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Research Article

EVALUATION OF PROBIOTIC CHARACTERISTICS OF BACTERIA ISOLATED FROM AQUAPONDS OF NELLORE DISTRICT AND THE ASSESSMENT OF ITS EFFICACY IN WHITE LEG SHRIMP INFECTED WITH VIRUS AND WATER QUALITY PARAMETERS IN EXPERIMENTAL CONDITIONS

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Abstract- In the present study, the probiotic characters of bacteria were determined which were isolated from shrimp cultured ponds. 10 bacterial strains were isolated and probiotic characters were determined. High cholesterol assimilation done by HL5 (27±1.2cfu/ml). V2-V3 region of 16srDNA was amplified for identification of Lactobacillus species. Then experiment was conducted having two phases to investigate the effects of dietary supplementation of probiotic bacterium on shrimp physiology. In first phase shrimp fed with probiotic supplemented feed for 21 days, in second phase challenged with White spot syndrome virus (WSSV and their physiological responses were investigated. In the first phase probiotic treated shrimp had significant ($P<0.01$) growth, THC, percentage of phagocytosis and phenoloxidase enzyme activity, IgG, IgA,and IgM like substances and antioxidant enzymes . Higher levels were observed in 10% treated shrimp. All three concentrations of Lactobacillus sps probiotic bacterium was effective in improving the resistance of shrimp against WSSV as they had higher THC, higher percentage of phagocytosis, phenoloxidase enzyme and immunoglobin like substances level.

Keywords- *probiotic bacteria, Litopenaeous vannamei, WSSV, lactobacillus*

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Introduction

Aquaculture, also known as aqua-forming, involves cultivating fresh water and salt water populations under controlled conditions and contrasted with commercial fishing which is the harvesting of wild fish. Aquaculture goes way beyond food production. Although aquaculture serves many purposes, the most important one is to supply food for humans. It also produce animal feed by producing algae and plant organisms. Last 50 years, there is an increase in the aquaculture production; around 59.9 million tons worth US $ 119 billion. Aquaculture will soon overtake cattle ranching as a global food resource, possibly signaling a basic shift in diets, and it is growing more rapidly than all other animal food producing sectors. According to FAO(2010) this sector has increased at average compound rate of 9.25% per year since 1970 when compared with 1.4% capture fisheries and 2.8% for terrestrial farmed meat production systems. Bacterial diseases are responsible for severe economic losses in aquaculture [47]. The indiscriminate use of antibiotics to control pathogenic microorganisms brings important changes in the microbiota of the aquaculture systems and surrounding environment, creating bacterial resistance to commonly used antimicrobials [38] and even affecting natural beneficial bacteria [22,23,24]. In agricultural and aquaculture, the use of antimicrobial drugs will lead to emergence of antibiotic resistant bacteria [1]. The transmission of these antibiotic resistance bacteria containing antibacterial resistant genes from aquaculture environments to the human environment of nonpathogenic bacteria can transfer such genes to human non pathogenic bacteria [42]. Considering the above factors the European Union and USA restricted the usage of antibiotics, Watson, 2008 [49]. Because of restriction on usage of antibiotic drugs, the new strategies in health management in aquaculture practice have received much attention [2]. A probiotic was a live microbial feed supplement or cultured product which beneficially affects the host by improving its intestinal (microbial) balance [18]. In this study, the effect of probiotics isolated from shrimp cultured ponds and their effect is evaluated in heathy *Litopenaeus vannamei* and in *WSSV* infection conditions.

Materials and Methods

Isolation and preliminary identification

All the bacterial strains were isolated from dried shrimp cultured ponds. Twenty six (26) soil samples were collected from the brakish water shrimp ponds from Mypadu, Ramudu palem and Kudithipalem of Nellore district, Andhra Pradesh, India. All soil samples were subjected for serial dilution under extreme sterile conditions using nutrient agar supplemented with 15% sodium chloride. All the prominent bacterial colonies obtained were subjected for pure culture isolation. Further the pure colonies were diluted in 0.85% NaCl and analyzed by spread inoculation. An inoculum (0.1 ml) of each decimal dilution of samples was plated onto the surface of MRS agar (Difco, Detroit, MI, USA) which were incubated in anaerobic jar (BBL, Gas Pak Plus), for enumeration of probiotic bacteria like lactobacillus bacteria. The isolates were gram-stained and characterized by carbohydrate utilization pattern using durham's tube. The isolates were further tested for catalase production, nitrate reduction and urease production. The probiotic bacteria such as lactic acid bacteria are gram positive so the gram positive strains HL1, HL2, HL3, HL4, HL5 and HL6 were made pure culture on MRS agar and these six strains were tested for further probiotic characteristics.

Table-1 Biochemical characteristics of isolates from shrimp cultured ponds

Test	HL1	HL2	HL3	HL4	HL5	HL6	HL7	HL8	HL9	HL10
Grams staining	+	+	+	+	+	+	-	-	-	-
Catalase	+	+	-	+	-	-	-	+	+	+
Urease	+	+	-	+	-	+	-	-	-	-
Nitrate reductase	+	+	+	+	-	-	-	-	-	-
Fermentation with glucose	+	+	+	+	+	+	+	+	+	+
With lactose	+	+	-	-	-	+	-	-	-	-
Xylose	+	-	-	+	+	+	-	-	-	-

Table-2 Growth in weight of Prawn *L.vannamei* after feeding with different concentrations of probiotics

Concentration of the Probiotic	Control (gms)	Probiotic treated (gms)	Weight gain (gms)	DWG[a] (g/d)	RGR[b] (%)	SGR[c] (%/day)
Control	5.74±0.04	7.24±0.07	1.50±0.03	0.07±0.01	26.1±0.06	5.90±0.25
5%	5.86±1.54	7.61±0.61	1.75±0.87	0.083±0.05	29.86±0.12	5.95±0.01
10%	6.10±0.58	9.12±0.14	3.02±0.44	0.143±0.36	49.5±0.83	7.16±0.02
15%	5.63±0.32	8.34±0.11	2.71±0.21	0.129±0.1	48.13±0.03	7.01±0.30

Probiotic characterization of isolates

The six isolates were further tested for probiotic characters such as tolerence to acid, bile tolerance tests were determined by the methods Hyronimus *et al*, 2000 [25] and Gilliland *et al*., 1975[19] respectively. *In vitro* determination of viability under conditions similar to those prevailing in the GUT was performed according to the method of Charteris *et al*. (1998) [14]. Assay of cholesterol assimilation was done by the method Pereira and Gibson, 2002 [36].

Identification of probiotic bacteria on the basis of 16s rRNA profile

DNA was isolated from bacterial isolates according to the method Dellaglio *et al*., 1975 [16]. The V2–V3 variable region (approx. 200 base pairs) of the 16S rRNA gene was amplified by using primers fD1 (5'-GAGTTTGATCCTGGCTCA-3) and rP2 (5'-ACGGCTACCTTGTTA CGACTT-3'), Naik *et al*., (2008) [34].

Animals

Healthy shrimp (wt of approximately 6 gms) were collected from a commercial farm and bring immediately to lab. The collected shrimp were acclimatized to culture conditions for one week in tanks containing filtered sea water and fresh water which is UV treated to bring salinity to 20ppm and continuous aeration was given.

Preparation of probiotic formulation

The strain lactobacillus HL5 was grown in sterile conditions using MRS broth until final density reaches to $1X10^6$ cells per ml. These cells were mixed with a commercial gel to attain different concentrations (5 to 15%) for using as feed supplement to study its effect on biochemical and immunological parameters.

Experimental design

To study each parameter a group of 24 animals were taken and they are acclimatized to laboratory conditions for a period of one to ten days. Four groups were made each one having 6 animals and each group was randomly assigned to triplicate plastic tanks. Three diets made with different doses (5%, 10% and 15%) of probiotic mixture were prepared. One group as control the remaining three groups were fed probiotic supplemented feed ($1X10^6$ @10gm gel/kg feed, 50gms, 100gms, 150gms of gel was added per Kg feed to make 5%,10% and 15%). For a period of three weeks the feed was given to each group. For control group the feed was supplied only by mixing with commercial gel without probiotic. In this experiment all shrimps were fed four times per day at 4% of the body weight. The immunological and biochemical parameters were studied before the initiation of study and also after three weeks of probiotic feed to assess the influence of probiotics on shrimp health. Daily 30% of culture water in all tanks was exchanged and the temperature of water was maintained at 28±1°C, the pH at 7.5-8.4 and the salinity was maintained at 20ppm. At the end of 4th week of study all groups of shrimp were infected with WSSV by feeding with macerated shrimp (mincemeat) which had been made from a severely WSSV-infected shrimp. Control shrimp were injected with PBS. The hemolymph was extracted and maintained at 70°C.

Sampling and analytical methods

The weights of all shrimps were determined at the start (Initial Weight) and at the end (Final Weight) of the 28 days experiment. The Daily Weight Gain (DWG; g d−1) was calculated as:

Final Weight(g) -Initial weight(g) / 28d

The Relative Gain Rate (RGR; %) was calculated as:

*Final weight(g) *Initial weight(g)/ Initial weight(g) ×100*

Study of immunological parameters

In order to study the effect of probiotics on immunological parameters about 0.8 ml of hemolymph was withdrawn from the ventral sinus in the first abdominal segment using a 26-gauge hypodermic needle on a 1-ml syringe. Hemocyte analysis was done according to the method Kondo, 2003[28]. Phagocytic activity was measured following the method described by Weeks-Perkins *et al*., 1995 [50] and Phenoloxidase activity was measured as detailed by Sung *et al*.,1994 [43]. 50 ul of serum was diluted using 1 ml of saline water and then the diluted serum was used for nephlometry. The concentrations of the immune factors in the serum such as immunoglobulin like substances were measured by the method of Wang *et al*., 1998[46].

Anti oxidant enzyme assays

Superoxide dismutase activity was measured as the inhibition of photo reduction of nitro blue tetrazolium (NBT) by the enzyme as described by Beauchamp and Fridovich, 1997 [8] and Catalase enzyme activity was measured following the method of Chance and Machly, 1995[13] Spectro photometrically.

Biochemical analysis of haemolymph

For glucose assay commercial kit GOD-POD, Merck-740393 was used, for lactate assay, Sigma-cat.73510 kit was used [5].

Protein estimation

protein determination was done by the method of Bradford ,1976 [6].

Estimation of nucleic acids

Nucleic acid was extracted from methanol insoluble tissue residue by the method of Schneider,1957 [41]. DNA was assayed by the method of Burton,1956 [7] and RNA was estimated by the method given by Ceriotti,1955[12].

Statistical analysis

The data were expressed as the arithmetic mean ± standard deviation and were analyzed by one- way –ANOVA.

Results

Isolation and characterization of Probiotic bacteria

10 samples were able to produce prominent colonies which were further subjected for repeated sub culturing for the isolation of pure colonies. Pure colonies obtained further streaked on MRS agar slants for further studies. Biochemical characteristics of all isolates were given in the [Table-1].

Molecular identification of lactobacillus species

The molecular analysis of the isolates tested using the 16S ribosomal RNA gene showed that all the bacterial DNA samples isolated were found to have the V2-V3 sequence of lactobacillus which is approximately 200bp.

Growth performance

A significant difference was observed between Weight of treatment groups and the Control. After 28 days, there was no significant difference between the mean weights of groups (average overall 1.71 (±0.06) g), [Fig-1] although the mean weight of each group increased with increasing concentration of probiotics. The mean weight of each treatment group was significantly higher than that of the Control (1.57 ±0.05 g) [Table-2].

Immune response

The total haemocyte count, phagocytic levels and phenol oxidase activity after three weeks in haemolymph of shrimp was significantly ($p<0.01$) higher in all the probiotic (5%, 10%, 15%) treated

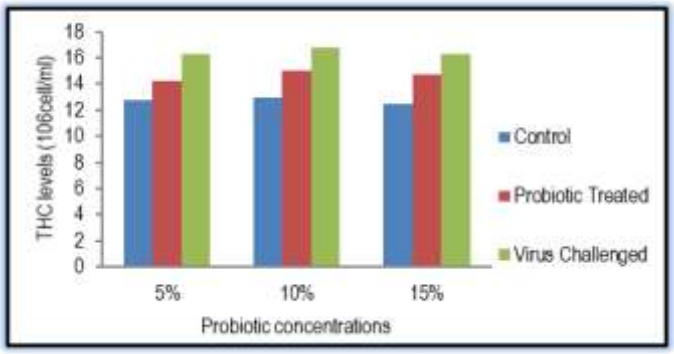

Fig-1 Growth in weight of prawn *Litopenaeus vanameii* by using different probiotic concentrations groups and in virus challenged shrimp.

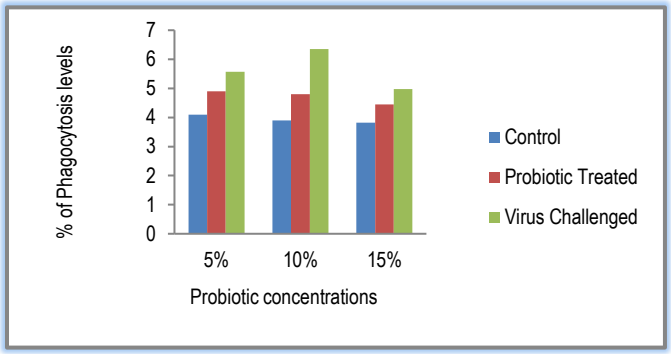

Fig-2 Total Hemocyte Count levels (106cell/ml) by using different probiotic concentrations

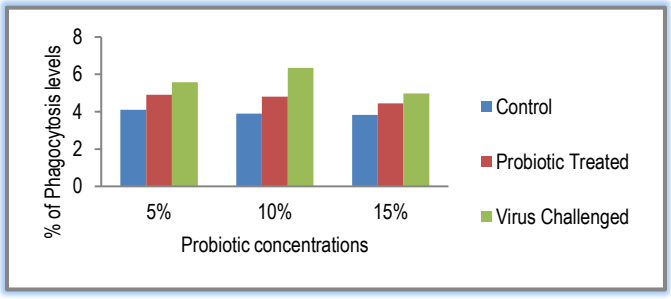

Fig-3 Effect of different probiotic concentrations on %of phagocytosis

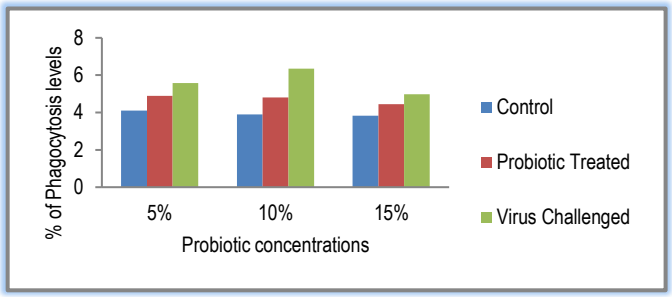

Fig-4 Effect of different probiotic concentrations on shrimp IgG levels in serum before and after challenge with WSSV

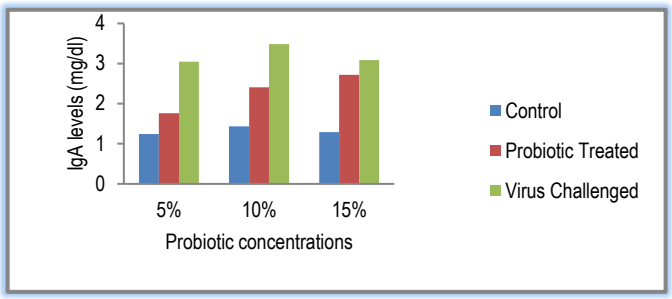

Fig-5 Effect of different probiotic concentrations on shrimp IgA levels in serum before and after challenge with WSSV

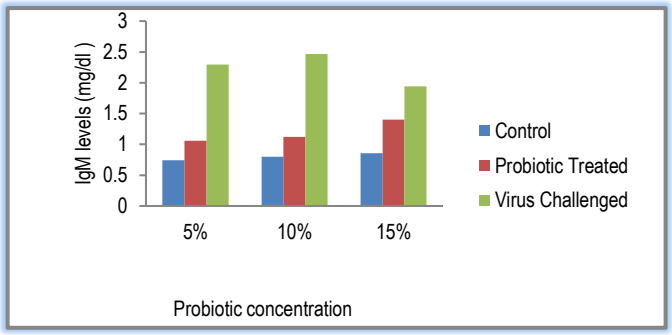

Fig-6 Effect of different probiotic concentrations on shrimp IgM levels in serum before and after challenge with WSSV

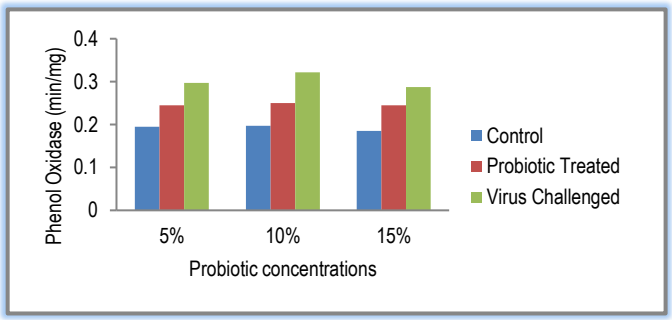

Fig-7 Effect of different probiotic concentrations on shrimp phenoloxidase enzyme levels in serum before and after challenge with WSSV

The high haemocyte count were observed in 10% group higher in all the probiotic (5%, 10%, 15%) treated groups and in virus challenged shrimp. The high haemocyte count were observed in 10% group (15±1.1 $5x10^6$/ml) when compared to control (13±0.8 $1x10^6$/ml) [Fig-2], phagocytic levels of the 5% treated group showed little higher percentage 4.9±0.43% than other groups and its control 4.1±0.47% [Fig-3]. The 10% and 15% treated groups resulted 4.8±0.71% and 4.45±0.34%, respectively. The virus challenged groups percentage of phagocytosis levels increased significantly when compared to control and probiotic treated groups, the highest phagocytosis levels 6.35±0.26%.were found in 10% group and the highest phenol oxidase activity 0.25±0.03 min/mg protein was observed in 10% treated group [Fig-4], the enzyme activity was increased significantly ($p<0.01$) in virus challenged groups than control group.

The highest activity was observed in 10% treated group 0.322±0.05 min/mg protein. IgG, IgA, IgM like substances in haemolymph are increased in all probiotic treated groups significantly (p<0.01) than that of control. High levels were observed in virus challenged shrimp than treated and the 10% diet fed shrimp showed high levels than 5% and 15% fed shrimp [Fig-5,6]

Antioxidant enzymes

The antioxidant enzymes super oxide dismutase and catalase activities increased significantly (P<0.01) in all the shrimp treated probiotics with as well as after virus challenge [Fig-8]. Superoxide dismutase activity exhibited an enhancing trend with increasing probiotic supplementation. The highest catalase activity 35.187±1.48 µmol/mg/min was observed in 10% treated group and its control showed 30.18±1.24 µmol/mg/min [Fig-9].

Biochemical parameters

The significant (p<0.01) increase in glucose levels were observed in all treated groups. 10% treated groups showed high glucose levels 26.45±0.43mg/dl when compared to control 25.35±1.23 mg/dl [Fig-10].

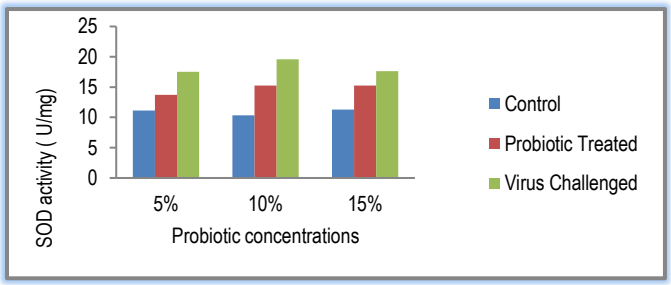

Fig-8 Effect of different probiotic concentrations on superoxide dismutase levels in serum before and after challenge with WSSV

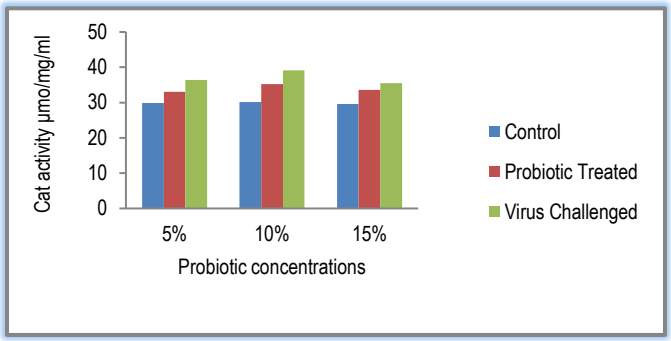

Fig-9 Effect of different probiotic concentrations on shrimp catalase levels in serum before and after challenge with WSSV

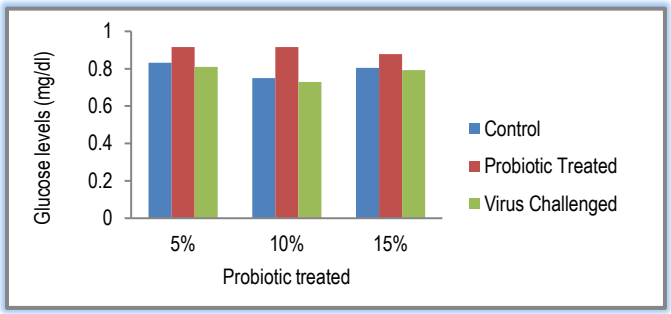

Fig-10 Effect of different probiotic concentrations on shrimp glucose levels in serum before and after challenge with WSSV

Lactic acid levels were significantly (P<0.01) increased in 10% and15% treated groups, high lactic acid level 14.75±0.5 mg/dl was recorded in 10% treated group [Fig-11]. Significant difference in triglyceride levels were not observed (p>0.05) between control and probiotic treated groups of 5% and 10% [Fig-12]. The 15% group displayed a higher level 43.25 ± 1.71mg/dl and showed significant difference with control. RNA levels increased in all the treated groups but significant increase (p<0.01) was observed in 10% group [Fig-13]. The treated shrimp groups do not show significant (p<0.01) increase in DNA levels than control groups [Fig-14]. All probiotic treated shrimp groups showed significant increase (p<0.05) in percentage of protein levels when compared to control groups. The 10% probiotic treated group showed highest protein leves 30.42 ± 0.74% than its control 23.1 ± 0.51% [Fig-15].

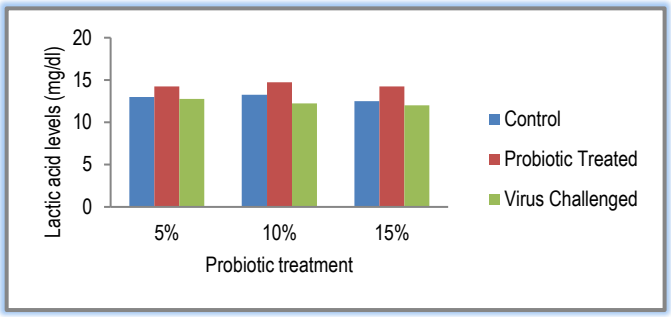

Fig-11 Effect of different probiotic concentrations on lactic acid levels in serum before and after challenge with WSSV

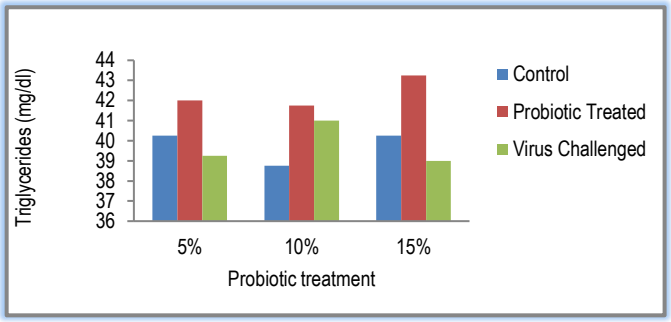

Fig-12 Effect of different probiotic concentrations on triglyceride levels in serum before and after challenge with WSSV

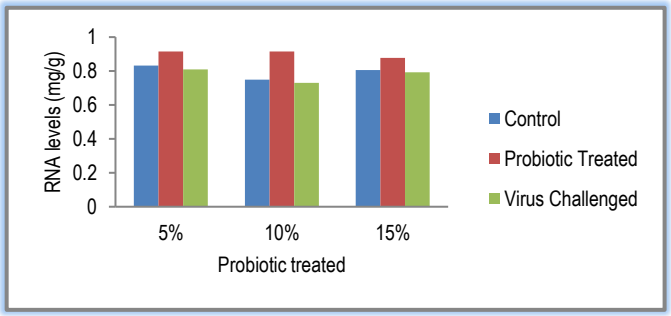

Fig-13 Effect of different probiotic concentrations on RNA levels in serum before and after challenge with WSSV

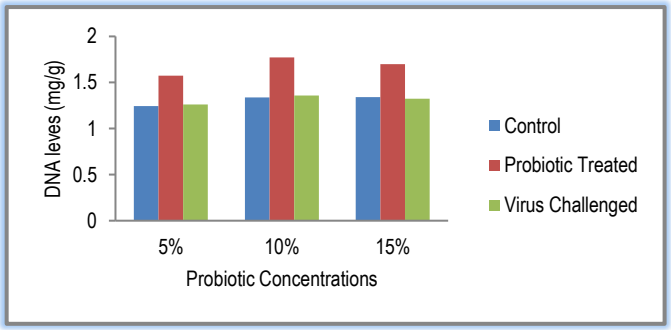

Fig-14 Effect of different probiotic concentrations on DNA levels in serum before and after challenge with WSSV

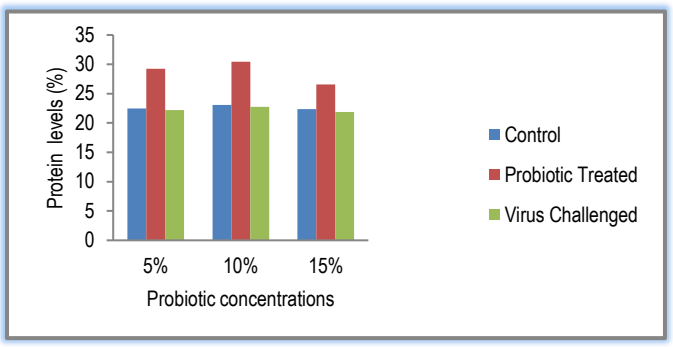

Fig-15 Effect of different probiotic concentrations on protein levels in serum before and after

Water quality parameters

Water quality parameters such as pH and salinity were not fluctuated much between control and probiotic treated tanks starting from first week to fifth week. However slight increase in pH was observed in 15% treated tank (8.4, 8.6, 8.4, 8.6) when compared to control (7.9, 8.3, 7.7, 8.5) from first to fourth week. Salinity foud to be same in both probiotic treated tanks (20 ppt) and its control tanks (20 ppt) up to four weeks. Carbonates in control and probiotic treated were fluctuated much. During the first and third week there were no carbohydrates were observed in control tanks (nil, 20 mg/L, nil, 42 mg/L). Carbonates were increased from first week to second week, decreased suddenly in third week in all the probiotic treated tanks. The total alkalinity in control tank (164mg/L,192mg/L,168mg/L,232mg/L) was increased from first week to fourth week and much fluctuations were observed between control and treated tanks. Among the treated tanks 10% showed fewer total alkalinities (186mg/L, 202mg/L, 206mg/L, 218mg/L) from first to fourth week. Mg hardness showed no high fluctuations between control and experimental groups from start of experiment to fourth week. These differences ranged from 424-444 ppm, 427-457 ppm, 422-467 ppm in 5%, 10%, 15% probiotic treated tanks respectively. Total hardness differences ranged from 495-518 ppm, 499-533 ppm, 493-542 ppm in 5%, 10%, 15% treated tank and in control tank it ranges 494-521 ppm. Slight high differences were observed in ammonia concentration in treated tanks compared to control.

Discussion

The major selection criteria for probiotic strains are resistance to low pH, resistance against bile salt and survival in gastric juice, Cakir, 2003 [9], In the present study all the six isolates were resistant to low pH for 1hr and 6hrs time duration. In the previous studies done by Jatindra *et al*.,2010 [26] 55 acid tolerant strains of LAB were selected in PBS buffer PH-2.5 for 3hrs. The other important criteria for colonization at pH3 and pH4 in gastric juice is resistance against bile salt and survival in gastric juices Strompfova and Laukova, 2007[45]. All isolated strains tolerated 0.3% bile salts concentration in 4hrs. The results of present study showed that all six isolates were resistant to low pH having high survival rate at 0.3% concentration as well as at 0.5% and 0.1% bile concentration for 4hrs. HL1 and HL5 exhibited high survival rate at all concentrations of bile. These two strains also have high survival rate at pH 8 in the simulated gastric and intestinal juice. A good probiotic bacterium should have cholesterol reduction efficiency. M.Bilige *et al*. 2009 [4] isolated 30 *lactobacillus* strains, MG2-1 have high cholesterol removal rate (51.74±0.04%). According to Nagpal *et al*., 2012 [33] probiotic have many health biological properties, one of them was anti cholesterol assimilation because elevated levels of certain blood lipids are a greater risk for cardiovascular disease. Lavanya *et al*., 2011[29] found two isolates16, 43 and *L.brevis* has ability to reduce the cholesterol level up to 80% in 24hrs. In the present study the highest assimilatory activity was 23±1.2µg/ml which was recorded in HL 5. This is because ability of organism to reduce cholesterol level was due to assimilation of cholesterol within bacterial cell and increased excretion of bile salts due to deconjuction by the bile salt hydrolase, Salminen *et al*., 2002[40]. All the selected samples for amplification were able to produce amplificons of 200 base pairs except HL6 which was failed in V2-V3 region amplification. Similar results were obtained by *L. plantarum L. fermentum, L. sakei* by Svetoslav *et al*., 2009[44] during the evaluation of *Enterococcus mundtii* ST4V (a potential probiotic and bacteriocin-producing strain), during its survival in commercial boza. In our study dietary administration of HL5 probiotic bacteria for 3 weeks increased the weight gain in treated shrimp. This showed when probiotic bacteria such as lactobacillus were supplemented in diets promote the weight gain which have resulted from secretion of digestive enzymes in the gastro intestinal tract. the probiotic bacterium *Bacillus subtilus* had beneficial effects on the final weight and weight gain, Hadizakaeifar *et al*., 2014 [21]. 10% treated shrimp have higher weight gain (9.12±0.14gms) when compare to others . This result agrees with the use of higher concentration of the probiotic did not always lead to better performances of growth, Ghosh, 2007 [20]. The total count of haemocytes and phenoloxidase activity levels were significantly increased in both probiotic treated and virus challenged shrimp. THC is used as a health indicator in shrimp and in other invertebrates because they are important non specific immunological parameter, during the periods of increased pathogen loads, higher THC numbers may provide improved immunity. Liu *et al* ., 2010 [30] who gave *B.subtilis* E.20 in the diet at a concentration of 10^8 CFU/kg to *L.vannamei* juvenile increases the disease resistance to the pathogenic bacterium, V.alginolyticus and immune responses including phagocytic activity. The enhanced phagocytosis was observed in the probiotic fed shrimp after Vibrio harvaeyi challenge in *L.vannamei*, Pope *et al*., 2011[37]. Phenoloxidase cascade plays a key role in the shrimp humoral response, Yeh *et al*., 2009[51]. Phenoloxidase activity was higher in shrimp when Bacillus cereus was fed along with diet than control diet shrimp. Navin Chandran *et al*., 2014 [35]. A significant alteration in PO activity was observed in probiotic treated shrimp when challenged with virus WSSV. In this study, we found a significant increase in immunoglobulin like substances in all group shrimp and after virus challenge. The 10% probiotic fed shrimp showed high IgG, IgM, IgA than 5% and 15% diet fed shrimp. According to Wang and Wang, 2013 [48] eleven pattern recognition particles which include immunoglobulin like proteins are present in shrimp haemolymph play a role in the immunity of shrimp against infection. The occurrence of higher immunoglobulin like substances in probiotic treated and virus challenged shrimp indicates possible immune reactive effect of probiotics against *WSSV*. High SOD activity was observed in the shrimp *L.stylirostris* which were fed with probiotic bacteria and infected with *V. nigripulchritudo*. As a result of pathogen pressure and environmental changes. aquatic animals were peculiarly susceptible to oxidative stress, Castex *et al*., 2009[10]. Shrimp have integrated antioxidant system includes enzymatic antioxidants, Castex, 2009[11]. In the present study increased SOD activity and catalase activity was observed both in probiotic fed shrimp and in virus challenged shrimp. This indicates that antioxidant system was enhanced by giving the diet contain probiotic bacteria. Administration of *L.Plantarum* in diet at 10^{10} CFU (Kg diet)-1 induced increased SOD activity significantly and enhanced the immune ability of *L.Vannamei* and increased its resistance to *V.alginolyticus* infection, Chieu *et al*., 2007 [15]. In our study higher SOD activity levels were observed in the treated shrimp feed and virus challenged than control group, high activity was observed in 10% when virus challenged to counterbalance this radical SOD appeared better activity. Better plasma glucose, lactate and tryglyceride levels were observed in *L.vannamei* maintained on the diet supplemented with probiotic bacteria showing significant differences ($P<0.01$) from the control and 10% Probiotic supplemented shrimp showed better glucose and lactate levels than other groups. High glucose levels were found by Ming-Chaoyu *et al*. 2008 [32] when they feed both *Bacillus* (0.2%) and medical herbs(0.3%) in shrimp *L. vannamei*. This indicates support for the suggestion that shrimp fed probiotic supplemented diets are healthier than control. According to Jorge Olmos *et al* , 2011[27]. increased glucose, lactate and cholesterol levels were observed when shrimp *Litopenaeous Vannamei* fed with probiotic bacteria *Bacillus* along with soyabean meal and carbohydrates. Zhou *et al*. 2009 [52] applied the Bacillus coagulans Sc8168 as a water additive found enhanced protein content of shrimp *P. vannamei*, the protein content (PC) was significantly high in probiotic treated groups, the highest PPC was observed in $5X10^5$ CFU concentration of probiotic fed shrimp when compared to that of control and groups. In our study we found that probiotics also have their effect on nucleic acids levels in shrimp tissue.

High nucleic acid levels were found in probiotic treated groups. This indicates that shrimp fed probiotic supplemented diets were healthier than control. The good water quality should maintain for survival and optimum growth of shrimp. Biological, chemical and physical parameters influenced the water quality increase or decrease of metabolic in water can have effect on growth. In the present study, the pH and salinity have high fluctuations between controlled and treated tanks and the pH ranged from 7.7 to 8.6. and salinity range of 10-35. This results are coincide with the work of Soundarapandian *et al*. (2010, 2008) [43,44], they noticed the pH range is in between 7.6 to 8.2 for probiotics treated and control ponds of P. monodon. pH is an important parameter which has its effect on the metabolism and other biological process of shrimp. As like alkalinity values total hardness values also increase from first week to fourth week. Similarly, Padmavathi *et al*., 2012, [36] observed the positive correlation between total hardness. Boyd,1982 [6] stated that the total hardness is usually related to total alkalinity because cations of hardness and anions of alkalinity are usually derived from the carbonate minerals in the solutions. In the present study, carbonate, bicarbonate, magnesium and calcium minerals increased from first week to fourth week in the treated tanks showed the increase in total alkalinity and total hardness in the tank. There was no high ammonia concentration was observed because of continuous aeration.

Application of research: The HL5 probiotic lactobacillus can be used in shrimp farms to increase the weight gain and disease resistance and it is ecofriendly which can avoid the use of antibiotics. This research is also lead to isolate noval probiotic bacteria from different sources and their molecular characterization.

Research Category: probiotics

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