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Performance of shrimp *Litopenaeus vannamei* with the addition of probiotics and bioflocs: A field study

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Abstract
An attempt has been made to study the culture performance of Pacific shrimp *Litopenaeus vannamei* with the addition of certain probiotic bacteria such as *Bacillus licheniformis* and *Lactobacilus rhamnorus* in the feed and addition of external carbon source from sugarcane molasses in the form of bioflocs for a period of 90 days. Water quality parameters and growth performance studies were conducted to evaluate the importance of probiotics and bioflocs addition in *L. vannamei*. Both probiotics and bioflocs, consequent upon its addition into the culture operations induces highest and best to keep the water quality to be most ideal for culture operations.

Keywords: *L. vannamei*, probiotics, bioflocs

1. Introduction

The Aquaculture industry is growing fast, at a rate of ~9% per year for the past three decades [1]. However, due to environmental concern, the requirement for more ecologically sourced management and culture practices is also growing fast. At present the expansion of aquaculture production is currently restricted due to the pressure it causes to the environment by the discharge of waste products to natural water bodies and by its strong dependence on feeding ingredients. Another important concern is water, its exchange is also considered to be an important factor contributing to several epizootic diseases on shrimp farms. In addition, water discharge of nutrient-rich effluent from the shrimp farms also contribute to eutrophication, potentially impacting both natural biota and also adjacent culture operations [2]. So to overcome the above constrains, certain alternatives such as Probiotics and Bioflocs usage came into existence in shrimp culture operation. McIntosh *et al.* [3] proposed that there are several methods to reduce water quality determination in aquaculture system i.e. (i) reducing nutrient waste by improving feed quality and feeding management (ii) applying water treatment to reduce waste discharged and (iii) reducing water volume used for aquaculture activities with minimum water use. Both Probiotics and Bioflocs have been considered to have beneficial effects on aquaculture production. The Probiotics were 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. The main principle of Bioflocs is the addition of organic carbon to stimulate the growth of heterotrophic bacteria was found to be beneficial to improve water quality in aquaculture system[4,5]. The objective of the present study was to monitor the effect of Probiotic bacteria addition and addition of sugarcane molasses as external carbon sources to maintain Carbon: Nitrogen (C:N) ratio, there by maintenance of water quality and production performance of shrimp *Litopenaeus vannamei* culture operation.

2. Materials and Methods

The present study was carried out in shrimp culture farms located in Ramayapatnam (Latitude 15^{0} 02^{1} 55^{11}N ; Longitude 80^{0} 02^{1} 50^{11} E) Prakasam District of Andhra Pradesh, India. The culture ponds selected in the present investigation are approximately with an average size of 1.0 ha and are rectangular in shape and soil is ideal for semi intensive type of culture operation. Water for culture operation was drawn with the help of motors from Buckingham canal and kept in a storage pond for further usage in the culture operation. All the experimental ponds were maintained a water depth of 1.2 mts.

2.1. Probiotic Feed Preparation
Probiotic supplemented Feeds were prepared as described by Naresh[6]. Probiotic bacterial species *Bacillus licheniformis* and *Lactobacilus rhamnorus* were obtained and maintained in the nutrient broth and were harvested by centrifuging at 10,000 rpm for 10 minutes subsequently washed with phosphate buffer, finally re-suspended in phosphate buffer saline (pH 7.4). These re-suspended bacteria were mixed uniformly to the feed pellets by spraying method. The Probiotic blended feed prepared was dried at 40 ^{0}C and packed in air tight polythene covers and stored in Refrigerator for further use. The Probiotic blended feed with *L. rhamnorus* and *B. licheniformis* (@ 10 billion cfu/kg) feed were prepared once in seven days.
Penaeid shrimps *Litopenaeus vannamei* of 0.79$\pm$0.04 g of uniform size were obtained from local aqua farms and pathogen free shrimp were selected and acclimatized in one of the experimental tanks with a salinity of 10$\pm$0.5 ppt. Feeding was done with a commercial feed obtained from local market @ 30% of the body weight in two times a day morning 6.00 AM and 6.00 PM in the evening. The present experiment was conducted for a period of 90 days.

2.2. Preparation of Biofloc
Sugarcane molasses was selected as a source for carbon and known to contains 36% carbon, 53% carbohydrate, 24% moisture content was incubated for 2 days in warm water at 40^{o} C and the same was added to the culture medium in the ratio of 1:3 Molasses: Water. To stimulate Nitrogen loading in an aquaculture system NH_4Cl, KH_2PO_4 and Na_2HPO_4 were added to each tank @ 96, 31 and 64 mg/lit, respectively[7]. The ratio between sugarcane molasses and feed to reach desired Carbon: Nitrogen (C: N) ratio was calculated based on assuming 50% nitrogen from feed eaten by the shrimp excreting in to the water environment[4]. On the above basis the formula of the ratio in weight between, the carbon source and feed can be given as follows

$$\frac{\Delta CH}{\Delta F} = \frac{((CN \times \%\,P(F) \times \%\,N\,(P)) - \%\,CF}{\%\,C_{CH}}$$

Where

ΔCH	:	Weight of Carbon Source
ΔF	:	Weight of the Feed
CN	:	C: N ratio need to be required
% P(F)	:	Protein content in Feed
% N(P)	:	Nitrogen content in Protein (15.5%)
% CF	:	Carbon content in the Feed (50%)
% C_{CH}	:	Carbon content in the Carbon Source

Carbon content was determined by adopting the method of Walkley and Black [8]. Total Ammonia Nitrogen (TAN) concentration and other water quality parameters were measured with the procedures according to APHA[9]. Growth parameters including, average body weights, average body growth rates, specific growth rates, feed conversion ratio, protein efficiency ratio, feed efficiency ratio and productivity rates were monitored and tabulated. All the above parameters were calculated by adopting the following Formulae Survival rate (%)

$$\frac{\text{Total number of live shrimp}}{\text{Total number of shrimps stocked}} \times 100$$

Weight Gain (g)
Weight of the shrimp (g) - Weight of the shrimp (g)
At the end of the Expt. at the start of the Expt.

Feed Conversion Ratio (FCR)

$$\frac{\text{Total amount of feed consumed (Kgs)}}{\text{Total biomass of shrimp (Kgs)}}$$

Average daily growth rates (ADGR)

$$\frac{\text{Weight of the shrimp (g) At the end of the Expt.} - \text{Weight of the shrimp (g) at the start of the Expt.}}{\text{Total number of days of Experiment}}$$

Specific growth rates (SGR)

$$\frac{\text{Log weight of the shrimp (g) At the end of the Expt.} - \text{Log weight of the shrimp (g) at the start of the Expt.}}{\text{Total number of days of experiment}} \times 100$$

$$\frac{(\text{Log } W_2 - \text{Log } W_1)}{T} \times 100$$

Where
W_1 : Weight of the shrimp at start of the experiment
W_2 : Weight of the shrimp at the end of the experiment
T : Total number of days of experiment

Vibrio Bacteria rate (%)

$$\frac{V}{H} \times 100$$

Where
V: Average density of vibrio bacteria
H: Average density of total bacteria in each Tank

Biofloc volume (FV)
$V_{Floc} / V_{collection}$
Where

V_{Floc}	:	Biofloc Volume (ml)
$V_{collection}$	:	Collected Sample Volume (ml)

The data obtained was analyzed statistically through Microsoft excel. The difference in variants between treatments was determined according to One-Way ANOVA using SPSS.

3. Results
In the present investigation an attempt has been made to study the water quality parameters periodically throughout the experimental period up to 90 days in different experimental

groups i.e. control group and another group fed with Probiotic blended feed fed and the other group added with Bioflocs were presented in Table 1. The water parameters like Salinity (10±0.4), Temperature (26-28 ^{0}C), pH (7.4-7.6), Dissolved Oxygen (DO) (6.3-6.5 mg/lit) were found to be almost static, no significant changes were noticed or observed throughout the experimental period of 90 days. The total ammonia nitrogen (TAN) content was found to be 2.14±0.12 mg/lit in control group compared to the shrimp groups fed with Probiotic blended feed (1.34±0.11 mg/lit) and Biofloc added group (0.37±0.04 mg/lit) showed a significant reduction in TAN content (Table 1). The reduction in TAN content was -37% in the group fed with Probiotic blended feed fed group compared to -84% in the shrimp fed with Biofloc added shrimp. Both Nitrate and Nitrite contents measured were found to be recorded maximum in the control group compared to a significant reduction in experimental groups. The reduction of Nitrite was significant i.e. -32% recorded with group fed with probiotic blended feed compared to -54% recorded with group added with Biofloc. Similarly Nitrate also recorded -25% with group fed with Probiotic blended feed fed group compared to -41% recorded with group added with Biofloc. The Alkalinity value was recorded to be 170 mg/lit in control group compared to 315 mg/lit and 503 mg/lit recorded with group fed with Probiotic blended feed group, group added with Biofloc, respectively. A significant increase was observed in Alkalinity values compared to control with Probiotic (+85) and Biofloc (+196) added groups. Similarly Total suspended solids (TSS) were also found to record lower in control group (260 mg/lit) compared to Probiotic (379 mg/lit) and Biofloc (449 mg/lit) fed groups. The TSS levels were found to be significantly increased in Probiotic (+46%) and Biofloc (+73%) added groups. The Biological Oxygen Demand (BOD) values recorded to be minimum with control group (18.15 mg/lit) compared to Probiotic (58.77 mg/lit) and Biofloc (80.77 mg/lit) fed groups. The BOD was found to be significantly increased and recorded +224% with Probiotic +345% with Biofloc added groups. The dissolved oxygen (DO) reduction rates recorded to be 0.46 mg/lit/hr, a minimum with control group compared to 1.04 mg/lit/hr with Probiotic and 1.35 mg/lit/hr with Biofloc added groups. The DO reduction rate was found to significantly increased i.e. +126% with probiotic, +193% with Biofloc added groups. The Biofloc volume was found to be 13.12, 29.77 and 33.39 mg/lit with control, Probiotic and Biofloc added groups, respectively. The Biofloc volume was also showed significant increase i.e. +127% with Probiotic, +155% with Biofloc added groups.

The performance detail of *L. vannamei* in the present investigation was presented in Table 2. In all the experimental ponds 50,000 nos. of PL's were stocked and experiment was conducted for 90 days. The per cent survival values were recorded to be 90%, 93% and 95% with control, Probiotic and Biofloc added groups, respectively. Soon after of the completion of the experiment, the final weights were recorded to be 22.13 g with control group, compared to 26.72 g with Probiotic fed and 29.18g with Biofloc added groups. The weight gain values recorded to be 21.34 g, 25.93 g and 28.39 g with control, Probiotic and Biofloc added groups, respectively. The daily growth rates observed to be 0.237 g minimum with control group followed by 0.288 g with Probiotic and 0.315 g with Biofloc added groups. Similarly the specific growth rates (SGR) were found to be 1.59, 1.70 and 1.73 recorded with control, Probiotic and Biofloc added groups, respectively. The protein efficiency ratio (PER) values were found to be 5.35, 7.24 and 7.92 with control, Probiotic and Biofloc added groups, respectively. The feed conversion ratio (FCR) values obtained in the present investigation found to be 2.69, 2.32 and 2.18 with control, Probiotic and Biofloc added groups, respectively. The feed efficiency ratio (FER) values obtained were 0.372, 0.431, 0.459 with control, Probiotic and Biofloc added groups, respectively. The average body weights obtained at the tag end of the experiment i.e. harvested shrimp size were found to be 13.22 g, 17.24 g and 18.95 g with control, Probiotic and Biofloc added groups, respectively. The Productivity or yield obtained after the completion of the experiment was found to be 595 kgs, 802 kgs and 900 kgs with control, Probiotic and Biofloc added groups respectively.

Total heterotrophic bacteria (THB) found to be increased significantly with an increase in the duration of the culture period in all the experimental conditions (Figures 1 and 2). Total vibrio bacteria (TVB) levels were found to be decreased significantly with an increase in the duration of the culture period (Figures 3 and 4). It is very clear that the results obtained in the present investigation pertaining to water quality parameters, and performance details were found to be more pronounced in Biofloc added group followed by Probiotic added group compared to control group.

4. Discussion

In the present investigation, an attempt was made to monitor the impact of addition of Probiotics and Bioflocs in the culture operation of *L. vannamei* to assess water quality parameters (Table 1) and performance of pond details in terms of productivity (Table 2). In the present investigations, all the water quality parameters recorded were found to be with in a suitable range for *L. vannamei* survival and growth for a period of 90 days and are within the accepted range for brackish water shrimp culture [10]. The TSS and Floc volume were found to be significantly increased in both Probiotic and Biofloc added shrimp culture operation. To date the probiotics can be considered to be a valid alternative to the use of antibiotics in Aquaculture and in particular in shrimp culture activity, to prevent high mortality and to improve yield by enhancing the growth rates. In the last two decades, many studies reported promising results using a beneficial bacterial strains on aquaculture of several candidate species. In the present investigation *Bacillus licheniformis* and *Lactobacilus rhamnorus* were added in to the feed and broadcasted in the culture operation, which not only protects water quality and also induces relatively higher growth potentials in *L. vannamei* culture operation. In the present investigation another group was maintained by adding blocs in to the culture media in the Carbon:Nitrogen (C:N) ratio 15:1 by the addition of external carbohydrate sources, which subsequently helps in the Biofloc production. Sugarcane molasses was used as carbon source for the successful augmentation of microbial growth. So, both Probiotics and Bioflocs added in to the culture operation are playing a vital role in controlling not only water quality parameters and also inducing growth potentials in *L. vannamei*. Due to adequate aeration in all the experimental ponds, the DO levels were maintained at sufficient levels. The alkalinity levels obtained in the present investigations were found to be relatively higher as it was recommended to be around 130 mg/lit for shrimp culture activity [11]. In the present investigation, due to the addition of sugarcane molasses as carbohydrate source to induce Biofloc production, significantly reduced the TAN levels in the water column of experimental ponds. The levels of TAN, Nitrate and Nitrite obtained were found to fall in the optimum range as recommended for juveniles of pacific white shrimp *L. vannamei* [12, 13]. Due to the addition of additional carbohydrate sources, the TAN levels were significantly reduced in the culture operation of fish Tilapia [14]; *P. monodon* [15, 16] and *L. vannamei* [17]. There were reports regarding the successful utilization of TAN and Carbon by the bacteria for the production of microbial floc with in the culture systems [4, 14]. In the present study, low level of Nitrite-N was recorded suggests the successful oxidation of ammonia to nitrate [18]. Due to the addition of Bioflocs in the culture operation, the Nitrite and Nitrates will be removed further they will be assimilated and yields in the production of protein [19, 20]. Avnihmelech [5] also reported that the addition of carbohydrate as carbon source, reduces the need of dietary protein concentration and also decrease the TAN concentrations in the culture systems. In the present study, the generation of ammonia and its transformed products such as TAN, Nitrate and Nitrite were founded to be significantly reduced during Biofloc addition into the culture, clearly revels that nitrogen was significantly transformed into protein component under the presence of Biofloc. The Biofloc volume, an index for production and density of plankton microbial content in the culture operation was also significantly increased, suggests the enhanced microbial growth. DO reduction can be taken as an index to assess the microbial load and occurrence of plankton quantity, the higher levels of results in the efficient reduction of DO reduction rates. In the present investigation DO reduction rates were significantly increased with the addition of both Probiotics and Bioflocs, which enhances the microbial load and phytoplankton quantity substantially, there by reduces the DO levels. Kumar *et al*. [16] also reported similar kind of observation with *P. monodon* culture operation after the addition of carbon sources as Bioflocs. The increased quantities of THB on the experimental ponds in the present study (Figures 1 and 2) suggests that toxic substances like NH_4^+ and NO_2^- were absorbed and subsequently counted into the bacterial biomass. The TVB contents were significantly reduced (Figures 3 and 4) with an increase in the survival rates due to Probiotic and Biofloc addition in the present investigation, Probiotics and Bioflocs facilitate the production of beneficial bacteria reduces the rate of development of vibrio bacteria in the culture operation.

The growth performance parameters recorded for *L. vannamei* after the addition of Probiotics and Bioflocs including average body weights, daily growth rates, specific growth rates, protein efficiency ratio, and related parameters were found to be significantly increased during Probiotic and Biofloc addition in the present study (Table 2). From the results obtained in the present study clearly indicates that due to addition of both Probiotics and Bioflocs, induces the growth potentials significantly in *L. vannamei*. Due to the addition of Probiotics and Bioflocs, the production of production was significantly increased and which in turns are being consumed results in the enhanced productivity in the present study. Similar kinds of observations were also updated by several authors [5, 21]. Due to the addition of carbon sources lead to higher protein utilization and subsequently higher productivity yields.

In the present investigation both Probiotics and Bioflocs added into culture operation are supposed to have different modes of action, but capable of inducing growth potentials and productivity rates in *L. vannamei*. The Probiotics used in the present study induces growth rates by adopting several modes of action, competitive exclusion of pathogenic bacteria through the production of inhibitory compounds; improvement of water quality, enhancement of immune response of the host species and enhancement of nutrition of host species through the production of supplemented digestive enzymes. Similarly due to addition of Bioflocs in to the culture operation facilitates the production of higher levels of plankton, and in consumption yields will end up with higher productivity rates.

Table 1: Water quality parameters during *L. vannamei* culture operation under Probiotics and Biofloc feeding trails

Parameter	Control	With Probiotics	With Bioflocs
Dissolved Oxygen (DO) (mg/lit)	6.27± 0.22 PDC	6.38± 0.21[a] (+21)	6.49 ± 0.22[a] (+32)
Total Ammonia Nitrogen (TAN) (mg/lit)	2.14 ± 0.12 PDC	1.34± 0.11[a] (-37)	0.37± 0.04[a] (-84)
Nitrite (mg/lit)	1.39 ± 0.11 PDC	0.94 ± 0.06[a] (-32)	0.64 ± 0.05[a] (-54)
Nitrate (mg/lit)	4.24 ± 0.22 PDC	3.18 ±0.15[b] (-32)	2.49 ± 0.13[a] (-54)
Alkalinity (mg/lit)	170.18 ± 26.77 PDC	315.14 ± 26.78[a] (+85)	503.13 ± 30.12[a] (+196)
Total Suspended Solids (TSS) (mg/lit)	260.13 ±29.13 PDC	379.14 ± 25.19[a] (+46)	7.92[a] (+48)
Biological Reduction rates (mg/lit)	18.15 ± 1.28 PDC	58.77 ± 2.72[a] (+224)	80.77 ± 2.89[a] (+345)
DO Reduction Rate (mg/lit/hr)	0.46 ± 0.05 PDC	1.04 ± 0.06[a] (+126)	1.35 ± 0.12[a] (+193)
Biofloc volume (mg/lit)	13.12± 0.59 PDC	29.77 ± 1.08[a] (+127)	33.39 ± 1.05[a] (+155)

All Values are Mean ± SD of six individual observations
Values presented in parenthesis are Percent Change over their respective Control
All values are Statistically Significant at [a]$P<0.001$; [b]$P<0.05$

Table 2: Performance details of *L. vannamei* under Probiotics and Biofloc feeding trails

Parameter	Control	With Probiotics	With Bioflocs
Shrimp stocked (nos)	50000	50000	50000
Percent survival (%)	90	93	95
Final Weight (g)	22.13 ± 0.32 PDC	26.72 ± 0.34[a] (+21)	29.18 ± 0.32[a] (+32)
Weight gain (g)	21.34 ± 0.34 PDC	25.93 ± 0.32[a] (+22)	28.39 ± 0.33[a] (+33)
Weight gain (%)	+2701	+3282[a]	+3594[a]
Daily Growth Rate (DGR) (g)	0.237 PDC	0.288[a] (+22)	0.315[a] (+33)
Specific Growth Rates (SGR)	1.59 PDC	1.70[c] (+7)	1.73[c] (+9)
Protein Efficiency Ratio (PER)	5.35 PDC	7.24[a] (+35)	7.92[a] (+48)
Feed Conversion Ratio (FCR)	2.69 PDC	2.32[b] (-14)	2.18[b] (-19)
Feed Efficiency Ratio (FER)	0.372 PDC	0.431[b] (+16)	0.459[a] (+23)
Harvest size (g)	13.12 PDC	17.24[a] (+30)	18.95[a] (+43)
Productivity (kgs)	595 PDC	802[a] (+35)	900[a] (+51)

Shrimp with an Average Weight of 0.79 ± 0.03 were stocked
Values presented in parenthesis are Percent Change over their respective Control
All values are Statistically Significant at [a]P<0.001; [b]P<0.05; [c]NS

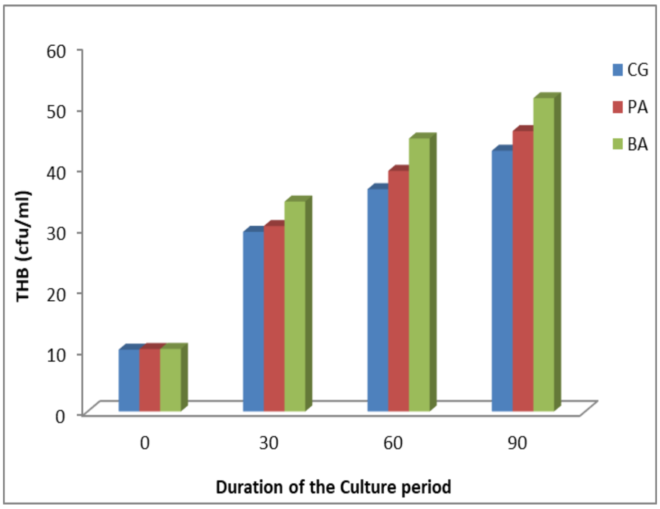


Fig 1: Total Heterotrophic Bacteria (THB) content in *L. vannamei* culture system

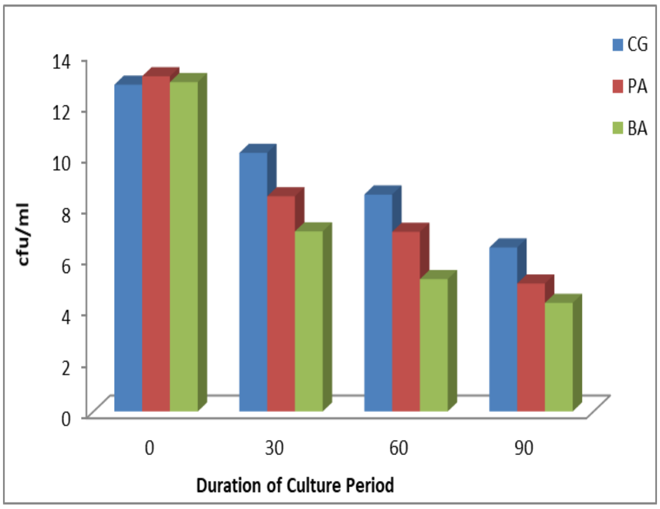


Fig 3: Total Vibrio Bacteria (TVB) content in *L. vannamei* culture system

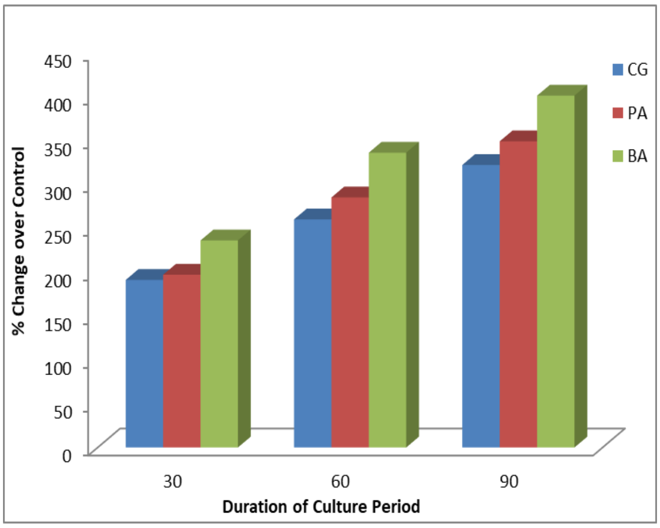


Fig 2: % Change of Total Heterotrophic Bacteria (THB) content in *L. vannamei* culture operation at different days

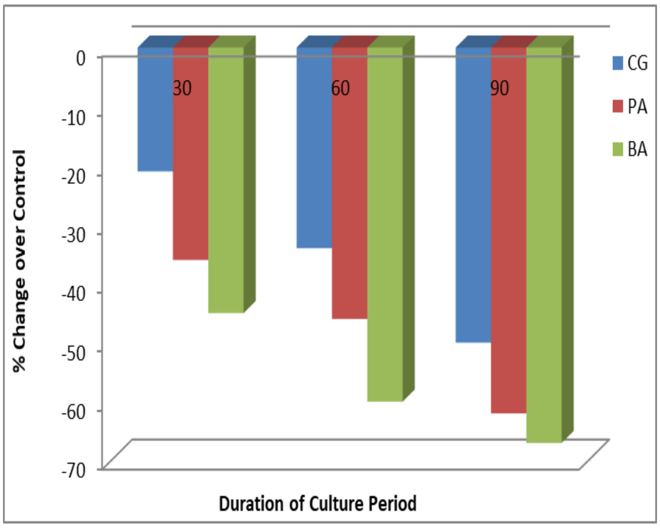
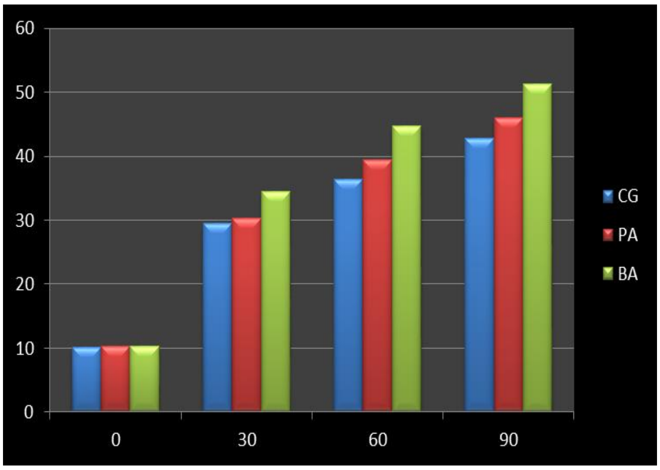


Fig 4: % Change of Total Vibrio Bacteria (TVB) content in *L. vannamei* culture operation at different days



Days of Culture
mg/lit

5. Conclusions

The present investigation may be concluded that, due to the addition of Probiotics and Bioflocs, not only keeping water quality at its best and also inducing highest and best growth rates in *L. vannamei* during culture operation. The results clearly suggests the use of Probiotics both *Bacillus licheniformis* and *Lactobacilus rhamnorus* and also Bioflocs in the form of sugarcane molasses for the addition of carbohydrate sources might be playing a promising role and substantially increasing the productivity in the culture operation of *Litopenaeus vannamei*.

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