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Review

Microbial Interactions, Growth, and Health of Aquatic Species in Biofloc Systems

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Abstract: Biofloc technology involves the manipulation of the culture system's carbon: nitrogen ratio to promote bacterial community growth to convert toxic nitrogenous wastes and organics into functional microbial protein; this protein can then be used as a food source and mediate water quality. Biofloc systems have several advantages, which include improved biosecurity, feed conversion, water use efficiency, and nutrient processing. Analyzing the nutritional value and the relationship between high production of aquacultural practices using biofloc is essential. Many studies have demonstrated that biofloc increases the growth of aquatic species by acting as a food source or providing bioactive compounds. Other than this, the beneficial micro-organisms in biofloc systems contain compounds such as organic acids that could resist the growth of pathogenic microbes. They will also serve as a natural probiotic and increase the immunity and survival of fish and shrimp. This technology could be useful for further integration within many aspects of aquaculture production when microbial interactions are considered. However, future studies must fully understand the principles and mechanisms behind the benefits of interactions between biofloc and cultured fish and crustacean species.

Keywords: bacterial communities; probiotics; recirculating aquaculture systems

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1. The Expansion of Intensive RAS in Aquaculture Production

Farming of aquatic animals and plants, known as aquaculture, dominates aquatic food production globally. Global aquaculture production was estimated at 122.6 million tonnes in 2020, with 87.5 million tonnes attributed to aquatic animal production [1]. The aquaculture sector represents a diverse group of plants and animals ranging from unicellular algae such as *Chlorella* sp. to large fish such as Atlantic salmon (*Salmo salar*) [2]. Freshwater aquaculture is known to have the highest industry production. Not only is this sector dominant, but it is also contributing to eliminating hunger and malnutrition by providing protein-rich food. As the aquaculture industry has grown and technologies have developed, there is a stronger focus on sustainable aquaculture practices to benefit production systems [3]

Interest in closed aquaculture systems is increasing rapidly due to biosecurity, environmental, and marketing advantages over other system types. Reusing water in aquacultural systems could control pollution, reduce or even eliminate the potential transfer of pathogenic organisms and mitigate the risk of escape for exotic or non-native organisms [4]. The biofloc systems can be considered an effective production tool with numerous advantages [5]. The primary goals of sustainable aquaculture include the production of more aquaculture products while minimizing water and land use, minimizing environmental pollution, and, lastly, expanding culture capabilities that support economically and socially sustainable production. All of this can be fulfilled by running an aquacultural system with biofloc technologies [6].

Among the various aquaculture technologies, water use varies considerably from flow through single-use systems (e.g., raceway setups) to limited discharge systems using minimal levels of water exchange. In a single pass system, clean water is passed through the culture tank, and then water is discharged back to the environment. The only technology controlling water quality is the rate of water exchange and/or supplemental aeration. At the same time, recirculating aquaculture systems (RAS) are land-based systems where water is recycled through a series of treatment processes, allowing reuse and minimizing water discharge. Thus, reducing water consumption, improving environmental control, and reducing the release of nutrients to the environment [7]. Biofilters, including heterotrophic and nitrification bacteria, are necessary for these systems to process nitrogen compounds and organic matter, respectively.

Further, inherent disinfection mechanics can be installed within RAS to further clarify the system. This can result in better health for aquatic animals, and disease management is better conserved than within flow-through systems where open water is required and discharged to maintain desired quality parameters. A primary rationale behind this potential health advantage is that opportunistic bacteria will not be as dominant in the system, and there is a greater level of control over the microbial load and inherent community dynamics [8]. The microbial communities in RAS may exhibit a greater level of stability than within flow-through systems. Moreover, the nitrifying and denitrifying bacteria in RAS help control the ammonium and nitrite concentration, and these parameters can be somewhat managed [9].

RAS fish production potential has significantly increased, and a variety of fish species are being propagated using this design. Some major freshwater species are catfish, tilapia, trout, and eel, and marine species such as sea bass [10]. The efficiency of RAS is more apparent than in flow-through systems, and production inputs and outputs may more easily be discerned. A study conducted by Bergheim et al. [11] stated that the smolt size ranged from 50–70 g in flow-through systems, but when fish were reared in RAS, an increase in size to 140–170 g was observed. Another study by Colson et al. [12] involved rainbow trout culture and demonstrated that RAS positively influences specific growth rates more than flow-through systems. Non-fish species, such as crustaceans and mollusks, are also cultured using RAS technology. It has been found that RAS is a feasible method of rearing juvenile abalones as growth rates (26.1 ± 15.96 μm day^{-1} in the recirculating systems and 22.21 ± 18.69 μm day^{-1} in flow-through systems). Survival rates (78.74% in RAS and 71.82% in the flow-through systems) were higher than in the flow-through system [13]. Thus, biofloc technology offers the potential for production yields and the growth of various cultured organisms.

2. Biofloc Technology and Production Aquaculture

The rearing technique that encompasses water quality by manipulating carbon and nitrogen and their inherent mixture of organic matter and microbes is known as biofloc technology [14]. This controlled addition of carbohydrates stimulates the growth of heterotrophic bacteria, and the production of bacterial proteins takes up nitrogen. The ammonia concentration in water rapidly reduces due to the uptake of nitrogen by bacterial growth. This way, carbon and nitrogen in water are well balanced [15]. The disadvantages, such as frequent maintenance and polluting the environments that are found in conventional technologies to manage and remove nitrogen compounds like nitrification reactors and solids removal, are reduced within this method. Aeration is another critical component of maintaining biofloc culture systems, with proper aeration needed to avoid the creation of anoxic zones that can be detrimental to the microorganisms [16]. With comparatively little maintenance or fine-tuning of water quality parameters, high fish survival can be achieved using a low or no water exchange rate in biofloc production. Thus, along with RAS systems, providing a better level of control over system inputs to better manage aquatic animal production. Retaining the same water for a long time allows the development of a dense and active biofloc, which offers further benefits for production applications in fish culture [17].

When considering the two most common types of closed aquaculture systems, RAS and biofloc systems, RAS usually have more filtration components leading to higher startup costs for producers. However, biofloc systems may have lower startup costs because less equipment is required to retain culture-ready tank conditions [18]. Although there may be less equipment overall, aeration equipment is still integral within biofloc systems as the microbial community may sometimes need more oxygen than the organisms being reared. Biofloc systems can be challenging to control at the start of system setup. They may have long establishment periods before adequate bacteria are present to process animal metabolites [19]. However, there may also be acclimation periods in RAS systems for sufficient biofiltration capabilities. Many advantages of biofloc systems over recirculating systems can be narrowed down. Some of these are improved biosecurity, feed conversion, water use efficiency, and improved water quality control [20]. However, the main potential benefit could be the capacity of the waste nutrients to be recycled through microbial protein into fish or shrimp for potential performance increases. Studies have shown that about 20–30 percent of the growth of aquatic organisms cultured in biofloc systems is derived from the consumption of digestion of microbial protein. Additionally, harvested biofloc may be processed via drying to create a powder, which may be integrated into fish diets directly [21]. No culture technique is without drawbacks; this biofloc technology has its disadvantages. Some of these less desirable system attributes are increased energy requirements for mixing and aeration, increased instability of nitrification, need for a startup period prior to animal introduction, and alkalinity supplementation or monitoring [22]. Additionally, as outdoor systems may require input from sunlight to maintain and create desired biofloc composition, seasonal changes may also be an impediment in some geographic locations [23]. Figure 1 summarizes the general overview of a biofloc system and its inherent advantages.

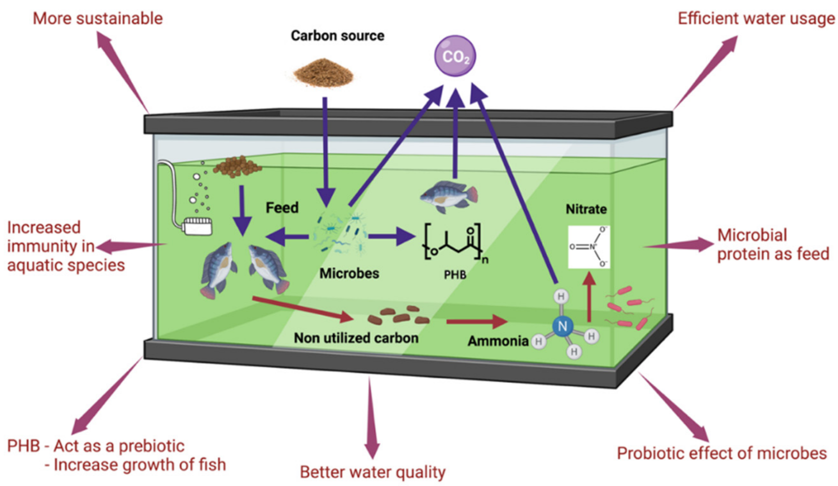

Figure 1. Overview of the inputs and outputs of a generalized biofloc system.

Previous research by [24] compared growth performance, hemato- immunological indices, water quality, and microbial communities of juvenile common carp (*Cyprinus carpio*) reared in both RAS and biofloc systems. During the experiment, it was discerned that the concentration of total ammonia nitrogen (TAN) demonstrated an increasing trend within RAS, but there was a sharp decrease in TAN in biofloc systems. This increment of TAN in RAS could be due to slower ammonia conversion rates, and the reduction of TAN in biofloc can be attributed to the higher growth of heterotrophic bacteria. TAN is toxic to aquatic organisms and requires a great deal of management when starting new intensive culture systems; thus, reduced TAN in system water is heavily promoted for advanced aquaculture technologies. Growth performance indices reflected good conditions in all treatments, although feed conversion was decreased significantly in one biofloc system as the daily feed was deducted because biofloc can be taken as a food source for common carp. Similar results were reported by Azim et al. [25], in a study that propagated Nile tilapia (*Oreochromis niltoticus*) under biofloc conditions. There was a 45% increase in fish production observed along with no differences in hematocrit or cortisol levels. These findings also indicate an appropriate fit for fish production within biofloc systems and no major physiological changes to the fish species during the rearing periods. The nutritional aspect of biofloc technology has also benefited shrimp production numbers. A study by Khatoon et al. [26] discerned that postlarval Pacific whiteleg shrimp (*Litopenaus vannamei*) could use biofloc directly as a feed source by assessing feed replacement ranging from 25–100% with dried waste biofloc from shrimp farm effluent.

Further defining factors contributing to the formation of floc structure are essential to understand the dynamics of biofloc aquaculture systems. However, more research in this area has yet to be done, and the knowledge pertaining to the promotion of floc formation in activated sludge could be applied to biofloc systems, as they are strongly interrelated. The mixing intensity rate through aeration is one of the critical factors that will influence the floc structure and the floc size. The equilibrium between the rate of aggregation and the rate of breakage will further determine the floc size. At the same time, the dissolved oxygen (DO) concentration in the water will also influence the floc structure [27]. Wilen and Balmer [28] found that when the DO is higher, the larger the compact flocs are. Organic carbon source and organic loading rates also have an influence mainly on the composition of biofloc, mainly on the storage polymers of the floc. Another factor that determines the structure of the floc is temperature. Wilen et al. [29] found that deflocculation could occur at very low temperatures (4 °C). This could be probably due to the slowdown of microbial activities.

Biofloc systems can be set up for exposure to natural light as outdoor ponds, tanks, and lined raceways, or these designs can be implemented within greenhouses. These greenhouse systems are often installed as closed systems inside buildings with little or no natural light. The outdoor systems are operated as green water bioflocs, and the indoor systems are employed as brown water bioflocs [27]. Identifying which target production species to be cultured in these systems is also a crucial decision before setting up this technology or determining system placement. Not all aquatic organisms are good candidates for biofloc systems. This may be due to feeding behaviors or water quality parameter tolerance that is not conducive to the dynamic yet static design. This technology works best with aquatic species that can withstand high solid concentrations and tolerate poor water quality. Shrimp species have long been used in biofloc system rearing, and they are an ideal species for researching system dynamics concerning enhancing production. Due to their adaptability, shrimp and tilapia are two commonly grown species in biofloc systems [30], although there is a great deal of potential for identifying novel biofloc species that hold high production value. Many different biofloc set ups have also been assess for shrimp culture. Shrimp have also been reared in multitrophic systems, along with tilapia and a hydroponic bench offset of *Sarcocornia ambigua* [31].

During a biofloc system startup, the water quality parameters are quite similar to that of a recirculating system in that there must be a high level of system manipulation and observation. The initial phase can be yet not fully predictable and therefore be risky to operate, so it is recommended that monitoring tools be implemented. Monitoring the concentration of total suspended solids which can be measured easily can be used and molecular monitoring tools also provide information on the condition of the biofloc [27]. The duration of a startup period depends upon many factors, and these include temperature, feeding rates, pre-seeding of biofiltration, and stocking density. As an example, if the feeding rate is increased rapidly, the ammonia concentration rises to a point that is toxic to the animals, reducing the growth rate, feed conversion ratio, and even animal survival. Nitrogen cycle-related peaks during a startup can be balanced by adding carbohydrates, and this gives time for the system to acclimatize and prepare to maintain animal biomass. Once the biofloc production system is more stable, carbohydrate additions are not always required, as nitrifying bacteria afford safe levels of ammonia [22].

The accumulation of solids within a biofloc system is typical; however, solids can settle at undesirable concentrations and disrupt the tank ecosystem with time. A suspended solid concentration of 200 to 500 mg/L is sufficient for a biofloc system to function efficiently. Settling cones effectively index settling solids and gauge solid accumulations within individual biofloc tanks or components. Graduated settling cones can be used to measure the volume of solids settled within a given time period, or solids can be measured with a turbidity meter for recordkeeping and to compare solid accumulation to biotic metrics or water quality. Operating a biofloc system with a suspended solid concentration within acceptable limits will reduce the risk of depletion of dissolved oxygen (DO). The use of DO monitoring is essential, and biofloc systems can deplete oxygen availability for the culture animals quickly if unbalanced. Simple gravity settling cones can control solid settling at higher feeding rates and allow for better water quality management during these demanding periods in the system. Reasonable control of the suspended solids can be achieved by operating the settling cones at a flow rate that turns over the rearing tank water every 3 to 4 days [22,32]. With the manipulations of solids within the system, the producer can control one aspect of water quality for propagation.

A significant problem in aquatic systems with animals is maintaining suitable levels of ammonia concentrations that promote growth and development. In biofloc systems, ammonia levels are modulated via algal uptake, bacterial assimilation, and nitrification [33]. Biofloc systems operating outside which are exposed to sunlight will have a good algal growth. These algae will uptake the decomposing organic matter such as fecal matter, uneaten solids, and dead algae and store them in algal cells and these processes will reduce the ammonia concentration in water. This method may be short- lived due to possibility of an algal population crash [34]. The heterotrophic bacteria living in biofloc systems immobilize ammonia in their cells as proteins, which later on may serve as a feed source. This conversion eventually controls the levels of toxic nitrogenous compounds and will be the dominating process later in the biofloc cycle. The success of this process largely depend on the C:N which is regarded as a control parameter [35]. Nitrification is the process of transforming harmful forms of nitrogen to less-toxic forms which minimizes the impact on aquacultural species. This is carried out by autotrophic nitrifying bacteria including ammonia-oxidizing bacteria and ammonia -oxidizing archaea and subsequently completed by nitrite oxidizing bacteria [36]. Unlike algal uptake and bacterial assimilation, this nitrification process is rather slow, but later this will be the major process converting toxic ammonia into less harmful compounds. Another major factor that controls ammonia is the C:N ratio. Feed with 30–35% protein levels have a low C:N ratio, and increasing the administered dietary ration will increase the growth of heterotrophic bacteria and control ammonia. Low C:N ratio feed can be balanced out by adding supplementary materials with a higher ratio or by reducing the amount of protein entering the system via diet [22].

One of the important goals of aquaculture production is to reduce production costs and achieve more profits. The main factor affecting production costs is the feed costs. The growth rate and feed conversion ratios play an important role. However, in biofloc systems, these are improved compared to other conventional systems. The key contributing factors of production costs, such as better feed conversion ratios, increased growth rates, and survival rates, are achieved through biofloc systems [37]. Some studies performed by Megahed [38] found that tilapia raised in biofloc systems had a 33% reduction in costs. The costs for organic and inorganic fertilizers are reduced though an extra cost should be allocated for the carbon source. As biofloc systems have reduced water exchanges, it considerably reduces water and water treatment expenses [39]. In saltwater biofloc systems, the use of salt can be a major expense for system operations. In a shrimp biofloc system, Pinto et al. [40] found that a blend of 25% commercial and 75% low-cost prepared salt to be financially optimal. Similarly, implementing sodium metasilicate enhanced the dominance of diatoms in the system, promoted biomass gain, and enhanced net profits and revenue compared to non-supplemented biofloc controls [41]. Some larger-scale economic evaluations of biofloc technology have also recently been performed. Betanco-Torres et al. [42] recently reviewed aspects of biofloc adoption for tilapia farming in Mexico and found that it had an immediate production commercialization potential, but more challenging to access the technology. The authors attributed a rate of return of 38% for this technology and deemed the technology to have a high cost–benefit ratio. Positive net incomes were also reported in biofloc-raised tilapia under various densities (60 and 80 fish m-3) and feeding regimes 2 and 3% [43]. Polyculture techniques within biofloc have also been evaluated for economic analyses. Sudirman et al. [44] recently investigated the concurrent culture of catfish and tilapia and found that a ratio of 60% catfish and 40% tilapia was most profitable, with a lowered payback period of 4.35 years for the model. Overall, as biofloc technology develops, more impacts related to economic benefits will undoubtedly aid in the adoption of this technology.

3. Microbial Communities and Biofloc Interactions

In biofloc systems, there's an aggregation of phytoplankton, diatoms, bacteria, algae, and protozoa, which are involved in various processes supporting the aquatic species [45]. These microbes can not only enhance or maintain water quality parameters but can also act as a potential food and nutrient source for the aquatic organisms and potentially reduce dietary protein inputs or operational expenses [46,47]. In a recent study by Sgnaulin et al. [48], Piracanjuba (*Brycon orbignyanus*) were reared in both clearwater recirculating and biofloc systems to evaluate growth and biofloc interactions. Protozoans, microalgae and rotifers were found to be most prevalent of twelve types of microorganisms found within the biofloc treatment group.The heterotrophic bacteria that are ammonia assimilative and chemoautotrophic nitrifying bacteria living in the water column assimilate inorganic nitrogen, balance the carbon and nitrogen content in water and improve water quality [49]. The carbon source has been found to influence aspects of bacterial load, abundance, and diversity within biofloc systems and the ability to control carbon inputs within a biofloc system may be a means of controlling bacterial interactions and potential production goals for systems [50]. Many mechanisms are involved in the formation of microbial biomass, and it's a complex process comprising physical, chemical, and biological processes. Surface coatings on the organism, forces of gravity and electrostatic repulsion forces help in microbial cell bonding to form masses [37]. Inorganic ions (i.e., calcium and aluminum) allow for stable flocs through bonding mechanics [37].

There are not many studies conducted about the types of bacteria living in biofloc systems and community complexes. The variety of microbial species in each biofloc can vary according to the kind of carbon source, salinity level, or the cultured species. Additionally, stocking density can also influence the microbial composition within a biofloc system, and this may be due to the influence of nutrient inputs being increased within the system or physiological outputs (i.e., increased fecal material), inclusive of nutrient dynamics [51]. The microbial community contains a higher percentage of phytoplankton in *Litopenaeus vannamei* cultured in biofloc systems than in bacteria biomass [52]. The most common bacterial phyla present in aquaculture are proteobacteria. Several studies done by various researchers also have found that proteobacteria is the phyla most commonly present within biofloc systems [53,54]. Proteobacteria is a group that is important in nutrient recycling and mineralization of organic components and has been reported within RAS [55]. Other than that, the system was also found to contain *Bacillus* spp. and Actinobacterium. *Roseobacter* sp. and *Cytophaga* sp represent other minor species that could be present in such biofloc systems [56]. Wei et al. [57] investigated the relationship of floc size with respect to bacterial communities using size fractionating sampling techniques. Study findings indicated that larger bioflocs (with constituent sized of >10 μm) demonstrated gene enrichment with respect to microbe motility and chemotaxis. Further, Family *Rhodobacteraceae* and *Flavobacteriaceae* constituents were found to be prevalent in the biofloc. Further, [58], reported a high degree of similarity between microbial communities within the biofloc and the gut of cultured shrimp. This further demonstrates a potential strategy for manipulating animal health via microbial components within the culture system.

Other than bacterial communities there can be various other microbial communities. The study done by Li et al. [59], on the performance and microbial community of combined denitrification and biofloc technology has found out the major eukaryotes present in water. From the results of 18S rRNA, the dominant phyla that were present in water were Rotifera (33.1%), Chlorophyta (23.5%) and Nematoda (20.7%). They further states that algae might facilitate the growth of these eukaryotes. The most abundant algae present in biofloc water from the study were *Tetraedron*, *Coelastrella* and *Selenastrum*. Shrimp bioflocs also have been associated with relatively diverse biofloc constituents, ranging from ciliates and copepods to nematodes [60]. The variation of microbial communities in biofloc systems throughout the experimental trial was documented by Tubin et al. [61]. The authors reared juvenile Nile tilapia in a biofloc system and were fed with diets containing mealworm. After bacterial communities, protozoan and phytoplankton groups seemed to be the most abundant. A low abundance of protozoans was observed in the first two weeks of the experiment and an increase was shown in the third week. They propose that this could be associated with ecological and chemical water quality parameters. Apart from protozoans, Monroy-Dosta et al. [62] recorded chlorophytes as the most abundant microalgae during the 3rd week of biofloc development.

Recent studies have been conducted to facilitate a more diverse microbial community in biofloc systems and determine which substrates enhance complex microbial communities. Complex microbial communities improve water quality, nitrogen removal, growth, and survival of the aquatic species in biofloc systems. Racz et al. [63] provide evidence that organic carbon source makes up a mixed culture of histotrophic and ammonia-oxidizing bacteria (AOB). The more complex the organic source, the more complex and diverse the bacterial community. Another study by Deng et al. [64] revealed that plant cellulose added as the carbonic source significantly affects the bacterial community, with changes to diversity and complexity. In these systems, the components of the flocs are more complex, provide enough nutrients for heterotrophic bacteria, and contain more inorganic nutrients for AOB. Another avenue of investigation for product quality related to system inputs is the relationship between feed amount sand substances that may cause off-flavor in fish fillets, as demonstrated by Schrader et al. [65] in channel catfish (*Ictalurus punctatus*) biofloc production. Together, the additions of nitrogen sources and their interactions with microbes within the biofloc require further investigation to define culture recommendations for aquatic species.

Microbial communities living in the water have self-generated bioremediation and a strong potential to improve water quality. These microbes also provide nutrients such as amino acids, proteins, fatty acids, and lipids in the form of different microorganisms [66]. Further, digestive capacity may also be enhanced using biofloc systems, via microbial interactions, further contributing to modified nutrient uptake for the cultured animals [67]. Protease and amylase enzymes may be elevated in biofloc systems when compared to clearwater, along with liver transaminases [68]. Together, these microbial community interactions are of great interest to further harness the potential of biofloc systems in aquaculture.

The interactions between the microbes in biofloc systems are complex and may be difficult to characterize. They can be harmonious interactions or competitive and intrinsic interactions between bacteria and algae that also demonstrate similar patterns. The tanks where bioflocs are dominated by algae present a greenish color, and the bioflocs dominated by heterotrophs are greenish-brown in coloration. The highest bioflocs densities are observed in heterotroph-dominated systems [17]. In a recent study by Dong et al. [69], the influence of *Platymonas* sp. added into a biofloc system was evaluated. Results concluded that this algal supplementation positively impacted nitrogen cycling and influenced microbial species diversity in the biofloc.

One example of the complementary relationship between algae and bacteria is the increment of bacterial productivity due to increased primary productivity in water. This is primarily because heterotrophic bacteria can utilize the organic carbon released by algae. This carbon availability and sink due to algal constituents should be considered when evaluating carbon cycling throughout a biofloc system [16]. Then, the bacteria can degrade this organic matter into nutrients such as vitamins and other bioactive compounds, which can influence the higher growth of phytoplankton [15]. Luo et al. [70] evaluated levels of carbohydrate additions into biofloc systems and found that a single 20:1 addition of glucose favored nitrification and that carbohydrate manipulations translated to differences in the compositions of bacterial communities within the biofloc.

The administration of aqueous substances like antibiotics and allelopathic substances can be an inhibitory effect between microalgae and bacteria. These substances can influence the chemical environment of the other. The production of glucosidases, chitinases, and cellulases by bacteria lysing microalgae cells can be an example of antagonistic growth [71]. Considering hurdles to both adoption and integration, there can be more possibilities for substrate competition, such as ammonia and nitrate within the rearing system. This depends on temperature and the amount of ammonia. In the summer months, phytoplankton will outcompete nitrifying bacteria for low ammonia, and the opposite is true in the colder months [72].

Effect of Carbon Source on the Microbial Community and Structure of Biofloc

Carbon sources added into biofloc systems have a high effect on microbial composition. Through high-throughput sequencing, many scientists have been able to characterize bacterial communities. The first information on the complex microbial community in biofloc using sugar cane molasses was described by Cardona et al. [73]. The authors further explained that the most frequently abundant phyla, such as Proteobacteria, Bacteroides, and Cyanobacteria need an organic substrate to grow and attach to surfaces and co-aggregate.

Another study by Wei et al. [53] used three carbon sources, glucose, starch, and glycerol, to demonstrate that the composition and microbial community of biofloc differs. The biofloc with starch as the carbon source had a higher density but a lower floc volume than the other two. Furthermore, varied carbon sources had different amounts of algae, where biofloc with starch had the most and the one with glycerol had the least. The microbial communities associated with glucose and glycerol carbon sources applied were similar and more diverse than the starch-supplemented system.

Generally, heterotrophic bacteria gain energy from organic carbon sources, and AOB requires inorganic carbon. However, studies have been conducted and indicated that even AOB communities are affected by the organic carbon addition. A study by Racz et al. [63] examined the effect of peptone and glucose on batch reactors. They identified that the peptone-fed reactor had a more significant proportion of AOB than the glucose-fed reactor. This increased proportions of AOB will help in faster nitrification, which will be useful in biofloc systems.

Carbon sources can impact on the immunological effects of aquatic organisms too. The study done by Eksari et al. [74] tested the disease resistance and immunity of Pacific white shrimp reared in different carbon sources against infectious myonecrosis virus (IMNV). In general, biofloc system had higher survival and higher resistance against the IMNV challenge than clear water systems however only slight differences were observed among the biofloc systems with different organic carbon sources. The total hemocyte count and phenoloxidase activity prior to the challenge in biofloc systems were higher and it was concluded that this higher activity appeared to be carbon source dependent.

In shrimp culture, modifications to carbon inputs have been evaluated for the early rearing periods and grow-out production stages. Molasses usage in a nursery setting was found to decrease ammonia levels while the use of dextrose in grow-out tanks was found to lower ammonia [75]. Thus, the selection of these carbon additions is important to consider for different life stages and within different biofloc system types. The cost of the carbon source can also be a factor for the system, with potential trade-offs for lower costs but with reduced performance. This was documented by García-Ríos et al. [76] in a tilapia biofloc, where estimated costs of wheat flour implementation as a carbon source were lower than that of corn flour or sugar, but the tilapia fingerlings did not grow well over a period of 31d.

4. Probiotic Applications and the Intestinal Microbiota of Aquatic Organisms

The close relationship between the microbes and other biotas in the water column and the microbiota in the digestive system of fish and other invertebrates in aquatic ecosystems are equally important. Studies have been conducted to discover these relationships in biofloc systems. A survey conducted by Cardona et al. [73] regarding the bacterial community characterization of the shrimp intestines (*Litopenaeus stylirostris)* in a biofloc system showed that the culture environment highly interacts with the intestinal microbial communities, and the bacterial composition was different from those cultured in clear seawater. The relative abundance of bacterial orders *Bacteroidia Bacteroidales, Flavobacteriia Flavobacteriales, and Mollicutes* was higher in the biofloc system than in clear seawater.

Though many studies examine shrimp within in situ biofloc systems and their constituent gut microbiota, research on tilapia is rare. However, it has been identified that in situ biofloc significantly increases the microbial diversity and richness in the gut of tilapia. Furthermore, it has been suggested that this increment is due to water contact or floc ingestion where live bacteria are thriving. More diverse gut microbiota is beneficial for fish in many aspects and can affect their physiological function and health [77,78]. Additionally, the gut microbiota of tilapia reared within in situ biofloc system has a distinct composition compared to clear water and *ex situ* biofloc supplementation. This is because the processing of the biofloc changes the microbial composition [77].

Some previous studies have also proven that the size of biofloc influences gut microbiota. The survey by Huang et al. [79] on shrimp gut microbiota has identified that large-sized bioflocs had higher community similarities and correlations than small bioflocs. It was further explained that shrimp are more prone to ingest large bioflocs as they are more recognizable, attractive, and perhaps more conducive to their feeding behavior. The family *Rhodobacteraceae,* the dominant core group in the shrimp gut, had the highest correlation between gut bacterial communities and the large bioflocs. The large-sized bioflocs harbored particle-attached bacteria such as *Planctomycetes, Bacteroidetes,* and *Alphaproteobacteria.* Thus, there may be potential for new technologies to separate biofloc components in an effort to control the production animal microbiomes.

Probiotics are widely used in aquaculture systems and also gaining momentum as production strives to navigate away from antibiotic usage. The probiotic applications are mainly used to modulate the gut microbiota of aquatic species, but it also helps in environmental bioremediation [80]. Many strains of bacteria such as *Bacillus* spp., *Paracoccus* sp. and *Lactobacillus* spp. are used as probiotics [81]. Not many studies previously performed compare the effect of probiotic applications on microbial community composition in biofloc systems. Kathia et al. [82] demonstrated that probiotic addition does not modify the microbial community, but this can be due to the probiotics used. However, probiotic application in containing *Bacillus subtilis* in biofloc positively affected the growth and survival of aquatic species [83,84].

5. Microbial Interactions in Biofloc and Implications on Health and Diseases of Cultured Species

Biofloc technology is mainly used in intensive aquaculture practices. Though intensive techniques offer many benefits, such as a reduction in land use of space, significant issues like disease outbreaks could lead to economic losses [85]. Yet, intensive aquaculture coupled with biofloc technology can minimize these types of problems. Diseases in aquatic species can be due to two main reasons. They could be microbial infections or because of environmental stress and/or when the water quality parameters are not acceptable [86]. The role of the organisms within biofloc technologies relies upon competitive between beneficial and pathogenic organisms within the system [51]. Thus, this natural model of interactions is conducive to mitigating traditional pathogen management strategies in aquaculture, including the use of antibiotics.

The immune system of fish consists of innate and adaptive immune responses. When the fish are injured and infected with a pathogen, they perform phagocytosis and other inflammatory reactions accompanied by non-specific immune cells like macrophages, neutrophils, etc. [87]. Many humoral factors are released from fish when the surrounding bacterial load is increased. Lysozyme is one such factor, and it is a preferred marker of the immune response. It has an anti-inflammatory effect, and many studies have found that biofloc systems have leading effects and enhancements of anti-inflammatory activities and increment of immunity of aquatic species, including fish and shrimps [6]. Fish in bloc systems have been found to have modulations in a variety of health-related metrics, including serum enzyme activities, lysozyme and complement content, and antioxidant status [88].

The most basic principle behind biofloc is maintaining standard water quality parameters. This will minimize stress on fish and increase biosecurity. Fish with minimum stress are less prone to diseases from pathogens. The larval tolerance to environmental stress could be enhanced by the consumption of live microbial flocs, which is present abundantly in biofloc systems. The tolerance to pH stress of sea breams increased when they were fed with Artemia enhanced with live microbes. The authors have mentioned that this was due to upregulated expression of the genes encoding for a heat shock protein (HSP70) that involves protecting the cells by binding and refolding damaged proteins [89]. The consumption of microbial flocs by aquatic species in biofloc systems has increased tolerance to environmental stress [90].

Numerous researchers have performed stress tests to assess the quality of species in aquaculture. A study on Nile tilapia broodstock raised in biofloc systems has identified that larviculture practices can improve the quality of the produced larvae and develop resistance when challenged with *Streptococcus agalactiae.* Two mechanisms explain this enhancement. The first involves the maternally derived immune protection potentially transferred to the offspring. When the female broodstock were reared in biofloc systems, they will confer some form of protection and immunity to the embryo with both innate and adaptive immunity by transferring factors like serine protease and various types of immunoglobins and macroglobulin. It is also described that if the broodstock are exposed to microbes, they will produce more immune factors and transfer to the offspring. The second mechanism is that consumption of micro flocs by larvae or the adult fish will expose them to an array of microbe-associated molecular patterns such as β-1-3-glucan, lipopolysaccharides, and peptidoglycan that will activate the non-specific immunity of aquatic species [90].

Bioflocs may also have a probiotic effect, reducing the number of pathogens in the water. The similarities between biofloc systems and probiotics are mainly because both contain live microbes [91]. Probiotics contribute positively to cultured species through bioremediation, competitive exclusion, immunomodulation, etc. Bioflocs also have some active compounds that have antibacterial properties. Poly-β-hydroxybutyrate (PHB) is a polymer present in bioflocs that has a prebiotic effect and benefits the growth of beneficial microbes in the colon of organisms. Other microbes commonly synthesize it in response to physiological stress [14]. Blocking or disrupting quorum sensing is another mechanism used by probiotics which can also be seen in bioflocs. Microbes in biofloc disrupt quorum sensing or cell-to-cell communication in infectious bacteria. A study done on the survival of brine shrimp when challenged with *Vibrio harveyi* has been able to test the impact on biofloc systems. Quorum sensing is known to regulate the virulence of *V. harveyi,* and Artemia showed a significant difference in survival and resulted in complete protection against the pathogen. The most common disruptions of quorum sensing are the production of quorum sensing antagonists and the employment of signal molecule degrading enzymes by microbes [92].

Lastly, the health of shrimp raised in biofloc systems also has a focal point of recent investigations. For instance, Tepaamorndech et al. [93] analyzed both immune gene expression and the gut microbiome of Pacific whiteleg shrimp. Findings demonstrated that the biofloc played a role in the maintenance of the gut bacteria and that Vibrio spp. were a significant constituent of the biofloc. Further, changes to the systemic immune system were attributed to biofloc rearing. Interestingly, Gustilatov et al. [94] also discerned that biofloc systems might have the potential to reduce concentrations of *Vibrio parahaemolyticus*, along with providing a reduction in the pathogen's biofilm activity. Few studies have been done on the physiological health of shrimp, focusing on immune and antioxidant defense when reared in biofloc systems. A study by Jang et al. [95] explained that *L. vannamei,* when reared in biofloc systems, significantly increased the expression of prophenol oxidase enzyme in hemocytes. Another study on the same species demonstrated that superoxide dismutase activity increases when cultured in bioflocs [96]. These enzymes are important in innate immunity due to their involvement in cellular and humoral defense. Jang et al. [95] further explained that this could be due to the entering microbes in the biofloc into the shrimp body and modulating the immune system. Thus, there is a comprehensive and dynamic role of the biofloc within shrimp culture systems when subjected to system inputs. These factors discussed may be manipulated to direct aspects of rearing performance and health.

6. Future Research and Optimization of Biofloc Systems in Fish and Shrimp Culture

Several beneficial features are associated with bioflocs and their microbial interactions. The advantages are lower feed and water input, less risk of pathogens and diseases, and increased biosecurity, growth, and survival. Some of the limitations for adoption include the availability of system inputs (water or energy), regulations, as well as the availability to technology for these systems [97]. Hence, it will be valuable to study more about the microbiome and its interaction with fish health and the mechanisms of quorum sensing and controlling the introduction of pathogens. Additional studies should be conducted to investigate the effects and combination of different carbon sources and the microbial community dynamics in biofloc to establish a healthy microbial community. More focus on microbial community-based protein production is needed so that the aquatic species can acquire microbial proteins as a feed source. Biofloc is a relatively new technology for aquaculture that could be applied to sustainable aquaculture practices, but more studies are needed in many areas to use it to obtain more production benefits.

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