Chapter 3

Study of growth and nutrient status of C. batrachus through the application of newly isolated probiotic-supplemented feed

3.1 Introduction

Aquaculture is a socio-economic activity, especially for rural communities, contributing to livelihoods, food security, income generation and employment. Utmost all natural occurring living resources in both marine and freshwater environments are considered overexploited, aquaculture has been playing a pivotal role to meet the future fish demand, which will continue to increase year after year due to population growth, protein demand and incrementing urbanization (Msangi and Batka 2015). In fact, to date aquaculture is the fastest growing food-producing industry worldwide (Tacon and Metian 2015).

Today, aquaculture is intending to produce quality fish stock to cope up with the growing demand. Feed digestibility in aquaculture sector highly affects fish growth and production cost due to increasing FCR, and can be improved by increasing the activity of digestive enzymes through the use of probiotic-supplemented feed (El-Haroun et al. 2006). Probiotics improve the quality of intestinal microflora that help the host to utilize variety of feed resources and many normally indigestible components become more easily assimilated (Abdelhamid et al. 2009; Liu et al. 2013; Wang et al. 2017).

Recently, the prevention and control of aquatic diseases have focused on the use of probiotics instead of chemotherapeutic agents and antibiotics. It confers protection against pathogens by competitive exclusion of adhesion sites or by production of antimicrobial substances. In addition, probiotics modulate physiological and immunological responses in fish (Balcazar et al. 2006). *Lactobacillus plantarum* JCM 1149 displayed immunostimulatory response against pathogenic *Aeromonas hydrophila* NJ-1 infection in hybrid tilapia (Ren et al. 2013).

Amino acids, the basic building blocks of proteins are essential precursors of a variety of biomolecules that participate in cell signaling, nucleotide biosynthesis, gene expression, heme production, hormonal regulation, nutrient transport, protein phosphorylation and stimulation of immune responses (Mohanty et al. 2014). Paul et al. (2016) obtained significantly higher amount of essential amino acids from *C. batrachus*. Haematological evaluations are also inevitable to assess the health status of fish and monitoring stress responses. It provides an accurate indication regarding changes that occur in the organism as a result of injuries to organs or tissues related to infectious diseases (Adeyemo 2007). Nwanna and Tope-Jegede (2016) reported that probiotic *Lactobacillus plantarum* administered to *C. gariepinus* had positive impact on blood profile, carcass protein and mineral composition of the catfish. Feed-probiotic *Lactobacillus acidophilus* administered to juvenile *C. gariepinus* increased the specific growth rate, relative growth rate, protein efficiency ratio, feed conversion ratio, hematological parameter and survival rate (Al-Dohail et al. 2009).

Hence, the present study is the first of its kind aimed to investigate the possible cellular modulation by dietary administration of the three bacterial isolates in Asian catfish *C. batrachus* to determine the growth performance, nutritional quality, hematological parameter and amino acid profile of the species.

3.2 Materials and Methods

3.2.1 Experimental set up

C. batrachus fingerlings were obtained from a farm in Eastern India's largest IMC spawn cultivation region, in the outskirts of Bankura district (23°06′ N, 87°15′ E), West Bengal, India. After a 15-day laboratory acclimatization (temperature 25-28 ºC; pH 7.0-7.5), the test organism was released into the 1600 l capacity continuous-flow experimental rectangular cemented tank (195 × 105 × 90 cm^3; 5 cm bottom mud). Each tank (Fig. 3.1) was stocked with 15 fish. The work was done in triplicates. Studies were conducted for 120 days during June-September' 2016. Water quality (such as temperature, pH, dissolve oxygen, total alkalinity, total ammonium, hardness) was monitored at weekly intervals (Barman et al. 2017). Fish were fed twice daily at 8 a.m. and 8 p.m. at 5% of their body weight in two equal installments (Bandyopadhyay and Das Mohapatra 2009). The length and body weight of the fingerlings were recorded in every month with measuring scale and electronic balance (Adair Dutt Instrument Private Ltd.).

3.2.2 Probiotic strains

The three bacterial isolates *Lysinibacillus sphaericus* PKA17 (GenBank Accession No. KX580190.1), *Bacillus cereus* PKA18 (GenBank Accession No. KX826079.1) and *Bacillus thuringiensis* PKA19 (GenBank Accession No. MF139049.1) were used as probiotic in the study.

Fig. 3.1: Design of the cemented culture tank: (A) Rearing tank (195 × 105 × 90 cm^3); (B) Tank floor and walls, provided with cow dung; (C) Bottom mud of 5 cm; (D) Rearing fish.

3.2.3 Formulation of experimental feed

The feed was prepared with ingredients including hydrolyzed fish meal (40%), ground nut cake (10%), cotton seed meal (5%), soybean meal (15%), mustard oil cake (10%), and wheat flour (10%) containing 40% protein for treating all experimental fish. The dough prepared by adding required amount of double distilled water to these ingredients was thoroughly sterilized (autoclaved at 121 ºC for 15 mins) and incorporated with a commercial vitamin-mineral mix (Karnataka Antibiotics & Pharmaceuticals Ltd., Bangalore, India) which contained 5,000,000 IU Vit. A; 2.0 g Vit. B2; 6.0 mg Vit. B12; 1,000,000 IU Vit. D3; 4.0 g calcium pantothenate; 800.0 g calcium; 150.0 g phosphorus; 27.5 g manganese; 1.0 g

iodine; 7.5 g iron; 15.0 g zinc; and 2.0 g copper at 2% (v/w). The feeds were initially sun-dried and then dried in an oven at 60 ºC for 12 h with 5-7% moisture content. It was then stored in air-tight sterile containers at room temperature for further use.

Bacterial isolates were centrifuged at 5000 g at 4 ºC for 10 min, washed thoroughly with sterile 1.0% NaCl solution and resuspended in sterile saline water. They were then added individually with the formulated feed (*L. sphaericus* PKA17- T1 feed; *B. cereus* PKA18- T2 feed; *B. thuringiensis* PKA19- T3 feed) at 2×10^5 cfu/100 g concentration daily (Bandyopadhyay and Das Mohapatra 2009) with the help of binder (Trubind; Biostadt India Limited, Mumbai, India). The control feed was not supplemented with any isolated strain. The final preparation was then shade dried for 2 h before application to the tank.

3.2.4 Morphometric measurement of the fingerlings

Dietary performance index like length gain (LG), live weight gain (LWG), average daily growth (ADG), specific growth rate (SGR), feed conversion ratio (FCR), protein intake (PI), protein efficiency ratio (PER), percentage weight gain (PWG) and survival rate (SR) were precisely calculated (Bandyopadhyay and Das Mohapatra 2009).

Length Gain:

$$\text{LG} = \text{Final length} - \text{Initial length}$$

Live Weight Gain:

$$\text{LWG (g)} = \text{Final weight} - \text{Initial weight}$$

Average Daily Growth:

$$\text{ADG (g)} = \frac{\text{Live weight gain}}{\text{Time (Days)}}$$

Specific Growth Rate:

$$\text{SGR (\%)} = \frac{\text{lnW2} - \text{lnW1}}{\text{Time (Days)}} \times 100$$

Where,

W1= Initial weight

W2= Final weight

Feed Conversion Ratio:

$$\text{FCR} = \frac{\text{Feed intake}}{\text{Fish weight gain}}$$

Where,

$$\text{Fish weight gain (g)} = (\text{Final weight} + \text{weight of the dead fish}) - \text{Initial weight}$$

Protein Efficiency Ratio:

$$\text{PER} = \frac{\text{Mean weight gain}}{\text{Protein intake}}$$

Protein Intake:

$$\text{PI (g)} = \text{Feed intake} \times \%\ \text{of Protein in diet}$$

Percentage Weight Gain:

$$\text{PWG (\%)} = \frac{\text{Mean weight gain}}{\text{Mean initial weight}} \times 100$$

Survival Rate:

$$\text{SR (\%)} = \frac{\text{Initial number} - \text{Final number}}{\text{Initial number}} \times 100$$

3.2.5 Proximate analysis of fish carcass

A good number of randomly selected fish of each group were sacrificed at the end of the feeding trial for nutritional assessment. The proximate composition of the fish carcass was thoroughly determined. The crude protein content was estimated by micro-Kjeldahl method (Bandyopadhyay and Das Mohapatra 2009). Carbohydrate content was measured following Dubois et al. (1956). Fat content was determined by FSSAI lab manual. Moisture and ash contents were also determined in muffle furnace by following FSSAI lab manual.

3.2.5.1 Determination of crude protein

The Kjeldahl method was performed to estimate the crude protein (Bandyopadhyay and Das Mohapatra 2009). 25-40 ml concentrated sulfuric acid (H_2SO_4), 15 g of potassium sulphate and 0.5 g of catalyst (copper sulphate) was added to the 1 g of sample. It was then heated gently to prevent foam formation and to carbonize the mass. The light green-blue colour eventually developed. It was allowed to cool. After cooling, 200 ml of water and 25 ml of sodium thiosulphate solution (80 g/l) was added to the hydrolysate to dissolve the sulfates before neutralization and titration. Methyl red indicator (5 drops) was added to the final preparation and titrated with 0.1 N NaOH. The content of protein was calculated through the formula:

Crude protein (g) = The amount of total nitrogen in the sample (N) × 6.25.

3.2.5.2 Determination of carbohydrate

The carbohydrate content was estimated according to Dubois et al. (1956) with slight modification. 5 ml of 1(N) NaOH was added to 10 mg of the grinded fish sample. It was then homogenized and centrifuged at 2500 g for 15 min. 0.5 ml of the solution was taken into a test tube made the volume to 1 ml with de-ionized water. 5 ml anthrone in sulfuric

acid was added to it and shaken vigorously and incubated for 15 min. After incubation, the test tubes were boiled in water bath for 15 min and cooled to normal temperature. It was then measured spectrophotometrically.

3.2.5.3 Determination of fat

Few drops of NH_4OH was added to the 3 g of homogenized sample and warmed. 10 ml of concentrated HCl was added to it and boiled for 30 min. It was then cooled and filtered. The filter paper was first washed with hot water, dried, rolled and placed in an extraction thimble. The fat was extracted in a soxhlet apparatus using ethyl ether and transferred to another flask. The flask was then kept in an air oven at 100 °C for 30 min to remove remaining residual solvent. The flask was then cooled in a desiccator. The residue was weighted and the total fat was calculated.

3.2.5.4 Determination of iron

The concentration of iron was estimated according to Adeyeye et al. (1996). The fish samples were defrosted for 2 h, dried and gradually ashed in the muffle furnace at 450±25 °C. The crucible was then cooled to room temperature in desiccators. The obtained white ash was then dissolved in 20% (v/v) nitric acid solution. The solution was carefully filtered and the filtrates were dissolved with de-ionized water. The concentration of iron was measured by atomic absorption spectrophotometer.

3.2.5.5 Determination of moisture

Moisture contents were estimated by following FSSAI lab manual. Fish muscles were subsequently grinded and homogenized. It was then transferred to an airtight container to prevent the loss of moisture. 5 g of sample was weighted in a moisture dish keeping slip on cover. It was then dried thoroughly in an air-oven at 100 °C for 5 h. The sample was cooled

in a desiccator by placing the lid on the dish. The weight the dish was measured. It was then dried for another half an hour, cooled in the desiccator and re-weighted.

$$\text{Moisture} = \frac{M_1 \times 100}{M_2}$$

Where,

M_1= Loss (g) in the mass of sample

M_2= Mass (g) of the sample taken for test

3.2.5.6 Determination of ash

The content of ash was also determined through FSSAI lab manual. The dried residue obtained after determination of moisture content was taken (2 g) in a platinum dish. The organic matter was then burned and transferred to a muffle furnace (550 °C) for few hours to obtain grey ash. It was then allowed to cool in a desiccator. The total ash was measured.

3.2.5.7 Determination of amino acid

The amino acid composition was determined following the protocol of Mohanty et al. (2014). The muscle protein was dissolved with 6(N) HCl at 110 °C for 24h under anaerobic condition. The sample was then neutralized with 6(N) NaOH; subsequently derivatized and analyzed through HPLC. The amino acids were identified with respect to the retention times and peak variation. For the analysis of tryptophan, the muscle protein was crushed and digested with 5% (w/v) NaOH for 24 h. 6(N) HCl was then added to neutralize it to pH 7.0. It was then measured spectrophotometrically.

3.2.5.8 Estimation of vitamins

The fat soluble vitamins were subsequently identified and quantified following the protocol of Paul et al. (2016). Fish tissue was first homogenized with anhydrous sodium sulfate. The

antioxidant butylated hydroxyanisole was then used followed by chloroform: methanol (2:1) solution to extract the oil. 25 ml of alcohol and 1.5 ml of 150% potassium hydroxide (KOH) was then added to the oil and reflux in water bath using a condenser for 30 min. After cooling, the contents were transferred into a separation funnel and allowed to separate. The aqueous layer was extracted and the solvent layer was thoroughly washed to exclude alkali. The non-saponifiable matter was processed, filtered and refrigerated. The fat soluble vitamins (A, D, E and K) were assayed with respect to the retention times and peak variation in Agilent 1260 Infinity High Performances Liquid Chromatography.

3.2.6 Hematological determination

Blood samples were collected from the caudal peduncle of fish from each tank with the help of a 2 ml plastic syringe. Ethylene diamine tetra acetate (EDTA) was used as anticoagulant. The hematological parameters analyzed include triglyceride, cholesterol, calcium, phosphorous and uric acid. The triglyceride level and calcium content were enumerated using enzymatic colorimetric (Sugiura et al. 1977) and Arsenazo III method respectively (Kavya et al. 2016). Photometry and phosphomolybdate methods were employed to determine the level of serum phosphorous (Dryer et al. 1957).

3.2.7 Statistical analysis of the results obtained

Data was statistically analyzed by one-way analysis of variance (ANOVA) taking replicate tank mean values using Microsoft Office Excel software. Significant differences ($\alpha = 0.05$) between the means of different groups in replicate were tested by Duncan Multiple Range Test (DMRT) through SPSS program version 19. The regression line, scatter diagram and mesh plot of different growth parameter of *C. batrachus* with control, T1, T2 and T3-supplemented feed were plotted by using MATLAB R2012a software.

The mesh (X,Y,Z) was drawn (spacing value h = 0.065) as a surface-plot graphics object with colour determined by Z. If X and Y were vectors, length(X) = n and length (Y) = m, where [m,n] = size(Z). In this case, ($X(j)$, $Y(i)$, $Z(i,j)$) were the intersections of the wireframe grid lines; X and Y correspond to the columns and rows of Z, respectively. If X and Y were matrices, ($X(i,j)$, $Y(i,j)$, $Z(i,j)$) were the intersections of the wireframe grid lines. The values in X, Y or Z were numeric or categorical values. The height Z was a single-valued function defined over a rectangular grid and color was proportional to the surface height. The control was represented in X axis, treatment in Y axis and f(x,y) in Z axis.

3.2.8 Dose level determination

The putative probiont *L. sphaericus* PKA17 was incorporated with basal feed to *C. batrachus* juveniles at different dose level: control (without probiotic), 2×10^4 (C1), 2×10^5 (C2) and 2×10^6 (C3) probiotic cells per 100 g feed for 60 days to determine the maximum efficient dose level. The same process of dose-level determination was also carried with the *B. cereus* PKA18. The morphometric measurements of the fish juveniles were examined at regular interval.

3.3 Result and Discussion

3.3.1 Morphometric measurement of the fingerlings

In the present study, the three aerobic Gram positive bacterial strains; isolated from the intestine of adult *C. batrachus* were evaluated as putative probiotics through a 120 days feeding trial. The result of growth performance of *C. batrachus* in relation to various feed is presented in Table 3.1. Although the initial body weight of *C. batrachus* was similar among all experimental groups, catfish fingerlings fed on probiotic-supplemented feed had faster

growth rate than those fed on control feed. Fish fed T2 displayed significantly (P≤0.05) higher LWG of 43.27±0.06 g followed by T1, T3 and control. The weight (g) of *C. batrachus* in relation to various feeds at different time interval is presented in Fig. 3.2. The T1 fed fish showed significantly (P≤0.05) lower FCR of 1.23±0.01 whereas the highest (2.25±0.03) was observed in control fed fish. FCR of all experimental groups differs significantly. The decrease in FCR also indicated that *C. batrachus* utilized dietary nutrients more efficiently with probiotic-supplemented feed which can subsequently reduce the production cost. Afrilasari et al. (2017) also have obtained improved FCR in *Clarias sp.* by supplementing the feed with *Bacillus megaterium* PTB 1.4. Application of viable probiotic *Lactobacillus acidophilus* improved the SGR, RGR, PER, FCR and survivability of *C. gariepinus* fingerlings (Ige 2013).

The probiotic supplemented fish also have resulted significantly (P≤0.05) higher SGR over the control group. Highest SGR was observed in T2 fed fish followed by T1, T3 and control. Similarly, Omenwa et al. (2015) obtained increased SGR in *C. gariepinus* fingerlings through the application of *Lactobacillus plantarum* and *Pseudomonas fluorescens.* Dey et al. (2017) utilized probiotic- (*B. aryabhattai* KP784311, *B. flexus* KR809411, *B. cereus* KR809412) encapsulated and ascorbic acid-enriched chironomid midge larvae and observed significantly higher (P<0.05) specific growth rate and survivability in *C. batrachus*.

Significantly (P≤0.05) higher PWG was observed in fish fed T2 followed by T1 and T3 whereas the fish fed control feed displayed lowest PWG. The PWG values varied significantly among different groups. The highest PI (23.60±0.47 g) and PER (2.05±0.01) were obtained in fish fed T2 and T1 feed respectively which indicates better utilization of

protein for growth and metabolism. The growth of *C. batrachus* in relation to control (Fig. 3.3.1), T1 (Fig. 3.3.2), T2 (Fig. 3.3.3) and T3-feed (Fig. 3.3.4) have been observed. High rate of survivability was observed in all of the test groups which established their non-pathogenicity and acceptance to the host.

In the present work, *L. sphaericus* PKA17, *B. cereus* PKA18 and *B. thuringiensis* PKA19 supplemented feed had resulted substantially better morphometric development than the control fed fish. *Bacillus* species were very much explored as probiotics in the aquaculture industry for enhancing enzymatic activity (Kumar et al. 2006), immunity (Nandi et al. 2017), feed conversion, fecundity and fry production (Ghosh et al. 2007). *Bacillus circulans* PB7 increased nonspecific-immunity and disease resistance in *Catla catla* fingerlings against *A. hydrophila* infection (Bandyopadhyay and Das Mohapatra 2009). Gomes et al. (2009) stated that incorporation of *Bacillus* sp. decreased handling stress during transport by reducing the cortisol levels of fish. El-Haroun (2007) observed increased growth performance, protein efficiency ratio, protein productive value and energy retention in *C. gariepinus* when the fish feed was supplemented with a commercial probiotic strain of *Bacillus.* Reneshwary et al. (2011) used *Bacillus thuringiensis* to enhance cellular non-specific immune response of *C. gariepinus.* Hapsari (2016) used fermented bioflocs inoculated with the bacterium *Bacillus cereus* to enhance the growth and feed utilization efficiency of juvenile catfish *C. gariepinus*.

Table 3.1: Growth performance of *Clarias batrachus* fingerlings with different feeds after 120 days of feeding trial.

Parameters	**Control**	**T1**	**T2**	**T3**
Initial length (cm)	3.88 ± 0.06	4 ± 0.02	3.93 ± 0.05	3.96 ± 0.02
Final length (cm)	15.24 ± 0.29	17.77 ± 0.56	19.4 ± 1.06	17.67 ± 0.21
Length gain (cm)	11.71 ± 0.11^{c}	13.77 ± 0.04^{b}	15.47 ± 0.1^{a}	13.70 ± 0.08^{b}
Initial weight (g)	4 ± 0.04	3.99 ± 0.03	4.02 ± 0.02	4.01 ± 0.02
Final weight (g)	24.71 ± 0.31^{d}	40.15 ± 0.35^{b}	47.29 ± 0.05^{a}	36.73 ± 0.08^{c}
Live weight gain (g)	19.37 ± 0.27^{d}	36.15 ± 0.33^{b}	43.27 ± 0.06^{a}	32.71 ± 0.09^{c}
Average daily growth (g)	0.16 ± 0^{d}	0.30 ± 0^{b}	0.36 ± 0^{a}	0.27 ± 0^{c}
Specific growth rate (%)	1.35 ± 0.15^{b}	1.92 ± 0.01^{a}	2.04 ± 0.01^{a}	1.84 ± 0.01^{a}
Feed conversion ratio	2.25 ± 0.03^{d}	1.23 ± 0.01^{a}	1.38 ± 0.03^{b}	1.84 ± 0.01^{c}
Protein efficiency ratio	0.99 ± 0.01^{d}	2.05 ± 0.01^{a}	1.83 ± 0.04^{b}	1.44 ± 0.02^{c}
Percentage weight gain (%)	484.83 ± 12.31^{d}	907.43 ± 8.03^{b}	1078.5 ± 6.17^{a}	818.23 ± 4.09^{c}
Protein intake (g)	18.28 ± 0.12^{b}	17.64 ± 0.13^{b}	23.60 ± 0.47^{a}	22.70 ± 0.15^{a}
Survival rate (%)	93.33	100	100	97.78

Control feed was not supplemented with any probiotic; T1= Feed supplemented with *Lysinibacillus sphaericus* PKA17; T2= Feed supplemented with *Bacillus cereus* PKA18; T3= Feed supplemented with *Bacillus thuringiensis* PKA19.
Results are given as (Mean±S.E)
Values in the same row with different superscripts denote a significant difference (P≤0.05)
Mean initial length and weight were (3.94±0.03) cm and (4.01±0.01) g respectively

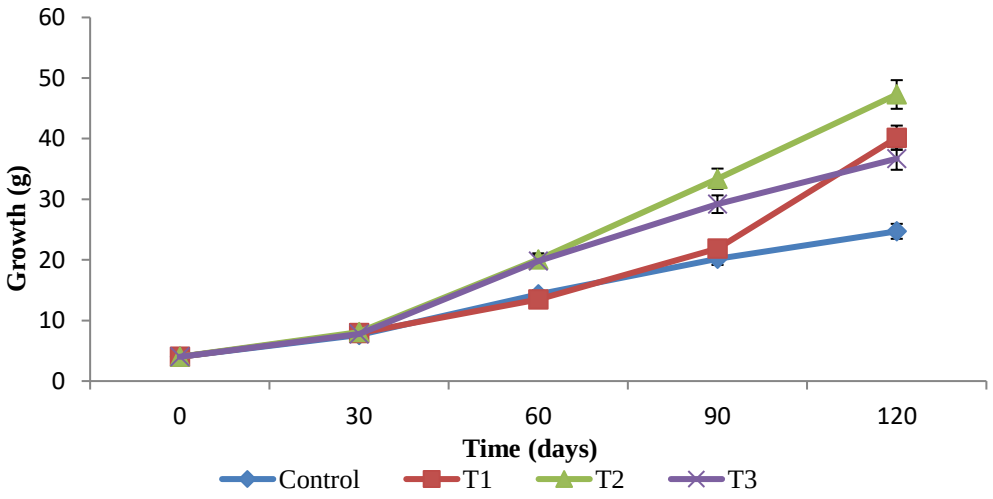


Fig. 3.2: Weight of *C. batrachus* in relation to various feeds at different time interval.

0 Day

30 Days time interval

60 Days time interval

120 Days time interval

90 Days time interval

Fig. 3.3.1: Effect of control feed on growth of *C. batrachus* fingerlings at different time interval.

0 Day

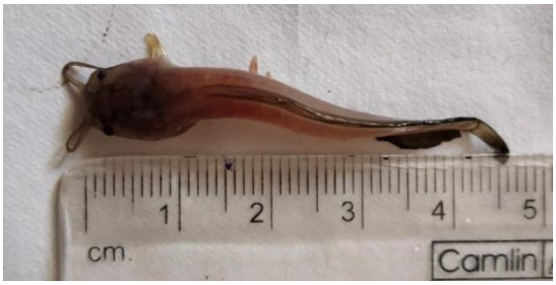
30 Days time interval

60 Days time interval

120 Days time interval

90 Days time interval

Fig. 3.3.2: Effect of probiotic-supplemented feed (T1) on growth of *C. batrachus* fingerlings at different time interval.



0 Day

30 Days time interval

60 Days time interval

120 Days time interval

90 Days time interval

Fig. 3.3.3: Effect of probiotic-supplemented feed (T2) on growth of *C. batrachus* fingerlings at different time interval.

0 Day

30 Days time interval

60 Days time interval

120 Days time interval

90 Days time interval

Fig. 3.3.4: Effect of probiotic-supplemented feed (T3) on growth of *C. batrachus* fingerlings at different time interval.

3.3.2 Assessment of growth parameters

The regression line and scatter diagram on different growth parameter of *C. batrachus* fed with control, T1, T2 and T3-supplemented feed were plotted (Fig. 3.4). The statistical analysis revealed that the regression coefficient values (r) of T1, T2 and T3-supplemented feed in respect to the control were 0.9386, 0.9469 and 0.9763 respectively. The result

indicated that the three test feeds (T1, T2, T3) were effective in their function in relation to the taken growth parameters of *C. batrachus*.

The mesh plot drew a wireframe of mesh with colour determined by the growth parameters of *C. batrachus* fed with different probiotic-supplemented feeds. It represented the proportionality of the different output obtained by various experimental feeds (T1, T2, T3) with respect to control. The height is a single-valued function of the contour parameter. The mesh plot was determined by 8 × 2 matrix through MATLAB. It performed a linear transformation on the 8 × 2 data matrix. The figures of the mesh plots of T1 against control (Fig. 3.5), T2 against control (Fig. 3.6) and T3 against control (Fig. 3.7) showed the deviation of *C. batrachus* production. This 3-D picture revealed that T2 had much peak variations and T1 had more flatten forms. From numerical point of view, it may be suggested that, the use of T1 with *L. sphaericus* PKA17 is most suitable and stable than the others regarding the productive value of *C. batrachus*.

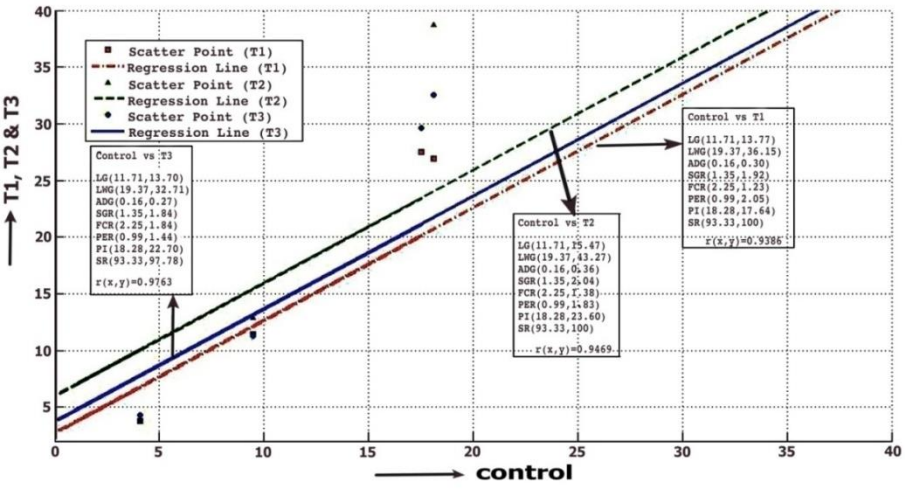


Fig. 3.4: Regression line and scatter diagram of different growth parameters of *C. batrachus* with respect to (control, T1), (control, T2) and (control, T3) feed.

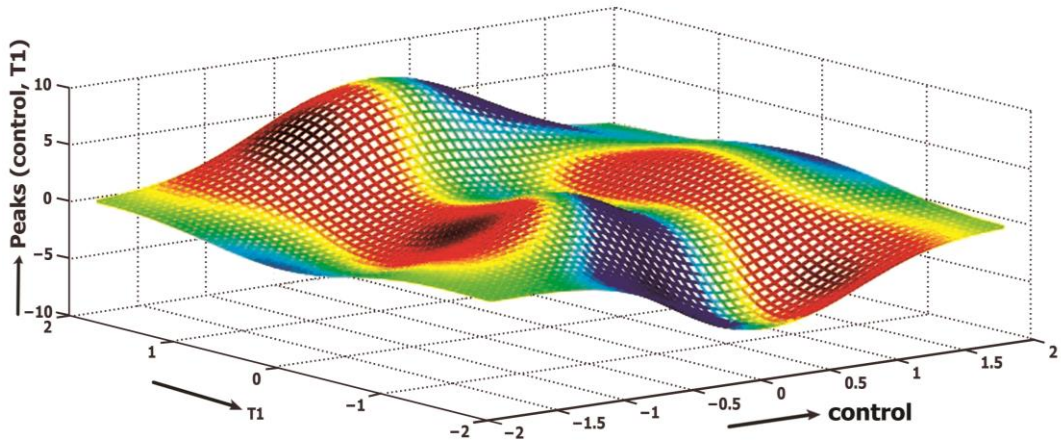


Fig. 3.5: Assessment of growth parameters of *C. batrachus* in T1 set in respect to control through mesh plot matrix.

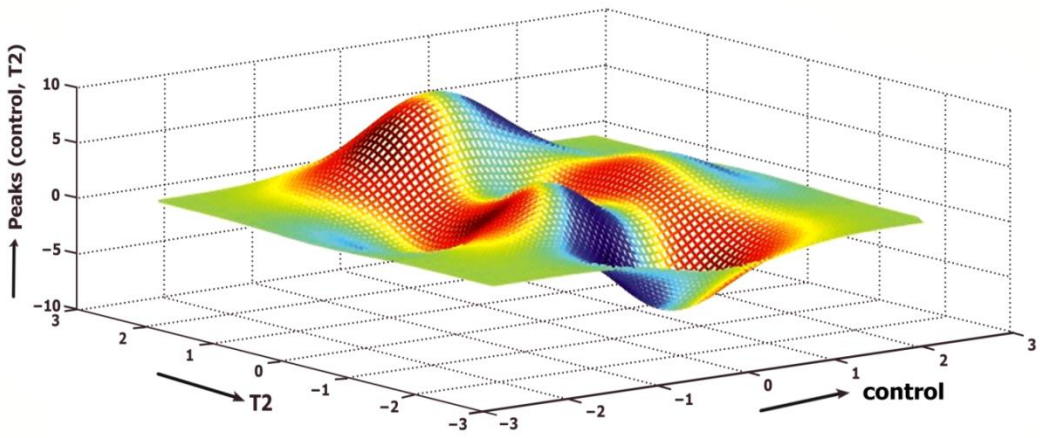


Fig. 3.6: Assessment of growth parameters of *C. batrachus* in T2 set in respect to control through mesh plot matrix.

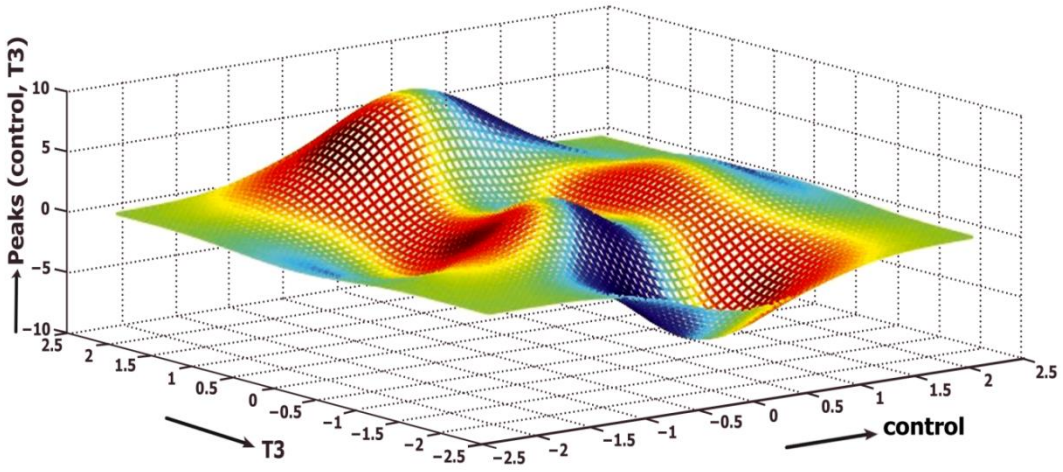


Fig. 3.7: Assessment of growth parameters of *C. batrachus* in T3 set in respect to control through mesh plot matrix.

3.3.3 Proximate analysis of the fish carcass

Proximate analysis of the whole carcass of *C. batrachus* of control, T1, T2 and T3 fed fish were carried out after 120 days of feeding trial. The result of nutritional indices of *C. batrachus* in relation to various feed is presented in Table 3.2. The carcass composition of the probiotic-supplemented fish revealed an apparent increase over the control fed fish. The carcass protein content was significantly ($P \leq 0.05$) higher (164.9 ± 0.23 g kg^{-1}) in T3 fed fish than the other experimental groups. The T1 and T2 fed fish also have resulted higher carcass protein content than the control fed fish. Nwanna and Tope-Jegede (2016) also observed enhanced content of carcass protein in *C. gariepinus* by supplementing the feed with probiotic *Lactobacillus plantarum*.

The carbohydrate content was significantly ($P \leq 0.05$) higher (2.8 ± 0.06 g kg^{-1}) in T1 fed fish than the other experimental groups. The content of carbohydrate among other groups didn't differ significantly. The study indicated a balanced carbohydrate concentration

among all the experimental groups. Wimalasena and Jayasuriya (1996) estimated the carbohydrate content of fresh water fishes. Sivakami et al. (1986) reported the gradual increase of muscle carbohydrate content in relation to maturity of *Cyprinus carpio*.

The fat content was considerably higher (75.5±0.46 g kg^{-1}) in control fed fish than the probiotic-fed fish. The fat content varied significantly among different groups. The high-quality, protein-enriched diet is essential for the building of muscle mass, neurological function, digestion, balancing of hormones and improvement of the immunity (Beveridge and McAndrew 2000).

The content of iron differs considerably among various experimental groups. The T1-fed fish produced significantly higher amount (2.21±0.01 mg $100g^{-1}$) of iron followed by T2, T3 and control. The content of iron in T1 fed fish was higher than the ICAR-CIFA standard (http://cifa.nic.in/pamphlets). The increased percentage of iron may help the host in synthesizing enzymes and generating cell signaling (Prabhulkar et al. 2012). The T1 fed fish displayed overall standard percentage of crude protein, carbohydrate and iron. The highest moisture (75.24%) and ash (4.73%) contents were observed in T1 and control respectively.

Table 3.2: Proximate composition of the whole carcass of *C. batrachus* after 120 days of feeding trial.

Proximate analysis of carcass	**Control**	**T1**	**T2**	**T3**
Carbohydrate (g kg^{-1})	1.7 ± 0.06^{b}	2.8 ± 0.06^{a}	1.6 ± 0.15^{b}	1.6 ± 0.10^{b}
Crude protein (g kg^{-1})	147 ± 1.73^{c}	159.2 ± 0.47^{b}	157.13 ± 0.19^{b}	164.9 ± 0.23^{a}
Fat (g kg^{-1})	75.5 ± 0.46^{a}	55.3 ± 0.12^{c}	69.7 ± 0.15^{b}	53.9 ± 0.21^{d}
Iron (mg $100g^{-1}$)	1.85 ± 0.01^{d}	2.21 ± 0.01^{a}	2.08 ± 0.02^{b}	1.98 ± 0.01^{c}
Moisture (g kg^{-1})	716.3 ± 0.25^{d}	752.4 ± 0.25^{a}	728.6 ± 0.21^{c}	733.1 ± 0.12^{b}
Ash (g kg^{-1})	47.3 ± 0.15^{a}	42.5 ± 0.15^{d}	43 ± 0.12^{c}	46.5 ± 0.11^{b}

Results are given as (Means ± S.E.)

Values in the same row with different superscripts denote a significant difference ($P \leq 0.05$)

The proximate composition of the vitamins of whole carcass of *C. batrachus* was thoroughly evaluated (Table 3.3). The content of vitamin A was significantly ($P \leq 0.05$) higher (7.19±0.04 I.U. 100 g^{-1}) in T1 fed fish followed by T2, T3 and control. The probiotic-fed fish vitamin A was considerably higher in compared to control. The content of vitamin D didn't vary widely among different experimental groups. The T1, T2 and control-fed fish contained almost equal amount of vitamin D. However, T3-fed fish displayed lower amount of vitamin D than the other groups. The vitamin E content was considerably higher in T1 (0.34±0.01 I.U. 100 g^{-1}) and T2 (0.29±0.01 I.U. 100 g^{-1}) fed fish. The content of vitamin K was higher (0.53±0.01 µg 100g^{-1}) in T1-fed fish and lower in T3-fed fish. The T1 fed fish overall displayed higher proportion of fat soluble vitamins than the control and other test diets. The higher amount of vitamin D and K in T1 fed fish can play a vital role in developing host innate immunity. The result was in accordance to Paul et al. (2016) who assessed the content of fat soluble vitamins in *Heteropneustes fossilis* and *C. batrachus*. The study revealed the content of vitamin A and D in indigenous *C. batrachus* were 6.03±0.01 and 44.73±0.31 (I.U. 100 g^{-1}) respectively. Fat-soluble vitamins are essential micronutrients obtained majorly through dietary resources. They have diverse biological functions. Vitamin A controls photoreception and regulates cell and tissue growth (Roos et al. 2003). Vitamin D enhances calcium and phosphorus metabolism. The catfishes generally contain substantial amount of vitamin D that plays a major role in developing immune system. Vitamin E functions as antioxidants where as vitamin K is required for the synthesis of blood coagulating proteins.

Table 3.3: Proximate composition of the vitamins of whole carcass of *C. batrachus* after 120 days of feeding trial.

Vitamin	Control	T1	T2	T3
Vitamin A (I.U. $100g^{-1}$)	6.01 ± 0.01^{d}	7.19 ± 0.04^{a}	7.01 ± 0.03^{b}	6.86 ± 0.06^{c}
Vitamin D (I.U. $100g^{-1}$)	44.24 ± 0.17^{a}	44.79 ± 0.05^{a}	44.73 ± 0.03^{a}	41.62 ± 0.53^{b}
Vitamin E (I.U. $100g^{-1}$)	0.15 ± 0.01^{c}	0.34 ± 0.01^{a}	$0.29 \pm 0.01^{a,b}$	0.25 ± 0.01^{b}
Vitamin K (µg $100g^{-1}$)	0.51 ± 0.01^{a}	0.53 ± 0.01^{a}	0.51 ± 0.01^{a}	0.46 ± 0.01^{b}

Results are given as (Means ± S.E.)
Values in the same row with different superscripts denote a significant difference ($P \leq 0.05$)

The fatty acid profile (Table 3.4) revealed higher content of polyunsaturated ω-3 fatty acids (1.7±0.12 g kg^{-1}) in T3-fed fish followed by T1, T2 and control. However, ω-6 fatty acid content was higher in control compared to formulated diets. The high content of ω-3 fatty acids in fishes fed with formulated diets compared to control feed may have significant value. Polyunsaturated ω-3 fatty acid reduces the risk of human cardiovascular diseases, insulin resistance in skeletal muscle, triglyceride level, rheumatoid arthritis, asthma, muscular degeneration and is vital to normal brain development of fetus and infants (Leaf and Kang 1996). The fresh water fish species mainly composed of low level of ω-3 with high content of ω-6 fatty acids and the ratio of 1:1 to 1:5 is recommended as a healthy human diet (Osman et al. 2001). Paul et al. (2016) obtained ω3 to ω6 ratio of 0.45:1 in *C. batrachus.* In the present study, the ω-3 and ω-6 ratio for *C. batrachus* was within 1:3 in T2, T1 and T3 fed fishes where as in fish fed with control diet it was > 1:5.

Table 3.4: The fatty acid profile of whole carcass of *C. batrachus* after 120 days of feeding trial.

Fatty acid	Control	T1	T2	T3
ω-3 fatty acids (g kg^{-1})	0.8±0.03	1.3±0.06	0.8±0.03	1.7±0.12
ω-6 fatty acids (g kg^{-1})	4.5±0.1	<0.01	2.7±0.03	0.2±0

Results are given as (Mean±S.E.)

The analysis of fish samples displayed significantly higher (P≤0.05) content of amino acids glycine, arginine, aspartic acid, glutamic acid, histidine, isoleucine, lysine, methionine, proline, threonine, tryptophan, tyrosine and valine in T1 fed fish than control and other test feeds (Table 3.5). The result has found homology (162.6 g kg^{-1}) with ICAR-CIFA standard (http://cifa.nic.in/pamphlets). The T1 fed fish contains significantly (P≤0.05) higher amount of essential amino acids compared to other experimental feeds. The content of arginine (1.06±0.01 g $100g^{-1}$ of muscle) was significantly (P≤0.05) higher in T1 fed fish followed by T2 and T3. The amino acid Arginine maintains immune function, regulates hormonal secretion, act as a precursor of neurotransmitter and removes nitrogenous waste (Mohanty et al. 2014). Isoleucine and valine were significantly higher (P≤0.05) in T1 fed fish whereas T3 fed fish contains high quantity (1.36±0.01 g $100g^{-1}$ of muscle) of leucine. Isoleucine, leucine and valine are branched chain amino acids that stimulate synthesis of muscle protein and repair damaged tissues (Mohanty et al. 2014). Significant differences were observed among different groups of diet-fed fish in the content of lysine and methionine. Lysine plays a crucial role in producing carnitine that transports fatty acid and generate essential energy. Methionine act as antioxidants, improves wound healing, prevents Parkinson's disease, regulates immune function, synthesizes and regulates protein metabolism (Mohanty et al. 2014). Threonine is essential in transepithelial permeability and maintenance of intestinal mucosa (Mao et al. 2011). Tryptophan functions as neurotransmitters and regulates neurobehavioral effect (Heine et al. 1995). The T1 fed fish contains significantly higher amount of all those essential amino acids. The carcass content of several amino acids of T1- fed fish was considerably higher comparing to the usual standard (http://cifa.nic.in/pamphlets). The amino acid glycine regulates cell signaling,

enhances protein synthesis, stimulates immune responses, stabilizes blood sugar, relaxes from mental stress, prevents autoimmune disorders and reduces muscle contraction (Wang et al. 2013). Glutamic acid modulates cellular metabolism, prevents muscular dystrophy and mental retardation (Paul et al. 2016). Histidine is needed for transport of metals, repairing of tissues, and maintenance of stomach content (Mohanty et al. 2014). The content of amino acid glycine, glutamic acid and histidine were also considerably higher in T1-fed fish in the present study. The Asian catfish, *C. batrachus* is attributed by high grade protein, iron and low fat content. It was observed in all fish with test diets that they have significantly lower amount of fat compared to fish with control diet. Their total energy is mainly dependent on protein and carbohydrate content rather than fat.

Table 3.5: Amino acid profile of whole carcass of *C. batrachus* after 120 days of feeding trial.

Amino acid (g 100g^{-1})	**Control**	**T1**	**T2**	**T3**
Glycine	2.02 ± 0.02^{c}	2.35 ± 0.05^{a}	2.14 ± 0.03^{b}	2 ± 0.01^{C}
Alanine	1.16 ± 0.01^{a}	0.97 ± 0.02^{d}	1.01 ± 0.02^{c}	1.08 ± 0.02^{b}
Arginine	0.72 ± 0.01^{d}	1.06 ± 0.01^{a}	0.96 ± 0.02^{b}	0.85 ± 0.01^{c}
Aspartic Acid	1.8 ± 0.06^{a}	1.84 ± 0.02^{a}	$1.75 \pm 0.02^{a,b}$	1.67 ± 0.01^{b}
Cysteine	0.02 ± 0^{a}	0.01 ± 0^{a}	0.02 ± 0^{a}	0.02 ± 0^{a}
Glutamic Acid	2.36 ± 0.03^{c}	2.54 ± 0.03^{a}	2.38 ± 0.01^{c}	2.42 ± 0.01^{b}
Histidine	0.66 ± 0.01^{d}	1.96 ± 0.02^{a}	1.26 ± 0.01^{b}	1.06 ± 0.01^{c}
Isoleucine	0.75 ± 0.01^{c}	0.82 ± 0.01^{a}	$0.80 \pm 0^{a,b}$	0.79 ± 0^{b}
Leucine	1.16 ± 0^{b}	0.98 ± 0^{d}	1.02 ± 0.01^{c}	1.36 ± 0.01^{a}
Lysine	0.59 ± 0.01^{c}	0.72 ± 0^{a}	0.61 ± 0.01^{c}	0.65 ± 0^{b}
Methionine	0.40 ± 0.01^{d}	0.47 ± 0^{a}	0.42 ± 0.01^{c}	0.44 ± 0^{b}
Phenylalanine	1.45 ± 0.01^{a}	0.70 ± 0.01^{d}	0.95 ± 0^{c}	1.14 ± 0.01^{b}
Proline	0.30 ± 0^{d}	0.71 ± 0.01^{a}	0.50 ± 0.01^{c}	0.65 ± 0^{b}
Serine	0.84 ± 0.01^{a}	0.73 ± 0.01^{c}	0.79 ± 0^{b}	$0.82 \pm 0.01^{a,b}$
Threonine	$0.80 \pm 0.02^{b,c}$	0.94 ± 0.02^{a}	0.82 ± 0.03^{b}	0.85 ± 0.02^{b}
Tryptophan	0.16 ± 0^{c}	0.21 ± 0^{a}	0.15 ± 0^{d}	0.18 ± 0^{b}
Tyrosine	0.12 ± 0^{d}	0.51 ± 0.01^{a}	0.35 ± 0.01^{c}	0.38 ± 0.01^{b}
Valine	0.96 ± 0.01^{d}	1.16 ± 0^{a}	1.03 ± 0.01^{c}	1.06 ± 0.01^{b}

Results are given as (Mean±S.E.)

Values in the same row with different superscripts denote a significant difference ($P \leq 0.05$)

3.3.4 Haematological parameters

The haematological parameters of *C. batrachus* of the control and probiotic-supplemented feed were evaluated in the present study (Table 3.6). The triglyceride content was significantly (P≤0.05) higher in control fed fish (140±1.15 mg/dl) compared to other groups. The higher triglyceride level may consequence with the risk of diabetes, blood pressure and obesity. Conversely, reduced level of triglyceride in probiotic-fed fish indicated their proper nutritional balance. Lambertsen (1977) evaluated the triglyceride level of 34 varieties of fish species and their byproducts. Elevated level of triglyceride increases the risk of heart disease, diabetes and stroke. On the contrary, high content of ω-3 fatty acid controls triglyceride to normal level. Similar kind of observations were noticed in the present study, where fish fed with probiotic-supplemented diet contained higher level of ω-3 fatty acid and reduced level of triglyceride.

Table 3.6: Hematological parameters of *C. batrachus* after 120 days of feeding trial.

Parameters	**Control**	**T1**	**T2**	**T3**
Triglyceride (mg/dl)	140 ± 1.15^{a}	70 ± 1.52^{b}	38 ± 0.76^{d}	58 ± 0.58^{c}
Total Cholesterol (mg/dl)	146 ± 1.15^{d}	210 ± 0.58^{c}	220 ± 1.15^{b}	238 ± 1.73^{a}
HDL (mg/dl)	24.33 ± 0.02^{c}	35 ± 1.15^{b}	36.67 ± 0.02^{b}	39.67 ± 0.45^{a}
LDL (mg/dl)	93.67 ± 0.25^{d}	161 ± 1.15^{c}	175.73 ± 0.64^{b}	186.73 ± 0.58^{a}
VLDL (mg/dl)	28 ± 0.94^{a}	14 ± 1.53^{b}	$7.6 \pm 1.52^{b,c}$	11.6 ± 1.93^{c}
Calcium (mg/dl)	9.52 ± 0.04^{c}	9.75 ± 0.04^{b}	9.83 ± 0.02^{b}	10.03 ± 0.08^{a}
Phosphorous (mg/dl)	15 ± 0.58^{a}	7 ± 0^{b}	5.7 ± 0.12^{c}	$6.4 \pm 0.15^{b,c}$
Uric Acid (mg/dl)	19.75 ± 0.02^{a}	19.25 ± 0.04^{c}	19.25 ± 0.02^{c}	19.4 ± 0.06^{b}

HDL: High density lipoprotein, LDL: Low density lipoprotein, VLDL: Very low density lipoprotein
Results are given as (Mean±S.E.)
Values in the same row with different superscripts denote a significant difference (P≤0.05)

The content of uric acid was also significantly higher ($P \leq 0.05$) in control fed fish (19.75±0.02 mg/dl). Uric acid may be associated with increased risk of cardiovascular diseases, gout and kidney stone (Borghi et al. 2015). Fatty fish may contain considerable levels of uric acid which should be avoided or limited in regular diet.

The high-density lipoprotein (HDL) cholesterol was significantly higher in fish fed T3-feed (39.67±0.45 mg/dl) followed by T2, T1 and control. The increased percentage of HDL cholesterol in probiotic-fed fish has significative therapeutic importance. Fisher et al. (2012) stated that HDL cholesterol maintains the diameter of blood vessel, transports cholesterol to liver and reduces the risk of cardiovascular disease.

The calcium content was considerably higher in T3-fed fish (10.03±0.08 mg/dl) followed by T2 and T1. The high content of calcium in probiotic-fed fish has added advantage as calcium is an essential component of bone and cartilage. It stimulates muscle contraction and regulates the transmission of nerve impulses. It also activates several key enzymes including pancreatic lipase, acid phosphatase, cholinesterase, ATPases and succinic dehydrogenase (Wu 2016). Larsen et al. (2000) observed the presence of high content of calcium in small indigenous fish *Amblypharyngodon mola*. Malde et al. (2010) stated that fish bone is a potential source of calcium. However, a good amount of phosphorous content was observed in control fed fish in the present study.

The haematological parameters often indicate the health status of aquatic animals. The present study revealed the improved level of haematological parameters in probiotic-fed fish compared to control. The result was in accordance to Olayinka and Afolabi (2013) who observed improved haematological parameters in *C. gariepinus* by supplementing the feed with *Lactobacillus acidophilus*. Ayo Olalusi et al. (2014) also have reported enhanced

haematological profile in *C. gariepinus* through the application of viable feed-probiotics. Nwanna and Tope-Jegede (2016) obtained increased haematological profile in *C. gariepinus* using *Lactobacillus plantarum*-supplemented feed. Fazio et al. (2013) investigated the hematological parameters of the gilthead sea bream (*Sparus aurata*) reared in different aquaculture systems.

3.3.5 Dose determination of *Lysinibacillus sphaericus* PKA17

The optimization of dose level of *L. sphaericus* PKA17 for the cultivation of *C. batrachus* juveniles are presented in Table 3.7. The application of probiotic in fish feed C2 has resulted significantly (P≤0.05) lower FCR than other diets. It not only indicated maximum feed utilization but also ensured better growth and development (Fig. 3.8). Significantly (P≤0.05) higher SGR (1.35±0.02%) and LWG (17.02±0.14 g) were observed in C2-fed diet. The PI (8.67±0.10 g) and PER (1.96±0.03) was also significantly (p≤0.05) higher in fish fed with C2 indicating better utilization of protein for growth and metabolism.

Table 3.7: Growth performance of *C. batrachus* juveniles after 60 days of feeding trial fed with different concentration of *L. sphaericus* PKA17 (C1=2 × 10^4, C2=2 × 10^5 and C3=2 × 10^6 probiotic cells per 100 g feed, control feed was not supplemented with probiotic).

Parameters	Control	C1	C2	C3
Live weight gain (g)	11.88 ± 0.07^{c}	15.81 ± 0.24^{b}	17.02 ± 0.14^{a}	16.28 ± 0.20^{b}
Average daily growth (g)	0.20 ± 0^{c}	0.26 ± 0^{b}	0.28 ± 0^{a}	$0.27 \pm 0^{a,b}$
Specific growth rate (%)	1.02 ± 0.01^{c}	1.29 ± 0.01^{b}	1.35 ± 0.02^{a}	1.31 ± 0.02^{b}
Feed conversion ratio	2.90 ± 0.01^{a}	2.17 ± 0.02^{b}	2.07 ± 0.05^{b}	2.16 ± 0.06^{b}
Protein efficiency ratio	1.44 ± 0.02^{c}	1.86 ± 0.02^{b}	1.96 ± 0.03^{a}	1.88 ± 0.05^{b}
Percentage weight gain (%)	87.13 ± 0.91^{c}	118.23 ± 1.85^{b}	126.8 ± 2.41^{a}	121.25 ± 2.51^{b}
Protein intake (g)	8.21 ± 0.09^{c}	8.53 ± 0.06^{b}	8.67 ± 0.10^{a}	8.65 ± 0.12^{a}

Results are given as (Mean±S.E); Values in the same row with different superscripts denote a significant difference (P≤0.05); Mean initial length and weight were (9.32±0.09) cm and (13.52±0.05) g respectively; Survival Rate (%) was 100 % in all cases

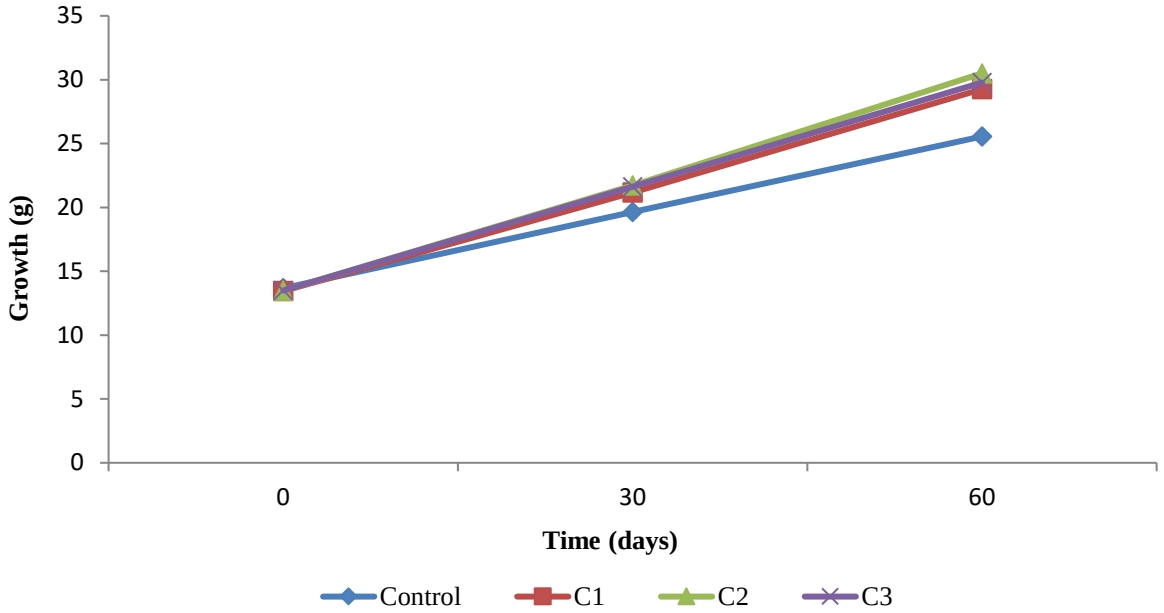


Fig. 3.8: Weight of *C. batrachus* in relation to various feeds (supplemented with different concentration of *L. sphaericus* PKA17; control feed was not supplemented with probiotic) at different time interval.

3.3.6 Dose determination of *Bacillus cereus* PKA18

The dose level determination of *B. cereus* PKA18 strain was carried out by supplementing the feed with different concentrations of the bacterial isolate (C1, C2, C3) for the cultivation of *C. batrachus*. The result was compared with the value of control-fed fish (Table 3.8). The application of probiotic-supplemented feed resulted enhanced growth compared to control (Fig. 3.9). The C2-fed fish was observed with considerably higher LWG (29.77±1.24 g), PER (2.34±0.12) and PWG (426.45±18.98). The FCR value also has declined drastically in C2-fed fish (1.72±0.11) followed by C3, C1 and control. High rate of survivability was observed among the all experimental groups. The result was in accordance to (Bandyopadhyay and Das Mohapatra 2009) who observed better growth and development in *Catla catla* by supplementing the feed with 2×10^5 *Bacillus circulans* PB7 probiotic cells per 100 g feed. So, the present study indicated concentration of C2 is best suited for the cultivation of indigenous *C. batrachus* using *B. cereus* PKA18 probiotic-supplemented feed.

Table 3.8: Growth performance of *C. batrachus* juveniles after 60 days of feeding trial fed with different concentration of *B. cereus* PKA18 (C1=2 × 10^4, C2=2 × 10^5 and C3=2 × 10^6 probiotic cells per 100 g feed, control feed was not supplemented with probiotic).

Feed	**Parameters**					
	LWG (g)	**FCR**	**PI (g)**	**PER**	**PWG (%)**	**SR**
Control	17.93±0.84	2.03±0.09	9.30±0.29	1.94±0.08	264.42±10.67	96
C1	24.95±0.80	2.18±0.06	13.98±0.19	1.78±0.05	359.32±13.09	97.33
C2	29.77 ± 1.24	1.72± 0.11	12.93±0.44	2.34± 0.12	426.45±18.98	98.67
C3	27.97 ± 0.92	1.97±0.03	14.27±0.34	1.95± 0.03	394.61±10.60	98.67

Results are given as (Mean±S.E.); Mean initial length and weight were (6.26±0.04) cm and (6.96±0.06) g respectively.

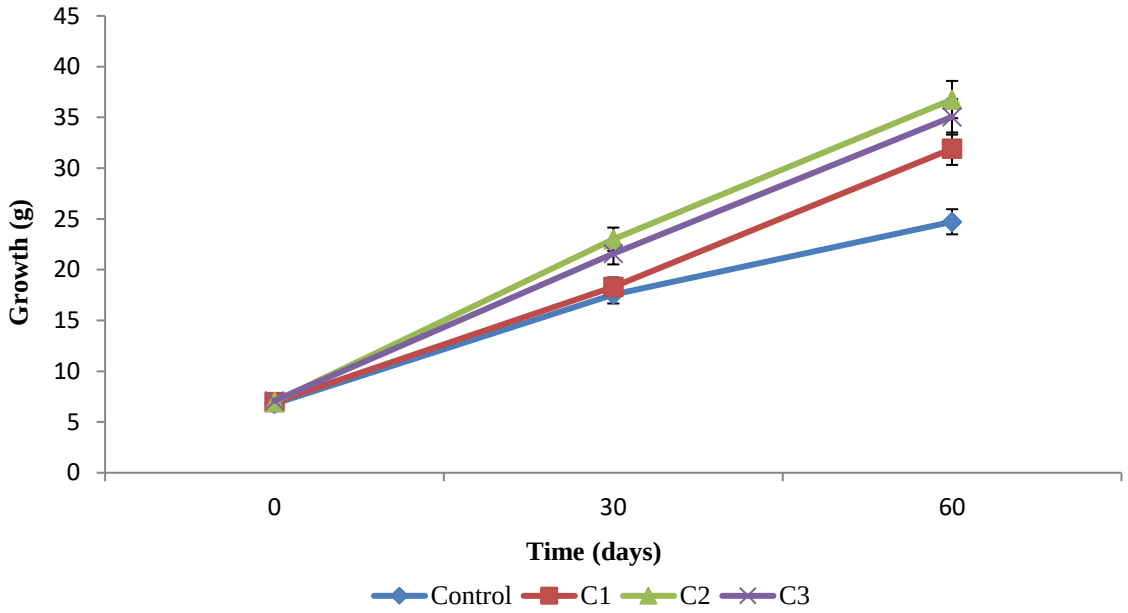


Fig. 3.9: Weight of *C. batrachus* in relation to various feeds (supplemented with different concentration of *B. cereus* PKA18; control feed was not supplemented with probiotic) at different time interval.

3.4 Conclusion

Lysinibacillus sphaericus PKA17, *Bacillus cereus* PKA18 and *Bacillus thuringiensis* PKA19, isolated from the intestine of adult *Clarias batrachus*, were evaluated to be used as candidate probiotics. *C. batrachus* fingerlings (4.01±0.01 g) were fed diets supplemented with 2×10^5 *L. sphaericus* PKA17 (T1 feed), *B. cereus* PKA18 (T2 feed) and *B. thuringiensis* PKA19 (T3 feed) probiotic cells per 100 g feed for 120 days at 5% of their body weight. The control feed was devoid of any probiotic. The fish fed T2 displayed significantly ($P \leq 0.05$) higher growth, specific growth rate and protein efficiency ratio. Significantly ($P \leq 0.05$) lower feed conversion ratio (1.23±0.01) was obtained from fish fed T1 followed by T2 and T3. Probiotic-supplemented feed resulted high content of protein, carbohydrate, iron, vitamins, amino acids and significantly lower amount of fat. The probiotic-supplemented fish exhibited increased level of calcium and HDL cholesterol. The results suggested that feed incorporated with these putative probiotics can be used during *C. batrachus* cultivation to enhance growth performance, feed efficiency and nutritional quality. The high survival rate of fish with probiotic-supplemented diet indicated that *L. sphaericus* PKA17, *B. cereus* PKA18 and *B. thuringiensis* PKA19 were not pathogenic to *C. batrachus.* The use of these probiotic organisms may consequence the cultivation of *C. batrachus* for the generation of high-quality livestock which may bring-about socio-economic development in various parts of South Asia.