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Establishing microbial communities to promote the growth of *Pleurotus ostreatus* through a top-down approach is hindered by the dominance of antagonistic interactions

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ABSTRACT Few attempts have been made to examine the diversity and functions that characterize microbial communities compatible with and beneficial to the biotechnologically relevant mushroom *Pleurotus ostreatus*. The quest for complementarity is complicated by the variable nature of beneficial traits, impairing the rational assembly of synthetic communities to improve bioprocesses. This study investigated whether the compatibility between *P. ostreatus* and microbial enrichments is lessened in conditions favoring saprophyte metabolism and is enhanced when a combination of recalcitrant and labile carbon is integrated in the enrichment. The microbial diversity of enrichment cultures and substrates colonized by *P. ostreatus* was analyzed through PCR amplicon sequencing, and the proliferation of *P. ostreatus* was assessed by quantitative image analysis. In general, co-inoculation of lignocellulosic substrate with microbial enrichment reduced the growth of *P. ostreatus*. The saprophytic enrichment conditions were more conducive to the development of antagonistic communities inhibiting *P. ostreatus* growth than enrichment cultures integrating labile carbon substrates. Both microbial community analyses and *in vitro* assays led to the identification of a single phylotype affiliated with *Brevundimonas* spp., which displayed neutral interaction with *P. ostreatus*. Recalcitrant and labile carbon degradation functions were not primary factors driving beneficial microbial communities for *P. ostreatus*. Additional functions beyond carbon metabolism are likely to promote beneficial interactions. Directed enrichment cultures, integrating the mushroom in the earliest stage of the procedure, are expected to promote more beneficial interactions than top-down approaches.

IMPORTANCE Lignocellulosic biomass upcycling biotechnologies integrating solid-state fermentation by fungi are aligned with sustainable development perspectives. While the recalcitrance of this biomass imposes a challenge for the implementation of these bioprocesses converting the lignocellulosic feedstock into bioenergy and bioproducts, pretreatment of lignocellulose biomass with fungi is efficient and generates fewer by-products than chemical approaches. Optimization and stabilization of this bioprocess by integrating microbial consortia has received little attention. The significance of our research is to bridge that knowledge gap by examining how interactions between the biotechnologically relevant basidiomycete *Pleurotus ostreatus* and microbial communities influence fungal growth in lignocellulosic substrate. Directed enrichment cultures integrating *Pleurotus ostreatus* as a selective agent are expected to trigger more beneficial interactions promoting mushroom growth than our top-down approaches, due to a dominance of antagonistic mushroom-bacteria interactions.

KEYWORDS top-down assembly of microbial consortia, *Pleurotus ostreatus*, microbial enrichment, antagonism, lignocellulose

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Interactions between bacteria and fungi (BFI) underpin major ecological processes, essential for ecosystem functioning, including the health of host organisms (animals and plants) and biogeochemical cycles. Thus, BFI are increasingly studied in medicine, agriculture, environmental science, food processing, and biotechnology (1, 2). Antagonistic relationships are particularly represented, with many well-characterized interactions ranging from competition for nutrients to interference competition (3). Despite the dominance of negative inter-kingdom interactions, positive interactions such as mutualism, commensalism, or symbiosis contribute to plant growth promotion through arbuscular mycorrhizal fungi (4, 5) and organic waste upcycling biotechnologies integrating solid-state fermentation (6). The latter is particularly aligned to sustainable development perspectives, integrating the valorization of lignocellulosic biomass generated by agro-industrial activities.

Due to its high carbon density and availability, lignocellulosic biomass could reduce dependency on non-renewable resources. The recalcitrance of the substrate imposes a challenge for the implementation of bioprocesses converting the lignocellulosic feedstock into bioenergy and bioproducts (7). Unlike chemical methods, pretreatment of lignocellulosic biomass using fungi that produce cellulase, hemicellulase, and lignin-modifying enzymes is more efficient and generates fewer by-products (8). However, this bioprocess is less commonly applied in industry, as microbial growth slows down the production rate. Other drawbacks relate to the energy costs associated with biomass sterilization requirements (9). Nevertheless, the demand for more environmentally friendly industrial processes, combined with the global challenge of biomass management, positions biological pretreatment as a promising solution for the future. Optimizing bioprocess performance through the characterization of microbial communities that are compatible with and beneficial to fungal growth is therefore essential to control the residual microbial community in the biomass after cost-effective pasteurization and to shorten the fungal growth period, respectively.

Lignocellulosic biomass is a highly resistant substrate when it comes to degradation because of its chemical composition, with lignin displaying a complex and irregular structure, and due to the crystallinity of cellulose (10). Despite this natural recalcitrance, bacteria and fungi developed several strategies supporting the carbon cycle, such as the production of extracellular enzymes and the deployment of oxidative processes for nutrient acquisition and for lignin and polysaccharide recycling (11). *Pleurotus ostreatus* is a saprophytic fungus with a broad ecological niche, with species from this complex found in both grassland and forest ecosystems, extracting nutrients from grasses and decaying wood (12, 13). Beyond the degradation of cellulose and hemicellulose, *P. ostreatus* and white rot fungi in general have the unique ability to mineralize lignin to CO_2 and water (14). Bacterial communities surrounding *P. ostreatus* also demonstrated cellulose and hemicellulose degradation capabilities (15, 16). Laccase-like genes were identified in *Pleurotus* sp. associated bacteria, suggesting that bacteria are also involved, at least in part, in lignin mineralization (17). This functional redundancy in the degradation of organic compounds has been observed not only in lignocellulosic biomass but also in other ecosystems such as litter (18) and soil (19). Consequently, there is competition between bacteria and fungi for the occupation of the ecological niche. Competition for carbon is stronger than for other resources due to the overlap between bacterial and fungal demand for organic compounds (19). While bacteria are more efficient for labile organic compounds assimilation, fungi outcompete the former for complex compounds thanks to their high catalytic enzyme efficiency (19). The extensive mycelial network of fungi allows nutrient bioprospection stored in recalcitrant organic pools (20), while the release of sugar monomers by fungal extracellular enzymes leads to inter-kingdom competition, the dominant soil interaction (19). This competition appears to be even more pronounced in an environment with limited resources, such as lignocellulosic biomass (21, 22).

Despite this competition, bacteria and fungi co-exist in ecosystems, and their metabolic dependency and complementarity shape their co-occurrence in nature (23).

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One mechanism is cross-feeding with lignocellulose-degrading synthetic communities (24, 25), for which bacteria and fungi can both benefit from the metabolites released by the partner. These observations drive studies for bacterial inoculation of mushroom growing substrate to enhance their biomass yield (26). Secondary metabolism functions and traits exerting a growth-promoting effect were examined in the literature (15, 26–30). The ability to fix nitrogen (31), or to produce 1-aminocyclopropane-1-carboxylic acid deaminase (32) and indole-3-acetic acid (33), is an example of beneficial bacterial traits for fungal growth. The ability to produce volatile organic compounds (34) and siderophores (35) was also reported to be involved in *Pleurotus* sp. growth promotion. The exact mechanisms underlying bacteria and fungi interactions, however, are largely uncertain because they are context-dependent (36) and sometimes conflicting (29). The identified traits are primarily based on correlations, without direct functional evidence, indicating that most mechanisms remain unproven.

Predicting beneficial interactions for *P. ostreatus* growth and defining an efficient enrichment strategy tailored to beneficial functions in bioprocesses is challenging. Isolating beneficial communities for *P. ostreatus* from natural environments is complicated by the unique nature of agricultural lignocellulosic waste integrated in biorefinery, i.e. microbial assembly in decaying wood or spent mushroom substrate (36) differs from those in coco fiber in a greenhouse containing plant tissues, fertilizers, and chemicals. In addition, this design is impeded by the poor understanding of the delicate trade-off delineating bacterial beneficial complementarity supporting vegetative growth of the fungus and competition reducing fungal biomass. Finally, bacterial communities surrounding fungi have been studied using high-throughput sequencing and imagery technologies (23, 37), but few attempts have been made to examine the diversity and functions that characterize microbial communities compatible with and beneficial to fungal growth. This study bridges that knowledge gap by examining how interactions between the biotechnologically relevant basidiomycete *Pleurotus ostreatus* and microbial communities influence fungal growth in coco fiber from planting substrates of a commercial greenhouse.

The absence of selective traits beneficial to the fungus impedes a targeted enrichment strategy. However, competition interactions for access to carbon and energy sources under saprophytic conditions are expected. Under this hypothesis, a microbial enrichment relying on lignocellulosic biomass as a sole carbon source is likely to be less compatible with *P. ostreatus* than enrichment strategies including additional labile carbon sources. In the latter, the enrichment of microbial consortia compatible with fungus growth would support beneficial traits. To test this hypothesis, contrasting microbial consortia were assembled through a top-down approach. Six microbial enrichments were first obtained under elevated or ambient CO_2 conditions and with different carbon amendments, ranging from recalcitrant only to a mixture of recalcitrant and labile carbon sources. *P. ostreatus* was then inoculated alone or with microbial enrichment cultures in the coco fiber retrieved from a commercial greenhouse. The microbial diversity in the enrichment cultures and in the substrate with *P. ostreatus* was analyzed to relate fungal growth performance with the composition and diversity of microbial communities.

MATERIALS AND METHODS

Sample collection and composite inoculum preparation

Soil, compost, and wood chips were sampled and aggregated into a composite inoculum for enrichment cultures. A soil mixture was first prepared with 100 g surface soil samples collected in a public park located in Montreal (Canada). Surface soil samples and decomposing wood chips comprising visible mycelium on the surface were collected in a maple forest located in Laval (Canada). Three samples of composted bean plant residues were supplied by a collaborator (Solinov Inc., Brossard, Canada). The different samples

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(soil, compost, and wood chips) were mixed in a ratio of 1:1:1 and used as inoculum in the enrichment cultures.

Coconut fiber retrieved from pepper production wastes (Les Fermes Luffa, Montréal, Canada), used as residual lignocellulosic biomass in this study, was also included as inoculum for enrichment cultures.

Enrichment cultures

Inoculum and non-sterile coconut fiber (1% wt/vol) were transferred into Erlenmeyer flasks containing Reasoner's 2A medium (R2A), peptone cellulose salt medium (PCS), and minimal salt medium (MSM) (Table S2) (38). The non-sterile coconut fiber was expected to promote the growth of endogenous lignocellulosic-degrading microorganisms and was the only source of carbon for the MSM medium. A sterilized cellulose paper strip (0.3 g) was added to PCS cultures. MSM cultures provided recalcitrant carbon sources, while R2A and PCS offered more labile carbon inputs, with PCS expected to promote the growth of cellulose-degrading microorganisms due to the presence of cellulose paper supplementation. Enrichments were incubated under two different atmospheres: ambient atmosphere through porous foam plugs placed on the apertum of the flasks (MSM_atm, R2A_atm, PCS_atm) or under a static headspace comprising elevated CO_2 concentration (MSM_CO_2, R2A_CO_2, PCS_CO_2) (Fig. 1). CO_2 was manipulated to promote complementary functions related to carbon fixation metabolism absent in mushrooms. Concentrations of approximately 10% CO_2 were achieved by injecting a defined volume of synthetic gas (99.9% CO_2, Bone Dry 3.0, absolute dry) using a gastight syringe (Precision Analytical Syringe, Pressure-Lok). The average CO_2 concentration was 9.2%, as measured by GC-FID (Agilent Technologies model 7890B) (39). All enrichment cultures were performed with three independent repetitions. Flasks were incubated at 25°C under agitation at 160 rpm for 10 days (3 media × 2 gaseous conditions × 3 repetitions = 18 enrichment cultures). After 10 days of incubation, the total volume of the microbial cultures was transferred to 250 mL of fresh sterile medium (R2A, MSM, or PCS) with 1% non-sterile coconut fiber and a cellulose filter paper strip in cultures with PCS medium. All flasks were incubated at 25°C under agitation at 160 rpm for 10 days. These enrichment cultures were sampled when the cell density reached at least 5 log cells/mL as measured by the most probable number method. Cellulose filter paper strips, used as an indicator for cellulase activity in PCS cultures, were visually degraded. Two sample fractions were collected from the cultures. The first fraction (1.8 mL) was centrifuged at

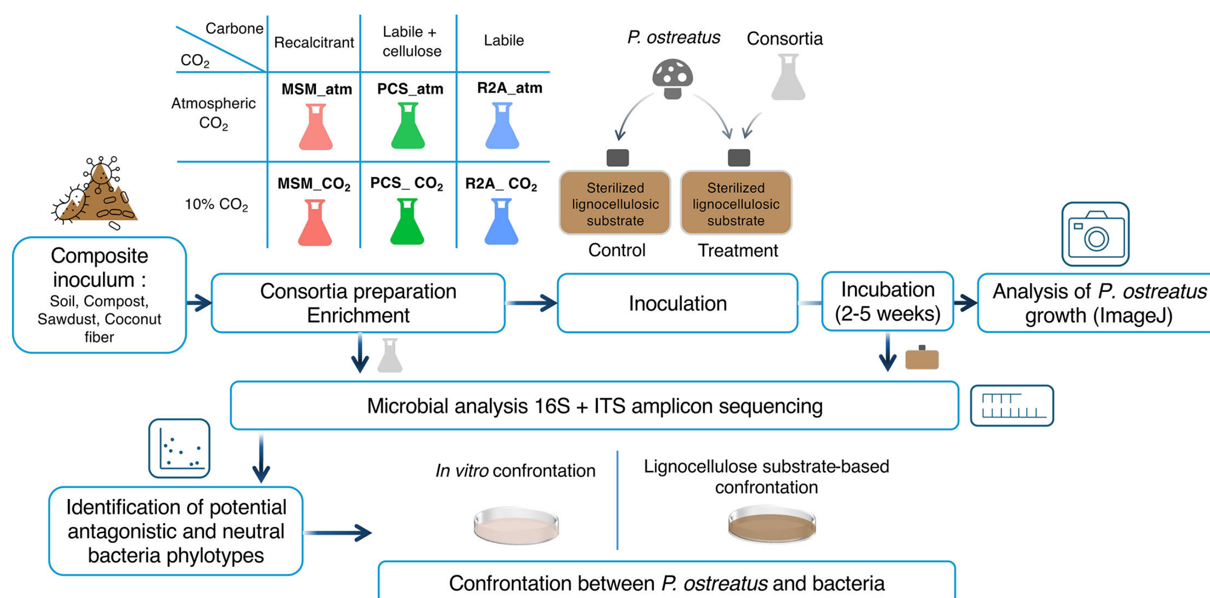

FIG 1 Schematic overview of the experimental design.

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10,000 × *g* for 10 min, and the pellets were stored in 2 mL tubes at −20°C for subsequent DNA extraction. The remaining volume was centrifuged at 5,000 × *g* for 10 min at 20°C. The supernatant was discarded, and the pellet was washed twice in phosphate-buffered saline solution (PBS) and resuspended in a final volume of 100 mL 1× PBS solution. These microbial suspensions were utilized to inoculate lignocellulosic substrate.

Lignocellulosic substrate preparation

The lignocellulosic substrate was prepared by mixing by hand 1,250 g sawdust with 1,250 g coconut fiber, 50 g calcium oxide, 250 g wheat bran, and 7.5 L reverse osmosis-filtered water to obtain a moisture content of 60% and a pH of 6.5. Then, 500 g lignocellulosic substrate was transferred into an autoclavable bag. The bags were closed with a plastic collar and a cap with a membrane to allow sterile air exchange (Fig. S1). A total of 24 bags were then sterilized and left to cool for 12 h before inoculation.

Inoculation

Fermentation bags were inoculated aseptically by adding 10 g solid fungal inoculum (mycelium of *P. ostreatus* cultivated in sterile rye grains) and 50 mL washed microbial suspension retrieved from the enrichment cultures. Sterile substrate inoculated with *P. ostreatus* without microbial inoculum and sterile substrate without *P. ostreatus* inoculum and without microbial inoculum were included as controls. This completely random design represented a total of 24 bags: (6 consortia + 2 controls) × 3 repetitions. Inoculated bags were randomly placed in an environmental chamber (Growth Cabinet, SANYO) in darkness, at 23°C and 70% humidity.

Fungal growth measurement

After 2 weeks, control bags inoculated with *P. ostreatus* without microbial inoculum were retrieved and stored at 4°C. The substrate surface of these control bags was completely covered with mycelium. Bags inoculated with *P. ostreatus* and microbial inocula were kept in the chamber for an additional 3 weeks to allow fungal growth. At the end of incubation, compact substrates were removed from their fermentation bags (control and treatment) for photograph capture of their front face and sampling. The substrate surface area covered by the mycelium of *P. ostreatus* was measured using ImageJ software (version 13.0.6) (40). The hue, the brightness, and the saturation were adjusted, and the area of the substrate not covered by mycelium was obtained after converting the threshold color into binary. The difference between the total surface area of the substrate and the surface area not covered by mycelium was used to deduce the substrate surface area covered with mycelium. The latter was converted into a percentage of mycelial growth relative to the substrate surface.

Sampling

Because of the heterogeneity of the lignocellulosic substrates, a composite sample of colonized substrate (20 g) was assembled by mixing subsamples collected from ten positions within the substrate. Composite samples representative of each experimental treatment were stored at −20°C for subsequent microbial diversity analyses.

Microbial diversity analysis

Total DNA was extracted from enrichment cultures and composite samples of lignocellulosic substrates with the DNeasy PowerLyzer Microbial kit and the DNeasy Plant Pro Kits (QIAGEN), respectively, following the manufacturer's instructions. Bacterial and fungal communities were examined by PCR amplicon sequencing. Universal primers 16S_520F (5′ AGCAGCCGCGGTAAT 3′) and 16S_799R2 (5′ CAGGGTATCTAATCCTGTT 3′) (41) were used to target the V4 region of bacterial 16S rRNA gene (279 bp), whereas the primers ITS1F (5′ CTTGGTCATTTAGAGGAAGTAA 3′) and 58A2R (5′ CTGCGTTCTTCATCGAT 3′) (42)

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were used for the fungal ITS1 region (300 bp). The libraries were prepared as described in reference 43. A single replicate of lignocellulosic substrates inoculated with the R2A_G enrichment was not included in the libraries due to insufficient PCR amplicon concentration. Pooled libraries were subjected to Illumina MiSeq PE-250 sequencing (Genome Québec). The raw sequence reads were processed in the software RStudio (version 4.2.0) (44). Illumina barcodes and primers were removed after trimming using Cutadapt (version 4.6) (45). The sequence data were then processed using the DADA2 package (version 1.26.0) (46). After dereplication, merging paired forward and reverse reads, and removing chimeras, an amplicon sequence variant (ASV) table was constructed. Taxonomy was assigned to ASVs using the reference database Silva version 138.1 for bacterial 16S rRNA genes (47) and the database UNITE version 29.11.2022 for fungal ITS (48). Only ASVs representing at least 0.005% of the whole ASV tables were kept for subsequent analyses (49). A total of 1,051 ASVs were obtained for the 16S rRNA gene, and 195 ASVs were obtained for the ITS (Tables S3 and S4).

Confrontation between *P. ostreatus* and bacteria *in vitro*

After the analyses of the microbial community in the lignocellulosic substrate, potential antagonistic, neutral, or auxiliary phylotypes on *P. ostreatus* growth were identified. These results were further tested in bioassays. Bacterial isolates affiliated with the same genera were selected to evaluate if potential antagonistic and auxiliary effects on *P. ostreatus* are conserved among different species. *Pseudomonas* sp. MB65 (PQ642331), *Rhizobium* sp. MB37 (PQ642303), and *Brevundimonas* sp. MB49 (PQ642315) was selected in an in-house collection of bacterial isolates. *Asticcacaulis endophyticus* ZFGT-14 (BCCM 27605) and *Shinella fusca* WF29 (BCCM 24714) were obtained from the Belgian Coordinated Collections of Microorganisms (BCCM) (Tables S5 and S6). A mycelial plug of *P. ostreatus* (75 mm diameter) was collected from the edge of the growing colony after seven incubation days on a PDA agar plate (Millipore) and was placed at the center of R2A agar plates (Millipore). Four drops (10 µL) of overnight bacterial cultures in R2A broth were inoculated at four points placed within a distance of 2.5 cm from the center of the mycelial plug. Each single bacterial strain and control (sterile R2A broth) was represented by three independent repetitions. All plates were incubated at 25°C, and the growth of *P. ostreatus* mycelium was evaluated by measuring the area of the mycelium (mm^2) with ImageJ. The mycelium surface area was then converted into the relative growth percentage of *P. ostreatus* mycelium co-cultured with each bacterial strain relative to *P. ostreatus* mycelium alone without bacteria. Interactions between *P. ostreatus* and *Brevundimonas* sp. MB49 was further examined in a lignocellulose substrate-based confrontation assay. The assay and the protocol to assess the growth of the bacteria by droplet digital PCR (ddPCR) in the presence and in the absence of *P. ostreatus* are described in the supplemental material (Fig. S2).

Statistical analysis

Statistical analyses were performed with the software RStudio (version 4.2.0) (44). Analysis of variance (ANOVA) and *post hoc* Tukey tests were performed to compare α-diversity among the different treatments, *P. ostreatus* mycelium in the fermentation bags among the different treatments, *P. ostreatus* mycelium area in the *in vitro* confrontation assay, and *Brevundimonas* sp. MB49 abundance in the lignocellulose substrate-based confrontation assay among the different treatments. The α-diversity was calculated using the sample size and coverage-based rarefaction and extrapolation (R/E) of the Hill numbers of species, richness ($q = 0$), Shannon index ($q = 1$), and inverse of Simpson's concentration index ($q = 2$) using the package iNEXT (version 3.0) (50). β-diversity analyses were computed on transformed ASV tables with the *decostand* function (Hellinger normalization). Principal coordinate analyses (PCoA) were performed on the Bray-Curtis distance matrix to examine dissimilarity of microbial community composition of the different enrichment cultures and lignocellulosic treatment conditions with the package phyloseq (version 1.42.0). Dissimilarities were

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further tested with permutational multivariate analysis (PERMANOVA) with the adonis2 function in the package vegan (version 2.6-4) (51). The differential abundance of ASVs between treatments was tested through the analysis of composition of microbiomes with bias correction (ANCOM-BC) with the package ANCOMBC (version 2.0.3) (52). Microbial communities were constrained by the culture medium used in enrichment (MSM, R2A, or PCS), the CO_2 condition, the relative proportion of ASVs affiliated to *Pleurotus* sp., and the percentage of *P. ostreatus* mycelial growth in a Bray-Curtis distance-based redundancy analysis (db-RDA). The function *forward.sel* from the package *adespatial* (version 0.3.23) was used for parsimonious db-RDA (53).

RESULTS

Diversity of the microbial enrichments

The integration of three different nutrient formulations, ranging from recalcitrant to labile carbon sources, with or without elevated CO_2, was successful in achieving contrasting microbial enrichments. The cultivation media significantly influenced the α-diversity of both bacteria and fungi, with bacteria showing the strongest response (Table S7). The MSM medium was the most favorable condition promoting bacterial species richness and Shannon diversity, while the labile carbon sources integrated in PCS and R2A were favorable for a few dominant ASVs, leading to a reduction of α-diversity. Neither bacterial nor fungal communities responded to CO_2 treatments.

The composition of bacterial communities was significantly influenced by the cultivation media (Fig. 2A), with community compositions on MSM, PCS, and R2A media being significantly different from one another (pairwise.adonis; Bray_Curtis, $R^2 = 0.20$, $P < 0.05$). More specifically, the ASV clustered at the Phylum level, contributing to discriminate MSM samples from both PCS and R2A samples, comprised 5 phyla, including potential oligotrophic bacteria affiliated to Acidobacteriota and Chloroflexi (Table S8) (54, 55). ASV aggregated at the genus level encompassing *Clostridium* was significantly higher in PCS compared to MSM enrichment cultures (ANCOM-BC, log-fold change > 1.49, $P < 0.05$). The enrichment of cellulolytic *Clostridium* bacteria and the degraded cellulose filter paper strips in PCS cultures suggested that these cultures were efficient for the enrichment of cellulolytic bacteria (56).

The impact of CO_2 on bacterial community composition was uneven among the three cultivation media. Bacterial communities in R2A and PCS were more responsive to CO_2 than MSM enrichment cultures (Fig. 2A). The ASV clustered at the genus level, contributing to discriminate samples R2A_CO_2 and PCS_CO_2 from all the other samples comprised 17 genera favored by CO_2 (Table S9). The most responsive taxa include potential anaerobic bacteria affiliated with *Clostridium* sp. and *Macellibacteroides* sp. (ANCOM-BC, log-fold change > 5, $P < 0.05$) (57, 58). Elevated CO_2 level combined with

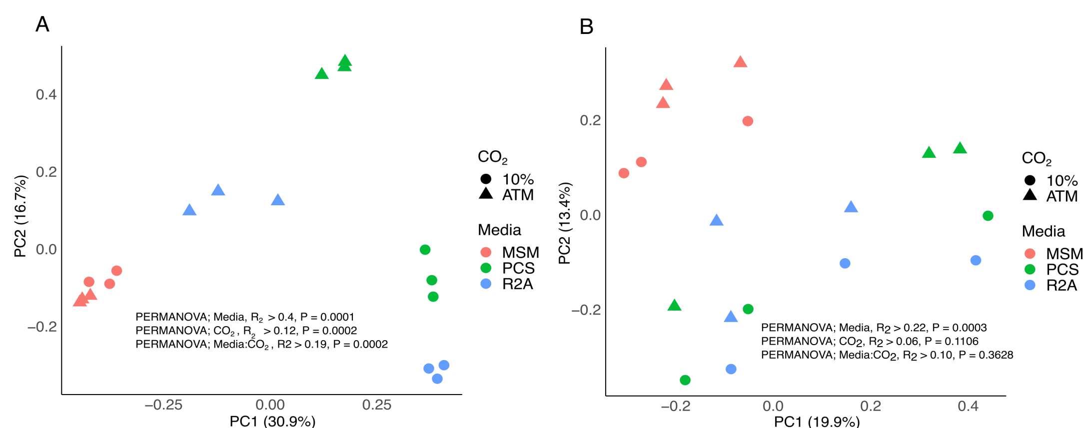

FIG 2 PCoA of the Bray-Curtis bacterial (A) and fungi (B) community distances between enrichment treatments. Colors indicate culture media, whereas circles represent enrichment cultures with 10% CO_2 and triangles represent enrichment cultures with atmospheric CO_2.

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biomass aggregates in the enrichment cultures was likely favorable to the formation of anaerobic microenvironments in R2A and PCS, while biomass aggregates displayed a lower density in MSM enrichments.

Fungal communities in the enrichment cultures were dominated by Ascomycota, represented by genera encompassing *Ovatospora* and *Chloridium*, followed by *Penicillium*, *Trichoderma,* and *Humicola* (Fig. S3A and B). The composition of the fungal community in MSM enrichments was distinct from R2A and PCS enrichment cultures (Fig. 2B). ASV aggregated at the genus level encompassing *Thermomyces, Tausonia, Mrakia,* and *Metarhizium* was significantly lower in MSM compared to the other enrichment cultures, whereas 12 genera were more abundant in MSM, including *Trichoderma* (Table S10). CO_2 exposure led to no coherent response at the community level (Fig. 2B), and no significant response was observed at the ASV level (ANCOM-BC, $P > 0.05$).

Microbial diversity in lignocellulosic substrate

The microbial diversity of the lignocellulosic substrate was analyzed after *P. ostreatus* growth, inoculated with the different microbial enrichment cultures. The species richness decreased significantly between enrichment culture and lignocellulosic substrate treatment for both bacterial and fungal diversity (Table S7).

The CO_2 treatments, the cultivation media, and the interaction among both factors were significant drivers of bacterial community composition (Fig. 3A). The composition of bacterial communities in lignocellulosic substrate inoculated with MSM enrichment cultures contrasted with those from PCS treatment (pairwise.adonis; Bray_Curtis, $R^2 = 0.17$, $P < 0.05$). The relative abundance of ASV affiliated to the genera *Mycobacterium, Flavobacterium, Chryseobacterium, Chitinophaga,* and *Asticcacaulis* was higher in MSM treatment compared to PCS (Table S11). Although the composition of the bacterial communities of R2A was initially different from PCS, they converged toward an indistinguishable community in the lignocellulosic substrate. A legacy effect of CO_2 was also noticed, in substrates inoculated with PCS_atm and PCS_CO_2 enrichment cultures, with the relative abundance of aggregated ASV at the genus level encompassing *Youhaiella, Pseudomonas, Pluralibacter,* and *Asticcacaulis* was favored in PCS_CO_2 (ANCOM-BC; $P < 0.05$).

Fungi were dominated by ASV encompassing the genus *Trichoderma* (Ascomycota) and *Pleurotus* (Basidiomycota) (Fig. S3B). The exclusion of most of the fungal diversity suggested a strong competition between *P. ostreatus* and the other fungi inoculated in the substrate. The cultivation media used during the enrichment stage were a significant driver of fungal community composition (Fig. 3B). ASV affiliated with *Trichoderma* sp.

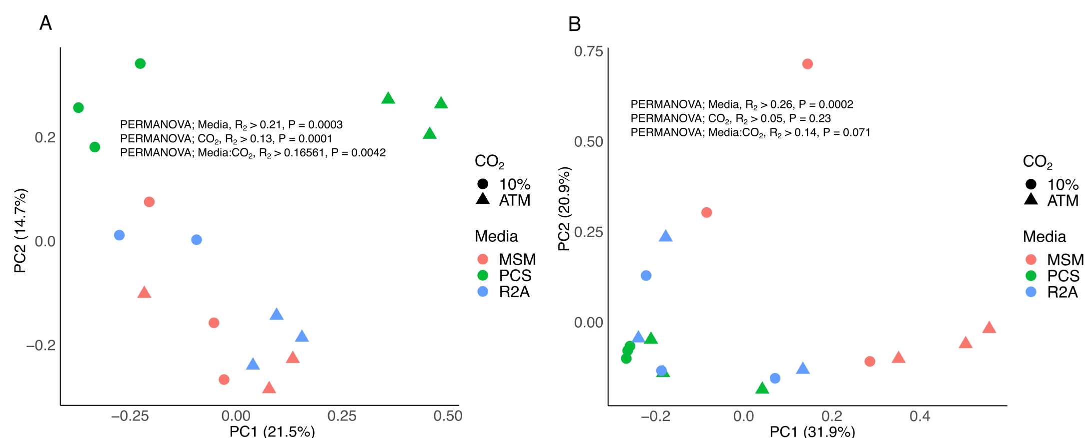

FIG 3 PCoA of the Bray-Curtis bacterial (A) and fungal (B) communities distances between lignocellulosic substrate treatments. Colors indicate culture media, whereas circles represent enrichment cultures with 10% CO_2 and triangles represent enrichment cultures with atmospheric CO_2. The third R2A replicate is missing due to an unsuccessful sequencing reaction.

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was the most abundant in MSM_atm, whereas *Pleurotus* sp. dominated in substrates inoculated with R2A and PCS enrichment cultures (ANCOM-BC; $P < 0.05$).

P. ostreatus mycelium growth in the lignocellulosic substrate

The relative abundance of ASV affiliated to *Pleurotus* sp. was proportional to the mycelium growth of *P. ostreatus* assessed by image integration (Pearson correlation, $\rho = 0.82$, $P = 5.581e\text{-}05$) (Table S12). Control substrates without microbial inoculum were visually completely covered with white mycelium after 14 days of incubation, while substrates comprising microbial inoculum presented lower mycelium growth restricted to the upper part of the substrate (Fig. S4). *P. ostreatus* growth was reduced with microbial inoculum, but after three additional weeks of incubation, the fungus achieved the same growth in substrates inoculated with the R2A and PCS enrichment compared to the control. MSM_atm and MSM_CO_2 treatments presented a lower mycelial growth compared to the control substrates (Fig. 4). Mycelial growth was significantly lower in MSM treatment compared to PCS treatment, but the difference was lost in enrichment culture from the same media exposed to elevated CO_2 (MSM_CO_2).

Covariation of microbial communities and *P. ostreatus* growth performance

The observed variance in growth rates of *P. ostreatus* across the different treatments (Fig. 4) could be ascribed to dissimilarities among bacterial community composition previously described. The CO_2 concentration in the headspace of enrichment cultures, the PCS medium, and the growth performance of *P. ostreatus* were the most significant features explaining the variation of the bacterial community structure in lignocellulosic substrate, with the PCS medium showing a positive correlation with the growth performance of *P. ostreatus* (Fig. 5). These results suggest that the composition of bacterial community in PCS enrichment culture was the most compatible with the growth performance of *P. ostreatus*. More specifically, the relative abundance of *Brevundimonas* sp. was significantly higher in PCS treatment compared to the other treatments (ANCOM-BC, log-fold change > 3, $P < 0.05$). Covariation of mycelium growth with the distribution of ASV 195 (*Brevundimonas* sp.) suggests a neutral or auxiliary effect of the bacterium (Fig. 5). The distribution of potential antagonistic phylotypes affiliated with *Pseudomonas* sp. (ASV 19), *Asticcacaulis* sp. (ASV 144), *Shinella* sp. (ASV 92), and *Rhizobium* sp. (ASV 186) displayed the opposite pattern (Fig. 5). Conservation of the potential antagonistic, neutral, or auxiliary effect of phylotypes was further tested with bacterial isolates affiliated to the same genera of available in public and in-house collections. The mycelial growth surface of *P. ostreatus* was significantly reduced in the presence of isolates affiliated to *Pseudomonas* sp. MB65, *Rhizobium* sp. MB37, *Asticcacaulis* sp. ZFGT-14, and *Shinella* sp. WF29 after 10 days on R2A agar (Fig. 6).

In contrast, the growth of *P. ostreatus* was neither reduced nor favored in the presence of the isolate *Brevundimonas* sp. MB49 for both *in vitro* (Fig. 6) and in lignocellulosic substrate-based bioassays (ANOVA, $P > 0.05$) (Tables S13 and S14). For the latter, bacterial abundance was significantly higher within the substrate area without mycelium or within the growing mycelium compared to the bacterial abundance assessed in the more senescent mycelial region (Fig. S5; Table S15). Together, these observations support conservation of neutral and antagonist bacteria-fungi interactions at the genus and species levels.

DISCUSSION

Although the diversity of microbial communities in the mycelium and in the growing environment of *P. ostreatus* has been studied (35, 59), the establishment of a compatible community beneficial to the growth of the fungus in lignocellulosic biomass appeared complex. Microbial communities were manipulated through enrichment by using different culture media and by modifying the CO_2 atmosphere. In the latter, headspace was modified to promote complementary functions related to carbon fixation metabolism absent in mushrooms. Formulation of cultivation media, comprising various

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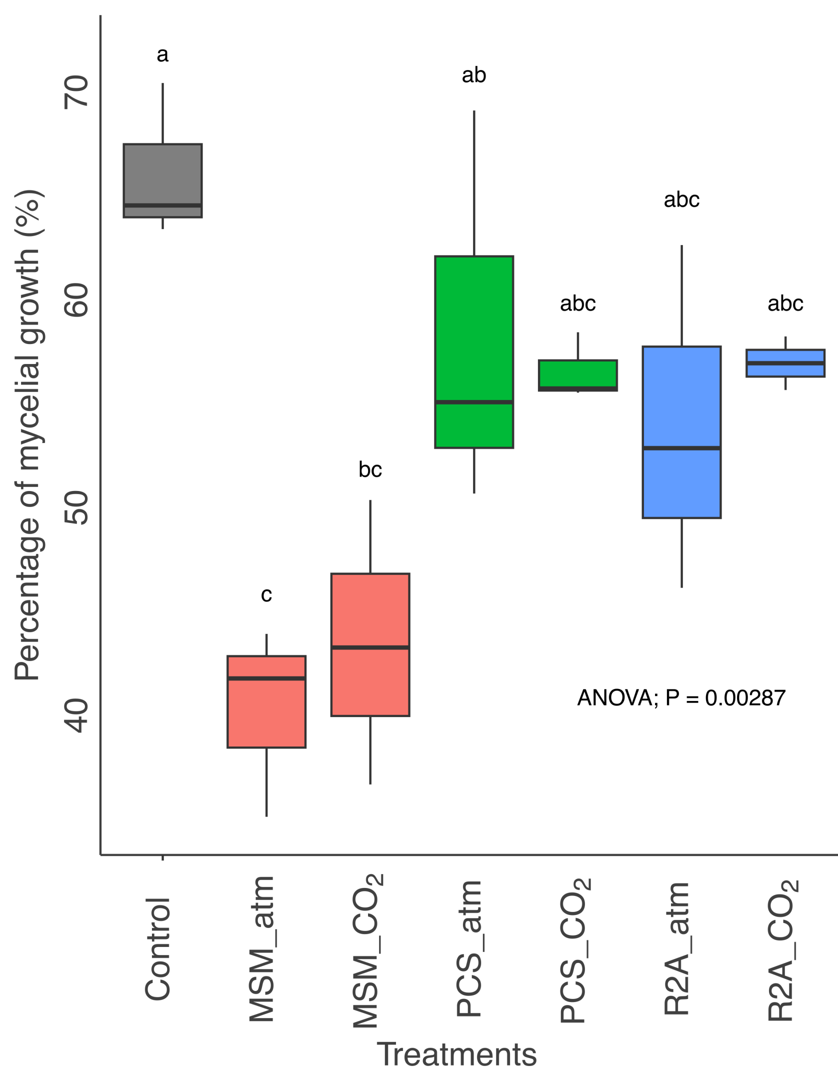

FIG 4 Percentage of *P. ostreatus* mycelial growth in the lignocellulosic substrate according to the different treatments. Control represents substrates inoculated with *P. ostreatus* without microbial inoculum. MSM_atm and MSM_CO_2 treatment presented a mycelial growth significantly lower compared to the control. Letters differ by Tukey's test ($P < 0.05$).

carbon amendments, ranging from recalcitrant (MSM) to labile carbon sources (PCS and R2A), exerted a greater effect on microbial diversity when compared to CO_2 favorable for chemolithoautotrophic growth of a few members of the microbial communities.

P. ostreatus growth was less efficient in lignocellulosic substrate co-inoculated with MSM enrichment cultures targeting saprotrophic communities than inoculated alone. Lignocellulosic biomass, as the sole energy and carbon source in MSM enrichment, likely accounted for the higher species richness compared to PCS and R2A media, comprising more labile nutrients, leading to the enrichment of dominant fast-growing micro-organisms (60). The richness of microbial enrichment in MSM likely induced a strong competition due to resource partitioning involving cheater species competing for the labile carbon released by the fungus (19). MSM enrichment conditions, close to those of the lignocellulosic environment, also enabled the MSM community to be stable and thus to survive and grow once in the substrate, key for niche occupancy success (61). Finally, oligotrophic phyla in the MSM enriched community, affiliated to Acidobacteriota and Chloroflexi, likely increased competition with the fungus due to their high substrate affinity (62, 63). Together, the functional redundancy related to substrate degradation

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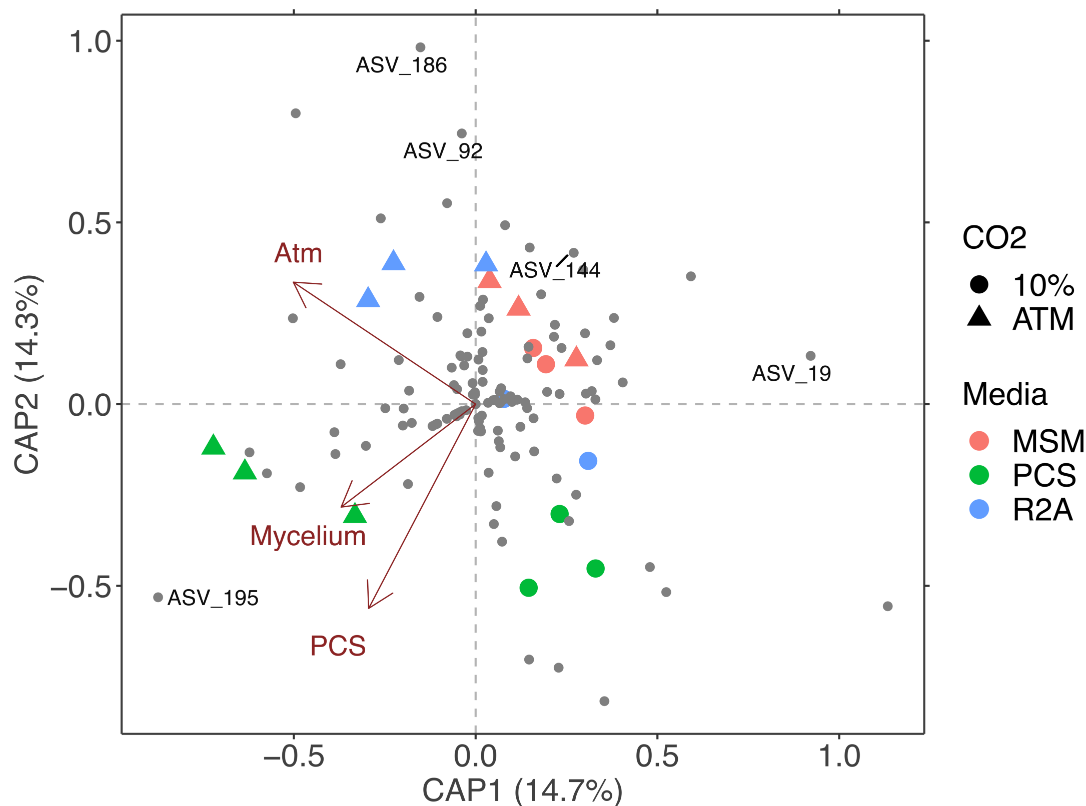

FIG 5 db-RDA based on Bray-Curtis distances showing the response of bacterial community composition, in lignocellulosic substrate treatments, to several parameters (the cultivation media, the headspace, the growth performance of *P. ostreatus,* and the relative proportion of ASVs *Pleurotus* spp. in the different treatments). The mycelium growth (P = 0.05), the PCS condition (P = 0.01), and the headspace (P = 0.05) contributed significantly (38%) to this variation (Bray-Curtis db-RDA, R_2 adj = 0.24, P = 0.001). Four ASVs (numbered 19, 92, 186, 144) are in the opposite direction of the "Mycelium" variable and represent the phylotypes *Pseudomonas* sp., *Shinella* sp., *Rhizobium* sp., *Asticcacaulis* sp., respectively, while ASV numbered 195 is in the same direction as the "Mycelium" variable and represents the phylotype *Brevundimonas* sp. The different ASVs are represented with small gray dots. Colors indicate culture media, whereas circles represent enrichment cultures with 10% CO_2. Arrows indicate significant variables (Mycelium; the growth performance of *P. ostreatus*, Atm; the headspace, and PCS; the PCS medium).

and the efficient niche occupancy of MSM enrichments promoted competition between the inoculum and *P. ostreatus*, leading to a reduced growth of the fungus.

The recalcitrant nature of lignocellulosic substrates is conducive to the division of metabolic labor and niche partitioning (61). Carbon labile sources integrated into PCS and R2A likely enriched carbon-degrading functions beyond saprotroph metabolism and could have improved consortia-*P. ostreatus* compatibility compared to MSM enrichments. However, substrate inoculated with R2A and PCS communities, with functions including cellulose breakdown for the latter, did not enhance fungal growth when compared to the control, suggesting that enzymatic synergy is not sufficient to enhance fungal biomass (27, 64). R2A and PCS communities also presented a lower microbial diversity compared to MSM's communities; this trade-off in diversity could have lowered the likelihood of auxiliary functions. Thus, nutrient and CO_2 manipulation of the enrichment media was not an effective strategy to drive a consortium beneficial to *P. ostreatus* growth. Taken together, these results suggested that functions linked to primary carbon metabolism are not those primarily involved in *P. ostreatus* promotion.

Analysis of microbial communities led to the identification of potential antagonistic and compatible bacteria and fungi for *P. ostreatus*. The reduced growth of the fungus in substrates inoculated with MSM enrichment could be a consequence of a strong competition with the dominant fungi *Trichoderma* spp., initially more abundant in the MSM enrichment compared to other enrichments. These fungi are efficient competitors,

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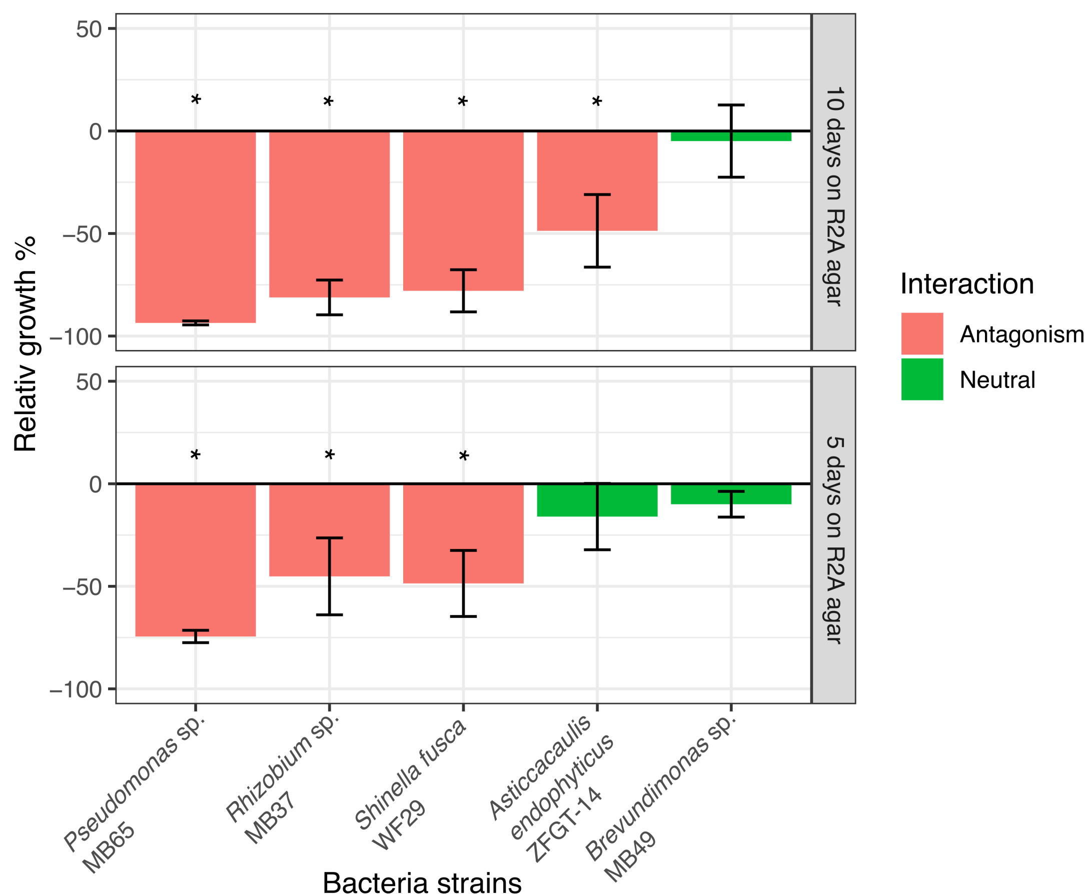

FIG 6 Growth effects of bacteria strains on mycelial growth of *P. ostreatus*. The relative mycelium growth of *P. ostreatus* co-cultured with bacterial isolates (retrieved from a culture collection) on Petri dishes containing R2A agar relative to *P. ostreatus* mycelium alone without bacteria (control) was measured after 5 and 10 days of incubation. Mycelium growth areas were measured after 4 and 8 days for the *in vitro* confrontation with *Brevundimonas* sp. * Significantly different compared to control, ANOVA, $P < 0.05$. Interactions were considered as antagonists if $P < 0.05$ and relative growth < 0, and considered as neutral if $P > 0.05$.

capable of rapidly colonizing lignocellulosic substrates. Known as green-mold disease, *Trichoderma* sp. is an issue in mushroom farms, causing significant loss in edible fungi crops (65). This taxon was also detected in R2A and PCS enrichments, ranking within the five most dominant fungal taxa in the enrichment cultures. Abundance of *Trichoderma* spp. alone was thus not sufficient to explain the variation of *P. ostreatus* growth performances. Specific bacteria likely contributed to *P. ostreatus* growth inhibition, as supported by correlations between *P. ostreatus* growth and the distribution of certain bacterial taxa in multivariate analyses. The causality of these correlations was supported by an isolate affiliated with *Asticcacaulis* sp.. The bacterial abundance of this genus was significantly higher in the MSM treatments, both in enrichment cultures and in the substrate, compared to other treatments. The potential antagonistic activity of *Asticcacaulis* spp. against *P. ostreatus* was validated *in vitro* along with isolates affiliated with *Pseudomonas* sp. MB65, *Rhizobium* sp. MB37, and *Shinella* sp. WF29. Conversely, indirect benefits for *P. ostreatus* may have contributed to higher performance in PCS and R2A through the deleterious effects of bacteria exerting antagonistic interaction with *Trichoderma* sp. In a previous study, bacteria of the *Bacillus* genus have demonstrated their ability to selectively inhibit the growth of *Trichoderma* sp. without affecting that of *P. ostreatus*, through the production of fengycin, an antifungal peptide (66). Finally, only one taxon, *Brevundimonas* sp., was compatible with *P. ostreatus*. ASV assigned in the genus *Brevundimonas* was more abundant in substrate inoculated with PCS compared to other treatments and *Brevundimonas* sp. MB49 was the sole isolate displaying no

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antagonistic effect in the *in vitro* confrontation test. *Brevundimonas* sp. strain was identified as a *P. ostreatus* growth-promoting bacterium in a previous report (67), but that trait is not conserved at the genus level, according to the *in vitro* and lignocellulosic-based assays conducted with *Brevundimonas* sp. MB49. Finally, interactions appeared to be driven by a trade-off between compatibility and colonization constraints imposed by unbalanced bacterial and fungal inocula. This is supported by neutral or negative impacts of *P. ostreatus* on the growth of *Brevundimonas* sp. MB49 in lignocellulosic-substrate-based assays. The difference in bacterial abundance between growing and senescent mycelium suggested that compatible inter-kingdom interactions could be constrained by the growth stage of the mycelium (68).

Microbial communities became more homogeneous in the lignocellulosic substrate across the different trials due to a filtering effect induced by environmental conditions and *P. ostreatus* itself. Indeed, bacteria compatible with fungal growth, such as *Brevundimonas* sp., were more abundant in trials with robust fungal growth, suggesting a selection by the fungus for less inhibitory bacterial communities (69). This is in line with previous findings that *P. ostreatus* can modify and select its surrounding microbial communities during growth (70), leading to a microbial succession (15). Due to its ability to influence microbial diversity, the fungus may be considered less as an individual within a community—where interactions are examined on a pairwise basis—but rather as a host, with the hyphosphere environment functioning similarly to the plant's rhizosphere (71). According to this concept, the establishment of a self-assembled consortium could be achieved through directed evolution. Co-culture of *P. ostreatus* with bacteria would be more conducive to auxiliary enrichments (72), whose beneficial functions are difficult to select using selective media. Key organisms that contribute to fungal growth promotion, along with the characterization of their auxiliary traits and their interplay with other species, could be further identified through isolation and assembly into synthetic communities (73).

Conclusion

Our results indicate that enrichment cultures only containing recalcitrant carbon sources are less compatible with *P. ostreatus* than enrichment cultures containing a combination of recalcitrant and labile carbon sources. The growth of *P. ostreatus* was nevertheless most efficient in the absence of microbial inoculum. Carbon degradation functions are not the main drivers of microbial communities beneficial to the growth of *P. ostreatus*. Other functions beyond the primary metabolism of carbon could be involved in its promotion. This knowledge will drive further studies on the assembly of compatible bacterial and fungal communities, as well as identifying ecological traits involved in fungal growth promotion. Ultimately, these future directions are expected to stabilize and optimize fungal biomass production and lignocellulosic feedstock pretreatments for bioproducts and bioenergy production.

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DATA AVAILABILITY

All raw data generated by this study have been deposited on NCBI under BioProject PRJNA1188180. This BioProject includes amplicon sequencing data of enrichment cultures and lignocellulosic substrates. The ASV table is available in the supplemental material. All code used for data processing and analyses is available in GitHub: https://github.com/LaboPC/Pleurotus-bacteria-enrichment_MarieBonduelle_2024

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental figures (AEM00898-25-S0001.pdf). Figures S1 to S5.
Supplemental tables (AEM00898-25-S0002.xlsx). Tables S2 to S15.

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