GUT MICROBIOME ANALYSIS IN ADULT TROPICAL GARS (*Atractosteus tropicus*)

Roberto Méndez-Pérez[1†], Rodrigo García-López[3,4†], J. Santiago Bautista-López[1], Jorge F. Vázquez-Castellanos[3,4], Emyr S. Peña-Marín[5], Rafael Martínez-García[5], Verónica I. Domínguez-Rodríguez[2], Randy H. Adams-Schroeder[2], Eduardo Baltierra-Trejo[2,6], Carolina Melgar Valdés[7], Andrés Moya[3,4,✉], Carlos A. Alvarez-González[5], Rodolfo Gómez-Cruz[1,✉]

[†] Equal contribution

[1] Biotech & Environmental Genomics and [2]Biorremedation Laboratories. Academic Body Environmental Sciences (CACIAM). Research Center for Conservation and Sustainable Use of Tropical Resources (CICART). Biological Sciences Academic Division (DACBiol). Juárez Autonomous University of Tabasco State (UJAT), Villahermosa, Tabasco, Mexico. [3] Department of Genomics and Health. Valencian Region Foundation for the Promotion of Health and Biomedical Research (FISABIO), Valencia, Spain. [4] Integrative Systems Biology Institute (I2Sysbio), University of Valencia, Valencia, Spain. [5] Tropical Aquaculture Laboratory. CICART-DACBiol-UJAT, Villahermosa, Tabasco, Mexico. [6] CONACyT-UJAT. [7]General Aquaculture Laboratory. Multidisciplinary Academic Division of the Rivers (DAMRios)-UJAT. Tenosique, Tabasco, Mexico.

✉ Corresponding authors: Rodolfo Gómez – rodolfo.gomez@ujat.mx, Andrés Moya – moya_and@gva.es

Abstract. Tropical gar (*Atractosteus tropicus*), is freshwater and estuarine fish that has inhabited the Earth since the Mesozoic era, undergoing limited physiological variation ever since. This omnivorous fish is endemic to southern Mexico and part of Central America. Besides its recognized cultural and scientific relevance, the species has seen remarkable growth in its economic impact due to pisciculture. Previous studies have highlighted the role of microbial communities in fish, particularly those in the gut microbiome, in maintaining their host homeostasis or disease. In this study, we present the first report of the whole taxonomic composition of microbial communities in gut contents of adults' *A. tropicus*, by sex (female/male) and origin (wild/cultivated). Using culture-independent techniques, we extracted metagenomic DNA that was used for high throughput 16S rDNA profiling by amplifying the V4 – V5 hypervariable regions of the bacterial gene. A total of 364,735 total paired-end reads were obtained on an Illumina MiSeq sequencing platform, belonging to 508 identified genera, with the most and least abundant are *Cetobacterium*, *Edwardsiella*, *Serratia*, *Clostridium sensu stricto*, *Paludibacter* and *Campylobacter*, *Snodgrassella*, *Albirhodobacter*, *Lentilitoribacter*, respectively. We detected that, by sex and origin, Proteobacteria, Fusobacteria, Firmicutes and Bacteroidetes phyla are the core gut microbiome of the adults' *A. tropicus*. We discover the Deinococcus-Thermus phylum sequence, wildtype males only, with extremophile capacity in another freshwater fish. We also identified the species *Lactococcus lactis* strains CAU929 and CAU6600, Cp6 and CAU9951, *Cetobacterium* strain H69, *Aeromonas hydrophila* strains P5 and WR-5-3- 2, *Aeromonas sobria* strain CP DC28 and *Aeromonas hydrophila* with probiotic potential in aquaculture within the three dominant phyla, especially in wild-type organisms.

Keywords: *Atractosteus tropicus*, gut microbiome, metagenomics, 16S rRNA profiling

1. Introduction. Aquaculture produces 76.6 million tons of fish for human consumption and economically ensures the livelihood of 10 to 12 % of the world population (FAO 2014, 2017). It has become the fastest growing food sector in the world, with an average annual growth rate of 8.9 % since 1970 (Subasinghe 2005) and there is a growing global trend towards diversifying the spectrum of cultured aquatic species. Biological diversity in Latin America is one of the richest on

the planet, including an important variety of its freshwater ichthyofauna (Flores-Nava y Brown 2010). Some of the greatest challenges for aquaculture during this millennium have been the creation of integral studies on pisciculture-revellant endemic species and the development of technologies that may allow for controlled production of these fish in a profitable, innocuous and environmentally-conscious approach (Márquez-Couturier y Vázquez-Navarrete 2015). Studies in other fish have explored the bacterial populations in varying habitats such as the skin, gills, eggs and gut emicrobiome (GMB) and the way they finfluence the host's general health and physiology (MacFarlane *et al*. 1986, Cahill 1990, Ringø & Tabachek 1995, Givens 2012, Austin & Austin 2016). They have reported a large variation of microbiota among the different niches and between species with the GMB as one of the most studied due to its high microorganism concentration. Of interest for quaculture aquaculture-relevant species are outbreaks of viral, bacterial and fungal infections as they may cause devastating economic losses worldwide due to poor environmental conditions in the farms, unbalanced feeding, generation of toxins and genetic factors (Martínez-Cruz *et al.* 2012).

Tropical gar (*Atractosteus tropicus*, also known as pejelagarto) is freshwater and brackish fish species of the Lepisosteidae family, which has an ample fossil record since the Cretaceous period of the Mezosoic Era (Wiley 1976, Reséndez-Medina & Salvadores-Baledón 1983). The morphology of these species has remained mostly unaltered, with current specimens having lengths between 1.0 and 1.2 m and weighing between 1000 and 3000 g in the wild. In their natural habitat, this species exists in coastal wetlands of the tropical rainy areas of southeastern Mexico, Belize, Guatemala, El Salvador, Honduras, Nicaragua and Costa Rica (Willey 1976, Bussing 1998, Miller *et al*. 2005, Nelson 2006). It is an omnivore, feeding on other fish, decomposing organic matter, crustaceans, plants, etc., depending on the availability, preferring carnivorous habits. This specially secluded species has seen a drastic decrease in wild populations, caused by anthropogenic activities that have led to the loss of habitats and severe ecological alterations (Méndez-Marín *et al*. 2012). Currently, *A. tropicus* is cultivated in fish farms for human consumption in Mexico (Márquez *et al*. 2015).

, The gastrointestinal tract of A. tropicus, is formed by the buccopharyngeal, esophagus, stomach, gut, pyloric blind, rectum and anus, which is rapidly developing during the larval period (Frías-Quintana *et al*. 2015). Having absorbed and secretory function, its intestine is a long tube, narrower than the stomach with an inner epithelial mucosa that forms long, and numerous folds comprised of high columnar cells with clearly defined brush border (Márquez-Couturier *et al*. 2006). Having absorbed and secretory function, its intestine is a long tube, narrower than the stomach with an inner epithelial mucosa that forms long, and numerous folds comprised of high columnar cells with clearly defined brush border (Márquez-Couturier *et al*. 2006). In the anterior and middle regions, long folds divide these regions into a series of compartments called a spiral valve. The shorter posterior region forms the rectum, which opens in the anus. The external muscle in the intestine is thinner than in the stomach. Early juveniles 20 days after hatching show a GMB like that of adults. toBefore colonization, microorganisms may access the GMB through food and water intake during the larval phase (Núñez de la Rosa 2011). The high abundance of certain groups of gastrointestinal bacteria in fish, when compared to the microbial composition of the surrounding water suggests that GMB poses as a unique niche for a specific, but diverse group of bacteria (Cahill 1990, Givens 2012). It has been reported that GMB fish has between 10^7 and 10^{11} bacteria per gram of feces (Nayak 2010). GMB fish plays an important role and directly influences the host's nutrition and general homeostasis. Normal GMB fish may contain both beneficial and potentially pathogenic bacteria. The loss of the microbiota equilibrium (dysbiosis) has been reported to impact the host's physiological state, potentially compromising immunity, growth, general development as well as

the overall quality of the aquaculture production due to an increase in fish morbidity and mortality (Núñez de la Rosa 2011, Al-Harbi and Udding, 2005).

Prior to 2005, microbial studies on fish relied exclusively on culture techniques for enumerating and identifying bacteria (Newman *et al*. 1972, MacFarlane *et al*. 1986, Spanggaard *et al*. 2000, Aschfalk & Müller 2002, Verner-Jeffreys *et al*. 2003, Al-Harbi & Uddin 2004, Martin-Antonio *et al*. 2007, Skrodenyté 2007), providing valuable information as isolates provide a suitable framework for studying individual culturable strains on a finer level but limited appreciation of the whole spectrum in microbial communities. In 2007, Izvekova and collaborators reviewed GMB fish studies published between 1929 and 2006 reporting 73 different bacteria. This is because culturable bacteria typically account for less than 1 % of the cells that are present by direct microscopic enumeration (Ferguson *et al*. 1984, Head *et al*. 1998).

The advent of culture-independent metagenomic studies during the mid-2000s enabled the simultaneous analysis of complex genomic information contained in a hundreds of microbial species in a single niche (Nielsen *et al*. 2014). These techniques circumvent most culturing requirements of microorganisms, avoiding collection and sampling biases, effectively representing the actual diversity of a microbial community. Under intensive production conditions for sustainable aquaculture, aquatic species are subjected to high-stress conditions, leading to an increased incidence of diseases that decrease productivity (Bondad *et al*. 2005). It has been proposed that microbiota dysbiosis may be avoided through the regulation of their microbiota (Verschuere *et al*. 2000).

Microbial studies in aquaculture focus on the understanding of the symbiotic or antagonistic interactions between microbes and their eukaryotic hosts such as fish, crustaceans and molluscs. In this sense, metagenomics can provide a deeper understanding of these relationships through information revealed by sequencing microbial DNA extracted from specific niches within host organisms and, in the case of 16S profiling, taxa are representative of the medium (Suttle 2007, Gianoulis *et al*. 2009). This latter consists of surveying the 16S rRNA gene of all present microorganisms as this marker is found in all prokaryotes with enough mutations to discern each taxon. Formerly, some bacteria had been difficult to isolate because some of them are obligate intracellular microorganisms that could only be cultured in semi-aqueous and/or cellular culture media (Avila-Villa *et al*. 2011). Current sequencing platforms and bioinformatic tools enable the research on the diversity of intracellular bacteria, but also to ignore culture other culture requirements altogether as the DNA from the whole community are sequenced as is, with majority and accessory species, in order to elucidate the relevant community configurations for the improvement of aquaculture techniques. Consequently, the objective of this research was to explore the bacterial composition in the gut of adults' *A. tropicus*, by analyzing the 16S rRNA gene profiles from adult male and female organisms, cultivated and wild, for biotechnological-relevant applications future.

2. Materials and methods

Specimen collection. Fifteen totals live adults' *A. tropicus* were collected for the study, 7 of them were cultivated in the Tropical Aquaculture Laboratory, Research Center for Conservation and Sustainable Use of Tropical Resources (CICART) at Biological Sciences Academic Division (DACBiol), Juárez Autonomous University of Tabasco State (UJAT), Mexico, and 8 wildtype, with an average weight and length of 5 kg and 1 m, respectively. This specimens were provided by fishermen of the municipalities of Nacajuca (18°14′50″N 92°49′58″O; n female, m male) and Centla (18°20′00″N 92°30′00″O; n female, m male), Tabasco, Mexico (Figure 1). All specimens were cut

lengthwise under sterile conditions to remove the intestine for extracting its contents with scissors disinfected in absolute ethyl alcohol, for storage at -20 °C in a 2 mL Eppendorf tubes.

Samples collection. All organisms were sacrificed according to the protocol was approved by Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (NOM-062-ZOO-1999) on 18 June 2001 by percussion stunning method, consisting of striking a quick blow on the head of the fish manually with a bat, to obtain fresh samples, squeezing the GMB and obtaining the feces separately, considering the geographical origin and sex by sterile conditions.

DNA extraction, pyrosequencing and sequence for metagenomic analysis. 200 mg of fresh feces were used and 2 mL were enough to ensure the target DNA with a capacity of the Eppendorf tubes selected. A whole genomic DNA (gDNA) extraction was carried out for each sample with a QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. DNA integrity and concentration was evaluated by electrophoresis submerged with 1.2 % agarose gel and by spectrophotometry with a GenovaNano spectrophotometer (Jenway, Stone, Staffs, UK), respectively. Universal primers 533F (5'-GTGCCAGCAGCCGCGGTAA-3') (Weisburg 1991) and 909R (5'-CCCCGYCAATTCMTTTRAGT-3') (Tamaki *et al*. 2011) were selected for the amplification a region including the V4 and V5 hypervariable region in the16S rRNA gene. PCR amplifications were carried out using Phusion High-Fidelity DNA Polymerase (Finnzymes OY, Espoo, Finland; Klindworth *et al*. 2013) and conditions were as follows: Library preparation and high throughput sequencing was performed through Research & Testing Labs (Lubbock, Texas, USA) services, with an Illumina MiSeq platform, using 16S rDNA profiling by amplifying the V4 – V5 hypervariable regions of the bacterial gene, reagents sequencing for 2x 300 bp paired-end reads.

Bioinformatic analysis. The sequences were subjected to the standard quality protocols, which included the sequencing adapters removal with the Cutadapt tool (1.18), the too short and low-quality sequences filtering based on the Phred quality score implemented in the PRINSEQ tool.lite (0.20.4). The paired-end were concatenated with -fastq_join command of the USEARCH program (V.11) and the chimeras were removed with - uchime2_ref command of the same program, using as reference the Gold database (microbiomeutil-r20110519). The sequences were clustered in OTUs with 97 % identity by usearch61_ref algorithm, using the SILVA132 database as a reference through the pick_otus.py command in QIIME (1.9) and OTUs were filtered with 3 or fewer sequences.

Alpha diversity was measured through the Chao1, Shannon and P. D. (Phylogenetic Diversity) diversity indexes, which were calculated by alpha_rarefaction.py command in QIIME (1.9). The Alpha diversity among samples and origin of the samples (wild and farmed) were compared using Kruskal-Wallis statistic (Multiple range test, Bonferroni post-hoc) and Mann-Whitney W statistic (Wilcoxon), respectively, both implemented in the ggpubr library of R software (V.3.4.4).

Beta Diversity was analyzed using a PCoA constructed from the Unweighted UniFrac distance, calculated with the beta_diversity_through_plots.py command from QIIME (1.9), the difference among groups was evaluated by ANOSIM statistical test, and the main coordinates of the PCoA were graphed in the factoextra library in R (V.3.4.4) and Differential Analysis of Abundance was compared the abundance patterns of the taxa between the groups of samples through LEfSe (Linear discriminant analysis effect size).

Phylogenetic analysis. A sequences base was constructed according to certain genera with probiotic potential in fish that we find in the literature sought (Table 1), 16S rRNA gene sequences were obtained from the NCBI GenBank database. The 6,266 sequences obtained were clustered to eliminate identical sequences and generate a table of OTUs with 97 % similarity, using the

usearch61 algorithm of QIIME (1.9). The database was indexed (Query Sequence) to be used in a local blast with the BLAST + tool (NCBI). The local blast was performed using the sequences of the samples (Subject sequences) clustered at 97 % as mentioned in the section on the processing of the sequences. The identity percentage was adjusted to 99 % (-perc_identity) and a coverage of 70 % (-qcov_hsp_perc 70). For phylogenetic analysis, the blast resulting Query and Subject sequences were subjected to a multiple alignment with the ClustalW algorithm, in MEGAX (Molecular Evolutionary Genetics Analysis), and the phylogenetic relationships were inferred by the Neighbor-joining method in MEGAx, with the predefined settings.

3. Results. The pyrosequencing method of the Illumina MiSeq platform was evaluated that uses the Research & Testing Labs (Lubbock, Texas, USA) services, applying 16S rRNA in V4 – V5 hypervariable regions. A total of 364,735 sequencing reads were generated, organized by sex and origin, according to Table 2. We can visualize in Table 2 that readings obtained of wildtype male organisms are far greater than of wildtype and cultivated females, and cultivated males. Sequences were clustered into 16,503 different OTUs (97 % identity), which were classified into 11 phyla, 22 class, 37 order, 86 families and 179 genera. Table 2 shows that about twice as many reads correspond to wild male.

Microbiota composition. The cultivated organisms, wildtype females and males' samples are dominated by the *Fusobacteria* (37.32 %), *Proteobacteria* (30.49 %) and *Firmicutes* (18.55 %) phyla, respectively (Figure 2a). In wildtype females the most abundant phyla were Proteobacteria (0.70 ± 0.28 SD), Firmicutes (0.38 ± 0.32 SD) and Fusobacteria (0.24 ± 0.29 SD) and in cultivated females are Proteobacteria (0.64 ± 0.20 SD), Fusobacteria (0.54 ± 0.25 SD) and Bacteroidetes (0.45 ± 0.006 SD). In wildtype and cultived males, the most abundant phyla are Fusobacteria (0.63 ± 0.39 SD), Proteobacteria (0.40 ± 0.26 SD) and Fusobacteria (0.76 ± 0.36 SD), Proteobacteria (0.19 ± 0.21 SD), respectively. Actinobacteria (0.16 ± 0.17 SD) and Deinococcus-thermus (0.003 ± 0.006 SD) were identified only in wildtype females and wildtype males, respectively. The same tendency is observed in male and female specimens, since the dominant phyla in both wild type and cultivated ones are Fusobacteria (0.63 ± 0.39 SD), Proteobacteria (0.70 ± 0.28 SD) and Fusobacteria (0.76 ± 0.36 SD) and Proteobacteria (0.64 ± 0.20 SD), respectively (Figure 2a). At the genus level, the most abundant in wildtype females are *Edwardsiella* (0.32 ± 0.53 SD) and *Cetobacterium* (0.24 ± 0.29 SD), and in cultivated females are *Serratia* (0.57 ± 0.17 SD) and *Cetobacterium* (0.54 ± 0.25 SD). In wildtype males, the most abundant genera are *Cetobacterium* (0.63 ± 0.39 SD) and *Clostridium sensu stricto* (0.32 ± 0.49 SD) in cultivated males *Cetobacterium* (0.75 ± 0.29 SD) and *Edwardsiella* (0.22 ± 0.30 SD) (Figure 2b). In the case *Cetobacterium* (37.34 %) and *Edwardsiella* (10.55 %) genera are the most abundant in cultivated organisms and wildtype females' samples, respectively (Figure 2b); whereas *Clostridium sensu stricto* (13.53 %) was more abundant in wildtype males' samples. The cultivated and wildtype organisms' samples are dominated by the Fusobacteria (38.95 %) and Firmicutes (19.57 %) phyla, respectively (Figure 2a). For the *Cetobacterium* (38.95 %) and *Clostridium sensu stricto* (15.09 %) genera they are more abundant in the cultured organisms and samples of wildtype organisms' samples, respectively (Figure 2b).

Likewise, the core microbiome composition at phylum and genus taxonomic levels, on a per-sample basis of *adults' A. tropicus* gut (figure 3), shows that the most abundant phyla per sample were Fusobacteria (47.01 %), Proteobacteria (29.02 %) and Firmicutes (12.90 %) (Figure 3a), while the most abundant genera were *Cetobacterium* (47.01 %), *Edwardsiella* (12.00 %) and *Serratia* (10.55 %) (Figure 3b). Although the primers are designed only to 16S rRNA gene amplify of bacteria, OTUs belonging to the archaea domain were also identified, represented by the genera *Salinirubrum*, *Salinigranum* and *Methanoculleus*, which together represent about 0.01 % of the total abundance.

Alpha diversity. In this work, diversity, dominance and richness were evaluated using the Shannon index about the total number of OTUs. Microbiota composition is influenced by source and sex

factors. The Shannon index indicates that the microbial types in adults' *A. tropicus* are more evenly distributed and thus may be more diverse in wild (5.32 ± 0.13 SD) and cultivated (3.75 ± 0.09 SD) female individuals than in the wild (3.14 ± 0.07 SD) and cultivated (3.38 ± 0.08 SD) male individuals (Table 3). We found significant differences in diversity indices and only some samples were not significant. The wild and cultivated females turned out to be the most diverse, but by origin, only the wild samples were the most diverse. In a total of 50 taxa, significant differences can be observed between cultivated and wild conditions.

Beta diversity. Bacterial communities beta diversity associated to TGI of adults' *A. tropicus* in origin conditions were measured through the ordination analysis from Principal Coordinates Analysis (PCoA), using Unweighted unifrac distances (UniFrac). The analysis produced an ordination of the dissimilarities, where similar individuals are close to one another and dissimilar ones are more distant (Figure 4). All ordination analysis showed a clear separation between wild and cultivated individuals samples. ANOSIM statistic (R: 0.60933; p-value <0.002) found significant statistical differences by origin, between the cultivated and wild groups (Figure 4).

Significant microbial components associated with the origin and sex. 8 genera were identified in wild organisms and these are *Methanoculleus, Flavobacterium, Psychrobacter, Acinetobacter, Pseudomonas, Paracoccus, Massilia* and *Shewanella.* Likewise, 13 genera were identified in cultivated organisms, which are *Paludibacter, Intestinibacter, Cellulosilyticum, Odoribacter, Turicibacter, Defluviitalea, Vallitalea, Acetivibrio, Terrisporobacter, Bacteroides, Acidaminobacter, Sporacetigenium* and *Macellibacteroides.*

Specific taxa distributed differentially between wild and cultivated organisms were identified by LEfSe tool. This allows to obtain statistical differences per each taxon, where linear discriminant analysis (LDA) score barplot is shown for both conditions, cultivated and wild organisms (female & male; figure 5a), cultivated and wild organisms (female; figure 5b), male organisms, cultivated & wild (Figure 6a), cultivated female organisms (Figure 6b) and wild female organisms (Figure 6c), representing each phylum, class, order, family and genus by a histogram.

*Phylogenetic reconstruction analysis***.** The sequences-base of rRNA 16S gene constructed of some organisms found in the literature with probiotic potential in fish, according to table 1, allowed us to find and identify possible organisms with probiotic potential in microbiome gut of adults' *A. tropicus*, adjusted to 99% identity and 70% coverage. In figure 7 we obtained a phylogenetic tree based on the identified sequences of the most abundant phyla per sample, such as Fusobacteria, Proteobacteria and Firmicutes. Besides, we *Methanosarcina thermophila* and *Archaeoglobus profundus* sequences data used thermophilic methanogens and sulfate-reducing archaea, respectively, as outgroups for the root identification, considering that they may be related only distantly with identified sequences in our work.

In phylogenetic tree reconstruction (Figure 7) we determined that the evolutionary conclusion of these relationships is that the two species of archaea or outgroups selected are ancestors of the nine species or ingroups identified with blue squares. Likewise, they belong to the three most dominant of phyla groups that integrate the core microbiome gut in adults' *A. tropicus*. On the other hand, these nine species, identified by blue squares, are those that have probiotic potential.

4. Discussion. We obtained the 16S rRNA Gene Amplicons preparation with high-throughput sequencing using Illumina MiSeq platform by Research & Testing Labs (Lubbock, Texas, USA) services, facilitating discovery and analysis of rare taxa and detection of previously unrecognized eukaryotic and prokaryotic microbiome (Paul *et al*. 2018). In this sense, we identified and compared, by indexes attachment of multiplexing samples and readings of sequencing products for 2x300 bp

paired-end, the core microbiome and microbial diversity, respectively, in the gut of adults' *A. tropicus* by origin and sex.

Previous studies have shown that the fish gut hosts an estimated 10^7 to 10^{11} bacteria per gram intestinal content (Nayak 2010) and the bacterial colonizers in fish gut include Proteobacteria, Fusobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Clostridia, Bacilli and Verrucomicrobia (Ringø *et al*. 2006, Desai *et al*. 2012, Li *et al*. 2013a,b, Carda-Diéguez *et al*. 2014, Ingerslev *et al*. 2014a,b) with the first 4 being the most abundant, depending on environmental conditions and the host's diet (Wang *et al*. 2017). Almost all Proteobacteria, Fusobacteria, Firmicutes, Bacteroidetes and Deinococcus-Thermus 16S rRNA sequences that we detect in CGMB of adults' *A. tropicus* belonged to the *Cetobacterium*, *Edwardsiella*, *Serrartia* and *Clostridium sensu stricto* genres. The observed bacterial profiles in the gut of adults' *A. tropicus*, by sex and origin, may reflect specific central microbiota, beyond the differences most likely attributable to feeding behavior. At the phylum level, almost 90 % of the total bacterial abundance only we were classified into total four phyla. Among these phyla, Proteobacteria and Fusobacteria were dominant in the fifteen fish species samples, female/male and cultivated/wild (Figs. 2, 3). The inferred physiological roles of the dominant prokaryotes are related to the metabolism of carbohydrates and nitrogenous compounds (Kormas *et al*. 2014). In contrast, dominant microbiota of marine fish is facultative anaerobes, including *Vibrio*, *Pseudomonas*, *Acinetobacter*, *Corynebacterium*, *Alteromonas*, *Flavobacterium* and *Micrococcus* (Onarheim *et al*. 1994, Blanch *et al*. 1997, Verner-Jeffreys *et al*. 2003).

Considering the above-mentioned, fish core gut microbiota (CGMB) can influence nutrition, growth, reproduction, general population dynamics and the host's vulnerability to diseases thus supporting a crucial role in aquaculture practice (Ghanbari *et al*. 2015). Current DNA sequencing technologies and bioinformatic analysis have contributed towards a deeper understanding of the complex microbial communities associated to diverse habitats, including CGMB of fish in response to a variety of factors affecting the host, including temperature variations, salinity, growth stage, digestive physiology and feeding strategy (Cahill 1990, Jammal *et al*. 2017). The concept 'core microbiota' referred to a set of abundant microbial lineages that are shared by all individuals from the same species (Wong *et al*. 2013). The concept of CGMB has been explored both in mammalian host's context and in freshwater fish (Turnbaugh *et al*. 2009, Roeselers *et al*. 2011, Nam *et al*. 2011, Wu *et al*. 2012, Wong *et al*. 2013).

On the other hand, Alpha diversity results indicate that the greatest gut microbiota abundance and richness is found in adults' *A. tropicus* wildtype female, rather than adult's wildtype males and the least gut microbiota abundance and richness is found in adults *A. tropicus*, female or male cultivated (Table 3). Ley *et al*. (2008) concluded that gut microflora of herbivorous mammals have the greatest richness and phylogenetic diversity and that both richness and phylogenetic diversity decreased among omnivores and decreased further among carnivores. We found the lowest richness and phylogenetic diversity (Table 3) in gut microbiomes from adults *A. tropicus* defined as top piscivores (carnivores; e.g., cultivated female and male *A. tropicus*). Indeed, MacFarlane *et al*. (1986) observed that farmraised fish had a simpler gut flora than their wild counterparts.

Likewise, our coefficients of similarity or dissimilarity of unweighted unifrac distances (UniFrac) by PCoA ordination analysis, indicate that gut microbiota of wild and cultivated adults *A. tropicus* are only similar by two wild samples and one cultivated and dissimilarity by most of the wild and cultivated samples (Figure 4). Principal coordinate analysis (PCoA) revealed that gut bacterial communities from adults' *A. tropicus* by origin formed different clusters. Cultivated and wild organisms formed distinctly clusters in PCoA space (Figure 4), suggesting that the enrichment and diversity of gut microbiota are affected by the origin. This result is similar to the research of Ni *et*

al. (2012) that the origin and host phylogeny they are quite related to the composition of adults' *A. tropicus* gut bacteria. Moreover, previous studies have shown that the microbiotic diversity content in all intestinal sections depends on the fish size (Moran *et al*. 2005, Bolnick *et al*. 2014, Clements *et al*. 2014).

Numerous works have built on this, demonstrating that many species of herbivorous and omnivorous fishes contain diverse intestinal communities (Rimmer & Wiebe 1987, Clements *et al*. 1989, Clements 1991, 1997, Martínez-Díaz & Pérez-Espana 1999, Ray *et al*. 2012) and that herbivorous and detritivorous fish species harbour distinctive microbial populations (Clements *et al*. 2014). Feeding habits are also an important factor that generally influences the microbial diversity in the fish CGMB, displaying a higher diversity in the following order: carnivores> omnivores> herbivores (Ward *et al*. 2009, Larsen *et al*. 2014, Li *et al*. 2014a,b, Miyake *et al*. 2015, Liu *et al*. 2016). He *et al*. (2013) study revealed that the herbivorous carp (*Ctenopharyngodon idellus*) reported a wider variety of bacterial species than the dark carnivorous carps and Gibel (*Carassius gibelio*) which are exclusively omnivorous, and also the sea bream, under the same culture conditions. This same tendency we identify in gut core microbiome of adults *A. tropicus* by sex and origin, because although these are omnivores, they prefer carnivorous habits. (Méndez-Marín *et al*. 2012, Figure 5a). At the sex level, we observed that core microbiome is more diverse in female organisms, and particularly in wild type organisms (5b, 6a,b)

More recent sequence-based approaches show that fish hindgut microbial communities much more closely resemble those of mammals than environmental microbial communities (Fidopiastis *et al*. 2006; Sullam *et al*. 2012), especially in the prevalence of Proteobacteria, Firmicutes and Bacteroidetes (Clements *et al*. 2007, Smriga *et al*. 2010, Sullam *et al*. 2012, Ye *et al*. 2014). These findings indicate that fish, like other vertebrates, harbour specialized gastrointestinal communities (Clements *et al*. 2014). We identified Proteobacteria as the most dominant phylum in gut core microbiome of the adults' *A. tropicus*. According to Rudi *et al*. (2018), Proteobacteria phylum is very characteristic in wildtype fish and denotes of a diet high in fat presence.

We identified Fusobacteria as the second dominant phylum in the gut of the male adults *A. tropicus*, wild and cultivated (Figure 2a). A few studies have shown Fusobacteria as dominant members of the gut microbiota of freshwater fishes (van Kessel *et al*. 2011, Di Maiuta *et al*. 2013). Fusobacteria is anaerobic, Gram-negative bacilli that produce butyrate (Bennett and Eley 1993), a short-chain fatty acid that is often the end product of the fermentation of carbohydrates including those found in mucins (Titus and Ahearn 1988, von Engelhardt *et al*. 1998). In mammals, butyrate provides many benefits to the host, including providing a majority of the energy supply to gastrointestinal cells (von Engelhardt *et al*. 1998, Collinder *et al*. 2003), enhancing mucus production, acting as an anti-carcinogen and anti-inflammatory, as well as playing a role in satiation (McBain *et al*. 1997, von Engelhardt *et al*. 1998, Andoh *et al*. 1999, Hamer *et al*. 2007). This fatty acid has been found in the gut of herbivorous and omnivorous fishes only (Clements *et al*. 1994, Clements and Choat 1995). Nuez-Ortin *et al*. (2012) demonstrated that the ability of butyric acid to inhibit potential freshwater fish pathogens, and sodium butyrate is currently sold as a food additive to promote fish health and growth. However, trials using blends of sodium butyrate and other additives have not proven beneficial (Owen *et al*. 2006, Gao *et al*. 2011).

OTUs sequences of the *Cetobacterium* genus were identified as dominant mainly in gut microbiome of the adults' *A. tropicus*, wild and cultivated male organisms. *Cetobacterium* genus is widely identified in freshwater and warm water fish species (Tsuchiya *et al*. 2007, Larsen *et al*. 2014, Li *et al*. 2017). *Cetobacterium* genus members' presence can perform fermentative metabolism of peptides and carbohydrates and produce vitamin B12 (cobalamin) (Larsen *et al*. 2014). *Cetobacterium* and

Bacteroides were reported as major producers of the vitamin B12 in the intestine (Tsuchiya *et al*. 2008, Vogiatzoglou *et al*. 2009) and they were the dominant genera in grass carp's intestine, with the abundance of more than 50 % (Li *et al*. 2015a). Animals, plants and fungi are incapable of cobalamin production and it is the only vitamin that is exclusively produced by microorganisms, particularly by anaerobes (Roth *et al*. 1996, Martens *et al*. 2002, Smith *et al*. 2007). Qi *et al*. (2017) showed that different concentrations of ammonia would affect the abundance of *Bacteroides* and *Cetobacterium* in gut fish, and the higher the concentration, the lower the abundance.

Despite we detected OTUs of Deinococcus-Thermus bacterial phyla non-dominant (0.003 ± 0.006 SD) in gut microbiome of the adults' *A. tropicus*, wildtype males only. However, Deinococcus-Thermus species are known for their resistance to extreme stresses, such as radiation, oxidation, desiccation and high temperature (Li *et al*. 2015b). The deeply branching Deinococcus-Thermus lineage is recognized as one of the most extremophilic phylum of bacteria (Theodorakopoulos *et al*. 2013). Sequence information from Deinococcus-Thermus phylum is presently available for only a limited number of species. However, the sequenced genomes include species from both the main families (i.e., *Deinococcaceae* and *Thermaceae*) within this phylum (Griffiths & Gupta 2007). In recent years, researchers have begun using Deinococcus spp in biotechnologies and bioremediation due to their specific ability to grow and express novel engineered functions. More recently, the sequencing of several Deinococcus spp and comparative genomic analysis have provided new insight into the potential of this genus. Features such as the accumulation of genes encoding cell cleaning systems that eliminate organic and inorganic cell toxic components are widespread among Deinococcus spp. Other features such as the ability to degrade and metabolize sugars and polymeric sugars make Deinococcus spp. an attractive alternative for use in industrial biotechnology (Gerber *et al*. 2015). That is why their functional role in the gut of the adults' *A. tropicus* deserves further research.

Bacteroidetes is a phylum composed of three large classes of Gram-negative, nonsporeforming, anaerobic or aerobic, and rod-shaped bacteria that are widely distributed in the environment, including in soil, sediments, and seawater, as well as in the guts and on the skin of animals (Ley *et al*. 2008), this was also present as the dominant phylum in adults *A. tropicus* female, wild and cultivated, usually; only this type of bacteria was detected as dominant in sample 4, cultivated male organism (Figure 2a). Likewise, a large part of the proteins synthesized by the genome of *Bacteroides*, a genus of Bacteroidetes, can break down polysaccharides and metabolize their sugars, playing a fundamental role in the degradation of complex molecules in the gut of the host. Their ability to harvest alternative energy sources from food could allow *Bacteroides* to be more competitive than other bacteria in CGMB of fish during starvation stage (Xu *et al*. 2003, Xia *et al*. 2014).

We identify *Clostridium sensu stricto* genus sequences in GMB of the adults' *A. tropicus*, wildtype males only. This genus has also been identified in GMB of carp fish (Li *et al*. 2015a). The members of the genera *Clostridium sensu stricto* that are dominant in the intestinal microbiota of grass carp (*Ctenopharyngodon idellus*), they are also versatile in their ability to utilise various polysaccharides, such as cellulose, xylan and hemicelluloses, which constitute the major part of vegetal fibres (Uffen 1997, Uz & Ogram 2006, Li *et al*. 2015a). Others also include not only species with saccharolytic and fiber-fermenting activities but also proteolytic species (Lubbs *et al*. 2009, Pikuta *et al*. 2009, Li *et al*. 2015a).

Serratia, *Edwardsiella*, *Plesiomonas* and *Reyranella* are genera belonging to Enterobacteriaceae family and Proteobacteria phylum. This isolated bacterial species are facultative pathogens for fish and humans and may be isolated from fish without apparent symptoms of the disease (Walczak *et al*.

2017). *Serratia* produces serrawettin that acts as a wetting agent to reduce the surface tension of the environment (Chan *et al*. 2013). *Edwardsiella* has been isolated from tortoises (Iveson 1971), crocodiles (Iveson 1971), aquarium water (Bartlett *et al*. 1977) and from seagull roosting areas (Berg & Anderson 1972). *Edwardsiella* was isolated on several occasions during the examination of dressed catfish for *Salmonella* (Wyatt *et al*. 1979). This report provides information on the isolation, identification, and incidence of *Edwardsiella* in freshwater catfish and their environment. There are many unanswered questions regarding their importance in freshwater fish. Liu *et al*. (2015) are reports of pathogenicity of *Plesiomonas shigelloides* to fish. *P. shigelloides* can occur as natural intestinal flora of fish, but in case of stress conditions, the following symptoms are observed: darkening of body, hemorrhaging, fin rotting, ascitic fluid in the abdominal cavity, and lesions in internal organs (Liu *et al*. 2015). Phylogenetically, *Reyranella* genus has an evolutionary lineage within the family Rhodospirillaceae in the class Alphaproteobacteria. The type species *Reyranella massiliensis* was originally identified by Pagnier *et al*. (2011). Subsequently, Kim *et al*. (2013) emended several characteristics (e.g., nitrate reduction, respiratory quinone information) into the genus description, and more recently, Cui *et al*. 2017 found that reduction of nitrate to nitrite is variable, the predominant isoprenoid quinone is ubiquinone-10 (Q-10), major polar lipids are PME, DPG, PG, PE and one unknown aminolipid. We detect these genera mainly in gut microbiome of the adults' *A. tropicus*, wildtype females and cultivated females and males organisms (Figures 2b, 3b). We assume that, although commonly known for their ability to cause deadly infectious diseases, this is populations of bacteria (identified as normal flora of the adults *A. tropicus*), which despite symbiotically living on and between wildtype females and cultivated females and males organisms of the adults' *A. tropicus*, really have a positive impact on host survival (Figures 2b, 3b).

Paludibacter genus was found exclusively in African microbiota. This is probably due to their increased fitness to grow on polysaccharides abundant in xylan or cellulose diets (De Filippo *et al*. 2010, Thomas *et al*. 2011). We identified OTUs of *Paludibacter* genus in core gut microbiome of the adults' *A. tropicus*, females and males cultivated organisms, only (Figure 2b).

In phylogenetic trees reconstruction (Figure 7) and according to Table 1, we identified the species *Lactococcus lactis* strains CAU929 and CAU6600, Cp6 and CAU9951, *Cetobacterium* strain H69, *Aeromonas hydrophila* strains P5 and WR-5-3- 2, *Aeromonas sobria* strain CP DC28 and *Aeromonas hydrophila* with probiotic potential within the three dominant phyla in core gut microbiome of the adults *A. tropicus*.

5. Conclusions. We conclude that adults' *A. tropicus* core gut microbiome is constituted by Proteobacteria, Fusobacteria, Firmicutes and Bacteroidetes phyla. Definitely, diversity and richness in core gut microbiome are higher of the female than males' organisms and wild than cultivated organisms of the adults' *A. tropicus*. We can also suppose that *Serratia*, *Edwardsiella*, *Plesiomonas* and *Reyranella* genera lives in symbiosis and has a positive impact on the survival of the adults' *A. tropicus*. There is great potential in the biotechnology industry with Deinococcus-Thermus phylum, due to its extremophile capacity. Further, adults' *A. tropicus* by sex and origin have been adapting to the environment and the diet due to their great capacity to saccharolytic, fiber fermentation and starvation activities. Finally, we can confirm that nine species can be found with the probiotic potential of 3 phyla that shape up the core gut microbiome of the adults *A. tropicus*. The CGMB of *A. tropicus* is increasingly regarded as an integral component of the host, due to important roles in the modulation of the immune system, the proliferation of the intestinal epithelium and the regulation of the dietary energy intake. Understanding the factors that influence the composition of these microbial communities is essential to health management, and the application to aquatic animals still requires basic investigation.

458 **Conflict of Interest**

459 The authors declare no conflict of interest.

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467 y juveniles de pejelagarto (*Atractosteus tropicus*) con base en técnicas histológicas, bioquímicas y
468 moleculares" CB-2016-01-282765 key.

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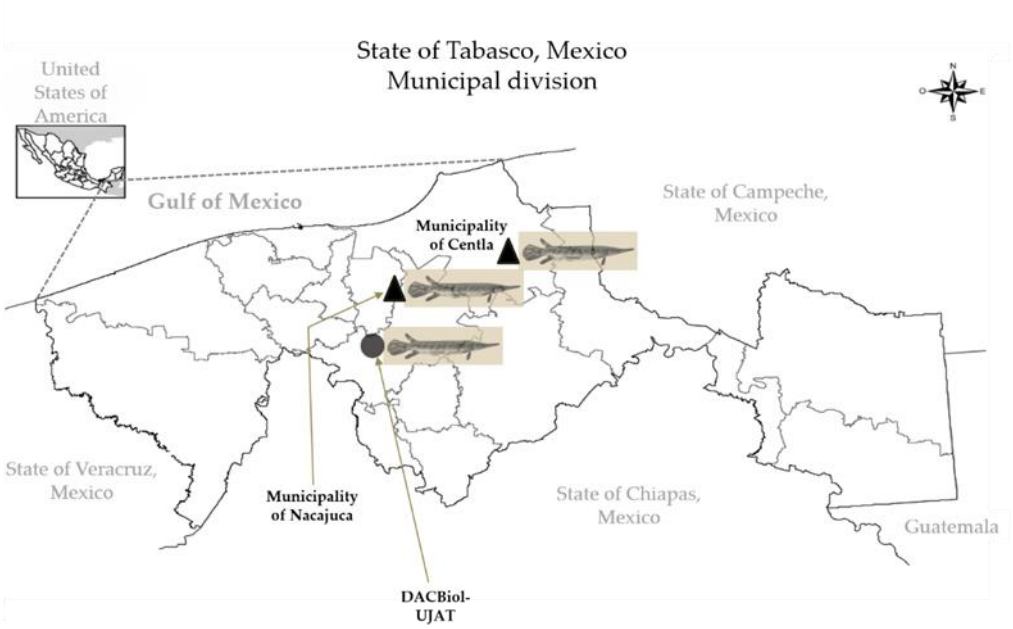

Figure 1. Collection sites of wild (triangle figures) and cultivated (circle figure) of the adults' tropical gars (*Atractosteus tropicus*)

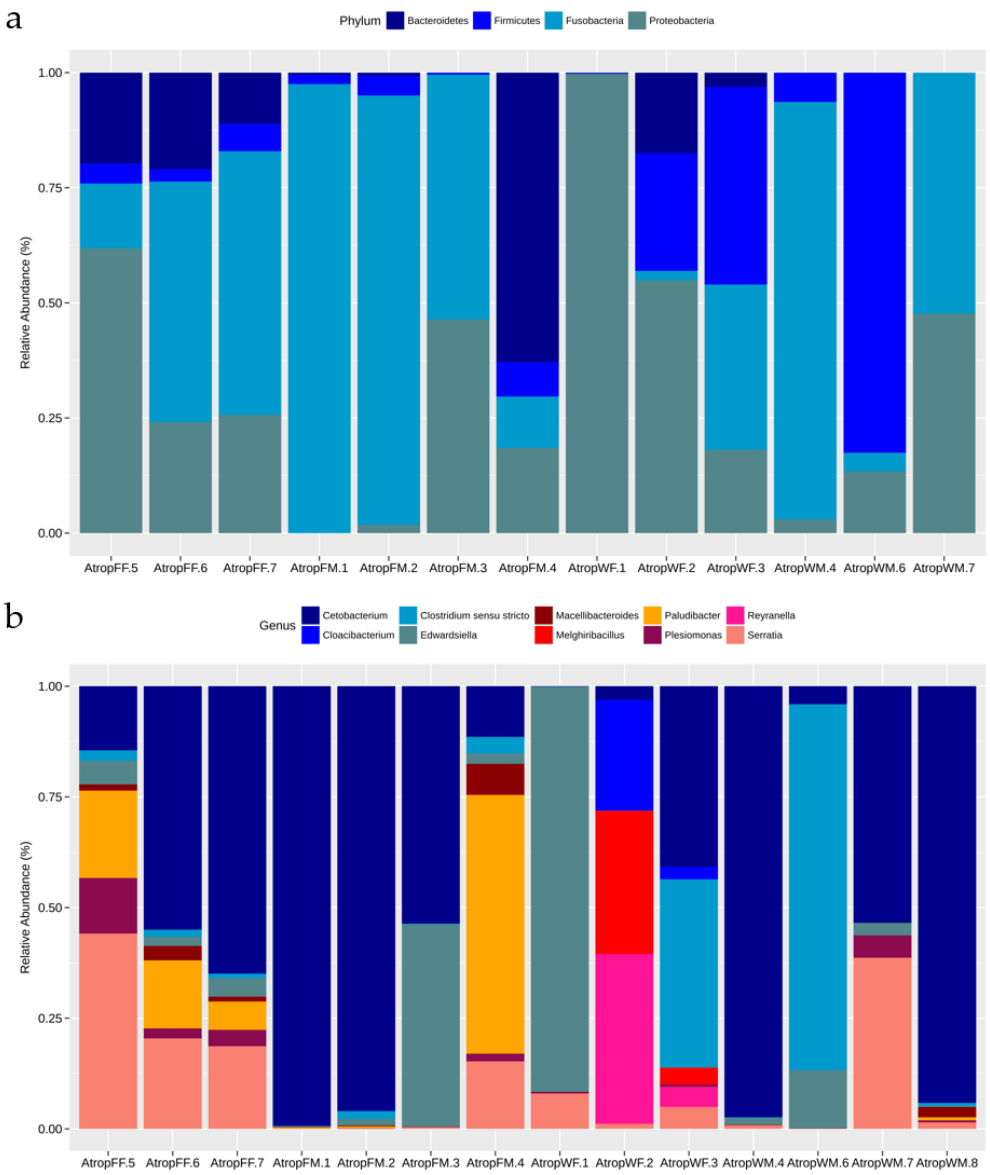

Figure 2. Taxonomic summary at different levels of relative bacterial abundance. (a) Phyla and (b) Genus. First letter W: wild or F: Farming individuals. Second letter sex M: male or F: female. Only taxa with relative abundance ≥ 1 % are shown.

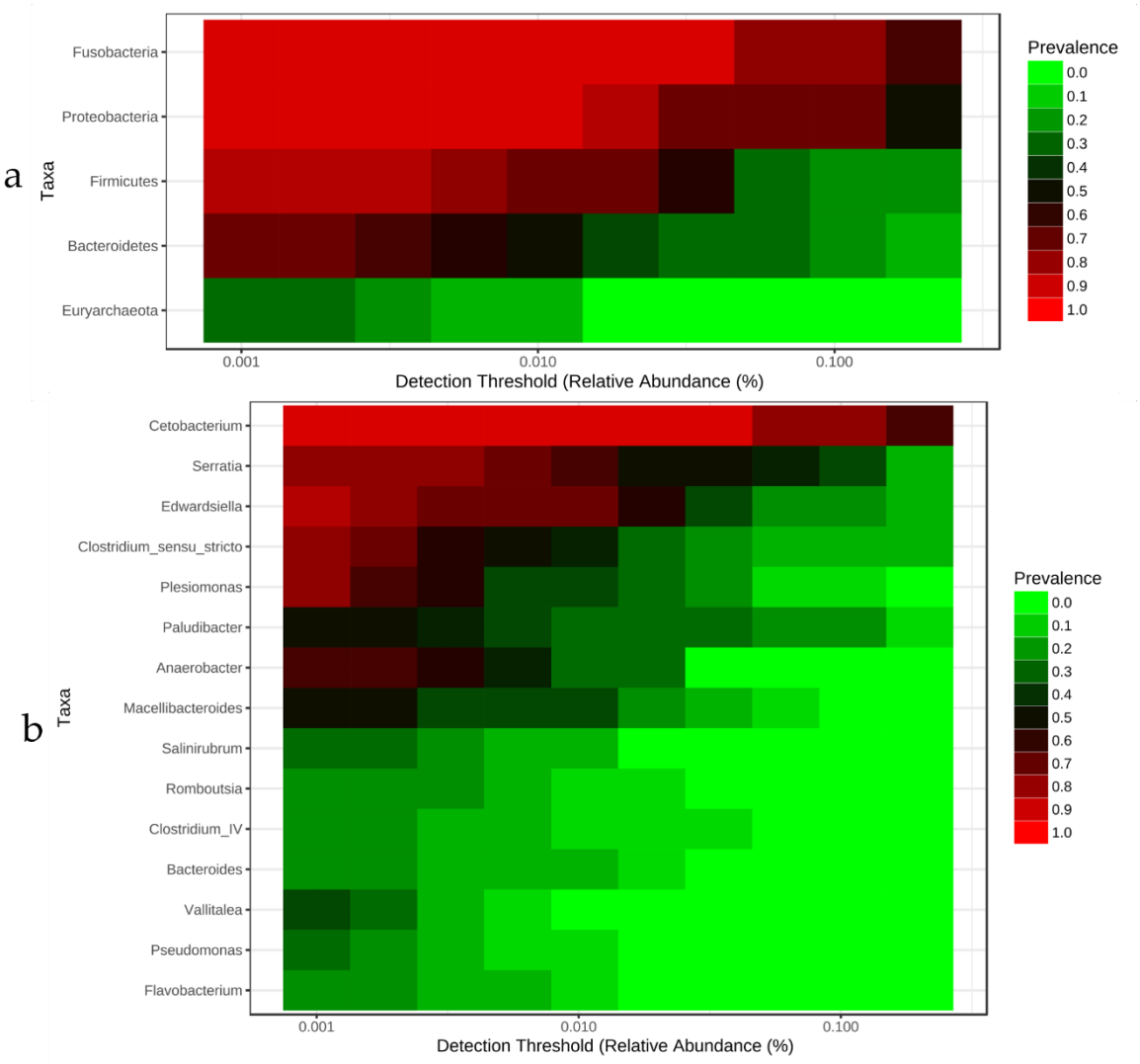

Figure 3. Core microbiome in gut of cultivated/wild and female/male adults' *A. tropicus*, at taxonomic class-level of (a) phylum and (b) genus.

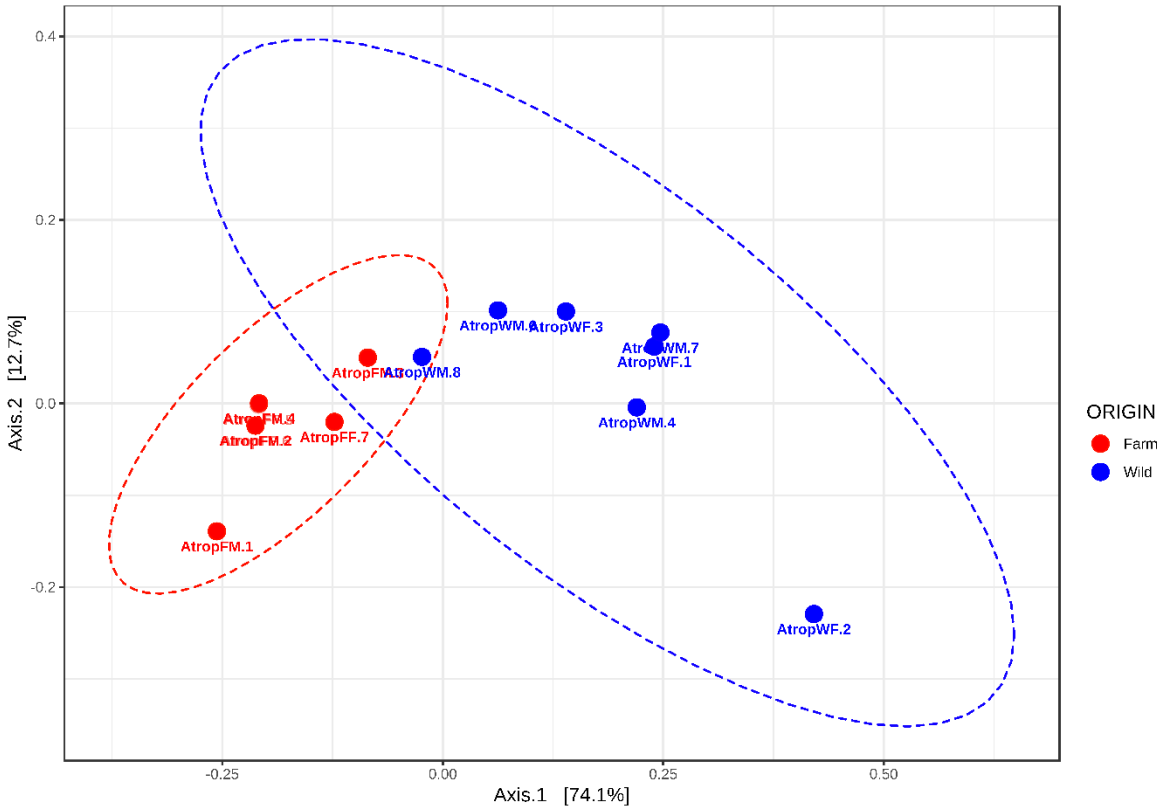

Figure 4. Similarity or dissimilarity coefficients of Unweighted UniFrac distances (UniFrac) by PCoA ordination analysis of the microbiota in adults' *A. tropicus*. Blue and red circles correspond to wild and cultivated individuals, respectively

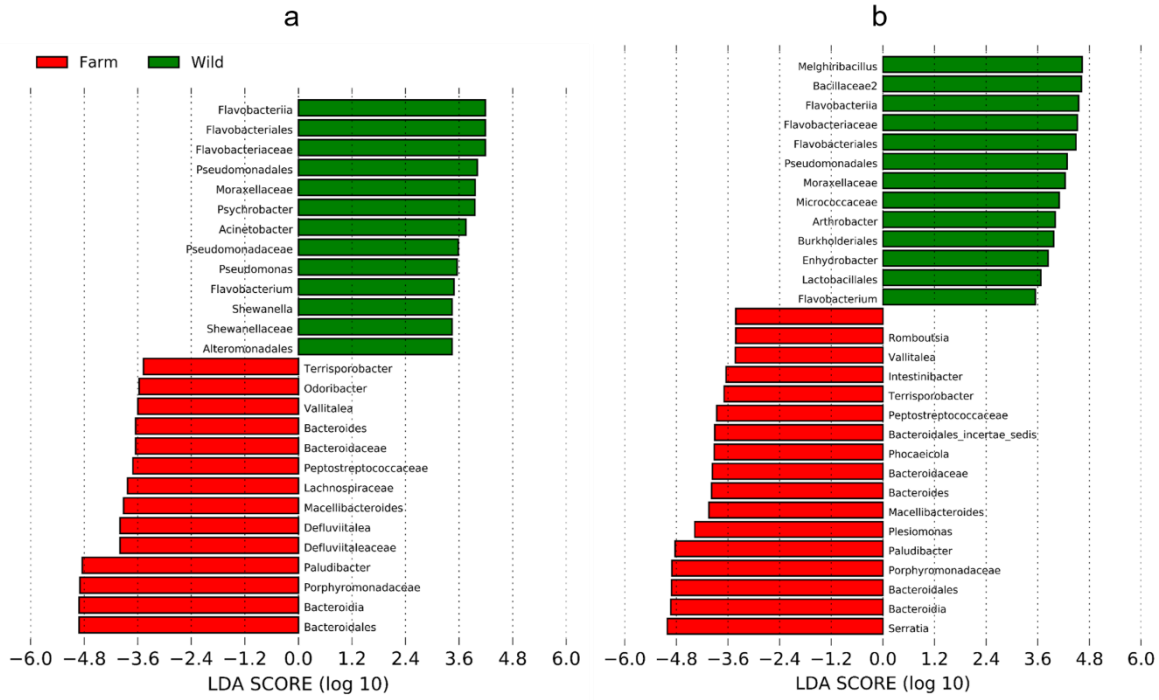

Figure 5. LEfSe analysis with the LDA score histogram of microbiota in adults' *A. tropicus* composition, (a) comparison among female and male organisms, cultivated and wild, and (b) comparison among female organisms cultivated and wild. Both show statistically significant differences depending on the origin. Red and green colors indicate cultivated and wild samples, respectively.

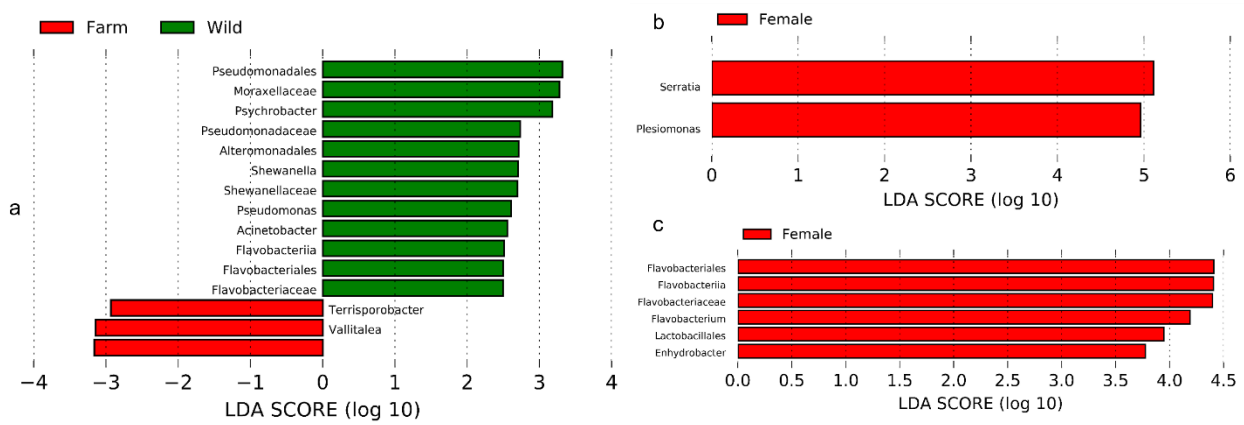

Figure 6. LEfSe analysis with the LDA score histogram of microbiota in adults' *A. tropicus* composition (a) male organisms, cultivated & wild, (b) female organisms cultivated and (c) wild female. All show statistically significant differences depending on the origin.

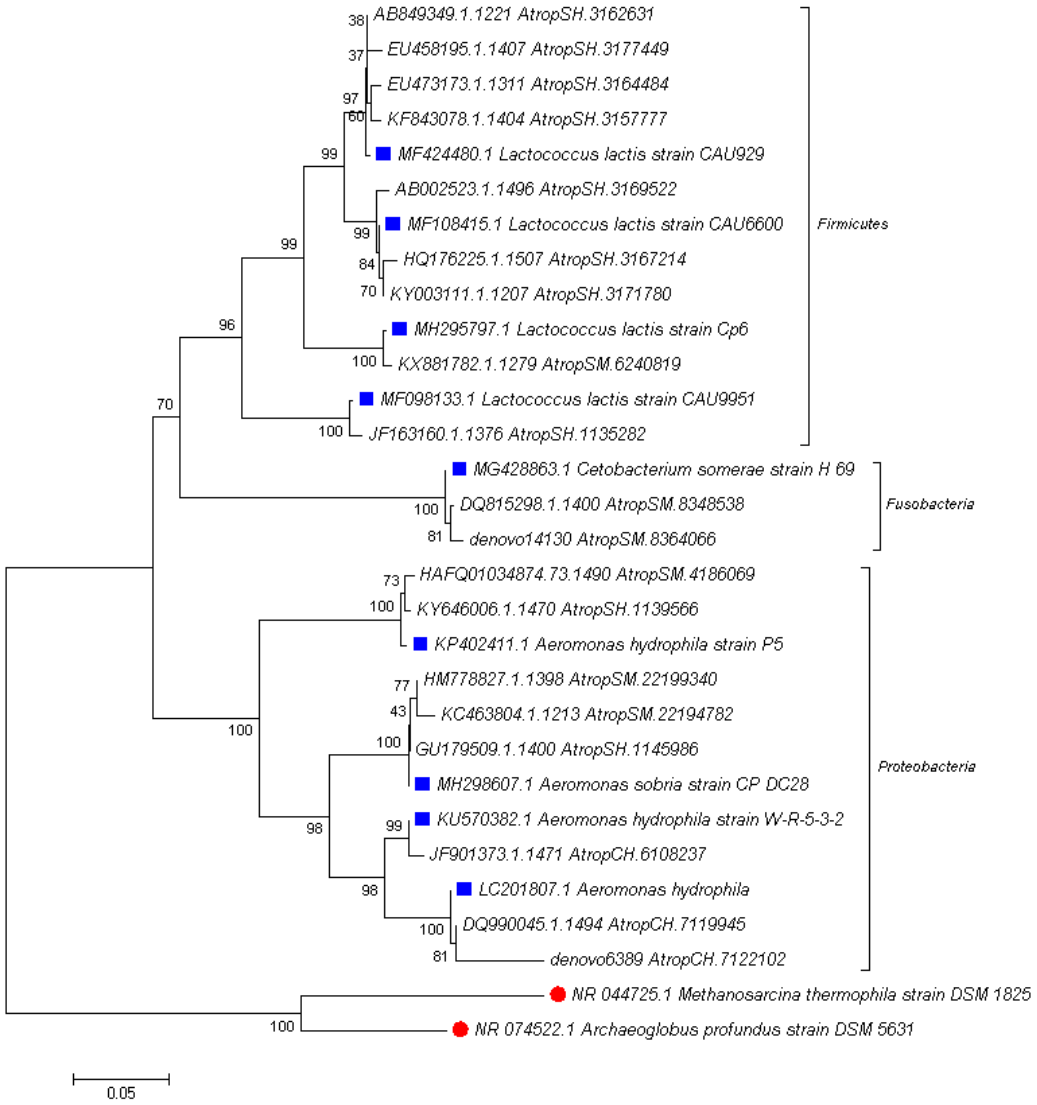

Figure 7. Phylogenetic tree from the rRNA 16S gene constructed of some organisms found in the literature with probiotic potential in fish (see table 1). Red circles and blue squares represent outgroup and reference sequences, respectively.