From networks to mechanisms: A cross-scale analysis of bacterial - fungal interactions in fungal necromass

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Abstract

Microbial community dynamics are dependent on interactions between the community members, yet studies of interactions across domains and with multiple experimental approaches are lacking. In this study, we explored interactions between bacteria and fungi associated with decaying fungal necromass using both field-based co-occurrence networks and laboratory-based pairwise interactions. The majority of field-derived bacterial-fungal correlations were negative, suggesting a potentially competitive environment within necromass compared to other systems. Laboratory experiments consisted of bacteria most often reducing fungal growth, while the fungal effect on bacterial growth was more varied and dependent on bacterial taxa. However, these interactions were not consistently predicted by field correlations, highlighting a disconnect between field-based and direct experimental approaches. Our findings suggest that using co-occurrence networks alone to predict BFI outcomes could be misleading, emphasizing the need for more comprehensive, multi-method studies to capture the dynamic and context-dependent nature of microbial interactions.

Introduction

Microbial communities are ubiquitous, vital to ecosystem functioning, and incredibly diverse (Fierer & Lennon, 2011). One gram of soil can harbor thousands of microbial species across all three domains of life (Rousk et al., 2010; Tecon & Or, 2017). These communities therefore provide a unique opportunity to study broad ecological phenomena including the generation and maintenance of diversity, community assembly, and interspecies interactions (Prosser et al., 2007). Despite this, research has historically focused on only one domain at a time (de Boer et al., 2005; Frey-Klett et al., 2011). This approach stifles a complete understanding of these diverse and complex communities, a gap in knowledge which has sparked interest in cross-domain interactions including those between bacteria and fungi (Kelliher et al., 2024; Robinson et al., 2023; Simon et al., 2015).

Fungi and bacteria are almost always found growing in the same environments and together, dominate the microbial communities found in soil (Thompson et al., 2017). When attention has focused on bacterial fungal interactions (BFIs), it has revealed important and novel processes. These include positive interactions (i.e., taxa of one or both domains benefitting from the other's presence) such as fungi facilitating bacterial motility (Worrich et al., 2016; Zhang et al., 2018) and bacterial nitrogen fixation that benefits fungal activity (Frey-Klett et al., 2011; Johnston et al., 2016; Rinne et al., 2017), as well as negative interactions (i.e., taxa of one or both domains being harmed by the other's presence) such as direct competition for simple carbon compounds

(Rousk et al., 2008a) and production of harmful bioactive compounds (Bahram et al., 2018; Fleming, 1929). Collectively, BFI-focused research has made it clear that bacteria and fungi frequently drive each other's evolution, niche requirements, and diversity, emphasizing the importance of studying their interactions across habitats and ecosystems.

Historically, much of the research on BFIs has utilized culture-dependent techniques to study individual strains that have been isolated from environmental samples (Pion et al., 2013; Worrich et al., 2016; Zhang et al., 2018). While this allows for a detailed understanding of the mechanisms of interactions, the scope of culture-based techniques is limited to a small subset of the full diversity of microbial communities (Torsvik & Øvreås, 2002). More recently, with advances in high throughput sequencing (HTS), there has been growing interest in assessing microbial interactions across domains through patterns of species co-occurrence (Carr et al., 2019; Matchado et al., 2021). Specifically, HTS data provides the opportunity to study co-occurrences between thousands of microbial taxa across multiple domains and to explore how they are shaped by various biotic and abiotic factors (Barberán et al., 2012; Gao et al., 2022; Kishore et al., 2023; Yuan et al., 2021). Calculating positive and/or negative correlations between pairs of taxa forms the basis of co-occurrence networks, which can then be analyzed with network theory to make inferences that would be obscured by analyzing only taxonomic composition.

Co-occurrence trends between bacteria and fungi have revealed that cross-domain interactions are a common feature of natural communities. Many studies have found that network structure changes depending on environmental factors such as warming, pH, N deposition, or precipitation. Specifically, more stressful environments may produce networks that are less dense, but have higher percentages of positive correlations (Cui et al., 2023; Gao et al., 2022; Hernandez et al., 2021; Wei et al., 2024; Yuan et al., 2021). Additionally, certain fungal taxa have been identified as 'hubs' (or those most connected to other taxa) of bacterial communities (Agler et al., 2016; Lee et al., 2022), indicating that they may have stronger biotic interactions with bacteria compared to other fungi. Taken together, this suggests that networks may be useful for providing insight into the interactions within microbiomes in a field setting.

While the aforementioned results are intriguing, their interpretation is dependent on a clear understanding of what co-occurrences represent. Ecologists studying macroorganisms have long demonstrated that positive and negative co-occurrences can be generated by other processes than biotic interactions (Connor & Simberloff, 1979; Diamond et al., 2015; Gotelli & Graves, 1996). For example, positive or negative co-occurrence can solely be due to habitat filtration (Barberán et al., 2012), as habitat specialists may have non-overlapping distributions that are unrelated to biotic interactions. Similarly, co-occurrence patterns can vary strongly by the scale of sampling, ranging from neutrally correlated at larger scales to more strongly correlated at finer scales (Bosch et al., 2023). Although it has been widely suggested that networks can serve as a 'starting point' to identify which taxa *may* be interacting biotically (Carr et al., 2019; Dundore-Arias et al., 2023), given these alternative processes it is important to investigate the extent to which co-occurrences reflect potential biotic interactions.

A thorough investigation of BFIs should not only identify taxa that interact, but also explore the mechanisms that lead to various outcomes. BFIs can can occur through two broad mechanistic categories: 1) 'chemical' mechanisms such as the exchange of metabolites and antimicrobial compounds and 2) 'physical' mechanisms dependent on association between bacterial cells and fungal hyphae, such as the use of hyphae for transport and the development of endosymbioses (Deveau et al., 2018). However, it has been historically difficult to untangle the effects of these mechanisms and their relative contributions to interaction outcomes remain unknown.

This study aims to investigate bacterial-fungal interactions across scales in a model microbial system: the decomposition of dead fungal biomass (hereafter referred to as "necromass"). Dead microbial matter in forest soils have been shown to contribute significantly to both short- and long- term carbon and nitrogen cycling (Wang et al., 2021; Zheng et al., 2019) and is commonly colonized by both bacteria and fungi (Beidler et al., 2020; Cantoran et al., 2023). Necromass is an environment where bacteria and fungi seem to be 'codominant', providing an ideal habitat to study BFIs (Brabcová et al., 2016). Understanding the outcome of these interactions is also ecologically important, as different microbes have different necromass decomposition capacities (Maillard, et al., 2023a), which ultimately affects how much dead fungal mycelium contributes to soil carbon persistence (Angst et al., 2021; Maillard et al., 2022). Despite suggestions that BFIs in necromass may be important, little is known about their frequency or mechanisms (Kennedy & Maillard, 2022). When they have been considered, fungi appear to facilitate bacterial growth without being affected themselves (Pérez-Pazos et al., 2024). This environment may facilitate competitive interactions, as it has been previously demonstrated that competition between bacteria and fungi is higher in plant-derived substrates that are more N-rich (Rousk et al., 2008a), and necromass has a significantly higher N content than plant derived matter. However, necromass provides a diverse array of compounds available, and transcriptomic analysis of necromass-associated bacteria suggests that taxa may specialize in degrading different compounds (Novak et al., 2024), in which case competition would be less likely.

Here, we study BFIs through a field-derived co-occurrence network of bacteria and fungi, laboratory-based pairwise interactions among selected taxa from the same genera, and a comparison of the results of these two methods. We hypothesize that interactions between bacteria and fungi on necromass depend on the taxa involved, but will overall be largely competitive due to high resource availability. This would be reflected in a co-occurrence network as a high percentage of negative edges and in pairwise interactions as reduced growth in both taxa when incubated together compared to alone. We expect that many positive interactions will consist of fungi facilitating bacterial growth, which would be reflected as positive interactions being driven by physical mechanisms, and negative interactions being driven by chemical mechanisms. Further, we hypothesize that positively correlated taxa in a co-occurrence network exhibit positive pairwise interactions in a laboratory environment, negatively correlated taxa exhibit negative interactions, and uncorrelated taxa exhibit asymmetric or insignificant interactions.

Methods

Necromass creation and field incubation, and microbial community characterization

To produce necromass for this study, *Hyaloscypha bicolor* (Hambl. & Sigler) Vohník, Fehrer & Réblová (formerly *Meliniomyces bicolor*) cultures were grown on half-strength potato dextrose (PD; HiMedia Laboratories, PA, USA) covered with a gel drying film (Promega, WI, USA). Five mm plugs were transferred to 125 mL Erlenmeyer flasks containing 100 mL of half-strength Potato Dextrose Broth (PDB), which had been adjusted to pH 5 using 10% hydrochloric acid, and grown for 30 days at 20ºC on orbital shakers at 150 rpm. Mycelium was harvested using sterile sieves, rinsed with sterile deionized water, and killed by lyophilization in a freeze dryer for 3 days at -50ºC under vacuum (Labcono, NH, USA). This method has been previously demonstrated to kill fungal hyphae (Maillard, et al., 2023b). The necromass was manually ground into a fine powder (<500 µm) using a ceramic mortar and pestle. Ground dry necromass (100 mg) was added to twelve polyethylene mesh bags with 53-µm pores (R510; ANKOM Technology, NY, USA). These bags were incubated in the top 5 cm of soil in a mixed-age white pine (*Pinus strobus*) forest at the Cedar Creek Ecosystem Science Reserve in East Bethel, MN, USA. The bags were buried in July 2020, harvested one month later, and immediately transported to the laboratory in a cooler at 4°C.

We suspended 1g of the field-incubated necromass in 20 mL sterile distilled water and vortexed the solution for two minutes before diluting it from 10^{-1} to 10^{-5} concentrations. For bacterial isolation, we plated 150 µL of diluted solutions on 1/10 diluted Tryptic Soy Agar (TSA) medium (3 g/L and agar, 12 g/L, pH 5) with 100 mg/L cycloheximide. For fungal isolation, we plated the same amount of solution on Modified Melin-Norkrans (MMN) medium (no malt extract, 0.5 g yeast extract, 0.23 g casein hydrolysate, agar 12 g/L, pH 5) with 150 mg/L Streptomycin sulfate. Petri dishes were incubated at 20°C. After 5 days, we randomly selected 50 bacterial colonies and isolated them by successively plating on 1/10 diluted TSA. We subcultured fungal colonies on MMN medium from the dilution plates daily for twelve days. Sequence identity was determined with Sanger sequencing. For bacteria, we used the 27F-1429R primer pair to amplify the 16S rRNA gene (Weisburg et al., 1991) and for fungi we used the ITS1F-ITS4 primer pair to amplify the ITS rRNA gene region (Gardes & Bruns, 1993).

A subset of 12 necromass bags were also assessed via HTS to characterize the bacterial and fungal communities associated with decaying necromass. The HTS methods matched those of Beidler et al., (2020), except that both the 16S and 18S datasets were rarefied to the same level (1159 reads per sample).

Creating microbial co-occurrence networks

From the HTS data, we selected all OTUs with a sequence match to a known genus and grouped each OTU at the genus level. Only genera that contributed to at least 1% of the total 16S or 18S community reads were retained. This dataset was used to construct the co-occurrence network using the NetCoMi package (Peschel et al., 2021). Briefly, Spearman correlations were calculated

between all taxa and correlations >0.4 or <-0.4 were used to construct the network. NetCoMi was used to calculate the edge density, the percent of edges that were positive, and the clustering coefficient, and to visualize the network.

Based on the co-occurrence network results and the taxa successfully isolated from necromass, we selected 20 pairs of bacterial and fungal taxa to study in our laboratory interaction assays. These consistented of four pairs in each of the following correlation categories: significantly positive (rho>0.6, p<0.05), marginally positive (rho>0.4, p<0.2), significantly negative (rho<-0.6, p<0.05), marginally negative (rho<-0.4, p<0.2), and uncorrelated (p>0.4, rho>-0.4 & <0.4). The taxa in each of these pairs and their associated statistics is shown in Table 1.

Experiment 1: Classifying Field-Inferred Interactions with Pairwise Laboratory Growth Assays

Preparing isolates

For all fifteen microbial genera in Table 1, we selected a random strain that had been isolated from necromass. Isolates were prepared for inoculation following the methods of Perez-Pazos et al., 2024. Briefly, bacterial isolates were prepared for the co-culture experiment by five days of growth in 20% (v/v) 5X Minimal Salts (M9) liquid media (Sigma Aldrich, St. Louis, MO, USA), modified with >0.001% (w/v) of $MnCl_2$, $ZnCl_2$, $CuCl_2$, $CoCl_2$, Na_2MoO_4, $CaCl_2$, $MgSO_4$, and FeCl3, 0.5 g/L of casein hydrolysate, supplemented with glucose and adjusted to a pH of 5.0 using 10% HCl (this media without the addition of glucose will hereafter be referred to as “modified M9 media”). After a five-day period, cultures were centrifuged at 4,500 g for five minutes. The pellet was rinsed twice and resuspended in the modified M9 media then adjusted to an OD of 0.30 (+/- 0.02) at 600 nm and diluted 1:10 to create the inocula used in the experiment. DNA extractions were performed (as described below) on the starting inoculum of each isolate.

Fungal isolates were prepared for the co-culture experiment by five days of growth on modified Norkan’s C (MNC) agar plates covered by a gel drying film. Isolates were transferred to a 2 mL Eppendorf centrifuge tube (Eppendorf, Hamburg, DE) and macerated using sterile plastic pestles connected to an electric motor. The optical density of each was adjusted to 0.30 (+/- 0.02) at 600 nm and diluted 1:10 in modified M9 media to create the inocula used in the experiment. DNA extractions were performed on the starting inoculum of each isolate.

Interaction setup

We used sterilized 96 well round bottom microtiter plates for the incubation (Celltreat Scientific Products, Pepperell, MA). Each microbial strain was inoculated alone, and in all 20 pairs (Table 1), each consisting of six replicate wells. Each inoculation contained 150 µL of modified M9 media containing 5g/L of powderized necromass and 25 µL of each microbial suspension. The treatments in which a microbial strain was inoculated alone were supplemented with 25 µL of modified M9 media to reach an equal volume and solute concentration. Experimental controls were included that were not incubated with any microbial isolate. The 96-well microtiter plates

were sealed with parafilm and incubated on an orbital shaker at 200 rpm at room temperature (21 - 23° C) for five days.

DNA extraction and quantification

After incubation, the contents of each well were transferred into a screw-cap tube containing 3 sterile 3 mm glass beads and rinsed with 200 µL of autoclaved deionized water. All samples were bead-beat for 30 seconds, then a 20 µL sample was taken for DNA extraction. DNA was extracted using the REDExtract-N-Amp method. Briefly, the 20 µL subsample was combined with 20 µL of REDE Extraction solution, heated for 10 minutes at 65 degrees C and 10 minutes at 95 degrees C, then combined with 50 µL of 3% (w/v) bovine serum albumin. DNA extracts were stored at -20° C and diluted tenfold for quantification.

The abundances of bacteria and fungi (both the starting inoculations and the abundances at the end of incubation) were measured using the identical methods of Perez-Pazos et al., 2024. Briefly, we used quantitative PCR (qPCR), in a StepOne Real-time PCR machine (Thermo Fisher Scientific) to amplify the bacterial 16S rRNA gene and fungal 18S rRNA gene using the 1401F/968R and FR1/FF390 primer sets, respectively (Cébron et al., 2008; Chemidlin Prévost-Bouré et al., 2011). Amplifications of the template DNA were compared to standard 16S and 18S linearized plasmids (10^9–10^3 gene copies/µl) to determine DNA concentration, and molecular grade water as a negative control. We used iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) for all qPCR reactions. qPCR yielded data describing the number of 16S or 18S copies per µL in the diluted and processed DNA extract. These were transformed to reflect the number of copies present per µL at the end of the incubation in the 96-well plate, yielding values ranging from 10^{11} to 10^5. To test if the presence of fungi affected the extraction efficiency of bacteria (and vice versa), we amplified an aliquot of each linearized plasmid combined with the DNA extract from the other domain and compared the results to when the plasmid was combined with water.

All isolates grown alone produced detectable levels of 16S or 18S genes. However, some co-inoculations showed no detectable gene copies. These interactions were crucial to include, as they resulted in significant growth changes. To incorporate these data without overestimating interaction effects, we used the lower detection limit from our qPCR standard curve (10^5 copies per µL) as substitute values. This approach preserved all data points while conservatively estimating interaction effects.

Experiment 2: Exploring interaction mechanisms

To determine the chemical and physical contributions to BFI's, unique, custom-made glass chambers were used to further examine one bacterial-fungal pair of particular interest: *Burkholderia* and *Metarhizium*. This pair was selected because *Burkholderia* was identified as a hub taxon, and *Metarhizium* had many positive correlations with bacteria in the co-occurrence network. Despite their positive correlation, their laboratory-derived interaction was mutually negative. According to our hypotheses, mutually negative interactions may provide the best opportunity to study chemical antagonism. These chambers consist of two detachable halves,

each with a screw cap and a volume of ~10 mL, that can be clamped together. A round filter of any pore size can be inserted between the halves, or they can be connected to make one continuous space.

To explore interaction mechanisms, *Burkholderia* and *Metarhizium* were prepared for inoculation exactly as described above. Each was inoculated into one half of the chambers under two conditions: 1) no filter present, allowing "full physical contact" between the microbes, and 2) a 0.2 µm mixed cellulose ester (MCE) membrane filter separating the microbes, allowing only "chemical contact" without growth through the filter. For both of these conditions we inoculated each microbe alone and the two in combination with three replicates each. All treatments contained 3 mL of modified M9 with necromass (for the chambers with a filter, 1.5 mL was put on either side). The 'together' treatments contained 500 µL of each inocula (for the filtered chambers, one on each side of the filter). The 'alone' treatments consisted of 500 µL of the microbial inocula (prepared as above) and 500 µL of modified M9 media with no carbon source on the other side, to create an equal volume as the 'together' treatment. We also included uninoculated controls. These were shaken on an orbital shaker for five days at 200 rpm. Processing, DNA extraction, and qPQR were done exactly as described above.

Statistical analyses

Statistical analyses were all performed using R software (v4.4.1, R Core Team, 2021).

Co-occurrence network

We performed a linear regression on the number of edges of each genus by its abundance in the dataset.

Experiment 1

To account for the differences in absolute growth across taxa, we analyzed each interaction in terms of how incubation with another microbe influenced biomass production relative to incubation alone. For each taxon, we first calculated the average amount of biomass (in 16S or 18S copies/uL, on a log scale) present when incubated alone. For each replicate of co-incubation, we calculated the difference in copies on a log scale compared to this average, hereafter referred to as the "interaction response" values.

The bacterial and fungal data were analyzed separately. We constructed two Type I two-way ANOVA models with bacterial or fungal response as the response variable and microbial identity (genus of the responding taxon) and microbial partner (genus of the co-inoculated taxon) as the predictor variables. Visual inspection of the residual plots and qq plots showed both datasets had homogenous variances and normal distributions. Within the main effect and interaction terms, differences among groups were determined using Tukey Honest Significant Difference (HSD) tests. Additionally, one-sample t-tests were performed on the responses of each genus to all interactions, as well as the response of each genus to each separate interaction, to determine whether the response significantly differed from zero.

Experiment 2

For each interaction chamber filter type, we compared the growth of each microbe alone versus in co-inoculation. We calculated the 'response' values for each replicate of the 'together' treatments as outlined above. We constructed a Type 1 one-way ANOVA model with bacterial or fungal response as the response value and chamber type as the predictor variable.

Comparing laboratory-based interactions with network-inferred interactions

To compare the field-based co-occurring relationships with empirically quantified interaction results from the laboratory assays, we grouped the pairs of taxa into their correlation categories (Table 1). We performed two ANOVAs with the bacterial or fungal response values as the response variable and the correlation category as the predictor variable, respectively.

Results

Co-occurrence network analysis

Between the 29 taxa in the co-occurrence network, there were 108 correlations. Slightly over half of these correlations, which are also referred to as "edges", were positive (54.6%) (Fig. 1). Of the 108 correlations, 46 were between bacterial genera, 14 were between fungal genera, and 48 were between a bacterial genus and a fungal genus (Table 2). The three most well-connected genera (hub taxa) were *Chaetomium*, *Burkholderia*, and *Luteibacter*. There was no significant relationship between abundance in the dataset and number of edges (F_{27}=1.59, P=0.218).

To choose which microbial co-occurrences to explore empirically in pairwise laboratory assays, we assessed the overlap in taxa in the network to those cultured from necromass post-field incubation. Overall, 45/108 (41.6%) of correlations were between two genera that were also found in the culture collection. Of these, almost half (46.67%) were between a bacterial and fungal genus (Table 2).

Experiment 1: Lab-based interaction results

Effect of fungi on bacterial growth

The bacterial response to fungal presence was significantly affected by the bacterial identity (ANOVA, $F_{6, 100}$ = 109.180, P < 0.001) but not fungal partner ($F_{7, 100}$=1.332, P=0.143). Additionally, there was a significant interaction between which fungus was present and bacterial identity ($F_{6, 100}$ = 7.936, P < 0.001) (Table 3, Figure S1). One-sample t-tests indicated that *Flavobacterium*, *Pseudomonas*, and *Sphingomonas* all significantly increased in growth when with a fungus relative to alone (t_{11}=8.42, P<0.001; t_{17}=4.33, P<0.001; t_{29}=5.59, P<0.0001, respectively), while *Luteibacter* and *Burkholderia* both significantly decreased (t_5=-5.79, P<0.01; t_{17}=4.70, P<0.001, respectively) (Figure 2). In contrast, *Rhizobium* was not significantly affected. *Chitinophaga* did not have a detectable amount of 16S genes in any of the co-inoculations, so all co-inoculation

data points were substituted with the lower detection limit of the qPCR standard curve. This yielded the largest average decrease of any bacterial isolate, but the data had no variation and therefore no statistical tests could be performed.

Effect of bacteria on fungal growth

The fungal response to bacterial presence was significantly affected by both fungal identity ($F_{7, 100} = 5.082$, $P < 0.001$) and bacterial partner ($F_{6, 100} = 4.355$, $P<0.001$). There was also a significant interaction between fungal identity and bacterial partner ($F_{6, 100} = 2.730$, $P = 0.0169$). While all fungal taxa displayed an average decrease in growth in the co-inoculation treatments, only that of *Trichoderma*, *Mucor*, and *Metarhizium* were significant decreases as determined by one-sample t-tests ($t_{11}=-7.54$, $P<0.001$; $t_5=-14.75$, $P<0.001$; $t_{23}=-4.76$, $P<0.001$, respectively) (Figure 2).

Experiment 2: Interaction mechanism for one focal bacterial-fungal pair

In Experiment 1 *Metarhizium* and *Burkholderia* displayed a mutually negative interaction (Figure S1), but the outcome was different in both treatments of Experiment 2. Without a filter, *Bukrholderia* increased slightly in growth in the co-inoculation relative to alone, and *Metarhizium* decreased slightly, though neither change was statistically significant. When co-inoculated but separated by a 0.2 μM filter, the effects on both isolates were more negative, though this difference was not statistically significant (Figure 4).

Comparing network co-occurrences with empirical interactions

Two one-way ANOVAs (one on bacterial 16S production and one on fungal 18S production) indicated no significant differences in the empirical interaction outcomes depending on the Spearman's correlation category (Table 1) of the taxa in the network (Figure 4).

Table 1. The pairs of bacterial and fungal taxa chosen for lab-based interactions, their correlation coefficients, and significance. The first four categories ('significant' and 'marginally significant' categories) are edges present in the network, while the 'uncorrelated' pairs are random selections of uncorrelated genera.

Correlation category	**Bacterium**	**Fungus**	**Spearman's rho**	**P value**
Significant positive	*Chitinophaga*	*Mortierella*	0.748	0.001
	Pseudomonas	*Cryptococcus*	0.682	0.014
	Burkholderia	*Cryptococcus*	0.608	0.035
	Flavobacterium	*Umbelopsis*	0.604	0.037
Marginally	*Sphingomonas*	*Metarhizium*	0.496	0.101

significant positive				
	Chitinophaga	*Metarhizium*	0.490	0.106
	Chitinophaga	*Trichoderma*	0.476	0.117
	Burkholderia	*Metarhizium*	0.467	0.126
Uncorrelated	*Chitinophaga*	*Cryptococcus*	0.373	0.232
	Flavobacterium	*Chaetomium*	0.273	0.390
	Pseudomonas	*Metarhizium*	0.264	0.405
	Sphingomonas	*Trichoderma*	-0.174	0.588
Marginally significant negative	*Sphingomonas*	*Absidia*	-0.422	0.172
	Sphingomonas	*Mucor*	-0.439	0.153
	Chitinophaga	*Chaetomium*	-0.451	0.140
	Rhizobium	*Chaetomium*	-0.501	0.097
Significant negative	*Sphingomonas*	*Chaetomium*	-0.585	0.046
	Pseudomonas	*Chaetomium*	-0.653	0.021
	Luteibacter	*Chaetomium*	-0.823	0.001
	Burkholderia	*Chaetomium*	-0.837	0.001

Table 2. Summary of network edges in terms of the domains of the taxa they connect. ‘Percent of total network edges’ indicates how many of the 108 total edges in the network are in each category, while ‘Percent of cultured edges’ indicates how many of the 45 edges that connect cultured genera are in each category.

Domains associated with edge	**Percent of total network edges**	**Percent of cultured edges**
Bacteria - bacteria	42.6%	31.1%
Fungi - fungi	13.0%	22.2%
Bacteria - fungi	44.4%	46.7%

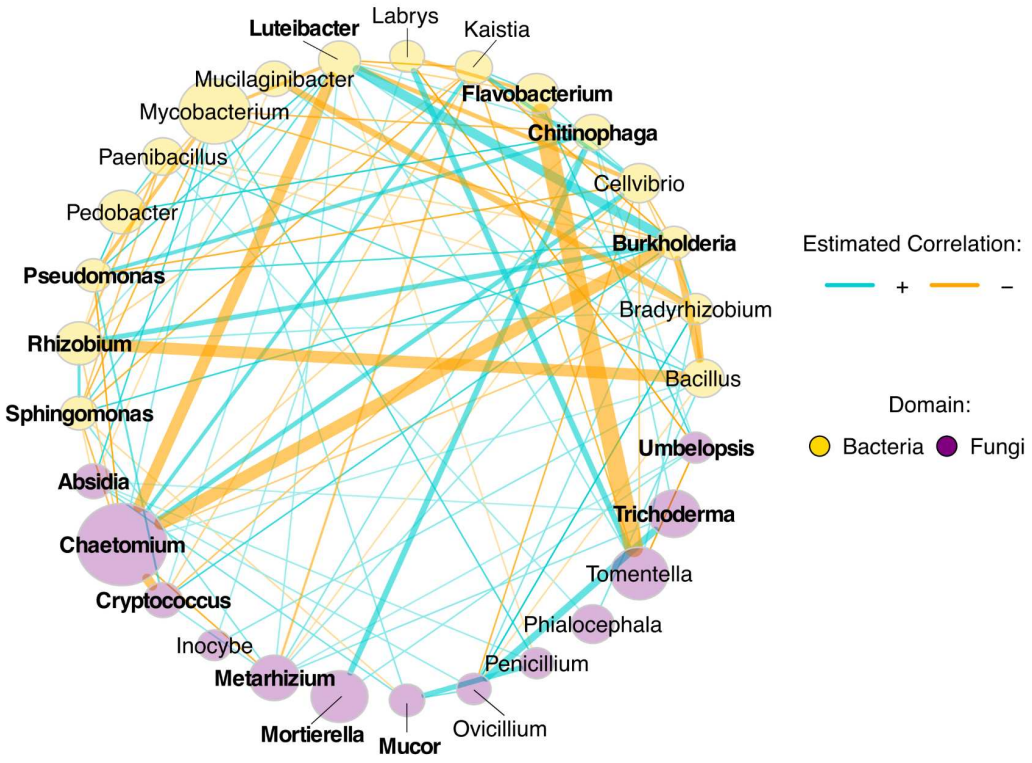


Figure 1. A co-occurrence network constructed for the bacterial and fungal communities present on field-incubated fungal necromass after 1 month based on HTS. Bolded taxa represent those chosen for interaction testing in the laboratory. Nodes represent all OTUs identified to bacterial (yellow) and fungal (purple) genera, and edges represent positive (blue) and negative (orange) Spearman's correlations between genus abundances. All correlations of which |rho| > 0.4 are included in the network. 55% of edges are positive. Edge width is proportional to the magnitude of the correlation coefficient. Node size is proportional to the abundance of the genus in the dataset. Bolded taxa represent those chosen for interaction testing in the laboratory.

Table 3. Two-way analysis of variance (ANOVA) summary table showing the effects of bacterial identity and fungal partner on bacterial response. Asterisks indicate statistical significance with * = $p \leq 0.05$, ** = $p \leq 0.01$, and *** = $p \leq 0.001$.

Source	df	SS	MS	F	Sig
Bacterial identity	6	18.142	3.024	109.180	<0.001***
Fungal partner	7	0.258	0.037	1.332	2.43E-01
Bacterial identity:Fungal partner	6	1.319	0.220	7.936	<0.001***
Residuals	100	2.769	0.028		

Table 4. Two-way analysis of variance (ANOVA) summary table showing the effects of fungal identity and bacterial partner on fungal response. Asterisks indicate statistical significance with * = $p \leq 0.05$, ** = $p \leq 0.01$, and *** = $p \leq 0.001$.

Source	df	SS	MS	F	Sig
Fungal identity	7	5.774	0.825	5.082	<0.001***
Bacterial partner	6	4.242	0.707	4.355	0.0167*
Fungal identity:Bacterial Partner	6	2.659	0.443	2.730	0.0169*
Residuals	100	16.232	0.162		

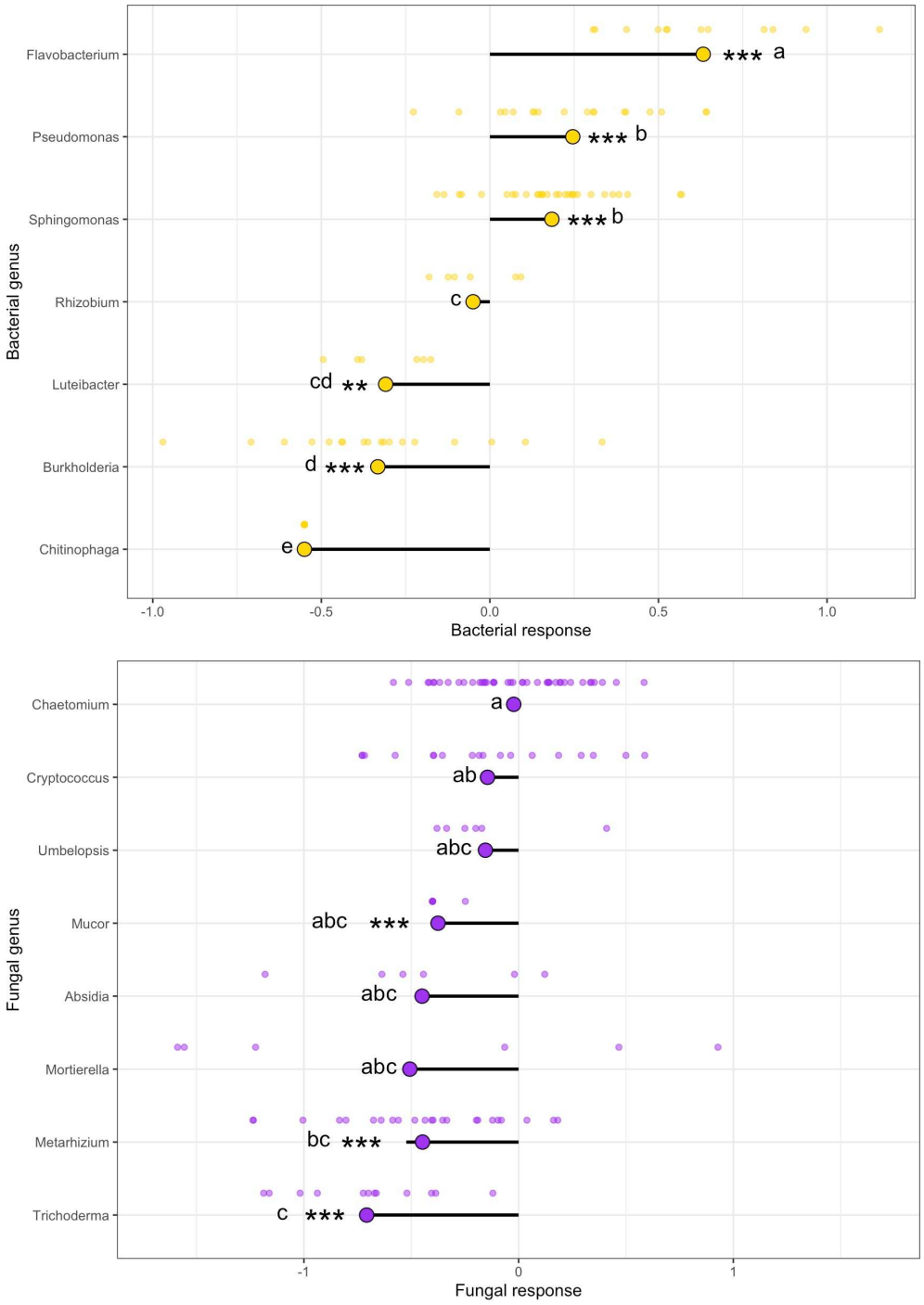


Figure 2. The response of bacterial (top) or fungal (bottom) genera to the laboratory-based pairwise interactions (Experiment 1). The response to each interaction was calculated by the change in 16S or 18S gene production when co-inoculated compared to alone. Treatments in which no gene copies were detected were assigned the lowest detection limit (e.x., *Chitinophaga*). Asterisks indicate whether each group is significantly different from zero as determined by one-sample t-tests.($P<0.001$(***), $P<0.01$(**), $P<0.05$(*)). Letters indicate which groups are significantly different from each other as determined by two-way ANOVAs.

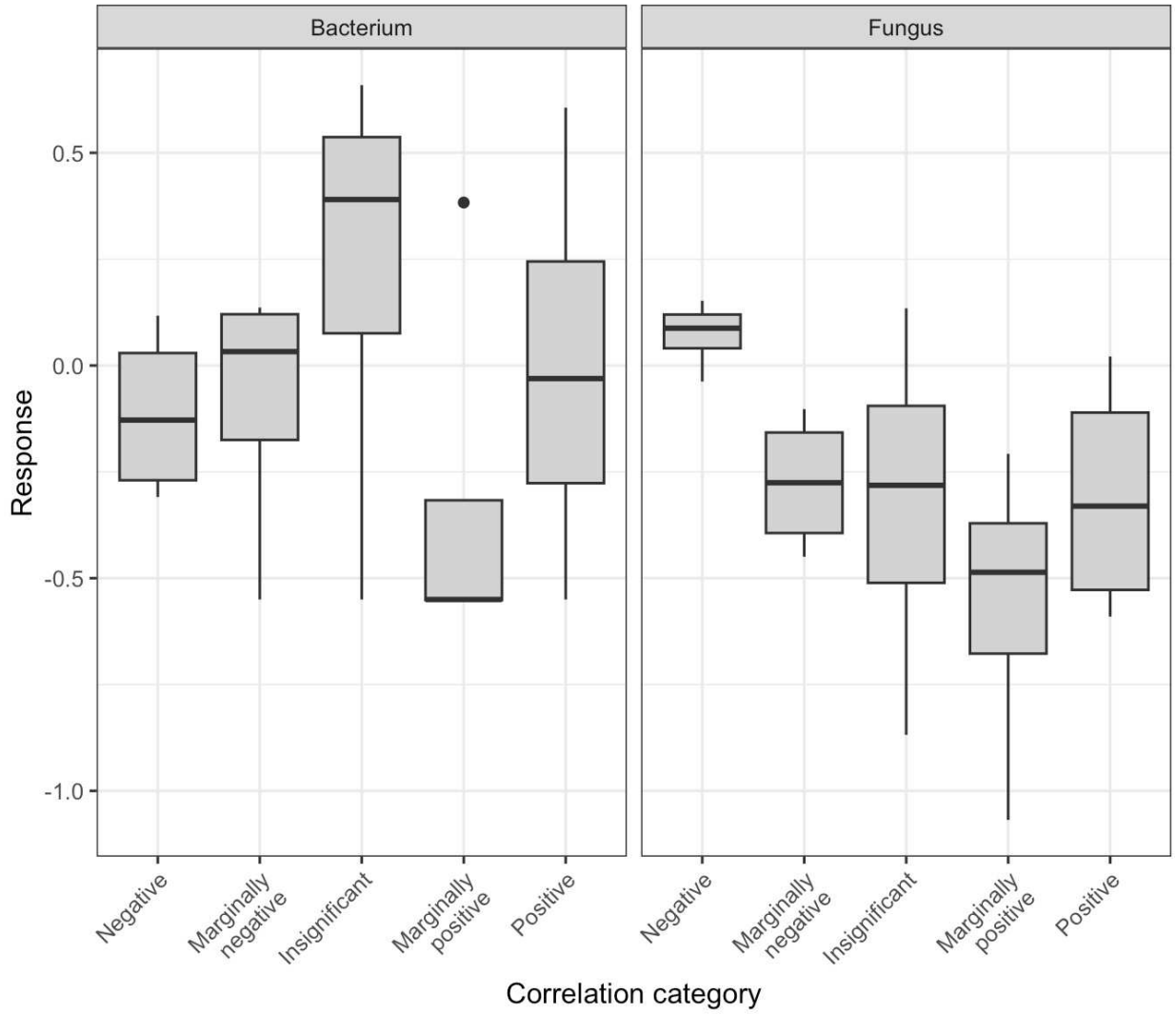


Figure 3. The response of bacteria and fungi to pairwise co-inoculations (Experiment 1) depending on the Spearman's correlation of the bacterial-fungal pair in the field-derived co-occurrence network. The response to each interaction was calculated by the change in 16S or 18S gene production when co-inoculated compared to alone. The pairs of taxa are binned by the Spearman's correlation coefficient between their abundances in the field (Table 1) (N = 4 per category). One-way ANOVAs on both domains indicate no significant differences in the empirical interactions of taxa with different statistical correlations.

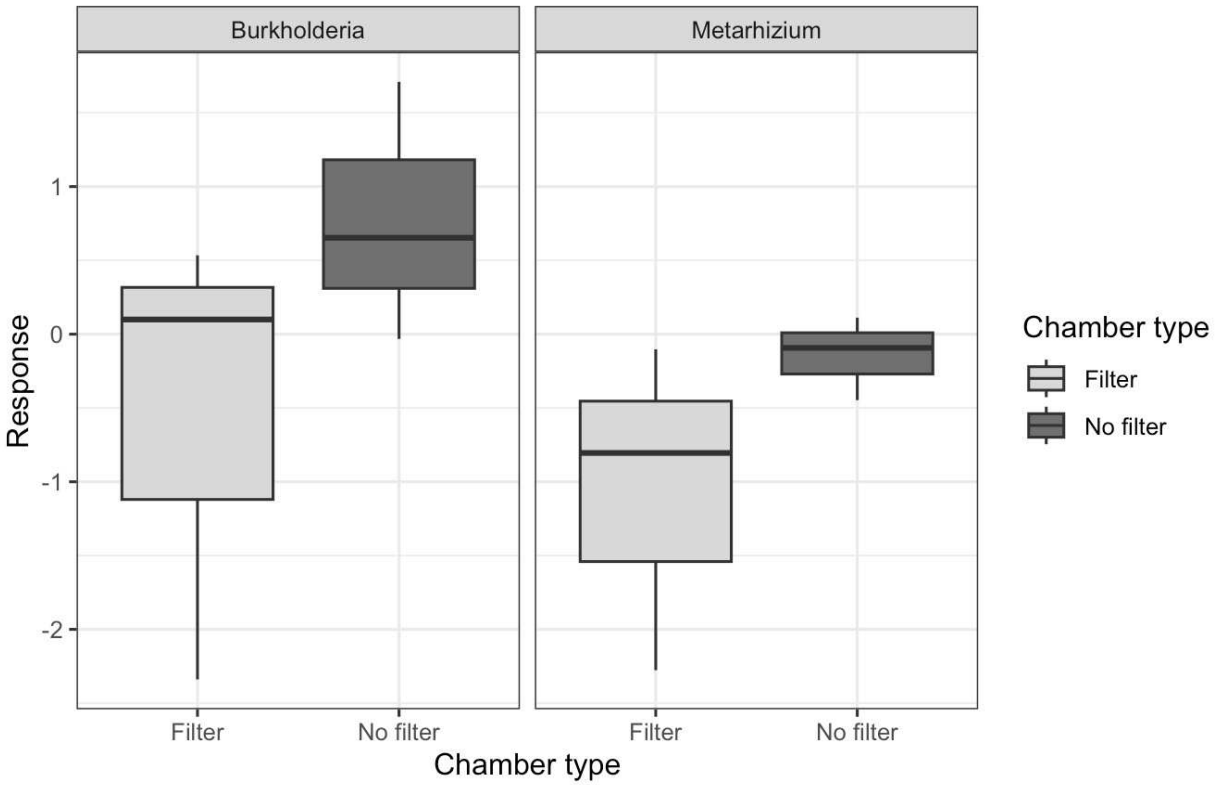
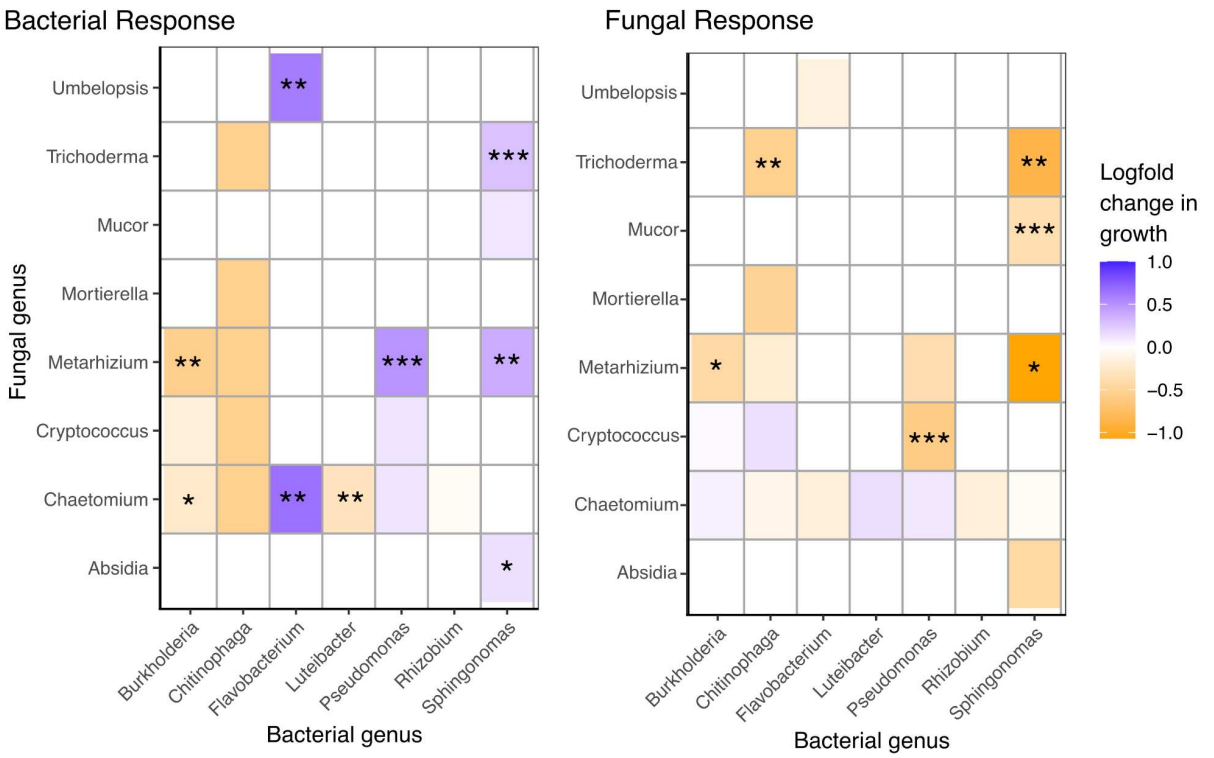


Figure 4. The response of *Burkholderia* and *Metarhizium* to co-inoculation in Experiment 2 depending on the presence of a 0.2 μM filter between them. The response to each interaction was calculated by the change in 16S or 18S gene production when co-inoculated compared to alone in the same chamber type. No differences between groups were statistically significant as determined by a two-way ANOVA.



Supplementary Figure 1. The outcomes of each pairwise interaction on the bacterial (left) and fungal (right) isolates from Experiment 1. The response to each interaction was calculated by the change in 16S or 18S gene production when co-inoculated compared to alone. The average change in growth of six replicates is represented by color, ranging from one log fold increase (purple) to one log fold decrease (orange). Asterisks indicate whether each group is significantly different from zero as determined by one-sample t-tests (P<0.001(***), P<0.01(**), P<0.05(*)).

Discussion

Interactions between bacteria and fungi are important drivers of microbial community activity but remain understudied relative to those within a single domain (Frey-Klett et al., 2011). Here, we examined bacterial-fungal interactions in necromass-associated microbial communities using both field-based co-occurrence networks and lab-based pairwise interactions. In agreement with our hypotheses, we found that field-derived bacterial-fungal correlations were less positive than typically found in other environments, while in the laboratory, pairwise interactions often reduced fungal growth and had mixed results on bacterial growth. Contrary to our hypotheses, the outcomes of pairwise interactions in our laboratory study were not predicted by correlations between the same genera in our field sampling. This latter result suggests that studies using only one type of method to assess bacterial-fungal interactions may limit understanding of their context-dependence.

Network results

The co-occurrence network of bacteria and fungi in necromass was 55% positive (Figure 1), which is notably lower than the 70-80% often observed in microbial co-occurrence networks (Kim et al., 2020; Tipton et al., 2018; Wei et al., 2024). If the network results are indicative of biotic interactions, it would suggest that necromass is a highly competitive environment between fungi and bacteria, which is well supported by other research. Both domains consume significant amounts of C and N from decaying necromass (Maillard et al., 2023a) and both fungal and bacterial genomic potential to degrade necromass has been widely reported (Brabcová et al., 2018; Martinović et al., 2022; Starke et al., 2020). Such an environment likely promotes competitive interactions similar to that of high nitrogen plant litter (Rousk et al., 2008b). If we interpret these negative edges as negative interactions, this also has implications for the necrobiome community stability. A community with more negative edges may indicate a higher stability than those with more positive edges (Gao et al., 2022; Hernandez et al., 2021).

Almost half (41.6%) of correlated pairs of genera overlapped with strains present in the culture collection. This is much higher than the expected percent of culturable soil microbial taxa, often estimated around 1%, (Amann et al., 1995; Staley, 1985; Vartoukian et al., 2010) and included three most prominent hub taxa in the network (*Chaetomium, Burkholderia,* and *Luteibacter*). While bacterial-bacterial correlations were the most abundant in the network overall, bacterial-fungal correlations were the most abundant among those in culture (Table 2). Together, this suggests that bacterial-fungal interactions, especially those in necromass, are a good opportunity to compare culture-independent and culture-dependent techniques. We recognize, however, that comparing field and laboratory-based studies in this way is not perfect, as single isolates in the laboratory may not be representative of the activities of an entire genus in the field.

The most well-connected fungal genus in the field-based network was *Chaetomium*. Interestingly, this genus has been found to be a network hub in at least two other microbial communities (Chen et al., 2019; Fan et al., 2018). The mechanisms of its interactions, however, have not been explored. In this network, many of its correlations with bacteria were negative, suggesting strong antagonistic tendencies. Surprisingly, *Chaetomium* did not respond significantly to any bacterial presence and did not have a consistent impact on bacterial growth (Figure 2). The HTS data also showed that *Chaetomium* was the most abundant fungal genus, which contrasts with previous studies in which *Chaetomium* has not typically among the most abundant fungal taxa (Beidler et al., 2020; Cantoran et al., 2023). Though within this study there is no significant relationship between a taxon's abundance and its number of edges, it is possible that the significance of *Chaetomium* in this network would not be found in other necromass studies where the genus is less abundant.

We acknowledge that this network is severely underpowered relative to most co-occurrence networks. There were only 12 replicates to form the basis of Spearman's correlations, while the recommended number is closer to 25 (Berry & Widder, 2014). Spearman's correlations themselves, though widely used, are not as robust to compositional effects as more complex methods such as SparCC or SPIEC-EASI (Carr et al., 2019). We chose to use Spearman's correlations in this study because, while imperfect, they provide a direct metric of the relationship

between two taxa in the dataset, providing the most applicable comparison to a lab-tested pairwise interaction. The use of Spearman's correlations and the small sample size may lead to correlation values that do not accurately represent the correlations between these taxa in the field, including both false positive and false negative correlations. At the same time, our network does have some strengths. Co-occurrences are more likely to represent biotic interactions when the environment between samples is more similar (Berry & Widder, 2014). All twelve replicates were buried in close proximity and contained the same starting materials. This type of network is therefore most appropriate to compare to laboratory-based interaction studies.

Interaction results

Our findings indicated that in pairwise, laboratory settings, fungal growth was often reduced by the presence of a bacterium (Figure 2). The results of experiment 2 suggest that these antagonistic effects were through chemical mechanisms in at least one case (Figure 4). These may include the production of bioactive compounds and/or the consumption of soluble resources. Though some fungi were unaffected, there were no cases where bacteria increased fungal growth. Bacteria can increase fungal growth under resource-limited or otherwise stressful environments through nitrogen fixation, vitamin production, and detoxification (Frey-Klett et al., 2011; Johnston et al., 2016). Since necromass is resource-rich, and sole fungal isolates are well-suited to grow on *H. bicolor* necromass in a laboratory setting (Pérez-Pazos et al., 2024), it is not surprising that bacteria did not increase fungal growth.

The impacts of fungal presence were more variable for bacteria. *Flavobacterium, Pseudomonas,* and *Sphingomonas* increased in growth, *Luteibacter, Burkholderia* and *Chitinophaga* decreased in growth, and *Rhizobium* was unaffected in response to a fungus (Figure 2). In at least one case, the negative impacts of fungi on bacterial growth may have been chemically driven, while physical contact ameliorated those negative effects (Figure 4). These positive physical mechanisms may include using hyphae as a method of transportation (de Boer et al., 2005; Kohlmeier et al., 2005). Interestingly, many of the specific interactions between these genera have been documented in different contexts and are consistent with what we found. *Flavobacterium* slightly benefitted from the presence of *Chaetomium* and has been previously observed as an endosymbiont of *Chaetomium* (Robinson et al., 2021). The same is true for *Sphingomonas* and its interactions with *Mucor* and *Trichoderma* (Robinson et al., 2021). Though it is unclear if endosymbiosis developed during our study, these bacterial genera may have conserved mechanisms that allow them to benefit from fungi across different environments. We also observed *Pseudomonas* slightly benefitting from *Cryptococcus*, while significantly reducing *Cryptococcus*' growth. This antagonistic relationship has been previously observed in a laboratory setting, with *Pseudomonas* producing harmful antimicrobials towards *Cryptococcus* (Nogueira et al., 2019). All four of the bacterial genera that were unaffected or negatively affected by a fungus do not have such documented interactions with those taxa (Robinson et al., 2023).

Interestingly, while some interactions were consistent with those in other contexts, the interaction between *Burkholderia* and *Metarhizium* differed between Experiment 1 and Experiment 2. In experiment 1, they displayed a mutually significant negative interaction (Figure S1) while in experiment 2 without the filter it was facilitative (Figure 4). This indicates that microbial interactions can be highly context dependent. Even small environmental differences (such as vial size and shape) can cause the interaction outcome to vary. Similarly, growth in liquid media (as opposed to on a solid surface) and our choice in media likely affected the secondary metabolites that could be produced by both fungi and bacteria, which can ultimately change interaction outcomes (Chao & Levin, 1981; Dos Santos et al., 2022; Hosida et al., 2018; Lazazzara et al., 2017). However, these caveats would be present no matter which media and spatial structure we chose to study the interactions. This highlights the complexity of studying microbial interactions in a laboratory setting, which can only be alleviated by studying them under many different media types and contexts or complementing lab-based work with field-based experiments.

Comparing bacterial-fungal co-occurrences and interactions

We did not find any relationship between the correlation observed between two taxa in the field-based network and their pairwise interactions in the lab-based experiment (Figure 3). While we expected to see such a relationship, especially when the field samples were from such similar environments (Berry & Widder, 2014), there are many possible explanations for these inconsistencies. For example, *Chitinophaga* was completely inhibited from growing in the presence of any fungal partner, yet did not have strong negative correlations with them in the field data and is still abundant on necromass. This suggests that members of this bacterial genus may require the presence of other bacteria or certain environmental conditions to co-exist with fungi. Similarly, *Chaetomium* was the most significant fungal hub in the field-based network yet had the weakest response to bacterial presence and did not affect bacterial growth in any consistent way in the lab-based experiments (Figure S1). This suggests that network hub taxa may not influence communities through direct, pairwise interactions, but rather play a mediating role between many taxa (Rawstern et al., 2023). Though the disagreement between the field and the lab data may not be surprising, it does have implications for our ecological interpretation of BFIs. While some have suggested using networks as a tool for selecting biocontrol agents for plant pathogens (Abdullaeva et al., 2024; Yang et al., 2024), our findings suggest that this strategy may not be successful. Even if correlations do reflect interactions in that environment, those interactions may not occur when shifted to a different context.

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