The microbiome profiling of fungivorous black tinder fungus beetle *Bolitophagus reticulatus* reveals the insight into bacterial communities associated with larvae and adults

Agnieszka Kaczmarczyk-Ziemba [Corresp., 1], **Grzegorz K. Wagner** [2], **Krzysztof Grzywnowicz** [3], **Marek Kucharczyk** [4], **Sylwia Zielińska** [5, 6]

[1] Department of Genetics and Biosystematics, Faculty of Biology, University of Gdansk, Gdansk, Poland
[2] Department of Zoology, Maria Curie-Sklodowska University, Lublin, Poland
[3] Department of Biochemistry, Maria Curie-Sklodowska University, Lublin, Poland
[4] Department of Nature Protection, Maria Curie-Sklodowska University, Poland
[5] Department of Bacterial Molecular Genetics, Faculty of Biology, University of Gdansk, Gdansk, Poland
[6] Phage Consultants, Gdansk, Poland

Corresponding Author: Agnieszka Kaczmarczyk-Ziemba
Email address: agnieszka.kaczmarczyk@biol.ug.edu.pl

Saproxylic beetles play a crucial role in key processes occurring in forest ecosystems and together with fungi, contribute to the decomposition and mineralization of wood. One of the group among them are mycetophilic beetles which associate with wood-decaying fungi and use the fruiting body for nourishment and development. Therefore, their feeding strategy (especially in the case of fungivorous species) requires special digestive capabilities to take advantage of the nutritional value of fungal tissue. Although polypore-beetle associations have been investigated in numerous studies, the detailed studies focused on microbiome associated with species feeding on fruiting bodies of polypores remain limited. Here we investigated the bacterial communities associated with larvae and adults of *Bolitophagus reticulatus* collected from *Fomes fomentarius* growing on two different host tree – beech (*Fagus* sp.) and birch (*Betula* sp.), respectively. Among 24 identified bacterial phyla, three were the most relatively abundant (Proteobacteria, Actinobacteria and Bacteroidetes). Moreover, we tried to find unique patterns of bacteria abundances which could be correlated with the long-term field observation showing that the fruiting bodies of *F. fomentarius,* growing on birch are more inhabited by beetles than fruiting bodies of the same fungus species growing on beech. Preliminary analyses showed that the level of protease inhibitors and secondary metabolites in *F. fomentarius* is higher in healthy fruiting bodies than in the inhabited ones. However, tested microbiome samples primarily clustered by developmental stage of *B. reticulatus* and host tree did not appear to impact the taxonomic distribution of the communities. This observation was supported by statistical analyses.

The microbiome profiling of fungivorous black tinder fungus beetle *Bolitophagus reticulatus* reveals the insight into bacterial communities associated with larvae and adults

Agnieszka Kaczmarczyk-Ziemba[1], Grzegorz K. Wagner[2], Krzysztof Grzywnowicz[3], Marek Kucharczyk[4], Sylwia Zielińska[5, 6]

[1] Department of Genetics and Biosystematics, Faculty of Biology, University of Gdansk, Wita Stwosza 59, 80-308 Gdansk, Poland, agnieszka.kaczmarczyk@biol.ug.edu.pl

[2] Department of Zoology, Maria Curie-Sklodowska University, Akademicka 19, 20-033 Lublin, Poland, grzegorz.wagner@umcs.lublin.pl

[3] Department of Biochemistry, Maria Curie-Sklodowska University, Akademicka 19, 20-033 Lublin, Poland, grzyw@poczta.umcs.lublin.pl

[4] Department of Nature Protection, Maria Curie-Sklodowska University, Akademicka 19, 20-033 Lublin, Poland, marek.kucharczyk@umcs.lublin.pl

[5] Department of Bacterial Molecular Genetics, Faculty of Biology, University of Gdansk, Wita Stwosza 59, 80-308 Gdansk, Poland, sylwia.zielinska@biol.ug.edu.pl

[6] Phage Consultants, Partyzantow 10/18, 80-254 Gdansk, Poland

Corresponding Author:

Agnieszka Kaczmarczyk-Ziemba[1]

Wita Stwosza 59, 80-308 Gdansk, Poland

Email address: agnieszka.kaczmarczyk@biol.ug.edu.pl

22

Abstract

Saproxylic beetles play a crucial role in key processes occurring in forest ecosystems and together with fungi, contribute to the decomposition and mineralization of wood. One of the group among them are mycetophilic beetles which associate with wood-decaying fungi and use the fruiting body for nourishment and development. Therefore, their feeding strategy (especially in the case of fungivorous species) requires special digestive capabilities to take advantage of the nutritional value of fungal tissue. Although polypore-beetle associations have been investigated in numerous studies, the detailed studies focused on microbiome associated with species feeding on fruiting bodies of polypores remain limited. Here we investigated the bacterial communities associated with larvae and adults of *Bolitophagus reticulatus* collected from *Fomes fomentarius* growing on two different host tree – beech (*Fagus* sp.) and birch (*Betula* sp.), respectively. Among 24 identified bacterial phyla, three were the most relatively abundant (Proteobacteria, Actinobacteria and Bacteroidetes). Moreover, we tried to find unique patterns of bacteria abundances which could be correlated with the long-term field observation showing that the fruiting bodies of *F. fomentarius,* growing on birch are more inhabited by beetles than fruiting bodies of the same fungus species growing on beech. Preliminary analyses showed that the level of protease inhibitors and secondary metabolites in *F. fomentarius* is higher in healthy fruiting bodies than in the inhabited ones. However, tested microbiome samples primarily clustered by developmental stage of *B. reticulatus* and host tree did not appear to impact the taxonomic distribution of the communities. This observation was supported by statistical analyses.

Introduction

Saproxylic beetles are directly or indirectly related to wood during at least one developmental stage (Speight, 1989). They play a critical role in key processes occurring in forest ecosystems and together with fungi, contribute to the decomposition and mineralization of wood (Gutowski & Buchholz, 2000). These species are a major component of forest biodiversity and help to maintain a specific homeostasis of the ecosystem. Saproxylic beetles can occupy several ecological nishes and therefore xylophages, cambiophages, predators, necrophiles, and finally mycetophiles can be distinguished (Gutowski et al., 2004).

Mycetophilic beetles associate with wood-decaying fungi and use the fruiting body for nourishment and development (Gutowski, 2006). Their feeding strategy requires special digestive capabilities to take advantage of the nutritional value of fungal tissue. Associated microorganisms play a crucial role in those processes. Although polypore-beetle associations have been investigated in numerous studies (Nikitsky & Schigel, 2004; Schigel, Niemelä & Kinnunen, 2006; Schigel, 2009, 2011, 2012), the detailed projects focused on their microbiome remain limited. Previous study was focused rather on microbiota associated with fungivorous insects (not only beetles, but also ants and termites) which cultivate fungi for food to take advantage of the nutritional value of fungal tissue (Aylward et al., 2014). However, the pilot investigations of microbiome of beetles associated with wood-decaying fungi also have been initiated. Recently, Wiater et al. (2018) identified bacteria *Paenibacillus* sp. in the gut of fungivorous darkling beetle *Diaperis boleti* (Tenebrionidae) feeding on polypore fungus *Laetiporus sulphureus*. These bacteria effectively degrade fungal α-(1→3)-glucan present in cell wall of fungi. More complex studies focused on profiling the microbiome of fungivorous beetles have not been performed yet.

The black tinder fungus beetle *Bolitophagus reticulatus* (Tenebrionidae) is a fungivorous species occurring widely throughout European forests (Fig. 1A). This beetle belongs to tribe Bolitophagini which represent feeding strategy of dwellers (Schigel, Niemelä & Kinnunen, 2006). *B. reticulatus* lives in close association with the perennial basidiocarps of *Fomes fomentarius* (L.) Fr. (commonly known as *the tinder fungus*; Fig. 1B) at all developmental stages and seems to be monophagous on this fungus species (Midtgaard, Rukke & Sverdrup-Thygeson, 1998 and references therein).

The long-term field observations have shown that *B. reticulatus* is more often found inside *F. fomentarius* fruiting bodies growing on birch (*Betula* sp.) compared with those growing on beech (*Fagus* sp.). Moreover, polypores growing on beech trees are much larger and less inhabited by insects than fruiting bodies growing on birch (Wagner, 2018). The growth of fungi is closely correlated with the amount of catechins utilized (Arunachalam et al., 2003). Catechins can be taken and metabolized mainly by wood degrading fungi (Rayner & Boddy, 1988). Derivatives of catechins are also present in fungi themselves (Zhou & Liu, 2010). Schwarze, Engels & Mattheck (2000) has shown that the mycelium growing on the tree accumulates the secondary metabolites of its host, especially in the parts covering the fruiting bodies. This process may be correlated with observed differences in colonization degrees. Fungal metabolites are of considerable interest and remarkable importance as new lead compounds for plant and animal or human protection. Importantly, fungal polyketides are one of the largest and most structurally diverse classes of naturally occurring compounds, ranging from simple aromatic metabolites to complex macrocyclic lactones. They are inhibitors of enzymes, including proteases (Shen et al., 2015). However, the state of knowledge about biological activity of substances derived from *F. fomentarius* remain limited. Antimicrobial activity of extracts

derived from the tinder fungus has been described by Dresch et al. (2015), while Chen et al. (2008) found that its exopolysaccharide (EPS) has direct antiproliferative effect *in vivo*. In turn, some active compounds (e.g. catalase, extracellular laccase, manganese-dependent peroxidase, carboxymethyl cellulose or xylanase) have been identified by Jaszek et al. (2006) and Elisashvili et al. (2009). Nevertheless, there is still little know about the secondary metabolites and inhibitors of proteases, especially with regard to fruiting bodies from the natural environment, not from *in vitro* cultures. Therefore, more detailed and complex studies are needed to test the hypothesis of the correlation between profiles of the secondary metabolites and inhibitors of proteases, and the degree of polypores colonization.

In the present study, we investigated the bacterial communities associated with larvae and adults of *B. reticulatus* collected from *F. fomentarius* growing on two different host tree – beech and birch, respectively. We used the Next Generation Sequencing (NGS) of the 16S rRNA gene to define whether the bacterial communities vary among the two tested developmental stages of *B. reticulatus*. Lastly, we investigated the potential differences between microbiome profiles of individuals collected from the tinder fungus growing on birch and beech and combined the results with those obtained from preliminary studies on the biochemical composition of the *F. fomentarius* fruiting bodies growing on wood of two different tree species.

Materials & Methods

Study area and sample collection

Materials for the study were collected in two National Parks: Poleski NP and Roztocze NP in south-eastern Poland. Field studies were approved by the Ministry of the Environment in Poland (field study approval numbers: DLP-III-4102-21/1728/15/MD for the field study in

Poleski National Park and DPL-LLL-4102-609/1699/14/MD for the field study in Roztocze National Park). Adults and larvae of black tinder fungus beetle *B. reticulatus* were caught in fruiting bodies of *F. fomentarius* (Figs. 1A and 1B, respectively). Five adults and five larvae were collected from the same fruit body growing on fallen birch trunk in Łowiszów (DMS: 51º26'57.762" N, 23º14'29.839" E), 10. November 2016, Poleski NP (Fig. 1C) and the same number of individuals were collected from one fungus growing on fallen beech stump in Obrocz (DMS: 50º34'32.403" N, 23º"24.388" E), 10. December 2016, Roztocze NP (Fig. 1D). Specimens were separately placed into tubes and stored at –30˚C. Afterwards the tubes with insects were sent for further analyses to the Department of Genetics and Biosystematics, University of Gdansk, Poland.

Studies on the occurrence of *F. fomentarius* fruiting bodies growing on birch and beech were performed during the years 2013-2016. In those studies four fallen tree trunks from each tree species were selected for further analyses. Chosen trunks were plentifully covered with sporocarps. Beech trunks were located in Roztocze NP (localization – Obrocz, DMS: 50º34'32.403" N, 23º0'24.388" E), while birch trunks were located in Poleski NP (localization: Lipniak DMS: 51º27'51.363" N, 23º6'28.062" E). Healthy and settled fruiting bodies were counted in the field and results are presented in Supplemental Table S1. For preliminary biochemical analyses, fruiting bodies of the tinder fungus (Fig. 1B.) were collected in July 2016 from fallen trunks of each tree species growing in two mentioned localities. In both sampling sites, 5 samples of healthy fruiting bodies and the same number of the inhabited fruiting bodies were taken. The fruiting bodies were inhabited by few species of mycophagous beetles with dominance of the studied species – *B. reticulatus*. Samples were cut out from the tissue above the hymenium.

136

137 *DNA extraction*

138 DNA was extracted from the whole bodies of *B. reticulatus* at two developmental stages
139 (larvae and adults, respectively). Insects were rinsed three times in sterile distilled water prior to
140 DNA extraction without soaking in ethanol. Specimens were homogenized with 0.7 mm garnet
141 beads in a high speed benchtop homogenizer FastPrep®-24 (MP Biomedicals, USA) at 4.5 m/s
142 for 20 s. The total DNA was then extracted using the Sherlock AX Purification Kit (A&A
143 Biotechnology, Poland). Thus, presented study resolves the complex microbial population
144 structure of two developmental stages of *B. reticulatus* collected from fruiting bodies growing on
145 different hosts. To avoid cross contamination of samples, the process was performed with sterile
146 equipment. The quantity and quality of the extracted DNA were evaluated by using a Nano Drop
147 ND-1000 spectrophotometer (Nano Drop Technologies). After extraction, the DNA was stored at
148 -20°C until further use. Twelve samples consisting of genetic material isolated from larvae and
149 adults (one individual per DNA isolate and three isolates per developmental stage collected from
150 fungi growing on different hosts) were used for microbiome analyses.

151

152 *16S rRNA gene sequencing and bacterial community analyses*

153 The V3-V4 hypervariable regions of bacterial 16S rRNA gene region were amplified
154 using the following primer set: 341F – 5'-CCTACGGGNGGCWGCAG-3' and 785R – 5'-
155 GACTACHVGGGTATCTAATCC-3'. The targeted gene region has been shown to be suitable
156 for the Illumina sequencing (Klindworth et al., 2013). Libraries were prepared with a two-step
157 PCR protocol based on Illumina's "16S metagenomic library prep guide" (15044223 Rev. B)
158 with NEBNext® Q5 Hotstart High-Fidelity DNA polymerase (New England BioLabs Inc.)

according to the manufacturer's protocol, using Q5® Hot Start High-Fidelity 2X Master Mix (NEBNext - New England BioLabs) and the Nextera Index kit (2x250bp). PCR was carried out under the following conditions: 98°C for 30 sec for initial denaturation of the DNA, followed by 25 cycles of 98°C for 10 sec, 55°C for 30 sec, 72°C for 20 sec and for additional 2 min at 72°C for final extension. Paired-end (PE, 2x250nt) sequencing with a 5% PhiX spike-in was performed with an Illumina MiSeq (MiSeq Reagent kit v2) at Genomed, Warsaw, Poland; following the manufacturer's run protocols (Illumina, Inc., San Diego, CA, USA). Automatic primary analysis and de-multiplexing of the raw reads were performed with MiSeq, with the use of MiSeq Reporter (MSR) v2.6 (16S Metagenomics Protocol).

The genetic material isolated from 12 individuals was sequenced separately. Samples were then marked as follows: L-*Fagus*-X and Im-*Fagus*-X for larva and adult collected from *F. fomentarius* fruiting body growing on beech stump; L-*Betula*-X and Im-*Betula*-X for larva and adult collected from *F. fomentarius* fruiting body growing on birch stump (X indicates the number of individual).

The samples were processed and analyzed using the Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.1) pipeline (Caporaso et al., 2010). Paired-end reads from MiSeq sequencing were quality trimmed and joined with PANDAseq version 2.8 (Masella et al., 2012) with a quality threshold of 0.9. The sequences that did not meet the quality criteria were removed from further analysis (mean quality >20). Chimeric reads detection was performed with VSEARCH, version 1.7.0 (Rognes et al., 2016), an open-source replacement of USEARCH software. Operational Taxonomic Unit (OTU) clustering was performed using UCLUST method, version 1.2.22q (Edgar, 2010) and taxonomic assignment performed at 97% against the SILVA v.132 database (Quast et al., 2013). The Biological Observation Matrix (BIOM) table was used

as the core data for downstream analyses (McDonald et al., 2012). Any sequences that were classified as Mitochondria or Chloroplast, as well as singletons were filtered out of the dataset. Sample reads were rarefied to 38,188 reads. OTU saturation was evaluated with rarefaction curves using Chao1 richness estimate. Moreover, the diversity indices were estimated, including the Chao1, PD (a quantitative measure of phylogenetic diversity), Shannon, and Simpson indices and also the number of observed OTUs. Comparison of the microbial community structures was performed with the use of UniFrac (Lozupone & Knight, 2005) and Emperor (Vázquez-Baeza et al., 2013). A two-sample *t*-test with a non-parametric Monte Carlo permutations (n = 999) and Bonferroni correction was used to test for statistically significant difference in alpha diversity between predefined groups (according to (1) developmental stage, (2) host tree species, (3) both mentioned factors). A two-dimensional Principal Coordinate Analysis (PCoA) was conducted from weighted UniFrac distances obtained in previous steps. In order to determine if observed clusters of samples were significantly dissimilar, an analysis of similarity (ANOSIM; Clarke, 1993) was performed in QIIME with 999 permutations.

Similarity percentage (SIMPER) analysis was performed to calculate the average dissimilarities in microbial community structures between particular samples and to access which family was responsible for the observed differences. Statistical analyses were performed using PAST 3.16 software (Ryan et al., 2001). Finally, to illustrate the most abundant bacterial families and community relationships across tested samples a heatmap and dendrogram was generated with Bray-Curtis dissimilarity index. Bacterial families whose relative read abundance was less than 3% of at least one sample were removed. Those analyses were performed in R v.3.4.3 (Neuwirth, 2014; Ploner, 2015; Oksanen et al., 2018; Warnes et al., 2019).

205 *Data availability*

206 Bacterial 16S reads for each sample were submitted to the European Nucleotide Archive
207 (ENA) database under accession number PRJEB23388.

208

209 *Preliminary biochemistry analyses*

210 With the use of thin layer chromatography (TLC), comparison of entomotoxic and
211 insecticidal features of fruiting bodies of *F. fomentarius*, enzyme analyses of the level of
212 protease inhibitors (Sabotič, Ohm & Künzler, 2016) and analyses of secondary metabolite
213 profiles was performed (Anke & Sterner, 2002).

214 Samples from 20 sporocarps of a known type (from beech and birch, healthy and
215 inhabited by beetles – separately) were mechanically ground and then homogenized in distilled
216 water (for inhibitor determinations and TLC analyses of secondary metabolites) or in methanol
217 (to TLC of secondary metabolites) in a Potter homogenizer; 100 mg of shredded the sporocarp in
218 5 ml of water or methanol. The homogenates were then centrifuged to give supernatants as assay
219 preparations (Sobczyk, 2010; Jaruga, 2013). Protein in water extracts was determined by
220 standard Bradford method (Bradford, 1976).

221 The level of protease inhibitors was determined according to Sobczyk (2010) and with
222 marker proteases (used to determine type of inhibitor and specific pH) according to Anson
223 (1938). 0.1 ml of the preparation was incubated with 0.1 ml marker enzyme solution (pepsin at
224 pH 5.0 for aspartate acid protease inhibitors, trypsin and papain at pH 7.0 for neutral serine and
225 cysteine protease inhibitors and trypsin at pH 9.0 for alkaline serine protease inhibitors) for 30
226 minutes at 37° C. After this time, 0.5 ml of the hemoglobin solution was added in buffer of
227 appropriate pH and incubated for 1 hour at 37° C. The reaction was stopped by adding 2.0 ml of

5% TCA (trichloroacetic acid). Samples were centrifuged and their absorbance measured at 280 nm. As controls, water instead of preparation (sample for % inhibition calculation) and marker enzyme with water instead of specimen (zero-sample to reset the spectrophotometer) were used. Percent inhibition was determined as the percentage of marker enzyme inhibition (Sobczyk, 2010).

During qualitative analysis of secondary metabolites by TLC, two types of extracts from the fruiting bodies were analyzed: aqueous and methanol. TLC chromatography was developed in two systems – ethanol : water (7:3) and ethyl acetate : acetic acid : water (2:1:1). Merck ready TLC plates (type: TLC Silica gel 60 F254) were used. Visualization of plates was done by UV light observation (254 nm and 365 nm), showing visible spots of secondary metabolites. Qualitative analysis were made by spot diameter and intensity of glaring, relative rating (standard TLC procedures). Then calculation of their retardation factor (Rf – defined as the ratio of the distance traveled by the center of a spot to the distance traveled by the solvent front) and estimation of their relative UV intensity was performed (Jaruga, 2013). To determine the identity of the compounds the Rf values were compared to the Rf value of compounds listed in databases (Clevenger et al., 2017).

Results

General description of 16S rRNA gene sequencing results

For each *B. reticulatus* sample, we obtained >38,000 good quality 16S rRNA gene sequences (V3-V4 region), ranging between 38,188 for L-*Betula*-2 and 73,856 for Im-*Betula*-3. Rarefaction curves with Chao1 diversity indices, indicating insect microbiome sampling depth and saturation is shown in Fig. 2A. Observed curves almost reached a plateau at this sequencing depth, suggesting that the sequencing was approximately deep. More details for sequence data

for each sample, as well as the number of the observed OTUs and the diversity indices are shown in Table 1. At least 319 OTUs, ranging from 319–1223, were observed in different samples of *B. reticulatus*, which indicates that the microbial population is complex.

In all samples, at least 98,94% of the reads could be classified to phylum level. Detailed taxonomic analyses on different ranks are available in supplementary data as sunburst charts (Supplemental Data S1) and also in a table (Supplemental Table S2).

Bacterial community composition

The analysis of bacterial community showed that for both larvae and adults of *B. reticulatus* >99.94% of total reads were represented by Bacteria (Supplemental Data S1 and Supplemental Table S2). The remaining percentage comprised *Archaea*. The microbiomes tested in this study contained 24 phyla. The most abundant phyla across all tested stages were Proteobacteria, Actinobacteria and Bacteroidetes. In each sample of *B. reticulatus* developmental stages collected from *F. fomentarius* fruiting bodies growing on both tree species, those phyla jointly accounted for more than 81.45% of the total microbial sequences obtained. Other bacterial and archaeal phyla were present in tested microbial communities with different relative abundances given in Supplemental Table S2.

The most abundant classes among bacterial communities of all samples were Alphaproteobacteria, Actinobacteria and Gammaproteobacteria, accounting for more than 69% of the total reads (Supplemental Data S1, Supplemental Table S2). The most abundant orders among analyzed microbiome profiles were Corynebacteriales, Betaproteobacteriales and Rhizobiales, accounting for more than 30% of the total reads. The most abundant family was Burkholderiaceae and among that family the most abundant genus was *Burkholderia-*

275 *Caballeronia-Parabirkholderia*, which accounted for 16.17% of the reads on average (ranging
276 from 0.82% in L-*Fagus*-2 to 38.87% in Im-*Fagus*-1).

277 Similarities among the bacterial community structures associated with tested samples are
278 illustrated with a heatmap (Fig. 3). We identified 27 families, which relative abundance was not
279 less than 3% of at least one sample. Tested samples primarily clustered by developmental stage
280 of *B. reticulatus* and host tree did not appear to impact the taxonomic distribution of the
281 communities (Fig. 2B). This observation was supported by statistical analyses (ANOSIM: R =
282 0.88, $p = 0.002$, and the alpha diversity indices were only significantly different ($p = 0.004$)
283 between samples grouped according to the developmental stage). In bacterial communities of
284 larvae Microbacteriaceae and Rhiziobiaceae were slightly more abundant, whereas
285 Acidobacteriaceae, Sphingomonadaceae, Rhodanobacteraceae, Mycobacteraceae and
286 Sphingomonadaceae were more abundant in microbiome of *B. reticulatus* adults. SIMPER
287 analysis showed that the last four families were primarily responsible for the differences between
288 microbial communities of larvae and adults.

289 Analyses of bacterial communities associated with *B. reticulatus* allow to identify the
290 presence of known endosymbionts. *Wolbachia* has been detected in 4 samples: all larvae
291 collected from *F. fomentarius* growing on beech (0.08% for both L-*Fagus*-1 and L-*Fagus*-2, and
292 0.14% for L-*Fagus*-3) and in one adult related to birch (<0.01% for Im-*Betula*-2). Moreover, two
293 other known endosymbiotic bacteria have been detected – *Arsenophonus* (0.06% for Im-*Fagus*-3
294 and L-*Betula*-2) and *Candidatus Cardinium* (0.01% for Im-*Betula*-1).

295

296 *Level of protease inhibitors and secondary metabolite profiles in F. fomentarius fruiting bodies*

297 The results of preliminary biochemical analysis of the collected fruiting bodies showed
298 that the level of inhibition was higher for aspartic acid protease inhibitors in fungus from beech,
299 and for cysteine inhibitors and serine neutral proteases in fungus from birch (Table 2). In the
300 case of inhibitors of basic serine proteases, the levels from both trees were similar (Table 2). The
301 level of inhibitors is higher for healthy fruiting bodies than for the inhabited ones.
302 Positive TLC results were obtained for both types of extraction (water and methanol)
303 (Table 3), but only in the ethanol-water system. In the ethyl acetate-acetic acid-water system, no
304 separation was obtained and only a spot corresponding to the initial application on the TLC plate
305 was apparent. The strongest spot of secondary metabolites, 0.11176, from a healthy beech tree,
306 has not been identified. There were no differences in the intensities of the weaker 0.9092 spot
307 from the healthy beech tree across samples and the 0.8235 spot, which was present in all fruiting
308 bodies. There is a visible decrease in the intensity of spots 0.7058, 0.1176 and 0.7882 on beech
309 between healthy and colonized fruit bodies. Spots 0.11176 (methanol), 0.9092 (water) and
310 0.7882 (water) are absent in the fruiting bodies growing on the birch, both healthy and inhabited.
311 The 0.7058 spot in all samples from the aqueous extract did not change its intensity, in
312 comparison to spots with the same Rf from the methanol extract.
313

314 **Discussion**

315 In the present study, we investigated the microbiome profiles of larvae and adults of
316 fungivorous beetle *B. reticulatus*. Obtained patterns of the abundance at both phylum and class
317 level remained in congruence with previous studies. Proteobacteria, Actinobacteria and
318 Bacteroidetes phyla, as well as Alpha- and Gammaproteobacteria classes have been listed as
319 most abundant bacterial groups in various insect species (e.g. Colman, Toolson & Takacs-

Vesbach, 2012; Jones, Sanchez & Fierer, 2013; Yun et al., 2014; Kim et al., 2017), also those cultivating fungi (Aylward et al., 2014). Moreover, identified patterns of the abundance remained in congruence with the results of our previous study focused on microbial communities associated with *Hoplothrips carpathicus* (Thysanoptera), which also inhabits fruiting bodies of *F. fomentarius* (Kaczmarczyk et al., 2018).

Interestingly, at the genus level *Burkholderia-Cabalerrinia-Paraburkholderia* (Burkholderiaceae) was one of the most dominant genera in all tested samples. In the study of bacterial communities associated with fungivorous *H. carpathicus* this genus was also noted (Kaczmarczyk et al., 2018), but it was not as abundant as in *B. reticulatus*. *Burkholderia* was also identified in bacterial communities associated with other insects e.g. in the larvae of the wood-feeding beetle *Prionoplus reticularis* (Reid et al., 2011), in longicorn beetle *Prionus insularis* (Park et al., 2007) or in members of Heteroptera (Kikuchi, Hosokawa & Fukatsu, 2010). This genus is linked with several functions – nitrogen fixation (Estrada-De et al., 2001), defence mechanisms (Santos et al., 2004), aromatic compound degradation (Laurie & Lloyd-Jones, 1999; Bugg et al., 2011), and detoxification of tree defence compounds (Smith et al., 2007; Adams et al., 2013). Furthermore, a symbiotic relationship between *Burkholderia* and white rot fungus *Phanerochaete chrysosporium* was described by Seigle-Murandi et al. (1996). This fungus species, similar to *F. fomentarius*, degrades lignocellulosic materials. Nevertheless, symbiotic relationships between *F. fomentarius* and microorganisms has not been investigated yet. Therefore, one may not exclude that *Burkholderia* identified in bacterial communities of *B. reticulatus* is connected also with the tinder fungus via a symbiotic relationship. However, its presence in microbiome profiles of different developmental stages of black tinder fungus beetle

342 is thought to be related to the potential of *Burkholderia* representatives to degradation of
343 aromatic compounds (e.g. lignin) present in *B. reticulatus* food source.

344 Besides *Burkholderia*, we found in tested bacterial communities also other genera, which
345 have been considered as degraders of aromatic compounds. *Pseudomonas* (Pseudomonadaceae)
346 and *Serratia* (Enterobacteriaceae) identified previously in microbiome of the mountain pine
347 beetle *Dendroctonus ponderosae*, have been described as terpene degraders (Adams et al., 2013).
348 In turn, *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*
349 (Sphingomonadaceae) have been recognised being involved in degradation of various
350 recalcitrant aromatic compounds and polysaccharides (Aylward et al., 2013). Moreover,
351 *Sphingomonas* has been identified in microbiome of wood-boring beetle, *Anoplophora*
352 *glabripennis* as genus involved in the degradation of lignocellulose, hemicellulose, and other
353 aromatic hydrocarbons (Geib et al., 2009a; Geib et al., 2009b; Scully et al., 2013). *Bosea*
354 (Beijerinckiaceae; also found in present study) was in turn identified being associated with
355 cuticles of plant-ant genera *Allomerus* and *Tetraponera* (Seipke et al., 2013) and described as
356 hydrocarbon degrader (Yang et al., 2016). Comprehensive analyses of the functional microbiome
357 of arthropods (e.g. honeybees, fruit flies, cockroaches, termites, ants and beetles) show that
358 *Burkholderia*, *Sphingomonas* and *Bosea* are together involved in the same processes e.g. aerobic
359 metabolism, reacting with cytochrome *c* or bypassing cytochrome *c* (Esposti & Romero, 2017).

360 The bacterial community associated with black tinder fungus beetle likely plays a role in
361 promotion of efficient digestion for extraction of maximum energy from ingested substrates.
362 Nevertheless, specific conditions in microenvironment of *F. fomentarius* fruiting body may
363 cause strong selective pressure against microorganisms that are not able to survive exposure to
364 defensive compounds produced by the tinder fungus. Recent study shed light on antimicrobial

activities of *F. fomentarius*. Kolundžić et al. (2016) found that the tinder fungus extracts of different polarity exhibit significant antimicrobial activity against nine bacterial strains (including *Staphylococcus aureus*, *Staphylococcus epidemidis, Bacillus subtilis* or *Klebsiella pneumonia*). In fact, the relative abundance of those bacteria in microbiome of *B. reticulatus* were low and in most samples tested did not exceed 1%. The observed antimicrobial activity of *F. fomentarius* may be linked e.g. with polyphenols and β-glucans which abundances are relatively high in its fruiting bodies (Seniuk et al., 2011; Zhao et al., 2013; Alves et al., 2013; Zhu et al., 2015) or with sesquiterpens which have been described as active compounds (identified enzyme inhibitors with antifungal, antibacterial and cytotoxic activities) (Abraham, 2001; Keller, 2018). These compounds may be considered as toxins for bacteria associated with fungivorous species inhabiting *F. fomentarius* fruiting bodies. Thus, associated bacterial communities need to overcome the presence of such substances through resistance or tolerance mechanisms.

Preliminary analyses performed in PICRUSt (Langille et al., 2013; Supplemental Figure S1) showed that in all tested bacterial communities genes involved in membrane transport and in metabolism of terpenoids and polyketides, as well as in xenobiotic biodegradation and metabolism should be elevated. Interestingly, terpenoids are main secondary metabolites of *F. fomentarius* (around 75%) (Grienke et al., 2014) what means that microorganisms associated with *F. fomentarius* feeding species remain exposed to these compounds. Moreover, the similar pattern of relative abundances were identified for predicted genes in the case of bacterial communities associated with thrips *H. carpathicus* (Kaczmarczyk et al., 2018). This might mean that similarities in predicted patterns of relative abundances of genes are characteristic for bacterial communities associated with fungivorous species inhabiting fruiting bodies of wood-

388 decaying fungi. However, the *in silico* predicted functions need to be validated *in vitro* in future
389 studies.

390 The additional aim of the present study was connected with the long-term field
391 observations showing that there is a difference between the amount of healthy and colonized
392 sporocarps growing on the trunks of both beech and birch (Supplemental Table S1). In the case
393 of beech, the *F. fomentarius* fruiting bodies inhabited by insects were collected less frequently
394 (~27% on average, in the range from 19% to 33% of all collected fruiting bodies growing on
395 selected trunks). In turn, the number of colonized sporocarps growing on birch was higher
396 (~66% on average) and ranged from 46% to 85% of all collected fruiting bodies growing on
397 these trees. To identify the potential factors related to the observed differences in degree of
398 fruiting bodies colonization by insects, we identified trends in biochemical profiles of fruiting
399 bodies growing on two different tree hosts.

400 We analyzed the level of protease inhibitors and secondary metabolites detected in *F.*
401 *fomentarius* fruiting bodies, which are involved in protection against fungivorous insects (Anke
402 & Sterner, 2002; Sabotič, Ohm & Künzler, 2016). Moreover, we investigated the bacterial
403 communities of larvae and adults of *B. reticulatus* for potential differences which could be
404 related to host-tree species.

405 The biochemical analysis showed that a higher level of protease inhibitors was observed
406 in healthy fruiting bodies than in colonized ones. Unfortunately, no previous research has been
407 done to investigate this phenomenon, so it is unclear why inhibitor levels are lower in colonized
408 fruiting bodies. Changes in levels of acidic, neutral, and alkaline proteases in colonized
409 sporocarps may resulted from the inherent properties of insect proteases, which are dominated by
410 serine and cysteine proteases over aspartic proteases (Terra & Ferreira, 1994). In turn, a high

411 level of acid inhibitors of aspartic proteases may be additionally associated with the protection of
412 fruiting bodies against pathogenic and saprophytic microscopic fungi (mainly molds) (Monod et
413 al., 2002), which have high levels of aspartic proteases in their proteolytic apparatus.
414 Moreover, performed analyses indicated the presence of unidentified secondary
415 metabolites in samples of non-colonized fruiting bodies collected from beech. Probably, these
416 substances are able to determine the susceptibility of fruiting bodies to be colonized by insects
417 and, generally, to be infected. It is worth noting that even dozen-year-old specimens of *F.*
418 *fomentarius*, growing on beech wood, are usually completely healthy, while fungi growing on
419 the birch, are colonized by insects and has signs of the presence of pathogenic fungi (e.g. mold).
420 Therefore, some obvious contributing factors to this phenomenon (e.g. size of fruiting body, sun
421 exposure or age) seem to be insignificant for determining the degree of fruiting bodies
422 colonization by insects. Some authors showed that *Fagus sylvatica* produces flavonoids and
423 organic acids which can be classified as repellents against insects (Harborne, 1997; Simmonds,
424 2003; Treutter, 2005; Podgórski & Podgórska, 2009). The accumulation of some flavonoids in
425 the fruiting bodies of *F. fomentarius* could be a barrier against the fungivorous beetles. At this
426 stage, it cannot be excluded that flavonoids are among those unidentified secondary metabolites
427 detected in present study. However, the identification of the secondary metabolites and their
428 potential impact on the relationship among host tree, fungi and insects require in-depth studies.
429 We are aware that these biochemical analyzes should be treated as preliminary. Fruiting
430 bodies for biochemical analyses were collected earlier than those from which *B. reticulatus*
431 specimens were collected. Both sets of samples were collected for different studies. Although the
432 obtained patterns needs to be tested in more complex studies where beetles would be collected
433 from sporocarps, which would then be used for further biochemical analyses, the results

presented here could be considered as insight in potential trends. More advanced biochemical analyses (e.g. using liquid chromatography or mass spectrometry) may provide a more complete insight into the biochemical profile of fruiting bodies. However, despite the observed trends in biochemical profiles, tested microbiome samples primarily clustered by developmental stage of *B. reticulatus* and host tree did not appear to impact the taxonomic distribution of the communities, what was supported by statistical analyses.

Additionally, the known endosymbionts has been identified in microbiome profiles of *B. reticulatus*. Among them *Wolbachia* should receive a special attention. It is a well-known endosymbiont, which is estimated to be present in more than 65% of all insect species (Hilgenboecker et al., 2008; Lewis & Lizé, 2015). *Wolbachia* is related to five commonly recognized manipulation schemes: feminization, parthenogenesis induction, early and late male killing, and cytoplasmic incompatibility (Engelstädter & Hurst, 2009). It appears that these phenomena do not occur in *B. reticulatus*, but more comprehensive studies should be performed to test this hypothesis.

Conclusions

In conclusion, this paper presents the insight into bacterial communities associated with two developmental stages of *B. reticulatus* beetle with the use of 16S rRNA sequence data. The approach based on NGS technique allowed us to characterize of tested microbiome. Moreover, it is the first approach to identification of factors which can be related to differences in degree of fruiting bodies colonization by insects. Results of this study show biochemical differences in fruiting bodies collected from birch and beech. We compared these preliminary results with those obtained during analyses of bacterial communities associated with *B. reticulatus*. However,

457 the host-tree appears to have no effect on the bacterial communities associated with tested
458 developmental stages of *B. reticulatus*. Despite the observed trends in biochemical profiles of
459 sporocarps collected from both tree species, tested samples primarily clustered by developmental
460 stage of *B. reticulatus*. Moreover, endosymbiotic Alphaproteobacteria *Wolbachia* was identified
461 for the first time in *B. reticulatus*.
462

References

Abraham W-R. 2001. Bioactive Sesquiterpenes Produced by Fungi are they Useful for Humans as Well. *Current Medicinal Chemistry* 8:583–606.

Adams AS., Aylward FO., Adams SM., Erbilgin N., Aukema BH., Currie CR., Suen G., Raffa KF. 2013. Mountain Pine Beetles Colonizing Historical and Naïve Host Trees Are Associated with a Bacterial Community Highly Enriched in Genes Contributing to Terpene Metabolism. *Applied and Environmental Microbiology* 79:3468–3475. DOI: 10.1128/AEM.00068-13.

Alves MJ., Ferreira ICFR., Froufe HJC., Abreu RMV., Martins A., Pintado M. 2013. Antimicrobial activity of phenolic compounds identified in wild mushrooms, SAR analysis and docking studies. *Journal of Applied Microbiology* 115:346–357. DOI: 10.1111/jam.12196.

Anke H., Sterner O. 2002. Insecticidal and nematicidal metabolites from fungi. In: *The Mycota - Industrial Applications*. Berlin: Springer Verlag, 109–135.

Anson ML. 1938. the Estimation of Pepsin, Trypsin, Papain, and Cathepsin With Hemoglobin. *The Journal of General Physiology* 22:79–89. DOI: 10.1085/jgp.22.1.79.

Arunachalam M., Mohan Raj M., Mohan N., Mahadevan A. 2003. Biodegradation of Catechin. *Proceedings of the Indian National Science Academy* B69:353–370. DOI: 10.1017/S000711451000396X.

Aylward FO., McDonald BR., Adams SM., Valenzuela A., Schmidt RA., Goodwin LA., Woyke T., Currie CR., Suen G., Poulsen M. 2013. Comparison of 26 Sphingomonad Genomes Reveals Diverse Environmental Adaptations and Biodegradative Capabilities. *Applied and Environmental Microbiology* 79:3724–3733. DOI: 10.1128/AEM.00518-13.

Aylward FO., Suen G., Biedermann PHW., Adams AS., Scott JJ., Malfatti SA., Glavina del Rio T., Tringe SG., Poulsen M., Raffa KF., Klepzig KD., Currie CR. 2014. Convergent bacterial microbiotas in the fungal agricultural systems of insects. *mBio* 5:e02077. DOI: 10.1128/mBio.02077-14.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72:248–254.

Bugg TD., Ahmad M., Hardiman EM., Singh R. 2011. The emerging role for bacteria in lignin degradation and bio-product formation. *Current Opinion in Biotechnology* 22:394–400. DOI: 10.1016/j.copbio.2010.10.009.

Caporaso JG., Kuczynski J., Stombaugh J., Bittinger K., Bushman FD., Costello EK., Fierer N., Gonzalez Pena A., Goodrich JK., Gordon JI., Huttley GA., Kelley ST., Knights D., Koenig JE., Ley RE., Lozupone CA., McDonald D., Muegge BD., Pirrung M., Reeder J., Sevinsly JR., Turnbaugh PJ., Walters WA., Widmann J., Yatsunenko T., Zaneveld J., Knight R. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* 7:335–336.

Chen W., Zhao Z., Chen S-F., Li Y-Q. 2008. Optimization for the production of exopolysaccharide from Fomes fomentarius in submerged culture and its antitumor effect in vitro. *Bioresource Technology* 99:3187–3194. DOI: 10.1016/J.BIORTECH.2007.05.049.

Clarke KR. 1993. Non-parametric multivariate analyses of changes in community structure. *Australian Journal of Ecology* 18:117–143. DOI: 10.1111/j.1442-9993.1993.tb00438.x.

Clevenger KD., Bok JW., Ye R., Miley GP., Verdan MH., Velk T., Chen C., Yang K., Robey MT., Gao P., Lamprecht M., Thomas PM., Islam MN., Palmer JM., Wu CC., Keller NP.,

509 Kelleher NL. 2017. A scalable platform to identify fungal secondary metabolites and their
510 gene clusters. *Nature Chemical Biology* 13:895–901. DOI: 10.1038/nchembio.2408.
511 Colman DR., Toolson EC., Takacs-Vesbach CD. 2012. Do diet and taxonomy influence insect
512 gut bacterial communities? *Molecular Ecology* 21:5124–5137. DOI: 10.1111/j.1365-
513 294X.2012.05752.x.
514 Dresch P., D'Aguanno MN., Rosam K., Grienke U., Rollinger JM., Peintner U. 2015. Fungal
515 strain matters: colony growth and bioactivity of the European medicinal polypores *Fomes*
516 *fomentarius*, *Fomitopsis pinicola* and *Piptoporus betulinus*. *AMB Express* 5:4. DOI:
517 10.1186/s13568-014-0093-0.
518 Edgar RC. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*
519 26:2460–2461. DOI: 10.1093/bioinformatics/btq461.
520 Elisashvili V., Kachlishvili E., Tsiklauri N., Metreveli E., Khardziani T., Agathos SN. 2009.
521 Lignocellulose-degrading enzyme production by white-rot Basidiomycetes isolated from the
522 forests of Georgia. *World Journal of Microbiology and Biotechnology* 25:331–339. DOI:
523 10.1007/s11274-008-9897-x.
524 Engelstädter J., Hurst GDD. 2009. The Ecology and Evolution of Microbes that Manipulate Host
525 Reproduction. *Annual Review of Ecology, Evolution, and Systematics* 40:127–49. DOI:
526 10.1146/annurev.ecolsys.110308.120206.
527 Esposti MD., Romero EM. 2017. The functional microbiome of arthropods. *PLoS One*
528 12:e0176573. DOI: 10.1371/journal.pone.0176573.
529 Estrada-De P., Santos L., Bustillos-Cristales RO., Caballero-Mellado JS. 2001. *Burkholderia*, a
530 Genus Rich in Plant-Associated Nitrogen Fixers with Wide Environmental and Geographic
531 Distribution. *Applied and Environmental Microbiology* 67:2790–2798. DOI:

10.1128/AEM.67.6.2790–2798.2001.

Geib SM., Jimenez-Gasco MDM., Carlson JE., Tien M., Hoover K. 2009a. Effect of host tree species on cellulase activity and bacterial community composition in the gut of larval Asian longhorned beetle. *Environmental Entomology* 38:686–699.

Geib SM., Del Mar Jimenez-Gasco M., Carlson JE., Tien M., Jabbour R., Hoover K. 2009b. Microbial Community Profiling to Investigate Transmission of Bacteria Between Life Stages of the Wood-Boring Beetle, *Anoplophora glabripennis*. *Microbial Ecology* 58:199–211. DOI: 10.1007/s00248-009-9501-4.

Grienke U., Zöll M., Peintner U., Rollinger JM. 2014. European medicinal polypores – A modern view on traditional uses. *Journal of Ethnopharmacology* 154:564–583. DOI: 10.1016/j.jep.2014.04.030.

Gutowski JM. 2006. Saproksyliczne chrząszcze. *Kosmos* 55:53–73.

Gutowski JM., Bobiec A., Pawlaczyk P., Zub K. 2004. *Drugie życie drzewa*. Warszawa-Hajnówka: WWF Polska.

Gutowski JM., Buchholz L. 2000. Owady leśne – zagrożenia i propozycje ochrony. *Wiadomości Entomologiczne* 18:43–72.

Harborne JB. 1997. *Ekologia biochemiczna*. Warsaw: Wydawnictwo Naukowe PWN.

Hilgenboecker K., Hammerstein P., Schlattmann P., Telschow A., Werren JH. 2008. How many species are infected with *Wolbachia*? A statistical analysis of current data. *FEMS Microbiology Letters* 281:215–20. DOI: 10.1111/j.1574-6968.2008.01110.x.

Jaruga A. 2013. Inhibitory metabolizmu alkoholu z grzybów wyższych. Master's Thesis. Maria Curie-Skłodowska Univeristy.

Jaszek M., Żuchowski J., Dajczak E., Cimek K., Grąz M., Grzywnowicz K. 2006. Ligninolytic

555 enzymes can participate in a multiple response system to oxidative stress in white-rot
556 basidiomycetes: *Fomes fomentarius* and *Tyromyces pubescens*. *International*
557 *Biodeterioration & Biodegradation* 58:168–175. DOI: 10.1016/J.IBIOD.2006.06.012.
558 Jones RT., Sanchez LG., Fierer N. 2013. A cross-taxon analysis of insect-associated bacterial
559 diversity. *PLoS One* 8:e61218. DOI: 10.1371/journal.pone.0061218.
560 Kaczmarczyk A., Kucharczyk H., Kucharczyk M., Kapusta P., Sell J., Zielińska S. 2018. First
561 insight into microbiome profile of fungivorous thrips *Hoplothrips carpathicus* (Insecta:
562 Thysanoptera) at different developmental stages: molecular evidence of Wolbachia
563 endosymbiosis. *Scientific Reports* 8:14376. DOI: 10.1038/s41598-018-32747-x.
564 Keller NP. 2018. Fungal secondary metabolism: regulation, function and drug discovery. *Nature*
565 *Reviews Microbiology*:1. DOI: 10.1038/s41579-018-0121-1.
566 Kikuchi Y., Hosokawa T., Fukatsu T. 2010. An ancient but promiscuous host–symbiont
567 association between *Burkholderia* gut symbionts and their heteropteran hosts. *The ISME*
568 *Journal* 5:446–460. DOI: 10.1038/ismej.2010.150.
569 Kim JM., Choi M-Y., Kim J-W., Lee SA., Ahn J-H., Song J., Kim S-H., Weon H-Y. 2017.
570 Effects of diet type, developmental stage, and gut compartment in the gut bacterial
571 communities of two Cerambycidae species (Coleoptera). *Journal of Microbiology* 55:21–
572 30. DOI: 10.1007/s12275-017-6561-x.
573 Klindworth A., Pruesse E., Schweer T., Peplies J., Quast C., Horn M., Glöckner FO. 2013.
574 Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-
575 generation sequencing-based diversity studies. *Nucleic Acids Research* 41:1–11. DOI:
576 10.1093/nar/gks808.
577 Kolundžić M., Grozdanić ND., Dodevska M., Milenković M., Sisto F., Miani A., Farronato G.,

578 Kundaković T. 2016. Antibacterial and cytotoxic activities of wild mushroom *Fomes*
579 *fomentarius* (L.) Fr., Polyporaceae. *Industrial Crops & Products* 79:110–115. DOI:
580 10.1016/j.indcrop.2015.10.030.
581 Langille MGI., Zaneveld J., Caporaso JG., Mcdonald D., Knights D., Reyes JA., Clemente JC.,
582 Burkepile DE., Vega Thurber RL., Knight R., Beiko RG., Huttenhower C. 2013. Predictive
583 functional profiling of microbial communities using 16S rRNA marker gene sequences
584 HHS Public Access. *Nature Biotechnology* 31:814–821. DOI: 10.1038/nbt.2676.
585 Laurie AD., Lloyd-Jones G. 1999. The *phn* Genes of *Burkholderia* sp. Strain RP007 Constitute a
586 Divergent Gene Cluster for Polycyclic Aromatic Hydrocarbon Catabolism. *Journal of*
587 *Bacteriology* 181:531–540.
588 Lewis Z., Lizé A. 2015. Insect behaviour and the microbiome. *Current Opinion in Insect Science*
589 9:86–90. DOI: 10.1016/J.COIS.2015.03.003.
590 Lozupone C., Knight R. 2005. UniFrac: a new phylogenetic method for comparing microbial
591 communities. *Applied and Environmental Microbiology* 71:8228–8235.
592 Masella AP., Bartram AK., Truszkowski JM., Brown DG., Naufeld JD. 2012. PANDAseq:
593 paired-end assembler for illumina sequences. *BMC Bioinformatics* 13:31. DOI:
594 10.1186/1471-2105-13-31.
595 McDonald D., Clemente JC., Kuczynski J., Rideout JR., Stombaugh J., Wendel D., Wilke A.,
596 Huse S., Hufnagle J., Meyer F., Knight R., Caporaso JG. 2012. The Biological Observation
597 Matrix (BIOM) format or: how I learned to stop worrying and love the ome-ome.
598 *GigaScience* 1:1–6. DOI: 10.1186/2047-217X-1-7.
599 Midtgaard F., Rukke BA., Sverdrup-Thygeson A. 1998. Habitat use of the fungivorous beetle
600 *Bolitophagus reticulatus* (Coleoptera: Tenebrionidae): Effects of basidiocarp size, humidity

and competitors. *European Journal of Entomology* 95:559–570.

Monod M., Capoccia S., Léchenne B., Zaugg C., Holdom M., Jousson O. 2002. Secreted proteases from pathogenic fungi. *International Journal of Medical Microbiology* 292:405–419.

Neuwirth E. 2014. RColorBrewer: ColorBrewer Palettes. R package version 1.1-2. https://CRAN.R-project.org/package=RColorBrewer.

Nikitsky NB., Schigel DS. 2004. Beetles in polypores of the Moscow region: checklist and ecological notes. *Entomologica Fennica* 15:6–22.

Oksanen J., Blanchet, F. Guillaume Friendly M., Kindt R., Legendre P., McGlinn D., Minchin PR., O'Hara RB., Simpson GL., Solymos P., Henry M., Stevens H., Szoecs E., Wagner H. 2018. vegan: Community Ecology Package. R package version 2.5-3. https://CRAN.R-project.org/package=vegan.

Park D-S., Oh H-W., Heo S-Y., Jeong W-J., Shin DH., Bae KS., Park H-Y. 2007. Characterization of an extracellular lipase in *Burkholderia* sp. HY-10 isolated from a longicorn beetle. *Journal of Microbiology* 45:409–417.

Ploner A. 2015. Heatplus: Heatmaps with row and/or column covariates and colored clusters. R package version 2.24.0. https://github.com/alexploner/Heatplus.

Podgórski A., Podgórska B. 2009. *Drzewa w pomniki zaklęte. Drzewa pomnikowe w Rudzie Śląskiej*. Katowice: KOS.

Quast C., Pruesse E., Yilmaz P., Gerken J., Schweer T., Yarza P., Peplies J., Glöckner FO. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Accids Research* 41:D590–D596. DOI: 10.1093/nar/gks1219.

Rayner ADM., Boddy L. 1988. *Fungal decomposition of wood. Its biology and ecology.* John

624 Wiley & Sons Ltd.
625 Reid NM., Addison SL., Macdonald LJ., Lloyd-Jones G. 2011. Biodiversity of active and
626 inactive bacteria in the gut flora of wood-feeding huhu beetle larvae (*Prionoplus*
627 *reticularis*). *Applied and Environmental Microbiology* 77:7000–6. DOI:
628 10.1128/AEM.05609-11.
629 Rognes T., Flouri T., Nichols B., Quince C., Mahé F. 2016. VSEARCH: a versatile open source
630 tool for metagenomics. *PeerJ* 4:e2584. DOI: 10.7717/peerj.2584.
631 Ryan PD., Hammer Ø., Harper DA., Paul Ryan DD. 2001. PAST: Paleontological statistics
632 software package for education and data analysis. *Palaeontologia Electronica 178kb. T.*
633 *Harper. Geological Museum* 4:5–7.
634 Sabotič J., Ohm RA., Künzler M. 2016. Entomotoxic and nematotoxic lectins and protease
635 inhibitors from fungal fruiting bodies. *Applied Microbiology and Biotechnology* 100:91–
636 111.
637 Santos A V., Dillon RJ., Dillon VM., Reynolds SE., Samuels RI. 2004. Ocurrence of the
638 antibiotic producing bacterium *Burkholderia* sp. in colonies of the leaf-cutting ant *Atta*
639 *sexdens rubropilosa*. *FEMS Microbiology Letters* 239:319–323. DOI:
640 10.1016/j.femsle.2004.09.005.
641 Schigel DS. 2009. Polypore assemblages in boreal old-growth forests, and associated Coleoptera.
642 University of Helsinki.
643 Schigel DS. 2011. Polypore – beetle associations in Finland. *Annales Zoologici Fennici* 48:319–
644 348. DOI: 10.5735/086.048.0601.
645 Schigel DS. 2012. Fungivory and host associations of Coleoptera: a bibliography and review of
646 research approaches. *Mycology* 3:258–272.

647 Schigel DS., Niemelä T., Kinnunen J. 2006. Polypores of western Finnish Lapland and seasonal
648 dynamics of polypore beetles. *Karstenia* 46:37–64.
649 Schwarze FWMR., Engels J., Mattheck C. 2000. *Fungal Strategies of Wood Decay in Trees*.
650 Springer.
651 Scully ED., Geib SM., Hoover K., Tien M., Tringe SG., Barry KW., Glavina del Rio T.,
652 Chovatia M., Herr JR., Carlson JE. 2013. Metagenomic Profiling Reveals Lignocellulose
653 Degrading System in a Microbial Community Associated with a Wood-Feeding Beetle.
654 *PLoS One* 8:e73827. DOI: 10.1371/journal.pone.0073827.
655 Seigle-Murandi F., Guiraud P., Croizé J., Falsen E., Eriksson KL. 1996. Bacteria Are
656 Omnipresent on *Phanerochaete chrysosporium* Burdsall. *Applied and Environmental*
657 *Microbiology* 62:2477–2481.
658 Seipke RF., Org Barke J., Heavens D., Yu DW., Hutchings MI. 2013. Analysis of the bacterial
659 communities associated with two ant–plant symbioses. *Microbiology Open* 2:276–283.
660 DOI: 10.1002/mbo3.73.
661 Seniuk OF., Gorovoj LF., Beketova G V., Savichuk HO., Rytik PG., Kucherov II., Prilutskay
662 AB., Prilutsky AI. 2011. Anti-infective properties of the melanin-glucan complex obtained
663 from medicinal tinder bracket mushroom, *Fomes fomentarius* (L.: Fr.) Fr.
664 (Aphyllophoromycetideae). *International Journal of Medicinal Mushrooms* 13:7–18.
665 Shen W., Mao H., Huang Q., Dong J. 2015. Benzenediol lactones: a class of fungal metabolites
666 with diverse structural features and biological activities. *European Journal of Medicinal*
667 *Chemistry* 97:747–777. DOI: 10.1016/j.ejmech.2014.11.067.
668 Simmonds MSJ. 2003. Flavonoid–insect interactions: recent advances in our knowledge.
669 *Phytochemistry* 64:21–30. DOI: 10.1016/S0031-9422(03)00293-0.

670 Smith DJ., Park J., Tiedje JM., Mohn WW. 2007. A Large Gene Cluster in *Burkholderia*
671 *xenovorans* Encoding Abietane Diterpenoid Catabolism. *Journal of Bacteriology* 189:6195–
672 6204. DOI: 10.1128/JB.00179-07.
673 Sobczyk K. 2010. Naturalne inhibitory proteaz asparaginianowych z grzybów rozkładających
674 drewno. D. Phil. Thesis. Maria Curie-Skłodowska University.
675 Speight MCD. 1989. *Saproxylic invertebrates and their conservation*. Strasbourg: Council of
676 Europe.
677 Terra WR., Ferreira C. 1994. Insect digestive enzymes: properties, compartmentalization and
678 function. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*
679 109:1–62.
680 Treutter D. 2005. Significance of Flavonoids in Plant Resistance and Enhancement of Their
681 Biosynthesis. *Plant Biology* 7:581–591. DOI: 10.1055/s-2005-873009.
682 Vázquez-Baeza Y., Pirrung M., Gonzalez A., Knight R. 2013. EMPeror: a tool for visualizing
683 high-throughput microbial community data. *Gigascience* 26:2–16.
684 Wagner GK. 2018. Chrząszcze mycetofilne zasiedlające wybrane gatunki hub na terenie
685 południowo-wschodniej Polski. D. Phil. Thesis. Maria Curie Sklodowska University.
686 Warnes GR., Bolker B., Bonebakker L., Gentleman R., Huber W., Liaw A., Lumley T.,
687 Maechler M., Magnusson A., Moeller S., Schwartz M., Venables B. 2019. gplots: Various
688 R Programming Tools for Plotting Data. R package version 3.0.1.1. https://CRAN.R-
689 project.org/package=gplots.
690 Wiater A., Pleszczyńska M., Pietrykowska-Tudruj E., Janczarek M., Staniec B., Szczodrak J.
691 2018. Isolation and characterization of α-(1→3)-glucan-degrading bacteria from the gut of
692 *Diaperis boleti* feeding on *Laetiporus sulphureus*. *Entomological Science*. DOI:

10.1111/ens.12332.

Yang S., Wen X., Shi Y., Liebner S., Jin H., Perfumo A. 2016. Hydrocarbon degraders establish at the costs of microbial richness, abundance and keystone taxa after crude oil contamination in permafrost environments. *Scientific Reports* 6:37473. DOI: 10.1038/srep37473.

Yun J-H., Roh SW., Whon TW., Jung M-J., Kim M-S., Park D-S., Yoon C., Nam Y-D., Kim Y-J., Choi J-H., Kim J-Y., Shin N-R., Kim S-H., Lee W-J., Bae J-W. 2014. Insect gut bacterial diversity determined by environmental habitat, diet, developmental stage, and phylogeny of host. *Applied and Environmental Microbiology* 80:5254–64. DOI: 10.1128/AEM.01226-14.

Zhao J-Y., Ding J-H., Li Z-H., Dong Z-J., Feng T., Zhang H-B., Liu J-K. 2013. Three new phenyl-ethanediols from the fruiting bodies of the mushroom *Fomes fomentarius*. *Journal of Asian Natural Products Research* 15:310–314. DOI: 10.1080/10286020.2013.764519.

Zhou ZY., Liu JK. 2010. Pigments of Fungi (Macromycetes). *Natural Product Reports* 27:1531–1570. DOI: 10.1039/c004593d.

Zhu F., Du B., Bian Z., Xu B. 2015. Beta-glucans from edible and medicinal mushrooms: Characteristics, physicochemical and biological activities. *Journal of Food Composition and Analysis* 41:165–173. DOI: 10.1016/J.JFCA.2015.01.019.

Table and Figure legends

Table 1. Summary of the sequencing data and statistical analysis of microbial communities.

Table 2. The level of inhibition, expressed in % inhibition of the marker enzyme, by protease inhibitors contained in *Fomes fometarius* fruiting bodies preparations.

Table 3. Rf of secondary metabolites spots contained in methanolic and aqueous preparations of fruiting bodies of *Fomes fometarius*. TLC made in the ethanol-water system. Visualization under a UV lamp at wave lengths of 254 and 365 nm.

Figure 1. *Bolitophagus reticulatus* individuals and *Fomes fomentarius* fruiting body with pictures of sampling sites where they were collected. **A** – adult and larva of *Bolitophagus reticulatus* L.; **B** – fruiting body of *Fomes fomentarius* (L.) Fr.; **C** – swampy birch forest, Poleski National Park; **D** – Carpathian beech forest, Roztocze National Park (phot. G. K. Wagner).

Figure 2. Rarefaction analysis and Principal Coordinate Analysis (PCoA) plot of the tested samples. **A** – Rarefaction curves with Chao1 diversity indices, indicating insect microbiome sampling depth and saturation. **B** – PCoA of bacterial communities associated in tested specimens based on weighted UniFrac distances. The dotted line indicates the sample clustering according to the developmental stage.

Figure 3. The heatmap showing bacterial families distributed across *B. reticulatus* samples. Only those families which relative abundance was not less than 3% of at least one sample were considered. Cell values were calculated proportionately across rows and dendrograms were estimated with Bray-Curtis dissimilarity index.

Supplemental Table S1. Number of *F. fomentarius* fruiting bodies growing on birch and beech trunks and being healthy or inhabited by mycetophagous beetles.

735 **Supplemental Table S2.** Detailed taxonomic analyses at different ranks with the data obtained
736 for the developmental stages tested. The table shows the relative abundance of microbial 16S
737 rDNA sequences for each stage, at different taxonomic levels.

738 **Supplemental Data S1.** Detailed taxonomic analyses at different ranks for DNA. Sunburst
739 charts show the relative abundance of microbial 16S rDNA sequences at different taxonomic
740 levels. The first level represents the kingdom, the second level represents all phyla present in a
741 particular sample; subsequent next levels represent the class, order, family and genus.

742 **Supplemental Figure S1.** Predicted functions of bacterial communities associated with tested
743 developmental stages of *B. reticulatus*. All of the predicted KEGG metabolic pathways are
744 shown at the second hierarchical level and grouped by major functional categories.

Figure 1

Bolitophagus reticulatus individuals and *Fomes fomentarius* fruiting body with pictures of sampling sites where they were collected.

A – adult and larva of *Bolitophagus reticulatus* L.; **B** – fruiting body of *Fomes fomentarius* (L.) Fr.; **C** – swampy birch forest, Poleski National Park; **D** – Carpathian beech forest, Roztocze National Park (phot. G. K. Wagner).

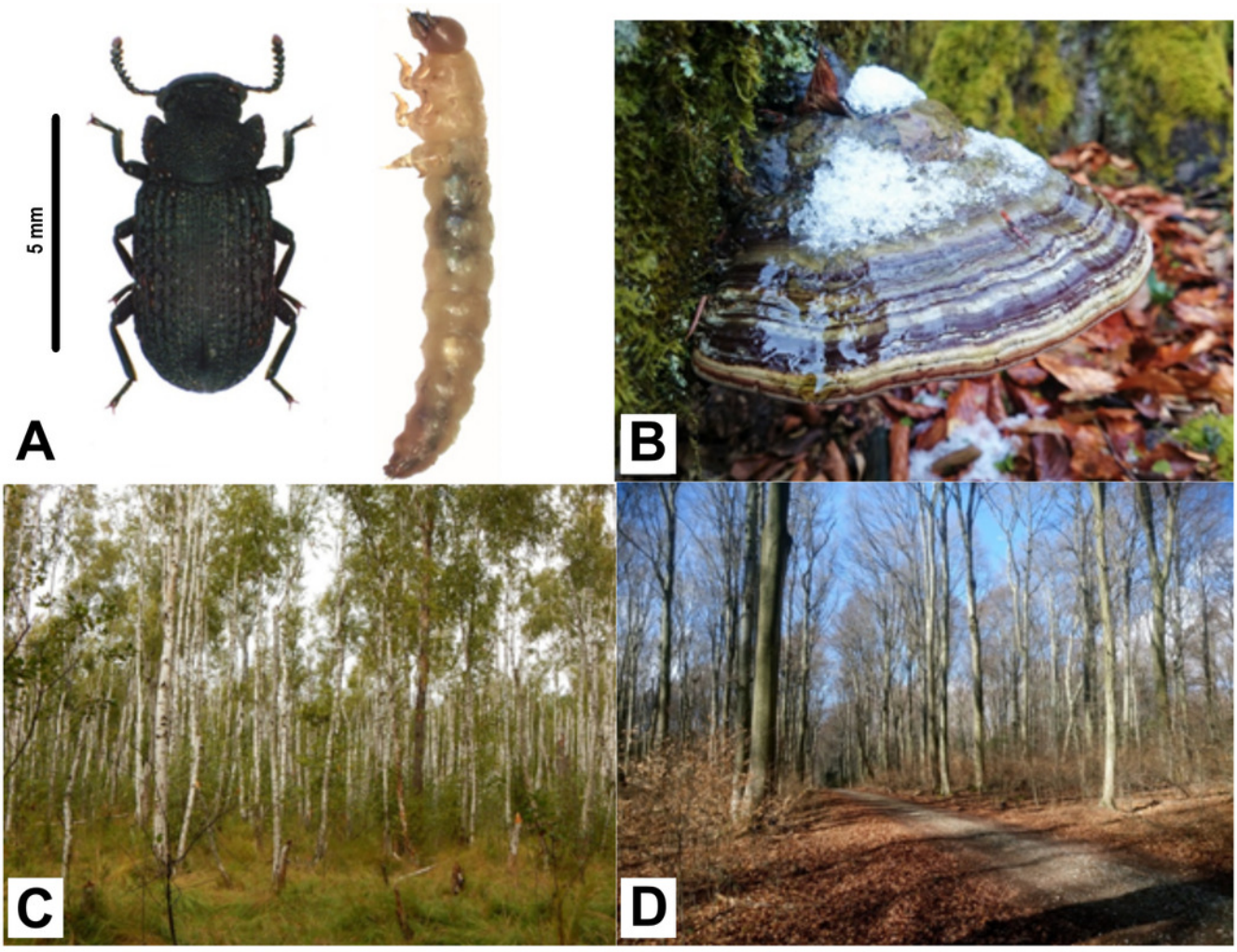

Figure 2

Rarefaction analysis and Principal Coordinate Analysis (PCoA) plot of the tested samples.

A – Rarefaction curves with Chao1 diversity indices, indicating insect microbiome sampling depth and saturation. **B** – PCoA of bacterial communities associated in tested specimens based on weighted UniFrac distances. The dotted line indicates the sample clustering according to the developmental stage.

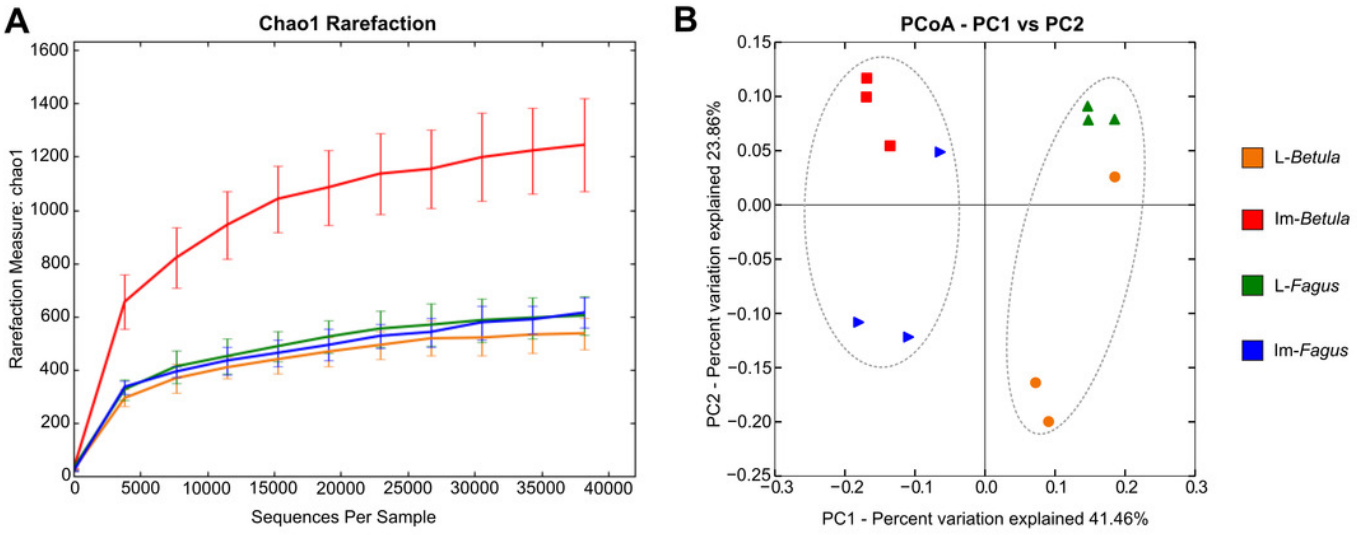

Figure 3

The heatmap showing bacterial families distributed across *B. reticulatus* samples.

Only those families which relative abundance was not less than 3% of at least one sample were considered. Cell values were calculated proportionately across rows and dendrograms were estimated with Bray-Curtis dissimilarity index.

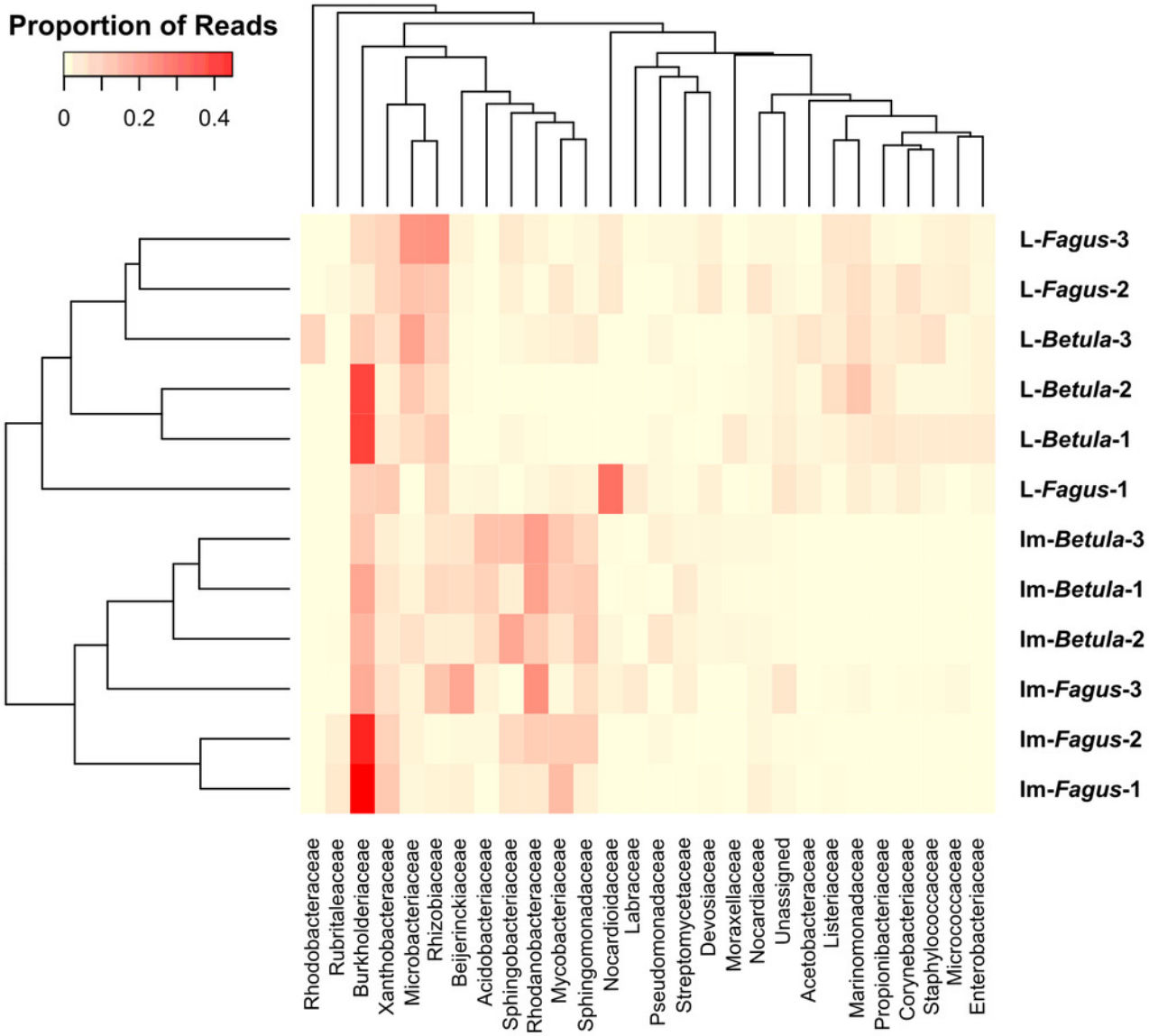

Table 1(on next page)

Summary of the sequencing data and statistical analysis of microbial communities.

Table 1. Summary of the sequencing data and statistical analysis of bacterial communities

ID	No. of bacterial reads	Average length (bp)	No. of observed OTU's	Chao1 index	Shannon index	Simpson index
L-*Betula*-1	53,423	454	463	552	5.86	0.95
L-*Betula*-2	38,188	451	319	453	5.30	0.94
L-*Betula*-3	71,060	450	527	594	6.04	0.96
Im-*Betula*-1	71,537	452	918	1017	5.96	0.95
Im-*Betula*-2	59,894	452	1195	1395	6.96	0.98
Im-*Betula*-3	73,856	452	1223	1236	6.72	0.97
L-*Fagus*-1	70,693	450	486	641	6.01	0.94
L-*Fagus*-2	47,193	449	433	573	6.39	0.98
L-*Fagus*-3	48,758	450	576	725	6.16	0.96
Im-*Fagus*-1	49,161	454	432	524	4.90	0.90
Im-*Fagus*-2	51,603	454	485	554	5.20	0.92
Im-*Fagus*-3	62,315	450	571	624	5.59	0.94
Total	697,681	451	2527	741	5.92	0.95

The ID abbreviations are defined in text. The number of OTUs (operational taxonomic units) was generated at the 97% sequence similarity cut-off. Diversity indices represent the randomly selected subsets for each sample normalized to 38,188 sequences.

Table 2(on next page)

The level of inhibition, expressed in % inhibition of the marker enzyme, by protease inhibitors contained in *Fomes fometarius* fruiting bodies preparations.

Table 2. The level of inhibition, expressed in % inhibition of the marker enzyme, by protease inhibitors contained in *Fomes fometarius* fruiting bodies preparations.

pH	Marker enzyme	*Fagus* sp.		*Betula* sp.	
		Healthy fungus	**Inhabited fungus**	**Healthy fungus**	**Inhabited fungus**
5.0	pepsin	6.20 ± 0.03	0.3	3.40 ± 0.02	0.10 ± 0.01
7.0	papain	0.20 ± 0.01	0	9.80 ± 0.03	5.70 ± 0.03
	trypsin	0	0	2.50 ± 0.02	0.20 ± 0.01
9.0	trypsin	0.10 ± 0.01	0	0.10 ± 0.01	0

Table 3(on next page)

Rf of secondary metabolites spots contained in methanolic and aqueous preparations of fruiting bodies of *Fomes fometarius*.

TLC made in the ethanol-water system. Visualization under a UV lamp at wave lengths of 254 and 365 nm.

Table 3. Retardation factor (Rf) of secondary metabolites spots contained in methanolic and aqueous preparations of fruiting bodies of *Fomes fometarius*. TLC plates were developed using the ethanol-water system and were visualized under a UV lamp at wavelengths of 254 and 365 nm.

Extract	UV wavelength (nm)	*Fagus* sp.		*Betula* sp.		
		Healthy fungus	**Inhabited fungus**	**Healthy fungus**	**Inhabited fungus**	
Methanol	365	0.8235 +	0.8235 +	0.8235 +	0.8235 +	**
	254	0.7058 ++	0.7058 +	0.7058 +	0.7058 +	
	254	0.1176 +++	-	-	-	
Water	365	0.9092 +	-	-	-	*
	254	0.7882 ++	-	-	-	
	254	0.7058 +	0.7058 +	0.7058 +	0.7058 +	**

+, ++, +++ - intensity of UV spots,

* - catechins and their derivatives,

** - sesquiterpenes lactones.