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Original Article

Effect of Probiotics on Survival and Growth of *Heteropneustes fossilis*

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ABSTRACT

Probiotics as live microbial food additives that improve the composition of the intestinal microflora in host have gained increased attention in aquaculture in recent decades. **Objective:** The aim of the present study was to evaluate the effects of three probiotic bacteria *viz*; *Bacillus subtilis*, *B. coagulans* and *Lactobacillus acidophilus* on survival and growth of *Heteropneustes fossilis*. **Results:** Test fish fed on 2 % and 3 % probiotic mixed diets showed better growth pergformances. The total heterotrophic bacterial count significantly differed among different treatments. *B. subtilis* (3 %) diet showed better values for weight gain (10.0 ± 0.95 g), SGR (0.8 ± 0.06 %), FCR (1.53 ± 0.15) and survival rate (100 %).The bacterial count was found to be highest for those fed on 3 % probiotic diet (9.8 x 10^7 ± 0.97 cfu/ml) and lowest in control fishes (8.3 x 10^4 ± 1.06 cfu/ml). *B. subtilis* count was found to be the maximum in 2% *B. subtilis* fed *H. fossilis* (9.5 x 10^5 ± 1.25 cfu/ml) followed by *B. coagulans* (2.8 x 10^5 ± 1.53 cfu/ml) and *L. acidophilus* (7.0 x 10^5 ± 1.10 cfu/ml) fed fishes. **Conclusion:** the incorporation of *B. subtilis* diets improved growth performance and survival, total heterotrophic bacterial count and total count of *B. subtilis* in *H. fossilis*. Hence, *B. subtilis* could be recommended for farmers practicing catfish culture in particular *H. fossilis* for successful growout culture.

Keywords: *H. fossilis,* Probiotics, *B. subtilis*, *B. coagulans, L. acidophilus*

1. INTRODUCTION

Aquaculture plays a pivotal role worldwide by offering better nutrition, employment and higher income. Fish is the main source of animal protein (more than 50%) and relatively cheaper for human consumption[1]. The production of healthy and high quality animal product is based on good nutrition systems. The benefits of live

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microbes (probiotics) on aquatic animal health have been well documented and scientifically reviewed by Gatesoupe [2], Verschuere *et al*.[3] and Irianto and Austin [4].

The use of probiotics in the culture of aquatic organisms is increasing with the demand for more environment-friendly aquaculture practices.[2] The term 'probiotics' is often used to describe a 'microbial formulation' responsible for biocontrol or bioremediation. The term probiotics was firstly coined by Parker [5] which mean "for life". Probiotics as live microbial food additives that improve the composition of the intestinal microflora in host [6] have gained increased attention in aquaculture in recent decades. [2] The first trial of probiotics in aquaculture was conducted in Japan in 1981. Spores of *Bacillus toyoi* were used in the feed to reduce the mortality of Japanese eel, *Anguilla japonica.* [7] Probiotics can be administered either as a food supplement or as an additive to the water. [8] The most commonly used probiotics in animal nutrition are lactic acid bacteria (LAB).

The *Bacillus* spores have been used as biocontrol agents to reduce *Vibrio* sp in shrimp culture. [9,10] These probiotics provide protection against pathogenic organisms by producing metabolites that inhibit the colonization or growth of other microorganisms or by competing with them for resources such as nutrients or space present in water medium and the guts of cultured animals. [11-17]

Heteropnuestes fossilis is an obligatory air breathing freshwater fish, highly tolerant to low oxygen conditions. It commands good consumer preference due to fewer intra muscular spines, tender flesh with delicious taste, high protein, iron and low fat contents [18] and is often recommended to convalescent people. [19] For the past two decades, this species is declining due to habitat loss, high fishing pressure and easily prone to EUS disease. [20] The present situation warrants enhancing the growth and survival of *H. fossilis*. Hence, the present study was attempted to evaluate the effects of three probiotic bacteria *viz*; *B. subtilis*, *B. coagulans* and *L. acidophilus* on survival and growth of *H. fossilis.*

2. MATERIALS AND METHODS

2.1 Sample collection

H. fossilis fingerlings (length: 11 ± 2 cm; and weight: 16 ± 0.73 g) were collected from Thamiraparani river fed systems and local fish market at Melapalayam, Tirunelveli (8.44^0 N, 77.44^0 E), Tamil Nadu, India. They were transported to CARE Aquafarm, stocked in an earthen pond (6m x 5m x 1.5 m) and fed a commercial pellet feed (CP Aquafeed, Chennai) for 10 days of acclimatization.

2.2 Experimental Design

The acclimatized fingerlings were randomly selected and distributed into 3m x 1m x 1m cement tanks filled with well water at a stocking rate of 45 fingerlings per tank and triplicates were maintained for each of the ten treatments. Ten diets were prepared for this experiment (Table 1). Diet 1 served as control diet (D1) and the remaining nine diets were D2 (1%), D3 (2%) and D4 (3%) of *Bacillus subtilis* (10^6 cells/g); D5 (1%), D6 (2%) and D7 (3%) of *B. coagulans* (10^6 cells/g) and D8 (1%), D9 (2%) and D10 (3%) of *Lactobacillus acidophilus* (10^6 cells/g). The experimental diets were grouped into four groups. The first group was control feed and the remaining three were *B. subtilis* (1%, 2%, 3%), *B. coagulans* (1%, 2%, 3%) and *L. acidophilus* (1%, 2%, 3%). The probiotic diets (1%, 2%, 3%) were partially replaced by the tapioca flour.

The bio-chemical analyses of the feeds were made following standard methods. [21] The fingerlings were fed 3% of their body weight with experimental diets twice a day for 60 days. Every third day, one third of water was partially changed in each tank. The mean

temperature (28 ± 1.5°C), dissolved oxygen (7.4 ± 0.6 mg/l), and total ammonia (0.5 ± 0.2 mg/l) were recorded during the feeding trial. Survival was recorded regularly and the fingerlings were weighed at every fortnight and feeding rate, weight gain, Specific Growth Rate (SGR) and Feed Conversion Ratio (FCR) were estimated. [22] On completion of the experimental period, gut samples were well homogenized and serially diluted in aseptic conditions. Heterotrophic bacteria were counted on MRS agar plates (Himedia) for the bacterial treatments [23] and the total counts were recorded as colony forming units (cfu)/ml.

2.3 Probiotics- B. subtilis

For culturing *B. subtilis*, *B. coagulans* and *L. acidophilus*, Sporulating agar, alkaline Bacillus medium and De Man Rogosa Sharpe (MRS) broth were used respectively. After the culture, cell suspension was serially diluted to a final concentration at 10^6cell/ml. They were harvested by centrifugation at 5000 rpm for 15 minutes, washed twice with sterile 0.85% NaCl solution and finally suspended in 10 ml diluent. This cell suspension after incorporation with the feed mix was used directly for fish feeding. [24] The culture was verified by subculturing and identification was based on their morphological and biochemical characteristics according to the Bergey's Manual of Systematic Bacteriology (1998). Pure cultures were stored at 4^0C in agar slants with sub culturing every 3 to 4 weeks.

2.4 Biochemical analyses of ingredients and test diets

About 2 ml of the log phase culture of *L. acidophilus*, *B. subtilis* and *B. coagulans* were centrifuged at 4000 rpm for 15 minutes. The pellets were washed in physiological saline with 250 µl of mix containing 10% SDS and 2% mercaptoethanol to lyse the cells and vortexed vigorously for 5 minutes and were taken for the estimation of protein, lipid, carbohydrate and dry matter content. [25] The ingredients of feeds were analyzed following standard procedures. [21]

2.5 Isolation and enumeration of microbiota from experimental fish

2.5.1 Screening of Total Heterotrophic Bacterial Population (THBP)

The probiotic treated *H. fossilis* were randomly sampled for gut content analysis during the experimental period (15 days intervals for 60 days). After surface sterilization the entire gut was carefully removed and ground in a mortar and pestle with sterile saline. The resultant aliquot was serially diluted and plated on nutrient agar after 24h of incubation at 37^0C for Total Heterotrophic Bacteria (THB) of gut samples. The bacterial populations of gut samples were expressed as number of colony forming units/ml (cfu/ml). [26]

2.5.2 Screening of B. subtilis, B. coagulans and L. acidophilus

Gut samples from the experimental fish were homogenized and serially diluted in aseptic condition, From the above prepared sample, 1 ml was taken individually and used for MRS agar (De Man Rogosa agar Sharpe) and incubated at 37^0C for 24 – 48 h to enumerate *L. acidophilus* [27] and same method was followed for isolation of *B. subtilis* in sporulating agar and *B. coagulans* in alkaline bacillus medium. The number of *L. acidophilus* and *B. subtilis* and *B. coagulans* in the guts of *H. fossilis* fed on control as well as experimental diets were counted and results were recorded.

2.6 Statistical analysis

Mean and standard deviations were compared by one-way ANOVA. SPSS software was used to find significant ($p<0.05$) differences in growth parameters.

3. RESULTS

3.1 Percentage composition of experimental diet

The control feed has Anchovy fish meal (26.9%), *Jawala acetes* (20%), wheat flour (10%), tapioca flour (10.9%), vegetable oil (5.8 ml), aqua savor (0.3%), vitamin C tablet (0.01%), vitamin premix (0.1%), mineral premix (0.5%) and monosodium phosphate (0.5%) (Table 1).

3.2 Proximate composition of ingredients

Among the ingredients, anchovy has the highest protein content (74.40%) followed by Jawala (55%). The carbohydrate level was found to be the highest in tapioca flour (44.9%) followed by wheat flour (29.5%) and soy flour (32%). The carbohydrate content was found to be the least in fish meal (1.7%). The maximum crude fat was estimated in Jawala (12.2%) whereas the least was recorded in tapioca flour (0.2%) as given in Table 2.

3.3 Growth performance

The test fishes readily accepted all the ten diets. The control fish showed lower growth and survival than fish fed with probiotic enriched diets (Table 3) and the differences were statistically significant ($p<0.05$). All the energy budget parameters showed better values in D4 (3 %) diet and D3 (2 %) probiotic mixed diets. *B. subtilis* D4 (3 %) diet showed better values for weight gain (10.0 ± 0.95 g), SGR (0.8 ± 0.06 %), FCR (1.53 ± 0.15) and survival rate (100 %) followed by D3 (2 %) diet with weight gain (9.5 ± 0.98 g), SGR (0.76 ± 0.06 %), FCR (1.54 ± 0.15) and survival rate (100 %). *B. coagulans* diet (3%) D7 showed better weight gain (8.9 ± 0.97 g), SGR (0.72 ±0.05 %), FCR (1.60 ± 0.17) and (95 ± 3.15 %) survival noticed. D10 *L. acidophilus* (3 %) mixed diet also showed better weight gain (7.9 ± 0.76 g), SGR (0.66 ± 0.04 %), FCR (1.77 ± 0.17) and (89 ± 2.98 %) survival. The lowest weight gain (6.5 ± 0.34 g) and SGR (0.58 ± 0.05 %) and highest FCR (2.21 ± 0.15) and lowest survival (82 ± 1.41 %) were recorded in the control diet (D1).

3.4 Isolation of probiotic bacteria from total heterotrophic count

According to the colony morphology, the different colonies were picked up and the results confirmed the presence of *B. subtilis*, *B. coagulans* and *L. acidophilus* using Bergey's manual classification (1998). The number of total bacterial colonies varied with the respective diets. The initial observation of bacterial colonies revealed $4.6 \times 10^2 \pm 1.01$ cfu/ml. At the end of the experimental period, the bacterial count was found to be the highest in (D4) $9.8 \times 10^7 \pm 0.97$ cfu/ml and the lowest in control (D1) $8.3 \times 10^4 \pm 1.06$ cfu/ml. In the present study, the diets D4, D3 and D7 were found to be highly significant (Table 4).

3.5 Screening of B. subtilis, B. coagulans and L. acidophilus

The number of *B. subtilis*, *B. coagulans* and *L. acidophilus* colonies per gram gut sample of *H. fossilis* were given in Table 5. *B. subtilis* was found to be maximum in D4 ($8.5 \times 10^6 \pm 1.19$ cfu/ml) and D3 ($9.5 \times 10^5 \pm 1.25$ cfu/ml) followed by *B. coagulans* in D7 ($2.8 \times 10^5 \pm 1.53$ cfu/ml) and *L. acidophilus* in D10 ($7.0 \times 10^5 \pm 1.10$ cfu/ml) fed fishes.

4. DISCUSSION

Probiotics play a major role in aquaculture by promoting growth, disease resistance by enhancing immunity and survival of fishes. Considerable work has been done in the field of probiotics, by a number of researchers *viz*; Queiroz and Boyd [28] in *Bacillus* sp on channel catfish; Irianto and Austin[29] in *Micrococcus luteus* A1-6 on *Oncorhynchus mykiss*; Suyanandana *et al*.[30] in *Lactobacillus* sp on *Oreochromis niloticus.* In later years, many other strains, including new strains from the aquatic environment were isolated and used for their better ability to colonize in the intestine of host thus enhancing growth of fish. One of the main successes of probiotic applications in aquaculture is the commercialization of *Bacillus*-based products for

shrimp culture. Mukhopadhay and Paul [31] worked on the advantages of probiotics as supplementary components in aquaculture feeds. In the present study, the addition of probiotics *B. subtilis* (10^6 cells/g), *B. coagulans* (10^6 cells/g) and *L. acidophilus* (10^6 cells/g) in different levels showed better performance (Table 3). Gatesoupe [32] proposed that the alternative feeding of rotifers enriched with probiotics i e., lactic acid bacteria or *Bacillus* spores with live feed decreased the amount of *Vibrio* sp and improved the survival rate of postlarvae and fry of turbot (*Scophthalmus maximus*) that were subsequently fed on them.

Porubcan [33] reported that addition of *Bacillus* sp in water reduced the COD and increased the shrimp harvest. He also added that biofilters with nitrifying bacteria increased survival of shrimps. Microbial strains such as *Lactobacillus* sp. [34], *Corynebacterium divergens* [35], *Vibrio alginolyticus* [36], *Pseudomonas fluorescens* [37] , *Streptococcus thermophilus* [32] and *Saccharomyces cerevisiae* [38] are used as probiotics in aquaculture. The *Lactobacillus* species viz., *Lactobacillus bulgaricus, L. acidophillus, L. sporogenes, L. casei* and *L. plantarum* are commonly used as single or mixed strain of probiotics. [39] Babitha *et al*. [40] reported *Artemia nauplii* enriched with *Lactobacillus* sp resulted in better growth and survival in *Macrobrachium rosenbergi. Bacillus* sp secretes many exoenzymes [8,41] aqnd these bacteria have been used widely as putative probiotics.

In our study, encouraging results were recorded when the experimental fishes were fed with diets D4, D3, D7, D6, D2 and D10, whereas the remaining fishes showed comparatively lesser growth. Among all experimental diets, significant weight gain was observed in diet D4 (10.0 ± 0.95), followed by diet D3 (9.5 ± 0.98) and diet D7 (8.9 ± 0.98) (Table 3). The maximum weight gain (10.0 ± 0.95) was observed in diet D4 fed fishes over the control diet. The present finding correlated with those of Moraity *et al*. [42] who stated significantly increased growth rate in shrimps fed with the sanolife *Bacillus* strains incorporated feed. Similarly, Wang [43] also reported significant growth rate (1.65 %) and daily weight gain (0.0384 g) in *Penaeus vannamei* fed with high concentration of photosynthetic *Bacillus* incorporated diet. In our study, no mortality was recorded in the diets D4 and D5 fed fishes. Similarly, Moriarty [41] noted an increase in prawn survival in ponds after the introduction of *Bacillus* sp. Rengipipat *et al*. [44] also showed better survival of black tiger shrimp (*Penaeus monodon*) fed with *Bacillus* incorporated diets. The above findings recommended for *Bacillus* sp for survival and growth also coincided with the present study.

A maximum weight gain of 7.9± 0.76g was observed in diet D10 fed fishes. Gatesoupe [31] found significant weight gain in *Scophthalmus maximus* (turbot) larvae fed with bio-encapsulated lactic acid bacteria and *B. toyoi*. The enhancement in growth could be due to the displacement of bacterial pathogens such as *Vibrio* sp in live larval feed (rotifer, *brachionus, plicatilis*) and also due to the improvement of the dietary value.

In the present study, diet D4 showed the significant SGR (0.8 ± 0.06%), weight gain (10.0 ± 0.95 g) and FCR (1.53 ± 0.15). Similarly, *B. coagulans* diet D7 showed significant growth (0.72 ± 0.05 %) and FCR (1.60 ± 0.17). Wang [43]has used *B. coagulans* with photosynthetic bacteria *Rhodobacter sphaeroides* in their study and has stated better performance of *B. coagulans* in shrimps. Their results support the present finding of *B. coagulans.*

L. acidophilus diet D10fed fishes showed lesser performance when compared with *Bacillus* sp fed fish. Among the *Lactobacillus* diets, D10 showed higher weight gain (7.9 ± 0.76 g), SGR (0.66 ± 0.04 %) and FCR (1.77 ± 0.17) which were not significant and all the three diets D8, D9 and D10 showed higher values

than the control diet (D1). Gatesoupe [45] reported that the lactic acid bacteria accelerated the growth by inhibiting the intestinal pathogens. Attack *et al*. [46] observed higher SGR (3.41 %), and optimum FCR (2.73) in bacteria incorporated feed. Their results also support our present findings.

The present study was extended to find out the total heterotrophic bacterial count besides *B. subtilis, B. coagulans* and *L. acidophilus.* In the initial stage of the experiment, the maximum heterotrophic bacterial count was found in D5, D6 and D7 groups ($2.1 \times 10^3 \pm 1.05$). At the end of the growth trial, the number of bacterial colonies were significant in diet D3 ($5.5 \times 10^7 \pm 1.01$), D4 ($9.8 \times 10^7 \pm 0.97$) and D7 ($6.8 \times 10^6 \pm 1.21$) (Table 4). Similarly, probiotics promote colonization of bacteria in the fish gut for a prolonged period and have the capacity to adhere and grow well in the intestinal mucus of turbot *in vitro.* [47] In our study, the total heterotrophic bacterial count was found to be significant in fish fed with diet D4. Similar to our findings, dietary incorporation of *S. cerevisiae* increased the gut microflora of *C. carpio.* [48].

In the present investigation, attempts were made to find out the total count of *B. subtilis, B. coagulans* and *L. acidophilus* in the probiotics fed fish. The diet D4 showed significant value ($8.5 \times 10^6 \pm 1.19$ cfu/ml) with respect to *B. subtilis* count (Table 5). Rengipipat *et al*. [44] reported the presence of the *Bacillus* sp in the gut flora of shrimps which increased survival (may be due to exclusion of other bacteria) particularly in the larval and early post larval stages where the *Bacillus* sp was dominant. In *P. monodon, Bacillus*, used as a probiotic, was found to colonize both in the culture water and the shrimp digestive tract replacing *Vibrio* sp in the gut of the shrimp, thereby increasing shrimp survival.

5. CONCLUSION

The survival of probiotics in the gut depends on colonization factors that they possess, such as secretion of enzymes which inhibits the entry of pathogens in the gut. In conclusion, the incorporation of *B. subtilis* diets improved growth performance and survival, total heterotrophic bacterial count and total count of *B. subtilis* in *H. fossilis*. Hence, *B. subtilis* could be recommended for farmers practicing catfish culture in particular *H. fossilis* for successful growout culture.

6. ACKNOWLEDGEMENT

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Table 1: Percentage composition of experimental diets and ingredients

Ingredients	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
Fish meal, Anchovy	26.9	26.9	26.9	26.9	26.9	26.9	26.9	26.9	26.9	26.9
Soy flour	25	25	25	25	25	25	25	25	25	25
Jawala acetes sp	20	20	20	20	20	20	20	20	20	20
Tapioca flour	10.9	9.9	8.9	7.9	9.9	8.9	7.9	9.9	8.9	7.9
Wheat flour	10	10	10	10	10	10	10	10	10	10
Sun flower oil	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Mineral premix[a]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Monosodium phosphate	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Aqua savor	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Vitamin premix[b]	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Ascorbic acid	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Bacillus subtilis	-	1%	2%	3%	-	-	-	-	-	-
Bacillus coagulans	-	-	-	-	1%	2%	3%	-	-	-
Lactobacillus acidophilus	-	-	-	-	-	-	-	1%	2%	3%

[a] Mineral premix to supply the following elements (mg kg-1 diet): zinc (as sulphate)72, iron (as sulphate) 36, manganese (as sulphate) 12, copper (as sulphate) 24, cobalt (as chloride) 0.6, iodine (as iodate)1.2, chromium (trivalent, as chloride) 0.8, selenium (as selenate) 0.2, and molybdenum (as molybadate) 0.2.

[b] Vitamin premix: (mg/kg-1) vitamin B12,0.1, nicotinic acid, 80.0: riboflavin, 50; pantothenic acid, 180; menadione, 40 folic acid, 6.0; biotin, 0.6, thiamine hydrochloride, 15; pyridoxine 60; thiamine, 40; inositol, 400; astaxanthin, 60; choline chloride, 20.0; vitamin C, 250 and (IU) vitamin A, 6000; vitamin D3, 2000; vitamin E, 6000 IU.

Table 2: Proximate Composition of Feed Ingredients

Ingredients	Crude Protein (%)	Crude Carbohydrate (%)	Crude Fat (%)	Crude Fibre (%)	Ash (%)
Fish meal, anchovy	74.40	1.7	10.3	1.5	10.9
Soy flour	50.05	32	0.1	7.8	13.8
Jawala acetes sp	55	1.9	12.2	4.9	12.9
Tapioca flour	13.9	44.9	0.2	9.6	11.4
Wheat flour	19.7	29.5	11.9	12.4	14.3

Table 3: Growth parameters of *Heteropnuestes fossilis* fed on experimental diets

Growth Parameter	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
Initial weight (g)	15.6 ± 0.73[a]	15.8 ± 0.82[a]	16.0 ± 0.89[a]	16.0 ± 0.89[a]	16.2 ± 0.90[a]	16.3 ± 0.89[a]	16.5 ± 0.89[a]	15.8 ± 0.82[a]	16.0 ± 0.89[a]	16.1 ± 0.90[a]
Final weight (g)	22.1 ± 0.91[a]	24.1 ± 0.92[a]	25.5 ± 0.97[b]	26.0 ± 0.99[b]	24.4 ± 0.85[a]	24.8 ± 0.89[a]	25.4 ± 0.98[b]	22.8 ± 0.92[a]	23.5 ± 0.95[a]	24.0 ± 0.96[a]
Weight gain (g)	6.5 ± 0.34[a]	8.3 ± 0.56[a]	9.5 ± 0.98[b]	10.0 ± 0.95[b]	8.2 ± 0.96[a]	8.5 ± 0.97[a]	8.9 ± 0.98[b]	7.0 ± 0.63[a]	7.5 ± 0.67[a]	7.9 ± 0.76[a]
SGR (%)	0.58 ± 0.05[a]	0.71 ± 0.07[a]	0.76 ± 0.06[b]	0.8 ± 0.06[b]	0.68 ± 0.06[a]	0.70 ± 0.05[a]	0.72 ± 0.05[b]	0.60 ± 0.04[a]	0.63 ± 0.05[a]	0.66 ± 0.04[a]
FCR	2.21 ± 0.15[b]	1.69 ± 0.16[a]	1.54 ± 0.15[a]	1.53 ± 0.15[a]	1.70 ± 0.17[b]	1.67 ± 0.16[a]	1.60 ± 0.17[a]	1.91 ± 0.19[b]	1.88 ± 0.18[b]	1.77 ± 0.17[b]
Survival rate (%)	82 ±1.41	90 ±2.42	100[a]	100[a]	89 ±2.91	92±3.16	95 ±3.15[b]	85 ±2.21	85 ±2.49	89 ±2.98

Calculation

Weight gain (%) = Final live weight – Initial live weight / Initial live weight (g) x 100

Specific Growth Rate (%/day) (SGR)=Final live weight – Log_e initial live weight/time (days)

Food Conversion Ratio (FCR) = Dry food consumed (g) / Wet weight gain (g)

Survival Rate = No. of final fish /No. initial fish x 100

Table 4: Total heterotrophic bacterial count in nutrient agar (cfu/ml)

Diets	Initial	15 days	30 days	45 days	60 days
D1 (Control)		$4.8 \times 10^2 \pm 1.05$	$6.5 \times 10^3 \pm 1.12$	$7.8 \times 10^4 \pm 1.15$	$8.3 \times 10^4 \pm 1.06$
D2		$6.5 \times 10^4 \pm 1.03$	$6.9 \times 10^4 \pm 0.8$	$2.2 \times 10^5 \pm 0.69$	$5.9 \times 10^{5b} \pm 1.03$
D3	$4.6 \times 10^2 \pm 1.01$	$4.5 \times 10^4 \pm 1.01$	$3.5 \times 10^5 \pm 0.18$	$3.7 \times 10^{6a} \pm 0.19$	$5.5 \times 10^{7a} \pm 1.01$
D4		$6.5 \times 10^4 \pm 1.33$	$4.2 \times 10^5 \pm 0.89$	$4.8 \times 10^{6a} \pm 0.91$	$9.8 \times 10^{7a} \pm 0.97$
D5		$1.5 \times 10^3 \pm 0.56$	$4.5 \times 10^3 \pm 0.17$	$6.5 \times 10^4 \pm 1.61$	$7.1 \times 10^{5b} \pm 1.12$
D6		$1.7 \times 10^3 \pm 0.63$	$3.5 \times 10^3 \pm 0.65$	$5.8 \times 10^5 \pm 1.08$	$6.7 \times 10^{5b} \pm 1.13$
D7		$1.6 \times 10^4 \pm 0.62$	$4.5 \times 10^4 \pm 0.52$	$6.3 \times 10^5 \pm 1.26$	$6.8 \times 10^{6a} \pm 1.21$
D8		$2.8 \times 10^3 \pm 0.91$	$5.1 \times 10^4 \pm 0.89$	$8.5 \times 10^5 \pm 0.91$	$9.1 \times 10^4 \pm 0.99$
D9		$3.3 \times 10^3 \pm 0.98$	$3.5 \times 10^5 \pm 0.18$	$4.7 \times 10^5 \pm 0.86$	$8.5 \times 10^{5b} \pm 0.91$
D10		$1.7 \times 10^4 \pm 0.88$	$2.7 \times 10^4 \pm 0.69$	$5.0 \times 10^4 \pm 1.12$	$6.5 \times 10^{5b} \pm 1.21$

Table 5: Total plate count in *H. fossilis* fed with *Bacillus subtilis, B.coagulans* and *L. acidophilus* using selective media (cfu/ml)

Diets	Initial	15 days	30 days	45 days	60 days
D1 (Control)	$2.6 \times 10^2 \pm 1.01$	$4.8 \times 10^2 \pm 1.05$	$7.3 \times 10^2 \pm 1.12$	$5.8 \times 10^3 \pm 1.10$	$9.3 \times 10^3 \pm 1.06$
D2	$3.2 \times 10^2 \pm 2.43$	$3.7 \times 10^3 \pm 1.32$	$7.5 \times 10^3 \pm 0.99$	$7.8 \times 10^4 \pm 0.99$	$8.9 \times 10^4 \pm .98$
D3		$4.3 \times 10^4 \pm 0.11$	$6.3 \times 10^5 \pm 1.18$	$7.5 \times 10^5 \pm 1.21$	$9.5 \times 10^{6a} \pm 1.25$
D4		$5.2 \times 10^4 \pm 1.09$	$6.5 \times 10^5 \pm 1.18$	$7.2 \times 10^5 \pm 1.21$	$8.5 \times 10^{6a} \pm 1.19$
D5	$2.1 \times 10^2 \pm 1.05$	$2.2 \times 10^3 \pm 0.56$	$3.2 \times 10^3 \pm 0.12$	$5.3 \times 10^4 \pm 1.06$	$8.1 \times 10^4 \pm 2.12$
D6		$1.2 \times 10^3 \pm 0.68$	$2.3 \times 10^3 \pm 0.53$	$6.2 \times 10^4 \pm 1.08$	$2.7 \times 10^{5b} \pm 1.56$
D7		$1.9 \times 10^3 \pm 0.68$	$7.2 \times 10^3 \pm 0.63$	$6.9 \times 10^4 \pm 1.66$	$2.8 \times 10^{6a} \pm 1.53$
D8	$3.4 \times 10^2 \pm 1.32$	$2.9 \times 10^3 \pm 1.11$	$3.5 \times 10^4 \pm 1.13$	$4.1 \times 10^4 \pm 1.19$	$8.8 \times 10^{5b} \pm 1.20$
D9		$3.5 \times 10^3 \pm 0.97$	$7.5 \times 10^3 \pm 0.99$	$8.5 \times 10^4 \pm 1.02$	$9.8 \times 10^{5b} \pm 1.30$
D10		$4.8 \times 10^3 \pm 1.12$	$6.1 \times 10^4 \pm 0.99$	$6.8 \times 10^4 \pm 1.10$	$7.0 \times 10^{5b} \pm 1.10$

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Conflict of Interest: None