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Research Article

Erwiniaceae bacteria play defensive and nutritional roles in two widespread ambrosia beetles

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Abstract

Ambrosia beetles are fungal-growing insects excavating galleries deep inside the wood. Their success as invaders increased scientific interest towards them. However, most studies on their microbiota targeted their fungal associates whereas the role of bacterial associates is understudied. To explore the role of abundant microbial associates, we isolated bacteria from active galleries of two widespread ambrosia beetles, *Xylosandrus crassiusculus* and *X. germanus*. These isolates were classified within the Erwiniaceae family and through a phylogenetic analysis including isolates from other insects we showed that they clustered with isolates obtained from ambrosia and bark beetles, including *Erwinia typographi*. The whole genome analysis of the isolate from active galleries of *X. crassiusculus* suggested that this bacterium plays both a nutritional role, by providing essential amino acids and enzymes for the hydrolysis of plant biomass, and a defensive role, by producing antibiotics. This defensive role was also tested *in vitro* against fungi, including mutualists, common associates, and parasites. The bacteria inhibited the growth of some of the common associates and parasites but did not affect mutualists. Our study supported the hypothesis of a mutualist role of Erwiniaceae bacteria in ambrosia beetles and highlighed the importance of bacteria in maintaining the symbiosis of their host with nutritional fungi.

Keywords: Ambrosia beetles; *Xylosandrus crassiusculus*; *Xylosandrus germanus*; *Erwinia*; Symbiosis; Mutualists

Introduction

Ambrosia beetles (Coleoptera: Curculionidae: Scolytinae and Platypodinae) are a polyphyletic group of fungus-growing insects that are mostly distributed in tropical and subtropical regions of the world (Hulcr et al. 2015, Kirkendall et al. 2015, Hulcr and Stelinski 2017). Several of them colonize stressed trees containing and emitting ethanol (Cavaletto et al. 2021, 2023, Ranger et al. 2021) by excavating galleries deep inside the wood, where they actively farm the nutritional fungal symbionts that they carry inside specific organs, called mycetangia (Skelton et al. 2019, Diehl et al. 2022, Mayers et al. 2022). In addition to being a common example of insect-microbial symbiosis (Hulcr et al. 2020), ambrosia beetles are also successful invaders. More than 50 species are established outside their native range (Lantschner et al. 2020), including a few serious pests in natural settings and orchards (Hughes et al. 2017, Gugliuzzo et al. 2021). Consequently, the interest in ambrosia beetles has increased exponentially over the last few decades.

Ambrosia beetles live in association with a wide range of microorganisms, including fungi and bacteria (Hulcr et al. 2012, Hulcr and Skelton 2023). Among fungi, nutritional symbionts, such as *Ambrosiella* spp., *Dryadomyces* spp. and *Raffaelea* spp. (Ascomycota: Ophiostomatales) (Hulcr and Skelton 2023, Osborn et al. 2023), are commonly present together with several other species that can be found both in the mycetangia and other body parts (Kostovcik et al. 2015, Bateman et al. 2016, Miller et al. 2019, Strzalka et al. 2021, Kolarik and Hulcr 2023). These additional fungi can be acquired from the tree hosts and the environment (Rassati et al. 2019, Morales-Rodriguez et al. 2021) and can be transient microbes, mutualists, or parasites of either the nutritional fungal symbionts (e.g., mycopathogens) or the beetles (e.g., entomopathogens) (Skelton et al. 2018). Among bacteria, Erwiniaceae, Enterobacteriaceae and Pseudomonadaceae represent the core part of the ambrosia beetle microbiome (Aylward et al. 2014, Ibarra-Juarez et al. 2020, Nones et al. 2021, Diehl et al. 2022, 2023), similarly to what is observed in other fungus-growing insects (Aylward et al. 2014, Barcoto et al. 2020). Their widespread presence on the beetle body, in the gut, inside the mycetangium, and in the galleries (Hulcr et al. 2012, Ibarra-Juarez et al. 2020, Diehl et al. 2022, 2023) clearly suggests that they can play a beneficial role for the beetle, potentially taking part as a third player in the bipartite beetle-fungus symbiosis.

Erwiniaceae bacteria found in association with other insects have been primarily assumed to play a nutritional role by hydrolyzing plant biomass and fixating nitrogen (Pinto-Tomás et al. 2009, Suen et al. 2010, Walterson and Stavrinides 2015). Nonetheless, they might play other important roles. As shown for *Erwinia typographi*, one of the most abundant bacterial phylotypes in the microbiome of different *Ips* bark beetle species (Skrodenytee-Arbaciauskiene et al. 2012, Chakraborty et al. 2020, Moussa et al.

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2023, Veselská et al. 2023), Erwiniaceae bacteria can also resist high concentrations of diterpenes (Skrodenytee-Arbaciauskiene et al. 2012) and can inhibit the growth of entomopathogenic fungi (Peral-Aranega et al. 2023). In addition, Erwiniaceae bacteria are known to produce antibiotics (Lim et al. 2014, Walterson et al. 2014, Smits et al. 2019). In systems characterized by an obligated symbiosis, the production of antibiotics is often involved in defending the host and its symbionts against competitors and parasites (Kroiss et al. 2010, Poulsen et al. 2005). Nonetheless, which functional roles are played by Erwiniaceae bacteria in ambrosia beetles is still unclear.

In this study, we aimed at investigating the presence and the functional roles of Erwiniaceae bacteria in ambrosia beetles. We hypothesized that these bacteria act as positive symbionts, providing different functions which include defensive and nutritional roles. To test these hypotheses, we first isolated Erwiniaceae bacteria from ambrosia beetles and we classified the isolates in a phylogenetic analysis of the partial 16S rRNA. Second, we sequenced the genome of one of the isolated Erwiniaceae bacteria and we screened for genes associated with different functional roles, including essential amino acids supplementation, plant biomass removal, nitrogen fixation, diterpene removal, and synthesis of antibiotics. Third, we carried out interaction assays between this Erwiniaceae bacterium isolate and fungi commonly found in association with ambrosia beetles to test whether the latter bacteria might play a defensive role. Our findings help to elucidate the main functions of bacteria that are part of the core microbiome of ambrosia beetles, aiding in the understanding of the multi-kingdom symbiotic web associated with this emerging insect group.

Materials and methods

Microbial isolation

Samples were obtained during an experiment conducted in a broadleaved-dominated forest in spring 2022 to compare the effect of plant stress on ambrosia beetle colonization (Riserva Naturale Integrale Bosco Nordio, Chioggia, Veneto, 45°07′30'' N, 12°15′47'' E), using 120 potted trees of various broadleaved tree species (diameter at the base 3.0–4.5 cm, height 2.0–3.0 m). One of these tree species, the European hornbeam *Carpinus betulus*, was selected for the isolation of the bacteria and fungi associated with two widespread ambrosia beetle species (i.e. *Xylosandrus crassiusculus* and *Xylosandrus germanus*). Bacteria were isolated from active galleries (i.e., galleries with living beetle colonies) in the xylem. On each tree, three active galleries per ambrosia beetle species were identified and dissected aseptically, the insects excised, and the walls scraped with a sterile scalpel. Wood tissue fragments from the gallery walls were individually placed in sterile vials and ground in 1 ml sterile NaCl 0.9% (m/v) solution using sterile plastic pestles. Consecutive decimal dilutions were performed in the same solution using the ground material. The decimal dilutions were then inoculated into three different growth media, potato-dextrose agar (PDA), Luria Bertani (LB) agar, and yeast extract/mannitol agar (YEMA) supplemented with antibiotics (2 mg/L cycloheximide and 1 mg/L streptomycin) (Biedermann et al. 2013), using Drigalski spatulas. Petri dishes were incubated at 28°C in the dark for two months. Every week, each morphotype per sample was further isolated on LB (bacteria) or PDA (fungi). Individual isolates were stored in glycerol 20% at -80°C.

Molecular identification of bacterial and fungal isolates

Isolates of bacteria and fungi were identified through barcoding. Genomic DNA was extracted using the Quick-DNA Miniprep Plus Kit (Zymo Research). For bacteria, PCR amplification of the partial 16S rRNA gene was performed according to the protocol described by Lane (1991), using the primers 27F/1492R. Fungal identification was performed using the primers ITS4/ITS5 (White et al. 1990) for all but Ophiostomatales, for which the primers SSUfungiF/SSUfungiR were used (Ibarra-Juarez et al. 2020). The ITS markers do not allow good barcoding results for fungi in this taxonomic group (Diehl et al. 2022, 2023). The PCR products were Sanger sequenced by BMR Genomics (Padova, Italy), using a 3730XL DNA Analyzer (Applied Biosystems). The sequences were edited with DNAbaser to remove low quality regions and identified by searching for close relatives using the BLAST tool (Altschul et al. 1990).

Phylogenetic analyses of Erwiniaceae bacteria

A phylogenetic analysis based on the partial 16S rRNA gene marker was performed using sequences obtained from the Erwiniaceae bacteria isolated from active galleries of ambrosia beetles and others retrieved from the NCBI GenBank database. The latter sequences belonged to microbes isolated from the body of ambrosia beetles, bark beetles and other Coleoptera (Herndon 1999, Cardoza et al. 2009, Morales-Jiménez et al. 2009, Skrodenytė-Arbačiauskienė et al. 2012, Ahmet and Hatice 2013; Biedermann unpublished data), Diptera (Pidiyar et al. 2004, Joyce et al 2011, Chandel et al. 2013, Valiente Moro et al. 2013), Hemiptera (Kenyon et al. 2015, Gonella et al. 2020), Hymenoptera (Harada et al. 1996, Pinto-Tomás et al. 2009, Suen et al. 2010, Adams et al. 2011, Vásquez et al. 2012), and Thysanoptera (De Vries et al. 2008). As references taxa we also included sequences of defined lineages used in taxonomic studies to describe the Erwiniaceae family (Walterson and Stavrinides 2015, Adeolu et al. 2016). All sequences were aligned using the MAFFT 7 CRBC Server (Katoh et al. 2019) and trimmed using MEGA11 (Tamura et al. 2021). The phylogenetic trees were inferred using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei 1993). The bootstrap consensus tree was inferred from 10 000 replicates, and the analysis involved 91 nucleotide sequences with 941 positions in the final dataset. Phylogenetic analyses were conducted using the MEGA11 software (Tamura et al. 2021).

Genome sequencing of an Erwiniaceae isolate from *X. crassiusculus*

A protocol combining Illumina and Oxford Nanopore sequencing was adopted to obtain the complete and assembled genome sequence of the isolate 1C4 obtained from active galleries of *X. crassiusculus*. We selected the latter isolate instead of the one from *X. germanus* because the geographic distribution of *X. crassiusculus* on a global scale is wider than that of *X. germanus* (Gugliuzzo et al. 2021). The isolate was grown to the late exponential phase in LB broth (25 °C, 150 r/m), cells were pelleted (10 min, 500 g), the supernatant was removed, cells were resuspended in cryopreservation fluid, and then stored in a Microbank tube (Pro-Lab Diagnostics, United Kingdom). The tube was sent to MicrobesNG (United Kingdom) for sequencing, following the Enhanced Service protocol. Briefly, genomic DNA libraries were prepared using a Nextera XT Library Prep Kit (Illumina, USA). DNA quantification and library preparation were performed using the Microlab

STAR system (Hamilton, USA). Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina (Roche, South Africa) on a Roche Light Cycler 96 qPCR system. Libraries were sequenced on an Illumina HiSeq using a 250 bp paired end protocol. Reads were adapter-trimmed using Trimmomatic 0.30 with a sliding window quality cut-off of Q15. Long read genomic DNA libraries were prepared with the Oxford Nanopore SQK-RBK004 kit and SQK-LSK109 kit with Native Barcoding EXP-NBD104/114 (ONT, United Kingdom) using 400–500 ng of HMW DNA. Then, twelve to twenty-four barcoded samples were pooled together into a single sequencing library and loaded into a FLO-MIN106 (R.9.4) flow cell in a GridION (Oxford Nanopore Technologies, United Kingdom).

De novo genome assembly was performed using Unicycler v0.4.0 (Wick et al. 2017). Assembly graphs were inspected with Bandage v0.8.1 (Wick et al. 2015) and contigs were annotated using Prokka v1.11 (Seemann 2014). Assembly quality and annotation completeness was evaluated using BUSCO analysis (Seppey et al. 2019). The OriTfinder server (Li et al. 2018) was used to determine the origin of transfer (oriT) and conjugation-related regions of the plasmid. The PATRIC phylogenomic service was used to construct a tree comparing the available sequenced genomes of Erwiniaceae bacteria and the isolate 1C4 obtained in this study from active galleries of *X. crassiusculus* (Wattam et al. 2017). Additional functional annotations were performed using the eggNOG-mapper against the eggNOG V 5.0 database (Huerta-Cepas et al. 2019), which includes the Carbohydrate-Active Enzymes (CAZymes) database (Cantarel et al. 2009). The PATRIC annotation service was used to predict genes associated to antibiotic resistance (Wattam et al. 2017). Biosynthetic gene clusters (BGC) annotation analysis was conducted using antiSMASH 5.0 (Blin et al. 2019) with default relaxed detection parameters. GC content and GC skew were determined using Artemis v18.1.0 (Carver et al. 2012). rRNA operons were identified using Barrnap (Seemann 2018). tRNA genes were identified using tRNAscan-SE (Lowe and Chan 2016). In order to look for genes corresponding to specific proteins of interest, single comparisons were carried out using pBLAST (Altschul et al. 1990). In particular, we investigated proteins involved in diterpene removal and nitrogen fixation, using respectively sequences from the *dit* gene cluster found in *Pseudomonas abietaniphila* BKME-9 (Martin et al. 1999), and the *nif* cluster of *Klebsiella pneumoniae* (Dixon et al. 1980). Protein candidates were excluded if they showed ≤30% of identity to the described sequences. Circular plots were generated using DNAplotter v18.1.0 (Carver et al. 2009).

In vitro interaction assays to test defensive role of an Erwiniaceae isolate from *X. crassiusculus*

To test the potential defensive role of Erwiniaceae bacteria, the isolate 1C4 obtained from active galleries of *X. crassiusculus* was confronted in co-culture using Sabouraud dextrose agar (SDA) to ambrosia beetle associated fungi that were isolated in this study from their active galleries. Other fungi used for the *in vitro* assay were obtained from a microbial collection maintained at the Department of Agronomy, Food, Natural resources, Animals, and Environment (DAFNAE), University of Padova. We selected fungi belonging to genera that are known as common associates of ambrosia beetles (McPherson et al. 2013, Malacrinò et al. 2017, Morales-Rodríguez et al. 2021, Diehl et al. 2023), including *Alternaria*, *Diplodia*, *Diaporthe*, and *Fusarium,* as well as the mycopathogen *Trichoderma atroviride* (Table S1). Additionally, we included commercially available entomopathogens (*Beauveria bassiana, Lecanicillium muscarium, Metarhizium brunneum, Paecilomyces lilacinus, Paecilomyces fumosoroseus*) and another mycopathogen (*Trichoderma harzianum*) (Table S2).

The interaction assays were conducted in a co-culture between the bacterial isolate and the different fungi. The bacterial inoculum was inoculated along a longitudinal streak of 50 μL of a late exponential culture (LB broth, 25°C, 150 r/m) across the center of the Petri dish and using a bacteriological loop. Each fungus was inoculated twice 2 cm away from each side of the bacterial streak. Fungal inocula were 6 mm circles of confluent solid media, which were prepared by incubating the fungi for 10 days at 25°C in the dark. For each fungus, a set of controls without bacterial inoculum were also used. The Petri dishes were incubated for 10 days at 25°C in the dark. After the incubation period, the total area of the dish colonized by the tested fungus (i.e., the sum of the colonized area in the two Petri dish halves) was calculated as a percentage using the ImageJ software (NIH). Each interaction assay was replicated six times. Per each set of fungi, a non-parametric Wilcoxon rank-sum test was used to compare the percentage of fungal colonization between Petri dishes with and without the bacterial inoculum. We used a non-parametric test instead of a parametric one because of the small sample size (i.e. 6 replicates) and because the data did not follow a normal distribution. The statistical analysis was performed using R (R Core Team 2021).

Results

Isolated microbes and phylogenetic analysis

Two Erwiniaceae bacteria were consistently isolated from active galleries of *X. crassiusculus* and *X. germanus*, that are 1C4 and 7G6, respectively (Table 1). The 16S rRNA phylogenetic study of these bacterial isolates showed that they are closely related to isolates from other Scolytinae species, including the ambrosia beetle *Xyleborinus saxesenii* and the bark beetles *Dendroctonus ponderosae, Dendroctonus valens*, and *I. typographus* (Fig. 1). All these bacteria clustered together and formed a monophyletic group isolated from other defined lineages of Erwiniaceae, which include bacteria isolated from different insect taxa. In addition, three fungi were consistently isolated from the active galleries of *X. crassiusculus*, i.e. *Dryadomyces* sp. and *Raffaelea* sp., which are both nutritional symbionts, and *Geosmithia pallida,* which is a common associate of ambrosia beetles (Table 1).

Whole genome sequencing of an Erwiniaceae isolate from *X. crassiusculus*

Whole genome sequencing was undertaken for isolate 1C4 from active galleries of *X. crassiusculus*, resulting in a 5.3 Mbp chromosome and a circular plasmid approximately 53.7 Kbp in length (Fig. S1), showing a BUSCO completeness score of 99.3%. A general representation of the chromosome is provided in Fig. 2; quality statistics are listed in Tables S3 and S4, and a further description of the genome can be found in the Supplementary Results. The phylogenomic analysis using a total of 100 genes (Table S5) revealed that the isolate 1C4 is more closely related to *Erwinia* spp. than to other genera in the family Erwiniaceae (Fig. S2). EggNOG V 5.0 annotation of the chromosome revealed a potential nutritional role through the presence of 65 CAZymes, including 24 glycosyl hydrolases (Table 2). Among them, we found enzymes with predicted activities related to cellulose (two β-glucosidases, and one endoglucanase), hemicellulose (α-xylosidase), and chitin (two chitinases) hydrolysis. Enzymes with α-amylase and trehalose-hydrolase activities were also annotated (Table 2). Additionally,

Table 1. List of bacterial and fungal isolates obtained in this study. Information on isolation source and barcoding is provided.

Isolate (NCBI accession number)	Source	Barcoding			
		Amplified gene	Kingdom	BLAST closest relative (NCBI accession number)	Identity bp (%)
Isolate 1C4	Active gallery of *X. crassiusculus*	16S rRNA	Bacteria	*Pantoea cedenensis* 16-CDF (FJ811867)	1017/1028 (99%)
Isolate 7G6 (OQ991373)	Active gallery of *X. germanus*	16S rRNA	Bacteria	*Pantoea cedenensis* 16-CDF (FJ811867)	911/924 (99%)
Dryadomyces sp. 2C1 (OQ991371)	Active gallery of *X. crassiusculus*	18S rRNA	Fungi	*Dryadomyces montetyi* PC06.001 (JF909512)	446/446 (100%)
Raffaelea sp. 1C2 (OQ991372)	Active gallery of *X. crassiusculus*	18S rRNA	Fungi	*Raffaelea* sp. Hulcr7507 (KX267141)	215/232 (93%)
Geosmithia pallida 4C1 (OR045359)	Active gallery of *X. crassiusculus*	ITS1-5.8S-ITS2 rRNA	Fungi	*Geosmithia pallida* U183 (HF546284)	550/550 (100%)

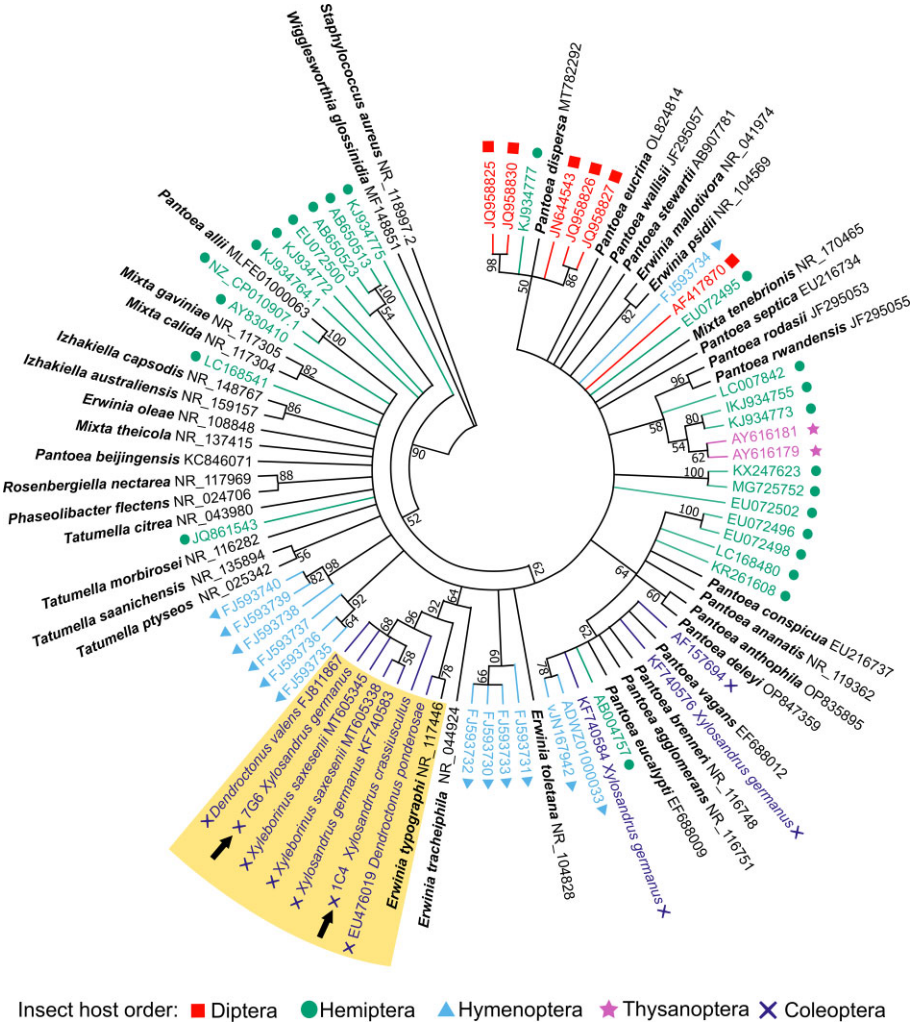

Figure 1. Phylogenetic analysis of 16S rRNA sequences of Erwiniaceae bacteria isolated in this study. Bacteria from active galleries of *X. crassiusculus* and *X. germanus* (i.e. 1C4 and 7G6, respectively) are pointed by black arrows. The clade grouping the latter two isolates and isolates from ambrosia and bark beetles obtained in other studies are highlighted in yellow. The numbers above the branches represent their calculated posterior probabilities. NCBI accession numbers and the insect host order (different colors/symbols) are also shown.

Prokka v1.11 annotation revealed genes for the synthesis of amino acids, including histidine and tryptophan. On the contrary, the pBLAST analysis showed the absence of sequences matching proteins described for the clusters *dit* and *nif*, thus not supporting roles in nitrogen fixation and diterpenes removal. The antiSMASH 5.0 *in silico* prediction revealed seven putative biosynthetic gene clusters (BGCs) including genes that encode for antibiotic synthesis, such as non-ribosomal peptide-synthetases (NRPS) and

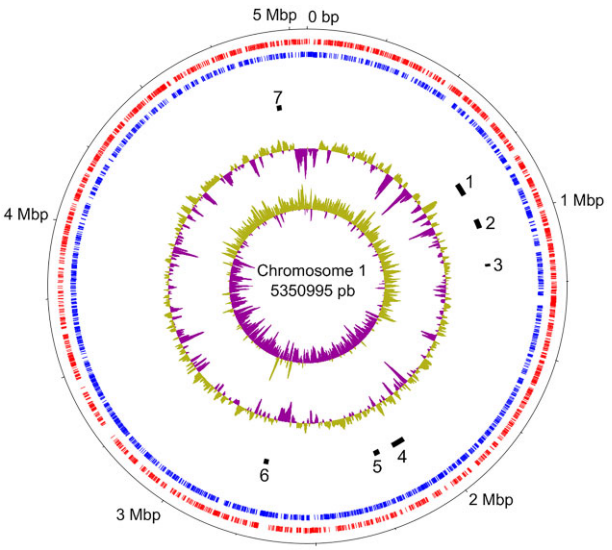

Figure 2. Chromosome map of the bacterial isolate 1C4 obtained from active galleries of *X. crassiusculus*. From outside to inside: open reading frames for the forward and reverse strands in red and blue; putative biosynthetic gene clusters from the Anti-SMASH analyses in black, showing the numbers that correlate with the information in Table 3; GC content (purple: below average, gold: above average); GC skew (purple: below average, gold: above average).

Table 2. Glycosyl hydrolases (GH) and putative activity detected in the genome of the Erwiniaceae isolate 1C4 obtained from active galleries of *X. crassiusculus*.

CAZy family	Closest relative	Predicted function
GH3	*bglX*	β-Glucosidase
GH3	*bglB*	β-Glucosidase
GH5, GH8, GH9	*bcsZ*	Endoglucanase
GH13	*amyA*	α-amylase
GH13	*ycjM*	Glucosyltransferase
GH13, GH31	*treC*	α-amylase
GH18	*ydhO*	Polysaccharide hydrolase
GH18	*chiA1*	Chitinase
GH19	*xylB*	Chitinase
GH20	*nahA*	Naphthalene dioxygenase
GH23	*emtA*	rRNA methyltransferase
GH23	*slt*	Peptidoglycan-modifying enzyme
GH23	*mltC*	Murein-degrading enzyme
GH31	*yicI*	α-xylosidase
GH37	*treA*	Trehalose hydrolase
GH37	*treF*	Trehalose hydrolase
GH77	*malQ*	4-alpha-glucanotransferase
GH102	*mltA*	Murein-degrading enzyme
GH103	*mltB*	Transglycosylase

polyketide synthases (T1PKS), supporting a defensive role. However, genes in these clusters showed variable similarity (13%–100%) to those for already described molecules (Table 3, Fig. 2). PATRIC annotation revealed the presence of multiple genes that would confer resistance to antibiotics, including 8 different efflux pumps (Table S5).

In vitro interaction assays to test defensive role of an Erwiniaceae isolate from *X. crassiusculus*

The isolate 1C4 from active galleries of *X. crassiusculus* did not affect the growth of the nutritional symbionts *Dryadomyces* sp. and *Raffaelea* sp. (Fig. 3A and B, respectively). For both fungi, the percentage of colonization did not differ between Petri dishes without or with the bacterial isolate (Table 4). On the contrary, most of the fungi from genera known to be common, but non-mutualistic associates of ambrosia beetles were inhibited by the bacterium, namely *Alternaria* sp. (Table 4, Fig. 3C), *Alternaria alternata* (Table 4, Fig. 3D), *Diplodia seriata* (Table 4, Fig. 3F), *Fusarium solani* (Table 4, Fig. 3G), and *G. pallida* (Table 4, Fig. 3H). The only associate from the culture collection which was not significantly affected by the presence of the bacterium was *Diaporthe foeniculina* (Table 4, Fig. 3E). Entomopathogens were differently affected by the bacterium, with a significant reduction for *Beauveria bassiana* (Table 4, Fig. 3I) and *Paecilomyces lilacinus* (Table 4, Fig. 3M) but no effects for *Lecanicillium muscarium* (Table 4, Fig. 3J), *Metarhizium brunneum* (Table 4, Fig. 3K), and *Paecilomyces fumosoroseus* (Table 4, Fig. 3L). Mycopathogens were also differently affected, with a significant reduction of *T. atroviride* (Table 4, Fig. 3N) but no effect on *T. harzianum* (Table 4, Fig. 3O).

Discussion

Erwiniaceae bacteria have been highlighted as part of the core microbiota of different ambrosia beetle species (Aylward et al. 2014, Nones et al. 2021, Diehl et al. 2022, 2023). Nonetheless, their functional roles in this emerging group of beetles were still unclear. Here we showed that one of these Erwiniaceae bacteria isolated from active galleries of *X. crassiusculus* is a common associate of ambrosia beetles and it may provide different benefits to their hosts, which range from nutrition, by supplementing with essential amino acids and removing plant polymers, to defense, by producing antibiotics that inhibit the growth of common associates and parasites. These results clearly highlighted that studying the ambrosia beetle bacteriome is essential to better understand the multi-kingdom interactions of this fungal-growing insect system.

Phylogenetic analysis of the bacteria isolated from active galleries of *X. crassiusculus* (i.e., 1C4) and *X. germanus* (i.e., 7G6) clearly assigned them to a clade including a few other isolates obtained from ambrosia and bark beetles, especially *E. typographi* associated with the Eurasian spruce bark beetle *I. typographus*. The latter bacterial species is the most abundant phylotype in the guts of different species of the bark beetle genus *Ips* (Chakraborty et al. 2020, Moussa et al. 2023), and the genus *Erwinia* is mentioned as part of the core microbiota of the gut and galleries of ambrosia beetles (Diehl et al. 2022, Diehl et al. 2023) as well as a common associate of other Curculionidae beetles (Berasategui et al. 2016). Nonetheless, isolates from previous studies that we showed to cluster within the clade of Erwiniaceae associated with ambrosia and bark beetles were identified as *Pantoea cedenensis* (Cardoza et al. 2009, Morales-Jiménez et al. 2009; Ahmet and Hatice 2013). This species has been isolated also from other organisms, including the pine wood nematode *Bursaphelenchus xylophilus* in Portugal (Vicente et al. 2011) and plant buds of *Prunus yedoensis* in Korea (Cheong et al. 2020). Furthermore, the reference bacteria *P. cedenensis* A34 (ATCC 700886) is reported to be an endophyte, a life habit that has not been reported for the Erwiniaceae associated with ambrosia and bark beetles so far. Our phylogenomic analysis

Table 3. Putative biosynthetic gene clusters (BGCs) in the genome of the Erwiniaceae isolate 1C4 obtained from active galleries of *X. crassiusculus*

BGC	Type	Position		Closest relative		
		From	To	Most Similar BGC	NCBI accession	Similarity (%)[a]
1	NRPS, T1PKS	779799	837887	Micacocidin	AL646052	40
2	T1PKS	957869	1000754	–	–	–
3	NI-siderophore	1164249	1176606	Desferrioxamine E	MH015039	100
4	Arylpolyene, hserlactone	2077797	2136607	Aryl polyenes	NZ_FO704550	94
5	Thiopeptide	2199226	2225445	O-antigen	AF035937	14
6	Hserlactone	2699139	2719798	–	–	–
7	Redox-cofactor	4919081	4941228	Lankacidin C	AB088224.2	13

[a]Similarity: percentage of genes that are similar to the closest known BGC.

Table 4. Percentage of fungal colonization in Petri dishes with and without the isolate 1C4 obtained from active galleries of *X. crassiusculus*. Results of the statistical analysis (i.e., W and p-value) are also included.

Fungal species	Without bacterial isolate	With bacterial isolate	W-statistics	P-value
Dryadomyces sp.	100.0 ± 0.0	100.0 ± 0.0	18	1.0000
Raffaelea sp.	32.8 ± 1.2	32.9 ± 1.7	18	1.0000
Alternaria sp.	79.04 ± 0.7	32.35 ± 1.6	36	0.0022
Alternaria alternata	29.8 ± 0.6	16.9 ± 7.2	36	0.0043
Diaporthe foeniculina	100.0 ± 0.0	100.0 ± 0.0	18	1.0000
Diplodia seriata	100 ± 0.0	83.2 ± 5.2	36	0.0028
Fusarium solani	100 ± 0.0	55.9 ± 4.0	36	0.0028
Geosmithia pallida	39.4 ± 0.6	28.4 ± 4.5	36	0.0022
Beauveria bassiana	6.9 ± 0.5	3.6 ± 0.2	36	0.0022
Lecanicillium muscarium	11.9 ± 0.6	11.7 ± 0.4	22	0.5887
Metarhizium brunneum	26.6 ± 3.4	25.9 ± 3.8	20	0.8089
Paecilomyces fumosoroseus	100.0 ± 0.0	100.0 ± 0.0	18	1.0000
Paecilomyces lilacinus	35.2 ± 1.1	22.1 ± 1.3	36	0.0022
Trichoderma atroviride	100.0 ± 0.0	53.1 ± 6.0	36	0.0028
Trichoderma harzianum	100.0 ± 0.0	100.0 ± 0.0	18	1.0000

of the isolate 1C4 from active galleries of *X. crassiusculus* placed it within the genus *Erwinia*, highlighting that further studies should revise the taxonomic classification of those isolates classified elsewhere as *P. cedenensis*.

The genome analysis of the bacterial isolates from active galleries of *X. crassiusculus* (i.e., 1C4) supported its nutritional role. In particular, CAZymes profile showed that this bacterium is adapted to interact with plants. The presence of sequences in the genome for α-amylases, cellulases (β-glucosidases, endoglucanases), and hemicellulose hydrolases (α-xylosidase) indicated that this bacterium can hydrolyse plant polymers, potentially aiding the insect host in the digestion of plant material. The same function was proposed for other Erwiniaceae symbionts of fungal-growing insects (Suen et al. 2010, Adams et al. 2011), even though the potential role of our bacterial isolate as an endophyte cannot be excluded. The presence of genes for the synthesis of essential amino acids, including histidine and tryptophan, also suggests a beneficial nutritional role which is known for other microbial insect symbionts (Douglas 2011, Hansen and Moran 2014). The presence of putative chitinases must be instead further investigated. Genes for chitinases might imply a negative effect on the insect host chitinous cuticula, as it has been proposed for other bacteria (Isaacson and Webster 2002, Hussin and Majid 2020), or a benefit for fungal growing insects that feed on fungi with chitinous cell walls. Second, the pBLAST analysis showed the absence of sequences matching proteins described for the clusters *dit* and *nif*, thus not supporting the roles in nitrogen fixation and diterpenes removal suggested by previous studies (Diehl et al. 2022, Diehl et al. 2023, Moussa et al. 2023). Third, BGC annotation highlighted that the isolate from *X. crassiusculus* might act as a protective symbiont. However, further research is needed to describe the antibiotic molecules produced by this bacterium. In fact, most of the putative clusters detected by the antiSMASH 5.0 database showed low similarity, suggesting that the molecules produced by the isolate 1C4 differ from those of previously described clusters for antibiotics on other Erwiniaceae bacteria (Lim et al. 2014, Walterson et al. 2014, Smits et al. 2019). Additionally, the presence of multiple resistance genes in the genome can be translated into an adaptive advantage that favors this bacterium competitiveness against other bacteria in the microbiome of the beetles.

Results of the interaction assays further supported the hypothesis that the isolate 1C4 from *X. crassiusculus* may act as a defensive symbiont in this species. The growth of *Dryadomyces* sp. and *Raffaelea* sp., known as ambrosia beetles' nutritional symbionts (Hulcr and Skelton 2023, Osborn et al. 2023), was not affected by the bacterium, which instead inhibited the growth of other common, non-mutualistic associates of ambrosia beetles and certain entomopathogens and mycopathogens. Among them, only *B. bassiana* can be regularly isolated from ambrosia beetles and their galleries (McPherson et al. 2013, Malacrinò et al. 2017, Morales-Rodríguez et al. 2021, Diehl et al. 2023), while all the others are probably not relevant in this multi-partite sym-

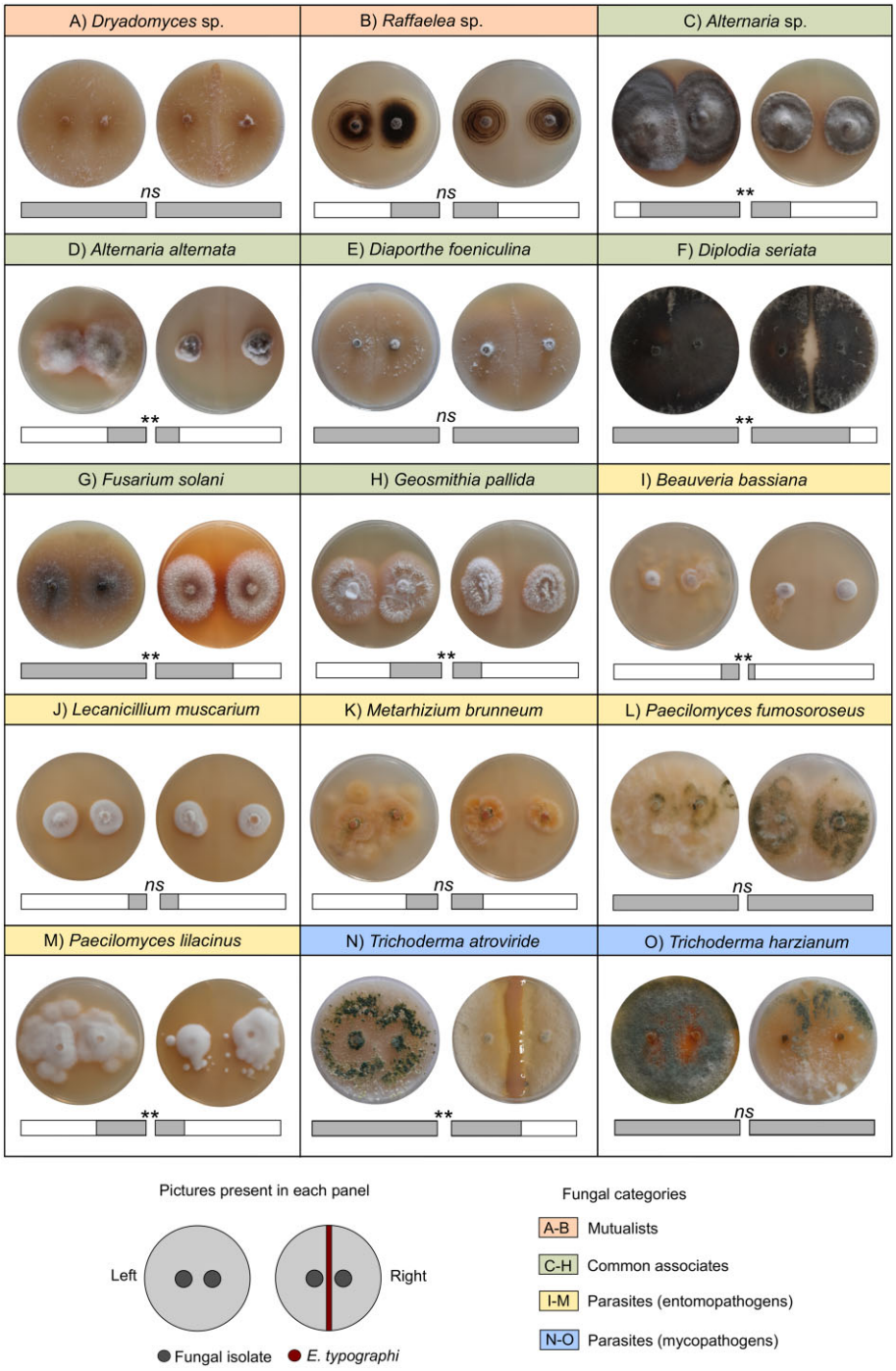

Figure 3. Results of the interaction assays between the bacterial isolate 1C4 obtained from active galleries galleries of *X. crassiusculus* and the different fungi tested in this study, divided per ecological category. Within each panel representative pictures of the control (left) and the co-culture assay (right) are shown together with bars showing the percentage of the Petri dish colonized by the fungus (grey portion). P-values: ** = 0.01–0.001; *ns* = not significant (>0.05).

biosis. The inhibitory effect can be attributed to the synthesis of secondary metabolites with antimicrobial activities (Smits et al. 2019), as suggested by the genome annotation. This defensive role can be particularly important from an ecological perspective. Ambrosia beetles come in contact with a number of fungi during their life cycle (Skelton et al. 2018, Rassati et al. 2019, Morales-Rodriguez et al. 2021), including endophytes living in host trees and parasites, and can potentially carry them on their body or inside the mycetangia (Kostovcik et al. 2015, Batemman et al. 2016). The capacity of the associated Erwiniaceae bacteria to protect the symbiosis between ambrosia beetles and their nutritional fungi can be a key advantage for the beetle, aiding in the likelihood of establishing and maintaining successful galleries. The defensive role can have important implications also for management strategies. Biocontrol agents can overgrow the nutritional symbionts of ambrosia beetles (Kushiyev et al. 2021, Gugliuzzo et al. 2022), but as we showed here this effect could be inhibited by bacterial associates, which could translate into con-

trol failure. However, in our study the inhibitory effect was evident only for certain entomopathogens or mycopathogens, which can help to explain why certain strains of *M. brunneum* show a stronger effect on ambrosia beetles than other strains of *B. bassiana* (Castrillo et al. 2011, Castrillo et al. 2013, Tuncer et al. 2019). This finding should be taken into account as selecting the right product or strain becomes basic to increase the likelihood of success in any biological control program against ambrosia beetles.

Conclusions

Despite the interest in ambrosia beetles is rapidly increasing worldwide most of the studies carried out so far are focused on their fungal rather than bacterial associates. In this study we showed that abundant bacteria associated with ambrosia beetles can play key nutritional and defensive roles aiding the beetle colonization success. These results support the idea that the bipartite beetle-fungus symbiosis is in fact multipartite, and highlight the importance of multi-domain microbial community studies in ambrosia beetles and other insects. Future studies should try to investigate how Erwiniaceae bacteria are transmitted through generations, and if the bacterium is associated to other plant inhabiting animals. In addition, it would be important to investigate the diversity of microbial species associated to ambrosia beetle species and the role of the most abundant ones, as they might be important for the beetle colonization success.

Author contributions

Juan Carlos Cambronero-Heinrichs (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing), Andrea Battisti (Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing), Peter Biedermann (Conceptualization, Supervision, Writing – review & editing), Giacomo Cavaletto (Methodology), Víctor Castro-Gutierrez (Data curation), Lorenzo Favaro (Supervision, Writing – review & editing), Giacomo Santoiemma (Data curation), and Davide Rassati (Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing)

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Supplementary data

Supplementary data is available at FEMSEC *Journal* online.

Conflict of interest: The authors declare that they have no conflicts of interest.

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Data availability

The genome sequences used in this manuscript were submitted to ENA under the BioProject accession number PRJEB62745.

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