THE INTERNATIONAL JOURNAL OF SCIENCE & TECHNOLEDGE

Dietary Supplementation of Potential Probiotics and Yeast Effect on Amino Acid Concentrations of Freshwater Fish, *Labeorohita* (Rohu)

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Abstract:
The effect ofdietary supplementation of potential probiotics andcommercial yeastwere evaluated in Labeorohita. Fish were fed for 30 and 60 days with control. Basal diet (BD) as Group I and 6 experimental diets containing GII- BD with S. cerevisiaeyeast 5mg/kg, G III- BD with mixture of L. sporogene, L. acidophilus& L. lactis5mg/kg, G IV- BD with mixture of B. subtilis, B. licheniformis& B. coagulans5mg/kg,G V-BD with mixture of L. sporogene, L. acidophilus, L. lactis&S. cerevisiaeyeast 5mg/kg G VI- BD with mixture of B. subtilis, B. licheniformis, B. coagulans&S.cerevisiaeyeast 5mg/kg and G VII- BD with mixture of L. sporogene, L. acidophilus, L. lactis, B. subtilis, B. licheniformis, B. coagulans&S. cerevisiaeyeast($1x10^{9}cfug^{-1}$) were fed 5mg/kg, respectively.The result sowed that total protein content, free aminoacids of essential and nonessential nature were significantly higher in the 30 and 60 days treatment groups when compared to control. But, these mixed probiotics and yeast were increasedpotentially from fish growth. These results indicate that dietary probiotics and yeast supplementation were effectively improved the total protein content and amino acid concentrationsin L. rohitamuscle.

Keywords: *Probiotics, yeast, Labeorohita, HPLC.*

1. Introduction

Aquaculture has emerged as one of the most promising and fastest-growing industries. It provides high-quality animal protein, raises nutritional levels, generate income and employment around the globe (FAO, 2010).While considering economical aspects of aquaculture, fermented feeds had been hypothesized to improve the assimilation efficiency of supplementary feeds (Mukhopadhyay and Ray, 1999; Skrede*et al.,* 2001 and2003).In fish farming the management is directed towards the realization of maximum fish biomass within a certain time. The fastest and the most reliable way to realize this objective is maximizing increase in weight of individual fish (Verreth, 1991). *Labeorohita*is an important freshwater fish species normally cultured in Asia particularly in the Indian subcontinent (Khan *et al.,*2004). The global production of *L. rohita* was approximately 1.2 million tons in 2005, out of which nearly 1million tons was contributed by India (FGIS, 2007).

Probiotics make up part of the resident microflora and contribute to the health or well-being of their host. Similarly, probiotics are used as dietary supplementations in aquaculture and their role in intestinal microbial balance, growth, nutrition, health status and resistance against infectious agents are already established (Gatesoupe*et al*., 1999). Microbes play a very important and critical role in aquaculture both at the hatcheryand the grow-out level, because water quality and disease control are directly affected by microbial activity. The common probiotics used in aquaculture belong to *Lactobacillus sp., Bacillussp.,Bifidobacterium sp., Vibrio sp., Saccharomyces sp*. and *Enterococcus sp*.(Rajesh Kumar, 2007).Commercial yeast, *Saccharomyces cerevisiae*is defined as unicellular eukaryotic micro-organisms (Kurtzman, 1998) and usually used in animal nutrition as an excellent source of protein and vitamins, especially B-complex vitamins, whose functions are related to metabolism.

Protein is frequently the most expensive nutritional factor. Therefore, using low-cost alternate sources of protein, which could provide better fish growth, is valuable for feed manufactures and aquaculture producers (Davis and Stickney, 1978). However, increase in cost and demand of feed protein from conventional sources requires fish culturists of the developing countries as ingredients in fish feeds (Sithara and Kamalaveni, 2008). The dietary protein link the amino acids released from the continual dissolution of structural and functional protein from the tissues and become a part of the amino acid pool.

The HPLC technique, combined with pre-column derivatisation of amino acids, has happened to a very significant method for the analysis of amino acids. One of the most well-liked derivatisation reagents was *o*-phthaldialdehyde (OPA), which ensures relatively

easy derivation and rapid reaction in aqueous solution at room temperature (Hanczko, 2007). Protein expresses the potentially of the fish to make use of its dietary proteins to increase its body protein.The amino acids from the hydrolysis of dietary protein join the amino acids free from the frequent collapse of structural and functional protein from the tissues and become a part of the amino acid pool. From the common amino acid pool, amino acids are full up by the cells, to be built into the cell structure as required (Anil Kumar, 2014).The present study was undertaken to the effect of different experimental diets and generates information on total protein content and amino acid composition through HPLC of *L. rohita*

2. Materials and Methods

2.1. Bacterial Strains

The potential probiotic bacterium culture (*Lactobacillus sporogene, Lactobacillus acidophilus* ATCC®4356™and*Lactobacillus lactis*ATCC®8000™) as well as(*Bacillus subtilis* ATCC®11774™, *Bacillus licheniformis* ATCC®12759™and*Bacillus coagulans* ATCC®7050™),*Saccharomyces cerevisiae*ATCC®9763™ as yeastwerepurchasedfrom HiMedia.These *Lactobacillus spp*., have been used as probiotic I and *Bacillus spp*., have been used as probiotic II.These bacterialcultureswere further maintained and grown in brain heart infusion broth for 24h at 37°C. Cell density was calculated from OD_{600} values and correlated with colony forming unit(cfu) counts using serial dilution and spread plating on tryptone soya agar (TSA). The quantified bacteria were maintained at 4°C in a suspended form and were used for feed preparation as required.

2.2. Diet Preparation

A basal diet comprising 34% groundnut oil cake, 40% rice bran, 19% soybean meal, 4% fish meal, 3% mineral and vitamin mixture (Every 250 gm of mineral-vitamin mixture provided vitamin A- 500,000 IU, vitamin D3- 100,000 IU, vitamin B2- 0.2 gm, vitamin E-75 units, vitamin K- 0.1 gm, calcium pantothenate- 0.25 gm, Nicotinamide- 0.1 gm, vitamin B12- 0.6 mg, choline chloride- 15 gm, calcium-75 gm, Manganese- 2.75 gm, iodine-0.1 gm, iron- 0.75 gm, zinc- 1.5 gm, copper- 0.2 gm and cobalt- 0.045 gm) was prepared. The basal dietwas used as control diet (G-I). In the 6 experimental groupsG-II, G-III, G-IV, G-V, G-VI and G-VII separated. Further, the individual and mixed probiotics(I and II)as well as *Saccharomyces cerevisiae*yeastwere added at a finaldose of 1×10^9cfu g^{-1} and 5gm/kgrespectively. To achieveaccurate final concentrations of the diet, the bacterial suspensionwas slowly added to dough, with gradual mixing in a drum mixer.The experimental diets were air-dried in a drying cabinet using anair blower at 38°C until moisture levels were around 10%. After airdrying,the diets were broken up and sieved into pellets of appropriatesize and stored at -20°C until use.

2.3. Experimental Animals

Rohu (*Labeorohita* Ham.) fingerlings of average weight 14gm were obtained from private fish farm at Thittai village, Thanjavur District, Tamilnadu, India. Twenty rohu fingerlings were maintained in (100×50×30) plastic tanks with constant aeration and two-thirds of energies water was exchanged with daily.The fish were acclimatized with 15 days period to the start of the experiment. De-chlorinated fresh water at 22°C±2 for 30 and 60 dayswas used throughout the course of experiment. During the investigation, different physico-chemical parameters in water such as temperature and pH were routinely monitored. The O_2 and ammonia concentrations ranged were from 6.5 to 7.5 mg L^{-1} and 0.5-0.9 mg L^{-1} ppm respectively and the waterpH as well as the temperature of the rearing pools was also varied from 7.4-7.8 and 26-29°C, respectively throughout the study period.The fish were randomly divided into 7 experimental groups with three replicates in each. Tank capacitywas 200 L and each tank contained 15 fish. Fish were fed one of 7 diets and used as a final dose.

Group-I	Basel diet only
Group-II	*S. cerevisiae*yeast (5gm) / kg^{-1} diet
Group-III	1×10^8cfu g^{-1}*L. sporogene*+ 1×10^8cfu g^{-1}*L. acidophilus* +1×10^8cfu g^{-1}*L. lactis*/ kg^{-1} diet
Group-IV	1×10^8cfu g^{-1} *B. subtilis* + 1×10^8cfu g^{-1} *B. licheniformis* +1×10^8cfu g^{-1} *B. coagulans*/ kg^{-1} diet
Group-V	1×10^8cfu g^{-1} *L. sporogene* +1×10^8cfu g^{-1} *L. acidophilus* + 1×10^8cfu g^{-1} *L. lactis* and 5gm *S. cerevisiae*yeast / kg^{-1} diet
Group-VI	1×10^8cfu g^{-1} *B. subtilis* + 1×10^8cfu g^{-1} *B. licheniformis* +1×10^8cfu g^{-1} *B. coagulans*and 5gm *S. cerevisiae*yeast / kg^{-1} diet
Group-VII	1×10^8cfu g^{-1} *L. sporogene* +1×10^8cfu g^{-1} *L. acidophilus* +1×10^8cfu g^{-1} *L. lactis* +1×10^8cfu g^{-1} *B. subtilis* + 1×10^8cfu g^{-1} *B. licheniformis* + 1×10^8cfu g^{-1} *B. coagulans*and 5gm *S. cerevisiae*yeast / kg^{-1} diet

Table 1

The feed rate was 3% of body weight per day, and equal rations were provided at 10.00 and 20.00 hr for 30 and 60 days. The amount of diet consumed was determined by daily recovery of excess feed, which was then dried and weighed (Sun *et al.,* 2011). Daily feed was adjusted for every 10 days by batch weighing after 24 h of starvation. During the experimental period, 25% of water was exchanged by fresh water in each tank every day.

2.4. Biochemical Composition of the Experimental Animals

The each group of fishes was sacrificed and muscle tissues were sampled. The samples were immediately processed for analyzing the total protein content. The analyses were made in triplicates. These diets were freshly produced after 30 and 60 days to ensure high probiotic viability throughout the duration of feeding trail. In the control diet, no probiotics were added.

2.5. Determination of Total Protein Content

Body muscle samples (free from skin and scales) of each group of fishes were collected and homogenized in a homogenizer individually, before the analysis of biochemical components. Protein assay was introduced by Lowry *et al*. (1951). Different groups of protein solutions are prepared by mixing stock protein solution (1mg/ ml) and water in the test tube. The final volume in each of the test tubes was 5 ml. The protein range was 5µg/ ml. From these different groups, pipette out 0.2 ml protein solution to different test tubes and add 2 ml of alkaline copper sulphate reagent (analytical reagent). Mix the solutions well. This solution was incubated at room temperature for 10mins. Then, add 0.2 ml of reagent FolinCiocalteau solution (reagent solutions) to each tube and incubate for 30 min. Zero the colorimeter with blank and take the optical density (measure the absorbance) at 660 nm.

2.6. Muscle Sample Preparation for Amino Acid Analysis

Muscle samples obtained by pooling the muscle of 15 fishes from each experimental treatment were blended. Then, 20ml of 6% trichloroacetic acid (TCA) was added. They were homogenized, sonicated for 3min (at 50% duty cycle), and centrifuged at 13,300rpm for 15min at 1^0C. After the supernatant was removed, the residue was re-extracted twice with TCA as above. All the supernatant was pooled, added to 30ml of ether, shaken for 30s, and the aqueous layer was concentrated in a low pressure rotary evaporator under 40^0C to a fairly highly viscous fluid, and then diluted with deionized double distilled water for amino acid analysis (Dalla via, 1986).

2.7. Amino Acid Analysis through HPLC

Amino acid analysis was carried out the method of Dalla via *et al*. (1986) using the procedure as suggested in the operational manual of the manufacturer. One hundred microliters of sample was diluted with an equal amount of sample dilution buffer A of 10 mM Sodium acetate adjusted to pH 6.4 with 6% acetic acid and buffer B of 10mM sodium acetate and 60% acetonitrile.Then half of the mixture was injected into a high performance liquid chromatography(HPLC) column (4.6×250mm) packed with Agilent TC-C18 high performance cation exchange resin. The HPLC column was eluted with four stages of lithium citrate buffer system (Beckman Co., USA) at the flow rate of 1ml/ml, 254nm wavelength and a column temperature of 34-72^{o}C. Finally, ninhydrin was used as a post column reactant with a flow rate of 10ml/h. Ammonia and urea concentration were also recorded at the same time by the amino acid analyzer. They were analyzed along with FAA due to their importance in amino acid metabolism.

2.8. Statistical Analysis

Data are expressed as mean ± standard error. Simple linear and non-linear regressions were performed to correlate the results obtained. A one-way analysis of variance (ANOVA) was used to determine differences among supplementary levels. Differences were considered significant at an alpha of 0.05 ($P<0.05$) (Finney, 1971).

3. Result and Discussion

The biochemical composition of the selected formulated feeds was determined and shown in Table. 1. These probiotics were formulated accordingly on the basis of crude proteinrequirement of *L. rohita*such that both the value added feeds would have similar proportion of nutrient content in 30 and 60 days.The proximate composition analysis of the formulated probiotics showed highest crude protein (45.63 and 45.93%) after the treatment of 30 and 60 daysas compared to conventional feed (3.5mg/100mg tissue) (Nalawade, 2014). Negligible variation was found in the protein content of the control group over the period. The actual was shown in graph. The crude proteincontent deposition in body meat of rohu was highest in themixed diet- G-VII (45.93±2.05) the results are agreed with findings of (Asad, 2005) they showed the highest digestibility ofmeat meal but some time these results not agree withfindings of other researchers because their might be anyproblem during procedure of feed preparation ordifference in feeding protocol (Noreen, 2008).In another study, the content of proteinwere also found high in animal, it wasmight be due to high concentration of minerals in animalprotein sources (Abid, 2009).

In Table.2, summarizes the aminoacid levels in treated muscle of *L. rohita*using different probiotics.The total amount of free amino acids, summed up in the tabulation. Among these, the essential and non essential amino acids amino acids were isoleusine, leucine, lysine, tryptophan, methionine, phenylalanine, threonine, valine, arginine, tyrosine and histidine and while others amino acids were phosphoserine, phosphoenolamine, aminoadipic acid, β-amino butyric acid, 3-methyl histidine and anserine, respectively. The amount of essential amino acids of leucine (3.77iµm/ml), threonine (4.83iµm/ml), arginine (2.84iµm/ml) was comparatively higher than other constitutes in effective treatment of G-VII. Glycine made up more than 27% of the total FAA pool, while the concentration of threonine, leucine all increased. The amount of arginine and valine increased promptly while the amount of OH lysine (0.69iµm/ml), tryptophan (0.61iµm/ml), phenylalanine (0.40iµm/ml), tyrosine (0.52iµm/ml) and methionine (0.31iµm/ml) decreased. The amino acid, tryptophan plays an important role in the brain as a precursor of the neurotransmitter, serotonin, which has a major effect on the feeding behavior of animals (Mullen and Mortin, 1992).

The amount of non essential amino acids of histidine(2iµm/ml) and arginine (2.38iµm/ml) increased when compared with control. These are the important substances for the regulation of osmotic pressure followed by isoleucinee, leucine, tryptophan, OH lysine, threonine, phenylalanine and tyrosine were detected in lower concentrations. The tested groups of amino acids peak values were

compared and analyzed with the standard table.3 which was effective growth treatment groups.Valine is involved in many metabolic pathways and is considered indispensable for protein synthesis and optimal growth (Wilson, 2002). Histidine is also an indispensable amino acid involved in many metabolic functions including the production of histamines, which take part in allergic and inflammatory reactions. It plays a very important role in maintaining the osmoregulatory process and is related to energy production or is used in other metabolic pathways during certain emergencies/ harsh conditions (Abe and Ohmama, 1987).This suggests sex differences and unique physiology of adult fish. The overall contents of these amino acids indicate that the growth of fish was progressed positively in the wild.

4. Conclusion

The present study proved that the supplementation of *L. sporogene*, *L. acidophilus,L. lactis, B. subtilis, B. licheniformis, B. coagulans*and *S. cerevisiae*yeastwere beneficial for *L.rohita.* It is argued that such probiotic has a role in protein growth promotion. Further studies are needed in this field to strengthenthis strategy and help understanding the role of probiotics looking for the alternative health management strategy for developing aquaculture productivity. Fish can give promising and favorable response to formulated diets if fed properly. The obtained results, we concluded that, the formulated feed will give better fish growth and nutritive content.

5. Acknowledgement

In this work was supported by UGC- RGNF at New Delhi, India.

Treatments	Crude protein (mg g^{-1})	
	30 days	60 days
G-I (Control)	40.12±1.85	40.21±1.88
G-II	43.53±1.92	43.98±1.93
G-III	43.98±1.99	44.32±2.00
G-IV	44.34±2.01	44.61±2.02
G-V	44.87±2.04	45.09±2.08
G-VI	45.05±2.07	45.24±1.89
G-VII	45.63±2.09	45.93±2.05

Table 2: Biochemical compositionof total protein contentof muscle of freshwater fish L. rohitafed used the formulated feed in the different experimental diet (g 100 g dry diet^{-1}). [a]Mean ± SE values (n=3) with different superscripts in each row are significantly different(P<0.05).

Amino acids	Standard	Ret time	G-I	G-II	G-III	G-IV	G-V	G-VI	G-VII
Isoleucine	54.62	54.86	0.13±0.00	0.31±0.01	0.53±0.02	0.82±0.04	1.03±0.05	1.19±0.05	1.30±0.06
Leucine	56.69	56.74	0.01±0.00	0.77±0.03	1.21±0.060	1.21±0.06	1.33±0.06	1.73±0.08	3.77±0.18
OH Lysine	57.465	57.37	-	-	-	-	0.22±0.01	0.51±0.02	0.69±0.03
Tryphtophan	58.48	58.70	-	0.23±0.01	0.25±0.01	0.29±0.01	1.11±0.05	3.11±0.15	0.61±0.03
Phenylalanine	60.031	60.37	-	-	0.13±0.00	0.16±0.00	0.23±0.01	0.38±0.01	0.40±0.02
Threonine	26.034	26.42	1.71±0.08	1.84±0.09	2.60±0.13	3.11±0.15	3.92±0.19	4.75±0.02	4.83±0.24
Histidine	27.631	27.89	1.00±0.05	1.38±0.06	1.52±0.07	1.79±0.08	1.80±0.09	1.83±0.09	1.86±0.09
Arginine	30.418	30.07	0.39±0.01	1.04±0.05	1.61±0.08	1.78±0.08	1.83±0.09	1.96±0.09	2.54±0.12
Tyrosine	44.234	44.51	0.05±0.00	0.14±0.00	0.22±0.01	0.28±0.01	0.31±0.01	0.45±0.02	0.52±0.02
Valine	45.772	45.66	-	0.51±0.02	0.72±0.03	0.84±0.04	0.98±0.04	1.04±0.05	1.43±0.07
Methionine	48.588	48.15	0.02±0.00	0.10±0.00	0.15±0.00	0.19±0.00	0.24±0.01	0.29±0.01	0.31±0.01
Essential			**3.31**	**6.32**	**8.94**	**10.47**	**13**	**17.24**	**18.23**
Carnosine	29.044	29.67	0.11±0.00	0.27±0.01	0.31±0.01	0.36±0.01	0.39±0.01	0.41±0.02	0.52±0.02
Aspartic acid	9.149	9.57	-	-	0.08±0.00	0.11±0.00	0.11±0.00	0.32±0.01	0.06±0.00
Glutamic acid	10.317	10.99	1.52±0.07	-	0.05±0.00	0.31±0.01	0.36±0.01	0.39±0.01	0.41±0.02
OH Proline	16.076	16.39	-	-	0.08±0.00	0.10±0.00	0.10±0.00	0.12±0.00	0.19±0.00
Serine	19.164	19.21	-	0.04±0.00	0.11±0.00	0.21±0.01	0.42±0.02	0.46±0.02	0.49±0.02
Glycine	20.544	19.81	-	0.02±0.00	0.09±0.00	0.12±0.00	0.19±0.00	0.25±0.01	0.31±0.01
Alanine	28.42	28.86	-	-	-	1.00±0.05	1.31±0.06	1.83±0.09	2.00±0.10
Proline	31.165	31.17	0.15±0.00	1.63±0.08	2.12±0.10	2.14±0.10	2.31±0.11	2.37±0.11	2.38±0.11
Cysteine	54.02	54.15	-	-	0.24±0.01	0.27±0.01	0.33±0.01	0.45±0.02	0.57±0.02
Non-Essential			**1.78**	**1.96**	**3.08**	**4.62**	**5.52**	**6.6**	**6.93**
Phoshoserine	7.694	7.63	0.13±0.00	0.19±0.00	0.66±0.03	0.71±0.03	0.79±0.03	0.85±0.04	0.88±0.04
Phosphoenolamine	17.157	16.77	-	-	-	0.01±0.00	0.11±0.00	0.16±0.00	0.19±0.00
Aminoadipic acid	29.46	29.33	1.48±0.07	3.11±0.15	3.62±0.18	3.90±0.19	4.16±0.20	4.83±0.24	4.91±0.24
β-amino butyric acid	29.46	29.93	-	0.82±0.04	-	-	-	-	0.57±0.02
3-Methylhistidine	32.319	32.43	0.17±0.00	0.13±0.00	1.06±0.05	1.12±0.05	1.16±0.05	1.19±0.05	2.05±0.10
Anserine	38.137	38.07	-	-	0.32±0.01	0.44±0.02	0.52±0.02	0.59±0.02	1.16±0.05
Others			**1.78**	**4.25**	**5.66**	**6.18**	**6.74**	**7.62**	**9.76**

Table 3: Amino acid profiles of L. rohita muscles using different treatments. Values are mean of triplicate analyses.

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