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Variations of the intestinal gut microbiota of farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum), depending on the infection status of the fish

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Running title: intestinal gut microbiota of rainbow trout

Abstract

Aims. The aim of the present study was to investigate the composition of the intestinal microbiota during the acute stage of a bacterial infection to understand how dysbiosis of the gut may influence overall taxonomic hierarchy and diversity, and determine if there exists a bacterial taxon(s) that serve as markers for healthy or diseased rainbow trout (*Oncorhynchus mykiss*).

Methods and Results. From July to September 2015 29 specimens of three-year-old (an average weight from 240.9±37.7 to 850.7±70.1 g) rainbow trout *Oncorhynchus mykiss* were studied. Next-generation high-throughput sequencing of the 16S ribosomal RNA genes was applied to stomach and intestinal samples to compare the impact of infection status on the microbiota of rainbow trout *Oncorhynchus mykiss* (Walbaum) from the northwest part of Eurasia (Karelian region, Russia). The alpha diversity (Chao1, Simpson and Shannon index) of the microbial community of healthy rainbow trout was significantly higher than in

unhealthy fish. The greatest contribution to the gut microbial composition of healthy fish was made by OTU's belonging to *Bacillus, Serratia, Pseudomonas, Cetobacterium*, and *Lactobacillus*. Microbiota of unhealthy fish in most cases was represented by the genera *Serratia, Bacillus*, and *Pseudomonas*. In microbiota of unhealthy fish there were also registered unique taxa such as bacteria from the family *Mycoplasmataceae* and *Renibacterium*. Analysis of similarities (ANOSIM test) revealed the significant dissimilarity between the microbiota of stomach and intestine ($p \leq 0.05$).

Conclusions. A substantial finding was the absence of differences between microbial communities of the stomach and intestine in the unhealthy groups if compared with healthy fish.

Significance and Impact of Study. These results demonstrated alterations of the gut microbiota of farmed rainbow trout, *O. mykiss* during co-infections and can be useful for the development of new strategies for disease control programs.

Keywords: bacterial community, salmonids, bacterial kidney disease, bacterial cold-water disease, freshwater cage aquaculture

Introduction

Aquaculture has been the world's fastest growing food producing industry during recent decades, producing up to 60.4 billion tons of fish products per year (FAO, 2010). Salmonids are among the most successful aquaculture species when measured by growth of production and one of the most important fish for aquaculture in the northern European region. According to the Food and Agriculture Organization, the share of salmon and trout in world trade has increased strongly in recent decades, becoming the largest single commodity by value in 2013 (FAO, 2016). Rainbow trout is one of the main aquaculture species in

Europe: its production exceeds 250 million tons with a value close to 700 million euros. In the Russian Federation the most important region for cage aquaculture of salmonids is Karelia located in the north-east part of Russia and has a climate similar to countries of northern Europe. At the present time, Karelia produces around 21 thousand tons of fish products where 97% of production is attributed to rainbow trout.

One of the most important problems in aquaculture is infectious diseases due to different pathological agents such as viruses, bacteria, fungi, and parasites (protozoan, cestoda, trematoda, crustacean etc.) that cause significant damage and great economical loss (Bebak-Williams et al., 2002). Unfortunately, studies regarding problems of fish diseases in cage aquaculture in Karelia are sporadic, do not have a wide coverage in the scientific literature and are represented by only a few works, which is under-representation for a region with highly developed trout farming and with future broad investment prospects. The main parasitic diseases in cage trout are ichthyobodosis (costiasis), ichthyophthiriasis (white spot disease), gyrodactylosis, digenean trematode infection, crustacean diseases (e.g. argulosis), among many others (Evseyeva, Dzubuk, 2016; Ieshko et al., 2016). Aggravating the situation with diseases of salmonids is the lack in Karelia of local brood stocks of rainbow trout for reproductive purposes to support the local aquaculture industry, which necessitates the spawn and fingerlings be bought from other regions of the Russian Federation (Murmansk Oblast, Leningrad Oblast, Arkhangelsk Oblast, Krasnodar Krai and North Ossetia-Alania) and other countries such as United Kingdom, USA, Belarus, Denmark, and Finland. It is very well known that with transport of fish species other organisms are introduced, some potentially pathogenic, including different pathogens that have never appeared in the local water bodies before. Such was the situation, for example, with triploid clone *Gyrodactylus salaris* RBT that was introduced in Karelia with fry imported from Finnish hatcheries in 2007. This has

become a problematic and harmful parasite species for both native and cultured salmonids, including rainbow trout.

In contrast to invasive pathogenic parasites of rainbow trout, in the Karelia region bacterial pathologies have been studied to a much lesser extent. The most studied bacterial pathogens for salmonids in Karelia are *Pseudomonas, Aeromonas, Flavobacterium* and *Yersinia* (Ryzhkov et al., 2007). The origin of these bacterial pathogens was intensive introduction of trout fry from Finland, Denmark, etc. during a period of active development of cage aquaculture in Karelia (Nechaeva, 2014).

The bacterial community of fish gut possesses different functions such as aiding in digestion and can play a key role in the defense against different pathogens (Verschuere et al., 2000, Araújo et al., 2014). There are described a plethora of biotic and abiotic factors that in nature are potentially able to affect the fish gut bacterial community. Under aquaculture conditions the number of factors is restricted if compared with conditions in nature, but includes among others the proximate composition of diet, fish age, ambient temperature, pH and chemical composition of water, etc (Heikkinen et al., 2006, Mansfield et al., 2010, Desai et al., 2012, Navarrete et al., 2012, Geurden et al., 2014, Ingerslev et al., 2014a, Llewellyn et al., 2016, Bruce et al., 2017, Lyons et al., 2017, Huyben et al., 2018, Mente et. al., 2018). It is very well known that the disorders caused by bacteria and parasites may lead to altered feeding behavior and dysfunction of the digestive system. These changes have not always been evaluated in terms of the potential resulting dysbiosis of the gut. Although several studies have focused on the structure of the gut microbial community of salmonids (among others Trust et al., 1974, Yoshimizu et al., 1976, Cahill, 1990, Ringø et al., 1995, 2008, Navarrete et al., 2009). Culture-dependent approaches are well-developed for salmonids but have revealed only around 1% of the microbial community (Amann et al., 1995) while the approaches based on genetic techniques, such as NGS, enable discovery of unculturable

taxonomical groups of bacteria (Ringø et al., 2016). What has been demonstrated is that the dominant core of the gut bacterial community from rainbow trout belongs to *Tenericutes, Firmicutes, Proteobacteria* and *Spirochaetae* (Wong et al., 2013, Ingerslev et al., 2014a, Lyons et al., 2015, 2016, Huyben et al., 2018, Mente et. al., 2018). Moreover, in others studies the dominance of γ-*Proteobacteria* among intestinal microbiota of rainbow trout was noted (Spanggaard et al., 2000, Huber at al., 2004, Pond et al., 2006, Dimitroglou et al., 2009, Etyemez et al., 2015). Unfortunately, studies that focus on the composition of the gut bacterial community of rainbow trout under the influence of different pathogens is almost non-existent (Reveco et al., 2014; Ingerslev et al., 2014b).

The first aim of the present study was to investigate the composition as well as richness and diversity estimates of the stomach and intestinal microbiota during the acute stage of ~~a~~ bacterial infection and, including a comparison with uninfected rainbow trout. The second aim was to compare the variability of the studied parameters of the bacterial community between stomach and intestine from healthy and unhealthy rainbow trout.

Materials and methods

Fish, water and diets. Twenty nine individuals (two years old) of rainbow trout (O. mykiss) were collected in the middle of July (fifteen fish) and August (eight fish), 2015 and in the beginning of September, 2015 (six fish) (Table 1). Water temperatures measured at time of collection in July, August and September (at a depth of 1 to 10 meters) were (mean±SE) 15.4±0.5, 15.3±0.4, and 14.2±0.04°C respectively. The fish farm is located on the northern part of Ladoga Lake, Karelia (Russia, 60°50′03″ N, 31°33′10″ E). Fish were kept in round cages (node-less nylon thread) with diameter of 17.8 m, area of 250 m2, height of 15 m, and volume of 2500 m3. Cages were placed in the open water of the lake at a depth of up to 15 m with average depth in this part of the lake of 20-25 m. The fish density was 2.7-3.5

kg/m3. All fish were fed twice per day (6.6-9.2 g per fish) by the same commercial diet with pellet size 6 mm according to the feeding table provided by the company (Italian feed Veronesi, Verona). The main composition of the diet (feed manufacturer) is given in Table 2. Special treatments were applied by local veterinarians in order to prevent the annual bacterial infections of rainbow trout in cages. The treatments included the following steps: fish were treated by the antibiotic ciprofloxacin from 1st to 14th of July and by MIDIVET (additive based on the fermented meat of clams) from 1st to 11th of July, and then with the probiotic additive Rybin A (probiotic based on Bacillus sp.) during two days (15 – 16th July).

The studied fish were humanely sacrificed by pithing with a needle before sampling. The standard length (cm) was measured and total body mass was weighed (g).

The degree of fullness of their digestive tract (stomach and intestinal regions) was ranked on a scale of 0-5 and assessed as follows: 0-1– gut is empty or containing a negligible amount of food particles (less than 1–2% of the total possible volume of food that could occupy the intestine); 2-3 – gut is moderately full and approximately filled at 50% capacity; and 4-5 – a high amount of food particles in the gut with some distention of the intestine wall. Prior to dissection fish skin was cleaned of mucus using a cotton wad disinfected with 70% ethanol; afterwards the abdomen was dissected using sterile instruments and the gut removed, put in a sterile zip-packet, covered by aluminum foil, and immediately frozen in liquid nitrogen. After one month storage in liquid nitrogen, the guts were thawed on ice and the mucosal layer from the stomach and the anterior, middle, and posterior intestine were taken separately using a spatula. Due to the mucosal layer being damaged by the freeze/thaw cycle the appropriate separation of mucosal layer and gut content was difficult. Thus, the DNA extraction was performed from mucosa layer and gut content together. After that the mucosa was stored in 70% ethanol in a separate sterile microcentrifuge tube at -20°C until DNA extraction.

At the time of sampling (June and September, 2015) the chemical composition of the water was also analyzed as a possible factor that may affect the structure of the gut bacterial community. Water samples were collected from the center of fish cages from an average depth of 5 m. Water samples were kept in glass and plastic bottles that were transported within a few hours to the Centre for Laboratory Analysis and Technical Measurements in Karelia (www.clatikarelia.ru) where the samples were analyzed. 15 physicochemical parameters were analyzed (mean±SE) such as pH (7.3±0.1), suspended solids (<5 mg/dm^3), color index (45.7±4.6 degree), biological oxygen demand in 5 days (BOD5) (1.3±0.2 mgO_2/dm^3), nitrite (<0.01 mg/dm^3), nitrate (0.2±0.1 mg/dm^3), ammonia (0.2±0.018 mg/dm^3) and total nitrogen (0.6±0.3 mg/dm^3), phosphate (<0.01 mg/dm^3), total phosphorus (<0.04 mg/dm^3), total iron (0.16±0.03 mg/dm^3), permanganate oxygen consumed (9.4±1 mgO_2/dm^3), sulphates (8.34±1 mg/dm^3), oil products (0.019±0.007 mg/dm^3) and phenol (<0.0005 mg/dm^3).

Healthy / unhealthy fish. Fish were classified as "healthy" or "unhealthy" according to external signs (skin surface e.g. ulcerations, lesions, haemorrhages, pigmentation, ectoparasites or fungi, mucous production), necroses of fins or gills, anaemia of gill, the condition of operculum, corneal opacity, exophthalmia, fatness, etc.) and internal signs (color of organ tissues, haemorrhages, ulcerations or petechiae in liver, muscles and perivisceral fat, size and consistent of spleen and kidney, gut fullness and inflammation, etc.) (https://www.necropsymanual.net/en/additional-info/fpa/) (Figures 1 and 2). Fish that belonged to the healthy group were divided into three subgroups (groups A and B included fish collected in the middle of July, 2015 whereas group C included fish collected in the middle of August, 2015).

Unhealthy fish were divided into two groups D and E that were collected in the middle of July and in the beginning of September, 2015. The first signs of the disease were observed from the middle of June 2015 and fish mortality in cages progressively increased after June 20th. For determination of the pathogens, twenty-one fish (group D) were transported to All-Russian Research Institute for Experimental Veterinary Medicine (Moscow, Russia). The fish from group E were not analyzed in the lab because of the visual signs (changes in behavior, the lack of appetite, ulcers and spots on the skin and organs, etc.) made clear that the fish were unhealthy.

Taxonomical identification of the pathogens from group D was determined by inspection of the isolates morphological characteristics and specific PCR techniques according to the OIE Diagnostic Manual for Aquatic Animal Diseases (2003). The bacterium *Renibacterium salmoninarum* is a Gram positive, small, non-motile diplobacillus. Growth on KDM-2 (Kidney Disease Medium 2) and SKDM (Selective Kidney Disease Medium) was slow, with pinpoint, opaque white colonies appearing after 14 days of incubation at 15°C. For identification of *R. salmoninarum* two pairs of oligonucleotide primers were used in a nested PCR protocol (Analytik Jena, Biometra TRIO, Germany). The primers used in the first round of amplification were: forward 75–93 (5'-AGC-TTC-GCA-AGGTGA-AGG-G-3' and reverse 438–458 (5'-GCA-ACA-GGT-TTA-TTT-GCC-GGG3; The primers used in the second round of amplification reaction were: forward 95–119 (5'- ATT-CTT-CCA-CTT-CAA-CAG-TAC-AAG-G-3' and reverse 394–415 (5'-CAT-TAT-CGT-TAC-ACC-CGA-AAC-C-3') (Chien et al., 1992). On Anacker and Ordal agar, a strain of *Flavobacterium psychrophilum* was isolated in pure culture from kidney. For identification of *F. psychrophilum* two pairs of universal oligonucleotide primers, 20F (AGA GTT TGA TCA TGG CTC AG) and 1500R (GGT TAC CTT GTT ACG ACT T), were used. The specific

primers used in the second round of amplification were: forward 190–206 (GTT GGC ATC AAC ACA CT) and reverse 1278–1262 (CGA TCC TAC TTG CGT AG) (Wiklund et al., 2000).

Isolation of bacterial DNA. In order to extract DNA from stomach and mucosa the Kit DNA sorb B (kit for DNA extraction, NekstBio, Central Research Institute of Epidemiology, Moscow, Russia) was applied. This kit is designed to extract DNA from a wide variety of clinical materials such as phlegm, faeces, blood, saliva and others. The method is based on the lysing and nuclease-inactivating properties of the chaotropic agent guanidiniumthiocyanate together with the nucleic acid-binding properties of silica particles or diatoms in the presence of this agent (Boom et al., 1990). Before DNA extraction, the tissue from each individual fish was mechanically homogenized by pestle for 1 min using a hand-held homogenizer. Tissue samples were fixed in a lysis buffer from the kit. Following the kit manufacturer's protocol DNA was extracted from 100 mg of sample.

16S r-RNA and sequencing. The V3-V4 region of the 16S rRNA gene (length 460 bp) was amplified in the Evrogen company (Moscow) with the primer pair 5'-TCG-TCG-GCA-GCG-TCA-GAT-GTG-TAT-AAG-AGA-CAG-CCT-ACG-GGN-GGC-WG-CAG-3' and 5'- GTC-TCG-TGG-GCT-CGG-AGA-TGT-GTA-TAA-GAG-ACA-GGA-CTA-CHV-GGG-TAT-CTA-ATCC- 3' (Klindworth et al., 2013). The concentration of the DNA was quantified on a Qubit (ThermoFisher, Waltham, MA, USA). Amplification of the V3-V4 region of the 16S rRNA gene was done according to the protocol from Illumina (16S Metagenomic Sequencing Library Preparation, Part # 15044223, Rev. B). After producing amplicons, the libraries were cleaned up and mixed in equimolecular portions using SequalPrep™ Normalization Plate Kit (ThermoFisher, Cat # A10510-01, Waltham, MA,

USA) and checked using capillary electrophoresis. Then, the pool of libraries was sequenced on Illumina MiSeq (250 cycles for forward and reverse read pairs) using MiSeq Reagent Kit v2 (500 cycles).

Sequence processing. Paired-end reads between forward and reverse read pairs were merged and quality filtered with Mothur 1.31.2 (Schloss et al., 2009). Any reads with ambiguous sites and homopolymers of more than eight bp and sequences shorter than 350 or greater than 500 bp were removed. QIIME 1.9.1 (Caporaso et al., 2010) was used for continued processing of the sequence data. *De novo* (abundance based) chimera detection using USEARCH 6.1 (Edgar, 2010) was applied to identify possible chimeric sequences ('identify_chimeric_seqs.py' with an option '-m usearch61' in QIIME). After chimera filtering, the QIIME script 'pick_open_reference_otus.py' with default options was used to perform open-reference OTU picking (by UCLAST; Edgar, 2010), taxonomy assignment (UCLAST), sequence alignment (PyNAST 1.2.2; Caporaso et al., 2010) and tree-building (FastTree 2.1.3; Price et al., 2010). This algorithm involves several steps of both closed-reference and open-reference OTU picking followed by taxonomy assignment, where the Greengenes core reference alignment (release 'gg_13_8'; DeSantis et al., 2006) was used as a reference. Chloroplast, mitochondria and nonbacterial sequences were removed from further analysis.

Analysis of alpha and beta diversity. The richness (Chao1 index) and diversity estimates (Shannon and Simpson index) per sample were calculated using QIIME. To test for group in the richness and diversity estimates, a nonparametric ANOVA (PERMANOVA) was conducted in PAST (Hammer et al., 2001). To explore the effect of various explanatory variables, i.e. type of tissue (intestine, stomach), health status of fish (healthy/unhealthy) on

the groupings of bacterial communities, two-way and one-way PERMANOVA based on the weighted UniFrac distance matrix (calculated in QIIME) were performed. To visualize differences among groups of samples non-metric multidimensional scaling (PCoA) was performed using phyloseq R-package (McMurdie, Holmes, 2013).

Functional analysis. The PICRUSt software package (Langille et al. 2013) was used to predict metagenome functional content of microbial communities. We generated the KEGG pathways (Kyoto encyclopedia of genes and genomes) and categorized functions to different gene categories at levels 1, 2, and 3. The categorized functions for different levels (frequency of occurrences of every group of genes in genomes) then were transformed to percentages from total quantity of genes obtained, and the differences between groups of samples were calculated by using PERMANOVA, at $p \leq 0.05$.

Nucleotide sequence accession numbers. Nucleotide sequences were deposited in the Sequence Read Archive (NCBI-SRA, BioProject PRJNA 482823).

Results

The mean weight (±SD) of the fish from the healthy group was significantly ($t=2.72$, $p=0.01$) higher (627±55.8 g) than in the unhealthy group (396±48 g). Fish from the healthy group (example Figure 1) could be characterized as fish with normal daily behavior and feeding activity (arbitrary scale of gut fullness of sacrificed fish was 4-5) during the entire period of observations, with transparent cornea, and viscera (heart, kidney, liver, and swim bladder) without signs of abnormality.

Unhealthy fish (group D) had fin rot and skin ulcers (example, Figure 2a). There was inflammation of the kidney especially in the posterior part. The gut was almost empty (arbitrary scale was 0-2). From the kidney of fish in group D there was isolated and identified gram-negative *Flavobacterium psychrophilum*, the causative agent of myxobacteriosis. From

the liver and kidney there was isolated gram-positive bacteria identified as *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease. Unhealthy fish from group E were characterized by exophthalmia, hemorrhages in muscles, viscera and fat (Figure 2 b,c). No specific aetiological agent was identified by culture on selective media. The liver had a pale color (Figure 2 b).

Rainbow trout were infected by *Caligus lacustris* (Steenstrup and Lütken, 1861) from July to August (groups A, B, C, D). *Caligus lacustris* is a parasitic copepod that belongs to *Caligidae* family from the *Crustacea* subphylum. In July the prevalence and intensity of this parasitic copepod was 15.4% and 0.85 ind. host-1 respectively. In August the prevalence had reached 90% with an intensity of 2.6 ind. host-1, whereas in September no individuals of *C. lacustris* were detected.

Microbiota composition of stomach and intestine of rainbow trout. All OTUs from stomach and intestine of rainbow trout belonged to twenty-two phyla of bacteria. Results of metagenomic sequencing from the healthy groups (A, B and C) have shown *Proteobacteria* (37.9, 59.7, and 30.8 %, correspondingly), *Firmicutes* (41.6, 38.3, and 58.7%, correspondingly), and *Fusobacteria* (17.7, 0.6, and 7.9%, correspondingly) to dominate the stomach of rainbow trout. In regard to unhealthy fish groups (D and E), the composition of bacteria was mostly presented by *Proteobacteria* (77.4 and 75.1%, correspondingly) and for group D by *Actinobacteria* (17.4%) and for group E by *Firmicutes* (22.7%) (Figure 3). The individual composition of the bacterial community for every fish at phylum level is represented in Supplementary File 1.

The taxonomical composition of the bacterial community from the intestine from healthy fish groups (A, B and C) was similar to the bacterial community from their stomach. The dominant groups for intestine in the majority of cases were *Proteobacteria* (35.6, 50.1, and 38.1%, correspondingly), *Firmicutes* (50.1, 33.5, and 33.5%, correspondingly), *Bacteroidetes* (11.4, 12.6, and 10.4%, correspondingly) and *Fusobacteria* (16.2%) for group C. In regard to the unhealthy fish groups (D and E), the composition of bacteria was presented by the following dominants: *Proteobacteria* (49.5 and 58.9%, correspondingly), *Firmicutes* (37.4 and 8.1%, correspondingly), *Actinobacteria* (9.9 %) for group D and *Tenericutes* (31.2%) for group E (Figure 4). The effect of the different health status was also reflected in the ratio of the most abundant bacterial phyla in stomach (Figure 5) and intestine (Figure 6).

On a lower taxonomic level, the microbial community from the stomach of rainbow trout from healthy fish was dominated by *Bacillaceae*, *Enterobacteriaceae*, *Pseudomonadacea, Fusobacteriaceae,* and *Lactobacillaceae* whereas in the stomach of unhealthy fish (group D and E) the microbiota was dominated by *Enterobacteriaceae*, *Pseudomonadaceae*, *Bacillaceae*, and *Micrococcaceae* (Figure 7). The individual composition of the bacterial community for every fish at the family level is represented in Supplementary File 2.

On a lower taxonomic level, the microbial community from the intestine of rainbow trout also showed differences that related to the health status of the groups (Figure 8). The microbiota of the intestine from the healthy fish (groups A, B and C) was dominated by *Bacillaceae* (40.0, 21.4, and 20.3 %), *Enterobacteriaceae* (12.1, 14.8, and 18.4 %), and *Pseudomonadaceae* (6.9, 9.7, and 11.0 %); whereas microbiota from the unhealthy fish (groups D and E) was dominated by *Enterobacteriaceae* (30.8 and 28.2 %), *Bacillaceae* (36.2

and 5.7 %), *Pseudomonadaceae* (9.2 and 15.0 %), and *Mycoplasmataceae* (31.2%) for *group E* (Figure 8).

On the lowest taxonomic level, the microbial community from the stomach (Figure 9) of healthy rainbow trout (groups A, B and C) was dominated by such genera as *Bacillus* (11.4, 29.5, and 27.6 %), *Serratia* (11.1, 26.6, and 4.3 %), *Pseudomonas* (11.0, 20.5, and 6.7 %), and *Cetobacterium* (12.6%) for group A. For unhealthy fish the genera *Serratia* (34.5 and 29.4 %), *Pseudomonas* (25.0 and 23.1 %), were detected and *Bacillus* for group E (18.1 %) was also detected. The individual composition of the bacterial community for every fish on the lowest taxonomical level is represented in Supplementary File 3.

The microbial community from the intestine of healthy (Figure 10) rainbow trout (groups A, B and C) was dominated by genera such as *Bacillus* (37.4, 19.7, and 19.5 %), *Serratia* (8.9, 9.5, and 13.9 %), *Pseudomonas* (6.9, 9.6, and 11.0 %), *Bacteroides* (6.5, 7.0, and 5.8 %) and *Cetobacterium* (14.5 %) for group C.

For unhealthy fish the genera *Serratia, Bacillus and Pseudomonas* were also detected. It has to be noted that for fish from group D collected during summer (July) *Renibacterium* was present in both stomach (17.0%) and intestine (9.6%), whereas for fish from group E collected during autumn (September) the dominant had changed to *Mycoplasmataceae* (31.2%) in the intestine (Figure 10).

Diversity analysis of the intestinal microbiota of rainbow trout. The species diversity and richness of the bacterial community in the intestine of rainbow trout from healthy fish (groups A, B and C) were significantly higher than from unhealthy fish (groups D and E) according to Chao1 and Shannon indices. There were no significant differences for the microbial community of the stomach between healthy and unhealthy fish (Table 3).

The maximum Simpson index values were detected in the microbial community in intestine of healthy fish (group B) in comparison with the microbial community of unhealthy fish (group E) where the Simpson index value was minimal (Table 3).

The health status was of fish was significant for Chao1 and Shannon index (two-way PERMANOVA, $p \leq 0.05$) (Table 4).

The PCoA using weighted UniFrac distance matrix between healthy and unhealthy fish showed grouping of samples into two big clusters (healthy and unhealthy). Interestingly, the cluster formed by unhealthy fish had no clear difference between microbiota of the stomach and intestine (Figure 11).

The analysis of similarities (ANOSIM) supported by these results and indicated that the "tissue" and "health status" were significant factors (P=0.0001 and P=0.0428 respectively) for forming the gut bacterial community (Table 5). No differences were found between stomach and intestine for unhealthy fish (P=0.1077) whereas for healthy fish these differences were significant (P=0.0098).

Predicted functional metagenomes of the gut microbiota from healthy and unhealthy rainbow trout using PICRUSt.

Metagenome data was analyzed using PICRUSt to determine the microbiome predicted functions of gut of healthy and unhealthy fish. At the level 1 there were no significant differences found between healthy and unhealthy fish for stomach and intestine (data not shown). The most abundant predicted function at the L2 level was "metabolism" with average value (mean±SE) 48.7±0.3 and 46.4±0.4% for healthy and unhealthy fish, respectively. The significant differences between healthy and unhealthy fish for stomach and intestine are shown in the Figure 7.

At the level 3 there were a few significantly different predicted functions (PERMANOVA, $p \leq 0.05$) for healthy (stomach – 0.0 and intestine – 2.4%) and unhealthy fish (stomach – 3.4 and intestine – 4.3%) collected in different time points. On other hand, the significantly different predicted functions (PERMANOVA, $p \leq 0.05$) between healthy and unhealthy fish for stomach and intestine were 80.8 and 38.0% respectively.

Discussion

The gut microbiota is a very important constituent of any vertebrate animal including fish. The bacterial microflora of fish has been associated with numerous functions, including nutrition, disease resistance and immunity (Austin 2002, Wang et al., 2018).

According to our results, the microbiota of the stomach of healthy rainbow trout was dominated by *Proteobacteria, Firmicutes, Fusobacteria* and in the intestine dominated by the same phyla but with the addition of *Bacteroidetes.* Similar results for healthy rainbow trout were obtained by Wong et al. (2013) and Lyons et al. (2016) who have demonstrated using an NGS approach that the phyla *Proteobacteria*, *Firmicutes* and *Bacteroidetes* were dominant in the intestine of rainbow trout. In lower taxonomical levels, the differences in microbial composition between stomach and intestine of healthy fish are related to high abundance of members of the genus *Cetobacterium* in the stomach and *Bacillus* in the intestine. Bacteria belonging to the genus *Cetobacterium* are widely distributed in the intestinal tracts of freshwater fish (Mente et al., 2018) and are able to produce vitamin B12 (Tsuchiya et al., 2008) making some member species good candidates as probiotic species for feed supplements. The genus *Bacillus* has been identified in many studies to be a common part of the core microbial community of the fish gut (Austin and Al-Zahrani 1988, Cahill, 1990, Ringø et al., 1995, Kim et al., 2007) and widely applied as a probiotic in aquaculture due to its ability to stimulate the host immune system, produce antimicrobial compounds, and other

useful substances such as digestive enzymes, amino acids, etc. (Newaj-Fyzul et al., 2007, Wang et al., 2008, Kamgar et al., 2014, Olmos and Paniagua-Michel, 2014).

At the same time, we present the findings of considerable differences among our results, and those obtained by Wong et al. (2013) and Lyons et al. (2016) at the taxonomic level of genus. Lyons et al. (2016) identified such genera as *Mycoplasma, Brevinema, Lactobacillus, Acetanaerobacterium, Catellicoccus, Streptococcus, Weissella, Leuconostoc, Lactococcus, Enterococcus* and *Bacillus* whereas other genera like *Lactobacillus, Streptococcus, Staphylococcus, Clostridia* were found by Wong et al. (2013). In the present work, the microbial community of healthy fish was dominated by bacteria from the genera *Bacillus, Serratia, Cetobacterium,* and *Pseudomonas*. Thus, the fish from our study have shared only one identical dominant genus (*Bacillus*) with those fish from Lyons's work and no identical dominant genus with Wong's work. Such great variability of results obtained by different research groups working with the same fish species may be explained by several factors such as 1) different rearing water conditions in all three studies e.g. water temperature was 9.4ºC (Lyons et al., 2016), 12.5ºC (Wong et al., 2013) and 14.2-15.4ºC (the present study); 2) different diets and premix; 3) different sample matrix/tissue used for DNA extraction (e.g. in the Lyons et al. (2016) study the source of genomic DNA was distal intestine (mucous+content), in the study by Wong et al. (2013) the genomic DNA source was the middle intestine, whereas in the present study the genomic DNA source was a mixture of mucosa from anterior, middle and posterior parts of the intestine); 4) different DNA extraction protocols may also affect the results as was previously shown by Kashinskaya et al. (2017); 5) life cycle stage Llewellyn et al. (2014); 6) differences over time Gonçalves et al. (2017). But at the same time the variability found could also be related to a great plasticity of the core microbial community of rainbow trout. To understand which of these features has greater influence on the microbial composition further investigations are needed. The

dominant genera absent in studies by Wong et al. (2013) and Lyons et al. (2016) (*Serratia*, *Pseudomonas*, and *Bacillus*), but registered in the present study were earlier found in a study in that used culture-dependent approaches (Nieto et al., 1984; Austin and Al-Zahrani, 1988; Ringø et al., 1995). However, the abundance of these genera and therefore whether or not they were truly dominant, is unknown due to the limitation of culture-dependent approaches.

It is well known that in gastric fish species (fish species that have stomach and acid stage of digestion), the different parts of the gut support different conditions such as pH values, activity and composition of digestive enzymes, etc (Solovyev et al., 2015, 2018). This can be seen as analogous to providing different types of selective media for culture and enrichment of specific bacteria; different parts of the gut form specific conditions like specific media in a culture-dependent approach for growing specific groups of bacteria. An improved understanding of the association between alterations in the diversity of gastrointestinal microbiota of fish due to infectious diseases will illuminate how gut microbes impact host health, which may aid development of more efficient strategies to prevent or treat diseases. But, unfortunately, it remains unclear whether changes in the intestinal microbiota composition are causes or consequences of different fish diseases.

Dysbiosis of the gut due to abiotic factors can lead to opportunistic infections that would be otherwise suppressed. In contrast, an aggressive pathogen producing powerful virulence factors may lead to disruption of the normal microbial balance and dysbiosis. Indeed, it is well known that different species/strains of bacteria may show predatory behavior and be able to produce various compounds with antibacterial activity. This activity was shown for such genera as *Myxobacteria*, *Cytophagales* and certain *Flavobacteria* (Linares-Otoya et al., 2017). Myxobacteriosis or Bacterial Cold-water Disease caused by *F. psychrophilum* is a bacterial disease that affects a broad host-range of fish species that inhabit

cold, fresh waters and occurs predominately at water temperatures of 16°C and below (Starliper, 2011).

The present study provides a description of the alterations of the gastrointestinal microbiota in farmed rainbow trout, *O. mykiss* during an epizootic of bacterial kidney disease and myxobacteriosis. Bacterial kidney disease (BKD) caused by the Gram-positive facultative intracellular bacteria *Renibacterium salmoninarum* is a chronic disease affecting a variety of wild and cultured salmonids including rainbow trout in Finland (Toranzo et al., 2005). This disease leads to the development of exophthalmia, anemia, ascites, and inflammation of the internal organs. This bacteria appears to have low tissue specificity and localizes in various organs and tissues, the ovarian fluid, but more often in clinical cases found in the kidney tissue disrupting the excretory and hematopoietic functions in fish. As seen in Figure 9 and Figure 10 species of *Renibacterium* were identified in unhealthy fish. The 16S library analysis does not enable identification to the level of species, but it can be that among species of this genera are low abundance of pathogenic strains living as commensals until conditions become less favourable for the host.

One of the main results of the present work is the difference in the bacterial communities between healthy and unhealthy fish. Although no association existed to water quality parameters, which remained relatively constant during the study period, the significant differences of the observed microbial diversity between healthy and unhealthy fish can enable some inferences to be made regarding dysbiosis and composition of the microbiota. Further, the pattern of change in the gut microbial diversity can be expected to differ with different etiology. In the present work in the group of unhealthy fish the dominant phyla were represented by *Proteobacteria, Actinobacteria, Firmicutes*, and *Tenericutes*. Apparently, with a high level of abundance of such genera as *Serratia, Bacillus, Pseudomonas, Renibacterium*, and bacteria from the family *Mycoplasmataceae*. The

occurrence of bacteria from the genus *Renibacterium* in the gut microbiota of unhealthy rainbow trout was early revealed and explained by horizontal transmission (Sakai et al., 1992; Balfry et al., 1996), and according to Bullock et al. (1978) *Renibacterium* may be found not only in the kidneys, but also in the intestine. In the present study, the clinical signs of the disease in fish, the high level of bacterial contamination and formation of *Renibacterium* colonies on dense nutrient media such as KDM-2 (Kidney Disease Medium 2), SKDM (Selective Kidney Disease Medium) on the 8^{th} day indicate an open form of infection rather than a latent carrier state.

The specific changes in the gut microbial community likely depend upon the species-specific bacterial infection affecting the fish host and, as a result, a great number host-specific reactions to every infection. One of the possible ways of changing the host microbial community is direct competition between pathogens and host microbiota for either resources or physical space for colonization. Thus, according to our results, the phylum *Tenericutes* (classified up to family *Mycoplasmataceae*) was dominant in the intestine of unhealthy rainbow trout only in group E. The prevalence of the phylotype *Mycoplasma* in the intestinal microbiome of salmon was first described in a study by Holben et al. (2002) of the core intestinal microbiota of salmonid fish. This observation has been confirmed by many researchers since then (Abid et al. 2013; Green et al. 2013; Zarkasi et al. 2014; Llewellyn et al. 2015; Lowrey et al. 2015; Ozorio et al. 2015, Villasante et al., 2017). The absence of this group of bacteria in the early studies could be explained by difficulties of their cultivation on artificial medias. At the present time, one of the possible roles of *Mycoplasma* is maintenance of the normal physiological status of animals, although earlier studies designated *Mycoplasma* was designated as a pathogen (Holben, 2002). Lyons et al. (2016) have determined *Tenericutes (*genus *Mycoplasma)* as a dominant taxa (81.59%) in the gut of healthy rainbow trout, and hypothesized there exists specificity of this bacterial taxa for

rainbow trout. Holben et al. (2002) revealed that *Mycoplasma* is able to produce different acids that are utilized by *Acinetobacter* as carbon sources. The high occurrence of several prokaryotes may also be caused by their active struggle against pathogenic microorganisms. As shown in Atlantic salmon (Reveco et al., 2014), the inclusion of soybean meal in feed induced enteritis and was simultaneously accompanied by a high number of *L. lactis* subsp. in the distal intestine, which are able to produce antimicrobial peptides and proteins to control infectious agents.

Another possible way of changing the host microbial community is by indirect influence of pathogens via, for example, alterations of fish feeding activity. In several studies the OTU's assigned to the phylum *Cyanobacteria* were considered potential plant chloroplast contaminants and removed from the analysis (Wong et al., 2013; Dehler et al., 2017). In our study we have eliminated *Cyanobacteria* from the bioinformatics analysis but before elimination the ratio of this group was up to 70.0% which could be interpreted as an indirect marker of fish feeding activity (Supplementary File 4). Moreover, the phylum *Cyanobacteria* did not alter the main findings when it was removed from analyses. While we have found a high abundance of *Cyanobacteria* spp. among healthy trout as compared with the unhealthy groups, this phylum was not the main source of significant differences obtained between healthy and unhealthy fish. It is known that blooms of *Cyanobacteria* microalgae occur every year (July-August) in Ladoga Lake (Voloshko et al., 2008). We assume that unhealthy fish had very low levels of feeding activity, which would have prevented ingestion of *Cyanobacteria* and explain their almost complete absence in the gut. The influence of the level of feeding activity, and the concomitant changes in hydrochloric acid secretion in stomach of fish, on the gut microbial community is also supported by the fact that there were no differences between bacterial communities of the stomach and intestine in unhealthy fish groups.

The questions associated with bacterial co-infections are still very weakly studied (Kotob et al., 2016) and further studies and additional approaches are required to advance this area of microbial pathology. Further, studies in which natural infections have occurred and healthy individuals can be contrasted with infected individuals are even more rare, and yet more relevant in some sense. Controlled challenge experiments utilize established doses and fish are routinely injected intraperitoneally. The high doses and intraperitoneal injection are not part of a normal infection process. The dosage and route of exposure can affect the severity, outcome, and prognosis for recovery of a disease. Herein we present interesting data on a natural infection with two distinct cohorts of the same species of differing health status. Summarizing the present data for farmed rainbow trout we have demonstrated there are significant differences in the gut bacterial community between healthy and unhealthy rainbow trout. In the group of unhealthy fish, the dominant phyla were represented by *Proteobacteria, Actinobacteria*, *Firmicutes*, and *Tenericutes*, whereas in healthy rainbow trout among the dominant OTUs were *Firmicutes, Fusobacteria, Proteobacteria,* and *Bacteroidetes*. Whether changes in the composition of the intestinal microbiota are causes or consequences of different fish diseases requires further study. Another substantial finding was the absence of differences between microbial communities of the stomach and intestine in the unhealthy groups when compared with the healthy fish groups, strongly implicating the influence that changes in the stomach pH can have on transmission of microbial flora along the digestive tract.

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Conflict of Interest. The authors declare that they have no competing interests.

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Figure 1 Fish from healthy group (group C)

Figure 2 Fish from unhealthy **group D:** a) extensive deep ulcers (ul), fin necrosis (fn); **group E:** b) petechiae in perivisceral fat (pt), pale-coloured liver (lv); c) exophthalmia; d) *Caligus lacustris* infection (cl), fin necrosis (fn).

Figure 3 Phylum composition of microbial community in stomach healthy (groups A, B and C) and unhealthy (groups D and E) of rainbow trout using 16S rDNA metagenomic sequencing (MiSeq). (■) Actinobacteria; (■) Bacteroidetes; (■) Firmicutes; (■) Fusobacteria; (■) Proteobacteria; (■) Others.

Figure 4 Phylum composition of microbial community in intestine healthy (groups A, B and C) and unhealthy (groups D and E) of rainbow trout using 16S rDNA metagenomic sequencing (MiSeq). (■) Actinobacteria; (■) Bacteroidetes; (■) Firmicutes; (■) Fusobacteria; (■) Proteobacteria; (■) Tenericutes; (■) Others.

Figure 5 Ratio of the most abundant bacterial phyla in stomach. (■) Healthy; (■) Unhealthy.

Figure 6 Ratio of the most abundant bacterial phyla in intestine. (■) Healthy; (■) Unhealthy.

Figure 7 Top OTUs at the family taxonomic level of the bacterial community from stomach of healthy (groups A, B and C) and unhealthy (groups D and E) rainbow trout. (■) Bacillaceae; (■) Enterobacteriaceae; (■) Fusobacteriaceae; (■) Lactobacillaceae; (■) Micrococcaceae; (■) Moraxellaceae; (■) Pseudomonadaceae; (■) Sphingomonadaceae; (■) Streptococcaceae; (■) Others.

Figure 8 Top OTUs at the family taxonomic level of the bacterial community from intestine of healthy (groups A, B and C) and unhealthy (groups D and E) rainbow trout. (■) Bacillaceae; (■) Bacteroidaceae; (■) Comamonadaceae; (■) Enterobacteriaceae; (■) Fusobacteriaceae; (■) Micrococcaceae; (■) Moraxellaceae; (■) Mycoplasmataceae; (■) Pseudomonadaceae; (■) Others.

Figure 9 Dominant groups of bacteria at the genus taxonomic level in stomach of studied rainbow trout. (■) Acinetobacter; (■) Bacillus; (■) Cetobacterium; (■) Fusobacterium; (■) Lactobacillus; (■) Lactococcus; (■) Pseudomonas; (□) Renibacterium; (■) Serratia; (■) Others.

Figure 10 Dominant groups of bacteria at the genus taxonomic level in intestine of studied rainbow trout. (■) Acinetobacter; (■) Bacillus; (■) Bacteroides; (■) Cetobacterium; (■) Pseudomonas; (□) Renibacterium; (■) Serratia; (■) Unknown Comamonadaceae; (■) Unknown Enterobacteriaceae; (■) Unknown Mycoplasmataceae; (■) Others.

Figure 11 PCoA based on weighted UniFrac distance matrix for all studied rainbow trout. ● circle denotes intestine; ▲ triangle denotes stomach. Group: (●) A; (●) B; (●) C; (●) D; (●) E.

Supplementation 1 Individual variability in stomach and intestine of bacterial phyla in studied fish groups. Group A (Healthy): (■) Bacteroidetes; (■) Firmicutes; (■) Fusobacteria; (■) Proteobacteria; (■) Others. Group B (Healthy): (■) Bacteroidetes; (■) Firmicutes; (■) Proteobacteria; (■) Others. Group C (Healthy): (■) Bacteroidetes; (■) Firmicutes; (■) Fusobacteria; (■) Proteobacteria; (■) Others. Group D (Unhealthy): (■) Actinobacteria; (■) Bacteroidetes; (■) Firmicutes; (■) Fusobacteria; (■) Proteobacteria; (■) Others. Group E (Unhealthy): (■) Firmicutes; (■) Proteobacteria; (■) Tenericutes; (■) Others. ∗ - fish number in every group; S - stomach; I – intestine.

Supplementation 2 Individual variability in stomach and intestine of bacterial families in studied fish groups. Group A (Healthy): (■) Bacillaceae; (■) Bacteroidaceae; (■) Comamonadaceae; (■) Enterobacteriaceae; (■) Fusobacteriaceae; (■) Lactobacillaceae; (■) Leuconostocaceae; (■) Pseudomonadaceae; (■) Sphingomonadaceae; (■) Streptococcaceae; (■) Vibrionaceae; (■) Others. Group B (Healthy): (■) Aeromonadaceae; (■) Bacillaceae; (■) Bacteroidaceae; (■) Comamonadaceae; (■) Enterobacteriaceae; (■) Moraxellaceae; (■) Pseudomonadaceae; (■) Streptococcaceae; (■) Unknown Rhizobiales; (■) Others. Group C (Healthy): (■) Aeromonadaceae; (■) Bacillaceae; (■) Bacteroidaceae; (■) Comamonadaceae; (■) Clostridiaceae; (■) Enterobacteriaceae; (■) Fusobacteriaceae; (■) Lactobacillaceae; (■) Porphyromonadaceae; (■) Pseudomonadaceae; (■) Shewanellaceae; (■) Streptococcaceae; (■) Vibrionaceae; (■) Others. Group D (Unhealthy): (■) Aeromonadaceae; (■) Bacillaceae; (■) Enterobacteriaceae; (■) Fusobacteriaceae; (□) Micrococcaceae; (■) Neisseriaceae; (■) Pseudomonadaceae; (■) Sphingomonadaceae; (■) Others. Group E (Unhealthy): (■) Aeromonadaceae; (■) Bacillaceae; (■) Enterobacteriaceae; (□) Moraxellaceae; (■) Mycoplasmataceae; (■) Pseudomonadaceae; (■) Shewanellaceae; (■) Others. ∗ - fish number in every group; S - stomach; I – intestine.

Supplementation 3 Individual variability of bacteria in stomach and intestine of studied fish groups at the lowest taxonomical level. Group A (Healthy): (■) Bacillus; (■) Bacteroides; (■) Cetobacterium; (■) Fusobacterium; (■) Lactobacillus; (■) Lactococcus; (■) Pseudomonas; (■) Serratia; (■) Streptococcus; (■) Unknown Comamonadaceae; (■) Unknown Enterobacteriaceae; (■) Unknown Leuconostocaceae; (■) Others. Group B (Healthy): (■) Acinetobacter; (■) Bacillus; (■) Bacteroides; (■) Pseudomonas; (■) Serratia; (■) Streptococcus; (■) Unknown Aeromonadaceae; (■) Unknown Comamonadaceae; (■) Unknown Enterobacteriaceae; (■) Unknown Rhizobiales; (■) Others. Group C (Healthy): (■) Bacillus; (■) Bacteroides; (■) Cetobacterium; (■) Clostridium; (■) Fusobacterium; (■) Lactobacillus; (■) Lactococcus; (■) Pseudomonas; (■) Serratia; (■) Shewanella; (■) Unknown Enterobacteriaceae; (■) Others. Group D (Unhealthy): (■) Bacillus; (■) Cetobacterium; (■) Clostridium; (■) Deefgea; (■) Janthinobacterium; (■) Pseudomonas; (■) Renibacterium; (■) Serratia; (■) Sphingobium; (■) Unknown Bacillaceae; (■) Unknown Enterobacteriaceae; (■) Others. Group E (Unhealthy): (■) Acinetobacter; (■) Bacillus; (■) Pseudomonas; (■) Serratia; (■) Shewanella; (■) Unknown Aeromonadaceae; (■) Unknown Enterobacteriaceae; (■) Unknown Mycoplasmataceae; (■) Others. ∗ - fish number in every group; S - stomach; I – intestine.

Supplementation 4 Stomach phyla with Cyanobacteria. (■) Actinobacteria; (■) Cyanobacteria; (■) Firmicutes; (■) Fusobacteria; (■) Proteobacteria; (■) Others.

Table 1 Characteristics of the investigated rainbow trout

Group	Status of fish	Number of individuals	Body weight (g)	Total length (cm)	Date of sampling
A	Healthy	8	510.8±25.7	32.8±0.5	17.07.2015
B	Healthy	3	342.0±22.4	29.3±0.8	18.07.2015
C	Healthy	8	850.7±70.1	27.1±0.8	16.08.2015
D	Unhealthy	4	240.9±37.7	27.4±1.4	18.07.2015
E	Unhealthy	6	499.5±32.9	32.8±0.5	09.09.2015

Table 2 Composition of the commercial diet (from the feed manufacturer)

Ingredients	**%**
Crude protein	42
Oils and fats	28
Crude fiber	1.6
Ash	8.3
Calcium	2
Phosphorus	1.3
Sodium	0.4
Vitamins (A, D3, E, C), trace elements, astaxanthin, citric acid, propyl gallate	16.4

Table 3 Alpha diversity analysis of microbiota of rainbow trout

Type of tissue	Fish	Group	Chao1 Mean±SE	ShannonH Mean±SE	Simpson Mean±SE
Intestine	Healthy	**A**	560.3±64.4	4.4±0.5	0.76±0.06
		B	788.2±22.5	5.9±0.8	0.89±0.08
		C	550.4±93.7	4.6±0.9	0.74±0.11
		mean	633.0±77.7*	5.0±0.5*	0.80±0.05
	Unhealthy	**D**	280.9±64.6	3.3±0.3	0.75±0.05
		E	296.9±71.2	3.2±0.7	0.68±0.14
		mean	288.9±8.0	3.3±0.05	0.72±0.04
Stomach	Healthy	**A**	452.8±67.3	5.0±0.8	0.80±0.11
		B	449.6±84.3	4.5±0.8	0.87±0.01
		C	548.1±36.4	5.7±0.5	0.88±0.05
		mean	483.5±32.3	5.1±0.3	0.85±0.03
	Unhealthy	**D**	319.5±39.5	3.8±0.6	0.80±0.08
		E	418.8±57.4	4.0±0.4	0.83±0.04
		mean	369.2±49.7	3.9±0.1	0.82±0.02

* - The differences between healthy and unhealthy groups were significant at $p \leq 0.05$

Table 4 Results of two-way PERMANOVA for richness and diversity estimates of microbiota of rainbow trout. Number of permutations, 10 000.

Factor	F	p-value
Chao		
Health status (healthy/unhealthy)	17.42	**0.0001**
Tissue (intestine/stomach)	0.45	0.49
Health status*tissue	3.35	0.49
Shannon		
Health status (healthy/unhealthy)	8.72	**0.003**
Tissue (intestine/stomach)	1.70	0.18
Health status*tissue	7.90	0.99
Simpson		
Health status (healthy/unhealthy)	0.50	0.48
Tissue (intestine/stomach)	1.94	0.16
Health status*tissue	4.87	0.69

Table 5 The impact of different factors on fish gut microbiota assessed by PERMANOVA test (with 10 000 permutations) on weighted UniFrac dissimilarity matrix

Test	F	p-value
Two-way PERMANOVA on all samples		
Health status (healthy/unhealthy)	8.15	**0.0001**
Tissue (intestine/stomach)	2.35	**0.0428**
Health status*tissue	-0.77	0.0715
One-way PERMANOVA on healthy fish		
Tissue (intestine/stomach)	4.531	**0.0041**
One-way PERMANOVA on unhealthy fish		
Tissue (intestine/stomach)	2.108	0.1077
One-way PERMANOVA on intestine samples		
Health status (healthy/unhealthy)	4.05	**0.0098**
One-way PERMANOVA on stomach samples		
Health status (healthy/unhealthy)	10.65	**0.0003**
One-way PERMANOVA on all samples		
Group (A, B, C, D, E)	2.521	**0.0034**