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Effect of Dietary Supplementation of *Bacillus subtilis* and *Terribacillus saccharophillus* on Innate Immune Responses of a Tropical Freshwater Fish, *Labeo rohita*

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Abstract

Objective: Indiscriminate use of antibiotics to control fish diseases has led to the development and propagation of antibiotic resistant bacteria in aquatic ecosystems. Amongst the various alternatives proposed, enhancing immunity in fish using probiotics is the most widely accepted approach. Several earlier studies indicated the possibility of more potent effects of bacteria on fish when administered in live condition than as inactivated cells. But very few studies have explored the effect of gut bacteria on innate immune responses of fishes. Hence an attempt is made to study immunostimulatory effects of dietary supplementation of *Bacillus subtilis* and *Terribacillus saccharophillus* isolated from the gut of an edible Indian freshwater fish, *Labeo rohita*, in the same species.

Methods: Long term administration (for 30 and 60 days) of *B. subtilis* or *T. saccharophillus* isolated from the gut wall of *L. rohita* were tested in the same species for their ability to improve innate immunity through measurement of the phagocytic activity, respiratory burst, myeloperoxidase activity, serum IgM levels, serum lectins, haemagglutination and haemolytic activity.

Results: Dietary administration of 107 cfu/g of *B.subtilis/T. saccharophillus* for 30 (B_1) or 60 (B_2) days resulted in a significant increase in immune and humoral responses of *L. rohita*. Serum phagocytic activity, respiratory burst activity, myeloperoxidase activity, serum lectins, haemagglutination and haemolytic activity increased upon administration of *B.subtilis / T. saccharophillus*, except serum IgM levels which increased only in B_1. A positive correlation was observed between the duration of supplementation of diets and the responses in both the cases.

Conclusion: The results suggest that *T. saccharophillus* can also be used as a potent probiont similar to *B.subtilis* and both will have significant applications in fish feed formulations and disease prevention.

Keywords: *B. subtilis*; *T. saccharophillus*; Immunity; IgM; Lectins; Respiratory burst; Phagocytic activity; Myeloperoxidase activity

Introduction

India is the second largest producer of fish in the world contributing to about 5.43% of global fish production and ranks second to China. Indian aquaculture is highly promising and has grown over six and half fold in the last two decades with freshwater aquaculture contributing over 95% of the total production [1]. Further it is dominated mainly by Indian Major Carps (IMCs) Viz. *Labeo rohita, Catla catla* and *Cirrhinus mrigala* which contribute nearly 87% of total production [2]. *L. rohita*, among these is widely cultured because of its market demand and thus contributes to 70% of total carps produced in India.

But diseases caused by parasites, viruses, bacteria and fungi have become primary constraints in the culture of most of these species, impeding both economic and social development [3-5]. Bacterial infections among these are considered to be the most common cause of fish mortality. Therefore, making aquaculture products disease-free and more acceptable to consumers has become the primary challenge. Accordingly during the last few decades several methods such as the use of non-specific immunostimulants (e.g. β-1, 3 glucan), probiotic supplemented diets, the development of genetically disease-resistant stocks, and restriction on the movement of infected stock have been put into practice as effective disease control strategies other than the traditional use of vaccines and antibiotics [6,7]. However, of late, the use of 'probiotics", "live micro-organisms that confer a health benefit on the host" is preferred over the rest to prevent proliferation of pathogens [8] and control disease outbreaks. Yasuda and Taga [9] as early as in 1980 first proposed the use of bacteria as a food source and biological control agents of fish diseases. *Bacillus, Lactobacillus, Enterococcus, Pediococcus, Bifidobacteria* and *Saccharomyces* are some of the known and commonly used probiotics [10].

Recent studies in cultured animals concerning the effects of probiotics have shown a reduction in mortality or, conversely, an increase in survival [11], improvement in disease resistance [12], enhancement in the ability of beneficial microbes to adhere and colonize in the gut to antagonize harmful organisms [13,14] and improve production of polyamines and digestive enzymes [15]. Nikoskelainen et al. [16] observed an increase in respiratory burst activity of blood cells and serum immunoglobulin levels in rainbow trout, *Oncorychus mykiss*, fed on *Lactobacillus rhamnosus*

supplemented diet for two weeks. Administration of heat-killed, live – sprayed and freeze- dried probiotics to rainbow trout was found to increase phagocytic activity, complement activity, plasma immunoglobulin levels, serum lysozyme activity, head-kidney leucocyte count and superoxide anion production [13]. Similarly an increase in phagocytic activity was noticed in fish fed on diets containing *Bacillus subtilis* [17] and a combination of *Lactococcus lactis,* Leuconostoc menenteriodes and *Lactococcus sakei* [18]. Some studies have clearly indicated the possibility of more potent effects of probiotic bacteria when administered as live cells rather than inactivated cells [13]. Apparently, however, very few studies have been conducted on the effects of *Bacillus subtilis*, a commonly used probiotic in relation to Indian major carps and practically nothing is known about the effects of *Terribacillus saccharophillus* on fishes.

In view of this, the present study is carried out to understand the immunomodulatory effects of dietary supplementation of viable *Bacillus subtilis,* a known probiont, and *Terribacillus saccharophillus*, isolated for the first time from the gut of a freshwater fish, *L. rohita*.

Methodology

Figure 1: Test Species: Juvenile Labeo rohita (*Hamilton, 1822*).

Preparation of *B. subtilis* and *T. saccharophillus* supplemented fish feed

B. subtilis and *T. saccharophillus* isolated from the gut wall of juvenile *L. rohita* were selected from a pool of 9 isolates. Primary cultures of *B. subtilis* and *T. saccharophillus* were prepared at 37°C for 24 hrs using Nutrient broth (Himedia). Plating on Nutrient agar was carried out using 100 ul of each pure culture. Two subsequent subcultures of the sample were established and freshly grown distinct colonies of these two strains were picked up, inoculated in 50 ml Nutrient broth separately and incubated at 37°C for 24 hrs. 1 ml each of the above samples, representing approximately 10^7 CFU/ml was centrifuged at 10,000rpm for 5 min; each pellet was resuspended in 1 ml PBS (pH 7.2) and stored at 40C for not more than 2 d for feed preparation.

The basal pelleted feed comprising 39% groundnut oil cake, 34% rice bran, 20% soybean meal, 5% fish meal, 2% minerals and vitamin mix was used as control diet (Diet 1). Diet 2 and 3 were prepared by adding 10^7 CFU/ml PBS culture of *B. subtilis* and *T. saccharophillus* respectively to 1 gm of basal feed since incorporation of 10^6 cfu / g or 10^7 cfu / g of *B. subtilis* / *T. sccharophillus* in the feed were found to remain viable for longer period of storage at 4°C [19]. Homogenous mixtures of above diets were spread over aluminum foil and dried for 10 min at 4°C in a hot air oven. The dried mixtures were stored at -20°C for further use.

Experimental design

Juvenile *L. rohita* were obtained from a Fish Hatchery located 19.7 kms, North West of Tirupati, Andhra Pradesh, India. 300 fishes were maintained in the laboratory in 3 concrete tanks of 200L capacity at a stocking density of 100 in each tank; fish were acclimated to laboratory conditions (28 ± 2°C and pH -7.8 ± 0.1) and fed on Diet 1 for a week with a replacement of 30% water every day. These fish were considered as control group (CG). Fish in tank 2 and 3 after having been acclimated for a week under above conditions were fed on Diet 2 (Group 'B') and 3 (Group 'T') respectively up to 60 days. All the groups were starved for 24 hrs before sampling for experimentation. 5 fish from each group were randomly sampled on days 0, 30 (B_1 or T_1) and 60 (B_2 or T_2) and were sacrificed by immersion in clove oil (20 mg/L) for 10min and used for the assessment of immunological parameters.

Determination of serum immune response parameters

Blood was drawn from caudal vein of 5 fishes from each group using a 1mL hypodermic syringe mounted with 28-guage needle prerinsed with 2.7% EDTA (anti-coagulant) solution and used immediately for analysis.

Serum was prepared by collecting another 1ml blood from each fish as explained above but without adding the anticoagulant. Samples were set aside for 2 hrs for clotting and centrifuged at 3,000 rpm for 5 min. Supernatants were collected into separate vials and stored at -20°C for further analysis.

Phagocytic activity

Phagocytic activity in the blood of control and treated groups was determined following the method of Park and Jeong [20]. 0.1 ml PBS consisting of 10^7 cells of freshly grown *A. hydrophila* was added to a sterile microplate containing the spread of 0.1 ml fresh blood drawn from each fish. After thorough shaking, plates were incubated at 25°C for 30min. Following incubation, the blood-bacteria suspension in the plates was mixed gently and slides were prepared by taking 50 µl suspensions from each well. Slides were air dried and fixed in 95% ethanol, re-dried and stained with Giemsa solution. The number of free phagocytic cells and those with engulfed bacteria were counted and phagocytic ratio was expressed using the following formula.

Phagocytic activity (%) = No. of phagocytic cells with engulfed bacteria/Total No. phagocytic cells × 100

Respiratory burst activity

Respiratory burst activity was measured based on the reduction of Nitro Blue Tetrazolium (NBT) by intracellular superoxide radicals [21]. 100µl blood collected from the fish of each group was mixed with 100 µl 0.2% NBT solution and incubated for 30 min by keeping in water bath maintained at 25°C. Following incubation, 1 ml N, N Diethylmethyl Formamide was added to 50 µl of the above mixture

and centrifuged at 6,000 g for 5 min. O.D. of the supernatant was measured at 540 nm against a blank comprising all the components except blood that was replaced with distilled water.

Myeloperoxidase activity

Myeloperoxidase activity was determined following the method of Quade et al. [22]. 15 µl serum was diluted with 135 µl Hanks Balanced Salt solution (Ca^{2+}, Mg^{2+} free) and the reaction was initiated by adding 50 µl substrate buffer (20 mM TMB and 5 mM H_2O_2) to it. The reaction was allowed to proceed for 2 min at room temperature and stopped by adding 50 µl 4 M Sulphuric acid. O.D was measured at 450 nm using UV-VIS spectrophotometer (Cecil, CE 2021) against a reagent TMB blank.

Immunoglobulin M level (IgM)

Total serum Immunoglobulin M level was estimated through Enzyme-linked immunosorbent assay [23]. Each well in flat bottomed 96-well plate was coated with 50 µl fresh serum, incubated at 37°C for 2 hrs and the liquid was pipetted out. 100 µl Biotin-Antibody was added to each well and incubated for 37°C for 1 hr to block the samples. Wells were aspirated and washed three times with 350 µl wash buffer. Later 100 µl Horseradish Peroxidase-Avidin (HRP-avidin) working solution was added to each well and incubated at 37°C for another 1 hr. Later 50 µl 3,3,5,5-Tetramethyl Benzidine (TMB) was added to each well and incubated for 30 min. The reaction was stopped by adding 50 µl 2 N sulfuric acid. The plate was read in the ELISA reader (Cyber Elisa R01, USA), at 450 nm against the negative control (without biotin-antibody). Mean absorbance of the negative control was subtracted from the optical density of the samples and expressed as mg ml^{-1} using human IgM as standard (100-1000 mg/ml).

Concentration of serum lectins

Serum lectins were extracted by purifying serum proteins through affinity chromatography using Feutin-agarose column. Anti-lectin antibodies (polyclonal antibodies) against purified lectins were raised using Wistar Albino rats (Ethical committee Ref. No. 1677/ PO/9/12/ CPCSEA/IAEC/DEC15/3 dt.23/12/2015).

Serum lectin concentration was also determined through ELISA [24]. 96-well microtiter plate (flat-bottom, polystyrene; Costar, Cambridge, MA) was used to perform ELISA. 200 µl purified serum samples were fixed to the bottom of each well by adding 150 µl 50 mM carbonate buffer (pH 9.6) and incubated at 4°C overnight. The plate was washed three times with PBST (Phosphate Buffer Saline Tween 20 buffer). Nonspecific binding sites were blocked by incubating the plate at room temperature for 2 hrs with 250 µl blocking buffer and washed again three times with PBST. Thereafter, 150 µl anti-lectin antibody (10 Ab) (1: 3,000 dilutions) was added to each well and incubated for 2 hrs at room temperature. The plate was then washed three times with PBST followed by the addition of 150 µl peroxidase-conjugated anti-rat antiserum (1:25,000 dilution) (20 Ab) per well and incubated for 2 hrs at room temperature. After washing again as described above, 150 µl substrate solution [0.4 mg/ml o- phenylenediamine (OPD) in 50 mM citrate buffer (pH 5) containing 0.012% H_20_2] was added to each well. The enzyme reaction was allowed to proceed for 30min and then stopped by the addition of 150 µl 2M H_2SO_4. The plate was read in the ELISA reader (Cyber Elisa R01, USA) at 450 nm against a blank comprising all the components except primary antibody. Standard curve was prepared using column purified lectins (30 to 300 ng).

Haemagglutination activity

Serum haemagglutination activity was measured following Blazer and Wolke [25]. Serial two - fold dilution of serum inactivated at 45°C for 30 min was taken in the titre plate having U-shaped wells in the range from 2 to 924. 50 µl fresh 1% red blood cell suspension of chick prepared in PBS was added to each well. The plate was kept at room temperature (28-30°C) for 2 hrs. Reciprocal of the highest dilution of serum titre showing complete agglutination of RBC was considered to represent the haemagglutination activity.

Haemolytic activity

Serum haemolytic activity was determined following the same procedure as explained for haemagglutination activity. However serial two fold dilution of 50 µl fresh serum was done instead of inactivated serum. 50 µl fresh 1% red blood cell suspension of chick prepared in PBS was added to each well. The plate was kept at room temperature (28 ± 2°C) for 2 hrs. Reciprocal of the highest dilution of serum titer showing complete lysis of RBC was considered to represent the haemolytic activity.

Statistical analysis

Mean values obtained using five fishes (n=5) from each group (Control / B_1/ T_1 / B_2 / T_2) were considered for final comparisons. Means and standard deviations of respective groups over three time periods (0, 30 & 60 days) were calculated and presented in a standard format of Mean ± SD. Repeated measures mixed factor ANOVA was calculated to know significant differences in parameters over the time period and also among the groups. Further pair-wise comparisons between groups were assessed with the help of Duncan's multiple range test (DMRT). Results were concluded at $P<0.01$ level of significance. Non-significant values in tabulated data are represented with similar marking.

Results and Discussion

Phagocytosis

Results on phagocytic activity measured in terms of percentage of phagocytic cells; respiratory burst activity (OD at 540 nm) measured in terms of release of superoxide radicals and hydrogen peroxide and myeloperoxidase activity (U/ml) measured in terms of release of hypochlorous acid from hydrogen peroxidase and chloride ions are presented in Tables 1, 2 and 3, respectively.

Group	0 Day	30 Days	60 Days
Control[a]	18.67 ± 1.5	25.33 ± 2.5	31 ± 2.6
B. subtilis (B)[b]		34.67 ± 3.1(B1)[c]	43 ± 4.6 (B2)[d]
T. saccharophillus(T) [b]		32 ± 3(T1)[c]	40.67 ± 3.8(T2)[d]
Superscripts 'a, b, c, d' - Values similarly marked are not significantly different from each other (DMRT). 5 fishes were used in each group.			

Table 1: Phagocytic activity (% cells) of *L. rohita* fed on *B. subtilis* (B) and *T. saccharophillus* (T) formulated diets for 30 (B_1/T_1) and 60 (B_2/T_2) days compared to control group fed on basal diet. (Values are Mean ± SD of 5 individual observations).

Group	0 Day	30 Days	60 Days
Control[a]		0.33 ± 0.1	0.36 ± 0.1
B. subtilis (B)[b]	0.14 ± 0	0.53 ± 0.1(B_1)[c]	0.69 ± 0.1(B_2)[d]
T. saccharophillus(T)[b]		0.48 ± 0.1(T_1)[c]	0.62 ± 0.1(T_2)[d]
Superscripts 'a, b, c, d' - Values similarly marked are not significantly different from each other (DMRT). 5 fishes were used in each group.			

Table 2: Respiratory Burst activity (RBA) of *L. rohita* fed on *B. subtilis* and (B) *T. saccharophillus* (T) formulated diets for 30 (B_1/T_1) and 60 (B_2/T_2) days compared to control group fed on basal diet. (Values are Mean ± SD of 5 individual observations).

Group	0 Day	30 Days	60 Days
Control[a]		2.22 ± 0.3	2.41 ± 0.3
B. subtilis (B)[b]	1.26 ± 0.1	2.81 ± 0.3(B1)[c]	4.14 ± 0.4(B2)[d]
T. saccharophillus(T) [b]		2.7 ± 0.3(T_1)[c]	4.03 ± 0.4(T_2)[d]
Superscripts 'a, b, c, d' - Values similarly marked are not significantly different from each other (DMRT). 5 fishes were used in each group.			

Table 3: Myeloperoxidase activity (U/ml) of *L. rohita* fed on *B. subtilis* (B) and *T. saccharophillus* (T) formulated diets for 30 (B_1/T_1) and 60 (B_2/T_2) days compared to control group fed on basal diet. (Values are Mean ± SD of 5 individual observations).

Phagocytic cells (=granulocytes, monocytes and macrophages, neutrophils) are specialized for the pursuit, capture, ingestion and intracellular destruction of invading microbes by phagocytosis, which is one of the most important process in all poikilothermic animals [26-28]. Neutrophils and macrophages are the main cells involved in phagocytosis in fish [29]. They remove bacteria through a process called "respiratory burst" or "oxidative burst" which is involved in the rapid release of reactive oxygen species (superoxide radical (O_2-) and hydrogen peroxide (H_2O_2)) as they come in contact with different bacteria or fungi.

In addition, neutrophils possess myeloperoxidase (MPO), a heme-based peroxidase enzyme in their cytoplasmic granules, which uses released reactive oxygen species or hydrogen peroxide and kills bacteria by halogenations of the bacterial cell wall releasing hypochlorous acid. Therefore MPO copiously expressed in stimulated neutrophils and granulocytes is responsible for antimicrobial activity against a wide range of organisms [30].

Thus significant ($p<0.01$) increases in phagocytic activity, respiratory burst and myeloperoxidase activity in B1 / T_1 or B_2/T_2 groups of *L. rohita* indicated the role of both *B. subtilis* and *T. saccharophillus* in promoting the activity of neutrophils and macrophages.

Dietary administration of *Bacillus sps.* to *L. rohit [31]*, *C. carpio* [32] and *Litopenaeus vannamei* [33] was earlier found to significantly increase the phagocytic activity. 39 and 27% increase in phagocytic activity in grouper fish fed on *B. pumilus* and *B. clausii* supplemented diets respectively [23], 62% increase in *O. niloticus* fed on *Micrococcus sps.* supplemented for 15d [34], and 34% increase in Nile tilapia fed on *L. plantarum* supplemented diets for 15d [35] demonstrated the role of probiotics in enhancing the phagocytic activity that helped to resist microbial infections [36,37]. Supplementation of diets with 3% probiotic mixture of *L. acidophilus* and *S. cerevisiae* for 105d were also found to result in 25% increase in phagocytic activity in the fingerlings of catfish [38].

Respiratory burst was also found to increase by 20% in a teleost, *Sparus aurata*, supplemented with *Lactobacillus delbrueckii* for 15d [39], by 25% in *L.rohita* supplemented with *B. subtilis* for 15d [40], by 206% in orange spotted grouper, supplemented with *S. cerevisiae* for 28d [41] and by 6, 16 and 50% in orange spotted grouper supplemented with *B. subtilis* for 10, 20 and 30d respectively [42], showing a positive correlation between bacterial pathogen killing by phagocytes through respiratory burst in fishes fed on probiotic diets [43]. A recent study on fingerlings of *L. rohita* fed on *B. subtilis*, *L. lactis* and *S. cerevisiae* for 30d showed 287% increase in MPO in consonance with respiratory burst [44].

The results of the present study also showed that *L. rohita* fed on *B. subtilis* supplemented diet for 30 (B_1) and 60 (B_2) days resulted in 36% (B_1) and 39% (B_2) increase in phagocytic activity, 60% (B_1) and 90% (B_2) increase in respiratory burst activity, 26% (B_1) and 71% (B_2) increase in myeloperoxidase activity (Figures 2, 3 and 4) compared to respective controls. Similarly, dietary supplementation with *T. saccharophillus* resulted in 26% (T_1) and 31% (T_2) increase in phagocytic activity, 45% (T_1) and 72% (T_2) increase in respiratory burst activity and 21% (T_1) and 67% (T_2) increase in myeloperoxidase activity (Figures 2, 3 and 4) compared to respective controls.

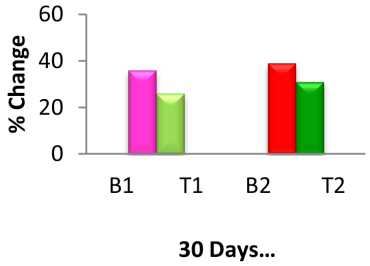

Figure 2: Percent change in Phagocytic cells of *L. rohita* fed on *B. subtilis* (B) and *T. saccharophillus* (T) formulated diets for 30 (B_1/T_1) and 60 (B_2/T_2) days compared to control group fed on basal diet.

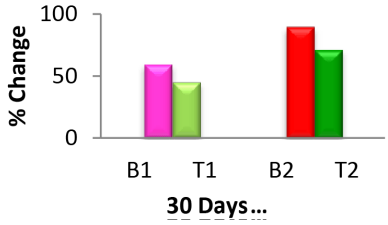

Figure 3: Percent Chang Chin-Chyuan e in Respiratory Burst activity of *L. rohita* fed on *B. subtilis* (B) and *T. saccharophillus* (T) formulated diets for 30 (B_1/T_1) and 60 (B_2/T_2) days compared to control group fed on basal diet.

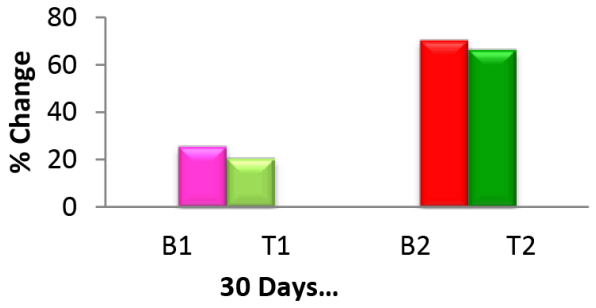

Figure 4: Percent change in Myeloperoxidase activity of *L. rohita* fed on *B. subtilis* (B) and *T. saccharophillus* (T) formulated diets for 30 (B_1/T_1) and 60 (B_2/T_2) days compared to control group fed on basal diet.

Beneficial effects of *B. subtilis*/ *T. saccharophillus* on MPO activity, respiratory burst and phagocytic response observed in the present study are in agreement with earlier observations of other researchers who studied the use of probiotics in aquaculture. For instance 130% increase in phagocytic activity, 50% increase in respiratory burst and 62% increase in myeloperoxidase activity was reported in rainbow trout fed on *B. subtilis* supplemented diet for 14d [17], 75% increase in respiratory burst and 50% increase in myeloperoxidase activity was observed in tilapia fed for 40d on *L. lactis* supplemented diet [45] and 29% increase in phagocytic activity and 25% increase in respiratory burst activity in *L. rohita* fed for 60d on *P. aeruginosa* supplemented diet [46]. The reactive O_2 species (ROS) produced by phagocytes upon activation (oxidative respiratory burst) (Table 2; Figure 3) are toxic to pathogens and hence their concentration can be thoroughly correlated to an increased killing ability of phagocytes [44,45].

Apparently increased levels of MPO activity (Table 3; Figure 4), in *B. subtilis* and *T. saccharophillus* fed groups represent one of the most immediate and key positive effects produced by these bacteria on the host immune system. Immunostimulatory effects of several species of *Bacillus* though demonstrated in different fish including *L. rohita* against different pathogens [17,42,44,47,48] similar response is detected with *T. saccharophillus* for the first time in this study.

Increased phagocytic activity (Table 1; Figure 3), the primary non-specific defense response, suggests that *B. subtilis* and *T. saccharophillus* can promote innate immunity to act against invading pathogenic organisms.

IgM (mg / ml^{-1})

Serum IgM levels of *L. rohita* fed on diets supplemented with *B. subtilis* (B_1 / B_2) and *T. saccharophillus* (T_1/T_2) are presented in table 4. It is clear that there is a significant ($p<0.01$) increase in serum IgM levels in B_1/T_1 groups without a significant change in B_2/T_2 groups compared to respective controls.

IgM is a tetrameric molecule and is, by far, the most prevalent immunoglobulin in plasma [49]. It is secreted mainly by plasmablasts and plasma-like cells that are located mostly in the head kidney and is a key to elicit memory responses [50]. Further fish IgM is reported to be important for pathogen recognition and activation of the innate immune system via the classical pathway of complement activation [51].

Group	0 Day	30 Days	60 Days
Control		0.29 ± 0.1	0.58 ± 0.2
B. subtilis (B)[b]	0.21 ± 0.1	0.57 ± 0.2(B_1)[c]	0.54 ± 0.1(B_2)
T. saccharophillus(T) [b]		0.54 ± 0.2(T_1)[c]	0.53 ≈ 0.1(T_2)
Superscripts 'a, b, c, d' - Values similarly marked are not significantly different from each other (DMRT). 5 fishes were used in each group.			

Table 4: Ig M (mg/ml^{-1}) levels of *L. rohita* fed on *B. subtilis* (B) and *T. saccharophillus* (T) formulated diets for 30 (B_1/T_1) and 60 (B_2/T_2) days compared to control group fed on basal diet. (Values are Mean ± SD of 5 individual observations).

The live yeast, *Debaryomyces hansenii* administered through diet for 5 weeks increased IgM levels by 15% in the leopard grouper, *Mycteroperca rosacea* [52], while *L. acidophilus* administered for 105d to *Catla catla* increased IgM levels by 4% [38]; Pediococcus acidilacticion, supplemented for 126 d enhanced the same by 24% in the ornamental fish Oscar [53]. On the other hand, serum IgM levels increased by 26 and 47% in grouper fish upon supplementation of *B. pumilus* and *B. clausii* respectively for 30d but decreased by 20 and 15% respectively with continued supplementation till 60 d [23]; *L. rohita* showed 24% increase and 9% decrease upon supplementation of *P. aeruginosa* for 30 and 60d respectively [46]; and the rainbow trout, O. mykiss, showed significant increase in plasma immunoglobulin upon 20d supplementation *L. rhamnosus* which decreased significantly thereafter with continued treatment [13].

Significant increase in serum IgM levels by 46% in B1 and 38% in T_1 groups compared to respective controls observed in the present study further indicated that supplementation with even a single probiont (*B. subtilis* or *T. saccharophillus*) for 30d can stimulate IgM production emphasizing the role of either of these bacteria in stimulating antibody production (Figure 5).

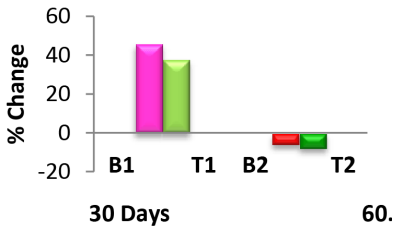

Figure 5: Percent change in Ig M levels of *L. rohita* fed on *B. subtilis* (B) and *T.saccharophillus* (T) formulated diets for 30 (B_1/T_1) and 60 (B_2/T_2) days compared to control group fed on basal diet.

However no change in IgM levels thereafter even with prolonged supplementation (up to 60d) of *B. subtilis* or *T. saccharophillus* clearly indicated that the stimulation of immunoglobulin production is a short-term phenomenon attributable to either of these bacteria but long term administration of the same may not enhance IgM production any further which has already reached threshold concentration. Since serum immunoglobulins are the major compounds of humoral immune system an increase in their levels in B_1 and T_1 revealed their role in clearly improving immune status of the fish that provides protection against infections.

Concentration of lectins (ng / ml), haemagglutination and haemolytic activities (titre values)

Tables 5, 6 and 7 present data on the levels of serum lectins, haemagglutination and haemolytic activities in *L. rohita* fed on diets supplemented with *B. subtilis* or *T. saccharophillus* for 30 (B_1/T_1) and 60d (B_2/T_2) compared to respective controls. Significant increases were observed in the levels of lectins, haemagglutination and haemolytic activities ($P<0.01$) in B_1/B_2 and T_1/T_2 groups.

Test samples	Concentration of lections (ng/ml)
C	80
B_1	120
T_1	100
B_2	180
T_2	160

Table 5: Serum lectin levels of *L.rohita* fed on *B. subtilis* (B) and *T. saccharophilus* (T) formulated diets for 30 (B_1/T_1) and 60 (B_2/T_2) days compared to control group fed on basal diet.

Group	0 Day	30 Days	60 Days
Control[a]		64.67 ± 3.1	128 ± 5.3
B. subtilis (B)[b]	34 ± 2	110 ± 7(B_1)[c]	252 ± 9(B_2)[d]
T. saccharophillus(T) [b]		109 ± 7.2(T_1)[c]	252 ± 9(T_2)[d]
Superscripts 'a, b, c, d' - Values similarly marked are not significantly different from each other (DMRT). 5 fishes were used in each group.			

Table 6: Haemagglutination activity (HA) of *L. rohita* fed on *B. subtilis* (B) and *T. saccharophillus* (T) formulated diets for 30 (B_1/T_1) and 60 (B_2/T_2) days compared to control group fed on basal diet. (Values are Mean ± SD of 5 individual observations).

Group	0 Day	30 Days	60 Days
Control[a]	7.33 ± 1.2	18 ± 3.5	128 ± 5.3
B. subtilis (B)[b]		32 ± 5(B1)[c]	254.00 ± 6.4(B2)[d]
T. saccharophillus(T) [b]		30.5 ± 4.2(T1)[c]	250.00 ± 4.2(T2)[d]
Superscripts 'a, b, c, d' - Values similarly marked are not significantly different from each other (DMRT). 5 fishes were used in each group.			

Table 7: Haemolytic activity (HA) of *L. rohita* fed on *B. subtilis* (B) and *T. saccharophillus* (T) formulated diets for 30 (B_1/T_1) and 60 (B_2/T_2) days compared to control group fed on basal diet. (Values are Mean ± SD of 5 individual observations).

Lectins are carbohydrate binding proteins found in viruses, prokaryotes and eukaryotes. These proteins are known to agglutinate foreign cells, precipitate membrane polysaccharides, glycoproteins or glycolipids mediating different biological processes such as cell-cell interaction, induction of apoptosis, antibacterial and antiviral actvitity etc. Haemagglutination and haemolytic activities are used to estimate lectin production that reflects innate immune response, which is mediated by natural agglutinin and complement proteins.

ELISA studies on hemolymph of freshwater prawns, *M. rosenbergii* using monoclonal IgG1 with kappa light chain antibodies raised against purified lectin showed a positive correlation between haemagglutination activity and concentration of lectin [24]. Sangvanich et al. [54] observed strongest haemagglutination activity against rabbit erythrocytes on using crude extract of Curcuma species, a phytochemical. Curcumin, a hydrophobic and polyphenolic compound of turmeric, *Curcuma longa*, has long been known to be a potent immunostimulatory agent in mice, horse, and other higher vertebrates including humans [55]. Zhang et al. [56] clearly demonstrated a sharp rise in spleen mannose-binding lectin expression in channel catfish challenged with a Gram -Negative bacterium.

Feed supplementation of *Achyranthes aspera* was found to increase haemagglutination and haemolytic activity in *L. rohita* [47] catla [57] and common carps [58]. But studies have not been conducted so far to understand the relation between serum lectins and probiotic diets, particulary in fishes and this is the first study made in this direction.

Serum lectin levels in *L. rohita* increased by 20 and 46% in B_1/B_2 groups respectively and by 18% and 45% in T_1/T_2 groups respectively (Figure 6).

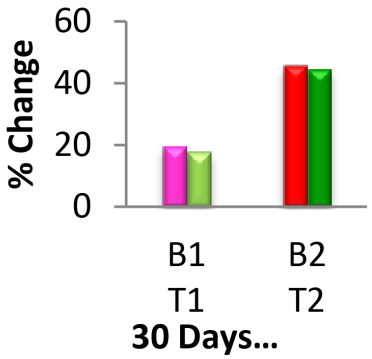

Figure 6: Percent change in serum lectin levels of *L. rohita* fed on *B. subtilis* (B) and *T. saccharophillus* (T) formulated diets for 30 (B_1/T_1) and 60 (B_2/T_2) days compared to control group fed on basal diet.

The results also showed that haemagglutination levels increased by 70 and 97% in B_1/B_2 groups respectively and by 68 and 97% in T_1/T_2 groups respectively (Figure 7) along with 77 and 98% increase in haemolytic activity in B_1/B_2 groups respectively and 69 and 95% in T_1/T_2 groups respectively (Figure 8). Further, the results clearly demonstrated that *T. saccharophillus* (Table 5, 6 and 7; Figures 6, 7 and 8) also significantly enhance the production of lectins, haemagglutination activity and haemolysis of pathogenic cells which may contribute to pathogen clearance similar to that exhibited by *B. subtilis*, a known probiont. Though lectins of invertebrates including crustaceans, have been studied and regarded as potential molecules involved in immune recognition of microorganisms and phagocytosis through opsonisation [59] more such studies are needed in fish especially in the context of probiotic diet supplementation.

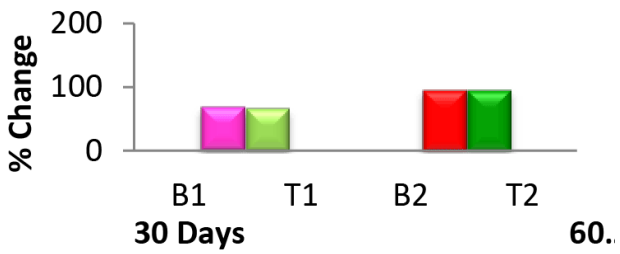

Figure 7: Percent change in Haemagglutination activity (HA) of *L. rohita* fed on *B. subtilis* (B) and *T. saccharophillus* (T) formulated diets for 30 (B_1/T_1) and 60 (B_2/T_2) days compared to control group fed on basal diet.

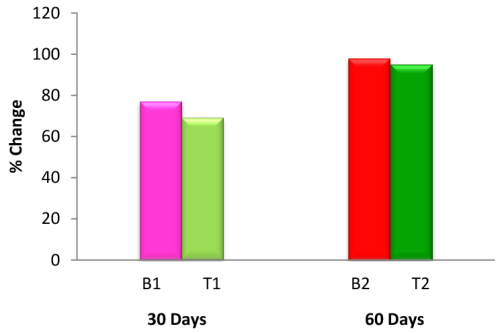

Figure 8: Percent change in Haemolytic activity (HL) of *L. rohita* fed on *B. subtilis* (B) and *T. saccharophillus* (T) formulated diets for 30 (B_1/T_1) and 60 (B_2/T_2) days compared to control group fed on basal diet.

Conclusions

This study clearly revealed that feeding L.rohita on diets supplemented with *B. subtilis* or *T. saccarophillus* can significantly improve fish innate immunity and promote resistance against bacterial infections. The findings of this study on immunostimulatory effects of *B. subtilis* and *T. saccarophillus* open new vistas in screening novel potential probiotic strains that may have extended use in aquaculture practices. Remission studies in order to understand the persistence of *B. subtilis/T. saccarophillus* in the gut after switching to control diet are under progress.

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