Wild apple-associated fungi and bacteria compete to colonize the larval gut of an invasive wood-borer *Agrilus mali* in Tianshan forests

Tohir Bozorov (tohirbozorov@yahoo.com)
Xinjiang Institute of Ecology and Geography https://orcid.org/0000-0002-8925-6533
Zokir Toshmatov
Plant Genetics Research Unit: Genetique Quantitative et Evolution Le Moulon
Gulnaz Kahar
Xinjiang Institute of Ecology and Geography
Daoyuan Zhang
Xinjiang Institute of Ecology and Geography
Hua Shao
Xinjiang Institute of Ecology and Geography
Yusufjon Gafforov
Institute of Botany, Academy of Sciences of Uzbekistan

Research

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Abstract

Background: The gut microflora of insects plays important roles throughout their lives. Different foods and geographic locations change gut bacterial communities. The invasive wood-borer *Agrilus mali* causes extensive mortality of wild apple, *Malus sieversii,* which is considered a progenitor of all cultivated apples, in Tianshan forests. Recent analysis showed that the gut microbiota of larvae collected from Tianshan forests showed rich bacterial diversity but the absence of fungal species. In this study, we explored the antagonistic ability of gut bacteria to address this absence of fungi in the larval gut.

Results: The results demonstrated that gut bacteria were able to selectively inhibit wild apple tree-associated fungi. However, *Pseudomonas synxantha* showed strong antagonistic ability, producing antifungal compounds. Using different analytical methods, such as column chromatography, mass spectrometry, HPLC and NMR, an antifungal compound, phenazine-1-carboxylic acid (PCA), was identified. Activity of the compound was determined by the minimum inhibitory concentration method and electron microscopy. Moreover, sequence alignment and cluster analysis showed that gut bacteria could originate from noninfested apple microflora during infestation.

Conclusions: Overall, the results showed that in newly invaded locations, *A. mali* larvae changed their gut microbiota and adopted new gut bacteria that prevented fungal colonization in the gut.

Introduction

Insects have diverse bacterial associations including relationships ranging from parasitism to mutualism [1]. There are a number of studies on the gut microbiota of wood-boring beetles, due to its essential roles in food digestion, compensation for dietary deficiencies, compound detoxification, nutrient production, and more, in the literature [1, 2]. In addition, insects have a very close relationship with their gut microbiome, and symbiotic interactions can result in host survival under extreme environmental conditions [3]. There is an unexplored question in invasion ecology, and a study highlighted by E Pennisi [4] raised a new hypothesis: Could the gut microbiome determine the invasion success of phytophagous insects? [5]. Recent reports support the crucial role of gut microflora in insect growth and development and in environmental adaptation [5, 6]. Gut-associated microorganisms such as bacteria, fungi, protozoa and viruses can be transiently or permanently transmitted to insects, and this relationship can be either beneficial or harmful [7, 8]. For example, the gut microflora has a symbiotic relationship with hosts to obtain nutrients, aid digestion, and promote host immunity by protecting against pathogens [1, 9, 10]. Therefore, studying the microbiome of the insect digestive tract is essential to improve beneficial insect digestive capabilities or pest management programs [11].

The interaction between fungi and bacteria in the invasive insect gut has not been thoroughly studied. Fungi and bacteria are frequent in most wood-feeding insect guts and likely play a role in the digestion of plant-cell polymers due to their symbiotic interaction with the host. Microbial composition and abundance in the insect gut vary; wood-feeding insects have a rich bacterial diversity, whereas sap-feeding insects have poor [10] due to insect gut compartmentalization [12]. The bacterial composition of the gut could either be specifically adapted to the gut, maternally transmitted or acquired from the environment [13]. Additionally, the microbial diversity of the gut also depends on geographic location, which has been shown for honeybees [14]. The composition of the myco- and microflora of the larval gut are dependent on the developmental stage of the insect and the location, as was shown for the citrus pest *Bactrocera minax* [15]. However, during larval growth, gut bacteria may influence fungal diversity [14-16]. Moreover, gut bacteria can compete with fungi during gut colonization [16].

Wild apple *Malus sieversii* (Ledeb.) Roem. is a species native to Tianshan forests and usually found in sub-mountain areas [17], and it is considered the primary progenitor of all cultivated apples [18]. Because of its rich genetic diversity, it remains a globally significant genetic resource. Unfortunately, wild apple faces extensive abiotic and biotic stresses. Among them, the invasive insect, *Agrilus mali* (Coleoptera: Buprestidae) has heavily attacked trees since its first detection in the early 1990s and has extensively damaged the wild apple forests of Tianshan [19, 20]; since then, 40% of the forest area has been damaged [21]. *In situ* or *ex situ* conservation of wild apple via propagation nurseries and the development of biotechnological tools to address this problem are necessary.

In our recent study, we showed the microbial diversity of the larval gut of invasive *A. mali* collected from a Tianshan wild apple forest using throughput sequencing and microbiological approaches to detect fungal species. However, the analysis demonstrated the absence of fungal species [22]. Recently, Zhang et al [23] demonstrated that both bacterial and fungal species can colonize adult and larval guts of *A. mali* when fed different diets. In this study, we explored the origin of *A. mali* larval gut bacteria and their antagonistic interaction with wild apple-associated fungi using microbiological, physiological and analytical chemistry approaches. We hypothesized that larvae-acquired gut bacteria compete with fungi for colonization in invaded locations. To our knowledge, this is the first study to show the origin of gut bacteria and their competition with fungi in new areas.

Methods

Plant material collection and bacterial species

The wild apple twigs were collected from forest at the Yili Botanical Research Station (43°22 N 83°34 W) of Xinyuan County of the Ili-Kazakh District of Xinjiang-Uyghur Autonomous region, China, which is located in the Ili Valley of the Tianshan Mountains. Uninfested, larvae infested, dead twigs and as well as larval frass were collected.

Gut bacterial species (*Pseudomonas synxantha* (#283), *Ps. orientalis (#24), Erwinia billingiae (#32), E. persicina (#12),* and four *Pantoea species strains (#2, 43, 153, 287)* were used from bacterial cryopreserved stock from previous study [22]. Bacteria were grown in Luria-Bertani Broth medium (Sigma).

Isolation of apple associated fungi and bacteria

Uninfested, infested and died wild apple twigs, and as well as larval frass were used to isolate fungi and bacteria. Larval frass was collected from serpentine galleries made by larvae after removal twig bark. Next, twigs and frass were grinded with home blender in sterile conditions. Ground tissues were placed on the respective potato dextrose agar (PDA) medium (potato starch 4 gm L^{-1}, dextrose 20 gm L^{-1}, and agar 15 gm L^{-1}, pH 5.6) (Potato Dextrose Agar, Solarbio, P8931-250G) and nutrient agar (NA) (0.5%peptone, 0.3% beef extract, 1.5% agar, pH 6.8) (Difco, France) for fungi and bacteria isolation and incubated at 28 ^{o}C. Single colonies were isolated and re-cultivated to classify their morphological features.

DNA extraction

Fungal isolates were cultivated on PDA medium for 7 - 14 days upon sufficient production of mycelia. Mini- preparation of fungal DNA method [24] with minor modification was used to extract fungal DNA. Briefly, cell walls of fungi mycelia were grinded with mortar and pestle in the presence of liquid nitrogen. One milliliter of lysis buffer (400mM Tris-HCl [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl, 1% sodium dodecyl sulfate) was added into fine powdered fungal mycelia. Next, mix was transferred into 2-mL Eppendorf tube, and thoroughly mixed, and left at room temperature for 10 min. Then, added 0.3 mL of potassium acetate (pH 4.8; which is made of 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid, and 28.5 ml of distilled water), vortexed briefly and centrifuged at 10 000 g for 1 min. The supernatant is transferred into new 2-mL Eppendorf tube and equal volume of isopropyl alcohol was added and mixed by inversion. Tubes were centrifuged at 10 000 g for 2 min and supernatant was discarded. Pellet washed with 0.3 mL of 70% ethanol and was spun at 10,000 g for 1 min, the supernatant was discarded. The DNA pellet was air dried and dissolved in 50 mL of 1 x Tris-EDTA buffer. Bacteria DNA extraction was carried out following our earlier study [22].

PCR analysis and sequence analysis

For apple associated fungi isolate identification, the internal transcribed spacer (ITS) region was amplified using primer pairs ITS1 (5¢-TCCGTAGGTGAACCTGCGG-3¢) and ITS4 (5¢-TCCTCCGCTTATTGATATGC-3¢) [25]. Amplifications were performed in a total volume of 50 µl containing 10 µl of PrimeSTAR HS (Premix) (Takara, Japan) with an appropriate concentration of dNTPs (0.2 mM) and Taq polymerase (5 U), 1 µl (0.2 µM) of each primer, and 1 µl of diluted DNA. The PCR conditions included 5 min at 95°C for the initial step followed by 35 cycles at 94°C for 15 s (denaturation), 55°C for 30 s (annealing), and 72°C for 2 min (elongation), with a final extension at 72°C for 10 min. PCR products were visualized on a 1.0% agarose gel. PCR products were sequenced bidirectionally with the Sanger method in Beijing Genomics Institute (Shenzhen, China).

PCR amplification of 20-fold-diluted bacterial DNA was performed on a Veriti thermocycler (Applied Biosystems, USA) using forward 27F 5'-AGAGTTTGATCATGGCTCAG-3' and reverse 1492R 5'-TACGGCTACCTTGTTACGACTT-3' primers [26]. PCR reaction and condition were performed following our earlier study [22].

Sequences were assembled using SeqMan (DNASTAR Lasergene 7). Sequences of ITS and 16S RNA were compared with respective other orthologous fungal and bacterial sequences deposited in GenBank using the BLASTN algorithm. Representative OTUs and sequences from the Sanger method were aligned with CLUSTALW. A phylogenetic tree was constructed based on the Neighbour-joining algorithm following the Tajima-Nei model with 1000 bootstrap replicates in MEGA7.

Extraction and purification of antifungal compounds

Each gut bacteria were cultured in 2 L Erlenmayer flasks with 5 L of LB liquid medium. After 5 days, the culture was centrifuged at 8000 rpm for 10 minutes to obtain cell-free supernatant. Supernatant were dehydrated under fume hood. Initially, small part of dried content were divided into three parts and dissolved in either petroleum ether, or dichlormethane or methanol to determine efficient extracting solvent for antifungal compound. Next, these extracts were vortexed and centrifuged at 10000 rpm for 5 min. Supernatants were concentrated with rotary evaporator and contents were dissolved in 1 mL of appropriate solution and examined its antifungal ability using agar diffusion assay against selected fungi.

Rest of dehydrated supernatant was extracted several time with effective dichlormethane. solvent, and phase was concentrated using a rotary evaporator (IKA RV8V, Germany). Crude extract was fractionated with silica gel or sephadex columns chromatogrpahy. Crude extract was mixed with equal mass of silica gel (200-300 mesh) (Qingdao Marine Chemical Company, China), mixed and loaded on top of the chromatography column (80 cm length and 5 cm diameter) containing 280 g of Silica gel. Chromatography column was washed with a mobile phase (v/v) with different proportion of petroleum ether : methanol (100:0, 36:1, 18:1, 9:1, 4:1, 2:1, 1:1, 0:100 v/v) and ethyl actetate : methanol (9:1, 4:1, 2:1, 1:1, 0:1 v/v). About 7 mL eluate was collected in glass vials, monitored with thin layer chromatography (TLC), and examined each fraction for antifungal activity by agar diffusion. Active fractions were combined based on TLC and diffusion agar results, and concentrated using the rotary evaporator. Next, combined fractions were loaded on to sephadex column (50 cm length and 1.5 cm diameter). Sephadex column (Sephadex LH-20, Amersham Pharmacia Biotech, Sweden), was washed with a mobile phase of chloroform : methanol proportion (1:1 v/v). Fractions with 7 ml was collected to glass vials and monitored with TLC. Antifungal activity was examined by agar diffusion against fungi. Next, based on TLC and activity results active fractions were combined and re-extracted again by silica gel column. Further, column was washed with a mobile phase with different proportion of hexane : ethyl acetate (100:0, 40:1,

30:1, 20:1, 15:1, 12:1, 10:1, 0:100 v/v). Fractions were examined by TLC and for antifungal activity. Positive fractions were purified with sephadex column by washing with different proportion of dichlormethane : methanol mobile phase (100:0, 70:1, 0:100).

Thin layer chromatography, HPLC, Mass spectrometry and NMR analyses

To investigate the qualitative compositions of the antifungal compound, TLC was applied. TLC analysis was used to monitor the fractions from column chromatography, and spots on Silica gel plates were visualized by spraying with solution (1.5 % of aluminum chloride in ethyl alcohol, ammonia vapor, 5% sulfuric acid in ethyl alcohol pre-heated at 105 °C). Next, purity of compound measured by an UV spectrophotometer (UV-2550 Shimadzu, Japan).

HPLC analysis was performed using a Hitachi Chromaster HPLC system consisting of an 1110 pump, DT-230 column oven, 1430 diode array detector and a YMC C18 column (250×4.6 mm, 5 µm). HPLC analysis was performed with EZChrom Elite software. Mobile Phases: water and acetonitrile and methanol. Mass spectra were measured in a 2690-ZQ 4000 Water-Alliance LC-MS spectrometer (Applied Biosystems/MDS Sciex Concord, ON, Canada). ^{1}H NMR, ^{13}C NMR, and 2D NMR spectra recorded on Varian MR-400, VNMRS-600 NMR spectrometers with TMS as an internal standard.

Agar diffusion

To determine antagonistic abilities of gut bacteria, mix of half of ISP2 medium (yeast extract 4 g L^{-1}, malt extract 10 g L^{-1}, Dextrose 4 g L^{-1}, agar 20 g L^{-1}, pH7.2) and half of PDA were used to pour onto plastic Petri dish (90 cm in diameter). Next, each gut bacteria were co-cultured with different fungi isolates. Co-cultivation experiment in single plate was repeated and the antagonistic ability of bacteria was determined by evaluation of distance between bacterial growth edge (from the fungal side) and fungi growth edge (from the bacterial side). Inhibition of fungal growth was calculated by using following equation [27]:

$$I=(1-a/b)*100$$

where *a* is the distance from the center of fungal colony to fungi growth edge (from the bacterial side and bacterial growth edge and *b* is the radius of control of fungal colony.

For examine antifungal activity of chromatography fractions, 3-mm in diameter wholes were punched out in PDA plates with whole puncher. Fraction from each column chromatography was loaded into well under sterile flow cabinet. 5 mm piece of fungi mycelium grown on PDA was punched out and transferred onto the middle of PDA plate. Plates were cultured for 7 days at 25 ^{o}C and fungi growth was recorded every day of post cultivation.

Determining enzymatic activities

To evaluate different enzymes activity of lignocellulolytic pathways that are involved in the degradation of plant cell-wall compounds such as cellulose, lignin, glucans, cellobiose and xylan. For cellulose degradation, ligninolytic activity and lignin oxidation assay were performed following reports by Vasanthakumar *et al*. and Machado *et al*. [28, 29]. Activities of xylanase, cellobiase and glucanase were determined by evaluation of coloration of respective substrates [30, 31]. For lipolytic and proteolytic activities, respective tween 20/80 and milk-powder were used as substrate. Enzymes activities of gut bacteria were evaluated by appearance of clear halos [32, 33].

Minimum Inhibitory Concentration (MIC)

The MIC for selected fungi where determined using a 10-fold serial dilution method. Petri dishes with PDA medium was prepared. Diluted pure compound with respective concentrations (20, 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08 µg) was mixed with PDA medium. The punched out fungi mycelium with 0.7-mm in diameter gel piece was transferred onto the middle of agar plate, and mycelial growth was evaluated after 3, 6, 8, 10, 12 days of post incubation at 25 ^{o}C. Each treatment was performed in triplicate, and diameter of mycelial growth inhibition was measured with calliper, and calculated according to following equation

$$MGI=[\frac{Dc-Dt}{Dc}x100$$

where Dc (mm) is mean of colony diameter in the control and Dt (mm) is mean of colony diameter of each treatment.

Scanning Electron Microscopy (SEM)

To examine the effect of antifungal compound on fungi hyphae structure, the wild apple associated *Dothiorella sarmentorum* fungi was selected. The aerial mycelium of fungi, a glass coverslip was placed on the surface of PDA medium inoculated with a mycelial plug and cultured for 4, 6 or 12 days. Glass coverslips covered with mycelium were directly coated with gold by an Iron Sputter Coater (SuPro, ISC 150, Germany) and viewed under a scanning electron microscope (Carl Zeiss Jena, SUPRA 55VP, Germany) as described earlier our study [34].

Statistical analysis

StatView software packages were used to perform Fisher's PLSD test following an ANOVA (SAS Institute Inc., Cary, NC, USA).

Results

Identification of cultivable wild apple-associated fungi and bacteria

To elucidate competition between wild apple-associated fungi and bacteria in terms of the larval gut of *A. mali* colonization, several molecular, microbiological, and analytical tools were applied. For this purpose, we developed an exploratory strategy to conduct stepwise identification of host tree-associated microorganisms and screen gut bacteria for antagonistic ability as well as determine the origin of gut bacteria, as depicted in Figure 1.

Based on the workflow, we first isolated fungi and bacteria from different states of twigs, such as noninfested, larvae-infested and dead twigs, as well as larval frass, to obtain as many species as possible. A total of 204 monoconidial fungal and 320 bacterial isolates were obtained. Each fungus and bacterium was individually isolated. Each fungal colony grown on PDA and bacterial colony on NA was characterized, and individual DNA was extracted from each isolate. For preliminary identification of fungal and bacterial isolates, the respective internal transcribed spacer (ITS) and 16S RNA regions were amplified. The traditional Sanger method was performed to sequence the regions. Next, sequences of host tree-associated bacterial isolates and sequences of gut bacteria from our earlier study [22] were compared to understand the origin of gut bacteria. Then, the antifungal activity of gut bacteria against host tree-associated fungi was determined. Antifungal compounds were isolated from the strongest gut bacteria, which were hypothesized to prevent fungal growth in the gut. Further, the activity of the antifungal compounds was analysed.

Sequence analysis among the 204 culturable fungal and 320 bacterial isolates demonstrated 23 and 54 operational taxonomic units (OTUs), respectively (Figure 2). Fungal isolates were distributed into 2 phyla, 7 classes, 13 orders, 15 families and 19 genera, whereas bacterial isolates corresponded to 4 phyla, 8 classes, 14 orders, 17 families and 27 genera (Figure 2; Tables 1 and 2).

Table 1. Fungal isolates identified from wild apple stems by ITS sequence analysis and their taxonomic status.

Phylum	Class	Order	Family	Genus	Predicted species	GenBank accession	Number of isolates
Ascomycota	Chaetothyriomycetidae	Chaetothyriales	Herpotrichiellaceae	*Capronia*	*Ca. coronata*	NR_154745***	1
	Dothideomycetes	Pleosporales	Pleosporaceae	*Alternaria*	*A. dactylidicola*	NR_151852*	1
					A. destruens	NR_137143	83
					A. forlicesenensis	NR_151853*	2
					A. sorghi	NR_160246*	1
			Melanommataceae	*Uzbekistanica*	*U. rosae-hissaricae*	NR_157549*	5
		not ranked	*not ranked*	*Tumularia*	*T. aquatica*	NR_145347**	8
	Eurotiomycetes	Eurotiales	Trichocomaceae	*Aspergillus*	*As. pseudonomius*	NR_137444*	1
				Penicillium	*P. raistrickii*	NR_119493*	4
	Leotiomycetes	Phacidiales	*not ranked*	*Pallidophorina*	*Pa. paarla*	NR_119749*	6
	Sordariomycetes	Hypocreales	Sarocladiaceae	*Sarocladium*	*S. strictum*	NR_111145*	1
			Nectriaceae	*Fusarium*	*F. petersiae*	NR_156397*	4
			not ranked	*Trichothecium*	*T. ovalisporum*	NR_111321*	1
		Dothideales	Dothioraceae	*Aureobasidium*	*Au. melanogenum*	NR_159598*	2
		Pleosporales	Didymellaceae	*Ascochyta*	*As. phacae*	NR_135942*	3
				Didymella	*D. keratinophila*	NR_158275*	36
		Capnodiales	Davidiellaceae	*Cladosporium*	*C. austroafricanum*	NR_152288*	1
					C. pini-ponderosae	NR_119730*	1
				Peyronellaea	*Pe. prosopidis*	NR_137836*	29
			Montagnulaceae	*Kalmusia*	*K. variispora*	NR_145165*	4
		Pleosporineae	Cucurbitariaceae	*Parafenestella*	*Par. pseudoplatani*	NR_165542*	8
		Botryosphaeriales	Botryosphaeriaceae	*Dothiorella*	*Do. sarmentorum*	NR_111166*	1
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	*Psathyrella*	*Ps. umbrosa*	NR_161031***	1

Asterisks indicate > 97%* > 95%** >85%*** similarities.

Table 2. Identified bacterial isolates from wild apple stems by 16S RNA sequence analysis and their taxonomic status.

Phylum	Class	Order	Family	Genus	Predicted species	GenBank accession	Number of isolates
Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	*Aeromicrobium*	*Ae. fastidiosum*	NR_044983.2*	9
		Corynebacteriales	Nocardiaceae	*Rhodococcus*	*Ro. sovatensis*	NR_156055.1*	1
					Ro. yunnanensis	NR_043009.1*	3
				Williamsia	*W. limnetica*	NR_117925.1*	3
					W. muralis	NR_037083.1**	1
		Micrococcales	Microbacteriaceae	*Agreia*	*A. pratensis*	NR_025460.2*	5
				Clavibacter	*Cl. michiganensis*	NR_134712.1*	1
				Curtobacterium	*Cu. flaccumfaciens*	NR_025467.1*	25
				Frigoribacterium	*Fr. endophyticum*	NR_134732.1****	2
				Frondihabitans	*Fh. sucicola*	NR_125644.1**	6
				Herbiconiux	*H. flava*	NR_113225.1**	4
				Plantibacter	*P. flavus*	NR_025462.1*	1
				Rothia	*R. dentocariosa*	NR_074568.1*	1
				Subtercola	*S. lobariae*	NR_156868.1***	1
	Thermoleophilia	Solirubrobacterales	Patulibacteraceae	*Patulibacter*	*Pa. americanus*	NR_042369.1*	3
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*B. amyloliquefaciens*	NR_117946.1**	1
					B. atrophaeus	NR_024689.1**	1
					B. flexus	NR_113800.1*	1
					B. methylotrophicus	NR_116240.1*	15
					B. nakamurai	NR_151897.1*	1
			Staphylococcaceae	*Staphylococcus*	*St. epidermidis*	NR_036904.1*	1
Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Flavobacterium*	*F. piscis*	NR_133746.1*	1
					F. saccharophilum	NR_112839.1*	4
	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	*Pedobacter*	*Pe. ginsenosidimutans*	NR_108685.1*	2
					Pe. petrophilus	NR_156885.1*	1
					Pe. westerhofensis	NR_042602.1*	2
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Rhizobium*	*Rh. huautlense*	NR_024863.1**	4
					Rh. smilacinae	NR_148270.1*	3
					Rh. soli	NR_115996.1*	4
		Sphingomonadales	Sphingomonadaceae	*Novosphingobium*	*N. barchaimii*	NR_118314.1*	1
				Sphingomonas	*Sp. faeni*	NR_042129.1**	2
	Betaproteobacteria	Burkholderiales	Comamonadaceae	*Variovorax*	*V. boronicumulans*	NR_114214.1*	1
					Sp. ginsenosidivorax	NR_117830.1**	1
					Sp. qilianensis	NR_146363.1*	1
	Gammaproteobacteria	Enterobacterales	Erwiniaceae	*Erwinia*	*E. billingiae*	NR_104932.1*	30
				Pantoea	*P. agglomerans*	NR_041978.1*	9
			Pectobacteriaceae	*Biostraticola*	*Bi. tofi*	NR_042650.1***	1
		Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	*Ps. arsenicoxydans*	NR_117022.1*	19
					Ps. azotoformans	NR_113600.1*	3
					Ps. caspiana	NR_152639.1*	13
					Ps. donghuensis	NR_136501.2**	11
					Ps. graminis	NR_026395.1*	36
					Ps. helmanticensis	NR_126220.1*	5
					Ps. koreensis	NR_025228.1*	3
					Ps. lurida	NR_042199.1	23
					Ps. lutea	NR_029103.1*	10
					Ps. orientalis	NR_024909.1*	5
					Ps. rhizosphaerae	NR_029063.1**	2
					Ps. silesiensis	NR_156815.1*	5
					Ps. synxantha	NR_113583.1*	1
					Ps. syringae	NR_074597.1**	8
					Ps. trivialis	NR_028987.1*	8
		Xanthomonadales	Rhodanobacteraceae	*Luteibacter*	*L. rhizovicinus*	NR_042197.1*	7
			Xanthomonadaceae	*Pseudoxanthomonas*	*Px. spadix*	NR_042580.1**	8

Asterisks indicate > 99%* > 98%** > 97*** >96%**** similarities.

The abundance and composition of apple twig fungi and bacteria differed depending on the twig state and larval frass (Figure 3). The abundance of fungal isolates was increased in infested twigs compared to noninfested and dead twigs, whereas bacterial diversity was richer in noninfested twigs than in either infested or dead twigs. Additionally, the number of fungi and bacterial isolates also differed in larval frass (Figure 3A). Fungi rather than bacteria mostly colonized dead twigs, whereas a higher abundance of bacterial isolates was found in larval frass.

The composition of isolates demonstrated that fungi and bacteria can colonize twigs commonly or specifically. The distribution of isolates by phylum, family and genus revealed diverse colonization of different twigs and larval frass (Figure 3B). For example, an additional fungal phylum, Basidiomycota, was found in dead twigs, whereas bacteria were reduced to one phylum. This suggests that increased saprophytic fungi in dead twigs could antagonistically reduce bacterial species.

Larval attack replaced the bacterial phylum Firmicutes by Bacteroidetes, and a similar replacement was observed in larval frass. The Bacillaceae, Staphylococcaceae and Comamonadaceae families were specific to noninfested twigs, whereas the Flavobacteriaceae, Nocardiaceae, Sphingobacteriaceae families were specific to infested twigs; Pectobacteriaceae, Rhodanobacteraceae and Xanthomonadaceae were specific to the larval frass. At the genus level, *Frondihabitans, Plantibacter, Bacillus, Staphylococcus, Sphingomonas* and *Variovorax* were replaced by *Rhodococcus, Clavibacter, Williamsia, Rothia, Flavobacterium, Pedobacter* and *Novosphingobium* in the infested twigs and *Luteibacter, Pseudoxanthomonas, Biostraticola, Subtercola, and Herbiconiux* in the larval frass. Dead twigs showed only one species, *Curtobacterium flaccumfaciens*, with the lowest abundance.

The distribution of fungal species by their taxonomy differed from that of bacteria. The genera *Cladosporium* and *Dothiorella* specifically colonized noninfested twigs while *Uzbekistanica, Tumularia, Sarocladium, Ascochyta, Didymella, Peyronellaea* and *Fusarium* were detected from infested twigs. *Trichothecium* and *Psathyrella* as well as *Aureobasidium* and *Capronia* were specific to dead twigs and larval frass, respectively. Distribution by species composition of fungi and bacteria revealed that 16 bacterial species were specific to non-infested twigs, 10 to infested twigs, and 9 to larval frass, whereas 6 fungal species were specific to non-infested twigs, 5 to infested twigs, 3 to dead twigs, and 3 to larval frass. Taken together, during larval infestation, host tree-associated bacterial composition was reduced, whereas fungal species were increased.

Determining the antagonistic ability of larval microflora

In our previous study, we demonstrated the rich diversity of the *Agrilus mali* larval gut microbiota, and approximately 99% of the taxa were cultivable bacteria [22]. To determine the antagonistic ability of the larval gut bacteria towards apple-associated fungi, *in vitro* screening was performed using cocultivation (Figure 4A). For this experiment, 25 fungi belonging to different species isolated from apple twigs were randomly selected. The results indicated that all gut bacteria demonstrated antagonistic ability in a selective manner (Figure 4B). The distribution of the proportion of total inhibition differed. Among them *Ps. synxantha* was able to inhibit fungal isolates with a 41.8% share (24 fungal species), *Ps. orientalis* with an 11.0% share (12 fungal species), *E. billingiae* with a 10.4% share (16 fungal species), *E. persicina* with a 13.3% share (17 fungal species), *Pantoea* sp. with 2.5% share (2 fungi species). Three *P. aqqlomerans* strains, 2, 153, and 43, inhibited fungi with a 14.0% (16 fungal species), 3.5% (6 fungal species), and 3.5% share (5 fungal species), respectively (Figure 4C and 4D). The results suggests that *Ps. synxantha* was the strongest antagonist in the larval gut and was able to inhibit 90.9% of the selected host tree-associated fungal species.

Determination of the enzymatic activities of gut bacteria

Gut bacteria perform various enzymatic functions in the degradation of cell wall compounds, lipids and proteins (Table 3). In a previous report, we examined the ability of gut bacteria to degrade plant cell wall components, but in this study, we showed the levels of bacterial lysocellulotic and other enzymatic activities. *Pantoea* species were able to highly degrade lignocellulosics such as cellulose, xylanase, glucanase and cellobiose but not lignin, lipids or proteins. Both *Erwinia* species showed weak cellulolytic activity, and only *E. persicina* demonstrated xylanase, glucanase, and lipase activities. *Pseudomonas* species were not able to degrade plant cell wall compounds but showed strong protease activity. Moreover, *Ps. orientalis* showed weak lignocellulolytic and strong lipolytic activities. Lignin oxidation was not observed by any gut bacteria.

Table 3. Enzymatic activities of gut bacteria from insect larvae.

Species	Cellulose	Xylanase	Glucanase	Cellobiase	RBBR[a]	Protease	Lipase	LAC
E. billingiae 32	+	-	-	-	-	-	-	-
E. persinia 12	+	++	+	-	-	-	++	-
P. aqqlomerans 2	+++	++	++	+	-	-	-	-
P. aqqlomerans 153	++	++	++	+	-	-	-	-
P. aqqlomerans 43	+++	++	++	+	-	-	-	-
Pantoea sp. 287	++	++	++	+	-	-	-	-
Ps. orientalis 24	-	+	-	-	+	+++	+++	-
Ps. synxantha 283	-	-	-	-	-	+++	-	-

[a]Lignin peroxidase activity in MEA-RBBR.

Bacterial antifungal compounds

To isolate antifungal metabolites from gut bacteria, dehydrated bacterial cultures were extracted using different solvents to determine the extraction efficiency. The results showed that methanolic extracts from *Ps. synxantha*, *Ps. orientalis*, *P. aqqlomerans* 2, *E. billingiae* and *E. persicina* demonstrated the highest antifungal activities compared to other solvents (Table 4). The strongest antifungal activity was observed for *Ps. synxantha* and *P. aqqlomerans* 2 compared to other gut bacteria. Since, *Ps. synxantha* was able to strongly inhibit almost all selected fungal species and could be a potential bacterium preventing fungal growth in the gut, further work along these lines will be continued. Moreover, it was reported that *P. aqqlomerans*, *Ps. orientalis* and *Erwinia* species can produce known antifungal compounds.

An estimated 280 mg crude methanolic extract from *Ps. synxantha* was re-extracted with silica and Sephadex gel column chromatographies using different solvent systems. Each fraction was examined with agar diffusion to determine its antifungal activity and monitored with TLC. For the final purification of the active fraction, Sephadex column chromatography was used with a dichloromethane mobile phase. The purity of the active fraction was analysed with preparative HPLC, showing a single symmetrical peak at 250 nm with a retention time of 9.1 min. The dried pure compound was greenish-yellow, needle-crystalline, and soluble in chloroform, DMSO and methanol but insoluble in water.

Table 4. Effect of different solvents on the extraction efficiency of active compounds.

Bacteria	Solvent		
	Methanol	Ethyl acetate	Petroleum ether
Ps. synxantha 283	+++++	++	-
Ps. orientalis 24	+	-	-
Pantoea sp. 287	-	-	-
P. aqqlomerans 43	-	-	-
P. aqqlomerans 153	-	-	-
P. aqqlomerans 2	+++	-	-
E. billingiae 32	+	-	-
E. persicina 12	+	-	-

Pure antifungal compounds were characterized by ESI-MS, ^{1}H-NMR and ^{13}C-NMR. The molecular mass spectra demonstrated ion peaks at *m/z* 225.06544 $[M+H]^+$ (base peak) and *m/z* 247.04738 $[M+NA]^+$ (Figure 5). The 1H and 13C NMR spectral data of the compounds and their assignments are shown in Table 5. After NMR and mass spectrometry analyses, the compound of interest was determined to be phenazine-1-carboxylic acid.

Table 5. ^{1} and ^{13} NMR chemical shifts of phenazine-1-carboxylic acid and HMBC data in $CDCL_3$, *δ*, ppm at 400 MHz.

Atom position	d_H (*J* in Hz)	d_C	HMBC (H→C)
1		125.06	
2	8.53, dd (8.7, 1.1)	135.25	4, 1a
3	8.05, m	130.42	1, 4a
4	8.98, dd (7.1, 1.1)	137.57	2, 1a, COOH
1a	-	140.19	
4a	-	143.51	
5a	-	144.22	
8a	-	139.97	
5	8.35, dd (8.1, 1.8)	130.21	7, 8a
6	7.97, dd (6.6, 1.3)	131.88	5a, 8
7	8.01, m	133.36	5, 8a
8	8.29, dd (8.0, 1.8)	128.11	5a, 6
COOH		166.07	

Minimum inhibitory concentration and scanning electron microscopy

To determine the minimum inhibitory concentration, a randomly selected filamentous fungus, *Dothiorella sarmentorum* #18, was chosen because of its faster growth on PDA. This species was also used during all purification steps to determine the antifungal activity of the fractions. Purified phenazine-1-carboxylic acid at an MIC of 12.5 mg mL^{-1} was able to clearly inhibit mycelial growth of *D. sarmentorum*. Serial dilutions of the compound showed that the effective doses at 50% and 80% were 1.25 and 5 mg mL^{-1}, respectively (Figure 6A and 6B).

To further understand the effect of phenazine-carboxylic acid on fungal growth, mycelial growth was observed with SEM. Mycelia obtained from the edge of the *D. sarmentorum* colony growing in antifungal compound-free medium (control) produced hyphae with smooth surfaces (Figure 6C). With the addition of several concentrations of PCA ranging from 0.312 µg mL^{-1} to 5 µg mL^{-1} into the medium, fungal hyphae lost smoothness depending on the PCA concentration. A high amount of PCA caused a greater decrease in fungal exopolysaccharide production and the failure to form a hyphal network. The results indicate that antifungal compounds damaged hyphal production, thus inhibiting fungal growth.

Origin of gut bacteria

After hatching, insect larvae can adopt bacteria either directly parentally or from the host during feeding. To understand the origin of gut bacteria, we compared 16 bacterial RNA sequences of the same species found in apple twigs and gut bacteria. For this purpose, we analysed bacteria from non-infested and infested twigs and larval frass. Since gut bacteria could be excreted, they could be found in larval frass. Sequence analysis and the neighbour-joining tree revealed that gut bacterial gene sequences were identical to those of related apple bacteria (Figure 7). *Erwinia billingiae, E. perscina, Ps. orientalis, Ps. synxantha,* and *P. agglomerans* specifically colonized noninfested twigs but were still found in infested twigs but not in dead twigs. *Erwinia billingiae* and *P. agglomerans* were detected in larval frass since these bacteria were highly abundant in the gut and thus could be excreted (Figure 6A).

Discussion

In nature, many microorganisms interact with each other to coexist, and their ecological relationships range from parasitism to mutualism [35]. Depending on the species, insects have symbiotic associations with diverse and complex resident and transient microorganisms [1, 2, 12, 36]. In previous work, the larval gut of the invasive wood-borer *A. mali* demonstrated a diverse bacterial community, but among them, the most abundant *Panthoea* spp. (99%), and some *Erwinia* species, but not *Pseudomonas* species, were able to degrade plant cell wall compounds. In this study, we explored the interaction between the gut bacteria of invasive wood-borer insect larvae and wild apple-associated fungi to elucidate the absence of fungi in the larval gut. The larval gut did not show any fungal species using either high-throughput sequencing or culture-dependent methods [22]. Moreover, we determined the origin of gut bacteria by comparison of their sequence analysis results.

Fungi and bacteria can be transiently or permanently associated with the host and transmitted to the gut from their parents or the host [7, 8]. In contrast to the results of Zhang et al. [23], who showed that *A. mali* larvae can be colonized by fungi and bacteria, our earlier report demonstrated the absence of fungi in the larval gut of *A. mali* [22]. However, both fungi and bacteria can colonize the congeneric *A. planipennis* [37]. Hypothetically, the initial invasion of Tianshan forests by *A. mali* could have involved both bacterial and fungal species that were transmitted maternally, but eventually, competition by local microorganisms within the gut could have orchestrated the gut community, resulting in the elimination of fungal species from the gut. This could result in insect larvae recompositing the gut microbiota to adapt to new environments for better insect survival. The diversity of insect gut microbiota can be determined by the environmental habitat, diet, developmental stage, and phylogeny of the host [14]. The gut microbiota could play an important role in the larval overwintering process at low temperature [38, 39], and the climate of *A. mali*'s natural habitat, the Far East, Eastern China and the Korean Peninsula, is not colder than that in Tianshan [40, 41]. The most abundant *Panthoea* bacteria of larval gut with strong lygnocellulosic ability might provide sugars as energy resources and as cryoprotectants. During overwintering, insects accumulate sugars and polyols [42, 43] that can act as cryoprotectants and enhance cold hardiness for winter survival [44].

Both fungal and bacterial compositions varied in different twigs. Bacterial abundance was high in noninfested twigs, but fungal abundance was higher in infested twigs. This indicates that plant immunity could be reduced because larvae feed on the phloem of trees by creating serpentine galleries, thus preventing nutrient movement. The transition from twig infestation to twig death thus creates the growth of saprophytic wood decaying fungi (WDF) [45, 46]. Lignin is known to be extensively degraded by WDF and other fungi [45, 46]; therefore, *A. mali* larvae gut bacteria specialized in degradation of cellulose but not lignin. Moreover, the lignin composition and content vary between xylem and phloem [47]. Wood-decaying saprophytic fungi are the majority of species belonging to Basidiomycota and Ascomycota [46]. Bacteria and WDF must interact with each other to cooccur, although WDF are well known for being highly competitive [48]. This is consistent with our result that bacterial composition was highly reduced in dead twigs. Likely, bacterial species from non-infested trees must be transmitted into the larval gut because the originated gut bacteria were reduced or not found in infested twigs (Figure 6B). Additionally, fungal isolates could also be transmitted into the *A. mali* gut, as reported by Zhang *et al.* [23], but our study showed that this might not have occurred in the Tianshan forests [22] because of the presence of gut antagonistic bacteria.

The current study demonstrated that all gut bacteria were able to antagonize tree-associated fungi with different inhibition levels. However, *Ps. synxantha* inhibited almost all fungal species with the highest inhibition rate. It is possible that this bacterium was the most competitive bacteria against fungi in the gut. Apparently, fungi also had a lower composition and low abundance in larval frass due to the presence of antifungal compounds produced by gut bacteria. However, no *Pseudomonas* species were detected in larval frass. It could be that *Pseudomonas* species might be localized in one of the upper compartments of the gut, such as the foregut or midgut. [49] demonstrated that a more diverse microbial community was found in the foregut than in the midgut and hindgut in silkworms (*Bombyx mori*).

Several studies have reported the antagonistic ability of *Pseudomonas* species against fungi by the production of various types of antifungal metabolites [50]. Phenazine carboxylic acid (PCA) is one of the antifungal compounds produced by *Pseudomonas chlororaphis, Ps. fluorescens* and *Ps. protegens* that are able to kill some lepidopteran larvae [51]. However, PCA produced from *Ps. synxantha* was not lethal to *A. mali* larvae but prevented fungal colonization of the larval gut, which likely explains the absence of fungi in the larval gut. This contrasts with the results of [23], who showed that both bacteria and fungi were found in *A. mali* larvae, including one *Pseudomonas* sp., but the species level identification was not clear. This species could be *Ps. orientalis* identified in our study as possessing weak inhibition of some apple-associated fungi. It was reported that these bacteria produce various types of antifungal compounds, such as pyoverdine, safracin, and phenazine, which are effective against bacteria and fungi [52]. This bacteria might be a true symbiont that has been isolated from most coleopteran insects due to its encoded cellulolytic enzyme involved in terpene transformation of plant resin compounds [30]; this is consistent with our result that *Ps. orientalis* play role in cell-wall degradation [22].

Other gut bacteria, such as *Panthoea* and *Erwinia,* also showed average antagonistic abilities against some apple-associated fungi despite their strong lignocellulolytic activities [22]. The most abundant gut bacteria, *P. aqqlomerans,* also had antifungal activity but was not able to inhibit all endophytic and saprophytic fungi. However, its antagonistic ability is reported in the literature [53, 54]. It was reported that *E. billingiae* also has antifungal ability against pathogenic *Heterobasidion annosum*, *Armillaria mellea* and *Fusarium circinatum* fungi infecting *Pinus radiata* trees [54, 55], and *E. persicina* has activity against *A. alternata* [56]. It is likely that bacterial species within the genus could produce similar antifungal compounds, such as herbicolin, pulicatin and pyrrolnitrin [53, 57, 58].

Conclusion

Taken together, we analysed wild apple-associated bacteria, and some of them were transmitted to the gut of *A. mali* larvae. Among them, *Ps. synxantha* produced an antifungal phenasine carboxylic acid compound that strongly inhibited apple-associated fungal species, thus preventing

fungal colonization in the larval gut. Other gut bacteria, *Panthoea* and some *Erwinia* species, participated in plant cell wall cellulose degradation. Taken together, apple-associated fungi and bacteria compete for food sources to colonize the larval gut. The gut bacterial community might participate in intensive plant cellulosic degradation to provide an energy source for larvae to better adapt and survive in new regions. To our knowledge, this is the first study to show the origin of gut bacteria and their competition with fungi to prevent gut colonization.

Abbreviations

PCA: Phenazine-1-carboxylic acid; PDA: Potato dextrose agar; NA: Nutrient agar; ITS: Internal transcribed spacer; TLC: Thin layer chromatography; SEM: Scanning Electron Microscopy; OUT: Operational taxonomic units.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

Not applicable

Competing interests

The authors declare no competing interests.

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Author contributions

T.A.B. wrote the manuscript and prepared all tables and figures; T.A.B.,Z.O.T. and G.K. contributed to the compound extraction and purification; Z.O.T. contributed to the compound analysing and structural determination; T.A.B. and Yu.G. contributed to fungi analysing; T.A.B, Z.D. and H.Sh. contributed to manuscript writing, and corrections. T.A.B. and Z.D. supervised the experiment and manuscript writing. All authors reviewed the manuscript.

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Figures

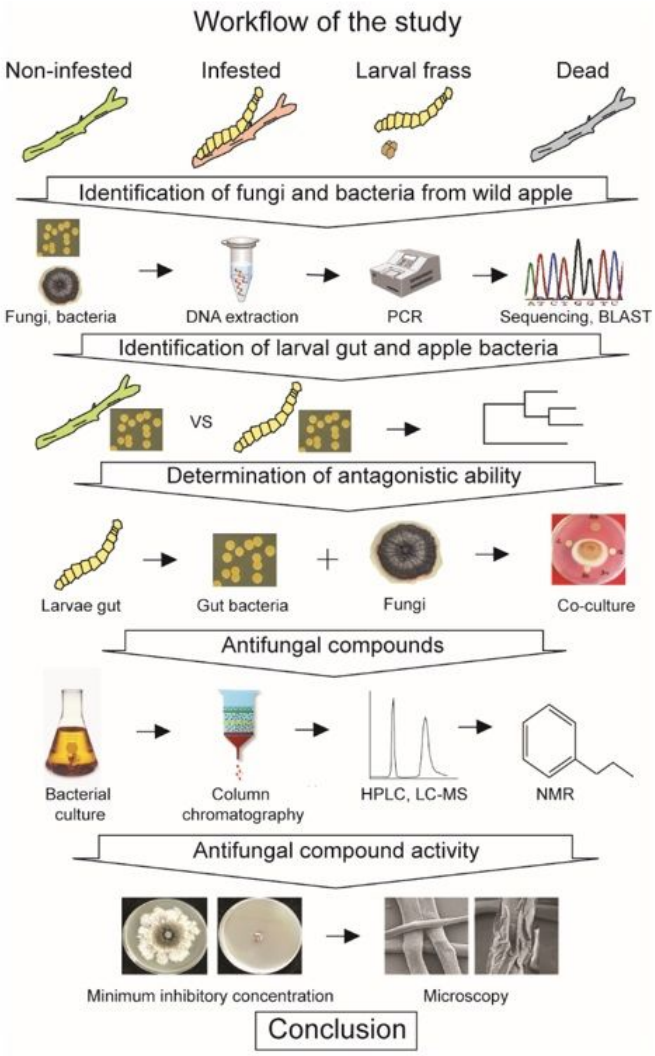

Figure 1

A workflow depicting the strategy used to investigate the gut microbiota against wild apple-associated fungi.

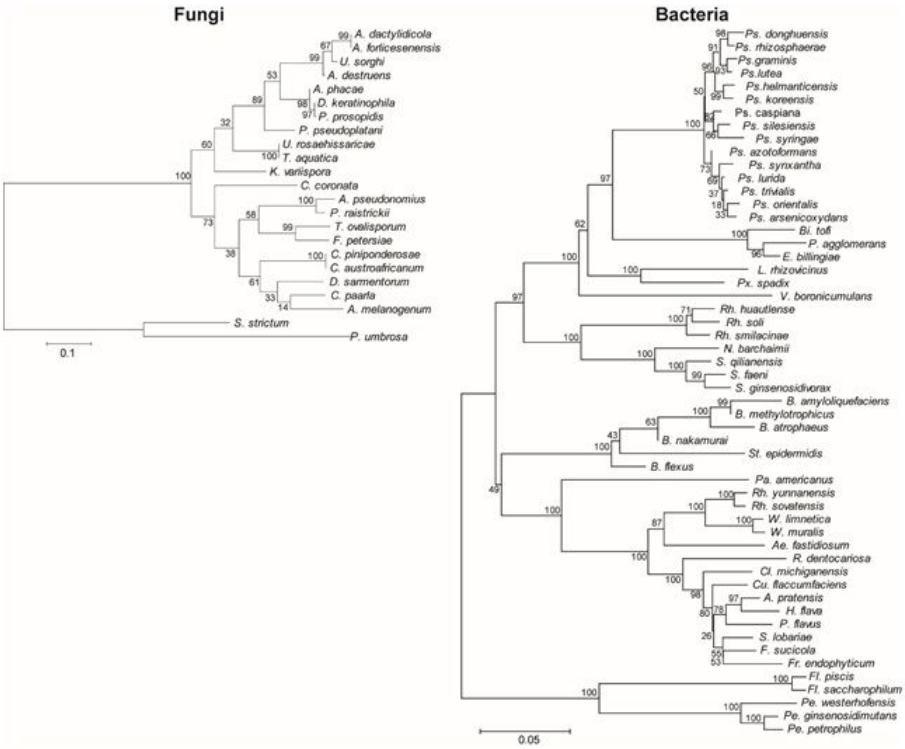

Figure 2

Clustering analysis of wild apple-associated fungal and bacterial diversity. The evolutionary history was inferred using the neighbour-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Tajima-Nei method and are in units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA 7.

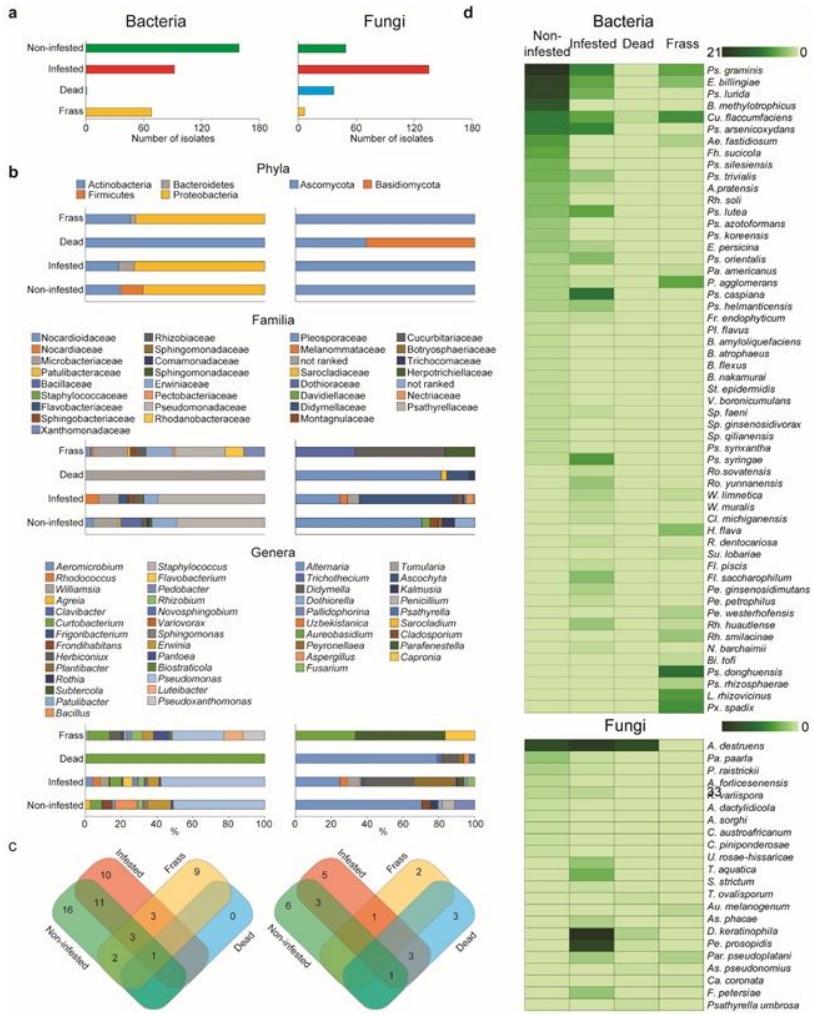

Figure 3

Fungal and bacterial diversity and abundance in different wild apple twigs and larval frass. A. Number of isolates of fungi and bacteria of different twigs and larval frass. B. Percentage distribution of isolates by their phylum, family and genus. C. Venn diagram summarizing the composition of fungal and bacterial isolates at the species level. D. Heat map analysis displaying a comparison of fungal and bacterial abundance by different twigs and larval frass.

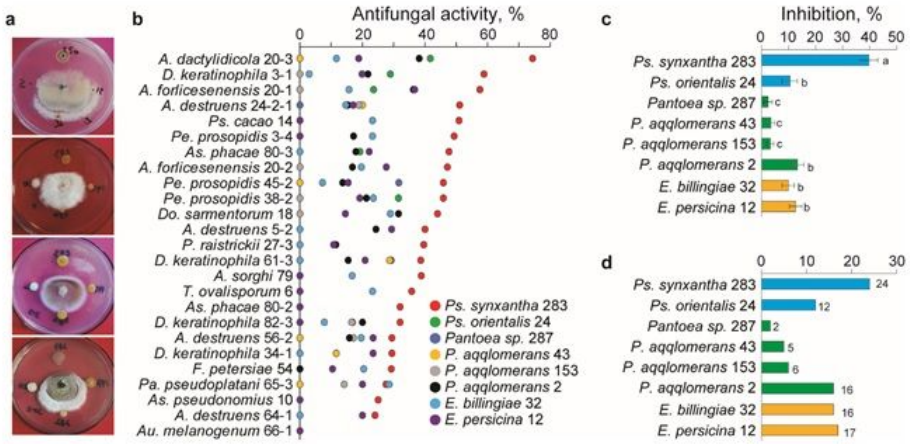

Figure 4

Antifungal activity of gut bacteria against different fungal species isolated from wild apple twigs A. Examples of gut bacterial inhibition of wild apple fungi. B. Antifungal activity of gut bacteria against selected fungi from the different types of apple twigs. C. Share of antifungal activity of each gut bacteria. Different letters indicate significant differences determined by one-way ANOVA, followed by a Fisher PLSD post hoc test ($P < 0.05$). D. Number of fungal isolates inhibited by each gut bacterium.

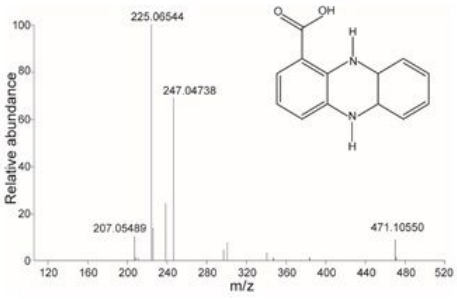

Figure 5

Mass spectra of antifungal compounds and the structure of phenazine-1-carboxylic acid (PCA)

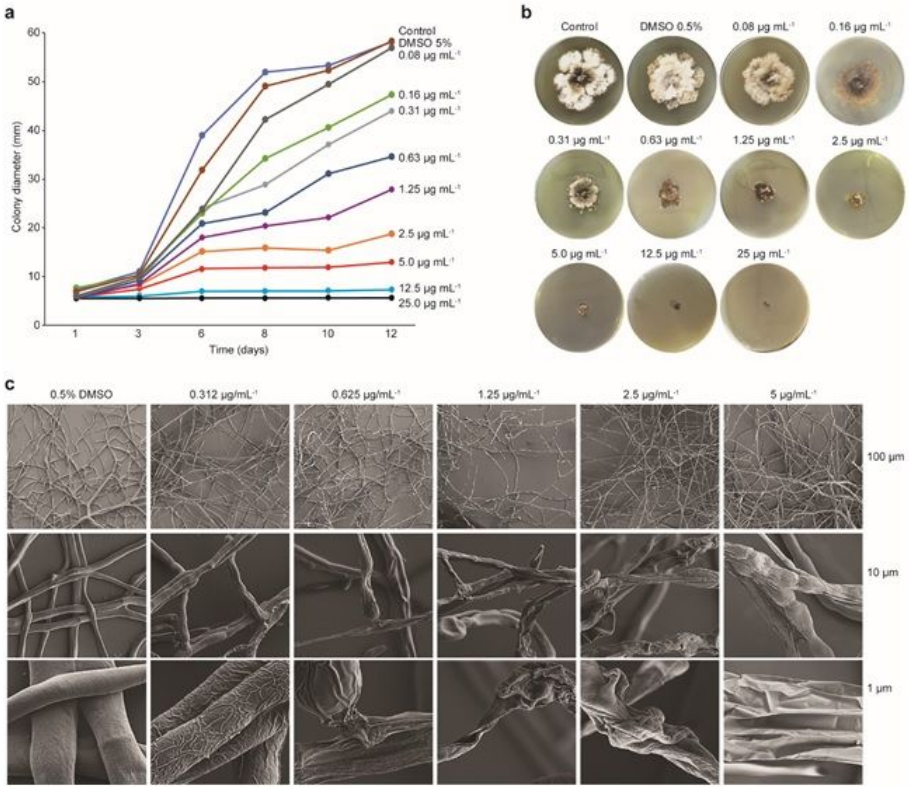

Figure 6

Minimum inhibitory concentration. Scanning electron microscopy. A. Minimum inhibitory concentration (MIC) of PCA. Graphic represents the effects of different PCA concentrations on fungal growth incubation time. Fungal growth decreased at a concentration of 0.08 mg mL-1 PCA, and complete inhibition of mycelial growth was observed at 25 and 12.5 mg mL-1. B. MIC assay after 12 d of incubation at 28oC. C. SEM images of the effect of PCA against D. sarmentorum.

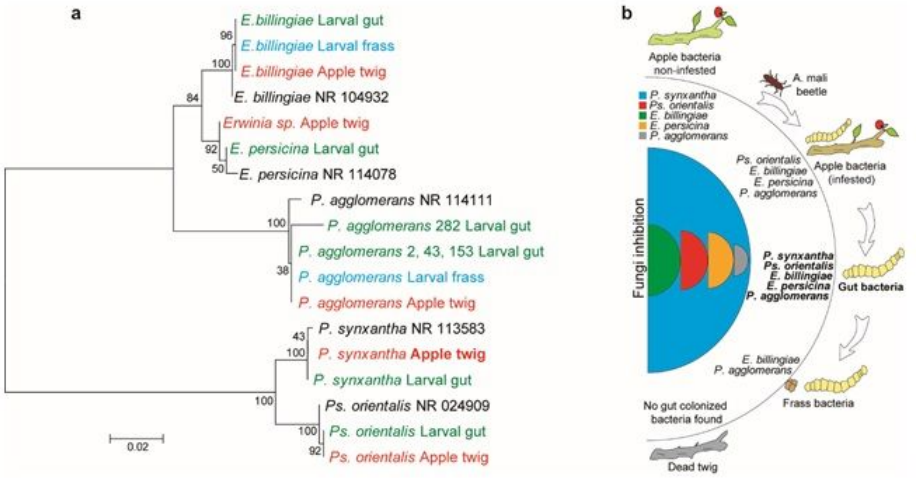

Figure 7

Identity analysis of gut bacteria and possible route of bacterial transmission to larvae A. Neighbour-joining tree of bacterial 16S RNA sequences from larval gut and apple twig-associated bacteria. Bacterial species in black, red and green words relate to GenBank, apple twigs and gut bacteria, respectively. B. Possible mechanism of transmission of tree bacteria to the larval gut.