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Use of five probiotic strains to determine sensitivity in vitro on pathogenic bacteria growth isolated from sick fishes.

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ABSTRACT

Ornamental aquaculture is an activity in clear economic growth, both globally and in Mexico where the development is particularly relevant to freshwater species. Infectious diseases produced by fungus, bacteria and virus are considered one of the principal limitations during the productive process. Between implemented strategies for reduction of antibiotic use, which are "living microorganisms that confer a health benefit to the host if they are given in adequate quantities"; lactic acid bacteria and yeast are among the most common used microorganism in aquaculture. This investigation, prove the effect of isolated probiotic bacteria from the digestive tract of healthy fish, belonging to specie: Bacillus sp., *Bacillus laterosporus*, *Bacillus subtilis*, *Lactobacillus* sp, and *Lactococcus lactis*, at different dilutions (10^9,10^8, 10^7,10^6, 10^5 and 10^4) in vitro growth of pathogenic bacteria: *Citrobacter freundii*, *Enterobacter sakasakii*, *Klebsiella oxytoca*, *Proteus vulgaris*, and *Vibrio fluvialis*, isolated from kidney of sick fish, cultured and purified through successive inoculations and identificated by the amplification of gene 16S of rRNA (PCR) using universal primers 8 for. (5'-AGACTTTGATCATGGCTCAG-3') and 1492 rev. (5'- TACGGCTACCTTGTTACGACTT-3') and comparison with GENEBANK sequences base. Probiotic strains were previously isolated from the digestive tract of different healthy fish in the laboratory. In order to perform in vitro challenge tests, pathogenic strains were inoculated three times each in BHI agar boxes at a concentration of $1x10^7$ CFU mL^{-1} and subsequently using the well diffusion method, 70 µL from a suspension with each of the probiotic strains were added. Agar boxes were incubated 24 h at 30ºC to observe the formation of inhibition halos. Obtained values from inhibition halos were transformed to qualitative data with the following premise: halo diameter < 2.0 mm negative effect; halo diameter > 2.0 mm positive effect. In this study, it was determined that probiotic strains *B. subtilis* was the one that gave better results to inhibit the growth of pathogenic bacteria *P. vulgaris*, *E. sakazakii*, *V. fluvialis*, *K. oxytoca* and *C. freundii* in most of used dilutions. Making it a strain with high potential in aquaculture.

Key words: Growth, halos, probiotics, bacteria, sensibility.

RESUMEN

La acuicultura de especies ornamentales es una actividad económica en franco crecimiento, tanto a nivel mundial como en México, en donde tiene particular desarrollo lo correspondiente a especies dulceacuícolas. Las enfermedades infeccionas producidas por hongos, bacterias y virus, están consideradas una de las limitantes principales durante el proceso productivo. Entre las estrategias implementadas para disminuir el uso de antibióticos para el control de patógenos, se encuentra el control biológico mediante el uso de organismos probióticos, los cuales son "microorganismos vivos los cuales, administrados en cantidades adecuadas, confieren un beneficio en la salud del hospedador"; entre los de uso más común en acuicultura se encuentran las lactobacterias y las levaduras. En el presente trabajo, se probó el efecto de bacterias probióticas aisladas del tracto digestivo de peces sanos, pertenecientes a las especies: *Bacillus* sp., *Bacillus laterosporus*, *Bacillus subtilis*, *Lactobacillus* sp, y *Lactococcus lactis*, a diferentes diluciones (10^9,10^8, 10^7,10^6, 10^5 y 10^4) en el crecimiento *in vitro* de las bacterias patógenas: *Citrobacter freundii*, *Enterobacter sakasakii*, *Klebsiella oxytoca*, *Proteus vulgaris* y *Vibrio fluvialis*, aisladas del riñón de peces enfermos, cultivadas y purificadas a través de resiembras sucesivas e identificadas mediante la amplificación del gen 16S del ARNr (PCR) utilizando los primers universales 8 for. (5'-

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AGACTTTGATCATGGCTCAG-3') y 1492 rev. (5'-TACGGCTACCTTGTTACGACTT-3') y su comparación con la base de secuencias GENEBANK. Las cepas probióticos fueron aisladas previamente del tracto intestinal de diversos peces sanos en el laboratorio. Para llevar a cabo las pruebas de desafío *in vitro*, las cepas patógenas se sembraron por triplicado en cajas de agar BHI a una concentración de $1x10^7$ UFC mL^{-1} y posteriormente, utilizando el método de difusión en pozos, se adicionaran 70 µL de una suspensión con cada una de las cepas probióticas Las placas se incubaron durante 24 h a 30ºC para observar la formación de halos de inhibición. Los valores obtenidos de los halos de inhibición fueron transformados a datos cualitativos con la siguiente premisa: diámetro halo < 2.0 mm efecto negativo; diámetro de halo > 2.0 mm efecto positivo. En este estudio, se determinó que la cepas probiótica *B. subtilis* fue la que dio mejores resultados al inhibir el crecimiento de las bacterias patógenas *P. vulgaris*, *E. sakazakii*, *V. fluvialis*, *K. oxytoca* y *C. freundii* en la mayoría de las diluciones utilizadas. Por lo que es una cepa con alto potencial en acuicultura.

Palabras clave: Crecimiento, halos, probióticos, bacterias, sensibilidad.

INTRODUCTION

Ornamental aquaculture has become into a productive activity with a high worldwide potential. México is not the exception, because in our country it is commercialized about 43 million of ornamental fishes primarily from freshwater, 46% of those fishes are imported and the rest (54%) are produced in over 250 national fish farms (SAGARPA 2012). Nevertheless, one of the principally difficulties that exist in ornamental species culture is the occurrence of infectious diseases as a result of bacteria, fungi and virus incidence, frequently associated with the increase in culture densities and deficiencies management methods, water quality, food nutritional values, among other factors (Paillard et al. 2004; Pruzzo et al. 2005).

Due to above, sanitary aspect is primordial in aquatic production systems that until today, chemicals and antibiotics are used for control of infectious process, even when this process is associated to negative environmental impacts, when such compounds are exposed to native wildlife, water bodies and surrounding sediments, also promotes bacterial resistance in cultured organisms (Carnevia et al. 2010). For that reason, replacement methods for pathogens control with antibiotic by biological control have been established, which represents an interesting opportunity from the scientific, environmental and economic point of view (López and Cruz 2011).

Thereby an interest emerges in use of probiotics, defined as "living microorganisms which administered in adequate amounts, confer to the host health benefit" (FAO 2006). Adequate probiotic development is not a simple task; it requires scientific investigation with large scale testing and development of appropriate monitoring instruments (World Gastroenterology Organization 2008; Castro et al. 2011). Probiotics commonly used in aquaculture include a high range of taxa, from acid lactic bacteria to other microorganisms such as yeast, however today studies focus in the obtainment of specific probiotics, because these could increase benefits (Monroy et al. 2012).

One of the most important characteristic that probiotic microorganism must have, is the capacity of pathogens microorganisms exclusion and, as a first step, it requires *in vitro* antagonistic evaluation that allows in further studies the evaluation of *in vivo* effect. So the goal of this study was to compare the inhibition *in vitro* growing of five isolated pathogenic bacteria from ornamental fishes with infection signs, with respect isolated probiotic strains from a healthy fish.

MATERIAL AND METHODS

Sick fishes

For this study, fifty ornamental fishes were obtained from Chinconcuac, Morelos State fish farm, which showed abnormal behavior such gasping, erratic swimming or limited mobility and injuries such as bleeding at fins, eyes or gills, exophthalmos, bulging abdomen and boils, which indicates that organisms were going throw an infectious process. The organisms were transferred to laboratory for processing.

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Isolation of pathogenic bacteria

The fishes were placed in dissecting trays and observed injury samples were took with the help of sterile swabs, subsequently they were anesthetized with sulfonyl methane of tricaine at 1% to perform a dissection by making an incision above lateral line from the operculum to caudal fin to leave exposed the internal organs and being able to take a kidney sample. Samples were inoculated in 9 mL of sterile saline solution and then 0.1 mL was taken and inoculated in Thiosulfate Citrate Bile Salts agar (TCBS), Brain Heart Infusion (BHI) and Eosin Methylene Blue (EMB) in agar boxes by duplicates. Boxes were incubated 24 h at 28ºC, after that, a colony forming units counting (CFU mL^{-1}) was performed and the colony morphology was characterized. Afterwards strains were isolated throw successive inoculations. Gram stain was made on the pure stains for the observation of cell morphology with an optical microscope.

Bacterial identification

Once strains were isolated, a bacterial identification was made throw conventional biochemical tests as mobility, cytochrome C and glucose and catalase oxide fermentation. Also the identification was confirmed by detection of 16S rRNA gene, performing an extraction of genomic DNA using Wizard Genomic DNA Purification Kit (PROMEGA ™) system, following the manufacturer's instructions. With the isolated DNA from the studied strains, amplification of rRNA 16S gene (PCR) was performed using universal 8 for. primers (5'-AGACTTTGATCATGGCTCAG-3') and 1492 rev. (5'-TACGGCTACCTTGTTACGACTT-3'). For removal residues of primers, nucleotides and polymerase, samples were purified with the QIAquick PCR Purification Kit (Qiagen). Purification products were sent to Macrogen DNA Corea sequencing service and obtained sequences were interpreted with the programs Chromas and Blast. Finally, obtained information was compared with the world sequences base (GENEBANK) and phylogenetic relations were obtained.

Probiotic bacteria obtainment

Probiotic bacteria used in this investigation were previously isolated from the digestive tract of diverse healthy fishes by Monroy et al. (2010). Which correspond to *Lactococcus lactis*. *Lactobacillus* sp, *Bacillus* sp. *Bacillus subtilis* and *Bacillus laterosporus* species.

In vitro antagonistic capacity of probiotic bacteria against pathogenic

Once identified the pathogenic strains, *in vitro* antagonistic challenge tests were made between probiotic and pathogenic bacteria, for which, pathogenic strains were inoculated three times each in BHI agar boxes at a concentration $1x10^7$ CFU mL^{-1}. Agar boxes were incubated 24 h at 30ºC. After that, using the well diffusion method, 70 µL from a suspension with each of the probiotic strains mentioned before, with absorbance at 620 nm corresponding to a bacterial population of 10^8 CFU mL^{-1} and dilutions up to 10^6 CFU mL^{-1} were added. Agar boxes were incubated 24 h at 30ºC, and after this period the formation inhibition halos for each dilution was observed, strains that presented halos above 2 mm diameter were considered as positive.

Conversion of quantitative data to qualitative

Obtained values of inhibition halos were changed to qualitative data with the next premise: halo diameter < 2.0 mm negative effect; halo diameter > 2.0 mm positive effect.

RESULTS

From table 1 to 5 pathogen sensitivity to the experimental probiotic dilution is shown. As it can be seen on Table 1, pathogen *P. vulgaris* present a positive sensitivity with all experimental probiotics that were used, regardless used dilution.

In table 2 it is observed that pathogen *E. sakazakii* does not show sensitivity (<2.0 mm of diameter) in the following probiotics: *B. laterosporus* (10^6, 10^5 and 10^4 dilutions); *Lactobacillus* sp. (10^9,

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10^6 and 10^4 dilutions) and *Bacillus* sp. (10^6 and 10^4 dilutions). Other dilutions with the used probiotic give a positive result in sensitivity against the employed pathogen.

Table 1. Positive (+) or negative (-) sensitivity of pathogen *Proteus vulgaris* exposed to experimental probiotic dilutions.

Experimental probiotic	Sensitivity in different dilutions					
	10^9	10^8	10^7	10^6	10^5	10^4
Bacillus subtilis	+	+	+	+	+	+
Bacillus laterosporus	+	+	+	+	+	+
Lactococcus lactis	+	+	+	+	+	+
Lactobacillus sp.	+	+	+	+	+	+
Bacillus sp.	+	+	+	+	+	+

Table 2. Positive (+) or negative (-) sensitivity of pathogen *Enterobacter sakazakii* exposed to experimental probiotic dilutions.

Experimental probiotic	Sensitivity in different dilutions					
	10^9	10^8	10^7	10^6	10^5	10^4
Bacillus subtilis	+	+	+	+	+	+
Bacillus laterosporus	+	+	+	-	-	-
Lactococcus lactis	+	+	+	+	+	+
Lactobacillus sp.	-	+	+	-	+	-
Bacillus sp.	+	+	+	-	+	-

Table 3 shows sensitivity of *V. fluvialis* pathogen with respect probiotic dilution. It is observed lack of sensitivity (<2.0 mm) in probiotic *B. laterosporus* (10^9, 10^5 and 10^4 dilutions); *Lactobacillus* sp. (10^6 dilution) and *Bacillus* sp. (10^5 dilution). The rest of probiotics and their dilutions, presents a positive sensitivity (>2.0 mm).

With respect *K. oxytoca* pathogen (Table 4) and its sensitivity response to experimental probiotics dilutions were observed a higher negative sensitivity against pathogen. Probiotics like *B. subtilis* (10^6 dilution); *B. laterosforus* (10^8, 10^7, 10^6, 10^5 and 10^4 dilutions); *Lactobacillus* sp. (10^9, 10^8, 10^7 and 10^6 dilutions) and *Bacillus* sp. (10^6 dilution) shown those negative sensitivity. Only probiotic *L. lactis* gave a positive sensitivity in all experimental dilutions.

Table 3. Positive (+) or negative (-) sensitivity of pathogen *Vibrio fluvialis* exposed to experimental probiotic dilutions.

Experimental probiotic	Sensitivity in different dilutions					
	10^9	10^8	10^7	10^6	10^5	10^4
Bacillus subtilis	+	+	+	+	+	+
Bacillus laterosporus	-	+	+	+	-	-
Lactococcus lactis	+	+	+	+	+	+
Lactobacillus sp.	+	+	+	-	+	+
Bacillus sp.	+	+	+	+	-	+

Table 4. Positive (+) or negative (-) sensitivity of pathogen *Klebsiella oxytoca* exposed to experimental probiotic dilutions.

Experimental probiotic	Sensitivity in different dilutions					
	10^9	10^8	10^7	10^6	10^5	10^4
Bacillus subtilis	+	+	+	-	+	+
Bacillus laterosporus	+	-	-	-	-	-
Lactococcus lactis	+	+	+	+	+	+
Lactobacillus sp.	-	-	-	-	+	+
Bacillus sp.	+	+	+	-	+	+

With *Citrobacter freundii* pathogen it is observed in Table 5 a negative sensitivity (<2.0 mm) with *B. laterosporus* (10^6, 10^5 and 10^4 dilutions) and with *Lactobacillus* sp. probiotic at same dilutions. *B. subtilis* show a positive sensitivity in all dilutions, as well as *L. lactis.*

Table 5. Positive (+) or negative (-) sensitivity of pathogen *Citrobacter freundii* exposed to experimental probiotic dilutions.

Experimental probiotic	Sensitivity in different dilutions					
	10^9	10^8	10^7	10^6	10^5	10^4
Bacillus subtilis	+	+	+	+	+	+
Bacillus laterosporus	+	+	+	-	-	+
Lactococcus lactis	+	+	+	+	+	+
Lactobacillus sp.	+	+	+	-	-	-
Bacillus sp.	+	+	+	+	+	+

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DISCUSSION

Acid lactic bacteria are used as probiotics not only in human being, but also in different mammals and recently these bacteria are used to eliminate the presence of pathogenic bacteria in fish and crustaceans culture systems, as mentioned by Gómez-Gil et al. (2000); Briones and Lozano (2003); Campaña et al. (2003) and Monrroy et al. (2009).

In this study, it was determined that *B. subtilis* inhibit the growth of pathogenic bacteria such as *P. vulgaris*, *E. sakazakii*, *V. fluvialis*, *K. oxytoca* and *C. freundii*. The inhibitory mechanism of interaction probiotic - pathogenic bacteria was not characterized in this study, but studies using acid lactic bacteria (ALB) suggest that the inhibitory mechanism of *Bacillus* sp. is through an alteration of pH in grow media, utilization of specific nutrients, and/or production of volatile compounds (Gullian et al. 2004; Chaurasia et al. 2005 and Yilmaz et al. 2006). In addition, several authors report that *Bacillus* sp. produce polypeptide antibiotics, such as bacitracin, gramicidin S, polymyxin and tyrothricin (Balcazar and Rojas, 2007).

Bacillus sp. shown a strong inhibitory activity against strains of *V. harveyi*, *V. alginolycticus* and *V. parahaemolyticus*; isolated bacteria *in vitro* and *in vivo* from a peneid shrimp culture, producing inhibition halos of 10 to 15 mm (Balcazar and Rojas 2007). Gómez-Gil et al. (1998) and Vaseeharan and Ramasamy (2003), found that *Bacillus* probiotic controls the growth of *V. harveyi* in *P. monodon* shrimp, by increasing resistance to pathogenic bacteria and reducing mortality by 90% in shrimps, constantly giving *Bacillus* as supplement in alimentation. In fish, *Bacillus subtilis* has shown effectivity to inhibit growth and control infections from pathogenic strains of *Aeromonas* sp. in tilapia (*Oreochromis* sp.) and rainbow trout (*Oncorhynchus mykiss*) (Newaj-Fyzul et al. 2006). Kesarcodi-Watson et al. (2008) mentioned that probiotics can improve the immunological system obtaining a positive effect on survival of cultured aquatic organisms in response to an adverse environment (Díaz et al. 2001).

Villamil et al. (2003) described the capacity that acid lactic bacteria *Lactobacillus brevis* has for the elimination of pathogen *Vibrio alginolyticus* from live food cultures. This activity was partially attributed to the production of lactic acid and acetic acid by the acid lactic bacteria, weakening the pathogenic bacteria cytoplasmic membrane and allowing bacteriocins input. The ability that *Bacillus* sp. and *Lactobacillus* sp. has to inhibit *in vitro* growth pathogenic bacteria tested in this study, suggests that they are good candidates to be considered as probiotics and eliminate pathogenic bacteria of fish and crustaceans in large-scale cultivation.

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