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Effect of Probiotic *Lactobacillus plantarum (NCDC LP20)* on Enhanced Innate Immunological Indices of Freshwater Fish.

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Abstract: In according to several reports, the advantageous effects of bacteria on fish have well been documented. Probiotics as an alternative strategy have been suggested to be used as reinstatement for antimicrobial drugs and growth promoters. Some researchers believe that Probiotics have an advantages for strengthening the health of fish in aquaculture and augmenting fish performance. The present study was designed to investigate the enhanced effects in innate immunological indices of by the oral administration of probiotic *Lactobacillus plantarum* (NCDC LP20) in freshwater fish, *Catla catla.* Probiotic was administered orally at three different doses 1×10^7 (T_2), 1.5×10^7 (T_3), 2×10^7 (T_4) cfu/g feed to *Catla catla.* for four weeks. The positive control group (T1) and negative control group (T_5) was fed feed without probiotic for the same period. On 29thday, blood and serum samples were collected to determine differential leukocyte counts (DLC), respiratory burst activity (NBT assay) and serum bactericidal activity. Fishes were challenged intra peritoneal injection with *Aeromonas hydrophila* after four weeks in the treatment groups (T_2, T_3 and T_4) and also in the positive control group (T_1), while the negative control group (T_5) was administered with phosphate buffer saline (PBS pH 7.2). The NBT assay and DLC were assessed on 7th day post challenge. Probiotic *Lactobacillus plantarum* (NCDC LP20) treated fish showed significantly higher ($P < 0.05$) respiratory burst activity and bactericidal activity during the pre-challenge compared with the control groups. The highest respiratory burst activity (0.42±0.01) and serum bactericidal activity was recorded in the group (T_4) fed feed containing *Lactobacillus plantarum* (NCDC LP20) at 2×10^7 cfu/g feed. Granulocytes numbers were significantly higher ($P < 0.05$) in treatment groups in comparison to the control in both the pre and post challenge periods. The result suggests that *Lactobacillus plantarum* (NCDC LP20) significantly enhance innate immune response in *Catla catla.*

Keywords: *Aeromonas hydrophila,* Differential leukocyte counts, *Catla catla*, *Lactobacillus plantarum* (NCDC LP20), Respiratory burst activity, Serum bactericidal activity.

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I. INTRODUCTION

The term probiotic is a Greek word, 'pro' meaning favour and 'bios' meaning life, i.e., favouring life or for life. The first person to describe the role of beneficial bacteria (probiotics) was Elie Metchnikoff who studied the effect of sour milk on human beings in 1907 [1]. 'Historically' the term 'probiotic' inevitably referred to gram-positive bacteria associated with the genus *Lactobacillus.* The original work on probiotic started with a *Lactobacillus* strain, *L. bulgaricus* and since then lactobacilli have remained the most commonly used probiotic organisms, as they are active against various gram positive and gram-negative bacteria.

Different strains of Lactobacilli currently used in probiotic preparations are *L. fructivorans* [2], *L. bulgaricus* [3], *L. acidophilus* [4, 5, 6], *L. reuteri* [7], *L. helveticus* [8], and *L. plantarum* [8,9,10]. Experiments with warm-blooded animals indicated that probiotics (lactic acid bacteria) administered orally might induce increased resistance to enteric infections [11]. There are many reports that bacterial compounds act as an immunostimulant in fish and shrimp, which has been reviewed by Sakai [12]. It has also been suggested that ingestion of bacteria and subsequent endocytosis in cod and herring larvae are involved in stimulation of the developing immune system [13]. Rengpipat *et al.* [14] reviewed the probiotic factors, which stimulate the immune system in shrimp. These include, (a) Dead bacterial cells (b) Yeast p-glucan and (c) Yeast zymosan [15, 16]. Many bacterial strains with probiotic potential are now being used as either single strain or multiple strain preparations. There has been increasing interest in the use of probiotics in aquaculture to control fish diseases [17, 18]. Hence, the present study was to investigate the effect of probiotic *Lactobacillus plantarum (NCDC LP20)* on enhanced innate immuno parameters in *Catla catla.*

II. MATERIAL AND METHODS

2.1 Source of fish

Fingerlings of *Catla catla* (Hamilton) collected from government fish breeding form at Kalyanidam, near to Tirupati, Chittoor district, Andhra Pradesh, were brought and acclimatized to the laboratory conditions for two weeks prior to the experiment and maintained on normal diet. Chlorine free tap water was used throughout the course of the experiment. The physico-chemical characteristics of the water were as follows: temperature, 29-31^{0}C; hardness, 80 mg/L (as $CaCO_3$); alkalinity, 82 mg/L; pH, 7.1 – 7.4; and dissolved oxygen concentration, 6.9 mg/L.

2.2 Culture of probiotic bacteria and feed preparation

The *Lactobacillus plantarum* (NCDC LP 20) was obtained as a gift from Dr. B. Vijay Kumar, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India. *Lactobacillus plantarum* (NCDC LP 20) was inoculated in to conical flask (1000ml) containing MRS broth and incubated at 37^{0}C for 24 hours in a shaker incubator. The culture was centrifuged at 10,000×g for 20 min at 4^{0}C and the supernatant discarded, while the pellet was resuspended in phosphate buffer saline (PBS; pH 7.2). The suspension was similarly washed and re-centrifuged four times and then quantified by spread plate technique (nutrient agar, incubated at 37^{0}C for 24 hours to determine the number of colony forming units, cfu). Purified and quantified bacteria were kept at 4^{0}C in suspended form and were used for feed preparation required. Feed was prepared thoroughly mixing the ingredients followed by steaming for 20 min, cooled, required bacterial culture was mixed thoroughly, and then pellets were made by a hand pelletizer. 10 ml, 15 ml, and 20 ml of *Lactobacillus plantarum* (NCDC LP 20) suspended in PBS at 10^7 cfu/ml/100g feed were used for treatment groups T2, T3 and T4 respectively. Feed was prepared every week and stored in a screw capped glass bottle at 4^{0}C.

2.3 *Aeromonas hydrophila* and challenge study

Aeromonas hydrophila was received as a gift from a doctor NIMS, Hyderabad. The isolate was verified by biochemical test and kept in nutrient agar slant at 4^{0}C for further use. *Aeromonas hydrophila* was inoculated in nutrient broth (Himedia Ltd, Chennai, India) and incubated at 37^{0}C, for 24 hours. The culture was centrifuged at 3000×g for 10 min. The supernatant was discarded and the pellet was re-suspended in sterile phosphate buffer saline (PBS, pH 7.4). Then the bacterial number was calculated by measuring optical density (OD) in a spectrophotometer and confirmed by plate count method. The final bacterial concentration was adjusted to 1×10^7 cfu/mL by serial dilution. The experimental fishes were challenged intraperitoneally with the bacterial suspension of 0.2, 0.3, 0.4 mL (1×10^7 to 2×10^7 cfu/mL) after 28 days of feeding the three treatment groups (T_2, T_3 and T_4) and positive control but the negative control (T_5) was injected intraperitoneally with PBS only. *Aeromonas hydrophila* was confirmed after re-isolating it from kidney of the fish of all groups, which are sacrificed on 7th day. Mortality was observed in control group (T1) and morbidity was observed in T2, T3 and T4 only.

2.4 Differential leukocyte count

Blood was drawn from the caudal peduncle region using sterile 2 ml syringes rinsed first with 2.7% EDTA solution and was collected in Eppendorf tubes coated with 20 ml of 2.7% EDTA solution. Methanol fixed blood smears were stainedwith Maye Grunewald Giemsa stain for 5 min. The slides were washed in tap water and allowed to dry before microscopic examination. Two slides per fish were taken for counting and two fishes were used from each tank at a time. Leukocytes were counted under microscope through many fields till it reached 100 cells per slide to find out the percentage of monocytes, granulocytes and lymphocytes in the blood. Absolute numbers of leukocytes were determined using a blood cell counter.

2.5 Respiratory burst activity

The respiratory burst activity of the phagocytes was carried out by nitro blue tetrazolium (NBT) assay following the method of Sercombes. Subsequently modified by Stasiack and Baumann [19].50µl of blood was placed into the wells of 'U' bottom microtitre plates and incubated at 37^{0}C for 1 hour to facilitate adhesion of cells. Then the supernatant was removed and the adhered cells were washed three times in PBS (pH 7.2). After washing, 50 µl of 0.2% NBT was added and the cells were incubated for a further 1 hour. The cells were then fixed with 100% methanol for 3 min and again washed thrice with 30% methanol. The plates were then air-dried and 60 µl 2 N potassium hydroxide (KOH) and 70 µl dimethyl sulphoxide were added into each well to dissolve the formazon blue precipitate. The optical density was then read in an ELISA reader at 540 nm.

2.6 Serum bactericidal activity

Blood was collected without anticoagulant, kept at room temperature for 1 hour, centrifuged at 2500 ×g for 10 min and the serum was collected by a micropipette. The serum was assayed for bactericidal activity following

Rainger and Rowley [20]. For this, *Aeromonas hydrophila* culture was centrifuged and the pellet was washed and suspended in PBS. The optical density (OD) of the suspension was adjusted to 0.7 at 540 nm. This bacterial suspension was serially diluted (1:10) with PBS five times. Serum bactericidal activity was determined by incubating 2 µl of the diluted *Aeromonas hydrophila* suspension with 20 µl of serum in a micro-vial for 1 hour at 37^0C. In the control group, PBS was used in place of the serum. After incubation, the number of viable bacteria was determined by counting the colonies grown on nutrient agar plate for 24 hours at 37^0C.

2.7 Statistical analysis

Significant differences among treatment groups were tested by one-way analysis of variance (ANOVA) and the comparison of any two mean values was made by Duncan's multiple range tests. A significance level of $P < 0.05$ was used. The mean values for pre- and post-challenge parameters were compared by Student's t-test. The statistical analysis was performed by using the software program SPSS (version 11.5).

III. RESULTS

3.1 Differential leukocyte count

The differential leukocyte counts of *Catla catla* of the experimental groups were shown in **Table 1.** The number of granulocytes and monocytes increased significantly ($P < 0.05$) in the treatment groups in comparison to the control group during pre-challenge. Treatment group T4 showed the maximum granulocyte (30±0.06) and monocyte (20±1.28) number during pre-challenge.

The granulocytes were significantly higher ($P < 0.05$) in all the treatment groups sampled post-challenge, compared to pre-challenge. The lymphocyte numbers post-challenge were significantly less ($P < 0.05$) than the pre-challenge. There was an increase in monocyte numbers during post-challenge.

3.2 Respiratory burst activity

The respiratory burst activities (NBT reduction) of neutrophils of *Catla catla* of the experimental groups were shown in **Table 2**. Respiratory burst activity increased significantly after oral supplementation of *Lactobacillus plantarum* (NCDC LP 20) in comparison to the control group during pre-challenge. The highest value of respiratory burst activity (0.39 ± 0.03) was found in T_4. Lower respiratory burst activity was observed post challenge in comparison to pre-challenge.

3.3 Serum bactericidal activity

The serum bactericidal activity increased significantly ($P<0.05$) in *Lactobacillus plantarum* (NCDC LP 20) fed group in comparison to the control group **Table 3.** The highest serum bactericidal activity was found in T_4 (*Lactobacillus plantarum* at 2×10^7cfu/g feed). The bactericidal activity of serum increased gradually as the quantity of *Lactobacillus plantarum* was increased in the diet. The number of bacterial colonies without addition of serum was 800. Incubation with control serum decreased the number of colonies to 590. The serum of *Lactobacillus plantarum* treated fish decreased the colony numbers to 462, 324 and 286 in T_2, T_3 and T_4 groups, respectively.

IV. DISCUSSION

In recent years, powerful vaccines have been established against many of the major bacterial diseases of fish in aquaculture. Nevertheless, considerable efforts are still being made to develop alternative or supplementary methods to improve fish health. Among such methods, the prophylactic use of immuno-stimulants and probiotics has attracted particular interest [21, 22]. Abraham and Banerjee [23] reported that the rearing of X. helleri in probiotic-enriched water have growth inducing ability and favorably influenced the reproductive performance in terms of high fecundity, high fry survival, reduced fry mortality and reduced fry deformity.

Now it has been established that several species of lactic acid bacteria (*Lactobacillus plantarum*) are part of the natural intestinal flora of healthy fish [22] and it is well known that Lactic acid bacteria often produce bacteriocins which may inhibit the growth of Gram negative fish pathogens. Apparently, Lactic acid bacteria are rarely present in juvenile fish reared on artificial feed, but may become dominant in the intestinal flora if they are supplemented in the feed [18, 24].

Lactobacillus plantarum is a Gram positive lactic acid bacteria usually found in fermented food and in the gastro intestinal tract and is commonly used in the food industry as a potential starter probiotic. Recently, the consumption of food alongwith with probiotics has extremely increased. Among the lactic acid bacteria, *Lactobacillus plantarum* attracted many researchers because of its extensive pharmaceutical applications. In the literature, it has been evident that, oral administration of *Bacillus subtilis* probiotic to *Labeo rohita* was considered to stimulate innate immuno parameters [25].

Literature claimed that, oral administration of probiotics to fish was considered to stimulate cellular rather than humoral immunity such as the increase in the number of monocytes and enhanced phagocytic activity [25]. The result from the present experiment also revealed an increase in granulocyte and monocyte in fishes fed with feed containing *Lactobacillus plantarum* LCDC LP20 probiotic compared to the fishes fed without probiotic.In the present study an effort has been made to provide an insight as to how *Lactobacillus plantarum* LCDC LP20 can be used as a probiotic for the prevention of diseases.

From the previous literature, it is evident that, non-specific immunity improved in fishes fed with feed containing *B. subtilis*. Similar results have been obtained in fishes fed with feed containing Lactobacillus plantarum LCDC LP 20 probiotic. Kim and Austin [26], also observed that the use of *Carnobacterium divergens* B33 in feed supplement to rainbow trout significantly increased respiratory burst activity. Nikoskelainen et al., [27] also observed that rainbow trout fed with *L. rhamnosus* demonstrated a significant increase in the respiratory burst activity of leukocytes.

The significant decrease in respiratory burst activity in the control group during post-challenge was probably due to exhaustion of the respiratory burst activity of the phagocytes following infection of the fish after *Aeromonas hydrophila* challenge. Contessie *et al..,* [28] also observed reduction in the respiratory burst activity during the outbreak of winter syndrome in farmed gilthead sea bream, Sparus aurata. Decreased respiratory burst activity could be observed in the treatment groups also during post-challenge, although the level was not significant. This indicated that *B. subtilis* enhances the immunity of *Catla catla* to overcome the stress caused by *Aeromonas hydrophila*. Previous studies demonstrated that administration of *Bacillus subtilis* to feed could reduce mortality of fish [25]. Our study showed that fishes fed with feed containing *Lactobacillus plantarum* probiotic (T_2, T3 and T_4) showed a significant increase in respiratory burst activity (NBT reduction assay), compared to the control.

Our study show the increase in resistance against *Aeromonas hydrophila* in fishes fed with *Lactobacillus plantarum* LCDC LP 20 probiotic, which is evident with the similar study of Rajesh Kumar *et al* ., [25], in *Bacillus subtilis*, can be explained on the basis of increased bactericidal activity of serum. Serum bactericidal activity increased in all the treatment groups with probiotic *Lactobacillus plantarum* (T2, T3 and T4) in comparison to the control group. The higher bactericidal activities can possibly be due to a higher concentration of lysosomal enzymes. Similar views were put forth by others who observed significant increase in complement bactericidal activity of rainbow trout fed with *Lactobacillus rhamnosus*.

V. CONCLUSION

From the above results, it is concluded that *Lactobacillus plantarum* LCDC LP20 is an effective probiotic to show enhanced immuno parameters in *Catla catla*. In future, some more experiments may be conducted using some other pathogenic bacteria and other species of aquatic organisms in order to establish the role of *Lactobacillus plantarum*.

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Table1. The effect of *Lactobacillus plantarum* LCDC LP20 probiotic on differential leukocyte counts of *Catla catla* during pre- and post-challenge with *Aeromonas hydrophila.*

Treatments	Granulocytes		Lymphocytes		Monocytes	
	Pre	Post	Pre	Post	Pre	Post
T_1	$12^c \pm 0.93$	$28^c \pm 0.83$	$83^a \pm 1.05$	$61^b \pm 0.63$	$9^c \pm 0.55$	$12^b \pm 1.33$
T_2	$19^b \pm 0.33$	$34^b \pm 1.03$	$70^b \pm 0.66$	$53^b \pm 1.56$	$12^c \pm 1.33$	$15^c \pm 1.05$
T_3	$28^a \pm 0.86$	$42^a \pm 1.33$	$57^c \pm 1.52$	$40^a \pm 2.33$	$16^b \pm 1.66$	$19^d \pm 0.86$
T_4	$30^a \pm 0.66$	$48^a \pm 0.56$	$51^d \pm 0.55$	$33^a \pm 1.66$	$20^a \pm 1.28$	$23^e \pm 1.63$
T_5		$15^d \pm 0.33$		$78^c \pm 0.33$		$10^a \pm 1.66$

Mean values in rows and columns containing the same superscript do not vary significantly ($P<0.05$). (T_1 = positive control, T_2 = *Catla catla* fed with feed containing *Lactobacillus plantarum* LCDC LP20 at 1× 10^7cfu/g feed, T_3 = *Catla catla* fed with feed containing *Lactobacillus plantarum* LCDC LP20 at 1.5× 10^7cfu/g feed, T_4 = *Catla catla* fed with feed containing *Lactobacillus plantarum* LCDC LP20 at 2× 10^7cfu/g feed, T_5 = negative control).

Table 2. Effect of *Lactobacillus plantarum* LCDC LP20 probiotic on respiratory burst activity of *Catla catla* before and after challenge with *Aeromonas hydrophila*

Treatment	Pre-challenge	Post-challenge
T_1	$0.23^c \pm 0.01$	$0.17^c \pm 0.02$
T_2	$0.29^b \pm 0.02$	$0.26^b \pm 0.01$
T_3	$0.36^a \pm 0.02$	$0.31^a \pm 0.01$
T_4	$0.39^a \pm 0.03$	$0.34^a \pm 0.02$
T_5		$0.24^b \pm 0.01$

Mean values in rows and columns containing the same superscript do not vary significantly ($P<0.05$). (T_1 = positive control, T_2 = *Catla catla* fed with feed containing *Lactobacillus plantarum* LCDC LP20 at 1× 10^7cfu/g feed, T_3 = *Catla catla* fed with feed containing *Lactobacillus plantarum* LCDC LP20 at 1.5× 10^7cfu/g feed, T_4 = *Catla catla* fed with feed containing *Lactobacillus plantarum* LCDC LP20 at 2× 10^7cfu/g feed, T_5 = negative control).

Table 3. Effect of *Lactobacillus plantarum* LCDC LP20 probiotic on serum bactericidal activity of *Catla catla* before challenge with *Aeromonas hydrophila.*

Treatment	Number of bacterial colonies
T_1	$590^b \pm 19.53$
T_2	$462^b \pm 21.32$
T_3	$324^c \pm 18.66$
T_4	$286^d \pm 16.03$
T_5	$800^a \pm 23.82$

Mean values in rows and columns containing the same superscript do not vary significantly ($P<0.05$). (T_1 = positive control, T_2 = *Catla catla* fed with feed containing *Lactobacillus plantarum* LCDC LP20 at 1× 10^7cfu/g feed, T_3 = *Catla catla* fed with feed containing *Lactobacillus plantarum* LCDC LP20 at 1.5× 10^7cfu/g feed, T_4 = *Catla catla* fed with feed containing *Lactobacillus plantarum* LCDC LP20 at 2× 10^7cfu/g feed, T_5 = negative control).

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