Cercomonas* spp. Hydrogen cyanide production (HCN) was also only weakly related to the resistance to predators. This is in line with the low toxicity reported for *Acanthamoeba castellanii,* which could survive exposure of up to 5 mM KCN (Jousset *et al.* 2010). Regarding antibiotic genes, the presence of the *prnD* gene (pyrrolnitrin) and the *phzF* gene (for phenazines) were only weakly associated with protist inhibition. This result coincides with the previously reported small contribution of pyrrolnitrin to protist predation resistance (Müller, Scheu and Jousset 2013). The potential contribution of phenazines, known to be toxic for nematodes (Cezairliyan *et al.* 2013), remains unknown. More surprising is the positive correlation between the *pltB* genes and *Cercomonas lenta* C5D3 and *Naegleria* sp. NL81, which was in contrast to previous studies reporting adverse effects of pyoluteorin against protists (Winding, Binnerup, and Pritchard 2004; Jousset *et al.* 2006). An additional candidate antibiotic compound, 7-hydroxytropolone, was recently reported for *P. donghuensis* SVBP6 to be at the origin of the broad-spectrum in vitro antifungal activity displayed by this bacterium (Muzio *et al.* 2020). Tropolone and products containing tropolonoid motifs display antimicrobial activities attributed to their metal-chelating and redox properties (Guo, Roman and Beemelmanns 2019) and derivatives have shown some anti-protozoan activities (Ren *et al.* 2003).

Production of ACC deaminase was associated with the inhibition of the two *Naegleria* spp. of our study. The production of ACC deaminase by bacteria typically reduces plant ethylene content, thereby promoting plant growth in the absence of stress (Glick 2014; Ravanbakhsh *et al.* 2018). A direct adverse effect of ACC deaminase against protists is rather unlikely. The mechanism behind the observed correlation is unclear and could be due to a covariate not included in our study. Nonetheless, if ACC deaminase is consistently correlated with negative protist density, bacteria producing this enzyme could be selected in a community exposed to protist predation.

Traits linked to root colonization and bacterial fitness, such as quorum sensing molecules and motility, were negatively associated with protist density. The production of N-acylhomoserine lactones (AHLs) was only weakly related to protist inhibition, despite previous reports of

quorum-sensing related traits for antagonistic interactions (Jones *et al.* 1993; Peng *et al.* 2018). In contrast, swimming motility was strongly correlated with the inhibition of both *Cercomonas* spp., *Naegleria clarki* P145-4 and *Vannella* sp. P147. High swimming speed has indeed been reported to provide bacteria with efficient protection against predation (Matz and Jürgens 2005).

Highly motile bacteria with low biocontrol activity could thus, nevertheless, have a selective advantage under predator pressure. Indeed, the bacterial isolate *P. putida* SVMP4 with low biocontrol activity but a high motility could efficiently escape predation of all protist isolates. In addition to exoprotease and biosurfactant production reported for *P. putida* SVMP4, motility could also contribute significantly to the resistance to predation of this strain. Showing the exact opposite trend, *P. chlororaphis* SVBP8 reported with an overall strong biocontrol activity, but only medium production of exoprotease and medium swimming motility, was not as successful as *P. putida* SVMP4 in inhibiting the protist isolates. The contribution of motility to resist predation is particularly relevant because biocontrol and plant-growth promotion activity presents a potential trade-off (Agaras *et al.* 2015). It is, however, worth noting that while motility has been shown to increase survival in aquatic system (Matz and Jürgens 2005) where possibilities for motility are high, similarly to our experimental setup, the benefits of motility may differ in a more heterogenous medium such as the soil depending on additional variables such as structure and moisture (Erktan, Or and Scheu 2020).

Our data support the hypothesis that bacterial isolates with functional traits related to pathogen suppression can also better resist predation. Further, we highlight the importance of swimming motility to escape predation. Recent results from field assays have also demonstrated that, within this set of probiotic *Pseudomonas*, those with high BPI values showed the highest effect on maize and wheat productivities, over three consecutive seasons and in different locations (Agaras *et al.* 2020). The correlation of BPI values and the resistance to predation supports the idea that biocontrol traits confer high adaptability and survival in complex environments, such as soil and rhizosphere, allowing the isolates to better display their plant growth promotion.

4.3 Conclusion and perspectives

In the present study, we show that several bacterial traits associated with plant growth and health are correlated with bacterial resistance to protist predation. This relationship appears to be rather general, offering better predictive capabilities than the phylogeny for either the prey or the predator. We show an important overlap between resistance to predation and pathogen suppression. Our correlation analysis especially suggests an important contribution of extracellular lytic enzymes such as exoproteases and highlights the important contribution of motility traits to resist predation.

Extrapolation to the complex soil system from our liquid system should be approached with caution, but we suggest that application of specific protist species can promote targeted functions in the soil microbiome. Depending on the resident bacterial community, the application of the *Cercomonas* spp. and *Naegleria* spp. has thus the potential to support bacteria with high biocontrol activity against fungi, while the application of the two Amoebozoa of our study might support exoprotease production. Not only biocontrol activity could be promoted, but also traits linked to direct plant growth promotion as illustrated by the association between ACC deaminase and inhibition of *Naegleria* spp. as well as the important contribution of motility to resist predation.

In conclusion, we suggest that understanding the linkage between bacterial fitness (here predation resistance) and traits related to pathogen inhibition allows strategically promoting/improving beneficial microbiome functions.

5. Aknowledgements

We would like to thank Yann Hautier for advices on the data analysis.

6. Conflicts of 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.

Supplementary Material

Supplementary tables

Table S1: Description of the bacterial isolates used in this study. The taxonomic assignment is as described in Agaras et al. (2015). The estimated densities at the day of inoculation (d0) of the present study are given in the table.

Strain code	Taxonomic assignment	Origin	Reference	Density at d0 [CFUs mL^{-1}]
OP50	*Escherichia coli*	-	Brenner (1974); NCBI:txid637912	7.00×10^8
RBBP4	*Pseudomonas fluorescens*	Soybean rhizosphere	Agaras *et al*. (2015)	2.33×10^8
SVBP6	*Pseudomonas donghuensis*	Bulk soil	Agaras *et al*. (2015, 2018)	4.00×10^8
SVMP4	*Pseudomonas putida*	Bulk soil	Agaras *et al*. (2015)	1.83×10^8
RPBP2	*Pseudomonas asplenii*	Maize rhizosphere	Agaras *et al*. (2015)	5.00×10^8
SVBP8	*Pseudomonas chlororaphis*	Bulk soil	Agaras *et al*. (2015)	2.17×10^8
SMMP3	*Pseudomonas chlororaphis*	Bulk soil	Agaras *et al*. (2015)	2.67×10^8
SVBP3	*Pseudomonas chlororaphis*	Bulk soil	Agaras *et al*. (2015)	3.83×10^8

Table S2: Estimation of the protist density and remaining Escherichia coli OP50 cells in the cultures used for predator-prey experiments.

Protist isolate	Active protists [cells mL^{-1}]	Encysted protists [cells mL^{-1}]	OP50 density [CFUs mL^{-1}]
Cercomonas lenta C5D3	7,500	0	9.33×10^6
Cercomonas sp. S24D2	7,500	0	3.33×10^6
Vannella sp. P147	5,000	0	1.32×10^7
Acanthamoeba sp. C13D2	1,000	0	8.33×10^6
Naegleria clarki NL81	0	7,500	7.67×10^6
Naegleria clarki P145-4	2,500	10	9.83×10^6

Table S3: Composition of the different wells for the co-inoculation.

	2%KB [µl]	Bacterial solution [µl][1]	Protist solution[µl][2]	Final volume [µl]
Co-cultures	125	15	10	150
Bacterial cultures	135	15	0	150
Protist cultures (No bacteria added)	140	0	10	150
Blank	150	0	0	150

[1] Bacterial densities were at 1 to 7 x 10^8 CFUs per mL.
[2] Protist densities was adjusted to 10^{2-3} active individuals per µL. The protist NL81 was inoculated as cysts.

Table S4. Biosurfactant activity of culture supernatants of Pseudomonas isolates used in the present study. The taxonomic assignment for the Pseudomonas is as described in Agaras et al. (2015). The protocol of the drop collapse assay to identify biosurfactant production is reported in Agaras, Iriarte, and Valverde (2018).

		Growth medium	
Strain code	**Bacterial isolate**	**OS-glucose**	**King's B**
RBBP4	*P. fluorescens*	-	-
SVBP6	*P. donghuensis*	-	-
SVMP4	*P. putida*	+	+
RPBP2	*P. asplenii*	-	-
SVBP8	*P. chlororaphis*	-	-
SMMP3	*P. chlororaphis*	+	+
SVBP3	*P. chlororaphis*	-	-

*Table S5: Estimated coefficients and significance tests for the negative binomial part and the logistic part of the hurdle model (pscl::hurdle and stats::summary) used to compare protist growth on each bacterial isolates at day 3, in 2%KB. We use as reference level for the model the protist density grown on remaining E. coli OP50 (No for No added bacterial cells). S.E.=standard error. Statistical significance is highlighted for p<0.001 with "***", for p<0.01 with "**", for p<0.05 with "*" and for p<0.1".".*

Abundance - Negative binomial regression part					
	Coefficient	**S.E.**	**z value**	***p* value**	**Statistical significance**
No (Intercept)[1]	11.024	0.244	45.168	< 0.001	***
OP50	0.048	0.339	0.142	0.887	
RBBP4	0.038	0.359	0.106	0.916	
RPBP2	-0.264	0.342	-0.773	0.440	
SMMP3	-3.451	0.384	-8.996	< 0.001	***
SVBP3	-2.439	0.363	-6.714	< 0.001	***
SVBP6	-5.157	0.445	-11.599	< 0.001	***
SVBP8	-1.094	0.355	-3.079	0.002	**
SVMP4	-3.139	0.363	-8.642	< 0.001	***
Presence/absence - Logistic regression part					
	Coefficient	**S.E.**	**z value**	***p* value**	
No (Intercept)[2]	2.639	0.732	3.606	< 0.001	***
OP50	15.927	1963.405	0.008	0.994	
RBBP4	-1.253	0.863	-1.452	0.146	
RPBP2	0.728	1.253	0.581	0.561	
SMMP3	-2.093	0.824	-2.539	0.011	*
SVBP3	-1.450	0.850	-1.706	0.088	.
SVBP6	-3.045	0.821	-3.707	< 0.001	***
SVBP8	-1.030	0.881	-1.169	0.242	
SVMP4	-1.450	0.850	-1.706	0.088	
Number of iterations in BFGS optimization: 19					
Log-likelihood: -2365 on 19 Df					

[1] The coefficients in the first row of the negative binomial regression part states if the model of our reference level (here protists grown on remaining E. coli OP50, no added bacterial cells) is significantly different from 0.

[2] The coefficient in the first row of the logistic regression part gives the probability of a non-zero count of our reference level.

Table S6: ANOVA table on protist density (square root transformed) after 72h incubation in 2% KB, expressed as a function of bacterial isolates identity.

		ANOVA	
Protist	**$F_{(8,45)}$**	***p* value**	**adj. R2**
C5D3	145.4	< 0.001	0.96
S24D2	17.33	< 0.001	0.75
P147	32.01	<0.001	0.85
C13D2	4.45	0.001	0.39
NL81	27.72	< 0.001	0.83
P145-4	220	< 0.001	0.98

*Table S7: Estimated coefficients and significance tests for the negative binomial part and the logistic part of the hurdle model (pscl::hurdle and stats::summary) used to compare protist growth on each bacterial isolates at day 3, in PAS. We use as reference level for the model the protist density grown on E. coli OP50. S.E.=Standard Error. Statistical significance is highlighted for p<0.001 with "***", for p<0.05 with "*" and for p<0.1".".*

Count model coefficients (truncated negative binomial with log link)					
	Coefficient	**S.E.**	**z value**	***p* value**	**Statistical significance**
OP50 (Intercept)[1]	10.889	0.296	36.765	< 0.001	***
No added bacteria	-3.185	0.457	-6.967	< 0.001	***
RBBP4	-0.005	0.425	-0.012	0.990	
SVBP6	-0.637	0.425	-1.499	0.134	
SVMP4	-1.079	0.419	-2.575	0.010	*
RPBP2	-0.790	0.425	-1.859	0.063	.
SVBP8	-0.925	0.425	-2.178	0.029	*
SMMP3	-0.629	0.448	-1.404	0.160	
SVBP3	-0.245	0.439	-0.557	0.577	
Log(theta)	-0.457	0.102	-4.460	< 0.001	***
zero hurdle model coefficients (binomial with logit link)					
	Coefficient	**S.E.**	**z value**	***p* value**	
OP50 (Intercept)[2]	19.570	4179.000	0.005	0.996	
No added bacteria	-18.610	4179.000	-0.004	0.996	
RBBP4	-16.730	4179.000	-0.004	0.997	
SVBP6	-17.430	4179.000	-0.004	0.997	
SVMP4	0.000	5910.000	0.000	1.000	
RPBP2	-16.730	4179.000	-0.004	0.997	
SVBP8	0.000	5996.000	0.000	1.000	
SMMP3	-18.310	4179.000	-0.004	0.997	
SVBP3	-17.960	4179.000	-0.004	0.997	
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					
Number of iterations in BFGS optimization: 16					
Log-likelihood: -1653 on 19 Df					

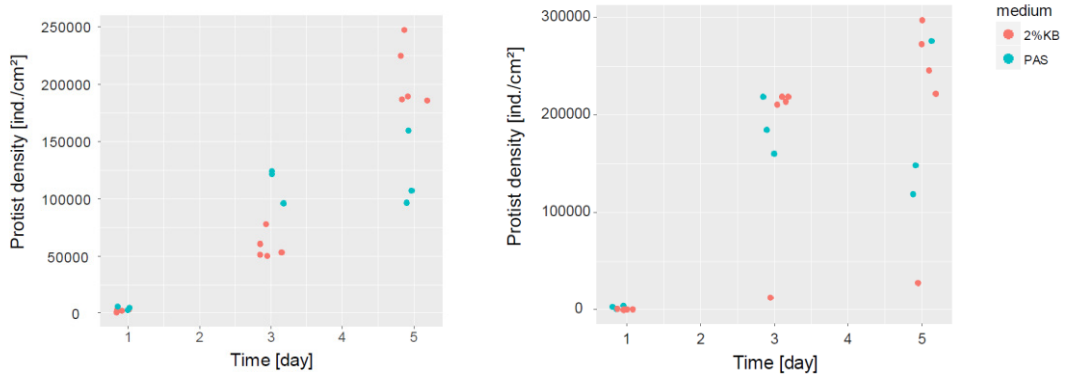
[1] The coefficients in the first row of the negative binomial regression part states if the model of our reference level (here E. coli OP50) is significantly different from 0.

[2] The coefficient in the first row of the logistic regression part gives the probability of a non-zero count of our reference level.

Table S8: Correlation coefficients and statistical test for the correlations between protist density (day 3, in 2%KB) and selected prey bacterial traits (Fig. 4). Only the treatments with p-values under 0.1 (yellow) or under 0.05 (red) are shown. The abbreviation "Nbre inhib. fungi" stands for "number of inhibited fungi".

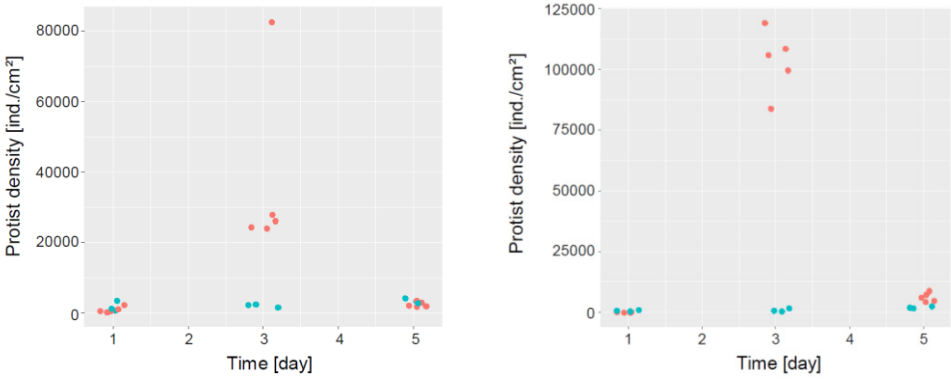
protist	Correlation type	bacterial trait	df	*p* value	Corr Coefficient
C5D3	Spearman	Nbre inhib. fungi	5	0.0477	-0.7594
C5D3	Pearson (point-biserial)	pltB-carrier	5	0.0067	0.8937
C5D3	Spearman	Swimming	5	0.0212	-0.8289
S24D2	Spearman	Nbre inhib. fungi	5	0.0424	-0.7709
S24D2	Spearman	Exoprotease	5	0.0938	-0.6786
S24D2	Spearman	Swimming	5	0.0068	-0.8929
P147	Spearman	Exoprotease	5	0.0137	-0.8571
P147	Spearman	Swimming	5	0.0522	-0.75
C13D2	Spearman	Exoprotease	5	0.0068	-0.8929
NL81	Spearman	Nbre inhib. fungi	5	0.0058	-0.8994
NL81	Spearman	Pythium inhib.	5	0.0626	-0.7298
NL81	Spearman	Exoprotease	5	0.0938	-0.6786
NL81	Pearson (point-biserial)	pltB-carrier	5	0.0073	0.8897
NL81	Spearman	ACC deaminase	5	0.0522	-0.75
P145-4	Spearman	Nbre inhib. fungi	5	0.0036	-0.9178
P145-4	Spearman	ACC deaminase	5	0.0522	-0.75
P145-4	Spearman	Swimming	5	0.0713	-0.7143

Supplementary figures



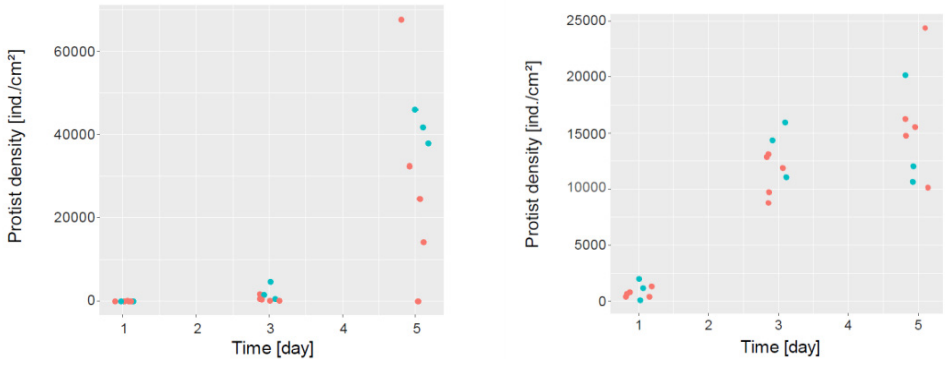
(A) *Cercomonas lenta* C5D3

(B) *Cercomonas* sp. S24D2



(C) *Naegleria clarki* NL81

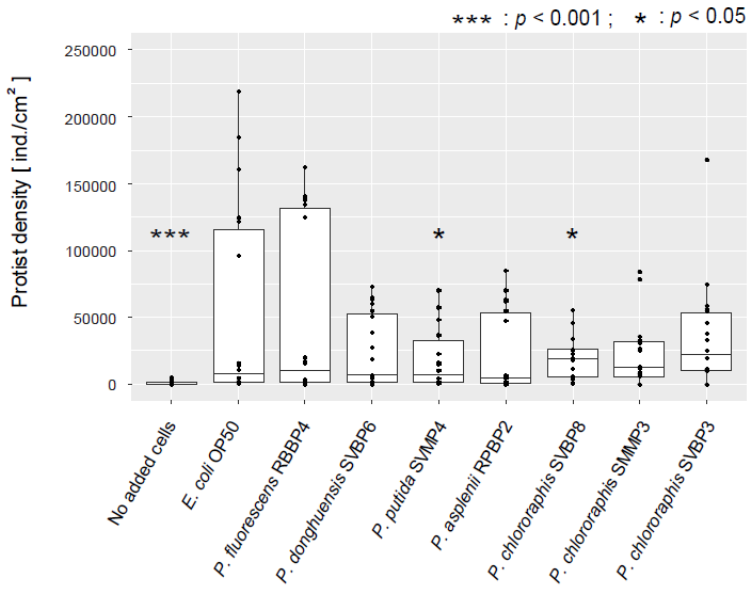
(D) *Naegleria clarki* P145-4



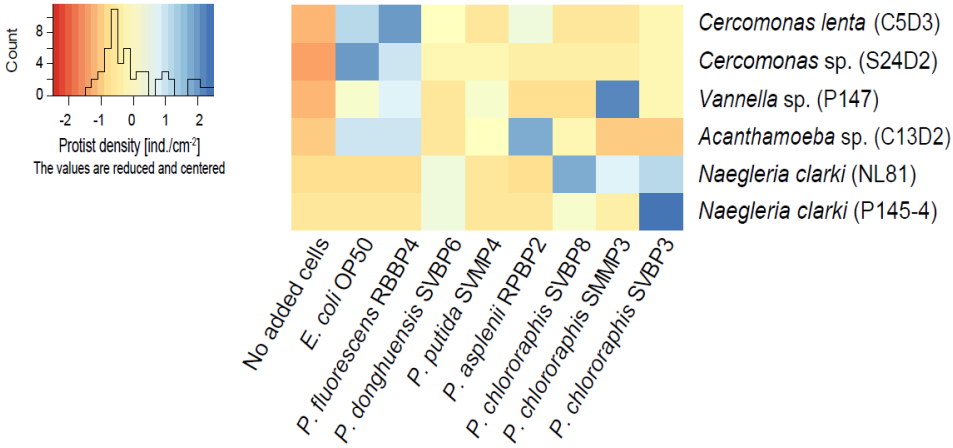
(E) *Acanthamoeba* sp.C13D2

(F) *Vannella* sp. P147

Figure S1 Temporal protist growth on E. coli OP50 in 2%KB and PAS – one graph per protist. Day 1 corresponds at day 1 after inoculation.



(A) General effect of the bacteria on the protists



(B) Species-specific effect of each bacteria on each protist

Figure S2: Active protist densities grown on different bacterial isolates (E. coli, No added cells and OP50, and Pseudomonas spp.) in PAS, shown for all protist isolates together (A), and individual predator-prey co-cultures (B). Asterisks indicate significant differences compared to the control (protist grown on E. coli OP50) reported from the negative binomial regression part of the hurdle model. The different colors of the heatmap represent the normalized protist density on each bacterial isolate; orange indicating lower density (i.e., lower growth compared to the row average) and blue indicating higher density (i.e., higher growth compared to the row average) than the overall mean (for each protist).

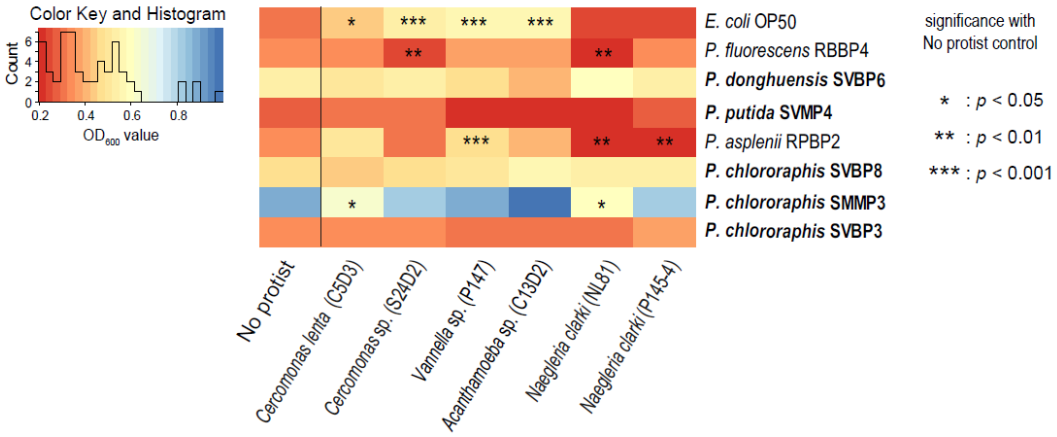


Figure S3: Mean optical densities measure at 600nm (OD600) for each bacteria in 2% KB for individual predator-prey co-cultures. Significant differences compared to the control group (no protist) are highlighted with asterisk, based on the ANOVA analysis (base::summary: : "", "**", "***" indicate p <0.05, p < 0.01 and p < 0.001 respectively. The five bacteria shown to inhibit all protists are highlighted in bold.*

Chapter 3

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

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(a modified version has been accepted in FEMS Microbiology Ecology)

Abstract

Predatory protists are major consumers of soil micro-organisms. By selectively feeding on their prey, they can reshape 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. Here, we used protist traits including the phylogenetic distance, growth rate and volume of eight isolates to investigate the underlying drivers of predation on 20 bacterial species in plate assays. We further described the emerging feeding patterns in terms of preferences and similarities between protist species. We then assessed 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 rate correlated well with the observed predatory impacts on the structure of soil bacterial communities. We conclude that *in vitro* screening may provide the base for protist selections for new strategies in agricultural biotechnology by reaching predictable impacts on the soil microbiome.

1. 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, Durán and Hacquard 2018), predatory soil protists are increasingly being recognized for their roles in nutrient turnover (Clarholm 1985; Bjørnlund *et al.* 2012a; Rønn, Vestergård and Ekelund 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). In addition, predator traits such as volume and/or cell flexibility have been related to the species-specific impact of protist on their prey community composition (Glücksman *et al.* 2010; Pedersen *et al.* 2010; Gao 2020). 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 to the thousands 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 range of favorable prey and discriminative potential (Glücksman *et al.* 2010; Gao 2020) and eventually their realized impact on the prey community composition (Jiang and Morin 2005; Ryberg, Smith and Chase 2012). Protists with low discriminative potential and low prey preferences would feed more or less equally on all bacteria, allowing stochastic processes to dominate bacterial community assembly. Protists with a stronger discriminative potential and clear prey preferences would display a narrower dietary niche breadth and feed mostly on a subset of preferred prey, leading to more deterministic shifts in the community (Ryberg, Smith and Chase 2012; Filip *et al.* 2014; Johnston, Pu and Jiang 2016). Protist species are thus likely to present different realized feeding preferences with relatively narrow to broad dietary niche breadth and impose relatively strong or weak predatory impact on their prey community, accordingly. 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 assessed in simplified laboratory settings has, however, never been linked to complex predation-induced shifts in soil bacterial communities.

In order to better understand the predatory impacts of soil 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). 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 stress, 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). We then 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 (1) each protist isolate would have a distinct feeding pattern, (2) 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, (3) protists with similar feeding patterns would exert similar impacts on the soil-borne bacterial prey community, and (4) predator traits related to protist size and prey traits related to biocontrol potential would be the most informative predictors of the observed patterns.

2. Materials & Methods

2.1 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), Heterolobosea sp. (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 (Gao 2020; see also Table 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 stress (low resource availability, high salinity, and oxidative stress).

2.2 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 Heterolobosea sp. S18D10 were a mixture of active and encysted individuals (1:1; Table S3). 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 S3).

2.3 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 rpm, 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 x 10^6 to 2.98 x 10^8 CFUs mL^{-1} (Table S4). Because the CFU assay failed for bacterial isolate 14, we used the theoretical value of 8.67 x 10^8 cells mL^{-1} based on our calibration curve for this strain.

2.4 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 S5 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 S6). As we previously identified that most relevant changes in the protist population would occur between 0-100 individuals per screen, we used four categories in this interval 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 S7).

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.

2.5 Determining protist feeding patterns based on in vitro plate assays

First, 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.

2.6 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 (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 hours 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 amplicon 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.

2.7 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). 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 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, Huber and Anders 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 (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.

3. Results

3.1 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 5.5x from the day 1, after which they generally decreased by day 5 to about 2.5 to 4.6x the initial population (Fig. S1). 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). 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 S8).

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<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 $t_{(51)}$= - 3.36, p=0.001, respectively; Table S9). On the prey side, however, we did not find any clear and significant correlations between bacterial traits and the observed protist feeding patterns (Fig. S2).

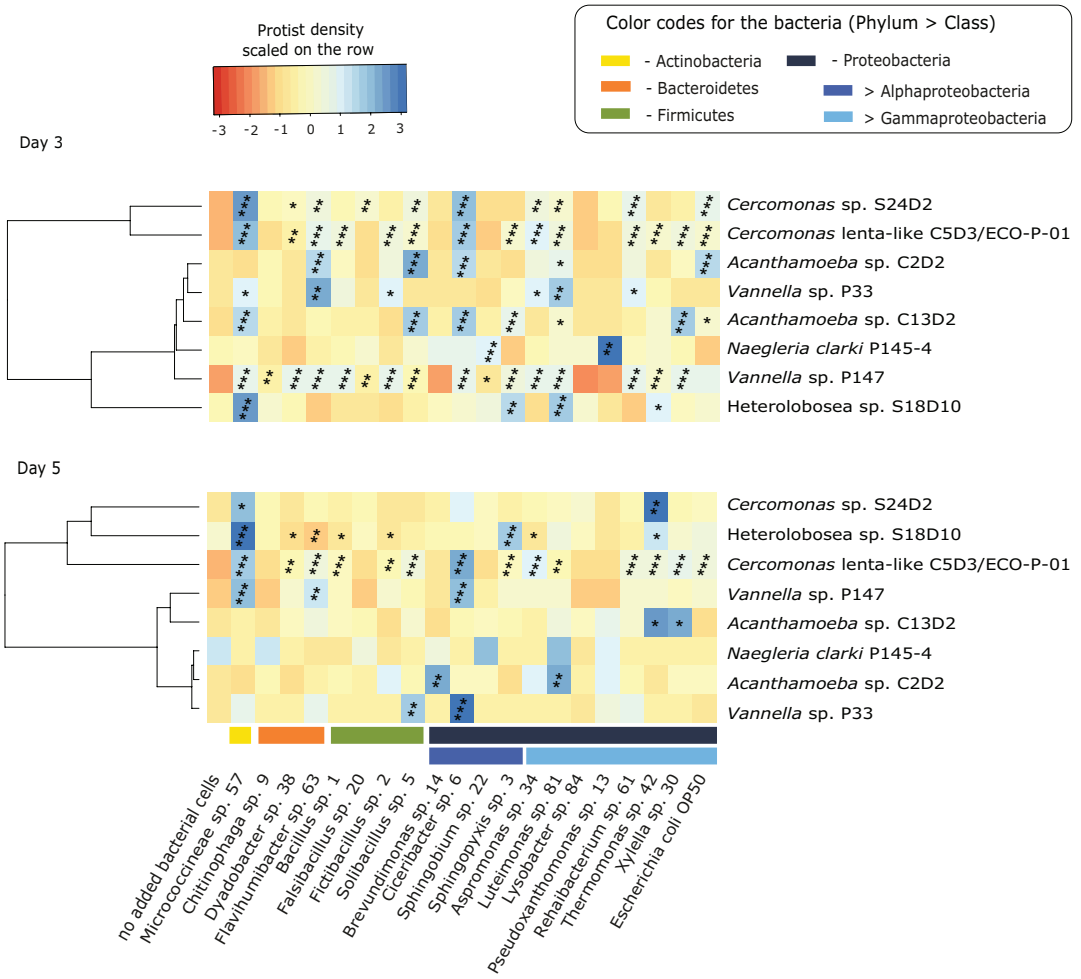

Fig. 1: Heatmap of the protist population density on each bacterial isolate representing the feeding patterns at days 3 (above panel) and 5 (below panel) after inoculation. The protist density has been scaled per row to facilitate comparison; each co-culture was set in triplicates (N=3). The y-axis is 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.*

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	- 0.167	$t_{(54)}$= - 1.396	0.168
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	- 0.201	$t_{(54)}$= - 1.511	0.137
Growth rate	**0.710**	**$t_{(54)}$= 7.406**	**< 0.001**

3.2 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 and Fig. S3 to S6 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 (Fig. S3-6).

3.3 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. 2 and Table S10 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 S11 for the weighted UniFrac). In addition, the coefficients 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,

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	R2	*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

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 Heterolobosea sp. S18D10 treatment compared to the no protist control, and significantly lower values for the ones exposed to the *Cercomonas* sp. S24D2 treatment (Fig. S7 and Table S12 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 S11).

3.4 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. 3): 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

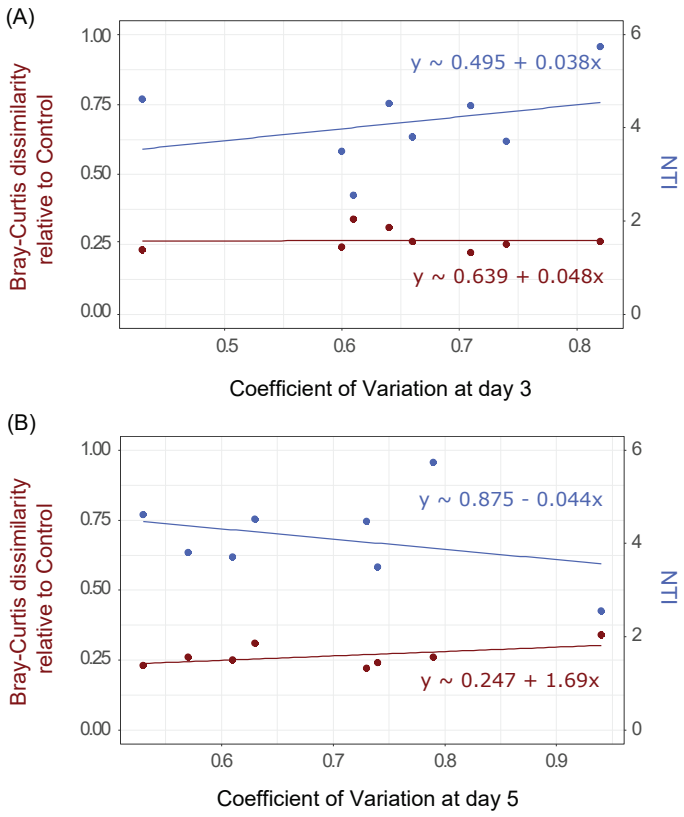

Fig. 2: Relation between the dietary niche breadth (coefficient of variation at days 3 and 5, upper and lower panel respectively) and the magnitude of the predatory impact on the bacterial prey community (Bray-Curtis dissimilarity of each treatment relative to control and NTI). The y-axis on the left 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.

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	-0.176	-0.131	0.677
Growth Rate	**0.898**	**0.774**	**< 0.01**

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. 3. Similarly, we only present the statistical results using the pair-wise Bray-Curtis dissimilarity. The results of the correlation analysis using the weighted UniFrac were generally similar and can be found in Table S11.

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	-0.179	0.014	0.178
Growth Rate	**0.723**	**0.513**	**< 0.01**

4. 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).

Similarly, each predatory protist exerted a distinct impact on its prey community composition. 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, Noll and Frenzel 2006; Rosenberg *et al.* 2009). Actinobacteria generally increased in relative

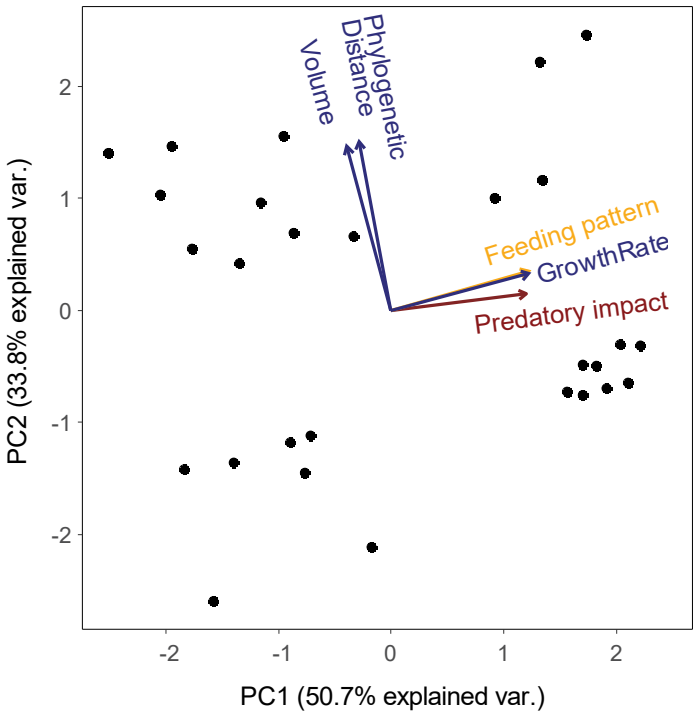

Fig. 3: Principal Component Analysis using the pair-wise Euclidean distances of protist traits (Volume, Phylogenetic distance, and Growth rate; blue color), pair-wise Euclidean distances between protist feeding pattern (plate assay, at day 3, yellow color) and the Bray-Curtis dissimilarity between bacterial 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.

abundance in response to protists, which was also in agreement with previous observations (Ekelund *et al.* 2009a; 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. S3-6). 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, Or and Scheu 2020; Xiong *et al.* 2021). Intriguingly, while Heterolobosea sp. 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, Pu and Jiang 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 Heterolobosea sp. 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, Smith and Chase 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, Brady and Tan 2011; Johnston, Pu and Jiang 2016). Note that we, here, primarily performed our NTI analyses in an exploratory manner and thus only superficially 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 (2451 μm^3) than the rest (115-578 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 (Matz and Kjelleberg 2005; Boenigk *et al.* 2001).

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).

5. Conclusions

Our in vitro feeding assays revealed the rather generalist feeding behavior of our selected protists, which nonetheless translated into species-specific feeding patterns that were, with protist growth rate, well correlated to their realized predatory impacts on soil bacterial communities. While our findings need to be extended to include additional protist and prey species, they highlight the potential of feeding assays to predict predatory impacts in soils and to investigate the multitrophic nature of the microbiome. Such knowledge is necessary to develop systematic strategies to steer the soil microbiome by inoculation of specific protist species.

7. Funding

This work was supported by NWO from the Netherlands Organisation for Scientific Research [ALWGR.2017.016]. Z. G was supported by Chinese Scholarship Council (CSC) [No. 201506350026]. J. H. was supported by NWO from the Netherlands Organisation for Scientific Research [ALW.870.15.050], TKI top-sector grant [KV1605 082] and Aide régionale aux projets H2020-MSCA-IF 2019 Seal of Excellence – Projet CORRiBIOM.

8. Conflicts of 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.

Supplementary Materials

Supplementary Tables

Table S1: Description of the protist isolates used in the present study. All protists were isolated and described by Gao (2020). The eukaryotic supergroups were assigned according to Burki and colleagues (2020). Note that eukaryotic supergroups are subject to changes over time and we only give them here to help the reader locate them in the eukaryotic phylogenetic tree and appreciate that, for instance, the Vannella spp. are closer related to the Acanthamoeba spp. as compared to Naegleria sp.

Code	**Eukaryotic supergroup**	**Taxonomic assignment**	**Morphotype**	**Growth rate [ind. day^{-1}]**	**Volume [µm^3]**	**NCBI Accession number**
C13D2	Amorphea	*Acanthamoeba* sp.	Amoeboid	25	123	NA
C2D2	Amorphea	*Acanthamoeba* sp.	Amoeboid	43	115	NA
P147	Amorphea	*Vannella* sp.	Amoeboid	21	262	Release upon acceptance
P33	Amorphea	*Vannella* sp.	Amoeboid	77	137	Release upon acceptance
P145-4	Excavates	*Naegleria clarki*	Amoebo-flag-ellate	14	2451	MT739326.1 (100% identity)
S18D10	Excavates	Heterolobosea sp.	Amoeboid	43	578	Release upon acceptance
C5D3*	TSAR‡	*Cercomonas* lenta-like	Flagellate	158	335	Release upon acceptance
S24D2	TSAR‡	*Cercomonas* sp.	Flagellate	166	278	Release upon acceptance

** This isolate is registered as Cercomonas lenta ECO-P-01 DSM 32401, safe deposit by ECOStyle BV*
‡ TSAR stands for telonemids, stramenopiles, alveolates, and Rhizaria (Burki et al. 2020)

Table S2: Description of the bacterial strains selected for the present study. All the bacterial isolates, except for E. coli OP50, were isolated from the rhizosphere of tomato plants (Lycopersicon esculentum, cultivar 'Jiangsu') in the seedling phase, as described by Hu and colleagues (Hu et al. 2020). For E. coli OP50, see ref (Brenner 1974) and registration number NCBI:txid637912.

Phylum	Order	Class	Family	Genus	Code
Actinobacteria	Actinobacteria	Actinobacteridae	Actinomycetales	*Micrococcineae*	57
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	*Chitinophaga*	9
Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	*Dyadobacter*	38
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	*Flavihumibacter*	63
Firmicutes	Bacilli	Bacillales	Bacillaceae 1	*Bacillus*	1
Firmicutes	Bacilli	Bacillales	Bacillaceae 1	*Falsibacillus*	20
Firmicutes	Bacilli	Bacillales	Bacillaceae 1	*Fictibacillus*	2
Firmicutes	Bacilli	Bacillales	Planococcaceae	*Solibacillus*	5
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Brevundimonas*	14
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Ciceribacter*	6
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingobium*	22
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingopyxis*	3
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Aspromonas*	34
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Luteimonas*	81
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Lysobacter*	84
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Pseudoxanthomonas*	13
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Rehaibacterium*	61
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Thermomonas*	42
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Xylella*	30
Proteobacteria	Gammaprroteobacteria	Enterobacteriales	Enterobacteriaceae	*Escherichia*	OP50

Table S3: Initial density of protist and remaining bacterial cells at the inoculation day (day 0) of the plate assay.

Protist	Active ind. mL^{-1}	Cysts mL^{-1}	CFUs mL^{-1}
P33	3,000	3,000	6.22×10^6
P147	10,000	0	1.31×10^7
C2D2	3,000 - 5,000	3,000- 5,000	1.47×10^7
C13D2	3,000 - 5,000	0	2.56×10^6
P145-4	3,000	3,000	1.40×10^7
S18D10	3,000	3,000	2×10^7
S24D2	5,000	0	5.56×10^6
C5D3	5,000	0	1.24×10^6

Table S4: Initial bacterial density in CFUs at the inoculation day (day 0) of the plate assay.

Bacterial isolate code	CFUs mL^{-1}
1	1.6×10^8
2	5.17×10^7
3	2.3×10^7
5	1.28×10^8
6	1.40×10^8
9	9.33×10^7
13	8.33×10^7
14	8.67×10^8 *
20	4.5×10^7
22	1.83×10^6
30	1.12×10^8
34	2.98×10^8
38	5.33×10^7
42	5.83×10^7
57	1.17×10^8
61	1.2×10^8
63	1.6×10^8
81	1.55×10^8
84	2.93×10^8
OP50	4.67×10^7

* *Theoretical value*

Table S5: Experimental design of the plate assay. PAS stands for Page's Amoeba Saline, a diluted phosphate buffer used to grow protists (Page 1976).

Treatment	PAS [µL]	Protist solution [µL]	Bacterial solution [µL]	Total [µL]
PAS Blank	150	0	0	150
Only Protist	140	10	0	150
Only Bacteria	135	0	15	150
Co-culture	125	10	15	150

Table S6: Categories used to estimate the protist density.

Category	ind./screen
0	0
1	1 - 10
2	11 - 50
3	51 - 100
4	101 - 400
5	401 - 800
6	> 800

Table S7: Explanatory table to translate category values per well to protist density in the plate assay.

Category per well	Mean category	Protist density [ind. cm^{-2}]
3x 0	0.0	0
2x 0, 1x 1	0.3	100
1x 0, 2x 1	0.7	200
3x 1	1.0	400
2x 1, 1x 2	1.3	1,600
1x 1, 2x 2	1.7	2,800
3x 2	2.0	4,000
2x 2, 1x 3	2.3	9,300
1x 2, 2x 3	2.7	14,600
3x 3	3.0	20,000
2x 3, 1x 4	3.3	26,600
1x 3, 2x 4	3.7	33,300
3x 4	4.0	40,000

Table S8: The coefficient of variation of the achieved densities on the 20 bacterial isolates tested in a plate assay, for each protist isolate, at days 3 and 5 after inoculation. The coefficient of variation is used as an estimate for protist dietary niche breadth.

Protist isolate	Coefficient of Variation at day 3	Coefficient of Variation at day 5
P33	0.74	0.61
P147	0.43	0.53
C2D2	0.71	0.73
C13D2	0.66	0.57
P145-4	0.6	0.74
S18D10	0.82	0.79
S24D2	0.61	0.94
C5D3	0.64	0.63

Table S9: Explanatory significance of protist traits for the observed feeding patterns (pair-wise Euclidean distance between the achieved densities on the 20 bacteria, per protist) at days 3 and 5, obtained from linear models. Significant results ($p < 0.05$) are highlighted in bold.

	T test	*p* value
Pair-wise euclidean distance at day 3 ; $F_{(4,51)}$=30.84, p < 0.001		
Coefficienf of variation at day 3	$t_{(51)}$= - 1.7	0.095
Phylogenetic distance	**$t_{(51)}$= 3.277**	**0.002**
Volume	**$t_{(51)}$= - 3.36**	**0.001**
Growth rate	**$t_{(51)}$= 9.495**	**<0.001**
Pair-wise euclidean distance at day 5; $F_{(4,51)}$=15.54, p < 0.001		
Coefficienf of variation at day 5	$t_{(51)}$= - 1.176	0.245
Phylogenetic distance	$t_{(51)}$= 1.337	0.187
Volume	$t_{(51)}$= - 1.795	0.079
Growth rate	**$t_{(51)}$= 7.045**	**<0.001**

Table S10: Statistical results of the linear models relating protist feeding range (dietary niche breadth) to the magnitude of predatory impact on soil bacterial community (Bray-Curtis to control and Nearest taxon index, NTI). The feeding range was assessed at days 3 and 5.

Formula	F test	*p* value	Adjusted R2
Bray-Curtis to control ~ Coefficient of variation at day 3	$F_{(1,6)}$=0.002	0.968	-0.166
NTI ~ Coefficient of variation at day 3	$F_{(1,6)}$=0.647	0.452	-0.053
Bray-Curtis to control ~ Coefficient of variation at day 5	$F_{(1,6)}$=2.176	0.191	0.144
NTI ~ Coefficient of variation at day 5	$F_{(1,6)}$=0.653	0.45	-0.052

Table S11: Correlation between the magnitude of the predatory impact on the soil bacterial community (weighted UniFrac relative to the control, soil microcosm) and protist traits. Significant results ($p < 0.05$) are highlighted in bold.

Protist Trait	Correlation coefficient	Adjusted R^2	*p* value
Coefficient of Variation (day 3)	-0.143	-0.143	0.736
Coefficient of Variation (day 5)	0.0797	-0.159	0.851
Volume	-0.395	0.015	0.333
Growth Rate	**0.859**	**0.694**	**<0.01**

Table S12: Nearest Taxon Index (NTI) values for each replicate of the soil bacterial community in a microcosm. "Rep" stands for Replicate.

Treatment	NTI values				
	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5
No protist control	4.301	4.391	4.312	2.456	2.788
Acanthamoeba sp. C13D2	4.885	4.835	2.898	3.632	2.760
Acanthamoeba sp. C2D2	5.719	4.840	4.739	3.845	3.233
Vannella sp. P147	5.161	5.082	5.373	3.582	3.858
Vannella sp. P33	3.232	3.986	3.012	4.285	4.028
Naegleria sp. P145-4	3.326	3.982	5.051	3.727	1.357
Heterolobosea sp. S18D10	5.940	5.918	5.716	5.622	5.506
Cercomonas lenta-like C5D3	4.512	4.432	4.172	4.970	NA
Cercomonas sp. S24D2	2.843	2.100	2.216	2.846	2.743

Supplementary Figures

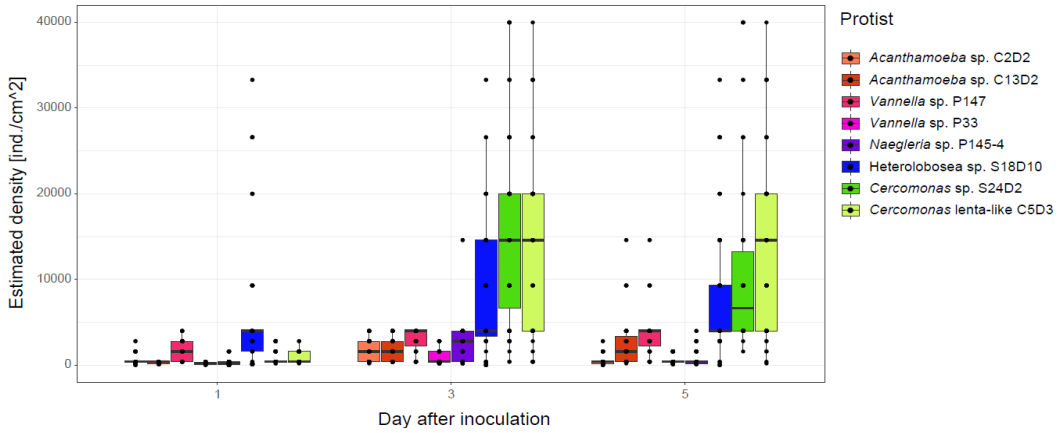

Fig. S1: Temporal pattern of each protist isolate without distinction between prey type. Note that one extreme value of the protist Cercomonas S24D2 grown on the bacterial isolate 42 was left out of this visualization (estimated density 131,900 ind. cm^{-2}).

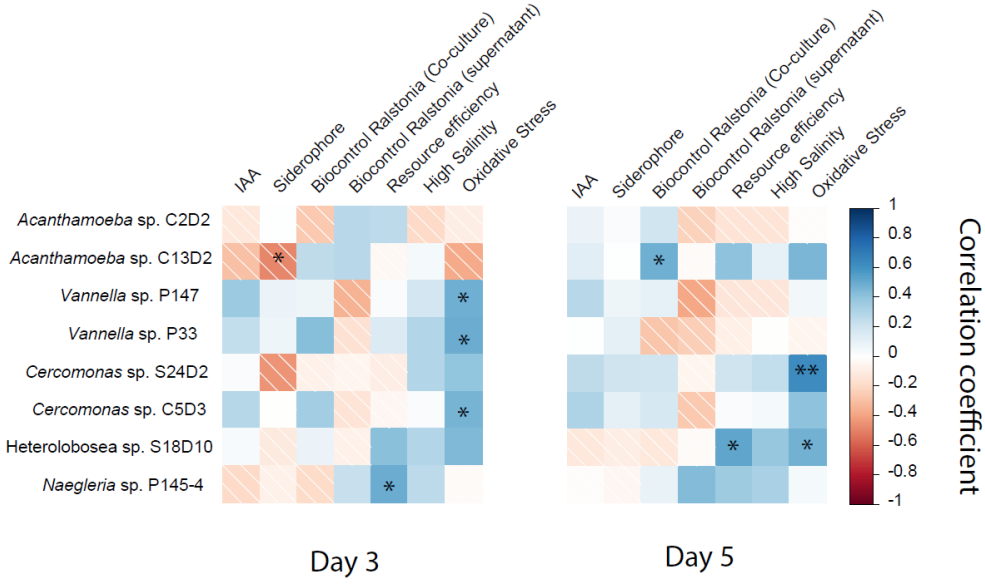

Fig. S2: Correlogram between protist density and bacterial traits. Asterisks highlight significantly correlations: "", "**" indicate p <0.05 and p < 001 respectively. Note that the positive correlation between some protist and oxidative stress is mostly driven by two bacterial isolates (Micrococcineae isolate 57 and Ciceribacter isolate 6).*

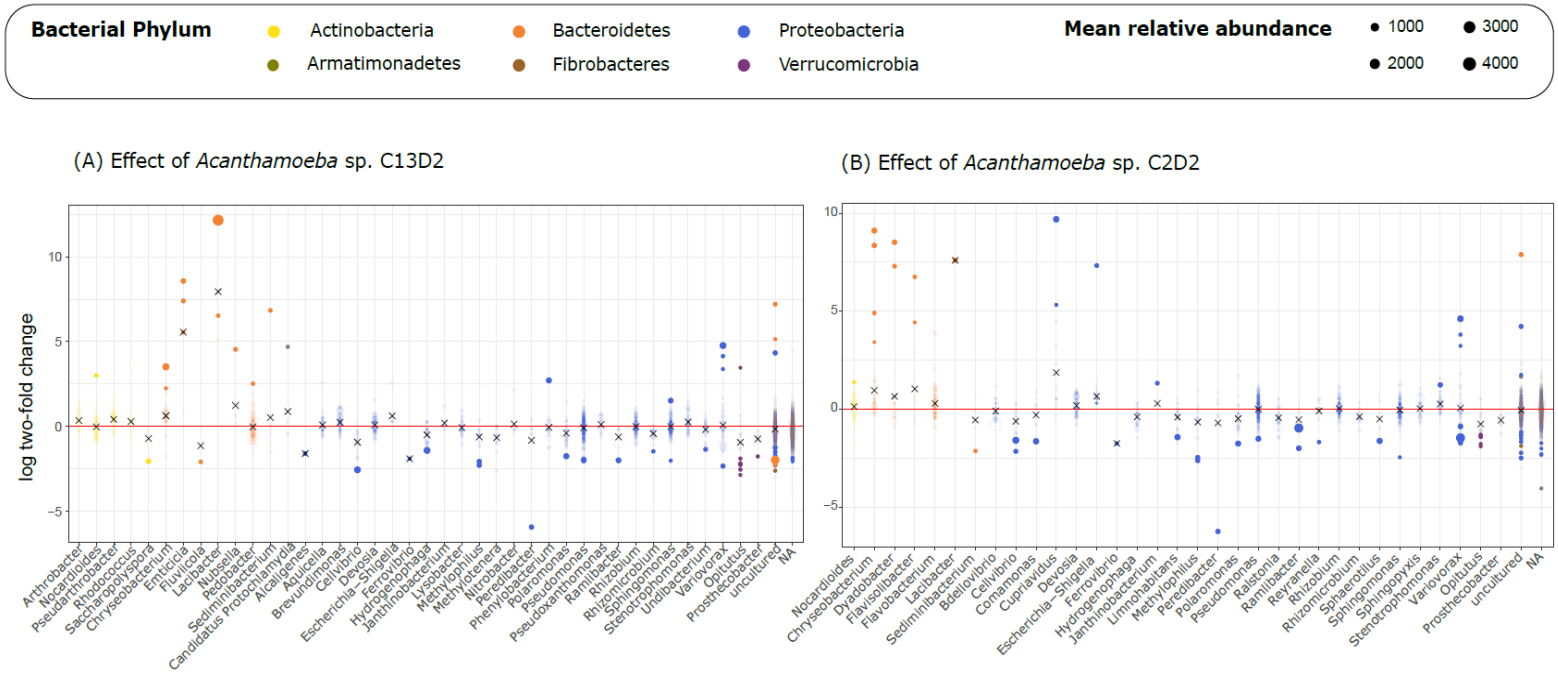

Fig. S3: Predatory impact of the Acanthamoeba spp. on the soil bacterial community, given as results from the DESeq analysis for each treatment against the control. Dots above the red line represent bacterial OTUs for which the relative abundance was higher in the treatment compared to the control, dots below the red line represent bacterial OTUS for which the relative abundance was lower in the treatment compared to the control. Open circles are the OTUs that were not significantly modified. The black crosses indicate the average values of the log two-fold changes of bacterial OTUs for each genus, including all non-significantly modified OTUs.

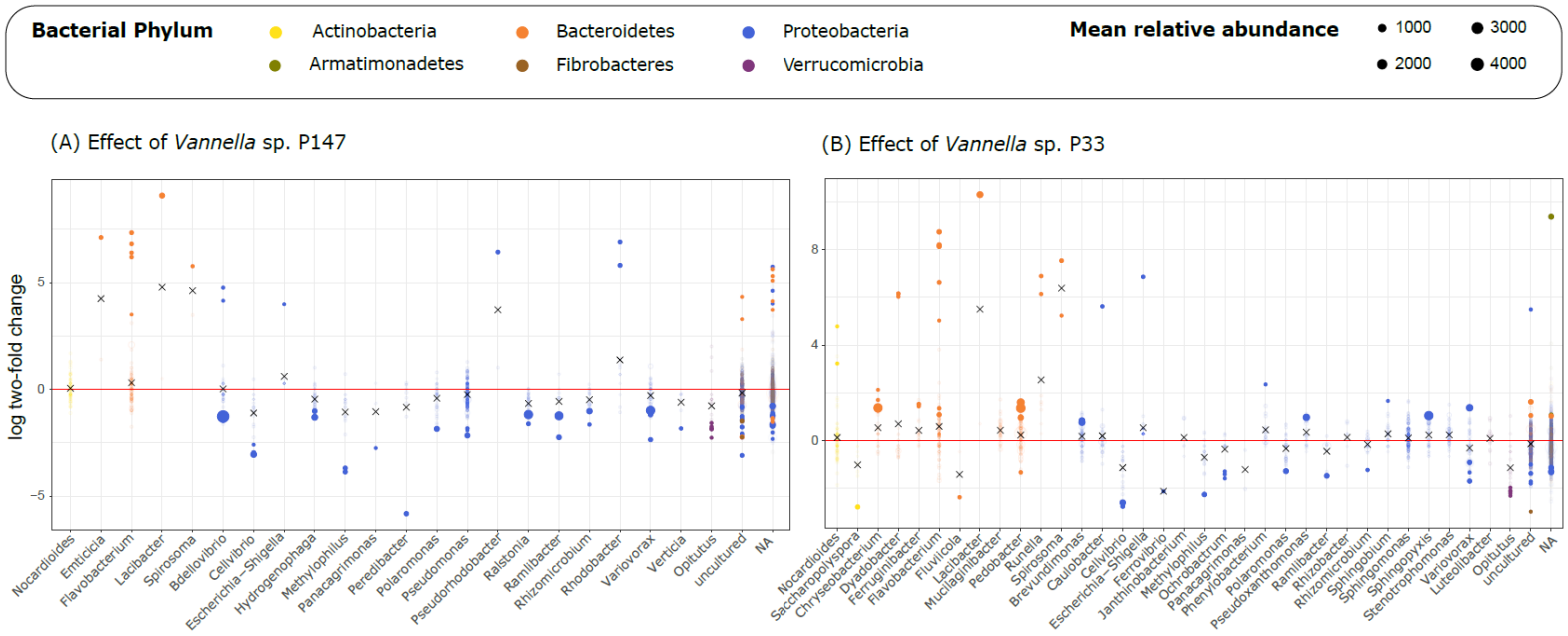

Fig. S4: Predatory impact of the Vannella spp. on the soil bacterial community, given as results from the DESeq analysis for each treatment against the control. Dots above the red line represent bacterial OTUs for which the relative abundance was higher in the treatment compared to the control, dots below the red line represent bacterial OTUS for which the relative abundance was lower in the treatment compared to the control. Open circles are the OTUs that were not significantly modified. The black crosses indicate the average values of the log two-fold changes of bacterial OTUs for each genus, including all non-significantly modified OTUs.

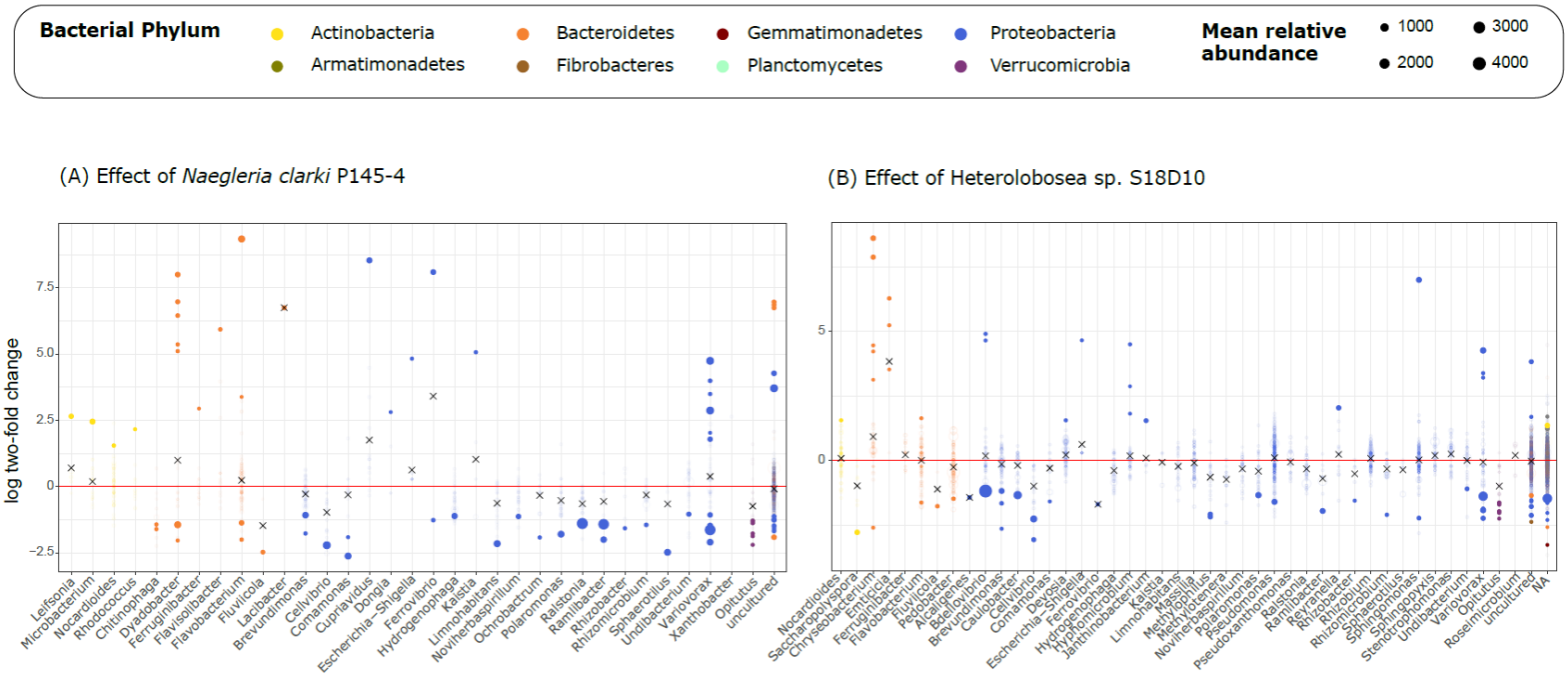

Fig. S5: Predatory impact profile of the protists Naegleria clarki P145-4 and Heterolobosea sp. S18D10 on the soil bacterial community, given as results from the DESeq analysis for each treatment against the control. Dots above the red line represent bacterial OTUs for which the relative abundance was higher in the treatment compared to the control, dots below the red line represent bacterial OTUS for which the relative abundance was lower in the treatment compared to the control. Open circles are the OTUs that were not significantly modified The black crosses indicate the average values of the log two-fold changes of bacterial OTUs for each genus, including all non-significantly modified OTUs.

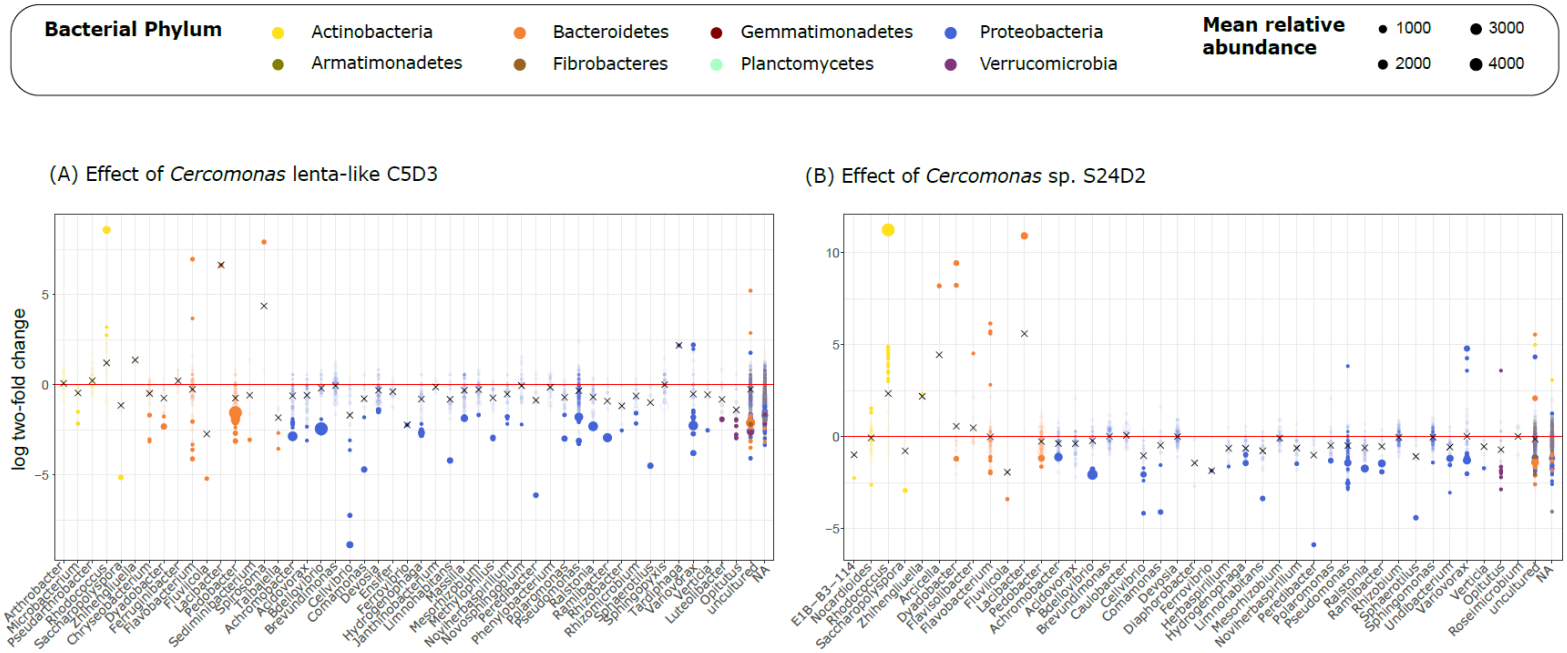

Fig. S6: Predatory impact profile of the Cercomonas spp. on the soil bacterial community, given as results from the DESeq analysis for each treatment against the control. Dots above the red line represent bacterial OTUs for which the relative abundance was higher in the treatment compared to the control, dots below the red line represent bacterial OTUS for which the relative abundance was lower in the treatment compared to the control. Open circles are the OTUs that were not significantly modified The black crosses indicate the average values of the log two-fold changes of bacterial OTUs for each genus, including all non-significantly modified OTUs.

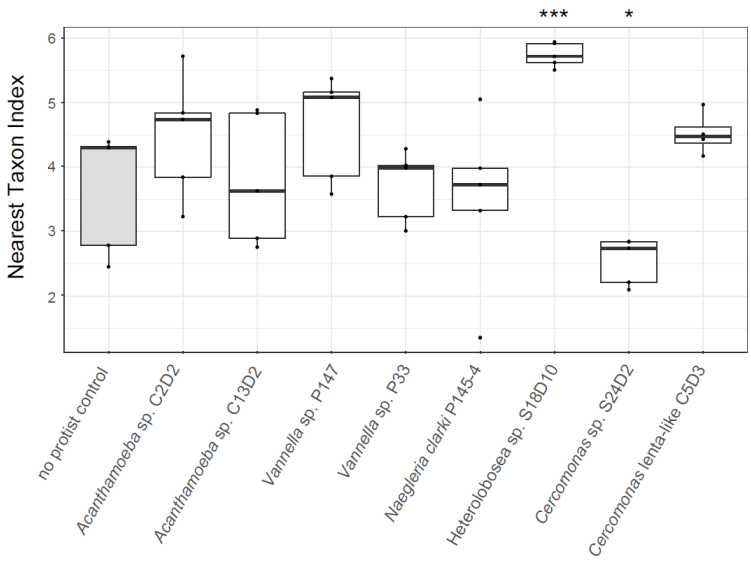

Fig. S7: Nearest taxon index of the soil bacterial communities with the different protist treatments. The asterisks indicate significant p-values compared to the control treatments; "" and "***" corresponding to p-value < 0.1 and < 0.001 respectively.*

Chapter 4

Taxon-specific impact of protist inoculation on plant properties and bacterial community structure

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Abstract

As the major bacterial predators in soils, free-living heterotrophic protists generally support plant growth by the release of excess nutrients from their consumption activities. Most protist species, however, do not feed equally on all bacteria, thus potentially leading to specific modifications of the prey community composition that could have consequences for plant performance. Elucidating the predatory impact of single protist species is the first step toward understanding the functional diversity of the numerous protists species co-existing in soils. Here, we examined the impact of six different protist species (from three lineages: Amorphea, TSAR and ´Excavates') on a range of plant properties including plant biomass and nutrient content and assessed the effect of each protist on soil-borne bacterial community structure via 16S rRNA gene amplicon sequencing. While protist inoculations did not significantly impact plant biomass or shoot nutrient content, two protist species (*Vannella* sp. and *Cercomonas* sp.) significantly increased the plant shoot-to-root ratio. Each protist induced subtle changes in bacterial community composition with taxon-specific enrichment/depletion of only certain bacterial taxa. Our results demonstrate that different protist species induce taxon-specific responses in the plant and soil community and highlight the importance of non-model species approaches when examining the ecological role of soil protists.

1. Introduction

Free-living, heterotrophic protists exhibit high density and diversity in most soils and are recognized as major consumers of bacteria (Oliverio *et al.* 2020; Xiong *et al.* 2021). Predatory protists can impact the bacterial community structure (Glücksman *et al.* 2010; Müller, Scheu and Jousset 2013), because they typically feed preferentially on some bacteria (Singh 1942; Pedersen *et al.* 2011). These changes in the prey community likely depend on predator identity (Glücksman *et al.* 2010). Prey preference may be due to specific protist traits such as cell volume and cell flexibility (Glücksman *et al.* 2010; Gao 2020), but also to bacterial traits such as toxicity (Jousset *et al.* 2009) and nutritional value (Weekers and Drift 1993; Bjørnlund *et al.* 2006). Prey selection further depends on the relative abundance of different prey types (Jürgens and DeMott 1995; Boenigk *et al.* 2002). Protists' predatory impact may thus differ depending on the fraction of the bacterial population, potentially having a stronger impact on the more easily available abundant (Jürgens and DeMott 1995) and competitive taxa (Kurm *et al*. 2019). Predation by protists may thus support higher bacterial diversity by preventing that competitive taxa become dominant and allowing the co-existence of otherwise rare taxa (Kurm *et al.* 2019).

The predatory activity of soil protists further typically benefits plant growth, because it liberates nutrients locked in the bacterial biomass (Clarholm 1984; Bonkowski 2004), including nitrogen (Bonkowski *et al.* 2000; Krome *et al.* 2009), phosphorous (Bonkowski, Jentschke, and Scheu 2001), magnesium and calciu m (Herdler *et al.* 2008). Such effects have not only been reported for the model plant *Arabidopsis thaliana* and the model amoeboid protist *Acanthamoeba castellanii* (Rosenberg *et al.* 2009), but also with single or multiple, identified or unidentified protists on crop plants such as wheat (Clarholm 1985; Kuikman and Van Veen 1989; Kuikman *et al.* 1990) and rice (Asiloglu *et al.* 2020). In the above mentioned research, the plants typically displayed increased shoot biomass and nitrogen content. In addition to the general benefit of unlocking nutrients, predation by protists might affect plant performance by inducing specific changes in the rhizosphere-associated bacterial community composition (Bonkowski and Clarholm 2012). Indeed, the composition of the rhizosphere bacterial community is closely related to plant development (Berendsen, Pieterse and Bakker 2012; Ikeda-Ohtsubo *et al.* 2018). Modifications in root morphology, such as the increase in lateral roots, have been observed in the presence of protists. Such changes in root morphology were explained by either the single or combined effect of the release of nutrients and changes in the bacterial community in favor of auxin-producing bacteria (Bonkowski and Clarholm 2012; Krome *et al.* 2010). The presence of protists was further associated with an increased survival and activity of plant-growth beneficial bacteria such as *Pseudomonas* spp. (Weidner *et al.* 2016) and *Azospirillum* sp. B510 (Asiloglu *et al.* 2020). The extent to which specific shifts in bacterial communities due to predation by different protist species could beneficially influence plant development is, however, largely unknown.

In a controlled pot experiment, we investigated the individual effect of six different protist species covering various lineages (Amorphea, TSAR and 'Excavates') and morphotypes (three amoeboid, two amoebo-flagellates, one flagellate) on the growth characteristics and nutrient content of lettuce plants (*Lactuca sativa*) growing on protist-amended soil. We also assessed the individual effect of protists on the soil bacterial community composition prior to plant transfer and on the rhizosphere-associated bacterial community at the end of the experiment via 16S rRNA gene amplicon sequencing. We hypothesized that adding protists would (1) induce phenotypic changes in the plant such as increased plant biomass, nitrogen content and root surface area. We further hypothesized that (2) protist amendment would alter both soil and rhizosphere bacterial community structure. We expected (3) the effects of protist inoculation on plant phenotype and bacterial communities to be protist taxon-specific. Finally, (4) we expected that changes in plant phenotype would be explained by protist taxon-specific induced shifts in bacterial community composition.

2. Materials and Methods

2.1 Preparation of protist-free, semi-natural soil bacterial community

Bacteria were extracted from a sandy soil collected at the Botanical Garden of Utrecht University (The Netherlands) and processed to obtain a protist-free bacterial community by following an adapted protocol from Rønn *et al.* (2002). Briefly, 75 g fresh soil were air-dried over 48 h before being resuspended in 250 mL 0.1% pyrophosphate buffer (1.68 $Na_4P_2O_7$ x 10 H_2O g L^{-1}). The resulting slurry was mixed for 1 min at maximum speed in a kitchen blender (Mix55, BCC Proline, Groupe Fnac Darty, Ivry-sur-Seine, France) and placed for 2 min on ice. The procedure was repeated five times. The obtained solution was subsequently centrifuged at 1,000 *g*, for 40 min at 4 °C using a fixed-angle rotor (Thermo Fisher Scientific, Langenselbold, Germany) with the lowest acceleration and deceleration settings to avoid resuspension. The supernatant was sieved over 50 µm mesh and vacuum filtered in a stepwise fashion using autoclaved Whatman® glass microfiber filters (Cytiva, Marlborough, USA) from 3 µm, 1.6 µm to 1.2 µm with a Büchner Funnel to remove micro-fauna including protists, while keeping the smaller size micro-organisms mostly constituted of bacteria. A volume of 10 mL of the bacterial suspension was inoculated in 250 mL CELLSTAR® cell culture flask culture flasks (Greiner Bio-One GmbH, Kremsmünster, Austria) filled with 30 mL of 0.13 mg L^{-1} TSB (BD, New Jersey, US) supplemented with 100 mg L^{-1} agar (BactoTM Agar, BD, New Jersey, US). After five days of incubation at 15°C in the dark, absence of contaminating protists was confirmed by direct observation at a 200x and 400x magnification under a Nikon Eclipse TS 100 inverted microscope with phase contrast (Nikon, Tokyo, Japan). By serial dilution plating on 3 g L^{-1} TSA plates for 12-24 hours at 28°C, we estimated the bacterial density to be ca 3.5 x 10^8 CFUs mL^{-1}.

2.2 Protist isolates

The protists were isolated from a range of environments (sandy soil and growth substrate)

in the Netherlands and characterized by Gao (2020). The selected protists represent some of the main lineages of soil-dwelling free living protists (*i.e.*, Amorphea, TSAR and "Excavates", according to Burki *et al.* (2020)) and covering various morphotypes (three amoeboid, two amoebo-flagellates, one flagellate, Table 1). In addition, we analyzed the 16S rRNA gene amplicon sequencing data from this previously performed soil microcosm study (Gao, 2020); we provide a summary of the study in the supplementary material) to estimate the predatory impact of the protists. We analyzed their impact on their prey community in terms of effects on the bacterial species observed richness, Pielou's evenness and community composition compared to a non-protist control (Bray-Curtis dissimilarity); the results of this analysis were used to select the isolates and a summary is given in Table 2 (detailed statistical results in Table S1).

The protist cultures were propagated in Page Amoeba Saline (PAS; Page 1976) on *Escherichia coli* OP50 as sole prey (*ca* 10^8 cells mL^{-1}) and kept at 15 °C in the dark. Prior the inoculation in the soil, we prepared the protist solution as follows. First, we detached the protists from the surface of the culture flasks by mild bath sonication of 1 min (220 Volts, cycles 50 s^{-1}, 0.25 Amp; Sonicor SC-50-22, Sonicor Instrument Corporation, New York, US). Second, we removed the spent medium and enriched the protists by three steps of gentle centrifugation at 800 *g* for 5 min (Heraeus Megafuge 40 Centrifuge, Thermo Fisher Scientific, Langenselbold, Germany), followed by careful removal of the supernatant. After centrifugation, the protists are concentrated in the lower part of the tube without forming a visible pellet, so that at each step we only discarded 75 % of the volume before resuspending the cells in the same volume of PAS. Because these washing steps are not sufficient to fully eliminate the presence of bacteria cells, we quantified the remaining bacteria by serial dilution plating 3 g L^{-1} TSA for 24h at 28°C (See Table 1 for bacterial load in each culture).

Protist density was estimated after transferring a volume of 100 µL in Clear Polystyrene 96-Well Microplates with flat bottom (Corning® 3370, Corning Incorporated, New York, US). The cells (active and encysted) were enumerated over three to five screens on a monitor connected to a phase contrast 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 estimated densities can be found in Table 1.

Table 1: Description of the protist isolates used in the present study. Taxonomic assignment, morphotype and volume are given as described in Gao (2020). The eukaryotic supergroups were assigned according to Burki et al. (2020).

Isolate code	Eukaryotic supergroup	Species	Morpho-type	Volume [μm^3]	Active ind. mL^{-1}	Cyst mL^{-1}	CFUs mL^{-1}
P1-1	Amorphea	*Didymium* sp.	amoeboid	504.64	3.05×10^4	0	1.32×10^6
P33	Amorphea	*Vannella* sp.	amoeboid	136.58	6.09×10^3	0	8.65×10^5
NL10	'Excavates'	*Allovahlkampfia* sp.	amoebo-flagellate	979.19	1.22×10^4	0	1.45×10^6
S24D2	TSAR*	*Cercomonas* sp.	flagellate	278.31	6.09×10^4	0	1.76×10^6
P147	Amorphea	*Vannella* new sp.	amoeboid	261.87	6.09×10^3	0	1.74×10^6
NL81	'Excavates'	*Naegleria* sp.	amoebo-flagellate	3359.57	3.66×10^4	3.66×10^4	6.75×10^4

** TSAR stands for telonemids, stramenopiles, alveolates, and Rhizaria (Burki et al. 2020); Note that the supergroup 'Excavates' is given in quotes as it lacks phylogenetic support (Burki et al. 2020); we still considered it useful to help the reader locate the protist isolates relative to each other and appreciate that, for instance, the Vannella spp. are phylogenetically closer to Didymium sp. compared to Naegleria sp.*

Table 2: Impact of the selected protist isolates on bacterial community in a soil microcosm experiment, in absence of plants. The soil microcosm was performed by Gao (2020). BH stands for the Benjamini and Hochberg correction for multiple testing. The arrows indicate the direction of the effect compared to control, when significative. NS = Not significant.

Isolate code	Bacterial Species richness (observed)	Pielou's Evenness Index	Bacterial structure (Bray-Curtis Dissimilarity relative to control)
P1-1	⬇ $p<0.001$	⬇ $p<0.001$	BH corrected $p=0.048$
P33	⬇ $p=0.016$	⬇ $p=0.017$	BH corrected $p=0.036$
NL10	⬇ $p=0.003$	⬇ $p=0.003$	BH corrected $p=0.036$
S24D2	NS	⬇ $p=0.014$	BH corrected $p=0.030$
NL81	NS	NS	BH corrected $p=0.048$
P147	NS	NS	BH corrected $p=0.06$

2.3 Experimental setup

The experimental setup followed a three-phase procedure as depicted in Figure 1: Phase 1, preparation and inoculation of a protist-free bacterial community to a gamma-sterilized soil; Phase 2, application and incubation of the protist treatment for three weeks; each treatment was set up in 13 replicates; Phase 3, germination, transfer of plant seedling and destructive harvest after twenty-one days. Soil samples were collected in each phase for the analysis of the bacterial communities via amplicon tag sequencing of the V4 region of the 16S rRNA gene. The effects of the treatments on plant properties included assessment of dry shoot and root biomass, shoot element content and root surface area.

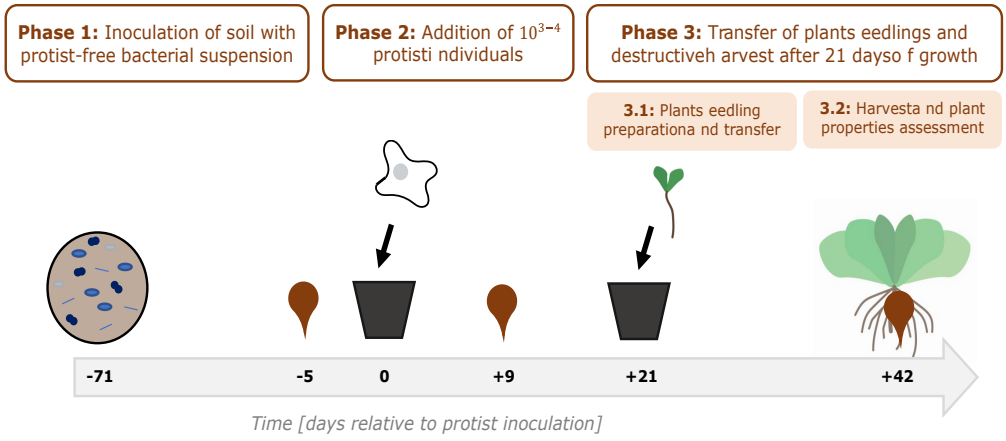

Figure 1: Illustration of the experimental setup and timeline. Day 0 indicates the day of protist inoculation. The brown signs at days -5, +9, and +42 indicate soil sampling times for bacterial community analysis (16S rRNA gene amplicon tag sequencing). In phase 1, a protist-free bacterial community (represented with blue icons of different size and shapes) is inoculated in a gamma-sterilized soil. In phase 2, protist (represented by an amoeboid shape) treatments are applied in the soil preliminary divided in pots; Note that six different protists were applied as single-species treatments and that we included a non-protist control treatment. In phase 3, lettuce seedlings are transferred in the pots and let grow for 21 days until the destructive harvest.

2.3.1 Phase 1: Inoculation of soil with protist-free bacterial suspension

Sandy soil was collected from the Botanical Garden of Utrecht University (The Netherlands), sieved (mesh size: 2 mm) and sterilized via gamma irradiation in 6 batches of ca 2 kg (>25 kGray; Isotron, Ede, The Netherlands).

The semi-natural protist-free bacterial community was inoculated into the bulk soil and let to establish over a period of 11 weeks at room temperature in the dark as follows: A volume of 20 mL of bacterial solution was diluted in 20 mL 0.9% NaCl and then added to *ca* 2 kg of gamma-sterilized soil. An additional 40 mL of demineralized water was added to ensure suitable soil moisture. The procedure was repeated six times in order to process all the required soil. A volume of 30 mL of autoclaved (20 min, 120°C, 100 kPa) demineralized

water was added every two weeks to maintain moderate moisture levels (*i.e.*, moderate clumping of the soil was seen upon compaction). The inoculated soil was mixed thoroughly each time by multiple inversion of the bag.

After 11 weeks, we sampled 1 g of soil from each soil batch (six samples in total) and froze them at -80°C for later DNA extraction to investigate the established bacterial community (referred to as initial soil bacterial community).

2.3.2 Phase 2: Pot preparation and application of protist treatment

We used Göttinger square pots (Width: 7 cm, Length: 7 cm, Height:8 cm; Lamprecht-Verpackungen GmbH, Göttingen, Germany). For optimal holding of the sandy soil in the pots, we first applied a thin layer of fine gravel at the bottom of each pot. To reduce contamination risk, the gravel was washed with water, autoclaved twice (20min, 120°C, 100 kPa) and treated under UV for 30 min. Before addition, the soil was visually checked for the presence of mycelium as indicator for fungal growth and soil samples were diluted in water before observation under microscope to further confirm the absence of protists and fungi. An amount of 200 g of soil was added to each pot and 2 ml of autoclaved demi water was applied to dampen the soil. The pots were distributed over eight different boxes with lids (12 pots per box, at least one replicate per treatment). The boxes were used to limit colonization by air-borne organisms. A hole of *ca* 1 cm depth was performed in the middle of each pot and 1 mL of protist solution was inoculated; the same volume of PAS was used for the non-protist control. To keep the soil moist and support the protist establishment a volume of 10 mL of autoclaved water was sprayed homogeneously on the soil surface. The soil was checked every day during the whole duration of the experiment (*i.e.*, before and after plant transfer) and water was added if required. Soil moisture, due to its importance for protist populations (Kuikman, Jansen, and Van Veen 1991; Geisen *et al.* 2014), was held constant. Due to strong variation between the boxes, this led to different watering effort for each box.

After 9 days, soil samples of ca 1 g were taken in the middle of each pot and frozen at -80°C for later DNA extraction. The duration of 9 days was chosen to be similar to a previous soil microcosm performed with the same protist isolates (Gao 2020).

2.3.3 Phase 3.1: Plant seedling preparation and transfer

Lettuce seeds were prepared according to an adapted protocol from Trinh *et al.* (2018) and transferred as seedlings 21 days after protist inoculation. First, we surface sterilized seeds of the lettuce *Lactuca sativa* Wonder der Vier Jaargetijden (De Bolster biologische zaden, Epe, The Netherlands) by placing them in 5% NaOCl for 10 min and subsequently rinsing them four times in 50 mL autoclaved demi water. To induce germination, the surface-sterilized seeds underwent a stratification step (three days at 4°C in the dark). The seeds were then transferred on Murashige and Skoog medium (Minimal Organic Powder Medium,

SERVA Electrophoresis GmbH, Heidelberg, Germany) with 3% sucrose (MS3; 8 to 10 seeds per petri dish) and incubated in a growth cabinet with a 16h/8h day/night regime, at 21°C for 4 days (Phytotron facility, Utrecht University, The Netherlands). Seedlings with comparable size (*i.e.*, 1 cm root length and visually healthy dicotyledons) were transferred to the middle of each pot. The plants were kept to grow in the growth cabinet with constant relative humidity at 70%, a 16h/8h day/night regime, at 21°C for 20 days. Every second day, the pots were watered with demineralized water to keep soil moisture constant, and both, pots within the boxes and the boxes within the growth cabinet, were randomized. The boxes themselves had a strong impact on the moisture content and were thus considered in the data analysis.

2.3.4 Phase 3.2: Destructive harvest and assessment of plant properties

After 21 days of growth, the plants were separated from the soil by inverting the pot on a paper, taking care to damage the roots as little as possible. The shoot was separated from the root by cutting at the shoot-root interface and dried at 70°C for 48h before weight measurement according to an internal manual and the Handbook of reference methods for plant analysis (Kalra 1998). Dried shoot material was further processed for Inductively Coupled Plasma Atomic Emission Spectrometry (ICP) and Elemental Analyzer (EA) analysis. The shoots were ground by use of 2 mL Eppendorf tube containing a metal ball placed on a Retsch MM200 mill (Retsch GmbH, Verder Scientific, Haan, Germany) for 2 min at frequency of 30 s^{-1}. The ground samples were dried at 70°C for 90 min. The procedure for sample preparation for ICP analysis described as in Isaac and Johnson (1998) was followed with a modification for lower amount. An amount of *ca* 50 mg of dried, ground shoot material was transformed to ash by a 2 hours exposition at 500°C in a muffle furnace, digestion in 300 µL of HNO_3 (65%), complete evaporation on a hot plate at 130°C, and 1 hour exposition at 500°C in the muffle furnace. We then added 100 µL HCl (32%) and 9.9 mL deionized water to the ash before processing the samples via the ICP Spectrometer iCAP 6000 Series (Thermo Fisher Scientific, Cambridge, United Kingdom) coupled with a AutoSampler ASX-520 (Teledyne CETAC Technologies, Nebraska, US). The concentration of the following elements was measured: Al, Ca, Cd, Cu, Fe, K, Mg, Mn, Na, P, Pb, S, and Zn. The values were converted from mg L^{-1} to the mg per plant based on the dry shoot weight initially measured. An amount of 2.5-3.5 mg dry ground shoot material was weighed on a Mettler Toledo MX5 microbalance (Mettler Toledo, Greifensee, Switzerland), placed into a small tin cup and arranged on a 96-wells plate as preparation for the elemental analyzer. Calibration standards employed included: an empty tin container, 25-(Bis(5-tert-butyl-2-benzo-oxazol-2-yl) thiophene (BBOT), acetanilide, and atropine. The samples were analyzed via an Interscience EA 1110 CHNS-O elemental analyzer (CE Instruments Ltd, Wigan, England) to obtain percentage of carbon and nitrogen within the samples. The obtained values were used to calculate the relative and absolute amount of carbon and nitrogen per plant as well as the C:N ratio.

The roots and the remaining soil around the roots were put in a Falcon tube and dry shaken. This step allowed to obtain the soil that was the most attached to the roots and considered as our rhizosphere soil. A sample of *ca* 0.5 g of rhizosphere soil was collected for DNA extraction. The roots were further washed with demineralized water over a sieve (mesh size: 2 mm) before being placed in petri dishes containing demineralized water, where they were carefully untangled with tweezers to facilitate image analysis with a WINRhizo scanner (Winrhizo, Regent Instruments Inc, Quebec, Canada). The root area was extracted from each image using ImageJ (Schneider, Rasband, and Eliceiri 2012). The roots were then dried at 70°C for 48h before weight measurement.

Some plant samples were lost during the procedure: one replicate of the treatments P147, NL10 and NL81 is fully missing of the plant analysis; the shoot samples of two replicates of the treatments S24D2 and P1-1 were lost due to mislabeling; the root samples of one replicate of the treatments S24D2 and P1-1 were lost. For the samples with the loss of the entire plant and/or the roots, no rhizosphere samples could be retrieved and are thus also missing from the bacterial community analysis.

We investigated the potential contamination by other protists as well as the presence of our selected protists at different steps under inverted microscope (Nikon Eclipse TS 100, Tokyo, Japan). We observed the presence of unidentified flagellates in the respective inoculants of *Allovahlkampfia* sp. NL10 and *Vannella* sp. P147 24-48 h after the application. We also tested for the presence of the inoculated protists after nine days and at the time of harvest. To this end, three grams of bulk/rhizosphere soil from six replicates per treatment were resuspended in 10 mL PAS, diluted ten folds and 2 mL was transferred into 6-wells NunclonTM Delta surface plates (Thermo Fisher Scientific, Roskilde, Denmark) for direct, qualitative observation or potential contaminants. Nine days after protist inoculation, mostly cysts were observed for *Allovahlkampfia* sp. NL10, *Vannella* sp P33, and *Naegleria* sp. NL81; few active individuals for *Didymium* sp. P1-1 and *Cercomonas* sp. S24D2, no amoebae were observed in the treatment with *Vannella* sp. P147, but some (unwanted) flagellates were observed. No protists were observed in the control treatment. The samples from the rhizosphere samples revealed some unidentified ciliates in all treatments, including the control.

2.4 Bacterial community analysis

We investigated the bacterial community composition at three different time points: before protist inoculation (initial soil, phase 1), 9 days after protist inoculation (prior plant transfer, phase 2) and at the final harvest (phase 3; see also Figure 1 for the overview of the experiment). A sample of *ca* 0.5 g of soil was used for DNA extraction using the DNeasy® PowerSoil® kit (Qiagen, Hilden, Germany) and following the manufacturer protocol with slight modifications previously shown to increase yield in our research group: an amount of 0.5 g of soil instead of 0.25 g was taken and lysis of the cells was obtained using a Retsch mill MM400 (Retsch GmbH, Verder Scientific, Haan, Germany) with Qiagen TissueLyser

Adapter (Qiagen, Hilden, Germany) fitting up to 24 2 mL Eppendorf tubes, at a frequency of 30 sec^{-1} for 6 min.

Estimates of total bacterial community abundance were obtained via qPCR targeting the V4 region of the 16S rRNA gene. Extracted environmental DNA was diluted twenty times and prepared for qPCR analysis with a Freedom EVO® Tecan robot (Tecan Trading AG, Männedorf, Switzerland). Bacterial DNA abundance was measured with an Applied Biosystems ViiATM7 PCR system (Thermo Fischer Scientific, Massachusetts, USA) using primers targeting the V4 region of the 16S rRNA gene (341F 5'-CCTACGGGNGGCWGCAG-3' and 805R 5'-GACTACHVGGGTATCTAATCC-3'; Herlemann *et al.*, 2011; Besemer *et al.*, 2012). The qPCR master mix was prepared with solutions of ItaqTM universal SYBR® green supermix (Bio-Rad Laboratories, Veenendaal, The Netherlands), forward primer (5 µM), reverse primer (5 µM), and milliQ water with the respective volume per sample: 5 µL, 0.5 µL, 0.5 µL, and 1.5 µL. A volume of 2.5 µL of template DNA was used per sample. The qPCR was performed by an initial denaturing step at 95°C for 30 s with subsequent cycling for 40 times with a 15 s denaturizing step at 95°C and a combining annealing/elongation steps at 60°C for 1 min. Melting curves were obtained based on a standard protocol and used to identify the characteristic peak of PCR product. Two independent technical replicates were performed for each sample.

The DNA extracts were further used as templates for high throughput 16S rRNA amplicon sequencing as carried out by Genome Quebec (Montréal, Canada). Before sending the extracted DNA solution, the obtained DNA yield was assessed on a DeNovix DS-11 Spectrophotometer (DeNovix, Wilmington, Delaware, US). The V4 region of the 16S was amplified on an Illumina MiSeq PE 250 bp sequencing machine (Illumina, California, US) using the same set of primers mentioned above (341F and 805R) with the MiSeq Reagent Kit v3 600 cycles from Illumina.

After sequencing, the primary analysis of raw FASTQ data was processed using QIIME2 pipeline (version 2020.6; Caporaso *et al.* 2010). The DADA2 workflow (Callahan *et al.* 2016) was followed with the default settings. QIIME2's q2-feature-classifier plugin (Bokulich *et al.* 2018) was used for taxonomy assignment against the SILVA 138 reference database at 99% OTUs from 515F/806R region of sequences (Glöckner *et al.* 2017). The version 4.0.3 of the open source statistical software R (R Core Team 2020) was used for the following processing of the datasets. After removing the sequences assigned to Eukaryota (n=4), chloroplast (n=52) and mitochondria (n=21), we obtained a total of 8,842 Amplicon Sequencing Variants (ASVs). To compare bacterial communities between the treatments, sequence read numbers were normalized to the minimum sequence number (12,400 reads) by random subsampling (phyloseq::rarefy_even_depth specify rngseed(12400) for reproducibility of the analysis; see also Figure S1 for the number of species in function of the sample size)

2.5 Data analysis

All data analyses were performed using the version 4.0.3 of the open source statistical software R (R Core Team 2020).

2.5.1 Plant properties

The effect of the protist treatment on the total dry biomass, the shoot dry weight, the root dry weight and root surface area was investigated with a sequential two-way analysis of variance (ANOVA, Type I Sum of Square), considering first the effect of the box (stats::lm, stats::anova). The ratio between the shoot and root was first log-transformed to address the skewness of the data, before the same sequential two-ways ANOVA was performed. We analyzed the nitrogen and carbon shoot content both as relative (% per plant) and as absolute amount (mg per plant). We calculated the carbon to nitrogen ratio based on the absolute values. We performed a sequential two-ways ANOVA as described above. Similarly to the other plant traits, significant differences in the shoot content of Al, Ca, Cd, Cu, Fe, K, Mg, Mn, Na, P, Pb, S, and Zn in the treatments compared to the control were assessed via a sequential two-ways ANOVA as described above. If the ANOVA was found significant, a Tukey post hoc test was performed to identify which groups were different from each other (stats::TukeyHSD).

2.5.2 Bacterial communities

We estimated bacterial abundances based on the copy numbers of the 16S gene from our qPCR analysis (Pfaffl 2001) and adjusted them to copy numbers per gram of soil. We calculated and plotted the species richness (phyloseq::plot_richness, with measures = c("Observed", "Chao1" and "Shannon"; McMurdie and Holmes, 2013) and calculated the Pielou's evenness index ($J'=H'/(H'_{Max})$) with H' being the Shannon Index and H'Max taking on the maximum value possible for the Shannon Index (*i.e.*, $H'_{Max} = \ln(S)$, S being the number of species) using the rarefied dataset to correct for differences in sequencing depth between samples.

The effect of the protist treatment on the estimated bacterial abundance, the bacterial species richness and evenness was investigated with a sequential two-way ANOVA (Type I Sum of Square) to take into account the box effect (stats::lm and stats::anova) for both time points. We performed a post hoc Tukey test in case of significant results of the ANOVA for the treatment (stats::TukeyHSD). We investigated the effect of the protist treatment on the change in community composition at both time points (prior plant transfer, phase 2, and at the harvest, phase 3) by performing a sequential two-way PERMANOVA (vegan::adonis; Box + protist) on the Bray-Curtis dissimilarity and phylogenetic aware UniFrac and weighted UniFrac distances calculated on the rarefied dataset.

Because the amplitude of predatory impact may vary depending on the protist species, in addition to the typical alpha and beta diversity analyses, we examined the effect of protists on different fractions of the bacterial community (rare, moderate, and abundant), on the ten most dominant phyla and further identified significant changes in the relative abundances of bacterial taxa for each protist taxon compared to the no-protist control. We investigated the effect of protists on different abundance categories defined as "rare" when corresponding to < 0.1% of the total reads, "moderate" between 0.1% and 1% and "abundant" when corresponding to > 1% (as described by Dai *et al.* 2016). Here, we also performed a sequential two-way PERMANOVA (vegan::adonis) on the Bray-Curtis dissimilarity, UniFrac and weighted UniFrac to investigate potential changes compared to the control. We also compared the relative abundance of the 10 dominant bacterial phyla with the sequential two-way ANOVA for both time points. We further explored the identity of bacterial taxa that increased or decreased in relative abundance upon the inoculation of protists. We subset our dataset in smaller datasets consisting of the treatment of interest and the control and removed rows containing only zeros. We plotted the log two-fold changes for each treatment compared to the control (DESeq; Love *et al.* 2014); we had to add an arbitrary one to each count due to the occurrence of zeros and we used an alpha of 0.001, 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).

Note that in order to obtain a reproducible data analysis, we set the root of the phylogenetic tree with set.seed(224) when using the UniFrac and weighted UniFrac distance. In case of significant PERMANOVA analysis, we did the pairwise test to identify which treatments were significantly different from each other; we controlled for multiple-testing by adjusting our p value with the Benjamini and Hochberg correction (Noble 2009); such correction is indicated as "BH corrected *p*" in the text.

2.5.3 Relations between rhizosphere-associated bacterial genera, plant properties and predatory impacts

We performed a correlation analysis between the relative abundance of rhizosphere-associated bacterial genera and plant properties to identify potential relations (stats::cor.test). We corrected the *p* values for multiple testing using the Bonferroni correction (stats::p.adjust) and visualize the significant correlations (corrected $p < 0.05$) in a heatmap (gplots::heatmap.2). We further took the average of the log two-fold values obtained from the DESeq analysis (when significant) to investigate the predatory impact of the protists on these identified bacteria. We visualized the direction of the protist impact (increased/decreased bacterial abundance) on the side of the heatmap. We provided a graphical reminder of the impact of protists on the plant properties on top of the heatmap.

3. Results

3.1 Impact of protists on plant properties

The total biomass, dry shoot and root weight were not significantly affected by the protist treatment (mean ± standard deviation; total biomass: 899.01mg ± 447.85mg, $F_{(6,68)}$=0.842, p=0.542 and Figure 2; dry weight for the shoot: 744.10mg ± 387.54mg, $F_{(6,70)}$=0.878; p=0.516; dry weight of the root: 161.29mg ± 118.60mg, $F_{(6,72)}$=1.673, p=0.14). Only the shoot-to-root ratio was affected by the protist treatment ($F_{(6,68)}$= 2.466, p=0.032) with a significant increase in the treatments exposed to *Vannella* sp. P147 ($t_{(67)}$=3.168, p=0.002) and *Cercomonas* sp. S24D2 ($t_{(67)}$=3.037, p=0.002) compared to the control (Figure 2). A trend was observed for the root surface area ($F_{(6,72)}$=2.084, p=0.066), where the area was significantly lower in the treatments *Vannella* sp. P147 ($t_{(71)}$= -2.234, p=0.029) and *Cercomonas* sp. S24D2 ($t_{(71)}$= -2.258, p=0.027) compared to the control (Figure 2). In addition to the experimental treatments, we observed an important impact of the box effect on the total plant biomass, dry shoot weight, dry root weight, shoot-to-root ratio and the root surface with p<0.001 (see also Table S2).

The treatments had no significant effect on shoot nitrogen content (relative: mean ± standard deviation; relative N content: 4.01% ± 0.78%, $F_{(6,70)}$=0.487, p= 0.816; absolute N content: 27.83 ± 11.01 mg, $F_{(6,70)}$=1.161, p= 0.337) and shoot carbon content (relative C content: mean ± standard deviation=37.51% ± 4.54%, $F_{(6,70)}$=0.748, p= 0.613; absolute C content: 272.77 mg ± 134.67 mg; $F_{(6,70)}$=1.601, p= 0.16), nor the shoot C/N ratio (mean ± standard deviation=9.59 ± 1.53, $F_{(6,70)}$=1.694, p= 0.135) (Table S2). Similarly, the ICP analysis reported significantly indifferent shoot content for all treatments compared to the control (Table S2).

3.2 Impact of protists on bacterial communities

The estimated bacterial abundance remained unchanged by the treatments both, prior plant transfer (average: 1.3 x 109 copies g^{-1}; Box: $F_{(7,75)}$=7.777, p<0.001, Treatment: $F_{(6,75)}$=0.264, p=0.952; note that one sample of P33 could not be used in the qPCR) and at harvest time (average: 8.9 x 108 copies g^{-1}; Box: $F_{(7,67)}$=0.684, p=0.685; Treatment: $F_{(6,67)}$=0.716, p=0.638).

Protist application did not significantly affect the bacterial alpha diversity and evenness prior plant transfer, but some effects on the bacterial alpha diversity were observed in the rhizosphere soil at harvest (Table 3 and Figure 3): the treatments with *Vannella* sp. P33 and *Cercomonas* sp. S24D2 induced an increase of the bacterial species richness compared to the control, while *Allovahlkampfia* sp. NL10 induced a decrease. From the *post hoc* Tukey test, *Vannella* sp. P33 and *Cercomonas* sp. S24D2 induced changes in bacterial species richness compared to all other treatments, but with each other (Table S3). The effects were less pronounced with the Chao1 for which the effect of S24D2 was indifferent from NL81

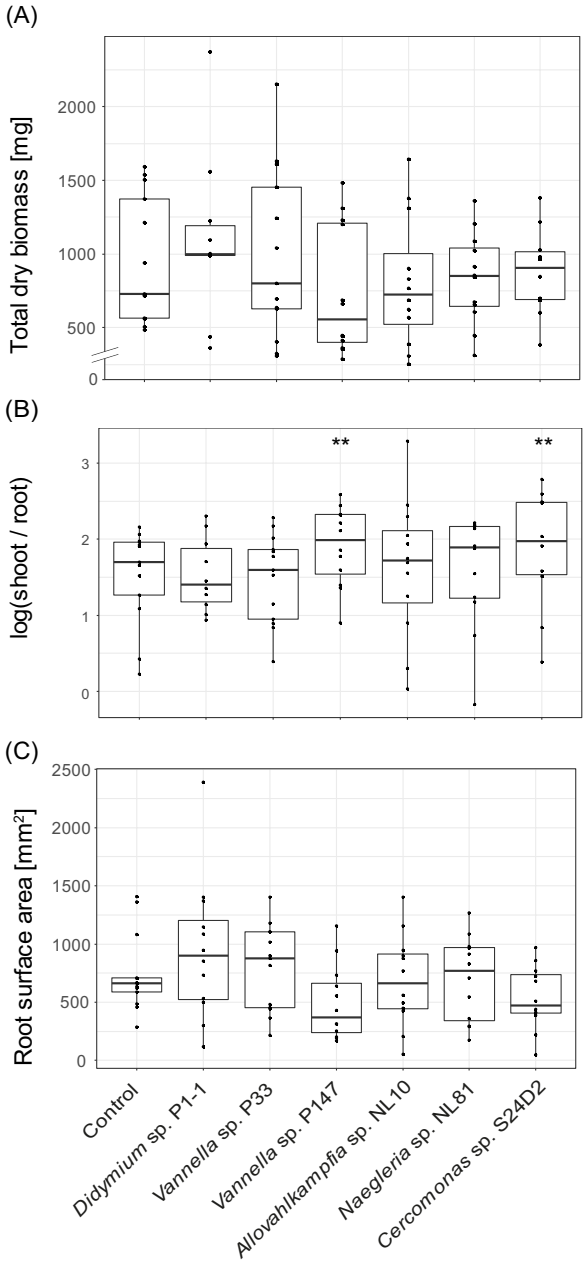

*Figure 2: Effect of protist inoculation on various properties of Lactuca sativa: panel A, total dry biomass, panel B, shoot-to-root ratio (log) and panel C, root surface area. The asterisks indicate significant differences compared to the control (no protist): **: p< 0.01.*

and Shannon indices for which the effect of P33 was indifferent from P1-1 and NL81 and the effect of S24D2 was not significantly different from the control anymore, but only from P147 and NL10 (Table S3). The bacterial evenness remained statistically indifferent across the treatments (Table 3).

Table 3: Impact of the box and protist treatments on the bacterial alpha diversity and evenness in the bulk soil (prior plant transfer) and in the rhizosphere soil (harvest). P values < 0.05 are considered significant and highlighted in bold.

Experimental Phase		Box		Protist Treatment	
	Index	**F test**	***p* value**	**F test**	***p* value**
Bulk soil, prior plant transfer	Species Richness	$F_{(7,76)}$=1.764	0.107	$F_{(6,76)}$=0.718	0.636
	Chao1	$F_{(7,76)}$=1.772	0.105	$F_{(6,76)}$=0.647	0.692
	Shannon	$F_{(7,76)}$=19.67	**<0.001**	$F_{(6,76)}$=0.534	0.781
	Pielou's evenness	$F_{(7,76)}$=26.643	**<0.001**	$F_{(6,76)}$=0.716	0.638
Rhizosphere soil, harvest	Species Richness	$F_{(7,67)}$=4.345	**<0.001**	$F_{(6,67)}$=9.909	**<0.001**
	Chao1	$F_{(7,67)}$=4.302	**<0.001**	$F_{(6,67)}$=10.102	**<0.001**
	Shannon	$F_{(7,67)}$=4.346	**<0.001**	$F_{(6,67)}$=5.56	**<0.001**
	Pielou's evenness	$F_{(7,67)}$=4.127	**<0.001**	$F_{(6,67)}$=2.200	0.054

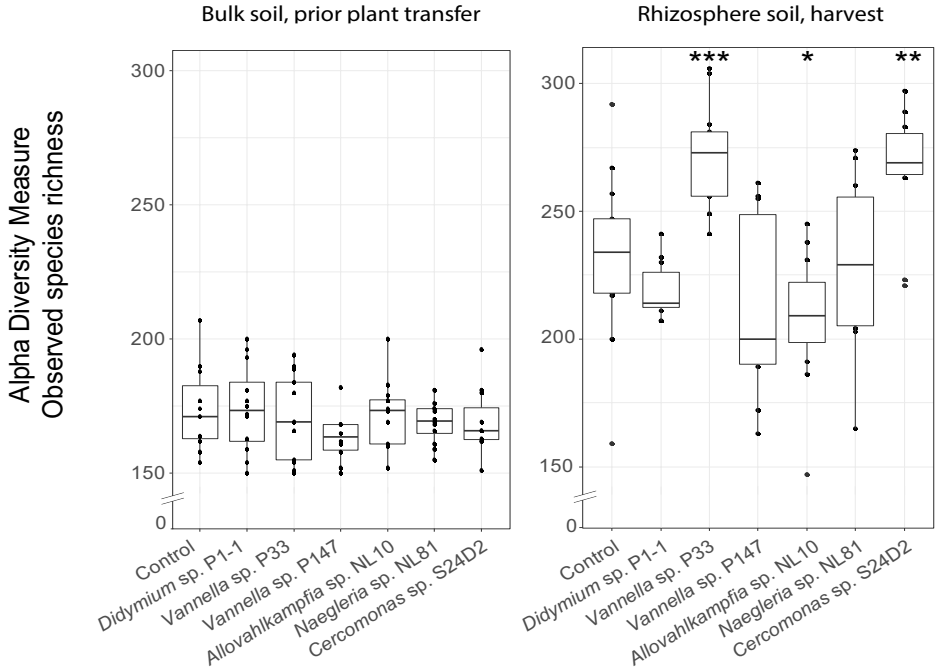

*Figure 3: Effect of protist inoculation on soil bacterial alpha diversity (observed species richness) prior plant transfer (left panel) and at the harvest (right panel). Asterisks indicate significant differences compared to control: ***: p < 0.001; **: p < 0.01; * < 0.05.*

Both the box and our protist treatment significantly affected the bacterial beta diversity for all distance metrics used (*i.e.*, Bray-Curtis dissimilarity, unweighted and weighted UniFrac distances) and at both time points (prior plant transfer and at harvest) (Table S4). After the Benjamini and Hochberg correction for multiple testing, we found for the time point prior plant transfer a significant difference only between the communities exposed to *Vannella* sp. P147 compared to the ones exposed to *Vannella* sp. P33, and this only with the Bray-Curtis Dissimilarity ($F_{(1,23)}$ = 1.991, BH corrected p = 0.021 and Table S5 for the other pairwise comparisons). In the rhizosphere soil at the harvest time, we observed significant differences in the communities exposed to *Cercomonas* sp. S24D2 compared to the control, *Didymium* sp. P1-1 and *Naegleria* sp. NL10, and for the ones exposed to *Vannella* sp. P33 compared to the control, *Didymium* sp. P1-1, *Vannella* sp. P147, *Naegleria* sp. NL10 and Heterolobosea sp. NL81, but only with the Bray-Curtis dissimilarity (Table 4 and S5).

Table 4: Significant pairwise comparison between treatments for the Bray-Curtis dissimilarity on the rhizosphere bacterial community composition (harvest time). Only the significant comparisons are given (BH p values < 0.05); the other pairwise comparisons are given in Table S5.

Comparison pair	F statistic	BH *p* values
S24D2 – Ctrl	$F_{(1,22)}$ = 1.476	0.021
S24D2 - P1-1	$F_{(1,20)}$ = 1.585	0.042
S24D2 – NL10	$F_{(1,21)}$ = 1.828	0.021
P33 – Ctrl	$F_{(1,24)}$ = 1.778	0.021
P33 – P1-1	$F_{(1,22)}$ = 1.892	0.021
P33 – P147	$F_{(1,21)}$ = 1.713	0.021
P33 – NL10	$F_{(1,23)}$ = 2.340	0.021
P33 – NL81	$F_{(1,22)}$ = 1.868	0.021

The PERMANOVA analyses revealed significant differences between the bacterial community composition prior plant transfer and at the harvest time for each abundance fraction (*i.e.*, rare, moderate and abundant) and for at least one of the dissimilarity/distance metric on community composition (Table S6). After applying the Benjamini and Hochberg correction for multiple testing, none of the pairwise comparisons was found significant for the soil bacterial communities (prior plant transfer) (Table S7). Significant differences were, however, observed in the rhizosphere-associated bacterial community composition in the moderately abundant fraction with the Bray-Curtis dissimilarity matrix and the weigthed UniFrac distance for the *Cercomonas* sp. S24D2 compared to the control and communities exposed to *Didymium* sp. P1-1 (Table 5 and S8). Similarly, the moderately abundant fraction of the bacterial communities exposed to *Vannella* sp. P33 were significantly different compared to the control and communities exposed to *Didymium* sp. P1-1, *Naegleria* sp. NL10 and Heterolobosea sp. NL81 based on the Bray-Curtis dissimilarity and/or the weighted UniFrac distance (Table 5 and S8).

The protist treatments further significantly affected the relative abundance of 5 of the 10 dominant bacterial phyla prior plant transfer (Proteobacteria, Bacteroidetes, Verrucomicrobia, Acidobacteria, and Myxococcota) and of 3 of the 10 dominant phyla at the harvest time (Actinobacteria, Verrucomicrobia and Gemmadimonadetes). Significant differences were, however, only found between treatments, but not compared to the control (Table S9 and Figure S2).

Table 5: Significant pairwise comparisons between treatments on the rhizosphere bacterial community composition (harvest time), only considering the moderately abundant taxa (0.1% < x < 1% of the total reads). The other pairwise comparisons are given in Table S7 and S8.

Comparison pair	**Metric**	**F statistic**	**BH *p* values**
S24D2 – Ctrl	Weigthed UniFrac	$F_{(1,22)}$= 4.144	0.042
S24D2 - P1-1	Bray-Curtis	$F_{(1,20)}$=1.858	0.021
P33 - Ctrl	Bray-Curtis	$F_{(1,24)}$= 1.826	0.021
	Weigthed UniFrac	$F_{(1,24)}$= 1.544	0.021
P33 – P1-1	Bray-Curtis	$F_{(1,22)}$=2.277	0.021
	Weigthed UniFrac	$F_{(1,22)}$=4.182	0.021
P33 – NL10	Bray-Curtis	$F_{(1,23)}$=2.291	0.021
P33 – NL81	Bray-Curtis	$F_{(1,22)}$=2.225	0.021

We further observed the emergence of specific patterns of enriched and reduced relative abundances of ASVs compared to the control for each protist treatment and at both sampling time points (Figure S3 for the samples prior plant transfer and Figure 4 for the rhizosphere samples). The Proteobacteria were systematically the most influenced, followed by the Actinobacteria, Planctomycetota and the Bacteroidetes.

3.3 Relation between relative abundance of specific bacterial taxa, plant properties and protist predatory impact

We identified seven bacterial taxa from our soil samples, prior plant transfer, and 37 bacterial taxa from our rhizosphere samples, at the harvest, for which the relative abundances were significantly correlated (positively or negatively) with plant properties (Figure 5). Most correlations prior plant transfer were related to the plant shoot-to-root ratio, with four correlations being negative and two positive. At harvest, we observed that bacteria tended to be similarly correlated to either the aboveground plant properties (including total biomass, shoot weight, shoot carbon and nitrogen content), the belowground properties (root biomass and surface area) or the plant shoot-to-root ratio (Figure 5). Seventeen of

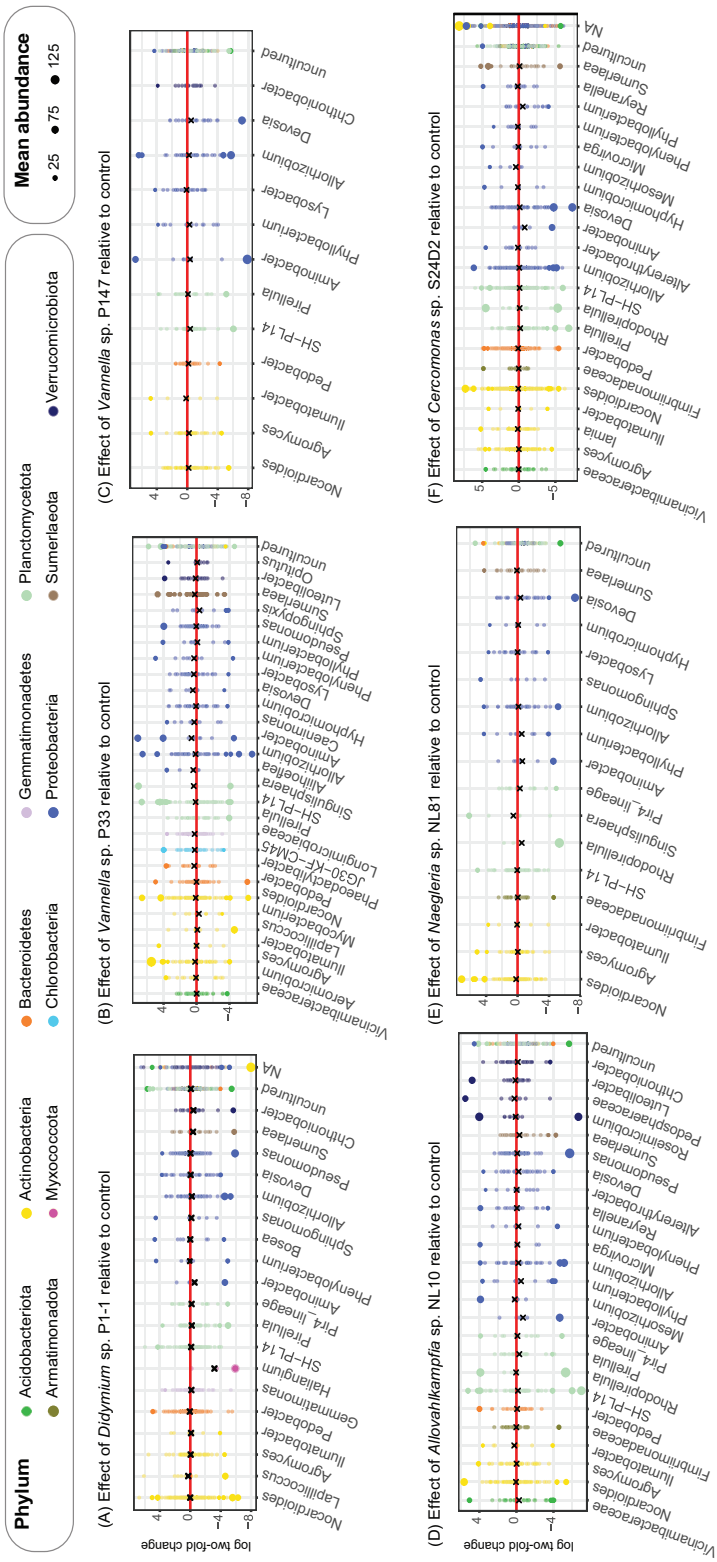

Figure 4: Impact of each protist on the relative abundance of different ASVs of the rhizosphere-associated bacterial communities, at the end of the experiment. The predatory impact is given as the statistically significant log two-fold changes compared to the no-protist control treatments. Dots above the red line represent bacterial ASVs that were more abundant in the treatment compared to the control, dots below the red line represent bacterial ASVs that were less abundant in the treatment compared to the control. Open circles are the ASVs not significantly modified. The cross indicate the log two-fold average for each genus, including all non-significantly modified ASVs. Note that we shorten the assigned Allorhizobium–Neorhizobium–Pararhizobium–Rhizobium to Allorhizobium.

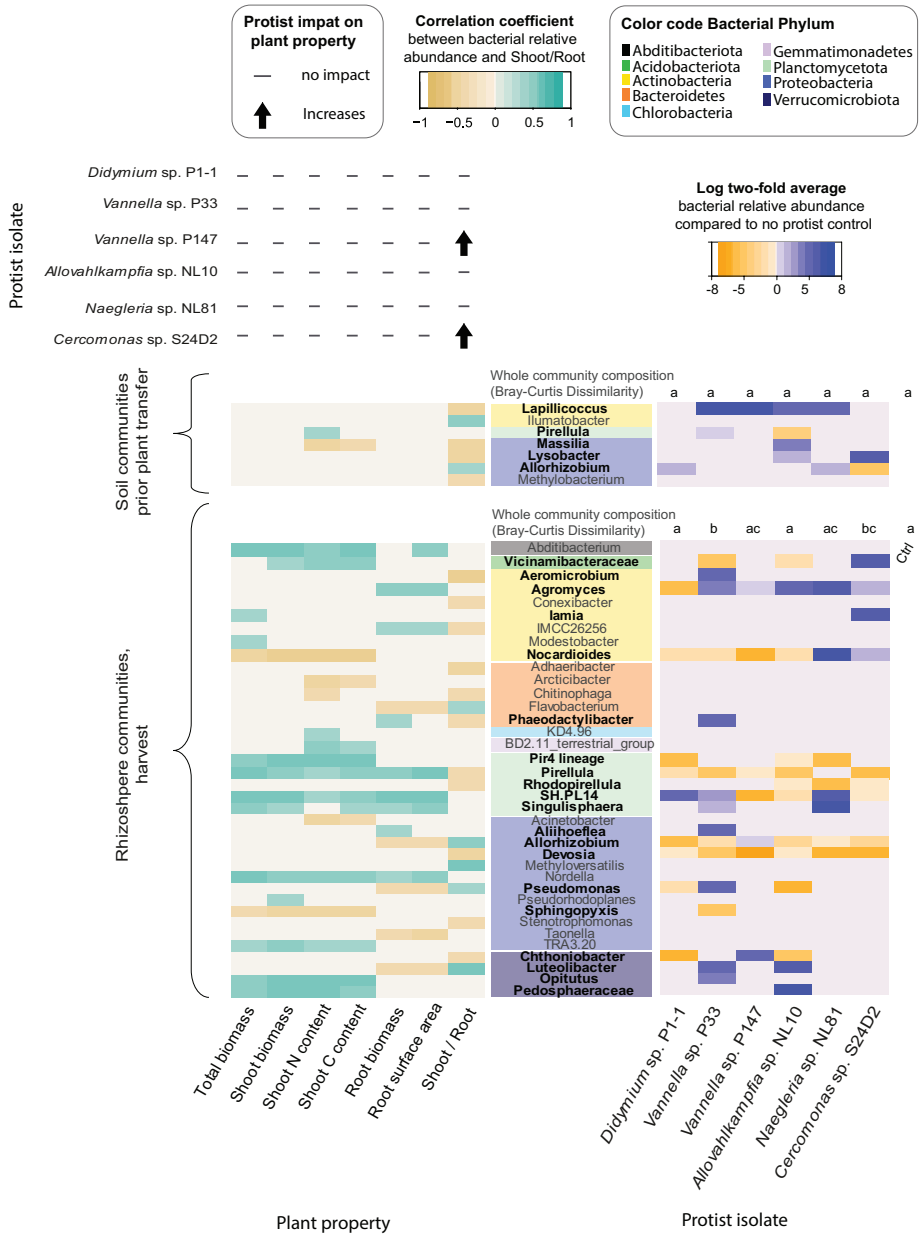

Figure 5: Impact of protists on plant properties (up left), correlogram between the abundance of rhizosphere-associated bacterial genera and plant properties (bottom left) and impact of protists on these bacteria (on the whole community and at the genus level, bottom right). Only the bacterial genera that were significantly correlated with at least one plant property are displayed. Bacterial genera impacted by at least one of the six protists are indicated in bold. Turquoise/blue color indicates increased abundance/positive correlation and beige/orange color indicates decreased abundance/ negative correlation. Grey/white indicate non-significant relations. The impact of protist on the whole community composition is given as the results from the pairwise comparisons of the Bray-Curtis dissimilarity on the community composition; the letter "a" indicates significantly indifferent from the control (Ctrl). The log two-fold average was calculated based on the significant values to summarize the direction of the predatory impact.

these bacterial taxa were not affected by any of the protist treatments, while the others were either positively or negatively affected. Some bacteria such as *Pirellula*, *Allorhizobium* or *Devosia* seemed to be particularly susceptible to predation, being negatively affected by at least three of the six protist treatments. In contrast, *Agromyces* was on average positively affected by protist inoculations.

4. Discussion

Most plant properties, including the total biomass and nitrogen content, remained similar across the treatments. Only the plant shoot-to-root ratio was increased and this with two of the six protist taxa. The inoculation of different protists led to subtle taxon-specific changes in the bacterial community structure by enriching/depleting some bacterial taxa compared to the control.

From the different plant properties investigated, we only observed a significant effect on the shoot-to-root ratio that was increased in two treatments (*Vannella* sp. P147 and *Cercomonas* sp. S24D2). This is contrary to our hypothesis and reports from previous studies that have shown direct effects on plant properties, such as increases of shoot biomass and nitrogen content upon protist amendment (as reviewed in Bonkowski 2004; Gao *et al.* 2019). The increase in shoot-to-root ratio was mostly driven by the trend toward smaller roots associated with the treatments with *Vannella* sp. P147 and *Cercomonas* sp. S24D2. Other studies also reported effects of protist inoculation on the root morphology, however the effects were in the opposite direction compared to our results, with typically greater root elongation and branching upon protist inoculation (Bonkowski and Brandt, 2002; Krome *et al.*, 2010; Kreuzer *et al.*, 2006).

After the initial 9-days incubation of soil after protist inoculation, we did not observe any significant changes in the soil bacterial alpha diversity and evenness. This result was contrary to our second hypothesis and expectations based on a previously performed soil microcosm, where three of the protists used in the present study were associated with a decrease in bacterial species richness (Table 2 and S1). At the harvest time, however, we observed a significant effect of protist inoculation on the bacterial observed richness, Chao1 index and Shannon index. In this line, a previous study also showed that protist inoculation tended to increase rhizosphere bacterial alpha diversity and evenness (Rosenberg *et al.* 2009). The application of protists, and of the protists used in the present study in particular (Table 2 and S1; Gao, 2020), had been related to significant modification of the overall bacterial community composition (Glücksman *et al.* 2010; Müller, Scheu and Jousset 2013). Modification of the bacterial community composition was also observed in our system, but only partially as discussed in the next section on taxon specificity of the observed effect. Interestingly, our protist inoculation especially affected the moderately abundant fraction of the rhizosphere bacterial community ($0.1\% < x < 1\%$). This result is contrary to previous studies suggesting that predatory impacts of protists mostly affect rare but competitive taxa (Kurm *et al.* 2019) or abundant taxa (Jürgens and DeMott 1995). This difference is

likely related to the constraints imposed by the soil physical structure that impacts trophic interactions by, for example, providing refuges (Erktan, Or and Scheu 2020), in comparison to the liquid culture conditions used in the mentioned studies.

In line with our third hypothesis, our protist inoculations led to modest but taxon-specific effects on plant properties and the bacterial community composition. The plant shoot-to-root ratio was increased only in the treatments with *Cercomonas* sp. S24D2 and *Vannella* sp. P147. Similarly, another study using different protist isolates reported taxon-specific effects of protists co-inoculated with the plant-beneficial bacteria *Azospirillum* sp. B510 on the growth of rice (Asiloglu *et al.* 2020). With respect to the impact on the bacterial communities and considering the alpha diversity, amendments with *Cercomonas* sp. S24D2 and *Vannella* sp. P33 increased bacterial species richness compared to the control, while *Allovahlkampfia* sp. NL10 decreased bacterial community diversity in the rhizosphere. The decrease of diversity is rather surprising, as protists are expected to feed on the more competitive taxa, providing an advantage to the other taxa and leading to higher species richness and evenness (Rosenberg *et al.* 2009; Kurm *et al.* 2019). With respect to the beta diversity, only *Cercomonas* sp. S24D2 and *Vannella* sp. P33 significantly modified the overall community composition (at harvest). For both sampling time points (prior plant transfer and at harvest), some of the bacterial phyla were, however, differently affected among the protist treatments (Figure S2, Table S9). In addition, each of the protists tested in our study led to distinct predatory impacts with taxon-specific patterns of enriched and depleted bacterial taxa (Figure 4 and S3). Similar to our results, Asiloglu *et al.* (2020) also reported the effect of protists only on the relative abundances of a few bacterial taxa, without modifying the overall bacterial community composition. Taxon-specific impacts of protists on bacterial communities are in line with previous observations, as even closely-related protist isolates can have differential impacts on their prey communities (Glücksman *et al.* 2010; Gao 2020). Our results further highlighted that these impacts can be rather subtle by affecting few bacterial taxa without modifying the overall bacterial community composition.

Because our protist inoculations led to taxon-specific but only modest effects on both the plant properties and bacterial community composition, we had relatively low power to address our fourth hypothesis. Nonetheless, we observed significant correlations, positive and negative, between certain plant properties and specific bacterial genera. The effect-size of protist predation on the relative abundance of these bacteria ranged from an absence of effect of any of the protists to the positive and/or negative effects of one or more protists. From these relations between plant properties and bacterial relative abundance and between bacterial relative abundance and protist treatment, we could not observe any clear patterns connecting the three. Identifying the interaction partners and assessing the importance of these relations for plant development remains typically challenging (Bonkowski and Clarholm 2012; Saleem and Moe 2014) and different, partially opposing, results have been reported. For instance, the inoculation of protists was previously shown

to modify plant properties both with or without significant modifications of the bacterial community composition (Bonkowski 2004; Ekelund *et al.* 2009a). Modifications of the bacterial community composition could, however, also be neutral or even detrimental in regard to the plant (Berendsen, Pieterse and Bakker 2012; Brugman *et al.* 2018). The plant itself further influences its rhizosphere associated bacterial community (Kröber *et al.* 2014; Shi *et al.* 2015a) and this could dampen the initial effect of introduced predatory protists. In addition, the relatively modest effects observed in our study may have been due to practical limitations of the experimental set-up, which included both box effects and inclusion of outside protists sources over the course of the experiment. Despite these limitations that likely reduced the effect size of our treatments, our results indicated potential relations between the rhizosphere partners.

5. Conclusions

The impact of protists on plant properties and bacterial community composition was taxon-specific. While the predatory impact of protists may only affect a subset of specific bacterial taxa without modification of the overall community composition, it could still have relevant consequences for microbiome functioning. The relations between predator, prey and plant properties remain very speculative, but are promising starting points for more targeted research efforts as required to address the role of multitrophic interactions in relation to plant development. Further understanding of the distinct impact of different predatory protist species will help assess their role in soils and potentially enable targeted application to steer their prey community toward a more plant-beneficial microbiome.

6. Acknowledgements

We thank Gerrit Rouwenhorst (Utrecht University) for the EA and ICP analysis as well as for his valuable help to prepare the samples and analyze the data. We thank Peter Veenhuizen (Utrecht University) for the qPCR and pre-processing of the 16S rRNA amplicon sequencing data on QIIME2, the advices and help with the laboratory work. Special thanks to Fabian Deply and Giovanna Lanius-Pascuzzi for part of the laboratory work.

7. Conflicts of 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.

Supplementary Material

Supplementary Method

We provide here a general description of the soil microcosm study from which we are using the data on the 16S rRNA gene amplicon sequencing to describe the predatory impact of the selected protist isolates of our study. Additional details can be found at Gao, 2020. In brief, protist-free soils were created using a microbial extraction from a natural sandy soil (Botanische Tuinen, de Uithof, Utrecht, The Netherlands) that was reinoculated back into sterilized soils. After 2 hours of incubation, the protist treatments were added by inoculating a volume of 400 µL of protist suspension (10^4 individuals mL^{-1}) or of 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 tag sequencing was carried out using a two-step PCR protocol and using modified primers (Caporaso *et al.* 2011) 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 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, corresponding to the smallest library size. The data analysis was performed with R version 4.0.3. We measured the bacterial species richness with phyloseq::estimate_richness and calculated the Pielou's eveness as described in the main text. Significant differences compared to the control were analyzed with a one-way ANOVA (stats::lm and basis::summary). We analyzed the predatory impact on the bacterial community structure by performing a PERMANOVA on the Bray-Curtis dissimilarity as a pairwise test for each treatment against the control with vegan::adonis; the obtained *p* values were adjusted for multiple testing with the Benjamini and Hochberg correction (stats::p.adjust).

Supplementary Tables

Table S1: Impact of the selected protist isolates on bacterial community in a soil microcosm experiment, in absence of plants. The soil microcosm was performed by Gao (2020). BH stands for the Benjamini and Hochberg correction for multiple testing.

Isolate code	**Species**	**Bacterial Species richness (observed)**	**Pielou's Evenness Index**	**Bacterial structure (Bray-Curtis Dissimilarity to control)**
No protist Control	F-statistiscs: Estimate:	$F_{(6,26)}$=6.26, p<0.001 1741	$F_{(6,26)}$=5.84, p<0.001 0.616	$F_{(6,26)}$=5.198, p=0.001
P1-1	*Didymium* sp.	-356; p<0.001	-0.054; p<0.001	$F_{(1,7)}$=7.968, p=0.004 BH corrected p=0.048
P33	*Vannella* sp.	-196; p=0.016	-0.031; p=0.017	$F_{(1,8)}$=3.353, p=0.006 BH corrected p=0.036
NL10	*Allovahlkampfia* sp.	-246; p=0.003	-0.040; p=0.003	$F_{(1,8)}$=5.046, p=0.006 BH corrected p=0.036
S24D2	*Cercomonas* sp.	NS	-0.032; p=0.014	$F_{(1,8)}$=10.74, p=0.005 BH corrected p=0.030
NL81	*Naegleria* sp.	NS	NS	$F_{(1,7)}$=4.072, p=0.008 BH corrected p=0.048
P147	*Vannella* new sp.	NS	NS	$F_{(1,8)}$=3.125, p=0.01 BH corrected p=0.06

Table S2: Results of the sequential two-way ANOVA addressing the main effect of the box and the protist treatments on various plant properties. The significant p-values (<0.05) are highlighted in bold

	Box		Protist treatment	
Plant property	**F test**	***p***	**F test**	***p***
Total plant biomass (dry weight, mg)	$F_{(7,68)}$=5.377	**< 0.001**	$F_{(6,68)}$=0.842	0.542
Shoot biomass (dry weight, mg)	$F_{(7,70)}$=7.685	**< 0.001**	$F_{(6,70)}$=0.878	0.516
Root biomass (dry weight, mg)	$F_{(7,72)}$=5.764	**< 0.001**	$F_{(6,72)}$=1.673	0.14
Shoot-to-root ratio (log)	$F_{(7,68)}$=11.730	**< 0.001**	$F_{(6,68)}$=2.466	**0.032**
Root surface area (mm2)	$F_{(7,72)}$=6.50	**< 0.001**	$F_{(6,72)}$=2.084	0.066
Shoot carbon content (%)	$F_{(7,70)}$=2.316	**0.035**	$F_{(6,70)}$=0.748	0.613
Shoot carbon content (mg)	$F_{(7,70)}$=10.794	**< 0.001**	$F_{(6,70)}$=1.601	0.16
Shoot nitrogen content (%)	$F_{(7,70)}$=2.698	**0.016**	$F_{(6,70)}$=0.487	0.816
Shoot nitrogen content (mg)	$F_{(7,70)}$=14.302	**< 0.001**	$F_{(6,70)}$=1.161	0.337
Shoot C/N ratio	$F_{(7,70)}$=3.455	**0.003**	$F_{(6,70)}$=1.694	0.135
Al content (mg per plant)	$F_{(7,63)}$=2.064	0.061	$F_{(6,63)}$=0.353	0.905
Ca content (mg per plant)	$F_{(7,63)}$=8.925	**<0.001**	$F_{(6,63)}$=0.226	0.967
Cu content (mg per plant)	$F_{(7,63)}$= 3.465	**0.004**	$F_{(6,63)}$=0.083	0.998
Cd content (mg per plant)	$F_{(7,63)}$= 1.836	0.096	$F_{(6,63)}$=0.726	0.63
Fe content (mg per plant)	$F_{(7,63)}$= 2.153	0.051	$F_{(6,63)}$=0.927	0.482
K content (mg per plant)	$F_{(7,63)}$= 9.405	**< 0.001**	$F_{(6,63)}$=0.55	0.768
Mg content (mg per plant)	$F_{(7,63)}$= 7.601	**< 0.001**	$F_{(6,63)}$=0.278	0.945
Mn content (mg per plant)	$F_{(7,63)}$= 0.599	0.755	$F_{(6,63)}$=0.215	0.971
Na content (mg per plant)	$F_{(7,63)}$= 6.914	**< 0.001**	$F_{(6,63)}$= 0.493	0.811
P content (mg per plant)	$F_{(7,63)}$= 7.632	**< 0.001**	$F_{(6,63)}$= 1.070	0.39
Pb content (mg per plant)	$F_{(7,63)}$= 1.643	0.14	$F_{(6,63)}$= 0.677	0.669
S content (mg per plant)	$F_{(7,63)}$= 9.801	**< 0.001**	$F_{(6,63)}$= 0.382	0.888
Zn content (mg per plant)	$F_{(7,63)}$= 4.928	**< 0.001**	$F_{(6,63)}$=0.201	0.975

Table S3: Comparisons of the effect of each treatment on the alpha bacterial diversity (Tukey test).

Treatment pair	diff	lwr	upr	*p* adj
Observed Richness				
P1_1-Control	-12.678	-46.540	21.183	0.915
P33-Control	40.923	8.503	73.343	0.005
P147-Control	-19.769	-54.536	14.997	0.603
NL10-Control	-24.103	-57.191	8.986	0.304
NL81-Control	-2.406	-36.267	31.456	1.000
S24D2-Control	34.594	0.733	68.456	0.042
P33-P1_1	53.601	19.740	87.463	<0.001
P147-P1_1	-7.091	-43.205	29.023	0.997
NL10-P1_1	-11.424	-45.926	23.078	0.952
NL81-P1_1	10.273	-24.971	45.517	0.974
S24D2-P1_1	47.273	12.029	82.517	0.002
P147-P33	-60.692	-95.459	-25.926	<0.001
NL10-P33	-65.026	-98.114	-31.937	<0.001
NL81-P33	-43.329	-77.190	-9.467	0.004
S24D2-P33	-6.329	-40.190	27.533	0.998
NL10-P147	-4.333	-39.724	31.057	1.000
NL81-P147	17.364	-18.751	53.478	0.769
S24D2-P147	54.364	18.249	90.478	<0.001
NL81-NL10	21.697	-12.805	56.199	0.483
S24D2-NL10	58.697	24.195	93.199	<0.001
S24D2-NL81	37	1.756	72.244	0.033
Chao1				
P1_1-Control	-13.821	-47.575	19.934	0.876
P33-Control	40.652	8.334	72.970	0.005
P147-Control	-20.487	-55.144	14.169	0.558
NL10-Control	-24.703	-57.687	8.281	0.272
NL81-Control	-3.178	-36.933	30.577	1.000
S24D2-Control	33.882	0.128	67.637	0.049
P33-P1_1	54.473	20.718	88.228	<0.001
P147-P1_1	-6.666	-42.667	29.334	0.998
NL10-P1_1	-10.882	-45.275	23.511	0.961
NL81-P1_1	10.643	-24.490	45.776	0.969
S24D2-P1_1	47.703	12.570	82.836	0.002
P147-P33	-61.139	-95.796	-26.483	<0.001
NL10-P33	-65.355	-98.339	-32.371	<0.001
NL81-P33	-43.830	-77.585	-10.075	0.003
S24D2-P33	-6.770	-40.524	26.985	0.996
NL10-P147	-4.215	-39.494	31.064	1.000

(the table continues on the next page)

Treatment pair	diff	lwr	upr	*p* adj
Chao1				
NL81-P147	17.309	-18.691	53.310	0.769
S24D2-P147	54.370	18.369	90.370	<0.001
NL81-NL10	21.525	-12.868	55.918	0.489
S24D2-NL10	58.585	24.192	92.978	<0.001
S24D2-NL81	37.060	1.927	72.193	0.032
Shannon				
P1_1-Control	0.042	-0.185	0.269	0.998
P33-Control	0.248	0.031	0.466	0.015
P147-Control	-0.089	-0.323	0.144	0.906
NL10-Control	-0.076	-0.298	0.146	0.942
NL81-Control	0.039	-0.188	0.266	0.998
S24D2-Control	0.197	-0.031	0.424	0.134
P33-P1_1	0.206	-0.021	0.433	0.101
P147-P1_1	-0.131	-0.374	0.111	0.655
NL10-P1_1	-0.118	-0.350	0.113	0.713
NL81-P1_1	-0.003	-0.239	0.233	1.000
S24D2-P1_1	0.155	-0.082	0.391	0.435
P147-P33	-0.337	-0.571	-0.104	0.001
NL10-P33	-0.324	-0.546	-0.103	0.001
NL81-P33	-0.209	-0.436	0.018	0.092
S24D2-P33	-0.051	-0.279	0.176	0.993
NL10-P147	0.013	-0.225	0.250	1.000
NL81-P147	0.128	-0.114	0.371	0.679
S24D2-P147	0.286	0.044	0.528	0.011
NL81-NL10	0.115	-0.116	0.347	0.737
S24D2-NL10	0.273	0.042	0.504	0.011
S24D2-NL81	0.158	-0.079	0.394	0.411

Table S4: Results of the sequential two-way PERMANOVA addressing the main effect of the box and the protist treatments on bacterial community composition (Bray-Curtis Dissimilarity, UniFrac and weigthed UniFrac) at the two different sampling timepoints.

Experimental Phase	Distance Used	Box		Protist Treatment	
		F test	***p* value**	**F test**	***p* value**
Phase 2, prior plant transfer	Bray-Curtis	$F_{(7,76)}$=1.945	0.001	$F_{(6,76)}$=1.213	0.014
	UniFrac	$F_{(7,76)}$=2.676	0.001	$F_{(6,76)}$=1.385	0.01
	weighted UniFrac	$F_{(7,76)}$=11.135	0.001	$F_{(6,76)}$=1.83	0.01
Phase 3, harvest	Bray-Curtis	$F_{(7,67)}$=1.411	0.001	$F_{(6,67)}$=1.425	0.001
	UniFrac	$F_{(7,67)}$= 2.866	0.001	$F_{(6,67)}$=1.384	0.001
	weighted UniFrac	$F_{(7,67)}$=7.006	0.001	$F_{(6,67)}$=1.874	0.004

Table S5: Pairwise comparison between all protist treatments on the bacterial community composition (based on Bray-Curtis Dissimilarity, UniFrac and weighted UniFrac (W UniFrac)). Because multiple testing was performed, the p values were adjusted per group (e.g., Bray-Curtis) with the Benjamini and Hochberg correction (BH p). The significant BH p-values (<0.05) are highlighted in bold.

Comparison pair		**Bulk soil, prior plant transfer**			**Rhizosphere soil, harvest time**		
		Bray-Curtis	**UniFrac**	**W UniFrac**	**Bray-Curtis**	**UniFrac**	**W UniFrac**
Ctrl: S24D2	F test	$F_{(1,24)}$= 1.199	$F_{(1,24)}$= 1.446	$F_{(1,24)}$= 1.133	$F_{(1,22)}$= 1.476	$F_{(1,22)}$= 1.183	$F_{(1,22)}$= 1.928
	p	0.133	0.025	0.331	0.001	0.145	0.071
	BH *p*	NA	0.399	NA	**0.021**	NA	NA
Ctrl: P1-1	F test	$F_{(1,24)}$= 0.93	$F_{(1,24)}$= 1.117	$F_{(1,24)}$= 0.773	$F_{(1,22)}$= 1.137	$F_{(1,22)}$= 0.952	$F_{(1,22)}$= 0.653
	p	0.612	0.272	0.603	0.193	0.505	0.696
	BH *p*	NA	NA	NA	NA	NA	NA
Ctrl: P33	F test	$F_{(1,24)}$= 1.366	$F_{(1,24)}$= 1.032	$F_{(1,24)}$= 0.393	$F_{(1,24)}$= 1.778	$F_{(1,24)}$= 1.315	$F_{(1,24)}$= 1.352
	p	0.038	0.377	0.874	0.001	0.086	0.214
	BH *p*	0.798	NA	NA	**0.021**	NA	NA
Ctrl: P147	F test	$F_{(1,23)}$= 1.554	$F_{(1,23)}$= 0.991	$F_{(1,23)}$= 1.172	$F_{(1,21)}$= 0.898	$F_{(1,21)}$= 1.051	$F_{(1,21)}$= 1.225
	p	0.007	0.489	0.341	0.743	0.336	0.252
	BH *p*	0.147	NA	NA	NA	NA	NA
Ctrl: NL10	F test	$F_{(1,24)}$= 1.122	$F_{(1,24)}$= 1.734	$F_{(1,24)}$= 1.261	$F_{(1,23)}$= 1.202	$F_{(1,23)}$= 1.319	$F_{(1,23)}$= 1.574
	p	0.228	0.014	0.27	0.087	0.094	0.127
	BH *p*	NA	0.294	NA	NA	NA	NA
Ctrl: NL81	F test	$F_{(1,24)}$= 1.207	$F_{(1,24)}$= 0.901	$F_{(1,24)}$= 0.358	$F_{(1,22)}$= 0.973	$F_{(1,22)}$= 0.791	$F_{(1,22)}$= 0.812
	p	0.108	0.614	0.933	0.56	0.854	0.557
	BH *p*	NA	NA	NA	NA	NA	NA
S24D2: P1-1	F test	$F_{(1,24)}$=0.674	$F_{(1,24)}$=1.442	$F_{(1,24)}$=1.167	$F_{(1,20)}$=1.585	$F_{(1,20)}$=1.476	$F_{(1,20)}$= 1.349
	p	0.988	0.064	0.319	0.002	0.042	0.168
	BH *p*	NA	NA	NA	**0.042**	0.882	NA
S24D2: P33	F test	$F_{(1,24)}$=0.905	$F_{(1,24)}$=1.132	$F_{(1,24)}$=0.983	$F_{(1,22)}$=1.020	$F_{(1,22)}$=1.225	$F_{(1,22)}$=1.248
	p	0.653	0.26	0.396	0.402	0.105	0.235
	BH *p*	NA	NA	NA	NA	NA	NA
S24D2: P147	F test	$F_{(1,23)}$=1.510	$F_{(1,23)}$=1.186	$F_{(1,23)}$=1.332	$F_{(1,19)}$=1.380	$F_{(1,19)}$=1.411	$F_{(1,19)}$=3.369
	p	0.018	0.182	0.254	0.006	0.063	0.016
	BH *p*	0.378	NA	NA	0.126	NA	0.336
S24D2: NL10	F test	$F_{(1,24)}$=1.062	$F_{(1,24)}$=1.533	$F_{(1,24)}$=0.778	$F_{(1,21)}$=1.828	$F_{(1,21)}$=1.931	$F_{(1,21)}$=2.820
	p	0.322	0.025	0.515	0.001	0.007	0.019
	BH *p*	NA	0.525	NA	**0.021**	0.147	0.399
S24D2: NL81	F test	$F_{(1,24)}$=0.876	$F_{(1,24)}$=1.240	$F_{(1,24)}$=0.723	$F_{(1,20)}$=1.388	$F_{(1,20)}$=1.249	$F_{(1,20)}$=2.185
	p	0.709	0.151	0.55	0.004	0.098	0.049
	BH *p*	NA	NA	NA	0.084	NA	NA

Comparison pair		Bulk soil, prior plant transfer			Rhizosphere soil, harvest time		
		Bray-Curtis	UniFrac	W UniFrac	Bray-Curtis	UniFrac	W UniFrac
P1-1: P33	F test	$F_{(1,24)}$=0.738	$F_{(1,24)}$= 0.628	$F_{(1,24)}$= 0.509	$F_{(1,22)}$= 1.892	$F_{(1,22)}$= 1.481	$F_{(1,22)}$= 0.797
	p	0.933	0.964	0.806	0.001	0.059	0.551
	BH *p*	NA	NA	NA	**0.021**	NA	NA
P1-1: P147	F test	$F_{(1,24)}$=1.378	$F_{(1,23)}$=0.048	$F_{(1,23)}$=0.641	$F_{(1,19)}$=1.035	$F_{(1,19)}$=1.226	$F_{(1,19)}$=1.197
	p	0.032	0.222	0.655	0.394	0.167	0.292
	BH *p*	0.651	NA	NA	NA	NA	NA
P1-1: NL10	F test	$F_{(1,24)}$=0.885	$F_{(1,24)}$=1.75	$F_{(1,24)}$=2.013	$F_{(1,21)}$=1.420	$F_{(1,21)}$=1.185	$F_{(1,21)}$=1.116
	p	0.728	0.026	0.082	0.014	0.202	0.309
	BH *p*	NA	0.546	NA	0.294	NA	NA
P1-1: NL81	F test	$F_{(1,24)}$=0.706	$F_{(1,24)}$=0.857	$F_{(1,24)}$=0.778	$F_{(1,20)}$=1.264	$F_{(1,20)}$=1.273	$F_{(1,20)}$=0.591
	p	0.963	0.631	0.547	0.042	0.125	0.755
	BH *p*	NA	NA	NA	NA	NA	NA
P33: P147	F test	$F_{(1,23)}$=1.991	$F_{(1,23)}$=0.775	$F_{(1,23)}$=0.935	$F_{(1,21)}$=1.713	$F_{(1,21)}$=1.690	$F_{(1,21)}$=2.406
	p	0.001	0.854	0.475	0.001	0.014	0.026
	BH *p*	**0.021**	NA	NA	**0.021**	0.294	0.546
P33: NL10	F test	$F_{(1,24)}$=1.153	$F_{(1,24)}$=1.298	$F_{(1,24)}$=0.873	$F_{(1,23)}$=2.340	$F_{(1,23)}$=2.070	$F_{(1,23)}$=2.722
	p	0.188	0.093	0.481	0.001	0.004	0.011
	BH *p*	NA	NA	NA	**0.021**	0.084	0.231
P33: NL81	F test	$F_{(1,24)}$=1.168	$F_{(1,24)}$=0.625	$F_{(1,24)}$=0.812	$F_{(1,22)}$=1.868	$F_{(1,22)}$=1.324	$F_{(1,22)}$=1.282
	p	0.196	0.962	0.513	0.001	0.082	0.241
	BH *p*	NA	NA	NA	**0.021**	NA	NA
P147: NL10	F test	$F_{(1,23)}$=1.687	$F_{(1,23)}$=1.263	$F_{(1,23)}$=2.619	$F_{(1,20)}$=0.999	$F_{(1,20)}$=1.260	$F_{(1,20)}$=0.932
	p	0.003	0.12	0.027	0.486	0.147	0.464
	BH *p*	0.063	NA	0.567	NA	NA	NA
P147: NL81	F test	$F_{(1,23)}$=0.853	$F_{(1,23)}$=1.164	$F_{(1,23)}$=1.071	$F_{(1,19)}$=1.04	$F_{(1,19)}$=0.909	$F_{(1,19)}$=0.798
	p	0.781	0.242	0.334	0.377	0.623	0.564
	BH *p*	NA	NA	NA	NA	NA	NA
NL10: NL81	F test	$F_{(1,24)}$=1.233	$F_{(1,24)}$=1.709	$F_{(1,24)}$=1.291	$F_{(1,21)}$=1.286	$F_{(1,21)}$=1.094	$F_{(1,21)}$=0.939
	p	0.136	0.019	0.262	0.026	0.304	0.451
	BH *p*	NA	0.399	NA	0.546	NA	NA

Table S6: Results of the sequential two-way PERMANOVA addressing the main effect of the box and the protist treatments on bacterial community composition according to different fraction of the community (rare: <0.1%; moderate: >0.1% and <1%; abundant: >1%) using Bray-Curtis Dissimilarity, UniFrac and weigthed UniFrac. The significant p-values (<0.05) are highlighted in bold.

Abundance Category	**Distance Used**	**Box**		**Protist Treatment**	
		F test	***p* value**	**F test**	***p* value**
Bulk soil, prior plant transfer					
Rare taxa	Bray Curtis	$F_{(7,76)}$=1.302	**0.001**	$F_{(6,76)}$=1.025	0.265
	UniFrac	$F_{(7,76)}$=2.403	**0.001**	$F_{(6,76)}$=1.311	**0.001**
	Weighted UniFrac	$F_{(7,76)}$=5.505	**0.001**	$F_{(6,76)}$=1.598	**0.008**
Moderate taxa	Bray Curtis	$F_{(7,76)}$=1.564	**0.001**	$F_{(6,76)}$=1.127	0.112
	UniFrac	$F_{(7,76)}$=1.042	0.421	$F_{(6,76)}$=1.028	0.440
	Weighted UniFrac	$F_{(7,76)}$=6.228	**0.001**	$F_{(6,76)}$=1.762	**0.005**
Abundant taxa	Bray Curtis	$F_{(7,76)}$=3.767	**0.001**	$F_{(6,76)}$=2.065	**0.007**
	UniFrac	$F_{(7,76)}$=2.057	**0.027**	$F_{(6,76)}$=3.047	**0.004**
	Weighted UniFrac	$F_{(7,76)}$=5.087	**0.001**	$F_{(6,76)}$=2.73	**0.016**
Rhizosphere soil, harvest					
Rare taxa	Bray Curtis	$F_{(7,67)}$=1.227	**0.001**	$F_{(6,67)}$=1.092	**0.002**
	UniFrac	$F_{(7,67)}$=2.570	**0.001**	$F_{(6,67)}$=1.361	**0.001**
	Weighted UniFrac	$F_{(7,67)}$=6.24	**0.001**	$F_{(6,67)}$=1.172	0.206
Moderate taxa	Bray Curtis	$F_{(7,67)}$=1.421	**0.001**	$F_{(6,67)}$=1.572	**0.001**
	UniFrac	$F_{(7,67)}$=1.811	**0.002**	$F_{(6,67)}$=1.045	0.374
	Weighted UniFrac	$F_{(7,67)}$=3.472	**0.001**	$F_{(6,67)}$=2.355	**0.001**
Abundant taxa	Bray Curtis	$F_{(7,67)}$=1.834	**0.007**	$F_{(6,67)}$=1.651	**0.025**
	UniFrac	$F_{(7,67)}$=1.137	0.343	$F_{(6,67)}$=1.318	0.230
	Weighted UniFrac	$F_{(7,67)}$=2.195	**0.014**	$F_{(6,67)}$=1.556	0.105

Table S7: Results of the pairwise PERMANOVA addressing the impact of protist treatments on the soil bacterial community composition (prior plant transfer) according to different fraction of the community (rare: <0.1%; moderate: >0.1% and <1%; abundant: >1%) using Bray-Curtis Dissimilarity, UniFrac and weigthed UniFrac (W UniFrac); only for the treatments reported significant in the previously performed sequential two-way PERMANOVA (Table S6).

Comparison pair		**Rare (<0.1%)**		**Moderate (0.1%< x <1%)**	**Abundant (> 1%)**		
		UniFrac	**W UniFrac**	**W UniFrac**	**Bray-Curtis**	**UniFrac**	**W UniFrac**
Ctrl: S24D2	F test	$F_{(1,24)}$= 1.296	$F_{(1,24)}$= 1.001	$F_{(1,24)}$= 1.585	$F_{(1,24)}$= 2.14	$F_{(1,24)}$= 6.35	$F_{(1,24)}$= 3.068
	p	0.066	0.418	0.128	0.077	0.013	0.076
	BH *p*	NA	NA	NA	NA	0.273	NA
Ctrl: P1-1	F test	$F_{(1,24)}$= 1.073	$F_{(1,24)}$= 0.802	$F_{(1,24)}$= 1.327	$F_{(1,24)}$= 1.129	$F_{(1,24)}$= 1.52	$F_{(1,24)}$= 0.554
	p	0.315	0.594	0.236	0.346	0.257	0.512
	BH *p*	NA	NA	NA	NA	NA	NA
Ctrl: P33	F test	$F_{(1,24)}$= 1.002	$F_{(1,24)}$= 0.704	$F_{(1,24)}$= 0.996	$F_{(1,24)}$= 1.989	$F_{(1,24)}$= 3.83	$F_{(1,24)}$= 1.073
	p	0.448	0.728	0.429	0.112	0.009	0.337
	BH *p*	NA	NA	NA	NA	0.189	NA
Ctrl: P147	F test	$F_{(1,23)}$= 0.934	$F_{(1,23)}$= 0.53	$F_{(1,23)}$= 2.249	$F_{(1,23)}$= 4.899	$F_{(1,23)}$= 4.691	$F_{(1,23)}$= 6.922
	p	0.613	0.851	0.063	0.003	0.008	0.013
	BH *p*	NA	NA	NA	0.063	0.168	0.273
Ctrl: NL10	F test	$F_{(1,24)}$= 1.681	$F_{(1,24)}$= 2.177	$F_{(1,24)}$= 1.991	$F_{(1,24)}$= 0.076	$F_{(1,24)}$= 1.045	$F_{(1,24)}$= 0.122
	p	0.009	0.035	0.069	0.966	0.412	0.89
	BH *p*	0.189	0.735	NA	NA	NA	NA
Ctrl: NL81	F test	$F_{(1,24)}$= 0.836	$F_{(1,24)}$= 0.356	$F_{(1,24)}$= 0.755	$F_{(1,24)}$= 2.108	$F_{(1,24)}$= 1.426	$F_{(1,24)}$= 3.016
	p	0.754	0.988	0.617	0.09	0.328	0.093
	BH *p*	NA	NA	NA	NA	NA	NA
S24D2: P1-1	F test	$F_{(1,24)}$=1.349	$F_{(1,24)}$=1.321	$F_{(1,24)}$=0.606	$F_{(1,24)}$=0.201	$F_{(1,24)}$=1.652	$F_{(1,24)}$=0.737
	p	0.089	0.213	0.762	0.901	0.219	0.407
	BH *p*	NA	NA	NA	NA	NA	NA
S24D2: P33	F test	$F_{(1,24)}$=1.121	$F_{(1,24)}$=0.682	$F_{(1,24)}$=0.505	$F_{(1,24)}$=0.446	$F_{(1,24)}$=2.453	$F_{(1,24)}$=1.214
	p	0.274	0.776	0.82	0.76	0.093	0.288
	BH *p*	NA	NA	NA	NA	NA	NA
S24D2: P147	F test	$F_{(1,23)}$=1.105	$F_{(1,23)}$= 0.823	$F_{(1,23)}$=2.102	$F_{(1,23)}$=4.311	$F_{(1,23)}$=3.328	$F_{(1,23)}$= 1.097
	p	0.273	0.574	0.053	0.009	0.058	0.289
	BH *p*	NA	NA	NA	0.189	NA	NA
S24D2: NL10	F test	$F_{(1,24)}$=1.438	$F_{(1,24)}$=0.726	$F_{(1,24)}$=0.765	$F_{(1,24)}$=1.164	$F_{(1,24)}$=5.908	$F_{(1,24)}$= 2.627
	p	0.035	0.702	0.588	0.342	0.011	0.109
	BH *p*	0.735	NA	NA	NA	0.231	NA

Comparison pair		Rare (<0.1%)		Moderate (0.1%< x <1%)	Abundant (> 1%)		
		UniFrac	W UniFrac	W UniFrac	Bray-Curtis	UniFrac	W UniFrac
S24D2: NL81	F test	$F_{(1,24)}$=1.152	$F_{(1,24)}$=1.343	$F_{(1,24)}$=0.734	$F_{(1,24)}$=0.867	$F_{(1,24)}$=1.27	$F_{(1,24)}$=0.28
	p	0.234	0.181	0.597	0.519	0.274	0.668
	BH *p*	NA	NA	NA	NA	NA	NA
P1-1: P33	F test	$F_{(1,24)}$=0.653	$F_{(1,24)}$=0.638	$F_{(1,24)}$=-0.045	$F_{(1,24)}$=0.121	$F_{(1,24)}$=0.376	$F_{(1,24)}$=0.093
	p	0.965	0.794	1	0.915	0.734	0.897
	BH p	NA	NA	NA	NA	NA	NA
P1-1: P147	F test	$F_{(1,24)}$=1.16	$F_{(1,24)}$=0.960	$F_{(1,23)}$=2.271	$F_{(1,23)}$=3.734	$F_{(1,23)}$=3.872	$F_{(1,23)}$=3.069
	p	0.212	0.437	0.043	0.007	0.44	0.077
	BH *p*	NA	NA	0.903	0.147	0.924	NA
P1-1: NL10	F test	$F_{(1,24)}$=1.664	$F_{(1,24)}$=1.897	$F_{(1,24)}$=0.943	$F_{(1,24)}$=0.82	$F_{(1,24)}$=2.693	$F_{(1,24)}$=0.743
	p	0.028	0.048	0.464	0.516	0.088	0.439
	BH *p*	0.588	1	NA	NA	NA	NA
P1-1: NL81	F test	$F_{(1,24)}$=0.872	$F_{(1,24)}$=0.587	$F_{(1,24)}$=1.059	$F_{(1,24)}$=0.614	$F_{(1,24)}$=0.717	$F_{(1,24)}$=0.927
	p	0.63	0.865	0.383	0.642	0.492	0.349
	BH *p*	NA	NA	NA	NA	NA	NA
P33: P147	F test	$F_{(1,24)}$=0.796	$F_{(1,24)}$=0.809	$F_{(1,23)}$=1.501	$F_{(1,23)}$=5.842	$F_{(1,23)}$=5.053	$F_{(1,23)}$=4.51
	p	0.853	0.585	0.174	0.003	0.014	0.031
	BH *p*	NA	NA	NA	0.063	0.294	0.651
P33: NL10	F test	$F_{(1,24)}$=1.346	$F_{(1,24)}$=1.099	$F_{(1,24)}$=0.448	$F_{(1,24)}$=1.155	$F_{(1,24)}$=4.239	$F_{(1,24)}$=1.100
	p	0.056	0.335	0.858	0.357	0.008	0.335
	BH *p*	NA	NA	NA	NA	0.168	NA
P33: NL81	F test	$F_{(1,24)}$=0.705	$F_{(1,24)}$=0.894	$F_{(1,24)}$=0.882	$F_{(1,24)}$=1.425	$F_{(1,24)}$=1.715	$F_{(1,24)}$=1.727
	p	0.913	0.551	0.48	0.243	0.196	0.184
	BH *p*	NA	NA	NA	NA	NA	NA
P147: NL10	F test	$F_{(1,23)}$=1.180	$F_{(1,23)}$=1.62	$F_{(1,23)}$=1.734	$F_{(1,23)}$=3.301	$F_{(1,23)}$=2.06	$F_{(1,23)}$=5.149
	p	0.18	0.112	0.022	0.012	0.153	0.03
	BH *p*	NA	NA	0.462	0.252	NA	0.63
P147: NL81	F test	$F_{(1,23)}$=1.118	$F_{(1,23)}$=0.776	$F_{(1,23)}$=1.962	$F_{(1,23)}$=1.585	$F_{(1,23)}$=0.778	$F_{(1,23)}$=0.747
	p	0.294	0.646	0.081	0.183	0.474	0.393
	BH *p*	NA	NA	NA	NA	NA	NA
NL10: NL81	F test	$F_{(1,24)}$=1.639	$F_{(1,24)}$=2.317	$F_{(1,24)}$=1.524	$F_{(1,24)}$=1.145	$F_{(1,24)}$=1.254	$F_{(1,24)}$=2.389
	p	0.018	0.013	0.143	0.344	0.309	0.128
	BH *p*	0.378	0.273	NA	NA	NA	NA

Table S8: Results of the pairwise PERMANOVA addressing the impact of protist treatments on the rhizosphere-associated bacterial community composition (harvest) according to different fraction of the community (rare: <0.1%; moderate: >0.1% and <1%; abundant: >1%) using Bray-Curtis Dissimilarity, UniFrac and weigthed UniFrac (W UniFrac); only for the treatments reported significant in the previously performed sequential two-way PERMANOVA (Table S6). The significant BH p-values (<0.05) are highlighted in bold.

Comparison pair		Rare (<0.1%)		Moderate (0.1%< x <1%)		Abundant (> 1%)
		Bray-Curtis	UniFrac	Bray-Curtis	W UniFrac	Bray-Curtis
Ctrl: S24D2	F test	$F_{(1,22)}$= 1.096	$F_{(1,22)}$= 1.211	$F_{(1,22)}$= 1.410	$F_{(1,22)}$= 4.144	$F_{(1,22)}$= 2.841
	p	0.117	0.111	0.054	0.002	0.037
	BH *p*	NA	NA	NA	**0.042**	0.777
Ctrl: P1-1	F test	$F_{(1,22)}$= 1.029	$F_{(1,22)}$= 1.023	$F_{(1,22)}$= 1.261	$F_{(1,22)}$= 0.400	$F_{(1,22)}$= 0.983
	p	0.339	0.385	0.159	0.954	0.415
	BH *p*	NA	NA	NA	NA	NA
Ctrl: P33	F test	$F_{(1,24)}$= 1.196	$F_{(1,24)}$= 1.325	$F_{(1,24)}$= 1.826	$F_{(1,24)}$= 1.544	$F_{(1,24)}$= 1.587
	p	0.005	0.059	0.001	0.001	0.163
	BH *p*	0.105	NA	**0.021**	**0.021**	NA
Ctrl: P147	F test	$F_{(1,21)}$= 1.036	$F_{(1,21)}$= 0.97	$F_{(1,21)}$= 0.876	$F_{(1,21)}$= 0.668	$F_{(1,21)}$= 0.581
	p	0.284	0.469	0.697	0.661	0.676
	BH *p*	NA	NA	NA	NA	NA
Ctrl: NL10	F test	$F_{(1,23)}$=1.02	$F_{(1,23)}$= 1.273	$F_{(1,23)}$= 1.384	$F_{(1,23)}$= 1.348	$F_{(1,23)}$= 0.938
	p	0.379	0.098	0.072	0.229	0.443
	BH *p*	NA	NA	NA	NA	NA
Ctrl: NL81	F test	$F_{(1,22)}$= 0.97	$F_{(1,23)}$= 0.741	$F_{(1,22)}$= 1.184	$F_{(1,22)}$= 1.006	$F_{(1,22)}$= 0.168
	p	0.65	0.939	0.232	0.395	0.923
	BH *p*	NA	NA	NA	NA	NA
S24D2: P1-1-	F test	$F_{(1,20)}$=1.141	$F_{(1,20)}$=1.518	$F_{(1,20)}$=1.858	$F_{(1,20)}$=2.781	$F_{(1,20)}$=2.546
	p	0.034	0.03	0.001	0.01	0.033
	BH *p*	0.714	0.63	**0.021**	0.21	0.693
S24D2: P33	F test	$F_{(1,22)}$=1.099	$F_{(1,22)}$=1.187	$F_{(1,22)}$=0.967	$F_{(1,22)}$=1.729	$F_{(1,22)}$= 1.037
	p	0.097	0.13	0.543	0.085	0.426
	BH *p*	NA	NA	NA	NA	NA
S24D2: P147	F test	$F_{(1,19)}$=1.133	$F_{(1,19)}$=1.434	$F_{(1,19)}$=1.218	$F_{(1,19)}$=2.072	$F_{(1,19)}$=1.702
	p	0.024	0.043	0.166	0.075	0.119
	BH *p*	0.504	0.903	NA	NA	NA
S24D2: NL10	F test	$F_{(1,21)}$=1.093	$F_{(1,21)}$=1.879	$F_{(1,21)}$=1.616	$F_{(1,21)}$=1.938	$F_{(1,21)}$=4.272
	p	0.095	0.008	0.009	0.091	0.005
	BH *p*	NA	0.168	0.189	NA	0.105

Comparison pair		Rare (<0.1%)		Moderate (0.1%< x <1%)		Abundant (> 1%)
		Bray-Curtis	UniFrac	Bray-Curtis	W UniFrac	Bray-Curtis
S24D2: NL81	F test	$F_{(1,20)}$=1.169	$F_{(1,20)}$=1.301	$F_{(1,20)}$=1.262	$F_{(1,20)}$=1.202	$F_{(1,20)}$=2.217
	p	0.005	0.064	0.103	0.275	0.058
	BH *p*	0.105	NA	NA	NA	NA
P1-1: P33	F test	$F_{(1,22)}$=1.191	$F_{(1,22)}$=1.558	$F_{(1,22)}$=2.277	$F_{(1,22)}$=4.182	$F_{(1,22)}$=1.97
	p	0.004	0.039	0.001	0.001	0.091
	BH *p*	0.084	0.819	**0.021**	**0.021**	NA
P1-1: P147	F test	$F_{(1,19)}$=0.991	$F_{(1,19)}$=1.236	$F_{(1,19)}$=1.054	$F_{(1,19)}$=0.609	$F_{(1,19)}$=0.999
	p	0.559	0.156	0.404	0.768	0.439
	BH *p*	NA	NA	NA	NA	NA
P1-1: NL10	F test	$F_{(1,21)}$=0.987	$F_{(1,21)}$=1.175	$F_{(1,21)}$=1.705	$F_{(1,21)=}$0.97	$F_{(1,21)}$=1.796
	p	0.571	0.194	0.008	0.425	0.164
	BH *p*	NA	NA	0.168	NA	NA
P1-1: NL81	F test	$F_{(1,20)}$= 0.998	$F_{(1,20)}$= 1.303	$F_{(1,20)}$= 1.811	$F_{(1,20)}$= 0.555	$F_{(1,20)}$= 0.449
	p	0.522	0.095	0.004	0.813	0.816
	BH *p*	NA	NA	0.084	NA	NA
P33: P147	F test	$F_{(1,21)}$=1.240	$F_{(1,21)}$=1.682	$F_{(1,21)}$=1.345	$F_{(1,21)}$=2.223	$F_{(1,21)}$=2.317
	p	0.003	0.014	0.074	0.062	0.039
	BH *p*	0.063	0.294	NA	NA	0.819
P33: NL10	F test	$F_{(1,23)}$=1.130	$F_{(1,23)}$=2.017	$F_{(1,23)}$=2.291	$F_{(1,23)}$=4.016	$F_{(1,23)}$=3.731
	p	0.058	0.003	0.001	0.004	0.004
	BH *p*	NA	0.063	**0.021**	0.084	0.084
P33: NL81	F test	$F_{(1,22)}$=1.170	$F_{(1,22)}$=1.367	$F_{(1,22)}$=2.225	$F_{(1,22)}$=2.276	$F_{(1,22)}$=1.509
	p	0.02	0.061	0.001	0.035	0.178
	BH *p*	0.42	NA	**0.021**	0.735	NA
P147: NL10	F test	$F_{(1,20)}$=0.920	$F_{(1,20)}$=1.231	$F_{(1,20)}$=1.312	$F_{(1,20)}$=0.988	$F_{(1,20)}$=0.696
	p	0.879	0.15	0.127	0.384	0.584
	BH *p*	NA	NA	NA	NA	NA
P147: NL81	F test	$F_{(1,19)}$=0.959	$F_{(1,19)}$=0.883	$F_{(1,19)}$=1.366	$F_{(1,19)}$=0.544	$F_{(1,19)}$=0.365
	p	0.698	0.692	0.081	0.744	0.827
	BH *p*	NA	NA	NA	NA	NA
NL10: NL81	F test	$F_{(1,21)}$=1.021	$F_{(1,21)}$=1.071	$F_{(1,21)}$=1.531	$F_{(1,21)}$=0.557	$F_{(1,21)}$=1.385
	p	0.383	0.326	0.019	0.738	0.269
	BH *p*	NA	NA	0.399	NA	NA

Table S9: Comparison of the effect of the different treatments on the abundance of the 10 most abundant bacterial phyla. Only the significant results are given. The results of the two-way ANOVA are given before the post hoc Tukey test results.

	diff	lwr	upr	p adj
Prior, Proteobacteria; Box: $F_{(7,76)}$=8.034, p < 0.001; Treatment: $F_{(6,76)}$=3.587, p = 0.003				
NL10-P1_1	568.989	7.276	1130.703	0.045
NL10-P147	622.317	49.021	1195.613	0.025
NL81-NL10	-609.228	-1170.941	-47.515	0.025
Prior, Bacteroidetes; Box: $F_{(7,76)}$=4.468, p < 0.001; Treatment: $F_{(6,76)}$=2.349, p = 0.039				
NL10-P147	-378.099	-730.381	-25.817	0.027
Prior, Verrucomicrobia; Box: $F_{(7,76)}$=4.4358, p < 0.001; Treatment: $F_{(6,76)}$=4.07, p = 0.001				
S24D2-P33	144.269	28.657	259.881	0.006
S24D2-P147	121.457	3.461	239.453	0.039
S24D2-NL10	153.707	38.095	269.319	0.002
Prior, Acidobacteria; Box: $F_{(7,76)}$=5.056, p < 0.001; Treatment: $F_{(6,76)}$=3.0497, p = 0.01				
S24D2-NL81	-27.697	-54.912	-0.481	0.043
Prior, Myxococcota; Box: $F_{(7,76)}$=3.912, p < 0.001; Treatment: $F_{(6,76)}$=2.6267, p = 0.023				
P147-P1_1	-27.400	-53.847	-0.952	0.037
Harvest, Actinobacteria; Box: $F_{(7,76)}$=10.857, p < 0.001; Treatment: $F_{(6,76)}$=3.539, p = 0.004				
P147-P33	632.974	46.209	1219.738	0.026
S24D2-P147	-759.772	-1369.287	-150.256	0.006
Harvest, Verrucomicrobia; Box: $F_{(7,76)}$=11.164, p < 0.001; Treatment: $F_{(6,76)}$=5.041, p < 0.001				
S24D2-Control	150.795	35.154	266.435	0.003
S24D2-P33	131.835	16.195	247.476	0.015
S24D2-P147	185.980	62.646	309.315	<0.001
Harvest, Gemmadimonadetes; Box: $F_{(7,76)}$=2.401, p = 0.03; Treatment: $F_{(6,76)}$=4.034, p = 0.002				
P147-P33	-111.802	-195.409	-28.196	0.002
NL10-P33	-84.292	-163.864	-4.721	0.031
S24D2-P147	90.181	3.332	177.029	0.037

Supplementary Figures

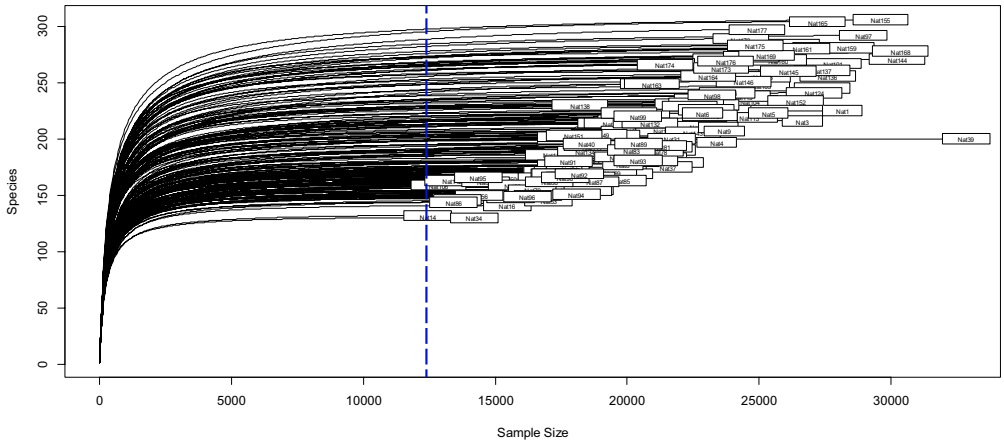

Figure S1: Collector's curve giving the number of species in function of the sample size. The blue dashed line represents the minimum obtained sample size, 12,4000, which was also used as threshold for the rarefied dataset.

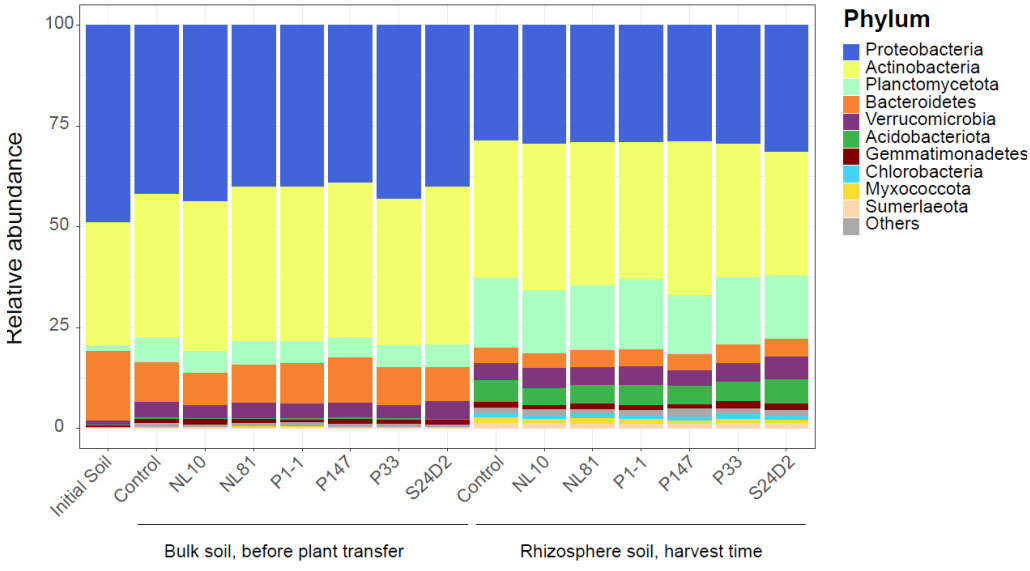

Figure S2: Distribution of the 10 most abundant bacterial phyla in the initial soil, in the bulk soil before plant transfer and in the rhizosphere soil at the harvest time, according to the different protist treatments.

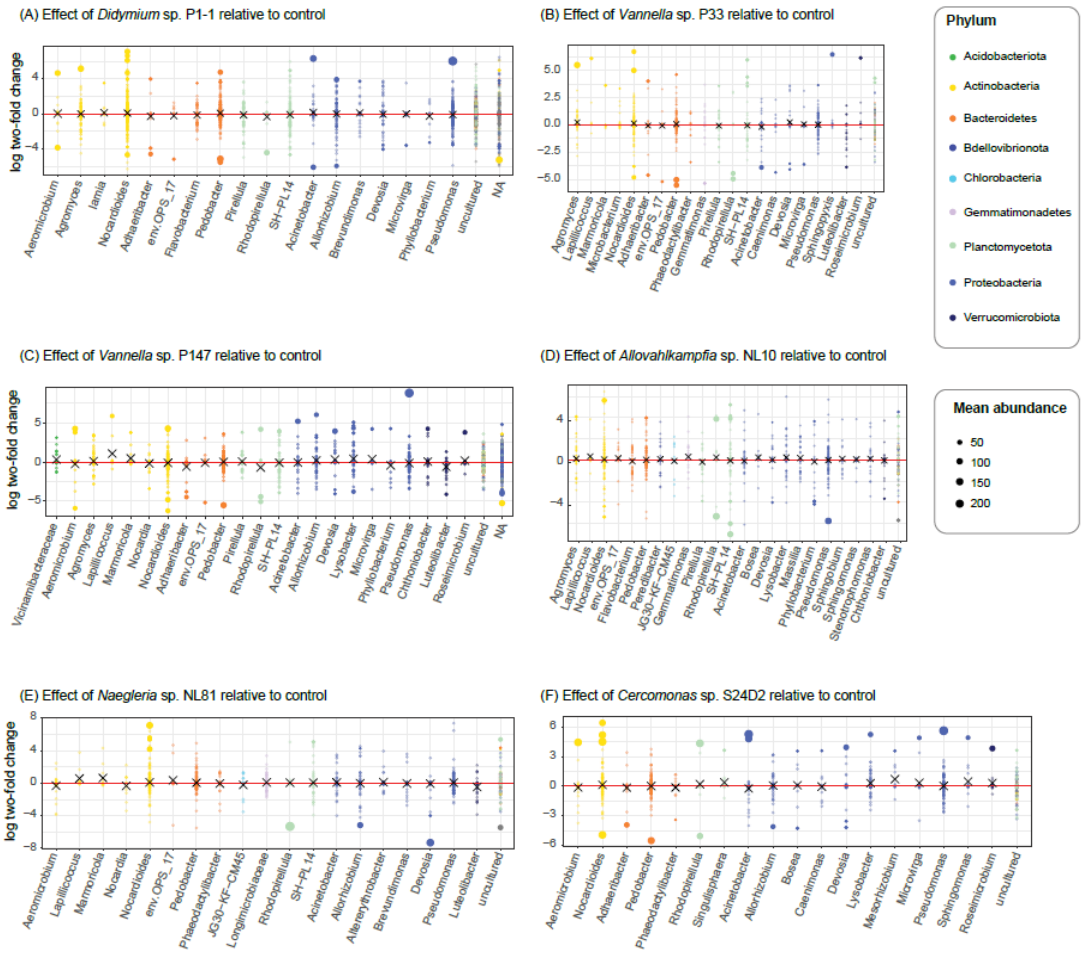

Figure S3: Impact of each protist on different ASVs prior plant transfer. The predatory impact is given as the statistically significant log two-fold changes compared to the no-protist control treatments. Dots above the red line represent bacterial ASVs that were more abundant in the treatment compared to the control, dots below the red line represent bacterial ASVs that were less abundant in the treatment compared to the control. Open circles are the ASVs not significantly modified. The cross indicate the log two-fold average for each genus, including all non-significantly modified ASVs. Note that we shorten the assigned Allorhizobium-−Neorhizobium−Pararhizobium−Rhizobium to Allorhizobium.

Chapter 5

Timing of protist inoculation affects plant performance with little impact on rhizosphere microbial community composition

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Abstract

Predatory soil protists have been linked to plant performance including increases in plant shoot biomass, nitrogen content, and root elongation and branching. Inoculation of protists has thus been proposed as a means of supporting plant development, but the optimal conditions of such amendments are generally not well known. To address this knowledge gap, we examined the impact of protist inoculation time (one week before seedling transfer, simultaneously with seedling transfer, and one week after seedling transfer) on plant performance and rhizosphere microbial communities in a greenhouse experiment with lettuce as a model crop. In addition, we compared the effect of inoculation with a single-species inoculum versus a three-species mixture. After 30 days of growth, plants were destructively harvested to analyze their biomass, nutrient content and rhizosphere bacterial and protist community composition (via 16S and 18S rRNA gene sequencing). We found that early protist inoculation provided the greatest increase in aboveground biomass compared to the non-inoculated control, with no differences observed between single- or mixed-species inoculations. Although the relative abundances of specific bacterial and protist taxa were found to correlate with various plant properties such as iron shoot content and root dry weight, the overall microbial community composition remained mostly unaffected by protist treatments.

1. Introduction

Soil predatory protists are abundant and diverse in terrestrial ecosystems (Singer *et al.* 2021; Xiong *et al.* 2021). They are one of the main consumers of bacteria, and their predatory activity can affect plant performance by releasing nutrients from bacterial biomass (Clarholm, 1985; Ekelund *et al.* 2009) and by modifying the bacterial community composition (Rosenberg *et al.* 2009; Müller, Scheu and Jousset 2013). The application of protists has been reported to lead to an increase in the shoot biomass (Bonkowski *et al.* 2000; Bonkowski and Clarholm 2012), a more elongated and branched root system (Kreuzer *et al.* 2006; Krome *et al.* 2010), a higher content of nitrogen (Kuikman and Van Veen 1989; Koller *et al.* 2013) and other elements in the shoot (Bonkowski, Jentschke and Scheu 2001; Herdler *et al.* 2008), as well as the enrichment of plant-beneficial taxa in the bacterial community (Bonkowski 2004; Bonkowski and Clarholm 2012). Based on these numerous beneficial effects on plants, the inoculation of protists has been suggested as a promising strategy to help support plant growth (Gao *et al.* 2019).

Studies investigating the effect of protist application on plant development have typically focused on one single inoculation time point, but the success of such amendments is likely strongly related to the timing of application. Significant increase in nutrient turnover may for instance only occur after several days due to the time required for the amended protist population to establish and grew sufficiently to exert a significant level of predatory activity (Anderson and Domsch 1978). Similarly, the impact of predatory protists on the bacterial community composition has been observed as early as two days after inoculation (Rosenberg *et al.* 2009). Such impact can, however, also influence bacterial community dynamics on a longer term as reported by the enrichment of certain bacterial taxa two to three weeks after protist inoculation (Müller, Scheu and Jousset 2013). Even if protist inoculation can have effects on the soil community composition for a relatively long time, the influence of the plant on rhizosphere community composition is likely to dominate over time (Kröber *et al.* 2014; Shi *et al.* 2015b), potentially overshadowing the predatory impacts of protists. If inoculation is too early or too late, the effectiveness of the treatment would likely be diminished, and it is thus desirable to identify the optimal time of application.

In addition, the inoculation of multiple protist species could be more effective than single-protist treatments in improving plant performance. In general, the inoculation of multiple protist species is expected to positively affect the prey bacterial diversity (Saleem *et al.* 2012, 2013). Higher bacterial diversity has been related to an increase in nitrogen mineralization, which in turn improve plant growth (Weidner *et al.* 2015).The preferential feeding typically reported for predatory protists (Singh 1942; Gao *et al.* 2019) may further lead to a higher diversity of plant-beneficial bacteria and their associated traits under the predatory pressure of a multispecies protist inoculant (Bell *et al.* 2005; Saleem *et al.* 2012). Increased protist diversity could thus have a larger and complementary impact on plant performance compared to a single-species inoculation.

Most studies investigating the effect of protist application have relied upon experimental approaches with sterilized soils and/or protist-free soils. Protists themselves are, however, part of a diverse plant-associated microbiome (Sapp *et al.* 2018) and they have been shown to respond as well to environmental changes, though in distinctive ways compared to other soil-borne microbes (Xiong *et al.* 2017; Zhao *et al.* 2019). It is therefore important to consider protist performance in the presence of the resident soil microbiome, as well as the impacts of protist inoculations on this resident microbiome.

Here, we investigated how different times of inoculation would affect lettuce growth with application of protists either one week before transferring plant seedlings, at the time of seedling transfer, or one week after seedling transfer. In addition, we inoculated with either a single-protist solution (*Cercomonas* sp. S24D2) or a three-species protist mixture solution (*Cercomonas* sp. S24D2, *Acanthamoeba* sp. C13D2 and Heterolobosea sp. S18D10; selected based on their impacts on plant traits and their prey consumption patterns). We assessed the effects of our treatments on plant performance by measuring biomass and nutrient content, and we further examined rhizosphere microbial communities at the time of harvest. Microbial community analyses targeted bacterial and protist communities via 16S and 18S rRNA gene amplicon sequencing, respectively. We hypothesized that (1) the inoculation of protists would improve plant performance and influence rhizosphere microbial community composition, (2) that the time of inoculation would influence the magnitude of effects observed on plant properties and on microbial community composition, (3) that the inoculation of multiple protist species would have a stronger impact on both the plant and the soil microbial community composition than single species additions, and (4) that the impacts on the plant properties could be partially explained by changes in the microbial community.

2. Material & Methods

2.1 Protist isolates and preparation

The protists used in our study were selected based upon previous results related to their impacts on plant traits and growth characteristics on different bacterial prey species. In a previously performed pot experiment, the application of *Cercomonas* sp. S24D2 to lettuce was shown to result in a greater shoot-to-root ratio when inoculated one week before plant transfer (chapter 4). Based on protist feeding plate assays, we selected *Acanthamoeba* sp. C13D2 and Heterolobosea sp. S18D10 as they showed distinct feeding patterns from each other and from *Cercomonas* sp. S24D2 (chapter 3). Combining three isolates with distinct feeding patterns was expected to have complementary impacts on the resident bacterial communities.

One week before application, each protist was grown separately on *Escherichia coli* OP50 (*ca* 10^8 cells per mL; adjusted to an OD_{600} of 0.5) in Page's Amoeba Saline (Page 1976; hereafter referred to as PAS) from an active stock culture. The day before the application,

we prepared the protist solution as follows: stock protist cultures were washed three times by gentle centrifugation at 800 *g* for 5 min (Heraeus Megafuge 40 Centrifuge, Thermo Fisher Scientific, Langenselbold, Germany) to remove spent medium and enrich the protists. Because no visible pellet is formed, we only discarded seventy-five percent of the volume before resuspending the cells in the same volume of PAS. The density was estimated by transferring three times a volume of 60 µL in Clear Polystyrene 96-Well Microplates with flat bottom (Corning® 3370, Arizona, USA). Cells were enumerated over five screens of the well surface on a monitor connected 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 solutions were further diluted when necessary to obtain similar final densities for each treatment at each time of application ($9 \times 10^3 \pm 3.5 \times 10^3$ ind. mL^{-1}; Table S3). Because these washing steps are not sufficient to fully eliminate the presence of bacteria cells, we quantified the remaining bacteria by serial dilution plating 3 g L^{-1} TSA for 24h at 28°C ($6.6 \times 10^6 \pm 2.5 \times 10^6$ CFUs mL^{-1} ; Table S1).

2.2 Plant seedling preparation

Lettuce seeds were prepared according to an adapted protocol from Trinh *et al.* (2018), as follows: First, we surface-sterilized seeds of the lettuce Lactuca sativa Wonder der Vier Jaargetijden (De Bolster biologische zaden, Epe, The Netherlands) by placing them in 5% NaOCl for 10 min and subsequently rinsing them four times in 50 mL autoclaved demi water. Second, to induce germination, the surface-sterilized seeds underwent a stratification step (three days at 4°C in the dark). Eventually, the seeds were transferred on Murashige and Skoog medium (Minimal Organic Powder Medium, SERVA Electrophoresis GmbH, Heidelberg, Germany) without sucrose (MS0; 8 to 10 seeds per petri dish). The petri dishes were incubated in a growth cabinet with a 16h/8h day/night regime, at 21°C for 4 days (Phytotron facility, Utrecht University, The Netherlands). We selected healthy seedlings with a similar phenotype (*i.e.*, *ca* 1 cm of main root with abundant root hairs) to be transferred in the pots.

2.3 Setting up of the greenhouse experiment

The experimental setup followed a two-factorial design with three different times of inoculation (seven days before plant transfer, at the time of plant transfer and seven days after plant transfer) and two different types of inoculation (single- (*Cercomonas* sp. S24D2) and mixed-species (*Cercomonas* sp. S24D2, *Acanthamoeba* sp. C13D2 and Heterolobosea sp. S18D10)) (Figure 1). Each treatment, including the no-protist treatment used as control, was set in 12 replicates, thereby leading to a total of 84 pots.

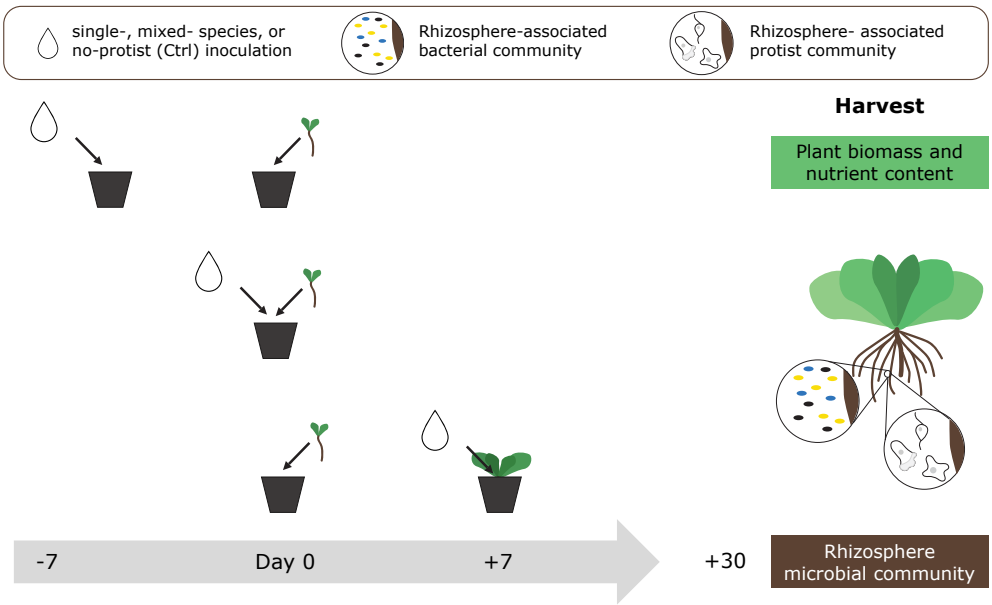

Figure 3: Diagram of the experimental design and timeline. The icon with a drop illustrates the treatment, being either single- (Cercomonas sp. S24D2) or mixed-species (Cercomonas sp. S24D2, Acanthamoeba sp. C13D2 and Heterolobosea sp. S18D10) inoculation, or no-protist control.

We prepared each pot (Pot 14 Cm 8° Lw YB, SKU 100.022.000, external diameter: 13.7 cm; height: 10.8 cm; volume: 0.95 L; Van Krimpen B.V., Standdaarbuiten, The Netherlands) by covering the bottom with a filter (Whatman No 1, Ø 90mm, Cat No 1001 090) and 70 mL of wet perlite (35 g ± 1 g). We then added 1,165 g ± 15 g of a sandy soil. The sandy soil was prepared as a 50%-50% mixture of river sand and sandy soil at SoilTech (Biezenmortel, The Netherlands); the characteristics of the soil were analyzed by Eurofins Agro Testing Wageningen BV (Wageningen, The Netherlands; Table S2). We also took four soil samples and froze them the same day at -80°C in Eppendorf tubes for subsequent DNA extractions. We placed the pots following a randomized scheme with an extra layer all around the experimental setup to ensure similar conditions for each plant. Each pot was watered with 10 mL of water on the first day and kept moist throughout the whole experiment.

We inoculated the protist solution in the center of each pot, as close as possible to the plants (or future location of the plant) to maximize the chance of the protist to establish close to the rhizosphere, where protist impact is expected to be highest (Elliott *et al.* 1984; Clarholm 1985). The first application time was performed one week before plant transfer. We created a small hole of *ca* 1 cm depth in the middle of the pot, where the plant would later be transferred, and pipetted 900 µL (single-protist treatment) or 3x 300 µL (mixture of protists) of the corresponding protist solution. We then added 10 mL of water to help protist establishment and closed the hole. The control treatment received 900 µL of PAS. For the second application time, we followed the same procedure but transferred the 5-days old lettuce seedlings before closing the hole. For the third application time, the protist solution was applied at the base of the plant shoot, as close as possible to the roots.

2.4 Harvest and plant properties measurement

Plants and rhizosphere soil were harvested after 30 days which allowed for sufficient above-ground biomass and limited the spread of an unidentified leaf disease that appeared 48 hours before harvest. The number of plants with disease symptoms was also monitored (Table S3); No significant patterns were detected regarding the distribution of disease symptoms and the different treatments of the experiment. During harvest, the shoot part was separated from the root and its fresh weight was recorded before being placed in a paper bag. On the same day, we put the shoot at 70°C to be dried. The rhizosphere soil was obtained by perforating with a metal core the center of each pot to obtain most of the main root and its surrounding soil, so to allow reproducible sampling between pots with different root biomass. The obtained samples were stored for 24h at 4°C, before being resuspended in a volume of 35 mL of 10 mM $MgSO_4$ by horizontal shaking for *ca* 10 min on (*ca* 90 rpm; Gerhardt Schüttelmaschine RO20, Gerhardt GmbH, Bonn, Germany). With sterilized tweezers, we recovered as many roots as possible into a labelled paper bag for weight measurement. The tubes were centrifuged at 3,000 *g* for 5 min, the supernatant discarded and the obtained rhizosphere soil samples stored at -20°C until further processing to extract DNA.

We further processed the remaining soils to retrieve as many roots as possible. The soil, dried at room temperature, was sieved (mesh diameter: 2 mm) and cleaned with water to eliminate soil and perlite particles. The obtained roots were combined in the paper bag with the main root previously obtained and dried at 70°C.

Roots and shoots were dried at 70°C for 48h in a paper bag, before dry weight was measured according to an internal manual and the Handbook of reference methods for plant analysis (Kalra 1998). Shoot samples were further processed for Inductively Coupled Plasma Atomic Emission Spectrometry (ICP) analysis to obtain Al, Ca, Cd, Cu, Fe, K, Mg, Mn, Na, P, Pb, S, and Zn shoot content. The shoots and roots were further processed for Elemental Analyzer (EA) analysis to obtain carbon and nitrogen content. The root samples did not provide enough material to allow for both analyses, and only carbon and nitrogen content were therefore analyzed. The roots were ground by use of 2 mL Eppendorf tube containing a metal ball placed on a Retsch MM400 mill (Retsch GmbH, Verder Scientific, Haan, Germany) for 4 min at frequency of 25 s^{-1}. The ground samples were dried at 70°C for 1.5h. The shoots were first cut into smaller pieces and fitted into bigger metal tubes containing a metal ball and ground similarly by use of a Retsch MM400 mill (Retsch GmbH, Verder Scientific, Haan, Germany) for 60 s at frequency of 25 s^{-1}. The ground shoots were transferred into 2 mL Eppendorf tubes. The ground shoots and roots were kept in a desiccator until further processing.

The procedure for sample preparation for ICP analysis described as in Isaac and Johnson (1998) was followed with a modification for lower amount. An amount of *ca* 50 mg of dried,

ground shoot material was transformed to ash by a 2 hours exposition at 500°C in a muffle furnace, digestion in 300 µL of HNO_3 (65%), complete evaporation on a hot plate at 130°C, and 1 hour exposition at 500°C in a muffle furnace. We then added 100 µL HCl (32%) and 9.9 mL deionized water to the ash before processing the samples via the ICP Spectrometer iCAP 6000 Series (Thermo Fisher Scientific, Cambridge, United Kingdom) coupled with a AutoSampler ASX-520 (Teledyne CETAC Technologies, Nebraska, USA). The concentration of the following elements was measured: Al, Ca, Cd, Cu, Fe, K, Mg, Mn, Na, P, Pb, S, and Zn. Values were converted from mg L^{-1} to the mg per plant, based on the dry shoot weight initially measured.

An amount of 2.5-3.5 mg dry ground shoot material was weighed on a Mettler Toledo MX5 microbalance (Mettler Toledo, Greifensee, Switzerland), placed into a small tin cup and arranged on a 96-wells plate as preparation for the elemental analyzer. Calibration standards employed included: an empty tin container, 25-(Bis(5-tert-butyl-2-benzo-oxazol-2-yl) thiophene (BBOT), acetanilide, and atropine. The samples were analyzed via an Interscience EA 1110 CHNS-O elemental analyzer (CE Instruments Ltd, Wigan, England) to obtain percentage of carbon and nitrogen within the samples. The obtained values were used to calculate the relative and absolute amount of carbon and nitrogen per sample, as well as the C:N ratio.

2.5 Rhizosphere microbial community analyses

The DNA was extracted from ca 0.5 g of the bulk soil (taken from the initial soil mixture) and rhizosphere soil samples (taken at the harvest time) by using the DNeasy® PowerSoil® kit (Qiagen, Hilden, Germany). We followed the manufacturer protocol with slight modifications previously shown to increase yield in our research group: an amount of 0.5 g of soil instead of 0.25 g was taken and lysis of the cells was obtained using a Retsch mill MM400 (Retsch GmbH, Verder Scientific, Haan, Germany) with Qiagen TissueLyser Adapter (Qiagen, Hilden, Germany) fitting up to 24 2 mL Eppendorf tubes at a frequency of 30 sec^{-1} for 6 min.

Estimates of total bacterial community abundance were obtained via qPCR targeting the V4 region of the 16S rRNA gene. Extracted environmental DNA was diluted twenty times and prepared for qPCR analysis with a Freedom EVO® Tecan robot (Tecan Trading AG, Männedorf, Switzerland). Bacterial DNA abundance was measured with an Applied Biosystems ViiATM7 PCR system (Thermo Fischer Scientific, Massachusetts, USA) using primers targeting the V4 region of the 16S rRNA gene (341F 5'-CCTACGGGNGGCWGCAG-3' and 805R 5'-GACTACHVGGGTATCTAATCC-3'; Herlemann *et al.*, 2011; Besemer *et al*., 2012). The qPCR master mix was prepared with solutions of ItaqTM universal SYBR® green supermix (Bio-Rad Laboratories, Veenendaal, The Netherlands), forward primer (5 µM), reverse primer (5 µM), and milliQ water with the respective volume per sample: 5 µL, 0.5 µL, 0.5 µL, and 1.5 µL. A volume of 2.5 µL of template DNA was used per sample. The qPCR was performed by an initial denaturing step at 95°C for 30 s with subsequent cycling for 40 times with a 15 s denaturizing step at 95°C and a combining annealing/elongation steps

at 60°C for 1 min. Melting curves were obtained based on a standard protocol and used to identify the characteristic peak of PCR product. Two independent technical replicates were performed for each sample.

In order to examine the microbial community structure, DNA extracts were used as template for high throughput 16S rRNA and 18S rRNA gene amplicon sequencing of bacterial and eukaryotic communities, respectively, as carried out by Genome Quebec (Montréal, Canada). Before sending the extracted DNA solution, the obtained DNA yield was assessed on a DeNovix DS-11 Spectrophotometer (DeNovix, Wilmington, Delaware, US), and the samples were sent in dried ice to maintain high quality of the DNA. The V4 region of the 16S was amplified on an Illumina MiSeq PE 250 bp sequencing machine (Illumina, California, US) using the same set of primers mentioned above (341F and 805R) with the MiSeq Reagent Kit v3 600 cycles from Illumina. The V9 region of the 18S was targeted with the primers 1391F and EUkBr (`5-GTACACACCGCCCGTC-3' and `5-TGATCCTTCYGCAGGTTCACCTAC-3'; Amaral-Zettler *et al.* 2009; Stoeck *et al.* 2010) and amplified on an Illumina MiSeq PE 250 bp sequencing machine (Illumina, California, US) with the MiSeq Reagent Kit v2 5,000 cycles from Illumina.

After sequencing, the primary analysis of raw FASTQ data was processed using the QIIME2 pipeline (version 2020.6; Caporaso *et al.*, 2010). The DADA2 workflow (Callahan *et al.* 2016) was followed with the default settings for error-correction, removal of forward and reverse primers, quality filtering, doubleton and chimera removal of the Illumina amplicon sequences with reads truncated at 200 bp for each single end read, corresponding to a quality score > 30, and allowing forward and reverse sequences to overlap>50 bp. QIIME2's q2-feature-classifier plugin (Bokulich *et al.* 2018) was used for taxonomy assignment against the SILVA 138 reference database using OTUs at a 99% similarity level from the 515F/806R region of sequences (Glöckner *et al.* 2017) for the bacterial community and against the PR2 database (Guillou *et al.* 2012) for the eukaryotic community. We further processed the dataset using the version 4.0.3 of the open source statistical software R (R Core Team 2020). From the 16S dataset, we removed the sequences assigned to chloroplast (n=361) and mitochondria (n=16) and obtained a total of 15,040 Amplicon Sequencing Variants (ASVs). To compare bacterial communities between the treatments, sequence read numbers were normalized to the minimum sequence number (16,048 reads) by random subsampling (phyloseq:: rarefy_even_depth specifying rngseed(1) for reproducibility of the analysis; Figure S1 for the collector's curve showing the number of species in function of the sample size); after rarefaction we obtained 14,258 ASVs. From the 18S dataset, we focused on the protist community and removed the sequences assigned to Streptophyta (n=683), Metazoa (n=595), Fungi (n=2,759), and the ones with no assigned Phylum (n=10,912). We obtained a total of 9,131 Amplicon Sequencing Variants (ASVs). To compare protist communities between the treatments, we normalized the sequences to 5,060 reads by random subsampling (phyloseq:: rarefy_even_depth specifying rngseed(224) for reproducibility of the analysis). With this threshold, we excluded one sample (nat46; single-protist treatment

inoculated after plant transfer, S3, replicate 12) with a particularly low library size (2,692), and all remaining samples had sufficient depth to approach an asymptote in the collector's curve (Figure S2). After rarefaction, we retained 8,849 ASVs.

2.6 Data analysis

All the data analysis was performed using the free, open-access software R, version 4.0.3 (2020-10-10) (R Core Team 2020).

2.6.1 Plant properties

We calculated the total biomass by summing up the dry weight of the shoot and of the root biomass. The shoot-to-root ratio was obtained from the ratio of the dry weights. We obtained from the Elemental Analyzer the relative content of carbon and nitrogen in both shoots and roots and calculated the absolute content in mg per plant, as well as the C:N ration on these absolute values. Similarly, we converted the values obtained from the ICP (mg mL^{-1}) into absolute shoot content per plant in mg for each element.

We investigated the main effects and potential interaction of our treatments by performing a two-way ANOVA (stats::lm) with a type II Sum of Square (car::Anova(type=c("II")) with the explanatory variables being the time point of application (1: seven days before plant transfer; 2: simultaneously with plant transfer; 3: seven days after plant transfer) and the treatment type (single- or mixed-species) on the following plant properties: fresh shoot weight, dry shoot weight, dry root weight, total biomass, shoot-to-root ratio, shoot carbon content, root carbon content, shoot nitrogen content, root nitrogen content, shoot CN ratio, root CN ratio, shoot Al content, shoot Ca content, shoot Cd content, shoot Cu content, shoot Fe content, shoot K content, shoot Mg content, shoot Mn content, shoot Na content, shoot P content, shoot Pb content, shoot S content, and shoot Zn content. The two-way ANOVA was followed by a *post hoc* Tukey test on the reported significant main effects ($p < 0.05$; stats::aov, stats:TukeyHSD) to identify which treatments were different from each other.

2.6.2 Microbial communities

We plotted the average relative abundance of the 10 dominant phyla per treatment for both bacterial and protistan communities from the rarefied dataset. We tested if the treatments had an effect on the relative abundance of taxa in each of these phyla with a two-way ANOVA (stats::lm, car::Anova(type-c("II")), followed by a *post hoc* Tukey test when significant ($p < 0.05$).

We calculated different species richness indices for both 16S and 18S using phyloseq::plot_richness and phyloseq::estimate_richness, using the rarefied dataset to control for differences in sequencing depth between samples. We considered the observed species richness, the Chao1 index, the Shannon index and the Pielou's evenness index (calculated

as the Shannon index divided by the logarithm of the observed species richness). We investigated the effects of our treatments on the microbial alpha diversity by performing a two-way ANOVA (stats::lm; car::Anova(type=c("II")) followed by a *post hoc* Tukey test on the reported significant main effect ($p < 0.05$; stats::aov, stats:TukeyHSD) to identify which treatments were different from each other.

We used the rarefied datasets to investigate the beta diversity between our samples. We used the Bray-Curtis dissimilarity and the two phylogenetic aware distances UniFrac and weighted UniFrac. We visualized the different samples based on these distance matrices using phyloseq::ordinate and statistically tested the effect of our treatments by use of a two-way PERMANOVA (vegan::adonis) with the explanatory variables being the time point of application (1: seven days before; 2: simultaneously; 3: seven days after plant transfer) or the treatment type (single- or mixed-species). In case of significant results, we further performed pairwise PERMANOVA tests on the significant main effect between the relevant treatments and corrected for multiple testing by using the Benjamini and Hochberg correction (indicated as "BH *p*"; Noble 2009). To enable replication of the analysis, we used set.seed(224) to force R to assign the same root in the phylogenetic tree when computing the UniFrac distances.

Because protist predation may in some cases not affect the whole bacterial community composition (*e.g.*, Asiloglu *et al.* 2020), we also investigated differential abundances on the unrarefied 16S and 18S dataset with the DESeq R package. Before the analyses, we subset our dataset in smaller datasets consisting of the treatment of interest and the control, and removed rows containing only zeros. We used a Differential expression analysis based on the Negative Binomial distribution (DESeq; Love *et al.* 2014) as basis to visually observed log two-fold changes at the genus level compared to the control. Because every sequence had at least one zero occurrence, which makes the calculation of the default logarithmic geometric mean to estimate the size factor impossible, we had to add an arbitrary value of one to every count before performing the analysis. In addition, to allow for meaningful visualization without too many ASVs, and as an attempt to control for the reported high false discover rate (Hawinkel *et al.* 2019), we set the alpha value to 0.001 (instead of the default 0.1).

2.6.3 Relation between impacts on the microbial community and plant properties

We merged the data of the sequencing datasets per treatments (phyloseq::merge_samples) to calculate the Bray-Curtis Dissimilarity and UniFrac/weigthed UniFrac distance (phyloseq::distance) relative to the non protist control as indicator of the magnitude of induced change on the microbial community composition. We further calculated the averaged difference for each plant property per treatment relative to the no-protist control as an indicator of the magnitude of the effect on the given plant property. We then performed correlation analyses between the magnitude of change induced on the microbial community and on the plant properties (stats::cor.test).

We further explored the potential link between the relative abundance of specific rhizosphere-associated bacterial and protist genera and plant properties by running a correlation analysis between them (stats::cor.test). We used the relative microbial abundance of the rarefied datasets. We corrected the p values for multiple testing using the Bonferroni correction (stats::p.adjust) and visualized the significant correlations (corrected $p < 0.05$) in a heatmap (gplots::heatmap.2).

3. Results

3.1 Plant properties

The inoculation of protists affected mostly the above-ground biomass and nutrient content, with clear differences between treatments (*i.e.*, time and type of inoculation) (Table S4). The time of inoculation affected plant above-ground properties including the shoot fresh and dry weight, carbon content, C:N ratio, and the shoot Al, Ca, Mg, Mn, Na, P, S and Zn contents (Figure 2; Table 1 and S4). All these properties were not affected by the treatment type (single- or mixed-species) or the interaction between the time of inoculation and the treatment type (Table S4). The *post hoc* Tukey tests revealed that the early inoculation time (1 week before the plant transfer) lead to the largest increase in above ground biomass and nutrient content (Figure 2; Table 1 and S5).

The treatment type (single- or mixed-species) only affected the shoot-to-root ratio ($F_{(1,75)}$=7.170, p = 0.009; Table S4) with a significant increase in the three-species mixture (mean ± sd: 5.66 ± 4.92) compared to the single-species inoculation (3.16 ± 2.62) (Table S5 and Figure S3). Here again, no significant interaction between the two main factors was observed. An interaction effect of the two main factors was only observed for the root C:N ($F_{(2,75)}$=3.803, p=0.027), which was a trait displaying no significant treatment effects (Time: $F_{(2,75)}$= 2.278, p=0.11; Type: $F_{(1,75)}$= 0.774, p=0.382; Table S4).

3.2 Microbial communities

In general, the treatments had little effect on the rhizosphere bacterial and protist communities. The relative abundance of the 10 most dominant bacterial phyla remained similar across treatments with the Proteobacteria (32.5%), Actinobacteria (20.4%) and Chloroflexi (13.8%) representing the most dominant phyla (Table S6, Figure S4). With regards to the protistan community, a dominance of Cercozoa (79.6%) was found in the soil prior the start of the experiment. The protist community has shifted by the end of the experiment with the following three phyla being most dominant: the Chlorophyta (46.5%), the Ciliophora (23.7%) and the Cercozoa (12.7%) (Figure S4). Only the Lobosa were significantly affected by the treatments. This group showed a relative increase in the treatment where the three-species mixture was inoculated simultaneously with the plant as compared to the three-species mixture inoculated before the plant and both treatment types (single- and mixed-species) inoculated after seedling transplantation (Figure S4; Table S7).

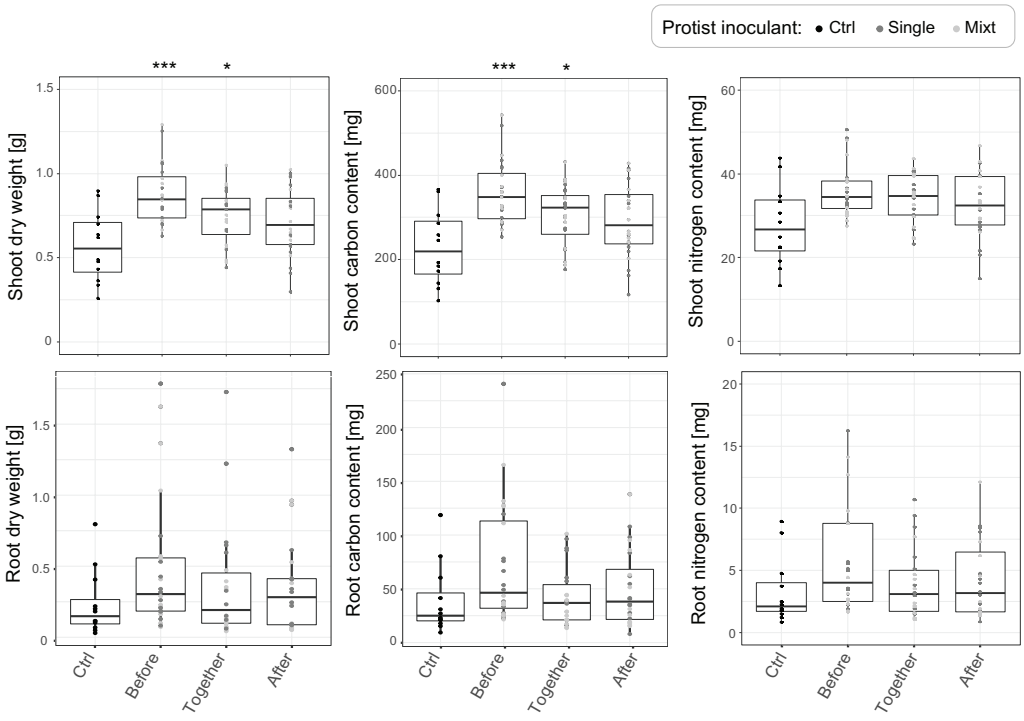

Figure 2: Effect of the inoculation time of protists (before, together or after plant transfer) on different plant properties: upper panels, from left to right: shoot dry weight, shoot carbon content, shoot nitrogen content; lower panels, from left to right: root dry weight, root carbon content, root nitrogen content. The treatment type, single or three-species mixture, is given by the colors dark gray and light gray, respectively; it had no significant effect on these plant properties. Asterisks indicate significant differences for the inoculation time compared to the non protist control with "" for p values < 0.05 and "***" for p values < 0.001.*

The estimated bacterial abundance, based upon 16S amplicon qPCR, was not significantly affected by any of the treatments (time of inoculation and treatment type; $2.7 \times 10^8 \pm 1.3 \times 10^8$ 16S copies per gram of soil; $F_{(6,75)} = 0.362$, $p = 0.9$). The timing of inoculation further did not significantly affect the bacterial alpha diversity nor the overall bacterial community structure (Figure 3 and Table S8), but the treatment type was associated with a reduction of the bacterial alpha diversity compared to the control (observed richness: $F_{(1,76)} = 4.152$, $p = 0.045$; Chao1: $F_{(1,76)} = 4.645$, $p = 0.034$; Shannon: $F_{(1,76)} = 4.185$, $p = 0.044$; Table S8). The *post hoc* Tukey test, however, did not reveal any difference between the single and three-species mixture in this regard (Table S9). No interaction effect between time of inoculation and protist treatment type was observed for any of the bacterial community characteristics investigated. With regards to protistan alpha and beta diversity, only the time of inoculation had a significant effect and this was only the case for the late inoculation time on the protistan community compared to the control (based on Bray-Curtis dissimilarity; $F_{(1,33)} = 1.025$, BH $p = 0.024$; Figure 3; Table S8 and S10). The treatment type had no significant effect (Table S8).

Table 1: Summary of statistics (mean ± standard deviation) for plant properties influenced by time of protist inoculation. Note that treatment type (single- or mixed-species) had no significant effect on these plant properties. Asterisks indicate significant differences compared to the control with "" for p values < 0.05 and "***" for p values < 0.001.*

	Control	**Before**	**Simultaneous**	**After**
Fresh shoot weight [g]	10.25 ± 3.52	15.06 ± 2.4 ***	12.86 ± 2.44	12.6 ± 3.16
Dry shoot weight [mg]	568.6 ± 208.91	877.95 ± 176.7 ***	746.93 ± 164.95 *	715.03 ± 203.1
Shoot C content [mg]	229.35 ± 88.35	358.48 ±75.25 ***	304.9 ± 70.03 *	292.85 ± 87.71
Shoot C:N	8.16 ± 0.52	9.95 ± 1.31 ***	8.85 ± 0.81	8.89 ± 0.9
Shoot Al [mg]	0.06 ± 0.03	0.09 ± 0.03	0.07 ± 0.03	0.07 ± 0.03
Shoot Ca [mg]	13.11 ± 4.37	22.14 ± 3.87 ***	18.57 ± 3.99 *	17.17 ± 4.35 *
Shoot Mg [mg]	8.89 ± 3.38	12.89 ± 2.48 ***	11.09 ± 2.25	10.4 ± 2.82
Shoot Mn [mg]	0.07 ± 0.03	0.12 ± 0.03 ***	0.1 ± 0.03 *	0.1 ± 0.03
Shoot Na [mg]	7.86 ± 3.84	12.5 ± 2.82 ***	10.84 ± 3.01 *	10.08 ± 3.14
Shoot P [mg]	7.16 ± 2.71	10.9 ± 1.88 ***	8.93 ± 2.14	8.7 ± 2.58
Shoot S [mg]	0.42 ± 0.13	0.58 ± 0.1 ***	0.54 ± 0.11 *	0.49 ± 0.13
Shoot Zn [mg]	0.05 ± 0.02	0.08 ± 0.02 ***	0.07 ± 0.02 *	0.07 ± 0.02 *

Our pairwise differential abundance analysis, however, did reveal significant changes in the relative abundance of specific bacterial genera between the different treatments compared to the control (Figures S5-7). In contrast, no protist genera were found to be significantly enriched/depleted in any of the treatments compared to the control.

3.3 Linking plant properties and microbial community changes

Due to the low impact of our treatments on the rhizosphere microbial community, we had little discriminatory power to address our fourth hypothesis. Nonetheless, we were able to identify potential relations between plant properties and associated microbial communities. The dissimilarity/distances of the bacterial community composition across treatments in relation to the control showed general positive trends with the magnitude of effects reported on the plants. The UniFrac distance related to the control on the bacterial community was positively correlated with the total plant biomass, the shoot fresh weight, Ca shoot content and root carbon content (Figure 4). The opposite trend was observed with the protistan community composition (Figure 4). The UniFrac distance related to the control on the protistan community was negatively correlated with plant total biomass, shoot biomass (fresh and dry weight), and shoot C, Al, Ca, Fe, Mg, Na, and P content.

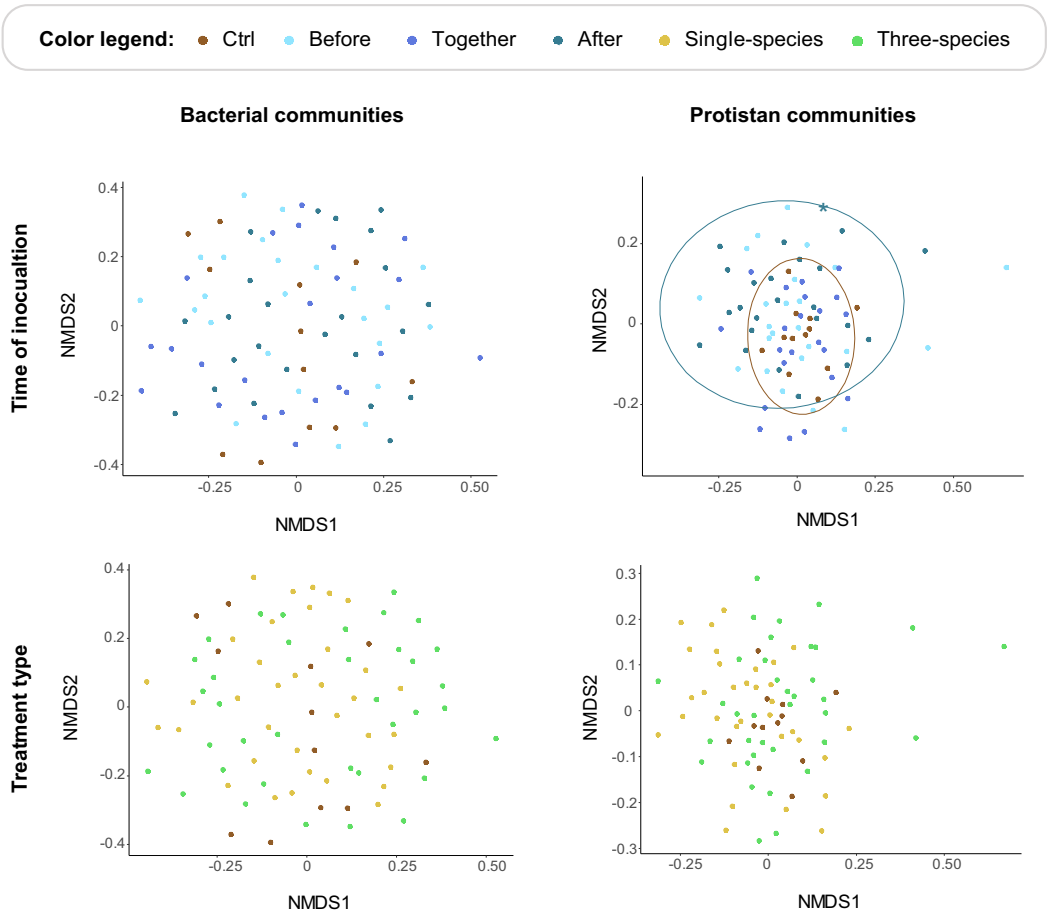

Figure 3: Effect of the treatments on the bacterial (left panel) and protistan community composition (right panel). The non-metric multidimensional scaling (NMDS) was performed using the Bray-Curtis dissimilarity. The upper panels display the effect of the time of inoculation (one week before seedling transfer, together with seedling transfer, one week after seedling transfer) on the communities and the lower panels display the effects of the treatment type (single- or mixed-species) on the communities. The only significant effect reported was observed between the protistan communities from the late inoculation time (one week after plant transfer) compared to the control; the effect is highlighted with an asterisk and by colored ellipses to help locate the points belonging to the control compared to the ones belonging to the late inoculation time.

We further found positive correlations between the relative abundances of some bacterial genera and most of the plant properties. Only N, Ca, Cu, P and S content in the shoot were not correlated with the relative abundance of any bacterial genera (Figure 5). Most correlations were found for cadmium and iron shoot content as well as for root dry weight. When investigating the potential effect of the protist treatment on these bacteria based on the log two-fold figures (Figure S5), we only found the Proteobacteria *Sphingopyxis* to be reduced in the single-protist treatment inoculated a week before plant transfer (S1, Log two fold value: -5.117). We also found eight protist genera to be positively correlated with the shoot aluminum, cadmium, copper, iron content or with the dry weight of the roots

(Figure 5). In particular, the cadmium shoot content was correlated with five protist genera. We summarized these findings and examined the plant-beneficial potential of the identified bacterial and protist genera by screening available literature (Table S11).

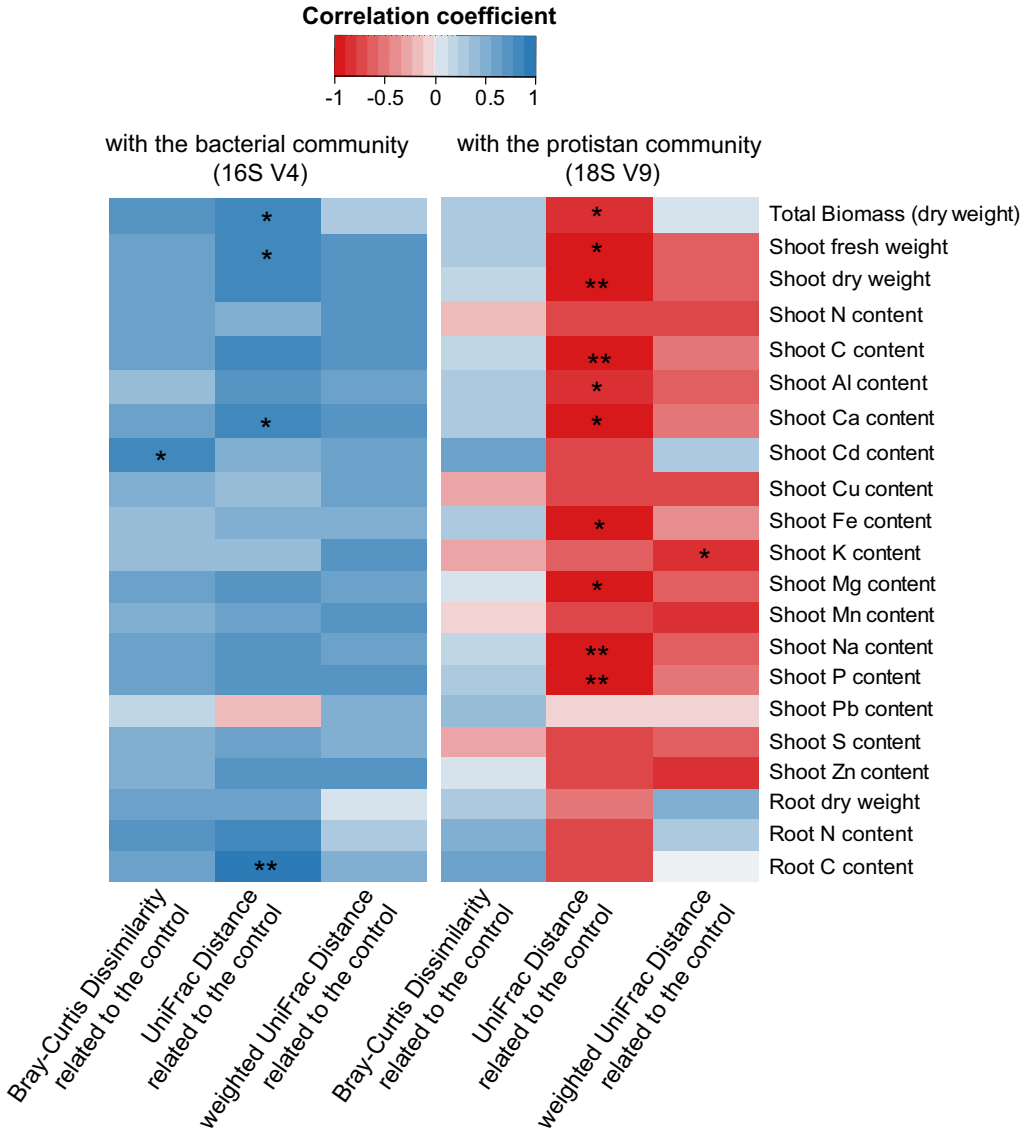

Figure 4: Relation between magnitude of changes on the microbial community composition (bacterial, left; protistan, right) and the magnitude of effect on the different plant properties measured. Asterisks indicate significant correlations with "" for $p < 0.05$ and "**" $p < 0.01$.*

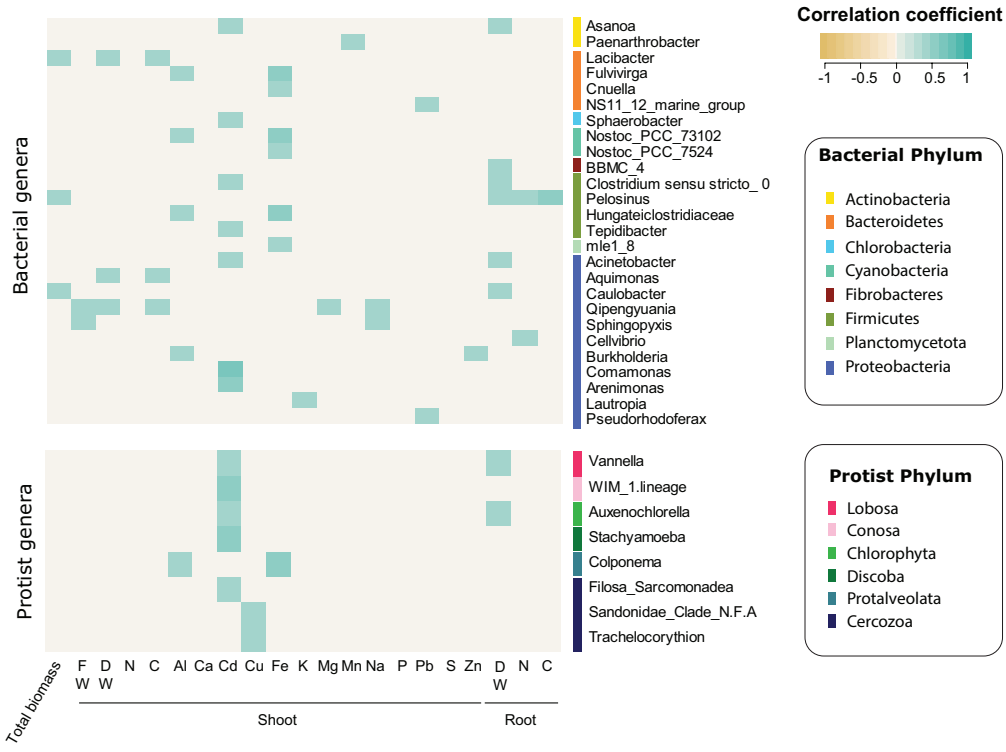

Figure 5: Correlogram between measured plant properties and relative abundance of bacterial genera (upper part) and protist genera (lower part). FW and DW stand for fresh and dry weight, respectively; the letters stand for the content of the corresponding element. Only correlations with Bonferroni corrected p values under 0.05 are given. Note that we shorten the assigned Burkholderia-Caballeronia-Paraburkholderia to Burkholderia (bacterial genera, Proteobacteria).

4. Discussion

According to our first hypothesis and in line with previous studies, we found an effect of the application of protists on the plant above ground biomass and nutrient contents (Bonkowski 2004; Gao *et al.* 2019); the observed impacts differed across treatments. In contrast, however, the inoculation of protists only had a subtle effect on the rhizosphere microbial community. While previous studies have reported clear modifications of the bacterial community upon protist inoculation (Kreuzer *et al.* 2006; Rosenberg *et al*. 2009; Bonkowski and Clarholm 2012), another recent study also observed relatively subtle effects of protist inoculation on the rhizosphere bacterial community (Asiloglu *et al.* 2020).

In line with our second hypothesis, we observed an important effect of the time of inoculation on the plant development. Early inoculation (one week before seedling transfer) led do the highest significant increase in plant fresh shoot biomass as compared to the control (1.5x), followed by the simultaneous inoculation (1.3x) and the late inoculation (no significant

difference); these plant properties were not significantly influenced by the inoculation type (single- or mixed-species). Such increases in aboveground biomass were mostly consistent with previous studies implementing before (Ekelund *et al.* 2009; Bjørnlund *et al.* 2012) or simultaneous protist inoculation with plant transfer (Kuikman *et al.* 1990; Asiloglu *et al.* 2020). Similarly to our study, such increase in the shoot biomass was usually accompanied by an increase in nutrient content (Clarholm 1985; Ekelund *et al.* 2009; Bjørnlund *et al.* 2012). Contrary to our first hypothesis, however, the time of inoculation only had very little influence on the microbial communities, with no significant effects on the bacterial community composition. We only observed some effects of the time of inoculation on the protistan community, with Lobosa being more abundant in the simultaneous inoculation compared to the early and late inoculation treatments, and the overall community composition being different in the late inoculation compared to the control. Interestingly, protistan communities have been suggested to respond more strongly to environmental changes and/or agricultural practices compared to bacterial communities (Xiong *et al.* 2017; Zhao *et al.* 2019). The time of sampling is, however, also relevant to adequately capture any impacts of protist inoculation on the microbial community. The lettuce plant itself is an important driver of the rhizosphere community composition (Schreiter *et al.* 2014), and the impacts of the predators is thus likely to get dampened over time by the increasingly dominant influence of the plant (Kröber *et al.* 2014). Early and multiple sampling time points should allow a better assessment of the predatory impact of protists (Kröber *et al.* 2014).

Contrary to our third hypothesis and previous studies (Bjørnlund *et al.* 2012; Asiloglu *et al.* 2020), we did not observe better plant performance in the three-species inoculum treatment compared to the single-species treatment. Similarly, while Asiloglu and colleagues (2020) reported a higher shoot biomass for rice in a four-species mixture treatment compared to most single-protist treatments, some single-protist treatments led to similar effects as observed for mixture. The identity of protist species in the mixture is very likely to influence the outcome (Canter *et al.* 2018). In our case, *Cercomonas* sp. S24D2 might have driven most of the effects observed on the plant in the three-species mixture. Interestingly, however, we observed a significant increase in the shoot-to-root ratio for the three-species mixture as compared to the single-protist treatment (1.8x). Shifts toward increased shoot-to-root ratio, indicating a shift of resource allocation into the shoot biomass, have been previously reported upon inoculation of two or more protist species compared to no-protist control (Kuikman and Van Veen 1989; Kuikman *et al.* 1990). Further and also contrary to our hypothesis, the three-species mixture treatments did not show a higher impact on the microbial communities. The impacts on the microbial community structure were generally relatively low in our experimental setup and we only observed a significant reduction of the bacterial alpha diversity compared to the control, without distinction between the two treatment types (single or three-species mixture). Such reduction in the bacterial alpha diversity contrasts with previous studies that indicated an increase in bacterial diversity upon protist predation (Rosenberg *et al.* 2009; Kurm *et al.* 2019). Independently of our

treatments, we further observed that the experimental conditions seemed to select against the Cercozoa, which is usually the dominant protist group in soils (Oliverio *et al.* 2020).

The modest impacts observed on microbial communities afforded rather little power to address our fourth hypothesis. However, we did observe positive correlations between the UniFrac distances relative to the control for the bacterial communities and the magnitude of effect on the plant biomass. Interestingly, the highest shoot biomass was previously observed for lettuce plants grown on soil associated with the highest rhizosphere effect (Schreiter *et al*. 2014). In contrast, the UniFrac distance on the protist community composition relative to the control was negatively correlated with the magnitude of effect on plant properties including total and shoot biomass, and shoot content of some elements (C, Al, Ca, Fe, Mg, Na and P). This suggests that, while modifications of the bacterial community composition might be beneficial for the plant, a stable community composition of the dominant protists might also have positive effects. Especially the community of predatory protists has been observed to be an important explanatory factor linked to plant health (Xiong *et al.* 2020). We further identified bacterial and protist genera that were correlated to specific plant properties. Of the bacterial genera identified, some are known to include plant beneficial microbes, such as *Caulobacter* spp. (Berrios 2022) or *Burkholderia* spp. (Parke and Gurian-Sherman 2001), but the potential plant-beneficial roles of others is much less likely (Table S11). We also observed a surprising positive correlation between five protist genera and the shoot content of cadmium, which can be toxic for plants if concentrations are above a certain threshold (Ismael *et al.* 2019). While protists have been reported to participate to various biogeochemical cycles for elements including carbon, nitrogen, phosphorous, magnesium, calcium and silica (Gao *et al.* 2019), to our knowledge no relation was found in relation to cadmium. Note that the above analyses attempting to link protist-induced modification of the microbial community structure to plant properties are exploratory, and any observed trends need to be further explored via follow up experiments targeting these microbial groups.

5. Conclusion

The application of protists was most successful when applied before plant transfer, and single-species inoculation performed as well as inoculation with a three-species protist mixture. The application of protists further did not have major effects on the rhizosphere microbial community, suggesting a relatively subtle impact of predation; If confirmed, this result is promising for safe application of protist inoculants without long-term, potentially undesirable effects on the microbial communities (Mawarda *et al.* 2020; Jack *et al.* 2021). Our results also suggest that the stability and composition of the rhizosphere protistan community might be an important component for the plant development. Our results should help steer future protist amendment strategies to improve effectiveness and application, while motivating further consideration of the role of protists in plant development.

6. Acknowledgements

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7. Conflicts of 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.

Table S1: Description of the protist isolates used in the present study. Taxonomic assignment is as described in Gao (2020). The density (active and cyst) of each protist is given for the different time point of application, as well as the number of E. coli OP50 transferred alongside the protists (given as CFUs).

				One week before plant transfer			Simultaneously with plant transfer			One week after plant transfer		
	Code	**Taxonomic assignment**	**Morpho-type**	**Active mL^{-1}**	**Cyst mL^{-1}**	**CFUs mL^{-1}**	**Active mL^{-1}**	**Cyst mL^{-1}**	**CFUs mL^{-1}**	**Active mL^{-1}**	**Cyst mL^{-1}**	**CFUs mL^{-1}**
Single protist	S24D2	*Cercomonas* sp.	Flagellate	1.08×10^4	2.80×10^3	1.83×10^7	1.19×10^4	1.13×10^2	1.02×10^6	1.31×10^4	2.49×10^2	6.00×10^5
	S24D2	*Cercomonas* sp.	Flagellate	9.13×10^3	1.03×10^4	1.50×10^7	8.59×10^3	1.13×10^2	1.07×10^6	7.41×10^3	1.36×10^2	3.67×10^5
Mixture of protists	S18D10	Heterolobosea sp.	Amoeboid	1.15×10^3	3.82×10^3	2.83×10^7	2.90×10^3	4.07×10^3	2.03×10^6	3.75×10^3	9.94×10^2	2.00×10^4
	C13D2	*Acanthamoeba* sp.	Amoeboid	2.03×10^3	3.69×10^3	6.33×10^6	1.12×10^3	3.39×10^3	2.00×10^6	2.67×10^3	3.30×10^3	9.17×10^5

Table S2: Characteristics of the sandy soil mixture obtained from SoilTech. The analysis was performed in August 2019 by Eurofins Agro Testing Wageningen BV (Wageningen, The Netherlands).

	mS cm^{-1} at 25°C	**mmol L^{-1}**			
pH	**EC**	**Si**			
5.3	0.5	0.08			
Cations mmol L^{-1}					
NH4	**K**	**Na**	**Ca**	**Mg**	
<0.1	0.2	0.2	1.3	0.3	
Anions mmol L^{-1}					
NO_3	**Cl**	**S**	**HCO_3**	**P**	
2.4	0.2	10.3	< 0.1	< 0.03	
Trace elements µmol L^{-1}					
Fe	**Mn**	**Zn**	**B**	**Cu**	**Mo**
<0.5	13	1.8	2.8	0.4	<0.1

Table S3 Disease incidence observed at the time of harvest

Treatment	**Diseased plant/total**
Control	7/12
Single protist, inoculation time 1 (S1)	11/12
Mixt protist, inoculation time 1 (M1)	12/12
Single protist, inoculation time 2 (S2)	11/12
Mixt protist, inoculation time 2 (M2)	9/12
Single protist, inoculation time 3 (S3)	10/12
Mixt protist, inoculation time 3 (M3)	11/12

Table S4: Effect of the treatments on plant properties including the main effects of the inoculation time (4 levels: control, before, simultaneously, after), the treatment type (3 levels: control, single and mixture) and the interaction between both. Note that the degree of freedom of the F-statistics do not correspond to the theoretical values (Inoculation time: k-1=3; Treatment type: k-1=2; n-1=83; with n the number samples and k the number of levels per factor); this is due to the loss of some samples, the use of the same control for both parameters and the ability of the model to use less degree of freedom. The p-values under 0.05 are highlighted in bold.

	Inoculation time		**Treatment type**		**Interaction**	
Plant trait	**F-statistic**	***p* value**	**F-statistic**	***p* value**	**F-statistic**	***p* value**
Fresh shoot weight	$F_{(2,77)}$= 5.458	**0.006**	$F_{(1,77)}$=0.511	0.477	$F_{(2,77)}$=0.607	0.548
Dry shoot weight	$F_{(2,77)}$=5.066	**0.009**	$F_{(1,77)}$=0.399	0.53	$F_{(2,77)}$=0.559	0.574
Dry root weight	$F_{(2,75)}$=0.925	0.401	$F_{(1,75)}$=1.146	0.288	$F_{(2,75)}$=2.511	0.088
Total Biomass	$F_{(2,75)}$=2.667	0.076	$F_{(1,75)}$=0.291	0.591	$F_{(2,75)}$=1.685	0.192
Shoot:Root ratio	$F_{(2,75)}$=0.853	0.43	$F_{(1,75)}$=7.170	0.009	$F_{(2,75)}$=2.086	0.131
Shoot C	$F_{(2,77)}$=4.530	**0.013**	$F_{(1,77)}$=0.374	0.543	$F_{(2,77)}$=0.427	0.654
Shoot N	$F_{(2,77)}$=1.56	0.217	$F_{(1,77)}$=0.441	0.509	$F_{(2,77)}$=1.926	0.153
Shoot C:N	$F_{(2,77)}$=9.852	**< 0.001**	$F_{(1,77)}$=0.106	0.745	$F_{(2,77)}$=1.959	0.148
Root C	$F_{(2,75)}$=3.152	**0.048**	$F_{(1,75)}$=0.357	0.552	$F_{(2,75)}$=0.567	0.569
Root N	$F_{(2,75)}$=1.872	0.161	$F_{(1,75)}$=0.443	0.508	$F_{(2,75)}$=1.574	0.214
Root C:N	$F_{(2,75)}$=2.278	0.11	$F_{(1,75)}$=0.774	0.382	$F_{(2,75)}$=3.803	**0.027**
Shoot Al	$F_{(2,77)}$=4.317	**0.017**	$F_{(1,77)}$=0.688	0.409	$F_{(2,77)}$=0.132	0.876
Shoot Ca	$F_{(2,77)}$=10.717	**< 0.001**	$F_{(1,77)}$=0.478	0.492	$F_{(2,77)}$=0.833	0.439
Shoot Cd	$F_{(2,77)}$=0.315	0.731	$F_{(1,77)}$=0.595	0.443	$F_{(2,77)}$=0.321	0.726
Shoot Cu	$F_{(2,77)}$=2.722	0.072	$F_{(1,77)}$=1.604	0.209	$F_{(2,77)}$=2.165	0.122
Shoot Fe	$F_{(2,77)}$=2.497	0.089	$F_{(1,77)}$=3.055	0.084	$F_{(2,77)}$=0.146	0.864
Shoot K	$F_{(2,77)}$=1.264	0.288	$F_{(1,77)}$=1.404	0.24	$F_{(2,77)}$=2.072	0.133
Shoot Mg	$F_{(2,77)}$=5.6	**0.005**	$F_{(1,77)}$=0.199	0.657	$F_{(2,77)}$=1.192	0.309
Shoot Mn	$F_{(2,77)}$=4.322	**0.017**	$F_{(1,77)}$=0.671	0.415	$F_{(2,77)}$=1.924	0.153
Shoot Na	$F_{(2,77)}$=3.700	**0.029**	$F_{(1,77)}$=0.501	0.481	$F_{(2,77)}$=0.377	0.687
Shoot P	$F_{(2,77)}$=6.761	**0.002**	$F_{(1,77)}$=2.587	0.112	$F_{(2,77)}$=0.755	0.474
Shoot Pb	$F_{(2,77)}$=0.188	0.829	$F_{(1,77)}$=2.985	0.088	$F_{(2,77)}$=0.043	0.958
Shoot S	$F_{(2,77)}$=3.418	**0.038**	$F_{(1,77)}$=0.005	0.943	$F_{(2,77)}$=2.138	0.125
Shoot Zn	$F_{(2,77)}$=6.944	**0.002**	$F_{(1,77)}$=0.706	0.403	$F_{(2,77)}$=2.923	**0.06**

Table S5: Results of the post hoc Tukey HSD for the significant main effects of either the temporal treatment (before, simultaneous, after) or the treatment type (single species, three-species mixture). The p-values under 0.05 are highlighted in bold.

Plant trait	Pairwise comparison	diff	lwr	upr	*p* adj
Fresh shoot weight	Before-Ctrl	4.810	2.197	7.424	**<0.001**
	Simultaneous-Ctrl	2.608	-0.006	5.221	0.051
	After-Ctrl	2.344	-0.269	4.958	0.095
	Simultaneous-Before	-2.203	-4.337	-0.069	**0.040**
	After-Before	-2.466	-4.600	-0.332	**0.017**
	After-Simultaneous	-0.263	-2.397	1.871	0.988
Dry shoot weight	Before-Ctrl	0.309	0.137	0.482	**<0.001**
	Simultaneous-Ctrl	0.178	0.006	0.351	**0.040**
	After-Ctrl	0.146	-0.026	0.319	0.125
	Simultaneous-Before	-0.131	-0.272	0.010	0.078
	After-Before	-0.163	-0.304	-0.022	**0.017**
	After-Simultaneous	-0.032	-0.173	0.109	0.934
Shoot : Root ratio	Single-Ctrl	-1.280	-4.402	1.842	0.592
	Mixt-Ctrl	1.225	-1.874	4.324	0.614
	Mixt-Single	2.505	0.282	4.728	**0.023**
Shoot Carbon content	Before-Ctrl	129.131	55.365	202.896	**<0.001**
	Simultaneous-Ctrl	75.548	1.782	149.313	**0.043**
	After-Ctrl	63.503	-10.263	137.269	0.117
	Simultaneous-Before	-53.583	-113.812	6.646	0.099
	After-Before	-65.628	-125.857	-5.398	**0.027**
	After-Simultaneous	-12.045	-72.274	48.185	0.953
Shoot C:N	Before-Ctrl	1.790	0.883	2.697	**<0.001**
	Simultaneous-Ctrl	0.691	-0.217	1.598	0.198
	After-Ctrl	0.734	-0.173	1.641	0.155
	Simultaneous-Before	-1.099	-1.840	-0.359	**0.001**
	After-Before	-1.056	-1.797	-0.315	**0.002**
	After-Simultaneous	0.043	-0.698	0.784	0.999
Shoot Al	Before-Ctrl	0.029	-0.001	0.059	0.066
	Simultaneous-Ctrl	0.005	-0.025	0.036	0.966
	After-Ctrl	0.004	-0.026	0.034	0.986
	Simultaneous-Before	-0.024	-0.048	0.001	0.067
	After-Before	-0.025	-0.050	0.000	**0.047**
	After-Simultaneous	-0.001	-0.026	0.023	0.999

Plant trait	Pairwise comparison	diff	lwr	upr	*p* adj
Shoot Ca	Before-Ctrl	9.036	5.219	12.854	**<0.001**
	Simultaneous-Ctrl	4.460	0.642	8.278	**0.015**
	After-Ctrl	4.060	0.242	7.878	**0.033**
	Simultaneous-Before	-4.576	-7.694	-1.459	**0.001**
	After-Before	-4.976	-8.094	-1.859	**<0.001**
	After-Simultaneous	-0.400	-3.517	2.717	0.987
Shoot Mg	Before-Ctrl	4.004	1.538	6.471	**<0.001**
	Simultaneous-Ctrl	2.199	-0.268	4.665	0.098
	After-Ctrl	1.511	-0.955	3.978	0.380
	Simultaneous-Before	-1.806	-3.819	0.208	0.095
	After-Before	-2.493	-4.507	-0.479	**0.009**
	After-Simultaneous	-0.687	-2.701	1.326	0.807
Shoot Mn	Before-Ctrl	0.045	0.020	0.071	**<0.001**
	Simultaneous-Ctrl	0.030	0.005	0.055	**0.013**
	After-Ctrl	0.023	-0.003	0.048	0.094
	Simultaneous-Before	-0.015	-0.036	0.006	0.226
	After-Before	-0.022	-0.043	-0.002	**0.027**
	After-Simultaneous	-0.007	-0.028	0.013	0.788
Shoot Na	Before-Ctrl	4.641	1.745	7.536	**<0.001**
	Simultaneous-Ctrl	2.975	0.079	5.870	**0.042**
	After-Ctrl	2.218	-0.677	5.114	0.193
	Simultaneous-Before	-1.666	-4.031	0.698	0.258
	After-Before	-2.422	-4.787	-0.058	**0.043**
	After-Simultaneous	-0.756	-3.121	1.608	0.836
Shoot P	Before-Ctrl	3.740	1.612	5.869	**<0.001**
	Simultaneous-Ctrl	1.769	-0.359	3.897	0.137
	After-Ctrl	1.541	-0.588	3.669	0.237
	Simultaneous-Before	-1.971	-3.709	-0.233	**0.020**
	After-Before	-2.200	-3.938	-0.462	**0.007**
	After-Simultaneous	-0.228	-1.966	1.509	0.986
Shoot S	Before-Ctrl	0.160	0.053	0.267	**0.001**
	Simultaneous-Ctrl	0.117	0.010	0.223	**0.026**
	After-Ctrl	0.074	-0.033	0.181	0.272
	Simultaneous-Before	-0.043	-0.130	0.044	0.562
	After-Before	-0.086	-0.173	0.001	0.054
	After-Simultaneous	-0.043	-0.130	0.044	0.574
Shoot Zn	Before-Ctrl	0.033	0.017	0.050	**<0.001**
	Simultaneous-Ctrl	0.017	0.001	0.034	**0.034**
	After-Ctrl	0.017	0	0.033	**0.042**
	Simultaneous-Before	-0.016	-0.029	-0.002	**0.014**
	After-Before	-0.016	-0.030	-0.003	**0.010**
	After-Simultaneous	-0.001	-0.014	0.013	1.000

Table S6: Effect of the treatments on number of taxa (ASVs) for the 10 most abundant bacterial and protistan phyla. Note that the degree of freedom of the F-statistics do not correspond to the theoretical values (Inoculation time: k-1=3; Treatment type: k-1=2; n-1=83; with n the number samples (84) and k the number of levels per factor); this is due to the loss of some samples, the use of the same control for both parameter and the ability of the model to use less degree of freedom. The p-values under 0.05 are highlighted in bold.

	Inoculation time		**Treatment type**		**Interaction**	
Bacterial phylum	**F-statistic**	***p***	**F-statistic**	***p***	**F-statistic**	***p***
Proteobacteria	$F_{(2,76)}$= 1.068	0.349	$F_{(1,76)}$=0.151	0.698	$F_{(2,76)}$=0.112	0.894
Actinobacteriota	$F_{(2,76)}$= 1.032	0.361	$F_{(1,76)}$=0.493	0.485	$F_{(2,76)}$=2.296	0.108
Chloroflexi	$F_{(2,76)}$= 0.051	0.951	$F_{(1,76)}$=0.924	0.34	$F_{(2,76)}$=0.343	0.711
Planctomycetota	$F_{(2,76)}$= 0.238	0.789	$F_{(1,76)}$=0.632	0.429	$F_{(2,76)}$=0.267	0.766
Acidobacteriota	$F_{(2,76)}$= 0.4	0.672	$F_{(1,76)}$=0.688	0.41	$F_{(2,76)}$=0.591	0.556
Gemmatimonadota	$F_{(2,76)}$= 0.236	0.791	$F_{(1,76)}$=2.131	0.149	$F_{(2,76)}$=0.371	0.691
Firmicutes	$F_{(2,76)}$= 0.179	0.837	$F_{(1,76)}$=0.281	0.598	$F_{(2,76)}$=0.39	0.678
Cyanobacteria	$F_{(2,76)}$= 0.656	0.522	$F_{(1,76)}$=0.569	0.453	$F_{(2,76)}$=1.562	0.216
Bacteroidota	$F_{(2,76)}$= 0.599	0.552	$F_{(1,76)}$=1.808	0.183	$F_{(2,76)}$=0.039	0.962
Patescibacteria	$F_{(2,76)}$= 0.777	0.463	$F_{(1,76)}$=0.42	0.519	$F_{(2,76)}$=1.005	0.371
Protistan phylum						
Chlorophyta	$F_{(2,74)}$= 1.065	0.35	$F_{(1,74)}$=0.19	0.665	$F_{(2,74)}$=0.515	0.6
Ciliophora	$F_{(2,74)}$= 0.956	0.389	$F_{(1,74)}$=2.511	0.117	$F_{(2,74)}$=0.727	0.487
Cercozoa	$F_{(2,74)}$= 7.391	0.255	$F_{(1,74)}$=0.22	0.641	$F_{(2,74)}$=0.47	0.627
Ochrophyta	$F_{(2,74)}$= 0.0001	0.999	$F_{(1,74)}$=0.073	0.788	$F_{(2,74)}$=0.541	0.585
Lobosa	$F_{(2,74)}$= 5.479	**0.006**	$F_{(1,74)}$=1.063	0.306	$F_{(2,74)}$=2.556	0.123
Conosa	$F_{(2,74)}$= 0.733	0.484	$F_{(1,74)}$=1.308	0.256	$F_{(2,74)}$=0.466	0.63
Apicomplexa	$F_{(2,74)}$= 0.262	0.77	$F_{(1,74)}$=0.074	0.786	$F_{(2,74)}$=0.224	0.8
Pseudofungi	$F_{(2,74)}$= 0.33	0.72	$F_{(1,74)}$=2.451	0.122	$F_{(2,74)}$=1.834	0.257
Centroheliozoa	$F_{(2,74)}$= 0.048	0.953	$F_{(1,74)}$<0.001	0.983	$F_{(2,74)}$=1.126	0.33
Discoba	$F_{(2,74)}$= 2.595	0.081	$F_{(1,74)}$<0.001	0.993	$F_{(2,74)}$=1.037	0.359

Table S7: Results of the post hoc Tukey HSD for the significant main effects of the temporal treatment (before, simultaneous, after) on the protist phylum Lobosa. The p-values under 0.05 are highlighted in bold.

Pairwise comparison	**diff**	**lwr**	**upr**	***p* adj**
Before-Ctrl	-0.013	-0.044	0.018	0.669
Simultaneous-Ctrl	0.014	-0.017	0.046	0.624
After-Ctrl	-0.015	-0.046	0.017	0.617
Simultaneous - Before	0.028	0.002	0.054	**0.030**
After-Before	-0.001	-0.027	0.025	0.999
After-Simultaneous	-0.029	-0.055	-0.003	**0.024**

Table S8: Effect of the treatments on the microbial community including the main effects of the inoculation time, the treatment type (single and mixture) and the interaction between both. Note that the degree of freedom of the F-statistics do not correspond to the theoretical values (Inoculation time: k-1=3; Treatment type: k-1=2; n-1=83; with n the number samples and k the number of levels per factor); this is due to the loss of some samples, the use of the same control for both parameter and the ability of the model to use less degree of freedom. The p-values under 0.05 are highlighted in bold. 16S stands for 16S rRNA gene amplicon sequencing and 18S stands for the 18S rRNA gene amplicon sequencing.

		Inoculation time		**Treatment type**		**Interaction**	
	Microbiome	**F-statistic**	***p***	**F-statistic**	***p***	**F-statistic**	***p***
Bacterial community	16S observed richness	$F_{(2,81)}$ = 0.504	0.606	$F_{(1,81)}$ = 4.152	**0.045**	$F_{(2,81)}$ = 1.479	0.234
	16S Chao1 index	$F_{(2,81)}$ = 0.523	0.595	$F_{(1,81)}$ = 4.646	**0.034**	$F_{(2,81)}$ = 1.328	0.271
	16S Shannon	$F_{(2,81)}$ = 0.112	0.894	$F_{(1,81)}$ = 4.185	**0.044**	$F_{(2,81)}$ = 2.14	0.125
	16S Pielou's index	$F_{(2,81)}$ = 0.448	0.641	$F_{(1,81)}$ = 1.264	0.265	$F_{(2,81)}$ = 1.482	0.234
	16S Bray-Curtis dissimilarity	$F_{(3,81)}$ = 1.037	0.231	$F_{(1,81)}$ = 0.983	0.559	$F_{(2,81)}$ = 0.993	0.574
	16S UniFrac Distance	$F_{(3,81)}$ = 1.012	0.3	$F_{(1,81)}$ = 0.929	0.969	$F_{(2,81)}$ = 1.044	0.072
	16S weigthed UniFrac Distance	$F_{(3,81)}$ = 0.214	0.084	$F_{(1,81)}$ = 1.066	0.323	$F_{(2,81)}$ =1.144	0.191

(the table continues in the next page)

	Microbiome	F-statistic	*p*	F-statistic	*p*	F-statistic	*p*
Protistan community	18S observed richness	$F_{(2,80)}$ = 0.281	0.756	$F_{(1,80)}$ = 0.541	0.465	$F_{(2,80)}$ = 0.207	0.814
	18S Chao1 index	$F_{(2,80)}$ = 0.282	0.755	$F_{(1,80)}$ = 0.437	0.511	$F_{(2,80)}$ = 0.195	0.823
	18S Shannon	$F_{(2,80)}$ = 2.152	0.123	$F_{(1,80)}$ = 2.728	0.103	$F_{(2,80)}$ = 0.22	0.803
	18S Pielou's index	$F_{(2,80)}$ = 1.689	0.192	$F_{(1,80)}$ = 0.769	0.383	$F_{(2,80)}$ = 0.447	0.641
	18S Bray-Curtis dissimilarity	$F_{(3,80)}$ = 1.014	**0.01**	$F_{(1,80)}$ = 0.996	0.669	$F_{(2,80)}$ = 1.005	0.149
	18S UniFrac Distance	$F_{(3,80)}$ = 0.991	0.562	$F_{(1,80)}$ = 0.917	0.928	$F_{(2,80)}$ = 0.024	0.818
	18S weigthed UniFrac Distance	$F_{(3,80)}$ = 1.176	0.188	$F_{(1,80)}$ = 0.844	0.67	$F_{(2,80)}$ = 1.007	0.469

Table S9: Results of the post hoc Tukey HSD for the significant main effects of either the treatment type (single species, three-species mixture) on the bacterial alpha diversity.

	Pairwise comparison	diff	lwr	upr	*p* adj
16S observed richness	Single-Ctrl	-13.807	-61.532	33.918	0.769
	Mixture-Ctrl	-42.500	-90.056	5.056	0.089
	Mixture-Single	-28.693	-62.559	5.173	0.113
16S Chao1 index	Single-Ctrl	-12.192	-61.055	36.672	0.823
	Mixture-Ctrl	-43.325	-92.015	5.364	0.091
	Mixture-Single	-31.134	-65.808	3.540	0.087
16S Shannon	Single-Ctrl	-0.005	-0.162	0.151	0.996
	Mixture-Ctrl	-0.100	-0.256	0.056	0.280
	Mixture-Single	-0.095	-0.206	0.016	0.109

Table S10: Results of the post hoc pairwise PERMANOVA analysis for the significant main effects of the temporal treatment (before, simultaneous, after) on the protistan beta diversity (Bray-Curtis dissimilarity).

Pairwise comparison	F-statistic	*p* value	BH *p* value
Before-Ctrl	$F_{(1,34)}$=0.011	0.064	NA
Simultaenous-Ctrl	$F_{(1,32)}$=0.994	0.651	NA
After-Ctrl	$F_{(1,33)}$=1.025	0.004	0.024
Simultaneous-Before	$F_{(1,44)}$=1.021	0.03	0.18
After-Before	$F_{(1,45)}$=1.005	0.121	NA
After-Simultaneous	$F_{(1,43)}$=1.024	0.029	0.174

Table S11: Description and/or putative functions of the bacterial and protist genera found to be correlated with plant properties of Lactuca sativa in our greenhouse experiment. Non-exhaustive literature research (the table continues on the two next pages

Phylum	Genus	Plant property	Ref	Comment
Actinobacteriota	Asanoa	Cd Shoot; Dry weight root	(Niemhom *et al.* 2016)	present in bulk and rhizosphere soil
	Paenarthrobacter	Mn Shoot	(Busse 2016; Riva *et al.* 2021)	potential plant beneficial activity under drought -> auxin production and degradation of ACC
Bacteroidota	Lacibacter	Total Biomass; dry weight shoot; C shoot;	(Yim *et al.* 2020)	positive correlation between lacibater and root and shoot dry weight
	Fulvivirga	Al Shoot; Fe Shoot	(Jung *et al.* 2016)	
	Cnuella	Fe Shoot	(Zhao *et al.* 2014)	
	NS11_12_marine_group	Pb Shoot	(Coclet *et al.*. 2019; Urban *et al.* 2021)	mainly composed of uncultured bacteria, mainly in marine habitats; without clear ecological implications; Correlated with Pb and Cu
Chloroflexi	Sphaerobacter	Cd Shoot	(Hugenholtz and Stackebrandt 2004)	
Cyanobacteria	Nostoc_PCC_73102	Al Shoot; Fe Shoot	(Rai, Söderbäck and Bergman 2000; Tsai *et al.* 2012)	N2 -fixing cyanobacteria, plant-symbiosis; N autotrophy; Nostoc has the widest host range from algae to angiosperm; iron in cytochrome
	Nostoc_PCC_7524	Fe Shoot		
Fibrobacteriota	BBMC_4	Dry weight root	NA	
Firmicutes	Clostridium_sensu_stricto_10	Cd Shoot; Dry weight root	(Gupta and Gao 2009)	cluster I includes many important human and animal pathogens
	Pelosinus	Total Biomass; dry weight root; N root; C root	(Shelobolina *et al.* 2007)	new genus and species,
	Hungateiclostridiaceae	Al Shoot; Fe Shoot	(Zhang *et al.* 2018)	new family; Obligately anaerobic chemoheterotrophs. Most type strains can utilize cellulose as sole carbon and energy source for growth.
	Tepidibacter	Cd Shoot	(Slobodkin 2015)	isolated from deep-sea hydrothermal vents.

Phylum	Genus	Plant property	Ref	Comment
Plancto-mycetota	mle1_8	Fe Shoot	NA	
Proteobacteria	Acinetobacter	Cd Shoot; Dry weight root	(Brady, Jamal and Perbin 2021)	found in soil and water, known to colonize intravenous fluids; low virulence, however but capable of causing infection in humans
	Aquimonas	dry weight shoot; C Shoot	(Saha *et al.* 2005)	new genus ; s isolated from a warm spring (India)
	Caulobacter	Total biomass; dry weight root	(Berrios 2022)	plant-growth promoting
	Qipengyuania	Fresh weight shoot; dry weight shoot; C shoot; Mg Shoot; Na Shoot;	(Liu *et al.* 2021)	
	Sphingopyxis	fresh weight shoot; Na shoot	(Sharma *et al.* 2021)	ability to survive under extreme environments; potential to degrade number of xenobiotics and other environmental contaminants
	Cellvibrio	N root	(Gardner and Keating 2012)	microbial plant cell wall degradation
	Burkholderia_Caballeronia. Paraburkholderia	Al shoot; Zn shoot	(Parke and Gurian-Sherman 2001; Depoorter *et al.* 2021)	antimicrobial activity ; siderophores; biological control and plant growth promoting; some cause disease in plant too
	Comamonas	Cd Shoot	(Wauters *et al.* 2003; Liu *et al.* 2020)	can be human pathogen
	Arenimonas	Cd shoot	(Yuan *et al.* 2014)	
	Lautropia	K shoot	(Gerner-Smidt *et al.* 1994)	members can be found in human oral cavities
	Pseudorhodoferax	Pb Shoot	(Bruland *et al.* 2009)	new genus in 2009; isolated from soil

Phylum	Genus	Plant property	Ref	Comment
Protists				
Lobosa	Vannella	Cd Shoot; dry weight root	(Maciver, De Obeso Fernandez Del Valle and Koutsogiannis 2017)	predator
Conosa	WIM_1.lineage_X	Cd Shoot	NA	
Chlorophyta	Auxenochlorella	Cd Shoot; dry weight root	https://www.algaebase.org/search/genus/detail/?genus_id=45154	autotroph
Discoba	Stachyamoeba	Cd Shoot	https://www.arcella.nl/stachy-amoeba-lipophora/	
Protalveolata	Colponema	Al Shoot; Fe Shoot	(Tikhonenkov *et al.* 2014)	obligate eukaryovore
	Filosa_Sarcomonadea_XXX	Cd Shoot	(Lupatini *et al.* 2019; Chen *et al.* 2021)	Abundant in (maize) soil
Cercozoa	Sandonidae_Clade_N.F.A	Cu Shoot	(Howe *et al.* 2011)	
	Trachelocorythion	Cu Shoot	https://www.arcella.nl/tra-chelocorythion (Chatelain *et al.* 2013)	shell-bearing

Supplementary figures

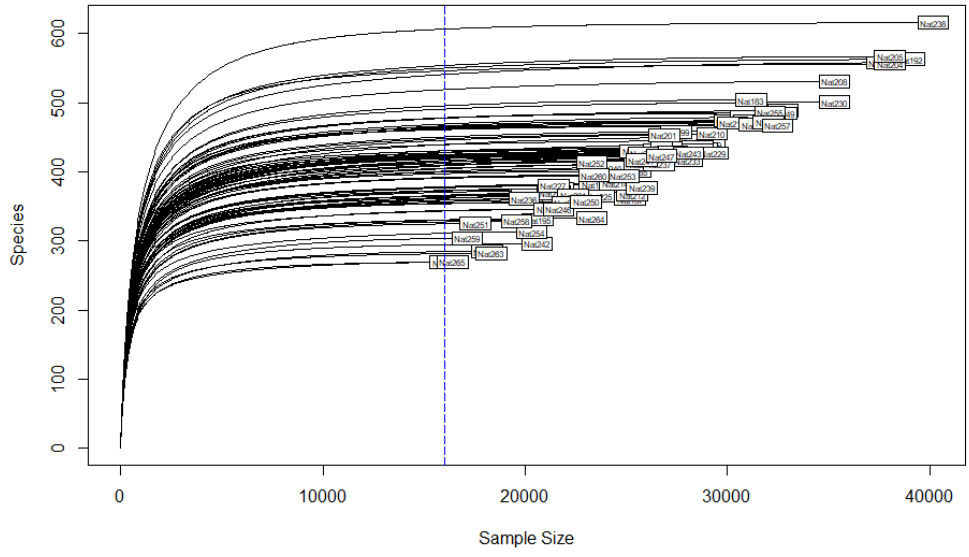

Figure S1: Collector's curve for the 16S amplicon sequencing; rarefaction was done at 16,000 reads.

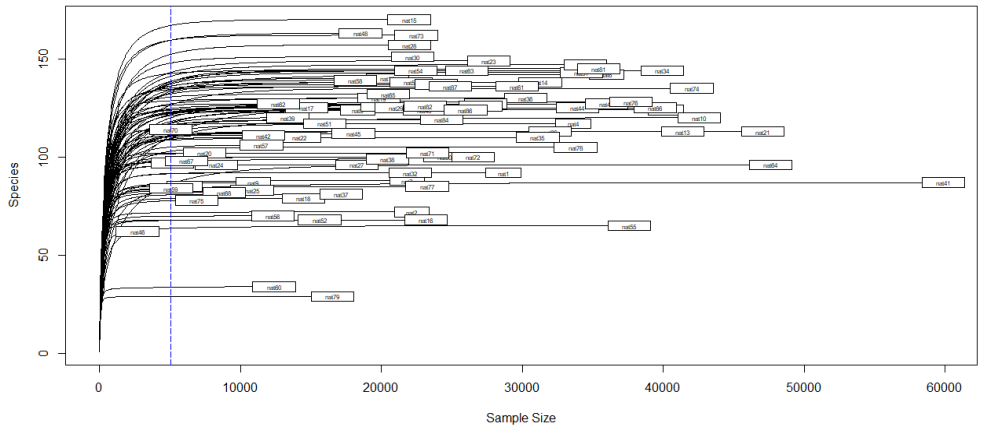

Figure S2: Collector's curve for the 18S amplicon sequencing; rarefaction was done at 5,060 reads and one sample (S3, replicate 12) was removed.

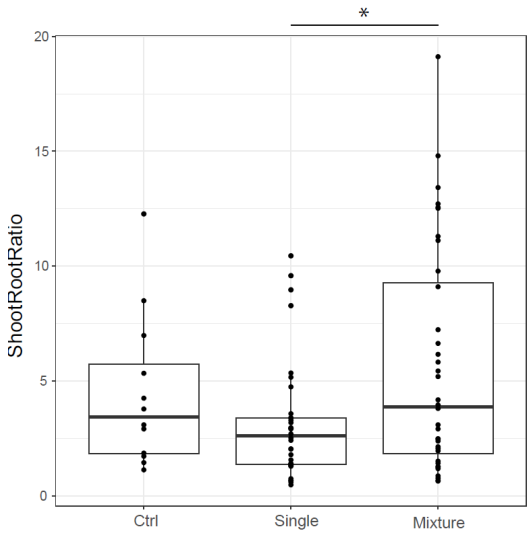

Figure S3: Effect of the treatment type on the shoot to root ratio. Note that the inoculation time had no significant effect on the shoot to root ratio, therefore, all three inoculation times for the single-species and mixed-species, respectively, are given without distinction. The asterisk indicates the significant difference ($p < 0.05$) between the single and three-species mixture treatments.

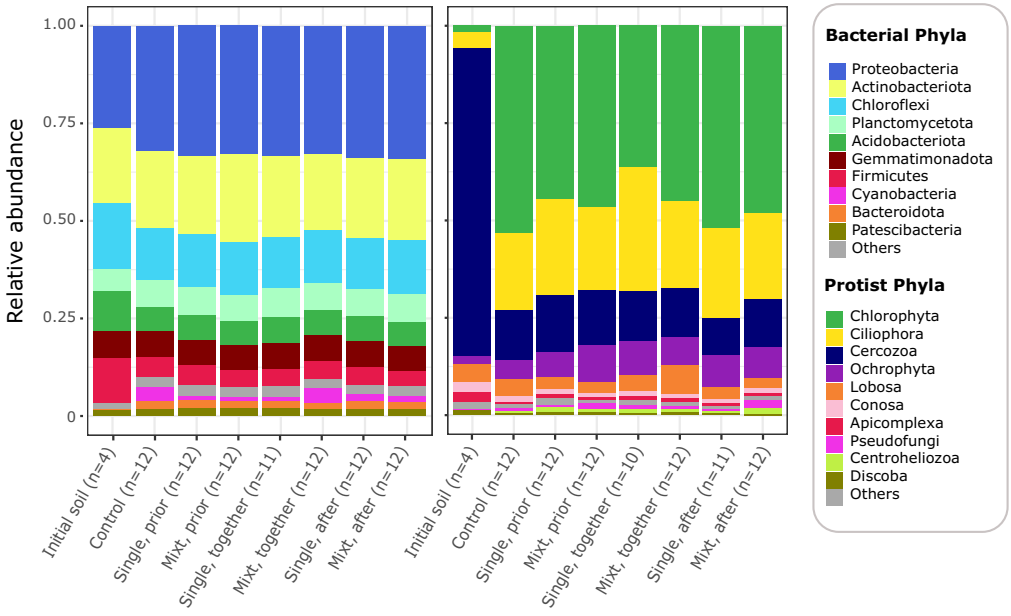

Figure S4: Effect of the protist inoculation on the bacterial (left panel) and protistan community composition (right panel) with single-species and three species mixture inoculation and prior, together with, or after seedling transfer transfer. The number in parenthesis indicates the number of replicates (n).

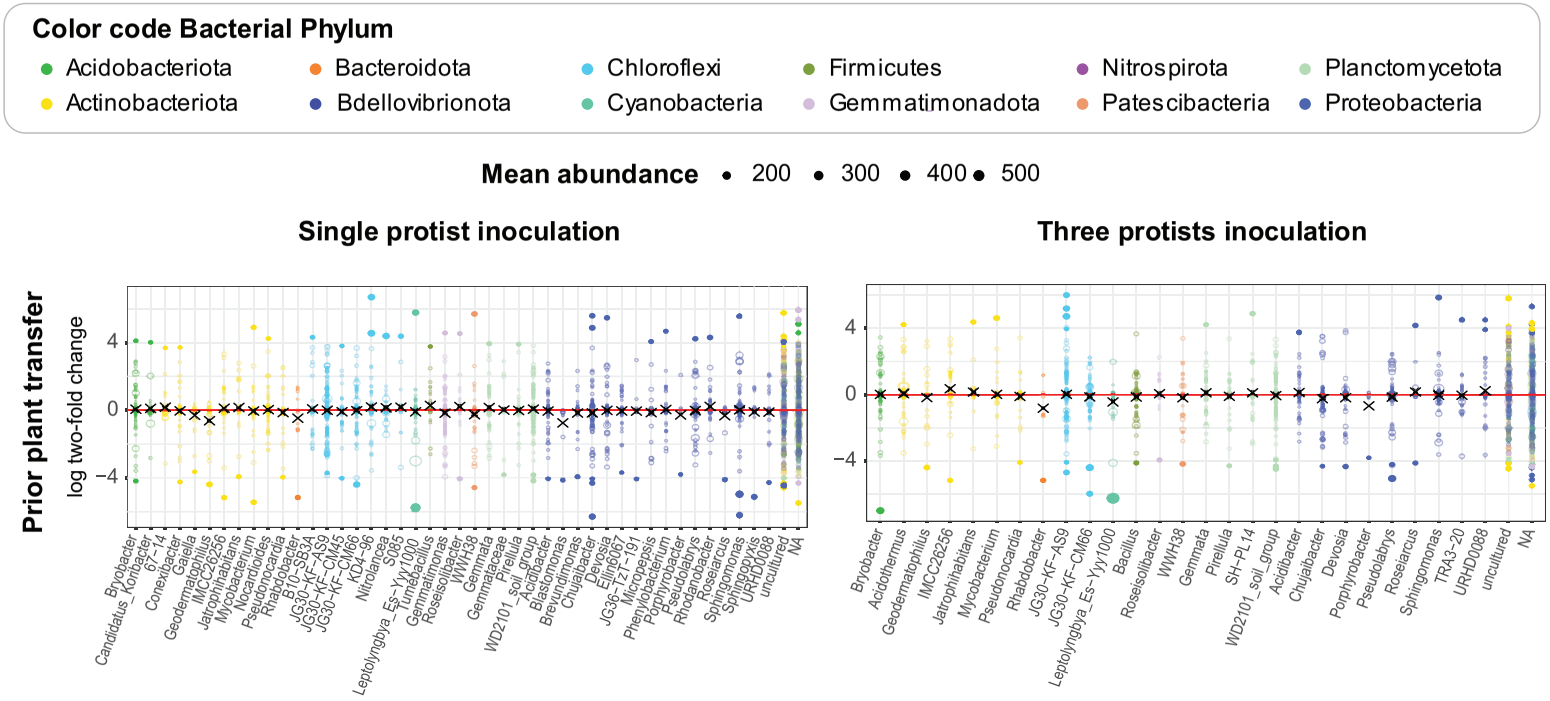

Figure S5: Impact of the protist inoculations applied one week before seedling transfer on bacterial ASVs. Dots above the red line represent bacterial ASVs that were significantly more abundant in the treatment compared to the control, dots below the red line represent bacterial ASVs that were significantly less abundant in the treatment compared to the control. Open circles are the ASVs showing no significant pattern. Crosses indicate the log two-fold average for each given genus, including all non-significantly modified ASVs.

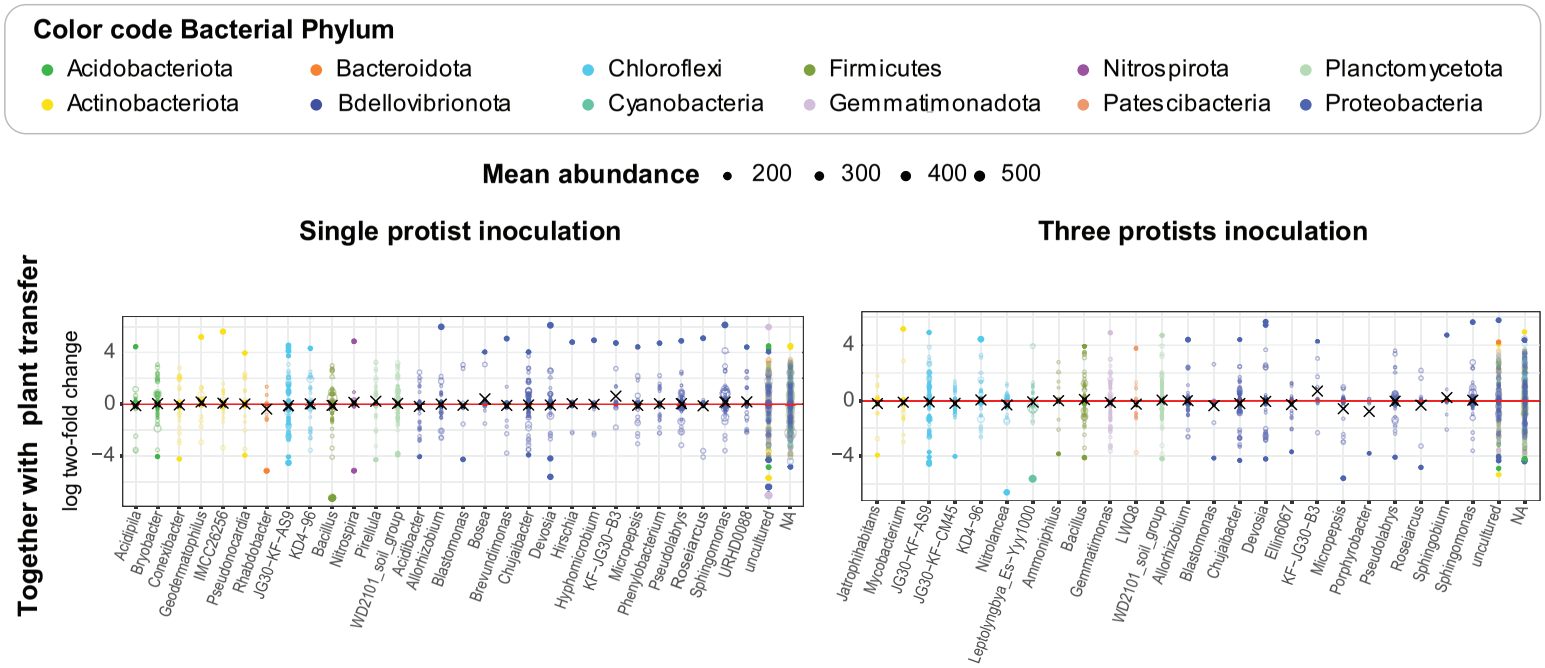

Figure S6: Impact of the protist inoculations applied simultaneously with seedling transfer on bacterial ASVs. Dots above the red line represent bacterial ASVs that were significantly more abundant in the treatment compared to the control, dots below the red line represent bacterial ASVs that were significantly less abundant in the treatment compared to the control. Open circles are the ASVs showing no significant pattern. Crosses indicate the log two-fold average for each given genus, including all non-significantly modified ASVs. Note that we shorten the assigned Allorhizobium-–Neorhizobium–Pararhizobium–Rhizobium to Allorhizobium.

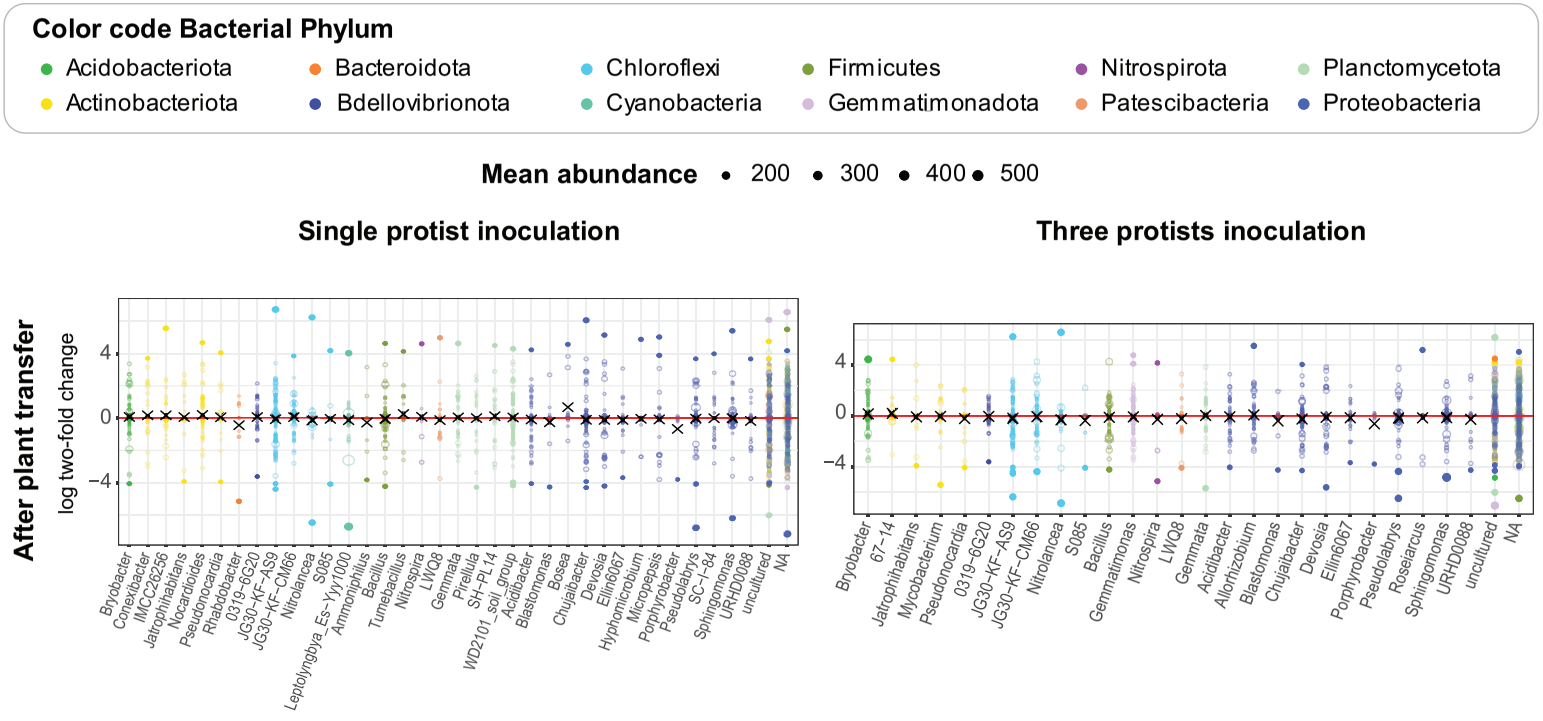

Figure S7: Impact of the protist inoculations applied one week after seedling transfer on bacterial ASVs. Dots above the red line represent bacterial ASVs that were significantly more abundant in the treatment compared to the control, dots below the red line represent bacterial ASVs that were significantly less abundant in the treatment compared to the control. Open circles are the ASVs showing no significant pattern. Crosses indicate the log two-fold average for each given genus, including all non-significantly modified ASVs. Note that we shorten the assigned Allorhizobium-–Neorhizobium–Pararhizobium–Rhizobium to Allorhizobium..

5

Chapter 6

Synthesis and General Discussion

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To adress the possibility of using predatory protists to support a beneficial soil microbiome and plant development, I first investigated relevant protist and bacterial traits to better understand and predict predator-prey interactions (**Chapters 2 and 3**). I then examined different aspects of protist application (*i.e.*, protist identity, diversity of inoculant and timing of inoculation) to best support plant development (**Chapters 4 and 5**). I hereafter synthesize and discuss our main findings, present some limitations of this work and suggest future directions and perspectives of protist application to benefit plant development.

1. Protist predation and biocontrol potential

Our results highlighted the general relation between bacterial biocontrol traits (*i.e.*, the potential to suppress (plant-) pathogens) and the resistance against predatory protists (**Chapter 2**). Numerous toxic compounds produced by bacteria such as 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide, pyrrolnitrin or phenazines, were long known for their antifungal activity before evidences of their role as defense mechanism against predatory protists were revealed (Jousset *et al.* 2006; Lugtenberg and Kamilova 2009). Such defense was found to be relatively specific or more general. On one hand and similarly to a previous study, we observed a great variation in the growth ability of different close-related protists on toxin-producing bacteria (Pedersen *et al.* 2011), making phylogenetic distance a rather poor predictor of the predator-prey interaction. On the other hand, three of the bacterial species used in our experiment (*Pseusomonas donghuensis* SVBP6, *Pseudomonas putida* SVMP4 and *Pseudomonas chlororaphis* SVBP3) were particularly successful in inhibiting all six protists tested. Such general inhibition suggested the production of a broad range of toxic compounds as reported for the isolate *P. donghuensis* SVBP6 (Agaras, Iriarte, and Valverde 2018). Interestingly, the biocontrol potential of bacteria was recently found to be a better predictor for the success of plant-beneficial bacteria in field conditions compared to direct plant growth promotion potential (Agaras *et al.* 2020). We suggested that this predictive power was related to higher chances of survival of biocontrol/predation-resistant bacteria in complex environments, such as soil and rhizosphere, where predation pressure may be high (**Chapter 2**). It is thus important to consider predation for the success of inoculated plant-beneficial microbes, but also as it is likely an initial selective agent for the occurrence and maintenance of biocontrol activity of bacteria (Jousset 2012; Müller, Scheu, and Jousset 2013).

2. Distinct predatory impact by different protist taxa

Similarly to the specific sensitivity of each protist toward toxin-producing *Pseudomonas* spp. (**Chapter 2**), the isolates showed distinct consumption patterns when grown on twenty soil bacteria separately (**Chapter 3**) and distinct predatory impacts on soil and rhizosphere bacterial community composition (**Chapter 3, 4 and 5**). All our results highlighted the taxon-specific nature of protist predation and thus also the poor explanatory value of phylogenetic distance in relation to predator-prey interactions. Such taxon-specificity of the effects also further promotes the necessity to continue isolating, cultivating and identifying

new species to enable assays with non-model protists (Montagnes *et al.* 2008; Geisen *et al.* 2017; Burki, Sandin and Jamy 2021). We also observed that protist predatory impact may not always be reflected on the whole community composition which is in contrast with most previous studies (Rønn, Vestergård, and Ekelund 2012; Bonkowski and Clarholm 2012; Gao *et al.* 2019). The predatory impact of protists might modify a subset of bacteria, without leading to significant changes of the overall community composition (**Chapters 4 and 5**; Asiloglu *et al.* 2020). Changes on the relative abundance of few bacterial taxa might, however, already lead to significant changes of the soil microbiome functioning and the plant development (Berendsen, Pieterse and Bakker 2012; Berendsen *et al.* 2018; Finkel *et al.* 2020). Application of protists could thus allow for very targeted application, only affecting the relative abundance of some bacterial taxa. It is, however, essential to further understand the context-dependency of such predatory impact (Box 1).

3. Functional and demographic traits of protists to predict their predatory impact

The use and identification of functional and/or demographic traits are useful to describe and predict the ecology of organisms (Violle *et al.* 2007; Krause *et al.* 2014). In studying soil protist ecology, I also investigated the potential of functional and demographic traits of protists to explain their interactions with their prey. In contrast with previous studies, we did not observe a significant relation between cell flexibility or volume and bacterial community composition (Glücksman *et al.* 2010; Gao *et al.* 2019). Similarly to these previous studies, the phylogenetic distance between protist isolates was also a poor indicator (Glücksman *et al.* 2010; Gao 2020). However, we observed the potential importance of growth rate in driving community composition (**Chapter 3)** in contrast with a previous study testing twenty protists isolates, including the taxa we used (Gao 2020). In addition to the more common traits mentioned above (cell flexibility, cell volume, phylogenetic distance and growth rate), I proposed that in vitro measured feeding patterns could be used as demographic traits to estimate protist dietary specialization and help predict their predatory impact (**Chapter 3**; Box 2). Because most protists do not feed equally on bacteria (Montagnes *et al.* 2008; Geisen *et al.* 2018), it is essential to consider their dietary specialization to adress their predatory impact on prey community composition (Montagnes *et al.* 2008; Devictor *et al.* 2010).

Box 1: Context dependency of predatory impact

The predatory impact of protists on bacterial community is likely to be highly context dependent, as different soils and plants host different microbial communities (Philippot *et al.* 2013; Bahram *et al.* 2018). I examined here the predatory impact of *Cercomonas* sp. S24D2 in three different setups ranging from soil microcosm with sterilized soil re-inoculated with a protist-free soil bacterial community to non-sterilized soil in a greenhouse experiment (**Chapters 3, 4, and 5**). By using a Venn diagram (Figure 1), I found that a total of 89 operational taxonomic units (OTUs) were shared between all three setups, corresponding to 7.8% of the total number of OTUs.

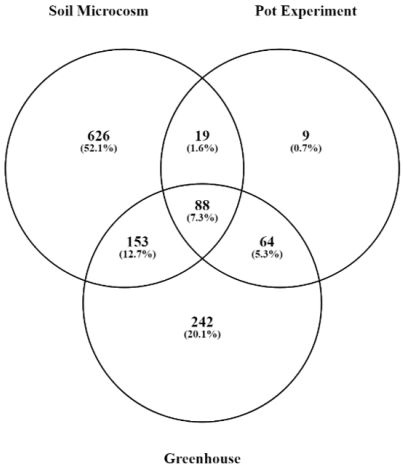

Figure 4: Venn diagram displaying the shared and unique operational taxonomic units based on the 16S rRNA gene amplicon sequencing from three experimental setups: Soil microcosm performed by Gao (2020; Chapter 3), the Pot experiment (Chapter 4), and the Greenhouse (Chapter 5).

(Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams. https://bioinfogp.cnb.csic.es/tools/ venny/index.html; visited in January 2022)

I then visualized in a heatmap the averaged log two-fold changes in relative abundance for each bacterial genera that was statistically significantly impacted by *Cercomonas* sp. S24D2 (Figure 2). Each experimental setup selected for a specific group of bacterial taxa and only little overlap existed across the setups (Figures 1 and 2), suggesting strong system dependency of the observed predatory impact. Nonetheless, eight bacterial taxa were affected by *Cercomonas* sp. S24D2 in all three setups for *Devosia*, in two of the three for *Nocardioides*, *Pedobacter* and *Sphigomonas*, in bulk soils for *Pseudomonas* and in rhizosphere soils for *Pirellula*, *Hyphomicrobium* and *Phenylobacterium* (Figure 2). The observed effects were not necessarily in one direction, suggesting the role of additional parameters affecting the final outcome.

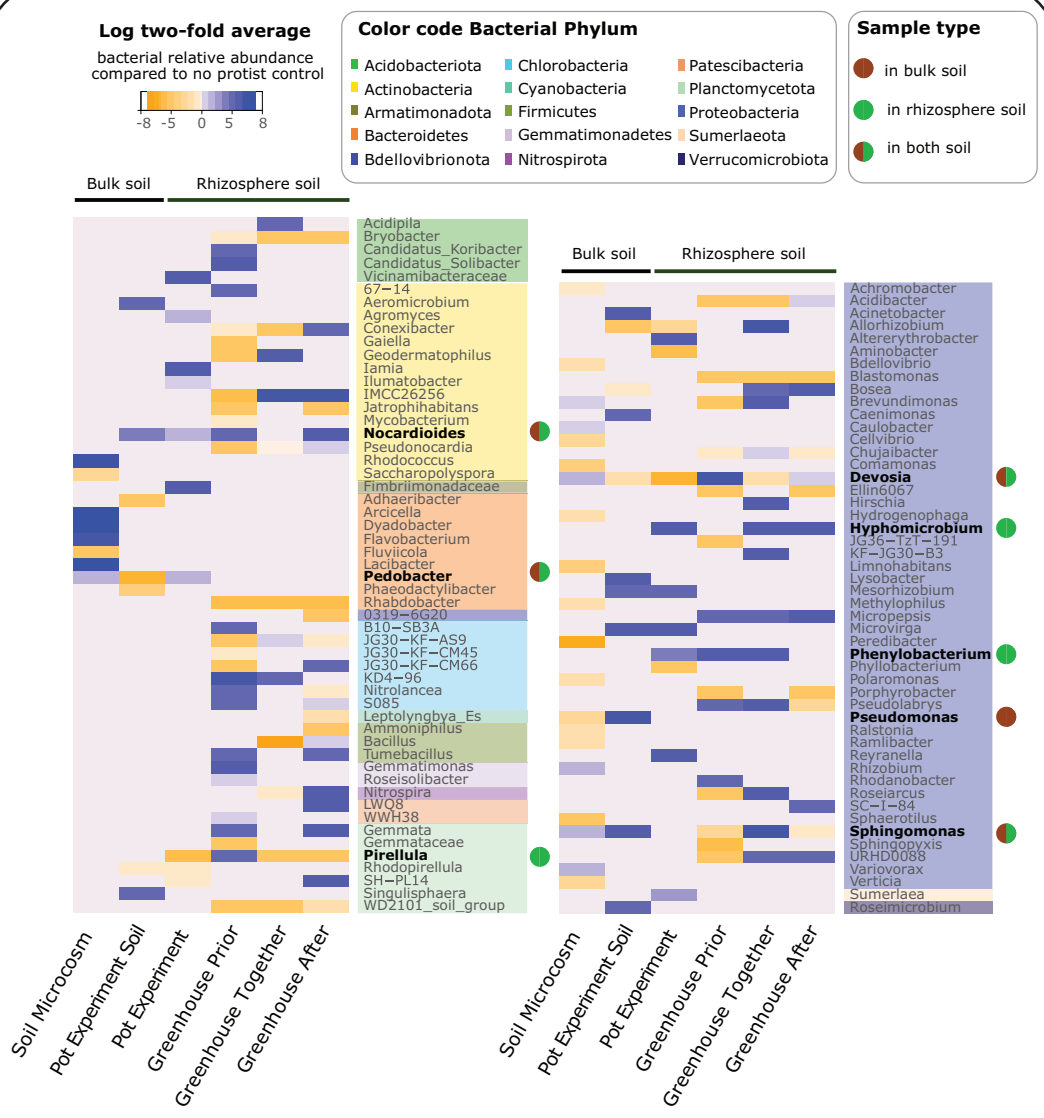

Figure 2: Predatory impact of Cercomonas sp. S24D2 on the relative abundance of bacteria in the different experiment setups used in the present thesis (Soil Microcosm, Chapter 3; Pot Experiment, Chapter 4; Greenhouse, Chapter 5). Bacteria affected in more than one setup are indicated in bold and with a dot color-filled depending of the sample type (bulk or rhizosphere soil). The values correspond to the averaged log two-fold values extracted from the different chapters.

Box 2: Potential of plate assays for soil protist ecology

Despite their artificial and simplistic nature, in vitro assays are still useful and even necessary to obtain a mechanistic understanding of microbial ecology as recently highlighted for fungal ecology (Crowther, Boddy, and Maynard 2018). Such assays are especially powerful when combined with other methods such as sequencing and/or modelling (Isaac Newton Institute Fellows *et al.* 2016; Vilanova and Porcar 2016). Recent studies have further successfully related results from field experiments with traits measured in laboratory assays (Agaras *et al.* 2020; Zanne *et al.* 2020).

For protist ecology, the development of high throughput plates is mostly limited by the time-consuming direct counting for density quantification. Various approaches could facilitate this step such as the implementation of automated image capture and analysis (Pennekamp and Schtickzelle 2013), the use of flow cytometry combined with dyes to estimate both prey and predators (Rose *et al.* 2004; Berney *et al.* 2007; Christaki *et al.* 2011) or also the measurement of respiration rate as proxy for consumption (*e.g.*, MicroRespTM; Campbell *et al.* 2003).

Such plate assays would be best used in combination with other experiments: a posteriori to confirm/reject interactions suggested by, for example, co-occurrence network analysis based on amplicon-sequencing methods and/or a priori to provide directions for more targeted research efforts.

4. Predatory impact of protists and plant development

Changes in bacterial community composition have been suggested as potential mechanism to explain beneficial effects of protist inoculation on plant performance (Bonkowski and Clarholm 2012; Gao *et al.* 2019). While we observed an increased shoot-to-root ratio (**Chapter 4**) and an important increase in aboveground biomass (**Chapter 5**) upon protist inoculation, the relation between protists, bacterial community composition and the plant performance remains mostly elusive (**Chapters 4 and 5**). Indeed, we did report positive correlations between plant properties and the relative abundance of some known plant-beneficial bacterial taxa such as *Caulobacter* spp. (Berrios 2022) or *Burkholderia* spp. (Parke and Gurian-Sherman 2001), but also with other bacterial taxa with a much less likely potential beneficial role for plants.

Predatory activity is further associated to an increased nutrient turnover that could lead to improved plant growth; This process is also referred to as the 'microbial loop' (Clarholm, 1985; Bonkowski 2004). While we did observe an increase in plant biomass (**Chapter 5**), we did not observe the typically associated increase in nitrogen content (Clarholm, 1985; Bonkowski 2004). Some other elements including Al, Ca, Mg, Mn, Na, P, S and

Zn were, however, enriched in the shoot of the plant (**Chapter 5**). Protist activity was also previously associated with increased phosphorous (Bonkowski, Jentschke, and Scheu 2001), magnesium and calcium content in plants (Herdler *et al.* 2008). Protist predation is thus likely not only important for nitrogen release but also for the release of other elements. Each protist species exerted an unique predatory pressure on its bacterial prey community, which was or not associated with changes in plant properties (Figure 3). The effect on plant properties (*e.g.*, increase in shoot-to-root ratio, increase in aboveground biomass, increase in nutrient content) was also dependent on the protist species. The two mechanisms mentioned above, change in bacterial community composition and increased nutrient turnover, are likely to co-occur and influence each other with potentially different intensity depending on the protist species.

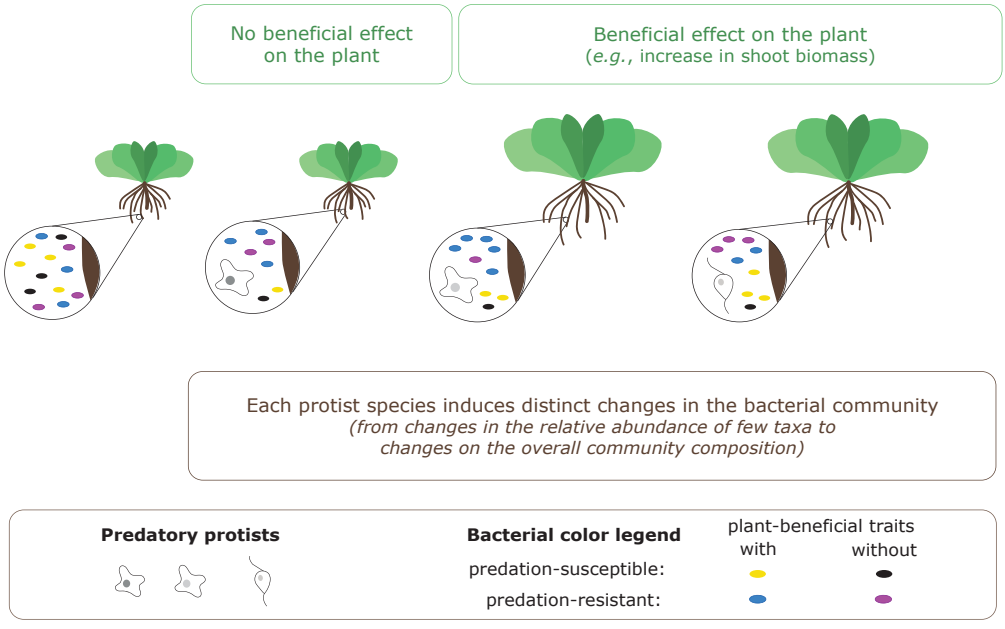

Figure 3:Schematic representation of the protist predatory impact on its prey community and its relation to plant performance, depending on the inoculated species. Each protist species has a specific impact on the bacterial community composition; The predation-susceptibility of a bacterium can vary depending on the protist species. The inoculation of protist species may or may not be associated with (beneficial) changes in plant properties.

5. Protist community composition and plant development

While I focused in the present thesis on the predatory impact of protists, we also observed the potential importance of a stable protistan community for the plants (**Chapter 5**). While protists are part of the plant microbiome (Sapp *et al.* 2018), still little is known about the relation between protist community structure and plant development. Both negative and

positive effects on plant development can be expected from the soil protist community (Hassani, Durán and Hacquard 2018). On one hand, the protist community includes some important plant-pathogens in the Oomycetes (Stramenopiles) and the phytomyxid Plasmodiophorida (Rhizaria) (Geisen *et al.* 2018). On the other hand, predatory protists have been suggested to play an important role in relation to plant health (Xiong *et al.* 2020): they may indeed directly feed on plant-pathogens (Chakraborty, Old and Warcup 1983; Long *et al.* 2018), can stimulate biocontrol agents (Jousset and Bonkowski 2010; Song *et al.* 2015) and increase the survival and activity of plant-beneficial bacteria (Weidner *et al.* 2016; Asiloglu *et al.* 2020; Bahroun *et al.* 2021). Thus, beneficial plant development is likely also dependent on its interactions with the soil protist community.

6. Limitations and future perspectives

In this thesis, I highlighted important taxon-specific impacts of soil protists. While I selected the protist isolates to span over three eukaryotic lineages, I am but scratching the surface of their enormous diversity (Burki, Sandin and Jamy 2021; Singer *et al.* 2021). While examining potential functional traits related to predatory impact, this limited selection may not have had sufficient analytical power to adress the importance of traits such as volume and/or cell flexibility previously associated with the influence of protists on their prey (Glücksman *et al.* 2010; Gao 2020). Future studies especially designed to address the influence of cell flexibility, volume or growth rate are thus required to confirm/reject our results.

One challenge faced when working with predatory protists is the necessity to provide them with food prey, that are typically difficult to eliminate prior experimentation. Thus, some prey bacterial cells get transferred alongside the protists and might influence the results. Each cultivation method has its own limitations (Montagnes *et al.* 2008) and reports showed that prey identity (Boenigk *et al.* 2001), density (Boenigk *et al.* 2002), and cell state (alive vs heat-killed; Pickup, Pickup and Parry 2007) of the stock cultures all influenced the later predator-prey interactions of interest. In addition, even on axenic cultures some bacteria may still survive inside the protist cell (Gong *et al.* 2016; Villanueva, Medina and Fernández 2016). In the present work, I decided to tackle this issue by including, when appropriate, controls with only the bacterial cells to examine their influence on the results. The effects of different cultivation conditions could be further addressed and would likely lead to optimized protocols to grow different protists.

The analyses performed to link protist predatory impact, bacterial community composition and plant properties, were mostly exploratory. Thus, rather than providing clear answers, they suggest directions for future research in the context of multitrophic interactions associated to plant development. Another promising approach would be to target functional genes of interest with specific primers (*e.g.*, Müller, Scheu, and Jousset 2013), inferred functions from amplicon sequencing (*e.g.*, PiCrust approach ; Douglas *et al.* 2019) and/or metagenomic (*e.g.*, Flues, Bass, and Bonkowski 2017) and link them with the predatory impact of the protists and with the plant properties.

While our experimental setups were mostly designed to investigate the potential role of the bacterial community composition in improving plant development, the release of nutrients upon protist predation could be equally or even more important (Gao *et al.* 2019). Further studies with different protists using isotopes and/or manipulating soil nutrient content could help adress the potential importance of the microbial loop (*e.g.*, Bjørnlund *et al.* 2012; Koller *et al.* 2013).

I also want to add a note of caution about the use of microbial inoculation and potential ecological risks associated: inoculation of microbes can lead to persistent modifications of the resident community and the associated consequences remain mostly unclear (Mawarda *et al.* 2020). In our experiments, the predatory impacts of protists were relatively subtle and, if confirmed, this could add to their quality to support plant development without strong, long-term, potentially harmful modifications of the soil microbiome. Future development and research should include long-term ecological assessment of microbial inoculation (Jack *et al.* 2021), which would likely have the positive side-effect to further inform on fundamental aspects of soil protist ecology.

7. Conclusion

The main findings of the present thesis are (1) that protist predatory impact is taxon specific, both on its prey community and on plant development, (2) that effects on plant development are not necessarily beneficial or related to an overall change in the bacterial community composition, and (3) that an application prior plant transfer yields the strongest effects on plant properties. In addition, soil predatory protists could be important regulators of pathogens in soils and should be considered when predicting the success of microbial inoculants in the field. I conclude that predatory protists are an essential component of the soil microbiome functioning and have the potential to support plant development.

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Summary

The application of micro-organisms has been increasingly suggested as sustainable agricultural approach. Soil micro-organisms include bacteria, archaea, fungi and protists. The majority of soil protists are free-living predators that feed, among others, on bacteria. Through their predatory activity, they typically unlock nutrients that can benefit plants. In addition, because soil protists do not equally feed on all bacteria, they influence their prey community structure which can further lead to an enrichment in plant-beneficial taxa. One challenge but also an opportunity of soil protist ecology lies in the high phylogenetic diversity of protists that likely mirrors an equally high functionality: each protist species could have a distinct impact on plant development. Most of the current understanding is, however, based on few model species and successful protist application typically follows a trial and error methodology with little a priori knowledges.

In the present work, we investigated the potential of phylogenetically diverse soil protists to support plant development, especially via their impact on the bacterial community composition. We first pursued to identify relevant protist and bacterial traits to better understand and predict predator-prey interactions. We were able to relate the ability of bacteria to inhibit their predators to their ability to suppress pathogens (**Chapter 2**). We identified for each of the protist tested distinct prey consumption patterns which were related to their predatory impacts on soil bacterial communities: protist species that could feed on the same set of bacteria in a plate assay had a similar impact on the bacterial community structure in the soil (**Chapter 3**). After investigating the predator-prey interactions, we examined different aspects of protist application to best support plant development by using lettuce, *Lactuca sativa*, as model plant. We found that the effect on the plant varied depending on the protist species inoculated (**Chapter 4**) and that application of protists before transferring the plant into the soil led to the best yield (**Chapter 5**). In **chapter 6**, I synthesized the obtained results, further examined the context-dependency of the predatory impact of protists and suggested plate assays to be promising in soil protist ecology.

The results of this work highlight that protist predatory impact is taxon specific, both on its prey community and on plant development. Further, effects on plant development are not necessarily beneficial or related to an overall change of the bacterial community composition. We conclude that predatory protists are an essential component of the soil microbiome functioning and have the potential to support plant development.

Samenvatting (Dutch summary)

De toepassing van micro-organismen wordt steeds vaker voor gebruik in duurzame landbouw geopperd. Bodemmicro-organismen omvatten bacteriën, archaea, schimmels en protisten. De meeste bodemprotisten zijn predatoren ("roofdieren") die zich met andere micro-organismen zoals bacteriën voeden. Door hun activiteit maken ze voedingsstoffen vrij die planten ten goede kunnen komen. Omdat protisten zich niet in gelijke mate met alle bacteriën voeden, beïnvloeden ze de structuur van hun prooigemeenschap en dit kan tot een verrijking van plantvriendelijke taxa leiden. Een van de uitdagingen, en ook mogelijkheden van bodemprotistenecologie ligt in de hoge fylogenetische diversiteit van protisten die waarschijnlijk een even hoge functionaliteit weerspiegelt: elke protist kan een specifieke impact op de ontwikkeling van planten hebben. Omdat het grootste deel van het recent inzicht op enkele modelsoorten gebaseerd is, zijn succesvolle toepassingen van protisten meestal het resultaat van een "*trial-and-error*" methodologie met weinig voorkennis.

In deze proefschrift hebben wij het potentieel onderzocht van fylogenetisch diverse bodemprotisten om de ontwikkeling van planten te ondersteunen, vooral via hun impact op de samenstelling van de bacteriële gemeenschap. Wij streefden in eerste instantie naar het identificeren van relevante eigenschappen van protisten en bacteriën om predator-prooi-interacties beter te begrijpen en te voorspellen. Wij konden het vermogen van bacteriën om hun predatoren te remmen relateren aan hun vermogen om pathogenen te onderdrukken (**Hoofdstuk 2**). Verder konden wij voor elke geteste protist een karakteristiek prooi consumptiepatroon identificeren en we konden deze consumptiepatronen relateren aan hun predatie impact op bacteriële gemeenschappen in de bodem: protistensoorten die zich voedden met dezelfde set bacteriën in een in vitro test hadden een vergelijkbare impact op de bacteriële gemeenschapsstructuur in de bodem (**Hoofdstuk 3**). Daarna hebben wij verschillende aspecten de toepassing van protisten om plantontwikkeling te ondersteunen onderzocht met sla, *Lactuca sativa*, als modelplant. Wij ontdekten dat de variatie in effect op de plant afhankelijk was van de gebruikte protistensoort (**Hoofdstuk 4**) en dat het toepassen van protisten vóór het overbrengen van de plant in de grond tot de beste opbrengst leidde (**Hoofdstuk 5**). In de **hoofdstuk 6** heb ik de verkregen resultaten bewerkt en de context-afhankelijkheid van de predatie impact van protisten verder onderzocht en gesuggereerd dat *in vitro* assays veelbelovend zouden zijn in de bodemprotistenecologie.

De resultaten van dit proefschrift benadrukken dat de predatie impact van protisten taxon specifiek is, zowel op de prooigemeenschap als ook op de ontwikkeling van planten. De effecten op de ontwikkeling van planten waren niet noodzakelijkerwijs gunstig en zijn niet altijd gerelateerd aan een algehele verandering van de samenstelling van de bacteriële gemeenschap. Wij concludeerden dat predatie protisten een essentieel onderdeel van het functioneren van het bodem microbioom zijn en dat ze potentie hebben om de ontwikkeling van planten te ondersteunen.

Résumé (French summary)

L'application de micro-organismes est de plus en plus suggérée comme approche durable en agriculture. Les micro-organismes du sol comprennent les bactéries, les archées, les champignons et les protistes. La majorité des protistes du sol sont des prédateurs qui se nourrissent des autres micro-organismes comme les bactéries. Ils libèrent des nutriments à travers leur activité de prédateur, les rendant ainsi accessible aux plantes. De plus, les protistes ne se nourrissent pas équitablement de toutes les bactéries. Un tel mode de consommation préférentiel influence la structure des communautés bactériennes, pouvant conduire à un enrichissement en taxons bénéfiques pour les plantes. L'un des défis mais aussi une opportunité de l'écologie des protistes du sol réside dans leur grande diversité phylogénétique qui reflète probablement une aussi grande diversité fonctionnelle : chaque protiste peut ainsi avoir un impact distinct sur le développement des plantes. Notre compréhension est cependant seulement basée sur quelques espèces modèles n'offrant ainsi qu'une fragile base pour une application réussie des protistes.

Dans ce travail, nous avons étudié le potentiel des protistes du sol à soutenir la croissance des plantes, en particulier à travers leur impact sur les communautés bactériennes. Nous avons tout d'abord cherché à identifier des caractéristiques protistes et bactériennes qui permettraient de mieux comprendre et prédire les interactions prédateur-proie. Nous avons observé que la capacité des bactéries à inhiber leurs prédateurs était associée à leur capacité à supprimer des agents pathogènes des plantes (**Chapitre 2**). Nous avons pu identifier pour chaque espèce de protiste testée un modèle distinct de consommation de ses proies qui était lié à son impact sur les communautés bactériennes du sol : les espèces de protistes se nourrissant de manière similaire sur un ensemble de bactéries proposées influencèrent de manière similaire la structure de la communauté bactérienne dans le sol (**Chapitre 3**). Nous avons ensuite examiné différent aspects de l'introduction de protistes dans le sol pour la croissance des plantes en utilisant la salade, *Lactuca sativa*, comme modèle. Nous avons trouvé que l'effet sur les plantes dépendait de l'identité du protiste utilisé (**Chapitre 4**) et qu'une introduction des protistes avant la mise en terre de la plante apportait les meilleurs résultats (**Chapitre 5**). Dans le **chapitre 6**, une synthèse des résultats obtenus est présentée ainsi qu'une étude sur la constance de l'impact prédateur dans les différents systèmes utilisés dans cette thèse. Les tests en microplaques y sont également discutés pour leur potentiel prometteur en l'écologie des protistes du sol.

Les résultats de cette thèse ont montrés que l'impact prédateur est spécifique à l'identité des protistes tant par rapport aux effets sur la communauté bactérienne que sur le développement des plantes. De plus, l'impact prédateur des protistes ne conduit pas nécessairement à un effet bénéfique pour la plante et n'est pas nécessairement lié à une modification de la composition globale de la communauté bactérienne. Nous concluons que les protistes prédateurs sont une composante essentielle du fonctionnement du microbiome du sol et qu'ils ont le potentiel de soutenir le développement des plantes.

Zusammenfassung (German summary)

Die Anwendung von Mikroorganismen um Pflanzenentwicklung zu unterstützen findet zunehmend Zuspruch in nachhaltiger Landwirtschaft. Bodenmikroorganismen umfassen Bakterien, Archaea, Pilze und Protisten. Die meisten Bodenprotisten sind freilebende Prädatoren („Raubtieren"), die sich unter anderem von Bakterien ernähren. Durch ihre räuberische Aktivität setzen sie Nährstoffe frei, die nützlich für Pflanzen sein können. Zudem bevorzugen Bodenprotisten unterschiedliche Bakterienarten als Nahrung. Eine solche diskriminierende Ernährung kann die Gemeinschaftsstruktur ihrer Beute beeinflussen und dadurch zu einer Anreicherung pflanzennützlicher Taxa führen. Eine der Herausforderungen, aber auch Chancen der Bodenprotistenökologie liegt der hohen phylogenetischen Vielfalt von Bodenprotisten, die eine ebenso hohe Funktionalität widerspiegeln kann: Jede Protistenart kann einen spezifischen Einfluss auf die Pflanzenentwicklung haben. Der aktuelle Wissenstand basiert jedoch hauptsächlich auf Studien weniger Modellorganismen und die erfolgreiche Anwendung von Protisten erfolgt meist nach einer *„Trial-and-Error"*-Methodik mit geringen Vorkenntnissen.

In dieser Doktorarbeit haben wir das Potenzial von Bodenprotisten zur Unterstützung der Pflanzenentwicklung untersucht, mit speziellem Augenmerk auf ihren Einfluss auf die Zusammensetzung der Bakteriengemeinschaft. Als erstes haben wir danach gestrebt Eigenschaften von Protisten und Bakterien zu identifizieren, um die Räuber-Beute-Interaktionen besser verstehen und vorhersagen zu können. Dadurch konnten wir einen Zusammenhang zwischen der Fähigkeit von Bakterien, ihre Fressfeinde zu hemmen, und ihrer Fähigkeit pflanzliche Krankheitserreger zu unterdrücken herstellen (**Kapitel 2**). Außerdem konnten wir für jeden der getesteten Protisten unterschiedliche Beuteaufnahmemuster identifizieren die in Zusammenhang mit ihren räuberischen Auswirkungen auf die Bodenbakteriengemeinschaften stehen: Protisten die sich von einem ähnlichen Anteil von angeboten Bakterienarten ernähren konnten, hatten eine ähnliche Auswirkung auf die Bakteriengemeinschaft im Boden (**Kapitel 3**). Als nächstes untersuchten wir verschiedene Aspekte der Anwendung von Protisten im Boden zur Unterstützung der Pflanzenentwicklung mit Salat, *Lactuca sativa*, als Modellpflanze. In unserem Experiment war das Wachstum der Pflanze abhängig von der Art des jeweiligen inokulierten Protisten (**Kapitel 4**). Zudem, konnten wir zeigen dass die Anwendung von Protisten in die Erde vor dem Transfer der Pflanze zum besten Ertrag führte (**Kapitel 5**). Im **Kapitel 6** sind alle Ergebnisse in einer Synthese dargestellt. Des Weiteren wird die Einheitlichkeit der räuberischen Wirkung von Protisten in drei der verschiedenen Experimente von dieser Arbeit untersucht. Außerdem wurden Tests mit Hilfe von Mikrotiterplatten wurden als vielversprechende Methode in der Bodenprotistenökologie diskutiert.

Diese Arbeit zeigt, dass der räuberische Einfluss von Protisten taxonspezifisch ist, bezogen auf seine Beutegemeinschaft und auf die Pflanzenentwicklung. Zudem ist dieser Einfluss nicht immer förderlich für die Pflanzenentwicklung und führt nicht zwangsläufig zu einer Gesamtveränderung der Bakteriengemeinschaft. Daraus schließen wir, dass räuberische Protisten ein wesentlicher Bestandteil für das erfolgreiche Funktionieren des Bodenmikrobioms sind, und dass sie das Potenzial haben, die Pflanzenentwicklung zu unterstützen.

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Thanks to all the people who were part of my life in one way or another during my PhD journey! Have some fun searching your name in the amoeba-shape word cloud in the next page; and, please, don't take it bad if you don't find it, the simplest explanation is that the amoeba ate it ;-) *big hug* everyone

Thanks, Bedankt, Merci, Danke, با سپاس , mulțumesc

Grazie, 謝謝, Спасибо, Gracias, Obrigada, ありがとう,

Teşekkürler, شکریہ, Хвала, Tack, ευχαριστώ, شکرا

A

This thesis is printed CO2 neural by planting four trees via Tree for All.

Publications related to this book

Peer-reviewed articles

Amacker, N., Hu, J., Gao, Z., Jousset, A., Kowalchuk, G., Geisen, S. 2022. "Protist feeding patterns and growth rate are related to their predatory impacts on soil bacterial communities." FEMS Microbiology Ecology. Accepted.

Amacker, N., Gao, Z., Agaras, B. C., Latz, E., Kowalchuk, G. A., Valverde, C. F., Jousset, A., and Weidner, S. 2020. "Biocontrol traits correlate with resistance to predation by protists in soil Pseudomonads." Frontiers in Microbiology 11 (December): 614194. https://doi.org/10.3389/fmicb.2020.614194.

Additional contributions

Geisen, S., **Amacker, N.**, Jousset, A. (*in press*) Soil Protists. Encyclopedia of Soils in the Environment, Second Edition.

Amacker, N., Gao, Z. (2021) Bodemprotisten en hun rol bij het onderdrukken van ziekteverwekkers. Gewasbescherming. 52(6), 182-184.

As a step toward more Open Science, all datasets and codes (R, for data analysis) are available on demand (nathalie.amacker@gmail.com) and/or are being put in the GitHub repositories of the author (NathAmack). They need some additional work to be readable and reproducible, and will probably still be a bit messy and far from ideal. Nonethless, they might be helpful :-)

About the author

Nathalie Amacker was born on the 18th of December 1990 in Sion (Valais/Wallis, Switzerland). In 2011, she started a Bachelor in Biology at the University of Zürich (Switzerland). Her interest for the microbial world and its interactions with the macroscopic world led her to join in 2014 the Master of Biogeoscience, jointly hosted by the University of Neuchâtel (UniNe) and the University of Lausanne (Unil; Switzerland), with the specialization in Geomicrobiology. One group of microbes in particular, the protists, got her attention as despite making up the vast majority of the eukaryotes they seemed to be still poorly studied. During her Master internship, she investigated the risk of a pesticide on one soil protist species under the supervision of Dr. Nathalie Chèvre (Unil) and Dr. Prof. Edward Mitchell (UniNe). After graduating from her Master, she wanted to explore the protist world further and did two internships in 2016 and 2017 in the group of Ecology and Biodiversity at the Utrecht University (UU; the Netherlands) under the supervision of Dr. Simone Weidner and Dr. Alexandre Jousset. During these internships, she explored the role of different soil protist species on the regulation of bacterial communities and the potential to suppress plant-pathogens. Following these internships, she started in 2018 her PhD to further dive in the potential of soil protists to support plant development under the supervision of Dr. Alexandre Jousset (UU), Dr. Stefan Geisen (Wageningen University & Research) and Prof. Dr. George A. Kowalchuk (UU).

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (4.5 ECTS)
- Intro of thesis & presentation in research group

Writing of project proposal (4.5 ECTS)
- Application of protozoa as next generation biofertilizer in sustainable agriculture

Post-graduate courses (4.1 ECTS)
- Frontiers in microbial ecology: eco-evolutionary dynamics of microbial-host interactions; RSEE, PE&RC & SENSE, WUR, the Netherlands (2018)
- Protists diversity and ecology in aquatic and terrestrial ecosystems; CUSO Conférence Universitaire de Suisse Occidentale, University of Neuchâtel, Switzerland (2019)
- Introduction to R and data; GSLS, Utrecht University, the Netherlands (2019)
- WIAS/PE&RC Advanced statistics course design of experiments; WIAS and PE&RC, WUR, the Netherlands (2019)
- Protist eDNA bio-informatics workshop; LPG-BIAF, Angers University, France (2021)

Invited review of journal manuscripts (8 ECTS)
- European Journal of Soil Biology: soil microorganism, nitrogen (2020)
- Soil Organisms: soil protists (2020)
- Soil Biology and Biochemistry: top down and bottom up effect on plant performance (2020-2021)
- Functional Ecology: protist, dormancy, prey scent (2020-2021)
- ISME J: soil protist, biogeography, agricultural soil (2021)
- Soil Biology and Biochemistry: rice paddy soil, soil protists (2021)
- Journal of European Protistology: selective grazing, protist, plant growth (2021)
- Soil Biology and Biochemistry: soil protist, soil aggregation (2021)

Competence strengthening / skills courses (4.5 ECTS)
- Supervision of masters students; GSLS (2018)
- This thing called science, includes ethical aspects; GSLS (2019)
- Research planning and time management; GSLS (2019)
- Intercultural communication; GSLS (2019)
- Breaking science; GSLS (2020)
- Writing a reproducible code, includes ethical aspects; GSLS (2020)
- Writing a scientific paper; GSLS (2020)
- Scientific artWork, data visualisation and infographics with Adobe Illustrator; GSLS (2021)
- InDesign; GSLS (2021)
- Motivation and negotiation workshop; UU PROUT (2021)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.1 ECTS)
- PE&RC First year weekend (2018)
- Symposium plant-soil feedback linkages between root traits and soil biota (2019)
- PE&RC Day (2019)
- PE&RC Last year weekend (2020)

Discussion groups / local seminars or scientific meetings (4.8 ECTS)
- Institute of environmental ecology PhD discussion group; IEB Utrecht (2018-2021)
- Joint meetings with the research group Gembloux Agro-Bio Tech, University of Liege in Belgium and in the Netherlands (2018)
- Joint meetings with the research group from Microbial Ecology Groningen in Groningen and in Utrecht (2018)
- Group for Dutch soil pathogen working (2018, 2020)
- Institute of environmental ecology PhD discussion group; IEB Utrecht (2018-2021)
- Creation, organisation and participation of EB PhD discussion group (2020-2021)
- Plant microbiome discussion group (2020-2021)

International symposia, workshops and conferences (5.1 ECTS)
- Workshop of the annual meeting of the German protozoological society DGP; Cologne; Germany (2018)
- Annual meeting of the German protozoological society; poster presentation; Cologne, Germany (2018)
- International Society for Microbial Ecology, ISME; poster presentation; Leipzig, Germany (2018)
- International Academic Conference for Graduate Students at Nanjing Agricultural University IACGS; oral presentation; Nanjing, China (2018)
- Rhizosphere; oral presentation; Saskatoon, Canada (2019)
- Participation to the workshop and annual meeting of the German protozoological society; oral presentation; online (2021)

Lecturing/supervision of practicals/tutorials (4.7 ECTS)
- Plant ecology (2018)
- Experiment and statistiek (2018, 2019, 2020, 2021)
- Ecology of natural resources; lecture (2020)

Supervision of MSc students (12 ECTS)
- Growth promotion of fungi by application of protists
- Effect of microplastics on soil protists
- Effect of selective feeding protist on plant performance
- Soil protist as biotechnological tool to improve plant growth

Protists and humans

Protists do not only contribute to various research fields including theoretical ecology and paleo-environment, but they are also, among others, at the basis of a societal model (the amoeba of cultural change by Alan AtKisson), the inspiration of a science fiction horror movie (The Blob, 1958) and even the subject of a lovely poem (see below).

"Ode To The Amoeba" by Arthur Guiterman (1871-1943)

Recall from Time's abysmal chasm
That piece of primal protoplasm
The First Amoeba, strangely splendid,
From whom we're all of us descended.
That First Amoeba, weirdly clever,
Exists today and shall forever,
Because he reproduced by fission;
He split himself, and each division
And subdivision deemed it fitting
To keep on splitting, splitting, splitting;
So, whatsoe'er their billions be,
All, all amoebas still are he.
Zoologists discern his features
In every sort of breathing creatures,
Since all of every living species,
No matter how their breed increases
Or how their ranks have been recruited,
From him alone were evoluted.
King Solomon, the Queen of Sheba
And Hoover sprang from that amoeba;
Columbus, Shakespeare, Darwin, Shelley
Derived from that same bit of jelly.
So famed is he and well-connected,
His statue ought to be erected,
For you and I and William Beebe
Are undeniably amoebae!

(1922)

As a finale note, I would say that studying the fascinating world of microbes makes one realize that life is everywhere and that we are never really alone.