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Original article

Intestinal microbiota of ornamental fish *Carassius auratus*

Dafne Itzel Orozco Rojas[a], María del Carmen Monroy-Dosta[a,*], Jorge Castro Mejía[a], Aida Hamdan Partida[b], Alberto Ramírez Torrez[b]

[a]*Departamento El Hombre y su Ambiente, Calzada del Universidad Autónoma Metropolitana-Xochimilco, Hueso No. 1100, Col. Villa Quietud, 04960 México DF, México.*

[b]*Departamento Atención a la Salud, Calzada del Universidad Autónoma Metropolitana-Xochimilco, Hueso No. 1100, Col. Villa Quietud, 04960 México DF, México.*

*Corresponding author; Departamento El Hombre y su Ambiente, Calzada del Universidad Autónoma Metropolitana-Xochimilco, Hueso No. 1100, Col. Villa Quietud, 04960 México DF, México.

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ABSTRACT

Intestinal microbiota is an essential component for any organism life, because it directly affects nutrient assimilation, growth and health processes. However, in many animals and in case of fish is unknown how Intestinal microbiota is formed, which species are dominant and which one has capacity probiotic for use in aquaculture. The goal of this study was to establish the bacterial load that dominates the back intestine of *Carassius auratus.* A batch of 200 healthy juvenile fishes were obtained from an ornamental fish farm in Mexico City. The fish were maintained in two culture beakers of 100 L during 15 day to acclimatization to ensure any sign of injury disease. To extract the back of gastrointestinal tract, which was rinse several times with distilled water for food and feces residues elimination. The sample was inoculated in 9 mL of sterile saline solution and from this, three dilutions in a 1:10 relation were made, inoculating 0.1 mL from each dilution in agar plates MRS, BHI and TCBS, and were incubated at 27ºC for 24 hours. Subsequently a colony forming units (CFU mL-1) count was made, with the help of a Quebec counter type. Colonies were purified through successive inoculations. The molecular identification was made out by sequencing the gene RNAr 16S using Wizard Genomic. Molecular

identification showed that the back region of the intestinal tract of *C. auratus* was dominated by phylum Proteobacteria and Firmicutes represented by the genus *Bacillus*, *Vibrio*, *Vagococcus*, *Brevibacillus*, *Aeromonas*, *Pseudomonas*, *Shewanella*, *Enterococcus*, *Paenibacillus* and *Morganella.* In relation to bacterial abundance by specie it was established that *Bacillus sp. and Pseudomonas stutzeri*were the most abundant reaching CFU mL^{-1} counts of 210 and 167 respectively, followed by*Paenibacillus lactis* with 103 CFU mL^{-1} and *Bacillus cereus* with 100 CFU mL^{-1} while *Enterococcus eurekensis* was the least abundant with an average value of 6 CFU mL^{-1}.

1. Introduction

Intestinal microbiota is an essential component for any organism life, because it directly affects nutrient assimilation, growth and health processes (Clements et al., 2014). It has been reported that fish microbiota is constituted by aerobic and facultative anaerobic bacteria which inhabit water and varies between different fish species, mainly due to food habits of each organism, genetic factors, growth stage, and environment (Tatsuro et al., 2004; Merrifield et al., 2009; Balcazar et al., 2006; Silva et al., 2011).Traditionally, it was considered that intestinal microbiota only affected in nutrient obtainmentnevertheless, recent studies have shown that it possess many important functions like: digestion, development of protective mucus, angiogenesis, immune system recognition and over 200 genes expression (Hooper et al., 2002; Macfarlane, 2007). That is why a strong interest is developing in knowing the composition and gastrointestinal microbial ecology of cultured fish, in order to recognize those who can possess probiotic characteristics and use them in aquaculture for improvement of fish and crustaceans culture. The interest lies in the need of prevent and control production problems associated to bacterial infections, as it has been shown that probiotic microorganisms are highly competitive and exclude diverse pathogenic microorganisms (Al-Harbi and Uddin, 2008; Ringo et al., 2003).

Manipulation of intestinal microbiota is a viable tool to reduce disease incidence in aquaculture production (Balcazar et al., 2006; Monroy et al., 2012). However, studies that describe fish intestinal microbiota are still incipient, but this is a fundamental aspect for bacteria strain selection with probiotic characteristics that can be use in fish and crustaceans culture, like *Carassius auratus* culture. This is anornamental fish with high commercial demand because of itsshowy aesthetics, easy adaptation and handling, and to its high reproductive rate, making possible to purchase in aquarium shops any of the 125 currently recognized varieties (Damas, 2005). Therefore the goal of this study was focused in isolation and identification of posterior intestine microbiota of *Carassius auratus,* a specie with high economic demand in ornamental aquaculture.

2. Materials and methods

2.1. Isolation of *Carassius auratus* bacteria

2.1.1. Fish supply

For this research, a batch of 200 healthy juvenile *Carassius auratus* fisheswere obtained from an ornamental fish farm in Mexico City. Fish were maintained in two culture beakers of 100 L during 15 day to acclimatization to ensure any sign of injury disease. Organisms were maintained at temperature of 23±2°C, a pH of 7, a dissolved oxygen concentration of 6 mg L^{-1} and 0.3 ppm of nitrates (NO_3) and nitrites (NO_2). Fish were daily fed *ad libitum* with *Artemia franciscana* in adult stage. After acclimation period, it was made the isolation of intestinal bacterial load.

2.1.2. Isolation of bacterial load from the back of the intestine of *Carassius auratus*

For obtainment of intestinal microbial load of *C. auratus*, 20 organisms were randomly selected, which were anesthetized following Perdikaris et al. (2010) modified method, using clove oil (150 mg per liter of water) during four minutes. Later, in sterile zone, the dissection was made by making a cut above lateral line until the base of caudal fin (Munro, 1982) to extract the back of gastrointestinal tract, which was rinse several times with distilled water for food and feces residues elimination. The sample was inoculated in 9 mL of sterile saline solution and from this, three dilutions in a 1:10 relation were made, inoculating 0.1 mL from each dilution in agar plates MRS (Man Rogosa Sharpe), BHI (Brain Heart Infusion agar) and TCBS (Thiosulfate Citrate Bile Sucrose), and were incubated at 27ºC for 24 hours (Sugita et al., 1988, Monroy, 2010). Subsequently a colony forming units (CFU mL^{-1}) count was made, with the help of a Quebec counter type. Colonies were purified through successive inoculations.

2.2. Molecular bacterial identification through the detection of RNAr 16S gene

2.2.1. DNA extraction

After obtaining pure strains, the molecular identification was made. From a bacterial culture of 24 hours, 1 mL was took and put in an Eppendorf sterile tube of 1.5 mL and centrifuged at 16 000 rpm during two minutes for pellet obtainment, which was used for DNA isolation using Wizard Genomic DNA Purification kit of PROMEGA™, following the manufacturer's instructions. Later for the determination of purity and integrity of genomic DNA of interest, it was made an electrophoresis in agarose gel at 1%.

2.2.2. Amplification of RNAr 16S gene through PCR

With the obtained DNA, the amplification of RNAr 16S gene was made, using the PCR kit "Flexi" of PROMEGA™ and universal primers 8 for (5'-AGACTTTGATCATGGCTCAG-3') and 1492 rev. (5'-TACGGCTACCTTGTTACGACTT-3'), with the following conditions: a preincubation period at 94ºC for five minutes, followed by 40 cycles of denaturalization at 94ºC for 38 seconds, hybridization at 52ºC during 40 seconds, pre extension at 72ºC for seven minutes, to end in a cooling process at 4ºC (Sambrook et al., 1989).

2.2.3. Electrophoresis detection of RNAr 16S gene from PCR products

PCR amplification products were subjected to electrophoresis in an agarose gel at 1% (TAE 1X), using as standard 1 µl of molecular marker of 100 bp to corroborate the size of resulting band in each sample. Samples were run on a power source BIO-RAD 4 Power Basic ® at 100 V during 30 minutes. Once the electrophoresis was done, the gel was dyed during 10 seconds with ethidium bromideand was analyzed in a BioRad ® gel image system (Sambrook et al., 1989; Hamdan, 2004).

2.2.4. Purification and sequencing

PCR products were purified with ExoSAP kit, following the manufacturer's instructions and were sent to the sequencing service Macrogen Korea. Obtained sequences were interpreted with BLAST program and were compared with the worldwide base of sequences of Gen Bank.

3. Results and discussion

3.1. Bacterial identification

A total of 75 strains from the posterior region of the digestive tract of *C. auratus* were isolated, from which 36% turn out to be Gram positive bacteria and 64% Gram negative. In 59% of the cases, it was observed bacillary forms and 41% coccus. Also the 68% of isolated strains grew in oxygen presence, while 32% were facultative anaerobic bacteria.

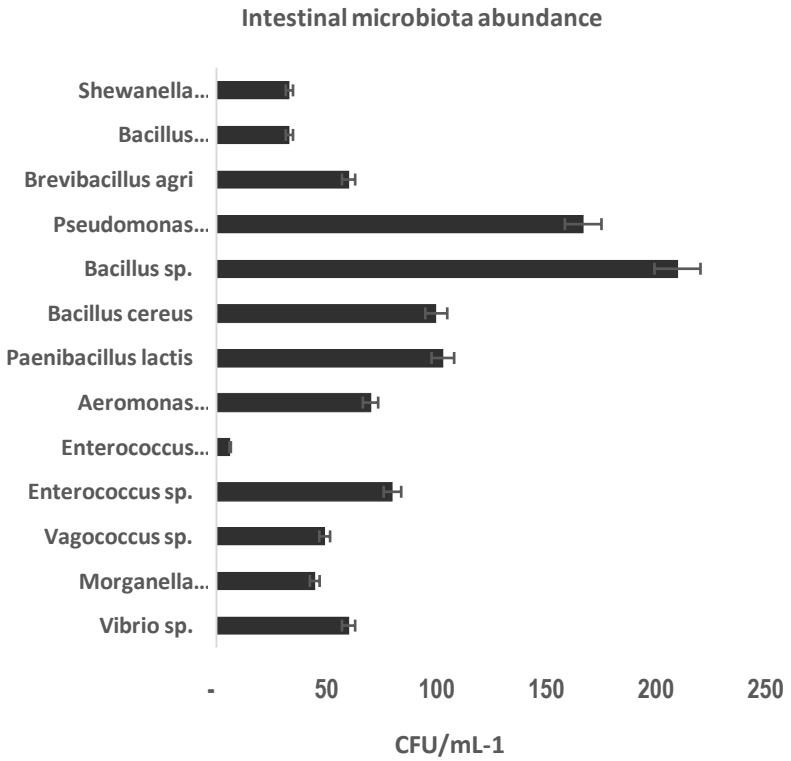
Molecular identification showed that the back region of the intestinal tract of *C. auratus* was dominated by phylum proteobacteria and firmicutes represented by the species that are shown in Table 1.

Table1
Identified species in the back of *C. auratus* TGI.

Specie	% Of identification	E Value	Phylum
Bacillus methylotrophicus	98	0.0	Firmicutes
Vibrio sp.	98	0.0	Proteobacteria
Vagococcus sp	97	0.0	Firmicutes
Bacillus cereus	99	0.0	Firmicutes
Brevibacillusagri	98	0.0	Firmicutes
Aeromonashydrophila	99	0.0	Proteobacteria
Pseudomonasstutzeri	97	0.0	Proteobacteria
Shewanella xiamensis	97	0.0	Proteobacteria
Enterococcus sp.	99	0.0	Firmicutes
Enterococcus eurekensis	97	0.0	Firmicutes
Paenibacillus lactis	98	0.0	Firmicutes
Bacillus sp	98	0.0	Firmicutes
Morganellamorganii	98	0.0	Proteobacteria

3.2. Bacterial abundance

In relation to bacterial abundance by specie it was established that *Bacillus sp*. and *Pseudomonas stutzeri*were the most abundant reaching CFU mL^{-1} counts of 210 and 167 respectively, followed by *Paenibacillus lactis* with 103 CFU mL^{-1} and *Bacillus cereus* with 100 CFU mL^{-1} while *Enterococcus eurekensis*was the least abundant with an average value of 6 CFU mL^{-1} (Figure 1).



3.3. Bacterial frequency

Variance analysis made to bacterial frequency of the different analyzed intestinal samples, indicates significant differences (P<0.05) but these differences are between *Enterococcus eurekensis*and *Vagococcus sp,* with*Paenibacillus lactis, Pseudomonas stutzeri* and *Shewanllaxiamnensis,* and between *Vagococcus sp* and *Vibrio sp,* according to multiple mean comparison (Tukey technique).

Obtained results in this investigation indicates that the back of *Carassius auratus* intestine, is colonized by bacteria of phylum proteobacteria and firmicutes, this matches with reports made by Nayak (2010) and Wu *et al.* (2013), where they point out that fish intestinal microbiota is dominated by these phylum, but they also mention that there might be variations between the described species (Kim et al., 2007), as it happened in this study because it was molecularly identified 13 species: *Bacillus sp, Bacillus cereus, Bacillus methylotrophicus, Enterococcus eurekensis, Vagococcus sp, Paenibacillus lactis, Pseudomonas stutzeri, Shewanllaxiamnensis, Vagococcus sp, Vibrio sp, Morganellamorgani, Aeromonashydrophila, Bacillus sp* and *Enterococcus sp*., and except for species *Aeromonashydrophila*and *Vibrio sp.,* the other species has not been reported as part of *Carassius auratus* intestinal microbiota. A study made by Wu et al. (2013), establish as part of Carassius *auratusgibelio*intestinal microbiota the species *Veilonella sp, Streptococcus sp, Lactobacillus sp, Rothia* sp, *Aeromonas* sp. and *Acinetobacter* sp. In the same way Suganya et al. (2014), report the presence of *Escherichia coli*, *Aeromonas*, sp, *Enterococcus* sp, *Pseudomonas* sp., *Streptococcus* sp., and *Proteus* sp. The difference among identified species between studies, can be due to the supplied diet to species, the stage, and type of culture, as indicated by Aguilera et al. (2013).

On other hand Sugita et al. (1999), and Silva et al. (2011), claim that in microbiota three categories are distinguished, transitory microbiota from diet and water, permanent or endogenous that is not affected by diet, and the mature microbiota that is established inside the intestine and developed when the organism is in its adult stage.

In identified strains, the genus Bacillus sp. was the most abundant and even though it was not reported for the studied specie, it is a frequent genus in other fish intestine Gunter and Jiménez-Montealegre (2004), and it has been prove beneficial when it is used as probiotic additive in diet, such is the case of the study of Monroy et al. (2012), who identified three strains of *Bacillus* sp. from the intestinal tract of *Pterophyllumscalare.* The strains were characterized as probiotics and made the *in vitro* inhibition of *Aeromonashydrophila*causative of fish septicemia. Also Mukherjee and Ghosh (2014) identified *Bacillus methylotrophicus* in the Indian carp (*Catlacatla*). The specie *Morganellamorganii*had not been described in fish, nevertheless, it has been characterized as normal inhabitant of marine and freshwater environments, which indicates its possible entrance to fish intestine (Moya et al., 2001; Herrera et al., 2000; Al-Harbi and Uddin, 2004; Diep et al., 2009). *Pseudomonas stuzeri*has been identified in water samples from a catfish culture Diep et al. (2009), proving its capacity to eliminate nitrogenous compounds, so it could be present in diverse aquatic organisms in culture as it happened in this study. The specie *Shewanella xiamenensis* had not been reported as part of ornamental fish microbiota, but it presents a wide distribution in marine and freshwater environments, being isolated from marine sediments in the coast of China, to which owes its name, which suggest that this bacteria is a common inhabitant of aquatic environments and can form part of fish transitory microbiota (Huang et al., 2010; Potron et al., 2011). In relation to genus *Brevibacillus,* it had not been isolated from fish, but it was previously isolated from an *Artemia* culture (Mahdhi et al., 2012), so it can be said that this bacteria could have reached the intestine through food, because during acclimatization process (15 days) it was daily administered *Artemia franciscana.*

Finally, various authors (Nikoskelainen et al., 2001; Balcazar et al., 2006; Jaffrès et al., 2010) mention lactic acid producing bacteria as part of subdominant microbiota, like genus *Lactobacillus* sp. and *Lactococcus* sp.; nevertheless, for this study they were represented by genus *Enterococcus* sp. and *Vagococcus* sp.

Obtained results allow us to establish bacterial genus that are housed in the back of the intestinal tract of *Carassius auratus;* however, more studies are required that allows to determine if identified species are transitory strains affected by alimentation, handling or by the studied fish stage or if they are part of the permanent intestinal microbial community, but above all, make studies that concern us whether the strains possess probiotic characteristics for their use in aquaculture.

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