BMC Microbiology

RESEARCH Open Access

Unravelling the complex relationship between *Suillus bovinus* and *Gomphidius roseus* through investigation of their sporocarps in *Pinus massoniana* forests

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Abstract

Background The co-occurrence of sporocarps has revealed many intimate associations between different ectomycorrhizal (ECM) fungi species. The co-occurrence of sporocarps of *Suillus bovinus* and *Gomphidius roseus*, two edible ECM fungi, is well recognized; however, the interactions between them remain largely unknown. This study investigated the relationship between these two fungi occurring in *Pinus massoniana* forests through phenological, microbiome, and metabolome analyses.

Results *Gomphidius roseus* sporocarps were always found alongside sporocarps of *S. bovinus*, but not vice versa. The ECM associated with *S. bovinus* sporocarps exhibited a long-distance exploration type, whereas the ECM associated with *G. roseus* sporocarps formed a contact exploration type. Both *S. bovinus* and *G. roseus* sporocarps and ECM contained the mycelia of both fungi. In contrast, different fungal sporocarps and ECM were dominated by distinct bacterial species. *Suillus bovinus* sporocarps were recorded in all ages investigated, ranging from 1 to 5 years old to over 30 years old. In contrast, *G. roseus* sporocarps were mainly found in forests older than 10 years. Previous studies suggested that *G. roseus* parasitizes *S. bovinus*; however, the occurrence of *G. roseus* sporocarps did not significantly affect *S. bovinus* sporocarp production or *P. massoniana* growth, challenging this assumption. Despite their intimate interactions, the metabolic profiles of *S. bovinus* sporocarps more closely resembled those of *S. luteus*, not *G. roseus*.

Conclusion Overall, our analyses showed both similarities and dissimilarities in phenology, microbiome, and metabolome features between the two fungi, and the genesis of *G. roseus* sporocarps is highly dependent on *S. bovinus*. These results further indicate that while the formation of ECM between *G. roseus* and the host may rely on ECM formed by *S. bovinus* and the same host, it is not parasitic.

Keywords Ectomycorrhiza, Interaction, Microbiome, Phenology, Stand age

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Introduction

Ectomycorrhizal (ECM) symbioses are among the most widespread associations between tree roots and soil fungi in forest ecosystems and contribute significantly to the sustainability of forest ecosystems through nutrient cycling and carbon sequestration [1]. In addition, some ECM fungal species produce edible sporocarps (i.e., edible mycorrhizal fungi) that have great gastronomic value, for example, *Tuber* spp., *Boletus* spp., *Tricholoma matsutake*, and *Lactarius deliciosus* [2, 3]. These edible mycorrhizal fungi are considered ecologically and economically important for forestry development, particularly in less developed regions worldwide [4].

Many intimate associations between different species of ECM fungi have been revealed by the co-occurrence of fruit bodies and mycelia in mycorrhizal root tips [1, 5]. For example, *Russula pulchella* consistently fruits in the presence of *Lactarius pubescens* [6]; basidiomes of *Xerocomus parasiticus* are occasionally found growing from basidiomes of *Scleroderma citrinum* [7]; and *Chroogomphus* species are commonly detected within ECM formed by *Rhizopogon* species [8]. However, the interactions between these fungal combinations are not well understood and are often attributed to fungal succession or competition for root colonization by different species [7, 9].

An association between *Suillus* species and members of the Gomphidiaceae has long been suspected because several Gomphidiaceae species only occur where their *Suillus* partner is present [10]. Both fungi have narrow host ranges among ECM plants and typically form mycorrhizas with members of the Pinaceae [11]. Among these fungal combinations, *Suillus bovinus* and *Gomphidius roseus* have attracted much attention. Both species produce edible sporocarps, widely collected in China for sale or home consumption (see Fig. S1; [12, 13]). Based on a combination of molecular and anatomical analyses, Olsson et al. [14] proposed that *G. roseus* behaves as a parasite of either *S. bovinus*, the plant host, or both. However, this assumption was based mainly on the haustorium-like structures observed in mixed mycorrhizas and detecting *S. bovinus* mycelium at the base of *G. roseus* sporocarps. If the assumption that *G. roseus* behaves as a parasite is correct, then host plant growth and/or the production of *S. bovinus* sporocarps may be constrained by the presence of *G. roseus*. Furthermore, given the intimate relationship between *S. bovinus* and *G. roseus*, the microbiome of their sporocarps as well as their metabolome may have some similarities.

Pinus massoniana is one of the most widespread tree species in South China (e.g., Guizhou, Guangxi, Jiangxi, and Hunan provinces) and is a typical ECM host [15, 16]. Our previous studies reported the presence of both *S. bovinus* and *G. roseus* sporocarps in *P. massoniana* forests [13, 17]. Analyses of sporocarp productivity and economic value indicate that a considerable amount of *S. bovinus* sporocarps could be produced in *P. massoniana* plantations, which merits further commercial investigation [17]. By contrast, the fruiting season of *G. roseus* is much shorter than that of *S. bovinus*, and sporocarp production is much lower [13]. To maintain forest health and mycosilvicultural needs, the relationship between *S. bovinus* and *G. roseus* and their relationship with their co-host should be clarified.

This study aimed to better understand the relationship between *S. bovinus*, *G. roseus*, and their co-host *P. massoniana* by investigating the morphology and phenology of *S. bovinus* and *G. roseus* sporocarps, as well as comparing their microbiomes and metabolomes. Our analyses indicate that a complex symbiotic relationship exists between *S. bovinus*, *G. roseus*, and their co-host, rather than a parasitic one.

Materials and methods

Sampling area description

Sporocarp collection and ECM root sampling were conducted in a pure stand of *P. massoniana* located in Mengguan state-owned forest farm, Guizhou Province, China (106°44′E, 26°21′N; altitude: 1,140 m above sea level). The forest covers an area of ca. 50 acres, and the stand was 22 years old at the start of the study. The basic physical and chemical properties of the topsoil (0–20 cm) were as follows: pH 4.4; soil organic matter, 18.65 g kg^{-1}; total nitrogen, 1.86 g kg^{-1}; available nitrogen, 115 mg kg^{-1}; total phosphorus, 0.75 g kg^{-1}; available phosphorus, 0.73 mg kg^{-1}; available potassium, 80.4 mg kg^{-1}. The climate in this region is classified as a humid subtropical climate, i.e., humid with a mean annual rainfall of 1,442 mm and a mean annual temperature of 14.9 °C.

Sporocarp and ectomycorrhiza sampling

Fresh sporocarps of both *S. bovinus* (growing in the presence of *G. roseus* sporocarps) and *G. roseus* were collected for microscopic, microbial diversity, and metabolic analyses in May 2021. Meanwhile, fine roots associated with sporocarps (underneath the sporocarps) and ectomycorrhizas were carefully traced and collected. Sporocarps and root samples were immediately taken back to the laboratory and analysed.

Morphological analysis of sporocarps and ectomycorrhizas

Macroscopic features (colour of cap, stipe, pores, and flesh; shape, size, and texture of both cap and stipe) were recorded based on fresh material and field photographs before transporting the fresh material to the laboratory for further observation and analysis. Sporocarps were either observed directly under a stereomicroscope (M205FA, Leica Microsystems, Wetzlar, Germany) or

sectioned by hand, mounted in filtered water (sterilized using a 0.22 μm filter membrane) and observed under a differential interference microscope (DM2500, Leica Microsystems). At least ten sporocarps of each fungal species were observed.

After the ectomycorrhizas had been carefully washed with tap water, they were observed under a stereomicroscope (M205FA, Leica Microsystems). The shape, colour, attachment, and surface texture were recorded. After that, longitudinal and transversal cross-sections of at least 20 independent ectomycorrhizas were obtained using a razor blade. Roots were cleared to make them transparent (5% KOH solution, 90 °C for 2 h), acidified (1% HCl solution w/v, 10 min at room temperature), and stained with 0.05% Trypan Blue dye (90 °C for 20 min). The stained sections were then mounted in glycerol, and the microscopic features were observed under a differential interference microscope (DM2500, Leica Microsystems). At least 30 ectomycorrhizas of each fungal species were observed.

Microbial diversity analysis

Fresh sporocarps of *Suillus bovinus* and *Gomphidius roseus* were separated into caps and stems using a scalpel to create four sample types: Sb.C (caps) and Sb.S (stems) for *S. bovinus*, and Gr.C (caps) and Gr.S (stems) for *G. roseus*. Mycorrhizas (Sb.M and Gr.M) were collected following the methods described in Sect. "Sporocarp and ectomycorrhiza sampling", and the mycorrhizosphere soils (Sb.MS and Gr.MS) were obtained by brushing the mycorrhizas. Caps or stems from at least five sporocarps were combined to form one sample. At least 30 ectomycorrhizas associated with at least five sporocarps of each fungal species were collected to make one ectomycorrhiza sample. The collections of adhered soil were combined to make one mycorrhizosphere soil sample. Each type of sample had five replicates.

Total genomic DNA was extracted using the CTAB method [14, 18]. DNA concentration and purity were assessed, and samples were diluted to 1 ng/μL. The V4 region (primer pair 515 F and 806R) of the 16 S rRNA gene and the ITS2 region (primer pair ITS3 and ITS4) of the ITS gene were amplified for bacterial and fungal analysis, respectively. PCR products were analysed by electrophoresis, and strong bands were selected for further processing. The resulting PCR products were purified using a Qiagen Gel Extraction Kit.

Sequencing libraries were prepared using a TruSeq® DNA PCR-Free Sample Preparation Kit, and quality was assessed using a Qubit® 2.0 Fluorometer and an Agilent Bioanalyzer 2100 system. Sequencing was performed on an Illumina HiSeq 2500 platform, generating 250-bp paired-end reads. FLASH software (V1.2.7) was used to merge reads, followed by quality filtering using QIIME (V1.7.0) to obtain clean tags. Chimera sequences were removed using the UCHIME algorithm.

Sequence analysis was performed using UPARSE software, clustering sequences with 97% similarity into operational taxonomic units (OTUs). Taxonomic annotation was conducted using the UNITE Database, and multiple sequence alignments were performed with MUSCLE software (V3.8.31) to study phylogenetic relationships. OTU abundance was normalized, and alpha and beta diversity analyses were conducted using QIIME (V1.7.0). Principal component analysis (PCA) was performed using R software. Potential metabolic functions of microbial communities were predicted using the Tax-4Fun package [19] based on 16 S rRNA sequences and the Kyoto Encyclopedia of Genes and Genomes (KEGG) [20], and fungal functional guilds were assigned using FUNGuild [21].

Raw sequence data have been deposited in the NCBI SRA database under accession numbers PRJNA1012362 (bacteria) and PRJNA1012375 (fungi).

Metabolome analysis of fungal sporocarps

To compare the metabolomes of *Suillus bovinus*, *Gomphidius roseus*, and *Suillus luteus*, sporocarps were collected in May 2021. For each species, at least five sporocarps were combined to create a single sample, with four independent replicates per species. Samples were freeze-dried and ground into powder using a mixer mill with zirconia beads. A 100 mg aliquot of each lyophilized sample was dissolved in 1.2 mL of 70% methanol, vortexed periodically, and refrigerated overnight. After centrifugation, the supernatant was filtered for liquid chromatography-mass spectrometry (LC-MS) analysis.

Metabolite analysis was conducted using an ultra-high-performance liquid chromatography (UHPLC) system coupled with electrospray ionization tandem mass spectrometry (ESI-MS/MS). The UHPLC was equipped with an Agilent SB-C18 column, using a mobile phase of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). A gradient elution program was employed, transitioning from 95% A to 95% B over 9 min. The flow rate was 0.35 mL/min, the column temperature was 40 °C, and the injection volume was 4 μL.

The mass spectrometer operated in positive ion mode with specific settings for ion source temperature, spray voltage, and gas pressures [22]. Data were acquired using multiple reaction monitoring (MRM) mode. Differentially accumulated metabolites (DAMs) were identified using orthogonal projection to latent structure discriminant analysis, with thresholds of fold change ≥ 2 or ≤ 0.5 and variable importance in projection ≥ 1. Metabolites were annotated using the KEGG Compound database, and pathway analysis was performed using metabolite set enrichment analysis.

Outbreak dynamics of *S. bovinus* and *G. roseus* sporocarps

Monthly surveys of sporocarp occurrence were carried out in several pure stands of *P. massoniana* of different stand ages from January 2022 until November 2023 (Table S1). At least one survey was performed each month, and between two and four surveys were performed monthly from May to September. All surveys were conducted during the day. For each survey, we walked along a linear census route (ca. 1 km), recording all *S. bovinus* sporocarps present within 2 m of the line on both sides.

To investigate whether the presence of *G. roseus* sporocarps influenced the development of *S. bovinus* sporocarps or *P. massoniana* growth, we weighed 50 *S. bovinus* sporocarps that were growing in the presence of *G. roseus* sporocarps and 50 that were growing in the absence of *G. roseus* sporocarps. We also determined the ratio between the diameter at breast height (DBH) of 20 *P. massoniana* trees with *S. bovinus* and *G. roseus* sporocarps growing beneath them and the DBH of 20 *P. massoniana* trees with only *S. bovinus* sporocarps growing beneath them.

Statistics

Mass spectrometry data were processed using Analyst 1.6.3 software (AB Sciex, https://sciex.com/); R version 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria; https://www.r-project.org/) was used for multiv ariate statistical analysis of the metabolome and microbiome data. SPSS 19.0.0 (SPSS Inc., http://www.spss.com.cn) was used for statistical analysis of sporocarp morphology, biomass, and DBH data, and a T-test was used to compare the means of comparable groups.

Results

Morphological features of sporocarps and ECM

Our sporocarp surveys revealed that *G. roseus* sporocarps were always found alongside sporocarps of *S. bovinus*, but not vice versa (Fig. 1a). *Gomphidius roseus* caps are often rosy red when young and turn brick red as they mature, and usually retain turned-down margins. *Gomphidius roseus* has thick decurrent gills with rod-like cheilocystidia. The dirty-white stem has a poorly defined glutinous ring, and the lower part of the stem is often

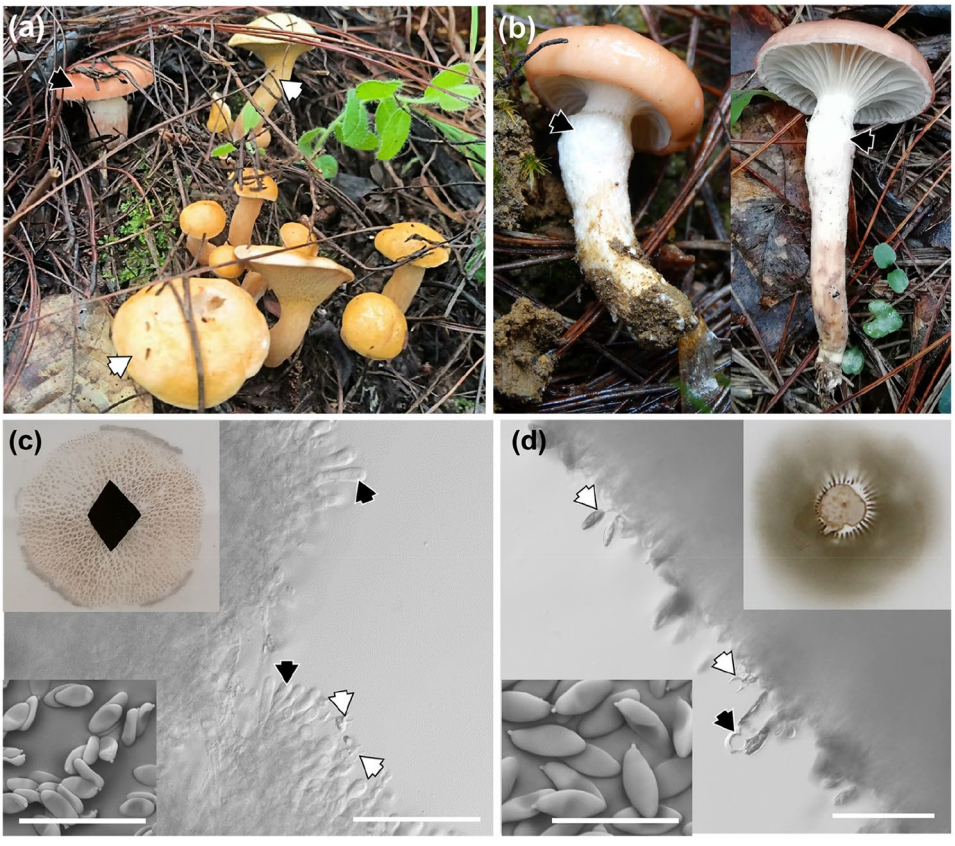

Fig. 1 Morphological features of sporocarps of *Suillus bovinus* and *Gomphidius roseus*. (**a**) The co-occurrence of sporocarps of *S. bovinus* (gregarious; indicated by white arrowheads) and *G. roseus* (black arrowhead); (**b**) sporocarps of *G. roseus*. Poorly defined glutinous rings are indicated by black arrowheads. (**c**) Rod-like cheilocystidia (black arrowheads) and basidium (white arrowheads) of an *S. bovinus* sporocarp. Scale bar = 50 µm. Top-left inset image, brown spore print of *S. bovinus* sporocarp; bottom-left inset image, long elliptical basidiospores (scale bar = 20 µm). (**d**) Rod-like cheilocystidia (black arrowhead) and basidium (white arrowheads) of a *G. roseus* sporocarp. Scale bar = 50 µm. Top-right inset image, dark-grey spore print of *G. roseus* sporocarp; bottom-left inset image, long elliptical basidiospores (scale bar = 20 µm)

tinged pink and tapers inwards. The spore print is greyish-green, and spores are subfusiform in shape (Fig. 1a, b,c, and Fig. S1b). By contrast, *S. bovinus* is a gregarious bolete. The colours of the cap vary from pale yellow to deep orange. Tubes terminate in large compound pores and have rod-like cheilocystidia. The sporocarp stipe is club-shaped and lacks a stem ring. The spore print is brown, and the basidiospores are subfusiform (Fig. 1a, d and Fig. S1).

ECMs associated with *S. bovinus* sporocarps were mainly coralloid in shape and yellowish to yellowish brown (Fig. 2a). The surface of the ECM is smooth, with extraradical mycelium and rhizomorphs attached to it and, hence, the ECM resembles a long-distance exploration type. ECMs associated with *G. roseus* sporocarps developed irregular dichotomous branching shapes. They were light yellowish to light yellowish brown (Fig. 2b). The surface of the ECM is smooth with few hyphae attached; hence, this ECM resembles a contact exploration type. Anatomical observations revealed that within the mantle of ECMs associated with *G. roseus* sporocarps, there are spore-like and haustorium-like structures (Fig. 2d and Fig. S2). These structures were absent in ECMs associated with *S. bovinus* sporocarps (Fig. 2c).

Fungal communities associated with different kinds of samples

After removing low-quality, non-fungi, potential chimeras, and singleton reads, the remaining fungal ITS2 sequences (2,601,876 reads in total) obtained from sporocarp, ectomycorrhiza, and mycorrhizosphere soil samples were grouped into 1583 non-singleton OTUs at a 97% sequence similarity level. Of these OTUs, 1460 (2,581,833 reads) were identified as fungal OTUs, of

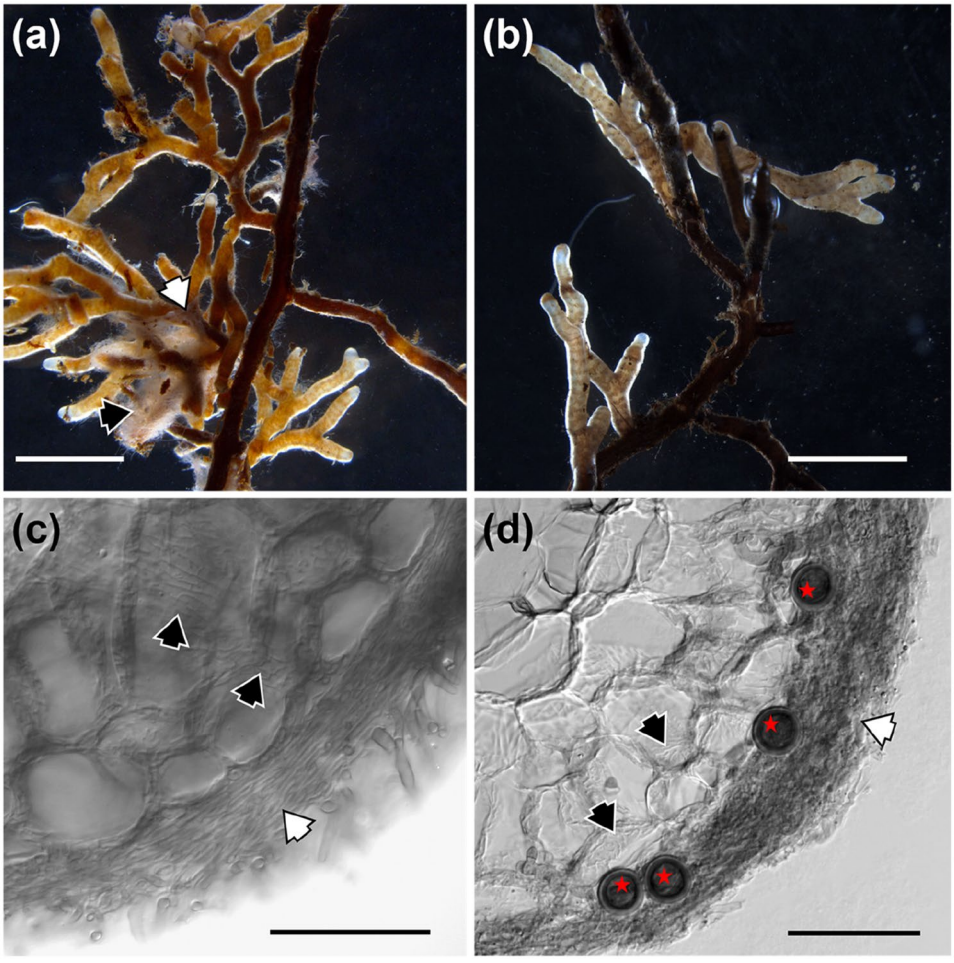

Fig. 2 Morphological features of ectomycorrhiza associated with sporocarps of *Suillus bovinus* and *Gomphidius roseus*. (**a**) Coralloid ectomycorrhizas collected in a *Pinus massoniana* forest that were associated with a sporocarp of *S. bovinus*. A black arrowhead indicates the extraradical mycelium, and a white arrowhead indicates the rhizomorph. Scale bar = 2 mm. (**b**) Irregular dichotomous ectomycorrhizas associated with a *G. roseus* sporocarp. Scale bar = 2 mm. (**c**) Transversal cross-section of ectomycorrhiza associated with an *S. bovinus* sporocarp. The white arrowhead indicates the mantle; the black arrowheads indicate the Hartig net. Scale bar = 20 µm. (**d**) Transversal cross-section of an ectomycorrhiza associated with a *G. roseus* sporocarp. The white arrowhead indicates the mantle; the black arrowheads indicate the Hartig net; the red pentagrams indicate spore-like structures. Scale bar = 20 µm

which 602 were classified as Ascomycota, 217 as Basidiomycota, 66 as Rozellomycota, 34 as Chytridiomycota, 29 as Glomeromycota, 60 belonged to other phyla, and 452 were unknown fungi.

The relative abundance of fungi detected varied among the different sample types (Fig. 3). Fungi detected in sporocarp samples aggregated together at general levels, and their differentiation distance with other samples was relatively large (Fig. 3a). In general, *S. bovinus* and *G. roseus* sporocarp samples mainly comprised *S. bovinus* or *G. roseus* mycelium, respectively (Fig. 3b). However, *S. bovinus* sporocarp samples also contained small amounts of *G. roseus* mycelium, and vice versa. *Gomphidius roseus* mycelium was detected in *S. bovinus* sporocarp caps and stems (accounting for 2.31% and 2.75% of reads, respectively, in terms of relative abundance), whereas *S. bovinus* mainly colonized *G. roseus* sporocarp stems (3.58%).

ECM samples collected underneath *S. bovinus* sporocarps also comprised species belonging to genera such as *Amanita*, *Clavulina*, and *Russula*, as well as *G. roseus* (0.95%). Furthermore, ECM samples collected beneath *G. roseus* sporocarps were dominated by ECM fungal species of *Russula*, *Amanita*, and *Calvulina*, as well as *S. bovinus* (2.87%). The fungal communities detected in the mycorrhizosphere soil samples were also diverse and dominated by *Russula* species.

Functional annotation revealed that all samples were dominated by either symbiotic fungi or pathogenic–symbiotic fungi (Fig. 3c). Many fungi with unknown functions were also detected. Sequence coverage of all samples was good (> 99.5%). Alpha indices varied among the different sample types (Fig. S3). The species diversity indices calculated for sporocarp samples were generally lower than those for mycorrhiza or mycorrhizosphere soil samples.

Bacterial communities associated with different kinds of sample

After removing low-quality, non-fungi, potential chimeras, and singleton reads, the remaining bacterial 16 S sequences (3,101,827 reads in total) obtained from sporocarp, ectomycorrhiza, and mycorrhizosphere soil samples were clustered into 8,213 non-singleton OTUs at a 97% sequence similarity level. Of these, 1,349 were

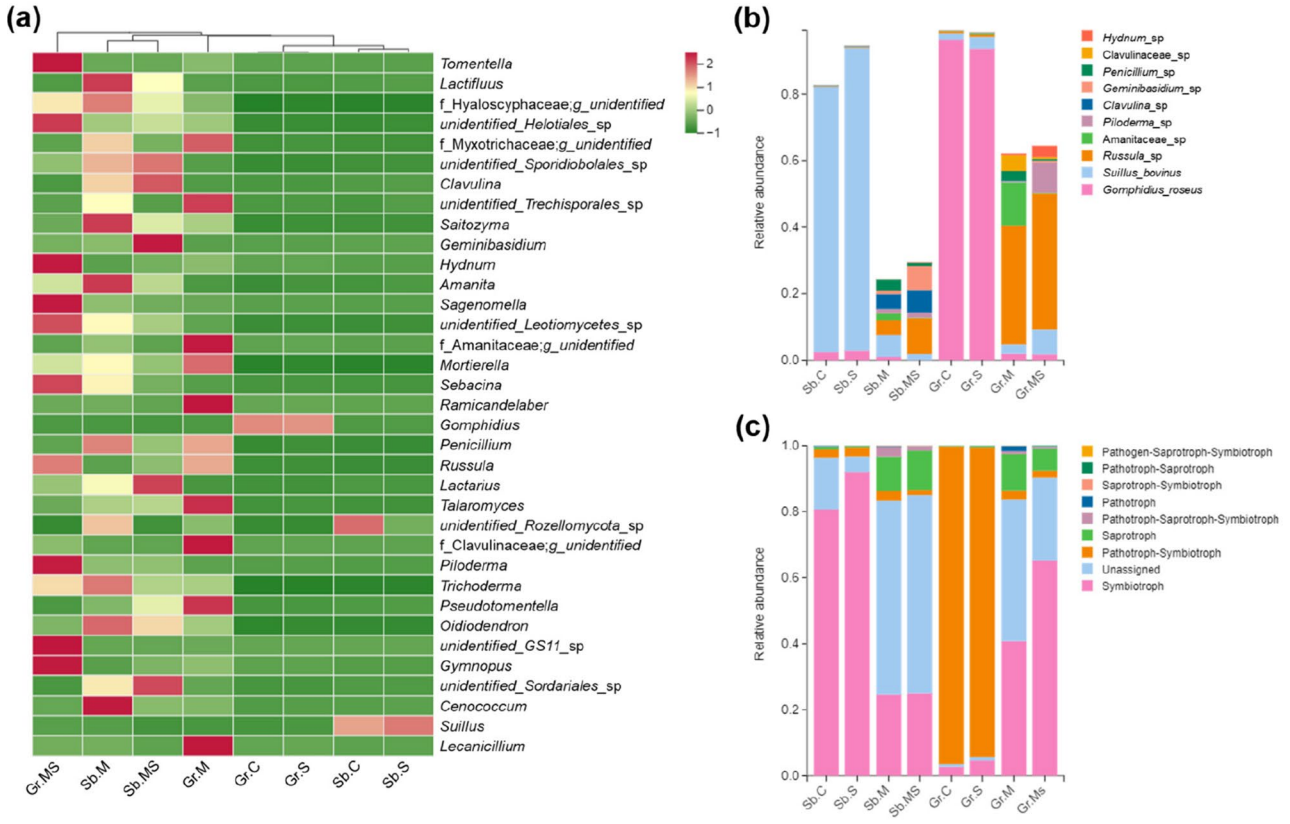

Fig. 3 Fungal diversity of sporocarps, ectomycorrhizas, and mycorrhizosphere soils. (**a**) Heatmap showing fungal associations among different sample types at the genus level. (**b**) Relative abundances of the top 10 most abundant fungal species among different sample types. (**c**) Functional annotation of fungi among different sample types. Sb.C and Sb.S, *Suillus bovinus* sporocarp caps, and stems, respectively; Gr.C and Gr.S, *Gomphidius roseus* sporocarp caps and stems, respectively; Sb.M and Sb.MS, mycorrhizas and mycorrhizosphere soils associated with *S. bovinus* sporocarps, respectively; Gr.M and Gr.MS, mycorrhizas and mycorrhizosphere soils associated with *G. roseus* sporocarps, respectively

classified as Proteobacteria, 1,071 as Acidobacteriota, 384 as Planctomycetota, 316 as Chloroflexi, 298 as Bacteroidota, and 4,795 as unknown bacteria.

A heatmap of the 35 most-abundant bacterial families (relative abundance) revealed that some bacterial families were more abundant in particular sample types (Fig. 4a). For example, bacteria belonging to the Sporolactobacillaceae, Sphingomonadaceae, and Oxalobacteraceae were mainly detected in *G. roseus* sporocarp caps; samples of mycorrhizosphere soil associated with *G. roseus* were mainly dominated by bacteria belonging to the Ktedonobacteraceae. At the species level, the dominant bacterial species of different types of samples also varied (Fig. 4b). Samples of *S. bovinus* sporocarps were dominated by *Ralstonia pickettii* and *Alcaligenes faecalis*, whereas *G. roseus* sporocarps were dominated by *Janthinobacterium svalbardensis* and *Novosphingobium rosa*. *Mycobacterium celatum* was the dominant species in mycorrhiza and mycorrhizosphere soil samples associated with *S. bovinus*. By contrast, mycorrhiza and mycorrhizosphere soil samples associated with *G. roseus* were dominated by *J. svalbardensis* and *M. celatum*. Functional annotation of bacterial strains revealed that bacterial communities of all sample types had similar functions, mainly metabolism and genetic information processing functions (Fig. 4c). Sequence coverage of all samples was good (>98%). Alpha indices varied among different sample types (Fig. S4). All the diversity indices for sporocarp samples were generally lower than those for mycorrhiza, or mycorrhizosphere soil samples.

Metabolic similarity between sporocarps of different ECM fungi

In total, 683 metabolites were detected in sporocarps of *S. bovinus*, *G. roseus*, and *S. luteus* (629, 622, and 632 metabolites, respectively), and 574 of these metabolites were detected in all three fungi. Lipids (151 metabolites), amino acids and derivatives (104), phenolic acids (95), and organic acids (78) were the most abundant metabolites (Fig. 5a and Table S2). Principal component analysis revealed that the first two principal components (PC1 and PC2) accounted for more than 72% of the variation in metabolic composition among samples. Samples of the three fungal species were aggregated together to form three separate clusters, revealing that each species showed distinct metabolite accumulation patterns (Fig. 5b). Correlation analysis further revealed that the metabolic profiles of the two *Suillus* species were more

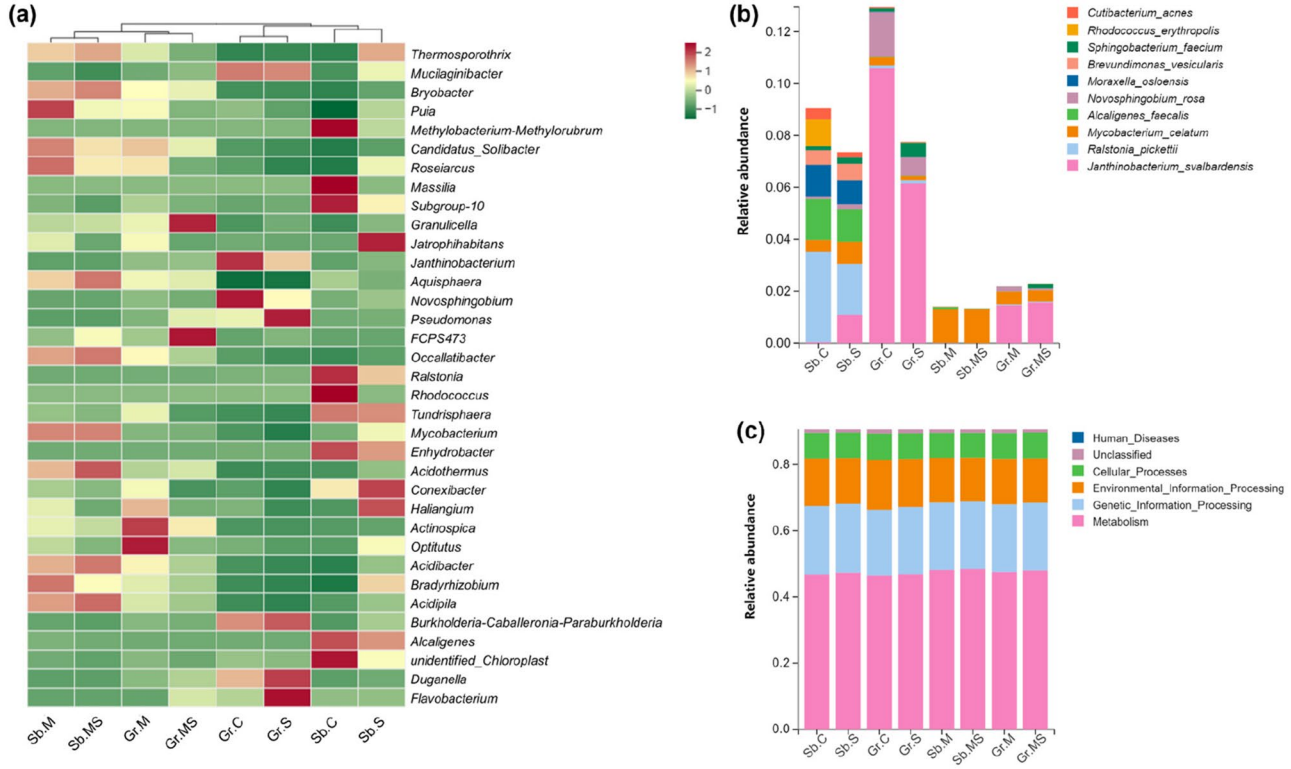

Fig. 4 Bacterial diversity of sporocarps, ectomycorrhizas, and mycorrhizosphere soils. (**a**) Heatmap showing bacterial associations among different sample types at the genus level. (**b**) Relative abundances of the top 10 most abundant bacterial species among different sample types. (**c**) Functional annotation of bacteria among different sample types. Sb.C and Sb.S, *Suillus bovinus* sporocarp caps and stems, respectively; Gr.C and Gr.S, *Gomphidius roseus* sporocarp caps and stems, respectively; Sb.M and Sb.MS, mycorrhizas and mycorrhizosphere soils associated with *S. bovinus* sporocarps, respectively; Gr.M and Gr.MS, mycorrhizas and mycorrhizosphere soils associated with *G. roseus* sporocarps, respectively

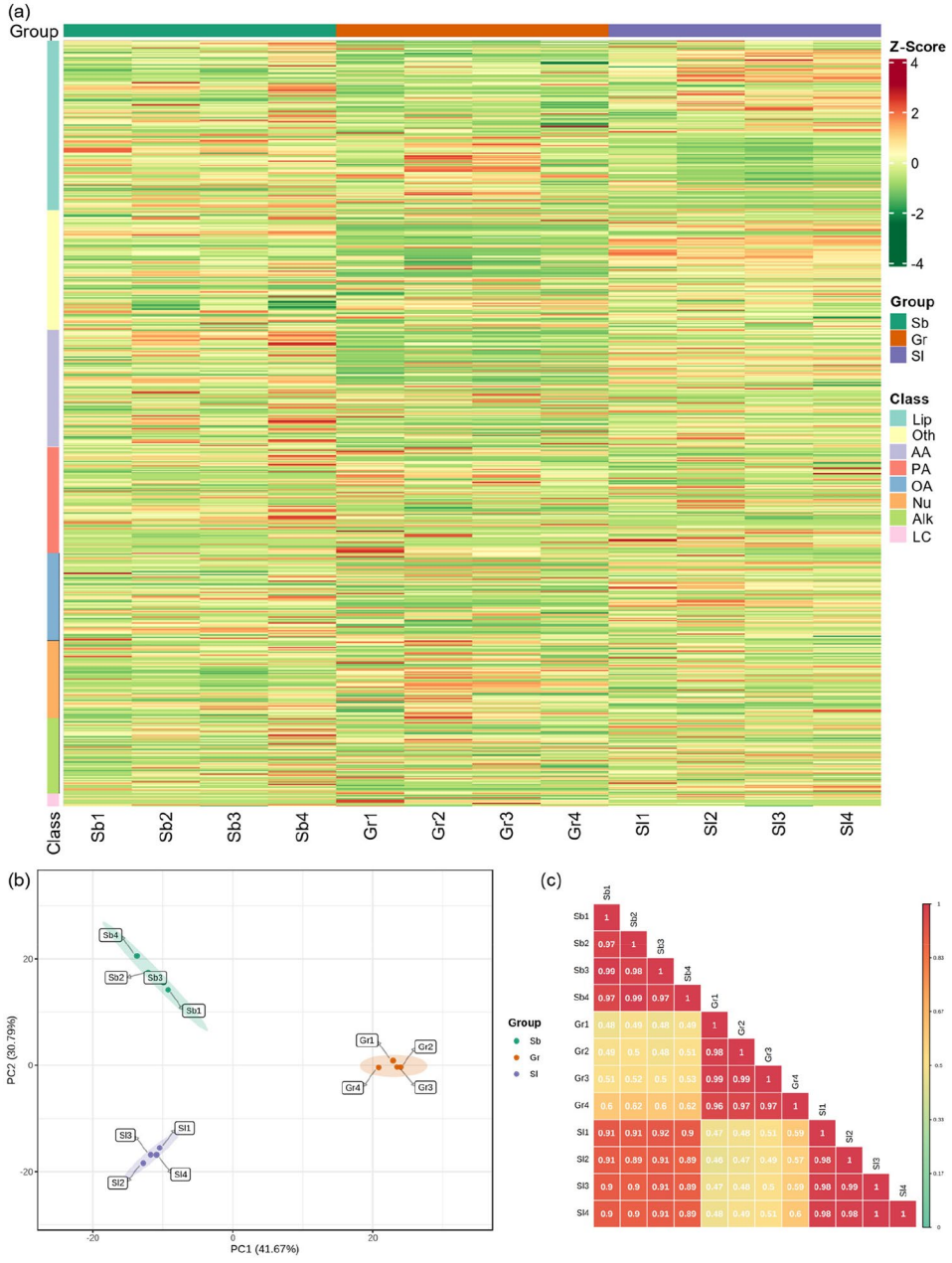

Fig. 5 Overview of widely targeted metabolome analysis of fungal sporocarps. (**a**) Heatmap visualization of metabolites. The content of each metabolite was normalized to complete linkage hierarchical clustering. Each example was visualized in a single column, and each metabolite is represented by a single row. Red indicates high abundance; metabolites with a low relative abundance are shown in green. The abbreviations Lip, Oth, AA, PA, OA, Nu, Alk, and LC refer to lipids, others, amino acids and derivatives, phenolic acids, organic acids, nucleotides and derivatives, alkaloids, and lignans and coumarins, respectively. (**b**) Principal component analysis (PCA) of metabolites. (**c**) Correlations among the metabolite profiles of different samples. Sb.C and Sb.S represent *Suillus bovinus* sporocarp caps and stems, respectively; Gr.C and Gr.S represent *Gomphidius roseus* sporocarp caps and stems, respectively; Sb.M and Sb.MS refer to mycorrhizas and mycorrhizosphere soils associated with *S. bovinus* sporocarps, respectively; Gr.M and Gr.MS refer to mycorrhizas and mycorrhizosphere soils associated with *G. roseus* sporocarps, respectively

similar to each other than they were to the metabolic profile of *G. roseus* (Fig. 5c).

Differentially accumulated metabolites (DAMs) analyses comparing *S. bovinus* sporocarp metabolites with those of *G. roseus* and *S. luteus* revealed 306 and 239 DAMs in *G. roseus* and *S. luteus* sporocarps, respectively (Fig. 6a, b; Table S3 and S4). The most common types of DAMs detected in *G. roseus* sporocarps were lipids (24 upregulated and 33 downregulated), amino acids and derivatives (18 upregulated and 37 downregulated), phenolic acids (20 upregulated and 21 downregulated), and organic acids (14 upregulated and 25 downregulated).

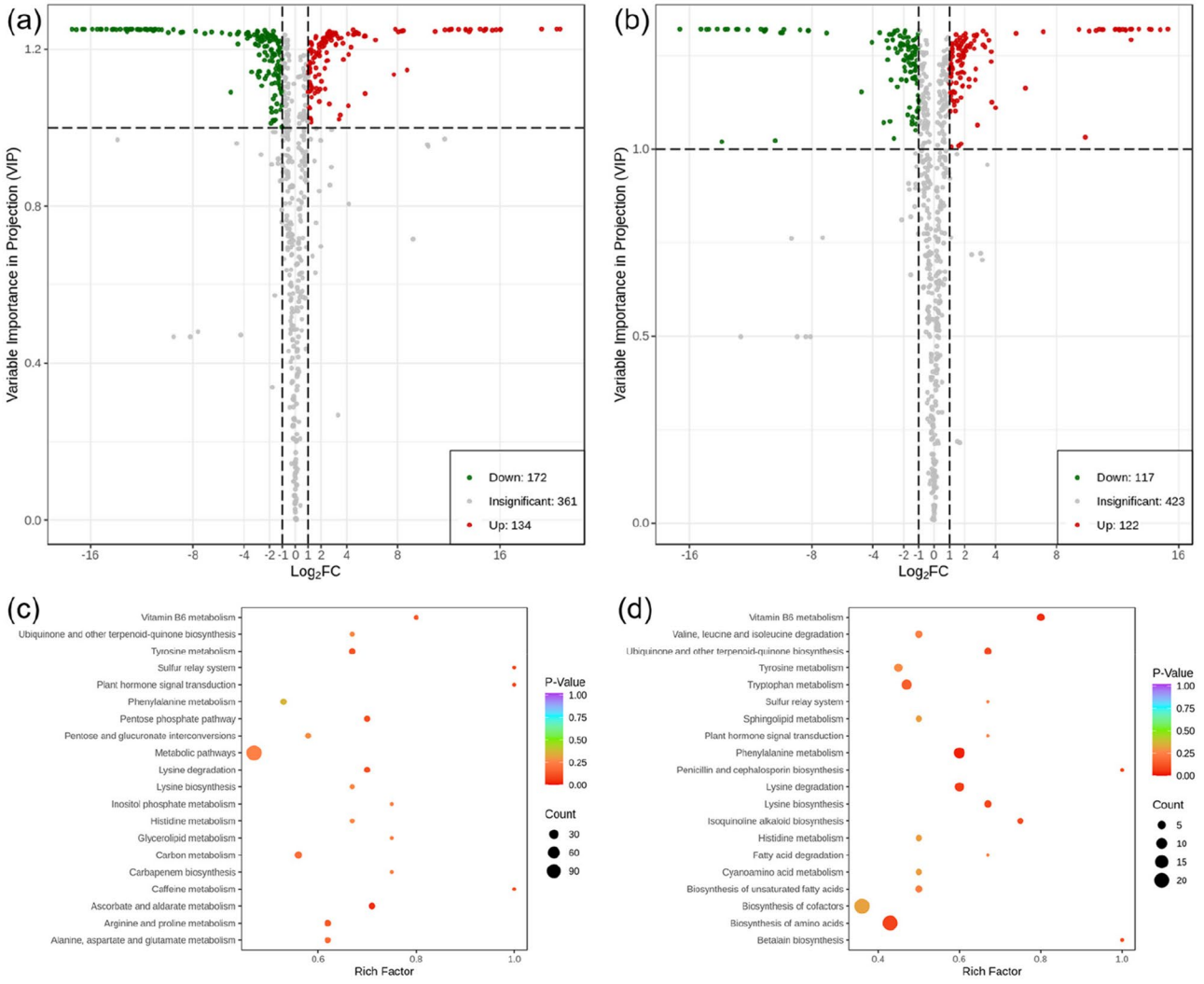

Fig. 6 Volcano plots and pathway analysis of differentially accumulated metabolites (DAMs) detected in *Gomphidius roseus*, *Suillus bovinus*, and *Suillus luteus* sporocarps. (**a**, **b**) Volcano plots showing the number of DAMs in *G. roseus* sporocarps vs. *S. bovinus* sporocarps, and in *S. luteus* vs. *S. bovinus* sporocarps. (**c**, **d**) KEGG enrichment of differential metabolites: (**c**) *G. roseus* sporocarps vs. *S. bovinus* sporocarps; (**d**) *S. luteus* vs. *S. bovinus* sporocarps. Each bubble in the plot represents a metabolic pathway. The abscissa and bubble size jointly indicate the magnitude of the impact factors of the pathway. A larger bubble indicates a larger impact factor. Bubble colours represent the *P*-values of the enrichment analysis, with darker colours showing a higher degree of enrichment

Similarly, in *S. luteus* sporocarps, most DAMs were lipids (22 upregulated and 38 downregulated), phenolic acids (23 upregulated and 23 downregulated), amino acids and derivatives (18 upregulated and 12 downregulated), or organic acids (14 upregulated and 16 downregulated) (Fig. S5). KEGG annotation revealed that DAMs synthesized in different fungal sporocarps involved several different biological processes (Fig. 6c, d). DAMs detected in *G. roseus* sporocarps are mainly involved in metabolic pathways; in contrast, DAMs detected in *S. luteus* sporocarps are mainly involved in the biosynthesis of amino acids, phenylalanine metabolism, and tryptophan metabolism.

Phenology of sporocarps of *S. bovinus* and *G. roseus*

A monthly survey of *S. bovinus* and *G. roseus* sporocarps was performed in *P. massoniana* forests in stands of different ages. *Suillus bovinus* sporocarps were recorded in all the stands investigated, ranging in age from 1 to 5 to > 30 years old. By contrast, *G. roseus* sporocarps were mainly found in forests that were > 10 years old (Table 1). The fruiting season of *G. roseus* occurred during the *S. bovinus* fruiting season and was shorter than that of *S. bovinus*, lasting between one and five months annually. Initially, the length of the fruiting season of both species increased as the stands developed before decreasing as *P. massoniana* aged.

Table 1 Monthly survey of *Suillus bovinus* and *Gomphidius roseus* sporocarp occurrence in *Pinus massoniana* forests of varying stand ages

Stand age (years)	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec
< 5	–	–	–	Sb	Sb	–	–	–	Sb	Sb	–	–
6–10	–	–	Sb	Sb, Gr	Sb	–	–	–	Sb	Sb	Sb	–
11–15	–	–	Sb	Sb, Gr	Sb	Sb	Sb	Sb	Sb	Sb	Sb, Gr	–
16–20	–	–	Sb	Sb, Gr	Sb, Gr	Sb, Gr	Sb	Sb	Sb	Sb, Gr	Sb, Gr	–
21–25	–	Sb	Sb	Sb, Gr	Sb, Gr	Sb, Gr	Sb	Sb	Sb	Sb, Gr	Sb, Gr	Sb
26–30	–	–	Sb	Sb, Gr	Sb, Gr	Sb, Gr	–	–	Sb	Sb, Gr	Sb, Gr	–
> 30	–	–	–	–	Sb	Sb	–	–	Sb	Sb, Gr	Sb	–

Note Sb indicates the occurrence of *S. bovinus* sporocarps, Gr indicates the occurrence of *G. roseus* sporocarps. '–' indicates that neither sporocarps of *S. bovinus* nor *G. roseus* were present

Gomphidius roseus sporocarps hardly influenced the growth of *S. bovinus* sporocarps or *P. massoniana* trees (as shown by the ratio between the DBH of *S. bovinus*-associated hosts and *G. roseus*-associated hosts) (Fig. 7).

Discussion

The co-occurrence of sporocarps of *S. bovinus* and *G. roseus* has been widely recognized [10, 11, 14]. In this study, we also found that the genesis of *G. roseus* sporocarps somewhat relies on the presence of *S. bovinus* sporocarps. We performed morphological, microbiome, metabolome, and phenological analyses to understand better the complex and intimate relationship between *G. roseus* and *S. bovinus*, and their relationship with their co-host.

Both *S. bovinus* and *G. roseus* sporocarps are edible, although the latter is smaller and sporocarp productivity is relatively low [13]. Previous research has indicated that *G. roseus* may be a parasite of either the fungal host (*S. bovinus*), the plant host (pine), or both [14]. If *G. roseus* does act as a parasite, the presence of *G. roseus* would be detrimental to the production of *S. bovinus* sporocarps and/or the growth of the plant host. However, we found that the occurrence of *G. roseus* sporocarps had little influence on the biomass of *S. bovinus* sporocarps or *P. massoniana* growth. These findings challenge the view that *G. roseus* has a parasitic relationship with *S. bovinus* and / or *P. massoniana*.

Different ECM exploration types may indicate differences in nutrient uptake and transfer capacities between fungi [23]. *Suillus bovinus* usually forms long-distance exploration ECMs, whereas species of *Gomphidius* form contact exploration ECM or medium-distance exploration ECM [23], which could explain why the nutrient uptake of *S. bovinus* is superior to that of *G. roseus* [23]. Functional complementation may exist between the two types of ECM. We observed spore-like and haustorium-like structures within ECM associated with *G. roseus* sporocarps. Olsson et al. [14] also reported the presence of chlamydospores and haustoria in the *P. sylvestris*–*S. bovinus*–*G. roseus* mycorrhizal system; however, the identity of these structures was unknown because their findings were mainly based on anatomic observations. Further investigations of the identity and functions of these structures would increase our understanding of the biological nature of the complex interactions that occur among *P. sylvestris*, *S. bovinus*, and *G. roseus*.

Bacteria and fungi have been recorded as associates of mycorrhizas, external mycelium, and fruiting bodies [24, 25]. Our analyses revealed that *S. bovinus* and *G. roseus* sporocarps contained the mycelial tissues of *G. roseus* and *S. bovinus*, respectively. By contrast, Olsson et al. [14] only detected the presence of *S. bovinus* on the stipe of *G. roseus* fruit bodies, which could be due to the low

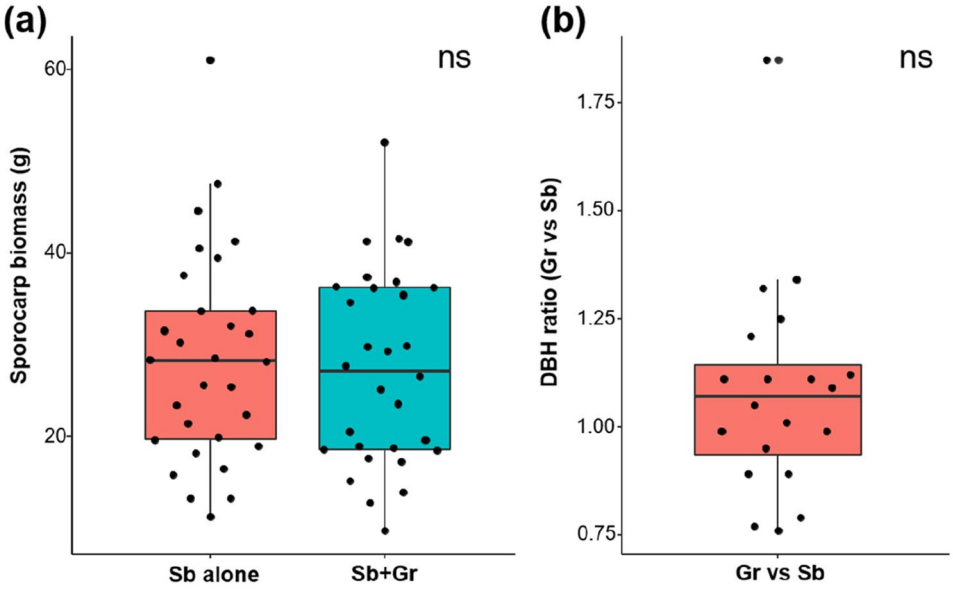

Fig. 7 Box plots showing the effects of *Gomphidius roseus* sporocarp genesis on *Suillus bovinus* sporocarp biomass and *Pinus massoniana* growth. (**a**) Biomass of *S. bovinus* sporocarps growing in the absence of *G. roseus* sporocarps (Sb alone) and the presence of *G. roseus* sporocarps (Sb + Gr). (**b**) Growth of *Pinus massoniana* based on the ratio of the diameter at breast height (DBH) of *P. massoniana* stands growing in the presence of *S. bovinus* and *G. roseus* sporocarps versus those with only *S. bovinus* sporocarps (Sb vs. Gr). 'ns' indicates no significant differences were found between sporocarps (**a**) (n = 50) or the DBH of *P. massoniana* (**b**) (n = 20) (T-test)

resolution of the technique that they used (e.g. detection of restriction fragment length polymorphisms (RFLPs) of the PCR-amplified ITS region of ribosomal DNA; the RFLP technique has a lower discriminatory power than high-throughput sequencing [26]. Our analyses showed that *G. roseus* mycelium colonized the caps and stems of *S. bovinus* sporocarps, whereas *S. bovinus* mainly colonized the stems of *G. roseus* sporocarps. These findings further challenge the idea that *G. roseus* has a parasitic relationship with *S. bovinus*, and the presence of *G. roseus* and *S. bovinus* mycelium in *S. bovinus* and *G. roseus* caps, respectively, further supports the hypothesis that these two species may be involved in each other's basidia and even spore formation.

Suilloid fungi (including *Suillus*, *Rhizopogon*, and members of the Gomphidiaceae) are very rarely detected on mycorrhizal roots in community structure studies, even when their fruit bodies are abundantly present [27, 28]. In this study, we found that mycorrhizas associated with *G. roseus* and *S. bovinus* were also dominated by other fungi and that the relative abundance of *S. bovinus* or *G. roseus* in these ECMs was relatively low (< 10%). This may explain why mycorrhizas associated with *G. roseus* and *S. bovinus* ECMs were hardly detected by Olsson et al. [14], particularly given that high-throughput sequencing techniques had not been fully developed at the time of their study. We also observed that *G. roseus* mycelium was present in *S. bovinus*-associated mycorrhiza, and vice versa. This suggests that *S. bovinus*, *G. roseus*, and their co-host formed a compound mycorrhiza. Furthermore, the relative abundance of *S. bovinus* mycelium was greater than that of *G. roseus* in both the mycorrhiza and the mycorrhizosphere soil associated with *G. roseus*. Based on these findings, we hypothesize that the formation of an ECM between *G. roseus* and the host relies on the ECM formed between *S. bovinus* and the same host.

ECM fungi can recruit specific bacterial populations in the mycosphere that can adhere to hyphae and even enter the fruiting body, performing various ecological functions, including promoting hyphal growth and assisting ECM fungi in nutrient absorption [29–31]. In this study, we detected various bacterial OTUs in sporocarps and ECMs. Samples of *S. bovinus* sporocarps were dominated by the bacteria *R. pickettii* and *A. faecalis*, whereas sporocarps of *G. roseus* were dominated by *J. svalbardensis* and *N. rosa*. These sporocarp-dwelling bacterial species may contribute to fungal nutrient absorption and sporocarp development [31, 32]. *Mycobacterium celatum* was the dominant species in mycorrhiza and mycorrhizosphere soil samples associated with *S. bovinus*. By contrast, mycorrhiza and mycorrhizosphere soil samples associated with *G. roseus* were dominated by *J. svalbardensis* and *M. celatum*. Although their exact functions are unknown, species of *Mycobacterium* are commonly found in the fungal hyphosphere of forests [33, 34, 35]. The presence of different dominant bacterial species in sporocarps and mycorrhiza of different ECM fungi may indicate that different bacteria are recruited for different roles by different symbiotic fungi at specific periods during their life cycle [25]. Further investigation of the

functions of these dominant bacterial species could help elucidate the mechanism of ECM establishment and sporocarp genesis.

The presence of *S. bovinus* or *G. roseus* mycelia in *G. roseus* or *S. bovinus* sporocarps, respectively, had limited influence on their metabolome composition, and the metabolic accumulation patterns of *S. bovinus* and *S. luteus* were more similar to each other than to *G. roseus*. Compared with *S. bovinus* sporocarps, the most common types of DAMs detected in *G. roseus* sporocarps were lipids, amino acids and derivatives, phenolic acids, and organic acids. KEGG annotation revealed that DAMs detected in *G. roseus* sporocarps are mainly involved in metabolic pathways. These DAMs may be at least partially responsible for the dependent nature of *G. roseus* on *S. bovinus*. The different chemical characteristics of *G. roseus* and *S. bovinus* sporocarps could also explain the different sporocarp bacterial communities to some extent because the chemical characteristics of sporocarps can influence the structure of endofungal bacterial communities [29]. In addition, differences between *G. roseus* and *S. bovinus* sporocarps in terms of the composition and content of amino acids, phenolic acids, and several vitamins may explain differences in the nutrition and flavor of these two edible mushroom species (Fig. S5; Table S3). These metabolites are important indicators when analyzing the nutrient composition of edible mushrooms [36, 37].

Species of *Suillus* are considered to be early succession ECM fungi in *Pinus* roots [38, 39]. Our investigation of sporocarp occurrence regarding stand age and monthly variation supports this view. We found *S. bovinus* sporocarps in all stands investigated, which ranged in age from 1 to 5 years old to >30 years old. Although *G. roseus* is also considered a suilloid fungus, *G. roseus* sporocarps emerged later than *S. bovinus* in *P. massoniana* forests. They were mainly found in forests that were >10 years old. The fruiting season of *G. roseus* was shorter than that of *S. bovinus* and occurred during the fruiting season of *S. bovinus*. Based on the microbiome data, it is reasonable to assume that the symbiosis between *G. roseus* and *P. massoniana* relies on an ECM formed with *S. bovinus* and *P. massoniana* and that a mixed ECM associated with *G. roseus*, *S. bovinus*, and *P. massoniana* is necessary for the genesis of *G. roseus* sporocarps. As *P. massoniana* aged, the length of the *G. roseus* and *S. bovinus* fruiting season showed an increasing trend and then decreased, following the growth rhythm of *P. massoniana*. The growth rate of *P. massoniana* decreases with stand age in *P. massoniana* forests that are more than 25 years old [15]. These changes may be at least partially ascribed to the replacement of earlier successional species by late-stage fungi [40]. Furthermore, studies have revealed that the vigor of the host tree is correlated with the production of ECM fungal sporocarps, and that lower growth rates may restrain sporocarp genesis [41].

Agerer [42] reported that *G. roseus* could not be isolated when co-cultivated with *S. bovinus*. Still, once established on a growth medium, growing pure cultures of *G. roseus* was possible. However, despite several attempts (including using *S. bovinus* or *P. massoniana* roots exudates), we failed to obtain a pure culture of *G. roseus* from *G. roseus* sporocarps (data not presented in this study). By contrast, it was much easier to obtain a pure culture of *S. bovinus* [17]. This could be because the ability of *G. roseus* to grow saprotrophically is weaker than that of *S. bovinus*.

Conclusions

Overall, regarding the succession sequence, the co-occurrence of sporocarps, the different ECM exploration types, and that ECM formation is a prerequisite for ECM fungal sporocarp genesis, we conclude that the formation of an ECM between *G. roseus* and the host *P. massoniana* may rely on the ECM formed by *S. bovinus* and *P. massoniana*. Our findings, which indicate that the occurrence of *G. roseus* sporocarps has minimal influence on *S. bovinus* sporocarp or *P. massoniana* growth, do not align with the hypothesis proposed by Olsson et al. [14] that *G. roseus* is parasitic on *S. bovinus*. Instead, a commensal relationship may exist between the two fungi, where *G. roseus* benefits from *S. bovinus* without affecting it. However, further investigations are urgently needed to fully understand these complex interactions. We need to explore the interactions between *G. roseus* and *S. bovinus* under pure culture conditions, the effects each fungal species has on each other's symbiotic process with *P. massoniana*, and their effects on *P. massoniana* growth. Additionally, our metabolome analysis has revealed nutritional and flavour differences between these two species of edible mushroom, which we believe warrants immediate attention from a food-development perspective.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-03881-0.

Supplementary Material 1
Supplementary Material 2
Supplementary Material 3
Supplementary Material 4
Supplementary Material 5
Supplementary Material 6
Supplementary Material 7
Supplementary Material 8
Supplementary Material 9

Supplementary Material 10

Supplementary Material 11

Author contributions
X.S. conceived the research idea; L.D., X.S., and G.Y. designed the study and collected data; X.S. processed data and wrote the draft. All authors reviewed the manuscript.

Funding
This research was supported by the National Natural Science Foundation of China under Grant [31971572 and 32360372] and the Cultivation Project of Guizhou University under Grant [(2020)47].

Data availability
Raw sequence data of both bacteria and fungi reported in this paper have been deposited in the NCBI SRA database under accession numbers PRJNA1012362 (bacteria) and PRJNA1012375 (fungi). Datasets of metabolomes presented in this study are listed in Table S2.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 5 August 2024 / Accepted: 10 March 2025
Published online: 26 March 2025

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