Trophic diversification and parasitic invasion as ecological niche modulators for gut microbiota of a sympatric pair of whitefish

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Abstract

The impact of parasites on gut microbiota of the host is well documented, but the role of the relationship between the parasite and the host in the formation of the microbiota is poorly understood. Using 16S amplicon sequencing and newly developed methodological approaches, we characterize the gut microbiota of the sympatric pair of whitefish Coregonus lavaretus complex and the associated microbiota of cestodes parasitizing their intestine. The essence of the proposed approaches is, firstly, to use the method of successive washes of the microbiota from the cestode's surfaces to analyze the degree of bacterial association to the tegument of the parasite. Secondly, to use a method combining the sampling of intestinal content and mucosa with the wash-out procedure from the mucosa to understand the real structure of the fish gut microbiota. Our results demonstrate that the trophic diversification of a sympatric pair of whitefish predetermines a segregation by ecological niches of their respective microbial communities within their intestine. Additional environmental niches for settlement of bacteria in the intestine are formed by the parasitic helminths that caused the restructuring of the bacterial community in infected fish compared to those uninfected. Using the desorption method in Ringer's solution, we have demonstrated that Proteocephalus sp. cestodes possess their own microbial community which is put together from "surface" bacteria received from the host, bacteria which are weakly and strongly associated with the tegument, and microbiota obtained after removal of the tegument from the cestodes.

Introduction

Any ecosystem consists of a number of ecological niches inhabited by different species and characterized by specific conditions. The digestive tract of fishes is a very specific ecosystem that receives regular contact from different external environmental compartments such as water, sediment, food items, etc. The fish gut is normally inhabited by multiple species of microorganisms forming its enteric microbiome. The host and microbiota form tandem close relationships that have developed during the course of a co-evolution and participate in digestion, synthesis of a number of vitamins and amino acids, as well as in the protection against pathogenic microorganisms (Nayak, 2010; Ghanbari, Kneifel, & Domig, 2015).

The gut of fish has a great variety in morphology among aquatic vertebrates. In general, all fishes can be divided into gastric and agastric species. In turn, the morphology of the intestine is also different, and may include various numbers of pyloric caeca situated in the anterior intestine and different septa and extensions in the posterior intestine. The relative length of the fish intestine can also vary significantly among different species. All these features determine the specific physical and biochemical conditions that are formed in the digestive tract of fishes, and therefore function as a physical-chemical barrier selecting specific microorganisms

from among the great taxonomic variety of microorganisms that are constantly ingested with water and diet, and are able to colonize the host gut. In nature, in parallel with the microbiota, the digestive tract of fish is normally inhabited by different classes of helminthes (Trematoda, Cestoda, Acanthocephala, or Nematoda) characterized by different seasonal activity and impact on the host. From this perspective the fish gut could be viewed as a multi-room apartment (different parts of the gut) where each "room" is inhabited by specific lodgers (microbiota and parasites) under specific physical-chemical conditions (pH, ion and gas composition and concentration, etc.). Moreover, both types of lodgers (microbiota and parasites) are replaced by different taxonomical groups during various seasons, host ontogeny stage, or host immune status. Like all living organisms the parasites produce a number of metabolites (protease inhibitors, hormone-like compounds, allergens, organic acids, lectins) forming the specific microenvironment that could be specified as a new ecological niche in the ecosystem of the host gut enabling colonization by specific microbiota. Indeed, it is known that cestodes may secret some organic acids (Izvekova, 2001) and, hypothetically, the pH values in the local microenvironment may be shifted to the acid side, and this can be a selective barrier for some bacterial groups. Such microenvironments permit survival on the cestode's tegument of those bacterial species that could not colonize the gut mucosa. Moreover, the tegument surface has microtriches (very similar to the host's microvilli), and this structure provides several layers where different parts of the bacterial community with various levels of adhesions may occur (Dalton et al., 2004; Poddubnaya, Izvekova, 2005; Korneva and Plotnikov, 2006). Such additional niches with specific microbiota that differs from microbiota of the host gut mucosa may increase the diversity of the total microbial community in the fish gut. Thus, the presence of infected fish in a population will significantly increase the populations overall bacterial diversity that potentially may help them to resist environmental disturbances such as natural outbreaks of some diseases.

To date, the versatile role of parasites in different aspects of fish physiology, immunology, behavior, etc. is well documented. Thus, ignoring parasitic invasions as a factor contributing to the diversity of the microbiota may lead to biases in the interpretations of results. Since the understanding of the functional roles of parasites in ecosystems is relatively new, the adequate approaches to investigate such roles are poorly developed, or absent. One such methodological "blind spot" at present is the studying of gut-parasite-microbiota interactions. From one perspective, the "omics" technology provides a great opportunity to study the taxonomical composition of the microbial community of the fish gut. However, there is still no scientifically-based, standardized approach for collecting samples from fish gut and its parasites, even though potential deviations from normality of results due to alternative methodologies has been documented (Kashinskaya et al., 2017). In many studies, the samples of mucosa and/or digesta are collected from fish gut in order to analyze the enteric microbiota. The main methodological restriction of this approach is to clean mucosa of microbial contamination from digesta and vice versa. One possible solution is to wash the intestinal segments (with mucosa attached) with saline solution. This approach was applied by Sevellec with co-authors (2018), but without robust experimental support, the outcome obtained from using this approach may be subject to bias (Solovyev et al. 2019).

The microbial community of fish, while studied by "omics" approaches for the last two decades and focused on several critically different methodological aspects, has almost no information on the microbiota of fish parasites. How to distinguish between the host microbiota and the microbial community associated with parasites, as well as to what degree the bacteria are associated to the helminth tegument is an area of study still under development, methodologically. At the present time there are no universal methodological approaches to explore "true indigenous" microbiota of helminthes, which are deprived of their own digestive system, but several authors have made attempts to explore bacteria associated with parasites. Izvekova and Lapteva (2004) described an approach based on serial washing of bacterial cells from the tegument of parasites via shaking of the whole parasite in buffers and their transfer through a series of those buffers with graded salinities. After that, the separate fractions of saline solution with bacterial cells were cultivated using nutrient mediums and counted. As a result of this culture-dependent approach to the study of the microbiota of cestodes, separate fractions were characterized by different levels of adhesion, but some bacterial cells could still exist in deep layers on microtriches even after many series of washings. In order to make advances towards a deeper understanding of the tapeworm's microbiome organization an improved method

is needed to separate the tegument from the cestode. The suitable approach was invented by Knowles and Oaks (1979) that, briefly, consisted of incubation of cestodes in a solution of detergent (Triton X-100) with subsequent shaking and thereafter portions fractionated by centrifugation. The combinations of these approaches are a prospective way to create the appropriate protocol for studying the complex microbial community of cestodes infesting the gut of vertebrates.

Teletskoye Lake (Western Siberia) is inhabited by a sympatric pair of whitefish: small "dwarf" planktivorous form *Coregonus lavaretuspravdinellus* (Dulkeit, 1949) and a large "normal" benthivorous form *C. l. pidschian* (Gmelin, 1789) (Bochkarev & Zuikova, 2006; Bochkarev, 2009; Solovyev et al., 2022). These whitefishes are infected by mature stages of *Proteocephalus* sp. (Cestoda) in the intestine with different levels of prevalence (100% for *C. l. pravdinellus*and 45% for *C. l. pidschian*) (Bochkarev & Gafina, 1993). In the present study we have used this sympatric pair of whitefish as a natural model of infected and uninfected fish with different feeding habits in order to gain a deeper insight into the structure of the enteric bacterial community.

The aim of the present study was to compare the composition of gut microbial communities of the sympatric pair of whitefish *C. l. pidschian* and *C. l. pravdinellus* and the associated microbiota of cestodes parasitizing their intestine using an approach described in the present study. This promising methodological approach to the study of the associated microbiota of parasites, in addition to the above mentioned protocols and methods of high-throughput sequencing can help to shed light on the relationships between parasite, fish and symbiotic microbiota.

In the present study, we have put forward several hypotheses focused on a new methodological approach and the structure of microbial communities in a host gut-parasite-microbiota system. First, we hypothesize that the primary wash-out procedure from the mucosa, as well as rinsing and shaking procedures for cestodes, are necessary to separate the microbiota that is weakly associated with these surfaces in order to understand in depth the real structure of the microbial communities of fish gut and cestodes. Secondarily, the gut microbiota of the sympatric pair of whitefish will be affected, not only by the differences of feeding habits and other biotic and abiotic factors, but also by cestode infestation. Thirdly, the microbial communities associated with fish gut and cestode will have specific taxonomic compositions. Fourthly, the associated microbiota of the parasite occupies different ecological niches within the cestode tegument and forms a parasite-specified microbial community, which on the one hand, will be similar to their host (surface microbiota) and, on the other hand, will form the microbiota of the deeper layer of the cestode´s tegument.

Materials and methods

Study area and sampling

Teletskoye Lake is a large (223 km^2) and deep (325 m) oligotrophic lake (basin of Ob River) in the Altai Mountains (Altai Republic, Russia). In August 2019 in the north part of Teletskoye Lake (51.79*N; 87.30*E) "pravdinellus" *C. l. pravdinellus* (total length, TL 158.8+-2.6 mm, n=14) infected by *Proteocephalus*sp.*,* as well as "pidschian" *C. l. pidschian* uninfected (TL 252.2+-6.4 mm, n=13) and infected by the same cestode (TL 241.3+-4.3 mm, n=9) were collected (Figure S1). For microbiota investigations of "*pravdinellus* " whitefish we used only infected individuals due to the high prevalence level (100%) of *Proteocephalus* sp. Fish were captured using gill-nets (mesh sizes 18-25 mm) and transported alive to the laboratory in plastic containers filled with water from the site of fish capture. All fish were sacrificed and samples were collected aseptically. Male and female fish were identified according to gonadal development (Table S1). The digestive tract (DT) was divided into three parts: stomach, anterior and posterior intestine and cut separately (Figure S2a). The content of each segment of DT were squeezed out by gentle stripping and collected separately. After collecting the content from the corresponding part of DT, the washing procedure was performed with sterile physiological saline solution (0.9% NaCl) to collect weakly adherent microbiota from mucosa of the stomach, anterior and posterior intestine of analyzed fish (Figure S2b). Five milliliters of the solution were taken by syringe and slowly squeezed out into the "proximal" part of a vertically fixed part of the DT (stomach, anterior or posterior intestine), then when the solution passed through this part of the DT the solution was collected in an empty sterile tube at the "distal end" part of the DT. Afterward, the collected

solution (washout) was stored at -80 degC until analysis.

Desorption of associated microbiota from the tegument of cestodes

Cestodes from "*pravdinellus* " were presented as a large tangled lump of small worms that could not be divided into separate individuals, whereas cestodes from the "*pidschian* " forms were larger and could be separated from each other. Due to this existence of significant phenotypic differences in size of the cestodes from "*pidschian* " and "*pravdinellus* ", we have collected "large" worms from "*pidschian* " whitefish only.

Associated microbiota of *Proteocephalus* sp. were analyzed by a method of desorption of bacteria from the tegument surfaces. The essence of the method consists in successive washings of the microbiota from the surface of the cestode's tegument in sterile Ringer's solution for cold-blooded animals (pH 7.4). The cestodes were removed immediately after dissection from nine infected fish intestine with a sterile needle or tweezers and placed in sterile Ringer's solution to remove fragments of the host's intestinal mucosa and content from their tegument. Depending on the size and number of worms, two to five individuals of worms were collected in one Eppendorf tube from each infected fish, the number of biological replicates from each fish were ranged from one to three. Following this step, the Ringer's solution fractions were frozen and also used for microbiological analysis (fraction D0). Then the first washout fraction (D1) was obtained after placing the cestodes in a new sterile Eppendorf tube with sterile Ringer's solution and vigorously shaking on a BIOSAN TS-100 vortex for 15 seconds at 900 rpm. Subsequent washings D2-D5 were obtained by sequential transfer of cestodes into a new Eppendorf tube with a sterile Ringer's solution and vigorous vortexing each for 15 minutes at 900 rpm. To separate the tegument from the cestodes after washing D5 the Triton X-100 detergent (0.2% w/v) was used. The volume of each fraction was 1 ml. After desorption with Triton X-100 detergent, the cestodes were transferred into new tubes for isolation of the bacterial DNA. The obtained washings containing fractions with bacteria expressing different degrees of attachment to the cestodes tegument were lyophilized and used for DNA isolation (Figure S2c).

Analysis of the 28S rRNA gene of *Proteocephalus* sp. from "*pidschian*" and "*pravdinellus*" whitefishes

For genetic analysis of seven individuals of *P. exigius* from "*pravdinellus* " and "*pidschian* ", characterized as populations of "small" and "large" individuals (four individuals for each population), the sequencing of part of 28S was conducted. To determine the species of cestodes, partial sequences of the nuclear large subunit of rRNA gene (28S) were amplified using primers LSU5 (TAGGTCGACCCGCTGAAYTTYAGCA) and 1500R (GCTATCCTGAGGGAAACTTCG) (Littlewood et al., 2000, 2008). PCR conditions and sequencing is described in Vlasenko et al. (2022). Sequences were deposited into GenBank (NCBI) under the following accession numbers: SUB11262576 ON133796- ON133802.

Scanning (SEM) and transmission (TEM) electron microscopy

To confirm and observe the effects of the desorption protocol, specimens of *Proteocephalus* sp. were sampled from "*pidschian* " whitefish using two methods. One way, immediately after dissection of the whitefish, several of the worms were removed from the fish intestine and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Additional specimens of *Proteocephalus* sp. were fixed with the same glutaraldehyde after desorption with 0.2% Triton X-100 detergent in order to confirm the elimination of the tegument from cestodes after this treatment. For SEM, after fixation in glutaraldehyde the specimens were dehydrated in a graded ethanol series, with a final change to absolute acetone. The worms were critical point-dried with liquid CO_2 and then mounted on stubs, sputter-coated with gold-palladium and examined using a JEOL JSM 6510LV scanning electron microscope operating at 30 kV.

For transmission electron microscopy (TEM), after fixation in glutaraldehyde, both additional specimens from whitefish and the specimens after desorption with Triton, were rinsed in 0.1 M sodium cacodylate buffer (pH 7.2) and post-fixed in 1% osmium tetroxide at 5degC for 1 h, The material was dehydrated as for the SEM and embedded in a mixture of different resins using a mixture of Araldite and Epon using an Araldite/Embed-812 EM Embedding kit (EMS). Ultrathin sections (40–90 nm in thickness) were cut using

a Leica Ultracut UCT ultramicrotome, double-stained with uranyl acetate and lead citrate, and examined in a JEOL 1010 transmission electron microscope operating at and 80 kV.

Histological observation of cestodes

Intestines of infested whitefish were dehydrated in a graded series of ethanol, embedded in paraffin and cut into serial sagittal sections (3 μm thick). Sections were stained by Harris' Haematoxylin and Eosin (HE) and Alcian Blue (AB) at pH 2.5 for general histomorphological observations and detection of carboxyl-rich and sulphated glycoconjugates in mucous cells, respectively (Pearse 1985). The histological sections were analyzed using an Olympus BX43 microscope and photographs were taken with a digital camera (Olympus UC90) with resolution of 300 dpi.

DNA extraction, and 16S rDNA metagenomic sequencing

Before DNA extraction, all samples (mucosa, content of stomach and intestine) were collected into sterile microcentrifuge tubes with lysis buffer (300 μl) for DNA isolation, then mechanically homogenized by pestle for 1 min. Washing of parasites and washing from mucosa of corresponding part of DT were lyophilized and used for DNA isolation. Following the kit manufacturer protocols, DNA was extracted from 100 mg of samples (excluding parasites and washings from mucosa) using a DNA-sorb B kit (NextBio, Russia) according to the protocol previously described (Kashinskaya *et al.* 2020).

Sequencing of the V3, V4 hypervariable regions of 16S rRNA genes was carried out on an Illumina MiSeq sequencing platform (500 cycles - 2×300 paired-end) by Evrogen (Moscow, Russia) using the primer pair S-D-Bact-0341-b-S-17, 5'-CCTACGGGNGGCWGCAG-3' and S-D-Bact-0785-a-A-21, 5'-GACTACHVGGGTATCTAATCC-3' (Klindworth *et al.* 2013). The amplification conditions, 16S sequence processing and statistical analyses is described by Kashinskaya *et al.* (2021a). Nucleotide sequences were deposited in the Sequence Read Archive (SRA NCBI), accession number PRJNA814856.

For details see section "Material and Methods" in the Supporting Information.

Results

1. 28S analysis, general histological view of Proteocephalus **sp.** and electron microscopy (SEM and TEM) description of its proglottid tegument before and after desorption with Triton X-100 detergent

A 1452 bp fragment of the 28S rRNA gene, was amplifed and sequenced from seven specimens of*Poroteocephalus* sp.; three sequences from cestodes from "*pidchian* " and four sequences from cestodes from "*pravdinellus* " were identical. Hence, we consider cestodes from both whitefishes to be the same species.

The histological sections of intestine of whitefish infested by*Poroteocephalus* sp. were shown in figure 1. Several tapeworms are attached by their scoleces to the folds of intestine with the strobilae lying within the intestinal lumen, other cestodes simply lying in the intestinal lumen.

By SEM, there was the typical highly dense arrangement of long, remarkably flexible filamentous microtriches revealed throughout the strobila in naturally infected *Proteocephalus* sp. (Figure 2A, B). The TEM images of these specimens shows that the tegument is composed of an external anucleate cytoplasmic layer (distal syncytial cytoplasm) covered with numerous long slender filamentous microtriches interspersed with individual spiniform microtriches (Figure 2D). Filamentous microtriches with a long, cylindrical base and shorter electron-dense pointed distal shaft are considered to increase the absorption area and thus facilitate uptake of nutrients. Spiniform microtriches have a shorter and wider cylindrical base and longer and wider electron-dense distal shaft. The distal tegumental cytoplasm lies on the basal lamina consisting of the outer dense homogeneous layer and the inner fibrillar extracellular layer (Figure 2D).

By SEM, the surface of *Proteocephalus* sp. after desorption with Triton X-100 detergent was smooth (Figure 2C) and devoid of surface microtriches (Figure 2E). Visible pores on the surface of the body are the places of the connection of sunken tegumental perikarya with distal syncytial cytoplasm via cytoplasmic processes

(Figure 2E). Shapeless, small fragments of residue or fragments of tegumental composition are visible on the surface (Figure 2E). TEM investigation of experimental specimens of *Proteocephalus* sp. distinguished only the occurrence of electron-dense basal lamina on the surface of these tapeworms (Figure 2F).

2. Alpha-diversity of microbial community associated with different parts of the digestive tract of whitefishes

"pidschian"

Stomach

The highest richness (Chao1=2503.4±589.6 and OTUs=1986.5±414.5) and diversity (Shannon=7.6±0.5 and Simpson=1.0±0.0) estimates were observed in samples of the stomach content of infected *"pidschian"* , while the lowest value was detected in the mucosa of infected fish (Chao1=483.7±62.7, OTUs=283.4±27.7, Shannon=5.8±0.2, Simpson=1.0±0.0). In uninfected *"pidschian"* similar tendencies of the values of indices were observed: the highest Chao1, OTUs, Shannon and Simpson were registered in stomach content (1181.9±187.2, 882.5±160.9, 6.1±0.6, and 0.9±0.1, correspondingly) and the lowest one in mucosa (234.4±32.2, 140.8±19.4, 3.1±0.3, 0.7±0.0) (Table 1). According to the Dunn's test (at p[?]0.05) the richness estimates of stomach mucosa of uninfected and infected *"pidschian"* were significantly low in comparison with their content (Table S2). There were no significant differences in the diversity estimates of the microbial community between mucosa and content of uninfected and infected *"pidschian"* (Dunn's test, p>0.05).

Intestine

The highest richness and diversity estimates (Chao1=1672.1+-358.9, OTUs=1367.4+-324.2, Shannon=7.6+-0.6, Simpson=1.0+-0.0) were observed in content of the posterior intestine of infected *"pidschian"* , while the lowest one was detected in the mucosa of anterior intestine of infected fish (Chao1=182.2+-46.2, OTUs=98.3+-26.9, Shannon=3.8+-0.6, Simpson=0.9+-0.1). The richness estimates in microbial community of mucosa of both anterior and posterior intestines of uninfected and infected *"pidschian"* were significantly low in comparison with their content (Dunn's test, p[?]0.05) with the exception of the mucosa of the posterior segment of infected *"pidschian"* . The Shannon index was also significantly low (Dunn's test, p[?]0.05) in the microbial community of mucosa in comparison with content of the anterior and posterior intestine of uninfected and infected *"pidschian"* . According to the Simpson index, the significant low diversity of mucosa in comparison with content was only estimated in the anterior intestine of infected *"pidschian"* and posterior intestine of uninfected *"pidschian"* (Dunn's test, p[?]0.05).

The segment of the digestive tract analyzed (stomach, anterior or posterior intestine) also had a significant determinative effect on the composition of the microbiota (Table S2). No significant differences in richness and diversity estimates were found between microbiota associated with anterior and posterior intestines (Dunn's test, p>0.05).

The infection status of fish (infected, uninfected) in almost all cases had no significant determinative effect on the composition of the microbiota (Dunn's test, p>0.05). The Simpson index value in stomach content of infected *"pidschian"* was significantly different in comparison with stomach content of uninfected *"pidschian"* (Dunn's test, z=2.3, p=0.043).

"pravdinellus"

Stomach

The highest richness and diversity estimates were observed in the stomach content (Chao1=449.1+-44.5, OTUs=268.9+-30.8, Shannon=4.1+-0.4, Simpson=0.8+-0.0), while the lowest ones were detected in the mucosa of infected *"pravdinellus"* (Chao1=277.6+-37.0, OTUs=165.5+-28.7, Shannon=3.4+-0.4, Simpson=0.7+-0.1) (Table 1). There are no significant differences in the alpha-diversity estimates of microbial community between the stomach mucosa and content of infected *"pravdinellus"* (Dunn's test, p>0.05).

Intestine

The highest richness and diversity estimates (Chao1=606.5+-90.8, OTUs=416.3+-69.0, Shannon=4.0+-0.5, Simpson=0.7+-0.1) were observed in content of posterior intestine, while the lowest ones were detected in the mucosa of anterior intestine of infected *"pravdinellus"*(Chao1=125.2+-20.6, OTUs=76.8+-11.5, Shannon=1.9+-0.4, Simpson=0.4+-0.1). Significant differences in richness estimates were found between the microbiota associated with the anterior and posterior intestinal content of *"pravdinellus"* (Dunn's test, p>0.05). The diversity estimates were only significantly different among mucosa of anterior and posterior intestines of *"pravdinellus"* (Table S2).

When comparing the different segments of the DT in infected *"pravdinellus"* significant difference of Shannon and Simpson values were only observed among microbiota from anterior intestinal mucosa and stomach mucosa (Dunn's test, p[?]0.05).

Between forms of whitefish.

The alpha-diversity of the microbiota in both forms were significantly varied depending on the type of sample (intestinal content, mucosa, or washout from the mucosa) and the different segment of digestive tract (stomach, anterior and posterior intestine). For both forms of whitefish progressive reduction in richness and diversity estimates were registered, as follows: stomach content, intestinal content, washout from stomach, stomach mucosa, intestinal mucosa, washout from intestine.

The differences in the highest richness and diversity estimates were significant between "*pidschian* " and "*pravdinellus* " for stomach content, mucosa and content of anterior intestine, and content of posterior intestine of both infected and uninfected fish (ADONIS, p[?]0.05), whereas for stomach mucosa and mucosa of posterior intestine the differences were insignificant (ADONIS, p[?]0.05) (Table S2).

3. Alpha-diversity of microbial community associated with washout from the stomach and intestinal mucosa of whitefish

"pidschian"

There were no significant differences in the richness and diversity estimates of the microbial community between the washout from the mucosa of anterior and posterior intestine in comparison with their intestinal mucosa of the corresponding part of DT of infected *"pidschian"*(Dunn's test, p>0.05). In uninfected *"pidschian"*the microbiota of the washout from the mucosa of the anterior intestine was significantly different from their intestinal mucosa only for Chao1 values (Dunn's test, p[?]0.05). The infection status of fish (infected, uninfected) had no significant determinative effect on the composition of the microbiota (Dunn's test, p>0.05) between infected and uninfected *"pidschian"* .

"pravdinellus"

There were also no significant differences in the richness estimates (OTUs, Chao1) of the microbial community between the washout from the mucosa of the anterior and posterior intestine in comparison with their intestinal mucosa of the corresponding part of DT of infected *"pravdinellus"* (Dunn's test, p>0.05). But, when comparing the diversity estimates (Shannon and Simpson) among washout samples from mucosa of the anterior intestine and their intestinal mucosa of infected *"pravdinellus",* significant differences were found (Dunn's test, p[?]0.05).

4. Alpha-diversity of microbial community associated with cestodes parasitizing the intestine of "pidschian"

Alpha-diversity of the microbial community associated with different fractions of cestodes obtained before and after desorption are shown in Figure S3a. According to Dunn's test (at p[?]0.05) the significantly highest richness (Chao1=894.2+-282.0, OTUs= 649.3+-229.9) and Shannon diversity estimates (4.7+-0.9) were observed in the D0 fraction in comparison with D1-D5 fractions (Chao1=410.6+-20.8, OTUs=210.6+-11.0, Shannon=2.7+-0.1). The number of OTUs in the microbiota of the D6 fraction (294.8+-29.5) was also significantly higher (Dunn's test, p[?]0.05) than in the D1-D5 fractions (210.6+-11.0) (Table 2).

5. Associated microbiota of different segments of digestive tract of whitefish

"pidschian"

The type of sample (mucosa, content) and the segment of the DT (stomach, anterior and posterior intestine) had a significant determinative effect on the composition of the *"pidschian"* microbiota. Thus, the differences in associated microbiota of the *"pidschian"* form were significant between mucosa and content for all segments of the DT (ADONIS test, UnWeighted UniFrac matrix, p[?]0.05). But, when comparing the microbiota among different segments of the DT, significant differences were not found (Table S3).

Forty-three phyla were registered in the microbiota associated with the stomach and intestine of the infected and uninfected *"pidschian"* . The dominant microbiota of the stomach mucosa and content from the infected "*pidschian*" was represented by Proteobacteria (65.7 and 61.4%, correspondingly), Bacteroidetes (10.2 and 2.5%, correspondingly), Verrucomicrobia (5.4 and 8.0%, correspondingly), Acidobacteria (6.5 and 1.7%, correspondingly), Actinobacteria (4.2 and 4.5%, correspondingly), and Planctomycetes (3.9 and 5.2%, correspondingly). In the anterior and posterior intestine of the infected "*pidschian*" , the relative abundance of Proteobacteria remained dominant and occupied from 46.1 to 67.4% of the total composition of phyla (Figure 3). Other dominant phyla in the anterior and posterior intestine of the infected "*pidschian*"were represented by Actinobacteria (2.1 - 8.4%), Firmicutes (1.5 - 14.3%), Planctomycetes (1.7 - 12.4%), and Tenericutes (0.4 - 15.5%). In the infected "*pidschian*" , the relative abundance of Tenericutes was insignificantly (Dunn's test, p[?]0.05) higher in microbiota associated with mucosa of the anterior intestine (15.5+-8.4%) in comparison with microbiota of the stomach mucosa (0.2+-0.01%), stomach content (0.01+-0.01%) and posterior intestine (0.71+-0.01%). Significant differences in relative abundances of the dominant phyla in microbiota of infected "*pidschian*" were only observed for Acidobacteria, in which the relative abundances were significantly higher (Dunn's test, p[?]0.05) in microbiota of the stomach content (6.5+-0.01%) than in mucosa of the anterior intestine (0.02+-0.01%).

In uninfected "*pidschian*" the dominant phyla were similarly represented in stomach and intestine with the exception of the phyla Acidobacteria and Tenericutes, in which relative abundances were low in comparison with infected fish.

The dominant OTUs at the lowest taxonomical level (Figure 4) in microbiota of the stomach mucosa and content of infected "*pidschian*" were ambiguous taxa (here and after "amt.") Burkholderiaceae (2.0+-0.7 and 4.6+-1.9%, correspondingly),*Comamonas* (3.9+-2.1 and 0.2+-0.04%, correspondingly),*Cyanobium* PCC-6307 (0.8+-0.8 and 0.9+-0.6%, correspondingly),*Pseudomonas* (20.1+-3.9 and 1.3+-0.9%, correspondingly), and uncultured (here and after "unc.") Rickettsiaceae (1.4+-1.2 and 2.1+-2.1%, correspondingly), whereas, the dominant microbiota of mucosa and content from the stomach of uninfected *pidschian* were represented by *Aeromonas* (3.7+-2.0 and 12.7+-6.9%, correspondingly), *Pseudomonas* (19.0+-6.2 and 0.9+-0.5%, correspondingly), *Silvanigrella* (5.2+-5.2 and 9.6+-7.3%, correspondingly), and amt. Burkholderiaceae (0.8+-0.2 and 7.6+-3.7%, correspondingly).

Included among the dominant microbial community of the mucosa and content of the anterior intestine of infected "*pidschian*" were*Comamonas* (5.6+-2.2 and 1.3+-0.5%, correspondingly),*Limnohabitans* (2.0+-2.0 and 8.0+-8.0%, correspondingly),*Mycoplasma* (13.6+-8.6 and 0.2+-0.1%, correspondingly),*Pseudomonas* (27.7+-9.2 and 1.2+-0.7%, correspondingly), and*Staphylococcus* (7.8+-7.4 and 1.2+-1.1%, correspondingly); whereas, the microbiota of the mucosa of the posterior intestine were dominated by *Negativibacillus* (10.3+-10.3%), *Pseudomonas*(34.7+-15.0%), and unc. Desulfovibrionaceae (9.9+-9.9%).*Comamonas* and unc. Pirellulaceae were the dominant microbiota of the content of the posterior intestine of infected fish (2.9+-1.0 and 3.1+-0.7%, correspondingly). The microbiota associated with anterior and posterior intestines of uninfected "*pidschian*" were dominated by *Aeromonas* , *Comamonas* , and *Pseudomonas.* According to the ADONIS test on UnWeighted UniFrac significant differences were obtained for stomach mucosa and content of anterior and posterior intestines between infected and uninfected fish (p[?]0.05) (Table S3).

"pravdinellus"

The type of sample (mucosa, content) had a significant determinative effect on the composition of the *"pravdinellus"* microbiota (Table S3). The differences in associated microbiota of the *"pravdinellus"* form

were significant between mucosa and content of the stomach (ADONIS test, UnWeighted UniFrac matrix, p[?]0.05). When comparing the microbiota of mucosa and content among different segments of the intestine (anterior and posterior), the differences were also significant.

Forty phyla were registered in the microbiota associated with the stomach and intestine of the *"pravdinellus"* , with Firmicutes (up to 57.6%) presenting as the dominant phylum in the stomach microbiota. In the mucosa of the posterior intestine from *"pravdinellus"* , the phylum Firmicutes was replaced by Proteobacteria (52.1%, correspondingly) and by Tenericutes (75.9%) in the mucosa of the anterior intestine. Firmicutes was present in a significant amount only in the intestinal content of the anterior and posterior intestine of the "*pravdinellus"* (37.6 and 43.3%, correspondingly). The relative abundance of Tenericutes was higher in microbiota associated with mucosa (75.9+-6.8%) and content (27.8+-8.3%) in comparison with microbiota of the stomach mucosa (4.3+-3.2%) and content (0.2+-0.1%) (Figure 3).

At the lowest taxonomical level, the microbiota associated with the stomach mucosa and content of infected "*pravdinellus"* were mainly represented by *Clostridium sensu stricto 1* (51.2+-11.2 and 46.9+-8.6%, correspondingly). The microbiota of mucosa and content of anterior intestine were dominated by *Clostridium sensu stricto 1*(1.4+-0.7 and 32.7+-10.8%, correspondingly), *Mycoplasma* (75.1+-6.7 and 25.4+-7.7%, correspondingly), and unc. Desulfovibrionaceae (8.1+-6.7 and 10.7+-8.7%, correspondingly); whereas, the microbiota of mucosa and content of the posterior intestine were dominated by *Clostridium sensu stricto 1* (4.4+-3.2 and 42.3+-9.4%, correspondingly),*Comamonas* (16.8+-3.4 and 2.5+-0.8%, correspondingly),*Cutibacterium* (8.8+-7.4 and 0.2+-0.06%, correspondingly),*Mycoplasma* (15.1+-4.9 and 3.5+-2.7%, correspondingly), and unc. Desulfovibrionaceae (8.7+-6.7 and 7.3+-6.6%, correspondingly) (Figure 4).

Between forms of whitefish.

The relative abundances of the dominant phyla and OTUs at the lowest taxonomical level in both forms varied depending on the different segment of the DT (stomach, anterior and posterior intestine) and the type of sample (content, mucosa, or washout from the mucosa). It is interesting to note that for the anterior intestine of both of the infected whitefish forms, the ratio of the phylum Tenericutes in the samples of the mucosa and content changed in a similar way. The highest abundance of this phylum was registered in the mucosa of the anterior rather than in the posterior intestines, and stomach mucosa and content.

The significant differences in microbiota associated with mucosa and content of all segments of the DT between infected "*pravdinellus"* , and infected and uninfected *"pidschian"* were obtained (ADONIS test, UnWeighted UniFrac matrix, p[?]0.05) by excluding the comparison of stomach mucosa among infected *"pravdinellus"* and uninfected "*pidschian"*(p>0.05) (Table S3).

6. Associated microbiota of washout fractions from the stomach and intestinal mucosa of whitefish

Variations in relative abundance of the several dominant OTUs between mucosa and their washout are presented in Table S4. According to the ADONIS test based on UnWeighted UniFrac matrix there were no significant differences between the microbiota of the washings from the intestinal mucosa from both anterior and posterior intestines of infected "*pidschian*" . In contrast to the infected "*pidschian*" , the microbiota of the mucosa of the anterior intestine from uninfected "*pidschian*" were significantly different in comparison with their washings (r^2=0.13, p=0.003). The significant differences were also observed between the mucosa and their washing from the posterior intestine of infected "*pravdinellus"*(r^2=0.13, p=0.008).

When comparing the microbiota of the washout between studied whitefishes (ADONIS test based on UnWeighted UniFrac matrix), significant differences in the stomach mucosa and mucosa of the anterior and posterior intestine were obtained in most cases (p[?]0.05). The washout from anterior intestine among infected *"pravdinellus"* and uninfected "*pidschian"* were not significant (p>0.05).

7. Desorption of associated microbiota from the tegument of cestodes parasitizing the intestine of "pidschian" and their relationship to the host

Microbiota of fractions D1, D2, D3, D4 and D5 after serial desorption of bacteria from the tegument of

cestodes were essentially replicates and did not differ significantly (ADONIS test, TableS5). For this reason, we combined these fractions and then compared it as a single sample with the other fractions obtained (from D0, D6 and D7). The principal coordinate analysis (PCoA) of D0, D1-D5, D6 and D7 fractions is shown in Figure S3b.

Thirty-nine phyla were registered in the microbiota associated with the cestodes parasitizing the intestine of *"pidschian"* . At the phylum level the dominant microbiota of all fractions were mainly represented by Proteobacteria (57.0 - 68.7%) and Tenericutes (19.4 - 39.0%). The relative abundances of these phyla did not significantly differ between various fractions from the tegument of cestodes (Dunn's test, p>0.05) (Figure 3). At the lowest taxonomical level, in the cestode's fraction D0 the dominant bacteria were similar to the microbiota of the intestinal mucosa of their host and represented by candidate genus*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (10.6+-8.9%),*Comamonas* (11.0+-4.6%), *Deefgea* (8.9+-8.8%), and*Mycoplasma* (19.3+-12.6%). The microbiota of fraction D1-D5 were dominated by *Acinetobacter* (13.6+-1.8%), *Mycoplasma*(30.1+-1.7%), and *Sphingobium* (13.1+-0.9%). The dominant position of these bacteria remained stable in fraction D6 with the exception of*Sphingobium* (0.11+-0.06%), which was replaced by more abundant bacteria from candidate genus*Allorhizobium -Neorhizobium -Pararhizobium -Rhizobium*(9.6+-3.0%) and *Pseudomonas* (13.4+-0.0%). The microbiota associated with fraction D7 were represented by *Comamonas*(15.3+-2.2%), *Delftia* (5.7+-1.6%), and *Mycoplasma*(42.2+-10.6%) (Figure S3d).

The significant increase of relative abundances of *Acinetobacter*and *Pseudomonas* were registered from the D0 to D6 fractions; whereas, the significant reduction in abundances of *Comamonas* and*Sphingobium* were noted from the D0 to D6, and D1-D5 to D6 fractions, correspondingly (Dunn's test, p[?]0.05) (Figure S3c).

According to the ADONIS test based on UnWeighted UniFrac matrix (Table 3) and using PCoA (Figure 5), significant differences in associated microbiota were found between all parts of the DT (stomach, anterior and posterior intestine) of infected and uninfected fish, and cestodes of D1-D5, D6 and D7 fractions (p[?]0.05). The associated microbiota of cestodes from fraction D0 (fraction before desorption) was similar to mucosa, their washout and content of anterior intestine and mucosa of posterior intestine of infected fish. In uninfected fish these comparisons were significantly different (p[?]0.05).

Test effect of host-related factor ¡¡Fish¿¿ on the microbial community of cestodes using the ADONIS test and PCoA on Weighted UniFrac matrix (Table S6) was also applied to degree of differences/similarity of microbiota of cestodes and the host in which they parasitize. The PCoA demonstrated a higher similarity of the microbial community of cestodes and their host (Figure S4). This means that for each host and their resident cestodes parasitizing their intestine there exists a unique microbial community that is significantly different, depending on the individual fish (ADONIS, p[?]0.05).

8. Linear discriminant analysis (LDA) effect size (LEfSe)

The LEfSE was performed to identify the bacterial OTU's that showed significant differences in relative abundances among the analyzed groups (Figure 6). The results show that unc. Rhizobiales was significantly different in the microbiota of the D0 fraction; whereas unc. Rhizobiacea, *Methylobacterium* , *Enterobacter* , and unc. taxa from Enterobacteriales order were significantly different in the microbiota of the D1-D5 fractions. In microbiota of the D7 fraction the significantly different bacterial taxa, compared to the other analyzed groups, were *Bosea* , *Comamonas* , *Corynebacterium* ,*Cualobacter* , *Cupriavidus* , *Paucibacter* ,*Pelomonas* , *Sphingopyxis* , *Thermus* , unc. Burkholderiaceae, Caulobacteriaceae, and Rhodospirillaceae. Compared to the other analyzed groups, unc. Micrococcaceae and unc. Burkholderiaceae were significantly different in content from the anterior intestine; whereas unc. Bacillales was significantly abundant in mucosa from the anterior intestine.

Discussion

Associated microbiota of different forms of whitefish

There is limited available data regarding the diversity of microbial communities in various sympatric pairs of salmonids (Sevellec et al., 2014; 2018; 2019; Belkova et al., 2017; Solovyev et al. 2019; Element et al.,

2020). Thus, the microbiota of the intestinal mucosa of a sympatric pair of *C. clupeaformis* was significantly different between "dwarf" and "normal" forms (Sevellec et al., 2018). For "dwarf" whitefish the genera *Stenotrophomonas* , and *Spartobacteria*were observed, whereas for the "normal" form of whitefish the bacteria from genera *Mycoplasma* , *Sarcina* , and *Serratia* were more abundant. At the same time the transient intestinal microbiota from the alimentary bolus obtained by Sevellec with co-authors (2019) in the same sympatric pairs of whitefish contained six dominant bacterial taxa:*Acinetobacter* , *Aeromonas* , *Clostridium* ,*Legionella* , *Methylobacterium* , and*Propionibacterium* . According to these results the authors concluded that the adherent microbiota is more preferable to study the effect of host species on gut microbiota than the analysis of transient microbiota. The major drawback of this approach was discussed by Solovyev with co-authors (2019), where they concluded that rinsing of intestine with sterile saline solution could eliminate the bacteria with weak adherence to their mucosa and thus biasing further analysis. Due to the lack of conclusive data regarding the methodology for collecting samples of the digestive tract, we used a more comprehensive approach to analyze gastrointestinal microbiota of fish, where the stomach, anterior and posterior intestine was subdivided to the mucosal layer and their content with a parallel study of the washout from their mucosa. As a result, significant differences were obtained between anterior mucosa and washout in uninfected *"pidschian"* and posterior mucosa and washout in infected *"pravdinellus"* . These results can be explained by the fact that the cestode, *Proteocephalus* sp. infested the anterior intestine and pyloric caeca of whitefish whereas in the posterior intestine these worms were almost absent. Live worms are constantly moving in the fish intestine due to the different layers within the intestinal content and mucus continuously being added. In parts of the intestine that are free from these parasites there is only intestinal contractions for mixing of layers, which is apparently not enough for deeply mixing different parts of the microbial community from intestinal mucus and content. Hence in uninfected parts, the difference in microbiota composition is significant between washout and mucosa due to lack of mixing by parasite movements. These results also indicate that the analysis of washing bacteria from the mucosa is more useful if it is necessary to assess the weakly associated intestinal microbiota.

A comparison of a sympatric pair of whitefish obtained in a previous study (Solovyev et al., 2019) to the present data, have shown that more stable microbial communities of whitefishes were observed in mucosa than in content. Thus, the shared OTUs in the microbiota of "*pravdinellus"* mucosa were *Comamonas* , *Mycoplasma* , and unclassified Desulfovibrionaceae and Comamonadaceae, whereas the shared OTUs in the microbiota of "*pidschian"* were*Aeromonas* , Aeromonadaceae and unclassified Desulfovibrionaceae. Microbiota of contents of both whitefishes analyzed by Solovyev with co-authors (2019) and in this study were different. These differences can be explained by fluctuations of the surrounding microbial community over lengths of time.

In a study similar to the current work, a comparison of a sympatric pair of whitefish from Canada analyzed microbiome samples from different sites among pooled samples obtained from *Salvelinus alpinus* and*C. clupeaformis* (Element et al. 2021). In that study, while it was noted that nearly half the samples had infestation with cestodes (among other parasite), there was no separation of the parasites from the host intestinal microbiota analyzed. The microbial profiles included members of the genera *Sphingomonas* and *Deefgea* as seen with the cestode samples herein, but in the study by Element et al. (2021) their data could not be used to assign any microbiota directly to the parasites of the host since no separation of parasite from host had been conducted. Some of the microbes therefore described in the study by Element et al. (2021) may in fact be restricted to the cestodes and other intestinal parasites noted. This is not to say that these taxa have no effect on the host, but it does call into question what constitutes the "normal core microbiome" of the host (Margarita et al., 2016).

In the present study the parasite infestation has significantly affected the microbial communities of the stomach and intestinal content of "*pidschian* " whitefish. Apparently, the gut tapeworms may have an effect on feeding regime and/or diet of infested fish, hence, such changes are reflected in the microbial composition. Indeed, it was shown that during co-infections (*F. psychrophilum* , *Renibacterium salmoninarum* and ectoparasite *Caligus lacustris*) in rainbow trout, *Oncorhynchus mykiss* the gut of unhealthy fish was almost empty. The dominant microbiota of the rainbow trout was represented by*Streptophyta* , *Bacillus* , *Serra-*

tia ,*Cetobacterium* , *Pseudomonas* and bacteria from the order Rickettsiales (Parshukov et al., 2019). The changes in gut microbiota of zebrafish *Danio rerio* during experimental *Pseudocapillaria tomentosa* infection was also revealed (Gaulke et al., 2019).

In this study, among "*pidschian* " and "*pravdinellus* " there was a notable increased abundance of *Mycoplasma* in the cestode-infected fish, but in the uninfected "*pidschian* " there was a clear increase in *Aeromonas* and reduction in*Mycoplasma* . *Aeromonas* has been described previously as a dominant OTU of the "core microbiome" from multiple fishes with different feeding habits in nature (Ofek et al., 2021). The appearance of *Aeromonas* as a dominant OTU in wild-caught whitefish has been noted previously as well (Sevellec et al. 2019). The abundance of*Mycoplasma* in the cestode samples (D0-D7) and also in cestode-infected whitefish strongly suggests, in this context, that this represents a dysbiosis due to the infestation by the cestodes. While cestodes may co-evolve with their hosts they may also be responsible for causing dysbiosis. However, curiously a study of *Mycoplasma*metagenomes collected from salmonids (among which whitefish are inclusive) suggested an underlying host benefit that is provided by*Mycoplasma* species; namely that their presence in the gut enables the juvenile salmonid to digest prey items enriched in long-chain polymers, such as chitin, which is often abundant in insects and crustaceans as a typical diet of whitefishes and the bacteria help to detoxify ammonia as well (Rasmussen et al., 2021). It has also been suggested that the presence of Mycoplasmas may be mutually exclusive for the presence of some potentially pathogenic *Vibrio* species (Rasmussen et al., 2021). These data could suggest that the abundance of Mycoplasmas is stage-specific for the fish host, and perhaps the abundance of *Aeromonas* is a more normal state for healthy adult whitefish. Additionally, studies with larger data sets are needed to improve clarity of this relationship.

Associated microbiota of cestode's

Data on the parasitic fauna of whitefishes from the lake Teletskoye is currently fragmentary (Bochkarev and Gafina, 1993; Solovyev et al., 2019; Kashinskaya et al., 2021b; Vlasenko et al., 2022 in press). The prevalence of invasion of the cestode *Proteocephalus* sp. in the intestine of both whitefish at the sampling date were 96.7% and 53.3%, for "*pravdinellus* " and "pidschian" forms, correspondingly, and correlates with early investigations ("*pravdinellus* " – 100% and "*pidschian* " – 45%) (Bochkarev and Gafina, 1993). While the specimens of *Proteocephalus* sp. obtained from "*pravdinellus* " and "*pidschian* " are very different in body size, we have shown based on 28S analysis, that these cestodes belong to the same species. Cestodes from the intestine of the whitefishes of Teletskoye Lake were previously identified as*Proteocephalus exiguus* (Titova, 1954, 1965, Bochkarev & Gafina, 1993). This species was later synonymized with *P. longicollis*(Scholz & Hanzelova, 1998). We also note the morphological similarity of the studied cestodes with *P. longicollis* ; however, in this work we define these species as *Proteocephalus* sp. The taxonomic position of these cestodes is uncertain and requires further study.

Microbiota and parasitic helminths also interact with each other and sharing the same niches within the fish host (Cortes et al., 2019). Parasite-associated microbiota have been described in different classes of Platyhelminthes (Turbellaria, Monogenea, Trematoda, Cestoda). Among these associations some bacteria are present on the tegument surface of the parasites (Hughes-Stamm et al., 1999; Cusack and Cone, 1985; Poddubnaya and Izvekova, 2005; Korneva and Plotnikov, 2006) and can be classified as ectosymbionts. Other bacteria that are present in helminth symbiotic organs (Gruber-Vodicka et al., 2011; Leisch et al., 2011; Caira et al, 2021), and intestinal tract (Morokuma et al., 2017; Jorge et al., 2020, 2021) are called endosymbiotic bacteria.

Bacterial associations with tapeworms are especially interesting because this group of platyhelminths lacks all elements of a digestive system except absorption. During co-evolution, intestinal cestodes adapted to the microenvironment of their host and use it as a resource for low molecular weight nutrients. It is known that the external surfaces of tapeworms are composed of a multifunctional syncytial tegument performing digestive-absorptive functions which are similar in structure and function to the brush border of the intestines of vertebrates (Halton, 1997; Dalton et al., 2014). The first ultrastructural description of the presence of bacteria on the tegument surfaces of cestodes was made by Poddubnaya and Izvekova, 2005. To date, the associated microbiota of cestodes has been studied using SEM and culture methods (Izvekova and Lapteva,

2004; Poddubnaya and Izvekova, 2005; Korneva and Plotnikov 2006; Caira et al, 2021).

Thus far there are only a few studies that have used a 16S rRNA sequencing approach to analyze the microbiota associated with fish cestodes (Hahn and Dheilly, 2018; 2021; Kashinskaya et al., 2020). Hahn and Dheilly (2018) characterized the microbiota of the tapeworm*Schistocephalus solidus* collected from the body cavity of threespine stickleback. In this study, parasites were shaken in sterile PBS to collect the surface microbiota of the *S. solidus* cestodes using culture-dependent methods. After rinsing in PBS solution the homogenate of cestodes was also sequenced. According to obtained results the authors suggested that *S. solidus* cestodes contain their own endomicrobiome showing the absence of cultivable bacteria on the surface and presence of *Polynucleobacter* as a dominant taxon in the homogenate of *S. solidus* (Hahn and Dheilly, 2018). The possible assumption of the absence of bacteria on the tegument surfaces of*S. solidus* can be explained by the presence of special attachment structures in bacteria, which help them to adhere to the tegument of the parasite and making release of the bacterial cells more difficult. Using the SEM, the clear evidence of a strong association of bacteria with the tegument of fish cestodes has been obtained. Attachment of the individual bacterial cells to the tegument surface of cestodes is carried out via a special holdfast structure (stalk-like tufts and filaments) (Poddubnaya and Izvekova, 2005). As for the internal microbiome described by Hahn and Dheilly (2018), if the observed bacteria were not represented by adherent external bacteria, they may have in fact been representing an endomicrobiome. The cestode gonopore connects the outside environment to internal cavities of the reproductive tract, such as the genital atrium below the surface of the tegument. As this is connected to the outside environment via the vagina and the common gonopore opening, though it is an internal space by definition, it is a region where the reproductive system is open to the external environment in the same way as our digestive system is open to the external environment. The presence of bacteria within the reproductive tract may not be a “normal” condition, but a type of infection of the tapeworm, just as vertebrates can get infections of their reproductive tract; however, it is also possible that this might be a “normal” microflora of the reproductive tract. Further validation of this finding is yet required.

In another study, Kashinskaya et al., (2020) estimated the structure of bacterial communities associated with the gastrointestinal tract of perch *Perca fluviatilis* with a parallel study of the microbiota associated with intestinal cestodes themselves. The bacteria from the genus *Mycoplasma* , *Serratia* , and *Pseudomonas* were the dominant taxa in the microbiota of cestodes of the genus*Proteocephalus* (Kashinskaya et al., 2020). Adding the “control” group (uninfected fish) and significantly improving the sample collection protocol for the cestode’s microbiota, we have analyzed the associated microbiota of the gut of whitefish as a complex multilevel system. According to the desorption method in Ringer’s solution, it was shown that for *Proteocephalus* sp. cestodes there are associations of several groups of microorganisms: 1) ”Surface” microbiota of cestodes, similar to the microbiota of the host (fraction D0), 2) weakly associated microbiota of cestodes (fraction D1-D5), 3) microbiota strongly associated with the tegument (fraction D6), and 4) microbiota obtained after removal of the tegument from cestodes (D7) (Figure 7). The presence of bacteria after Triton treatment can be explained by the fact that cestodes have no digestive system, but they do have a reproductive tract and excretory organs (as noted above). Moreover, the specialized symbiotic organ in the form of infoldings of the dorsal and ventral surfaces of cestodes body *Elicilacunosus dharmadii* from eagle ray (*Aetomylaeus nichofii*) has been demonstrated to accommodate their bacterial symbionts (Caira et al, 2021). Hughes-Stamm and co-authors identified 7 microbial morphotypes, including*Eubacteria* , and *Spirochaetes* , associated with the dorsal surface and excretory papillae regions of the trematode*Gyliauchenn nahaensis* isolated from *Siganus doliatus* ,*S. orallines* , *S. puellus* and *S. lineatus*(Hughes-Stamm *et al* ., 1999). According to these findings we do not exclude the presence of bacteria in the reproductive tract of cestodes.

Specific microenvironments of the intestine provide specific conditions for colonization by different groups of bacteria with a wide spectrum of functional and biochemical activity. The data from figures 3 and 4 clearly shows a unique microbial signature from the cestodes as compared to the fish hosts. This is seen in the PCoA and also in the microbiome profiles from the separate fractions D0–D7. Among the distinct differences, several OTUs are worth noting. *Delftia* ,*Acinetobacter* , *Azospirillum* , *Deefgea* , and*Sphingobium* are among those taxa more abundant in the cestode samples. The presence of these particular taxa may be

indicating certain adaptations suitable for a symbiotic opportunist. Some species of*Delftia* produce potent sidirophores that have antimicrobial activity as well. While not unusual among bacteria generally, these features of sidirophores (chelation of metals for detoxification and antimicrobial activity) can be of benefit in a competitive environment like the host gut (Margarita et al., 2016).

Acinetobacter spp., are noted to possess a CRISPR/Cas system that positively influences biofilm production (Sarshar et al., 2021) that can in turn enhance persistence within the host, which may explain the close association with the cestode tegument in this study, predominating in fraction D6 (Figure S3c), even during removal and cleaning of the cestodes from fish gut. They are a group of species common in the natural environment, but are increasing in importance in human clinical settings due to increasing antibiotic resistance. From figure S3c we can see that much of *Sphingobium* is released early and also some*Comamonas* . This result is suggestive that these bacteria are not likely to be in the subsurface within the gonopore or parts of the reproductive tract. Others such as *Delftia* and Mycoplasma (especially Mycoplasma since these are characterized by having very small cells and frequently exist as commensals due to their reduced genome) are more likely among cells that are located in more internal sites and removal and collection of these cells requires increasingly more stringent treatments. This persistence may be facilitated by biofilms or other mechanisms of adherence. The findings of Mycoplasma with parasites of the intestinal tract of fish is a possible indication of coevolution with this host with specific adaptations for survival such as receptor mediated surface attachment to the tegument or internal surfaces of the cestode (and/or the host). Symbiotic associations between cestodes and mycoplasma have been noted previously (Margarita 2016) and so the benefits such as increased ATP production noted previously may also be at work imposing selective pressures on the host-pathogen relationship.

In summary, based on previous hypotheses we may make several conclusions: first, the rinsing procedure from the mucosa (as well as rinsing and shaking procedures for cestodes) are suitable approaches for broadly different ecological, biological, physiological, etc. studies, where detailed deep insight of the gut bacterial community structures and functions is needed because it permits separation of the microbial community into different subcommunities. But it has to be noted that this approach is based on features of adherence of different bacterial groups, and separation based on some other bacterial features (for example cell size, sedimentation velocity, or others) that may give different results. Since the aforementioned second, third, and fourth hypotheses were partially or completely supported because the gut microbiota of whitefish was affected by their cestode infestation, thus, such factor as parasite infestation has to be taken into account in studies focused on a "normal" vertebrate microbiome where different groups of parasites are also a normal part of the ecosystem.

In general, the ecosystem of the vertebrate gut is a very complex system that needs suitable and advanced technological methods and approaches for correct investigation and interpretation of results. The obtained results in the present study support an ecological approach, where all possible parts of the natural ecosystems have to be considered in order to describe, understand, and in the future, potentially manage any ecosystem properly.

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Data availability statement. Raw sequence reads have been made available in the Sequence Read Archive (SRA) in NCBI (BioProject: PRJNA814856).

Benefit-sharing statement.

Benefits from this research accrue from the sharing of our data and results on public databases as described above.

Author contribution

Elena N. Kashinskaya and Mikhail M. Solovyev conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, contributed reagents, wrote the paper, approved the final draft. Evgeniy Simonov performed the bioinformatics analysis, analyzed the data, prepared figures and/or tables, approved the final draft. Larisa Poddubnaya performed scanning and transmission electron microscopy, prepared figures and approved the final draft. Pavel Vlasenko performed analysis of the 28S rRNA gene of*Proteocephalus* sp. and approved the final draft. Anastasya Shokurova designed graphs, prepared figures and/or tables. Aleksey Parshukov participated in the extraction of DNA from samples and approved the final draft. Karl Andree analyzed the data, wrote the paper and approved the final draft.

Animal approval

The following information was supplied relating to ethical approvals (i.e., approving institutional body and any reference numbers): The present research has met the requirements guided by the order of the High and Middle Education Ministry (care for vertebrate animal included in scientific experiments, text number 742 from 13-11-1984) and additionally by the Federal Law of the Russian Federation text number 498 FL (from 19-12-2018) with regard to the humane treatment of animals.

Conflict of interest. The authors declare no conflict of interest.

Figures:

Figure 1. Histological dissections of whitefish intestine infected by*Proteocephalus* sp. (A, B) - sections were stained by Alcian Blue at pH 2.5; (C, D, E, F) by Harris' Haematoxylin and Eosin.

Figure 2. SEM and TEM observation of *Proteocephalus* sp. surface before (A, B, D) and after (C, E, F) Triton desorption. A, B. SEM view of arrangement of filamentous microtriches on strobila surface. C. SEM view of smooth surface of strobila. D. TEM view of the tegument showing distal syncytial cytoplasm covered with microtriches and supported by basal lamina and fibrillar extracellular layer. E. SEM view of the surface losing distal syncytial cytoplasm, note places of the connection of distal cytoplasm with sunken perikarya. F. TEM of a portion of the tegument losing distal cytoplasm with microtriches, note basal lamina with fibrillar extracellular layer along the border of the tapeworm. Abbreviations: bl, basal lamina; cb, cylindrical base of microtriches; dc, distal syncytial cytoplasm; ds, distal shaft of microtiches; em, extracellular matrix; fm, filamentous microtriches; mf, muscle fibers; p, pores; sm, spiniform microtriches; ss, smooth surface.

Figure 3. Phylum composition of the microbial communities of different segments of the digestive tract of whitefish and the microbiota associated with cestodes parasitizing the intestine of *C. l. pidschian* .

Figure 4. Dominant OTUs at the lowest taxonomical level within the microbial communities from different segments of the digestive tract of whitefish and microbiota associated with cestodes parasitizing the intestine of *C. l. pidschian* .

Figure 5. Principal coordinates analysis (PCoA) for microbial communities of different segments of the digestive tract of uninfected and infected *C. l. pidschian* and cestodes parasitizing the intestine of fish.

Figure 6. LEfSe results presenting the identified OTUs that showed significant differences in abundances between the analyzed groups.

Figure 7. Different microbial communities associated with infected*C. l. pidschian* and the cestodes, *Proteocephalus* sp. parasitizing the intestine of fish.

Figure S1. Sympatric pair of whitefish of the *Coregonus lavaretus*complex inhibited the Lake Teletskoye (Russia). a) benthivorous *C. l. pidschian* , b) planktivorous *C. l. pravdinellus* .

Figure S2. Schematic view of sample collection. a) Organizaton of gastriontestinal tract of different forms of whitefish. b) Washing of mucosa from different segment of gastrointestinal tract of fish. c) desoption of bacteria from tegument of cestodes.

Figure S3. Diversity analysis of the microbial community associated with different fractions of cestodes. a) The richness and diversity estimates of microbial communities. b) Principal coordinates analysis (PCoA). c) The dominant OTUs of associated microbiota of cestodes. The asterisk character indicates significance at p [?] 0.05 using Dunn's test.

Figure S4. Test effect of factor ¡¡Fish¿¿ on microbial community of cestodes using ADONIS test on Weighted UniFrac matrix.

Tables:

Table 1. Metrics of richness and diversity estimates of the microbial community associated with different parts of the digestive tract of whitefish and cestodes parasitizing the intestine of *C. l. pidschian* .

Fish	Segment of digestive tract	Type of sample	Chao1	Number of OTUs	Number of OTUs	Shannon	Shannon	Simpson	Si
Infected *C. l. pravdinellus*	Stomach	Content	449.1±44.5	268.9±30.8	268.9±30.8	4.1±0.4	4.1±0.4	0.8±0.0	0.8
		Mucosa	277.6±37.0	165.5±28.7	165.5±28.7	3.4±0.4	3.4±0.4	0.7±0.1	0.7
		Washout	489.1±58.1	264.9±37.1	264.9±37.1	5.0±0.3	5.0±0.3	0.9±0.0	0.9
		Mean±SE	**405.3±46.5**	**233.1±32.2**	**233.1±32.2**	**4.2±0.4**	**4.2±0.4**	**0.8±0.0**	**0.8**
	Intestine (anterior)	Content	353.5±107.9	200.7±64.7	200.7±64.7	2.9±0.5	2.9±0.5	0.7±0.1	0.7
		Mucosa	125.2±20.6	76.8±11.5	76.8±11.5	1.9±0.4	1.9±0.4	0.4±0.1	0.4
		Washout	152.1±12.2	73.7±6.4	73.7±6.4	3.8±0.2	3.8±0.2	0.8±0.0	0.8
		Mean±SE	**210.2±46.9**	**117.1±27.5**	**117.1±27.5**	**2.8±0.4**	**2.8±0.4**	**0.6±0.1**	**0.6**
	Intestine (posterior)	Content	606.5±90.8	416.3±69.0	416.3±69.0	4.0±0.5	4.0±0.5	0.7±0.1	0.7
		Mucosa	326.3±72.4	170.4±27.9	170.4±27.9	3.8±0.3	3.8±0.3	0.8±0.1	0.8
		Washout	317.2±34.1	170.4±18.0	170.4±18.0	5.1±0.2	5.1±0.2	0.9±0.0	0.9

Fish	Segment of digestive tract	Type of sample	Chao1	Number of OTUs	Number of OTUs	Shannon	Shannon	Simpson	Si
		Mean±SE	**416.6±65.7**	**252.3±38.3**	**252.3±38.3**	**4.3±0.4**	**4.3±0.4**	**0.8±0.0**	**0.8**
Uninfected *C. l. pidschian*	Stomach	Content	1704.4±238.0	1288.1±183.5	1288.1±183.5	5.4±0.5	5.4±0.5	0.8±0.1	0.8
		Mucosa	473.8±60.0	276.6±46.6	276.6±46.6	4.4±0.4	4.4±0.4	0.8±0.0	0.8
		Washout	729.4±102.4	479.5±70.9	479.5±70.9	5.2±0.6	5.2±0.6	0.9±0.0	0.9
		Mean±SE	**969.2±133.5**	**681.4±100.3**	**681.4±100.3**	**5.0±0.5**	**5.0±0.5**	**0.8±0.0**	**0.8**
	Intestine (anterior)	Content	1181.9±187.2	882.5±160.9	882.5±160.9	6.1±0.6	6.1±0.6	0.9±0.1	0.9
		Mucosa	234.4±32.2	140.8±19.4	140.8±19.4	3.1±0.3	3.1±0.3	0.7±0.0	0.7
		Washout	474.5±52.8	260.5±28.4	260.5±28.4	4.1±0.3	4.1±0.3	0.8±0.0	0.8
		Mean±SE	**630.3±90.7**	**427.9±69.6**	**427.9±69.6**	**4.4±0.4**	**4.4±0.4**	**0.8±0.0**	**0.8**
	Intestine (posterior)	Content	1103.6±167.1	801.4±143.1	801.4±143.1	5.2±0.6	5.2±0.6	0.8±0.1	0.8
		Mucosa	226.1±31.5	124.0±17.3	124.0±17.3	3.4±0.2	3.4±0.2	0.8±0.0	0.8
		Washout	251.9±41.2	147.1±22.5	147.1±22.5	2.9±0.3	2.9±0.3	0.7±0.1	0.7
		Mean±SE	**527.2±80.0**	**357.5±61.0**	**357.5±61.0**	**3.8±0.4**	**3.8±0.4**	**0.8±0.1**	**0.8**
Infected *C. l. pidschian*	Stomach	Content	2503.4±589.6	1986.5±414.5	1986.5±414.5	7.6±0.5	7.6±0.5	1.0±0.0	1.0
		Mucosa	483.7±62.7	283.4±27.7	283.4±27.7	5.8±0.2	5.8±0.2	1.0±0.0	1.0
		Washout	895.8±146.6	602.1±108.1	602.1±108.1	5.8±0.8	5.8±0.8	0.9±0.1	0.9
		Mean±SE	**1294.3±266.9**	**857.3±183.4**	**857.3±183.4**	**6.4±0.5**	**6.4±0.5**	**0.9±0.0**	**0.9**
	Intestine (anterior)	Content	1392.8±279.6	1119.8±265.3	1119.8±265.3	7.0±0.9	7.0±0.9	0.9±0.1	0.9
		Mucosa	182.2±46.2	98.3±26.9	98.3±26.9	3.8±0.6	3.8±0.6	0.9±0.1	0.9
		Washout	319.8±56.7	174.6±31.6	174.6±31.6	3.6±0.5	3.6±0.5	0.8±0.1	0.8
		Mean±SE	**631.6±127.5**	**464.2±108.0**	**464.2±108.0**	**4.8±0.7**	**4.8±0.7**	**0.8±0.1**	**0.8**
	Intestine (posterior)	Content	1672.1±358.9	1367.4±324.2	1367.4±324.2	7.6±0.6	7.6±0.6	1.0±0.0	1.0
		Mucosa	318.0±44.5	203.3±35.8	203.3±35.8	3.9±0.7	3.9±0.7	0.8±0.0	0.8
		Washout	405.1±53.4	212.3±56.3	212.3±56.3	3.7±.0.4	3.7±.0.4	0.8±0.1	0.8
		Mean±SE	**798.4±152.3**	**594.3±138.8**	**594.3±138.8**	**5.1±0.6**	**5.1±0.6**	**0.8±0.0**	**0.8**
Cestodes	Cestodes	D0	894.2±282.0	894.2±282.0	649.3±229.9	649.3±229.9	4.7±0.9	4.7±0.9	0.7
		D1	481.2±48.6	481.2±48.6	236.4±21.7	236.4±21.7	3.0±0.3	3.0±0.3	0.7
		D2	405.9±49.1	405.9±49.1	209.3±26.6	209.3±26.6	2.4±0.3	2.4±0.3	0.6
		D3	405.0±50.5	405.0±50.5	206.6±26.1	206.6±26.1	2.7±0.3	2.7±0.3	0.6
		D4	402.3±47.4	402.3±47.4	212.5±28.9	212.5±28.9	2.7±0.3	2.7±0.3	0.6
		D5	358.5±36.0	358.5±36.0	188.3±19.4	188.3±19.4	2.8±0.3	2.8±0.3	0.6
		Mean±SE	**410.6±20.8**	**410.6±20.8**	**210.6±11.0**	**210.6±11.0**	**2.7±0.1**	**2.7±0.1**	**0.6**
		D6	538.0±53.9	538.0±53.9	294.8±29.5	294.8±29.5	2.7±0.3	2.7±0.3	0.6
		D7	499.4±35.7	499.4±35.7	240.9±20.6	240.9±20.6	3.4±0.3	3.4±0.3	0.7

Table 2. Alpha- and beta-diversity of microbial community of different fractions of cestodes obtained before and after desorption. The bold character indicates significance at p [?] 0.05.

Combination	Alpha-diversity	Alpha-diversity	Alpha-diversity	Alpha-diversity	Alpha-diversity
	Richness estimates	Richness estimates	Richness estimates	Richness estimates	Diversity estimates
	OTU	OTU	Chao1	Chao1	Shannon
	Z statistic	adjusted p-value	Z statistic	adjusted p-value	Z statistic
D0 vs. D1-D5	2.58	**0.015**	2.06	0.060*	2.20
D0 vs.D6	0.82	0.206	0.56	0.345	1.96
D0 vs. D7	-1.55	0.122	-0.71	0.358	-0.72
D1-D5 vs. D6	-2.71	**0.020**	-2.33	**0.059**	-0.01
D1-D5 vs. D7	1.34	0.135	2.01	**0.044**	2.22
D6 vs. D7	-1.03	0.182	-0.21	0.415	1.73

Table 3. Comparison of the associated microbiota between different types of samples using ADONIS test based on UnWeighted UniFrac matrix. The bold character indicates significance at p [?] 0.05.

Combination	Combination	Combination	R^2	P-value corrected	R^2
			Infected *C. l. pidschian*	Infected *C. l. pidschian*	Unin
D0	vs.	Stomach content	0.17	**0.004**	0.15
	vs.	Stomach mucosa	0.19	**0.002**	0.20
	vs.	Washout from stomach mucosa	0.18	**0.041**	0.21
	vs.	Anterior content	0.09	0.341	0.16
	vs.	Anterior mucosa	0.08	0.360	0.16
	vs.	Washout from anterior mucosa	0.00	0.978	0.39
	vs.	Posterior content	0.18	**0.007**	0.15
	vs.	Posterior mucosa	0.17	0.146	0.17
	vs.	Washout from posterior mucosa	0.32	**0.013**	0.38
D1-D5	vs.	Stomach content	0.06	**0.012**	0.06
	vs.	Stomach mucosa	0.07	**0.007**	0.10
	vs.	Washout from stomach mucosa	0.06	**0.009**	0.10
	vs.	Anterior content	0.05	**0.015**	0.08
	vs.	Anterior mucosa	0.05	**0.017**	0.08
	vs.	Washout from anterior mucosa	0.01	0.349	0.15
	vs.	Posterior content	0.06	**0.013**	0.09
	vs.	Posterior mucosa	0.06	**0.010**	0.08
	vs.	Washout from posterior mucosa	0.09	**0.003**	0.14
D6	vs.	Stomach content	0.23	**0.013**	0.23
	vs.	Stomach mucosa	0.26	**0.010**	0.30
	vs.	Washout from stomach mucosa	0.24	**0.011**	0.31
	vs.	Anterior content	0.19	**0.020**	0.28
	vs.	Anterior mucosa	0.15	**0.035**	0.26
	vs.	Washout from anterior mucosa	0.05	0.341	0.41
	vs.	Posterior content	0.23	**0.014**	0.29
	vs.	Posterior mucosa	0.23	**0.014**	0.26
	vs.	Washout from posterior mucosa	0.31	**0.003**	0.39
D7	vs.	Stomach content	0.33	**0.003**	0.33
	vs.	Stomach mucosa	0.36	**0.003**	0.41
	vs.	Washout from stomach mucosa	0.34	**0.003**	0.41
	vs.	Anterior content	0.26	**0.009**	0.37
	vs.	Anterior mucosa	0.21	**0.012**	0.36
	vs.	Washout from anterior mucosa	0.09	0.173	0.52
	vs.	Posterior content	0.32	**0.005**	0.37

Combination	Combination	Combination	R^2	P-value corrected	R^2
	vs.	Posterior mucosa	0.31	**0.004**	0.36
	vs.	Washout from posterior mucosa	0.43	**0.002**	0.51

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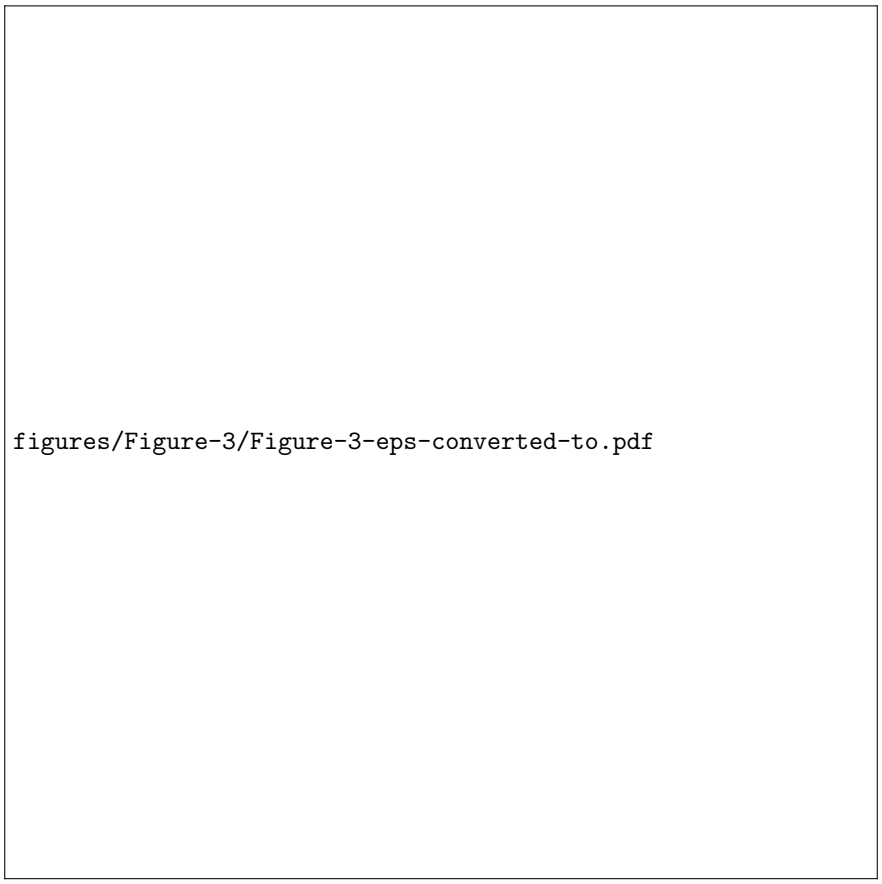

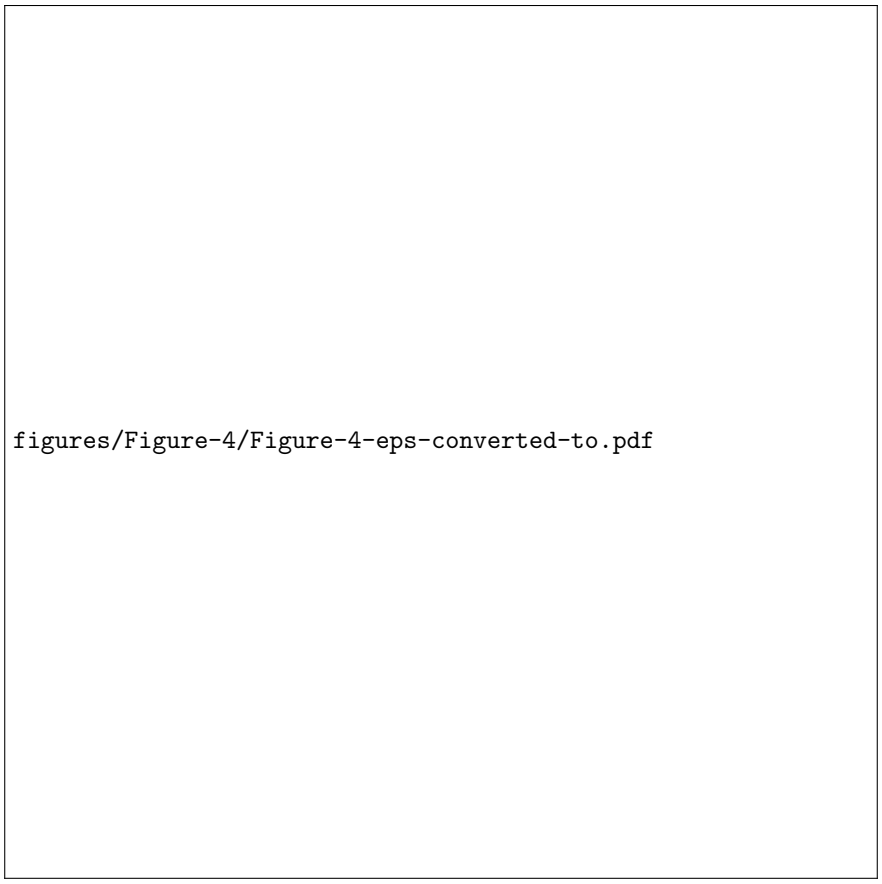

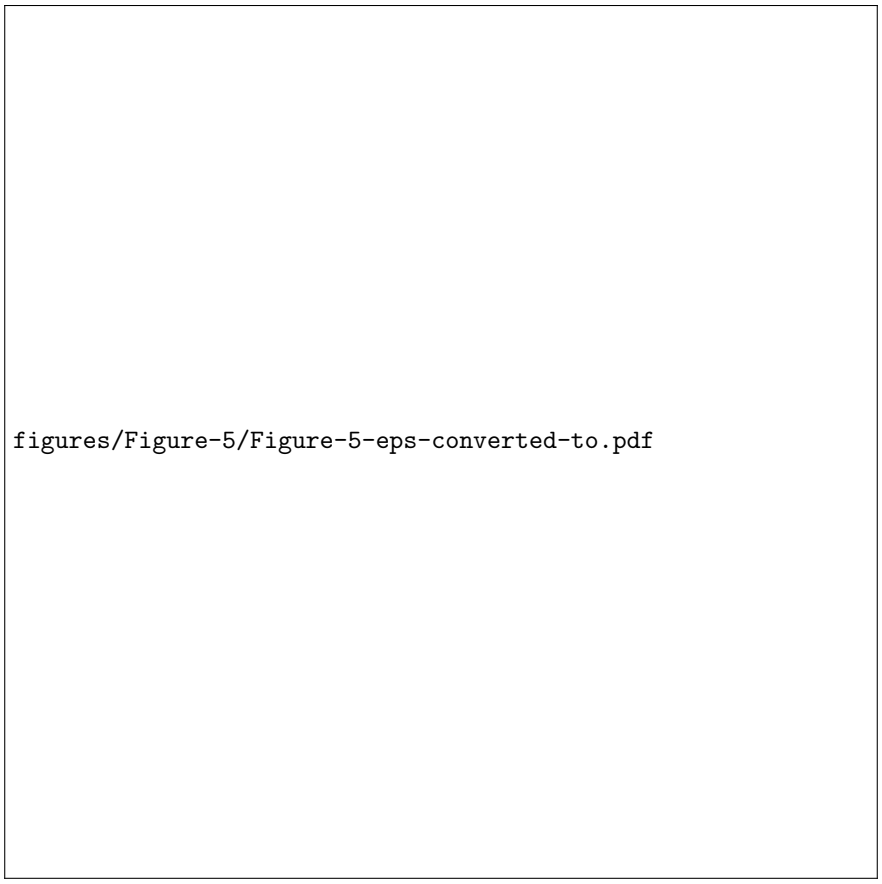

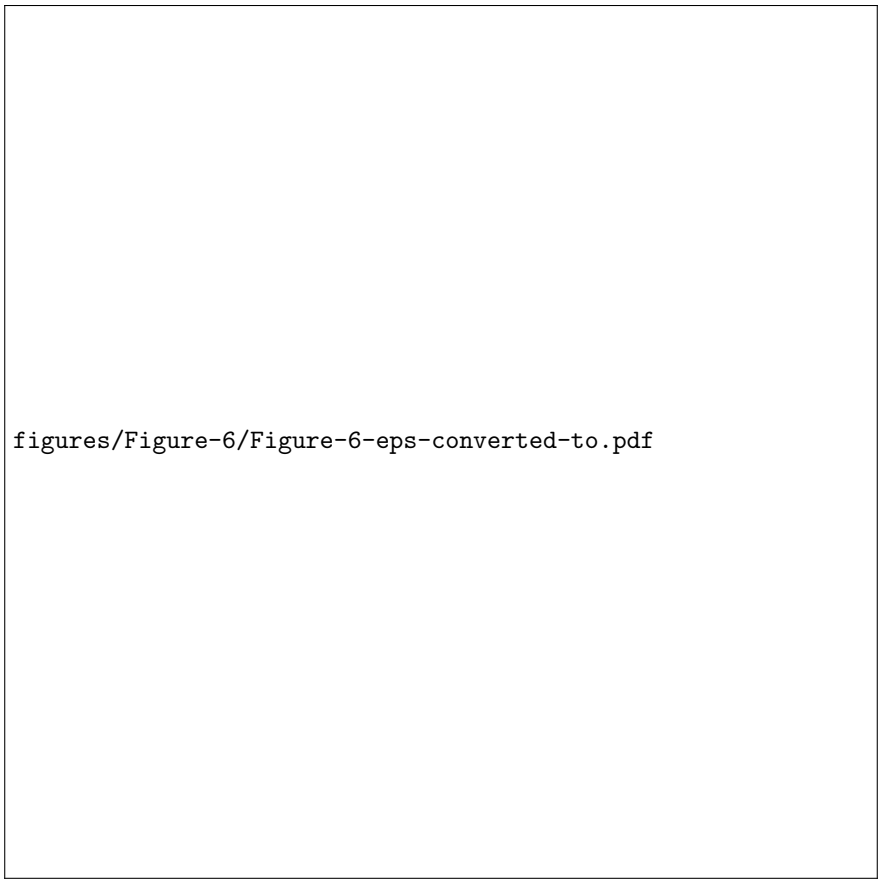

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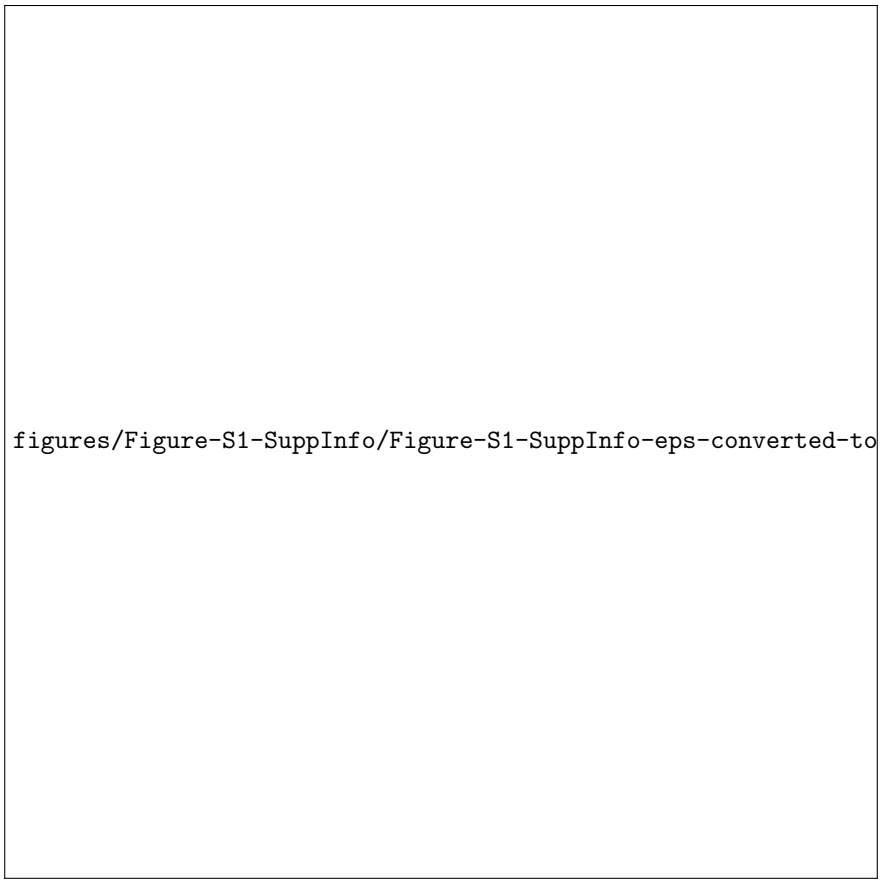

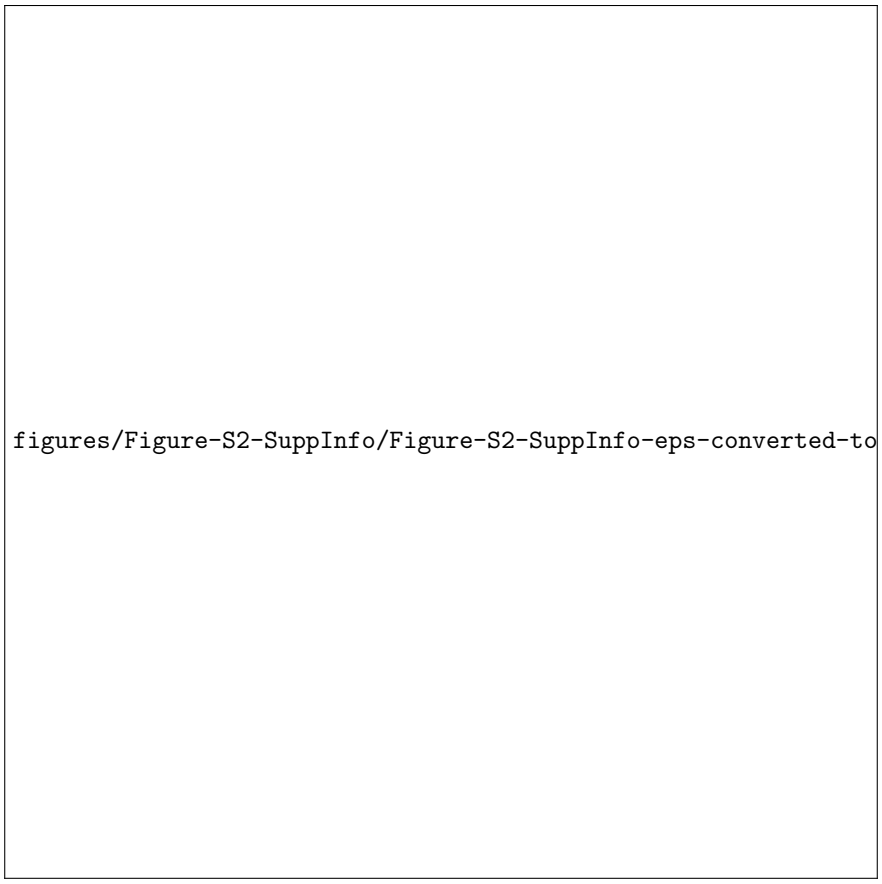

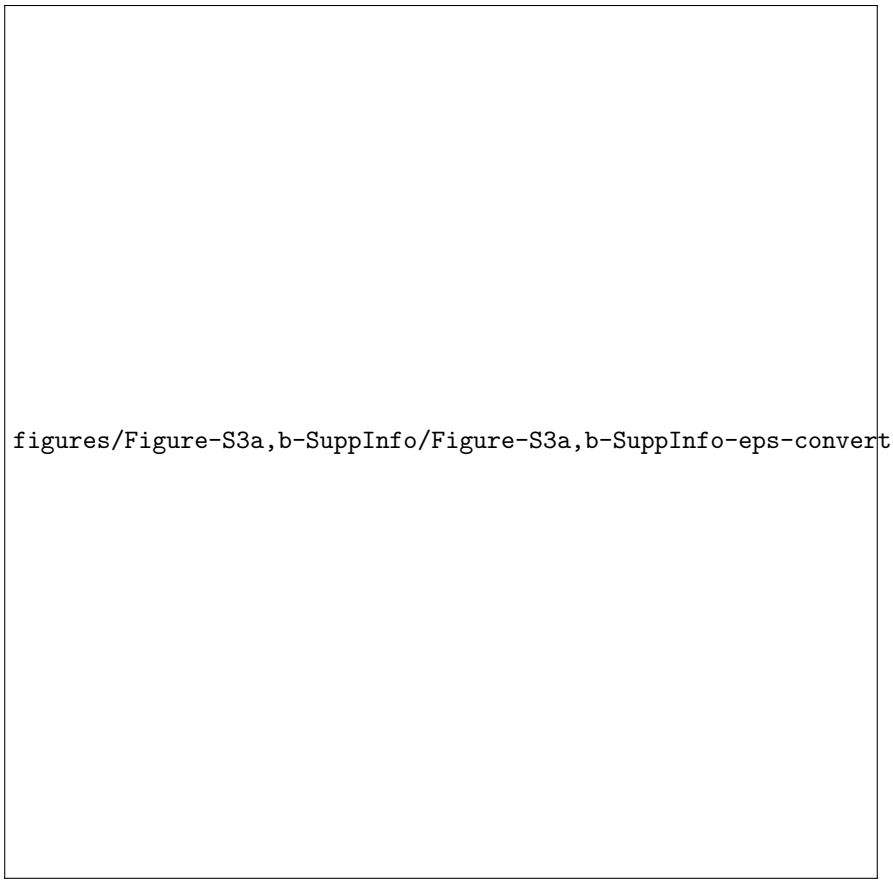

figures/Figure-S3c-SuppInfo/Figure-S3c-SuppInfo-eps-converted-to.pdf