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Growth performance, nutrient digestibility and digestive enzyme activity in Asian seabass, *Lates calcarifer* juveniles fed diets supplemented with cellulolytic and amylolytic gut bacteria isolated from brackishwater fish

Debasis De, T. K. Ghoshal, R. Ananda Raja & Sujeet Kumar

Kakdwip Research Centre of Central Institute of Brackishwater Aquaculture (ICAR), Kakdwip, West Bengal, India

Correspondence: D De. Sr. Scientist, KRC of CIBA (ICAR), Kakdwip, South 24 Pgs, WB-743347, India. E-mails: debasiskrc@yahoo.com; dedebasis47@gmail.com

Abstract

Asian seabass, *Lates calcarifer* juveniles were fed for 30 days to evaluate the efficacy of cellulolytic (*Bacillus* sp. DDKRC1.) and amylolytic (*Bacillus subtilis* DDKRC5.) bacteria supplemented diets. Four experimental groups were maintained in triplicate of which group I was the control group and received no bacterial supplementation. Groups II, III and IV were fed diet supplemented with *B. subtilis* DDKRC5. (14.25×10^7 CFU/mL), *Bacillus* sp. DDKRC1. (2.94×10^7 CFU/mL) and mixture of both the microbes (1:1 ratio), respectively, @ 1% (v/w). After 30 days of feeding, fish reared as group IV showed significantly higher ($P < 0.01$) weight gain (141.42%), survival (91.50%), protein efficiency ratio (1.18) and significantly lower ($P < 0.01$) FCR (2.15) than that of other groups.

Nutrient digestibility parameters were significantly ($P < 0.01$) higher in group IV followed by groups III, II and I. Digestive cellulase, amylase and protease activities in the gastrointestinal tract were also significantly ($P < 0.01$) higher in group IV. Amylolytic and cellulolytic microbial count in the gastrointestinal (GI) tract were significantly ($P < 0.01$) higher in groups II, III and IV as compared with that of control. It can be concluded that feed supplemented with mixture of *B. subtilis* DDKRC5. and *Bacillus* sp. DDKRC1. showed better performance in Asian seabass juveniles.

Keywords: amylolytic bacteria, Asian seabass, cellulolytic bacteria, digestibility, gut microbiota

Introduction

Feed constitute 50–60% of total operational cost of intensive aquaculture (Sinha, Kumar, Makkar, De Boeck & Becker 2011). For successful and profitable culture of Asian seabass (*Lates calcarifer*), good quality formulated feed is essential. Asian seabass is a carnivorous fish and need high protein (40–50%) diet for their growth (Davis 1987; Sakaras, Boonyaratpalin, Unpraser & Kumpang 1988; Sakaras, Boonyarapalin, Unpraser & Kumpang 1989; Boonyaratpalin 1997; Williams, Barlow, Rodgers, Hockings, Agcopra & Ruscoe 2003; Ali, Ambasankar, Syamadayal, Thirunnavukarasu, Kailasam, Sundaray & Ponniah 2012). Fish meal is the major ingredient in practical diet of seabass to meet the high protein requirement. Due to uncertainty in supply of good quality fish meal, increasing competition from livestock sector and rising cost, the efforts have been made to replace fish meal by alternative plant protein (Dabrowski, Poczyczynski, Kock & Berger 1989; Olli, Berg-Lea & Krogdahl 1989; Wee & Shu 1989; Pongmaneerat & Watanabe 1993; Kaushik, Covès, Dutto & Blanc 2004; Torstensen, Espe, Sanden, Stubhaug, Waagbø, Hemre, Fontanillas, Nordgarden, Hevrøy, Olsvik & Berntssen 2008; Hemre, Amlund, Aursand, Bakke, Olsen, Ringø & Svihus 2009). However, protein source of plant origin is rich in cellulose, which being monogastric fish cannot utilize efficiently (Sinha *et al.* 2011). Moreover, carnivorous fish utilize starch less efficiently compared with

herbivores due to low endogenous amylase activity (Sarbahi 1951; Dhage1968). Cellulolytic microbes break complex ligno-cellulosic bond in feed ingredients of plant origin and amylolytic microbes degrade starch to a simple sugar by secreting digestive enzymes in the gut (Verschuere, Rombaut, Sorgeloos & Verstraete 2000; Balcazar, de Blas, Ruiz-Zarzuela, Cunningham, Vendrell & Muzquiz 2006; Kesarcodi-Watson, Kaspar, Lategan & Gibson 2008). Use of microbial supplement improves nutrient utilization and growth of African catfish, *Clarias gariepinus* (Al-Dohail, Hashim & Aliyu-Paiko 2009), Senegalese sole, *Solea senegalensis* (Saenz de Rodriganez, Diaz-Rosales, Chabrillon, Smidt, Arijo, Leon-Rubio, Alarcon, Balebona, Morinigo, Cara & Moyano 2009), tilapia, *Oreochromis niloticus* (Lara-Flores, Olvera-Novoa, Guzmán-Méndez & López-Madrid 2003; El-Haroun, Goda & Kabir Chowdhury 2006), Japanese flounder, *Paralichthys olivaceus* (Taoka, Maeda, Jo, Jeon, Bai, Lee, Yuge & Koshio 2006), gilthead seabream, *Sparus aurata* and Seabass, *Dicentrarchus labrax* (Carnevali, de Vivo, Sulpizio, Gioacchini, Olivotto, Silvi & Cresci 2006).

Cellulolytic and amylolytic activity of gut microbes have been reported from the gastrointestinal tract of different fresh water fish (Bairagi, Sarkar Ghosh, Sen & Ray 2004; Saha, Roy, Sen & Ray 2006; Mondal, Roy, Sen & Ray 2008; Ghosh, Roy, Kar & Ringø 2010; Ray, Roy, Mondal & Ringø 2010) and brackishwater fish (Rani, Garg, Sabhlok & Bhatnagar 2004). Reduction in the crude fibre, cellulose, hemicellulose and antinutritional factors were observed in *Labeo rohita* due to fermentation with gut microbes (Bairagi *et al.* 2004; Saha & Ray 2011;). *In vitro* fermentation with bacterial strain secreting cellulase, amylase and protease reduces the crude fibre and soluble carbohydrate level and increase the protein content in feed ingredients (Saha & Ray 2011; De, Ghoshal & Ananda Raja 2012). Supplementation of these microbes with fish feed may help to utilize cellulose and starch more efficiently and improve the growth performance of fish. However, there is dearth of information with regard to application of live cellulolytic and amylolytic gut microbes in Asian seabass. In view of this, present study was intended to evaluate the efficacy of the selected cellulolytic and amylolytic bacterial strains as feed supplement for *L. calcarifer* juveniles fed plant protein incorporated diets.

Materials and methods

Bacterial strain

Bacillus sp. DDKRC1. (JN641289), isolated from the distal intestine of *L. calcarifer*, served as potential cellulolytic bacteria. Another bacterial strain *B. subtilis* DDKRC5. (JN641293), isolated from the distal intestine of Milk fish (*Chanos chanos),* acted as potential amylolytic bacteria. These two bacterial strains were supplemented in the diet of Asian seabass.

Weaning and acclimatization of seabass fry

Asian seabass (*Lates calcarifer*) juveniles were collected from Muriganga estuary at Kakdwip (Lat. 21°51′15.01″–21°51′30.77″N, Long. 88°10′58.44″–88°11′12.09″E), South 24 Parganas, West Bengal, India. The seabass juveniles were stocked for weaning in fibre-reinforced plastic (FRP) tanks (100 L) filled with strained dechlorinated brackishwater. Before starting the experiment, seabass fries were fed weaning diet (semi moist) containing boiled fish flesh (*Liza parsia*) and wheat flour (80:20) for 30 days. The weaning diet contained 63.74% crude protein and 5.55 Kcal/g gross energy. Subsequently, fish were acclimatized on control diet for 7 days before starting the experiment.

Experimental diets

Four isonitrogenous experimental diets (39.5% CP) were formulated (Table 1). All the feed ingredients, except bacterial culture, mineral-vitamin mixture, amino acid mixture and fish oil, were mixed with water and cooked in autoclave for 20 min with exposure at 15 psi, 121 °C for 5 min. The remaining ingredients including bacterial culture were mixed after cooling the dough. Dough was passed through a mincer with a die (2 mm diameter) to get spaghetti-like strings. Feed strings were air dried for 1 h at ambient temperature and fed to fish. Feed was prepared twice daily before feeding to maintain the efficacy of bacterial culture.

The control diet (D1) had no bacterial supplementation. Diets D2, D3 and D4 were formulated with all the ingredients similar to control diet but supplemented with *B. subtilis* DDKRC5. (14.25×10^7 CFU/mL), *Bacillus* sp. DDKRC1. (2.94×10^7 CFU/mL) and mixture of *B. subtilis* DDKRC5. and *Bacillus* sp. DDKRC1. (1:1) respectively. Both the

Table 1 Composition of practical diet (on dry weight basis) for Asian seabass

Ingredient (% in feed)	D1	D2	D3	D4
Fish meal	20	20	20	20
Poultry offal	24	24	24	24
Groundnut oil cake	10	10	10	10
Mustard oil cake	5	5	5	5
Soybean meal	17	17	17	17
Wheat flour	12.5	12.5	12.5	12.5
Lecithin	3	3	3	3
Fish oil	3	3	3	3
Mineral & Vit Mix. & Vit C*	3	3	3	3
Amino acid mix†	3	3	3	3
Cr_20_3	0.5	0.5	0.5	0.5
B. subtilis DDKRC5. (10^7 CFU/mL)	–	1% (v/w)	–	–
Bacillus sp. DDKRC1. (2.94×10^7 CFU/mL)	–	–	1% (v/w)	–
B. subtilis DDKRC5. (14.25×10^7 CFU/mL)+ *Bacillus* sp. DDKRC1 (2.25×10^7 CFU/mL) (1:1)	–	–	–	1% (v/w)

*Vitamin mix (mg/100 g) Vitamin A 2.0, Vitamin D 0.4, Vitamin E 12.0, Vitamin K 6.0, Choline chloride 600.0, Thiamine 18.0, Riboflavin 24.0, Pyridoxine 18.0, Niacin 108.0, Pantothenic acid 72.0, Biotin 0.2, Folic acid 3.0, Vitamin B12 0.015, Inositol 150.0, Vitamin C 900.0. Mineral mix (g/kg) CaCO3 28.0, K2SO410.0, MgSO4 12.5, CuSO4 0.2, FeCl3 0.5, MnSO40.5, KI 0.01; ZnSO4 1.0, CoSO4 0.01, Cr2SO4 0.05, Bread flour 7.14.

†Amino acid mix(g/100 g dry diet) L- Phenylalanine 0.6, L- Arginine HC 1.3, L- Cystine 0.7,L-Tryptophan 0.2, L- Histidine HC 0.2, DL- Alanine 1.3, L- Aspargine Na 1.0, L- lysine HC 0.7, L- Valine 0.7, Glycine 0.4.

bacteria were grown in nutrient broth for 24 h at 34 °C and used for incorporation in diet. The 34 °C temperature selected based on maximum growth of these two microbes at this temperature (Bairagi, Sarkar Ghosh, Sen & Ray 2002; Mondal *et al.* 2008; De, Ghoshal & Kundu 2012; De *et al.* 2012).

Experimental set up

The feeding trial was conducted for 30 days with four groups of weaned *L. calcarifer* fries (3.70 ± 0.01 g). The experiment was performed in triplicates with 10 fish per replicate, in 12 FRP tanks filled with 100 L dechlorinated brackish-water. Brackishwater was taken from nearby creek connected with Muriganga estuary. Uninterrupted aeration was provided in all the tanks.

Fish of groups I, II, III and IV were fed diet D1, D2, D3 and D4 respectively (Table 1). Feeding was done twice daily at 10:00 hours and 17:00 hours, at 10% of the total body weight. The daily ration was adjusted every tenth day after weighing the fish from each replicate. Everyday 20% water was exchanged for maintaining the water quality. The uneaten feed was syphoned out 2 h after each feeding, and oven dried at 60 °C for 24 h to calculate the diet conversion ratio. The faecal samples voided by the fish were collected daily from each tank by pipetting (Spyridakis, Metailler, Gabaudan & Riaza 1989). The oven dried (60 °C) faecal samples were analysed for proximate principles. Water quality parameter were analysed (APHA 1998) at weekly interval. At the end of the feeding trial, fish from each treatment were sacrificed to study total heterotrophic counts, amylolytic, cellulolytic and proteolytic bacterial count in gut, intestinal enzyme activity and body composition of fish of different groups.

Chemical analysis of feed, faecal and fish tissue samples

The proximate principles of feed, faecal and fish tissue samples were determined following AOAC (Association of Official Analytical Chemists) (1995). Hemicellulose and cellulose content of feed and faecal samples were measured according to the Van Soest (1967) method. Chromic oxide levels in the diets and faecal samples were estimated by wet digestion method (Furukawa & Tsukahara 1966). Average live weight gain (%), specific growth rate (SGR% day^{-1}), feed conversion ratio (FCR) and protein efficiency ratio (PER) were calculated using standard methods.

The apparent digestibility coefficients (ADC) of nutrients were calculated using the following formula (De Silva & Anderson 1995):

$$\text{Digestibility coefficient (\%)} = 100 - \frac{\%\ Cr_2O_3 \text{in diet}}{\%\ Cr_2O_3 \text{in faeces}} \times \frac{\%\ \text{nutrient in faeces}}{\%\ \text{nutrient in diet}} \times 100$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Feed distributed in dry weight}}{\text{Increase in live weight}}$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{Live weight gain}}{\text{Protein consumed in dry weight}}$$

Digestive enzyme assay

Fish from each experimental group were dissected on an ice tray to remove the gastrointestinal tract (GI) to determine the digestive enzyme activities at the end of the feeding trials. After evisceration, the whole GI tract was homogenized with five times (w/v) of sterile chilled phosphate-buffered saline with 0.9% NaCl (pH 7.2). Homogenate was centrifuged at 10 000 *g* for 1 h at 4 °C. The supernatant was collected and used for enzyme assay. Cellulase activity was assayed using 1% Carboxy Methyl Cellulose in citrate buffer (0.1 M, pH 6.75) as substrate (Denison & Koehn 1977). Amylase activity was measured using 1% soluble starch in phosphate buffer (0.02 M; pH 6.9 containing 0.0067 M NaCl) as substrate (Bernfeld 1955). Protease activity was detected by caseinase assay method using 0.5% casein in Tris-HCl buffer (0.02 M, pH 7.0) as substrate (Walter 1984).

Quantification of cellulolytic, amylolytic, proteolytic and culturable heterotrophic bacteria

Five fish from each experimental group were dissected on an ice tray to remove the GI tract to determine the intestinal microbial population at the end of the feeding trials. The entire GI tract was homogenized with five times (w/v) of sterile chilled phosphate-buffered saline with 0.9% NaCl (pH 7.2). The homogenate of the intestine of each test fish was 10-fold serially diluted and 0.1 mL of each dilution was aseptically poured on tryptone soya agar (HiMedia, Mumbai, India), carboxymethyl cellulose agar, starch agar and peptone gelatin agar plates in duplicate for total heterotrophic, cellulolytic, amylolytic and proteolytic bacterial count (Bairagi *et al.* 2002). Plates were incubated at 30 °C for 48 h and colony-forming units were determined (Rahmatullah & Beveridge 1993). At the end of the experiment, total *Vibrio* count of water was done using thiosulphate citrate bile salts sucrose (TCBS) agar.

Statistical analysis

The experimental data were subjected to analysis of variance (ANOVA) to test the significance among the treatments. One-way analysis of variance, followed by Duncan's multiple range test (Duncan 1955) was applied to find out the significant difference between the treatments, using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

The proximate composition (Table 2) of four formulated diets did not vary significantly ($P > 0.05$). The crude protein, hemicellulose, cellulose, lipid, and ash content varied from 39.48 to 39.59, 19.22 to 19.39, 8.67 to 8.81, 9.18 to 9.22, and 5.52 to 5.57% respectively.

The dissolved oxygen, pH, temperature, salinity, alkalinity, nitrate, nitrite and total ammonia nitrogen level of water in experimental tanks did not differ significantly between the groups and were within the normal range. At the end of experiment, culturable heterotrophic *Vibrio* count was significantly ($P < 0.05$) higher in control group ($1.24 \pm 0.01 \times 10^2$ CFU/mL) as compared with that of groups II, III and IV. Furthermore, among the treatment groups, group IV had significantly ($P < 0.05$) lower *Vibrio* count $0.03 \pm 0.00 \times 10^2$ CFU/mL as compared with that of groups II ($0.423 \pm 0.03 \times 10^2$ CFU/mL) and III($0.423 \pm 0.03 \times 10^2$ CFU/mL).

From growth performance study, it was observed that final body weight, total weight gain, average daily gain and weight gain per cent were significantly higher ($P < 0.01$) in group IV as compared with those of groups III, II and I (Table 3). Total weight gain and average daily gain did not differ significantly ($P > 0.05$) between group II and group III, but was significantly higher than those of group I.

Fish of group IV had significantly higher ($P < 0.01$) growth (weight gain: 141.42%), survival (93.33%), protein efficiency ratio (1.18) and significantly lower ($P < 0.01$) FCR (2.15) than those of other groups (Table 3). Fish of groups II

Table 2 Proximate composition (% of dry weight) of experimental diets

	Diets			
Parameters	**D1**	**D2**	**D3**	**D4**
Dry Matter	54.59 ± 0.04	54.58 ± 0.11	54.59 ± 0.05	54.59 ± 0.16
Crude protein	39.48 ± 0.03	39.54 ± 0.29	39.53 ± 0.14	39.59 ± 0.24
Hemi cellulose	19.31 ± 0.07	19.39 ± 0.11	19.22 ± 0.01	19.29 ± 0.12
Cellulose	5.60 ± 0.03	5.62 ± 1.05	5.67 ± 0.88	5.61 ± 0.05
Ether extract	9.21 ± 0.07	9.18 ± 0.05	9.22 ± 0.02	9.21 ± 0.06
Ash	12.57 ± 0.03	12.52 ± 0.03	12.57 ± 0.14	12.57 ± 0.11
NFE	35.52 ± 0.02	35.61 ± 0.15	35.49 ± 0.05	35.55 ± 0.22

D1, control diet; D2, diet supplemented with live bacteria *B. subtilis* DDKRC5; D3, diet supplemented with live bacteria *Bacillus* sp. DDKRC1; D4, diet supplemented with mixture of live bacteria (*B. subtilis* DDKRC5. & *Bacillus* sp. DDKRC1.); NFE, nitrogen-free extract.

Table 3 Growth performance of Asian seabass fed live microbial supplemented diet

	Treatment groups			
Parameters	**Group I**	**Group II**	**Group III**	**Group IV**
Initial body wt. (g)	3.70 ± 0.01	3.70 ± 0.02	3.70 ± 0.02	3.70 ± 0.02
Final body wt. (g)**	7.16 ± 0.04^{a}	8.23 ± 0.08^{b}	8.41 ± 0.05^{b}	8.93 ± 0.11^{c}
Total wt. gain (g)**	3.46 ± 0.03^{a}	4.53 ± 0.05^{b}	4.71 ± 0.03^{b}	5.23 ± 0.08^{c}
ADG (mg/d)**	115.45 ± 1.22^{a}	150.89 ± 1.95^{b}	157.11 ± 1.11^{b}	174.45 ± 2.89^{c}
Weight gain per cent**	93.60 ± 0.75^{a}	122.33 ± 0.76^{b}	127.39 ± 0.52^{c}	141.42 ± 2.76^{d}
FCR**	3.25 ± 0.03^{d}	2.49 ± 0.03^{c}	2.38 ± 0.01^{b}	2.15 ± 0.03^{a}
SGR (%)**	2.20 ± 0.01^{a}	2.66 ± 0.0^{b}	2.74 ± 0.00^{c}	2.93 ± 0.02^{d}
PER**	0.78 ± 0.01^{a}	1.02 ± 0.01^{b}	1.06 ± 0.01^{b}	1.18 ± 0.01^{c}
Survival (%)**	70.00 ± 5.77^{a}	83.33 ± 6.67^{ab}	86.67 ± 3.33^{b}	93.33 ± 3.33^{b}

Group I, fed diet D1; Group II, fed diet D2 and Group III, fed diet D3; and Group IV, fed diet D4. a, b, c: values bearing different superscript in a row differ significantly.
$^{**}P < 0.01$.

Table 4 Apparent nutrient digestibility in *L. calcarifer* fed different diets

	Treatment groups			
Digestibility (%)	**Group I**	**Group II**	**Group III**	**Group IV**
Dry matter**	68.71 ± 0.24^{a}	71.47 ± 0.15^{b}	71.64 ± 0.18^{b}	73.78 ± 0.24^{c}
Organic matter**	69.82 ± 0.20^{a}	72.31 ± 0.13^{b}	72.52 ± 0.18^{b}	74.46 ± 0.20^{c}
Hemi cellulose**	63.07 ± 0.80^{a}	64.12 ± 0.27^{b}	64.74 ± 0.10^{c}	65.10 ± 0.09^{d}
Cellulose**	59.21 ± 0.15^{a}	62.31 ± 0.22^{b}	64.08 ± 0.23^{c}	64.77 ± 0.51^{c}
Crude protein**	91.39 ± 0.17^{a}	93.49 ± 0.11^{b}	94.07 ± 0.12^{c}	95.86 ± 0.17^{d}
Crude lipid**	97.12 ± 0.06^{a}	98.02 ± 0.04^{b}	98.29 ± 0.06^{c}	98.78 ± 0.03^{d}
NFE**	75.33 ± 0.24^{a}	80.91 ± 0.29^{bc}	78.33 ± 0.45^{b}	84.33 ± 0.21^{c}

Group I, fed diet D1; Group II, fed diet D2; Group III, fed diet D3; and Group IV, fed diet D4.
$^{**}P < 0.01$; a, b, c, d: values bearing different superscript in a row differ significantly.

and III also showed higher ($P < 0.01$) growth, PER, SGR and significantly lower ($P < 0.01$) FCR than those of the control group. SGR and FCR were significantly ($P < 0.01$) better in group III as compared with that of group II. Survival was significantly ($P < 0.01$) higher in groups III and IV compared with the control.

Dry matter, organic matter, hemicellulose, lipid and protein digestibility were significantly ($P < 0.01$) higher in group IV as compared with

those of other groups (Table 4). Cellulose digestibility was significantly ($P < 0.01$) higher in groups III and IV than that of group II, which was again significantly ($P < 0.01$) higher than that of group I. Similarly, protein, lipid and hemicellulose digestibility were significantly ($P < 0.01$) higher in group IV followed by groups III, II and I.

Digestive cellulase, amylase and protease activity in the intestinal tract were significantly ($P < 0.01$) higher in group IV as compared with other groups (Fig. 1). Furthermore, significantly higher ($P < 0.01$) amylase, cellulase and protease activity was observed in groups II and III compared with control.

The highest heterotrophic and amylolytic bacterial count was observed in the GI tract of groups II and III, respectively (Table 5). However, the highest cellulolytic and proteolytic count was observed in group IV. The cellulolytic bacterial count in groups III and IV was significantly ($P < 0.01$) higher as compared with other two groups. Amylolytic bacterial count was significantly ($P < 0.01$) higher in group IV followed by groups II, III and I with significant difference between each other.

Carcass composition of *L. calcarifer* at the end of the experiment (Table 6) revealed that body protein and lipid content of group IV was significantly ($P < 0.01$) higher compared with other three groups. However, the crude fibre content was higher ($P < 0.01$) in group I compared with other three groups fed with bacterial supplemented diet.

Discussion

Asian seabass is a carnivorous fish and need high-protein diet (40–50%) for their growth (Williams *et al.* 2003; Ali *et al.* 2012). Most of this protein requirement is fulfilled by costly fish meal (Kaushik *et al.* 2004; Torstensen *et al.* 2008). To reduce the feed cost, several efforts have been made to replace the fish meal by plant proteins (Kaushik *et al.* 2004; Torstensen *et al.* 2008; Hemre *et al.*2009). The plant-based ingredients are rich in cellulose and starch which carnivorous fish like Asian Seabass cannot utilize efficiently (Sinha *et al.* 2011). Therefore, feed was supplemented in the present study with either *Bacillus* sp. DDKRC1., a potential cellulolytic bacteria or *B. subtilis* DDKRC5., a

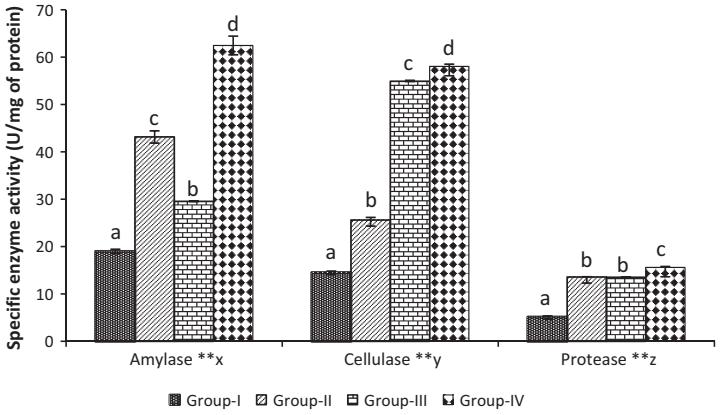

Figure 1 Specific amylase, cellulase and protease activity in gut of *Lates calcarifer* fed different diets. Bar bearing different superscripts differ significantly ($P < 0.01$). x-μg of maltose liberated/mg of protein/min, y- μg of D-glucose liberated/mg of protein/min z- μg of L-tyrosine liberated/mg of protein/min.

Table 5 Microbial count (CFU) in gut of *L. calcarifer* after 30 days of feeding.

	Treatment groups			
Parameter	**Group I**	**Group II**	**Group III**	**Group IV**
Total count ($\times 10^5$)**	19.65 ± 0.82^a	40.65 ± 0.99^b	43.51 ± 0.71^b	51.09 ± 1.41^c
Amylolytic count ($\times 10^4$)**	12.36 ± 0.07^a	31.53 ± 1.06^c	21.29 ± 0.76^b	40.27 ± 0.65^d
Cellulolytic count ($\times 10^4$)**	6.04 ± 0.57^a	23.36 ± 1.42^b	40.27 ± 0.65^c	44.55 ± 2.48^c
Proteolytic count($\times 10^4$)**	6.50 ± 0.78^{ab}	6.02 ± 0.12^a	6.15 ± 1.51^a	9.18 ± 0.35^b

Group I, fed diet D1; Group II, fed diet D2; Group III, fed diet D3; and Group IV, fed diet D4.
**$P < 0.01$; a, b,c,d: values bearing different superscript in a row differ significantly.

Table 6 Carcass composition (% of dry weight) of *Lates calcarifer* fed different diets.

Parameter	Treatment groups			
	Group I	Group II	Group III	Group IV
Crude protein**	60.21 ± 0.07[a]	62.09 ± 0.21[b]	62.07 ± 0.42[b]	63.28 ± 0.33[c]
Crude fibre**	2.12 ± 0.10[b]	1.32 ± 0.26[a]	1.16 ± 0.05[a]	1.03 ± 0.20[a]
Crude lipid	4.35 ± 0.05	4.22 ± 0.10	4.42 ± 0.20	4.21 ± 0.12
Ash**	14.32 ± 0.13[a]	14.59 ± 0.13[ab]	14.87 ± 0.04[b]	14.67 ± 0.05[ab]

Group I, fed diet D1; Group II, fed diet D2; Group III, fed diet D3; and Group IV, fed diet D4.
**$P < 0.01$; a, b: values bearing different superscript in a row differ significantly.

potential amylolytic bacteria or mixture of both. All the treatment groups supplemented with these bacteria recorded higher weight gain, improved nutrient digestibility and survival. Furthermore, the bacterial supplemented group had significantly higher ($P < 0.01$) PER, SGR and significantly lower ($P < 0.01$) FCR compared with control. Improved survival and growth were reported after supplementation of probiotics in different shrimp and fish species like Indian white shrimp, *Fenneropenaeus indicus* (Ziaei-Nejad, Rezaei, Takami, Lovett, Mirvaghefi & Shakouri 2006), American white shrimp, *Litopenaeus vannamei* (Wang 2007), common carp (Wang & Xu 2006), rohu (Ghosh, Sen & Ray 2003), Japanese flounder (Taoka *et al.* 2006), gilthead seabream (Suzer, Coban, Kamaci, Saka, Firat, Otgucuoğlu & Küçüksari 2008) and Pursean sturgeon and beluga (Askarian, Kousha, Salma & Ringø 2011). Bacterial supplementation helps in the establishment of favourable microbiota in fish gut (Essa, EL-Serafy, El-Ezabi, M Daboor, A Esmael & P Lall 2010). This leads to increased digestive enzyme activities, better nutrient digestibility and higher nutrient absorption (Al-Dohail *et al.* 2009; Essa *et al.* 2010).

Microorganisms and their enzymes have an important role in the digestion process by increasing the total enzyme activity (Ding, Li, Chen, Lin, Yang & Yang 2004; Ziaei-Nejad *et al.* 2006). The microbial components also stimulate the endogenous enzyme secretions (Ochoa-Solano & Olmos-Soto 2006; Wang 2007). In the present study, increased level of cellulase and amylase activity was observed in microbial supplemented groups compared with control. Several reports are available regarding the cellulolytic and amylolytic activity of gut microbes in fresh water fishes such as tilapia (*Oreochromis mossambica*), Chinese grass carp (*Ctenopharyngodon idella*) and common carp (*Cyprinus carpio*) (Bairagi *et al.*2004; Saha *et al.* 2006). Earlier, *Bacillus sp.* such as *B. subtilis* P6 and *B. velesensis* P11 have shown strong cellulolytic activity (Peixoto, Cladera-Olivera, Daroit & Brandelli 2011). Many others *Bacillus* sps such as, *B. circulans*, *B. pumilus* and *B. cereus* from rohu had strong amylolytic activity (Ghosh, Sen & Ray 2002). Bairagi *et al.* (2004) reported that cellulolytic and amylolytic activities in *B. subtilis* and *B. circulans* reduced the crude fibre, cellulose and hemicellulose contents and anti-nutritional factors, such as tannin, phytic acid and mimosine in the *Leucaena* leaf meal. Apart from digestive enzymes, supplementation of cellulolytic and amylolytic bacteria provides additional nutrients such as vitamins, essential amino acid and fatty acids (Ray, Ghosh & Ringø 2012). Overall, increased digestive enzyme activities and availability of additional nutrients from microbes may have enhanced the nutrient digestibility and growth performance in microbial supplemented groups. In the present study, the cellulase and amylase activity were significantly higher in group supplemented with both cellulolytic and amylolytic bacteria (group IV), compared with treatment supplemented with only amylolytic (group II) or cellulolytic (group III) bacteria. This probably resulted from synergistic response of both the type of bacteria. The overall higher digestive enzyme activities in the treatment group IV were probably the reasons behind the better feed utilization, lower FCR and better PER compared with all other treatment groups.

The total heterotrophic, amylolytic, cellulolytic and proteolytic bacterial count were higher in the gut of treatment groups compared with control. The synergistic response was observed in group IV, as the group had higher cellulolytic and amylolytic bacterial population compared with other treatment groups. These favourable bacteria, apart

from secreting the digestive enzymes and essential nutrients, colonize within the gut and so prevent the colonization by pathogenic microbes (Vine, Leukes & Kaiser 2004). This may have caused the better survival in microbe supplemented groups. A Gram-negative bacterium such as *Vibrio* is dominant in the gut and aquatic environment of crustaceans (Moriarty 1990) and marine fish (Sakata 1990). It is also reported as a major bacterial pathogen in the brackishwater fish and shrimp (Gatesoupe 1999). In the present experiment, low *Vibrio* count in water was observed in the group fed either cellulolytic or amylolytic bacteria or both. However, these supplemented bacterial population was not measured in water, but it seems that reduced number of *Vibrio* resulted from the competitive exclusion by bacteria fed to Asian seabass. Although inclusion of chromic oxide in diet affects the gut microbial composition resulting in dominance of *Streptococcus* sp. and *Lactobacillus* sp. bacteria (Ringø 1993), in the present study, no negative impact on cellulolytic and amylolytic bacterial population, enzyme activity, digestibility of nutrients and performance of fish was observed due to use of chromic oxide as inert marker for nutrient digestibility studies as reported by Lara-Flores and Olvera-Novoa (2013) in tilapia fry.

Carcass composition of experimental fish revealed that protein content was significantly ($P < 0.01$) higher in microbial supplemented groups compared with the control. Furthermore, the highest carcass protein level was observed in group IV, supplemented with both cellulolytic and amylolytic bacteria. This might be due to higher conversion of feed protein to carcass protein as reflected by lower FCR, better protein digestibility and protein efficiency ratio (De, Ghoshal, Kundu & Ali 2011; De *et al.* 2012).

Conclusion

Supplementation of cellulolytic and amylolytic bacteria revealed that mixture of *B. subtilis* DDKRC5. and *Bacillus* sp. DDKRC1. with plant protein based diet improved the growth performance, nutrient digestibility, gut microbial status and digestive enzyme activity in Asian seabass. These findings have practical significance towards development of feed probiotic for brackishwater aquaculture. There is a scope for refinement with regard to dose and form of supplementation and working out the economic return.

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