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ELIMINATION OF PATHOGENIC BACTERIUM, *Aeromonas hydrophila* BY THE USE OF PROBIOTICS

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Abstract: The present investigation was carried out to study the elimination of pathogenic, chloroamphinicol resistant *Aeromonas hydrophila* (*A. hydrophila*^{Chr}) by the use of single probiotics; Probiotic 1 (*Lactobacillus sporogenes*), Probiotic 2 (*Saccharomyces boulardii*) or mixture of probiotics; Probiotic 3 (*Nitromonas* spp., *Rhodococcus* spp., *Bacilus megaterium*, *Lecheni formis*, *Desulphovibrio sulphuricum*, *Psuedomonas* spp., *Chromatium* spp., *Chlorobium* spp., *Thiobacillus* spp., *Thioxidants* spp., *Thiobacilus ferroxidant*, *Methylomonas methyanica*, *Glucon acetobactor*, *Azospirillum* spp., *Trichoderma* spp., *Shizophyllum commune* and *Sclertium gluconicum*); *in vitro* as well as *in vivo*. For this purpose probiotics 1, 2 and 3 were tested against the pathogenic *Aeromonas hydrophila*^{chr}. *In vitro* experiment revealed that the zones of inhibition of probiotic 1 were highest than probiotic 3 followed by probiotic 2 with values of 19.67 ± 0.67, 19.33 ± 0.33 and 17.00 ±0.58 mm, respectively. *In vivo* experiment also showed that the elimination of pathogenic *A. hydrophila*^{chr} from 1.54 x 10^{11} CFU/mL to 1.90 x 10^{1}, 2.30 x 10^{1} and 5.33 x 10^{1} CFU/mL lasted four weeks by probiotic 1, probiotic 3 and probiotic 2, respecticvely. In conclusion, the viable counts of pathogenic bacterium were the highest in the fish inoculated only with the pathogenic bacteria 6.07x10^{12} cells/mL in four weeks. Probiotic cultures used had significantly reduced the viable count of *A. hydrophila* in fish. The numbers of viable counts was the lowest in catfishes treated with probiotic 1 followed by probiotic 3 and probiotic 2 over a period of four weeks.

Keywords: Indian Catfish, *C. batrachus*, Probiotic, Pathogen, *A. hydrophila*, Bacteria

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Özet: **Patojen bir bakteri olan *Aeromonas hydrophilia*' nın probiyotik kullanılarak eliminasyonu**

Bu çalışmada, *Chloramhinicol*'e dirençli *Aeromonas hydrophilia*'nın tekil probiyotikler; Probiyotik 1 (*Lactobacillus sporogenes*), Probiotic 2 (*Saccharomyces boulardii*) veya karışım Probiyotik 3 (*Nitromonas spp., Rhodococcus spp., Bacilus megaterium, Lecheniformis, Desulphovibrio sulphuricum,Psuedomonas spp.,Chromatium spp., Chlorobium spp.,Thiobacillus spp., Thioxidants spp., Thiobacilus ferroxidant,Methylomonasme thyanica, Glucon acetobactor, Azospirillum spp., Trichoderma spp., Shizophyllum commune* ve *Sclertium gluconicum*); kullanılarak eliminasyonu in vitro ve in vivo olarak incelenmiştir. Bu amaçla, probiyotik 1, 2 ve 3 patojenik *Aeromonas hydrophilia*^{chr} üzerinde test edilmiştir. In vitro deneyler sonunda probiyotik 1'in inhibisyon zonu en yüksek olarak tespit edildi, onu probiyotik 3 ve 2 takip etti, elde edilen değerler sırasıyla 19.67 ± 0.67, 19.33 ± 0.33 ve 17.00 ±0.58 mm oldu. In vivo deneylerde ise *A. hydrophilia*'nın 1.54 x 1011CFU/mL değerinden probiyotik 1, 2 ve 3 için sırasıyla 1.90 x 101, 2.30 x 101 and 5.33 x 101 CFU/mL değerlerine indirilmesi dört hafta sürdü. Sonuç olarak, patojenik bakteriler dört hafta sonunda en yoğun 6.07x1012 Hücre/mL olarak sadece bakteri inoküle edilen balıklarda görüldü. Kullanılan probiyotikler balıklarda *A. hydrophila* canlı sayısında önemli azalmaya neden olmuştur. Dört haftalık denemenin sonunda canlı sayıları probiyotik 1 kullanılan kedi balıklarında en düşük tespit edildi, bunu probiyotik 3 ve probiyotik 2 takip etti.

Anahtar Kelimeler: Hint kedi balığı, *C. batrachus*, Probiyotik, Patojen, *A. hydrophilia*, Bakter

Introduction

Aeromonads are the major pathogens in the fisheries sector. *A. hydrophila* is an opportunistic pathogen of a wide variety of hosts. This bacterium inflicts serious damage in pond and aquarium cultures. The pathogenesis and histopathology of the red-sore disease was extensively studied in the common carp (*C. carpio*) and the Channel catfish (*Ictalurus punctatus*) (Harikrishnan *et al.*, 2009). Probiotics microorganisms may release chemical substances that have a bactericidal or bacteriostatic effect on other microbial populations; they do so by altering interpopulation relationships like competition for chemicals or available energy rich compounds, producing inhibitory substances in the intestine of the host, on its body surface, or in culture medium where organism live and create a barrier against the proliferation of opportunistic pathogens [Watson et al, 2008]. The major taxonomic groups contributing to the healthy intestinal flora of fish species include *Vibrio* [Austin et al, 1995], *Lactobacillus* [Kacem and Karam, 2006], *Acinetobacter*, and *Achromobacter*, followed by *Bacillus* and representatives from the family Enterobacteriaceae [Ringo and Strom, 1994]. The probiotic bacteria generally present in the soil, pond bottom and water and grow well at temperature range 25-37 ^{0}C. In aquaculture, non-pathogenic strains of identified bacteria have been successfully used as probiotics to control the diseases in fish [Gomez-Gil et al, 2002]. These probiotic bacteria suppress proliferation of pathogenic and opportunistic bacteria in the mucus in intestine as well as ambient environment of the fishes simultaneously [Balcazar et al, 2006]. Consequently the probionts reduce the incidence of diseases. Earlier studies, Dahiya et al [2009], observed pathogenicity of *A. hydrophila*, and disease symptoms caused in Indian magur (*Clarius batrachus* L.). So, keeping above facts in mind; present investigation was proposed to investigate the elimination of pathogenic bacterium *Aeromonas hydrophila* by the use of three probiotics.

Materials and Methods

Probiotics 1 and 2 or Probiotic 3 cultures were used to observe *in vitro* and *in vivo* antagonism effects against pathogenic bacterium, *A. hydrophila*. Probiotics 1 contained only single bacterium named lactic acid bacteria (*Lactobacillus sporogenes*); Probiotics 2 contained single fungus (yeast) *Saccharomyces boulardii* while probiotics 3 contained a mixture of many bacteria including, *Nitromonas* spp., *Rhodococcus* spp., *Bacilus megaterium*, *Lecheni formis*, *Desulphovibrio sulphuricum*, *Psuedomonas* spp., *Chromatium* spp.,

Chlorobium spp., *Thiobacillus* spp., *Thioxidants* spp., *Thiobacilus ferroxidant*, *Methylomonas methyanica*, *Glucon acetobactor*, *Azospirillum* spp., *Trichoderma* spp., *Shizophyllum commune* and *Sclertium gluconicum*.

In vitro test of probiotics against the pathogenic bacteria.

In vitro antagonism tests of three probiotics against *A. hydrophila* were carried out by using agar well diffusion method [Gram et al, 1999], which is based on inoculation of culture medium with pathogenic bacteria and then allowing probiotic to grow on medium in the bored well. Zone of inhibition were measured (in millimeters) by agar well diffusion method to observe the antagonism of three probiotics against pathogenic bacterium. The following procedure was followed step by step:

i) Pathogenic bacteria (*A. hydrophila*) of 10^6 CFU (colony forming units) was poured into melted nutrient agar (beef extract 3g, peptone 5g, Sodium chloride 5g, agar 15g for one liter volume at pH 7.0±0.2), at 60-62 ^{0}C; and mixed well by shaking then poured into petri plates, and allowed to solidify in laminar flow.

ii) Three well were bored in solidified NA containing pathogenic bacterium *A. hydrophila* by the well borer and every time the borer pipe was sterilized on the flame.

iii) Then 5-10 µl melted water agar (15 g Agar + 1 litre distilled water) was added at the bottom of the each well with micropipette; to prevent the seepage of the probiotic bacterial suspension to the bottom of Petri plates.

iv) Then the probiotics 5x 10^{11} (50 µl) were added to each well and plates were incubated in B.O.D. at 35-37 ^{0}C for 18-24 h. The Zone of Inhibition was measured with simple scale and recorded.

In vivo tests of probiotics

Antibiotic chloroamphinicol resistant *A. hydrophila* (*A. hydrophila*^{chr}) was taken intraperitoneally as pathogenic organism for inoculation from catfishes (*Clarius batrachus* L.). The treatments of experiment are given in Table 1. Each treatment has three replicates and one Indian magur from each replicate was sacrificed at weekly interval, and viable counts of *A. hydrophila*^{chr} were worked out using serial dilution method. The obtained results were analyzed statistically using completely randomized design (CRD) to evaluate differences among different treatments means at 0.05 significant levels [Snedecor et al, 1989].

Table 1. CFU of pathogenic bacterium *A. hydrophila*^{chr} for *in vitro* and *in vivo* antagonism with probiotics.

Sr. no.	Treatments		CFU per mL
1	T	Control	200µl of PBS
2	T1	Control + *A. hydrophila*^{chr}	5 x 10^{11}
3	T2	Control + *A. hydrophila*^{chr} + probiotic 1	5 x 10^{11}
4	T3	Control + *A. hydrophila*^{chr} + probiotic 2	5 x 10^{11}
5	T4	Control + *A. hydrophila*^{chr} + probiotic 3	5 x 10^{11}

Results and Discussion

Inhibition zone of probiotic against *A. hydrophila*^{chr} was found to be different in each treatment. Probiotic 1 showed bigger inhibition zone as compared to probiotic 2 and probiotic 3 against each bacterium. From these results, it is concluded that probiotic 1 was better than probiotic 3 and probiotic 3 was better than the probiotic 2, in gushing out the pathogenic bacteria- *A. hydrophila*^{chr} from diseased catfishes (Table 2; Figure 1, 2, 3).

Table 2. Inhibition zones (in millimeters) of three probiotics against pathogenic bacteria *A. hydrophila*^{chr}

Sr.No.	Probiotics	Inhibition zones against *A. hydrophila* ^{chr} (in mm)
1	Probiotics	19.67 ± 0.67
2	Probiotics	17.00 ± 0.58
3	Probiotics	19.33 ± 0.33
C.D. Value	1.92	

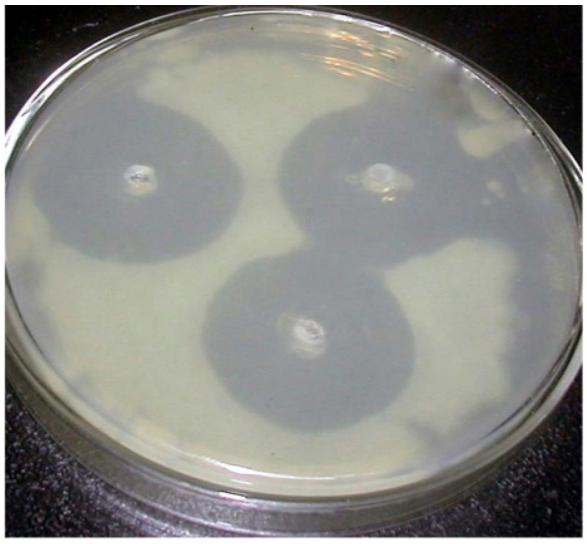

Figure 1. Probiotics 1 (*Lactobacillus sporogenes*) showing zone of inhibition against *A. hydrophila*^{chr}

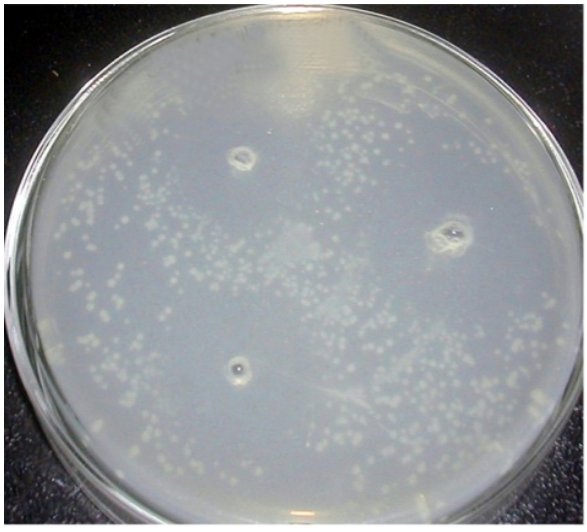

Figure 2. Probiotics 2 (*Saccharomyces boulardii*) showing zone of inhibition against *A. hydrophila*^{chr}

The results of viable counts of pathogenic bacterium *A. hydrophila*^{chr} under different treatments over a period of four weeks are presented in Table 3. The number of viable counts of catfishes injected with *A. hydrophila* (Control-T1) *increased* from initial value of 1.54 x 10^{11} to 6.07 x10^{12} cells/mL in four week. The viable counts of *A. hydrophila*^{chr} became so high in fifth week that the catfishes could not tolerate and subsequently showed mortality. But the catfishes inoculated with *A. hydrophila* along with the three probiotics 1, 2, and 3, showed progressive decline in the viable counts of *A. hydrophila* from initial value of 1.54 x $10^{11}$to 1.90 x10^{1} in four week, whilst the bacterial load in T3 and T4 dropped from initial value of 1.54 x $10^{11}$to 5.33x10^{1} and 2.30x10^{1} cells/mL in four week, respectively. *A. hydrophila* was successfully eliminated by the all of three probiotics 1, 2 and 3. Comparatively, probiotics 1 showed best results among these three probiotics (Table 3).

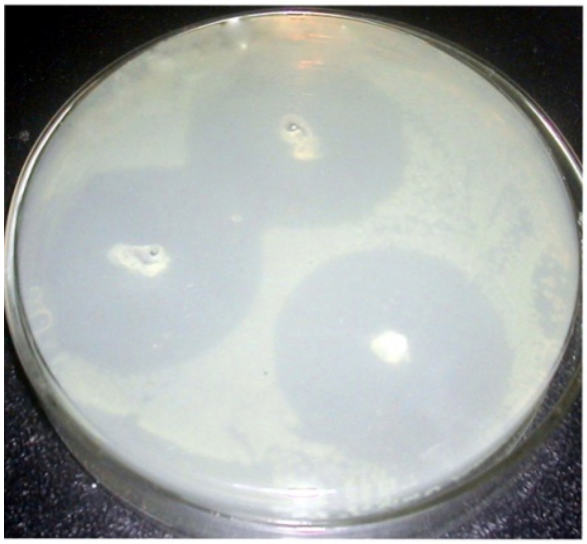

Figure 3. Probiotics 3 (mixture of many bacteria) showing zone of inhibition against *A. hydrophila*^{chr}

The mode of action of the probiotics is rarely investigated, but possibilities include competitive exclusion principle i.e. the probiotics actively inhibit the colonization of potential pathogens in the digestive tract by antibiosis or by competition for nutrients and space, alteration of microbial metabolism, and by the stimulation of host (human and animals) immunity [Irianto and Austin, 2002 and Dahiya et al, 2011]. There are several modes of probiotic action in the aquatic environment, these may include improved feed conversion efficiency and feed utilization, higher adhesion capacity to the intestinal mucosa and reduc-

tion of adherence of pathogenic bacteria, production of extra-cellular antibiotics or iron chelating agents (siderophores) which prevent the growth of almost all pathogenic bacteria [Verschuere et al, 2000] and improvement of water quality (bio-remediation) especially in the pond and reducing the problem of red tide planktons [Watson et al, 2008].

Probiotic strains of *V. alginolyticus* found very effective in reducing disease caused by the *A. salmonicida*, *V. anguillarum* and *V. ordalli* in aquatic animals [Austin et al , 1995]. A culture of *V. alginolyticus* with *Chaetoceros meulleri* is also used as a potent probiotics [Gomez-Gil, 2002]. A similar study was conducted in shrimp farming, in which a marine strain of *Pseudomonas* was found to inhibit pathogenic *Vibrio* bacteria [Chythanya et al, 2002]. In the present investigation the viable counts of pathogenic bacterium *A. hydrophila* were the highest in the catfishes without probiotic inoculations. However, these counts decreased in the presence of probiotic in catfishes. The numbers of viable counts were the lowest in probiotic 1 compared to probiotic 3 and probiotic 2 over a period of four week. Similar results were observed by Nimrat and Vuthiphandchai [2011], they found the inhibition ability of probiotic, *Lactococcus lactis* RQ516, against *A. hydrophila*, *in vitro* with 14.77 ± 1.17 mm zones of inhibition and; immunostimulator and growth promoter, *in vivo* in tilapia, *Oreochromis niloticus*. Although, their study was on different fish with different probiotics and pathogenic bacterium, but pattern of inhibition in both in *vitro* as well as *in vivo* was found same. The study results were in agreement with results of Zhou et al [2010] who observed inhibition effect of twelve commercial probiotic products against pathogenic bacterium *Vibrio harveyi* in marine shrimp. In the present investigation the similar results were obtained i.e. all three probiotics inhibit the pathogenic *A. hydrophila*^{chr}; both *in vitro* and *in vivo* experiments.

Conclusions

In conclusion, the viable counts of pathogenic bacterium were the highest in the fish inoculated with only pathogenic bacteria. However, these counts decreased in the presence of probiotic in fish. Probiotic 1 was found to be more inhibition effect on the growth of *A. hydrophila*, followed by Probiotic 3. The numbers of viable counts decreased more in probiotic 1 as compare to probiotic 3 followed by probiotic 2 over a period of four weeks.

Table 3. CFU of *A. hydrophila*^{chr} under *in vivo* induced pathogenicity over a period of four weeks.

Treatment	**Viable counts of *A. hydrophila*^{chr} bacterium in different weeks**					
	0	**1**	**2**	**3**	**4**	
T1	*A. hydrophila*	1.54×10^{11}	2.10×10^{11}	3.10×10^{12}	3.15×10^{12}	6.07×10^{12} ¥
T2	*A. hydrophila* + probiotic 1	1.54×10^{11}	3.64×10^{4}	1.96×10^{3}	8.60×10^{1}	1.90×10^{1}
T3	*A. hydrophila* + probiotic 2	1.54×10^{11}	6.53×10^{5}	6.07×10^{4}	5.75×10^{2}	5.33×10^{1}
T4	*A. hydrophila* + probiotic 3	1.54×10^{11}	4.50×10^{6}	8.27×10^{4}	9.27×10^{2}	2.30×10^{1}
¥ After four weeks catfishes in treatment T1 died						

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