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Panchagavya Dietary Supplementation with Probiotic *Lactobacillus*: Impact of Immunity and Disease Resistance in *Oreochromis mossambicus*

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Abstract

In the current investigation suggested that the immune stimulatory effect of panchagavya probiotics *Lacto bacillus* on the growth, immunity and disease resistance of Tilapia, *Oreochromis mosaambicus* and improve the disease resistance against *Edward siella tarda* and *Aeromonas hydrophila*. The efficacy of basal diet (BD) of the following experimental diets were treated with *O. mosaambicus*: T_1 (Basal diet + Panchagavya); T_2 (Basal diet + *Lactobacillus*); T_3 (Basal diet + Panchagavya + *Lactobacillus*) and control. Various immune parameters were examined at 7, 14, 21 and 28 days post-feeding. Fishes were challenged with *A. hydrophila* and *E. tarda* 30 days post-feeding, and mortalities were recorded over 14 days post infection. Results showed that administration of panchagavya with probiotics of *Lacto bacillus* combination for 28 days had significant effects ($p<0.05$) on the specific and non-specific immune response of *O. mossambicus*. Dietary administration of *O. mossambicus* from panchagavya with probiotic feed significantly increased the neutrophil, lysozyme, antiprotease and myeloperoxidase activities were gradually increased respectively. The highest ROS and RNS ($p<0.05$) were observed in the fish group feed a diet containing panchagavya and *Lacto bacillus*. Furthermore, fish feed a panchagavya-enriched diet with *Lactobacillus* had significantly higher ($p<0.05$) post-challenge relative percent survival (RPS). Considering these promising benefits, the panchagavya with probiotic *Lacto bacillus supplementation* in equal proportions for 30 days can effectively impair the immunity of *O. mossambicus*.

***Key words*:** *Aeromonas hydrophila, Edwardsiella tarda,* feed, *Lactobacillus, O. mossambicus,* Panchagavya

The aquaculture sector has shown a rapid growth over the last 30 years with an associated increase in disease problems as result of rapid expansion and amongst other factors high stocking densities. In order to maintain fish health and to improve performance immune stimulants have been used as dietary additives and improve weight gain, feed efficiency and disease resistance in cultured fish. Over the years, intensive aquaculture has been expanded and is emerging as one of the most viable and promising sectors for providing nutritional and food security to human [1]. In intensive aquaculture, fish are subjected to stress conditions that weaken fish immune system, leading to the emergence of diseases [2]. These diseases have resulted in production losses and remain as one of the major cause of concern in fish farms [3]. Traditionally, vaccines, antibiotics and chemotherapeutics have been employed for disease control. However, the application of antibiotics causes many problems such as development of drug resistant pathogens, environmental hazards and food safety problems [1]. Traditional Agriculture has been generally considered everywhere as a joint effort of man and cattle. In recent past, a great deal of importance has been given to individual animal product and formulation. Among the formulations, the most widely mentioned and discussed is Panchagavya, which literally means a mixture of five products originating from cow. Panchagavya is the formulation mentioned in Ayurveda prepared using five components derived from cow viz. milk, curd, ghee, urine and dung [4]. The liquid organic manures such as Panchagavya and cow urine are commonly used in organic farming to provide balanced nutrition to the crop. [5] observed the presence of naturally occurring beneficial microorganisms, predominantly bacteria, yeast, actinomycetes, photosynthetic bacteria and certain fungi in organic liquid manures. Another important product that is being extensively used in traditional agriculture is cow's urine, which has been known to be used by

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various sectors of people in India from Vedic period for medicinal and agricultural purposes. The traditional knowledge also recognizes the importance of cow urine in agriculture and is being sprayed on plants to control fungal/bacterial diseases. It helps in the management of pests (25%) of sweet corn at tasseling and cob formation stages. Maize is the third most important cereal grown in India after wheat and rice. The intensive use of antibiotics to prevent and control bacterial diseases in aquaculture has led to an increase in antibiotic-resistant bacteria [6-7]. Therefore, several alternative strategies to the use of antimicrobials have been proposed, such as the use of probiotics as biological control agents. Probiotics, live microbes that may serve as dietary supplements to improve fish growth and immune responses, have received some attention in aquaculture [8-10].

Lactobacillus, is used for the bakers' industry that contains various immune stimulating compounds such as β-glucans, nucleic acids as well as mannan oligosaccharides, and it has the capability to enhance immune responses [11-13] as well as growth [14-19] of various fish species. However, the administration of yeast has been recognized to have important effects on immune stimulant functions [20]. The maintenance of fish health and welfare, which can be greatly influenced by administrated feed and environmental conditions, is presently the main concern in aquaculture [21]. There is currently a significant of scientific and commercial interest in the manipulation of gut microbiota through the use of dietary supplements enriched with beneficial microbes (probiotics). The beneficial microorganisms multiply and colonize the gut of the host, having a number of beneficial effects on both the host and the environmental conditions [22]. However, there are no reports on the Panchagavya as a fed with probiotic *Lactobacillus*. Hence, the study was carried out to explore the effects of Panchagavya and probiotics due to administration for shorter duration on immune mechanism to *E. tarda* and *A. hydrophila*.

MATERIALS AND METHODS

Experimental animal and their maintenance

Healthy Tilapia wet weight: 12.5 ± 0.10 g; length: 8.3 ± 0.5 cm were bought from a nearby fish farm, Kumbakonam, Tamil Nadu, India and refined from dechlorinated water. Before the treatment, all experimental fishes were acclimatized at pH 7.0 ± 0.2 at a constant temperature of 22 ± 1˚C and a photo-period of 16 h : 8 h (light: dark) for two weeks and fed once every a day with control feed.

Preparation of Panchagavya

The Panchagavya was prepared by using cow dung (5 %), urine (3 %), milk (2 %), curd (2 %) and ghee (1%) obtained from indigenous cow (*Bos indicus*) along with other ingredients viz. sugarcane juice - 3 parts, tender coconut juice - 3 parts, ripened banana - 12 numbers and toddy - 2 parts as per the methods of [23]. The fresh cow dung was thoroughly mixed with ghee in a wide mouth mud pot and kept for three days. The above mixture was thoroughly mixed once in daily. On the fourth day, other ingredients were added to the mud pot, mixed properly and covered with nylon net to prevent entry of flies into the pot. The pot was placed in shade and mixed thoroughly twice a day for 30 days.

Yeast

Lacto bacillus MTCC 172 (from the IMTCH, Chandigarh, India) was grown for 48 h in 500 mL of nutrient broth (4.0 g yeast extract; 5.0 g casein digest, 50.0 g dextrose, 0.55 g monopotassium phosphate, 0.425 g potassium chloride, 0.125 g calcium chloride, 0.125 g magnesium sulfate, 0.0025 g ferric chloride, 0.0025 g manganese sulfate, 0.0022 g bromocresol green at 30 °C in shaking bottles. The overnight culture was centrifuged at 5000 rpm for 15 min at 4°C, repeated thrice with sterile 0.1% NaCl solution washing. The pelleted cells were added at the rate of 1% to the feeds [24].

Diet preparation

A basal diet was obtained commercially which formulated *Lactobacillus* with contain 13% moisture, 47% protein, 12% oil, 16% crude ash and 3,500 kcal/g energy for tilapia. The basal diet served as the control diet (without *Lactobacillus* and Panchagavya). T_1 (Basal diet+Panchagavya-1%); T_2 (Basal diet + *S. cerevicieae*1%); T_3 (Basal diet + Panchagavya (1%) + *Lactobacillus* 1%) and control (Basal diet). The commercial feed was blended first, then combined with the Panchagavya and *Lactobacillus* with water (100 mL of water/kg of diet) to form a paste, which passed through a meat grinder and pelleted again to produce 2.0-mm pellets. The feed was air dried at room temperature (25°C) for at least 48 h and it was stored at 4°C until further use [25].

Experimental design

Experiments were performed in rectangular plastic tubs (95 × 70 × 60 cm, 180-L) with lids and the water use for rearing was drawn from dechlorinated water. Tilapia fishes (*n*=375) were distributed into 15 tanks with each tank containing 25 fishes were maintained. The fish were given a basal diet without supplementation of Panchagavya with *Lacto bacillus* as a control diet T_1 (Basal diet + Panchagavya-1%); T_2 (Basal diet + *Lactobacillus*-1%); T_3 (Basal diet + Panchagavya (1%) + *Lactobacillus*-1%) for a duration of 28 days. The fishes were fed with the investigational diet at the rate of 4% of body weight twice per day, at 09.00 and 17.00 h. for 30 days. Six fishes from each experimental group were sampled and blood was drawn on 7, 14, 21 and 28 days for various immunological parameters analyzed left over fishes were challenged with virulent *A. hydrophila* and *E. tarda* 30 days after feeded and the relative percentage survival (%) was recorded after 14 days post treated [26].

Blood collection

Blood samples were collected from fishes on 7, 14, 21 and 28 days treatment. Blood was drawn from the caudal vein of each fish using a 1.0 ml hypo-dermal syringe and 24-gauge needles which was rinsed with 2.7% EDTA solution before use. The collected blood was immediately transferred to a test tube coated with a thin layer of EDTA (as an anticoagulant) and shaken to prevent hemolysis and blood clotting. Serum was collected without using anticoagulant and was separated from the remaining blood by keeping the tubes in a slanted position for about 2 h and then centrifuged at 3500 rpm for 15 min at 4°C for further analysis.

Immunological parameters

Neutrophil activity

The modified [27] for the NBT (Nitro Blutetrazolium) assay was followed. To facilitate the adhesion of the cells, 100 µl of blood was placed into the wells of flat bottom micro plates and incubated at 37 °C for 1 hour. The supernatant was removed and the loaded wells were washed three times using phosphate buffer saline (PBS, pH 7.5). After washing, 100 washinl of 0.2% NBT was added and the plate was further incubated for 1 hour. The cells were mixed with 100% methanol for 2-3 minutes and then washed three times with 70% methanol. The

plates were then air dried. 120 Dl 2N KOH and 140 Ol DMSO (dimethyl sulfoxide) were added to each well to form a pharmasone blue precipitate. The Optical density was read into a microplate reader (Systronics, India) at 620 nm.

Serum lysozyme activity

Lysozyme activity was measured by the turbidimetric assay of [28] with the microplate optimization of [29]. In this technique, 0.05% lyophilized *Micrococcus luetus* in 0.05 mM sodium phosphate buffer (pH 6.2) was used as the substrate. Ten microliters of fish serum were added to a 250 µm bacterial suspension in a microplate and a reduction in absorbance was determined at 490 nm after 0.5 and 4.5 minutes of incubation at 22 °C using a microplate reader. One unit of lysozyme activity was defined as a decrease in absorbance of 0.001 per minute.

Myeloperoxidase activity

The total myeloperoxidase activity of the serum was described by [30] and partially modified by [31]. Briefly, 10 mL serum was diluted with 90 mL of HBSS with Ca_2 + or Mg_2 + in a 96-well microtitre plate containing 25µl 20 mM 3,3 5,5-Tetramethylbenzidine (TMB) (Genie, India) and 5 m H_2O_2 with H_20_2 added at 1:20 dilution. After 2 minutes of incubation, 50µl of 4 M sulfuric acid (H_2SO_4) was added to stop the reaction. The optical density was read at 450 nm in a microtitre plate reader (Cyper Lab).

Serum antiprotease activity

The serum antiprotease assay was performed [32]. Briefly, 10 µl serum incubation (10µl) was performed with 100 mL trypsin (type 1 from bovine pancreas) for 30 min at 25°C. Two blank (110 µl PBS) and three references (10 mL PBS / 100 mL trypsin) were also taken. Each of the reaction mixtures was added with 1 µl casein for 15 min. The reaction was stopped by adding 500 added with 10% trichloroacetic acid (TCA) to all. The mixture was centrifuged at 5,551 xg for 5 min and the optical density of the supernatant at 280 nm was taken. Percent blocking was calculated using the following formula:

$$\text{Percentage of prohibition (\%)} = \frac{\text{OD reference} \times \text{OD sample}}{\text{OD reference}} \times 100$$

Preparation of viable leukocytes from peripheral blood

For the dissociation of peripheral blood leukocytes, fish should be fed 5–5 ml syringe-filled 2 ml blood collecting medium (RPMI-1640 supplemented with 50,000 IU-1 sodium heparin, 1,00,000 IU-1 penicillin and 100 mg) was used with. L-1 streptomycin). Diluted blood was carefully visualized on the same volume of lymphocyte dissociation medium (lymphosep, ICN Biomedicals, Inc., USA) and cells were reduced to 800 g for 20 min. Leukocytes were collected at the interface and washed twice with medium (RPMI-1640 supplemented with 10,000 IU-1 sodium heparin, 1,00,000 IU-1 penicillin, and 100 mg L-1 streptomycin) and RSMI through the culture medium. Resuspended in 1640 supplemented with 3% (v / v) of tilapia serum, 1,00,000 IU-1 penicillin, 100 mg L-1 streptomycin and 4 mM L-glutamine, Biochrome AG, Germany). The number of viable cells was enumerated using the trypan blue exclusion method and adjusted to 4 x 107 cells through ml-1use culture medium [33].

Production of reactive oxygen species

Intracellular respiratory burst activity was measured with minor modifications. Peripheral blood leukocytes (1x106 cells / well) were incubated with 25 µl of 25 µM nitrobluetetrazolium (NBT, 1 g L-1) in 175 forl culture medium at 28 °C for 2 hours. The supernatant was carefully removed and the cells were mixed in 100% (v / v) methanol for 5 min. Each well was washed twice with 125 µl of 70% (v / v) methanol. The fixed cells were allowed to air-dry overnight. Low NBT (as formazan) was dissolved using 125 N 2 L potassium hydroxide (KOH) and dimethyl sulfoxide (DMSO) per 150 DLT per well. The optical density was measured in a microplate reader at 650 nm [34].

Production of reactive nitrogen species

Nitric oxide (NO) released by peripheral blood leukocytes in the medium was measured using the Griess reagent [35]. Leukocytes produce nitric oxide which is rapidly converted to more stable nitrate. The nitrite present in the culture supernatant can be measured calorically by adding Grimase reagent and converting it to pink. Peripheral blood leukocytes were cultured in a chamber in 175 h culture medium with 96% 1% copper sulfate solution at 28 °C. Fifty microliter supernatants of the culture were collected and transferred to a separate microtitre plate. For each well containing the culture supernatant, 50 g of grease reagent (1% sulfalamide, 0.1% N-naphthul-ethylenediamine, 2.5% phosphoric acid) was added. After 10 minutes of incubation, molar concentrations of NO_2 were read from a standard curve generated before a hierarchical series of $NaNO_2$ concentration in the culture medium.

Post challenge

After the immunostimulation trial for 28 days, 50 fishes in duplicate tanks under each treatment group were pooled together. Then the fish were distributed in four tanks under two subgroups for challenge study each containing 12 fish. Fish under first and second group were injected with the pathogens obtained from IMTECH, Chandigarh, India at a level of 0.1 mL suspension of *A. hydrophila* ($1.7x10^8$cfu/per fish, 10 times of the LD_{50} concentration) in PBS and 0.1 mL suspension of *E. tarda* ($1.3x10^8$cfu/per fish, 10 times of the LD_{50} concentration) in PBS, respectively. The cumulative mortality and relative percent survival (RPS) of challenged fish was observed up to the 14 days after challenge [36].

$$\text{Relative percentage survival (RPS)} = \frac{\text{No. of surviving fish after challenge}}{\text{No. of fish infected with bacteria}} \times 100$$

Statistical analysis

The data was statistically analyzed using the statistical package SPSS version 21 in which data were subjected to two-way ANOVA; Duncan's multiple range test (DMRT) was used to determine the significant differences between the means (S.E.M). The level of significance was set at $p<0.05$.

RESULTS AND DISCUSSION

The neutrophil activity was least on day 7 which treated with control group, and maximum on 14 and 21 days, gradually decreased in Panchagavya and *Lactobacillus* treated groups when compared to control (Fig 1). The lysozyme level of treated and control was similar on day 7 of post treatment. Lysozyme levels were significantly ($p<0.05$) higher in the treated group than the control group on 14 and 21 and there was no significant difference between these groups (Fig 2). The maximum level of lysozyme was found in T_3 treated groups. The maximum level of antiprotease was found in T_3 treated groups when compared to control and significant higher ($p<0.05$). The minimum antiprotease activity was noted in

control. The highest antiprotease level was found on 14 and 21 days of post treatment (Fig 3). The same trend was found in myeloperoxidase activity (Fig 4). There was a significant increase in ROS and RNS level from 7 to 14. The increased level was maintained up to day 21, then decreased by day 28, which treated 0.5% AgNPs treated group (Fig 5-6). However, there was no significant difference of ROS and RNS level between the treated groups and control group. Mortality was observed from 2nd day of the post challenge which was confirmed due to the challenge infection with *E. tarda* and *A. hydrophila*. The mortality rate was continued up to 14 days. Relative percent survival of *O. mossambicus* after challenging *E. tarda* and *A. hydrophila* in differential experimental groups (Fig 7). The highest survival was recorded in T_3 group followed by T_2 group, lowest survival was observed in T_4 and followed by control group.

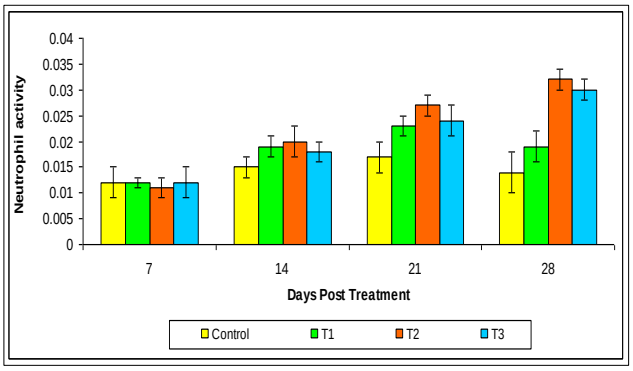

Fig 1. Efficacy of Panchagavya and *Lacto bacillus* probiotics with different intervals of neutrophil activities, *O. mossambicus* T_1 (Basal diet+Panchagavya-1%); T_2 (Basal diet+ *Lacto bacillus* -1%); T_3 (Basal diet+Panchagavya-1%) + *Lactobacillus* (1%) and control (Basal diet). Values are expressed as mean ± SE

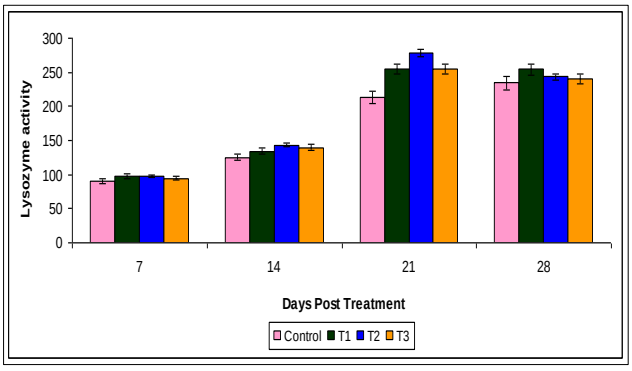

Fig 2 Analysis of Lysozyme activities of Tilapia by the effect of diet supplements, *Oreochromis mossambicus* T_1 (Basal diet+Panchagavya-1%); T_2 (Basal diet+ *Lactobacillus*-1%); T_3 (Basal diet+Panchagavya-1%) +*Lactobacillus* (1%) and control (Basal diet). Values are expressed as mean ± SE

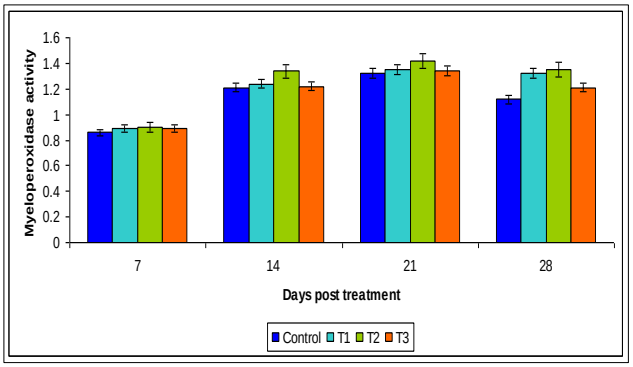

Fig 3 Efficacy of fed supplement on the myeloperoxidase activity of tilapia fish, *Lacto bacillus* on the, *Oreochromis mossambicus* T_1 (Basal diet+Panchagavya-1%); T_2 (Basal diet+ *Lactobacillus*-1%); T_3 (Basal diet+Panchagavya-1%) +*Lactobacillus* (1%) and control (Basal diet). Values are expressed as mean ± SE

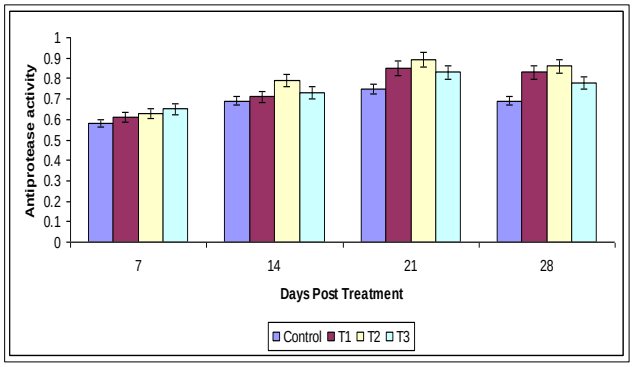

Fig 4 Analysis of antiprotease activity on the growth of Tilapia with different intervals, *Oreochromis mossambicus* T_1 (Basal diet+Panchagavya-1%); T_2 (Basal diet+ *Lactobacillus* 1%); T_3 (Basal diet+Panchagavya-1%) +*Lactobacillus* (1%) and control (Basal diet). Values are expressed as mean ± SE

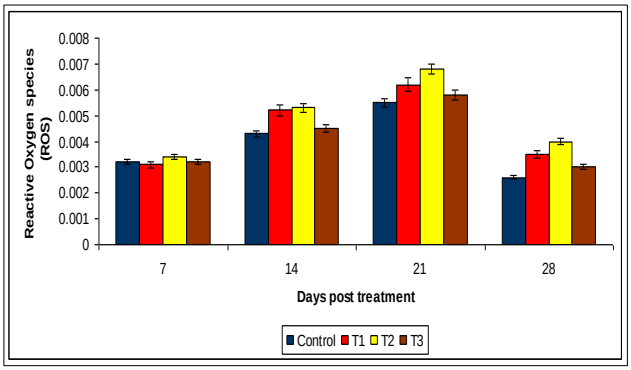

Fig 5 Determination of ROS (Reactive Oxygen Species) of Tilapia with some fed supplement, *Oreochromis mossambicus* T_1 (Basal diet+Panchagavya-1%); T_2 (Basal diet+ *Lactobacillus* 1%); T_3 (Basal diet+Panchagavya-1%) + *Lactobacillus* (1%) and control (Basal diet). Values are expressed as mean ± SE

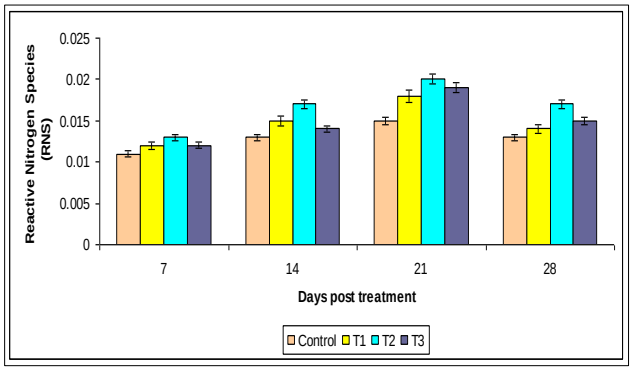

Fig 6 Determination of RNS (Reactive Nitrogen Species) of Tilapia with different interval, *Oreochromis mossambicus* T_1 (Basal diet+Panchagavya-1%); T_2 (Basal diet+ *Lactobacillus*-1%); T_3 (Basal diet+Panchagavya-1%) +*Lactobacillus* 1%) and control (Basal diet). Values are expressed as mean ± SE

Fish culture is increasing to compensate the shortage of animal protein all over the world. Fish under intensive culture conditions will be badly affected and often fall prey to different microbial pathogens that have been treated with chemotherapeutic substances of which antibiotics were intensively used. These curative substances produce the

problem of bacterial drug fastness on one hand and the public health hazards on the other hand [37]. These awaited drawbacks enforced the fish pathologists to seek for other alternatives; the use of natural immune stimulants in fish culture for the prevention of diseases is a promising new development and could solve the problems of massive antibiotic use. Natural immune stimulants are biocompatible, biodegradable and safe for both the environment and human health. Moreover, they possess an added nutritional value [38].

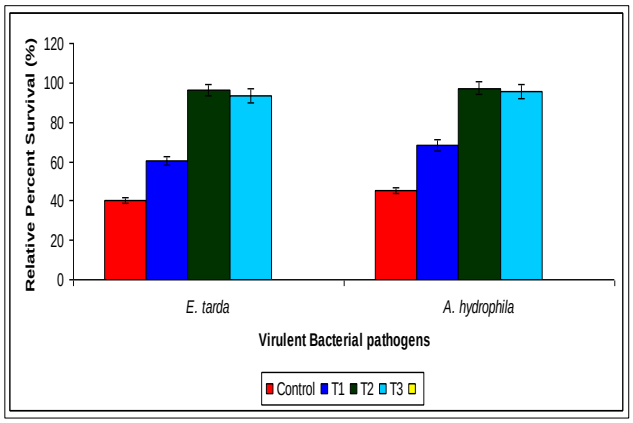

Fig 7 Percentage of survival rate of Tilapia against diseases, *Oreochromis mossambicus*
T_1 (Basal diet+Panchagavya-1%); T_2 (Basal diet+ *Lactobacillus*-1%); T_3 (Basal diet+Panchagavya-1%) +*Lactobacillus* (1%) and control (Basal diet). Values are expressed as mean ± SE

In the past few years, the trend of environment friendly aquaculture has been evolving globally to overcome the shortage of fish vaccines and drawbacks of chemotherapeutics. Numerous biotic forms have been widely used to boost both growth in the probiotic/symbiotic feed additives which enhance the digestibility through providing fish with certain essential nutrients, vitamins and digestive enzymes.

In the present study not only support the use of *Lactobacillus* for better growth, and proper nutrient utilization but also support its stimulating effect on the nonspecific immune response. The activation mechanisms involved could be related to the carbohydrates, derived from the yeast cell wall. [39] stated that the yeast cell wall is constructed almost entirely of two classes of non-soluble polysaccharides, mannose polymers linked with peptide (nano protein) and glucose polymers (glucan). The glucan and nano protein occur in equal amounts in the wall. The third sugar is chitin but is present only in minor amount. Not only sugar but also nucleic acid especially yeast RNA act as immune system enhancers. In the investigation to explore the effects of Panchagavya and probiotics of *Lactobacillus* administrated for shorter duration on immune mechanism against *E. tarda* and *A. hydrophila*.

Phagocytic activity is responsible for early activation of the inflammatory response before antibody production and plays an important role in antibacterial defenses. The highest Phagocytic activity and index were recorded in symbiotic and probiotic treated fish groups followed by prebiotic group. These findings supported those of [40-41]. These could be attributed to the stimulatory effect of yeast cell wall components mainly b-glucan, mannoprotein; chitin and yeast RNA on the circulating and tissue macrophages. The presence of glucan and mannose are not only stimulating the phagocytosis but also increasing their destructive and killing ability [42-43].

The ability of macrophages to kill pathogenic microbes is probably one of the most important mechanisms of protection against diseases among fish. The oxygen radicals and nitric oxide are the most destructive products produced by activated macrophages. Increase of respiratory burst activity and reactive nitrogen species can be correlated with increase of oxygen and nitric oxide radicals' production and increase of killing activity. In the present study, the three used biotic forms showed significant enhancement of the neutrophils/macrophages activity than in control group, which coincides with those previous publications presented by [44-46].

Lysozyme is considered as one of the important bactericidal enzymes and an indispensable tool of fish to fight infectious agents. *Lactobacillus* is found to trigger the serum lysozyme level in teleost. Our research showed that the use of such biotic forms have a significant increase on the lysozyme activity in all treated fish [44]. Lysozyme is constitutively expressed, synthesized and secreted by neutrophils, monocytes and macrophages. The greatest concentration of lysozyme was directly proportional to the leukocytic count. In the current research, the addition of *Lactobacillus* in fish diet has remarkably increased the leukocytic count, which in turn elevated lysozyme concentration and activity [47]. In the present study Pachagavya with *S. cerevisae* treated group found in lysozyme activity. The similar results were observed in Labeorohita [48], Cirrhinusmrigala [49].

In the present study, myeloperoxidase activity, lysozyme activity, respiratory burst activity was statistically enhanced by diets containing *Lactobacillus* with Panchagavya, while serum peroxidases and ROS and RNS activity were inhibited. Among the humoral responses studied (natural complement and lysozyme activities and peroxidase content) no difference was observed as a consequence of the dietary intake of Panchagavya with yeast cells. These observations agree with previous studies in which the dietary intake of purified b-glucans, chitin or even the whole *Lactobacillus* did not affect complement or lysozyme activities [39], [50-51], although such activities were enhanced by injected b-glucans or chitin [42], [52].

The obvious decline in fish mortalities among the three treated groups could be results of immune system activation against all kinds of pathogenic as well as opportunistic bacterial invaders. Further, the whole yeast supplement could have played a critical role in the sharp decrease of the competitor bacterial loads encircling treated fish throughout the experiment by what is called 'competitive exclusion theory' [53-54].

CONCLUSION

In conclusion, the results of this study revealed that Panchagavya and *Lactobacillus* frequently used probiotics supplement s to *O. mossambicus* for preventing the fish pathogens. Also, the diets supplemented with Panchagavya and *Lactobacillus* improve the non-specific immune response which reflected on the stimulation of macrophage cells and increasing their neutrophil activity. Finally, these probiotics could provide healthy and safe fish production from aquaculture replacing the Xenobiotics (antibiotics) for both fish and fish consumers.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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