Caterpillar Gut Microbiome: Fungi Are More Transient Than Bacteria

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Abstract

Despite increasing studies on caterpillar (Insecta: Lepidoptera) gut microbiota, bacteria have been emphasized more than fungi. Therefore, we lack data on whether fungal microbiota is resident or transient and shaped by factors similar to those of bacteria. We sampled nine polyphagous caterpillar species from several tree species at multiple sites to determine the factors shaping leaf and gut bacterial and fungal microbiota as well as the extent to which caterpillars acquire microbiota from their diet. We performed 16S and ITS2 DNA metabarcoding of leaves and guts to determine the composition and richness of respective microbiota. While spatial variables shaped both the bacterial and fungal microbiota of the leaves, they only affected fungi in the guts, whereas bacteria were shaped primarily by caterpillar species, with some species harboring more specific bacterial consortia. Leaf and gut microbiota significantly differed; in bacteria, this difference was much more pronounced. Moreover, the leaf–gut similarity of bacterial but not fungal components differed significantly among caterpillar species, suggesting that some species have more transient bacterial microbiota than others. Our results suggest the complexity of the factors shaping the gut microbiota, while pointing out interspecific differences in microbiota residency within the same insect functional group.

Introduction

Interactions between insect herbivores and host plants are among the most important ecological associations on Earth[1]. The role of mediating such interactions is played by associated gut microbiota (bacteria, archaea, fungi, protozoa, and viruses)[2,3]. These microorganisms may, among other roles, provide their herbivore hosts with nutrients; aid in the digestion and detoxification of plant tissues; synthesize pheromones; modulate immune responses and communication; govern reproduction; and provide protection against pathogens, predators, and parasitoids[4–6]. Lepidoptera are one of the largest insect herbivore orders; their evolutionary success may depend on their beneficial relationship with microorganisms[5,7].

Similarly, living leaves harbor abundant epiphytic (epiphytes)[8] and endophytic (endophytes)[9] microbial communities. Leaf–microbiota relationships range from clearly negative for the plant[10] to strongly positive[11]. The leaf microbiota is diverse with bacteria and especially fungi playing the most prominent roles[12,13], providing an interactive potential for leaf consumers[14]; however, caterpillar midgut represents a hostile environment for diet-derived microbes because of its simple tube-like structure, extreme alkalinity (pH 8–12), and high plant secondary metabolite (allelochemicals) content from ingested plant tissues[5,15,16]. Species-specific digestive enzymes are adapted to the gut physiochemical conditions; thus, they may act as filters for specific gut microbial communities[5,17].

There is an ongoing debate concerning the residency and ecological role of caterpillar gut microbiota[18]. Transiency is supported by the fact that diet best explains the dissimilarity in microbiomes[19,20] and that gut changes during the life cycle may prevent the establishment of specific assemblages[21]. Residency is

supported by the fact that microbiota removal may reduce caterpillar fitness[22]. Moreover, certain bacterial populations may persist throughout the life cycle, despite extensive gut changes during pupation and metamorphosis[19,23]. Furthermore, despite the absence of specialized gut structures and rapid food transition[24], bacteria may form a biofilm, suggesting their ability to colonize the gut[25]. Thus, the caterpillar microbiome is likely a multilayer system composed of core taxa and a more flexible non-core microbiome[26,27] with a controversial functional role.

Caterpillar gut microbiomes are dynamic and variable with differences in community composition depending mainly on host phylogeny, life stage, physiological environment, and diet[4,7,16,19,28–30]. In contrast, the diet (i.e., leaf) of microbiota depends on plant identity; plant species differ in their capacity to harbor microbial communities[12,13,31]. Nutrient availability varies in space and time[32] depending on environmental factors and the associated physiological activity and productivity[10,33]. Thus, we expect host-interspecific and spatial differences in the diversity and community composition of gut microbiota.

To determine the factors shaping the diversity and composition of leaf and gut microbiota and to identify the core and transient components of the gut, it is necessary to analyze both environments simultaneously. Many metabarcoding studies have focused on a single factor[7,34–36], neglecting possible multifactorial effects. Similarly, studies on the leaf microbiomes of multiple plant species are rare (see[12,13,37,38] for exceptions) and the same applies to caterpillars[35,39,40]. Such studies have only focused on either the bacterial or fungal components. The fungal microbiome has been neglected, especially in caterpillars, although it may be richer than the bacterial microbiome[40].

Using an extensive dataset of caterpillars from nine polyphagous species sampled from five tree species at multiple sites in three geographically distant temperate forests, we aimed to (i) determine the factors shaping the composition and species richness of bacterial and fungal microbiota of leaves and caterpillars, and (ii) compare the composition and richness of leaf and gut microbiota to elucidate the origin/host fidelity of the gut microbiota. We hypothesized that in the case of transient microbiota, the composition and richness of the gut microbiota of caterpillars feeding on different plant populations or species is different. If the community structure and richness were either affected by caterpillar species or constantly differed from the leaf microbiota, we expected the existence of specific gut microbiota.

Results

Dataset

Regarding the leaf samples, bacterial (μ = 9281 reads per sample; interquartile range (IQR) 2525–13 335) and fungal reads (μ = 4738; IQR 1702.5–6418) were represented by 10 965 and 4034 ASVs, respectively. On average, we classified 166.8 (SD ± 120.4) bacterial and 72.3 (SD ± 36.2) fungal ASVs per leaf sample.

The bacterial (μ = 7935 reads per sample; IQR 1571.5–9757) and fungal reads (μ = 5922; IQR 2028–7911) in the guts were represented by 12 004 and 9378 ASVs, respectively. On average, we classified 104.2 (SD

± 81.8) bacterial and 90.7 (SD ± 39.8) fungal ASVs per gut sample. The hierarchical taxonomic composition of the leaf and gut microbiota is shown in **Fig. S1** (bacteria) and **Fig. S2** (fungi).

Factors Shaping Leaf Microbiota

Bacterial composition was shaped primarily by locality (explaining 10.94% of variability; df = 262, F = 19.36, p = 0.001), tree species (8.86%; df = 262, F = 6.27, p = 0.001) and their interactions (5.24%; df = 262, F = 2.65, p = 0.001) (**Fig. S3**), and irradiation (0.90%; df = 262, F = 3.18, p = 0.001). Fungal composition was shaped by tree species (explaining 18.03% of variability; df = 264, F = 15.20, p = 0.001), locality (12.20%; F = 25.70, p = 0.001), and their interactions (7.12%; F = 4.29, p = 0.001) (**Fig. S3**; Fig. 1).

Bacterial richness mainly depended on locality (df = 262, F = 40.22, p < 0.001), tree species (df = 262, F = 8.88, p < 0.001) and their interactions (df = 262, F = 4.74, p < 0.001) (Fig. 2a), and irradiation (df = 262, F = 5.02, p = 0.026). With increasing irradiation, the richness decreased (df = 262, F = 5.41, p = 0.021). Fungal richness mostly depended on locality (df = 262, F = 48.54, p < 0.001), tree species (df = 262, F = 11.84, p < 0.001), and their interactions (df = 262, F = 8.58, p < 0.001) (Fig. 2b) and decreased significantly with increasing irradiation (df = 262, F = 5.45, p = 0.005) (Fig. 1).

Factors Shaping Gut Microbiota

The bacterial composition was primarily shaped by caterpillar species (explaining 21.10% of variability; df = 866, F = 30.36, p = 0.001; Fig. 3a; **Fig. S4**), then by caterpillar body length (1.62%; df = 866, F = 18.62, p = 0.001), locality (0.94%; df = 866, F = 5.39, p = 0.001), tree species (0.91%; df = 866, F = 2.62, p = 0.001), and irradiation (0.20%; df = 866, F = 2.33, p = 0.011). The fungal composition was primarily shaped by locality (explaining 8.40% of variability; df = 882, F = 5.16, p = 0.001) and caterpillar species (4.02%; df = 882, F = 5.16, p = 0.001; Fig. 3b; **Fig. S4**), followed by tree species (3.24%; df = 882, F = 6.65, p = 0.001) (Fig. 1).

Bacterial richness depended on caterpillar species (df = 864, F = 62.47, p < 0.001; Fig. 4a), sampling plot (df = 864, F = 4.05, p < 0.001), and irradiation (df = 864, F = 6.64, p = 0.001) with peak bacterial richness at 60% of the irradiated crown. There was a significant interaction between caterpillar species and the sampling plot (df = 861, F = 3.49, p < 0.001). Fungal richness depended on the sampling plot (df = 861, F = 18.06, p < 0.001), followed by caterpillar species (df = 861, F = 10.32, p < 0.001; Fig. 4b) and tree species (df = 861, F = 5.52, p < 0.001) (Fig. 1).

Comparison Of Leaf And Gut Microbiota

The composition of the bacterial microbiota of leaves and guts significantly differed, explaining 7.40% of the variability (df = 1160, F = 92.66, p = 0.001; Fig. 5a). The composition of fungal microbiota also differed, explaining 1.14% of the variability (df = 1161, F = 13.43, p = 0.001; Fig. 5b). The betadispersion

of samples was greater for guts than for leaves in bacteria (df = 1159, $F = 16.90$, $p = 0.001$) but not in fungi (df = 1160, $F = 3.06$, $p = 0.087$; **Fig. S5**). The quantitative similarity between leaves and guts was higher for fungal microbiota than for bacterial microbiota (V = 8091, $p < 0.001$; Fig. 5a,b and **Fig. S6**). The leaf–gut similarity did not differ among host plants (df = 877, $\chi^2 = 1.45$, $p = 0.835$ and df = 878, $\chi^2 = 8.97$, $p = 0.062$ for bacteria and fungi, respectively) or among caterpillar species for the fungal microbiome (df = 874, $\chi^2 = 9.15$, $p = 0.329$) but differed among caterpillar species for the bacterial microbiome (df = 873, $\chi^2 = 19.70$, $p = 0.012$) and was significantly lower in *A. aescularia* and *O. brumata* (df = 873, z = −2.68, $p = 0.007$ and df = 873, z = −2.01, $p = 0.045$, respectively).

Among the 10 most abundant bacteria and fungi, *Streptococcus* (bacteria) was significantly associated with guts ($p < 0.05$), whereas *Sphingomonas* (bacteria) and *Erysiphe* (fungus) were significantly associated with leaves ($p < 0.05$) (Fig. 6a,b). All indicator taxa are shown in **Table S1.**

Rarefied bacterial genera richness was higher in the leaves than in the guts ($\chi^2 = 9.82$, $p = 0.007$; 47.3 [SD ± 20.2] and 40.3 [SD ± 21.7] genera per sample in the leaves and guts, respectively), whereas the rarefied fungal species richness was higher in the guts than in the leaves ($\chi^2 = 15.91$, $p < 0.001$; 30.0 [SD ± 9.2] and 25.9 [SD ± 9.3] species per sample in the guts and leaves, respectively). In both the leaves and guts, bacterial genera richness was higher than fungal species richness (V = 36 614, $p < 0.001$; V = 279 457, $p < 0.001$) (**Fig. S7**).

Discussion

Leaves harbor diverse and abundant microbial communities–both bacterial[12,38] and fungal[13,41]. However, the high overall richness in the caterpillar guts contradicts previous findings reporting species-poor bacterial communities[7,30,39] Contrary to our previous study[40], bacterial microbiome was richer than fungal microbiome, especially considering that bacterial richness was estimated at the genus level and fungal richness at the species level. The accurate quantification of the 16S and ITS2 DNA in the initial samples is necessary to assess the proportional representation of bacteria and fungi in the gut microbiota. Moreover, the extent to which microbial communities obtained by metabarcoding are composed of legacy DNA from dead or dormant cells and spores remains to be determined[42].

Both the bacterial and fungal components of leaves were significantly affected by tree species, which is a typical pattern[43]; however, they always significantly interacted with locality, implying that trees at different localities harbored specific microbial assemblages. This contradicts a recent study suggesting low variability in leaf microbial diversity among individual sites[44], although it accords with the fact that the leaf microbes are acquired from the environment, but their survival is filtered by the plant[45]. The microbiomes of a focal host species may be affected by local plant community composition and diversity[31,37,44]; these measurements were beyond the scope of our study but could be responsible for the observed spatial differences.

Irradiation significantly affected the fungal richness and bacterial composition and richness. Sun exposure is a good predictor of fungal abundance[46] and diversity[47]. For bacteria, Stone and Jackson[48] found distinct community compositions across canopy positions, with speculative attributability to radiation; however, shaded leaves are less exposed to rain, which changes bacterial composition. Moisture availability may have a substantial impact on leaf microbiota[49]; desiccation, especially in combination with UV radiation, strongly limits microbial populations[50]. However, in leaf bacteria, UV radiation has been found to have no effect on species richness[51].

In caterpillars, bacterial composition and richness were primarily shaped by caterpillar species. In fungi, the role of the host species is only secondary; they are shaped primarily by spatial variables. Great interspecific differences were quite surprising, given that numerous studies have reported similar communities shared among caterpillar species, both implying the low importance of the host physiological environment in structuring microbial communities and emphasizing the dietary effect[19,29,35,39]. In contrast, independent of diet, there are interspecific differences in the physiochemical conditions of the gut, which exert strong selection pressure on microbiota[5,17]. From this point of view, the associated differences in gut microbiota were expected.

Bacterial composition was significantly affected by caterpillar body length. Changes in community composition are attributed to the increasing importance of gut filtering throughout the caterpillar life cycle[7,28,52]. As caterpillars grow, less oxygen penetrates the gut lumen, which promotes the development of facultative anaerobic bacteria, mainly Enterobacteriaceae, decreasing diversity[23]. This was not reflected here; we sampled only similarly sized caterpillars (3–4th instar) to suppress the effect of developmental stage. This protocol did not result in a dataset composed of same-instar caterpillars, and some effect of body length may be attributable to the above-mentioned effect.

Apart from the strong effect of caterpillar species, the differences in the composition and richness of gut bacterial microbiome were shaped by irradiation, spatial variables, and their interaction with caterpillar species. The climatic and ecological factors of host habitats are known to affect the insect gut bacterial composition[53,54]. However, as individual conditions of individual plots affect individual caterpillar species differently, the effect of environmental conditions remains unsolved. Alternatively, bacterial richness and composition may be strongly affected by biotic conditions, especially parasitoid infection[26]. Spatial differences in parasitism rate and parasitoid community composition are well known[55,56]. Although we tried to eliminate all parasitized samples before processing, parasitoid juvenile stages could have been overlooked and may have contributed to the differences in bacterial composition and richness.

Host tree species also significantly affected gut fungal and bacterial microbiomes. Bacterial gut communities are greatly influenced by diet[57]. Here, however, leaf bacteria were primarily affected by spatial variables. Thus, bacterial gut content may be affected not by the microbial composition of the diet, but rather by its quality (protein and carbohydrate content)[20] and plant secondary metabolites[25],

which places major selective pressure on the gut microbiota. This pressure seems to be less important for fungal components, which are primarily affected by spatial variables, either in the diet or gut.

The compositions of both bacterial and fungal gut components significantly differed from those of leaves; however, in bacteria, this difference was much more pronounced. Caterpillars move over relatively long distances when feeding[58], and *Orthosia* spp. are even occasional entomophages[59]. Thus, they probably sampled a much greater microbial pool than that reflected in the relevant leaf sample, which may have contributed to their low similarity. Bacterial richness was higher in the leaves than in the guts, whereas fungal components showed an opposite pattern, suggesting that fungi may be less filtered than bacteria. The composition of the leaf bacteria was rather balanced, whereas in the guts there was a great host-interspecific variability. Contrarily, fungal component of both leaves and guts was dominated by *Aureobasidium pullulans*, *Ramularia*, and *Dothiora*, again pointing out environmental acquisition greater than in the case of bacteria.

This finding was corroborated by the analysis of the factors that form individual microbial components of the gut. In fungi, the caterpillar species was only a secondary factor explaining less variability than the spatial variables (locality and sampling plot), which predominantly shaped the richness and composition of the diet. However, the bacterial component of the guts was shaped primarily by caterpillar species and was affected by the caterpillar body length, indicating a greater involvement of gut filtering. Bacteria in the diet varied greatly among individual localities; however, gut bacteria did not reflect this. The spatial variability of bacteria manifested only at the level of individual localities, which may reflect the host's adaptation to local conditions. These results indicate that the fungal component of the gut is more transient, whereas bacteria form a core component.

The leaf–gut similarity of bacterial—but not fungal—components differed significantly among caterpillar species, suggesting that some species have lower leaf–gut similarities than others (specifically *A. aescularia* and *O. brumata*). This suggests a higher involvement of resident (core) bacterial taxa or stronger environmental filtering. This may be explained by their similar life histories and dispersal strategies. Unlike the rest of the study species, adults remain active during winter, occurring in high abundances[60]. For both species, ballooning dispersal, in which caterpillars use silk to move through the air, was documented[61]. Both strategies could contribute to sampling different microbial pools from the environment. Moreover, extreme winter conditions may alter adult gut microbiota, which may be vertically transferred to the offspring through the contamination of the egg surface[23,28]. Furthermore, while bacterial microbiota of some hosts (*P. munda*, *A. aescularia*, *P. pilosaria*, *O. brumata*) was quite similar to each other, other species (*E. defoliaria*, *L. dispar*) hosted more specific bacterial consortia, suggesting diversity in directions of environmental filtering.

Our study indicates the complexity of the factors shaping leaf and caterpillar gut microbiota, which makes it difficult to draw conclusions, even with a large dataset. Other important factors were not accounted for in this study, namely interactions within and among microbial groups that are abundant and of great importance, in both the leaves[62,63] and gut[3,64]. The relatively static physiochemistry of the

host as well as dynamic microbe-microbe, microbe-host and host-mediated microbe-microbe interactions are likely the drivers of microbiota community composition[43], as applied to both environments. Future studies using the community-level approach may clarify the relative importance of stochastic and deterministic processes in governing gut microbiota assembly and how this importance varies through space and time. A functional approach using transcriptomics, which identifies biologically active taxa, would complement these studies and elucidate the link between the core component of the gut and its significance for the host.

Materials And Methods

Sampling of leaves and caterpillars

Field sampling was conducted at the end of April to mid-May, 2018 at three remote localities in the temperate floodplain forests of Moravia, Czech Republic: southern (48.8926N, 17.0700E; 170 m a.s.l.), central (49.6932N, 17.1399E; 225 m a.s.l.), and eastern (49.7918N, 18.2061E; 220 m a.s.l.) (**Fig. S8**). At each locality, we sampled the leaves of five tree (Fagales: *Quercus robur*, *Q. petraea*, *Corylus avellana*, *Carpinus betulus*, and *Alnus glutinosa*) and nine caterpillar (polyphagous leaf-chewers, Lepidoptera—Noctuidae: *Orthosia cerasi*, *O. cruda*, and *Perigrapha munda*; Geometridae: *Agriopis marginaria*, *Alsophila aescularia*, *Erannis defoliaria*, *Operophtera brumata*, and *Phigalia pilosaria*; and Erebidae: *Lymantria dispar*) species. Within each locality, we set three remote sites (sampling plots) containing all tree species, each represented by six to seven individuals (Fig. 1). Caterpillars were sampled manually or by using 1-m^2 beating sheets. Each individual was captured using sterilized tweezers, transferred to a 1.5-ml centrifuge tube with 98% ethanol, and post-mortem length was measured. To minimize the combined effect of the developmental stage and locality (localities were sampled consecutively) on the composition and diversity of microbiota[7], we sampled 3–4th instars of the given species at each sampling plot.

Simultaneously, we sampled host tree leaves. Within each tree individual, we randomly selected five leaves, cut the middle parts (2 cm^2 segments; i.e., 10 cm^2 per sample) using sterilized tweezers and scissors, and transferred them to a 1.5-ml centrifuge tube with 98% ethanol. As herbivory is known to generate variations in within-host microbial fitness and consequently, alter the structure of the leaf microbiota[65,66], we assessed the effect of herbivory by selecting approximately half of the samples (n = 136) from leaves with herbivory damage and half (n = 143) from pristine leaves. For each individual tree, from which we had leaf and caterpillar samples, we estimated (based on three collectors) the irradiated proportion of the crown, measured the sampling height above the ground using a digital laser distance meter (HECHT® 2006; Hecht Motors Inc., Prague, Czech Republic), and measured the diameter at breast height. Tubes with caterpillar and leaf samples were stored at –32°C. To maintain a balanced sampling design, leaf samples from 279 tree individuals and 883 caterpillars with the best overlap among tree individuals, tree species, sampling plots, and localities were selected for further processing (**Table S2**).

Identification of caterpillars

Caterpillars were identified at the morphospecies level using standard identification keys, field guides, and online databases (**Table S3**). Specimens that could not be reliably assigned to a morphospecies (e.g., congeneric species; 231 individuals) were subjected to the DNA barcoding of cytochrome oxidase subunit I (COI) following Hrcek et al.[67]. We used DNA extracted from the guts (see below). PCR products were sequenced in the forward or reverse direction using an ABI 3730XL sequencer (Macrogen Europe, Amsterdam, Netherlands). Specimen records with sequences are accessible on BOLD (dataset DS-SYMB; DOI dx.doi.org/10.5883/DS-SYMB).

Processing of caterpillars and leaves

Each caterpillar was washed by vortexing in a 1.5-ml tube with 98% ethanol at 2100 rpm for 90 s, transferred to a clean 1.5-ml tube, and washed in a 1-ml sterile solution of 1% Tween 80 and phosphate-buffered saline (PBS) (Sigma-Aldrich, Saint Louis, MO, USA) at 2100 rpm for 45 s to minimize contamination by surficial microbiota. The gut content was separated using a sterilized scalpel, needle, and minute pins onto paraffin wax sterilized with flamed ethanol[68] and transferred to a new 1.5-ml tube with 100 µl of 1× PBS. The leaf samples in 1.5-ml tubes were vortexed at 2,100 rpm for 45 s and centrifuged at 5400 × *g* for 15 min at 4°C. The supernatant was discarded, and the residual ethanol was evaporated at 55°C for 45 min. Subsequently, samples were resuspended in 200 µl of 1× PBS solution and stored at −32°C for subsequent DNA isolation.

DNA metabarcoding of bacteria and fungi

DNA was extracted from the guts (n = 883) and leaves (n = 279) using a NucleoSpin Tissue DNA Isolation Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. The samples were repeatedly crushed in 1.5-ml tubes using plastic pestles and liquid nitrogen before cell lysis. In samples with higher amounts of the input tissue, we adequately increased the volume of enzymes and buffers used for (pre)lysis and subsequent DNA binding steps. To ensure broad bacterial and fungal diversity recovery, we used highly degenerate primers, which can significantly reduce the recovery of plant-originating sequences (chloroplasts): for the amplification of the fungal ITS2 rRNA region, we used ITS3_KYO2 5′-GATGAAGAACGYAGYRAA-3′ (forward) and ITS4_KYO3 5′-CTBTTVCCKCTTCACTCG-3′ (reverse)[69], and for the bacterial V5–V6 16S rRNA region, we used 799F 5′-CMGGATTAGATACCCKGG-3′ (forward) and 1115R 5′-AGGGTTGCGCTCGTTG-3′ (reverse)[70,71] with barcodes added to the 5′ end of both primers, enabling the identification of each sample. All PCRs were performed in triplicate to minimize the effects of stochastic amplification. The amplification of the ITS2 rRNA gene region was performed as described by Toju et al.[69] with minor modifications consisting of initial denaturation at 95°C for 3 min; 35 cycles at 94°C for 30 s, 55°C for 60 s, 72°C for 60 s; and a final extension at 72°C for 10 min. The amplification of the 16S rRNA gene region consisted of initial denaturation at 94°C for 4 min; 35 cycles at 94°C for 45 s, 50°C for 60 s, 72°C for 75 s, and a final extension at 72°C for 10 min. Each PCR reaction (25 µl) consisted of 9.4 µl of molecular biology grade water (New England BioLabs, Ipswich, MA, USA), 0.5 U KAPA2G Robust HotStart DNA Polymerase, 5 µl of 5× KAPA2G Buffer B, 5 µl of 5× KAPA2G Enhancer (all Kapa Biosystems, Wilmington, NC, USA), 0.5 µl of 10 mM dNTP Mix (Thermo Fisher Scientific, Waltham, MA, USA), 0.8 µM of each primer, and 2 µl of genomic DNA. All PCR products were

analyzed using 1.5% agarose gel. We pooled triplicate PCR reactions of individual samples within each “plate library” (96 samples). The amplicons of specific length from individual libraries were excised from the 2% agarose gel and purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). DNA concentration was measured using a Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific), and we equalized concentrations within all libraries to 20 ng/μl. Individual “plate libraries” were subjected to DNA ligation of sequencing adapters and library-unique multiplex identifiers using KAPA Hyper Prep Kit, and subsequently quantified using a KAPA Library Quantification Kit (both Kapa Biosystems). Further, the equimolar proportions of individual “plate libraries” were pooled, creating one final library of fungal samples and a second of bacterial samples at 7.5 ng/μl. The fungal library was subjected to paired-end sequencing on a MiSeq instrument, producing 2 × 300 bp reads (four runs in total), whereas the bacterial library was subjected to single-end sequencing on NextSeq 500 (Illumina Inc., San Diego, CA, USA) (one run), producing 1 × 150 bp read at the Genomics Core Facility, CEITEC (Masaryk University, Brno, Czech Republic). In this study, bacterial sequences represented 39.0% and fungal sequences represented 39.8% of the total NextSeq and MiSeq sequencing outputs. Raw demultiplexed sequencing data with sample annotations are available at the NCBI Bioproject website (https://www.ncbi.nlm.nih.gov/bioproject/) under the accession number PRJNA694554.

Dna Metabarcoding Data Processing

Sequencing data were processed using QIIME 2.0 2020.2[72]. Raw reads were demultiplexed and quality filtered using the q2-demux plugin, and in the case of fungal datasets, the ITS region was extracted using the q2-ITSxpress plugin[73]. Afterwards, reads were denoised using the DADA2 algorithm[74] and a feature table with counts of amplicon sequence variants (ASVs) per sample was produced. Taxonomy was assigned using the q2-feature-classifier classify-sklearn[75] using a trained naïve Bayes classifier against the SILVA_138_SSURef_Nr99 bacterial reference database[76] and UNITE QIIME release for Fungi version 8.0[77,78]. We obtained an ASV table with 27 552 665 bacterial and 6 679 221 fungal reads. Further, we identified contaminant ASVs using the “decontam” package[79] based on the prevalence method with extraction controls as negatives (three per each 96-well plate). The probability threshold below which the null hypothesis of non-contamination was rejected was 0.1. We discarded 353 bacterial and 291 fungal ASVs (2.92% of reads; **Table S4**) and removed unassigned reads (3.04%) and those associated with the chloroplasts and mitochondria (46.9%). Finally, 9 586 289 bacterial (7 006 293 gut; 2 579 996 leaf) and 6 551 306 fungal reads (5 229 511 gut; 1 321 795 leaf) were used for analysis.

Statistical analyses

Data were analyzed using R 4.0.2[80] and Canoco 5.0[81]. For hierarchical visualization of the recovered fungal and bacterial composition of the leaf and gut microbiota, we used Krona charts[82]. Bacterial ASVs were analyzed at the genus level (only a small number of ASVs could be classified to the species level), whereas fungal ASVs were analyzed at the species level. For bacterial and fungal taxa, number of reads,

and variables entering the analyzes, see **Table S5**. To compare bacterial genera/fungal species richness, the number of reads in each sample was rarefied to 400. Rickettsiales (i.e., *Rickettsia* and *Wolbachia*) were excluded from the analyses of gut composition as they are intracellular parasites, most probably originating from gut cells instead of the lumen; however, they were not excluded from leaf analyses, in which they commonly survive[83]. However, this group was excluded from both datasets for the comparison of leaf and gut composition. For the final generalized linear models, we checked the possible collinearity of variables by generalized variance inflation factor from the "car" package[84] adjusted to the given degrees of freedom, potentially excluding variables exceeding the threshold of >2.

Declarations

Ethics statement

No specific permissions were required to collect insect and plant specimens, because the study species do not include any species at the risk of extinction (according to the IUCN Policy Statement on Research Involving Species at Risk of Extinction) or endangered species of wild fauna and flora (according to the Convention on the Trade in Endangered Species of Wild Fauna and Flora). Voucher specimens for all plant and insect species described in the manuscript are deposited in the collection of the University of Ostrava.

Factors shaping leaf and gut microbiota

To determine the most important factors shaping the composition of leaf and gut microbiota, we performed permutational multivariate analysis of variance (PERMANOVA) using the "vegan" package[85] with 999 permutations and distance matrices calculated by the Bray–Curtis method for bacterial and fungal datasets separately. For leaf microbiota, we used two groups of explanatory variables, characterizing the host tree (species, diameter at breast height, sampling height, irradiation of crown, and herbivory damage) and environment (locality, sampling plot, and day in the season). For gut microbiota, we added a third group of variables characterizing the host caterpillar (species, family, and body length). Final models were built by stepwise forward selection based on Akaike's information criterion (AIC). The resulting models were accompanied by principal coordinate analyses (PCoA) of both datasets tested by Monte Carlo tests with 999 permutations. The caterpillar species was used as an explanatory variable, and for the fungal dataset, locality was used as a covariate, which explained more variability than caterpillar species in PERMANOVA.

We analyzed the rarefied richness by generalized linear models with Gamma distribution (with the exception of a linear model for gut microbiota fungal dataset), all built by stepwise forward selection based on AIC from the above-defined set of explanatory variables characterizing the tree, environment, and caterpillar (for gut microbiota only). For gut microbiota, we compared the distributions of rarefied richness for bacterial and fungal datasets using the paired Wilcoxon signed-rank test.

Comparison of leaf and gut microbiota

We used PERMANOVA to compare the bacterial/fungal composition between the leaves and guts. As multivariate variation among the test groups may, in the case of an unbalanced number of samples, compromise PERMANOVA results, we added Marti Anderson's PERMDISP2 procedure for the analysis of multivariate homogeneity of group dispersions (variances) based on Bray–Curtis distance, measuring the distance to group centroids[86]. The models were accompanied by PCoA for both datasets, with each tested using the Monte Carlo test with 999 permutations. The analyses were supplied by barplots depicting the 10 most abundant bacterial genera and fungal species. We calculated the quantitative similarity between each gut and its host tree leaf sample using the Renkonen index[87]. We compared the distributions of leaf–gut similarity for bacterial and fungal datasets using paired Wilcoxon signed-rank tests. We analyzed which explanatory variables affected similarity using generalized linear models with binomial distribution built by stepwise forward selection. For the final model, contrasts were set to sum, which compares the mean of a dependent variable for a given level to the overall mean of the dependent variable.

The rarefied bacterial genera/fungal species richness of the gut and leaf microbiota was compared by generalized linear mixed models with Gamma distribution and sampling plot as random terms using the "lme4" package[88]. The significance of the models was determined by comparing with relevant null models. We identified the indicator bacterial genera/fungal species for leaves and guts and separately for the gut microbiota of each caterpillar species and leaf microbiota of each tree species using the IndVal method from the "labdsv"[89] and "indicspecies"[90] packages, which generates a value indicating the frequency and relative abundance of reads[91] and by using multi-level pattern analysis[89,90] with adjusted *p*-values to correct for multiple comparisons using Benjamini–Hochberg corrections[92].

Data Availability

Caterpillar specimen records with sequences are accessible on BOLD (dataset DS-SYMB; DOI dx.doi.org/10.5883/DS-SYMB). Bacterial and fungal raw demultiplexed sequencing data with sample annotations are available at the NCBI Bioproject website (https://www.ncbi.nlm.nih.gov/bioproject/) under the accession number PRJNA694554. An overview of bacterial and fungal taxa, number of reads, and variables entering the analyzes is included in Supplementary Information (Table S5).

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Author Contributions

PD, MKol, PP and MŠ conceived the main ideas and designed the study; NK, OD, PP, MŠ, HŠ and DV performed field sampling; MŠ, DV, PP, OD and NK performed laboratory processing; MKos carried out bioinformatic analyses; PP performed statistical analyses with a major contribution of PD; MŠ and HŠ drafted the manuscript; PD, PP, MKol and MKos revised it critically for important intellectual content.

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Figures

Figure 1

Sampling scheme of leaves and caterpillars with an overview of the effect of individual variables on the composition and richness of associated bacteria and fungi. For significant variables, the order of their significance in the respective analyses is given based on Akaike's information criterion (AIC), stepwise forward selection from permutational multivariate analysis of variance (PERMANOVA) (richness), and generalized linear models with Gamma distribution (composition). Only significant interactions are shown.

Figure 2

Rarefied richness of **a)** bacterial genera and **b)** fungal species at tree species level within sampling localities.

Figure 3

Principal coordinates analysis (PCoA) plots showing differences in **a)** bacterial and **b)** fungal microbiota composition among the guts of nine polyphagous caterpillar species.

Figure 4

Accumulation curves (mean ± SD) of **a)** bacterial genera and **b)** fungal species associated with the guts of nine polyphagous caterpillar species.

Figure 5

Similarity comparison of **a)** bacterial and **b)** fungal microbiota composition between caterpillar guts and host tree leaves.

Figure 6

Composition of **a)** bacterial and **b)** fungal microbiota associated with caterpillar guts and host tree leaves. The 10 most abundant bacterial genera and fungal species are shown.

Supplementary Files

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