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Immune response and disease resistance of shrimp fed biofloc grown on different carbon sources

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ABSTRACT

The objective of this study was to document the immunological effects of growing shrimp in biofloc systems. The experiment consisted of four types of biofloc systems in which bioflocs were produced by daily supplementation of four different carbon sources, i.e. molasses, tapioca, tapioca-by-product, and rice bran, at an estimated C/N ratio of 15 and a control system without any organic carbon addition. Each biofloc system was stocked with Pacific white shrimp (*Litopenaeus vannamei*) juveniles that were reared for 49 days. The use of tapioca-by-product resulted in a higher survival (93%) of the shrimp as compared to the other carbon sources and the control. The highest yield and protein assimilation was observed when tapioca was used as the carbon source. After 49 days, phenoloxidase (PO) activity of the shrimp grown in all biofloc systems was higher than that of the shrimp from the control system. Following a challenge test by injection with infectious myonecrosis virus (IMNV), the levels of PO and respiratory burst (RB) activity in the shrimp of all biofloc treatments were higher than that of the challenged shrimp from the control treatment. An increased immunity was also suggested by the survival of the challenged shrimp from the experimental biofloc groups that was significantly higher as compared to the challenged shrimp from the control treatment, regardless of the organic carbon source used to grow the bioflocs. Overall, this study demonstrated that the application of biofloc technology may contribute to the robustness of cultured shrimp by immunostimulation and that this effect is independent of the type of carbon source used to grow the flocs.

1. Introduction

Disease remains a limiting factor for the aquaculture industry [1]. With respect to the shrimp culture industry, disease outbreaks have been the primary cause of production loss during the last two decades [1]. Disease outbreaks not only result from the mere presence of a pathogen in the system, a compromised health status of the cultured animals in combination with suboptimal environmental conditions are also factors facilitating disease outbreaks [2,3]. Therefore, disease prevention and control should not only focus on implementing biosecurity measures, but must be performed in an integral approach involving, among others, adequate nutrition, enhancing the immunity of the cultured animals and maintaining a good water quality.

Biofloc technology (BFT) has been studied at several occasions and contributes to the maintenance of good water quality in the system and to the nutrition of the cultured animals [4]. The basic principle of the biofloc system is to recycle waste nutrients, in particular nitrogen, into microbial biomass that can be used *in situ* by the cultured animals or be harvested and processed into feed ingredients [5–9]. Heterotrophic microbial aggregates are stimulated to grow by steering the C/N ratio in the water through the modification of the carbohydrate content in the feed or by the addition of an external carbon source [4], so that the bacteria can assimilate the waste ammonia for new biomass production. Biofloc systems have been shown not only to maintain ammonia below toxic levels and to improve the feed nutrient utilization efficiency of the cultured animals [4,9,10], but also to provide extra nutrients [11] and exogenous digestive enzymes [12]. Biofloc application can also lead to increased growth, survival and reproductive performance of the cultured animals [13,14].

So far, very few studies [15–18] investigated the immunological potential of the biofloc technology although it is widely known that

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microorganisms, their cell components or their metabolites can act as immunostimulants that enhance the shrimp innate immune system and provide improved protection against pathogens [19,20]. Xu and Pan [16] reported that the total haemocyte count and phagocytic activity of the haemocyte of the shrimp from biofloc containing culture units were significantly higher than those of the shrimp in the non-biofloc control group. Furthermore, the authors also noted that shrimp grown in a biofloc environment harbored a higher total antioxidant capacity both in the plasma and hepatopancreas. A recent study reported that the expression of six selected genes (prophenoloxidase [ProPO1 and ProPO2], serine protease [SP1], prophenoloxidase activating enzyme [PPAE1], masquerade-like serine protease [mas] and Rat-sarcoma-related nuclear protein), directly and indirectly related to the shrimp immune response, were significantly upregulated in biofloc-grown shrimp [17]. Immune stimulation may thus be a very important feature in biofloc-grown shrimp contributing to disease control. It could for example (partly) explain the lower prevalence of acute hepatopancreatic necrosis disease (AHPND) observed in farms that apply BFT [21]. AHPND is currently causing very large problems in the culture of shrimp post larvae in Asia [22].

The objective of this study was to perform a study on biofloc-grown shrimp. The water quality was monitored over a 49-day period in biofloc systems supplied with different organic carbon sources (molasses, tapioca, tapioca by-products, and rice bran). The shrimp growth performance, immune responses and resistance to the infectious myonecrosis virus (IMNV) were also verified. The results of this study provide information on the immunostimulatory nature of biofloc for shrimp and how this varies depending on the carbon source supplied.

2. Materials and methods

2.1. Experimental design

Twenty glass tanks (90 cm × 40 cm × 35 cm) filled with 100 L seawater were used as the experimental culture units. Temperature in all tanks was maintained in the range of 27.3–28.3 °C during the entire experiment, aeration was provided in each aquarium using an air blower and the light regime was set at 12 h light/12 h dark. Inter-molt phase Pacific white shrimp juveniles, previously acclimatized collectively to the experimental room and conditions for 1 week, at an initial average body weight of 2.02 ± 0.05 g were randomly distributed in the tanks at a density of 30 shrimp/tank (83 shrimp m^{-2}). Four times daily, a commercial pellet containing 30% of crude protein (Feng Li, PT Matahari Sakti, Indonesia) was provided for 49 days to all tanks. The feeding level was determined at 7% on wet body weight per day and the daily feed amount was adjusted to the biomass in the tanks.

The experiment consisted of five treatments (four replicate tanks per treatment): one control treatment without organic carbon addition and with a weekly water exchange of 50%, and four treatments with different organic carbon sources added for biofloc development (molasses, tapioca, tapioca by-products, and rice bran, respectively). Freshwater was regularly added only to make up for water loss due to evaporation. All organic carbon sources were locally purchased. Organic carbon was added daily two hours after feeding at an estimated C/N ratio of 15 [5]. Proximate composition and organic carbon content in the different types of organic carbon source were determined according to Takeuchi [23] and Walkley and Black [24] (Table 1).

Table 1
Proximate composition and total organic carbon content of molasses, tapioca, tapioca-by-product, and rice bran (all values, except moisture, are expressed as percentage on dry weight).

	Molasses	Tapioca	Tapioca-by-product	Rice bran
Moisture (%)	31.9	10.0	13.8	9.6
Ash (%)	5.9	0.3	0.6	7.4
Protein (%)	3.8	1.6	nd	6.6
Lipid (%)	0.4	nd	nd	9.9
Fibre (%)	nd	nd	7.9	13.3
Nitrogen free extract (%)	58.1	88.1	77.7	53.4
Organic carbon (%)	38.0	50.3	48.8	43.5

nd: not detectable.

2.2. Water quality

Temperature, dissolved oxygen (DO), pH, and salinity were daily measured *in situ* using a portable DO meter (Lutron DO-5519, Taiwan), pH meter (Lutron YK2001PH, Taiwan) and refractometer (ATAGO 2491-MASTER S, USA). Biochemical oxygen demand (BOD), alkalinity, dissolved inorganic nitrogen (total ammonium nitrogen, NO_2–N, and NO_3–N), and total suspended solids (TSS) were determined weekly following the procedures in the Standard Methods for the Examination of the Water and Wastewater [25].

2.3. Zootechnical performance of the shrimp

Survival was expressed as the percentage of live shrimp on the final day of the experiment relative to the total initially stocked shrimp. Shrimp growth was monitored by weekly sampling and restocking of the measured animals. Specific growth rate was calculated according to Huisman [26] with the following formula:

$$\mathrm{SGR}\left(\% \Big/ \mathrm{day}\right) = \left(\sqrt[t]{\frac{\mathrm{wt}}{\mathrm{wo}}} - 1\right) \times 100$$

SGR = specific growth rate (%/day)
wt = final average shrimp body weight (g)
wo = initial average shrimp body weight (g)
t = experimental period (day)

The food conversion ratio (FCR) was expressed as the ratio of the total feed given relative to the shrimp biomass gain, whereas the input/output ratio was measured as the summed weight of feed and carbon source given per unit of biomass gain. These parameters were calculated for each tank at the end of the culture period.

2.4. Protein and lipid assimilation

Shrimp protein and lipid content were determined according to the Folch and Kjehdahl method as described in Takeuchi [23]. The assimilation of protein and lipid originating from the feed by the shrimp (%) were subsequently calculated according to the following formula [23]:

$$\text{Protein assimilation}(\%) = \frac{\text{Final protein content} - \text{Initial protein content}}{\text{Protein input}} \times 100$$

$$\text{Lipid assimilation } (\%) = \frac{\text{Final lipid content} - \text{Initial lipid content}}{\text{Lipid input}} \times 100$$

2.5. IMNV challenge test

An IMNV challenge test was performed at the end of the experimental period. The IMNV was obtained from IMNV infected Pacific white shrimp (as determined by a IQ2000 IMNV detection kit, Genereach, Taiwan) from the Brackish Water Aquaculture Development Institute (BBAP Situbondo, East Java Indonesia). This stock was free of taura syndrome virus (TSV), white spot syndrome virus (WSSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV) as verified by polymerase chain reaction using IQ2000 detection kits for TSV, WSSV and IHHNV, respectively. The preparation of the IMNV stock and the determination of virus titre were conducted according to Escobedo-Bonilla et al. [27]. Briefly, muscle tissue of the naturally infected shrimp was suspended and grinded in phosphate buffer saline (PBS, 10×) and centrifuged at 3000 × *g* for 20 min at 4 °C. The supernatant was transferred into a new tube and centrifuged again at 13 000 × *g* (4 °C for 20 min). The supernatant was then filtered over a 0.45 μm syringe filter and stored in −80 °C until further use. Prior to the actual challenge test, a preliminary experiment was performed to determine the LD_{50} and the effective period of infection (LT_{50}).

Ten healthy appearing inter-molt stage shrimp were kept in each carbon source treatment replicate tank while the remaining shrimp were removed. Sixty healthy inter-molt stage shrimp from the control treatment tanks were randomly selected and redistributed over 6 tanks containing new seawater to make up the negative (non-challenged) and positive (challenged) control for the challenge test ($n = 3$, each). The challenge test was performed by injecting 100 μL of virus suspension (100 μL of PBS for negative control) in between the third and fourth abdominal segment. The challenge test was run for 6 days during which feed was given four times a day to visual satiation according to feeding tray observation. Shrimp mortality was determined daily; most of the dead shrimp showed clinical signs of IMNV infection and this was further confirmed by PCR using a IQ2000 IMNV detection kit. PCR conditions were applied according to the manufacturers protocol.

2.6. Immune parameters

Immune parameters were measured at the end of the rearing period (day 49) and 6 days after IMNV injection. The measurement of total haemocyte count (THC), phenoloxidase activity and respiratory burst were performed for two inter-molt stage shrimp from each replicate tank according to Liu and Chen [2]. Briefly, 200 μL of haemolymph sample was taken with a 1 mL syringe containing 200 μL of precooled anticoagulant solution (30 mM trisodium citrate, 0.34 M sodium chloride, 10 mM EDTA, 0.12 M glucose, pH 7.55). For total haemocyte counting, duplicates of 50 μL of diluted haemolymph were counted for the number of haemocytes using a haemocytometer under a light microscope.

Phenoloxidase activity measurement was performed by adding 200 μL of the diluted haemolymph into 1 mL with anticoagulant solution followed by centrifuging at 700 × *g* for 20 min at 4 °C. The supernatant was discarded and the pellet was rinsed and resuspended in cacodylate-citrate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.10 M trisodium citrate, pH 7.0) and centrifuged again. The pellet was resuspended in 200 μL cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.26 M magnesium chloride, pH 7.0), and a 100 μL aliquot was incubated with 50 μL trypsin (1 mg mL^{-1}) as an activator for 10 min at 25 °C, followed by adding 50 μL of L-dihydroxyphenylalanine (L-DOPA) and 800 μL of cacodylate buffer 5 min later. A no-activation control measurement was prepared at the same time consisting of 100 μL cell suspension in cacodylate buffer, 850 μL cacodylate buffer, and 50 μL L-DOPA. The optical density of the shrimp's phenoloxidase activity was expressed as dopachrome formation in 100 μL of haemolymph at 490 nm.

Respiratory burst activity (production of superoxide anion O_2^-) was determined by reduction of nitroblue tetrazolium (NBT) to formazan according to Song and Hsieh [28] with some modifications. Fifty μL of the diluted haemolymph was incubated for 30 min at room temperature, followed by centrifugation at 1000 × *g* for 20 min at 4 °C. The pellet was then incubated with 100 μL nitroblue tetrazolium (0.3% in Hank's balanced salt solution) for 2 h at room temperature. The suspension was subsequently centrifuged at 1000 × *g* for 10 min, and fixed with 100 μL of absolute methanol. The formazan pellet was then rinsed with 70% methanol for three times and air-dried. Formazan was dissolved with the addition of 120 μL KOH (2 M) and 140 μL dimethylsulfoxide (DMSO). The optical density was measured at 630 nm using a microplate reader, and respiratory burst was expressed as NBT-reduction in 10 μL of haemolymph.

2.7. Bacterial quantification

After 49 days of rearing, a total viable bacterial count and estimated *Vibrio* count from tank water and shrimp intestine was determined by the spread-plate technique on sea water complete agar [29] and thiosulfate citrate bile salts sucrose (TCBS) agar, respectively. Three shrimp from each replicate tank were collected and aseptically dissected. The intestine was removed, pooled and homogenized in PBS.

Table 2
The range and mean value (between brackets, $n = 4$) of water quality parameters in Pacific white shrimp culture water in biofloc technology systems supplied with different carbon sources. Dissolved oxygen (DO), pH, and salinity were measured daily, whereas biochemical oxygen demand (BOD), alkalinity, and total suspended solids (TSS) were measured weekly.

	Control	Molasses	Tapioca	Tapioca-by-product	Rice bran
DO (mg L^{-1})	5.9–7.2 (6.3)	5.8–7.3 (6.0)	5.6–7.3 (6.1)	5.8–7.2 (6.1)	5.9–7.0 (6.1)
BOD (mg L^{-1})	1.05–3.88 (2.57)	0.93–4.38 (2.87)	1.08–4.20 (2.81)	0.85–4.60 (2.91)	1.13–4.20 (2.83)
pH	7.2–8.1 (7.4)	7.0–8.1 (7.4)	6.9–8.1 (7.4)	6.9–8.1 (7.4)	6.9–8.1 (7.4)
Salinity (g L^{-1})	29–30 (30)	29–30 (30)	29–30 (30)	29–30 (30)	29–30 (30)
Alkalinity (mg L^{-1})	77–160 (121)	55–167 (107)	58–161 (118)	52–148 (107)	69–154 (114)
TSS (mg L^{-1})	48–97 (93)	82–241 (180)	67–196 (155)	66–204 (160)	68–230 (184)

2.8. Statistical analyses

Correlation coefficients between the protein and lipid content of the carbon sources and the protein and lipid assimilation by the shrimp was calculated using Pearson's Product–Moment Correlation. All survival data was arcsine transformed. Homoscedasticity and normality of all data were assessed using Levene's test and a Kolmogorov–Smirnov test, respectively. As all data was normally distributed and the variances of the variables were equal, the data were analysed using one-way ANOVA. Repeated measures ANOVA using the linear model of two factors (C source and time) was used to analyse the post challenge survival data [30]. Statistical analyses were conducted using SPSS statistics version 18 for windows (SPSS Inc.) at a significance level of 0.05. Significant differences between treatments were determined using a post-hoc Duncan test.

3. Results

3.1. Water quality

The water quality parameters DO, BOD, pH, salinity, and alkalinity were found to be highly similar among treatments (Table 2). While there was no significant difference observed amongst carbon source treatments, the TSS levels in these treatments were significantly higher than in the control, in particular from day 28 onward. Total ammonium nitrogen concentration in the control was generally higher than that of the carbon source treatments except for rice bran (Fig. 1). In most sampling weeks, the dissolved inorganic N concentrations in the control appeared to be higher than in the treatments, and the difference was significant ($P < 0.05$) in week 2, 5, and 6. In comparison to the other organic carbon source used in this study, dosing rice bran resulted in higher concentrations of total ammonium nitrogen (TAN), nitrite-N and nitrate N.

3.2. Survival and growth performance

There was a trend towards a higher survival in the biofloc treatments as compared to the control, although the difference was only significant for the tapioca-by-product treatment (Table 3). No significant differences were observed in the final body weight and specific growth rate among treatments. The protein assimilation was significantly higher for the tapioca and rice-bran treatments relative to the control while the lipid assimilation was significantly higher for the molasses and tapioca treatments. The addition of organic carbon source resulted in a significantly higher shrimp yield and significantly lower food conversion ratio for the tapioca and tapioca-by-product treatments as compared to the control. In term of input/output ratio, the rice bran treatment resulted in the highest value as compared to the other C source treatments.

3.3. Immune parameters

After 49 days in the experimental period, PO activity of the shrimp from the biofloc treatments was higher than that of the

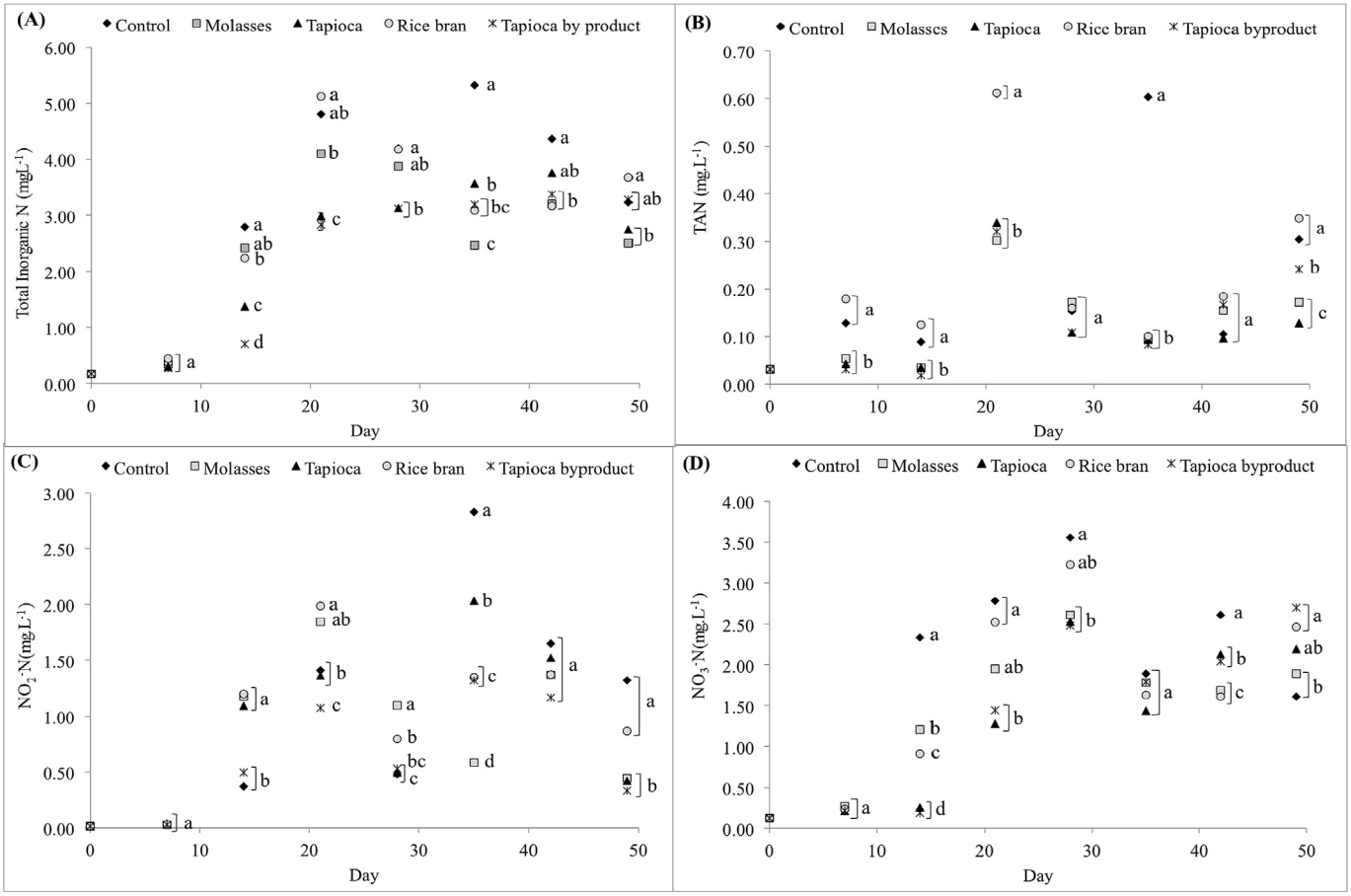

Fig. 1. Dissolved inorganic nitrogen concentrations in Pacific white shrimp culture water in biofloc technology systems supplied with different carbon sources, A) total dissolved inorganic N; B) total ammonium nitrogen (TAN); C) nitrite nitrogen (NO_2^--N); and D) nitrate nitrogen (NO_3^--N). Values are means and standard deviations are not presented for clarity of the figure ($n = 4$). For each day, values marked with a different letter are significantly different ($P < 0.05$).

Table 3
Mean values ± standard deviation of the growth parameters of Pacific white shrimp cultured in biofloc technology systems supplied with different carbon sources ($n = 4$). Values for the same parameter marked with a different superscript letter are significantly different ($P < 0.05$).

	Control	Molasses	Tapioca	Tapioca by-product	Rice bran	*P* value
Survival (%)	84 ± 3^{a}	87 ± 3^{ab}	88 ± 3^{ab}	93 ± 5^{b}	85 ± 3^{ab}	0.030
Final body weight (g)	7.14 ± 0.53	7.26 ± 0.34	7.75 ± 0.54	7.22 ± 0.30	7.36 ± 0.30	0.295
SGR* (% day^{-1})	3.06 ± 0.19	3.08 ± 0.15	3.24 ± 0.13	3.05 ± 0.10	3.15 ± 0.17	0.391
Protein assimilation (%)	30.63 ± 1.85^{a}	32.90 ± 1.98^{ab}	37.61 ± 3.38^{b}	32.37 ± 3.03^{ab}	35.74 ± 1.05^{b}	0.007
Lipid assimilation (%)	12.93 ± 1.08^{a}	20.22 ± 1.50^{c}	16.18 ± 1.84^{b}	11.66 ± 1.61^{a}	13.08 ± 0.69^{a}	0.000
Yield (kg m^{-2})	0.50 ± 0.02^{a}	0.52 ± 0.02^{ab}	0.57 ± 0.04^{b}	0.56 ± 0.04^{b}	0.52 ± 0.01^{ab}	0.030
FCR**	1.67 ± 0.10^{a}	1.56 ± 0.09^{ab}	1.41 ± 0.13^{b}	1.44 ± 0.13^{b}	1.56 ± 0.05^{ab}	0.021
Input/output ratio	1.67 ± 0.10^{a}	2.77 ± 0.15^{b}	2.81 ± 0.24^{b}	3.01 ± 0.22^{b}	3.46 ± 0.12^{c}	0.000

*SGR: specific growth rate.
**FCR: food conversion ratio.

control shrimp and the differences were significant for the molasses and tapioca treatments (Table 4). There was no significant difference observed in THC. Respiratory burst activity of the carbon source treatments was not significantly different from the control. However, it can be observed that the RB activity was influenced by the carbon source used for biofloc culture as indicated by the significantly higher activity of RB in shrimp of the molasses treatment relative to the tapioca-by-product treatment. Following the IMNV challenge, a significant lower level of THC was observed for all treatments as compared to the negative control (the latter being non-challenged shrimp). Among these treatments, THC did not show significant differences. The IMNV challenge induced a decrease in the PO activity of the shrimp of all treatments. Nonetheless, the activity of PO in the challenged shrimp cultured in the tapioca treatment was significantly higher than the shrimp from the positive control. A similar pattern was observed for the RB activity. The RB activity in the challenged shrimp of all treatments with organic carbon addition was significantly higher than in the shrimp from the positive control.

During the first 3 days of challenge a significant effect of time ($P = 0.03$) was observed, however, the survival of shrimp from the carbon treatments did not show significant differences ($P = 0.572$) relative to the shrimp from the positive and the negative control (Fig. 2). In contrast, a significant effect of time ($P = 0.00$) and a significant interaction between time and C source ($P = 0.012$) were observed on day 4–6. A sharp decrease in survival was observed in all treatments in the period of day 3–5. On day 5, the survival in the positive control (23%) was significantly lower ($P = 0.01$) than in all other treatments. Although there was no significant difference amongst carbon treatments, the survival of the challenged shrimp in these treatments were significantly higher than the positive control. Similar pattern was observed on day 6, only now the survival of the shrimp from the molasses and tapioca treatments were not significantly different from that of the shrimp from the positive control. No significant differences in shrimp survival between the carbon treatments were observed after the IMNV challenge.

3.4. Bacterial counts in water and shrimp intestines

The total viable bacterial count in the water of the control treatment was significantly lower than that in the water of the biofloc treatments ($P < 0.05$; Fig. 3A). The number of presumptive vibrios in the shrimp intestines of the control treatment was significantly higher than that in the shrimp intestines of the biofloc treatments, irrespectively of the organic carbon sources used ($P < 0.05$; Fig. 3B). There was no significant difference observed in the total viable bacterial count and the presumptive *Vibrio* count in the shrimp intestines and in the water, respectively, between the different organic carbon source treatments.

4. Discussion

In this research, we illustrate that the application of biofloc technology for Pacific white shrimp culture significantly affects the shrimp immune response, a currently underexplored feature of bioflocs, and may increase the robustness of shrimp to resist infection. These effects seem to be independent of the type of carbon source used to grow the bioflocs.

Each type of bioflocs grown on a different carbon source (molasses, tapioca, tapioca by-product, rice bran) was adequate in maintaining the overall water quality parameters in a normal range for shrimp growth. The lower level of dissolved inorganic nitrogen generally observed in the tanks with biofloc treatment as compared to the control with regular water replacement confirmed previous reports regarding the effect of BFT application on water quality in shrimp culture. Avnimelech [6] pointed out different effects of

Table 4
Mean values ± standard deviation of immune parameters of Pacific white shrimp in biofloc technology systems supplied with different carbon sources prior to IMNV challenge ($n = 4$) and post IMNV challenge ($n = 4$ for biofloc treatments; $n = 3$ for negative and positive control). Values within the same column marked with a different superscript are significantly different ($P < 0.05$).

Treatment	Pre-challenge			Post–challenge		
	THC ($\times 10^6$ cells mL^{-1})*	PO (OD_{490} 100 L^{-1}μ)**	RB (OD_{630} 10 L^{-1}μ)***	THC ($\times 10^6$ cells mL^{-1})	PO (OD_{490} 100 L^{-1}μ)**	RB (OD_{630} 10 L^{-1}μ)***
Negative control****	11.90 ± 0.69^{a}	0.160 ± 0.026^{a}	0.223 ± 0.115^{ab}	11.68 ± 0.64^{a}	0.144 ± 0.039^{a}	0.257 ± 0.155^{ab}
Positive control****				6.81 ± 2.40^{b}	0.071 ± 0.019^{b}	0.084 ± 0.007^{c}
Molasses	12.03 ± 3.06^{a}	0.491 ± 0.224^{b}	0.470 ± 0.147^{a}	6.80 ± 2.54^{b}	0.090 ± 0.020^{ab}	0.317 ± 0.020^{a}
Tapioca	16.61 ± 4.21^{a}	0.603 ± 0.224^{b}	0.214 ± 0.055^{ab}	5.10 ± 0.81^{b}	0.192 ± 0.090^{a}	0.215 ± 0.052^{ab}
Tapioca by-product	16.21 ± 2.50^{a}	0.277 ± 0.084^{ab}	0.189 ± 0.114^{b}	7.15 ± 1.23^{b}	0.132 ± 0.008^{ab}	0.194 ± 0.055^{b}
Rice bran	15.08 ± 4.48^{a}	0.435 ± 0.094^{ab}	0.459 ± 0.183^{ab}	6.49 ± 1.26^{b}	0.127 ± 0.003^{ab}	0.160 ± 0.016^{b}
P value	0.255	0.036	0.048	0.007	0.009	0.003

*THC: total haemocyte count.
**PO: phenoloxidase activity.
***RB: respiratory burst.
****The negative and positive controls prior to challenge are the same non-biofloc control treatment and therefore have the same value.

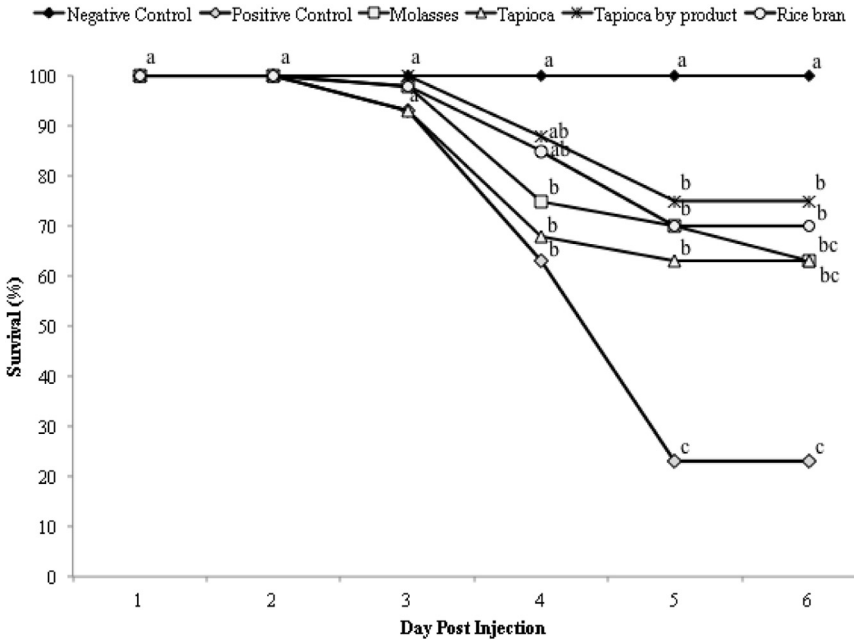

Fig. 2. Mean values ± standard deviation of survival (%) of Pacific white shrimp cultured in biofloc systems supplied with different carbon sources following a challenge test with infectious myonecrosis virus (IMNV) ($n = 4$ for biofloc treatments; $n = 3$ for negative and positive control). Standard deviations are not presented for clarity of the figure. At each time point, values marked with a different letter are significantly different ($P < 0.05$).

simple versus more complex carbohydrates applied as the carbon source in biofloc-based ponds. Simple sugars, such as sucrose, result in a faster ammonia removal, while more complex carbohydrates require more time for decomposition into simple sugars, thereby resulting in slower ammonia removal. This may explain the higher TAN levels at several time points during the experimental period for the rice bran treatment, the carbon source that contained the highest fibre level, as compared to the other carbon source treatments. In this regards, fermentation of the complex carbon sources prior to application in biofloc system could be an alternative solution and interesting subject for further investigation for the use of complex or fibrous carbon sources.

In earlier studies, it was shown that the application of BFT in general results in an increased growth performance, FCR and survival of the cultured shrimp [9,10,31,32]. In our study, there also seemed to be a trend towards a slightly increased growth rate and survival for the BFT treated shrimp, although no significant differences were observed in comparison to the shrimp from the control treatment. Also, no differences were observed between the different carbon source treatments. The different organic carbon sources, however, appeared to have an effect on the assimilation of protein and lipid by the shrimp. The protein assimilation by the shrimp in the biofloc treatments was higher than that of the control shrimp but only significantly in case of the tapioca and rice bran treatments. A similar observation was obtained for the lipid assimilation that was significantly higher for the molasses and tapioca treatment. As the shrimp in all treatments received the same dietary supplementation of proteins and lipids contained in

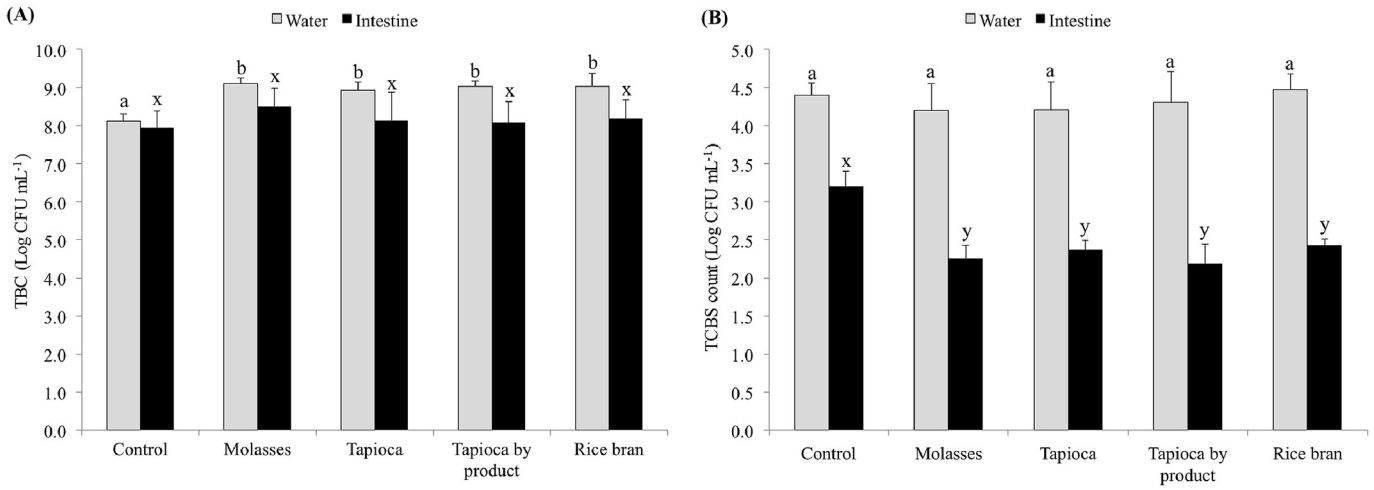

Fig. 3. Mean values ± standard deviation of (A) total viable bacterial counts (TBC) and (B) presumptive *Vibrio* counts (TCBS) in the water and intestines of Pacific white shrimp cultured in biofloc systems supplied of different carbon source ($n = 4$). Bars of the same series (water and intestine, respectively) with different superscript letters are significantly different.

the commercial diet, and only little correlations ($r = 0.17$ and $r = -0.33$, respectively) was observed between the shrimp protein and lipid assimilation and the protein and lipid content in the carbon sources, the altered assimilation values can only result from protein and lipid utilization in the form of biofloc biomass. One of our recent studies [33] showed that according to the essential amino acid composition, biofloc can be considered a good quality protein source. Xu et al. [11] suggested that the improvement of protein assimilation by animals reared in BFT systems is also related to the increase in digestive proteinase activity in the intestinal tract as a result of the contribution of both exogenous digestive enzymes by the microbes in the biofloc and the endogenous digestive enzymes production as stimulated by the biofloc. The enhancement of protein and lipid assimilation in the biofloc treatments clearly showed a positive contribution of bioflocs biomass generated from nutrient waste as a food source for the cultivated animal. This in turn can result in a lower feed conversion ratio in the biofloc systems [10,11,34,35], which was also observed in the present study. Furthermore, the input/output ratio that represents the gain in biomass relative to the combined input of feed and C source indicates the effectiveness of the source of organic C in relation to biomass gain. In this regard, it can be observed that the use of rice bran as an organic C source was the least effective as compared to the other C sources in this study.

The biofloc clearly affected the shrimp innate immune response. For shrimp reared in a biofloc system, the total haemocyte count and phenoloxidase activity prior to challenge showed higher values as compared to the control. This stimulation effect seems to be a general feature of bioflocs, although the extent of the stimulation seemed to be carbon source dependent. The circulating haemocytes of crustaceans and other invertebrates are essential in immunity, performing functions such as phagocytosis, encapsulation, and storage and release of the prophenoloxidase system [36]. Phenoloxidase is an enzyme of the crustacean defense mechanisms that leads to melanisation of foreign cells to inactivate them and prevent their spread throughout the body. This enzyme is highly stimulated by microbial cell wall components such as lipopolysaccharides (LPS) and β-1,3-glucans [37–39]. As the shrimps were cultured in BFT-based systems they evidently consumed the microbial floc *in situ* [33,40] so that the increases in total haemocyte number and PO activity point in the direction of a stimulatory effect of the (digested) biofloc on shrimp immunity. When considering biofloc-based stimulation of PO activity, Kim et al. [17] clearly showed that the expression levels of proPO1, proPO2 and PPAE1 genes, which regulate the phenoloxidase activation systems, were significantly higher in biofloc-cultured shrimp than those of the shrimp from a control treatment. The absence of significant effects in terms of respiratory burst activity, a measure to determine the generation of reactive oxygen species associated with phagocytosis by shrimp haemocytes [28], indicates that actual phagocytosis might not occur more in comparison to the control. It can thus be that the immune system is stimulated by a yet uncharacterized variety of immunostimulatory microbial cell wall material resulting in a higher immune response capability [41,42]. Similar observations were obtained by Xu and Pan [16]. These authors described that bioflocs stimulated the release of haemocytes into the circulation but that antibacterial and bacteriolytic responses were not significantly affected.

Following IMNV challenge, a decrease in the levels of THC, PO and RB activity was observed for the positive control, which is a normal physiological response in case of infection [2,43–46]. The recovery of the shrimp immune system from viral infections, if not mortal, can take rather long (9–12 days) [45]. Although the level of THC in all challenged treatments was similar 6 days after infection, the higher levels of PO and RB activity for the biofloc treatments as compared to the positive control pointed towards a faster recovery, or a more constant activity of the immune system in these treatments. The increased activity or efficiency of the immune system of the shrimp from the biofloc treatments was also illustrated by the shrimp survival after infection that was significantly higher than in the positive challenge control.

It is also interesting to note that the application of biofloc in shrimp culture results in similar effects in terms of growth, feeding efficiency, pathogenic bacteria inhibition and immune responses as the application of probiotics [47–52]. For instance, Zokaeifar et al. [47,48] reported that adding *Bacillus subtilis* into water or the feed of white shrimp resulted in better growth and survival, inhibition of *Vibrio* growth in the intestine, enhanced protease and amylase activities, as well as up-regulation of immune related genes such as LGBP, proPO, peroxinectin, and serine protease. The mechanisms by which probiotic bacteria affect shrimp performance have been reviewed by several authors [3,53,54]. They include immunomodulation, competitive exclusion, bioremediation, providing a source of nutrients and enzymatic contribution to digestion and quorum sensing blocking. The effects that biofloc can have for shrimp culture as shown in the present study, as well as in recently reported studies [11,12,15–17] strongly suggests that the beneficial effects associated with biofloc run at least partly parallel to those observed by addition of probiotics.

In conclusion, the present study showed that bioflocs have positive effects on the immune response of white shrimp leading to a higher resistance against IMNV challenge. Only slight differences were observed amongst the different organic carbon treatments. Overall, this study has demonstrated that the potential of applying biofloc technology to achieve disease control and management in the shrimp culture industry.

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