Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2020; 8(4): 125-128

Received: 07-05-2020
Accepted: 09-06-2020

Nesara KM
Department of Aquaculture, College of Fisheries, Mangalore, Karnataka, India

Jayaraj EG
Department of Aquaculture, College of Fisheries, Mangalore, Karnataka, India

Sandeep CV
Department of Aquaculture, College of Fisheries, Mangalore, Karnataka, India

Corresponding Author:
Nesara KM
Department of Aquaculture, College of Fisheries, Mangalore, Karnataka, India

Effects of *Lactobacillus plantarum* and citric acid individually and in combination on certain immunological parameters of Indian major carp *Labeo rohita* challenged to *Aeromonas hydrophila*

Nesara KM, Jayaraj EG and Sandeep CV

Abstract
The present study was carried out to evaluate the influence of dietary supplementation of probiotic and organic acid on immunological parameters of *Labeo rohita*. The feeding trial was conducted for about 120 days with fingerlings of length and weights ranging from 8.55-9.21cm and 5.55-6.65g respectively. The fishes were administered with diets containing different level of *Lactobacillus plantarum* (LP) (10^8 CFU/g) and citric acid (CA) (3%) individually and in combination 10^8 CFU/g LP+ 3% CA and without *Lactobacillus plantarum* and citric acid served as control. Higher super oxide anion production was evident in *Lactobacillus plantarum* (10^8 CFU/g) followed by citric acid (3%) individually while LP+CA (combination) showed significant difference between the groups (P<0.05). The total serum protein was higher in LP+CA followed by LP alone, citric acid alone and control with significance (P<0.05). Similarly, lysozyme activity also showed significant difference (P<0.05) but observed to be higher in LP alone followed by LP+CA, citric acid alone and control diet. The fishes challenged to *Aeromonas hydrophila* at the end of growth study showed significant difference in cumulative survival in different treatment groups. However, highest cumulative percent of survival and relative percent survival was evident in fish fed with LP+CA incorporated diets.

Keywords: *Lactobacillus plantarum*, citric acid, *Labeo rohita*, *Aeromonas hydrophila*

Introduction
The rapid increase of the world's population has witnessed the corresponding increase in the demand for the food production in general and the protein requirement in particular. Indian freshwater aquaculture constitutes mainly the culture of Indian major carps (IMC) namely, Catla (*Catla catla*), rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigal*). Among these IMC, rohu (*Labeo rohita*) is the most preferred fish for freshwater aquaculture. It is widely cultured in Indian subcontinent mainly in India and Bangladesh. In the present scenario aquaculture have continued to follow the trend where in capture fisheries production reaching stagnate on the aquaculture output expanding faster than any other animal based food sector. Aquaculture has been increasing in recent decades as a consequence of increase in fish consumption, since capture fisheries have possibly reached their maximum due to overexploitation [1].

The rapid expansion and intensification of aquaculture production had led to the outbreaks of new pathogens and infectious diseases caused by viruses, bacteria and parasites, inflicting major problems in the fish farming industry [2, 3]. Frequent use of antibiotic in the aquaculture system harmful for aquatic animals which majorly affect the beneficial microbiota in gastrointestinal system of animals and in turn unsafe for human consumption. This resulted in a new trend in aquaculture that paved a way towards use of probiotic in aquaculture which exert a beneficial effect via a wide array of actions.

Probiotics are defined as live bacteria which are beneficial microbes that can be consumed for the betterment of the health benefits of host [4]. Probiotics can produce inhibitory compounds, which are mainly competing for chemicals and adhesion sites and can modify the immune function by improving the microbial adhesion [5]. Commonly used probiotics in aquaculture are *Carnobacterium*, *Saccharomyces*, *Lactobacillus*, *Bacillus*, *Clostridium*, *Leuconostoc*, *Lactococcus*, *Aeromonas* and several other species [6]. Many published reports showed positive responses of the addition of *Lactobacillus plantarum* as a probiotic which can produce bacteriocin and can inhibit the growth of Gram-positive and Gram-negative bacteria [7].

Feed based incorporation of *L. plantarum*as a dietary supplement in aquaculture has been employed to protect from various fresh water infectious diseases [8]. In culturing ecosystem adhesion of *Aeromonas hydrophila* and *Aeromonas salmonicida* significantly reduced when the fish consume *L. plantarum* incorporated feed [9].

Another replacement to antibiotics can be organic acids employed in aqua feed like citric acid, propionic acid, acetic acid, lactic acid and oxalic acid have been evaluated for their effects on the feeding and immune response in fish. Incorporation of such acidifiers in the fish feed mainly reduce the pH in the gastrointestinal tract (GI), increased the phytate hydrolysis, nutrient digestibility, improved nitrogen retention, mineral absorption and resulting in killing the GI pathogenic microorganisms and resulting in decrease in emptying time of the GI tract. Citric acid which are used widely in aquaculture and pharmaceuticals industry for its unique flavor and high buffering capacity. Although several scientific publications have focused on immunological parameters, pertaining to citric acid very few publications have delineated their effects on the immunological parameters. Thus, the present study focused on supplementing *L. plantarum* and citric acid individually and in combination on immunological parameters of *L. rohita* and further challenging with a bacterial pathogen *A. hydrophila* to understand the innate immunity responses.

Materials and Methods

Experimental set up

The experiment was carried out in triplicates in 2×2×1m soil based cement cisterin. The spawn of *Labeo rohita* was procured from Bhadra Reservoir Project Fish Seed Production Centre, Department of Fisheries, Government of Karnataka, Shivamogga and further reared in the Research and Instructional Fish Farm of the College of Fisheries, Mangaluru for about 7- 8 months till it attain fingerling in the range of 8.55-9.21 cm and weight 5.55-6.65g. After experimental period of 120 days fish were subjected to immunological assay. The whole blood (0.5 ml) was collected from the caudal vein of each fish, using syringes (1ml) and 27-gauge needles that were rinsed in heparin (15 unit ml^{-1}).

Experimental feed preparation

Four experimental diets namely F1: *Lactobacillus plantarum* (10^8 cfu/g), F2: citric acid 3%, F3 *L. plantarum*+citric acid (10^8 cfu/g + 3%) and F0: control without supplementing *Lactobacillus plantarum* and citric acid were prepared using locally available feed ingredients like ground nut oil cake, rice bran, fish meal, tapioca, vitamin- minerals mix were used for feed preparations. Feeds were formulated according to Pearson's square method to achieve approximately 30% protein in the diet. All the feed ingredients were ground (except probiotic and vitamin- mineral mix) mixed with sufficient quantity of water (1:0.8) and hand kneaded to dough which was further cooked under pressure at 105 ºC for 20-30 min. The cooked feed was cooled to ambient temperature and mixed with probiotic and organic acid. This dough mixture was further extruded using a pelletizer having 3 mm dia. Pellets were dried in a hot air oven at 60 ^{0}C till the moisture content was reduced to less than 10%.

Immune parameters of *Labeo rohita*

a) Super oxide anion production assay (Nitro blue tetrazolium assay (NBT)): Blood (0.1 ml) was placed in micro titer plate wells, to which an equal amount of 0.2% NBT solution was added and incubated for 30 min at room temperature. A sample of NBT blood cell suspension (0.05 ml) was added to a glass tube containing 1 ml N, N-dimethyl formamide and centrifuged for 5 min at 3000 rpm. The supernatant fluid was measured in a spectrophotometer at 620 nm in 1 ml cuvettes [10].

b) Lysozyme (Turbidimetric method) assay: Serum lysozyme activity of fish in each group was measured with the turbidimetric method as described[11] using 0.2 mg mL^{-1} lyophilized *Micrococcus luteus* as the substrate in 0.05M phosphate buffer (pH 6.2). Various amounts of serum (25-100µl) were added to 2 ml of the suspension and the absorbance was measured at 0.5 and 4.5 min intervals at 530 nm (25 ^{0}C). A lysozyme unit was defined as the activity of enzyme producing a decrease in absorbance of 0.001 min^{-1}.

c) Total serum protein: Total serum protein was measured by using GeNei™ protein analysis kit (by Lowry's method).

Susceptibility of *Labeo rohita* to *Aeromonas hydrophila* infection:

The *A. hydrophila*, AH04, was grown in nutrient broth and incubated at 37 C for 24 h. Lethal dose 50% (LD50) of *A. hydrophila* (i.e., able to kill 50% of the *Labeo rohita* population) was determined by intraperitoneal injection of 60 fish with different doses of *A. hydrophila* (10^6, 10^7, 10^8 and 10^9 CFU/fish) at 25 ºC. LD50 were calculated using the method by consideration of Koch's postulate [12]. Sixteen days after experimental feeding, the fish from each group were divided into two subgroups (45) fish in each group), one as a challenge group to be infected with *A. hydrophila* and the other as an uninfected control. The challenge group was injected intra peritoneal with 0.2 ml PBS containing the LD50 dose of live *A. hydrophila* determined above, while control fish received only 0.2 ml PBS. The cumulative mortality was determined after 10 days post-infection.

Relative percent survival (RPS) was calculated as detailed below [13].

RPS = [1-(% mortality of treatment group / % mortality in control] × 100

Statistical analysis

The data obtained from this study were subjected to statistical tools. The differences in the means (± SD) between groups were assessed using one way Anova.

Results and Discussion

In present study, the feed was supplemented with *L. plantarum* and citric acid individually and in combination; so far no clear evidence in literature about the immunological parameters influenced by combined effect of LP+CA in fish are available. Higher superoxide anion production noted in the group fed with F1 (1.120) followed by F2 (0.976), F3 (0.802) and F0 (0.076) statistically there was no significant difference (P<0.05) (Table 1) was observed between the groups. Study reported that increase in respiratory burst activity of sea bream (*Sparus aurata*) leucocytes with 51M6, *L. delbrueckii* subsp. lactis and *B. subtilis* increase in trend was noticed in all the three probiotic groups[14]. Similarly, in a study improved in NBT level in *O. mykiss* fed with *L. rhamnosus* [15]. In the similar way, significant increase in

respiratory burst activity by supplementing rainbow trout fed with probiotic *Carnobacterium divergens* B 33 [16]. These results proved that by supplementing probiotic for longer time to the fish as a feed additive will be resulting in activation of phagocytic cells throughout the experimental period to achieve disease resistance.

Lysozyme is one of the most indispensable tools to fight against infectious agents in the innate immunity responses in the aquatic environment [17]. In the present study, higher in F1 (1763.6) followed by F3 (1454.33), F2 (1312.00) and F0 (1103.00) lysozyme activity was found in (Table 1). Similarly reports were found by feeding *L. plantarum* VSG3 [18] at the concentration of 10^8 and 10^{10}cfu g^{-1} fed to *L. rohita* showed significantly higher than the control diet after 60 days of feeding regime. Similar results were also noted by authors in kelp grouper, *E. bruneus* [19] and *E. coioides* [20]. Likewise, feeding humic acid to *common carp* at concentrations of 0.4, 0.8, and 1% and increased in lysozyme activity was noted in 6th week of the feeding experiment but there were no any statistical significant changes [21]. In present study citric acid showed increase in lysozyme activity than the control group. In reference to above authors, in present study results fish fed at 10^8cfu g^{-1} *L. plantarum* showed significantly enhanced serum lysozyme activity after 120 days of the feeding period.

Total serum protein is an indicator of nutritional condition and fish health which are monitored and indicated by total serum protein in the fish body [22]. In the present study, higher serum protein was noted in the group F3 (34.49) followed by F1 (26.83), F2 (23.33) and F0 (17.29) (Table 1) with significant difference (P<0.05) between the groups. In relation to present study increase in serum protein, albumin and globulin levels are thought to be associated with a stronger innate immune response of fish [23]. Increased in serum protein, globulin content and a lower A/G ratio in *L. rohita* treated with *B. subtilis* [24]. In the present study decrease in total serum protein was observed in the group fed with citric acid compare to LP+CA and *L. plantarum* alone, these results shows that a greater immunomodulatory effect was enhanced and defence mechanism was observed in the group fed with LP+CA. Decrease in trend of serum protein was observed in control group than the supplemented groups showed lowest defence mechanism and the immunity level.

After 120 days of experimental period *L. rohita* was challenged to understand the immune status *Aeromonas hydrophila.* The cumulative percentage survival and relative percentage survival was notably increased in the group fed with F3 (71.42) followed by F1 (47.61), F2 (42.85) and F0 (19.04) with no significant difference (P<0.05) (Table 2). The relative percentage survival (RPS) (Table 3) showed increase in RPS was in F3 (82.40) followed by F2 (35.60), and decrease in RPS value was noted in the group fed with the diet F1 (35.29) (Table 3). Similar observation has been represented on *L. vannamei* fed with *L. plantarum* proved to be beneficial by increasing humoral and cellular immune response after challenged with *V. alginolyticus* [25]. High cumulative mortality of fish fed with no organic acids as compared to fish fed with organic acid supplemented diets at 16 days of post challenge indicates the possible role of organic acids in immunomodulation [26].

Table 1: Immunological parameter of control and treated group

Treatment	Super oxide anion production (OD at 620 nm)	Lysozyme activity (10^3 Units/l)	Total serum protein(mg/ml)
F0	0.076 ±0.00	1103.00±89.67	17.29±1.54
F1	1.120±0.10	1763.60±5.231	26.83±0.70
F2	0.976±0.33	1312.00±24.00	23.33±1.49
F3	0.802±0.15	1454.33±25.77	34.49±1.32

Table 2: Cumulative percentage of survival of *Labeo rohita* recorded in different treatments and control group after challenged with *Aeromonas hydrophila.*

Treatments	Cumulative percentage of survival of *Labeo rohita*
F_0	19.04
F_1	47.61
F_2	42.85
F_3	71.42

Table 3: Relative percentage of survival (RPS) of *Labeo rohita* recorded in different treatments and control group after challenged with *Aeromonas hydrophila.*

RPS / Treatments	Relative percentage of survival against control (%)
F_1	35.29
F_2	35.60
F_3	82.40

Conclusion

Our findings showed that combined use of lactic acid and organic acid would give better results with immunity than single use of any other in the fish feed diet.

References

1. Ferreira JG, Andersson HC, Corner RA, Desmit X, Fang Q, De Goede ED. Sustainable Options for People, Catchment and Aquatic Resources. The SPEAR Project, an International Collaboration on Integrated Coastal Zone Management. Institute of Marine Research (IMAR), European Commission. 2008, 180.
2. Gatesoupe FJ. Updating the importance of lactic acid bacteria in fish farming: natural occurrence and probiotic treatments. J Molecular Microbiology and Biotechnology. 2008; 14:107-114.
3. Geng X, Dong XH, Tan BP, Yang QH, Chi SY, Liu HY *et al.* Effects of dietary probiotic on the growth performance, nonspecific immunity and disease resistance of cobia, *Rachycentron canadum.* Aquaculture Nutrition, 2012; 18(1):46.
4. Food and Agriculture Organization/World Health Organization (FAO/WHO), 2001. Health and nutritional properties of Probiotics in food including powder milk with live lactic acid bacteria, Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live lactic acid bacteria, Co´rdoba, Argentina.
5. Fuller R. Probiotics in man and animals. J. Applied Bacteriology, 1989; 66:365.
6. Abumourad IMK. Enterococcus faecium probiotic as a growth promoter and its impact on the expression of the host innate immune in cultured Oreochromis niloticus.

Research Journal of Pharmaceutics, Biology Chemical Sciences, 2014; 63(1):1.
7. Lash BW, Tami H, Mysliwiec, Gourama H. Detection and partial characterization of a broad-range bacteriocin produced by *Lactobacillus plantarum* (ATCC 8014). Food Microbiol, 2005; 22:199.
8. Geng X, Dong XH, Tan BP, Yang QH, Chi SY, Liu HY *et al*. Effects of dietary probiotic on the growth performance, non-specific immunity and disease resistance of cobia, *Rachycentron canadum*. Aquaculture Nutrition, 2012; 18(1):46.
9. Denev SY, Staykov R, Moutafchieva, Beev G. Microbial ecology of the gastrointestinal tract of fish and the potential application of probiotics and prebiotics in finfish aquaculture. International journal of Aquatic Research. 2009; 1:1.
10. Siwicki AK, Studnicka M, Ryka B. Phagocytic ability of neutrophils in carp (*Cyprinus carpio*). Bamidgeh. 1985; (37)123.
11. Parry RM, Chandau RC, Shahani RM. A rapid sensitive assay of muramidase. Proceedings of the Society for Experimental Biology. 1965; 119:384.
12. Amend DF, Potency testing of fish vaccines. Dev Biology Studies. 1981; 49:447.
13. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. American J Hygiene. 271(1938) 493.
14. Salinas I, Diaz-Rosales P, Cuesta A, Meseguer J, Chabrillon M, Morinigo MA *et al*. Effect of heat-inactivated fish and non-fish derived probiotics on the innate immune parameters of a teleost fish (*Sparus aurata* L.). Veterinary Immunology Immunopathology. 2006; 111:279.
15. Nikoskelainen S, Ouwehand AC, Bylund G, Salminen S, Lilius E. Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotics bacteria (*Lactobacillus rhamnosus*). Fish Shellfish Immunology, 2003; 15:443.
16. Kim DH, Austin B. Innate Immune Responses in Rainbow trout *Oncorhynchus mykiss* Induced by Probiotics. Fish Shellfish Immunology, 2006; 21:513.
17. Yang JL, Chen HC. Effects of gallium on common carp (*Cyprinus carpio*): acute test, serum biochemistry and erythrocyte morphology. Chemosphere, 2003; 53:877.
18. Yano T, Matsuyama H, Mangindaan REP. Polysaccharide induced protection of carp *Cyprinus carpio* against bacterial infection. Journal of Fish Disease. 1991; 14:577.
19. Nayak SK, Swain P, Mukherjee SC. Effect of dietary supplementation of probiotic and vitamin C on the immune response of Indian Major Carp, *Labeo rohita* (Ham.). Fish Shellfish Immunology, 2007; 23:892.
20. Lindsay GJH. The significance of chitinolytic enzymes and lysozyme in rainbow trout (*Salmo gairdneri*) defense. Aquaculture, 1986; 51:169.
21. Giri SS, Sukumaran V, Oviya M. Potential, Probiotic *Lactobacillus plantarum* VSG3 Im- proves the Growth, Immunity, and Disease Resistance of Tropical Freshwater Fish, Labeo rohita. Fish Shellfish Immunollogy. 2013; (34):660.
22. Harikrishnan R, Balasundaram C, Heo MS. Lactobacillus sakei BK19 enriched diet enhances the immunity status and disease resistance to *Streptococcosis* infection in kelp grouper, *Epinephelus bruneus*. Fish Shellfish Immunology. 2010; 29:1037.
23. Son VM, Chang CC, Wu MC, Guu YK, Chiu CH, Cheng WT. Dietary administration of the probiotic, *Lactobacillus plantarum*, enhanced the growth, innate immune responses, and disease resistance of the grouper Epinephelus coioides. Fish Shellfish Immunology. 2009; 26(5):691.
24. Ahmed M, Abdel-Wahab, Ahmed ME, El-Refaee, Ayman A. Ammar, Effects of Humic Acid as Feed Additive in Improvement of Nonspecific Immune Response and Disease Resistance in Common Carp (*Cyprinus carpio*). Egyptian Journal of Aquaculture. 2012; 2(1).
25. Norha CBR, Marysol SR, Ariane MG, Cristhiane G, Juliana RR, Walter QS *et al*. Effect of dietary supplementation with butyrate and probiotic on the survival of Pacific white shrimp after challenge with *Vibrio alginolyticus*. Revista Brasileira de Zootecnia, 2017; 46(6):471.
26. Wing KN, Koh CB, Sudesh K, Siti Z, Abdullah. Effects of dietary organic acids on growth, nutrient digestibility and gut microflora of red hybrid tilapia, *Oreochromis* sp., and subsequent survival during a challenge test with *Streptococcus agalactiae*. Aquaculture Research. 2009; 40:1490.