Microbial community structure in a host-parasite system: the case of Prussian carp and its parasitic crustaceans

Running title: skin microbiota during ectoparasite infestation

DOI: 10.1111/jam.15071

Kashinskaya E.N.¹, Simonov E.P.¹,², Andree K.B.³, Vlasenko P.G.¹, Polenogova O.V.¹, Kiryukhin B.A.² and M.M. Solovyev¹,⁴

¹Institute of Systematics and Ecology of Animals of Siberian Branch of Russian Academy of Sciences; Research group of physiology and genetics of hydrobionts; Frunze St. 11, Novosibirsk, 630091, Russia.

²University of Tyumen; Institute of Environmental and Agricultural Biology; 25 Lenina St., Tyumen, 625003, Russia.

³Instituto de Investigación y Tecnología Agroalimentarias; Cultius Aquàtics; San Carlos de la Rapita, Tarragona, ES 08140, Spain.

⁴Tomsk State University; Biological Institute; 36 Lenin Ave, Tomsk, 634050, Russia.

Correspondence: Elena N. Kashinskaya, Institute of Systematics and Ecology of Animals of Siberian Branch of Russian Academy of Sciences; Research group of physiology and genetics of hydrobionts; Frunze St. 11, Novosibirsk, 630091, Russia. e-mail: elena.kashinskaya@inbox.ru

Key words: skin microbiota; 16S rRNA; freshwater fish; A. foliaceus, L. cyprinacea; fish pathogens.
Abstract

Aims. The aim of the study was to investigate the skin microbiota of Prussian carp infested by ectoparasites from the genus Argulus and Lernaea.

Methods and Results. Associated microbiota of skin of Prussian carp and ectoparasites were investigated by sequencing of the V3, V4 hypervariable regions of 16S rRNA using Illumina MiSeq sequencing platform.

Conclusions. According to the Spearman rank correlation test, the increasing load of ulcerations of the skin of Prussian carp was weak negative correlated with reduction in the abundance of the following taxa: Acrobacter, bacteria C39 (Rhodocyclaceae), Rheinheimera, Comamonadaceae, Helicobacteraceae, and Vogesella. In the present study, the microbiota of ectoparasites from genus Lernaea and Argulus were characterized for the first time. The microbiota associated with L. cyprinacea was significantly different from microbial communities of intact skin mucosa of both infested and uninfested fish and skin ulcers (ADONIS, p≤0.05). The microbiota associated with parasitic crustaceans L. cyprinacea were dominated by unclassified bacteria from Comamonadaceae, Aeromonadaceae families, and Vogesella. The dominant microbiota of A. foliaceus were represented by Flavobacterium, Corynebacterium, and unclassified Comamonadaceae.

Significance and Impact of Study. Results from these studies indicate that ectoparasites have the potential to alter skin microbiota, which can play a possible role in transmission of secondary bacterial infection in fish, caused by pathogenic bacteria.

Introduction

Water is a dense media where all organisms including fish are in direct contact throughout their lives with a multitude of biotic and abiotic elements, including a wide variety of
pathogenic and non-pathogenic microbes. Fish have developed innate immune mechanisms against potential pathogens to which they are continuously exposed. The normal structure and features of the fish mucosa (skin, gills, and intestine, inclusive) functions towards differentiating the host response to the plethora of physical, chemical, and biological stimuli of the aquatic environment (Esteban and Cerezuel, 2015). One of the host’s protective mechanisms is the external epithelia that prevents entry of pathogenic organisms or allergens, and maintains osmotic balance. Mucous glands of the fish skin produce the mucous layer that, in turn, is colonized by different bacterial species (commensal and/or opportunistic), which play many different roles, including the priming of immune memory for the development of the host immune system (Minniti et al. 2017). Fish mucus has been demonstrated to possess antimicrobial properties against different pathogenic bacteria and viruses. Among different immune molecules, lysozyme is one of the main antibacterial agents that hydrolyzes cell walls of both gram-positive and gram-negative bacteria. Some proteases are also able to protect skin surfaces against pathogens (Esteban and Cerezuel, 2015).

The majority of bacteria detected in the skin-mucosal surfaces of fish belong to the phylum Proteobacteria, Actinobacteria, Bacteriodetes, Firmicutes, Cyanobacteria, Chlorobi, and TM7 (Boutin et al. 2013; Larsen et al. 2013; Leonard et al. 2014; Wang et al. 2010; Minniti et al. 2017). To date, there are many studies focused on the fish skin microbiome under normal conditions, whereas studies associated with fish skin subjected to different pathogens (bacteria, viruses, fungi, protozoa and metazoan ectoparasites, etc) are limited (Llewellyn et al. 2017; Li et al. 2019). It is well known that different pathogens can provoke a direct damage to fish skin (Austin and Austin 2016). In addition, indirect damage can also be caused by parasite during the course of life cycle in host tissue, which creates a "gateway" for secondary infections (Bandilla et al. 2006). In the places where disruption of the integrity of the host skin has occurred, there is a risk of infections caused by different groups of microorganisms, mainly by bacteria and fungi (Bandilla et al. 2006; Wafer et al. 2015). An example of that mentioned above is the case of
Lepeophtheirus salmonis infestation in Atlantic salmon Salmo salar where multiple pathogenic genera (Vibrio, Flavobacterium, Tenacibaculum, Pseudomonas) were registered in the skin microbiota of infested fish. A significant reduction in microbial richness and highly significant destabilization of microbiota of infested fish, in comparison with control fish, was also observed (Llewellyn et al. 2017).

In freshwater ecosystems the diseases caused by crustaceans from the genus Argulus (Family: Argulidae) and the genus Lernaea (Family: Lernaeidae) are an acute problem in the management of pond aquaculture. In Russia there are three Argulus species, commonly known as fish lice, which infest and cause skin diseases in fish (Steckler and Yanong 2012a). The most widely known is Argulus foliaceus, whereas A. coregoni and A. japonica occur in freshwater fish of the northern regions and in the basin of the Amur river, respectively (Zharikova et al. 2002). Another crustacean ectoparasite of fish in Russia belongs to the genus Lernaea commonly called anchor worms. Fish from different waterbodies in Russia are mainly parasitized by two species of Lernaea: L. cuprinacea and L. elegans. In Russia the studies of these fish ectoparasites are mainly focused on the different aspects of population biology such as abundance and prevalence and their interannual variability under natural and artificial conditions (Zharikova et al. 2002). Whilst there is an increasing understanding of fish immunity in response of ectoparasitic infestations, and secondary infections (bacteria, fungi, viruses, etc.), little is known about the possible role of microbes that are associated to such parasites. Previous studies have shown that infestation of fish by L. cyprinacea and A. foliaceus results in deep ulcers on the skin surfaces at the site of attachment with peripheral inflammation noted by reddening of the skin and irregular, bumpy edges. A number of studies have shown that bacterial pathogens like Aeromonas salmonicida, Flavobacterium columnare and Saprolegniales moulds, which are the causative agents of dermatomycosis, were isolated from fish after infestation with A. coregoni (Bandilla et al. 2006; Steckler and Yanong 2012a; Shahraki et al. 2014). Also, it is known that Lernaea can cause intense inflammation, leading to different secondary bacterial (e.g., Aeromonas...
*hydrophila* and fungal infections (Steckler and Yanong 2012b). Experimental study of parasites, their microbiota, and interactions among them and the host has become more feasible with newer metagenomic approaches. Although, in some studies no changes in the structure of the bacterial community upon infection were found (Baxter *et al.* 2015; Cooper *et al.* 2013; Reynolds, 2015), in others significant alterations were noted (Baxter *et al.* 2015; Kreisinger *et al.* 2015). Therefore, the determination of the bacterial community structure local to parasite attachment and in places of skin penetration likely inducing antimicrobial properties of fish skin mucus are the key aspects for understanding the mechanisms of the development of secondary infections and, consequently, for investigating effective methods of prevention and treatment of fish.

Prussian carp, *Carassius gibelio*, is one of the oldest cultured fish species in the world. For example, various carp species are the primary fish species produced in industrial aquaculture, and their production in recent years has accounted for more than 80 percent of the total (FAO, 2020). Moreover, the eutrophic Chany Lake, being one of the largest lakes in western Siberia and epidemiologically unfavorable for ectoparasitic infestations is an appropriate model to study the interactions between parasites and host-associated microbiota. (Given that the lake is overall epidemiologically unfavorable to ectoparasite infestations there is a better probability of acquiring infested samples together with uninfested fish samples as controls from the same water body.) In this aspect, the relationship between parasites, an economically important host species and host-associated microbiota are important for disease progression and morbidity. Knowledge gained in this area can provide potential points for intervention.

The aim of the study was to investigate the microbial community structure of skin mucus of infested and uninfested Prussian carp caused by parasitic crustaceans from the genus *Argulus* and *Lernaea* in a eutrophic lake with a parallel study of the associated microbiota of their parasites and environmental compartments.

**Materials and methods**
Study area and sampling

35 individuals of Prussian carp *Carassius gibelio* (Linnaeus 1758) with total length 225.2±0.14 mm and their ectoparasites *Lernaea cyprinacea* and *Argulus foliaceus* were collected from July 30 to August 01, 2017 in the area of Malye Chany Lake in west Siberia (Russia, 54°36’56.3”N, 78°12’5.9”E). Fish were captured using gill-nets (mesh sizes 35 and 45 mm) and transported alive to the laboratory in plastic containers filled with water from the site of fish capture (duration approximately 15 min). All fish were sacrificed and samples were collected aseptically. In order to avoid contamination during sampling, one fish per container was transported to the laboratory. Fishes were touched only by one assistant wearing sterile gloves and handled only by the mouth and caudal fin. In this time, skin mucus samples were collected by another assistant equipped with a sterile cell-scraper.

Skin samples comprised mucus and scales along the right and left full length surfaces of fish from the posterior of the gill operculum to the base of the caudal fin (Figure 1). To avoid any contradictory interpretation and mislead, we stipulate that the sampled mucosa is from scales of the fish and indicated as “skin mucosa”. Skin mucosa and ulcer samples were collected by gently scraping of the external surfaces of a fish using a cell scraper. Ectoparasites were retrieved aseptically from the skin surfaces of Prussian carp using a sterile forceps.

Male and female fish were identified according to gonadal development (Table 1). All fish were divided into three groups depending on their infestation level. Six individuals of uninfested fish were collected for group A, while group B (fish only with ulcers) and C (fish with ulcers and parasites) included 13 and 16 individuals of infested fish, correspondingly. A total number of 80 samples from 35 individuals of fish and environment were analyzed: six samples of intact skin mucosa, or ISM from uninfested and 29 samples from infested fish. In addition, ulcers from skin surfaces from infested Prussian carp at the site of *L. cyprinacea* attachment (n=10), ulcers without *L. cyprinacea* (n=11), ectoparasites *L. cyprinacea* (n=11) and
A. foliaceus (n=4) were sampled (Figure 1). Water (n=3), sediment (n=3) and common reed (Phragmites australis, n=3) were also collected nearby from the fish capture sites. Microorganisms from the water, sediment, and common reed were collected according to Kashinskaya et al. (2018).

**Parasitological analysis**

During sampling time (July-August of 2017) 35 individuals of Prussian carp collected in order to estimate the prevalence of L. cyprinacea and A. foliaceus infestation. The number of parasites and number of ulcers are given in Table 1. The prevalence (P) and mean intensity of parasite infestation were calculated according to the definitions by Bush et al. (1997).

The prevalence (P) of parasite infestation (in %) was calculated as:

\[ P = \frac{I \times 100}{N} \]

Where \( I \) is number of infested host, and \( N \) is total number of host examined. The error of prevalence index (E) was calculated by the following formula:

\[ E = \sqrt{\frac{P \times (100 - P)}{N}} \]

where \( P \) is prevalence, and \( N \) is total number of host examined.

Mean intensity of invasion (I) was assessed as the average of number of individuals of a particular parasite species (K) in a single infested host (n):

\[ I = \frac{K}{n} \]

Error of intensity indice (SE) was calculated according to:

\[ SE = \frac{SD}{\sqrt{n}} \]

Where \( SD \) is standard deviation of row of number of individuals of a particular parasite species in a single infested host, and \( n \) is total number of infested hosts.

**Sample preparation, DNA extraction, and 16S rDNA metagenomic sequencing**

Before DNA extraction, ectoparasites, scrapings from skin mucosa and ulcers were collected into sterile microcentrifuge tubes with lysis buffer (300 µl) for DNA isolation and mechanically homogenized by pestle for 1 min. Following the kit manufacturer protocols, DNA was extracted from 100 mg of samples (excluding parasites) by DNA-sorb B kit (NextBio, Russia). Ectoparasites were rinsed three times in sterile deionized water and used for DNA extraction. The DNA extraction protocol was previously described in Kashinskaya et al. (2020).
DNA from a sample containing only sterile deionized water was extracted and included in PCR as a negative control. Sequencing of the V3, V4 hypervariable regions of 16S rRNA genes was carried out on an Illumina MiSeq sequencing platform (500 cycles - 2x250 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 and other methods were applied according to the original manufacturer’s protocol (https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16smetagenomic-library-prep-guide-15044223-b.pdf). The PCR reaction contained at least 2.5 µl of DNA (5 ng µl⁻¹), 5 µl of reverse primer (1 µM), 5 µl of forward primer (1 µM) and 12.5 µl of 2x KAPA HiFi Hotstart ReadyMix (KAPA Biosystems, Wilmington, MA, USA) in a total volume of 25 µL. The PCR reaction was performed on a 96-well 0.2 ml PCR plate (Life Technologies) using the following program: 95°C for 3 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s and a final extension step at 72°C for 5 min. After producing amplicons, the libraries were cleaned up and mixed in equimolecular portions using SequalPrep™ Normalization Plate Kit (ThermoFisher, Cat # A10510-01) and checked using capillary electrophoresis. Samples were multiplexed using a dual-index approach with the Nextera XT Index kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer’s instructions.

**Molecular identification of ectoparasites**

For molecular characterization, *L. cyprinacea* (n=2) and *A. foliaceus* (n=4) were preserved in 96% ethanol and stored at 4°C until extraction. Total DNA was extracted from single ethanol-preserved individuals of ectoparasites using the DNA-sorb B kit manufacturer’s protocols (kit for DNA extraction, Central Research Institute of Epidemiology, Russia). The 28S ribosomal RNA (28S rRNA) was amplified using the following primers (forward 5’-CCCSCGTAARTTAAGCATAT-3’, reverse 5’-TCCGGAAGGAAACCAGCTACTA-3’) and PCR
conditions as described in Ruiz et al. (2017). Double-stranded DNA was amplified using BioMaster HS-Taq PCR-Color (2x) kit (Biolabmix, Novosibirsk, Russia) according to the manufacturer’s instructions (http://biolabmix.ru/products/klassicheskaja_pcr/biomaster_hs-taq_pcr-color__2_/). The PCR products were purified by adsorption on Agencourt Ampure XP (Beckman Coulter, Indianapolis, IN, USA) columns and subjected to Sanger sequencing using the BigDye Terminator V.3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA) with subsequent unincorporated dye removal by the Sephadex G-50 gel filtration (GE Healthcare, Chicago, IL, USA). The Sanger products were analyzed on an ABI 3130XL Genetic Analyzer (Applied Biosystems). The purification and sequencing of PCR products were performed in the SB RAS Genomics Core Facility (Novosibirsk, Russia). Manual edition, alignment of sequences and phylogenetic analysis were performed with MEGA 10 (Kumar et al., 2018). Sequences were deposited into GenBank (NCBI) under the following accession numbers: SUB8819858, SUB8819858, SUB8819881, SUB8819881, SUB8819881, SUB8819881.

Identification of opportunistic pathogens

Identification of opportunistic pathogens were based on comparison of the generated OTU’s table after QIIME processing before “core” analysis (OTU’s tables are available at http://doi.org/10.6084/m9.figshare.11967729) to an available list of bacterial fish pathogens according to Austin and Austin (2016). The OTU’s table after QIIME processing includes a list of identified bacteria at the lowest taxonomic level where several OTU’s were identified at the species level.

Activity of trypsin

For quantifying the activity of mucosal skin trypsin the samples were homogenized for 30 sec using ultrasonic homogenizer (Bandelin Sonopuls HD 2070, Germany) in 2.5 volumes (w/v) of ice-cold distilled water, centrifuged at 10000 ×g for 5 min at 4 °C. Then, the supernatant removed for trypsin quantification and kept at −80 °C until further analysis. Trypsin (E.C. 3.4.21.4) activity was assayed at 25 °C using BAPNA (N-α-benzoyl-DL-arginine p-nitroanilide)
as substrate in 100 mM Tris-HCl, 150 mM NaCl, 20 mM CaCl₂ buffer (pH 8.0). One unit of trypsin per ml (U) was defined as 1 μmol BAPNA hydrolyzed per min per ml of enzyme extract at 407 nm (Holm et al. 1988). Enzymatic activity was expressed as specific activity defined as units per milligram of protein (mU mg protein⁻¹). Soluble protein of crude enzyme extract was quantified by means of the Bradford's method (Bradford 1976) using bovine serum albumin as standard. All measurements were made in three methodological replicates. The absorbance read using a spectrophotometer (Power Wave XS2, BioTek) and data presented in total and specific activity units.

Lysozyme-like activity

Analysis of Lysozyme-like activity were performed using a diffusion test on Nutrient agar (NA) (Himedia, India). Lysozyme-like-induced growth inhibition of Micrococcus lysodeicticus (from collected microorganisms of ISEA SB RAS) was tested on 9-mm Petri dishes. Bacterial cultures (1×10⁷ cells ml⁻¹) were grown in 10 ml after cooling the nutrient agar to 45 °C. After solidification (~30 min) wells with 1,5-mm diameter were made. A test sample (3 μl) was placed in each well and were incubated 24 h at 37 °C. Samples were tested in triplicated on plates. Activity from homogenate of skin mucosa was standardized against a serial dilution of chicken egg white lysozyme (EWL) (Sigma-Aldrich) at known concentrations. Antimicrobial activity was determined by measuring clear zone around wells. A value was expressed as equivalent to EWL (mg ml⁻¹) (Mohner and Messner, 1968).

16S sequence processing

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 were removed, as well as sequences shorter than 350 or greater than 500 bp. QIIME 1.9.1 (Caporaso et al., 2010) was used for the further processing of the sequences. 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 UCLUST (Edgar, 2010), taxonomy assignment (UCLUST), 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 non-bacterial sequences were removed from further analysis.

The richness (number of OTU’s and Chao1 index) and diversity estimates (Shannon and Simpson index) per sample were calculated using QIIME 1.9.1 (Caporaso et al. 2010). Then, the samples were rarified to the lowest sequencing effort (1494 sequences) using QIIME. Such sequencing effort allowed us to include as many samples as possible in further analyses without reducing the power of the statistical methods. Increasing the sequencing effort did not change the obtained results, but reduced the number of analyzed samples (Figure S1).

In addition, shared OTUs between all samples and a core microbiome was computed. The “core” microbiome was calculated by identification of OTUs observed in at least 50% of all samples (‘compute_core_microbiome.py’ in QIIME). Due to specific composition of the microbiota associated with environmental compartments we excluded water, reed and sediment when selecting the “core” OTUs and in the downstream analysis of the core microbiome. Nucleotide sequences were deposited in the Sequence Read Archive (SRA NCBI), accession number PRJNA596590.

**Statistical analyses**

All data are presented as a mean ± standard error (SE). STATISTICA 8.0, PAST v. 3.16, and Excel 2019 were used to create bar chart and box plot graphs with mean and standard error. For estimating the differences between the richness and diversity estimates, a non-parametric Kruskal-Wallis test with Dunn's multiple comparisons test was applied. The Kruskal-Wallis test
with Dunn’s multiple comparisons test was also done to compare the differential abundance of
dominant OTUs between different types of samples in control and infested fish using in PAST,
3.16 (Hammer et al. 2011). In the same program among infested fish, Bonferroni-corrected
correlation using Spearman rank tests were explored between alpha and beta diversity of
microbial communities and numbers of ulcers. A weighted UniFrac (Lozupone and Knight,
2005) dissimilarity matrix was calculated in QIIME and used for downstream analyses. The
matrix was used to perform principle coordinates analysis (PCoA) to visualize differences
among groups of samples. Permutational multivariate analysis of variance using distance
matrices was used as implemented in the ‘adonis’ function of the vegan R package (Oksanen et
al. 2018). Pairwise comparisons for all pairs of levels of used factors were performed using
‘adonis.pair’ function of the EcolUtils R package (Salazar, 2018). Analysis of multivariate
homogeneity of group dispersions (variances) to test if one or more groups is more variable than
the others, was performed using the ‘betadisper’ function of the vegan R-package. In all the
aforementioned tests statistical significance was determined by 10 000 permutations. To estimate
the differences between the lysozyme- and trypsin-like activities of healthy and unhealthy fish
the Kruskal-Wallis test was applied using PAST.

Results

The prevalence and mean intensity of parasite infestation

The prevalence and intensity (mean±SE) of *L. cyprinacea* and *A. foliaceus* infestation
were 31.4±7.8, 2.2±0.4, and 20.0±6.8, 1.9±0.6%, correspondingly.

The alpha diversity estimates of microbial communities

According to Dunn’s post hoc test the Chao1 index value and number of observed OTU’s
in the microbial community of environmental compartments (water, sediment, and common
reed) were significantly higher than in the microbiota associated with ulcers in both with *L.
cyprinacea* and without *L. cyprinacea* (p≤0.05). Significant differences were also observed in
Shannon and Simpson index values between sediment and ulcers with *L. cyprinacea* (Shannon, Z=3.6, p=0.015; Simpson, Z=0.03, p=0.025, correspondingly). The number of OTU’s and Chao1 value were significantly different between ulcers with and without *L. cyprinacea* and the microbiota associated with *L. cyprinacea* (OTU’s, Z=3.6, p=0.015 and Z=3.3, p=0.038; Chao1, Z=3.6, p=0.014 and Z=3.2, p=0.07, correspondingly). No significant differences in richness and diversity estimates were found between microbiota associated with ectoparasites and intact skin mucus of both uninfested (group A) and infested fish (group B and C). Also, there were no significant differences found in intact skin mucus of uninfested fish (group A) in comparison with the infested fish (group B and C). The a-diversity of intact skin mucosa from group B was not significantly different from microbiota of intact skin mucosa from group C (Figure 2).

**The beta diversity estimates of microbial communities**

Due to absence of significant differences (ADONIS, p>0.05) in the microbiota of intact skin mucosa of infested fish between group B (fish only with ulcers) and C (fish with ulcers and parasites) as well as between microbiota of ulcers at the site of *L. cyprinacea* attachment and ulcers without *L. cyprinacea*, we combined these data to the group “intact skin mucosa of infested fish” and group “ulcers”, correspondingly. Further statistical analysis was made according to these criteria.

The microbial community of intact skin mucosa of uninfested and infested fish, parasites, and environmental compartments was dominated by Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Verrucomicrobia, Chloroflexi, and Cyanobacteria (Figure 3).

**Dominant microbial taxa in skin of uninfested and infested fish**

The dominant skin microbiota of uninfested fish was represented by *Arcobacter* (12.57±3.49%), unclassified bacteria from the Comamonadaceae family (6.40±1.13%); unclassified bacteria from the Helicobacteraceae family (4.37±1.47%); *Polynucleobacter* (3.44±0.78%), and unclassified bacteria from the order Sphingobacteriales (3.40±1.23%). Additional genera were present at high abundances globally included *Acinetobacter*,...
Corynebacterium, Rheinheimera, Staphylococcus, Vogesella, unclassified bacteria from the family Aeromonadaceae. The skin microbiota of infested fish was mainly represented by Corynebacterium (10.03±1.82%), Arcobacter (5.47±0.97%), unclassified bacteria from Comamonadaceae family (4.44±0.48%), Rheinheimera (4.08±1.03%), and Staphylococcus (3.91±0.88%). The microbiota of ulcers was dominated by Corynebacterium (17.31±3.20%), unclassified bacteria from Comamonadaceae (7.67±2.58%) and Aeromonadaceae (6.86±2.85%) families, Staphylococcus (6.67±1.40%), and Acinetobacter (3.66±1.44%) (Figure 3). As shown in figure 4, the dominant microbiota in skin of uninfested and infested fish did not significantly differ between each other with the exception of Arcobacter and unclassified Helicobacteraceae. The relative abundance of these bacteria were significantly higher in skin microbiota of uninfested fish compared to ulcers (Dunn's post hoc, z=3.3, p=0.024 and z=4.1, p=0.0013, correspondingly).

**Distribution of dominant microbial taxa in environmental compartments**

Microbiota associated with environmental compartments (water, sediment, and common reed) was dominated by Arcobacter, Crenothrix, Rhodobacter, Synechococcus, unclassified genera from the family Comamonadaceae and unclassified bacteria from the order Bacteroidales (Figure 3). Arcobacter was significantly more abundant in water community (14.80±1.60%) in comparison with microbiota associated with reed (0.13±0.1%) and sediment (0.01±0.01%) (Dunn's post hoc, z=3.5, p=0.014 and z=3.7, p=0.005, correspondingly). Other abundant taxa in the microbiota of water were unclassified bacteria from the family Comamonadaceae (7.93±1.26%) and Synechococcus (6.33±2.02%). Microbiota of common reed were dominated by Crenothrix (17.04±12.42%) and unclassified bacteria from the family Comamonadaceae (17.04±3.37%). Unclassified Bacteroidales (6.43±0.47%), Crenothrix (3.56±0.99%), and Thiobacillus (3.24±0.74%) were dominant in microbiota of sediment.

**Dominant microbial taxa in parasitic crustaceans and their relation to fish skin and microbiota of environmental compartments**
The microbiota associated with parasitic crustaceans *L. cyprinacea* were dominated by unclassified Comamonadaceae (33.93±9.18%), Aeromonadaceae (8.44±5.97%), and *Vogesella* (4.27±3.32%), whereas the dominant microbiota of *A. foliaceus* were represented by *Flavobacterium* (17.95±16.19%), *Corynebacterium* (10.61±5.0%), and unclassified bacteria from the Comamonadaceae family (3.81±1.60%). No significant differences in associated microbiota of ectoparasites were found when comparing *L. cyprinacea* and *A. foliaceus* between each other (Dunn's post hoc, p>0.05).

The abundances of *Arcobacter*, *Corynebacterium*, *Polynucleobacter* were significantly lower in microbiota of *L. cyprinacea* (1.64±0.49; 2.89±1.01, and 0.10±0.06%, correspondingly) in comparison with microbiota of intact skin mucosa of uninfested fish (12.57±3.49; 3.13±0.84, and 3.44±0.78%, correspondingly). The significant differences in abundances of *Polynucleobacter* in intact skin mucosa of infested fish (1.69±0.40%) was also significantly different in comparison with associated microbiota of *L. cyprinacea* (0.1±0.06%) (Dunn's post hoc, z=3.2, p=0.036). The relative abundance of individual genera containing *Corynebacterium* was significantly lower in *L. cyprinacea* (2.89±1.01%) as compared to ulcers (17.31±3.20%) of infested fish (Dunn's post hoc, z=3.6, p=0.01). Opposite trends were observed for unclassified bacteria from Comamonadaceae family (Figure 4) where abundance was significantly higher in microbiota of *L. cyprinacea* (33.93±9.18%) then in skin ulcers (7.67±2.58%), intact skin mucosa of infested fish (4.44±0.48%), and sediment (2.10±0.41%) (Dunn's post hoc, z=3.6, p=0.007; z=4.0, p=0.002, and z=3.3, p=0.032, correspondingly).

The significant differences in dominant microbiota of *A. foliaceus* were noted only for *Thiobacillus* and unclassified Bacteroidales where abundance was significantly lower in microbiota of *A. foliaceus* (0.003±0.003 and 0.07±0.06%) when comparing with sediment (3.43±0.74 and 6.43±0.47) (Dunn's post hoc, z=3.5, p= 0.032 and z=3.2, p= 0.046, correspondingly).
The higher proportion of the microbial communities of skin mucosa of Prussian carp and ectoparasites were mainly represented by bacteria which abundance were less than one percent and these minor bacteria ranged from 40 to 80% of the total community. For this reason, we made statistical comparisons with a “core” microbiota.

**Analysis of “core” microbiome**

We identified 131 OTUs as shared by at least 50% of samples. Moreover, all these OTU’s are present in each experimental group, and 100 of them have statistically significant differential abundance between groups (G-test, p≤0.05 after FDR correction, Tables are available at [http://doi.org/10.6084/m9.figshare.13580627](http://doi.org/10.6084/m9.figshare.13580627)). According to the ADONIS test (Table 2) the microbiota associated with *L. cyprinacea* was significantly different from microbial communities of intact skin mucus of both infested and uninfested fish and ulcers (p≤0.05). Intact skin mucosa of uninfested fish was also significantly different from the same of infested fish ($R^2 =0.089$, p=0.035) and ulcers ($R^2 =0.229$, p=0.001). The principal coordinates analysis (PCoA) based on “core” calculations showed a clear grouping of intact skin mucus of Prussian carp in comparison with microbiota associated with *A. foliaceus*, *L. cyprinacea* and ulcers of fish (Figure 5).

**Relative abundance of known opportunistic pathogens**

The number of reads that belonged to genera of potentially opportunistic pathogens (Figure 6) were significantly higher in microbiota associated with *A. foliaceus*, (47.24±10.90%), ulcers (37.81±4.51%) and intact skin mucosa of infested fish (27.85±2.94%) in comparison to *L. cyprinacea* (47.24±10.90%), reed (2.00±0.47%) and sediment (0.80±0.16%) (Dunn's post hoc at p≤0.05). The ratio of potential pathogens known from existing literature was represented by *Acinetobacter lwofii*, *A. johnsonii*, *Myroides odoratimimus*, *Flavobacterium succinicans*, *F. columnare*, *Janthinobacterium lividum*, *Plesiomonas shigelloides*, *Staphylococcus epidermidis*, and *Lactococcus garvieae*. Relative abundance of pathogenic species in associated microbiota of intact skin mucosa of both uninfested and infested Prussian carp, ulcers and ectoparasites were mainly represented by *A. johnsonii*, *F. columnare*, and *S. epidermidis*. The dominant potential
pathogens found in environmental compartments were represented by F. succinicans, F. columnare, P. shigelloides, and L. garvieae.

Correlation between opportunistic pathogens and number of ulcerations

In order to estimate possible correlation between parasite load (number of parasites), the number of ulcers and alpha and beta diversity of the skin microbial community of fish, the Spearman rank correlation test was applied. No correlation was observed between branchiuran and copepod ectoparasite load (number of parasites) and alpha diversity of microbial community of intact skin mucosa of infested fish (Spearman Rank correlations, p>0.05). When analyzed, the beta diversity in intact skin mucus of infested fish, there was observed a weak negative correlation between relative abundance of Acrobacter (Spearman \( p=-0.48; p=0.008 \)), genus C39 from Rhodocyclaceae family (\( p=-0.43; p=0.021 \)), Corynebacterium (\( p=-0.38; p=0.044 \)), Rheinheimera (\( p=-0.46; p=0.012 \)), unclassified Comamonadaceae (\( p=-0.38; p=0.040 \)), unclassified Helicobacteraceae (\( p=-0.52; p=0.003 \)), Vogesella (\( p=-0.55; p=0.002 \)), and the number of ulcerations (Figure S2). No significant correlation was found between the abundance of dominant microbiota of intact skin mucosa of infested fish and parasite load (number of parasites) (p>0.05).

Lysozyme- and trypsin-like activities of skin mucosa of Prussian carp and ectoparasites infestation

To estimate the antimicrobial potential of skin mucosa of Prussian carp infested by ectoparasites the lysozyme- and trypsin-like activity of skin mucosa was determined. No significant differences were found in the lytic activity of lysozyme and activity of trypsin-like enzymes in skin mucosa of uninfested fish in comparison to infested fish (Kruskal-Wallis test at \( p>0.05 \)) (Supplementary Figure S3 and Figure S4).

Discussion
The mucous secreted on the external surface of the fish epidermis is one of the most distinctive features of fish skin. The mucous secreted by mucous glands of the epithelial layer of skin provides a protective barrier against abrasive external environmental surfaces and pathogenic organisms. Several bacterial phyla reported as typical components of the microbiota of fish skin include Proteobacteria, Actinobacteria, Bacteriodetes, Firmicutes, Cyanobacteria, Chlorobi, and TM7 (Wang et al. 2010; Boutin et al. 2013; Larsen et al. 2013 among others). These results were observed in our study, including Proteobacteria, Actinobacteria, Firmicutes, Bacteriodetes, Verrucomicrobia and Acidobacteria. The most common bacterial genera detected in fish skin were represented by Acinetobacter, Arcobacter, Bacillus, Flavobacterium, Lysobacter, Methylobacterium, Noviherbaspirillum, Polynucleobacter, Pseudomonas, Sphingomonas, Vibrio (Austin, 2006; Wang et al. 2010; Svanevik et al. 2011; Boutin et al. 2013; Larsen et al. 2013; Leonard et al. 2014; Minniti et al. 2017; Sylvain et al. 2017).

Despite the shared groups of bacteria found in the skin microbiota of uninfested fish, some variations were observed due to dietary, environmental (temperature, salinity, and sesonality), and others factors (stocking density, stress, etc.). Different approaches towards determination of the skin microbiota may also affect the reported microbial community structure. The accurate determination of the diversity of skin-associated microbiota is complicated by different methods that are applied to collection of material and distinctly different sampling sites. The skin mucosa is most often sampled by using sterile swabs (Wang et al. 2010; Boutin et al. 2013, 2014; Minniti et al. 2017; Llewellyn et al. 2017; Legrand et al. 2018; Tarnecki et al. 2019), or by gentle scraping of the fish external surfaces using a cell-scaper or spatula (Svanevik et al. 2011; Sylvain et al. 2017). Other methods to collect fish mucus include rinsing the entire external surface of the fish or section of fish skin with saline buffers (Leonard et al. 2014; Li et al. 2019). Several authors used plastic bags filled with different saline solutions (Ross et al. 2000; Subramanjan et al. 2007). The skin sample sites are not always described precisely in different studies and, for this reason, there may be variations in the microbial
Communities described from different locations of the fish body surface. Moreover, variation in the skin microbiota of fish and data interpretation can also occur when researchers used a method rinsing the whole fish in a buffer solution; in this case it is not clear that the skin microbiota did not also include a flushing of skin mucosa from other sites like the nasal rosette, the buccal cavity and gills (Leonard et al. 2014). Hence, lack of standard methods of skin sample collection creates difficulties for comparison and interpretation of skin microbiota results. At the same time, results on skin microbiota obtained by different researchers, even when using the same collection methods will not always be comparable. Moreover, the differences in sample storage, DNA extraction protocol and methods of sequencing can also be a source of variation (Kashinskaya et al., 2017; Tarnecki et al., 2017).

**Microbiota of skin mucosa of Prussian carp during ectoparasitic infestation**

In the present study we obtained data on the microbial community associated with skin mucosa from fish infested and uninfested by parasitic crustaceans from the genus *Argulus* and *Lernaea*. According to our results, the microbiota of intact skin mucosa of uninfested (group A) and infested (group B and C) Prussian carp was represented by *Arcobacter*, *Corynebacterium*, and unclassified genera from the Comamonadaceae and Helicobacteraceae families. It is known that *Arcobacter* includes microaerophilic and anaerobic opportunistic pathogenic organisms, which inhabit a wide range of ecosystems including freshwater and marine habitats, sewage, and the host mucosa (Legrand *et al.* 2018; Ur Rahmann *et al.* 2020). The second dominant OTU, *Corynebacterium*, is often found in the environment and usually colonizes the blood, skin and mucous membranes of humans and animals (Baya *et al.* 1992, Tarnecki *et al.* 2018). At the same time, this genus includes one known fish pathogen – *C. aquaticum*. To date this pathogenicity was registered only for striped bass *Morone saxatilis* and rainbow trout *Oncorhynchus mykiss* (Baya *et al.* 1992).
Another interesting member of the skin-mucus microbiota of Prussian carp was *Rheinheimera*, which is a potentially important genus as it provides protection against pathogens, and is known for possessing beneficial antibacterial activity (Boutin *et al.* 2013).

The skin surfaces of fish at the site of attachment of the parasite from the genus *Lernaea* and *Argulus* there formed a deep ulcer with reddish irregular, bumpy edges. Fish without lice may show nonspecific signs of infestation, including spots or pinpoint hemorrhages, “flashing” behavior, and poor body condition. In some cases, there may be no obvious signs of disease other than presence of the parasite (Wafer *et al.* 2015). In the present study, during ectoparasite infestations, the skin surfaces of Prussian carp were characterized by different levels of petechial hemorrhages (number of ulcers ranged from one to 16). According to the Spearman rank correlation test, the increase of ulceration in skin of Prussian carp were significantly correlated with the reduced abundance of *Acrobacter*, *Corynebacterium*, bacteria C39 from Rhodocyclaceae family, *Rheinheimera*, unclassified bacteria from Comamonadaceae and Helicobacteraceae families, and *Vogesella*. Other findings have also shown that parasitic infestations can modify the skin microbiome of fish. Thus, during infestation in *S. salar* by *Lepeophtheirus salmonis* a significant reduction in microbial richness of infested fish in comparison with control fish was observed. Moreover, a network analysis of microbial taxa on skin mucosa of *S. salar* revealed an association of high louse burdens to multiple pathogenic genera (*Vibrio*, *Flavobacterium*, *Tenacibaculum*, *Pseudomonas*) (Llewellyn *et al.* 2017). Thus, summarizing the above, our results indicate that ectoparasite infestations significantly affects the abundance/diversity of the bacterial community associated with skin of infested Prussian carp, as compared to uninfested fish.

**Lysozyme- and trypsin-like activities of skin mucosa of Prussian carp with and without ectoparasite infestation**

In previous studies the activity of trypsin has been reported in rainbow trout mucus (Hjelmeland *et al.* 1983) and in mucus-secreting cells of *S. salar* (Braun *et al.* 1990). Total
protease activity in the mucus of *S. salar* infested with *L. salmonis* was reduced in comparison with uninfested fish. This effect was found for both studied infestation levels (20±13 and 178±67 lice per fish) (Ross *et al.* 2000). Our data have shown that no significant differences were found in lysozyme-like and trypsin-like activities between intact skin mucosa of uninfested (group A) and infested fish (group B and C). As mentioned above, the microbiota of fish with ulcers and parasites and from fish only with ulcers (group B and C, correspondingly) did not significantly differ. It was demonstrated that antimicrobial components in skin mucosa of infested Prussian carp were similar to intact skin mucosa of uninfested fish and probably can act equally as a medium with antimicrobial properties that protect the skin from pathogenic organisms. This assumption requires additional verification, because we acknowledge that such a low level of louse load (one to five) in the present study may have been insufficient to affect the trypsin-like activity in skin mucosa of Prussian carp, as was shown for *S. salar* infested with *L. salmonis* (Ross *et al.* 2000).

**The microbiota of environmental compartments**

The composition of the bacterial community inhabiting the water and subcompartments of the surrounding environment was also investigated in order to evaluate its correlation with the community present on the fish skin. Our findings imply that colonization of skin mucosa of fish by *Arcobacter* and unclassified genera from the Comamonadaceae family is probably enhanced in a natural water environment, but other groups of bacteria associated to this environmental microbiota were specific and significantly different from those from intact skin mucosa of Prussian carp. Based on published data there are contradicting suggestions in terms of the similarity of skin microbiota and their environment. For instance, Larsen *et al.* (2015) has reported changes in the skin microbiota of *Fundulus grandis* depending on the environmental origin. In its freshwater habitat the microbiota was represented by Gammaproteobacteria and Betaproteobacteria, whereas the marine microbiota is dominated by Cyanobacteria and Alphaproteobacteria (Larsen *et al.* 2015). However, some authors claim that the skin microbiota
is a reflection of their environment (Horsley, 1973). Similarity of water and skin mucosa was also observed in farmed *S. salar*, but the authors indicate that interpretation of obtained results need to be viewed with caution due to the low biomass of starting material used for study (Minniti *et al.* 2017).

**The microbiota of ectoparasites**

In early studies the parasitic crustaceans *A. foliaceus* and *L. cyprinacea* in Chany Lake were detected in a taxonomically close species, *C. carassius*, and their prevalence in summer was 6.6 and 26.4%, correspondingly; although the prevalence of ectoparasites in Prussian carp was not reported (Sous and Rostovsev, 2006). In other data the lower values of *L. cyprinacea* prevalence (2.2%) was observed in Prussian carp *C. gibelio* from the Anzali wetland in the southwest of the Caspian Sea (Daghigh Roohi *et al.* 2014). The prevalence of *L. cyprinacea* in *C. gibelio* from Manisa Province, Western Turkey was the highest (48.3%) in the summer (Demir and Karakisi, 2016). Thus, the level of parasite infestation obtained for Prussian carp in Chany Lake was moderate by comparison.

The evolutionary origins of the microbiota of fish skin remain largely unstudied as an aspect of host-parasite-microbiota associations, therefore whether or not ectoparasites harbor unique microbiota and are able to play a significant role in transmission of opportunistic pathogens, or drive evolutionary processes is poorly understood. It is well known that microorganisms which can be a causative agent of infectious diseases of fish are natural inhabitants of microbial communities and widely distributed in aquatic ecosystems. It is assumed that the entry of pathogenic microbiota can be either from water and / or bottom sediments, or transmitted directly upon contact with parasitic organisms. It is suggested that the direct route of transmission of infections caused by *Argulus* can lead to increased susceptibility of fish to these diseases, for example, when an entry pathway is created for bacteria due to epidermal damage (Kanno *et al.* 1990, Buchmann and Bresciani, 1997), or when the parasite acts as a vector (Cusack and Cone, 1985). In addition, the parasite can cause bacterial infections indirectly by
reducing the host's immunocompetence (Bowers et al. 2000). A number of studies have shown that the bacterial pathogens *A. salmonicida, F. columnare* and the fungus *Saprolegnia spp.*, the causative agents of ringworm in fish, were isolated from fish infested with *A. coregoni* (Bandilla et al. 2006; Steckler and Yanong 2012a; Shahraki et al. 2014). Among the representatives of the genus *Flavobacterium*, a whole spectrum of microorganisms pathogenic for fish is known: *F. psychrophilum, F. columnar, F. branchiophilum*. Bacterial cold-water disease (BCWD), the causative agent of which is *F. psychrophilum*, causes ulcers on the body and fin rot of salmoninds at low temperatures (Castillo et al. 2012). Previous studies have shown that the Spring viraemia of carp (SVC) is also recorded in fish infested with crustaceans from the genus *Argulus* (Steckler and Yanong, 2012a). From our data, the microbiota associated with parasitic *A. foliaceus* were dominated by bacteria from the genera *Flavobacterium* and *Corynebacterium*. The microbiota associated with *L. cyprinacea* was represented by unclassified bacteria from the Comamonadaceae and Aeromonadaceae families.

Diseases caused by branchiuran crustaceans from the genus *Argulus*, and secondary infections arising from them, are recorded in various countries of the world, and are an acute problem in the management of pond aquaculture. Similar works about infectious diseases of fish of a bacterial, viral and fungal nature arising with *Argulus* infestation are not numerous (Bandilla et al. 2006; Steckler and Yanong 2012a; Shahraki et al. 2014). A study regarding the diversity and composition of microbiota from *S. salar* parasitized on their skin by the copepod *L. salmonis* has also revealed the association of multiple, potentially pathogenic bacterial genera such as *Vibrio, Flavobacterium, Tenacibaculum, and Pseudomonas* (Llewellyn et al. 2017). According to our data *A. johnsonii, F. succinicans, F. columnare, J. lividum, P. shigelloides*, and *S. epidermidis* were found in microbiota associated with *L. cyprinacea* and *A. foliaceus* ectoparasites. These results indicate that ectoparasites such as *A. foliaceus* can play a possible role in transmission of secondary bacterial infection in fish either by creating a portal of entry by wound formation and/or acting as a vector. In this aspect, understanding the mechanism of
occurrences of secondary bacterial infections in fish can be of value and interest in future laboratory studies in terms fish-parasites and pathogenic bacteria relationships.

In conclusion, we investigated the microbiota of skin mucosa of fish during branchiuran and copepod crustacean infestations in natural habitats, with a parallel study of the microbiota of the parasites themselves, water, reed and sediments as possible sources of pathogenic microorganisms. Our result revealed a significant perturbation of the microbial community in skin mucosa of infested Prussian carp in comparison with uninfested fish. During ectoparasite infestations the increase of ulcerations in the skin of Prussian carp was significantly correlated with reduced relative abundances of several dominant groups of bacteria. In line with previous studies of microbiota associated to ectoparasites, several pathogenic species of bacteria (A. johnsonii, F. succinicans, F. columnare, J. lividum, P. shigelloides, and S. epidermidis) were revealed. In future studies, a possible pathogenic role of associated bacteria should be demonstrated and their presence confirmed using multiple tests for each suspected pathogen. These findings expand our current knowledge regarding the relationship between host-associated microbiota and ectoparasites.

Acknowledgement. This work was supported by the Russian Science Foundation, project no.17-74-10054.

Conflict of interest. None declared.

Animal Ethics. 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.


**Author contributions**

Conceptualization: Kashinskaya E.N., Solovyev M.M.; Data analysis and interpretation: Kashinskaya E.N., Simonov E.P., Polenogova O.V., Kiryukhin B.A., Solovyev M.M.; Writing - original draft preparation: Kashinskaya E.N., Solovyev M.M.; Writing - review and editing: Kashinskaya E.N., Andree K.B., Solovyev M.M.
Figure legend

**Figure 1.** Study site and sampling approach. Sample site of intact skin mucosa denoted by blue dashed lines, ulcers denoted by red circles.

**Figure 2.** Diversity analysis of microbial community of skin surfaces of Prussian carp, ectoparasites and environmental compartments. The lower case character indicates significance at \( p \leq 0.05 \). ISM – intact skin mucosa.

**Figure 3.** Microbial communities at the phylum and the lowest taxonomical level identified from skin mucosa of Prussian carp, their ectoparasites and environmental compartments. Upper case character indicates “I” as intact skin mucosa, or ISM. **Phylum:** (■) Acidobacteria, (■) Actinobacteria, (■) Bacteroidetes, (■) Chlorobi, (■) Chloroflexi, (■) Cyanobacteria, (■) Firmicutes, (■) Nitrospirae, (■) Planctomycetes, (■) Proteobacteria, (■) Spirochaetes, (■) Verrucomicrobia, (■) Others. Bacteria with a relative abundance less than 1% were pooled and indicated as “Others.” **The lowest taxonomical level:** (■) g_Acinetobacter; (■) g_Anaerococcus; (■) g_Arcobacter; (■) g_Candidatus Xiphinematobacter; (■) g_Corynebacterium; (■) g_Crenothrix; (■) g_C39 from the Rhodocyclaceae family; (■) g_Flavobacterium; (■) g_Polynucleobacter; (■) g_Rheinheimera; (■) g_Rhodobacter; (■) g_Staphylococcus; (■) g_Synechococcus; (■) g_Thiobacillus; (■) g_Vogesella; (■) f_Unclassified Aeromonadaceae; (■) f_Unclassified Helicobacteraceae; (■) f_Unclassified Myxococcales; (■) f_Unclassified Sinobacteraceae; (■) o_Unclassified Bacteroidales; (■) o_Unclassified Sphingobacteriales; (■) Others. Bacteria with a relative abundance less than 3% were pooled and indicated as “Others”.

**Figure 4.** The dominant microbiota in skin mucosa of Prussian carp, their ectoparasites and environmental compartments. The lower case character indicates significance at \( p \leq 0.05 \). ISM – intact skin mucosa.
Figure 5. Principal coordinates analysis (PCoA) for core skin microbiota of Prussian carp and their ectoparasites. *A. foliaceus* – empty black circles; *L. cyprinacea* – empty red triangles; ISM (Uninfested) – green plus; ISM (Infested) – blue cross; Ulcers – light blue diamond.

Figure 6. Number of reads that belonged to potentially pathogenic genera (a) and relative abundance pathogenic genera in associated microbiota of skin of Prussian carp during ectoparasites infestation (b). ISM – intact skin mucosa. (■) *Acinetobacter johnsonii*; (■) *Myroides odoratimimus*; (■) *Flavobacterium succinicans*; (■) *Flavobacterium columnare*; (■) *Janthinobacterium lividum*; (■) *Plesiomonas shigelloides*; (■) *Staphylococcus epidermidis*; (■) *Lactococcus garvieae*;

Figure S1. The rarefaction curves.

Figure S2. Relationship between the relative abundance of known opportunistic pathogens found in skin of infected Prussian carp during ectoparasites infestations. Correlation between relative abundance of different opportunistic pathogens and number of ulcers were evaluated using Spearman rank correlation test at p≤0.05.

Figure S3. Lysosome-like activity of skin mucosa of Prussian carp infected by ectoparasites.

Figure S4. Activity of trypsin in skin mucosa of Prussian carp infected by ectoparasites.
<table>
<thead>
<tr>
<th>Number of fish</th>
<th>Sex</th>
<th>Body weight, g</th>
<th>Total Length, sm</th>
<th>Standard Length, sm</th>
<th>L. cyprina cea</th>
<th>A. foliaceus</th>
<th>Number of ulcers</th>
<th>Samples for microbiome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>394.8</td>
<td>27.9</td>
<td>23.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>I</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>312.0</td>
<td>25.3</td>
<td>20.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>I</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>300.0</td>
<td>24.9</td>
<td>20.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>I</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>378.6</td>
<td>27.3</td>
<td>22.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>I</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>309.0</td>
<td>26.2</td>
<td>21.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>I</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>414.2</td>
<td>28.7</td>
<td>23.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>I</td>
</tr>
<tr>
<td><strong>Mean±SE</strong></td>
<td></td>
<td><strong>351.4±20.5</strong></td>
<td><strong>26.7±0.61</strong></td>
<td><strong>21.8±0.51</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Uninfested fish (Group A)**

**Infested fish (without parasites, only ulcers – Group B)**

<p>| 7             | F   | 378.6          | 27.9             | 23.0                | 0             | 0           | 1                | I                     |
| 8             | F   | 385.0          | 27.3             | 22.4                | 0             | 0           | 1                | I                     |
| 9             | F   | 339.0          | 26.5             | 22.2                | 0             | 0           | 1                | I                     |
| 10            | F   | 371.6          | 27.0             | 22.1                | 0             | 0           | 1                | I                     |
| 11            | F   | 364.0          | 27.1             | 22.1                | 0             | 0           | 1                | I                     |
| 12            | F   | 385.6          | 27.7             | 22.5                | 0             | 0           | 2                | I                     |
| 13            | F   | 390.4          | 28.4             | 22.9                | 0             | 0           | 1                | I, U                  |
| 14            | F   | 380.0          | 27.7             | 23.4                | 0             | 0           | 2                | I, U                  |
| 15            | M   | 336.8          | 27.2             | 22.5                | 0             | 0           | 3                | I, U                  |
| 16            | F   | 405.0          | 27.8             | 22.7                | 0             | 0           | 4                | I, U                  |
| 17            | F   | 401.8          | 27.8             | 22.7                | 0             | 0           | 2                | I, U                  |
| 18            | F   | 386.2          | 27.9             | 22.7                | 0             | 0           | 1                | I, U                  |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>F</td>
<td>528.4</td>
<td>29.4</td>
<td>24.1</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>I, U</td>
</tr>
<tr>
<td><strong>Mean±SE</strong></td>
<td>388.7±13.0</td>
<td>27.7±0.20</td>
<td>22.7±0.16</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Fish with ulcers and parasites (Group C)*

<p>| | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>F</td>
<td>345.2</td>
<td>27.4</td>
<td>22.1</td>
<td>4</td>
<td>0</td>
<td>8</td>
<td>I, UL, L</td>
</tr>
<tr>
<td>21</td>
<td>F</td>
<td>367.8</td>
<td>27.3</td>
<td>22.5</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>I, UL, L</td>
</tr>
<tr>
<td>22</td>
<td>F</td>
<td>380.8</td>
<td>28.8</td>
<td>23.4</td>
<td>3</td>
<td>0</td>
<td>13</td>
<td>I, UL, L</td>
</tr>
<tr>
<td>23</td>
<td>M</td>
<td>342.0</td>
<td>26.5</td>
<td>21.9</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>I, UL, L</td>
</tr>
<tr>
<td>24</td>
<td>F</td>
<td>380.0</td>
<td>28.2</td>
<td>23.0</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>I, UL, L</td>
</tr>
<tr>
<td>25</td>
<td>F</td>
<td>417.0</td>
<td>28.7</td>
<td>23.9</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>I, UL, L</td>
</tr>
<tr>
<td>26</td>
<td>F</td>
<td>366.0</td>
<td>28.1</td>
<td>23.0</td>
<td>1</td>
<td>0</td>
<td>7</td>
<td>I, UL, L</td>
</tr>
<tr>
<td>27</td>
<td>-</td>
<td>334.8</td>
<td>27.3</td>
<td>22.4</td>
<td>5</td>
<td>0</td>
<td>9</td>
<td>I, UL, L</td>
</tr>
<tr>
<td>28</td>
<td>F</td>
<td>360.0</td>
<td>26.9</td>
<td>22.0</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>I, UL, L</td>
</tr>
<tr>
<td>29</td>
<td>F</td>
<td>320.8</td>
<td>26.5</td>
<td>21.6</td>
<td>3</td>
<td>1</td>
<td>16</td>
<td>I, U, UL, L, A</td>
</tr>
<tr>
<td>30</td>
<td>M</td>
<td>448.0</td>
<td>28.9</td>
<td>24.1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>I, U, L</td>
</tr>
<tr>
<td>31</td>
<td>F</td>
<td>368.0</td>
<td>27.7</td>
<td>22.7</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>I, U, A</td>
</tr>
<tr>
<td>32</td>
<td>F</td>
<td>386.4</td>
<td>28.4</td>
<td>23.4</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>I, U</td>
</tr>
<tr>
<td>33</td>
<td>F</td>
<td>334.8</td>
<td>26.1</td>
<td>21.8</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>I, A</td>
</tr>
<tr>
<td>34</td>
<td>-</td>
<td>353.4</td>
<td>27.1</td>
<td>22.4</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>I, A</td>
</tr>
<tr>
<td>35</td>
<td>F</td>
<td>381.8</td>
<td>26.7</td>
<td>21.9</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>I</td>
</tr>
<tr>
<td><strong>Mean±SE</strong></td>
<td>367.3±8.05</td>
<td>27.5±0.22</td>
<td>22.7±0.19</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Upper case character indicates “I” as intact skin mucosa, or ISM; “U” – ulcers without *L. cyprinacea*; UL” – ulcers with *L. cyprinacea*; “A” – *A. foliaceus*; “L” – *L. cyprinacea*.

Table 2 Comparison (ADONIS test) of “Core” microbiota associated with skin surfaces of Prussian carp and their ectoparasites. Extra bold indicates significance at p≤0.05.
<table>
<thead>
<tr>
<th>Combination</th>
<th>$R^2$</th>
<th>p-value FDR corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. foliaceus</em> vs. <em>L. cyprinacea</em></td>
<td>0.163</td>
<td>0.091</td>
</tr>
<tr>
<td><em>A. foliaceus</em> vs. Intact skin mucosa (Uninfested)</td>
<td>0.231</td>
<td>0.058</td>
</tr>
<tr>
<td><em>A. foliaceus</em> vs. Intact skin mucosa (Infested)</td>
<td>0.005</td>
<td>0.987</td>
</tr>
<tr>
<td><em>A. foliaceus</em> vs. Ulcers</td>
<td>0.029</td>
<td>0.678</td>
</tr>
<tr>
<td><em>L. cyprinacea</em> vs. Intact skin mucosa (Uninfested)</td>
<td>0.326</td>
<td>0.003</td>
</tr>
<tr>
<td><em>L. cyprinacea</em> vs. Intact skin mucosa (Infested)</td>
<td>0.171</td>
<td>0.001</td>
</tr>
<tr>
<td><em>L. cyprinacea</em> vs. Ulcers</td>
<td>0.163</td>
<td>0.004</td>
</tr>
<tr>
<td>Intact skin mucosa (Uninfested) vs. Intact skin mucosa (Infested)</td>
<td>0.089</td>
<td>0.035</td>
</tr>
<tr>
<td>Intact skin mucosa (Uninfested) vs. Ulcers</td>
<td>0.229</td>
<td>0.001</td>
</tr>
<tr>
<td>Intact skin mucosa (Infested) vs. Ulcers</td>
<td>0.069</td>
<td>0.035</td>
</tr>
</tbody>
</table>

*Carassius gibelio, n=35*

Fig. 1
Fig. 2
a) Number of reads belonged to potentially pathogenic genera, %

- A. foliaceus
- Ulcers
- ISM (Infested)
- ISM (Uninfested)
- Water
- L. cyprinacea
- Reed
- Sediment

b) Relative abundance of pathogenic genera in associated microbiota of skin of Prussian carp during ectoparasites infestation

Fig. 6
Fig. S1

**Arcobacter sp.**
- \( r = -0.48; \) 
- \( p = 0.008 \)

**Corynebacterium sp.**
- \( r = -0.38; \) 
- \( p = 0.044 \)

**Rheinheimera sp.**
- \( r = -0.46; \) 
- \( p = 0.012 \)

**Vogesella sp.**
- \( r = -0.55; \) 
- \( p = 0.002 \)

---

Fig. S2

**Genus C39 from Rhodocyclaceae family**
- \( r = -0.43; \) 
- \( p = 0.021 \)

**Unclassified Comamonadaceae**
- \( r = -0.38; \) 
- \( p = 0.040 \)

**Unclassified Helicobacteraceae**
- \( r = -0.52; \) 
- \( p = 0.003 \)

---

Fig. S3

**Area of lysis zone, cm²/mg protein⁻¹**

- Uninfested (Group A)
- Infested (Group B)
- Infested (Group C)
Table S1. Shared OTU’s between groups after “Core” microbiome calculation.