water

MDPI

Article

Evaluation of Biofloc-Based Probiotic Isolates on Growth Performance and Physiological Responses in *Litopenaeus vannamei*

Meenakshisundaram Menaga [1], Perepi Rajasulochana [2,†], Sugantham Felix [3], Shanmugam Sudarshan [1], Ashish Kapoor [4], Kumaraswamy Gandla [5], Moustafa M. Saleh [6], Adel Ehab Ibrahim [7] and Sami El Deeb [8,9,*,†]

1 Department of Aquatic Environment Management, Dr. MGR Fisheries College and Research Institute, Vettar River View Campus, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Nagapattinam 611 002, Tamil Nadu, India; menaga@tnfu.ac.in (M.M.); sudars30@gmail.com (S.S.)
2 Department of Microbiology, Saveetha Medical College and Hospitals, SIMATS, Saveetha University, Chennai 602 105, Tamil Nadu, India; rajasulochanap.smc@saveetha.com
3 Department of Aquaculture, Vettar River View Campus, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Nagapattinam 611 002, Tamil Nadu, India; felixfisheries@gmail.com
4 Department of Chemical Engineering, Harcourt Butler Technical University, Kanpur 208 002, Uttar Pradesh, India; ashishkapoorchem@gmail.com
5 Department of Pharmaceutical Analysis, Chaitanya (Deemed to Be University), Hanamkonda 522 302, Telangana, India; drkumaraswamygandla@gmail.com
6 Microbiology and Immunology Department, Faculty of Pharmacy, Port-Said University, Port Said 42511, Egypt; mostafa.mohamed@pharm.psu.edu.eg
7 Natural and Medical Sciences Research Center, University of Nizwa, P.O. Box 33, Birkat Al Mauz, Nizwa 616, Oman; adel@unizwa.edu.om
8 Institute of Medicinal and Pharmaceutical Chemistry, Technische Universitaet Braunschweig, 38106 Braunschweig, Germany
9 Institute of Pharmacy, Freie Universität Berlin, Königin-Luise-Str. 2+4, 14195 Berlin, Germany
* Correspondence: s.eldeeb@tu-braunschweig.de
† These authors contributed equally to this work.

check for updates

Citation: Menaga, M.; Rajasulochana, P.; Felix, S.; Sudarshan, S.; Kapoor, A.; Gandla, K.; Saleh, M.M.; Ibrahim, A.E.; El Deeb, S. Evaluation of Biofloc-Based Probiotic Isolates on Growth Performance and Physiological Responses in *Litopenaeus vannamei*. *Water* **2023**, *15*, 3010. https://doi.org/10.3390/w15163010

Academic Editor: Kuntong Jia

Received: 14 June 2023
Revised: 8 August 2023
Accepted: 11 August 2023
Published: 21 August 2023

Abstract: A comparison of the growth performance of *Penaeus vannamei* was ascertained by supplementing the potential probiotics isolated from a biofloc system incorporated through feed. Post-larvae shrimp (0.045 ± 0.005 g) were stocked at a density of 500/m^3 in FRP tanks (500 L) in triplicates for a period of 60 days. A total of 40 bacterial strains were isolated from previous biofloc culture trials and tested for their antimicrobial activity against the pathogen *Vibrio parahaemolyticus*. Among these, *Bacillus megaterium*, *Exiguobacterium profundum*, *Pseudomonas balearica*, and *Pseudomonas stutzeri* showed higher antimicrobial activity. The treatment groups included clear water with no probiotics (CW), clear water + isolated probiotic (CW + IP), biofloc alone (BFT), and biofloc + isolated probiotic (BFT + IP), in triplicates. Distillery spent wash was used as a carbon source for biofloc development and maintenance. A probiotic concentration of 1×10^9 cfu/g was supplemented throughout the trial. The recorded water quality parameters (pH, alkalinity, calcium, and magnesium) were observed to be significant among the experimental groups ($p \leq 0.05$). The highest weight gain (2.43 g), SGR, PER, and lower FCR values were recorded in BFT + IP. The lowest values of total *Vibrio* were found in BFT. The histology analysis revealed that there was a mild increase in the B and R cell vacuoles in the hepatopancreas of CW and BFT + IP, whereas mild degeneration was found in the intestine of CW and CW + IP. Microbiome analysis of the shrimp gut revealed that *Proteobacteria* was the most abundant phylum in all experimental groups. *P. balearica*, *K. pneumoniae*, *P. stutzeri*, and *E. profundum* were present in the gut of C, whereas *P. balearica*, *K. pneumonia*, and *P. stutzeri* were present in the gut of CW + IP and BFT + IP. The results proved that the probiotics isolated from biofloc colonized in shrimp gut could play a promising role in aquaculture.

Keywords: aquaculture; shrimp; biofloc; colonies; probiotics

1. Introduction

The aquaculture industry, a fast-growing sector, meets the demand of the growing population to balance the decline in capture fisheries. Globally, India is the second largest country in aquaculture production [1], and shrimp farming has gained positive attention due to its export potential compared to other aquatic species. In recent times, Litopenaeus vannamei has attracted farmers with its characteristics of fast growth, resistance to native diseases, availability, and tolerance to a wide salinity range [2]. Despite the prospects, this industry faces hindrances related to the availability of natural resources, feed raw materials, and disease outbreaks.

Deterioration of water quality paves the way for disease outbreak, which affects production and productivity. In addition to other interventions to overcome the issue, biofloc technology, which involves zero or limited water exchange technology, is gaining momentum. Biofloc is an aggregate of biotic and abiotic factors, which in turn acts as a feed to the culture animal [3]. Biofloc, with various advantages, has also been reported with the presence of bacteria that can induce a probiotic effect to the culture animal internally or externally against *Vibrio* sp. and ectoparasites [4]. Kuhn et al. [5] explained that the healthiest shrimp and their performance in the system were associated with various algae and bacteria. The usage of antibiotics, prebiotics, and probiotics in aquaculture, particularly in shrimp farming, has widely increased for proper growth and maintenance [6]. However, antibiotic use in aquaculture has now been restricted as the pathogenic bacteria have become resistant toward the numerous antibiotics available in the market [7]. The use of antibiotics has been replaced by the use of prebiotics and probiotics due to their inefficiency in treating bacterial diseases [8]. The function of probiotics in aquaculture for disease prevention and nutritional enhancement has been reported by several investigators [9–11]. Probiotics act on the principle of rapid multiplication, thereby reducing the levels of pathogenic bacteria. Probiotics supplemented through feed act by rapidly multiplying in the intestine, thereby reducing the infections caused by the pathogens. The supplementation of probiotics in the feed will also help in improving the organ development and intestinal equilibrium of the culture animal [12]. Numerous authors have documented the positive effects of probiotics on growth and survival in various studies. These effects are attributed to their ability to enhance nutrient digestibility and absorption, combat Vibrio pathogens, and stimulate immune components [13–18]. In the realm of shrimp aquaculture, several commercial probiotic products are readily available, with a focus on multi-species bacterial preparations, notably lactic acid bacteria (LAB). According to Ninawe and Selvin [19], probiotics are restricted because of their shelf life and sustainability across the entire culture cycle. In contrast, Hauville et al. [20] claimed a higher efficiency of multi-strain probiotics than single-strain probiotics in aquaculture.

Several studies have been reported using *Bacillus* as a mode of probiotic biofloc to improve shrimp growth and immune response [18–20]. As biofloc has proven its various advantages, the isolation of bacteria from biofloc culture water has also been a major challenge. Very few research projects have been carried out on isolating probiotic bacteria from biofloc culture systems [21]. Studies on isolating probiotic bacteria from biofloc systems and microbial characterization of the intestine are less common. Therefore, the proposed study is aimed at proving the advantage of using isolated probiotic strains from a biofloc culture system and its application in *L. vannamei* culture.

2. Materials and Methods

2.1. Biofloc Production and Isolation of Probiotic Bacteria

According to Nyan Taw [22], one month was required to maintain the biofloc in 50 ton raceways (DSW) obtained from MS Biosolutions. Coimbatore was used as source of carbon to maintain the C:N ratio at 10:1 [23]. The bacterial isolates were derived from biofloc culture systems and screened for probiotic potential by checking antimicrobial activity against the common pathogen *Vibrio parahaemolyticus* (ATCC® 17802™). Isolates from shrimp were procured from the State Referral Lab for Aquatic Animal Health, TNJFU,

Chennai. Morphological and biochemical characterization confirmed the bacterial species as a probiotic according to Bergey's Manual of Determinative Bacteriology [24]. The zone of inhibition criterion was utilized for selecting the bacterial strains for further testing.

2.2. Molecular Confirmation of Identified Probiotic Bacterial Isolates

The genomic DNA isolation from probiotic bacteria was carried out using the phenol–chloroform method. PCR amplification was carried out using the universal forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-CGGTTACCTTGT TACGACTT-3′) [24]. The PCR was performed using the standard protocol. The products of PCR were sequenced and confirmed through BLAST analysis. Once the sequence was confirmed, it was submitted to the NCBI GenBank, and the related numbers were obtained.

2.3. Medium Optimization

A Lark Innovative, Chennai brand in situ fermenter was used, to which we added various sources of carbon at the rate of 1%. It was inoculated with probiotic strains in nutrient broth supplemented with DSW under aerobic conditions. The most suitable carbon sources were identified to detect the cell growth and dry weight of probiotic strains. The probiotic strains were further inoculated in 0.5, 1, 2, 4, and 8% DSW to identify the optimal carbon percentage. A bioreactor of 5 L volume was used to incubate the bacteria consortia. The bioreactor in this experiment had a microporous tube at the bottom receiving vigorous aeration.

To sterilize the water, sodium hypochlorite solution was employed after filtering it through microns. For 36 h, these probiotics were incubated at 30 °C. The viability of different bacteria species was attained at 1×10^9 CFU/mL under these ideal conditions.

Water (33–35 °C) was sterilized with sodium hypochlorite, filtered through a 5-micron filter, and then neutralized with sodium thiosulfate after 12 h. For 36 h, the probiotics were incubated at 30 °C. The viable heterotrophic bacteria reached 1×10^9 CFU/mL under these circumstances.

2.4. Experimental Design

Post-larvae *Litopenaeus vannamei* (0.045 ± 0.005 g) were stocked at 500/m^3 density in triplicate in 500 L FRP tanks for a period of 60 days. The treatment groups included clear water with no probiotics (CW), clear water + isolated probiotic (CW + IP), biofloc alone (BFT), and biofloc + isolated probiotic (BFT + IP), in triplicates. In the biofloc tanks, the carbon-to-nitrogen ratio was maintained at 10:1 [25], using DSW as a carbon source.

The isolated potential probiotics grown in 0.5% DSW as a carbon source were supplemented in the feed. The isolated probiotics were supplemented at the concentration of 1×10^9 cfu/g once a week throughout the trial period following the method of Kesselring et al. (2019) [25]. The experimental feeds were incorporated with 35% crude protein content. Feed was prepared, once every five days, to ensure the maximum survival of probiotics in the experimental diets.

2.5. Water Quality Parameters

Physicochemical parameters such as temperature, pH, dissolved oxygen, alkalinity, ammonia (NH_4-N), nitrite (N-NO_2), calcium, and magnesium ion concentrations were measured on a weekly basis, according to APHA [26].

2.6. Growth Parameters

Different parameters were calculated including weight gain, feed conversion ratio (FCR), specific growth rate (SGR) per day, protein efficiency ratio (PER), and survival percentage using the below formulae.

$$\text{Weight gain} = \text{Wt} - \text{W}_0 \quad (1)$$

$$\left(\text{SGR}, \%\ \text{day}^{-1}\right) = \frac{[\ln W_t - \ln W_0]}{t} \times 100 \quad (2)$$

where W_0 and W_t are the initial weight and final weight (in grams) of shrimp, and t is the culture time in days.

$$\text{FCR} = \frac{\text{Feed intake}}{\text{Weight gain}} \quad (3)$$

$$\text{PER} = \frac{\text{Weight gain}}{\text{Amount of feed given} \times \text{Protein content in feed}} \quad (4)$$

$$\text{Survival}(\%) = \frac{\text{Total number of shrimp harvested}}{\text{Total number of shrimp stocked}} \times 100 \quad (5)$$

2.6.1. Total Vibrio Count

The total Vibrio count in the water samples of experimental tanks was conducted on a weekly basis by plating on thiosulfate–citrate–bile salts–sucrose (TCBS) agar, Hi Media Laboratories, India, at room temperature for 24 h, and this was expressed as CFU.

2.6.2. Histological Analysis

The histological analysis was performed in the Department of Pathology, Madras Veterinary College, Chennai for the hepatopancreas and intestine of *P. vannamei* from various treatments [27].

2.6.3. Shrimp Gut Microbiome Analysis

In our research experiment, we analyzed the gut of each experimental animal from each tank and then analyzed the microbiome. Prior to sample collection, the feeding of the experimental animals was stopped before 24 h. To isolate the DNA samples, we used the EXpure Microbial DNA kit. Then, using this DNA, PCR amplification was carried out. A Qubit Fluorometer 3.0 instrument was used to determine the quality and quantity of PCR products. On the basis of the nanopore sequencing and 16s sequences using the NCBI DATABASE, we classified the sequences in terms of percent coverage and identity [28].

2.6.4. Statistical Analysis of Experiment

Results are presented as the mean and standard deviation (SD), and data were analyzed using one-way ANOVA, followed by a Duncan test, with the help of SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). We compared each dataset to the relevant control. The differences were deemed significant at $p = 0.05$.

3. Results

3.1. Molecular Confirmation

Out of 40 bacterial isolates screened on the basis of the zone of inhibition, the following were found to possess probiotic properties: *Bacillus megaterium* (577 bp)—MH424904, *Pseudomonas balearica* (736 bp)—MH997474, *Exiguobacterium profundum* (1001 bp)—MH424898, and *Pseudomonas stutzeri* (611 bp)—MK332605.

3.2. Medium Optimization

The growth performance of probiotic strains under various carbon sources is given in Figure 1. DSW was found to be an ideal carbon source when compared with the other commercially available carbon sources. All probiotic isolates showed good performance in terms of animal growth and dry weight of the cell when inoculated with 0.5% distillery spent wash. The OD value at 600 nm was observed, and the wet weight and dry weight of the cell are shown in Figure 2.

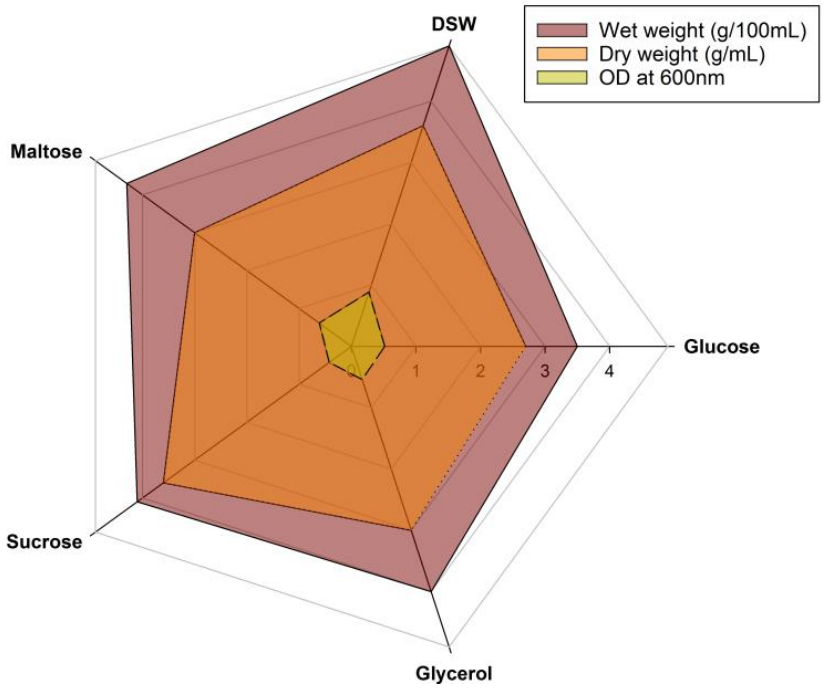

Figure 1. Growth performance of probiotic strains under various carbon sources.

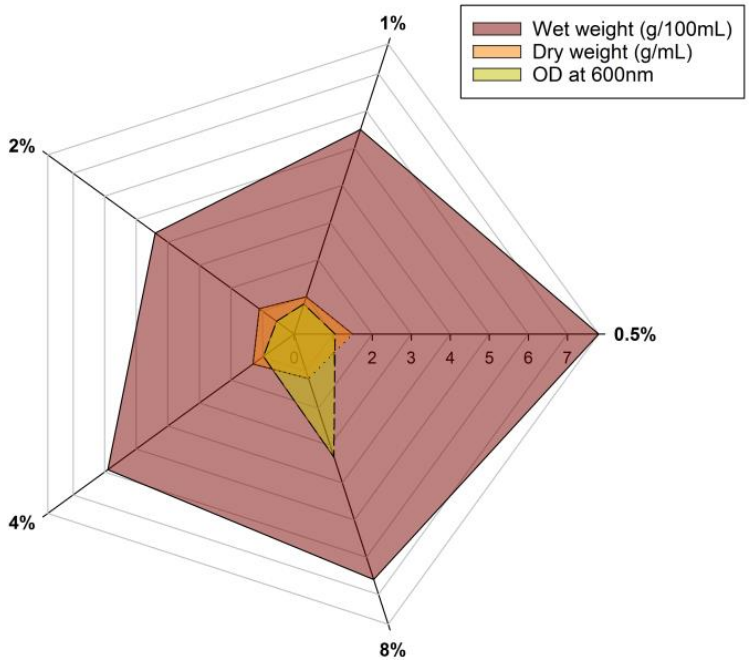

Figure 2. Growth performance of probiotic strains with various concentrations of DSW.

3.3. Water Quality Parameters

3.3.1. Parameters of Water

Various water quality parameters were recorded during the culture trial. The parameters included pH and alkalinity, and a significant difference was observed in calcium and magnesium among the treatments. There was no significant difference in water temperature among the various treatments, as presented in Table 1.

Table 1. Comparison of water quality parameters recorded during the experimental trial ($p > 0.05$).

Parameters	CW	CW + IP	BFT	BFT + IP
pH	7.41 ± 0.93^{a} (6.85–8.34)	7.76 ± 0.76^{b} (7.00–8.52)	7.82 ± 0.69^{c} (6.46–8.51)	7.50 ± 1.25^{d} (6.72–8.75)
Temperature (°C)	30 ± 0.5 (29.9–30.3)	30 ± 0.5 (29.9–30.5)	30 ± 0.5 (29.9–30.1)	30 ± 0.5 (29.9–30.3)
Alkalinity(mg/L)	110 ± 5^{a} (80–118)	143 ± 7^{b} (80–152)	135 ± 9^{c} (84–176)	105 ± 13^{d} (76–110)
Nitrite (NO_2-N) (mg/L)	0.052 ± 0.017 (0.011–0.069)	0.02 ± 0.015^{a} (0.001–0.037)	0.028 ± 0.005 (0.002–0.045)	0.042 ± 0.015^{b} (0.004–0.062)
Ammonia (NH_3-N) (mg/L)	0.047 ± 0.017 (0.011–0.069)	0.052 ± 0.005 (0.001–0.065)	0.049 ± 0.005 (0.002–0.055)	0.050 ± 0.01 (0.004–0.062)
DO (mg/L)	4.91 ± 0.51 (4.10–5.64)	4.77 ± 0.27^{a} (4.32–5.43)	4.87 ± 0.83^{b} (4.56–5.32)	4.7 ± 0.8^{c} (4.33–5.21)
Calcium (mg/L)	360 ± 4^{a} (220–420)	196 ± 13^{b} (156–252)	190 ± 11^{c} (140–264)	181 ± 20^{d} (140–236)
Magnesium (mg/L)	40 ± 23^{a} (21.6–62.4)	48 ± 14^{b} (24–67.8)	38.7 ± 11^{c} (21.6–60)	35.2 ± 11^{d} (24–55.2)

Notes: Values within the same row having different superscripts are significantly different—ANOVA and Kruskal-Wallis test ($p \leq 0.05$).

3.3.2. Growth Parameters

Various growth indices were observed during the culture period. A difference was observed in the experimental animals with respect to final weight, weight gain, PER, and SGR among treatments such as CW, BFT, and BFT + IP, and a significant difference in survival between CW and CW + IP was also recorded, as presented in Table 2.

Table 2. Growth parameters of *L. vannamei* reared under various treatment conditions ($p > 0.05$).

Treatment	Final Weight (g)	Weight Gain (g)	SGR *	FCR **	PER ***	Survival (%)
CW	1.03 ± 0.005^{a}	0.98 ± 0.004^{a}	5.21 ± 0.015^{a}	2.55 ± 0.05^{a}	1.3 ± 0.05	92^{a}
CW + IP	1.7 ± 0.007^{a}	1.65 ± 0.002	6.05 ± 0.012	1.51 ± 0.015	2.2 ± 0.02^{a}	95^{b}
BFT	2.25 ± 0.015^{b}	2.20 ± 0.006^{b}	6.52 ± 0.012^{b}	1.13 ± 0.01^{b}	2.9 ± 0.01^{b}	95
BFT + IP	2.47 ± 0.003^{c}	2.43 ± 0.005^{c}	6.67 ± 0.004^{c}	1.02 ± 0.012^{c}	3.24 ± 0.02^{c}	97

Notes: * SGR—specific growth rate; ** FCR—feed conversion ratio; *** PER—protein efficiency ratio. Values within the same row having different superscripts are significantly different—ANOVA and Tukey's test or Kruskal-Wallis test ($p \leq 0.05$).

3.3.3. Total Vibrio Count

To evaluate the various bacterial concentrations, we used the logarithm values of our experimental data. The total count of *Vibrio* sp. observed in the water sample of various treatments is listed in Figure 3. The population of *Vibrio* sp. gradually increased with the days of the culture period up to the 49th day and decreased afterward.

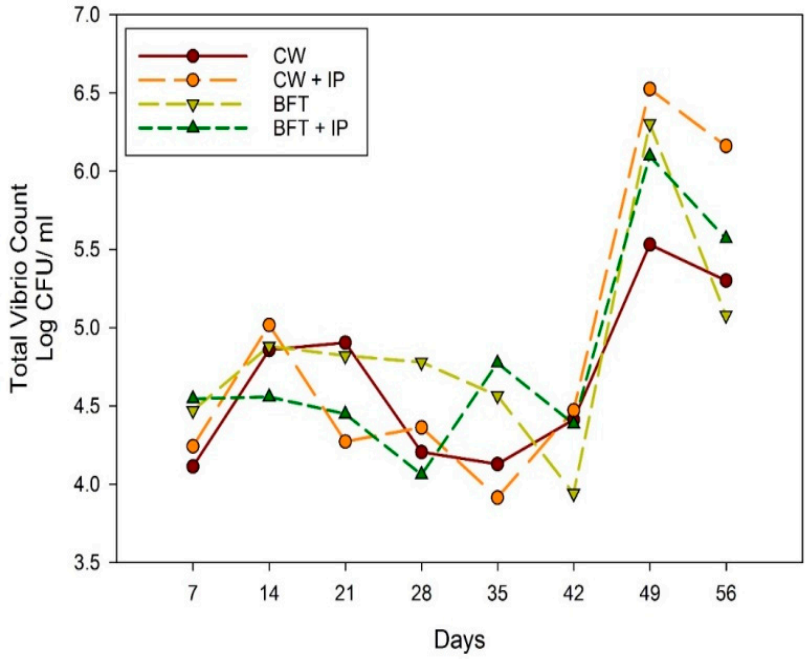

Figure 3. Periodic Vibrio count under various treatments (log CFU/mL).

3.4. Histological Analysis

Figures 4 and 5 show various histological changes in the shrimp intestine and hepatopancreas system reared under various treatments (10× magnification).

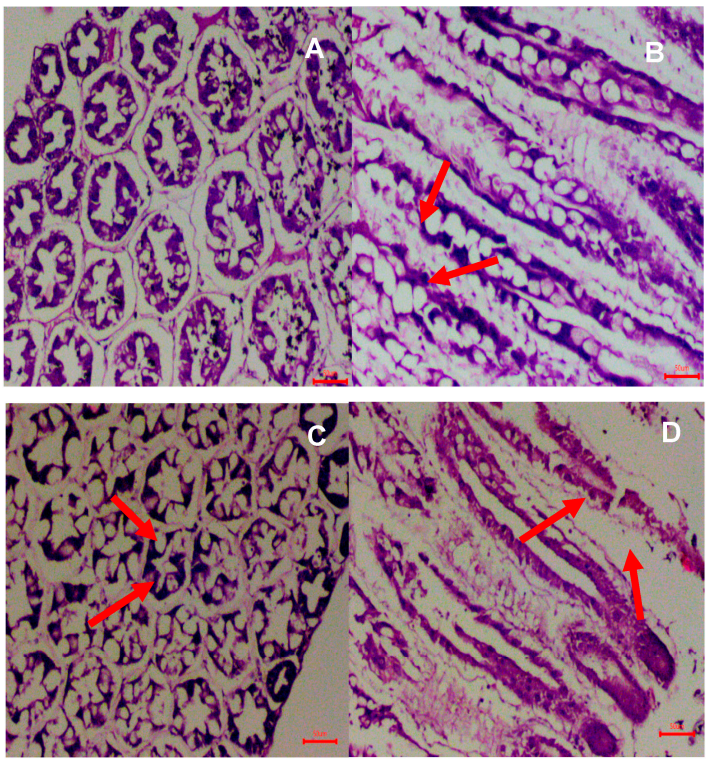

Figure 4. Histology of hepatopancreas: (**A**) no abnormality was found; (**B**) CW + IP—increased B and R cell vacuoles and relatively larger vacuoles were observed, along with mild degeneration of hepatopancreatic tubules; (**C**) BFT—mild degeneration of hepatopancreatic tubules; (**D**) BFT + IP—mild degeneration and mild increase in B and R cell vacuoles.

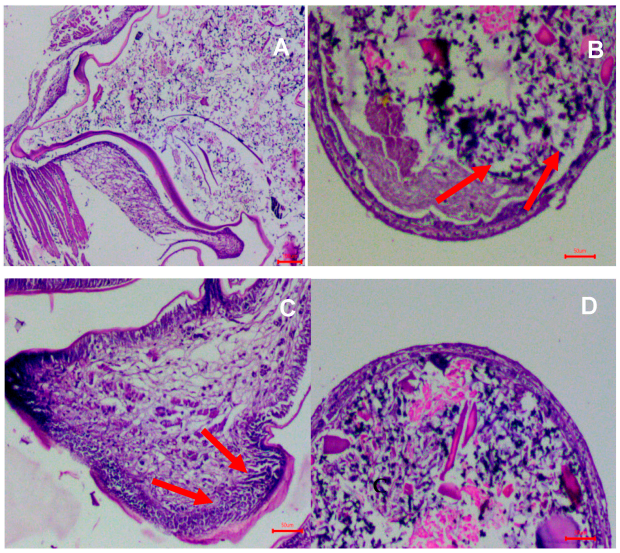

Figure 5. Histology of intestine: (**A**) no anomaly was seen in the CW; (**B**) CW + IP—mild degeneration was discovered, but other abnormalities such as hemocytic infiltration and lumen disintegration were absent; (**C**) BFT—mild epithelial mucosal layer thickening and degeneration; (**D**) IP + BFT—NAD.

3.5. Microbiome Content of Shrimp Gut

After the microbiome study of prawn guts raised under various treatments, CW had a classification of 59%, CW + IP had a classification of 64%, BFT had a classification of 77%, BFT + IP had a classification of 82%, and BFT alone had a classification of 18%. In our research and experiments, we identified *Proteobacteria* as the major phylum. The cumulative reads for *Proteobacteria* were as follows: CW-13250, CW + IP-801, BFT-5044, and BFT + IP-4961. The relative abundance of the microbiome is depicted in Figure 6.

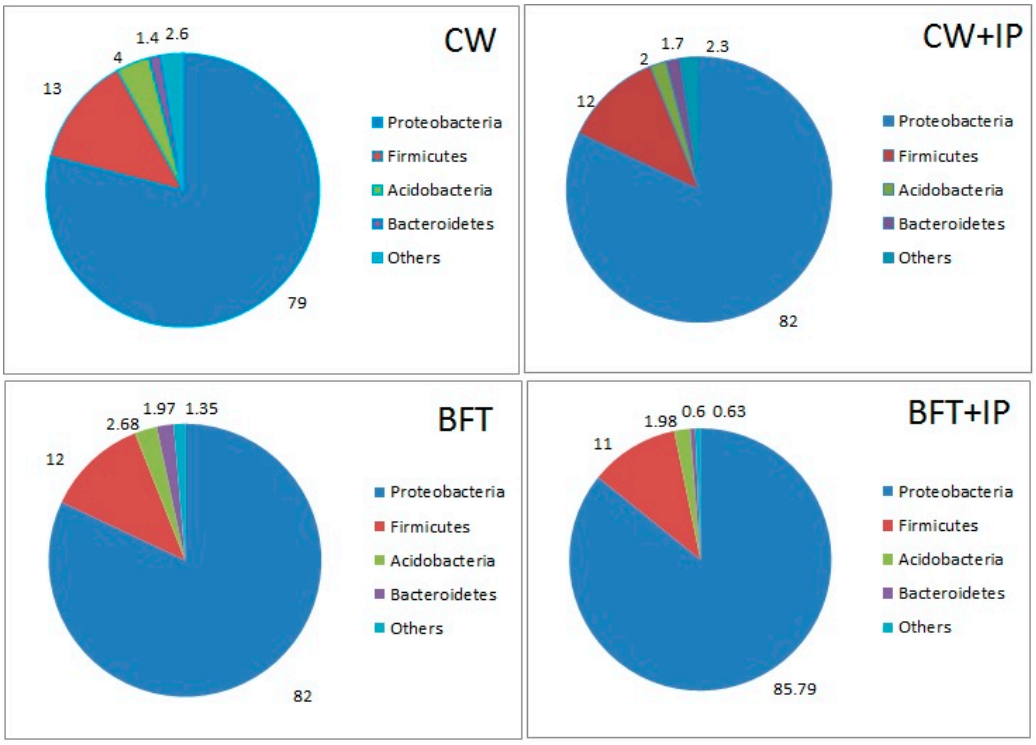

Figure 6. Gut microbial flora in shrimp reared under various treatments.

Pseudomonas stutzeri, *Pseudomonas balearica*, and *Exiguobacterium profundum* were detected at the species level, indicating that the isolated bacterial strains present in the CW also had the capacity to colonize prawn guts. Animals raised in CW + IP and BFT + IP were found to have supplemental *Pseudomonas stutzeri*, *Klebsiella pneumonia*, and *Pseudomonas balearica* in their guts. See the Supplementary Materials for the full results.

4. Discussion

In this study, we evaluated the probiotic bacteria isolated from the biofloc system and their colonization ability in the shrimp gut. Among the 40 bacterial strains, *Bacillus megaterium*, *Pseudomonas balearica*, *Exiguobacterium profundum*, and *Pseudomonas stutzeri* developed a major zone of inhibition against the tested pathogen *Vibrio parahaemolyticus*. The presence of these isolated strains in the biofloc culture systems at the genus level was previously reported [29,30]. Martínez Cruz et al. [31] described that microbes act as probiotics such as Pseudomonas and *Bacillus* sp. in an aquatic environment, helping to maintain the water quality and improve animal growth in the aquaculture environment. In biofloc systems of *L. vannamei*, Panigrahi et al. [32] identified *Exiguobacterium* sp. in the biofloc system. Kumar and Suresh [33] determined the extracellular protease activity of these potential probiotics in the biofloc system.

Further, these isolates were inoculated in medium supplemented with different carbon sources such as glycerol, as well as carbohydrates such as glucose, maltose, and sucrose substances. DSW was found to have a better growth performance.

Although the study used simple soluble carbon sources, there may have been major differences in the carbon utilization by the bacteria, and the benefits derived would have varied accordingly. Further dose optimization of the DSW revealed that an increase in the percentage supplementation of DSW showed no improvement in bacterial growth. Panigrahiet al. [34] reported that higher levels of carbon addition have no effect on stimulating the heterotrophic bacterial population. Yuniasari and Ekasari [35] and Krummenauer et al. [36] reported the importance of maintaining optimal levels of basic variables such as pH, DO, temperature, and salinity to maintain the quality of water in all experimental tanks. Adding a carbon source to the system can help to minimize the exchange of water in BFT and BFT +IP. Ebeling et al. [37] stated that a biofloc system including bacteria with a high metabolic rate can reduce pH and alkalinity in BFT + IP and BFT. This study mentioned these factors are reduced because of the inorganic carbon consumption by the various heterotrophic and probiotic nitrifying bacteria. Probiotic addition in the CW + IP treatment had no effect on the pH and alkalinity levels. Ammonia was very low in all treatments. Ebeling et al. [37] described ammonia assimilation in the CW + IP, BFT, and BFT + IP treatments by different bacteria present in the biofloc system, which helped to reduce the ammonia and nitrite concentrations in the water environment. Boyd [38] and Avnimelech [3] described the calcium (Ca) levels in various tanks, identifying that Ca levels were higher in CW because of $CaCO_3$ precipitation and its concentration in the water culture; sustaining the calcium ions in the water system may be a result of the micro-aggregates of the biofloc acids.

The levels of magnesium were lower in the case of BFT + IP than other treatments, which might be due to the higher levels of uptake by the culture animals compared to other treatments for their growth and survival. However, optimum concentrations of magnesium were maintained throughout the culture trial (10–30 mg/L) [38]. The highest weight gain with survival was found in the BFT + IP treatment, in which the isolated potential probiotic was supplemented in the feed along with the biofloc. Improved growth performance (weight gain, SGR, FCR, and PER) was found in both BFT and BFT + IP, compared to other treatments. This may have been due to the effect of rearing the animals in biofloc systems, as well as the supplementation of probiotics, which would have improved the equilibrium of intestinal activity.

The total Vibrio count was found at reduced levels in CW + IP, BFT, and BFT + IP. The results of the present study are in agreement with the findings of Aguilera-Rivera et al. [39] and

Sundaram et al. [40], who recorded a reduction in the population of *Vibrio* in the rearing system. Zhao et al. [41] also reported that inoculating *Bacillus* into BFT water resulted in a decrease in the population of total *Vibrio*. This may have been due to the fact that biofloc displayed bio-control activity against *Vibrio*, the virulence of which can be regulated by the process of quorum sensing. The study reiterated the fact that biofloc not only maintained the water quality but also helped in reducing the spread of harmful pathogens. Further, supplementation of potential probiotics through the feed helped in the plausible stimulation of heterotrophic bacteria, thereby reducing the levels of pathogens.

As a result of the histological examination, various types of cells were identified in the tissue of the hepatopancreatic tubules. The hepatopancreas sections showed significant changes between the control and the treatment groups. A normal hepatopancreas was observed in the control group. However, the hepatopancreas of the culture animals reared under various treatments showed degeneration and increased B and R cells, especially in the case of BFT + IP. In the experimental animals, the proximal tubule portion showed a greater quantity of ingested material by the animals, indicating a larger proportion of cells with intracellular digestion (B) and absorption (R). However, no such abnormalities were found in the shrimp's intestine in all experimental groups, except CW, where a positive thickening of the epithelial mucosal layer was found. Won et al. [27] also revealed thickening of the muscle layer of shrimp fed with isolated potential probiotics in shrimp culture [25].

According to Pilotto et al. [42], some phyla such as *Proteobacteria*, *Bacteroidetes*, *Acidobacteria*, and *Firmicute* were identified as important in the intestine of *L. vannamei.* This was also confirmed in our study. Pilotto et al. [42] stated that the microbiome digestive tract was influenced by the environment in which the animals were reared. *Proteobacteria* was the abundant taxonomic group at the phylum level, but differed in frequency distribution among the experimental groups, similar to the findings of Sha et al. [43]. This phylum is predominantly found in the marine environment, and it contributes to the nutrient and mineral processing of organic compounds [43]. The relative abundance of *Proteobacteria* (13,250, 82%) in CW may have been due to the nature of its abundance in biofloc, biofilms, and recirculation systems. Its ability to efficiently degrade complex organic compounds in biofloc systems plays a significant role in maintaining the system's ecological balance and nutrient cycling, which, in turn, sustains its growth and abundance within the microbial community. The lower abundance of this phylum in CW was due to regular water exchange. Firmicutes was present in CW at 12% (1955), CW + IP at 13% (133), BFT at 14.7% (923), and BFT + IP at 11% (643). *Acidobacteria* was present in CW at 2% (352), CW + IP at 4% (43), BFT at 3% (212), and BFT + IP at 1.98% (115). *Bacteriodetes* was present in CW at 1.7% (276), CW + IP at 1.4% (15), BFT at 0.85% (54), and BFT + IP at 0.6% (35). *Firmicutes*, *Acidobacteria*, and *Bacteriodetes* were the most abundant phyla with respect to relative abundance, along with *Proteobacteria*. Vargas-Albores et al. [44] found that biofloc bacterial communities were mainly *Planctomycetes*, *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*, whose various pathways are essential for nitrogenous and carbonaceous compound processing, similar to our experimental results. Wei et al. [45] reported that *Acidobacteria* associated with the biofloc system is involved in the ammonia assimilation process. Wobken [46] reviewed that *Bacteriodetes* is frequently found in colonizing macroscopic organic matter particles. These bacteria use organic and nitrogen compounds for their growth. These two parameters were satisfied in the BFT system, enabling the biofloc-associated bacteria to utilize the organic matter and attach to surfaces. The isolated probiotic bacteria were found in the CW gut, while we also observed species of *Pseudomonas* in BFT and BFT + IP, such as *P. balearica* and *P. stutzeri*, in the diets of shrimp.

Some probiotic bacteria such as *Bacillus megaterium* and *Exiguobacterium profudumin* were absent in the intestine walls due to a lower or lack of adherence capacity. The main feed was supplemented with isolated potential probiotics, which may have been due to the competitive inhibition among the various probiotics supplemented in the feed.

Vargas-Albores et al. [44] stated that *P. stutzeri* acts as a commercial probiotic, revealing its role in the CW, BFT, and BFT + IP guts. The biofloc system is more advantageous, as it is made of rich organic matter and stimulates the population of microbes, thereby acting as a source of nutrients to maintain the culture animals. According to Aguilera et al. [39], this system is activated through colonization by bacteria, phytoplankton, and zooplankton. Tzuc et al. [47] described that the animal intestine populated by microbes gains additional potential benefits toward growth and metabolism. In this research, we aimed to isolate the probiotic bacteria in the biofloc system, which support the development and rearing efficiency of animals in this system. This study proved that we could isolate potential probiotics from the biofloc system and use them to promote the growth and immune response of shrimp.

5. Conclusions

This study aimed to characterized the probiotics derived from biofloc and determine their efficacy. Distillery spent wash proved to be highly beneficial as it promoted the development of a diverse bacterial community within the biofloc environment. In addition to improved growth performance, the biofloc and isolated probiotic treatments also exhibited reduced levels of ammonia and a decreased abundance of Vibrio. Further, the biofloc treatments demonstrated an increase in B and R cells in the shrimp's hepatopancreas, indicating potential improvements in the shrimp's immune response and nutrient utilization efficiency. The high abundance of Proteobacteria observed in the biofloc system is particularly noteworthy, as these bacteria are known for their ability to consume organic compounds, which contributes to the overall nutrient cycling and bioremediation capacity of the biofloc system. Overall, this study contributes to advancing sustainable aquaculture practices, and the application of biofloc systems and probiotics may hold promising prospects for the future of shrimp farming.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w15163010/s1, Table S1: Morphological and biochemical characteristics of four different *Bacterial* sp. Isolated from the biofloc system; Table S2: Determination of antagonism of selected *Bacterial* sp., against shrimp pathogens (*Vibrio parahaemolyticus*).

Author Contributions: Conceptualization, M.M. and S.F.; methodology, P.R.; software, S.S.; validation, A.K., K.G. and M.M.S.; formal analysis, M.M.; investigation, S.F.; resources, P.R.; data curation, M.M.; writing—original draft preparation, A.E.I.; writing—review and editing, S.E.D.; visualization. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Data are available from the corresponding author upon request.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. FAO. *The State of World Fisheries and Aquaculture 2022*; Towards Blue Transformation; FAO: Rome, Italy, 2022.
2. Liao, I.C.; Chien, Y.H. The pacific white shrimp, *Litopenaeus vannamei*, in Asia: The world's most widely cultured alien crustacean. In *In the Wrong Place-Alien Marine Crustaceans: Distribution, Biology and Impacts*; Springer: Dordrecht, The Netherlands, 2011; pp. 489–519.
3. Avnimelech, Y. *Biofloc Technology: A Practical Guide Book*; World Aquaculture Society: Baton Rouge, LA, USA, 2009.
4. Lezama-Cervantes, C.; Paniagua-Michel, J. Effects of constructed microbial mats on water quality and performance of *Litopenaeus vannamei* post-larvae. *Aquac. Eng.* **2010**, *42*, 75–81. [CrossRef]
5. Kuhn, D.D.; Boardman, G.D.; Lawrence, A.L.; Marsh, L.; Flick, G.J., Jr. Microbial floc meal as a replacement ingredient for fish meal and soybean protein in shrimp feed. *Aquaculture* **2009**, *296*, 51–57. [CrossRef]
6. Van Hai, N.; Fotedar, R. A review of probiotics in shrimp aquaculture. *J. Appl. Aquac.* **2010**, *22*, 251–266. [CrossRef]
7. Defoirdt, T.; Halet, D.; Vervaeren, H.; Boon, N.; Van de Wiele, T.; Sorgeloos, P.; Bossier, P.; Verstraete, W. The bacterial storage compound poly-β-hydroxybutyrate protects *Artemia franciscana* from pathogenic *Vibrio campbellii*. *Environ. Microbiol.* **2007**, *9*, 445–452. [CrossRef] [PubMed]
8. Gatesoupe, F.J. The use of probiotics in aquaculture. *Aquaculture* **1999**, *180*, 147–165. [CrossRef]

9. Moriarty, D.J.W. Probiotics and bioremediation in aquaculture. *Asian Shrimp News* **1996**, *26*, 3.
10. Utiswannakul, P.; Sangchai, S.; Rengpipat, S. Enhanced growth of black tiger shrimp *Penaeus monodon* by dietary supplementation with Bacillus (BP11) as a probiotic. *J. Aquac. Res. Dev.* **2011**, *1*, 2. [CrossRef]
11. Verschuere, L.; Rombaut, G.; Sorgeloos, P.; Verstraete, W. Probiotic bacteria as biological control agents in aquaculture. *Microbiol. Mol. Biol. Rev.* **2000**, *64*, 655–671. [CrossRef]
12. Zokaeifar, H.; Babaei, N.; Saad, C.R.; Kamarudin, M.S.; Sijam, K.; Balcazar, J.L. 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.* **2014**, *36*, 68–74. [CrossRef]
13. Sha, Y.; Wang, L.; Liu, M.; Jiang, K.; Xin, F.; Wang, B. Effects of lactic acid bacteria and the corresponding supernatant on the survival, growth performance, immune response and disease resistance of *Litopenaeus vannamei*. *Aquaculture* **2016**, *452*, 28–36. [CrossRef]
14. Interaminense, J.A.; Vogeley, J.L.; Gouveia, C.K.; Portela, R.W.; Oliveira, J.P.; Andrade, H.A.; Peixoto, S.M.; Soares, R.B.; Buarque, D.S.; Bezerra, R.S. In vitro and in vivo potential probiotic activity of *Bacillus subtilis* and *Shewanella* algae for use in *Litopenaeus vannamei* rearing. *Aquaculture* **2018**, *488*, 114–122. [CrossRef]
15. Cai, Y.; Yuan, W.; Wang, S.; Guo, W.; Li, A.; Wu, Y.; Chen, X.; Ren, Z.; Zhou, Y. In vitro screening of putative probiotics and their dual beneficial effects: To white shrimp (*Litopenaeus vannamei*) postlarvae and to the rearing water. *Aquaculture* **2019**, *498*, 61–71. [CrossRef]
16. Tepaamorndech, S.; Chantarasakha, K.; Kingcha, Y.; Chaiyapechara, S.; Phromson, M.; Sriariyanun, M.; Kirschke, C.P.; Huang, L.; Visessanguan, W. Effects of *Bacillus aryabhattai* TBRC8450 on vibriosis resistance and immune enhancement in Pacific white shrimp, *Litopenaeus vannamei*. *Fish Shellfish Immunol.* **2019**, *86*, 4–13. [CrossRef] [PubMed]
17. Geier, M.S.; Butler, R.N.; Howarth, G.S. Inflammatory bowel disease: Current insights into pathogenesis and new therapeutic options; probiotics, prebiotics and synbiotics. *Int. J. Food Microbiol.* **2007**, *115*, 1–11. [CrossRef]
18. Donkor, O.N.; Nilmini, S.L.I.; Stolic, P.; Vasiljevic, T.; Shah, N.P. Survival and activity of selected probiotic organisms in set-type yoghurt during cold storage. *Int. Dairy J.* **2007**, *17*, 657–665. [CrossRef]
19. Ninawe, A.S.; Selvin, J. Probiotics in shrimp aquaculture: Avenues and challenges. *Crit. Rev. Microbiol.* **2009**, *35*, 43–66. [CrossRef]
20. Hauville, M.R.; Zambonino-Infante, J.L.; Gordon Bell, J.; Migaud, H.; Main, K.L. Effects of a mix of *Bacillus* sp. as a potential probiotic for Florida pompano, common snook and red drum larvae performances and digestive enzyme activities. *Aquac. Nutr.* **2016**, *22*, 51–60. [CrossRef]
21. Decamp, O.; Moriarty, D.J.; Lavens, P. Probiotics for shrimp larviculture: Review of field data from Asia and Latin America. *Aquac. Res.* **2008**, *39*, 334–338. [CrossRef]
22. Taw, N. *Intensive Shrimp Farming Systems in Asia: Commercial Implementation of Biosecure and Biofloc Production Systems to Help Control Shrimp Farming Diseases*; SPS Publishers: Hyderabad, India, 2018.
23. Tong, R.; Chen, W.; Pan, L.; Zhang, K. Effects of feeding level and C/N ratio on water quality, growth performance, immune and antioxidant status of *Litopenaeus vannamei* in zero–water exchange bioflocs-based outdoor soil culture ponds. *Fish Shellfish Immunol.* **2020**, *101*, 126–134. [CrossRef]
24. Schubert, R.H.; Buchanan, R.E.; Gibbons, N.E. Genus II *aeromonas*. In *Bergey's Manual of Determinative Bacteriology*, 8th ed.; Buchanan, R.E., Gibbons, N.E., Eds.; Williams and Wilkins: Philadelphia, PA, USA, 1974; p. 354.
25. Kesselring, J.C.; Gruber, C.; Standen, B.; Wein, S. Continuous and pulse-feeding application of multispecies probiotic bacteria in whiteleg shrimp, *Litopenaeus vannamei*. *J. World Aquac. Soc.* **2019**, *50*, 1123–1132. [CrossRef]
26. APHA (American Public Health Association). *Standard Methods for the Examination of Water and Wastewater*; American Public Health Association: Washington, DC, USA, 2008.
27. Won, S.; Hamidoghli, A.; Choi, W.; Bae, J.; Jang, W.J.; Lee, S.; Bai, S.C. Evaluation of Potential Probiotics *Bacillus subtilis* WB60, *Pediococcus pentosaceus*, and *Lactococcus lactis* on Growth Performance, Immune Response, Gut Histology and Immune-Related Genes in Whiteleg Shrimp, *Litopenaeus vannamei*. *Microorganisms* **2020**, *8*, 281. [CrossRef] [PubMed]
28. Edwards, U.; Rogall, T.; Blöcker, H.; Emde, M.; Böttger, E.C. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res.* **1989**, *7*, 7843–7853. [CrossRef]
29. Manan, H.; Moh, J.H.Z.; Kasan, N.A.; Suratman, S.; Ikhwanuddin, M. Identification of biofloc microscopic composition as the natural bioremediation in zero water exchange of Pacific white shrimp, *Penaeus vannamei*, culture in closed hatchery system. *Appl. Water Sci.* **2017**, *7*, 2437–2446. [CrossRef]
30. Pérez-Fuentes, J.A.; Pérez-Rostro, C.I.; Hernández-Vergara, M.P.; Monroy-Dosta, M.D.C. Variation of the bacterial composition of biofloc and the intestine of Nile tilapia *Oreochromis niloticus*, cultivated using biofloc technology, supplied different feed rations. *Aquac. Res.* **2018**, *49*, 3658–3668. [CrossRef]
31. Martínez Cruz, P.; Ibáñez, A.L.; Monroy Hermosillo, O.A.; Ramírez Saad, H.C. Use of probiotics in aquaculture. *Int. Sch. Res. Netw. ISRN Microbiol.* **2012**, *2012*, 916845. [CrossRef]
32. Panigrahi, A.; Esakkiraj, P.; Jayashree, S.; Saranya, C.; Das, R.R.; Sundaram, M. Colonization of enzymatic bacterial flora in biofloc grown shrimp *Penaeus vannamei* and evaluation of their beneficial effect. *Aquac. Int.* **2019**, *27*, 1835–1846. [CrossRef]
33. Kumar, P.A.; Suresh, P.V. Biodegradation of shrimp biowaste by marine *Exiguobacterium* sp. CFR26M and concomitant production of extracellular protease and antioxidant materials: Production and process optimization by response surface methodology. *Mar. Biotechnol.* **2014**, *16*, 202–218. [CrossRef]

34. Panigrahi, A.; Saranya, C.; Sundaram, M.; Kannan, S.V.; Das, R.R.; Kumar, R.S.; Rajesh, P.; Otta, S.K. Carbon: Nitrogen (C: N) ratio level variation influences microbial community of the system and growth as well as immunity of shrimp (*Litopenaeus vannamei*) in biofloc based culture system. *Fish Shellfish. Immunol.* **2018**, *81*, 329–337. [CrossRef]
35. Yuniasari, D.; Ekasari, J. Nursery culture performance of *Litopenaeus vannamei* with probiotics addition and different C/N Ratio under laboratory condition. *HAYATI J. Biosci.* **2010**, *17*, 115–119.
36. Krummenauer, D.; Peixoto, S.; Cavalli, R.O.; Poersch, L.H.; Wasielesky, W., Jr. Superintensive culture of white shrimp, *Litopenaeus vannamei*, in a biofloc technology system in southern Brazil at different stocking densities. *J. World Aquac. Soc.* **2011**, *42*, 726–733. [CrossRef]
37. Ebeling, J.M.; Welsh, C.F.; Rishel, K.L. Performance evaluation of the Hydrotech belt filter using coagulation/flocculation aids (alum/polymers) for the removal of suspended solids and phosphorus from intensive recirculating aquaculture microscreen backwash effluent. *Aquacult. Eng.* **2006**, *35*, 61–77. [CrossRef]
38. Boyd, C.E. Practical aspects of chemistry in pond aquaculture. *Progress. Fish-Cult.* **1997**, *59*, 85–93. [CrossRef]
39. Aguilera-Rivera, D.; Prieto-Davó, A.; Escalante, K.; Chávez, C.; Cuzon, G.; Gaxiola, G. Probiotic effect of FLOC on Vibrios in the pacific white shrimp *Litopenaeus vannamei*. *Aquaculture* **2014**, *424*, 215–219. [CrossRef]
40. Sundaram, M.; Panigrahi, A.; Ganesh, J.; Rekha, P.N.; Sivagnanam, S.; Rajamanickam, S.; Gopal, C. Evaluation of Different Probiotic Strains for Growth Performance and Immunomodulation in Pacific White Shrimp Penaeus vannamei Boone, 1931. *Indian J. Fish.* **2017**, *64*, 89–96. [CrossRef]
41. Zhao, P.; Huang, J.; Wang, X.H.; Song, X.L.; Yang, C.H.; Zhang, X.G.; Wang, G.C. The application of bioflocs technology in high-intensive, zero exchange farming systems of *Marsupenaeus japonicus*. *Aquaculture* **2012**, *354*, 97–106. [CrossRef]
42. Pilotto, M.R.; Goncalves, A.N.; Vieira, F.N.; Seifert, W.Q.; Bachère, E.; Rosa, R.D.; Perazzolo, L.M. Exploring the impact of the biofloc rearing system and an oral WSSV challenge on the intestinal bacteriome of *Litopenaeus vannamei*. *Microorganisms* **2018**, *6*, 83. [CrossRef]
43. Sha, Y.; Liu, M.; Wang, B.; Jiang, K.; Qi, C.; Wang, L. Bacterial population in intestines of *Litopenaeus vannamei* fed different probiotics or probiotic supernatant. *J. Microbiol. Biotechnol.* **2016**, *26*, 1736–1745. [CrossRef]
44. Vargas-Albores, F.; Porchas-Cornejo, M.A.; Martínez-Porchas, M.; Villalpando-Canchola, E.; Gollas-Galván, T.; Martínez-Córdova, L.R. Bacterial biota of shrimp intestine is significantly modified by the use of a probiotic mixture: A high throughput sequencing approach. *Helgol. Mar. Res.* **2017**, *71*, 5. [CrossRef]
45. Wei, Y.; Liao, S.A.; Wang, A.L. The effect of different carbon sources on the nutritional composition, microbial community and structure of bioflocs. *Aquaculture* **2016**, *465*, 88–93. [CrossRef]
46. Wöbken, D. Diversity and Ecology of Marine Planctomycetes. Doctoral Dissertation, Universität Bremen, Bremen, Germany, 2007.
47. Tzuc, J.T.; Escalante, D.R.; Herrera, R.R.; Cortés, G.G.; Ortiz, M.L.A. Microbiota from Litopenaeus vannamei: Digestive tract microbial community of Pacific white shrimp (*Litopenaeus vannamei*). *SpringerPlus* **2014**, *3*, 280. [CrossRef]