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Synergetic effect of probiotic, molasses and immunostimulant supplementation on the production of white leg shrimp *Litopenaeus vannamei* Boone, 1931

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The aim of the study is to find out a suitable combination of supplements for the successful production of shrimp *Litopenaeus vannamei*. The experiment was performed in two different shrimp farms located in the same area at Athirampattinam (Farm-A) and (Farm-B), Tamil Nadu, East coast of India. Each group consisted of three ponds, farm-A supplemented with probiotic, immunostimulant and molasses. Subsequently, farm-B was supplemented with chemicals and probiotics. The shrimp average body weight (23.2 ± 0.41 g) survival (84 ± 0.05 %) and production (6.98 tons/ha) was significantly higher ($P < 0.05$) in farm-A than the farm-B. The water parameters such as temperature, pH, salinity, transparency, dissolved oxygen, total ammonia, nitrite, total plate count and *Vibrio* population were significantly varied between these two farms. The safe water quality parameter values were obtained in farm-A. Henceforth, the present study was concluded that the supplementation of probiotic, immunostimulant and molasses combination enhanced the shrimp production.

[Key words: Immunostimulant, *Litopenaeus vannamei*, Molasses, Probiotic]

Introduction

Shrimp culture is a fast-growing food producing sector in the world, moving in newer directions, intensifying and diversifying[1]. In the past few decades, shrimp aquaculture has faced many challenges, due to over-intensification and inappropriate management practices, resulting in the outbreak of many viral diseases in *Penaeus monodon* and causing huge losses to farmers[2-4]. To overcome this issue, an alternate species of white shrimp (*Litopenaeus vannamei)* was introduced and intensively cultured in many Asian countries. This is an important shrimp species in aquaculture throughout the world due to its many advantages over the *P. monodon*. In India, *L. vannamei* was introduced for commercial production from 2008 and now it has become a monopoly. However, recently there has been an increasing fear about the environmental impacts on shrimp farms due to the occurrence of diseases. This has led to the development of various management practices using various chemicals, probiotic and other feed supplements.

Generally, chemical and a combination of drugs are used to prevent and control the diseases in aquaculture. However, the continuous use of these agents may develop drug-resistance in pathogens. Moreover, there is a possibility of accumulation of drug residues in the shrimp, which may cause harmful effect to human health through the food chain. Although, the application of beneficial bacteria in animal husbandry as a nutrient supplement has long been recognized, the use of such probiotics in aquaculture is a relatively new concept[5]. The effectiveness of these products in commercial shrimp farming is yet to be clearly established. In addition, immunostimulant and prebiotics also used as a feed additive along with probiotics to overcome the diseases. In aquaculture ponds, during the culture period the quality of soil and water may deteriorate due to the accumulation of metabolic wastes, unutilized feed and dead and decayed biotic materials. Moreover, supplementation of probiotic bacteria directly uptake or decompose the organic waste or toxic substances and improved the soil and water quality. In recent years, there has been a growing interest in the use of probiotic bacteria in aquaculture practice to improve the pond ecosystem and to combat pathogens. This may indirectly

promote the growth of farmed organisms[6]. Sugarcane molasses is an organic by-product that is used in the livestock industry as a nutritional supplement to enhance appetites of the rearing organisms. This molasses is rich in minerals and vitamins that act as a good substrate for beneficial bacteria. This molasses can be applied through feed or with other supplements. Recently, shrimp farmers have been using molasses as a supplement to enhance the production without awareness of the scientific information. Hence, this study aims to determine the suitable combination of health supplements for the successful shrimp culture (*L. vannamei*) by comparing the pond environmental parameters, growth, survival and production of two different nearby farms, supplement with probiotic, immunostimulant and molasses (Farm-A) and administrated with chemicals and probiotic (Farm-B).

Material and Methods

Experimental design

This study was carried out on two different nearby shrimp farms (Farm-A and Farm-B) located in Athirampattinam, Tanjavur (10°47' N, 79°10' E) district, Tamil Nadu, East coast of India. Both farms hold three culture ponds, each with water spread over an area of 0.8 ha and one reservoir (Farm-A; 1 ha and Farm-B; 1.2 ha). Seawater was pumped into the reservoir and used for the water exchange in the culture ponds. Farm-A was supplemented with probiotic from pond preparation (through water and feed), immunostimulant (through feed) and molasses (through water and feed) and Farm B-chemicals and administration of probiotic through water and feed from 40th days of culture (Table 1).

Pond preparation and water culture

After the previous harvest, 500 Kg ha^{-1} of agricultural lime and 75 Kg ha^{-1} of bleaching powder were applied uniformly over the pond bottom. Subsequently, the drainage, shutter, screen and objects used in the production were treated with liquid chlorine at the rate of 30 ppm. After 30 days of curing, the soil pH was measured at different locations using soil pH cone and agricultural lime was applied based on the soil pH level. One ton of lime was applied to augment the soil pH to 1. Initially, 50 % of the required lime was applied over the soil and tilling was done. After 5 days, the remaining lime was applied along with zeolite at 150 kg ha^{-1} and second tilling was done. After 2 days, the soil pH was measured once again using pH cone at various locations to confirm the increase in the soil pH. The water was pumped from the reservoir to the pond and filled up to 50 cm. Then to develop a phytoplankton bloom, inorganic fertilizer urea (N) and superphosphate (P) were dissolved in pond water at the ratio of 5(N):1(P), and sprinkled over the pond water in the early morning and observed for colour change. Once the water colour changed to yellowish green (approximately 4 days) the water level was increased

Table 1 — Application schedule of supplements to the experimental shrimp ponds (*Litopenaeus vannamei)*

Experimental pond	Supplements quantity	DOC
Farm-A	Probiotic in water – 750 g	0
	Probiotic in water – 500 g	11,23,43,53,63,73,83,93
	Molasses - 5 l/one feeding	4,51,61
	Water culture	20,40,60
	Molasses - 101/pond	76,81,89
	Immunostimulant – 500 g	39,40,41,66,67,68,90,91,92
Farm-B	Probiotic in Water – 500 g	40, 68,80
	Phytomin - 10 kg	0,25,50,75,
	Extramin - 3 kg	10,22,31,38,
	Phottash - 5 kg	20,59,69
	Powermine - 10 kg	44,55,
	Powermine - 20 kg	65,71
	Powermine - 25 kg	82,93
	For-AM - 2 kg	61,77
	Proton s - 1 kg	78
	Zoothamnicide - 250	89
	Iodine – 10 %	62,91

DOC - days of culture, Immunostimulant - 1, 3 and 1, 6 beta glucon, extramin - essential minerals, powermin - multimineral supplements, proton S - bottom cleaner, AM - ammonia reducer, zoothamnicide - clear *Zoothamnium*

to 1.1 to 1.2 meters and the second dose of fertilizer was applied at the same ratio to maintain the phytoplankton bloom. In farm-A, in addition to the inorganic fertilizer, molasses slurry (25 kg) and followed by probiotic at 500 g pond^{-1} were also applied. On the other hand, in farm-B, 10 kg of phytomin was applied to enhance the phytoplankton bloom.

Stocking

Fourteen day-old-specific pathogen-free post larvae (SPF-PLs) of *L. vannamei* were purchased from CP hatchery, Marakanam, Tamil Nadu, India. The PLs were transported in oxygenated double layered polythene bags (3500 PLs bag) in a controlled temperature using crushed ice bags inside the corroborated box. The PLs were brought to the farm site and acclimatized to the pond environment by sprinkling the pond water into the PL bag for 30-40 min. Then, all the bags were evenly distributed to the ponds and the larvae were released slowly into the water. In each pond 3,20,000 (40/m^2) shrimp post larvae (PLs) were stocked. Farm-A was stocked first and farm-B was stocked after three days from the date of stocking of farm-A. To estimate the initial larval survival before stocking, post larvae were randomly collected from each larvae bag and a total of 200 shrimp larvae were stocked in the hapa (2 m length × 1 m width × 1.5 m H) in each pond. The PL survival in the hapa was estimated after 48 h from the stocking day.

Water quality analysis

After 30 days, once in every 15 days, the pond water samples were collected from the middle of each experimental pond in sterile bottles to estimate the water quality parameters such as temperature, pH, salinity, transparency, dissolved oxygen, ammonia and nitrite. The temperature was measured using a graduated thermometer from 0 to 110 °C, pH was recorded using the pH pen (IR-50, Roy instrument, Chennai), salinity was measured using a refractometer, transparency was measured using secchi disk, the dissolved oxygen content was estimated by Winkler's method[7], and the total ammonia and nitrite levels were determined by the standard methods[8,9].

Microbiological analysis

Soil and water samples were collected from the centre of the pond using sterile containers and transported immediately to the laboratory for bacterial analysis. The total plate count (TPC) and *Vibrio* species were estimated by pour plate method on the sterile Zobell marine agar and TCBS medium (Himedia Laboratories Pvt Ltd, Mumbai, India), respectively, with suitable dilutions. After inoculation, the plates were incubated in an inverted position and maintained at 28 ± 1 °C. After the 24 – 48 h of incubation the bacterial colonies were counted and their density was expressed as a log colony forming unit (CFU) per milliliter (water) and gram (soil).

Water exchange

Water was pumped from the reservoir periodically to compensate the water loss due to evaporation and seepage in farm-A. In farm-B, all the ponds at different period the water exchange was carried out for four times throughout the culture period at an average rate of 20 %.

Application of probiotic and molasses

Commercial probiotic (*Bacillus* sp., *Lactobacillus bacillus* and *Bifidobacterium* combination at 10^{-9}) were soaked in the pond water at a ratio of 1 g/200 ml and mixed thoroughly. After activation, the slurry was sprinkled uniformly over the surface of the pond water. Subsequently, the paddle wheel aerators were operated to achieve proper mixing. Similarly, feed probiotic and immunostimulant 1, 3 and 1, 6 beta glucon were sprayed and mixed in the feed along with egg albumin for proper binding and kept for 1 h in the shade prior to feed application. The molasses was mixed with feed, shade dried and applied into the pond. In farm-A probiotic was applied periodically from the water culture (before stocking) to till harvest, whereas in farm-B the probiotic application was started from the 40th day of culture and applied only thrice during the entire culture period. In farm-A molasses slurry (rice bran, molasses and yeast were added to seawater and kept in closed containers for 24 – 48 h, after every 12 h the slurry was mixed thoroughly) was mixed with pond water and sprinkled over the pond water during early in the morning.

Chemicals applications

The chemicals applied in farm-B were extramin (essential minerals, powermin and multimineral supplements), phytomin, potash, proton S bottom (cleaner), Am (ammoina reducer) and zoothamnicide (to clear *Zoothamnium*). In farm-B, phytomin was applied to develop the phytoplankton bloom and zoothamnicide was applied on the 98th day of culture.

Feeding schedule

In both farms (A&B), CP Blanca pellet feed (CP Aquaculture India Pvt Ltd) was used to feed the shrimp as per the CP feeding chart. Initially, from 1 to

10 days shrimp was fed with two times in a day, 3 times in a day from the 11th to 20th days and 4 times a day from the 25th day onwards. In the total feed, 40 % of the ration was fed during day time and 60 % during the evening and night time. For the first 10 days, feeding was done along the peripheral dyke of the ponds. After the 11th day for each feeding schedule, 60 % of the feed was distributed along the periphery dyke and the remaining 40 % was distributed in the middle of the pond using floats. After 30 days, feed was distributed throughout the pond using floats for effective feeding.

Estimation of survival and growth rate

In both farms, from the 40th day of culture sampling was done with the help of fishermen using cast net. Totally, seven hauls were made in each pond and the number of shrimps caught per haul was weighed, counted and recorded. The health status, percentage of survival, average body weight of the shrimps, average daily growth and food conversion ratio were estimated through sampling as follows.

Survival = Number of shrimps in the sampling/Initial stocking ×100

Average body weight (ABW) = Total weight of the shrimps (g)/ Number of shrimps

Food conversion ration = Total feed given/ total biomass

Harvest

The harvest was performed after confirming the soft-shelled shrimp (below 3 %) through sampling. Harvesting was done by draining the entire pond water and collected the shrimps in a netted bag which drained the water. The harvested shrimps were ice killed, packed, weighed and sold.

Statistical analysis

Data were expressed as mean ± standard deviation. The result on shrimp growth, water quality parameter, bacterial population of cultured pond, total production was compared and analyzed using one-way analysis of variance (One-way ANOVA) and significance of differences between the farms were assessed by Duncan multiple range test[10,11]. The level of significance was accepted at $P < 0.05$. All statistical analysis was performed using SPSS (Version 16.0) program.

Results

Initially, at the time of pond preparation the average soil pH of the farms was varied between 4.5 ± 0.20 and 4.6 ± 0.1. To improve the pond soil pH to 7.0, 2.5 tonnes of agricultural lime was applied per pond in two equal doses at the rate. The water salinity and temperature were varied between 32 ± 0.05 and 35.5 ± 0.66 ppt and 26 ± 0.15 to 29 ± 0.77 °C respectively for both the farms (Figs. 1a & b).The water pH remained on the alkaline side throughout the culture period in all the ponds of both Farm-A and B it was ranged from 7.7 ± 0.06 to 8.4 ± 0.15, respectively. The highest and the lowest pH values of 8.4 ± 0.15 and 7.7 ± 0.06 were recorded in farm-B on 90th and 75th days of culture respectively (Fig. 1c). The dissolved oxygen level was significantly differed between Farms-A & B and it was varied from 4 ± 0.30 to 5.8 ± 0.1 ppm in both farms (Fig. 1d). The highest level of dissolved oxygen 5.8 ± 0.1 ppm was registered in farm-A and the lowest level of 4 ± 0.30 was recorded in farm-B. In farm-B the transparency level was varied between 39.5 ± 0.15 and 48 ± 0.70 cm and it was fluctuated whereas a stable transparency level was observed in farm-A, shrimps treated with probiotic, immunostimulant and molasses and it was varied from 33 ± 0.25 to 39 ± 0.15 cm (Fig. 1e). The transparency level was improved whenever probiotics and phytomin were applied in farm-B. In both the farms, the total ammonia level was increased gradually from 0 to 0.28 ± 0.02 ppm. In farm-A, the total ammonia level was varied between 0 and 0.15 ± 0.02 ppm, whereas in farm-B the total ammonia level diverse from 0 to 0.28 ± 0.02 ppm. Similarly, the highest nitrite level (0.0056 ± 0.0003 ppm) was observed in farm-B on 90th day of culture, whereas on the same day, 0.0037 ± 0.0002 ppm of nitrite level was registered in farm-A (Fig. 2). The total plate count (TPC) of the pond water was varied between 3.8 ± 0.25 and 6 ± 0.1 log CFU ml^{-1} in farm-A, whereas in farm-B the TPC level ranged between 4 and 6.8 CFU ml^{-1}, respectively. The highest green and yellow colonies (in TCBS agar plate) at 0.58 ± 0.06 and 3.1 ± 0.11 log CFU ml^{-1} were observed respectively in farm-B. Whereas the probiotic, immunostimulant and molasses treated group (farm-A), demonstrates the lowest green and yellow *Vibrio* colonies of 0.17 ± 0.01 log CFU ml^{-1} (90 DOC) and 1.7 ± 0.1 (30 DOC) log CFU ml^{-1} respectively. Similarly, the soil TPC

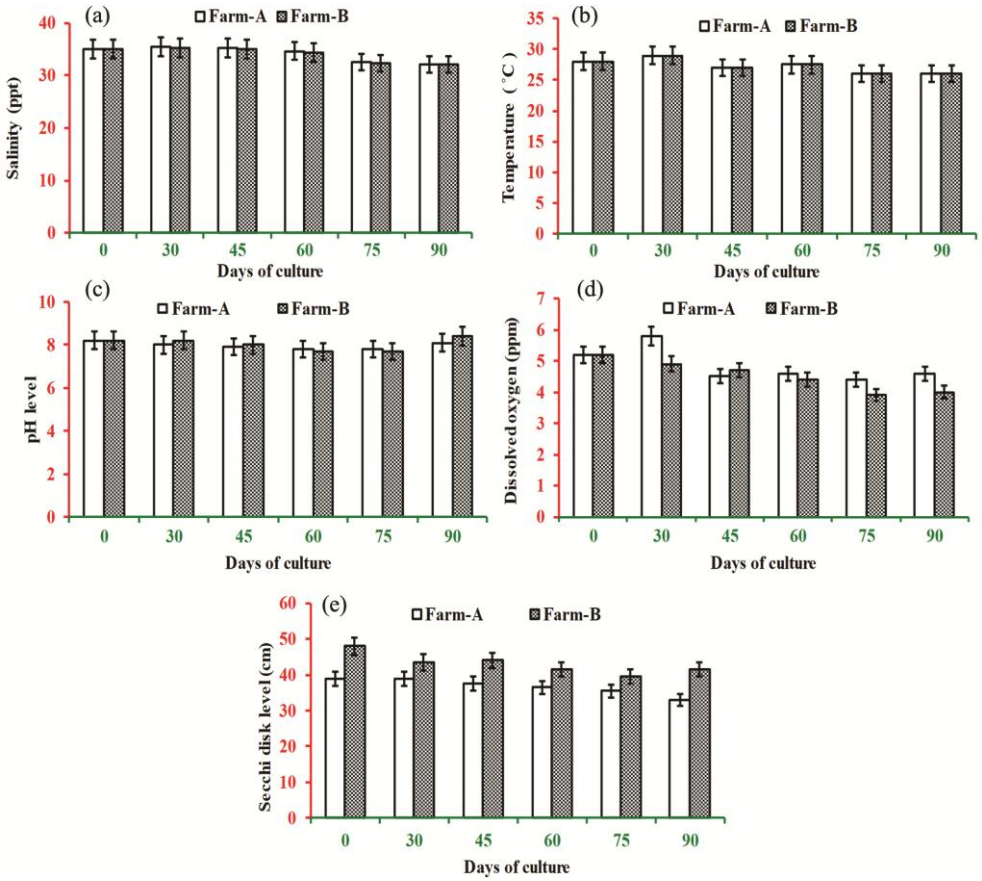

Fig. 1 — Average mean ± S.D value of (a) Salinity (ppt), (b) Temperature (°C), (c) pH, (d) Dissolved oxygen (ppm) and (e) Secchi disk level (cm); in shrimp farm-A and B.

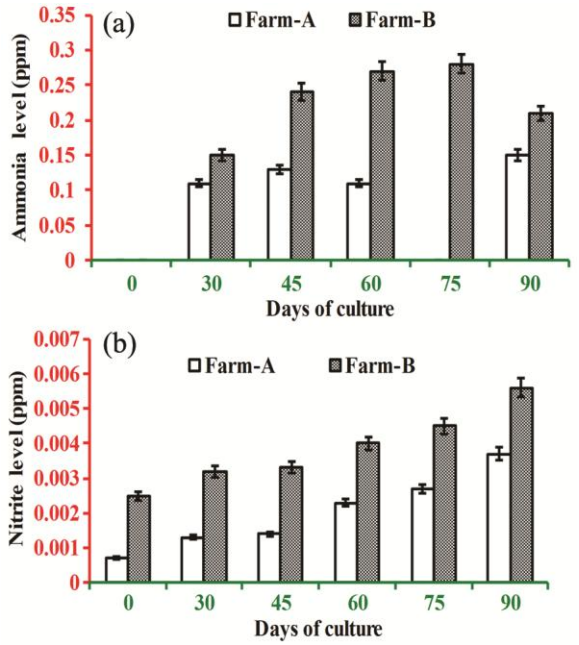

Fig. 2 — Average mean ± SD value of (a) Ammonia (ppm) and (b) Nitrite (ppm) level in shrimp farm-A and B.

level was diverse between 4.7 ± 0.1 and 7.15 ± 0.02 log CFU/g for both farms. The highest TPC level was observed in farm-B 7.15 ± 0.02 log CFU g^{-1} and the lowest log value of 4.7 ± 0.1 was recorded in farm-A. The highest green and yellow colonies of 0.56 ± 0.03 and 4.0 ± 0.09 log CFU ml^{-1} were observed respectively in farms-B on 60th DOC (Fig. 3) whereas the lowest level of 0.37 ± 0.01 and 1.5 ± 0.15 CFU ml^{-1} were recorded in farm-A.

The survival rate was higher (84 ± 0.05 %) in farm-A than the farm-B (74.5 ± 0.70 %) and were significantly differed ($P < 0.05$) between the farms. Similarly, the highest growth (Average Body Weight of 23.2 ± 0.41 g) and production (6.98 tons/ha) was observed in farm-A than farm-B ($P < 0.05$) and the lowest growth (Average Body Weight of 19.6 ± 0.1 g) and production (5.117 tons/ha) was recorded in farm-B (Fig. 4). Farm-A showed a better food conversion ratio of 1:1 than the farm-B at 1:1.37. Moreover, in farm B *Zoothamnium* outbreak and mortalities were observed in two ponds at the end of the culture period.

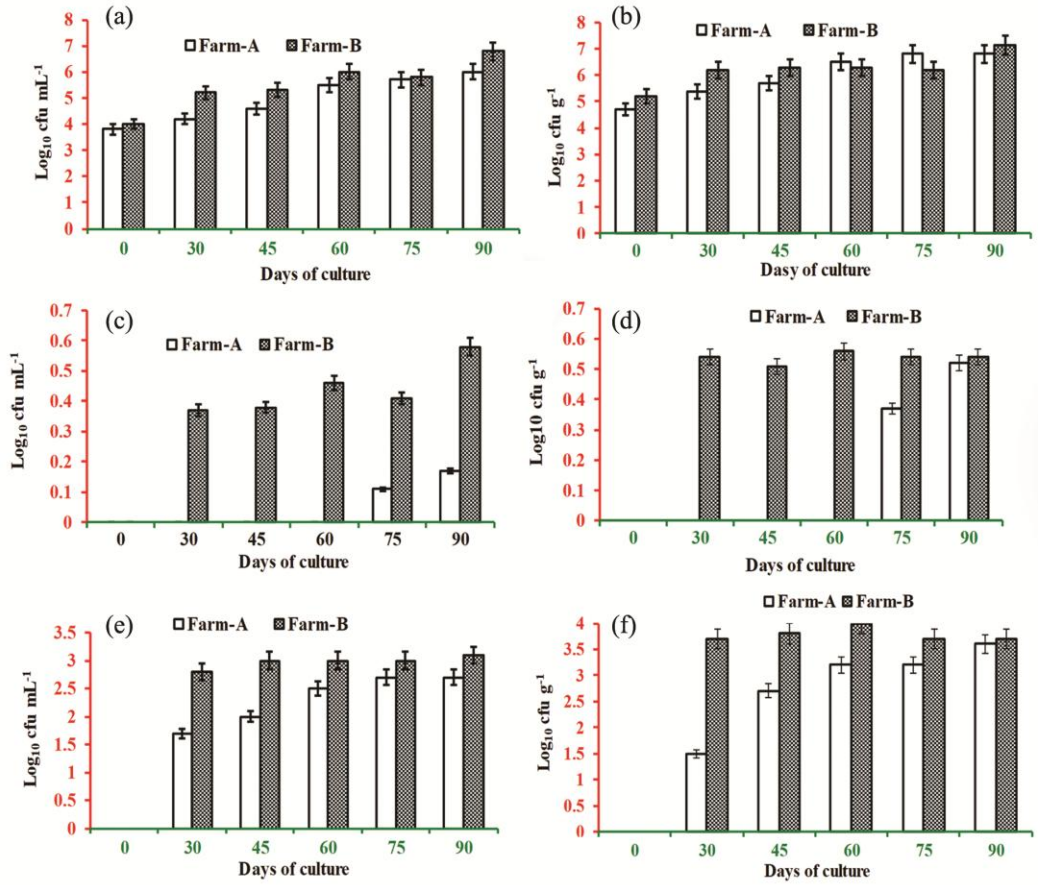

Fig. 3 — Mean ± S.D value of bacterial populations of water and soil in different experimental shrimp ponds: (a) Total plate count in water, (b) Total plate count in soil, (c) *Vibrio* (green) colonies in water, (d) *Vibrio* (green) colonies in soil, (e) *Vibrio* (yellow) colonies in water, (f) *Vibrio* (yellow) colonies in soil

Discussion

Generally, shrimp farmers use different types of commercial health supplements such as chemicals, vitamins, probiotic and immunostimulant etc. for successful shrimp production. However, the scientific background and the efficacy of these products are not fully understood by farmers. Keeping the above facts in mind, this study was carried out in two different groups of shrimp farms for one culture period from pond preparation to harvest. The efficacy of different combination of supplements such as probiotic, immunostimulant and molasses (Farm-A) and chemicals and Probiotic (Farm-B) were evaluated by analyzing the water and soil quality parameters (physiochemical and bacterial population) shrimp survival, growth, production and FCR.

The ideal soil pH was 7 to 8 of the shrimp pond[12]. The lime ($CaCO_3$) is a better neutralizing agent than CaOH or CaO(ref. 13). The application of agricultural lime at the rate of 0.6 to 1-ton ha^{-1} increase the soil pH of 1and the use of zeolite has enhanced the pond bottom condition in intensive farming[13,14]. In this study, to raise the soil pH, in each pond 2.5 tons of agricultural lime was applied in two equal doses during the pond preparation. At the time of the second dose, along with lime 150 kg of zeolite was applied to improve the soil quality. The salinity level was remained constant and there was no much difference between farms.

Temperature plays a vital role in metabolism of shrimp and the safe level for shrimp culture is between 25 °C and 30 °C[13]. In this study, the temperature was within the limits and it was ranged between 26.1 ± 0.15 and 29 ± 0.77 °C. The water pH of the pond is directly related to the shrimp physiological process. Development of low pH has increased the nitrite toxicity to the cultured organism while a high pH has increased the unionized ammonia and a toxic form of sulphide[8,15]. In this study, during the culture period, the pH level was within the limit

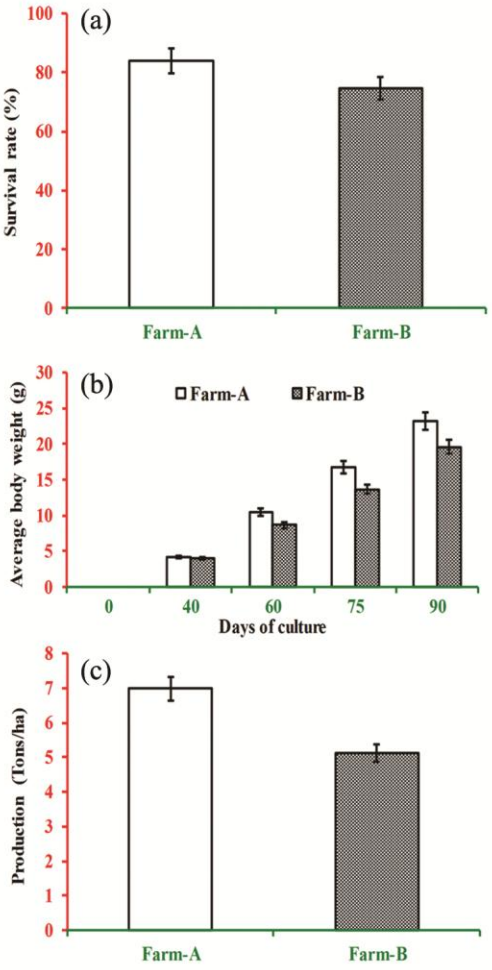

Fig. 4 — Average mean ± SD value of (a) Shrimp survival in %, (b) Average body weight (g) and (c) Production (tons/ha) in shrimp farm-A and B.

in both farms, however, farm supplemented with probiotic, immunostimulant and molasses demonstrate the stable pH whereas the high pH fluctuation was observed in the farm-B, supplemented with chemicals and Probiotic ($P < 0.05$). It shows that application of probiotics, immunostimulant and molasses indirectly supports to maintain the water pH (farm-A). In both farms, 16 HP aerators were used to manage the oxygen and water quality parameters in all the ponds. The dissolved oxygen level was above 4 mg/l in both the farms due to the aeration. However, the oxygen level was slightly high in farm-A than B. This could be attributed to the beneficial effect of the probiotic and molasses through phytoplankton production and photosynthesis activity.

The colour of the pond water is also a good indicator, dull green or yellowish green or brownish green colour is associated with green algae and diatoms. The greenish yellow to yellow colour was noticed in farm-A. However, the water colour of the pond was varied in farm-B. Transparency is an important parameter to measure the phytoplankton bloom. The pond supplemented with probiotic, immunostimulant and molasses combination exhibited 33 ± 0.25 to 39 ± 0.15 cm of transparency level throughout the culture period and showed a yellow-green to green colour. In contrast, the transparency level was highly fluctuated between 39.5 ± 0.15 and 48 ± 0.70 cm in the farm-B ponds, treated with probiotic (partial) and chemicals, even after the application of phytomin (phytoplankton inducing chemical). In farm-A, molasses was applied to improve the phytoplankton bloom and to enrich the probiotic bacterial activity, whereas in farm-B phytomin was applied to improve the bloom. Molasses is a carbon source which supports the growth of plankton, whereas commercial phytomin induces the plankton bloom immediately however, the stability was less, this clearly indicated that the molasses and probiotic combination has enhanced the phytoplankton. Nitrogen and phosphate supplementation with minerals play an important role in the development of the diatom population in shrimp ponds[16]. All the ponds of farm-A and B were administrated with inorganic fertilizer at a 5 N: 1 P ratio from water culture during preparation. In addition to the inorganic fertilizer, phytomin, a plankton inducer was applied in farm-B ponds, whereas probiotic, immunostimulant and molasses slurry was administered in the farm-A ponds. This exhibited better result in the transparency levels and the ideal transparency level of the shrimp pond is varied from 25 to 40 cm[17]. Interestingly, the similar level was observed in the farm-A.

The ammonia level was gradually increased in all the ponds (farm-A & B), but it was within the limits. The lowest level was observed in farm-A, supplemented with probiotic, immunostimulant and molasses, compared with the chemical applied pond even after application of ammonia binder in farm-B. These results clearly indicated that the combined application of probiotic, immunostimulant and molasses has effectively controlled ammonia levels in the pond. On the other hand, nitrite is an intermediate product of the ammonia cycle and probiotic microorganisms converts this nitrite into useful nitrate. In probiotic, immunostimulant and molasses applied ponds (farm-A) the nitrite level was less as compared to the other ponds of farm-B, which clearly

indicated that the application of probiotic might be supported to eliminate the toxic substances such as ammonia and nitrite.

The bacterial colonies developed in the TPC are a group of pathogenic and beneficial microorganisms that require carbon for multiplication. In the culture environment, the basis of carbon is uneaten feeds and animal metabolic wastes that contribute to a relatively higher bacterial load in the culture ponds[18,19]. In general, THB always dominates the other groups in the natural environment[20]. In this study, the TPC count was increased in all the ponds of farm-A except in the farm-B, which may be due to the multiplication of beneficial microorganism. The water and soil *Vibrio* species log value green and yellow colour colonies were high in farm-B. In contrast, the *Vibrio* colonies were less in farm-A ponds, showing that application of probiotic, immunostimulant and molasses significantly ($P < 0.05$), enhance the beneficial bacterial groups in the culture environment and reduced the pathogenic microorganism. In farm-A, seawater was pumped from the reservoir to compensate the water loss due to evaporation and seepage. However, in farm-B, water was exchanged due to water quality problems and application of chemicals.

Growth rate of *L. vannamei* has significantly improved while increasing the feeding frequency from one to four times per day[21]. Asian shrimp farmers who place 1 % to 3 % of the scheduled feed ratio in each feeding tray exhibited better feed management[22]. In this study, the feeding frequency was initially two times in a day from 1 to 10 days, 3 times from the 11th day and 4 times in a day from the 25th day onwards. The probiotic, immunostimulant and molasses applied ponds (Farm-A) showed a better food conversion ratio of 1:1 than the other farm-B at 1:1.37. This shows that the probiotic indirectly support to the feed digestion and effectively absorb the nutrient content, which reflected in the better shrimp growth and FCR in farm-A. At the end of the culture, highest survival (84 ± 0.05 %), ABW (23.2 ± 0.41 g) and production (6.98 tons/ha) were obtained in farm-A, whereas the lowest survival, ABW and production were registered in the farm-B. Moreover, protozoan disease was also observed in the farm-B. The results clearly shows that application of probiotic, immunostimulant and molasses has notably improved the soil and water quality parameters and enhances the survival, growth and the production of shrimp. Hence, the present study concludes that the combined application of probiotic and molasses from pond preparation and immunostimulant supplementation from the initial days to the end of the culture period might improve the shrimp production.

Conclusion

The present study was accomplished in two different shrimp farms (Farm-A: probiotic, immunostimulant and molasses; Farm-B: chemicals and Probiotic) for one culture period from pond preparation to harvest to ascertain a suitable combination of supplements for the successful shrimp production. The temperature, pH, salinity, transparency, dissolved oxygen, total ammonia, nitrite, total plate count of bacterial populations and *Vibrio* spp were within the safe limit in farm-A, supplemented with probiotic, immunostimulant and molasses combination. Hence, the present findings conclude that the supplementation of probiotic and molasses from pond preparation to harvest and application of immunostimulant through a feed from the initial days to end of the culture might prevent the disease and enhance the shrimp production.

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Conflict of Interest

We declare that authors have no conflict of interest.

Author Contributions

PR and SS: Conceptualization, Methodology, Investigation, Writing-Original Draft. TM: Formal analysis, Writing-Review and Editing. BB and GS: Writing-Review and Editing. NMP: Methodology, Supervision and Writing-Review and Editing.

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