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Bacillus pumilus as a potential probiotic for shrimp culture

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Abstract
The present study was primarily based up on testing and evaluation of the bacteria *Bacillus pumilus.* It was used as a feed supplement in the diet for *Litopenaeus vannamei* (*L. vannamei*), which were left to be acclimated to laboratory conditions for a period of one week. Shrimp were divided into 4 groups consisting of 6 shrimp and all the experiments were run in triplicate. Three diets were formulated containing different doses (5%, 10% and 15%) of the probiotic bacteria. Keeping one group as control and the remaining three groups were fed with probiotic supplemented feed ($1X10^6$ at10g gel/kg feed, 50gms, 100g, 150g of gel was added per Kg feed to make 5%, 10% and 15%). After feeding the shrimp with probiotic bacteria, different parameters such as antagonistic property, growth performance and hemolymph parameters such as the total Hemocyte Count levels, Glucose levels, Protein levels, Triglycerides levels. Water quality assessment was carried out to test the potentiality of bacteria *Bacillus pumilus* as probiotic.

Keywords: Probiotics, shrimp culture, water quality, *Litopenaeus vannamei*, *Bacillus pumilus*

1. Introduction
The aquaculture is mainly practiced as fresh water aquaculture and brackish water aquaculture. The most widely cultivated species of aquaculture include; Rohu (*Labeo rohita*), Catla (*Catla catla*), Grass carp (*Cenopharyngodon idellus*), Mrigal (*Cirrhinus mrigala*), Common Carp (*Cyprinus carpio*), Magur (*Clarius batrachus*), Silver carp (*Hypophalmichthys molitrin*), Singhi (*Hetero pneustes fossilis*), Rainbow trout (*Salmogairdneri*) and Gaint prawn (*Macrobrachium rosenbergii*).
Despite of enormous growth in aquaculture, there are outbreaks that effect enormously the economic growth, especially due to diseases. Diseases increase risk, deterring investment and economic growth. Usage of antibiotics leads to development of multi drug resistance strains of disease causing pathogens. Apart from lose of sensitivity to drugs there is also a potential risk of Aqua products rejection in International market if antibiotics are used.
In this contrast there is a strong need for developing the natural, non-toxic components which can ameliorate the diseases caused by pathogenic, diverse micro-organisms. Therefore, alternative methods are necessary to maintain the health of aquatic organisms. Administrating the aquaculture ponds and feed with probiotics is one of such methods that are having importance in controlling potential pathogens and also have the multi-dimensional functions in aquatic animals such as stimulating the immune system [1], maintaining the health, improving the aquatic animals quality, growth and environment etc., [2]
In our previous study we have isolated bacterial strain from river banks of Krishna, Andhra Pradesh. The isolated strain was subjected to different biochemical and molecular level characterization to identify the isolated strain at Genus and species level. The bacterium was identified as *Bacillus pumilus*, as it was isolated from natural habitat, it was used to test its potentiality to be used in aquaculture ponds by testing its efficacy when supplemented as probiotic strain in diet of shrimp *L. vannamei.* Different water quality parameters were also studied to identify *Bacillus pumilus* strain role in and around the shrimp environment.

2. Materials and Methods
2.1 Antagonistic Property
A pure colony of isolate was inoculated into a flask containing 200ml Nutrient Broth and incubated on a shaker at 200 rpm for 48 hours at 30 °C. The broth was centrifuged at 2,000×*g* for 10 minutes. The bacterial pellet was re suspended in sterile water and adjusted to 1×10^7,

1 × 10^8 and 1 ×10^9 CFU/ml. These stocks were used for analyzing the antagonistic property. For this purpose *E. coli* was cultured on nutrient agar plates. After incubation for 24 hours at 37 °C, the plates were added with isolated strain at the middle. Again incubated for further at room temperature and results were observed.

2.2 Experimental design

2.2.1 Experimental animals

For each parameter to study *L. vannamei* of a weight of 6g±0.2g each were taken and left to be acclimated to laboratory conditions for a period of one week. These animals were divided into 4 groups each consists of 6 shrimp aquatic animals, all groups were run in triplicate.

2.2.2 Diets

Three diets containing different doses (5%, 10% and 15%) of probiotic mixture were prepared. Keeping one group as control the remaining three groups were fed with probiotic supplemented feed (1X10^6 at10g gel/kg feed, 50g, 100g, 150g of gel was added per Kg feed to make 5%, 10% and 15%). The feed was given to each group for a period of three weeks, for control group the feed was supplied only by mixing with commercial gel without probiotic. In this feeding trial all shrimps were fed diets four times a day at 4% of the body weight. All the biochemical parameters mentioned below were studied just before the initiation of study and also after three weeks to assess the influence of probiotic on shrimp health. During the experiment 30% of the water was exchanged with sea water daily and the water temperature was maintained at 28±1 ^{0}C, the pH at 7.5-8.4 and the salinity was maintained at 20 ppm.

2.3 Preparation of probiotic formulation

The isolated, biochemical and molecular level identified strain was grown in sterile condition using Nutrient agar broth until the final concentration of bacteria reaches to 1X10^6 cells per ml. These cells were mixed with a commercial gel (Him C, Himalaya chemicals) to attain different concentrations of 5%, 10% and 15%. The different concentrations of bacteria are used as feed supplement to study its effect on different biochemical parameters.

2.4. Growth performance

The first sampling was taken just before starting the study. Individuals and average body weight (ABW) was measured. Sampling was regularly performed after every week days not only to assess the growth but also to check the healthiness of animals [3]. At the end of the 28 day experiment the mean weight, weight gain daily weight gain (DWG) and relative gain rate (RGR), specific growth rate (SGR) was calculated.

2.5. Hemolymph parameters

2.5.1 Triglycerides assay

The triacylglycerol assay was carried out by enzymatic GPO-PAP method, Merck, cat. 14354. The kit contains Reagent 1 (Good's Buffer pH 7.2, 50 mmol/l, 4-Chlorophenol 4 mmol/l, ATP 2 mmol/l, Mg2+ 15 mmol/l, Glycerokinase-0.4 kU/l, Peroxidase-2 kU/l, Lipoprotein lipase 4 kU/l, 4-Aminoantipyrine 0.5 mmol/l, Glycerin phosphatoxidase (GPO) 1.5 kU/l) and standard**:** 200 mg/dl (2.26 mmol/l). All the components were added as per suppliers instructions and then incubate for 5minutes at room temperature. Absorbance was measured at 500 nm [4].

2.5.2 Protein level

Plasma was isolated and further diluted 1:500 for protein determination by the method of Bradford [5]. 100 µL of plasma sample was made up to 1 mL and added with 5mL of coomassie blue, mixed well and incubated for 5 minutes. The Absorbance was measured at 595 nm. Bovine serum albumin was used as a standard.

2.5.3 Glucose level

The Glucose level from plasma was estimated using the GOD-POD method. The 1 ml of GOD-POD reagent was added with 10 µL of sample, mixed well, incubated at room temperature for 15 minutes. The absorbance was measured at 505 nm, a standard with glucose was also used to estimate the concentration.

2.5.4 Total Hemocyte Count

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. Each syringe was filled with 0.2 ml of pre cooled anticoagulant (10 mM Tris–HCl, 250 mM sucrose, 100 mM sodium citrate, pH 7.6). More anticoagulant was added to make an equal volume ratio of hemolymph to anticoagulant. Twenty hemolymph samples from each treatment were analyzed individually. A volume of 50 µL anti coagulated hemolymph was fixed with an equal volume of neutral buffered formalin (10%) for 30 min to measure the total hemocyte count (THC). Total hemocytes were counted using a hemocytometer (Boeco, Germany) and light microscope at 100X.

2.6 Water quality monitoring

The water quality parameters were monitored regularly in both control and treated ponds. The water level was measured by using a standard scale with centimeter marking. The water salinity was measured using a hand refractometer (Earma, Japan) and the pH was measured using a digital pH meter (Elico Ltd.). The total alkalinity, hardness and ammonia levels were estimated as per the standard protocols [6]. The water temperature was measured in the pond itself using a thermometer. Dissolved oxygen meter was used to estimate the D. O levels. Transparency was measured in terms of light penetration using a secchi disc.

3. Results and discussion

3.1 Antagonistic property

Image 1: Antagonistic property with *E. coli*

The *Bacillus pumilus* showed good antagonistic property against bacterial strain *E. coli.*

3.2 Growth performance

Table 1: Growth in weight of Prawn *L. vannamei* by using different concentrations of probiotic bacteria *Bacillus pumilus*.

Concentration of the Probiotic	Control	Probiotic treated	Weight gain (g)	DWG (g/d)	RGR (%)	SGR (%/day)
Control	5.71±0.04	7.12±0.07	1.39±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

3.3 Total Hemocyte count level

Table 2: Total Hemocyte Count

Concentration of the Probiotic	Control	Probiotic treated
5%	11.75±0.96 PDC	13.25±0.96 11.76
10%	12±0.81 PDC	14±1.15 15.38
15%	11.5±1.73 PDC	13.75±0.96 18

The Hemocyte count level was represented in table 1. At 10% of probiotic concentration there is significance of good count. At 10% concentration *L. vannamei* showed a Hemocyte count of 14±1.15.

3.4 Glucose levels

Glucose levels were estimated in plasma of control and probiotic treated groups. The significant difference ($p<0.01$) was observed in 5% and 10% groups. Among three groups 10% treated groups showed high glucose levels 26.45±0.43 mg/dl when compared to control 22.0±0.62 mg/dl, other two groups 5% and 15% showed 23.1±0.93mg/dl and 25.8±0.57mg/dl, respectively.

3.5 Protein levels

After three weeks of probiotic feeding the percentage of protein levels were measured in plasma of shrimp. There are significant increase in all probiotic treated shrimp ($p<0.01$) in percentage of protein levels compared to control groups. The 10% probiotic treated group showed highest protein levels 31.42 ± 0.74% than its control 25.1±0.51%, 5% treated group 29.25±1.26% and the least protein levels were recorded in 15% treated group 26.575±1.5% .

3.6 Triglycerides levels

The triglyceride levels were measured in plasma of control and probiotic treated shrimp groups. There was no significant difference ($P>0.05$) between control and 5%, 10% probiotic treated groups, but 15% group showed significant difference. The triglyceride levels were increased in all probiotic treated groups when compared to control groups. The 15% group displayed a higher level 42.25±1.71 mg/dl and the control diet fed group showed 37.75±2.06 mg/dl, whereas lower levels 41±1.82 mg/dl and 40.75±1.75 mg/dl were noticed in 5% and 10%, respectively.

3.7 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 fourth week. However slight increase in pH was observed in 15% treated tank when compared to control. Salinity found to be same in both probiotic treated tanks and in control tank in all the four weeks. Carbonates in control and probiotic treated were fluctuated much. During the first and third week there were no significant carbohydrate levels were observed in control tanks. Carbonates were increased from first week to second week, decreased suddenly in third week in all the probiotic treated tanks. The bicarbonate concentrations in control were increased in second week, decreased in fourth week. But the levels of bicarbonates were increased in the probiotic treated tanks in third and fourth week The total alkalinity in control tank was increased from first week to fourth week and much fluctuations were observed between control and treated tanks. Among the treated tanks 10% showed less total alkalinities from first to fourth week, and high alkalinities were shown in 5% from first to fourth week respectively. Very less fluctuations in calcium hardness was observed in control as well as in probiotic treated tanks. Calcium hardness was increased from first week to fourth week in all the tanks and the differences were ranged. All the values of water quality parameters are represented in Table No's 3, 4, 5 and 6 respectively.

Table 3: Water quality parameters for control tank

No. of Week	1	2	3	4
pH	7.7	8.3	7.7	8.5
Salinity (ppt)	20	20	20	20
Carbonates (ppm)	-	20	-	42
Bicarbonates (ppm)	160	170	170	190
Total alkanity (ppm)	164	192	168	232
Mg hardness (ppm)	4242	4624	4178	4468
Ca hardness (ppm)	700	729	727	745
Total hard ness (ppm)	4942	5354	4906	5214
Ammonia (ppm)	0.08	0.10	0.14	0.12

Table 4: Water quality parameters for 5% treated

No of Week	1	2	3	4
pH	8.3	8.7	8.4	8.5
Salinity (ppt)	20	20	20	20
Carbonates (ppm)	20	40	31	40
Bicarbonates (ppm)	174	180	188	180
Total alkanity (ppm)	194	220	220	220
Mg hardness (ppm)	4248	4634	4270	4448
Ca hardness (ppm)	712	753	737	739
Total hard ness (ppm)	4950	5392	5013	5177
Ammonia (ppm)	0.05	0.06	0.12	0.1

Table 5: Water quality parameters for 10% treated

No of Week	1	2	3	4
pH	8.2	8.4	8.4	8.5
Salinity (ppt)	20	20	20	20
Carbonates (ppm)	20	30	20	34
Bicarbonates (ppm)	166	172	180	184
Total alkanity (ppm)	186	202	206	218
Mg hardness (ppm)	4218	4544	4498	4578
Ca hardness (ppm)	718	744	738	752
Total hard ness (ppm)	4996	5288	5236	5330
Ammonia (ppm)	0.06	0.08	0.04	0.06

Table 6: Water quality parameters for 15% treated

No of Week	1	2	3	4
pH	8.4	8.6	8.4	8.6
Salinity (ppt)	20	20	20	20
Carbonates (ppm)	24	40	36	44
Bicarbonates (ppm)	174	170	182	188
Total alkanity (ppm)	198	210	208	222
Mg hardness (ppm)	4228	4538	4494	4678
Ca hardness (ppm)	710	738	760	740
Total hard ness (ppm)	4938	5236	5234	5424
Ammonia (ppm)	0.04	0.10	0.08	0.11

4. Summary and Conclusion

The present research work was aimed at the identifying the potentiality of bacterium *Bacillus pumilus* as probiotic strain in the aquaculture. When this bacteria was supplemented with feed at different concentrations for a period of four weeks. After particular period of feeding, when parameters such as glucose level, protein level and triglycerides levels are assessed, it showed potential good results at a concentration of 10%. It showed best triglycerides level increase at a concentration of 15%. Apart from this different parameters such as salinity, pH and bicarbonates were assessed to evaluate the role of probiotic supplemented in maintaining the water quality and found good results. Based up on all these results, it can be concluded that the bacteria *Bacillus pumilus* can be used as the potential probiotic in aquaculture, especially as feed supplement to *L. vannamei* at a concentration of 10% and 15%.

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