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EFFICACY OF FARM ISOLATED PROBIOTIC SUPPLEMENTATION ON IMMUNE INDICES AND HISTOLOGICAL STATUS OF SHRIMP *L. VENNAMEI* INFECTED WITH WSSV

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ABSTRACT:

Probiotic feed supplementation for 21 days followed by an injection with inoculum of White Spot Syndrome Virus were performed to assess the outcome of farm isolated feed probiotic (LAB) on some immune indices and histological status of some tissues of the white leg shrimp. *L vennamei*. Haemolymph (100μL) was extracted from the pericardial sinus of each shrimp at the starting of the study, after feeding with probiotics and after exposing the shrimp to WSSV virus to determine the immune parameters. The specimen was immersed in 100ml of fixative for 24 hours, and collected the Hepatopancreas, intestine tissues carefully to examine histological changes. The probiotic supplemented shrimp group showed significant increase in immunoglobulin substances and improved histological status of the tissues by lessening the histopathological indices due to WSSV infection in the marine shrimp.

Key Words: Probiotics, Immune indices, Feed Supplementation, Histological status, WSSV.

INTRODUCTION

Asia is the cradle of aquaculture and is also established as the most important area for shrimp culture since it has produced more than 80% of the cultured shrimp annually for the world market over the past several years. Currently some 14 species of marine, brackish water and freshwater crustaceans are cultured for food production in many Asian countries, accounting for 89% of the world's production of edible crustaceans (Achuthan kutty, 1998; Yao et al., 2018)

The intensification of aquaculture and globalization of the sea food trade have led to remarkable developments in the aquaculture industry (Kutty, 2005). However, the economies of most modern aquaculture operations require that hydrobionts be cultured at high densities and a consequence is the increased probability of exposure of the hydrobionts to elevated stressful conditions. The diseases and deterioration of environmental conditions often occur and result in serious economic losses (Dsouza and Colvalkar, 2001; Bondad-Reantaso et al., 2005; Bricknell and Dalmo, 2005).

In recent decades, disease prevention and control have led to a substantial increase in the use of chemical additives and veterinary medicines. The utility of antimicrobial agents as a preventive measure has been questioned, given extensive documentation of the evolution of antimicrobial resistance among pathogenic bacteria (Nomoto, 2005; Mehdi and Mojtaba, 2009). Bacterial infections cause more disease complications than any other infection and rank number one among etiological agents. Septicaemias, cutaneous abrasions, and obliteration of the shell are among the manifestations of bacterial infections (Karunsagar and Indrani, 1995). Viral pathogens also establish one of the most serious disease risks to the successful rearing of marine species and larvae. In Asian countries, the authentic economic loss due to diseases in the aquaculture industry was assessed as 9 billion U.S dollars per year (Bondad-Reantaso et al., 2005).

WSSV is discovered in almost all shrimp producing countries and is harmful to all commercially cultivated Penaeid shrimp species (Sung et al., 1996; Balcazar et al.,2006). For the past 16 years, WSSV has been recognized as a severe and upsetting pathogen of the farmed shrimp worldwide as it is concerned in a serious economic effect on the shrimp aquaculture industry due to 80 to 100% mortality within 3 to 10 days post-infection (Sablok et al., 2012; Ramos-Carreño et al., 2014). Infection by Vibrio and WSSV generates oxidative stress through the liberation of reactive oxygen species that are toxic to cells. Shrimp produce a large amount of antioxidant enzymes during a pathogenic infection, which are potential indicators of oxidative stress (Mohana Kumar and Ramasamy, 2006).

In aquaculture antimicrobials are regularly added to the feed. Sometimes antimicrobials may be added directly to the water. These procedures put on selective pressure in the exposed environment (Ratchana and Rengpipat, 2015). The use of probiotics as a means of reducing disease is widely recognized on the principle of competitive exclusion of potentially pathogenic microorganisms, digestion improvement by participating in digestion process, delivering fatty acids and vitamins, and supplying necessary growth factors, immune response enhancement through modifying non-specific immune responses, water quality improvement by enhancing the power of heterotrophic bacteria and antiviral effects through the production of antagonistic compounds (Vine et al., 2006; Ghosh et al., 2016).).

Appropriate management on the use of probiotics in aquaculture will avoid the potential risk of transfer of antibiotic resistant genes to other microbes in the culture systems and successively to the other microflora of shrimp that has a possible risk of transfer to the consumers. The application of terrestrial bacterial species as probiotics for aquaculture have had partial success because bacterial strain characters are reliant on environment in which they thrive. So, the better method is isolating probable probiotic bacteria from the marine or pond environment in which they grow to use it as feed supplement in shrimp culture to attain better production (Cakir, 2003).

Our research was intended to isolation of bacteria from the soil samples of brackish water shrimp ponds of Kolanakuduru, Bestapalem, and Tippaguntapalem villages of Gudur coastal division, Nellore district, Andhra Pradesh, India. Geographically these are located in Gudur division at 140-47'N and 790-03'E. All these areas are nearer (5-10 km) to Bay of Bengal where *L. vannamei* being cultured extensively for the last 6-8 years. After bacterial isolation, their characterization under various physical and functional conditions to select an efficient antibacterial probiotic strain (LAB) for using as feed supplement in order to study its effect on, immunological efficiencies, histopathological studies to attain better yields in *L. vennamei* shrimp farming.

MATERIALS AND METHODS

The selected *Lactobacillus* strain DZ4 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 (Bindex gel, Matrix Sea foods India Pvt. Ltd, Hyd) to attain different concentrations (5 to 15%) for using as feed supplement to study its effect on biochemical and immunological parameters.

Haemolymph (100µL) was extracted from the pericardial sinus of each shrimp into a 1ml sterile syringe (24 guage) comprising 0.9ml anticoagulant solution (30mM trisodium citrate, 0.34 M sodium chloride, 10mM EDTA, at a pH of 7.55

and with the osmolality adjusted with glucose to 780mOsm kg^{-1}). The haemolymph of each shrimp was analysed individually for Total Haemocyte Count (THC), levels of Immunoglobulin like substances and Phenol oxidase activity.

The technique for preparation of histological sections and staining was followed as per the method described by Rajendran (1999). The shrimps were injected with 1ml of Davidson's fixative into hepatopancreas, region anterior to hepatopancreas, cephalothorax, anterior abdominal and posterior abdominal regions. These tissues were dehydrated through ascending rates of alcohol, clearing of tissue, impregnation with paraffin and embedding. The sections were deparaffinized and dehydrated and then stained with haematoxylin and eosin (H & E). The tissue sections were examined under trinocular microscope at 400X magnification for histological changes.

RESULTS AND DISCUSSION

The activity of immunological parameters in selected shrimp groups were analysed in the control, after supplemented with probiotic feed for three weeks and after challenging with WSS virus respectively. Mean values of the various immunological indices such as Total Haemocyte Count, levels of Immunoglobulin like substances and activity of Phenol Oxidase system are computed in Tables (1-4) and percent change over control have shown graphically in Figures (1-4).

Total haemocyte count (THC):

After probiotic supplementation for 3 weeks all the treated animals have shown an increase in total haemocyte count when compared with controls (Tab-1). Among the treated shrimp groups, T10 group showed highest increase from $13.00 \pm 0.89 \times 10^6$/ml to $17.00 \pm 1.10 \times 10^6$/ml($P<0.05$); whereas $12.67 \pm 0.82 \times 10^6$/ml to $15.33 \pm 1.03 \times 10^6$/ml in 5% treated animals and $12.03 \pm 0.98 \times 10^6$/ml to$14.50 \pm 0.84 \times 10^6$/ml in 15% treated animals. After virus challenge total haemocyte count was slightly increased in 10% treated group followed by 5% and 15% treated groups.

Table-1: Total Haemocyte Count (10^6/ml) in the haemolymph of *L. vannamei* before and after fed with *Lactobacillus* supplement diet at three concentrations and after virus challenge.

	T5	T10	T15
Before Probiotic	12.67±0.82	13.00±0.89	12.03±0.98
After Probiotic	15.33±1.03	17.00±1.10**	14.50±0.84
After challenging with virus	16.50±0.55*	16.83±0.98*	14.50±0.84

* Significant at (P<0.05) ** significant at (P<0.01)

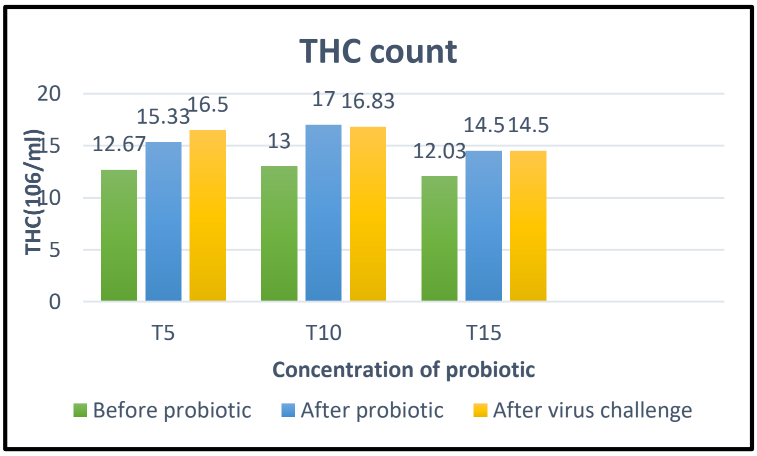

Fig-1: Total Haemocyte Count (106/ml) in the haemolymph of *L. vannamei* before and after fed with *Lactobacillus* supplement diet at three concentrations and after virus challenge.

IgA: The levels of IgA like substances in probiotic treated groups and virus challenged shrimps were significantly higher(P<0.001) than the control group shrimps (Tab-17). Among the three groups,10% treated group showed high IgA levels (3.80±0.17mg/dl) followed by 5% group (3.62±0.16mg/dl) and 15% group (3.46±0.17mg/dl). In post infected groups also, these IgA levels were increased in all the groups including control (Tab-2).

Table-2: IgA levels (mg/dl) in the haemolymph of *L. vannamei* before and after fed with *Lactobacillus* supplement diet at three concentrations and after virus challenge.

	T 5	T10	T15
Before Probiotic	0.95±0.01	0.95±0.02	0.95±0.02
After Probiotic	3.62±0.16***	3.80±0.17***	3.38±0.17***
After challenging with virus	3.69±0.15	3.94±0.24	3.46±0.17

*** significant at (p<0.001)

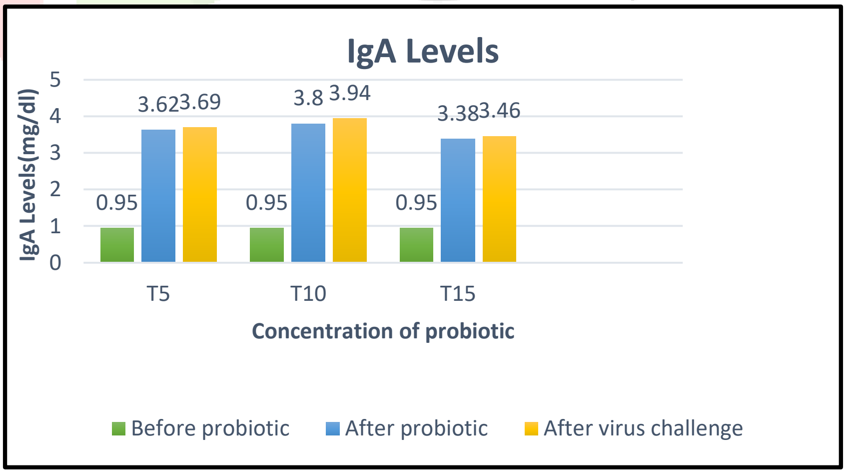

Fig-2: IgA levels (mg/dl) in the haemolymph of *L. vannamei* before and after fed with Lactobacillus supplement diet at three concentrations and after virus challenge.

IgM: The IgM like substances increased in all the probiotic treated shrimp groups as well as in virus challenged shrimps. Among the three treated groups T10 group has shown high increase in levels of IgM like substances (4.86±012 mg/dl) followed by T5 (4.63±0.22mg/dl) and T15 (4.43±0.23mg/dl) groups ($P<0.001$). After challenging with virus these IgM levels were increased in all the groups including control (Tab-3)

Table-3: IgM levels (mg/dl) in the haemolymph of *L. vannamei* before and after fed with *Lactobacillus* supplement diet at three concentrations and after virus challenge.

	T 5	T10	T15
Before Probiotic	2.57±0.13	2.57±0.13	2.56±0.12
After Probiotic	4.63±0.22**	4.86±0.12***	4.43±0.23**
After challenging with Virus	4.81±0.25	5.08±0.18	4.63±0.23

** indicates significant at ($P<0.01$) *** indicates significant at ($P<0.001$

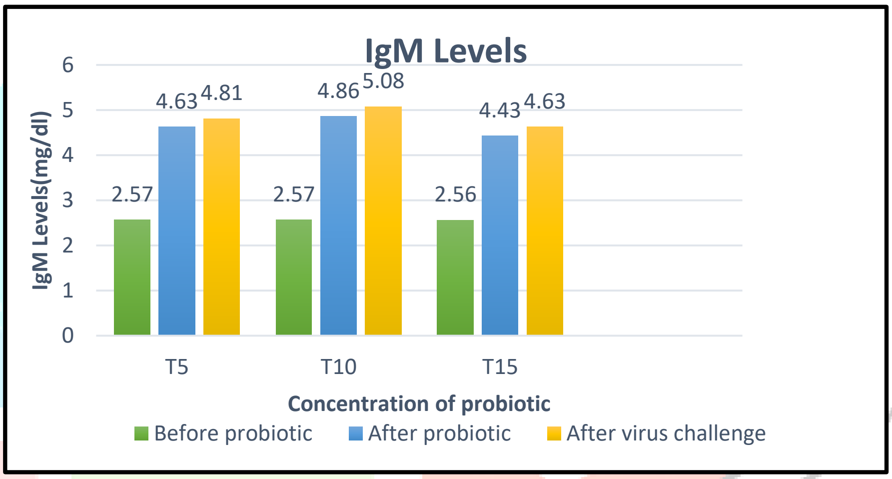

Fig-3: IgM levels (mg/dl) in the haemolymph of *L. vannamei* before and after fed with *Lactobacillus* supplement diet at three concentrations and after virus challenge.

IgG: After supplementation with probiotic for 3 weeks all the shrimp groups showed highly significant increase ($P<0.001$) in IgG like substances over the control (Tab-19). Among the treated groups T10 group showed high increase(4.75±0.28mg/dl) followed by T5 (4.26±0.20mg/dl) and T15(4.22±0.18mg/dl). In post infected also there was an increase in levels of IgG like substances in all the groups including control. It is evident from the results that the T10 group has shown significant increase($P<0.001$) than the other groups.

Table-4: IgG levels (mg/dl) in the haemolymph of *L. vannamei* before and after fed with *Lactobacillus* supplement diet at three concentrations and after virus challenge.

	T5	T10	T15
Before Probiotic	1.34±0.06	1.34±0.06	1.32±0.05
After Probiotic	4.26±0.20***	4.75±0.28***	4.22±0.18***
After challenging with Virus	4.36±0.16	4.93±0.26***	4.33±0.17

*** significant at ($p<0.001$).

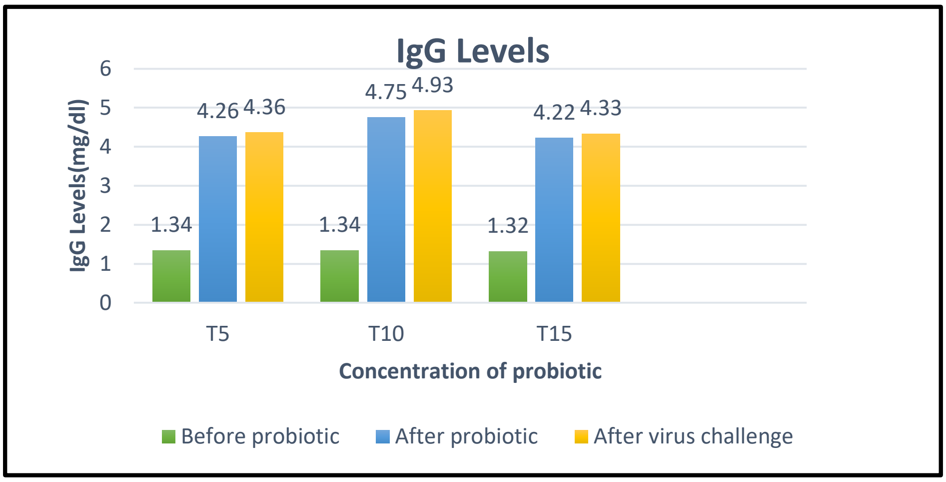

Fig-4: Phenol Oxidase enzyme activity levels(min/mg) in the haemolymph of *L. vannamei* before and after fed with *Lactobacillus* supplement diet at three concentrations and after virus challenge. Hepatopancreas:

HISTOLOGICAL STATUS

The hepatopancreas (HP) is a vital and chief organ of a decapod crustacean that performs many of the functions of the liver, pancreas, intestine, and other organs in the vertebrates (Boonyaratpalin et al., 2001), Wang et al., (1999a). Our observations (Fig:22-24) shown that the infected hepatopancreas exhibited sloughing, karyomegaly or enlargement of the nuclei, collapse of the hepatopancreas tubules, and degeneration of the tubule lumens, whereas healthy hepatopancreas showed intact E, B, R epithelial cells, and still rounded epithelial tubule instead of degraded or in necrosis conditions. The destruction of hepatopancreas tissue observed may be due to the disease affected the healthy shrimp.

Similar histopathological result was also obtained by Prachumwat et al., (2012), who recognized dysfunction of the tubule epithelial cells that progress from proximal to distal ends of hepatopancreatic tubules. However, in treated animal severity of infection is less when compared with control. Hypertrophied nuclei are less in number in treated animal compared to control which may be due to increased resistance through probiotic supplementation.

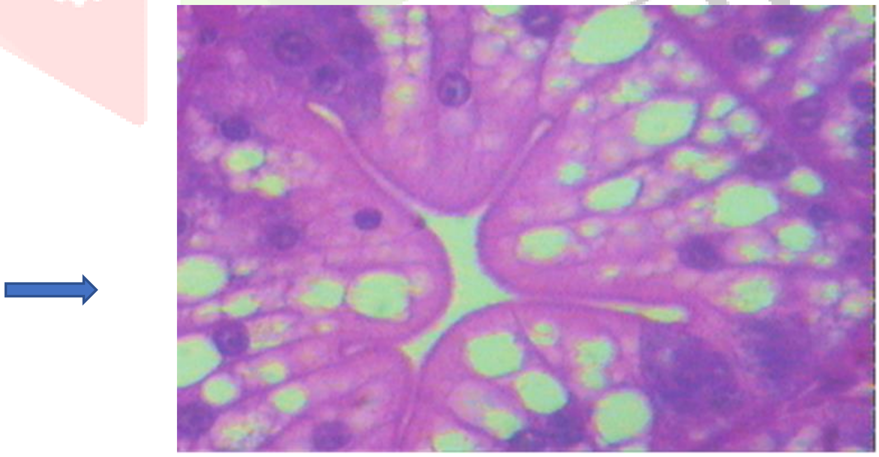

Fig-5 : Cross section of Hepatopancreas of Control animal with star shaped lumen (arrow indicates) (40X).

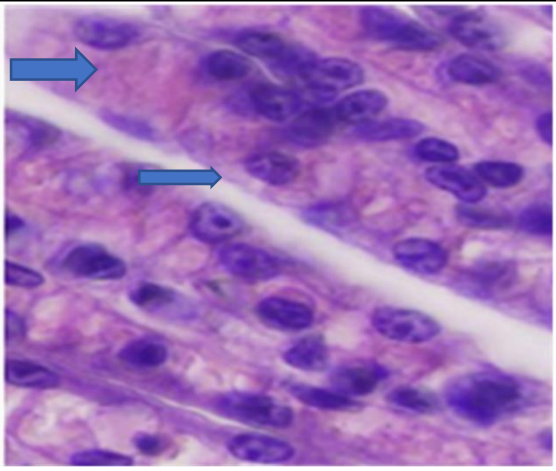

Fig-6: Cross section of WSSV infected hepatopancreatic tissue in high magnification (400X) showing Karyomegaly and Hypertrophied nuclei (shown in arrows)

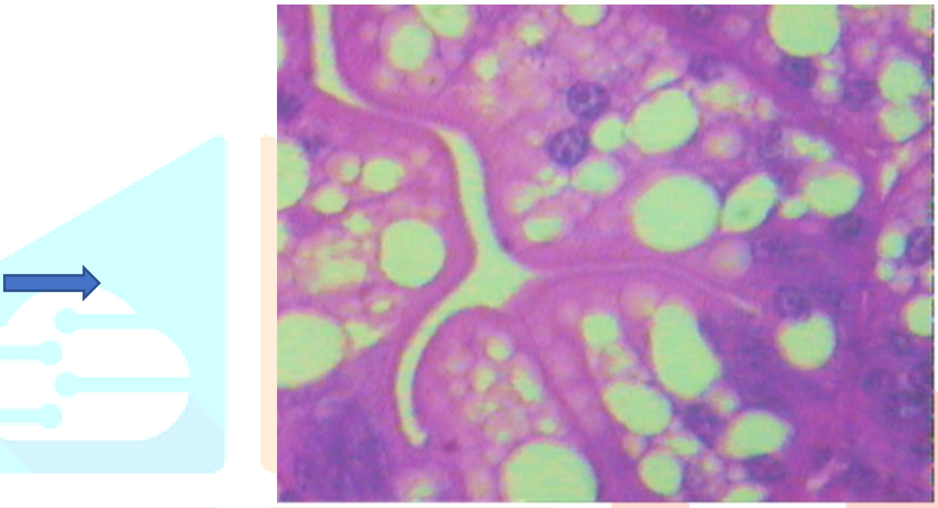

Fig-7: Cross section of Hepatopancreas of treated animal (40X) with increased lumen and delayed infection (arrow indicates).

CONCLUSION

From our studies it is evident that the mean values of haemocytes were increased significantly before and after challenge in all probiotic supplemented shrimps. The total haemocyte count (THC) is used as health indicator and provide immune efficiency during the periods of heavy pathogen loads. Higher levels of IgG, IgM, IgA like substances and phenol oxidase enzyme activity both in probiotic treated and virus challenged shrimps indicates the possible immune reactive effect of LAB against WSSV. These Ig-like proteins present in shrimp haemolymph plays a critical role in the immunity of shrimp against infection. The histopathological observations in the tissues of Hepatopancreas of *L. vennamei* indicate that the probiotic treated shrimp exhibited minimal pathological alterations compared to controls. It was observed that of less karyomegaly and decreased hypertrophied nuclei in hepatopancreas. Hence, we can conclude that the application of probiotic *Lactobacillus* species is more beneficial to the shrimp industry.

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