P R E P R I N T

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Influence of Dietary Carbohydrate Types on Growth Outcomes and Microbial Community Structure in Biofloc-Reared Stinging Catfish (*Heteropneustes fossilis*)

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Abstract

Aquaculture plays a crucial role in global food and nutritional security, with biofloc technology (BFT) emerging as a sustainable approach to enhance productivity and environmental health. BFT improves water quality and feed efficiency by converting waste nutrients into microbial biomass beneficial to cultured species. *Heteropneustes fossilis*, a hardy and high-value catfish, holds strong potential for integration into BFT systems to promote growth and gut health. The present study evaluated the effects of different carbohydrate sources on water quality, growth performance, and microbial communities in *H. fossilis* culture. The 120-day experiment was conducted in 450 L tanks with five treatments: three biofloc groups (T1-molasses, T2-rice flour, and T3-molasses+rice flour), one probiotic group, and a single control. Fingerlings (2.86/ ±/ 0.5/ g) were stocked at a density of 700/ m{ ^{3} (n/ =/ 210 per tank). Results indicated significant improvement in water quality parameters across all biofloc treatments compared to the control, with treatment T3 (a combination of molasses and rice flour) exhibiting the most pronounced effects. T3 optimised floc volume (34.77 ± 0.30), enhanced microbial diversity, and supported the highest growth and survival rates (1.90 ± 0.02 & 96.35 ± 0.7). Key performance indicators, including percentage weight gain and feed conversion ratio (782.52 ± 25.52 & 1.60 ± 0.10), were superior in T3, highlighting the synergistic benefits of mixed carbon sources. Moreover, T3 promoted greater proliferation of *Bacillus* spp. and total heterotrophic bacteria in both water and gut samples, improving nutrient utilisation and fish health. These findings demonstrate that mixed carbohydrate-based biofloc systems can sustainably improve water quality, microbial stability, and growth performance in *H. fossilis* aquaculture.

Keywords: Biofloc technology (BFT), carbon sources, probiotic bacteria, growth performance, aquaculture productivity

Introduction

Aquaculture is a key driver in achieving sustainable food supply, contributing to the nutritional security of approximately 800 million people worldwide (FAO, 2019). It is increasingly recognised as a key sector for meeting the current and future demands for high-quality animal protein (FAO, 2024). However, achieving this goal requires adopting eco-friendly farming practices that promote higher yields with minimal environmental impact (Avnimelech, 2009; Panigrahi et al., 2020). Such practices must benefit both the environment and farmers (Dauda, Ajadi, Tola-Fabunmi, & Akinwole, 2019; David, Pinho, Keesman, & Garcia, 2021; Jiang,

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Bhattarai, Pahlow, & Xu, 2022). One such innovative and sustainable approach is biofloc technology (BFT), which has gained popularity among farmers over the past two decades. BFT effectively addresses critical challenges related to feed efficiency, water quality, and microbial dynamics. BFT is characterised by little to no replacement of culture water, relying instead on the development of microbial aggregates (bioflocs) that utilise externally added carbon sources. This system maintains water quality by promoting the microbial assimilation of excess nitrogen, particularly ammonium, through maintaining an effective carbon-to-nitrogen (C:N) ratio. As a result, BFT offers a high-yield, eco-friendly solution that enhances biosecurity and fish health (Khanjani & Sharifinia, 2020; Habib, Batool, Rehman, & Naz, 2022; Khanjani, Sharifinia, & Hajirezaee, 2022). Often described as a "blue revolution" in aquaculture, BFT addresses critical challenges including environmental degradation, water scarcity, and disease management while contributing to sustainable animal protein production (Akange, Aende, Rastegari, Odeyemi, & Kasan, 2024; Minaz, Yazici, Sevgili, & Aydin, 2024). The core principle of biofloc technology lies in the transformation and recycling of waste nutrients, particularly inorganic nitrogen compounds such as ammonia (NH_3-N) and nitrite (NO_2-N) originating from uneaten feed and faeces. These compounds are converted into microbial biomass through the activity of heterotrophic bacteria, which utilise both organic carbon from waste and supplementary carbon sources to regulate the system's C:N balance (Silva, Falcon, Pessôa, & Correia, 2017; Minabi, Sourinejad, Alizadeh, Ghatrami, & Khanjani, 2020; Panigrahi et al., 2020). The resulting microbial biomass (biofloc) is rich in protein and can be directly consumed by farmed animals, serving as an *in-situ* protein supplement. This not only improves feed efficiency but also enhances water quality and gut health, thereby contributing to the overall sustainability and profitability of aquaculture production (Yu et al., 2023).

Heteropneustes fossilis, commonly known as 'singhi', is a catfish belonging to the family Heteropneustidae and the order Siluriformes. It is a commercially important native species cultured across various Asian countries, including India, Thailand, Bangladesh, Pakistan, Nepal, Sri Lanka, Myanmar, Indonesia, and Cambodia (Burgess, 1989; Akand, Hasan, & Habib, 1991; Hossain et al., 2015). Under optimal conditions, *H. fossilis* can attain a weight of 80–100 g within a 10-month culture period and is typically marketed at sizes above 50 g (Das, Ferosekhan, Choudhary, Kumari, & Mohanty, 2024). It is highly regarded for its tender flesh, pleasant taste, absence of pin bones, and low-fat content, in addition to its traditional medicinal value. Notably, the species contains a notably high level of iron, measuring 226 mg per 100 g, surpassing that of many other freshwater fish species (Chakraborty & Nur, 2012), and is thus advised as a dietary supplement for people suffering from anaemia (Kohli & Goswami, 1989; Alok, Krishnan, Talwar, & Garg, 1993). These nutritional qualities drive its strong market demand and high economic worth, with prices typically ranging from USD 6 to USD 8 per kg. Furthermore, *H. fossilis* is recognised for its ability to tolerate adverse environmental conditions, including low dissolved oxygen levels and high stocking densities, as well as its adaptability to pelleted feeds. These characteristics make it a promising candidate for diversifying freshwater aquaculture in India (Tharakan & Joy, 1996; Kutty, 2001). The species has also shown encouraging growth performance in indoor tank systems, reinforcing its potential for integration into biofloc technology (BFT) systems (Zafar & Khan, 2019; Sahoo, Ferosekhan, Sahoo, Das, & Giri, 2022; Shamsuddin et al., 2022). The biofloc system critically relies on maintaining an appropriate C:N ratio, which is achieved through the regulated supply of carbon sources and feed inputs. The addition of an external carbon source promotes the growth of heterotrophic bacteria, thereby enhancing nitrogen assimilation and maintaining the desired C:N balance in the system (Avnimelech, 1999; Hargreaves, 2006; Crab, Defoirdt, Bossier, & Verstraete, 2012). Different organic carbon sources likely influence the makeup of biofloc communities (Deng et al., 2018). Carbon sources are essential for balancing the carbon-to-nitrogen ratio by promoting the growth of heterotrophic bacteria, which leads to biofloc formation, reduces Total Ammonia Nitrogen (TAN) levels, and consequently enhances water quality. Therefore, this study aimed to assess the growth and gut health of *H. fossilis* cultured in biofloc technology (BFT) systems supplemented with various carbon sources.

Despite the growing application of biofloc technology in aquaculture, most studies have focused on single carbon sources and commercially dominant species such as tilapia and shrimp, with limited attention to freshwater catfish species like *H. fossilis*. Furthermore, the influence of combined carbohydrate sources on microbial community dynamics and their subsequent effects on gut health and growth performance remains poorly understood. Addressing these gaps, the present study evaluates the synergistic effects of different carbon sources, including a mixed carbohydrate strategy, on water quality, microbial communities, and growth performance of *H. fossilis* cultured under biofloc conditions. By integrating growth, water quality, and gut–environment microbial interactions, this study provides a more comprehensive understanding of carbon-driven biofloc dynamics, offering practical insights for optimising sustainable aquaculture practices.

Materials and Methods

The study was carried out for 120 days in 450 L circular fibre-reinforced plastic (FRP) tanks with a working water volume of 300 L at the Germplasm Resource Centre of ICAR- National Bureau of Fish Genetic Resource, Lucknow, India. The experiment was laid out in a Completely Randomized Design (CRD) consisting of five treatments in triplicate: T1 (molasses), T2 (rice flour), T3 (molasses + rice flour; 1:1), T4 (probiotic only; Blueweight Everfresh Pro 500 GM Aqua Probiotics, India), and a control (without carbon source or probiotic). All the experimental tanks were filled with dechlorinated freshwater and were provided with adequate aeration by a 2 HP blower. To stimulate floc production, a mixture of calcium carbonate (0.08 g L^{-1}), molasses (0.26 g L^{-1}), rice flour (0.26 g L^{-1}), probiotic (0.06 g L^{-1}), and common salt (1 g L^{-1}) was added to the respective tanks. After 7 days, when the water became turbid, fingerlings (2.86 ± 0.5 g) were stocked at a density of 700 fish m{ ^{3} (n = 210 per tank) with a working volume of 300 L. The fish were fed with a commercial balanced floating catfish diet (Growel Feeds Pvt. Ltd., India, 40% crude protein and 6% crude fat) at the rate of 3% of the body weight thrice a day (at 9.00 h, 13.00 h and 17.00 h) for 120 days. The feeding rate of the fish was adjusted every fortnight. A carbon source was added every alternate day to maintain a target C:N ratio of 15:1 to promote heterotrophic bacterial growth. The quantity of carbon source added was calculated relative to the daily feed ration and its crude protein content, following the method described by Perez-Fuentes, Hernández-Vergara, Pérez-Rostro, and Fogel (2016). Nitrogen input was calculated from the feed only. The nitrogen content of the feed was determined by dividing the crude protein percentage by 6.25 (i.e., assuming crude protein contains 16% nitrogen). The amount of carbon required to achieve the desired 15:1 C:N ratio was then calculated based on this nitrogen input, and the corresponding quantity of the selected carbon source was added accordingly.

Nitrogen from feed (g) = [Daily feed amount (g) × Crude protein (%)] / 6.25

Carbon required (g) = Nitrogen from feed (g) × 15

A 30% weekly water exchange was maintained in the control tanks, whereas the biofloc tanks were maintained without routine water exchange, except for the fortnightly replenishment of water lost through evaporation (≤ 5%).

Water quality parameters such as temperature (°C), pH, total dissolved solids (TDS, mg L^{-1}), and dissolved oxygen (DO, mg L^{-1}) were regularly monitored during the experiment using a Hanna multi-parameter probe (HI98194, Leighton Buzzard, Bedfordshire, UK). Total ammonia nitrogen (TAN) (mg L^{-1}) and nitrate-nitrogen (mg L^{-1}) were measured with a Hanna Multiparameter Photometer (HI83300, Leighton Buzzard, Bedfordshire, UK). Floc volume (mL L^{-1}) was measured weekly using 1,000 mL Imhoff cones after 20 min of sedimentation. The concentration of total suspended solids (TSS) was measured following Standard Methods 2540 D. A known volume of water was filtered through a 0.45 μm cellulose acetate membrane filter, and the dried residue retained on the filter was weighed. The TSS concentration was then calculated using the following formula:

$$TSS\ (mg\ L^{-1}) = \frac{(Wf - Wi)\ (g)\ X\ 1,000,000}{V}$$

Where:

Wf = final weight (g)

Wi = initial weight (g)

V = sample volume (mL)

Length and weight of the fish (n=20) were randomly recorded every month to estimate growth from each treatment and control. At the end of the experiment, the total number of surviving fish was counted to determine the survival rate. Growth performance indices, including weight gain (WG%), Net biomass (kg m^{-3}), survival (%), specific growth rate (SGR), and feed conversion ratio (FCR), were calculated using the following formulae (De Silva & Anderson, 1995; APHA, 2017):

Weight gain (%) = $\frac{(W_f - W_i)}{W_i} \times 100$

Where:

W_f = average final weight (g)

W_i = average initial weight (g)

Specific growth rate (% day^{-1}) =

$$\frac{[\ln(W_f) - \ln(W_i)]}{t} \times 100$$

Where:

W_f = final weight (g)

W_i = initial weight (g)

t = duration of rearing period (days)

Survival rate (%) = $\frac{N_s}{N_i} \times 100$

Where:

N_s = number of survived fish

N_i = number of fish stocked

Apparent FCR = $\frac{F}{B_g}$

Where:

F = total weight of feed provided (g)

B_g = net biomass gain (g)

Total heterotrophic bacteria (THB) and probiotic bacteria were enumerated at 30-day intervals. Biofloc samples were collected from each replicate by thoroughly mixing the water and transferring 50 mL into sterile Eppendorf tubes. The samples were homogenised and then serially diluted tenfold in sterile normal saline (0.85% NaCl) as diluent, following standard microbiological procedures for bacterial enumeration (APHA, 2017; ISO, 2017). Bacterial counts were determined by spread plating 100 µL of the diluted samples, in triplicate, onto tryptone soya agar (TSA) for total bacteria, and HiCrome™ Bacillus selective agar (HBSA) for *Bacillus* species (HiMedia Laboratories Pvt. Ltd., Mumbai, India). Similarly, bacterial enumeration from gut samples was performed monthly. Gut tissue (1 g) was aseptically removed (euthanising the fish by pithing), diluted (10% w/v) in normal saline and homogenised, thereafter serially diluted and 100 µL from each dilution was spread onto TSA/HBSA medium. After 24 hours of incubation, colonies ranging between 30 and 300 CFU were counted. Bacterial colonies, based on their colour, morphology, and Gram-staining appearance, were preserved in 20% glycerol stocks.

The cryopreserved stock of gut and biofloc isolates was cultured on TSA for molecular identification of *Bacillus* bacteria by 16S rRNA PCR amplification and sequencing. Genomic DNA from the isolates was extracted using PureLink™ Genomic DNA Mini Kit (Invitrogen, USA) following the manufacturer's instructions. The universal primers of 16S rRNA 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (TACGGCTACCTTGTTACGACTT) were used (Weisburg, Barns, Pelletier, & Lane, 1991). PCR was carried out using master mix according to the manufacturer's instructions (Takara Taq PCR mini kit) in HiMedia Prima (HiMedia Laboratories Pvt. Ltd., Mumbai, India) 96-well thermal cycler, and amplification products were visualised using a gel documentation system (NuGenius, Syngene, Cambridge, UK). Amplified PCR products were sequenced by Sanger sequencing. The obtained sequences were compared with GenBank database entries using the BLASTn algorithm for identification.

The data generated from the experiment were statistically analysed using SPSS (version 26). Data were first tested for normality using the Shapiro-Wilk test and for homogeneity of variances using Levene's test. All data met the assumptions of normality and homogeneity of variance ($p > 0.05$), so no data transformations were required. One-way analysis of variance (ANOVA) was then performed to determine significant differences among treatments and the control group, followed by Tukey's HSD post hoc test for multiple comparisons where significant differences were detected. Results were considered statistically significant at $p < 0.05$. Exact p-values are presented in the tables. CFU values were log-transformed before statistical analysis.

Results and Discussion

Different carbon sources in biofloc systems significantly influence floc composition, size, and nutritional quality, thereby affecting water quality and fish growth. In this study, various carbon sources and their combinations were evaluated to understand their impact on water quality and growth performance of *H. fossilis*.

The physico-chemical parameters measured in the experimental tanks across treatments (T1, T2, T3, T4) and the control group are summarised in Table 1, including pH, temperature, TDS, TSS, floc volume, nitrite-nitrogen, and total ammonia-nitrogen (TAN).

The pH remained stable across all treatments with no significant differences ($p > 0.05$), ranging from 7.2 to 8.2, within the optimal range (7.0–8.0) reported for *H. fossilis* culture in biofloc systems. T1 exhibited a slightly higher mean pH (8.04 ± 0.06) and T4 the lowest (7.90 ± 0.04); however, no significant difference was observed ($p > 0.05$). Similarly, temperature did not differ significantly among treatments, ranging from 26 to 32 °C, remaining within the optimal physiological range (26–30 °C), supporting efficient metabolic activity and microbial processes in biofloc systems. This consistency reflects the controlled experimental conditions, ensuring that treatment effects were accurately evaluated. In contrast, TDS and TSS varied significantly ($p < 0.05$) among treatments. T1 exhibited the highest TDS (932.2 ± 45.7 mg L^{-1}) and TSS (364.88 ± 20.9 mg L^{-1}), whereas the control recorded the lowest values of 317 ± 13.1 mg L^{-1} and 63.0 ± 5.84 mg L^{-1}, respectively. These elevated levels in biofloc treatments indicate increased suspended particulate matter, which supports microbial colonisation and nutrient recycling. Similar observations have been reported by Avnimelech (2009) and De Lima, de Souza, Girao, Braga, and Correia (2018), highlighting the importance of suspended solids in enhancing microbial activity and nitrogen cycling efficiency.

Table 1. Water quality parameters across all the treatments and control

Parameters	T1	T2	T3	T4	Control	p-value
pH	8.04 ± 0.06^{a}	7.98 ± 0.05^{a}	7.97 ± 0.04^{a}	7.90 ± 0.04^{a}	8.00 ± 0.02 ^{a}	0.082
Temperature (°C)	26.56 ± 0.05^{a}	26.41 ± 0.09^{a}	26.38 ± 0.06^{a}	26.27 ± 0.29^{a}	26.50 ±0.06 ^{a}	0.171
TDS	932.2 ± 45.7^{b}	872.9 ± 29.1^{b}	871.1 ± 25.4^{b}	364.2 ± 13.9^{a}	317±13.1^{a}	0.000
TSS	364.88 ± 20.9^{c}	219.7 ± 28.4^{b}	272.4 ± 25.7^{b}	92.5 ± 4.13^{a}	63.0±5.84^{a}	0.000
Floc volume	37.40 ± 1.44^{a}	29.15 ± 0.72^{c}	34.77 ± 0.30^{b}	9.00 ± 0.38^{d}	4.19 ±0.53^{e}	0.000
Nitrite nitrogen (mg L^{-1})	0.23 ± 0.01^{c}	0.14 ± 0.0^{a}	0.15 ± 0.0^{a}	0.19 ± 0.0^{b}	0.24 ±0.0^{c}	0.000
Total ammonia nitrogen (mg L^{-1})	1.06 ± 0.03^{ab}	0.80 ± 0.07^{a}	0.71 ± 0.04^{a}	0.81 ± 0.29^{a}	1.31 ±0.04^{b}	0.002

Values presented as mean ± SE; mean values in the same column with different superscripts (a, b) differ significantly ($p < 0.05$).

Table 2. Mean values of growth parameters in the treatments and control group

Treatment	Average Initial weight (g)	Harvested weight (g) (kg)	Net weight (g) (%)	Net biomass (kg)	Percentage weight gain (%)	Specific growth rate (%)	Survival (%)	Apparent FCR
T1	2.8	22.14 ± 0.79^{b}	19.28 ± 0.79^{b}	3.79 ± 0.03^{c}	574.13 ± 27.62^{b}	1.70 ± 0.03^{b}	93.80 ± 1.7^{ab}	1.80 ± 0.00^{ab}
T2	2.8	16.51 ± 0.68^{c}	13.65 ± 0.68^{c}	2.55 ± 0.03^{b}	377.27 ± 23.78^{c}	1.46 ± 0.03^{c}	89.52 ± 3.6^{ab}	2.20 ± 0.30^{cd}
T3	2.8	28.10 ± 0.73^{a}	25.24 ± 0.73^{a}	5.10 ± 0.04^{d}	782.52 ± 25.52^{a}	1.90 ± 0.02^{a}	96.34 ± 0.7^{b}	1.60 ± 0.10^{a}
T4	2.8	15.41 ± 0.08^{d}	12.55 ± 0.08^{cd}	2.43 ± 0.02^{b}	338.81 ± 2.80^{cd}	1.40 ± 0.00^{cd}	92.38 ± 1.0^{ab}	2.00 ± 0.00^{bc}
Control	2.8	13.95 ± 0.76^{e}	11.09 ± 0.76^{e}	2.00 ± 0.1^{a}	287.76 ± 26.57^{e}	1.32 ± 0.05^{e}	85.71 ± 1.1^{a}	2.10 ± 0.00^{bc}
p-value		0.000	0.000	0.000	0.000	0.000	0.123	0.003

Values presented as mean ± SE; mean values in the same column with different superscripts (a, b) differ significantly ($p < 0.05$).

Floc volume, a key indicator of biofloc system performance, was highest in T1 (37.40 ± 1.44 mL L^{-1}), followed by T3 (34.77 ± 0.30 mL L^{-1}), whereas the control exhibited minimal floc formation (4.19 ± 0.53 mL L^{-1}). These findings align with Wei, Liao, and Wang (2016), who demonstrated that carbon source complexity significantly affects floc structure and microbial diversity. The formation of well-developed flocs is essential for improving water quality, enhancing nutrient retention, and maintaining a stable and functional microbial ecosystem.

Efficient nitrogen cycling is essential for minimising the accumulation of toxic ammonia and nitrite in aquaculture systems (Hargreaves, 2006). In the present study, the control group exhibited the highest nitrite-N (0.24 ± 0.00 mg L^{-1}) and TAN (1.31 ± 0.04 mg L^{-1}), whereas biofloc treatments significantly reduced these levels. The measured parameter, total ammonia nitrogen (TAN), includes both ionized (NH„ z) and unionized ammonia (NH*f*). The lowest TAN levels were observed in T3 (0.71 ± 0.04 mg L^{-1}) and T2 (0.80 ± 0.07 mg L^{-1}). These results are consistent with findings by Azim and Little (2008) and Cardona et al. (2016), who reported reduced ammonia concentrations in biofloc systems compared to controls. This reduction is primarily due to the assimilation of ammonia by heterotrophic bacteria and its nitrification by autotrophic microbes. The enhanced microbial diversity in carbon-supplemented treatments likely contributed to improved nitrogen removal efficiency. All TAN values remained within safe limits for catfish culture (< 1.0–2.0 mg L^{-1} at pH 7–8), with negligible risk from unionised ammonia (NH_3), indicating effective microbial regulation of nitrogen dynamics.

Growth performance parameters (Table 2) showed significant variation among treatments. Treatment T3 recorded the highest harvested weight (28.10 ± 0.73 g), net weight gain (25.24 ± 0.73 g), net biomass increase (5.10 ± 0.04 g), percentage weight gain (782.52 ± 25.52%), and specific growth rate (SGR) (1.90 ± 0.02 % day^{-1}), all significantly higher ($p < 0.05$) than other treatments and the control. The control group consistently showed the lowest performance across all growth metrics.

Survival rate was also significantly higher in T3 (96.35 ± 0.7%) ($p < 0.05$) compared to the control (85.71 ± 1.1%), although no significant differences were observed among biofloc treatments. The feed conversion ratio (FCR) was lowest in T3 (1.60 ± 0.10), followed by T1 (1.80 ± 0.00), T4 (2.00 ± 0.00), control (2.10 ± 0.00), and T2 (2.20 ± 0.30), indicating superior feed efficiency in T3.

The enhanced growth performance and feed utilisation observed in T3 can be attributed to the continuous availability of microbial biomass in the biofloc system. Bioflocs serve as a natural supplementary feed source rich in proteins, essential amino acids, fatty acids, and bioactive compounds, improving nutrient assimilation and metabolic efficiency. This results in improved SGR, higher survival, and reduced FCR, which have also been reported in previous studies (Xu & Pan, 2013; Ekasari et al., 2014; Ahmad, Rani, Verma, & Maqsood, 2017; Das et al., 2025).

The lower FCR observed in biofloc treatments further supports the role of microbial flocs in enhancing feed efficiency by partially substituting the formulated feed and enhancing metabolic activity and nutrient utilisation (Khanjani & Sharifinia, 2020; Dos Santos et al., 2021; Soliman & Abdel-Tawwab, 2022). Additionally, improved survival rates may be linked to better water quality and the immunostimulatory effects of microbial communities (Zafar & Khan, 2019; Shamsuddin et al., 2022). These findings highlight the economic and biological advantages of biofloc technology in high-density aquaculture systems.

The study evaluated gut-associated *Bacillus* spp. (Fig. 1) and total heterotrophic bacteria (THB) in both water and gut samples across treatments (T1–T4) and the control (Fig. 2). All values were expressed as $\log_{10}$ CFU/mL. Treatment T3 consistently showed the highest bacterial proliferation.

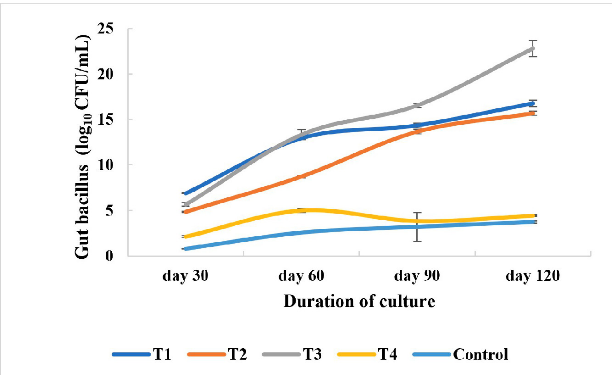

Fig. 1. Variation in gut Bacillus count during different culture durations under various treatment and control conditions.

In gut samples, *Bacillus* counts were highest in T3 (14.58 ± 3.56 CFU/mL), significantly higher than the control (2.51 ± 0.63 CFU/mL). Similarly, in water samples, T3 recorded the highest *Bacillus* counts (31.82 ± 6.98 CFU/mL), while no detectable counts were observed in the control. The THB levels followed a similar trend, with T3 showing the highest counts in both gut (17.58 ± 0.01 CFU/mL) and water (70.57 ± 4.54 CFU/mL) samples (Fig. 2a, b).

These results indicate that T3 provided optimal conditions for microbial proliferation. This observation aligns with Asaduzzaman et al. (2010), who reported that increasing the C:N ratio enhances heterotrophic bacterial growth by providing sufficient carbon sources. Azim, Beveridge, van Dam, and Verdegem (2005) also highlighted the synergistic interaction between autotrophic and heterotrophic bacteria in nutrient recycling and productivity enhancement.

Higher THB levels in T3 suggest more efficient organic matter decomposition and nutrient cycling, supporting improved system productivity. Similar findings were reported by Hari, Kurup, Varghese, Schrama, and Verdegem (2004), who observed increased bacterial biomass at elevated C:N ratios, contributing to reduced nitrogenous waste and improved system health.

In contrast, T4 exhibited comparatively lower bacterial counts, suggesting suboptimal environmental conditions for microbial growth. This highlights the importance of optimising carbon inputs to sustain beneficial microbial communities (Asaduzzaman et al., 2008). Additionally, probiotic *Bacillus* spp. is known to enhance digestive enzyme activity and feed utilisation, reducing organic waste accumulation (Hura, Zafar, Borana, Prasad, & Iqbal, 2018).

In both gut and biofloc water samples, *Bacillus* was selectively isolated using specific HiCrome™ Bacillus selective agar. The colonies appeared convex, blue-coloured, with smooth margins. Presumptive identification of *Bacillus* was based on morphological and biochemical characteristics, including Gram-positive, rod-shaped cells, and positive reactions for catalase and citrate utilisation. Molecular identification revealed 99–100% similarity of the amplified 16S rRNA gene fragments with *B. cereus*, based on 1508 bp sequences. The negative control in the PCR reaction included a reaction without template DNA and was also used as a quality control for the amplified products before sequencing. The sequences were deposited in the NCBI database under accession numbers Seq1-PQ632428, Seq2-PQ632429, and Seq3-PQ632430 (Fig. 3) (water samples), and Seq 4-PQ901980, Seq 5-PQ901981, and Seq 6-PQ901982 (gut samples). Collectively, these findings underscore the direct influence of treatment strategies on microbial community dynamics. This is consistent with the conclusions of Browdy, Bratvold, Stokes, and McIntosh (2001), who emphasised adjustment of nutrient inputs to support microbial processes in aquaculture. Optimising nutrient availability and environmental parameters can foster beneficial microbial communities, enhance nutrient utilisation, and contribute to the sustainability of aquaculture operations (Tanaka, Ootsubo, Sawabe, Ezura, & Tajima, 2004). Future research should further investigate microbial community interactions under different carbon sources to refine management strategies. Additionally, incorporation

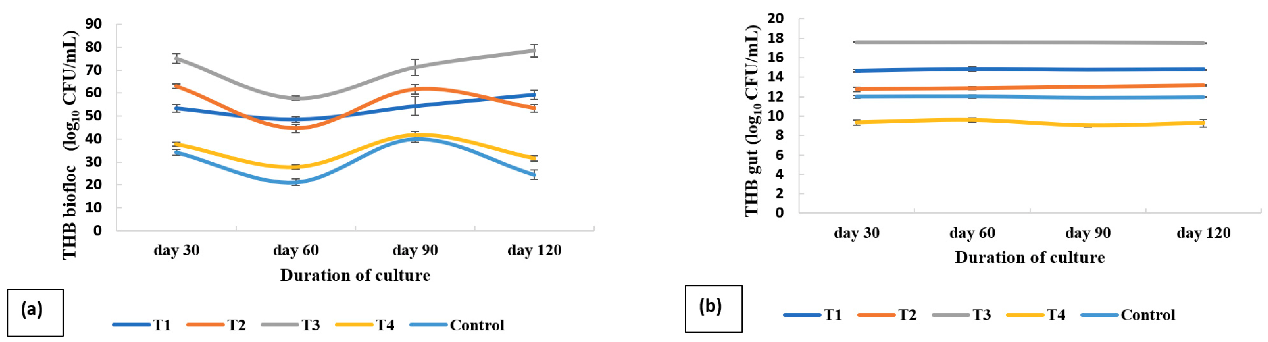

Fig. 2. Dynamics of total heterotrophic bacterial (THB) counts in (a) biofloc and (b) gut during the culture cycle under different treatment conditions

of substrates such as periphyton has demonstrated potential to enhance both bacterial and autotrophic productivity in aquaculture systems (Verdegem, Bosma, & Verreth, 2006; Ruby, Ahilan, & Prabu, 2018).

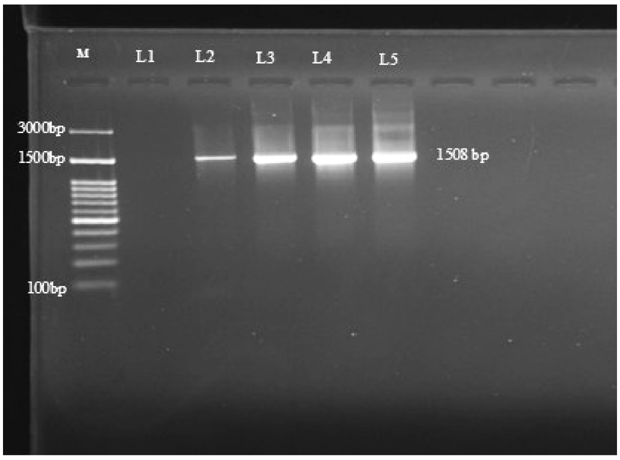

Fig. 3. PCR amplification of *Bacillus cereus* isolates showing ~1508 bp 16S rRNA gene fragments. M =DNA Ladder(100-3000bp). L1= Negative control, L2= Positive control, L3-L5 = PCR amplification of biofloc water and gut isolates

Although virulence gene screening was not performed, no adverse effects were observed during the feeding trial, indicating the safety of the isolates. Previous studies have reported similar findings, where probiotic *B. cereus* strains were safely used in aquaculture without pathological effects (Amenyogbe, Huang, Chen, & Wang, 2021; James et al., 2025). Additional safety assessments such as haemolytic activity and antibiotic susceptibility profiling are commonly employed (Shokrak, Khairi, Hazrin-Chong, Mohamed, & Abdella, 2024). For instance, weak á-haemolysis is generally associated with lower pathogenicity than â-haemolytic strains. Notably, even some â-haemolytic *Bacillus* strains have been safely used as probiotics without adverse effects (Wang et al., 2017; Elsabagh et al., 2018; Xia, Wang, Gao, Lu, & Chen, 2020)

This study highlights the significant role of different carbon sources in optimising biofloc systems to enhance water quality, growth performance, feed conversion efficiency, and overall fish health. Among all treatments, T3 showed that incorporating rice flour alongside molasses substantially reduces molasses consumption without adversely affecting biofloc production. These findings suggest that mixed carbon-source strategies can improve system productivity while maintaining economic feasibility. Future research should investigate the long-term effects of biofloc application on fish health and physiological performance, and evaluate the economic viability of scaling this technology across diverse species and production systems.

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