ISSN: 2347-5129
IJFAS 2015; 2(4): 362-369
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www.fisheriesjournal.com
Received: 13-11-2014
Accepted: 27-12-2014

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Immunostimulatory effect of *Lactobacillus sporogenes* on the nonspecific defense mechanisms of *Oreochromis mossambicus* (Peters)

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Abstract
The concept of using beneficial whole microorganisms is termed as **"probiotics**" and is gaining importance, as an eco-friendly disease management tool. In addition to disease resistance, they also improve water quality and growth of farmed fish. *Lactobacillus sporogenes* has been reported as one such beneficial bacteria in man and mammals. In order to explore the effect of this probiotic three groups of fish were treated with different number of vegetative cells in the log phase of growth for seven days. After this period of probiotic treatment fish were immunized with heat killed *Aeromonas hydrophila.* Then the fish were bled serially on 2nd, 4th, 6th, 8th, 10th, and on the 12th day of immunization for various non-specific immuno assays like activated neutrophils, peripheral blood leukocyte count, and number of lymphocytes, granulocytes and monocytes. The results showed that *L. sporogenes* is an effective nonspecific immunostimulant in aquaculture.

Keywords: Probiotics, Aquaculture, *Lactobacillus sporogenes, Aeromonas hydrophila,* Immunostimulant, Nonspecific immune mechanisms.

1. Introduction
Farmed fishes are inevitably subjected to various stresses like handling, transportation, crowding, infections, exposure to pollutants, and physiological changes that may lead to immunosuppression and consequent infections. Even though vaccines are available in developed countries against a few diseases, aquaculture still experiences high loss of fish stocks due to outbreak of diseases. Part of this is because even efficient vaccines lose their effect one year after vaccination, and new pathogens are constantly gaining territory [1]. Further, it seems unlikely that cultured fish can be vaccinated against all potential diseases. Hence, the significance of a suitable client in preserving the health of living organisms is widely recognized [2].

In recent years, the increasing consumer concern about the residues of antibiotics, hormones, growth promoters, and the danger of development of antibiotic resistant strains has led to the use of **immunostimulants** in aquaculture. By definition, immunostimulants are substances that can enhance the nonspecific defense mechanisms as well as specific immune response if the treatment is followed by infection or vaccination [3]. Many natural and synthetic substances have been reported that potentiate the fish immune system and increase disease resistance [4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15]. The search for new immunostimulants continues as an attempt to improve intensive fish farming. According to Rodriguez [16] such new products should possess two characteristics.

1. Provide general stimulation and
2. Economically affordable.

These two characters are well fulfilled by whole microorganisms.

1. First, they are rich sources of immunostimulant substances such as β -glucans, chitin, vitamins, genetic material etc. At the same time, they act as a source of nutrients and micronutrients that affect the general fish physiology.
2. They are cheap sources of immunostimulants. New strains can be generated by genetically manipulating strains with a high content of specific substances.

This concept of using beneficial whole microorganisms is termed as **"probiotics**" and is gaining importance, as an eco-friendly disease management tool. In addition to disease resistance, they also improve water quality and growth of farmed fish. Probiotics are defined as microbial dietary adjuvants that beneficially affect the host physiology by modulating mucosal and systemic immunity as well as improving nutritional and microbial balance in the intestinal tract [17]. Beneficial bacteria in the best cases could be used to substitute the use of antibiotics as preventive agents of disease [18] and as growth promoters [19]. Immunomodulation by probiotics have been well documented in mammals including man [20, 21, 22, 23, 24, 25, 26, 27].

Recently, this theory has been applied to aquaculture. Many researchers attempt to use some kind of probiotics in aquaculture water to regulate the micro flora of aquaculture water, control pathogenic microorganisms, to enhance decomposition of the undesirable organic substances in aquaculture water, and improve ecological environment of aquaculture. In addition, the use of probiotics can increase the population of food organisms, improve the nutrition level of aquacultural animals and improve immunity of cultured animals to pathogenic microorganisms. In addition, the use of antibiotics and chemicals can be reduced and frequent outbreaks of diseases can be prevented [28].

There are a few studies that explore the immunomodulatory role of probiotics in fish. The non-specific immunostimulation and colonizing efficiency in gut, skin, mucous of *Lactobacillus rhamnosus* were studied in rainbow trout [29] and in turbot [30]. The probiotic yeast cells *Saccharomyces cerevisiae* was recorded for its immunostimulatory activities in rainbow trout [31] and in gilt head seabream [16-32]. Investigated the effects of various levels of dietary *Bacillus subtilis* and chitosan on the growth performance, non-specific immunity and protection against *Vibrio harveyi* infection in cobia, *Rachycentron canadum*.

Although we already have a broad knowledge base with regards to the effect on host innate immunity at the systemic level, our understanding of the important host-microbe interactions at the mucosal interface and the subsequent localised immunological responses is lacking [33].

However, during the last few years a number of papers have revealed important information on the localised host response to gut microbes and probiotics with respect to the gene expression of pro- and anti-inflammatory cytokines (e.g. IL-1β, IL-8, IL-10 and TNFα), mucosal antibodies (i.e. IgT/IgZ), TLR's, various other important immunological proteins and proteins involved in the regulation of cellular activity and apoptosis (e.g. PCNA and Hsp70) [33].

Hence the present study has been aimed at exploring the immunostimulatory effect of the microbial probiotic *Lactobacillus sporogenes* on non-specific defence mechanisms of *Oreochromis mossambicus.* When administered directly in the medium as vegetative cells.

2. Materials and methods

2.1. Animal maintenance

Oreochromis mossambicus a common fresh water cichlid fish was used for the study. Fish procured from river cauvery were stocked in large fiber tanks. The experiments were carried out in plastic tubs of 70 lt capacity. Fish of both sexes weighing 20-25 gm were used in the study. Water was changed frequently to avoid stress due to ammonia accumulation. The animals were fed *ad libitum* with a balanced fish diet prepared in our laboratory.

2.2. Culture of *Lactobacillus sporogenes*

Lactobacillus sporogenes spores commercially available as the pharmaceutical product SPORLAC were used for the present study. The culture and maintenance of the probiotic was done using MRS agar and nutrient broth (Titan Biotech, India.). SPOROLAC tablet was first opened under aseptic conditions and soaked in physiological saline solution for overnight to get a starter culture. This step initiated the germination of the spores. Then it was inoculated in MRS broth and cultured at room temperature (32 ± 1 ºC) in a rotatory shaker. From the 16 hr culture using an inoculation loop a streak culture was made in MRS agar plates. After 24 hrs, a single colony was taken and inoculated in MRS broth to culture the probiotic bacteria in required quantity.

2.3. Experimental protocol

Three groups of fish were administered with different numbers of the probiotic cells - 2.5 x 10^4 (T1) 5 x 10^4 (T2) 1 x 10^5 (T3). The lactobacilli obtained from the 16 hrs culture were washed well and required number of cells suspended in PBS and introduced in the tank water. A seperate control group was maintained to which physiological saline was added. Seven days after the probiotic treatment, water was changed and fish were immunized with intra peritoneal injection of 10^9 cells of heat killed *Aeromonas hydrophila.* The immune parameters were assayed on different days based on the period of response.

2.4. Number of Activated Neutrophils by NBT assay

The NBT assay followed was that of Anderson [34] except that distilled water was used instead of saline to prepare the NBT solution [35]. Fifty µl of blood was bled from the common cardinal vein using a syringe with 50 µl of heparinised saline. This 100 µl of heparinised blood was placed on a glass cover slip. The cover slips were placed on moist cotton in a petridish for 30 minutes. The excess cells were washed off with a stream of PBS from a Pasteur pipette. Blotting the edge of the coverslip with a paper towel drained off the PBS. The cover slip was turned upside down onto a drop (50 µl) of the NBT solution in a glass microscope slide. The slide was incubated for 30 minutes. Then the slides were examined under a light microscope (400 x). Five random fields of positive dark blue stained cells were observed for each cover slip. Activated neutrophils in each field were added together to give a total number of cells per slide. The NBT assay was done on 2nd, 4th, 6th, 8th, 10th and 12th days post immunization.

2.5. Total and differential white blood cell counts

Total WBC was counted in a Neubauer counting chamber using Natt-Herrings solution as the diluting fluid [36]. 0.1 ml of blood was diluted to eight times using Natt-Herrings solution and kept for five minutes. The stained cells are counted in four large squares of Neubauer counting chamber. Differential count was done using Leishman stained blood smears. Cover the smear with stain and leave for 1-3 minutes. Add PBS and allowed to mix on slide and leave for five minutes. Rinse in distilled water. Blot dry the slide and examine under 100x magnification of a binocular microscope. 100 cells were counted and the number of cells was expressed in percentage.

2.6. Statistical analysis

One-way ANOVA was performed using Minitab (SPSS Inc, Chicago, Illinois, USA) software for analyzing the significance between means and Microsoft Excel was used for graphical presentation of data.

3. Result and Discussion

3.1. Activated Neutrophils

L. sporogenes has significantly ($p < 0.05$; Table 1) stimulated the number of activated neutrophils (Fig. 1) in a dose dependent manner. The peak day of for control was Day 6, while T1 stimulated the number of activated neutrophils significantly and at the same time advanced the peak day to day 4. $1x10^5$ cells/ml concentration has no significant effect on the number of activated neutrophils.

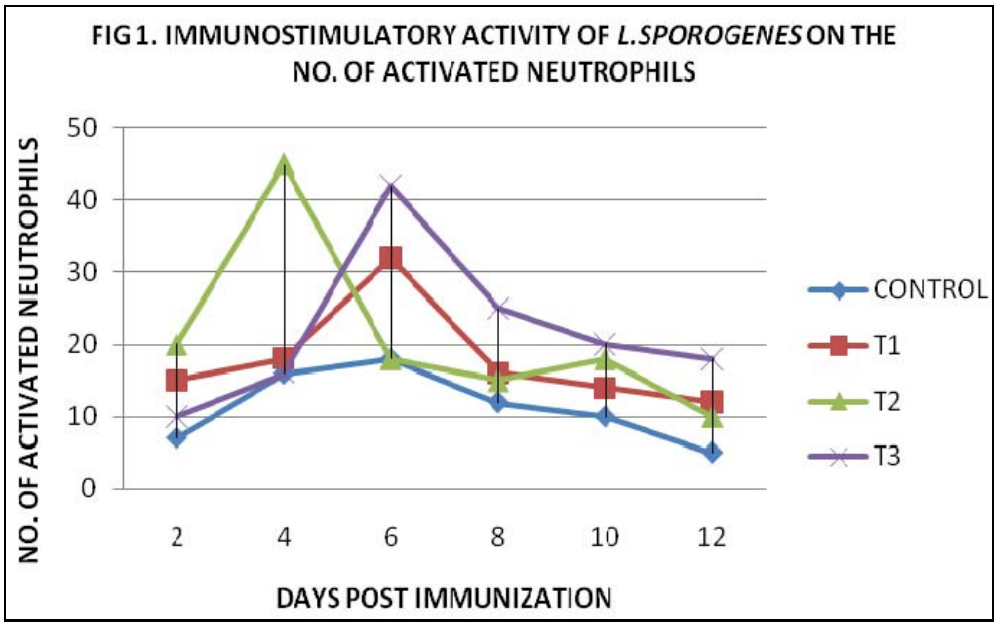

Table 1: One-Way Analysis of Variance showing the overall effect of *Lactobacillus sporogenes* on Number of activated neutrophils when administered as water additive

Analysis of Variance					
Source	**DF**	**SS**	**MS**	**F**	**P**
Factor	3	2425.92	148.97	10.59	0.000
Error	140	10685.1	3.97		
Total	143	13110.1			

Level	**N**	**Mean**	**St De**
C	36	11.472	4.699
T1	36	17.972	6.627
T2	36	21.111	11.434
T3	36	21.889	10.419

Pooled St Dev = 8.736

Individual 95% CIs For Mean
Based on Pooled St Dev

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10.0        15.0        20.0        25.0
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The results of the present study clearly shows that exposure to the probiotic bacteria increases the number of activated macrophages. The results are in conformation with the earlier studies on fish [30, 29, 37, 16, 32, 38]. Many immunostimulants have been reported for their stimulatory action on activity of the neutrophils in fish [6, 3, 9, 8, 11, 39]. *L. rhamnosus* is recorded to have the activity of interferon and interleukins IL - 4 & IL -5, and monokines (IL-12, IL-18) [40]. It has been shown that certain probiotic bacteria are able to stimulate phagocytic activity in humans [41, 42]. Immunomodulation by *Lactobacillus* sp. in improving non-specific defenses has been well documented in mammals [20, 21, 22].

The water additive route was most effective in comparison with the other two routes of administration studied earlier (feed supplementation and immobilized cells) [43, 38] in enhancing the number of activated neutrophils. The magnitude of the response is in the order of water additive > feed supplement > immobilized cells.

3.2. Total peripheral blood leukocyte count

Figure 2 reveals that lactobacillus increased the number of white blood cells when administered as water additive. Treatment T1 has number effect on the WBC count. T2 and T3 enhanced the peripheral leukocytes count significantly ($P < 0.05$; Table 2). T2 enhanced maximally on 6th day and T1 showed minimum effect.

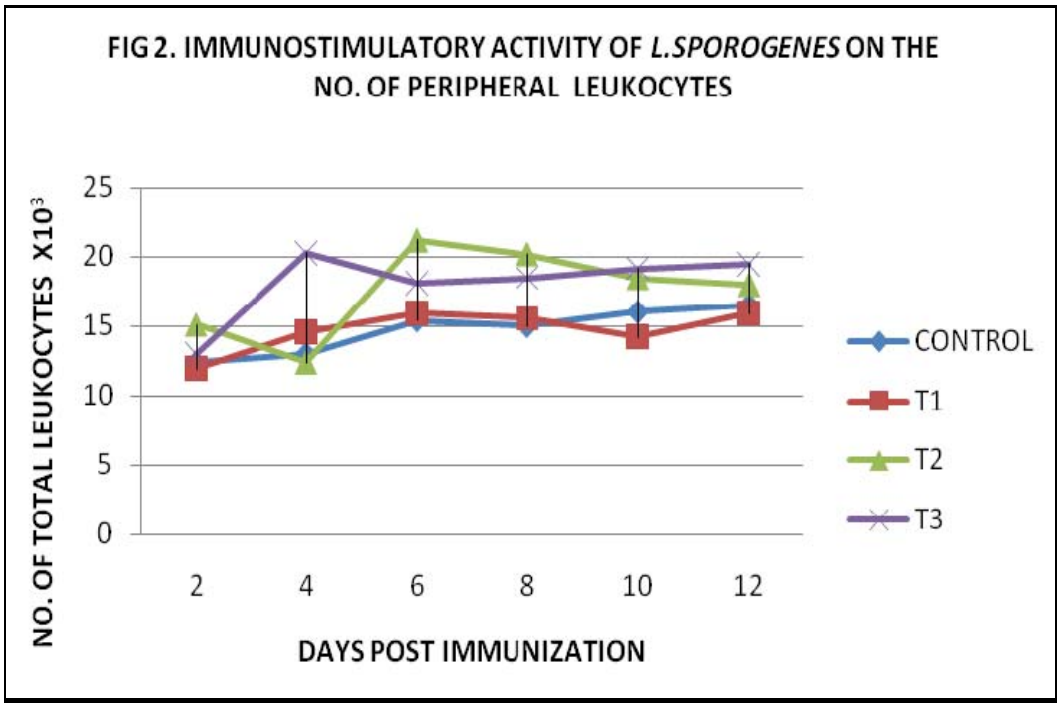

Table 2: One-Way Analysis of Variance showing the overall effect of *Lactobacillus sporogenes* on Number peripheral blood leukocytes when administered as water additive

Analysis of Variance					
Source	**DF**	**SS**	**MS**	**F**	**P**
Factor	3	459783069	153261356	30.12	0.000
Error	140	712284931	5087750		
Total	143	1.172E+09			

Level	N	Mean	St De
C	36	13995	1795
T1	36	14654	1431
T2	36	17582	3044
T3	36	18114	2411

Pooled St Dev = 2256

Individual 95% CIs For Mean
Based on Pooled St Dev
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14400 16000 17600

Blood monitoring though considered less sensitive [44], is one of the most popular method for immunological assays because the animal need not be sacrificed. Although tilapia is the second most frequently cultured fish in the world, there are surprisingly few reports on normal blood values for this species. Our results on total WBC count and differential count conform well with observations done elsewhere [45, 46, 47]. An increase in the number of leukocytes (leukocytosis) is a normal mode of innate defense response of the fish to a pathogenic attack. Hari krishnan *et al.,* [48] showed that *A. hydrophila* infection caused an increase in WBC count. Herbal immunostimulants have also been shown to stimulate the proliferation of leukocytes [49]. However in the field of probiotics, studies on the effect of probiotics on hematological parameters are lacking. The vegetative cells (water additive) show a similar pattern of enhancement in cell counts as observed for activated neutrophil responses. In this group T2 showed maximum enhancement

3.3. Differential leukocyte count

3.3.1. Lymphocytes

There is significant stimulation of lymphocytes by all the treatments (Fig 3). A dose dependent effect was observed with T3 having the highest effect and T1 with lowest stimulatory effect. ($P < 0.05$; Table 3). The number of lymphocytes was maximum on day 12 post immunization.

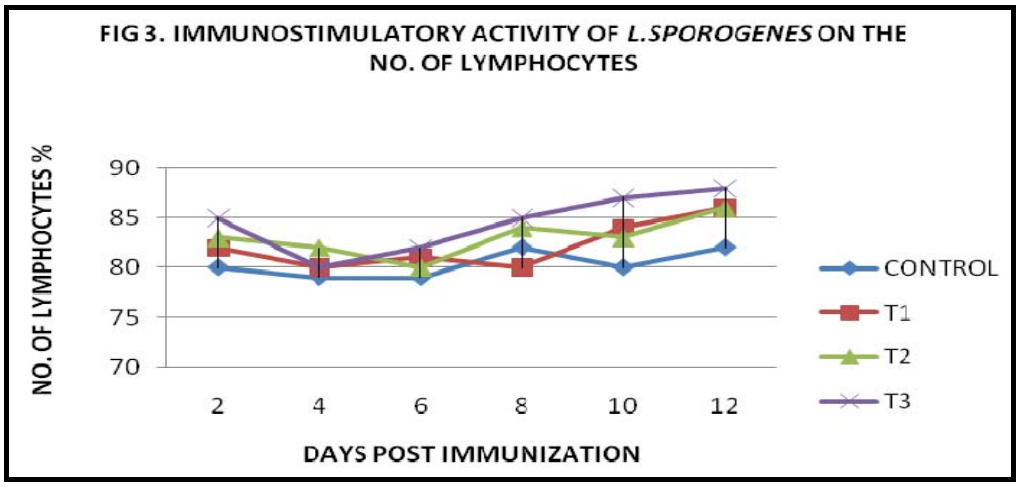

Table 3: One-Way Analysis of Variance showing the overall effect of *Lactobacillus sporogenes* on Number of lymphocytes when administered as water additive

Analysis of Variance					
Source	**DF**	**SS**	**MS**	**F**	**P**
Factor	3	446.92	148.97	37.48	0.000
Error	140	556.39	3.97		
Total	143	1003.31			
Level	**N**	**Mean**	**St De**	Individual 95% CIs For Mean Based on Pooled St Dev	
C	36	80.306	v	-- + --------- + --------- + --------- + ----	
T1	36	81.944	1.582	(--- * --)	
T2	36	82.278	1.912	(--- * --)	
T3	36	85.194	1.579	(-- * ---)	
			2.692	(-- * --)	
Pooled St Dev = 1.994				-- + --------- + --------- + --------- + ----	
				80.0 82.0 84.0 86.0	

The present investigation shows a significant enhancement in lymphocyte count among probiotic treated fish compared to the control. The probiotics seem to stimulate the proliferation of lymphocytes either directly or indirectly. Observations of Kitazawa *et al.*, [50] that probiotics enhanced multiplication of B lymphocytes by stimulating the expression of CD molecules (Cluster of Differentiation system), could be a possible mechanism for the enhancement observed in the present study.

3.3.2. Granulocytes

There is significant stimulation of granulocytes by all the treatments (Fig 4) on the peak day (Day 4). Maximum enhancement was done by T3 and minimum by T2. When the overall response was analyzed, the effect was seemed to be suppressive. ($p < 0.05$; Table 4).

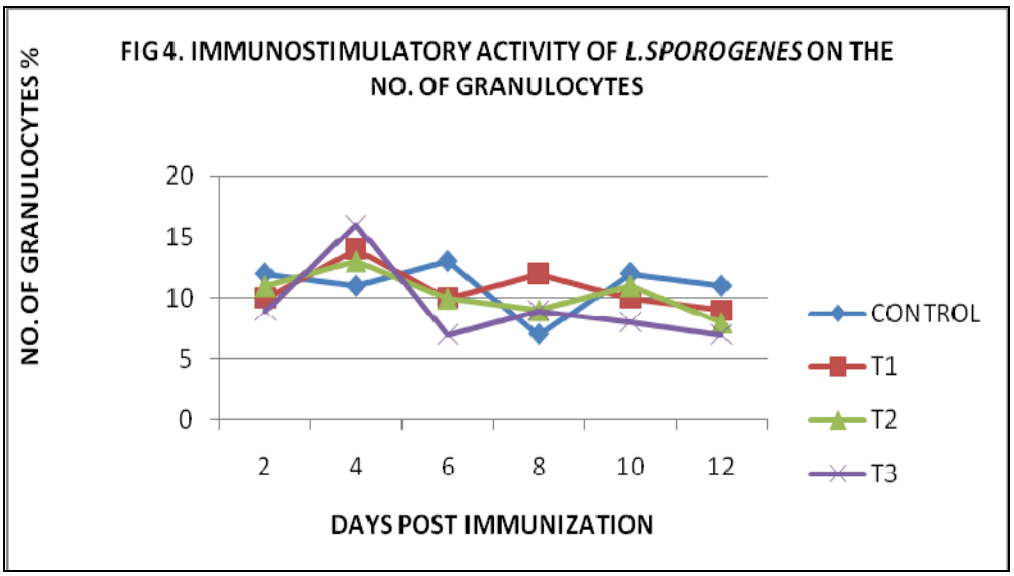

Table 4: One-Way Analysis of Variance showing the overall effect of *Lactobacillus sporogenes* on Number of granulocytes when administered as water additive

Analysis of Variance					
Source	**DF**	**SS**	**MS**	**F**	**P**
Factor	3	155.80	51.93	13.91	0.000
Error	140	522.86	3.73		
Total	143	678.66			
Level	N	Mean	St Dev	Individual 95% CIs For Mean Based on Pooled St Dev	
C	36	10.889	2.025		
T1	36	11.000	2.191		
T2	36	10.389	1.946		
T3	36	8.417	1.500		
Pooled StDev = 1.933					

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8.4 9.6 10.8 12.0

In fish the major granulocytes are neutrophils. Granulocytes in blood can be greatly increased within 24 hrs after subjecting fish to stress [51]. In the present study there is a significant reduction in the number of granulocytes in the probiotic treated fish groups. This could be attributed to the fact that granulocytes are attracted to the site of infection by chemotaxis [52]. Being highly motile these phagocytic cells are the first to arrive at the site of infection. This might result in a reduction of circulating granulocytes in blood. The reduction in granulocytes count in blood could thus be related to an increased disease resistance.

3.3.3. Monocytes

There is significant stimulation of monocytes by the treatments T2 and T3 (Fig 5) on the peak day. (Day 6). Maximum enhancement was done by T3 and minimum by T2. The lowest treatment T1 was suppressive in the monocytes count. When the overall response was analyzed, the effect was seemed to be suppressive by all three treatments. ($p < 0.05$; Table 5).

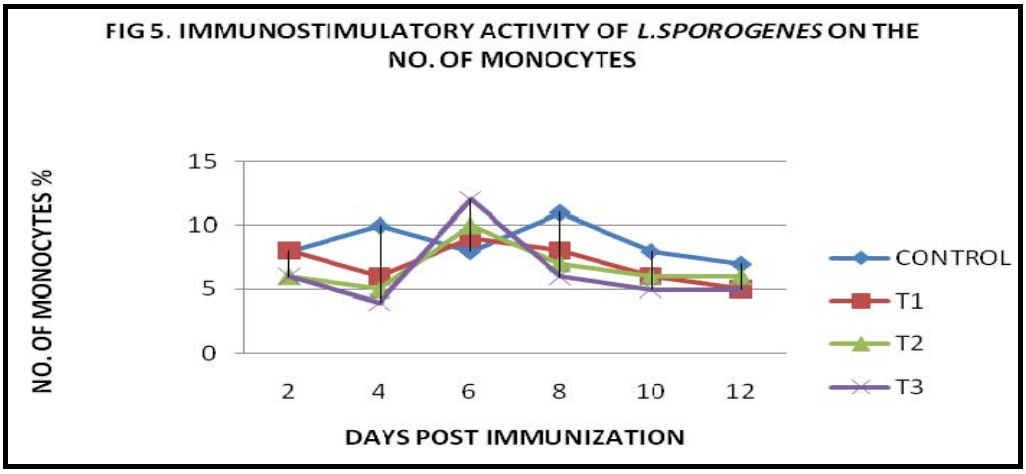

Table 5: One-Way Analysis of Variance showing the overall effect of *Lactobacillus sporogenes* on Number of monocytes When administered as water additive

Analysis of Variance					
Source	**DF**	**SS**	**Ms**	**F**	**P**
Factor	3	100.47	33.49	8.63	0.000
Error	140	543.28	3.88		
Total	143	643.75			
Level	**N**	**Mean**	**StDe**	Individual 95% CIs For Mean Based on Pooled StDev	
C	36	8.667	1.621		
T1	36	6.972	1.576		
T2	36	7.111	1.753		
T3	36	6.417	2.708		
Pooled StDev = 1.045					

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4.0 4.7 5.2 5.8

Monocytes are the largest circulating leukocyte population. In the present study there is a significant decrease in the number of monocytes in fish treated with probiotic bacteria. The decrease in number of monocytes in blood could be justified by the fact that the monocytes are differentiated into tissue macrophages and they migrate towards the site of infection or inflammation [52], for phagocytosis and subsequent antigen processing and presentation. A decrease in monocyte count could therefore be an indication of the stimulation of the specific and non-specific defense mechanism.

4. Conclusion

The microbial probiotic *L. sporogenes* could be used as an effective cheap source for prophylactic measures in aquaculture. The mode application is highly simple and requires minimum labour. Since the strain used in the present study is meant for human consumption, there is no need for discussing its safety issues.

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