



The wild solitary bees *Andrena vaga*, *Anthophora plumipes*, *Colletes cunicularius*, and *Osmia cornuta* microbiota are host specific and dominated by endosymbionts and environmental microorganisms

Amanda Hettiarachchi¹ · Margo Cnockaert¹ · Marie Joossens¹ · Antoine Gekière² · Ivan Meeus³ · Nicolas J. Vereecken⁴ · Denis Michez² · Guy Smagghe³ · Peter Vandamme¹

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Abstract

We characterized the microbial communities of the crop, midgut, hindgut, and ovaries of the wild solitary bees *Andrena vaga*, *Anthophora plumipes*, *Colletes cunicularius*, and *Osmia cornuta* through 16S rRNA gene and ITS2 amplicon sequencing and a large-scale isolation campaign. The bacterial communities of these bees were dominated by endosymbionts of the genera *Wolbachia* and *Spiroplasma*. Bacterial and yeast genera representing the remaining predominant taxa were linked to an environmental origin. While only a single sampling site was examined for *Andrena vaga*, *Anthophora plumipes*, and *Colletes cunicularius*, and two sampling sites for *Osmia cornuta*, the microbiota appeared to be host specific: bacterial, but not fungal, communities generally differed between the analyzed bee species, gut compartments and ovaries. This may suggest a selective process determined by floral and host traits. Many of the gut symbionts identified in the present study are characterized by metabolic versatility. Whether they exert similar functionalities within the bee gut and thus functional redundancy remains to be elucidated.

Keywords Solitary bee · Gut microbiota · Amplicon sequencing · Cultivation · Endosymbionts

Introduction

Solitary bees represent the majority of approximately 20,000 known bee species [1]. Their high species diversity is reflected in a vast variation in morphological traits, phenology, geographic distribution, nesting behavior, and foraging preferences [2]. As pollinators, they are major contributors

to a key ecosystem service required for global food security and survival of flowering plants [3]. The importance of bees as pollinators and the interest to improve bee health has intensified the research on bee gut microbiota. A better understanding of the microbial communities associated with bees may offer new insights into microbiome acquisition and evolution, its contribution to host health and resilience, and the factors influencing its composition [4].

Despite their high species diversity, the gut microbiome of solitary bees has been studied less frequently than that of social bees [5]. The microbiota of solitary bee larvae and their pollen provisions are best studied so far. A diverse range of bacteria have been identified and were mostly associated with floral resources [6–11]. The microbiome of pollen provisions appeared to change over time of storage [12], with the development of larvae [13] and with pollen diet across landscapes [14]. In addition, larvae displayed different bacterial communities at each developmental stage [15–17], and early-stage larvae had a microbiome composition comparable to their pollen provisions [18].

✉ Peter Vandamme
Peter.Vandamme@ugent.be

¹ Laboratory of Microbiology, Department of Biochemistry and Microbiology, Faculty of Sciences, Ghent University, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium

² Laboratory of Zoology, Research Institute for Biosciences, University of Mons, Place du parc 20, 7000 Mons, Belgium

³ Laboratory of Agrozoology, Department of Plants of Crops, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Gent, Belgium

⁴ Agroecology Lab, Université libre de Bruxelles (ULB), Boulevard du Triomphe CP 264/02, 1050 Brussels, Belgium

Gut microbiota studies of adult solitary bees are more scarce. Their gut microbial composition was reported to be distinct from that of social bees [19] and included several environmental microbes, such as Bacillaceae, Enterobacteriaceae, Lactobacillaceae, Moraxellaceae, Pseudomonadaceae, and Staphylococcaceae [7, 10, 12, 17, 18, 20–24]. The solitary bee gut also commonly harbors endosymbiotic bacteria of the genus *Wolbachia* [17, 19, 25]. Unlike social bees, solitary species lack the transmission routes to acquire gut symbionts from nest mates and are directly exposed to the environment upon emergence [5]. As a result, the adult solitary bee probably acquires its symbionts from nest material, pollen provisions or from visited flowers. Additionally, the variety of life history traits displayed by solitary bees likely influences their gut microbiome composition as well [4, 5]. Thus, solitary bee species appear to harbor a highly diverse gut microbial community that is shaped by the environment. Yet, some solitary bee species associate with specific microbial taxa suggesting that these poorly known colonization mechanisms can also lead to host-specific microbes that persist in the solitary bee gut [12, 17, 26].

In the present study, we characterized the gut microbial composition of *Andrena vaga* (Hymenoptera: Andrenidae), *Anthophora plumipes* (Hymenoptera: Apidae), *Colletes cunicularius* (Hymenoptera: Colletidae), and *Osmia cornuta* (Hymenoptera: Megachilidae), four common solitary bee species in Belgium. The bacterial and fungal compositions of the crop, midgut, and hindgut compartments and of ovaries were studied through 16S rRNA gene and ITS2 amplicon sequencing, respectively. The composition and diversity of the microbial communities between compartments and bee species were assessed, and the microbial composition of *O. cornuta* from two different sampling locations was evaluated. A large-scale cultivation campaign was performed to isolate and identify the predominant, cultivable symbionts of each gut compartment.

Methods

Bee sampling and processing

Wild solitary bees were sampled from an urban environment in Etterbeek (Jardins Participatifs d'Etterbeek, 50° 50' 59.17" N, 4° 23' 28.32" E) and a semi-natural environment in Ave-et-Auffe, Rochefort (50° 6' 34.11" N, 5° 7' 57.28" E) in Belgium, in the spring of 2018. The urban location was a garden built by the municipality of Etterbeek to promote biodiversity and gardening in an urban context. The semi-natural location was a woodland surrounded by agricultural fields. *Andrena vaga* ($n=16$), *An. plumipes* ($n=21$) and *O. cornuta* ($n=23$) bees were sampled from the urban location and *C. cunicularius* ($n=49$) and *O. cornuta* ($n=21$) from the

semi-natural location. Only *O. cornuta* could be collected from both sampling sites. Sampling was performed during multiple visits to the sites as the bee species have different foraging periods within spring. Specimens were immediately frozen at -20°C upon arrival in the laboratory. Bees were surface-sterilized with Umonium38 Medical Spray and dissected under sterile conditions. The gut was extracted and separated into crop, midgut, and hindgut when a clear differentiation of the sections was possible. Ovaries were collected as well. Gut sections and ovaries were collected in 250 μl of physiological saline (0.85% NaCl, 0.1% peptone, 0.1% Tween80) and homogenized using sterile micro-pestles. A total of 125 μl of each cell suspension was stored immediately at -80°C until DNA extraction, while the remaining 125 μl of cell suspension was mixed with an equal volume of 40% glycerol and stored at -80°C using Nalgene® Mr. Frosty™ Freezing containers until cultivation.

16S rRNA and ITS2 amplicon sequencing

Bacterial and fungal DNAs were extracted using a phenol-chloroform-based DNA extraction protocol as described before [27]. DNA quality was evaluated with NanoDrop and 1% agarose gel electrophoresis, and DNA yield was assessed using a Quantus™ Fluorometer. Samples with DNA concentrations $>1\text{ ng}/\mu\text{l}$ (Table S1) were sent to BaseClear B.V. (Leiden, The Netherlands) for library preparation and amplicon sequencing. The 16S rRNA V3–V4 region was amplified using forward primer 341F (5'-CCTACG GGNCGCWGCAG-3') and reverse primer 785R (5'-GAC TACHVGGGTATCTAATCC-3'). The eukaryotic internal transcribed spacer 2 region (ITS2) was amplified using forward primer ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATAT GC-3'). The library was sequenced on an Illumina MiSeq platform generating 300 bp paired-end reads.

16S rRNA and ITS2 raw reads were analyzed separately with the DADA2 pipeline version 1.14.1 [28]. The bacterial forward and reverse reads were trimmed to 280 bp and 210 bp, respectively, upon read quality inspection and primers were removed from the respective reads using the trimLeft parameter. Merged paired reads with a length between 400 and 428 bp were retained for further analyses. ITS2 primers were removed using cutadapt version 3.4 [29] and reads with a minimum length of 50 bp were retained through the rest of the pipeline. Taxonomy was assigned to the resulting 16S rRNA and ITS2 amplicon sequence variants (ASVs) using the DADA2 formatted training FASTA files of the SILVA SSU database version 132 [30] and the general FASTA release files from the UNITE ITS database version 8.3 (all eukaryotes) [31], respectively. Sequences classified as chloroplasts, mitochondria, Archaea, Eukarya, and unclassified phyla were removed from the 16S rRNA ASV

dataset, whereas only fungal ASVs were retained in the ITS2 dataset for further analyses.

Data analyses of the microbial community

All analyses were performed in R version 4.1.0. Alpha and beta diversity measures were calculated and plotted using phyloseq package version 1.36.0 [32]. Alpha diversity estimates (observed richness and Shannon diversity) were compared between gut compartments per bee species and between bee species per compartment using Kruskal–Wallis tests. Statistical tests were applied using the ggpvr package version 0.4.0. Bray–Curtis distance matrices were calculated for beta diversity analyses and were visualized through principal coordinates analysis (PCoA) plots. Community dissimilarity between the above-mentioned groups were compared with the PERMANOVA statistical test (“adonis” with 9999 permutations) and analyzed for homogeneity of group dispersions (“betadispr”) using the vegan package version 2.5-7 [33]. Pairwise PERMANOVA tests were performed using the pairwise Adonis package version 0.4. Differentially abundant ASVs ($p_{\text{adj}} < 0.05$) between the analyzed groups were identified with the DESeq2 package version 1.32.0 [34] considering only ASVs with >1% relative read abundance and occurring in at least two samples. For all pairwise statistical tests, p values were adjusted for multiple hypothesis testing with the Benjamini–Hochberg method.

Isolation of the microbial community

Six cell suspensions of each compartment were pooled per bee species. These pools were diluted to 10^{-1} for crop and midgut and to 10^{-4} for hindgut and ovary pools. Fifty microliters of each dilution was directly plated onto multiple agar media in order to isolate a maximal diversity of bacteria and yeasts (Table S2). Additionally, an enrichment of acetic acid bacteria was performed by adding 50 μl of undiluted sample to 10 ml of enrichment medium 1 and 2 broth [35] which was incubated aerobically for 3 days at 28°C. This enrichment culture was plated after serial dilution on four agar media for the isolation of acetic acid bacteria (Table S2). All media were supplemented with either 10 ppm cycloheximide or 20 ppm chloramphenicol to inhibit fungal or bacterial growth, respectively. After 7 days, colonies were picked randomly and subcultivated twice using the respective isolation conditions.

Third generation axenic isolates were used for protein extraction and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis for isolate identification as described earlier [36]. MALDI-TOF MS was performed for all isolates in duplicate using a Bruker Microflex® LT/SH s-Smart platform (Bruker Daltonics, Bremen, Germany). Mass spectra were

dereplicated into operational isolation units (OIUs), i.e., mass spectrometry-defined independent strains, using the SPeDE algorithm with default settings [37] and a single representative isolate per OIU (i.e., a “reference”) was selected for further identification analyses when needed (see below).

Identification of isolates

The obtained mass spectra were compared to the Bruker BDAL MSP database using MBT Compass Explorer software (Bruker Daltonics), and identifications were assigned based on a scoring system provided by the manufacturer. After dereplication, Bruker log scores ≥ 2.0 were considered to represent high-confidence identifications and therefore references with a log score ≥ 2.0 were considered identified at the species level. References with a Bruker log score < 2.0 were considered to represent low-confidence identifications and were further identified through sequence analysis. To this end, bacterial DNA was extracted by heating 1 μl loop of cell material in 20 μl of alkaline lysis buffer (0.25% (w/v) SDS and 0.05 M NaOH) for 15 min at 95°C. The resulting lysate was diluted with 180 μl of Milli-Q water and the suspension was collected after centrifugation for 5 min at 13,000 rpm at 4°C. The 16S rRNA gene was PCR amplified as described before using forward primer 5'-AGAGTTTGATCCTGGCTGAG-3' and reverse primer 5'-AAGGAGGTGATCCAGCCGCA-3' [38]. Yeast genomic DNA was extracted as previously described [39], and the 26S rRNA gene sequence was amplified using primers LROR and LR6 [40].

The resulting PCR products were purified using a NucleoFast 96 PCR clean-up kit (Macherey-Nagel, Eupen, Belgium) and were submitted for Sanger sequencing by a commercial provider (Eurofins Genomics, Ebersberg, Germany). Near-to-full length 16S rRNA gene sequences were obtained using the forward primers 5'-CTCCTACGGGAGGCAGCA GT-3' and 5'-AACTCAAAGGAATTGACGG-3' and the reverse primers 5'-GTATTACCGCGGCTGCTGGCA-3' and 5'-GTTGCGCTCGTTGCGGGACT-3'. 26S rRNA gene sequences were determined using the PCR primers [40]. Sequences were assembled using BioNumerics version 7.6 (Applied Maths, Sint-Martens-Latem, Belgium). Taxonomy of the isolates was determined based on the 16S and 26S rRNA gene consensus sequences using the EzBioCloud webserver [41] or the NCBI RefSeq-targeted loci database (<https://www.ncbi.nlm.nih.gov/refseq/targetedloci/>), respectively. References with 16S rRNA gene sequences that shared >98.65% sequence identity to only one taxonomic type strain were considered tentatively identified at the species level [42]. When 16S rRNA gene sequences shared <98.65% sequence identity with established type strains of a single genus or when multiple type strains of the same genus shared >98.65% sequence identity, isolates were considered

identified at the genus level only. A family level identification was given if 16S rRNA gene sequences shared <98.65% sequence identity with established type strains of different genera. Yeast isolates were identified at the species or genus level through 26S rRNA gene sequence analysis, based on the reported 1% intra-species divergence for ascomycetous yeasts [43]. 16S rRNA gene sequences of references were aligned and clustered using BioNumerics (Applied Maths). Clusters of references with 16S rRNA gene sequence similarity >98.65% were considered to represent the same species. All 16S and 26S rRNA gene sequences of references with a Bruker log score < 2.0 are provided in Table S3.

Results

Microbial community composition

For several samples, DNA extraction of individual compartments resulted in a low DNA yield (<1 ng/μl). As a consequence, amplicon sequencing could not be consistently performed for each of the examined compartments. In case the DNA yield was insufficient for both 16S rRNA gene and ITS2 amplicon sequencing, we prioritized amplicon sequencing of the former. This resulted in a total of 141 samples for 16S rRNA gene amplicon sequencing, and 52 samples for ITS2 amplicon sequencing (Table S1). Analysis of the raw data with DADA2 and data cleaning yielded 3,016,474 bacterial and 756,674 fungal high-quality reads attributed to 5763 and 285 ASVs, respectively.

The majority of the bacterial reads were assigned to the phylum Pseudomonadota (67.3%), followed by Actinobacteriota (14.7%), Bacillota (11.5%), Bacteroidota (3.5%), and Mycoplasmatota (2.5%). The bacterial community was dominated by *Wolbachia* ASVs and different *Wolbachia* ASVs prevailed in different bee hosts. *Wolbachia* ASV1 was dominant in *C. cunicularius*, *Wolbachia* ASV2 and ASV6 in *A. vago*, and *Wolbachia* ASV3 in *An. plumipes* (Fig. S1). Two *Spiroplasma* ASVs (ASV15 and ASV16) represented the dominant endosymbionts in *O. cornuta* samples whereas 2 other *Spiroplasma* ASVs (ASV58 and ASV61) were detected in *C. cunicularius*. *Wolbachia* ASVs in *O. cornuta* samples were either absent or detected in very low relative abundances. In addition to endosymbiont ASVs, dominant ASVs assigned to the genera *Cutibacterium*, *Streptomyces*, *Stenotrophomonas*, *Sphingobacterium*, *Staphylococcus*, *Streptococcus*, and *Enhydrobacter* were present in all four bee species. The prevalence of other dominant ASVs was host-specific. Figure 1 shows the 20 most abundant bacterial ASVs in samples for each solitary bee species. The relative abundance of the predominant bacterial ASVs without the endosymbiont ASVs is shown in Fig. S2.

The two most abundant fungal ASVs (ASV1 and ASV2 comprising 81.3% of the fungal reads) in each bee species were assigned to the genus *Meyerozyma* and were detected in all samples (Fig. 2). *Andrena vago* samples were additionally dominated by *Starmerella* and *Malassezia* reads, *An. plumipes* samples by *Metschnikowia* reads, *C. cunicularius* samples by *Metschnikowia*, *Starmerella*, *Cladosporium*, and *Malassezia* reads, and urban *O. cornuta* samples by *Metschnikowia* and *Starmerella* reads (Fig. 2).

Diversity across compartments within bee species

Bacterial richness differed significantly between the analyzed compartments of *A. vago* and semi-natural *O. cornuta*, as crop samples hosted a lower richness than midgut and hindgut samples. In contrast, the bacterial Shannon diversity was similar between the compartments of each host (Fig. S3). Both alpha diversity indices for the fungal communities were similar between compartments of each bee species (Fig. S4).

Furthermore, the bacterial communities differed significantly between the analyzed sections of each bee species (PERMANOVA tests, $p < 0.05$, Table S4), except for urban *O. cornuta*, while fungal communities within the compartments of only *A. vago* were significantly different ($R^2 = 0.57$, $p < 0.05$). Analysis of group dispersions showed that the bacterial data were heterogeneously dispersed for most bee species, whereas the fungal data were homogeneously dispersed (Table S4). Pairwise PERMANOVA tests demonstrated that the bacterial communities of crop samples differed significantly from midgut samples in *A. vago*, and the bacterial communities of the ovaries differed significantly from those of the gut sections in *An. plumipes* and semi-natural *O. cornuta* (Table S5). Pairwise PERMANOVA tests indicated that the fungal communities did not differ significantly between the compartments of each bee species (Table S5). The bacterial communities of the ovaries of *An. plumipes* and semi-natural *O. cornuta* clustered separately from the gut sections (Fig. S5), whereas no clear clustering by compartment was observed on the PCoA plots for the fungal community (Fig. S6).

Using DESeq2, bacterial and fungal ASVs that were differentially abundant within a compartment with respect to all other compartments were identified for each bee species. Bacterial ASVs were more abundant in ovary samples compared to the gut sections in *An. plumipes*, *C. cunicularius*, and semi-natural *O. cornuta* (Table S6). In the latter, two bacterial ASVs were also more abundant in the midgut. One *Starmerella bombicola* ASV was more abundant in the crop of urban *O. cornuta* compared to its other compartments (Table S7).

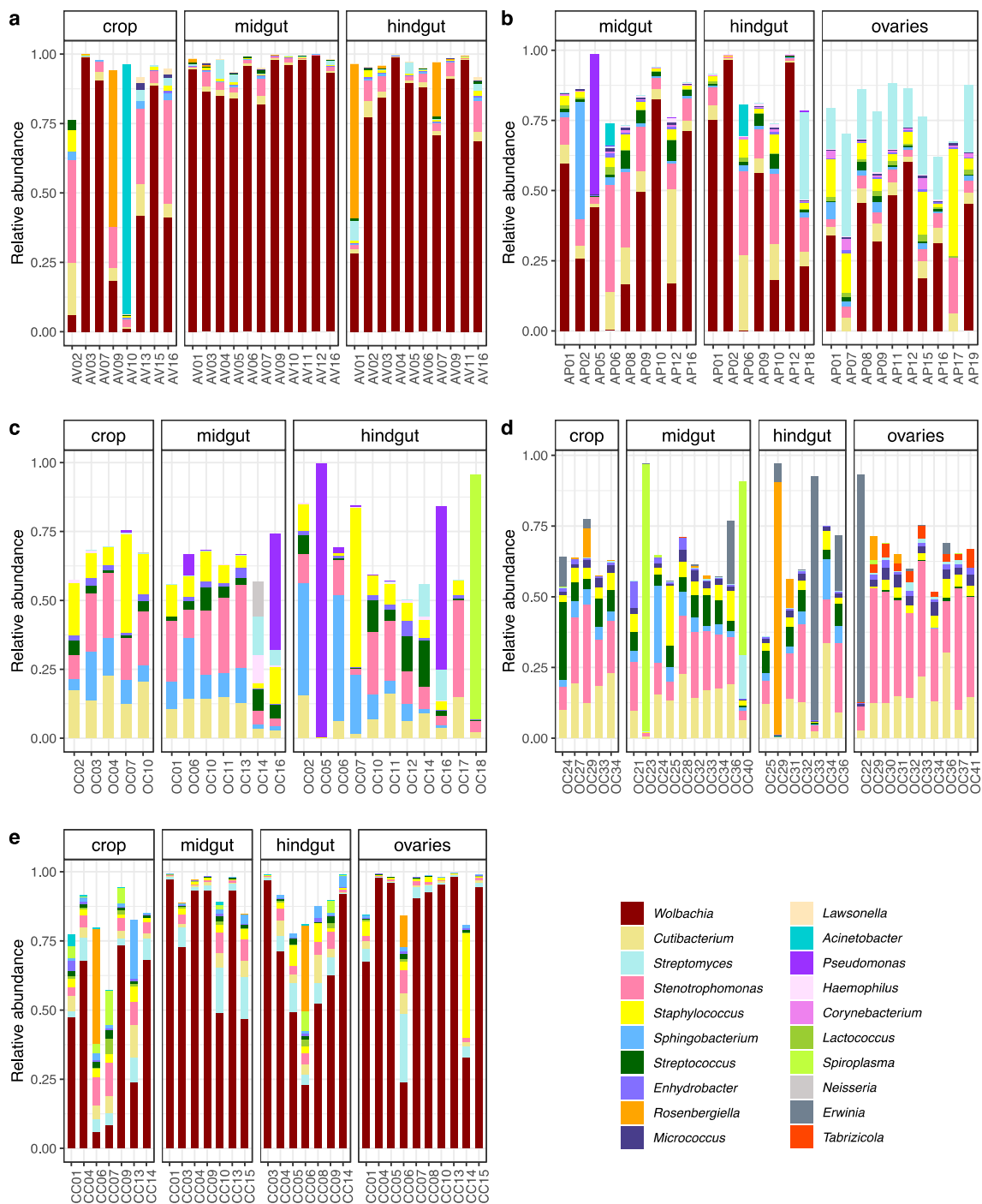


Fig. 1 Relative abundance of predominant bacterial ASVs in the crop, midgut, hindgut, and ovaries as revealed through 16S rRNA gene amplicon sequencing. The 20 most abundant ASVs of **a**

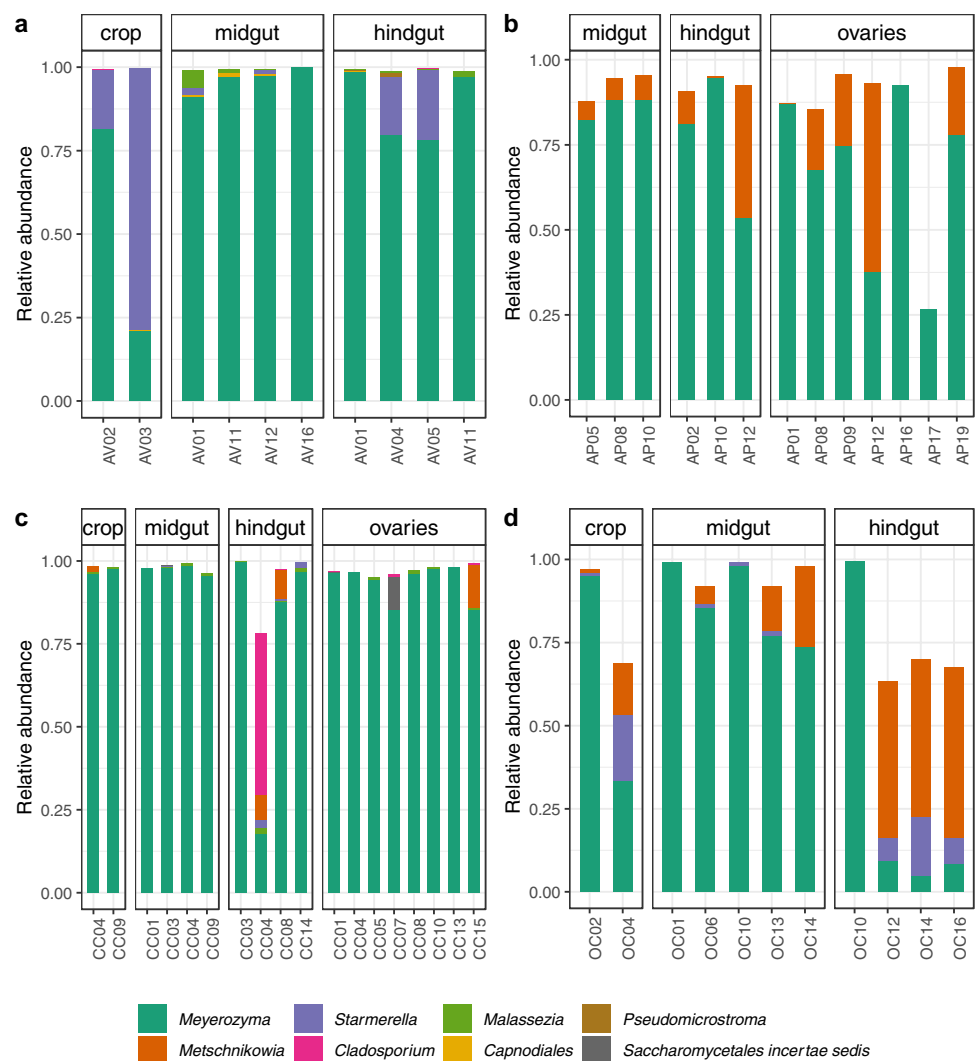
Andrena vaga, **b** *Anthophora plumipes*, **c** urban *Osmia cornuta*, **d** semi-natural *Osmia cornuta*, and **e** *Colletes cunicularius* are summarized at genus level

Diversity across bee species per compartment

Both bacterial richness and Shannon diversity of the crops, midguts, and ovaries differed between bee species (Fig. S7). Fungal richness and Shannon diversity of only the ovaries differed between *An. plumipes* and *C. cunicularius* (Fig. S8).

PERMANOVA analyses indicated that the bacterial communities within every compartment (all $p < 0.001$) and the fungal communities within the midguts ($R^2 = 0.32$, $p < 0.05$), hindguts ($R^2 = 0.40$, $p = 0.05$), and ovaries ($R^2 = 0.17$, $p < 0.01$) differed significantly between bee species (Table S4). The bacterial datasets were heterogeneously

Fig. 2 Relative abundance of predominant fungal ASVs in the crop, midgut, hindgut and ovaries as revealed through ITS2 amplicon sequencing. The 10 most abundant ASVs of **a** *Andrena vaga*, **b** *Anthophora plumipes*, **c** *Colletes cunicularius*, and **d** urban *Osmia cornuta* are summarized at genus level



dispersed, as well as the fungal data for hindgut and ovaries (Table S4), reducing the explanatory power of the PERMANOVA test. Pairwise PERMANOVA tests demonstrated that the compartment-specific bacterial communities also differed significantly between each host species (Table S5). Pairwise PERMANOVA tests demonstrated that the compartment-specific fungal communities were not significantly different between bee species, except for the fungal communities within the ovaries of *An. plumipes* and *C. cunicularius* (Table S5). PCoA ordinations confirmed that bacterial communities of each compartment clustered separately according to host species (Fig. S9), whereas the clustering by host species was less clear for the fungal community (Fig. S10). PCoA ordinations of the bacterial community without endosymbionts (Fig. S11) revealed a less apparent clustering per bee species compared to Fig. S9. Nevertheless, PERMANOVA tests revealed that the bacterial communities within every compartment remained significantly different between the analyzed bee species (all $p < 0.001$).

Significantly differentially abundant ASVs within a bee species compared to all other bee species were identified for each compartment (Table S6 and S7). *Wolbachia* ASV2 and ASV6 were more abundant in the three gut sections of *A. vaga*, whereas *Wolbachia* ASV3 was more abundant in all sections of *An. plumipes*. In addition, the ovary samples of *An. plumipes* included five *Metschnikowia* ASVs that were more abundant than in the ovary samples of *C. cunicularius* (the ovary fungal community of other species could not be determined). *Colletes cunicularius* hosted multiple bacterial and fungal ASVs that were more proportionally abundant within its compartments, with *Wolbachia* ASV1 being more abundant in every compartment. Only bacterial ASVs were more abundant in the *O. cornuta* compartments.

Diversity across *O. cornuta* populations

Pairwise PERMANOVA analyses showed that the bacterial communities of urban and semi-natural *O. cornuta* were

similar within each compartment (Table S5) which was confirmed by ordination on PCoA plots as urban and semi-natural samples clustered together (Fig. S9). Yet, bacterial ASVs that were differentially abundant in the two *O. cornuta* populations were identified using DESeq2 (Table S6). In the crop samples, two *Brevibacterium* ASVs (ASV51 and ASV75) were more abundant in semi-natural *O. cornuta*, while one *Stenotrophomonas* ASV was more abundant in the urban samples. In the midgut, five differentially abundant ASVs were identified, and all were more abundant in the semi-natural location, i.e., two *Spiroplasma* ASVs, one *Staphylococcus* ASV, and two *Brevibacterium* ASVs (ASV51 and ASV75). *Brevibacterium* ASV51 and ASV75 and one *Erwinia* ASV were more abundant in the hindgut of semi-natural *O. cornuta*, whereas four ASVs were more abundant in urban *O. cornuta* hindguts (i.e., *Staphylococcus*, *Corynebacterium*, *Streptococcus*, and *Pseudomonas*). *Brevibacterium* ASV51 and ASV75 were consistently more abundant in all gut compartments of semi-natural *O. cornuta*.

Isolation of the microbial community

A total of 1510 isolates were collected using a diverse range of isolation conditions (Table S2) and were dereplicated into 546 OIUs. References of 241 OIUs had Bruker log scores ≥ 2.0 and were considered identified at the species level, while references of 305 OIUs with Bruker log scores < 2.0 were further identified via comparative sequence analysis (Table S3). The identification result for each reference was subsequently applied to all isolates within the same OIU (Table S8).

Isolates assigned to 55 genera and 128 species were obtained (Table S8 and Fig. 3). Bacterial isolates belonged to 51 genera and represented three phyla, i.e., Bacillota (30.9%), Pseudomonadota (29.3%), and Actinobacteriota (26.7%), whereas the four yeast genera belonged to the phylum Ascomycota (13.2%) (Fig. 4). Over half of the isolates were attributed to only five genera, i.e., *Cutibacterium* ($n=254$), *Pseudomonas* ($n=207$), *Pantoea* ($n=143$), *Bacillus* ($n=136$), and *Staphylococcus* ($n=97$) (Fig. 4). While *Cutibacterium* and *Pantoea* were represented by only two species each, other predominantly isolated genera, such as *Bacillus* (seven spp.), *Paenibacillus* (eight spp.), *Pseudomonas* (eight spp.), *Staphylococcus* (eleven spp.), and *Streptomyces* (twelve spp.) showed a high species diversity (Table S8).

The highest number of species were isolated from *C. cunicularius* ($n=44$), *An. plumipes* ($n=43$), and semi-natural *O. cornuta* ($n=43$), while *A. vaga* yielded 31 species and urban *O. cornuta* only 21 species. *Cutibacterium* was the most dominantly isolated genus and represented major fractions of *An. plumipes*, *C. cunicularius*, and semi-natural *O. cornuta* isolates (Fig. S12). *Pantoea* spp. were only isolated from *An. plumipes*, whereas *Lactococcus garvieae* was isolated from semi-natural *O. cornuta* only. *Micrococcus* and *Rosenbergiella* were predominantly isolated from *Colletes cunicularius*. *Rosenbergiella* was also dominantly isolated from semi-natural *O. cornuta* samples. *Bacillus* spp., *Enterococcus faecalis* and *Cytobacillus horneckiae* were most dominantly isolated from *A. vaga*, whereas *Pseudomonas* spp. were dominantly isolated from urban *O. cornuta* samples. *Cutibacterium*, *Bacillus*, *Staphylococcus*, *Streptomyces*, and *Paenibacillus* were the genera isolated from every

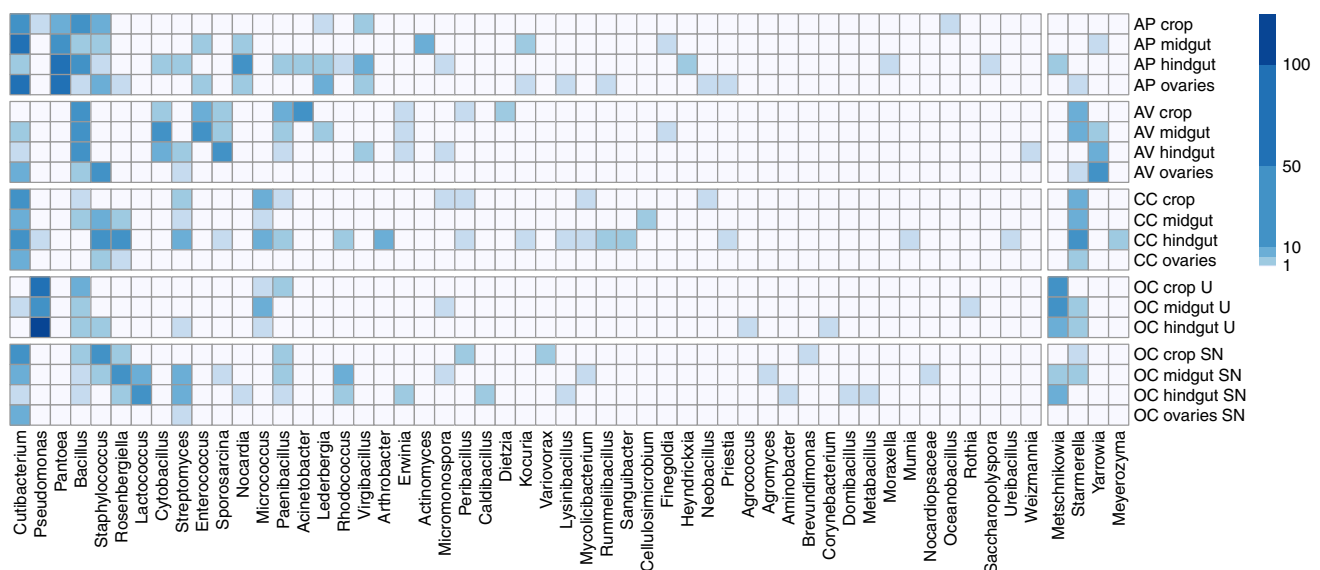


Fig. 3 Distribution of the isolated genera over the analyzed bee species and their compartments. Bacterial and yeast genera are arranged from the highest number of isolates to the lowest. AP, *Anthophora*

plumipes; AV, *Andrena vaga*; CC, *Colletes cunicularius*; OC, *Osmia cornuta*; U, urban; SN, semi-natural

Fig. 4 Taxonomic distribution of the 1510 isolates over bacterial and fungal genera and corresponding phyla: **A**, Bacillota; **B**, Pseudomonadota; **C**, Actinobacteriota; **D**, Ascomycota. The different species isolated per genus can be found in Table S8



bee species (Fig. 3). *Metschnikowia* was the most dominantly isolated yeast genus (Fig. 4), whereas *Starmerella* was isolated from every bee species (Fig. 3).

Discussion

Microbial community of the analyzed solitary bees

16S rRNA gene amplicon sequencing revealed that the bacterial communities of the solitary bees *A. vaga*, *An. plumipes*, *C. cunicularius*, and *O. cornuta* were dominated by endosymbionts (Fig. 1). Endosymbiont infection is common in insect hosts and *Wolbachia* and *Spiroplasma* are the most frequently detected endosymbiotic bacteria [44], as confirmed in the present study (Fig. 1). The four dominant

Wolbachia ASVs were each associated with a certain bee host: *Wolbachia* ASV1 occurred only in *C. cunicularius*, *Wolbachia* ASV2 and ASV6 in *A. vaga*, and *Wolbachia* ASV3 in *An. plumipes* (Fig. S1). The detection of different *Wolbachia* ASVs in different host species and the degree of sequence divergence between *Wolbachia* ASVs of different bee species (98.0–99.7%) likely suggest the occurrence of different *Wolbachia* species in different solitary bee hosts. *Wolbachia* is typically associated with the host reproductive system, but also occurs in other tissues [45]. It is mostly vertically transmitted to offspring which might have aided in the coevolution of some *Wolbachia* species with their host [46, 47]. The role of *Wolbachia* infection in solitary bees is unclear. In other insects both negative and positive effects have been reported, including male feminization or killing and cytoplasmic incompatibility, and the provision

of essential nutrients and defense against pathogens [48]. *Spiroplasma* was the second most dominant endosymbiont and again different ASVs occurred in *C. cunicularius* and *O. cornuta*. Previous studies of pathogens revealed the occurrence of *Spiroplasma* in the solitary bees *Osmia bicornis* [49], *Osmia cornifrons*, *Andrena*, and *Anthophora* species [50]. *Spiroplasma* may cause several diseases, mortality, or gender-ratio distortions in progeny [51], or were shown to provide pathogen protection [52, 53]. Other insect endosymbionts, such as *Arsenophonus*, *Cardinium*, *Rickettsia*, and *Sodalis* [44], were either absent or were detected in very low relative abundances.

In addition to endosymbionts, a diverse set of environmental microorganisms dominated the solitary bee gut microbiota (Fig. 1 and Fig. 3), as reported in previous studies of solitary bees [7, 10, 12, 17, 18, 20–24]. Several of these predominant bacteria such as *Streptomyces* [54], *Stenotrophomonas* [55], *Sphingobacterium* [56], *Enhydrobacter* [57], *Pseudomonas* [58], *Bacillus* [59], and *Pantoea* [60] primarily occur in environmental samples such as soil, water, and plants. Others are common members of human and animal microbiomes but have also been detected in environmental sources. These include *Cutibacterium* which occurs as an endophyte in grapevine and plant seeds [61–63], *Staphylococcus* which can commonly be detected in water and soil samples [64], and *Streptococcus* which occurs in plants [65]. Also, the predominant fungi (Fig. 2) could be linked to environmental sources and had also previously been reported in other insect species. *Meyerozyma* yeasts have been associated with various insects [66]. *Metschnikowia* and *Starmerella* commonly inhabit flowers and flower-visiting insects such as bees, flies, and beetles [67–69]. *Malassezia* has been reported in the olive fruit fly [70] but occurs in a broad diversity of habitats and can be pathogenic [71] and *Cladosporium* fungi are mostly saprobic, are commonly found on soil and plant material, but some can also act as plant and animal pathogens [72].

We provided an inventory of the cultivable microbiota from the different compartments of each analyzed bee species (Fig. 3 and Table S8). Large-scale cultivation studies can provide an image of the gut microbiota that is complementary to that obtained through amplicon sequencing and can provide a more accurate identification and reveal a higher species-level diversity [40, 73–76]. Moreover, the cultures provided by such studies can be used for genome and biochemical analyses to elucidate the functional roles of dominant members of the gut microbiota. Isolates identified as *Cutibacterium*, *Streptomyces*, *Staphylococcus*, *Pseudomonas*, *Lactococcus*, *Acinetobacter*, *Micrococcus*, *Rosenbergiella*, and *Erwinia* represented most of the dominant ASVs. However, isolates corresponding to other dominant ASVs such as *Wolbachia*, *Spiroplasma*, *Stenotrophomonas*, *Sphingobacterium*, *Streptococcus*, and *Enhydrobacter* were

not obtained. While species of the endosymbiotic genera *Wolbachia* and *Spiroplasma* require highly specific isolation conditions [77, 78], species of the other genera should be cultivable on the media used. In contrast, some genera such as *Bacillus* and *Pantoea* were isolated in high numbers (Fig. 4), but were not detected among the most predominant ASVs, while *Nocardia*, *Enterococcus*, *Sporosarcina*, and *Paenibacillus* ASVs were only detected at a relative read abundance lower than 0.1%. We used multiple media for the isolation of lactic acid bacteria, bifidobacteria, and acetic acid bacteria, including an enrichment for the latter group. However, neither bifidobacteria nor acetic acid bacteria were isolated, which corresponded to the sporadic detection of such ASVs, and only a few lactic acid bacteria were isolated, i.e., *Lactococcus garvieae*, *Enterococcus faecalis*, and *Enterococcus faecium*. All four isolated yeast genera, i.e., *Metschnikowia*, *Starmerella*, *Yarrowia*, and *Meyerozyma*, were detected through amplicon sequencing as predominant ASVs. However, the dominance of *Meyerozyma* as revealed through amplicon sequencing was not reflected in the number of yeast isolates, as only two *Meyerozyma guilliermondii* isolates were obtained (Fig. 4). No *Cladosporium* and *Malassezia* isolates were retrieved. The former, an environmental mold, was not isolated as we targeted only yeast species, while the latter requires specific culture media [71].

As described above, most dominant gut microbes detected or isolated in the present study have been reported in flowers, soil, or plant material. Flowers provide nectar and pollen to solitary bees, which furthermore use soil and plant material for nesting or nest construction [2]. This likely ensures a continuous transmission of microorganisms from these environmental sources to the bee gut. Yet, our data showed that the bacterial communities differed between the analyzed host species (Table S4, Table S5, and Fig. S9), reinforcing a host-specific selection process recently described as microbial filtering [79]. Architecture of the local plant-bee network, phylogenetic predisposition and specialization of bee foraging, and microbial filtering by plant and bee hosts were suggested by Keller and colleagues as the three components driving the microbiomes associated with bee hosts [79]. The former two elements shape the transmission of microbes between environmental sources and bees, whereas both floral and host traits filter the incoming microbes which impacts their further transmission in the network [79]. Life history traits of the host, but also its innate immune system and gut conditions may influence either elimination or selection and proliferation of some of the acquired microbes leading to a host-specific gut microbiota [79, 80]. To our knowledge, the gut microbiome of adult solitary bees within the genera *Andrena*, *Anthophora*, and *Colletes* was not reported before. Conversely, the gut microbiota of several other *Osmia* species were studied before: *Apilactobacillus michenieri* dominated in *Osmia chalybea* and *Osmia subfasciata*

[7], *Acinetobacter* and *Erwinia* in *Osmia lignaria* [21], and Pseudomonadaceae, Moraxellaceae, and *Spiroplasma* OTUs in *Osmia brevicornis* and *Osmia caerulea* [81]. These *Osmia* species were therefore all dominated by bacterial taxa that differed from the dominant taxa detected in *O. cornuta* in the present study (Fig. 1 and Fig. S2), thus reinforcing host-specificity of the gut microbiota of solitary bees. Unlike in social bees, the microbiota in the gut sections of solitary bees were not commonly studied, except for one study in the carpenter bee *Xylocopa tenuiscapa* [82]. The variation in bacterial community between the compartments observed in this study (Table S4) corresponded with the observation of a compartment-specific bacterial community in social bees [83, 84] and in *X. tenuiscapa* [82]. We observed similar alpha diversity indices between the compartments (Fig. S3) as recently demonstrated in the honey bee [83], but our data differed from the higher diversity observed in the hindgut of *X. tenuiscapa* [82].

Plant-bee network characteristics can differ between environments. As a result, the microbiomes of bee species can vary by habitat, as observed in previous studies with bumble bees and pollen provisions of solitary bees [14, 85, 86]. In the present study, however, bacterial communities in semi-natural and urban *O. cornuta* samples were overall similar (Table S5). Yet, several differentially abundant ASVs were detected between the two *O. cornuta* populations (Table S6). Remarkably, two *Brevibacterium* ASVs were consistently more abundant in all gut sections of semi-natural *O. cornuta*. Although *Brevibacterium* can be found in soil and nectar of apple blossoms [87, 88], which likely explains its occurrence in *O. cornuta*, it is unclear how the semi-natural location contributes to a higher abundance of *Brevibacterium*.

The dominant gut microbiota are known for their metabolic versatility

Gut symbiont bacteria are generally considered to shape bee fitness as they provide functions that include pathogen inhibition, degradation of pollen and toxic compounds, and immune activation [4, 80, 84]. Several of the dominant genera detected in the present study have the potential to fit this paradigm as they are well-known for their remarkable metabolic versatility.

No less than 45 Actinobacteriota species, including 12 *Streptomyces* and 2 *Cutibacterium* spp. were isolated in the present study, and both genera also represented the two most abundant non-endosymbiont ASVs (Fig. 1). Actinobacteriota species commonly produce many secondary metabolites and degrade complex biopolymers, two functions that are beneficial to the insect host [89]. Streptomyces are well-known antimicrobial producers, and some *Streptomyces* species are beneficial symbionts in insects, providing protection against pathogens through antibiotic production [90, 91].

Likewise, *Cutibacterium acnes* was recently identified as a core gut bacterium of a marine worm providing its host with essential nutrients [92].

Bacillus, *Pseudomonas*, *Stenotrophomonas*, and *Sphingobacterium* species have a high genetic and metabolic diversity and are well-known producers of antimicrobials, toxins, lytic enzymes, and volatile organic compounds that can inhibit or interfere with other organisms [55, 58, 59]. While some bacilli are insect pathogens [93], bee-associated *Bacillus* isolates showed enzymatic activity against carbohydrates, proteins, and lipids [20, 94] and have been reported to degrade pectin [40]. *Pseudomonas* bacteria are well-known for their catabolic and biocontrol potential [58] and have been reported in several bee studies [6, 24], yet their specific function remains to be elucidated. The eight *Pseudomonas* species isolated in the present study may suggest a variety of functions or reveal functional redundancy. *Stenotrophomonas* or *Sphingobacterium* have well-documented capacities for degrading pollutants and other xenobiotics, including a neonicotinoid insecticide [95].

Meyerozyma, the most predominant yeast detected through ITS2 amplicon sequencing, has various beneficial potentials including inhibition of plant pathogens and bioremediation [96–98]. Whereas *Metschnikowia* and *Starmerella*, the two most dominantly isolated yeasts, may be involved in protection against pathogens through production of antimicrobial compounds [99–101].

Conclusion

The bacterial and fungal communities of the crop, midgut, hindgut, and ovaries of four common solitary bees in Belgium were identified through cultivation-dependent and independent approaches and were dominated by endosymbionts of the genera *Wolbachia* and *Spiroplasma* and environmental bacteria and yeasts. While only a single sampling site was examined for *Andrena vaga*, *Anthophora plumipes* and *Colletes cunicularius*, and two sampling sites for *Osmia cornuta*, especially the bacterial communities varied between compartments and bee species. While the present study was not configured to provide absolute abundances of symbiont species, our findings reinforced the recent understanding of host-specific gut microbiota in solitary bees. Metabolic versatility appeared as a hallmark of many of these gut symbionts. Whether they exert similar functionalities within the solitary bee gut and thus functional redundancy remains to be elucidated.

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Author contribution AH, IM, and PV conceptualized the study. NJV, DM, GS, and PV acquired the funding. NJV provided the study

material. AH, MC, and AG performed the experiments. AH and MJ performed the data analysis. AH and PV wrote the original draft of the manuscript. All authors reviewed and contributed to the final version of the manuscript.

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Data availability All 16S rRNA and ITS2 amplicon sequencing data generated for the present study are archived at the European Nucleotide Archive under BioProject accession numbers PRJEB60082 and PRJEB60083, respectively.

Declarations

Ethical approval No approval of research ethics committees was required.

Competing interests The authors declare no competing interests.

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