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Extracts of *Talaromyces purpureogenus* Strains from *Apis mellifera* Bee Bread Inhibit the Growth of *Paenibacillus* spp. In Vitro

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Abstract: Honey bees coexist with fungi that colonize hive surfaces and pollen. Some of these fungi are opportunistic pathogens, but many are beneficial species that produce antimicrobial compounds for pollen conservation and the regulation of pathogen populations. In this study, we tested the in vitro antimicrobial activity of *Talaromyces purpureogenus* strains isolated from bee bread against *Paenibacillus alvei* (associated with European foulbrood disease) and three *Aspergillus* species that cause stonebrood disease. We found that methanol extracts of *T. purpureogenus* strains B18 and B195 inhibited the growth of *P. alvei* at a concentration of 0.39 mg/mL. Bioactivity-guided dereplication revealed that the activity of the crude extracts correlated with the presence of diketopiperazines, a siderophore, and three unknown compounds. We propose that non-pathogenic fungi such as *Talaromyces* spp. and their metabolites in bee bread could be an important requirement to prevent disease. Agricultural practices involving the use of fungicides can disrupt the fungal community and thus negatively affect the health of bee colonies.

Keywords: *Apis mellifera*; honey bee; fungi; bee bread; *Talaromyces*; antimicrobial activity; biocontrol; natural product

1. Introduction

The reproduction of most flowering plants and ~30% of all crops is dependent on pollination [1,2]. The estimated value of pollinators to the agricultural economy was USD 164 billion in 2009 [3] and now stands somewhere between USD 235 and USD 577 billion annually [4,5]. This value increases in line with crop production, and so does our dependence on pollinators for the maintenance of food security [2,4,6,7]. However, there has been a recent, dramatic decline in natural pollinator abundance and diversity [8,9]. Accordingly, managed pollinators such as honey bees (*Apis mellifera*, Linné 1758) (Hymenoptera: Apidae) play a key role in agroecosystems by augmenting wild pollinators and thus ensuring crop quality and yield stability. However, an array of biotic and abiotic stressors associated with agricultural intensification, parasites, and pathogens negatively affect the health of managed bees [10–12]. The deterioration in bee health results in the loss of colonies, particularly in the northern hemisphere [13–15]. Optimizing the health of individual bees is necessary to improve colony fitness, correlating directly with their benefits to humans, including pollination services as well as the production of honey.

Microorganisms that colonize animals (topically and internally) and their environments often fulfill essential functions [16]. The microbial communities associated with honey bees influence host metabolism, health, and stress tolerance [17–22]. Research has focused on the conserved core gut microbiome, which is dominated by nine clusters of bacterial species that develop 4–6 days after enclosure [23] and are present in almost all individuals regardless of geographic location or season [24–27]. In contrast, less attention has been paid to the composition and function of in-hive microbial communities that colonize the bee cuticle, hive surfaces, and food stores [28]. These consist of bacteria, yeast, and several genera of filamentous fungi that form the core mycobiome of pollen and bee bread, including *Aspergillus*, *Cladosporium*, *Botrytis*, *Penicillium*, *Alternaria*, *Mucor*, and *Rhizopus* [29]. Fungi are important for bee nutrition because they participate in pollen fermentation and its transformation into bee bread [30]. They also break down toxins [31] and confer resistance to fungal pathogens such as *Ascosphaera apis* (Maassen ex Claussen, L.S. Olive, and Spiltoir, 1955), the cause of chalkbrood disease, by producing antifungal compounds [32–34]. Some strains also reduce the viral load in the colony [35,36] and act as probiotics to promote the growth of symbiotic bacteria [37].

The genus *Talaromyces* (Benjamin, 1955) (Eurotiales: Trichomaceae) is a source of natural compounds with applications in medicine and the food industry (as producers of *Monascus*-like pigments) [38–41]. These fungi are known to antagonize plant pathogens and to be associated with insects [42]. The interactions within such complex ecological relationships are mediated by specialized microbial metabolites, and *Talaromyces* species produce diverse natural products that can be used for biological control [38,39].

Only a few studies have reported the presence of *Talaromyces* spp. associated with bees [34,36,42,43]. Here, we characterized *Talaromyces* strains recently isolated from honey bee bread [36] using a combination of colony morphology, DNA barcoding, and phylogenetic analysis. Bee-related *Talaromyces* strains have already been shown to inhibit human pathogens (*T. versatilis*) [43], mammalian and bee viruses (*T. purpureogenus*) [36], and fungal bee pathogens (*T. scorteus* and *T. dendriticus*) [34]. We tested the activity of organic crude extracts against the *Aspergillus* species (Eurotiales: Aspergillaceae) that cause stonebrood disease [44] and the bacterial opportunistic pathogen *Paenibacillus alvei* (Bacillales: Paenibacillaceae). This spore-forming bacterium is often isolated from colonies affected by European foulbrood disease (EFB), which is caused primarily by *Melissococcus plutonius* [45,46]. Both *Aspergillus* and *P. alvei* are also present in healthy colonies [47,48], but stonebrood disease is prevented by hygienic beekeeping practices and EFB has, until recently, been prevented by the use of oxytetracycline [49]. This is becoming less effective due to the ban on apiary antibiotics in many countries and the emergence of antibiotic-resistant pathogens, leading to heavy EFB infestations that often require the destruction of the colony to avoid disease spread [50]. The same measures might be needed for colonies severely infected with stonebrood disease, due to the health risk for beekeepers and consumers [51]. Defining the function of bee bread fungi such as *Talaromyces* strains and their metabolites in the control of these diseases would facilitate the development of new and more effective honey bee health protection strategies.

2. Materials and Methods

2.1. Bee Bread Collection and Fungal Cultivation

The fungal strains were isolated from *A. mellifera* bee bread collected in Kamenny Malikov, Czech Republic (KM Zirovnice; 49°12′51.533″ N, 15°7′5.129″ E) in March/April 2019 as previously described [36]. Briefly, hive frames containing stored pollen of various ages were cut out, and bee bread from 10 randomly selected cells was suspended in 0.9% NaCl containing 1% Tween-80 (Sigma-Aldrich, St. Louis, MO, USA). The suspension was then inoculated onto potato dextrose agar (PDA, VWR International, Radnor, PA, USA) at 25 °C. Fungi were subcultured several times until axenic isolates were obtained and identified. The *Talaromyces* strains were deposited in the Fraunhofer strain collection (EXT111748–EXT111754); other fungal genera were excluded from this study.

To obtain spore suspensions, pure colonies were washed with 2 mL of 0.2% agar (Carl Roth, Karlsruhe, Germany) containing 0.05% Tween-80 [52], filtered through three layers of miracloth and stored at 4 °C. Three 1-μL drops were inoculated onto solid media: Czapek yeast autolysate (CYA) medium, malt extract agar (MEA), yeast extract supplemented (YES) medium, creatine sucrose agar (CREA) (according to [52]), and Sabouraud's dextrose agar (SDA)—30 g/L Sabouraud dextrose broth (Merck Millipore, Burlington, MA, USA) and 15 g/L agar (Carl Roth, Karlsruhe, Germany). After 7 or 10 days, colonies were transferred to liquid malt peptone (MP) medium—30 g/L malt extract (Thermo Fisher Scientific, Dreieich, Germany), 5 g/L mycological peptone from meat (Carl Roth, Karlsruhe, Germany) as previously described [36]. The workflow is summarized in Figure 1. Images of fungal colonies were captured using an EOS 450D camera (Canon, Tokyo, Japan) and edited in GIMP ver. 2.10.34 [53].
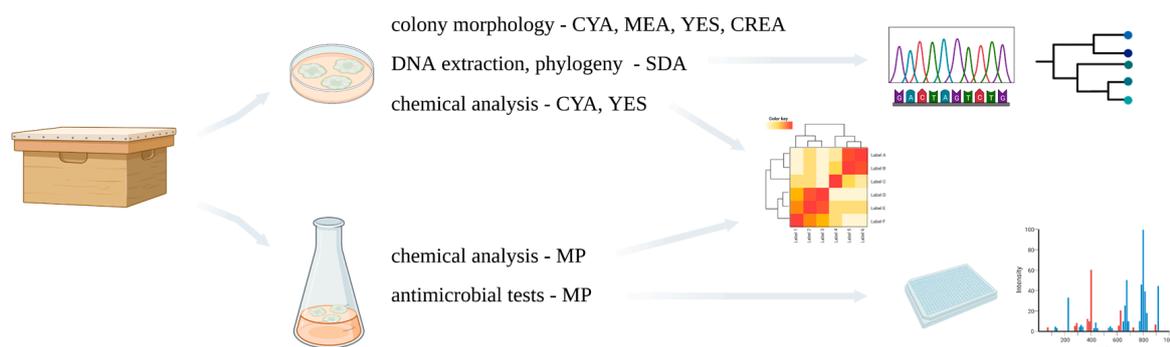
Figure 1. Workflow showing the use of cultivation media prior to colony morphology assessment, molecular barcoding, and phylogenetic analysis (top right), chemical analysis (cosine similarity, center), antimicrobial assays and bioactivity-guided dereplication (bottom right). Figure created with BioRender.com.

2.2. Extract Preparation for Chemical Analysis and Antimicrobial Tests

Extracts were prepared from cultures grown on solid CYA and YES medium for 10 days [52]. Briefly, three plugs were cut from the center of single colonies and transferred to 2 mL screw cap vials. We added 0.8 mL ethyl acetate containing 1% formic acid and incubated in an ultrasonic bath (35 kHz) for 30 min [52]. The liquid phase was transferred to a fresh vial and evaporated under nitrogen, and the residues were redissolved in 40 μL methanol and stored at 4 °C. The fungal strains were also incubated in liquid MP medium and were lyophilized and extracted in methanol as previously described [36]. The crude extracts were redissolved in methanol to a concentration of 100 mg/mL and were stored at –20 °C. Before UHPLC-HR-MS analysis, all samples were centrifuged ($8000 \times g$, 1 min, room temperature), and 30–40 μL was transferred to 2-mL HPLC vials with glass micro-inserts.

2.3. Molecular Barcoding and Phylogenetics

We collected mycelia from 7-day-old colonies grown on SDA using a sterile scalpel and homogenized the tissue under liquid nitrogen with a mortar and pestle. The ground tissue (50–80 mg) was mixed with 490 μL 2 × CTAB buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris of pH 8) preheated to 60 °C. We then added 10 μL of proteinase K (50 mg/mL) and incubated at 60 °C for 1 h on a shaking platform. The lysate was extracted by adding 500 μL 24:1 chloroform:isoamylalcohol, and the aqueous layer (400 μL) containing was transferred to a new 1.5 mL Eppendorf tube. The DNA was precipitated by adding 270 μL isopropanol. After centrifugation ($14,000 \times g$, 10 min, room temperature), the pellet was washed with 1 mL ice-cold 70% ethanol and dried in a vacuum concentrator for 10 min. The DNA was dissolved in 20 μL of TE buffer with 2 μL of RNase A (10 mg/mL) for 3 h at 37 °C. Samples were then stored at –20 °C. The concentration and quality of DNA was determined using a NanoDrop UV spectrophotometer and by 1% agarose gel electrophoresis.

To create the barcodes, we amplified four DNA regions: β-tubulin (BenA), calmodulin (CaM), the internal transcribed spacer (ITS), and RNA polymerase II second largest subunit (RBPII), which are often used for the molecular identification and phylogenetic analysis of fungal isolates [52,54] (Table 1). Each 20 μL PCR mix contained 10 μL Taq 2× Master Mix, 0.25 μM of each primer, 100–200 ng of template DNA, and PCR-grade water to top up. The reaction conditions are summarized in Table 2. PCR products were purified using ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions but using modified thermal conditions (37 °C for 35 min, 85 °C for 15 min, and 10 °C infinite hold). The cleaned PCR products were sent with forward and reverse primers for bidirectional sequencing (Eurofins, Val Fleuri, Luxembourg).

Table 1. Primer pairs used for the amplification of the four gene regions.

Target	Name	Orientation	Sequence (5′→3′)	Reference
BenA	Bt2a	For	GGTAACCAAATCGGTGCTGCTTTC	[55]
	Bt2b	Rev	ACCCTCAGTGTAGTGACCCTTGGC	
	Bt1a	For	TTCCCCCGTCTCCACTTCTTCATG	
	Bt1b	Rev	GACGAGATCGTTCATGTTGAACTC	
ITS	ITS1F	For	CTTGGTCATTTAGAGGAAGTAA	[56]
	ITS4	Rev	TCCTCCGCTTATTGATATGC	[57]
CaM	CF1	For	GCCGACTCTTTGACYGARGAR	[58]
	CF4	Rev	TTTYTGCATCATRAGYTGGAC	
RBPII	5F2	For	GGGGWGAYCAGAAGAAGGC	[59]
	7CR	Rev	CCCATRGCTTGYTTRCCCAT	

Table 2. PCR thermal profiles for the used primers.

	ITS and CaM	Ben1A and Ben2A	RBPII
Initial denaturation	95 °C/5 min	94 °C/5 min	95 °C/5 min
Denaturation	94 °C/45 s	94 °C/45 s	94 °C/45 s
Annealing	55 °C/45 s	50 °C/45 s	52 °C/45 s
Extension	72 °C/1 min	72 °C/1 min	72 °C/1 min
Final extension	72 °C/7 min	72 °C/7 min	72 °C/7 min

Sequence reads were analyzed and assembled using Geneious v10.2.6, and the assembly consensus sequences of the barcodes were compared to the sequences in the International Sequence Database (INSD) using the GenBank basic local alignment search tool (BLAST) [60]. The search was adjusted for comparison with the type and reference material and the RefSeq Targeted Loci database (available only for ITS sequences). For species assignment based on ITS sequences, we used the recommended identity threshold of ≥97% [61]. Due to the lack of available Ben1A sequences from the type material in the database (December 2022), phylogenetic analysis was carried using the Ben2A, CaM, ITS, and RBPII sequences (Supplementary Table S1).

Sequence alignments were prepared using MUSCLE in Geneious v10.2.6, and single gene trees (Supplementary Figures S1–S5) were constructed using IQtree with default settings [62,63]. From the concatenated alignment, uninformative sites were removed using Gblocks 0.91b [64,65]. Next, we used partition finder in Cipres Science Gateway v3.3 [66] to determine best-fitting models of molecular evolution and partition schemes. Two simultaneous phylogenetic analyses were carried out using MrBayes v3.2 [67–69] with four Markov chains (three heated, one cold) for 10 million generations with a sampling frequency of 1000 and a 25% burn-in. From the resulting trees, a 65% majority-rule consensus tree was constructed and visualized using iTol v6 [70].

2.4. Detection of Talaromyces spp. at Different Time Points and Locations

Samples were collected in July 2019 and April and July 2020 from five apiaries in the South Bohemia region of Czech Republic as described in Section 2.1. The collection sites were Kamenny Malikov village (KM Zirovnice and KM agro) and three apiaries in Ceske Budejovice (CB campus, CB Litvinovice, and CB Kroclov) from urban and suburban areas and the city periphery, respectively. The locations were ranked by anthropogenic influence, focusing on the level of urbanization and the agricultural landscape within ~6 km (Supplementary Figure S6). ITS sequences were used for species assignment.

2.5. UHPLC-HR-MS Analysis and Metabolic Fingerprinting

Samples were processed for metabolic fingerprinting as previously described [71]. Briefly, the samples were fractionated on a 1290 UHPLC system (Agilent, Santa Clara, CA, USA) equipped with DAD, ELSD, and a maXis II (Bruker, Billerica, MA, USA) ESI-qTOF-HRMS. We used a gradient of 0.1% formic acid in water (buffer A) and 0.1% formic acid in acetonitrile (buffer B) at a flow rate of 600 μL/min. The gradient began at 95% A and was held for 0.30 min before a transition to 4.75% A over 18.00 min and 0% A over 18.10 min, with a hold for 22.50 min. The gradient then increased to 95% A over 22.60 min followed by a hold for 25.00 min. The column oven temperature was set at 45 °C, and we used an Acquity UPLC BEH C18 1.7 μm column (2.1 × 100 mm) with an Acquity UPLC BEH C18 1.7 μm VanGuard pre-column (2.1 × 5 mm).

For micro-fractionation [42], extracts were injected into the UHPLC-HR-MS system described above. However, the flow path was changed so that 90% of the flow was diverted to a custom-made fraction collector (Zinsser–Analytik, Frankfurt, Germany) while the rest was analyzed in MS/MS mode in the maXis II. Collision-induced fragmentation at 28.0–35.05 eV was achieved using argon at 10^{-2} mbar. Micro-fractionation assay plates were prepared by injecting 2 or 5 μL of extract. For each injection, 159 fractions were generated and collected on a 384-well plate (fraction length: 7 s). Before screening, the plates were dried under a vacuum using an HT12-II centrifugal concentrator (Genevac, Ipswich, UK) at 35 °C.

For metabolic fingerprinting, raw MS data were processed using DataAnalysis v5.3 (Bruker) including recalibration with sodium formate, followed by RecalculateLinespectra with a threshold of 10,000 and subsequent FindMolecularFeatures (0.5–25 min, S/N = 0, minimal compound length = 8 spectra, smoothing width = 2, correlation coefficient threshold = 0.7). Bucketing was achieved using ProfileAnalysis v2.3 (30–1080 s, 100–6000 m/z, advanced bucketing for 12 s at 5 ppm, no transformation, bucketing basis = H+). We then constructed a cosine similarity heat map.

2.6. Evaluation of Antimicrobial Activity

2.6.1. Antifungal Activity Test

Antifungal activity was tested against three *Aspergillus* species associated with stone-brood disease (*A. flavus* ATCC9170, *A. fumigatus* ATCC10894, and *A. niger* ATCC10549) from Fraunhofer strain collection (STO20519-STO29521) using CLSI M51-A parameters [72]. The *Aspergillus* strains were cultivated on PDA (VWR International, Radnor, PA, USA) for 7 days at 25 °C. The spores were washed by 5–6 mL of 0.05% Tween-80 (Sigma-Aldrich, St. Louis, MO, USA) and filtered through three layers of miracloth to remove the hyphal structures. The inoculum was adjusted to OD_{530} = 0.14–0.46 based on spore morphology [73]. The inoculum was spread evenly over the surface of Mueller–Hinton agar (Carl Roth, Karlsruhe, Germany) using a sterile cotton swab. The depth of the medium was consistent in each Petri dish (4 mm). The extracts from the liquid *T. purpureogenus* strains cultures (Figure 1) were diluted with methanol to 10 mg/mL, and 25 μL of the crude extract was applied to 6 mm cellulose disks and left in the laminar flow cabinet for 30 min to dry. Methanol was used as a control. The disks were placed on the inoculated media and pressed down with sterile forceps. The zones of inhibition were evaluated in triplicate after incubation at 35 °C for 24 h.

2.6.2. Antibacterial Activity Test

We tested the antibacterial activity of the extracts in a dilution series (25–0.39 mg/mL) in triplicate using a micro-broth dilution test against *P. alvei* CCM 2051 provided by the Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic) as previously described [74] with modifications. Briefly, extracts were diluted 1:1 with Mueller–Hinton broth, yeast extract, potassium phosphate, glucose, and pyruvate (MYPGP), and 100 µL of the solution was pipetted to the first row of the microdilution 96-well plate. A two-fold dilution series was prepared by transferring the extract solutions (50 µL) to the next rows containing 50 µL of the MYPGP. Dilutions of methanol were included as a solvent growth control. The lyophilized bacteria were cultivated at 37 °C for ~24 or ~48 h on MYPGP agar. The inoculum was diluted in sterile water and adjusted to 0.5 McFarland units using the DEN-1 McFarland densitometer (BioSan, Riga, Latvia). The bacterial suspension was diluted 1:150 with the MYPGP (~10^6 CFU/mL), and 50 µL was added to each well within 15 min. Bacterial growth was measured at 625 nm using an xMark spectrophotometer (Bio-Rad, Hercules, CA, USA). Minimum inhibitory concentrations (MICs) were defined as the lowest concentration of extract or standard that inhibited growth by at least 80% relative to the control (bacterial suspension with no extract/solvent).

2.6.3. Bioactivity-Guided Dereplication

The potency of the *T. purpureogenus* strains extracts was determined using micro-broth dilution assays as previously described [75–77]. Briefly, extracts were screened in a 12-point dilution series (2–0.001 mg/mL) in triplicate. In addition to four *Paenibacillus* strains (*P. lautus* DSM3035, *P. lactis* FH1832, and two unspecified taxa from the Fraunhofer strain collection ST133196 and ST514408 [78]), we also included the control strain *Escherichia coli* ATCC35218 and the type strain *Bacillus subtilis* DSM 10. For all bacteria, the density of the overnight pre-cultures, incubated in cation-adjusted Mueller–Hinton II medium (BD) at 37 °C while shaking at 180 rpm, was adjusted to 5×10^5 cells/mL. The assay plates were set up by pipetting 100 µL of adjusted bacterial suspension in each well, except the medium control wells H01-H05 (blank or "low"). Wells H06-H12 contained bacterial suspensions without extracts or antibiotics (growth control, "high"). On each assay plate, we also assessed the MIC of three reference antibiotics (ciprofloxacin 0.5–0.0002 µg/mL, cefotaxime, and gentamicin both 64–0.03 µg/mL). The dilution series of pure MeOH, the sample triplicate solved in MeOH, and the reference antibiotics was prepared by adding additional 98 µL of bacterial suspension plus 2 µL of the respective test samples to the first well of rows A–G. Hence, the maximal solvent concentration per well was 1%. Next, the 1:2 dilution series were prepared by transferring 100 µL suspension. After the last dilution step in column 12, the remaining 100 µL were discarded. Assay plates were incubated for 18 h at 37 °C and 85% relative humidity, shaking at 180 rpm, before we measured the turbidity at 600 nm on a LUMIstar Omega microplate spectrophotometer (BMG Labtech, Ortenberg, Germany) as a proxy for cell growth. The relative growth inhibition was calculated based on the absorbance units (AU) of the sample and the controls (see above) using the following formula:

$$\text{rel. inh.\%} = 100 * \left[1 - \frac{\text{AU sample} - \text{AU Low}}{\text{AU High} - \text{AU Low}} \right]$$

MICs were defined as above. Extracts with bioactivity observed over at least three dilution steps (≤0.5 mg/mL) were selected for micro-fractionation. Selected extracts were injected twice into our UHPLC-HRMS-MS system (2 and 5 µL µL, see Section 2.5). The dried assay plate was re-screened against *P. lautus* by adding 50 µL of adjusted pre-culture (see above) to each well except column 1 (media blank). Columns 2 and 3 contained no fractions, but a dilution series of gentamicin ranging from 256–0.008 µg/mL. Column 4 contained only bacterial suspension (growth control), while the remaining 320 wells contained 2×159 fractions collected from the two injection and an unfractionated extract

control of the respective volume. Incubation, read-out, and calculation of the relative growth inhibition was carried out as described above.

3. Results

3.1. Colony Morphology

The use of four standard media enabled us to distinguish the fungal strains based on specific morphological traits: size, sporulation color, and pigment production (Figure 2). The size of the cultures on MEA was comparable (Table 3) but differences in red pigment production (*Monascus*-like red pigments) were observed in the fruiting body (B18 > B13 > B195 on reversed MEA and YES) and media (B13 > B195 > B18 on CYA). The production of low levels of acid on CREA was observed only for strain B49 (Figure 2, Table 3).

Figure 2. Colony morphology of *Talaromyces purpureogenus* strains isolated from bee bread (columns) when grown on different types of media (rows).

Table 3. Comparison of colony morphology for *Talaromyces purpureogenus* strains isolated from bee bread and species characterization.

Strain	Diameter on CYA (mm)	Diameter on MEA (mm)	Soluble Pigment on CYA	Colony Texture on MEA	Acid Production on CREA	Exudates on MEA
Talaromyces sp. B11	21–25	33–37	Weak red	Velvety to floccose	Absent	Yellow
Talaromyces sp. B13	25–26	25–30	Red	Floccose, wrinkled	Absent	Absent
Talaromyces sp. B18	23–27	35–37	Red	Velvety, floccose	Absent	Orange
Talaromyces sp. B30	21–24	33–40	Absent	Floccose	Absent	Absent
Talaromyces sp. B49	25–30	33–36	Absent	Velvety, floccose	Weak	Colorless
Talaromyces sp. B69	24–28	26–36	Absent	Velvety	Absent	Absent
Talaromyces sp. B195	26–29	27–34	Red	Floccose, funiculous	Absent	Red
T. purpureogenus [54]	20–25	30–45	Red	Velvety, floccose	Absent	–

Our cultivation-dependent method detected isolates of the genus *Talaromyces* in bee bread from three of the five apiaries, but only in a few hives in spring (Table 4). Based on ITS sequencing, we identified *T. purpureogenus* in apiaries in areas with a low anthropogenic influence (CB Kroclov and KM Zirovnice) and two species (*T. purpureogenus* and *T. piceae*) in urban samples (CB campus). We did not detect any *Talaromyces* spp. in the other apiaries (KM agro and CB Litvinovice). Images of the isolates are provided in Supplementary Table S2. Fungal isolates representing other genera were not considered.

Table 4. Presence of *Talaromyces* strains in honey bee bread collected from different sampling sites (apiaries) from 2019 to 2020. The values represent the number of hives where *Talaromyces* strains were found relative to the total amount of hives from which the bee bread was collected.

| Apiary	Area	April 2019	July 2019	April 2020	July 2020
KM Zirovnice	Rural	1/6	0/6	1/6	0/6
KM agro	Rural	0/10	0/10	0/10	0/10
CB Kroclov	Periphery	No data	No data	1/3	0/3
CB campus	Urban	No data	No data	2/4	0/4
CB Litvinovice	Suburban	No data	No data	0/4	0/4

3.2. Molecular and Phylogenetic Characterization

We assigned all strains to the species *T. purpureogenus* based on ITS sequence similarity, thus excluding other species in the RefSeq ITS database (Table 5). The sequences were uploaded to the NCBI database (OR192894–OR192900, Supplementary Table S1). By comparing these sequences with the database, we also observed the highest identity (>98%) with *T. purpureogenus* for the other three barcodes (Ben2A, CMD, and RBPII). Only one Ben1A sequence from *Talaromyces* spp. type material was available in the database—*T. stipitatus* (query cover/identity (QC/I) = 88/96.45%; XM_002341495.1). In the standard database, the Ben1A sequences (OR327661–OR327667) showed the highest QC/I to *T. marneffei* (>99/>93.48%; CP045656.1) and less than 90% identity to *T. purpureogenus* isolated from medicinal plants (QC/I = >95/89.65–89.82; HM596783.1) [79].

Table 5. Five sequences with the highest identity in the ITS region based on BLAST analysis. The ITS sequence was identical in all seven strains. QC = query cover, I = identity.

ITS Region Seq.	Database Hits	Score	QC/I [%]	Accession
Talaromyces strains, 577 bp	*T. purpureogenus* CBS 286.36	1000	97/98.93	NR_121529.1
	T. rufus CBS 141834	953	100/96.54	NR_170773.1
	T. thailandensis CBS 133147	942	100/96.19	NR_147428.1
	T. zhenhaiensis CGMCC 3.16102	937	96/96.96	NR_177565.1
	T. aspriconidius CBS 141835	935	97/96.64	NR_170774.1

The resulting phylogeny supports the monophyletic nature of *Talaromyces* and is composed of several subclades containing different members of this genus (Figure 3). General support for the *Talaromyces* clade was high, but several of the deepest clades were only

weakly supported (see Supplementary Figure S1 for numeric bootstrap values). However, the overwhelming majority of shallow clades relevant for the taxonomic placement of our isolates were sufficiently supported to draw firm conclusions.
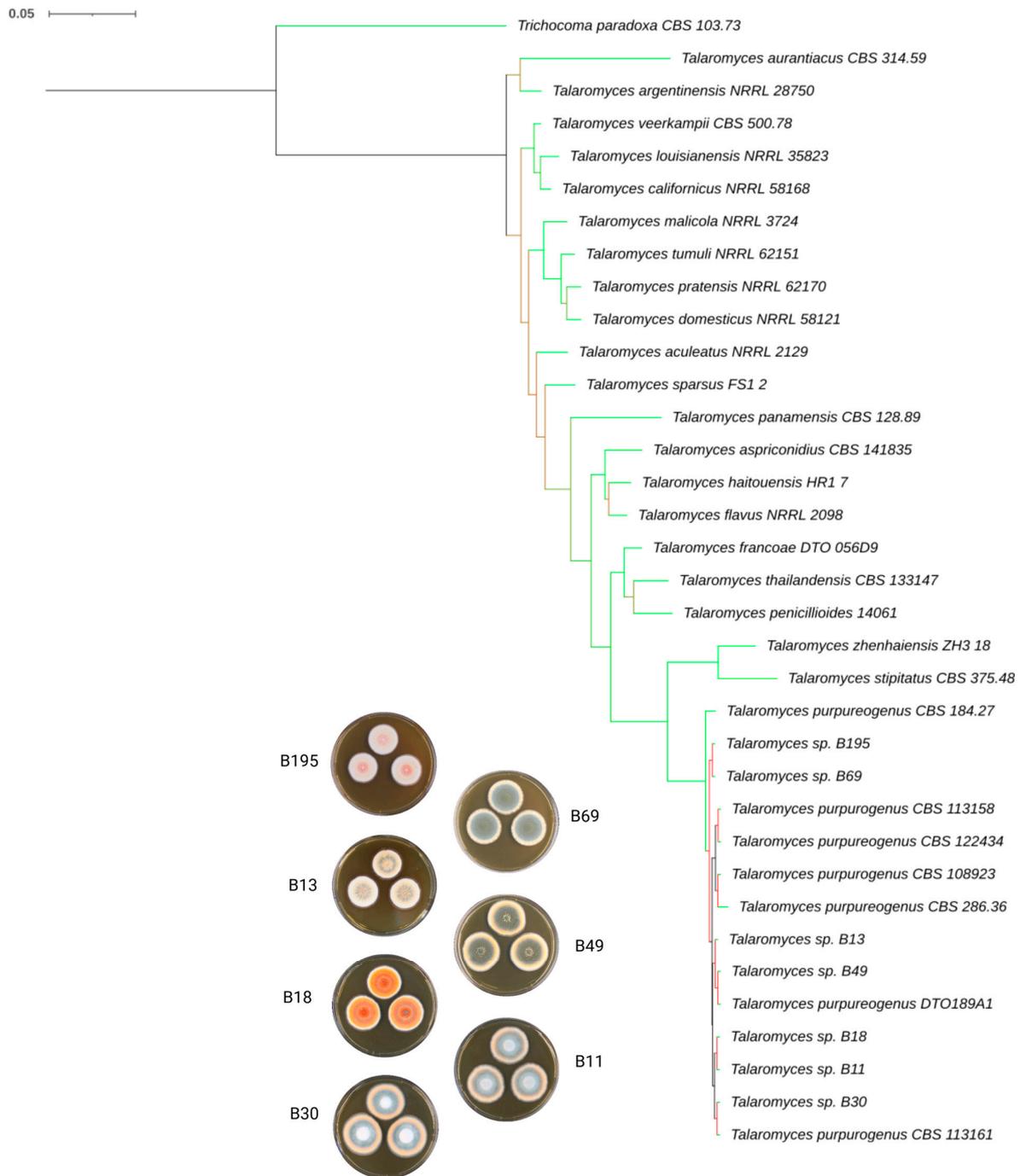
Figure 3. Phylogenetic tree based on ITS, Ben2A, CaM, and RBPII gene regions showing the relationship between the seven newly described *T. purpureogenus* strains and other members of the genus *Talaromyces*. The bootstrap values are indicated using a green–red color model (green = high support and red = low support). The numeric values can be found in Supplementary Figure S1. *Trichocoma paradoxa* was used as the outgroup.

The most ancestral clade within our phylogeny contained two taxa (*T. aurantiacus* and *T. argentinensis*) and was followed by a three-taxon clade (*T. veerkampii*, *T. louisianensis*, and *T. caifornicus*). The next clade contained four taxa (*T. malicola*, *T. tumui*, *T. pratensis*,

and *T. domesticus*) with others splitting off subsequently. The first taxon to split off was *T. aculeatus*, followed by *T. sparsus* and *T. panamensis*, then a clade containing *T. aspriconidius*, *T. haitouensis*, and *T. flavus*, and another with *T. francoe*, *T. thalandensis*, and *T. penicilloides*. The remaining taxa included in our analysis formed the two youngest clades. The first contained two taxa (*T. zhenhaiensis* and *T. stipitatus*) and was placed as a sister to a clade containing all our isolates and different strains of *T. pupurogenus* (CBS 184.27, CBS 113158, CBS 122434, CBS 108923, CBS 286.36, CBS 113161, and DTO189A1). This larger clade, containing the *T. zhenaiensis/T. stipitatus* clade plus the *T. purpureogenus* clade, received substantial support (99%). This also applied to both included subclades, which received 100% support for their monophyletic composition. Similar topologies of our isolates in close proximity to *T. purpureogenus* were consistently recovered across all single-gene trees (Supplementary Figures S2–S5). Overall, our phylogenetic analysis supports the assignment of our isolated fungi to the species *T. purpureogenus* as suggested by our ITS DNA barcoding data (Table 5).

3.3. Metabolic Fingerprinting

The extracts showed a broad distribution of compounds over the polarity range (Supplementary Figures S7–S9). The pairwise cosine similarities of all samples were plotted as a heat map (Figure 4). The main grouping correlated with the solid and liquid media, as expected. The clustering order in the solid media was less influenced by the medium (YES and CYA) and the metabolic features of the solid medium controls were therefore very similar (cosine 0.93). The medium controls and strains B11, B13, B30, and B195 clustered in pairs (consisting of one sample each from CYA and YES). Strains B49 and B69 were merged into one cluster. Strains B18 and B195 clustered together in YES, but the metabolic profile of B18 on CYA was distinct from the other strains in this cluster.

In liquid media, the highest similarity in metabolic features was observed between two pairs – strains B30 and B49 (0.91) and strains B11 and B69 (0.83). Whereas all five strains (B11, B13, B30, B49, and B69) were merged into one cluster, strains B18 and B195 were clustered more closely (0.60) and the metabolic features were more distant from those of the other strains. The clustering order of the strains and the corresponding pairwise cosine similarities are summarized in Supplementary Table S3.

3.4. Antimicrobial Activity and Bioactivity-Guided Dereplication

The crude extracts showed no ability to inhibit the growth of any of the *Aspergillus* species (no zone of inhibition) at a concentration of 0.25 mg of per disc. Extracts B195 and B18 inhibited the growth of *P. alvei* at an MIC of 0.39 mg/mL, whereas extracts B11 and B69 showed activity only at higher concentrations, with MICs of 6.25 and 3.13 mg/mL, respectively (Table 6).

Table 6. Minimal inhibitory concentrations of crude methanol extracts from *T. purpureogenus* strains tested at 0.001–2 mg/mL against *B. subtilis*, *P. lautus,* and *P. lactis*, and at 0.39–6.25 mg/mL against *P. alvei* (* using different protocol).

| Strains	*B. subtilis*	*P. alvei* *	*P. lautus*	*P. lactis*
B11	>2.00	6.25	>2.00	2.00
B13	>2.00	>2.00	>2.00	>2.00
B18	>2.00	0.39	0.50	0.50
B30	>2.00	>2.00	>2.00	2.00
B49	>2.00	>2.00	>2.00	>2.00
B69	>2.00	3.13	2.00	>2.00
B195	2.00	0.39	2.00	2.00
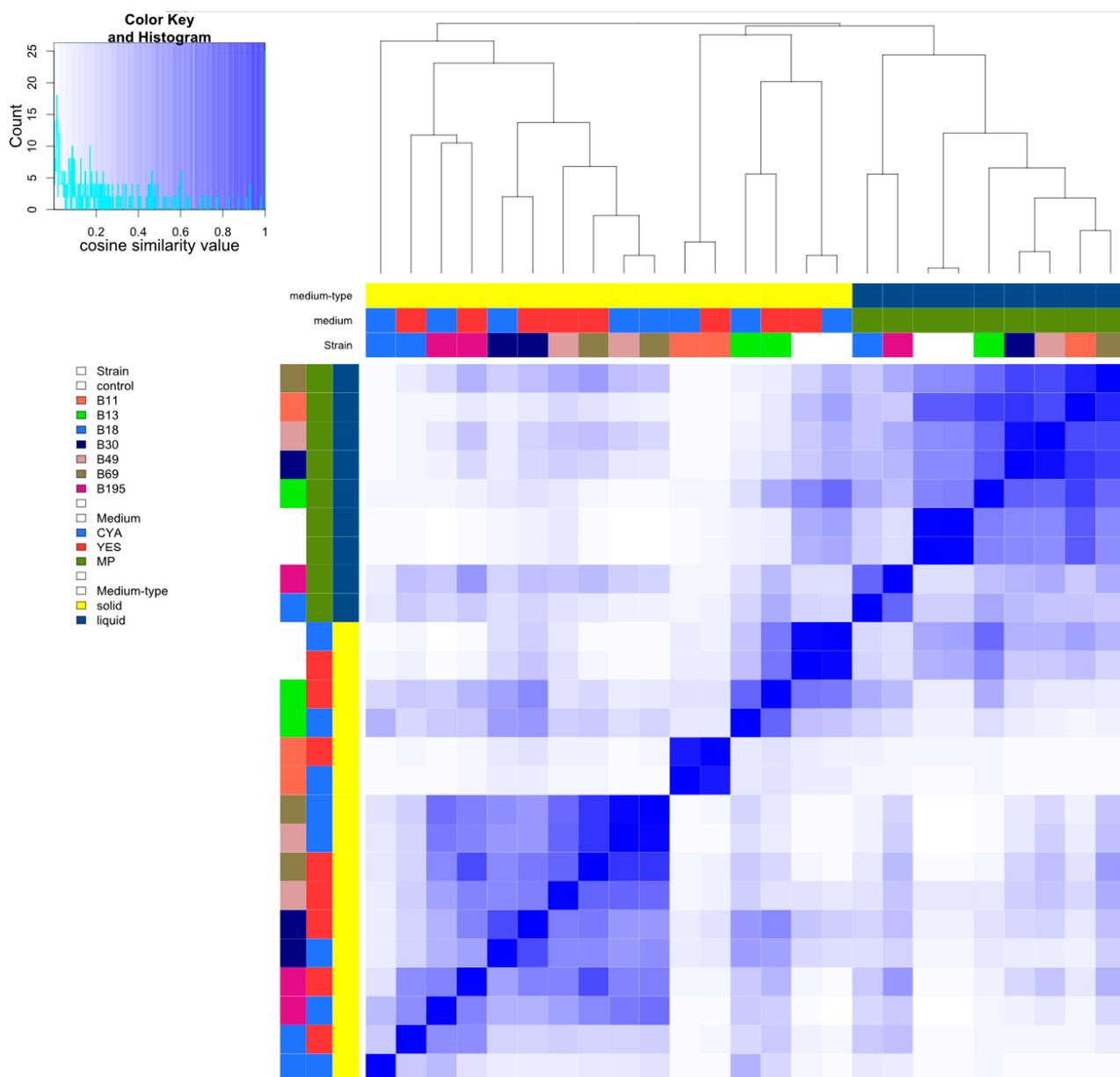
Figure 4. Cosine similarity heat map of extracts analyzed by mass spectrometry.

To support the results of the antimicrobial activity against the bee pathogen *P. alvei*, we expanded the screening panel to include further *Paenibacillus* species as well as the quality-control strain *E. coli* ATCC35218 and type strain *B. subtilis* DSM 10. Interestingly, extract B18 inhibited the growth of *P. lautus* and *P. lactis* at MIC = 0.5 mg/mL (Table 6). The previously observed ability of extract B195 to inhibit *P. alvei* was also observed against *P. lautus* and *P. lactis,* but only at the highest tested concentration (MIC = 2 mg/mL). Extract B195 also displayed moderate activity against *B. subtilis* at 2 mg/mL. None of the extracts inhibited *E. coli* at the concentrations we tested. The full dataset is provided in Supplementary Table S4. Based on the above results, extract B18 was used for microfractionation and re-screened against *P. lautus*, for which optimal growth conditions have been established in our laboratory.

The B18 extract yielded five zones of consecutive fractions that inhibited the growth of the test strain, namely fractions 26–28, 30–31, 33–34, 39, and 114 (Table 7). The fractionation, MS, and MS/MS spectra are provided in Supplementary Figures S10–21. Dereplication was achieved by aligning the major ions in active fractions to commercial and in-house MS/MS reference databases. The two main signals in the average mass spectrum of fractions 26–28 were the single protonated ions at *m/z* 245.1283 $[M+H]^+$ and *m/z* 211.1438 $[M+H]^+$, corresponding to the molecular formulae $C_{14}H_{16}N_2O_2$ and $C_{11}H_{18}N_2O_2$, respectively. The compounds were identified as the diketopiperazines cyclo-Phenyl- alanyl-Prolyl and cyclo-Leucyl-Prolyl (Figure 5: 1–2). Fractions 30–31 contained a group of stereoisomers of cyclo-Phenylalanyl-Prolyl (Figure 5: 1) as well as *m/z* 543.2176 $[M+H]^+$, which was assigned the molecular formula $C_{24}H_{34}N_2O_{12}$. Interestingly, this compound did not match any reference compound in our internal MS/MS database, and a molecular formula search in commercial natural product databases (DNP, Dictionary of Natural Products and AntiBase) also retrieved no hits. The identity of the compound remains unknown. An isomer of the same compound was identified in fractions 33–34. In addition, these fractions contained 5,6,8-trihydroxy-3-methyl-2-benzopyran-1-one ($C_{10}H_8O_5$, ionizing at *m/z* 191.0334 $[M-H_2O+H]^+$, *m/z* 209.0434 $[M+H]^+$ and *m/z* 417.0807 $[2M-H_2O+H]^+$) (Figure 5: 3). Fraction 39 contained the siderophore L-ornithine, N^2-acetyl-N^5-hydroxy-N^5-(5-hydroxy-3-methyl-1-oxo-2-pentenyl)-, trimol. ester, (Z,Z,Z)-(9CI) ($C_{39}H_{62}N_6O_{16}$, ionizing at *m/z* 436.5186 $[M+2H]^{2+}$ and 871.4286 $[M+H]^+$) (Figure 5: 4). A second major ion in fraction 39 (*m/z* 516.2204 $[M+H]^+$) could not be dereplicated. Ultimately, fraction 114 contained one major ion (*m/z* 281.2482 $[M+H]^+$) and a minor ion (*m/z* 379.3368 $[M+H]^+$). Neither compound aligned with any reference compound spectrum. The predicted molecular formula search of the major ion ($C_{18}H_{32}O_2$) retrieved >100 candidates in the DNP, including ubiquitous linolenic acid. However, the nonspecific fragmentation did not allow structural assignment.

Table 7. Summary of the dereplication of active fractions derived from the methanol extract of the *T. purpureogenus* strain B18. The structures are shown in Figure 5.

Fraction	*m/z*	Adduct	Formula	Name	Structure
26–28	245.1283	$[M+H]^+$	$C_{14}H_{16}N_2O_2$	cyclo-(Phenylalanyl-Prolyl)	1
	211.1438	$[M+H]^+$	$C_{11}H_{18}N_2O_2$	cyclo-(Leucyl-Prolyl)	2
30–31	245.1283	$[M+H]^+$	$C_{14}H_{16}N_2O_2$	cyclo-(Phenylalanyl-Prolyl)	1
	543.2176	$[M+H]^+$	$C_{24}H_{34}N_2O_{12}$	unknown	
33–34	543.2176	$[M+H]^+$	$C_{24}H_{34}N_2O_{12}$	unknown	
	191.0334	$[M-H_2O+H]^+$	$C_{10}H_8O_5$	5,6,8-trihydroxy-3-methyl-2-benzopyran-1-one	3
39	516.2204	$[M+H]^+$	$C_{22}H_{33}N_3O_{11}$	unknown	
	436.5186	$[M+2H]^{2+}$	$C_{39}H_{62}N_6O_{16}$	L-ornithine, N^2-acetyl-N^5-hydroxy-N^5-(5-hydroxy-3-methyl-1-oxo-2-pentenyl)-, trimol. ester, (Z,Z,Z)-(9CI)	4
114	379.3368	$[M+H]^+$	$C_{28}H_{42}$	unassigned	
	281.2482	$[M+H]^+$	$C_{18}H_{32}O_2$	unassigned	
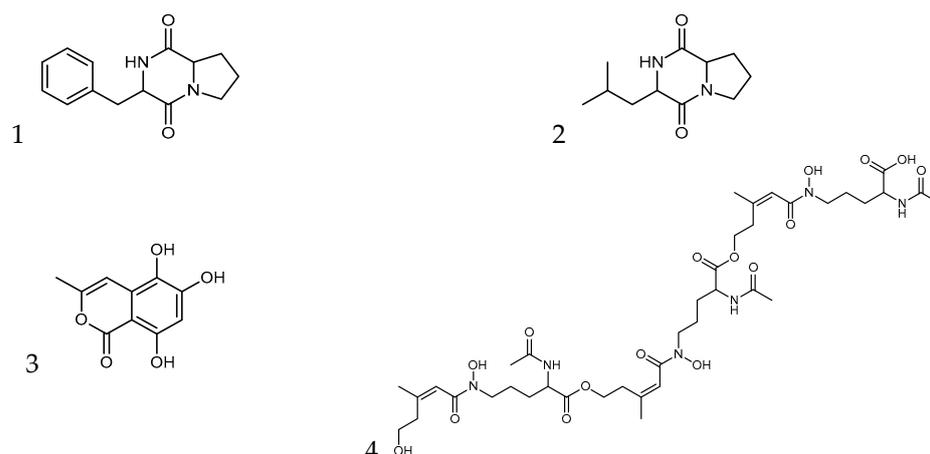
Figure 5. Chemical structures of the compounds identified in the active fractions. 1 and 2—diketopiperazines cyclo-(Phenylalanyl-Prolyl) and cyclo-(Leucyl-Prolyl), resp.; 3—5,6,8-trihydroxy-3-methyl-2-benzopyran-1-one; 4—siderophore L-ornithine, N^2-acetyl-N^5-hydroxy-N^5-(5-hydroxy-3-methyl-1-oxo-2-pentenyl)-, trimol. ester, (Z,Z,Z)-(9CI).

4. Discussion

The role of non-pathogenic fungi as regulators of pathogens in the hive and in bee bread has been addressed in several studies [32–34]. Indeed, bee bread is colonized by many filamentous fungi whose potential as biological control agents is yet to be explored. Here, we characterized seven strains of *Talaromyces* recently found in honey bee bread and tested the antimicrobial activity of methanol extracts against bacteria associated with EFB (*P. alvei*) and opportunistic pathogens of the genus *Aspergillus*.

Based on morphology, DNA barcoding, and phylogenetic analysis, we assigned all seven strains to the species *T. purpureogenus* (Samson, Yilmaz, Frisvad, and Seifert 2011), formerly known as *Penicillium purpureogenum* (Stoll, 1904). *Talaromyces* species have previously been recovered from *A. mellifera* honey [80], *A. cerana* bee bread [34], dead adults of *A. dorsata* (Fabricius, 1793) [43], and stingless bees (*Melipona* spp.) [81]. However, to the best of our knowledge, the species *T. purpureogenus* had not been identified in honey bees before, although it has been found in Coleoptera (bark beetles), Diptera (mosquitoes), Hymenoptera (ants), Hemiptera, and Trichoptera [42].

Although few studies have reported the presence of *Talaromyces* in bee bread, its presence is not surprising because this ubiquitous genus can be collected by bees from various sources [54]. Bees passively or actively collect fungi during foraging and incorporate them into the corbicular pollen [82,83]. Interestingly, some fungi are probably introduced or eliminated by the bees during the collection and storage of pollen [30]. We isolated *Talaromyces* strains from some of the hives in one rural location, an urban area, and the city periphery. Seasonal factors may be relevant because we found the *Talaromyces* strains only in spring. Other studies exploring the composition of fungi in bee bread in Europe did not find any *Talaromyces* species, but samples were collected in the summer [29,84], when the absence of *Talaromyces* is consistent with our results. In worker bees, the prevalence of fungi was higher in fall and winter [32]. The composition of fungi in bee bread during months with no flight activity has not been studied.

We did not find any *Talaromyces* species in apiaries located directly on agricultural land. The foraging distance of *A. mellifera* ranges from several hundred meters to several kilometers, depending on the available pasture [85,86]. The presence of agricultural land (such as a canola field) within the foraging range increases the risk of fungicide residues in the food stores that influence the fungal community. The negative effect of agricultural pressure and fungicide contamination on the bee bread fungal community has been confirmed by sequence-based [29] and cultivation-based analysis [87]. Our results suggest that seasonal effects and agricultural pressure determine the presence of *Talaromyces* in bee

bread. However, more research is needed to correlate the presence and seasonal prevalence of *T. purpureogenus* and other *Talaromyces* species in bee bread.

Our in vitro assays revealed that extracts of *Talaromyces* strains B18 and B195 showed antibacterial activity against the pathogen *P. alvei* and other tested strains of *P. lautus* and *P. lactis*. Bioactivity-guided dereplication in assays against *P. lautus* revealed multiple fractions and corresponding inhibitory compounds (siderophores, diketopiperazines, and three unknown compounds) that are also likely to show activity against the other *Paenibacillus* species. Siderophores are metal chelators that facilitate the uptake, intracellular transport, and storage of iron in plants, fungi, and bacteria, making them useful for both medical and environmental applications [88]. Most fungal siderophores are acylated hydroxamates [89], such as the coprogen-type talarazines produced by *Talaromyces* [90]. The biosynthesis of siderophores plays a key role in fungal virulence and influences their interactions with other microbes [91]. The ability to produce iron-chelating molecules can starve some microbes of this essential nutrient and thus cause growth suppression while providing a source of iron for others, such as yeasts, thus promoting their growth [91]. Several fungal compounds have been associated with regulation of the bee bread microbiome, including organic acids [34] and lovastatin [92]. Given that siderophores inhibited the growth of bacteria in our study, it would be interesting to explore the use of siderophores to regulate EFB and AFB (American foulbrood) in vivo. The unknown active compounds should also be investigated as novel anti-infectives.

Most sequence-based studies identify fungi to the genus or species level. Our results highlight the metabolic diversity of different strains of the species *T. purpureogenus*. Depending on the strain and cultivation conditions, *T. purpureogenus* isolated from the bee bread can affect colony health and pathogen resistance in different ways, such as the production of antimicrobial compounds as discussed here, as well as mycotoxins such as the rubratoxins found in our previous study [36]. The genus *Talaromyces* may consist of an assemblage of species/strains with various metabolic activities, as described for bee-related *Aspergillus* species [93]. These fungi are found in the bee hive as a mixture of atoxigenic and toxigenic strains, and some of them are opportunistic pathogens [93]. Similarly, strains of other genera associated with bee bread, such as *Penicillium* and *Alternaria* [94,95], are assumed to be beneficial but can produce both the antimicrobial compounds and mycotoxins that are lethal to bees. Therefore, the nature of the bee bread mycobiome depends on the balance between different fungal strains, which under ideal circumstances are beneficial to the health of bees and the colony as a whole.

5. Conclusions

Fungi in bee bread play an important role in the regulation of pathogens, conferring resistance and promoting colony survival. Many bee bread fungi, including *T. purpureogenus*, can produce protective antimicrobial compounds as well as lethal mycotoxins. The strain-level balance is therefore important for the beneficial function of the mycobiome. Anthropogenic activity, such as the use of fungicides in agriculture, can disrupt this balance and negatively affect colony health.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms11082067/s1, Table S1: Sequences used for phylogenetic analysis. Table S2: *Talaromyces* spp. isolates from *A. mellifera* bee bread collected from different locations in South Bohemia. Table S3: Cosine similarities and grouping of the *T. purpureogenus* strain extracts on different cultivation media. Table S4: Minimum inhibitory concentration (µg/mL) of the crude methanol extracts from *T. purpureogenus* strains. Table S5: Minimum inhibitory concentration (µg/mL) of the reference antibiotics. Figures S1–S5: Phylogenetic trees. Figure S6: Locations of the apiaries, from which the bee bread samples were collected. Figures S7–S9: MS chromatograms of the crude extracts of *T. purpureogenus* strains from different cultivation media. Figures S10–S21: MS and MS/MS spectra of the active fractions and the precursor ions.

References

1. Ollerton, J.; Winfree, R.; Tarrant, S. How Many Flowering Plants Are Pollinated by Animals? *Oikos* **2011**, *120*, 321–326. [CrossRef]
2. Klein, A.M.; Vaissiere, B.E.; Cane, J.H.; Steffan-Dewenter, I.; Cunningham, S.A.; Kremen, C.; Tscharntke, T. Importance of Pollinators in Changing Landscapes for World Crops. *Proc. R. Soc. B Biol. Sci.* **2007**, *274*, 303–313. [CrossRef] [PubMed]
3. Gallai, N.; Salles, J.; Settele, J.; Vaissière, B.E.; Pollinisation, L.; Abeilles, E.; Abeilles, U.M.R.; Cedex, A.; Lameta, U.M.R.; Cedex, M. Economic Valuation of the Vulnerability of World Agriculture Confronted with Pollinator Decline. *Ecol. Econ.* **2009**, *68*, 810–821. [CrossRef]
4. Lautenbach, S.; Seppelt, R.; Liebscher, J.; Dormann, C.F. Spatial and Temporal Trends of Global Pollination Benefit. *PLoS ONE* **2012**, *7*, e35954. [CrossRef] [PubMed]
5. Potts, S.G.; Imperatriz-Fonseca, V.; Ngo, H.T.; Aizen, M.A.; Biesmeijer, J.C.; Breeze, T.D.; Dicks, L.V.; Garibaldi, L.A.; Hill, R.; Settele, J.; et al. Safeguarding Pollinators and Their Values to Human Well-Being. *Nature* **2016**, *540*, 220–229. [CrossRef]
6. Aizen, M.A.; Garibaldi, L.A.; Cunningham, S.A.; Klein, A.M. Long-Term Global Trends in Crop Yield and Production Reveal No Current Pollination Shortage but Increasing Pollinator Dependency. *Curr. Biol.* **2008**, *18*, 1572–1575. [CrossRef]
7. Aizen, M.A.; Harder, L.D. The Global Stock of Domesticated Honey Bees Is Growing Slower than Agricultural Demand for Pollination. *Curr. Biol.* **2009**, *19*, 915–918. [CrossRef]
8. Potts, S.G.; Biesmeijer, J.C.; Kremen, C.; Neumann, P.; Schweiger, O.; Kunin, W.E. Global Pollinator Declines: Trends, Impacts and Drivers. *Trends Ecol. Evol.* **2010**, *25*, 345–353. [CrossRef]
9. Zattara, E.E.; Aizen, M.A. Worldwide Occurrence Records Suggest a Global Decline in Bee Species Richness. *One Earth* **2021**, *4*, 114–123. [CrossRef]
10. Goulson, D.; Nicholls, E.; Botías, C.; Rotheray, E.L. Bee Declines Driven by Combined Stress from Parasites, Pesticides, and Lack of Flowers. *Science.* **2015**, *347*, 1255957. [CrossRef]
11. Smith, K.M.; Loh, E.H.; Rostal, M.K.; Zambrana-Torrelio, C.M.; Mendiola, L.; Daszak, P. Pathogens, Pests, and Economics: Drivers of Honey Bee Colony Declines and Losses. *Ecohealth* **2013**, *10*, 434–445. [CrossRef]
12. Vilcinskas, A. Pathogens Associated with Invasive or Introduced Insects Threaten the Health and Diversity of Native Species. *Curr. Opin. Insect Sci.* **2019**, *33*, 43–48. [CrossRef]
13. vanEngelsdorp, D.; Meixner, M.D. A Historical Review of Managed Honey Bee Populations in Europe and the United States and the Factors That May Affect Them. *J. Invertebr. Pathol.* **2010**, *103*, S80–S95. [CrossRef] [PubMed]
14. Bruckner, S.; Wilson, M.; Aurell, D.; Rennich, K.; vanEngelsdorp, D.; Steinhauer, N.; Williams, G.R. A National Survey of Managed Honey Bee Colony Losses in the USA: Results from the Bee Informed Partnership for 2017–2018, 2018–2019, and 2019–2020. *J. Apic. Res.* **2022**, *62*, 429–443. [CrossRef]
15. Gray, A.; Noureddine, A.; Arab, A.; Ballis, A.; Brusbardis, V.; Bugeja Douglas, A.; Cadahía, L.; Charrière, J.D.; Chlebo, R.; Coffey, M.F.; et al. Honey Bee Colony Loss Rates in 37 Countries Using the COLOSS Survey for Winter 2019–2020: The Combined Effects of Operation Size, Migration and Queen Replacement. *J. Apic. Res.* **2022**, *62*, 204–210. [CrossRef]
16. Dale, C.; Moran, N.A. Molecular Interactions between Bacterial Symbionts and Their Hosts. *Cell* **2006**, *126*, 453–465. [CrossRef] [PubMed]
17. Schwarz, R.S.; Moran, N.A.; Evans, J.D. Early Gut Colonizers Shape Parasite Susceptibility and Microbiota Composition in Honey Bee Workers. *PNAS* **2016**, *113*, 9345–9350. [CrossRef]
18. Kwong, W.K.; Mancenido, A.L.; Moran, N.A. Immune System Stimulation by the Native Gut Microbiota of Honey Bees. *R. Soc. Open Sci.* **2017**, *4*, 170003. [CrossRef]

19. Dharampal, P.S.; Carlson, C.; Currie, C.R.; Steffan, S.A. Pollen-Borne Microbes Shape Bee Fitness. *Proc. R. Soc. B* **2019**, *286*, 20182894. [CrossRef]

20. Raymann, K.; Moran, N.A. The Role of the Gut Microbiome in Health and Disease of Adult Honey Bee Workers. *Curr. Opin. Insect Sci.* **2018**, *26*, 97–104. [CrossRef]

21. Janashia, I.; Alaux, C. Specific Immune Stimulation by Endogenous Bacteria in Honey Bees (Hymenoptera: *Apidae*). *J. Econ. Entomol.* **2016**, *109*, 1474–1477. [CrossRef] [PubMed]

22. Emery, O.; Schmidt, K.; Engel, P. Immune System Stimulation by the Gut Symbiont *Frischella perrara* in the Honey Bee (*Apis mellifera*). *Mol. Ecol.* **2017**, *26*, 2576–2590. [CrossRef] [PubMed]

23. Powell, J.E.; Martinson, V.G.; Urban-Mead, K.; Moran, N.A. Routes of Acquisition of the Gut Microbiota of the Honey Bee *Apis mellifera*. *Appl. Environ. Microbiol.* **2014**, *80*, 7378–7387. [CrossRef] [PubMed]

24. Moran, N.A.; Hansen, A.K.; Powell, J.E.; Sabree, Z.L. Distinctive Gut Microbiota of Honey Bees Assessed Using Deep Sampling from Individual Worker Bees. *PLoS ONE* **2012**, *7*, e36393. [CrossRef] [PubMed]

25. Martinson, V.G.; Danforth, B.N.; Minckley, R.L.; Rueppell, O.; Tingek, S.; Moran, N.A. A Simple and Distinctive Microbiota Associated with Honey Bees and Bumble Bees. *Mol. Ecol.* **2011**, *20*, 619–628. [CrossRef] [PubMed]

26. Ellegaard, K.M.; Engel, P. Genomic Diversity Landscape of the Honey Bee Gut Microbiota. *Nat. Commun.* **2019**, *10*, 446. [CrossRef] [PubMed]

27. Kwong, W.K.; Moran, N.A. Gut Microbial Communities of Social Bees. *Nat. Rev. Microbiol.* **2016**, *14*, 374–384. [CrossRef]

28. Smutin, D.; Lebedev, E.; Selitskiy, M.; Panyushev, N.; Adonin, L. Micro"bee"ota: Honey Bee Normal Microbiota as a Part of Superorganism. *Microorganisms* **2022**, *10*, 2359. [CrossRef]

29. Dimov, S.G.; Zagorchev, L.; Iliev, M.; Dekova, T.; Ilieva, R.; Kitanova, M.; Georgieva-Miteva, D.; Dimitrov, M.; Peykov, S. A Snapshot Picture of the Fungal Composition of Bee Bread in Four Locations in Bulgaria, Differing in Anthropogenic Influence. *J. Fungi* **2021**, *109*, 1474–1477. [CrossRef]

30. Gilliam, M.; Prest, D.B.; Lorenz, B.J. Microbiology of Pollen and Bee Bread: Taxonomy and Enzymology of Molds. *Apidologie* **1989**, *20*, 53–68. [CrossRef]

31. Berenbaum, M.R.; Johnson, R.M. Xenobiotic Detoxification Pathways in Honey Bees. *Curr. Opin. Insect Sci.* **2015**, *10*, 51–58. [CrossRef]

32. Gilliam, M. Identication and Roles of Non-Pathogenic Microflora Associated with Honey Bees. 1997, 155, 1–10. [CrossRef]

33. Gilliam, M.; Taber, S.; Lorenz, B.J.; Prest, D.B. Factors Affecting Development of Chalkbrood Disease in Colonies of Honey Bees, Apis Mellifera, Fed Pollen Contaminated with Ascosphaera Apis. *J. Invertebr. Pathol.* **1988**, *52*, 314–325. [CrossRef]

34. Disayathanoowat, T.; Li, H.; Supapimon, N.; Suwannarach, N.; Lumyong, S.; Chantawannakul, P.; Guo, J. Different Dynamics of Bacterial and Fungal Communities in Hive-Stored Bee Bread and Their Possible Roles: A Case Study from Two Commercial Honey Bees in China. *Microorganisms* **2020**, *8*, 264. [CrossRef]

35. Stamets, P.E.; Naeger, N.L.; Evans, J.D.; Han, J.O.; Hopkins, B.K.; Lopez, D.; Moershel, H.M.; Nally, R.; Sumerlin, D.; Taylor, A.W.; et al. Extracts of Polypore Mushroom Mycelia Reduce Viruses in Honey Bees. *Sci. Rep.* **2018**, *8*, 508. [CrossRef]

36. Vocadlova, K.; Lamp, B.; Benes, K.; Matha, V.; Lee, K.-Z.; Vilcinskas, A. Crude Extracts of *Talaromyces* Strains (*Ascomycota*) Affect. *Viruses* **2023**, *15*, 343. [CrossRef]

37. Hsu, C.K.; Wang, D.Y.; Wu, M.C. A Potential Fungal Probiotic *Aureobasidium melanogenum* Ck-Csc for the Western Honey Bee, Apis Mellifera. *J. Fungi* **2021**, *7*, 508. [CrossRef]

38. Zhai, M.M.; Li, J.; Jiang, C.X.; Shi, Y.P.; Di, D.L.; Crews, P.; Wu, Q.X. The Bioactive Secondary Metabolites from *Talaromyces* Species. *Nat. Products Bioprospect.* **2016**, *6*, 1–24. [CrossRef]

39. Nicoletti, R.; Salvatore, M.M.; Andolfi, A. Secondary Metabolites of Mangrove-Associated Strains of *Talaromyces*. *Mar. Drugs* **2018**, *16*, 12. [CrossRef]

40. Mizuno, K.; Yagi, A.; Takada, M.; Matsuura, K.; Yamaguchi, K. Letter: A New Antibiotic, Talaron. *J. Antibiot.* **1974**, *27*, 560–563. [CrossRef]

41. Mapari, S.A.S.; Meyer, A.S.; Thrane, U.; Frisvad, J.C. Identification of Potentially Safe Promising Fungal Cell Factories for the Production of Polyketide Natural Food Colorants Using Chemotaxonomic Rationale. *Microb. Cell Fact.* **2009**, *8*, 24. [CrossRef]

42. Nicoletti, R. Talaromyces—Insect Relationships. *Microorganisms* **2022**, *10*, 45. [CrossRef]

43. Sandeepani, H.P.; Ratnaweera, P.B. Antibacterial Activity of Entomopathogenic Fungi Isolated from *Vespa affinis* and *Apis dorsata* in Sri Lanka. Proceedings of 1st International Conference on Frontiers in Chemical Technology, Colombo, Sri Lanka, 20–22 July 2020; p. 29.

44. Lopes, L.Q.S.; Quatrin, P.M.; De Souza, M.E.; Vaucher, R.D.A.; Santos, R.C.V. Fungal Infections In Honey Bees. *Fungal Genom. Biol.* **2015**, *5*, 1000118. [CrossRef]

45. Djordjevic, S.P.; Forbes, W.A.; Smith, L.A.; Hornitzky, M.A. Genetic and Biochemical Diversity among Isolates of *Paenibacillus alvei* Cultured from Australian Honeybee (*Apis mellifera*) Colonies. *Appl. Environ. Microbiol.* **2000**, *66*, 1098–1106. [CrossRef]

46. WOAH. European Foulbrood of Honey Bees (Infection of Honey Bees with *Melissococcus plutonius*). In *OIE Terrestrial Manual*; WOAH: Paris, France, 2018; pp. 736–743.plutonius). In *OIE Terrestrial Manual*; WOAH: Paris, France, 2018; pp. 736–743.

47. Jensen, A.B.; Hughes, W.O.H.; Foley, K. The Distribution of *Aspergillus* Spp. Opportunistic Parasites in Hives and Their Pathogenicity to Honey Bees. *Veter Microbiol.* **2014**, *169*, 203–210. [CrossRef]

48. Roetschi, A.; Berthoud, H.; Kuhn, R.; Imdorf, A. Infection Rate Based on Quantitative Real-Time PCR of *Melissococcus plutonius*, the Causal Agent of European Foulbrood, in Honeybee Colonies before and after Apiary Sanitation. *Apidologie* **2008**, *39*, 362–371. [CrossRef]

49. Richards, E.D.; Tell, L.A.; Davis, J.L.; Baynes, R.E.; Lin, Z.; Maunsell, F.P.; Riviere, J.E.; Jaberi-Douraki, M.; Martin, K.L.; Davidson, G. Honey Bee Medicine for Veterinarians and Guidance for Avoiding Violative Chemical Residues in Honey. *J. Am. Vet. Med. Assoc.* **2021**, *259*, 860–873. [CrossRef]

50. Wilkins, S.; Brown, M.A.; Cuthbertson, A.G.S. The Incidence of Honey Bee Pests and Diseases in England and Wales. *Pest Manag. Sci.* **2007**, *63*, 1062–1068. [CrossRef]

51. Seyedmousavi, S.; Guillot, J.; Arné, P.; De Hoog, G.S.; Mouton, J.W.; Melchers, W.J.G.; Verweij, P.E. Aspergillus and Aspergilloses in Wild and Domestic Animals: A Global Health Concern with Parallels to Human Disease. *Med. Mycol.* **2015**, *53*, 765–797. [CrossRef]

52. Samson, R.A.; Houbraken, J.; Thrane, U.; Frisvad, J.C.; Andersen, B. *Food and Indoor Fungi*, 2nd ed.; CBS-KNAW Fungal Biodiversity Centre: Utrecht, The Netherlands, 2010; ISBN 9789491751189.

53. GIMP. The GIMP Develompment Team. Available online: https://www.gimp.org (accessed on 15 December 2020).

54. Yilmaz, N.; Visagie, C.M.; Houbraken, J.; Frisvad, J.C.; Samson, R.A. Polyphasic Taxonomy of the Genus *Talaromyces*. *Stud. Mycol.* **2014**, *78*, 175–341. [CrossRef]

55. Glass, N.L.; Donaldson, G.C. Development of Primer Sets Designed for Use with the PCR to Amplify Conserved Genes from Filamentous Ascomycetes. *Appl. Environ. Microbiol.* **1995**, *61*, 1323–1330. [CrossRef]

56. Gardes, M.; Bruns, T.D. ITS Primers with Enhanced Specificity for Basidiomycetes-Application to the Identification of Mycorrhizae and Rusts. *Mol. Ecol.* **1993**, *2*, 113–118. [CrossRef]

57. White, T.J.; Bruns, S.; Lee, S.; Taylor, J. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*; Academic Press: San Diego, CA, USA, 1990; pp. 315–322.

58. Peterson, S.W.; Vega, F.E.; Posada, F.; Nagai, C. *Penicillium coffeae*, a New Endophytic Species Isolated from a Coffee Plant and Its Phylogenetic Relationship to *P. Fellutanum, P. Thiersii and P. Brocae Based on Parsimony Analysis of Multilocus DNA Sequences*. *Mycologia* **2005**, *97*, 659–666. [CrossRef]

59. Liu, Y.J.; Whelen, S.; Hall, B.D. Phylogenetic Relationships among Ascomycetes: Evidence from an RNA Polymerse II Subunit. *Mol. Biol. Evol.* **1999**, *16*, 1799–1808. [CrossRef]

60. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic Local Alignment Search Tool. *J. Mol. Biol.* **1990**, *215*, 403–410. [CrossRef]

61. Raja, H.A.; Miller, A.N.; Pearce, C.J.; Oberlies, N.H. Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community. *J. Nat. Prod.* **2017**, *80*, 756–770. [CrossRef]

62. Nguyen, L.T.; Schmidt, H.A.; Von Haeseler, A.; Minh, B.Q. IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Mol. Biol. Evol.* **2015**, *32*, 268–274. [CrossRef]

63. Minh, B.Q.; Nguyen, M.A.T.; Von Haeseler, A. Ultrafast Approximation for Phylogenetic Bootstrap. *Mol. Biol. Evol.* **2013**, *30*, 1188–1195. [CrossRef]

64. Castresana, J. Selection of Conserved Blocks from Multiple Alignments for Their Use in Phylogenetic Analysis. *Mol. Biol. Evol.* **2000**, *17*, 540–552. [CrossRef]

65. Talavera, G.; Castresana, J. Improvement of Phylogenies after Removing Divergent and Ambiguously Aligned Blocks from Protein Sequence Alignments. *Syst. Biol.* **2007**, *56*, 564–577. [CrossRef]

66. Miller, M.A.; Pfeiffer, W.; Schwartz, T. Creating the CIPRES Science Gateway for Inference of Large Phylogenetic Trees. In Proceedings of the 2010 gateway computing environments workshop (GCE), New Orleans, LA, USA, 14 November 2010; pp. 1–8.

67. Ronquist, F.; Teslenko, M.; van der Mark, P.; Ayres, D.L.; Darling, A.; Höhna, S.; Larget, B.; Liu, L.; Suchard, M.A.; Huelsenbeck, J.P. MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice across a Large Model Space. *Syst. Biol.* **2012**, *61*, 539–542. [CrossRef]

68. Huelsenbeck, J.P.; Ronquist, F. MRBAYES: Bayesian Inference of Phylogenetic Trees. *Bioinformatics* **2001**, *17*, 754–755. [CrossRef] [PubMed]

69. Ronquist, F.; Huelsenbeck, J.P. MrBayes 3: Bayesian Phylogenetic Inference under Mixed Models. *Bioinformatics* **2003**, *19*, 1572–1574. [CrossRef] [PubMed]

70. Letunic, I.; Bork, P. Interactive Tree of Life (ITOL) v5: An Online Tool for Phylogenetic Tree Display and Annotation. *Nucleic Acids Res.* **2021**, *49*, W293–W296. [CrossRef]

71. Marner, M.; Hartwig, C.; Patras, M.A.; Wodi, S.I.M.; Rieuwpassa, F.J.; Ijong, F.G.; Balansa, W. Sustainable Low-Volume Analysis of Environmental Samples by Semi-Automated Prioritization of Extracts for Natural Product Research (SeaPEPR). *Mar. Drugs* **2020**, *18*, 649. [CrossRef]

72. Espinel-Ingroff, A.; Cantón, E.; Pemán, J. Antifungal Susceptibility Testing of Filamentous Fungi. *Curr. Fungal Infect. Rep.* **2012**, *6*, 41–50. [CrossRef]

73. Petrikkou, E.; Rodríguez-Tudela, J.L.; Cuenca-Estrella, M.; Gómez, A.; Molleja, A.; Mellado, E. Inoculum Standardization for Antifungal Susceptibility Testing of Filamentous Fungi Pathogenic for Humans. *J. Clin. Microbiol.* **2001**, *39*, 1345–1347. [CrossRef]

74. Balouiri, M.; Sadiki, M.; Ibnsouda, S.K. Methods for in Vitro Evaluating Antimicrobial Activity: A Review. *J. Pharm. Anal.* **2016**, *6*, 71–79. [CrossRef]

75. Kresna, I.D.M.; Wuisan, Z.G.; Pohl, J.M.; Mettal, U.; Otoya, V.L.; Gand, M.; Marner, M.; Otoya, L.L.; Bohringer, N.; Vilcinskas, A.; et al. Genome-Mining-Guided Discovery and Characterization of the PKS-NRPS-Hybrid Polyoxyperuin Produced by a Marine-Derived Streptomycete. *J. Nat. Prod.* **2022**, *85*, 888–898. [CrossRef]

76. Marner, M.; Patras, M.A.; Kurz, M.; Zubeil, F.; Forster, F.; Schuler, S.; Bauer, A.; Hammann, P.; Vilcinskas, A.; Schaberle, T.F.; et al. Molecular Networking-Guided Discovery and Characterization of Stechlisins, a Group of Cyclic Lipopeptides from a Pseudomonas Sp. *J. Nat. Prod.* **2020**, *83*, 2607–2617. [CrossRef]

77. Wang, L.; Linares-Otoya, V.; Liu, Y.; Mettal, U.; Marner, M.; Armas-Mantilla, L.; Willbold, S.; Kurtan, T.; Linares-Otoya, L.; Schaberle, T.F. Discovery and Biosynthesis of Antimicrobial Phenethylamine Alkaloids from the Marine Flavobacterium *Tenacibaculum discolor* sv11. *J. Nat. Prod.* **2022**, *85*, 1039–1051. [CrossRef]

78. Fox, J.L. Fraunhofer to Mine Sanofi Microbial Collection. *Nat. Biotechnol.* **2014**, *32*, 305. [CrossRef] [PubMed]

79. Bhagobaty, R.K.; Joshi, S.R. Multi-Loci Molecular Characterisation of Endophytic Fungi Isolated from Five Medicinal Plants of Meghalaya, India. *Mycobiology* **2011**, *39*, 71–78. [CrossRef]

80. Rodríguez-Andrade, E.; Stchigel, A.M.; Terrab, A.; Guarro, J.; Cano-Lira, J.F. Diversity of Xerotolerant and Xerophilic Fungi in Honey. *IMA Fungus* **2019**, *10*, 20. [CrossRef] [PubMed]

81. Barbosa, R.N.; Bezerra, J.D.P.; Souza-Motta, C.M.; Frisvad, J.C.; Samson, R.A.; Oliveira, N.T.; Houbraken, J. New *Penicillium* and *Talaromyces* Species from Honey, Pollen and Nests of Stingless Bees. *Antonie van Leeuwenhoek* **2018**, *111*, 1883–1912. [CrossRef]

82. Parish, J.B.; Scott, E.S.; Hogendoorn, K. Collection of Conidia of *Podosphaera xanthii* by Honey Bee Workers. *Australas. Plant Pathol.* **2020**, *49*, 245–247. [CrossRef]

83. Shaw, D.E. The Incidental Collection of Fungal Spores by Bees and the Collection of Spores in Lieu of Pollen. *Bee World* **1990**, *71*, 158–176. [CrossRef]

84. Friedle, C.; D'Alvise, P.; Schweikert, K.; Wallner, K.; Hasselmann, M. Changes of Microorganism Composition in Fresh and Stored Bee Pollen from Southern Germany. *Environ. Sci. Pollut. Res.* **2021**, *28*, 47251–47261. [CrossRef]

85. Waddington, K.D.; Herbert, T.J.; Visscher, P.K.; Richter, M.R. Comparisons of Forager Distributions from Matched Honey Bee Colonies in Suburban Environments. *Behav. Ecol. Sociobiol.* **1994**, *35*, 423–429. [CrossRef]

86. Beekman, M.; Ratnieks, F.L.W. Long-Range Foraging by the Honey-Bee, *Apis mellifera* L. *Funct. Ecol.* **2000**, *14*, 490–496. [CrossRef]

87. Yoder, J.A.; Jajack, A.J.; Rosselot, A.E.; Smith, T.J.; Yerke, M.C.; Sammataro, D. Fungicide Contamination Reduces Beneficial Fungi in Bee Bread Based on an Area-Wide Field Study in Honey Bee, *Apis mellifera*, Colonies. *J. Toxicol. Environ. Health Part A Curr. Issues* **2013**, *76*, 587–600. [CrossRef]

88. Kurth, C.; Kage, H.; Nett, M. Siderophores as Molecular Tools in Medical and Environmental Applications. *Org. Biomol. Chem.* **2016**, *14*, 8212–8227. [CrossRef]

89. Haas, H. Molecular Genetics of Fungal Siderophore Biosynthesis and Uptake: The Role of Siderophores in Iron Uptake and Storage. *Appl. Microbiol. Biotechnol.* **2003**, *62*, 316–330. [CrossRef] [PubMed]

90. Kalansuriya, P.; Quezada, M.; Espósito, B.P.; Capon, R.J. Talarazines A-E: Noncytotoxic Iron(III) Chelators from an Australian Mud Dauber Wasp-Associated Fungus, *Talaromyces* Sp. (CMB-W045). *J. Nat. Prod.* **2017**, *80*, 609–615. [CrossRef] [PubMed]

91. Haas, H. Fungal Siderophore Metabolism with a Focus on *Aspergillus fumigatus*. *Nat. Prod. Rep.* **2014**, *31*, 1266–1276. [CrossRef] [PubMed]

92. Paludo, C.R.; Pishchany, G.; Andrade-Dominguez, A.; Silva-Junior, E.A.; Menezes, C.; Nascimento, F.S.; Currie, C.R.; Kolter, R.; Clardy, J.; Pupo, M.T. Microbial Community Modulates Growth of Symbiotic Fungus Required for Stingless Bee Metamorphosis. *PLoS ONE* **2019**, *14*, e0219696. [CrossRef] [PubMed]

93. Becchimanzi, A.; Nicoletti, R. Aspergillus-Bees: A Dynamic Symbiotic Association. *Front. Microbiol.* **2022**, *13*, 968963. [CrossRef]

94. Frisvad, J.C.; Smedsgaard, J.; Larsen, T.O.; Samson, R.A. Mycotoxins, Drugs and Other Extrolites Produced by Species in *Penicillium* subgenus *Penicillium*. *Stud. Mycol.* **2004**, *49*, 201–241.

95. Lou, J.; Fu, L.; Peng, Y.; Zhou, L. Metabolites from Alternaria Fungi and Their Bioactivities. *Molecules* **2013**, *18*, 5891–5935. [CrossRef]