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Biological control of *Aeromonas salmonicida* in *Puntius conchonius* culture using probiotics under laboratory and fish farm conditions

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Abstract
The aim of the present study was to evaluate the use of probiotics bacteria as biological control of *Aeromonas salmonicida* in the culture of *Puntius conchonius* in laboratory conditions and farm. The results of this investigation show variations between treatments, because in laboratory conditions not development the infectious process, although in some cases it was observed injury and signs of diseases, these signs disappeared and the organisms were maintained alive until experiment finished. Opposite case in fish farming conditions, wherein the infectious process was developed in all treatments since 24 hours of being fed enriched *Artemia* with pathogen. However, in the treatments with the probiotic bacteria: *Citrobacter* sp, *Exiguobacterium* sp and *Enterobacter* sp, the fishes resisted infection and presented a survival of 100, 88 and 44% respectively. In the treatment where probiotic did not was supplied 100% mortality was observed.

Keywords: *Aeromonas salmonicida*, bacteria, probiotic, *Puntios conchonius*

1. Introduction

In Mexico around 43 million of freshwater ornamental fish are marketed, including farming of commonly known fish as Barbo rosi (*Puntios conchonius*), high demanded specie due to aesthetics shape, highly reproductive potential and easy handling [1], but because of its high demand has caused that is cultivated in high densities with different water quality conditions and has resulted in an increase of number diseases, focusing on indiscriminate use of therapeutic agents as most effective diseases control but propitiate the appearance of antimicrobial resistance [2, 3]. The bacterial genders that propitiate the common infectious processes in fish culture are: *Aeromonas, Vibrio, Pseudomonas, Yersinia*, *Flexibacter, Mycobacterium, Nocardia*, *Edwuarsiella* and *Pasturella* [4]. Specifically, the bacteria *Aeromonas salmonicida* causing furunculosis, a chronic disease that can kill 100% of cultured organisms, is very common in production systems [5]. Furunculosis is characterized by lethargy, and multiple hemorrhages in fins, anus and muscle tissue. Into the body fishes produced hemorrhages in liver, spleen and kidney necrosis.

One of implemented strategies for reducing pathogens in aquaculture is biological control using probiotic microorganisms, which administered in adequate amount through water or food vehicles can avoids the pathogens adhesion by competitive exclusion in the gut and enhance the nutrient assimilation and improve water quality culture system [6]. So manipulation of intestinal microbiota in aquatic organisms, using probiotic microorganisms, is a viable for decrease the diseases incidence in aquaculture production [7].

2. Materials and Méthods

2.1 Experimental fishes obtainment

For the present study 300 fishes of *Puntius conchonius* were obtained from México City Production Center.

2.2 Experimental design

The fishes were maintained in laboratory acclimation during 15 days, at a pH 7, 5 mg mL^{-1} oxygen concentration, 28 °C temperature and 0.3 ppm of nitrates and nitrites. The fishes were fed daily with biofloc and their behavior was observed during 15 experimental days.

2.3 Probiotic strains obtainment

The probiotic strains used in this research were isolated previously from *Puntius conchonius* intestinal tract and tested to characterize as probiotic in laboratory conditions and used in live challenge test.

The obtained bacterial strains were *Exiguobacterium* sp, *Citrobacter* sp and *Enterobacter* sp, which were suspended in Brain-Heart Infusion Suspension (BHI) liquid culture medium and maintained at $1x10^8$ CFU mL^{-1}.

2.4 Antagonism test in laboratory and fish farm

To evaluate the probiotic antagonism *in vivo* effect against pathogen bacteria *A. salmonicida*, 150 fishes were randomly distributed in 15 fish tanks of 40 L capacity (10 fishes per fish tank). The fishes were fed with enriched *Artemia* with the probiotic strains and subsequently were inoculated with *Aeromonas salmonicida* pathogen at $1x10^8$ CFU mL^{-1} concentration. Each treatment was made by triplicate. The experimental design has two control experiments: 1) *Artemia* without probiotic; and 2) Positive control (*Artemia* + pathogen without probiotic). The experimental duration was of 30 days. The same experimental treatments were made in a fish farming ornamental freshwater fishes in Guadalajara State in their own working conditions.

Once inoculated pathogen strain in fish tanks, daily monitoring was registered: mortality, disease signs and injuries. Samples of produced injuries and kidney were taken to verify by PCR the inoculated pathogen presence and determined the furunculous disease.

Processing data

The clinic picture data were capture in Excel 2010 base data and tested with analysis of variance (ANOVA) to determined significant differences ($P<0.05$) between treatments. When significant differences were observed a mean multiple comparison method with Tukey test statistical analysis was made by using Systat 10.2 program. At same time, a discriminate analysis test was made to stablish what kind of signs and injuries have biggest statistical weight in the development of infection process.

3. Results

3.1 Laboratory conditions

The infection process not developed in laboratory conditions, not even in treatment where pathogen was inoculated without probiotic presence. Only few fishes showed diseases signs like: skin lesions, fin frayed and hemorrhagic gills (Table 1). However, the fishes remained alive during all experiment period.

Fish farming conditions

In farm conditions were carried out extensive manipulation of organisms, the infection process begins after 24 hours of pathogen administration at different treatments. They showed characteristics disease signs. These were more evident as time progressed (Table 1).

Table 1: Signs and injuries frequency showed in infected fishes with *A. salmonicida* (10^7 UFC mL^{-1}) under laboratory and fish farming conditions.

Affected organs	Observed signs and injuries	Laboratory conditions				Fish farming conditions			
		Treatment frequency				Treatment frequency			
		Exiguobacterium	***Citrobacter***	***Enterobacter***	**Control**	***Exiguobacterium***	***Citrobacter***	***Enterobacter***	**Control**
Skin	ulcerated with petechiae	3	2	0	0	40	32	44	54
Scales	Scaly	4	3	3	0	48	40	43	48
Fins and tail	Hemorrhagic	3	4	2	2	50	48	46	52
Mouth	Open	0	0	0	3	15	5	12	62
Gills	Hemorrhagic	2	3	3	0	25	5	12	56
Eyes	Hemorrhagic, exophthalmic	0	1	2	0	40	20	25	58
Appetite	Anorexic	0	1	1	1	38	30	35	49
Behavior	Passive	0	0	0	3	20	5	20	52
Swimming	Erratic	0	1	2	0	5	5	0	54
Digestive tract	Inflamed	0	0	0	0	10	12	11	30
Kidney	Hemorrhagic	0	0	0	0	2	0	9	25
Air bladder	Trapping	0	0	0	0	0	0	13	23

With respect to pathogen without probiotic treatment, the fishes began to die early and 100% mortality was showed in 48 hours. At *Citrobacter* sp. used like probiotic experiment, the fishes begin to show signs of injuries, but after 36 hours, the fishes began to improve until the illness signs disappeared. The survival was 100% in the treatment with *Citrobacter* sp, for *Exiguobacterium* sp. 88% and *Enterobacter* sp. it only reached 44% survival and for Positive Control (*Artemia* without pathogen) 86% survival (Fig. 1).

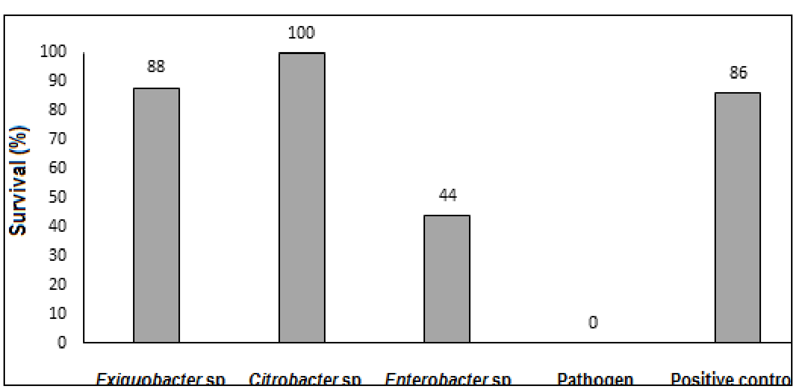
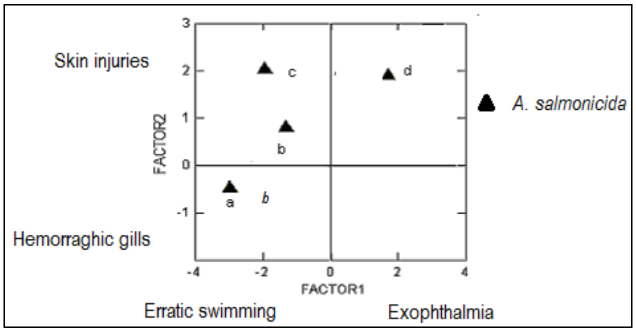


Fig 1: Survival percentage between experimental treatments.

The Discriminant analysis (standardized with variance) showed with that infection signs of influence mayor in the experiment were: skin injuries, hemorrhagic gills, erratic swimming and exophthalmia (Fig. 2).



Note: a: Fishes without probiotic and inoculated with *A. salmonicida* 10^7 UFC mL^{-1} b, c, and d: replica

Fig 2: Discriminant analysis about clinic picture showed for *P. conchonius*.

3.2 Recovery from inoculated pathogen

The causal agent of infection process was *A. salmonicida* determined in 100% of samples by PCR technique. The samples were taken from infection injuries. A 450 bp fragment was amplified, corresponding to Positive control treatment (*Aeromonas salmonicida* ATTC 33658), thereby confirming the pathogen presence inoculated and their relationship with infection process showed by fishes which were not fed with probiotic strains (Fig.3).

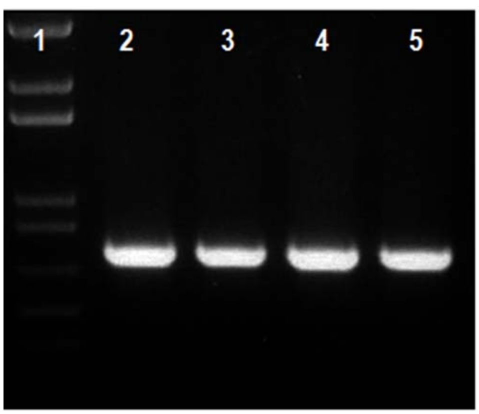


Fig 3: PCR products: 1) Marker molecular weight (1kb), 2) Positive control, 3 y 4) Skin samples, 5) Kidney samples.

4. Discussion

One of the most important aspect which enable to defined a microorganism like probiotic was their capacity to inhibit *in vivo* in front a potential pathogen. In this study, this evaluation was made in two steps: a) at laboratory condition and b) in a fish farming production of *P. conchonius*, where different observations were recorded, because in laboratory conditions it was not triggered the infection process in any experimental treatment although in some cases signs or diseases were observed, whishes disappeared and the organisms were maintained alive until experiment finished. Otherwise, in fish farming conditions, the infection process developed in all experimental treatments from the first 24 hours of being fed with enriched *Artemia* with the pathogen bacteria. Nevertheless, as experimental period progressed it was observed that experimental treatments with bacteria: *Citrobacter* sp, *Exiguobacterium* sp and *Enterobacter* sp, the fishes can resist the experimental infection because they maintained 100, 88 and 44% of survival respectively. In the experimental treatment where probiotic did not was supplied, 100% mortality was observed.

To understand the fact that in laboratory conditions failed the infection process, it is necessary to mention the point of view other authors [8], who explains that for infection propitiate and thereby the disease in an aquatic system, the host must be susceptible, the pathogen must be virulent and unfavorable environmental conditions for the host. This is important because in laboratory optimal conditions were maintained for *P. conchonius* and therefore the disease never occurred, unlike conditions in fish farming where the infection process occur with the characteristic furuncolosis clinical as: skin injuries, petechiae, hemorrhagic fins and gills and erratic swimming [9, 10], indicated that at fish farms the diseases occur in response to environmental variations, which provoke stress in cultured species, making them susceptible to those diseases occurred by opportunistic pathogens like *Aeromonas salmonicida*. Other studies [11], reported that *Citrobacter* sp. allows better resistance to infection caused by *Aeromonas* sp. in *Oreochomis niloticus*, as it happened in this study. Likewise, where the infection process occurs with the characteristic furuncolosis clinical. Likewise, it has been used *Exiguobacterium* sp. like probiotic bacteria in *Artemia* sp. with positive results [12].

5. Conclusions

In this study it can be concluded that the *Exiguobacterium* sp, *Citrobacter* sp strains can be used as biological control of *Aeromonas salmonicida* in the cultivation of ornamental fish *Puntios conchonius* as a strategy to eliminate the use of chemicals and antibiotics overall environmental impact and low efficiency in the control of infectious processes fish.

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