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Original article

Impact of *Bacillus subtilis* supplemented feed on growth and biochemical constituents in *Labeo rohita* fingerlings

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ABSTRACT

The study investigated the potential of probiotic isolate *Bacillus subtilis* AsCh-A4 (Accession No. MF543124) fermented feed to improve growth profile of *Labeo rohita* fingerlings. Morphometric measurements and nutritional indices of control and experimental groups were compared fortnightly in 90 days experiment. The formulated fish feed was fermented by *Bacillus subtilis* AsCh-A4 up to seven days and administered at 3% (b/w) with live (G1) and dead (G2) bacterium. G1 group showed significant higher values of DG (Daily gain) and RGR% (percent relative growth rate) in wet body weight than control and G2 while Specific Growth Rate (0.08 – 0.11%) in G1 within phase IV – VI followed by G2 (0.07%) at phase IV over control group. In all groups, the computed condition factor was 1.13, 0.91, 1.09 indicating isometric fish growth. Significantly increased body contents i.e., total protein (phase IV – VI), total carbohydrate (phase V) in G1 and DNA (phase IV) in G1 as well as G2 (phase II – III). Moreover, no significant change was assessed in total lipids, cholesterol, RNA contents, Alkaline phosphatase, Alanine aminotransferase, protease and somatic indices in all groups. Aspartate aminotransferase and acid phosphatase were significantly decreased in G1 (phase II – III) and G2 (phase III) while amylase increased in G1 (phase II). These results clearly demonstrated the growth promoting effect of the probiotics for rohu fish.

1. Introduction

In developing and under developed countries, massive increase in human population will lead to acute food shortage. While the aquatic resources possess great potential to help meeting the increasing demands for high biologic value protein. Aquaculture contributes as animal protein source to overcome the food shortage. Now a day advanced rearing techniques and nutrition improvement practices will promote aquaculture at large scale in Pakistan (Javaid, 1990; Sandhu, 2005). Among these practices,

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the major restraint is the cost of feed that is going to raise expenses of fish rearing. There is a dire need to adopt supplementary feeding as substitute technique which is considered promising to enhance production and carrying capacity of fish culturing (Razvi, 2006).

Now a days, Probiotics have been employed as dietary supplement to enhance fish growth and improve resistance against disease at every stage (Gatesoupe, 2008; Bagheri et al., 2008; Suzer et al., 2008; Essa et al., 2010; Faramarzi et al., 2011; Allameh et al., 2017). Probiotics are considered as microbes to be administered deliberately to improve gut flora, health of host and to withstand acidity of stomach, bile salts and anti-microbial compounds (Siuta-Cruce and Goulet 2001; Nikoskelainen et al., 2001; Yanbo and Zirong 2006). Besides this, animal feed can also be improved by enhancing nutritional values by addition of probiotics (Ibrahim et al., 2004).

Probiotics have direct influence to promote fish growth by improving nutrient uptake and digestibility, or being source of vitamins and nutrients (Ringo and Gatesoupe, 1998; Chaudhary and Qazi, 2007). The experimental endorsement of probiotics proved to be a fish growth promoter either as feed supplement or administered directly or in water (Bogut et al., 1998). The principal bacterial groups tested as probiotic are *Lactobacilli, Bacillus, Pseudomonas, Bifidobacteria* and *Vibrio* (Kolndadacha et al., 2011). The present study, therefore, observed the use of probiotic as dietary supplement as growth promoter in *Labeo rohita* fingerlings.

2. Materials and methods

2.1. Composition of fish feed (25% protein)

Fish feed comprised the following ingredients (%);fish meal 5.0, ground nut oil cake 53.7, rice polishing 34.3, molasses 4.0, table salt 1.0, di calcium phosphate 1.0, and vitamin premix 1.0.

2.2. Probiotic isolate

Probiotic *Bacillus subtilis* AsCh-A4 was selected as test isolate that was capable to cause 100% increase in different contents of the fermented formulated (Chaudhary and Qazi, 2006). Formulate fish feed was subjected to solid state fermentation in an apparatus designed by following Hofrichter et al. (1999). Solid state fermentation of sterilized fish feed was performed in apparatus consisting of screwed capped glass container. The substrate should be up to 2 cm in height in container. Fish feed was inoculated with 288×10^5 CFU/mL (10% inoculum), aerated, incubated at 37 – 40 °C for 7 days and moisture contents (70% v/w) was maintained by addition of autoclaved water on daily basis. The isolate *Bacillus subtilis* AsCh-A4 caused 93.79, 130.59 and 131.78 % increment in different contents such as total protein, lipids and total carbohydrates correspondingly on 7th day of fermentation (Chaudhary and Qazi, 2006).

2.3. Identification of the bacterial isolate

The probiotic isolate AsCh-A4 was examined for colonial as well cell morphological characteristics and different tests to identify biochemically (Pelczar et al., 1986; Benson, 1994; Collins et al., 1995; Weyant et al., 1996; Merk, 1996 – 1997). However, selected strain AsCh-A4 was identified taxonomically and characterized molecularly using 16S rRNA gene sequencing.

2.4. Molecular characterization of probiotic isolate

Total DNA of AsCh-A4 isolate was extracted by heating a loopful fresh culture in 50 mM NaOH (45 μL) in water bath (95 °C). Bacterial lysate was mixed with Tris HCl (5 μL, 1 M, pH 8) and centrifuge to get supernatant for 10 min (5000 rpm). The gene 16 S rRNA was amplified with DNA polymerase (KOD FX) using PCR. The reaction mixture included 2 mM dNTP, 2 μL of AsCh-A4 isolate's DNA, 50 μM of each primer i.e., 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) as well as 1492R (5′- AGGCTACCTTGTTACGACTT-3′). Polymerase chain reaction (Applied Biosystems' (ABI) GeneAmp™ PCR System 2700) was carried out up to 35 cycles and each cycle involved following steps; denaturation (98 °C, 10 sec), annealing (53 °C, 30 sec) and extension (72 °C, 1 min). The amplified product was sequenced using automated sequencer after purification.

The BLAST-querying the GenBank database http://www.ncbi.nlm.nih.gov/blast was used to check the homology of sequences and was applied to procure the accession number.

2.5. Experimental set up

Labeo rohita fingerlings with average total length 5 – 7.5 cm were acclimatized for 10 days in laboratory conditions according to experimental parameters between their arrival and the start of the inoculation. The fish were reared in glass aquaria (2′ × 1′ × 1′) in University animal house following animal standards (The Pakistan Prevention of Cruelty to Animals Act, 1890). During acclimatization period, the fish were observed to check health status. Control feed was supplied to fingerling according to 3% of fish body weight once a day throughout experiment. Temperature of water was maintained at 24 to 27 °C. Two third water was changed daily to clean glass aquaria partially.

The experiment was designed with three experimental groups running in triplicates and each replicate consist of 30 fingerlings. Experimental work had been performed by following General principles for the care and use of animals for scientific purposes mentioned in "Guidelines on the Care and Use of Animals for Scientific Purposes, 2004 by National Advisory Committee for Laboratory Animal Research. Sterilized formulated fish was administered to control fish group. *Labeo rohita* fingerlings were provided with fermented feed containing live bacteria in the Experimental group G1, whereas the animals in Experimental group G2 were fed sterilized fermented feed. The fermentation of fish feed was carried out by selected probiotic isolate *Bacillus subtilis* AsCh-A4 in both experimental groups except control. Morphometric measurement was recorded as zero readings while stocking. Experimental duration was 90 days.

2.6. Feeding regime

Fishes were provided with their specific feed by following 3% of wet body weight daily in the morning to control and experimental groups. Calculation for administered feed was done per aquarium after 15 days based on total wet body weight. And the feeding was continued for 90 days.

2.7. Fish growth parameters

At every 15th day of the sampling period, all fingerlings of one aquarium were measured morphometrically for, total, standard and fork length, width and wet body weight by using scale on graph paper and weighing balance (Shimadzu, ELB300). For morphometric measurement, fishes were sampled in separate container having water. Fish was taken outside water to weigh and released back to respective aquaria. Utmost care was taken to avoid handling stress to the fish. Fingerlings were shifted back to their respective aquaria after collecting data. Average weight, total length (TL), standard Length (SL), fork length (FL), width, WG (wet body weight gain), K (Condition factor) DG (Daily weight gain), % RGR (Percent relative gain rate), SGR (Specific growth rate), and

FCR (Feed conversion ratio) were assessed by the following expressions (Halver, 1972; Austreng, 1978; Busacker, 1990; Ahmad et al., 2002; Abdel Tawwab et al., 2008; USAID, 2011).

Average body weight = W2 (g)/total number of fishes (n)
Average TL = Final TL (cm)/total number of fishes (n)
Average SL = Final SL (cm)/total number of fishes (n)
Average FL = Final FL (cm)/total number of fishes (n)
Average width = Final width (cm)/total number of fishes (n)
K = K = 100 × W/L^3
DG (g) = [W2 (g) – W1 (g)]/Days
WG (g) = [W2 (g) – W1 (g)]/W1 (g)
%RGR = [W2 (g) – W1 (g)/Days] × 100
SGR = [(log W2–log W1)/Experimental periods in days] × 100
FCR = Total feed consumption (g)/WG (g)
Where;
W2 = Final body weight
W1 = Initial body weight

Percent increment in weight than control = [(wt of experimental fishes–wt of control fishes) × 100]/wt of control fishes

In the same way, percent increment was calculated for total length, total protein, lipid and cholesterol contents.

2.8. Sampling of fish

After every 15 days, five fingerlings per aquarium were sampled randomly in the morning. The sampled fish per aquarium were netted gently and rapidly anesthetized using clove oil (50 μL/L) to collect muscle tissue (Shamna et al., 2017). After Anesthesia, fingerlings were handled carefully by wearing gloves to place in clean zipped polyethylene bags and labelled well by assigning sampling numbers. Their weight, total, fork, standard length, and width were recorded accordingly.

2.9. Organ/body weight indices

After anesthesia, five fishes per replicate were sampled and dissected within 3 h of sampling. Three organs i.e., liver, kidneys and heart of the sampled fish (n = 5) were separated after dissection and weighed to be nearest 0.001 g. Organ body indices were computed accordingly to following expression;

Relative organ weight = [Weight of organ (g) × 100]/wet body weight (g)

2.10. Biochemical analysis of muscle tissue

After anesthesia, five fishes per replicate were dissected to sample muscle tissue within 3 h of sampling. White muscle between dorsal and caudal fin above lateral line was sampled after removing skin. Muscle of sampled fishes was homogenized to extract protein, carbohydrate and enzymes (Anwar et al., 2004), lipids, RNA and DNA (Shakoori and Ahmad, 1973). The extraction was carried out for spectrophotometric estimation of total protein (Lowry et al., 1951), total lipids (Zollner and Kirsch, 1962) and total carbohydrates (Dubois et al., 1956). Nucleic acid i.e, DNA, RNA were estimated by the methods of Schneider (1957). Muscle tissues of all groups were heated in muffle furnace (550 °C for 3 – 4 h) to determine ash contents whereas tissue samples dried in convection oven up to 105 °C till constant weight to calculate moisture contents (AOAC, 2012).

AkP, Alkaline phosphate (Orthophosphoric monoester phosphohydrolase, alkaline optimum), AcP (Orthophosphoric monoester phosphohydrolase, acid optimum) activities were assessed by following protocol of Kind and King (1954). While activity of Asparatate aminotransferase (AST) alanine aminotrasferase (ALT), Amylase (1, 4 a-D glucanhydrolase) and protease were measured according to Reitman and Frankel (1957), Stegbauer (1974) and Anson (1938), respectively.

2.11. Statistical analysis

Experimental data was recorded as mean ± S.E.M for five fish per replicate. One way ANOVA using Duncan's multiple range test was applied to analyze the fish morphometric and nutritional data (SPSS ver.16.0, Chicago, IL, USA).

3. Results

3.1. Identification and characterization of bacterial AsCh-A4 isolate

According to colonial characteristics on nutrient agar, AsCh-A4 was white in appearance and 5 mm in diameter. The colony was round, flattened, with filiform margins, butyreus (consistency) and opaque. The isolate was stained as G-positive. The cellular characteristics include slender rods with rounded ends (3 × 1 μm). Probiotic isolate was motile having polar flagella (1–2), facultative anaerobe, required 25, 37 and 45 °C temperature for growth and have 6% salt tolerance. The bacterium grew at nitrate by reducing to produce no gas and MacConkey agar but not at Simmon's citrate and cetrimide agar. AsCh-A4 showed negative result for methyl red and urease tests while showed positive oxidase, catalase, and Voges Proskauer tests.

Based on colonial, cellular and biochemical as well as molecular characteristics, the strain AsCh-A4 was similar up to 98% to 16 S rRNA gene sequence of *Bacillus subtilis*. The accession numbers MF543124 was assigned to the isolate *Bacillus subtilis* AsCh-A4 by GenBank database (http://www.ncbi.nlm.nih.gov/blast).

3.2. Effect of B. Subtilis AsCh-A4 supplemented feed on growth parameters of Labeo rohita fingerlings

Average body weights of the G1 fishes showed significant increase at the last three phases (IV – VI) of experiment over the respective control. The percent increment in weight was observed as 32.83 and 19.02 for G1 as well as G2 fishes than control group. Similarly regarding average total, standard, fork lengths and width, same group at the same phases showed significant elevations over the respective control. Experimental group G2 showed non-significant differences from control and G1 (Table 1). At termination of experiment, fishes of G1 and G2 showed percent increase in total length as 18.40 and 7.34 than control group.

Condition factor for all phases was calculated. The K factor varies from 1.03 to 1.13 (control), 0.91–1.07 (G1) and 0.99–1.50 (G2) from the start to end of experiment. When wet body weight gain (WG) and daily weight gain (DG) of control and experimental fishes were compared, it appeared that G1 group at the last four phases (III – VI) had significantly higher values of the parameter as compared to both the control and G2 groups. Percent Relative weight gain rate (%RGR) also indicated significant increase in G1 at the last four phases (III – VI) and in G2 at last two phases (V – VI) of the experiment as compared to control level. Regarding percent specific growth rate (SGR), significant increase was observed in G1 group in last three phases (IV – VI) while same increasing trend was only observed in G2 at phase IV (60 days) than control values as described in Table 1.

Feed conversion rate (FCR) values of both experimental groups were lower than control but significant at phase V than control and G2 groups (Table 1). Growth of control and experimental groups were showed in Figs. 1–6.

Table 1
Growth Profile of *L. rohita* fingerlings administered with *B. subtilis* AsCh-A4 fermented feed in control and experimental groups at different phases.

Aspects	Parameters	Groups	Phases (Days)						
			Zero	I(15)	II(30)	III(45)	IV(60)	V(75)	VI(90)
Morphometry	Average weights (g)	Cont	5.29 ± 0.06	5.92 ± 0.12	6.36 ± 0.16	6.78 ± 0.28	7.17 ± 0.27 ^{a}	7.78 ± 0.20 ^{a}	8.62 ± 0.16 ^{a}
		G1	5.26 ± 0.13	5.99 ± 0.16	6.51 ± 0.13	7.30 ± 0.15	8.31 ± 0.22^{b}	9.65 ± 0.31^{b}	11.45 ± 0.38^{b}
		G2	5.30 ± 0.18	6.09 ± 0.12	6.54 ± 0.20	7.09 ± 0.22	7.86 ± 0.11 ^{ab}	8.93 ± 0.17 ^{ab}	10.26 ± 0.04 ^{ab}
	Average total length (cm)	Cont	8.02 ± 0.05	8.22 ± 0.05	8.42 ± 0.07	8.60 ± 0.12	8.64 ± 0.05 ^{a}	8.89 ± 0.10 ^{a}	9.13 ± 0.09 ^{a}
		G1	8.00 ± 0.06	8.24 ± 0.07	8.53 ± 0.006	8.86 ± 0.07	9.26 ± 0.03^{b}	9.70 ± 0.01^{b}	10.81 ± 0.07^{b}
		G2	8.11 ± 0.09	8.30 ± 0.08	8.54 ± 0.08	8.74 ± 0.10	9.07 ± 0.06 ^{ab}	9.38 ± 0.03 ^{ab}	9.80 ± 0.00 ^{ab}
	Condition Factor (K)	Cont	1.03	1.07	1.07	1.07	1.11	1.11	1.13
		G1	1.03	1.07	1.05	1.05	1.05	1.06	0.91
		G2	0.99	1.07	1.05	1.06	1.50	1.08	1.09
	Average standard length (cm)	Cont	6.24 ± 0.05	6.44 ± 0.05	6.64 ± 0.07	6.81 ± 0.11	6.96 ± 0.12 ^{a}	7.11 ± 0.10 ^{a}	7.35 ± 0.09 ^{a}
		G1	6.22 ± 0.06	6.46 ± 0.07	6.75 ± 0.06	7.15 ± 0.01	7.48 ± 0.03^{b}	7.92 ± 0.01^{b}	8.38 ± 0.08^{b}
		G2	6.14 ± 0.03	6.51 ± 0.08	6.76 ± 0.08	7.96 ± 0.01	7.28 ± 0.04 ^{ab}	7.60 ± 0.03 ^{ab}	7.97 ± 0.05 ^{ab}
	Average fork length (cm)	Cont	6.74 ± 0.05	6.94 ± 0.05	7.14 ± 0.07	7.32 ± 0.12	7.46 ± 0.12 ^{a}	7.61 ± 0.1 ^{a}	7.85 ± 0.09 ^{a}
		G1	6.72 ± 0.06	6.96 ± 0.07	7.25 ± 0.06	7.58 ± 0.07	7.98 ± 0.03^{b}	8.42 ± 0.01^{b}	8.90 ± 0.07^{b}
		G2	6.83 ± 0.09	7.02 ± 0.08	7.26 ± 0.08	7.46 ± 0.10	7.79 ± 0.06 ^{ab}	8.10 ± 0.03 ^{ab}	8.42 ± 0.10 ^{ab}
	Average width(cm)	Cont	1.71 ± 0.02	1.82 ± 0.01	1.86 ± 0.01	1.96 ± 0.02	2.02 ± 0.03 ^{a}	2.11 ± 0.01 ^{a}	2.19 ± 0.01 ^{a}
		G1	1.72 ± 0.03	1.85 ± 0.02	1.96 ± 0.03	2.06 ± 0.02	2.22 ± 0.03^{b}	2.37 ± 0.04^{b}	2.50 ± 0.02^{b}
		G2	1.71 ± 0.01	1.83 ± 0.02	1.93 ± 0.02	2.01 ± 0.02	2.14 ± 0.03 ^{ab}	2.25 ± 0.03 ^{ab}	2.35 ± 0.02 ^{ab}
Nutritional Indices	Wet body weight gain fortnightly (WG)	Cont	–	18.96 ± 2.24	10.30 ± 1.03	10.49 ± 0.88 ^{a}	7.06 ± 1.34 ^{a}	6.28 ± 0.43 ^{a}	4.34 ± 0.06 ^{a}
		G1	–	22.11 ± 3.15	10.97 ± 0.26	13.96 ± 0.91^{b}	12.85 ± 0.35^{b}	11.91 ± 0.55^{b}	9.26 ± 0.68^{b}
		G2	–	23.48 ± 1.68	11.41 ± 1.34	10.57 ± 0.38 ^{a}	9.16 ± 0.30 ^{a}	8.31 ± 0.33 ^{a}	6.03 ± 0.56 ^{a}
	Daily weight gain (DG)	Cont	–	1.26 ± 0.15	0.68 ± 0.07	0.70 ± 0.06 ^{a}	0.47 ± 0.09 ^{a}	0.41 ± 0.03 ^{a}	0.28 ± 0.00 ^{a}
		G1	–	1.47 ± 0.21	0.73 ± 0.02	0.93 ± 0.06^{b}	0.86 ± 0.03^{b}	0.79 ± 0.04^{b}	0.62 ± 0.04^{b}
		G2	–	1.57 ± 0.11	0.76 ± 0.09	0.70 ± 0.003 ^{a}	0.61 ± 0.02 ^{a}	0.55 ± 0.02 ^{a}	0.41 ± 0.03 ^{a}
	% Relative weight gain rate (% RGR)	Cont	–	11.94 ± 1.34	8.0 ± 1.28	6.07 ± 2.06 ^{ab}	5.64 ± 1.28 ^{a}	8.62 ± 1.57 ^{a}	10.62 ± 1.44 ^{a}
		G1	–	14.03 ± 1.99	8.69 ± 1.07	12.03 ± 0.53 ^{a}	13.78 ± 0.92^{b}	16.09 ± 0.65^{b}	19.36 ± 0.45^{b}
		G2	–	14.83 ± 1.51	8.18 ± 1.65	7.70 ± 0.50^{b}	10.62 ± 2.49 ^{ab}	13.35 ± 1.16^{c}	13.13 ± 2.32^{c}
	Specific growth rate (SGR)	Cont	–	0.33 ± 0.04	0.11 ± 0.02	0.06 ± 0.02	0.04 ± 0.01 ^{a}	0.05 ± 0.08 ^{a}	0.05 ± 0.006 ^{a}
		G1	–	0.38 ± 0.05	0.12 ± 0.01	0.11 ± 0.005	0.09 ± 0.006^{b}	0.08 ± 0.005^{b}	0.08 ± 0.003^{b}
		G2	–	0.4 ± 0.04	0.11 ± 0.02	0.07 ± 0.005	0.07 ± 0.016^{b}	0.07 ± 0.007 ^{ab}	0.06 ± 0.01 ^{ab}
	FCR	Cont	–	2.86 ± 0.36	5.03 ± 0.54	3.95 ± 0.21	4.67 ± 0.96	3.23 ± 0.19 ^{a}	2.20 ± 0.03
		G1	–	2.54 ± 0.37	4.70 ± 0.28	3.29 ± 0.23	2.96 ± 0.15	2.39 ± 0.03^{b}	1.68 ± 0.08
		G2	–	2.32 ± 0.24	4.64 ± 0.45	4.13 ± 0.30	3.81 ± 0.24	3.12 ± 0.05^{c}	2.28 ± 0.23
	PER%	Cont	–	4.21 ± 0.68 ^{a}	0.81 ± 3.56	−9.88 ± 3.86 ^{a}	−3.75 ± 1.55 ^{a}	−0.124 ± 1.053	−2.67 ± 1.20 ^{a}
		G1	–	1.69 ± 0.23^{b}	0.48 ± 0.01	0.831 ± 0.12^{b}	0.64 ± 0.06^{b}	0.54 ± 0.05	0.43 ± 0.04^{b}
		G2	–	1.39 ± 0.12^{b}	0.70 ± 0.17	1.40 ± 0.04^{b}	0.63 ± 0.00^{b}	0.30 ± 0.01	0.26 ± 0.01^{b}

All values represent means of three replicates ± S.E.M. Values within respective column not sharing a common alphabet differ significantly from each other at $p \leq 0.05$ at single factor analysis of variance.

3.3. Somatic indices

Somatic indices of kidney, heart, and liver from the control and two experimental groups at different phases were recorded in Table 2. Non-significant difference in somatic indices were evaluated in control as well as experimental groups at all phases of the experiment.

3.4. Biochemical constituents of muscle tissues of control and experimental fishes

Biochemical components such as total protein, total lipids, total carbohydrates, RNA, DNA, moisture and ash contents were analyzed in control as well as experimental fishes at different phases of experiment (Table 3). No-significant difference was observed in total lipids, RNA and moisture contents in control and experimental groups. Regarding total protein, experimental group G1 showed significant increase at phase V and VI (75 – 90 days). Percent elevation in protein contents were 30.27 (G1) and 6.85 (G2) than respective control. In Lipids and cholesterol contents, percent increase in experimental groups than control was calculated as 4.42 and 2.09 (G1), 1.93 and 1.09 (G2) respectively at end of experiment. Significant increase in total carbohydrates was recorded in experimental group G2 at phase I, II while significant decrease was evaluated at phases IV – VI. Significant difference in total carbohydrates was noted in G1 at Phase I and V. Group G2 showed increase in DNA contents to more/less decrease in phase I and V. No difference was seen at phase V and VI in both G1 and G2. Significant difference in ash contents was evaluated in both experimental groups than control values at phases I, IV – VI while both experimental groups differed significantly at last two phases. Non-significant difference was observed in fat, RNA and moisture contents.

The activities of different enzyme i.e., AkP (Alkaline phosphatase), AcP (Acid phosphatase), AST, ALT, protease and amylase in muscle tissue were also investigated and results were tabulated in Table 3. Significant decrease was recorded in group G1 in acid phosphatase and ALT at phase II, III while in AST at phase II. Significant increase in G1 at phase II was investigated. Group G2 showed significant difference in alkaline phosphatase and protease (phase II), AST (phase III), acid phosphatase (phase IV) than control and experimental groups. In Later phases, enzymes stabilizes and no significant differences were noted.

4. Discussion

In the present study, the results revealed the beneficial effect of *Bacillus subtilis* AsCh-A4 supplementation in fish feed on growth efficiency in *L. rohita* fingerlings. The beneficial incorporation of microbes in fish feed or water to improve the health status of the

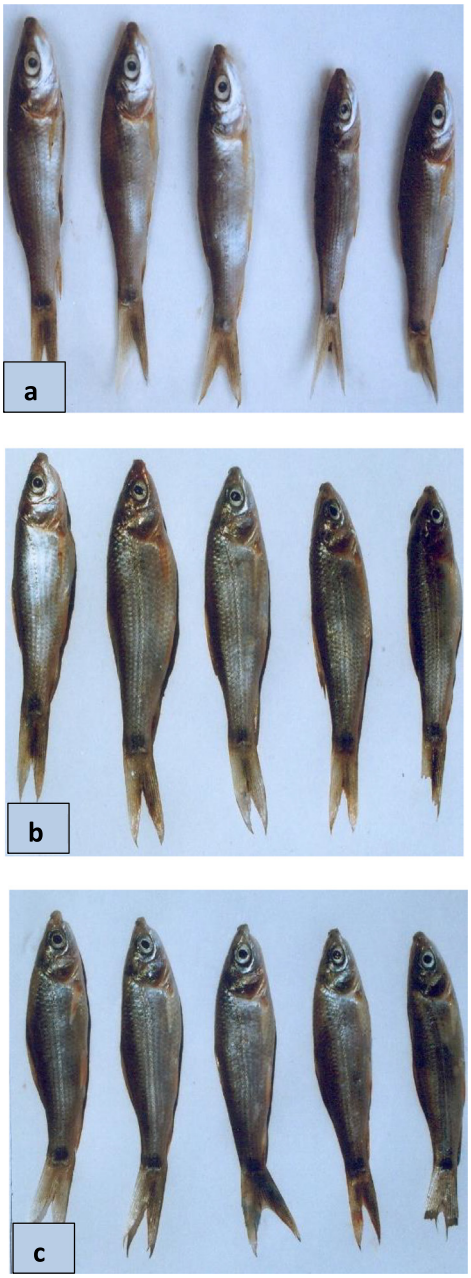

Fig. 1. Photographs of control (a), G1 (b) and G2 (c) fish groups at start of experiment i.e. phase 0 (zero days).

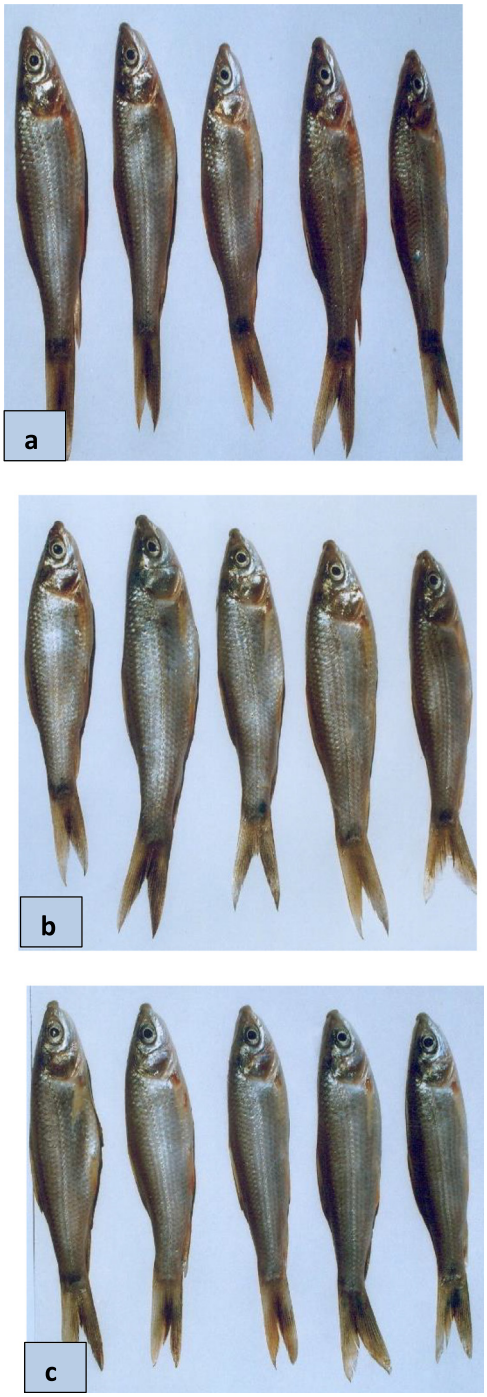

Fig. 2. Photographs of control (a), G1 (b) and G2 (c) fish groups at phase II (30 days).

animal can be termed as probiotics (Moriarty, 1998). The variety of probiotics can be incorporated to fish culture such as yeast, bacteria (gram positive as well gram negative), unicellular algae, and bacteriophages (Irianto and Austin, 2002a).

The study dealt with the effect of fermented fish feed by *Bacillus subtilis* AsCh-A4 that was administered to *Labeo rohita* fingerlings in two forms i.e., live and dead bacterium. The useful application of administration of live probiotics has been commented by many investigators (Lara-Flores et al., 2010; Essa et al., 2010; Boonthai et al., 2011; Seenivasan et al., 2011, 2012; Reda and Selim, 2015). According to Siuta Cruce and Goulet (2001), viability of probiotic stains should be maintained in order to get better results regarding host' health and should be incorporated in high enough numbers. Similar trend were also observed by Noh et al. (1994) in carps, Gildberg et al. (1995, 1997) in Atlantic cod and Metaillier and Hollocou (1993) in Atlantic salmon. The percent increment in weight was observed as 32.83 and 19.02 for G1 as well as G2 fishes than control group. At termination of experiment, fishes of G1 and G2 showed percent increase in total length as 18.40 and 7.34 than control group. The present investigation also corroborated the results of Ringpipat et al. (1998), Maeda and Liao. (1992) and Garriques and Arevalo (1995) which revealed a significant increase in growth parameters of *Penaeus* when provided with probiotic supplemented diets. According to Ding et al. (2004), probiotic augmented diets enhanced fish growth that may be related to better digestive activity by improved vitamin synthesis and digestive enzyme activity.

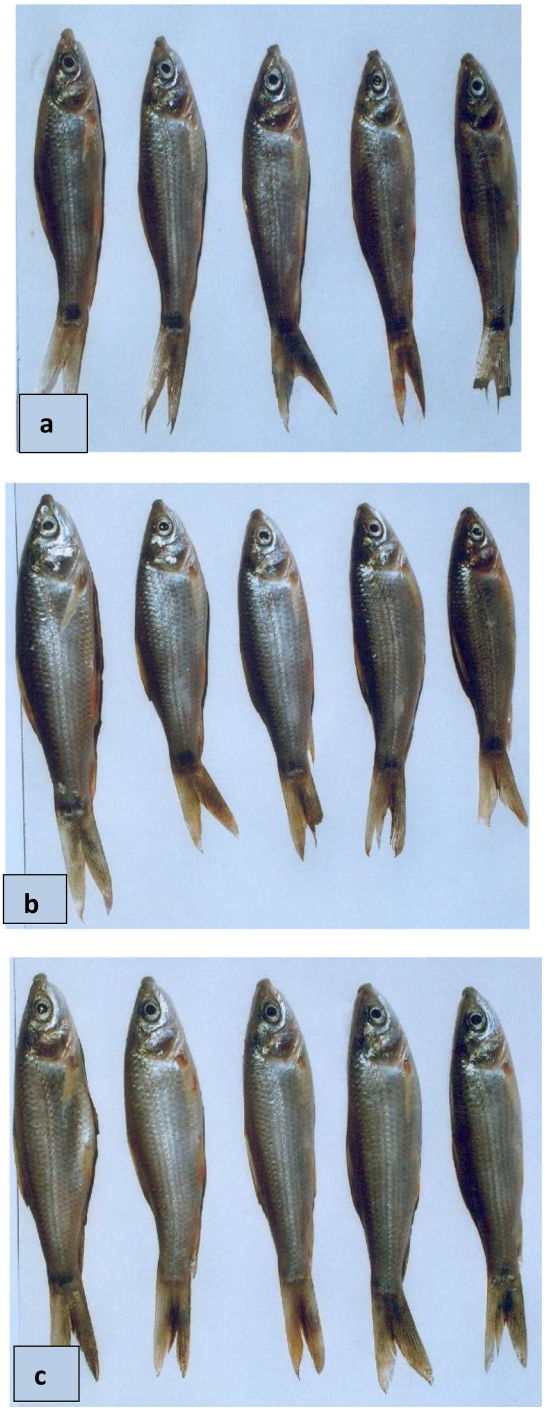

Fig. 3. Photographs of control (a), G1 (b) and G2 (c) fish groups at phase III (45 days).

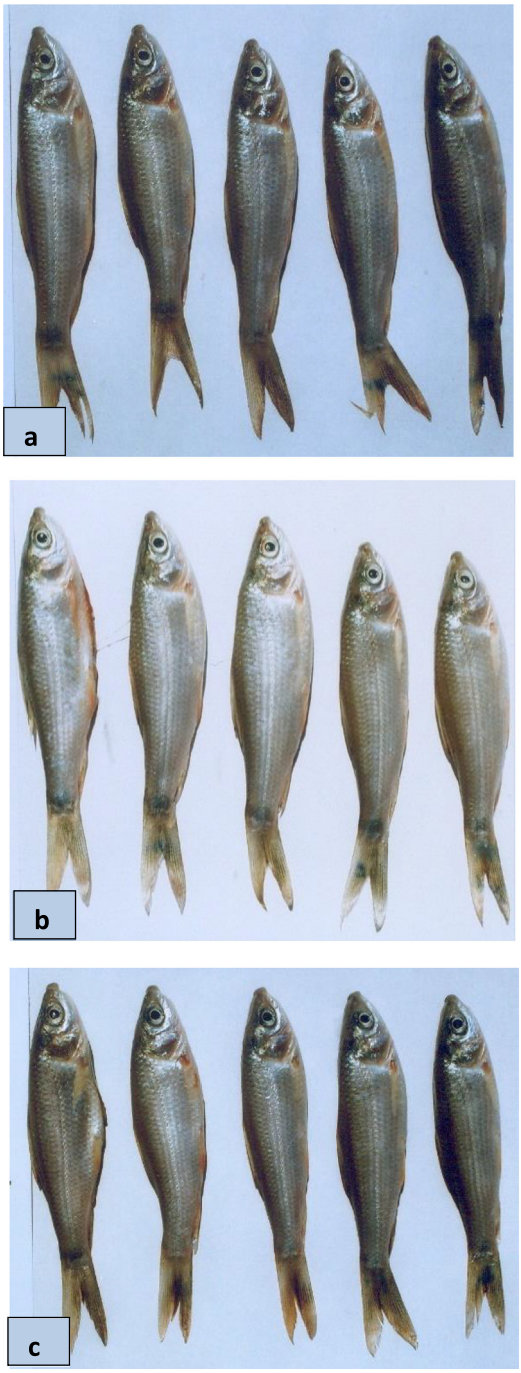

Fig. 4. Photographs of control (a), G1 (b) and G2 (c) fish groups at phase IV (60 days).

Condition factor is considered a standard practice representing good experimental conditions and isometric growth indicator (Ayode, 2011). Computation of factor is based on length weight data analysis. The study reflected the isometric growth in control (1.03–1.13) as well as experimental groups (G1, 0.91–1.07, G2, 0.99–1.50) because condition factor was observed near 1. Same results (1.64–1.79) were reported in *Oreochromis niloticus* (Ighwela et al., 2011).

Supplementation of probiotics in basal and formulated feed of fish influenced different morphometric parameters such as body weight, body length, weight gain and SGR. Such potential was reported in Nile tilapia, *Oreochromis niloticus* using *Bacillus subtilis* (Soltan and El-Laithy, 2008) and with *Enterococcus faecium* (Wang et al., 2008). Lactobacillus acidophilus improved growth profile than control group of African catfish *Clarias gariepinus* (Al-Dohail et al., 2009). Probiotics boosted the nutritional value of fish diet by vitamin production such as vitamin B_{12} and biotin and detoxification of detrimental compounds. It promote immunity by inhibiting pathogen colonization in host gut, triggering for nutrient competition and adjusting metabolism of microbes (Gibson et al., 1997; Gatesoupe, 1999).

The usage of biochemical constituent measurement of fish muscle is considered as a reliable index to assess growth nutritional

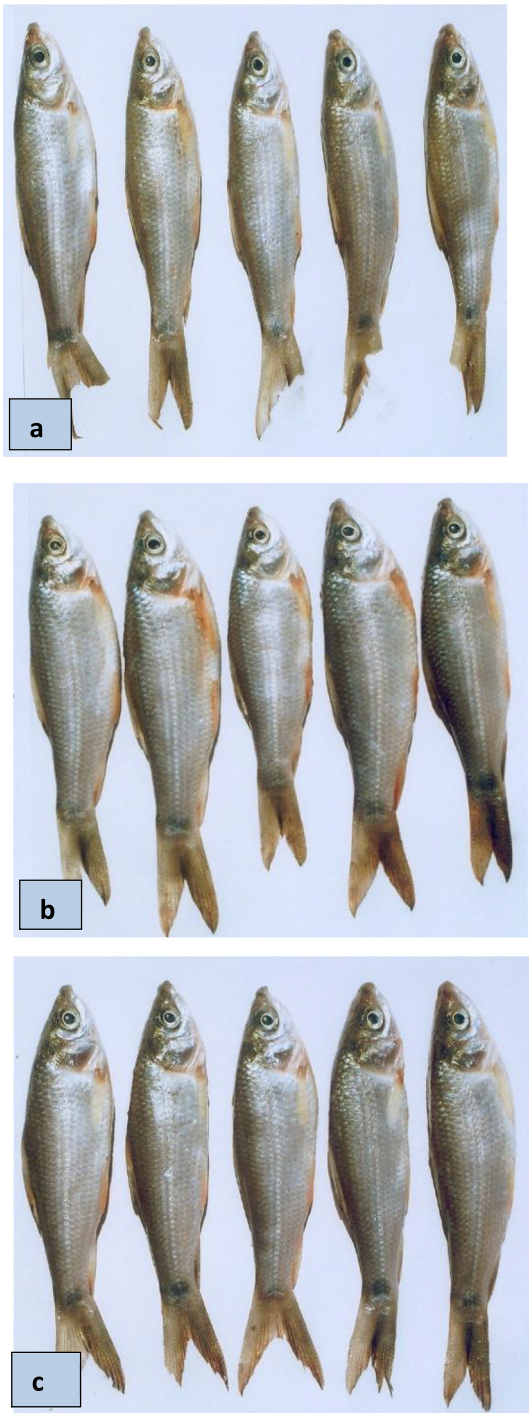

Fig. 5. Photographs of control (a) and experimental groups G1 (b), G2 (c) fishes representing phase V (75 days).

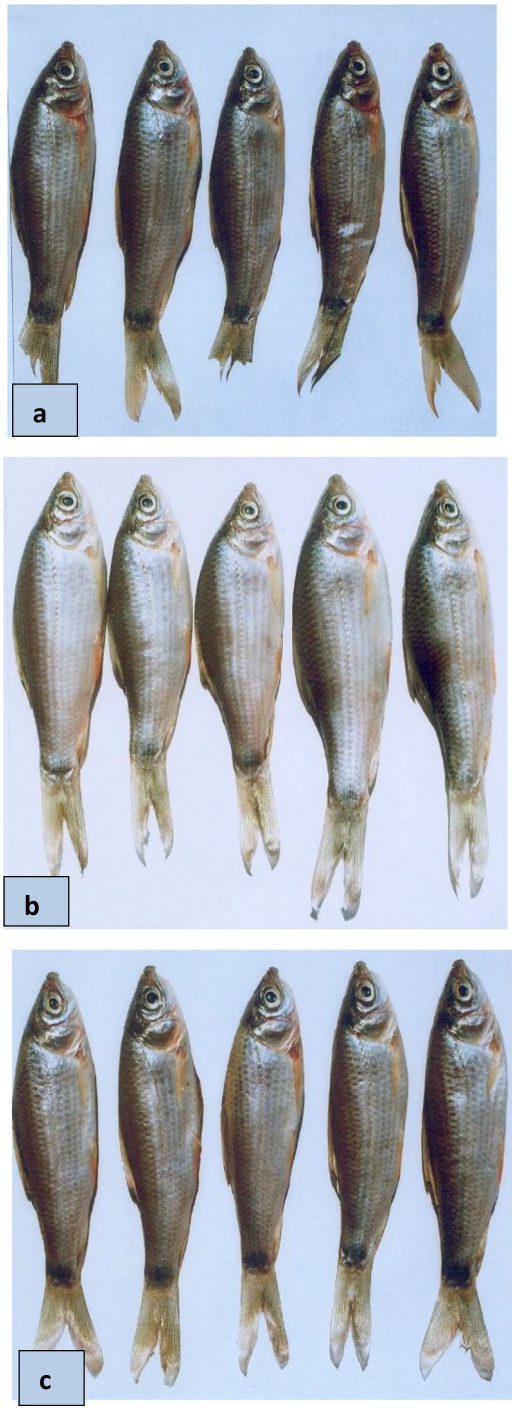

Fig. 6. Photographs of control (a) and experimental groups G1 (b), G2 (c) fishes representing phase VI (90 days).

indices and growth profile of fish fingerlings (Ghosh et al., 2003; Cnaani et al., 2004; Krishnaveni et al., 2013). *Bacillus subtilis* AsCh-A4 improved the factors to assess growth profile such as average weight, length, width of fish, Daily gain, Relative gain rate, Specific growth rate, and reduced FCR etc. Feed with live bacterium (G1) reduced the FCR in all phases and to less than 2 at end of experiment than control and G2. While PER% were accelerated in G1 and G2. The reduction of FCR is supported by PER which is evident attribute of tested probiotic (USAID 2011). Inclusion of live probiotic in fish may lead to improve GIT microflora and greater GIT activity as well as FCE and PER (Munir et al., 2016). The artificial and probiotic incorporated fish feed can be considered as good source to fulfill the nutritional requirements. The rate of growth, FCR, and chemical composition of fish may be exaggerated by feed qualitatively and quantitatively (Jena et al., 1998; Erfanullah and Jafri, 1998).

Dietary probiotics and prebiotics when administered to fish will lead to increase in muscle protein and crude lipids. Fish with high protein contents and low lipids is considered beneficial to compensate feed shortage (Wee, 1982; Annasari et al., 2012). In last phases, probiotics improved body composition from control i.e., higher deposition of protein with more or less difference in ash

Table 2
Somatic indices (%) of different organs of the sampled fish fingerlings administered with *B. subtilis* AsCh-A4 fermented feed from control and experimental groups at different phases.

Parameters	Groups	Phases (Days)						
		Zero	I(15)	II(30)	III(45)	IV(60)	V(75)	VI(90)
Kidney index	Cont	11.72 ± 0.54	11.80 ± 0.48	12.21 ± 0.41	17.60 ± 0.28	17.92 ± 0.34	19.53 ± 0.34 [a]	22.53 ± 0.26 [a]
	G1	11.27 ± 0.61	12.73 ± 0.53	13.10 ± 0.57	18.02 ± 0.33	18.19 ± 0.35	20.86 ± 0.24[b]	24.35 ± 0.23[b]
	G2	11.78 ± 0.33	12.76 ± 0.45	13.01 ± 0.61	18.03 ± 0.33	18.08 ± 0.27	19.61 ± 0.27 [a]	23.07 ± 0.21 [a]
Heart index	Cont	74.63 ± 2.29	77.92 ± 0.81 [a]	87.28 ± 1.12 [a]	92.82 ± 1.43	92.76 ± 1.46 [a]	97.44 ± 1.15 [a]	101.39 ± 1.73 [a]
	G1	74.88 ± 1.45	84.83 ± 1.41[b]	88.97 ± 1.43 [ab]	95.26 ± 2.19	96.47 ± 1.45 [ab]	99.68 ± 1.46[b]	107.33 ± 1.86 [a]
	G2	74.00 ± 1.45	86.52 ± 1.24[b]	92.89 ± 1.44[b]	94.91 ± 1.87	92.09 ± 1.49[b]	78.46 ± 1.74 [a]	81.15 ± 2.00[b]
Liver index	Cont	276.69 ± 28.77	350.75 ± 23.04	369.83 ± 17.66	380.58 ± 35.13	395.42 ± 27.69	423.29 ± 26.73	454.94 ± 30.33
	G1	275.44 ± 13.08	386.18 ± 20.89	383.76 ± 18.58	422.83 ± 13.08	387.39 ± 28.33	436.46 ± 16.12	475.08 ± 26.53
	G2	275.79 ± 16.12	385.27 ± 25.42	360.89 ± 10.63	398.30 ± 23.87	381.79 ± 23.29	427.53 ± 24.49	463.72 ± 21.13

All values represent means of three replicates ± S.E.M. Values within respective column not sharing a common alphabet differ significantly from each other at $p \leq 0.05$ at single factor analysis of variance.

contents. The result coincides with findings of Lara-Flores et al. (2003) as well as Alizade et al. (2011). Higher body protein contents implies the fact that probiotic incorporated feed was converted to structural protein more effectively and leads to more body muscle production. Non-significant difference was observed in fat, RNA and moisture contents. Similar results i.e., no difference

Table 3
Effect of probiotic bacterial isolate *B. subtilis* AsCh-A4 on biochemical components (mg/g) and enzyme activities of muscle tissues of *L. rohita* fingerlings from control and experimental groups at different phases.

Parameters		Groups	Phases (Days)						
			Zero	I(15)	II(30)	III(45)	IV(60)	V(75)	VI(90)
Biochemical Constituents	Total protein (mg/g)	Cont	11.72 ± 0.54	11.80 ± 0.48	12.21 ± 0.41	17.60 ± 0.28	17.92 ± 0.34	19.53 ± 0.34 [a]	22.53 ± 0.26 [a]
		G1	11.42 ± 0.33	12.73 ± 0.53	13.10 ± 0.57	18.02 ± 0.33	18.19 ± 0.35	24.86 ± 0.24[b]	29.35 ± 0.23[b]
		G2	11.77 ± 0.43	12.76 ± 0.45	13.01 ± 0.61	18.03 ± 0.33	18.08 ± 0.27	19.61 ± 0.27 [a]	24.07 ± 0.21 [a]
	Total carbohydrates (mg/g)	Cont	74.63 ± 2.29	77.92 ± 0.81 [a]	87.28 ± 1.12 [a]	92.82 ± 1.43	92.76 ± 1.46 [a]	97.44 ± 1.15 [a]	101.39 ± 1.73 [a]
		G1	75.66 ± 2.10	84.83 ± 1.41[b]	88.97 ± 1.43 [ab]	95.26 ± 2.19	96.47 ± 1.45 [ab]	99.68 ± 1.46[b]	107.33 ± 1.86 [a]
		G2	75.00 ± 1.80	86.52 ± 1.24[b]	92.89 ± 1.44[b]	94.91 ± 1.87	92.09 ± 1.49[b]	78.46 ± 1.74 [a]	81.15 ± 2.00[b]
	Total lipids (mg/g)	Cont	276.69 ± 28.77	350.75 ± 23.04	369.83 ± 17.66	380.58 ± 35.13	395.42 ± 27.69	423.29 ± 26.73	454.94 ± 30.33
		G1	276.11 ± 11.85	386.18 ± 20.89	383.76 ± 18.58	422.83 ± 13.08	387.39 ± 28.33	436.46 ± 16.12	475.08 ± 26.53
		G2	276.00 ± 15.07	385.27 ± 25.42	360.89 ± 10.63	398.30 ± 23.87	381.79 ± 23.29	427.53 ± 24.49	463.72 ± 21.13
	Cholestrol (mg/g)	Cont	69.05 ± 14.17	75.57 ± 7.05	79.27 ± 5.67	78.03 ± 6.33	98.32 ± 2.56	113.99 ± 13.87	150.61 ± 8.96
		G1	69.89 ± 3.55	80.13 ± 4.77	90.83 ± 4.45	84.25 ± 5.66	100.54 ± 4.61	143.57 ± 6.70	153.76 ± 11.77
		G2	70.44 ± 11.87	77.61 ± 5.96	79.69 ± 4.67	84.40 ± 4.09	99.68 ± 3.99	147.88 ± 12.08	152.26 ± 8.78
	DNA (μg/g)	Cont	0.45 ± 0.02	0.83 ± 0.42	0.34 ± 0.03 [a]	0.49 ± 0.05[a]	0.53 ± 0.02 [a]	0.71 ± 0.04	0.59 ± 0.08
		G1	0.44 ± 0.08	0.47 ± 0.04	0.44 ± 0.03 [ab]	0.50 ± 0.007 [a]	0.51 ± 0.04[b]	0.51 ± 0.04	0.70 ± 0.04
		G2	0.45 ± 0.02	0.80 ± 0.06	0.49 ± 0.06[b]	0.69 ± 0.04[b]	0.59 ± 0.05 [ab]	0.63 ± 0.06	0.70 ± 0.05
	RNA (μg/g)	Cont	2.93 ± 0.05	2.99 ± 0.31	2.59 ± 0.13	3.10 ± 0.13	3.31 ± 0.08	3.62 ± 0.06	4.01 ± 0.04
		G1	3.15 ± 0.07	2.88 ± 0.05	2.26 ± 0.11	3.08 ± 0.13	3.27 ± 0.08	3.70 ± 0.05	3.99 ± 0.05
		G2	3.22 ± 0.18	3.01 ± 0.13	2.59 ± 0.15	3.06 ± 0.12	3.21 ± 0.06	3.58 ± 0.04	3.97 ± 0.05
	Ash contents (%)	Cont	4.68 ± 0.27	5.73 ± 0.25 [a]	6.32 ± 0.19 [a]	8.96 ± 0.21 [a]	9.20 ± 0.26 [a]	10.14 ± 0.32 [a]	11.03 ± 0.20 [a]
		G1	4.44 ± 0.10	6.90 ± 0.19[b]	7.68 ± 0.23 [a]	9.80 ± 0.24[b]	10.65 ± 0.20[b]	12.15 ± 0.30[b]	14.84 ± 0.18[b]
		G2	4.03 ± 0.38	7.05 ± 0.20[b]	8.53 ± 0.28[b]	9.11 ± 0.18 [ab]	8.24 ± 0.25[c]	10.38 ± 0.44 [a]	11.54 ± 0.21 [a]
	Moisture contents (%)	Cont	69.05 ± 14.17	75.57 ± 7.05 [a]	79.27 ± 5.67	78.03 ± 6.33 [a]	78.32 ± 2.56	73.99 ± 13.87 [a]	70.61 ± 8.96
		G1	68.77 ± 2.87	80.13 ± 4.77[b]	80.83 ± 4.45	74.25 ± 5.66[b]	80.54 ± 4.61	73.57 ± 6.70 [a]	71.76 ± 11.77
		G2	69.23 ± 3.09	77.61 ± 5.96 [ab]	79.69 ± 4.67	74.40 ± 4.09[b]	79.68 ± 3.99	77.88 ± 12.08[b]	71.26 ± 8.78
Enzyme Profiling	Acid phosphatase IU/g	Cont	0.04 ± 0.01	0.07 ± 0.007	0.12 ± 0.02[a]	0.12 ± 0.02 [a]	0.24 ± 0.03 [a]	0.14 ± 0.03	0.15 ± 0.02
		G1	0.04 ± 0.03	0.05 ± 0.006	0.06 ± 0.01[b]	0.07 ± 0.01[b]	0.18 ± 0.03 [ab]	0.11 ± 0.01	0.14 ± 0.02
		G2	0.04 ± 0.02	0.05 ± 0.004	0.08 ± 0.01 [ab]	0.09 ± 0.01 [ab]	0.11 ± 0.02[b]	0.13 ± 0.01	0.13 ± 0.02
	Alkaline phosphatase IU/g	Cont	0.11 ± 0.03	0.09 ± 0.02	0.15 ± 0.03 [ab]	0.06 ± 0.01	0.16 ± 0.05	0.21 ± 0.01	0.24 ± 0.05
		G1	0.10 ± 0.01	0.19 ± 0.03	0.23 ± 0.05 [a]	0.06 ± 0.01	0.17 ± 0.03	0.18 ± 0.04	0.22 ± 0.03
		G2	0.11 ± 0.01	0.19 ± 0.03	0.08 ± 0.01[b]	0.07 ± 0.01	0.23 ± 0.10	0.20 ± 0.11	0.23 ± 0.05
	AST IU/g	Cont	5.48 ± 0.48	5.81 ± 2.26	5.22 ± 0.28 [a]	7.46 ± 0.46 [a]	6.99 ± 0.81	5.98 ± 0.70	6.22 ± 0.46
		G1	5.23 ± 0.51	5.78 ± 0.79	3.69 ± 0.34[b]	5.22 ± 0.28[b]	6.68 ± 0.57	5.65 ± 0.29	5.98 ± 0.24
		G2	4.21 ± 0.12	4.96 ± 0.33	4.37 ± 0.20 [ab]	4.99 ± 0.35[b]	6.79 ± 0.77	5.53 ± 0.38	6.15 ± 0.22
	ALT IU/g	Cont	1.63 ± 0.48	2.66 ± 1.06	2.42 ± 0.30 [a]	3.78 ± 1.09	5.29 ± 1.13	6.47 ± 1.41	7.0 ± 0.78
		G1	1.22 ± 0.11	1.95 ± 0.26	5.12 ± 0.79[b]	3.30 ± 0.42	5.10 ± 1.09	6.91 ± 0.45	7.45 ± 0.81
		G2	1.59 ± 0.22	2.41 ± 0.15	4.71 ± 0.76 [ab]	1.87 ± 0.24	3.21 ± 0.66	5.26 ± 1.42	9.06 ± 0.74
	Protease μM/g	Cont	0.09 ± 0.01	0.11 ± 0.06	0.24 ± 0.02 [ab]	0.19 ± 0.09	0.12 ± 0.03	0.17 ± 0.02	0.24 ± 0.04
		G1	0.10 ± 0.04	0.19 ± 0.02	0.31 ± 0.06 [a]	0.11 ± 0.02	0.11 ± 0.01	0.13 ± 0.01	0.20 ± 0.03
		G2	0.08 ± 0.01	0.15 ± 0.02	0.13 ± 0.03[b]	0.09 ± 0.02	0.10 ± 0.02	0.13 ± 0.01	0.23 ± 0.03
	Amylase mM/g	Cont	2.46 ± 0.52	1.88 ± 0.45	1.93 ± 0.21 [a]	1.57 ± 0.18	0.90 ± 0.20	1.69 ± 0.26	2.36 ± 0.16
		G1	2.16 ± 0.21	2.50 ± 0.24	3.52 ± 0.53[b]	1.50 ± 0.58	1.22 ± 0.28	1.65 ± 0.21	2.33 ± 0.16
		G2	2.44 ± 0.01	1.78 ± 0.22	1.61 ± 0.28 [a]	1.45 ± 0.51	0.91 ± 0.12	1.34 ± 0.17	1.95 ± 0.27

All values represent means of three replicates ± S.E.M. Values within respective column not sharing a common alphabet differ significantly from each other at $p \leq 0.05$ at single factor analysis of variance.
n = 15 for each control and experimental groups.

in body moisture and fat were reported by Eid and Mohmad (2008) and Hassaan et al. (2014) in *Oreochromis niloticus* fingerlings. Highest DNA in two phases were recorded which is corroborated with Krishnaveni et al. (2013) and Khan and Jafri (1991). DNA and RNA are considered a good indicator for fish growth assessment (Smith and Buckley, 2003; Mukherjee and Jana, 2007).

The fluctuation in protein, fat and carbohydrates in fish muscle tissue can be associated to the deposition and synthesis rate in fish (Abdel-Tawwab et al., 2006). In present study, the role of *Bacillus subtilis* AsCh-A4 in fish feed cannot be ignored in enhancement of feed intake, growth and body composition. This genus has ability of producing enzymes, antibiotics and amino acids and is not associated with fish pathologies (Moriarty, 1998; Sanders et al., 2003; Gullian et al., 2004). Ray et al. (2012) reported the *Bacillus* sp. as immunostimulant and posing adhesion and bacteriocin producing abilities. Growth, survival and carcass composition improvement by dietary provision of Bacillus has been recorded in rainbow trout fry and fresh water prawn *Macrobrachoum rosenbergii* post larvae (Alizadeh et al., 2011; Seenivasan et al., 2013).

In later phases of experiment, enzymes stabilizes and no significant differences were noted. Decreased ALT and AST levels were observed in Nile tilapia when administered with probiotic enriched diet (Soltan and El-Laithy 2008) and diet supplemented pseudomonas as well as *Micrococcus luteus* and *Pseudomonas* sp. consortium (Wache¢ et al., 2006. Similarly, fish fed with probiotics i.e., dead *S. cerevisae* as well as live *B. subtilis* and *S. cerevisae* mixture reflected decrease in ALT and AST than control (Marzouk et al., 2008).

Aminotransferases indicated the damage at cellular level and tissue amelioration due to stress. It is considered to identify dysfunction in fish tissue or organ as an enzymatic biomarker. Aspartate aminotransferases are considered to be involved in transamination process that are helpful in protein and carbohydrate metabolism by converting alanine and α-ketoglutarate to glutamic and pyruvic acid (Salah El-Deen and Rogers, 1993; Philip and Rajarsee, 1996; Palanivelu et al. 2005; Gabriel and George, 2005). Alanine aminotransferases are indicator for ameliorative changes in tissues by assessing physiological and biochemical dysfunction and enzyme leakage from liver to cytosol than blood (Kumar et al., 2012). Phosphatases are considered multifunctional enzyme biomarkers to probe the response of cells against stresses in form of toxic pollutants. Alkaline phosphatase mediated mineralization in aquatic animal's skeleton (Lan et al. 1995; Lohner et al., 2001; Zikic et al. 2001). Probiotics and prebiotics supplemented feeds mediate intestinal micro flora to secrete major enzymes such as amylase to stimulate digestion (Xu et al., 2003; Yanbo and Zirong 2006; Essa et al., 2010; Askarian et al., 2011; Sang et al., 2011; Wu et al., 2014).

5. Conclusion

The tested probiotic established the effectiveness in *L. rohita* fingerlings growth, high PER, increased protein contents, low FCR and fat when incorporated in feed with 10% (288×10^5 CFU/mL) inoculum. The feed with live *Bacillus subtilis* AsCh-A4 may be proved economically viable. However, this study will provide base to explore more about nutrient digestibility, gut microflora, blood parameters, and immunity etc. for *L. rohita* fingerlings.

Ethical Statement

It is to certify that during experimental research entitled "Impact of *Bacillus subtilis* supplemented feed on growth and biochemical constituents in *Labeo rohita* Fingerlings", none of the *Labeo rohita* fingerlings was subjected to killing unless international guidelines for rearing and care of fish were strictly followed.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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