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Effect of dietary protein source and probiotic inclusion on pattern of excretion of ammonia and orthophosphate in holding water in *Catla catla* culture system

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Abstract
This study was performed to determine the daily periodicity of ammonia and orthophosphate excretion in the fish *Catla catla* with different levels of inclusion of probiotics *Bacillus coagulans* in processed soybean and Duck weed based diets (approx. 40 percent protein). Two experiments were conducted. In experiment 1, a feeding trial of 90 days was conducted: *C. catla* fingerlings (average. BW 0.30±0.03g) were fed on Soybean supplemented feed with 1000 (diet D1), 2000 (diet D2), 3000 (diet D3) and 5000 (diet D4) CFUg^{-1} of *B. coagulans* as probiotic bacterium. In experiment 2, fishes (average BW 1.79± 0.03 g) were fed on artificial diet containing duckweed (@199.5 g Kg^{-1}) with no probiotic bacterium (C1), *B. coagulans* in proportion of 1000(C2), 3000(C3) and 5000 (C4) cells g^{-1} of duckweed supplemented feed. At the end of 90 days feeding trial, water sample from each aquaria were collected at 2 hour interval to estimate the excretory levels of total ammonia and reactive phosphate. The peak values for ammonia excretion occurred approximately at 6th and 12th hour post feeding while orthophosphate production showed an initial high level at 2 hours post feeding and 2nd peak at 8 hours post feeding. The low excretion of metabolites was found in group of fishes fed on diet supplemented @ 3000 CFU g^{-1} of probiotic irrespective of the dietary protein source (Soybean and Duck weed) indicating better dietary utilization of nutrients with optimum inclusion of probiotics. However, the values of metabolites were low in fishes fed on duck weed supplemented diets in comparison to soybean supplemented diets. The low values of metabolite excretion also coincided with higher growth performance. These results clearly indicate that protein source, its level of inclusion and dietary addition of probiotics at optimum level play a significant role in the management of excretory levels of ammonia and phosphorous and thus in turn improving growth performance of fish.

Keywords: Periodicity, Processed Soybean, Duckweed, *B. coagulans, C. catla*, Metabolites.

1. Introduction
Expansion of aquaculture production is largely the result of intensification of farming activities with a higher degree of feed input. Phosphorus and nitrogen are the two excretory products of farmed fish which are of great concern. Dietary phosphorus not absorbed and deposited in body tissues of farmed fish is excreted into rearing water either in feces or in urine (phosphorus absorbed in excess of need for growth). Dietary protein that is not digested is excreted in the form of ammonia in the holding water. Excretion of these metabolites i.e., Ammonia (NH_4-N) and orthophosphate (o-PO_4) depends on dietary intake and show rhythmic pattern in fishes. The postprandial ammonia excretion rate of culture fish has been found to be dependent on dietary protein levels [1, 2, 3, 4], water temperature [5, 6] and combined effects of water temperature, dietary protein level and dietary energy source in cultured fish species [7, 8]. Protein is considered as the main source of nitrogen and all essential amino acid that made its high cost have been studied by Pillai and Sollows [9]. Optimum level of protein should be supplied to the fish [10] for maximizing the nutrient utilization and minimize the solid and soluble waste load. Later, Beveridge and Phillips [11] studied that nutrients absorbed in excess may be excreted as ammonia and urea. High food wastage and poor nitrogen retention and assimilation indicate that major portion of nitrogen added to the culture system pollute aquatic environment [12]. Phosphorus is excreted by fishes in soluble and particulate forms [13, 14, 15, 16]. The ortho phosphate (o-PO_4), is soluble fraction which is most available for plant growth [17]. Rychly and Marina [18] studied the diurnal pattern of ammonia excretion in fed and starved

rainbow trout (29 to 70 g at 14 °C). The authors further reported two peaks in the diurnal graph when ammonia was sampled every 2 h for the general pattern of ammonia remains fluctuating. Kalla [19] and Garg *et al*. [20] studied the postprandial pattern of ammonia and phosphate excretion and reported that their production in the holding water was greater in diets with animal origin protein in comparison to plant origin proteins. Most of the studies [4, 19-23] further reported that when ammonia and phosphorus excretion is high in holding water, the growth performance/rate is less. Bhatnagar and Sushma [24] and Bhatnagar and Raparia [25] have evaluated that incorporation of probiotics in the diet further decrease the excretion of ammonia and phosphate in the holding water because of better nutrient utilization. Rajaharia [23] has reported that even in plant protein based diets optimum level of calcium and magnesium are essential for decreasing nitrogen and phosphorus excretion in holding water, in case of culture of *Cirrhinus mrigala*. Moreover, to reach sustainable aquaculture, cheaper protein sources originating from plant or animal sources has become inevitable to enhance stable aqua feed production [26, 27]. Among these, processed soybean [7, 28] and duckweed [29, 30, 31] both have potential to be incorporated in fish diets. However, to combat disease resistance due to excess of antibiotics and excess of pollution in holding waters, the focus instead has turned considerably to the use and contribution of probiotics (beneficial bacteria that fight against pathogenic bacteria) as an alternative technique to improve fish health, combat pollution in the holding water and to improve fish growth.

Therefore, in the present studies attempt has been made to determine diurnal excretory patterns of phosphorus and ammonia in the holding water that might help in evaluating the potential waste load of farm effluents to increase fish production.

2. Methodology

The various feed ingredients used in present investigation were processed Soybean (*Glycine max*), Duckweed (*Lemna minor*), Groundnut Oil Cakes (GNOC), Rice Bran (RB), Wheat Flour (WF) and mineral mixture. Soybean was subjected to hydrothermal treatment at 15 lbs. at 121 °C for 15 minutes for the removal of Anti-Nutrient Factors (ANF_s) [20], prior to incorporation in the diets. Soybean has been used as main protein source. Duckweed was used as soybean replacer in one of the experiments as it contains all essential nutritive components and for economic benefits. Mineral mixture was incorporated as dietary supplement. Pelleted diets were prepared for Experiment 1(Table 1) and Experiment 2 (Table 2). Chromic oxide was used as digestibility marker.

2.1 Experimental set up

C. catla (Hamilton) fingerlings were obtained from "Sultan Fish Seed Farm" in village Butana (29°18'N latitude and 76°62'E longitude), Nilokheri, District Karnal, Haryana, India for experiment 1 and 2.

The experiments 1 was conducted under laboratory conditions (25±1 °C) in glass aquarium (30 L capacity) in chlorine free water, kept in the laboratory where the temperature was maintained at 25±1ºC. Each aquarium was stocked with 20 fish fingerlings with average BW 0.30±0.03 g. Five dietary treatments (DC, D1, D2, D3 and D4) were performed with three replicates of each treatment (Table-1). In treatment 1 (DC), fishes were fed on artificial diet without probiotic bacteria (i.e., control diet). In treatment 2 (D1), fishes were fed on artificial diet containing *B. coagulans* isolated from the gut of mature *C. catla* and then mass cultured in laboratory in proportion 1000 cells g^{-1} of feed. In treatment 3 (D2), fishes were fed on artificial diet containing *B. coagulans* in proportion of 2000 cells g^{-1} of feed. In treatment 4 (D3), fishes were fed on artificial diet containing *B. coagulans* in proportion of 3000 cells g^{-1} of feed and with proportion of 5000 cells g^{-1} of feed in treatment 4 (D4). All these diets were isocaloric and isoproteic with approximately 40% proteins after spraying the feed was air dried at room temperature and the bacterial concentration of feed (CFU/g) was calculated. Finally, the feed was stored in vacuumed plastic container at 4 °C. All groups of fish were fed daily at 4% BW in 2 installments at 08:00 and 16:30 hours for 90 days.

Table 1: Ingredient composition (g Kg^{-1}) of experimental feeds for *Catla catla*

Ingredients (g Kg^{-1})	**Dietary treatments**				
	DC	**D1**	**D2**	**D3**	**D4**
Groundnut oil cake	650.0	650.0	650.0	650.0	650.0
Rice bran	42.0	42.0	42.0	42.0	42.0
Processed soybean*	266.0	266.0	266.0	266.0	266.0
Wheat flour	32.0	32.0	32.0	32.0	32.0
Chromic oxide(Cr_2O_3)	10.0	10.0	10.0	10.0	10.0
Mineral mixture **	10.0	10.0	10.0	10.0	10.0
Probiotic bacterium *Bacillus coagulans*	0.0	1000 cells g^{-1}	2000 cells g^{-1}	3000 cells g^{-1}	5000 cells g^{-1}

*Soybean was hydrothermically processed in an autoclave at 121 °C (15 lbs for 15 minutes) to eliminate antinutrient factors [20].
****** 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%.

The experiment 2 was conducted under laboratory conditions (25±1 °C) in plastic tubs (HDPE) of 50 L capacity. Each plastic tub was filled with de-chlorinated tap water and then stocked with 20 fish (fingerlings with average BW 1.79± 0.03 g). Four dietary treatments (CC, C1, C2 and C3) were performed with three replicates of each treatment (Table-2). In treatment 1 (TC), fishes were fed on artificial diet containing 75% of soybean replaced with duckweed (control diet with duckweed @199.5 g Kg^{-1}). In treatment 2(C1), 3(C2) and 4(C4), fishes were fed on artificial diet containing *B. coagulans* in proportion of 1000, 3000 and 5000 cells g^{-1} of feed. All these diets were almost isocaloric and isoproteic with approximately 40% proteins. All groups of fish were fed daily at 4% BW in 2 installments at 08:00 and 16:30 hours for 90 days. The diets are kept in a refrigerator at 4 ºC until use.

Table 2: Ingredient composition (g Kg^{-1}) of different experimental feeds with duckweed and B. coagulans for Catla catla

Ingredients (g Kg^{1})	**Dietary treatments**			
	CC	**C1**	**C2**	**C3**
Groundnut oil cake	650.0	650.0	650.0	650.0
Rice bran	42.0	42.0	42.0	42.0
Processed soybean*	66.5	66.5	66.5	66.5
Duckweed	199.5	199.5	199.5	199.5
Wheat flour	32.0	32.0	32.0	32.0
Chromic oxide (Cr_2O_3)	10.0	10.0	10.0	10.0
Mineral mixture**	10.0	10.0	10.0	10.0
Probiotic bacterium *Bacillus coagulans*	0.0	1000 cells g^{-1}	3000 cells g^{-1}	5000 cells g^{-1}

*Soybean was hydrothermally processed in an autoclave at 121 °C (15 lbs for 15 minutes) to eliminate ant nutrient factors [20].
****** 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%.

2.2 Analytical procedures
The initial and final weight of fishes is recorded for experiment 1 and 2 and live weight gain was calculated using standard method of Steffens [32]. At end of 90 days feeding trials, fishes were offered same diet in sufficient quantity, and waited for 2 hours to be consumed. Then, fixed levels of water maintained for both experiments and excess of feed was removed. After that, water samples from each aquaria/tub were collected at 2h intervals to estimate the excretory levels of total ammonia ($N\text{-}NH_4$) and reactive orthophosphate ($o\text{-}PO_4$) following APHA [33], and calculated following Sumagaysay-Chavoso [34]. The quantity of nitrogen and phosphate excreted by fish in holding water was calculated as follows:

$$\text{Total } N\text{-}NH_4/o\text{-}PO_4 \text{ excretion } (mg\ kg^{-1} BW\ 2h^{-1}) = \frac{[(N\text{-}NH_4/o\text{-}PO_4)_{120} - (N\text{-}NH_4/o\text{-}PO_4)_0] \times a}{\text{Fish biomass/ kg}}$$

$(N\text{-}NH_4/o\text{-}PO_4)_0$ and $(N\text{-}NH_4/o\text{-}PO_4)_{120}$ = concentration at times 0 and 120 min (2h) post feeding.

a = amount of holding water (L) in which fishes were kept.

2.3 Analysis of metabolites
Total ammonia (NH_4-N) and orthophosphate were analyzed following APHA [33] to investigate the influence of compounded feeds on quality of holding water.

2.3.1 Determination of total ammonia (NH_4-N)
The ammonium ions reacted with an alkaline solution of Nessler's reagent (Potassium mercury iodide) to form a yellow brown colored complex of ammonium mercury iodine. The light absorption was measured at wavelength of 425 nm. 50 ml. of filtered water sample was taken in Erlenmeyer flask and 2 drops of Rochelle salt (sodium potassium tartarte) solution was added to it, mixed well and then 2 ml of Nessler's reagent was added. After 10-20 minutes (color development) the absorbance was noted at 425 nm. The concentration of NH_4-N was deduced from the standard curve prepared by dissolving NH_4Cl (E.Merk) in distilled water to prepare standard ammonia solution of 1.0 mg L^{-1}(concentrations 0.2-1.0mgL^{-1}).

2.3.2 Determination of orthophosphate (soluble reactive phosphorus)
The orthophosphate ($o\text{-}PO_4$) react with an acidified ammonium molybdate solution and form molybdophosphoric acid, which was then reduced to a blue complex in the presence of stannous chloride. Intensity of blue color was noted at 690 nm on spectrophotometer.25 ml of water sample was taken in a conical flask (100 ml). Distilled water was used as blank. 1 ml of ammonium molybdate solution was added followed by 3 drops of stannous chloride solution (freshly prepared) and was shaken well. Blue color appeared, after 10 minutes the absorbance was recorded at 690 nm. The value of $o\text{-}PO_4$ (mg L^{-1}) was deduced with help of standard curve prepared by using standard phosphate solution of 1.0 mg L^{-1}(concentration 0.2-1.0 mg L^{-1}).

2.4 Statistical analysis
Analysis of variance (ANOVA) followed by Duncan's multiple range test [35] for all the experiments was used to determine the significant variation between the different treatments. Statistical significance was settled at a probability value of $P<0.05$. All statistics were performed using SPSS Version 11.5 for Windows.

3. Results
3.1 Experiment 1 (Soybean based dietary treatments)
Postprandial excretory pattern after 90 days of feeding revealed significantly ($P<0.05$) low values in total ammonia excretion and reactive orthophosphate production (mg Kg^{-1} BW d^{-1}) in holding water, in fish fed on diet D3 supplemented with 3000 cellsg^{-1} of feed (Table-3). Peak values of ammonia excretion occurred approximately 6h after feed was given to fish and second peak at 12h after feeding while $o\text{-}PO_4$ production showed an initial high level at 2h post feeding and second peak at 8h post feeding (Fig- 1).

Table 3: Effect of fish fed on soybean based diets with different proportion of probiotic bacterium *Bacillus coagulans* supplementation on Metabolite Excretion

Metabolite Excretion	Dietary treatments				
	DC (control)	**D1 (1000 CFUg^{-1})**	**D2 (2000 CFUg^{-1})**	**D3 (3000 CFUg^{-1})**	**D4 (5000 CFUg^{-1})**
Total NH_3-N excretion (mg Kg^{-1} BW day^{-1})	1890.2±32.74^{A}	1287.31±19.4^{C}	752.8±16.36^{D}	619.3±13.4^{E}	1326.3±19.9^{B}
Total O-PO_4 production (mg Kg^{-1} BW day^{-1})	766.02±11.3^{A}	472.62±7.55^{C}	335.16±8.07^{D}	278.55±13.1^{E}	473.15±13.6^{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)

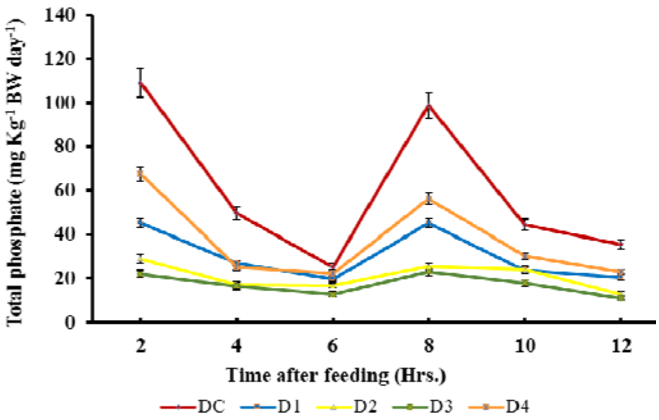

Fig-1-A

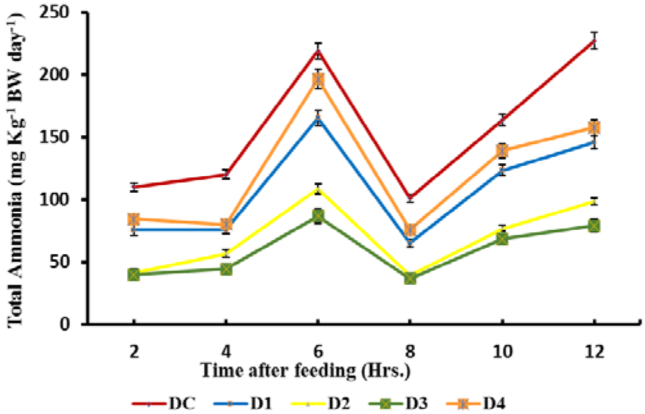

Fig-1-B

Fig 1: Post prandial excretory patterns of orthophosphate (1 A) and total ammonia (1B), (mg Kg^{-1} Body weight of fish) in holding water for fish *Catla catla* in different dietary treatments containing varying proportion of *B. coagulans* (DC=control, D1=1000 cells g^{-1}, D2=2000 cells g^{-1}, D3=3000 cells g^{-1} and D4=5000 cells g^{-1} of diet).

3.2 Experiment 2 (Duckweed based dietary treatments)

Postprandial excretory patterns of total ammonia and reactive orthophosphate in the holding water revealed significantly (P<0.05) low values in total ammonia excretion and reactive phosphate production (mg Kg^{-1} BW d^{-1}) in fish fed on diet C2 supplemented with 3 ×10^{5} cells 100 g^{-1}, i.e., 3000 cells g^{-1} of feed (Table-4). Irrespective of the protein level and source, ammonia (N–NH_4) excretion showed two peak values, one at 4 h and second at 12 h post-feeding, while o-PO_4 production showed an initial high level at 2 h post-feeding and a second peak at 10 post-feeding (Fig.2).

Table 5: Effect of fish fed on Duckweed based diets with different proportion of probiotic bacterium *Bacillus coagulans* supplementation on Metabolite Excretion

Metabolite Excretion	Dietary treatments			
	CC (control)	**C1 (1000 CFU g^{-1})**	**C2 (3000 CFU g^{-1})**	**C3 (5000 CFU g^{-1})**
Total Ammonia excretion (mg Kg^{-1} BW day^{-1})	856.30 ± 11.42^{A}	784.15±11.76^{B}	609±14.2^{D}	835.19±13.65^{C}
Total phosphate production (mg Kg^{-1} BW day^{-1})	398 ± 9.59^{A}	304.14±11.19^{B}	233.66±4.2^{D}	346±13.44^{C}

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)

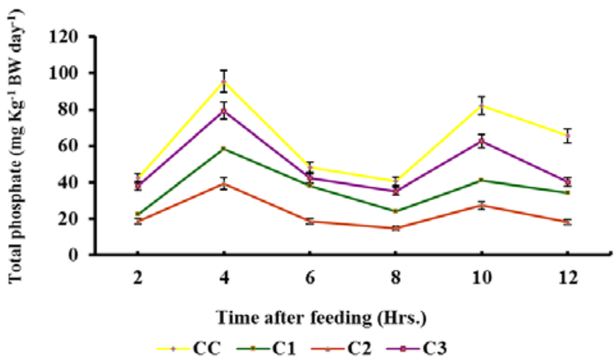

Fig-2-A

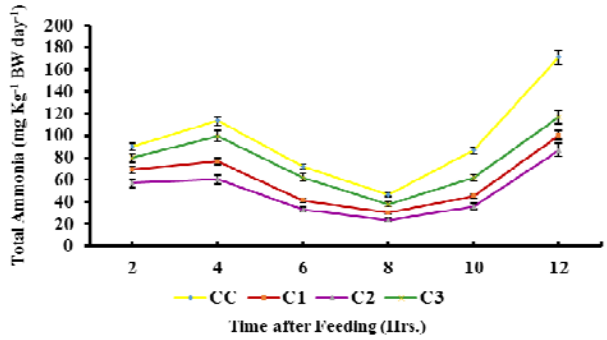

Fig-2-B

Fig 2: Post prandial excretory patterns of orthophosphate (2A) and total ammonia (2B), (mg/Kg Body weight of fish) in holding water for fish *Catla catla* fed on diets with duckweed and varying proportion of *Bacillus coagulans* (CC=control, C1=1000 cells g^{-1}, C2=3000 cells g^{-1}and C3=5000 cells g^{-1} of diet).

3.3 Relation between weight gain and excretion of metabolites

The orthogonal polynomial fit curve shows a clear dose dependent trend line curve, fitting to the data of Weight gain (Fig-3 and 4) which was significantly (P<0.05) high in treatment D3 and C2 respectively, where fishes were fed on diet containing probiotics (*B. coagulans*), in proportion of 3 $\times 10^5$ cells 100g^{-1} (3000 cells g^{-1}) of feed. However, lesser nitrogen and phosphate excretion were also observed in dietary treatment D3 and C2 which can be attributed to proper probiotic concentration in these specific feeds whereas lesser carcass protein and greater nitrogen and phosphate excretion were observed in dietary treatment D4 and C3 (containing *B. coagulans*, supplemented at the rate of 5000 CFU g^{-1} of diet) which could have been due to the overall low feed utilization level.

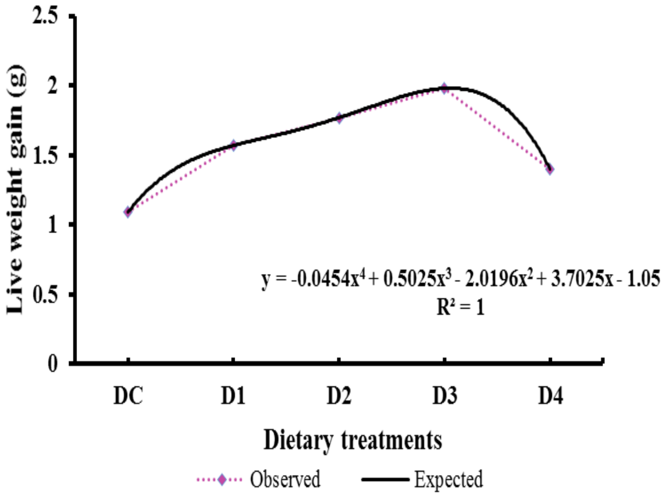

Fig 3: Polynomial fit curve to show effect of *B. coagulans* supplementation (DC=control, D1=1000 cells g^{-1}, D2=2000 cells g^{-1}, D3=3000 cells g^{-1} and D4=5000 cells g^{-1} of diet) fitting to the data of live weight gain in the fingerlings of *Catla catla*.

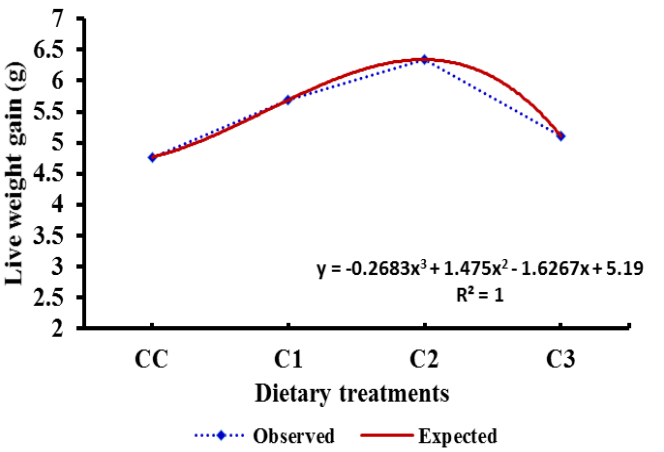

Fig 4: Polynomial fit curve to show effect of diets with duckweed and varying proportion of *Bacillus coagulans* (CC=control, C1=1000 cells g^{-1}, C2=3000 cells g^{-1}and C3=5000 cells g^{-1} of diet) fitting to the data of live weight gain in the fingerlings of *Catla catla* in a 90 days feeding trial.

4. Discussion

In aquaculture, water quality deteriorates mainly due to the accumulation of metabolic wastes such as ammonia and orthophosphate excretion in the holding water. *Bacillus* sp. reduces the quantity of ammonia and nitrite in the water as it degrades the organic matter and facilitates nutrients recycling [36, 37]. Similarly, in the present study, *B. coagulans* supplementation at 3000 CFU g^{-1} improved the water quality parameters and also reduced pathogenic bacteria load to significant levels. However, the excretion of metabolites, i.e., $N\text{-}NH_4$ and $o\text{-}PO_4$ in the holding water also increased with the increase in the inclusion level of probiotic bacteria (B. coagulans) above the optimum dose, i.e, at 5000 cells g^{-1} of feed in treatment D4 and C3. This may again be attributed to low feed utilization and when dietary utilization is low, deamination of unutilized feed protein occurs and excretion of metabolites in the holding water increases. These findings of experiments are in agreement with earlier reports [38, 39, 40, 41]. Although it is said [42] that mode of action of probiotic is ecological also as it optimizes the nitrification and de-nitrification rates in the water. However, when the value of the probiotics exceeds the optimum limit, there is negative impact on nutritive physiology decreasing growth performance and increasing excretion of metabolites in holding water. The use of plant protein as a means to reduce total ammonia and phosphorus content in the holding water has already been suggested by many workers [14, 43]. Duckweeds have been widely used in effluent water treatment as they have capacity to reduce the ammonia and phosphate concentration to significant levels [44] and when used as a protein source in diets, the phosphorus contents are utilized by fishes properly and less excretion take place in holding water. Further the ability of duckweed to sequester ammonia, nitrate and phosphorus and therefore, helping in the cleaning of the water, has been widely discussed in the literature [29, 45, 46, 47]. Also, Zimmo *et al.* [48] stated that the nutrient uptake is the main mechanism of nitrogen and phosphorus removal in duckweeds systems and thus, duckweed is mainly used to reduce chemical load of facultative sewage ponds during waste water treatment [49, 50, 51]. In the present study also, low levels of ammonia and phosphate excretion in the holding water were found in duckweed based diets when compared with soybean based diets, revealing that diets with duckweed are environment friendly.

The growth and digestibility parameters were thus found to be negatively correlated with ($N\text{–}NH_4$) and $o\text{-}PO_4$. A comparison of weight gain in fish groups of Experiment 1 and 2 show that the dietary treatments with low excretion of metabolites show maximum growth performance. However, duckweed as a dietary protein source is a better option as of low cost and less excretion of metabolites in the holding water.

5. Conclusion

Determination of diurnal excretory patterns of phosphorus and ammonia in the holding water is essential for evaluating the potential waste load of farm effluents to increase fish production. Better growth performance and nutrient retention requires the reduction of discharge ($N\text{-}NH_4$ and $o\text{-}PO_4$) in holding water. The use of probiotics bacterium such as *B. coagulans* and suitable plant protein source like duckweed also alleviates the pollution problems associated with intensive aquaculture system.

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