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Expression of immune-related gene from African mud catfish *Clarias gariepinus* reared in bioflocs systems after *Aeromonas hydrophilia* infection

Omoniyi Michael Popoola[1,2]*, Ayomide Miracle Oyelade[1], Success Taiwo Torhukerijho[1]

[1]Department of Fisheries and Aquaculture, Federal University of Technology, PMB 704, Akure, Nigeria.
[2]Aquatic Environmental Biotechnology & Nanotechnology Division ICAR-Central Inland Fisheries Research Institute, Indian Council of Agricultural Research, Barrackpore, Kolkata-700 120 West Bengal, India.

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Corresponding Author
Tel.: +234 803 834 9325
E-mail: ompopoola@futa.edu.ng

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Abstract

The influence of various carbon sources as bioflocs on relative immunological gene expression, haematology, growth, and microbial community in *Clarias gariepinus* juvenile culture is investigated in this study. The bioflocs groups (four) were created by daily supplementation with four carbon sources (cassava peel, tapioca, wheat offal, and brewery waste) with a carbon-nitrogen ratio of 20 and the control without carbon addition. The juvenile *Clarias gariepinus* (8.16 ± 0.2 g) was stocked into each bioflocs system and reared for 72 days. The results revealed that the water quality parameter and survival rate differed significantly across the treatments. The microbial community revealed that there were differences in bacterial intensity and diversity among the various culture systems. The haematological parameters between the treatments showed a significant difference $p<0.05$ in the challenged test. qRT-PCR was used to assess immune-related gene expression, and four immune genes (*IL-10*, *TNF-α*, *TGF-β*, *IL-1β*) were shown to be increased. As a result, the bioflocs system can be considered to boost innate immunity and immune-related gene expression. Overall, this research found that using bioflocs technology can help with immunostimulation, and that the effect is independent of the organic carbon utilised to keep the fish alive.

Introduction

An ecologically important and commercially valued fish for sub-Sahara Africa especially Nigerian aquaculture industry is the African catfish, *Clarias gariepinus,* belonging to the family Claridae (Ita, 1980). *Clarias gariepinus* is generally and widely cultivated in ponds because of their ability to survive in captivity, they also occur freely in natural freshwaters in Nigeria (Adebayo & Daramola, 2013). The current demand for fish protein has led to the intensification of aquaculture to make fish available to the growing population, therefore, increasing productivity per unit space is accomplished by increasing the stocking density of fish. Due to limited control over pathogens, health protection and biosecurity are major challenges to production intensification (Xie & Yu, 2007). Attaining sustainability in feed management is also an important aspect of the production intensification of any aquatic organism.

The scientific community's understanding of the genus *Aeromonas* has also evolved. Initially, aeromonads were only recognised as producing systemic disorders in poikilothermic animals. The genus *Aeromonas* is now recognised not only as a significant disease-causing pathogen of fish and other cold-blooded animals, but also as the causative agent for a variety of infectious problems in both immunocompetent and immunocompromised people (Janda & Abbott, 2010). Mesophilic species (*A. hydrophila* and *A. veronii*) infect fish with a similar range of diseases, including motile *Aeromonas septicemia* (hemorrhagic septicemia) in carp, tilapia, perch, catfish, and salmon, and a red sore disease in carp, tilapia,

perch, catfish, and salmon, and ulcerative infections in catfish, cod, carp, and goby (Joseph & Carnahan, 1994; Ture et al., 2018). Over the last decade, mesophilic *Aeromonas* species, most notably *A. hydrophila*, have been connected to massive die-offs and fish kills all over the world, resulting in enormous economic losses. *A. hydrophila* is a pathogenic bacterium that is found all over the world, especially in warm water. They are gram-negative, motile rods with oxidase and catalase activity, as well as fermentative (Sabur, 2006). MAS is caused by the bacterium *A. hydrophila* (motile *Aeromonas septicemia*). This disease affects both farmed and wild fish, however fish in intensive culture systems are more sensitive to the disease condition caused by *A. hydrophila.*

Bioflocs technology is a method of improving water quality by adding extra carbon to the aquaculture system, either from an external source or by increasing the carbon content of the feed. This approach encourages bacterial growth to take up nitrogen, resulting in a faster decrease in ammonium content than nitrification (Hargreaves, 2006). This biotechnological approach has proved efficient in improving water quality and feed usage efficiency compared to conventional practices for farming of Tilapia and *Litopenaeus vannamei* (Avnimelech, 1999; Milstein et al., 2001). The bioflocs rearing system has been developed to implement the use of minimum water exchange and usage in aquaculture ponds, through maintaining adequate water quality within the culture unit as well as producing heterotrophic media and a multiphasic feed source that can serve as food for aquatic organisms (Avnimelech, 1999; Crab et al., 2007; Crab, 2010), invented the bioflocs rearing system to execute minimum water exchange and water utilisation in aquaculture ponds, which improves aquatic creatures' nutritional and reproductive performance (Emerenciano et al., 2013). It has been hypothesised that biofouling could be reduced as a result of the minimum water exchange.

Bioflocs keeps pathogens out of ponds and disease outbreaks from spreading between farms (Crab et al., 2010). Recent studies have evaluated probiotic effects of bioflocs and role in immune response mechanisms in an attempt to demonstrate its benefits to aquatic organisms' health (Wang et al., 2013; Ahmad et al., 2016), suggesting that disease can be prevented in fish using this sustainable rearing system (Wang et al., 2013; Ahmad et al., 2016; Liu et al., 2016). In terms of conferring immunological benefits, C/N ratios in bioflocs improved the innate immune response and antioxidant status in various species of shell and finfishes when challenged with a disease-causing organism (bacteria) (Xu & Pan, 2013; Ekasari et al., 2014; Ahmad et al., 2016), implying that disease can be prevented in fish using this sustainable rearing system. The study aimed at determining how different carbon sources used in bioflocs affect immunological parameters and immune-related gene expression in the *C. gariepinus* challenged with *A. hydrophilia*.

Materials and Methods

Experimental design

Approximately 1000 *Clarias gariepinus* Juvenile (Initial weight 8.21 ± 0.3g) was obtained from the Department of Fisheries and Aquaculture Technology Teaching and Research Farm, the Federal University of Technology Akure and acclimated in a water volume of 100L. Acclimation to experimental conditions was carried for 14 days within the facilities of the institution, and during this period, the juveniles were fed with a commercial diet two times daily (08.00 and 18.00 h) at 3% of their body weight.

T1 (Clearwater), T2 (Cassava peel as a carbon source), T3 (Tapioca as a carbon source), T4 (Wheat offal as a carbon source), T5 (Brewery waste as a carbon source) were the five treatment groups, four bioflocs treatments, and one control in triplicate with a water volume of 500 L. Collecting Pond bottom soil from a reputed fish farm was used to make the inoculum. In glass tanks (5L), inoculum was made by mixing 20g of pond bottom soil with 1 L of well-aerated water containing 10mg L^{-1} ammonium sulphate $(NH)_4SO_4$ and 400 mg L^{-1} of various carbon sources (tapioca, wheat offal, brewery, and cassava peel). For 24 h, the suspension was incubated for the development of microbial growth.

The prepared inoculum was added to the respective experimental groups after floc formation, the tank was aerated for 7 days to ensure optimum floc formation, and carbon sources were added at a rate of 20 g of carbon source per 1 g of TAN as described by (Avnimelech, 1999) who assumed that 20 g of carbon source is required to convert 1 g of TAN, and (Ebeling et al., 2006) to provide an initial substrate and stimulate the growth of all the treatment groups, 100 fish per tank (2 x 1 x 1 m^3) were stocked. Every week, 70% of the water in the control group was replaced with fresh water, whereas there was no water exchange in the bioflocs based groups. Evaporation losses were corrected with dechlorinated fluids to account for the bioflocs' variation and achieve optimal experimental conditions. The experimental fish were fed with commercial diets at 3% of their body weight under continuous aeration using air blower installed at 10 lines (5 l/min per line).

The determination of the required amount of carbon to reduce the total ammoniacal nitrogen was calculated as follows:

Calculation 1: (Carbon Nitrogen (C: N) content in the feed)

$$C: \frac{\text{Kg of feed} \times 0.9(90\%\ \text{dry matter}) \times 0.7(30\%\ \text{of fish assimilation}}{2(\text{Carbon content of the feed} \sim 50\%\ \text{based on dry matter}}$$

$$N: \frac{\text{Kg of feed} \times 0.9(90\%\ \text{dry matter}) \times 0.7(30\%\ \text{of fish assimilation} \times \text{Crude protein of feed}(\%)}{6.25(constant)}$$

Calculation 2: (Adjusting the Carbon Nitrogen Ratio (C: N)

$$C{:}N\ of\ 20{:}1 = (N(Nitrogen\ content\ in\ feed) \times 20) - C(Carbon\ content\ in\ feed) \times 2$$

Haematological indices

Blood samples were obtained by caudal vein-puncture using a syringe and dispensed into a sample bottle containing ethylene diamine tetra-acetic (5 mL EDTA) as an anticoagulant after five experimental fish were taken from each experimental tank and sedated with clove oil (50 mg L^{-1}).

Svobodova et al. (1991) described a method for analysing blood parameters (RBC, HB, WBC, PCV, MCV, MCHC, and MCH). The haematocrit (Ht) was evaluated using the microcentrifuge technique, and the white blood cell (WBC) and red blood cell (RBC) were determined using a haemocytometer. The Ht, HB, and RBC values were used to determine the MCV, MCH, and MCHC.

Microbial community analysis

The APHA (1998) approach was used to characterise the bioflocs and hindgut bacterial populations of the experimental fish under each treatment. 1 mL of the sample was transferred into 9 mL of sterile distilled water in a bottle at the end of the experiment to obtain dilutions 10^{-1}, and 1 mL from the previous dilution was transferred into another 9mL of sterile distilled water to obtain dilutions 10^{-2} and up to 10^{-5}, respectively, using a sterile pipette. After that, 1mL of each sample from dilutions, 10^{-4} and 10^{-5} was put into sterile Petri-dishes. Each plate was then filled with 20 mL of molten sterile nutritional agar that had been cooled to 45 ^{o}C. After gently swirling the plates and allowing them to harden, they were incubated at 37 °C for 24 h. The plates were checked for growing colonies after 24 h. The colonies were counted and their morphological features were recorded. Representative colonies were chosen and sub-cultured on new nutrient agar multiple times until pure cultures were produced. The bacteria were identified using morphological characteristics of the colonies and biochemical assays was performed on each isolate. To establish pure cultures for identification of fungi, representative colonies were chosen and sub-cultured onto fresh potato dextrose agar. Fungal taxonomic identification was used to identify isolates. The hyphae of the fungal isolates were mounted aseptically on a microscopic slide and given a drop of lactophenol cotton blue before being covered with a coverslip. The slide was examined under the microscope with an x40 objective lens.

Challenge test

The experimental fish in different bioflocs system were exposed to pathogenic strain of *A. hydrophilia* (MPSTR 2143), mildly pathogenic strain (Animal care Laboratory Ogere), grown on a brain heart infusion broth (EM Science, Darmstadt Germany) in a shaking bath 27 ^{0}C overnight in the Department of microbiology Federal University of Technology, Akure. The concentration of bacterial suspension was determined by the serial plate count method and diluted to $9.3x10^{5}$ CFU. Prior to challenge test, lethal dose ($LD_{50\text{-}96h}$) of *A. hydrophilia* (MPSTR 2143) against *C. gariepinus* was determined. Fifteen individuals of *C. gariepinus* were distributed in four tanks in duplicate (120 fish) and maintained without feed till 96 h. The fishes were injected intraperitoneally with 1mL of *A. hydrophilia* (MPSTR 2143) suspension with the concentrations: 1×10^{9} $CFUmL^{-1}$, 1×10^{8} $CFUmL^{-1}$, 1×10^{7} CFU/mL and 0 $CFUmL^{-1}$ (control, using 500µL of physiological solution 0.9% NaCl). The percentage of mortality was calculated at 24 h, 48 h, 72 h, and 96 h, respectively as described by Abbott (1925) and the LD_{50} was determined with Probit analysis. For the challenge test, fifty fish were challenged by *A. hydrophila* (10 fish/ group) by intra-peritoneal injection of 0.5 mL of bacterial suspension using 2 ml Insulin syringe at a concentration of 1.7×10^{9} $CFUmL^{-1}$ of bacterial culture/fish for *A. hydrophila* obtained from lethal dose ($LD_{50\text{-}96h}$). Injected fish were observed for up to 14 days for daily monitoring of symptoms. Mortality and survival were monitored and recorded. Survival at the end of 14 days post-infection was calculated using the following formula (Amend, 1981).

$$Relative\ percentage\ survival\ (RPS) = \frac{Number\ of\ surviving\ fishes\ after\ challenge}{Number\ of\ fish\ bathed\ with\ bacteria} \times 100$$

Tissue sampling

After 14 days of being challenged with *A. hydrophila*, liver samples were taken from the various treatments by slicing the fish to reveal the visceral organs. About 50mg of liver was sliced off and placed in 1.5 mL microcentrifuge tubes, which were kept on ice to prevent RNA denaturation owing to temperature changes. The collected materials were transported to the lab, fixed in RNA, and kept at -20 ^{o}C until RNA extraction was completed.

RNA extraction

Total RNA was isolated from liver tissue of *A. hydrophila* treated and control *C. gariepinus* using Trizol reagent. The purity and quantity of isolated total RNA was quantified using Nanodrop™ Thermo Scientific at the 260:280 ratio. After, the extracted RNA was kept at -20 °C till further use.

cDNA synthesis and quantitative Real Time-PCR

Subsequently, complementary DNA (cDNA) was produced according to the manufacturer's procedure using a cDNA synthesis kit (Bio-iScript Rad's cDNA synthesis kit). With *β-actin* and *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as a housekeeping gene, specific primers for gene expression were constructed to amplify the targeted genes. The qRT-PCR analysis was performed using the Bio-Rad CFX96 Touch Real time PCR detection equipment and Sso Advanced

Universal SYBR green supermix (Bio-Rad) according to the manufacturer's instructions and modified by Plaza-Diaz et al. (2014) technique.

Data and statistical analysis

For all genes and samples, the Ct was calculated. The comparative Ct method was used to calculate the relative quantitation of *TNF-α, IL-1β*, *IL-10*, and *TGF-β*. The target gene's relative quantification value was standardised to an endogenous control and expressed as 2-Ct relative to the calibrator. Ct is equal to the Ct of the target gene (*TNF-α*, *IL-1β*, *IL-10*, and *TGF-β*) minus the Ct of the endogenous control gene (*GAPDH* and *β-actin*) in this investigation. Ct is equal to the sample's Ct minus the calibrator's Ct. The fold change in expression levels was computed by dividing the treated sample's relative gene expression value by the negative control's relative gene expression value. Statistical analyses were performed with one-way analysis of variance (ANOVA) and Dunnett's test to compare differences between the treatment groups and the negative control group, and the data were tested for normality and heterogeneity of variance using the Kolmogorov-Smirnov test and Levene's test, respectively. Multiple comparisons were taken into account when calculating the p-values. Graphpad Prism version 8.0 was used to visualise the results.

All data were subjected to an analysis of variance (ANOVA) with a significance level of 0.05 (95% confidence), and the findings were given as Mean SE. To look for significant changes between the treatments, Duncan's multiple range tests were utilised. IBM SPSS statistics version 22 for Windows was used to conduct all of the analyses. Prior to statistical analysis, Log 2 Transformation was performed.

Results

The growth performance of *Clarias gariepinus showed* no significant difference ($p<0.05$) in the weight gain, FCR and SGR (Table 1), although a significant difference $p<0.05$ was observed in the relative survival recorded in tapioca-based treatment 76.00±1.50 and lowest in control 50±10.00 (Figure 1).

The haematological parameters (PCV, HB, RBC, WBC, MCH, MCV, and MCHC) of *Clarias gariepinus raised* in bioflocs systems before and after challenge with *A. hydrophilia* is shown in Table 2. The haematological parameters were significantly different $p<0.05$ but tapioca was observed to be the lowest compared to the control and other bioflocs systems. WBC showed no significant difference among the treatments group.

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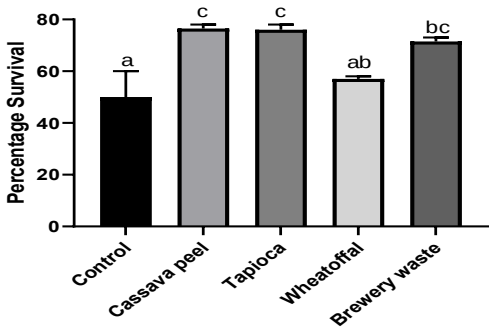

Figure 1. Relative percentage survival of *C. gariepinus in* different treatment groups.

The haematological parameters (PCV, HB, RBC, WBC, MCH, MCV, and MCHC) of *Clarias gariepinus raised* in bioflocs systems before and after challenge with *A. hydrophilia* is shown in Table 2. The haematological parameters were significantly different $p<0.05$ but tapioca was observed to be the lowest compared to the control and other bioflocs systems. WBC showed no significant difference among the treatments group.

Gram-negative bacilli bacterial colonies from the Enterobacteriaceae family were detected, including *Proteus* sp., *Klebsiella* sp., *Shigella* sp., and *Escherichia coli* (Table 3). A group of sporulated gram-positive bacilli linked to lactobacillus and bacteria that are functionally referred to as cocci has been discovered. The presence or absence of the indicated bacterium species in the culture media, as well as heterotrophic bacteria, was a common occurrence.

Colonies from the family Hypocreaceae, which included *Trichoderma* sp., the family Trichomaceae, which included colonies from *Aspergillus* sp. and *Penicillum chysogenum* were among the groups detected (Table 4). A group of filamentous fungi was discovered, including *Fusarium* sp. colonies and the

Table 1. Growth performance of *C. gariepinus juveniles* raised in bioflocs systems

Treatments	Control	Cassava peel	Tapioca	Wheat offal	Brewery waste
Initial (g)	8.13±0.48	8.07±0.56	8.18±0.25	8.61±0.01	8.62±0.07
Final (g)	88.45±5.45	81.30±2.20	90.35±3.25	81.70±22.16	77.10±2.20
Weight gain (g)	80.31±5.93	73.23±2.76	82.16±3.50	73.08±22.09	68.47±2.14
SGR	2.98±0.15	2.89±0.12	3.00±0.08	2.76±0.35	2.74±0.03
FCR	0.66±0.05	0.72±0.03	0.64±0.03	0.79±0.24	0.77±0.02

SGR: Specific growth rate, FCR: Feed conversion ratio

Table 2. Haematological Parameters of *C. gariepinus* raised in bioflocs systems

	Treatment	Control	Cassava peel	Tapioca	Wheat offal	Brewery waste
HB(g/100ml)	Pre challenge	11.00±0.30^{b}	9.80±0.50^{ab}	9.55±0.15^{a}	10.20±0.20^{ab}	10.20±0.50^{ab}
	Post challenge	12.40±0.30^{b}	10.65±0.65^{a}	11.20±0.50^{ab}	11.15±0.15^{ab}	10.15±0.15^{a}
Ht (%)	Pre challenge	33.00±1.00^{b}	29.50±1.5^{ab}	28.50±0.50^{a}	30.50±0.50^{ab}	30.50±1.50^{ab}
	Post challenge	37.00±1.00^{b}	32.00±2.00^{a}	33.50±1.50^{ab}	33.50±0.50^{ab}	30.50±0.50^{a}
WBC (x103/mm3)	Pre challenge	5250±450.00^{a}	5850±450.00^{a}	6800±300.00^{a}	5150±650.00^{a}	5325±725.00^{a}
	Post challenge	4500.00±300.00^{a}	5725.00±325.00^{a}	5100.00±700.00^{a}	4750.00±250.00^{a}	6250.00±950.00^{a}
RBC	Pre challenge	3.68±0.13^{b}	3.25±0.15^{ab}	3.15±0.05^{a}	3.35±0.05^{ab}	3.38±0.18^{ab}
	Post challenge	4.1±0.10^{b}	3.53±0.23^{a}	3.73±0.18^{ab}	3.73±0.08^{ab}	3.35±0.05^{a}

Values are expressed as mean ± SE. The mean values (n=2) with different superscripts within the same row are significantly different (p<0.05). HB: haemoglobin, Ht: haematocrit, RBC: red blood cell, WBC: white blood cell

saprophytic fungus *Rhizopus* sp. The identification of fungus species and their qualitative characterization to their presence or absence in the culture media was noticed.

Table 3. Characterization of bacteria isolate in biofloc systems

Treatment	Control	Cassava peel	Tapioca	Wheat offal	Brewery waste
Bacillus sp.	-	+	+	+	+
Shigella sp.	+	+	-	-	-
Proteus sp.	-	+	+	+	-
Escherichia coli	+	-	+	-	+
Staphylococcus epidermidis	+	-	-	+	-
Staphylococcus aureus	+	-	-	+	+
Klebsiella sp.	+	-	-	-	+

Changes in the expression of all immunological genes investigated as a result of *A. hydrophilla* infection compared to the calibrator sample (Control). When standardised against *GAPDH*, the expression levels of target genes *TNF-α*, *IL-1β*, *TGF-β*, and *IL-10* revealed a considerable down-regulation (Figure 2). There was a substantial difference between the treatments, with cassava peel notably different from the other treatments in terms of *TNF-α* and *IL-1β* expression.

When normalised against *β-actin*, *TNF-α*, and *IL-1β* are down-regulated relative to the calibrator sample, but there is a statistically significant difference (p<0.05) among the bioflocs treatment, but *IL-10* and *TGF-β* are up-regulated with no significant difference among the bioflocs treatment (Figure 3).

Discussion

The non-significant differences in fish growth performance in the treatment groups, Weight gain, SGR, and FCR, could indicate that the bioflocs system did no influence on fish development and that the feed was efficiently utilised by fish produced in the bioflocs system at a Carbon Nitrogen Ratio of 20. (Avnimelech, 2007). There is a sliver of evidence suggesting the biofloc helped to the fish's development and production, which contrasts with the findings of Azim & Little (2008), who found that bioflocs had a low FCR in *Oreochromis niloticus*. Increased turbidity owing to bioflocs, which limits visibility and thus artificial feed intake, could be attributed to the increased FCR and reduced weight gain of fish species in the CP+BFT, WO+BFT, and BW+BFT when compared to the control. When compared with the control, the survival rate of the fish in the bioflocs systems were considerably greater (p<0.05). Several pathogenic species, such as bacteria, fungi, and viruses have been identified as major limiting factors in fish and other aquatic animals' growth. *A. hydrophilia* is the most prevalent and commonly seen bacterial disease in tropical regions, causing serious harm to fish output (Karunasagar et al., 1991). BFT has been demonstrated to boost fish immune responses, but just a few types of research have looked in the resistance of aquatic animals cultivated in bioflocs-based systems to infectious disease, with no promising results thus far. Bioflocs-based systems greatly boosted resistance of *L. vannamei* to infectious myonecrosis virus and *L. rohita* to *A. hydrophila,* according to Ekasari et al. (2014) and Ahmad et al. (2016). This is attributable to their better innate immune responses. Before the bacterial challenge test, there were no significant differences in haematological parameters across the treatments in this study. Although the WBC of the different treatments showed no significant difference to the control, a higher value of WBC was observed in the BFT compared to the control, which could be the result of the destruction of WBC during macrophagosis in the control group, the bioflocs group were able to release more antibody to

Table 4. Characterization of Fungi isolate in biofloc systems

Treatment	Control	Cassava peel	Tapioca	Wheat offal	Brewery waste
Trichoderma viride	+	+	+	-	-
Fusarium sp.	-	+	+	-	-
Aspergillus niger	+	-	+	+	-
Trichoderma sp.	-	-	+	-	-
Aspergillus flavus	-	-	-	+	-
Fusarium oxysporium	-	-	-	+	-
Penicillium chrysogenum	+	-	-	+	+
Rhizopus stolonifer	-	-	-	-	+

counter the effect. Bioflocs have been shown to be an effective way of reducing *A. hydrophila*, a widespread disease-causing bacterium found all over the world, especially in intensive freshwater systems (Saavedra et al., 2004; Zmyslowska et al., 2009). Ulcers, depigmentation, fraying, and reddening of fins are all symptoms of *A. hydrophila* infections, which can be fatal to *C. gariepinus* and result in significant economic losses (FAO, 2016; Kusdarwati et al., 2017). As a result, BFT could be a good way to protect catfish from *A. hydrophila* infection during intensive culture.

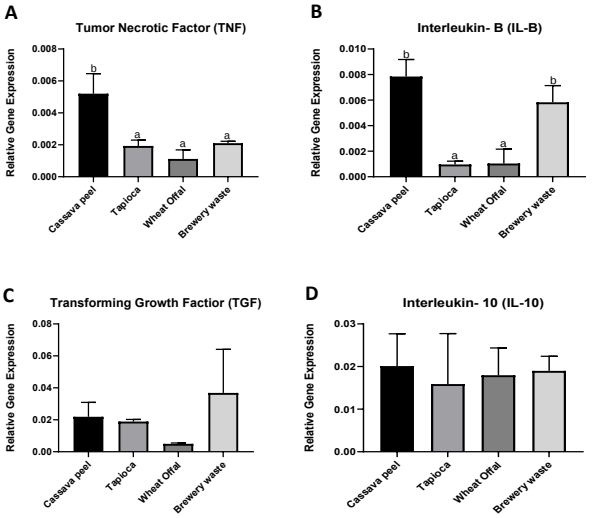

Figure 2. Mean fold change in gene expression + standard error in four treatment groups for four different genes A, B, C, D relative to the calibrator sample normalized against *GAPDH* ($p<0.05$).

Microorganisms such as bacteria and protozoa produce microbial proteins in ponds under aerobic and anaerobic conditions by decomposing organic debris and uneaten feed. According to Reddy and Patrick (1975), the aerobic decomposition process is usually faster than the anaerobic decomposition process because the presence of oxygen speeds up the rate of breakdown. Natural production, nutrient cycling, water quality, and the nutrition of farmed animals are all influenced by microorganisms (Moriarty, 1997; McIntosh et al., 2000). In many cases, the microorganisms identified as being involved in the above process are restricted. So yet, only about a quarter of naturally occurring bacteria have been isolated and characterised (Muyzer et al., 1993). The classification of the groups as microorganisms is common in most BFT culture investigations (Ballester et al., 2010; Ray et al., 2010; Loureiro et al., 2012; Emerenciano et al., 2013). As a result, the formation of a distinct, dominant, uniform, and diverse microbial community with traits including reproductive strategy, small size, short life cycle, and broad tolerance to environmental influences is postulated. These bacteria can also establish themselves as a result of system variables such as carbon supply, initial inoculum, and, as demonstrated in this study, the cultured species' behavior and nutritional habits.

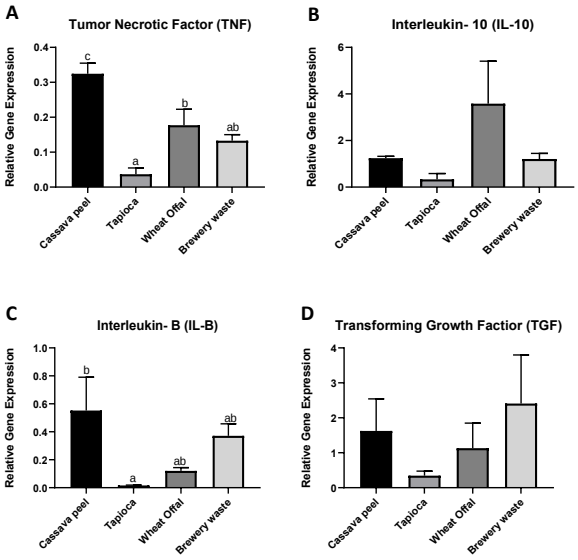

Figure 3. Mean fold change in gene expression + standard error in four treatment groups for four different genes A, B, C, D relative to the calibrator sample normalized against *Actin* ($p<0.05$).

The microbiota in the system has an impact on nutrient dynamics; these were characterised for Enterobacteriaceae, *Bacillus*, *Coccus*, and heterotrophic organisms, which agrees with the findings of Monroy-Dosta et al., (2013) who discovered heterotrophic bacteria such as *Pseudomonas, Bacillus, Vibrios, Enterobacter*, and *Micrococcus* in the microbial community connected with the bioflocs in tilapia culture. In farming settings, this species of bacteria promotes established channels for the elimination of hazardous nitrogen compounds. According to Ebeling et al. (2006), the main routes within the flow of nutrients in BFT culture systems are nitrogen elimination by photoautotrophic algae, nitrogen immobilisation by heterotrophic bacteria of the microbial protein biomass, and nitrogen chemo-autotrophic oxidation in nitrate by nitrifying bacteria. Depending on the type and intensity of the manufacturing system, the relative importance of each one varies. Although heterotrophic bacteria were encouraged to immobilise, the system was dominated by nitrifying bacteria in this study.

The importance of *Bacillus* colonies, which have probiotic properties, in the development of fish culture is underlined because these genera emit a wide range of exoenzymes and polymers that create a hostile environment for pathogenic bacteria (Monroy et al., 2010). Although routes of nitrification and recycling of nitrogen compounds exist under the minimum requirements of nutrients and the management of water quality in optimal ranges for the cultivation of the species, the system is maintained by established bacterial communities (Ayazo-Gene et al., 2019). The results of this study show that the presence of specific

microorganisms varied across treatments, and that a significant difference in CFU of total bacterial and SFU of total fungi could be attributed to differences in the composition of the carbon sources (simple and complex carbohydrate) used in the experiment, allowing us to conclude that the fungi and bacterial communities varied across all treatments. The current microbiota is linked to the system's dynamics, with a higher proportion of enterobacteria and heterotrophic bacteria. The immune system is divided into two parts: innate (non-specific) and adaptive (specific). Innate immunity serves as a barrier against infections, foreign substances, chemical agents, and environmental changes, and fish rely heavily on it. The innate immune system of fish is made up of several different components, each of which serves a specific purpose. Many research has been conducted to determine and understand the behavior of immune-related genes in both normal and pathologic states on *Acipenser dabryanus* (Dabry's sturgeon) (Zhang et al., 2018), on (*Carassius auratus*) (Qihecrucian carp) (Wang et al., 2013, 2016), on *Ctenopharyngodon idella* (grass carp) (Gou et al., 2018), on *Pelteobagrus fulvidraco* (yellow catfish) (Liu et al., 2016), on channel catfish (Prideon et al., 2013), and on *Oncorhynchus mykiss* (rainbow trout) (Yarahmadi et al., 2016). Inadequate research on *C. gariepinus* immune system is a key stumbling block to understanding immune system development, vaccine development, and immune stimulant evaluation. In addition, using marker assisted selection or selective breeding, bacterial resistance strains can be selected. There is still a lack of information about *C. gariepinus* immune-related genes, as well as evaluation of the immune response based on immune genes on the mRNA level following stress induction and bacterial infection. Due to the current state of *C. gariepinus* immune response, it is required to expand research on the expression of immune-related genes following a bacterial challenge. This paper reports on the expression of immune-related genes in the liver (a significant immunological organ in fish) of *C. gariepinus* maintained in bioflocs systems and challenged with *A. hydrophilia*, which will aid in a better understanding of the disease resistance mechanism.

When determining the biological significance of changing gene expression profiles, it's crucial to consider the amount of expression in comparison to the control (Gorgoglione et al., 2013) Relative quantification is a widely used approach for analysing gene expression, and as the name implies, it is an analysis based on the target gene's expression being normalised relative to the expression of a control gene (Kheirelseid et al., 2010). Changes in cytokine gene expression were detected between treatments and between the reference genes employed (Table 5). *TNF-α* and *IL-1β* were observed in the liver to generate a statistically significant increase in the fold among the treatments. In the cascade signaling of pro-inflammatory genes, *TNF-α* is most commonly the first cytokine secreted, which eventually leads to the downstream of *IL-1β* and other chemokines. In parasite and bacterial infections, cytokines such as *IL-1β* and *TNF-α* are key and crucial mediators of pro-inflammatory responses, and they are frequently co-expressed with other macrophage-derived inflammatory mediators such as *IL-1β*. The target genes' fold changes in all treatments, adjusted against *GAPDH*, were significantly lower than in the calibrator sample (Untreated), which could be due to the presence of probiotic bacteria in the bioflocs culture medium. These findings matched those of Xiao et al. (2019), who looked into the effect of *Clostridium butyricum* (CB) diet on yellow catfish (*Pelteobagrus fulvidraco*).

Table 5. Mean fold change in gene expression for four different genes in *C. gariepinus* raised in biofloc systems

Gene	Treatment	*Beta-Actin*	*GAPDH*
TGF-β	Control	1.05±0.74	**6.60±3.53** *
	Cassava peel	0.63±0.92	1.02±0.43
	Tapioca	**2.71±0.58**	0.87±0.07
	Wheat offal	1.24±1.07	1.06±0.11
	Brewery waste	0.08±0.95	1.60±0.84
TNF-α	Control	**3.82±0.83** **	**11.47±1.97****
	Cassava peel	**2.19±0.14***	**3.84±0.35** *
	Tapioca	1.12±0.79	**2.42±0.28**
	Wheat offal	1.27±0.38	1.45±0.80
	Brewery waste	0.89±0.19	**2.57±0.08**
IL-B	Control	0.23±0.76	**7.42±3.55** *
	Cassava peel	1.24±0.67	0.42±0.18
	Tapioca	**6.22±0.23****	**2.64±0.28**
	Wheat offal	**3.32±0.29** **	**3.14±1.47**
	Brewery waste	1.70±0.34	0.02±0.23
IL-10	Control	**6.54±0.23****	**14.19±2.57****
	Cassava peel	**6.85±0.10****	**8.50±0.40** **
	Tapioca	**4.40±1.36** **	**8.00±0.85** **
	Wheat offal	**8.16±0.81****	**8.34±0.37** **
	Brewery waste	**6.78±0.30****	**8.45±0.19** **

The value for the negative control group for each gene and treatment is always 1 and is therefore not shown. Greater than two-fold increases or decreases in gene expression relative to the negative control appear in bold, and statistically significant changes are marked with asterisks: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

The results showed that downregulation of *IL-10* expression in CB treated fish could have resulted in the anti-inflammatory effect of the probiotic administered after bacterial challenge. Probiotics have been shown to be effective against bacterial pathogens in aquaculture species (Chinabut & Puttinaowarat, 2005; Rendueles et al., 2012; Plaza-Diaz et al., 2014). The presence of this probiotic in the bioflocs culture system can increase the organism's tolerance to pathogenic invasion by inducing the secretion of anti-inflammatory cytokines such as IL-10 and TGF-β as standardised against Actin relative to the calibrator sample, the fold change seen in this study was down-regulated (*TNF-α* and *IL-1β*), with the tapioca-based treatment being the most down-regulated among the treatments when compared to the control. This could explain why, in response to the inflammatory response, pro-inflammatory genes were activated more in the tapioca-

based bioflocs than in the other treatments. Other immune-related signaling pathways and enzyme activity production are affected by these inflammatory substances. As a result, *IL-1β* and *TNF-α* are two major pro-inflammatory molecules that can trigger an inflammatory response by controlling the expression of other cytokines (Zhang et al., 2018). It's worth noting that the bioflocs culture technique drastically lowered *TNF-α* and *IL-1β* expression in this study.

The immune response genes *TGF-β* and *IL-10* in *C. gariepinus* were up-regulated in the bioflocs-based treatments, with the exception of the tapioca-based bioflocs system, which was down-regulated compared to a calibrator sample (Untreated). The up-regulation of these anti-inflammatory cytokines shows that the pro-inflammatory response began early in the challenge, since *IL-10* was up-regulated in this study compared to the negative control. *IL-10* regulates the immunological response of the fish by preventing macrophages from releasing pro-inflammatory cytokines such as *TNF-α*, *IL-2*, and *IL-3*. *TNF*, *IL-1β* may have been down-regulated because of this. The regulating effect of *IL-10* on *IL-1β* expression has been found in Indian main carp (*Catla catla*) (Moore et al., 2001; Swain et al., 2011), *IL-10* modulates inflammatory responses and potently inhibits the production of various cytokines including interleukin1 and TNF-α.

TGF-β is a pleiotropic cytokine that regulates inflammatory response onset and resolution. *TGF-β* expression is up-regulated in rainbow trout when parasite pathogens are produced, according to research, and infection with IHNV on rainbow trout expression (Holland et al., 2003; Lindenstrom et al., 2004; Purcell et al., 2004). In this study, the bioflocs system increased *TGF-β* expression in all treatments when compared to the calibrator sample, indicating that the fish grown in the bioflocs system had a higher level of resistance. Although the fold change was normal in magnitude, it was statistically significant between the control and treatment groups.

Conclusion

Due to its numerous advantages, bioflocs technology is one of the most widely used advanced culture methods in shrimp and fish farming. It provides nourishment for the cultured animals and increases the farm's biosecurity while requiring little or no water exchange. This study and its findings have shed light on the impact of bioflocs consumption on *C. gariepinus* immunological performance and gene expression. This provides important information about bioflocs supplementation in feed and its development in culture ponds for the maintenance of optimal water quality parameters, growth performance, and immune gene regulation in *C. gariepinus* grow-out culture systems.

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Author Contributions

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