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Original article

Intestinal microbiota composition with probiotic potential of three species of the genus *Chirostoma*

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ABSTRACT

The aim of this research was to identify the microbiota that was found in *C, jordani, C. humboldtianum* and *C. estor* to obtain those strains with probiotic capacity and can be used in *Chirostoma* sp. culture. Pre-adult stages were obtained for dissection and gain an intestinal tissue portion and get pure strains throughout consecutive reseeding in NHI and TSA agar plates. Then proceeded to the molecular identification with RNAr 16S sequencing gen and establish their probiotic capacity growing them in acid pH, bile salts, pathogen inhibition to *Aeromonas hydrophila* and *Vibrio fluvialisin vitro* and with antibiotics according to FAO (2006) and Balcázar*et al*. (2006) techniques. Results shown that analyzed *Chirostoma* sp. from intestinal tract were dominated by Proteobacteria, Actinobacteria and Firmicutes identifying 60 strains, 40% of them were from *C. jordani*; 30% from *C. humboldtianum* and 20% of strains were from *C. estor*. The three analyzed species share some bacterial groups even if they were grown in different environmental conditions. The

common species strains were *Lactobacillus* sp., *L.crispatus*, *Bacillus subtilis*, B. sp., *Aeromonashydrophila, A.*hydrophila caviae, *Vibrio fluvialis* and *Pseudomonaluteola*. The only bacterial species that resisted stress experiments, correspond to *Bacillus* sp. genus represented by *Bacillus subtilis*, *Bacillus* sp. and *Bacillus laterodporus*, so they are good probiotics candidates for culture of *Chirostoma* sp. genus.

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1. Introduction

The intestinal study of microbiota of any organism gained importance in recent years due to retailed studies have shown that microbiota possess many important functions like digestion, protective mucosa development, angiogenesis, immune system recognition and expression of more than 200 genes (Hooper et al., 2002; Macfarlane, 2007). In aquatic species cases, there is a close relationship with intestinal microbiota and the one in the surrounding environment; some may remain definitively being part of beneficial intestinal flora. However, there are affected by various environmental factors (temperature, oxygen concentration, salinity and food quality and quantity), as well as chemical or antibiotics pollutants which can harmful and allow pathogen organisms get and show them virulent causing infection process in culture fishes and crustaceans (Verschuerer et al., 2000; Ringo et al., 2007; Sun et al., 2010).

The beneficial microbiota equilibrium can be optimized by intestinal ecosystem manipulation, using probiotic organism, which positively affect in different process like nutrient assimilation, immunity and disease resistance (Al-Harbi and Uddin, 2005). These characteristics give an interesting strategy in cultured commercial species and those in risk category like *Chirostoma* sp. genus case, conformed by fish group which include “white fish” and “charales”, since prehispanic times were conformed the artisanal fisheries of Mexico Central Zone and several of their species besides being endemics, also were found at risk species according to rule NOM 059. So different strategies were required to improve their cultivation and conservation (Elias- Fernández et al., 2008; Martínez-Palacios et al., 2002).

Taking into consideration that one of the first steps to follow in the search for interested aquatic specie of suitable a specific probiotic; the microbial community characterization from the target specie gut and later, determined the microbiota transient of that who were dominant and beneficial to organism’s, with probiotic characteristics to be used in aquaculture. The goal of this research was to identify the microbiota community, with probiotic potential, at intestinal tract from three *Chirostoma* sp. genus species.

2. Materials and methods

2.1. Intestinal bacterial load isolation from healthy fishes of *Chirostoma*genus

Adult stage batches were obtained from Centro Regional de InvestigaciónPesquera (CRIP) of Pátzcuaro, Michoacán (*Chirostoma estor*); from Centro de Investigaciones Acuícola de Cuemanco (CIBAC) (*Chirostoma jordani*); and *Chirostoma humboldtianum* was obtained from Villa Victoria dam in State of Mexico. The fishes were maintained fasted during 24 hours to make bacterial isolation from rear gut. From each species, nine specimens were taken by spoon-shaped net following Perdikaris (2010) technique. Fishes were anaesthetized during four minutes with clove oil (150 mg L^{-1} concentration). Later the fishes were dissected making a cut above lateral line to caudal fin base (Munro, 1980). A rear gastrointestinal tract (GIT) portion was extracted, washed several times with sterile water to eliminate faces waste, inoculated in a sterile tube with 9 mL of sterile salt solution and homogenized during five minutes in a vortex.Of original sample, three dilutions were made (1:10) and 0.1 mL inoculated in agar plates MRS, BHI, and TBS per triplicate. Agar plates were incubated at 27°C temperature during 24 hours. After this time, the colony formed units (CFU mL^{-1}) were counted from each dilution test. Colony morphology was characterized and strains were purified with successive sowings.

2.2. Molecular bacterial identification by 16S RNAr gen detection

2.2.1. DNA isolation

To identify the isolated bacteria, DNA extraction was made taking out 1 mL of bacterial medium, cultured during 24 hours in soy broth, which was introduced in 1.5 mL Eppendorf sterile tube and centrifuged at 13 000 rpm during the two minutes to obtain a pellet, from which DNA isolated was made using genomic DNA extraction kit - Wizard Genomic DNA Purification Kit (PROMEGA ™) following manufacturer's instructions. Later an electrophoresis in 1% agarose gel were made to determine purity and integrity of genomic DNA isolated (Hamdan, 2004).

With DNA isolated of bacterial strains in the study, amplification of 16S RNAr gen was made using universal primers 8 for. (5'-AGACTTTGATCATGGCTCAG-3') and 1492 rev. (5'-TACGGCTACCTTGTTACGACTT-3'), with following conditions: preincubation at 95°C, during 10 minutes and 30 cycles, denaturalization at 95°C during 60 seconds, hybridization at 53°C during 60 seconds and extension at 72°C during 30 seconds. Thirty cycles were made finished in one step at 4°C (Hamdan, 2004). To remove primers, nucleotides and polymerase wastes, the samples were purified using purification QIAquick PCRKit (Qiagen), following manufacture's instructions. The purification products were sent to sequence service Macrogen Corea DNA and sequences obtained were interpreted with Chromas and Blast programs. Finally, the information obtained were compared with a worldwide sequence database (GENEBANK).

2.2.2. Tests to characterize a microorganism as probiotic

To identify bacterial strains bioassays were made to determine their probiotic capacity throughout different tests: pH acid growth, growth in bile salts, *Aeromonas hydrophila* and *Vibrio fluvialis* fishes pathogen inhibition *in vitro* and antibiotic as FAO (2006) stipulated and according to Balcázar et al. (2006) as follows.

Resistance to pH acid

Modified protocol by Dunne et al. (2001), was used to simulated gastric barrier and make microorganisms grow in acid culture medium, pH 1.5, 2.5 and 3.0 range, checking bacterial growing during 24 hours using a spectrophotometer with 620 nm wavelength (Ávila et al., 2010). The bacterial strains that did not survive to these stress conditions were rejected.

Resistance evaluation of isolated bacteria's from bile salts

Later, a growth test was effected with bile salts for which three Erlenmeyer flasks were used with 100 mL MRS broth plus 0.1%, 0.5% and 1.0% fish bile salts fresh, respectively. The flasks was inoculated with 1 mL of survival strains from acid conditions test. The samples were incubated to 37°C temperature during three hours contact. As control was used culture viability on MRS (Oxoid) broth without bile. Sample lectures were taken in spectrophotometer with wavelength of 620 nm (Ávila et al., 2010).

Antagonist capacity *in vitro*

Strains which showed positive results in previous tests, went through *in vitro* inhibition tests using pathogen strains for fishes per triplicate *Aeromonas hydrophila* and *Vibrio fluvialis* ($1x10^7$FCU mL^{-1}). Later, using diffusion wells method 100 µLwas added from each isolated gut bacteria suspension at a $1x10^7mL^{-1}$ concentration. The Petri dishes were incubated during 24 hours at 30°C. After this time, the inhibition halos formation was observed (Dopazo et al., 1988). Strains that showed halos wider than 1 cm diameter were considered positive.

Susceptibility to antimicrobial agents

For antibiotic sensibility test BD BBL Sensi-Disc Antimicrobial Susceptibility Test Discs were used, which follow the diffusion agar method, using filter paper discs impregnate with antimicrobial agents with known concentration (Standard method for Clinical and Laboratory Standards Institute), in this case ampicillin and ciprofloxacin following manufacture's instructions.

3. Results and discussion

3.1. *C. jordani, C. humboltianum* and *C. estor* gut microbiota identification

The results showed that *Chirostoma* sp. genus gut analyzed in this study, are dominated by Phyla Proteobacteria, Actinobacteria and Firmicutes. Sixty strains were identified and 40% corresponding to *C. jordani* gut, 30% from *C. humboldtianum* gut, and 20% from *C. estor* gut. The three analyzed species share some bacterial groups even if they are cultivated in different environmental conditions. The common microbiota among them were conformed for strains of species like *Lactobacillus* sp, *L. crispatus*, *Bacillus* sp, *B. subtilis*, *Aeromonas hydrophila*, *A. hydrophila caviae*, *Vibrio fluvialis* and *Pseudomonaluteola.* It should be noted that *C. estor* showed the highest gut bacterial abundance in all cases. However, this specie showed lowest bacterial diversity with 11 dominant strains in comparison with *C. jordani* which obtained 18 dominant strains (Fig. 1.).

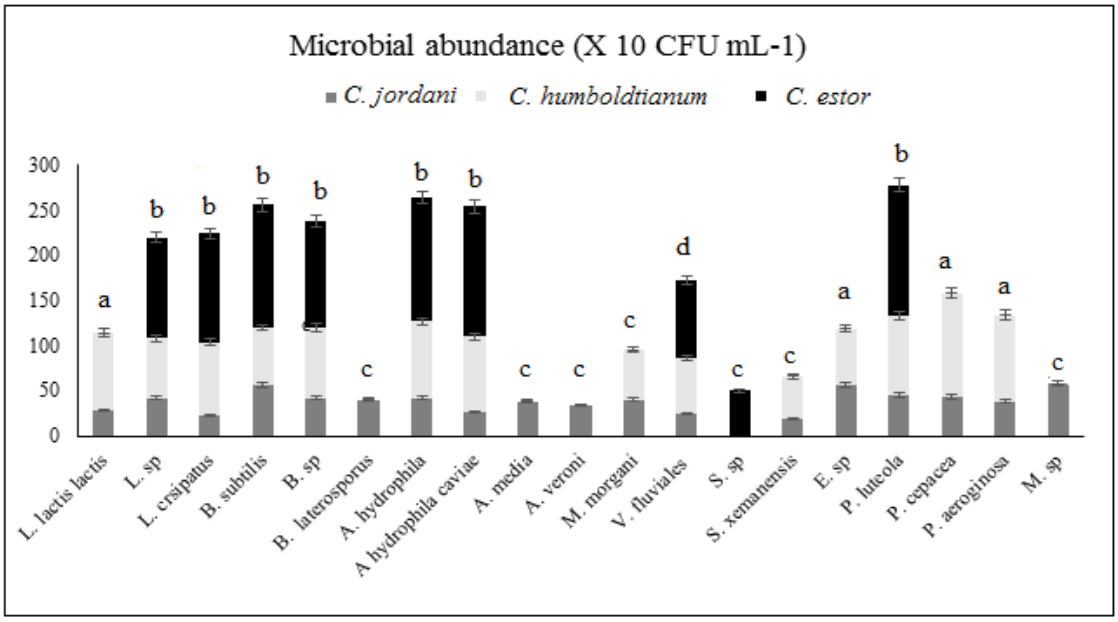

Fig. 1. Intestinal tract bacterial abundance in *C. jordani*, *C. humboltianum* and *C. estor*.

*Different letters show significant differences (P<0.05).

3.2. Tests to characterize bacteria's like probiotics

3.2.1. pH acid resistance

To identify strains were apply tests to characterize like probiotic strains. However, this test was applied only to those that were dominant and showed in Fig.1 which were identified dominant in three studied species. Furthermore, were discarded those which reported as fish opportunistic pathogens or those who have resistance to different antibiotics since not be considered as probiotic strains like gender cases as: *Aeromonas, Enterobacter, Micrococcus, Shewanella* and *Morganella.*

Species under stress in different pH conditions, the strains from *Pseudomonas*genus did not resist acid conditions tested in this study, meanwhile *Bacillus*genus survival under low pH conditions between 1.5 to 3.0 in comparison with *Lactococcus* and *Lactobacilos*which showed low growth (Table 1 and Fig. 2.).

3.2.2. Bile salt resistance

Those bacteria which had good growth in acid pH were made a bile salt growth test. This important factor inhibits intestinal microbial growth. The results in Fig. 3. show that all strains have a resistance to bile salts. However, *Bacillus* sp, and *Lactobacillus crispatus* obtained better growth.

Table 1
Mean values of optical density registered for each strain at different treatments.

Strain	Time	pH 1.5	pH 2.5	pH 3.0
Bacillus sp.	Initial	0.389 ± 0.002	0.555 ± 0.002	6.231 ± 0.017
	Final	0.513 ± 0.004	0.757 ± 0.006	0.881 ± 0.005
Bacillus subtilis	Initial	0.380 ± 0.003	0.471 ± 0.005	0.490 ± 0.003
	Final	0.414 ± 0.004	0.423 ± 0.011	0.563 ± 0.002
Bacillus laterosforus	Initial	0.307 ± 0.002	0.302 ± 0.004	0.3181 ± 0.006
	Final	0.408 ± 0.004	0.454 ± 0.007	0.484 ± 0.004
Lactococcus lactis	Initial	0.308 ± 0.007	0.330 ± 0.005	0.257 ± 0.009
	Final	0.368 ± 0.012	0.371 ± 0.005	0.300 ± 0.005
Lactobacillus sp	Initial	0.327 ± 0.06	0.294 ± 0.03	0.290 ± 0.04
	Final	0.0460 ± 0.003	0.0588 ± 0.006	0.0602 ± 0.010
Pseudomonas luteola	Initial	0.0227 ± 0.006	0.0294 ± 0.003	0.0290 ± 0.004
	Final	0.060 ± 0.003	0.048 ± 0.006	0.0102 ± 0.010
Pseudomonas cepacia	Initial	0.0027 ± 0.006	0.0029 ± 0.003	0.02090 ± 0.004
	Final	0.010 ± 0.003	0.088 ± 0.006	0.0102 ± 0.010

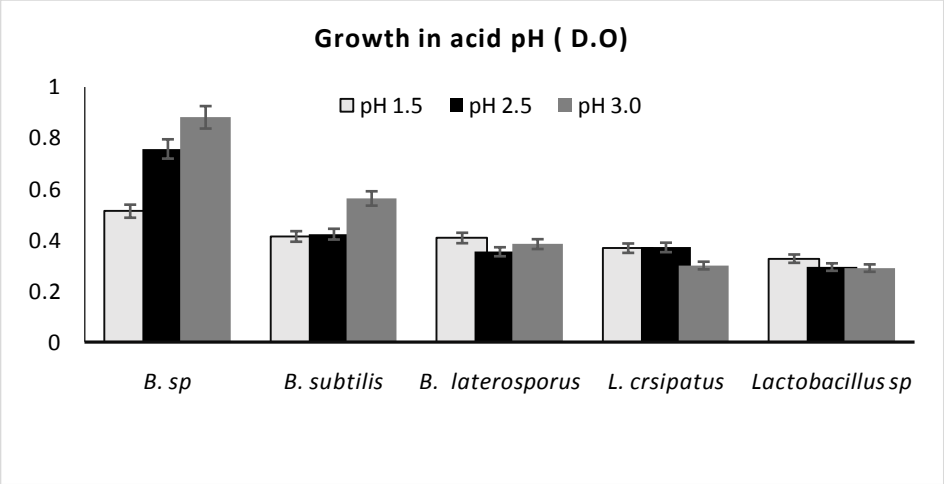

Fig. 2. Bacterial growth in acid pH presence.

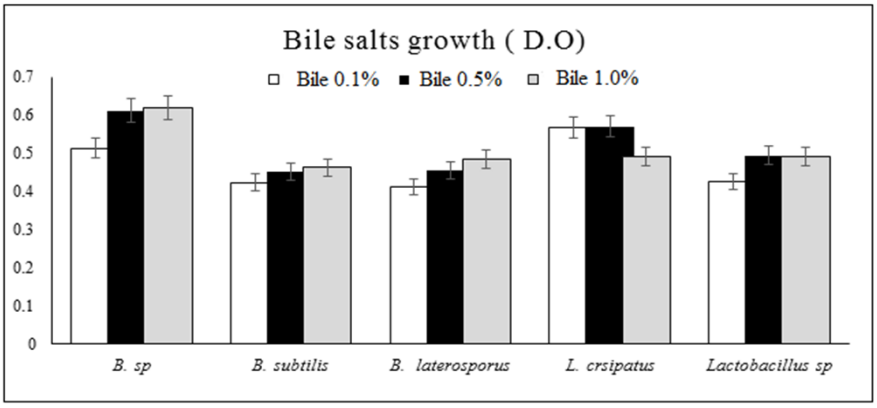

Fig. 3. Bacterial growth in bile salts presence.

3.2.3. *In vitro* Inhibition

With respect *in vitro* inhibition can observe that isolated bacterial strains from intestinal fish tract inhibit the pathogens growth with both antimicrobial compounds. However, only *Bacillus subtilis*, *Bacillus* sp and *Bacillus laterosporus* can obtain halos above 1cm of diameter, meanwhile *Lactobacillu* sp and *L. crispatus* were maintained below considering positive values in this test (Fig. 4.)

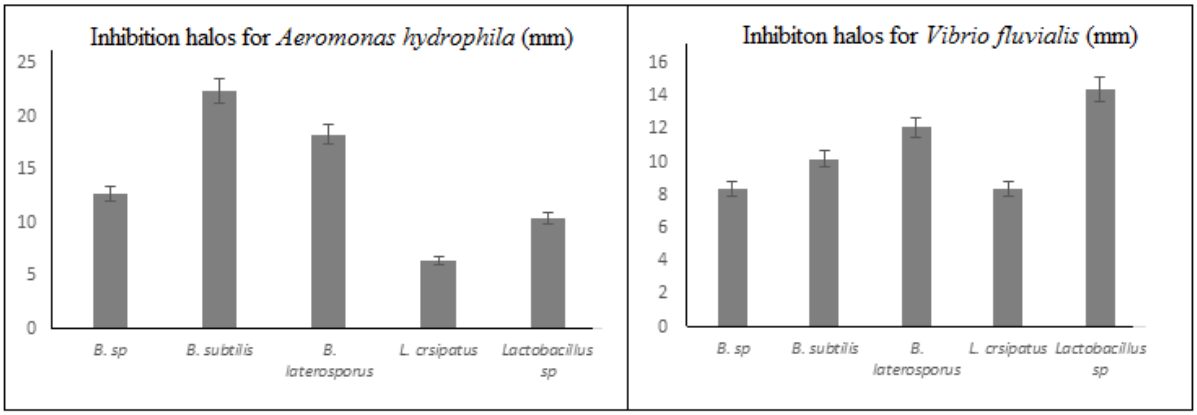

Fig. 4. Inhibition of *Aeromonas hydrophila* and *Vibrio fluvialis* for bacterial strains isolated from the intestinal tract of Chirostoma sp.

3.2.4. Susceptibility to microbial agents

The diameter halos obtained by their sensibility to antibiotic tested are shown in Table 2. The antibiotics ampicillin and ciprofloxacin did not allow bacterial growth until 48 hours of exposition. Bigger halos were obtained with *Bacillus subtilis*, *Bacillus* sp. and *Bacillus lateroporus*in comparison with *Lactobacillus* sp and *Lactobacillus crispatus* with significant difference values (P<0.001).

The results showed that *Bacillussubtilis*, *Bacillus* sp and *Bacilluslaterosporus*strains passed all tests and can be considered as probiotic strains candidates. These strains survival all stress tests (gastric barrier simulation) like acid pH and bile salts. Also fail to inhibit pathogens like *Aeromonas hydrophila* and *Vibrio fluvialis* and showed ampicillin and ciprofloxacin sensibility, requirements to be considered as probiotic strains.

Table 2
Bacterial inhibition halos diameter registered (mm) when were exposed to antimicrobial compounds.

Antimicrobialcompund	*Bacillus sp.*	*B. subtilis*	*B. laterosporus*	*Lactobacillus sp.*	*L. crispatus*
Ampicillin	30.71	23.90	20.40	10.10	10.20
	± 1.60	± 1.50	± 1.00	± 1.10	± 2.10
Ciprofloxacin	26.10	24.60	20.60	14.70	13.50
	± 1.7	± 1.22	± 2.10	± 1.40	± 1.10

The obtained results in this research show that intestinal microbial of *C. jordani*, *C. humboltianum* and *C. estor,* is dominated by bacteria from phyla Proteobacteria, Actinomycetes and Firmicutes, these results confirm what was reported by Nayak (2010) and Wu et al. (2013), which showed that these phyla were representative of intestinal microbiota of most fishes. The molecular identification determined 60 strains presence of which 40% corresponding from intestinal tract of *C. jordani*, 30% from *C. humboldtianum* and 20% from *C. estor*. The three analyzed species share some bacterial groups although they were cultivated at different environment conditions. The common microbiota among them were formed by strains of species *Lactobacillus* sp., *Lactobacillus crispatus*, *Bacillus subtilis*, *Bacillus* sp, *Aeromonas hydrophila*, *Aeromonas hydrophila caviae, Vibrio fluvialis* and *Pseudomonaluteola*. These results were relevant because until this moment there was not registered about the bacteria that make part of fish intestinal tract microbiota. Some studies, like Paniagua et al. (2006), focused on isolation of pathogen bacteria genders in *C. jordani* and founded *Aeromonas hydrophila*, *Enterobacter* sp and *Citrobacterfreundi*, but these beneficial bacteria have not been studied. The identified strains in this study differ from those reported in other fish species, as Lin et al. (2014), study which found at intestinal carp microbiota different gender like *Clostridium, Streptococcus, Lactococcus sp* and *Bacillus* sp. Befring et al. (2007), reported *Lactococcus* sp, *Photobacterium* sp, *Acetinobacter* sp, *Peudomonas* and *Vibrio*presence in salmonids. The differences found between the identified strains at different studies can be due to food diet apply to species, stages and culture type, as indicated in Aguilera et al. (2013).

Within identified strains, the *Bacillus*genus was most abundant and although it has not been reported for study specie, is a frequent genus in the intestinal tract of other fishes (Ghosh et al., 2002). Such as Monroy et al. (2010), which identified three *Bacillus* sp. strains from *Pterophyllumscalare* intestinal tract. Likewise, Mukherjee and Ghosh (2014), identified *Bacillus methylotrophicus* from Indian carp (*Catlacatla*).

Other researchers like Balcazar et al. (2006) and Jaffrès et al. (2010), showed that part of subdominant microbiota were acid lactic producers bacteria like gender *Lactobacillus* sp. and *Lactococcus* sp.; which were represented in this study by *Lactobacillus* sp. and *Lactobacillus crispatus* species.

The probiotic characterization tests performed in this study were good criteria to distinguish two bacteria types, the transitional which proceed from water, food and surrounding environment to cultured fish and cannot stablished in organisms gut, because cannot tolerated physical and chemical host conditions; and those bacteria type called permanent, although they are present in environment, they persist and achieve to colonized the fish intestinal tract, evading stomach acid conditions and also digestive process bile salts as mentioned by Sugita et al. (1988). Because of this, it is possible to mention that most studied strains are not permanent residents from *Chirostoma* sp. intestinal tract, because they cannot pass the exposed tests, excluding *Bacillus* and *Lactobacillus* gender, which can grow in stress conditions and can easily reach hindgut and maintained viable in this ecosystem (Balcázar et al., 2006). The result matchs with reported by Sim et al. (2015), which reported that *Bacillus methylotrophicus* bacteria can be used as probiotic, checking their survival capacity above 80% in pH of 2.5 and 75% survival to 0.5% bile salt concentrations.

With respect to bacteria capacity to inhibit *Aeromonas hydrophila* and *Vibrio fluvialis*pathogens, this study showed a success from both pathogens. However, only *Bacillus subtilis*, *Bacillus* sp. and *Bacillus laterosporus*could obtained halos upper to one centimeter, positive value considering for this experiment. The results were similar to those founded by Sugita et al. (2002), which test inhibit capacity from isolated bacteriafrom seven fishes versus *Vibriovulnificus* pathogen. These authors determined that *Bacillus* sp. strains were highly active in this, because their antimicrobial substances production and siderophores with a molecular weight less than 5kDa. Several studies mentioned that strains with probiotic bacteria showed qualities to produce antimicrobial and fungi agents like antibiotics, siderophores, toxins and enzymes which provide them the capacity to inhibit proliferation of some pathogens (Prasana et al., 2010).

According to FAO (2006), an important aspect is that any bacteria which has been considered as probiotic must be controlled with some chemical or antibiotic agent to contrast any adverse effect during their application in culture fishes or the environment. Due that, the isolated strains from fish gut from *Chirostoma*sp. genus in this study, were proved to sensibility test versus Ampicillin and Ciprofloxacin. The results showed that all isolated strains, only *Bacillus subtilis*, *Bacillus* sp., *Bacillus lateroporus*, *Lactobacillus* sp. and *Lactobacillus crispatus* were sensitive to the used antibiotics. However, *Bacillus* sp. strains showed greater inhibition versus antibiotics so it was easy to control strains.

Because of all the above, the results obtained in this study were very important, because now there is information about bacterial groups that form part of intestinal microbiota of three genders of *Chirostoma* species.

At the same time, they are encouraging because three *Bacillus* sp. strains were identified by the characterization selection *in vitro*as probiotic bacteria and be used in fishes culture from *Chrirostoma* sp. gender, species which were considered in the ecological risk category in their natural environment and their successfull cultivation with these probiotic must be considered as a conservation strategy and any biotechnological application can help to increase their production must be important. However, *in vivo* test must be done to obtain same *in vitro* results or better, if increasing survival, growth and immune response of *Chirostoma* sp. species using these three *Bacillus* sp. identified strains in this study.

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