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RESEARCH

Chironomus riparius Larval Gut Bacteriobiota and Its Potential in Microplastic Degradation

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Abstract

Chironomus riparius are sediment-dwelling invertebrates in freshwater ecosystems and are used as indicators of environmental pollution. Their habitat is threatened by high levels of contaminants such as microplastics and organic matter. A promising strategy for the eco-friendly degradation of pollutants is the use of bacteria and their enzymatic activity. The aim of this study was to characterize for the first time bacteriobiota associated with the gut of *C. riparius* larvae from nature and laboratory samples, to compare it with sediment and food as potential sources of gut microbiota, and to assess its ability to degrade cellulose, proteins, and three different types of microplastics (polyethylene, polyvinyl chloride, and polyamide). The metabarcoding approach highlighted *Proteobacteria*, *Firmicutes*, *Bacteroidota*, and *Actinobacteriota* as most abundant in both gut samples. Culturable microbiota analysis revealed *Metabacillus idriensis*, *Peribacillus simplex*, *Neobacillus cucumis*, *Bacillus thuringiensis/toyonensis*, and *Fictibacillus phosphorivorans* as five common species for nature and laboratory samples. Two *P. simplex* and one *P. frigoritolerans* isolates showed the ability for intensive growth on polyethylene, polyvinyl chloride, and polyamide. Both cellulolytic and proteolytic activity was observed for *Paenibacillus xylanexedens* and *P. amylolyticus* isolates. The characterized strains are promising candidates for the development of environmentally friendly strategies to degrade organic pollution and microplastics in freshwater ecosystems.

Keywords *Chironomus* · Metabarcoding · Larval gut bacteriobiota · Biodegraders · Microplastics

Introduction

The genus *Chironomus* is comprised of members that are widely distributed in freshwater aquatic ecosystems. Their life cycle includes complete metamorphosis with four stages: egg, larva, and pupa, which develop in water, and adults as short-living aerial forms [1]. During the larval stage, *Chironomus* are sediment-dwelling and exposed to various stressful environmental conditions, including temperature, salinity, desiccation, pH, toxic substances, ultraviolet light (UV), and gamma radiation, to which they are highly resistant [2]. One of the most important components that enable Chironomids to survive in harsh conditions is certainly their microbiota, which plays an important role in the defense of insects under various stress conditions [1, 3]. Endosymbiotic microorganisms are often essential for the growth and development of insect species as they are involved in the reproduction, supply of essential nutrients, and digestion [4]. Recently, the microbiomes of *C. transvaalensis*, *C. ramosus*, and *C. sancticaroli* gut microbial community of larvae have been described [2, 3, 5], but to the best of our knowledge, the microbiome of *C. riparius* from natural habitat still has not been reported.

As the natural habitat of *Chironomus* spp., freshwater ecosystems are of immense importance to humans and biodiversity, but they are threatened by high levels of pollution originating from a variety of sources, including municipal, industrial and, agricultural wastes, sewage and nutrient runoff, energy production, and heavy industry [6]. One of the pollutants impacting freshwaters through these sources that has recently become to the focus of attention is

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microplastics. These ecosystems are affected by microplastic pollution worldwide, which negatively impacts aquatic biota [7]. The small dimensions and slow biodegradation rate facilitate microplastic ingestion by organisms and eventually spread through food webs [8]. Synthetic plastics are widely used, and their accumulation as a consequence of poor recycling and low circular use is a major threat to human and environmental health. According to Plastics Europe [9], the polymers most commonly used for plastics production are polyethylene (PE), polyvinyl chloride (PVC), and polyamide (PA), which belong to the group of thermoplastics and are the most frequently detected as pollutants. Recently, it was confirmed that *C. riparius* can be used as a suitable indicator of microplastic pollution in an aquatic ecosystem [7]. They are highly effective biondicators, as chironomids are among the most abundant non-selective feeders in the sediments of aquatic ecosystems and constitute a significant proportion of the macroinvertebrate community in polluted areas [10]. In addition, *C. riparius* is routinely used in bioassays and toxicity tests as an important tool to evaluate the level of response to environmental pollution stressors at the biochemical and physiological levels [11–13].

Several studies have examined the effects of microplastic ingestion on the gut of *C. riparius* larvae. At high concentrations of microplastics, the intestinal epithelium is damaged, triggering proteolysis, and the inflammation process is initiated followed by the activation of the phenoloxidase system [14]. Stanković et al. [7] reported that environmentally relevant concentrations of the microplastic mixture, including PVC and PA, have a negative impact by disrupting the developmental processes of *C. riparius* and cause deformities o female wings and larval mandibles and mentums. Ingestion of small-sized PE was also found to negatively affect life-history traits [15] and induce oxidative damage in *C. riparius*, making it an early indicator of acute stress caused by microplastic pollution of aquatic ecosystems [16].

The accumulation of microplastics is a global problem that affects the planet's health. Therefore, it is of great importance to find ecologically appropriate measures to reduce microplastics to create a healthier environment. A promising strategy for the degradation of plastics is using microbial activity or their enzymes to depolymerize plastics into monomers or mineralize them to water, carbon dioxide, and new biomass [17]. Microbiomes of invertebrates have recently been recognized as possible sources of plastic-degrading bacteria. The invertebrates first break down larger pieces of plastic into smaller ones, which increases the surface area where microorganisms from the gut can now attach and degrade the plastic [18]. For instance, *Bacillus* sp. has been identified as the bacterium responsible for the degradation of polyethylene in Indian mealworms [19, 20]. Bacterial representatives within the genera *Citrobacter* and *Kosakonia* were identified as the main degraders for polystyrene and polyethylene in mealworm guts [21]. In addition to microplastics, organic pollution is a common problem for aquatic ecosystems coming from domestic, agricultural, and industrial wastewater and characterized by high levels of cellulose and proteins [22, 23]. Protease and cellulase-producing bacteria play an important role in the degradation of organic matter in aquatic ecosystems [24]. They could be used as a strategy to degrade organic pollution in an environmentally friendly manner and to improve the quality of freshwater ecosystems.

Considering *C. riparius* larvae as a member of a macrodetritivore community with a lifestyle as deposit feeders, their associated gut microbiota could be investigated as a potential source of microplastic biodegraders. This study aimed to characterize for the first time the gut microbiota associated with *C. riparius* larvae from a river habitat and a laboratory culture, to compare it with sediment and fish food as potential sources of microbiota colonization and to further evaluate its ability to degrade different substrates, including cellulose, proteins, and three different types of microplastics (PE, PVC, and PA). The data obtained could be further used to develop environmentally friendly strategies for the degradation of organic and microplastic pollution in freshwater ecosystems.

Material and Methods

Chironomid Sampling and Identification

C. riparius larvae were collected from the natural habitat (Nišava River, Serbia) and from the stock culture at the laboratory of the Faculty of Science and Mathematics, University of Niš (Niš, Serbia), which were reared under the Organisation for Economic Co-operation and Development [25] guideline. Samples from the natural habitats were sorted and identified up to species level using a morphology-based key [26]. From each habitat were finally obtained five replicas, each containing pulled ten guts dissected from *C. riparius* larvae. Samples were stored in Luria–Bertani (LB) medium supplemented with glycerol (final concentration 20%) at − 20 °C until further use.

To validate the morphology-based identification, DNA was extracted using the QIAGEN Dneasy® Blood & Tissue Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol. The barcoding region of the mitochondrial cytochrome oxidase subunit I (COI) gene was amplified using the universal primers LCO1490 and HCO2198 [27]. The PCR reaction was carried out in a volume of 20 µL, including 1 µL of extracted DNA, 11.8 µL H_2O, 1 µL of each primer 0.5 µM, 2 µL High Yield Reaction Buffer A with 1 × Mg, 1.8 µL of $MgCl_2$ 2.25 mM, 1.2 µL of dNTP 0.6 mM, 0.2 µL DNA polymerase 0.05 U/µL. The amplification

protocol included the following: (I) initial denaturation for 5 min at 95 °C; II) 35 cycles of 1 min at 94 °C, 1 min at 54 °C, and 30 s at 72 °C; and (III) final extension at 72 °C for 7 min. Amplified products were run on 1% agarose gel, stained with Midori Green Advanced DNA safe stain (NIPPON Genetics Europe, GmbH, Düren, Germany), and visualized under a UV transilluminator. Barcoding COI fragments were sequenced using automated equipment (Macrogen Europe, Amsterdam, Netherlands). The obtained sequences were manually checked and trimmed using FinchTV chromatogram viewer (Geospiza, Inc.) and compared to the closest ones in the National Center for Biotechnology Information (NCBI) database using the BLAST function method (Basic Local Alignment Search Tool). The analyzed sequence matched with the *Chironomus riparius* vouchers in the GenBank with a percentage of identity over 99.5% in more than ninety cases, and one case with 100% identity. Therefore, the previously identified specimen of *Chironomus riparius* based on the morphological characters was confirmed also on the molecular level.

Isolation of Microorganisms

From obtained larval gut samples, 50 µL of suspension was plated in duplicate on LB agar plates (LA; composition g/L: tryptone 10.0, yeast extract 5.0, sodium chloride 5.0, agar 15.0), De Man, Rogosa, and Sharpe agar (MRS; Lab M, UK), minimal medium (MM; K_2HPO_4 10.5 g, KH_2PO_4 4.5 g, $(NH_4)SO_4$ 1 g, Na-citrate × $2H_2O$ 0.42 g, 20% $MgSO_4$ 0.5 mL, thiamine (5 mg/mL) 0.4 mL, 40% glucose 5 mL, agar 15.0), King's B agar (King's medium B base 43 g, Glycerol 10 mL) and glucose yeast calcium carbonate (GYC; glucose 50.0, yeast extract 10.0, $CaCO_3$ 20.0, agar 20.0) growth media. One set of plates was incubated aerobically for 48 h at 30 °C. In parallel, the second set was grown in an Anaerobic jar (Schuett-biotec GmbH, Göttingen, Germany) supplemented with AnaeroGen™ sachets (Thermo Fisher Scientific, Waltham, MA, USA) thus the anaerobic conditions have been achieved. Pure cultures of the selected bacterial isolates were maintained in LB glycerol stocks at − 20 °C until further use.

Molecular Identification of Bacterial Isolates

The total genomic DNA of bacterial isolates was extracted as described earlier [28]. After overnight culturing on LB agar plates at 30 °C, a full loop of bacterial cultures was harvested and washed in TE buffer (10 mmoL L^{-1} Tris, pH 8; 1 mmol L^{-1} EDTA). Then, the pellet was re-suspended in 500 µL of lysis buffer (50 mmoL L^{-1} Tris, pH 8; 1 mmoL L^{-1} EDTA, pH 8; 25% sucrose) which contained 200 µg/mL final concentration of lysozyme (Serva GMBH, Heidelberg, Germany) for DNA isolation of Gram-positive isolates. Gram-negative isolates were re-suspended in 567 µL of TE buffer with 100 µg/mL final concentration of proteinase K (Sigma, Sent Louis, USA) in 0.5% sodium dodecyl sulfate (SDS). After 30 min of incubation at 37 °C, 100 µL of 5 M NaCl was added to the samples and further treated with 300 µL 3% (w/v) CTAB + PVP buffer. The mixture was incubated at 65 °C for 10 min, followed by chloroform extraction. The DNA was precipitated by cold isopropanol and ethanol and re-dissolved in 50 µL of TE buffer containing 1 µL of RNase (10 mg/mL). Molecular identification of bacterial isolates was performed by amplifying partial 16S rRNA gene sequence with universal primers fD1Funi–16SF (5′-AGAGTTTGATCCTGGCTCAG-3′) and rP2Runi–16SR (5′-ACGGCTACCTTGTTAGGACTT-3′). PCR amplifications were performed in a 25 µL reaction mixture containing 1 µg of template DNA, 1 µL of each primer, 12.5 µL of FastGene Taq ready mix with dye (NIPPON Genetics Europe, GmbH, Düren, Germany), and PCR H_2O to final volume. The PCR reactions were performed as usual with primer annealing at 54 °C for 40 s. The PCR products were purified using the Euroclone spinNAker Gel&PCR DNA Purification Kit (Milano, Italy) according to the manufacturer's protocol, and later sequenced by Eurofins Genomics Europe Sequencing GmbH (Cologne, Germany). PCR amplicons were sequenced using the 907R-16S primer (5′-CCGTCA ATTCMTTTRAGTTT-3′). All sequences were searched for homology using BLAST within NCBI GenBank Nucleotide collection 16S database. All sequences were aligned and manually checked with reference strain sequences from the GenBank database using CLUSTAL W implemented in BioEdit 7.2.6 software, while phylogenetic trees were constructed in MEGA X software using the Neighbor-joining method based on a pairwise distance matrix with the Kimura two-parameter nucleotide substitution model.

Amplicon Sequencing of Unculturable Bacteriobiota

Total DNA was isolated from the samples each containing ten guts dissected from *C. riparius* larvae. Also, sediment samples collected in natural habitat and commercial TetraMin® fish food flakes mixture were included in metabarcoding analysis. Extraction was performed using the ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. The DNA was quantified using Qubit fluorometric quantitation (Qubit 4 fluorometer, Invitrogen™, Waltham, MA, USA). Thereafter, the DNA samples were commercially sequenced by Novogene Co., Ltd. (Beijing, China) using a 2 × 300-bp paired-end run on a MiSeq Sequencer, according to the manufacturer's instructions (Illumina, San Diego, CA, USA). The 16S rRNA gene-specific sequences to target the V3 and V4 regions were used in this study, with the defined

forward (5′-CCTACGGGNGGCWGCAG-3′) and reverse (5′-GACTACHVGGGTATCTAATCC-3′) primers [29].

Reprocessing, Sequence Inference, Taxonomy Annotation, and Statistical Analysis

Data analysis was conducted using an ad hoc DADA2 R package pipeline for denoising, paired-end joining and chimera depletion, starting from the paired-ends data [30]. All sequences having more than 2 for forward and 2 for reverse strand expected errors (calculated as sum(10^(-Q/10))—where Q is the quality score) were discarded (argument: maxEE = c(2, 2)) as well as sequences shorter than 150 nt. Additionally, sequence pairs with a minimum overlap of 12 nt were merged. Taxonomic affiliations were assigned using the RDP Naive Bayesian classifier [31] with an assignment to the SILVA 138. Taxonomy assignment up to the genus level was performed using IDTAXA [32] with default options. For species-level annotation, exact sequence matching was used using the species assignment SILVA 138 set (https://zenodo.org/record/3986799). In addition, amplicon sequence variants (ASVs) with high abundance and ambiguous taxonomy assignments were annotated based on the BLAST best hit in the National Center for Biotechnology Information (NCBI) nucleotide database up to the species level of annotation. Sequences assigned to chloroplasts and mitochondria were excluded from further analysis.

Alpha diversity of bacterial communities was determined by analysis of phylum, family, genus, species, and ASV level and was shown through estimators of the Shannon, Simpson, and Fisher indices. Observed and estimated richness was determined according to the following estimators: observed (OBS), Chao1, and ACE. The Wilcoxon rank-sum test was used for testing significance among the two groups of samples and the p-values obtained were adjusted for multiple comparisons using Benjamini and Hochberg method. P-adjusted values < 0.05 were considered significant. Beta diversity was performed using Double Principle Coordinate Analysis (DPCoA). The betadisper test was used to check if the two groups have significant compositional dissimilarity homogeneity of group dispersions. Analysis of sums of squares using distance matrices was performed by permutational multivariate analysis of variance (PERMANOVA). Also, p-adjusted values < 0.05 were considered significant. Differential abundance testing was performed using prevalence-filtered taxa aggregated to the genus level with DESeq2 [33]. To estimate differentially abundant taxa a log2 fold change threshold of 2 was used, and for p-adjustment, Benjamini and Hochberg [34] methods were applied using values of $p < 0.01$ considered as statistically significant. Furthermore, to explain the differences in taxonomic composition between CL and CN samples differential abundance testing was performed. The package Metacoder was used to convert the genus aggregation counts to per-taxon counts for ranks from kingdom to genus level and the obtained matrix was used to visualize differentially abundant representatives over mentioned taxonomic ranks.

All data were deposited within the NCBI database as BioProject ID: PRJNA89878.

Enzymatic Assays

Qualitative Determination of Exoenzyme Production

The hydrolytic activity of isolates was monitored on a growth medium with an appropriate substrate for each enzyme. On each plate was spotted 5 µL overnight cultures. Proteinase activity detection was performed on a plate with the following composition per liter of distilled H_2O: nutrient broth 8.0, NaCl 4.0, gelatine 4.0, and agar 10.0. After incubation at 30 °C, plates were overflowed with a 1% solution of tannic acid and strains with a clear halo around colonies were considered to be producers of proteinase. Cellulase production was detected on plates containing tryptone 10.0, yeast extract 5.0, NaCl 5.0, carboxymethyl cellulose 10.0, and agar 15.0 per liter of distilled H_2O [35]. After incubation at 30 °C, plates were overflowed with 0.1% solution of Congo red, incubated for 15 min, and washed with 1 M solution of NaCl several times. Strains with a halo around colonies were considered cellulase producers. Each experiment was performed in triplicate. Enzymatic activity was calculated according to the formula $\text{Enzymatic index (EI)} = \frac{\text{diameter of hydrolysis zone (in mm)}}{\text{diameter of the colony (in mm)}}$ [36].

Microplastics Degradation Ability

The potential of the bacterial isolates originating from *C. riparius* gut to degrade plastics was tested according to Brunner et al. [37]. The degradation assay using PE, PVC, and PA as a plastic source was performed on the agar medium with further composition g/L: NH_4NO_3 3.0, K_2HPO_4 5.0, NaCl 1.0, $MgSO_4 \times 7H_2O$ 0.2, Tween 20 0.25 mL, and agar 15.0. Immediately after autoclaving, 10 g L^{-1} of PE, PA, and PVC powder (Sigma-Aldrich, Buchs, Switzerland; particle size 125 µm) were added to make each microplastic agar plate. Thus, the medium contained the nutrients nitrogen, phosphorus, sulphur, potassium, magnesium, sodium, and chlorine whilst microplastics were the only source of carbon. The Petri dishes were visually inspected every 7 days for 1 month period. According to the intensity of observed growth, the ability of isolates to use microplastics as only carbon source was evaluated as high (+++), medium (++), and low (+).

Results

Total Bacteriobiota of *C. riparius* Larvae

The phylogenetic composition of bacterial communities associated with *C. riparius* larvae was analyzed using 22 DNA samples isolated from laboratory, nature, fish food, and sediment samples by amplification and sequencing of the V3-V4 region of the 16S rRNA gene. After trimming and quality filtering, 70,475 (CL), 77,186 (CN), 58,859 (SE), and 55,244 (HR) classifiable paired-end sequence reads were retained (Table S1).

Estimation of alpha diversity using Chao1 and Fisher indices and observed features (OBS) detected statistically significantly higher diversity in the nature samples (CN) compared with laboratory culture (CL) at all taxonomic levels assessed (Fig. 1, Table S2). Beta diversity analysis was performed using DPCoA at the ASV level. The DPCoA diagram with group centroids and the distances of the individual samples to the centroids was shown in Fig. 2. According to axis 1 (which explains 89% of the variability), the samples from nature and from the laboratory were significantly different ($P = 0.004$). Sample CL5 was identified as an outlier according to PCoA1. Homogeneity of variances between groups was confirmed by the betadisper test ($P = 0.55$). A PERMANOVA test revealed statistically significant variability (R^2 0.82; $P = 0.004$).

According to the differential abundance analysis (Fig. 3), the differences in the most abundant or unique taxa among the analyzed samples are significant and include a large number of genera as discriminants. Representatives of *Paucibacter*, *Aeromonas*, *Legionella*, *Bdellovibrio*, *Pir4* lineage, *Roseomonas*, *Roseococcus*, *Sandaracinobacter*, *Roseovarius*, *Rhodobacter*, *Bosea*, *Xanthobacter*, *Kaistia*, *Bacillus*, and *Pseudoxanthomonas* were identified as unique in CL samples. In CN samples, the unique were representatives of *Sphaerotilus*, *OM60*(*NOR5*) clade, *BD1-7* clade, *Acinetobacter*, *Desulfovibrio*, *Micrococcus*, *Cutibacterium*, *Sphingomonas*, *Clostridium* sensu stricto, *Cetobacterium*, *Lactococcus*, *Trichococcus*, *Staphylococcus*, *Dechloromonas*, and *Arenimonas* (Table S3).

The Composition of Total Bacteriobiota

According to the analysis of the 16S rRNA gene sequences obtained from larval gut samples in both habitats studied, a total of 31 phyla were detected. Mainly, *Proteobacteria*, *Firmicutes*, *Bacteroidota*, and *Actinobacteriota* were detected in both gut samples (Fig. 4A). In the CN samples, the phylum *Fusobacteriota* was additionally detected in

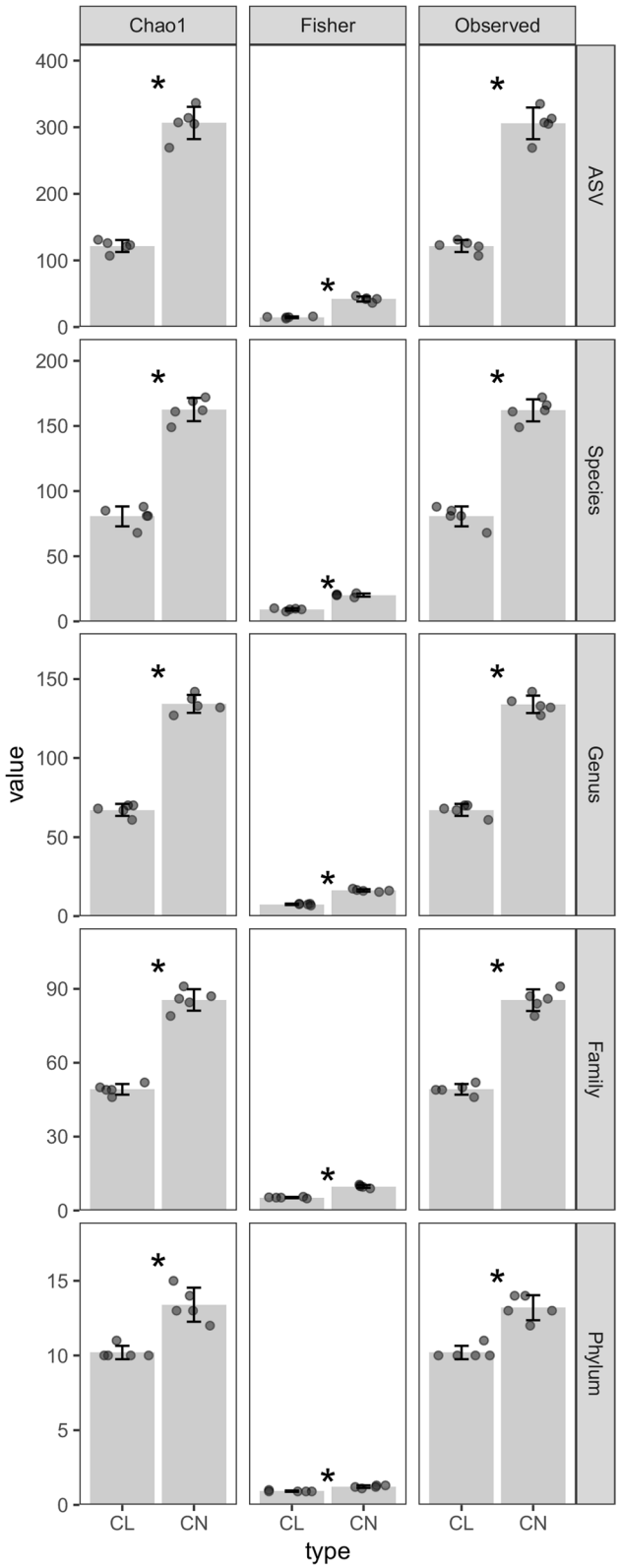

Fig. 1 Potential differences in alpha diversity were examined in the two groups of samples CN1-5 and CL1-5, at all tax-level aggregations using Wilcoxon rank-sum test. The *p*-values obtained were adjusted for multiple comparisons using Benjamini and Hochberg method. The asterisk denotes statistical significance at $p < 0.05$

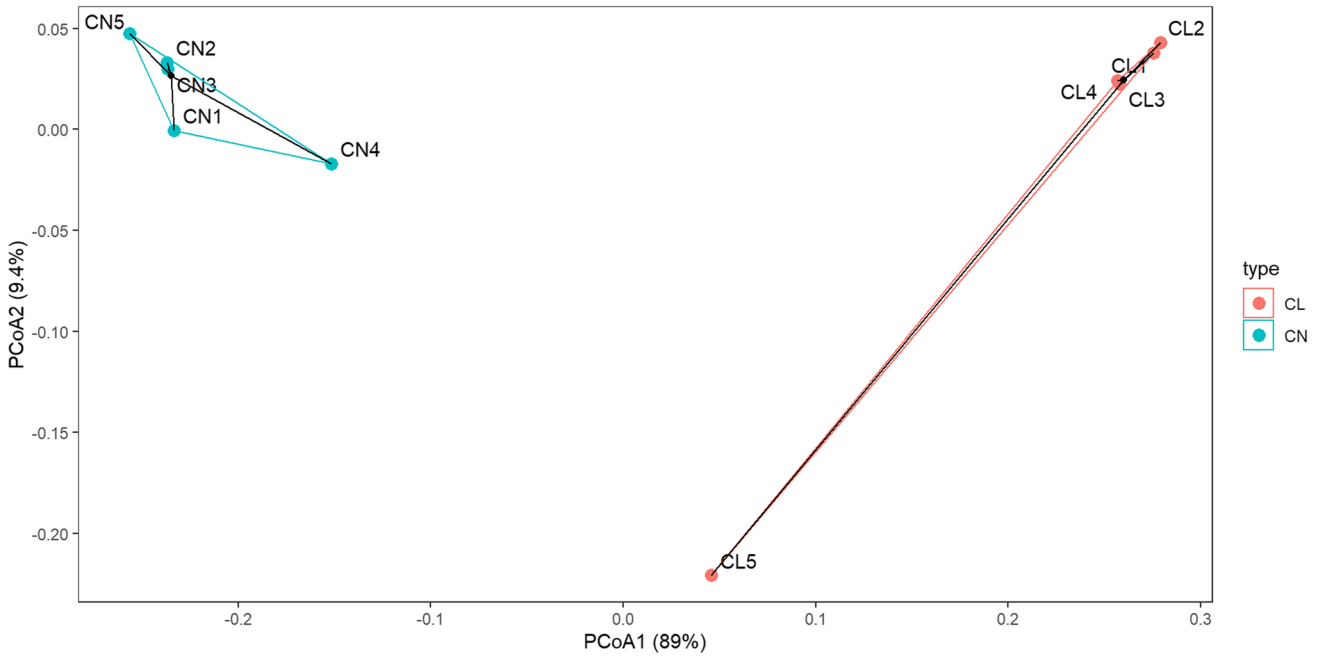

Fig. 2 Beta diversity of bacterial communities of *C. riparius* larval gut from laboratory and nature was performed using DPCoA analysis

a high percentage (37%). At the genus level, a total of 674 different genera were detected. The most abundant members of microbiota in both CN and CL samples were *Paludicola* with the identified species *P. psychrotolerans*, *Dysgomonas* (*D. alginatilytica*), *Pseudomonas* (*P. pohangensis*), *Tabrizicola* (*T. aquatica*), *Acidovorax (A. monticola)*, and *Wohlfahrtiimonas* (Fig. 4B). In CN samples, the genus *Cetobacterium* was predominantly observed with the identified species *C. somerae*, (37%). Above 1%, the genera *Andreesenia* (7%), *Paludicola* (*P. psychrotolerans*, 7%), *Dysgonomonas* (*D. alginatilytica*, 5%), *Wohlfahrtiimonas* (3%), *Dechloromonas hortensis* (3%), *Anaerotignum* (3%), *Staphylococcus* (2%), *Trichococcus* (2%), *Arenimonas* (1%), *Pseudomonas* (1%), *Cutibacterium* (1%), and *Desulfovibrio* (1%) were also detected. In the laboratory culture samples, the most abundant were genera belonging to *Phnomibacter* (*P. ginsenosidimutans*, 18%) followed by *Dysgonomonas* (*D. alginatilytica*, 12%), *Roseovarius* (*Phycocomes zhengii*, 11%), *Gemmobacter* (*G. lanyuensis*, 9%), *Paludicola* (*P. psychrotolerans*, 5%), *Acidovorax* (*A. monticola* 5%), *Rhodobacter* (*Tabrizicola aquatica*, 4%), and *Aeromonas* (*Pseudaeromonas pectinilytica*, 3%) (Table S4).

Furthermore, sediment (SE) sampled from the natural habitat of *C. riparius* and fish food (HR) used to feed the laboratory culture of *C. riparius* larvae were analyzed as potential primary sources of gut microbiota. The most prevalent phyla in the sediment samples, *Proteobacteria*, *Firmicutes*, *Bacteroidota*, and *Actinobacteria*, were also the most abundant in the analyzed gut samples. Distinctively, HR samples had a lower diversity of detected phyla and were mainly characterized by *Firmicutes* (92%), followed by *Actinobacteriota*, *Cyanobacteria*, and *Proteobacteria*. When analyzed at the genus level, SE samples were characterized by a high prevalence of *Acidovorax* (9%), *Dechloromonas* (6%), *Sphaerotilus* (8%), *Trichococcus* (4%), *Pseudomonas* (3%), *Aquabacterium* (3%), *Arenimonas* (3%), *Staphylococcus* (3%), *Acinetobacter* (2%), and *Thiothrix* (2%). In the HR samples above 1% only four genera were identified: *Weissella* (58%), *Streptococcus* (17%), *Lactococcus* (14%), and *Corynebacterium* (2%).

Culturable Bacteriobiota

From larval gut samples of *C. riparius* collected in the laboratory, 64 potentially different isolates were cultured using a conventional culturable approach—43 selected under aerobic and 21 under anaerobic conditions. From CN samples, 33 potentially different isolates were cultured, of which 18 were grown under aerobic and 15 under anaerobic conditions. Based on the 16S rRNA gene sequences, the isolates were identified as representatives of *Peribacillus*, *Metabacillus*, *Neobacillus*, *Bacillus*, *Fictibacillus Paenibacillus*, *Lysinibacillus*, *Gottfriedia*, *Rummeliibacillus*, *Mesobacillus*, *Sporosarcina*, and *Priestia*. The phylogenetic relationships of the identified species are shown in Fig. 5. Of the total 41 determined bacterial species isolated from larval samples, five identified species were common for CN and CL samples including *Metabacillus idriensis*, *Peribacillus simplex*, *Neobacillus cucumis*, *Bacillus thuringiensis/toyonensis*, and

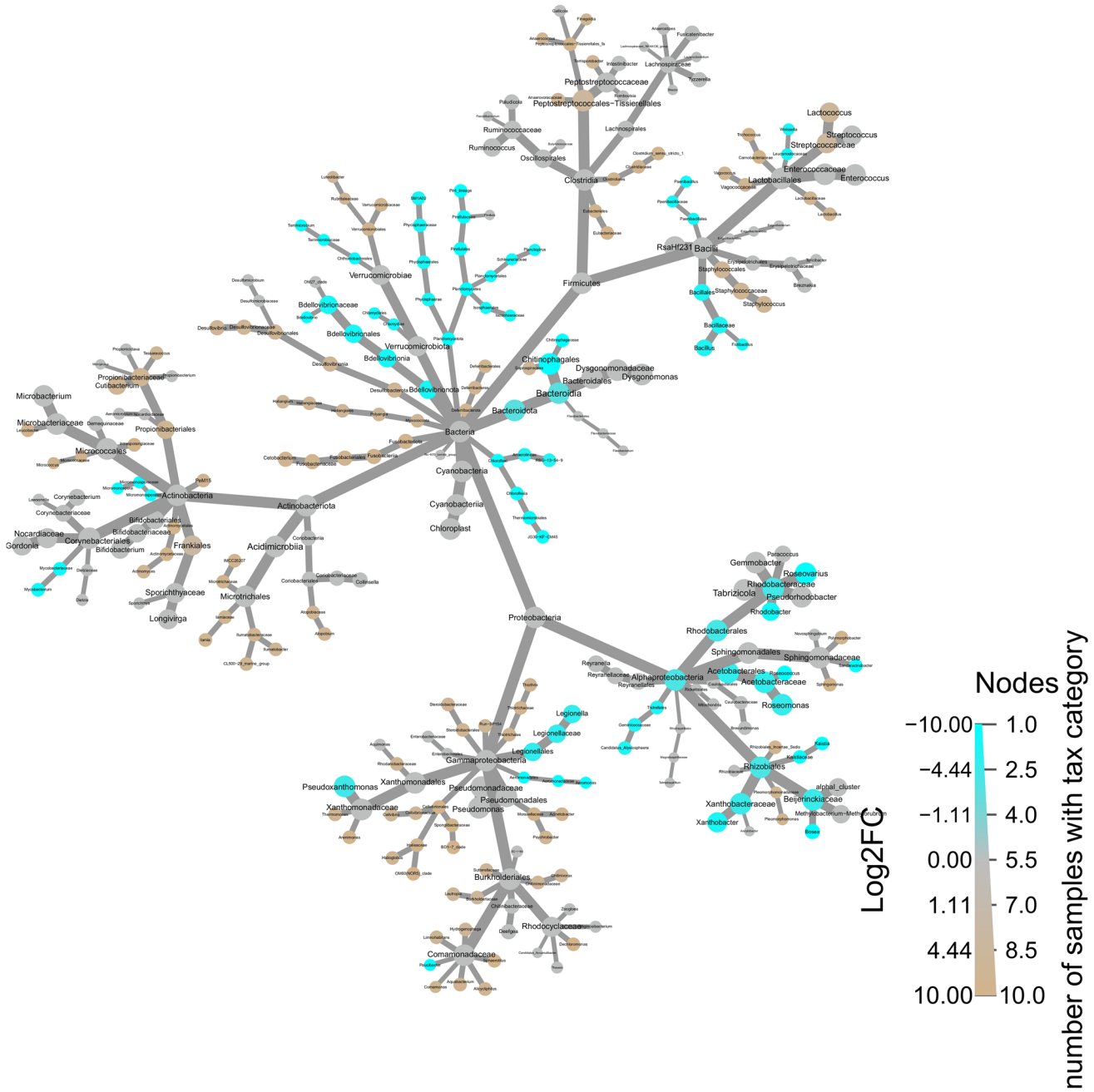

Fig. 3 Differential abundance analysis presented in metacoder heat tree. Cyan-colored taxa are significantly more abundant in CL samples while tan-colored taxa are significantly more abundant in CN samples. The intensity of the color corresponds to log2 fold change as indicated in the legend, while the size of the nodes corresponds to the number of samples where the corresponding tax category had a non-zero count

Fictibacillus phosphorivorans. In the natural habitat, a more diverse bacterial community was detected with a total of 33 species identified. Among them, representatives of the genus *Bacillus* were the most abundant with 13 identified species, followed by eight *Paenibacillus* species. The laboratory culture was less diverse with only 13 species detected, with the genera *Peribacillus*, *Paenibacillus*, and *Bacillus* being the most abundant with two species each. According to Venn diagram analysis, the unique and common species between two sampled *C. riparius* habitats, under two growth conditions, are shown in Table 1.

Enzymatic Activity

The ability to degrade proteins, cellulose, and three types of microplastics (PE, PA, and PVC) was tested on 13 selected

Fig. 4 Relative abundance (RA) of bacterial phylum (**A**) and genera (**B**) associated with *C. riparius* larval gut sampled from nature (CN) and from laboratory culture (CL), sediment (SE), and food (HR)

isolates from CL samples and 24 from CN samples. Based on the diameter of the hydrolysis zone and the calculated enzymatic index, the most effective proteolytic activity was detected for the isolates *Fictibacillus phosphorivorans* (1.5), *B. cereus/tropicus/paramycoides/nitratireducens/luti/albus* (1.43) followed by *Paenibacillus xylanexedens* (1.33) and *P. amylolyticus* (1.35). The highest cellulolytic activity was observed also for *P. xylanexedens* (1.92) and *P. amylolyticus* (2.00). The ability to use microplastics as a sole carbon source was evaluated based on observed growth after 1 month of incubation. Intensive growth (+++) was observed for two *Peribacillus simplex* isolates and one *P. frigoritolerans* isolate on all three types of microplastics (PE, PA, and PVC). Medium ability (++) was also observed for *B. wiedmanii/proteolyticus/fungorum* and two isolates of *B. thuringiensis/toyonensis* on PE, PA, and PVC. Only the isolate of *P. xylanexedens* was detected to have both, the ability of low growth on plates supplemented with PE, PA, and PVC and high proteolytic and cellulolytic activity (Table 2).

Discussion

The present study evaluated for the first time the diversity of bacterial communities associated with the gut of *C. riparius* larvae in natural habitat and was compared to laboratory culture. In addition, the potential of endogenous bacteria to degrade various substrates including microplastics (PVC, PA, and PE) was investigated. The microbiota of nature samples indicated higher diversity and abundance compared to laboratory-reared *C. riparius* larvae. A natural habitat with complex ecological factors may influence a more diverse microbiome in the gut of *C. riparius* compared to laboratory cultures. For instance, several studies have reported that the transition of insects from the wild habitat to a laboratory environment leads to a decrease in microbiome diversity associated with their gut [38, 39]. Differential abundance analysis indicated significant differences in the composition of the microbiota in the two analyzed habitats, with many representatives at the genus level found to be specific to each

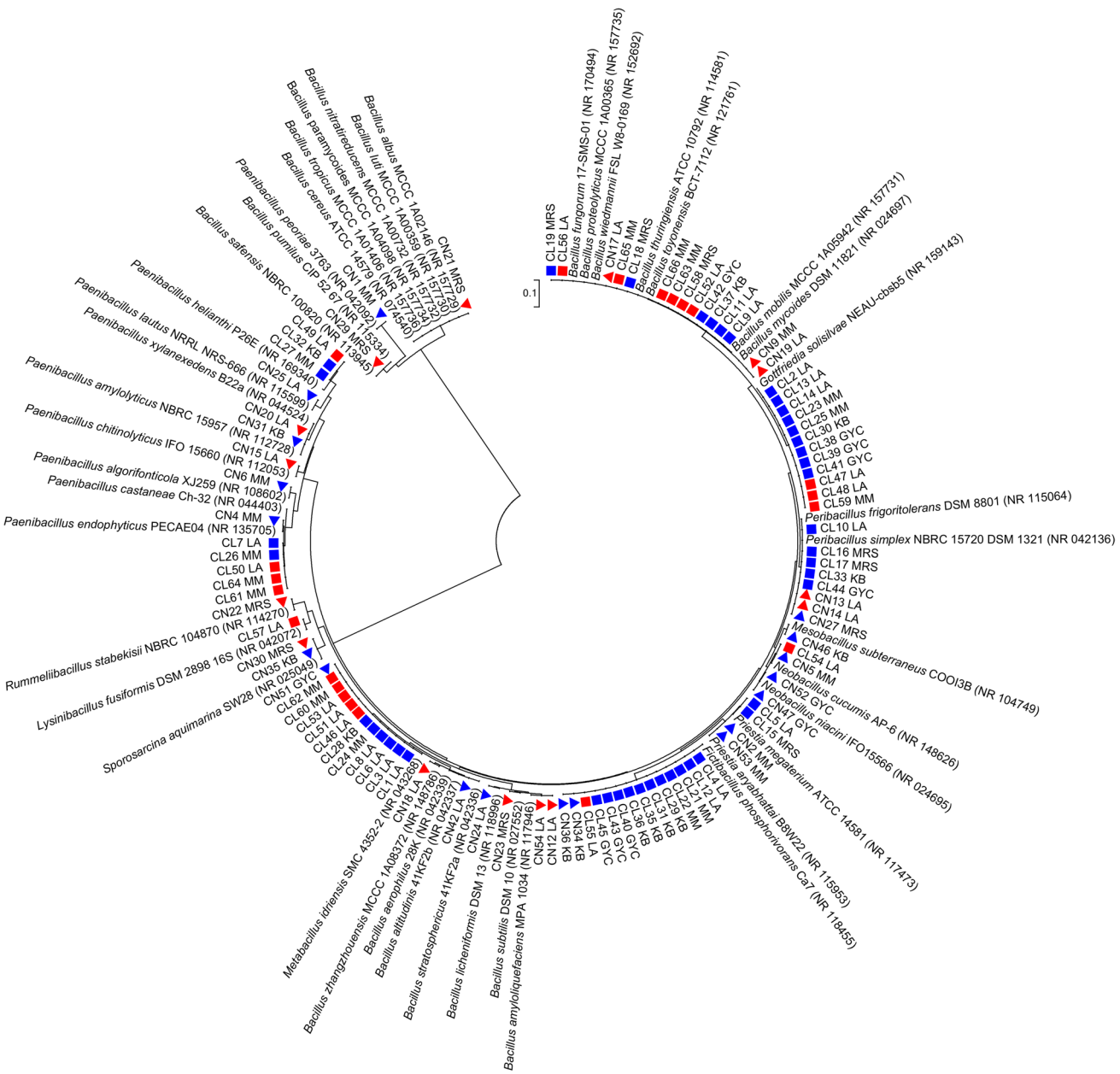

Fig. 5 Phylogenetic relationships of bacterial isolates from *C. riparius* (CL ■ and CN ▲) based on the 16S rRNA sequence. Isolates grown under aerobic conditions were marked as blue, and those grown anaerobically were marked as red. A phylogenetic tree was constructed by the Neighbor-joining method and the distances were calculated with the Kimura two-parameter model. Bootstrap values are given for each node, with 1000 replicates

gut sample type. Selective environmental factors have been shown to be more important than host species in shaping the insect gut microbiome [38].

The phyla *Proteobacteria*, *Fusobacteriota*, *Firmicutes*, *Bacteroidota*, and *Actinobacteriota*, which were the most prevalent, differed in their abundance in the analyzed CN and CL samples. The trend of higher abundance of *Fusobacteriota* and *Firmicutes* in the natural habitat and higher abundance of *Proteobacteria* and *Bacteriodetes* in the CL samples was in accordance to the recent study comparing the endogenous microbiome of *C. ramosus* larvae from the Mutha River and laboratory culture [2]. Laviad-Shitrit et al. [40] also detected *Fusobacteriota* as the dominant phylum in *C. circumdatus* larvae collected from the natural environment. The two most prevalent representatives at the genus/species level in both the CN and CL samples are associated with aquatic ecosystems. *Paludicola psychrotolerans* was described as a psychrotolerant chitinolytic bacterium first

Table 1 The overlap of culturable microbiota of *C. ramosus* larvae from laboratory and nature samples selected under aerobic and anaerobic growth conditions

Origin	Unique species	Species
CL aerobes	2	*Peribacillus frigoritolerans, Priestia megaterium*
CL anaerobes	1	*Lysinibacillus fusiformis*
CN aerobes	11	*Paenibacillus peoriae, Paenibacillus lautus, Paenibacillus endophyticus, Paenibacillus amylolyticus, Bacillus idriensis, Neobacillus niacini, Mesobacillus subterraneus, Bacillus licheniformis, Paenibacillus algorifonticola, Priestia aryabhattai Bacillus altitudinis/aerophilus/stratosphericus*
CN anaerobes	11	*Paenibacillus xylanexedens, Bacillus zhangzhouensis, Bacillus zhangzhouensis/pumilus/safensis, Paenibacillus chitinolyticus, Rummeliibacillus stabekisii, Bacillus subtilis, Bacillus thuringiensis, Bacillus mycoides, Bacillus amyloliquefaciens, Bacillus toyonensis, Bacillus cereus/tropicus/paramycoides/nitratireducens/luti/albus*
CL aerobes and CL anaerobes	7	*Paenibacillus castaneae, Metabacillus idriensis, Bacillus mobilis, Gottfriedia solisilvae, Bacillus wiedmannii/proteolyticus/fungorum, Paenibacillus helianthi, Bacillus toyonensis/thuringiensis*
CL anaerobes and CN aerobes	1	*Neobacillus cucumis*
CN aerobes and CN anaerobes	1	*Sporosarcina aquimarina*
CL aerobes and CL anaerobes and CN aerobes	1	*Fictibacillus phosphorivorans*
CL aerobes and CN aerobs and CN anaerobes	1	*Peribacillus simplex*

isolated from a wetland [41] and *Dysgomonas alginatilytica* was previously isolated from an alginate-degrading microbial consortium acclimated from sea sand [42]. Furthermore, the CN samples were characterized by a high prevalence of *Cetobacterium* spp. Similarly, the genera *Cetobacterium* and *Dysgonomonas* were detected in previous studies as the most abundant in samples of *C. ramosus* [2] and *C. circumdatus* larvae [40] collected from India Rivers. Considering the geographical distance between our two sampling sites, it could be discussed that *Cetobacterium* and *Dysgonomonas* potentially are core members of *Chironomus* larval gut in the natural environment. It has been shown that *Cetobacterium* spp. is widely distributed in the gastrointestinal tract of various fishes [43, 44]. As the most abundant in CL samples, in addition to the previously mentioned *P. psychrotolerans* and *D. alginatilytica*, representatives associated with aquatic ecosystems were identified as a potential source of larval gut colonization. *Phnomibacter ginsenosidimutans* is a newly described glycoside hydrolase-positive bacterial strain with ginsenoside hydrolysing activity [45]. *Phycocomes zhengii* was recently described as a marine bacterium of the family *Rhodobacteraceae* isolated from the phycosphere of *Chlorella vulgaris* [46], and *Gemmobacter lanyuensis* was isolated from a freshwater spring source for the first time [47]. Of the most abundant genera, only *Acidovorax* was previously identified as a member of the egg mass microbiota of *Chironomus* spp. [48].

Considering that diet was previously reported as an important factor in shaping the gut microbiome [49], the microbial communities of the sediment samples and the fish food used to feed the laboratory cultures were analyzed in parallel with the gut samples. Comparing microbial community structure at the phylum and genus levels, CL samples and fish food did not share taxa with more than 1% of relative abundance, indicating that fish food did not affect the structure of the gut microbiome of laboratory-reared *C. riparius* larvae. Distinctively, SE and CN samples shared representatives of the genera *Acidovorax*, *Dechloromonas*, *Arenimonas*, and *Staphylococcus*, potentially indicating that bacteria inhabiting sediment in the natural habitat colonized the *C. riparius* larval gut. The differences in relations of CL-fish food and CN-sediment could be explained by different environmental conditions [50].

In addition to characterizing the total microbiota of *C. riparius* larvae, bacteria were also isolated from the same CN and CL samples to discover potential candidates that can enzymatically degrade different substrates. Both the CN and CL samples examined were characterized by a high diversity of *Bacillus* spp. with 13 species identified, and several genera recently separated from *Bacillus* spp. Studies using a culturable approach mainly report bacterial communities associated with the *Chironomus* egg mass, whereas the culturable microbiota of the larval gut is scarcely reported. *Bacillus* spp. was also identified as a common genus among the culturable microbiota of *C. plumosus* and *C. circumdatus* larvae [1, 51]. For comparison, as main representatives of *C. plumosus* and *C. circumdatus* larvae microbiota besides *Bacillus* were revealed also *Klebsiella*, *Enterobacter*, *Escherichia, Delftia*, *Aeromonas*, *Acinetobacter*, *Citrobacter*, *Clostridium*, *Corynebacterium*, *Micrococcus*,

Table 2 Enzymatic activity of cellulase and protease after 24 h of incubation. The enzymatic activity index (Ei) was calculated for each enzyme. Growth on a medium supplemented with microplastics was evaluated after 1 month of incubation and evaluated as high (+++), medium (++), and low (+)

Species	Label	Ei celulase	Ei protease	PVC	PE	PA
Metabacillus idriensis	CL1_LA	0.00	0.00	-	-	+
Gottfriedia solisilvae	CL2_LA	0.00	0.00	-	-	-
Fictibacillus phosphorivorans	CL4_LA	0.00	1.33	-	-	+
Priestia megaterium	CL5_LA	0.00	0.00	+	+	+
Paenibacillus castaneae	CL7_LA	0.00	0.00	-	-	-
Bacillus mobilis	CL9_LA	0.00	1.07	++	+	+
Peribacillus frigoritolerans	CL10_LA	0.00	0.00	+++	+++	+++
Peribacillus simplex	CL16_MRS	0.00	0.00	+++	+++	+++
Bacillus thuringiensis/toyonensis	CL18_MRS	0.00	0.00	++	++	++
Bacillus wiedmannii/proteolyticus/fungorum	CL19_MRS	0.00	0.00	++	++	++
Paenibacillus helianthi	CL27_MM	1.50	0.00	-	-	-
Neobacillus cucumis	CL54_LA	0.00	0.00	-	-	-
Lysinibacillus fusiformis	CL57_LA	0.00	1.14	-	-	-
Paenibacillus peoriae	CN1_MM	0.00	1.23	-	-	-
Priestia aryabhattai	CN2_MM	0.00	0.00	+	-	+
Paenibacillus endophyticus	CN4_MM	0.00	1.23	-	-	-
Neobacillus cucumis	CN5_MM	0.00	0.00	-	-	-
Paenibacillus algorifonticola	CN6_MM	0.00	0.00	-	-	-
Bacillus mycoides	CN9_MM	0.00	0.00	++	+	+
Bacillus amyloliquefaciens	CN12_LA	1.38	0.00	-	-	-
Peribacillus simplex	CN13_LA	0.00	0.00	+++	+++	+++
Paenibacillus chitinolyticus	CN15_LA	0.00	0.00	-	-	-
Bacillus thuringiensis/toyonensis	CN17_LA	0.00	0.00	++	++	++
Bacillus zhangzhouensis	CN18_LA	1.19	0.00	-	-	+
Paenibacillus xylanexedens	CN20_LA	1.92	1.33	+	+	+
Bacillus cereus/tropicus/paramycoides/nitratireducens/luti/albus	CN21_MRS	0.00	1.43	+	-	++
Rummeliibacillus stabekisii	CN22_MRS	0.00	0.00	-	-	-
Bacillus subtilis	CN23_MRS	1.38	0.00	++	+	++
Bacillus licheniformis	CN24_LA	0.00	0.00	++	+	++
Bacillus zhangzhouensis/pumilus/safensis	CN29_MRS	1.27	0.00	-	-	-
Sporosarcina aquimarina	CN30_MRS	0.00	0.00	-	-	-
Paenibacillus amylolyticus	CN31_KB	2.00	1.35	+	-	+
Fictibacillus phosphorivorans	CN34_KB	0.00	1.50	+	-	+
Bacillus altitudinis/aerophilus/stratosphericus	CN42_LA	1.13	1.05	-	-	-
Mesobacillus subterraneus	CN46_KB	0.00	0.00	-	-	-
Bacillus idriensis	CN51_GYC	0.00	0.00	+	+	+
Neobacillus niacini	CN52_GYC	0.00	0.00	-	-	+

Pseudomonas, *Serratia*, *Providencia*, *Yersinia*, and *Staphylococcus* as genera that not detected in our study. Furthermore, the samples from CL and CN were characterized by a high diversity of *Paenibacillus* genera. Representatives of *Paenibacillus* spp. have been detected in a freshwater environment [52], but until now was not detected in *Chironomus* spp. microbiome. Another aquatic bacterium, *Sporosarcina aquimarina*, was detected only in CN samples. The typical habitat of *S. aquimarina* is marine water [53], but its habitat could be broader as we have detected it in a freshwater ecosystem. Common species detected for CN and CL samples were *Metabacillus idriensis*, *Peribacillus simplex*, *Neobacillus cucumis*, *Bacillus thuringiensis/toyonensis*, and *Fictibacillus phosphorivorans*. Only *B. thuringiensis/toyonensis* has been previously detected in *C. circumdatus* larvae [51], while other identified species have not been previously detected in the microbiome of *Chironomus* spp. The higher diversity of nature samples with 28 unique species compared

to the CL samples (8 unique species) might be related to different food sources and habitat conditions. Laboratory cultures are grown under optimized conditions with stable biotic and abiotic factors, in contrast to natural habitats with high selection pressure, fluctuating environmental conditions, and competition for nutrients, all of which affect the composition of the microbiota [50].

For further testing, 37 isolates were selected to evaluate the ability to degrade different substrates, excluding clonal ones from the collection. Two *Peribacillus simplex* isolates (from CN and CL samples) and one *P. frigoritolerans* isolate (CL sample) showed high potential to grow on plastics. The bacterial community isolated from the digestive tract of earthworms, which also contained *Peribacillus simplex* (formerly *Bacillus simplex*), was able to reduce the size of polyethylene microplastics [54]. To the best of our knowledge, there has been no previous report on the ability of *P. frigoritolerans* to degrade microplastics. Moderate activity has also been reported for *B. thuringiensis/toyonensis* and *B. wiedmanii/proteolyticus/fungorum*. The *Bacillus* genus is an important part of the *Chironomus* spp. bacteriobiota, and besides genera *Klebsiella*, *Enterobacter*, *Acinetobacter*, *Paenibacillus* is involved in the detoxification of various types of xenobiotics including microplastics in the water [55, 56]. Also, our results confirmed previous statements that invertebrate microbiomes are sources of plastic-degrading bacteria [20, 50, 51]. In most cases, isolates with the high ability to metabolize microplastics did not possess proteolytic and cellulolytic activity. *Paneibacillus xylanexedens* was only species isolated with high proteolytic and cellulolytic activity, followed by a low ability to grow on PE, PA, and PVC-supplemented plates. Also, *P. amylolyticus* was dominant in producing both enzymes, followed by *Fictibacillus phosphorivorans* and *B. cereus/tropicus/paramycoides/nitratireducens/luti/albus* with the highest proteolytic activity. Enzymes such as protease and cellulase play an important role in ecosystems by hydrolyzing polymers to monomers and returning them to the upper trophic network [23]. Further study should test the compatibility between the most efficient microplastic degraders and strains with high proteolytic and cellulolytic activity so that the bacterial consortium can be selected for the degradation of this type of pollution. The selected consortia, which include efficient producers of hydrolytic enzymes, could have important application for treating lakes and rivers polluted with the organic matter and microplastics.

In conclusion, this is the first study to analyze the gut bacterial diversity of *C. riparius* larvae from both natural habitat and laboratory culture using culture-dependent and culture-independent approaches. A significant difference in microbial diversity was found between natural and laboratory-reared *C. riparius* larvae. In parallel, analysis of the culturable microbiota showed it to be an important source of bacteria with the potential to grow on PE, PVC, and PA microplastics, and also identified strains with cellulolytic and proteolytic activity. Anthropogenic pressure on freshwater ecosystems by organic and microplastic pollution is a problem of modern society, and finding efficient bioremediation measures is of great importance. Therefore, in a future perspective, our strains could be tested for bioremediation of sites polluted with plastics and high organic input.

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Data Availability The data presented in this study are openly available as BioProject ID: PRJNA89878 in the (NCBI) repository, (https://www.ncbi.nlm.nih.gov/sra/PRJNA898781).

Declarations

Ethics Approval A study did not require ethics approval.

Competing Interests The authors declare no competing interests.

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