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Isolation of Bacteriocin Producing Lactic Acid Bacteria from Fish Gut and Probiotic Activity Against Common Fresh Water Fish Pathogen *Aeromonas hydrophila*

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Abstract: Lactic acid bacteria (LAB) produce many kinds of metabolites, which might affect the other microbes in the fish mid gut. Lactic acid produced by both homolactic acid and heterolactic strains, which will reduce pH in the luminal contents in the stomach of neonatal piglets of fish. Isolated LAB was used as a probiotic in fresh water fish tilapia (*Oreochromis mossambicus*) against the most common fish pathogen *Aeromonas hydrophila.* Higher antagonistic activity recorded from extra cellular protein (ECP) or bacteriocin compared to the intra cellular protein (ICP) against *A. hydrophila.* After feeding with the potential probiotics for 25 days, challenge by immersion indicated effectiveness at reducing disease caused by *A. hydrophila* in fishes. Tilapia exhibited no significant difference in growth, survival nor external appearance between the probiotic fed combine probiotic treatments, but significant differences ($p<0.05$) occurred between probiotic and control groups. The use of LAB also enhances the production rate of rotifers, which act as biocarriers of probiotics and when fed to fish, they showed increased growth rate and weight of the animal. LAB is highly appreciated as a biological enhancers-probiotics, because of beneficial effect of live microorganisms

Key words: Lactic acid bacteria, inhibitory activity, probiotic, immersion study, *Oreochromis mossambicus*

INTRODUCTION

Lactic acid bacteria are gram-positive, non-sporulating and catalase negative rods or cocci that ferment various carbohydrates mainly to lactate and acetate. Various amino acids, vitamins and minerals are essential for their growth (Kandler and Wise, 1986). Various authors have shown that lactic acid bacteria are also part of the normal intestinal flora of fish (Ringo and Gatesoupe, 1998). Most of the evidence comes from salmonid species like Arctic charr *(Salvelinus alpinus*) and Atlantic salmon (*Salmo salar*) (Ringo *et al*., 1995; Gonzalez *et al*., 2000). Few studies have described lactic acid bacteria in other freshwater fish (Kvasnikov *et al*., 1977; Cai *et al*., 1999). Kvasnikov *et al*. (1977) described the presence of lactic acid bacteria, including *Lactobacillus* in the intestines of various fish species at larval, fry and fingerling stages inhabiting ponds. However, it was discussed that some human activities like artificial feeding in ponds would have had an effect on the bacterial composition and load in some fish, like carp (*Cyprinus carpio*) which showed the highest content of lactic acid bacteria in the intestines.

Probiotics can be defined as a food (feed) or drug containing live microbes that, when ingested, is expected to give beneficial physiologic effects to the host animal through microbial actions (Ishibashi and Yamazaki, 2001). Most of the probiotics are marketed as foodstuffs or drug. Consideration of the safety of probiotics is therefore of extreme importance. The safety of the microbes that have been used traditionally in probiotics can be conformed through a long period of experience (Mayra-Makien and Bigert, 1993). Yasuds and Taga (1980) suggested that probiotic bacteria would be found to be useful not only as food but also as biological controllers of fish disease and activators of nutrient regeneration. In the biological control in aquaculture emerge and since then the research effort has continually increased. *Bacillus* sp. is often antagonistic against other fresh water fish pathogenic bacteria (Gatesoupe, 1999; Rengipipat *et al*., 2000). Generally bacteria play two major roles as beneficial bacteria and pathogenic forms, beneficial bacteria are helpful in nutrient recycling and organic matter degradation and thus clear the environment (Moriarty, 1997). Pathogenic bacteria are the causative agents of bad water quality, stress and diseases as they act as primary and secondary pathogens (Karunasagar *et al*., 1996). Among fish pathogenic *Aeromonas hydrophila* and direct cause the syptecimea diseases and indirectly affect the fish health through developing poor water quality, high cyclone and stress (Lightner *et al*., 1992).

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In this present study investigated the inhibitory activity of *Bacillus* sp. isolated from fish digestive tract, identify and evaluate bacteria which could be used in disease prevention in *Oreochromis mossambicus.* To test bacterial isolates for antagonistic activity against putative fresh water pathogenic bacteria *Aeromonas hydrophila.*

MATERIALS AND METHODS

Lactic acid producing bacteria isolated from fish digestive tract: The tilapia *Oreochromis mossambicus* fish was collected from the Cauvery, fresh water river, Erode district, Tamilnadu, India. The fish were washed with sterile distilled to remove the unwanted particles. Then the animals were dissected to remove the digestive tracts by the sterilization condition. The digestive tracts were homogenized in the same sterile distilled water for centrifugation. After centrifugation the supernatant was taken and serially diluted in sterile distilled water in the test tubes to 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} dilution and were pour plated on nutrient agar plate and incubated for 24 h at room temperature. Individual colonies were taken and inoculated in Elliker's broth. Then the broths were incubated for 18-24 h at 37°C. One hundred microliter of culture was inoculated in *Bacillus* selective medium *Lactobacillus bulgaricus* agar plate and different colonies were selected and subjected to confirmatory tests for (LAB).

Conformation of *Bacillus* sp.

Grams staining: Markedly available Gram's staining kit purchased from Himedia, heat-fixed smears should be stained for 1 min with crystal violet, washed in tap water, covered with Grams iodine for 1 min re-washed, decolorized by a few seconds in acetone-alcohol and counterstained for 30 sec in safranin. The smears are washed thoroughly and gently air-dried and observed under the oil immersion objective.

Methyl Red test (MR): Inoculate the isolated *Bacillus* with buffered glucose broth and incubate at 37°C for 48 h. After incubation add a few drops of methyl red solution to the culture, read immediately.

Catalase test: A loopful of the culture was placed on a slide and few drops of 10% hydrogen peroxide were added. The slides were observed for effervescence.

Detection of antagonistic activity: Isolated probiotic *Bacillus* sp. was assayed by the agar well diffusion method according to Benkerroum *et al.* (1993). The plates were examined for lysis around the wells at different time intervals for a total of 24 h. The agar well disc diffusion assay was used by two different isolated bacteriocin such as extra cellular protein (ECP) and intra cellular protein (ICP), the zone of inhibition was observed against common fish pathogen *A. hydrophila.*

Probiotic study-Maintenance of fish: The fish employed in this study were common fish *Oreochromis mossambicus* with weight range between 20±2 g fish was collected from the Cauvery fresh water river, Erode district, Tamilnadu, India. The major physical and chemical factors affect the growth of fish in a closed system. The fish were feed with commercially available artificial diet twice a day. The parameters were maintained, temperature (26-30°C), salinity (1-2 ppm), dissolved oxygen (6-7 mg L^{-1}), pH (6.5-8) and light.

Immersion study of *Bacillus* sp. against Aeromonas hydrophila: Tilapias (*Oreochromis mossambicus*) had not been exposed to fish diseases and were deemed specific pathogen free. The tilapias were acclimated for one week in four tanks to laboratory conditions before the start of the trial. After the acclimation period the average weight of the fish was 22±2 g and the fish were randomly divided into twenty 500 L capacity polypropylene tanks with three treatments and three replicates per treatment, each containing 50 tilapia fish. The water temperature was held at 26-30°C during the whole trial. One treatment served as the control and was fed with regular diet (without the probiotics) during the entire trial period.

In order to obtain the bacterial suspensions, the probiotic strains were grown in MRS broth in a shaking incubated at 30°C overnight. After incubation, the cells were harvested by centrifugation (2000 rpm), washed twice with PBS buffer and re-suspended in the same buffer. The absorbance at 600 nm was adjusted to standardize the number of bacteria (10^5-10^6 CFU mL^{-1}). Commercial artificial feed was used as the basal diet for the supplementation of probiotic strains. Probiotic diets were prepared with cells resuspended in 5 mL of PBS to 10^6 CFU mL^{-1} and mixed. Tilapia was fed two times daily at 5% body weight per day with a 50% water change every day, during 25 day feeding trials and survival was estimated visually each morning. *A. hydrophila* was used as an infectious agent in the experiment. The strain was grown for 12 h at 30°C in nutrient broth. Directly immersion of the tank 3 and 4, treatments and control were exposed to pathogenic *A. hydrophila* at a level of 10^5-10^6 CFU mL^{-1} at 28°C by adding the bacteria to the water. Dead fish were collected and recorded daily.

Statistical analysis: The results were analyzed using one-way analysis of variance (ANOVA). Fisher's range test was used to determine differences ($p<0.05$) between tested groups. All statistics were performed using SPSS 10 for Windows.

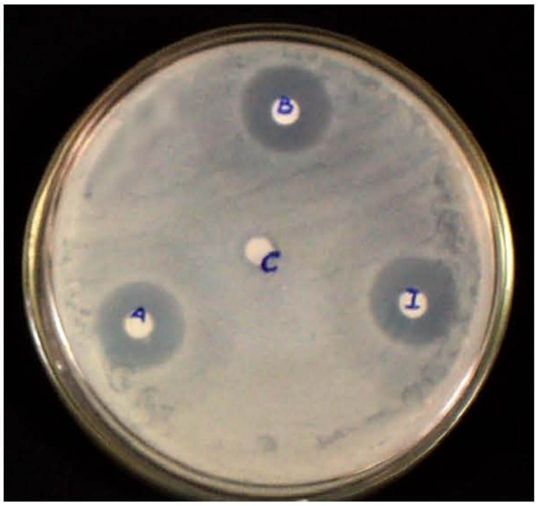

Fig. 1: Antagonistic activity against *Aeromonas hydrophila*, A: Antibiotic, B: Bacteriocin (Extra cellular protein), I: Intra cellular protein, C: Control

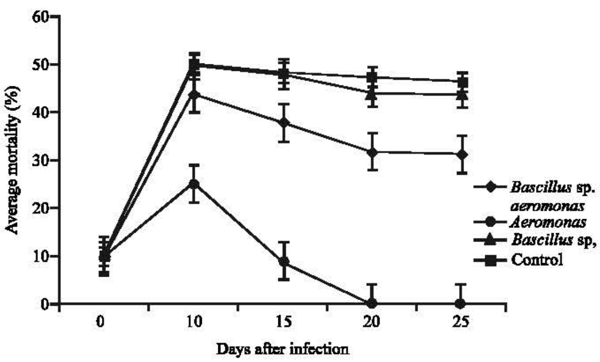

Fig. 2: Accumulated mortality of *Bacillus* sp. infected with 10^5-10^6 CFU mL^{-1} of pathogenic *A. hydrophila* for 25 days with probiotic treatment

RESULTS

The *Bacillus* sp. colonies were obtained from the *Oreochromis mossambicus* fish gut by the 10^{-6} and 10^{-7} dilutions; these were conformed as the initial screening for grams staining. Lactic acid bacteria are cocci or bacilli forms, non-motile, gram positive, Methyl red test positive and Catalase negative. Inhibition zone obtained the fish pathogen by antagonistic activity assay.

The inhibition zone 8 mm was observed on the plates inoculated with *Aeromonas hydrophila* by *Bacillus* sp. indicates the production of secondary metabolite of antimicrobial substances of bacteriocin against *A. hydrophila* (Fig. 1) by agar well diffusion method. Immersion study was observed in the four different groups of tanks contain *O. mossambicus* with 10^5-10^6 CFU mL^{-1} cultures of *Bacillus* sp. and *A. hydrophila* and mortality was observed (Table 1). Isolated bacillus strains were inoculated second tank 87.5% of mortality rate was observed by the 25 days experiment. At the same mass culture of common fish pathogen *A. hydrophila* (10^5-10^6 CFU mL^{-1}) were inoculated third tank was observed zero % of mortality rate. Both two stains were immersed fourth tanks survival rates were increased compare to the third tank. 65% of mortality rates were observed by probiotic stains using fourth tank (Fig. 2).

Table 1: Immersion study of *A. hydrophila* by *Bacillus* sp.

No. of tanks	Mortality rate of fishes (Days) 10	15	20	25
[1]Tank 1	50.00±7.22	48.11±6.66	47.33±6.35	46.21±7.60
[2]Tank 2	49.44±6.48	47.77±7.00	44.00±6.67	43.72±6.90
[3]Tank 3	25.16±5.91	9.00±40.80	0.00	0.00
[4]Tank 4	43.66±6.47	37.72±7.21	31.66±7.09	31.27±8.07
Significance	NS	4, 2 Vs 3	4, 2, 1 Vs 3	4, 2, 1 Vs 3

Values are given as mean±SD for three tanks in each group, Significant at ($p<0.05$), NS: Non significant, [1]Tank 1. Control, [2]Tank 2. *Bacillus* sp. (10^5-10^6 CFU mL^{-1}), [3]Tank 3. *Aeromonas hydrophila* (10^5-10^6 CFU mL^{-1}), [4]Tank 4. *Aeromonas hydrophila* (10^5-10^6 CFU mL^{-1}) + *Bacillus* sp. (10^5-10^6 CFU mL^{-1})

DISCUSSION

The concept of biological control for health maintenance has received widespread attention during the last few years, driven in large part by consumers and the lay press. The first report of the existence in freshwater of bacteria with an inhibitory effect against a *Aeromonas* sp. has been attributed to Ochoa and Olmos (2006) Subsequently, Rosenfeld and ZoBell (1947) described a study of antibiotic-producing marine microorganisms and since then research has begun to develop biological control strategies based on the application of these bacteria.

The isolated LAB species conformed by the methyl red and catalyzed negative test by Jack *et al.* (1995). Gatesoupe *et al.* (1999) observed that *L. lactis* showed an inhibitory activity against fish pathogen. This observation was confirmed by an inhibition zone of 8 mm as observed in this study. The culture media (supernatant) ECP were used, which showed highest inhibition against *A. hydrophila*. The inhibitory effects of *Bacillus* sp. may be due to production of antibiotics, bacteriocins, lysozymes, proteases and/or hydrogen peroxide and the alteration of pH values by the production of organic acids (Verschuere *et al.*, 2000). All the LAB species produced by secondary metabolite of bacteriocin compounds against *A. hydrophila* (Santos *et al.*, 1996). Adolfo (2004) was reported to the isolated *Lactobacillus* sp., both homofermenters and heterofermenters, were able to inhibit the human and fish pathogens by acid production when using a high glucose concentration. A few strains also inhibited both gram-positive and gram-negative fish and human pathogens with low (0.2%) concentration of glucose in the medium. Inhibitory activities of these strains have been usually detected against related species such as *Staphylococcus aureus*, *Clostridium* and other fish pathogenic bacteria (Schillinger and Lucke, 1989). But, only few *Bacillus* growing at low glucose concentration have been reported to inhibit a broader range of microorganisms, including gram negative foodborne and human pathogens. Some of these are currently used as probiotics (Jacobsen *et al*., 1999).

Bacteriocins are bactericidal or bacteriostatic peptides that are mostly active against bacteria closely related to the producer (Klaenhammer, 1988). Immersion application against pathogenic bacteria, *O. mossambicus* fish mortality rate was increased by the above experiments. *A. hydrophila* produces extracelluar hemolysis compounds after 24 h 10^5-10^6 CFU mL^{-1} culture inoculations. *In vivo* condition *A. hydrophila* hemolytic toxins were produced at the same time as *Bacillus* sp. had produced by the secondary metabolite of extracelluar bacteriocin compounds. The isolated *Bacillus* sp. produces hydrogen peroxidase, other organic acids and bacteriocin (Klaenhammer, 1988; Daeschel, 1989). A growing concern about the high consumption of antibiotics in aquaculture has initiated a search for alternative methods of disease control (Gildberg *et al*., 1997) and growth promotion (Byun *et al*., 1997). The isolated *Bacillus* sp. was checked the disc diffusion assay and to improved resistance against infectious diseases can be achieved by the use of probiotics (Gildberg *et al*., 1997). Probiotic are living preparations of microbial cells that, when ingested in high enough concentration, beneficially affect the host's health and growth by improving the intestinal microbial balance (Fuller, 1989; Havenaar *et al*., 1992). Selection of probiotic strains is achieved by screening procedures for several characteristics *in vitro*, such as inhibitory activities against several fish pathogens and gastric and intestinal secretions (Byun *et al*., 1997; Joborn *et al*., 1997). Cai *et al*. (1998) was reported to the similar experiments have shown that the inoculation of some probiotic strains, mainly lactic acid bacteria, increase fish survival after being challenged with fish pathogens while lactic acid bacteria populations in the gut increase and in one study lactic acid bacteria inoculation was related with an increase of fish growth rate.

The present study suggests that it is possible to maintain artificially the gut isolated *Bacillus* sp. treatment of fish by the immersion techniques proved successful as against fish pathogen of *A. hydrophila.* It is apparent that lactic acid producing bacteria may be used to control the *A. hydrophila* infection in fresh water fishes. Statistical analysis displayed significant differences ($p<0.05$) in the mortality of the tilapia between the treatment and control groups. Mortality in probiotic treatments and control were observed starting on the third day of contact with the pathogen, but the probiotic treatments were significantly different from the control when the mortality stabilized ending on the 25th day (Fig. 2). The high dose of the *Bacillus* sp. did not observe any negative side effects and this dose was associated with some improvements in the specific growth rate. However, further studies in which dosages of *Bacillus* sp. are repetitively fed to fish and to evaluate the biological effects of such treatments.

REFERENCES

Adolfo, B.G., 2004. *Lactobacillus plantarum* 44A as a live feed supplement for freshwater fish. Ph.D Thesis, The Netherlands with Summaries in English, Dutch and Spanish, Wageningen Universiteit, Wageningen. The Netherlands ISBN., 90-5808-943-6.

Benkerroum, N., Y. Ghouati, W.E. Sandine and A. Tantaoui-Elaraki, 1993. Methods to demonstrate the bactericidal activity of bacteriocins. Lett. Applied Microbiol., 17: 80.

Byun, J.W., S.C. Park, Y. Ben and T.K. Oh, 1997. Probiotic effect of *Lactobacillus* sp. DS-12 in flounder (*Paralichthys olivaceus*). J. Gent. Applied Microbiol., 43: 305-308.

Cai, Y., Y. Benno, T. Nakase and T.K. Oh, 1998. Specific probiotic characterization of *Weissella hellenica* DS-12 isolated from flounder intestine. J. Gent. Applied Microbiol., 44: 311-316.

Cai, Y., P. Suyanandana, P. Saman and Y. Benno, 1999. Classification and characterization of lactic acid bacteria isolated from the intestines of common carp and freshwater prawns. J. Gent. Applied Microbiol., 45: 177.

Daeschel, A.M., 1989. Antimicrobial substances from lactic acid bacteria for use as food as preservatives. Food Technol., 43: 91.

Fuller, R., 1989. Probiotics in man and animals. A review. J. Applied Bacteriol., 66: 365-378.

Gatesoupe, F.J., 1999. The use of Probiotics in aquaculture. Aquaculture, 180: 147.

Gildberg A., H. Mikkelsen, E. Sandaker and E. Ringo, 1997. Probiotic effect of lactic acid bacteria in the feed on growth and survival of fry of Atlantic cod (*Gadus morhua*). Hydrobiologia, 352: 279-285.

Gonzalez, C.J., J.P. Encinas, M.L. Garcia-Lopez and A. Otero, 2000. Characterization and identification of lactic acid bacteria from freshwater fishes. Food Microbiol., 17: 383.

Havenaar, R. and J.H.J. Huis in't Veld, 1992. Probiotics: A General View. In: The Lactic Acid Bacteria. The Lactic Acid Bacteria in Health and Disease. Wood, B.J. (Ed.), Vol. 1. Elsevier Applied Sci., London, pp: 151-170.

Ishibashi, N. and S. Yamazaki, 2001. Probiotics and safety. Am. J. Clin. Nutr., 73: 465.

Jack, R.W., J.R. Tagg and B. Ray, 1995. Rev. Bacteriocins of gram positive bacteria. Microbiology, 59: 171.

Jacobsen, C.N., V. Rosenfeldt Nielsen, A.E. Hayford, P.L. Moller, K.F. Michaelsen, A. Pærregaard, M. Sandström, Tvede and M. Jakobsen, 1999. Screening of probiotic activities of forty-seven strains of *Lactobacillus* sp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. Applied Environ. Microbiol., 65: 4949-4956.

Joborn A., J.C. Olsson, A. Westerdahl, P.L. Conway and S. Kjelleberg, 1997. Colonization in the fish intestinal tract and production of inhibitory substances in intestinal mucus and faecal extracts by *Carnobacterium* sp. strain K1. J. Fish Dis., 20: 383-392.

Kandler, O. and W.N. Regular, 1986. Non-Sporing Gram-Positive Rods. In: Bergey's Manual of Systematic Bacteriology. Sneath, P.H.A., N.S. Mair, M.E. Sharpe and J.G. Holt (Eds.), Vol. 2, Williams and Wilkins, Baltimore MD., pp: 1208.

Karunasagar, I., S.K. Otta, I. Karunasagar and K. Joshua, 1996. Applications of *Vibrio* vaccine in shrimp culture. Fish Chim., 16: 49.

Klaenhammer, T.R., 1988. Bacteriocin of lactic acid bacteria. Biochimie, 70: 337.

Kvasnikov, E.I., N.K. Kovalenko and L.G. Materinskaya, 1977. Lactic acid bacteria of freshwater fish. Mikrobiologiya, 46: 619.

Lightner, D.V., T.A. Bell, R.M. Redman, L.L. Mohney, J.M. Natividad, A. Rukyani and A. Poernomo, 1992. A review of some major diseases of significance in penaeid prawn/shrimps of the America and Indo-Pacific. Dis. Asian Aquacult., pp: 57.

Mayra-Makien, A. and M. Bigret, 1993. Industrial Use and Production of Lactic Acid Bacteria. In: Lactic Acid Bacteria. Salminen, S.V. and A. Wright (Eds.), Marcel Dekker, New York, pp: 65.

Moriarty, D.J.W., 1997. The role of microorganisms in aquaculture ponds. Aquaculture, 151: 333.

Ochoa, S.J.L. and S.J. Olmos, 2006. The functional property of *Bacillus* for shrimp feeds. Food Microbiol., 23: 519-525.

Rengipipat, S., S. Rukpratanporn, S. Piyatiratitivorakul and P. Menasaveta, 2000. Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiotic bacterium (*Bacillus* S11). Aquaculture, 191: 271.

Ringo, E., E. Strom and J.A. Tabachek, 1995. Intestinal microflora of salmonids: A review. Aquacult. Res., 26: 773.

Ringo, E. and F.J. Gatesoupe, 1998. Lactic acid bacteria in fish: A review. Aquaculture, 160: 177.

Rosenfeld, W.D. and C.E. ZoBell, 1947. Antibiotic production by marine microorganisms. J. Bacteriol., 54: 393-398.

Santos, J.T., M.L. Lopez-Diaz, Gareia-Fernandez, Gareia-Lozez and A. Otero, 1996. Effect of lactic starter culture on the growth and protease activity of *Aeromonase hydrophila*. J. Applied Bacteriol., 10: 13.

Schillinger, U. and F.K. Lucke, 1989. Antibacterial activity of *Lactobacillus sakei* isolated from meat. Applied Environ. Microbiol., 55: 1901-1906.

Verschuere, L., G. Rombaut, P. Sorgeloos and W. Verstraete, 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiol. Mol. Biol. Rev., 64: 655-671.

Yasuds, K. and N.A. Taga, 1980. Mass culture method for *Artemia salina* using bacteria as food. Mer., 18: 53.