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Research Article

STUDIES ON ANTIOXIDANT RESPONSE, IMMUNE EFFICIENCY AND DISEASE COMPATIBILITY OF THE MARINE SHRIMP LITOPENAEUS VANNAMEI FED WITH THE PROBIOTIC LACTOBACILLUS SPECIES ISOLATED FROM THE CULTURED PONDS

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ABSTRACT

Aquaculture is the world worth coming expansion to compensate the shortage of animal protein. Feed in aquaculture plays an important role in the production and feed additive sectors are expanding day after day to achieve better growth. At the same time wide and discriminate use of antibiotics has resulted in serious threat to aqua industry. As an alternative, the probiotic technology provides a solution and proved its success in feeding practices and beneficial effects in disease control and growth of the cultured species. In the present study the effect of the farm isolated lactobacillus species on Antioxidant response, Immune efficiency and disease compatibility of *L.vannamei* has been investigated. In the first phase of the experimental set up, the shrimp are fed with probiotic supplemented diet for 21 days and in the second phase challenged with WSSV and their response were investigated for 2 days post challenged. The probiotic bacterium Lactobacillus species was added to the formulated basal diet at 5%, 10%and 15% concentration. The treated shrimp group showed significant increase in antioxidant enzyme activity and immunoglobulin substances when compared to control. Both antioxidants and immune efficiency levels are higher in 10% treated shrimp. It is also evident from the investigation that all the three concentrations of the *Lactobacillus* probiotic bacterium were effective in enhancing the resistance of the shrimp against WSSV.

INTRODUCTION

Aquaculture is considered as one of the major food production sectors practiced worldwide due to its increased availability of proteins for human consumption. According to FAO, the supplies of fish, crustaceans, and mollusks from aquaculture increased from 3.9% of total production by weight in 1970 to 27.3% in 2000 and is growing more rapidly than all other animal-food-producing sectors (FAO, 2002). Shrimp farming is one of the aquaculture business that involves production of shrimp in marine waters and prawn in fresh waters. Over the past 5 years there have been major and innovative developments in shrimp culture. Fastidious expansion and intensification of aquaculture has led to the outbreak of many diseases caused by infectious pathogens like viruses, bacteria, protozoans, etc. Hence, diseases have become a critical limiting factor in shrimp culture and processing. In order to find a solution to the outbreak of diseases, wide use of antibiotics came into existence. The usage of these antimicrobial agents has increased enormously, and tones of antibiotics are distributed in the biosphere during an antibiotic era of only about 60-year duration. The emergence of antibiotic-resistance among shrimp pathogens undermines the effectiveness of the prophylactic use of antibiotics in aquaculture (Labee-Lund and Sorum, 2001; Sorum *et al*., 2006) and increases the possibilities for passage not only of these antibiotic-resistant bacteria but also of their antibiotic resistance determinants to bacteria of terrestrial animals and human beings, including pathogens. Another problem created by the excessive use of antibiotics in industrial aquaculture is the presence of residual antibiotics in commercialized fish, shrimp, and shellfish products (Gold burg *et al*., 2001; Angulo *et al*., 2004; Cabello, 2004;).

So, in order to manage the shrimp health, usage of non-antimicrobial agents like probiotics which are known to control infectious pathogens has gained importance in shrimp industry. Besides controlling pathogens, probiotics are also known to act as natural immune enhancers which provoke the disease resistance system in shrimp. Exploitation of probiotics in

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shrimp also facilitates prevention of viral infections in shrimp industry. Probiotic agents also exert a beneficial effect via a wide array of actions; including competition for adhesion sites, resistance to colonization and competition for essential nutrients. The present study focuses on the probiotic efficiency of the *lactobacillus* bacterium on the antioxidant response and immune status of *L.vennamei* and resistance against WSSV.

MATERIALS AND METHODS

Preparation of probiotic formulation: The strain lactobacillus DZ4 collected from the soil samples of the coastal areas of Gudur division, Nellore district, Andhra Pradesh, India located at 14◦.47'N and 79◦.03'E 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 (Bendix gel, Matrix sea foods India Pvt. Ltd, Hyd) to attain different concentrations (5%, 10% and 15%) for using as feed supplement to study its effect on biochemical and immunological parameters.

Experimental design

For each parameter to study a group of 24 animals (approximately 6g wt.) were taken and acclimatized to laboratory conditions for a period of one week. These animals were divided into 4 groups each consists of 6 animals. Keeping one group as control the remaining three groups were fed probiotic supplemented feed (VAM RISE) at different concentrations (5%,10%,15%) for each group respectively for a period of three weeks. For control group the feed was supplied only by mixing with commercial gel without probiotic. The antioxidant response and immunological parameters mentioned below were studied just before the initiation of study and after three weeks to assess the influence of probiotic on shrimp health just before challenging with virus and after challenging.

Challenging with virus

At the end of 3rd week of study all groups were infected with WSSV by feeding with macerated shrimp (mincemeat) that had been prepared from a severely WSSV-infected shrimp obtained from a shrimp farm in the coastal area of Gudur, Nellore Dt., and were tested positive by nested PCR. A small piece of abdominal tissue (ca.1g) was macerated in 10mL of phosphate buffer saline (PBS) 1:10 (w:v) and centrifuged at 3000X g for 20min at 4^0C. The supernatant was centrifuged again under the same conditions (Sahul Hameed, *et.al*.1998) and the final supernatant was injected (0.2mL) into the second abdominal segment of healthy shrimp. Control shrimp were injected with PBS. All shrimp injected with infected supernatant were moribund and were dead by 72 h post challenge (hpc). The hemolymph was extracted and maintained at -70^0 C for studying the antioxidant status and immune parameters.

Antioxidant Enzyme assays

Catalase (EC: 1.11.1.6)

Activity was measured following the method of Chance and Machly, (1955) Spectrophotometrically. The reaction mixture contained 2 ml of phosphate buffer (pH 7.0), 0.45 ml of hydrogen peroxide (30 mM H_20_2) in 0.25 ml of enzyme source. The absorbance was read at 240 nm against a reagent blank. The enzyme activity was expressed as µ moles of H_2O_2 metabolized / mg protein / min.

Super oxide dismutase (EC: 1.15.1.1)

Super oxide dismutase activity was measured as the inhibition of photo reduction of nitro blue tetrazolium (NBT) by the enzyme as described by Beauchamp Fridovich (1971). The total reaction mixture consisted of 100 mM phosphate buffer (pH 7.5), 10 mM EDTA, 130 mM methionine, 750 mM NBT, 60 mM riboflavin and the enzyme source. The reaction was initiated by the addition of riboflavin and the samples were placed under a tube light (fluorescence) for 30 minutes and the resulting color was read against a reagent blank kept in dark place. The activity of the enzyme was expressed as units / mg protein. One unit defined as the amount of enzyme causing 50% decrease in the photo reduction of Nitro Blue Tetrazolium (NBT). The Mn containing type of the enzyme was proved by inhibition analysis with 2 mM KCN (Galler and Winge, 1984.

Determination of effect of probiotics on immune parameters of Litopenaeus vannamei in the laboratory

The immune parameters of the shrimp following feeding with different concentrations of probiotics were determined at the beginning, after feeding with probiotics and after exposing the shrimp to WSS virus.

Collection of hemolymph

Hemolymph (100µl) was withdrawn from the pericardial sinus of each shrimp into a 1ml sterile syringe (24 gauge) containing 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 780m Osm kg^{-1}).

They were divided into two parts. A drop of the anticoagulant-hemolymph mixture (100 ml) was placed on a hemocytometer to measure the THC using an inverted phase-contrast microscope (Leica DMIL, Leica Microsystems, Wetzlar, Germany). The remainder of the hemolymph mixture was used for subsequent tests.

Total Hemocyte Count (THC)

After collecting hemolymph, a drop of the anticoagulant-hemolymph mixture was placed on a hemocytometer to measured and calculated the number of blood cells (total hemocytes per cubic millimeter) using an inverted phase-contrast microscope and expressed as 10^6 cell/ml.

Preparation of serum samples

The hemolymph sample was collected from the heart of each shrimp into a 1ml sterile syringe (24 gauge). The hemolymph was transferred to the Eppendorf tube and centrifuged for about 6min at 3000rpm and 4^0C. The supernatant liquid was used as serum.

Measurement of concentration of Immunoglobulin- like substances (IgG-like, IgA-like and IgM-like) in the serum

The concentration of immunoglobulin like substances in the serum was measured with the help of kits and was expressed as mg/dl.

Measurement of Phenol oxidase activity

Phenol oxidase activity was estimated spectrometrically using L-3, 4-dihydrophenylalanine (L-DOPA; Sigma-Tau Pharmaceuticals Inc., Gaitherburg, MD, USA) as substrate (Hernandez-Lopez *et al*. 1996) and trypsin (Amresco, Dallas, TX, USA) as elicitor following the modified method described by Smith and Soderhall (1991). Briefly, a total of 50µl of serum was incubated with 50µl of 0.1% trypsin in CAC buffer at 25^0C f was or 10min and then 50µl of L-DOPA (0.3% in CAC buffer) was added, mixed and optical density measured at 490nm. One unit of enzyme activity was defined as an increase in absorbance of 0.001/min/mg protein (Soderhall and Unestam 1979). Protein content in serum was measured by the Bradford method (Bradford 1976), using bovine serum albumin (Amresco) as a standard protein.

Respiratory bursts of hemocytes were quantified using the reduction of nitro blue tetrazolium (NBT) to formazan as a measure of superoxide anion ($O2^-$) formation as described previously. The optical density at 630 nm was measured using a microplate reader (Model VERSAmax, Molecular Devices, Sunnyvale, CA, USA). Respiratory bursts were expressed as NBT-reduction per 10 ml of hemolymph.

Statistical Analysis

The data were expressed as the arithmetic mean $\pm$ standard deviation and subjected to two- way ANOVA followed by Tukey multiple comparison test using the SPSS statistical software package. Levels of $p<0.01$ and $p<0.05$ were considered significant

RESULTS

The activity of antioxidant enzymes and immunological parameters in selected groups of shrimps were analyzed at the initiation of the study, after supplemented with probiotic feed at three different concentrations (5%,10%&15%) respectively for three weeks and after challenging with WSS virus. The results obtained are presented in tables 2 and 3 and the corresponding percent changes over controls shown graphically in figures 1 to 7.

Anti-oxidant enzymes

Catalase activity

The catalase activity increased significantly ($P<0.05$) in all the shrimps fed with 5%.10%.ad 15% diets and after virus challenge also.

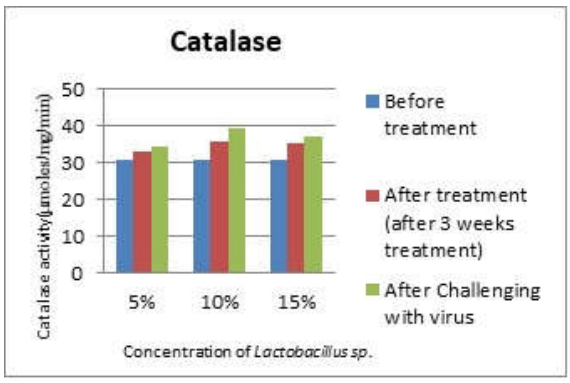

Fig 1 Effect of different Probiotic concentrations on Catalase activity

Among the three diet fed shrimps ,10% fed shrimp showed highest activity 35.74±1.46 µ moles/mg/min and after virus challenge also high enzyme activity 39.39±0.85 µ moles/mg/min (Figure:1)

Superoxide dismutase [SOD]

Significant ($P<0.05$) increasing SOD activity was observed in probiotic treated as well as virus challenged shrimp.10% probiotic diet fed shrimp showed relatively high activity15.10±1.27U/mg protein and also after virus challenge 19.52±0.85U/mg protein, compare to 5% and 15% concentrations (Figure:1).

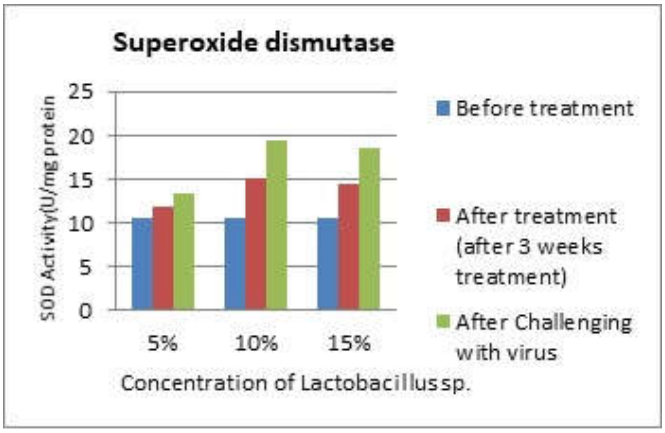

Fig 2 Comparison of SOD levels under different probiotic concentrations

Immune Parameters

Total hemocyte count: Among all the treated ones, significant increase in the number of blood cells was observed in all the probiotic treated groups and in virus challenged shrimp. However, no significant difference was found in all the three treated groups. (Figure 3).

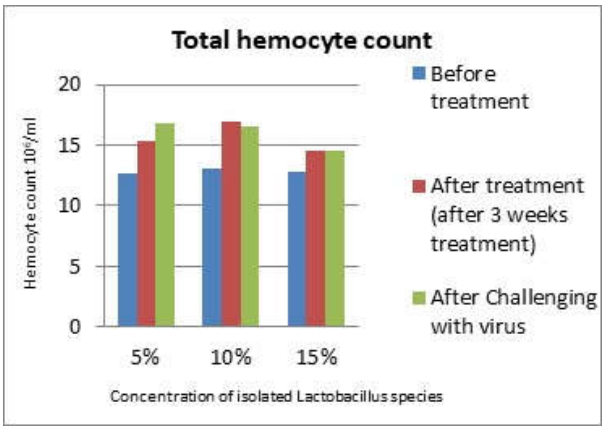

Fig 3 Effect of different probiotic concentrations on THC levels

Immunoglobulin like substances

IgG like substances

All the probiotic treated groups showed significant increase ($P<0.01$) in IgG like substances over the control before and after virus challenged shrimps.10% diet fed shrimp showed high IgG levels than 5% and 15% fed shrimps. (Figure: 4)

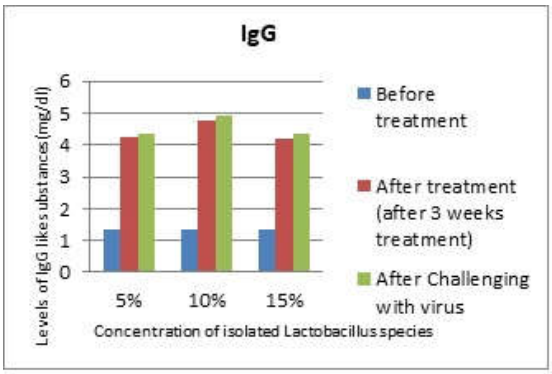

Fig 4 IgG like substances in control and treated groups

IgA like substances

The levels of IgA like substances in probiotic treated groups and virus challenged shrimps were significantly higher($P<0.01$) than that of control group shrimps. In all the three groups,10% treated group showed high IgA levels 3.80±0.17mg/dl(Fig 5)

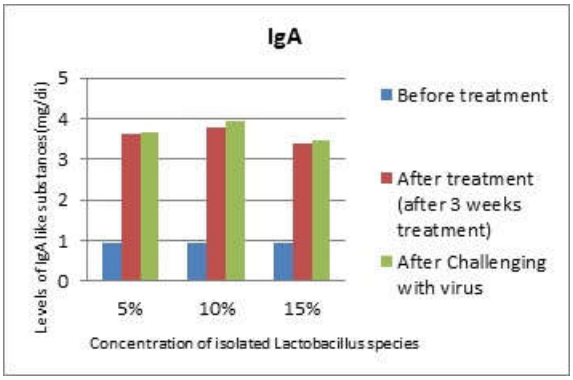

Fig 5 IgA like substances in control and treated groups

IgM like substances

In all the probiotic treated groups as well as virus challenged shrimps, the IgM like substances increased significantly ($P<0.01$). High IgM was observed in virus challenged shrimp. Among the three related groups 10% showed high IgM levels (4.86±012 mg/dl) (Fig 6)

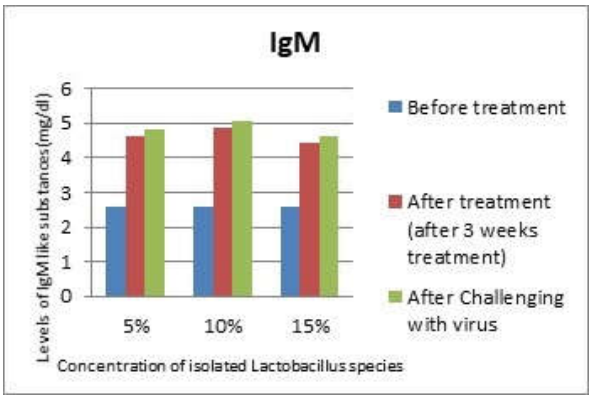

Figure 6 IgM like substances in control and treated groups

Phenol oxidase activity

Among the three groups. high phenol oxidase activity was recorded in 10% treated group (0.25±0.02 min/mg). After challenging with virus also there is maximum increase in phenol oxidase activity in 10% treated group (0.32±0.04 min/mg) (Fig 7)

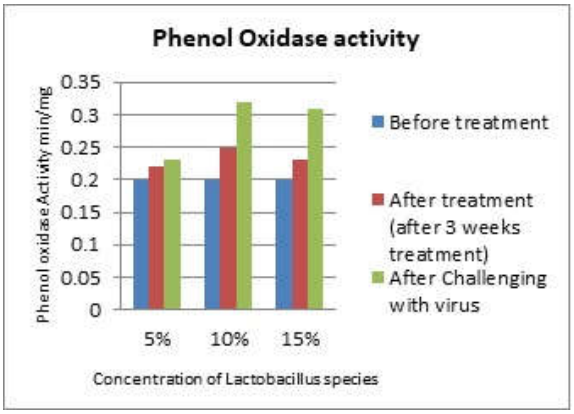

Fig 7 Comparison of PO activities under different probiotic concentrations

Table 1 Antioxidant and Immune parameters of shrimp after supplementation with different probiotic concentrations

Antioxidant and Immune parameters	Control	5% treated	10%treated	15%treated
Catalase activity(µmol/mg)	30.74±1.30	32.82±1.44	35.74±1.46	35.36±1.57
SOD Activity(U/mgprotein	10.58 ±1.15	11.89±0.99	13.10±1.27	14.84±1.30
Totalhemocytecount(10^{6}/ml)	12.50±1.05	15.33±1.03	17.00±1.10	14.50±0.84
IgG (mg/dl)	1.34±0.06	4.26±0.20	4.75±0.28	4.22±0.18
IgA(mg/dl)	0.95±0.02	3.62±0.16	3.80±0.17	3.38±0.17
IgM(mg/dl)	2.57±0.13	4.63±0.22	4.86±0.12	4.43±0.23
PO(min/mg)	0.20±0.01	0.22±0.01	0.25±0.02	0.23±0.02

Table 2 Antioxidant and Immune parameters of shrimp after challenging with WSSV

Antioxidant and Immune parameter	Control	5% treated	10%treated	15%treated
Catalase activity(µmol/mg)	33.61±1.84	34.50±0.98	39.39±0.85	37.05±1.91
SOD Activity(U/mgprotein	16.65±0.88	13.34±0.64	19.52±0.85	18.59±1.09
Total hemocyte count(10^{6}/ml)	13.00±0.89	16.83±0.98	16.30±0.53	14.30±0.84
IgG (mg/dl)	3.17±0.25	4.36±0.16	4.93±0.26	4.33±0.17
IgA(mg/dl)	2.48±0.17	3.69±0.15	3.94±0.24	3.46±0.17
IgM(mg/dl)	4.31±0.26	4.81±0.25	5.08±0.18	4.63±0.23
PO(min/mg)	0.20±0.01	0.23±0.01	0.32±0.04	0.31±0.04

DISCUSSION

Application of live probiotics in aquaculture is currently displaying an increasing trend. It is considered as an alternative and healthy approach to depressing the diseases in the host animal to enhance their immune power and to reduce the risk from pathogen in aquaculture. In the present study we have tried to evaluate the changes in the antioxidant response and immune efficiency before, after probiotic feed supplementation for three weeks and after challenging with virus

Antioxidant enzymes

In marine aquatic organisms, it has been stated that the main antioxidant enzymes are usually with higher activity in biotransformation organs as digestive tissues (Livingstone *et al*., 1992; Lemaire and Livingstone, 1993). In this study we have evaluated SOD and CAT activity in the hemolymph, since the blood cells of invertebrates are the primary effectors in host defense and are involved in various immune responses such as phagocytosis (Söderhäll and Cerenius, 1998). These anti-oxidant enzymes can destroy foreign invaders proficiently if directed at the right target.

In the present study all the three probiotic supplemented groups showed significant increase in catalase and SOD levels after

three weeks of probiotic feed supplementation than control, but highest activity was unveiled by 10% probiotic treated group. The results of the present studies clearly indicate that addition of probiotic increases the respiratory burst activity, and this supports the findings of Liu *et al* (2010) in *L. vannamei* and Zhang *et al*. (2012) in *P. japonicus*. Studies of Ankita Nandi*et.al* (2017) on *Labeo rohita* also revealed that both SOD and CAT activities of fish fed with probiotic diets were considerably ($p < 0.05$) higher from that of the control group after 70 days of feeding period. Accumulation of antioxidant enzymes in response to oxidative stress caused by biological agents is one of the main antioxidant defense pathways. Elevated levels of SOD have been linked to induce oxidative stress (Fridovich, 1995). In our study also SOD and CAT activities increased significantly after post challenge with WSSV. To overcome the stress conditions, cell has its own defense mechanisms like production of SOD and CAT to neutralize the toxic effect of free radicals and to reduce the lipid peroxidation rate (PrustyAK *et.al* 2011; Banarjee *et.al*., 2016 ; Ankita Nandi *et.al*., 2017). In our present study higher catalase and SOD levels were observed in the probiotic supplemented shrimp and virus challenged than the control group.

Prevention and control of diseases are the first priority for the development and durability of the shrimp industry. Shrimp immunology played a key role in establishing strategies for the control and prevention of diseases in shrimp farming. Shrimps lack adaptive and specific immune system; hence they depend on innate immune system. (Vazquez *et.al*,2009). In the immune defense system circulating hemocytes play a major role. In crustaceans, haemocytes take part in the reaction called immediate defence reaction that includes, modulation, encapsulation, and phagocytosis (Bachere, 2000) In the present study the mean values of hemocytes were increased significantly before and after virus challenge in all probiotic diet fed shrimps. Higher hemocyte count may provide immune efficiency during the periods of heavy pathogen loads. The occurrence of WSSV infection in inner side of the cell would interrupt the metabolism and production of haemocytes. The total count of hemocytes are used as a health indicator since they are important nonspecific immunological parameter. (Bachere *et.al*,2003)

Some proteins which are like IgG, IgA, and IgM were measured in the present study in the hemolymph of the probiotic diet fed and control group shrimp. Our findings reveal a significant increase of these immunoglobulin like substances in all the treated groups including the virus challenge.10% probiotic diet fed shrimp shown higher IgG, IgA, and IgM levels than the other two groups. These immunoglobulin like proteins present in shrimp hemolymph play a critical role in the immunity of shrimp against infection. (Wang&Wang,2013). Higher levels of immunoglobulin like substances both in probiotic treated and virus challenged shrimp may indicate possible immune reactive effect of *Lactobacillus* against WSSV.

The Phenol oxidase(PO) cascade constitutes a major component of the shrimp humoral response. (Hernández-López *et al.*, 1996; Söderhäll and Cerenius,1998.) In the present study significant increase in phenol oxidase was observed in both probiotic diet fed shrimp and in virus challenged shrimp. Previous studies of Vieira*et.al*,2010; Naveen Chandran *et.al*,2014, also coincide with our findings. The PO activity was significantly altered in probiotic treated shrimp when challenged with WSSV.

CONCLUSION

From our studies it is evident that the 10% probiotic concentration supplemented group shown highest levels of antioxidant status and immune response followed by 15% and 5%. The *Lactobacillus* probiotic supplemented diet produced favourable outcomes in terms of antioxidant response and immune parameters when challenged with WSSV compared to control diet. Hence, we can conclude that the application of probiotic *Lactobacillus* species is very more beneficial to the shrimp industry.

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