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**Md. Amirul Islam**
Principal Scientific Officer, Bangladesh Fisheries Research Institute, Riverine Sub-Station, Khepupara, Patuakhali-8650, Bangladesh and PhD Fellow, Fisheries and Marine Resource Technology Discipline, Khulna University, Khulna, Bangladesh

**Shikder Saiful Islam**
Associate Professor, Fisheries and Marine Resource Technology Discipline, Khulna University, Khulna, Bangladesh

**Prosenjit Debnath**
Farm Manager, Department of Fisheries (DoF), Ministry of fisheries and Livestock, Bangladesh

**Joyanta Bir**
Associate Professor, Fisheries and Marine Resource Technology Discipline, Khulna University, Khulna, Bangladesh

**Khandaker Anisul Huq**
Professor, Fisheries and Marine Resource Technology Discipline, Khulna University, Khulna, Bangladesh

**Corresponding Author:**
**Md. Amirul Islam**
Principal Scientific Officer, Bangladesh Fisheries Research Institute, Riverine Sub-Station, Khepupara, Patuakhali-8650, Bangladesh and PhD Fellow, Fisheries and Marine Resource Technology Discipline, Khulna University, Khulna, Bangladesh

# Effect of different C/N ratios on volume and potential microbial composition of flocs in freshwater prawn *Macrobrachium rosenbergii* culture system

**Md. Amirul Islam, Shikder Saiful Islam, Prosenjit Debnath, Joyanta Bir and Khandaker Anisul Huq**

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**Abstract**
The present study was conducted to evaluate the effects of C/N ratio on the volume of floc formation as well as on the load of several potential microbes of the fresh water prawn (*Macrobrachium rosenbergii)* culture tanks. The experiment was designed with three different C/N ratios *viz.* 10:1, 15:1, and 20:1 as $T_1$, $T_2$ and $T_3$ were applied with a control (C) at laboratory condition. It was observed that C/N ratio 15:1 (12.22 ± 1.08 ml/L) and 20:1 (15.33 ± 1.37 ml/L) gave higher floc development compared to control C (5.22 ± 0.62 ml/L) and $T_1$ (7.22 ± .46 ml/L) ($P$<0.05). The loads of *Enterococcus* spp.was found to be highest in $T_2$, followed by $T_1$, C and $T_3$ ($P$<0.05). Higher *Lactobacilllus* spp. load was found in $T_1$ and $T_2$ compared to $T_3$ and C, which indicates that growth of *Lactobacillus* spp. require a fair range of carbon source. No *Salmonella* spp. was found in any treatment. The present findings indicate that C/N ratio 15:1 could be applied in sustainable freshwater prawn culture.

**Keywords:** C/N ratios, volume, microbial, composition, flocs

## 1. Introduction
Freshwater giant prawn (*Macrobrachium rosenbergii*) farming is currently one of the crux sectors of the national economy in Bangladesh with its diverse contributions such as food production, employment opportunity and valuable foreign currency earnings. As a sub-tropical country, Bangladesh has a vast water bodies and suitable ecological condition for the culture of freshwater prawn. The average prawn production in Bangladesh is still remained very low (300-600kg/ha/year) compared to other neighboring countries [38]. Biofloc-based aquaculture systems have three pathways for nitrogen conversion: photoautotrophic uptake by algae, chemoautotrophic bacterial conversion of ammonia-nitrogen to nitrate-nitrogen, and heterotrophic bacterial assimilation of ammonia-nitrogen directly to bacterial biomass [21]. Biofloc systems are a unique type of RAS that maintain a community of suspended microalgae, autotrophic and heterotrophic bacteria which develop in limited-exchange systems [20, 26, 31, 42]. Thebiofloc is adding of carbon sources to regulate the ratio of C:N that naturally varies between 15:1 and 20: [5]. The ammonium in the water is decreased by the bacteria of biofloc, which, in turn, significantly improves the quality of the water for cultivation [10, 16]. Biofloc technology has been successfully tested for crustacean like shrimp [15] and to a lesser extent tilapia [18]. Biofloc in these systems have been reported to confer many beneficial effects on shrimp culture including: (1) improved water quality through removal of toxic nitrogen species such as ammonia and nitrite [19, 21, 41, 57]; (2) improved feed utilization and shrimp performance from natural productivity [11, 51, 54, 56]; and (3) enhanced shrimp health through possible probiotic effects [17, 28, 55, 58]. Prawns, which are a detritivorous, opportunistic species that feeds on bacteria, fungi, and decomposing material [36, 47] can be grown efficiently in biofloc. In BFT, microorganisms present a key role in nutrition of cultured animals. The macroaggregates is a rich protein-lipid natural source available "*in situ"* 24 hours per day [9]. The consumption of biofloc by shrimp or fish has demonstrated innumerous benefits such as improvement of growth rate, decrease of FCR and associated costs in feed [15]. The biofloc (microorganisms) has two major roles: (i) maintains water quality by the uptake of nitrogenous

compounds to generate microbial proteins on-site; and (ii) increases culture feasibility by reducing feed conversion ratio through higher protein utilization and lower inputs of commercial feed, hence decreasing feed cost [20]. The C/N ratio of the culture water is thought to be one of the critical factors affecting growth rate of different microbial communities, thereby generating different substrate utilization pathways and microbial biomass yields [21, 26]. Previous studies showed that increasing the C/N ratio from feed and/or direct organic carbon supplementation can induce a shift of the biofloc community from photoautotrophic or chemoautotrophic to heterotrophic-dominated communities [8, 13, 21]. This transformation can have very significant impacts on water quality and biofloc biomass production [21], both of which can eventually affect feed utilization and shrimp performance. Maintaining carbon-nitrogen ratio (C/N) in the aquatic environment converts toxic inorganic nitrogen compounds into useful bacterial cells (single-cell protein) that may be a direct source of food for the cultured organisms [8]. Immobilization of inorganic nitrogen takes place when the C/N ratio of the organic matter is higher than 10 [33]. Carbon-nitrogen ratio causes shifting of autotrophic to a heterotrophic system [8, 12]. As a result TAN and $NO_2$-N concentrations can be effectively controlled by either heterotrophic assimilation or autotrophic nitrification that helps to maintain their concentrations at acceptable ranges for the cultured organisms even at higher stocking densities [52]. C/N ratio of 10 or more in the feed is required for the growth of heterotrophic microorganisms [8, 32] observed that carbon supplementation enhanced the removal rates of TAN at 26% per hour compared to 1% per hour in a control system. The C/N ratio of around 10 is maintained in most of the feeds used in semi-intensive aquaculture ponds, but bacteria require about 20 units of carbon per unit of nitrogen assimilated [8]. So, when C/N ratio is low in the feed, carbon becomes the limiting nutrient for the growth of heterotrophic bacterial populations in the aquaculture ponds [6] and hence the heterotrophic bacterial population will not inflate beyond a certain point due to the limited availability of carbon in the system. Different organic carbon sources (glucose, cassava, molasses, wheat, corn, sugar bagasse, sorghum meal, etc.) are used to enhance production and to improve the nutrient dynamics through altered C/N ratio in shrimp culture [8], and C/N ratio is also widely used as a guide for analyzing the decomposition of organic matter [2]. The biofloc system maintained with C/N ratio of higher than 15–20 will be developing sufficient microbial floc to assimilate toxic nitrogenous species under intensive farming with limited discharge [7]. The excretion of nitrogenous metabolic wastes and their assimilation by heterotrophic bacteria maintain a balance by manipulating the carbon-to-nitrogen ration (C/N ratio) by the addition of carbon sources in the water. High carbon to nitrogen ratio is required to guarantee optimum heterotrophic bacteria growth [9]. The C/N ratio of most of the feeds used in semi-intensive aquaculture ponds is around 10:1, but bacteria require about 20 units of carbon per unit of nitrogen assimilated [8]. Therefore, in case of low C/N ratio, carbon is the limiting nutrient for heterotrophic bacteria populations in aquaculture ponds. So, the growth of bacterial population will be hampered due to the limited availability of carbon. The C/N ratio in the pond can be increased by adding different locally available cheap carbon sources [26]. If the C/N ratio is increased by adding a carbohydrate source such as molasses now available in Bangladesh in addition to the regular feed, the increased availability of carbon allows the heterotrophic bacterial population to grow to a dense mass. Therefore, manipulation in the C/N ratio may result in a shift from an autotrophic to a heterotrophic system [8, 13]. The heterotrophic bacteria population utilizes the ammonium in addition to the organic nitrogenous wastes to synthesize new cells (single cell microbial protein) [46], and it may be utilized as a natural food source by freshwater prawn [5]. Currently, there is a little information on production of prawns with biofloc technology in Bangladesh except [5], so it is difficult to establish the relevance of this technology and critical activities to improve production of prawn. However, biofloc development and its relationship to water quality dynamics and prawn performance due to C/N ratio manipulation remains poorly understood in Bangladesh. Therefore, the present study was conducted to optimize suitable C/N ratio for prawn production by assessing the volume and potential microbial composition of flocs in freshwater prawn *Macrobrachium rosenbergii* culture system.

## 2. Materials and Methods

### 2.1. Experimental design

The experiment was conducted in 12 (twelve) fiberglass tanks for close observation of the biofloc technology. The experimental tanks were treated with the C/N=10:1 (denoted as $T_1$), C/N=15:1 (denoted as $T_2$) and C:N=20:1 (denoted as $T_3$) to develop the biofloc. In addition, a control group was also maintained without any carbon addition. The heterotropic bacteria were developed in the flocs, which were collected and determined from each of the experimental groups. Afterwards, macro and microbiological composition of the flocs were determined by laboratory analysis. Macroscopic organisms were determined by using light microscope and the potential bacterial composition was determined by microbiological plate assay. The floc samples were examined to observe the load of *Enterococcus* spp., *Clostridium* spp., *Lactobacillus* spp. and *Salmonella* spp.

### 2.2. Development of biofloc and rearing of prawn in the tanks

The trials for biofloc development were carried out in the fiber glass tank in water chemistry laboratory of Fisheries and Marine Resource Technology (FMRT) Discipline, Khulna University and in the Molecular Biology Laboratory of same Discipline from 02, August to 15, August, 2017. Biofloc volume was determined at the end of the tank trials and samples determined for next one months for the bacterial composition of the flocs. For the determination of the bacterial composition, the freshwater prawn *Macrobrachium rosenbergii* were cultured with BFT treatment in experimental tank and then the samples were taken in the Biochemistry and Molecular Genetics Laboratory of FMRT Discipline, Khulna University for culture and enumeration of bacterial species. Twelve tanks were set randomly in the water chemistry laboratory for developing bioflocs in prawn culture system. Before stocking the prawn juveniles were weighed and feed was given at 5% of their body weight. From the amount of feed, the proper amount of molasses was calculated to maintain proper C/N ratio (10, 15 and 20) in different treatments. After that, specific amount of feed and molasses were provided to the experimental tanks twice a day to adjust the feed nitrogen for targeted microbial growth. Besides, continuous aeration was provided in all the tanks for homogenous oxygen and nutrients distribution for flocs. The

carbon nitrogen ratio was calculated according to the following equation:

$$\text{The equation is}\Delta CH=\frac{(\text{Quantityoffeedx \%Ninfeedx \%Nexcretion})}{0.05}$$ [9]

### 2.3. Collection and Determination of floc

Samples of floc were collected for three times from each of the experimental tank for each and every enumeration at every three days interval. So, nine samples were collected under each treatment. As a result thirty six samples were collected from twelve experimental tanks under four treatment groups. Then collected sample was carried immediately to the Molecular Biology Laboratory of FMRT Discipline, Khulna University. All the samples were instantly used for microbial analysis and also properly labeled and stored in -20ºC temperature for further analysis. Total volume of floc per liter water was measured at first by using specific cone. Then the proportion or percentage of different types of flocs such as protozoa, plankton, copepods, etc. in the collected sample was determined at first by observing them under a light microscope.

### 2.4. Enumeration of beneficial and harmful bacteria in the flocs

Test tubes, petri dishes, conical flask, plastic tips and other glassware were sterilized by autoclaving at a temperature of 121 °C and a pressure of 15 pound per square inch for 15 minutes. Petri dishes, tips box was subjected to dry heat sterilization at 155 °C for 1 hour in electric oven [48]. Peptone water, Absolute alcohol, Ethyl alcohol (75%), distilled water and detergent powder were used as chemical and reagent during the study periods [24]. Plate counting agar was used for standard plate count. For culture and normal counting of *Salmonella* spp., Broth Medium I (Tetrathionate Bile Brilliant Green Broth) was used. This media was specific or selective for harmful *Salmonella* spp. For culture and normal counting of *E. faecalis*, SF Broth Medium was used. *Lactobacillus spp.* bacteria were cultured in *Lactobacillus* MRS Agar medium. For culture and normal counting of *Clostridium butyricum*, selective Reinforced Clostridial Agar (M154) was used. Accurate amount (1 ml) of pooled sample was measured by pipette. Then the sample was taken into a sterile test tube containing 9 ml 0.1% sterile peptone water for vortex mixing. The mixture was homogenised for 2 min. This serial dilution was continued up to $10^{-5}$. Samples from each of the experimental tank were taken and prepared in the same way. The prepared samples of the six tanks were kept in separate test tube. At first the samples were cultured in the standard plate count agar media. Then the colonies of the total heterotrophic bacteria were counted by using colony counting equipment.

#### 2.4.1. Determination of *Enterococcus* spp.

*Enterococcus* spp. bacteria were cultured in the SF Broth media. SF Broth was produced by Hi Media Laboratories according to the formulation developed by [25]. 36.032 g media **(**SF Broth) was mixed in 1000 ml of distilled/deionized water. Heat was provided to dissolve the medium completely. Then it was dispensed in tubes and sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 minutes. The Final pH of the media was adjusted to 6.9 ± 0.3 at 25 ºC before autoclaving. The media was stored below 30 °C in tightly closed container and the prepared medium was at 2 – 8 °C. Then the bacteria were cultured in the prepared culture media (SF broth). The bacteria were cultured in five-fold dilutions and each dilution had three tubes. So, fifteen test tubes were needed for one stock solution. Twelve stock solutions were prepared from the twelve experimental tanks and used for bacterial analysis. (12×15) = 180 test tubes were needed for the culture of bacteria of twelve tanks. The test tubes were incubated at specific temperature (45.5 °C) for bacterial growth.

Cultural characteristics were observed after incubation at 45-46 °C for 18-48 hours. The color of the tubes were changed from purple to yellow giving the positive result in which the growth of *Enterococcus* spp. was luxuriant. Some tubes remained unchanged (purple color) which gave the negative result due to lack of growth. As the SF broth media is selective for Enterococci and the presence of *Enterococcus* spp. was identified by the color change (from purple to yellow) at the time of culture, so any further confirmation test was not necessary.

In the tube culture method, the number of bacteria cannot be found directly. Only the number of positive and negative tube for each dilution were found and recorded. Then an established Most Probable Number (MPN) chart was used to estimate the number of bacterial cell by using the number of positive tube for each dilution. The MPN chart was prepared on the basis of poisson counting statistics regarding the number of positive tubes out of all the cultured tubes. The bacterial culture was done in a five-fold dilution each of with three replication tubes. So, three tubes MPN chart was used for the estimation of the load of *Enterococcus* spp. Then the Most Probable Number (MPN) bacterial data was recorded, accumulated, grouped and interpreted according to the objectives as well as parameters.

#### 2.4.2. Determination of *Clostridium* spp.

*Clostridium* spp. bacteria were cultured in the Reinforced Clostridial Agar (M154) media. 51.0 gm of the media was dissolved in 1 L of distilled water, followed by heat sterilization by autoclaving at 15 lbs pressure (121 °C) for 15 minutes. By pour plating the bacteria was inoculated into the media and incubated at anaerobically at 37 °C for 24 hours. *Clostridium* spp. was cultured by Pour plating method. The appropriate dilutions were selected and for every dilution 1.0 ml aliquot were transferred into sterile petri dishes. Approximately 20.0 ml portion of Reinforced Clostridial agar was poured into each of these sterile petri dishes. The plates were then rotated 5 times clockwise, 5 times anticlockwise, 5 times back and forth. Care was taken not to splash agar on the lid of the dish. Plates were left to solidify the plates and were inverted. Then the petri dishes with solidified agar were incubated at 37 °C for 24 hours in the incubator.

#### 2.4.3. Determination of *Lactobacillus* spp.

*Latobacillus* spp. bacteria were cultured in the Lactobacillus MRS Agar medium. About 67.15 g of the media was dissolved in 1 L of distilled water, followed by heat sterilization by autoclaving at 15 lbs pressure (121 °C) for 15 minutes. *Lactobacillus spp.* was cultured by pour plating method same as the *Clostridium* spp. culture and incubated for 18-48 hours at 35-37 °C.

#### 2.4.4. Determination of *Salmonella* spp.

*Salmonella* spp. bacteria were cultured in the Broth Medium I (Tetrathionate Bile Brilliant Green Broth media).The composition of the media has been given in the section of appendix-1. 63.07 gm of the media was dissolved in 1 L of distilled water, followed by heat sterilization by autoclaving

at 15 lbs pressure (121 °C) for 15 minutes. By pour plating method the bacteria were inoculated into the media and incubated at anaerobically at 41-43 °C for 18-24 hours.

#### 2.4.5. Counting of colonies
After the specified period of incubation, the bacterial colonies of each Petri dish were counted, using the colony counting equipment. Plates with 10-300 colonies on the surface were only counted. To calculate the number of bacteria per mL of diluted sample following equation was used [30]:

$$\frac{\text{Number of CFU}}{\text{Volume plated (mL) x total dilution used}} \rightarrow \frac{\text{Number of CFU}}{\text{mL}}$$

#### 2.4.6. Biochemical tests of bacterial composition
*Enterococcus* spp., *Clostridium* spp., and *Lactobacillus spp.* were confirmed by following specific biochemical tests. Such as D- Sorbitol test, D- Xylose test, L- Arabinose test, Sulphur Indole Motility (SIM) test, Triple Sugar Iron (TSI) agar test, Indole test, Methyl Red test, Voges Proskauer test, Catalase test, Oxidase Test, Starch hydrolysis test.

### 2.5. Statistical analysis
The data were recorded during the experiment and collected data were stored, explored and analyzed using Microsoft Excel, SPSS and Microsoft Word program to present the results and discussion. The normality test of the data was done in SPSS at first. After verifying the normality of flocs volume and log transformed bacterial data, one-way analysis of variance (one-way ANOVA) was performed to check the significant differences among the treatments. After detecting it ($P$<0.05), the Tukey test of means comparison was used in SPSS to compare the data of control and treated tanks.

## 3. Results
### 3.1. Development of Floc
The volume of floc per liter was measured for three times from each of the tank of treatment groups at every three days interval. Higher floc development was found in C/N ratio of 15 and 20 ($T_2$ and $T_3$) compared to C and $T_1$ (C/N ratio 10) ($P$<0.05). The lowest volume of floc was determined in C group (5.22 ± 0.62 ml/L) followed by $T_1$ (7.22 ± 0.46 ml/L), $T_2$ (12.22 ± 1.08 ml/L) and $T_3$ (15.33 ± 1.37 ml/L) respectively (Figure 1).

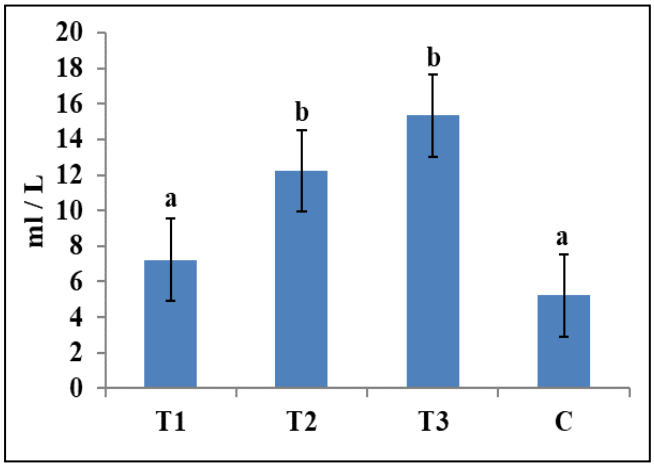


**Fig 1:** Volume of flocs at different C/N ratios in biofloc based prawn culture system. The error bars represent the standard deviations of nine replicates. Different letters indicate significant difference among the treatments (one-way ANOVA, $P$<0.05).

### 3.2. Total bacterial count
The mean number of total heterotrophic bacteria (THB) in control group was (1.56 ± 0.07) ×$10^7$ CFU/ ml) which was lower than $T_2$ ((2.30 ± 0.21) × $10^8$ CFU/ ml) and $T_3$ ((3.90 ± 1.33) × $10^8$ CFU/ ml) (P<0.05) (Figure-2). The treatment with C/N, 10 also showed significantly lower THB ((1.55 ± 0.28) ×$10^7$ CFU/ ml) compared to $T_2$ and $T_3$ (P<0.05) (Figure-2).

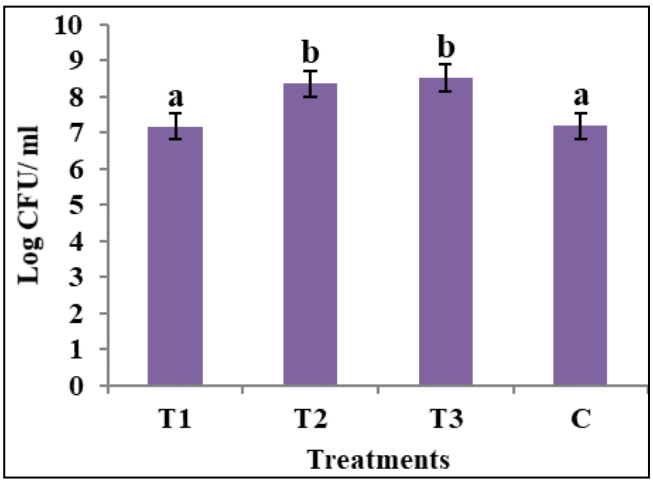


**Fig 2:** Total heterotrophic bacterial count (Log CFU/mL) in the flocs at different C/N ratios in biofloc based prawn culture system. The error bars represent the standard deviations of nine replicates. Different letters indicate significant difference among the treatments (one-way ANOVA, P<0.05).

### 3.3 *Enterococcus* spp. count
The highest mean number of *Enterococcus* spp. (2.74 ± 0.30) × $10^3$ CFU/ ml) was found in $T_2$ (C/N ratio 15) compared to other treatments ($P$<0.05). The load of this beneficial bacteria was found higher also in $T_1$ ((1.29 ± 0.21) × $10^3$ CFU/ ml) and C ((1.19 ± 0.09) × $10^3$ CFU/ ml) compared to $T_3$ ((4.18±0.88) ×$10^2$ CFU/ ml) ($P$<0.05) (Figure 3).

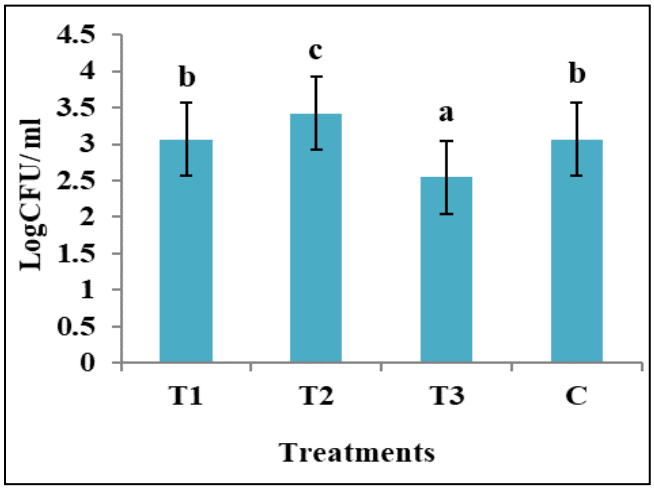


**Fig 3:** *Enterococcus* spp. count (Log CFU/mL) in the flocs at different C/N ratios in biofloc based prawn culture system. The error bars represent the standard deviations of nine replicates. Different letters indicate significant difference among the treatments (one-way ANOVA, $P$<0.05).

### 3.4. *Clostridium* spp. count
The highest mean number of *Clostridium* spp. was found in $T_1$ ((8.64± 1.74) × $10^5$ CFU/ ml), followed by $T_2$ ((6.89 ± 2.16) × $10^5$ CFU/ ml), $T_3$ ((4.74 ± 1.75) × $10^5$ CFU/ ml) and Control group ((3.59 ± 0.87) × $10^5$ CFU/ ml) respectively. The

treatments did not show significant difference among them in respect of *Clostridium* spp. load in the flocs (Figure 4) ($P$<0.05).

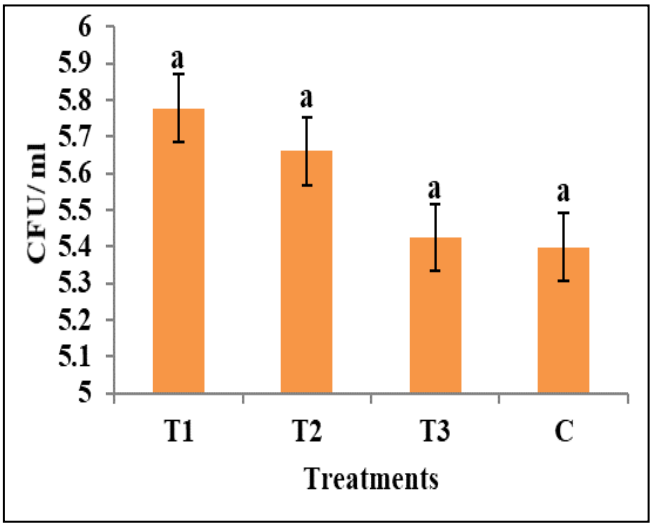


**Fig 4:** *Clostridium* spp. count (LogCFU/mL) in the flocs at different C/N ratios in biofloc based prawn culture system. The error bars represent the standard deviations of nine replicates. Different letters indicate significant difference among the treatments (one-way ANOVA, $P$<0.05).

### 3.5. *Lactobacillus* spp. count

The mean number of *Lactobacillus spp.* was found to be higher in $T_1$ compared to $T_3$ and C ($P$<0.05). $T_1$ had the highest number of *Lactobacillus* spp. count ((1.15 ± 0.12) × $10^6$ CFU/ ml) among the treatments, followed by $T_2$ ((9.65 ± 2.48) × $10^5$ CFU/ ml), C ((3.02 ± 0.95) × $10^5$ CFU/ ml) and $T_3$ ((2.53 ± 0.25) × $10^5$ CFU/ ml) (Figure 5).

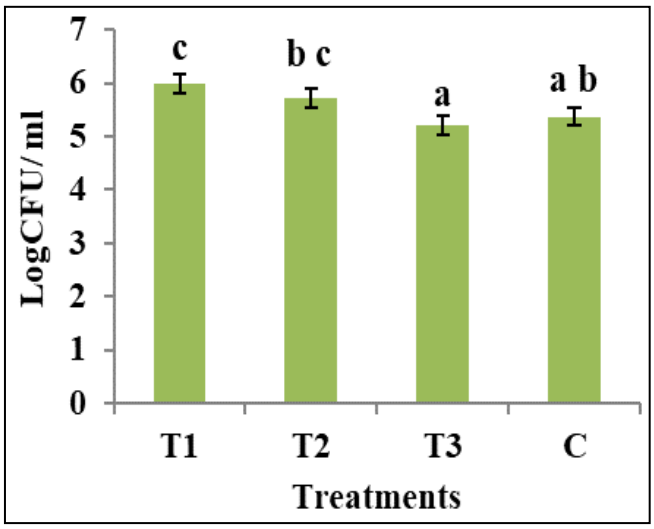


**Fig 5:** *Lactobacillus* spp. count (LogCFU/mL) in the flocs at different C/N ratios in biofloc based prawn culture system. The error bars represent the standard deviations of nine replicates. Different letters indicate significant difference among the treatments (one-way ANOVA, $P$<0.05)

### 3.6. *Salmonella* spp. count

No colony forming units of *Salmonella* spp. was found in any of the treatment in this study.

### 3.7. Biochemical tests

Different biochemical tests were conducted to confirm the specific bacterial species. Result of biochemical tests found for the confirmation of *Enterococcus* spp., *Clostridium* spp. and *Lactobacillus spp.* bacterial species has been furnished in the table 1.

**Table 1:** Biochemical tests results for indication of specific group of bacteria.

| Name of the Tests | Results for *Enterococcus* spp. | Results for *Clostridium* spp. | Results for *Lactobacillus spp.* |
|---|---|---|---|
| D- Sorbitol test | Positive (+) | Positive (+) | Positive (+) |
| D- Xylose test | Negative (-) | Negative (-) | Positive (+) |
| L- Arabinose test | Positive (+) | Negative (-) | Negative (-) |
| Sulphur Indole Motility (SIM) test | Negative (-) | Negative (-) | Negative (-) |
| Triple Sugar Iron (TSI) agar test | Positive (+) | Positive (+) | Positive (+) |
| Indole test | Negative (-) | Negative (-) | Positive (+) |
| Methyl Red test | Negative (-) | Negative (-) | Negative (-) |
| Voges Proskauer test | Negative (-) | Negative (-) | Negative (-) |
| Catalase test | Negative (-) | Negative (-) | Negative (-) |
| Oxidase Test | Negative (-) | Negative (-) | Negative (-) |
| Starch hydrolysis test | Positive (+) | Positive (+) | Negative (-) |

## 4. Discussion

In this study, the C/N ratio was necessarily correlated with the floc volume. The volume was increased with the increasing rate of C/N ratio. But the density of floc was lower in C/N 20. The best quality floc and the highest volume were found in the C/N 15. This also appears to be supported by the biofloc appearing more closely aggregated and being less dense in the C/N 10 and 20 groups compared to the biofloc particles in the C/N 15 treatment, which is consistent with filamentous bacteria formation [53]. The floc volume was also between 7 to 15 ml/L in the treatments that have similarities with the statement of [8]. Proliferation of bacterial colonies and microorganisms generates an increase in the biofloc biomass, it increases must have a density between 10 and 15 mL so the system can keep functioning properly between 7 to 15 ml/L in the treatments that have similarities with the statement of [8].

The addition of carbohydrate to the water column increased the C/N ratio and resulted in a significant increase in the total heterotrophic bacterial (THB) count [14, 27]. Mean THB count of the flocs varied from $41.5\times10^7$ to $53.9\times10^7$ CFU/ml revealed the positive effect of carbohydrate addition on the bacterial population of the pond sediment. The number of bacteria showed a significant increase from the initial sampling date to the second and then stabilized for the entire culture period [27]. The increase in C/N ratio benefited the growth of both potential probiotics and pathogenic bacteria. The high C/N (14) ratio enhanced the bacterial functions of chemoheterotrophy and hydrocarbon degradation [59] that supports the findings of the present study. The trend of pathogenic bacteria dominance decreased with the increase in C/N ratios and thus confirming the dominance of heterotrophic bacteria in high C/N ratio groups [40].

Generating a nitrogen cycle in a closed water system by stimulating the microbial growth is the basic principle of biofloc technology [22]. Molasses, which is a by-product of sugar production that contained 49-50% carbon was used in the experiment. The higher bacterial population in the carbohydrate added treatments revealed that the molasses is a good source of organic carbon as it was well utilized by the heterotrophic bacterial population. Wheat flour as a carbon source stimulates microbial growth and found the main microbes to be *Vibrio*, *Lactobacillus*, *Bacillus* and fungi [3]. *Bacillus* from a highly enclosed culture system of *Litopenaeus vannamei* was isolated [23]. [45] used molasses as source of carbon for developing biofloc for *Penaeus indicus* post larvae

rearing in tank system that showed floc volume of 12 ml/L very close to the present study.

In the present study, the highest load of *Enterococcus spp.* was found in $T_2$ and it had significant difference with the control and other C/N treated systems. *Enterococcus spp.* load was lowest in $T_3$ at the C/N, 20 might be due to over organic load which might cause development of toxic gases, lowering the pH of the system, thereby creating unfavorable condition for growth of different bacterial population. Similarly, load of *Lactobacillus spp.* was also lower in $T_3$ compared to other treatments, which was found to be highest in $T_1$ and $T_2$. These findings have similarities with the study of [39]. They reported that the total count of viable heterotrophic bacteria of the genus *Bacillus* spp. in the water of shrimp cultures demonstrated that during the culture that *Bacillus* spp. presented the greatest bacterial load in both treatments. [35] also found that in the biofloc tanks, *Bacillus subtilis* and *Lactococcus* could utilize the nitrogenous waste and accumulated carbon as a nutrient source to preserve shrimp growth in the water. The growth of *Bacillus subtilis* and *Lactococcus* was restricted in the control tanks because of an inadequate carbon source. Therefore, biofloc technology played an important role in this study in maintaining a relatively high proportion of *Bacillus subtilis* and *Lactococcus* in the biofloc rearing water. *Bacillus subtilis* and *Lactobacillus* can successfully colonize the intestine and affect the intestinal microbial community [35]. The microbial community in the host intestine has been reported to play a key role in improving the immunity of the host by forming a physical barrier to provide a defense against pathogens or by occupying an ecological niche [29, 34].

However, although C/N ratios 10 and 15 gave higher count of *Clostridium spp.*, but the load was not significant with either $T_3$ or Control group. This phenomenon might be due to the short culture period for the proper development of *Clostridium spp.* bacteria. As the C/N ratio of bacterial cells is 5:1 [44] and the conversion efficiency of bacteria is 40–60%, C/N ratio of 10 or more in the feed is required for the growth of heterotrophic microorganisms [8]. [40] found C/N ratio 15 suitable for enhancing the production of Total Heterotrophic Bacteria (THB) in a biofloc-based rearing system of Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931). Beneficial microbial bacterial floc and its derivative compounds such as organic acids, polyhydroxy acetate and polyhydroxy butyrate, could resist the growth of other pathogens, thus serves as a natural probiotic and immunostimulant [1].

A few researchers worked on the effect of supplementation of external carbon source on competition between probiotics bacteria and pathogens in aquaculture systems. [50] found *Flavobacterium* and *Pseudomonas* as pathogens where as *Bacillus*, *Bdellovibrio*, *Lactococcus* and *Synechococcus* were reported as probiotics [37]. We found that total heterotrophic bacteria were increased with increase of C/N ratio, suggesting that increasing C/N ratio could benefit both potential probiotics and opportunistic pathogens at the same time. In the present study the no *Salmonella spp.* was found. It assumes that the environment of the culture system was good and hygienic and the beneficial microbes might inhibit the growth of the pathogenic agent. Like as, the association of flocs acts as a bio-regulator of water quality and as an inhibitory agent to prevent the growth of *Vibrio* spp. during the cultivation of shrimp in the biofloc system [39]. Moreover, the addition of lactic acid bacteria in shrimp diets has been suggested to decrease the adherence and colonization of pathogenic bacteria and to improve fish health [43]. So, the *Lactobacillus* spp. may be the cause of inhibiting the *Salmonella* spp. Many other studies have also found that biofloc technology led to significantly different microbial composition in the rearing water [4, 49, 23].

## 5. Conclusion

Biofloc technology (BFT) is a novel modern aquaculture farming technique used to reduce toxic nitrogen concentration, act as in situ food source and eradicate pollutants using carbon and therefore to control C/N ratio in an aquaculture system [40]. The driving force of BFT systems is the development of biofloc, which is responsible for water quality control, waste assimilation, and nutrient cycling, which contribute to improved performance of cultured shrimp [7] and C/N ratio manipulation is the prerequisite to develop flocs in biofloc based system. The results of the present study showed that manipulating input C/N ratio had significant influence on production of flocs and probiotic bacterial species that are very much useful for culture of freshwater prawn *Macrobrachium rosenbergii* in biofloc based culture systems. From the findings of this study two significant conclusions can be drawn as follows: (1) Increased C/N ratios enhance the volume of floc production in biofloc based *M. rosenbergii* culture system. C/N, 15 might have better option in this culture system considering the cost of carbon sources and environmental degradation, i.e. huge organic load; (2) C/N, 15 and 20 had the higher heterotrophic bacterial count; however, 15 had the highest *Enterococcus* spp., which was lowest in 20. Although not significant, but C/N, 10 and 15 had higher *Clostridium* spp. and *Lactobacillus* spp.

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