Growth promotion and immunomodulation with autochthonous probiotic bacteria in *Labeo calbasu* (Hamilton, 1822)

Dr Oshin Dhillon
Assistant Professor, Department of Zoology
Akal University, Bathinda
Punjab (India)
https://www.researchgate.net/profile/Oshin-Dhillon
https://scholar.google.com/citations?user=d7qizcoAAAAJ&hl=en

Introduction

- Intensification of Aquaculture satisfies the growing demand of animal protein as wild capture fishery is decreasing
- Nutrition and disease free environment are important aspects for introducing a new species for the diversification
- Dietary protein determines fish growth rate, yield and survival
- Optimum protein requirement essential
- Cost effective supplementary feed using plant protein source instead of fish meal
- Essential amino acids required for significant growth

- Methionine and Lysine as supplement to foodstuffs to increase the biological value of low value plant proteins (Murthy and Varghese, 1997)
- Deficiency causes retardation in growth
- Emergence of diseases with commercialization of aquaculture
- Antibiotics reduced diseases but led to increased antibiotic resistance
- Commercially available immunostimulant and probiotics are often less effective

- **Use of autochthonous probiotic bacterial strain is the best approach**
- **Improve growth, immunity and feed utilization efficiency**

Labeo calbasu
State fish of Haryana

Lower Risk/Near Threatened

CAMP, 1998. Report of the workshop on "Conservation Assessment and Management Plan (CAMP) for Freshwater Fishes of India." Zoo outreach organization and NBFGR, Lucknow. 22-26 September 1997, pp: 156.

Objective

To assess the effect of probiotic supplementation and its comparison with other feed supplements on *Labeo calbasu* fingerlings

Methodology

- Selection, procurement and acclimation of fish:

  L. calbasu fingerlings (weighing 1.92±0.08 g, 5.43±0.2 cm in length) were procured from **Fish Seed Production Centre, Pawarkhera, Hoshangabad (Madhya Pradesh)** and acclimated in the **Aquaculture Research Unit** of Department of Zoology, Kurukshetra University, Kurukshetra (29°58’ N, 76°51’ E).

- Experimental/feeding trial

Four dietary treatments

- **T1** – **Soybean**
- **T2** – **Duckweed**
- **T3** – **Duckweed, Lysine, Methionine**
- **T4** – **Duckweed, *Aneurinibacillus aneurinilyticus***

Experimental setup

Growth parameters: (Garg *et al*. 2002)

PARAMETERS	FORMULA
Live weight gain	$W_2 - W_1$
Growth per cent gain in body weight	$\frac{W_2 - W_1}{W_1} \times 100$
Growth per day in percentage body weight	$\frac{2(W_2 - W_1)}{t(W_1 + W_2)} \times 100$
Specific Growth Rate (SGR)	$\frac{\ln W_2 - \ln W_1}{t} \times 100$
Feed Conversion Ratio (FCR)	$\frac{\text{Feed offered (Dry weight)(g)}}{\text{Body weight gain (Wet weight)(g)}}$
Gross Conversion Efficiency (GCE)	$\frac{\text{Body weight gain (Wet weight)(g)}}{\text{Feed offered (Dry weight)(g)}}$
Protein Efficiency Ratio (PER)	$\frac{\text{Wet weight gain (g)}}{\text{Crude protein fed (\%)}}$
Apparent protein Digestibility (APD)	$100 - \frac{\text{\% marker in diet}}{\text{\% marker in feces}} \times \frac{\text{\% nutrient in feces}}{\text{\% nutrient in diet}}$

Where, W_1 = **Initial Weight (g),** W_2 = **Final Weight (g)**
t = **Duration of experiment (Number of days)**

Determination of intestinal enzymatic activity

Protease	Walter (1984)
Amylase	Sawhney and Singh (2000)
Cellulase	Sadasivam (1996)

Biochemical Analysis (AOAC, 1995)

Moisture	**Oven dry**
Crude protein	**Kjeldahl method**
Crude lipid	**Soxhlet**
Ash	**Muffle furnance (AICIL furnance)**
Nitrogen free extract	**100 - (% of crude protein + % crude lipid + % moisture + % ash)**
Gross energy	**0.2364 × protein (%) + 0.3954 × fat (%) + 0.1715 × carbohydrate (%)**
Phosphorus	**Spectrophotometer**

Hematological diagnosis: Hemocytometer using a Neubaur's counting chamber (Dacie and Lewis,1971)

Challenge Trial: Challenged with pathogenic strain ***Aeromonas hydrophila*** (Ellis, 1988)

Immunohistochemistry Analysis

(Dosta *et al*. 2012)

Cross-sections of the intestinal tissue were removed

The samples were fixed in 10% formaldehyde

Fixed treated with 3% 3-aminopropylethoxysilane and 5µm cuts made

Tissue sections dewaxed at 60°C for 10 minutes, immediately three xylol washing performed for 5 minutes each

Tissue sections soaked in 10% alcohol and washed twice with 70% alcohol, and a final wash with distilled water for 5 minutes

For immuno-detection, an Immuno Cruz Staining System used

***Bacillus* Spore Antigen Polyclonal Antibody (Invitrogen PA1-7203) at a 1:20 dilution used, and Grill's haematoxylin applied for 5 seconds**

Standard Safety Measures (Christian, 2007)

Statistical Analysis

- All assays were performed in triplicate and data were represented as **Mean ± S. E. of mean** for three sets of each experimental sub-groups
- Significant differences among treatment groups have been tested using **ANOVA** followed by **Duncan's Multiple Range Test** for the experiments at a probability value of **$p<0.05$**

Growth performance of *Labeo calbasu* fed on the diets

Table 1: Ingredient and proximate composition (g Kg^{-1}) of experimental diets

Parameters	T1 40% soybean (Control)	T2 40% duckweed	T3 40% duckweed + lysine + methionine	T4 40% duckweed + *Aneurinibacillus aneurinilyticus*
Ingredient composition (g Kg^{-1})				
Ground nut oil cake	650	650	650	650
Rice bran	32	32	32	32
Wheat flour	32	32	32	32
Processed soybean*	266	-	-	-
Duckweed	-	266	266	266
Lysine	-	-	4	-
Methionine	-	-	7	-
***Aneurinibacillus aneurinilyticus* (CFU ml^{-1})**	-	-	-	2000
Mineral mixture**	10	10	10	10
Chromic oxide (Cr_2O_3)	10	10	10	10
Proximate composition (% dry weight basis)				
Moisture (%)	4.56 ± 0.035^{B}	2.94 ± 0.035^{E}	3.48 ± 0.046^{A}	3.03 ± 0.118^{A}
Crude Protein (%)	39.71 ± 0.246^{C}	40.81 ± 0.365^{A}	40.95 ± 0.945^{A}	40.65 ± 0.559^{A}
Crude Fat (%)	9.26 ± 0.061^{AB}	9.13 ± 0.043^{A}	9.30 ± 0.061^{A}	9.11 ± 0.050^{A}
Crude Fiber (%)	6.29 ± 0.018^{C}	6.40 ± 0.032^{D}	6.52 ± 0.033^{A}	6.80 ± 0.109^{A}
Total Ash (%)	7.50 ± 0.187^{BC}	7.19 ± 0.023^{B}	7.38 ± 0.243^{A}	7.46 ± 0.038^{AB}
Nitrogen free extract (%)	32.68 ± 0.498^{C}	33.52 ± 0.400^{AB}	32.37 ± 0.922^{A}	33.95 ± 0.477^{A}
Gross energy (kJ g^{-1})	18.65 ± 0.019^{BC}	19.01 ± 0.018^{A}	18.91 ± 0.084^{A}	19.03 ± 0.024^{A}
Feed Phosphorus (%)	1.41 ± 0.021^{B}	1.40 ± 0.019^{B}	1.46 ± 0.019^{A}	1.44 ± 0.019^{A}

*Soybean was hydrothermically processed in an autoclave at 121°C (15 lbs for 15 minutes) to eliminate anti-nutritional factors (Garg *et al*., 2002).

**Each kg has nutritional value: copper 312 mg, cobalt 35 mg, magnesium 2.114g, iron 979 mg, zinc 2 mg, iodine 15 mg, DL-methionine 1.920 g, L-lysine monohydrochloride 4.4 g, calcium 30%, phosphorous 8.25%.

All values are Mean ± S. E. of mean.

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

Table 2: Growth performance and the intestinal enzyme activities of *Labeo calbasu* fed on diets

Growth parameters	T1 40% soybean (Control)	T2 40% duckweed	T3 40% duckweed + lysine + methionine	T4 40% duckweed + *Aneurinibacillus aneurinilyticus*
Live weight gain (g)	1.18 ± 0.039^{d}	1.37 ± 0.018^{C}	2.30 ± 0.064^{B}	2.36 ± 0.023^{A}
Survival rate (%)	100	100	100	100
Growth (%) gain in BW	64.29 ± 1.505^{C}	66.31 ± 2.134^{C}	76.18 ± 2.414^{AB}	77.40 ± 0.420^{A}
Growth/day (%) in BW	0.55 ± 0.004^{A}	0.56 ± 0.010^{A}	0.61 ± 0.014^{A}	0.62 ± 0.002^{A}
Specific Growth Rate (SGR) (% BW d^{-1})	2.4 ± 0.004^{A}	2.5 ± 0.006^{A}	2.7 ± 0.007^{A}	2.8 ± 0.002^{A}
Feed Conversion Ratio (FCR)	1.36 ± 0.109^{B}	1.49 ± 0.104^{B}	1.92 ± 0.069^{C}	1.86 ± 0.005^{C}
Gross Conversion Efficiency (GCE)	0.75 ± 0.056^{A}	0.68 ± 0.048^{A}	0.52 ± 0.018^{A}	0.54 ± 0.001^{A}
Protein Efficiency Ratio (PER)	1.61 ± 0.038^{A}	1.66 ± 0.053^{A}	1.90 ± 0.060^{A}	1.94 ± 0.010^{A}
Apparent Protein Digestibility (APD) (%)	79.53 ± 0.145^{A}	82.23 ± 0.348^{A}	85.73 ± 0.067^{A}	86.33 ± 0.067^{A}
Specific protease activity[1]	2.42 ± 0.015^{A}	2.79 ± 0.021^{A}	2.84 ± 0.015^{A}	2.96 ± 0.018^{A}
Specific amylase activity[2]	1.37 ± 0.024^{A}	1.57 ± 0.007^{A}	1.75 ± 0.013^{A}	1.79 ± 0.009^{A}
Specific cellulase activity[3]	1.86 ± 0.031^{A}	2.05 ± 0.024^{A}	2.57 ± 0.009^{A}	2.62 ± 0.009^{A}

All the values are Mean ± S. E. of mean. Means with different letters in the same row are significantly ($p<0.05$) different (Duncan's Multiple Range Test).

[1]mg of tyrosine liberated mg of protein^{-1} h^{-1}

[2] mg of maltose liberated mg of protein^{-1} h^{-1}

[3] mg of glucose liberated mg of protein^{-1} h^{-1}

Table 3: Proximate carcass composition of *Labeo calbasu* fed on different diets

Proximate composition	T1 40% soybean (Control)	T2 40% duckweed	T3 40% duckweed + lysine + methionine	T4 40% duckweed + *Aneurinibacillus aneurinilyticus*
Moisture (%)	63.68 ± 0.205^{B}	62.85 ± 0.392^{B}	65.14 ± 0.989^{AB}	63.23 ± 1.355^{B}
Crude Protein (%)	14.10 ± 0.208^{B}	14.82 ± 0.110^{B}	14.58 ± 0.290^{B}	15.16 ± 0.293^{A}
Crude Fat (%)	7.27 ± 0.273^{A}	6.90 ± 0.153^{A}	10.25 ± 0.144^{A}	7.40 ± 0.208^{C}
Total Ash (%)	2.86 ± 0.183^{B}	3.73 ± 0.193^{AB}	2.20 ± 0.147^{C}	2.44 ± 0.301^{B}
Nitrogen free extract (%)	11.95 ± 0.159^{A}	11.54 ± 0.092^{C}	7.83 ± 0.844^{B}	11.77 ± 1.809^{A}
Gross energy (kJ g^{-1})	8.26 ± 0.059^{A}	8.26 ± 0.043^{A}	8.85 ± 0.218^{A}	8.53 ± 0.252^{A}
Phosphorus (%)	0.64 ± 0.015^{B}	0.65 ± 0.012^{A}	0.72 ± 0.033^{A}	0.55 ± 0.009^{B}

All values are Mean ± S. E. of mean.
Means with different letters in the same row are significantly ($p<0.05$) different (Duncan's Multiple Range test)

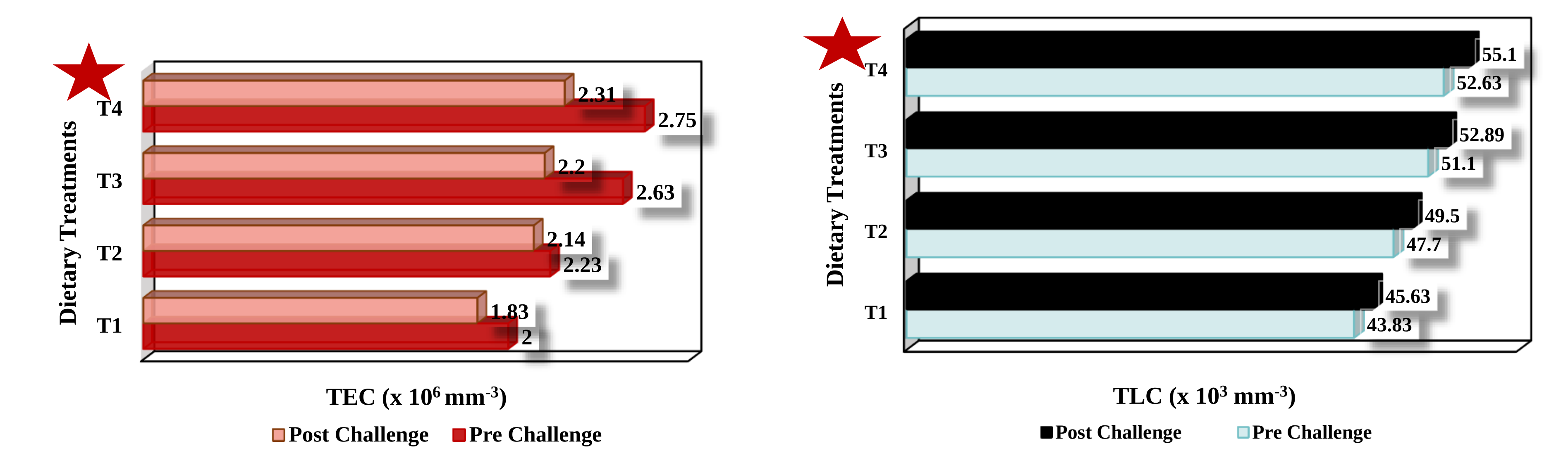

Fig. 1: Total erythrocyte count (TEC) and Total leucocyte count (TLC) of *Labeo calbasu* (Mean ± S. E. of mean) in different dietary treatments

Immunohistochemistry

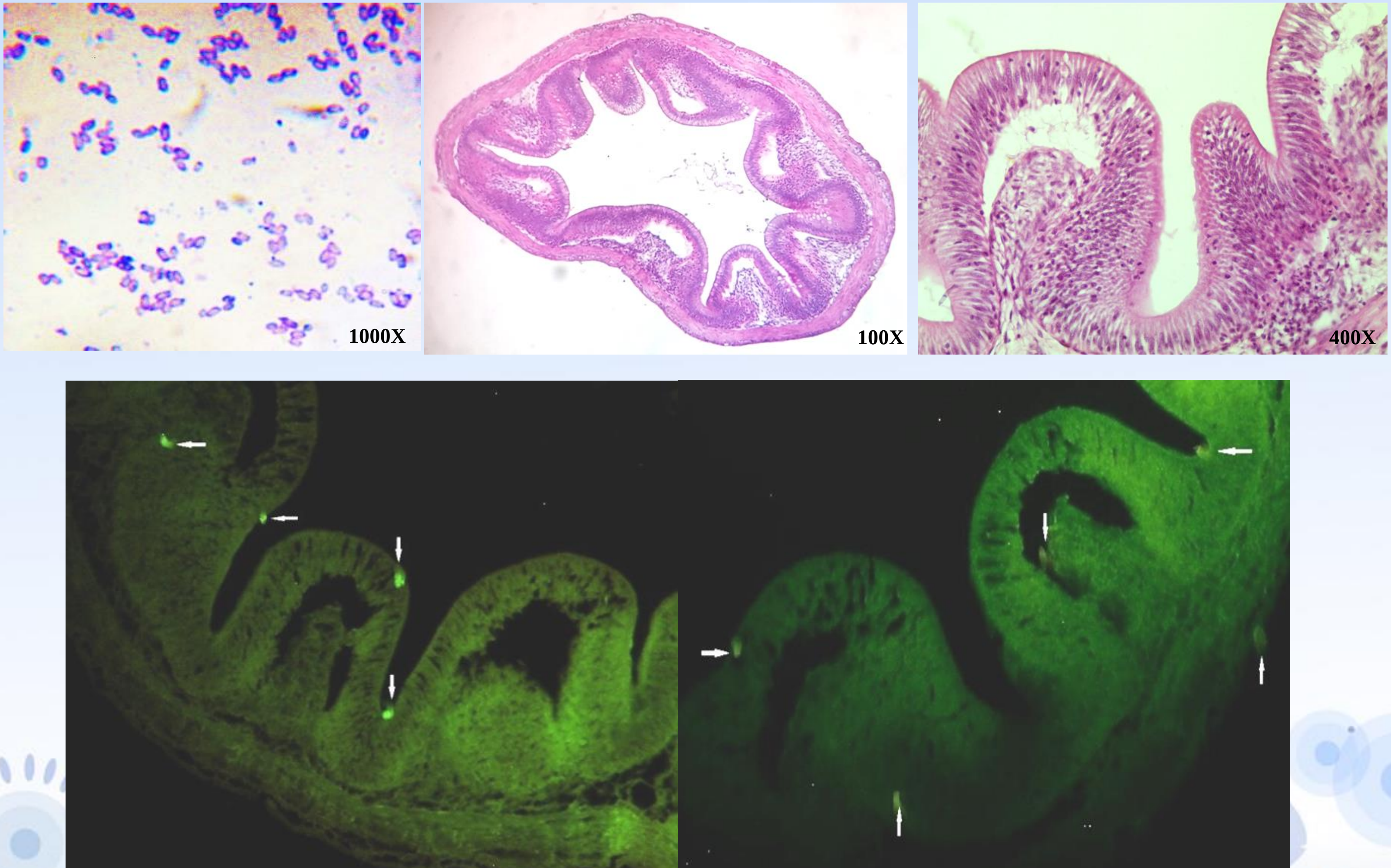

Fig. 2: Location of probiotics in transverse sections of gastrointestinal tract marked with antibodies to *Aneurinibacillus aneurinilyticus*, in the microvilli and in the gut lumen (*arrow indicates the immunolabelling positive. To highlight marking, a dark filter was used in these images) (400X).

Salient Findings

- Duckweed **eco-friendly**, easily available at **farmers' doorstep** making it **pocket-friendly protein-rich source**.
- The supplementation of **lysine and methionine** for well-balanced diet.
- ***Aneurinibacillus aneurinilyticus*** exhibiting **potential probiotic properties e**nhanced **growth performance, nutrient retention, digestibility**, **immune response.**
- Survival after the challenge trial with pathogenic bacteria *A. hydrophila* indicated its **resistance against the disease risk**. **Presence and colonization of bacteria** observed in the fish intestine.
- The basis for additional exploration for elucidating the **formulation of fish feed** for **economic and sustainable aquaculture**.

Significance

The fish feed utilizing autochthonous probiotic bacterial strain, ***A. aneurinilyticus*** in **40% protein** containing **duckweed** based diet may

- Serve as **ideal diet for *Labeo calbasu*** for **optimum growth** without **deterioration of water**
- Enhances the **immune response** and the use of natural product may prevent disease risk and eliminate the use of **ineffective, immoderate and extravagant antibiotics and chemotherapeutics**
- May aid in the **conservation** and **protection** of the fish species in addition to providing **food security to the nation**
- The formulation of appropriate feed using **cost-effective** and **eco-friendly** **plant protein source** at an **ideal protein level** with the supplementation of **probiotic** will be useful in inciting **productive and sustainable aquaculture**.

Acknowledgements

- University Grants Commission (MANF)
- The Chairman, Department of Zoology, Kurukshetra University
- Administrative Authorities, Kurukshetra University


Thank
you