Fungal Ecology 77 (2025) 101455

Contents lists available at ScienceDirect

Fungal Ecology

journal homepage: www.elsevier.com/locate/funeco

Endohyphal bacterial communities modulate fungal responses to hyphosphere-associated bacteria in *Fusarium*

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ARTICLE INFO

Handling Editor: Prof. Benjamin E. Wolfe

ABSTRACT

Fungi and bacteria form complex associations in soil environments, which can impact their symbiotic relationships. While previous studies have examined either endohyphal bacteria or hyphosphere-associated bacterial community interactions with fungi independently, a critical knowledge gap remains related to how hyphosphere-associated bacteria modulate the endohyphal bacterial communities and fungal responses. This study investigated the impact of hyphosphere-associated bacteria co-culture with three *Fusarium* isolates containing natural endohyphal bacteria (composed primarily of *Cutibacterium*, *Acinetobacter*, *Pelomonas*, *Achromobacter*, *Citrobacter,* and other less abundant species) and their antibiotic-treated counterparts with reduced endohyphal bacterial communities. The study determined the changes in fungal growth and microbiome composition when co-cultured with individual or a bacterial mix of four rhizosphere bacterial isolates (*Pseudomonas*, *Bacillus*, *Stenotrophomonas*, and *Rhizobium*). Results showed that endohyphal bacteria modulated fungal responses to hyphosphere-associated bacteria co-culture, with wild-type fungi typically exhibiting lower growth inhibition than their counterparts with reduced diversity and quantity of endohyphal bacteria. However, when the fungi were exposed to a synthetic hyphosphere-associated bacterial mix, all fungal isolates stimulated the endohyphal bacterial community leading to higher endohyphal bacterial diversity compared to single-species hyphosphere-associated bacterial treatments. These findings highlight the importance of considering complex interactions within the fungal microbiome, demonstrating that endohyphal bacteria respond dynamically to the composition of hyphosphere-associated bacterial communities. Our results also provide new insights into how the interplay between endohyphal and hyphosphere-associated bacterial communities shape fungal biology in soil environments.

1. Introduction

Fungi and bacteria are widely acknowledged for their symbiotic interplay and ability to establish intimate and dynamic associations in the environment (Scherlach et al., 2013; Deveau et al., 2018). These interactions have gained substantial attention in recent years, particularly within soil matrices, since they play critical roles in nutrient turnover, maintenance, and promotion of soil and plant health (Aylward et al., 2014; Rashid et al., 2016; Deveau et al., 2018; Jansson and Hofmockel, 2020). The associations between fungi and bacteria can be highly specific and diverse (Bianciotto et al., 1996; Robinson et al., 2021).

Studies have revealed the coexistence of two distinct bacterial communities associated with fungi: endosymbiotic bacteria residing within the fungal hyphae (*i.e.* endohyphal bacteria) and extracellular bacteria that colonize and live in close association with the external surface of fungal hyphae (*i.e.* hyphosphere-associated bacteria) (Warmink et al., 2009; Ghodsalavi et al., 2017; Robinson et al., 2021; Lupini et al., 2023). The endohyphal bacteria have specialized mechanisms to survive within the intracellular environment of fungal cells and often cannot be removed by surface sterilization techniques. In contrast, hyphosphere-associated bacteria colonize and survive on the external

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https://doi.org/10.1016/j.funeco.2025.101455
Received 12 November 2024; Received in revised form 12 June 2025; Accepted 2 July 2025
Available online 15 August 2025

surface of fungal hyphae, forming biofilms or other adherent communities in the immediate vicinity of the fungal cell wall (Warmink et al., 2009, 2011; Simon et al., 2017). These external bacterial communities can use fungal hyphae as highways for dispersal through soil matrices and may engage in metabolic exchanges with their fungal partners through the cell wall interface. (Partida-Martinez et al., 2007; Salvioli et al., 2016, 2017; Ghodsalavi et al., 2017; Uehling et al., 2017; Lastovetsky et al., 2020). Together, the fungus and its associated microbiome form a "holobiont", which is a cohesive ecological unit that can also include other associated organisms such as mycoparasitic fungi and arthropods. These close interactions among fungi and bacteria emphasize the importance of considering the fungus and its bacterial partners as an integrated system (Bordenstein and Theis, 2015; Partida-Martínez, 2017; Deveau et al., 2018). Therefore, in this study, we focus specifically on the bacterial components of the holobiont system.

Despite the proliferation of studies elucidating the diversity and specificity of bacterial-fungal interactions (BFI), there are knowledge gaps related to the roles and benefits of bacterial microbiomes (*e.g.* presence of endo- and hyphosphere-associated bacteria) that are part of fungi. Only recently has the presence of endohyphal bacteria been recognized as more common than initially anticipated (Bonfante and Desirò, 2017; Robinson et al., 2021; Lupini et al., 2022). Few studies have investigated specific bacterial-fungal interactions and have demonstrated the importance of the function-structure of the fungal endo-microbiota. For instance, studies comparing isogenic cultures of fungi lacking endo-bacteria with their wild-type counterparts have demonstrated the role of endosymbionts in modifying host metabolism (Lumini et al., 2007; Uehling et al., 2017) and conferring resistance to different metals (Lupini et al., 2022). Although groundbreaking, most of the experimental studies primarily rely on model organisms or pairwise interactions excluding the hyphosphere bacterial community.

Previous studies, only considering hyphosphere-associated bacteria, have also shown their impact on fungi. For instance, the bacterium *Streptomyces* sp. AcH 505 promoted hyphal extension and increased the branching of the mycorrhizal fungus *Amanita muscaria*, indicating that their interaction was able to influence gene expression and cell growth of their fungal partners (Schrey et al., 2005). In another example, the interaction between *Bacillus thuringiensis* and *Fusarium roseum* (now known as *Fusarium sambucinum*) led to the inhibition of the fungus due to the chitinolytic action and volatile antifungal compounds produced by the bacterium (Sadfi et al., 2001). Furthermore, the presence of *Pseudomonas monteilii* was described to be necessary for the formation of mycorrhizae and the establishment of a symbiosis between the fungus *Pisolithus* and the plant *Acacia holosericea* (Founoune et al., 2002). However, no previous studies have considered holistically the fungus network including both its endohyphal and hyphoshere-associated bacterial partners.

The overarching goals of this study were: 1) evaluate how endohyphal bacteria affect fungal competitiveness during interactions with hyphosphere bacteria, particularly examining whether the presence of endosymbionts influences fungal growth performance; 2) determine whether exposure to complex bacterial consortia in the hyphosphere affects fungal-bacterial interaction patterns differently than single-species interactions, particularly focusing on how interkingdom competition moderates impact on the fungal host; 3) investigate how hyphoshere-associated bacterial consortia impact the composition and concentration of endohyphal bacterial communities. Based on previous research and preliminary observations, we propose three hypotheses regarding bacterial-fungal interactions. The first hypothesis is that the presence of endohyphal bacteria would harm fungal growth during competition with hyphosphere bacteria, as maintaining endosymbionts could reduce the fungal host's competitive performance. Second, both the composition and concentration of the endohyphal bacterial community would be affected by external bacterial consortia, reflecting the dynamic nature of these microbial interactions. Third, we hypothesized that exposure to more complex bacterial consortia in the hyphosphere would reduce the competitive burden on both the fungus and its endohyphal bacteria, as the interkingdom competition among the external bacteria would moderate their overall impact on the fungal host. To test these hypotheses, we used three *Fusarium* isolates as a model system, comparing wild-type fungi containing natural endohyphal bacteria with antibiotic-treated counterparts with reduced loads of endohyphal bacteria. We investigated their interactions with hyphosphere-associated bacteria including *Pseudomonas*, *Bacillus*, *Stenotrophomonas*, and *Rhizobium*. This approach allowed us to examine both the individual and combined effects of endohyphal and hyphosphere-associated bacteria on fungal biology. This systematic investigation aimed to determine how distinct microbial communities, both endosymbiotic and hyphosphere-associated bacteria, collectively shape BFI.

2. Materials and methods

2.1. Media

The media used to grow the fungi and the bacteria for the experiments were Reasoner's 2A agar (R2A) and Reasoner's 2A broth without antibiotics (Reasoner and Geldreich, 1985). Both media were purchased from Thermo Fisher Scientific (Waltham, MA, USA). While R2A medium may not maximize *Fusarium* growth compared to traditional fungal media, our choice was driven by the need to support both fungal and bacterial growth in our interaction studies while using a minimum medium, to better simulate the low nutrient conditions in the soil where these fungi were isolated. The media was autoclaved at 121 °C for 15 min. The media used for the endohyphal bacterial reduction consisted of the R2A media supplemented with a mix of antibiotics from stock solutions (10X) of ampicillin (1 g/L), kanamycin (5 g/L), streptomycin (1 g/L), ciprofloxacin (1 g/L) and chloramphenicol (2 g/L). All antibiotic stock solutions were filter-sterilized using a 0.22 μm syringe filter.

The antibiotic mix was prepared by combining the stock solutions to achieve the following final concentrations in the media: ampicillin (100 mg/L), kanamycin (100 mg/L), streptomycin (100 mg/L), ciprofloxacin (1 mg/L) and chloramphenicol (200 mg/L) (Uehling et al., 2017; Desirò et al., 2018). The antibiotic mix was added to the cooled R2A agar and R2A broth after autoclaving, and the media were gently mixed to ensure an even distribution of the antibiotic mix.

2.2. Bacterial and fungal cultures

The fungal and bacterial collections used in this study were previously obtained using the "microcosm fungal highway" isolation method from rhizoplanes of six different plants representing both trees (*Citrus sinensis*, *Diospyros kaki*, *Cycas revoluta*) and shrubs (*Ilex vomitoria*, *Myrica cerifera*, *Buxus sempervirens*) (Pilar et al., 2021; Lupini et al., 2023). The selected plants represented both common urban landscape species and economically important fruit trees in the Houston area, chosen to capture different root architectures and rhizosphere environments.

The molecular identification of the fungal isolates used in this work was previously obtained in our earlier study using both ITS and LSU markers. Phylogenetic relationships of the selected *Fusarium* isolates to reference species are shown in Fig. S1. Molecular identification was performed using ITS1F/ITS4 and LR0R/LR5 primers for ITS and LSU regions, respectively, with sequences deposited in NCBI BioProject PRJNA644907 as detailed in Lupini et al. (2023). The hyphosphere-associated bacteria used in this study (primarily *Pseudomonas*, *Bacillus*, *Stenotrophomonas*, and *Rhizobium*) were previously isolated alongside the fungal strains using the microcosm fungal highway method. These bacteria were originally identified through 16S rRNA gene sequencing of the V3/V4 region using primers F341/R806, with sequences deposited under accession numbers MZ374450-MZ374743; BioProject PRJNA644907.

The presence of endohyphal bacterial communities in the fungal isolates was previously confirmed through scanning electron

microscopy (SEM) analysis (Lupini et al., 2023). Furthermore, the consistent detection of bacterial DNA through both qPCR and sequencing analyses, despite the absence of external bacteria, confirmed that the detected bacteria were residing within the fungal hyphae. Note that our previous work (Lupini et al., 2023) over 80 % of the hyphosphere-associated bacterial community was different than the endohyphal bacterial community in these fungi, further confirming the unique composition of these microbial communities.

To analyze potential associations between fungi and hyphosphere-associated bacteria, we first constructed a co-occurrence matrix based on presence/absence data of fungi-bacteria pairs that were co-isolated and identified on the same original isolation plates across all sampling locations in our previous work (Lupini et al., 2023). The matrix was refined and weighed according to the frequency of co-occurrence between fungi and bacteria. This approach provided a quantitative foundation for the subsequent network analyses (Fig. S2). Network visualization and analysis were performed using the *R-studio igraph* package (Csardi, 2013), with the Kamada Kawai algorithm employed for layout optimization to ensure clear visualization of relationship patterns. (Kamada and Kawai, 1989; Kamada and Kawai, 1989). For fungal candidates, we prioritized organisms demonstrating high network centrality scores, indicating their potential importance in the microbial community (K-core decomposition, representative of the level of structural interconnection) (Bader and Hogue, 2003). Additional selection factors included consistent presence across multiple sampling sites and demonstrated ability to be successfully cultivated under laboratory conditions. Bacterial candidates were selected based on their strong co-occurrence patterns with the selected fungi, ensuring the representation of different functional groups within the microbial community. This comprehensive analysis led to the identification of key microorganisms for this study: ten *Fusarium* isolates obtained from five distinct rhizospheres, and four bacterial genera (*Bacillus*, *Stenotrophomonas*, *Rhizobium*, and *Pseudomonas*) that demonstrated the strongest connectivity patterns in our network analysis. These organisms formed the foundation for our subsequent experimental investigations into bacterial-fungal interactions. Since there were multiple isolates obtained from each petri dish in the microcosm fungal highway experiments, we cannot say with certainty that the selected *Fusarium* isolates carried the specific bacterial isolates chosen for this study. However, the network analysis suggests a high level of potential association between these fungal and bacterial genera based on their co-occurrence patterns. We note that the taxonomic concept of *Fusarium sensu lato* is currently subject to debate in the field, with some researchers proposing its division into several genera while others argue for maintaining it as a single genus. For consistency with our previous work (Lupini et al., 2023), we maintained the traditional genus designation while acknowledging this ongoing taxonomic discussion.

2.3. Antibiotic treatment for generation of treated isogenic lines of fungi

The fungal isolates belonging to the genus *Fusarium* previously characterized to contain diverse endohyphal bacterial communities (Lupini et al., 2023) were selected to remove the endohyphal bacteria via antibiotic treatments. The fungal cultures underwent ten cycles (every 5 days) of alternation between solid and liquid media supplemented with a mix of antibiotics, which included ampicillin (100 ng/ml), kanamycin (100 ng/ml), streptomycin (100 ng/ml), and chloramphenicol (200 ng/ml) (Uehling et al., 2017). At the end of the ten cycles, the fungi were grown on R2A agar without antibiotics. The fungus growth on the plates was determined after 5 days as the radius of the mycelium. A growth comparison between the antibiotic-treated and untreated fungi was performed in triplicate and used to determine phenotypical change in their growth rates. The isogenic lines characterized by a statistically significant difference in growth between treated and wild type ($p < 0.05$, Student T-test) were selected as candidates for the study. Following the removal or significant load reduction of endohyphal bacteria, the fungi were only cultured on antibiotic-free media. All subsequent experiments were conducted in the absence of antibiotics to avoid any antibiotic influence in the analysis. For simplification, in the present study, we will refer to the wild-type (WT) lines that were not treated with antibiotics using their strain name followed by "+" (*e.g.*, F3+, F13b+, F31+), and control isogenic fungi lacking or with reduced loads of endohyphal bacteria were abbreviated with a "-"sign (*e.g.*, F3-, F13b-, F31-).

2.4. DNA extraction, quality control, and holobiont inhibition determination

Sterile cellophane paper disks (Gel Company cellophane sheet 35 × 45cm PK100, Fisher Scientific) were positioned on top of agar plates before the inoculation with either fungal plugs or bacterial suspensions to facilitate the collection of fungal mycelia for DNA extraction.

An agar plug containing fungal mycelium was then transferred to the center of the petri dish containing cellophane and incubated until the fungal growth reached approximately 1/2 to 2/3 of the plate diameter. As controls, petri dishes with sterile cellophane papers were also incubated at the same time. Post-incubation, biomass processing, and DNA extraction were conducted using the Zymo extraction kit for the controls and samples (Zymo Quick-DNA Fungal/Bacterial Kit, D6005). To determine the amount of biomass for extraction, the fresh weight of the fungus was calculated by subtracting the weight of the empty collection tube from the weight of the tube containing the harvested fungal biomass.

2.5. Fungal-hyphosphere-associated bacterial co-culture and fungal inhibition

To assess the impact of hyphosphere-associated bacterial interactions on fungal growth and quantify fungal inhibition, we conducted confrontation assays. To compare the influence of endosymbionts on fungal susceptibility to growth inhibition by hyphosphere-associated bacteria, both wild-type (containing endosymbionts) and antibiotic-treated (with reduced or eliminated endosymbionts) isogenic fungal lines were tested against individual bacterial strains (*Bacillus* sp., *Stenotrophomonas* sp., *Rhizobium* sp., and *Pseudomonas* sp.) and their mixture following an adapted dual culture plate assay (where the fungus is inoculated in agar plug form rather than liquid culture) (Kerr, 1999). The selected fungal lines were maintained in R2A media at 4 °C and transferred to a new plate that was freshly prepared before each set of experiments. The selected bacterial isolates (*Bacillus*, *Stenotrophomonas*, *Rhizobium,* and *Pseudomonas*) were grown overnight at 28 °C in R2A broth until they reached the exponential growth phase. The growth curve was previously determined by the standard plate count method and correlated with the absorbance readings. After reaching the desired growth phase, bacterial cells were harvested by centrifugation (5000×*g* for 10 min at room temperature) and washed three times in phosphate-buffered saline (PBS) solution. After washing, the bacterial cells were centrifugated again, to achieve a final concentration of 10^8 Colony Forming Units per milliliter (CFU/mL). To prepare the bacterial mix, equal volumes (1 ml) of each bacterial suspension (*Bacillus*, *Stenotrophomonas*, *Rhizobium*, and *Pseudomonas*) at 10^8 CFU/mL were combined in a sterile tube and gently mixed by pipetting.

For the confrontation assays, a volume of 20 μL of the bacterial solution was dispensed in the central region of the R2A agar plate in a biohood. Subsequently, a plug of the fungal hyphae was transferred to a new plate by cutting the periphery of a 5-day-old fungal agar culture with the wider aperture of a sterile 200 μL pipette tip. The plug was deposited on top of the bacterial droplet to allow direct interaction between the bacterium or bacterial mix and the fungus. The plate was then incubated at 28 °C. Controls included the fungus in the absence of bacteria, each bacterium and the bacterial mix in the absence of the

fungus, and the culture medium without fungi or bacteria. Each experimental condition and their corresponding controls were conducted in triplicate. The experiments were monitored for 5 days (or until the mycelium completely colonized the plate (Fig. S3). The radial growth of the fungal colony was determined using a digital camera and measured using ImageJ. To quantify the inhibitory effect of the bacterial isolates in the fungus, the inhibition percentage (I%) was calculated as follows:

$$I\% = (Rc\text{-}Rt) / Rc \times 100$$

where Rc represents the mean radius of the fungal mycelium in the control plates, and Rt represents the mean radius of the fungal colonies in the presence of bacteria (Ji et al., 2013).

2.6. Holobiont inhibition and effects on endohyphal bacterial load via qPCR

To assess the impact of antibiotic treatment on the fungal endosymbiotic bacterial community, we quantified the bacterial load in both wild-type and treated fungal isolates. This quantification allowed us to determine the effectiveness of the antibiotic treatment in reducing or eliminating the endosymbiotic bacteria. For all fungal specimens investigated, F13b, F3, and F31, the quantification of the endosymbiotic community was performed according to the method described (Azarbad et al., 2022) using qPCR assays targeting the 16S rRNA gene (E8-F, E533-R) (Nguyen et al., 2017). The endohyphal bacterial load was estimated utilizing a calibration curve correlating copy gene number to Ct values (cycle threshold). A five-point standard curve was generated from a ten-fold dilution series of the *E. coli* K12 DNA and used as a template for the amplification of 16S rRNA ($R^2 = 0.9893$) (Fig. S4). The estimated copy number was further normalized to the amount of extracted biomass (mg) and expressed as copy genes per milligram (copy gene/mg) (Whelan et al., 2003). The reduction in microbial load between treated and wild-type lines was then calculated as follows: Reduction % = (BLw- BLt)/BLw × 100 where BLw represents the mean of the determined bacterial load in the wild type, and BLt represents the mean of the determined bacterial load in the treatment (Fig. S5).

Moreover, to quantify the inhibitory effect imposed by co-culture with the different bacteria on the fungal holobiont, the holobiont inhibition percentage (I%) was calculated as follows:

$$I\% = ((Hc + Hb)\text{-}Ht) / (Hc + Hb) \times 100$$

Where Hc represents the total bacterial load in the control plates (without bacteria), Hb represents the bacterial load associated with the bacterial control (without fungi) and Ht represents the total bacterial load of the fungal colonies in the presence of bacteria (Ji et al., 2013).

2.7. DNA barcoding and sequencing

Before the identification of the fungal-associated holobiont, a nested PCR targeting its 16S rRNA was performed to increase the sensitivity of detection. The two subsequent polymerase chain reactions were intended to amplify the V1/V5 region (EUB9-27F: 5′-GAGTTTGATCCTGGCTCAG-3′; 907r: 5′-CCGTCAATTCCTTTGAGTTT-3′) (Stackebrandt, 1993; Muyzer et al., 1995) and the V3/V4 region (F341: 5′-ACTCCTACGGGRSGCAGCAG-3′; R806: 5′-GGACTACVVGGGTATCTAATC-3′) (Klindworth et al., 2013).

The DNA barcoding was then performed through PCR with each template incorporating unique barcodes via the Barcoding Kit 96 (SQK-RBK110.96) from Oxford Nanopore Technologies (ONT). For each sample, a 10 μL PCR reaction consisting of 9 μL of 50 ng template (obtained from the Nested PCR) and 1 μL of Rapid Barcodes (RB01-96, one per sample). Thermal cycling conditions were 30 °C for 2 min followed by 80 °C for 2 min. PCR product purification was done according to the kit's protocol using a magnetic rack, with elution performed in 10 μL of 10 mM Tris-HCl pH 8.0 with 50 mM NaCl. Quantification of each library was conducted using an Invitrogen Qubit Fluorometer to enable equal concentration for pooling. A volume of 75 μL of the pooled libraries was loaded onto the R9.4.1 flow cell of the ONT MinION Mk1C device (ONT, London, UK), followed by priming of the flow cell as per the kit's manufacturer's instructions. To ensure sequencing data quality and control for potential contamination, negative controls were included. Sequencing reads from control samples was used to establish contamination thresholds - any bacterial taxa appearing in controls were flagged as potential contaminants and removed from the dataset before statistical analysis.

2.8. Fungal microbiome analysis

The sequencing data underwent comprehensive quality control and analysis through a standardized pipeline EPI2ME WIMP (default settings). Base calling was performed using the Guppy basecaller in high-accuracy mode, followed by stringent read filtering with a minimum quality score threshold of 7. This workflow allows for cloud-based processing of the data that includes demultiplexing, quality control, and taxonomic assignment, achieved through the utilization of the BLAST algorithm and Centrifuge against reference database NCBI and SILVA (Benítez-Páez et al., 2016). For each experimental condition, three biological replicates were analyzed, generating an average of 10, 000 reads per sample, which provided sufficient depth for reliable community profiling based on established protocols for bacterial 16S rRNA gene sequencing.

2.9. Statistical analysis and data processing

The data obtained from the sequencing was exported as rarefied abundance at the genus level and analyzed in R Studio. A non-metric multidimensional scaling (NMDS) analysis (Kruskal, 1964; Faith et al., 1987; Dixon, 2003) was conducted to compare the similarities in the fungal microbiome across the different fungal-bacterial co-cultures. The distance matrix was calculated as Bray-Curtis dissimilarities using the vegan package (Dixon, 2003) in R with 2 dimensions (k), and results were visualized with the ggplot2 package (Wickham, 2016). NMDS was complemented with Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson, 2001) analysis using the adonis2 function in the vegan package (R-Studio).

Diversity indices (alpha and beta diversity) were calculated for bacterial endosymbionts using the vegan package in R. Indicator species analysis was performed to determine characteristic functions associated with endo- and hyphosphere-associated bacterial communities. Network analysis was conducted using NetCoMi (Peschel et al., 2020) in R-studio to visualize associations between fungi and associated holobiont. The Fruchterman-Reingold layout algorithm was utilized for network visualization. The different nodes were color-coded based on identified clusters in the data, and the size was represented as degree centrality (representative of the number of connected nodes) (Freeman, 2002). The edges between the nodes were color-coded based on the correlation matrix (positive correlation = green, negative correlation = red, neutral correlation = grey), and the thickness of the lines was plotted based on the strength of the correlation.

3. Results and discussion

3.1. Comparative growth between isogenic lines of Fusarium

A total of seven fungal isolates belonging to the genus *Fusarium*, previously characterized to contain diverse endohyphal bacterial communities (Lupini et al., 2023), were selected for the removal of the endohyphal bacteria via antibiotic treatments. Comparative growth between antibiotic-treated and untreated fungi was performed in triplicate and used to determine phenotypical change in growth rate, as previously reported (Li et al., 2017; Desirò et al., 2018; Uehling et al.,

2017). The treated and wild-type isogenic lines characterized by statistically significant different growth rates ($P < 0.05$, Student T-test) were selected as candidates for the study (Fig. 1).

Three *Fusarium* isolates, namely F3, F31, and F13b, showed notable phenotypic variations between their isogenic lines and were further selected for the present study. Both F31 and F13b antibiotic-treated isolates exhibited a 255 % and 125 % increase in growth rate, compared to their wild-type counterparts, while F3 showed a reduced growth rate of 50 % compared to the control. This difference in terms of fungal growth between the isogenic lines suggested that the treatment might have impacted the endohyphal bacteria and substantially affected the fungal growth.

To confirm that the treatment eliminated or reduced the endohyphal bacterial load, we performed qPCR. All wild-type *Fusarium* isolates (F3, F13b, and F31) presented a higher endohyphal bacterial load compared to their treated counterparts. The results showed that there was a reduction of 90 % in the number of endohyphal bacteria in all three isolates (Fig. 2). These results indicate that the reduction of endohyphal bacteria substantially impacted their growth, either positively or negatively (Fig. 2). Regarding the growth increase found for the F13b and F31 lines following treatment with antibiotics, similar findings have been reported in other fungal-bacterial symbioses. For example, after the removal of the endobacterium *Mycoavidus cysteinexigens* from the fungal host *Mortierella elongata*, the treated fungal strain exhibited a higher growth rate compared to the strain harboring the endohyphal bacteria (Uehling et al., 2017). In contrast, the treated F3 isolate presented a reduced growth rate compared to its wild-type, indicating that the presence of endohyphal bacteria has a beneficial effect on the growth of this particular isolate. Similarly, in the arbuscular mycorrhizal fungus *Gigaspora margarita*, the removal of its endobacterium *Candidatus Glomeribacter gigasporarum* resulted in a decreased fungal biomass (Lumini et al., 2007) due to the capacity of the bacteria to increase the bioenergetic potential of the fungal host (Salvioli et al., 2016).

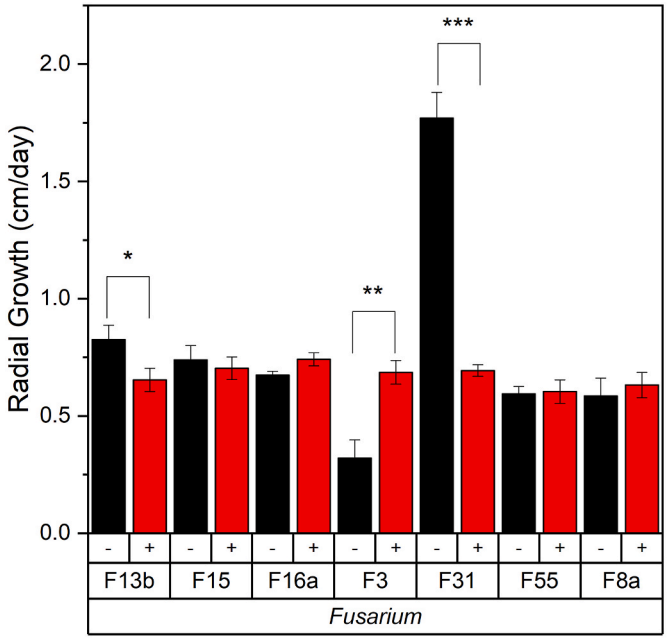

Fig. 1. Comparative growth between wild type and antibiotic treated lines of the different Fusarium isolates. The radial growth of the isolates belonging to the genus *Fusarium* was monitored over 5 days of incubation at 28 °C for 24 h in R2A media without antibiotics. After 5 days, images were taken and measured using the software ImageJ. The radial growth was calculated and expressed as cm per day (cm/day). The graph was divided by isogenic lines and represented as "+" for the wild-type isogenic lines while "-" for those treated with antibiotics. The significance among the isogenic lines was assessed using the Student t-test. P-values <0.05 (*), <0.01 (**), <0.001 (***).

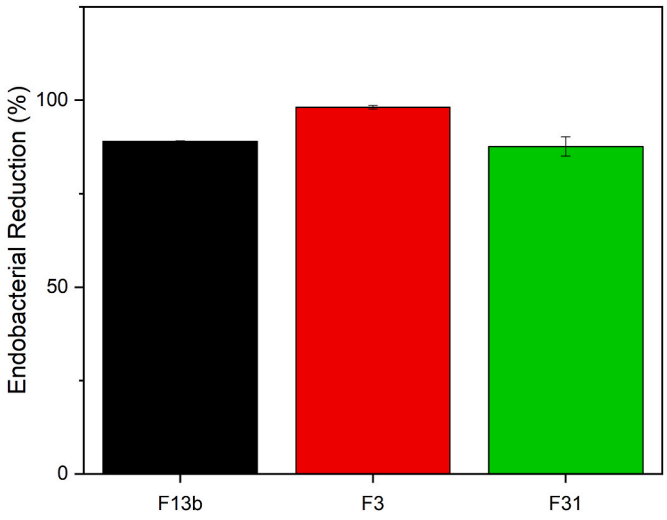

Fig. 2. Reduction of the Endohyphal bacterial load in the isogenic lines of *Fusarium*. The endohyphal bacterial load was estimated with qPCR and expressed as a percentage reduction between the isogenic line treated with antibiotics and its respective wild type.

In this study, the aim was to completely eliminate the endohyphal bacterial population from the fungi. However, we achieved a 90 % reduction of the endohyphal bacterial load across all fungal isolates by treating them with antibiotics. It is worth pointing out that this 90 % reduction was still sufficient to show a significant change in the fungal growth, either by improving the fungal growth or reducing it. These findings show that even though the fungal isolates belong to the same genus, their microbiota can have divergent responses to the antibiotic treatment and affect the fungal growth differently.

To further investigate which pervalent bacterial genera commonly found in the *Fusurium* lines (representing 85 % of total microbial abundance) were the most significantly impacted by the antibiotic treatment, regardless of the fungal strain, a multilevel pattern analysis using the indicator species analysis was performed comparing wild-type and antibiotic-treated fungi. Using the genera identified by the indicator species analysis, a heatmap was generated showing the percentage reduction of genera between the treated fungal lines and their respective wild type (Fig. 3). Based on the top 25 abundant genera (representing 85 % of total microbial abundance), four bacterial genera emerged as being significantly associated with the WT group, while no genera were found to be significantly associated with the antibiotic-treated group (Table 1). The four genera showing strong association with the WT group were *Bacillus* ($p = 0.001$), *Pseudoxanthomonas* ($p = 0.002$), *Xanthomonas* ($p = 0.006$), and *Luteimonas* ($p = 0.012$). After antibiotic treatment, *Pseudoxanthomonas* disappeared or significantly reduced in all three fungi (F3-: 14 %, F13b-: 88 %, and completely removed from F31-). Similarly, *Xantomonas* and *Luteimonas* were also completely removed in F3 and F13, while they were minimally reduced in F31 (7 % and 8 % respectively). Finally, *Bacillus* was almost completely removed from F13 (98 %) but not from the other two (Fig. 3). These findings indicate that the antibiotic treatment affected differently the abundance of these bacteria within the fungal microbiome but overall made them less prevalent and less strongly associated with fungi that were antibiotic-treated compared to the wild type.

Having identified the top abundant genera (part of the 85 % of total microbial abundance) in *Fusarium* that were affected by the antibiotic treatment, we then proceeded to understand why the three *Fusarium* isolates exhibited markedly different growth responses to the antibiotic treatment (with F31 and F13b showing enhanced growth, while F3 showed reduced growth). To address this question, we performed a second, complementary analysis focusing on the overall microbial

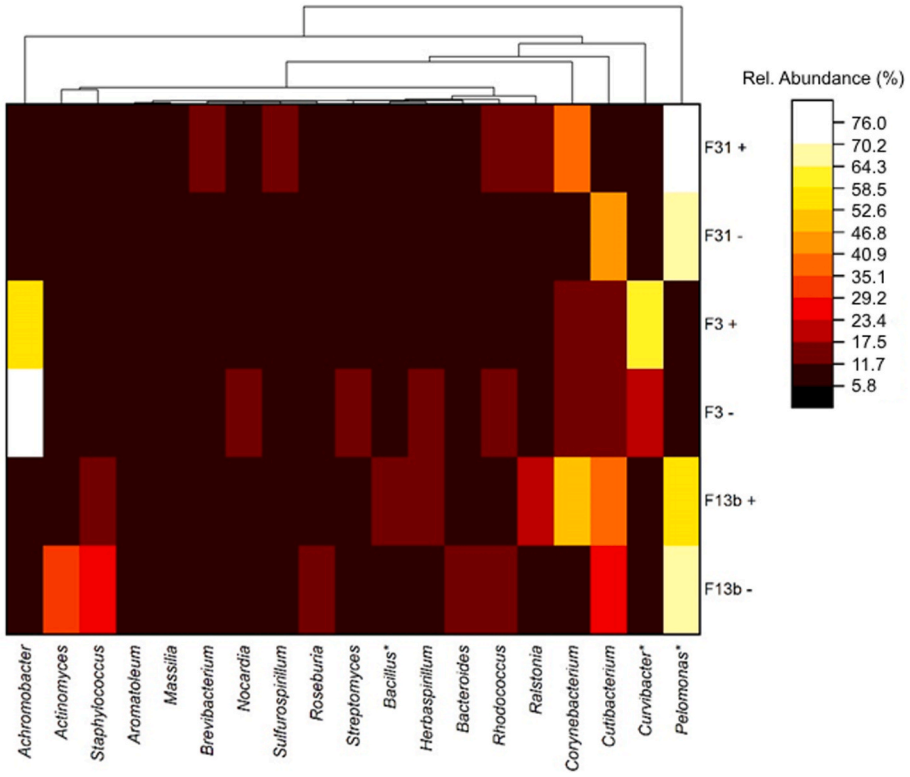

Fig. 3. Heatmap representing microbial composition between the treated lines and their respective wild types. This heatmap visualizes the relative abundance of different bacterial genera across various *Fusarium* isolates, comparing wild-type (+) and antibiotic-treated (−) lines. The genera represented in the figure have been identified with the indicator species analysis. The genera statistically associated (p-value <0.05) with the different isolates were marked with *.

Table 1
Indicator species analysis comparing the genus associated between treated and wildtype isogenic lines of Fusarium (F3, F13b and F31). Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

List of bacteria associated with WT and Antibiotic treated (Multilevel pattern analysis)		
Group wild type #sps. 4	stat	p. value
Bacillus	0.998	0.001 ***
Pseudoxanthomonas	0.907	0.002 **
Xanthomonas	0.870	0.006 **
Luteimonas	0.853	0.012 *

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

community composition differences between the three non-antibiotics treated (wild type) *Fusarium* lines (F3, F13b, and F31) and their antibiotic treated counterparts. This approach allowed us to identify strain-specific endohyphal bacterial associations that might explain the divergent growth responses observed following antibiotic treatment (Table 2). In this analysis we performed the indicator species analysis and found that both F13b and F31 lines were statistically associated with the endobacterium *Pelomonas* ($p = 0.033$). The genus *Pelomonas* has been previously reported as an endosymbiont in various fungi, including isolates belonging to the same family as *Fusarium* (*Nectriaceae*) (Shaffer et al., 2016), such as *Pleurotus ostreatus* (Adamski and Pietr, 2019) and *Metarhizium* (Ying et al., 2022). *Pelomonas* is known for its ability to thrive in oligotrophic environments and utilize multiple carbon sources (Gomila et al., 2005). Moreover, it has been identified as one of the most abundant components of complex microbial systems, such as those found in food waste (Wang et al., 2020) suggesting its ability to withstand both intra- and interkingdom competition. Therefore, it is possible that the presence of *Pelomonas* could have imposed a growth burden on the fungal hosts, potentially competing for nutrients and energy resources. The reduction of *Pelomonas* as endohyphal bacteria in the

Table 2
Indicator species analysis comparing the genus associated with the Fusarium (F3, F13b and F31). Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' '

List of bacteria associated with each isolate (Multilevel pattern analysis)		
Group F13b #sps. 1	stat	p. value
stat p.value		
Bradyrhizobium	1	0.032 *
Group F3 #sps. 1		
Curvibacter	1	0.033 *
Group F31 #sps. 3		
Rickettsia	1	0.045 *
Buttiauxella	1	0.045 *
Modestobacter	1	0.045 *
Group F13b + F3 #sps. 2		
Cutibacterium	1	0.045 *
Acinetobacter	0.952	0.045 *
Group F13b + F31 #sps. 1		
Pelomonas	1	0.033 *
Group F3+F31 #sps. 2		
Achromobacter	1	0.032 *
Citrobacter	1	0.032 *

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

Fusarium isolates F13b and F31 (respectively reduced by 63.9 % and 49.4 % compared to the WT) through the antibiotic treatment could have alleviated this growth burden on the fungal hosts, allowing them to redirect their resources toward faster growth (Fig. 3).

In the case of the F3 fungus, this fungus was statistically associated

with the bacterium *Curvibacter* (p-value = 0.033). *Curvibacter* abundance was reduced by over 80 % compared to the wild type following antibiotic treatment, coinciding with the observed reduction in F3 growth rate (Fig. 3). This bacterium was also previously found to be an endosymbiont of *Pleurotus ostreatus* (Adamski and Pietr, 2019) and it is also well-known for its capacity to grow in oligotrophic conditions (Lesaulnier et al., 2017). Previous studies have demonstrated that *Curvibacter* does not exhibit antifungal activity against *Fusarium* (Fraune et al., 2015). Moreover, *Curvibacter* has been reported to engage in carbon fixation by utilizing CO_2, coupled with sulfur and iron oxidation (Gulliver et al., 2019). While the specific functional relationship between *Curvibacter* and F3 growth performance remains to be determined, this association suggests that certain endohyphal bacteria may contribute differently to fungal fitness across strains.

Another genus that was affected differently by the antibiotic treatment was *Achromobacter*, a genus known for its nitrogen-fixing capabilities (Proctor and Wilson, 1959). This genus was affected in the fungal isolates F3 (increased by 88 % in the cured compared to the wild type) and F31 (reduced by 75 %). Moreover, other bacterial genera impacted by the antibacterial treatment were *Corynebacterium* (removed by the treatment in the F13 and F31 fungi but enriched by 45 % in the F3 fungus) and (removed by the treatment in the F3, by 74 % in F31 and by 60 % in the F3 fungus) (Fig. 2). Isolates belonging to this genus have previously been characterized for their ability to use chitin and glucans as carbon sources, which are components of the fungal cell wall (Hyeon et al., 2011; Lee et al., 2022). The reduction of *Corynebacterium* abundance in F13b and F31 coincided with increased growth rates in these isolates following antibiotic treatment. Although not statistically significant, these alterations in *Achromobacter* and *Corynebacterium* abundance may have contributed to the observed divergent growth responses, with F3 exhibiting increased growth and F31 showing decreased growth following the treatment (Fig. 1). Finally, among the bacteria found to be affected by the antibiotics, we noted that there is a good portion of bacteria that were not completely removed. Among them, we found *Nocardia* (increased by the treatment in the F3 fungus), *Bacteroides* (increased by treatment in fungus F13b), and *Rhodococcus* (increased by treatment in fungus F13b but reduced in fungus F31). These findings suggest that the antibiotic treatment may have impacted differentially the various bacteria associated with the fungi, either by removing or promoting their presence within the fungal microbiome. This alteration in the bacterial composition could have potentially resulted in an imbalanced microbiota, known as dysbiosis (Belizário and Faintuch, 2018). Such a disturbance in the microbial equilibrium would influence the growth of the fungal host, as the complex interactions between fungi and their associated bacteria are likely to play a significant role in maintaining the overall well-being and functionality of the fungal organism in the environment (Salvioli et al., 2017; Uehling et al., 2017).

3.2. Quantification of fungal inhibition by hyphosphere-associated bacterial isolates

In this study, we aimed to quantify the inhibitory effects of various hyphosphere-associated bacterial isolates on the growth of fungal isolates, both WT and antibiotic-treated fungi. By comparing the growth of WT fungal isolates containing their natural endohyphal bacterial communities with those treated with antibiotics, we sought to elucidate the role of these endosymbionts in modulating the host response to the co-culture with hyphosphere-associated bacterial isolates. The hyphosphere-associated bacterial isolates used in this study included *Pseudomonas*, *Bacillus*, *Stenotrophomonas*, and *Rhizobium*. In addition to the individual bacterial isolates, a mixed bacterial co-culture containing a combination of these isolates was also used to investigate the effects of a more complex microbial community on the fungal isolates. The fungal growth inhibition percentage (I%), calculated by comparing the mean radius of fungal colonies in the control plates with the mean radius of fungal colonies in the co-culture plates, was used to assess the complex interplay among the fungal isolates, their endohyphal bacterial communities, and the hyphosphere-associated bacterial isolates, providing insights into the role of endosymbionts and hyphosphere-associated bacteria in modulating the fungal growth rates.

When comparing the antibiotic-treated and wild-type fungal isolates, a general trend emerged. The treated fungal isolates, which had their endohyphal bacterial communities reduced, exhibited higher growth inhibition percentages compared to their WT counterparts for both the F3 (towards co-culture with bacteria *Pseudomonas* B2, *Bacillus* B6a, and *Rhizobium* B59b) and the F31 (under all co-culture conditions) (Fig. 4). This suggests that the presence of endohyphal bacteria can potentially provide some level of resistance against the inhibitory effects of external bacteria. For example, the antibiotic-treated F31 isolate showed growth inhibition percentages ranging from 68 % with the highest level of inhibition against *Pseudomonas* B2, *Bacillus* B6a, and the bacterial mix (76.44 %, 75.07 % and 70.57 % respectively), while the F31 wild-type had lower inhibition percentages (*Pseudomonas* B2 = 38.52 %, *Bacillus* B6a = 16.46 %, *Stenotrophomonas* B28b = -0.53 %, *Rhizobium* B59b = 0.41 % and Mix = 24.91 %) (Fig. 4). This reduced inhibitory effect conferred by the endohyphal bacteria to the wild-type lines highlights the importance of the endohyphal bacterial communities in maintaining the growth and adaptability of their fungal hosts when faced with external competition (Partida-Martinez and Hertweck, 2005; Salvioli et al., 2016; Vannini et al., 2016).

Although this result appears to be in contrast with what was previously discussed regarding the increase in growth of the fungus without the endohyphal bacteria and hyphosphere-associated bacteria, it is important to note that the co-culture between fungi and bacteria creates different competitive conditions that would not occur in the absence of the hyphosphere-associated bacteria (Brakhage and Schroeckh, 2011; Scherlach and Hertweck, 2020). These results highlight the importance of investigations in complex conditions, which is demonstrated by the two different responses of the fungi in the presence of endohyphal bacteria and hyphosphere-associated bacteria. For this reason, the two results, *i.e.*, with and without hyphosphere-associated bacteria, should be considered separately, as the complex interactions induced by the co-culture between fungus and hyphosphere-associated bacteria could have completely changed the growth trade-offs concerning the maintenance of the endosymbiont and related fitness of the fungal host (Saikkonen et al., 2002; Salvioli et al., 2016; Vannini et al., 2016; Pawlowska et al., 2018).

Moreover, in addition to the trend found between treated and untreated, we also noticed that the response to different bacterial isolates and mixed cultures varied for each of the different fungal isolates. For instance, *Pseudomonas* (B2) generally induced higher inhibition percentages across the fungal isolates, with an average inhibition of 30 % for the treated antibiotic-treated lines and 28 % for the wild-type lines (Fig. 4). Similarly, the co-culture with the *Bacillus* (B6a) also presented an inhibitory effect on all fungi. In this case, however, the reduction of the endobacterium played a crucial role in the interaction. For instance, the fungi treated with the antibiotic presented an average inhibitory effect of 36 %, compared to 5 % for their respective wild types. On the other hand, *Rhizobium* (B59b), generally resulted in lower inhibition percentages in the treated lines (14 %) and even induced growth by 7 % for the wild-type lines (Fig. 4). Interestingly, the mixed bacterial co-culture, which contained a combination of the bacterial isolates, often resulted in intermediate inhibition percentages compared to the individual bacterial co-cultures. This validates our initial hypothesis regarding the fact that in a more complex system such as a synthetic

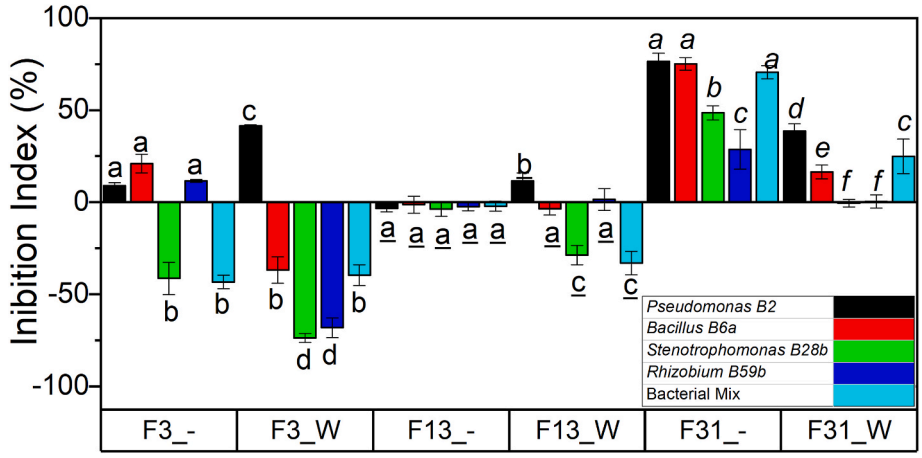

Fig. 4. Fungal inhibition index values for the interaction between three *Fusarium* isolates (F3, F13b, F31) and various bacterial strains (*Pseudomonas* B2, *Bacillus* B6a, *Stenotrophomonas* B28b, *Rhizobium* B59b, and a bacterial mix, comparing wild-type (+) and antibiotic-treated (−) lines. Letters above bars indicate significant differences ($p < 0.05$, Tukey's HSD test), using normal letters for F3 (*i.e.* a,b), underscored letters for F13b (*i.e.* a,b) and italicized letters for F31 comparisons (*i.e. a,b*).

bacterial mix, the interkingdom competition between bacteria would have had a positive impact concerning the growth of the fungus (Partida-Martinez and Hertweck, 2005; Salvioli et al., 2016; Vannini et al., 2016). This finding underscores the need to investigate the fungal microbiome as a complex network of interactions rather than focusing solely on pairwise interactions between fungi and bacteria (Agler et al., 2016).

3.3. Impact of Co-culture on bacterial holobiont

In this study, we investigated the impact of co-culture between fungal isolates and hyphosphere-associated bacterial isolates on the bacterial holobiont. The holobiont concept considers the fungal host and its associated microbial communities (both inside and outside the fungal hyphae) as a single ecological unit, recognizing the complex interactions and symbiotic relationships between them (Bosch and McFall-Ngai, 2011; Deveau et al., 2018). By examining the holobiont, we aim to gain a more comprehensive understanding of how the presence of endohyphal bacteria influences the fungal host's response to external microbial challenges. To determine the impact of co-culture between fungus and bacteria on the holobiont, the number of copies of the 16S rRNA gene per gram of biomass was quantified compared to the control. The control samples consisted of the fungal isolates grown in the absence of hyphosphere-associated bacterial isolates and the bacterial cultures without the fungus. The combination of these two factors represents a baseline in which the two microbial populations, representing the fungal holobiont, have no external challenges.

The comparative analysis of the antibiotic-treated with the WT allowed the determination of the overall changes in the abundance of the holobiont in the presence of hyphosphere-associated bacteria *Pseudomonas* B2, *Bacillus* B6a, *Stenotrophomonas* B28b, *Rhizobium* B59b. The results showed significant inhibition of the holobiont when comparing antibiotic-treated fungi versus wild-type fungi in the presence of different bacterial strains: B2 (*Pseudomonas*) caused 40 % inhibition in antibiotic-treated fungi compared to 25 % in wild-type fungi; B6a (*Bacillus*) caused 44 % inhibition in antibiotic-treated fungi versus 27 % in wild-type fungi; B28b (*Stenotrophomonas*) resulted in 44 % inhibition in antibiotic-treated fungi versus 35 % in wild-type fungi; B59b (*Rhizobium*) led to 51 % inhibition in antibiotic-treated fungi versus 36 % in wild-type fungi; and the bacterial mix caused 39 % inhibition in antibiotic-treated fungi compared to 28 % in wild-type fungi (Fig. 5). Overall, all holobiont that were antibiotic-treated presented higher inhibition than their wild-type fungus counterparts.

In addition to determining the direct effects of the hyphosphere-associated bacteria in the endohyphal bacteria, we also determined the inhibition effects of the holobiont across the different *Fusarium* lines. In the case of F3, we observed that the holobiont inhibition index was consistently lower in the wild-type (WT) specimens compared to

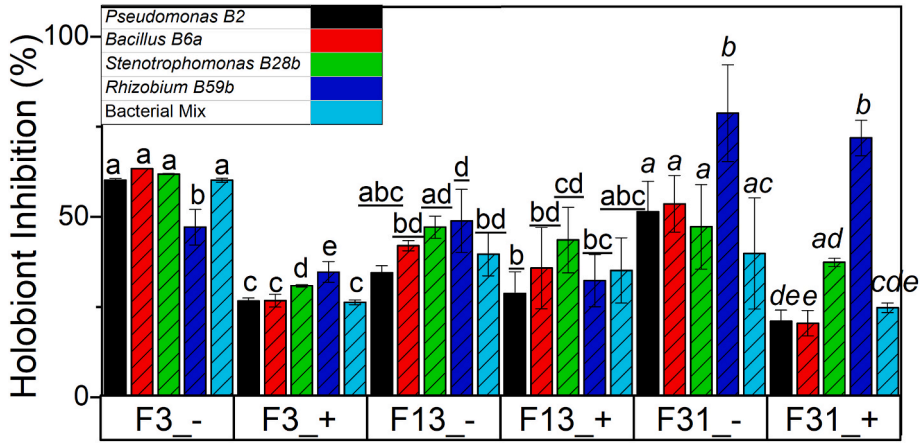

Fig. 5. Holobiont inhibition index values for the interaction between three *Fusarium* isolates (F3, F13b, F31) and various bacterial strains (*Pseudomonas* B2, *Bacillus* B6a, *Stenotrophomonas* B28b, *Rhizobium* B59b, and a bacterial mix, comparing wild-type (+) and antibiotic-treated (−) lines. Letters above bars indicate significant differences ($p < 0.05$, Tukey's HSD test), using normal letters for F3 (*i.e.* a,b), underscored letters for F13b (*i.e.* a,b) and italicized letters for F31 comparisons (*i.e. a,b*).

antibiotic-treated specimens across all bacterial exposures. Specifically, when exposed to *Pseudomonas* B2, the antibiotic-treated F3 exhibited 60 % inhibition compared to only 27 % inhibition in the WT F3. Similarly, when exposed to *Bacillus* B6a, the antibiotic-treated F3 showed 63 % inhibition versus 27 % in WT F3. This pattern continued with *Stenotrophomonas* B28b (44 % inhibition in antibiotic-treated F3 versus 31 % in WT F3), *Rhizobium* B59b (47 % inhibition in antibiotic-treated F3 versus 34 % in WT F3), and the bacterial mix (60 % inhibition in antibiotic-treated F3 versus 26 % in WT F3). These findings suggest that the endophytic bacterial community present in the F3 strain provides a protective effect against external bacterial competition. When this endophytic community was reduced through antibiotic treatment, the fungus became significantly more susceptible to inhibition by hyphosphere-associated bacteria. This demonstrates that intact endophytic bacterial populations can modulate and mitigate the inhibitory effects of external bacteria on the fungal holobiont. (Salvioli et al., 2016; Vannini et al., 2016). For F13b, only in the case of co-culture with *Rhizobium* (B59b) the inhibition of the holobiont was statistically significant (treated: 48.9; wild type: 32.3 %) (Fig. 5). In the case of co-culture with other bacteria although not statistically significant, the WT performed better compared to antibiotic-treated fungi (Fig. 5). The fungus F31 presented a similar scenario as F13b, where, although a statistically significant difference was not found, we can see how the treatment with antibiotics led to a general inhibition of the holobiont compared to the wild-type line. (Fig. 5). The inhibition index values for both the antibiotic-treated and WT against *Rhizobium* (B59B) are relatively similar across the two isogenic lines. These findings suggest that the presence of the endobacterium not only can buffer the fungal host against inhibition by hyphosphere-associated bacteria but can partially protect the holobiont. This result reflects what was previously observed with animals and plants, which suggests that the role of holobionts is important also for fungi (Belizário and Faintuch, 2018; Arnault et al., 2023). The alteration of the microbial composition is known to negatively affect the fitness of the host itself (Vandenkoornhuyse et al., 2015). To the best of our knowledge, this is the first study to determine the impact that dysbiosis of the fungal-associated microbiota can have on the host and its holobiont.

3.4. Holobiont composition and identification of hub species

The fungal holobiont, comprising the total microbial communities associated with the fungus, plays a crucial role in the ecology and functioning of an organism (de Menezes et al., 2017; Deveau et al., 2018). Understanding how interkingdom interactions involving fungi and bacteria can share ecological niches is essential for elucidating the factors that shape their structure, functionality, and stability. While previous studies have demonstrated that bacterial communities can be associated with fungi (Bonfante and Desirò, 2017; Robinson et al., 2021; Lupini et al., 2023), there is still a lack of knowledge regarding how the complex network of associations related to the holobiont is influenced by interactions between the fungus and its hyphosphere-associated bacterial community. One approach to unraveling this complex system of associations is to construct network analyses and, through structural characterization of the different components, identify potential hubs (Agler et al., 2016). Bacterial hubs are those that have a disproportionately large influence on the structure and function of the microbial community (Agler et al., 2016; Banerjee et al., 2019). These bacteria often engage in multiple interactions with other members of the community and play critical roles in maintaining the stability and resilience of the holobiont. In this study, we aimed to address this knowledge gap by investigating the composition of the fungal holobiont and identifying potential bacterial hubs within the bacterial community.

To investigate the composition of the wild-type fungal holobiont and identify potential bacterial hubs within the microbial community, we focused exclusively on wild-type *Fusarium* strains (F3+, F13b+, and F31+) rather than their antibiotic-treated counterparts. This methodological choice was deliberate, as our objective was to characterize natural holobiont networks under different bacterial challenges. The antibiotic-treated strains, having significantly reduced endohyphal bacterial abundance and altered community structures (as demonstrated in Figs. 2 and 3), would not provide meaningful insights into natural hub species and network relationships. We employed a combination of heatmap analysis, indicator species analysis, and network analysis. The heatmap analysis allowed us to visualize the differences in the fungal holobiont composition across various experimental settings, specifically: (1) fungal strain differences (comparing the three wild-type *Fusarium* isolates F3+, F13b+, and F31+), and (2) bacterial exposure treatments (comparing control fungi with those exposed to individual bacterial species *Pseudomonas* B2, *Bacillus* B6a, *Stenotrophomonas* B28b, *Rhizobium* B59b, or the synthetic bacterial mixture). The indicator species analysis helped to identify bacterial genera that were significantly associated with specific groups or treatments. Finally, the network analysis enabled us to determine the interactions and co-occurrence patterns among the bacterial and fungal members of the holobiont, as well as to identify potential bacterial hub species that play a central role in shaping the holobiont structure and function. Additionally, to investigate how the presence of interkingdom competition within a synthetic bacterial mix influences the holobiont associations compared to co-culture with individual bacterial isolates, we compared the bacterial network reconstructions of the synthetic microbial community (Mix) with those of the wild-type fungal holobiont co-cultured with each individual bacterial isolate.

The diversity of the fungal holobiont, measured by the Shannon index, was generally higher in co-culture conditions compared to their respective controls, although the differences were not statistically significant. Among the fungal isolates, F3 exhibited the most pronounced increase in holobiont diversity when co-cultured with different bacterial isolates, ranging from 5.4 % (*Pseudomonas* B2) to 44.8 % (Mix) (Fig. 6).

In contrast, F31 showed a different response when compared to the control without the external bacterial challenge. For instance, when F3 was co-cultured with the bacterial mix (Mix), the holobiont diversity increased by 9.7 % as opposed to a decrease in diversity when co-cultured with individual bacterial isolates. It is noteworthy mentioning that the co-culture with the bacterial mix consistently led to an increase in the diversity of the fungal holobiont across all fungal isolates. This suggests that exposure to a diverse consortium of external bacteria may reduce competitive exclusion dynamics among endohyphal bacteria. When multiple bacterial species interact in the hyphosphere, their competitive interactions with each other moderate the selective pressure any single species exerts on the endohyphal community, allowing for greater endohyphal bacterial diversity compared to exposure to single bacterial species (Zhang et al., 2021).

To further understand the differences in the endophytic bacterial community of the holobiont in response to different hyphosphere-associated bacteria inoculant treatments, we employed a combination of heatmap analysis (Fig. 6) and Network Analysis (Table 3, Figs. S6–10). The heatmap revealed the presence of important endohyphal bacterial genera after exposure to individual hyphosphere-associated bacteria and bacterial mix, which include the genera *Citrobacter*, *Erwinia*, *Buttiauxella*, *Cutibacterium*, *Pelomonas*, *Pseudoxanthomonas*, *Xanthomonas*, *Lysobacter*, and *Luteimonas*. Some of these genera were also identified as potential hubs of the holobiont following co-culture with different bacteria (Fig. 6).

Network analysis using the NetCoMi tool (Peschel et al., 2020) revealed that the percentage of positive interactions between bacterial taxa within the fungal holobiont (including both endohyphal and hyphosphere-associated bacteria) varied depending on the co-culture condition. When fungi were co-cultured with different bacterial treatments, we observed distinct network structures. For instance, *Bacillus* B6a and *Stenotrophomonas* B28b presented the highest percentage of positive edges (88.7 % and 90 %, respectively), while *Pseudomonas* B2 presented the lowest (65 %) (Table 3).

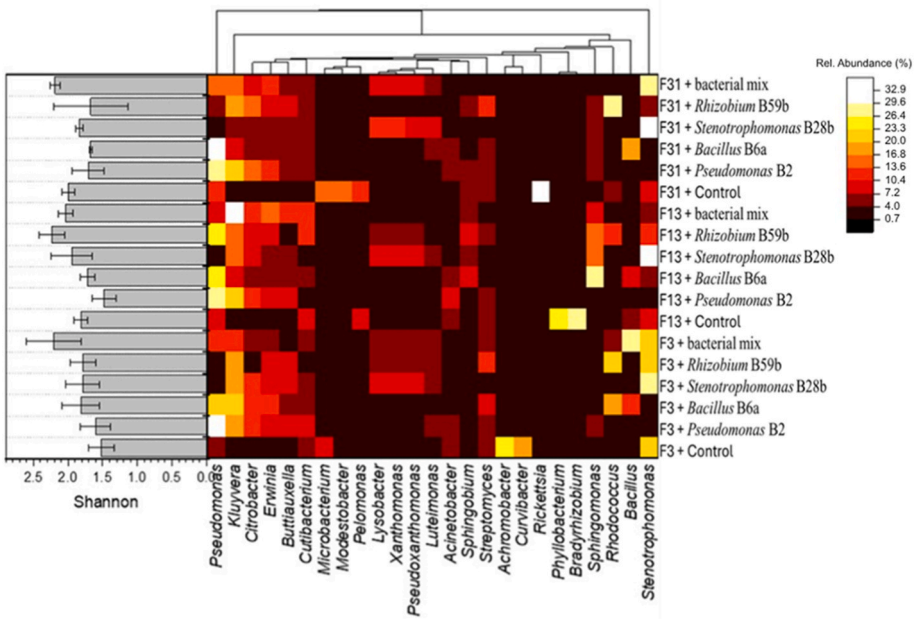

Fig. 6. Heatmap showing the fungal holobiont differences across conditions. The top 25 bacterial genera across different conditions for easy visualization. The clustering was based on the similarities of relative abundance of bacterial genera in the different co-cultures. The bar graph represents the bacterial diversity associated with the different conditions.

Table 3
Properties of networks built through NetCoMi in different co-culture conditions.

	Pseudomonas (B2)	*Bacillus (B6)*	*Stenotrophomonas (B28b)*	*Rhizobium (B59b)*	*Bacterial mix (Mix)*
Clustering coefficient	***0.27924***	***0.31874***	***0.35515***	***0.1863***	***0.32055***
Modularity	***0.71556***	***0.68158***	***0.67671***	***0.64514***	***0.69852***
Positive edge percentage	***65.47619***	***88.70968***	***89.65517***	***76.72414***	***77.95276***
Hubs	***Citrobacter***	***Buttiauxella***	***Buttiauxella***	***Buttiauxella***	***Inhella***
	Erwinia	***Erwinia***	***Citrobacter***	***Ideonella***	***Luteimonas***
	Rahnella	***Pectobacterium***	***Franconibacter***	***Kluyvera***	***Pseudoxanthomonas***
	Yersinia	***Serratia***	***Klebsiella***	***Pectobacterium***	***Xanthomonas***

Clustering Coefficient: the tendency of a node's neighbors to form tightly connected clusters (Saramäki et al., 2007). Modularity: quantifies the quality of a proposed division of a network into distinct communities or modules, >0.3 it is defined as the good presence of significant structures present within the network (Clauset et al., 2004).

The most observed genera across all fungal holobionts were *Klebsiella*, *Kluyvera*, *Bacillus*, *Pectobacterium*, and *Buttiauxella*, which have been previously described to be associated with the rhizosphere (Table 3) (Sachdev et al., 2009; Krzyzanowska et al., 2012; Mussa et al., 2018; Lupini et al., 2023). Despite the significant compositional overlap between the holobionts associated with different *Fusarium* isolates, our hub analysis revealed only three bacterial genera (*Buttiauxella*, *Erwinia*, and *Citrobacter*) that consistently functioned as network hubs across multiple bacterial co-culture conditions (Table 3). These genera are likely closely associated with these fungal isolates, and their persistence as network hubs across different experimental conditions suggests they are actively maintained by the fungi for their potential metabolic or ecological benefits. While their specific functional roles in the *Fusarium* holobiont require further experimental verification, previous literature suggests that these genera may provide beneficial properties such as phosphorus solubilization, auxin production, and ligninolytic activity (Patel et al., 2008; Wang et al., 2022; Xie et al., 2023), potentially contributing to fungal fitness and adaptation to environmental challenges (Jiang et al., 2021).

By comparing the networks of the holobionts associated with the co-culture of the synthetic bacterial mix (Mix) and the individual bacterial isolates, we can observe how the interkingdom competition within the bacterial mix influenced the bacterial associations within the holobiont (Figs. S11–S14). For instance, co-culture with *Pseudomonas* (B2) and *Rhizobium* (B59b) resulted in more changes in holobiont associations compared to the Mix, with only a few associations remaining unaffected. In contrast, co-culture with *Bacillus* (B6a) and *Stenotrophomonas* (B28b) had a lesser impact on the bacterial associations, with most of the associations remaining unchanged compared to the Mix. These findings suggest that the impact of co-culture on the holobiont composition varies depending on the specific bacterial isolates involved, with some isolates having a more pronounced effect on the bacterial associations than others (de Boer, 2017; Deveau et al., 2018).

4. Conclusions

This study provides novel insights into how endohyphal bacterial communities influence fungal interactions with hyphosphere-associated bacteria, advancing our understanding of complex microbial relationships in several ways. First, while previous research has examined either endohyphal bacteria or hyphosphere-associated bacterial interactions with fungi independently, our work demonstrates that these communities act synergistically to shape fungal responses to microbial communities. The observation that wild-type fungi containing intact endohyphal bacterial communities showed enhanced resilience against external bacterial competitors compared to their antibiotic-treated counterparts suggests a previously unrecognized protective role of endohyphal bacteria.

Furthermore, our findings that different *Fusarium* isolates exhibited distinct responses to bacterial challenges, despite belonging to the same genus, highlights the strain-specific nature of these interactions. This suggests that bacterial-fungal interaction patterns observed in model systems may not be broadly generalized across fungal taxa. However, the consistent pattern of enhanced resilience conferred by endohyphal bacteria across all tested strains points to fundamental mechanisms that may extend beyond *Fusarium* to other fungal genera.

The increased bacterial community diversity observed in response to our synthetic bacterial mix, compared to single-species treatments, suggests that more complex external bacterial communities may help stabilize the holobiont through competitive interactions. This raises intriguing questions about how fungal holobionts might respond to even more diverse bacterial communities found in natural soil environments. Future research should explore these interactions using more complex synthetic communities and soil microbiome transplants to better understand how endohyphal bacteria influence fungal ecology in natural settings.

Our work also revealed potential bacterial hub species within fungal holobionts, including *Buttiauxella*, *Erwinia*, and *Citrobacter*, whose presence strongly influenced overall community structure. Understanding the roles of these key taxa could provide new approaches for manipulating bacterial-fungal interactions in agricultural and environmental applications.

While our experimental approach using a simplified agar medium provided valuable insights into fungal-bacterial interactions, we acknowledge several important limitations of this reduced system. The artificial growth conditions and nutrient availability on agar plates differ substantially from the complex physicochemical environment of natural soils, where factors such as pH gradients, nutrient heterogeneity, moisture fluctuations, and the presence of other microorganisms likely influence bacterial-fungal interactions. Although this simplified system was necessary to isolate and study specific aspects of bacterial-fungal relationships, future research should validate these findings under more naturalistic conditions that better reflect the complexity of soil ecosystems. This might include examining these interactions in soil microcosms or using lower bacterial concentrations more representative of natural populations.

Despite the possible limitations due to the simplification of a complex system such as the rhizosphere, this study establishes a framework for investigating how internal and external bacterial communities collectively shape fungal biology. Future research should extend these findings by examining these interactions under more naturalistic conditions, incorporating spatial and temporal heterogeneity, and exploring how these patterns manifest across diverse fungal taxa. Such work will be crucial for understanding the ecological importance of bacterial-fungal interactions in soil environments and their implications for ecosystem functions.

CRediT authorship contribution statement

Simone Lupini: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Lúrima Uane Soares Faria:** Methodology, Investigation. **Claudio Augusto Oller do Nascimento:** Supervision, Resources, Investigation. **Debora F. Rodrigues:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Availability of data and materials

All data derived from this work is publicly available in the NCBI-GenBank database under the following accession numbers: 16s rRNA gene sequences: BioProject PRJNA1134346.

Consent for publication

All authors have read and participated in the preparation of the manuscript. All authors consent to the publication.

Funding

This study was funded by the NSF award #2125480.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2025.101455.

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