

## Gut colonization and permanence of the probiotics in *Labeo calbasu* (Hamilton, 1822)

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### ABSTRACT

The gnotobiotic approach utilizing autochthonous probiotics enhance the growth and immunity by fastening to persist in the fish's gastrointestinal tract. The present work was conducted to examine the adherence and colonization of probiotic, *Aneurinibacillus aneurinilyticus* and *Stenotrophomonas* sp. fed to *Labeo calbasu*. The juveniles were fed on Diet C (control), Diet 1 (*A. aneurinilyticus*), Diet 2 (*Stenotrophomonas* sp.) and Diet 3 (*A. aneurinilyticus* and *Stenotrophomonas* sp.) maintained in triplicate with ten individuals each for a duration of 90 days. The fingerlings depicting significant growth performance ( $p < 0.05$ ) were subjected to intestinal enzymatic, haematological and immunohistochemical assays. Diet 1 fed group revealed significantly ( $p < 0.05$ ) high values of enzymes, blood corpuscles and respiratory burst activity. The results showed the permanence of probiotic colonies in gastrointestinal tract lumen and on the microvilli edges. This clearly reveals that probiotic *A. aneurinilyticus* enhanced growth, digestibility and immune response providing an appreciable approach towards sustainable aquaculture.

### INTRODUCTION

Intensification of aquaculture requires effective fish management and culture practices for appeasing the consistently expanding need of developing populace. The requirement for upgraded disease resistance, feed proficiency, and growth development of cultured organisms are significant for different divisions of this industry. Probiotics could be presented in commercial aquaculture by consolidating them into formulated fish diets or in the form of bacteria biofilm to accomplish colonization in the fish gastrointestinal (GI) tract at a higher degree. These tiny organisms may be helpful in diminishing the occurrence of ailment or decreasing the seriousness of outbreaks. Establishment of defensive gut microflora is a key segment in barring potential trespassers and looking after wellbeing and maintaining health. Symbiotic bacteria assist in digestion, absorption and consumption of food by the production of various enzymes (Carnevali *et al.*, 2006). This prompts the utilization of probiotics to avert infection hazard and provide immune-stimulation in the candidate species.

Distinctive scrutinizations have demonstrated the probiotic's incorporation to enhance the growth efficiency, weight gain and efficient conversion of feed into flesh in fish (Wang *et al.*, 2008; Son *et al.*, 2009; Daniels *et al.*, 2010; Bisht *et al.*, 2012; Bhatnagar and Raparia, 2014; Bhatnagar and Lamba, 2017; Bhatnagar and Dhillon, 2019). The wellbeing status of fishes is interestingly identified with their prompt surroundings that comprise a high convergence of microorganisms. Gatesoupe (1999) stated that probiotics aid in maintaining the health and wellness of the host organism by contributing to gut microbiota. These are ought to adhere well to the intestinal tract or develop sufficiently quick so as to remain within the host gut to prevent their removed with food's movement in gastrointestinal tract. Probiotics can expand their time inside the gut in this way impacting the gastrointestinal microflora of their host by attaching to the intestinal mucosa (Vine *et al.*, 2004). The antagonistic activity against the disease-causing organisms is known to be enhanced by the colonization of probiotics to the gut of host (Coconnier *et al.*, 2003).

The permanence and colonization of the probiotic in the gut can be visualized by the means of microscopy techniques (Salinas *et al.*, 2008). Thus, using the technique of immunohistochemistry, the present study aimed to address the ability of the supplemented probiotics enhancing the growth, intestinal enzymatic activity and immune response by adhering and making a permanence colonization in the gastrointestinal tract of *Labeo calbasu* juveniles.

## MATERIALS AND METHODS

The present study had the same experimental set up as described by Bhatnagar and Dhillon (2019) with four experimental feeds (Diet C, Diet 1, Diet 2, and Diet 3) containing probiotic bacterial strains isolated from the gut of *L. calbasu*, *Aneurinibacillus aneurinilyticus*, and *Stenotrophomonas* sp. The fingerlings (initial weight 3.01-3.05 g) were fed with the respective pelleted feed twice daily (4% body weight) for 90 days. Ration was adjusted by weighing the fingerlings fortnightly using top pan balance (AFCOSET FX-1200) and water was renewed daily with dechlorinated water ( $25 \pm 1^\circ\text{C}$ ). Weight gain was measured (Garg *et al.*, 2002). The juveniles were analysed for intestinal enzymatic activity, haematological diagnosis and respiratory burst activity at the end of 90 days feeding experiment. A challenge trial was also conducted at the end where ten fingerlings from each treatment were challenged with fish pathogenic strain *Aeromonas hydrophila* (MTCC 1739) (Austin *et al.*, 1995) following standard safety measures while handling and discarding bacteria (Christian, 2007).

### Determination of intestinal enzymatic activity

Intestinal protease, amylase and cellulase activities were determined by following the standard procedures given by Walter (1984), Sawhney and Singh (2000) and Sadasivam and Manickam (1996), respectively, by processing intestine of few fingerlings from each feeding group. Specific enzymatic activities were calculated as follows:

$$\begin{aligned} & \text{Specific protease activity (mg of tyrosine mg protein}^{-1}\text{h}^{-1}) \\ &= \frac{\text{Enzyme activity (mg g}^{-1}\text{h}^{-1})}{\text{Protein mg g}^{-1}} \end{aligned}$$

$$\begin{aligned} & \text{Specific amylase activity (mg of maltose mg protein}^{-1}\text{h}^{-1}) \\ &= \frac{\text{Enzyme activity (mg g}^{-1}\text{h}^{-1})}{\text{Protein mg g}^{-1}} \\ & \text{Specific cellulase activity (mg of glucose mg protein}^{-1}\text{h}^{-1}) \\ &= \frac{\text{Enzyme activity (mg g}^{-1}\text{h}^{-1})}{\text{Protein mg g}^{-1}} \end{aligned}$$

### Haematological diagnosis

To study the changes in profile of different hematological and immunological parameters, the blood sample was collected in vacutainers. Total erythrocyte and leucocyte count, before and after the challenge was done using hemocytometer and Neubaur's counting chamber by standard protocol of **Dacie and Lewis (1971)**.

TEC = total number of cells in five small squares  $\times 10000 \text{ mm}^{-3}$

TLC = Total number of cells in four large squares  $\times 500 \text{ mm}^{-3}$

The phagocytic assay was performed according to the method of **Siwicki et al. (1994)** and **Park and Jeong (1996)**. Phagocytic ratio (PR) and phagocytic index (PI) were determined by enumerating 100 phagocytes per slide under a microscope and average of three slides was calculated.

$$\begin{aligned} & \text{Phagocytic ratio (PR; } i. e. \text{ percentage of cell with engulfed bacteria)} \\ &= \frac{\text{Number of phagocytic cells with engulfed bacteria}}{\text{Number of phagocytic cells}} \times 100 \end{aligned}$$

$$\begin{aligned} & \text{Phagocytic Index (PI; } i. e. \text{ number of engulfed bacteria per cell)} \\ &= \frac{\text{Number of engulfed bacteria}}{\text{Number of phagocytic cells}} \times 100 \end{aligned}$$

The respiratory burst activity is the production of oxygen radical production by blood phagocytes. It was measured by recording the light absorption of the sample at wavelength of 540 nm spectrophotometrically by nitroblue tetrazolium (NBT) assay (**Anderson and Siwicki, 1995**) after the challenge trial with *A. hydrophila*.

### Immunohistochemical analysis

The feeding group showing significant result were further considered for histological study of intestine. The tissue was fixed in Bouin's fixative and was further processed with different alcohol grades (**Pearse, 1968**). Thin section (5 $\mu\text{m}$ ) cutting was done with rotary microtome. The eosin-haematoxylin stained tissue was examined for histology under light microscope.

The existence of the probiotic supplemented through the fish feed was perused by the immunohistochemical analysis of intestinal cross-sections. The intestine samples of the fish with best results after the feeding trial were placed for fixation in 10% formalin. The tissue pieces were then dehydrated by immersing in different grades of alcohol to avoid contortion. Tissue was not placed in paraffin and 5 $\mu\text{m}$  cuts were made after a treatment with 3% 3-aminopropylethoxysilane (Sigma Laboratories). Tissue dewaxing was done at 60°C followed by xylol washing thrice. Soaking of the tissue was done with 10% alcohol following twice washing with 70% and a final wash with deionised water. Immunodetection was carried out following the manufacturer's instructions by Immuno Cruz Staining System (Santa Cruz Biotechnology, USA). *Bacillus* Spore Antigen Polyclonal Antibody (Invitrogen PA1-7203) at a 1:20 dilution (Affinity Bioreagents, USA) was employed as primary antibody with Grill's haematoxylin as a contrast

medium. The tissue was then observed and photographed at  $\times 400$  under Leica DM 6000 microscope fitted with Leica DFC 480C camera (Leica, Germany) using a dark contrast for better visibility of the images.

### Statistical analysis

One Way Analysis of variance (ANOVA) followed by Duncan's multiple range tests (**Duncan, 1955**) was used for analysing the significant differences among treatment groups. Statistical significance was settled at a probability value of  $p < 0.05$ . All statistics were performed using SPSS Version 18.0

## RESULTS

The fingerlings sufficiently acknowledged the trial diet and kept up normal conduct all through the experimental frame without any mortality for all dietary treatments. ANOVA followed by Duncan's Multiple Range Test revealed Diet 1 fed group consolidated with probiotic *Aneurinibacillus aneurinilyticus* with significantly high growth of fish in terms of weight gain ( $p < 0.05$ ).

### 1. Intestinal digestive enzyme activities

The intestinal enzymatic activity was observed to be significantly ( $p < 0.05$ ) high in the probiotic supplemented groups as compared to control (without probiotic incorporation). The fingerlings in Diet 1 fed group (supplemented with *A. aneurinilyticus*) manifested significantly ( $p < 0.05$ ) high specific protease ( $2.96 \pm 0.018$  mg of tyrosine liberated mg of protein<sup>-1</sup> h<sup>-1</sup>), specific amylase ( $1.79 \pm 0.009$  mg of maltose liberated mg of protein<sup>-1</sup> h<sup>-1</sup>) and specific cellulase ( $2.62 \pm 0.009$  mg of glucose liberated mg of protein<sup>-1</sup> h<sup>-1</sup>) (Table 1).

### 2. Haematological parameters

Blood samples were collected for haematological diagnosis reporting erythrocyte and total leucocyte count (TEC and TLC) of *L. calbasu* fingerlings fed with different dietary feeds (Figure 1, Table 2). Significant ( $p < 0.05$ ) increase in TEC ( $10^6$  mm<sup>-3</sup>) and TLC ( $10^3$  mm<sup>-3</sup>) was observed in the fingerlings fed with probiotic supplemented dietary feed as to the fingerlings group fed with Diet C ( $2.14 \pm 0.044$  and  $43.83 \pm 0.145$ ). Phagocytic ratios and phagocytic indices in the fingerlings fed with various selected strain of probiotics were significantly ( $p < 0.05$ ) higher than control fingerlings during the assay period. Maximum values of phagocytic ratio ( $82.38 \pm 1.097\%$ ) and phagocytic index ( $2.03 \pm 0.033$ ) were observed in Diet 1 fed group (Table 2). After challenge trial, the haematological values were slightly lower for TEC and higher for TLC than pre-challenge values depicting a possible expanded contagiousness and provocative response intervened by leucocyte, being highest for Diet 1 juveniles with significant difference among groups ( $p < 0.05$ ). The intracellular superoxide radicals produced by leucocytes in response to the pathogens was restrained by the reduction of Nitroblue tetrazolium (NBT) and was perceived to be significantly influenced by the probiotic diets. The statistical analysis resulted in significant ( $p < 0.05$ ) variation in the NBT activity (Units min<sup>-1</sup> mg<sup>-1</sup> of protein) with superlative increase to be observed in *A. aneurinilyticus* supplemented fingerlings ( $0.58 \pm 0.015$ ) (Figure 2).

### 3. Colonization and permanence of *Aneurinibacillus aneurinilyticus* strain in *L. calbasu* by immunohistochemical analysis

The fingerlings fed with Diet 1 incorporated with gram positive probiotic *A. aneurinilyticus* showed significantly high growth, intestinal enzymatic activity and immune response. The results of the histological studies displayed conventional framework of intestine with circular muscles, longitudinal muscles, serosa, villi and microvilli. To observe the presence of colonization and permanence of the incorporated probiotics through feed, the gastrointestinal tissue of the fingerlings was analysed by immunohistochemical analysis. The existence of the probiotic augmented to the juveniles was contemplated in *Bacillus* Spore Antigen Polyclonal Antibody (Invitrogen PA1-7203) labelled histological cuts in the gastrointestinal lumen and on the microvilli edges with positive marking and fluorescence delineated the ubiquity of probiotic colonies when visualized under fluorescent microscope (Figure 3).

**Table 1: Growth and the intestinal enzyme activities of *Labeo calbasu* fed on the diets containing autochthonous probiotic bacterial strain (Diet C=Control, Diet 1= *A. aneurinilyticus*, Diet 2= *Stenotrophomonas* sp., Diet 3= *A. aneurinilyticus* + *Stenotrophomonas* sp.)**

Growth parameters	Diet C (Control)	Diet 1 ( <i>A. aneurinilyticus</i> )	Diet 2 ( <i>Stenotrophomonas</i> sp.)	Diet 3 ( <i>A. aneurinilyticus</i> + <i>Stenotrophomonas</i> sp.)
Initial weight (g)	3.01 ± 0.018 <sup>A</sup>	3.05 ± 0.015 <sup>A</sup>	3.03 ± 0.017 <sup>A</sup>	3.05 ± 0.009 <sup>A</sup>
Final weight (g)	4.49 ± 0.022 <sup>D</sup>	5.42 ± 0.038 <sup>A</sup>	4.93 ± 0.023 <sup>C</sup>	5.11 ± 0.015 <sup>B</sup>
Weight gain (g)	1.48 ± 0.032 <sup>D</sup>	2.36 ± 0.023 <sup>A</sup>	1.90 ± 0.019 <sup>C</sup>	2.06 ± 0.009 <sup>B</sup>
Survival rate (%)	100	100	100	100
Specific protease activity <sup>1</sup>	2.27 ± 0.014 <sup>D</sup>	2.96 ± 0.018 <sup>A</sup>	2.51 ± 0.014 <sup>C</sup>	2.67 ± 0.014 <sup>B</sup>
Specific amylase activity <sup>2</sup>	1.60 ± 0.014 <sup>D</sup>	1.79 ± 0.009 <sup>A</sup>	1.71 ± 0.009 <sup>C</sup>	1.76 ± 0.009 <sup>B</sup>
Specific cellulase activity <sup>3</sup>	2.09 ± 0.012 <sup>D</sup>	2.62 ± 0.009 <sup>A</sup>	2.46 ± 0.012 <sup>C</sup>	2.54 ± 0.009 <sup>B</sup>

All the values are Mean ± S.E.M.

Means with different letters in the same row are significant ( $p < 0.05$ ) different.

(Data were analysed by Duncan's Multiple Range Test)

<sup>1</sup>mg of tyrosine liberated mg of protein<sup>-1</sup> h<sup>-1</sup>

<sup>2</sup>mg of maltose liberated mg of protein<sup>-1</sup> h<sup>-1</sup>

<sup>3</sup>mg of glucose liberated mg of protein<sup>-1</sup> h<sup>-1</sup>

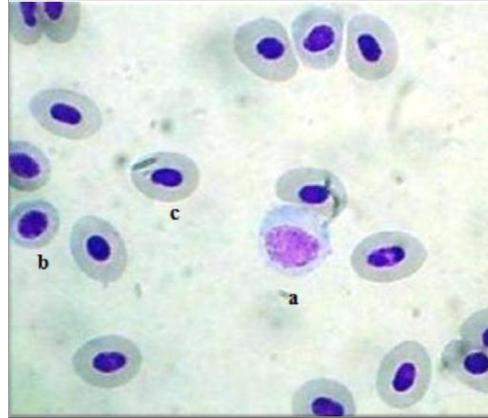


Figure 1: Lymphocyte (a), monocytes (b) and erythrocyte (c) of *Labeo calbasu* observed in different dietary treatments with probiotics ( $\times 1000$ ).

Table 2: Haematological values, phagocytic ratio and phagocytic index of *Labeo calbasu* fed on diets containing autochthonous probiotic bacterial strain (Diet C=Control, Diet 1= *A. aneurinilyticus*, Diet 2= *Stenotrophomonas* sp., Diet 3= *A. aneurinilyticus* + *Stenotrophomonas* sp.)

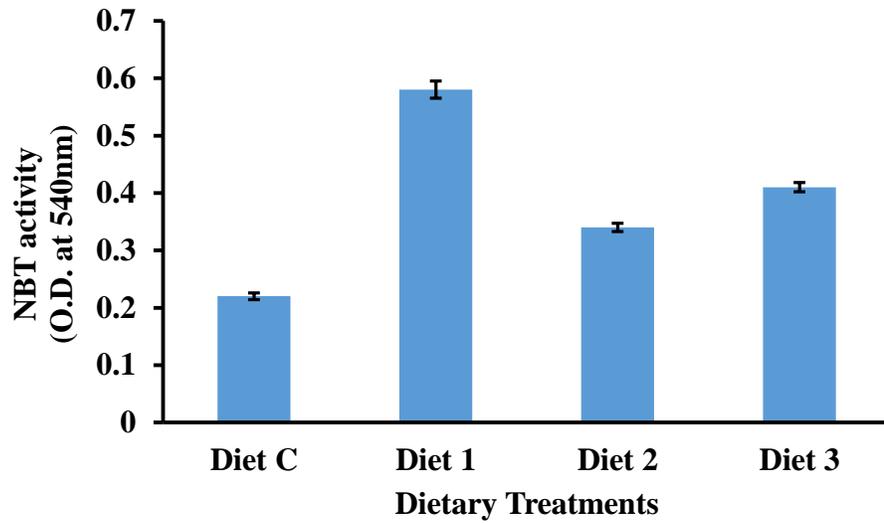
Haematological Parameters	Diet C (Control)	Diet 1 ( <i>A. aneurinilyticus</i> )	Diet 2 ( <i>Stenotrophomonas</i> sp.)	Diet 3 ( <i>A. aneurinilyticus</i> + <i>Stenotrophomonas</i> sp.)
<b>TEC* (<math>\times 10^6 \text{ mm}^{-3}</math>)</b>				
Pre-Challenge	2.14 $\pm$ 0.044 <sup>C</sup>	2.75 $\pm$ 0.017 <sup>A</sup>	2.53 $\pm$ 0.023 <sup>B</sup>	2.60 $\pm$ 0.018 <sup>B</sup>
Post Challenge	1.48 $\pm$ 0.023 <sup>C</sup>	2.31 $\pm$ 0.035 <sup>A</sup>	2.08 $\pm$ 0.233 <sup>B</sup>	2.16 $\pm$ 0.012 <sup>B</sup>
<b>TLC** (<math>\times 10^3 \text{ mm}^{-3}</math>)</b>				
Pre-Challenge	43.83 $\pm$ 0.145 <sup>D</sup>	52.63 $\pm$ 0.233 <sup>A</sup>	48.63 $\pm$ 0.318 <sup>C</sup>	50.73 $\pm$ 0.202 <sup>B</sup>
Post Challenge	47.83 $\pm$ 0.223 <sup>D</sup>	55.10 $\pm$ 0.173 <sup>A</sup>	50.73 $\pm$ 0.437 <sup>C</sup>	52.97 $\pm$ 0.120 <sup>B</sup>
<b>Phagocytic assay</b>				
Phagocytic Ratio (%)	55.96 $\pm$ 1.586 <sup>D</sup>	82.38 $\pm$ 1.097 <sup>A</sup>	60.21 $\pm$ 0.373 <sup>C</sup>	65.97 $\pm$ 0.944 <sup>B</sup>
Phagocytic Index	1.25 $\pm$ 0.041 <sup>D</sup>	2.03 $\pm$ 0.033 <sup>A</sup>	1.39 $\pm$ 0.019 <sup>C</sup>	1.60 $\pm$ 0.012 <sup>B</sup>

\*TEC- Total Erythrocyte Count

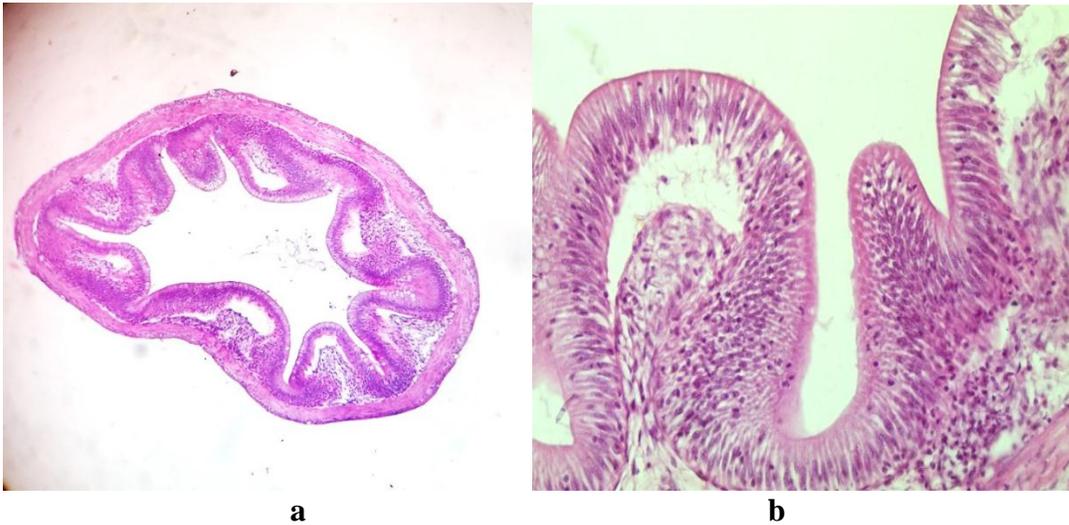
\*\*TLC- Total Leucocyte Count

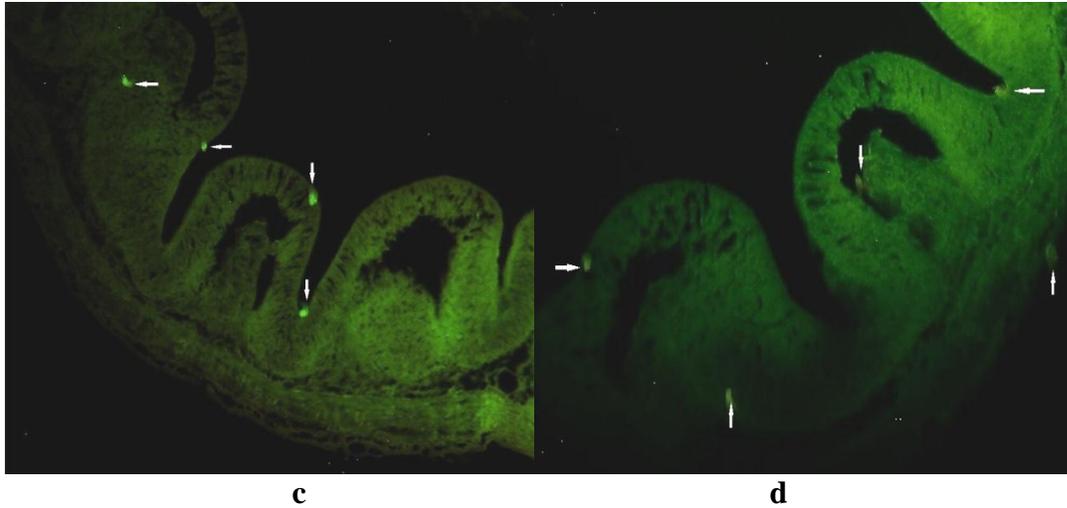
All values are Mean  $\pm$  S.E.M.

Means with different letters in the same row are significantly ( $p < 0.05$ ) different (Duncan's Multiple Range test).



**Figure 2:** Nitroblue tetrazolium (NBT) activity (O.D. at 540 nm) of *Labeo calbasu* fed on diets containing autochthonous probiotic strain in a challenge trial with *Aeromonas hydrophila* for 10 days (Diet C=Control, Diet 1= *A. aneurinilyticus*, Diet 2= *Stenotrophomonas* sp., Diet 3= *A. aneurinilyticus* + *Stenotrophomonas* sp.)





**Figure 3:** (a) and (b) Photomicrograph showing **histological** section of fish intestine fed on Diet 1 showing normal architecture ( $\times 100$  and  $\times 400$ ) (c) and (d) Location of probiotics in transverse sections of gastrointestinal tract marked with antibodies to *Aneurinibacillus aneurinilyticus*, in the villi, microvilli and in the gut lumen (\*arrow indicates the immunolabelling positive. To highlight marking, a dark filter was used in these images) ( $\times 400$ ).

## DISCUSSION

Dietary manipulations with the incorporation and supplementation of different natural ingredients have been proved to enhance growth and immunity and have become the priority for sustainable aquaculture. Probiotics have grabbed incredible consideration for its commitment as a non-traditional source in the aquaculture for giving different wellbeing and growth benefits. Various studies have reported the existence of numerous probiotic bacterial strains in the fish gut that can be incorporated to the diet for enhanced growth and development purposes (Bairagi *et al.*, 2002; Bondad-Reantaso *et al.*, 2005; Balcazar *et al.*, 2006; Kesarcodi-Watson *et al.*, 2008; Bhatnagar and Khandelwal, 2009; Daniels *et al.*, 2010; Bisht *et al.*, 2012; Bhatnagar and Raparia, 2014; Bhatnagar and Lamba, 2017; Bhatnagar and Dhillon, 2019). The present scrutinization with the principal endeavour was led for assessing the colonization and permanence of probiotic enhancing intestinal enzyme activity and immune response, in the terms of blood cell count and respiratory burst activity. Zero mortality revealed no negative impact of the isolated strains on the fingerlings and clearly demonstrated their utilization to be safe. The evaluation of the presence and activity of intestinal enzymes might be utilized as a marker of growth and development of fish, acceptability towards the feed offered and an indicator of their future survival (Cara *et al.*, 2003). It is notable that the intestinal enzymatic activity can denote the acceptance and digestive efficacy of feed offered to the fingerlings (Uberschar, 1993; Hakim *et al.*, 2006).

Probiotics have been recognized to improve the nourishment by fabricating some vitamins, fatty acids, amylase, protease and by detoxifying the conceivably detrimental constituents in the diet (Gatesoupe, 1999). Significant ( $p < 0.05$ ) high digestive enzymatic activities were reported in the Diet 1 fed fingerlings (containing *Aneurinibacillus*

*aneurinilyticus*) than the other dietary groups. High enzymatic activities may be accredited to the presence of probiotics in the feed that colonized in the gut of the fish in the present investigation. Exogenous and extracellular production of protease, amylase and cellulase enzymes by *Bacillus* has been divulged by distinctive investigations (Bairagi *et al.*, 2004; Rani *et al.*, 2004; Sudesh, 2008). The upgraded nutrient and enzyme supplementation by probiotic prompted expanded food assimilation and retention which thus persuaded better fish growth. Moreover *A. aneurinilyticus* belonging to class Bacilli is a gram-positive, endospore forming bacteria with thiamine degrading activity for enhanced utilization of the feed. Significant enzymatic activity have been reported by gastrointestinal isolated bacteria such as cellulolytic activity in rohu, tilapia, catla, mrigal, grass carp (Ghosh *et al.*, 2002; Saha *et al.*, 2006; Ray *et al.*, 2010; Jiang *et al.*, 2011); amylolytic in grass carp, rohu, murrel, bata (Das and Tripathi, 1991; Kar and Ghosh, 2008; Mondal *et al.*, 2010); proteolytic in salmon (Morita *et al.*, 1998; Askarian *et al.*, 2012); and lipolytic in atlantic cod (Askarian *et al.*, 2013). The contribution of intestinal enzymes to the digestion empowered by gut bacteria show their capacity to help in the assimilation of plant feedstuffs concluding in better growth execution.

Blood corpuscles exemplifies the wellbeing of the organism attributable to immune response. The probiotic supplemented experimental diets showed a significant effect on the erythrocyte and leucocyte count. Verschuere *et al.* (2000) defined immunostimulants as immune response activating chemicals that delineates more resistance against pathogens. Probiotics have the ability to produce such substances that alert the immune system against pathogen agents (Aguirre-Guzman *et al.*, 2012). The commenced study concedes that probiotic's inclusion significantly enhanced erythrocytes and leucocytes count as to the control group without probiotics. Later on, challenging the experiments fingerlings with pathogenic bacteria *A. hydrophila*, a decrease in erythrocytes and an increase in leucocytes were observed displaying positive immune response in the fingerlings fed with probiotics. Augmented immunity and combat to challenge trial with potent fish pathogen *A. hydrophila* were observed in all the probiotic incorporated groups while significant ( $p < 0.05$ ) high value of these immunity parameters coincided with Diet 1 fed group in which significant weight gain and enzymatic activities were also observed. Leucocytes have the ability to derive their bactericidal power from a vast range of cellular functions, including phagocytosis. It was perceived that Diet 1 fed fingerlings showed significant ( $p < 0.05$ ) high number of phagocytic cells revealing to high phagocytic ratio and phagocytic index than the other dietary groups. This may be appertained to the point that the probiotics included in the diet elicited an immune response that defended the body of host on acquaintance to the disease-causing pathogens. Similar has been observed in *C. catla* (Bhatnagar and Raparia 2014), *C. mrigala* (Bhatnagar and Lamba, 2015; 2017) depicting a possible effective response mediated by leucocyte against pathogenic bacteria, *A. hydrophila*. Phagocytes illustrate respiratory burst activity while attacking the intrusive pathogens which can be assessed by nitro blue tetrazolium (NBT) assay portraying host defence capabilities. Significantly ( $p < 0.05$ ) high value of NBT was depicted by Diet 1 fed group. Comparative patterns were seen in phagocytosis and free-radical production with the dietary supplementation of probiotic supporting the result of the current work (Kumar *et al.*, 2008; Harikrishanan *et al.*, 2010; Giri *et al.*, 2013; Bhatnagar and Lamba, 2017; Bhatnagar and Dhillon, 2019).

The colonization and persistence of the probiotic in the host actuate its adequacy. The results of the immunohistochemistry done for the intestine of Diet 1 fed juveniles affirmed the existence and placement of the probiotic supplemented with its binding with anti-*Bacillus* antibody indicating affirmative markings in the intestinal lumen and microvilli of *L. calbasu*. **Bengmark (1998)** and **Sansawat and Thirabunyanon (2009)** stated the ability of bacterial stain to form colony and tolerate colonization only with its symbiotic bacteria. The autoaggregation and co-aggregation ability exhibited by the probiotics used in the present studies justify it to initiate a strong host protective mechanism in fish gut by colonizing to the intestinal surfaces (**Bhatnagar and Dhillon, 2019**). **Makridis et al. (2001)** and **Dosta et al. (2012)** showed similar presence of the bacterial strain incorporated in the diet with immunohistochemical techniques which were in agreement with the present work. Significant results observed in Diet 1 fed group are attributable to the presence of probiotic related to Bacilli class that provides positive physiological and biological changes in the gut of the fish. The colonization and adherence owing to greater hydrophobicity, aggregation and co-aggregation parameters shown by the isolated strain *A. aneurinilyticus* (**Bhatnagar and Dhillon, 2019**). Growth performance was significantly high in the group fed on *A. aneurinilyticus* supplemented diets than those containing *Stenotrophomonas* sp., and both *A. aneurinilyticus* and *Stenotrophomonas* sp. This may be due to the competition between both the bacterial strains for colonizing and adhering in the gut of the fingerlings. Moreover, *A. aneurinilyticus* belonging to Class Bacilli is an endospore forming gram positive aerobic rod-shaped bacteria, which are resistant to chemical and physical agents while *Stenotrophomonas* sp. belonging to family Gammaproteobacteria do not produce any endospore which makes it less resistant and thus conferring low growth and immunity to the fish when incorporated as dietary supplement. This clearly reveals that the autochthonous probiotic *A. aneurinilyticus* proved to enhance weight, intestinal enzymatic activity and immune response against the pathogenic bacteria in terms of respiratory burst activity, when incorporated in the diet providing a gnotobiotic approach towards sustainable aquaculture.

## CONCLUSION

Aquaculture is assuming utmost importance in satisfying the developing interest for the sustenance of consistently expanding human populace by providing opportunities for the production of wide varieties of aquatic foods including fish and shellfish. The presented gnotobiotic approach of utilizing autochthonous probiotic bacterial strain for proving immunity and better growth performance is a suitable appeal for the administration and implementation of the assets beneficially. The results of the present work may further provide assistance in the prevention of disease risk and growth enhancement which further may lead to the elimination of ineffective chemical substances, immoderate and extravagant immunostimulants.

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