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In Vivo Test to Eliminate *Aeromonas hydrophila* (Bacteria) and *Aphanomyces invadans* (Fungi) by the use of Probiotics

Abstract

In vitro and *In vivo* test were carried out to find the effect of probiotics in the elimination of the EUS (Epizootical Ulcerative Syndrome) disease caused by agents i.e *Aeromonas hydrophilla* (bacteria) and *Aphanomyces invadans* (fungi) in fish. *In vitro* experiment revealed that the zone of inhibition to inhibit the growth of bacteria and fungi was occurred in both the probiotics; although probiotic 2 had higher zone of inhibition than probiotic 1. *In vivo* experiment also revealed that the elimination of pathogenic organisms observed in the form of colony forming units (cfu)/ mL i.e $8.0x10^{11}$ to $3.0x10^{5}$ cfu/ mL by probiotic 2 was higher as compared to probiotic 1 i.e $1.8x10^{5}$cfu/ml. In conclusion, the present investigation showed that the viable counts of pathogenic bacterium were the highest in the fish inoculated only with the pathogenic organisms' i.e. 6.5x1012cells/mL after a three weeks period. Probiotic cultures that were used had considerable reduction in the viable count of Aeromonas hydrophila in fish. The numbers of viable counts was the lowest in mrigal (*C. mrigala*) treated with probiotic 2 followed by probiotic 1 over a period of four weeks.

Keywords: Fish, *Cirrihinus mrigala, Aeromonas hydrophila, Aphanomyces invadans*, Colony forming unit

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Parvati Sharma
Department of Zoology and Aquaculture, CCS Haryana Agricultural University, India

Correspondence: Parvati Sharma, Department of Zoology and Aquaculture, CCS Haryana Agricultural University, Research Associate, National Research Centre on Equines, Hisar, Haryana, India, Email parvati.hsr@gmail.com

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Abbreviations: Cfu, Colony Forming units; NA, Nutrient Agar; EUS, Epizootic Ulcerative Syndrome

Introduction

Aeromonas hydrophilla and *Apanomyces invadens* is the main causing agent of EUS disease. Probiotics that are the live microorganisms may release the chemical substances that have bactericidal and bacteriostatic effect on microbial population.[1,2] A probioticis defined as a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance.[3] Probiotics which have the property to colonize of the potential pathogens in the digestive tract and alteration of microbial metabolism or by the stimulation of host.[4-12] The common healthy intestinal flora of fish species include *Vibrio,*[5] *Lactobacillus,*[13] *Acinetobacter, Achromobacter* and by *Bacillus* and representatives from the family Enterobacteriaceae.[14] Keeping these in mind, the present investigation proposed to investigate the elimination of pathogenic organisms (*Aeromonas hydrophila and Aphanomyces invadans*) by using two probiotics (probiotic 1 and probiotic 2).

Material and methods

The probiotic 1 was composed by *Nitrosomonas, Azosporillium, Lcithiniformes, Bacillus subtilis, Nitrobacter, Trichoderma, Bacillus megetherium.* The probiotic 2 was composed by *Lactobacillus sporogenes, Lactobacillus acidophilus, Bacillus subtilis, Bacillus licheniformis, Saccharomyces cervirial,* Sea weed extract, Enzyme complex contains, Amylase, Phylase, Protease, Cellulose, Beta-galactosidase, Lipase, Vitamins (Vitamin C = 20g, Vitamin B6 = 1g). Both probiotics were used to find out the *In vitro* and *In vivo* antagonism against pathogenic organism.

In vitro test for determination of the antimicrobial activity

Bacterial cultures of probiotic 1and probiotic 2were examined for inhibitory effects against the pathogenic bacteria, *A. hydrophila* isolated from diseased fish.[15] The *In vitro* antimicrobial activity was assessed using agar diffusion method and the inhibition zone was determined according to.[16] The two probiotic bacteria were inoculated in the center of petri dishes, containing Nutrient agar (NA agar) and incubated at 30 °C for 24h. Subsequently, fresh inoculums of the pathogenic *A. hydrophila*, was spread over the plates (by pour plate method), previously inoculated with probiotic bacterial culture. The plates were further incubated at 30 °C for 24h, and then checked for the appearance of inhibition zone.[12]

In vivo tests of probiotics

The healthy individuals of mrigal fish weighing 20g were used to perform *In vivo* pathogenicity tests following.[17] Nine fish were kept in each tub and the experiment was done in triplicate. The fish was inoculated with the pathogenic organisms say *Aeromonas hydrophilla* (bacterium) and *Aphanomyces invadans* (fungus). The causative nature of these pathogens to induce EUS was earlier tested by *Sharma*.[18] All treatments given to the fish with pathogens and probiotics is shown in table 1. One fish from each replicate was sacrificed at weekly intervals and the bacterial flora from intestine; liver and kidney were taken after maceration of tissues. The viable counts of the bacterial pathogens were worked out. The difference between treatments means (at 0.05 significant levels) was investigated by following the method described by Snedecor et al.[19]

Table 1 Viable count of bacteria during the experiment in different weeks

Treatment	Viable Count of Bacteria in Different Weeks							
	1	2	3	4	5	6	7	8
Bacteria	$5.5x10^{11}$	$7.8x10^{11}$	$8.7x10^{11}$	-	-	-	-	-
Bacteria+Fungus	$8.0x10^{11}$	$4.7x10^{12}$	$6.5x10^{12}$	-	-	-	-	-
Bacteria+Probiotic1	$7.6x10^{9}$	$4.3x10^{9}$	$4.3x10^{8}$	$2.5x10^{8}$	$5.0x10^{6}$	$3.0x10^{6}$	$5.7x10^{5}$	$2.8x10^{5}$
Bacteria+Fungus+Probioti1	$7.2x10^{9}$	$7.4x10^{8}$	$6.1x10^{8}$	$3.6x10^{8}$	$3.0x10^{7}$	$5.8x10^{6}$	$8.5x10^{5}$	$5.3x10^{5}$
Bacteria+Probiotic2	$6.4x10^{9}$	$3.0x10^{9}$	$3.2x10^{8}$	$1.9x10^{8}$	$2.0x10^{6}$	$5.9x10^{5}$	$2.1x10^{5}$	$1.8x10^{5}$
Bacteria+Fungus+Probioti2	$7.0x10^{9}$	$5.1x10^{8}$	$4.1x10^{8}$	$3.0x10^{8}$	$2.6x10^{6}$	$8.2x10^{5}$	$3.6x10^{5}$	$3.0x10^{5}$

Results

In vitro antagonistic test

Probiotic cultures, viz., Probiotic 1and Probiotic 2 exhibited *In vitro* antagonistic activity against the pathogenic *A. hydrophila*. Probiotic 2 developed larger inhibition zone than probiotic 1 against *A. hydrophila* on NA agar plates.

The *In vivo* results of viable counts of *A. hydrophilla* and *A. invadans* under different treatments over a period of eight weeks are presented in table 1. In the first treatment with bacteria, the number of viable counts progressively from $5.5x10^{11}$ in first week to $8.7x10^{11}$ in the third week when fish died. The situation became more critical when fish injected with both the pathogenic organisms (bacteria+fungi) together. The viable counts increased from $8.0x10^{11}$ in first week to $6.5x10^{12}$ in the third week. The viable count of the pathogenic organism became so high that the fish could not tolerate and subsequently also died. However, when the treatment was given with probiotics then it indicated a progressive decrease in the viable counts. The viable counts of bacteria with probiotic 1 declined from $7.6x10^{9}$ in first week to $2.8x10^{5}$ in eighth week. The viable counts of bacteria and fungi with probiotic 1 declined from $7.2x10^{9}$ in first week to $5.3x10^{5}$ in eighth week. The viable counts of bacteria with probiotic 2 decreased from $6.4x10^{9}$to $1.8x10^{5}$ in the eighth week. The viable count of bacteria and fungi together with probiotic 2 declined from $7.0x10^{9}$ in first week to $3.0x10^{5}$ in eighth week (Table 1). The tests results therefore show that pathogenic bacteria were eliminated successfully by both the probiotics.

Probiotic bacteria on the aspect of safety

Both probiotics were found to be harmless to *C. mrigala* as any clinical signs and mortalities were noticed during the probiotic treatments.

Discussion

In the present investigation, the viable counts or colony forming units (cfu) of pathogenic organism were high in the inoculated fish. However, these counts decreased when treated along with probiotics. The results showed that the number of viable counts decreased more when using probiotic 2 than when using probiotic 1over a period of eight weeks. Similar results were observed by *Zhou et al.*[20] in their study on the inhibition ability of probiotic, *Lactococcus lactis* RQ516, against *A. hydrophila; In vitro* with 14.77 ± 1.17 mm zones of inhibition and; immune stimulator and growth promoter, *In vivo* in tilapia, *Oreochromis niloticus.* The study of Abd El Rhman et al.[21] showed that combination of two probiotic bacteria (*Micrococcus luteus* and *Pseudomonas sp.*) gave adverse effect against *A. hydrophila* in Nile tilapia, Oreochromis niloticus. Although, their study was on different fish, with different probiotics and pathogenic bacterium, the pattern of inhibition in both *In vitro* as well as *In vivo* was found to be the same. Nimrat & Vuthiphandchai[22] also observed similar results in marine shrimp, where they used 12 commercial probiotic products against shrimp pathogenic bacterium *Vibrio harveyi*. Probiotic enhanced immune system observed in a number of earlier studies[23-25] showing a decrease in the number of colony forming units of bacteria in fish. Salini et al.[26] observed that the diversity and intensity of microbial flora got reduced when treated with culture of medicated diet having probiotic bacteria (*Bacillus*). Behera & Nayak[27] also found that the bacterial load present inside the culture ponds does not show any harm due to presence of probiotics which helped for suppression and maintaining of a clean and hygienic environment for sustainable shrimp culture.

Conclusion

In conclusion, the pathogenic organisms that cause disease in fish can be effectively eliminated by the use of probiotics.

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Conflicts of interest

None.

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