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Probiotics Enhance the Zootechnical, Biochemical, and Water Quality Aspects of Culturing the Whiteleg Shrimp, *Litopenaeus vannamei* in the Biofloc System

Wael Abo El-Naga[1], Zaki Zaki Sharawy[2*], Al-Azab Tahoun[1], Ahmed El-Ashram[3], Ashraf Suloma[4]
[1]Aquaculture Department, Faculty of Fish Resources, Suez University, Egypt
[2]National Institute of Oceanography and Fisheries (NIOF), Egypt
[3]Fish Health and Diseases Department. Central Lab for Aquaculture Research, Agriculture Research Center, Egypt
[4]Fish Nutrition Laboratory, Animal Production Department, Faculty of Agriculture, Cairo University, Giza, 12613, Egypt
[*]Corresponding Author: zaki_sharawy@yahoo.com

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ABSTRACT
The study investigated the impacts of dietary supplementation with two probiotics, PondToss® and LactéolFort®, on the growth performance and biochemical indices of the whiteleg shrimp (*Litopenaeus vannamei*) in clear water (CW) and biofloc technology (BFT) systems. Six different treatments were applied: T1 (CW without probiotics, control), T2 (BFT without probiotics), T3 (BFT with PondToss® at 0.5g g^{-1}), T4 (BFT with PondToss® at 1g kg^{-1}), T5 (BFT with LactéolFort® at 0.5gkg^{-1}), and T6 (BFT with LactéolFort® at 1g kg^{-1}). Over 90 days, shrimps with initial average weights of 3.9 to 4.10g were fed the experimental diets to satiety twice daily. The study found that neither rearing system nor probiotic treatment affected water temperature or dissolved oxygen (DO) levels. However, the treatments significantly influenced water pH, dissolved nitrogen forms (ammonia, nitrite, and nitrate), total suspended solids (TSS), total alkalinity (as $CaCO_3$), and floc volume (VF). Biofloc and probiotic-enriched diets stimulated growth in terms of weight gain, specific growth rate (SGR), and survival rate. In biofloc conditions, shrimps fed with probiotics showed an increased feed intake and a better feed and protein utilization. Biochemical indices such as albumin, total protein, and globulin levels varied significantly among the groups, with the control group (T1) recording the lowest survival rate (66.89%), while the BFT and probiotic-enriched groups had higher survival rates, ranging from 87.35 (T6) to 90.21% (T2). The findings suggest that probiotic-supplemented diets in BFT systems improve water quality, boost shrimp growth, enhance nutrient utilization, and positively affect biochemical characteristics. Thus, combining probiotics with biofloc technology appears to be a promising strategy for enhancing shrimp performance.

INTRODUCTION

Among marine shrimp species, the whiteleg shrimp (*Litopenaeus vannamei*), often known as the Pacific shrimp, is widely farmed for aquaculture programs. Among the important traits that make it a highly desirable species for aquaculture is its ability to survive a wide range of water salinities from fresh, saline, to seawater, ranging from 0.5 to 45ppt. The whiteleg shrimp is known for its rapid growth rate and efficiency as an aquaculture species as well as its disease resistance, making it a more reliable and viable option. Moroever, there is a high demand globally for the whiteleg shrimp since it is a popular seafood item and is widely consumed **(Supono *et al*., 2022)**.

Probiotic supplements improve the competitive exclusion of diseases aquaculture system and boost the immunological characteristics of shrimp, without harming them **(Newaj-Fyzul & Austin, 2015; Soto, 2017; Tahoun, 2022)**. However, antibiotic and chemical disinfectant applications are no longer suggested since they harm the environment and cause the evolution of bacterial resistance **(Maron *et al.*, 2013; Goh *et al.*, 2023)**. Certain probiotics are recognized as an eco-friendly approach to growth-boosting **(Muthu *et al.*, 2024)**. Probiotics-enriched diets have been shown to significantly improve crustacean biological activities such as growth performance, oxidant/antioxidant action, immunity, and disease resistance **(Muthu *et al*., 2024; Noman *et al*., 2024)**.

Biofloc technology (BFT) is an aquaculture approach that is both sustainable and cost-effective. This method produces aquatic animals without the need for water exchange. The process leads to the accumulation of organic substrates and the establishment of a dense microbial biomass. Bioflocs are made up of many suspended species, such as bacteria, fungi, detritus, and microalgae **(Sharawy *et al*., 2022)**. These bacteria have a role in nourishing farmed aquatic species and eliminating surplus nutrients **(Azim & Little, 2008)**. In aquaculture, BFT improves water quality by providing extra external carbon sources while maintaining a high level of aeration, resulting in substantial volumes of biofloc **(Hassan *et al*., 2022a)**. In this system, it is critical to keep a C/N ratio above 10. You can achieve this by adding carbon-containing resources like wheat flour, molasses, rice bran, and starch, or by reducing the diet's protein content to boost the activity of heterotrophic bacteria. When choosing a carbon source, factors such as carbohydrate availability and digestibility, protein content, and cost-effectiveness should be considered **(Hassan *et al*., 2022b)**. Ingestion of BF boosts the non-specific immune system and improves shrimp's ability to fight viral and bacterial infections **(Hassan *et al*., 2023)**. The presence of many microorganisms and bioactive chemicals in biofloc systems is believed to enhance shrimp's immune response and reduce mortality **(Mansour *et al*., 2022)**.

The current study aimed to evaluate the effects of probiotic supplements on shrimp maintained under biofloc conditions, including water quality, growth and nutrient utilization, and biochemical characteristics.

MATERIALS AND METHODS

1. Experiment design

The trial was carried out in eighteen 100L glass aquaria at the facility of the invertebrate Lab, National Institute of Oceanography and Fisheries (NIOF), Suez, Egypt. The Eighteen aquariums were cleaned and filled with seawater through a plankton net (50µm) to prevent unwanted debris and suspended particles. Next, freshwater was added to the salinity to adjust it to 32ppt. All aquaria were supplied with a diffuser system that is connects to the air blower and works similarly to air stone pipes. The aeration system ran continuously (24hrs day^{-1}) to provide the appropriate levels of dissolved oxygen and robust water mixing for ensuring better bio-flocculation throughout the whole experimental period. Every aquarium was equipped with an air diffuser for aerating end with a stone pipe that was connected to the air blower.

2. Shrimp rearing

Healthy *L. vannamei* shrimps were purchased from the private shrimp hatchery (Sayed Abo Omar shrimp hatchery) located in El-Diba province, Port Said Governorate, Egypt. Moreover, shrimps were transported in a well-oxygenated tank. When the shrimps arrived at the invertebrate lab (NIOF), they were immediately placed in the acclimatization reservoir tank (salinity, 32ppt). Additionally, initial samples of shrimp juveniles were taken immediately after they arrived from the hatchery.

For three weeks, the shrimps were allowed to acclimate to lab conditions by feeding them the control diet 2 times a day (8:00 am and 2:00 pm). After acclimation, shrimp samples (3.9- 4.1g) from six treatments in triplicate were randomly stocked in eighteen 100L aquaria at a density of 15 juveniles per tank in triplicates. Experimental diets were given to the animals two times a day (8:00 am and 2:00 pm) until they appeared satiated. Daily cleanings were performed on the aquariums allocated to the clear water system (control group); waste and half of the water in each aquarium were siphoned out and replaced with brand-new, salted water from a storage reservoir tank (32ppt). While other glass aquaria were managed and operated following the BFT protocol, molasses (carbon source) was added to achieve a C:N ratio of 10:1.

3. Preparation of experimental diets

Two commercial probiotics PondToss® (Keeton Industries Inc. USA) containing 109 CFU g^{-1} *Bacillus* species (i.e., *subtilis*, *amyloliquefaciens*, *pumilus* and *licheniformis*) and LactéolFort® (*Lactobacillus delbruekii* (10^{13} CFU g^{-1}) and *L. fermentum* (1013 CFU g^{-1}) were purchased from the local market and were supplemented to the baseline diet, as described by **Abdel-Tawwab *et al.* (2021)** at 0.0 (control), 0.5, and 1g/ kg. Before being used, the dough was pelleted in a meat grinder, then air dried with a fan, and kept in a refrigerator (4°C). The basal diet contains 40% crude protein (CP), 3810.5 gross energy (kcal kg^{-1}) 9% ether extract (EE), 35% nitrogen free extract (NFE), and 12.7% ash. Each probiotic's lyophilized powder form was combined with maize oil and sprayed into the diet. The diets were given 2 times a day (8:00 am and 2:00 pm) at a rate of 2% of shrimp biomass, with a biweekly ration size adjustment. To stimulate biofloc formation and growth, molasses (a carbon source) was added to BFT tanks at a carbon/nitrogen (C/N) ratio of 10:1.

4. Water quality parameters

To examine water quality, samples were collected from each aquarium to determine 8 physicochemical variables: water pH, temperature, and DO (once daily), while water NH_3-N, NO_2-N, NO_3, total alkalinity, floc volume (FV), and total suspended solids (TSS) were recorded biweekly. A digital thermometer was used to measure the temperature of the water and a pH meter was used to assess the pH of the water. A photometer and multi-test kit were used to measure the water's DO, NH_3-N, NO_2-N, NO_3-N, and TSS. Using an Imhoff cone, FV at the bottom of the cone was observed after 15min of settling and was recorded as described by **Avnimelech (2007)**. The water's total alkalinity was measured by titrating it to a pH point of 4.5 **(APHA, 1998)**.

5. Optimizing growth, survival, nutrient, and energy utilization

The shrimp from every tank were gathered, numbered, and collectively weighed at the end of the experiment. After about 3 months of the feeding trials, the total amount of diet for each tank was determined. The following equations were used to calculate the growth, for survival and nutrient utilization:

Average Weight Gain (AWG, g) = W2 − W1.

Specific Growth Rate (SGR; % day^{-1}) = 100 [*ln*W2 − *ln*W1] / Time (in days);

where W2 and W1 are the final and the initial weight (g), respectively; *ln* is the natural logarithm

Feed Conversion Ratio (FCR) = Feed Intake / Weight Gain;

Protein Efficiency Ratio (PER) = Weight Gain / Protein Intake;

Protein Productive Value (PPV, %) =100 [Retained Protein / Protein Intake];

Energy Utilization (EU, %) = 100 [Retained Energy / Energy Intake];

Shrimp survival (S, %) = 100 [Survived Shrimp Number (at the end)/ Initial Shrimp Number (at the start)].

6. Biochemical analyses

Commercial test kits (Bio-Diagnostic Company, Cairo, Egypt) were used to measure the total protein (TP), albumin (ALB), and globulin (GLO). The amounts of ALB and TP were quantified using **Doumas *et al.* (1971)** procedures. The amount of GLO was determined by subtracting ALB from TP.

7. Statistical analysis

All statistical analyses were performed using Statistical Package for Social Science Software V.22 **(SPSS, 2013)** to identify statistically significant differences among treatments; data from each treatment (mean± standard error, SE) were analyzed using the one-way-ANOVA. The mean differences were analyzed utilizing the Duncan multiple range test **(Duncan, 1955)**, with a significance level of $P \leq 0.05$.

RESULTS

1. Water quality indices

During the 90-day experiment, water concentrations of NH_3-N, NO_2-N, and NO_3-N, as well as total alkalinity ($CaCO_3$, FV, and TSS), were monitored at regular intervals and they showed significant differences ($P \leq 0.05$) in water-dissolved nitrogenous compounds, total alkalinity, FV, and TSS (Table 1). In all BFT aquaria, the flocculated biofloc was developed. Throughout the study, the FV and TSS values increased. Brownish bioflocs with suspended organic macro-aggregate particles were observed. These flocculated macroaggregates, also known as bioflocs, were colonized by a large number of heterotrophic bacteria, microalgae, and protozoans. During the whole trial period, all water quality parameters were determined and were within limits that are appropriate for *L. vannam*ei cultivation.

Table 1. Water quality criteria f of *L. vannamei* fed probiotics-enriched diets for 90 days with different culture systems

	Temp.	DO	pH	NH_3-N	NO_2-N	NO_3-N	Alkalinity	FV	TSS
T1	29.41±0.15[a]	5.03±0.02[a]	7.91±0.04[a]	0.36±0.01[a]	0.59±0.03[a]	36.69±1.19[a]	236.66±20.56[a]	1.00±0.31[b]	117.69±6.40[b]
T2	29.68±0.12[a]	5.04±0.02[a]	7.94±0.04[a]	0.28±0.02[b]	0.47±0.02[b]	31.35±0.76[b]	250.04±17.25[a]	12.99±2.06[a]	180.93±21.16[a]
T3	29.64±0.12[a]	5.06±0.04[a]	7.93±0.07[a]	0.26±0.01[b]	0.42±0.03[b]	29.34±1.37[b]	248.52±19.73[a]	16.57±1.72[a]	200.04±23.10[a]
T4	29.54±0.14[a]	5.00±0.00[a]	7.96±0.06[a]	0.27±0.01[b]	0.42±0.02[b]	29.29±2.00[b]	244.14±19.37[a]	15.99±1.75[a]	202.43±24.65[a]
T5	29.66±0.12[a]	5.00±0.00[a]	7.95±0.05[a]	0.28±0.02[b]	0.41±0.02[b]	29.67±1.62[b]	239.24±18.32[a]	16.11±1.70[a]	198.29±23.18[a]
T6	29.39±0.13[a]	5.00±0.00[a]	7.97±0.04[a]	0.26±0.02[b]	0.39±0.02[b]	29.57±1.45[b]	242.57±19.39[a]	16.31±1.69[a]	198.14±25.08[a]

Different superscript letters in the same column are significantly different ($P \leq 0.05$).

2. The growth and survival of shrimp

Table (2) shows juvenile growth and survival. Probiotic-supplemented diets with biofloc conditions significantly ($P \leq 0.05$) improved shrimp growth compared to CW and non-probiotic groups. All BFT and BFT-enriched groups had significantly higher survival % than the control.

Table 2. Growth and SR% of shrimp fed probiotics-enriched diets for 90 days with different culture systems

	W_2	WG	SGR	SR
T1	9.67±0.20[b]	5.63±0.28[b]	1.04±0.06[b]	66.98±3.39[b]
T2	11.48±0.12[a]	7.58±0.07[a]	1.29±0.003[a]	90.21±0.95[a]
T3	11.48±0.25[a]	7.37±0.16[a]	1.22±0.12[a]	87.80±1.93[a]
T4	11.44±0.22[a]	7.41±0.35[a]	1.24±0.06[a]	88.22±4.16[a]
T5	11.43±0.16[a]	7.38±0.19[a]	1.23±0.03[a]	87.90±2.27[a]
T6	11.28±0.21[a]	7.34±0.20[a]	1.23± 0.02[a]	87.35±2.39[a]

Different superscript letters in the same column are significantly different ($P \leq 0.05$).

Diets containing probiotics PondToss® or LactéolForte® responded similarly in terms of growth performance. Shrimp survival % ranged from 66.98 to 90.2%. Moreover, shrimp reared in CW had the lowest survival rate %. No significant differences in shrimps' survival % were detected among BFT (T2) and BFT-administered probiotics (T3, T4, T5, and T6). Additionally, shrimps fed probiotic-supplemented diets consumed more feed than the control diet. The highest feed intake value (11.83g) was observed in T5: BFT+ LactéolFort® at 1g Kg^{-1}, while the lowest value (11.16g) was recorded for T3 (Table 3). Since the feed intake, FCR, PER, PPV, and EU are common measurements for calculating the efficiency of utilizing nutrients, data also showed significant ($P \leq 0.05$) differences among various treatments (Table 3).

Table 3. Feed and protein utilization parameters of *L. vannam*ei fed probiotics-enriched diets for 90 days under different culture systems

	FI (g)	FCR	PER	EU (%)	PPV (%)
T1	11.5±0.29^{ab}	2.05±0.13^{a}	1.34±0.09^{b}	20.973±1.67^{b}	33.35±1.73^{c}
T2	11.83±0.17^{ab}	1.56±0.04^{b}	1.74±0.04^{a}	28.660±0.38^{a}	44.45±0.58^{ab}
T3	11.16±0.16^{b}	1.52±0.04^{b}	1.79±0.04^{a}	30.260±0.51^{a}	47.30±0.63^{a}
T4	12.00±0.29^{ab}	1.63±0.09^{b}	1.68±0.08^{a}	28.093±0.65^{a}	43.70±0.99^{b}
T5	12.17±0.44^{a}	1.64±0.03^{b}	1.65±0.03^{a}	28.077±0.73^{a}	43.67±1.11^{b}
T6	11.83±0.17^{ab}	1.61±0.04^{b}	1.68±0.04^{a}	28.663±0.62^{a}	44.81±0.96^{ab}

Different superscript letters in the same column are significantly different ($P \leq 0.05$).

3. Biochemical indices

Total protein, albumin, and globulins levels (mg/L) were significantly higher in all BFT and probiotic-enriched BFT groups when compared to the CW group. T6 (BFT+ LactéolFort®) had the highest values, followed in a descending order by T5, T4, and T3, when compared to T2 (BFT without probiotic addition).

Table 5. Biochemical variables of *L. vannamei* fed probiotics-enriched diets for 90 days under different culture systems

	Total protein (mg/L)	Albumin (mg/L)	Globulin (mg/L)
T1	5.73±0.067^{d}	3.23±0.033^{c}	2.50± 0.058^{c}
T2	6.93±0.120^{c}	3.87±0.033^{b}	3.07±0.088^{b}
T3	7.23±0.067^{b}	4.00±0.058^{b}	3.23±0.033^{ab}
T4	7.20±0.058^{bc}	3.90±0.058^{b}	3.30±0.100^{a}
T5	7.30±0.100^{b}	4.03±0.089^{b}	3.27±0.033^{ab}
T6	7.70±0.100^{a}	4.27±0.089^{a}	3.43±0.033^{a}

Different superscript letters in the same column are significantly different ($P \leq 0.05$).

DISCUSSION

The continuous aeration in experimental CW, BFT and BFT probiotic-supplemented aquaria was responsible for the adequate amounts of dissolved oxygen levels found in this study, which were within the permissible ranges required for shrimp survival and growth. The concentrations of water DO, and estimated values of water dissolved nitrogenous compounds (ammonia, nitrite, and nitrate) were within the recommended limits for the Pacific shrimp, *L. vannamei*. Studies evaluating water quality in BFT revealed reduced water ammonia and nitrite levels in biofloc compared to clear water **(Mabroke *et al*., 2021; Ogello *et al*., 2021;; Hassan *et al*., 2022a; Tahoun, 2022; Omran *et al.*, 2024)**. The much better water quality in all biofloc treatments compared to the clear water group in this experiment confirms heterotrophic bacteria's absorption of nitrogen, with the majority of the ammonia in the culture system being absorbed. Concerning the nitrogenous constituents in BFT groups, it should be noted that they were suitable for shrimp culture **(Cardona *et al*., 2016)**, and were within acceptable concentrations **(Hassan *et al*., 2022b)**. The observed FV and TSS values in our study were also within the recommended range and consistent with the findings of **Tahoun (2022)**, who investigated the effects of probiotics in different systems (CW and BFT). PondToss® probiotics have shown promising effects in sustaining good water quality for shrimp pond farming. They can efficiently minimize nitrogen wastes, resulting in a cleaner aquatic habitat for shrimp. Probiotics help to decompose organic waste, maintain the ecosystem's balance in the pond, and regulate the nitrogen cycle. Several studies **(Hai, 2015)** have found that supplementing shrimp feed with *Lactobacillus* probiotics can eliminate harmful nitrogenous effluents which influence the culture water environment.

According to **Li *et al*. (2020)**, adding *Lactobacillus* probiotics to the water decreased the ammonia content and improved overall water quality in a shrimp farm. The probiotics (*Lactobacillus*) have been shown to improve water quality metrics in shrimp cultivation systems. Similarly, adding probiotics to the water reduced ammonia concentrations, improved the overall water quality, and decreased ammonia and nitrite levels in culture water **(Soto, 2017; Li *et al*., 2020; Tahoun, 2022; Omran *et al*., 2024)**. Moreover, the findings of our study were also confirmed by the work of **Jena *et al*. (2023)**, who showed how adding probiotic *Bacillus* and *Lactobacillus* spp. improved the water quality in shrimp aquaculture **(Prasertphan *et al*., 2019; Panigrahi *et al*., 2020)**.

Many studies have shown that biofloc and probiotics improved shrimp growth, feeding efficiency through improved digestive enzyme activities, and nutrient absorption **(Karimi *et al*., 2019; Mabroke *et al*., 2021; Hien *et al*. 2022, Tahoun, 2022; Muthu *et al*., 2024; Noman *et al*., 2024; Omran *et al*., 2024)**. The probiotics *L. plantarum* and *L. acidophilus* have been found to promote faster growth and higher survival rates in various shrimp species. Species such as *B. subtilis* and *L. acidophilus* positively affected the growth of shrimp species **(Chen *et al*., 2022)**. Similar studies indicated that probiotics improved the growth of shrimp by increasing their survival and weight gain **(Kim *et al*., 2015; Lee *et al*., 2022)**. The mechanisms behind these effects include improved nutrient digestion, absorption, and utilization, leading to better growth outcomes **(Karimi *et al*., 2019; Kesselring *et al*., 2019; Xie *et al*., 2019; Vivek *et al*., 2023; Muthu *et al*., 2024; Noman *et al*., 2024)**. Poly-hydroxybutyrate produced by heterotrophic bacteria has been shown to have antibacterial properties that help enhance disease resistance in the biofloc system **(Emerenciano *et al*., 2013; Raza *et al*., 2024)**. The improved survival % in probiotics-fortified diets in the current study was verified by **Hien *et al*. (2022)**, who provided shrimp diets treated with Pro-A, a probiotics mixture including a blend of *Bacillus* sp. and reported fewer mortalities.

In BFT, heterotrophic bacteria are abundant, recycling nutrients and effluents into microbial protein. Shrimp may graze on this protein-rich feed source for the entire day **(Omran *et al*., 2024)**. **Wang *et al*. (2019)** experimented on *M. japonicus* and found that *Bacillus* probiotics led to better nutrient utilization via improved digestive enzyme activities. **Li *et al*. (2020)** found that probiotics improved the nutritional efficiency of shrimp by increasing the absorption of nutrients and reducing the excretion of nitrogen and phosphorus. Comparable results were confirmed by our work on dietary probiotics PondToss® and LactéolFort® administered to shrimp for supporting shrimp growth which was considerably enhanced in the BFT and BFT enriched probiotics compared to control groups. The significant differences in growth and survival rates between BFT and BFT+ probiotics-enriched group and the control (CW+ no probiotic) treatment shows the benefits of probiotics and efficient BFT in maintaining stable water quality, reducing nutrient requirements, serving as a reliable source of nutrient for shrimp, and lowering feed protein needs **(Tacon *et al*., 2002; Tahoun, 2022; Amiin *et al*., 2023; Arshad *et al*., 2023)**.

According to **Abdel-Tawwab *et al*. (2021, 2022)**, biochemical analyses are essential for evaluating fish health and nutrition and their capacity for environmental adaptation. It was discovered in this study that adding probiotics (PondToss® and LactéolFort®) to shrimp diets greatly improved total protein. The total protein content may be a crucial indicator of the humoral defense system, in addition to serving as a general indicator for all metabolites, stress hormones, and protective enzymes in the body's critical fluid **(Shiry *et al*., 2019)**. **Proespraiwong *et al*. (2023)** reported that adding probiotics (PondToss® and LactéolFort®) to the diet significantly enhanced TP, ALB, and GLO levels.

CONCLUSION

It is believed that oral administration of exogenous microbes enhances shrimp growth performance. The whiteleg shrimp *L. vannamei* cultivated in a biofloc system and fed probiotics PondToss® and LactéolForte® at a rate of 1g kg^{-1} diet outperformed the control group in terms of growth rates. Probiotics and the probiotic-enriched biofloc system have been proposed as shrimp-friendly diets that improve water quality, growth, nutrition utilization, and biochemical and protein responses.

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