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1 **Microbial community structure in a host-parasite system: the case of Prussian carp**
2 **and its parasitic crustaceans**
3
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22 **Key words:** skin microbiota; 16S rRNA; freshwater fish; *A. foliaceus*, *L. cyprinacea*; fish
23 pathogens.
24
25

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, $p \leq 0.05$). 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

52 pathogenic and non-pathogenic microbes. Fish have developed innate immune mechanisms
53 against potential pathogens to which they are continuously exposed. The normal structure and
54 features of the fish mucosa (skin, gills, and intestine, inclusive) functions towards differentiating
55 the host response to the plethora of physical, chemical, and biological stimuli of the aquatic
56 environment (Esteban and Cerezuel, 2015). One of the host's protective mechanisms is the
57 external epithelia that prevents entry of pathogenic organisms or allergens, and maintains
58 osmotic balance. Mucous glands of the fish skin produce the mucous layer that, in turn, is
59 colonized by different bacterial species (commensal and/or opportunistic), which play many
60 different roles, including the priming of immune memory for the development of the host
61 immune system (Minniti *et al.* 2017). Fish mucus has been demonstrated to possess
62 antimicrobial properties against different pathogenic bacteria and viruses. Among different
63 immune molecules, lysozyme is one of the main antibacterial agents that hydrolyzes cell walls of
64 both gram-positive and gram-negative bacteria. Some proteases are also able to protect skin
65 surfaces against pathogens (Esteban and Cerezuel, 2015).
66 The majority of bacteria detected in the skin-mucosal surfaces of fish belong to the
67 phylum Proteobacteria, Actinobacteria, Bacteriodetes, Firmicutes, Cyanobacteria, Chlorobi, and
68 TM7 (Boutin *et al.* 2013; Larsen *et al.* 2013; Leonard *et al.* 2014; Wang *et al.* 2010; Minniti *et*
69 *al.* 2017). To date, there are many studies focused on the fish skin microbiome under normal
70 conditions, whereas studies associated with fish skin subjected to different pathogens (bacteria,
71 viruses, fungi, protozoa and metazoan ectoparasites, etc) are limited (Llewellyn *et al.* 2017; Li *et*
72 *al.* 2019). It is well known that different pathogens can provoke a direct damage to fish skin
73 (Austin and Austin 2016). In addition, indirect damage can also be caused by parasite during the
74 course of life cycle in host tissue, which creates a "gateway" for secondary infections (Bandilla
75 *et al.* 2006). In the places where disruption of the integrity of the host skin has occurred, there is
76 a risk of infections caused by different groups of microorganisms, mainly by bacteria and fungi
77 (Bandilla *et al.* 2006; Wafer *et al.* 2015). An example of that mentioned above is the case of

78 *Lepeophtheirus salmonis* infestation in Atlantic salmon *Salmo salar* where multiple pathogenic
79 genera (*Vibrio*, *Flavobacterium*, *Tenacibaculum*, *Pseudomonas*) were registered in the skin
80 microbiota of infested fish. A significant reduction in microbial richness and highly significant
81 destabilization of microbiota of infested fish, in comparison with control fish, was also observed
82 (Llewellyn *et al.* 2017).

83 In freshwater ecosystems the diseases caused by crustaceans from the genus *Argulus*
84 (Family: Argulidae) and the genus *Lernaea* (Family: Lernaeidae) are an acute problem in the
85 management of pond aquaculture. In Russia there are three *Argulus* species, commonly known as
86 fish lice, which infest and cause skin diseases in fish (Steckler and Yanong 2012a). The most
87 widely known is *Argulus foliaceus,* whereas *A. coregoni* and *A. japonica* occur in freshwater fish
88 of the northern regions and in the basin of the Amur river, respectively (Zharikova *et al.* 2002).
89 Another crustacean ectoparasite of fish in Russia belongs to the genus *Lernaea* commonly called
90 anchor worms. Fish from different waterbodies in Russia are mainly parasitized by two species
91 of *Lernaea*: *L. cuprinacea* and *L. elegans*. In Russia the studies of these fish ectoparasites are
92 mainly focused on the different aspects of population biology such as abundance and prevalence
93 and their interannual variability under natural and artificial conditions (Zharikova *et al.* 2002).
94 Whilst there is an increasing understanding of fish immunity in response of ectoparasitic
95 infestations, and secondary infections (bacteria, fungi, viruses, etc.), little is known about the
96 possible role of microbes that are associated to such parasites. Previous studies have shown that
97 infestation of fish by *L. cyprinacea* and *A. foliaceus* results in deep ulcers on the skin surfaces at
98 the site of attachment with peripheral inflammation noted by reddening of the skin and irregular,
99 bumpy edges. A number of studies have shown that bacterial pathogens like *Aeromonas*
100 *salmonicida*, *Flavobacterium columnare* and Saprolegniales moulds, which are the causative
101 agents of dermatomycosis, were isolated from fish after infestation with *A. coregoni* (Bandilla *et*
102 *al.* 2006; Steckler and Yanong 2012a; Shahraki *et al.* 2014). Also, it is known that *Lernaea* can
103 cause intense inflammation, leading to different secondary bacterial (e.g., *Aeromonas*

104 *hydrophila*) and fungal infections (Steckler and Yanong 2012b). Experimental study of parasites,
105 their microbiota, and interactions among them and the host has become more feasible with newer
106 metagenomic approaches. Although, in some studies no changes in the structure of the bacterial
107 community upon infection were found (Baxter *et al.* 2015; Cooper *et al.* 2013; Reynolds, 2015),
108 in others significant alterations were noted (Baxter *et al.* 2015; Kreisinger *et al.* 2015).
109 Therefore, the determination of the bacterial community structure local to parasite attachment
110 and in places of skin penetration likely inducing antimicrobial properties of fish skin mucus are
111 the key aspects for understanding the mechanisms of the development of secondary infections
112 and, consequently, for investigating effective methods of prevention and treatment of fish.

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

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

128

129 **Materials and methods**

130

131 **Study area and sampling**

132 35 individuals of Prussian carp *Carassius gibelio* (Linnaeus 1758) with total length 133 225.2±0.14 mm and their ectoparasites *Lernaea cyprinacea* and *Argulus foliaceus* were collected 134 from July 30 to August 01, 2017 in the area of Malye Chany Lake in west Siberia (Russia, 135 $54^0$36'56.3''N, $78^0$12'5.9''E). Fish were captured using gill-nets (mesh sizes 35 and 45 mm) and 136 transported alive to the laboratory in plastic containers filled with water from the site of fish 137 capture (duration approximately 15 min). All fish were sacrificed and samples were collected 138 aseptically. In order to avoid contamination during sampling, one fish per container was 139 transported to the laboratory. Fishes were touched only by one assistant wearing sterile gloves 140 and handled only by the mouth and caudal fin. In this time, skin mucus samples were collected 141 by another assistant equipped with a sterile cell-scraper.

142 Skin samples comprised mucus and scales along the right and left full length surfaces of 143 fish from the posterior of the gill operculum to the base of the caudal fin (Figure 1). To avoid 144 any contradictory interpretation and mislead, we stipulate that the sampled mucosa is from scales 145 of the fish and indicated as "skin mucosa". Skin mucosa and ulcer samples were collected by 146 gently scraping of the external surfaces of a fish using a cell scraper. Ectoparasites were 147 retrieved aseptically from the skin surfaces of Prussian carp using a sterile forceps.

148 Male and female fish were identified according to gonadal development (Table 1). All 149 fish were divided into three groups depending on their infestation level. Six individuals of 150 uninfested fish were collected for group A, while group B (fish only with ulcers) and C (fish 151 with ulcers and parasites) included 13 and 16 individuals of infested fish, correspondingly. A 152 total number of 80 samples from 35 individuals of fish and environment were analyzed: six 153 samples of intact skin mucosa, or ISM from uninfested and 29 samples from infested fish. In 154 addition, ulcers from skin surfaces from infested Prussian carp at the site of *L. cyprinacea* 155 attachment (n=10), ulcers without *L. cyprinacea* (n=11), ectoparasites *L. cyprinacea* (n=11) and

156 *A. foliaceus* (n=4) were sampled (Figure 1). Water (n=3), sediment (n=3) and common reed
157 (*Phragmites australis*, n=3) were also collected nearby from the fish capture sites.
158 Microorganisms from the water, sediment, and common reed were collected according to
159 Kashinskaya *et al.* (2018).

160 **Parasitological analysis**

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

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

166 $$P=I*100/N,$$

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

169 $$E=\sqrt{[P\times(100-P)/N]},$$

170 where P is prevalence, and N is total number of host examined.

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

173 $$I=K/n,$$

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

175 $$SE=SD/\sqrt{n},$$

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

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

179 Before DNA extraction, ectoparasites, scrapings from skin mucosa and ulcers were
180 collected into sterile microcentrifuge tubes with lysis buffer (300 µl) for DNA isolation and
181 mechanically homogenized by pestle for 1 min. Following the kit manufacturer protocols, DNA
182 was extracted from 100 mg of samples (excluding parasites) by DNA-sorb B kit (NextBio,
183 Russia). Ectoparasites were rinsed three times in sterile deionized water and used for DNA
184 extraction. The DNA extraction protocol was previously described in Kashinskaya *et al.* (2020).

185 DNA from a sample containing only sterile deionized water was extracted and included in PCR
186 as a negative control. Sequencing of the V3, V4 hypervariable regions of 16S rRNA genes was
187 carried out on an Illumina MiSeq sequencing platform (500 cycles - 2×250 paired-end) by
188 Evrogen (Moscow, Russia) using the primer pair S-D-Bact-0341-b-S-17, 5'-
189 CCTACGGGNGGCWGCAG-3' and S-D-Bact-0785-a-A-21, 5'-
190 GACTACHVGGGTATCTAATCC-3' (Klindworth *et al.* 2013).

191 The amplification conditions and other methods were applied according to the original
192 manufacturer's protocol
193 (https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16smetag
194 enomic- library- prep- guide- 15044223- b.pdf). The PCR reaction contained at least 2.5 µl of
195 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
196 KAPA HiFi Hotstart ReadyMix (KAPA Biosystems, Wilmington, MA, USA) in a total volume
197 of 25 µL. The PCR reaction was performed on a 96- well 0.2 ml PCR plate (Life Technologies)
198 using the following program: 95°C for 3 min, followed by 25 cycles of 95°C for 30 s, 55°C for
199 30 s and 72°C for 30 s and a final extension step at 72°C for 5 min. After producing amplicons,
200 the libraries were cleaned up and mixed in equimolecular portions using SequalPrepTM
201 Normalization Plate Kit (ThermoFisher, Cat # A10510-01) and checked using capillary
202 electrophoresis. Samples were multiplexed using a dual-index approach with the Nextera XT
203 Index kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions.

204 **Molecular identification of ectoparasites**

205 For molecular characterization, *L. cyprinacea* (n=2) and *A. foliaceus* (n=4) were
206 preserved in 96% ethanol and stored at 4℃ until extraction. Total DNA was extracted from
207 single ethanol-preserved individuals of ectoparasites using the DNA-sorb B kit manufacturer's
208 protocols (kit for DNA extraction, Central Research Institute of Epidemiology, Russia). The 28S
209 ribosomal RNA (28S rRNA) was amplified using the following primers (forward 5'-
210 CCCSCGTAARTTAAGCATAT-3', reverse 5'-TCCGGAAGGAACCAGCTACTA-3') and PCR

211 conditions as described in Ruiz *et al.* (2017). Double-stranded DNA was amplified using
212 BioMaster HS-Taq PCR-Color (2x) kit (Biolabmix, Novosibirsk, Russia) according to the
213 manufacturer's instructions (http://biolabmix.ru/products/klassicheskaja_pcr/biomaster_hs-
214 taq_pcr-color__2_/). The PCR products were purified by adsorption on Agencourt Ampure XP
215 (Beckman Coulter, Indianapolis, IN, USA) columns and subjected to Sanger sequencing using
216 the BigDye Terminator V.3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA)
217 with subsequent unincorporated dye removal by the Sephadex G-50 gel filtration (GE
218 Healthcare, Chicago, IL, USA). The Sanger products were analyzed on an ABI 3130XL Genetic
219 Analyzer (Applied Biosystems). The purification and sequencing of PCR products were
220 performed in the SB RAS Genomics Core Facility (Novosibirsk, Russia). Manual edition,
221 alignment of sequences and phylogenetic analysis were performed with MEGA 10 (Kumar *et al.*,
222 2018). Sequences were deposited into GenBank (NCBI) under the following accession numbers:
223 SUB8819858, SUB8819858, SUB8819881, SUB8819881, SUB8819881, SUB8819881.

224 **Identification of opportunistic pathogens**

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

231 **Activity of trypsin**

232 For quantifying the activity of mucosal skin trypsin the samples were homogenized for 30
233 sec using ultrasonic homogenizer (Bandelin Sonopuls HD 2070, Germany) in 2.5 volumes (w/v)
234 of ice-cold distilled water, centrifuged at 10000 ×g for 5 min at 4 °C. Then, the supernatant
235 removed for trypsin quantification and kept at−80 °C until further analysis. Trypsin (E.C.
236 3.4.21.4) activity was assayed at 25 °C using BAPNA (N-α-benzoyl-DL-arginine p-nitroanilide)

237 as substrate in 100 mM Tris-HCl, 150 mM NaCl, 20 mM $CaCl_2$ buffer (pH 8.0). One unit of
238 trypsin per ml (U) was defined as 1 μmol BAPNA hydrolyzed per min per ml of enzyme extract
239 at 407 nm (Holm et al. 1988). Enzymatic activity was expressed as specific activity defined as
240 units per milligram of protein (mU mg protein^{-1}). Soluble protein of crude enzyme extract was
241 quantified by means of the Bradford's method (Bradford 1976) using bovine serum albumin as
242 standard. All measurements were made in three methodological replicates. The absorbance read
243 using a spectrophotometer (Power Wave XS2, BioTek) and data presented in total and specific
244 activity units.

245 **Lysozyme-like activity**

246 Analysis of Lysozyme-like activity were performed using a diffusion test on Nutrient
247 agar (NA) (Himedia, India). Lysozyme-like-induced growth inhibition of *Micrococcus*
248 *lysodecticus* (from collected microorganisms of ISEA SB RAS) was tested on 9-mm Petri dishes.
249 Bacterial cultures (1×10^7 cells ml^{-1}) were grown in 10 ml after cooling the nutrient agar to 45 °C.
250 After solidification (~30 min) wells with 1,5-mm diameter were made. A test sample (3 µl) was
251 placed in each well and were incubated 24 h at 37 °C. Samples were tested in triplicated on
252 plates. Activity from homogenate of skin mucosa was standardized against a serial dilution of
253 chicken egg white lysozyme (EWL) (Sigma-Aldrich) at known concentrations. Antimicrobial
254 activity was determined by measuring clear zone around wells. A value was expressed as
255 equivalent to EWL (mg ml^{-1}) (Mohner and Messner, 1968).

256 **16S sequence processing**

257 Forward and reverse read pairs were merged and quality filtered with Mothur 1.31.2
258 (Schloss et al., 2009). Any reads with ambiguous sites and homopolymers of more than eight bp
259 were removed, as well as sequences shorter that 350 or greater than 500 bp. QIIME 1.9.1
260 (Caporaso et al., 2010) was used for the further processing of the sequences. *De novo*
261 (abundance based) chimera detection using USEARCH 6.1 (Edgar, 2010) was applied to identify
262 possible chimeric sequences ('identify_chimeric_seqs.py' with an option '-m usearch61' in

263 QIIME). After chimera filtering, the QIIME script 'pick_open_reference_otus.py' with default
264 options was used to perform open-reference OTU picking by UCLUST (Edgar, 2010), taxonomy
265 assignment (UCLUST), sequence alignment (PyNAST 1.2.2; Caporaso et al., 2010) and tree-
266 building (FastTree 2.1.3; Price et al., 2010). This algorithm involves several steps of both closed-
267 reference and open-reference OTU picking followed by taxonomy assignment, where the
268 Greengenes core reference alignment (release 'gg_13_8'; DeSantis et al., 2006) was used as a
269 reference. Chloroplast, mitochondria and non-bacterial sequences were removed from further
270 analysis.

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

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

284 **Statistical analyses**

285 All data are presented as a mean ± standard error (SE). STATISTICA 8.0, PAST v. 3.16,
286 and Excel 2019 were used to create bar chart and box plot graphs with mean and standard error.
287 For estimating the differences between the richness and diversity estimates, a non-parametric
288 Kruskal-Wallis test with Dunn's multiple comparisons test was applied. The Kruskal-Wallis test

289 with Dunn's multiple comparisons test was also done to compare the differential abundance of
290 dominant OTUs between different types of samples in control and infested fish using in PAST,
291 3.16 (Hammer *et al.* 2011). In the same program among infested fish, Bonferroni-corrected
292 correlation using Spearman rank tests were explored between alpha and beta diversity of
293 microbial communities and numbers of ulcers. A weighted UniFrac (Lozupone and Knight,
294 2005) dissimilarity matrix was calculated in QIIME and used for downstream analyses. The
295 matrix was used to perform principle coordinates analysis (PCoA) to visualize differences
296 among groups of samples. Permutational multivariate analysis of variance using distance
297 matrices was used as implemented in the 'adonis' function of the vegan R package (Oksanen *et*
298 *al.* 2018). Pairwise comparisons for all pairs of levels of used factors were performed using
299 'adonis.pair' function of the EcolUtils R package (Salazar, 2018). Analysis of multivariate
300 homogeneity of group dispersions (variances) to test if one or more groups is more variable than
301 the others, was performed using the 'betadisper' function of the vegan R-package. In all the
302 aforementioned tests statistical significance was determined by 10 000 permutations. To estimate
303 the differences between the lysozyme- and trypsin-like activities of healthy and unhealthy fish
304 the Kruskal-Wallis test was applied using PAST.

305

306 **Results**

307 **The prevalence and mean intensity of parasite infestation**

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

310 **The alpha diversity estimates of microbial communities**

311 According to Dunn's post hoc test the Chao1 index value and number of observed OTU's
312 in the microbial community of environmental compartments (water, sediment, and common
313 reed) were significantly higher than in the microbiota associated with ulcers in both with *L.*
314 *cyprinacea* and without *L. cyprinacea* ($p \leq 0.05$). Significant differences were also observed in

315 Shannon and Simpson index values between sediment and ulcers with *L. cyprinacea* (Shannon,
316 Z=3.6, p=0.015; Simpson, Z=0.03, p=0.025, correspondingly). The number of OTU's and Chao1
317 value were significantly different between ulcers with and without *L. cyprinacea* and the
318 microbiota associated with *L. cyprinacea* (OTU's, Z=3.6, p=0.015 and Z=3.3, p=0.038; Chao1,
319 Z=3.6, p=0.014 and Z=3.2, p=0.07, correspondingly). No significant differences in richness and
320 diversity estimates were found between microbiota associated with ectoparasites and intact skin
321 mucus of both uninfested (group A) and infested fish (group B and C). Also, there were no
322 significant differences found in intact skin mucus of uninfested fish (group A) in comparison
323 with the infested fish (group B and C). The a-diversity of intact skin mucosa from group B was
324 not significantly different from microbiota of intact skin mucosa from group C (Figure 2).

325 **The beta diversity estimates of microbial communities**

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

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

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

336 The dominant skin microbiota of uninfested fish was represented by *Arcobacter*
337 (12.57±3.49%), unclassified bacteria from the Comamonadaceae family (6.40±1.13%);
338 unclassified bacteria from the Helicobacteraceae family (4.37±1.47%); *Polynucleobacter*
339 (3.44±0.78%), and unclassified bacteria from the order Sphingobacteriales (3.40±1.23%).
340 Additional genera were present at high abundances globally included *Acinetobacter*,

341 *Corynebacterium, Rheinheimera*, *Staphylococcus*, *Vogesella*, unclassified bacteria from the
342 family Aeromonadaceae. The skin microbiota of infested fish was mainly represented by
343 *Corynebacterium* (10.03±1.82%), *Arcobacter* (5.47±0.97%), unclassified bacteria from
344 Comamonadaceae family (4.44±0.48%), *Rheinheimera* (4.08±1.03%), and *Staphylococcus*
345 (3.91±0.88%). The microbiota of ulcers was dominated by *Corynebacterium* (17.31±3.20%),
346 unclassified bacteria from Comamonadaceae (7.67±2.58%) and Aeromonadaceae (6.86±2.85%)
347 families, *Staphylococcus* (6.67±1.40%), and *Acinetobacter* (3.66±1.44%) (Figure 3). As shown
348 in figure 4, the dominant microbiota in skin of uninfested and infested fish did not significantly
349 differ between each other with the exception of *Arcobacter* and unclassified Helicobacteraceae.
350 The relative abundance of these bacteria were significantly higher in skin microbiota of
351 uninfested fish compared to ulcers (Dunn's post hoc, z=3.3, p=0.024 and z=4.1, p=0.0013,
352 correspondingly).

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

354 Microbiota associated with environmental compartments (water, sediment, and common
355 reed) was dominated by *Arcobacter*, *Crenothrix*, *Rhodobacter*, *Synechococcus*, unclassified
356 genera from the family Comamonadaceae and unclassified bacteria from the order Bacteroidales
357 (Figure 3). *Arcobacter* was significantly more abundant in water community (14.80±1.60%) in
358 comparison with microbiota associated with reed (0.13±0.1%) and sediment (0.01±0.01%)
359 (Dunn's post hoc, z=3.5, p=0.014 and z=3.7, p=0.005, correspondingly). Other abundant taxa in
360 the microbiota of water were unclassified bacteria from the family Comamonadaceae
361 (7.93±1.26%) and *Synechococcus* (6.33±2.02%). Microbiota of common reed were dominated
362 by *Crenothrix* (17.04±12.42%) and unclassified bacteria from the family Comamonadaceae
363 (17.04±3.37%). Unclassified Bacteroidales (6.43±0.47%), *Crenothrix* (3.56±0.99%), and
364 *Thiobacillus* (3.24±0.74%) were dominant in microbiota of sediment.

365 **Dominant microbial taxa in parasitic crustaceans and their relation to fish skin and**
366 **microbiota of environmental compartments**

367 The microbiota associated with parasitic crustaceans *L. cyprinacea* were dominated by
368 unclassified Comamonadaceae (33.93±9.18%). Aeromonadaceae (8.44±5.97%), and *Vogesella*
369 (4.27±3.32%), whereas the dominant microbiota of *A. foliaceus* were represented by
370 *Flavobacterium* (17.95±16.19%), *Corynebacterium* (10.61±5.0%), and unclassified bacteria
371 from the Comamonadaceae family (3.81±1.60%). No significant differences in associated
372 microbiota of ectoparasites were found when comparing *L. cyprinacea* and *A. foliaceus* between
373 each other (Dunn's post hoc, p>0.05).

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

387 The significant differences in dominant microbiota of *A. foliaceus* were noted only for
388 *Thiobacillus* and unclassifed Bacteroidales where abundance was significantly lower in
389 microbiota of *A. foliaceus* (0.003±0.003 and 0.07±0.06%) when comparing with sediment
390 (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,
391 correspondingly).

392 The higher proportion of the microbial communities of skin mucosa of Prussian carp and
393 ectoparasites were mainly represented by bacteria which abundance were less than one percent
394 and these minor bacteria ranged from 40 to 80% of the total community. For this reason, we
395 made statistical comparisons with a "core" microbiota.

396 **Analysis of "core" microbiome**

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

407 **Relative abundance of known opportunistic pathogens**

408 The number of reads that belonged to genera of potentially opportunistic pathogens
409 (Figure 6) were significantly higher in microbiota associated with *A. foliaceus*, (47.24±10.90%),
410 ulcers (37.81±4.51%) and intact skin mucosa of infested fish (27.85±2.94%) in comparison to *L.*
411 *cyprinacea* (47.24±10.90%), reed (2.00±0.47%) and sediment (0.80±0.16%) (Dunn's post hoc at
412 $p \leq 0.05$). The ratio of potential pathogens known from existing literature was represented by
413 *Acinetobacter lwoffii*, *A. johnsonii*, *Myroides odoratimimus*, *Flavobacterium succinicans*, *F.*
414 *columnare*, *Janthinobacterium lividum*, *Plesiomonas shigelloides*, *Staphylococcus epidermidis*,
415 *Lactococcus garvieae*. Relative abundance of pathogenic species in associated microbiota of
416 intact skin mucosa of both uninfested and infested Prussian carp, ulcers and ectoparasites were
417 mainly represented by *A. johnsonii, F. columnare*, and *S. epidermidis*. The dominant potential

418 pathogens found in environmental compartments were represented by *F. succinicans, F.*
419 *columnare*, *P. shigelloides*, and *L. garvieae*.

420 **Correlation between opportunistic pathogens and number of ulcerations**

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

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

436 To estimate the antimicrobial potential of skin mucosa of Prussian carp infested by
437 ectoparasites the lysozyme- and trypsin-like activity of skin mucosa was determined. No
438 significant differences were found in the lytic activity of lysozyme and activity of trypsin-like
439 enzymes in skin mucosa of uninfested fish in comparison to infested fish (Kruskal-Wallis test at
440 $p>0.05$) (Supplementary Figure S3 and Figure S4).

441

442 **Discussion**

443

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

456 Despite the shared groups of bacteria found in the skin microbiota of uninfested fish,
457 some variations were observed due to dietary, environmental (temperature, salinity, and
458 sesonality), and others factors (stocking density, stress, etc.). Different approaches towards
459 determination of the skin microbiota may also affect the reported microbial community structure.
460 The accurate determination of the diversity of skin-associated microbiota is complicated by
461 different methods that are applied to collection of material and distinctly different sampling sites.
462 The skin mucosa is most often sampled by using sterile swabs (Wang *et al.* 2010; Boutin *et al.*
463 2013, 2014; Minniti *et al.* 2017; Llewellyn *et al.* 2017; Legrand *et al.* 2018; Tarnecki *et al.*
464 2019), or by gentle scraping of the fish external surfaces using a cell-scraper or spatula
465 (Svanevik *et al.* 2011; Sylvain *et al.* 2017). Other methods to collect fish mucus include rinsing
466 the entire external surface of the fish or section of fish skin with saline buffers (Leonard *et al.*
467 2014; Li *et al.* 2019). Several authors used plastic bags filled with different saline solutions
468 (Ross et al. 2000; Subramanjan *et al.* 2007). The skin sample sites are not always described
469 precisely in different studies and, for this reason, there may be variations in the microbial

470 communities described from different locations of the fish body surface. Moreover, variation in
471 the skin microbiota of fish and data interpretation can also occur when researchers used a method
472 rinsing the whole fish in a buffer solution; in this case it is not clear that the skin microbiota did
473 not also include a flushing of skin mucosa from other sites like the nasal rosette, the buccal
474 cavity and gills (Leonard *et al.* 2014). Hence, lack of standard methods of skin sample collection
475 creates difficulties for comparison and interpretation of skin microbiota results. At the same
476 time, results on skin microbiota obtained by different researchers, even when using the same
477 collection methods will not always be comparable. Moreover, the differences in sample storage,
478 DNA extraction protocol and methods of sequencing can also be a source of variation
479 (Kashinskaya et al., 2017; Tarnecki et al., 2017).

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

481 In the present study we obtained data on the microbial community associated with skin
482 mucosa from fish infested and uninfested by parasitic crustaceans from the genus *Argulus* and
483 *Lernaea*. According to our results, the microbiota of intact skin mucosa of uninfested (group A)
484 and infested (group B and C) Prussian carp was represented by *Arcobacter*, *Corynebacterium*,
485 and unclassified genera from the Comamonadaceae and Helicobacteraceae families. It is known
486 that *Arcobacter* includes microaerophilic and anaerobic opportunistic pathogenic organisms,
487 which inhabit a wide range of ecosystems including freshwater and marine habitats, sewage, and
488 the host mucosa (Legrand *et al.* 2018; Ur Rahmann et al. 2020). The second dominant OTU,
489 *Corynebacterium*, is often found in the environment and usually colonizes the blood, skin and
490 mucous membranes of humans and animals (Baya *et al.* 1992, Tarnecki *et al.* 2018). At the same
491 time, this genus includes one known fish pathogen – *C. aquaticum*. To date this pathogenicity
492 was registered only for striped bass *Morone saxatilis* and rainbow trout *Oncorhynchus mykiss*
493 (Baya *et al.* 1992).

494 Another interesting member of the skin-mucus microbiota of Prussian carp was
495 *Rheinheimera,* which is a potentially important genus as it provides protection against pathogens,
496 and is known for possessing beneficial antibacterial activity (Boutin *et al.* 2013).
497 The skin surfaces of fish at the site of attachment of the parasite from the genus *Lernaea*
498 and *Argulus* there formed a deep ulcer with reddish irregular, bumpy edges. Fish without lice
499 may show nonspecific signs of infestation, including spots or pinpoint hemorrhages, "flashing"
500 behavior, and poor body condition. In some cases, there may be no obvious signs of disease
501 other than presence of the parasite (Wafer *et al.* 2015). In the present study, during ectoparasite
502 infestations, the skin surfaces of Prussian carp were characterized by different levels of petechial
503 hemorrhages (number of ulcers ranged from one to 16). According to the Spearman rank
504 correlation test, the increase of ulceration in skin of Prussian carp were significantly correlated
505 with the reduced abundance of *Acrobacter*, *Corynebacterium*, bacteria C39 from
506 Rhodocyclaceae family, *Rheinheimera,* unclassified bacteria from Comamonadaceae and
507 Helicobacteraceae families, and *Vogesella*. Other findings have also shown that parasitic
508 infestations can modify the skin microbiome of fish. Thus, during infestation in *S. salar* by
509 *Lepeophtheirus salmonis* a significant reduction in microbial richness of infested fish in
510 comparison with control fish was observed. Moreover, a network analysis of microbial taxa on
511 skin mucosa of *S. salar* revealed an association of high louse burdens to multiple pathogenic
512 genera (*Vibrio*, *Flavobacterium*, *Tenacibaculum*, *Pseudomonas*) (Llewellyn *et al.* 2017). Thus,
513 summarizing the above, our results indicate that ectoparasite infestations significantly affects the
514 abundance/diversity of the bacterial community associated with skin of infested Prussian carp, as
515 compared to uninfested fish.

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

518 In previous studies the activity of trypsin has been reported in rainbow trout mucus
519 (Hjelmeland *et al.* 1983) and in mucus-secreting cells of *S. salar* (Braun et al. 1990). Total

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

533 **The microbiota of environmental compartments**

534 The composition of the bacterial community inhabiting the water and subcompartments
535 of the surrounding environment was also investigated in order to evaluate its correlation with the
536 community present on the fish skin. Our findings imply that colonization of skin mucosa of fish
537 by *Arcobacter* and unclassified genera from the Comamonadaceae family is probably enhanced
538 in a natural water environment, but other groups of bacteria associated to this environmental
539 microbiota were specific and significantly different from those from intact skin mucosa of
540 Prussian carp. Based on published data there are contradicting suggestions in terms of the
541 similarity of skin microbiota and their environment. For instance, Larsen et al. (2015) has
542 reported changes in the skin microbiota of *Fundulus grandis* depending on the environmental
543 origin. In its freshwater habitat the microbiota was represented by Gammaproteobacteria and
544 Betaproteobacteria, whereas the marine microbiota is dominated by Cyanobacteria and
545 Alphaproteobacteria (Larsen *et al.* 2015). However, some authors claim that the skin microbiota

546 is a reflection of their environment (Horsley, 1973). Similarity of water and skin mucosa was
547 also observed in farmed *S. salar*, but the authors indicate that interpretation of obtained results
548 need to be viewed with caution due to the low biomass of starting material used for study
549 (Minniti *et al.* 2017).

550 **The microbiota of ectoparasites**

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

560 The evolutionary origins of the microbiota of fish skin remain largely unstudied as an
561 aspect of host-parasite-microbiota associations, therefore whether or not ectoparasites harbor
562 unique microbiota and are able to play a significant role in transmission of opportunistic
563 pathogens, or drive evolutionary processes is poorly understood. It is well known that
564 microorganisms which can be a causative agent of infectious diseases of fish are natural
565 inhabitants of microbial communities and widely distributed in aquatic ecosystems. It is assumed
566 that the entry of pathogenic microbiota can be either from water and / or bottom sediments, or
567 transmitted directly upon contact with parasitic organisms. It is suggested that the direct route of
568 transmission of infections caused by *Argulus* can lead to increased susceptibility of fish to these
569 diseases, for example, when an entry pathway is created for bacteria due to epidermal damage
570 (Kanno *et al.* 1990, Buchmann and Bresciani, 1997), or when the parasite acts as a vector
571 (Cusack and Cone, 1985). In addition, the parasite can cause bacterial infections indirectly by

572 reducing the host's immunocompetence (Bowers *et al.* 2000). A number of studies have shown
573 that the bacterial pathogens *A. salmonicida*, *F. columnare* and the fungus *Saprolegnia spp.*, the
574 causative agents of ringworm in fish, were isolated from fish infested with *A. coregoni* (Bandilla
575 *et al.* 2006; Steckler and Yanong 2012a; Shahraki *et al.* 2014). Among the representatives of the
576 genus *Flavobacterium*, a whole spectrum of microorganisms pathogenic for fish is known: *F.*
577 *psychrophilum*, *F. columnar*, *F. branchiophilum*. Bacterial cold-water disease (BCWD), the
578 causative agent of which is *F. psychrophilum*, causes ulcers on the body and fin rot of
579 salmoninds at low temperatures (Castillo *et al.* 2012). Previous studies have shown that the
580 Spring viraemia of carp (SVC) is also recorded in fish infested with crustaceans from the genus
581 *Argulus* (Steckler and Yanong, 2012a). From our data, the microbiota associated with parasitic
582 *A. foliaceus* were dominated by bacteria from the genera *Flavobacterium* and *Corynebacterium*.
583 The microbiota associated with *L. cyprinacea* was represented by unclassified bacteria from the
584 Comamonadaceae and Aeromonadaceae families.

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

598 occurrences of secondary bacterial infections in fish can be of value and interest in future
599 laboratory studies in terms fish-parasites and pathogenic bacteria relationships.

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

613

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620 animal included in scientific experiments, text number 742 from 13.11.1984) and additionally by
621 the Federal Law of the Russian Federation text number 498 FL (from 19.12.2018) with regard to
622 the humane treatment of animals.

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828 **Author contributions**

829 Conceptualization: Kashinskaya E.N., Solovyev M.M.; Data analysis and interpretation:
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832 Kashinskaya E.N., Andree K.B., Solovyev M.M.

833 **Figure legend**

834 **Figure 1.** Study site and sampling approach. Sample site of intact skin mucosa denoted by 835 blue dashed lines, ulcers denoted by red circles.

836 **Figure 2.** Diversity analysis of microbial community of skin surfaces of Prussian carp, 837 ectoparasites and environmental compartments. The lower case character indicates significance at p 838 ≤ 0.05. ISM – intact skin mucosa.

839 **Figure 3.** Microbial communities at the phylum and the lowest taxonomical level identified 840 from skin mucosa of Prussian carp, their ectoparasites and environmental compartments. Upper 841 case character indicates "I" as intact skin mucosa, or ISM. **Phylum:** (■) Acidobacteria, (■) 842 Actinobacteria, (■) Bacteroidetes, (■) Chlorobi, (■) Chloroflexi, (■) Cyanobacteria, (■) 843 Firmicutes, (■) Nitrospirae, (■) Planctomycetes, (■) Proteobacteria, (■) Spirochaetes, (■) 844 Verrucomicrobia, (■) Others. Bacteria with a relative abundance less than 1% were pooled and 845 indicated as "Others". **The lowest taxonomical level:** (■) g_*Acinetobacter*; (■) g_*Anaerococcus*; 846 (■) g_*Arcobacter*; (■) g_Candidatus Xiphinematobacter; (■) g_*Corynebacterium*; (■) 847 g_Crenothrix; (■) g_C39 from the Rhodocyclaceae family; (■) g_*Flavobacterium*; (■) 848 g_*Polynucleobacter*; (■) g_*Rheinheimera*; (■) g_*Rhodobacter*; (■) g_*Staphylococcus*; (■) 849 g_*Synechococcus*; (■) g_*Thiobacillus*; (■) g_*Vogesella*; (■) f_Unclassified Aeromonadaceae; (■) 850 Unclassified Chromatiaceae; (■) f_Unclassified Comamonadaceae; (■) f_Unclassified 851 Helicobacteraceae; (■) f_Unclassified Myxococcales; (■) f_Unclassified Sinobacteraceae; (■) 852 o_Unclassifed Bacteroidales; (■) o_Unclassified Sphingobacteriales; (■) Others. Bacteria with a 853 relative abundance less than 3% were pooled and indicated as "Others".

854 **Figure 4.** The dominant microbiota in skin mucosa of Prussian carp, their ectoparasites and 855 environmental compartments. The lower case character indicates significance at p ≤ 0.05. ISM – 856 intact skin mucosa.

857 **Figure 5.** Principal coordinates analysis (PCoA) for core skin microbiota of Prussian carp and
858 their ectoparasites. *A. foliaceus* – empty black circles; *L. cyprinacea* – empty red triangles; ISM
859 (Uninfested) – green plus; ISM (Infested) – blue cross; Ulcers – light blue diamond.

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

866 **Figure S1.** The rarefaction curves.

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

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

872 **Figure S4.** Activity of trypsin in skin mucosa of Prussian carp infected by ectoparasites.

873 Table 1 Sample information

Number of fish	Sex	Body weight, g	Total Length, sm	Standard Length, sm	*L. cyprinacea*	*A. foliaceus*	Number of ulcers	Samples for microbiome data
Uninfested fish (Group A)								
1	F	394.8	27.9	23.1	0	0	0	I
2	F	312.0	25.3	20.2	0	0	0	I
3	M	300.0	24.9	20.8	0	0	0	I
4	F	378.6	27.3	22.6	0	0	0	I
5	F	309.0	26.2	21.1	0	0	0	I
6	F	414.2	28.7	23.0	0	0	0	I
Mean±SE		**351.4±20.5**	**26.7±0.61**	**21.8±0.51**	**-**	**-**	**-**	**-**
Infested fish (without parasites, only ulcers – Group B)								
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

19	F	528.4	29.4	24.1	0	0	4	I, U
Mean±SE		**388.7±13.0**	**27.7±0.20**	**22.7±0.16**	**-**	**-**	**-**	**-**
Fish with ulcers and parasites (Group C)								
20	F	345.2	27.4	22.1	4	0	8	I, UL, L
21	F	367.8	27.3	22.5	3	0	4	I, UL, L
22	F	380.8	28.8	23.4	3	0	13	I, UL, L
23	M	342.0	26.5	21.9	1	0	2	I, UL, L
24	F	380.0	28.2	23.0	1	0	3	I, UL, L
25	F	417.0	28.7	23.9	1	0	2	I, UL, L
26	F	366.0	28.1	23.0	1	0	7	I, UL, L
27	-	334.8	27.3	22.4	5	0	9	I, UL, L
28	F	360.0	26.9	22.0	1	1	2	I, UL, L
29	F	320.8	26.5	21.6	3	1	16	I, U, UL, L, A
30	M	448.0	28.9	24.1	1	0	2	I, U, L
31	F	368.0	27.7	22.7	0	5	2	I, U, A
32	F	386.4	28.4	23.4	0	1	2	I, U
33	F	334.8	26.1	21.8	0	3	3	I, A
34	-	353.4	27.1	22.4	0	1	1	I, A
35	F	381.8	26.7	21.9	0	1	1	I
Mean±SE		**367.3±8.05**	**27.5±0.22**	**22.7±0.19**	**-**	**-**	**-**	**-**

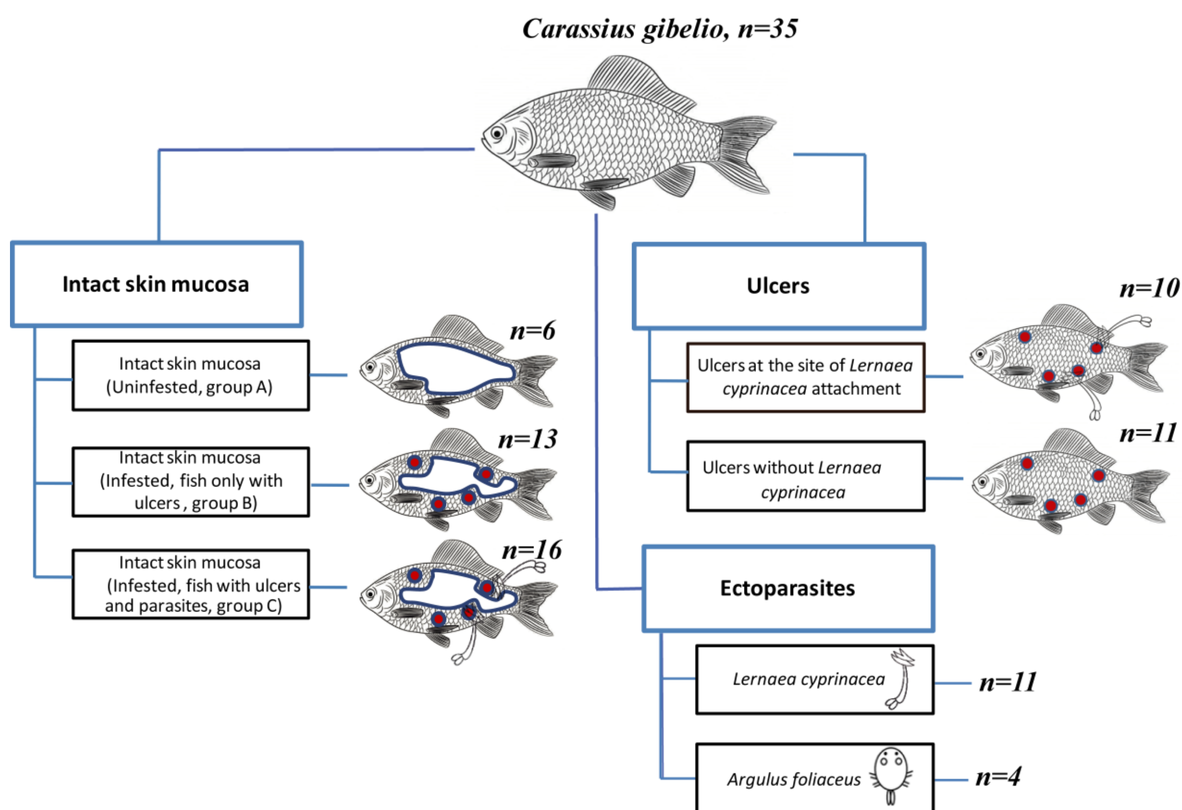
874 Upper case character indicates "I" as intact skin mucosa, or ISM; "U" – ulcers without *L.*
875 *cyprinacea*; UL" – ulcers with *L. cyprinacea*; "A" – *A. foliaceus*; "L" – *L. cyprinacea*

876

877 Table 2 Comparison (ADONIS test) of "Core" microbiota associated with skin surfaces of Prussian
878 carp and their ectoparasites. Extra bold indicates significance at p≤0.05.

Combination	R^2	p-value FDR corrected
A. foliaceus vs. *L. cyprinacea*	0.163	0.091
A. foliaceus vs. Intact skin mucosa (Uninfested)	0.231	0.058
A. foliaceus vs. Intact skin mucosa (Infested)	0.005	0.987
A. foliaceus vs. Ulcers	0.029	0.678
L. cyprinacea vs. Intact skin mucosa (Uninfested)	0.326	**0.003**
L. cyprinacea vs. Intact skin mucosa (Infested)	0.171	**0.001**
L. cyprinacea vs. Ulcers	0.163	**0.004**
Intact skin mucosa (Uninfested) vs. Intact skin mucosa (Infested)	0.089	**0.035**
Intact skin mucosa (Uninfested) vs. Ulcers	0.229	**0.001**
Intact skin mucosa (Infested) vs. Ulcers	0.069	**0.035**

879



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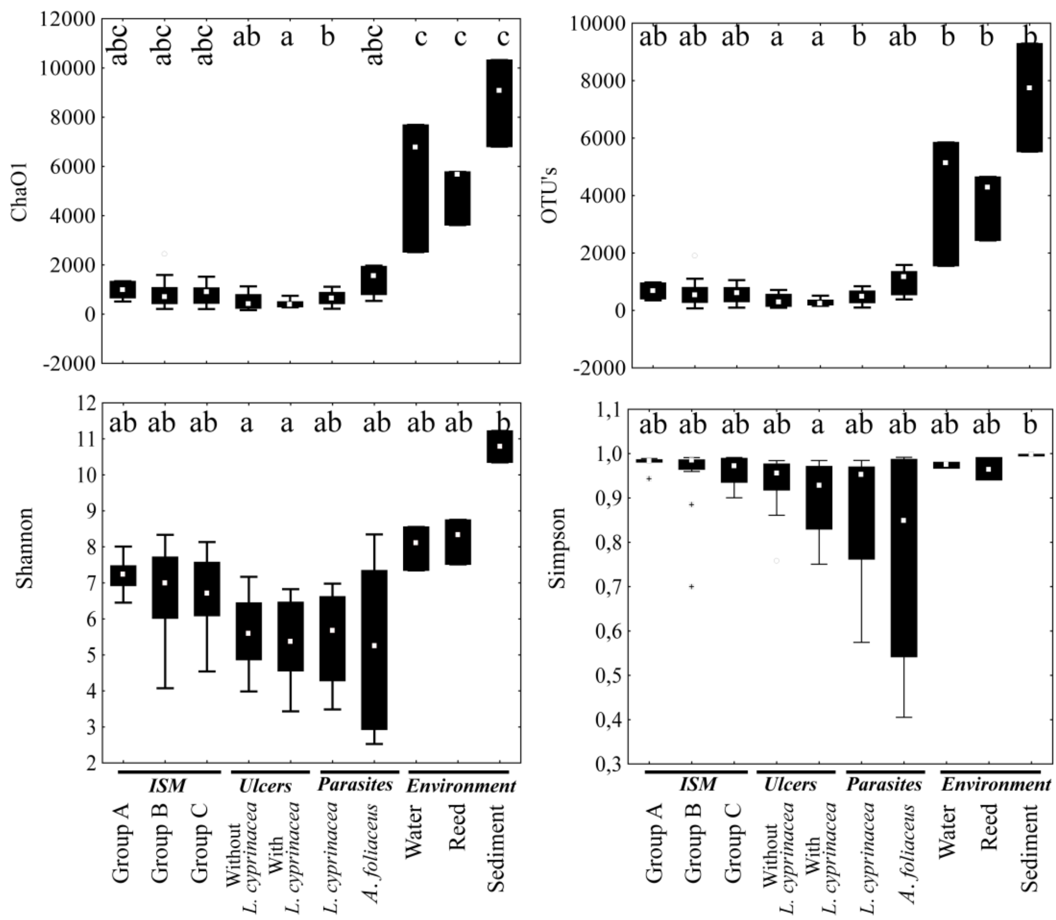
881 Fig. 1



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883 Fig. 2

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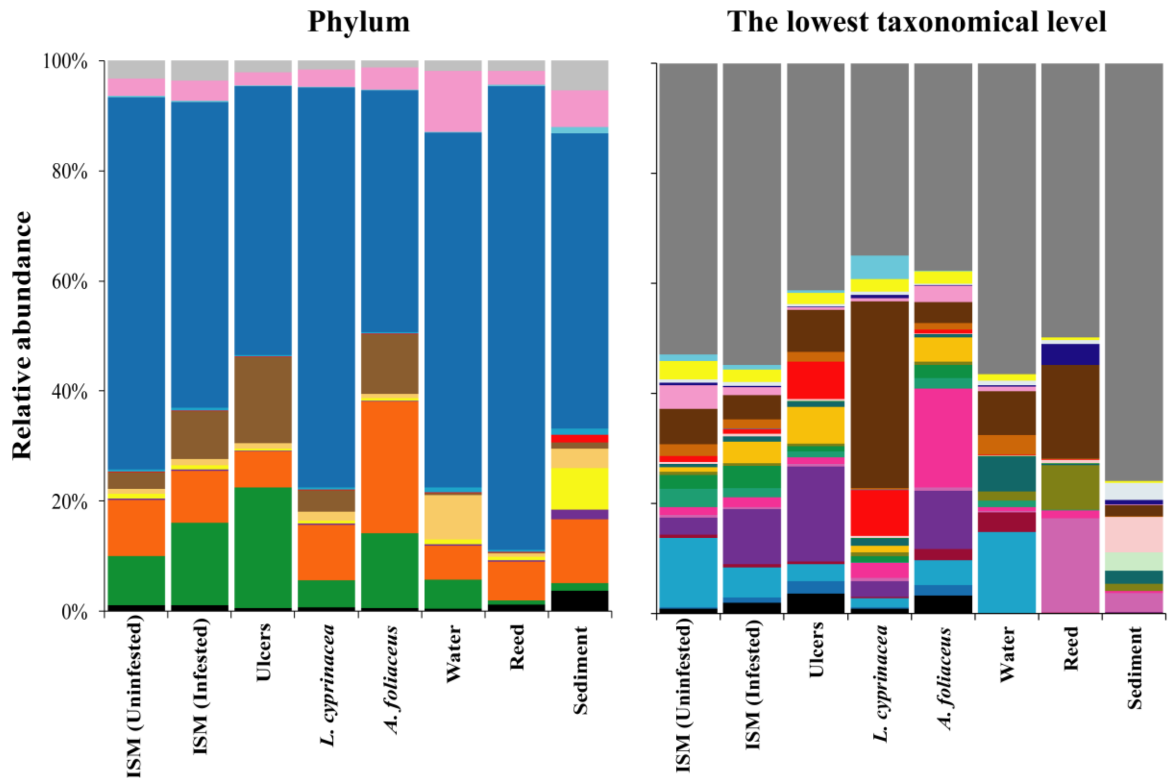

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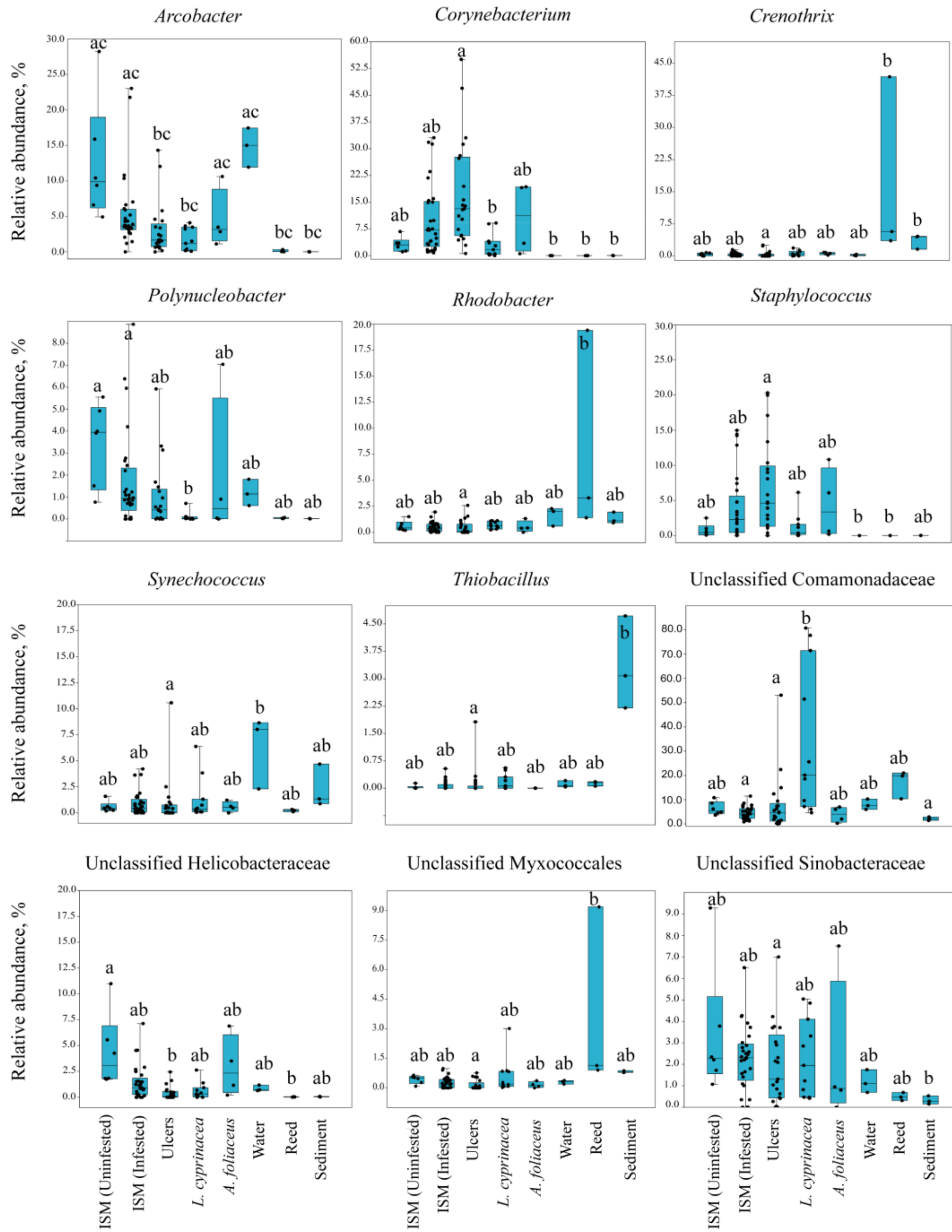
886 Fig. 3



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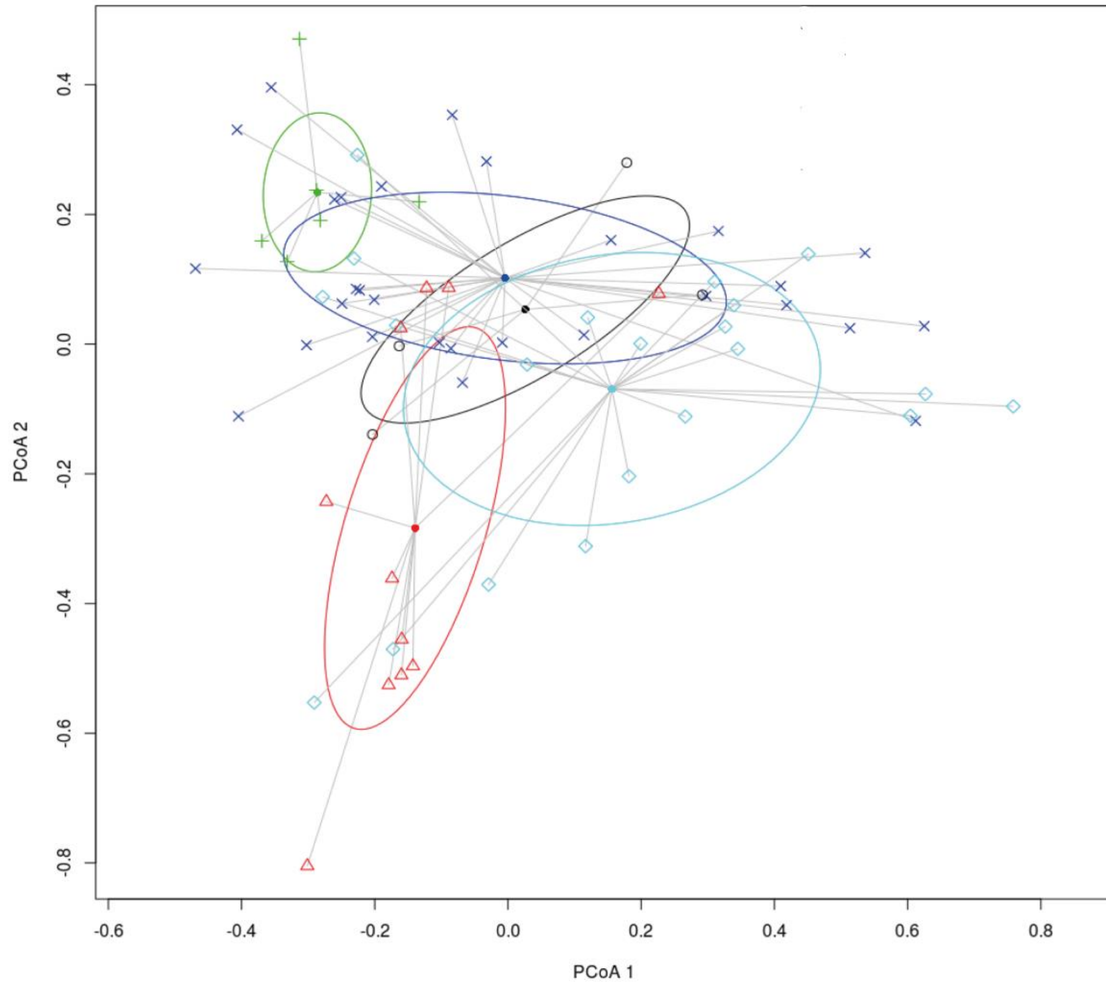

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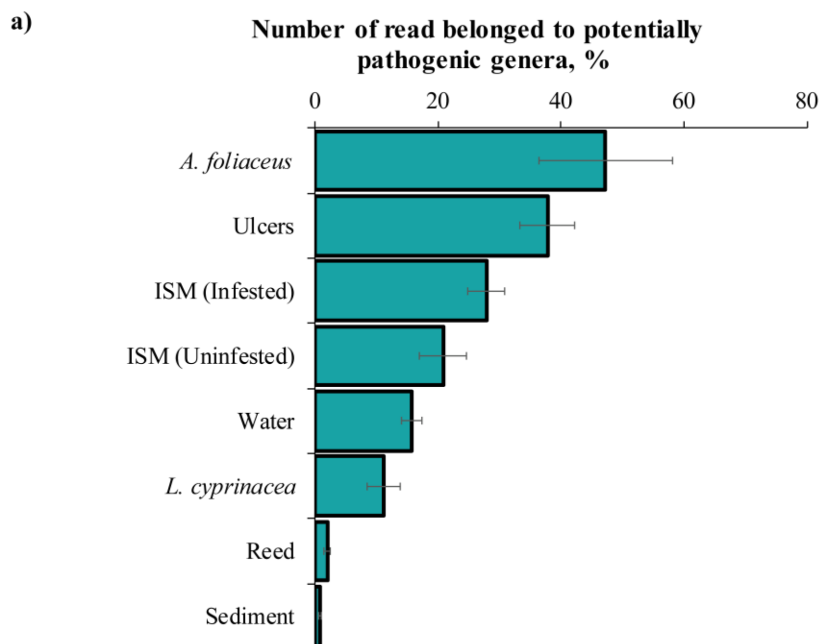
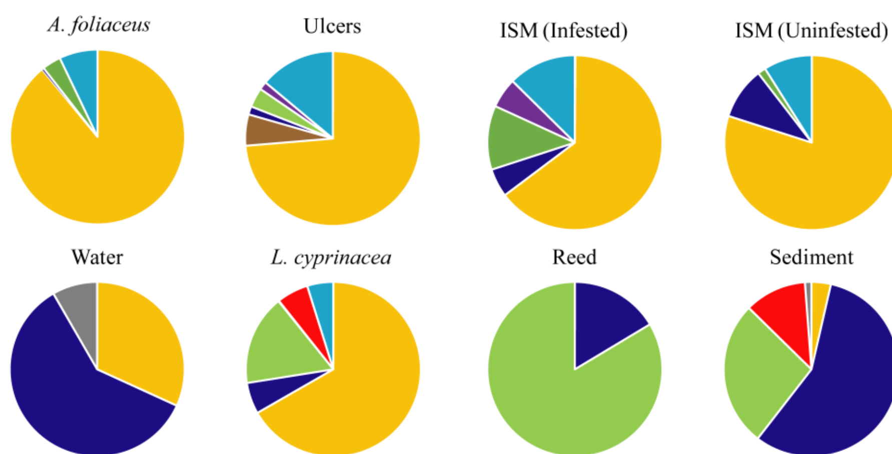
890 Fig. 5





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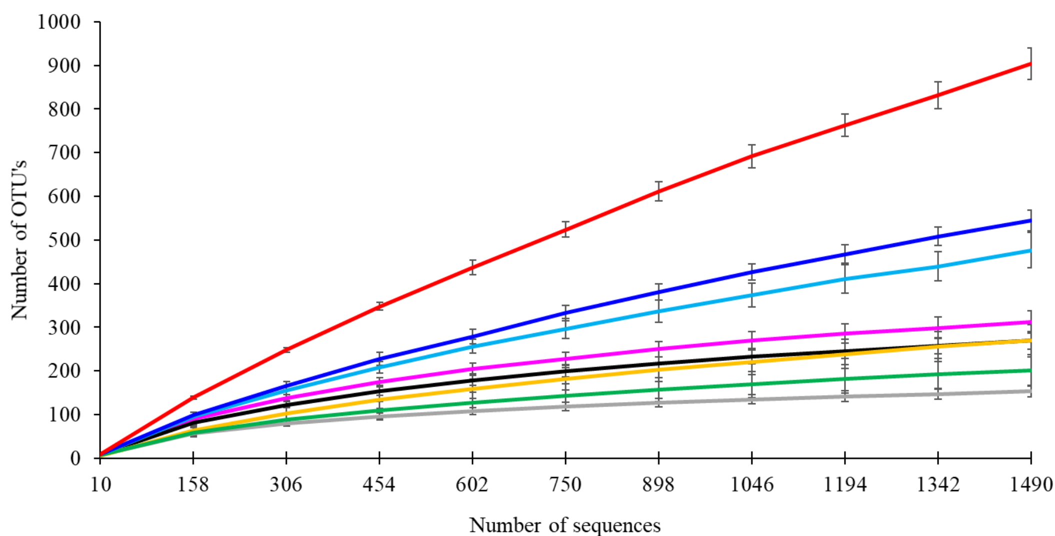
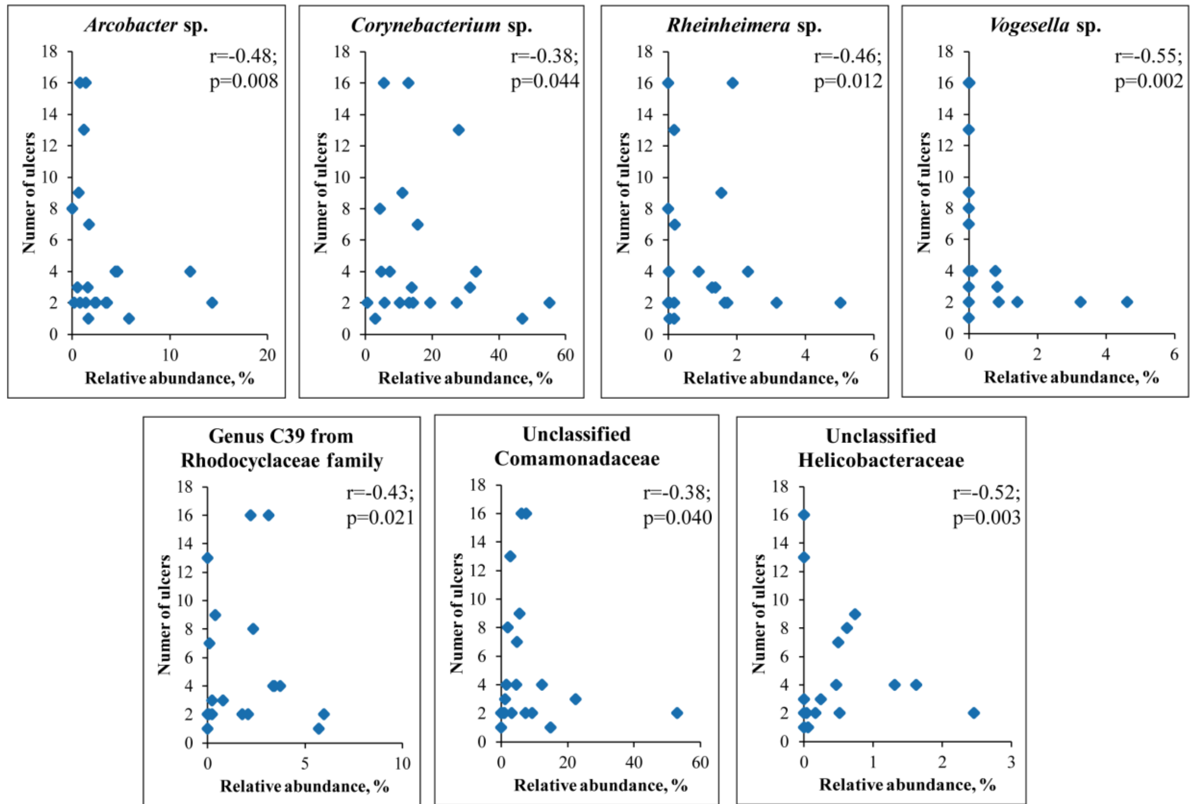
893 Fig. 6



894

895 Fig.S1

896



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898 Fig.S2

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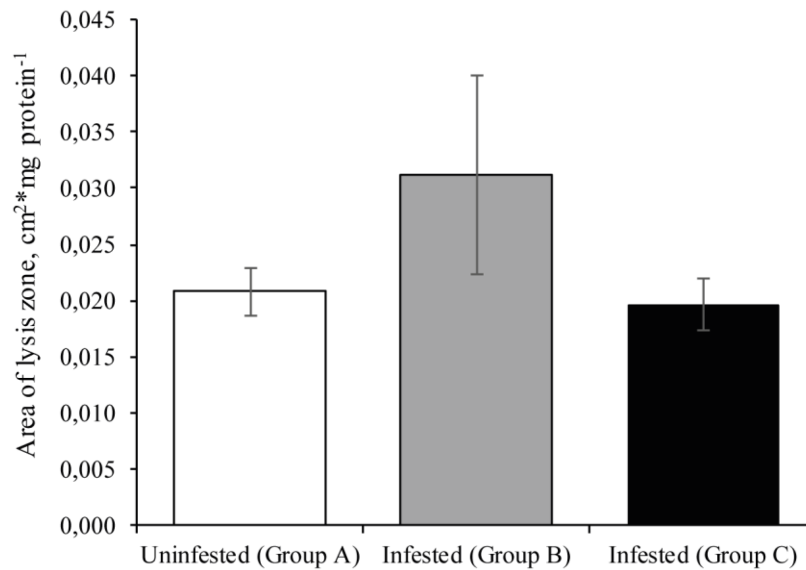

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901 Fig.S3

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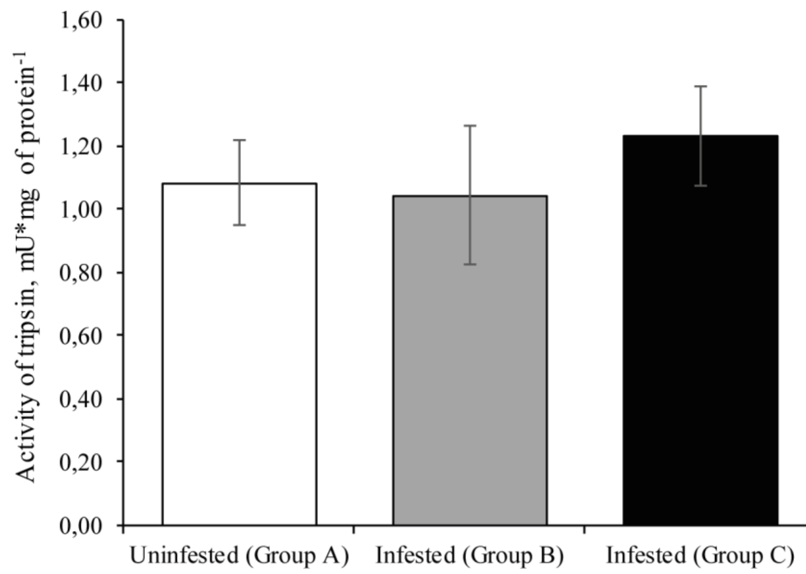

903

904 Fig.S4

905 Table S1. Shared OTU's between groups after "Core" microbiome calculation.

906