Predictive functional profiling and surveying the microbiome of fungivorous black tinder beetle *Bolitophagus reticulatus*

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Saproxylic beetles play an important 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 for which the fruiting bodies of wood-decaying fungi are the food base and/or a place for 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 adaptations to 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 27 identified bacterial phyla, four were the most relatively abundant (*Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and TM7). PICRUSt analysis predicted that each bacterial community should be rich in genes involved in membrane transport, amino acids metabolism and carbohydrate metabolism. Moreover, we tried to propose the explanation for long-term field observation showing that the fruiting bodies of *F. fomentarius,* growing on birch are more infested 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 for healthy fruiting bodies than for the inhabited ones. Although tested microbiome profiles of *B. reticulatus* collected from polypores growing on both tree species did not differ significantly for pairwise comparisons, subtle differences connected with host tree species can be seen in distribution of identified OTUs. Among OTUs which were unique for sample sets associated to birch and beech trees, differences were seen primarily in proportion of

Actinobacteria and *Bacteroidetes*.

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Abstract

Saproxylic beetles play an important 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 for which the fruiting bodies of wood-decaying fungi are the food base and/or a place for 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 adaptations to 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 27 identified bacterial phyla, four were the most relatively abundant (*Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and TM7). PICRUSt analysis predicted that each bacterial community should be rich in genes involved in membrane transport, amino acids metabolism and carbohydrate metabolism. Moreover, we tried to propose the explanation for long-term field observation showing that the fruiting bodies of *F. fomentarius,* growing on birch are more infested 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 for healthy fruiting bodies than for the inhabited ones. Although tested microbiome profiles of *B. reticulatus* collected from polypores growing on both tree species did not differ significantly for pairwise comparisons, subtle differences connected with host tree species can be seen in distribution of identified OTUs. Among OTUs which were unique for

sample sets associated to birch and beech trees, differences were seen primarily in proportion of *Actinobacteria* and *Bacteroidetes*.

Introduction

Saproxylic beetles are an extremely important ecological group of forest entomofauna. They include species in which at least one developmental stage is directly or indirectly related to wood (Speight, 1989). Saproxylic beetles play an important 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 (Gutowski et al., 2004). Depending on the ecological niche occupied, a number of saproxylic groups of Coleoptera can be distinguished: xylophages, cambiophages, predators, necrophiles, and finally mycetophiles (Gutowski et al., 2004).

Mycetophilic beetles associated with decaying-wood fungi are presented by species for which the fruiting body is the food base and/or a place for development (Gutowski, 2006). They are able to use both persistent and ephemeral fruiting bodies of polypores as their microsettlements (e.g. (Matthewman & Pielou, 1971; Jonsell & Nordlander, 2002; Lik & Barczak, 2005; Schigel, Niemelä & Kinnunen, 2006; Łakomy & Kwaśna, 2008; Thakeow et al., 2008)). Mycetophiles can be placed in one of two major groups: either those with fungivorous larvae (i.e. *dwellers*), or those which larvae are not fungivorous (i.e. *visitors*) (Schigel, Niemelä & Kinnunen, 2006). Beetles described as dwellers spend most of their life cycle inside the fruiting body and leave the fungus usually for mating and dispersal only. Their feeding strategy requires special digestive capabilities to take advantage of the nutritional value of fungal tissue.

Although polypore-beetle associations have been investigated in numerous studies (e.g. (Nikitsky & Schigel, 2004; Schigel, Niemelä & Kinnunen, 2006; Schigel, 2009, 2011, 2012), the detailed studies focused on adaptations to feeding on fruiting bodies of polypores remaining limited. Previous studies were focused rather on adaptations of fungivorous insects (not only beetles, but also ants and termites) which cultivate fungi species for food to take advantage of the nutritional value of fungal tissue (e.g. (Aylward et al., 2014)). However, studies of feeding adaptations in beetles associated with wood-decaying fungi also have gained the raising interest. 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* (Wiater et al., 2018). 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.

One of the widely distributed fungivorous beetle is black tinder beetle *Bolitophagus reticulatus* (Tenebrionidae) (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. (Fig. 1B) at all developmental stages and seems to be monophagous on tinder fungus (Midtgaard et al., 1998 and references therein). Previous studies have suggested that *B. reticulatus* has limited dispersal abilities (Sverdrup-Thygeson & Midtgaard, 1998; Rukke & Midtgaard, 1998; Knutsen et al., 2000). However, Jonsell et al. (2003) found that the dispersal ability of black tinder beetle may have been underestimated previously (Jonsell, Schroeder & Larsson, 2003).

Long-term field observations have shown that *B. reticulatus* was more often found inside *F. fomentarius* fruiting bodies growing on birch (*Betula* sp.) compared with those growing on

beech (*Fagus* sp.). Moreover, it was noticed that polypores growing on beech trees were much larger and less inhabited by insects than fruiting bodies growing on birch (Wagner, 2018). However, the reasons of these observation remain unclear. Previous studies (e.g. (Schwarze, Engels & Mattheck, 2000)) have 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. However, the state of knowledge about biological activity of substances derived from *F. fomentarius* remain limited (e.g. (Jaszek et al., 2006; Chen et al., 2008; Elisashvili et al., 2009; Dresch et al., 2015) and 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 NGS of the 16S rRNA gene to define whether the bacterial communities varies among the two tested developmental stages of *B. reticulatus*. We predicted the metabolic activity of the microorganisms associated with tested developmental stages of black tinder beetle. Lastly, we investigated the differences between microbiome profiles of individuals collected from 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 research 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 (approval numbers: DLP-III-4102-21/1728/15/MD for field study in Poleski National Park and DPL-LLL-4102-609/1699/14/MD for 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 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 beech stump in Obrocz (DMS: 50º34'32.403" N, 23º0'24.388" 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.

Studies on occurrence of *F. fomentarius* fruiting bodies growing on birch and beech were performed during the years 2013-2016. In those studies four 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 Table S1. For preliminary biochemical analyses, fruiting bodies of tinder fungus *F. fomentarius* (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

136 fruiting bodies and the same number of the inhabiting fruiting bodies were taken. The fruiting
137 bodies were inhabited by few species of mycophagous beetles with dominance of the studied
138 species – *B. reticulatus*. Samples were cut out from the tissue above the hymenium.
139
140 *DNA extraction*
141 DNA was extracted from the whole bodies of *B. reticulatus* at two developmental stages
142 (larva and imago, respectively) by the Sherlock AX Purification Kit (A&A Biotechnology,
143 Poland). Insects were rinsed three times in sterile distilled water prior to DNA extraction without
144 soaking in ethanol. To avoid cross contamination of samples, the process was performed with
145 sterile equipment. The quantity and quality of the extracted DNA were evaluated by using a
146 Nano Drop ND-1000 spectrophotometer (Nano Drop Technologies). After extraction, the DNA
147 was stored at -20°C until further use.
148
149 *16S rRNA gene amplification and sequencing*
150 Twelve samples consisting of larval instars and adults were used for further microbiome
151 analyses (one DNA isolate per sample and three isolates per developmental stage). The V3-V4
152 hypervariable regions of bacterial 16S rRNA gene region were amplified using the following
153 primer set: 341F – 5'-CCTACGGGNGGCWGCAG-3' and 785R – 5'-
154 GACTACHVGGGTATCTAATCC-3'. The targeted gene region has been shown to be suitable
155 for the Illumina sequencing (Klindworth et al., 2013). Libraries were prepared with a two-step
156 PCR protocol based on Illumina's "16S metagenomic library prep guide" (15044223 Rev. B)
157 with NEBNext® Q5 Hotstart High-Fidelity DNA polymerase (New England BioLabs Inc.)
158 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 data obtained for the 3 independent DNA extractions for each developmental stage of *B. reticulatus* were merged and considered as one sample in further taxonomic analyses. The intention of this approach was to obtain a more reliable view into the "average" bacterial communities' structure (Kaczmarczyk et al., 2018). Samples were then marked as follows: L-*Fagus* and Im-*Fagus* for larva and adult collected from *F. fomentarius* fruiting body growing on beech stump; L-*Betula* and Im-*Betula* for larva and adult collected from *F. fomentarius* fruiting body growing on birch stump.

The samples were processed and analyzed using the Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.1) pipeline (Caporaso et al., 2010b). 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. Clustering of operational taxonomic units (OTUs) at 97% similarity was performed by using the uclust method, version 1.2.22q (Edgar, 2010). OTUs were assigned to taxa using the

GreenGenes, release 13.5, as the reference (DeSantis et al., 2006), with the taxonomy assignment tool PyNAST (Caporaso et al., 2010a). 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. The completeness of microbial communities sampling was estimated using trends in rarefaction analysis of the obtained data (plateau curves). Based on clusters, 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 membership Venn diagram was computed using the MetaCoMET (Wang et al., 2016) web platform to identify specific and shared OTUs across the developmental stages of *B. reticulatus* collected from *F. fomentarius* growing on two different hosts. Similarity percentage (SIMPER) analysis was performed to calculate the average dissimilarities in microbial community structures between particular samples and to access which phylum was responsible for the observed differences. The differences in microbial community structures were tested by the χ^2 test. Statistical analyses were performed using PAST 3.16 (Ryan et al., 2001) and PopTools 3.2 (Hood, 2010) software. The bipartite network was visualized using Cytoscape 2.8.0 software (Shannon et al., 2003). This approach provided an additional tool for exploring the interrelationship between host lineage, and shared and unique microbial taxa (Ley et al., 2008). Tested developmental stages of *B. reticulatus* and microbial OTUs were used as nodes in a bipartite graph, with edges connecting OTU nodes to the hosts in which they were found. NGS data are deposited and fully available under study accession number PRJEB23388 in ENA – the European Nucleotide Archive.

205 Metabolic capacity of the microbiome contained in 16S libraries was predicted using
206 PICRUSt software (Phylogenetic Investigation of Communities by Reconstruction of
207 Unobserved States; (Langille et al., 2013)). This approach exploits the relationship between
208 phylogeny and function by combining 16S data with a GreenGenes database of reference
209 genomes (DeSantis et al., 2006) to predict the presence of gene families. The inferences on
210 metabolic capacities of microorganisms associated with tested developmental stages of *B.*
211 *reticulatus* were used for further identification of similarities and dissimilarities in identified
212 metabolic capacities of associated microorganisms, which could be linked to the differences in
213 species of *F. fomentarius* host tree. The final result was an annotated table of gene counts per
214 sample that can be linked to the Kyoto encyclopedia of genes and genomes (KEGG) orthology
215 (KO) accession numbers (Kanehisa et al., 2014). The functions were categorized at levels 2 and
216 3.
217
218 *Preliminary biochemistry analyses*
219 With the use of thin layer chromatography (TLC), comparison of entomotoxic and
220 insecticidal features of fruiting bodies of *F. fomentarius*, enzyme analyses of the level of
221 protease inhibitors (Sabotič, Ohm & Künzler, 2016) and analyses of secondary metabolite
222 profiles was performed (Anke & Sterner, 2002).
223 Samples from 20 sporocarps of a known type (from beech and birch, healthy and
224 inhabited by beetles – separately) were mechanically ground and then homogenized in distilled
225 water (for inhibitor determinations and TLC analyses of secondary metabolites) or in methanol
226 (to TLC secondary metabolites) in a Potter homogenizer; 100 mg of shredded the sporocarp in 5
227 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 cysteine protease inhibitors and trypsin at pH 9.0 for alkaline serine protease inhibitors) for 30 minutes at 37° C. After this time, 0.5 ml of the hemoglobin solution was added in buffer of 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, more than 184 000 good quality 16S rRNA gene sequences (V3-V4 region) were obtained. These sequences ranged between 184 132 for L-*Betula* and 276 230 for Im-*Betula*. At least 1280 OTUs, ranking from 1280–3648, were observed in different developmental stages of *B. reticulatus*, what indicates that the microbial population is highly complex. Moreover, rarefaction analysis of the obtained data revealed trends indicating that sampling of microbial communities varied in their degree of completion by life stage (data not shown). More details for sequence data, as well as the number of the observed OTUs and the diversity indices, are shown in Table 1.

Venn diagram (Fig. 2) shows shared OTUs among tested developmental stages of *B. reticulatus* (at 97% similarity). This analysis revealed a total of 3360 OTUs that were unique to one sample and a microbial core comprised of 246 OTUs that were found in all samples.

In all samples, at least 99.69% of the reads could be classified to phylum level. Detailed taxonomic analyses on different ranks are available in supplementary data as sunburst charts (Data S1) and also in a table (Table S2).

Bacterial community composition

The analysis of bacterial community showed that for both larvae and adults of *B. reticulatus* more than 99.69% of total reads were represented by *Bacteria* (Data S1 and Table

S2). The remaining percentage comprised *Archaea* and unassigned records. The highest percentage of bacterial reads was observed in specimens (both larva and imago) collected from *F. fomentarius* fruiting body growing on beech stump. The microbiomes tested in this study contained 27 phyla (Fig. 3, Data S1, Table S2).

The most abundant phyla across all tested stages were *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and TM7. In each developmental stage of *B. reticulatus* collected from *F. fomentarius* fruiting bodies growing on both tree species, those phyla jointly accounted for more than 82.88% of the total microbial sequences obtained. Separately, 54.67% of the reads on average were derived from *Proteobacteria* (ranging from 40.98% in L-*Fagus* to 65.92% in Im-*Fagus*) and 23.82% from *Actinobacteria* (ranging from 14.53% in Im-*Betula* to 39.57% in L-*Fagus*). *Bacteroidetes* comprised on average 6.53% (ranging from 3.01% in L-*Betula* to 14.63% in Im-*Betula*) and TM7 on average 5.57% (ranging from 1.06% in L-*Betula* to 8.35% in Im-*Fagus*) of the total reads. The remaining reads were derived from phyla shown on Fig. 3, which were present in different abundances within the populations. SIMPER analysis showed that the differences in the relative abundances of *Proteobacteria*, *Actinobacteria* and *Cyanobacteria* were primarily responsible for the differences between samples (Table 2). Bacterial community structures of both larvae and adults did not differ significantly between each other (χ^2: 12.5–32.4, $df = 27$, $P > 0.05$).

The most dominant classes among bacterial communities of all samples were *Alphaproteobacteria*, *Actinobacteria*, *Betaproteobacteria* and *Gammaproteobacteria,* accounting for more than 60% of the total reads (Data S1, Table S2). The most dominant orders among analyzed microbiome profiles were *Actinomycetales*, *Burkholderiales* and *Rhizobiales,* accounting for more than 38% of the total reads. The most dominant family was

297 *Burkholderiaceae* and among that family the most dominant genus was *Burkholderia*, which
298 accounted for 14.08% of the reads on average (ranging from 3.49% in L-*Fagus* to 24.57% in
299 Im-*Fagus*). In the larval stage collected from fungus growing on beech stump, *Wolbachia* was
300 detected and accounted for 0.07% of the total reads obtained for this sample. *Wolbachia* was not
301 observed in the rest of the analyzed samples.

302 Similarities among the bacterial community structures associated with tested samples are
303 illustrated with a heatmap (at the phylum level) and as a bipartite network demonstrating the
304 abundance of microorganisms for each sample (Fig. 4, part A and B, respectively). This analysis
305 showed that the samples primarily clustered by developmental stage and that host tree did not
306 appear to impact the taxonomic distribution of the communities.

307 Detailed analysis of Venn diagram (Fig. 2) constructed on the basis of OTU table,
308 allowed to identify OTUs which were unique for bacterial communities associated with tested
309 developmental stages of black tinder fungus beetle collected from *F. fomentarius* growing on
310 birch and those collected from fruiting bodies growing on beech. Among core OTUs common to
311 all samples, the majority were categorized as *Proteobacteria* (60% of common OTUs) while
312 relatively abundant phyla included *Actinobacteria* (16% of common OTUs), *Bacteroidetes* (9%
313 of common OTUs), *Firmicutes* (4% of common OTUs), and TM7 and *Acidobacteria* (3% of
314 common OTUs for each). *Proteobacteria* were also abundant among OTUs uniquely associated
315 with birch and beech, which represented 43% and 53% of uniquely associated OTUs,
316 respectively. Differences were seen primarily in proportion of *Actinobacteria* (10% of uniquely
317 associated OTUs in birch vs. 17% in beech) and *Bacteroidetes* (24% of uniquely associated
318 OTUs in birch vs. 5% in beech).

PICRUSt analysis showed predictive functional potentials of the bacterial communities associated with larvae and adults of *B. reticulatus*. For all tested samples, there were no major differences in relative abundances of different functional categories (Fig. 5). At the second level of KEGG pathways organization, tested bacterial communities were thought to be rich in genes involved in membrane transport, amino acids metabolism and carbohydrate metabolism. Additionally, in all samples, high relative abundances of predicted genes linked to replication and repair, energy metabolism and xenobiotics biodegradation and metabolism were observed. Analyses at third level of KEGG pathways organization predicted that all tested bacterial communities were thought to be primarily rich in genes connected with transporters.

Statistical analysis of level 2 KEGG pathways showed no differences between developmental stages of *B. reticulatus* (χ^2 test: χ^2: 0.11–1.08, $df = 32$, $P > 0.05$).

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

As a result of preliminary biochemical analysis of the obtained fruiting bodies, it was found 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 3). In the case of inhibitors of basic serine proteases, the levels from both trees were similar (Table 3). The level of inhibitors is higher for healthy fruiting bodies than for the beetle inhabited ones.

Positive TLC results were obtained for both types of extraction (water and methanol) (Table 4), but only for the ethanol-water developing system. In the ethyl acetate-acetic acid-water system, no separation was obtained and only a spot corresponding to the initial application of the preparation to the TLC plate was apparent. The strongest spot of secondary metabolites, 0.11176, from a healthy beech tree, has not been identified. There were no differences in the

intensities of the weaker 0.9092 spot from the healthy beech tree across samples and or the 0.8235 spot, which was present in all fruiting bodies. There is a visible decrease in the intensity of spots 0.7058, 0.1176 and 0.7882 on beech 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. The 0.7058 spot in all samples from the aqueous extract did not change its intensity, in comparison to spots with the same Rf from the methanol extract.

Discussion

Saproxylic beetles are an important ecological group of forest entomofauna and they play essential roles in key processes occurring in forest ecosystems. Together with fungi, they have roles in the decomposition and mineralization of wood (Gutowski & Buchholz, 2000). Saproxylic beetles, especially those being fungivorous and inhabiting fruiting bodies of polypores, are exposed to many biologically active compounds which are present in their habitats. These compound affect not only direct insects, but primarily microorganisms associated with those invertebrates. In the present study, we investigated the microbiome associated with two developmental stages of *B. reticulatus* inhabiting fruiting bodies of *F. fomentarius*. Substances produced by tinder fungus have a variety of interesting properties and can function as antioxidants, anti-inflammatories, or inducers of apoptosis (Ito, Sugiura & Miyazaki, 1976; Park et al., 2004; Lee, 2005; Chen et al., 2008; Kim, Jakhar & Kang, 2015). Some of them (e.g. antioxidant activity) can be considered either 'pro-biotic' or 'anti-biotic' depending on dose and context. Recent studies shed light on antimicrobial activities of *F. fomentarius*. (Kolundžić et al., 2016) found that tinder fungus extracts of different polarity exhibit significant antimicrobial activity against nine bacterial strains (including *Staphylococcus aureus*, *Staphylococcus*

365 *epidemidis, Bacillus subtilis* or *Klebsiella pneumonia*). This activity may be linked e.g. with
366 polyphenols and β-glucans whose abundances are relatively high in *F. fomentarius* fruiting
367 bodies (Seniuk et al., 2011; Zhao et al., 2013; Alves et al., 2013; Zhu et al., 2015). In turn, these
368 compounds may be considered as toxins for bacteria associated with fungivorous species
369 inhabiting *F. fomentarius* fruiting bodies. Thus, associated bacterial communities need to
370 overcome the presence of such substances through resistance or tolerance mechanisms.
371 Nevertheless, without specifying the profile of the microbiome associated with fungivorous
372 species and its predictive functional profiling, it is difficult to comprehensively assess the
373 resistance or tolerance mechanisms.

374 Detailed analyses of microbial communities associated with tested developmental stages
375 of *B. reticulatus* showed that 4 phyla were the most abundant across samples – *Proteobacteria*,
376 *Actinobacteria*, *Bacteroidetes* and TM7. Previous studies have demonstrated that especially
377 *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* are among the most abundant phyla in tested
378 microbial communities associated with insect species (e.g. (Colman, Toolson & Takacs-
379 Vesbach, 2012; Jones, Sanchez & Fierer, 2013; Yun et al., 2014; Kim et al., 2017)). Recent
380 studies on bacterial communities associated with selected insect species which cultivate fungi
381 have shown that the most abundant phylum in these communities was *Proteobacteria* (>75% in
382 all tested samples) (Aylward et al., 2014). Although in *B. reticulatus Proteobacteria* were among
383 four most abundant bacterial phyla, their relative abundances in tested host's developmental
384 stages were lower (ranging from 40.98% in L-*Fagus* to 65.92% in Im-*Fagus*). Aylward et al.
385 (2014) found also that in microbiome of adult ambrosia beetles *Xyleborinus saxesenii*
386 *Proteobacteria* were not the most abundant phylum and comprised only ~25% of total bacterial
387 community, whereas *Bacteroidetes* comprised ~50% of it. This relative abundance is much

higher than those calculated in presented study. In *B. reticulatus* the highest relative abundance of *Bacteroidetes* was observed in adults collected from fruiting body growing on birch (14.63%).

At the class level, *Alpha*-, *Beta*- and *Gammaproteobacteria* are listed as the most abundant classes across various insect species (Yun et al., 2014). In insect species which cultivate fungi, *Gammaproteobacteria* were the most abundant class in associated bacterial communities (>75% in almost all tested microbiomes) (Aylward et al., 2014). However, in the case presented here, *Gammaproteobacteria* were less abundant (up to 16.21% in Im-*Betula*).

Although Aylward et al. (2014) analysed bacterial communities associated with fungivorous insects, observed results are different than those presented for *B. reticulatus*. However, identified microbiomes were associated with insects which do not inhabit fruiting bodies of wood-decaying fungi. Therefore, observed differences in relative abundances of bacterial groups may be related to specific conditions prevailing inside the polypores. Our previous study have been focused on microbiome associated with *Hoplothrips carpathicus* (Thysanoptera), which also inhabits fruiting bodies of *F. fomentarius*. Analysis of bacterial communities associated with four developmental stages of this thrips species revealed that the same bacterial phyla and classes were generally the most abundant, as in the case of *B. reticulatus* (Kaczmarczyk et al., 2018). Differences can be seen in proportions of classes classified in *Proteobacteria.* In the case of black tinder fungus beetle *Alphaproteobacteria* were less abundant (on average 23% vs. 38% of associated bacterial communities) and *Betaproteobacteria* were more abundant (on average 18% vs. 4% of associated bacterial communities) comparing with *H. carpathicus*.

At the genus level, *Burkholderia* (*Betaproteobacteria*) was the most dominant 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 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). 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 tinder fungus via a symbiotic relationship. Nevertheless, its presence in microbiome profiles of different developmental stages of black tinder fungus beetle is thought to be related to the potential of *Burkholderia* representatives to degradation of aromatic compounds (e.g. lignin) present in *B. reticulatus* food source.

Additionally, among most abundant genera identified in tested bacterial communities, *Sphingomonas* and *Bosea* belonging to *Alphaproteobacteria*, were also observed. These genera have been also detected in bacterial communities associated with other insect species (e.g. (Xiang et al., 2006; Alonso-Pernas et al., 2017)). Moreover, *Sphingomonas* has been previously implicated in the degradation of lignocellulose, hemicellulose, and other aromatic hydrocarbons in wood-boring beetle, *Anoplophora glabripennis* (Geib et al., 2009b,a; Scully et al., 2013). Comprehensive analyses of the functional microbiome of arthropods (e.g. honeybees, fruit flies,

cockroaches, termites, ants and beetles) show that these three genera (*Burkholderia*, *Sphingomonas* and *Bosea*) are involved in the same processes e.g. aerobic metabolism, reacting with cytochrome *c* or bypassing cytochrome *c* (Esposti & Romero, 2017). Similar to *Sphingomonas*, *Bosea* is also included into hydrocarbon degrader genera (Yang et al., 2016). This genus was identified as associated with cuticles of plant-ant genera *Allomerus* and *Tetraponera* (Seipke et al., 2013). In the study presented here, DNA was extracted from whole bodies of tested individuals (both larvae and adults) and therefore, it is impossible to clearly distinguish internal and external symbionts. Although taking into account results described by (Seipke et al., 2013), one may not unambiguously exclude the association of *Bosea* with the cuticle of *B. reticulatus*.

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

Properties of microorganisms associated with *B. reticulatus* which inhabit and feed on *F. fomentarius* fruiting bodies are related to the presence of its compounds which are biologically active. In the study presented here, the functional potentials of bacterial communities associated with tested developmental stages of black tinder fungus beetle were predicted. PICRUSt analysis showed, that in all tested bacterial communities, genes involved in membrane transport, and amino acids and carbohydrate metabolism should be elevated (Fig. 5). Relative abundance of predicted genes involved in membrane transport was slightly higher in the case of intensively feeding larvae collected from tinder fungus growing on both birch and beech. These predicted

457 genes were connected with multiple types of transporters, especially ABC-transporters.
458 Transporters of this type can be related to antibiotic resistance, because ATP-binding cassette
459 (ABC)-type multidrug transporters use a free energy of ATP hydrolysis to pump drug molecules
460 out of cells (Putman, van Veen & Konings, 2000). In the case of genes thought to be involved in
461 amino acids and carbohydrate metabolism, the slightly higher relative abundance was observed
462 in bacterial communities associated with adults. Similarly, the higher level of predicted genes
463 involved in metabolism of terpenoids and polyketides, as well as in xenobiotic biodegradation
464 and metabolism, was observed in the case of adult *B. reticulatus*. The latest group of genes are
465 predicted to be related to metabolism of terpenoids – main secondary metabolites of *F.*
466 *fomentarius* (around 75%) (Grienke et al., 2014). Similar pattern of relative abundances were
467 identified for predicted genes in the case of bacterial communities associated with thrips *H.*
468 *carpathicus* (Kaczmarczyk et al., 2018). This might mean that similarities in predicted patterns
469 of relative abundances of genes are characteristic for bacterial communities associated with
470 fungivorous species inhabiting fruiting bodies of wood-decaying fungi.
471 In the future studies some of predicted functions might be confirmed under laboratory
472 conditions. Such studies have been performed for beetles and flies associated with fruiting bodies
473 of *L. sulphureus* (Wiater et al., 2018). The cell wall of its mature fruiting bodies is a rich source
474 of α-(1→3)-glucan (Grün, 2003). Therefore, it can be assumed that the insects may contain an
475 associated bacterial communities producing enzymes capable of decomposing the main
476 components of the *L. sulphureus* mycelium. Detailed analyses under laboratory conditions
477 confirmed α-(1→3)-glucanase activity in homogenates prepared for beetle *D. boleti*. Similar
478 studies, focused not only on this activity, but also on other predicted functions are planned for *B.*
479 *reticulatus*.

The additional aim of presented study was investigation of the differences between microbiome profiles of individuals collected from 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. 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).

Fruiting bodies for biochemical analyses were collected earlier than those from which *B. reticulatus* specimens were obtained. The samples were collected for different projects, hence the difference in the time of their collection. Although the correlation among the structures of *B. reticulatus* bacterial communities and the levels of protease inhibitors and secondary metabolites derived from *F. fomentarius* fruiting bodies needs further detailed analyses, the presented analyses could be the basis for further research. More complex biochemical analyses (e.g. using liquid chromatography or mass spectrometry) may provide a more complete insight into the biochemical profile of fruiting bodies.

The biochemical analysis showed that a higher level of inhibitors was observed for healthy fruiting bodies than for inhabited ones. This may be a direct result of feeding by insects or from enzyme inhibitors binding to beetle digestive enzymes. Unfortunately, no previous research has been done to investigate this phenomenon, so it is unclear why inhibitor levels are lower in infested fruiting bodies. Changes in levels of acidic, neutral, and alkaline proteases in infested fruiting bodies may result from the inherent properties of insect proteases, which are dominated by 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

503 protection of fruiting bodies against pathogenic and saprophytic microscopic fungi (mainly
504 molds) (Monod et al., 2002), which have high levels of aspartic proteases in their proteolytic
505 apparatus.

506 Moreover, performed analyses indicated the presence of unidentified secondary
507 metabolites in samples of non-inhabited fruiting bodies collected from beech. It is probable that
508 these may also be the substances that determine the susceptibility of fruiting bodies to
509 colonization by insects, and generally to infection. It is worth noting that even dozen-year-old
510 specimens of *F. fomentarius*, growing on beech wood, were usually completely healthy, while
511 fungi growing on the birch, were colonized by insects and had signs of the presence of
512 pathogenic fungi also (mold). Therefore, some obvious contributing factors to this phenomenon
513 (e.g. size of fruiting body, sun exposure or age) seem to be insignificant for determining the
514 degree of fruiting bodies colonization by insects. Some authors showed that *Fagus sylvatica*
515 produces flavonoids and organic acids, several of which can act as repellents against insects in
516 angiosperm plants (Harborne, 1997; Simmonds, 2003; Treutter, 2005; Podgórski & Podgórska,
517 2009). It is possible that accumulation of some flavonoids in the fruiting bodies of *F.*
518 *fomentarius* could be a barrier against the fungivorous beetles. Substances of this group could be
519 the unidentified secondary metabolites detected in this research. However, further attempts to
520 identify secondary metabolites and their effect on the relationship of tree-mushroom-insects
521 require in-depth research.

522 Long-term field observations showed that there is a difference between the share of
523 healthy and inhabited sporocarps in the trunks of both beech and birch (Table S1). On beech, the
524 share of settled fungi was in the range from 19% to 33% of all collected fruiting bodies growing
525 on selected trunks. In turn, on the birch the share of settled sporocarps was higher and amounted

526 the range from 46% to 85% of all collected fruiting bodies growing on selected trunks. After
527 total summation of collected and analyzed fruiting bodies, the share of those which were
528 colonized on beech was ~27%, while on the birch ~66%. To identify the potential factors related
529 to the differences in degree of fruiting bodies colonization by insects, the identified trends in
530 biochemical profiles of fruiting bodies growing on two different tree hosts were combined with
531 the potential differences in bacterial communities associated with two developmental stages of *B.*
532 *reticulatus* inhabiting *F. fomentarius* fruiting bodies. However, differences among microbiome
533 profiles were not significant, except one comparison – L-*Betula* and Im-*Betula*. Functional
534 profiles between other comparisons did not differ significantly. Clustering of tested samples
535 according to similarity of associated bacterial communities showed that samples are grouped
536 according to developmental stages rather than tree species (Fig. 4A). Relationships of bacterial
537 communities resolved as a bipartite network were congruent with those described above, but the
538 complexity of these associations was more visible (Fig. 4B).
539 Although tested microbiome profiles did not differ significantly for most pairwise
540 comparisons, subtle differences can be seen in distribution of identified OTUs. Among unique
541 OTUs, which were characteristic for bacterial communities associated with samples connected
542 with birch (L-*Betula* and Im-*Betula*, respectively), a lower relative abundance of *Actinobacteria*
543 and a higher relative abundance of *Bacteroidetes* were observed, compared to samples connected
544 with beech (L-*Fagus* and Im-*Fagus*, respectively). The difference in relative abundances of
545 *Bacteroidetes* was surprisingly high, as in the case of *Betula*-associated samples this phylum was
546 almost five times more abundant than in *Fagus*-associated samples. The increase of this phylum
547 is thought to be related with higher ability to *F. fomentarius* colonization by *B. reticulatus*.
548 *Bacteroidetes* are regarded as specialists for the degradation of high molecular weight organic

549 matter, i.e. proteins and carbohydrates and recent studies based on their genomes sequencing
550 confirm the presence of numerous carbohydrate-active enzymes covering a large spectrum of
551 substrates (Thomas et al., 2011). Members of this phylum are well known degraders of
552 polymeric organic matter, in particular polysaccharides (e.g. cellulose, pectin or xylan).
553 Therefore, our prediction seems to be likely. Nevertheless, the wide scale studies should be
554 performed to verify the correctness of this assumption.

555 Additionally, the presence of an OTU derived from *Wolbachia* was observed in the larval
556 developmental stage of *B. reticulatus* collected from fruiting bodies growing on beech. This is
557 the first report on *Wolbachia* endosymbiosis in black tinder beetle. *Wolbachia* belongs to
558 *Alphaproteobacteria*. It is a well-known endosymbiont, which is estimated to be present in more
559 than 65% of all insect species (Hilgenboecker et al., 2008; Lewis & Lizé, 2015). *Wolbachia* is
560 related to five commonly recognized manipulation schemes: feminization, parthenogenesis
561 induction, early and late male killing, and cytoplasmic incompatibility (Engelstädter & Hurst,
562 2009). Recent studies have showed that conventional PCR can fail to detect low-level *Wolbachia*
563 infections (Mee et al., 2015) and therefore, more sensitive sequencing techniques, such as next
564 generation sequencing are helpful for endosymbiont detection. Here, we identified low level of
565 endosymbiosis (<1% of *Wolbachia* relative abundance). However, the degree of *Wolbachia*
566 endosymbiosis in fungivorous insects may vary. In our previous study *Wolbachia* have been also
567 identified in *H. carpathicus* inhabiting *F. fomentarius* fruiting bodies, but the relative
568 abundances of this endosymbiont in analysed samples were higher (1.25-69.95%) (Kaczmarczyk
569 et al., 2018). More complex studies are needed to estimate the degree of *Wolbachia*
570 endosymbiosis in other fungivorous insects associated with wood-decaying fungi. Although
571 *Wolbachia* was identified in microbiome profiles of *Fagus*-associated larvae, its presence was

572 not observed in tested adults of *B. reticulatus* connected with beech. Therefore, it cannot be
573 excluded that observed pattern of *Wolbachia* presence in tested developmental stages of *B.*
574 *reticulatus* is distorted by the sample size and further analyses focused on endosymbiont
575 detection should be performed in wider scale.
576
577 **Conclusions**
578 In conclusion, this paper presents data of bacterial community analysis of two
579 developmental stages of *B. reticulatus* beetle with the use of NGS 16S rRNA sequence data. This
580 approach allowed us to nearly fully characterize its microbiome. Moreover, it is the first
581 approach to identification of factors which can be related to differences in degree of fruiting
582 bodies colonization by insects. Results in this study show biochemical differences in fruiting
583 bodies collected from birch and beech. We compared these preliminary results with those
584 obtained during analyses of bacterial communities associated with *B. reticulatus*. However, the
585 predicted impact of tree host on bacterial communities associated with tested developmental
586 stages of *B. reticulatus* seems to be more subtle. The exceed of unique OTUs identified as
587 *Bacteroidetes* in *Betula*-associated samples may be related to higher degree of infestation of
588 *Betula*-growing fruiting bodies of *F. fomentarius* by *B. reticulatus* beetles. Moreover,
589 endosymbiotic *Alphaproteobacteria Wolbachia*, identified here for the first time in *B.*
590 *reticulatus*, was observed only in instars collected from *F. fomentarius* growing on beech.
591 Nevertheless, the direct impacts of levels of defensive compounds on bacterial community
592 composition and structure need further analyses in wider scale.
593
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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).

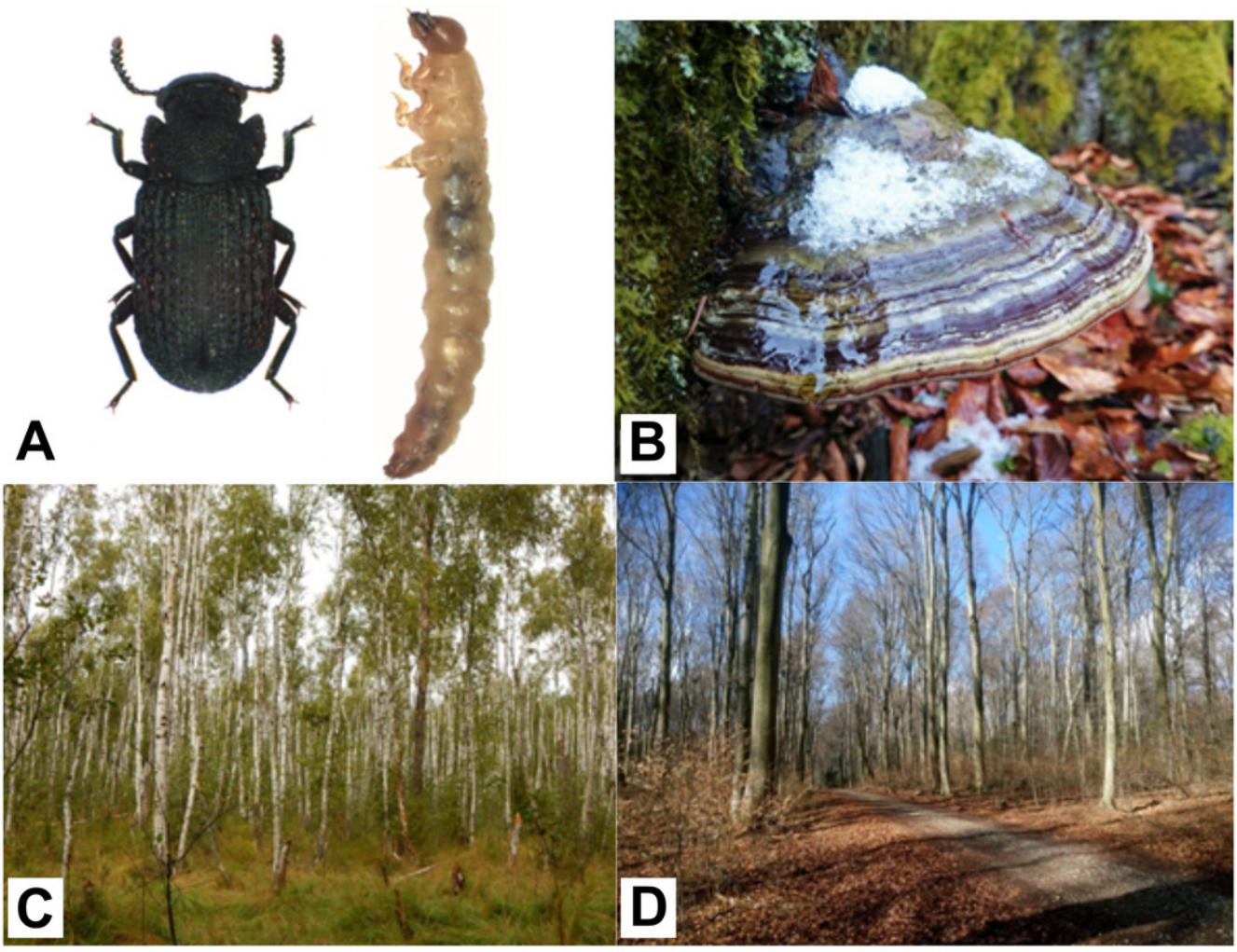


Figure 2

Analysis of OTUs at 97% similarity among tested developmental stages of *B. reticulatus.*

Venn diagram shows distribution of OTUs among samples and indicates 254 OTUs shared among all samples.

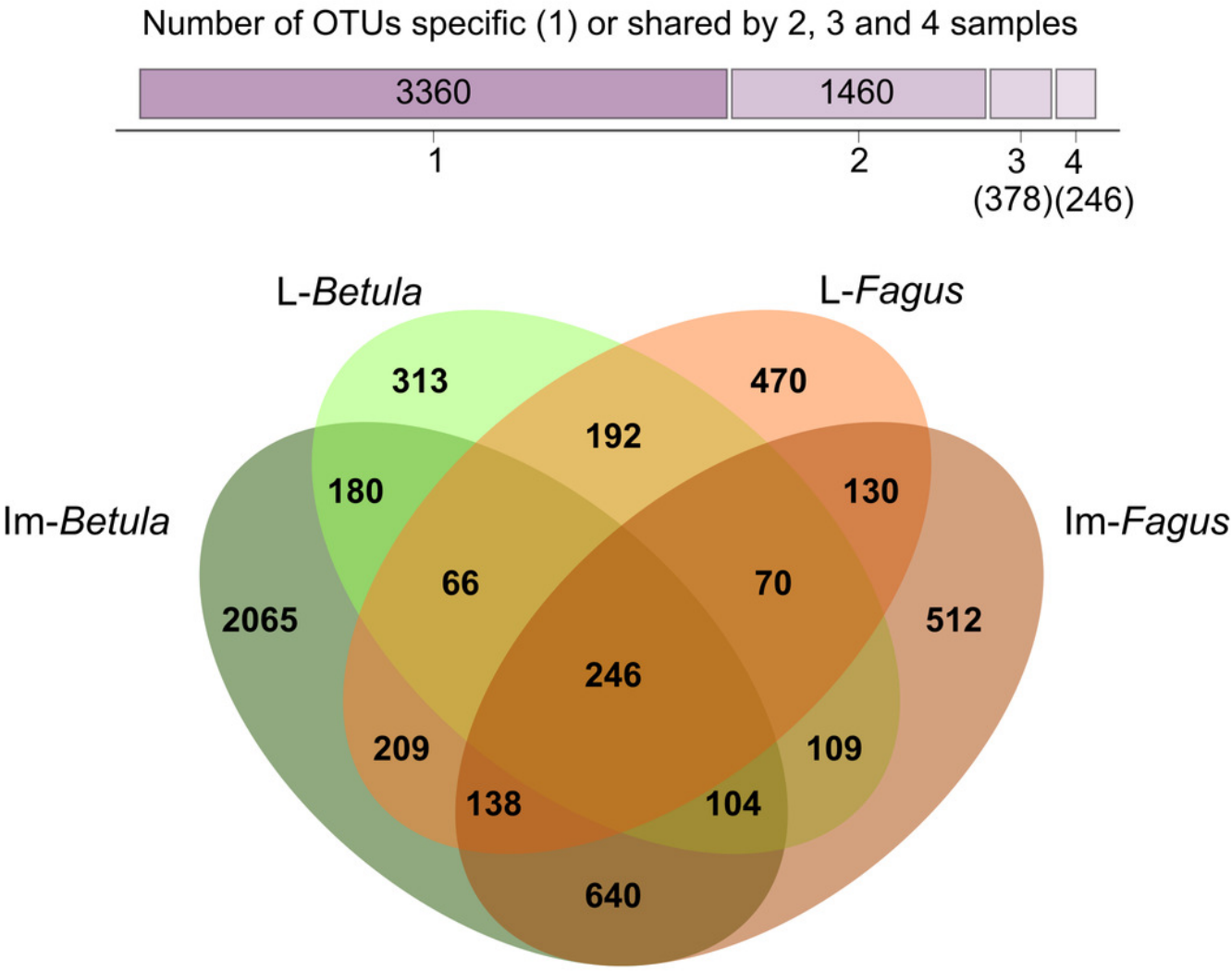


Figure 3

Abundance of bacterial 16S rRNA gene sequences at the phylum level.

Analyses of the microbial community structures at two developmental stages of *B. reticulatus* collected from *F. fomentarius* fruiting bodies growing on birch and beech stumps.
Abbreviations: L-*Betula* and Im-*Betula* – larva and imago collected from fruiting body growing on birch stump; L-*Fagus* and Im-*Fagus* – larva and imago collected from fruiting body growing on beech stump.

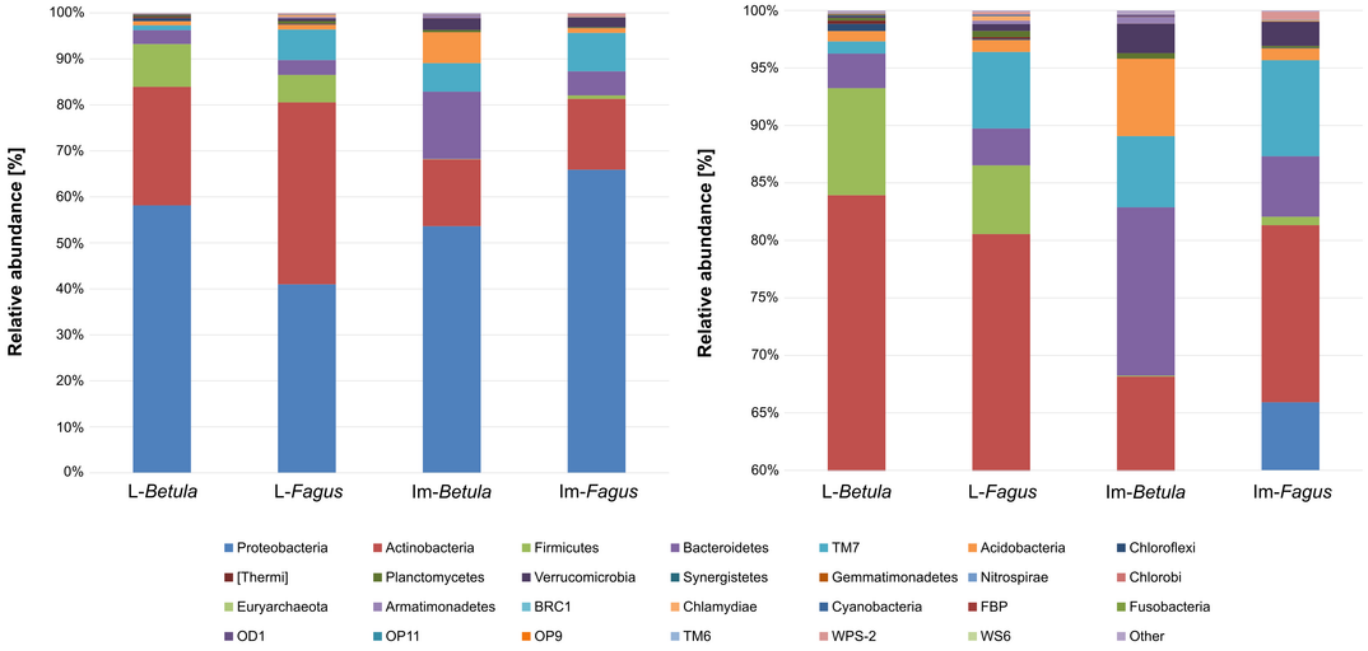
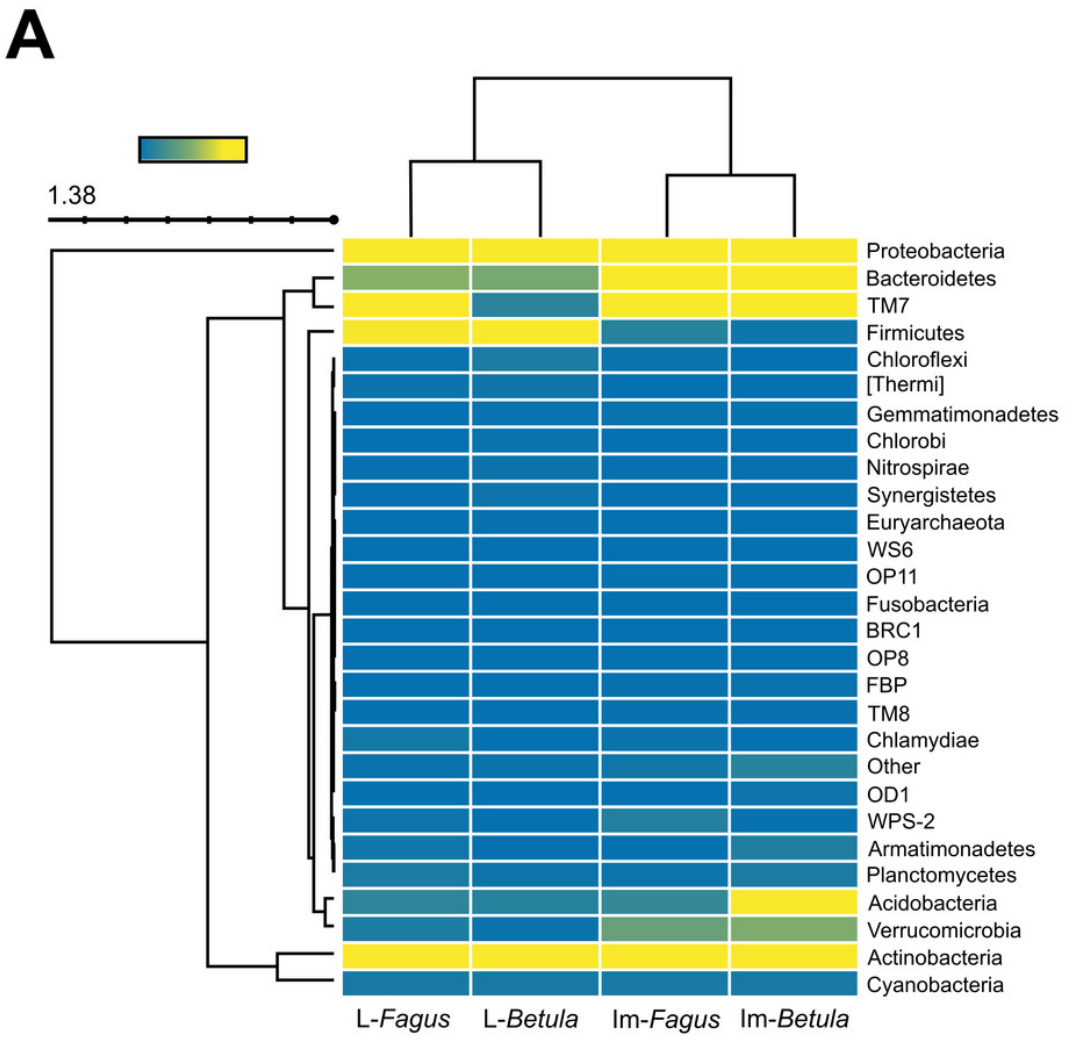


Figure 4

Similarities in microbial population structure associated with developmental stages of *B. reticulatus* collected from two tree species.

A – The cluster heatmap shows relative abundances of each phylum. Lighter colors indicate higher relative abundance; **B** – The bipartite network of microbial 16S rRNA derived OTUs showing relationships of microbial communities associated with tested developmental stages of *B. reticulatus*.


A
1.38
Proteobacteria
Bacteroidetes
TM7
Firmicutes
Chloroflexi
[Thermi]
Gemmatimonadetes
Chlorobi
Nitrospirae
Synergistetes
Euryarchaeota
WS6
OP11
Fusobacteria
BRC1
OP8
FBP
TM8
Chlamydiae
Other
OD1
WPS-2
Armatimonadetes
Planctomycetes
Acidobacteria
Verrucomicrobia
Actinobacteria
Cyanobacteria
L-Fagus
L-Betula
lm-Fagus
lm-Betula

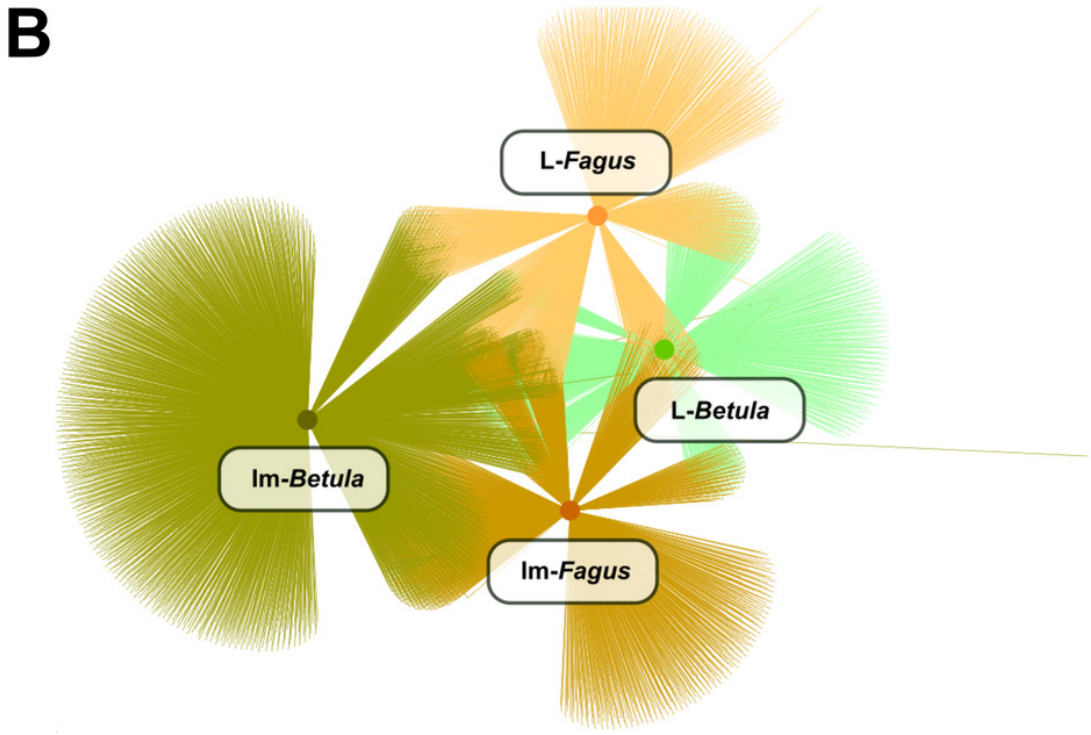

B
L-Fagus
L-Betula
lm-Betula
lm-Fagus

Figure 5

Inferred functions of bacterial communities associated with tested 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.

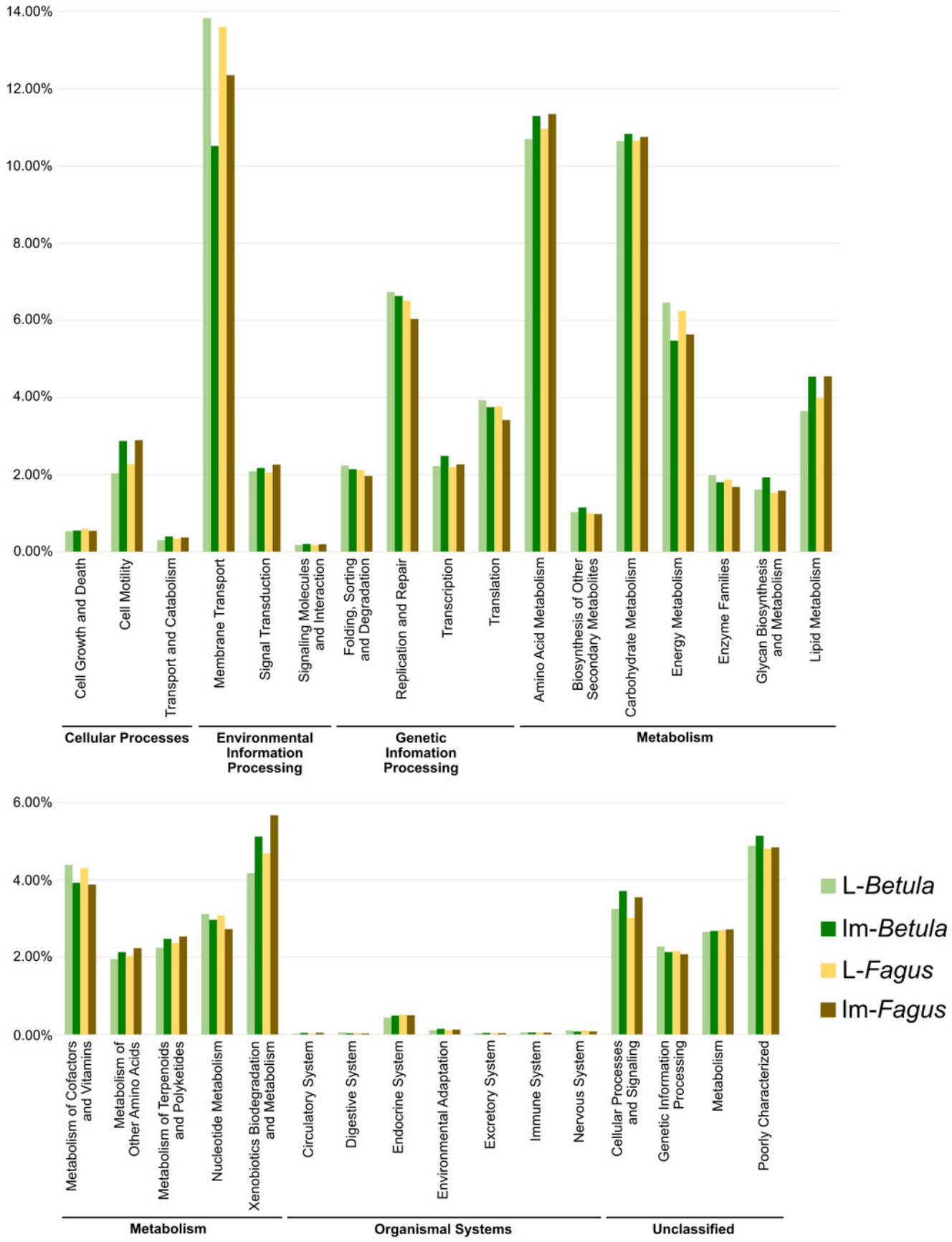

14.00%
12.00%
10.00%
8.00%
6.00%
4.00%
2.00%
0.00%
Cell Growth and Death
Cell Motility
Transport and Catabolism
Membrane Transport
Signal Transduction
Signaling Molecules and Interaction
Folding, Sorting and Degradation
Replication and Repair
Transcription
Translation
Amino Acid Metabolism
Biosynthesis of Other Secondary Metabolites
Carbohydrate Metabolism
Energy Metabolism
Enzyme Families
Glycan Biosynthesis and Metabolism
Lipid Metabolism
Cellular Processes
Environmental Information Processing
Genetic Infomation Processing
Metabolism
6.00%
4.00%
2.00%
0.00%
Metabolism of Cofactors and Vitamins
Metabolism of Other Amino Acids
Metabolism of Terpenoids and Polyketides
Nucleotide Metabolism
Xenobiotics Biodegradation and Metabolism
Circulatory System
Digestive System
Endocrine System
Environmental Adaptation
Excretory System
Immune System
Nervous System
Cellular Processes and Signaling
Genetic Information Processing
Metabolism
Poorly Characterized
Metabolism
Organismal Systems
Unclassified
L-Betula
lm-Betula
L-Fagus
lm-Fagus

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 microbial communities

ID	No. of bacterial reads	Average length (bp)	No. of observed OTU's	Chao1 index	Shannon index	Simpson index
L-*Betula*	184,132	452	1280	1617	6.45	0.95
Im-*Betula*	276,230	453	3648	3944	7.28	0.98
L-*Fagus*	222,980	450	1521	1734	7.04	0.98
Im-*Fagus*	207,899	453	1949	2231	5.87	0.93
Total	891,241	452	5444	2382	6.66	0.96

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 184,132 sequences.

Table 2(on next page)

Average dissimilarity in microbial community structure.

1 Table 2. Average dissimilarity in microbial community structure.

2

	Average dissimilarity (%)			
	Betula larva vs. imago	*Fagus* larva vs. imago	larva *Fagus* vs. *Betula*	imago *Fagus* vs. *Betula*
Proteobacteria	2.26	12.47	8.59	6.14
Actinobacteria	5.63	12.09	6.90	0.44
TM7	2.57	0.85	2.80	1.08
Firmicutes	4.61	2.63	1.66	0.33
Chloroflexi	0.31	0.03	0.26	0.03
Verrucomicrobia	1.20	0.75	0.23	0.23
Chlamydiae	0.01	0.18	0.20	0.02
Planctomycetes	0.13	0.19	0.15	0.17
Armatimonadetes	0.27	0.14	0.15	0.27
WPS-2	0.02	0.23	0.11	0.33
Bacteroidetes	5.81	1.03	0.10	4.69
Acidobacteria	2.91	0.01	0.07	2.85
[Thermi]	0.15	0.08	0.07	0.00
Cyanobacteria	0.01	0.03	0.04	0.00
Gemmatimonadetes	0.04	0.02	0.04	0.02
Synergistetes	0.04	0.00	0.04	0.00
Nitrospirae	0.04	0.00	0.04	0.00
Chlorobi	0.03	0.00	0.03	0.00
OD1	0.09	0.01	0.02	0.09
TM6	0.01	0.01	0.02	0.00
Euryarchaeota	0.01	0.00	0.01	0.00
WS6	0.00	0.01	0.01	0.00
BRC1	0.00	0.00	0.00	0.00
FBP	0.02	0.01	0.00	0.01
Fusobacteria	0.00	0.00	0.00	0.00
OP11	0.00	0.01	0.00	0.01
OP9	0.00	0.00	0.00	0.00
Other	0.07	0.01	0.03	0.10
Overall	26.20	30.74	21.52	16.75

Table 3(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 3. 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 4(on next page)

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.

Table 4. 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.