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INHIBITION OF *Aeromonas hydrophila* BY PROBIOTIC STRAINS ISOLATED FROM THE DIGESTIVE TRACT OF *Pterophyllum scalare*

INHIBICIÓN DE *Aeromonas hydrophila* POR CEPAS PROBIÓTICAS AISLADAS DEL TRACTO DIGESTIVO DE *Pterophyllum scalare*

M.C. Monroy-Dosta[1*], T. Castro-Barrera[1], F.J. Fernández-Perrino[2] and L. Mayorga-Reyes[3]

[1] *Laboratorio de Alimento Vivo, Departamento El Hombre y Su Ambiente, Universidad Autónoma Metropolitana-Xochimilco, Calzada del Hueso 1100, Colonia Villa Quietud, C. P. 04960, México, D. F.*
[2] *Departamento de Biotecnología,Universidad Autónoma Metropolitana-Iztapalapa Av. San Rafael Atlixco 186 Colonia Vicentina. C. P. 09340 México D. F.*
[3] *Departamento de Sistemas Biológicos, Universidad Autónoma Metropolitana-Xochimilco, Calzada del Hueso 1100, Colonia Villa Quietud, C. P. 04960, México, D. F.*

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Abstract

The effect of three different probiotic strains of *Bacillus* (B1, B2 and B3) isolated from the digestive tract of *Pterophyllum scalare* against *Aeromonas hydrophila* was evaluated. All the fish that where fed with the different probiotic strains obtained higher resistance to the pathogen inoculation since they did not developed any signs of illness nor lesions and they stayed healthy until the end of the experiment, with an observed survival of 100 %. On the other hand, the fish where the probiotic bacteria were not administrated developed the infectious process caused by *Aeromonas hydrophila* with signs of illness and lesions in the gills and hemorrhagic eyes, irregular swim, and injuries on the skin. The survival in these treatments was barely up to 8 %.

Keywords: *Aeromonas hydrophila*, *Bacillus sp*, Probiotics, *Pterophyllum scalare*.

Resumen

El efecto antagonista de tres diferentes cepas probioticas de *Bacillus* (B1,B2 y B3) aisladas del tracto digestivo de *Pterophyllum scalare* frente a Aeomonas hydrophila fue evaluado. Todos los peces que fueron alimentados con las diferentes cepas probióticas obtuvieron alta resistencia a la inoculación del patógeno y no mostraron signos de lesiones o enfermedad y permanecieron saludables hasta el final del experimento con una sobrevivencia del 100 %. Los peces que no fueron alimentados con probióticos desarrollaron el proceso infeccioso observándose signos y lesiones de enfermedad como branquias y ojos hemorrágicos, nado irregular y lesiones en la piel. La sobrevivencia en estos tratamientos fue escasamente del 8 %.

Palabras clave: *Aeromonas hydrophila*, *Bacillus sp*, probióticos, *Pterophyllum scalare*.

1. Introduction

During the last few years, several papers have been directed to the use of probiotic microorganisms with the objective of reducing the amount of diseases during the culture of ornamental fish and restrict or reduce the use of antibiotics, as these compounds have provoked bacterial resilience, destruction of ecosystems, and high cost of production (Westerdahl *et al.*, 1991; Maeda 1994; Abraham *et al.* 2001; Nikoskelainen *et al.*, 2003). The probiotics are microorganisms that adhere to the gastrointestinal tract forming a thin biofilm and have benefic effects on the host, including improvements on digestion, immunity, and resistance

* *Corresponding author. E-mail*: monroydosta@hotmail.com
Tel. (52) 55-5483-7151 Fax (52) 55-5483-7469

against diseases as they produce substances like bacteriocins, acetic acid and lactic acid that inhibit the proliferation of pathogenic bacteria (Rengpipat *et al.*, 2000; Irianto and Austin, 2002; Vine *et al.*, 2004; Gullian *et al.*, 2004; Balcazar *et al.*, 2006).

Amongst the most studied probitic strains, we find: the lactic bacteria, bifidobacteria, and yeasts (Abraham *et al.*, 2001; Singh *et al.*, 2001; Jameson, 2003). However, one of the problems on the use of probitics is the method to select them, according to Gómez and Roque (1998) in most of the cases this process is based only on empiric observations and with limited scientific evidence as the bacteria used on fish culture are isolated from the digestive tract of humans or other mammals. Thus, the use of strains isolated from fishes might be an interesting possibility to obtain better results. The objective of the present work was to evaluate the response of Pterophylum scalare fed with three strains of *Bacillus* (isolated from fish) against *Aeromonas hydrophila*, a common bacteria found on infectious processes in aquatic organisms.

2. Materials and methods

2.1. Microorganisms

The three strains that were used in this study were previously isolated from the digestive tract of health fish *P. scalare* in the laboratory. It is noteworthy that the molecular evidence to date indicates that these are three different strains of *Bacillus*, so to ease the handling during the experiment they were assigned with the nomenclature B1, B2 and B3.

2.2. Isolation and identification of A. hydrophila obtained from the fish's kidney.

The strain of *A. hydrophila* was isolated from an ornamental fish farm during an infectious process. Samples of kidney and injuries on the skin, gills, fins, and eyes were collected and were placed in Petri dishes with TBCS agar. Colonies were purified by successive re-seeding in BHI agar until a homogeneous cellular morphology was obtained. Gram staining and some confirmative biochemical test were performed (motility, citocrome C, glucose oxide fermentation, NaCl tolerance, catalase and resistance against the vibrostatic agent 0/129).

The molecular identification of *A. hydrophila* was done by DNA isolation (16s rDNA) with the DNA-EASY kit (Qiagen) by following the manufacturer's instruction. To establish the presence of *A. hydrophila* in the samples, PCR technique was performed by using the sequence oligo Aer8-5'-TGCTGGCTGTGACGTTACTCGCAG-3' and Aer9-5'-TTCGCCACCGGTATTCCTCCAGATC-3' (Martinez-Murcia *et al.*, 1992). Amplification reactions were done on a thermocycler (Amplitron II Thermolyne Barnstead International) under the following conditions: pre-incubation: 95°C during 10 min; denaturalization: 30 cycles at 95°C for 1 min; aligning: 68°C for 1 min, extension: 72°C for 30 seconds and pause of 4°C. The PCR products were analyzed on 1% agarose gel with a photodocumentator GelDoc 2000 (Bio-Rad, Hercules, California). A DNA sample of the strain *A. hydrophila* ATCC356554A was used as positive control.

2.3. Probiotic preparation

A sample of each *Bacillus* was added to 500 mL of TSA broth, incubating them at 30°C for 48 hrs or until achieving a 10^7 CFU/mL concentration. To measure the required bacterial concentration, a JENWAY 6400® spectrometer was used using a 620nm wave length. Also an CFU/mL count was done. The relationship between the obtained value by spectrometry and the CFU/mL was done according to Gullian *et al.* (2004).

2.4. Enrichment Artemia franciscana adults

In a 200 mL beaker previously sterilized, 50 adults of *A. franciscana* were placed and 10mL of the different probiotic strains were added at a concentration of 10^7 CFU/mL. Enrichment was performed during 30 min with continuous aeration and after this period, the adults of *Artemia* were observed on a stereoscopic microscope to ensure that digestive tract was full with the bacteria, and them Artemia was washed with tap water and fed to the fish. Same procedure was followed to enrich the Artemia adults with the rest of the probiotic bacteria and the pathogen *Aeromonas hydrophila*.

2.5. Treatments

150 healthy juvenile fish (that not presented signs of infection or lesions) of the species *Pterophyllum scalare* were used. After a period of acclimatization of 30 days the experimental phase began. In

12 aquariums, each with a 40L capacity, 10 juvenile fish were distributed, on each of the aquariums at temperature of 28°C, pH 7, and 5 mg/L of dissolved oxygen and afterwards the following treatments were carried out:

Treatment 1 (control): Fish fed with *Artemia* adults (without any probiotics and *A. hydrophila*) during 30 days.

Treatment 2: Fish fed with *Artemia* adults inoculated with *A. hydrophila*.

Treatment 3: Fish fed with the combination of the probiotic bacteria (B1, B2y B3) during 7 days, on the eighth day *A. hydrophila* was inoculated in the *Artemia* and then fed to the fish.

For treatments 4, 5, and 6, the fish were fed with *Artemia* adults enriched with the different probiotic bacteria and *A. hydrophila* was administrated in the same way that in the previous treatment.

2.6. Characterization of the signs of injuries observed on the fish

24 hrs after the administration of the pathogen, mortality, changes in behavior, and signs of injuries on the skin were observed carefully, with the objective of developing a clinic history.

2.7. Pathogen recovery

To ensure that *A. hydrophila* was the actual agent that produced the infection and/or the fish's death, samples were taken from the injuries and kidneys of the diseased animals. Samples were placed on a TCBS medium and were isolated afterwards on BHI medium. The presence of *A. hydrophila* was confirmed on samples using the PCR technique described previously.

2.8. Statistical analysis

To determine which organ of the fish were the most affected by the inoculation of *A. hydrophila* a discriminate analysis was performed. The following variables were considered: coloration, skin, scales, fins and tail, mouth, gills, eyes, body, swimming, behavior, digestive tube, kidney, liver, swimming bladder, bile vesicle, heart, gonads and the grade of injuries: 0 when no damage or injuries were observed, 1 was associated with minor damage, 2 with major damage and 3 with severe damage.

Table 1. Signs and lesions observed in fish fed *Artemia* inoculated with *A. hydrophila* (10^7ufc/mL) and without administered probiotics

Affected organs	Signs and lesions
**Skin	Ulcerated, discolored, with mucus
Scales	Eroded, desquamation
Fins and tail	Hemorrhagic
Mouth	Open
**Branchia	Hemorrhagic
**Eyes	Haemorrhage, exophthalmia
Body	*
Appetite	Anorexia
Behavior	Inmmobile
**Swimming	Erratic
Digestive tract	Inflamed
Kidney	Hemorrhagic
Liver	*
Gallbladder	*
Bladder	Destroyed
Heart	*

* No signs were shown
** The most common signs of infection

3. Results

3.1. Signs and lesions presented by the fish

24h after the pathogen administration, the infectious process began in the treatments where no probiotic was administrated. Characteristic signs of lesions and illness that were observed are shown on Table 1. When making the statistical analysis using the Canonical discriminant functions (standardized by within variances), it was considered that the infection signs that developed the most after the pathogen administration were: hemorrhagic gills and eyes, irregular swim and skin lesions.

3.2. Survival

The inoculation of *A. hydrophila* in the fish that were not fed with the probiotics caused a mortality of more than 90 % of the organisms, while the fish tried with the different *Bacillus* strains resisted the pathogen administration better with a survival between 88 and 100 % (Fig. 1).

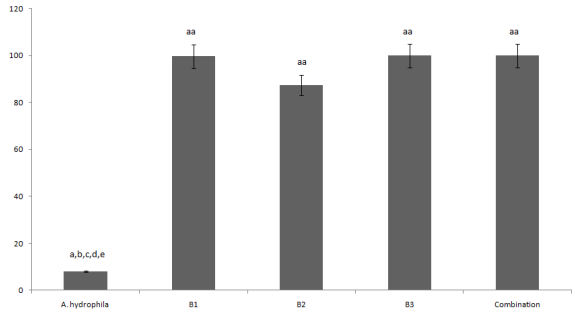

Fig. 1: Survival of *P. scalare* fed probiotic strains and inoculated with *A. hydrophila*.

3.3. Pathogen recovery

A. hydrophila was found in every sample gathered from the infected lesions. This microorganism was identified by PCR by amplifying a 400-bp fragment that corresponded to an *A. hydrophila* positive control, thereby confirming the presence of the pathogen and its relationship with the infectious process in the fish not fed the probiotic strains (Figs. 2 and 3).

4. Discussion

Different studies have demonstrated the capacity of different microorganisms to improve the fish survival during their culture (Gatesoupe, 1994; Gullian *et al.*, 2004; Venkant *et al.*, 2004; Bagheri *et al.*, 2008). In agreement with the present work the use of the probiotic strains B1, B2, and B3, isolated from the digestive tract of *P. scalare*, used in an individual way or in a combined way they remarkably improved the survival of this cichlid, showing higher resilience to the inoculation of *A. hydrophila*. The survival rate was of 100 % in the treatments B1, B3, and the combination of the three bacteria. These results improved those obtained by Martínez *et al* (2008) who infected tilapias with different pathogens, after the challenge with the pathogenic bacteria, the highest survival was obtained in those treatments that the supplement *Bacillussp.* and *Lacto Bacillus casei* in comparison the treatment that was not given any bacteria (control). Gatesoupe (1994), improved the survival of *Scophthalmus maximus* larvae when gave them acid lactic bacteria; Lara (1999) carried out an investigation on the effect of three different probiotics fed to *Tilapia nilótica* (*Oreochromis niloticus*) subjected to different stress conditions, obtaining the best results in growth and survival with the addition of *Saccharomyces cerevisae*.

On investigations on the use of probiotics for the pathogens exclusion in aquaculture Aly *et al.*,

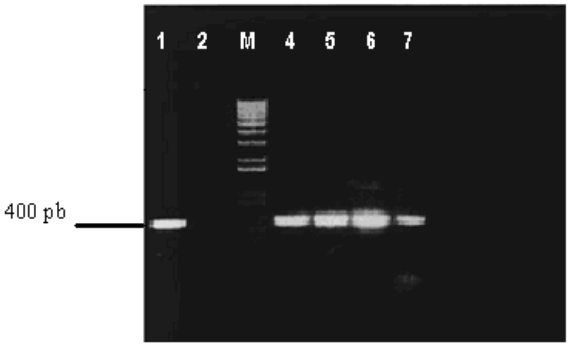

Fig. 2: PCR for the detection of *Aeromonas hydrophila* 1) positive control, 2) negative control, 3) molecular weight marker, 4 -6) amplified DNA fragments of *A. hydrophila* isolated from kidney fish.

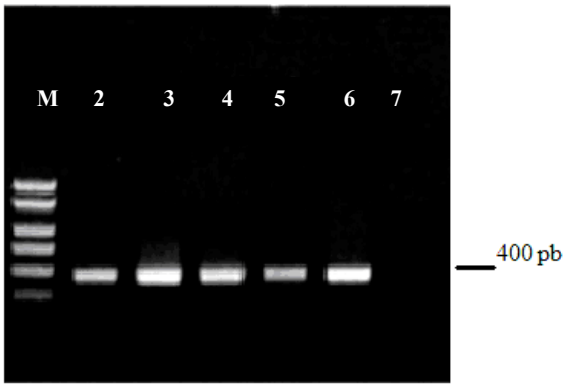

Fig. 3: Identification of *Aeromonas hydrophila* 1) molecular weight marker, 2) positive control, 3 and 4) samples of branchia lesions, 5) sample of digestive tract, 6) sample of kidney, 7) Negative control.

(2008), mention the exclusion of *Aeromonas hydrophila* with *Bacillus subtilis* and *Lacto Bacillus acidophillus* in *Tilapia nilotica*. However concerning to the use of specific probiotic strains for ornamental fish few studies have been made. This investigation reports the first advances on the use of bacteria isolated from the digestive tract of *P. scalare* with probiotic capacities; ornamental fish of great commercial importance that has been affected by infectious processes in those that frequently has been isolated bacterias like *A. hydrophila* (Cipriano *et al.*, 1984; Dixon and Issvoran, 1993, Baez *et al.*, 2008). The results obtained in this work are very encouraging because they demonstrate the live antagonistic effect of the three strains of *Bacillus sp.* setting them as specific probiotics for *P. scalare* due to the fact that they do not allow the development of the infection process in the treatments where this bacteria was

administrated, also, after concluding the experiment their presence was verified inside the digestive tract of the fish which confirms their capacity to adhere to the digestive tract and their antagonistic potential. Therefore it is recommended the use of this strains that where isolated to battle against illnesses like hemorrhagic septicemia caused by *A. hydrophila* which occupies a highlighted place due to the economical losses of those that produce species that are specially important such as salmons, flounders, basses or sea basses, amongst some other and a great number of ornamental species (Sumawidjaja *et al.*, 2001; Rodriguez, 2002; Harikrishnan and Balasundaram, 2005).

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