Impact of *Bacillus subtilis* on growth, biochemical constituents and energy utilization of the post larvae of shrimp, *L. vannamei*

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ABSTRACT

The present study was carried out to evaluate the effect of probiotic bacteria Bacillus subtilis on survival, growth, biochemical constituents and energy utilization in Pacific whiteleg shrimp, *Litopenaeus vannamei*. Experimental diets were prepared with different concentrations of *B. subtilis* at the rate of 2%, 4% and 6% and the feed without probiotic bacteria ,0%, considered as control feed. The above diets were fed to *L. vannamei* for a period of 90 days. The feed influenced on various growth parameters such as survival rate, weight gain, specific growth rate, feed conversion efficiency, and protein efficiency and all are increased significantly at 4% *Bacillus subtilis* assimilated diet fed PL of shrimp. The biochemical constituents of proteins, amino acids, carbohydrates, lipid and ash and moisture contents were also significantly increased (P <0.05) at 4% *B. subtilis* assimilated diet in PL of shrimp. From the above results, we can conclude that *Bacillus subtilis* might have a promising role in shrimp culture. This kind of perspective can be the better option for the establishment of sustainable aquaculture.

Keywords: *Litopenaeus vannamei*, *Bacillus subtilis*, *Post Larvae*, growth parameters, biochemical constituents, Promising role.

INTRODUCTION

It is well known that the human consumption of animal protein has rapidly increased, which is mainly depend upon the terrestrial and also on aquatic farm animals. World aquaculture production has increased significantly in the last years (Yan-Bo, 2007). Among them, shrimp farming is one of the fastest growing aquaculture sectors in many tropical countries; however, this development has been accompanied by adverse impacts due to the intensification, such as problems related to diseases and deterioration of environmental conditions

The Pacific whiteleg shrimp, *Litopenaeus vannamei* (Boone, 1931) is a natural inhabitant of tropical marine waters along the Eastern Pacific coast, from the Gulf of California (Mexico) to Tumber (North of Peru), where the year-round water temperature normally exceeds 20°C. Presently, it is the most widely cultured shrimp species all over the world. It's farmed area is expanding mainly due to the availability of specific pathogen-free (SPF) seeds in the international market and its phenomenal success in farming systems due to the ability to tolerate wide salinity ranges (0–45 ppt), fast growth rate, low dietary protein requirement (30–35%), column feeding habit and amenability for crowding and very high stocking densities (Briggs et al. 2004; Ravichandran et al. 2009).

Aquaculture, however, is an increasingly important option in animal protein production. This activity requires high-quality feeds with a high protein content, which should contain not only the necessary nutrients but also complementary additives to keep organisms healthy and promote favourable growth. Some of the most utilized growth-promoting additives include hormones, antibiotics, ionophores and some salts (Fuller, 1992; Gongora, 1998; Klaenhammer and Kullen, 1999). Although these do promote growth, their improper use can result in adverse effects in the animal and the final consumer, as well as lead to resistance in pathogenic bacteria in the case of antibiotics. This situation paved the way for the search for an alternative to the antibiotics. Success of quality seed production depends mainly on the availability of suitable food that is readily consumed, efficiently digested and that provides the required nutrients to support good growth and health (Alokesh kumar et al. 2016; Janardanareddy et al.2018; Tovar-Ramirez et al., 2002; Wache et al., 2006).

Shrimp farming is a major production system contributing about 70% of India's total seafood in value. In India, large scale culture of *L. vannamei* commenced from 2009 with the import of SPF broodstock for revitalization of the ailing shrimp farming sector, which was constantly incurring heavy losses owing to the outbreak of white spot disease in *Penaeus monodon*.

Probiotics are defined as a live microbial adjunct which has a beneficial effect on the host by modifying the host associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment (Verschuere, 2000). Probiotic bacteria could produce digestive enzymes and essential growth nutrients such as vitamins and amino acids, which are benefit for enhancing the best growth, also they could benefit to their invertebrate host by competitive exclusion against pathogens (Gomez-Gil et al., 2000) or by increasing the host resistance and immunity (Austin et al., 1995) which are benefit to achieve the higher survival rate and healthier animals.

Appropriate uses of probiotic in aquaculture industry were shown to improve intestinal microbial balance, and also to improve feed absorption, thus leading to increased growth rate (Fuller, 1989; Rengpipat et al., 1998) and also reduced feed conversion ratio (FCR) during the cultural period (Wang, 2007).

Vannamei shrimp (*Litopenaeus vannamei*) is one of the shrimp varieties that are currently widely cultivated due to several advantages including among others, fast growth, ability to be cultivated in high density and relatively high market price (Nuraini et al. 2007). Factors that influence the success of vannamei cultivation are fish health, good water environment and quality feed. Probiotic use is currently a popular innovation in aquaculture because the microbes contained in the probiotics have the ability to give advantages to the host by modifying microbe community or through association with the host, by improving nutritional values and feed use, by improving the host response to diseases and by improving the environment quality (Widanarni et al., 2012).

Probiotics are Gram-positive, lactic acid-producing bacteria that cause an increase in enzymatic activity, leading to a better feed digestibility. The criteria for lactic acid bacteria strains to be used as "probiotics" include (Sharma et al.2014):

(i) exert a beneficial effect on the host
(ii) endure into a food product at high cell counts
(iii) survive during passage through the gastrointestinal tract
(iv) adhere to the intestinal epithelium
(v) produce antimicrobial substances
(vi) have antagonistic activity against pathogenic bacteria
(vii)stabilize the intestinal microflora
(viii) stable against bile, acid, enzyme and oxygen and
(ix) safety assessment (non-pathogenic, non-toxic and non-allergic)

A probiotic organism must possess certain properties reported by Sugitha et al. (1998) and Kesarcodi-Watson et al. (2008):

(i) to be highly accepted by the host
(ii) should not be harmful to the host
(iii)work in vivo as opposed to in vitro findings
(iv) reach the location where the effect is required to take place and
(v) should not contain virulence resistance genes

In general the Probiotics are used in:

(i) animal feed, as growth promoters and competitive exclusion agents
(ii) human nutrition, as dietary supplements and
(iii)Aquaculture systems are also used for-disease-resistance and enhancing the growth (Buruiana et al., 2014; Cha et al., 2013; Dong et al., 2009).

Following methods have to be selected probiotic bacteria for use in the aquaculture industry, might be included the following steps (Buruiana et al., 2014; Sahu et al., 2008).

(i) acquisition of potential probiotics
(ii) collection of background information
(iii) evaluation of pathogenicity and survival test
(iv) economic cost/benefit analysis.
(v) evaluation of the effect of the potential probiotics in the host (in vivo evaluation) and
(vi) evaluation of the ability of potential probiotics to out-compete pathogenic strains (in vitro evaluation) (Buruiana et al., 2014).

The mechanisms of action regarding the beneficial effect of probiotics on the host are as follows (Balcazar et al., 2006; Mohapatra et al., 2013):

(i) improvement of water quality

(ii) modulation of immune responses of host

(iii)enzymatic contribution to digestion

(iv)competition for chemicals or available energy

(v) competition for adhesion sites

(vi)antagonistic activities (antibacterial, antiviral and antifungal activity)

(vii) competition for nutrients and

(viii) production of inhibitory compounds

The genus *Bacillus* has been isolated from crustacean intestine (Rengpipat et al., 2000). Some species of this genus have shown inhibitory activity against various pathogens and also increase of mean weight and survival rate of *Penaeus monodon* larvae and post larva (Sugitha et al., 1998; Rengpipat et al., 2000). The effect of probiotic, *Bacillus coagulans,* as water additive on larvae shrimp (*Penaeus vannamei*) based on water quality, survival rate and digestive enzyme activities was investigated at different larvae stage and different concentration [29]. Ziaei-Nejad et al., (2006) examined the effect of commercial Bacillus probiotic by three experiments on the digestive enzyme activity, survival and growth of *Fenneropenaeus indicus* at various ontogenetic stages.

Bacillus subtilis is also a Gram-positive, lactic acid-forming bacterial species. A strain of B. subtilis 2335 has the ability to produce the antibiotic, Amicoumacin, which showed an in-vitro activity against Helicobacter pylori (Morikawa et al. 1992).

In the present study, *B. subtilis* was individually incorporated at different concentrations with formulated diets and fed to L. vannamei PL for assessing their ability on promotion of growth and survival by enhancing the contents of basic biochemical constituents, and activities of digestive. This was further to recommend the aquaculture industry with their optimum concentrations. Furthermore, to evaluate their competitive exclusion abilities of pathogenic bacteria, their colony establishments in the gut of PL were confirmed biochemically.

The objective of this study was to investigate the role of probiotic *Bacillus subtilis* isolated (Avakh, 2006) from digestive tract of *M. rosenbergii (*freshwater prawn) on growth and survival rate of Pacific white shrimp, *Litopenaeus vannamei.*

RESEARCH METHODOLOGY

The post larvae of shrimp, *Litopenaeus vannamei* (PL 19) were obtained from Sri Venkateswara Hatchery, Nellore, India and were stocked in cement tanks (1000 L capacity). The PL were acclimatised at usual laboratory conditions for two weeks (left over up to PL 34) and starved for 24 h prior to the commencement of the feeding.

Table - 1: The quality parameters are assessed and maintained as:

S No	Parameters	Values
1	pH	7.05 ± 0.10
2	total dissolved solids	0.94± 0.08 g/L1
3	dissolved oxygen	7.20 ±0.20 mg/L1
4	COD	130.00± 10.00 mg/L1
5	BOD	1.18 ± 0.54mg/L
6	ammonia	0.028 ± 0.006 mg/L1
7	Temperature	28.36 ± 2.72ºC
8	Salinity	19.95 ± 3.79%
9	Dissolved Oxygen	6.65 ± 1.09mg/L
1	Turbidity	9.63 ± 3.41NTU

Diet preparation

The composition of the experimental diet is given in Table 1.The probiotic *B. subtilis*, one gram of lyophilized powders contains 12.25 cfu cells. *B. subtilis* was incorporated in to the test diets at different concentrations individually at 2%, 4% and 6% respectively. Control animal group was also maintained simultaneously without incorporating the bacteria in the diet. Diet formulation was done basically by ‘‘Pearson’s square-method’’ using determined values of 40% protein content (Table-2). The proportion of each ingredient required was calculated precisely providing allowance for the premix. The dough was steam cooked and cooled to room temperature. After that different concentration of *B. subtilis* was mixed with the dough and the diets were pelletized separately with a locally made hand pelletizer. The pellets were dried in a thermostatic oven (Tamilnadu Engineering Instruments, Chennai) at 40ºC until it reached constant weight and stored in airtight jars at room temperature. The proximate biochemical composition of the experimental diets, total protein [33] (Lowry et al., 1951), amino acid [34] (Moore and Stein 1948), lipid [35] (Folch et al., 1957), and carbohydrate [36] (Roe, 1955) were determined. These diets were freshly prepared after every 30 days to ensure high probiotic viability throughout the duration of trail. In the control diet, no *B. subtilis* was found throughout the duration of feeding trail.

Feeding experiment

L.vannamei (PL-20) with the length and weight of 1.25 ± 0.21 cm were used for feeding experiment. 60 PL for each diet in triplicate were maintained in plastic tanks with 20 L water. The PL was maintained at the stocking density of 2/L. One group served as control (without Probiotics) (0%). All the experimental groups were fed with the respective concentration of B. subtilis incorporated diets. The shrimp were fed with feeding two times a day (6:00 am and 6:00 pm). The daily ration was given at the rate of 20% of the body weight of PL with two equal half parts throughout the experimental period. The unfed feed, faeces and moult if any were collected after completion respective hours of feeding. The feeding experiment was prolonged and continued up to 90 days.

During the experimental period continuous aeration was provided, in order to maintain the optimum level of dissolved oxygen.

Determination of growth parameters

At the end of feeding trial, the growth parameters such as survival rate (SR), weight gain (WG), specific growth rate (SGR), feed conversion rate (FCR), feed conversion efficiency (FCE) and protein efficiency rate (PER) were individually determined by following equations.

SR (%) = (Total Number of live animals) / (Total Number of initial animals) X 100

WG (g) = Final weight (g) - Initial weight (g)

SGR (%) = (log w2 - log w1) / t X 100

(**where, w1 & w2** = Initial and Final weight respectively (g),

And

t= Total number of experimental days

FCR (g) = Total Feed intake (g) / Total weight gain of the prawn (g).

FCE (g) = Biomass (g) / X Total Feed intake (g) X100

PER (g) = Total Weight gain of PL (g) = Total Protein consumed (g)

Energy utilization

The energy content of whole prawns, feeds, moult and faeces was measured using Parr 1281 **Oxygen Bomb Calorimeter.**

The energy utilization was calculated using the equation

(C = (P+ E) +R +F + U)

whereas:

C is the energy consumed in food;

P is the growth

R is the material lost as heat due to metabolism

F is the energy lost in faeces

U is the energy lost in excretion and

E is the energy lost in exuvia.

$$\textbf{Feeding Rate} = \frac{\text{Mean Food Consumption kcal / day}}{\text{Initial live weight of the prawn (g)}}$$

$$\textbf{Mean Absorption} = \frac{\text{Mean Food Consumption kcal}}{\text{Mean Food Excreted as Faeces k cal}}$$

Mean Absorption (k cal /day)

Absorption Rate = --

Initial live weight of the prawn (g)

Mean Conversion = Mean weight gain (kcal / day) + Mean exuvial weight (kcal/day)

$$\textbf{Conversion rate} = \frac{\text{Mean Conversion (kcal / day)}}{\text{Initial live weight of the prawn (g)}}$$

$$\textbf{NH}_3\textbf{ Excretion Rate} = \frac{\text{Mean } NH_3 \text{ Excretion (k cal / day)}}{\text{Initial live weight of the prawn (g)}}$$

Metabolic rate = AR (kcal / g / day) - CR (kcal / g / day) + NH_3 ER (kcal / g / day)

(whereas, AR = Absorption rate; CR = Conversion rate; ER = Excretion rate)

Biochemical constituents of the experimental animals

In the initial and final days of the experiment, the biochemical constituents of PL were determined. The biochemical constituents are estimated by standard methods.

Water quality management

Water quality was monitored weekly, temperature and pH was measured using pH and temperature meters respectively. Dissolved oxygen was estimated by DO and temperature meter and ammonium and nitrite was estimated by an ammonia meter respectively.

The shrimp aquaria were daily cleaned by siphoning out the material and unconsumed feed. Water exchanging was done 30% daily. All of the aquaria were supported by perfect and strong aeration system.

Statistical analysis

Data on growth parameters, bacteria count from digestive tract and rearing water between replicates and treatments were analyzed by using one-way analysis of variance and significance of differences between treatments were assessed by Duncan multiple range test [37]. (Sokal and Rohlf, 1995). The level of significance was accepted at $P < 0.05$. All statistical analysis was performed using SPSS, Released 15 software.

Results and Discussions

It is important to provide healthy shrimp with higher production and probiotics has a great deal of potential (Gomez et al., 2000; Goncalves et al., 2011). Moriarty (1998) added Bacillus spp. as probiotic in the penaeid shrimp ponds and the result of this study shows increasing survival rate in the pond water.

Biochemical constituents of experimental diet

The biochemical constituents, ingredients and proximate composition of experimental diet incorporated with *B. subtilis* are presented in Table-2. It is evident that the protein, carbohydrates and lipid levels are approximately 43%, 24% and 8.0% respectively. The levels of ash and moisture contents are in equal concentrations (Table-2). The digestible energy of these diets was about approximately 3267 $kcal/kg^{-1}$.

It has been reported that the *L. vannamei* required 37 – 45% protein, 5–9% lipid and 25–35% carbohydrates are as optimum (Table-2) in the diet of shrimp (Chaucheyras-durand et al., 2010).

Survival and Growth Rates

The result on the survival rate of *Bacillus subtilis* supplemented diet fed PL group of *L. vannamei* is represented in Table 3. After 90 days of feeding experiment, the survival rate was significantly higher ($P < 0.05$) in 4% probiotic bacteria incorporated diet fed PL, followed by other experimental groups such as 2% and 6% than the control. Similar results have also reported in PL of *M. rosenbergii* fed with *Saccharomyces cerevisiae, Lactobacillus sporogenes* and *B. subtilis* supplemented diets have improved the percentage of survival rate (Seenivasan et al., 2012). Improvement in survival rate has been reported in juveniles of *P. indicus* fed with Lactic acid bacteria supplemented diets (Fernandez et al., 2011). It has been observed that Bacillus supplemented diets induced the survival rate in tiger shrimp, *Penaeus monodon* (Wang, 2007).

Effects of commercial probiotic on aquaculture has been investigated by researchers, and some of these research have not shown any positive effects on growth parameters or survival rate or any promising result on the cultural condition. For instance, Shariff et al. (2001) found that treatment of *P. monodon* with a commercial Bacillus probiotic did not significantly increase survival.

McIntosh et al. (2000) found that treatment of *Litopenaeus vannamei* with a commercial Bacillus probiotics did not significantly increase ($P>0.05$) either survival or growth.

Zokaeifar et al. (2014) reported that the administration of *Bacillus subtilis* at a dose of 108 CFU/mL in the rearing water of shrimp (*Litopenaeus vannamei*) for 8 weeks confers the following beneficial effects for shrimp culture, such as: (i) water quality; (ii) growth performance; (iii) digestive enzyme activity; (iv) immune response; and (v) disease resistance. Besides that administration of *Bacillus subtilis* strains increased total protein, protease and amylase activity of treated shrimp after the colonization in the gastro-intestinal tract. Ziaei-Nejad et al. (2006) investigated the effects of some commercial Bacillus probiotic strains (*Bacillus subtilis, Bacillus licheniformis, Bacillus polymyxa, Bacillus laterosporus* and *Bacillus circulans*) on the digestive enzyme activity, survival and growth of white shrimp (*Fenneropenaeus indicus*).

Seenivasan et al. (2012) found that Bacillus subtilis can be used as a probiotic at 3% inclusion in the experimental diet of *Macrobrachium rosenbergii* culture to enhance the survival, growth, nutritional indices, tissue biochemical components and energy utilization performance.

In this study, *B. subtilis* supplemented diet fed *L. vannamei* PL resulted in significant increase ($P < 0.05$) of weight gain, specific growth rate, feed conversion efficiency and protein efficiency ratio. In support to these the feed conversion ratio was found to be decrease ($P < 0.05$) in B. subtilis incorporated diet fed *L. vannamei* PL (Table-3). Therefore *B. subtilis* incorporated diets exhibited superior growth than that of control diet. Among different level of BS the overall growth was elevated particularly in 4% *B. subtilis* incorporated diet fed shrimp. This indicates the fact that this much quantity of B. subtilis addition was required to attain superior growth rate in PL of *L. vannamei*.

Liu et al.(2010) showed that *Bacillus subtilis* E20 has probiotic potential for white shrimp *L. vannamei* larval breeding by adding the probiotic to larval rearing water at a level of 109 CFU/L, thus improving the larval survival rate, development, stress resistance, and immune status.

Bacillus bacteria are able to out-compete other bacteria for nutrients and space and can exclude other bacteria through the production of antibiotics (Moriarty, 1998; Verschuere et al., 2000). Many different antibiotic compounds are produced naturally by a range of Bacillus species, and it appears that other bacteria would be unlikely to have resistance genes to all of the antibiotics produced by the Bacillus probionts (Moriarty, 1998). Bacillus administration also has been shown to increase shrimp survival by enhancing resistance to pathogens by activating both cellular and humoral immune defenses in shrimp (Rengpipat et al., 2000).

Apart from this growth in crustaceans is closely associated with moulting and the frequency of moulting is positively influenced by the maintenance of favorable environmental conditions (Ravi et al. 1998).

Boonthai et al. (2011) showed that *P. monodon* PL fed with probiotic (Bacillus sp,) supplemented diets had enhanced the growth performance. It has also been reported that Bacillus spp., supplemented diets have improved the growth performance of the rainbow trout, *O. mykiss* (Bagheri et al., 2008).

Ghosh et al. (2008) has also reported that the significant growth was recorded by some ornamental fishes fed with *B. subtilis* supplemented diets. Dhanaraja et al. (2010) reported that probioitics *L. acidophilus* and yeast *S. cervisiae* supplemented diets had improved the growth performance of Koi Carp and *common carp.*

Biochemical Indices

The results on biochemical indices such as proteins, amino acids, carbohydrates, lipids, ash and moisture of *B. subtilis* supplementation diet fed PL group are represented in Table 3. The variations of these constituents except moisture were found to proportionately higher in PL fed with B. subtilis incorporated diet. These elevations were better in the 4% *B. subtilis* incorporated diet fed shrimps followed by the other experimental diets, when compared with control (Table-5).

Yu et al. (2009) pointed out that Bacillus spp. At different concentrations incorporated diets had significantly increased the biochemical proximate composition in white shrimp. Fernandez et al. (2011) reported that Lactic acid bacteria enhanced the biochemical proximate composition in juveniles of *Penaeus indicus.* It has been reported that Bacillus spp., supplemented diets had significantly improved the carcass biochemical proximate composition of the rainbow trout (Bagheri et al. 2008). Hidalgo et al. (2006) also reported that probiotic *B. toyoi* (@ 0.5, 1 and 2 g kg-1 and *B. cereus* @ 0.5, 1 and 2 g kg-1, incorporated diets had significantly improved the biochemical proximate composition of juvenile, *Dentex dentex*. Merrifield et al. (2009) reported that two probiotics *B. subtilis* and *B. licheniformis* incorporated diets, both have caused to elevate the body biochemical proximate composition of trout fish.

CONCLUSIONS

The present investigation clearly indicate that, interestingly the effect of probiotic bacteria treatment induced a highly significant increase in all the growth indices of white shrimp *Litopenaeus vannamei* in culture pond environment. *B. subtilis* on individual from at optimized concentrations was found to be enhanced the survival, growth, nutritional indices, tissue biochemical components and energy utilization of reared shrimp. Further research on the diets produced with optimized concentration of the chosen probiotic organisms may be evaluated under field condition in the candidate species *L. vannamei.* Thus, *Bacillus subtilis* might have a promising role used as probiotic in shrimp culture. This kind of approach can be a better option for the development of sustainable aquaculture.

REFERENCES

Alokesh Kumar Ghosh(Assistant professor), Joyanta Bir(Assistant professor), Md. Abul KalamAzadPhD student(Senior Upazilla Fisheries Officer), Abul Farah Md.Hasanuzzaman(Assistant professor),Md. SanaulIslam(Associate professor), and Khandaker AnisulHuq(Professor) (2016). Impact of commercial probiotics application on growth and production of giant fresh water prawn (*Macrobrachium Rosenbergii* De Man, 1879), Aquaculture Reports, Volume 4, November 2016, Pages 112-117.

Austin, B., Stuckey LE., Robertson PAW., Effendi I., and Griffith DRW (1995). A probiotic strain of Vibrio alginolyticus effective in reducing disease caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalli*. J. Fish Dis. 18: 93-96.

Avakh, K.M (2006). Assessment of putative bacteria as probiotics in juvenile freshwater prawn, *Macrobrachium rosenbergii.* Doctoral dissertation, University of Putra, Malaysia.

Bagheri, T., Hedayati, S., Yavari, V., Alizade, M., and Farzanfar, A., (2008). Growth, survival and gut microbial load of rainbow trout (*Onchorhynchus mykiss*) fry given diet supplemented with probiotic during the two months of first feeding. Turkish Journal of Fisheries and Aquatic Science 8, 43–48.

Balcazar, J.L., de Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., and Múzquiz, J.L. (2006). The role of probiotics in aquaculture. Veterinary Microbiology, 114, 173–186.

Boone (1931) *Litopenaeus vannamei* (Boone, 1931), the Pacific whiteleg shrimp in River Cauvery, Current Science, 2018, VOL. 115, NO. 8, 25 October.

Boonthai, T., Vuthhiphandchai, V., and Nimrat, S. (2011). Probiotic bacteria effects on growth and bacterial composition of black tiger shrimp (*Penaus monodon*). Aquaculture Nutrition. 1365-2095.2011.00865.x

Briggs, M., Funge-Smith, S., Subasinghe, R. and Phillips, M.,(2004). Introductions and movement of Penaeus vannamei and Penaeus stylirostris in Asia and the Pacific, Food and Agriculture Organization of the United Nations, RAP Publication, p. 92. 4.

Buruiana Cristian-Teodor, Alina Georgiana Profir, and Camelia Vizireanu.(2014). Effects of probiotic bacillus species in aquaculture – an overview. AUDJG – Food Technology 10, 38(2), 9-17.

Cha, J.H., Rahimnejad, S., Yang, S.Y., Kim, K.W., and Lee, K.J. (2013). Evaluations of Bacillus spp. as dietary additives on growth performance, innate immunity and disease resistance of olive flounder (*Paralichthys olivaceus*) against *Streptococcus iniae* and as water additives. Aquaculture, 402–403, 50–57.

Chaucheyras-Durand, F. and H Durand. .(2010). Probiotics in animal nutrition and health. Benef. Microbes, 1(1): 3–9.

Dhanaraja, M., Haniffaa, M.A., Arun Singha, S.V., Jesu Arockiarajb, A., Muthuramakrishanana, C., Seetharamana, S., and Arthimanjua, R., (2010). Effect of probiotics on growth performance of koi carp (*Cyprinus carpio*). Journal of Applied Aquaculture 22, 202–209.

Dong, T.C., Van, P.H., and Cutting, S.M. (2009). Bacillus Probiotics. Nutra Foods, 8, 7–14.

Fernandez R, Sridhar M, and Sridhar N. (2011). Effect of Lactic acid bacteria administered orally on growth performance of *Penaeus indicus* (H. Milne Edwards) juveniles. Res J Microbiol. 6, 466-479.

Folch, J., Lees, M., and Bloane-Stanley, G.H., (1957). A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry 266, 497–509.

Fuller, R. (1989). Probiotics in man and animals. Journal of Applied Bacteriology, 66, 365–378.

Fuller, R., (1992). History and development of probiotics. In: Fuller, R. (Ed.), Probiotics: The Scientific Basis. Champman and Hall, London, England, pp. 1–18.

Ghosh, S., Sinha, A., and Sahu, C., (2008). Dietary probiotic supplementation in growth and health of live-bearing ornamental fishes. Aquaculture Nutrition 14, 289–299.

Gomez-Gil, B., A. Roque, and J. F. Turnbull. (2000). The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. Aquaculture, 191: 259–270.

Goncalves, A. T., M. Maita, K. Futami, M. Endo, and T. Katagiri. (2011). Effects of a probiotic bacterial *Lactobacillus rhamnosus* dietary supplement on the crowding stress response of juvenile Nile tilapia (*Oreochromis niloticus*). Fish. Sci., 77: 633–642.

Gongora, C.M., 1998. Mecanismos de resistencia bacteriana ante lamedicina actual. McGraw-Hill, Barcelona, Spain, pp. 156–201.

Hidalgo, M.C., Skalli, A., Abella, N.E., Arizcun, M., and Cardenete, G., (2006). Dietary intake of probiotics and maslinic acid in juvenile dentex (*Dentex dentex* L.): effects on growth performance, survival and liver proteolytic activities. Aquaculture Nutrition 12, 256–266.

Janardana Reddy S, Vineela D and B. Kiran Kumar (2018). Effect of Probiotic *Lactobacillus plantarum* (NCDC LP20) on Enhanced Innate Immunological Indices of Freshwater Fish. IOSR Journal of Pharmacy. Volume 8, Issue 10, Version I, pp. 30 - 35.

Kesarcodi-Watson, A., Kaspar, H., M. Lategan, J., and Gibson, L. (2008). Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. Aquaculture, 274, 1–14.

Klaenhammer, T.D., and Kullen, M.J., (1999). Selection and design of probiotics. International Journal of Food and Microbiology 50, 45–57.

Liu, K.F., Chiu, C.H., Shiu, Y.L., Cheng, W., and Liu, C.H. (2010). Effects of the probiotic, *Bacillus subtilis* E20, on the survival, development, stress tolerance, and immune status of white shrimp, *Litopenaeus vannamei* larvae. Fish & Shellfish Immunology, 28, 837–844.

Lowry, O.H., Rosenbrough, W.J., Fair, A.L., and Randall, R.J., (1951). Protein measurement with the folin phenol reagent. Journal of Biological Chemistry 193, 265–275.

Mcintosh, D., T.M. Samocha, E.R. Jones, A.L. Lawrence, D.A. McKee, S. Horowitz and A. Horowitz. (2000). The effect of a commercial bacterial supplement on the high-density culturing of *Litopenaeus vannamei* with a low-protein diet in an outdoor tank system and no water exchange. Aquac. Eng. 21: 215-227.

Merrifield, D.L., Bradley, G., Baker, R.T.M., and Davies, S.J., (2009). Probiotic application for rainbow trout (*Oncorhynchus mykiss walbaum*) II. Effects on growth performance, feed utilization, intestinal microflora and related health criteria post antibiotic treatment. Aquaculture Nutrition., 1365-2095.2009.00688.x.

Mohapatra, S., Chakraborty, T., Kumar, V., DeBoeck, G., Mohanta, K.N.(2013). Aquaculture and stress management: a review of probiotic intervention. Journal of Animal Physiology and Animal Nutrition, 97, 405–430.

Moore, W., and Stein, H., (1948). In: Olowick, S.P., Kaplan, N.D. (Eds.), Methods in Enzymol. Academic press, New York, p. 468.

Moriarty, D. (1998). Control of luminous Vibrio species in penaeid aquaculture ponds. Aquaculture, 164, 351–358.

Morikawa M, Ito M, and Imanaka T. (1992). Isolation of a new surfactin producer *Bacillus pumilus* A-1, and cloning and nucleotide sequence of the regulator gene, psf-1. J Fermrnt Bioeng, 74: 255-61.

Nuraini YL, Bambang H., Subyakto S. and dan Gemi T.(2007). Active Surveilance of Infectious Myonecrosis Virus (IMNV) in Pond-Cultured White Shrimp (*Litopenaeus vannamei*) in East Java and Bali, Jurnal Perikanan UGM, 9(1), 25-31.

Ravi VS, Ajmalkhan S, and Rajagopal S.(1998). Influence of Probiotics on growth of Indian white prawn Penaeus indicus. J. Sci. Ind. Res. 57(10-11): 752–756.

Ravichandran, P., Panigrahi, A. and Kumaran, M. (2009). Biology and Culture of Litopenaeus vannamei vis-à-vis *Penaeus monodon* – Handbook on Seed Production and Farming of *Litopenaeus vannamei* 46, CIBA Special Publication, Chennai, p. 70.

Rengpipat S, Phianphak W, Piyatiratitivorakul S, and Menasaveta P (1998). Effects of a probiotic bacterium in black tiger shrimp *Penaeus monodon* survival and growth. J. Aquacult. 167: 301-313.

Rengpipat S, Rukpratanporn S, Piyatiratitivorakul S, and Menasaveta (2000). Immunity enhancement in black tiger shrimp (Penaeus monodon) by a probiont bacterium (Bacillus S11). J. Aquacult. 191: 271-288.

Roe, J.H., (1955). The determination of sugar and blood and spinal fluid with anthrone reagent. Journal of Biological Chemistry 212, 335–343.

Sahu, M.K., Swarnakumar, N.S., Sivakumar, K., Thangaradjou, T., and Kannan, L. (2008). Probiotics in aquaculture: importance and future perspectives. Indian Journal of Microbiology, 48, 299–308.

Seenivasan, C., Radhakrishnan, S., Muralisankar T, and Bhavan PS. (2012). *Bacillus subtilis* on survival, growth, biochemical constituents and energy utilization of the freshwater prawn *Macrobrachium rosenbergii* post larvae. Egyptian Journal of Aquatic Research, 38, 195–203.

Shariff, M., F.M. Yusoff, T.N. Devaraja and S.P. Srinivasa Rao. (2001). The effectiveness of a commercial microbial product in poorly prepared tiger shrimp, *Penaeus monodon* (Fabricius), ponds. Aquac. Res. 32:181-187.

Sharma, P., Tomar, S.K., Goswami, P., Sangwan, V., and Singh, R. (2014). Antibiotic resistance among commercially available probiotics. Food Research International, 57, 176–195.

Sokal, R.R. and Rohlf, F.J. (1995). Biometry. 3rd edition. WH Freeman and Co., New York NY.

Sugita H, Hirose Y, Matsuo N, and Deguchi Y (1998). Production of the antibacterial substance by Bacillus sp. strain NM12, an intestinal bacterium of Japanese coastal fish. J. Aquacult. 165: 269-280.

Tovar-Ramirez, D., Zambonino, J., Cahu, C., Gatesoupe, F.J., Vazquez-Juarez, R., and Lesel, R., (2002). Effect of live yeast incorporation Incorporation in compound diet on digestive enzyme activity in sea bass (*Dicentrarchus labrax*) larvae. Aquaculture 204, 113–123.

Venkat HK, Sahu NP, and kamal K Jain (2004). Effect of feeding Lactobacillus-based probiotic on the gut microflora, growth and survival of postlarva of *Macrobrachium rosenbergii* (de Man). J. Aquacult. Res. 35: 501-507.

Verschuere L., Rombaut G. and Verstraete W.(2000). Probiotic bacteria as biological control agents in aquaculture, Journal Microbiology and Molecular Biology Reviews, 64(4), 665-666.

Wache, Y., Auffray, F., Gatesoupe, F.J., Zambonino, J., Gayet, V., Labbe, L., and Quentel, C., (2006). Cross effects of the strain of dietary Saccharomyces cerevisiae and rearing conditions on the onset of intestinal microbiota and digestive enzymes in rainbow trout, *Onchorynchus mykiss,* fry. Aquaculture 258, 470–478.

Wang YB. (2007). Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei.* Aquaculture 2007;269: 259 - 264.

Widanarni D., Wahjuningrum and dan Puspita F., (2012). Aplikasi bakteri probiotik melalui pakan buatan untuk meningkatkan kinerja pertumbuhan udang windu *Penaeus monodon,* Jurnal Sains Terapan, 2(1), 32 – 49.

Yu MC, Li ZJ, Lin HZ, Wen GL, and Ma S. (2009) Effects of dietary medicinal herbs and Bacillus on survival, growth, body composition, and digestive enzyme activity of the white shrimp *Litopenaeus vannamei.* Aquacul. Int.17, 377-384.

Zhou X, Wang Y, and Li W (2009). Effect of probiotic on larvae shrimp (Penaeus vannamei) based on water quality, survival rate and digestive enzyme activities. Aquaculture, 287: 349-353.

Ziaei-Nejad S, Rezaei MH, Takami GA, Lovett DL, Mirvaghefi A-R, and Shakouri M. (2006). The effect of Bacillus spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus.* Aquaculture, 252: 516 - 24.

Zokaeifar H, Babaei N, Saad CR, Kamarudin MS, Sijam K, and Balcazar JL (2014). . Administration of *Bacillus subtilis* strains in the rearing water enhances the water quality, growth performance, immune response, and resistance against Vibrio harveyi infection in juvenile white shrimp, *Litopenaeus vannamei.* Fish Shellfish Immunol. 36(1):68-74.

Table -2: Ingredients and proximate composition of experimental diets.

Ingredients (g/kg¯-1)	**Control (BI)**
Fish meal	26.43
Groundnut oilcake	26.41
Soybean meal	27.00
Corn flour	5.00
Egg albumin	4.06
Topica flour	6.10
Cod liver oil	3.00
B-complex Vitamin mix	2.00
B. subtilis (BS)	0
Total	**100**
Proximate composition	
Protein (%)	43.16
Carbohydrate (%)	23.52
Lipid (%)	8.25
Ash (%)	11.26
Moisture (%)	10.52
Digestible energy (kcal/kg_1)	3267.34

Table - 3: Variations in the growth performances of *L. vannamei* PL fed with control and different concentrations (2-6%) of *Bacillus subtilis* supplemented experimental diets for 90 days.

Parameters	Control Diet	Experimental Diets of *Bacillus subtilis* bacteria		
		2% Probiotics	4% Probiotics	6% Probiotics
Survival Rate of fish , SR (%)	75.25 ±2.85	79.55±2.00	85.75±3.00	92.75±2.50
WG (g)	0.70±0.06	0.79±0.08	1.52±0.05	1.25±0.15
SGR (%)	0.618±0.18	0.739±0.19	0.879±0.54	0.852±0.062
FCR (g)	2.965±0.25	2.823±0.32	2.715±0.23	2.516±0.35
FCE (%)	0.69±0.06	0.89±0.05	1.23±0.22	0.95±0.08
PER (g)	0.568±0.12	0.735±0.18	0.966±0.13	0.865±0.12

S, Survival; WG, Weight gain; SGR, Specific growth rate; FCR, Food conversion rate; FCE, Food conversion efficiency; PER, Protein efficiency rate.
Values are expressed as kcal/g/day; Each value is a mean± SD of six individual observations.
values are significant at P< 0.05.

Table - 4: Variations in the energy utilization of *L. vannamei* PL fed with control and different concentrations (2-6%) of *Bacillus subtilis* supplemented experimental diets for 90 days.

Parameters	Control Diet	Experimental Diets of *Bacillus subtilis* bacteria		
		2% Probiotics	4% Probiotics	6% Probiotics
Feeding Rate FR	0.526±0.05	0.655±0.08	0.795±0.09	0.655±0.05
Absorption Rate AR	0.588±0.22	0.695±0.15	0.759±0.15	0.688±0.13
Conversion Rate CR	0.257±0.06	0.486±0.05	0.685±0.06	0.635±0.04
Ammonia Excretion Rate AER	0.015±0.005	0.016±0.006	0.017±0.005	0.016±0.004
Metabolic Rate MR	0.325±0.005	0.358±0.005	0.457±0.006	0.366±0.003

FR, feeding rate; AR, absorption rate; CR, conversion rate; AE, NH3 excretory rate; MR, metabolic rate.

Values are expressed as kcal/g/day; Each value is a mean± SD of six individual observations.
values are significant at P< 0.05.

Table - 5: Variations in the Biochemical Constituents of *L. vannamei* PL fed with control and different concentrations (2-6%) of *Bacillus subtilis* supplemented experimental diets for 90 days.

Parameters (%)	Control Diet	Experimental Diets of *Bacillus subtilis* bacteria		
		2% Probiotics	4% Probiotics	6% Probiotics
Proteins	65.22±1.25	67.16±1.35	69.26±3.46	72.76±2.57
Amino Acids	26.16±2.34	28.22±2.36	31.54±3.22	33.63±3.54
Carbohydrates	8.95±0.66	12.35±2.85	14.76±1.29	17.96±2.48
Lipids	5.39±0.86	7.68±2.65	9.38±2.54	12.53±2.48
Ash	14.25±0.86	15.64±0.28	16.57±1.26	17.38±1.24
Moisture	65.37±2.86	65.18±2.18	64.17±1.31	63.28±1.25

Values are expressed as kcal/g/day; Each value is mean ± SD of six individual observations.
Values are significant at $P < 0.05$.