ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 7, Issue, 12, pp. 14606-14612, December, 2016

International Journal of Recent Scientific Research

Research Article

BENEFICIAL MICROBES AS PROBIOTICS ON AQUACULTURE TO BRING SUSTAINABILITY IN BLUE REVOLUTION

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ARTICLE INFO

Article History:

Received 17th September, 2016
Received in revised form 21th October, 2016
Accepted 28th November, 2016
Published online 28th December, 2016

Key Words:

Shrimp Culture, Probiotics, White Spot Syndrome Virus, Immune System, Litopenaeus vannamei.

ABSTRACT

The use of probiotics or beneficial bacteria, which control pathogens through a variety of mechanisms, is increasingly viewed as an alternative to antibiotic treatment, which cause attendant problems like drug residue in tissues, export rejection etc. The present study was aimed to determine the probiotic effectiveness of Bacillus sp, *Lactobacillus sp* and *Arthrobacter sp* (isolated from marine samples) on Litopenaeus vannamei culture for the prevalence of white spot syndrome virus (WSSV) under laboratory scale conditions. All the three selected isolates were included in the diet of juvenile shrimp at different combination and concentration. Two bioassays were conducted with treatments by triplicate. Based on the, Initial mean weight, Mean weight gain, FCR, DWG, Yield, Survival rate and Vibrio load, the test group T-5, where the shrimps fed with all the three different probiotic strains (Bacillus sp, *Lactobacillus sp* and *Arthrobacter sp*) incorporated feed (in the range of 5×106 cfu mL) showed significant changes in regard to the mentioned biometric parameters than other groups. In bioassay II, after 21 days of culture, the maximum shrimp survival (46%) was observed in treatment II comparatively than control (28%). The consortium of three potential beneficial probiotic bacterial consortiums didn't eliminate the WSSV in cultured shrimps, but increase the survival rate and decrease the vibrio load in the culture systems and water.

INTRODUCTION

Intensive development of these shrimp industries and extensive culture of these aqua farms has created various ecological, economical and social problems. During the last few years white spot syndrome virus (WSSV) disease has spread worldwide and caused large scale mortalities and economic loss in shrimp culture particularly in Asia (Danya and Jagadish, 2014). Due to the continuous outbreak of this WSSV disease in *Penaeus monodon* culture leading to loss of shrimp culture in India the farmers are seriously looking for alternative shrimp species for culture (Karthik *et al*., 2014). In 2008, the Coastal Aquaculture Authority of India (CAA) introduced a new shrimp species *Litopenaeus vannamei* as an alternative to Penaeid species in India to culture and export. Since the *Litopenaeus vannamei* exhibits fast growth rate and its culture period is significantly shorter compared to *Penaeus monodon*. Several maritime countries have switched over to *Litopenaeus vannamei* culture instead of *Penaeus monodon* as a prospective species in terms of economical gain and standing top production in short periods (Karthik *et al*., 2015 a&b; Karuppasamy, *et al*., 2013).

The newest attempt to improve water quality in aquaculture is the application of probiotics and/or enzymes to ponds. This approach of biotechnology is also known as bioremediation, which involves manipulation of microorganisms in ponds to enhance mineralization of organic matter and get rid of undesirable waste compounds. The concept of biological disease control, particularly using microbiological modulator for disease prevention has received widespread attention (Karthik *et al*., 2016). A bacterial supplement of a single or mixed culture of selected non-pathogenic bacterial strains is termed as probiotics. Thus this chapter of the study, focused to evaluate the effectiveness of potentially selected and/or

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developed suitable microalgae and a mixture of beneficial and/or probiotic bacterial consortium along with nitrifying and denitrifying bacteria on prevalence of WSSV on (*Litopenaeus vannamei*) culture under laboratory scale experimental conditions.

MATERIALS AND METHODS

Mass culture of potential bacterial strains and preparation of probiotic feed

The bacterial strains namely Bacillus sp, *Lactobacillus sp* and *Arthrobacter sp* were separately grown in nutrient broth in a shaking incubator at 30°C for 24 hours. After the incubation period, the cells were harvested by centrifuging at at 4000 rpm for 15 min at 4ºC. The obtained pellet washed twice with phosphate-buffered saline (pH 7.2) and re-suspended in the same buffer and the number bacteria (5×10^{13} CFU/ml) was standardized by dilute plating method. Spraying method was used to prepare the diet according to Gildberg and Mikkelsen (1998). For feed preparation, according to Avakh (2006), 1 kg of the commercial pellet feed containing 38% crude protein was sprayed (Individually or in a combine) by 3 ml of bacteria solution followed Gildberg and Mikkelsen (1998). Then the feed was oven-dried at 35ºC for 1 - 2 hours. The amount of bacterial load in the feed was determined by standard plate count method using respective media (Ajitha *et al*., 2004).

Mass culture of Nitrosomonas sp and Nitrobacter sp

The potential *Nitrosomonas* sp and *Nitrobacter* sp strains were mass cultured in a 2L fermentor by using sodium nitrite (0.25mg NaNO2/l Winogradsky broth) and ammonium sulphate (5.0mg (NH4)2SO4/l Winogradsky broth) as medium for *Nitrobacter* sp and *Nitrosomonas* sp respectively with proper pH (8), temperature (28°C), agitation (200 rpm) and aeration (at the rate of 0.6 L min"1). The fermentor was covered with a black cloth to protect the culture from light inactivation. When the rate of substrate uptake and product formation declined (indicating the attainment of stationary phase) the culture was harvested by centrifuging at 8000 rpm for 20 minutes at 4°C. The obtained culture filtrate was washed with fresh medium and re suspended in corresponding medium (containing 10 µg mL^{-1} substrate) and it was stored in air tight container at 4°C. Then, the absorbance at 600 nm was adjusted to 0.25 ± 0.05 in order to standardize the number of bacteria (5×10^2 CFU/ml) it was subjected to dilute plating method.

Experimental animal

Litopenaeus vannamei (nauplii 24 h) seeds were obtained from a commercial shrimp hatchery located in Marakanam, Kanchipuram District, Tamil Nadu, India. They were kept in seawater with aeration for a period of 6 h in order to avoid any stress to the animals and then used for further experiments.

Feeding schedule from zoea to post larvae

The Zoea of *Litopenaeus vannamei* kept in the experimental tanks was fed with *Cheatoceros calcitrans*. On 1st day of the Zoea (Z) of I- III stages were fed thrice with 30×10^4 cells/mL of algal cells. On the 2nd day of the Mysis (M) (I- III/Postlarve 1) of *Litopenaeus vannamei* were fed thrice with 40×10^4 cells/mL of algal cells. From 3rd day up to 20th day they were fed thrice with 3-8 No/mL of *Artemia salina* nauplii enriched with *Cheatoceros calcitrans*. The 24 h old *Artemia salina* nauplii enriched with *Cheatoceros calcitrans* for a period of 24 h was fed to *Litopenaeus vannamei*. This experiment was conducted up to when the larvae reach PL15 stage. Then from PL15 to PL25 animals were fed with *Artemia salina* and commercial pellet feed at daily rate of 8% body weight, three times daily.

Shrimp acclimation for experimental conditions

A total of six hundred and fifty shrimps were individually weight and placed into thirteen batches (each containing 50 shrimps) and acclimated to culture and/or experimental conditions for five days. During the first five days of experimental condition, animals were fed with commercial feed. Two bioassays were conducted to evaluate the effect of feed supplemented with different probiotic bacteria to evaluate in terms of growth performance, survival and prevalence of WSSV.

Experimental design for Bioassay I

After acclimation of five days, shrimps in the control tank were fed only with commercial feed and the shrimps in the experimental tank 1 to 12 were fed with probiotic bacterial isolates mixture of two or three probiotic bacteria) supplemented feed (Table). The experiment carried for 60 days, during the culture period shrimp in all groups were fed twice daily at 9am and 5pm. Shrimps were fed twice daily and half of the water was exchanged at day three and the uneaten food and waste matter were removed daily before feeding.

Table 1 Experimental setup for probiotics administration (Bioassay I)

Treatments	Feed incorporated with mixture of multi probiotic isolates @ ratio 1:1	Experiments	Dosage	Water Probiotics in the range of 5×10^2 CFU/ml
T – 1	Commercial feed	Control	-	-
		E – 1	10^2 CFU mL^{-1}	Once in a week
T – 2	Bacillus sp + *Lactobacillus sp*	E – 2	10^4 CFU mL^{-1}	Once in a week
		E – 3	10^6 CFU mL^{-1}	Once in a week
		E – 4	10^2 CFU mL^{-1}	Once in a week
T – 3	Bacillus sp + *Arthrobacter sp*	E -5	10^4 CFU mL^{-1}	Once in a week
		E – 6	10^6 CFU mL^{-1}	Once in a week
		E – 7	10^2 CFU mL^{-1}	Once in a week
T – 4	*Lactobacillus sp* + *Arthrobacter sp*	E – 8	10^4 CFU mL^{-1}	Once in a week
		E – 9	10^6 CFU mL^{-1}	Once in a week
		E -10	10^2 CFU mL^{-1}	Once in a week
T – 5	Bacillus sp + *Lactobacillus sp* + *Arthrobacter sp*	E – 11	10^4 CFU mL^{-1}	Once in a week
		E – 12	10^6 CFU mL^{-1}	Once in a week

During the culture period, both the potentially selected nitrifying and denitrifying bacterial strains such as, *Nitrosomonas* sp and *Nitrobacter* sp (each in the range of 5 × 10^2 CFU/ml) were added (through water) in all the experimental tanks (7, 14, 21, 28, 35, 42, 49 and 56th day). The growth parameters were calculated according to Robertson *et al*. (2000), Felix and Sudharsan (2004) and Venkat *et al*. (2004).

1. Weight gain (g/shrimp) = Final weight (g) – Initial weight (g)
2. Weight gain (%) = Final weight (g) – initial weight (g) X 100
3. Initial weight (g)
4. Food conversion ratio (FCR) = Total feed given (g)
5. Wet weight gain (g)
6. Daily weight gain (DWG; g/days) = Final weight (g) x Initial weigh t (g)
7. Days
8. Yield of shrimps (g) = Mean body weigth (g) x Total viable shrimps at harvest
9. Survival rate (%) =Total number of larvae survived Initial number of larvae stocked X 100

Experimental design for Bioassay II

Total of one experimental with one control trial were conducted in aerated 120-L indoor plastic tanks containing 80 L of filtered (20 µm) sea water (34 to 35 g/l) and constant aeration in groups of 50 animals per tank. The second bioassay experiment was conducted for 21 days. After acclimation of five days (Similarly to Bioassay I), shrimps in the control tank were fed only with commercial feed and the shrimps in the experimental tank were fed with probiotic bacterial isolates mixture of two or three probiotic bacteria) supplemented feed (Table) for the first seven days . Shrimp were fed twice daily and half of the water was exchanged at day three and the uneaten food and waste matter were removed daily before feeding. Meanwhile, WSSV infected shrimps were obtained from a disease spread out pond and confirmed for positive by RT-PCR. One gram of homogenised WSSV positive shrimp paste was fed to the animals in both the treatments (control and experiment) on day eight. Then from day nine to 21 animals were fed as the first seven days. During the culture, both the potentially selected nitrifying and denitrifying bacterial strains such as, *Nitrosomonas* sp and *Nitrobacter* sp (each in the range of 10^5 ml^{-1}) were added (through water) in the experimental tank (7, 14and 21st day). At the end of 21st day, shrimp survival in the control and experimental groups were determined and shrimp samples were taken for WSSV analysis.

Table 2 Experimental setup for probiotics administration (Bioassay II)

Treatments	Feed incorporated with mixture of multi probiotic isolates @ ratio 1:1	Experiments	Dosage	Water Probiotics in the range of 5×10^2 CFU/ml
T – 1	Commercial feed	Control	-	-
T – 2	Bacillus sp + *Lactobacillus sp* + *Arthrobacter sp*	E -10	10^2 CFU mL^{-1}	Once in a week

Water quality analysis

Water quality analysis has done using following standard methods. pH pen (Scan – 2- Eutech cybernetics PTE Ltd, Singapore) used to measure the water pH and handy refractometer (Atago, Japan) for estimating salinity. Dissolved oxygen and temperature together were measured with the help of handy D.O meter (YSI 55 model). Ammonium and nitrite was estimated by an ammonia meter respectively. The shrimp culture systems were cleaned daily by siphoning out the wastes and uneaten feed. Water exchanging was done 30% daily. All the control and experimental tanks were supported by well-built aeration.

Microbiological analysis

Shrimps and the water samples were taken from all the control and experimental tanks during the first bioassay at every 15 days time intervals to enumerate and check the *Vibrio* sp on TCBS agar medium (Sivakumar *et al*., 2012; Akponah *et al*., 2014).

RESULTS AND DISCUSSION

The farming of whiteleg shrimp (Litopenaeus vannamei) is an important economic activity in India. However, in the last decade, this industry has been threatened by viral diseases that have affected its production performance. While white spot syndrome virus (WSSV) can cause cumulative mortalities of up to 100% within 3–10 days (Chou *et al*., 1995; Wang *et al*., 1995). One of the strategies involved in aquaculture to prevent losses caused by diseases are the basic practices of good management, chemotherapy and vaccination (Subasinghe and Barg, 1998). However, in the last years, biological control has become a useful technique in aquaculture. It consists in the use of probiotic bacteria capable of stimulating growth and improving animal health (Farzanfar, 2006). In aquaculture, the term probiotic is defined as a microbial supplement formed by a single or a mixed culture of selected microorganisms that are added to a culture system in order to manipulate the microbial communities present in the pond (Balcázar, 2002). Probiotics have multiple mechanisms of action to inhibit pathogens which include competitive exclusion, production of substances that inhibit growth of opportunistic pathogens (antagonism), stimulation of the immune response, antiviral effects, increase of digestive function through production of enzymes, improved nutrition by providing essential nutrients, and improved water quality (Balcázar, 2002; Balcázar *et al*., 2006; Farzanfar, 2006). In the two last decades, many studies reported promising results using a single beneficial bacterial strain in the culture of many finfish species (Avella *et al*., 2010a). The application of probiotics
against viruses in shrimp cultivation is a novel and safe approach. Looking for novel approach, Hence, this chapter of the study, focused to evaluate the effectiveness of potentially selected and/or developed suitable microalgae and a mixture of beneficial and/or probiotic bacterial consortium (at different combination and concentration) along with nitrifying and denitrifying bacteria on prevalence of WSSV on *Litopenaeus vannamei* culture under laboratory scale experimental conditions.

Water quality parameters

During the experimental period, the temperature (26 – 28°C), salinity (25 – 28%), total ammonium (0 – 0.1 mgl−1), nitrite (0 – 0.05 mgl−1) and pH (7.0 – 7.6) were maintained in the suitable range and found to be stable in all the control and experimental tanks. Shan and Obbard (2003) reported that reduction of TAN in aquaculture system can be facilitated by providing and maintaining an optimum environment condition for nitrifying bacteria. Nitrification is the aerobic oxidation of ammonia to nitrite followed by the aerobic oxidation of nitrite to nitrate. Nitrification is a two step process in which ammonia is oxidized to nitrite by ammonia oxidizing bacteria (AOB) or ammonia oxidizing archaea (AOA) and nitrite is then oxidized to nitrate by nitrite oxidizing bacteria (NOB). Hence, in this study during the culture, both the potentially selected nitrifying and denitrifying bacterial strains *Nitrobacter* sp and *Nitrosomonas* sp were added (through water) in all the experimental tanks on weekly intervals. While, checking the Ammonia (NH_4^+), Nitrite (NO_2) and Nitrate (NO_3) in all the tank water on day the ammonia and nitrite concentration decreased and the nitrate concentration increased in the both *P.monodon* and *L.vannamei* culture experimental tanks.

Effectiveness of bacterial mixtures on Bioassay I

The first bioassay was carried out for 60 days. In this, study all the three potentially selected probiotic bacterial strains viz., Bacillus sp, *Lactobacillus sp* and *Arthrobacter sp* were incorporated in to feed at different combination and concentration and fed to the shrimps. At the beginning of study there were no significant differences for initial mean weight of shrimps calculated between all the control and experimental groups. After 60 days of culture, there were significance differences for survival rate between and the other treated and control groups and also the mean yield. There were significance differences in final mean weight (10.2±0.20 g), Mean weight gain (6.17±17 g), Mean weight gain (153.10±18%), FCR (2.65±0.07), DWG (0.68±0.05 g/days), Yield (265.31±15.11 g) and Survival rate (86.1±1.18) in Treatment T-5, where the shrimps fed with all the three different probiotic strains such as, Bacillus sp, *Lactobacillus sp* and *Arthrobacter sp* incorporated feed (1×10^6 CFU mL^{-1}) followed by T-3, T-4, T-2 treatments and control groups.

The first bioassay results indicates that, incorporating a mixture (consortium) of Bacillus sp, *Lactobacillus sp* and *Arthrobacter sp* as feed probiotics in the concentration of 1×10^6 CFU mL^{-1} along with *Nitrobacter* sp and *Nitrosomonas* sp each in the concentration of 5×10^2 CFU/ml through water (Weekly once) will absolutely provide additional support against the unfavorable conditions or pathogen attacks during the culture and increased significant survival and yield. Other studies previously demonstrated enhanced protection with multi-species probiotics (Timmerman *et al*., 2007; Zoppi *et al*., 2001), based on the theory that multiple species-specific benefits possessed broaden spectrum of probiotic effect. Indeed, three LAB probiotics were effective against *Vibrio harveyi*, *V. parahaemolyticus* and *Pseudoalteromonas piscicida* in an *in vitro* assay (Talpur *et al*., 2012). The higher survival of shrimp fed with probiotic supplemented feed might be related to an immune reactive effect of probiotics on the host immune system, by producing extracellular compounds to stimulate the non specific immune response in vertebrates (Marteau *et al*., 2002; Gill, 2001).

Effectiveness of bacterial mixtures on control of Vibrio load

While checking the vibrio load in the culture water and shrimp (in both control and experimental groups) on 7th, 14th and 21st day, the higher Vibrio load was observed in shrimp intestine and culture water where the shrimps fed with control diet, and it was decreased in treatment (T- II), where the shrimps were fed with all the three different probiotic strains such as, Bacillus sp, *Lactobacillus sp* and *Arthrobacter sp* incorporated feed (10^6 CFU mL^{-1}). Far *et al*., 2009 as also suggested that the use of Bacillus sp as a probiotic in shrimp culture will colonize both the culture water and the shrimp digestive tract and also replace *Vibrio* spp. in the gut of the shrimp, thereby increasing shrimp survival. Abrashev *et al.* (1998), have reported that, some *Arthrobacter sp*ecies have the ability to produce a number of valuable substances like amino acids, vitamins, enzymes, specific growth factors, and polysaccharides and its possess many advantageous properties and nutritional benefits to be probiotics in aquaculture (Li *et al*., 2006). Recently, Amnah, (2013) have reported that the *Arthrobacter sp* can be regarded as a probiotic bacterium for the culture of shrimp while *β*-1,3 glucan and, *Moringa oleifera* leaf were considered as immunostimulants for cultured of shrimp *Penaeus indicus* Juvenile against pathogenic vibrios Juvenile. Natesan *et al*., 2012 also observed the maximum zone of inhibition (16mm) against *V. alginolyticus* using their strain *L. acidophilus* 04. The previous authors also described that, the antibacterial activity of *Lactobacillus sp* against the pathogenic microbes may be due to the production of its metabolites such as, organic acids (lactic and acetic acid), hydrogen peroxide, diacetyl and bacteriocins (Valenzuela *et al*., 2010).

Effectiveness of bacterial mixtures on Bioassay II

WSSV is a large dsDNA virus infecting crustaceans and is the most important viral pathogen of cultured penaeid shrimp worldwide. In cultured shrimp, WSSV causes a cumulative mortality of up to 100% within 3-10 days. Due to its rapid spread and high associated mortality rates, WSSV is an extremely virulent pathogen in shrimp culture (James *et al*., 2010). Hence, in this study, the second bioassay was conducted for 21 days with shrimp weighing (12.8 ± 1.8 g). After acclimation of seven days with probiotic diet, animals in the control and experimental treatments were fed with only 1 g per tank of muscle shrimp paste positive for WSSV and confirmed positive for the White Spot Syndrome Virus. During the experimental period, the temperature (26 – 28°C), salinity (25 – 28%), total ammonium (0 – 0.1 mgl−1), nitrite (0 – 0.05 mgl−1) and pH (7.0 – 7.6) were maintained in the suitable range and found to be stable in all the control and experimental tanks. After 21 days of culture, the maximum shrimp survival (46%) was observed in treatment II comparatively than control (28%), and the shrimps from both control and experiments were confirmed with WSSV positive. As the same beneficial microbes also play a vital role in aqua ponds therefore using commercially available water and soil probiotic products such as, Grobac, Good Earth, Prorich and Nature - 365 (Guybro Chemical Pvt Ltd, Mumbai) which enhances the crop

production in aquaculture sector. Farmers feedback and trial reports of these products also have proven that the product works effectively *viz.*, oxidize organic matter into enzymes and available nutrients, produce antimicrobial compounds and eliminates pathogens, removes bad odor, sludge and black soil from the pond bottom, biological oxidation of ammonia and ammonium to nitrite, biological reduction of nitrite into useful nitrate and improves the growth of beneficial microbes and plankton under different regions and farming situations in India and as well as in other countries.

From the results, it has been suggested that the, the consortium of these potential and/or beneficial probiotic bacterial consortium have not eliminated the WSSV infection in cultured shrimps, but increased the survival rate and decreased the vibrio load in the culture systems and water.

A perusal of literature revealed thus far no reports on the effect of incorporating a mixture (consortium) of Bacillus sp, *Lactobacillus sp* and *Arthrobacter sp* as probiotics in shrimp feed and WSSV prevalence in *L. vannamei* or other penaeid shrimp. Similarly, Partida Arangure *et al*., 2012, reported that, inulin and probiotic bacteria increased the shrimp survival of 100% and a decrease in the prevalence of WSSV (22.2%) in shrimp fed inulin (8.0 g/kg feed) and bacteria (1 x 105 CFU/g feed) compared with control (44.4 and 51.8%).

However, study of the antiviral activity of the probiotics is an upcoming research which needs a deep insight. Further research works should be needed to adapt the WSSV to the BHK cell lines, to study their antiviral efficacy and cytopathic effect (CPE) in the BHK cell lines for the qualitative assessment of antiviral efficacy of the probiotic bacteria strains used in this study.

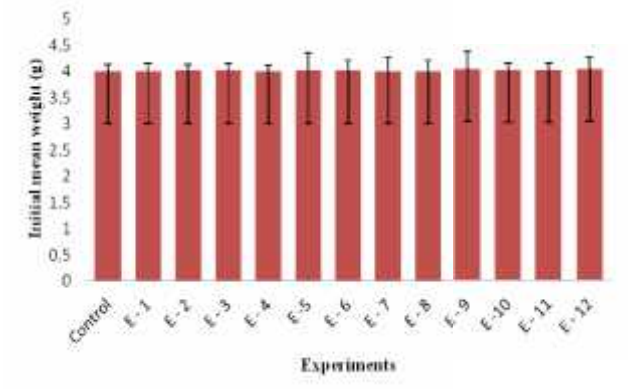

Fig 1 Initial mean weight (g) of *L. Vannamei*

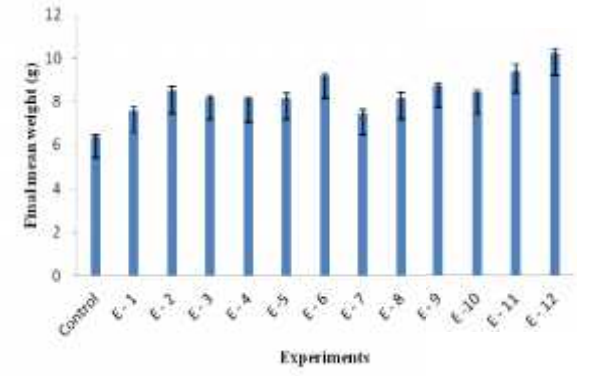

Fig 2 Final mean weight (g) of *L. vannamei*

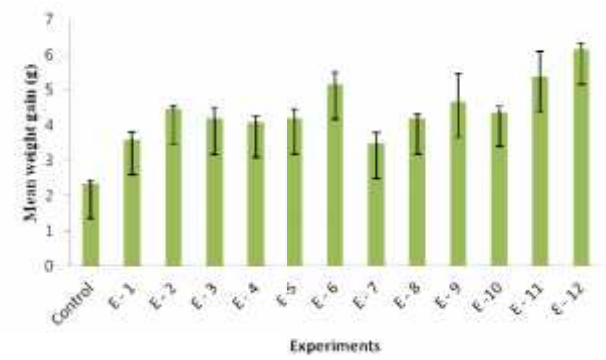

Fig 3 Mean weight gain (g) of *L. vannamei*

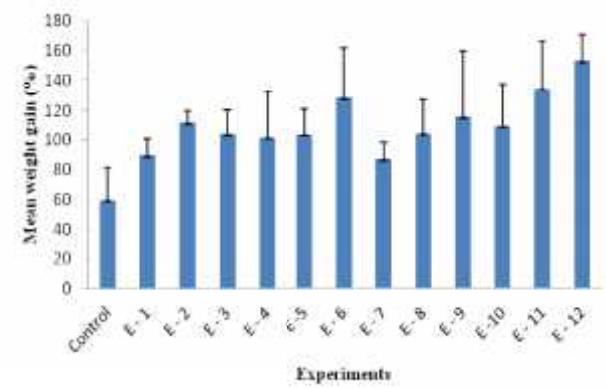

Fig 4 Mean weight gain (%) of *L. vannamei*

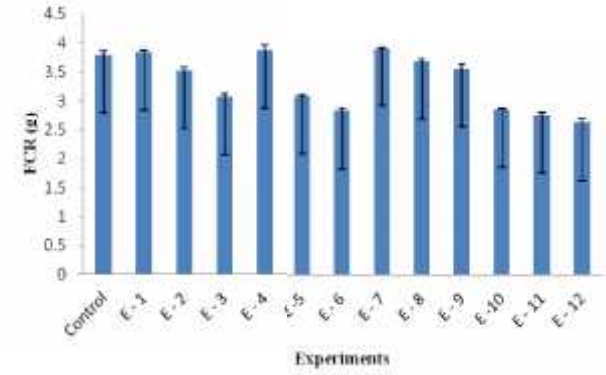

Fig 5 FCR (g) of *L. vannamei*

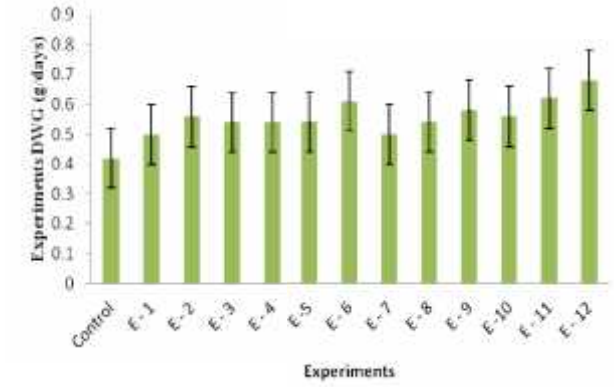

Fig 6 DWG (g/days) of *L. vannamei*

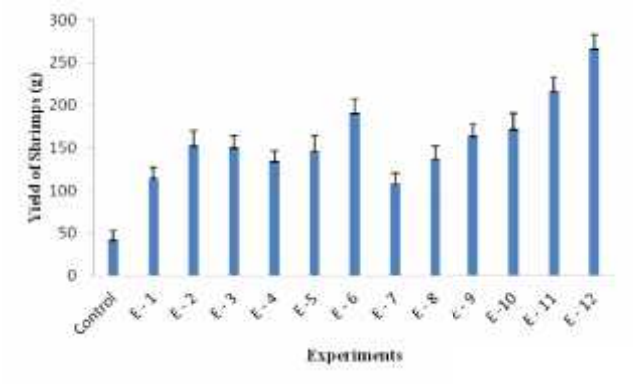

Fig 7 Yield of Shrimps (g) of *L. vannamei*

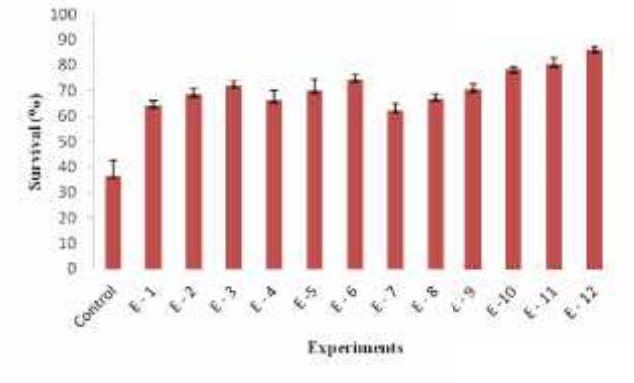

Fig 8 Survival (%) of *L. vannamei*

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How to cite this article:

Karthik Ramachandran *et al*.2016, Beneficial Microbes As Probiotics on Aquaculture to Bring Sustainability In Blue Revolution. *Int J Recent Sci Res.* 7(12), pp. 14606-14612.