Microbiome differences between river-dwelling and cave-adapted populations of the fish *Astyanax mexicanus* (De Filippi, 1853)

Patricia Ornelas-García Corresp., [1] , **Silvia Pajares** [2] , **Víctor M. Sosa-Jiménez** [1] , **Sylvie Rétaux** [3] , **Ramsés A. Miranda-Gamboa** [4]

[1] Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City, Mexico City, Mexico

[2] Unidad Académica de Ecología y Biodiversidad Acuática, Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Mexico City, Mexico City, Mexico

[3] Paris-Saclay Institute of Neuroscience, Université Paris Sud, CNRS UMR9197, Université Paris-Saclay, Gif-sur-Yvette, France, France

[4] Instituto de Energías Renovables, Universidad Nacional Autónoma de México, Temixco, Morelos, Mexico

Corresponding Author: Patricia Ornelas-García
Email address: patricia.ornelas.g@ib.unam.mx

Symbiotic relationships between host and microbiome can play a major role in local adaptation. Previous studies with freshwater organisms have shown that microbiome performs numerous important biochemical functions for the host, playing a key role in metabolism, physiology or health. Experimental studies in fish groups have found an effect of enzymatic activity of gut microbiota on a variety of metabolic processes. The goal of this study was to compare stomach microbiome from cave and surface *Astyanax mexicanus,* in order to evaluate the potential response of microbiota to contrasting environmental conditions and physiological adaptations of the host. Stomach microbiota was obtained from three different populations: Pachón cave, and two surface rivers (Rascón and Micos rivers). The stomach microbiome was analyzed using the Ion 16S Metagenomic kit considering seven variable regions: V2, V3, V4, V6-7, V8 and V9. A high diversity was observed across samples, including 16 phyla, 120 families and 178 genera. Gammaproteobacteria, Firmicutes, Bacteroidetes and Betaproteobacteria were the most abundant phyla across the samples. Although the relative abundance of the core OTUs at genus level were highly contrasting among populations, we did not recover differences in stomach microbiome between contrasting habitats (cave vs surface rivers). Rather, we observed a consistent association between β-diversity and dissolved oxygen concentration in water. Therefore, and unexpectedly, the microbiota of *A. mexicanus* is not linked with the contrasting conditions of the habitat considered here but is related to water parameters.

1   **Microbiome differences between river-dwelling and cave-adapted populations of the fish**

2   *Astyanax mexicanus* **(De Filippi, 1853)**

4   **Claudia Patricia Ornelas-García**[a]§ **Silvia Pajares**[b]**, Víctor Manuel Sosa-Jiménez**[a]**, Sylvie**

5   **Rétaux**[c] **and Ramsés Alejandro Miranda-Gamboa**[d]

6   [a]Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México.

7   [b]Unidad Académica de Ecología y Biodiversidad Acuática, Instituto de Ciencias del Mar y

8   Limnología, Universidad Nacional Autónoma de México.

9   [c]Paris-Saclay Institute of Neuroscience, Université Paris Sud, CNRS UMR9197, Université

10 Paris-Saclay.

11 [d]Instituto de Energías Renovables, Universidad Nacional Autónoma de México.

14 §Correspondence: patricia.ornelas.g@ib.unam.mx

16   **Abstract**

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18   Previous studies with freshwater organisms have shown that microbiome performs numerous

19   important biochemical functions for the host, playing a key role in metabolism, physiology or

20   health. Experimental studies in fish groups have found an effect of enzymatic activity of gut

21   microbiota on a variety of metabolic processes. The goal of this study was to compare stomach

22   microbiome from cave and surface *Astyanax mexicanus,* in order to evaluate the potential

23   response of microbiota to contrasting environmental conditions and physiological adaptations of

24   the host. Stomach microbiota was obtained from three different populations: Pachón cave, and

25   two surface rivers (Rascón and Micos rivers). The stomach microbiome was analyzed using the

26   Ion 16S Metagenomic kit considering seven variable regions: V2, V3, V4, V6-7, V8 and V9. A

27   high diversity was observed across samples, including 16 phyla, 120 families and 178 genera.

28   Gammaproteobacteria, Firmicutes, Bacteroidetes and Betaproteobacteria were the most abundant

29   phyla across the samples. Although the relative abundance of the core OTUs at genus level were

30   highly contrasting among populations, we did not recover differences in stomach microbiome

31   between contrasting habitats (cave vs surface rivers). Rather, we observed a consistent

32   association between β-diversity and dissolved oxygen concentration in water. Therefore, and

33   unexpectedly, the microbiota of *A. mexicanus* is not linked with the contrasting conditions of the

34   habitat considered here but is related to water parameters.

36   Key words: Microbiome, symbiotic interactions, cavefish, *Astyanax*.

38 **Introduction**

39 Microbe-host associations are essential drivers of evolution and can play a role in adaptation and

40 speciation. Across taxa, the microbiome is essential for regulating metabolism, physiology, and

41 health (Brucker & Bordenstein 2012; Franchini et al. 2014; Sevellec et al. 2014). In fish groups

42 for example, microbiota alter enzymatic activity in the gut to influence polysaccharides

43 metabolism or fat storage (e.g. Bäckhed et al. 2004; Hongoh 2010; Nicholson et al. 2012; Sullam

44 et al. 2012; Tetu et al. 2013; Franchini et al. 2014). The microbiome – host evolution has been

45 suggested as a 'hologenome' model, with the host microbiome viewed as an extension of its own

46 genome, but with a faster evolution, and the possibility to exchange microorganisms (with their

47 genes and associated functions) with the environment (Shapira et al. 2016).

48   Several functional relationships have been proposed between bacteria and their host: 1)

49 commensal bacteria (Cahill 1990), which can be neutral or beneficial to the host (Prescott,

50 Harley & Klein 2005); 2) symbiotic obligatory relationships, with a mutual benefit (Perru 2006);

51 3) opportunistic bacteria, which could be pathogenic under certain circumstances; and 4)

52 pathogenic bacteria, which are responsible for infectious diseases (Falkow 1997). The

53 knowledge of metagenomes can provide unique insights about how the relationship between

54 microbial communities, environmental variation and evolutionary processes shapes organism's

55 niche adaptation to particular habitats and can have a prominent role in the host speciation

56 (Franchini et al. 2014; Sevellec et al. 2014; Liu et al. 2016; Shapira et al. 2016).

57   The technological advances in high throughput sequencing have given an unprecedented

58 opportunity to comprehensively characterize bacterial communities (Hugenholtz & Tyson 2008).

59 Additionally, the methods for characterizing microbiome assemblages are changing rapidly, with

60 a variety of technologies being used to assay DNA sequence variation. Among such methods,

61   16S rRNA gene amplicons is the method most commonly used to characterize bacterial

62   community composition (Kent et al. 2004; Nemergut et al. 2011; Poretsky et al. 2014; Liu et al.

63   2016), particularly through the study of hypervariable V3 and V4 regions (Yu & Morrison 2004;

64   Wang et al. 2007). However, there is an ongoing debate regarding which region is the most

65   convenient to get the best characterization of the microbiome diversity (Zwart et al. 2002;

66   Chakravorty et al. 2007; Youssef et al. 2009; Guo et al. 2013; Barb et al. 2016). Previous studies

67   have shown differences in diversity estimates among 16S rRNA regions (Youssef et al. 2009),

68   while "universal" primers have shown preferential amplification across certain bacterial groups

69   (Ahantarig et al. 2013; Gofton et al. 2015).

70   Populations inhabiting caves provide a unique opportunity to study how extreme

71   environments can shape the evolution of organisms adapted to dark conditions. The troglobite

72   populations of the fish genus *Astyanax*, which inhabit the karstic Sierra Madre Oriental in

73   northeastern Mexico, are among the most studied cavefish groups because they represent an

74   outstanding opportunity to understand local adaptation and trait evolution (Jeffery 2009; Gross

75   2012; Yoshizawa et al. 2012; Gross et al. 2013; Hinaux et al. 2013; Keene et al. 2015; Ornelas-

76   García & Pedraza-Lara 2015). From its discovery by Salvador Coronado in 1936, this emerging

77   model organism has brought light into the study of the evolution of unique characteristics

78   associated to living in dark conditions. The occurrence of its sister surface species inhabiting the

79   same region allows the comparison of adaptations under contrasting environmental pressures

80   (cave *vs*. surface). Additionally, this model provides a unique opportunity to evaluate the role of

81   the evolutionary history in the microbiome biodiversity.

82   Among the unique characteristics that distinguish hypogean systems are the absence of

83   light and primary production, as well as stable annual temperature comparatively to the

84 photosynthetically active epigean systems (Culver & White 2005; Tabin et al. 2018). Hence, in

85 comparison to their closely-related populations from surface habitat, *Astyanax* cavefish have

86 evolved a series of adaptations to local conditions that could be considered as either regressive or

87 constructive. For example, loss of eyes and pigments are considered as regressed characteristics,

88 but they may confer a selective advantage for life in the dark (Bilandžija et al. 2013; Moran,

89 Softley & Warrant 2015), while an increase of the number and size of neuromasts (Yoshizawa et

90 al. 2012), as well as augmented olfactory capabilities (Protas et al. 2008; Bibliowicz et al. 2013;

91 Hinaux et al. 2016; Blin et. al. 2018) are considered as constructive adaptations. Metabolic

92 adaptations, such as higher body fat and blood glucose content, have also been described in

93 *Astyanax* cave populations (Riddle et al. 2018; Xiong et al. 2018). This turn-over of adaptive

94 traits across alternative environments could also occur regarding inter-species associations, like

95 those established between host and microbiome. A fundamental aspect in the study of such

96 associations is to distinguish between two different groups of microbiota, one host-adapted core

97 and one environmentally-determined group (Shapira 2016).

98   The main goal of the present study was to compare the microbiome of cave and surface

99 *A. mexicanus,* in order to evaluate the potential response of this microbiome to contrasting

100 environmental conditions and physiological adaptations of the host. To this end, we sampled one

101 cave and two surface populations in the wild that came from different river systems in order to

102 (a) characterize microbiome structure across seven hypervariable regions of the 16S rRNA gene

103 (Ion Torrent PGM™ 16S kit: V2, V3, V4, V6-V7, V8, and V9), and (b) identify relationships

104 between microbial community structure and contrasting environments.

105

106 **MATERIALS AND METHODS**

107

108   **Study area**

109   We included one cavefish population (Pachón) from the Sierra de El Abra, a mountain range

110   running north to south along the eastern margin of the Sierra Madre Oriental, a karstic region in

111   northeastern Mexico (Elliott 2016). El Abra is considered as one of the highest flowing karst

112   springs worldwide (Gary & Sharp 2006) and the presence of *A. mexicanus* cavefish populations

113   have been reported in at least 30 caves in the region (Elliott 2016; Espinasa et al. 2018).

114   Additionally, we included two surface populations located at the vicinity of El Abra, between the

115   Sierras Las Crucitas and Sierra de la Colmena (San Luis Potosí, Figure 1). All locations form

116   part of the Pánuco river basin, which drains to the Gulf of Mexico (Hudson 2003).

117

118   **Sample collection and DNA extraction**

119   Sampling was made from August 5th to 8th, 2016, during the wet season. A total of eight samples

120   were collected in cave and surface habitats (Figure 1): Pachón cave (n=3, two females and one

121   male), Rascón river (n=2, both males), and Micos river (n=3, two females and one male). All the

122   samples were collected under the auspices of the permit SGPA/DGVS/02438/16, delivered by

123   SEMARNAT. Fish specimens were captured using hand nets, kept alive and rapidly transported

124   in their environment water to a place with semi-sterile conditions to avoid environmental and

125   cross-contamination. Fish were euthanized following conditions stated in the collecting permit

126   SGPA/DGVS/02438/16. The complete digestive tract was dissected and rinsed with sterile saline

127   solution to remove any adherent material and preserved in 2 ml cryovials with Invitrogen™

128   RNAlater™ Stabilization Solution. The samples were kept in liquid nitrogen during sampling (5

129   days), and once in the laboratory were stored at -72ºC. All dissected specimens were preserved

130 as vouchers in 95% ethanol and deposited in the Colección Nacional de Peces, IBUNAM

131 (Mexico). Under sterile conditions and in a laminar flow hood, the stomach sack was excised

132 from the rest of the intestine (approximately 1 cm^2) and immediately transferred into the lysing

133 matrix provided by the FastDNA Isolation Kit for Soil (MP Bio®). The genomic DNA was

134 extracted and purified following manufacturer's instructions. DNA quality and quantity were

135 assessed by both, agarose gel electrophoresis and spectrophotometrically with a NanoDrop 2000

136 (Thermo Fisher Scientific, DE).

137   At each sampling point, seven physicochemical parameters were measured: pH, water

138 temperature, dissolved oxygen (DO), electrical conductivity, total dissolved solids, salinity, and

139 oxido-reduction potential (ORP), using a multiparameter Hanna HI9828 (HANNA Instruments).

140

141 **16S rRNA library preparation and sequencing**

142 The stomach microbiome for the eight *A. mexicanus* samples were assayed using the Ion 16S

143 Metagenomic kit (LifeTech A2616), which uses seven hypervariable regions: V2, V3, V4, V6-7,

144 V8 and V9. Amplification occurred in two multiplex pools (one for the V2, V4, and V8, and one

145 for the V3, V6-7 and V9), with 25 PCR cycles, using the Ion Xpress Barcoded adapters.

146 Amplicon sizes of the 6 fragments averaged 254 bp, within the range of 215 – 295 bp in *E. coli*

147 (Ion Torrent, 2014). Libraries were quantified using Real-Time PCR, normalized and pooled.

148 Emulsion PCR used Ion PGM Template OT2 400 kit. Sequencing was performed on an Ion

149 Torrent PGM, using Ion 316 chip kit v2 BC with 4 and 8 chips.

150

151 **Sequences analysis**

152   Bacterial taxonomic assignments were performed using Ion Reporter Metagenomics 16S

153   software v5.2 (Life Technologies) with the following analysis pipeline: a) a minimum Phred

154   score of Q20; b) only reads with no mismatches in the barcode, up to 3 mismatches in the primer

155   sequence, and a minimum length of 150 bp were included, and c) reads were trimmed by primers

156   at both ends. After primer trimming and length checking, reads were assigned to a hash table to

157   get unique sequences and their abundance. Taxonomic assignment for unique sequences with a

158   minimum of 10 copies was performed using MicroSEQ ID 16S Reference Library v2013.1

159   (Thermo Fisher Science) and Greengenes v13.5 (McDonald et al. 2012) databases. Reads were

160   grouped into two bins: 90–97% match (approximating the family level) and >97–99% identity

161   (genus level). This tool also provided information on relative taxonomic abundance by consensus

162   and by primer within the dataset.

163   Rarefaction curves and alpha diversity indices were calculated using the alpha diversity

164   script in the Quantitative Insights into Microbial Ecology software (QIIME v. 1.9.0; (Caporaso et

165   al. 2010). For the diversity estimates, sequences were rarefied at 88,076 reads, which was the

166   smallest read count across all fish samples (sample Micos female 1). The species richness was

167   estimated with the Chao index, and diversity was determined by Shannon index (that quantifies

168   the uncertainty in predicting the species identity in a random sampling) and Simpson index (that

169   quantifies evenness and dominance of the species in the sample) (Hill et al. 2003).

170   The number of unique and shared OTUs (Operational Taxonomic Units) at genus level

171   between sexes and populations was visualized with Venn diagrams using the Euleer package in

172   R software (Core 2015). We evaluated the capability of each sequenced region to describe the

173   stomach microbiota composition through the survey of the relative proportion of bacterial

174   taxonomic groups retrieved by each hypervariable region of the 16S rRNA gene. Posteriorly, a

175   non-metric multidimensional scaling (NMDS) ordination plot using Bray-Curtis dissimilarity

176   matrix was constructed with the vegan package in R to depict community structure patterns in

177   two dimensions. Permutation tests (n = 1000) were used to fit significant water physico-chemical

178   properties (P < 0.1) onto the ordination plot as vectors. Analysis of similarity (ANOSIM) was

179   used to test for significant differences in microbiome structure between fish samples (999

180   permutations, P < 0.001). To analyze the distribution of OTUs at family level with relative

181   abundances >1% of the total reads across all the samples, a heatmap was constructed using the

182   Manhattan dissimilarity matrix and Ward's hierarchical clustering algorithms with the plots

183   package in R. The 16S rRNA sequences were deposited in the NCBI Sequence Read Archive

184   (SRA) under the Bioproject PRJNA487659 (SAMN09907737-SAMN09907742).

185

186   **Results**

187   **Diversity and composition of bacterial community in wild populations obtained by 16S**

188   **rRNA variable regions**

189   A total of 1,969,556 reads were recovered, with an average length of ~200 bp. We observed that

190   all rarefaction curves from fish samples attain an asymptote (Supplementary material 1),

191   indicating that the sequencing effort was sufficient to cover the bacterial community. Alpha

192   diversity indices varied among the fish samples, but didn't show a clear pattern among

193   populations (Table 1). In terms of species richness, samples from Pachón cave showed the

194   highest Chao index values, followed by Micos and finally Rascón. Diversity indices across

195   samples were similar, with a range for the Shannon diversity index between 2.26 and 3.65, while

196   for the Simpson diversity index the samples varied between 0.614 and 0.884, with the lowest

197   values in both indices for the Rascón male 1. Finally, sex did not seem to impact biodiversity

198   indexes (Supplementary material 2).

199   In total, we recovered 16 phyla, 120 families, and 178 genera, where the four following

200   lineages accounted for 93.8% of total sequences across all the samples: Gammaproteobacteria

201   (31.7%), Firmicutes (29.9%), Bacteroidetes (16.2%) and Betaproteobacteria (15%). Differences

202   in composition and distribution of phylogenetic groups across populations were recovered

203   (Figure 2). The stomach microbiome of surface Rascón samples was dominated by Bacteroidetes

204   (61.4% male 1 and 29.5% male 2) and Firmicutes (29% male 1 and 63.5% male 2), with a lesser

205   proportion of Actinobacteria, Verrucomicrobia and Fusofacteria. In contrast, a different

206   microbiota was recovered from Pachón cave samples, which were dominated by

207   Gammaproteobacteria (37.6% male 1 and 47.9% female 1), Betaproteobacteria (17% male 1, and

208   34.5% female 1) and Firmicutes (26.3% male 1 and 11.9% female 2). The microbiome from

209   Micos surface population was more similar to the Pachón cave population than to the Rascón

210   surface population, being Gammaproteobacteria (68.5% female 1 and 32.2% female 2),

211   Betaproteobacteria (19.4% female 1 and 13.6% female 2) and Firmicutes (7.8% female 1 and

212   40.5% female 2) the most abundant groups, with a lesser proportion of other groups such as

213   Cyanobacteria and Fusobacteria.

214

215   **Comparison of 16S rRNA hypervariable regions for assignment of microbial diversity**

216   The percentage of mapped reads was not homogenous among the hypervariable regions

217   (Supplementary material 3). The region with the highest percentage of mapped reads was V3

218   (from 31.8% in female 1 to 44.9% in female 2, both from Micos river), followed by V8 (from

219   9.5% in Micos female 2 to 29.6% in Micos female 1), V4 (from 11.7% in Rascón male 2 to 18%

220   in Rascón male 1 and Pachón female 1), and V6-V7 (from 6.6% in Pachón female 1 to 20.9% in

221   Rascón male 1). The regions with the lowest percentage of mapped reads were V2 (from 1.4% in

222   Rascón male 2 to 10.9% in Pachón female 1) and V9 (from 0.8% in Rascón male 1 to 12% in

223   Micos female 1). In addition, not all the regions could distinguish the different taxonomic groups

224   in the same way. For instance, V3 was the region with the greatest coverage of taxonomic groups

225   (from 6 to 9 groups), allowing the detection of the four most abundant groups

226   (Gammaproteobacteria, Firmicutes, Betaproteobacteria, and Bacteroidetes) across all the fish

227   samples. On the other hand, V9 covered the least number of taxonomic groups (from 1 to 2

228   groups) and could only detect Gammaproteobacteria and Betaproteobacteria groups.

229

230   **Shared and dominant stomach microbiome between *A. mexicanus* populations**

231   The core stomach microbiome shared by the three *A. mexicanus* populations was formed by 8

232   genus OTUs (Figure 3) belonging to *Acinetobacter, Aeromonas, Citrobacter* and *Klebsiella*

233   (class Gammaproteobacteria)*, Prevotella* (Bacteroidetes)*, Vogesella* (class Betaproteobacteria)*,*

234   *Clostridium* (Firmicutes)*,* and *Cetobacterium* (Fusobacteria). The abundance distribution of the

235   core microbiome differs among the three populations: Rascón river had the highest abundance of

236   *Prevotella* (89.9%), while Micos river had more *Acinetobacter* (60.6%) and *Vogesella* (11.8%),

237   and Pachón cave had more *Vogesella* (40.4%) and *Acinetobacter* (34.3%). Although they come

238   from contrasting environments, Micos river and Pachón cave specimens shared more OTUs

239   between them, than with samples from Rascón river. In addition, the most abundant bacterial

240   groups per locality showed a clear difference across populations (Table 2): the dominant genus

241   OTUs in Pachón cave samples belonged to Proteobacteria, Firmicutes and Actinobacteria, in

242   Micos river samples belonged to Proteobacteria and Firmicutes, and in Rascón river samples

243   belonged to Proteobacteria, Firmicutes and Bacteroidetes.

244

245 **Environmental determinants for the microbiome composition across populations**

246 As the contrast between environments (cave *vs*. surface) did not seem to account for the observed

247 differences in stomach microbiota, we assessed the association of water physicochemical

248 variables with respect to β-diversity by means of NMDS analysis (Figure 4), which grouped the

249 samples in two clusters: bacterial community from the Rascón river samples formed a unique

250 cluster, and those from Micos river and Pachón cave formed another cluster. This strong

251 clustering trend was also confirmed by the ANOSIM (p < 0.001) analysis. Fitting the

252 physicochemical variables onto the ordination plot showed that dissolved oxygen (DO) was the

253 only significant explanatory variable for the bacterial community structure. The DO values were

254 considerably lower in both Pachón cave (2.97 mg/L) and Micos river (4.43 mg/L) relative to that

255 from Rascón river (8.2 mg/L). Interestingly, observed differences in other variables such as

256 conductivity, for which contrasting values were obtained (e.g. 226 μS/cm and 580 μS/cm for

257 Rascón and Micos rivers, respectively), did not emerge as important factor as DO did for

258 stomach microbiota community distribution (Table 3).

259   The heatmap of family OTUs with relative abundance >1% of the total reads showed the

260 same pattern of grouping as the NMDS analysis (Figure 5), separating the stomach microbiome

261 in two groups: the first one included the samples from Rascón river, and the second one included

262 a mixture of both surface and cave environments (Micos river and Pachón cave). Moraxellaceae

263 (Gammaproteobacteria) was the most abundant family with 14.5% of the total reads, followed by

264 Enterobacteriaceae (Gammaproteobacteria) with 11.5% of the total reads and Prevotellaceae

265 (Bacteroidetes) with 11.1% of the total reads. In general, fish samples from Micos river and

266 Pachón cave showed greater abundance of bacteria belonging to Moraxellaceae and

267 Enterobacteriaceae, while fish samples from Rascón showed greater abundance of bacteria

268 belonging to Prevotellaceae.

269

270 **Discussion**

271 **Different hypervariable regions of the 16S rRNA show a differential bacterial identification**

272 **of the stomach microbiome of *A. mexicanus***

273 Next generation sequencing techniques have revolutionized the way of studying microbial

274 diversity. However, inherent biases arise due to insufficient coverage of primers (Hong et al.

275 2009; Ghyselinck et al. 2013), primer-template mismatches (Sipos et al. 2007), and unequal

276 amplification (Polz & Cavanaugh 1998), distorting the comprehensive view of complex bacterial

277 communities. The choice of the primer sets and covered regions can deeply affect the profile of

278 microbiome composition (Kumar et al. 2011; Soergel et al. 2012). Therefore, the use of multiple

279 primers targeted to amplify different hypervariable regions of the 16S rRNA gene is an

280 innovative strategy in metagenomic protocols that allows detecting a larger array of bacterial

281 groups and enabling a more accurate microbiome description (Milani et al. 2013; Barb et al.

282 2016).

283   Previous studies in fish microbiota only targeted one or two hypervariable regions

284 (typically V1-V3, V4, V6 or V8) (Rawls et al. 2006; Kim, Brunt & Austin 2007; Sun et al. 2009;

285 Sullam et al. 2012; Franchini et al. 2014; Liu et al. 2016). Here, we reported the first fish

286 microbiota characterization incorporating the sequencing of seven hypervariable regions of the

287 16S rRNA gene (V2, V3, V4, V6-7, V8, and V9) using the Ion 16S ™ Metagenomics Kit. This

288 methodology has been recently reported for other models (Barb et al. 2016; Cornejo-Granados et

289 al. 2017; Sperling et al. 2017), suggesting that more than one hypervariable region is needed to

290 provide reliable diversity inferences (Barb et al. 2016).

291 In this study, the V3, V4 and V8 regions were the most accurate both for number of assigned

292 reads per sample and for the OTUs distribution, while the other regions seem to underestimate

293 the bacterial diversity. Among the hypervariable regions of the 16S rRNA gene, V3 and V4 are

294 the less biased and the most used for microbiome studies (Cai et al. 2013; Milani et al. 2013).

295 Additionally, studies using the Ion 16S ™ Metagenomics Kit showed differences in the

296 microbiome diversity depending on the region analyzed, recovering similarly to our results that

297 the regions V3, V4 and V8 were the most informative-less biased regions (Cornejo-Granados et

298 al. 2017; Sperling et al. 2017). Our results in the stomach microbiome characterization of *A.*

299 *mexicanus*, as previous studies, showed the benefits of using different non-overlapping regions,

300 and the potential biases of single marker identification in the microbiome characterization, which

301 could affect not only the richness but also diversity in microbiome studies.

302

303 ***Environmental factors influencing microbiota diversity of A. mexicanus***

304 Vertebrate microbiome is a complex microbial ecosystem containing diverse and abundant

305 bacteria, archaea and fungi, whose structure can be influenced by several factors, such as host

306 genetics, type of environment, trophic level, fish density, physicochemical conditions and

307 seasonality (Sullam et al. 2012; Liu et al. 2016).

308 In this context, we compared stomach microbiome from surface and cave-dwelling *A.*

309 *mexicanus,* to evaluate the potential response of stomach microbiota under contrasting

310 environmental conditions. Unexpectedly, the results show that patterns of microbial diversity are

311 more related to local environmental conditions rather than to the contrasting nature of habitats

312   (cave *vs*. surface). That is, water DO concentration seems to play a major role in the stomach

313   microbiome diversity.

314   Emerging research suggests that the core microbiota in *A. mexicanus* is related to

315   environmental water and sediment bacteria, represented by Bacteroidetes, Proteobacteria,

316   Firmicutes and Actinobacteria (Zwart et al. 2002; Austin et al. 2012; Liu et al. 2016), which are

317   abundant in microbiota of other vertebrates as well (Franchini et al. 2014; Sevellec et al. 2014;

318   Liu et al. 2016; Xiong et al. 2018). This is interesting because these taxonomic groups are shared

319   across a wide diversity of marine and freshwater fish, suggesting the existence of a core

320   microbiota shared between a broad range of species (Franchini et al. 2014; Sevellec et al. 2014;

321   Liu et al. 2016).

322   Previous studies have suggested a correlation between fish microbiome and evolutionary

323   history (Ley, Peterson & Gordon 2006), diet (Liu et al. 2016), and/or environment (e.g. salinity,

324   (Sullam et al. 2012). Thus, even when environment can affect the ecology and population

325   dynamics of microorganisms (Babich, Stotzky & Ehrlich 1980), the effect of abiotic factors

326   remains understudied in fishes' microbiota (Sullam et al. 2012). We did not find evidence that

327   contrasting conditions such as those represented by cave and surface environments have a

328   significant effect on the stomach microbiota diversity of *A. mexicanus*, suggesting that other

329   factors could be the main determinants in the bacterial consortia in this study system. Previous

330   studies have reported the lack of differentiation of microbiota of fish species inhabiting different

331   habitats (freshwater *vs*. saltwater), finding a significant interaction between trophic level and

332   habitat conditions with the gut microbiome diversity (Sullam et al. 2012). Previous studies have

333   also found evidence that water quality, temperature, salinity and seasonality are among the

334   abiotic conditions affecting fish microbiome diversity (Cahill 1990).

335

336   ***Commensals and pathogens in A. mexicanus microbiome***

337   Even if major differences in stomach microbiota structure could be explained by DO

338   concentration, differences in richness and diversity across fish samples may reflect some

339   symbiotic interactions between the microbiome communities and nutrients assimilation from

340   host's diet, as reported in previous studies (e.g. (Liu et al. 2016)). It was suggested that cellulose-

341   degrading bacteria such as *Citrobacter, Leptotrichia, Bacillus* and *Enterobacter* are the dominant

342   groups in herbivorous fish, while *Clostridium* has been reported at higher abundances in

343   omnivorous and filter-feeding fishes. Protease-producing bacteria, such as *Cetobacterium* and

344   *Halomonas*, have been reported in fish with piscivorous habits (Liu et al. 2016). In this study,

345   fish samples from Micos river and Pachón cave showed a larger abundance of bacteria belonging

346   to *Vogesella*, *Acinetobacter*, *Cetobacterium* and *Citrobacter*, tentatively associated to cellulose-

347   degrading metabolism. Similar microbiome composition was reported for omnivorous fish (Liu

348   et al. 2016).

349   In the fish samples from Pachón cave *Citrobacter* was the OTU with the largest relative

350   abundance, which seems counter-intuitive regarding the lightless and vegetal-free environment

351   of their hosts. However, *Citrobacter* could play a role in polysaccharides metabolism, since

352   cellulose digestion in fish depends on exogenous cellulose activity (i.e. diet-dependent),

353   suggesting stomach microbiota could contribute to their digestion (Saha & Ray 1998).

354   Additionally, as a part of the core microbiome in fish samples from Pachón cave, we observed

355   the presence of *Klebsiella* genus, which is associated to tanniase metabolism (tannin acyl

356   hydrolase). Tannin is a substance found in different plants groups (Ray, Ghosh & Ringø 2012).

357   In this regard, vegetal material could be introduced to the Pachón cave during the rainy season,

358   corresponding to a resource for the fish nutrition, and which digestion could be microbiome-

359   dependent. Further analyses of the carbon and nitrogen metabolism could shed more light about

360   the trophic levels of these populations. Contrastingly, *Prevotella* was the most abundant genus in

361   the Rascón river population. Previous studies have reported *Prevotella* in the intestinal mucus of

362   the rainbow trout *Oncorhynchus mykiss* (Kim, Brunt & Austin 2007), and it has been linked to

363   plant-rich diets, as well as with chronic inflammatory conditions in other fish species (Ley,

364   Peterson & Gordon 2006).

365   In general terms, the microbiome diversity found in *Astyanax* surface and cave-adapted

366   fish was similar to that previously reported for another omnivorous fish (Liu et al. 2016), in

367   which stomach microbiome contributes to several enzymatic activities related to polysaccharides

368   digestion. In this respect, considering the omnivorous trophic habits of a closely-related species,

369   *Astyanax aeneus*, which has been shown to display a high capability to exploit a wide variety of

370   trophic resources (Ornelas-García et al. 2018), it is possible that a similar trophic strategy is

371   depicted by surface *A. mexicanus*. Several studies have documented trophic habits of the

372   troglobitic populations of *A. mexicanus*, with evidence of detritivore habits (Wilkens 1972;

373   Mitchell et al. 1977; Hüppop 1987). By contrast, a recent study evaluated in detail the food

374   regime in individuals from the Pachón cave, and found an ontogenetic change in trophic habits.

375   During post-larvae stage *Astyanax* fish are active predators of water fleas (Cladocera), copepods,

376   ostracods, isopods and other insects. In contrast, in the adult stage, the stomach content was

377   dominated by detritus (possibly bat guano), and preys as complete flies and beetles, which

378   tentatively were ingested alive (Espinasa et al. 2017). Further studies are required including

379   additional cave populations in order to better explore the diversity in stomach microbiome across

380   caves and surface populations, and its relevance for the host metabolism.

381   Finally, we recovered some groups of bacteria that have been reported as pathogenic or

382   opportunistic, which can cause a disease once the fish is exposed to stressful conditions. Among

383   the exclusive OTUs within Pachón cave population, we recovered the genera *Mycobacterium*,

384   *Rothia* (both Actinobacteria), and *Providencia* (Proteobacteria), all of them reported as

385   pathogenic bacteria in freshwater fish (Austin et al. 2012). Additionally, *Acinetobacter*,

386   *Aeromonas* and *Cetobacterium,* have been reported as dominant microbiota in the lake whitefish

387   (*Coregonus clupeaformis*) and several other species, in which a pathogenic role has been

388   suggested (Sevellec et al. 2014, and references within). Further studies are required to better

389   understand the contrasting dynamics of the microbiome between surface and cave-dwelling

390   *Astyanax* fish, as well as those opportunistic bacterial groups.

391

392   **Conclusions**

393   *Astyanax mexicanus* stomach microbiome resembles that observed in other fish groups, and

394   further that reported for other vertebrates, and is composed of Bacteroidetes, Proteobacteria,

395   Firmicutes and Actinobacteria. Although we did not recover differences in stomach microbiome

396   between contrasting habitats (caves vs surface), the relative abundance of the core OTUs at

397   genus level was highly contrasting among populations. We observed a consistent association

398   between stomach β-diversity and water DO concentration. Therefore, microbiota in *A. mexicanus*

399   could result from a combination between physicochemical water conditions and its diet. Further

400   investigation including other cave fish populations, and focus on seasonal variation, as well as

401   diet characterization, could shed light to our understanding of the microbiome-host symbiotic

402   interactions under contrasting environments.

403

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412

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598   lakes and rivers. *Aquatic microbial ecology* 28:141-155.
599
600

601 **Figures**

602 **Figure 1.** Map of the sampling localities at the Sierra Madre Oriental (Mexico): **1**) Pachón cave

603 (El Abra region), **2**) Micos river (at Otates locality) and **3**) Rascón river (at Rascón locality).

604 Map modified from Elliott (2016). Red lines correspond to the surface-dwelling populations.

605 Ciudad Valles is in the yellow polygon, while blue polygons correspond to dams or water

606 reservoirs.

607

608 **Figure 2.** Relative abundance of bacterial taxonomic groups (at the level of phylum, and class

609 for the Proteobacteria) from the fish samples. Symbol abbreviations: R, Rascón; M, Micos; P,

610 Pachón; M, Male; F, female.

611

612 **Figure 3.** Venn diagrams showing the numbers of shared and unique genus OTUs (at 97% of

613 similarity) among populations. Also shows the relative abundance of bacterial genera shared

614 among the three populations (core microbiome). Symbol abbreviations: R, Rascón; M, Micos; P,

615 Pachón.

616

617 **Figure 4.** NMDS (Non-metric Multidimensional Scaling) plot of the samples (see symbols)

618 based on Bray-Curtis distance matrix of genes OTUs from fish stomach microbiome.

619 Physicochemical properties were fitted onto the NMDS ordination and only significant variables

620 are shown (p < 0.1). Symbol abbreviations: RM1 = R, Rascón; M, Micos; P, Pachón; M, male; F,

621 female.

622

623 **Figure 5.** Heatmap of family OTUs with relative abundances >1% of the total reads. The

624 Manhattan dissimilarity matrix and Ward's hierarchical clustering algorithms were applied in

625 this analysis. The color key scale represents the number of sequences detected for each fish

626 sample from lower (red) to higher (blue) abundance. Rows indicate family OTUs and columns

627 indicate the different fish samples. Symbol abbreviations: R, Rascón; M, Micos; P, Pachón; M,

628 male; F, female.

629

630   **Supplementary material**

631

632   **Figure S1.** Rarefaction curves of observed species of 16S rDNA genes for the different fish

633   samples. Symbol abbreviations: R, Rascón; M, Micos; P, Pachón; M, male; F, female.

634

635   **Figure S2.** Venn diagrams showing the numbers of shared and unique genus OTUs (at 97% of

636   similarity) from stomach microbiome among fish samples from the same population: a) Rascón,

637   b) Micos, and c) Pachón. Symbol abbreviations: R, Rascón; M, Micos; P, Pachón; M, male; F,

638   female.

639

640   **Figure S3.** Distribution of bacterial taxonomic groups from mapped reads among the different

641   hypervariable regions of 16S rRNA for each fish sample. The number of reads for each region is

642   indicated in parentheses. Symbol abbreviations: R, Rascón; M, Micos; P, Pachón; M, male; F,

643   female.

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Table 1.

Alpha diversity indices of 16S rDNA gene sequences (at 97% of similarity) for the fish
samples.

1   Table 1. Alpha diversity indices of 16S rDNA gene sequences (at 97% of similarity) for the fish

2   samples.

Samples	No. reads	Observed genera	Chao1	Shannon	Simpson
Rascon ♂1	336,118	30	30	2.26	0.614
Rascon ♂2	353,478	53	53	3.64	0.884
Micos ♀1	273,178	30	30	3.13	0.809
Micos♀2	299,903	55	55	3.30	0.825
Pachon♂1	329,915	65	65	3.65	0.864
Pachon♀2	384,847	64	64	3.58	0.874

Table 2.

Relative abundance of dominant OTUs at genus level expressed as mean values of the percentage of total bacteria.

1 Table 2. Relative abundance of dominant OTUs at genus level expressed as mean values of the

2 percentage of total bacteria.

Population	OTUs (Genus level)	Abundance (Typical % of total bacteria)
Pachón	*Dolosigranulum* (F)	27.6
	Mycobacterium (A)	22.3
	Providencia (P)	17.8
	Enterococcus (F)	12.7
Micos	*Sarcina* (F)	21.3
	Legionella (P)	16.6
	Neorickettsia (P)	13.8
	Limnohabitans (P)	8.1
Rascón	*Faecalibacterium* (F)	13.3
	Rosevuria (F)	12.9
	Blattabacterium (B)	10.9
	Sutterella (P)	9.9

3 F= Firmicutes; P = Proteobacteria; A= Actinobacteria and B = Bacteroidetes.

Table 3.

Values of physicochemical parameters for the three localities and statistical analysis (p-values) of the non-metric multidimensional scaling (NMDS) for the two ordination scores.

1   Table 3. Values of physicochemical parameters for the three localities and statistical analysis (p-

2   values) of the non-metric multidimensional scaling (NMDS) for the two ordination scores.

	Rascón	Micos	Pachón	NMDS1	NMDS2	r2	Pr(>r)
pH	8.2	8.19	7.34	-0.710	-0.703	0.481	0.230
Temperature (ºC)	26.75	26.24	25.09	-0.867	-0.497	0.696	0.070
Conductivity (µS/cm)	580	226	478	-0.667	0.745	0.458	0.281
Salinity	0.28	0.11	0.23	-0.672	0.740	0.463	0.273
Dissolved oxygen (mg/L)	8.2	4.41	2.97	-0.991	-0.133	0.961	0.010 **
Total dissolved solid (ppt)	290	113	239	-0.667	0.745	0.458	0.281

3   Significance codes: **, 0.05.

4   *P* values based on 1000 permutations.

Figure 1

Figure 1.

Map of the sampling localities at the Sierra Madre Oriental (Mexico): 1) Pachón cave (El Abra region), 2) Micos river (at Otates locality) and 3) Rascón river (at Rascón locality). Map modified from Elliot (2016).
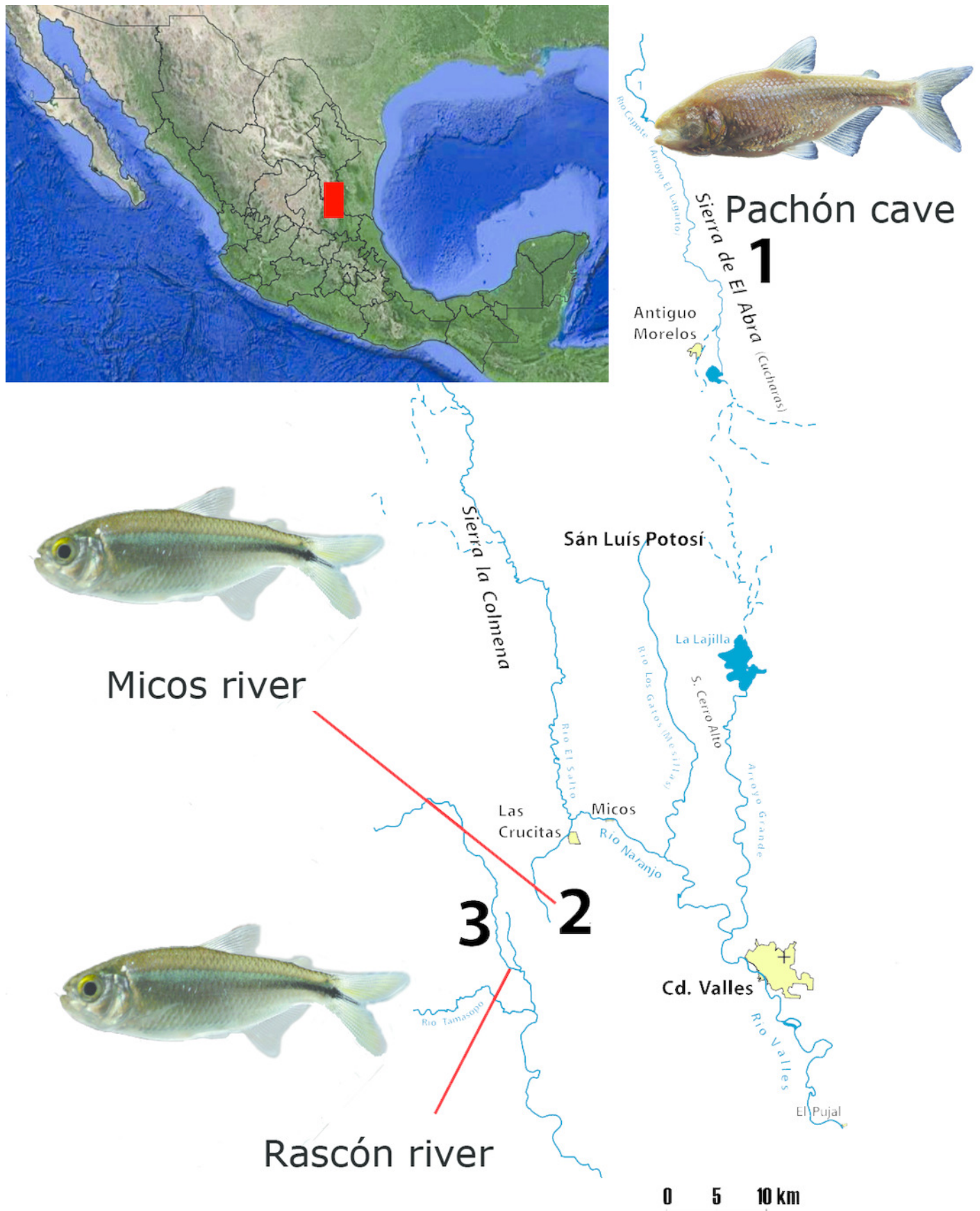
Figure 2

Figure 2.

Relative abundance of bacterial taxonomic groups (at the level of phylum, and class for the Proteobacteria) from the fish samples. Symbol abbreviations: R, Rascón; M, Micos; P, Pachón; M, Male; F, female.
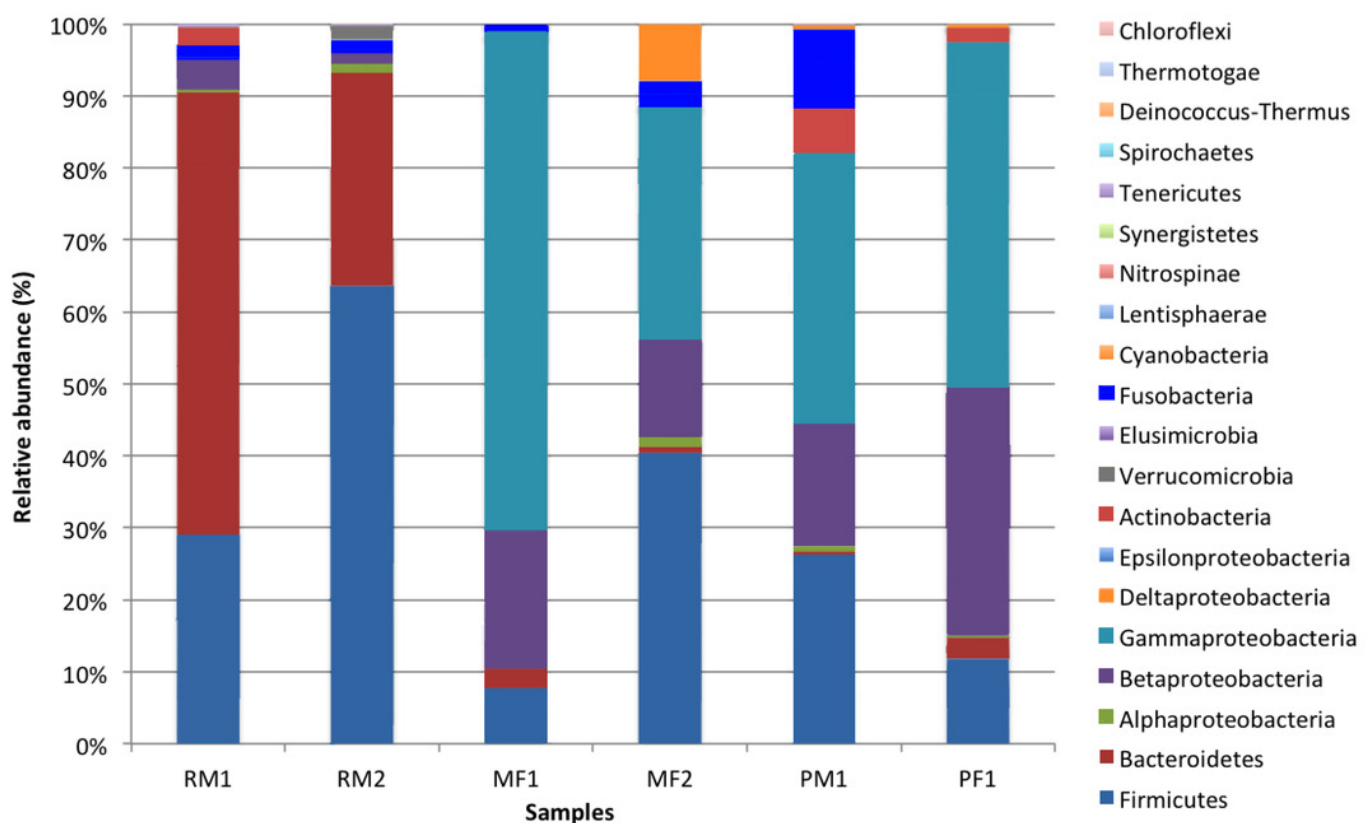
Figure 3

Figure 3.

Venn diagrams showing the numbers of shared and unique genus OTUs (at 97% of similarity) among populations. Also shows the relative abundance of bacterial genera shared among the three populations (core microbiome). Symbol abbreviations: R, Rascón; M, Micos; P, Pachón.
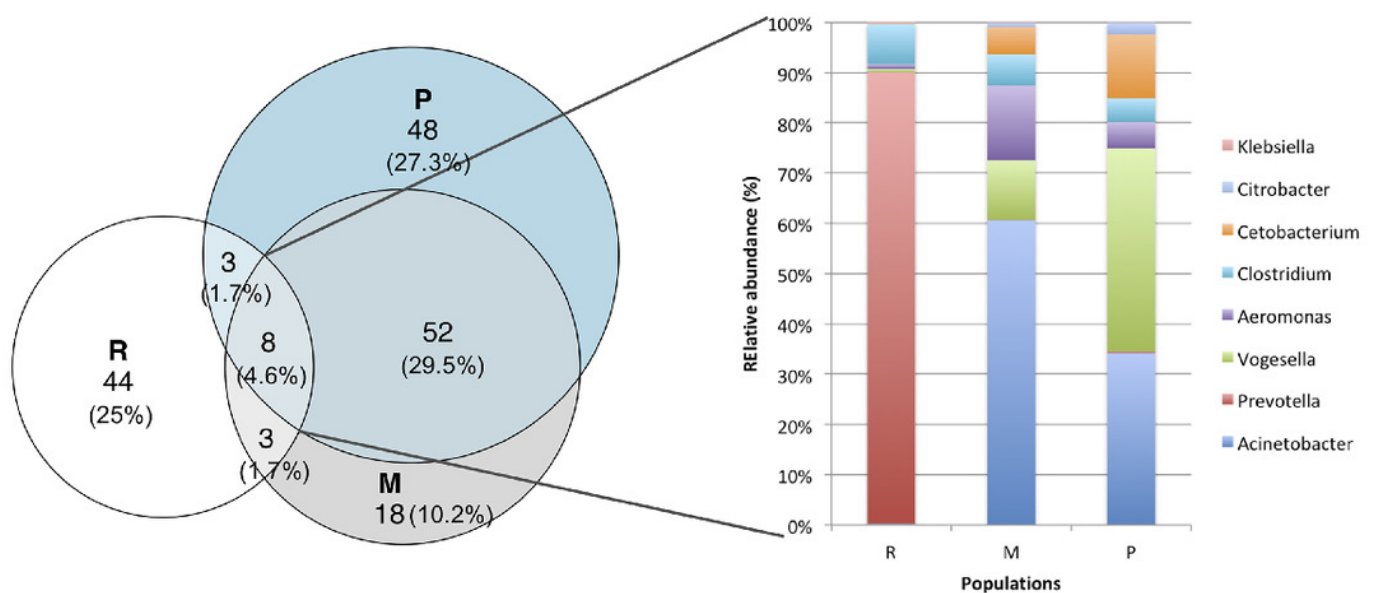
Figure 4

Figure 4.

NMDS (Non-metric Multidimensional Scaling) plot of the samples (see symbols) based on Bray-Curtis distance matrix of genes OTUs from fish stomach microbiome. Physicochemical properties were fitted onto the NMDS ordination and only significant variables are shown (p < 0.1). Symbol abbreviations: RM1 = R, Rascón; M, Micos; P, Pachón; M, male; F, female.
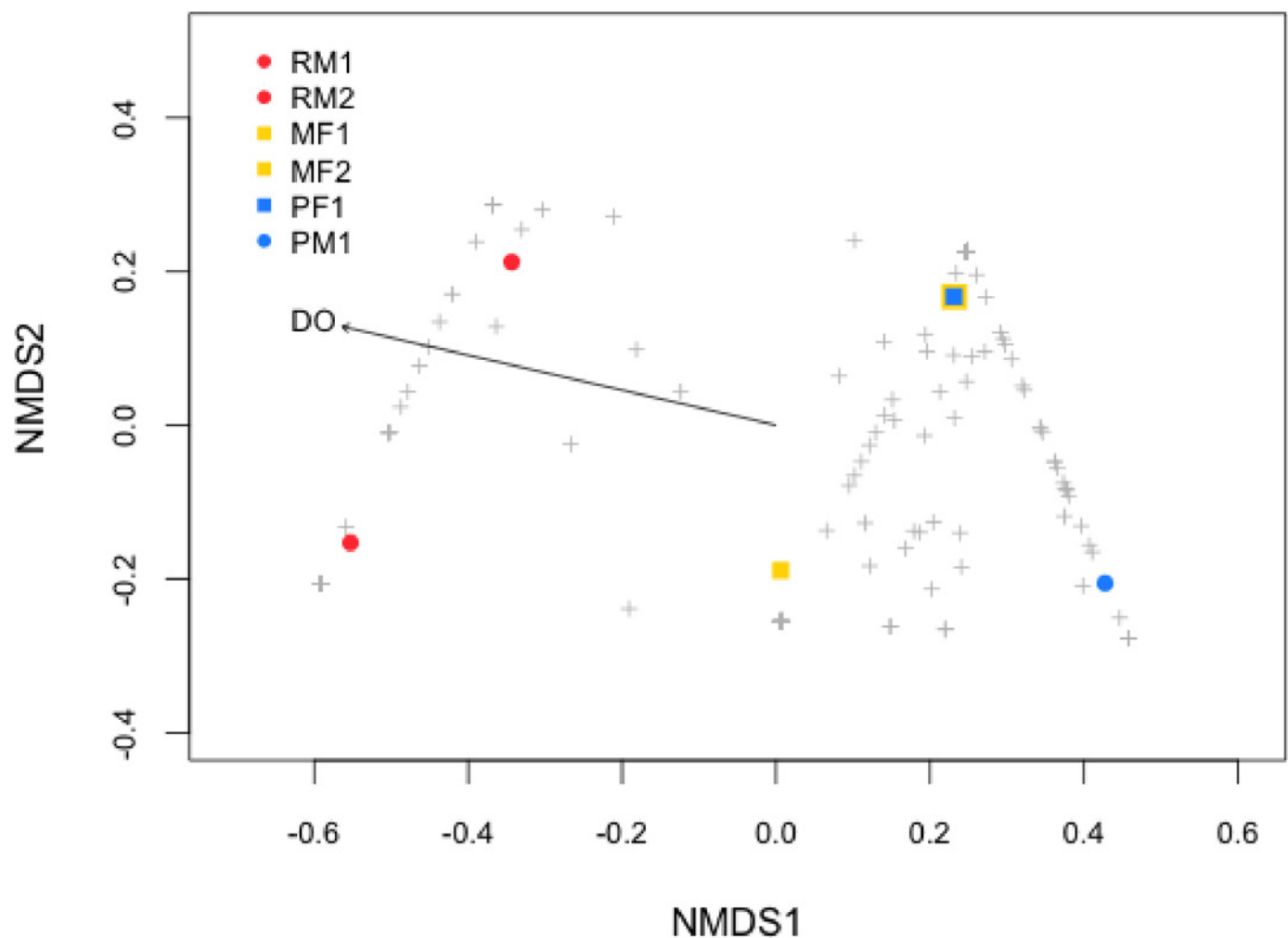
Figure 5

Figure 5.

Heatmap of family OTUs with relative abundances >1% of the total reads. The Manhattan dissimilarity matrix and Ward's hierarchical clustering algorithms were applied in this analysis. The color key scale represents the number of sequences detected for each fish sample from lower (red) to higher (blue) abundance. Rows indicate family OTUs and columns indicate the different fish samples. Symbol abbreviations: R, Rascón; M, Micos; P, Pachón; M, male; F, female.

Color Key

0   20000   40000   60000

Number of sequences

Moraxellaceae
Enterobacteriaceae
Prevotellaceae
Bacillaceae
Neisseriaceae
Lachnospiraceae
Ruminococcaceae
Clostridiaceae
Aeromonadaceae
Burkholderiaceae
Fusobacteriaceae
Comamonadaceae
unclassified_Burkholderiales
Peptostreptococcaceae
Porphyromonadaceae
Chromobacteriaceae
Chromatiaceae
Lactobacillaceae
Staphylococcaceae

RM2 RM1 PM1 MF2 MF1 PF1