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Cite this article: Tan J, Wei N, Turcotte MM. 2024 Trophic interactions in microbiomes influence plant host population size and ecosystem function. *Proc. R. Soc. B* **291**: 20240612.
<https://doi.org/10.1098/rspb.2024.0612>

Received: 14 March 2024

Accepted: 8 April 2024

Subject Category:

Ecology

Subject Areas:

ecology

Keywords:

parasitism, plant–microbiome interactions, predation, ecosystem functions

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Electronic supplementary material is available online at <https://doi.org/10.6084/m9.figshare.c.7218574>.

Trophic interactions in microbiomes influence plant host population size and ecosystem function

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Plant microbiomes that comprise diverse microorganisms, including prokaryotes, eukaryotes and viruses, are the key determinants of plant population dynamics and ecosystem function. Despite their importance, little is known about how species interactions (especially trophic interactions) between microbes from different domains modify the importance of microbiomes for plant hosts and ecosystems. Using the common duckweed *Lemna minor*, we experimentally examined the effects of predation (by bacterivorous protists) and parasitism (by bacteriophages) within microbiomes on plant population size and ecosystem phosphorus removal. Our results revealed that the addition of predators increased plant population size and phosphorus removal, whereas the addition of parasites showed the opposite pattern. The structural equation modelling further pointed out that predation and parasitism affected plant population size and ecosystem function via distinct mechanisms that were both mediated by microbiomes. Our results highlight the importance of understanding microbial trophic interactions for predicting the outcomes and ecosystem impacts of plant–microbiome symbiosis.

1. Introduction

Plant–microbiome symbiosis plays a vital role in ecological communities, driving critical ecosystem functions [1–3]. There is growing recognition that plant microbiomes, which comprise diverse microorganisms associated with plants, serve as essential players that connect different components of ecosystems [4–6]. Indeed, both microbiomes and plants can be directly involved in ecosystem functions, including altering nutrient cycling, participating in pollutant uptake, and contributing to contaminant degradation, whereas microbiomes can also influence ecosystem functions indirectly by affecting plant hosts' physiology, growth and reproduction [7–13]. Nevertheless, the extent to which species interactions within plant microbiomes (microbe–microbe interactions) affect plant populations and ecosystem functions remains unclear [4,14,15]. As microbe–microbe interactions have often been studied in pairs or synthetic communities *in vitro* [14,16,17], they often do not fully capture the complexity of natural microbiomes that comprise producers, consumers, decomposers and more. Ignoring the emergent impacts of common species interactions within microbiomes may limit our understanding of how microbiomes influence their macroscopic hosts and ecosystem functions.

Trophic interactions, which involve the transfer of energy and materials through different trophic levels, are pervasive in nature [18–21]. Bacteria play a foundational role in ecosystems, supporting diverse consumers from macroorganisms such as metazoans to microorganisms such as protists and viruses. For example, in terrestrial plant and soil microbiomes, metazoan predators, including nematodes and microarthropods, have been shown to influence microbiome composition and the fitness of plant hosts [22–24]. Similarly, metazoan predators in aquatic ecosystems, including common filter feeders, like rotifers

and microcrustaceans, contribute to microbial consumption, potentially influencing microbial communities, akin to their terrestrial counterparts [25–27]. Similar to these macroscopic predators, microorganisms, including bacteriophages and protists (e.g. flagellates, ciliates and amoebae), which are widespread in both terrestrial and aquatic ecosystems, are also important bacterivorous consumers [28–30]. Nevertheless, our knowledge on the role of trophic interactions between microbes, especially within plant microbiomes, in mediating microbiome structure and function on plant population dynamics and ecosystem function remains limited.

In plant microbiomes, microorganisms, including prokaryotes, viruses and eukaryotes, form complex networks [7,31] via horizontal resource-mediated interactions (e.g. competition and mutualism) and via trophic interactions that vertically connect microbial hosts with parasites and predators with prey (e.g. parasitism and predation) [8]. Among them, viruses that infect bacteria (bacteriophages or phages) [32] and protists [33] represent two prominent types of bacterivorous consumers with their impacts on microbiomes potentially varying markedly. Phages show considerable variation in their life history. For example, lytic phages can trigger acute bacterial mortality by lysing bacterial cells, leading to the transfer of energy and materials within microbiomes. On the other hand, temperate phages, often persisting as prophages by integrating into bacterial genomes during lysogenic cycles, may contribute to lower engagement in trophic interactions [34]. Compared to protists that are often viewed as generalist microbial predators, phages function as more specialized microbial parasites. Phages display varying degree of host specificity, selectively targeting one or several groups of microbes, potentially leading to a reduction in microbiome richness and a shift in composition [32,35]. While phages can decrease the abundance of their target bacteria, their overall impact on the microbiome abundance varies. This variability depends on factors, such as whether phages target dominant bacterial taxa and/or foster the growth of rare bacterial taxa, serving as keystone species. Unlike phages, protists, as more generalist predators, can graze across a broader spectrum of bacterial taxa, often exhibiting density-dependent foraging behaviours [33]. As a result, protists may contribute to a reduction in overall microbiome abundance and serve as a stabilizing mechanism, suppressing dominant species and promoting species coexistence within microbiomes.

Moreover, phages and protists can directly influence plants and ecosystem functions or indirectly impact them by affecting bacteria within microbiomes. For example, phages and protists may influence plant populations and ecosystem functions by facilitating the recycling of limiting nutrients and releasing growth-stimulating substances to the environment [36]. Through trophic interactions with bacteria, phages and protists may also have the potential to alter the properties of microbiomes, such as microbial abundance, richness and composition, thereby indirectly impacting host plant populations and ecosystem functions. It is important to note that diet preferences of consumers span on a continuous spectrum, several possible scenarios mentioned above regarding how microbial consumers impact microbiomes, plant populations and ecosystem functions may occur simultaneously.

Here, we examined how trophic interactions within plant microbiomes influenced plant populations and ecosystem functions using the common duckweed, *Lemna minor*, and their natural microbiomes. By experimentally manipulating the initial abundance of lytic phages and protists in the bacterial microbiomes of *L. minor*, we examined the effects of trophic interactions on bacterial microbiomes and plant population size. Moreover, we assessed the responses of phosphorus removal, which serves as a crucial ecosystem function that reflects the collaborative efforts of microbes and plants in freshwater ecosystems. Our research aimed to gain a deeper understanding of the contributions of microbe–microbe and microbe–plant interactions to the overall functioning of ecosystems.

2. Materials and methods

(a) Study system and sampling

We used *L. minor* as the plant host for our experiment. Two *L. minor* genotypes that were genetically differentiated by two polymorphic microsatellite loci [37,38] were collected from lakes in Boyce Mayview Park (40.33566° N, 80.11269° W) and Deer Lakes State Park (40.62245° N, 79.82246° W) in Pennsylvania, USA. To create axenic *L. minor* lines, we first rinsed *L. minor* in 4.5 ml sterile water in glass test tubes (16 × 150 mm) and then transferred into 8 ml 0.025 M phosphate buffer saline (PBS) and sonicated for 5 min. We then bleached *L. minor* with 1% sodium hypochlorite for 20 s before transferring into sterile media (8 ml 1 : 4 Schenk and Hildebrandt (SH) basal salt medium; Sigma-Aldrich, St Louis, Missouri, USA) for propagation under 24 h light. This bleaching process was repeated multiple times until no microbes growing in the duckweed medium. To further verify the axenic condition of the *L. minor* cultures, we tested the presence or absence of microbes using the sequencing services at Azenta US, Inc. (South Plainfield, New Jersey, USA). No bacterial 16S or fungal ITS sequences were detected [14].

We used duckweed microbiomes that were naïve to the two focal *L. minor* genotypes to avoid pre-existing local adaptation of microbiomes to host plants that might cause genotypic differences in fitness. Bacterial communities and phages were extracted from the natural microbiomes of *L. minor* collected from the City Park Lake (30.42900° N, 91.16747° W; microbiome source 1) and College Lake (30.40714° N, 91.16888° W; microbiome source 2) in Louisiana, USA four days prior to the experiment. We collected 50 clusters of *L. minor* (approx. 150 individuals) at each location and vortexed them gently in 4.5 ml sterile water for 10 s to remove the microbial residents from lake water. We then transferred the clusters to 8 ml PBS and sonicated at 40 kHz for 5 min to release duckweed microbiomes into PBS. We further removed eukaryotes by filtering 6 ml of the microbiome extracts using 1.2 µm PTFE sterile syringe filters (Acrodisc, Pall Laboratory, New York, USA), and confirmed the depletion of eukaryotes by screening 0.1 ml of the filtered microbiome extracts using optical microscopy.

The filtered microbiome extracts contained bacteria and viruses. To pellet the bacteria, we centrifuged the filtered microbiomes at 3000 rpm for 15 min and discarded the supernatant that contained mostly viruses. To further purify the bacteria and remove remaining extracellular viruses, we re-suspended the bacterial pellets in 1 ml PBS by shaking at 500 rpm for 30 min, and centrifuged at 3000 rpm for 15 min to concentrate the bacterial pellets in 0.05 ml PBS. We repeated these steps six times, resulting in approximately 6.4×10^7 times dilution of viruses, especially the lytic phages, while prophages that were integrated into host genomes could not be removed by rinsing and remained inside some bacterial cells. We suspended the final bacterial pellets in 1 ml 15% glycerol solutions and preserved them at –80°C.

To isolate extracellular phages, we filtered 2 ml of the original microbiome extracts using 1.2 µm (Acrodisc, Pall Biotech, Westborough, Massachusetts, USA) and then 0.2 µm (Whatman Uniflo, Cytiva, Massachusetts, USA) PTFE sterile syringe filters. The virus extracts were

preserved with 15% glycerol at -80°C before the experiment. The presence of lytic phage particles was confirmed by screening the filtered extracts using plaque formation assays (see electronic supplementary material). Using epifluorescence microscopy [39,40], 52 000 and 24 000 virus-like particles per ml were detected in microbiome sources 1 and 2, respectively. Note that the concentration of virus-like particles in duckweed microbiomes was considerably lower compared to that reported in other studies and in natural microbiomes [36,41]. Due to insufficient viral genetic material, we did not characterize the phages in the microbiome sources.

Our observation showed that in the *L. minor* microbiomes, the most common protists are ciliated bacterivorous generalists, which could consume a large spectrum of bacterial species [42]. We chose one of them, *Tetrahymena pyriformis*, as a representative of the protist communities. We acquired axenic *T. pyriformis* from the Carolina Biological Supply (Burlington, North Carolina, USA), and maintained it in 6 ml 10 g l^{-1} peptone water at 24°C prior to the experiment.

(b) Trophic interaction experiment

We used a three-way factorial design that manipulated host plant genotypes (A and B), microbiome sources (1 and 2), and trophic interactions (control, phage addition and protist addition). We replicated the experiment three times with 36 total microcosms. These microcosms were loosely capped glass test tubes ($16 \times 150\text{ mm}$) with 8 ml autoclaved 1:4 SH salt medium.

For the experiment, we first introduced one cluster (with three to four individuals) of axenic *L. minor* into each microcosm. We then inoculated the bacterial microbiomes by thawing the frozen bacterial isolates, diluting in 8 ml 1:4 SH salt medium, and transferring 0.1 ml of approximately $10^5\text{ cells ml}^{-1}$ into the designated microcosms. In the phage addition treatment, we thawed the frozen virus isolates and transferred 10 μl of the isolates to the designated microcosms. The virus isolates from microbiome sources 1 and 2 were added to microcosms that contained the bacteria from the same source. In the protist addition treatment, we diluted the axenic culture of *T. pyriformis* in 1:4 SH salt medium and inoculated roughly 100 individuals (10 μl) into each of the designated microcosms. The control treatment with lytic phages and protists depleted received 10 μl autoclaved 1:4 SH salt medium instead. The microcosm experiment was carried out in a Percival light incubator (I-36LL; Percival Scientific, Perry, Iowa, USA) under the 12 h photocycle and $24^{\circ}\text{C}/16^{\circ}\text{C}$ day/night temperatures. The photosynthetic photon flux level was maintained at $75\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ during the day. Microcosms were randomized once a week. As *L. minor* populations reached equilibrium in four weeks, the experiment was terminated on day 35.

The treatments of phage and protist additions have the potential to directly affect *L. minor* population size and ecosystem function in the absence of bacterial microbiomes. For example, plant viruses might remain in phage extracts, driving a possible direct effect on plant hosts. To examine this possibility, we set up a follow-up experiment where we manipulated host genotypes (A and B) and trophic interaction types (control, phage addition and protist addition) without bacterial microbiomes. This follow-up experiment was also replicated three times and followed the settings as described above.

(c) Plant population size and phosphorus concentration in the microcosms

We measured plant population size by counting the number of *L. minor* individuals in each microcosm at the end of the experiment. For phosphorus concentration, we measured total phosphorus concentration remained in the medium, following Wetzel and Likens [43]. As each microcosm started with the same amount of phosphorus at the beginning of the experiment, a lower remaining phosphorus concentration indicated a higher phosphorus removal rate by organisms in the microcosms. Briefly, we gently vortexed the microcosms, collected 1 ml from each microcosm, and removed particles (such as plant tissues and bacteria and protist cells) by filtration through 0.2 μm PTFE filters (Whatman Uniflo). The phosphorus in the 1 ml sample was first oxidized by 0.16 ml 5% persulfate solution and then reacted with 0.1 ml composite reagent of 0.6% ammonium molybdate, 7.5% sulfuric acid, 1.2% ascorbic acid, and 0.014% potassium antimonyl-tartrate, prior to reading under OD885 against the standard solutions of potassium dihydrogen phosphate ($0\text{--}2\text{ mg l}^{-1}$) using a microplate reader (EPOCH2, Biotek, Santa Clara, California, USA).

(d) Measuring microbiome properties

We measured the bacterial abundance, richness and community composition in each microcosm at the end of the experiment. To measure bacterial abundance, we sonicated the microcosms for 5 min to release duckweed microbiomes to the liquid. Due to the presence of other particles (e.g. plant tissues and protist cells) in the microcosms, we stained the bacterial cells in a 0.15 ml sample with 5% crystal violet. The cells were then pelleted and rinsed twice with 95% ethanol and screened under OD570. We approximated bacterial cell concentration as $2 \times 10^7\text{ cells ml}^{-1}$ per one OD570 unit (see electronic supplementary material) and determined the final bacterial abundance in each microcosm. We also counted the protist abundance using a haemocytometer with a stereomicroscope at the end of the experiment, but not phage abundance due to logistical constraints.

To measure bacterial richness and community composition, we conducted microbiome sequencing. We pelleted bacterial cells from a 4.5 ml sample taken from each microcosm, which contained a fraction of bacteria from all duckweed individuals, by centrifuging at 3000 rpm for 30 min. We then extracted bacterial DNA with a Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, California, USA). Two negative controls that contained the SH salt medium alone were included in the process of DNA extraction. The 36 samples and two negative controls were sent to Novogene Corporation (Sacramento, California, USA) for library preparation (16S rRNA V5–V7 region; with primers 799F: AACMGGATTAGATACCKG, 1193R: ACGTCATCCCCACCTTCC) and sequencing on an Illumina MiSeq paired-end 250 bp. While the two negative controls failed in sequencing, we obtained 81 913 raw reads on average per sample (range = 51 411–98 957).

These paired-end (PE) raw reads were used for detecting bacterial amplicon sequence variants (ASVs) using the package DADA2 v1.14.0 [44] in R v3.6.2 [45]. Following previous pipelines [46–48], the PE reads were trimmed and quality filtered [$\text{truncLen} = \text{c}(220, 220)$, $\text{maxN} = 0$, $\text{truncQ} = 2$, $\text{maxEE} = \text{c}(2, 2)$], prior to specific variant identification that took into account sequence errors. The PE reads were then end joined ($\text{minOverlap} = 20$, $\text{maxMismatch} = 4$) for ASV detection and chimera removal. ASVs were assigned with taxonomic identification based on the SILVA reference database (132 release NR 99) implemented in DADA2. A rooted phylogenetic tree of the ASVs was built using QIIME 2 v2019.10 [49]. These ASVs were further filtered using the package phyloseq [50]. First, we removed non-focal ASVs (Archaea, chloroplasts and mitochondria). Second, we conducted rarefaction analysis using the package iNEXT [51] to confirm that the sequencing effort was sufficient to capture the bacterial richness (electronic supplementary material, figure S1). We further normalized per-sample reads by rarefying to the lowest number of the filtered reads (28 343) across samples. Lastly, we removed low-frequency ASVs (less than 0.001% of total

observations). The final bacterial community matrix consisted of 497 ASVs across 36 samples. Bacterial richness and diversity were quantified as the number of ASVs and the Shannon diversity index, respectively. Both measures provided qualitatively similar results. We used bacterial richness as a more direct measure of diversity for the subsequent analyses.

(e) Statistical analyses of plant population size and phosphorus concentration

To evaluate how trophic interactions, microbiome sources and host plant genotypes influence plant population size and total phosphorus concentration, we conducted general linear models (LMs) in R. The predictors of the LMs included trophic interaction (control, phage addition and protist addition), microbiome source (1 and 2), and host genotype (A and B), as well as their two-way and three-way interactions. The response variables were natural log transformed to improve normality. The data from the main and follow-up experiment (in the absence of bacterial microbiomes) were analysed separately. Statistical significance (type III sums of squares) and least-squares means (LS-means) of predictors were assessed using the packages *emmeans* [52] and *phia* [53].

(f) Statistical analyses of bacterial abundance, richness and community composition

To evaluate how trophic interactions, microbiome sources and host plant genotypes influence bacterial abundance (OD570) and richness, the predictors of the LMs included trophic interaction (control, phage addition and protist addition), microbiome source (1 and 2) and host genotype (A and B), as well as their two-way and three-way interactions. The response variable (bacterial abundance or richness) of each LM was power transformed if necessary to improve normality, with the optimal power parameter determined using the Box–Cox method in the package *car* [54] (power parameter = 1, no transformation, for both bacterial abundance and richness here).

Bacterial community composition was assessed based on Bray–Curtis dissimilarity using constrained principal coordinates analysis (cPCoA) with *capscale()* in the package *vegan* [55]. To assess the significance of the main effects, the predictors of cPCoA included trophic interaction, microbiome source and host genotype. To assess the significance of two-way interactions, the predictors of cPCoA included both the main effects and their two-way interactions. Likewise, to assess the significance of the three-way interaction, the predictors of cPCoA included the main effects, two-way interactions and the three-way interaction following our previous work [56]. To further pinpoint the bacterial ASVs that affected the cPCoA, we focused on ASVs with large influence (scores ≥ 0.01 or ≤ -0.01) on the cPCoA axes.

(g) Structural equation modelling

To evaluate how trophic interactions influence plant population size and ecosystem function via bacterial microbiomes, we conducted structural equation modelling (SEM) to link trophic interactions to bacterial abundance (OD570), richness (ASVs) and community composition (cPCoA 2, which was strongly influenced by trophic interactions) to plant population size and total phosphorus concentration. The SEM did not include microbiome source (1 and 2) as an exogenous variable, because microbiome source showed weak effects on bacterial abundance, richness and bacterial cPCoA 2, although it influenced bacterial cPCoA 1. Likewise, the SEM did not include host genotype (A and B) as an exogenous variable, due to its weak effects on bacterial microbiomes (abundance, richness and composition) and plant population size, especially compared to trophic interactions. For trophic interactions, we coded the control treatment as the reference level so that the effects of all other treatments (phage addition and protist addition) were relative to the control. Plant population size and total phosphorus concentration were natural log transformed as described in the LMs above. The full model of SEM (electronic supplementary material, figure S2) was fitted using maximum likelihood with robust Huber–White standard errors using the package *lavaan* [57]. To reduce model complexity, the SEM was re-fitted with only notable paths ($p < 0.10$) present. Model fit was confirmed (comparative fit index, CFI > 0.9 ; root mean squared error of approximation, RMSEA, the lower bound of 90% confidence interval < 0.05 ; standardized root mean squared residual, SRMR < 0.1). To account for the potential influence of limited sample size, we further re-fitted the SEM using bootstrapping in *lavaan*. The two estimators (maximum likelihood and bootstrapping) resulted in consistent results.

3. Results

(a) Contrasting effects of parasitism and predation on plant population size and phosphorus concentration

Duckweed population size was significantly lowered in the phage addition treatment but increased in the protist addition treatment relative to the control where consumers were depleted (figure 1a; electronic supplementary material, table S1; LM: trophic interaction, $F = 129.6$, $p < 0.001$; planned contrasts within the LM: control versus phage addition, $F = 138.7$, $p < 0.001$, control versus protist addition, $F = 13.1$, $p = 0.001$). We observed an opposite pattern for the concentration of remaining phosphorus: higher in the phage addition treatment but lower in the protist addition treatment relative to the control (figure 1b; electronic supplementary material, table S2; trophic interaction: $F = 82.9$, $p < 0.001$; control versus phage addition, $F = 56.4$, $p < 0.001$, control versus protist addition, $F = 28.1$, $p < 0.001$). According to our follow-up experiment, in the absence of the bacterial microbiomes, phage and protist additions did not influence duckweed population size ($F = 0.20$, $p = 0.66$) and phosphorus concentration ($F = 0.11$, $p = 0.74$), indicating that the effects of parasitism and predation via phage and protist additions on duckweed populations and phosphorus concentrations were mediated through the bacterial microbiomes.

(b) The mechanisms by which parasitism and predation influence plant population size and phosphorus concentration

To examine how trophic interactions within the microbiomes influenced duckweed population size and phosphorus concentration, we sequenced the microbiomes using 16S rRNA sequencing. We found that trophic interactions had a significant impact on bacterial abundance, richness and community composition (figure 2), with phage and protist additions affecting different aspects of the microbiomes. Specifically, protist addition reduced bacterial abundance (LM, $F = 4.45$, $p = 0.045$; figure 2a; electronic supplementary material, table S3). Such reduction was primarily caused by the decrease of the most abundant bacterial ASVs (electronic supplementary material, figure S3 and table S4), despite that the most abundant ASVs differed between microbiome source 1 (*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* sp.; ANPR) and source 2 (*Burkholderia-Caballeronia-Paraburkholderia*

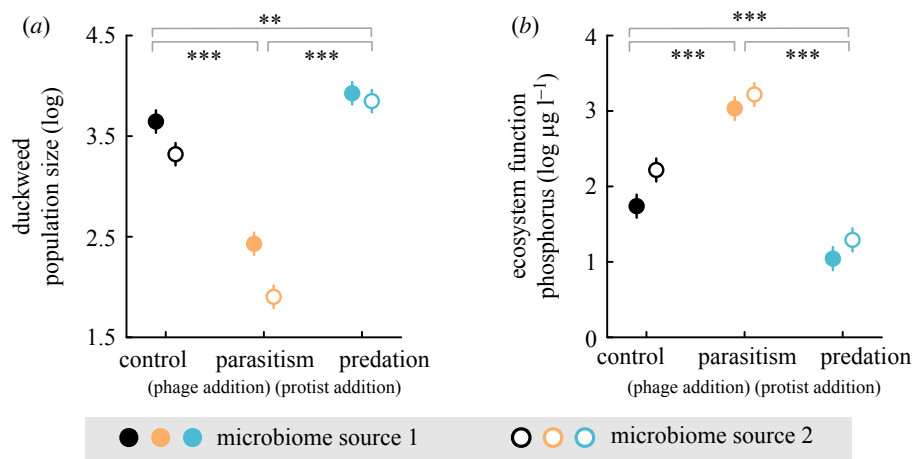


Figure 1. Plant population size and ecosystem function differ strongly among trophic interaction treatments. The least-squares means (LS means) of natural log transformed (a) duckweed population size and (b) ecosystem phosphorus concentration are plotted with error bars (SE) based on general linear models (predictors: interaction treatment, microbiome source, genotype, and their two-way and three-way interactions). Significant LS mean contrasts between treatments are denoted: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. Duckweed population size decreased and phosphorus concentration increased in the parasitism treatment (phage addition) relative to the control. The opposite pattern was observed in the predation treatment (protist addition) relative to the control. See statistical details in electronic supplementary material, tables S1 and S2.

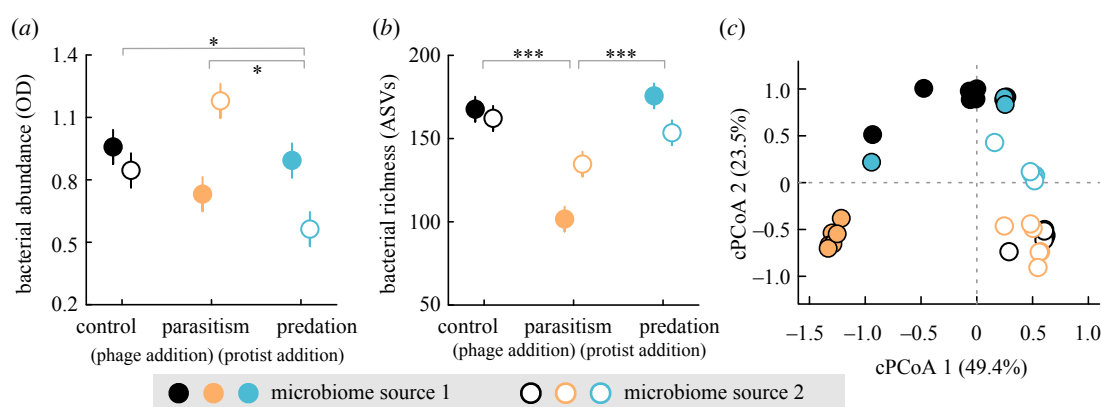


Figure 2. Bacterial microbiomes differ strongly among trophic interaction treatments. Bacterial microbiomes were characterized using (a) optical density (OD) at 570 nm to indicate bacterial abundance, (b) amplicon sequence variants (ASVs) to represent bacterial richness, and (c) constrained principal coordinates analysis (cPCoA) to characterize community composition. The least-squares means (LS means) of bacterial OD and ASVs are plotted with error bars (SE) based on general linear models (predictors: interaction treatment, microbiome source, genotype, and their two-way and three-way interactions). Significant LS mean contrasts between treatments are denoted: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. The cPCoA revealed a strong effect of microbiome source ($F = 24.5$, $p = 0.001$) on composition along the first axis (cPCoA 1), and a strong effect of interaction treatment ($F = 10.6$, $p = 0.001$) along the second axis (cPCoA 2). See statistical details in electronic supplementary material, tables S3–S7.

sp.; BCP). Protist abundance reached approximately 5×10^4 from 100 in the protist addition treatment consistently across microbiome sources.

Different from protist addition, phage addition led to different responses in bacterial abundance between microbiome sources 1 and 2 (figure 2a; electronic supplementary material, table S3). Specifically, phage addition did not significantly affect bacterial abundance in microbiome source 1 ($F = 3.81$, $p = 0.06$; electronic supplementary material, table S3), because the decline in some bacteria, including the most abundant ASV (ANPR sp.), was compensated by the increase in other bacteria (electronic supplementary material, figure S3). In contrast, phage addition significantly increased bacterial abundance in microbiome source 2 ($F = 8.24$, $p = 0.008$; electronic supplementary material, table S3), because the most abundant ASV (BCP sp.) was little affected, whereas another ASV (*Falschrobacterium* sp.) increased dramatically (electronic supplementary material, figure S3).

On the other hand, phage addition resulted in a decline in bacterial richness consistently across microbiomes ($F = 39.4$, $p < 0.001$; figure 2b; electronic supplementary material, table S5). Phage addition also drove the separation of the community composition from the control and protist addition treatments along the second axis of the constrained principal coordinates analysis (cPCoA 2: 23.5% of total variation; trophic interaction; $F = 10.6$, $p = 0.001$; figure 2c; electronic supplementary material, table S6), especially in Beijerinckiaceae, Burkholderiaceae, Enterobacteriaceae, Microbacteriaceae, Mycobacteriaceae, Rhizobiaceae, Sphingomonadaceae and Xanthobacteraceae (electronic supplementary material, table S7).

We further conducted SEM to explore how the impacts of trophic interactions on different properties of microbiomes influenced duckweed population size and phosphorus concentration (figure 3). Our results suggested three possible mechanisms through which phage addition could influence duckweed population size (figure 3; electronic supplementary material, table S8). First, by reducing bacterial richness, phage addition indirectly increased plant population size ($r = -0.71 \times -0.18 = 0.13$). Second, by changing bacterial

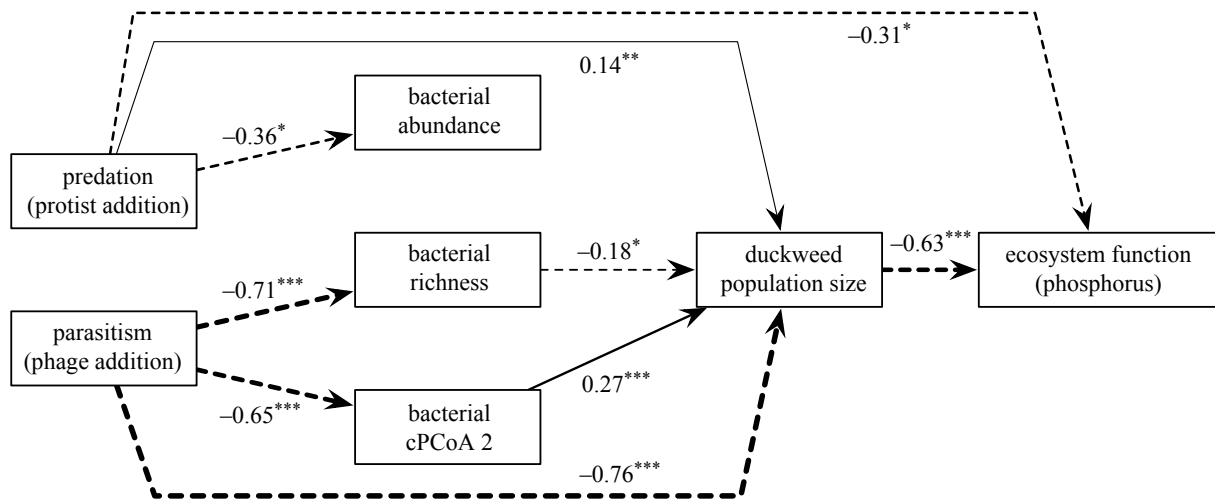


Figure 3. Structural equation model of trophic interactions in microbiomes explaining duckweed population size and ecosystem function. Arrows indicate significant positive (solid) and negative (dashed) relationships (for the original full model of SEM, see electronic supplementary material, figure S2). The SEM was estimated with both Huber–White robust standard errors (electronic supplementary material, table S8) and bootstrapping (shown here), and yielded consistent results. Numbers adjacent to arrows indicate standardized path coefficients. Trophic interactions were coded using the control treatment as the reference level in the SEM. Bacterial cPCoA 2 represented the second axis of the constrained principal coordinates analysis of bacterial community composition (figure 2c). Significance levels: $***p < 0.001$; $**p < 0.01$; $*p < 0.05$. See statistical details in electronic supplementary material, table S8.

community composition, phage addition indirectly decreased plant population size ($r = -0.65 \times 0.27 = -0.18$). Third, phage addition was directly, negatively linked to plant population size ($r = -0.76$, $p < 0.001$), which was probably influenced by unmeasured properties of the bacterial communities, as phage addition without bacterial microbiomes showed no effect on plant population size (the follow-up experiment; see Results above). As a result, there was a net negative effect of phage addition on plant population size ($r = -0.76 + -0.18 + 0.13 = -0.81$), consistent with the results of the LM (figure 1a).

The SEM also indicated that protist addition affected plant population size through different mechanisms compared to phage addition (figure 3). While protist addition reduced bacterial abundance ($r = -0.36$, $p = 0.025$; figure 3), consistent with the finding of the LM (figure 2a). Such reduction in bacterial abundance did not significantly affect plant population size ($r = -0.12$, $p = 0.106$). Instead, we found a direct link between protist addition and plant population size ($r = 0.14$, $p = 0.006$), which was probably influenced by other unmeasured properties of the bacterial communities, as protist addition without bacterial microbiomes showed no effect on plant population size per the follow-up experiment (see Results above).

The SEM revealed different mechanisms by which phage and protist additions influenced phosphorus concentration (figure 3). Phage addition showed a net positive effect on phosphorus concentration, mediated by a reduction in plant population size caused by changes in the microbiomes ($r = (-0.76 + -0.65 \times 0.27 + -0.71 \times -0.18) \times -0.63 = 0.51$). Protist addition, on the other hand, showed a net negative effect on phosphorus concentration ($r = 0.14 \times -0.63 + -0.31 = -0.40$) through affecting plant population size ($r = -0.63 \times 0.14 = -0.09$) and through microbiomes directly ($r = -0.31$, $p = 0.015$).

4. Discussion

Trophic interactions shape ecological populations and communities and drive ecosystem function [24,58–60]. In this study, we specifically explored the effects of two important types of trophic interactions in plant microbiomes—predation by protists and parasitism by phages. When compared to the consumer-depleted control, protist addition was found to reduce bacterial abundance while promoting plant population size and phosphorus removal. On the other hand, phage addition reduced bacterial richness and shifted community structure, suppressing plant population size and phosphorus removal. Furthermore, our SEM analysis indicated that the distinct outcomes of predation and parasitism were influenced by mechanisms that extend beyond the factors of microbiome abundance, richness and composition.

Our results showed that predation and parasitism influenced different properties of microbiomes. On the one hand, predation by protists reduced bacterial abundance, especially the most abundant bacteria (electronic supplementary material, table S4 and figure S3), consistent with a density-dependent foraging behaviour often exhibited by generalist predators [61]. Although rarer bacteria could increase in abundance (electronic supplementary material, figure S3) probably by colonizing the vacated niches of the abundant bacteria, this was yet insufficient to compensate for the loss of overall bacterial abundance, resulting in a net negative effect of predation on bacterial abundance. On the other hand, our study revealed that parasitism by phages reduced bacterial richness, probably due to the presence of different lytic phages targeting specific bacterial species in our phage extracts from the natural duckweed microbiomes. Interestingly, this finding contrasted with the study of Morella *et al.* [32], which focused on the interaction between phages and bacteria in tomato phyllosphere and found relative neutral influence of phages on bacterial richness with a negative effect on bacterial abundance at day 1 and no effect on bacterial abundance at day 7. The depleted bacterial species in our study were not among the most abundant ones in the microbiomes (electronic supplementary material, figure S3 and table S4). However, the effect of phage addition on the overall bacterial abundance was microbiome source dependent, indicating complex outcomes influenced by the diversity and composition of phage communities in duckweed microbiomes.

The distinct effects of the two major categories of consumers on microbiomes contributed to our understanding of the principles that govern microbiome assembly and the roles of different types of trophic interactions.

Predation and parasitism not only showed opposite effects on plant population size but also acted via different mechanisms. For example, relative to the control, protist addition reduced bacterial abundance. However, this mechanism was improbably responsible for the significant impact of protists on duckweed population size. Instead, protist addition increased duckweed population size by influencing aspects of the microbiomes (the direct link, $r = 0.14$) other than abundance, richness and composition. It should be noted that our amplicon sequencing-based approach was unable to definitively determine these unmeasured aspects of the microbiomes. One potential factor to be considered in future research is microbial physiological or evolutionary changes. Predation could increase the overall activity level of the microbiomes to affect plant population size by increasing the proportion of active relative to dormant bacteria. One specific situation could involve protists consuming dormant bacteria (different from parasitic phages that preferentially target active bacteria) [62] with the vacated niches being occupied by active bacteria that recover more quickly than dormant bacteria from predation. Moreover, predation might drive the rapid evolution of consumer-resisting traits in bacteria, for example, forming biofilm against predation [63] that could also alter microbiome function and plant growth [64].

In contrast to predation, when compared to the control, phage addition showed a net negative effect on duckweed population size by influencing bacterial richness and community composition, as well as other aspects of the microbiomes. The shifted community composition by phage addition led to a negative effect on duckweed population size. This was probably because phage addition changed the composition of many bacteria that belong to the bacterial groups that often promote plant growth (e.g. *Burkholderia*, *Methylobacterium*, *Rhizobium* and *Sphingomonas*; electronic supplementary material, table S7) [65–68]. In addition to composition, the direct link between phage addition and duckweed population size ($r = -0.76$) indicated the role of other mechanisms. It is possible that phages reduced bacterial activity in the microbiomes via hijacking the molecular machinery of bacteria in the microbiomes to reproduce themselves [62]. It is also possible that like predation, parasitism might drive the evolution of bacteria to develop defence traits, for example, toxin-antitoxin systems [69] that can potentially suppress plant growth as an off-target effect [70]. It is worth noting that SEM suggested a potential positive effect of phage addition on duckweed population size through reducing bacterial richness, although this effect appeared to be overshadowed by mechanisms driving a negative effect. Nevertheless, our results demonstrate predation and parasitism within microbiomes influence plant populations differently and highlight the need to elucidate the physiological and evolutionary mechanisms in understanding microbiome function on plants.

Phosphorus is one of the most important limiting factors in freshwater ecosystems [71]. While both plants and microbes have been found to be important in mediating phosphorus cycling [72], our results disentangled their relative importance in phosphorus removal. Compared to microbiomes, duckweeds played a more important role in phosphorus removal in this system through direct phosphorus intake, as phosphorus is an important nutrient for plants. In addition to this mechanism, the photosynthesis of duckweeds might be able to change the environmental pH [73], which could influence phosphorus solubility and lower phosphorus availability [74]. While phosphorus is also important for bacterial growth as a cellular component for lipid and nucleic acid synthesis [75], bacterial abundance, richness and composition did not influence phosphorus concentration directly (electronic supplementary material, figure S2). Instead, the effects of bacterial richness and composition on phosphorus concentration were mediated through influencing duckweed population size. Furthermore, the opposing effects of predation and parasitism on phosphorus concentration were mediated by distinct mechanisms. While both predation and parasitism influenced phosphorus concentration via affecting duckweed population size, predation also reduced phosphorus concentration by altering other aspects of the microbiomes (the direct link, $r = -0.31$). This is probably because eukaryotic protists reduced phosphorus concentration by uptaking it through consuming bacteria, and this amount of phosphorus uptake is expected to be substantially higher than phages that transform a very small amount of bacterial phosphorus into their own nucleotides [75]. Overall, our results emphasize that the effects of predation and parasitism on phosphorus removal are primarily mediated via host plants, adding significantly to our knowledge of within-microbiome trophic interactions mediate ecosystem function.

5. Conclusion

Our study provides the first experimental demonstration of how trophic interactions within microbiomes influence plant population size and ecosystem function. By leveraging the duckweed-microbiome symbiosis system, we show that not only do microbial predator-prey and host-parasite interactions operate in opposite directions in influencing plant population size and ecosystem function but also act via distinct mechanisms. It is important to distinguish between different types of trophic interactions when examining the role of trophic interactions for plant-microbiome symbiosis. We emphasize that our findings are based on a single general predator species and a group of more specialized parasites. As such, several critical aspects, especially regarding the diversity and composition of phage communities, remain unexplored. Future research on linking the host range and life history (e.g. lytic versus lysogenic cycles) of diverse phages [34] to microbial community structure and dynamics and the outcome on plant population and ecosystem function is needed. Nevertheless, our findings provide important insights into the host and ecosystem-level function of microbiomes and call for the need of incorporating the knowledge of species interactions across multiple kingdoms and domains and the need of exploring diverse microbiome properties (including physiology and evolution) for better predicting the outcomes of complex host-microbiome systems.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. Data and analyses that support the findings of this study are included in this published article, in the electronic supplementary material [76] and on Mendeley Data (<https://data.mendeley.com/datasets/bc2dvv2c4p/1>). The raw sequencing data can be found on NCBI (<https://www.ncbi.nlm.nih.gov/sra/PRJNA1096745>).

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. J.T.: conceptualization, data curation, writing—original draft, writing—review and editing; N.W.: conceptualization, formal analysis, visualization, data curation, writing—original draft, writing—review and editing; M.M.T.: conceptualization, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

Funding. This project was supported by Gordon and Betty Moore Foundation Symbiosis in Aquatic Systems Initiative (10635 to JT), Holden Arboretum (030869 to NW), National Science Foundation (MCB-2300057 to J.T., MCB-2300058 to N.W., and DEB-1935410 to M.M.T.), and Louisiana Board of Regents (LEQSF(2023-26)-RD-A-14 to JT and NW).

Acknowledgments. We thank Jennifer Brum, Chen Chen, Jae Kerstetter, Yifeng Cao, Jada Daniels and Xiyan Feng for assistance with the experiment, and Michael Hellberg and William Doerrler for allowing us to use their lab space and equipment.

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