Chapter 10

Bacteria with Probiotic Capabilities Isolated from the Digestive Tract of the Ornamental Fish *Pterophyllum scalare*

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http://dx.doi.org/10.5772/45954

1. Introduction

Aquaculture has made significant advances in recent years in the production of a wide range of aquatic organisms, both for human consumption and as ornamental species (Balcazar et al., 2006; Kesarcodi-Watson et al., 2008). One of the most successful freshwater ornamental species is *Pterophyllum scalare* (angelfish), a cichlid native to the Amazon that has adapted throughout the world and has great economic potential; it is one of the most in-demand species on the market (Agudelo;2005; Soriano and Hernández, 2002; Zilberga et al., 2004). This species is grown in intensive and semi-intensive systems, where its nutritional requirements are met with artificial diets. However, due to growth conditions such as high seeding densities and limited amounts of water, the organisms are subjected to constant stress, which translates into low growth rates and diseases (Auró & Ocampo, 1999; Verjan, 2002; Akinbowale et al., 2006). Therefore, there is an ongoing search for alternatives, such as the use of nutritional supplements, to prevent the rise of diseases and improve production. One interesting strategy focuses on the use of probiotics microorganisms that promote the welfare of the host they inhabit by improving its digestion and immune response as well as by inhibiting the growth of pathogenic microorganisms (Riquelme et al., 2000; Verschuere et al.,2000; Planas et al.,2006; Wang & Xu, 2006; Vine et al., 2006; Wang, 2007; Gatesoupe, 2007).

The presence of probiotic bacteria in the digestive tracts of fish is subject to several factors such as their ability to adhere to the surface of the intestinal epithelium and the production of substances that antagonise pathogenic microorganisms (Boris et al., 1997; Del Re et al., 2000; Reid et al., 1988; Balcázar, 2002;). Difficulties involved in the study of in vivo bacterial colonisation have led to the development of new *in vitro* techniques, such as sweeping electron microscopy and molecular analyses (PCR, FISH and DAPI). The objective of this

work was to isolate and identify by the isolation of 16Sr DNA, bacteria with probiotic capabilities from the digestive tract of *Pterophyllum scalare* and evaluate their ability to adhere to the epithelium intestinal using immunohistochemical techniques and bacteriological analysis.

2. Materials and methods

2.1. Isolation of microorganisms of digestive tract de *Pterophyllum scalare*

A batch of 200 healthy young fish (15 cm in length) of *P. scalare* (angel fish) was obtained from a production center in Xochimilco, Mexico City. The fish were introduced to a growth tank equipped to hold them during an acclimation period of 15 days under the same growth conditions of the production center: 28°C, pH 7, 5 mg/L dissolved oxygen and 0.3 ppm of nitrates and nitrites. Once the acclimation period had passed, the fish were starved for 24 hours. Next, 20 fish were randomly taken and dissected with a cut above the lateral line from the operculum to the base of the caudal fin. The digestive tracts of the fish were extracted and homogenised in 90 mL of sterile saline solution. They were diluted ten-fold and inoculated in 0.1 mL aliquots onto MSR, BHI and TCBS agar plates in triplicate. The plates were incubated at 35°C for 24 h. After the incubation was done counting colony forming units for each dilution (CFU / mL), was characterized colony morphology and subsequent reseeding strains were purified. Immediately was performed Gram staining to observe cell morphology using an Olympus microscope SZX12. Additional biochemical tests were performed (mobility, cytochrome C, glucose fermentation oxide, catalase, Voges Proskauer and indole) prior to molecular identification by DNA isolation 16Rs.

2.2. Tests to characterise a microorganism as probiotic

2.2.1. Resistance to acidic pH

To show the resistance of the bacteria to acidic pH, the gastric barrier was simulated by placing the isolated microorganisms in acidic growth media with pH values of 1.5, 2.5 and 3.0, and the strains that did not survive these stress conditions were discarded.

2.2.2. Growth in bile salts

To perform the growth in bile salts test, three 150 mL Erlenmeyer flasks were each filled with 100 mL of MRS broth plus 0.1%, 0.5% or 1.0% fresh bile. The flasks were inoculated with 1 mL of the microorganism strains that survived the acidic conditions and were incubated at 37°C for 3 h. The viability of the culture in MRS (Oxoid) broth medium was used as a control.

2.2.3. In vitro antagonistic capability

The strains that yielded positive results in the previous studies were used in vitro inhibition tests. For this experiment, was used the pathogen *Aeromonas hydrophila* ATCC356554A and

was seeded in triplicate onto BHI agar plates, which were incubated for 24 h at 30°C. Next, using the well diffusion method, 70 μL of a suspension of the beneficial strains isolated in sterile water was added, with concentration of CFU 10^7 (colony forming units per mL). The plates were incubated for 24 h at 30ºC, after which we observed the formation of inhibition halos. The strains that showed halos larger than 2 mm were considered positive.

2.3. Molecular identification of bacteria with the isolation of 16Sr DNA

2.3.1. DNA Isolation

The Wizard Genomic DNA Purification Kit (Promega™ Madison, U.S A) was used to extract genomic DNA for the molecular identification of the bacteria that showed probiotic capabilities, following the manufacturer's instructions.

To determine the purity and integrity of the genomic DNA of interest, samples were subjected to electrophoresis in a 1% agarose gel.

2.3.2. Polymerase Chain Reaction (PCR)

PCR was performed with the isolated genomic DNA of the bacteria that showed probiotic capabilities using the universal primers 9F (5'-GAGTTTGATCCTGGCTCAG-3') and E939R (5'- CTTGTGCGGGCCCCCGTCAATTC-3') in a Biometra® TGradient thermocycler under the following conditions: pre-incubation at 95°C for 10 minutes; 30 cycles of denaturation 124 at 95°C for 30 seconds, hybridisation at 55°C for 30 seconds and elongation at 72°C for 1 minute; and refrigeration at 4°C. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen), following the manufacturer's instructions. Finally, the genetic sequence of each strain was determined and compared to sequences in the GenBank database using the similarity search program BLAST.

2.4. Determination of the location and permanence of the probiotic bacteria in the digestive tract of *P.scalare*

The fish were fed with the isolated probiotic bacterial strains to establish the strains' adhesion capabilities. The genomic DNA analysis indicated that these microorganisms were three different strains of the *Bacillus* genus, which were assigned the labels *Bsp1*, *Bsp2* and *Bsp3*.

2.4.1. Preparation of the probiotic strains

A sample of each bacillus was taken with a bacteriological loop, and each sample was seeded into 500 mL of TSA broth and incubated at 30°C for 48 h or until there was a starting concentration of 107 CFU/mL. A Jenway 6400® Spectrophotometer with a 620-nm wavelength was used to measure the required bacterial concentration, and CFU/mL counts were performed. The relationship between the values obtained with spectrophotometry and the number of CFU/mL was determined according to the method established by Gullian (2001).

2.4.2. Feeding the fish with Artemia enriched with the isolated bacteria

Four fish tanks (60L) were prepared with 20 fish each and were kept at 28ºC and pH 7, with 5 mg/L of dissolved oxygen and a 0.2 ppm nitrite concentration. The fish were fed daily for 60 days with *Artemia franciscana* adults (50 *Artemia* per fish) enriched with 2 x 107 CFU/mL of each of the probiotic strains.

The fish were distributed in each of the four tanks arranged in the following way. Tank 1 was used as a control in which the fish were fed with *Artemia* adults without probiotics. The fish in tanks 2, 3 and 4 were fed with *Artemia* enriched with the *Bsp1, Bsp2* and *Bsp3* strains, respective,, each treatment was performed in triplicate. Food residues and faeces were removed from the fish tanks to maintain the quality of the water, and the physicochemical parameters were monitored (temperature, pH, dissolved oxygen, nitrites and nitrates) using a Hach DR/850 colourimeter.

2.4.3. Incorporation of the probiotic strains into Artemia franciscana adults

To incorporate the bacteria into the fish, 50 *Artemia franciscana* adults were placed in 200 mL of 149 sterile water that had been inoculated with 3 mL of the bacterial strains, to a concentration of 1×10^7 CFU/mL, for 30 min. After, an Olympus ZX12 stereo microscope was used to verify that the digestive tract of *Artemia* was completely filled with the bacteria. Next, the sample was passed through a light sieve with a 2.0-mm grid aperture size, and *Artemia* were fed to the fish.

2.4.4. Bacteriological analysis of the GIT of P. scalare during feeding in probiotics

The location and viability of the probiotics within the digestive tract of the fish were evaluated by analyzing bacteriological a portion of the GIT every 15 days for the 60 days of the administration of bacteria in the diet, using the methods of Riquelme et al. (2000).

2.4.5. Analysis of the faecal matter samples

After discontinuing the bacillus-containing feed, a bacteriological analysis of the faeces was performed to establish the permanence time of the bacteria in the digestive tract. Each week, 10 to 50 mg of faecal matter from the fish was sampled, and the presence of the administered strains was determined by quantifying them with the seeding of decimal dilutions into specific culture media (Thitaram et al., 2005). Twenty-four hours after incubation, the CFU were counted, and the morphology and Gram staining characteristics were corroborated for each bacterial group. All of the tests were performed in duplicate, and counting was performed during the 10 weeks following cessation of feeding with bacilli-enriched food.

A database was created in Excel that contained the bacterial count (CFU/mL) data from the microbiological analysis of the GIT and faeces, and descriptive statistics techniques, along with an analysis of variance (ANOVA), were applied to obtain the mean and

standard deviation. When significant differences were found between the treatments (<0.005), the multiple means test with the Tukey method was performed with Systat 10.2 software.

2.5. Immunohistochemistry

Cross-sections of the intestinal tissue of the fish were removed for the immunohistochemistry analysis. The samples were placed in 10% formaldehyde in phosphate-buffered saline (PBS). Once the samples were fixed, they were processed using routine histology techniques and placed in paraffin, and 5µm cuts were made. The cuts were pre-treated with 3% 3-aminopropylethoxysilane (Sigma Laboratories). Next, the tissue sections were dewaxed at 60°C for 10 minutes, and three xylol washes of 5 minutes each were immediately performed. The tissue sections were soaked in 10% alcohol and washed twice with 70% alcohol, and a final wash with distilled water was performed for five minutes. An Immuno Cruz Staining System (Santa Cruz Biotechnology, USA) was used for Immunodetection, following the manufacturer's instructions. As a primary antibody, anti-*Bacillus*. (HRP) was used at a 1:20 dilution (Affinity Bioreagents, USA), and Grill's haematoxylin was applied for five seconds as a contrast medium.

2.6. Growth assessment of *P. scalare* fed probiotic strains isolated

In the laboratory, was prepared 15 aquaria (40 L) with 20 fish each, which were maintained for 15 days in a period of acclimation. Later the fish were fed daily for 60 days with *Artemia* adults (50 Artemia / fish) inoculated with 2 x 107 CFU/ mL of the isolated bacteria. The fish were distributed in each of the aquaria arranged as follows: the treatment 1 is assigned as a control, in this; the fish were fed *Artemia* adults without probiotics, treatment 2 to 4 were fed with enriched *Artemia* with *Bsp1, Bsp2* y *Bsp* respectively and treatment 5 fish fed with a combination of these. There were three replicates per treatment. To evaluate the growth of the fish were taken every 15 days biometric parameters (length, height, width and weight). A biometric data tests were applied descriptive statistics for the mean and standard deviation are also performed an analysis of variance (ANOVA). When significant differences were found between treatments (<O.OO5) was tested multiple mean comparison by Tukey method, with the program Systat 10.2. Also we calculated condition factor (Km), for which we used the following equation:

$$\text{Condition Factor Km} = 100\ (W) / L^3$$

3. Results

3.1. Bacterial isolation

A total of 108 strains were isolated from the digestive tract of *P. scalare*, only 20 of which grew in an acidic pH in the presence of bile salts.

3.2. *In vitro* inhibition activity

Only 20 of the strains resisted the acidic pH and bile salts conditions, and 3 showed the ability to inhibit *Aeromonas hydrophila*. However, no significant differences were observed between the three strains because in all cases, inhibition halos with mean values between 19 and 24 mm were formed (Figure 1a and b).

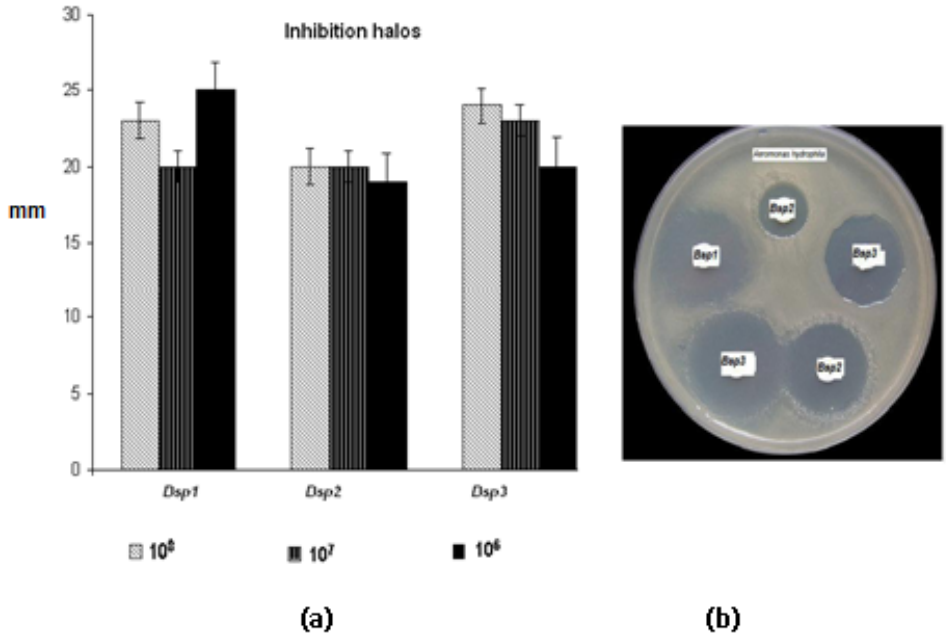


Figure 1. *In vitro* inhibition halos of *A. hydrophila* with the *Bsp1, Bsp2* and *Bsp3* strains, 10 with mean values between 19 and 24 mm.

3.3. Molecular identification of the isolated probiotic strains of *P. scalare*

The genomic DNA sequence obtained from strain 1 was composed of 885 bp (Figure 2) and coincided with 22 types of *Bacillus sp*. and one type of *Acetobacter pasteurianus*, all with 99% sequence homology. Strain 2 yielded a sequence of 860 bp, which coincided with 51 types of *Bacillus sp*. and *Acetobacter pasteurianus*, all with 99% homology. The 900 bp sequence of strain 3 matched 100% with the synthetic construct of *Bacillus sp*. clone and showed 84% agreement with *B. weihenstephanensis.* Therefore, the three strains could only be assigned with certainty at the genus level to *Bacillus* and were labelled *Bsp1, Bsp2* and *Bsp3* (Figure 3).

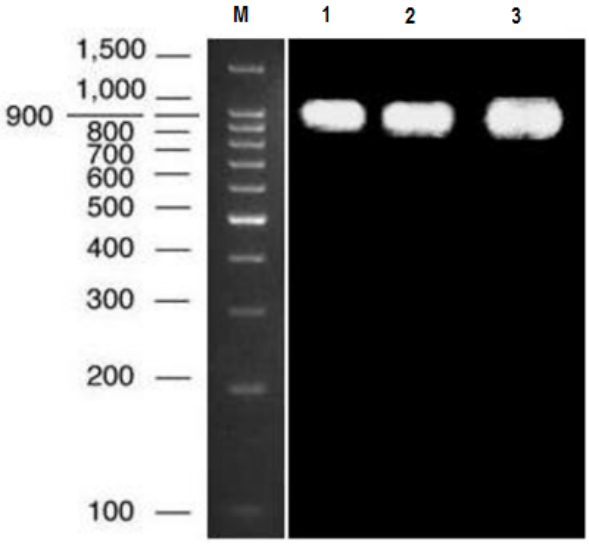


Figure 2. Comparison of the PCR product bands with the 9F and E939F universal primers from the three strains to the 100 bp molecular marker from Promega™ (M). Line 1 *Bsp1*, Line 2 *Bsp2*; Line 3 *Bsp*3.

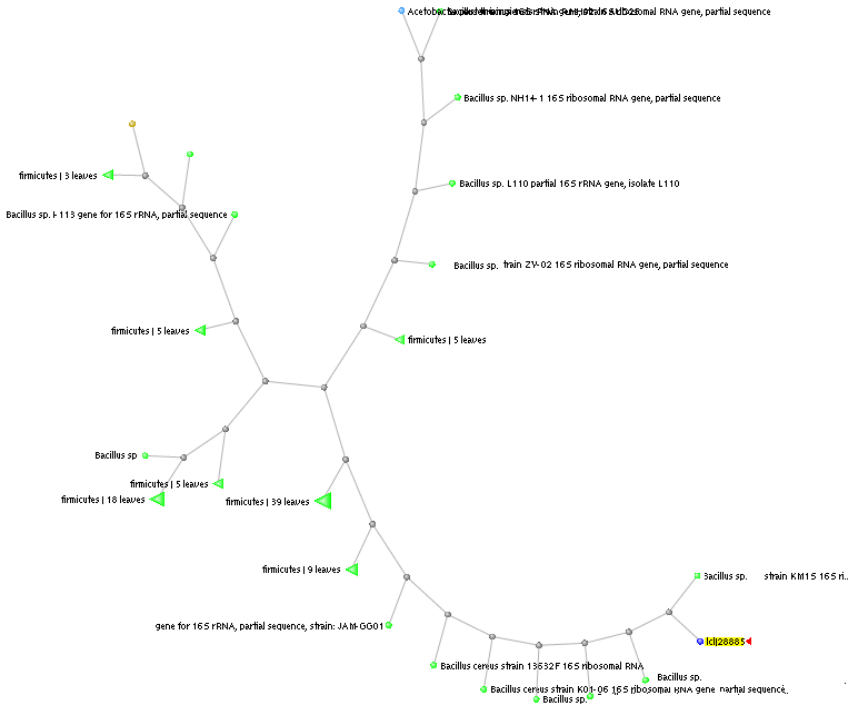
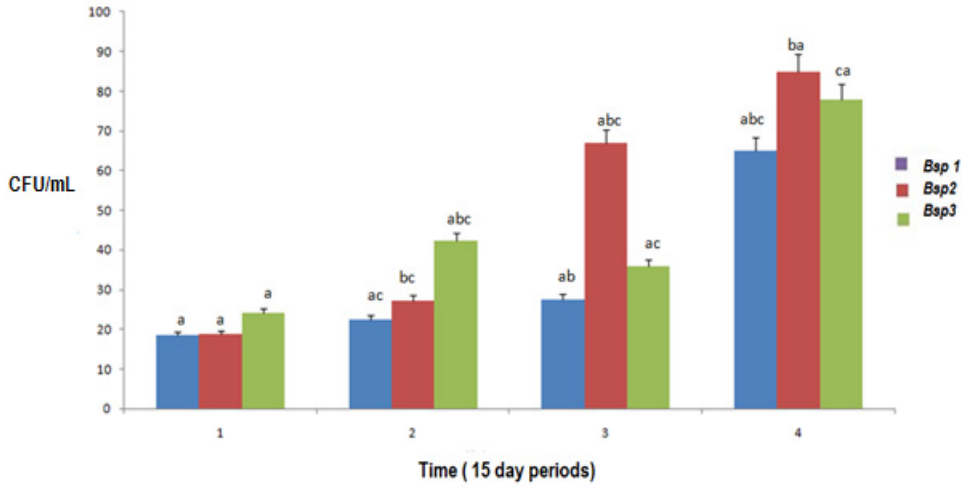


Figure 3. Phylogenetic tree of the *B. sp3* strain. Euclidean distance O.75

3.4. Colonisation and permanence of *Bacillus sp.* strains in the epithelial tissue *of P. scalare*

3.4.1. Bacteriological analysis of the digestive tract of P. scalare

The bacteriological analysis of the digestive tract of the fish during feeding with the different strains of *Bacillus* indicated that the three strains colonised the digestive tract of *P. scalare*, which was visible when we isolated the characteristic morphotypes of the bacteria supplied in the TSA media. Over the course of the experiment, it was established that the *Bsp2* strain showed the highest mean CFU/mL values (Figure 4).

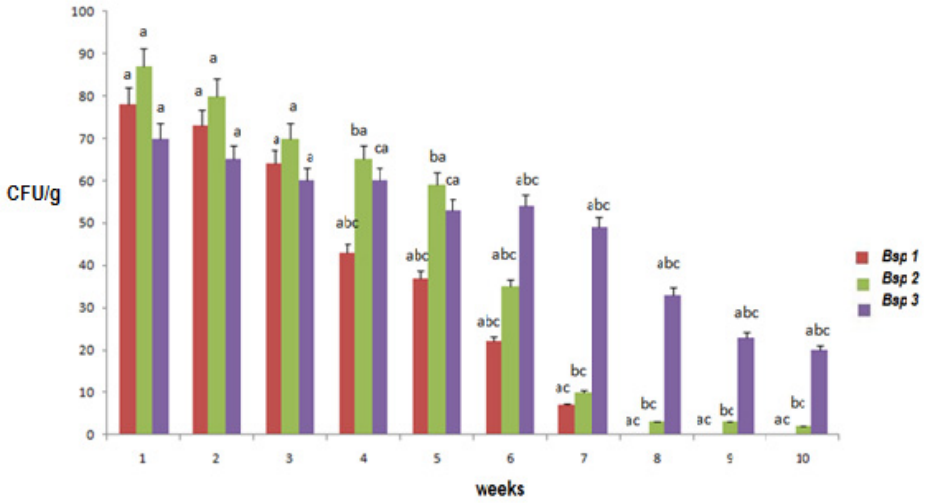


*Different letters show significant differences between the groups at each time point ($p<0.05$).

Figure 4. CFU/mL counts of the probiotic bacteria in the digestive tract of *P. scalare* over 60 days (four 15-day periods).

3.4.2. Bacteriological analysis of the faeces

During the bacteriological analysis of the faeces, it was established that the *Bsp3* strain had a high degree of colonisation and competition in the digestive tract of *P. scalare* because mean counts above 120 CFU/mL were obtained up to the sixth week. After concluding the feeding tests, the *Bsp3* strain was observed up to the tenth week, whereas the *Bsp1* and *Bsp2* strains had CFU/mL counts with mean values of 70 and 30, respectively, in the sixth week. From the eighth weeks on, no colonies characteristic to these strains were obtained, and bacterial growth of a different morphotype was observed (Figure 5).

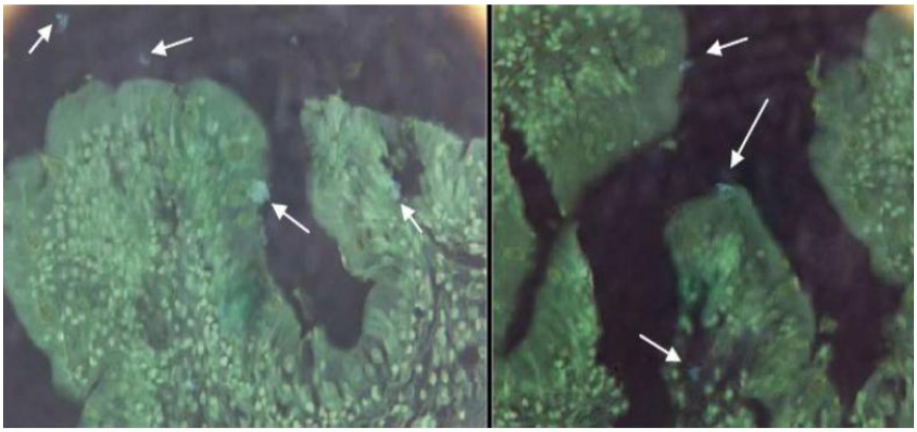


* Different letters show significant differences between groups at each time ($p < 0.05$)

Figure 5. Counts of CFU/mL of faeces of *P. scalare*, ten weeks after discontinuing feeding of fish

3.5. Immunohistochemical analysis

In the figure 6a and b, shows the presence of the probiotics supplied to the fish. Was observed in histological cuts labeled with *Bacillus* antibodies in the intestinal lumen and on the edges of the microvilli to positive marking, a dark filter was used in these images.



* The arrow indicates the Immunolabelling positive. To highlight marking, a dark filter was used in these images.

Figure 6. 6a and b. Location of probiotics in transverse sections of digestive tract marked with antibodies to *Bacillus*, in the microvilli and in the gut lumen.

3.6. Survival and growth of *P. scalare*

3.6.1. The survival of fish fed the probiotic strains was 100% compared with 80% survival of fish fed without probiotic.

3.6.1.1. Total length

The analysis of variance for total length indicated that there are significant differences between treatments (F = 15,656, df = 4, P <0.005). When making multiple mean comparison by Tukey test, it was found that treatment of fish fed *Bsp3* achieve the highest total length (4.5 cm), while fish in the control group received only a length of 3 cm (Figure 7).

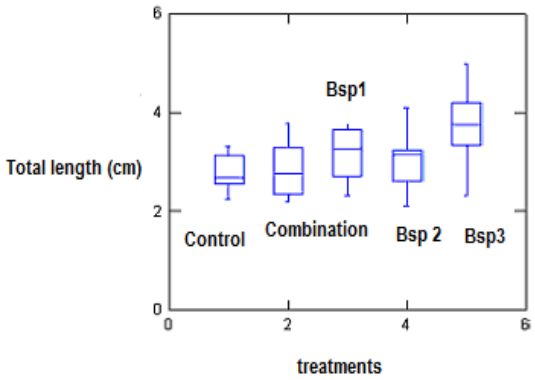


Figure 7. Comparison of the total length of fish between treatments.

3.6.1.2. Width

In regard to width of the fish we observed no significant differences between treatments fed with probiotics which reached values of 1.10 and 1.25 cm, however if there are differences with the control values obtained as 0.63cm (Figure 8).

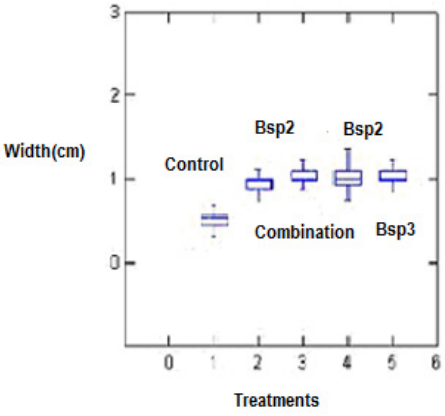


Figure 8. Comparison of the width of the fish between treatments.

3.6.1.3. Weight

With regard to weight, the analysis of variance indicated significant differences between treatments (F = 17,394, df = 4, P <0.001). In the analysis of multiple means by Tukey's method shows that the treatment provides greater weight is *Bsp3*, with an average weight of 1.90 g, while the combination and the control group provided weights below 1 g (Figure 9).

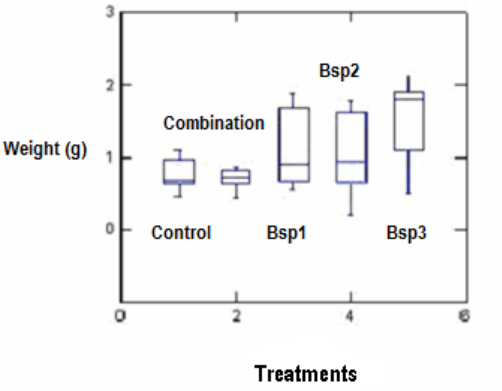


Figure 9. Comparison of the variation in weight of fish with different treatments.

3.6.2. Condition Factor (Km)

The results of the Condition Factor indicate that fish fed *Bsp2, Bsp3* strains, and the combination, get better a weight - length relationship to obtain values above the initial Km compared to fish fed *Bsp1* strain and the control, that values were below the initial km (Figure 10).

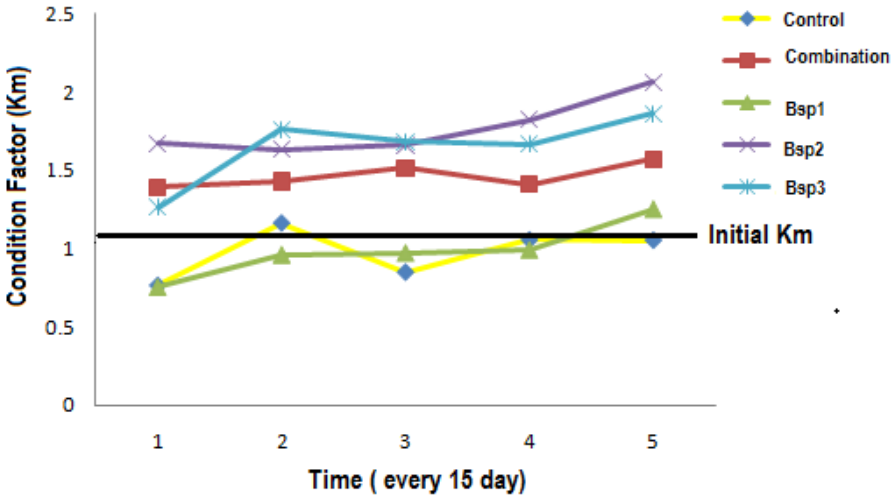


Figure 10. Condition factor of fish fed the different probiotic strains

4. Discussion

The results obtained from the molecular analysis place the three bacterial strains isolated in this work in the Bacillus genus. Although there have been studies on the use of bacteria from this genus as probiotics, there are no reports of its isolation from the digestive tract of fish, with the exception of the work of Gullian et al., (2004) in which the presence of this genus in shrimp (*Penneus vannamei*), is mentioned.

The use of universal primers such as 9F and E939R of 16S rDNA proved to 278 be adequate to amplify the 16S rDNA of the unknown strains. These results agree with those of Heyndrickx et al. (2004) and Rodicio & Mendoza (2004). The analysis of the 16S rDNA sequence of the different phylogenetic groups revealed the presence of one or more characteristic sequences, which are denoted signature oligonucleotides: short, specific sequences that are found in all (or most) of the members of a particular phylogenetic group and are never (or only on occasion) present in other groups (including the closest ones). However, despite the certain inclusion of the three stains in the *Bacillus genus*, not a single one could be identified at the species level, due to variations that were found in their sequences with respect to the sequences of known species. This identification difficulty is in agreement with the results reported by Woo et al. (2008), who explain that this variation can occur when isolating 16S rDNA because when two different bacterial species share almost all of their 16S rDNA sequence, this technique is not able to distinguish between the two; only the genus can be determined with certainty. These results imply that these could be previously unidentified species because there is no report of their isolation in samples from the digestive tract of fish. In the present study, the bacteriological analysis showed that the three probiotics were capable of colonising the digestive tract. However, there were differences in the number of cells from the 30th day of the experiment, where the number of strain *Bsp3* cells was higher than the others; however, at 45 days, the *Bsp2* strain had higher counts, averaging 65 CFU/mL and dominating both of the other two strains until the end of the experiment. These higher counts indicate that the *Bsp2* strain was better to colonize the digestive tract of the fish ($p< 0.05$) and will thrive as long as this probiotic is provided. Studies performed with aquatic organisms have also shown that, when supplying different strains of probiotics, even if they all colonise, there will always be one strain that dominates or varies its number of cells over time (Gildberg et al., 1997; Ringo and Vadstein, 1998;Ringo & Olsen., 1999; Rengpipat et al., 2000; Nikoskelainen et al., 2003; Gullian *et al*., 2004; Macey & Coyne, 2006;). When testing the persistence of probiotics in the digestive tract of the fish, the *Bps3* strain maintained a higher cell count up to the tenth week after suspending the food-containing probiotics. The permanence of the probiotics in the faeces evidenced the great colonizing power of the digestive tract of the fish in contrast with other aquatic organisms, such as the *Abalone* mollusc, which show a marked decrease in probiotic cells during the first and second days after ceasing probiotic feed and show low amounts of these cells ($p<0.05$) in their faeces 15 days later (Macey & Coyne, 2006).

The immunodetection tests performed confirmed the presence and location of the *Bacillus* bacteria added to the fish food (*Artemia*), displaying positive markings in the microvilli and in the intestinal lumen of the front part of the angelfish intestine. Makridis et al. (2001) also showed with immunohistochemical techniques that there was Vibrio in the lumen and in the microvilli of the intestinal tube of *Hippoglossus hippoglossus* (sheer) fish larvae up to 10 days later after providing the bacteria, which were also bioencapsulated in *Artemia*. According to the results obtained in the growth of fish fed with the probiotic bacteria isolated in this study, we observed that the use of food fish was higher in treatments in which they contain added probiotic strains, especially with Bs3 strain in which the fish were much higher growth in total length, weight and width (with almost 50% increase compared to the control group and the combination of probiotic strains). These results agree with the study by Ghosh et al., (2008), which reported significant differences in the growth of ornamental fish species *Poecilia reticulata*, *Poecilia sphenops*, *Xiphophorus maculatus* and *Xiphophorus hellieri*, after being fed with feed enriched *Bacillus sp* for a period of 60 days, compared with a control treatment without probiotic.

5. Conclusion

The genetic sequence of probióticos strains isolated from *P scalare* only allowed us locate these bacteria within the genus Bacillus, because it was not possible to identify the specie, due to the variations found in the sequences of the three strains with respect to the sequences of species known until today.

The three strains of Bacillus (*Bsp1, Bsp2* and *Bsp3*) survived the gastric barrier of the intestine and had high colonization of the intestinal epithelium as well as the ability to inhibit *Aeromonas hydrophila in vitro.*

The Bacillus *Bsp3* promoted better growth in *P scalare:* total length, width and weight with almost 50% compared with control fish.

The results of this work show that the three strains used are capable of colonizing the digestive tract of angelfish. The *Bsp2* strain has the greatest capacity, although the Bsp3 strain remains longest. Thus, it could be proposed to ornamental fish producers, specifically those that grow angelfish, to use mixed *Bsp2* and *Bsp3* strains to achieve better results and indicate them the time required to provide the food probiotics again.

Although other studies have reported that the combination of probiotics provides better results in terms of growth, but in this study the combination did not give better results than those obtained with single strain.

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