

Integrated metabolomic and genomic characterization of antifungal *Streptomyces halstedii* S14 from *Melipona mondury* larval food

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Abstract

Although the larval food of stingless bees harbors microorganisms with potential for natural-product discovery, the chemical and genomic potential of actinobacteria associated with *Melipona mondury* remains poorly explored. We investigated the antagonism of *Streptomyces halstedii* S14 against *Trichoderma atroviride* by integrating co-culture, spatial UHPLC-HRMS/MS, Feature-Based Molecular Networking, and genome mining with antiSMASH and BiG-SCAPE. S14 produced an inhibition zone of 1.28 ± 0.38 cm. Of 771 molecular features evaluated, 515 showed significant spatial variation across the co-culture gradient after accounting for plate identity. Pre-specified contrasts retained seven robust features associated with the inhibition zone (IZ) and 11 with the halo–fungus interface (TP). Among annotated candidates, m/z 295.1294, putatively assigned as glutamylphenylalanine, was associated with IZ, whereas m/z 522.3565, putatively assigned as 1-oleoyl-lysophosphatidylcholine, was associated with TP. Genome mining identified 59 biosynthetic regions. The strongest genome–metabolome convergence involved m/z 256.0608, putatively annotated as caboxamycin, and NODE_598, closely related to the caboxamycin reference BGC (BiG-SCAPE distance 0.03). A family-level association linked a bafilomycin C1-like signal (m/z 743.399) to NODE_118 (distance 0.34). These findings reveal a spatially structured chemical interaction and show how regional metabolomics combined with genome mining can prioritize biosynthetic hypotheses without assigning the antifungal phenotype to a single compound.

Introduction

Stingless bees (Hymenoptera: Apidae: Meliponini) harbor microbial communities associated with both the intestinal tract and various biological matrices within the colony—including honey, stored pollen, and larval food—the composition of which can vary depending on the host species and the matrix analyzed [1–3]. Among these matrices, larval food stands out as a biologically significant microenvironment, as it hosts microbial communities directly linked to offspring development. Previous studies on various stingless bee species have demonstrated the presence of structured microbial communities within this matrix and revealed that cultivable bacteria recovered from larval food can exhibit antimicrobial activity [2, 4]. In *Melipona scutellaris*, for instance, a *Paenibacillus polymyxa* strain isolated from larval food produced phenyllactic acid and fusaricidins with activity against microorganisms relevant to bees [5]. In contrast, recent studies on *Melipona mondury* have focused primarily on the gut microbiota across different developmental stages and on the microbiological and metabolic profiles of honey [6–8]. Although these investigations expand microbiological knowledge regarding the species, the potential of cultivable actinobacteria from *M. mondury* larval food remains poorly understood. This lack of systematic study represents a significant gap, limiting our understanding of the chemical ecology and biological diversity of microorganisms associated with this specific niche [9].

Among bacteria associated with organisms of the order Hymenoptera, actinobacteria—particularly representatives of the genus *Streptomyces*—have been recovered from various hosts and intracolony compartments, representing promising sources for the discovery of natural products [10, 11]. Large-scale comparative studies have also indicated that certain insect-associated *Streptomyces* isolates exhibit significant biosynthetic diversity and antimicrobial activity, reinforcing interest in microbiomes for bioprospecting [12]. This potential has also been demonstrated in stingless bees. Microorganisms associated with *Tetragonisca angustula* showed antibacterial and antifungal activity in antagonism assays [13]. Additionally, in *M. scutellaris*, actinobacteria isolated from adult bees produced metabolites characterized as meliponamycin depsipeptides, lobophorin glycosylated terpenoids, and anthracycline-type polyketides, with some exhibiting activity against the colony pathogen *Paenibacillus larvae* [14, 15]. Collectively, these studies indicate that stingless bee-associated actinobacteria possess biosynthetic potential for extensive chemical diversity as well as associated antimicrobial activity. In contrast, studies on *M. mondury* have focused primarily on characterizing the microbiota and colony products, while the chemical and biosynthetic potential of cultivable actinobacteria associated with larval food remains underexplored [6–8]. Importantly, microbial interactions can influence the expression of specialized metabolism; co-culture studies have demonstrated that interactions between *Streptomyces* and fungi can induce quantitative and qualitative changes in the metabolic profiles of the organisms involved [16, 17]. Among these systems, interactions involving *Trichoderma* species are particularly relevant, given that these fungi possess recognized antagonistic capabilities and are widely used as biological control agents; furthermore,

exposure of bees to formulations containing *Trichoderma* spp. has been linked to physiological changes and potential risks to these organisms [18]. In a system involving *Streptomyces* sp. and *Trichoderma harzianum*, for instance, the antifungal activity associated with bafilomycin C1 was accompanied by pronounced changes in the fungus's metabolism [19]. In solid media, this dynamic may also exhibit spatial organization, as different regions of the interaction can display distinct metabolic profiles [20, 21]. This aspect is especially pertinent to *T. atroviride*, for which regional heterogeneity in metabolite production and distribution in solid culture has already been demonstrated [22]. In this context, sampling spatially defined regions of the interaction preserves spatial information that would otherwise be lost through the homogenization of the entire co-culture, enabling the identification and prioritization of features differentially enriched in regions of biological interest [17, 20, 23].

Integrating metabolomic and genomic data via metabologenomics approaches enables the prioritization of candidate associations between detected metabolites and biosynthetic gene clusters (BGCs), guiding the investigation of natural products toward subsequent stages of experimental validation [24, 25]. Currently, the distribution of chemical profiles obtained through regional metabolomic sampling during *Streptomyces*-mediated fungal antagonism—involving strains isolated from the larval food of *M. mondury*—remains poorly characterized, as does the direct correlation of these profiles with the isolate's genomic potential. To address this issue, this study aimed to characterize the antagonistic interaction between *Streptomyces halstedii* S14 (isolated from the larval food of *M. mondury*) and the filamentous fungus *Trichoderma atroviride*. The hypothesis is that regional metabolomic sampling, when systematically integrated with genomic mapping, allows for the mapping of spatial variations in chemical substances and the revelation of convergences consistent with specific biosynthetic gene clusters. Thus, this work presents a strategy for prioritizing features spatially associated with the inhibition zone and investigating convergences with BGCs predicted in the genome. The specific objectives of the study were: (i) to apply regional metabolomic sampling to characterize the spatial distribution of chemical features across the co-culture and prioritize candidates associated with the inhibition zone; and (ii) to explore, in an integrated manner, the convergences between the detected metabolomic evidence and the biosynthetic potential encoded in the *S. halstedii* S14 genome.

Materials and methods

Sample collection and processing

Samples of larval food from the stingless bee *Melipona mondury* were collected at the Paraitinga Meliponary (Fig. 1), located in Natividade da Serra, São Paulo, Brazil (23°19'26.4"S, 45°29'06.0"W). Three combs containing brood cells with larvae at the larval development stage were collected from three independent, healthy colonies and aseptically transferred to sterile Petri dishes. Immediately after collection, the samples were stored at 4°C in a portable cooling system to preserve their chemical and microbiological integrity. Transport to the Multidisciplinary Health and Environment Laboratory (LABMSMA) was completed within approximately two hours.

All processing of the biological material was conducted under strict aseptic conditions. Brood cells were opened individually using sterilized forceps, and larval food was collected with sterile pipettes. Approximately 1.5 mL of larval food was recovered from each brood comb. To mitigate specific biological variation among individual colonies and ensure a homogeneous, representative volume from the local meliponary, the larval food volumes from each of the three independent colonies were combined into a single biological pool of approximately 4.5 mL, which was then homogenized via gentle mechanical agitation. Aliquots of 500 µL from this homogeneous pool were immediately withdrawn for use in microbiological enrichment procedures.

Isolation of actinobacteria associated with larval food

To enrich the microorganisms, 500 µL aliquots of the larval food pool were separately inoculated into 50 mL of ISP-9, NYDB (D-glucose 10.0 g/L, beef extract 3.0 g/L, yeast extract 5.0 g/L, monobasic sodium phosphate 0.3 g/L, dibasic sodium phosphate 1.2 g/L, and peptone 5.0 g/L), and ISP-2 (D-glucose 4 g/L, yeast extract 5.0 g/L, malt extract 10.0 g/L, and starch 5 g/L). To selectively inhibit the growth of fast-growing contaminating fungi and endogenous yeasts present in the sample, the

culture media were supplemented with cycloheximide (0.05 g/L) and nystatin (0.02 g/L), and the cultures were incubated at 30°C with agitation at 120 rpm for 6–12 days.

Negative controls consisting of the respective media inoculated with 500 µL of sterile 0.9% saline solution were incubated under the same experimental conditions to rigorously monitor the sterility of the analytical process. After enrichment, serial dilutions ranging from 10^{-1} to 10^{-3} were prepared, and 100 µL aliquots were plated in biological triplicate onto solid NYDA (NYDB with 20 g/L agar) and ISP-2 agar (ISP-2 with 20 g/L agar) media. The plates were incubated at 30°C for 7–14 days, and isolated colonies were successively streaked onto NYDA and ISP-2 until pure cultures were obtained. The actinobacteria were preserved under refrigeration at 4°C, following the method described by Castelanni [26]. Morphological characterization of the isolates was performed via macroscopic colony evaluation, Gram staining, and scanning electron microscopy (SEM). Macroscopic evaluation assessed growth characteristics, color, texture, and colony morphology. Gram staining was performed using a standard procedure involving primary staining with crystal violet, treatment with iodine solution, decolorization, and counterstaining with safranin, followed by optical microscopy to determine the Gram reaction and cell morphology [27]. For SEM analysis, cultures of isolate S14 were prepared according to standard procedures for biological samples, including fixation, dehydration in an increasing ethanol series, drying, mounting on appropriate stubs, and coating with conductive material prior to micrograph acquisition [28] (Fig. 2).

DNA extraction, sequencing, genome assembly, and taxonomic analysis

For DNA extraction, cultures of isolate S14 were activated in Tryptic Soy Broth (TSB: tryptone 17 g/L, soy peptone 3 g/L, sodium chloride 5 g/L, D-dextrose 2.5 g/L, dipotassium hydrogen phosphate 2.5 g/L). DNA was obtained using the Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA), following the manufacturer's protocol for Gram-positive bacteria with specific modifications for cell lysis. The pellet from 2 mL of active culture—obtained by centrifugation in an Eppendorf 5424 R refrigerated centrifuge operating at 16,000 rpm for 2 minutes—was subjected to enzymatic lysis with 120 µL of lysozyme (10 mg/mL; Sigma-Aldrich) for 60 minutes at 37°C in a Techne TC-312 water bath. The pellet was resuspended in 600 µL of Nuclei Lysis Solution (Promega), incubated at 80°C for 5 minutes, and treated with 3 µL of RNase Solution (Promega) at 37°C for 60 minutes. After protein precipitation (200 µL of Protein Precipitation Solution, Promega), the DNA was precipitated with isopropanol (Merck), washed with 70% (v/v) ethanol, air-dried at room temperature, and resuspended in 100 µL of DNA Rehydration Solution (Promega). Purity and concentration were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific), while integrity was assessed via 0.8% agarose gel electrophoresis (Bio-Rad Mini-Sub Cell GT).

Whole-genome sequencing (WGS) of isolate S14 was performed using Illumina NextSeq 550 platform. Libraries were prepared using the Nextera XT protocol (Illumina), and sequencing was carried out in paired-end mode using a 300-cycle run, generating 2×150 -bp reads. The sequencing data were processed using the Varsmetagen bioinformatics pipeline, including read quality control, filtering, removal of reads not assigned to the microorganism of interest, and genome assembly. Taxonomic classification of the recovered genome was performed using the Genome Taxonomy Database (GTDB), and assembly completeness was estimated using CheckM. Average nucleotide identity (ANI) and alignment fraction (AF) were calculated using FastANI, with the *Streptomyces halstedii* reference genome GCA_000720535.1 was obtained from the National Center for Biotechnology Information (NCBI).

Antagonism assay against *Trichoderma atroviride* in co-culture

The antifungal activity of isolate S14 was evaluated in a co-culture assay against the filamentous fungus *Trichoderma atroviride* CMLD 34 (GenBank PZ207909—previously isolated from mycelium growing on geopropolis (*batume*) and wood from *Melipona marginata* stingless bee colonies and available in the microbial isolate bank of LabMINP (Laboratory of Chemical Biology of Microorganisms, Insects, and Plants) at UNIFESP, Diadema Campus. The microorganisms were initially cultured separately on NYDA at 30°C for seven days. Subsequently, an agar plug (approximately 1 cm²) containing the growth of isolate S14 was transferred to the center of a new NYDA plate and incubated at 30°C in the dark for seven days. Following the

initial growth of the actinobacterium, 100 µL of a *T. atroviride* suspension (7,000 spores/mL, prepared in peptone water) was distributed along the edge of the plate. The co-cultures were incubated under the same conditions for an additional seven days, totaling a 14-day experiment. The assay was performed using three independent plates. Axenic cultures of isolate S14 and *T. atroviride*, as well as plates containing only the NYDA medium, were maintained under the same experimental conditions as controls.

Antifungal activity was evaluated based on the formation of inhibition zones between the actinobacterium colony and the filamentous fungus. The distance between the two colonies was measured using a digital caliper at three positions on each plate. The average of the three measurements was used as the representative value for each plate.

At the end of the antagonism assay, agar fragments of approximately 1 cm² were aseptically collected from five regions of the co-culture plates: the center of the S14 colony (AD), the interface between the actinobacterium colony and the inhibition zone (AP), the center of the inhibition zone (IZ), the interface between the inhibition zone and the fungal growth (TP), and the center of the *T. atroviride* colony (TD).

Each region was sampled across the three independent plates, totaling three samples per group. Equivalent fragments were collected from the axenic cultures of the actinobacterium and the fungus, and from plates containing only the NYDA medium, corresponding to the AC, TC, and MC controls, respectively.

The samples were transferred to 1.5 mL vials and subjected to metabolite extraction for subsequent analysis by UHPLC-HRMS. A description of the regions and controls used is presented in Table 1.

Table 1
Spatial sampling scheme used for UHPLC-HRMS analysis of the actinobacterium-fungus co-culture assay.

Sample code	Sample type
AD	Actinobacterial Distal region
AP	Actinobacterial Proximal region
IZ	Inhibition Zone
TP	<i>T. atroviride</i> Proximal region
TD	<i>T. atroviride</i> Distal region
AC	Actinobacterium Control
TC	<i>T. atroviride</i> Control
MC	Medium Control

Extraction of metabolites from solid cultures

Agar fragments collected from different regions of the co-culture plates and experimental controls were subjected to ultrasound-assisted extraction following a previously described method Smedsgaard [29], with modifications. For each sample, the agar discs or fragments containing microbial material were transferred to 1.5 mL vials and extracted with 1 mL of a methanol/dichloromethane/ethyl acetate mixture (1:2:3 v/v/v) using analytical-grade solvents. The samples were sonicated in an ultrasonic bath for 30 min at room temperature. Following extraction, the supernatants were collected, and the solvents were evaporated in a fume hood until the extracts were completely dry. The dried micro-extracts were resuspended in 1 mL of HPLC-grade methanol, sonicated for 2 min, and centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to vials with inserts and stored at 4°C pending analysis by liquid chromatography coupled with high-resolution mass spectrometry.

UHPLC-HRMS analysis and data processing

Analyses were performed using a Nexera X2 UHPLC system coupled to an LCMS-9030 qTOF mass spectrometer (Shimadzu), equipped with an electrospray ionization (ESI) source operating in both positive and negative modes. Chromatographic separation was carried out on a Kinetex C18 column (100 × 2.1 mm, 2.6 µm) equipped with a guard column, maintained at 50°C, with a flow rate of 0.35 mL/min and an injection volume of 2 µL. The mobile phases consisted of ultrapure water (A) and acetonitrile (ACS Científica, Sumaré, SP, Brazil) (B), both containing 0.1% formic acid (LiChropur™, Merck KGaA, Darmstadt, Germany). The gradient used was: 0–2 min (15% B), 2–12 min (15–95% B), 12–16 min (95% B), 16–18 min (95–15% B), and 18–20 min (15% B). Data were acquired in the *m/z* range of 50–1200, using 2–6 MS/MS-dependent events per cycle, with an acquisition time of 0.1 s for MS and 0.1–0.2 s for each MS/MS event, resulting in a recommended loop time between 0.8 and 1.4 s. Collision energy was applied automatically by the instrument (Collision Energy Auto; CE Spread ± 35). Precursor ion selection was performed automatically via DDA, using a configurable minimum intensity filter (Threshold). The main ESI source parameters were: interface voltage of 1.5 kV, nebulizer gas flow of 3 L/min, heating gas flow of 10 L/min, interface temperature of 100°C, desolvation temperature of 160°C, DL temperature of 200°C, and heat block temperature of 300°C. Mass calibration was performed using sodium formate.

Raw files were processed in MZmine 4.9.14 using MZWizard with default parameters for LC-MS/MS data, including mass detection, chromatogram construction, deisotoping, cross-sample alignment, and generation of the molecular feature table. Files were imported in mzML format. Cross-sample alignment was performed using the standard MZWizard algorithm, followed by filtering to retain features detected in the samples of interest. Information on feature occurrence in analytical blanks was retained for subsequent exclusion of background-associated signals during candidate prioritization.

Samples QCMIX1, QCMIX2, QCMIX3, QCD1, QCD2, and QCD3 were used for analytical-run quality control and assessment of data processing and were not included as biological samples. QCMIX1, QCMIX2, and QCMIX3 corresponded to mixtures of extracts from the co-culture regions, specifically from vials 1–5, 6–10, and 11–15, respectively. QCD1, QCD2, and QCD3 corresponded to mixtures of extracts from the actinobacterial and fungal controls, specifically from vials 16/19, 17/20, and 18/21, respectively.

The feature table generated in MZmine was subsequently imported into the MetaboAnalyst workflow [30]. Among the 1,690 initial molecular features, those detected in fewer than two of the 24 biological samples were removed as non-informative signals, eliminating 405 features and leaving 1,285. The remaining features were ranked according to interquartile range (IQR), and the 60% with the highest IQR were retained, resulting in a final dataset of 771 features. Missing values were imputed using one-fifth of the minimum positive value of each feature, followed by sample median normalization and log₁₀ transformation. Pareto scaling was applied for multivariate analyses and visualization, whereas inferential analyses were performed on the median-normalized, log₁₀-transformed intensities before Pareto scaling. Analytical blanks and QC samples were excluded from the biological data matrix, while information on blank occurrence was retained for candidate filtering.

For molecular networking, MS/MS spectra and the corresponding feature table were submitted to the GNPS2 platform using the Feature-Based Molecular Networking (FBMN) workflow. A precursor-ion mass tolerance of 0.02 Da, fragment-ion mass tolerance of 0.05 Da, minimum cosine score of 0.7, and minimum of six shared fragments were used to establish connections between network nodes; all other parameters were maintained at platform default values. Compound annotations were obtained by comparison of MS/MS spectra against spectral libraries available through GNPS2. Features matching reference spectra were assigned confidence level 2 according to the Metabolomics Standards Initiative (MSI) recommendations[31]. For prioritized features without spectral-library matches, SIRIUS 6.3.4[32] was used for molecular-formula prediction, and putative chemical classes were assigned using CANOPUS [33]

Genomic mining of biosynthetic clusters using antiSMASH

Genome mining was performed to identify biosynthetic gene clusters (BGCs) related to the production of specialized metabolites in the genome of isolate S14. The preliminary genome assembly of isolate S14 was submitted to antiSMASH

bacterial version 8.0.4 [34] using the genome file in FASTA format. The predicted biosynthetic regions were classified according to BGC type, including nonribosomal peptide synthetases (NRPS), polyketide synthases (PKS), terpenes, ribosomally synthesized and post-translationally modified peptides (RiPPs), and other specialized metabolites. For each predicted BGC, the cluster type, position on the contig, presence at a contig edge, and similarity to previously described BGCs were evaluated. Initial comparison with known clusters was conducted using antiSMASH similarity modules, including ClusterBlast, KnownClusterBlast, and comparison with regions deposited in MIBiG 3.1 [35]. These annotations served as an initial screen and were interpreted conservatively, as similarity to a known BGC does not confirm the production of the same metabolite by the analyzed strain. A systematic comparison of the S14 regions with reference BGCs was subsequently performed using BiG-SCAPE.

Comparative analysis of BGCs using BiG-SCAPE

The 59 .region.gbk files generated by antiSMASH for isolate S14 were analyzed using BiG-SCAPE 2.0.3 in conjunction with the MIBiG 3.1 database. The analysis was performed on the cluster workflow, using each antiSMASH region as the unit of comparison (record type: region), with classification by biosynthetic category, automatic alignment, and the legacy extension strategy. Distance cutoffs of 0.3, 0.4, 0.5, 0.6, and 0.7 were evaluated, including singletons. In the main analysis, networks were examined without mixing between biosynthetic categories. For each BGC, the following data were retrieved: direct comparisons with MIBiG BGCs; BiG-SCAPE distance metrics (Jaccard, adjacency, and domain sequence similarity [DSS]); and association with gene cluster families (GCFs) at each cutoff. Relationships were classified as: direct connection to MIBiG; membership in the same GCF without a direct edge; GCF containing only S14 BGCs; or singleton. The closest reference BGC was defined based on the lowest BiG-SCAPE distance observed in the full network. For the primary interpretation, the 0.3 cutoff was considered the most restrictive level, while the subsequent cutoffs were used to assess relationship stability at progressively more permissive distance levels.

Comparison of gene organization and synteny analysis

To investigate the relationship between selected BGCs from S14 and their reference BGCs in MIBiG at the gene level, pairwise comparisons were performed using Clinker [36]. The region.gbk files generated by antiSMASH were compared with the corresponding GenBank files from MIBiG 3.1, preserving the original gene order. Proteins encoded within the regions were compared using a minimum identity threshold of 30%, and homologous gene groups were used to assess the conservation of gene content, order, and orientation. Identities between homologous proteins and the relative gene organization were visualized in Clinker. For nodes extending to the end of the contig, the analysis was interpreted as a partial synteny comparison rather than a reconstruction of the complete BGC.

Statistical analyses

Microbiological growth data obtained from the antagonism assay against the fungus *T. atroviride* were evaluated using univariate analyses. The three measurements taken at different positions on each plate were treated as technical subsamples and used to calculate a mean value per plate. The overall mean and standard deviation were calculated based on the means of the three independent plates. The range of variation was determined by considering all individual measurements. The data were analyzed descriptively, as the plates represented replicates of the same treatment rather than independent experimental groups. Analyses of the microbiological assay were performed using R software version 4.4.1, utilizing standard program functions and the ggplot2 package for graph generation.

The metabolomic dataset comprised 24 biological samples distributed among AD, AP, IZ, TP, TD, AC, TC, and MC, with three samples per group. For the primary analysis of spatial variation, only the 15 co-culture samples were included in the inferential model because AD, AP, IZ, TP, and TD were sampled from the same three independent co-culture plates. Plate identity was therefore included as a blocking factor. AC, TC, and MC were retained as biological reference groups for descriptive interpretation and for determining the group in which each feature showed its highest mean abundance.

For each of the 771 retained molecular features, spatial inference was performed on median-normalized, log10-transformed intensities using a plate-blocked linear model of the form $\text{abundance} \sim \text{region} + \text{plate}$, fitted using the limma framework with robust empirical-Bayes variance moderation. The overall effect of the region across AD, AP, IZ, TP, and TD was evaluated using a moderated F-test. Benjamini–Hochberg correction was applied across the 771 features, with BH-FDR < 0.05 considered statistically significant.

Two biologically pre-specified, two-sided contrasts were evaluated using the same blocked model: IZ versus the mean of AD, AP, TP, and TD, and TP versus the mean of AD, AP, IZ, and TD. BH-FDR correction was applied separately to each set of 771 contrasts. Positive regional enrichment required a positive estimated contrast. A feature was classified as an IZ- or TP-associated candidate when the corresponding contrast showed BH-FDR < 0.05, the estimated contrast was positive, the target region showed the highest mean abundance among all eight biological groups, and the feature was not detected in any of the three analytical blanks before imputation. The overall blocked region test was considered complementary evidence and was not required as an additional criterion when the pre-specified regional contrast fulfilled the candidate-selection criteria.

Sample-level PCA, feature-level PCA, PLS-DA, VIP scores, and Random Forest rankings were used as exploratory analyses. Multivariate visualizations were based on the Pareto-scaled matrix. Hierarchical clustering heatmaps were generated using row scaling, Euclidean distance, and Ward hierarchical clustering. PLS-DA, VIP scores, and Random Forest rankings were not used as criteria for defining IZ- or TP-associated candidates. For feature-level visualization, the first PCA included all 771 retained features, whereas the second was restricted to the 515 features showing a significant overall region effect in the plate-blocked analysis (BH-FDR < 0.05).

All inferential tests were two-sided unless otherwise stated, and adjusted p values are reported as BH-FDR. Candidate-level statistics, effect estimates, fold ratios, and blank-occurrence information are provided in Supplementary Table S3.

Results

Isolation and taxonomic identification

An actinobacterium isolate, designated S14, was recovered from the larval food pool of *Melipona mondury* through enrichment in the NYDB culture medium.

On agar, the isolate exhibited dry, rugose colonies with a powdery appearance (Fig. 3a). Gram staining revealed Gram-positive filamentous structures (Fig. 3b), while scanning electron microscopy demonstrated the isolate's filamentous mycelial organization (Fig. 3c–d).

Genomic sequencing analyses yielded 3,346,845 raw reads, of which 3,098,593 remained after quality filtering and 2,687,392 after the removal of reads not assigned to the microorganism of interest. More than 80% of the reads exhibited a Phred quality score greater than 30.

The preliminary genome assembly comprised 7,509,532 bp distributed across 739 contigs, with an estimated completeness of 97.78%. GTDB-based classification assigned the isolate to the species *Streptomyces halstedii*. Compared to the reference genome GCA_000720535.1, the assembly showed an average nucleotide identity of 96.5065% and an alignment fraction of 0.85, supporting the identification of the isolate as *S. halstedii* S14.

Antifungal activity against *Trichoderma atroviride*

The *Streptomyces halstedii* S14 isolate inhibited the mycelial growth of *Trichoderma atroviride* CMLD 34 in solid-medium co-culture (Fig. 4). An inhibition zone was observed between the actinobacterium colony and the fungal mycelium (Fig. 4-IZ) on all three independent plates evaluated. The mean inhibition zone widths across the three plates were 0.86, 1.38, and 1.60 cm, resulting in an overall mean of 1.28 ± 0.38 cm. Individual measurements ranged from 0.54 to 1.74 cm, and the complete values are presented in Supplementary Table S1.

Based on the consistent formation of the zone of inhibition, the AD, AP, IZ, TP, and TD regions were used to evaluate the spatial distribution of molecular features during the interaction between *S. halstedii* S14 and *T. atroviride*. Axenic cultures of the actinobacterium and the fungus, along with the culture medium control, were included as analytical references and are presented in Supplementary Figure S1.

Spatial metabolomic profile of co-culture regions by UHPLC-HRMS

Metabolomic analysis by UHPLC-HRMS was performed on spatially defined regions of the co-culture between *S. halstedii* S14 and *T. atroviride*. The initial matrix contained 1,690 molecular features defined by *m/z*–retention-time pairs. Removal of 405 features detected in fewer than two biological samples left 1,285 features. Retention of the 60% with the highest interquartile range (IQR) yielded a final set of 771 molecular features for downstream statistical and multivariate analyses (Table 2).

Table 2
Summary of UHPLC-HRMS feature filtering steps used for metabolomic analyses.

Processing step	Number of features
Initial features in the processed data matrix	1,690
Features detected in fewer than two biological samples	405
Features remaining after removal of non-informative signals	1,285
Features retained after top-60% IQR filtering	771
Features used for statistical analyses	771

The overall structure of the metabolomic dataset was initially examined using sample-level PCA and PLS-DA, which showed separation patterns between the controls and the co-culture regions (Supplementary Figure S2). PLS-DA was used only as an exploratory supervised visualization. VIP scores and Random Forest variable-importance rankings are presented in Supplementary Figure S3 and were used as exploratory feature-ranking approaches.

The filtered dataset was then examined at the feature level according to the biological group in which each molecular feature showed its highest mean abundance. Features preferentially associated with AD, AP, IZ, TP, TD, AC, TC, and MC occupied different regions of the multivariate space, illustrating the heterogeneous chemical distribution across the interaction system.

Feature-level PCA was first performed using all 771 retained molecular features (Fig. 5a). A second PCA was restricted to the 515 features showing a significant overall spatial region effect in the plate-blocked analysis after BH-FDR correction (Fig. 5b). This subset represents the spatially responsive portion of the metabolomic dataset and retained a structured distribution across the co-culture gradient and biological controls. The two PCAs therefore provide complementary views of the complete retained feature set and of the subset showing statistically supported spatial variation.

The plate-blocked spatial analysis identified 515 of the 771 molecular features as showing a significant overall region effect across AD, AP, IZ, TP, and TD after BH-FDR correction. To visualize the features with the strongest statistical evidence of spatial variation, the 50 features with the lowest BH-adjusted FDR values for the overall blocked region effect were selected for hierarchical clustering (Fig. 6).

The heatmap revealed distinct abundance profiles across the five co-culture regions and provided additional descriptive context relative to AC, TC, and MC. These controls were displayed as biological references but were not included in the primary inferential test of within-plate spatial variation. The observed patterns further illustrate the heterogeneous spatial organization of the co-culture metabolome and provide context for the subsequent IZ- and TP-focused candidate prioritization.

Spatial distribution and prioritization of features along the interaction gradient

To integrate the quantitative results with the biological interpretation of the assay, each of the 771 features was assigned to the biological group in which it showed the highest mean relative abundance. This classification was descriptive and does not imply exclusive occurrence in a given region, since individual features could be detected across multiple co-culture regions and controls. Instead, it summarizes the position along the AD–AP–IZ–TP–TD gradient, or among AC, TC, and MC, in which each feature reached its highest mean abundance (Table 3).

Table 3
Distribution of UHPLC-HRMS molecular features according to the experimental group of highest mean relative abundance.

Region	Description	Features assigned by highest abundance	GNPS annotated features	Robust spatial candidates
AD	Actinobacterial Distal region	51	5	—
AP	Actinobacterial Proximal region	53	0	—
IZ	Inhibition Zone	14	1	7
TP	<i>T. atroviride</i> Proximal region	15	1	11
TD	<i>T. atroviride</i> Distal region	25	2	—
AC	Actinobacterium Control	202	0	—
TC	<i>T. atroviride</i> Control	138	1	—
MC	Medium Control	273	8	—

The highest-mean classification revealed a markedly heterogeneous distribution of molecular features across the co-culture regions and controls (Table 3). Final IZ- and TP-associated candidates were then defined using the pre-specified statistical criteria described in the Methods.

Eleven features satisfied the criteria for association with TP (Supplementary Table S3). The strongest TP contrast was observed for *m/z* 351.2514 (BH-FDR = 2.58×10^{-5} ; fold ratio = 24.85). The feature *m/z* 522.3565 also showed a strong TP association (BH-FDR = 2.11×10^{-4} ; fold ratio = 29.20) and had a putative spectral-library annotation compatible with 1-oleoyl-lysophosphatidylcholine. Additional TP-associated features were *m/z* 380.1710, 343.3325 (RT 6.90 min), 371.3640, 310.2380, 611.4297, 458.2987, 343.3325 (RT 6.97 min), 341.3169, and 198.1856. Some of these features also showed elevated abundance in TD or TC and were therefore interpreted as TP-associated rather than exclusive to TP.

Seven features satisfied the corresponding criteria for association with IZ: *m/z* 678.5083 (RT 11.87 min), 692.5239 (RT 13.72 min), 678.5069 (RT 12.10 min), 179.0792 (RT 0.87 min), 704.5241 (RT 13.02 min), 295.1294 (RT 1.11 min), and 288.1274 (RT 3.72 min) (Supplementary Table S3). Among these, *m/z* 295.1294 also showed a putative GNPS/MassBank annotation compatible with glutamylphenylalanine-like chemistry. Its IZ contrast was 0.483 log₁₀ units, corresponding to an approximately 3.04-fold increase relative to the mean of the other four co-culture regions (BH-FDR = 0.016). The remaining IZ-associated candidates did not require a specific spectral-library annotation for retention.

Although AP and TD also contained distinct sets of preferentially distributed molecular features, no additional region-specific candidate category was defined for these regions because the targeted contrasts were focused on IZ and TP. The analysis therefore reduced the region-focused set to 18 molecular features: seven associated with IZ and 11 with TP. These candidates were subsequently examined together with molecular-network position and chemical annotation to provide additional chemical context.

Molecular networking and putative annotation of selected features

In addition to univariate and multivariate statistical analyses, UHPLC-HRMS data were evaluated using molecular networking and spectral annotation inspection—in both positive (<https://gnps2.org/status?task=eeca1187c8e84c1f88099393206a528a>) and negative (<https://gnps2.org/status?task=52924dd90eba411ea2eec93624cbceba>) modes—to complement the chemical interpretation of signals detected in the different regions of the co-culture. This approach enabled the organization of features into molecular components defined by MS/MS spectral similarity, facilitating the identification of chemical families and related molecular candidates.

Molecular network analysis was integrated with the spatial distribution of signals on the plate, alongside the controls. Thus, candidates were interpreted not only based on spectral annotation but also on their relative abundance patterns along the interaction gradient. The key molecular components discussed in this section are presented in Fig. 7, along with boxplots showing the abundance of the selected ions. The main putatively annotated features selected for chemical interpretation are summarized in Table 4.

Table 4

Main putatively annotated UHPLC-HRMS/MS features selected for chemical and spatial interpretation of the interaction between *S. halstedii* S14 and *T. atroviride*.

Feature / <i>m/z</i>	RT (min)	Putative annotation/chemical class	Evidence	GNPS score	Spatial or quantitative relevance	Interpretation level
256.0608	7.79	Caboxamycin	Putative UHPLC-HRMS/MS annotation; independent genomic support from NODE_598/BGC0001444	—	Predominantly associated with the actinobacterial side of the co-culture and AC	Strongest genome–metabolome correspondence; not a robust IZ/TP candidate
522.3565	9.98	1-oleoyl-lysophosphatidylcholine	GNPS/NIST putative spectral-library annotation	0.78	Highest mean abundance in TP, with elevated values also toward TD, TC, and IZ	Robust TP-associated candidate; BH-FDR = 2.11×10^{-4} ; fold ratio = 29.20
295.1294	1.11	Glutamylphenylalanine	GNPS/MassBank putative annotation	0.74	Highest mean abundance in IZ with a broader IZ–TP distribution	Robust IZ-associated candidate; BH-FDR = 0.016; fold ratio = 3.04
269.0926	7.82	methyl 1-oxo-4-(1H-pyrrol-2-yl)-2H-isoquinoline-3-carboxylate	Molecular networking and putative chemical annotation	0.72	Higher abundance in AD, AP, and AC, with lower abundance toward the fungal side	Actinobacteria-associated exploratory candidate; not a robust IZ/TP candidate
430.3337	12.63	N-oleoyl-phenylalanine	GNPS putative annotation	0.89	Highest abundance in TD, with elevated values also in TP and TC	Fungal-side/interface-associated annotated feature; not a robust TP candidate
743.399	11.85	bafilomycin C1	GNPS spectral annotation; complementary genomic evidence from NODE_118/BGC0000028	—	Enriched in AC and AD rather than IZ or TP	Bafilomycin-family-level genome–metabolome correspondence; no specific congener confirmed
227.0817	4.87	Carboline/isoquinoline-like candidate	GNPS/molecular networking and spectral interpretation	0.85	Co-culture-associated nitrogen-containing signal	Secondary chemical candidate
344.0918	1.57	Pyridine and derivatives	Molecular class prediction	—	Nitrogen-containing molecular class detected in the co-culture dataset	Exploratory chemical class

Component 3 contained signals predominantly associated with the actinobacterial side of the co-culture. Within this component, *m/z* 256.0608, annotated as caboxamycin, showed higher abundance in regions close to the actinobacterium and

in AC, whereas m/z 269.0926, annotated as methyl 1-oxo-4-(1H-pyrrol-2-yl)-2H-isoquinoline-3-carboxylate, showed higher abundance in AD, AP, and AC. These features were retained as chemically annotated features associated with the actinobacterial side of the interaction rather than as robust IZ- or TP-associated candidates.

Component 19 contained signals distributed across the inhibition-zone/interface axis. m/z 295.1294, putatively annotated as glutamylphenylalanine, was a robust IZ-associated candidate while retaining a broader IZ–TP abundance pattern. In the same component, m/z 430.3337, putatively annotated as N-oleoyl-phenylalanine, was most abundant in TD, with elevated values also in TP and TC, and was therefore interpreted as a fungal-side/interface-associated annotated feature rather than a robust TP candidate.

In Component 23, m/z 522.3565, putatively annotated as 1-oleoyl-lysophosphatidylcholine, satisfied the robust TP-associated criteria and showed its highest mean abundance in TP. Component 37 contained three robust TP-associated features: m/z 343.3325 at RT 6.90 min, m/z 343.3325 at RT 6.97 min, and m/z 341.3169. The two signals with m/z 343.3325 were retained as distinct molecular features because they occurred at different retention times.

The remaining robust spatial candidates occurred as singleton nodes under the FBMN similarity threshold. Together with the robust candidates located within Components 19, 23, and 37, these features constitute the complete set of seven IZ- and 11 TP-associated candidates identified in the spatial analysis (Supplementary Table S3). Selected singleton candidates are highlighted in Fig. 7.

The integrated molecular network and abundance profiles therefore distinguish three complementary patterns: actinobacteria-associated annotated features such as m/z 256.0608 and 269.0926; robust spatial candidates associated with IZ or TP, including the annotated m/z 295.1294 and 522.3565; and fungal-side/interface-associated annotated features such as m/z 430.3337. These categories represent different evidence layers and do not assign the observed antifungal phenotype causally to any single molecular feature.

Genomic mining and comparative analysis of BGCs using antiSMASH and BiG-SCAPE

Combined analysis using antiSMASH and BiG-SCAPE revealed that *S. halstedii* S14 possesses an extensive biosynthetic repertoire, albeit one with heterogeneous relationships to previously characterized BGCs. AntiSMASH 8.0.4 identified 59 biosynthetic regions, predominantly featuring architectures containing NRPS and PKS components, as well as terpenes, RiPPs, and other classes of specialized metabolites. Since 57 of the 59 regions were detected at contig edges, their relationships to known clusters were interpreted as evidence of biosynthetic potential rather than confirmation of the production of specific metabolites. The complete list of regions predicted by antiSMASH and their distribution across the main biosynthetic classes are presented in Supplementary Table S2 and Supplementary Figure S4, respectively.

When compared to MIBiG 3.1, only a fraction of the repertoire showed high proximity to reference BGCs under BiG-SCAPE's most restrictive cutoff. At a cutoff of 0.3, eight of the 59 S14 BGCs established direct connections with characterized references, whereas relaxing the cutoff to 0.7 increased this figure to 43 BGCs. The variation in S14–MIBiG connectivity across the different cutoffs is presented in Supplementary Table S4. This pattern indicates a continuum of biosynthetic similarity, with some regions close to known clusters and others more distant from the space currently represented in MIBiG. The relationships of greatest interest are summarized in Table 5.

Among these relationships, NODE_8 stood out due to its hybrid NRPS/T1PKS architecture and proximity to BGCs associated with polycyclic tetramate macrolactams (PTMs). Its closest MIBiG neighbor was BGC0001043, associated with SGR PTMs (distance 0.14), while BGC0002365, associated with the 10-epi-HSAF/maltophilin family, showed a distance of 0.18. The agreement between the architecture predicted by antiSMASH and the relationships recovered by BiG-SCAPE supports the presence of a BGC related to this biosynthetic space in S14, although it does not allow for assignment to a specific product.

The primary convergence between genomic mining and metabolomic analysis was observed for caboxamycin. The PKS-like BGC NODE_598 showed a direct connection to BGC0001444-the caboxamycin reference from *Streptomyces* sp. NTK 937- a BiG-SCAPE distance of 0.03, the lowest among the highlighted S14-MiBiG relationships. Comparative analysis at the gene level revealed partial synteny between NODE_598 and BGC0001444. The three genes present in NODE_598 showed homology to *cbxA*, *cbxB*, and *cbxC*, respectively, maintaining the same order and orientation observed in the reference cluster. The CbxA-like, CbxB-like, and CbxC-like proteins exhibited 97.6%, 97.2%, and 96.6% amino acid sequence identity, respectively, with their homologs in BGC0001444 (Fig. 8). Among these genes, the *cbxB* homolog-containing a KSIII domain-was classified by antiSMASH as a core biosynthetic gene, whereas the *cbxA* and *cbxC* homologs were classified as additional biosynthetic genes. Independently, UHPLC-HRMS/MS analysis detected the feature *m/z* 256.0608, putatively annotated as caboxamycin and predominantly associated with the actinobacterium in the co-culture. A comparison of the gene organization of NODE_598 with the reference BGC is presented in Fig. 8. This concordance supports a hypothesis regarding caboxamycin biosynthesis in S14, without confirming the metabolite's structural identity or establishing a causal link to the observed antifungal activity.

A second, more cautious match was observed for the bafilomycin family. NODE_118 showed BGC0000028-associated with bafilomycin B1-as the closest MiBiG reference, with a BiG-SCAPE distance of 0.34 and an initial direct connection at the 0.4 cutoff. In parallel, the LC-MS/MS dataset contains the feature *m/z* 743.399, putatively annotated via spectral comparison as *bafilomycin* C1. Since the genomic and metabolomic evidence points to distinct congeners and the biosynthetic relationship is less close than that observed for caboxamycin, this match was interpreted only at the bafilomycin family level.

Table 5
Selected BiG-SCAPE relationships between S14 biosynthetic gene clusters and MiBiG 3.1 reference BGCs. Node labels correspond to the antiSMASH region identifiers used throughout the BiG-SCAPE analysis.

S14 BGC	antiSMASH product	Direct MiBiG reference / product(s)	BiG-SCAPE distance(s)	First direct cutoff
NODE_598	PKS	BGC0001444 (caboxamycin)	0.03	0.3
NODE_216	Ectoine	BGC0000853; BGC0002052 (ectoine)	0.05; 0.12	0.3
NODE_8	NRPS; T1PKS	BGC0001043 (SGR PTMs); BGC0002365 (10-epi-HSAF)	0.14; 0.18	0.3
NODE_17	Terpene	BGC0001181 (geosmin)	0.21	0.3
NODE_113	T2PKS	BGC0000215 (curamycin)	0.23	0.3
NODE_577	Terpene	BGC0000664 (isorenieratene)	0.25	0.3
NODE_1	NRPS	BGC0000953 (amicetin)	0.26	0.3
NODE_147	T1PKS	BGC0001348 (JBIR-100)	0.29	0.3
NODE_118	T1PKS	BGC0000028 (bafilomycin B1)	0.34	0.4

Discussion

The isolation of *S. halstedii* S14 from the larval food of *Melipona mondury* expands knowledge regarding the cultivable fraction of the microbiota present in this matrix and reveals larval food as a potential source of actinobacteria capable of producing specialized metabolites. Recent studies on *M. mondury* have characterized bacterial communities in various biological matrices—such as honey and the intestinal tracts of individuals at different developmental stages—demonstrating that microbiota composition varies according to the matrix analyzed, as well as the individuals' ontogeny and social role [6, 8]. Although these studies contributed to characterizing the microbiota associated with *M. mondury*, they did not specifically investigate the isolation of cultivable actinobacteria from larval food with the aim of prospecting for specialized metabolites. Thus, the recovery of a bioactive *Streptomyces* strain from this niche not only fills this gap but also draws a consistent parallel with studies on *Melipona scutellaris*, in which *Streptomyces* sp. and *Micromonospora* sp. strains associated with adult bees

produced bioactive natural products [14, 15]. From a broader perspective, our findings support the hypothesis that insect microbiomes harbor highly specialized biosynthetic diversity, establishing the larval food of *M. mondury* as a strategic microenvironment for the bioprospecting of new molecules involved in specialized metabolite production.

In this study, the consistent and reproducible formation of an inhibition zone averaging 1.28 ± 0.38 cm between isolate *S. halstedii* S14 and *T. atroviride* CMLD 34 in solid co-culture directly demonstrates that this actinobacterium exhibits antagonistic and antifungal activity under the tested conditions. This antagonism phenotype aligns with the antifungal potential previously described for other insect-associated *Streptomyces* lineages, as well as for the *S. halstedii* species itself—whose antifungal metabolites, characterized in reference strains such as K122, have demonstrated inhibitory action against various filamentous fungi [37]. However, the interaction dynamics established in the Petri dish go beyond the mere passive secretion of a single antimicrobial compound by the bacterium. In co-culture interactions, physicochemical confrontation often triggers mutual metabolic reprogramming, driving quantitative and qualitative changes in the metabolic profiles of both partners and inducing the accumulation of molecules that would not be detected in their respective axenic cultures [38]. This phenomenon is particularly pronounced in interactions involving the genus *Trichoderma*, where exposure to *Streptomyces* metabolites can induce a metabolic stress response and trigger the fungus's own defense mechanisms [19]. Consequently, the chemical signatures found within the exclusion zone may reflect not only diffusible bacterial substances but also chemical responses induced in the fungus under localized stress. This complexity imposes a crucial limitation on interpretation: while the assay visually demonstrates biological antagonism between the two partners, macroscopic observation of the inhibition zone does not allow the inhibitory effect to be causally attributed to any single, specific metabolite. Nevertheless, this visible phenotype played a fundamental methodological role in the study, serving as a precise spatial reference for physically segmenting the plate and guiding metabolomic sampling. This strategy enabled a detailed chromatographic investigation of the bacterial colony, the zone of exclusion, and the fungal growth, paving the way to unravel the chemical heterogeneity governing this interaction arena. In this study, the physical dissection of the pairing arena into five biologically defined regions (AD, AP, IZ, TP, and TD) showed that the co-culture plate constitutes a chemically heterogeneous environment. By isolating the inhibition zone (IZ) itself and its biological transition boundaries (AP and TP) prior to UHPLC-HRMS/MS analysis, we were able to mitigate the dilution effect inherent in conventional whole-plate extractions, allowing us to prioritize these compartments as spatially segregated chemical signatures associated with the exclusion phenotype. This approach extends the principle of earlier strategies that demonstrated the value of preserving spatial information in microbial interactions through mass spectrometry imaging, analysis of specific interaction zones, or regional agar sampling [17, 20, 21, 23].

While previous investigations of *Streptomyces*–fungus interactions have visualized bacterial metabolites within inhibition zones and identified numerous features altered relative to monocultures by LC-MS/MS [15,19], the present design preserves the physical structure of the active confrontation by sampling five linked regions from each independent plate. This distinction is particularly important for *T. atroviride*, which itself shows regional heterogeneity in metabolite production and distribution on solid medium [22]. The plate-blocked analysis confirmed extensive spatial heterogeneity, with 515 of 771 features showing a significant overall region effect after BH-FDR correction. Targeted contrasts further retained seven robust IZ-associated and 11 robust TP-associated features. Among the robust candidates, *m/z* 295.1294 and *m/z* 522.3565 provide useful chemical anchors because each combines reproducible spatial association with an independent putative spectral annotation. Interestingly, glutamylphenylalanine has recently been identified among peptide-related biomarkers negatively associated with the development of the phytopathogenic fungi *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum* in antifungal low-molecular-weight biomolecule mixtures [39]. Although this association did not establish direct antifungal activity of the isolated compound, it provides an independent precedent linking this metabolite to chemically complex antifungal systems. Similarly, lysophosphatidylcholines have been implicated in fungal morphogenesis: a lyso-PC preparation containing 1-oleoyl-lysophosphatidylcholine among its major constituents suppressed the yeast-to-hypha transition in several *Candida* species through a MAP kinase-associated mechanism, without directly inhibiting fungal growth [40]. These observations provide biological context for the spatial enrichment of the two annotated candidates observed here, while not demonstrating that either compound is responsible for the inhibition of *T. atroviride*. Several other robust candidates remain chemically unresolved. Their lack of a spectral-library match prioritizes them for structural characterization but does not, by itself,

demonstrate chemical novelty. Feature-Based Molecular Networking, SIRIUS, and CANOPUS therefore provide complementary organizational and predictive information rather than structural confirmation [31, 32, 41, 42]. Most importantly, enrichment in IZ or TP establishes a reproducible spatial association with the antagonistic phenotype, not direct antifungal causality; functional assignment will require subsequent isolation, bioactivity-guided testing, and/or targeted genetic validation [23].

Genomic analysis of *S. halstedii* S14 revealed 59 biosynthetic regions predicted by antiSMASH, including BGCs related to NRPS, PKS, RiPPs, terpenes, and other classes of specialized metabolites. This repertoire is consistent with the well-known biosynthetic capacity of the *Streptomyces* genus and reinforces the potential of *Streptomyces halstedii* S14 as a source of natural products. However, BiG-SCAPE comparison with experimentally characterized BGCs in MIBiG showed that only a fraction of the *S. halstedii* S14 regions exhibit high similarity to known clusters, while others occupy more distant positions in the biosynthetic similarity space; this suggests a repertoire combining regions related to known pathways with others less represented among currently characterized BGCs [34, 35, 43]. Nevertheless, 57 of the 59 regions extend to the edges of the contigs and may be incompletely represented in the draft assembly, limiting the detailed reconstruction of their biosynthetic architectures. Thus, the detected BGCs should be interpreted as evidence of biosynthetic potential rather than a demonstration of the production of the respective metabolites under the experimental conditions evaluated [34, 43].

The integration of metabolomic and genomic data revealed varying levels of evidence for caboxamycin and bafilomycins. The feature with m/z 256.0608, putatively annotated as caboxamycin, showed the most consistent convergence with genome mining results, as NODE_598 displayed high similarity to the reference caboxamycin BGC, with a BiG-SCAPE distance of 0.03. This concordance between an independent metabolomic annotation and a BGC strongly related to an experimentally characterized biosynthetic pathway supports a convergent caboxamycin-related biosynthetic hypothesis for *S. halstedii* S14 [44, 45]. However, this convergence neither confirms the molecule's structural identity nor establishes a causal link to antifungal activity, particularly given that existing studies on caboxamycin primarily describe antibacterial activities and offer only limited evidence of antifungal effects. In contrast, bafilomycin C1 has a functional precedent more directly linked to the genus *Trichoderma*; in another system, the molecule strongly inhibited *T. harzianum* growth and induced a pronounced metabolic response in the fungus [19]. Furthermore, bafilomycins B1 and C1 have previously been structurally characterized in another *S. halstedii* strain [37]. However, in *S. halstedii* S14, NODE_118 showed a more distant genomic relationship to the reference bafilomycin B1 BGC, whereas the metabolomic signal was annotated as bafilomycin C1. Therefore, this convergence should be interpreted as indicating membership in the bafilomycin family rather than specific identification of the congener.

In addition to the hypotheses regarding caboxamycin and bafilomycins, NODE_8 showed similarity to BGCs associated with polycyclic tetramate macrolactams (PTMs), including 10-epi-HSAF, thereby expanding the repertoire of biosynthetic pathways potentially encoded by *S. halstedii*. Hybrid PKS/NRPS BGCs related to this family have previously been experimentally linked to PTM production in other *Streptomyces* strains, demonstrating the utility of comparative BGC analysis in guiding the investigation of natural product families [46]. In the case of *S. halstedii* S14, however, the lack of an independent metabolomic match—comparable to that observed for caboxamycin—means that NODE_8 remains primarily a genome-based hypothesis. Taken together, these results establish a phenotype-driven prioritization strategy in which antifungal activity spatially defines regions of interest, metabolomics reveals the chemical heterogeneity of these regions, and genome mining provides an independent layer for assessing the biosynthetic plausibility of candidates. Previous approaches have shown that progressing from spatial associations to robust functional evidence can be achieved through bioactivity-guided fractionation and analysis of isolated compounds, while the link between a BGC and its product can subsequently be evaluated using specific genetic strategies [23, 45]. Thus, the present study not only demonstrates the antagonism of *S. halstedii* S14 against *T. atroviride* but also progressively narrows the pool of chemical candidates associated with the interaction by combining the inhibition phenotype, the spatial resolution of the metabolome, and the isolate's biosynthetic potential.

Conclusion

Streptomyces halstedii S14, isolated from *Melipona mondury* larval food, reproducibly inhibited *Trichoderma atroviride* under the tested co-culture conditions. Spatial metabolomics showed that this interaction is chemically heterogeneous: 515 of 771 molecular features varied significantly across the co-culture gradient after accounting for plate identity, and pre-specified contrasts retained seven robust IZ-associated and 11 robust TP-associated candidates. Putatively annotated m/z 295.1294 (glutamylphenylalanine) and m/z 522.3565 (1-oleoyl-lysophosphatidylcholine) provided chemical anchors for IZ and TP, respectively, while genome mining revealed 59 BGC regions and supported a convergent caboxamycin-related hypothesis involving NODE_598. These results establish a phenotype-guided framework for integrating spatial metabolomics, molecular networking, and genome mining, while emphasizing that spatial association and BGC similarity require subsequent chemical and functional validation.

Declarations

Competing interests

The authors declare no competing interests.

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Author Contribution

YRD: Conceptualization, Methodology, Investigation, Formal analysis, Data acquisition, Data curation, Visualization, Writing – original draft, Writing – review & editing. LTDC: Conceptualization, Methodology, Investigation, Supervision, Writing – review & editing. KDLL: Investigation, Formal analysis, Writing – review & editing. ACC: Data acquisition, Methodology, Writing – review & editing. NGS, VGV, JKCS, and FKK: Investigation, Methodology, Data acquisition. LSM: Data acquisition (LC–MS), Formal analysis, Data curation, Writing – review & editing. TMF: Conceptualization, Investigation, Resources, Project administration, Writing – review & editing. SPV: Conceptualization, Supervision, Resources, Project administration, Writing – review & editing. MMM: Conceptualization, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data acquisition, Data curation, Visualization, Writing – original draft, Writing – review & editing.

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Data Availability

The draft genome assembly of *Streptomyces halstedii* S14 generated in this study has been deposited in DDBJ/ENA/GenBank under accession number JCDVFI000000000. The raw DNA sequencing reads used for genome assembly have been deposited

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47. Use of artificial intelligence.
48. Generative artificial intelligence. tools were used during manuscript preparation to assist text organization and editorial revision. All AI-assisted content was critically reviewed, and verified by the authors, who take full responsibility for the accuracy, integrity, and scientific content of the manuscript.

Figures

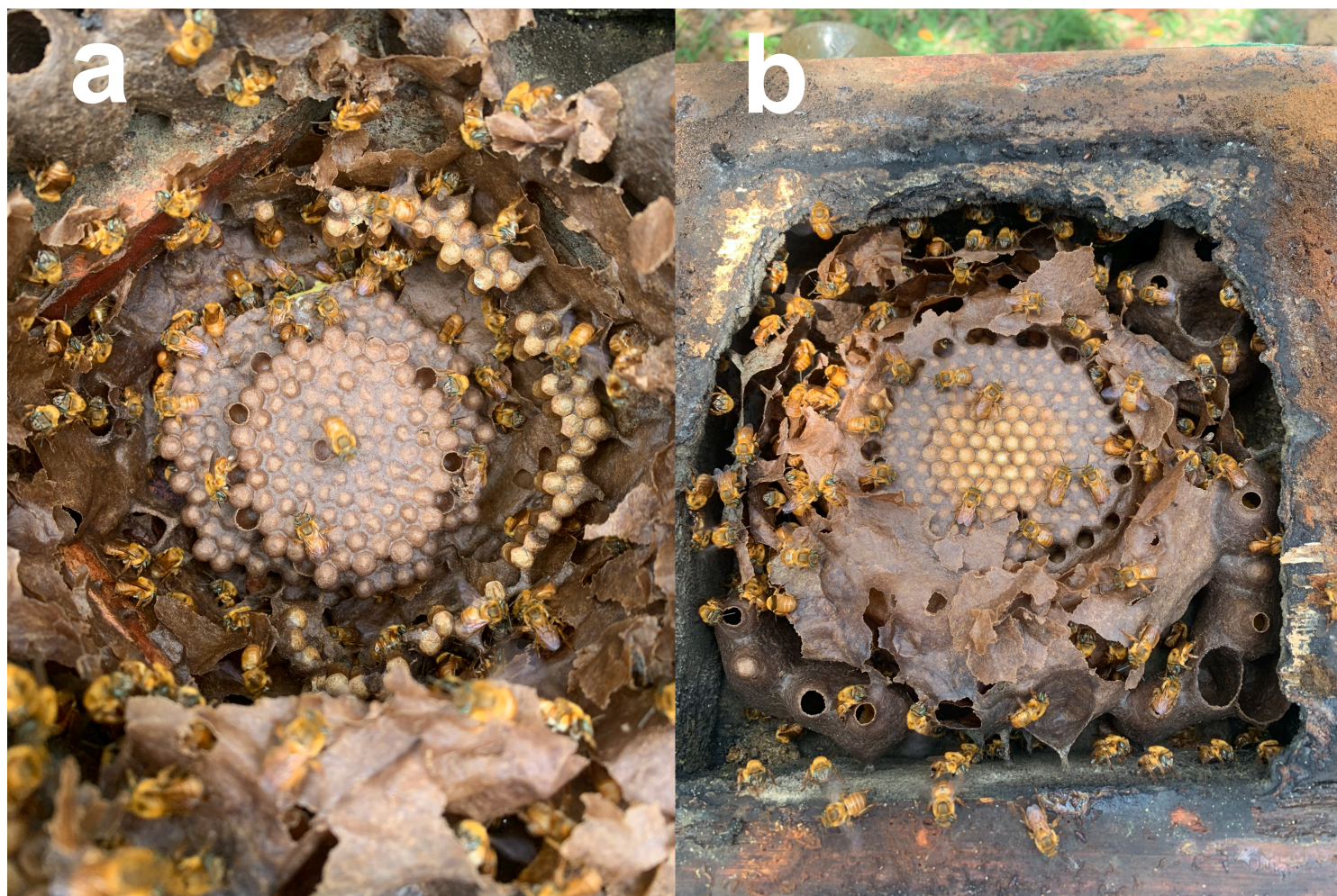


Figure 1

Melipona mondury colony used for larval food sampling. (a) Close-up view of the brood area showing brood cells and workers inside the nest. (b) Overview of the opened colony, showing the brood disc surrounded by nest structures and workers.

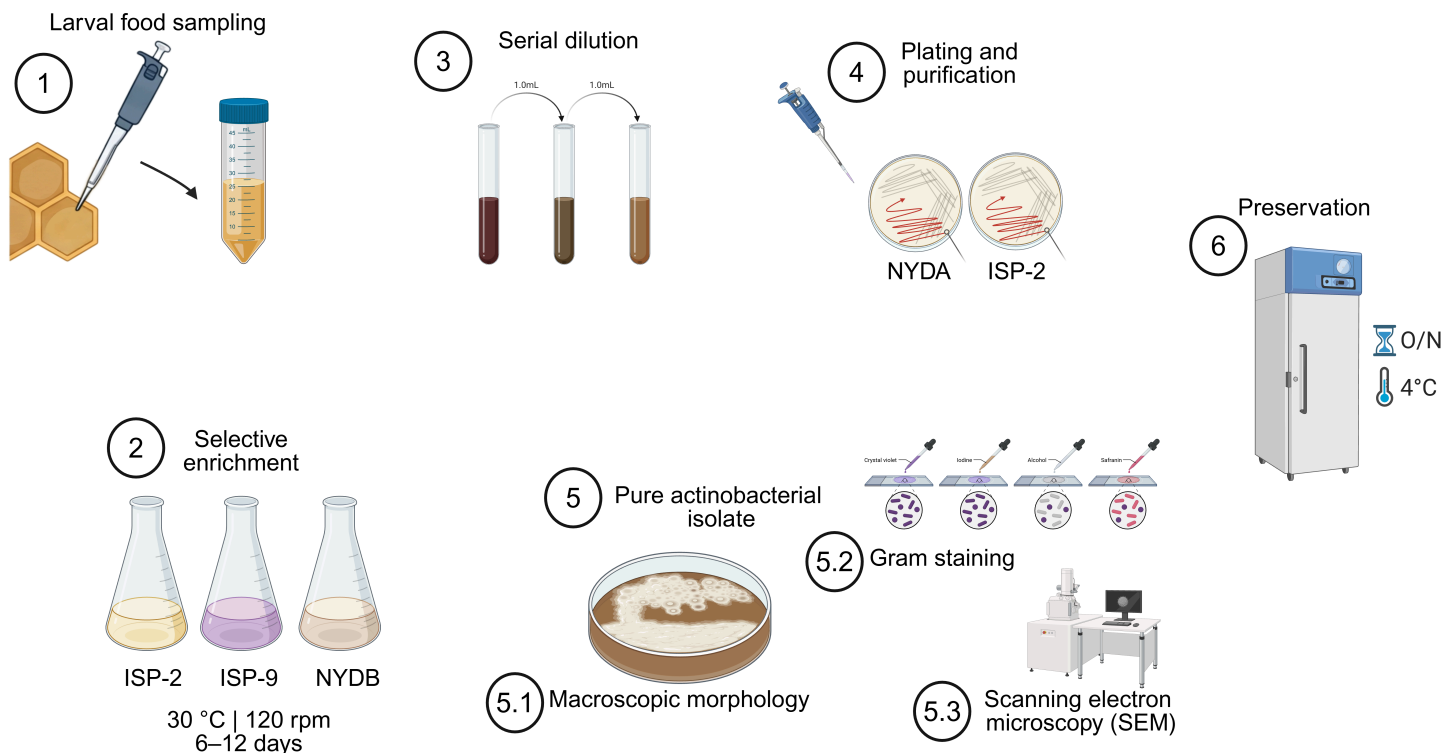


Figure 2

Experimental protocol for the enrichment, isolation, characterization, and preservation of actinobacteria from stingless bee larval food. The workflow includes: (1) collection of the larval food sample; (2) selective enrichment in ISP-2, ISP-9, and NYDB media at 30 °C and 120 rpm for 6–12 days; (3) serial dilution of the enriched cultures; (4) plating on NYDA and ISP-2 solid media for colony isolation and purification; (5) selection of a pure actinobacterial isolate followed by characterization based on (5.1) macroscopic colony morphology, (5.2) Gram staining, and (5.3) scanning electron microscopy (SEM); and (6) preservation of the isolate at 4 °C.

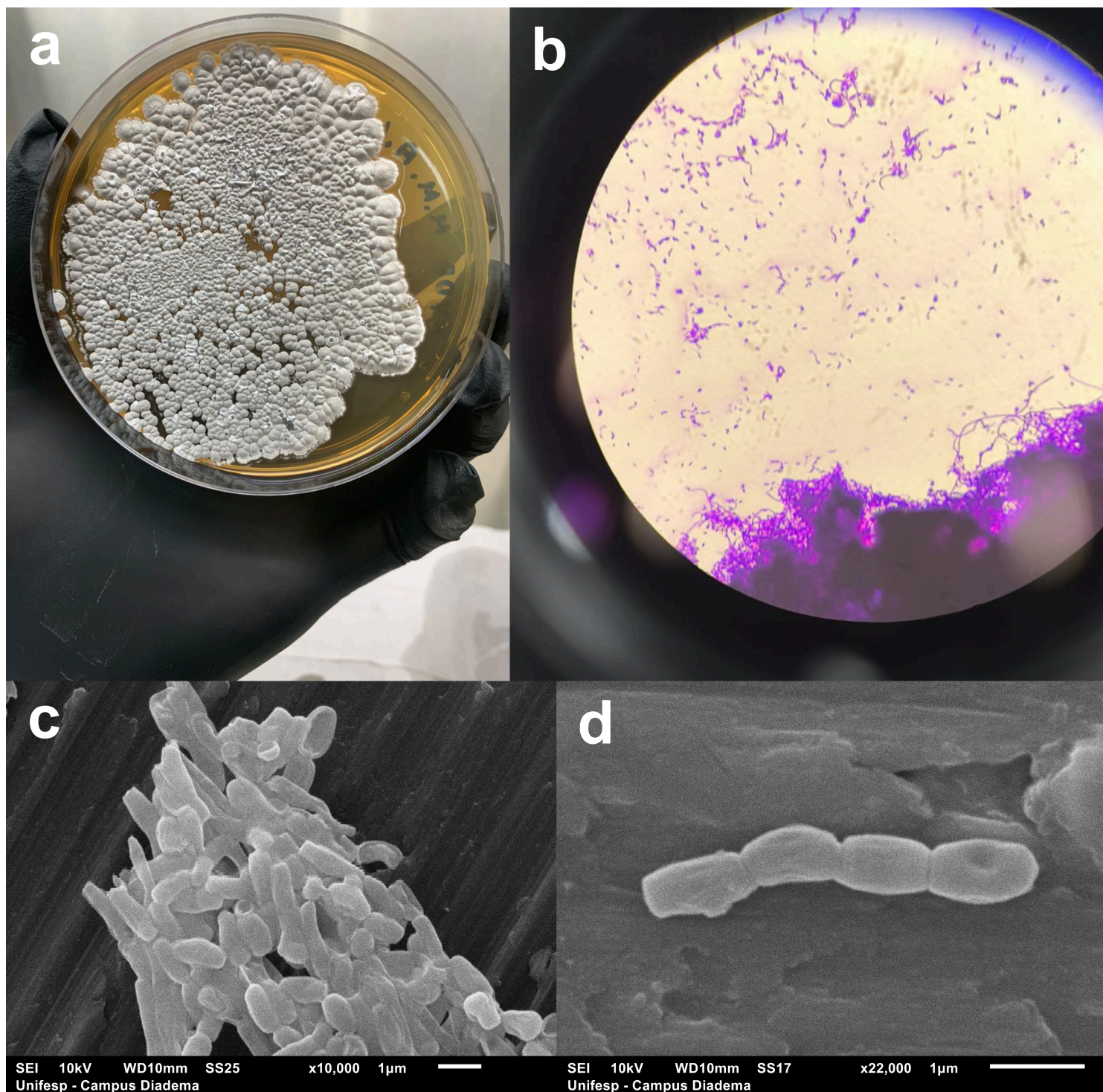


Figure 3

(a) The obverse of the plate of actinobacteria isolated from the larval food of *M. mondury*. (b) Gram stain and identification of the filaments. (c) Actinobacteria at x10000 magnification. (d) Actinobacteria at 22000 magnification.

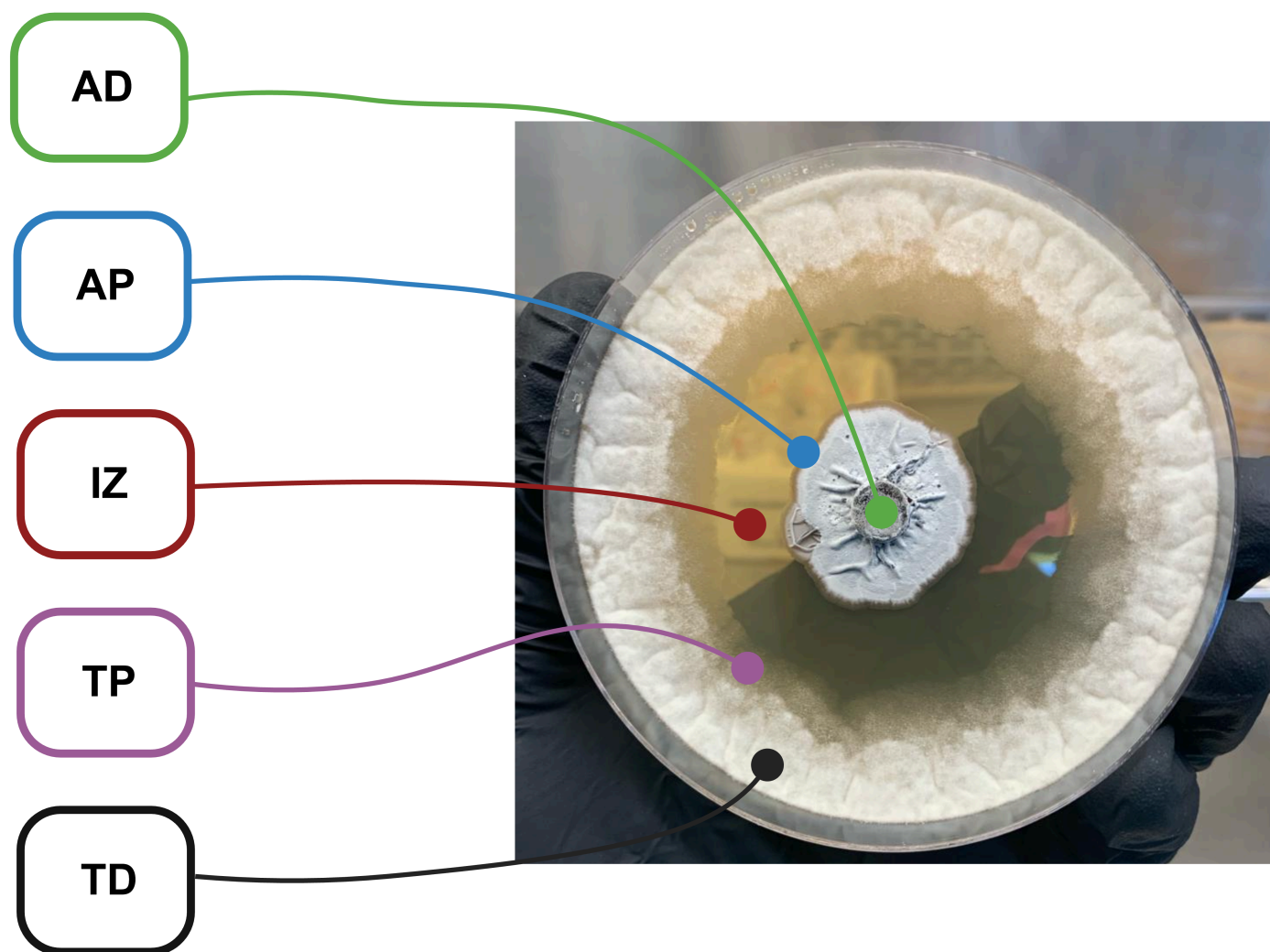


Figure 4

Spatial sampling strategy used for UHPLC-HRMS analysis of the actinobacterium-fungus co-culture assay. The image shows the 14-day co-culture plate. Colored points indicate the sampled regions along the interaction gradient, including the actinobacteria-side region, the inhibition halo, the halo-fungus interface and the fungal-side region.

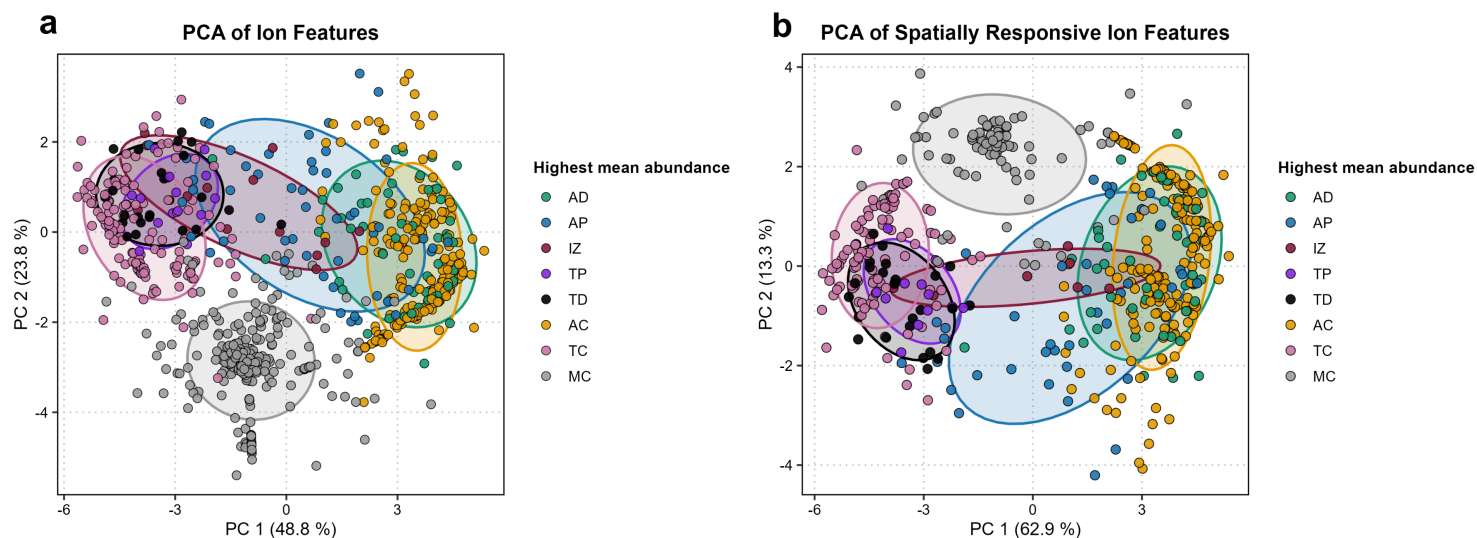


Figure 5

Feature-level principal component analysis of UHPLC-HRMS molecular features from the co-culture regions and controls. (a) PCA of all 771 retained ion features, with each feature colored according to the biological group in which it showed the highest mean abundance. (b) PCA of the 515 spatially responsive ion features showing a significant overall region effect after accounting for plate identity (BH-FDR < 0.05). Feature colors indicate the group of highest mean abundance across AD, AP, IZ, TP, TD, AC, TC, and MC. The analyses provide exploratory visualization of the chemical organization of the complete and spatially responsive feature sets.

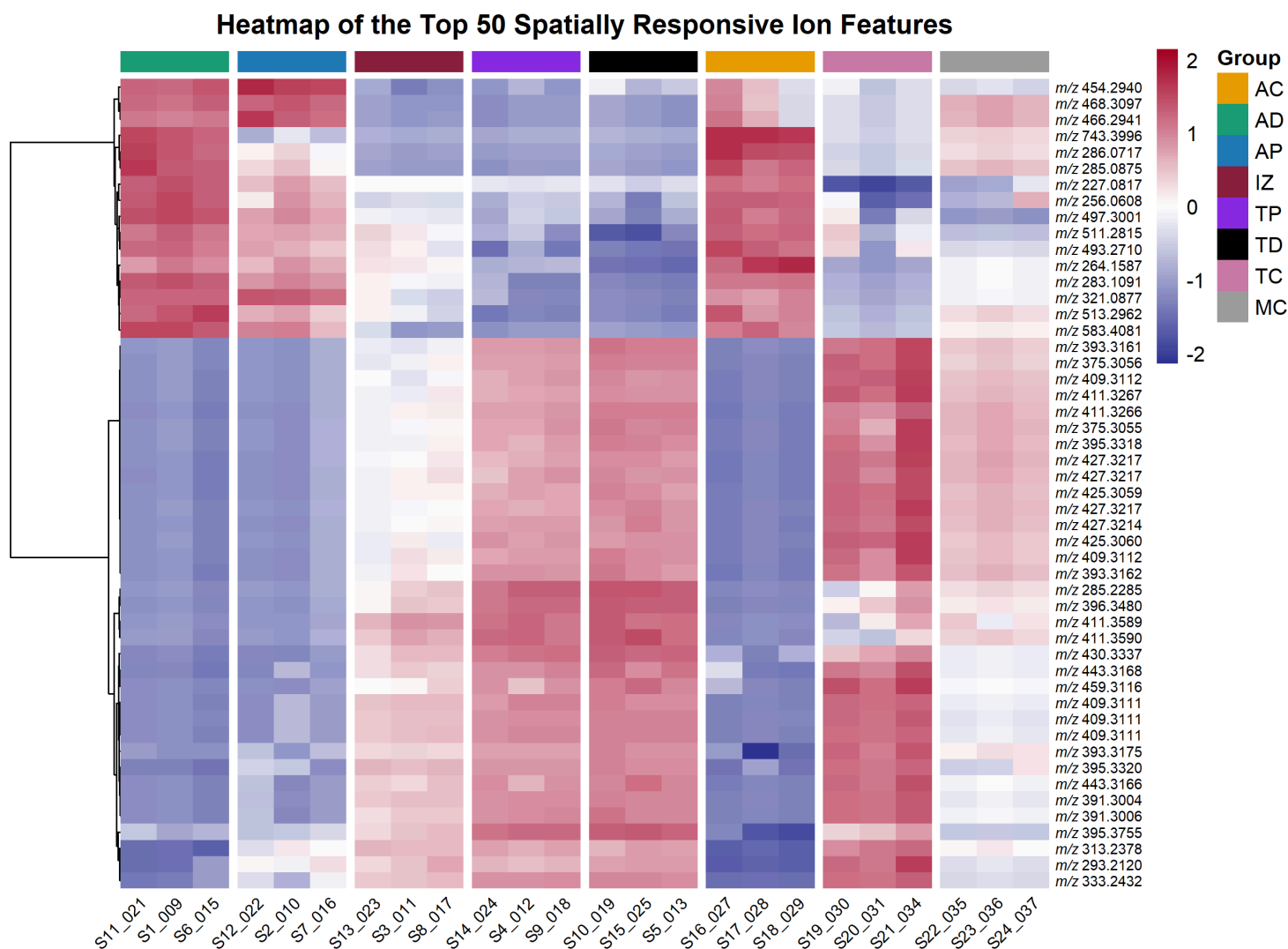


Figure 6

Heatmap of the Top 50 Spatially Responsive Ion Features. The 50 molecular features with the lowest BH-adjusted FDR values for the plate-blocked spatial region effect are shown. Rows represent molecular features and columns represent individual experimental samples. Molecular-feature labels are presented as *m/z* values. The upper annotation bar indicates the experimental group of each sample. The heatmap was generated from the processed UHPLC-HRMS matrix using row scaling, Euclidean distance, and Ward hierarchical clustering. AC, TC, and MC are shown as biological references but were not included in the primary blocked test of spatial variation within the co-culture plates.

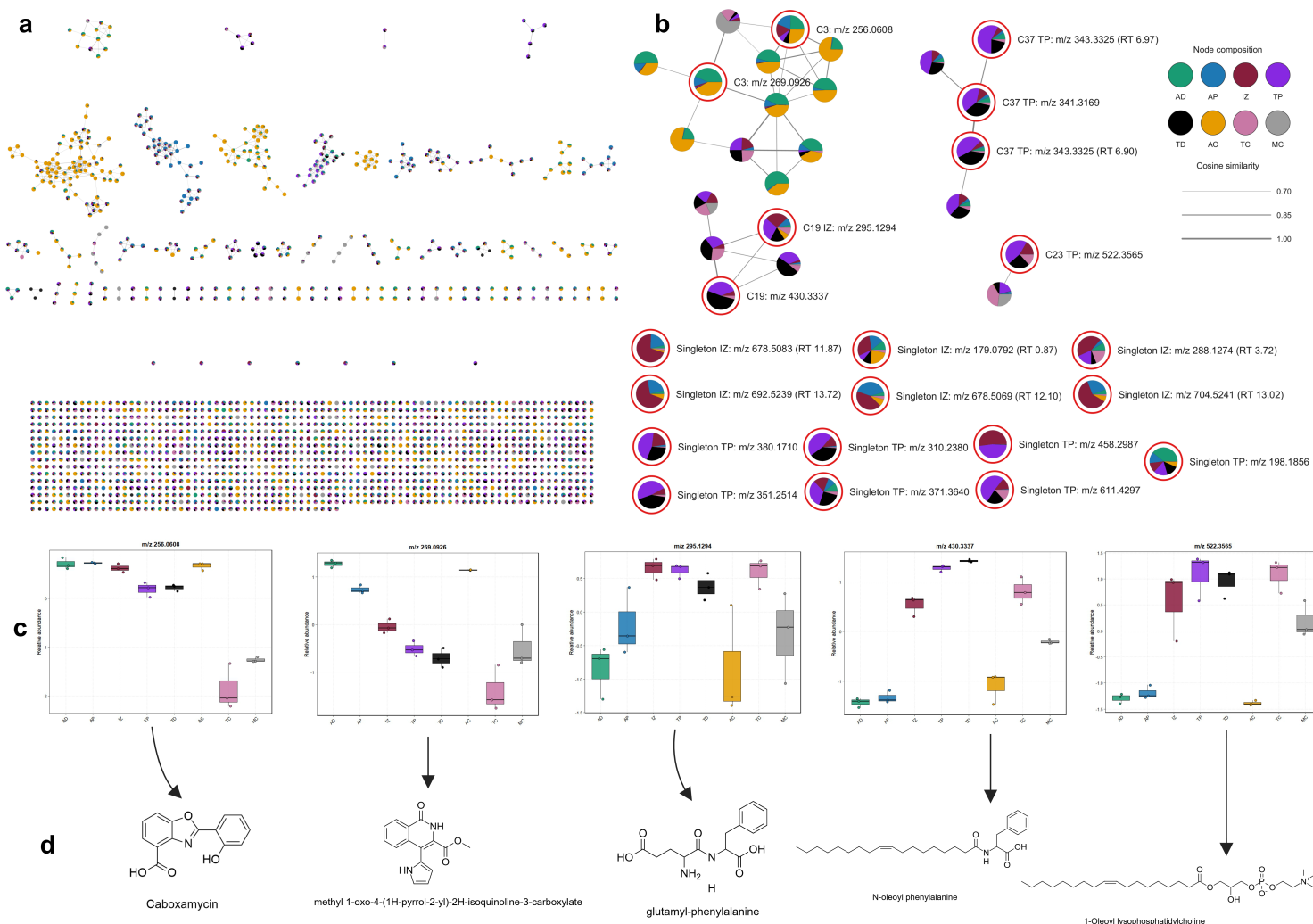


Figure 7

Feature-based molecular networking, spatial distribution, and putative annotation of selected UHPLC-HRMS features detected during the co-culture of *Streptomyces halstedii* S14 and *Trichoderma atroviride*. **(a)** Global Feature-Based Molecular Network (FBMN) representing molecular features detected across the five spatial regions of the co-culture plate: AD (Actinobacterial Distal region), AP (Actinobacterial Proximal region), IZ (Inhibition Zone), TP (*T. atroviride* Proximal region), and TD (*T. atroviride* Distal region), together with the corresponding controls AC (Actinobacterium Control), TC (*T. atroviride* Control), and MC (Medium Control). Each node represents an MS/MS molecular feature, and node pie charts indicate the relative contribution of each experimental group. Edges represent MS/MS spectral similarity between connected features, with edge thickness proportional to the cosine similarity score. Highlighted nodes identify features discussed in the text and indicate their corresponding molecular component or singleton status. **(b)** Enlarged subnetworks and selected singleton candidates. Subnetworks show Component 3, containing m/z 256.0608 and 269.0926; Component 19, containing m/z 295.1294 and 430.3337; Component 23, containing m/z 522.3565; and Component 37, containing the TP-associated features m/z 343.3325 (RT 6.90 min), m/z 343.3325 (RT 6.97 min), and m/z 341.3169. Selected IZ- and TP-associated features that did not form connections with other molecular features under the FBMN filtering criteria are displayed separately as singletons. In total, seven IZ-associated and 11 TP-associated candidate features were retained and are listed in Supplementary Table S3. **(c)** Boxplots showing the normalized relative abundance across experimental groups of five selected features for which putative chemical annotations were assigned (m/z 256.0608, 269.0926, 295.1294, 430.3337, and 522.3565). **(d)** Corresponding putative chemical structures of the annotated features shown in panel (c).

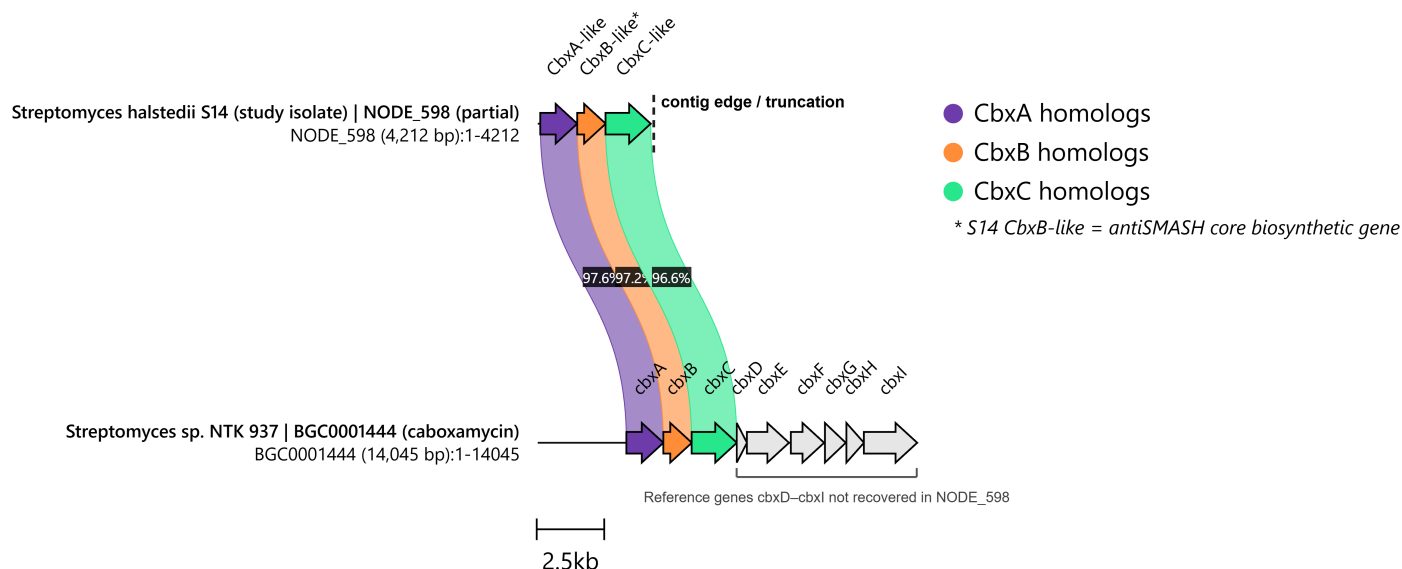


Figure 8

Clinker comparison between the S14 NODE_598 region and the caboxamycin biosynthetic gene cluster BGC0001444 from *Streptomyces* sp. NTK 937. Matching colours indicate homologous gene groups, and connecting links show pairwise amino acid sequence identity. The asterisk marks the S14 gene classified by antiSMASH as a core biosynthetic gene. NODE_598 terminates at the contig boundary immediately downstream of the CbxC-like gene.

Supplementary Files

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