

Degradation capacities of bacteria and yeasts isolated from the gut of *Dendroctonus rhizophagus* (Curculionidae: Scolytinae)

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Abstract Bark beetles (Curculionidae: Scolytinae) feed on the xylem and phloem of their host, which are composed of structural carbohydrates and organic compounds that are not easily degraded by the insects. Some of these compounds might be hydrolyzed by digestive enzymes produced by microbes present in the gut of these insects. In this study, we evaluated the enzymatic capacity of bacteria (*Acinetobacter lwoffii*, *Arthrobacter* sp., *Pseudomonas putida*, *Pseudomonas azotoformans*, and *Rahnella* sp.) and yeasts (*Candida piceae*, *Candida oregonensis*, *Cyberlindnera americana*, *Zygoascus* sp., and *Rhodotorula mucilaginosa*) isolated from the *Dendroctonus rhizophagus* gut to hydrolyze cellulose, xylan, pectin, starch, lipids, and esters. All isolates, with the exception of *C. piceae*, showed lipolytic activity. Furthermore, *P. putida*, *P. azotoformans*, *C. americana*, *C. piceae*, and *R. mucilaginosa* presented amylolytic activity. Esterase activity was shown by *A. lwoffii*, *P. azotoformans*, and *Rahnella* sp. Cellulolytic and xylanolytic activities were present only in *Arthrobacter* sp. and *P. azotoformans*. The pectinolytic activity was not recorded in any isolate. This is the first study to

provide evidence on the capacity of microbes associated with the *D. rhizophagus* gut to hydrolyze specific substrates, which might cover part of the nutritional requirements for the development, fitness, and survival of these insects.

Introduction

Bark beetles (Curculionidae: Scolytinae) constitute a highly diverse group of weevils associated with major groups of terrestrial plants (gymnosperms and angiosperms; Wood 1982), and they are among the most ecologically and economically important insects in coniferous forests (Raffa et al. 2008). The evolution of bark beetles is associated with the diversification of their host trees (Farrell et al. 2001). However, a key factor that has driven the adaptation and diversification of bark beetles is their symbiotic association with a broad spectrum of microbes, which increased their metabolic capacity and their success to exploit novel habitats and food sources (Six 2013).

Bark beetles feed on the xylem and phloem of their host and construct galleries under the bark of trees, where they lay eggs and their broods develop (Wood 1982). These substrates are rich in organic compounds, non-essentials amino acids, and soluble sugars, as well as structural carbohydrates such as hemicelluloses and cellulose (Thornber and Northcote 1962; Hodges and Lorio 1969; Sudachkova et al. 2004). The availability of these substrates changes once the colonization of the host trees starts and their quality declines as the life cycle of the insect proceeds (Six and Klepzig 2004). It has been demonstrated that bark beetles and their associated filamentous fungi from the genera *Ceratotsytopsis*, *Entomocorticium*, *Grosmannia*, and *Ophiostoma* are capable of producing a variety of enzymes (e.g., glucosidase, amylase, pectinase, peptidase, galactosidase, trehalose, and tryptase) involved in the hydrolysis of substrates present in the wood

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and phloem (Balogun 1969; Valiev et al. 2009). In addition, the enzymatic capacities of these fungi contribute substantially to the nutrition necessary for the development of bark beetles (Ayres et al. 2000; Bentz and Six 2006). However, to our knowledge, the enzymatic activities of the bacteria and yeasts associated with these insects have not been directly tested, except for those related to cellulose degradation, nitrogen fixation, recycling of uric acid, and assimilation and fermentation of certain single carbohydrates (Morales-Jiménez et al. 2009, 2012, 2013; Rivera et al. 2009; Hu et al. 2014). Determining the metabolic capacity of the gut-associated bacteria and yeasts of bark beetles is significant; in that, phloem is a potential source of nitrogen and carbon compound that these insects could easily assimilate.

Dendroctonus rhizophagus Thomas and Bright is an endemic bark beetle to Mexico, which colonizes the lower stem, root collar, and lateral roots of seedlings and young pine trees (<3 m, 10-cm diameter). It is an atypical species within the *Dendroctonus* genus because it does not carry out massive attacks as some other species do; instead, only one or two couples usually colonize a host. The life cycle of this species is univoltine, synchronous, and is largely regulated by the conditions of temperature and humidity (Cibrián-Tovar et al. 1995). The microbial community of the gut of *D. rhizophagus* was previously characterized using traditional culture and culture-independent molecular techniques (i.e., denaturing gradient gel electrophoresis and library clones; Rivera et al. 2009; Morales-Jimenez et al. 2012). These studies demonstrated that the diversity of the cultivable microbial community is scarce and similar to that found in other bark beetles species (Vasanthakumar et al. 2006; Yilmaz et al. 2006; Adams et al. 2010; Yaman et al. 2010; Hu et al. 2013; Lou et al. 2014).

The aim of the present study was to evaluate the enzymatic capacity of the bacteria and yeasts isolated from the gut of *D. rhizophagus* throughout its life cycle. In particular, we assessed the capacity of these microorganisms to degrade complex substrates such as polysaccharides, which include cellulose, xylan, pectin, and starch, as well as esters and lipids, which are organic compounds commonly available in the host trees.

Material and methods

Insect collection and dissection

Larvae (fifth instar), pupae, teneral adults, pre-emerged adults, and emerged adults (female and male adults that colonize the underside of the bark) of *D. rhizophagus* were collected by hand using forceps from naturally infested Arizona pines, *Pinus arizonica* Engelm. The collection was performed in San Juanito, Bocoyna Municipality, Chihuahua State, Mexico (27° 45' 11" N, 107° 38' 06" W) throughout the life cycle of the insect. These collections were carried out from late November

2011 (larvae) to mid-July 2012 (emerged adults). Three Arizona pines (<60 cm high, 10-cm diameter) were sampled for each development stage, except for the emerged adults, for which 20 trees were sampled. A total of 40 individuals from each stage were obtained from the sampled trees, stored at 4 °C in plastic containers, and transported to the laboratory for further analysis.

All the individuals were surface disinfested by sequential rinses with sterile distilled water for 1 min; with a detergent solution (10 mmol/L Tris-HCl pH 8, 1 mmol/L EDTA, 10 mmol/L NaCl, 1 % SDS, 2 % Triton X-100) for 1 min; with 1 % sodium hypochlorite solution for 1 min; with 70 % ethanol solution for 1 min; and finally, repeated washes with sterile distilled water. To assess the efficiency of the disinfestation, the last washing water was inoculated in Petri dishes with yeast extract-peptone-dextrose (YPD) agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose, 15 g/L agar; BD Difco, USA) for yeasts and tryptic soy agar (TSA; 15 g/L tryptone, 5 g/L soytone, 5 g/L sodium chloride, 15 g/L agar; BD Difco, USA) for bacteria. Plates were incubated at 28 °C for 48–72 h. Insects were dissected in a drop of phosphate-buffered solution (PBS; pH 7.4; 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L NaHPO₄, 2 mmol/L KH₂PO₄) under sterile conditions using fine-tipped forceps. To remove the gut, a longitudinal incision was made on the body of each of the immature stages; in the case of pre-emerged and emerged adults, the elytra, wings, and tergites were removed before the longitudinal incision was performed. Each gut was transferred into a 1.5-mL microcentrifuge tube and was homogenized in 1 mL of PBS with sterile pistils under an aseptic condition.

Bacteria and yeast enumeration and isolation

Viable counts of microorganisms were performed with one gut for each developmental stage. Serial tenfold dilutions were carried out in PBS solution, and 100 µL of each dilution was spread on YPD plates for yeasts (supplemented with streptomycin 0.5 mg/mL and kanamycin 0.5 mg/mL to inhibit bacteria) and TSA plates for bacteria. The assays were performed in triplicate. Cultures were incubated at 28 °C for 24 h for bacteria and 48 h for yeast. The viable count was expressed as colony-forming unit per milliliter (CFU/mL).

For the isolation of bacteria and yeasts, a pool of 30 guts in each developmental stage, from 40 collected samples, were used. Serial tenfold dilutions were performed as previously described. According to their morphological characteristics, 60 colonies of bacteria and 60 colonies of yeasts were randomly isolated from the plates of each developmental stage. To obtain pure cultures, the isolates were repeatedly streaked on agar plates. Axenic cultures were stored at –70 °C in 40 % glycerol.

DNA extraction, PCR amplification, and identification

The bacterial DNA was extracted from the pure colonies following the protocol of Hoffman and Winston (Hoffman and Winston 1987). A bacterial fragment ~1500 bp was amplified using universal primers 27F (5'-AGAGTTTGTATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'; Lane 1991). The yeast DNA was obtained using the method described by Cenis (1992). A region that ranged from 300 to 500 bp for the ITS was amplified with primers ITS1 (5'-TCCG TAGGTGAACCTGCGG-3') and ITS4 (5'-TCCT CCGCTTATTGATATGC-3'; White et al. 1990), and another fragment of ~600 bp for the 26S gene was amplified with primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3'; O'Donnell 1993). The PCR amplifications were performed in a TC-5000 thermocycler (Techne, Staffordshire, UK) using a total reaction volume of 25 μ L, which contained 50–100 ng DNA template, \times 1 reaction buffer, 2.0 mM MgCl₂, 0.4 μ M each primer, 0.4 mM dNTPs, and 1 U Taq polymerase (Invitrogen Life Technologies, Sao Paulo, BR). The reaction conditions were the following: initial denaturation at 94 °C for 5 min, 25 cycles at 94 °C for 1 min, annealing temperature 58 °C (16S gene) and 55 °C (ITS and 26S gene) for 1 min, 72 °C for 1 min, and final extension at 72 °C for 5 min. The PCR products were purified with a GeneJET PCR Purification Kit (Thermo Scientific, Waltham, MA) according to the manufacturer's protocol and sequenced in an ABI 3130xl Genetic Analyser (Hitachi, Tokyo, Japan).

The taxonomic identification of the bacteria and yeast isolates was based on the similarity level with respect to reference sequences from the GenBank and on a phylogenetic analysis performed by maximum likelihood in PhyML (<http://atgc.lirmm.fr/phyml/>) using the 16S ribosomal ribonucleic acid (rRNA) gene for bacteria and the intergenic spacer region (ITS1–5.8S–ITS2) and the D1/D2 domain of the 26S rRNA gene for yeasts. In addition, we followed the Rosselló-Mora and Amann criteria (2001) in the case of bacteria. The sequences were deposited in the GenBank, under the accession numbers KU143968–KU144267 for the 16S rRNA gene, KU144268–KU144567 for the ITS region, and KU144568–KU144579 for the 26S rRNA gene.

Enzymatic activities

The capacity to degrade cellulose, lipids, pectin, starch, polysorbate compounds (Tween 20 and Tween 80), and xylan was tested for 300 isolates from both bacteria and yeasts (60 bacteria and 60 yeast isolates for each developmental stage) at three different pH levels (6, 7, and 8). The tested pH range was previously determined using pH strips. To obtain active cultures, bacteria and yeasts were grown in YPD and TSA plates, respectively. After 24–48 h, single colonies were point inoculated in the solid media supplied with the below-mentioned compounds. The assays were

performed in triplicates for each pH level. The cultures were incubated at 28 °C for 48–72 h.

The amylase, esterase, lipase, and pectinase assays were performed with the Castañeda medium (0.625 g/L dibasic ammonium citrate, 0.375 g/L KH₂PO₄, 1.0 g/L yeast extract, 0.375 g/L Na₂CO₃, 0.250 g/L NaCl, 0.275 g/L MgSO₄·7H₂O, and 1.5 % agar; Castañeda-Agulló 1956) supplemented separately with 2 % (w/v) starch, 2 % Tween 20 or Tween 80, 0.5 % butter with 0.2 g/L resazurin, and 1 % citrus pectin to evaluate the amylolytic, esterase, lipolytic, and pectinolytic activities, respectively. The cellulolytic and xylanolytic activities were assessed using Congo red agar (0.50 g/L KH₂PO₄, 0.25 g/L MgSO₄·7H₂O, 2 g/L gelatin, 0.2 g/L Congo red, 1.5 % agar) supplemented with 1.88 g/L carboxymethyl cellulose (CMC) for cellulases and 1.88 g/L xylan from beechwood for xylanases (Wood 1980). The amylase activity was shown by the addition of 1 % iodine solution to the plates, and the presence of a clear zone around a colony was defined as a positive result (Hols et al. 1994). The esterase activity was identified by observing halos of precipitation around the inoculum under transmitted light (Gessner 1980). The lipase activity was considered positive in the presence of a pink halo on a purple background. The pectinase activity was recognizable by the presence of a clear ring around a colony by the addition of a 1 % cethyl trimethyl ammonium bromide (CTAB) solution (Hankin et al. 1971). Both cellulolytic and xylanolytic activities were identified by a clear zone around the colonies (Wood 1980).

Statistical analysis

Statistically significant differences among and within developmental stages were determined using one-way ANOVA test. The substrate degradation index (DI) was calculated for each culture as the diameter of the halo (or precipitation) divided by the diameter of the colony in each culture (Hankin et al. 1971). It was expressed as the mean of triplicates \pm their standard deviation. The DI values were subjected to a Kruskal-Wallis test to detect the statistical significance among pH levels both in bacterial and yeast species.

Results

Microbial load in the gut of *D. rhizophagus*

The microbial loads of bacteria and yeasts in the different developmental stages were variable (Table 1). The CFU per gut for bacteria varied from $4.40 \pm 1.10 \times 10^2$ for pupae to $1.70 \pm 0.43 \times 10^8$ for larvae. No statistically significant differences were found within stages but yes among developmental stages. For yeasts, the CFU varied from $5.90 \pm 1.60 \times 10^1$ for pupae to $3.75 \pm 0.25 \times 10^4$ for pre-emerged adults. No

Table 1 Microbial load of bacteria and yeasts in the gut of *Dendroctonus rhizophagus*

Developmental stage	Larvae	Pupae	Teneral adults	Pre-emerged adults	Emerged adult
Bacteria	$1.70 \pm 0.43 \times 10^8$ *	$4.40 \pm 1.10 \times 10^2$ *	$1.97 \pm 0.54 \times 10^6$ *	$3.13 \pm 0.17 \times 10^6$ *	$9.46 \pm 5.46 \times 10^6$ *
Yeasts	$1.71 \pm 0.42 \times 10^3$	$5.90 \pm 1.60 \times 10^1$	$2.14 \pm 1.66 \times 10^3$	$3.75 \pm 0.25 \times 10^4$ *	$6.7 \pm 1.7 \times 10^1$

Colony forming unit/mL (mean \pm standard deviation)

*Statistically significant difference ($P < 0.05$, based on one-way ANOVA test) among stages

statistically significant differences were also found within stages but yes among developmental stages.

Taxonomic identification of bacteria and yeasts from *D. rhizophagus*

The pairwise similitude and phylogenetic inference analyses showed that the cultivable bacterial community was composed of *Acinetobacter lwoffii*, *Arthrobacter* sp., *Pseudomonas putida*, *Pseudomonas azotoformans*, and *Rahnella* sp. Likewise, the yeast community was composed of *Candida oregonensis*, *Candida piceae*, *Cyberlindnera americana*, *Zygoascus* sp., and *Rhodotorula mucilaginosa*. Whereas *Rahnella* sp. and *C. americana* were the dominant and most microbial isolated in all the developmental stages, the other species were isolated only from specific developmental stages with a variable relative abundance (Table 2 and Online Resource Figs. 1 and 2).

Enzymatic profile of bacteria and yeasts isolated from the gut of *D. rhizophagus*

Lipolytic activity was observed in all the bacterial species (Table 3). All the isolates of these species displayed this activity in the developmental stages from which they were isolated. Likewise, all the isolated yeasts species, except for *C. piceae*, showed the same activities in the sites from where they were isolated (Table 4). Significant differences in the mean DI among pH levels ($P < 0.05$) were observed for certain bacterial and yeasts (Tables 3 and 4).

The amyolytic activity was observed only in *P. azotoformans* and *P. putida*, and all of their isolates presented it. The mean DI was statistically significant among different pH levels ($P < 0.05$) only for the second species (Table 3). *C. americana*, *C. piceae*, and *R. mucilaginosa* yeast isolates (100 %) showed this activity in the different developmental stages from which they were isolated. No significant difference was found in the mean DI at different pH levels

Table 2 Taxonomic assignment and abundance of bacteria and yeasts isolated from the gut of *Dendroctonus rhizophagus* per developmental stage

Closest known species in GenBank (accession number)	Similarity (%) ^a	Phylogenetic affiliation	Developmental stage				
			Larvae	Pupae	Teneral adults	Pre-emerged adults	Emerged adults
Bacteria							
<i>Rahnella inusinata</i> (U90758)	97.0–99.0	<i>Rahnella</i> sp.	43	60	58	51	59
<i>Pseudomonas putida</i> (EU439424)	98.8–98.9	<i>Pseudomonas putida</i>	10	0	0	0	0
<i>Pseudomonas azotoformans</i> (AB680322)	99.8	<i>Pseudomonas azotoformans</i>	0	0	0	0	1
<i>Acinetobacter lwoffii</i> (HE651921)	98.0–98.3	<i>Acinetobacter lwoffii</i>	7	0	0	9	0
<i>Arthrobacter oxidans</i> (KC934831)	98.5–99.6	<i>Arthrobacter</i> sp.	0	0	2	0	0
Yeasts							
<i>Candida piceae</i> (EF090824)	98.6–100	<i>Candida piceae</i>	0	0	0	0	24
<i>Candida oregonensis</i> (HM156520)	99.7–100	<i>Candida oregonensis</i>	0	0	0	0	6
<i>Cyberlindnera americana</i> (EF093384)	99.8–100	<i>Cyberlindnera americana</i>	60	60	60	60	13
<i>Zygoascus hellenicus</i> (KP241903)	97–97.5	<i>Zygoascus</i> sp.	0	0	0	0	16
<i>Rhodotorula mucilaginosa</i> (KC881070)	99.6	<i>Rhodotorula mucilaginosa</i>	0	0	0	0	1

^a Similarity based on comparisons with 16S rRNA for bacteria and ITS (ITS1–5.8S rRNA-ITS2) for yeasts

Table 3 Enzymatic profile of bacteria isolated from the gut of *Dendroctonus rhizophagus* at different developmental stages

Enzymatic activity	Larvae			Pupa		Teneral adults		Pre-emerged adults		Emerging adults	
	<i>Rahnella</i> sp.	<i>P. putida</i>	<i>A. lwoffii</i>	<i>Rahnella</i> sp.	<i>Arthrobacter</i> sp.	<i>Rahnella</i> sp.	<i>Rahnella</i> sp.	<i>A. lwoffii</i>	<i>Rahnella</i> sp.	<i>P. azotoformans</i>	
Lipases	6	43 (2.3 ± 0.02)	10 (2.2 ± 0.06)	7 (2.4 ± 0.09)	60 (1.8 ± 0.04)	58 (1.9 ± 0.02)	2 (2.0 ± 0.16)	51 (1.9 ± 0.01)	9 (1.9 ± 0.02)	59 (2.1 ± 0.01)	1 (1.7 ± 0.04)
	7	43 (2.2 ± 0.02)	10 (1.9 ± 0.07)	7 (2.1 ± 0.08)	60 (1.9 ± 0.05)	58 (1.9 ± 0.02)	2 (1.8 ± 0.09)	51 (1.7 ± 0.03)	9 (1.7 ± 0.04)	59 (1.9 ± 0.01)	1 (2.0 ± 0.01)
	8	43 (2.1 ± 0.01)	10 (2.0 ± 0.07)	7 (2.2 ± 0.03)	60 (1.8 ± 0.04)	58 (1.9 ± 0.02)	2 (2.3 ± 0.20)	51 (1.9 ± 0.01)	9 (1.7 ± 0.03)	59 (2.0 ± 0.01)	1 (1.5 ± 0.05)
Amylases	6	0	10 (1.3 ± 0.15)	0	0	0	0	0	0	0	1 (1.4 ± 0.02)
	7	0	10 (2.0 ± 0.05)	0	0	0	0	0	0	0	1 (1.7 ± 0.06)
	8	0	10 (1.7 ± 0.04)	0	0	0	0	0	0	0	1 (1.4 ± 0.02)
Esterases in Tween 20	6	35 (1.4 ± 0.02)	0	3 (1.0 ± 0.0)	60 (1.0 ± 0.01)	58 (1.2 ± 0.01)	0	49 (1.2 ± 0.02)	5 (1.6 ± 0.10)	56 (1.5 ± 0.01)	1 (2.2 ± 0.0)
	7	16 (1.1 ± 0.03)	0	7 (0.9 ± 0.03)	58 (1.0 ± 0.01)	58 (1.2 ± 0.03)	0	44 (1.3 ± 0.02)	5 (1.5 ± 0.08)	56 (1.7 ± 0.01)	1 (2.0 ± 0.0)
	8	16 (0.8 ± 0.03)	0	7 (0.9 ± 0.01)	58 (1.0 ± 0.0)	58 (1.2 ± 0.03)	0	46 (1.3 ± 0.02)	9 (1.6 ± 0.06)	56 (1.6 ± 0.01)	1 (2.0 ± 0.0)
Esterases in Tween 80	6	34 (1.0 ± 0.0)	0	7 (1.0 ± 0.0)	60 (1.0 ± 0.0)	58 (1.3 ± 0.01)	0	21 (1.0 ± 0.01)	0	54 (1.0 ± 0.01)	0
	7	34 (1.0 ± 0.0)	0	7 (1.0 ± 0.0)	58 (0.9 ± 0.01)	58 (1.3 ± 0.01)	0	46 (1.1 ± 0.01)	0	54 (1.0 ± 0.01)	0
	8	34 (0.9 ± 0.01)	0	7 (1.0 ± 0.0)	58 (1.0 ± 0.0)	58 (1.3 ± 0.01)	0	36 (1.1 ± 0.02)	0	54 (1.1 ± 0.01)	0
Cellulases	6	0	0	0	0	0	2 (3.1 ± 0.29)	0	0	0	1 (8.0 ± 0.0)
	7	0	0	0	0	0	2 (3.3 ± 0.36)	0	0	0	1 (8.0 ± 0.0)
	8	0	0	0	0	0	2 (3.7 ± 0.25)	0	0	0	1 (8.0 ± 0.0)
Xylanases	6	0	0	0	0	0	2 (2.6 ± 0.07)	0	0	0	1 (10.0 ± 0.0)
	7	0	0	0	0	0	2 (2.2 ± 0.17)	0	0	0	1 (10.6 ± 0.66)
	8	0	0	0	0	0	2 (2.3 ± 0.16)	0	0	0	1 (10.0 ± 0.0)

A total of 60 isolates for each developmental stage were tested. The number before parenthesis represents the number of isolates for each species that showed the activity. The value in parenthesis is the DI mean ± standard deviation, which was estimated from three replicates. The DI was calculated for each culture as the diameter of the halo (mm) divided by the diameter of the colony (mm). Significant differences ($P < 0.05$, based on Kruskal-Wallis test) among pH levels of enzymatic activity for bacterial species at each developmental stage are shown in *italic*

Table 4 Enzymatic profile of yeasts isolated from the gut of *Dendroctonus rhizophagus* at different developmental stages

Enzymatic activity	Larva		Pupa		Teneral adults		Pre-emerged adults		Emerging adults		<i>Zygoascus</i> sp.	<i>R. mucilaginosa</i>
	<i>C. americana</i>	<i>C. americana</i>	<i>C. americana</i>	<i>C. americana</i>	<i>C. americana</i>	<i>C. americana</i>	<i>C. americana</i>	<i>C. piceae</i>	<i>C. americana</i>	<i>C. oregonensis</i>		
Lipases	pH 6	60 (2.3 ± 0.02)	60 (2.7 ± 0.02)	60 (1.9 ± 0.02)	60 (1.9 ± 0.02)	60 (1.9 ± 0.02)	60 (1.9 ± 0.01)	0	13 (2.4 ± 0.05)	6 (2.5 ± 0.07)	10 (2.1 ± 0.04)	1 (2.1 ± 0.08)
	pH 7	60 (2.3 ± 0.01)	60 (2.8 ± 0.03)	60 (1.9 ± 0.02)	60 (1.9 ± 0.02)	60 (1.9 ± 0.03)	60 (1.9 ± 0.03)	0	13 (2.2 ± 0.04)	6 (2.2 ± 0.10)	10 (2.1 ± 0.07)	1 (2.5 ± 0.0)
	pH 8	60 (2.5 ± 0.02)	60 (2.5 ± 0.02)	60 (1.9 ± 0.02)	60 (1.9 ± 0.03)	60 (1.9 ± 0.02)	60 (1.9 ± 0.01)	0	13 (2.1 ± 0.04)	6 (2.0 ± 0.06)	10 (2.0 ± 0.03)	1 (2.1 ± 0.11)
Amylases	pH 6	60 (1.5 ± 0.02)	0	60 (1.9 ± 0.02)	60 (1.9 ± 0.02)	60 (1.9 ± 0.02)	60 (1.0 ± 0.0)	23 (1.6 ± 0.05)	13 (1.6 ± 0.05)	0	0	1 (1.3 ± 0.0)
	pH 7	60 (1.5 ± 0.01)	60 (1.4 ± 0.01)	60 (1.9 ± 0.03)	60 (1.9 ± 0.03)	60 (1.9 ± 0.03)	60 (1.0 ± 0.0)	22 (1.5 ± 0.03)	13 (1.5 ± 0.03)	0	0	1 (1.4 ± 0.05)
	pH 8	60 (1.4 ± 0.01)	0	60 (1.9 ± 0.02)	60 (1.9 ± 0.02)	60 (1.9 ± 0.02)	60 (1.0 ± 0.0)	22 (1.6 ± 0.04)	13 (1.4 ± 0.02)	0	0	1 (1.3 ± 0.0)

A total of 60 isolates for each developmental stage were tested. The number before parenthesis represents the number of isolates for each species that showed the activity. The value in parenthesis is the DI mean ± standard deviation, which was estimated from three replicates. The DI was calculated for each culture as the diameter of the halo (mm) divided by the diameter of the colony (mm). Significant differences ($P < 0.05$, based on Kruskal-Wallis test) among pH levels of enzymatic activity for bacterial species at each developmental stage are shown in *italic*

($P > 0.05$), except for *C. americana*, where the enzymatic activity was recorded only at pH 7 in the pupal stage (Table 4).

Esterase activity was present in *A. lwoffii*, *P. azotoformans*, and *Rahnella* sp. (Table 3). However, not all the isolates of the different developmental stages showed this activity. In addition, Tween 20 was hydrolyzed by the isolates of the majority of these species. Instead, differences in the hydrolysis of Tween 80 were evident. The mean DI for Tween 20 was statistically significant for the different pH levels ($P < 0.05$) with *A. lwoffii* and *Rahnella* sp. in larvae, pupae, and emerged adults (Table 3).

Cellulolytic and xylanolytic activities were present in *Arthrobacter* sp. isolated from teneral adults and in *P. azotoformans* isolated from emerged adults, which showed a higher level of enzymatic activity (DI). Statistically significant differences were only found in *Arthrobacter* sp. among different pH levels ($P < 0.05$; Table 3). Finally, pectinolytic activity was not observed in bacterial or yeast species.

Discussion

The cultivable microbial community associated with the different developmental stages of *D. rhizophagus* was composed of five bacterial species, i.e., *A. lwoffii*, *Arthrobacter* sp., *P. putida*, *P. azotoformans*, and *Rahnella* sp., as well as five yeast species, i.e., *C. oregonensis*, *C. picea*, *C. americana*, *Zygoascus* sp., and *R. mucilaginosa*. Some of these microorganisms have also been isolated and reported in other bark beetles (Delalibera et al. 2005; Vasanthakumar et al. 2006; Yilmaz et al. 2006; Morales-Jiménez et al. 2009; Rivera et al. 2009; Hu et al. 2013, 2014; Lou et al. 2014). It is noteworthy the absence of some of these microbes in certain life cycle stages of this bark beetle. This outcome must be due to the culture conditions used, the sample size analyzed, and the low abundance of certain microbe groups.

The microbial loads of these bacteria and yeasts in the gut of *D. rhizophagus* were variable throughout the different developmental stages (Table 1). In particular, the bacterial load (4.40×10^2 in pupae and 1.7×10^8 in larvae) was higher than that of the yeasts (5.90×10^1 in pupae and 3.75×10^4 in pre-emerged adults), which may be due to different factors such as the higher bacterial reproduction rate than that of yeasts, the microenvironmental conditions of the gut (e.g., pH and O_2) that might favor the bacterial growth (Kurtzman et al. 2011; Madigan et al. 2015), and a broader physiological profile observed in bacteria isolated from bark beetles than that of yeasts. The low microbial load observed in the pupal stage is interesting and is in accordance with previous reports from other bark beetles, including *D. rhizophagus* (Delalibera et al. 2005; Morales-Jimenez et al. 2012; Hu et al. 2013). This result may be due to the decreased

metabolic level that insects experience in this developmental stage and the morphostructural, physicochemical, and hormonal changes that the gut suffer during metamorphosis (Engel and Moran 2013).

The abundance of certain bacteria and yeasts across the different life stages of *D. rhizophagus* could be indicative of their functional importance in the gut (Tables 3 and 4). Even though insects, including bark beetles, possess certain enzymes already examined (Horne et al. 2009; Arrese and Soulages 2010), the lipolytic capacity showed by all the bacteria and yeasts isolated in this study suggests a role in the nutritional processes, as it has been reported for isolates of other insects (Park et al. 2007; Feng et al. 2011). In the larval stages of *D. rhizophagus*, the microbial lipases might contribute to hydrolyze fatty acids, phospholipids, and triacylglycerols present in the trees resin and phloem (Sudachkova et al. 2004). These organic compounds may be stored as an energy reserve in the insects' fat body to be used later during metamorphosis, diapause, or latency during pupal and teneral stages. Likewise, they can be used during dispersion, host colonization, pheromone signaling, and reproduction in the adult stage (Horne et al. 2009; Arrese and Soulages 2010).

The functional characterization of amylases has been widely studied in insects, such as Coleoptera, and in microorganisms (Naidu and Saranraj 2013; Kaur et al. 2014). However, little is known about the amylases of bark beetles and their associated microbes (Viktorinova et al. 2011). Our results show that *Pseudomonas* sp. and the *C. americana*, *C. piceae*, and *R. mucilaginosus* yeasts have this ability, which might be relevant for the growth of *D. rhizophagus*. A considerable quantity of starch is accumulated in grains inside the living cells (axial and ray parenchyma) of the sapwood and root phloem cells (Pallardy 2008), where this species spends most of its life cycle, i.e., feeding on the phloem of seedlings or young pine trees. In addition, it has been demonstrated that the bark, needles, and other tissues of pines have amylase inhibitors (Kim et al. 2004), which might act on the catalytic sites of the insect digestive amylase. The presence of microorganisms with this ability in the gut of *D. rhizophagus* suggests that they could contribute greatly to the hydrolysis of starch, which might decrease the potential effect that such inhibitors could have on the insect amylase.

The detection of esterase activity in most of the isolates from *Rahnella* sp., *P. azotoformans*, and *A. lwoffii* suggests that they might contribute to the digestive processes, the degradation of xenobiotics (Dowd 1992), and endogenous compounds derived from the insect metabolism (e.g., hormones) or from the action of toxic terpenes on the gut epithelial cells (e.g., cell wastes). Microbial esterases have been associated with the degradation of glycerol esters, short-chain triglycerides, and partially xylan as well as with the degradation of aromatic hydrocarbons, insecticides, aromatic esters, phenolic acids, and resin acids (Bomscheuer 2002). In insects, esterases of the carboxylesterase gene family are clustered into the following three classes: the neuro/developmental class, the hormone/semiochemical

processing class, and the dietary/detoxification class (Montella et al. 2012). The two first classes are associated with development and behavior, and the third class is related to the xenobiotic metabolism and digestive processes. This latter class is likely a shared function with the gut microbes. Future studies are necessary to determine the functional role of microbial esterases in bark beetles.

The xylanolytic and cellulolytic activities were only detected in *Arthrobacter* sp. and *P. azotoformans* from teneral and emerged adults, respectively. To our knowledge, xylan-degrading bacteria have not been reported from bark beetles; however, xylanolytic bacteria of the genera *Bacillus*, *Citrobacter*, *Klebsiella*, *Proteus*, and *Serratia* have been previously isolated from the larva and adult gut of wood-feeding insects (Anand et al. 2010), which probably contribute to the breakdown of hemicellulose into disaccharides or monosaccharides that might be easily assimilated by the insects.

Cellulolytic bacteria of the genera *Bacillus*, *Brevundimonas*, *Methylobacterium*, *Kocuria*, *Paenibacillus*, *Ponticoccus*, *Pseudomonas*, *Pseudoxanthomonas*, *Serratia*, and *Stenotrophomonas* have been isolated from *Dendroctonus armandi* and *D. rhizophagus* using enriched media (Morales-Jimenez et al. 2012; Hu et al. 2014); however, they have not been isolated in other bark beetle species (Delalibera et al. 2005). The absence or low abundance of these microbes in the gut of bark beetles has been explained by the fact that the phloem contains adequate concentrations of single carbohydrates, which suggests that the degradation of cellulose could not be necessary to feed the insects (Six 2013). However, this hypothesis should be taken with caution, because the absence of cellulolytic activity could be an artifact associated with the methodology, i.e., the isolation, culture, and growth conditions of the microbes.

The same consideration might be applied to other enzymatic activities. For example, the genome sequencing of certain bacteria and yeasts isolated in this study (e.g., *Rahnella* sp. and *C. oregonensis*), which did not show some of the in vitro specific activities (e.g., cellulolytic, amylolytic, xylanolytic, and pectinolytic) harbored genes that codified related enzymes (e.g., α - and β -glycosidase, α -amylase, α -xylosidase, endo-xylanase, and pectinesterase).

In brief, this is the first study to provide evidence that bacteria and yeasts associated with the gut of the bark beetle *D. rhizophagus* hydrolyze compounds such as lipids, starch, esters, and xylan, as well as cellulose. The high proportion of bacteria and yeasts in the gut with amylolytic and lipolytic activities might be a consequence of the large amounts of starch and triacylglycerides present in the tree tissue that insects ingest. The esterase production by bacterial isolates could be related not only to digestive processes but also to xenobiotic degradation processes. Although insects could possess their own enzymes for some of these activities, microbes might cover part of the nutritional requirements necessary for the development, fitness, and survival of these insects.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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