Loss of gut microbial diversity in the cultured, agastric fish, Mexican pike silverside (*Chirostoma estor*: Atherinopsidae)

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Abstract

Teleost fish are the most diverse group of extant vertebrates and have varied digestive anatomical structures and strategies, suggesting they also possess an array of different host-microbiota interactions. Differences in fish microbiota have been shown to affect host development, the process of gut colonization, and the outcomes of gene-environment or immune system-microbiota interactions. There is generally a lack of studies on the digestive mechanisms and microbiota of agastric short-intestine fish however, meaning that we do not understand how changes in gut microbial diversity might influence the health of these types of fish. To help fill these gaps in knowledge, we decided to study the Mexican pike silverside (*Chirostoma estor*) which has a simplified alimentary canal (agastric, short-intestine, 0.7 gut relative length) to observe the diversity and metabolic potential of its intestinal microbiota. We characterized gut microbial populations using high-throughput sequencing of the V3 region in bacterial 16S rRNA

39 genes while searching for population shifts resulting associated with fish development in
40 different environments and cultivation methods. Microbiota samples were taken from the
41 digesta, anterior and posterior intestine (the 3 different intestinal components) of fish that grew
42 wild in a lake, that were cultivated in indoor tanks, or that were raised in outdoor ponds. Gut
43 microbial diversity was significantly higher in wild fish than in cultivated fish, suggesting a loss
44 of diversity when fish are raised in controlled environments. The most abundant phyla observed
45 in these experiments were Firmicutes and Proteobacteria, particularly of the genera *Mycoplasma*,
46 *Staphylococcus*, *Spiroplasma*, and *Aeromonas*. Of the 14,161 OTUs observed in this experiment,
47 133 were found in all groups, and 17 of these, belonging to *Acinetobacter*, *Aeromonas*,
48 *Pseudomonas,* and *Spiroplasma* genera*,* were found in all samples suggesting the existence of a
49 core *C. estor* microbiome. Functional metagenomic prediction of bacterial ecological functions
50 using PICRUSt2 suggested that different intestinal components select for functionally distinct
51 microbial populations with variation in pathways related to the metabolism of amino acids,
52 vitamins, cofactors, and energy. Our results provide, for the first time, information on the
53 bacterial populations present in an agastric, short-gut teleost with commercial potential and show
54 that controlled cultivation of this fish reduces the diversity of its intestinal microbiota.

55 Keywords:
56 environmental metagenomics, microbe-higher organism interactions, microbial ecology,
57 microbial communities, microbe-fish symbiosis, fish microbiome
58

59
60

Introduction

62 Gut microbiota, defined as the collective community of microorganisms that inhabit the intestine,
63 are known to contribute to host physiology like digestion, nutrient absorption, and immune
64 system function, significantly impacting animal health, welfare, growth, and behavior (reviewed
65 by Douglas, 2019). In fish, the density, composition, and function of intestinal microbial
66 communities are influenced by internal and external factors, including genotype, life stage,
67 trophic level, diet, season, habitat (chemical and physical factors), sex, and phylogeny (reviewed
68 by Butt & Volkoff, 2019). It is estimated that the fish gut contains between 10^7 and 10^{11} bacterial
69 cells per gram of intestinal content, and these microbes can be either residents or transients
70 (*Nayak, 2010; Navarrete et al., 2012*). A total of about 145 different teleost fish species have
71 been involved in microbiome studies, however, these only include the zebrafish model and
72 others important for commercial aquaculture (*Perry et al., 2020*).

73

74 Teleosts are the most diverse group of vertebrates, occupying many different habitats, employing
75 many varied feeding strategies, and possessing distinctive digestive specializations/anatomical
76 configurations, which has resulted in their having a wide array of host-microbiota interactions
77 (*Lescak & Milligan-Myhre, 2017*). For example, some agastric fish like cyprinids compensate for
78 their lack of stomach-based acid digestion by increasing the length of their intestines and
79 presumably depend to a greater extent on microbial activity to help break down their food
80 (*Manjakasy et al., 2009; Egerton et al., 2018*). Little is known about the microbiota of agastric
81 short-intestine teleosts lacking a true stomach, except for the zebrafish model species *Danio*
82 *rerio*, which is an agastric short-intestine species of no commercial value. The study of microbial
83 community dynamics in both wild and controlled environments studies is relatively simple with
84 teleosts because in captivity, most fish species develop rapidly and in high quantities (*Lescak &*
85 *Milligan-Myhre, 2017*). Such studies have previously demonstrated that gut microbiome
86 composition affects host development, the process of microbiome colonization and succession,
87 and gene-environment or immune system-microbiota interactions (reviewed by *Nayak*, 2010 and
88 *Butt & Volkoff*, 2019). As omics techniques (metagenomics, metatranscriptomics,
89 metaproteomics, and metabolomics) continue to be developed and improved, our ability to study
90 the ecology and function of fish microbiota is likewise evolving.

91

92 To help fill in the knowledge gap about the importance and function of agastric fish gut
93 microbiota, we propose studying the Mexican pike silverside, *Chirostoma estor* due to its
94 simplified digestive tract lacking a stomach and consisting instead of a short-intestine that is only
95 0.7 the fish's length (relative gut length - rgl) (*Ross et al., 2006*). The Mexican pike silverside
96 has been described by the FAO of the United Nations, as a species with high aquaculture
97 potential due to its regional importance, low trophic level, and high nutraceutical value (high
98 docosahexaenoic acid (DHA) content) (*Fonseca-Madrigal et al., 2014*; *Martínez-Palacios et al.,*

99   *2019, Martínez-Palacios et al., 2020*). This teleost fish consumes mostly zooplankton and
100   insects, which are finely ground by chewing before swallowing (*Martínez-Palacios et al., 2019,*
101   *Ross et al., 2006*). Due to its simplified intestinal configuration, we hypothesize that the Mexican
102   pike silverside is likely to depend more heavily on its gut microbiota for aid in digestion and
103   nutrient absorption, compared to teleosts with stomachs or more sophisticated digestive systems.
104   If we are to successfully develop methods of aquaculture for agastric fish such as the Mexican
105   pike silverside, it will be important to better understand how their intestinal microbiomes are
106   established, how these microbes influence their health and nutrition, and what conditions are
107   necessary to optimize the interactions between bacteria and host.
108   In this study, we analyze and compare the gut microbiomes of *C. estor* harvested from a wild
109   environment (Lake Patzcuaro), cultivated in indoor tanks, and raised in outdoor ponds. DNA was
110   extracted from each fish's digesta, anterior intestine, and posterior intestine, followed by
111   amplification of the bacterial 16S and sequencing on the Illumina Miniseq platform. To attempt
112   and predict the metabolic activities associated with the different gut bacterial populations, we
113   used taxonomic information to estimate metabolic functions.

114

115   Materials & Methods

116

117   Sample Collection

118   Adult *Chirostoma estor* were collected from Lake Patzcuaro (LP), México, in the spring of 2018
119   (March) (19° 36' 14'' N, 101° 37' 56'' W, 22 °C, altitude 2,040 masl, N = 13). In May of the
120   same year, cultured adult fish of similar size to those from the lake were also harvested from
121   fiberglass indoor tanks (tank culture = C) and outdoor earth ponds (extensive culture = E) (N=20
122   and N=19, respectively) located in the aquaculture biotechnology laboratory at Universidad
123   Michoacana de San Nicolás de Hidalgo in Morelia, Michoacan, Mexico (19° 41' 22'' N, 101°
124   14' 56'' W, 23 °C, altitude 1,896 masl). Populations of intensively cultured fish were established
125   twenty years ago using stock from Lake Patzcuaro. Fish from the laboratory were released into
126   the outdoor earth ponds 8 months before sampling, and are designated as E.
127   At harvest, individual fish from each environment were measured and weighed (Table S1).
128   Immediately after collection, the abdominal cavities of fish were dissected under aseptic
129   conditions, the intestines (guts) extracted and their contents (digesta = D) were gently squeezed
130   out and placed into sterile tubes with 96% ethanol. The guts of *C. estor* consist of a short
131   intestine with a single loop in the midgut (Fig. S1), and were divided into two sections (anterior
132   = A, and posterior = P gut) and placed in sterile tubes containing 96% ethanol and stored at 4 °C
133   until later DNA extraction. All specimens were treated following EU Directives 2010/63/EU for
134   animal experimentation and 2007/526/EC accommodation and care of animals used for
135   experimental and other scientific purposes.

136

137   DNA extraction and sequencing

138 Metagenomic DNA of fish intestines (D, A, and P) from the 3 different populations was
139 extracted with a modified CTAB extraction protocol (*Doyle & Doyle, 1987*) by adding lysozyme
140 (100 mg/mL), proteinase k (20 mg/mL), lithium chloride (5M), and sodium acetate (3 M, pH 5.2)
141 to the extraction buffer. After DNA extraction, the V3 variable region of the 16S rRNA gene was
142 PCR-amplified with the primer pair V3-338f and V3-533r (*Huse et al., 2008*). PCR products
143 were paired-sequenced (300 cycles, 2X150) on an Illumina Miniseq, following Illumina standard
144 protocol at Centro de Investigaciones en Alimentación y Desarrollo, A.C. (CIAD), Mazatlan,
145 Sinaloa, Mexico.
146
147 **Bioinformatics**
148 All amplicon sequence data derived from this work was submitted to NCBI Sequence Read
149 Archive (SRA) (SRX11596261 to SRX11596334) under BioProject accession ID:
150 PRJNA750495. After demultiplexing, CIAD sent back data as FastQ files which were
151 preprocessed with PrinSeq (*Schmieder & Edwards, 2011*). Reads were then assembled and
152 quality filtered with Flash v.1.2.7 software (*Magoc & Salzberg, 2011*), and VSEARCH (*Rognes*
153 *et al., 2016*) was used for further processing, obtaining an abundance matrix of bacterial OTUs
154 (operational taxonomic units clusterized at 97% identity) that was normalized using the
155 metagenomeSeq method (*McMurdie & Holmes, 2014*). Taxonomic annotation was performed
156 using VSEARCH (*Rognes et al., 2016*) against the SILVA138 database (*Quast et al., 2013*). The
157 abundance matrix at the genus taxonomic level was used to calculate Good's coverage and alpha
158 diversity indexes (i.e., Chao 1 and Shannon indexes), which were estimated using the R Phyloseq
159 library (*McMurdie and Holmes*, 2013). The genus-level abundance matrix was normalized using
160 the metagenomeSeq method (*McMurdie and Holmes*, 2014), and the beta diversity distance
161 matrix was calculated using Bray-Curtis dissimilarity. Beta diversity distance matrices were
162 visualized with Non-metric Multidimensional Scaling plots (NMDS) of bacterial data grouped
163 by environments and intestinal components, while ordination was based on between-sample
164 dissimilarities calculated by Bray-Curtis distance and 999 permutations using library vegan 2.5-6
165 (*Oksanen et al., 2019*) in R software (*R Core Team, 2013)*.
166 Although many groups define a core microbiome as those microbes with 100% occupancy across
167 all samples (*Turnbaugh et al., 2007*), we considered (like other researchers) that a more relaxed
168 threshold of greater than 80% occupancy would allow us to include more physiologically
169 important bacteria in our analysis (*Baldo et al., 2015; do ValePereira et al 2017; Sweet &*
170 *Bulling, 2017; Rimoldi et al., 2019*). Core microbiota was identified at the level of the genus
171 when OTUs were present in at least 80% of samples per defined group (LP, C, E, D, A, and P)
172 and at the level of OTU when present in at least one sample of each group (DLP, ALP, PLP, DC,
173 AC, PC, DE, AE, PE). To compare microbiota between environments or digestive components,
174 data was visualized with Venn diagrams (*Chen & Boutros, 2011*), in UpSet plots made with the
175 package UpSetR (*Conway, Lex & Gehlenborget, 2017*).
176
177 **Statistical analysis**

178   All statistical analyses were performed using R software (*R Core Team, 2013*) and R library
179   vegan 2.5-6 (*Oksanen et al., 2019*). Alpha diversity and relative abundance data were tested for
180   normality and homoscedasticity by Shapiro–Wilk's and Bartlett's tests, respectively. Data sets
181   grouped by intestinal component (Digesta, Anterior, and Posterior gut) or environment (Lake
182   Patzcuaro, Intensive Culture, and Extensive Culture) were found to not be normally distributed.
183   To compare beta diversity, environment, and intestinal component groups were statistically
184   analyzed using non-metric dimensional scaling (NMDS) based on Bray-Curtis distances (*Bray &*
185   *Curtis, 1957*), Analysis of Similarities (ANOSIM), as well as Adonis tests (Permutational
186   Multivariate Analysis of Variance, PERMANOVA) using the Bray-Curtis index at 999
187   permutations. The number of reads across samples was normalized by sample size, and each
188   taxon's relative abundance (%) was calculated. Although results were generated for each
189   taxonomic level, only Phylum and Genus levels are shown. Only taxa with the highest relative
190   abundance were considered for statistical analysis, totaling 10 for phylum and 20 for genus.
191   Phylum and genus abundance data were not normally distributed. Non-normal data were
192   analyzed using nonparametric Kruskal–Wallis followed by Mann-Witney-Wilcoxon's post hoc
193   tests with statistical significance set at $p < 0.05$. Additional analysis to detect differential
194   abundance was performed using the Linear discriminant analysis Effect Size (LEfSe) method
195   (*Segata et al., 2011*) integrated within the Galaxy framework
196   (https://huttenhower.sph.harvard.edu/galaxy/). In particular, the non-parametric Kruskal-Wallis
197   sum-rank test was used to detect differentially abundant taxa, while Linear Discriminant
198   Analysis (LDA) was used to estimate the effect size.
199

Predictive functional analysis by PICRUSt2
201   The functional metabolic profiles of 16S rDNA data were predicted using the Phylogenetic
202   Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) v2.3.0 beta
203   software that uses HMMER (www.hmmer.org) to place OTUs into a reference phylogeny,
204   followed by the castor R package to predict gene family abundances using hidden-state
205   prediction. Pathway abundances are inferred based on the predicted sample functional profiles
206   linked to pathway reactions using a modified version of MinPath (*Douglas et al., 2019*). Pathway
207   abundance was then inferred using KEGG (Kyoto Encyclopedia of Genes and Genomes)
208   (pathway_pipeline.py).
209   The output file (path_abun_unstrat_descrip.tsv) of predicted pathway abundance was loaded to R
210   software (*R Core Team, 2013*). The 60 most abundant functional pathways (taking into account
211   the total average of the samples) were visualized using a heatmap created by the
212   ComplexHeatmap package in R (*Gu, Eils & Schlesner, 2016*). To determine specific differences
213   between each of the predicted paths, the output file of PICRUSt2
214   (path_abun_unstrat_descrip.tsv) was loaded into the MEGAN5 package (*Huson et al., 2016*)
215   which allowed us to define the metabolic categories predicted in these bacteria. These metabolic
216   predictions were then analyzed using STAMP (statistical analysis of taxonomic and functional
217   profiles) (*Parks et al., 2014*) software to further interrogate all predicted functional datasets and

218   produce graphical depictions of key functional pathways. To contrast group metabolic
219   predictions by environment (LP-C-E) and intestinal component (D-A-P) samples were compared
220   using an ANOVA and Tukey-Kramer test. Pathways with a p-value < 0.05 were considered to be
221   statistically significant. For paired analysis of all 9 metabolic predictions (DLP, ALP, PLP, DC,
222   AC, PC, DE, AE, PE) simultaneously, environmental and intestinal components were compared
223   using a two-sided Welch's t-test. Pathways with a p-value < 0.05 were considered to be
224   statistically significant.
225
226   ## Results
227
228   **Bacterial diversity in the gut of *C. estor***
229   The bacterial community profile found in *C. estor* gut is highly variable from individual to
230   individual, especially at the level of genus (Fig. 1A). At the level of phylum (Fig. 1B; Table S2)
231   most samples contained Firmicutes (56.27% ± 4.28) and Proteobacteria (28.68% ± 3.47), of
232   which the two most abundant classes were Gammaproteobacteria (24.35% ± 3.16) and
233   Alphaproteobacteria (4.33% ± 1.25). Other common phyla were Cyanobacteria (4.69% ± 1.57),
234   Actinobacteriota (3.51% ± 1.35), Bacteroidota (1.78% ± 0.37), Desulfobacterota (1.43% ± 0.44),
235   Fusobacteriota (1.39% ± 1.05), Planctomycetota (0.97% ± 0.40), Verrucomicrobia (0.38% ±
236   0.10) and Acidobacteriota (0.29% ± 0.16).
237   Of the 1,207 annotated genera (Fig. 1A; Table 1), the most abundant bacterial genera observed
238   within individual fish included *Mycoplasma* (19.95% ± 4.11), *Staphylococcus* (10.90% ± 3.30),
239   *Spiroplasma* (10.23% ± 2.80), *Aeromonas* (7.51% ± 1.87), *Pseudomonas* (6.11% ± 1.86),
240   *Planomicrobium* (4.75% ± 1.90), *Clostridium* (4.67% ± 1.32), *Foliisarcina* (2.37% ± 1.36) and
241   *Cutibacterium* (2.16% ± 1.25). At the genus level, all digesta and Lake Patzcuaro samples
242   contained significantly less *Mycoplasma* spp. than other intestinal components or environments
243   (Table 1). Other differences were observed in the abundance of *Clostridium*, *Romboutsia*,
244   *Prochlorococcus*, *Acinetobacter*, *Cetobacterium*, *Anaerobacter*, *Flavobacterium*,
245   *Methylobacterium-Methylorubrum,* and *Dongia* within lake samples, while *Staphylococcus* was
246   most abundant in the intensive culture and *Planomicrobium*, *Erwinia,* and *Dechloromonas* were
247   most abundant in the extensive culture (Table 1).
248   Alpha-diversity varied considerably but not significantly by both environment and intestinal
249   compartment, except for the Shannon index of fish digesta from Lake Patzcuaro (LP) which was
250   significantly higher than the Shannon index of digesta from cultivated fish ($p = 0.021$) (Fig. 1C;
251   Table S3). Bacterial diversity and Shannon index values from Lake Patzcuaro samples were
252   significantly higher ($p = 0.00477$) than those of fish from tank cultures or outdoor ponds (Table
253   S3).
254
255   **Environmental influence on microbial communities**
256   NMDS of the gut microbiota in fish from different environments (Fig. 2A) revealed a nearly
257   separate cluster of the LP samples concerning the other two environments (C and E), the latter

258   which largely overlapped instead. These observed differences were statistically supported by
259   ANOSIM: $R = 0.3119$, $p = 0.001$ (Fig. 2A; Table S4), and PERMANOVA (Table S4). Repeating
260   this analysis with data from the intestinal components showed complete overlapping of the three
261   different groups (Fig. 2B), which surprisingly appeared to be significantly different from each
262   other judging by ANOSIM (R-value of 0.06763, $p =0.004$ (Fig. 2B; Table S5)). PERMANOVA
263   supported this result, showing a significant difference between digesta and anterior gut with an F
264   value of 2.2341, $p =0.001$ (Table S5). ANOSIM and PERMANOVA analyses were stratified by
265   intestinal component or environment, showing differences in digesta taken from fish harvested
266   from the three environments (DLP, DC, and DE), differences in anterior guts from fish harvested
267   in ALP vs AC and AE, while in the posterior gut, there was a significant difference only between
268   PLP vs PE (Table S5).
269
270   **Core microbiota analysis**
271   Analysis to determine the core microbiota suggested that the genera *Mycoplasma*, *Spiroplasma*,
272   *Aeromonas*, *Pseudomonas,* and *Acinetobacter* were present in all intestinal components of fish
273   harvested from in all three environments (Fig. 3A). In addition to the aforementioned core
274   genera, digesta samples from all three environments also contained *Vibrio* and *Bacillus* (Fig.
275   3B); all anterior intestine samples contained *Mycoplasma*, *Staphylococcus*, *Spiroplasma*,
276   *Aeromonas*, *Pseudomonas*, *Acinetobacter*, *Vibrio,* and *Weissella* genera (Fig. 3C), while
277   posterior intestine samples contained *Mycoplasma*, *Spiroplasma*, *Aeromonas*, *Pseudomonas* and
278   *Acinetobacter* genera (Fig. 3D).
279   An UpSet plot was made to show OTUs (summed across replicates in 9 different groups) shared
280   between the different environments and intestinal components (Fig. 3E). There was a total of 133
281   OTUs (Fig. 3E), of which only 42 were annotated to the level of genus using the SILVA
282   database (Table S6). The two other UpSet plots show the core microbiome consisting of 4
283   different genera (*Acinetobacter*, *Aeromonas*, *Pseudomona*s, and *Spiroplasma*), as well as one
284   OTU which was present in more than 80% of all the samples (Fig. S2).
285
286   **Differential abundance of taxonomic groups**
287   A total of 11 phyla and 48 genera were present in different samples, with significant taxonomic
288   variation when comparing LP vs C, LP vs E, and C vs E (Fig. 4).
289   LEfSe analysis showed that fish present in the LP environment had a greater diversity of phyla
290   than fish grown in C (7 vs 2) or E (4 vs 1) and that in general, fish grown in the E environment
291   had a greater diversity of phyla than fish grown in the C environment (2 vs 1) (Fig. 4A, B and
292   C). Zooming into the level of genus, there were 17 and 15 genera that were more highly
293   abundant in LP than in C (4) or E (7) groups, respectively (Fig. 4D and E). The comparison
294   between C or E fish microbiota suggests that only 4 or 5 genera were abundant in each (Fig. 4F).
295
296   **Functional metabolic prediction using PICRUSt2**

297   A total of 440 functional pathways were predicted for the 74 different samples (from all
298   components and environments) that were analyzed. Of these, the 60 most abundant and with
299   statistical differences between samples were re-analyzed, with metabolic predictions shown in
300   Fig. S3. It was predicted that certain metabolic pathways would be over-represented in digesta
301   samples relative to others (DLP, D, and DE). The majority of the functional KO pathways
302   predicted to occur in all 9 groups belonged to four main categories: (i) Metabolism, (ii) Genetic
303   Information Processing, (iii) Environmental Information Processing, and (iv) Cellular Processes
304   (Fig. 5).
305   Within these predicted metabolic pathways, genes associated with the metabolism of energy,
306   cofactors, vitamins, nucleotides, carbohydrates, and amino acids were overrepresented in
307   cultured anterior intestines and digesta samples harvested from all environments (DLP, DC, AC,
308   and DE) (Fig. 5). Predicted differences in metabolic pathways and their regulation are shown in
309   Fig. S3 and S4.
310
311
312
313   **Discussion**
314
315   Gut microbiota has the potential to influence the health and welfare of fish raised in commercial
316   aquaculture, making it important to understand what factors may influence their microbiome
317   diversity. Hypothesizing that captive conditions would alter the gut microbiome of the Mexican
318   pike silverside, we compared the microbiota in wild fish to those raised in captivity. We
319   characterized the microbiota found in the digesta and intestines of *C. estor* collected from
320   different environments using high-throughput DNA sequencing.
321   The most abundant bacterial phyla in *C. estor* guts harvested from all three environments were
322   Proteobacteria and Firmicutes, which are both common and abundant in most fish (*Egerton et*
323   *al., 2018*). Despite numerous published studies on fish microbiomes, few publications report
324   their results at the genus level as we have here in the current study. In agreement with other
325   freshwater teleosts, the following genera were abundant in many of our samples:
326   *Propionibacterium* (or *Cutibacterium*), *Staphylococcus*, *Clostridium*, *Flavobacterium*,
327   *Cetobacterium*, *Pseudomonas*, *Romboutsia*, *Spiroplasma*, *Vibrio*, *Aeromonas*, and *Mycoplasma*
328   (e.g. *Roeselers et al., 2011; Gajardo et al., 2016; do Vale-Pereira et al., 2017; Rimoldi et al.,*
329   *2019; Ruzauskas et al., 2021*). Although it is hard to predict physiological roles for these
330   microbes, some bacteria of the genera *Staphylococcus*, *Vibrio*, and *Aeromonas* can be
331   opportunistic pathogens, while others have been used as probiotics in fish diets promoting
332   positive immune and growth responses (*Austin et al., 1995; Taoka et al., 2006; Pieters et al.,*
333   *2008; Abd El-Rhman, Khattab & Shalaby, 2009; Korkea-aho et al., 2012; Gao et al., 2016; Qi et*
334   *al., 2020; Tran et al., 2020*). *Undibacterium* spp. was abundant in wild *C. estor,* and has also
335   been isolated from zebrafish (*Danio rerio)* and Korean shiner (*Coreoleuciscus splendidus)*
336   (*Kämpfer et al., 2016; Lee et al., 2019*). Wild *C. estor* contained abundant *Anaerobacter*,

337 *Methylobacterium-Methylorubrum,* and *Hyphomicrobium,* and while their influence on fish
338 physiology is unknown, they are also known to be abundant in other aquatic organisms such as
339 coral (*Maire et al., 2021*).
340 Interestingly, our results suggest that the microbiota of *C. estor* is very similar to that of other
341 freshwater omnivorous fish (*Sullam et al.*, 2012), containing both high levels of Actinobacteria
342 while lacking Bacteroidetes. *C. estor* also contained a high relative abundance of Firmicutes and
343 Cyanobacteria, a low relative abundance of Proteobacteria, and an absence of Enterobacteriales
344 which are strongly characteristic of herbivorous marine fish (*Sullam et al.*, 2012). These
345 particularities of the *C. estor* microbiome might be diagnostic for a fish with a specialized
346 zooplanktonic and planktonic diet and may be linked to its specialized form of agastric digestion.
347 *C. estor* gut samples had high inter-individual variability (Fig. 1A and B), which could reflect the
348 stochastic instability of microbial diversity in the rapidly changing environment of the short *C.*
349 *estor* intestine. Despite this high variability, we were able to predict the existence of a core
350 microbiome in *C. estor* (Fig. 3 A-D and Fig. S2 above). Core microbiota included 4 genera and
351 133 OTUs, which is a number similar to the core microbiota for other fish species (*Dehler et al.*
352 *2017; Rimoldi et al., 2019*).
353 Most significantly, the diverse bacterial genera observed exclusively in Lake grown fish samples
354 (24 genera and 166 OTUs), suggests that fish grown in captivity lose some of their microbial
355 diversity. Future experiments will be required to study the impact that this loss of bacterial
356 diversity might have on fish digestion, health, and growth. Similar to other published
357 experiments and aiming to find bacteria with probiotic potential, such experiments might include
358 transplanting of microbial consortia or cross inoculation with individual strains isolated from the
359 intestines of wild fish, farmed fish, or autochthonous aquatic microbial communities (*Robertson*
360 *et al., 2000*; *Wang & Xu, 2006*; *Abd El-Rhman, Khattab & Shalaby, 2009*; *Essa et al., 2010*;
361 *Korkea-aho et al., 2012*; *Dias et al., 2018*; *Li et al., 2019*; *Mohammadian et al., 2019*;
362 *Mukherjee, Chandra & Ghosh, 2019, Xia et al., 2020*).
363 Identification of a core microbiome and the results of the differential abundance analysis (LEfSe)
364 showed that samples from Lake Patzcuaro had higher prevalent and abundant genera compared
365 to fish samples from Intensive and Extensive Cultures. This could be explained by the
366 zooplanktonic feeding habits of the species (*Martínez-Palacios et al., 2007*) and the greater
367 variety of food items found in the wild. Our results are in line with the previously published
368 theory that a wild environment is a greater source of bacterial diversity than a controlled
369 environment (*Dehler, Secombes & Martin, 2017*).
370 Bacterial diversity seemed to be higher in the anterior vs the posterior intestine (8 vs. 5 core
371 genera), which could be related to differences in digestive physiology (pH) along the digestive
372 canal as has been reported previously (*Martinez-Palacios et al., 2002*). Nevertheless, individual
373 gut components didn't develop significantly distinct microbial communities (Fig. 2B), explained
374 perhaps by the constant turnover of intestinal contents in fish that feed very frequently.
375

376 Prediction of differential microbiome metabolic potential using PICRUSt2 suggested that genes
377 associated with amino acid, vitamins and cofactors, and energy metabolic pathways would be
378 over-represented in certain compartments of the digestive system in *C. estor*, implying (as also
379 observed by others for other fish species) a link between microbial diversity and fish
380 physiological function (*Geraylou et al., 2012*; *Li et al., 2017*; *Agus, Planchais & Sokol, 2018*). It
381 is important to remember that results from PICRUSt2 are only bioinformatic predictions based
382 on the taxonomy of 16S rRNA genes and should be interpreted with caution. It will be important
383 to follow up our experiment with more in-depth functional studies such as metatranscriptomics,
384 metabolomics, and microbiome transplantation to validate predictions about microbiota
385 functionality in this fish species.
386
387

388 ## Conclusions
389
390 This is the first study on the Mexican pike silverside *Chirostoma estor* (a short-gut agastric
391 model) to employ high-throughput sequencing of 16S rRNA genes to define the diversity of
392 intestinal bacteria while attempting to predict their function. Microbial diversity was lowest in
393 fish cultured in fiberglass tanks, intermediate in fish cultured in earth ponds, and highest in wild
394 fish. These results suggest that husbandry can influence Mexican pike silverside microbiota, with
395 possible implications for its commercial aquaculture. Restoration or optimization of lost
396 intestinal microbiota, perhaps through autochthonous probiotic diet supplementation, may
397 become a technology to promote better growth and health of captive fish in the future.
398 Microbial profiles showed high inter-individual variation that could be due to the dynamic nature
399 of this fish's digestive tract. Finally, a core microbiome was identified in *C. estor,* although few
400 of the genera identified were similar to those of other fish species. Compared to other fish
401 species, this agastric short-intestine fish also possesses a unique digestive microbiota. The
402 development of culturing methods of similar agastric fish with aquaculture potential such as
403 anchovies, sardines, other atherinopsids, and hemyranphids may benefit from this intestinal
404 microbiome data on the model agastric fish *Chirostoma estor*.
405
406

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431

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Figure 1

Gut microbiota diversity.

(A) Normalized profiles of bacterial genera in fish guts. (B) Normalized profiles of bacterial phyla in fish guts. Legends display only the 30 most abundant taxa (genus and phylum). (C) Shannon diversity index of gut bacteria grouped by environments or intestinal components, calculated using OTU data. Asterisk indicates the significant ($p < 0.05$) statistical difference between digesta from Lake Patzcuaro (DLP) and digesta from intensive culture (C).

(A)

Lake Patzcuaro (LP) **Intensive culture (C)** **Extensive culture (C)**

Mycoplasma
Staphylococcus
Spiroplasma
Aeromonas
Pseudomonas
Planomicrobium
Clostridium
Foliisarcina
Cutibacterium
Romboutsia
Prochlorococcus
Acinetobacter
Cetobacterium
Anaerobacter
Erwinia
Flavobacterium
Dechloromonas
Methylobacterium
Dongia
Desulfomicrobium
Massilia
Burkholderia
Vibrio
Thermosilla
Tolypothrix
Niveispirillum
Desulfovibrio
Incertae
Shimwellia
Comamonas
Plesiomonas
Achromobacter
Stenotrophomonas
Iphinoe
Brevundimonas
Caulobacter
Streptococcus
Novosphingobium
Vicinamibacter
Undibacterium
Terrimicrobium
Rhodoligotrophos
Sphingomonas

(B)

Firmicutes
Gammaproteobacteria
Cyanobacteria
Alphaproteobacteria
Actinobacteriota
Bacteroidota
Desulfobacterota
Fusobacteriota
Planctomycetota
Verrucomicrobiota
Acidobacteriota
Chloroflexi
Spirochaetota
Myxococcota
Gemmatimonadota
Deinococcota
Campylobacterota
Deferrisomatota
Nitrospirota
Armatimonadota
Bdellovibrionota
Synergistota
Dictyoglomota
Thermotogota
Crenarchaeota
Aquificota
Caldisericota
Chrysiogenetota
Fibrobacterota
Elusimicrobiota
Thermosulfidibacterota
Halanaerobiaeota
Thermodesulfobiota

(C)

Intestinal component
Anterior gut
Digesta
Posterior gut

Lake Patzcuaro   Intensive culture   Extensive culture

Figure 2

Beta diversity analysis.

Nonmetric multidimensional scaling (NMDS) plot of the Bray–Curtis beta-diversity from *C. estor* microbiota profiles, estimated from the OTU abundance matrix. The two groupings were by environment (A) and by intestinal component (B).

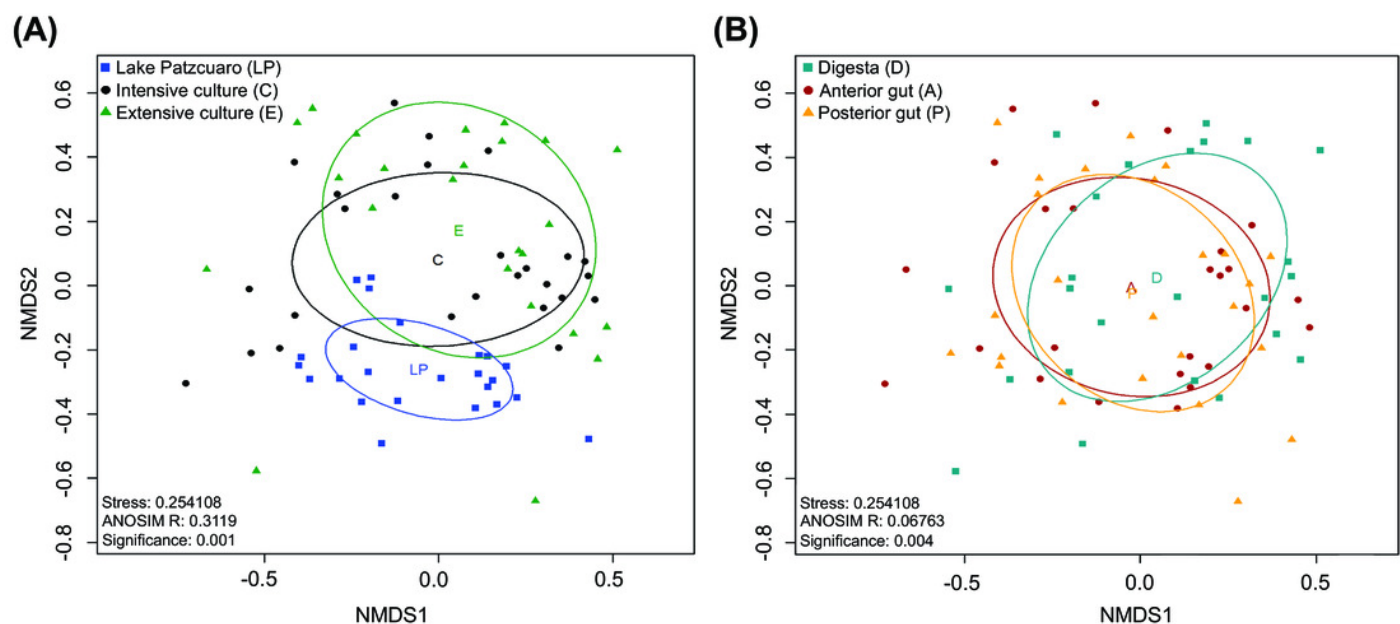



Figure 3

Core microbiota amongst different environmental groups.

(A) Core microbiota were defined at the level of genera as present in at least 80% of the samples of a group. All three environments (LP - Lake Patzcuaro; C - Intensive Culture; E - Extensive Culture) shared 5 genera regardless of the intestinal compartment (5/36 genera). (B) In samples of digesta, there were 6 core bacterial genera (6/89 genera). (C) In samples of the fish anterior intestine, there were 8 genera shared between environments (8/69 genera). (D) In samples of the fish posterior intestine, there were 5 genera shared between environments (5/49 genera). (E) An UpSet plot based on the presence/absence of OTUs after summing up across replicates. Presence means OTUs occurred in at least one sample of each group (DC - digesta of intensive culture; PC - posterior intestine of intensive culture; DLP - digesta of Lake Patzcuaro fish; PLP - posterior intestine of Lake Patzcuaro fish; ALP - anterior intestine of Lake Patzcuaro fish; AE - fish anterior intestine of extensive culture; PE - fish posterior intestine of extensive culture; DE - fish digesta of extensive culture; AC - fish anterior intestine of intensive culture). The set size of each group is plotted in horizontal bars (in blue). Bars show the number of OTUs present uniquely in a specified group and dark circles while connecting bars indicate shared OTUs between multiple samples.

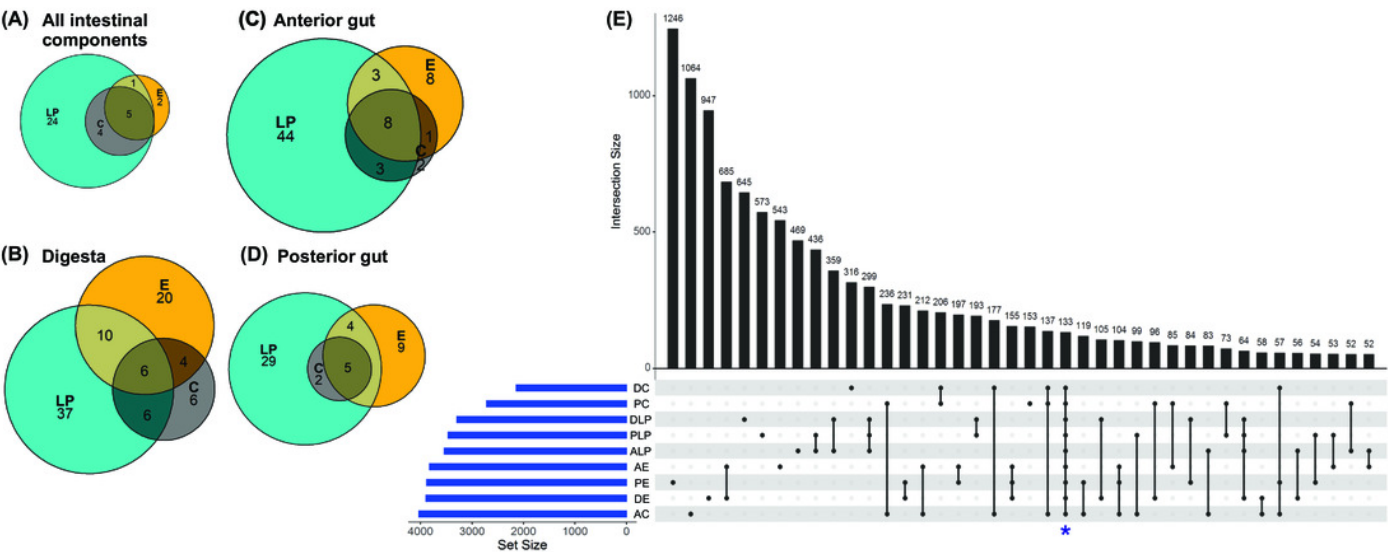



Figure 4

Genera prevalence deduced by LEfSe analysis.

Significant differences in the relative abundance of microbial phyla and genera by environment group (Lake Patzcuaro, LP; Intensive culture, C; Extensive culture, E). (A-C) Differences in phylum abundance between LP & C, LP & E, and C & E. (D-F) Differences in genus abundance between LP & C, LP & E, and C & E. Statistical significance of LDA effect size was evaluated in the Kruskal-Wallis test with p-value cutoff = 0.05.

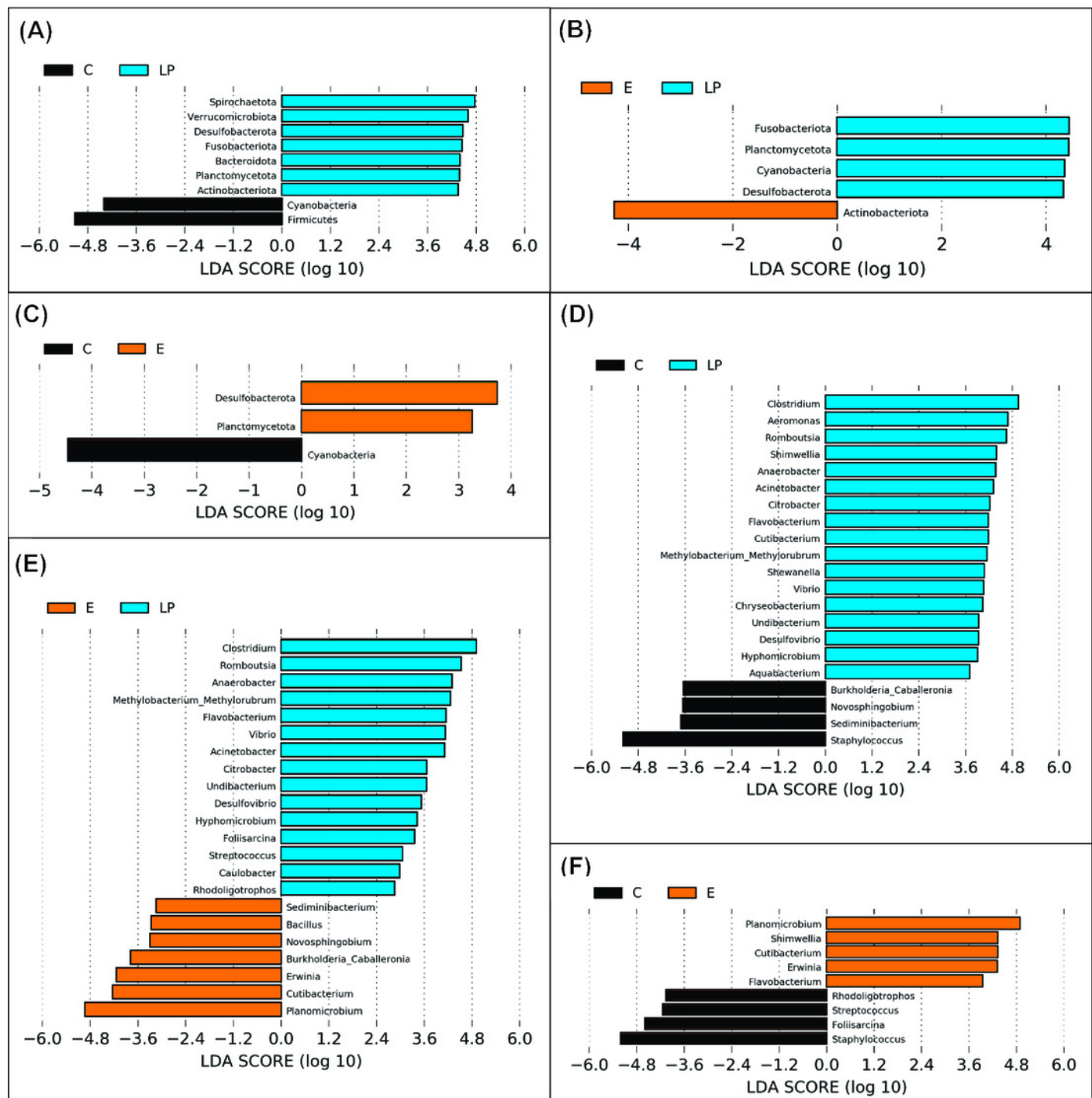
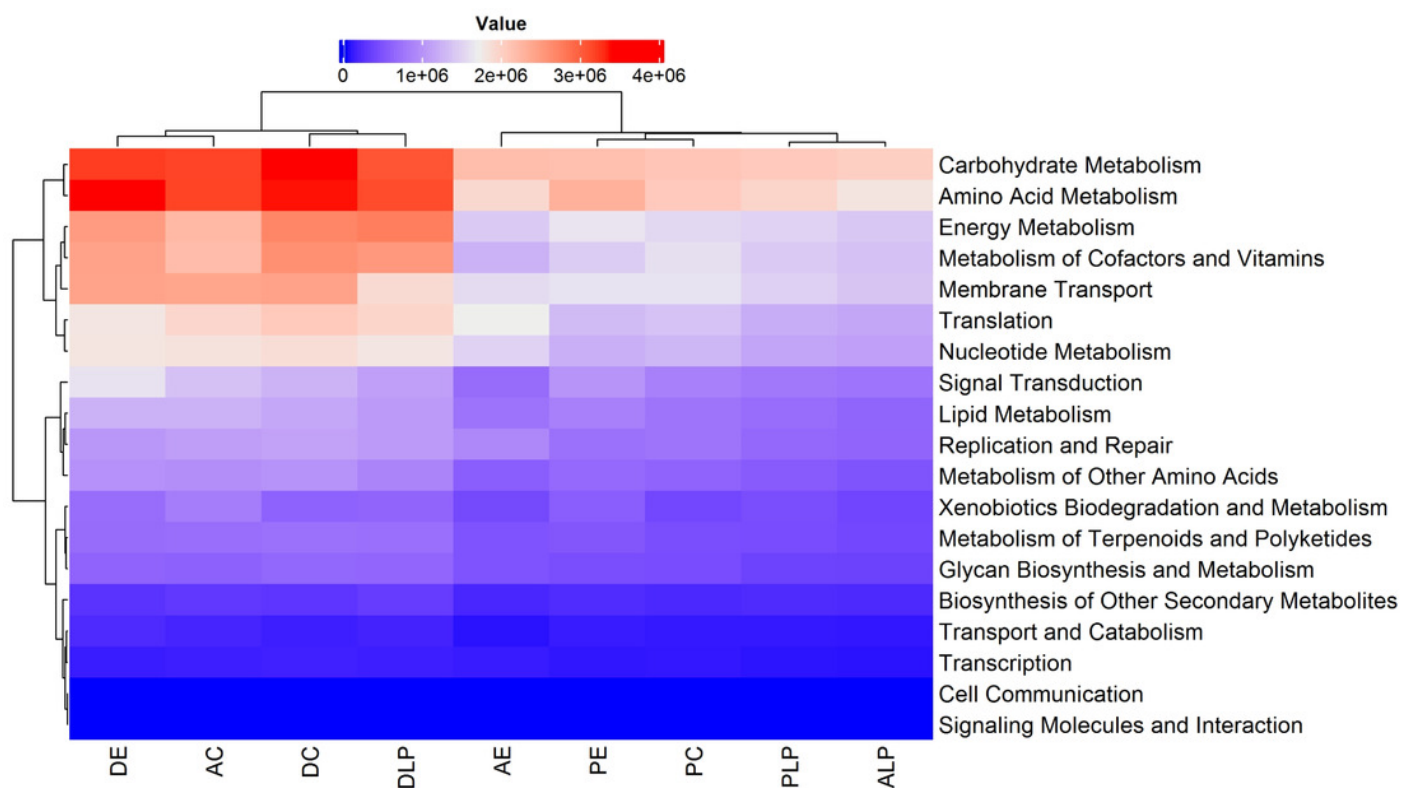



Figure 5

Heatmap and dendrogram of KOs pathways in 9 different groups.

Prediction by PICRUSt2 is shown at KEGG´s first level. AE - anterior intestine/extensive culture; ALP - anterior intestine/Lake Patzcuaro; PLP - posterior intestine/Lake Patzcuaro; PC - posterior intestine/intensive culture; PE - posterior intestine/extensive culture; DLP - digesta/Lake Patzcuaro; DC - digesta/intensive culture; AC - anterior intestine/intensive culture; DE - digesta/extensive culture.

Total mean relative abundance (%) (first column) ± SE, and mean relative abundance ± SE of the 20 most prevalent genera found in guts from different environments.

Data of digesta, D; anterior intestine, A; and posterior intestine, P of pike silverside in the three environments (Lake Patzcuaro, LP; Intensive Culture, C; Extensive Culture, E). Statistical comparisons were performed separately by intestinal components (D, A, P) and environments (LP, C, E). Different letters indicate statistical significance between taxonomic group abundance in a row ($p < 0.05$).

Genus	Total abundance (N=74)	Intestinal component			Environment		
		D (N=24)	A (N=25)	P (N=25)	LP (N=24)	C (N=26)	E (N=24)
Mycoplasma	19.95 ± 4.11	1.55 ± 1.11 **B**	35.88 ± 8.59 **A**	21.68 ± 7.25 **A**	7.17 ± 3.87 **b**	19.94 ± 6.45 **ab**	32.76 ± 9.31 **a**
Staphylococcus	10.9 ± 3.30	18.08 ± 7.28	4.64 ± 3.98	10.28 ± 5.44	0.09 ± 0.03 **b**	30.71 ± 8.14 **a**	0.25 ± 0.21 **b**
Spiroplasma	10.23 ± 2.80	5.49 ± 1.79	10.67 ± 5.31	14.35 ± 6.15	11.68 ± 5.47	14.48 ± 6.01	4.18 ± 1.29
Aeromonas	7.51 ± 1.87	10.62 ± 4.18	6.96 ± 3.23	5.08 ± 2.09	11.59 ± 3.88	4.72 ± 2.25	6.44 ± 3.48
Pseudomonas	6.11 ± 1.86	6.31 ± 3.55	3.93 ± 1.46	8.10 ± 4.14	1.86 ± 0.89	7.56 ± 3.36	8.79 ± 4.32
Planomicrobium	4.75 ± 1.90	11.03 ± 5.09	0.25 ± 0.21	3.23 ± 2.44	0.02 ± 0.01 **b**	0.05 ± 0.04 **b**	14.57 ± 5.39 **a**
Clostridium	4.67 ± 1.32	4.18 ± 1.79	6.88 ± 2.94	2.92 ± 1.95	13.77 ± 3.42 **a**	0.08 ± 0.06 **c**	0.53 ± 0.28 **b**
Foliisarcina	2.37 ± 1.36	4.55 ± 3.63	0.21 ± 0.13	2.44 ± 2.01	0.22 ± 0.10 **b**	6.53 ± 3.77 **ab**	0.02 ± 0.01 **c**
Cutibacterium	2.16 ± 1.25	4.49 ± 3.77	1.23 ± 0.70	0.87 ± 0.53	2.21 ± 0.77 **b**	0.63 ± 0.49 **c**	3.78 ± 3.78 **a**
Romboutsia	2.06 ± 0.62	2.68 ± 1.31	2.32 ± 1.11	1.21 ± 0.77	5.41 ± 1.60 **a**	0.03 ± 0.01 **b**	0.92 ± 0.67 **b**
Prochlorococcus	1.43 ± 0.70	3.34 ± 2.00	0.95 ± 0.71	0.08 ± 0.03	4.25 ± 2.07 **a**	0.01 ± 0.01 **b**	0.14 ± 0.08 **b**
Acinetobacter	1.37 ± 0.34	0.59 ± 0.14	1.41 ± 0.43	2.09 ± 0.89	2.66 ± 0.92 **a**	0.68 ± 0.27 **b**	0.83 ± 0.33 **b**
Cetobacterium	1.37 ± 1.05	0.32 ± 0.16	0.43 ± 0.40	3.32 ± 3.07	4.05 ± 3.20 **a**	0.11 ± 0.10 **b**	0.04 ± 0.03 **b**
Anaerobacter	1.17 ± 0.35	1.07 ± 0.62	1.67 ± 0.69	0.77 ± 0.50	3.49 ± 0.92 **a**	0.01 ± 0.01 **c**	0.11 ± 0.05 **b**
Erwinia	0.98 ± 0.70	2.50 ± 2.16	0.18 ± 0.13	0.33 ± 0.20	0.02 ± 0.01 **c**	0.14 ± 0.12 **bc**	2.86 ± 2.15 **a**
Flavobacterium	0.91 ± 0.31	1.50 ± 0.82	0.53 ± 0.17	0.73 ± 0.45	2.53 ± 0.87 **a**	0.07 ± 0.05 **c**	0.20 ± 0.08 **b**
Dechloromonas	0.89 ± 0.49	1.14 ± 0.94	1.48 ± 1.15	0.05 ± 0.03	0.01 ± 0.01 **b**	0.01 ± 0.01 **b**	2.72 ± 1.47 **a**
Methylobacterium-Methylorubrum	0.73 ± 0.62	0.02 ± 0.01	0.12 ± 0.05	2.02 ± 1.83	2.08 ± 1.91 **a**	0.09 ± 0.07 **b**	0.08 ± 0.05 **b**
Dongia	0.71 ± 0.37	1.04 ± 0.84	0.98 ± 0.72	0.12 ± 0.06	2.17 ± 1.08 **a**	0.01 ± 0.00 **b**	0.01 ± 0.01 **b**
Desulfomicrobium	0.66 ± 0.35	0.58 ± 0.41	0.53 ± 0.50	0.88 ± 0.85	1.99 ± 1.06 **a**	0.00 ± 0.00 **b**	0.06 ± 0.03 **a**