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# Effect of probiotic (*Bacillus subtilis*) on the immune system of fingerlings of Grass carp, *Ctenopharyngodon idella*

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**Abstract**
In the modern high intensity aquaculture, probiotics offer an encouraging substitute to chemicals and antibiotics in aquatic animals, one such important application of probiotics is their use as Immune enhancer in aquaculture, in addition to growth and water quality management. So the present study was carried to evaluate the effect of dietary incorporation of probiotic - *Bacillus subtilis* on the hematology of grass carp (*Ctenopharyngodon idella*. The probiotic - *Bacillus subtilis* was mixed with the basal diet (Protein 32%) in three different concentrations (0.5, 1.0 and 1.5% designated as $T_1$, $T_2$, and $T_3$). The basal feed with no probiotic was used as control. The study was conducted for a period of 60 days. Feeding was done twice a day at the rate of 5% of their body weight. Hematological parameters (TLC and DLC) were also examined. The probiotic treated fish ($T^3$, 1.5%/100g) displayed highest WBC count as compared to the control.

**Keywords:** *Ctenopharyngodon idella*, *Bacillus subtilis*, TLC, DLC

## Introduction
The grass carp is one of the member of the largest family Cyprinidae and is the only member of the genus *Ctenopharyngodon* (Bozkurt *et al*., 2017) [2]. It is one of the four well-known "Chinese farm fish" and nourishes on aquatic grass and also land grass. Grass carp is cultivated almost in any water body such as ponds, lakes, rivers and reservoir throughout China.

However, grass carp is also susceptible to various kinds of diseases and this makes it difficult to culture particularly in fingerling stage. Survival of as low as 5% has been recorded in grass carp (Nie and Pan, 1985) [8].

Researchers are interested in finding environmentally friendly solution to fish diseases where probiotics seemed as a good substitute to antibiotics due to adverse impact of antibiotics such as alterations in the microbiota of the aquatic systems which result in bacterial resistance to frequently used antimicrobials which in turn effects the natural useful bacterial population (Kavitha *et al*., 2018) [5].

Probiotics also influence the mucosal barrier by their trophic effect on intestinal epithelium and stimulate both specific and non-specific components of the immune system (Vendrell *et al*., 2008) [10]. They also contribute to higher growth and feed efficiency, avoid intestinal ailments and pre-digestion of anti-nutritional factors existing in the ingredients thus improving nutrients utilization. Probiotics may also detoxify the possibly harmful compounds in feeds, by denaturing the potentially indigestible components in the diet by hydrolytic enzymes such as amylase and protease.

As a result numerous probiotics such as *Arthrobacter*, *Bacillus*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Micrococcus*, *Pediococcus*, *Aeromanas*, *Burkholderia*, *Enterobacter*, *Vibrio*, *Pseudomonas*, *Rhodo pseudomonas*, *Roseobacter* and *Shewanella* have been discovered and used to augment growth and immunity of aquaculture species over the years (Kuebutoryne *et al*., 2019) [6].

*Bacillus* species also possess immunostimulatory effects and stimulation of beneficial gut microflora thus improve the host's innate and adaptive immunity (Kuebotoryne *et al*., 2019) [6]. Thus this study was designed to evaluate the Effect of probiotic (*Bacillus subtilis*) as an immune stimulant in Grass carp (*Ctenopharyngodon idella*).

## Material and Methods

### Experimental setup

Healthy and disease free fingerlings of *Ctenopharyngodon idella* average weight of 8.30±0.006 gm. were collected from Fisheries Instructional Research Farm , Faculty of Fisheries located at Shuhama campus of SKUAST-K and were acclimatized to laboratory conditions for a week, before the start of experiment. During acclimatization the mixture of soybean and wheat bran (1:1) pellet feed in granular forms were fed to the fish. A completely randomized design was developed. The experiment was carried out in sixteen plastic tubs of eighty liters capacity. Fifteen *Ctenopharyngodon idella* fishes were stocked in each plastic tub with four replicates for four experimental diets.

### Haematological studies

For haematological studies blood samples were collected from live fishes by heart puncture (Lucky, 1977) [7]. Before collection of blood, the fishes were anaesthesized using clove oil (Javahery et al., 2012) [4]. For determining haematological parameters, samples were collected in glass vials containing EDTA as anticoagulant at an approximate concentration of 5mg/ml of blood (Blaxhall and Daisley, 1973) [1].

### Total leukocyte count (TLC)

The Leucocyte Count (TLC) was determined by the Haemocytometer (Stevens, 1997) as shown in. Anticoagulated non haemolized blood was drawn into the erythrocyte pipette up to 0.5 mark and WBC dilution fluid was also drawn into the pipette slowly until the mixture reaches at 11 mark. The sample was mixed far at least 3 minutes to facilitate hemolysis of RBCs. The Neubaurs counting chamber was then charged with the mixture after discarding the liquid into the capillary portion of pipette. The counting of the WBC was taken under 40 x objectives.

Calculation: $WBC/mm^3 = N \times 500$

Where, N denotes the total number of WBC counted in the four squares of counting chamber and 500 is the dilution factor

### Differential Leukocyte count (DLC)

DLC was performed as per the methodology of Hudson and Hay (1991) [3]. A small drop of fresh, anticoagulated non haemolysed blood was poured on the grease free slide using a spreader slide, a thin tongue shaped smear was made. The blood smear was air dried, fixed with methanol for 2 minutes and stained with Giemsa stain for 5 minutes. Then the slides were washed by running water directly to the Centre of the slide to prevent a residue of precipitated stain, dried and observed under oil immersion objective (100X). The values of all hematological parameters under study were properly recorded and subjected to statistical analysis.

**Fig A1:** Anaesthetizing fish using clove oil

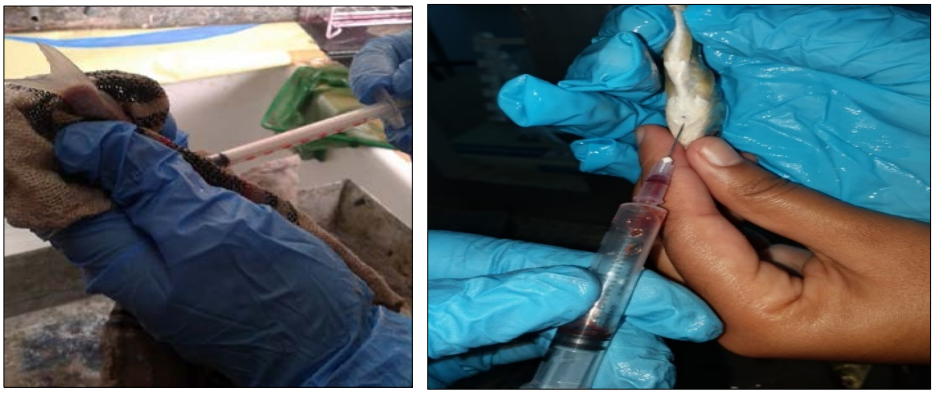


**Fig A2:** Blood collection in fish by heart puncture

## Results And Discussion

### Effect of probiotic on Total leucocyte count (TLC) ($\times 10^3$/µl)

The Total leucocyte count (TLC) of probiotic treated fish samples was estimated and compared with the control ($T_0$). The mean value of TLC of control group ($T_0$) was found to be 6.25±0.08 while as in probiotic treated fish it significantly increased ($P \leq 0.05$) with highest value of 8.07±0.08 in treatment 3 fed with 1.5% probiotic.

**Table A1:** Mean ± SE of Total leucocyte count of fingerlings of *Ctenopharyngodon idella* fed with different experimental diets

| Treatments | Total leucocyte count (TLC) ($\times 10^3$/µl) ±SE |
|---|---|
| Control ($T_0$) | 6.25±0.08 |
| $T^1$ | 6.72±0.10 |
| $T^2$ | 7.17±0.04 |
| $T^3$ | 8.07±0.0.08 |
| P value | < 0.05 |

### Effect of probiotic on differential leucocyte count (DLC)

In this study, differential leucocyte count was carried out in probiotic treated fish and compared with the control. Lymphocyte count was found to be increased in probiotic

treated fish as compared to the control group ($T_0$). Mean value of lymphocyte count recorded in $T_3$ group was 79.3±0.108 and that of control group ($T_0$) it was 75.05±0.064

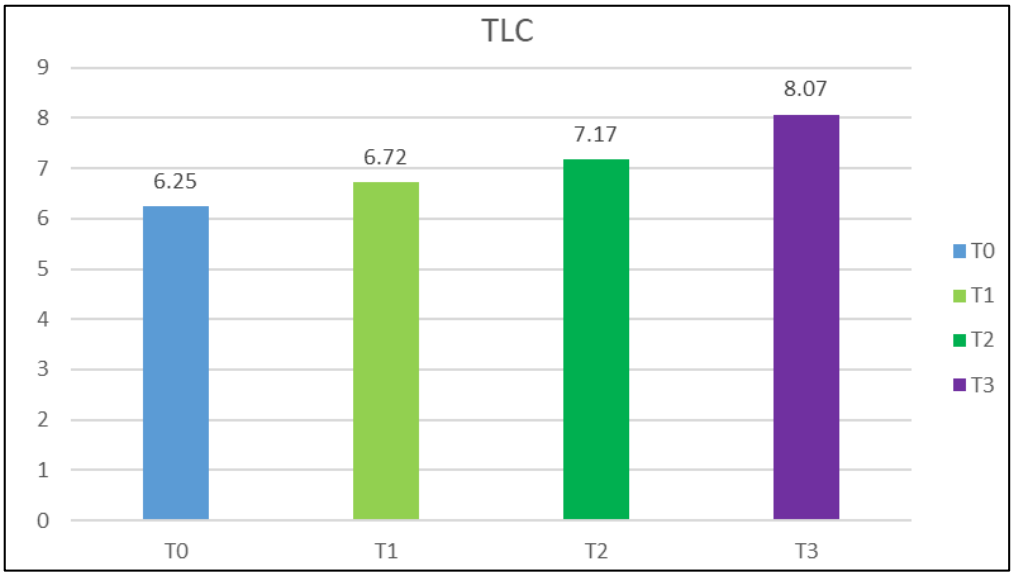
Neutrophils count was found to be decreased in probiotic treated fish when compared with the control. Mean value of neutrophil count recorded in control group ($T_0$) was 22.3±00.108 and that of in probiotic treated fish it was 20.0±0.057

Monocytes, eosinophils and basophils showed no significant difference as compared to the control group ($T_0$) with no probiotic

**Table A2:** Mean ± SE of Differential leucocyte count of fingerlings of *Ctenopharyngodon idella* fed with different experimental diets

| Treatments | Lymphocytes | Neutrophils | Eosinophils | Basophils | Monocytes |
|---|---|---|---|---|---|
| Control ($T_0$) | 75.05±0.064 | 22.3±0.108 | 0.62±0.011 | 0.32±0.006 | 1.60±0.002 |
| $T_1$ | 77.5±0.040 | 20.1±0.025 | 0.31±0.006 | 0.34±0.007 | 1.14±0.004 |
| $T_2$ | 78.05±0.028 | 20.2±0.002 | 0.31±0.006 | 0.33±0.004 | 1.31±0.006 |
| $T_3$ | 79.3±0.108 | 20.0±0.057 | 0.31±0.004 | 0.33±0.010 | 1.30±0.002 |
| P value | < 0.05 | < 0.05 | > 0.05 | > 0.05 | > 0.05 |

T1 = Feed supplemented with *B. subtilis* @ 0.5%/100g
T2 = Feed supplemented with *B. subtilis* @ 1%/100g
T3 = Feed supplemented with *B. subtilis* @ 1.5%/100g




T 1 = Feed supplemented with *B. subtilis* @ 0.5%/100g
T 2 = Feed supplemented with *B. subtilis* @ 1%/100g
T3 = Feed supplemented with *B. subtilis* @ 1.5%/100g
Control (T0) = Feed with no supplementation

**Fig A4:** Differential leucocyte count of different treatments and control fed with different experimental diets. Data is presented as Mean ± S.E.

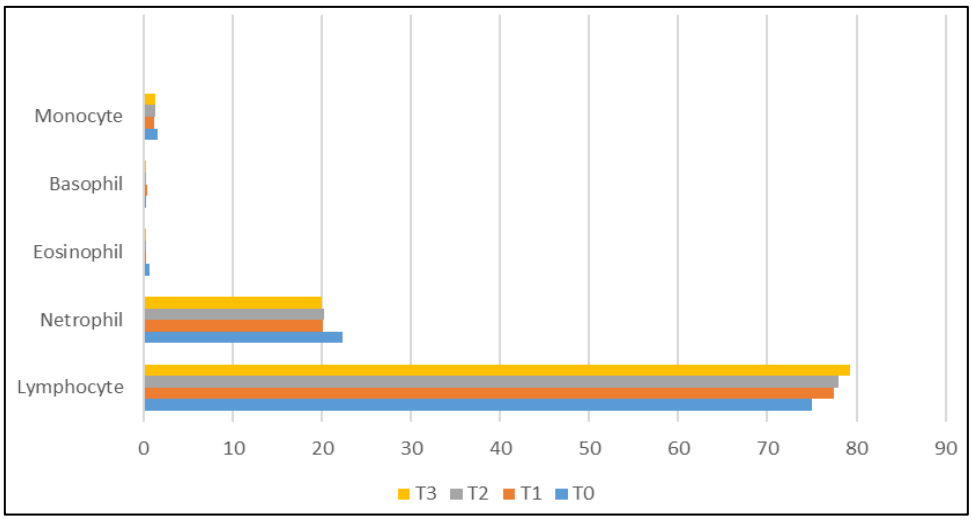



**Fig A4:** DLC

## Conclusion

The present study revealed the immunostimulatory effect of *Bacillus subtilis*. The results demonstrated that probiotic incorporated diets significantly enhanced the immune system of *Ctenopharyngodon idella*. It can be concluded that the incorporation of *Bacillus subtilis* in the feed @ 1.5%/100g

resulted in better immune system in terms of increased WBC count as compared to the control.

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