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

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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. Among this group 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, detailed studies focusing on the 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. Biochemical 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.

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23 Abstract

Saproxylic beetles play a crucial role in key processes occurring in forest ecosystems and 24 together with fungi, contribute to the decomposition and mineralization of wood. Among this 25 group are mycetophilic beetles which associate with wood-decaying fungi and use the fruiting 26 body for nourishment and development. Therefore, their feeding strategy (especially in the case 27 28 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 29 numerous studies, detailed studies focusing on the microbiome associated with species feeding 30 31 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 32 growing on two different host tree – beech (Fagus sp.) and birch (Betula sp.), respectively. 33 Among 24 identified bacterial phyla, three were the most relatively abundant (Proteobacteria, 34 Actinobacteria and Bacteroidetes). Moreover, we tried to find unique patterns of bacteria 35 abundances which could be correlated with the long-term field observation showing that the 36 fruiting bodies of F. fomentarius, growing on birch are more inhabited by beetles than fruiting 37 bodies of the same fungus species growing on beech. Biochemical analyses showed that the level 38 of protease inhibitors and secondary metabolites in F. fomentarius is higher in healthy fruiting 39 bodies than in the inhabited ones. However, tested microbiome samples primarily clustered by 40 developmental stage of *B. reticulatus* and host tree did not appear to impact the taxonomic 41 42 distribution of the communities. This observation was supported by statistical analyses.

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43 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).

51 Mycetophilic beetles associate with wood-decaying fungi and use the fruiting body for nourishment and development (Gutowski, 2006). Their feeding strategy requires special 52 digestive capabilities to take advantage of the nutritional value of fungal tissue. Associated 53 microorganisms play a crucial role in those processes. Although polypore-beetle associations 54 have been investigated in numerous studies (Nikitsky & Schigel, 2004; Schigel, Niemelä & 55 Kinnunen, 2006; Schigel, 2009, 2011, 2012), the detailed projects focused on their microbiome 56 remain limited. Previous study was focused rather on microbiota associated with fungivorous 57 insects (not only beetles, but also ants and termites) which cultivate fungi for food to take 58 59 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 60 61 initiated. Recently, Wiater et al. (2018) identified bacteria *Paenibacillus* sp. in the gut of 62 fungivorous darkling beetle Diaperis boleti (Tenebrionidae) feeding on polypore fungus *Laetiporus sulphureus*. These bacteria effectively degrade fungal α -(1 \rightarrow 3)-glucan present in cell 63 64 wall of fungi. More complex studies focused on profiling the microbiome of fungivorous beetles 65 have not been performed yet.

66 The black tinder fungus beetle *Bolitophagus reticulatus* (Tenebrionidae) is a fungivorous species occurring widely throughout European forests (Fig. 1A). This beetle belongs to tribe 67 Bolitophagini which represent feeding strategy of dwellers. Larvae of beetles described as 68 dwellers are fungivorous. In turn, their adults spend most of their life cycle inside the fruiting 69 body and leave the fungus usually for mating and dispersal only (Schigel, Niemelä & Kinnunen, 70 71 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 72 and seems to be monophagous on this fungus species (Midtgaard, Rukke & Sverdrup-Thygeson, 73 74 1998 and references therein).

The long-term field observations have shown that *B. reticulatus* is more often found 75 inside F. fomentarius fruiting bodies growing on birch (Betula sp.) compared with those growing 76 on beech (Fagus sp.). Moreover, polypores growing on beech trees are much larger and less 77 inhabited by insects than fruiting bodies growing on birch (Wagner, 2018). The growth of fungi 78 79 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). 80 Derivatives of catechins are also present in fungi themselves (Zhou & Liu, 2010). Schwarze, 81 82 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 83 84 process may be correlated with observed differences in colonization degrees. Fungal metabolites 85 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 86 87 structurally diverse classes of naturally occurring compounds, ranging from simple aromatic 88 metabolites to complex macrocyclic lactones. They are inhibitors of enzymes, including

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proteases (Shen et al., 2015). However, the state of knowledge about biological activity of 89 substances derived from F. fomentarius remain limited. Antimicrobial activity of extracts 90 derived from the tinder fungus has been described by Dresch et al. (2015), while Chen et al. 91 (2008) found that its exopolysaccharide (EPS) has direct antiproliferative effect in vivo. In turn, 92 some active compounds (e.g. catalase, extracellular laccase, manganese-dependent peroxidase, 93 94 carboxymethyl cellulose or xylanase) have been identified by Jaszek et al. (2006) and Elisashvili et al. (2009). Nevertheless, there is still little known about the secondary metabolites and 95 inhibitors of proteases, especially with regard to fruiting bodies from the natural environment, 96 not from in vitro cultures. Therefore, more detailed and complex studies are needed to test the 97 hypothesis of the correlation between profiles of the secondary metabolites and inhibitors of 98 proteases, and the degree of polypores colonization. 99

In the present study, we investigated the bacterial communities associated with larvae and 100 adults of *B. reticulatus* collected from *F. fomentarius* growing on two different host tree – beech 101 and birch, respectively. We used the Next Generation Sequencing (NGS) of the 16S rRNA gene 102 to define whether the bacterial communities vary among the two tested developmental stages of 103 B. reticulatus. Lastly, we investigated the potential differences between microbiome profiles of 104 individuals collected from the tinder fungus growing on birch and beech and combined the 105 results with those obtained from studies on the biochemical composition of the F. fomentarius 106 107 fruiting bodies growing on wood of two different tree species.

108

109 Materials & Methods

110 Study area and sample collection

Materials for the study were collected in two National Parks: Poleski NP and Roztocze 111 NP in south-eastern Poland. Field studies were approved by the Ministry of the Environment in 112 Poland (field study approval numbers: DLP-III-4102-21/1728/15/MD for the field study in 113 Poleski National Park and DPL-LLL-4102-609/1699/14/MD for the field study in Roztocze 114 National Park). Adults and larvae of black tinder fungus beetle *B. reticulatus* were caught in 115 116 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: 117 51°26'57.762" N, 23°14'29.839" E), 10. November 2016, Poleski NP (Fig. 1C) and the same 118 number of individuals were collected from one fungus growing on fallen beech stump in Obrocz 119 (DMS: 50°34'32.403" N, 23°'24.388" E), 10. December 2016, Roztocze NP (Fig. 1D). Specimens 120 were separately placed into tubes and stored at -30° C. Afterwards the tubes with insects were 121 sent for further analyses to the Department of Genetics and Biosystematics, University of 122 Gdansk, Poland. 123

124 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 125 tree species were selected for further analyses. Chosen trunks were plentifully covered with 126 127 sporocarps. Beech trunks were located in Roztocze NP (localization – Obrocz, DMS: 50°34'32.403" N, 23°0'24.388" E), while birch trunks were located in Poleski NP (localization: 128 129 Lipniak DMS: 51°27'51.363" N, 23°6'28.062" E). Healthy and settled fruiting bodies were 130 counted in the field and results are presented in Supplemental Table S1. For biochemical analyses, fruiting bodies of the tinder fungus (Fig. 1B.) were collected in July 2016 from fallen 131 132 trunks of each tree species growing in two mentioned localities. In both sampling sites, 5 133 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.

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138 DNA extraction

139 DNA was extracted from the whole bodies of *B. reticulatus* at two developmental stages (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 149 150 adults (one individual per DNA isolate and three isolates per developmental stage collected from fungi growing on different hosts) were used for microbiome analyses. 151

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153 16S rRNA gene sequencing and bacterial community analyses

The V3-V4 hypervariable regions of bacterial 16S rRNA gene region were amplified using the following primer set: 341F - 5'-CCTACGGGNGGCWGCAG-3' and 785R - 5'-

156 GACTACHVGGGTATCTAATCC-3'. The targeted gene region has been shown to be suitable

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® O5 Hotstart High-Fidelity DNA polymerase (New England BioLabs Inc.) 159 according to the manufacturer's protocol, using O5® Hot Start High-Fidelity 2X Master Mix 160 (NEBNext - New England BioLabs) and the Nextera Index kit (2x250bp). PCR was carried out 161 162 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 163 for final extension. Paired-end (PE, 2x250nt) sequencing with a 5% PhiX spike-in was 164 performed with an Illumina MiSeq (MiSeq Reagent kit v2) at Genomed, Warsaw, Poland; 165 following the manufacturer's run protocols (Illumina, Inc., San Diego, CA, USA). Automatic 166 primary analysis and de-multiplexing of the raw reads were performed with MiSeq, with the use 167 of MiSeq Reporter (MSR) v2.6 (16S Metagenomics Protocol). 168 The genetic material isolated from 12 individuals was sequenced separately. Samples 169 were then marked as follows: L-Fagus-X and Im-Fagus-X for larva and adult collected from F. 170 fomentarius fruiting body growing on beech stump; L-Betula-X and Im-Betula-X for larva and 171 adult collected from F. fomentarius fruiting body growing on birch stump (X indicates the 172 number of individual). 173

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, 180 version 1.2.22q (Edgar, 2010) and taxonomic assignment performed at 97% against the SILVA 181 v.132 database (Quast et al., 2013). The Biological Observation Matrix (BIOM) table was used 182 as the core data for downstream analyses (McDonald et al., 2012). Any sequences that were 183 classified as Mitochondria or Chloroplast, as well as singletons were filtered out of the dataset. 184 185 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 186 the Chao1, PD (a quantitative measure of phylogenetic diversity), Shannon, and Simpson indices 187 and also the number of observed OTUs. Comparison of the microbial community structures was 188 performed with the use of UniFrac (Lozupone & Knight, 2005) and Emperor (Vázquez-Baeza et 189 al., 2013). A two-sample *t*-test with a non-parametric Monte Carlo permutations (n = 999) and 190 Bonferroni correction was used to test for statistically significant difference in alpha diversity 191 between predefined groups (according to (1) developmental stage, (2) host tree species, (3) both 192 mentioned factors). A two-dimensional Principal Coordinate Analysis (PCoA) was conducted 193 from weighted UniFrac distances obtained in previous steps. In order to determine if observed 194 clusters of samples were significantly dissimilar, an analysis of similarity (ANOSIM; Clarke, 195 196 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

203 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

206 *Data availability*

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

209

210 Biochemical analyses

With the use of thin layer chromatography (TLC), comparison of entomotoxic and
insecticidal features of fruiting bodies of *F. fomentarius*, enzyme analyses of the level of
protease inhibitors (Sabotič, Ohm & Künzler, 2016) and analyses of secondary metabolite
profiles was performed (Anke & Sterner, 2002).
Samples from 20 sporocarps of a known type (from beech and birch, healthy and

inhabited by beetles – separately) were mechanically ground and then homogenized in distilled
water (for inhibitor determinations and TLC analyses of secondary metabolites) or in methanol
(to TLC of secondary metabolites) in a Potter homogenizer; 100 mg of shredded the sporocarp in
5 ml of water or methanol. The homogenates were then centrifuged to give supernatants as assay
preparations (Sobczyk, 2010; Jaruga, 2013). Protein in water extracts was determined by
standard Bradford method (Bradford, 1976).

The level of protease inhibitors was determined according to Sobczyk (2010) and with marker proteases (used to determine type of inhibitor and specific pH) according to Anson (1938). 0.1 ml of the preparation was incubated with 0.1 ml marker enzyme solution (pepsin at pH 5.0 for aspartate acid protease inhibitors, trypsin and papain at pH 7.0 for neutral serine and

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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 228 5% TCA (trichloroacetic acid). Samples were centrifuged and their absorbance measured at 280 229 nm. As controls, water instead of preparation (sample for % inhibition calculation) and marker 230 231 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, 232 2010). 233

During qualitative analysis of secondary metabolites by TLC, two types of extracts from 234 the fruiting bodies were analyzed: aqueous and methanol. TLC chromatography was developed 235 in two systems – ethanol : water (7:3) and ethyl acetate : acetic acid : water (2:1:1). Merck ready 236 TLC plates (type: TLC Silica gel 60 F254) were used. Visualization of plates was done by UV 237 light observation (254 nm and 365 nm), showing visible spots of secondary metabolites. 238 239 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 240 of the distance traveled by the center of a spot to the distance traveled by the solvent front) and 241 242 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 243 244 (Clevenger et al., 2017).

245

246 Results

247 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.

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Rarefaction curves with Chao1 diversity indices, indicating insect microbiome sampling depth 250 and saturation is shown in Fig. 2A. Observed curves almost reached a plateau at this sequencing 251 depth, suggesting that the sequencing was sufficient for microbiome characterization. More 252 details for sequence data for each sample, as well as the number of the observed OTUs and the 253 diversity indices are shown in Table 1. At least 319 OTUs, ranging from 319–1223, were 254 255 observed in different samples of *B. reticulatus*, which indicates that the microbial population is complex. 256 In all samples, at least 98,94% of the reads could be classified to phylum level. Detailed 257 taxonomic analyses on different ranks are available in supplementary data as sunburst charts 258

259 (Supplemental Data S1) and also in a table (Supplemental Table S2).

260

261 Bacterial community composition

The analysis of bacterial community showed that for both larvae and adults of B. 262 reticulatus >99.94% of total reads were represented by Bacteria (Supplemental Data S1 and 263 Supplemental Table S2). The remaining percentage comprised Archaea. The microbiomes tested 264 in this study contained 24 phyla. The most abundant phyla across all tested stages were 265 266 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 267 jointly accounted for more than 81.45% of the total microbial sequences obtained. Other 268 269 bacterial and archaeal phyla were present in tested microbial communities with different relative abundances given in Supplemental Table S2. 270 The most abundant classes among bacterial communities of all samples were 271

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

Similarities among the bacterial community structures associated with tested samples are 279 illustrated with a heatmap (Fig. 3). We identified 27 families, which relative abundance was not 280 less than 3% of at least one sample. Tested samples primarily clustered by developmental stage 281 of *B. reticulatus* and host tree did not appear to impact the taxonomic distribution of the 282 communities (Fig. 2B). This observation was supported by statistical analyses (ANOSIM: R =283 0.88, p = 0.002, and the alpha diversity indices were only significantly different (p = 0.004) 284 between samples grouped according to the developmental stage). In bacterial communities of 285 larvae Microbacteriaceae and Rhiziobiaceae were slightly more abundant, whereas 286 Acidobacteriaceae, Sphingomonadaceae, Rhodanobacteraceae, Mycobacteraceae and 287 Sphingomonadaceae were more abundant in microbiome of B. reticulatus adults. SIMPER 288 289 analysis showed that the last four families were primarily responsible for the differences between microbial communities of larvae and adults. 290

Analyses of bacterial communities associated with *B. reticulatus* revealed the presence of known endosymbionts. *Wolbachia* has been detected in 4 samples: all larvae collected from *F. fomentarius* growing on beech (0.08% for both L-*Fagus*-1 and L-*Fagus*-2, and 0.14% for L-

294 Fagus-3) and in one adult related to birch (<0.01% for Im-Betula-2). Moreover, two other known

- endosymbiotic bacteria have been detected *Arsenophonus* (0.06% for Im-*Fagus*-3 and L-*Betula*-2) and *Candidatus Cardinium* (0.01% for Im-*Betula*-1).
- 297

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

The results of biochemical analyses of the collected fruiting bodies showed that the level of inhibition was higher for aspartic acid protease inhibitors in fungus from beech, and for cysteine inhibitors and serine neutral proteases in fungus from birch (Table 2). In the case of inhibitors of basic serine proteases, the levels from both trees were similar (Table 2). The level of inhibitors is higher for healthy fruiting bodies than for the inhabited ones.

Positive TLC results were obtained for both types of extraction (water and methanol) 304 (Table 3), but only in the ethanol-water system. In the ethyl acetate-acetic acid-water system, no 305 separation was obtained and only a spot corresponding to the initial application on the TLC plate 306 was apparent. The strongest spot of secondary metabolites, 0.11176, from a healthy beech tree, 307 has not been identified. There were no differences in the intensities of the weaker 0.9092 spot 308 from the healthy beech tree across samples and the 0.8235 spot, which was present in all fruiting 309 bodies. There is a visible decrease in the intensity of spots 0.7058, 0.1176 and 0.7882 on beech 310 311 between healthy and colonized fruit bodies. Spots 0.11176 (methanol), 0.9092 (water) and 0.7882 (water) are absent in the fruiting bodies growing on the birch, both healthy and inhabited. 312 313 The 0.7058 spot in all samples from the aqueous extract did not change its intensity, in 314 comparison to spots with the same Rf from the methanol extract.

315

316 **Discussion**

In the present study, we investigated the microbiome profiles of larvae and adults of 317 fungivorous beetle B. reticulatus. Obtained patterns of the abundance at both phylum and class 318 level remained in congruence with previous studies. Proteobacteria, Actinobacteria and 319 Bacteroidetes phyla, as well as Alpha- and Gammaproteobacteria classes have been listed as the 320 most abundant bacterial groups in various insect species (e.g. Colman, Toolson & Takacs-321 Vesbach, 2012; Jones, Sanchez & Fierer, 2013; Yun et al., 2014; Kim et al., 2017), and also in 322 those cultivating fungi (Aylward et al., 2014). Moreover, identified patterns of the abundance 323 remained in congruence with the results of our previous study focused on microbial communities 324 associated with Hoplothrips carpathicus (Thysanoptera), which also inhabits fruiting bodies of 325 F. fomentarius (Kaczmarczyk et al., 2018). 326

Interestingly, at the genus level Burkholderia-Cabalerrinia-Paraburkholderia 327 (Burkholderiaceae) was one of the most dominant genera in all tested samples. In the study of 328 bacterial communities associated with fungivorous H. carpathicus this genus was also noted 329 (Kaczmarczyk et al., 2018), but it was not as abundant as in *B. reticulatus*. Burkholderia was 330 also identified in bacterial communities associated with other insects e.g. in the larvae of the 331 wood-feeding beetle Prionoplus reticularis (Reid et al., 2011), in longicorn beetle Prionus 332 333 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), 334 defence mechanisms (Santos et al., 2004), aromatic compound degradation (Laurie & Lloyd-335 336 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 337 white rot fungus *Phanerochaete chrysosporium* was described by Seigle-Murandi et al. (1996). 338 339 This fungus species, similar to F. fomentarius, degrades lignocellulosic materials. Nevertheless,

symbiotic relationships between F. fomentarius and microorganisms has not been investigated 340 yet. Therefore, one may not exclude that Burkholderia identified in bacterial communities of B. 341 reticulatus is connected also with the tinder fungus via a symbiotic relationship. However, its 342 presence in microbiome profiles of different developmental stages of black tinder fungus beetle 343 is thought to be related to the potential of *Burkholderia* representatives to degradation of 344 345 aromatic compounds (e.g. lignin) present in *B. reticulatus* food source. Besides Burkholderia, we found in tested bacterial communities also other genera, which 346 have been considered as degraders of aromatic compounds. Pseudomonas (Pseudomonadaceae) 347 and Serratia (Enterobacteriaceae) identified previously in microbiome of the mountain pine 348 beetle *Dendroctonus ponderosae*, have been described as terpene degraders (Adams et al., 2013). 349 In turn, Sphingomonas, Sphingobium, Novosphingobium and Sphingopyxis 350 (Sphingomonadaceae) have been recognised being involved in degradation of various 351 recalcitrant aromatic compounds and polysaccharides (Aylward et al., 2013). Moreover, 352 353 Sphingomonas has been identified in microbiome of wood-boring beetle, Anoplophora 354 glabripennis as genus involved in the degradation of lignocellulose, hemicellulose, and other aromatic hydrocarbons (Geib et al., 2009a; Geib et al., 2009b; Scully et al., 2013). Bosea 355 356 (Beijerinckiaceae; also found in present study) was in turn identified being associated with cuticles of plant-ant genera Allomerus and Tetraponera (Seipke et al., 2013) and described as 357 hydrocarbon degrader (Yang et al., 2016). Comprehensive analyses of the functional microbiome 358 359 of arthropods (e.g. honeybees, fruit flies, cockroaches, termites, ants and beetles) show that Burkholderia, Sphingomonas and Bosea are together involved in the same processes e.g. aerobic 360 361 metabolism, reacting with cytochrome c or bypassing cytochrome c (Esposti & Romero, 2017).

The bacterial community associated with black tinder fungus beetle likely plays a role in 362 promotion of efficient digestion for extraction of maximum energy from ingested substrates. 363 Nevertheless, specific conditions in microenvironment of F. fomentarius fruiting body may 364 cause strong selective pressure against microorganisms that are not able to survive exposure to 365 defensive compounds produced by the tinder fungus. Recent study shed light on antimicrobial 366 367 activities of F. fomentarius. Kolundžíć et al. (2016) found that the tinder fungus extracts of different polarity exhibit significant antimicrobial activity against nine bacterial strains 368 (including Staphylococcus aureus, Staphylococcus epidemidis, Bacillus subtilis or Klebsiella 369 pneumonia). In fact, the relative abundance of those bacteria in microbiome of B. reticulatus 370 were low and in most samples tested did not exceed 1%. The observed antimicrobial activity of 371 F. fomentarius may be linked e.g. with polyphenols and β -glucans which abundances are 372 relatively high in its fruiting bodies (Seniuk et al., 2011; Zhao et al., 2013; Alves et al., 2013; 373 Zhu et al., 2015) or with sesquiterpens which have been described as active compounds 374 (identified enzyme inhibitors with antifungal, antibacterial and cytotoxic activities) (Abraham, 375 2001; Keller, 2018). These compounds may be considered as toxins for bacteria associated with 376 fungivorous species inhabiting F. fomentarius fruiting bodies. Thus, associated bacterial 377 378 communities need to overcome the presence of such substances through resistance or tolerance mechanisms. 379

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

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

The additional aim of the present study was connected with the long-term field 392 observations showing that there is a difference between the amount of healthy and colonized 393 sporocarps growing on the trunks of both beech and birch (Supplemental Table S1). In the case 394 of beech, the F. fomentarius fruiting bodies inhabited by insects were collected less frequently 395 $(\sim 27\%)$ on average, in the range from 19% to 33% of all collected fruiting bodies growing on 396 selected trunks). In turn, the number of colonized sporocarps growing on birch was higher 397 (~66% on average) and ranged from 46% to 85% of all collected fruiting bodies growing on 398 these trees. To identify the potential factors related to the observed differences in degree of 399 fruiting bodies colonization by insects, we identified trends in biochemical profiles of fruiting 400 401 bodies growing on two different tree hosts.

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

The biochemical analysis showed that a higher level of protease inhibitors was observed 407 in healthy fruiting bodies than in colonized ones. Unfortunately, no previous research has been 408 done to investigate this phenomenon, so it is unclear why inhibitor levels are lower in colonized 409 fruiting bodies. Changes in levels of acidic, neutral, and alkaline proteases in colonized 410 sporocarps may resulted from the inherent properties of insect proteases, which are dominated by 411 412 serine and cysteine proteases over aspartic proteases (Terra & Ferreira, 1994). In turn, a high level of acid inhibitors of aspartic proteases may be additionally associated with the protection of 413 fruiting bodies against pathogenic and saprophytic microscopic fungi (mainly molds) (Monod et 414 al., 2002), which have high levels of aspartic proteases in their proteolytic apparatus. 415 Moreover, performed analyses indicated the presence of unidentified secondary 416 metabolites in samples of non-colonized fruiting bodies collected from beech. Probably, these 417 substances are able to determine the susceptibility of fruiting bodies to be colonized by insects 418 and, generally, to be infected. It is worth noting that even dozen-year-old specimens of F. 419 fomentarius, growing on beech wood, are usually completely healthy, while fungi growing on 420 the birch, are colonized by insects and has signs of the presence of pathogenic fungi (e.g. mold). 421 Therefore, some obvious contributing factors to this phenomenon (e.g. size of fruiting body, sun 422 423 exposure or age) seem to be insignificant for determining the degree of fruiting bodies colonization by insects. Some authors showed that Fagus sylvatica produces flavonoids and 424 425 organic acids which can be classified as repellents against insects (Harborne, 1997; Simmonds, 426 2003; Treutter, 2005; Podgórski & Podgórska, 2009). The accumulation of some flavonoids in the fruiting bodies of F. fomentarius could be a barrier against the fungivorous beetles. At this 427 428 stage, it cannot be excluded that flavonoids are among those unidentified secondary metabolites

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detected in present study. However, the identification of the secondary metabolites and their 429 potential impact on the relationship among host tree, fungi and insects require in-depth studies. 430 The biochemical analyses presented here should be treated with caution since the fruiting 431 bodies sampled for these analyses were collected earlier than those from which *B. reticulatus* 432 specimens were collected. Both sets of samples were collected for different studies. Although the 433 434 obtained patterns needs to be tested in more complex studies where beetles would be collected from sporocarps, which would then be used for further biochemical analyses, the results 435 presented here could be considered as insight in potential trends. More advanced biochemical 436 analyses (e.g. using liquid chromatography or mass spectrometry) may provide a more complete 437 insight into the biochemical profile of fruiting bodies. However, despite the observed trends in 438 biochemical profiles, tested microbiome samples primarily clustered by developmental stage of 439 B. reticulatus and host tree did not appear to impact the taxonomic distribution of the 440 communities, what was supported by statistical analyses. 441 Additionally, known endosymbionts have been identified in microbiome profiles of B. 442 reticulatus. Among them Wolbachia should receive a special attention. It is a well-known 443 endosymbiont, which is estimated to be present in more than 65% of all insect species 444 445 (Hilgenboecker et al., 2008; Lewis & Lizé, 2015). Wolbachia is related to five commonly recognized manipulation schemes: feminization, parthenogenesis induction, early and late male 446 447 killing, and cytoplasmic incompatibility (Engelstädter & Hurst, 2009). It appears that these 448 phenomena do not occur in *B. reticulatus*, but more comprehensive studies should be performed to test this hypothesis. 449

450

451 Conclusions

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In conclusion, this paper presents the insight into bacterial communities associated with 452 two developmental stages of *B. reticulatus* beetle with the use of 16S rRNA sequence data. The 453 approach based on NGS technique allowed us to characterize of tested microbiome. Moreover, it 454 is the first approach to identification of factors which can be related to differences in degree of 455 fruiting bodies colonization by insects. Results of this study show biochemical differences in 456 fruiting bodies collected from birch and beech. We compared these results with those obtained 457 during analyses of bacterial communities associated with B. reticulatus. However, the host-tree 458 appears to have no effect on the bacterial communities associated with tested developmental 459 stages of *B. reticulatus*. Despite the observed trends in biochemical profiles of sporocarps 460 collected from both tree species, tested samples primarily clustered by developmental stage of B. 461 reticulatus. Moreover, endosymbiotic Alphaproteobacteria Wolbachia was identified for the first 462 time in *B. reticulatus*. 463

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713	Table and Figure legends						
714	Table 1. Summary of the sequencing data and statistical analysis of microbial communities.						
715	Table 2. The level of inhibition, expressed in % inhibition of the marker enzyme, by protease						
716	inhibitors contained in Fomes fometarius fruiting bodies preparations.						
717	Table 3. Rf of secondary metabolites spots contained in methanolic and aqueous preparations of						
718	fruiting bodies of Fomes fometarius. TLC made in the ethanol-water system. Visualization under						
719	a UV lamp at wave lengths of 254 and 365 nm.						
720							
721	Figure 1. Bolitophagus reticulatus individuals and Fomes fomentarius fruiting body with						
722	pictures of sampling sites where they were collected. A – adult and larva of <i>Bolitophagus</i>						
723	reticulatus L.; B – fruiting body of Fomes fomentarius (L.) Fr.; C – swampy birch forest, Poleski						
724	National Park; D – Carpathian beech forest, Roztocze National Park (phot. G. K. Wagner).						
725	Figure 2. Rarefaction analysis and Principal Coordinate Analysis (PCoA) plot of the tested						
726	samples. $A - Rarefaction$ curves with Chao1 diversity indices, indicating insect microbiome						
727	sampling depth and saturation. \mathbf{B} – PCoA of bacterial communities associated in tested						
728	specimens based on weighted UniFrac distances. The dotted line indicates the sample clustering						
729	according to the developmental stage.						
730	Figure 3. The heatmap showing bacterial families distributed across <i>B. reticulatus</i> samples. Only						
731	those families which relative abundance was not less than 3% of at least one sample were						
732	considered. Cell values were calculated proportionately across rows and dendrograms were						
733	estimated with Bray-Curtis dissimilarity index.						
734							
735	Supplemental Table S1. Number of <i>F. fomentarius</i> fruiting bodies growing on birch and beech						
736	trunks and being healthy or inhabited by mycetophagous beetles.						

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

- 740 Supplemental Data S1. Detailed taxonomic analyses at different ranks for DNA. Sunburst
- charts show the relative abundance of microbial 16S rDNA sequences at different taxonomic
- 742 levels. The first level represents the kingdom, the second level represents all phyla present in a
- 743 particular sample; subsequent next levels represent the class, order, family and genus.
- 744 Supplemental Figure S1. Predicted functions of bacterial communities associated with tested
- 745 developmental stages of *B. reticulatus*. All of the predicted KEGG metabolic pathways are
- 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.

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- <i>Betula</i> -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

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

2 The ID abbreviations are defined in text. The number of OTUs (operational taxonomic units) was

3 generated at the 97% sequence similarity cut-off. Diversity indices represent the randomly selected

4 subsets for each sample normalized to 38,188 sequences.

5



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.

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

3

11	Marker	Fag	<i>us</i> sp.	<i>Betula</i> sp.		
рн	enzyme	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	

4

5



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.

1 Table 3. Retardation factor (Rf) of secondary metabolites spots contained in methanolic and

2 aqueous preparations of fruiting bodies of *Fomes fometarius*. TLC plates were developed using

3 the ethanol-water system and were visualized under a UV lamp at wavelengths of 254 and 365

4 nm.

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

5

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

- 7 * catechins and their derivatives,
- 8 ** sesquiterpenes lactones.

9