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Effect of addition of carbon and probiotics on water quality, production performance, and health of catfish (*Clarias gariepinus*) in biofloc systems

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Abstract
This study aimed to evaluate the use of organic carbon and probiotics on water quality, production performance, and haematological status in sangkuriang catfish. This experiment used a completely randomized design with four treatments: addition of organic carbon (C), the addition of probiotics (P), the addition of organic carbon and probiotics (CP), and control (K), each treatment with three replications. Catfish seeds measuring 1.86±0.60g 500 tails were stocked in a 1m3 pond. The seeds were fed commercial feed with a dose of 5% of the biomass. The results showed that the four treatments pH, dissolved oxygen, nitrite, and the bacterial population, had no significant effect. In contrast, TSS, TAN, and floc volume had a significant effect but were still in a good range of growth catfish. Specific growth rate, absolute growth, and final weight were higher in adding organic carbon (C) and (CP), survival, and the best feed conversion ratio in addition to organic carbon and probiotics than in control. Low haemoglobin and erythrocyte values were indicated as the cause of fish death. Based on the study's results, it can be concluded that adding organic carbon to the experimental pond can increase the specific growth rate. Adding organic carbon and probiotics increased survival and final weight but decreased the best feed conversion ratio compared to controls. Therefore, from the study results, it is enough for fish cultivators to utilize the addition of organic carbon to provide the best growth for fish cultured in a biofloc system.

Keywords: biofloc, organic carbon, probiotics, production performance, hematology status

Introduction
Intensive aquaculture technology in the current fisheries sector has the most promising prospects, one of which is the type of fish consumption business. Catfish have advantages: fast growth, more disease resistance, and higher production yields (Simanjuntak *et al*., 2020) [28]. However, many fish cultivators encounter various problems in the form of decreased water quality, high water demand, limited land, accumulation of organic matter, feces and feed residue in culture (Sukardi *et al*., 2018) [30]. Biofloc technology with low water replacement, high stocking density and most of their habitat microbiota are formed as complementary food sources (Souza *et al*., 2019; Zaidy & Eliyani, 2021) [29, 39].
Cultivation with high stocking density must be accompanied by adequate feeding according to the fish biomass supplied to the culture (J. Ekasari, 2009) [13]. Organisms can utilize only about 30-40% of the feed for growth and energy sources, and some will be wasted in the form of feces. Residual feed and metabolism accumulate in ammonia, which is toxic to fish (Avnimelech & Kochba, 2009) [6]. According to (Liu *et al*., 2018) [24] biofloc technology in fish and shrimp culture significantly removes toxic nitrogen, such as ammonia and nitrite levels. Increasing the C/N ratio with organic carbon supplementation causes communities in biofloc to lead to the dominance of heterotrophic bacteria (Kamilya *et al*., 2017) [23] Deep biofloc technology Aquaculture is currently used to neutralize toxic nitrogen concentrations, act as an in situ food source, and eradicate pollutants using carbon (Panigrahi *et al*., 2018) [26]. The system in biofloc technology is to balance carbon and nitrogen sources (Crab *et al*., 2012) [9]. Meanwhile, (De Schryver *et al*., 2008) [11] states that the main principle in biofloc technology is how to assimilate inorganic nitrogen (ammonia, nitrite and nitrate) in cultivation

activities by utilizing microorganisms as food sources. The growth of heterotrophic bacteria with the addition of organic carbon ammonia nitrogen substrate will be assimilated directly into cellular proteins (Ebeling *et al*., 2006) [12]. Using technology in the biofloc system that has been tested affects increasing growth and feed efficiency in shrimp (Xu & Pan, 2012) [36]. In addition, biofloc also stimulates the activity of protease enzymes in the digestion of fish & shrimp (Xu & Pan, 2012) [36] and has been successfully applied to rearing tilapia broodstock, thereby improving the quality of larval production (Julie Ekasari *et al*., 2015) [15].

With the addition of a carbon source in the biofloc system, when compared to the control, the number of suspended solids and nitrite was lower (Zaidy, 2022) [38]. (Wijaya *et al*., 2016) [34] Also stated that by rearing catfish by adding molasses, the C/N ratio 12 grew faster. Meanwhile (D. Zhao *et al*., 2016) [40] in the working group biofloc system, the carbohydrate source of molasses and 50% wheat had the best growth performance. In intensive system cultivation, the addition of carbohydrates can reduce inorganic nitrogen.

The use of probiotics on water quality is expected to be able to suppress or degrade the elements that influence the cultured water media. (Zokaeifar *et al*., 2014) [43] Stated that in various fishery commodities, the application of various stages of aquaculture and biological control and the provision of probiotic *Bacillus* positively contributed to the cultivation media. The use of probiotic applications with modified Microbiota increases nutrition for fish through immune response mechanisms, inhibits the growth of pathogenic microorganisms and enzymes and reduces compounds in aquaculture systems (Cienfuegos *et al*., 2017) [8]. In adding probiotics to heterotrophic bacteria that play a good role in improving water quality, there are several probiotic application systems, including *Bacillus* sp, *Bacillus subtilis*, *Pseudomonas*, *Bacillus licheniformis*, *Bacillus pumillus* and *Bacillus megaterium* (Otari & Ghosh, 2009) [25]. The use of probiotics in aquaculture other than as an alternative solution to overcome water quality problems and increase productivity (Avnimelech & Kochba, 2009) [6].

Applying biofloc technology with the addition of carbon sources and probiotics simultaneously in catfish (*Clarias gariepinus*) cultivation is still minimal, and further research needs to be done.

Materials and Methods

Experimental Design

The experiment used a completely randomized design, consisting of four treatments, control (K) addition of probiotics (P), the addition of organic carbon (C) addition of organic carbon and probiotics (CP), each treatment with three replications. The fish used was Sangkuriang Catfish (*Clarias gariepinus)* weighing 1.86±0.60g from the Gelam River Freshwater Cultivation Fisheries Center, Jambi. Organic carbon comes from molasses with a concentration of 51.6 Organic C. Commercial probiotics containing bacteria *Bacillus subtillis, Bacillus p olymixa, Bacillus megaterium, Bacillus coagulans, Bacillus cereus, Bacillus alvei, Bacillus amyloliquefaciens, Bacillus brevis, Bacillus circulans firmus, Bacillus circulans, Bicillus pumilus.*

The research pond was prepared for 12 round tarpaulin tubs with a volume of 1 m3 and equipped with aeration. In the preparation of the treatment pond, 8.16 ml/ m3 of molasses was added, and probiotics of 2 g/m3 were added. After seven days, Sangkuriang catfish seeds were stocked with as many as 500 fish/m ^{3}. Feed with a protein content of 32.66% (Table 1) was given by feeding rate of 5%/day of biomass weight, given three times a day. Adding molasses was carried out every day for as much as 17.6% of the feed, added 2 hours after feeding. The addition of probiotics was carried out every four days, as much as 2 g/m3. The amount of feed was adjusted every ten days. Water changes were carried out in the control treatment pond (K) every four days as much as 60-70%, and in the biofloc treatment pond, when the volume of biofloc was more than 100 ml/L, as much as 60-70% of the total volume. The research time was carried out for 60 days.

The results of the proximate test of the feed given can be seen in Table 1.

Table 1: Proximate results of feed

Component	Unit	Rate
Water content	%	8.88
Crude protein	%	32.66
Crude fat	%	4.74
Ash	%	9.31
Coarse fiber	%	6.56

Water quality

Water quality parameters are measured periodically according to Official Method part 4 (AOAC, 2005) [3] including dissolved oxygen, and temperature measurement using a digital DO meter (AMTASH EC900) and pH with a digital pH meter (Lutron WA-2017SD). Ammonia and nitrite are tested using the *spectrophotometric* method. Meanwhile, total suspended solids (TSS) using the *gravimetric method*.

Floc Volume and Consumption

Calculating the abundance of *Bacillus* bacteria in water was carried out at the end of the study. Sampling using the total plate count (TPC) technique on trypticase soy agar (TSA) media, the bacteria were incubated for 24 hours, after which the number of colonies that grew was calculated using the formula:

$$TKB = \Sigma \text{ Colony x } \frac{1}{\text{Spread Volume}} \text{x } \frac{1}{fp} \quad (1)$$

Measurement of floc volume was carried out every week in each pond to determine the density of floc particles in the water. A sample of 1000 ml of water was deposited for 1 hour in an *Imhoff cone measuring cup*, then recorded and calculated the amount of precipitated floc volume using the formula:

$$Vol.Flok = \frac{Vol.Endapan}{Vol.Sampel\ Air} \quad (2)$$

Measurement of the proximate test floc quality parameters was carried out in the laboratory using the official method in section 4 (AOAC, 2005) [3]. The ash content was tested using an ashing furnace at a temperature of 600 ^{o} C for 4 hours. The protein content was tested using the *Kjeldahl method.* The crude fiber was tested using an electric furnace at 600 ^{o}C for 1 hour, the fat content was analyzed by using *Soxhlet fat extraction*, and the moisture content was using an oven at 65 ^{o} C for 24 hours.

Production Performance

Specific growth rate, absolute growth, final weight, survival and feed conversion ratio using the following formula:

$$SGR = \frac{(Ln(Wt)-(Ln(Wo)}{Wo} x\ 100\% \quad (3)$$

The absolute growth calculation is calculated using the formula:

$$W_m = W_t - W_0 \quad (4)$$

The final weight is calculated using the following formula:

$$G = B_{t-B_0} \quad (5)$$

Survival (SR) is calculated using the formula:

$$SR\ (\%) = \frac{N_t}{N_0} X\ 100 \quad (6)$$

Feed conversion ratio (FCR) is calculated using the formula:

$$FCR = \frac{F}{(W_t - D) - W_0} \quad (7)$$

Haematological status

Observation of haemoglobin using the Sahli method using a Sahlinometer (Wedemeyer & Yasutake, 1977) [33]. The level of hematocrit blood samples was taken using a microhematocrit tube and stirred using a *centrifuge* at 6000 rpm for 5 minutes according to the equation (Anderson & Siwicki, 1995) [2]:

$$He = \frac{a}{b}\ x\ 100\% \quad (8)$$

The total erythrocytes were measured using the procedure (Blaxhall & Daisley, 1973) [7]. Blood samples were taken using a pipette containing red grains on a 0.5 scale. Then the *hayem reagent solution was added*, then stirred by shaking the pipette for 3-5 minutes until the blood with the hayem solution was evenly mixed, the first drop was removed, and the subsequent drop was dripped on a hemacytometer and observed under a microscope with 400 times magnification. Calculation of total erythrocytes using the formula:

$$TE = \Sigma TE\ x\ \frac{1}{Vol.kotak}\ x\ \frac{1}{fp} \quad (9)$$

Data analysis

Statistical software analyzed the data using RAL one-way ANOVA with a 95% confidence level (sig. 0.05). To determine whether the dose of carbon and probiotics affected the specific growth rate, absolute growth, final weight, survival and feed conversion ratio, and continued with the Duncan Multiple Range Test (DMRT). For water quality data, flock volume and haematological status were analyzed descriptively.

Results and Discussion

Water quality

Table 2 shows that dissolved oxygen, pH, and nitrite were not significantly different between treatments (P>0.05), while TAN and TSS were significantly different between treatments (p<0.05). The highest TAN was in the pond adding probiotics (p) and adding organic carbon (C), while the lowest was in the control pond (K). TSS in the treatment pond with the addition of organic carbon and probiotics (CP), the addition of organic carbon (C), and the addition of probiotics (P) were higher than in the control pond (K). TAN and TSS in the control treatment were lower because of the change of water every four days, so TAN and TSS were wasted. TAN and TSS in the treatment ponds were higher because there was no water change during the experiment.

The study by (Zaidy, 2022) [38] showed that the TAN levels in the biofloc system's maintenance medium without water replacement were 2.11 – 2.33 times higher than changing the water. The water quality of each treatment is still within the acceptable range (Augusta, 2016) [4] states water quality is between water temperature 25 – 33 °C, dissolved oxygen 3.3 – 4.4 mg/l (Avnimelech, 2007) [5] and pH 6.4-9.5. The results of research by (Sumitro *et al*., 2021) [31] showed that in three catfish ponds with densities of 500, 750, and 1000 fish/m3, the concentration of dissolved oxygen in the first week and then in the third week the oxygen concentration decreased to 1.8-2.0 mg/L and the specific growth rate relatively high 5.81-6.01% / day.

Table 2: Water quality

S. No.	Parameter	Treatment			
		(K)	(P)	(C)	CP
1.	Temperature (°C)	26.3-29.0	26.2 – 30.0	26.1 – 29.0	26.0 – 29.0
2.	DO (mg/L)	2.92±0.28 ^{a}	3.09±0.62 ^{a}	3.23±0.37 ^{a}	3.63±0.28 ^{a}
3.	pH	5.67±0.16 ^{a}	5.6±0.13 ^{a}	5.66±0.03 ^{a}	5.59±0.02 ^{a}
4.	TAN (mg/L)	7.3±3.0 ^{a}	17.6±1.9 ^{b}	14.4±1.4 ^{b}	12.1±5.6 ^{ab}
5.	Nitrite (mg/L)	0.3±0.17 ^{a}	0.56±0.2 ^{a}	0.36±0.11 ^{a}	0.46±0.25 ^{a}
6.	TSS	506.66±98.6 ^{a}	993.33±142.2 ^{b}	1046±58.27 ^{b}	1118.66±122.9 ^{b}

During the 60-day maintenance period, the TAN value increased, and the nitrite value was still within the tolerance threshold. Based on the results of (Zaidy & Eliyani, 2021) [39] research, the tolerance value for TAN levels is 0.05±0.02 to 0.11 mg/l and nitrite <1 mg/l is 0.082±0.009 to 0.79. This condition can occur because it is supported by several test parameters that can affect nitrifying bacteria's growth and development, such as DO, pH, and the appropriate temperature. Nitrifying bacteria are aerobic bacteria, which in their growth process, always require oxygen. To be able to oxidize 1mg of ammonia, the nitrifying bacteria requires 2mg/l - 4.6 mg/l of dissolved oxygen to work optimally (Eliyani *et al*., 2015) [16]. The high value of TSS in the biofloc treatment compared to the control was thought to be due to the addition of molasses as a carbon source, causing the abundance of bacterial colonies to increase (Wang *et al*.,

2015) [32]. In cultivation media, the more flocs formed, the TSS value will increase. In biofloc-based aquaculture technology, the recommended TSS value is 200-1000 mg/l (De Schryver *et al*., 2008) [11].

Biofloc and Bacteria Volume

Table 3. The results of the analysis of floc volume in each treatment showed a significant effect *($p<0.05$)*, while the bacterial population had no significant effect between treatments *($p>0.05$).*

Table 3: Parameters of biofloc and bacteria in each treatment

S. No.	Parameter	Treatment			
		(K)	(P)	(C)	CP
1.	Floc Volume (ml/L)	17.6±1.5 [a]	32.3±2.5 [b]	111±3.6 [cd]	106±1.7 [c]
2.	Bacteria (Cfu/ml)	0.26 10^6±0.02 [a]	0.26 10^6±0.02 [a]	0.26 10^6±0.02 [a]	0.41 10^6±0.24 [a]

The highest floc volume was found in the addition of organic carbon (C), and the addition of organic carbon and probiotics (CP) compared to the control. Floc volume is one way to see the abundance of organisms contained in biofloc media. Adding organic carbon to the media positively affects the volume of the floc. (Dauda *et al*., 2018; Imron *et al*., 2014; Xu & Pan, 2012) [36, 10, 19] stated that suspended solids in the floc volume are composed of phytoplankton and zooplankton types. According to (Xu *et al.*, 2013) [37], Floc formation positively correlates with adding organic carbon into the culture medium.

The addition of probiotic bacteria from the type of *Bacillus* sp in this study did not give a significantly different value ($p>0.05$). However, the abundance of bacteria increased with the addition of organic carbon and probiotics (CP). This result is because the nitrifying bacteria added to the test media with the addition of organic carbon and probiotics can grow and reproduce, so adding bacteria to the test media can increase the number of bacterial populations. This condition can occur because it is supported by several test parameters that can affect nitrifying bacteria's growth and development, such as DO, pH, and the appropriate temperature. Nitrifying bacteria are aerobic bacteria, which in their growth process, always require oxygen. Nitrifying bacteria to oxidize 1 mg of ammonia requires 2 mg/l - 4.6 mg/l of dissolved oxygen to work optimally (Eliyani *et al*., 2015) [16].

The use of nitrifying bacteria of the *Bacillus* type, which functions as nitrifying bacteria in this study, follows the research of (Eliyani *et al*., 2015) [16] stated that to improve water quality by using a variety of bacteria from *Bacillus* sp.

Biofloc Nutrition

The results of the proximate test analysis of floc composition are in Table 4. The protein content of the floc in treatment (C) showed a relatively high value of 25.92% and fat content of 4.2%. This result is following research conducted by Anand *et al.* (2014) [1] that the composition contained in biofloc consists of 24.30% protein, essential fats such as palmitic (46.54%), linoleic (10.67%), cis vaccenic (15.37%) and oleic (9.19%). It contains nutrients that are good enough for fish growth. In line with this, Jimoh *et al.* (2014) [20] stated that the compounds in flocs affect catfish growth which is omnivorous and tends to be carnivorous, so that bioflocs can increase the intake of protein sources in feed.

Table 4: Proximate test results of biofloc nutrients in each treatment

Component	Treatment			
	(K)	(C)	(P)	(CP)
Crude protein (%)	23.04	23.21	25.92	24.9
Crude fat (%)	1.24	3.53	4.2	3.55
Ash (%)	6.93	7.16	5.59	6.85
Crude fiber (%)	16.83	20.64	17.43	17.04
Carbohydrate (%)	51.96	45.46	46.86	47.66

Biofloc Consumption

Based on the research results, the composition of plankton species in the rearing media and fish intestines can be seen in Table 5. Comparing the types of plankton in the media and intestines have the same types, showing that catfish consume plankton.

Table 5: Results of identification of density and types of plankton in water media and fish intestines in each treatment

Treatment	Density and type Plankton on medium (Ind/ml)	Types of food in the intestines
(K)	Bacillariophyceae	Bacillariophyceae
	Chlorophyceae	Chlorophyceae
	Cyanophyceae	Cyanophyceae
	Zooplankton	Zooplankton
(P)	Bacillariophyceae	Bacillariophyceae
	Chlorophyceae	Chlorophyceae
	Cyanophyceae	Cyanophyceae
	Zooplankton	Zooplankton
(C)	Bacillariophyceae	Bacillariophyceae
	Chlorophyceae	Chlorophyceae
	Cyanophyceae	Cyanophyceae
	Zooplankton	Zooplankton
(CP)	Bacillariophyceae	Bacillariophyceae
	Chlorophyceae	Chlorophyceae
	Cyanophyceae	Cyanophyceae
	Zooplankton	Zooplankton

This study is in line with research (Julie Ekasari *et al*., 2014) [14] which found evidence that fish consumed biofloc. According to (Hargreaves, 2013) [18], the biofloc system consists of algae, bacteria, protozoa, zooplankton, and other

microorganisms. (Xu *et al*., 2016) [40] Stated that the dominance of microalgae and heterotrophic bacteria was higher in the cultivation of biofloc systems. Table 5 shows the types of plankton identified in the catfish intestine, which consist of Phytoplankton (Class Bacillariophyceae, Chlorophyceae, and Cyanophyceae) and Zooplankton. This result follows the presence of plankton in the rearing media detected with the same class. Based on each rearing, it showed that the type of plankton was more abundant in the addition of probiotics (P), the addition of organic carbon (C) and the addition of organic carbon and probiotics (CP) compared to the control treatment pond (K)

Haematology Status

Table 6: Haematological parameters of catfish (*Clarias gariepinus)* in each treatment

Parameter	Standard Healthy (Normal)	Treatment			
		(K)	(P)	(C)	(CP)
Haemoglobin (Hb/100 ml)	12.- 14	8.53±4.78 [a]	7.63±2.82 [a]	7.43±1.51 [a]	8.43±0.55 [a]
Haematocrit (%)	30.8- 45.5	30.47±7.32 [a]	30±10.78 [a]	33.33±4.12 [a]	34.76±4.12 [a]
Erythrocytes (X10^6 cells/mm^3	3.18	1.73 x 10^6 ±0.39 [a]	1.92 x10^6±0.70 [a]	2.16 x10^6±0.63 [a]	1.92 x10^6±0.69a

Production Performance

Specific growth rate, absolute growth pointed, and final weight showed a significant effect between treatments (p<0.05). Meanwhile, survival and feed conversion ratio had no significant effect between treatments (p>0.05) Table 6

Table 7: Production Performance

Parameter	Treatment			
	(K)	(P)	(C)	CP
Specific growth rate (%)	3.24±0.34 [a]	3.36±0.85 [a]	3.55±0.16 [b]	3.50±0.15 [a]
Growth (g)	23.89±0.93 [a]	27.30±2.40 [a]	33.90±6.02 [b]	32.13±5.07 [a]
Final weight (Kg)	9.56±0.58 [a]	11.4±1.3 [a]	13.13±2.4 [b]	13.46±2.27 [a]
Survival (%)	74.26±4.23 [a]	78.66±12.2 [a]	73.33±1.50 [a]	79.20±4.58 [a]
Feed Conversion Ratio (FCR)	0.89±0.09 [a]	0.99±0.04 [a]	0.90±0.03 [a]	0.88±0.04 [a]

Values in rows with the same letter notation have no significant effect (*p>0.05*) Duncan's test showed that the best specific growth rates were obtained from adding organic carbon (C) and organic carbon and probiotics (CP) compared to controls. As for the final weight, the best results were obtained from adding organic carbon and probiotics (CP) compared to the control.

This result is in line with several studies that have been conducted (Gunadi *et al*., 2011 [17]; Jimoh *et al*., 2014 [21]; Sgnaulin *et al*., 2018) [27] that with the use of molasses in fish farming using a biofloc system can produce higher biomass. (P. Zhao *et al*., 2012) [41] stated that the biofloc system treatment on shrimp resulted in 41.3% higher production. The specific growth rate with adding organic carbon (C) resulted in the highest value of 3.55 ± 0.16% with a significance (0.011). This result follows the research conducted by (Joseph *et al*., 2014) [22], which stated that the carbon source of molasses and wheat bran had the best 50% growth rate. This is thought to be due to the many factors in the biofloc system that stimulate growth in the form of amylase, protease, lipase, and undetectable extracellular enzymes (Zhao *et al*., 2014) [42].

Significant influence on the specific growth rate, absolute growth and final weight caused by nutrient intake other than feed, which is from floc nutrition. The volume of floc in the treatment of addition of organic carbon (C) and treatment of addition of organic carbon and probiotics (CP) has a higher value (Table 2), so the amount of nutrient availability is more. The low survival rate of fish was due to the high mortality of fish during the 31 - 40 day rearing period for the control treatment (K) and the addition of probiotics (P), while for the treatment (C) and (CP), the high mortality rate of fish seen in age 51 - 60 days. This condition follows the data on the haematological status of fish, where all treatments showed unhealthy fish conditions.

Conclusion

The treatment of adding organic carbon and probiotics to pH, dissolved oxygen, ammonia, and nitrite was still within the range for catfish growth. Floc volume, TSS, TAN and types of plankton in the catfish intestine were more found in the organic carbon addition pond than in the control. The specific growth rate and absolute growth in the organic carbon addition pond were not different from the organic carbon addition, and probiotic ponds were higher than the control pond. In addition to organic carbon and probiotic ponds, biomass was higher than the control. Based on research data, the production performance of ponds fed with organic carbon was no different from ponds fed with a mixture of organic carbon and probiotics. Therefore, the biofloc system of fish farming with organic carbon added saves more costs and labour compared to that added with a mixture of organic carbon and probiotics.

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