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Running Title: Protists stimulate litter breakdown

Abstract

Soil bacteria and fungi are key drivers of carbon released from soils to the atmosphere through decomposition of plant-derived organic carbon sources. This process has important consequences for the global climate. While global change factors, such as increased temperature, are known to affect bacterial- and fungal-mediated decomposition rates, the role of trophic interactions in affecting decomposition remains largely unknown. We designed synthetic microbial communities consisting of eight bacterial and eight fungal species and tested the influence of predation by a model protist, *Physarum polycephalum*, on litter breakdown at 17 and 21°C. Protists increased CO_2 release and litter mass loss by ~35% at 17°C lower temperatures, while they only had minor effects on microbial-driven CO_2 release and mass loss at 21°C. We found species-specific differences in predator-prey interactions, which may affect microbial community composition and functioning and thus underlie the impact of protists on litter breakdown. Our findings suggest that microbial predation by fast-growing protists is of under-appreciated functional importance, as it affects decomposition and, as such, may influence global carbon dynamics. Our results indicate that we need to better understand the role of trophic interactions within the microbiome in controlling decomposition processes and carbon cycling.

Main text

Soil microorganisms, mainly bacteria and fungi, are major drivers of soil carbon cycling through their decomposing activity of plant-derived carbon [1,2] and their role in soil carbon stabilization [3,4]. This has important consequences for atmospheric carbon concentrations and thereby, for ongoing climate change [5,6]. It is well established that large-scale abiotic factors, such as

climate, affect microbial activity and thereby, decomposition rates [7]. More recently it was shown that climate-independent variation in local-scale factors can drive broad-scale variation in decomposition rates [8]. Among these might be microbial predators that vary and affect microbial community composition and functioning at the local scale [9]. However, how microbial predators alter litter breakdown remains largely unknown.

Protists are major microbial predators of soil bacteria and to some extent fungi [10]. Protists are the taxonomically most diverse eukaryotes and occupy all key functional roles in soil food webs [10]. Most soil protists are phagotrophic [11] and prey on bacteria and fungi, which leads to changes in microbial biomass, activity and community structure [10]. This is likely to have important functional consequences, including impacts on litter decomposition processes and thereby, the global carbon cycle. However, there is little experimental evidence underpinning how protists impact decomposition. Moreover, both protist and microbial activity are affected by temperature [9,12], but whether temperature also modifies protist-induced changes in microbial functioning remains unknown.

To test the role of protist predation on microbial-driven decomposition we inoculated microcosms of synthetic microbial communities consisting of sixteen bacterial and fungal species (Table S1, S2) to sterilized oak litter (*Quercus robur*) at both 17 and 21°C. After one week we added protists of the model species *Physarum polycephalum* at three different concentrations (no protists, and low, medium, and high concentration). This resulted in a full-factorial design with 16 treatments: 2 microbial inocula (yes/no) × 2 temperatures (17/21°C) × 4 protist concentrations (Table S3) and we used six replicates per treatment. Microcosms without microbial inocula were established to test for successful establishment of the synthetic microbial community and were not used for further analyses as they did not remain sterile. For

each microcosm, we measured CO_2 production, litter mass loss and litter nitrogen and carbon content of the remaining litter. See supplementary methods for further details.

Before the addition of protists, microcosms with bacteria and fungi produced more CO_2 than microbial-free ones ($F_{1,92} = 431.16$, $p < 0.001$), and this effect was not different between temperatures ($F_{1,92} = 0.04$, $p = 0.846$; Fig. S1), indicating successful establishment of a synthetic microbial community after inoculation. After protistan addition, there was no interactive effect of protists and temperature on CO_2 production ($F_{3,40} = 1.48$, $p = 0.234$). However, both increased temperature ($F_{1,40} = 14.96$, $p < 0.001$) and presence of protists irrespective of their concentration ($F_{3,40} = 3.24$, $p = 0.032$) increased CO_2 production (Fig. 1a). A posthoc analysis indicated that protist addition effects appeared stronger at lower than at higher temperatures (Fig. 1; please note that boxplots highlight medians while posthoc tests compare means). An interaction between the protist and temperature treatment affected litter mass loss ($F_{3,40} = 10.50$, $p < 0.001$; Fig. 1b), indicating that the addition of protists at all concentrations increased litter mass loss at 17°C by more than 35% on average, but not at 21°C (Fig. 1b). The addition of protists did not affect litter carbon (C) ($F_{3,40} = 0.55$, $p = 0.653$) and nitrogen (N) content ($F_{3,40} = 0.03$, $p = 0.993$) and the litter C:N ratio ($F_{3,40} = 0.04$, $p = 0.990$) at the end of the experiment (Fig. S2). Litter N content was higher at 21 than at 17°C, indicating higher N loss during decomposition at lower temperatures ($F_{1,30} = 7.42$, $p = 0.010$; Fig. S2b), resulting in higher C:N ratios at 17°C than at 21°C ($F_{1,40} = 8.08$, $p = 0.007$).

Interaction-assays in split-petri dishes to test for volatile-induced microbial effects (Fig. S3) showed that protist growth (plasmodial length) was affected by bacterial ($F_{5,23} = 63.22$, $p < 0.001$) and fungal volatiles ($F_{5,24} = 12.29$, $p < 0.001$; Fig. 2). Presence of *Collimonas pratensis* T91, *Pseudomonas* sp. AD21 and *Trichoderma citrinoviride* reduced protist growth most strongly (Fig. 2). The overall negative effects of bacteria and fungi on protists likely through volatiles

contradict with the variable effects of volatiles on other protist species which ranged from stimulation to inhibition[13]. But as inhibition differed between microbial species, some potentially efficient decomposers might benefit through a reduction of competition from more easily preyed microbes, which could explain the observed increased decomposition rates. Yet, other mechanisms are likely to contribute to increased decomposition in presence of predators, such as predation-induced increased microbial activity or alternative enzyme production- details to be explored in future studies.

Our results support previous findings showing that predator-prey interactions within the microbiome affect microbial-derived CO_2 production [14], but we extend this knowledge and show that this effect tends to of lower importance at higher temperature. Furthermore, we now show that microbial predators alter litter decomposition in a temperature-dependent manner, with an increased importance at lower temperature. This result extends the known importance of larger-sized soil animals in increasing litter decomposition [15,16] and contrasts previous findings that microscopic predators (mostly protists and nematodes) have a limited effect on litter breakdown [16]. Mechanistically, protists might increase decomposition via microbe-specific predator-prey interactions [10] that change microbial community composition and functioning [17]. Our interaction-assays suggests that microbial predator-prey interactions mediated by volatiles could differ, which might benefit some efficient microbial decomposers.

The effect of protists on litter decomposition was strongest at lower temperatures, contradicting previous findings that larger soil animals have increased effects on decomposition at higher temperatures [18]. This discrepancy might be explained by the higher microbial diversity in our model communities compared to often single-decomposer model species used before, in which predation might favor metabolically active microorganisms [10]. The effect of predation on microbial-driven decomposition seems to differ between protists and soil animals,

as soil animals were shown to have limited effects on decomposition rates [16]. The increased importance of protist predation on microbial decomposition at lower temperatures suggest a more profound role of predation on carbon cycling in colder, non-tropical climates that host most microbial biomass [19] and store most carbon [20]. If this pattern can be confirmed with a wider range of protists, and in natural soils rather than this simplified laboratory assay, these microbial predators may play a key role in accelerating the global carbon cycle. Further studies should test exactly those by using realistic climate scenarios, more diverse protists and microbial decomposers, and in natural settings to untangle the importance of protists on decomposition and the carbon cycle. In turn, even more detailed laboratory analyses are needed to unreliably determine the exact mechanisms of how protists affect decomposition.

In summary, we reveal microbiome predation by protists as a key driver of microbial-driven decomposition with potential impacts on the global carbon cycle. Further integrated microbiome analyses are needed to investigate how and under which conditions microbial predation affects litter decomposition and if and how protists contribute to the global carbon cycle.

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Competing Interests

There are no conflicts of interests.

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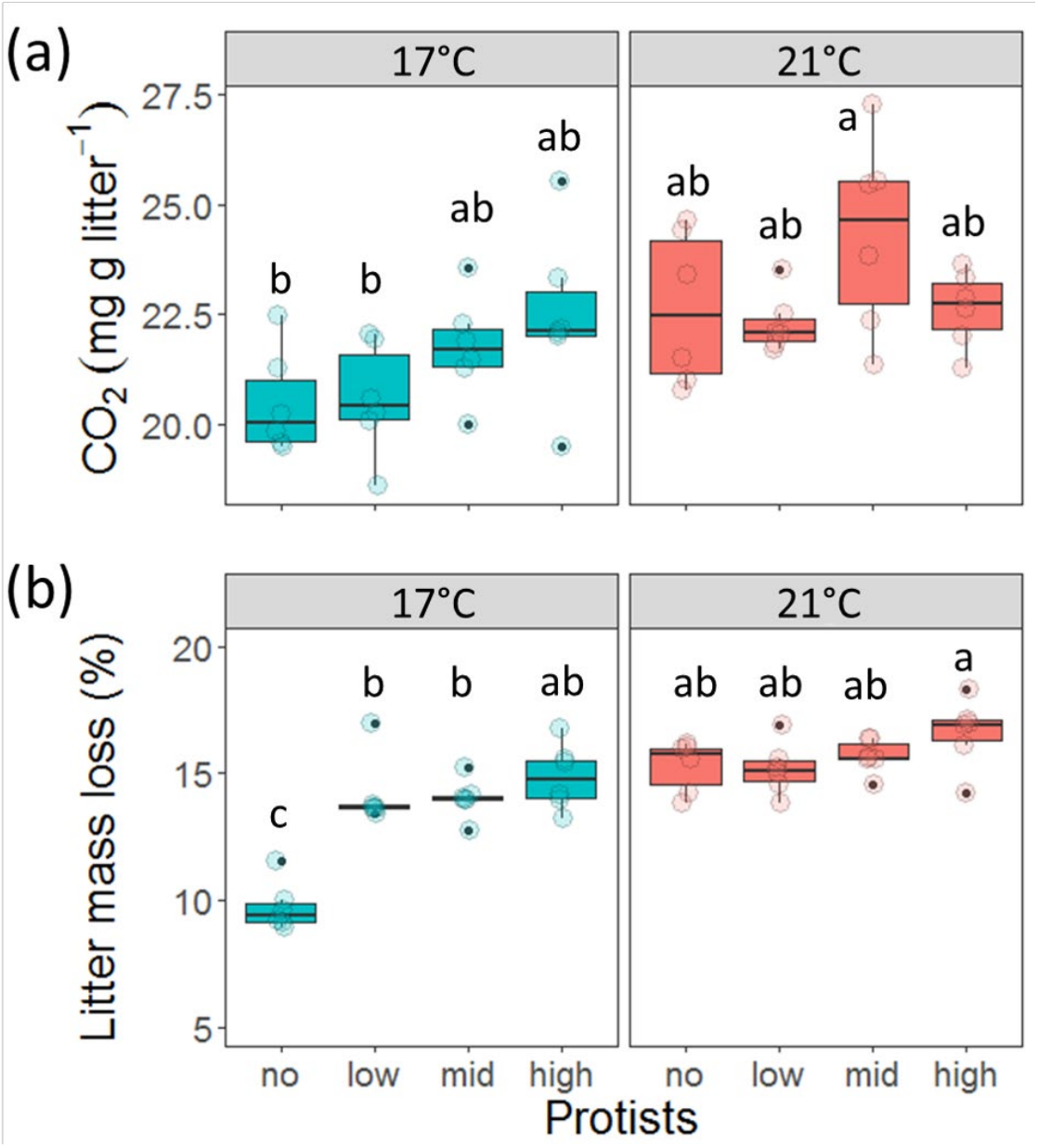
188 **Figures**

189

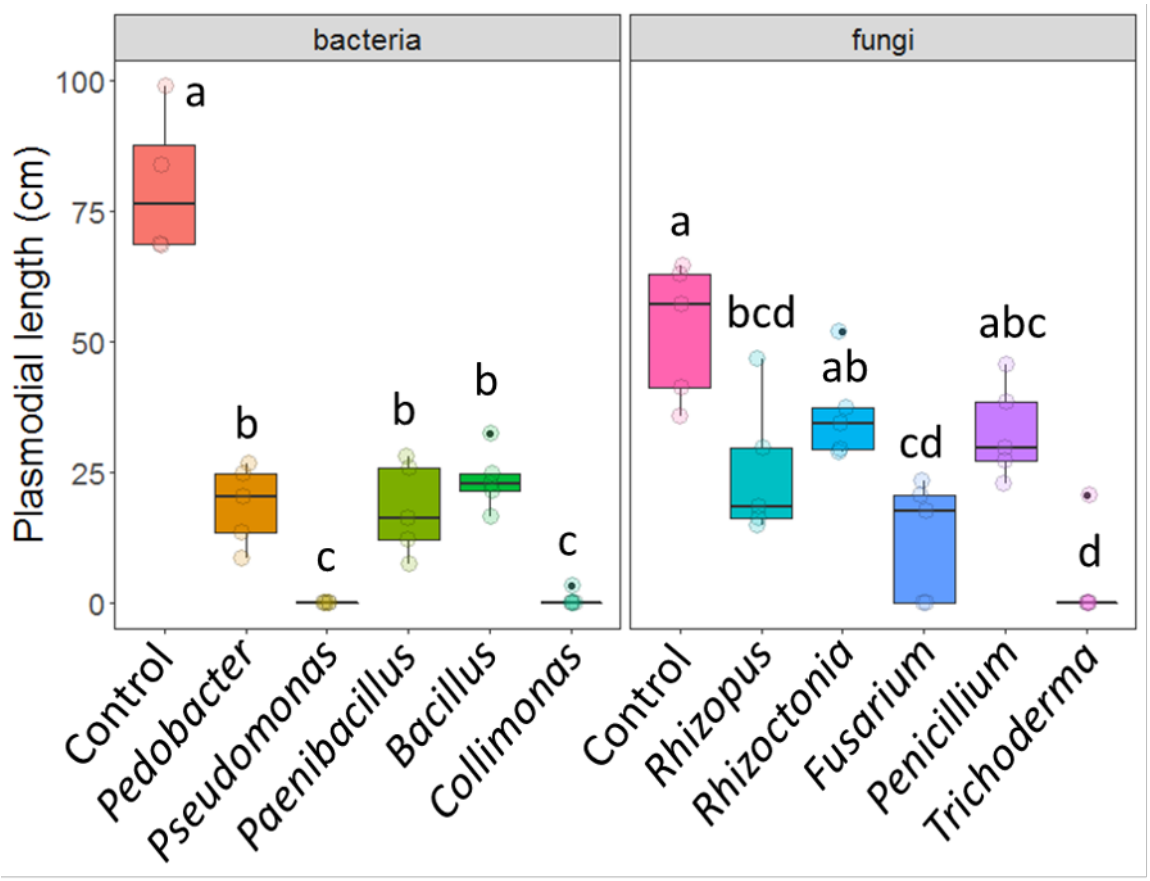


190

191 Fig. 1. Boxplots showing (a) cumulative CO_2 respiration (measured from the addition of protists
192 until the end of the experiment) and (b) litter mass loss for microcosms with no protists or low,
193 medium (mid) or high concentrations of protists (x-axis) at 17° and 21°C. Different letters above
194 the boxes indicate significant differences ($p < 0.05$) between treatments, as was indicated in a

195 Tukey HSD posthoc test. Tukey tests were carried out across the protists × temperature

196 interactions, so letters can be compared across facets.



197

198 Fig. 2. Boxplots showing plasmodial length of the model protist *Physarum polycephalum* in

199 response to different (a) bacterial and (b) fungal taxa (x-axis) that were part of the microbial

200 decomposer communities (Table S1 and S2). C is the control with only nutrient agar without

201 bacteria (left) or potato dextrose agar without fungi (right). Different letters above the bars

202 indicate that protist responses differed significantly ($p < 0.05$) between the microbial species in a

203 TukeyHSD test. TukeyHSD tests were carried out for bacteria and fungi separately, therefore

204 letters should be compared within panels only.

205

Supplementary information

Materials and Methods

Preparation of microorganisms

A total of 8 bacterial species, including 5 Gram-negative and 3 Gram-positive bacteria and differing widely in their phylogeny and in traits were used in the experiments (Supplementary Table 1). Well-grown bacterial cultures on 0.8% H_2O Agar with 16 ml/l Artificial Root Exudates (ARE) [1] were enriched by suspension in 3 ml Neff's Modified Amoebae Saline (NMAS) buffer [2] that was carefully scraped off the agar plates and vortexed in centrifuge tubes. Optical densities of all bacterial cultures were measured at 320 nm and adjusted to an OD of 0.5 by adding NMAS to cultures with higher OD.

Eight common soil fungi from the three major phylogenetic groups of Mucoromycotina, Ascomycota and Basidiomycota were used in the experiments (Supplementary Table 2). Well-grown cultures on universal fungal media plates containing 2% agar and 1.5 % malt-extract were suspended by adding 5 ml sterile demineralized H_2O, carefully scraping off spores and mycelia and vortexing in centrifuge tubes. Optical densities of all fungal cultures were measured at 546 nm and adjusted to an OD of 0.5 by adding sterile demineralized H_2O.

The plasmodium-forming protist *Physarum polycephalum* was grown on 1 % H_2O agar with 2% powdered oatmeal in the dark at 22-26 °C for 2-3 days. An agar block (6 mm in diameter) was cut from the plasmodial front to be used in the experiments.

Decomposition experiment under different temperatures

We set up a microcosm experiment in April 2019 to test the effect of microbial predation by the model protist *P. polycephalum* on litter decomposition by microorganisms. These microcosms consisted of 50-ml Falcon tubes to which we added 0.5 g leaf litter (*Quercus robur*), which was collected in the field in October 2017 immediately upon senescence and then was air-dried, sterilized by gamma irradiation (25 kGray) and cut into 1-cm pieces.

We created a microbial inoculum by equally mixing suspensions of all eight bacterial and fungal species. One day after adding the leaf litter, the microbial inoculum (40 µl) was added to half of the tubes. NMAS buffer (40 µl) was added to the other half as control to adjust potential effects of added nutrients. We allowed for microbial establishment for one week and then protists were added to the microcosms by transferring agar pieces containing plasmodium of *P. polycephalum*. Agar pieces without protist plasmodium were added to microbe-only treatments or controls (i.e., no microbial inoculum). We established four protist treatments: no protists (control), low, medium, or high concentrations (Supplementary Table 3). Half of the tubes were incubated at low temperature (17˚C for 16 hours by day and 13˚C for 8 hours at night) and the other half at high temperature (21˚C for 16 hours by day and 16˚C for 8 hours at night). This resulted in a total of 16 treatments: 2 microbial inocula (yes/no) × 4 protist treatments (no, or low, mid, or high concentration) × 2 temperature treaments (17/21 ˚C). We used six replicates per treatment, resulting in 96 microcosms in total. As we could not avoid contamination by airborne microbes

when opening the tubes for measurements throughout the experiment, the non-microbe controls were only used to check whether initial microbial inoculation was successful (i.e., resulted in increased respiration). As such, we compared non-microbial treatments with microbial treatments in a separate analysis to evaluate the success of initiating a decomposer microbiome. For the latter analyses, the non-microbe treatments were excluded to allow a balanced analysis to test for the impact of protists on decomposition. Microcosms were incubated in the climate chambers for nine weeks. Throughout the 9 weeks of the experiment, we watered the tubes four times using sterilized water (a total of 2.5 g microcosm^{-1}, equaling approx. 60% water holding capacity) to maintain a moist environment to sustain microbial activity. Microcosms were incubated with a cap loosely placed on top of them, to allow for CO_2 exchange.

We measured the microbial respiration (CO_2 efflux) to investigate microbial activity throughout the experiment. Measurements were collected at day 3, 8, 10, 15, 17, 23, 30, 44 and 58 of the experiment. Measurements at day 3 and 8 were before the addition of protists (establishment phase of microbial inocula), day 10 was two days after the addition of protists. When starting the sampling procedure, we closed all microcosms using lids with a septum. We included four empty tubes (no litter, no inocula) as measurement controls. All tubes were flushed with CO_2-free air for 2 min at 2 bar, incubated for 4 h at 17˚C and 21˚C, respectively. After 4 h we collected 12 ml of headspace CO_2 with a syringe and transferred this into Exetainers (Labco, Lampeter, United Kingdom). Exetainers were stored at 4 °C until measurement on a CH_4/CO_2 analyser (Thermo Scientific Trace Ultra Gas Chromatography system using Tri-Plus RSH injection Robot (Thermo Scientific). CO_2 concentrations (µg/g litter) were calculated using Chromeleon 7.2 (Thermo Scientific).

We determined litter mass loss by comparing the initial litter mass (g) and litter mass (g) after freeze-drying (Manual Labconco Freezone 12 Freeze-dryer) the samples for four days at the end of the experiment. A subsample of the litter was ground and used to determine the content of carbon (C) and nitrogen (N) on an Element Analyzer (Thermo Scientific Flash EA 1112).

Interaction experiment through volatiles between *Physarum* and other microbes

Two-compartment petri plates (90 mm) were filled half with ARE agar to be inoculated in the center of one side with a 6 mm *Physarum* plasmodium-containing agar blocks. The other half was filled with Nutrient Agar (3 g beef extract, 5 g peptone, 15 g agar) for inoculating 20 µl suspension of individual bacteria or with Potato Dextrose Agar (4 g potato extract, 20 g dextrose, 15 g agar) for inoculating 20 µl of individual fungi. Bacterial and fungal suspensions were equally spread across the surface with a sterile plastic L rod (Fig. S3). Uninoculated culture media served as controls. Five replicate plates were prepared per treatment and incubated at 20°C in the dark. Then, the distance travelled by the plasmodia was measured after 3 days of incubation. Controls were established where no bacteria or fungi were added on the other side of the protist. All plates were sealed with air-permeable parafilm to reduce an accumulation of air in the containers. As the protists and bacteria/fungi were inoculated to different sides of the petri dishes and no contamination of any microbe to the non-inoculated side was observed throughout the experiment, we used the setup to test for volatile-induced effects of the bacteria and fungi on the protist, as often these volatile effects mirror those of direct predation interactions [3].

Data Analyses

Data were analysed in R [4].

To test the effect of the microbial inoculum on initial CO_2 production (first week after addition of microbial inocula) we used a general linear model with microbial inoculum (yes or no microbes added), temperature treatment (17 or 21^{o}C) and their interaction as fixed factors and cumulative CO_2 production in the first week as a response variable. This test was used to check whether the microbial inoculum was effective, i.e., resulted in CO_2 respiration. For further analyses we focused only on the microcosms with microbial inocula because we were not able to maintain microcosms without microbial inocula sterile and because our main aim was to test how predation by protists affected decomposition processes. To test how protist addition affected cumulative CO_2 production (total CO_2 production in the period after protist addition), litter mass loss and litter C and N content in the remaining litter we used general linear models with protist concentration (no protists and low, medium and high concentrations) and temperature treatment as fixed factors. To test the interaction between the model protist and other microbes, we used a general linear model with plasmodial length as a response variable and bacterial and fungal species as respective fixed factors.

Supplementary Tables

Supplementary Table 1. Bacteria used in the experiment

Code	Gram stain	Bacterial family	Bacterial strain
B1	Gram (-)	Sphingobacteriaceae	*Pedobacter* sp. VuD
B2	Gram (-)	Oxalobacteraceae	*Janthinobacterium* sp. ADdo
B3	Gram (-)	Pseudomonadaceae	*Pseudomonas* sp. AD21
B4	Gram (+)	Paenibacillaceae	*Paenibacillus* sp. Add7
B5	Gram (-)	Rhodanobacteraceae	*Dyella* sp. AD56
B6	Gram (+)	Bacillaceae	*Bacillus* sp. V102
B7	Gram (-)	Enterobacteraceae	*Serratia plymuthica* PR1-2C
B8	Gram (-)	Oxalobacteraceae	*Collimonas pratensis* T91

Supplementary Table 2. Fungal cultures used in the experiments

Code	Fungal	Fungal (sub)phylum	Fungal strain
F1	Saprotroph	Mucoromycotina	*Rhizopus oryzae*
F2	Saprotroph	Mucoromycotina	*Mucor hiemalis*
F3	Plant pathogen	Basidiomycota	*Rhizoctonia* sp.
F4	Saprotroph	Basidiomycota	*Ceratobasidium* sp.
F5	Plant pathogen	Ascomycota	*Fusarium oxysporum*
F6	Plant pathogen	Ascomycota	*Phoma exigua*
F7	Saprotroph	Ascomycota	*Penicillium chrysogenum*
F8	Saprotroph	Ascomycota	*Trichoderma citrinoviride*

Supplementary Table 3. The communities of protists with (+) or without (–) fungi and bacteria. Communities 1, 3, 5 and 7 were established as an initial control to check for successful inoculation but were not further analysed as the containers were not maintained sterile during measurement. All communities were incubated at 17 and 21°C, resulting in 16 experimental treatments in total.

Communities	*P. polycephalum* concentration	*P. polycephalum* plasmodium-containing agar blocks (1 cm^2) added to microcosms	Bacteria & Fungi
1	No protists	Circular area of sterile agar	–
2			+
3	Low	¼ area of agar with plasmodium + ¾ of sterile agar	–
4			+
5	Medium	½ area of agar with plasmodium + ½ of sterile agar	–
6			+
7	High	Full circular area of agar with plasmodium	–
8			+

Supplementary Figures

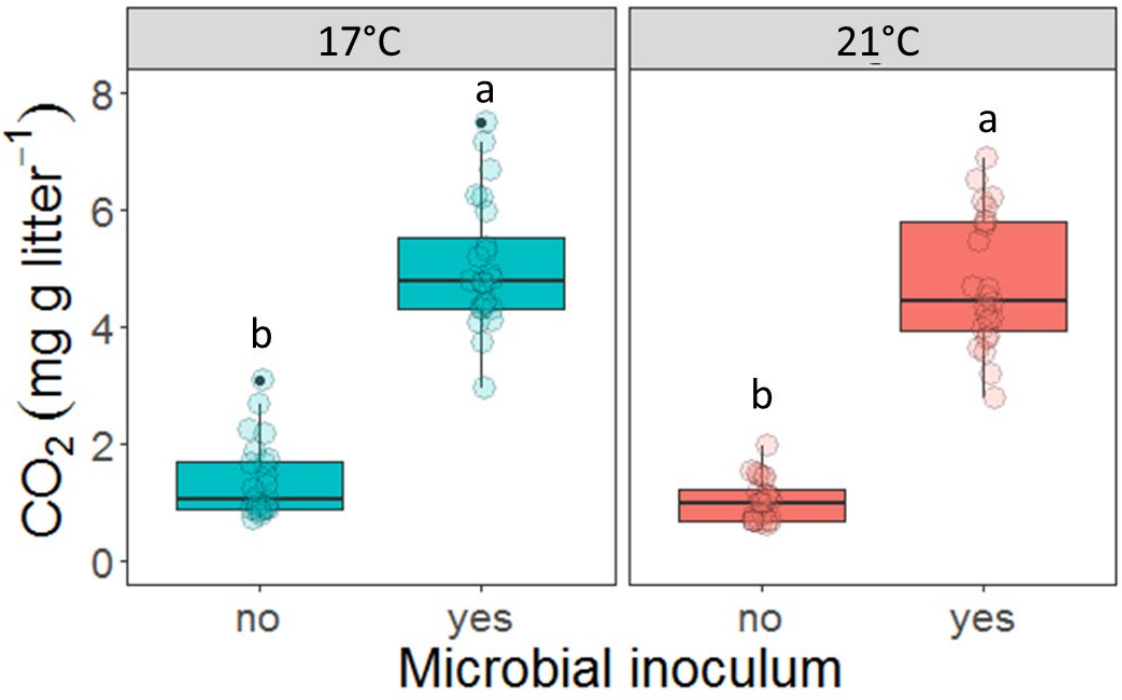
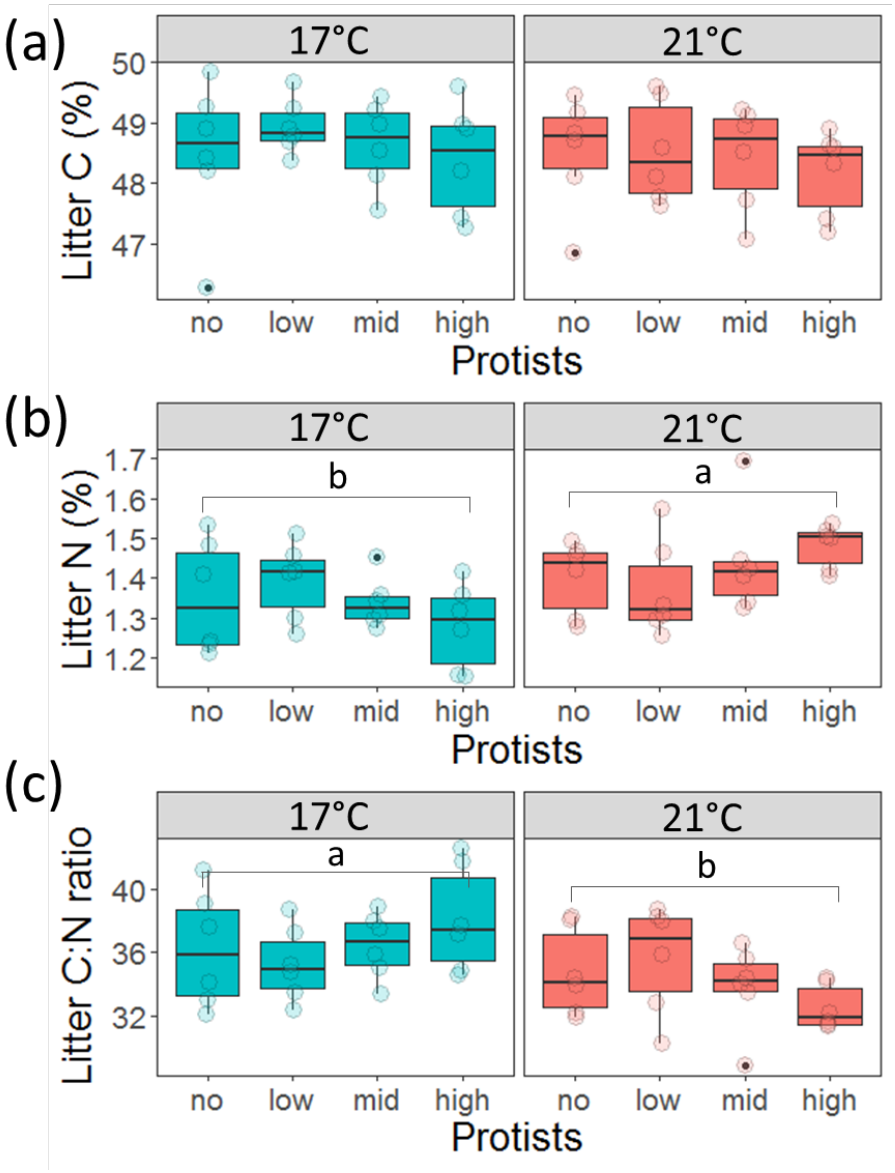


Fig. S1. Boxplots showing cumulative CO_2 respiration measured in the first week after the addition of the microbial inoculum (before the addition of the protists) at 17° and 21°C. Different letters above the boxes indicate significant differences ($p < 0.05$) between treatments, as was indicated in a Tukey HSD posthoc test. Tukey tests were carried out across inoculum × temperature interactions, so letters can be compared across facets.



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327 Fig. S2. Boxplots showing (a) litter carbon content (C), (b) litter N (N) content and (c) litter C:N
328 ratio at 17° and 21°C at the end of the experiment. Different letters above the boxes indicate
329 significant differences ($p < 0.05$) between treatments, as was indicated in a Tukey HSD posthoc
330 test. Tukey tests were carried out across temperature treatments, so letters can be compared
331 across facets.

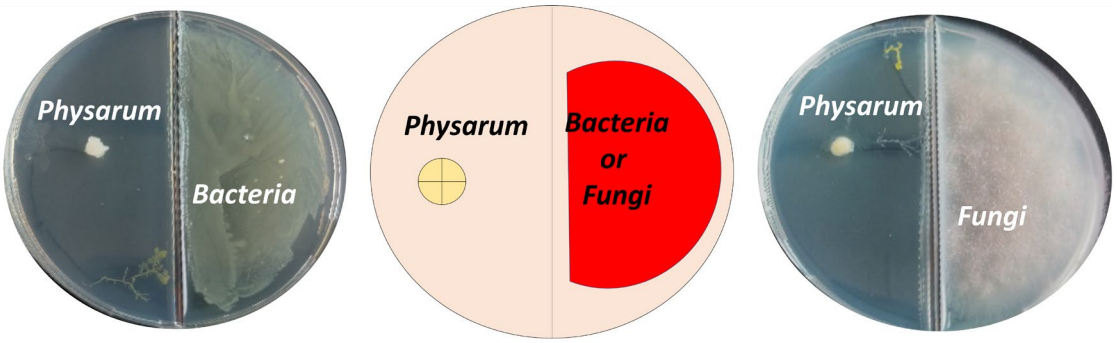

Fig. S3. Setup of the interaction experiment between *Physarum polycephalum* and individual bacterial and fungal species.

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