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Using a combination of phospholipid fatty acids profiles and DNA-based sequencing analyses to detect shifts in the biofloc microbial community in different carbon sources and carbon/nitrogen ratios

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Abstract

A 35-day study investigated the impact of carbon sources and carbon/nitrogen (C/N) ratios on the microbial community of biofloc. For this purpose, we utilized a combination of phospho-lipid fatty acids (PLFAs) profiles and DNA-based sequencing methods to investigate changes in the microbial community composition and structure. The experiment involved three carbon sources including Dextrin (DEX), corn starch (CS) and wheat bran (WB) at two C/N ratios (19 and 30). The results indicated that WB and CS were found to decrease nitrogen metabolite concentration while increasing total suspended solids and bacterial density compared to DEX. The treatments exhibited variations in microbial communities and the use of polymerase chain reaction/ denaturing gradient gel electrophoresis analysis revealed distinct dominant bacterial species linked to carbon sources and C/N ratios. Furthermore, the highest levels of bacteria and protozoa PLFAs biomarkers were observed in the C/N30 ratio and WB treatment while the ratio for poly-β-hydroxybutyrate/PLFAs and fungi biomarkers displayed a decrease. Also, by incorporating the results of PLFAs profile and conducting a principal component analysis, the treatments were categorized into distinct groups based on both the carbon source and C/N ratios. Overall, both methods yield consistent results. PLFAs offered additional insights into the microbial composition beyond bacterial structure while DNA-based analysis provided finer taxonomic resolution.

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Introduction

The decline in global fishing has led to intensive aquaculture systems, causing wastewater generation.[1] This wastewater contributes to water eutrophication. Recirculating aquaculture systems are being explored as a solution to reduce effluent discharge; however, high costs hinder their wide adoption. Researchers are actively looking for cost-effective alternatives to address this issue.

An inventive aquaculture technique called biofloc achieves twofold benefits by utilizing inexpensive carbon sources and adjusting the carbon/nitrogen (C/N) ratio. These actions support the development of micro-organisms by providing more nourishment and efficiently removing toxic metabolites such as ammonia and nitrite.[2-4] Important studies highlight how important carbon sources and the C/N ratio are in determining the microbial makeup and structure of the biofloc system.[5-7] Moreover, the influence on this system is significantly affected by the nature of carbon sources, irrespective of their simplicity or complexity.[7-9] Different investigations have shown different results: Microbial diversity between treated and untreated water was similar for simple carbohydrates like sugar cane molasses, while it was much higher for complex sources like plant cellulose.[7,10] However, because most bacterial populations cannot be cultured, basic bacterial counting methods are insufficient and expensive molecular techniques such as polymerase chain reaction/denaturing gradient gel electrophoresis (PCR-DGGE) are required for a thorough investigation of the microbial community.[11,12]

In recent years, the use of phospholipid fatty acids (PLFAs) profiles to study the soil microbial community was pioneered by Frostegård *et al.*,[13] Since then, this technique has become one of the popular ways to gain insights into the biomass, structure and metabolic status of microbial communities in different ecosystems.[13-15] This method allows for the distinction and discrimination of

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bacteria (Gram-positive and Gram-negative) and other microorganisms through the use of specific PLFAs biomarkers.[16] Furthermore, the analysis of PLFAs in the samples is more cost-effective than those of other microbial constituents such as RNA, DNA and proteins making it an efficient methodology to assess microbial community changes in response to environmental conditions.[13,17] In a study by Chen *et al.*,[16] the effects of simulated spring precipitation on the soil microbial community were investigated over a short-term period using PLFA and DNA-based high-throughput sequencing analyses. The study found that the combination of these methods which rely on different microbial biomarkers produced consistent results. The PLFA-based method showed greater sensitivity in detecting short-term changes in the soil microbial community in response to the precipitation event while the DNA-based method provided more detailed taxonomic information about the microbial taxa at a finer resolution. Fanin *et al*.,[17] demonstrated an association between soil microbial response to carbon limitation and classification of bacteria. Gram-negative bacteria favored simple carbon while Gram-positive adhered to complex carbon.

No studies have examined the impact of varying carbon sources including dextrin (DEX): Simple structure, corn starch (CS): Medium and wheat bran (WB): Complex structure) and C/N ratios (19 and 30) on the structure. The authors believed that using PLFAs and PCR-DGGE techniques could confirm this theory, a hypothesis they tested in their research.

Materials and Methods

Experiment design. In a 35-day study, we examined three carbon sources (CS, DEX, and WB) and two C/N ratios (19 and 30) across six treatments: CS19, CS30, DEX19, DEX30, WB19, WB30. We introduced 180 common carp (*cyprinus carpio*) fingerlings (initial weight 40.00 ± 3.20 g) into 35.00 L rectangle tanks at a density of 10 fish per replicate. They were fed a commercial diet (36.00% protein, 6.00% lipid and 9.00% ash) three times daily and maintained under a 12-hr light/12-hr dark cycle at a constant 25.00 ˚C. The project utilized water sourced from a well located near the experiment location. Following aeration through an air pump, this water was employed for the experiment. Additionally, replacing 20.00% of the water in the tankers was a regular practice.

Microbial flocs formation. Fish were fed a commercial food (Pre-Grower Feed for Common Carp, Faradaneh, Tehran, Iran) to start the growth of microbial flocs and the nitrogen content and tank discharge of the diet were used to calculate the nitrogen metabolites. This work used a postulated hypothesis ratio (19, 30) to calculate nitrogen metabolites by taking into account food intake, nitrogen released within tanks and the addition of carbon sources. An elemental analyzer (Vario MAX cube; Elementar, Langenselbold, Germany) was used to determine the organic carbon contents of DEX, WB and CS which were 39.20%, 41.40%, and 40.00%, respectively. The 75.00% of nitrogen was released into the environment as ammonia, nitrite and nitrate as a result of the food which contained 35.00% protein and 6.25 g of nitrogen per 100 g. Every day, the carbon sources for every gram of food and the resulting nitrogen metabolites for every tank were calculated according to predetermined ratios. Every day, 100 mL of tank water were used to dissolve the weighted carbon source and released it into the environment.[2]

Water quality parameters. Daily monitoring of water temperature, dissolved oxygen (8403; AZ Instrument Corp., Taichung, Taiwan) and pH (CP-411; Elmetron, Zabrze, Poland) was performed during the experimental period along with weekly measurements of certain nitrogen compounds. A 100 mL water sample was collected from each tank for analysis with half used for nitrogen metabolite measurements, and the remaining half filtered and dried to determine total suspended solids (TSS) content.[5] Nitrogen metabolites concentration nitrite (NO_2-N, total ammonia, and NO_3-N) was determined using a commercial kit (Palintest, Gateshead, UK).

Microbial flocs PLFAs profiles and poly-β-hydroxy-butyrate (PHB) concentration. At the end of the experiment, 1.00 L of water was collected from each replication for the analysis of PLFA profiles. The water was centrifuged at 5,000 rpm for 5 min and the resulting pellets were used for PLFA profile analysis. A gas chromatography instrument (7890A; Agilent Technologies, Santa Clara, USA) equipped with a flame ionization detector and a cyanopropyl phenyl capillary column (30.00 m × 0.25 mm, DB225MS; Agilent) was used to carry out the methyl esterification process as described by Lepage and Roy.[18] The saturated PLFAs were used as a signature for the general bacterial marker, mono-unsaturated PLFAs for Gram-negative bacteria, and the sum of 18:1n9, 18:2n6, and 18:3n3 for fungi. The sum of all PLFAs was expressed as total bacteria.[19] Also, PHB was quantified by the method described by Laranja *et al*., [20] in microbial flocs.

Microbial community analysis. The enumeration of total bacteria and *Lactobacilli* was conducted following standard procedures.[21] For this purpose, every week, the viable total bacterial and estimated *Lactobacilli* count in the tank water were determined using the spread-plate technique on Trypticase Soya Agar (TSA) (Formedium Ltd., Norfolk, UK) and DeMan-Rogosa-Sharpe agar (MRS; Formedium Ltd.) medium, respectively. To enumerate total bacteria, 100 µL of water samples were aseptically plated on TSA medium under aerobic conditions. Similarly, for the enumeration of total *Lactobacilli*, 100 µL of the samples were plated on MRS medium. The plates were then

incubated at 30.00 ˚C for 48 hr in aerobic and anaerobic condition after which the colonies were counted. Anaerobic conditions were established within the chambers using Anaerocult™ A (Sigma-Aldrich, St. Louis, USA).

DNA extraction and PCR-DGGE. As part of the investigation to look at the composition of the bacterial community, water samples were collected. For this reason, three samples were drawn from each tank. After that, these samples were pooled to form a single sample that was utilized for the genetic analyses for each replication (three samples for each treatment). The DNA was isolated using the cetyltrimethylammonium bromide buffer procedure.[22] The V3 region was amplified using primer 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) with a 40-base GC clamp attached to its 5′ end and primer 518R (5′-ATTACCGCGGCTGCTGG-3′). The study of DGGE made it easier to assess the genetic patterns which were further analyzed using unweighted pair group method with arithmetic mean (UPGMA) clustering analysis in Bio-Numerics (version 6.5; BioMérieux SA, Craponne, France).

Statistical analysis. To determine the connections between DGGE profiles, a cluster analysis was performed. We evaluated the similarity between the PCR-DGGE band patterns using Pearson correlation coefficient and represented the similarities graphically using UPGMA clustering algorithm. Dendrogram construction and cluster analysis were performed using NTSYSpc (version 2.10; Exeter Software, Setauket, USA). Principal component analysis (PCA) was used to identify PLFAs that explained the variation in the data, and forward stepwise discriminant analysis was used to determine whether these variables could be used to distinguish between the treatments.

Results

Water quality parameters. Figure 1 displays dissolved inorganic nitrogen concentrations (Ammonia, NO_2-N, and NO_3-N) during the experiment. Fluctuations varied among treatments. CS treatments had minor fluctuations with the lowest ammonia at the end.

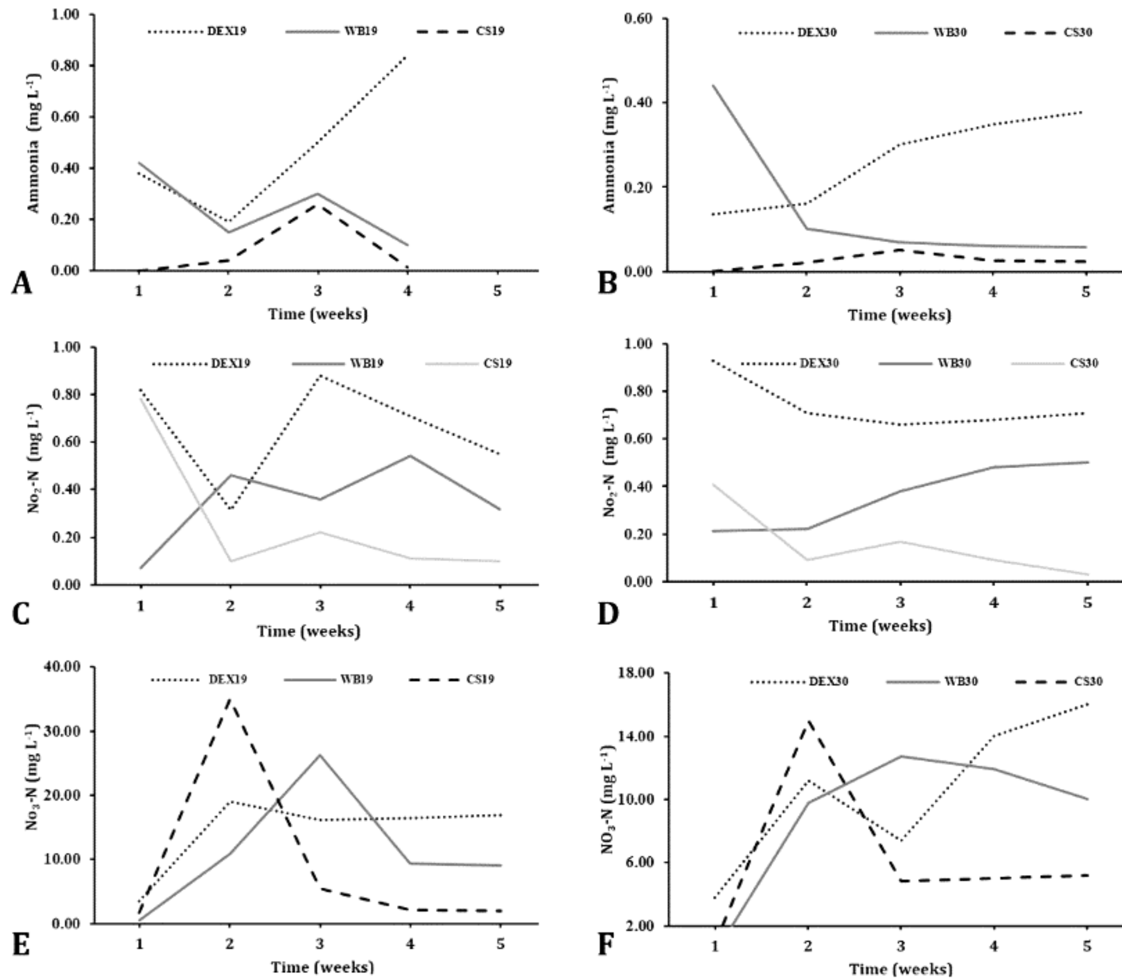

Fig. 1. Changes in **A** and **B)** total ammonia nitrogen, **C** and **D)** nitrite (NO_2-N), and **E** and **F)** nitrate (NO_3-N) were monitored throughout the experimental period. The treatments included dextrin (DEX) 19, DEX30, wheat bran (WB) 19, WB30, corn starch (CS) 19 and CS30 each using a specific carbon source (dextrin, wheat bran, or corn starch) with varying ratios (19 or 30).

In C/N19 and 30, ammonia levels in WB and DEX treatments behaved differently. In C/N19, ammonia was decreased in both carbon sources, however, increased in DEX19. In C/N30, ammonia was slightly increased, while WB30 decreased, with the lowest in CS30. Nitrite concentration fluctuations was depended on C/N ratio. DEX19 showed a sharp rise in the second week, while WB19 had minor fluctuations. The CS19 was decreased significantly. In C/N30, a similar pattern emerged, however, with fewer fluctuations. The NO_3-N concentration in CS treatments for both ratios was higher in C/N19. Initially, it was increased and then dramatically decreased. Both carbon sources showed increased nitrate concentration in weeks two and three with the highest in DEX and then WB.

Community composition, PLFAs, PHB. The results of total PLFAs, PHB (%) on cell dry weight (CDW), PHB/ total PLFAs, monounsaturated PLFAs/total PLFAs (biomarker of Gram-negative bacteria) and fungi biomarkers of PLFAs are presented in Figure 2. Our results indicated that the total PLFAs changed in experimental treatments and the higher C/N ratio in all carbon sources indicated significantly higher PLFAs when compared to C/N19 ($p < 0.05$). The highest PHB percentage (%CDW) was seen in DEX treatments when compared to the others ($p < 0.05$). No significant difference was observed between other treatments ($p > 0.05$). The PHB relative to total PLFAs was calculated in all treatments and DEX treatment showed a higher value when compared to the others. Furthermore, the C/N19 in each carbon source was found to be higher than the C/N30, although no significant difference was observed. The ratio of monounsaturated PLFAs/total PLFAs was investigated and the results indicated that the carbon sources with a lower C/N ratio showed significantly lower values ($p < 0.05$). An adverse pattern was seen in fungal PLFAs biomarkers and C/N19 exhibited a higher relative in all carbon sources.

Microbial community analysis. The dynamics of total cultivable bacterial density during this study are shown in Figure 3. This part also includes the results pertaining to pH and TSS taking into account the connection between these variables and the bacterial density debate. In contrast to the fluctuating inorganic nitrogen levels, TSS showed only slight alterations during the experiment with the highest TSS observed in WB followed by CS and DEX in C/N19. However, a different pattern was observed in C/N30 where TSS was decreased by week 3 in all treatments followed by higher levels in WB, DEX and CS, respectively. The pH was changed in all treatments, decreasing in week 2 and then tending to increase until week 3 after which it was remained constant until day 35.

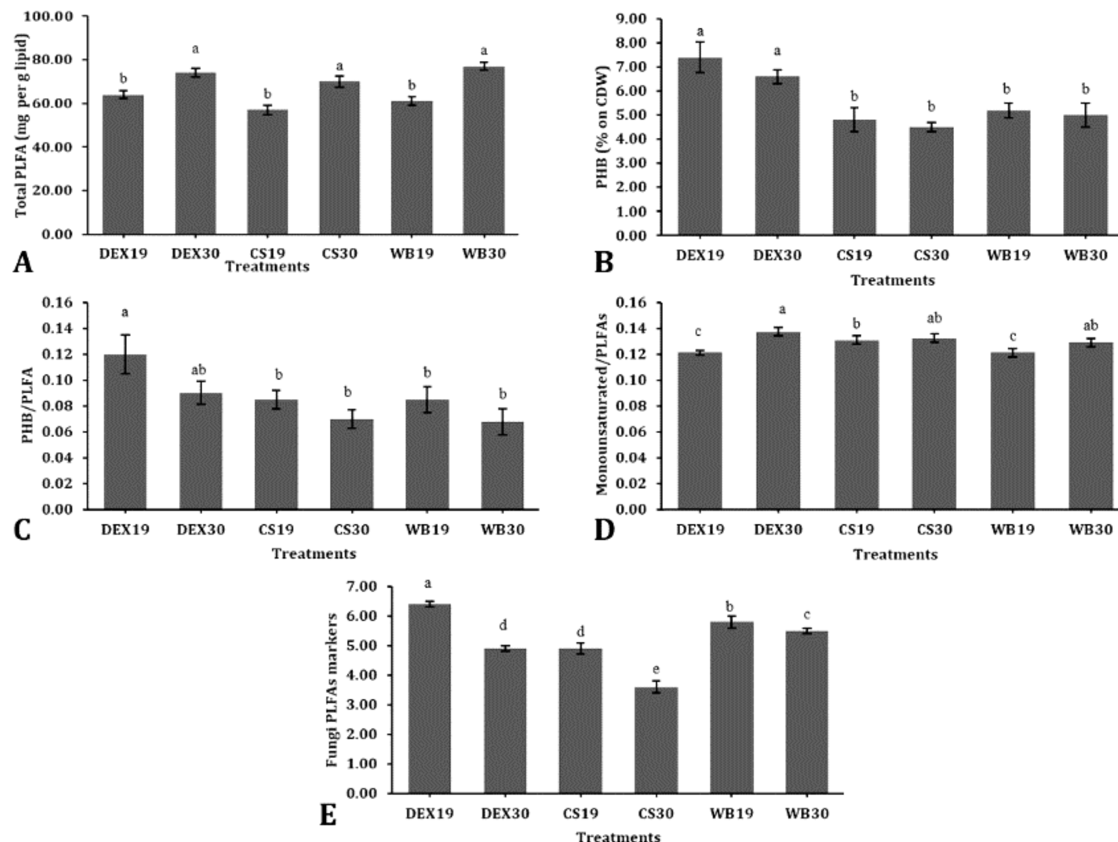

Fig. 2. The amounts of **A)** total phospholipid fatty acids (PLFAs), **B)** poly-β-hydroxybutyrate (PHB), **C)** PHB/total PLFA, **D)** mono-unsaturated/saturated PLFA, and **E)** saturated PLFA to total PLFA as well as the fungi biomarker (18:1n9 + 18:2n6 + 18:3n3) that were measured in the treatments (n = 3, means ± SD). Lower case letters indicate significant differences ($p < 0.05$) according to the Duncan test.

At the end of the experiment, a higher pH was observed in WB19 followed by CS19 and DEX19. A similar pattern was observed for C/N30 with the lowest pH seen in DEX30 compared to the other treatments. In C/N 19, bacterial density was increased in week 2, then decreased in week 3 except for DEX19 where a dramatic increase occurred in WB19 and CS19 treatments.

A different pattern was observed in C/N30 with bacterial density increasing slightly in CS30 and WB30 treatments until week 5, while a decrease was observed from week 3 in DEX30. The dynamics of total *Lactobacilli* density in biofloc treatments throughout the experimental period are presented in Figure 3 The results indicated that the pattern of lactobacilli dynamics in C/N19 was similar to that observed in total bacterial density in C/N19. At the end of the experiment, the highest lactobacilli density was observed in WB19 followed by CS19 and DEX19. The changes in total lactobacilli dynamics in C/N30 were different with density increasing in week 2 then experiencing a slight decrease in the following weeks. The highest density was seen in WB30 and CS30.

DNA extraction and PCR-DGGE. The results of the genetic distance analysis due to nucleotide diversity of the *16S rRNA* gene region are presented in Figure 4A. In this dendrogram, twelve dominant bacterial species were identified from all groups. In each treatment, two dominant bacteria were isolated and identified, while in the DEX19, three dominant bacteria were isolated. In contrast to all treatments, CS30 showed only one dominant bacteria. The WB30, CS19 and CS30 each using a specific carbon source (DEX, WB, or CS) with varying ratios (19 or 30).

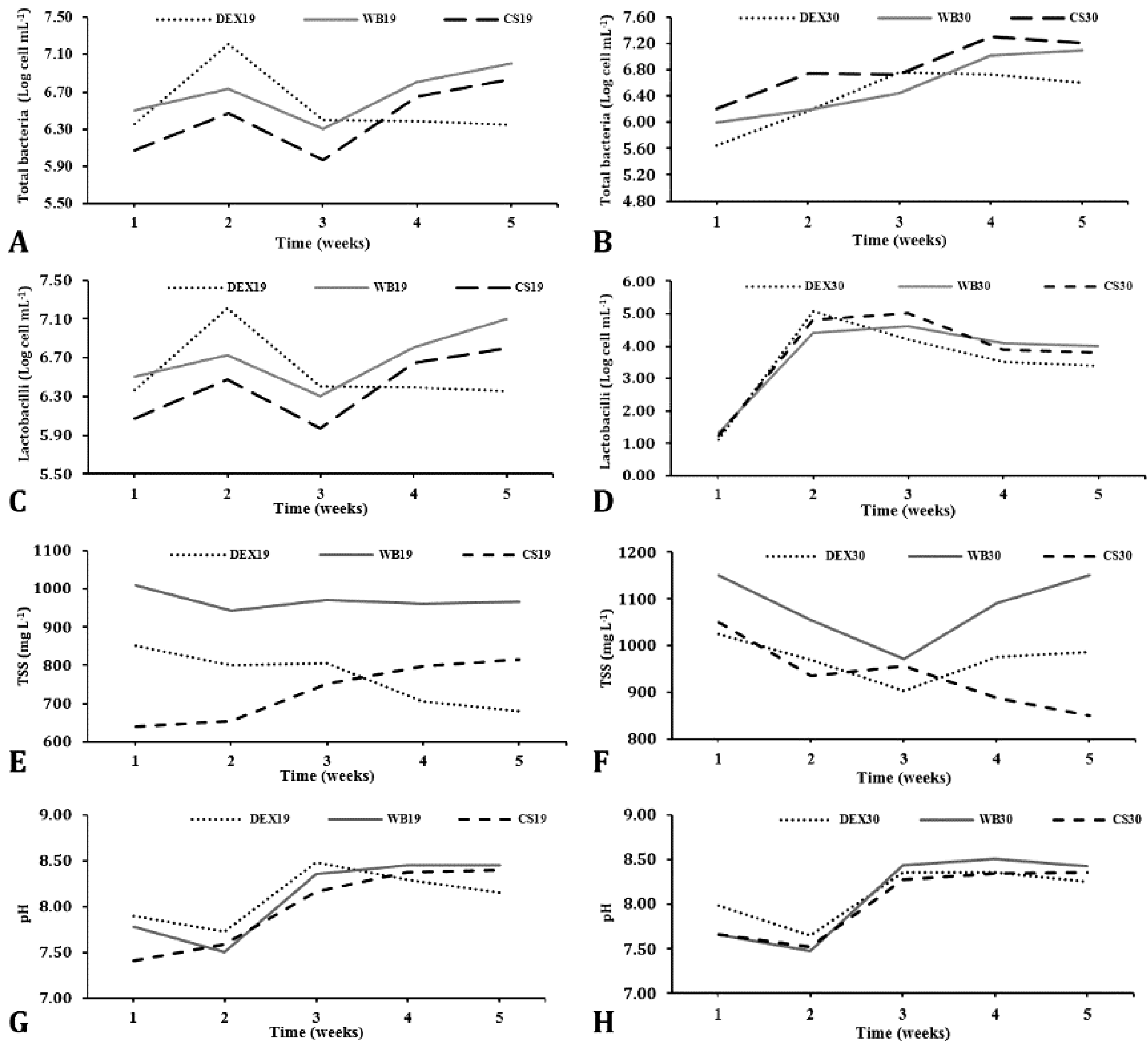

Fig. 3. The dynamic changes of **A** and **B)** total bacteria, **C** and **D)** *Lactobacilli*, **E** and **F)** total suspended solids (TSS), and **G** and **H)** pH in the biofloc system during the experimental period.

Furthermore, based on the observed nucleotide diversity among bacteria, two separate branches were seen among the bacteria. One branch includes C/N19 and another belongs to C/N30. In the set of bacteria in C/N19, *Acinetobacter hemolyticus* and *Aureimonas* sp. (in DEX), *Bacillus cereus* and *Acinetobacter* sp. (in WB), *Enterococcus gallinarum* and *Actinomyces* sp. (in CS) were observed. Furthermore, in C/N30, *Cellulomonas* sp. (in DEX), *Pseudomonas* sp. (in CS), *Rhodobacterales bacterium* and *Cellulomonas* sp. (in WB) were identified.

Multivariate analyses. The results of the (PCA are presented in Figure 4B, C. The first principal component (PC1) and the third principal component (PC3) accounted for 30.34% and 18.01% of the variance, respectively, capturing a total of 48.35% of the data variability. The C/N30 ratio exhibited a positive correlation with PC1, while, C/N19 showed a negative correlation with PC1. The DEX showed a negative correlation with PC3, whereas, CS and WB exhibited a positive correlation with PC3. The PLFAs biomarkers C14:1n5, C16:1n9, total mono-unsaturated fatty acids and C181n7 had positive values for PC1 and negative for PC3. The PLFAs biomarker C20:1n9 had a positive value for both PC. The PLFAs biomarker C18:3n3 was negative for PC1 and positive for PC3. The PLFAs biomarker C18:2n6 cis, total n6, total n3, C22:6n3, C18:1n9, C14:0 was negatively loaded for PC1 and PC3.

Discussion

The study findings showed that both PCR-DGGE and PLFAs analysis methods provided consistent results in assessing changes in the microbial community including structure and composition. These changes were influenced by the carbon sources and C/N ratios, enabling researchers to observe variations specific to different carbon sources and ratios.

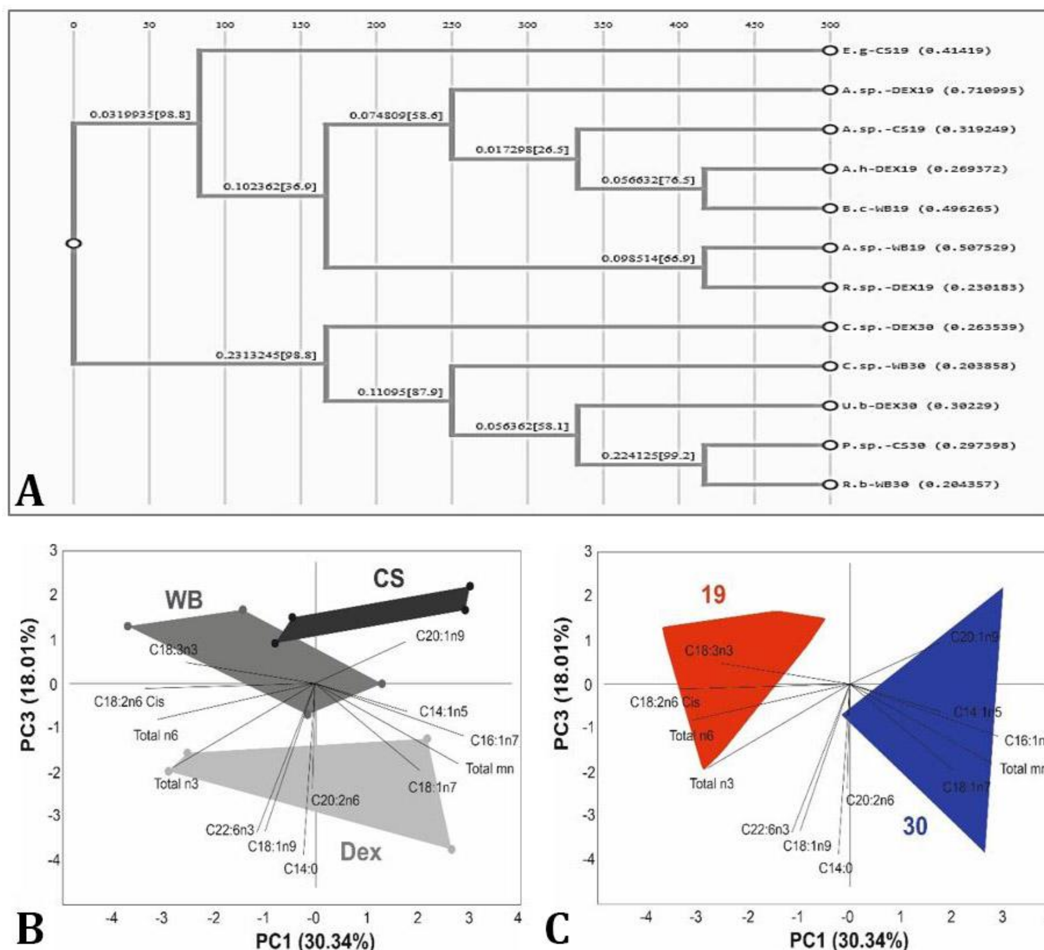

Fig. 4. A) Cluster analysis of polymerase chain reaction/denaturing gradient gel electrophoresis band patterns for experimental treatments. Phylogenetic tree showing the relationship of predominant *16S rRNA* sequences of bacteria in biofloc system to reference sequences obtained from the GenBank database. **B** and **C)** Ordination by principal component analysis (PCA) of PLFA biomarkers in different carbon sources (Dex for Dextrin, WB for wheat bran, CS for corn starch) and carbon/nitrogen ratios (19, 30). Variance explained by PCA axis 1 (PC1) and axis 2 (PC3) were 30.34% and 18.01%, respectively. The main PLFAs that accounted for the separation of treatments are shown in all plots. Each data point is a mean of three replicates.

Our study found that nitrogen metabolite patterns were similar between both C/N ratios, however, the concentrations varied across different carbon sources. The highest ammonia levels were observed in the DEX treatment after two weeks and overall ammonia concentration was higher in C/N19 compared to C/N30. This was in agreement with Avnimelech[2] observation that carbon sources with simple structures removed ammonia nitrogen faster than those with complex structures. Complex carbon sources promote microbial proliferation and aid in ammonia reduction, while, simpler structures like DEX decompose rapidly and may hinder ammonia elimination. Furthermore, complex carbohydrates like WB provide a favorable surface area for bacterial growth and lead to more diverse bacteria resulting in ammonia decomposition.[23] The pH, TSS and total bacterial density in the treatments support these findings with DEX treatment exhibiting the lowest levels compared to WB and CS treatments. Ammonia concentration toxicity also reduces the total microbial community in the DEX treatment.[24] The overall concentration of NO_3-N was lower in C/N30 compared to C/N19 indicating that a higher C/N ratio stimulated denitrification and reduced NO3-N concentration. The C/N ratio is crucial in aerobic denitrification and higher ratios increase process efficiency.[3] In both C/N ratios, the DEX treatment showed the highest levels of ammonia, NO_2-N, and NO_3-N followed by WB and CS treatments. PCR-DGGE analysis revealed that dominant bacteria in the treatments played significant roles in nitrification and denitrification processes. *Acinetobacter hemolyticus*, found in the DEX treatment, performs aerobic denitrification and utilizes various nitrogen sources.[25] Acinetobacter sp. and Rhodobacterales bacterium, found in the WB treatment, are involved in nitrogen cycling.[26] Pseudomonas sp. in the CS treatment plays a significant role in nitrogen nitrification and denitrification processes with certain strains possessing enzymes for ammonia oxidation and nitrite reduction.[23]

In our experiment, TSS was measured and the results indicated that TSS in C/N30 was higher than the C/N19 which indicated that C/N30 increased microbial biomass or organic particles. Also, the highest value was observed in WB treatment in both C/N ratios. These findings were in agreement with our total PLFAs, bacteria and protozoa PLFAs biomarkers results. Analysis of PLFAs and specific biomarkers gives a quantitative estimation of the microbial biomass in the environment[27,28] and determination of total PLFAs in the natural ecosystems is used for microbial biomass estimation.[29] Based on obtained results, higher organic materials (C) stimulate microbial community growth resulting in more microbial biomass in aquatic reservoirs,[30] marine sediments,[31] and soil.[32] Figure 2 illustrates the results of the biochemical analyses conducted on microbial flocs in the experimental treatments. Due to the lack of sufficient research on the topic within the biofloc system, the authors have relied on findings from other environmental studies such as soil and sequencing batch reactors to compensate for this gap. By incorporating insights from these related studies, the authors aim to enhance the interpretation of their results and provide a broader understanding of the subject matter. Previous research indicated that the PLFA profile served as a valuable tool for estimating the biomass of microbial communities in diverse ecosystems.[15, 17, 27] In the present study, the total amount of PLFAs in the groups indicated that C/N30 had higher PLFAs levels across all carbon sources which was consistent with the findings presented in Figure 3. The results also revealed an increase in the total cultivable bacterial density during the experimental period with the highest bacterial biomass observed in the WB30 and CS30 treatments at the end of the trial. Furthermore, PHB is considered an endogenous carbon storage substance that various bacteria produce under unbalanced conditions when nitrogen is limited, but carbon is available.[32,33] According to Wang *et al.,*[34] PHB granules serve as a carbon source that can stimulate the denitrification process in the environment. In our experiment, the DEX treatments displayed the highest levels of PHB and NO3-N concentration, while, the WB and CS treatments exhibited lower values. These results indicated that the microbial community utilized PHB granules during the denitrification process resulting in a reduction of PHB concentration in the WB and CS treatments and a corresponding decrease in NO3-N levels. These findings were in agreement with those of Barak and van Rijn[35] who found that bacterial communities could lower NO3-N levels using PHB. The PHB/PLFAs ratio was calculated for each treatment and it was observed that the C/N19 group displayed a higher ratio compared to C/N30. McKinley *et al.,* [14] have previously reported a negative correlation between the PHB/PLFA ratio and both the C/N ratio and organic matter. Our results were consistent with these findings with the higher PHB/PLFAs ratio being observed in treatments with a lower C/N ratio. Additionally, a lower ratio of monounsaturated/total PLFAs and an increase in fungal biomarkers are commonly associated with environmental stress in microbial communities, particularly when nutrients and/or organic carbon are limited.[36] Lange *et al.,*[37] noted that compared to Gram-positive bacteria, Gram-negative bacteria were generally considered fast-growing but less tolerant to environmental changes and stress due to their thin cell wall. In our study, we found that microbial flocs in the C/N19 ratio in each carbon source had a lower monounsaturated/total PLFAs ratio, indicating a lower density of Gram-negative bacteria and higher fungal biomarkers compared to the C/N30 ratios. This suggested higher nutritional and physiological stress in the lower C/N ratio. Conversely, the efficient nitrogen recycling observed in the C/N30 ratio suggested less nutritional and

physiological stress and a more stable and diverse microbial community in the C/N30 ratio. These findings were supported by the PCR-DGGE results which revealed that most of the dominant Gram-negative bacteria were sequenced in the C/N30 ratio.

The PCR-DGGE analysis of dominant bacteria in the treatments revealed that the majority of sequenced bacteria play major roles in nitrification and denitrification processes. *Acinetobacter hemolyticus*, identified in the DEX treatment, is an active bacteria involved in these processes performing aerobic denitrification and utilizing various nitrogen sources.[34,38] *Acinetobacter* sp. (performing both nitrification and denitrification)[38] and *Rhodobacterales bacterium*, identified in WB treatment are bacteria involved in nitrogen cycling.[39,40] In the CS treatment, *Pseudomonas* sp. is a genus of bacteria that includes species known to play significant roles in nitrogen nitrification and denitrification processes. Some strains of Pseudomonas have the capability to oxidize ammonia to nitrite (nitrification) through the action of enzymes like ammonia monooxygenase. Additionally, certain Pseudomonas species possess nitrite reductase enzymes that enable them to further convert nitrite to nitrogen gas (denitrification) completing the nitrogen cycle.[41,42]

The PLFA profiles provide valuable insights into the structure of microbial communities as the relative abundance of PLFAs that varies significantly among different microorganism groups.[43] Our results demonstrated significant differences in PLFA species and contents based on carbon sources and C/N ratios. The PCA of the 17 identified PLFAs revealed distinct clustering of samples based on carbon sources and C/N ratios. PC1 explained 30.34% of the total data variability and highlighted the influence of C/N ratio on PLFA profiles while PC3 explained 18.01% of the variability and primarily reflected the effect of carbon sources. In the score plot, samples with C/N19 were grouped on the negative side of PC1, while samples with C/N30 were spread on the positive side. These findings suggested that the PLFAs profiles differed between the two C/N ratios indicating distinct metabolic pathways and nutrient utilization patterns.[15,17] The PC3 showed a negative correlation with DEX, while, WB and CS showed a positive aspect. Also, the factor loadings of identified PLFAs was distributed across four quadrants. Bacterial PLFAs mostly are situated in the fourth quadrant (C/N30 and DEX), while, fungi PLFA biomarkers are found in the first and second quadrants (In WB and CS, particularly at a C/N19 and to a lesser extent at a ratio of 30). The findings indicated a dominance of fungi biomarkers in the WB and CS treatments, whereas, bacterial and protozoan biomarkers prevail in DEX. This suggested that WB and CS created favorable conditions for microorganisms associated with these specific PLFA types, influencing the development of microbial communities within the system.

In summary, this experiment demonstrated that different carbon sources with C/N ratios could significantly impact the water quality, microbial composition and structure of the biofloc system. The treatments with complex structure (CS, WB) and higher C/N ratio exhibited lower levels of nitrogen metabolism compared to DEX. Both molecular and PLFA analyzed methods yielded consistent results in terms of microbial flora changes with the DNA-based method providing insights into dominant bacteria and the fatty acid analysis offering information on the abundance of other microorganisms like fungi and protozoa, in addition to bacteria. The bacterial density was found to be higher in treatments with higher C/N ratio, while the density of fungi was higher in carbon sources with complex structures compared to DEX. These findings enhanced our understanding of the microbial ecology and nutrient dynamics in the biofloc system providing insights for its optimization.

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Conflict of interest

None declared.

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