

*Valorising plant-based residues
for shrimp production through biofloc technology*

Apriana Vinasyam



Propositions

1. Fertilizing the biofloc microbiota through the shrimp gut is as effective as via the water.
(this thesis)
2. In biofloc systems, shrimp production is not affected by the digestibility of dietary carbohydrate.
(this thesis)
3. The consistent growth in contribution of ponds to global aquaculture production is not reflected in the scientific output.
4. Waste is just an un-exploited resource.
5. To do a PhD one needs to be a jack of all trades.
6. Social media is nowadays our digitalised curriculum vitae.

Propositions belonging to the thesis, entitled

Valorising plant-based residues for shrimp production through biofloc technology

Apriana Vinasyam

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for shrimp production through biofloc technology**

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**Valorising plant-based residues for shrimp production
through biofloc technology**

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to my family
untuk keluargaku

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1

General introduction

1. Overview of biofloc system: promises and challenges

Biofloc technology (BFT) started more than 35 years ago as a promising zero-water exchange system that can contribute to the ecological intensification of aquaculture (Avnimelech, 2015; Zaki et al., 2020). BFT allows to maintain water quality, to increase animal growth and production, and to improve nutrient utilization efficiency (Crab et al., 2007; Hargreaves, 2013; Kumar et al., 2018; Panigrahi et al., 2019). Moreover, the carrying capacity of BFT systems is higher than in conventional pond systems, allowing farmers to increase the stocking density without compromising system performance and animal welfare (Widanarni et al., 2012; Zaki et al., 2020; Oliveira et al., 2022; Tinh et al., 2023).

Biofloc is a heterogenous mixture of microorganisms (e.g. bacteria, archaea, algae, protists, zooplankton, fungi) and organic detritus aggregated together in a polysaccharide matrix (extracellular polymeric substances, EPS) (Hargreaves, 2006; De Schryver et al., 2008; Wilen et al., 2008; Xu and Pan, 2012; More et al., 2014). Heterotrophic bacteria play an important role in water quality management by immobilizing organic and inorganic nitrogen into biofloc biomass using organic carbon as energy source (Hargreaves, 2013; Martins et al., 2020).

In aquaculture, the C:N ratio of pelleted shrimp feed typically varies between 6.5 – 9.5 (Hardy and Kaushik, 2021). After feeding, the C:N ratio of the feed waste drops, making it too low to allow heterotrophic bacteria to immobilize all the nitrogen waste into bacterial biomass. This happens because the shrimp respire a high fraction of the dietary carbon as CO₂ while nearly all the dietary nitrogen not retained in growth enters the biofloc rearing tank, mainly as inorganic ammonia, which becomes toxic, even at low concentration. Therefore, a C:N ratio between 15 – 20 of the nutrient input (pelleted feed plus carbohydrate) is recommended, to make sure the C:N ratio is high enough for the shrimp and for the heterotrophic bacteria to avoid ammonia intoxication (Avnimelech, 1999; De Schryver et al., 2008; Xu and Pan, 2012).

A BFT system is created by maintaining metabolic wastes in suspension which is mostly done by keeping the water column well mixed and oxygenated through continuous aeration. Various types of carbohydrate are used to maintain a sufficiently high C:N ratio to maintain water quality, such as molasses, tapioca or corn starch (Ekasari et al., 2014b; Rajkumar et al., 2016; Xu et al., 2016; Panigrahi et al., 2019; Khanjani et al., 2021; Tinh et al., 2021b). BFT can be applied in fresh, brackish or marine water, and used to culture a wide range of species, including the herbivorous piracanjuba (*Brycon orbignyanus*) (Sgnaulin et al., 2018), herbivorous/omnivorous species like crucian carp (*Carassius auratus*), common carp (*Cyprinus carpio*) (Wang et al., 2015; Ebrahimi et al.,

2020), Nile tilapia (*Oreochromis niloticus*) (Choo and Caipang, 2015; El-Husseiny, 2018; Deng et al., 2021; Khanjani, 2021), and more omnivorous/carnivorous species such as black tiger shrimp (*Penaeus monodon*) (Kumar et al., 2017) and Pacific white shrimp (*Litopenaeus vannamei*) (Panigrahi et al., 2019; Silva et al., 2021; Tinh et al., 2021b; Xu et al., 2022; Tinh et al., 2023).

Besides controlling total ammonia nitrogen (TAN) and nitrite (NO₂-N) toxicity (Crab et al., 2007; Ray et al., 2011; Avnimelech, 2015), biofloc can serve as an *in situ* produced natural food produced on recycled waste nutrients (Cardona et al., 2015). In doing so, it can reduce the feed conversion ratio by 30% (Hargreaves, 2006; Wasielesky et al., 2006). In addition, recycling nutrients using protein-rich biofloc allows to reduce the dietary protein concentration in pelleted feed (Xu and Pan, 2013b; Jatobá et al., 2014). Up to 37% of the nitrogen retained by the shrimp in BFT systems can be provided by biofloc (Cardona et al., 2015), while for carbon, this can be up to 60% retained in body mass gain (Avnimelech and Kochba, 2009; Cardona et al., 2015; Emerenciano et al., 2017; Ray et al., 2017). Other advantages of BFT systems include the presence of beneficial bacteria in biofloc who outcompete pathogenic bacteria through competition for space and nutrients (Emerenciano et al., 2013; de Souza Valente et al., 2020), high antioxidant compounds that may improve disease resistance (Liu and Fang, 2003; De Schryver and Verstraete, 2009), and provision of external digestive enzymes that help fermentation in the gut (Xu and Pan, 2013b; Wang et al., 2016; Chakrapani et al., 2021).

Despite the abovementioned benefits, BFT requires a continuous and high energy supply to keep the system running. In addition, BFT systems are complex and challenging to manage effectively, which partly explains a still limited adoption throughout the industry in spite of a very large research effort since its introduction 35 years ago (Khanjani and Sharifinia, 2020).

2. Biofloc development

2.1. Biofloc development until maturity

BFT involves intentional cultivation and manipulation of the microbiota within the culture environment, aiming to convert feed waste into biofloc to maintain water quality and to provide supplementary natural food. During development, a dense aggregation of microbiota, organic matter, and detritus results in the formation of biofloc. Sometimes, biofloc development is stimulated by introducing inorganic fertilizers, calcium carbonate (CaCO₃) and easily degradable carbon sources such as molasses or starch into a start-up culture environment of fish or shrimp. The latter is to supply energy to bacteria utilizing the post-feeding nitrogen waste (79-94% of feed nitrogen) (Jackson et al., 2003; Hari

et al., 2006). Post-feeding waste includes waste from uneaten feed and spillage (5% of feed), excretion (faeces; 13.8%) and egestion (branchial & urinary excretion; 56.2%) (Hu et al., 2012). Furthermore, decaying microbiota may be categorized as an indirect post-feeding waste, as they grew utilizing waste nutrients.

When carbohydrate is added to a biofloc tank, it is uniformly distributed over the water surface after feeding (Khanjani et al., 2019). During start-up of a biofloc system, sometimes urea or ammonium sulphate is added to increase the nitrogen levels in the water and accelerate microbial biomass development (Nisar et al., 2021). An inoculum, existing of biofloc-rich water from another BFT system, can also be added to stimulate microbiota community development (Widanarni et al., 2012; Martins et al., 2020; Ferreira et al., 2021; Tinh et al., 2021c). In some cases, the diatom *Thalassiosira weissflogii* (Emerenciano et al., 2012a) or a commercial probiotic mixture mainly containing lactic acid bacteria (e.g. *Pseudomonas* or *Bacillus*) can be also added (Nisar et al., 2021). Moreover, to provide a surface for microbial growth, tiny substrates like clay or straw can be added (Addo et al., 2021; Nisar et al., 2021).

Visual, physical, and chemical water quality parameters like water colour, temperature, dissolved oxygen (DO), pH, salinity, alkalinity, total ammonia nitrogen (TAN), nitrite (NO₂), and nitrate (NO₃) concentrations are regularly assessed. These measurements help to monitor the system's dynamics and determine whether any actions are necessary to maintain biofloc development. Biofloc development is indicated by the observed increase in biofloc volume, measured in Imhoff cones (Sgnaulin et al., 2018). When starting a culture with a low shrimp biomass, as the culture progresses, the biofloc microbiota can develop within 1 – 4 weeks, enough to deal with the increasing feed load and to maintain water quality (Emerenciano et al., 2012b; Widanarni et al., 2012).

Mature biofloc is defined as the state in which biofloc has already established a functional microbial community, capable of effectively managing the post-feeding waste in the system. This involves photo-autotrophic uptake of inorganic nutrients by microalgae, conversion of ammonia and nitrite into nitrate by chemo-autotrophic bacteria through nitrification and assimilation by heterotrophic aerobic bacteria (Ebeling et al., 2006; Xu et al., 2016; Martins et al., 2020; Ferreira et al., 2021). Mature biofloc develops within 30-50 days starting with clear water, reaching a floc volume density of 5 mL/L (Emerenciano et al., 2012b), or a total suspended solid (TSS) concentration above 100 mg/L (Emerenciano et al., 2012a). Once the biofloc is mature, the carbon input can be temporarily reduced since senescent biofloc partially decomposes providing extra carbon for maintaining the heterotrophic microbial community (Xu et al., 2016). Despite these suggestions, in reality establishing a standard time to reach maturity remains

difficult, as the time required to achieve it depends on culture conditions and nutrient load. Furthermore, quantifying the microbial functionality in such a complex system like biofloc presents challenges. Which processes dominate in maintaining the water quality in the biofloc system depends on numerous factors, including culture intensity, the type and amount of nutrient input, and the biofloc age and density. More research exploring how these factors interact is needed.

2.2. Biofloc microbial pathways

There are several processes involved in the nitrogen waste remediation in a biofloc system, which are: photoautotrophic assimilation, chemoautotrophic oxidation and heterotrophic assimilation.

a. *Photoautotrophic assimilation*

Phytoplankton (microalgae) play a pivotal role in this pathway, producing biomass by absorbing inorganic carbon (CO_2) and utilizing nitrogen waste, mainly ammonium (NH_4^+) due to its low energy requirement (Kumar and Bera, 2020). When they take CO_2 from air, a carbon influx is introduced to the system, which is not always taken considered when making a nutrient budget. This pathway thrives in an environment with a C:N ratio of 2-3 and requires little dissolved oxygen (Ebeling et al., 2006; McCusker et al., 2023).

b. *Chemoautotrophic oxidation*

Nitrifying bacteria dominate the chemoautotrophic pathways, converting ammonia into nitrate while consuming alkalinity (CaCO_3) (Ebeling et al., 2006). Nitrification consists of two steps: ammonia conversion to nitrite by ammonia oxidizing bacteria (AOB) like *Nitrosomonas* and *Nitrosococcus*, and nitrite conversion to nitrate by nitrite oxidizing bacteria (NOB) like *Nitrospira* (Preena et al., 2021). To counteract the alkalinity consumption, sodium bicarbonate or sodium hydroxide is commonly added to the system (Ebeling et al., 2006). Moreover, nitrifiers require a low C:N ratio (1-2) and sufficient DO (Zhu and Chen, 2001; Ebeling et al., 2006); for the latter a well aerated biofloc system is essential. A different chemoautotrophic pathway, other than nitrification, is the inorganic nitrogen removal via denitrification, converting nitrate into gaseous nitrogen (N_2) (Hu et al., 2013). While aerobic denitrification may also occur (Deng et al., 2020a; Deng et al., 2020b), anaerobic ammonium oxidation (anammox) is limited in an aerated biofloc system (Kumar and Lin, 2010).

c. *Heterotrophic assimilation*

Heterotrophs are organisms that fulfill their energy requirements by utilizing organic matter to immobilize organic and inorganic nitrogen into bacterial biomass (Schneider et al.; Ebeling et al., 2006; Schneider et al., 2006). An important role of the biofloc is to keep the ammonia and nitrite concentrations low. For that, approximately 20 g of carbohydrate (50% carbon content) is needed to immobilize 1 g of ammonia or nitrate nitrogen into bacterial biomass, achieving a C:N ratio of 4-6 (Avnimelech, 1999). Overall, this pathway requires C:N ratios (g/g) above 10 in the nutrient input (De Schryver et al., 2008; Ray and Lotz, 2014; Avnimelech, 2015; Xu et al., 2016; McCusker et al., 2023) and 2-6 in the biofloc tank. When the carbon input drops, autotrophic bacteria are required to prevent ammonia build up in the biofloc system (Ebeling et al., 2006). In addition, bacterial genera such as *Bacillus*, *Paracoccus*, *Pseudomonas*, might perform heterotrophic nitrification, and thus contribute in the ammonia and nitrite removal (Preena et al., 2021).

An additional microbial group that can assist in N mobilization is the zooplankton, which can accumulate up to 71% protein (dry matter basis) in their biomass when grazing on microalgae, bacteria and organic matter in the system. This activity is done aerobically, as these organisms need oxygen for respiration (Khanjani et al., 2022). Moreover, aquatic fungi and yeast can heterotrophically assimilate inorganic nitrogen and synthesize EPS (extracellular polysaccharides), also known as polymeric substances (Ravella et al., 2010). *Aspergillus* can convert nitrogen via nitrification, while *Candida* assimilates nitrogen and produces EPS in an aquaculture system (Gao et al., 2019a; Preena et al., 2021).

In a biofloc culture system, chemoautotrophic and heterotrophic assimilation and chemoautotrophic oxidation occur side by side, making it a mixotrophic system. During early development, one process might dominate in the biofloc, but as the biofloc reaches maturity it becomes more mixotrophic (Ferreira et al., 2021). Overall, a biofloc system is a mixotrophic system in which various microbial photoautotrophic, chemoautotrophic, and heterotrophic pathways interact.

2.3. Biofloc microbial dynamics and composition

The initiation phase of biofloc system is associated with a low nutrient load, during which the microbial composition is predominantly governed by microalgae in the presence of light. Microalgae can outcompete bacteria due to their ability to absorb CO₂ in absence of organic carbon. Furthermore, due to their large biomass, initially algae outcompete bacteria in clean water, but when the organic matter load in the culture

system increases, the bacteria community develops. While algae development is surface limited, bacteria are not. Heterotrophic bacterial biomass will continue to grow as the daily feed and carbohydrate input increases and outcompete the microalgae (Xu et al., 2016).

In comparison to chemoautotrophic bacteria, heterotrophic bacteria have a shorter generation time (30 minutes vs. 12 hours) and 40 times more biomass per unit substrate (Ebeling et al., 2006; Avnimelech and Kochba, 2009). As a result, with sufficient organic carbon availability, heterotrophic bacteria can outcompete the growth of chemoautotrophic bacteria and limit nitrification in the system (Hargreaves, 2006). However, although slowly, chemoautotrophic bacteria will accumulate in the biofloc system over time. As the system matures and becomes more mixotrophic, nitrogen waste is utilized through diverse pathways by various microbiota, rendering the system more stable and resilient in accommodating a high nutrient loading (Xu et al., 2016). Overall, previous studies reported that an increased C:N ratio of the nutrient input shifts microbial dominance from photoautotrophic or chemoautotrophic to heterotrophic (Avnimelech, 1999; Ebeling et al., 2006; De Schryver and Verstraete, 2009). This transition can be observed by a change in water color from green to brown when algae become less dominant in the rearing system (Nisar et al., 2021).

Studying a freshwater African catfish biofloc system, Yun et al. (2017) reported that microbiota consisted of 35% bacteria, 29% microalgae, 24% fungi and 12% zooplankton. However, the ratio between the different groups of microorganisms is highly variable and situation dependent (Ray et al., 2010). Biofloc collected from Pacific white shrimp reared at salinities ranging between 5 and 32 ppt, comprised 25% phytoplankton (dominated by diatoms), 3% bacterial biomass, a minor amount of protozoan community (comprising 98% flagellates, 1.5% rotifers, and 0.5% amoeba), and 33% detritus; the residual 39% was ash (Ju et al., 2008). Reported dominant phyla in the microbial community of Pacific white shrimp biofloc culture systems are presented in **Table 1.1** It is important to highlight that most studies focused on the prokaryotic community in biofloc, and so far, few studies have explored the eukaryotic, community composition and functionality, thus resulting in an important knowledge gap.

3. Biofloc studies: current state and what can be improved

Studies have been done so far to simplify and enhance the efficiency of biofloc management. Parameters studied include the nutrient input level and C:N (g/g) ratio (Pérez-Fuentes et al., 2016; Xu et al., 2016; Panigrahi et al., 2018), the type of carbohydrate (Serra et al., 2015; El-Husseiny, 2018; Chakrapani et al., 2021; Tinh et al., 2021b), the carbon addition method (Tinh et al., 2021a; Tinh et al., 2021c), the biofloc

Table 1.1. Biofloc microbial composition in various Pacific white shrimp biofloc systems with different carbon type addition.

Carbon type*	Observed microbial phyla**	Source
Sucrose	<i>Proteobacteria</i> (38–60%), <i>Bacteroidota</i> (15–28%), <i>Actinobacteriota</i> , (8–14%), <i>Cyanobacteria</i> (2–8%), <i>Firmicutes</i> (3–6%), <i>Chloroflexi</i> (1–3%)	(Chen et al., 2021)
Glucose	<i>Proteobacteria</i> (45.8%), <i>Bacteroidetes</i> (21.1%), <i>Planctomycetes</i> (13.5%), <i>Chlamydiae</i> (10.3%) and <i>Firmicutes</i> (6.8%)	(Huang et al., 2022a)
Mollasses	Phytoplankton: <i>Cyanobacteria</i> (78%), <i>Chlorophyta</i> (20%), <i>Heterokontophyta</i> (2%), <i>Euglenophyta</i> (0.1%) and <i>Dinophyta</i> (0.02%)	(G'álvez et al., 2015)
	Zooplankton: <i>Rotifers</i> (43%), <i>Copepoda</i> (33%), <i>Protozoa</i> (14%) and <i>Cladocera</i> (10%)	
	<i>Bacteroidetes</i> and <i>Proteobacteria</i> (>74%)	(Jiang et al., 2020)
	Nitrification bacteria: <i>Nitrosopumilus</i> , <i>Nitrosopelagicus</i> , <i>Nitrosomonas</i> (a total of 97%), <i>Nitrobacter</i> , <i>Planctomyces</i>	(Xu et al., 2020)
	<i>Proteobacteria</i> , <i>Bacteroidetes</i> , <i>Planctomycetes</i> , <i>Chloroflexi</i> , <i>Actinobacteria</i> , <i>Latescibacteria</i> , <i>Gemmatimonadetes</i> , <i>Patescibacteria</i> , <i>Acidobacteria</i> , <i>Verrucomicrobia</i> , <i>Cyanobacteria</i> , nitrification bacteria (<i>Nitrosomonas</i> , <i>Nitrosococcaceae</i> , <i>Unclassified</i> , <i>Nitrobacter</i> , <i>Nitrococcus</i> and <i>Nitrospina</i>)	(Xu et al., 2021)
	<i>Tropicibacter</i> , <i>Saprospiraceae_uncultured</i> , <i>Planctomyces</i> , <i>Rhodobacteraceae_uncultured</i> , <i>Pir4 lineage</i> , <i>Ardenticatenia_norank</i> , <i>Microbacterium</i> and <i>Candidatus Alysiosphaera</i>	(Xu et al., 2022)
Corn starch	Prokaryotic: <i>Proteobacteria</i> (35%), <i>Planctomycetote</i> (15%), <i>Bacteroidota</i> (15%), <i>Cyanobacteria</i> (12%) and <i>Chloroflexi</i> (10%)	(Tinh et al., 2021c)
	<i>Vibrionaceae</i> <i>Enterobacteriaceae</i> , <i>Alteromonadaceae</i> and <i>Micrococcaceae</i>	(Luis-Villaseñor et al., 2016)

This table is modified from Khanjani et al. (2022). *Order: from simple to complex carbon source. ** Order: from the highest relative abundance among the microbial groups.

concentration (Ray et al., 2010; Schweitzer et al., 2013; Gaona et al., 2017) and the biofloc particle size (Ekasari et al., 2014a).

Under the conventional method, the biofloc is managed by adding simple sugars or starch-rich carbon sources (molasses, corn starch, wheat flour) to maintain a high C:N ratio during culture. Continuous administration of carbohydrate supports the continuous production of new biofloc mainly through heterotrophic bacterial growth. Although this practice helps to maintain water quality, it also increases the biofloc concentration, measured as total suspended solids (TSS), which can eventually become too high (Ray and Lotz, 2014). It has been reported that a TSS concentration that exceeds 800 mg/L may cause clogging of the shrimp gills (Ray et al., 2010; Schweitzer et al., 2013). A simple way to reduce the TSS concentration is by diluting the biofloc through water exchange. However, in doing so, important nutrients and beneficial microbiota are lost from the system (Avnimelech, 2015). This can be especially challenging for chemoautotrophic bacteria, which are slow-growers and need time to regrow in the system. Thus, dilution may temporarily reduce the nitrification capacity of the biofloc system (Ebeling et al., 2006), and as a consequence force the farmer to reduce temporarily the feed input to prevent ammonia intoxication.

On the other hand, maintaining a high TSS concentration exerts a high biological oxygen demand (BOD) due to the microbial respiration, thus, additional aeration is required. This can facilitate the development of pathogenic microorganisms in the flocs (Alonso-Rodríguez

and Páez-Osuna, 2003; Brune et al., 2003; Hargreaves, 2006; Avnimelech, 2009a; Hu et al., 2014; Nisar et al., 2021). Moreover, maintaining a high biofloc concentration in the system requires an unnecessary high carbohydrate input, which besides being costly, also raises the carbon loss through CO₂ emissions. For example, a previous study by Tinh et al. (2021a) reported a 5-fold increase in CO₂-loss, when carbohydrates were added in biofloc systems, compared to no carbohydrate addition. As such emissions contribute to global warming (Hu et al., 2014), alternative practices should be considered to reduce carbon loss.

The contribution of aquaculture to global greenhouse gases (GHG) emissions, such as CO₂, has aroused great attention in recent years (Williams and Crutzen, 2010; Hu et al., 2012; Hu et al., 2014). One possibility to reduce the CO₂ loss from biofloc systems, is to decrease the dietary protein input. In this way, less carbohydrate addition would be needed to maintain a sufficiently high C:N ratio in the nutrient input (Avnimelech, 1999; De Schryver et al., 2008; Emerenciano et al., 2017). In turn, the biofloc would act as a supplemental protein-rich natural food, mitigating the negative impact of the reduced

dietary protein input on shrimp growth (Xu and Pan, 2014; da Silva et al., 2018; Nguyen et al., 2021). Such an approach is possible, because the microbiota in the biofloc can upgrade the inorganic post-feeding nitrogen waste into bacterial protein (Ebeling et al., 2006; Crab et al., 2007; Avnimelech, 2015). However, how efficiently Pacific white shrimp can compensate a reduced protein supply through the feed, in a zero-water exchange biofloc tank system, still needs to be evaluated. This approach gave good results with the herbivorous-omnivorous Nile tilapia (Azim and Little, 2008; Mansour and Esteban, 2017; da Silva et al., 2018; Nguyen et al., 2021), but is it not clear whether it would work similarly for the Pacific white shrimp. It may be possible that shrimp prefers more protein-rich feed compared to Nile tilapia, as the latter can also feed on algae and detritus. Therefore, such an approach needs to be evaluated.

Although not yet fully explored, an additional way to counteract the reduction of carbon input in BFT systems is by utilizing the existing microbial biomass as additional carbon source. With the use of outdoor mixotrophic systems which contain both algae and bacteria (Xu et al., 2016; Ferreira et al., 2021), additional carbon is introduced by algae (Ebeling et al., 2006). Moreover, the possible contribution of decaying microbial biomass as an *in situ* carbon supply when using mature biofloc has not been explored in depth. Therefore, working with mature biofloc, either indoor or outdoor, may allow to reduce the carbohydrate input to the biofloc system.

Despite its potentials, farmers often avoid working with mature biofloc, because it complicates system management. Mature biofloc has a high ash content and the biofloc TSS concentration in the rearing tank might become too high (Ju et al., 2008). In addition, conventional methods often apply an easily degradable carbon source (e.g. molasses, starch) (De Schryver et al., 2008; Hargreaves, 2013). The latter tends to be quickly consumed by heterotrophic bacteria for biomass production or ammonia removal, causing fluctuations in dissolved oxygen (DO), pH, as well as concentrations of ammonia, nitrite, and nitrate (Ekasari et al., 2014b; Serra et al., 2015). Furthermore, applying easily degradable carbohydrates stimulates nitrogen immobilization and growth of heterotrophic bacteria, and thus reduces the nitrogen available for algal growth; this may lead to a reduction in the CO₂ uptake by algae and *in situ* carbon production. Moreover, administering a carbohydrate besides the feed requires extra labour to monitor the water quality, to calculate daily the required amount of carbohydrate to be added, weighing the carbohydrate and applying it to the biofloc system (Tinh et al., 2021c). This results in feeding the system twice: first the pelleted feed followed by the carbohydrate, requiring a higher labour intensity and resulting in a higher carbon losses.

The proposed approaches — reducing the nitrogen and carbon input and making better use of mature biofloc and *in situ* carbon supply — might make biofloc systems more nutrient-efficient, while decreasing the nutrient input costs and preventing biofloc overproduction. Additionally, both approaches can mitigate the overall organic carbon loss while recycling post-feeding nitrogen waste as edible biofloc for the shrimp. Furthermore, the use of carbohydrate sources which would reduce the costs for the farmer and promote circularity aspects, such as cheap, low-quality ingredients (i.e. dietary fiber or indigestible polysaccharides), in combination with lowering labour intensity with alternative management practices such as carbon addition via the pelleted feed, are intriguing aspects to consider.

4. Proposed approach

Manipulating the C:N ratio of the nutrient input in a biofloc system by adding carbohydrate enhances water quality management and nutrient recycling (Pérez-Fuentes et al., 2016; Xu et al., 2016; Miao et al., 2017; Panigrahi et al., 2018; Panigrahi et al., 2019). An alternative approach, previously reported (Kabir et al., 2020; Tinh et al., 2021c), is to apply a dual-purpose feed that contains sufficient carbon and nutrients to feed the shrimp (purpose 1: good quality shrimp feed), and support the microbial community in the rearing system (purpose 2: quality nutrient and energy input for biofloc microbiota to maintain water quality). For this purpose, addition of carbohydrates via the feed that are less digestible by the culture animal, such as for example the non-starch polysaccharides (NSP), would result in faeces with a high organic carbon content (Amirkolaie et al., 2005; Sinha et al., 2011; Maas et al., 2020). Complex NSP-rich carbohydrate sources will pass relatively unharmed through the gastro-intestinal tract (GIT) (Amirkolaie et al., 2005; Maas et al., 2020). This is more pronounced in Pacific white shrimp which has a short GIT compared to Nile tilapia, leaving less room and time for fermentation to occur (Beseres et al., 2006; Kabir et al., 2020). Thus, the assumption is that by incorporating NSP in the feed, its carbon content will only increase slightly, while it will result in a higher faecal C:N ratio. The faeces can then provide energy to the heterotrophic bacteria of the system, allowing them to utilize nutrients provided by the post-feeding waste.

Decreasing the overall C:N input in the system can have implications for the biofloc performance and water quality. However, by using mature biofloc already at the start of a new culture cycle, extra protein and energy source can be provided to the culture system via the decaying biomass (Azim and Little, 2008; Jatobá et al., 2014; Xu and Pan, 2014; da Silva et al., 2018; Nguyen et al., 2021). By following the above approach,

it might be possible to reduce the carbon and nitrogen input without a negative effect on biofloc production and water quality maintenance in biofloc systems. This approach might also allow better control on excessive biofloc accumulation in the biofloc system (Ray et al., 2010; Schweitzer et al., 2013; Gaona et al., 2017).

Though still subject of debate, indications are that complex carbohydrates with a high NSP content, like rice bran, wheat bran and cellulose, are used by heterotrophic bacteria, although more slowly than when feeding simple sugars like molasses. Consequently, their administration can reduce rapid bacterial biomass production, thereby slowing down the increase in TSS concentration. In consequence, the organic load in the biofloc rearing tank will increase slowly, maintaining room for algae to contribute to water quality maintenance and organic carbon input via photosynthesis. In addition, a slowly degradable carbohydrate also tempers rapid fluctuations in water quality, making the system more stable (Serra et al., 2015).

Finally, by administering a dual-purpose feed, the nutrients and carbon are provided in the right proportions required by the microbiota, which will eventually mobilize the post-feeding waste and maintain water quality. Furthermore, the faeces may already contain some microbiota after passing through the GIT, facilitating their further breakdown when entering the system. Nevertheless, the proposed approach has been insufficiently explored; there are still concerns regarding potential negative impacts of feeding an NSP-rich diet to shrimp, on nutrient digestion within the gut, water quality and total shrimp production at system level (Amirkolaie et al., 2005; Sinha et al., 2011).

5. Aim of this thesis

5.1. Scope and context

This thesis is a continuation of the PhD project of Tran Huu Tinh (2022), exploring the potential of dual-purpose feeds for biofloc systems of Pacific white shrimp. One observation was that biofloc technology application caused a 5-fold increase of carbon loss compared to a system with no added carbon ((Tinh et al., 2021a). In addition, the type of carbohydrate applied affects the nutrient utilization efficiency in the biofloc system (Tinh et al., 2021b). Therefore, reducing carbon loss and developing a better understanding of the impact different carbon sources have on biofloc system functioning are important research priorities.

In this thesis, the feasibility of using indigestible carbohydrate sources as organic carbon supply for biofloc systems of Pacific white shrimp is explored. Specifically, alternative strategies to carbon addition by testing dual-purpose feeds were evaluated, while aiming at reducing the overall carbon input in the system. As read-outs, shrimp

performance, water quality, biofloc quantity and quality, and microbiota composition were evaluated. This strategy was based on a previous study (Kabir et al., 2020), where feeding Nile tilapia with NSP-rich diets in semi-intensive ponds showed positive effects on both fish and food web productivity. Considering little is known about the capacity of shrimp to digest NSP-rich ingredients, such as wheat bran, palm kernel meal and corn cob meal, the feasibility of this approach for shrimp biofloc systems is worth exploring.

The choice of working with Pacific white shrimp in this thesis is because this species continues to be a highly valued seafood commodity worldwide (Zhang et al., 2017). Pacific white shrimp holds a major share of global crustaceans production (51.7%), with approximately 5.8 million metric tons produced in 2020 (FAO, 2022). To meet future demand, the industry also seeks to intensify in a sustainable way. One possible way to achieve this is through biofloc technology (Khanjani et al., 2023; McCusker et al., 2023). However, biofloc technology is still not a leading technology in the industry, mainly because the complex interactions within the microbial community in biofloc systems are insufficiently understood (Salin and Vinh, 2023).

Therefore, the aim of this PhD project was to investigate the effects of NSP-rich carbohydrate addition, either via the pelleted feed or externally, as carbon source, on the overall system performance, including both shrimp and biofloc production. The overall objective is to identify alternative strategies that will improve the performance of the shrimp-biofloc system and simplify the biofloc management practices. Moreover, the use of mature biofloc was also explored. The principal parameters I evaluated were shrimp performance in terms of nutrient digestibility, growth, and production, as well as the system performance in terms of water and biofloc quality, microbial composition, nutrient distribution, and the system's ability to mitigate the carbon loss.

The central emphasis on shrimp growth performance focuses more on production at system scale rather than on individual growth. This study is not a conventional dietary nutrition-based research endeavour which observes the effect of the feed as a sole nutrient source for the shrimp. Rather the distinct involvement of natural biota within the biofloc system is explored within this thesis, which provides supplementary food to the shrimp, and thus we consider the overall shrimp production in the biofloc system to be more relevant than the individual growth. This study aims at understanding the mechanisms underlying the utilization of low-quality carbohydrate sources through biofloc technology, administered via the diet, in a controlled-mesocosm environment. Further research will be still necessary to determine the applicability of this concept under practical fields conditions.

5.2. Thesis outline

Chapter 2 investigates the effects of supplementation of wheat bran, which is an NSP-rich ingredient, in a commercial-like feed for Pacific white shrimp (control diet) on nutrient digestibility, shrimp and system performances. The hypothesis investigated is that the wheat bran supplementation will reduce the apparent digestibility coefficient (ADC) of nutrients in the shrimp gut, causing an increase in the shrimp faecal C:N ratio. Such increase in faecal organic carbon is then expected to stimulate the growth of heterotrophic bacteria, leading to higher biofloc mass production achieved in a commercial-like system. To evaluate this hypothesis, two trials were conducted: a digestibility experiment in a clear-water system, and a growth experiment in biofloc mesocosm tanks. The diets were administered at iso-nitrogenous basis. Shrimp performance included individual growth and total production, while system performance involved biofloc quality, quantity, and activity, in addition to water quality. Furthermore, the distribution of nutrients (carbon, nitrogen, and phosphorus) in different system compartments (shrimp, biofloc, and water) was evaluated, together with the unaccounted nutrient loss.

To comprehensively understand the effect of wheat bran supplementation on the biofloc microbial compositions and dynamics, **Chapter 3** explored both prokaryotic and eukaryotic microbial groups including fungi, within biofloc samples (**from Chapter 2**). Predicted prokaryotic functionalities were also examined to gain insights into how wheat bran supplementation influenced biofloc performance.

Chapter 4 focuses on comparing two methods of adding wheat bran to a Pacific white shrimp biofloc system as a proof of concept: the conventional method by direct addition and addition via pelleted feed. This chapter aims to determine the feasibility of supplementing wheat bran via the feed in order to simplify biofloc management practice, compared to the conventional approach. Like in **Chapter 2**, **Chapter 4** investigates the effects of the treatments on the nutrient digestibility, shrimp performance, system performance and nutrient balance. The experimental set-up mirrored that of **Chapter 2**, with the key difference being that input nutrients (such as protein, fat, carbohydrate) were maintained at similar levels in this chapter, as opposed to being based solely on nitrogen level (iso-nitrogenous) as in **Chapter 2**.

The feasibility of incorporating NSP via diet to feed both shrimp and biofloc led to a more extensive investigation in **Chapter 5**, exploring the use of various NSP ingredients with different fermentability levels, mainly due to their differences in cellulose and lignin levels, on similar aspects as explored in the previous chapters. **Chapter 5** explores the addition of various NSP-containing ingredients with two different fermentability

levels (poor versus moderate) to the diet fed to a Pacific white shrimp biofloc system. The potential for reducing protein input was also evaluated by applying the two experimental diets at two different input levels, resulting in a 2x2 factorial design. As a point of comparison, a high-starch-low-NSP diet was also formulated. Overall, this experiment analysed the same parameters as in the previous chapters. Limited observations on the compositions of prokaryotic and fungal communities in the biofloc were also performed.

In **Chapter 6**, the experimental results are placed in a wider scientific context and its relevance to the society is explored.



2

Effects of high wheat bran input on the performance of a biofloc system for Pacific white shrimp (*Litopenaeus vannamei*)

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Abstract

Applying an external carbohydrate source to stimulate heterotrophic bacteria growth in biofloc systems is a common practice which could be simplified by combining the carbohydrate source with the feed into one pellet. In the current study, such an approach was tested in Pacific white shrimp, *Litopenaeus vannamei*. Wheat bran (WB) containing non-starch polysaccharides (NSP), a hardly digestible carbohydrate, was mixed with a commercial-like shrimp diet (control; CONdiet) to create a wheat bran rich diet (WBdiet). Shrimp were fed isonitrogenously, resulting in the WBdiet group receiving equal absolute amount of nitrogen but more carbon than the CONdiet group. A digestibility trial was performed in a clear water recirculating aquaculture system for 35 days, using 6 replicates aquaria per diet. Compared to the CONdiet, shrimp fed with the WBdiet had a reduced overall digestibility and an increased faecal C:N ratio (from 12 to 20). Following the digestibility trial, a second experiment was performed to monitor the growth performance of the shrimp reared in biofloc systems for 42 days, using three replicate tanks per diet. The dissolved nitrogen species concentration was lower in the tanks fed the WBdiet, while the dissolved carbon concentration was similar between the diets. From a system perspective, similar amounts of nitrogen accumulated across the system compartments with both diets, with maximum 3% total nitrogen loss reached at the end of the experiment. The percentage of carbon loss per kg feed was similar between diets. Overall, diluting the CONdiet with wheat bran and feeding both diets isonitrogenously did not reduce shrimp survival and had minor effect on growth (2% reduction of the specific growth rate). Therefore, incorporating NSP-rich ingredients, such as wheat bran applied as one pellet has a potential to simplify the carbon addition management in a biofloc system.

Keywords: biofloc, shrimp, carbon, non-starch polysaccharides, system performances

1. Introduction

Biofloc technology has been widely studied and applied in shrimp culture (Bossier and Ekasari, 2017; Panigrahi et al., 2020; Tinh et al., 2021b). In a biofloc system, the rearing tank is also an active sludge tank, in which metabolic wastes and uneaten feed are converted into biofloc. The principal actors in a biofloc system are heterotrophic bacteria, which break down organic matter for the synthesis of their own biomass (Khanjani et al., 2022). In this process, also potentially harmful inorganic nitrogen is converted into bacterial biomass, thus contributing to maintaining good water quality. These heterotrophic bacteria produce an extracellular polymeric substances (EPS) matrix, which aggregates microbiota into biofloc particles (Wilén et al., 2008; Osemwegie et al., 2020). Biofloc can be *in situ* consumed by the shrimp as an additional food source, enhancing production and lowering the feed conversion ratio (FCR) (Burford et al., 2004; Tacon and Metian, 2015; Ray et al., 2017). A biofloc system improves organic matter retention at system level in shrimp and biofloc biomass, as well as dissolved nutrients in the water (De Schryver et al., 2008; Cardona et al., 2015; Khanjani et al., 2023). In a biofloc system, carbon is required to assimilate nitrogen into bacterial biomass. The optimal carbon to nitrogen ratio (C:N ratio) in a biofloc system has been reported to be between 15 and 20 (Avnimelech, 2009; Wei et al., 2016). To achieve the optimal C:N ratio, the current common practice is to add a carbon source directly into the water besides the feed, which is labor intensive and prone to errors. Another approach can be to incorporate the carbon source directly into the pelleted feed. The feed then acts as a single-nutrient-input-package for both shrimp and biofloc, and as such simplifies biofloc management and reduces the workload of the farmer.

The type of carbon source, supplemented via the diet, may differently affect biofloc development and shrimp performance. A previous study showed that combining an easily digestible carbon source (e.g. corn starch) with feed into one pellet did not improve the growth and the nutritional content of biofloc, compared to feed without corn starch addition (Tinh et al., 2021c). Corn starch is easily digested by shrimp leading to quick carbon dioxide (CO₂) release (Rosas et al., 2001), resulting in limited carbon left in the shrimp faeces. Consequently, insufficient energy is available for heterotrophic respiration in the biofloc tank. Wheat bran, on the other hand, includes non-starch polysaccharides (NSP), which are complex carbohydrates that shrimp hardly digests (Kaushik et al., 2022). Therefore, dietary inclusion of wheat bran in the pelleted diet, could be an alternative way to provide organic carbon to the biofloc via the undigested fraction of NSP in the faeces. Dietary inclusion of NSP, however, can reduce the digestibility of other macronutrients, which was shown for Nile tilapia and rainbow trout (Sinha et al., 2011; Haidar et al., 2016; Staessen et al., 2019). To date, information

on NSP digestibility in shrimp is still lacking, while knowledge on the effect of a high dietary NSP input on biofloc system performance is limited.

Biofloc studies performed during the last two decades have demonstrated that the type of carbohydrate influences biofloc formation and activity (Wang et al., 2016; Xu et al., 2016) to a large extent due to differences in the degradation velocity between types of carbon sources (Vilani et al., 2016). Molasses, which contains simple sugars, dissolves and mineralizes faster than rice bran, which contains more complex carbohydrates in the form of NSP (Serra et al., 2015). Variation in the degradation rates of the different types of carbon sources can induce non-simultaneous nutrient availability, thereby affecting bacterial growth. For instance, adding a carbon source which is slowly degraded may slow down the immobilization of inorganic, potentially harmful, nitrogen waste, while an easily degraded carbon source may lead to excess biofloc production and rapid fluctuations in water quality. Therefore, the types of carbon can affect biofloc production and water quality dynamics (Ekasari et al., 2014; Rajkumar et al., 2016).

Here, we hypothesized that NSP is indigestible by shrimp and therefore dietary wheat bran inclusion will increase the C:N ratio of the faeces. The higher availability of organic carbon relative to nitrogen entering the system through the faeces and branchial and urinary loss by the shrimp will provide more energy for bacterial mineralization and thus stimulate biofloc formation. Stimulation of the biofloc production will raise natural food availability and enhance immobilization of inorganic (toxic) nitrogen species and help to maintain good water quality during culture. We mimicked the biofloc management practice in the field by giving additional carbon input to the system. However, we supplemented carbon through the feed, as opposed to the conventional approach which deliver it separately from feeding. Therefore, this study investigated the effects of increasing carbon input via dietary wheat bran supplementation in pelleted diet (wheat bran diet; WBdiet) on nutrient digestibility of Pacific white shrimp (*Litopenaeus vannamei*) and on system performance (shrimp, water quality, biofloc, and nutrient mass balances). Mature biofloc water was used to ensure that the system receiving no extra carbon (control diet; CONdiet) would not collapse when exposed to nitrogen waste from the shrimp excreta. Our study focused not only on animal growth, but also on the system's components, which included both biotic and abiotic factors, providing a comprehensive understanding of the system's dynamics. While the CONdiet might appear to be better for shrimp, the WBdiet would be better in terms of system performance.

2. Materials and methods

The experiment followed the protocol from Carus Animal Research Facilities of Wageningen University and Research (WUR), which assured the animal welfare. The stocking density and water quality conditions were maintained according to water quality limits for shrimp (Mohanty et al., 2018). Two experiments were executed: a digestibility experiment with daily faeces collection in clear water aquaria without biofloc and a growth performance experiment in biofloc mesocosm tanks. The treatment consisted of wheat bran-rich diet (WBdiet), which was compared to a control diet (CONDiet). The digestibility experiment used aquaria as experimental units with 6 replicates per treatment, while the growth experiment involved mesocosm tanks with 3 replicates for each treatment. Both experiments used completely randomized designs.

2.1. Experimental animals and diets

Pacific white shrimp were obtained from Crevetec, Ternat, Belgium. Two diets were formulated (**Table 2.1**). One diet served as the control diet with a composition representative for a commercial shrimp diet, further referred to as the CONDiet. The second diet was formulated diluting the ingredient mix of the CONDiet with wheat bran, an ingredient rich in NSP. This diet is further referred to as the WBdiet (**Table 2.1**). The percentages of soya lecithin, monocalcium phosphate ($\text{Ca}(\text{H}_2\text{PO}_4)_2$), calcium carbonate (CaCO_3), cholesterol, vitamin and mineral-mix (premix), and yttrium (Y) in the WBdiet were kept similar to the concentrations in the CONDiet to make sure the nutrient requirement of Pacific white shrimp was also met with the WBdiet (**Table 2.1**). Both diets contained 0.02% yttrium oxide (Y_2O_3) as an inert marker for digestibility measurement.

Shrimp were fed daily continuously using mechanical belt feeders for 10-12 hour starting at 16.00. In the WBdiet treatment, the daily feeding rate gradually declined as the shrimp grew from 11.9 to 3.3 % body weight (BW), assuming that the FCR gradually increased from 0.6 to 1.1 during the experiment. Diluting the CONDiet with wheat bran reduced the diet protein content. The amount of CONDiet fed daily to the shrimp was calculated aiming to feed the same absolute amount of protein as with the WBdiet. Consequently, the dietary input of protein was similar, but the input of carbohydrate was higher in the WBdiet.

2.2. Digestibility experiment

Determining digestibility is the first step in evaluating the usefulness of feed ingredients in formulated diets for target species (Ayisi et al., 2017). Apparent digestibility was determined as first described by (Cho et al., 1982). The feed digestibility experiment

Table 2.1. Diet formulation used in this experiment

Parameters	Diet	
	CONDiet	WBDiet
Ingredients (%)		
Fishmeal	21	12
Soya bean meal	12	7
Wheat	20	12
Wheat flour	30	17
Wheat bran	0	40
Wheat gluten	10	6
Soya lecithin	2.0	2.0
Salmon oil	1.0	0.6
Ca(H ₂ PO ₄) ₂	1.2	1.2
Chalk (CaCO ₃)	0.5	0.5
Cholesterol	0.3	0.3
Premix	1	1
L-Lysine HCl	0.2	0.12
DL-Methionine	0.3	0.17
L-Threonine	0.2	0.12
Yttrium oxide	0.02	0.02
Nutrient composition on dry matter basis (g/kg)		
Dry matter (DM, g/kg diet)	900	906
Ash	72	74
Crude protein	384	296
Fat	60	67
Energy (kJ/g DM)	20.0	19.9
Carbohydrate*	484	562
Phosphorus	11.3	13.3
Calcium	14.6	10.9
Magnesium	1.9	3.4
Yttrium	0.16	0.17
Carbon	433	434
Nitrogen	56	44
C:N ratio (mass, g/g)	7.79	9.81

Values are the mean of each diet (CONDiet = control diet, WBDiet = wheat bran diet. C:N ratio = carbon to nitrogen ratio. *Calculated by subtracting ash, crude protein and fat from dry matter.

was executed in clear-water recirculating aquaculture system (RAS) in absence of biofloc. Shrimp were grown in 120-L aquaria connected to the water purification unit of one RAS, with 6 replicate aquaria per diet. Per aquarium, 25 to 30 shrimp of 13 ± 2 g were stocked. Each aquarium was continuously aerated to maintain the dissolved oxygen concentration above $6.5 \text{ mg O}_2 \text{ L}^{-1}$ and the water temperature at 27 ± 1.0 °C. Shrimp were fed overnight as explained in section 2.1. To collect faeces, each morning per aquarium 1-1.5 g extra feed was administered, observing a 10-minute time interval between the aquaria. One hour after feeding, all uneaten feed, faeces and carapaces were removed from each aquarium by siphoning. Again, one hour later, the shrimp faeces in each aquarium were collected and stored in a collection bottle that was put on ice in a bucket, to slow down deterioration until the faeces from all tanks were collected. De-ionized water was added to the bottle to dilute remaining culture water containing salt from siphoning process, and to rinse off salt that was attached to the faeces. The de-ionized water was then removed from the bottle, eliminating the salt from the faeces sample. Per treatment, the rinsed faeces were pooled into one aluminum tray and stored at -20 °C before drying at 70 °C for 72 hours. The dried faeces were then manually ground using a porcelain mortar, and stored in a plastic jar at room temperature, until proximate analysis. The experiment lasted 35 days.

2.3. Growth experiment

Biofloc inoculum water was taken from the previous culture cycle. Prior the stocking day, inoculum was pooled in a large tank, mixed, and kept in suspension through aeration. Subsequently, from the large tank filled with mature biofloc, 450-L water was transferred to each of 6 mesocosm tanks. The water volume in each tank was increased by adding a mix of salt and fresh water until reaching a volume of 750 L and a salinity of 23 ± 1.0 ppt. The shrimp were stocked the next day at stocking density of 100 ind/mesocosm tank. Besides the diet, no external carbon input was added to the mesocosm tanks. Freshwater was added every week to each tank to compensate for evaporation and splashing of small water droplets due to aeration (84 ± 7 L / week). During the experiment, water quality was checked daily and maintained at 27.2 ± 0.6 °C, 7.0 ± 0.1 mg $\text{O}_2 \text{ L}^{-1}$, 8.0 ± 0.1 pH, 23.2 ± 1.0 ppt salinity and 36.7 ± 1.5 $\mu\text{S cm}^{-1}$ conductivity. These water quality parameters were measured using a multi-parameter electronic meter (WTW Multi3630IDS™). In addition, each day in each biofloc tank the ammonium (N-NH₄) and nitrite (N-NO₂) concentrations were measured using colorimetric test kits (mg/L, MColorTest™, EMD Millipore), while the nitrate (N-NO₃) concentration was checked with colorimetric test strips (mg/L, MQuant®, EMD Millipore).

2.4. Sampling and analysis

2.4.1. Sample collection and preparation

In each biofloc tank, feed, shrimp, biofloc (particulate matter retained on a 1.5 μm glass fiber filter) and water that passed through the glass fiber filter were sampled. Samples were collected at the beginning of day-1 (D1), at the end of day-21 (D21) and at the end of day-42 (D42). To avoid stressing the shrimp, no shrimp samples were taken at D21.

To sample biofloc and water, 2-L closed jars were positioned in the middle of the tank, opened to fill and closed when full. Per biofloc tank, ten 2-L jars were filled, and subsequently pooled in a 50-L plastic bucket, and mixed at 300 rpm. While still mixing, four 2-L jars were filled with mixed sample. Of these, the water from 2 jars was filtered in batches of 100 mL through a 1.5 μm pore size filter to collect biofloc. The 100 mL samples were collected while continuously mixing the water in the jar with a magnetic stirrer (Heidolph Mr Hei-Mix L, Heidolph Instruments, Germany) at 300 rpm. Once the biofloc-water was filtered, 100 mL of demi water was poured into the filtration chamber and filtered to dilute and remove the salt from the filter. When no more water in the filter could be removed by the vacuum pump, the filtration was stopped. The filter having the biofloc was folded and stored in a clean, dry tube. Samples were then kept in $-20\text{ }^{\circ}\text{C}$ freezer. Biofloc accumulated on the pore filters was used to determine dry matter (DM), ash, calcium (Ca), magnesium (Mg), total phosphorus (TP), total suspended solid (TSS) and volatile suspended solid (VSS), protein, energy, C:N ratio, chemical oxygen demand (COD) and chlorophyll-a (Chl-a). In addition, for Chl-a analysis, also 3 unfiltered water samples were collected. Each analysis was performed in triplicate. Filters containing biofloc were dried in the oven at $70\text{ }^{\circ}\text{C}$ for 3 days before being analyzed. For COD and Chl-a parameters, biofloc-containing filters were not dried prior the analysis.

The filtrate after sampling was pooled and thoroughly mixed before collecting a 50-mL sample that was acidified with 3N hydrogen chloride (HCl) to reduce the pH to less than 3. A volume of 12 mL of acidified water was then filtered using 0.45 μm pore size syringe filter. The final filtrate was then stored in a plastic tube with cap and put aside until analysis within the next 24 hours. Of the remaining two jars, one jar was acidified with 3N HCl, while the other jar was kept neutral. They were stored at $-20\text{ }^{\circ}\text{C}$ as back-up sample sources.

A representative 100 g shrimp sample was collected from the base population at the start of the experiment (D1), while at the end of experiment (D42), all shrimp in each biofloc tank were collected, counted and batch weighed. Shrimp samples were kept at $-20\text{ }^{\circ}\text{C}$ until being freeze-dried for 5 days and then ground for the proximate analysis (AOAC, 2020). Twenty g of the CONdiet and WBdiet was added weekly to 1 large

container per diet and used to determine the proximate composition of the feed at the end of the experiment. Sample of dried faeces, shrimp and feed, were stored in plastic containers at room temperature until further analysis.

2.4.2. Sample analysis

The proximate composition (DM, ash, protein, fat, and energy) of shrimp, feed, faeces and biofloc were determined by the Aquaculture and Fisheries (AFI) laboratory of WUR. The DM concentration was calculated after oven-drying each sample at 103 °C for at least 4 hours until constant weight following ISO-6496 (1999). After DM determination, the samples were incinerated at 550 °C for at least 4 hours until constant weight following ISO-5984 (2002). Ash-free dry matter (AFDM) is calculated by subtracting ash from DM. The TSS concentration was the amount of DM per liter culture water, while the VSS concentration was the weight loss after incineration of the TSS per liter of culture water (APHA 1995). The ash was analyzed for total phosphorus, calcium, magnesium, and yttrium using plasma-mass spectrophotometry (ICP OES) following the NEN 15510 procedure. The crude protein analysis was determined by the Kjeldahl method according to ISO-5983 (2005), while energy content was determined using bomb calorimetry by direct combustion (IKA® werke C7000; IKA Analysentechnik, Weikershem Germany) (ISO-9831, 1998). The fat analysis was carried by Soxhlet (ISO-6492, 1999). The carbon (C) and nitrogen (N) content were determined using DUMAS analyzer (Leco CN 628, Leco Instrumente GmbH., Germany). The C:N ratio (mass) was calculated by dividing the C content with and N content.

Water samples were analyzed using a segmented flow analyzer (SAN++, Skalar Analytical B.V., The Netherlands), measuring total organic carbon (TOC), total inorganic carbon (TIC), total carbon (TC), ammonium N-(NH₄), nitrate+nitrite N-(NO₃+NO₂), total organic nitrogen (TON), total nitrogen (TN), and orthophosphate (P-PO₄). The biological oxygen demand (BOD₅) analysis was performed according to APHA (1995) with a modification of the incubation time. A dark-colored glass bottle was filled with 500 ml biofloc water from the culture tank. A magnetic stir rod was put into each glass bottle. The bottle was then incubated on an underwater magnetic stir plate in an open water bath at 27 ± 1 °C, mimicking the temperature in the culture tank. Water in the bottle was continuously stirred to maintain the biofloc in suspension. During preliminary incubation runs, all dissolved oxygen (DO) in the bottle was consumed in less than 5 days. Diluting the biofloc water with autoclaved-artificial saltwater did not result in consistent and logical DO concentration values, probably due to oxygen consumption by salt. Therefore, the analysis was done in duplicate, filling two 500 ml DO bottles: one bottle (replicate A; replicate A) was kept unopened until the end of the incubation, and the other (replicate B; Rep B) was opened and closed every hour to measure the DO concentration. Before

closing bottles of Rep A and Rep B at the start of the incubation, the DO concentration was recorded. When the DO concentration in Rep B dropped below 2, the incubation of Rep A was terminated, and the DO concentration was measured. The time period between the start to end of incubation of each sample was recorded. Only the dissolved oxygen consumption in Rep A was considered to calculate the BOD₅ by extrapolating the observed DO consumption to a 5-day period.

The COD concentration was analyzed based on protocol of ISO-6060 (1989), meanwhile the Chl-a was measured according to the standard protocol 10200H issued by APHA (1995). The biodegradability index (BI) is calculated as BOD₅/COD (Rojas-Tirado et al., 2018). The microbial activity was determined using hydrogen peroxide (H₂O₂) degradation assay according to Pedersen et al. (2019).

2.4.3. Calculation and data analysis

Parameters of shrimp's performances, such as the apparent digestibility coefficient (ADC), the specific growth rate (SGR), shrimp production, feed conversion ratio (FCR) with and without wheat bran (FCR carbon excl.), protein efficiency ratio (PER), as well as protein and energy retention efficiency, were calculated as follows:

$$\text{ADC}_{(\%) } = 1 - \left\{ \left(\text{Nut}_{\text{faeces (g/kg)}} / \text{Nut}_{\text{diet (g/kg)}} \right) \times \left(\text{Ytrr}_{\text{diet (g/kg)}} / \text{Ytrr}_{\text{faeces (g/kg)}} \right) \right\} \times 100 \quad (1)$$

$$\text{SGR}_{(\%/ \text{day})} = (\ln W_{\text{day-42 (g)}} - \ln W_{\text{day-1 (g)}}) / t \times 100 \quad (2)$$

$$\text{Production}_{(\text{kg}/\text{m}^3)} = (W_{\text{day-42 (g)}} - W_{\text{day-1 (g)}}) / (\text{water volume}_{(\text{m}^3)} \times 1,000) \quad (3)$$

$$\text{FCR} = \text{diet input}_{(\text{g})} / (W_{\text{day-42}} - W_{\text{day-1}}) \quad (4)$$

$$\text{FCR carbon excl.} = (\text{diet input}_{(\text{g})} - \text{wheat bran input}_{(\text{g})}) / (W_{\text{day-42 (g)}} - W_{\text{day-1 (g)}}) \quad (5)$$

$$\text{PER}_{(\%) } = (W_{\text{day-42 (g)}} - W_{\text{day-1 (g)}}) / \text{protein input}_{(\text{g})} \quad (6)$$

$$\text{The protein retention efficiency}_{(\%) } = (\text{retained protein}_{(\text{g})} / \text{protein input}_{(\text{g})}) \times 100 \quad (7)$$

$$\text{The energy retention efficiency}_{(\%) } = (\text{retained energy}_{(\text{kJ})} / \text{energy input}_{(\text{kJ})}) \times 100 \quad (8)$$

$$\text{Total nutrient input}_{(\text{g})} = \text{Nut}_{\text{diet (g)}} + (\text{Nut}_{\text{shrimp day-1 (g)}} + \text{Nut}_{\text{biofloc day-1 (g)}} + \text{Nut}_{\text{water day-1 (g)}}) \quad (9)$$

$$\text{Nutrient loss}_{(\text{g})} = \text{Total nutrient input}_{(\text{g})} - (\text{Nut}_{\text{shrimp day-42 (g)}} + \text{Nut}_{\text{biofloc day-42 (g)}} + \text{Nut}_{\text{water day-42 (g)}}) \quad (10)$$

$\text{Nut}_{\text{faeces (g/kg)}}$ and $\text{Nut}_{\text{diet (g/kg)}}$ are the nutrient concentrations contained in faeces and diet, in g/kg; $\text{Ytrr}_{\text{diet (g/kg)}}$ / $\text{Ytrr}_{\text{faeces (g/kg)}}$ are the Yttrium concentrations contained in faeces and diet, in g/kg; $W_{\text{day-42 (g)}}$ and $W_{\text{day-1 (g)}}$ are the biomass of shrimp at the end of day-42 and at the beginning of day-1 in each aquarium or biofloc tank, in g (wet); water volume (m³) is the total water volume in the biofloc tank in m³, diet input_(g) and wheat bran input_(g) are the total absolute amount of diet and additional wheat bran given per tank within 42 days culture period, in g (wet); retained protein_(g) and retained energy_(kJ) are the

protein and energy retained in shrimp within 42 days culture period, in g (dry) and kJ, respectively; protein input and energy input are the total absolute amount of protein and energy from diet given per tank during the 42 days culture period, in g (dry) and kJ, respectively.

Parameters of system's nutrient balance, such as total nutrient input and nutrient loss were calculated using formulas:

$$\text{Total nutrient input}_{(g)} = \text{Nut}_{\text{diet } (g)} + (\text{Nut}_{\text{shrimp day-1 } (g)} + \text{Nut}_{\text{biofloc day-1 } (g)} + \text{Nut}_{\text{water day-1 } (g)}) \quad (9)$$

$$\text{Nutrient loss}_{(g)} = \text{Total nutrient input}_{(g)} - (\text{Nut}_{\text{shrimp day-42 } (g)} + \text{Nut}_{\text{biofloc day-42 } (g)} + \text{Nut}_{\text{water day-42 } (g)}) \quad (10)$$

$\text{Nut}_{\text{diet } (g)}$ is total nutrient in the diet; $\text{Nut}_{\text{shrimp day-1 } (g)}$, $\text{Nut}_{\text{biofloc day-1 } (g)}$, and $\text{Nut}_{\text{water day-1 } (g)}$ are the nutrient present in the shrimp, biofloc, and water, at the beginning of day-1, respectively; $\text{Nut}_{\text{shrimp day-42 } (g)}$, $\text{Nut}_{\text{biofloc day-42 } (g)}$, and $\text{Nut}_{\text{water day-42 } (g)}$ are the nutrient present in shrimp, biofloc, and water, at the end of day-42, respectively.

Statistical analyses were performed using the IBM Statistics 26 software (IBM Corporation, NY, USA). The effect of diet on ADC, faecal composition, water quality, as well as shrimp growth and body composition were analyzed using one-way ANOVA (GLM). The biofloc performance, including quality, quantity and activity, were analyzed using repeated-measure ANOVA (GLM). The nutrient distribution between each compartment in each biofloc tank at each sampling time was compared between the diets with one-way ANOVA (GLM). The normality and homoscedasticity assumptions were checked using the Shapiro-Wilk and Levene's tests. If the assumptions of the ANOVA were not met, then the data were analyzed nonparametrically using a ranking test. However, ranking test done showed similar results as those obtained using ANOVA. When there was significant interaction ($P < 0.05$) in the result of the repeated measure ANOVA, a one-way ANOVA was performed combining all possible factor combinations followed by a post-hoc Tukey analysis.

3. RESULTS

3.1. Digestibility experiment

The ADC and the proximate composition of the faeces of shrimp reared in biofloc tanks fed the CONdiet and WBdiet are listed in **Table 2.2** and **Table 2.3**, respectively. The ADCs of all measured parameters including protein, fat, energy, carbohydrate, phosphorus, calcium, magnesium and carbon, were lower in shrimp fed the WBdiet compared to shrimp fed the CONdiet ($P < 0.05$) (**Table 2.2**). In addition, the faecal

proximate composition of shrimp fed the WBdiet was different from shrimp fed the CONdiet ($P < 0.05$), except for energy and carbon ($P > 0.05$). The concentrations of crude protein, phosphorus, calcium and magnesium were higher in the faeces of shrimp fed the CONdiet than in faeces of shrimp fed the WBdiet ($P < 0.05$), while the concentrations of fat and carbohydrate in faeces were lower ($P < 0.05$) (**Table 2.3**).

3.2 Growth experiment

3.2.1. Shrimp

The shrimp's growth performance and proximate composition are summarized in **Table 2.4** and **2.5**, respectively. The amounts of feed and protein fed were controlled; therefore, no statistical analysis was performed. No significant differences were observed in the final individual weight, individual weight gain, biomass gain and production between diets ($P > 0.05$). The specific growth rate was higher in CONdiet ($P = 0.05$), even though the difference with the WBdiet was only 2%. No differences in protein efficiency ratio, survival and protein retention efficiency were observed between the diets ($P > 0.05$). The FCR of the WBdiet (1.70) was 44% higher than the CONdiet FCR (1.18) ($P < 0.05$). The shrimp's body composition was also similar between the dietary treatments. Exceptions were the crude protein body content of shrimp, which was 3.4% higher in shrimp fed the CONdiet, and the phosphorus body content which was 7.9% higher in shrimp fed the WBdiet ($P < 0.05$).

3.2.2. Biofloc

The nutritional quality of biofloc in terms of AFDM, crude protein, energy, carbon content and C:N ratio, were similar in tanks fed either the CONdiet or WBdiet ($P > 0.05$, **Table 2.6**). Diet also did not affect the TSS and VSS concentration in rearing tanks ($P > 0.05$, **Table 2.6** and **Figure 2.1**). However, TSS and VSS concentrations changed over time ($P < 0.05$). The TSS concentration decreased between D1 and D21 and remained similar thereafter (**Table 2.6**). There was a significant diet by time interaction for VSS, with a higher concentration in tanks fed the WBdiet than in tanks receiving the CONdiet at the end of the experiment on D42, while differences between the diets were less pronounced during the first half of the experiment ($P < 0.05$) (**Figure 2.1**). The BOD₅ measurement on D1 samples were not successful. Based on data collected in D21 and D42, the BOD₅ was higher in mesocosms tanks fed the WBdiet ($P < 0.05$). Organic matter degradability, as indicated by the biodegradability index, was similar between tanks fed the CONdiet and WBdiet ($P < 0.05$). Similarly, no differences in Chl-a concentrations were observed between the CONdiet and WBdiet ($P > 0.05$). This was also the case for microbial activity

Table 2.2. Effect of the dietary NSP supplementation on the apparent digestibility coefficient (ADC) of nutrient in the diet of *Litopenaeus vannamei*

Parameters	Unit	CONdiet		WBdiet		P-value
		Mean	sd	Mean	sd	
Ash-free dry matter (AFDM)	%	85	0.3	61	0.2	0.000
Crude protein	%	91	0.2	81	0.3	0.001
Fat	%	92	0.6	72	0.1	0.000
Energy	%	85	0.2	61	0.6	0.000
Carbohydrate*	%	78	0.1	46	0.3	0.000
Phosphorus	%	19	0.3	-1	0.4	0.000
Calcium	%	-37	4.3	-73	1.1	0.007
Magnesium	%	-9	0.9	-44	4.1	0.007
Carbon	%	85	0.2	61	0.4	0.000

Values are the mean and the standard deviation (sd) of each diet (CON = control diet, WBdiet = wheat bran diet). The data was observed from digestibility trial. P-value = probability value. *Calculated by subtracting ash, crude protein, and fat from dry matter.

Table 2.3. Effect of the dietary NSP supplementation on the proximate quality of faeces of the *Litopenaeus vannamei*

Parameters	Unit	CONdiet		WBdiet		P-value
		Mean	sd	Mean	sd	
Ash-free-dry matter (FDM)	g/kg dm	578	2	684	3	0.000
Crude protein	g/kg AFDM	256	3.0	152	0.2	0.000
Fat	g/kg AFDM	33	2.2	52	0.2	0.000
Energy	kJ/g AFDM	21.4	0.12	21.4	0.08	0.885
Carbohydrate*	g/kg AFDM	762	4.7	828	0.5	0.000
Carbon	g/kg AFDM	469	3	466	2	0.252
C:N ratio (mass)	g/g	12	0.4	20	0.1	0.000
Phosphorus	g/kg dm	41.6	0.5	28.2	0.3	0.001
Calcium	g/kg dm	90.4	0.4	39.6	0.7	0.000
Magnesium	g/kg dm	9.6	0.02	10.4	0.01	0.000

Values are the mean and the standard deviation (sd) of each diet (CONdiet = control diet, WBdiet = wheat bran diet). The data was observed from digestibility trial. C:N ratio = carbon to nitrogen ratio, P-value = probability value. *Calculated by subtracting ash, crude protein and fat from dry matter.

Table 2.4. Dietary input and effect of the dietary NSP supplementation on the growth performance of the *Litopenaeus vannamei* at biofloc system level

Parameters	CONdiet		WBdiet		P-value
	Mean	sd	Mean	sd	
<i>Dietary nutrient input</i>					
Total diet g	560		747		
Ash g	36		50		
Crude protein g	194		201		
Fat g	30		46		
Energy kJ	10		13		
Carbohydrate* g	244		380		
<i>Performances</i>					
Initial individual weight g	0.5	0.02	0.5	0.01	0.954
Final individual weight g	6.0	0.15	5.8	0.18	0.107
Individual weight gain g	5.5	0.13	5.3	0.18	0.093
Biomass gain g	477	43	444	52	0.439
Production g/m ³	636	57	591	69	0.444
Growth rate g/d	0.132	0.003	0.125	0.004	0.093
Specific growth rate % bw/d	5.93	0.03	5.82	0.07	0.050*
FCR	1.18	0.10	1.70	0.20	0.016
Protein efficiency ratio	2.46	0.22	2.21	0.26	0.274
Survival %	87	7.0	86	6.7	0.780
Protein retention efficiency %	44	3.8	38	4.3	0.142
Energy retention efficiency %	23	2.0	16	1.9	0.012

Values are the mean and the standard deviation (sd) of each diet (CONdiet = control diet, WBdiet = wheat bran diet). FCR = feed conversion ratio, P-value = probability value, d = day, bw = body weight. *the unrounded value is smaller than 0.050. *Calculated by subtracting ash, crude protein and fat from dry matter.

Table 2.5. Effect of the dietary NSP supplementation on the body composition of the *Litopenaeus vannamei* in biofloc system

Parameters	D1		D42		P-value	
	CONdiet		WBdiet			
	Mean	Mean	sd	Mean		sd
DM g /kg wet	201	233	0.1	232	0.8	0.054
Ash g/kg dm	180	143	13	152	4	0.310
Crude protein g/kg dm	729	752	6	727	13	0.037
Fat g/kg dm	26	37	3.9	32	0.2	0.074
Energy kJ/g dm	19.2	20.1	0.1	20.1	0.2	0.631
Carbohydrate* g/kg dm	65	68	15	90	10	0.098
Phosphorus g/kg dm	13	9.1	0.03	9.9	0.17	0.002
Calcium g/kg dm	40	32	5.3	36	1.4	0.292
Magnesium g/kg dm	3.4	2.9	0.2	3.1	0.1	0.140
Carbon g/kg dm	438	460	1	458	6	0.337
C:N ratio (mass) g/g	3.8	3.9	0.04	4.0	0.08	0.503

Values are the mean and the standard deviation (sd) of each diet (CON = control diet, WBdiet = wheat bran diet). C:N ratio = carbon to nitrogen ratio, P-value = probability value. *Calculated by subtracting ash, crude protein, and fat from dry matter.

in the biofloc tanks ($P > 0.05$), even though the microbial activity decreased between D1 and D21 and subsequently increased between D21 and D42 ($P < 0.05$) (**Table 2.6**).

3.2.3. Water quality

No impact of the diet was observed on the concentrations of TOC, TIC and TC in water ($P > 0.05$), while time had a significant effect on the concentration of TIC, the latter increasing between D1 and D21 and subsequently being lowest on D42 ($P < 0.05$, **Table 7**). The $N-NH_4$ concentration in the water was unaffected by diet ($P > 0.05$) but decreased over time ($P < 0.05$). In contrast, the concentrations of TON, TIN, TN and $N-(NO_3+NO_2)$ were lower in tanks fed the WBdiet, as compared to tanks fed the CONdiet ($P < 0.05$). In addition, the concentrations of all dissolved nitrogen species increased over time ($P < 0.05$). The dynamics of TON, TIN, and TN are displayed in **Figure 2**. A diet by time interaction was only observed for the $P-PO_4$ concentration. Data show that the difference between the two diets gradually increased over time ($P < 0.05$; **Table 7**).

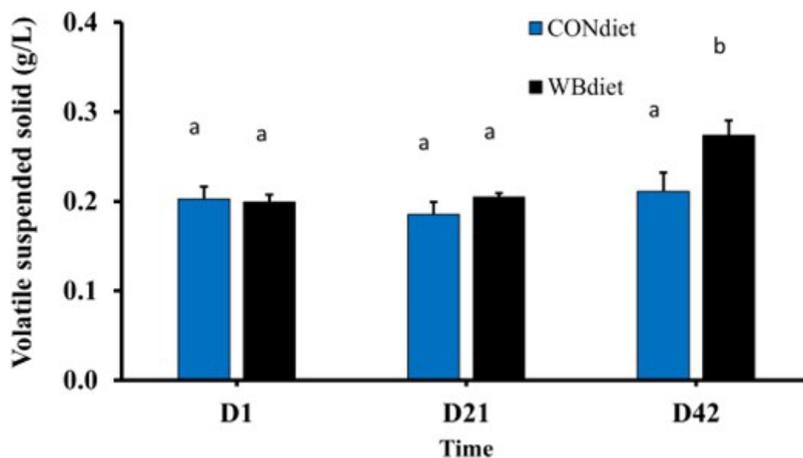


Figure 2.1. Effect of wheat bran addition in the diet on volatile suspended solid (VSS) concentration in the water. Values are the mean and the standard deviation of each diet (CONdiet = control diet, and WBdiet = wheat bran diet) at each sampling times (D1 = day-1, D21 = day-21, and D42 = day-42). Data were analyzed with 2-way ANOVA, with diet as main factor and time as repeated measure: P-value of diet=0.041, time=0.000 and interaction=0.003. Different letters on top of each bar indicate significant difference ($P < 0.05$) based on one-way ANOVA considering the 6 diet x time combinations, followed by Tukey's test

Table 2.6. Effect of the dietary NSP supplementation on biofloc quality, quantity, and activity during the experiment

Parameters	Diet		SE		Time		SE		P-value		
	CONdiet	WBdiet	D1	D21	D42	Time	SE Time	SE Diet*Time	Diet	Time	Diet*Time
Ash-free dry matter (AFDM)	611	624	28	494c	729a	629b	24	34	0.768	0.000	0.357
Crude protein	395	391	15	330b	426a	424a	20	28	0.863	0.013	0.724
Energy	18.5	18.1	0.7	14.9b	19.9a	20.2a	0.7	1.0	0.734	0.001	0.381
Carbon	346	359	23	394a	388a	275b	15	21	0.688	0.001	0.915
C:N ratio (mass)	7.12	7.22	0.05	6.74a	7.35a	7.42b	0.06	0.09	0.230	0.000	0.215
Phosphorus	11.7	11.9	0.3	4.9c	12.1b	12.1b	0.5	0.7	0.688	0.000	0.232
Calcium	40.4	36.3	2.0	33.0b	49.6a	32.5b	2.0	2.8	0.228	0.000	0.156
Magnesium	15.0	16.1	0.9	23.1a	12.9b	10.6c	0.7	1	0.446	0.000	0.018
TSS	0.33	0.36	0.02	0.41a	0.31b	0.31b	0.02	0.02	0.365	0.004	0.129
COD	253y	312x	12	252b	335a	261b	15	22	0.025	0.009	0.473
BOD ₅	110y	133x	6	nm	127	115	6	9	0.049	0.257	0.065
Biodegradability index	0.41	0.41	0.02	nm	0.38	0.45	0.03	0.04	0.984	0.178	0.282
Chl-a total	777	773	29	504b	955a	866a	35	50	0.925	0.000	0.744

Table 2.6 Effect of the dietary NSP supplementation on biofloc quality, quantity, and activity during the experiment (Continue)

Parameters	Diet		SE Diet	Time		SE Time	SE Diet*Time	P-value		
	CONdiet	WBdiet	D1	D21	D42	Diet	Time	Diet	Time	
Chl-a biofloc mg/m ³	566	637	24	334 c	808 a	20	42	0.099	0.000	0.401
Chl-a water mg/m ³	212	136	26	170	147	26	36	0.111	0.332	0.071
Microbial activity	-0.013*	-0.016*	0.001	-0.024* a	-0.008* c	0.001	0.001	0.076	0.000	0.023

Values are the mean of three sampling times of each diet (CON = control diet, and WBdiet = wheat bran diet) and the mean of two diets of each sampling times (D1 = day-1, D21 = day-21, and D42 = day-42). AFDM = ash free dry matter, TSS = total suspended solid, COD = chemical oxygen demand, BOD₅ = biological oxygen demand, Chl-a = chlorophyll-a, d = day, SE = standard error. nm = not measured. P-value = probability value. For each factor (diet or time), different letters in bold show significant difference (P<0.05). Minus value can be ignored as it only showed a decline in H₂O₂ concentration during the analysis, indicating the occurrence of the microbial activity.

3.2.4. Nutrient balances (carbon, nitrogen and phosphorus)

The amounts of nitrogen, phosphorus and carbon present in shrimp, biofloc, and water in the biofloc rearing tanks on D1, D21 and D42 are shown in **Figure 2.2**. The dietary nitrogen input was similar between the CONdiet and WBdiet, whereas more phosphorus and carbon were administered with the WBdiet. At the start of the experiment on D1, similar concentrations of nitrogen, phosphorus and carbon were present in shrimp, biofloc and water in the biofloc tanks of both dietary treatments ($P>0.05$). This was also the case for nitrogen on D21 and D42 ($P>0.05$), and no differences in nitrogen input and nitrogen loss were observed during the experiment (**Figure 2.2a**). Meanwhile, more phosphorus accumulated in biofloc and water in tanks fed the WBdiet ($P<0.05$, **Figure 2.2b**). The unaccounted phosphorus refers to phosphorous species other than $PO_4\text{-P}$, as only the latter was measured in filtrate water. The amounts of carbon present in shrimp, biofloc and water on each sampling date were always similar between diets ($P>0.05$, **Figure 2.2c**), but considering the whole experimental period, more carbon was lost from biofloc tanks fed the WBdiet than from tanks fed the CONdiet ($P<0.05$).

The distribution of nitrogen, phosphorus and carbon, as percentage of the total amount present at each sampling date in tanks fed the CONdiet and WBdiet, is shown in **Figure 2.3**. During the experiment, most nitrogen was present in the water (**Figure 2.3a**), whereas phosphorus was mainly present in biofloc (**Figure 2.3b**). For carbon, the highest fraction was lost (**Figure 2.3c**). The percentages of nitrogen, phosphorus and carbon retained in the biofloc tanks on D42 were 97-101%, 78-82%, and 39-44% respectively, concurring with losses of 0-3% for nitrogen, 18-22% for phosphorus and 56-61% for carbon (**Figure 2.3**).

4. DISCUSSION

4.1. Effect of high wheat bran input on nutrient digestibility

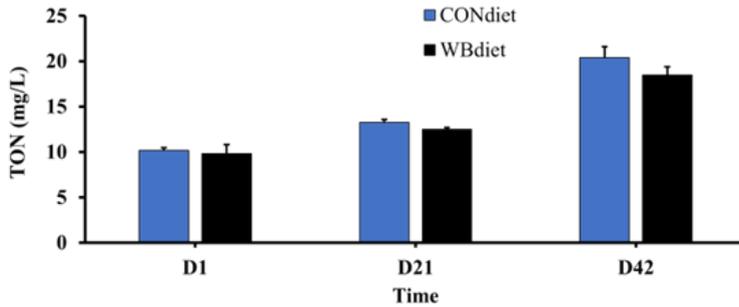
In this study, the aim of increasing the carbon input through dietary wheat bran inclusion was to raise the C:N ratio in the shrimp faeces and the availability of organic carbon to stimulate biofloc formation and activity. Adding wheat bran in the diet indeed decreased the overall ADC of nutrients (**Table 2.2**) and altered the shrimp faeces composition (**Table 2.3**). This was suggested to be related with their NSP contents. Wheat bran contains 56% NSP (dry), mostly in the form of arabinoxylans (70%) and cellulose (24%) (Maes and Delcour, 2002; Stevenson et al., 2012; CVB, 2022). Including 40% wheat bran into the CONdiet increased the dietary NSP content by 22%, and this resulted in a 28%

Table 2.7. Effect of the dietary NSP supplementation on the water quality

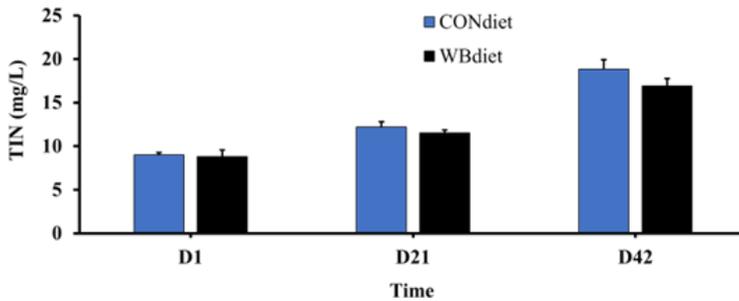
Parameters	Unit	Diet		Time			SE		SE Diet * Time	Pvalue		
		CONDiet	WBdiet	D1	D21	D42	SE Time	Diet		Time	Diet*Time	
TON	mg/L	14.6 x	13.6 y	0.2	10.0 c	12.9 b	19.5 a	0.3	0.5	0.019	0.000	0.291
N-NH ₄	mg/L	0.06	0.05	0.004	0.07 a	0.06 a	0.04 b	0.002	0.003	0.760	0.001	0.812
N-(NO ₃ +NO ₂)	mg/L	13.3 x	12.4 y	0.2	8.8 c	11.8 b	17.8 a	0.3	0.4	0.039	0.000	0.174
TIN	mg/L	13.3 x	12.4 y	0.2	8.9 c	11.9 b	17.9 a	0.3	0.4	0.037	0.000	0.173
TN	mg/L	28.0 x	26.0 y	0.4	18.9 c	24.8 b	37.3 a	0.6	0.9	0.026	0.000	0.226
TOC	mg/L	15.0	13.2	0.2	9.7	19.1	13.5	2.7	3.8	0.599	0.102	0.638
TIC	mg/L	0.20	0.18	0.01	0.10 b	0.40 a	0.06 c	0.01	0.01	0.117	0.000	0.150
TC	mg/L	15.2	13.4	2.3	9.8	19.5	13.6	2.8	3.8	0.596	0.091	0.634
P-PO ₄	mg/L	1.77 y	2.43 x	0.04	1.13 b	1.83 b	3.35 a	0.04	0.06	0.000	0.000	0.000

Values are the mean of three sampling times of each diet (CON = control diet, and WBdiet = wheat bran diet) and the mean of two diets of each sampling times (D1 = day-1, D21 = day-21, and D42 = day-42). TON = total organic nitrogen; TIN = total inorganic nitrogen; TN = total nitrogen; IC = inorganic carbon; TOC = total organic carbon; TIC = total inorganic carbon; TC = total carbon, SEM = standard error of the mean, P-value = probability value. For each factor (diet or time), different letters in bold show significant difference (P<0.05).

(a)



(b)



(c)

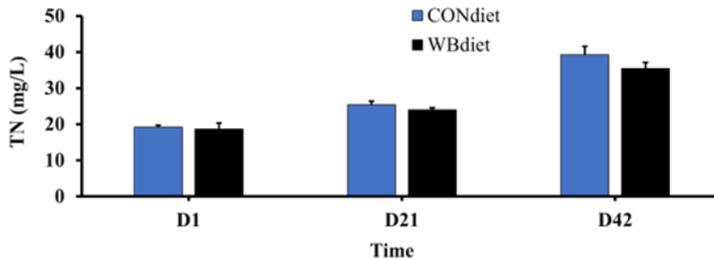


Figure 2.2. Effect of wheat bran addition in the diet on concentrations of dissolved nitrogen: total organic nitrogen (TON) (a), total inorganic nitrogen (TIN) (b), and total nitrogen (TN) (c). Values are the mean and the standard deviation of each diet (CONdiet = control diet, and WBdiet = wheat bran diet) at each sampling times (D1 = day 1, D21 = day 21, and D42 = day 42). No significant differences between treatments are shown in the figure because there was no interaction between the dietary treatments x time. P-values for the factors diet and time and their interactions (diet x time) are given in Table 7.

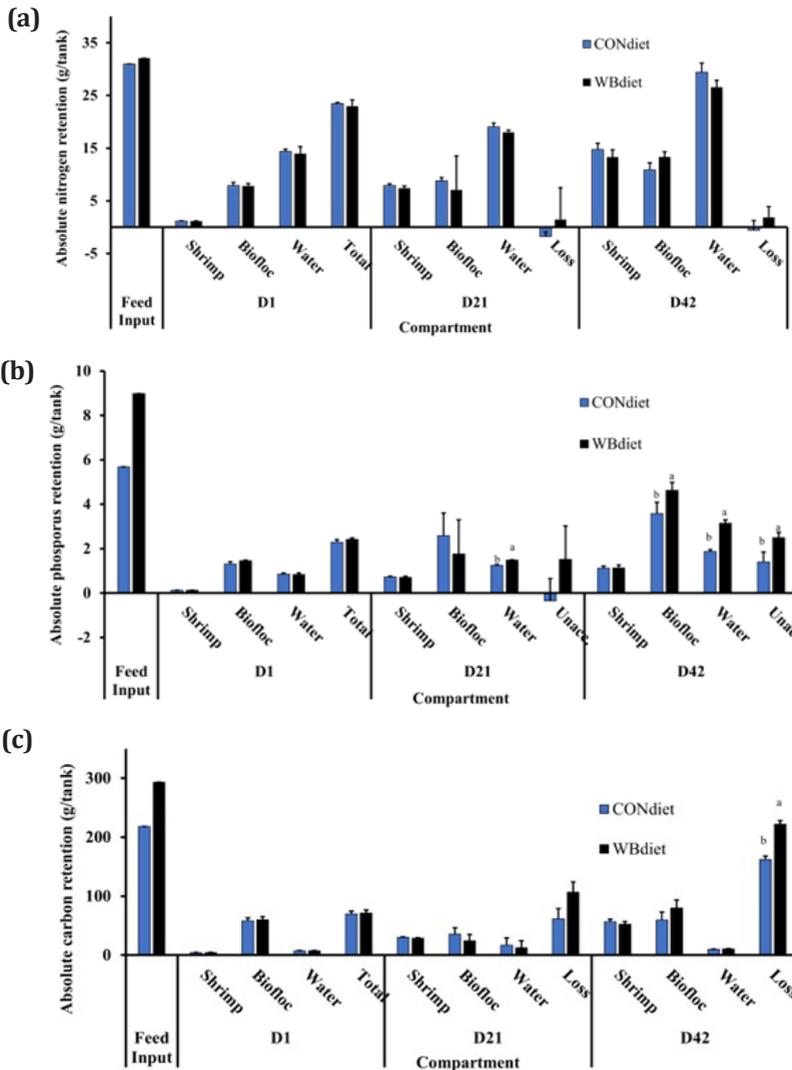


Figure 2.3. Effect of wheat bran addition in the diet on nitrogen (a), carbon (b) and phosphorous (c) distribution in the biofloc system (g absolute amount / tank). Values are the mean and the standard deviation of each diet (CONdiet = control diet, and WBdiet = wheat bran diet) at each sampling times (D1 = day-1, D21 = day-21, and D42 = day-42). Loss = (total nutrient input – total nutrient present in the system) at the particular day. The total nutrient input = total nutrient from feed + total initial nutrient present in the system. Feed input = total nutrient input from feed given in 42-day experiment, unacc. phosphorus = unaccounted phosphorus in the water, in the form other than orthophosphate. Different letters on top of the bar showed significant difference ($P < 0.05$) between the diet. The feed input has no variation within diet treatment, and therefore was not statistically analyzed.

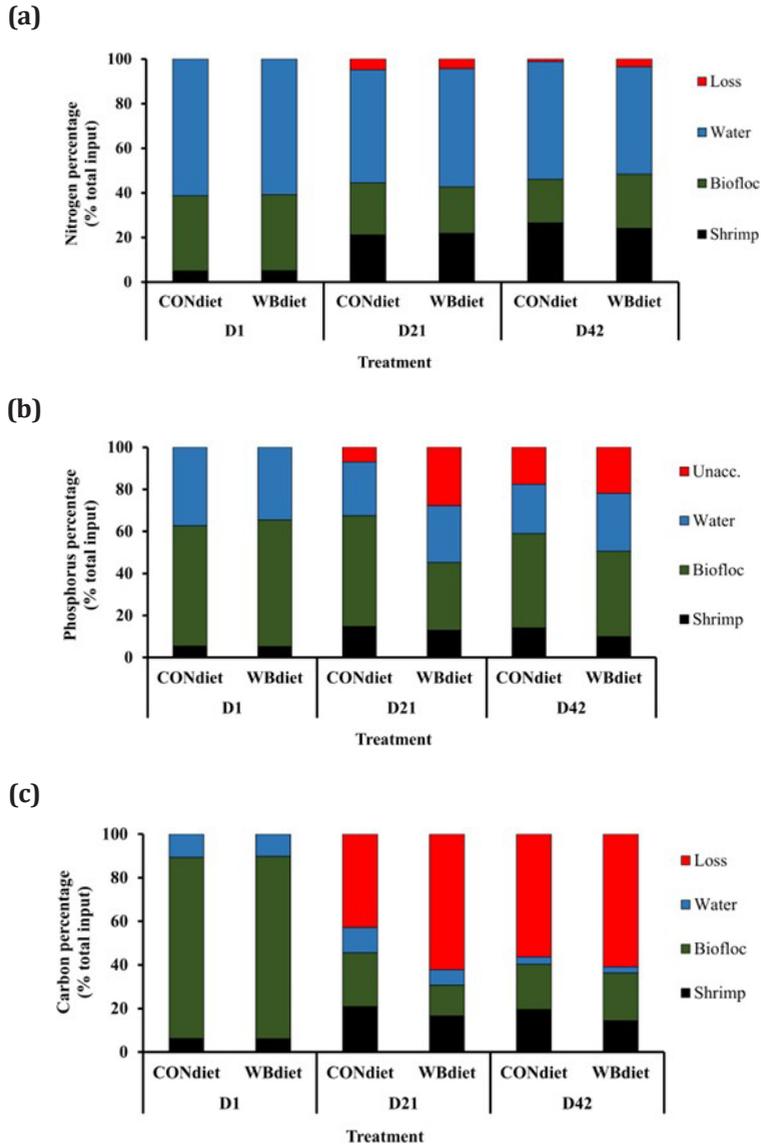


Figure 2.4. The effect of wheat bran addition in the diet on the distribution of nitrogen (a), phosphorous (c), and carbon (c) in the biofloc system (in percentage of total particular nutrient at each sampling times). Values are the mean of each diet (CON = control diet, and WBdiet = wheat bran diet) at each sampling times (D1 = day-1, D21 = day-21, and D42 = day-42). Loss = percentage of absolute loss relatively to total nutrient present in the system, at the particular day. Unacc. phosphorus = unaccounted phosphorus in the water, in the form other than orthophosphate.

reduction of ADC of AFDM, 11% of crude protein, 22% of fat, 28% of energy and 41% of carbohydrate (**Table 2.2**). Due to these differences in ADC, the C:N ratio in the faeces increased from 12 in the CONdiet to 20 in the WBdiet (**Table 2.3**), which was also what this inclusion was aiming for. Negative values for the ADC of phosphorus, calcium and magnesium might be due to salt contamination of the collected faecal samples even after rinsing with de-ionized water.

Based on earlier studies in fish, NSP affects digestion, absorption, and metabolic processes by altering digesta viscosity, gastric emptying rate, gut morphology and the intestinal microbiota composition (Sinha et al., 2011). In addition, in fish the production of NSP-digestive enzymes such as β -glucanases or β -xylanases is low, and NSP degradation is assumed to be mainly due to microbial fermentation in the gut (Sinha et al., 2011; Maas et al., 2020). It remains, however, unknown whether NSP degrading enzymes are synthesized in shrimp and if NSP fermentation is taking place. A decrease in the ADC of shrimp fed with diets containing NSP-rich ingredients such as lupin kernel meal (Molina-Poveda et al., 2013), high protein distiller's dried grains (Qiu et al., 2017) and soyabean meal (Cruz-Suárez et al., 2009; Fang et al., 2016) has been reported, although the magnitude to which NSP reduces nutrient digestibility varies between studies. This may be affected by NSP type, NSP dietary inclusion level, the pre-treatment method applied on NSP-rich dietary ingredients, or by a combination of these factors (Cruz-Suárez et al., 2009; Sinha et al., 2011). Further research is needed to understand the mechanism how WBdiet reduced the ADC in Pacific white shrimp.

4.2. Effect of high wheat bran input on system performance

4.2.1. Shrimp

Shrimp in both treatments were fed equal amounts of protein, and showed a lower protein digestibility when fed the WBdiet, the similar protein retention in shrimp most likely was due to shrimp grazing on biofloc. The specific growth rate of shrimp fed the WBdiet was statistically lower than the growth of shrimp fed the CONdiet, although in absolute terms the difference was small (1.8%; **Table 4**). For the other performance parameters (biomass gain, production, and growth rate), no significant differences were observed. This suggested that supplementing a high input of complex carbon source did not give major impact on the shrimp growth, when protein and phosphorus input did not limit the shrimp growth (Prabhu et al., 2013). Eating biofloc as an additional food might compensate for potentially reduced shrimp growth due to wheat bran inclusion in the diet. Previous studies suggested that biofloc can improve feed utilization in shrimp by either stimulating synthesis of endogenous digestive enzymes or by using exogenous digestive enzymes obtained by eating biofloc (Wang et al., 2016; Panigrahi et al., 2021).

In this study, biofloc consumption by shrimp resulted in similar production and protein retention efficiency between both diets (**Table 4**). The level of biofloc contribution to shrimp growth, however, could not be calculated in this study. To carry out such analysis, one can compare the growth of shrimp reared in biofloc and clear water systems or conduct a nutrient isotope study. Another limitation of this study was that the nutrient digestibility of the feed might have been underestimated because the digestibility trial was done in clear water system with no biofloc consumption by shrimp.

4.2.2. Water quality and biofloc

We hypothesized that the higher input of carbon-rich faeces coming from the WBdiet would stimulate a higher biofloc formation, in terms of TSS and VSS, compared to the CONdiet. A decline of TSS observed after the start of the experiment in both treatments may be related to a reduction in the organic load via lower feed load in the systems compared to where the biofloc were matured before. Another explanation might be owing to the age of the biofloc, where mature biofloc aggregation could degrade after a period of time (Martins et al., 2020). At the start of the experiment, the unforeseen influx of organic carbon from decaying biofloc and photosynthetic carbon assimilation were larger than the extra influx through shrimp faeces in tanks fed the WBdiet, which could have masked the effect of the latter (Tinh et al., 2021c). This could also explain why the WBdiet did not increase the final TSS concentration or lead to differences in biofloc composition compared to the CONdiet (**Table 2.6**). Mature biofloc had the *in situ* carbon source to maintain the biofloc needs (Samocha et al., 2007; Xu et al., 2016; Martins et al., 2020). However, we hypothesized that this independence would only remain shortly without external carbon supplies, as the part of the *in situ* carbon loss out of the system in form of CO₂ via microbial respiration. This was supported by our finding, the VSS concentration started to be higher at the end of experiment (D42) in tanks fed the WBdiet compared to the CONdiet (**Figure 2.1**). In CONdiet, with a low external carbon input, the biofloc decaying rate outcompeted its development rate. Meanwhile, in WBdiet tanks, with higher external carbon input, the opposite occurred. This, therefore, could explain why biofloc VSS concentration was higher on D42 in tanks fed the WBdiet compared to the CONdiet (**Figure 2.1**).

At the end of the experiment on D42 the N-content in biofloc was 41% and 75% higher in the CONdiet treatment and WBdiet treatment, respectively, than on D1 (**Table 2.6**). This concurs with the higher organic matter accumulation shown by BOD₅ and a trend ($P < 0.1$) for higher microbial activity in the WBdiet than in the CONdiet tanks (**Table 2.6**). An increase in organic matter correlates positively with an increase in microbial biomass, consisting of mainly heterotrophic bacteria in the biofloc (Ray and

Lotz, 2014; Xu et al., 2018). At the end of the experiment, the higher organic matter accumulation in WBdiet tanks suggested greater biomass of heterotrophic bacteria, compared to the CONdiet tanks. In addition, there was an indication of more intensive heterotrophic mobilization of nitrogen in the WBdiet tanks, as seen from the lower final TON concentration, despite the higher faecal nitrogen input (due to lower dietary protein ADC). Despite these differences, the concentrations of TON and ammonium were maintained low in both treatments, throughout the experiment.

Like heterotrophic bacteria, autotrophic bacteria also contributed to the water quality maintenance, but it is expected that this group has a low biomass consisting of only a minor fraction of the total biofloc (Ebeling et al., 2006). An increase in the TIN concentration in both treatments during the experiment, mainly as nitrate, the final product of nitrification, indicated the presence of active autotrophic bacteria in both treatments. This finding confirms previous studies that showed active nitrifying bacteria in mature biofloc (Krummenauer et al., 2014; Emerenciano et al., 2017). Adding external carbon input to a system with mature biofloc, the expectation is that heterotrophic bacteria would gradually reduce the contribution of autotrophic bacteria in controlling the ammonia while producing nitrite and nitrate (Xu et al., 2016). This actually happened in our study, as seen from the lower accumulation of N-(NO₃+NO₂) (**Table 2.7**) in the WBdiet tanks compared to the CONdiet. However, it was slower than expected as the difference was small (7%, **Table 2.7**). Adding more carbon to the diet to make a major change in the nitrite and nitrate concentrations may risk the shrimp performances. Further research is needed to investigate an adequate C:N ratio of the WBdiet for both shrimp and system performances.

In biofloc, algae can produce in-situ organic carbon and immobilize nutrients (Emerenciano et al., 2017). In this study, we measured Chl-a to get an indication of algae development in the biofloc tanks. The total Chl-a concentrations were statistically similar between the dietary treatments (**Table 2.6**). However, the trend showed that diet affected the Chl-a distribution within the system. In the WBdiet system, Chl-a tended to be more profound in the biofloc aggregate than in the water, contrasting the condition observed in the CONdiet (**Table 2.6**). This could be because WBdiet had more abundant EPS matrix due to the increased number of heterotrophic bacteria or that diets generated different types of algae in terms of size and aggregation ability. Further in-depth analysis of the algal composition is necessary to confirm the second notion.

Our study was the first to apply the H₂O₂ degradation analysis for microbial activity analysis in the biofloc water sample. It measures the enzyme activity of bacteria, algae, and protozoans (Rojas-Tirado et al., 2018; Pedersen et al., 2019), and shows it to be a promising technique to measure microbial activity in biofloc systems. The measurement

is significantly considerably less time-consuming than BOD₅ analysis (half day vs 5 days). More trials were still needed to establish a standardized protocol for biofloc sample.

4.2.3. Nutrient mass balances

By feeding iso-nitrogenous, similar amounts of nitrogen were fed to all biofloc tanks during the experiment. Nevertheless, with the two diets, similar amounts of nitrogen were present in all tank compartments during the experiment. The overall nitrogen retention relatively to the nitrogen input in the system in both treatments was 97-100% (**Figure 2.4a**). This retention was higher compared to the 77-87% reported in previous studies (Tinh et al., 2021a; Tinh et al., 2021c). The high retention efficiency of nitrogen could be due to the low ammonia-N concentrations, keeping ammonia volatilization low. In addition, the dissolved oxygen concentrations remained high throughout the study, reducing the room for anoxic and anaerobic conditions, favorable to the loss of nitrous oxides from the biofloc tanks, to develop (Ebeling et al., 2006).

Based on previous study, the phytic acid content in wheat bran, fluctuates between 3.0 and 8.5 mg g⁻¹ (Pramitha et al., 2021), which is higher than for most other dietary ingredients, causing the P-PO₄ content in the WBdiet to be higher than in the CONdiet (**Table 7**). Two grams more phosphorus was fed per kg feed on a dry matter basis with the WBdiet than with the CONdiet. Unfortunately, because total phosphorus was not measured during this experiment, the phosphorus loss (P-loss) from the system could not be accurately measured and is therefore referred to as unaccounted phosphorus (**Figure 2.3b**).

For carbon, the amount lost was higher in tanks fed the WBdiet, yet the percentage loss was similar between diets (56-61%). This makes it likely that NSP was biodegraded by the microbiota resulting in a similar biodegradability index (0.41), observed for both diets (**Table 2.6**). The 0.41 biodegradability observed in this experiment is in the same range as reported by Rojas-Tirado et al. (2017). A biodegradability index ranging between 0.3 and 0.6 indicates that the biodegradability of the organic matter in the biofloc tanks was average (Srinivas, 2008).

5. CONCLUSIONS

Incorporating wheat bran in the diet and feeding isonitrogenously decreased the overall nutrient digestibility and increased the faecal C:N ratio in Pacific white shrimp. However, this study concluded that no major differences were observed in biofloc formation or shrimp performance during a 42-day culture period. From this perspective, the WBdiet treatment was not an improvement, but it could serve as alternative and simplified method for biofloc management practices. Nevertheless, it is worth noting that the

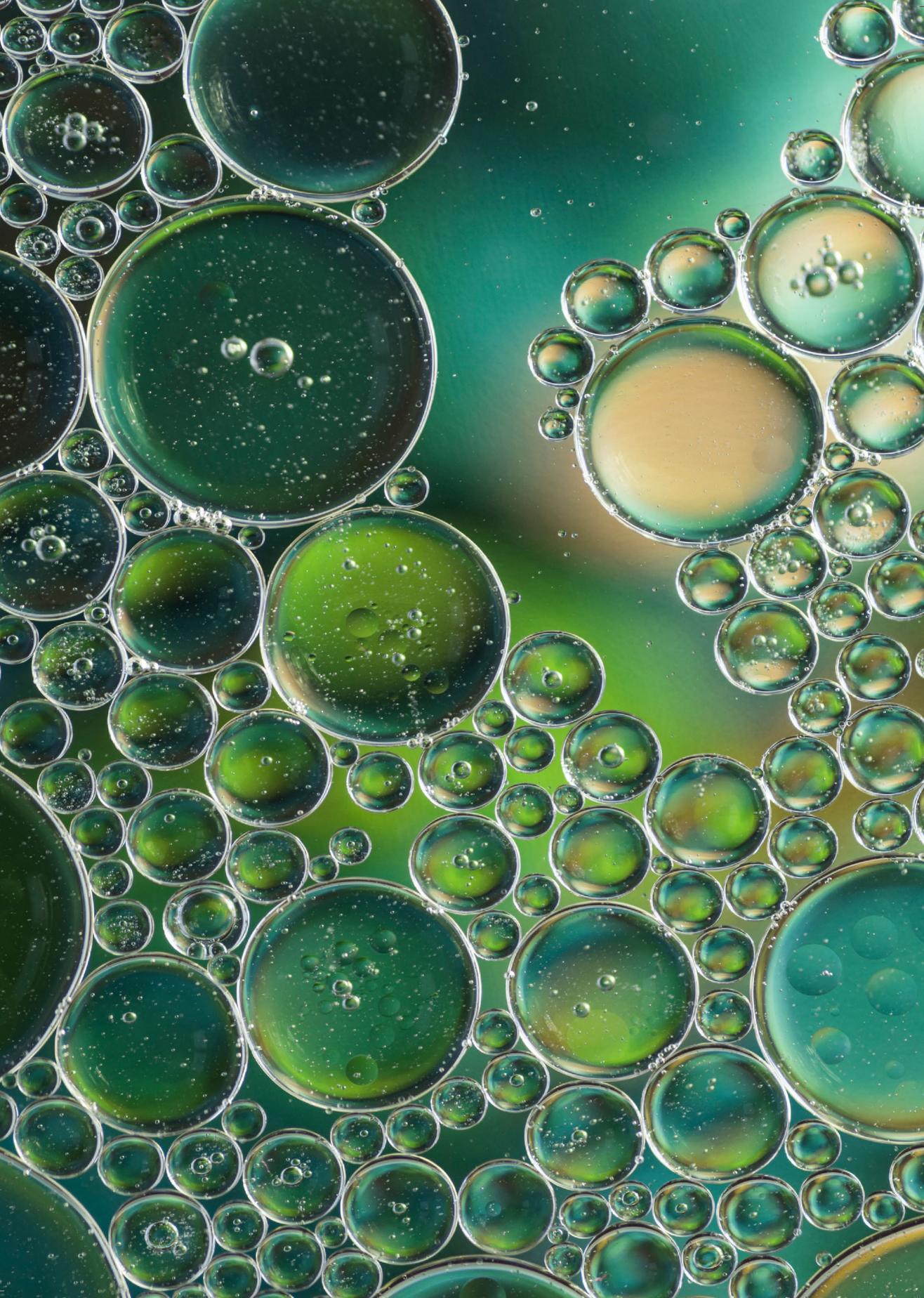
organic matter accumulation in biofloc fed WBdiet started to surpass that in the CONdiet at the end of culture period, suggesting a potential long-term effect of WBdiet on biofloc formation. Additionally, in situations where new biofloc develops during the culture period and no algae are present, the WBdiet may perform better than the CONdiet in biofloc formation. It would lead to a higher availability of biofloc as additional food for shrimp growth. No difference in protein retention efficiency was observed in this study between the dietary treatments, suggesting that biofloc contributed to shrimp nutrition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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The background of the cover features two petri dishes. The one in the foreground is filled with a vibrant blue agar medium, showing a dense, textured surface with numerous small, light-colored spots and streaks, likely representing bacterial colonies or biofilms. The second petri dish is partially visible in the upper right corner, containing a similar blue agar medium with some yellowish-green spots. The overall lighting is bright and even, highlighting the colors and textures of the microbial systems.

3

Prokaryotic and eukaryotic microbial community dynamics in biofloc systems supplemented with non-starch polysaccharides

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Abstract

Biofloc technology has been developed as a sustainable system in aquaculture, which uses microbial processes to remove nutrients from the water and convert them into microbial biomass. For the optimal performance, the addition of carbohydrates is essential, while their type can regulate the microbial activity and compositions. Specifically, we hypothesize that dietary supplementation of wheat bran, containing indigestible carbohydrates such as non-starch polysaccharides, can provide complex substrates supporting diverse microbial communities within the biofloc. In this study, we investigated how higher dietary wheat bran input affected the biofloc prokaryotic and eukaryotic microbial communities in Pacific white shrimp (*Litopenaeus vannamei*) culture. Two pelleted diets were made: a control diet (CONdiet) with a composition similar to a commercial shrimp diet, and a diet rich in non-starch polysaccharides, which was created by adding wheat bran to the dietary ingredient mix (WBdiet) of the CONdiet, before pelleting. These two diets were fed isonitrogenously for 42 days to the shrimp. Sampling of the biofloc was performed every three weeks. The results showed that the alpha and beta diversity in biofloc evolved during the experiment. Diet had a significant effect on the prokaryotic community composition in the biofloc at the end of the culture period, with several genera being enriched in tanks fed the WBdiet such as *Muricauda*, *Pirellula*, and *Cyanobacteriaceae*. Regarding the eukaryotic communities, overall, only few taxa were significantly affected belonging to *Trebouxiophyceae* and *Suillus* groups within the WBdiet. Interestingly, when feeding the WBdiet, the microbial communities in biofloc exhibited functionalities that were more abundant in carbohydrates metabolism, and more specifically related to pentose, fructose, mannose, and galactose metabolism. These results provide a basis for the control of microbial communities by using ingredients rich in plant-derived non-starch polysaccharides which shrimp cannot digest but are a good energy source for microbiota in the biofloc.

Keywords: biofloc, non-starch polysaccharides, wheat bran, shrimp, microbiota

1. INTRODUCTION

Biofloc technology (BFT) has been developed as an efficient, eco-friendly, and sustainable aquaculture system (De Schryver et al., 2008; Emerenciano et al., 2017). The principle of BFT involves the recycling and conversion of waste excreted and egested by the culture animals into microbial biomass. Biofloc are aggregates of suspended particles and microorganisms held together by extracellular polymeric substances (EPS) produced *in situ* by the microbiota (Bossier & Ekasari, 2017). Biofloc form a complex system in which various nitrogen metabolic pathways are present; photoautotrophic uptake by algae, heterotrophic and autotrophic bacterial respiration and assimilation, as well as fungal assimilation (Ebeling et al., 2006; Xu et al., 2016). So far, studies about the microbiota in biofloc systems mostly focused on the prokaryotic group, since heterotrophic bacteria play an important role in the biofloc system (Addo et al., 2021; Cardona et al., 2016; Chakrapani et al., 2021; El-Husseiny, 2018; Ferreira et al., 2021; Guo et al., 2022; Jiang et al., 2020; Panigrahi et al., 2018; Tinh et al., 2021; Xu et al., 2022; Zhu et al., 2022). Studies investigating eukaryotic communities, mainly focused on the composition of microalgae communities and their abundance as indicated by the chlorophyll-a concentration (Chakrapani et al., 2021; Jiang et al., 2020; Kabir et al., 2020; Rajkumar et al., 2016; Tinh et al., 2021). Despite their importance on biofloc functionality, the number of studies looking at eukaryotic community taxonomic composition is much lower compared to the ones focusing on the prokaryotic communities, while for the fungal community in biofloc, studies are nearly non-existent.

It is well established that the microbial composition in the biofloc can be steered by increasing the carbon (C) to nitrogen (N) ratio by adding organic carbon to the culture system (Addo et al., 2021; Deng et al., 2018; Panigrahi et al., 2018). Not only the quantity but also the types and complexity levels of carbon sources can affect the microbial community composition and functionality (Chakrapani et al., 2021; El-Husseiny, 2018; Kabir et al., 2020; Serra et al., 2015). Typically, carbon addition following feeding is the driving force in biofloc management which enters usually in the form of rich in carbohydrate (CHO) sources such as starch, molasses, or cassava; however, such conventional application is labour-intensive under farming conditions, as the right quantity, quality and timing of carbohydrates addition require daily attention from the farmer (Tinh et al., 2021). A proposed solution is to incorporate ingredients in the diet rich in carbohydrates which are less digestible by the animals such as non-starch polysaccharides (NSP), thus resulting in NSP-rich faeces rich in carbon ending up in the system in the form of NSP. The latter can provide a carbon and energy source to the biofloc microbiota. In turn, the biofloc will maintain the water quality and provide an additional feed source for the culture organisms (Hargreaves, 2013; Rajkumar et

al., 2016; Ray et al., 2017). Wheat bran is a carbon source commonly used as a feed ingredient for shrimp, containing 56% NSP (CVB 2022). Therefore, we hypothesize that dietary wheat bran addition will increase the carbon to nitrogen (C:N) ratio of the shrimp faeces, providing extra energy to the microbiota in the biofloc.

Opposing the conventional practice of biofloc management which is providing the carbon supply directly to the culture system, in this study, our aim was to incorporate the extra CHO as input to the pelleted diet. The experiment investigated the effect of a high dietary wheat bran input to a biofloc system for Pacific white shrimp, *Litopenaeus vannamei*, on the prokaryotic and eukaryotic microbial community composition, and how its composition evolved over time. Furthermore, we also looked specifically at the fungal community, considering that fungi contribute significantly to the metabolic processes in biofloc (Grossart et al., 2019). Our study outcomes on eukaryotes and fungi communities may shed light on their complementary roles in biofloc systems besides the prokaryotic community.

2. MATERIALS AND METHOD

2.1. Experimental design

Two pelleted diets were made: a control diet (CONdiet) with a composition similar to a commercial shrimp diet, and a diet rich in non-starch polysaccharides, which was created by adding wheat bran to the dietary ingredient mix (WBdiet) of the CONdiet, before pelleting. To meet the nutritional needs of Pacific white shrimp, the concentrations of essential amino acids, monocalcium phosphate, chalk, cholesterol, vitamin, premix, and yttrium in both diets were kept constant (**Table S3.1**). The additional carbon source in this study, wheat bran, was included in the diet rather than administered separately as in the conventional approach of biofloc management. It resulted in the higher amount of carbon input in the WBdiet tank, while the protein amount was comparable (iso-nitrogenous feeding). The experiment used 1000-L indoor mesocosm tanks as biological unit, equipped with a 600-watt Schego stick heater, an aeration ring placed at the bottom of the tanks, an aeration stone hanging in the center of the tanks, and net cover (mesh size ~1,5 x 1,5 cm). To mimic the sunlight and to stimulate the photosynthesis of algae, a pair of lights were set in between 2 tanks, namely Light Emitting Plasma Grow (LEP, Gavita Pro 270e), High-Intensity Discharge (HID) High-pressure Sodium (HPS) bulbs (400W) with reflector (Gavita HortiStar 600 SE EU, 96% efficiency). To compare the two diets, three replicate tanks were used per diet. The study was carried out in the Carus Animal Research Facilities of Wageningen University and Research (WUR).

2.2. Biofloc and shrimp stocking

The experiment started with mature biofloc in order to prevent water quality to deteriorate if no CHO was added, as in the case of the CON. It has been reported in previous studies that once the biofloc system is mature (30-50 days), its functionality will not be affected by no CHO addition for a few weeks (Martins et al., 2020; Samocha et al., 2007; Xu et al., 2016). A volume of 450-L biofloc inoculum water from the previous culture cycle was mixed with both freshwater and saltwater water until reaching a volume of 750 L and a salinity of 23 ± 1.0 ppt. The biofloc water was continuously aerated before and after the water stocking to the 6 mesocosm tanks. The Pacific white shrimp (0.05 ± 0.02 g/ind) were stocked at the stocking density of 100 ind/tank the next day. The shrimp were supplied by Crevetec in Ternat, Belgium.

2.3. Shrimp culture and system maintenance

Mechanical belt feeders were used to feed shrimp continuously during 10-12 hours per day starting at 16.00. The daily feeding rate in the WBdiet gradually decreased from 11.9 to 3.3% BW, assuming the FCR increased from 0.6 to 1.1 throughout the experiment. The diets were fed isonitrogenously, assuring that shrimp in each diet got the same amount of protein.

Each tank received freshwater weekly to compensate for the evaporation and water droplet loss from aeration (84 ± 7 L / week). Water quality was monitored daily and maintained to reach temperature range of 27.2 ± 0.6 °C, DO 7.0 ± 0.1 mg O₂ L⁻¹, pH 8.0, salinity 23.2 ± 1.0 ppt and conductivity 36.7 ± 1.5 μS cm⁻¹, measured using a multi-parameter electronic meter (WTW Multi3630IDSTM). Furthermore, the concentrations of ammonium (NH₄⁺, mg/L) and nitrite (NO₂⁻, mg/L) were measured using MColorTest™, EMD Millipore, while the concentration of nitrate (NO₃⁻, mg/L) was checked using MQuant®, EMD Millipore).

2.4. Sample collection and preparation

Biofloc was sampled at day-1, day-21 and day-42. Water containing biofloc was taken from the center of each tank using a 2-L jar for 10 times. A total volume of 20 L of shrimp culture water from tank was then pooled in a 50-L plastic bucket. Water was then mixed using IKA T-25 Ultra-Turrax dispersing instrument at speed of 300 rpm for 30 seconds. A 2-L of stirred water was then transferred to four 2-L jars.

To collect biofloc, a total 50-ml of the biofloc water was filtered through a 0.45 μm pore-size filter using a vacuum-pump and a glass filtration chamber (Tinh et al., 2021). Prior the filtration, water containing biofloc in jar was continually mixed with a magnetic stirrer (Heidolph Mr. Hei-Mix L, Heidolph Instruments, Germany) at 300 rpm, to ensure

a homogenous biofloc sample across filtration batches. After the filtration, a two times 50-ml demi water was also allowed to pass the filter to rinse the remaining salt. Filter containing biofloc particle was folded and stored in a closed-sterile tube. Sample was then temporarily stored in the liquid nitrogen, before being stored in the -80 freezer.

2.5. Biofloc microbial DNA extraction

For microbiota analysis, DNA was extracted from the biofloc samples. The samples were extracted using the DNeasy PowerSoil kit (Qiagen, Valencia, CA). The harvested DNA was quantified using the Nanodrop spectrophotometer. Sequencing of the PCR-amplified V4 region of the 16S rRNA (prokaryotic microbial communities), using primers 515 F (CTAGTCCAGCMGCCGCGGTAA) and 806 R (CTAGGACTACHVGGGTWTCTAAT), of the 18S SSU (eukaryotic microbial communities), using primers 3Ndf-CS1F (GGCAAGTCTGGTGCCAG) and V4-Euk-CS2R (ACGGTATCTRATCRTCCTTCG), and of the ITS (eukaryotic fungal communities), using primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC), was performed using a MiSeq PE300 Next Generation system (Illumina) by Genome Quebec, following the company's protocol. Sequencing data can be found at the NCBI (SRA) database under the study accession code PRJNA953186.

2.6. Microbial communities data analysis

For the microbial community analysis, an open-source software package, DADA2 (Callahan et al., 2016), was applied to model and correct Illumina-sequenced amplicon errors. Data were demultiplexed into forward and reverse reads according to the barcode sequence into sample identity, and trimming was performed, according to (Kokou et al., 2020). DADA2 resolves differences at the single-nucleotide level and the end product is an amplicon sequence variant table, recording the number of times each exact sequence variant (ESV) was observed in each sample (100% sequence identity). Taxonomy was assigned using the *assignTaxonomy* function of DADA2 package, which provides a native implementation of the naive Bayesian classifier method for this purpose (Wang et al., 2007). Silva reference databases against the 16S gene [138 version; (McLaren, 2020)] for the prokaryotes and the 18S gene [128 version; (Morien & Parfrey, 2018)] for the eukaryotes were used, while for the fungi the UNITE ITS database was used (Pölme et al., 2020). Owing to the variation in sequence depths between samples, all samples were normalized to the lowest depth by subsampling (18,052 reads for 16S; 14,080 for the 18S; 2,000 for the ITS).

For the alpha-diversity analysis, richness (observed taxa) and Shannon H' diversity were calculated for all microbial communities using *microbiome* package (Lahti et al., 2017). Non-parametric tests (Wilcoxon test) and linear mixed-effect models (*nlme* R

package, [Pineiro et al., 2012]) were used to assess alpha diversity. Non-parametric permutational multivariate analysis of variance [PERMANOVA; (Anderson, 2001)] was used to assess beta diversity. Principal coordinate analysis (PCoA) and the similarity index comparison were performed using Bray Curtis distance metric. To investigate the distribution of the microbial community on day-42, distance-based redundancy analysis (dbRDA) was performed, using Primer software (version 6; Informer Technologies), on the relative abundance of ASVs using TSS (total suspended solid), VSS (volatile suspended solid), P (phosphorous), Ca (calcium) and Mg (magnesium) composition of the biofloc as explanatory variables (**Figure 2.3**). Exact sequent variants which were statistically different between diets were detected using linear discriminant analysis effect size (LEfSe tool) (Segata et al., 2011). To examine the unique and shared amplicon sequence variants (ASVs) of prokaryotic, eukaryotic, or fungi, we defined the presence of an ASV in the diet group when its prevalence was 100%, meaning that it is present in the three tanks per diet. Core genera were defined as taxa that had higher than 95% prevalence and more than 1% in relative abundance.

To predict the functional content of the biofloc prokaryotes, we used the PICRUST2 tool (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; (Douglas et al., 2020). The exact sequence variant (ESV) sequences were aligned to the reference sequences and the Nearest Sequenced Taxon Index (NSTI) score was used to evaluate the availability of reference genomes that are closely related to the most abundant microorganisms in the samples. Sequences with NSTI scores >2 were removed from the dataset (103 of 7,056 ESVs), as predictions would be of low accuracy. The functional profile results were then analyzed using the DESeq2 tool (Love et al., 2014).

3. RESULTS

3.1. Microbial diversity dynamics of prokaryotic and eukaryotic biofloc communities

Diversity analysis (richness and Shannon H' index) of the prokaryotic, eukaryotic, and fungal communities in the biofloc samples, did not reveal significant differences between dietary diets (**Figure 3.1, Table S3.2**). However, richness significantly changed over time ($P < 0.05$), as indicated by linear mixed-effects (LME) analysis (**Table S3.2**), showing a decrease in all microbial groups (**Figure 3.1A-C.i**). Time also showed a significant effect ($P < 0.05$) on the Shannon H' diversity index (**Table S3.2**), increasing for the prokaryotic community (**Figure 3.1A.ii**) while decreasing for eukaryotic and fungal communities (**Figure 3.1B.ii, and Figure 3.1C.ii**).

To investigate the structure of the microbial community in the biofloc in the different diets, principal coordinate analysis (PCoA) using the Bray-Curtis metric was performed based on the relative abundance of ASVs for prokaryotic (**Figure 3.2A**), eukaryotic (**Figure 3.2B**) and fungal communities (**Figure 3.2C**). In all communities tested, the communities became more separate by day-42 (**Figure 3.2**). Prokaryotes showed a distinct cluster between diets was observed for the prokaryotic communities (**Figure 3.2A**). Time and interaction effects between time and diet showed a significant effect on community structure for prokaryotic and eukaryotic groups as indicated by the Permanova analysis ($P < 0.05$; **Table 3.1**). Meanwhile in the fungal community, only time showed a significant effect ($P < 0.05$; **Table 3.1**). The first axis of PCoA explained 61%, 69%, and 34% of the total variation while the second axis explained 18%, 21%, and 11% for prokaryotic, eukaryotic, and fungal communities, respectively (**Figure 3.2**).

The similarity index bar plots using Bray-Curtis analysis for prokaryotic, eukaryotic, and fungi were presented in **Figure 3.3**. The trend showed that the similarity level of all microbiota in both diets decreased over time, with the CONdiet suggested a greater decline than the WBdiet. Furthermore, the discrepancy of similarity level between diet in fungi and prokaryotic were higher than the eukaryotes. (**Figure 3.3**). Distance-based linear model (DistLM) analysis revealed that dissolved concentration of total nitrogen (TN) and chlorophyll-a concentration in filtered water (ChlWat) together explained 58% (R^2 sequential) of the observed total variation in the profiles of the prokaryotic community (**Table S3.3**). In the eukaryotic community, 84% of the total variation was explained by TN, ChlWat, microbial activity (MicAct), the crude protein content of biofloc (CP), C:N ratio of biofloc (C:N ratio), biological oxygen demand (BOD), the dissolved concentration of total carbon (TC) (**Table S3.3**). Specifically in fungal community, TN, ChlWat, BOD and C:Nratio showed 56% correlation with the total variation (**Table S3.3**). In all measured communities, ChlWat was correlated with differences in microbial profiles between diet (WBdiet and CONdiet), while TN was with differences between time points (day-21 and day-42) (**Figure 3.4**).

Venn diagram analysis indicated that in total 208 ASVs (41.3% of total ASVs) were shared between the CONdiet and WBdiet across day-21 and day-42 (**Figure S3.1A**). On day-42, two core genera namely *Pirellula* and *OM27 clade* were found solely in biofloc in tanks fed the WBdiet, and *Calorithrix* was the only core genus present in biofloc tanks fed the CONdiet. The shared core genera in biofloc between the two diets were *Woeseia*, *Pseudoalteromonas*, *Pleurocapsa*, *Maricauda*, *Candidatus nitrosopumilus* and *Bythopirellula*, besides one non assigned genus (**Figure S3.1B**).

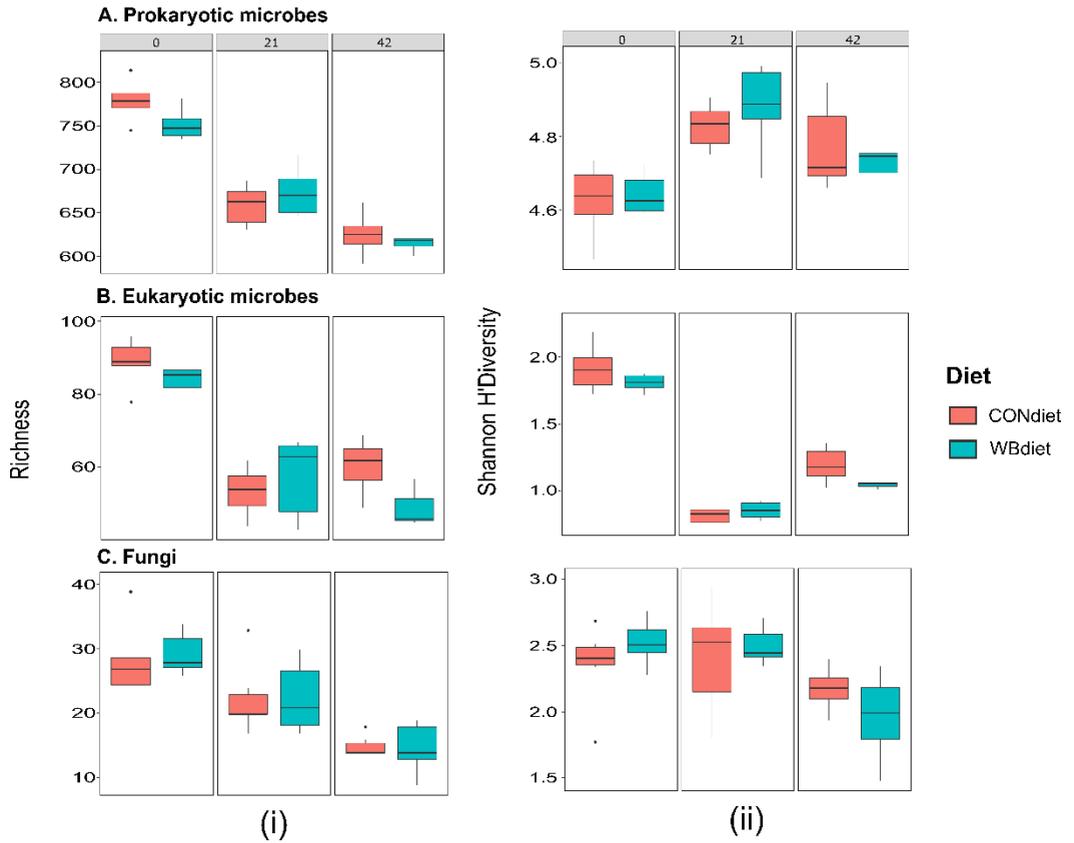
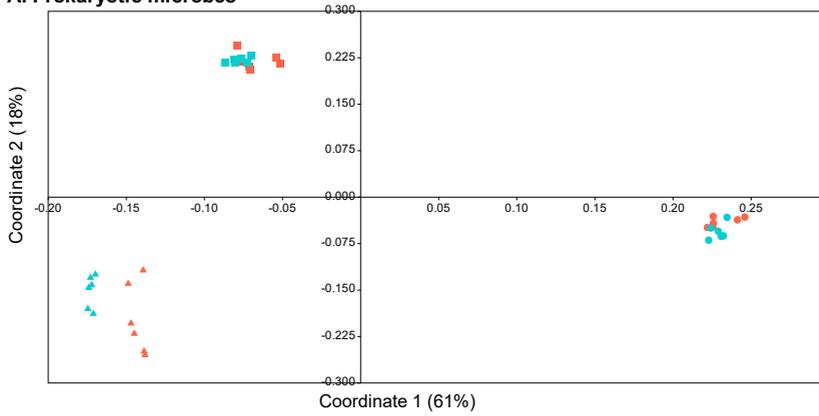
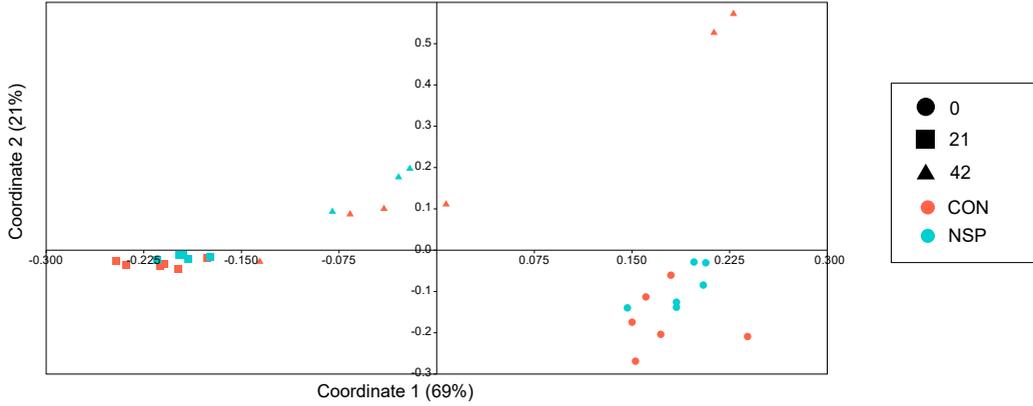


Figure 3.1. Alpha diversity of the Prokaryotic (16S rRNA) (A), Eukaryotic (18S rRNA) (B) and Fungal (ITS) (C) microbial communities, coming from the CONdiet or WBdiet over time, including (i) richness and (ii) Shannon diversity index. CONdiet = control diet, WBdiet = wheat bran diet, 0 = day-1, 21 = day-21, 42 = day-42.

A. Prokaryotic microbes



B. Eukaryotic microbes



C. Fungi

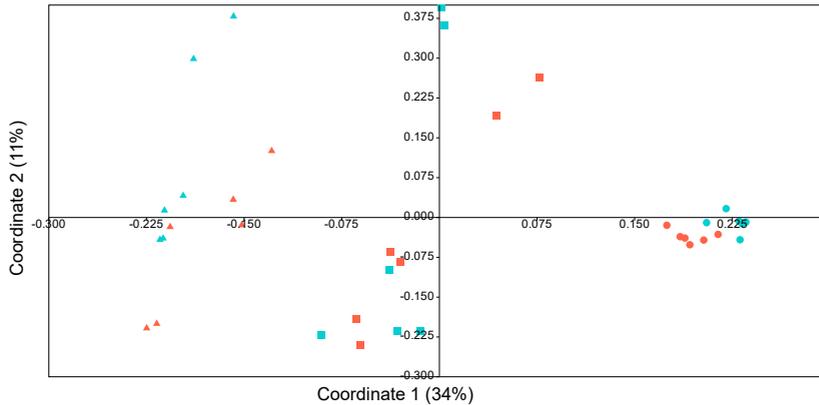


Figure 3.2. Principal coordinate analysis (PCoA) using Bray-Curtis metric at the ASV level for prokaryotic (16S rRNA) (A), eukaryotic (18S rRNA) (B) and fungal (ITS) (C) microbial communities. CONdiet = control diet, WBdiet = wheat bran diet, 0 = day-1, 21 = day-21, 42 = day-42.

Table 3.1. Two-way Permanova analysis for biofloc microbial communities based on Bray–Curtis distances

Permanova main effects						
Effect	Degree of freedom	Sum of Squares	Mean sum of Squares	Pseudo-F	P(perm)	perms
<i>Prokaryotic</i>						
Diet	1	407	407	2.53	0.124	10
Tank (Diet)	4	643	161	1.23	0.143	999
Time	2	7483	3742	29	0.001	997
Diet x time	2	467	234	1.8	0.013	998
Residuals	8	1048	131			
Total	17	10048				
<i>Eukaryotic</i>						
Diet	1	212	212	1.95	0.308	10
Tank (Diet)	4	435	109	1.48	0.074	999
Time	2	5403	2701	37	0.001	998
Diet x time	2	231	115	1.57	0.133	997
Residuals	8	586	73			
Total	17	6866				
<i>Fungi</i>						
Diet	1	1104	1104	0.62	1.000	10
Tank (Diet)	4	7142	1785	2.02	0.004	997
Time	2	14979	7490	8.46	0.001	999
Diet x time	2	2597	1298	1.47	0.111	999
Residuals	8	7084	886			
Total	17	32905				
Pairwise comparisons on Time (day)						
Groups	t	P(perm)	Unique perms			
<i>Prokaryotic</i>						
D1, D21	5.60	0.002	968			
D1, D42	6.47	0.005	985			
D21, D42	3.76	0.002	974			
<i>Eukaryotic</i>						
D1, D21	7.98	0.002	974			
D1, D42	7.44	0.004	974			
D21, D42	2.90	0.005	983			
<i>Fungi</i>						
D1, D21	2.64	0.011	972			
D1, D42	3.35	0.004	978			
D21, D42	2.59	0.007	968			

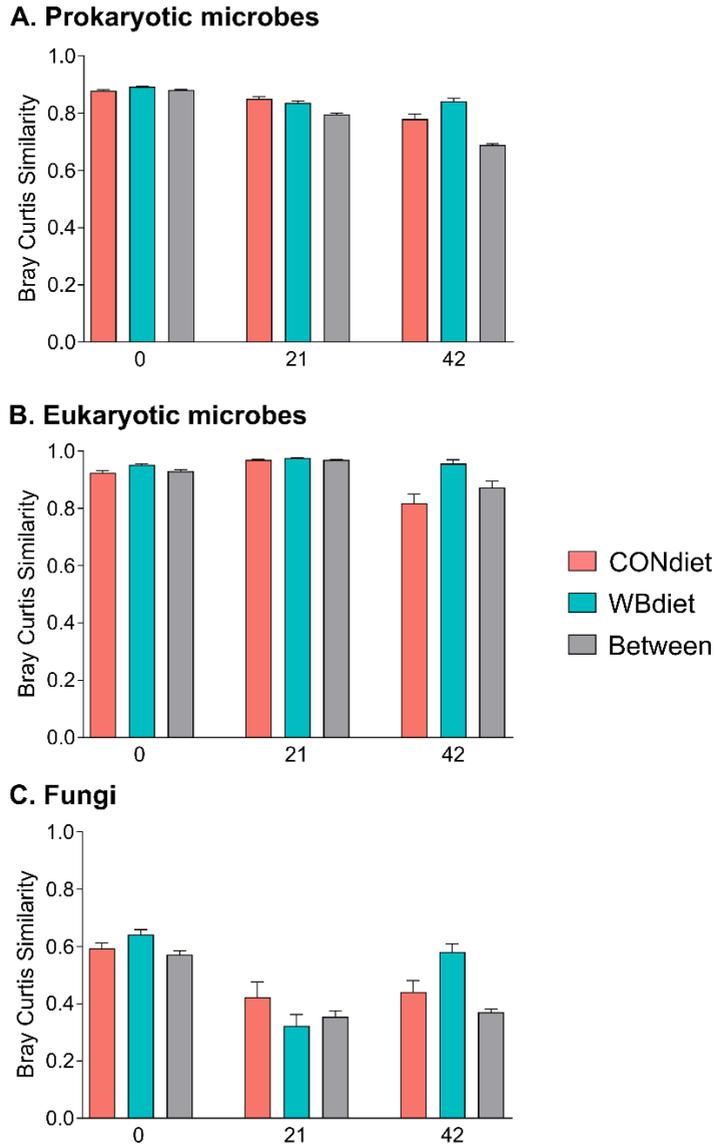


Figure 3.3. Similarity index based on Bray-Curtis distance analysis on prokaryotic (A), eukaryotic (B) and fungal (C) microbial communities. CONdiet = control diet, WBdiet = wheat bran diet, Between = pooled data of CONdiet and WBdiet, D1 = day-1, D21 = day-21, D42 = day-42. Each bar showed the means and standard error of the means of the Bray-Curtis similarity index of a diet per sampling time.

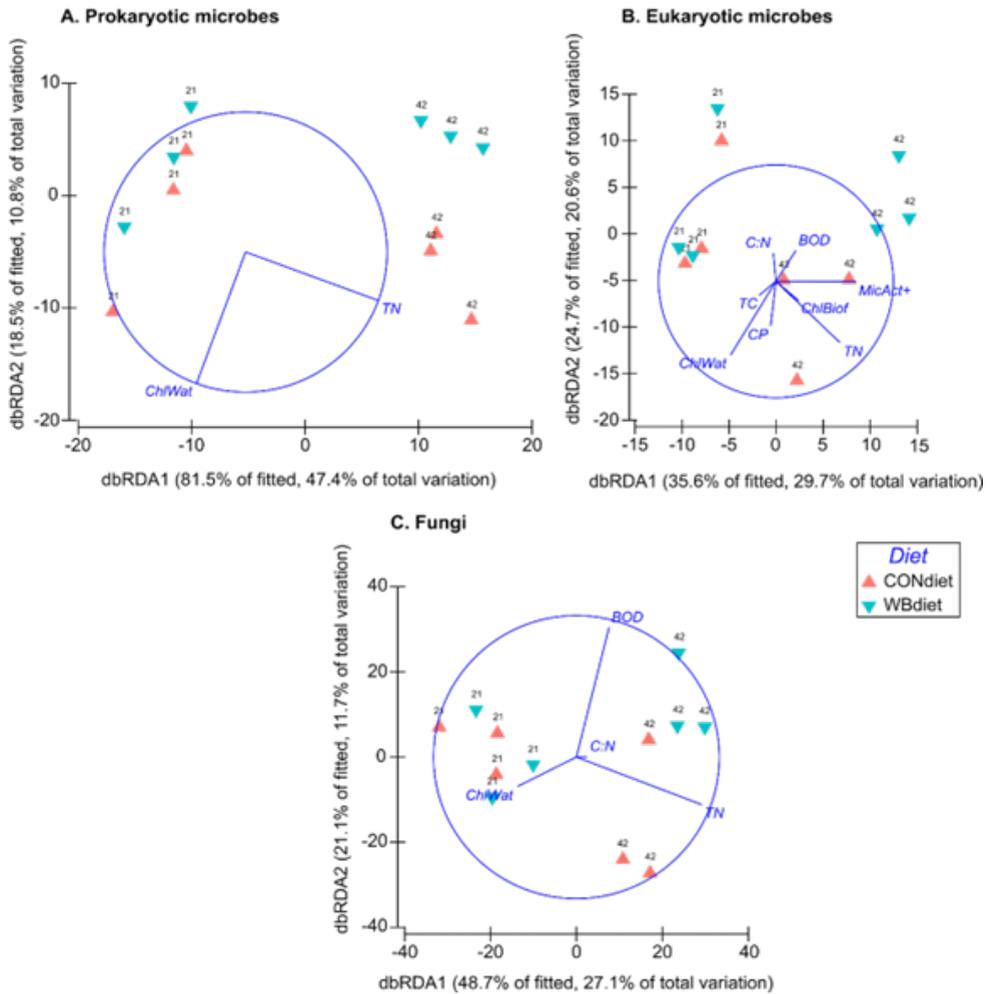


Figure 3.4. Distance based Redundancy Analysis (dbRDA) of biofloc prokaryotic (A), eukaryotic (B) and fungal (C) microbial communities. Relative position of biofloc samples in the biplot is based on Bray Curtis similarity of square root transformed relative abundance at the ASV level. Vectors indicate the weight and direction of the different biofloc composition parameters that were best predictors of the microbial composition as suggested by the results of the distance-based linear model (distLM). The dbRDA axes describe the percentage of the fitted or total variation explained by each axis while being constrained to account for group differences. Sample IDs indicate the sampling day (21 and 42) and the diet (WBdiet and CONdiet). TC: total carbon; BOD: Biological Oxygen Demand; C:N: Carbon to Nitrogen ratio; MicAct+: Microbial Activity; ChlWat: Chlorophyll in the water; ChlBiof: Chlorophyll in the biofloc; TN: Total Nitrogen; CP: Crude Protein.

3.2. Microbial composition of prokaryotic and eukaryotic biofloc communities

When looking at the prokaryotic communities, the most abundant phyla on all sampling days were *Proteobacteria* and *Chloroflexi*, followed by *Planctomycetote*, *Bacteroidota*, and *Cyanobacteria* (**Figure 3.5A**). In both diet groups, the relative abundance of *Proteobacteria* increased, while the *Chloroflexi* decreased over time (**Figure 3.5A**). About genera (**Figure 3.5B**), a large portion of the prokaryotic community could not be assigned to a genus (approximately 60% of the relative abundance). *Candidatus nitrosopumilus*, *Muricauda*, *Calorithrix*, *Pleurocapsa*, *Pseudoalteromonas*, *Woeseia*, and *Bythopirellula* had a relative abundance > 1% in both diets across the three sampling days (**Figure 3.5B**). In both diets, the relative abundance of *Calorithrix* decreased, while in contrast, *Muricauda* and *Pleurocapsa* increased over time (**Figure 3.5B**).

At the phylum level of the eukaryotic communities, *Chlorophyta* occupied the highest proportion (more than 75% of the relative abundance) and showed an increasing trend over time in both diets (**Figure 3.6A**). Besides, *Nematoda* and *Rotifera* were also found relatively abundant regardless of diet (**Figure 3.6A**). A large proportion of the eukaryotic genera was not assigned to genus level (up to 75%). *Nannochloris* and *Limnias* were found dominant on day-42, and their relative abundance was higher in tanks fed the CONdiet than in tanks fed the WBdiet (**Figure 3.6B**). In contrast, the relative abundance of *Anurofeca*, *Rhabdolaimus*, and *Picochiorum* genera decreased over time (**Figure 3.6B**).

In the fungal community, the main phyla were *Ascomycota* and *Basidiomycota*, while a large portion of the community was not assigned to any taxonomic composition (**Figure 3.7A**). Throughout the experiment, *Ascomycota* increased in relative abundance, while the *Basidiomycota* group decreased, as seen in both diets (**Figure 3.7A**). At genus level (**Figure 3.7B**), *Trichosporon*, *Engyodontium*, *Apiotrichum*, and *Didymella* were initially amongst the most abundant groups, while on day-42, *Penicillium* became more abundant, followed by *Phoma*, *Fusarium*, *Hortaea*, and *Aspergillus*. The proportion of *Penicillium* and *Aspergillus* increased throughout the experiment and had a higher relative abundance in tanks fed the CONdiet than in tanks fed the WBdiet (**Figure 3.7B**).

The differentially enriched taxa on day-42 between diets were determined using LefSe analysis (**Figure 3.8**). Biofloc in tanks fed the WBdiet were enriched with the prokaryotes *Caldilineaceae*, *Muricauda*, *Pirellula*, and *Cyanobacteriaceae*, eukaryotes *Trebouxiophyceae*, and fungi *Suillus*. Meanwhile, in tanks fed the CONdiet the biofloc was enriched with the prokaryotes *Pleurocapsa*, *Candidatus Nitrosopumilus*, *Chloroflexi*, *Motilimonas* and *Bdellovibrionaceae*, the eukaryote *Nannochloris*, and the fungus *Debaromyces*.

3.3. Predictive functionality of prokaryotic microbial community

We showed that the microbial composition is affected by diet, which was more evident on day-42 within the prokaryotes (**Figure 3.2**). Therefore, we evaluated the microbial community attributes of this group. We used the PICRUSt2 tool to understand the impact of dietary NSP supplementation on the functional diversity in the prokaryotic microbial community in biofloc (**Figure 3.9**); the detailed pathways are listed in **Table S3.4**. The supplementation of NSP promoted functionalities related to CHO metabolism, which became more evident with time, and more specifically, we found higher abundance of pathways related to the metabolism of pentose, fructose, mannose and galactose.

We observed a higher abundance of genes associated with the synthesis of fucI; L-fucose/D-arabinose isomerase, algL; poly(beta-D-man urinate) lyase and GMPP; mannose-1-phosphate guanylyltransferase, in tanks fed the WBdiet compared to tanks fed the CONdiet. Moreover, in the CONdiet, we observed an enrichment of various microbial functionalities related to the metabolism of energy (via photosynthesis and methane metabolism); nucleotide (purine); amino acid (cysteine, methionine, alanine, aspartate, glutamate); cofactors and vitamins (porphyrin, pantothenate, and CoA) and CHO (fructose and mannose).

4. DISCUSSION

Our study explored the microbial dynamics within biofloc aggregates when supplemented with dietary NSP-containing ingredients, in form of wheat bran. We showed that such supplementation affected the biofloc prokaryotic and eukaryotic microbial composition during the 42-day culture period. In addition, the predictive prokaryotic functionalities were also affected, indicating that a shift towards utilization of NSP-containing ingredients such as wheat bran may be possible.

4.1. Microbial diversity dynamics

We examined the microbial communities of biofloc in shrimp culture fed different amounts of dietary carbon input for 42 days. Time significantly affected both the richness and Shannon index of the prokaryotes and eukaryotes (**Table S2**), as in agreement with Tinh et al. (2021). The alpha diversity indices decreased over time in all tested communities, except for the Shannon diversity index of the prokaryotes (**Figure 1**), suggesting the instability of microbial diversity across the culture stages. These findings contradicted the earlier studies looking at bacterial dynamics in the biofloc for *L. vannamei* shrimp culture (Ferreira et al., 2021; Xu et al., 2022). Xu et al. (2022) demonstrated that in the early stage of biofloc formation, the rise in bacterial diversity derived from constant carbon addition indicated a time of adaptation and maturation

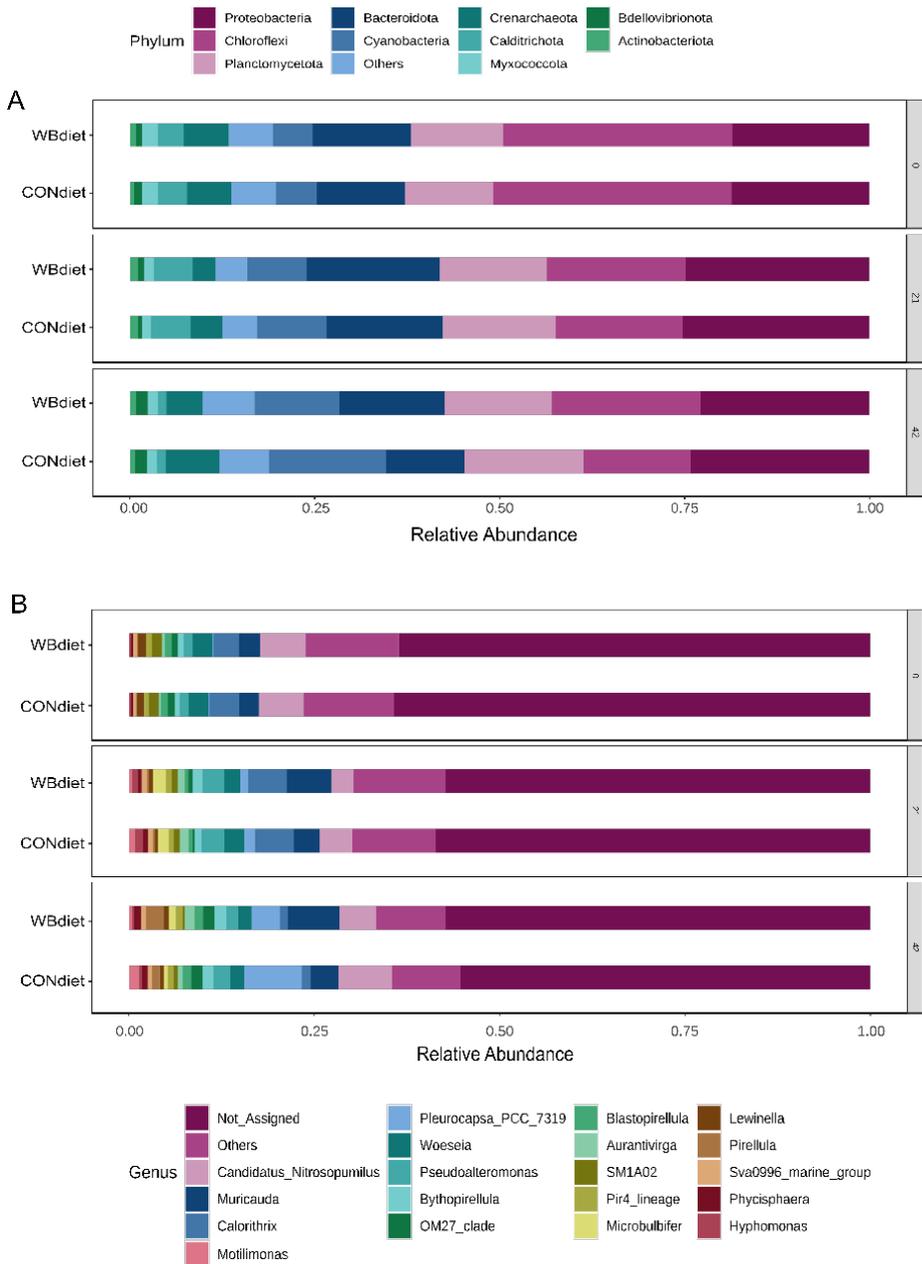


Figure 3.5. Prokaryotic microbial composition of the biofloc samples between the CONdiet and WBdiet over time at phylum (A) and genus level (B). CONdiet = control diet, WBdiet = wheat bran diet, 0 = day-1, 21 = day-21, 42 = day-42.

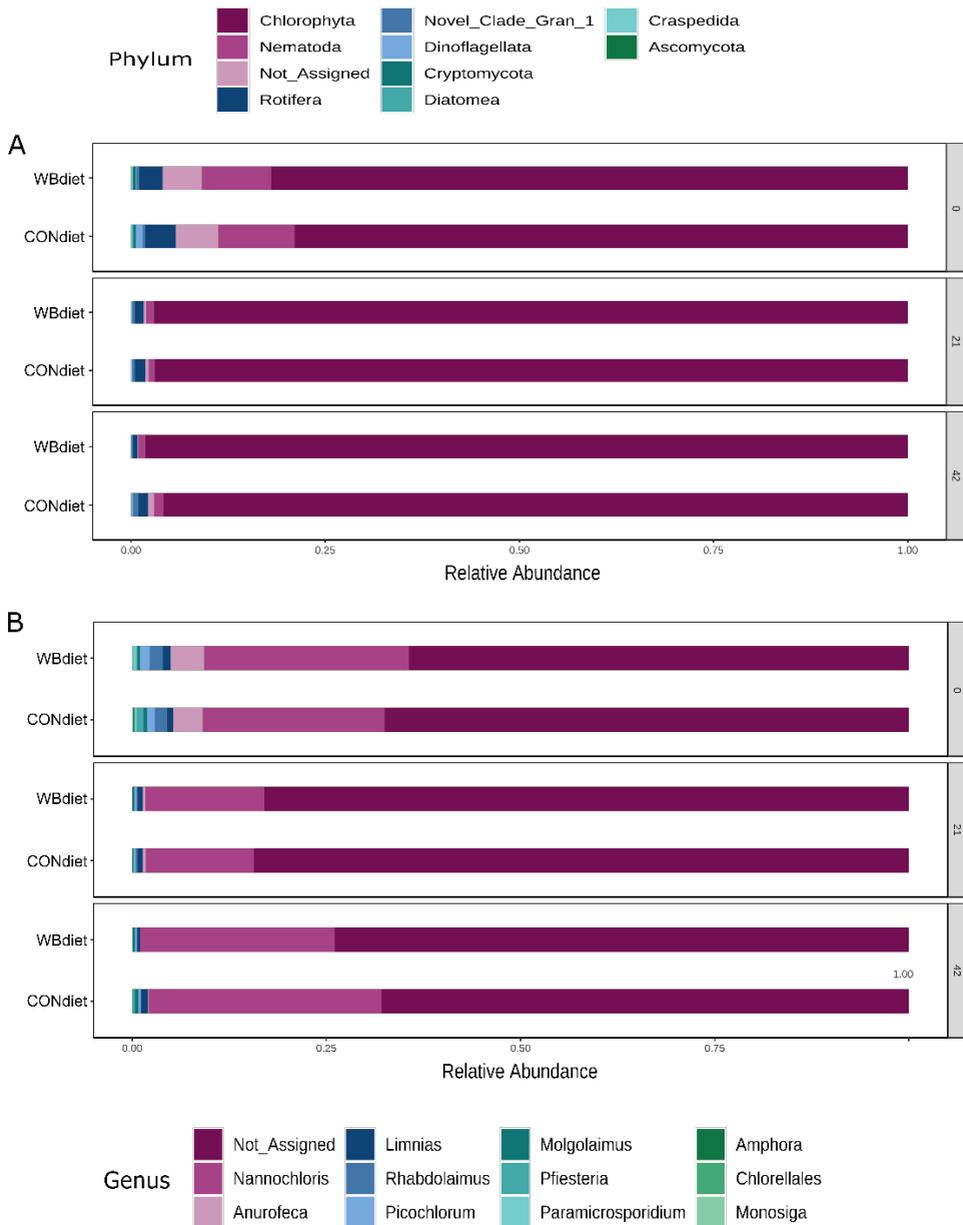


Figure 3.6. Eukaryotic microbial composition of the biofloc samples between the CONdiet and WBdiet over time at phylum (A) and genus level (B). CONdiet = control diet, WBdiet = wheat bran diet, 0 = day-1, 21 = day-21, 42 = day-42.

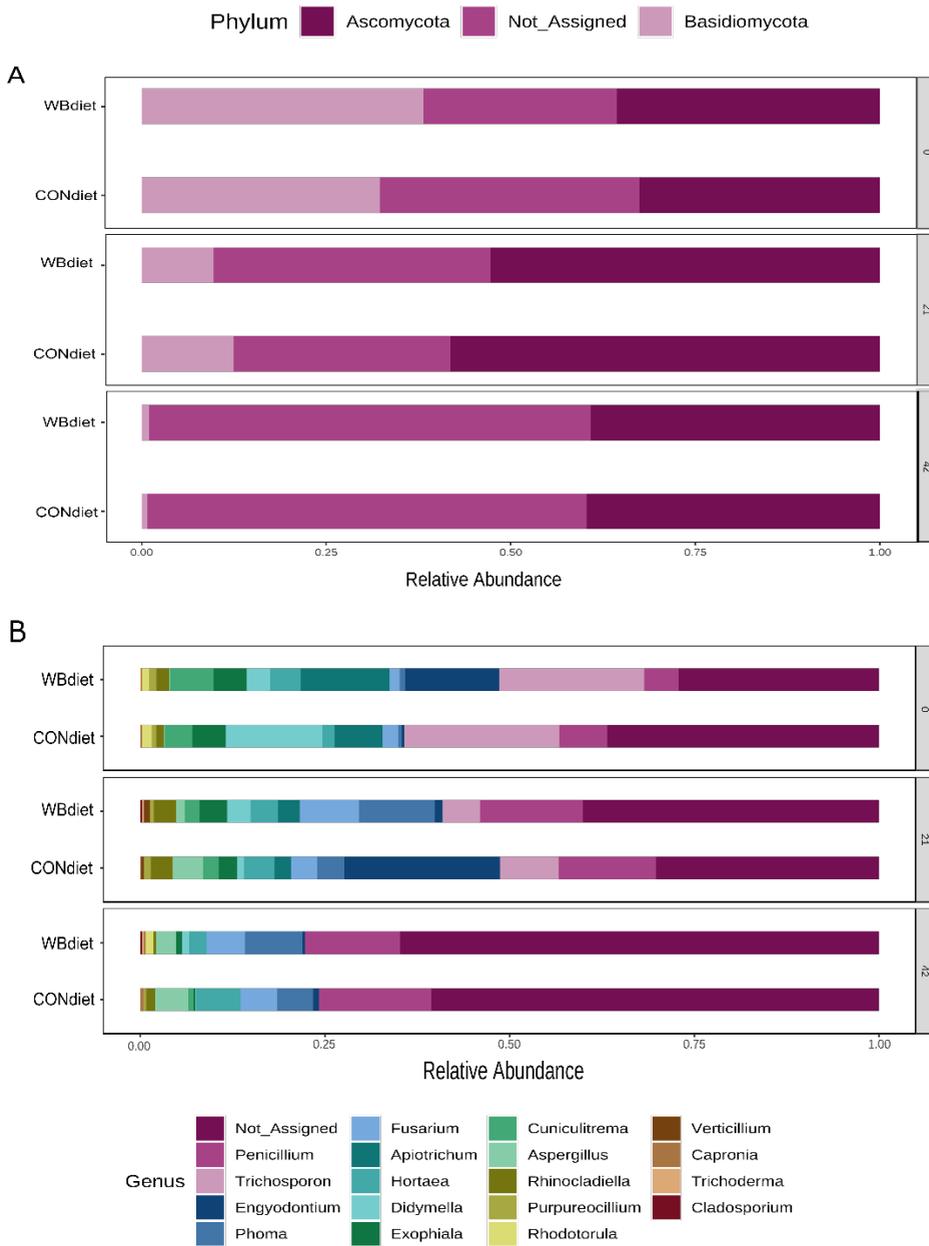


Figure 3.7. Fungal microbial composition of the biofloc samples between the CONdiet and WBdiet over time at phylum (A) and genus level (B). CONdiet = control diet, WBdiet = wheat bran diet, 0 = day-1, 21 = day-21, 42 = day-42.

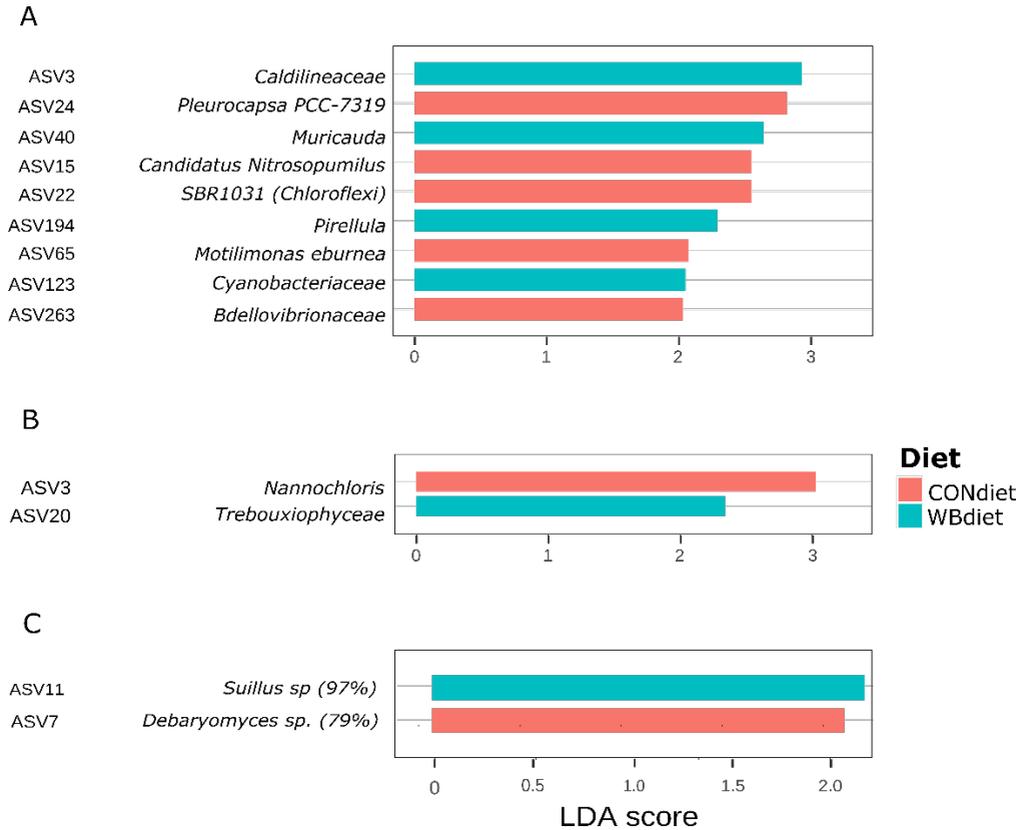


Figure 3.8. Lefse analysis (Linear discriminant analysis Effect Size) on prokaryotic (A), eukaryotic (B) and fungal (C) taxa, showing the linear discriminant analysis (LDA) score of the differentially enriched taxa affected by diet on day 42. For the fungal ASV, manual BLAST was performed in order to identify the closest genus; similarity percentage is included in brackets. CONdiet = control diet, WBdiet = wheat bran diet.

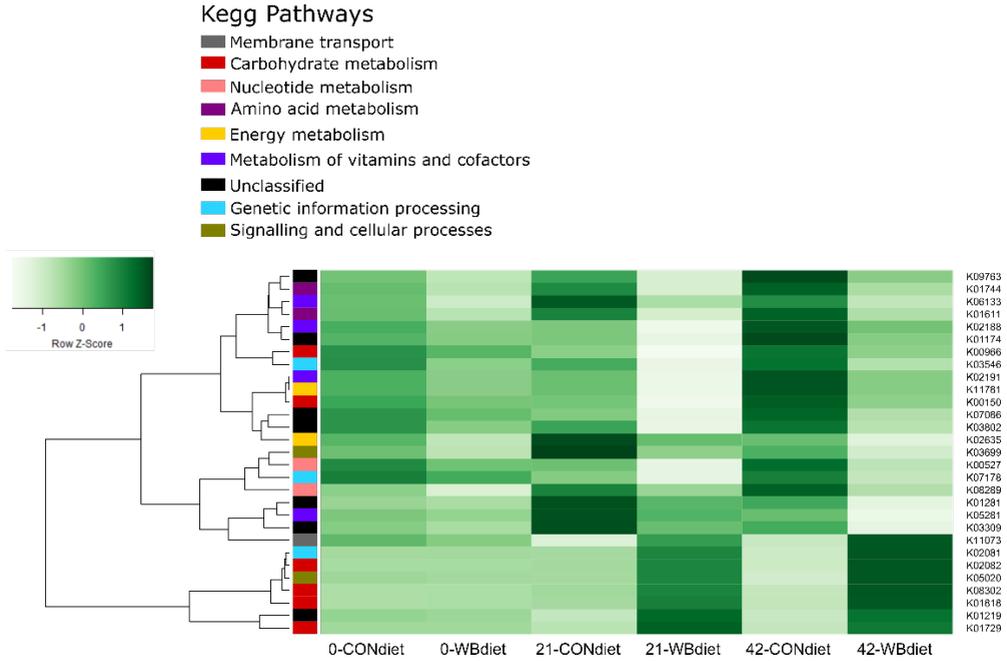


Figure 3.9. Predictive functionality analysis using PICRUST2 based on KEGG (Kyoto Encyclopaedia of Genes and Genomes) orthologs between the CONdiet and WBdiet biofloc groups. Significant pathway enrichment was tested using DESeq analysis. D1-CONdiet = control diet at D1, D1-WBdiet = wheat bran diet at D1, D21-CONdiet = control diet at D21, D21-WBdiet = wheat bran diet at D21, D42-CONdiet = control diet at D42, D42-WBdiet = wheat bran diet at D42.

for bacterial growth and development. However, the increasing trend was also found even when the biofloc has matured (Ferreira et al., 2021). Mature biofloc was defined as when biofloc has been developed between 30-50 days and the suspended solid concentration reached at least 5 mL/L (Emerenciano et al., 2017), which fit the biofloc condition that we used during the culture period. Our results suggested that the trend in the microbial diversity dynamic could vary despite maintaining a biofloc system with carbon addition, and regardless of the maturity status of the biofloc.

Higher dietary carbon input via the WBdiet did not significantly affect alpha diversity indices in all tested microbial communities ($P > 0.05$; **Table S3.2**), opposing previous studies (Chakrapani et al., 2021; Gou et al., 2019; Guo et al., 2022; Martins et al., 2020; Michaud et al., 2014; Tinh et al., 2021; Xu et al., 2022; Zheng et al., 2018). The decreasing trend of alpha diversity from beginning to the end in both diets demonstrated that the constant dietary carbon addition could not contribute to stable microbial diversity, and that positive selection pressure on certain microbial groups could occur, leaving only those more adapted to factors changing with time (Martins et al., 2020).

Looking at the beta diversity of the prokaryotic community, the significant effect of time and interaction between time and diet ($P < 0.05$; **Table 3.1**) demonstrated that bacterial structures and profiles developed differently across culture stages and the discrepancy between diets distanced further at the end of culture period (**Figure 3.2A**). At the beginning of the experiment, the in situ carbon input from decaying microbiota in mature biofloc was higher than the carbon from shrimp feces in tanks fed the WBdiet. This, therefore, concealed the effect of higher dietary wheat bran input via the the WBdiet. Previous studies revealed mature biofloc provides an internal carbon source from the decayed microbiota, and might require less external carbon to maintain water quality (Martins et al., 2020; Samocha et al., 2007; Xu et al., 2016). As the shrimp grew and the feeding level increased, so did the faecal load; the WBdiet started to differentiate the bacterial profiles from those in the CONDiet, explaining the significant interaction between diet and time ($P < 0.05$, **Table 3.1**). In the eukaryotic community, diet also did not affect the beta diversity ($P < 0.05$; **Table 3.1**), supporting earlier finding by Tinh et al. (2021). A similar trend as in the prokaryotic community was also observed in the eukaryotic and fungal communities (**Figure 3.2B** and **Figure 3.2C**). In our opinion, this study still supported the idea that the quantity of carbon input is a pivotal factor in steering the bacterial structure in a biofloc system (Gou et al., 2019; Martins et al., 2020; Zheng et al., 2018). However, a longer period of culture may be required to see the significant effect of diet on the bacterial community structure.

When comparing the three microbial communities observed, the prokaryotes and fungi had more distinct microbial clusters between diets than the general eukaryotes (mainly consisting of algae) (**Figure 3.2**). Supporting this finding, the decrease in the similarity index between diet during the experiment was higher in the prokaryotes and the fungi than in the eukaryotes (**Figure 3.3**). It was thought to be associated with the ability of bacteria and fungi to directly utilize organic carbon via heterotrophic and saprophytic pathways (Grossart et al., 2019; Manan et al., 2016), as well as to act as intermediate degraders for NSP. Higher dietary carbon and NSP content given to the system via WBdiet was suggested to influence both the prokaryotes and the fungi more intensively than the general eukaryotes. Bacteria and fungi broke the complex structure of shrimp faecal NSP into simple sugars, which were more easily degraded by other microbes. Consequently, more variety of carbon types were present in the WBdiet tanks, which potentially induced higher microbial diversity and numbers of microbial species (Chakrapani et al., 2021; El-Husseiny, 2018; Tinh et al., 2021). However, in this study, WBdiet did not significantly affect either of the parameters mentioned above (**Table S3.2**), and a precise mechanism could not be drawn due to the limitations of our study. Therefore, further research may look deeper into how different carbon substances influence the microbial diversity and microbial profiles in the biofloc systems.

Time significantly altered the alpha and beta diversity of prokaryotic and eukaryotic communities, supporting the earlier findings by Tinh et al. (2021). Time might be related to the dynamic factors within the system, such as the water quality and microbial biomass (Cardona et al., 2016; Pekkoh et al., 2022; Tinh et al., 2021; Xu et al., 2022). Similarly, our study also found that environmental factors such as the dissolved concentrations of total nitrogen (TN) and Chlorophyll-a explained the variations in the microbial profiles in all tested communities (**Figure 3.4**). Other factors were also found to be responsible specifically for the eukaryotic communities: biological oxygen demand (BOD), microbial activity, dissolved concentrations of total carbon, as well as the crude protein (CP) content and C:N ratio of biofloc (**Figure 3.4B** and **3.4C**). Yun et al. (2022) reported that the proportion of biofloc mainly consisted of eukaryotes (49-73%), followed by prokaryotes (27-51%). It then made sense that higher organic matter in the form of the eukaryotes influenced the level of BOD and the system's microbial activity. The shifts of the microbial communities between day-21 and day-42 in all observed communities were associated with the increasing concentration of dissolved TN over time, resulting from the increasing dietary nitrogen input and the accumulation of nitrogen from microbiota. Nitrogen was more conserved in the system than carbon because as the carbon is degraded and lost via microbial respiration, part of nitrogen may be converted into biomass and ammonia (Gou et al., 2019). The nitrogen

lost was due to denitrification, and this process was minimal in a fully-suspended and continuously-aerated environment like the biofloc system. The effect of dissolved TN had less effect in explaining variations of the microbial profiles between diets because both diets were fed isonitrogenously to the shrimp.

4.2. Microbial taxonomic composition

The relative abundance of the most abundant prokaryotic phyla (*Proteobacteria*, *Chloroflexi*, *Planctomycetote*, *Bacteroidota*, and *Cyanobacteria*) fluctuated over time, but still remained independent of diet (**Figure 3.5A**, **Table 3.1**). This suggested that the differences between the WBdiet and CONdiet in terms of carbon input quantity and complexity could not drastically alter the diversity of the most abundant phyla in biofloc. It was consistent with previous studies observing that these phyla are reported dominant across a wide range of CN ratios ranging from 10 to 20 (Cardona et al., 2016; Deng et al., 2019; Gou et al., 2019). Moreover, similar prokaryotic phyla has been reported with various carbon sources differing in structure complexities such as sucrose (Chen et al., 2021), molasses (Cardona et al., 2016; Jiang et al., 2020; Panigrahi et al., 2018), rice straws (Addo et al., 2021), corn starch (Tinh et al., 2021), wheat (Martínez-Córdova et al., 2018) and vegetal floating substrates such as amaranth, oat and wheat (Peiro-Alcantar et al., 2022), indicating those as core prokaryotic phyla within biofloc.

Biofloc from WBdiet showed a higher abundance of *Caldilineaceae*, *Muricauda*, *Pirellula*, and *Cyanobacteriaceae* classes compared to the CONdiet (**Figure 3.8**). *Caldilineaceae* is a filamentous bacteria from the *Chloroflexi* phylum (Yu et al., 2018). *Chloroflexi* species form the floc skeleton by producing extracellular polysaccharides (EPS) (Yu et al., 2018; Zhu et al., 2022). *Muricauda* has been reported as a dominant biofloc-associated bacteria, which increased with higher carbon availability (Tinh et al., 2021; Zhu et al., 2022). These taxa were thought to contribute to the yellow-brownish color of biofloc (Bruns et al., 2001; Oh et al., 2015). *Pirellula* has roles in global carbon and nitrogen cycles, for example, as the nitrate reducer (Fraser, 2004; Op den Camp et al., 2007). Overall, our study reported genera which have been previously commonly found in biofloc systems (Deng et al., 2019; Tinh et al., 2021; Xu et al., 2022)

With regard to eukaryotic communities, the dominant phyla found in this study (*Chlorophyta*, *Nematoda* and *Rotifera*; **Table 3.6A**) have been commonly found in biofloc systems (Chakrapani et al., 2021; Maicá et al., 2012; Manan et al., 2016; Ray et al., 2010). *Chlorophyta* had higher relative abundance in WBdiet on day-42 (**Figure 3.6A**), which was associated due to the higher organic carbon availability within the system as also stated by Gao et al. (2019). However, some species of *Chlorophyta* were also an obligate autotroph that could not utilize organic carbon because of the absence of genes

encoding organic carbon transporters (Celente et al., 2022; Zaslavskaia et al., 2001). *Nematode* and *Rotifer* are classified as algae grazing zooplanktons whose abundance increased along with the abundance of algae in the biofloc system (Hargreaves, 2013). In our study, we found the opposite pattern, with *Rotifer* and *Nematode* abundance decreased while algae abundance increased, possibly indicating the shrimp's grazing activities on zooplankton over time (**Figure 3.6**). Furthermore, the declines in both zooplankton abundance were more intensive in WBdiet than in CONdiet tanks (**Figure 3.6**). *Nematode* and *Rotifer* were a nutritious live prey for shrimp (Silva et al., 2021). Our study concluded that the eukaryotic community was dominated by algae belong to *Chlorophyta* phylum, followed zooplanktons groups (*Nematoda* and *Rotifera*).

Inorganic carbon availability is a determining factor in the algal productivity and their photosynthesis activity. The increasing abundance of *Nannochloris* over time in both diets indicated that no inorganic carbon limitation condition occurred (Tamburic et al., 2015; Zavrel et al., 2018). The addition of organic carbon such as ethanol and glucose had no effect on the net photosynthetic rate but increased the biomass of *Nannochloris* (Fang et al., 2004). The fact that the relative abundance of *Nannochloris* in the WBdiet was lower than in the CONdiet (**Figure 3.6**; **Figure 3.8B**) was probably due to the increase in growth of un-assigned algal genera from the *Chlorophyta* phylum. This study was linear to previous research, showing that *Nannochloris* was one of the most identified eukaryotic genera in biofloc system (Yun et al., 2022). However, majority of genus were still not identified due to the limited database.

In the fungal community, the dominant phyla observed were only *Ascomycota* and *Basidiomycota* since a high proportion of species were unknown (**Figure 3.7A**). Diet showed a comparable effect on the dynamics in the relative abundances of these two phyla. *Phoma* and *Rhodoturolo* were genera showing higher relative abundances with the WBdiet than with the CONdiet (**Figure 3.7B**). In an *in vivo* culture, *Phoma* species grew more vigorously using simple sugars compared to NSP-containing carbon source such as in sucrose and soluble starch compared to cellulose, and in glycerol rather than in rice bran media (Cao & Li, 2022; Luft et al., 2021). The higher the relative abundance of the *Phoma* genus in WBdiet tanks at day-21 and day-42 suggested the higher availability of simple sugars in the system. The latter might have been sourced from the degradation of complex carbon in the shrimp's faeces by an intermediate microbiota. *Rhodoturolo* species was reported able to utilize lignin-containing ingredients (Gupta et al., 1990; Hainal et al., 2012; Martins et al., 2021). Lignin is categorized as an NSP. Overall, we discovered that the dominating genera of the fungal communities were associated with carbon availability.

Phoma and *Rhodoturoloa* species can produce biopolymers composed of CHO synthesized via extracellular pathways during the process of growth and metabolism, creating an extracellular polysaccharides-enriched fractions also known as exopolysaccharides (EPSs) (Luft et al., 2021; Mahapatra & Banerjee, 2013; Osemwegie et al., 2020). This fraction was hypothesized to have a similar function as the EPS matrix secreted by heterotrophic bacteria due to its roles in cellular aggregation, nutrient attachment, and substrate adhesion (Breitenbach et al., 2022; More et al., 2014). However, unlike bacterial EPS production in a biofloc system via the heterotrophic pathway, fungal EPS is commonly produced via fermentation (Mahapatra & Banerjee, 2013). Microbial fermentation was thought to be hardly present in a full-aerated environment with continuous water suspension such as biofloc system. A follow-up study may investigate the occurrence and role of fungal EPS in biofloc systems.

4.3. Predictive functionality of prokaryotic microbial communities

We observed the effect of high dietary wheat bran input on microbial functionalities, specifically on the prokaryotic community. Adding wheat bran increased the abundance of prokaryotic predicted functionalities related to CHO metabolism (**Figure 9**). Higher carbon metabolism was thought to be associated with the higher absolute amount of carbon input in WBdiet than in CONdiet tanks. The total CHO in WBdiet tanks was 380 g, higher than the 244 g in CONdiet. More specifically, we observed a significant enrichment of pathways related to the metabolism of pentose, fructose, mannose, and galactose in the WBdiet than in the CONdiet (**Table S4**). Wheat bran contains 56% of NSP, mainly in the form of arabinoxylans (70%) and cellulose (24%) (CVB, 2022; Maes & Delcour, 2002; Stevenson et al., 2012). Including 40% wheat bran in the WB diet increased the content of NSP, arabinoxylan, and cellulose by 22%, 16%, and 5.4% respectively, which also increased the NSP content in the shrimp faeces. The increasing metabolism of simple sugars mentioned above was most likely related to the intermediate degradation of NSP by bacteria, breaking the complex CHO into simpler substances that were more easily degradable by other microbes. Biofloc is a metabolic network in which cross-feeding occurs, with by-products of one organism serving as essential nutrient substrates for others. Some bacteria act as generalists, capable of degrading a wide range of polysaccharides, while others are specialists that can only target a few types of carbon substances (Tepaamorndech et al., 2020). Gradual degradation of NSP done by various groups of microbiota resulted in similar overall biodegradability indices and microbial activity levels in both diets (Vinasyiam et al. under review). It emphasized that the biofloc could utilize NSP (Chakrapani et al., 2021; Ekasari et al., 2014), confirming that microbes can use various types of carbon, from simple to complex sugars (Thomsen,

2005). However, we did not measure the NSP content of the faeces nor the microbial gene expression via transcriptomic analysis, which should be investigated in follow-up studies.

The overall microbial functionalities in maintaining potential toxic nitrogen substances were improved by using the WBdiet compared to the CONdiet, as the dissolved total organic nitrogen (TON), nitrate+nitrite, and total nitrogen (TN) were lower in WBdiet tanks at the end of the culture period (Vinasiam et al. under review). This suggested that the NSP-containing wheat bran, was effectively controlling inorganic toxic N-species in the biofloc system. Previous studies demonstrated, that NSP-containing carbon sources, when given directly to the system after feeding, absorbed ammonia up to 62-67% (Chakrapani et al., 2021), were equally effective as molasses (El-Husseiny, 2018), and maintained shrimp performance (Serra et al., 2021). This provides a good starting point for discussions and conceptualizing further research on microbiota functionality in biofloc systems fed different types of CHO, as started by Tinh et al. (2021) for corn starch. In addition, functionality needs to be elucidated at the specific functional genus level to understand the relationship between microbial community and environmental parameters such as water quality (Zheng et al., 2018).

5. CONCLUSION

Our study evaluated the effect of higher dietary wheat bran input on the microbial composition dynamics and functionalities of the biofloc for *L. vannamei* culture. Until today, most studies focused on prokaryotic and only few looked into the eukaryotic and fungal microorganisms in biofloc systems, thus our study provide novel insights on the taxonomic composition and dynamics of these communities. Overall, we showed that the dynamics of all microbial communities in response to wheat bran addition differed and fluctuated significantly over time. Wheat bran addition markedly affected prokaryotic communities to become more distinct from the control group, while it also modulated their potential functionalities to shift towards carbohydrate metabolism (pentose, fructose, mannose, and galactose). Eukaryotic community composition was less affected from the wheat bran addition compared to prokaryotic communities, but was mainly shaped by culture period. The dynamics of both prokaryotic and eukaryotic microbial community, including fungi, are affected by the dietary wheat bran and the culture time.

6. ACKNOWLEDGMENTS

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Supplementary Table S3.1. Diet formulation used in this experiment

Parameters	Diet	
	CONdiet	WBdiet
Ingredients (%)		
Fishmeal	21	12
Soya bean meal	12	7
Wheat	20	12
Wheat flour	30	17
Wheat bran	0	40
Wheat gluten	10	6
Soya lecithin	2.0	2.0
Salmon oil	1.0	0.6
Monocalcium phosphate	1.2	1.2
Chalk (CaCO ₃)	0.5	0.5
Cholesterol	0.3	0.3
Premix	1	1
L-Lysine HCl	0.2	0.12
DL-Methionine	0.3	0.17
L-Threonine	0.2	0.12
Yttrium oxide	0.02	0.02
Nutrient composition on dry matter basis (g/kg)		
Dry matter (DM, g/kg diet)	900	906
Ash	72	74
Crude protein	384	296
Fat	60	67
Energy (kJ/g DM)	20.0	19.9
Carbohydrates*	484	562
Phosphorus	11.27	13.28
Calcium	14.59	10.87
Magnesium	1.9	3.4
Yttrium	0.16	0.17
Carbon	433	434
Nitrogen	56	44
Dietary C:N ratio** (mass, g/g)	7.79	9.81
Faecal C:N ratio** (mass, g/g)	12	20

Values are the mean of each diet (CONdiet = control diet, WBdiet = wheat bran diet. C:N ratio = carbon to nitrogen ratio. *Calculated by subtracting ash, crude protein and fat from dry matter. **Measured using DUMAS analysis method.

Supplementary Table S3.2. Linear mixed-effects model by restricted maximum likelihood (REML) for time and dietary treatment on richness and diversity of prokaryotes, eukaryotes, and fungi

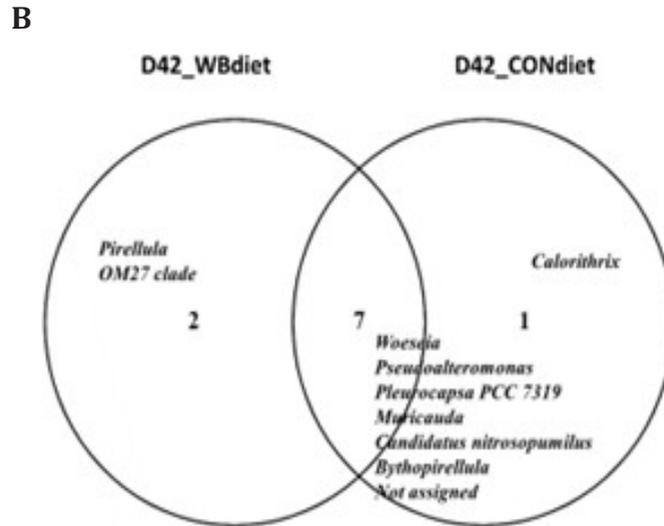
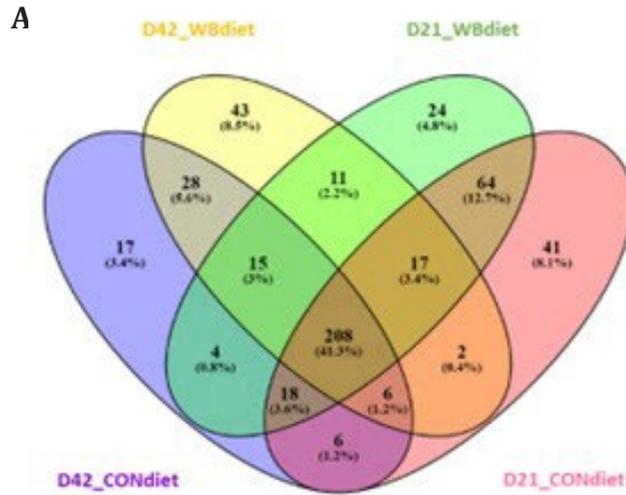
	d.f.	F	P-value	Significant contrast
<i>Prokaryotic</i>				
<u><i>Richness</i></u>				
Diet	32	0.813	0.374	
Time	32	185.981	<0.001***	Decreasing with time
Diet x time	32	0.626	0.435	
<u><i>Diversity</i></u>				
Diet	32	0.07	0.796	
Time	32	5.92	<0.021*	Increasing with time
Diet x time	32	0.30	0.586	
<i>Eukaryotic</i>				
<u><i>Richness</i></u>				
Diet	28	0.8817	0.356	
Time	28	41.6911	<0.001***	Decreasing with time
Diet x time	28	0.8522	0.363	
<u><i>Diversity</i></u>				
Diet	28	0.5444	0.467	
Time	28	25.8588	<0.001***	Decreasing with time
Diet x time	28	0.3538	0.554	
<i>Fungi</i>				
<u><i>Richness</i></u>				
Diet	32	0.0683	0.795	
Time	32	57.6536	<0.001***	Decreasing with time
Diet x time	32	0.2092	0.621	
<u><i>Diversity</i></u>				
Diet	32	0.0195	0.890	
Time	32	10.1437	0.003**	Decreasing with time
Diet x time	32	2.6607	0.113	

d.f. degrees of freedom, SS sum of Squares, MS mean Squares. *Statistical significance at $p < 0.05$; ** statistical significance at $P < 0.01$; *** statistical significance at $P < 0.001$.

Supplementary Table S3.3. Distance Based Linear Model (DistLM) - Sequential tests

Sequential tests							
<i>Prokaryotic community</i>							
Variable	AIC	SS(trace)	Pseudo-F	P	Prop.	Cumul.	res.df
TN	67.52	1848	7.74	0.002	0.436	0.436	10
ChlWat	65.93	617	3.14	0.003	0.146	0.582	9
AIC	R ²	RSS	No. Variables				
65.93	0.582	1771	2				
<i>Eukaryotic community</i>							
Variable	AIC	SS(trace)	Pseudo-F	P	Prop.	Cumul.	res.df
MicAct	67.55	861	3.60	0.001	0.265	0.265	10
TN	66.80	489	2.31	0.016	0.150	0.415	9
ChlWat	66.41	344	1.77	0.078	0.106	0.521	8
CP	65.64	322	1.82	0.099	0.099	0.619	7
ChlBiof	65.38	212	1.24	0.318	0.065	0.685	6
C:N ratio	64.48	221	1.37	0.244	0.068	0.753	5
BOD	64.41	128	0.754	0.587	0.039	0.792	4
TC	63.60	141	0.789	0.525	0.043	0.835	3
AIC	R ²	RSS	No. Variables				
63.60	0.835	536	8				
<i>Fungal community</i>							
Variable	AIC	SS(trace)	Pseudo-F	P	Prop.	Cumul.	res.df
TN	89.973	4591.300	2.960	0.003	0.228	0.228	10
ChlWat	89.619	2763.500	1.951	0.021	0.137	0.366	9
BOD	89.391	2160.400	1.632	0.093	0.107	0.473	8
C:N ratio	89.320	1677.800	1.318	0.235	0.083	0.557	7
AIC	R ²	RSS	No. Variables				
89.32	0.557	8910	4				

Relative position of biofloc samples in the biplot is based on Bray Curtis similarity of square root transformed relative abundance at the ASV level. Vectors indicate the weight and direction of the environmental parameters that were best predictors of microbial compositions as suggested by the results of the distance-based linear model (distLM). The dbRDA axes describe the percentage of the fitted or total variation explained by each axis while being constrained to account for group differences. TN: the dissolved concentration of total nitrogen, ChlWat: chlorophyl-a concentration in filtered water, MicAct: microbial activity, CP: crude protein content of biofloc, C:N ratio: carbon to nitrogen ratio of biofloc, BOD: biological oxygen demand, TC: the dissolved concentration of total carbon.



Supplementary Figure S3.1. Shared and unique core genera of prokaryotes, eucaryotes and fungi in tanks fed the CONdiet and WBdiet at D21 and D42 (A) and venn diagram showing the shared ASV (prevalence = 100% in each treatment at D42) (B). CONdiet = control diet, WBdiet = wheat bran diet, D21 = day-21, D42 = day-42. Core genera have a prevalence of >95% and a relative abundance >1%.

Supplementary Table S3.4. Prokaryotic functionalities

	Function	KEGG category	Membrane transport	ABC transporters	Description
WBdiet	K11073	Environmental Information Processing			potF; putrescine transport system substrate-binding protein
WBdiet	K01818	Metabolism	Carbohydrate metabolism	Pentose and glucuronate interconversions	fucl; L-fucose/D-arabinose isomerase [EC:5.3.1.25 5.3.1.3]
WBdiet	K01729	Metabolism	Carbohydrate metabolism	Fructose and mannose metabolism	algL; poly(beta-D-mannuronate) lyase [EC:4.2.2.3]
WBdiet	K01219	Metabolism	Unclassified	Metabolism- Unclassified	E3.2.1.81; beta-agarase [EC:3.2.1.81]
WBdiet	K02082	Metabolism	Carbohydrate metabolism	Galactose metabolism	agaS; tagatose-6-phosphate ketose/aldose isomerase [EC:5.-.-.]
WBdiet	K08302	Metabolism	Carbohydrate metabolism	Galactose metabolism	gatY-kbaY; tagatose 1,6-diphosphate aldolase GatY/KbaY [EC:4.1.2.40]
WBdiet	K02081	Genetic information processing	-Protein families	Transcription factors	agaR; DeoR family transcriptional regulator, aga operon transcriptional repressor
WBdiet	K05020	Signaling and cellular processes	Signaling and cellular processes-Protein families	Transporters	opuD, betL; glycine betaine transporter
CONDiet	K08289	Metabolism	Nucleotide metabolism	Purine metabolism	purT; phosphoribosylglycinamide formyltransferase 2 [EC:2.1.2.2]

Supplementary Table S3.4. Prokaryotic functionalities (continue)

	Function	KEGG category		Description
CONdiet	K05281	Metabolism	Biosynthesis of other secondary metabolites	E1.3.1.45; 2'-hydroxyisoflavone reductase [EC:1.3.1.45]
CONdiet	K03309	Signaling and cellular processes	Unclassified	TC.DAACS; dicarboxylate/amino acid:cation (Na+ or H+) symporter, DAACS family
CONdiet	K00527	Metabolism	Nucleotide metabolism	rtpR; ribonucleoside-triphosphate reductase (thioredoxin) [EC:1.17.4.2]
CONdiet	K03699	Signaling and cellular processes	Protein families	tlyC; putative hemolysin
CONdiet	K07178	Genetic Information Processing	Translation	RIOK1; RIO kinase 1 [EC:2.7.11.1]
CONdiet	K01281	Metabolism	Protein families	pepX; X-Pro dipeptidyl-peptidase [EC:3.4.14.11]
CONdiet	K03802	Metabolism	Enzymes	cphA; cyanophycin synthetase [EC:6.3.2.29 6.3.2.30]
CONdiet	K02635	Metabolism	Energy metabolism	petB; cytochrome b6
CONdiet	K07086	Uncharacterized	NA	K07086; uncharacterized protein
CONdiet	K01611	Metabolism	Amino acid metabolism	speD, AMD1; S-adenosylmethionine decarboxylase [EC:4.1.1.50]

Supplementary Table S3.4. Prokaryotic functionalities (continue)

Function	KEGG category	Description
CONdiet	Metabolism	Pantothenate and CoA biosynthesis LYS5, acpT; 4'-phosphopantetheinyl transferase [EC:2.7.8.-] cofH; FO synthase subunit 2 [EC:2.5.1.77]
CONdiet	Metabolism	Methane metabolism
CONdiet	Metabolism	Porphyrin metabolism
CONdiet	Metabolism	Glycolysis / Gluconeogenesis cbiT; cobalt-precorrin-6B (C15)-methyltransferase [EC:2.1.1.196] gap2; glyceraldehyde-3-phosphate dehydrogenase (NAD(P)) [EC:1.2.1.59]
CONdiet	Metabolism	Porphyrin metabolism
CONdiet	Genetic information processing	DNA repair and recombination proteins cbiD; cobalt-precorrin-5B (C1)-methyltransferase [EC:2.1.1.195] sbcC, rad50; DNA repair protein SbcC/Rad50
CONdiet	Metabolism	Alanine, aspartate and glutamate metabolism aspA; aspartate ammonia-lyase [EC:4.3.1.1]
CONdiet	Uncharacterized	Uncharacterized-protein K09763; uncharacterized protein nuc; micrococcal nuclease [EC:3.1.31.1]
CONdiet	Metabolism	Metabolism-Unclassified
CONdiet	Metabolism	Fructose and mannose metabolism GMPP; mannose-1-phosphate guanylyltransferase [EC:2.7.13]

CONdiet = control diet, WBDiet = wheat bran diet.



4

Non-starch polysaccharides addition method in Pacific white shrimp (*Litopenaeus vannamei*) - biofloc system

Apriana Vinasyiam

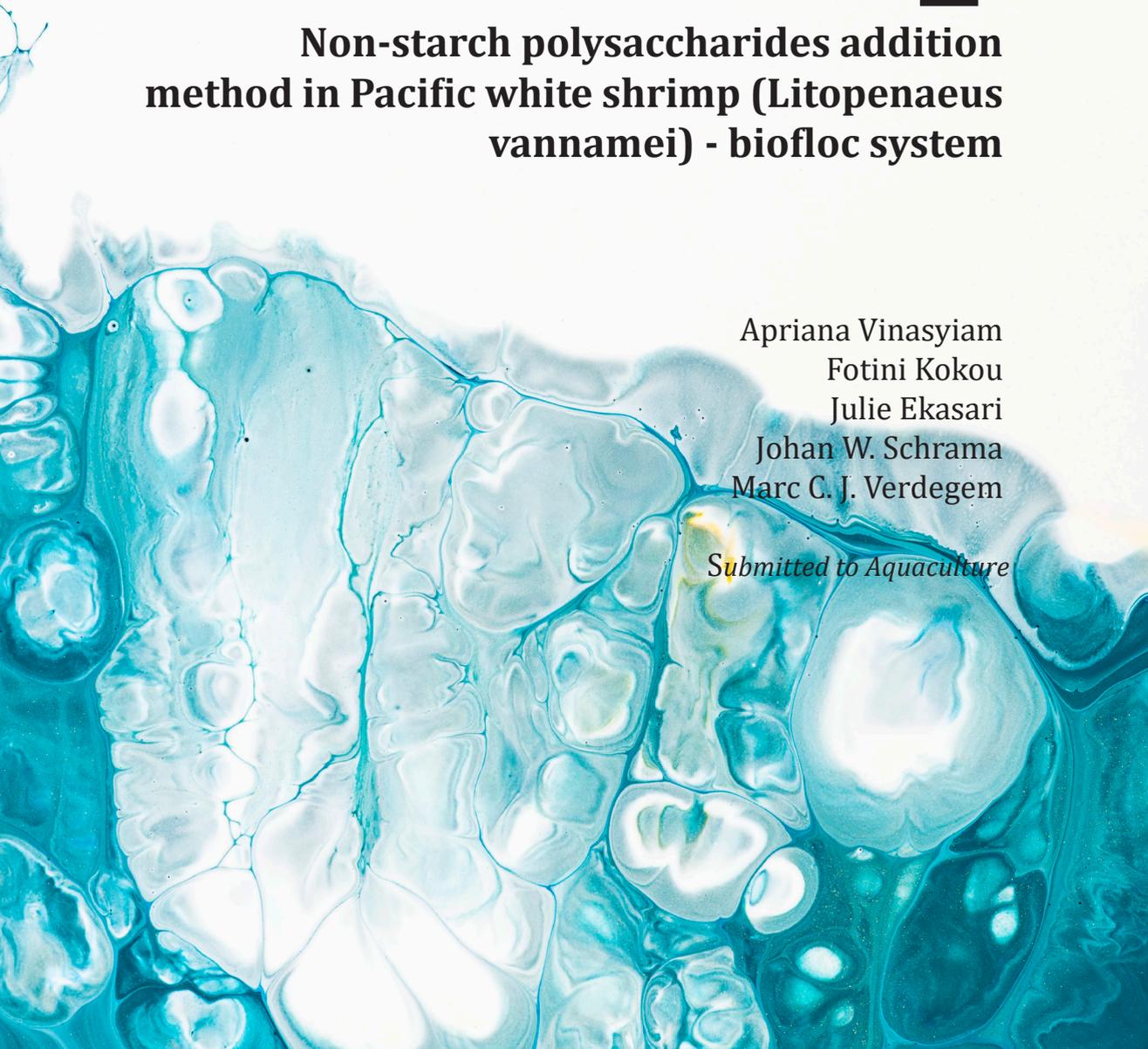
Fotini Kokou

Julie Ekasari

Johan W. Schrama

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Submitted to Aquaculture



Abstract

Biofloc system management can be done by adding complex carbon source, such as non-starch polysaccharides (NSP) directly to the water after feeding shrimps. Alternatively, NSP can also be incorporated through the feed, which may affect nutrient availability in the system and biofloc formation, by altering shrimp nutrient digestibility and faeces composition. This study evaluated the effect of two methods of addition of an NSP-rich carbon source, wheat bran, to a Pacific white shrimp (*Litopenaeus vannamei*) biofloc system; supplementation via feed (wheat bran diet; WBdiet) and direct addition to the water (CONDiet+WB). The amount and composition of the total nutrient input to each mesocosm of the treatments was the same. A digestibility and a growth experiment were carried out separately for 35 and 42 days, respectively. The nutrient digestibility was overall lower in shrimp fed the WBdiet compared to the CONDiet, but without an effect on shrimp final weight, production, and survival. When comparing the methods of addition, neither of the approaches altered the shrimp nutritional quality. As expected, a difference in faecal C:N ratio was observed; 21 vs. 17 for WBdiet and CONDiet, respectively. The difference in faeces composition did not affect biofloc quantity, quality, and chlorophyll-a concentrations, and resulted in comparable water quality and microbial activity. Our study concluded that both NSP-addition methods, either via feed or via the water, were equally effective. When compared to the traditional addition of carbohydrate to the water, supplementing NSP-containing carbon sources via the feed can be a more efficient approach in terms of labour and feeding management practices.

Keywords: carbon supplementation, *Litopenaeus vannamei*, digestibility, growth performance, microbial activity

1. Introduction

Zero water exchange biofloc technology (BFT) systems have been employed the past 35 years to maintain water quality, improve nutrient use efficiency and promote shrimp growth (Avnimelech, 2015; Kumar et al., 2018; Panigrahi et al., 2019; Tinh et al., 2021c). This cultivation method relies mainly on heterotrophic bacteria to immobilize toxic inorganic nitrogen species into bacterial biomass, using organic carbon as energy source. These microorganisms produce extracellular polysaccharides (EPS), aggregating different microbial groups (bacteria, archaea, algae, protists, zooplankton, fungi) and particles into biofloc (Wilén et al., 2008; More et al., 2014). The carbon to nitrogen (C:N) ratio of a 38 – 42% protein shrimp diet is smaller than 10, while an optimal growth of heterotrophic bacteria requires a C:N ratio of 10-20 (Avnimelech, 2012). Therefore, an external carbon source is usually added to the water in the biofloc system to increase the C:N ratio of the feed to avoid carbon deficiency.

Under conventional biofloc management, extra carbohydrates such as molasses or starch are added directly into the water to stimulate biofloc formation and maintain water quality (Miao et al., 2017; Xu et al., 2018; Panigrahi et al., 2019; Khanjani, 2021). Administrating these carbohydrates, however, requires extra labour and skills from the farmer to provide the right amount of carbon to maintain the water quality in line with the feed input. A more simple method can be to feed one single pellet that combines both the diet and the carbon source (Tinh et al., 2021c). If an easily digestible carbon source is incorporated in the pelleted feed, most will be digested by the culture species. When using a source like wheat bran that contains a high fraction of indigestible non-starch polysaccharides (NSP), a major fraction of the NSP will be excreted with the faeces and become available to heterotrophic bacteria in the water column, stimulating biofloc development (López-Elías et al., 2014; Braga et al., 2016). Moreover, administrating easily digestible carbohydrate to the water will rapidly induce excessive biofloc production (Serra et al., 2015; El-Husseiny, 2018). By providing carbohydrates rich in NSP through the pelleted feed, the carbon input may become better synchronized with the total organic matter flow through the biofloc system, as it requires more time for decomposition into simple sugars (Avnimelech, 2012; Ekasari et al., 2014b; Serra et al., 2015). Therefore, comparing the input of NSP-rich carbohydrates via the feed or via the water is worth investigating to optimize BFT management practices.

In this experiment, addition of wheat bran to a Pacific white shrimp biofloc rearing system, either via the feed or via the water column, was compared. First, a digestibility trial was conducted to assess the feed digestibility for the shrimp and the composition of the faeces. Subsequently, in a growth experiment, shrimp performance (growth,

production, survival, body composition) and biofloc system performance (biofloc quantity and quality, water quality, carbon C, nitrogen N and phosphorus P mas balances) of the two wheat bran administration methods (treatments) were compared. The nutrient input per mesocosm was the same in both treatments.

2. Material and Methods

2.1. Experimental feed and general design

The experiment involved two treatments differing in the supply of wheat bran (WB) to the ponds. In the first treatment, all wheat bran was included in the diet and thus all WB was assumed to pass through the shrimp ('WBdiet' treatment). In the second treatment, 30% of the WB was taken out of 'WBdiet', which resulted in the CON diet. This 30% WB was added directly to the water of the biofloc system. Therefore, the two experimental diets had an identical composition except for the inclusion level of wheat bran (WB) (**Table 4.1**). The CONdiet was aimed to be representative for a commercial shrimp diet regarding ingredients used and nutrient content. Yttrium oxide (Y_2O_3) was used as a marker for nutrient digestibility. The diet formulation and nutritional compositions of both diets are shown in **Table 4.1**. Both diets were produced by steam pelleting at a die of 2mm. This was done by Research Diet Service (Wijk bij Duurstede, The Netherlands). The diets were stored at 4 °C before and during the experiment.

Two experiments were conducted at the animal research facility Carus of Wageningen UR, including a digestibility study and a shrimp and biofloc system performance assessment. The digestibility study compared the apparent digestibility coefficient and faeces composition when feeding equal rations of the WBdiet and CONdiet to Pacific white shrimp (*Litopenaeus vannamei*). In the shrimp and biofloc system performance experiment the treatments were WBdiet and CONdiet+WB, as explained above.

2.2. Digestibility experiment

Fourteen aquaria, part of one recirculating aquaculture system (RAS) containing a sedimentation unit for particle removal, a trickling filter, a degassing unit, a sump, and a tube with UV light for water sterilization. Each aquarium was equipped with an aeration stone and an aquarium pump to create continuous water movement. The inflow of water was sourced from the sump. Thirty shrimp (6.0 ± 2.0 g/ind) were stocked per aquarium and reared for five weeks. Shrimp were fed overnight for 8-10 hours using a mechanical belt feeder. The amount of feed fed daily was calculated using the equations 1* and 2* (Tinh et al., 2021c). Faeces was collected in the morning, five days a week. There was no water exchange during faeces collection. Prior to faeces collection at 07.00 in the morning, shrimp were fed the assigned diet at 1-2 g/aquarium. One hour

Table 4.1. Diet formulation and analysed nutrient composition

	CONDiet	Wheat bran	WBdiet
<i>Ingredients</i>			
Fishmeal %	17.36	-	12.15
Soyabean meal %	9.93	-	6.95
Wheat %	16.77	-	11.74
Wheat flower %	24.8	-	17.36
Wheat bran %	14.29	100	40.00
Wheat gluten %	8.27	-	5.79
Soya lecithin %	2.86	-	2.00
Salmon oil %	0.83	-	0.58
Monocalcium phosphate %	1.71	-	1.20
Limestone (CaCO ₃) %	0.71	-	0.50
Cholesterol %	0.43	-	0.30
Premix %	1.43	-	1.00
L-Lysine HCl %	0.17	-	0.12
DL-Methionine %	0.24	-	0.17
L-Threonine %	0.17	-	0.12
Yttrium oxide %	0.03		0.02
Total %	100	100	100
<i>Nutritional composition</i>			
Dry matter g/kg	927	891	919
Ash g/kg dm	71	54	68
Crude protein g/kg dm	348	194	303
Fat g/kg dm	65	52	61
Carbohydrate* g/kg dm	515	700	568
Energy kJ/g dm	19.8	19.5	19.5
Phosphorus g/kg dm	13.0	13.3	13.4
Calcium g/kg dm	13.4	1.1	10.4
Magnesium g/kg dm	2.8	4.7	3.4
Yttrium g/kg dm	0.17	0.00	0.24
C:N ratio (mass) g/g	8.2	14.5	9.4

C:N ratio = carbon to nitrogen ratio. *Calculated by subtracting ash, crude protein, and fat from dry matter.

later, any carapaces, faeces, and debris were siphoned out. Shrimp were given an additional hour to excrete new faeces. Afterwards, faeces were siphoned from the aquarium bottom, and were collected in 500-mL glass bottles placed on ice. After rinsing with demi-water to remove salt, faeces from the same diet were pooled into an aluminium container and stored at -20 °C. To remove water, faeces were dried at 70°C for 72 hours, then kept in an open container at room temperature for another six hours, and later used as a faeces sample for the proximate analysis.

(1 *) *Feeding rate (% Individual Body Weight (IBW))*

$$= 0.0861 * (IBW (g \text{ wet weight})^{-0.428}) * 100$$

(2 *) $FCR = 0.001 * IBW (g \text{ wet weight})^2 + 0.0681 * IBW (g \text{ wet weight}) + 0.5386$

2.3. Growth experiment

2.3.1. Biofloc inoculant production

Three weeks prior to the start of the experiment, post-larvae (PL) shrimp from CreveTec, Belgium, were stocked in three 1.000-L tanks, containing 750 L each, at a density of approximately 500 shrimp per tank. Shrimp were fed a commercial shrimp starter diet (CP 41%) twice daily to satiation. Corn starch was added to the water following feeding to raise the C:N ratio (g/g) of the total nutrient input to 20:1 to promote biofloc formation. The rearing tanks had a 12h dark/light cycle using artificial lighting (LEP, Gavita Pro 270e; HI, Gavita Hortistar 600 SE EU). Continuous aeration was provided from one aeration stone hanging in the centre of the tank and from a circular aeration pipe close to the bottom, maintaining the dissolved oxygen level above 6 mg/L. Temperature was maintained at 24±1 °C using an aquarium heater Schego® (600 W) placed in each tank, while salinity was kept at 23±2 ppt during the inoculant production period.

2.3.2. Mesocosm set-up and water quality monitoring

Six identical mesocosm tanks were used (3 per treatment). At the start of the experiment, shrimp from the biofloc inoculant production tanks were harvested for the growth experiment. Meanwhile, the pre-matured biofloc water was pooled in one large tank with continuous aeration and water circulation to ensure homogenous mixing, as well as to keep the biofloc in suspension. The biofloc water was evenly divided among six clean 1.000-L mesocosm tanks, resulting in a volume of ca. 350 L per tank. Subsequently, 400 L brackish water was added to each tank, bringing the final water volume to 750 L per tank. Shrimp with a body weight of 0.27±0.05 g was stocked at a density of 150 individuals per tank. During the experiment, the water was continuously aerated and exposed to a 12h dark/light cycle.

Water temperature, salinity, pH, and oxygen were monitored daily at 08:00 am using electronic probes. The dissolved oxygen (DO) concentration was maintained above 6 mg/L, salinity at 25 ± 1 ppt and the pH above 7.5. At the start of the experiment, the temperature was increased approximately 1 °C daily, from 24 ± 1 °C to 27 ± 1 °C. During the experiment, 12 L of fresh water was added weekly to each tank to compensate evaporation loss. The concentrations of dissolved ammonia, nitrite and nitrate were checked daily using Merck MQuant® kits.

2.3.3. Feeding management

Shrimp were fed each day for 8-10 hours using mechanical belt feeders. The amount the CONdiet fed daily was calculated using formula 1* (Tinh et al., 2021c). The daily shrimp weight gain was calculated by dividing the feed input by the FCR using formula 2* (Tinh et al., 2021c). The daily feeding rate of the CONdiet declined from 15.1 % BW on day 1 (D1) to 3.9% BW on D42. In CONdiet+WB treatment tanks, wheat bran was added to the water from a mechanical belt feeder approximately one hour after feeding. Each day, the mass of WBdiet fed was equal to the mass of CONdiet+WB fed.

2.3.4. Sample collection during grow-out experiment

Shrimp was collected for proximate composition analyses at the start (D1) and end of the growth experiment (D42). Two hundred shrimp were collected as initial sample, while on D42 all the shrimp in each tank were harvested, counted, and weighed. The samples were subsequently freeze-dried 72 hours and reweighed. Throughout the experiment, 20 g portions from the WBdiet, the CONdiet, and the wheat bran were collected each week. Each collected portion was then placed in a designated container assigned to its respective feed type. These containers were consistently maintained at a temperature of 4 °C. At the end of the experiment, the proximate composition of both the diets and wheat bran was determined.

Biofloc was sampled on D1, D21 and D42. Before sampling, 1.5 µm pore size fiber glass filters were dried at 70 °C for minimum 3 hours. Dried filters were stored in a desiccator for 1 hour and then weighed to determine the empty filter weight. To collect biofloc sample, 10 L of biofloc water was siphoned to a plastic bucket from the centre of each biofloc tank. Collected biofloc water was mixed thoroughly using an electric disperser IKA Ultra-Turrax® at 300 rpm for 30 seconds and distributed into four 1.5-L plastic jars of which two were stored for back-up. The other jars were immediately processed. While processing, the water in each jar was continuously mixed at 250 rpm using a magnetic stirrer. Batches of 100 ml biofloc water were filtered through 1.5 µm pore size fiber glass filters using a vacuum pump to collect biofloc. Subsequently, 100 ml

demi water was passed twice through the fibre glass filters to remove salt and to collect biofloc particles stuck on the inner wall of the filtration chamber. After filtration, each filter with biofloc was folded and stored in a clean plastic tube at -20 °C until further analysis.

Prior to proximate analysis, biofloc-filled filters with biofloc were dried at 70 °C for minimum 72 hours, transferred to a desiccator for 1 hour and weighed. The biofloc mass was obtained by deducting the weight of the full and empty filter. Unfiltered biofloc samples were used to measure chlorophyll-a and microbial activity. Fifty ml of biofloc water from the center of each tank was collected by siphoning and stored in a sterile plastic tube. For chlorophyll-a measurement, samples were stored at -20 °C until analysis. Samples to measure biofloc microbial activity were analyzed immediately after collection.

The filtrate water from the biofloc filtration was used as water sample. Fifty ml of filtrate water of each tank was acidified to a pH of 2-3 by adding 2 N HCl. Subsequently, 10 ml of acidified water was filtered using a 0.45 µm syringe filter and stored in a clean plastic tube at room temperature. Samples were analysed within 24 hours after collection.

2.4. Sample analysis

Proximate analysis was conducted on shrimp, faeces, diet and biofloc samples to determine their nutrient composition, including dry matter (DM), ash, minerals, crude protein, fat, energy, carbon, and nitrogen content. To measure the DM content, each sample was oven-dried at 103°C for at least 4 hours until a constant weight (ISO-6496, 1999). After DM determination, the samples were incinerated at 550°C for minimum 4 hours until constant weight (ISO-5984, 2002). The ash samples were analysed for phosphorous (P), calcium (Ca), magnesium (Mg), copper (Cu) and yttrium (Y) using plasma-mass spectrophotometry (ICP OES), following the NEN-15510 (2017) procedure. Crude protein analysis was determined using the Kjeldahl method according to (ISO-5983, 2005), while the energy content was determined by bomb calorimetry through direct combustion (IKA® werke C7000; IKA Analysentechnik, Weikershem, Germany) (ISO-9831, 1998). The fat analysis was performed according to the Soxhlet method (ISO-6492, 1999). The carbon and nitrogen content were determined using a DUMAS analyzer (Leco CN 628, Leco Instrumente GmbH, Germany) (Tinh et al., 2021c).

The total suspended solids (TSS) represents the mass of biofloc particles (DM) per liter of rearing water, while volatile suspended solids (VSS) specifically refers to the organic portion (ash-free DM) of the VSS (APHA, 1995). Chlorophyll-a was determined following standard methods for examination of water and wastewater (APHA, 1995). The microbial activity was determined by performing H₂O₂ degradation assays according to

Pedersen et al. (2019). Dissolved nutrients were analysed using a segmented analyzer (SAN ++, Skalar Analytical B.V.) measuring concentrations of total carbon (TC), total inorganic carbon (TIC), total organic carbon (TOC), total nitrogen (TN), total inorganic nitrogen (TIN), total organic nitrogen (TON), total ammonia nitrogen (TAN), nitrate-nitrite nitrogen ($\text{NO}_x\text{-N}$) and orthophosphate ($\text{PO}_4\text{-P}$).

2.5. Data calculation and analysis

Table 4.2 presents the formulas used to calculate the apparent digestibility coefficient (ADC), shrimp growth and feed utilization parameters, and nutrient balances. Statistical analysis was performed with IBM SPSS Statistics 26 software (IBM Corporation, NY, USA). The data homogeneity was assessed using Levene's test, while normality distribution was assessed using Shapiro-Wilk's test. Statistical analysis comparing faeces compositions, ADC, shrimp growth, and body composition between treatments was conducted using one-way ANOVA. Meanwhile, biofloc parameters and water quality were analysed using repeated measure ANOVA with treatment as main factor and sampling day as repeated factor. A significance level (α -value) of 0.05 was employed to determine significant differences. Post-hoc analysis was performed using Tuckey HSD. The correlation between microbial activity and VSS as well as between microbial activity and chlorophyll-a concentration was determined.

3. Results

3.1. Shrimp performance and digestibility

Diet treatment effects on shrimp faecal composition and ADC are presented in **Table 4.3** and **Table 4.4**, while in **Table 4.5** and **Table 4.6** the effects on shrimp growth performance and body composition are shown. The shrimp faecal composition was affected by treatment ($P < 0.05$). Shrimp consuming the WBdiet had 14% less protein and 5% more carbohydrate in the faeces than shrimp eating the CONDiet. This resulted in a faecal C:N ratio of 21 and 17 in shrimp consuming the WBdiet and CONDiet, respectively (**Table 4.3**). Consumption of the WBdiet reduced the ADC of crude protein by 4%, fat by 10% and carbohydrate by 14% (**Table 4.4**). No differences in shrimp individual growth and total production parameters were observed between dietary treatments ($P < 0.05$, **Table 4.5**), except for FCR and EUE: 1.24 vs. 0.89 and 21 vs. 27 for the WBdiet and CONDiet+WB treatments, respectively ($P > 0.05$). To be noted, FCR and EUE only considered the amounts of WBdiet and CONDiet fed, not the amount of WB fed separately. When summing the amounts of CONDiet and WB fed into CONDiet+WB treatment tanks, the resulting $\text{FCR}_{\text{system}}$ and $\text{EUE}_{\text{system}}$ were similar between treatments ($P > 0.05$). The PER showed a trend ($P = 0.051$) for being higher in CONDiet+WB fed treatment tanks, while

Table 4.2. Formula for data calculation

Parameters	Formula
ADC (%)	$1 - \left\{ \frac{\text{nut.conc}_{\text{faeces (g/kg)}}}{\text{nut.conc}_{\text{diet (g/kg)}}} \times \left(\frac{\text{Y.conc}_{\text{diet (g/kg)}}}{\text{Y.conc}_{\text{faeces (g/kg)}}} \right) \right\} \times 100$
SGR (% BW/day)	$(\ln W_f - \ln W_i) / t * 100$
Production (kg/m ³)	$(\text{BM}_{f(g)} - \text{BM}_{i(g)}) / (\text{water volume}_{(m^3)} \times 1,000)$
Survival (%)	Final number of shrimps / initial number of shrimps * 100
FCR	total diet input _(g) / (BM _{f(g)} - BM _{i(g)})
FCR _{system}	(total diet input _(g) + total wheat bran input _(g)) / (BM _{f(g)} - BM _{i(g)})
PER	(BM _{f(g)} - BM _{i(g)}) / dietary protein input _(g) × 1000
PER _{system}	(BM _{f(g)} - BM _{i(g)}) / total protein input (diet + wheat bran) _(g)
PUE (%)	(retained protein _(g) / dietary protein input _(g)) × 100
PUE _{system} (%)	(retained protein _(g) / total protein input (diet + wheat bran) _(g)) × 100
EUE (%)	(retained energy _(kj) / dietary energy input _(kj)) × 100
EUE _{system} (%)	(retained energy _(kj) / total energy input (diet + wheat bran) _(kj)) × 100
Total nutrient input _(g)	$\text{nut}_{\text{diet (g)}} + (\text{nut}_{\text{shrimp}_i (g)} + \text{nut}_{\text{biofloc}_i (g)} + \text{nut}_{\text{water}_i (g)})$
Nutrient loss _(g)	$\text{total nutrient input}_{(g)} - (\text{nut}_{\text{shrimp}_f (g)} + \text{nut}_{\text{biofloc}_f (g)} + \text{nut}_{\text{water}_f (g)})$

ADC = apparent digestibility coefficient, GR = growth rate, SGR = specific growth rate, FCR = feed conversion ratio, PER = protein efficiency ratio, PUE = protein utilization efficiency, EUE = energy utilization efficiency, Nut.conc_{faeces} and Nut.conc_{diet} represent the nutrient concentrations in faeces and diet, Y.conc_{diet} (g/kg) and Y.conc_{faeces} (g/kg) indicate the yttrium concentration in diet and faeces, W_i = initial body weight, W_f = final body weight, BM = biomass, BM_i = initial BM, BM_f = final BM, t = number of days, total diet input and total wheat bran input refer to total amount of diet and wheat bran given during experiment, water volume = total volume of water in the tank, nut_{diet} (g) is total nutrient in the diet, nut_{shrimp_i} (g), nut_{biofloc_i} (g), and nut_{water_i} (g) are the initial nutrient present in the shrimp, biofloc, and water, respectively, nut_{shrimp_f} (g), nut_{biofloc_f} (g), and nut_{water_f} (g) are the final amount of nutrient present in shrimp, biofloc, and water, respectively.

PER_{system} was similar between the dietary treatments (P<0.05). On average, survival was 87%, with a higher variability observed in WBdiet fed tanks (standard deviation - sd of 10) than in CONdiet+WB fed tanks (sd of 3). In both treatments, shrimp grew on average from 0.27 g to 5.5 g within 42 days, realizing an SGR of 7.15 % body weight day⁻¹ and a total harvested shrimp biomass of 710 g (P>0.05, **Table 4.5**). No differences were observed in body composition between the dietary treatments, except for magnesium content, which was 6% smaller in shrimp fed the WBdiet, compared to shrimp fed the CONdiet with separate WB addition to the water (P<0.05, **Table 4.6**).

Table 4.3. Effect of the wheat bran addition method on the proximate composition of faeces of Pacific white shrimp

Parameters	CONdiet		WBdiet		P-value
	Mean	sd	Mean	sd	
Crude protein g/kg AFDM	185 ±	2.02 a	159 ±	0.61 b	0.000
Fat g/kg AFDM	84 ±	0.96 a	75 ±	1.06 b	0.001
Energy kJ/g AFDM	23 ±	0.18	23 ±	0.14	0.052
Carbohydrates* g/kg AFDM	731 ±	2.79 b	766 ±	1.13 a	0.000
Phosphorus g/kg DM	29 ±	0.23 a	25 ±	0.46 b	0.000
Calcium g/kg DM	53 ±	0.51 a	36 ±	0.66	0.000
Magnesium g/kg DM	9 ±	0.07 b	10 ±	0.12 a	0.003
Yttrium g/kg DM	1 ±	0.01 a	0.3 ±	0.01 b	0.000
Carbon (C) g/kg AFDM	540 ±	7.29	551 ±	8.99	0.171
Nitrogen (N) g/kg AFDM	31 ±	0.46 a	26 ±	0.46 b	0.000
C:N ratio (mass) g/g	17 ±	0.48 b	21 ±	0.69 a	0.002

Values are the mean ± standard deviation (sd) of each diet (CONdiet = control diet and WBdiet = wheat bran diet). The data are obtained from the digestibility experiment. DM = dry matter, C:N ratio = carbon to nitrogen ratio, P-value = probability value. Per row, different letters in bold indicate significant difference (P<0.05). *Calculated by subtracting ash, crude protein, and fat from ash dry matter.

Table 4.4. Effect of the dietary wheat bran addition on the apparent digestibility coefficient (ADC) of nutrients in Pacific white shrimp

Parameters	CONdiet		WBdiet		P-value
	mean	sd	mean	sd	
Crude protein %	84 ±	0.60 a	81 ±	1.79 b	0.001
Fat %	62 ±	0.70 a	56 ±	1.11 b	0.005
Energy %	66 ±	0.58 a	58 ±	1.42 b	0.000
Carbohydrates %	50 ±	2.44 a	43 ±	2.72 b	0.001
Phosphorus %	5 ±	6.20	8 ±	3.80	0.188
Carbon (C) %	65 ±	0.27	57 ±	1.78	0.024
Nitrogen (N) %	83 ±	0.15	81 ±	0.81	0.056

Values are the mean and the standard deviation (sd) of each treatment (CONdiet+WB = control diet + addition of wheat bran to the water and WBdiet = wheat bran diet), P-value = probability value. Per row, different letters in bold show significant difference (P<0.05).

Table 4.5. Effect of the wheat bran addition method on the growth performance of Pacific white shrimp reared in a biofloc system

Parameters	CONdiet+WB		WBdiet		P-value
	Mean	sd	Mean	sd	
Initial weight g/ind	0.27		0.27		
Final weight g/ind	5.6 ± 0.3		5.4 ± 0.4		0.901
Final biomass g	719 ± 55		701 ± 57		0.722
Specific growth rate (SGR) %/day	7.2 ± 0.1		7.1 ± 0.2		0.886
Production kg/m ³	0.90 ± 0.07		0.88 ± 0.08		0.725
Survival %	88 ± 3		87 ± 10		0.867
Feed conversion ratio (FCR)	0.89 ± 0.07 b		1.24 ± 0.11 a		0.009
FCR _{system}	1.21 ± 0.10		1.24 ± 0.11		0.723
Protein efficiency ratio (PER)	3.5 ± 0.3		2.9 ± 0.3		0.051
PER _{system}	2.9 ± 0.2		2.9 ± 0.3		0.894
Protein utilization ratio (PUE) %	58 ± 5		49 ± 4		0.066
PUE _{system} %	49 ± 4		49 ± 4		0.919
Energy utilization ratio (EUE) %	27 ± 2 a		21 ± 2 b		0.016
EUE _{system} %	20 ± 2		21 ± 2		0.782

Values are the mean and the standard deviation (sd) of each treatment (CONdiet+WB = control diet + addition of wheat bran to the water and WBdiet = wheat bran diet), P-value = probability value. Different letters in bold show significant difference (P<0.05)

Table 4.6. Effect of the wheat bran addition method on the body composition of the Pacific white shrimp reared in a biofloc system

Parameters		D1		D42		P-value
		CONdiet+WB		WBdiet		
		Mean	sd	Mean	sd	
Dry matter (DM)	g/kg	226 ± 0.02	223 ± 4.99	227 ± 1.43	0.245	
Ash	g/kg DM	173 ± 1	148 ± 4	141 ± 4	0.088	
Crude protein	g/kg DM	710 ± 2	745 ± 20	744 ± 1	0.903	
Fat	g/kg DM	38 ± 1	39 ± 3	43 ± 5	0.285	
Energy	g/kg DM	18.4 ± 0.1	19.9 ± 0.1	20.3 ± 0.3	0.066	
Carbohydrates*	g/kg DM	79 ± 1	67 ± 19	72 ± 2	0.309	
Phosphorus	g/kg DM	14.0 ± 0.1	12.2 ± 0.1	11.9 ± 0.3	0.101	
Calcium	g/kg DM	42 ± 0.4	36 ± 2.5	33 ± 0.5	0.071	
Magnesium	g/kg DM	3.2 ± 0.05	3.3 ± 0.02 a	3.1 ± 0.10 b	0.021	
Carbon (C)	g/kg DM	427 ± 2	454 ± 4	457 ± 2	0.252	
Nitrogen (N)	g/kg DM	121 ± 1.2	121 ± 0.8	120 ± 0.4	0.102	
C:N ratio (mass)		3.5 ± 0.01	3.7 ± 0.05	3.8 ± 0.00	0.104	

Values are the mean and the standard deviation (sd) of each treatment (CONdiet+WB = control diet + addition of wheat bran to the water and WBdiet = wheat bran diet). C:N ratio = carbon to nitrogen ratio, P-value = probability value. Per row, different letters in bold show significant difference ($P < 0.05$). *Calculated by subtracting ash, crude protein, and fat from dry matter.

3.2. Biofloc

Biofloc composition is summarized in **Table 4.7** and microbial activity is shown in **Figure 4.1**. No differences were observed between the WB addition treatments in biofloc quantity (e.g., TSS and VSS) and quality, nor in biofloc chlorophyll-a content and microbial activity ($P < 0.05$). In addition, there was no treatment x time interaction effect observed for all biofloc parameters. However, the biofloc composition changed during the experiment ($P < 0.05$) for all parameters, except for the calcium content ($P > 0.05$). Overall, all parameter values on the nutritional content of biofloc showed an increasing trend. TSS, VSS, chlorophyll-a content and biofloc microbial activity increased faster between D1 and D21 than between D21 and D42 (**Table 4.7, Figure 4.1**). Correlation analysis showed that microbial activity was positively associated with the VSS concentration ($R^2 = 0.82 - 0.84$) and with total chlorophyll-a ($R^2 = 0.72 - 0.74$) (**Figure 4.2**).

Table 4.7. Effect of the wheat bran addition method on biofloc quantity, quality, and chlorophyll-a concentration during the experiment

Parameters	WB addition method (Treatment)				Time		P-value	
	CON+WB	WBdiet	D1	D21	D42	Treatment	Time	Treatment*time
Crude protein	697	703	532 b	814 a	753 a	0.863	0.000	0.609
Energy	28.3	27	15.1 b	36.0 a	31.5 a	0.479	0.001	0.538
Phosphorus	13.7	13.6	14.4 b	11.4 b	15.2 a	0.822	0.003	0.135
Calcium	50	51	50	49	52	0.957	0.700	0.381
Magnesium	11.8	12.4	4.7 b	17.2 a	14.4 a	0.520	0.000	0.567
Carbon (C)	553	541	349 a	641 b	650 b	0.448	0.000	0.193
Nitrogen (N)	93	92	62 b	108 a	107 a	0.692	0.000	0.307
C:N ratio (mass)	5.9	5.9	5.6 b	5.9 a	6.1 a	0.503	0.002	0.912
TSS	585	597	340 c	651 b	781 a	0.611	0.000	0.676
VSS	356	373	269 c	377 b	448 a	0.398	0.000	0.532
Chl-a water	183	193	104 c	206 b	254 a	0.594	0.000	0.943
Chl-a particulate	196	205	110 c	226 b	265 a	0.450	0.000	0.290
Chl-a total	378	398	214 c	432 b	519 a	0.487	0.000	0.628

Values are the mean of three sampling times of each treatment (CONdiet+WB = control diet + addition of wheat bran to the water and WBdiet = wheat bran diet) and the mean of two treatments of each sampling times (D1 = day-1, D21 = day-21, and D42 = day-42). DM = dry matter, WB = wheat bran, TSS = total suspended solid, VSS = volatile suspended solid, Chl-a = chlorophyll-a, P-value = probability value. Per row, for each factor (diet or time), different letters in bold show significant difference (P<0.05).

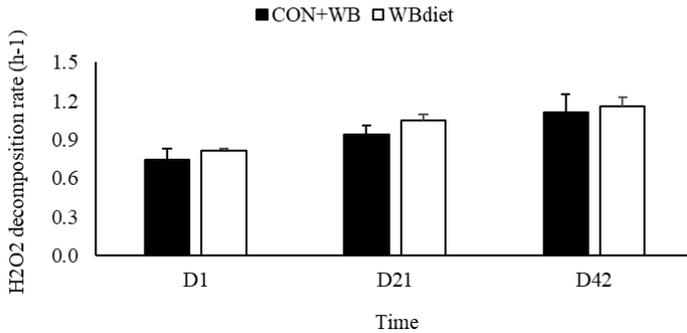


Figure 4.1. Effect of the wheat bran addition method on biofloc microbial activity during the experiment. Values are the mean and the standard deviation of each treatment (CONdiet+WB = control diet + direct addition of wheat bran and WBdiet = wheat bran diet) at each sampling times (D1 = day-1, D21 = day-21, and D42 = day-42). P-value of diet=0.662, time=0.002 and interaction=0.527 based on the result of one-way ANOVA in which diet at each time sampling was the factor.

3.3. Water quality

The effect of WB addition method on water quality is summarized in **Table 4.8**. No differences were observed between WB addition treatments in dissolved nutrient concentrations ($P>0.05$), but the water quality changed over time for all parameters ($P<0.05$). The $\text{NH}_4\text{-N}$ concentration was low overall and still decreased over time, meanwhile other dissolved nitrogen substances including $\text{NO}_x\text{-N}$ (which mostly consisted of $\text{NO}_3\text{-N}$), TIN and TON increased ($P<0.05$). The dissolved carbon substances (TIC, TOC, and TC) showed a declining trend during the culture period ($P<0.05$). The $\text{PO}_4\text{-P}$ concentration doubled during the experiment, increasing faster towards the end of the experiment ($P<0.05$, **Table 4.8**).

3.4. Nutrient balance

The nutrient mass balances (carbon, nitrogen and phosphorous) of the biofloc rearing systems on each sampling day (D1, D21 and D42) are shown in **Figure 4.3**. The same information, expressed as a percentage to the total nutrient input (feed + initial nutrient present in the system) is shown in **Figure 4.4**. No treatment effect was observed on the amount of carbon and nitrogen preserved in the system compartments (shrimp, biofloc and water) on each sampling day ($P>0.05$). Meanwhile for phosphorus, biofloc accumulated faster in the WBdiet treatment than in the CONdiet+WB treatment ($P<0.05$). At the end of culture period on D42 the main fraction of carbon, nitrogen and phosphorous present was in the biofloc ($P>0.05$). In addition, no differences in

unaccounted nutrients were observed on D42 ($P > 0.05$, **Figure 4.3** and **Figure 4.4**). On average 38%, -4% and 3% for carbon, nitrogen and phosphorous, respectively, was unaccounted at the end of the experiment. Without considering initial nutrient presence in the system, in both treatments, shrimp accumulated 20%, 49% and 18% of the carbon, nitrogen and phosphorous fed during the 42-day culture period.

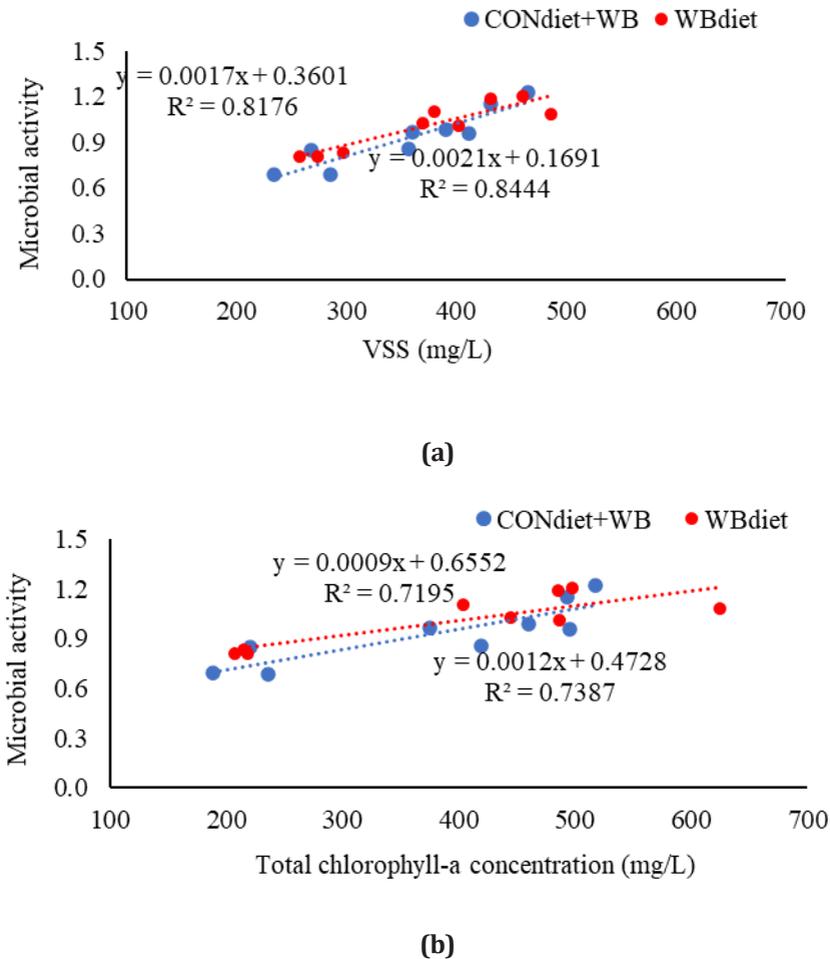


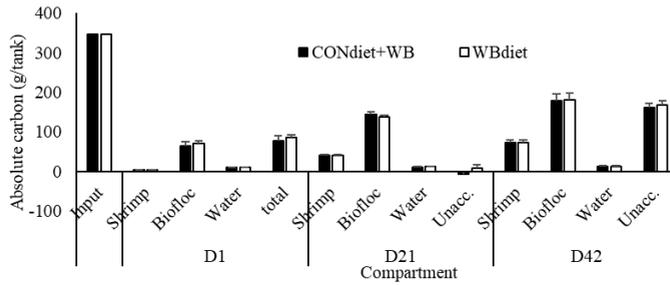
Figure 4.2. Linear regression between (a) microbial activity and volatile suspended solid (VSS); (b) microbial activity and total chlorophyll-a concentration. Values are the mean of each treatment (CONdiet+WB = control diet + direct addition of wheat bran and WBdiet = wheat bran diet) on all sampling times (D1 = day-1, D21 = day-21, and D42 = day-42). R^2 = coefficient of determination.

Table 4.8. Effect of the wheat bran addition method on the water quality during the experiment

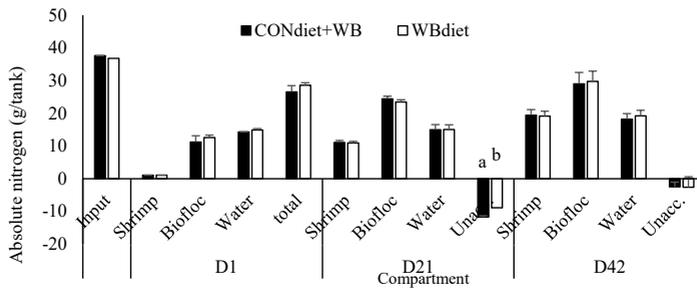
Parameter	Unit	WB addition (treatment)				P-value			
		CON+WB	WBdiet	D1	D21	D42	Treatment	Time	Treatment*time
NH ₄ -N	mg/L	0.026	0.023	0.039 a	0.019 b	0.018 b	0.633	0.000	0.514
NO _x -N	mg/L	19.3	20.2	18.2 b	18.5 b	22.6 a	0.467	0.001	0.755
TIN	mg/L	19.4	20.2	18.2 b	18.5 b	22.6 a	0.467	0.001	0.755
TON	mg/L	1.71	1.68	1.26 b	1.55 b	2.29 a	0.746	0.000	0.910
TN	mg/L	21.1	21.9	19.5 b	20.0 b	25.0 a	0.459	0.001	0.785
TIC	mg/L	12.7	12.5	13.8 b	15.5 a	8.5 c	0.856	0.000	0.459
TOC	mg/L	28.4	28.4	27.9 b	30.9 a	26.4 b	0.925	0.001	0.874
TC	mg/L	15.7	15.9	14.0 c	15.5 a	17.9 b	0.574	0.000	0.958
P-PO ₄	mg/L	3.36	3.27	2.1 c	2.7 b	5.2 a	0.501	0.000	0.602

Values are the mean of three sampling times of each treatment (CONdiet+WB = control diet + direct addition of wheat bran and WBdiet = wheat bran diet) and the mean of two treatments of each sampling times (D1 = day-1, D21 = day-21, and D42 = day-42). WB = wheat bran, NO_x-N = total of NO₂-N and NO₃-N, TON = total organic nitrogen; TIN = total inorganic nitrogen; TN = total nitrogen; TIC = total inorganic carbon; TOC = total organic carbon; TC = total carbon, P-value = probability value. Per row, for each factor (treatment or time) different letters in bold show significant difference (P<0.05).

(a)



(b)



(c)

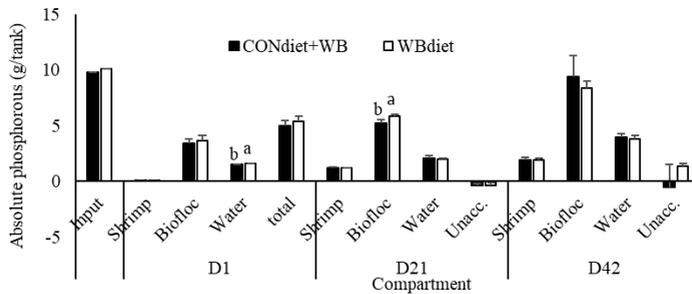


Figure 4.3. Effect of the wheat bran addition method on (a) nitrogen, (b) carbon and (c) phosphorous distribution in a biofloc system (g absolute amount / tank). Values are the mean and the standard deviation of each treatment (CONdiet+WB = control diet + addition of wheat bran to the water and WBdiet = wheat bran diet) at each sampling times (D1 = day-1, D21 = day-21, and D42 = day-42). Unacc. = unaccounted amount of nutrient (total nutrient input – total nutrient present in the system) at a sampling day. Input = nutrient input from feed and wheat bran during the experiment, total = total initial nutrient present in the system. Different letters on top of the bar showed significant difference ($P < 0.05$) between the diets. The input has no variation within diet treatment, and therefore was not statistically analyzed.

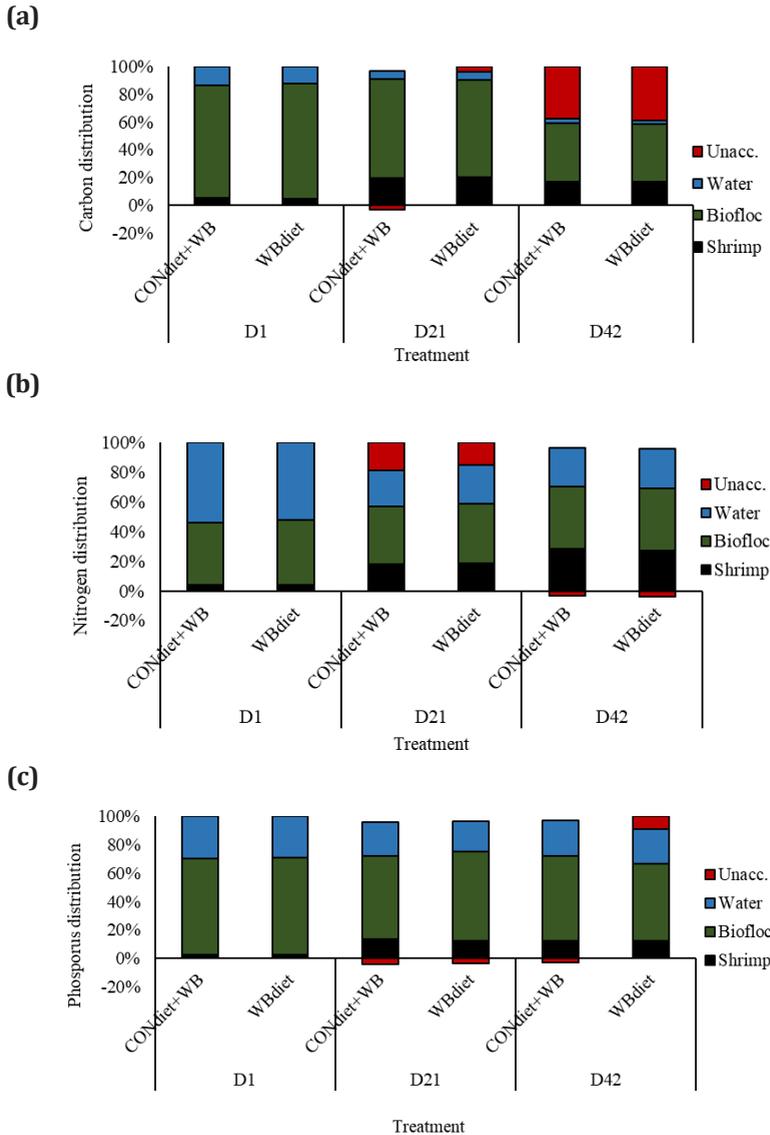


Figure 4.4. Effect of the wheat bran addition method on (a) nitrogen, (b) carbon and (c) phosphorous distribution in biofloc a system (in percentage of total nutrient in the system at each sampling times). Values are the mean and the standard deviation of each treatment (CONdiet+WB = control diet + direct addition of wheat bran and WBdiet = wheat bran diet) at each sampling times (D1 = day-1, D21 = day-21, and D42 = day-42). Unacc. = unaccounted amount of nutrient (total nutrient input – total nutrient present in the system).

4. Discussion

4.1. Nutrient digestibility and shrimp performance

The inclusion of wheat bran increased the NSP level in the diet which reduced the apparent digestibility. According to Sinha et al. (2011), dietary NSP increases the digesta viscosity and the digesta passage velocity, and reduces the mixing of digestive enzymes with feed in the fish gut. Yang et al. (2009) reported that 30% incorporation of various plant-based ingredients, such as soyabean meal, peanut meal and wheat gluten meal reduced the nutrient ADCs in Pacific white shrimp. However, the ADC values reported in their study were higher than in our study because we included more NSP in the diet.

Differences in ADC of protein and carbohydrate were small (4-15%; **Table 4.4**). In our study, 14% wheat bran was also included in the CONdiet, to reduce the difference in digestibility with the WBdiet, which partially explains the small differences in protein and carbohydrate apparent digestibility between diets. The average carbohydrate ADC for Pacific white shrimp in this study (43-50%) was lower than reported for Nile tilapia (65-72%), averaged from studies using diverse feed formulations (Maas et al., 2020). Related to the longer gut, the gut passage time is much longer in Nile tilapia than in Pacific white shrimp and consequently there is a longer exposure time for fermentation of carbohydrates in Nile tilapia, which might explain the difference in ADC between both species (Beseres et al., 2006; Kabir et al., 2020). Therefore, Nile tilapia might cope better with NSP than Pacific white shrimp. More research is needed on NSP digestibility in shrimp, to be able to finetune the formulation of NSP-rich shrimp diets.

Assuming 100% diet intake, the protein intake of shrimp fed the WBdiet and CONdiet was estimated. Shrimp fed the WBdiet via water consumed 18% more protein (**Table S4.1**) with a 4% lower apparent digestibility (**Table 4.4**), resulting in a 13% higher digestible protein intake compared to shrimp in the CONdiet+WB treatment (**Table S4.2**). However, the shrimp production and shrimp protein content were similar ($P>0.05$), most likely because shrimp in CONdiet+WB treatment tanks consumed more biofloc. Krummenauer et al. (2020), using stable isotopes, estimated that biofloc contributed 35 - 86% to the N-retention by Pacific white shrimp in biofloc systems. That biofloc contributes to shrimp production is also suggested comparing biofloc and non-biofloc studies. In biofloc studies the reported SGR fall in the range of 2.1-10.1% body weight gain/day, FCR in the range of 0.9-2.6, and PER in the range of 2.2-3.8 g body weight gain/g protein intake (Braga et al., 2016; Rajkumar et al., 2016; Panigrahi et al., 2018; Panigrahi et al., 2020; Tinh et al., 2021a; Tinh et al., 2021c). In contrast, in non-biofloc studies on average the performance is less, with reported ranges for SGR of 1.6-4.1 % body weight gain/day, for FCR of 1.5-2.8 and for PER of 1.3-1.8 g body

weight gain/g protein intake (Rajkumar et al., 2016; Panigrahi et al., 2018; Panigrahi et al., 2020; Ruvalcaba-Márquez et al., 2021; Mansour et al., 2022).

Tinh et al. (2021c) did a similar study, comparing inclusion in the pelleted feed versus separate addition of corn starch. In contrast to our results, including the carbohydrate in the pelleted diet was less effective than separate addition. Corn starch is highly digestible, and when included in the pelleted feed, the shrimp digest it, resulting in faeces with a low C:N ratio providing less energy for the microbiota in the biofloc system. When applying the corn starch via the water, the microbiota in the biofloc tank benefit, and contribute more to water quality maintenance, and shrimp and biofloc production. By incorporating an NSP-rich carbohydrate in the pelleted feed, however, a large fraction of the dietary carbohydrate may be transferred to the biofloc tank through the faeces, benefiting biofloc performance and contribution to shrimp growth. As a result, there was no difference in shrimp and system performance when administrating the NSP-rich carbohydrate through either the pelleted feed (WBdiet treatment) or by introducing it into the water in the biofloc tank (CONDiet+WB treatment) (**Table S4.1**).

4.2. Biofloc quantity, quality, and activity

A high fibre content in the diet increases the TSS concentration in the rearing system (López-Elías et al., 2014; Braga et al., 2016). In this study, the way wheat bran was introduced in the rearing system did not affect the quantity (e.g., TSS and VSS) of biofloc present ($P>0.05$; **Table 4.6**). Tinh et al. (2021c) also found similar TSS and VSS concentrations between dietary treatments with four times higher stocking density than in our experiment using corn starch instead of wheat bran as carbon source. The TSS concentration, regardless of the treatment, reached up to 781 mg/L at the end of experiment (**Table 4.7**). However, shrimp coped well with the biofloc concentration in the biofloc rearing system, as seen from a high survival (above 85%; **Table 4.5**) for the type of culture system used Hamidoghli et al. (2018). If the culture period would have been longer causing the TSS concentration to raise above 800 mg/L then water exchange or partial biofloc harvesting might become necessary (Ray et al., 2010; Schweitzer et al., 2013; Gaona et al., 2017; Ray et al., 2017).

The proximate content of the biofloc, including ash, crude protein, energy and minerals in this study (**Table 4.7**) was similar between wheat bran addition method and comparable with reported contents in literature (Tacon et al., 2002; Ekasari et al., 2014a; Kumar et al., 2018). The nutritive value of biofloc makes it a good supplemental natural food (Wang et al., 2015; Kuhn et al., 2016), although it should be checked for deficiencies. For instance, in a study by Ju et al. (2008) biofloc was deficient in arginine and lysine (Ju et al., 2008). In our study, the concentration ranges of Cu (0.09-2.1 g/kg DM), Mg (4.1-12.3 g/kg DM), and P (12.3-31.5 g/kg DM) aligned closely with the ranges

documented by Kuhn et al. (2009) and Rajkumar et al. (2016), except for the Ca content, which was twofold higher in our study. This discrepancy could potentially be attributed to difference in salt removal during sample washing.

The chlorophyll-a concentrations (in water and biofloc; **Table 4.7**) and the microbial activity (**Figure 4.1**) were similar between treatments, indicating that both wheat bran addition methods did not affect the algal growth and the microbial activity in the system. Our study measured microbial activity using H₂O₂ degradation analysis, principally measuring microbial enzymatic activity (peroxidases and catalases) (Mishra and Imlay, 2012; Iwase et al., 2013; Rojas-Tirado et al., 2018). Dead microbiota are not contributing, as suggested by Arvin and Pedersen (2015) who measured no H₂O₂ degradation after autoclaving water samples showing previously microbial activity. We observed significant correlations between microbial activity and TSS, VSS and chlorophyll-a concentration (**Figure 4.2**). Pedersen et al. (2019) reported a significant correlation between microbial activity and biological oxygen demand (BOD) and Rojas Rojas-Tirado et al. (2018) between microbial activity and feed load. In our study, the concentrations of both VSS and chlorophyll-a were positively correlated with bacterial activity. The latter is confusing, as algae under stress produce extracellular reactive oxygen species (ROS) and hydrogen peroxide (Diaz et al., 2018), which might reduce the net H₂O₂ degradation recorded with the H₂O₂ test. A possible explanation is that epiphytic bacteria on algae contribute to the antioxidative defences of the algae, which is beneficial to the growth of both algae and bacteria (Hunken et al., 2008). More work is needed to fine-tune the hydrogen peroxide test for use in mixotrophic biofloc systems, with algae and heterotrophic and nitrification bacteria present.

If measurements of microbial activity would be further standardized and results better interpreted, then it is a simple, cost-effective, rapid, and relatively accurate approach for assessing the microbial activity in water samples, including biofloc samples. Microbial activity measurements are less time consuming than TSS, VSS and BOD₅ analyses, and thus this will allow to obtain insight in biofloc development and activity faster than presently possible.

The biofloc ash content increased over time while the protein level decreased ($P < 0.05$; **Table 4.6**). This trend was consistent with previous studies (Ju et al., 2008). Post-feeding waste, shrimp and plankton exoskeletons, and minerals (e.g. acid soluble oxides and mixed silicates) mainly contribute to ash accumulation (Tacon et al., 2002). The chlorophyll-a concentration, an indicator of algal presence, doubled during the first half of the culture period, and continued to increase until the end of the experiment, although more slowly. A similar pattern was observed in the biofloc for the amount Mg,

an important component of chlorophyll-a, although the increase stabilized during the second half of the culture period (Marchand et al., 2018; Salman et al., 2023). In spite of observed increase in chlorophyll-a, the authors observed a change in colour from green to brownish, during the culture period, concurring with observations of Ju et al. (2008), who reported a negative correlation between the brownness of biofloc water and the alga: bacteria ratio.

Besides the algae, both autotrophic and heterotrophic bacteria contributed to water quality management in the biofloc rearing system, as seen from the low TON and the increasing accumulation of NO_x-N throughout the culture period, as suggested by (Correia et al., 2014). Here too, more research is needed on how to keep a mature biofloc system maintaining the water quality, finding a balance between formation and degradation of biofloc, and keeping nutrients in the system. In this study, working with mature biofloc, the nitrogen and phosphorous supplied stayed in the system and carbon loss was low (Tinh et al., 2021a and 2021b). The challenge is to keep the nutrients accumulated in biofloc and water biological available and to steer them through the microbial food web towards shrimp production.

5. Conclusion

When feeding a biofloc system with the same nutrient inputs, it does not matter if carbohydrate to raise the C:N ratio of nutrient inputs is provided via the feed or via the water. Shrimp production and biofloc system performance were not affected by the way the carbohydrate was administrated. Simplifying biofloc system management for farmers to administrating daily one input, in this case the WBdiet, reduces on-farm labour and room for human error, while ensuring homogenous carbon and energy delivery to the microbiota in the biofloc rearing tank through shrimp faeces.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary Table S4.1. Absolute nutrient input from feed and wheat bran

Parameters	WBdiet	CONdiet+WB		
		CONdiet	WB	Total
Feed g DM	752	556	194	750
Ash g DM	51	40	11	50
Crude protein g DM	228	194	38	231
Fat g DM	46	36	10	46
Carbohydrates* g DM	427	286	136	422
Energy MJ	15	11	4	15

CONdiet+WB = control diet + direct addition of wheat bran and WBdiet = wheat bran diet, WB = wheat bran. *Calculated by subtracting ash, crude protein, and fat from dry matter.

Supplementary Table S4.2. Theoretical calculation of digested nutrient from feed assuming 100% feed intake

Parameters	WBdiet	CONdiet
Feed g AFDM	404	336
Crude protein g	185	163
Crude fat g	26	23
Energy MJ	8	7
Carbohydrates g	182	143

CONdiet = control diet, WBdiet = wheat bran diet, afdm = ash-free dry matter



5

Effects of different sources of dietary non-starch polysaccharides on Pacific white shrimp (*Litopenaeus vannamei*) performance, water quality and biofloc nutritional and microbial composition

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In preparation

Abstract

Inclusion of ingredients rich in non-starch polysaccharides (NSP) in pelleted feed can serve as a carbon-rich source to manage water quality and production of Pacific white shrimp (*Litopenaeus vannamei*) in biofloc systems. However, the inclusion, source and type of NSP (i.e., fermentability) can affect the feed quality by reducing the dietary protein content and nutrient utilization. This study evaluated the effects of replacing dietary starch in a commercial-like diet (STdiet), with either poorly fermentable (PF) or moderately fermentable (MF) NSP-rich ingredients. Moreover, two feeding approaches were tested to counteract with the lower protein content of the NSP-rich diets: feeding either iso-nitrogenously (IN) or at *as-is* basis, resulting in five dietary treatments: IN-PF, IN-MF, IB-PF, IB-MF and STdiet. Digestibility (30-day trial) and growth performance (42-day trial) were assessed, as well as the biofloc nutritional and microbial composition. Pacific white shrimp had a lower nutrient digestibility when fed NSP diets compared to the STdiet. The NSP type did not affect shrimp performance, biofloc formation and water quality. Feeding iso-nitrogenously compared to *as-is* basis resulted in similar performance between treatments and increased the protein content in both shrimp and biofloc. With regard to the biofloc microbiota composition, inclusion of NSP-rich ingredients in the diets moderated the prokaryotic community by decreasing *Cyanobacteria* and increasing *Actinobacteria* abundance. In conclusion, when fed iso-nitrogenously, the type of NSP-rich ingredients did not affect biofloc system performance and worked as effectively as the STdiet in maintaining water quality.

Keywords: feeding approach, NSP-rich ingredients, apparent digestibility, nitrogen budget, carbon budget, prokaryotic communities

1. Introduction

In a biofloc system, carbon is an essential nutrient required for the microorganisms associated with the flocs, in order to transform or immobilize organic and inorganic post-feeding nitrogen waste associated with shrimp egestion (e.g. CO₂, NH₃), excretion (faeces), decayed biota (plankton, bacteria, etc.) and detritus (Avnimelech, 2009b; Panigrahi et al., 2018; Khanjani et al., 2022). Under conventional biofloc system management, an external carbon source is added to raise the carbon-to-nitrogen (C:N) ratio (g/g) to a range between 10 to 20 (Dauda et al., 2017; Silva et al., 2017; Oliveira et al., 2022). A previous study showed that both external carbohydrate addition as carbon source, and addition through the feed pellet, can assist in water quality maintenance in biofloc systems (Tinh et al., 2021c). In a similar approach, our previous work showed that inclusion of ingredients containing non-digestible carbohydrates, such as non-starch polysaccharides (NSP), in the shrimp diet, instead of adding it externally, can actually increase the excretion of carbon-rich faeces (Vinasyiam et al., under review). Such faeces can serve as an energy substrate for microorganisms in a biofloc system, which will then promote the degradation of post-feeding wastes and assist in water quality management. Moreover, a practical advantage of rearing shrimp in such systems is that the flocs can become an additional feed source; thus, it is possible to lower the dietary nutrient content without reducing shrimp growth and production (Avnimelech, 2007). Although inclusion of less digestible NSP-rich ingredients is expected to reduce nutrient digestibility, in fact, when reared in biofloc systems, the flocs can provide additional feed to the shrimp, thus compensating for the lower nutrient uptake from the pelleted feed (Avnimelech, 2007; De Schryver et al., 2008).

Plant-based crop residues, rich in NSP are ideal carbon sources for biofloc systems (Ekasari et al., 2014b; Serra et al., 2015; El-Husseiny, 2018), because they are cheaper and available in larger quantities than simple sugars and starch. The use of NSP-rich ingredients such as wheat bran was shown to improve the nutritional value of the biofloc (El-Husseiny, 2018) and the production of Nile tilapia (*Oreochromis niloticus*) (Zaki et al., 2020), common carp (*Cyprinus carpio*) (Ebrahimi et al., 2020), Pacific white shrimp (*Litopenaeus vannamei*) (Wang et al., 2016) and giant tiger shrimp (*Penaeus monodon*) (Kumar et al., 2017). In all the above-mentioned studies, NSP was applied separately from the feed. Nevertheless, as mentioned above, few studies have shown that it is possible to include NSP-rich ingredients directly in the feed, even when partially replacing dietary protein. Specifically, Kabir et al. (2020), working with Nile tilapia, showed that NSP-rich feed improved the natural food production in ponds, leading to a higher production, compared to an NSP-poor commercial feed. In addition, Kabir et al. (2020) showed that a diet rich in poorly fermentable NSP (lignin and cellulose) enhanced the growth of fish

and natural biota in ponds more than a feed containing mainly moderately fermentable NSP (pectin and hemicellulose). Although such results come mainly from Nile tilapia, a question remains whether the use of different type of NSP added directly in the pelleted feed affect other species reared in biofloc systems. Therefore, the first objective of this study is to verify if an effect of poorly and moderately fermentable NSP occurs also in shrimp biofloc rearing systems.

Addition of NSP in the pelleted feed results in a reduction of dietary nutrients, including protein, and as a result also reduced nutrient utilization (Amirkolaie et al., 2005). It has been shown for Pacific white shrimp that reducing the dietary protein level from 35-36% to 30-25% did not affect performance when feeding diets with easily digestible carbohydrates such as rice grits, wheat flour or wheat meal in a biofloc system (Xu and Pan, 2013a; Jatobá et al., 2014). However, so far it has not been investigated whether the type and fermentability of NSP-containing ingredients has an impact on shrimp nutrient retention. Therefore, the second objective of this study is to evaluate whether different feeding approaches, feeding iso-nitrogenously or at as-is basis, will result in similar production. For the two abovementioned objectives, nutrient digestibility, biofloc system performance (shrimp performance, biofloc growth and water quality), as well as nutrient (C and N) mass balances were assessed. In addition, the biofloc microbial community composition was assessed.

2. Material and Methods

Two experiments were executed: a digestibility experiment in clear water aquaria with daily faeces collection, and a growth experiment in mesocosm biofloc tanks. The experiments were carried out at the IPB (Institut Pertanian Bogor) Fisheries and Marine Observation Station (IFMOS) Ancol research station, IPB University, DKI (*Daerah Khusus Ibukota* – The Special Capital Region) Jakarta Province, Indonesia.

2.1. Experimental animal procurement and adaptation to culture environment

For the digestibility experiment, Pacific white shrimp with an average weight of 7.3 ± 0.3 g, were obtained from a grow-out pond in Karawang, Indonesia. The shrimp were transferred to continuously aerated 700-L tanks, containing clear water (salinity 25 ± 1 ppt) and allowed to adapt to the culture environment for two weeks until the start of the experiment.

For the growth experiment, 0.45 ± 0.004 g Pacific white shrimp larvae were obtained from the CV (*Commanditaire Vennootschap*) Benur Lampung, Lampung Province, Indonesia. The larvae were grown for three weeks in a 4,000-L biofloc tank (salinity 25 ± 1 ppt) until the start of the experiment. During that period, shrimp were fed a 40%

protein crumble diet Feng Li (MS-Matahari Sakti®). To mature the biofloc systems, 50 ml molasses (total sucrose inverted = 48.5%) were added to the biofloc maturation tank every 3 days, for three weeks, to maintain water quality by promoting the growth of heterotrophic bacteria.

2.2. Experimental diets

Three diets were formulated. One diet, which was used as basal, had low NSP content and contained about 37% crude protein, while a major part of the carbohydrates was starch (STdiet; **Table 5.1**). Specifically, STdiet contained 19% cornstarch and 19% tapioca starch. For the two NSP-rich diets, the STdiet was diluted with different types of NSP: a poorly fermentable mixture consisting of 28% palm kernel meal and 10% corn straw (PFdiet), and a moderately fermentable mixture, consisting of 22% wheat bran and 22% distillers dried grains with soluble (DDGS; MFdiet). Consequently, these diets had a lower crude protein content and a higher NSP content compared to the STdiet. All diets contained similar amounts lecithin, cholesterol, premix, monocalcium phosphate, L-lysine, DL-methionine, L-threonine (**Table 5.1**). Chromium oxide was added as inert marker for digestibility measurements. The analyzed nutrient content of the diets is given in **Table 5.1**.

2.3. Digestibility experiment

The digestibility experiment was carried out in twelve 90 x 40 x 35 cm³ aquaria of 80-L each. Twenty juvenile shrimp were stocked per aquarium, and four replicate aquaria were randomly assigned to each experimental diet. All aquaria were fed the same amount of feed for 30 days. Each aquarium had one air stone and its own filter system, which was continuously working except during faeces collection. Water was pumped from the aquarium bottom through a sponge in the filter system to trap particulate matter. The sponge was daily replaced by a clean sponge. The dissolved oxygen and temperature were maintained minimum at 6.5 mg O₂ L⁻¹ and 27±1.0 °C, respectively. The shrimp were fed at 06:00, 11:00, 16:00 and 21:00. Faeces were collected between the first and second feeding of the day, six days per week.

2.4. Growth experiment

At the end of the maturation phase, 250-L water was transferred from the biofloc maturation tank to each of fifteen 1,000-L outdoor biofloc tanks. The water volume in the tanks was increased to 700 L by adding a mixture of freshwater and marine water. The salinity was maintained at 25±1 ppt. Tanks had a tarpaulin roof to reduce fluctuations in temperature due to direct sunlight during midday and to prevent water

Table 5.1. Diet formulation and composition

Diet composition	Unit	Diet		
		PF	MF	ST
<i>Ingredients</i>				
Soybean meal	%	15.9	12	26
Wheat gluten	%	6	5	10
Wheat bran	%	0	21.5	0
Lecithin	%	2	2	2
Fishmeal 58-63%	%	12	10	20
Cholesterol	%	0.3	0.3	0.3
Corn starch	%	11	12	19
Tapioca starch	%	11	11.9	18.9
Palm kernel meal	%	28	0	0
DDGS (distiller`s dried grains with soluble)	%	0	21.5	0
Corn cob meal	%	10	0	0
Premix shrimp	%	1	1	1
Monocalcium phosphate (CaH ₄ P ₂ O ₈)	%	1.2	1.2	1.2
L-lysine HCl	%	0.5	0.5	0.5
DL-methionine	%	0.3	0.3	0.3
L-threonine	%	0.3	0.3	0.3
Chromium oxide (Cr ₂ O ₃)	%	0.5	0.5	0.5
Total	%	100	100	100
<i>Analyzed proximate compositions</i>				
Dry matter (DM)	g/kg	886	888	889
Ash-free DM	g/kg	810	816	805
Ash	g/kg DM	85	81	95

Table 5.1. Diet formulation and composition (continue)

Diet composition	Unit	Diet		
		PF	MF	ST
<i>Analyzed proximate compositions</i>				
Crude protein	g/kg DM	284	274	366
Crude fat	g/kg DM	72	66	67
Crude fibre	g/kg DM	109	46	55
Energy	kJ/g DM	19.6	20.6	20.9
Nitrogen-free extract	g/kg DM	450	533	417
Carbohydrate	g/kg DM	559	579	472
Nitrogen (N)	g/kg DM	45	44	59
Carbon (C)	g/kg DM	463	465	455
C:N ratio (mass)	g/g	10.2	10.6	7.8
Calcium	g/kg DM	12.7	12.1	23.2
Magnesium	g/kg DM	2.8	3.3	1.7
Phosphorous	g/kg DM	7.8	10.2	8.2
N:P ratio (mass)	g/g	4.3	7.2	5.9
Chromium	g/kg DM	6.8	6.0	6.7

PF = poorly fermentable non-starch polysaccharide diet; MF = moderately fermentable non-starch polysaccharide diet; ST = starch diet.

influx and overflow during heavy rain. Each biofloc tank had five air stones for aeration and suspension of the biofloc in the water column.

Hundred fifty (150) shrimp larvae were stocked in each biofloc tank. The water in each biofloc tank was not changed during the 42-day culture period. During the experiment, water temperature was maintained at 28 ± 3 °C, pH at 7.7 ± 0.3 and dissolved oxygen (DO) at 6.5 ± 0.5 mg L⁻¹ (multi-parameter WTW Multi3630IDS™ meter). Total ammonia nitrogen (TAN) and nitrite (NO₂-N) were measured once every three days using colorimetric test kits (mg/L, MColorTest™, EMD Millipore) and nitrate (NO₃-N) was tested using colorimetric test strips (mg/L, MQuant®, EMD Millipore).

The amount fed daily to STdiet treatment tanks was calculated using a standard feeding table normally used in shrimp pond culture (Tinh et al., 2021c). The FCR gradually adjusted from 0.6 on day one (D1) to 1.1 at harvest (D42). The PFdiet and MFdiet were fed either iso-nitrogenously (IN) or at *as-is* basis (iso-biomass; IB) as for the STdiet, resulting in five treatments: IN-PF, IN-MF, IB-PF, IB-MF and STdiet. There were three replicate tanks per treatment. The biofloc tanks were fed daily at 06:00, 11:00, 16:00

and 21:00 o'clock for 42 days. Every two weeks, a subsample of the shrimp population in STdiet treatment was batch weighed to estimate the average individual weight, in order to adjust the feeding tables. The amounts to be fed to the IN and IB treatments were calculated based on the amount fed to the STdiet treatment.

2.5. Sampling and sample preparation

2.5.1. Digestibility experiment

The filter in each aquarium was turned off during faeces collection. One hour after the morning feeding, all visible wastes were removed by siphoning, and collected in a glass bottle placed on ice. Faeces were then rinsed using demi-water to remove salt from the fish-rearing water and stored in an aluminum tray at -20° C until further analysis. One tray was assigned to each aquarium. To remove water before the proximate analysis, faeces were dried at 70 °C for 72 hours. Afterward, faeces were ground manually with mortar and pestle, and stored at room temperature until analysis.

2.5.2. Growth experiment

Shrimp and diet

The weight and proximate composition of shrimp at the start (D1) and end (D42) of the experiment were recorded. Before stocking, 150 shrimp were randomly removed from the base population and batch weighed. At stocking, per biofloc tank, 150 shrimp were batch weighed, while on D42 all shrimp in each biofloc tank were harvested, counted and batch weighed. The collected shrimp were paper-dried to remove the excess water and stored at -20 °C. Before the proximate composition analysis, the pre-stocking sample and shrimp harvested from each tank were grounded and homogenized. For the proximate analysis of the diets, 40-50 g of each diet was added weekly to one jar per diet kept at 4° C. Prior to proximate composition analysis, the feed samples were grounded, homogenized, and stored in an airtight closed jar at room temperature.

Biofloc and water quality

Samples were collected at D1, D21 and D42. Water was taken from the center of each biofloc tank. The unfiltered water was used to determine biofloc biomass and proximate composition (2 x 1.5 L), and BOD₅ and COD (2 x 1.5 L). The biofloc was collected immediately after taking the water sample. For BOD₅ and COD, the water was kept at 4 °C before and during transport to the laboratory (3-4 hours). BOD and COD samples were properly prepared and stored until analysis, but because the lab was closed during a nationwide Covid-19 lockdown, the samples collected on D21 expired before the labs reopened.

To collect biofloc, 50 mL was first filtered through a 1.5 µm pore size Whatman® filter 934 AH 47 mm, using a vacuum pump. Afterwards, the filter was rinsed two times with 50 ml demi water to rinse the salt and collect biofloc particles sticking on the glass of the filtration chamber. The filters with biofloc were folded, put each in a clean tube and stored at -20 °C until further analysis. The biofloc filters were used to determine total suspended solids (TSS), volatile suspended solids (VSS) and biofloc proximate composition. Before the analysis, biofloc filters were dried at 70 °C for a minimum of 72 hours. Afterward, filters were cooled down in the desiccator before weighing.

Total alkalinity, total ammonia-nitrogen (TAN), nitrite-nitrogen (NO₂-N), nitrate-nitrogen (NO₃-N), orthophosphate (PO₄-P), carbon dioxide (CO₂), total inorganic nitrogen (TIN), and total inorganic carbon (TIC) were measured in filtrate water from biofloc sampling. Per biofloc tank, the filtrate water was homogenized with a magnetic stirrer, before preparing 25-mL samples for water quality analysis.

2.6. Sample analysis

2.6.1. Proximate composition

Dry matter (DM), ash, ash-free DM (AFDM), crude protein, crude fat, crude fiber, energy, carbohydrate, and the C, N, Ca, Mg, P content were determined. For faeces and diet samples, Cr content was analyzed to calculate the apparent digestibility coefficient (ADC). Insufficient biofloc was collected to be able to measure energy content. In faeces, Ca, Mg and P were not determined. Special attention was given to washing out salt from faeces samples, although it is difficult to judge if this worked equally well across treatments. Therefore, the AFDM data are presented in tables, but not discussed in depth.

The DM concentration was estimated by oven-drying samples at 105 °C for at least 3 hours until constant weight (ISO-6496, 1999). Following DM determination, the samples were incinerated at 600 °C for at least 4 hours until a constant weight was achieved to determine the ash content (ISO-5984, 2002). The nitrogen and crude protein content were determined by the Kjeldahl method (ISO-5983, 2005). The crude fat analysis was done by the Folch method (ISO-6492, 1999). The energy level was measured using bomb calorimetry by direct combustion method (ISO-9831, 1998). The crude fiber content was analyzed according to ISO-5498 (1981). The Ca, Mg and P content were analyzed using plasma-mass spectrophotometry (ICP OES) following the NEN-15510 (2017) procedure, while the Cr concentration was analyzed according to ISO-15192 (2021). The carbohydrate content was calculated by subtracting the dry matter with ash, crude protein, and fat, while nitrogen-free extract (NFE) was calculated by subtracting the crude fiber content from the carbohydrate content.

2.6.2. Dissolved matter sample

The concentrations of TAN, NO₂-N, and NO₃-N in the filtrate water sample and BOD₅ and chlorophyll-a in the unfiltered water sample from the growth experiment were analyzed according to APHA (1995). The chemical oxygen demand (COD) was analyzed based on the protocol of ISO-6060 (1989). The biodegradability index (BI) is calculated as BOD/COD (Rojas-Tirado et al., 2018).

2.7. Microbiota in Biofloc

2.7.1. Sample collection and preparation

To collect biofloc water from each tank, the same procedure as in biofloc sampling was carried out. Fifty ml biofloc water was filtered through a 0.45 µm pore-size filter according to Tinh et al. (2021). To ensure homogenous biofloc water samples were collected, biofloc water was mixed at 300 rpm using a magnetic stirrer (Heidolph Mr. Hei-Mix L, Heidolph Instruments, Germany). After filtration, 50-ml demi-water was passed through the filter twice to remove the remaining salt that had been trapped on the filter. Then, each filter with biofloc was folded and placed in a closed-sterile tube and stored at -80 °C.

2.7.2. Biofloc microbial DNA extraction

The sample processing, library preparation and sequencing were performed by Novogene (China). The DNA from 3 pooled biofloc samples per treatment was extracted using CTAB/SDS method. DNA concentration and purity was monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1 ng/µL using sterile water. 16S rRNA and ITS genes of distinct regions (16S V3-V4 and ITS2) were amplified and barcoded. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). The PCR product detection was carried out by mixing same volumes of 1X loading buffer (contained SYB green) and PCR products, and operating electrophoresis on 2% agarose gel. Samples with bright main strip between 400bp-450bp were chosen for further analysis. PCR products were mixed at equal density ratios, then were purified with Qiagen Gel Extraction Kit (Qiagen, Germany). The libraries generated with NEBNext® Ultra™ DNA Library Prep Kit for Illumina and quantified via Qubit and Q-PCR, were analysed by Illumina PE250 platform.

2.7.3. Sequencing data processing

Paired-end reads was assigned to samples based on their unique barcodes and truncated by cutting off the barcode and primer sequences. Paired-end reads were merged using FLASH (V1.2.7) (Magoc and Salzberg, 2011), which was designed to merge paired-end reads when at least some of the reads overlap the read generated from the opposite end

of the same DNA fragment, and the splicing sequences were called raw tags. Quality filtering on the raw tags were performed under specific filtering conditions to obtain the high-quality clean tags (Bokulich et al., 2013) according to the Qiime (V1.7.0) quality-controlled process (Caporaso et al., 2010). The tags were compared with the reference database (Gold database for the 16S and Unite database for the ITS) using UCHIME algorithm to detect chimera sequences (Edgar et al., 2011). The chimera sequences were removed to obtain the effective tags (Haas et al., 2011).

2.7.4. OTU cluster and Taxonomic annotation

Sequences analysis was performed by Uparse software (Uparse v7.0.1001) using all the effective tags (Edgar, 2013). Sequences with $\geq 97\%$ similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation. For each representative sequence, Mothur software was performed against the SSUrRNA database of SILVA Database for species annotation of the prokaryotic community at each taxonomic rank (Threshold:0.8~1) (kingdom, phylum, class, order, family, genus, species) (Wang et al., 2007; Quast et al., 2013). Meanwhile for the fungal community, sequences analysis was performed by blast with Qiime (Version 1.7.0) and Unite database (Altschul et al., 1990; Koljalg et al., 2013). To obtain the phylogenetic relationship of all OTUs representative sequences, multiple sequences were compared using MUSCLE software (Version 3.8.31) (Edgar, 2004). OTUs abundance information were normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity was performed based on the output normalized data. Alpha diversity indices: Observed-species, Shannon and the Phylogenetic Diversity Index were calculated with QIIME (Version 1.7.0).

2.8. Data calculation

For the digestibility experiment, the apparent digestibility coefficient (ADC; %) of dietary macronutrients was calculated as $ADC (\%) = 100 \times [1 - (Y_d \times \text{amount nutrient in faeces}) / (Y_f \times \text{amount nutrient in diet})]$, where Y_d (g/kg DM) is the inert marker content in the diet and Y_f (g/kg DM) is the marker content in the faeces.

For the growth experiment, biomass or individual weight gain were the differences between total biomass or individual weight gain of shrimp between D42 and D1. The individual growth rate ($g d^{-1}$) was the individual weight gain divided by the number of days of the culture period. Specific growth rate (SGR; % BW d^{-1}) was calculated as $(\ln W_t - \ln W_0) / t \times 100$, where W_0 and W_t are the initial and final individual body weight (g) of shrimp and t is the number of culture days. The feed conversion ratio was calculated as $FI / (W_t - W_0)$, where FI is feed input (g). The protein efficiency ratio (PER) was calculated as shrimp biomass gain divided by the total dietary protein input. Protein and energy

retention efficiencies (PUE and EUE) (%) were calculated as $N_s/N_i \times 100$, where N_s (g) is the nutrient retained in shrimp (protein or energy) and N_i (g) is the dietary nutrient input. Production (kg m^{-3}) was calculated as the total shrimp biomass gain divided by the water volume. Survival (%) was calculated as $N_t/N_0 \times 100$, where N_t and N_0 were the numbers of shrimp on D42 and D1, respectively.

For system nutrient balance estimation in the growth experiment, the total nutrient input (g) was calculated as the sum of the nutrient from the diet and from the system compartments (shrimp, biofloc and water) at D1. Unaccounted loss (g) was the difference between the total nutrient input and the nutrient in the system compartments (shrimp, biofloc and water) at D42. The biodegradability index (BI) was calculated as BOD/COD (Rojas-Tirado et al., 2018).

2.9. Data analysis

Statistical analyses were performed using the IBM Statistics 26 software (IBM Corporation, NY, USA). The collected data were analyzed based on the factors being compared:

a. *Digestibility experiment*

One-way ANOVA, comparing the effect of 3 diets (PFdiet, MFdiet and STdiet).

b. *Growth experiment*

- Two-way ANOVA, comparing the effect of NSP type (PF and MF) and the effect of feeding level (IN and IB) as factors.
- Two-way repeated measure ANOVA, with sampling day (D1 and D42) as repeated measure, and NSP type (PF and MF) and feeding level (IN and IB) as main factors.
- Repeated measure ANOVA, with sampling day (D1 and D42) as repeated measure and treatment (IN-PF, IN-MF, IB-PF, IB-MF and STdiet) as main factor. Meanwhile, for parameters having only one sampling time (D42), the effect of 5 treatments were analyzed with one-way ANOVA.
- The absolute nutrient deposition within each compartment at each sampling time was compared between the dietary treatments with one-way ANOVA.
- Significant differences ($P < 0.05$) were further analyzed by post-hoc Tukey HSD range test.

3. Results

3.1. Shrimp growth performance

Diet significantly affected the proximate composition of the faeces (**Table 5.2**). Overall, the faeces of shrimp fed the PFdiet and the STdiet were different ($P < 0.05$), except for the fat content ($P > 0.05$). The faeces collected from shrimp fed the MFdiet and STdiet had equal crude protein, crude fibre, NFE, N and C contents, as well as C:N ratio ($P > 0.05$). The faeces of shrimp fed the PFdiet and MFdiet were similar in composition in terms of DM, AFDM, crude fat, energy, C:N ratio, and chromium content ($P > 0.05$).

With regard to nutrient digestibility (**Table 5.3**), the crude protein ADC of the PFdiet was lower than the protein ADC of the MFdiet ($P < 0.05$); while the ADCs of AFDM, fat and carbohydrate were similar between these diets ($P > 0.05$). In addition, the ADCs of the PF and MF diets were lower than the ADC of the STdiet ($P < 0.05$), except for carbohydrate ($P > 0.05$).

In **Table 5.4** and **Table S5.1**, the effects of diet on shrimp growth performance are summarized as determined by two-way and one-way ANOVA, respectively. No effects of 'NSP type' and of 'NSP type x feeding' were observed on shrimp performance ($P > 0.05$, **Table 5.4**). Although not significantly different ($P > 0.05$), however, numerically the PFdiet consistently resulted in better growth performance than the MFdiet except for protein utilization efficiency. Feeding IN resulted in higher individual growth (final individual body mass, individual body mass gain, GR, and SGR) and system performance (final biomass, total biomass gain, total protein gain and production) than feeding at *as-is* basis (IB) ($P < 0.05$). The FCR, PER, PUE, EUE, and survival were not different between IN and IB feeding ($P > 0.05$), although here too these parameters were numerically better when feeding IN.

Comparing the 5 treatments by one-way ANOVA, diet significantly affected the final individual weight, protein gain, energy gain, GR, SGR, and production ($P < 0.05$, **Table S5.1**), mainly showing differences between the STdiet and the PF and MF diets fed at *as-is* basis (IB-PF and IB-MF). However, no differences with the STdiet were observed when the NSP-rich diets were fed iso-nitrogenously (IN-PF and IN-MF) ($P > 0.05$). The FCR, PER, PUE, EUE, and survival, were not affected by diet ($P > 0.05$). Although not statistically different, the survival of shrimp fed NSP-rich diets were numerically higher compared to survival of shrimp raised in STdiet tanks.

3.2. Shrimp body composition

Two-way ANOVA showed that for the NSP-rich dietary treatments feeding iso-nitrogenously resulted in a higher crude protein content and lower crude fat content in

the body at harvest than when feeding equal biomass ($P < 0.05$, **Table 5.5**). In addition, feeding the PFdiet led to a higher crude body fat content than when feeding the MFdiet ($P < 0.05$). No other differences for the factors ‘NSP’ and ‘Feeding’ were observed ($P > 0.05$).

Based on one-way ANOVA, the crude protein content in shrimp in the IN-MF and IN-PF treatments was higher than in shrimp in the STdiet treatment ($P < 0.05$), while the shrimp protein content was intermediate in the IB-PF and IB-MF treatments (**Table S5.2**). The crude fat body content in shrimp harvested from IB-PF and STdiet treatment tanks was higher than in other treatments ($P < 0.05$). The energy content was higher in shrimp fed the PFdiet regardless of the “feeding” level (IN-PF and IB-PF treatments) compared to STdiet ($P < 0.05$), and intermediate for the other treatments. The C:N ratio was highest in shrimp harvested from STdiet treatment tanks, intermediate for IB treatment tanks and lowest for IN treatment tanks ($P < 0.05$). For the other parameters, the shrimp body composition was similar between treatments ($P > 0.05$).

3.3. Biofloc

The effects of “NSP” type and “feeding” level on biofloc quantity and quality related parameters were measured on D21 and D42, with and without the STdiet treatment are shown in the **Table 5.6** and **Table S5.3**, respectively. For the biofloc parameters for which the D21 samples could not be analysed, the D42 data comparing “NSP” type and “feeding” level are presented in **Table 5.7**, while the results of one-way ANOVA with the 5 treatments are shown in **Table S5.4**.

The “NSP” type did not affect the biofloc quality (proximate analysis) and quantity (TSS and VSS concentration), as well as chlorophyll-a concentration, COD, BOD₅ and the biodegradability index ($P > 0.05$) (**Tables 5.6** and **Table 5.7**). Feeding NSP-rich diets iso-nitrogenously (IN; IN-PF and IN-MF) showed higher crude protein content of biofloc compared to feeding at *as-is* basis (IB: IB-PF and IB-MF) ($P < 0.05$, **Table 5.6**). Comparing the NSP-rich diets and STdiet, the biofloc crude protein content in tanks fed the STdiet was higher than in tanks fed NSP-rich diets at *as-is* basis (IB-PF and IB-MF), while similar to the other treatments (**Table S5.3**). The VSS concentration in tanks fed with STdiet was higher than in IB-MF treatment tanks, while other treatments were intermediate (**Table S5.3**). Although not statistically different, the chlorophyll-a content in biofloc collected in tanks fed the STdiet was nearly 75% higher than in the other treatment tanks ($P > 0.05$, **Table S5.3**). Between day 21 and 42, the biofloc TSS, VSS and chlorophyll-a content increased ($P < 0.05$, **Table S3**). The BOD₅ and the biodegradability index among the 5 treatments were similar ($P > 0.05$, **Table S5.4**).

Table 5.2. Effect of the dietary NSP and starch addition on the proximate quality of faeces of the Pacific white shrimp (*Litopenaeus vannamei*)

Parameters		Diet						P-value
		PF		MF		ST		
Crude protein	g/kg AFDM	156	± 8 a	125	± 5 b	109	± 11 b	0.001
Crude fat	g/kg AFDM	53	± 0.4 ab	54	± 1 a	51	± 0.7 b	0.020
Crude fibre	g/kg AFDM	358	± 13 a	269	± 22 b	246	± 14 b	0.000
Energy	kJ/g AFDM	15.3	± 0.2 b	15.6	± 0.3 b	16.3	± 0.2 a	0.003
Nitrogen-free extract (NFE)	g/kg AFDM	433	± 17 b	552	± 23 a	594	± 10 a	0.000
Carbohydrate	g/kg AFDM	791	± 7 c	821	± 5 b	840	± 6 a	0.000
Nitrogen (N)	g/kg AFDM	25	± 1 a	20	± 0.7 b	17	± 2 b	0.001
Carbon (C)	g/kg AFDM	506	± 0.3 b	507	± 0.3 a	507	± 0.5 a	0.015
C:N ratio (mass)	g/g	20	± 1 b	25	± 0.9 ab	29	± 3 a	0.005
N:P ratio* (mass)	g/g	1.3		1.0		1.8		
Chromium	g/kg DM	8.9	± 0.8 b	8.4	± 0.5 b	12.0	± 0.4 a	0.000

Values are means and the standard deviation of the mean (SD) of each diet. PF = poorly fermentable diet, MF = moderately fermentable diet, ST = starch diet, dm = dry matter, AFDM = ash-free dry matter, N:P ratio = nitrogen to phosphorous. Data were analyzed using one-way ANOVA. Different letters in bold in the same row show significant difference ($P < 0.05$). *Calculated from the faecal N content and the estimated faecal P content (faecal P content = dietary P input \times (100 - DM ADC%).

Table 5.3. Effect of the dietary NSP and starch addition on the apparent digestibility coefficient (ADC) of nutrient in the diet of Pacific white shrimp (*Litopenaeus vannamei*)

Parameters	Diet			P-value
	PF	MF	ST	
Ash-free dry matter (AFDM) %	31 ± 5b	36 ± 3b	48 ± 2a	0.003
Crude protein %	66 ± 4c	74 ± 1b	87 ± 2a	0.000
Crude fat %	55 ± 3b	53 ± 4b	66 ± 0.5a	0.003
Carbohydrate %	13 ± 6	18 ± 5	20 ± 2	0.265

Values are means and the standard deviation of the mean (sd) of each diet. PF = poorly fermentable diet, MF diet = moderately fermentable diet, ST = starch diet. P-value = probability value. Data were analyzed using one-way ANOVA. Different letters in bold in the same row show significant difference ($P < 0.05$).

Table 5.4. Effect of the dietary NSP type and feeding level on the growth performance of the Pacific white shrimp (*Litopenaeus vannamei*) in biofloc systems

Parameters	NSP		SE NSP	Feeding		SEM	P-value	
	PF	MF		IN	IB		NSP	Feeding
Total diet input g	692	676		764	604		---	---
Total protein input g	174	164		189	149		---	---
Total fat input g	44	40		47	37		---	---
Total energy input kJ	12.0	12.4		13.6	10.8		---	---
Total carbohydrate input	343	348		386	305		---	---
								Day 42 (D42)
Final individual body mass g	3.56	3.26	0.10	3.73x	3.09y	0.10	0.07	0.066 0.002
Final biomass g	487	445	27	522x	410y	27	19	0.300 0.018
Individual body mass gain g	3.11	2.81	0.10	3.28x	2.64y	0.10	0.07	0.066 0.002
Total biomass gain g	419	377	26	455x	342y	26	19	0.299 0.018
Total protein gain g	57	56	4	67x	46y	4	3	0.903 0.009
Individual Energy gain kJ	7.5	6.7	0.5	8.1x	6.1y	0.5	0.3	0.299 0.018
Individual daily growth rate (GR) ind	0.074	0.067	0.002	0.078x	0.063y	0.002	0.002	0.065 0.002
Specific growth rate (SGR) %/d	4.91	4.68	0.08	5.03x	4.56y	0.08	0.06	0.080 0.004

Table 5.4. Effect of the dietary NSP type and feeding level on the growth performance of the Pacific white shrimp (*Litopenaeus vannamei*) in biofloc systems (continue)

Parameters	NSP		SE NSP		Feeding		SE feeding	SEM	P-value		
	PF	MF	SE	NSP	IN	IB			NSP	Feeding	NSP*feeding
Feed conversion ratio (FCR)	1.65	1.98	0.25	0.25	1.68	1.95	0.25	0.18	0.376	0.460	0.343
Protein efficiency ratio (PER)	2.42	2.27	0.18		2.41	2.29	0.18	0.13	0.582	0.651	0.337
Protein utilization efficiency (PUE)	33	34	3		36	31	3	2.1	0.837	0.272	0.262
Energy utilization efficiency (EUE)	62	54	4		59	57	4	3.1	0.203	0.658	0.331
Production	0.60	0.54	0.04		0.65 ^x	0.49 ^y	0.04	0.03	0.299	0.018	0.448
Survival	91	90	5		93	88	5	3	0.817	0.400	0.843

Values of D1 are means while values of D42 are means of each treatment. NSP = non-starch polysaccharides, PF = poorly fermentable diet, MF = moderately fermentable diet. SE = standard error, SEM = standard error of the mean. P-value = probability value. Data were analyzed using two-way ANOVA. For each factor (NSP or feeding), different letters in bold in the same row show significant difference (P<0.05).

Table 5.5. Effect of the dietary NSP type and feeding level on the body composition of the Pacific white shrimp (*Litopenaeus vannamei*) in biofloc systems

Parameters	Unit	D1	D42										P-value
			NSP		SE NSP		Feeding		SE feeding		SEM		
			PF	MF	IN	IB	IN	IB	NSP	Feeding	NSP	Feeding	
Dry matter (DM)	g/kg	243 ± 0.5	220	226	6	219	227	6	4.0	0.496	0.360	0.506	
Ash-free DM	g/kg	202 ± 1.1	182	188	5	183	187	5	3.7	0.515	0.562	0.575	
Ash DM	g/kg	170 ± 6.4	172	170	4	167	176	4	2.8	0.823	0.145	0.917	
Crude protein DM	g/kg	559 ± 59.3	617	641	17	666x	592y	17	12.0	0.344	0.015	0.726	
Crude fat DM	g/kg	51 ± 14.4	55a	47b	2	45y	58x	2	1.4	0.016	0.002	0.056	
Crude fibre DM	g/kg	46.7 ± 5.94	85.2	87.5	6.0	83.5	89.3	6.0	4.27	0.797	0.520	0.527	
Energy DM	kJ/g	77.2 ± 1.39	82.2	78.5	1.8	82.0	78.7	1.8	1.27	0.179	0.228	0.853	
Nitrogen-free extract DM	g/kg	174 ± 61.3	71	54	22	39	86	22	15.6	0.608	0.175	0.758	
Carbohydrate DM	g/kg	220 ± 67.3	156	142	18	123	175	18	13.0	0.596	0.080	0.567	
Carbon (C) DM	g/kg	408 ± 4.3	407	405	2	406	406	2	1.7	0.607	0.925	0.841	

Table 5.5. Effect of the dietary NSP type and feeding level on the body composition of the Pacific white shrimp (*Litopenaeus vannamei*) in biofloc systems (continue)

Parameters	D1	D42								P-value			
		NSP		SE		Feeding		SEM					
		PF	MF	NSP	SE	IN	IB	feeding	SE		NSP	Feeding	NSP*Feeding
C:N ratio (mass)	4.59 ± 0.439	4.14	3.98	0.12	0.12	3.82	4.30	4.30	0.12	0.082	0.348	0.019	0.837
Calcium g/kg DM	193 ± 8.1	183	188	11	11	187	184	184	11	7.7	0.780	0.872	0.253
Magnesium g/kg DM	18.62 ± 0.438	19.46	19.62	0.92	0.92	19.88	19.21	19.21	0.92	0.648	0.903	0.619	0.248

Values of D1 are means and the standard deviation of the mean (sd) of each diet, while values of D42 are means of each diet. D1 = day-1, D42 = day-42, NSP = non-starch polysaccharides, PF = poorly fermentable diet, MF = moderately fermentable diet, SE = standard error, SEM = standard error of the mean, P-value = probability value. Data of D42 were analyzed using two-way ANOVA. For each factor (NSP or feeding), different letters in bold in the same row show significant difference ($P < 0.05$).

Table 5.6. Effect of the dietary NSP type and feeding level on biofloc quality, quantity, and activity during the experiment

Parameters	D1			NSP			Feeding			Time			SEM			P-value		
	Mean	sd		PF	MF	IN	IB	D21	D42	NSP (N)	Feeding (F)	Time (T)	N*F	T*N	T*F	T*N*F		
Ash-free DM	533	13		607	585	594	598	597	615	7	0.147	0.878	0.966	0.139	0.506	0.292		
Ash	454	13		370	391	383	379	378	364	6	0.130	0.832	0.697	0.222	0.665	0.401		
Crude protein	261	9		321	289	327 b	283 a	307	325	9	0.115	0.207	0.066	0.271	0.066	0.171		
Crude fat	0	0		159	145	149	155	169 a	115 b	12	0.566	0.822	0.632	0.349	0.518	0.740		
Carbohydrate	739	9		520	566	524	562	524	560	17	0.215	0.225	0.468	0.228	0.153	0.335		
Carbon (C)	492	0		522	520	520	522	525 a	513 b	2	0.690	0.012	0.470	0.400	0.666	0.864		
C:N ratio (mass)	12	0		11	12	10	12	11	10	0.4	0.220	0.219	0.128	0.149	0.147	0.326		
Calcium	209	35		166	171	152 b	185 a	209 a	118 b	6	0.714	0.000	0.607	0.965	0.443	0.943		
Magnesium	68	13		44	44	41	47	63 a	22 b	2	0.894	0.077	0.682	0.801	0.094	0.763		
Phosphorous	4	1		6	7	7	6	7	6	0.5	0.560	0.227	0.965	0.090	0.213	0.821		

Table 5.6. Effect of the dietary NSP type and feeding level on biofloc quality, quantity, and activity during the experiment (continue)

Parameters	D1				Feeding				Time		P-value					
	Mean	sd	PF	MF	IN	IB	D21	D42	SEM (N)	NSP (N)	Feeding (F)	Time (T)	N*F	T*N	T*F	T*N*F
Total suspended solid (TSS)	147	25	201	197	210	188	167 b	242 a	6	0.791	0.102	0.000	0.662	0.356	0.832	0.899
Volatile suspended solid (VSS)	97	3	129	123	129	122	108 b	157 a	4	0.422	0.338	0.001	0.758	0.392	0.364	0.876
Chlorophyll-a	472	9	367	353	352	369	338 b	485 a	26	0.797	0.747	0.006	0.892	0.588	0.331	0.939
COD	200	14	459	506	528	437	444	496	34	0.507	0.209	0.689	0.398	0.294	0.304	0.606

Values of D1 are means and the standard deviation of the mean (sd) of each diet, while values of D42 are means of each diet. D1 = day-1, D42 = day-42, dm = dry matter, AFDM = ash free dry matter, NSP = non-starch polysaccharides, IN-PF = poorly fermentable diet fed at iso-nitrogenous, IN-MF = moderately fermentable diet fed at iso-nitrogenous, IB-PF = poorly fermentable diet fed at equal biomass, IB-MF = moderately fermentable diet fed at equal biomass, N*F = interaction effect between NSP and feeding, T*N = interaction effect between time and NSP, T*F = interaction effect between time and feeding, T*N*F = interaction effect between time, NSP and feeding. SEM = standard error of the mean, P-value = probability value. Data of D21 and D42 were analyzed using repeated measure ANOVA with two between-subject factors. For the factors (NSP, feeding or time time), different letters in bold in the same row show significant difference (P<0.05).

Table 5.7. Effect of the dietary NSP type and feeding level on the crude fibre, energy, nitrogen free extract, biological oxygen demand, and biodegradability index of biofloc during the experiment

Parameters	D1		D42					P-value		
	mean	sd	IN-PF	IN-MF	IB-PF	IB-MF	SEM	NSP	Feeding	NSP*feeding
Crude fibre g/kg dm			427a	232b	239b	280ab	20	0.094	0.122	0.019
Energy g/kg dm			16b	21a	19ab	17ab	0.5	0.139	0.580	0.008
NFE mg/L			61b	319ab	395a	301ab	36	0.295	0.062	0.042
BOD ₅ mg/L	99	5	167	230	137	217	29	0.255	0.720	0.890
Biodegradability index			0.35	0.33	0.34	0.50	0.04	0.401	0.336	0.275

Values of D1 are means and the standard deviation of the mean (sd) of each diet, while values of D42 are means of each diet. D1 = day-1, D42 = day-42, dm = dry matter, BOD₅ = biological oxygen demand, IN-PF = poorly fermentable diet fed at iso-nitrogenous, IN-MF diet = moderately fermentable diet fed at iso-nitrogenous, IB-PF = poorly fermentable diet fed at equal biomass, IB-MF diet = moderately fermentable diet fed at equal biomass. P-value = probability value. Data of D42 were analyzed using one-way ANOVA. Different letters in bold in the same row show significant difference (P<0.05).

3.4. Water quality

The alkalinity in tanks fed the MFdiet was 9% lower than in tanks fed the PFdiet ($P < 0.05$), while other water quality parameters were not affected by the “NSP” type (**Table 5.8**). When NSP-rich diets were fed iso-nitrogenously (IN-PF and IN-MF), the TIN concentration was higher than when feeding at *as-is* basis (IB-PF and IB-MF) ($P < 0.05$, **Table 5.8**). When comparing NSP-rich diets with the STdiet, no differences in $\text{NO}_2\text{-N}$, TAN, CO_2 , PO_4 and TIC were observed ($P < 0.05$, **Table S5.5**). However, the $\text{NO}_3\text{-N}$ concentration in STdiet treatment was higher than in the IB-PF treatment, while intermediate for the other treatments ($P < 0.05$; **Table S5.5**). Overall, water quality parameters changed during the experiment, with alkalinity, PO_4 and TIC decreasing ($P < 0.05$), and $\text{NO}_3\text{-N}$, TAN, CO_2 , and TIN increasing between D21 and D42 in all 5 treatments ($P < 0.05$; **Table S5.5**).

3.5. Nutrient balance

The nitrogen and carbon mass balances of biofloc tanks per treatment on D1, D21 and D42 are shown in **Figure 5.1**. The same data are shown as a percentage of the total nitrogen or carbon input in **Figure 2**. The nitrogen input through feeding in the IN (IN-PF and IN-MF) and STdiet treatment tanks was 21-22% higher than tanks fed at *as-is* basis (IB-PF and IB-MF) (**Figure 5.1a**). In contrast, the carbon input to STdiet treatment tanks was similar with the carbon input to tanks fed at *as-is* basis (**Figure 5.1b**). At the end of the culture period, the amount of N present in shrimp biomass in IN-MF treatment tanks was higher than that in IB-MF treatment tanks, while intermediate for the other treatments ($P < 0.05$; **Figure 5.1a**).

Up to 84-94% of the nitrogen fed + present in the system on D1 was still present on D42, mostly in shrimp and water (**Figure 5.2a**), with the highest retention in the STdiet and IN-MF treatments. The carbon loss was higher in the NSP-rich dietary treatments than in the STdiet treatment (**Figure 5.1b**). On average, 30-35% and 45% of the carbon input was still present on D42 in the NSP-rich diet and STdiet treatment tanks, respectively, mainly in biofloc and shrimp biomass (**Figure 5.2b**).

Table 5.8. Effect of the dietary NSP type and feeding level on the water quality

Parameters	D1	NSP				Feeding				Time			P-value			
		PF	MF	IN	IB	IB	IN	D21	D42	SEM	NSP (N)	Feeding (F)	Time (T)	N*F	T*N	T*F
Alkalinity mg/L	359	184a	168b	170	183	221a	131b	3	0.023	0.064	0.000	0.377	0.424	0.017	0.430	
NO ₃ -N mg/L	0.02	10.1	11.4	11.4	10.1	6.9b	14.6a	0.3	0.056	0.051	0.000	0.851	0.726	0.085	0.196	
NO ₂ -N mg/L	0.02	1.0	1.0	1.2	0.8	0.9	1.0	0.1	0.990	0.101	0.583	0.377	0.736	0.422	0.145	
TAN mg/L	0.04	0.8	0.9	0.9	0.8	0.6b	1.1a	0.1	0.623	0.524	0.002	0.876	0.380	0.397	0.388	
CO ₂ mg/L	22.1	11.6	15.0	13.3	13.3	10.6b	16.0a	0.9	0.083	0.998	0.009	0.296	0.380	0.334	0.296	
PO ₄ mg/L	0.01	7.2	7.1	7.2	7.0	7.7a	6.5b	0.2	0.822	0.603	0.011	0.500	0.732	0.499	0.409	
TIN mg/L	0.07	11.9	13.2	13.4a	11.7b	8.4b	16.7a	0.3	0.068	0.024	0.000	0.664	0.738	0.029	0.386	
TIC mg/L	49	25.3	24.3	24.0	25.5	29.5a	20.1b	0.5	0.348	0.181	0.000	0.903	0.156	0.003	0.133	

Values are means and the standard deviation of the mean (sd) of each diet. D1 = day-1, D42 = day-42, IN-PF = poorly fermentable diet fed at iso-nitrogenous, IN-MF diet = moderately fermentable diet fed at iso-nitrogenous, IB-PF = poorly fermentable diet fed at equal biomass, IB-MF diet = moderately fermentable diet fed at equal biomass, N*F = interaction effect between NSP and feeding, T*N = interaction effect between time and NSP, T*F = interaction effect between time and feeding, T*N*F = interaction effect between time, NSP and feeding. P-value = probability value, TAN = total ammonia nitrogen, TIN = total inorganic nitrogen, TIC = total inorganic carbon. Data of D21 and D42 were analyzed using repeated measure ANOVA with two between-subject factors. For the factors (NSP, feeding or time time), different letters in bold in the same row show significant difference (P<0.05).

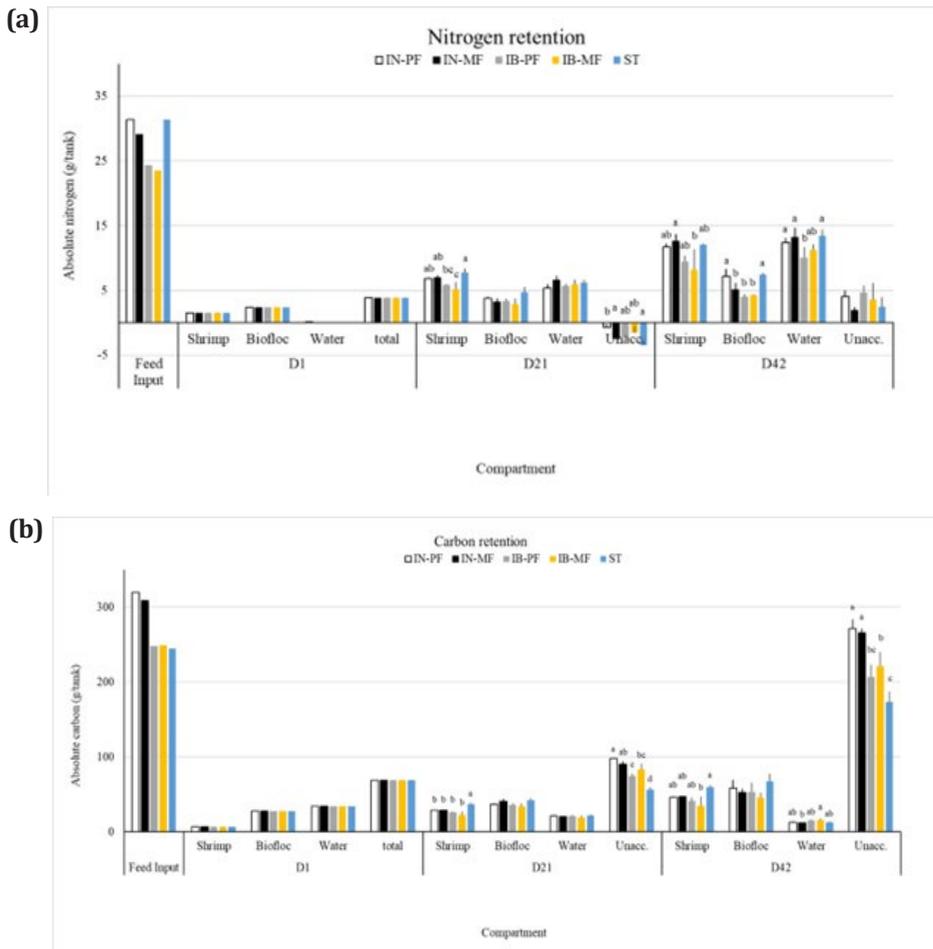


Figure 5.1. Effect of the dietary NSP supplementation on (a) nitrogen and (b) carbon distribution in the biofloc systems (g absolute amount/tank). Values are means and standard deviations of the mean of each diet at each sampling time. D1 = day-1, D21 = day-21, D42 = day-42, IN-PF = poorly fermentable diet fed at iso-nitrogenous, IN-MF diet = moderately fermentable diet fed at iso-nitrogenous, IB-PF = poorly fermentable diet fed at equal biomass, IB-MF diet = moderately fermentable diet fed at equal biomass, ST = starch diet. Unacc. = unaccounted nutrient loss (total nutrient input – total nutrient present in the system) at a sampling time. The total nutrient input = total nutrient from feed + total initial nutrient present in the system. Feed input = total nutrient input from feed given. Data were analyzed using one-way ANOVA, comparing treatment at each system compartment in a sampling time. Different letters on top of the bar showed significant difference ($P < 0.05$). The feed input has no variation within diet treatment, and therefore was not statistically analyzed.

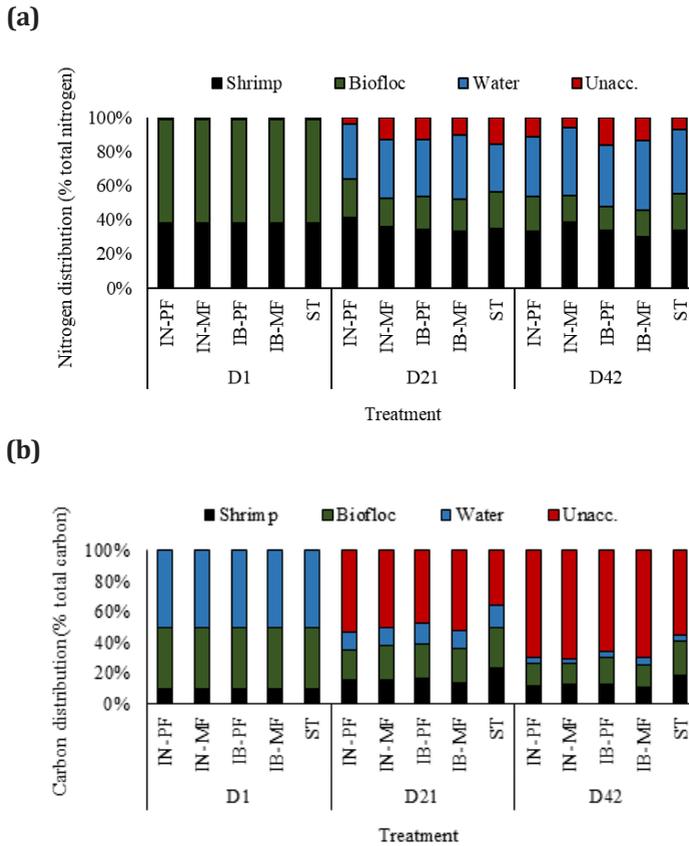


Figure 5.2. The effect of the NSP supplementation on the distribution of nitrogen (a) and carbon (b) in biofloc systems (in percentage of total particular nutrient at each sampling time). Values are means of each diet at each sampling time. D1 = day-1, D21 = day-21, D42 = day-42, IN-PF = poorly fermentable diet fed at iso-nitrogenous, IN-MF = moderately fermentable diet fed at iso-nitrogenous, IB-PF = poorly fermentable diet fed at equal biomass, IB-MF = moderately fermentable diet fed at equal biomass, ST = starch diet. Unacc. = percentage of unaccounted nutrient loss to total nutrient present in the system.

3.1. Microbial composition

The number of reads and the alpha diversity indices are presented in **Table S5.6** and **Table S5.7**, respectively. After removing the low-quality read, for prokaryotes, the effective reads varied between 107,992-121,360, while for fungi they varied between 125,801-168,713 (**Table S5.6**). The Shannon diversity index and the phylogenetic diversity for prokaryotes varied between 6.48-7.36 and 122-187, respectively, while for fungi between 0.39-2.96 and, 17-28, respectively (**Table S5.7**). The IB-PF and IN-MF showed highest diversity indices in the prokaryotic and fungal communities,

respectively. However, a statistical analysis for comparing treatments could not be performed because the biological replicates were pooled due to budget constraints. A total of 740 OTUs were shared among the 5 treatments in the prokaryotic community, while 25 OTUs were shared in the fungal community (**Figure S5.1**). The STdiet had a relatively high number of unique OTUs in the prokaryotic community, but a relatively low number in the fungal community compared to the NSP-rich diet treatments (**Figure S5.1**).

The relative abundance of the most abundant phyla and genera of the prokaryotic and the fungal communities are presented in **Figure 5.3** and **Figure S5.2**, respectively. In all treatments, the most abundant phyla in the prokaryotic community were *Proteobacteria*, *Cyanobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Verrucomicrobia*, *Firmicutes*, *Acidobacteria*, *Gemmatimonadetes* and *Planctomycetes*; comprising 98.9-99.4% of the total abundance. Cyanobacteria was more abundant, while *Chloroflexi* and *Actinobacteria* were less abundant in the STdiet treatment tanks compared to NSP-diet treatment tanks (**Figure 5.3**). In the fungal community, most OTUs were not identified, and from those that were characterized *Ascomycota* and *Xeromyces* were the most abundant phylum and genus, respectively (**Figure S5.2**).

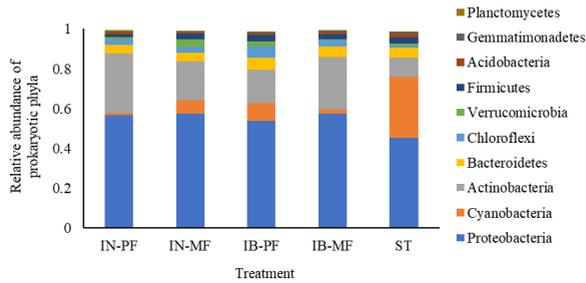
4. Discussion

4.1. Nutrient digestibility and faecal composition

The digestibility of crude protein and crude fat of the NSP-rich diets (PFdiet and MFdiet) were lower than that of the STdiet, as observed by (González-Peña et al., 2002) for cellulose in fresh water prawn (*Macrobrachium rosenbergii*) and by (Smith et al., 2007) for lupin kernel meal, an NSP-rich feed ingredient, in diets for giant tiger shrimp. The reason behind this may be due to similar mechanisms reported in fish; NSP reduces the nutrient digestibility by increasing digesta viscosity and reducing the mixing of digestive enzymes and the chyme in the gut (Sinha et al., 2011). The NSP-rich ingredients were also a source of dietary protein with wheat-DDGS containing 16%, palm kernel meal 32% and wheat bran 15% protein, which might have been less digestible considering the NSP content of these ingredients, causing the overall protein ADC to drop compared to the STdiet (CVB, 2022).

Surprisingly, the carbohydrate digestibility was low for all experimental diets, ranging from 13–20%. This was unexpected considering the STdiet contained corn and tapioca starch, which are normally better digested than fiber-rich ingredients (Brunson et al., 1997). Such low carbohydrate digestibility might be due to the high starch content in the STdiet, as it contained in total 38% starch-based ingredients (corn starch and tapioca starch, **Table 1**). Velurtas et al. (2011) observed a decrease in digestibility of protein

(a)



(b)

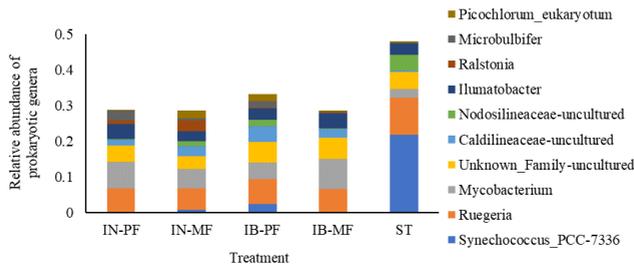


Figure 5.3. Top ten taxa relative abundance in (a) phylum and (b) genera of the prokaryotes; IN-PF = poorly fermentable diet fed at iso-nitrogenous, IN-MF = moderately fermentable diet fed at iso-nitrogenous, IB-PF = poorly fermentable diet fed at equal biomass, IB-MF = moderately fermentable diet fed at equal biomass, ST = starch diet.

from 72% to 8% as the ratio between the percentage of starch and the percentage of cellulose in the diet changed from 30:0 to 0:30 in diets for Argentine red shrimp (*Pleoticus muelleri*). Although starch and cellulose were not measured in our study, we expected the STdiet to be quite low in cellulose (only from soybean as main source), and thus this may explain the low carbohydrate digestibility.

For biofloc, the desirable C:N ratio for optimal growth is above 10 (Avnimelech, 1999; Dauda et al., 2017; Silva et al., 2017; Oliveira et al., 2022). In the present study, we achieved such ratio, as the C:N ratios in the faeces of shrimp fed the PFdiet, MFdiet and STdiet were 20, 25 and 29, respectively. Surprisingly, STdiet had the highest C:N ratio. This is explained by the high protein digestibility in the STdiet, which was approximately 20% higher compared to the NSP-rich diets, while at the same time, the faecal C content was similar between the three diets.

4.2. Effect of NSP type and feeding approach on biofloc system performance

4.2.1. Effect of NSP type

The first objective of the study was to investigate the effect of NSP type in the diet for a shrimp-biofloc system, focusing on system performance parameters.

Comparing the digestibility between diets, the protein ADC of the PFdiet was lower than of the MFdiet, which concurs with our hypothesis. The PFdiet contained palm kernel meal and corn straw, ingredients with β -mannan as the main NSP component, followed by cellulose, lignin, xylan, and arabinoxylan (Abdollahi et al., 2016; Sundu et al., 2019). Meanwhile, the MFdiet contained wheat bran and DDGS which have arabinoxylan and cellulose as principal NSP components (Maes and Delcour, 2002; Stevenson et al., 2012; Pedersen et al., 2014). In general, mannan is less soluble in water and less susceptible to hydrolysis by enzymes than arabinoxylan and cellulose, suggesting that the PFdiet will be more difficult to be broken down in the shrimp digestive tract than the MF diet.

Pavasovic et al. (2006) found that in absence of natural food a dietary cellulose content above 12% severely reduced survival, individual growth and feeding efficiency of freshwater red claw crayfish (*Cherax quadricarinatus*) and that inclusion of 30% cellulose in the diet reduce the protein ADC. This was also the case in our experiment, showing on average a 20% lower protein and 18% lower fat ADC for the NSP-rich diets than for the STdiet (**Table 5.3**). However, shrimp performance is less predictable in presence of biofloc. Several studies have shown (Ekasari et al., 2014b; Wang et al., 2016) that adding molasses or tapioca to the biofloc system not via the feed resulted in better culture performance than when doing the same with wheat bran. In contrast, Serra et al. (2015) comparing the addition of wheat bran or dextrose directly to the water of the biofloc system reported no effect on the growth of Pacific white shrimp. Next to the diet composition and C:N ratio, many other factors influence biofloc system performance including feeding level, culture intensity, biofloc concentration, age of biofloc, water quality, the ratio between heterotrophic and autotrophic processes in the microbial community, presence of algae, and water and biofloc turnover rate (Ebeling et al., 2006; Khanjani and Sharifinia, 2020; Tinh et al., 2023). This makes it difficult to compare the results of different studies. To elucidate the role of NSP in aquaculture systems, feeding purified or semi-purified diets might help to get better insight in the mechanisms involved.

Kabir et al. (2020), compared the effect of NSP-rich diets, containing mainly either lignin-cellulose or pectin-hemicellulose, growing Nile tilapia in semi-intensive ponds. The authors found that in absence of natural food, fish biomass gain was similar between diets, but in ponds, feeding the lignin-cellulose diet resulted in a higher contribution of

natural food and a higher total production than when feeding the pectin-hemicellulose diet. The PF and MF diets used in our experiment are both lignin-cellulose rich diets, with the PF diet containing ingredients that are more difficult to digest than the ingredients in the MF diet, as explained above. However, the gut passage time of food in Nile tilapia is much longer than in Pacific white shrimp, and therefore less fermentation might occur in the shrimp gut, affecting the faecal composition (Beseres et al., 2006; Uscanga et al., 2010) and its contribution to natural food production. Due to the much lower ADC of a NSP-rich diet in Pacific white shrimp than in Nile tilapia, a higher fraction of the diet enters the biofloc tank as faeces with shrimp than would have been the case with Nile tilapia. In turn, high post-feeding loading with organic matter through excretion in the well aerated biofloc tank stimulates heterotrophic biofloc formation, which is an excellent natural food (Deng et al., 2021 ; Khanjani et al., 2023). This study shows that despite the lower protein ADC of the PF diet compared to the MF diet, the shrimp performance was not affected (**Table 5.4**). This suggests that the difference in protein digestibility between the PF and MF diets might have been compensated by consumption of biofloc, confirming findings of previous studies (Burford et al., 2004; da Silva et al., 2013; Jatobá et al., 2014).

An additional observation in our study was that MF diet treatments on average resulted in lower alkalinity in the rearing tanks than PF diet treatments (**Table 5.8**). However, no differences were observed in NO₃-N, NO₂-N, TAN, and TIN, or VSS, BOD₅ and the biodegradability index of biofloc (**Tables 5.6 and Table 5.7**). The cumulative interplay between nitrification, denitrification, and immobilization of N species with the MF diet treatments consumed more alkalinity compared to the PF diet treatments (Deng et al., 2020a; Deng et al., 2020b). The faecal C:N ratio of the MF diet was higher than the ratio of the PF diet. In addition, the NSP of the MF diet was more easily fermentable than the NSP in the PF diet and could stimulate more heterotrophic bacterial activity. However, this was not visible in our biofloc and water quality data as also highlighted by other researchers (John et al., 2020; Preena et al., 2021; Ray and Lotz, 2014). Krummenauer et al. (2020) using stable isotopes, showed that biofloc contributed up to 86% to the growth of Pacific white shrimp in a fed intensive biofloc system, and that the percentage biofloc contribution increases with animal size. Using this stable isotope approach in our study might help in better understand the contribution of biofloc to production.

4.2.2. Feeding iso-nitrogenous or as-is basis

More feed was fed iso-nitrogenously resulting in a 23%, 33% and 13% higher individual daily growth rate, production and crude protein body content, respectively, than feeding at *as-is* basis (**Tables 5.4 and Tables 5.5**). With an average of 28% of crude protein per kg DM, the NSP-rich diets contained 7 to 36% less protein than recommended (Rosas

et al., 2001; Tacon and Metian, 2008; Jatobá et al., 2014; Xu and Pan, 2014) (**Table 5.1**). In addition, the crude protein ADC of the PFdiet and MFdiet were respectively 24% and 15% lower than the ADC of the STdiet, reducing the amount of dietary protein available to support direct shrimp growth. The reported biofloc contribution to growth fluctuates between 18 – 86%, depending on system design and shrimp size (Burford et al., 2004; Krummenauer et al., 2022). However, it remains unclear how much biofloc contributed to production. Our expectation was that biofloc would have contributed more to production in treatments fed at *as-is* basis (IB-PF and IB-MF). The VSS biofloc concentration in our study reached 157 mg/L at harvest. Maybe this concentration was too low. More research is needed on the relation between biofloc concentration and feeding efficiency of biofloc for Pacific white shrimp.

4.3. Nutrient balance

The amount of nitrogen retained in biofloc and water at the end of culture period reflected the dietary nitrogen input (**Figure 5.1a**). The nitrogen mainly accumulated in shrimp biomass and as inorganic nitrogen in the water column, mostly as $\text{NO}_3\text{-N}$. The percentage of the nitrogen retention within the system ranged between 84-94% of the nitrogen input, which was high compared to previous studies (77-87%) (Tinh et al., 2021a; Tinh et al., 2021c). Our findings suggest that feeding less nitrogen resulted in higher nitrogen loss (13-16% when feeding at *as-is* basis compared to 6-11% when feeding iso-nitrogenously and 7% in the STdiet treatment; **Figure 5.2a**). In addition, the STdiet also showed the lowest carbon loss compared to the NSP-rich diets: 55% versus 65-70%. This might have been due to the higher carbon retention through algal production which was more abundant in tanks fed the STdiet (**Figure 5.2b**).

4.4. Microbial composition

Microbial composition analysis revealed *Proteobacteria*, *Cyanobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Verrucomicrobia*, *Firmicutes*, *Acidobacteria*, *Gemmatimonadetes* and *Planctomycetes* as the most abundant phyla within the biofloc in this study, as also found in previous studies (Deng et al., 2019; Tinh et al., 2021c; Xu et al., 2022). *Actinobacteria* was found more abundant with the NSP-rich diet treatments compared to the STdiet; this phylum has been associated with NSP-degrading enzymes, such as cellulose (Opazo et al., 2012). On the other hand, a higher abundance of *Cyanobacteria* phyla was found in the STdiet, which could be also correlated with the high concentration of chlorophyll-a content in the biofloc.

A very large proportion of the fungal OTUs could not be identified with the databases used. In addition, the abundance of the most dominant phyla or genera was highly different between our treatments. The most abundant genera observed in our study

(*Xeromyces* and *Purpureocillium*) were also identified in other biofloc studies (Khanjani et al., 2022). The importance of fungi in biofloc formation and biofloc system performance remains underexplored due to limited information and their lower observed abundance compared to bacteria in biofloc (Yun et al., 2022), as also observed in this study.

5. Conclusion

In this study, the fermentability of NSP-containing ingredients did not affect shrimp growth, biofloc quality and quantity, as well as the water quality in the system. In addition, there was a tendency for MFdiet to promote more nitrification and heterotrophic bacterial growth within the system. When feeding iso-nitrogenously, all diets resulted in a similar shrimp growth and biofloc protein content. However, lower unaccounted carbon loss was found in the STDiet (55%) compared to the NSP diets (65-70%) which could have been due to higher CO₂ retention via algal photosynthesis. To explain differences in system performance between NSP-rich dietary treatments, analysis of the contribution of biofloc to shrimp production, for instance by stable isotope analysis, might be the next step towards developing a better understanding of the role of NSP-rich feed ingredients in biofloc systems.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary Table S5.1. Effect of the dietary NSP and starch addition on the growth performance of Pacific white shrimp (*Litopenaeus vannamei*) reared in biofloc tanks

Parameters		DIET					SEM	P-value
		IN-PF	IN-MF	IB-PF	IB-MF	ST		
Total feed input	g	780	749	604	604	604		
Total protein input	g	196	182	152	147	196		
Final individual weight	g	3.77b	3.69b	3.35bc	2.83c	4.80a	0.32	0.001
Final biomass	g	528	516	446	373	543	32	0.051
Protein gain	g	64ab	70a	50bc	42c	66ab	5	0.039
Energy gain	g	8.2a	8.0a	6.7ab	5.4b	8.5a	0.6	0.050
Growth rate (GR)	g/day/ ind	0.079b	0.077b	0.069bc	0.057c	0.103a	0.008	0.001
Specific growth rate (SGR)	%/day	5.06ab	5.01ab	4.77bc	4.35c	5.60a	0.20	0.001
Feed conversion ratio (FCR)		1.69	1.67	1.61	2.30	1.27	0.17	0.418
Protein efficiency ratio (PER)		2.35	2.46	2.49	2.08	2.42	0.07	0.764
Protein utilization efficiency (PUE)	%	33	38	33	29	33	2	0.548
Energy utilization efficiency (EUE)	%	61	58	64	49	75	4	0.156
Production	kg/m ³	0.66a	0.64a	0.54ab	0.44b	0.68a	0.05	0.050
Survival	%	94	93	89	86	76	3	0.477

Values are means of replicates in each treatment. IN-PF = poorly fermentable diet fed iso-nitrogenously, IN-MF diet = moderately fermentable diet fed iso-nitrogenously, IB-PF = poorly fermentable diet fed iso-biomass, IB-MF diet = moderately fermentable diet fed iso-biomass, ST = starch diet, bw = bodyweight. Data were analyzed using one-way ANOVA. Different letters in bold in the same row show significant difference ($P < 0.05$).

Supplementary Table S5.2. Effect of the dietary NSP and starch addition on the body composition of the *Litopenaeus vannamei* in biofloc systems

Parameters	Unit	D1	D42					SEM	P-value
			IN-PF	IN-MF	IB-PF	IB-MF	ST		
Dry matter (DM)	g/kg wet	243 ± 1	214 b	225 b	227 b	227 b	266 a	9	0.019
Ash-free DM	g/kg wet	202 ± 1	178 b	187 ab	187 ab	188 ab	221 a	7	0.039
Ash	g/kg dm	170 ± 6	167	166	176	175	170	2	0.577
Crude protein	g/kg dm	559 ± 59	650 a	683 a	584 ab	600 ab	520 b	28	0.013
Crude fat	g/kg dm	51 ± 14	46 b	44 b	65 a	50 b	74 a	6	0.000
Crude fibre	g/kg dm	47 ± 6	85	82	85	93	93	2	0.841
Energy	kJ/g dm	77 ± 1	84 a	80 ab	80 a	77 ab	68 b	3	0.023
Nitrogen-free extract	g/kg dm	174 ± 61	52	26	89	82	143	20	0.215
Carbohydrate	g/kg dm	220 ± 67	138 ab	108 b	174 ab	175 ab	236 a	22	0.081
Carbon (C)	g/kg dm	408 ± 1	406	405	407	405	414	2	0.392
C:N ratio (mass)		5 ± 1	3.9 b	3.7 b	4.4 ab	4.2 ab	5.0 a	0.2	0.008
Calcium	g/kg dm	193 ± 6	194	179	172	196	169	5	0.698
Magnesium	g/kg dm	19 ± 59	20.6	19.2	18.3	20.1	16.4	1	0.294
Phosphorous	g/kg dm	46 ± 14	54	49	50	52	45	1	0.288

Values are means and the standard deviation of the mean (sd) of each diet. D1 = day-1, D42 = day-42, IN-PF = poorly fermentable diet fed at iso-nitrogenous, IN-MF diet = moderately fermentable diet fed at iso-nitrogenous, IB-PF = poorly fermentable diet fed at equal biomass, IB-MF diet = moderately fermentable diet fed at equal biomass, ST = starch diet. P-value = probability value. Data of the D42 were analyzed using one-way ANOVA. Different letters in bold in the same row show significant difference (P<0.05).

Supplementary Table S5.3. Effect of the dietary NSP and starch addition on biofloc quality and quantity during the experiment

Parameters	D1		Treatment					Time			P-value		Treatment* time
	mean	sd	IN-PF	IN-MF	IB-PF	IB-MF	ST	D21	D42	SEM	Treatment	Time	
Ash-free DM	533	12.8	605	584	610	587	646	597	615	6	0.091	0.303	0.129
Ash	454	13.1	375	391	366	392	330	378	364	5	0.051	0.449	0.262
Crude protein	261	9.4	362a	292bc	280c	286c	358ab	307	325	9	0.040	0.270	0.140
Crude fat	0	0.0	150	148	168	141	103	169a	115b	11	0.564	0.022	0.636
Carbohydrate	739	9.4	488	560	552	572	539	524	560	17	0.544	0.239	0.300
Carbon (C)	492	0.3	519	521	525	520	510	525a	513b	2	0.384	0.015	0.692
C:N ratio (mass)	11.8	0.42	9.1	11.5	12.0	11.7	9.0	11.0	10.3	0.4	0.076	0.278	0.209
Calcium	209	35.1	153	151	180	190	142	209a	118b	5	0.064	0.000	0.936
Magnesium	68	12.7	41	40	46	48	37	63a	22b	1	0.184	0.000	0.314
Phosphorous	4.5	1.03	6.7	7.3	5.5	6.2	8.0	7.4	6.1	0.6	0.726	0.126	0.276

Supplementary Table S5.3. Effect of the dietary NSP and starch addition on biofloc quality and quantity during the experiment (continue)

Parameters	D1		Treatment					Time			P-value		
	mean	sd	IN-PF	IN-MF	IB-PF	IB-MF	ST	D21	D42	SEM	Treatment	Time	Treatment * time
Total suspended solid (TSS)	147	25	209	211	192	184	226	167 b	242 a	5	0.217	0.000	0.917
Volatile suspended solid (VSS)	97	3.1	131 ab	128 ab	126 ab	118 b	160 a	108 b	157 a	3	0.048	0.000	0.491
Chlorophyll-a	472	9.5	362	341	372	366	618	338 b	485 a	25	0.060	0.002	0.554
COD	200	14.1	475	582	443	430	420	444	496	33	0.535	0.408	0.506

Values of D1 are means and the standard deviation of the mean (sd) of each diet, while values of D42 are means of each diet. D1 = day-1, D42 = day-42, dm = dry matter, AFDM = ash-free dry matter, IN-PF = poorly fermentable diet fed at iso-nitrogenous, IN-MF diet = moderately fermentable diet fed at iso-nitrogenous, IB-PF = poorly fermentable diet fed at equal biomass, IB-MF diet = moderately fermentable diet fed at equal biomass, ST = starch diet. SEM = standard error of the mean, P-value = probability value. Data of D21 and D42 were analyzed using repeated measure ANOVA. For the factors (treatment and time), different letters in bold in the same row show significant difference ($P < 0.05$).

Supplementary Table S5.4. Effect of the dietary NSP and starch addition on the crude fiber, energy, nitrogen free extract, biological oxygen demand, and biodegradability index of biofloc during the experiment

Parameters	D1		D42				SEM	P-value	
	mean	sd	IN-PF	IN-MF	IB-PF	IB-MF			ST
Crude fibre g/kg DM			427a	232ab	239ab	280ab	186b	41	0.030
Energy g/kg DM			16b	21a	19ab	17ab	15b	1.1	0.013
NFE mg/L			61	319	395	301	361	59	0.105
BOD ₅ g/kg AFDM	99	5	167	230	137	217	170	17	0.255
Biodegradability index			0.35	0.33	0.34	0.50	0.34	0.03	0.401

Values of D1 are means and the standard deviation of the mean (sd) of each diet, while values of D42 are means of each diet. D1 = day-1, D42 = day-42, BOD₅ = biological oxygen demand, dm = dry matter, IN-PF = poorly fermentable diet fed at iso-nitrogenous, IN-MF diet = moderately fermentable diet fed at iso-nitrogenous, IB-PF = poorly fermentable diet fed at equal biomass, IB-MF diet = moderately fermentable diet fed at equal biomass, ST = starch diet. P-value = probability value. Data of D42 were analyzed using one-way ANOVA. Different letters in bold in the same row show significant difference (P<0.05).

Supplementary Table S5.5. Effect of the dietary NSP and starch addition on the water quality

Parameters	Unit	D1	Treatment							SEM	P-value	Treatment * time	
			IN-PF	IN-MF	IB-PF	IB-MF	STdiet	D21	D42				
Alkalinity	mg/L	359	176	165	193	172	180	225a	129b	3	0.105	0.000	0.056
NO ₃ -N	mg/L	0.02	10.7ab	12.0ab	9.6b	10.7ab	12.4a	6.9b	15.2a	0.3	0.042	0.000	0.148
NO ₂ -N	mg/L	0.02	1.07	1.26	0.89	0.70	0.64	0.85	0.97	0.09	0.248	0.441	0.474
TAN	mg/L	0.04	0.87	0.91	0.77	0.85	0.98	0.74b	1.02a	0.06	0.869	0.010	0.054
CO ₂	mg/L	22.1	12.6	14.0	10.7	15.9	11.2	10.7b	15.1a	0.7	0.238	0.013	0.360
PO ₄	mg/L	0.01	7.40	7.03	6.91	7.09	7.45	7.80a	6.55b	0.18	0.836	0.004	0.799
TIN	mg/L	0.07	12.7ab	14.2a	11.2b	12.2ab	14.0ab	8.5b	17.2a	0.3	0.032	0.000	0.122
TIC	mg/L	49	24.5	23.6	26.1	25.0	24.6	29.9a	19.6b	0.5	0.574	0.000	0.005

Values are means and the standard deviation of the mean (sd) of each diet. D1 = day-1, D42 = day-42, IN-PF = poorly fermentable diet fed at iso-nitrogenous, IN-MF diet = moderately fermentable diet fed at iso-nitrogenous, IB-PF = poorly fermentable diet fed at equal biomass, IB-MF diet = moderately fermentable diet fed at equal biomass, ST = starch diet. P-value = probability value, TAN = total ammonia nitrogen, TIN = total inorganic nitrogen, TIC = total inorganic carbon. Data of D21 and D42 were analyzed using repeated measure ANOVA. For the factors (treatment and time), different letters in bold in the same row show significant difference (P<0.05).

Supplementary Table S5.6. Clean data of the prokaryotic and fungal communities

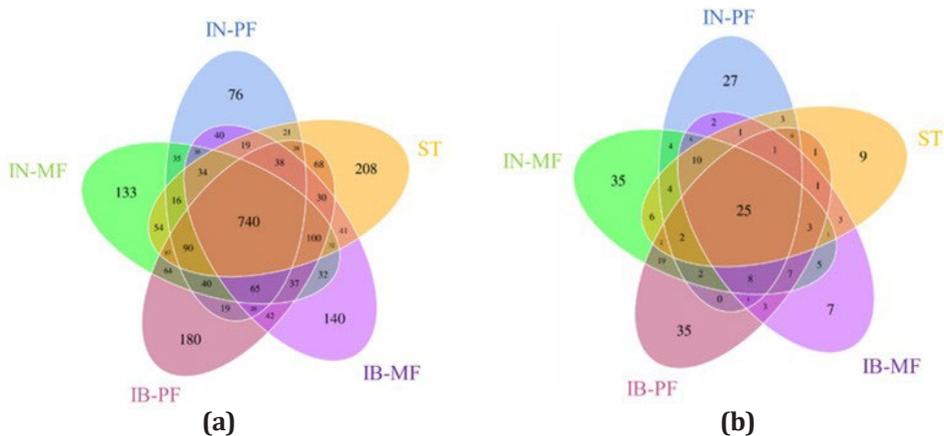
Sample	Raw PE (#)	Combined (#)	Qualified (#)	Nochime (#)	Base (nt)	AvgLen (nt)	Q20	Q30	GC%	Effective %
<i>Prokaryotic</i>										
IN-PF	164,972	162,032	160,410	120,766	49,741,547	412	99.03	96.57	55.69	73.2
IN-MF	178,847	174,453	172,513	121,360	49,961,335	412	98.95	96.34	55.21	67.86
IB-PF	167,783	163,694	161,942	107,992	44,513,817	412	98.98	96.39	55.07	64.36
IB-MF	174,609	171,685	169,840	120,852	49,789,175	412	99.04	96.56	55.35	69.21
ST	173,520	170,680	168,875	117,844	48,353,896	410	98.99	96.45	54.77	67.91
<i>Fungi</i>										
IN-PF	168,983	163,903	163,569	162,923	54,302,796	333	99.02	96.63	58.72	96.41
IN-MF	177,909	161,250	160,283	159,891	59,152,941	370	98.3	94.37	57.91	89.87
IB-PF	162,260	148,295	125,825	125,801	46,093,069	366	98.36	94.63	57.2	77.53
IB-MF	171,355	162,819	162,477	162,058	54,078,292	334	98.82	96.14	58.78	94.57
ST	175,949	169,182	168,875	168,713	55,772,788	331	98.99	96.69	58.42	95.89

IN-PF = poorly fermentable diet fed at iso-nitrogenous, IN-MF diet = moderately fermentable diet fed at iso-nitrogenous, IB-PF = poorly fermentable diet fed at equal biomass, IB-MF diet = moderately fermentable diet fed at equal biomass, ST = starch diet. Raw PE represents the original PE reads after sequencing; Raw Tags represents tags merged from PE reads; Clean Tags represents tags after filtering; Effective Tags represents tags after filtering chimera and can be finally used for subsequent analysis; Base is the number of bases of the Effective Tags; AvgLen represents average length of Effective Tags; Q20 and Q30 are the percentages of bases whose quality value in Effective Tags is greater than 20 (sequencing error rate is less than 1%) and 30 (sequencing error rate is less than 0.1%); GC (%) represents GC content in Effective Tags; Effective (%) represents the percentage of Effective Tags in Raw PE.

Supplementary Table S5.7. Alpha diversity index of the prokaryotic and fungal communities

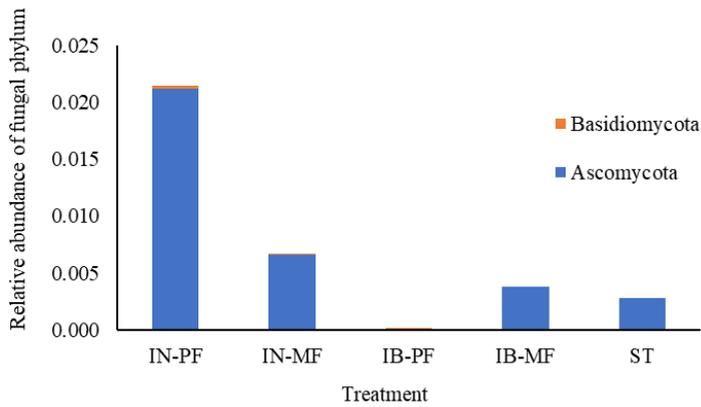
	Observed species*	Shannon index	Phylogenetic diversity
<i>Prokaryotic</i>			
IN-PF	1311	6.96	136.0
IN-MF	1569	7.04	186.1
IB-PF	1628	7.36	212.6
IB-MF	1440	7.11	122.3
ST	1586	6.48	186.7
<i>Fungi</i>			
IN-PF	96	1.18	19.7
IN-MF	139	2.96	28.0
IB-PF	110	2.81	27.6
IB-MF	84	1.57	19.0
ST	72	0.39	16.5

IN-PF = poorly fermentable diet fed at iso-nitrogenous, IN-MF diet = moderately fermentable diet fed at iso-nitrogenous, IB-PF = poorly fermentable diet fed at equal biomass, IB-MF diet = moderately fermentable diet fed at equal biomass, ST = starch diet. *OTUs generated at 97% sequence identity are homologous in species.

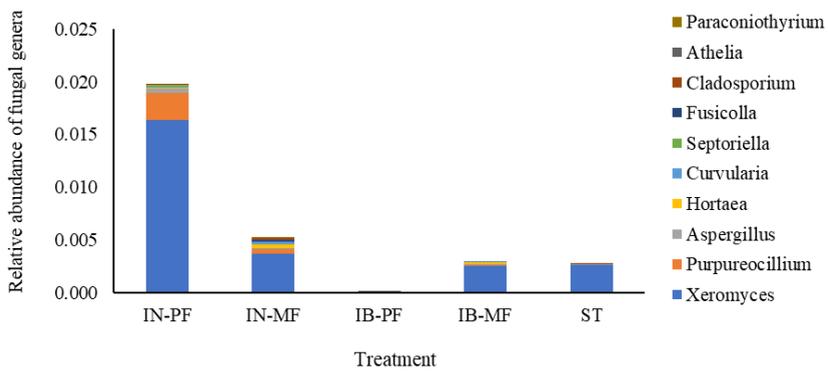


Supplementary Figure S5.1. Venn diagram of (a) the prokaryotes and (b) the fungi. IN-PF = poorly fermentable diet fed at iso-nitrogenous, IN-MF diet = moderately fermentable diet fed at iso-nitrogenous, IB-PF = poorly fermentable diet fed at equal biomass, IB-MF diet = moderately fermentable diet fed at equal biomass, ST = starch diet.

(a)



(b)



Supplementary Figure S5.2. Top ten taxa relative abundance in (a) phylum and (b) genera of the fungi. IN-PF = poorly fermentable diet fed at iso-nitrogenous, IN-MF = moderately fermentable diet fed at iso-nitrogenous, IB-PF = poorly fermentable diet fed at equal biomass, IB-MF = moderately fermentable diet fed at equal biomass, ST = starch diet.





6

General discussion

Shrimp culture started by collecting post larvae (PL) and juveniles in estuaries and stocking them in coastal ponds. Pond production was enhanced by introducing allochthonous nutrients from inexpensive sources, such as poultry and agricultural by-products, to enhance the amount of natural food available for the shrimp (Kang'ombe et al., 2007). Relying solely on natural biota to feed the shrimp allowed farmers to produce on average 300 kg/ha stocking 4-5 PL/m² (Hariati et al., 1998). Further intensification involved formulated feed administration, which started around 1980, and since then gradually expanded. The expectation is that by 2025, the shrimp industry will administrate 10.5 million metric ton of commercial feed, involving nearly all shrimp farms (Tacon, 2020). The optimal protein level in commercial feed for Pacific white shrimp varies, depending on animal size, between 30-35% of which between 34-44% is retained in shrimp body mass gain (Saraswathy et al., 2013; Lee and Lee, 2018). The rest, including uneaten feed, faeces and egestion accumulates in the culture system, including inorganic and organic nitrogen substances. Without sanitation, accumulated waste reduce the water quality, and toxicity might develop, potentially killing the shrimp.

Various culture systems have been developed to keep the water quality supportive to shrimp welfare and production, such as continuous water replacement (i.e. raceways, cages), *in situ* waste treatment (e.g. extensive ponds, biofloc ponds, recirculation systems), or by *ex situ* waste (re)utilization (e.g. integrated multitrophic systems, aquaponics, hydroponics) (Verdegem et al., 2023). Shrimp is predominantly produced in ponds in presence of natural biota. Biofloc technology has been considered the holy grail of shrimp farming, because it allows intensification, while maintaining good water quality, and because it turns waste into biofloc, a nutritious natural food for Pacific white shrimp. As such, biofloc systems facilitate *in situ* nutrient recycling. However, despite being studied for over 35 years, biofloc technology has not emerged as the leading technology for global shrimp production, although it is still considered a promising tool to improve aquaculture production (Khanjani and Sharifinia, 2020). The principal aim of this research project was to simplify the system management by formulating pelleted shrimp feeds that nourish both the shrimp and the biofloc. In doing so, some of the key bottlenecks facing biofloc system technology were addressed, including (1) the need to manage a complex mixotrophic microbial system, requiring high quality management skills and labour, (2) the need to avoid excessive CO₂ losses, requiring to reduce carbohydrate inputs and to improve carbohydrate type selection, (3) getting a better understanding of the microbial ecology in order to develop a better grip on microbiota management, and (4) enhanced waste re-utilization through selection of feed ingredients and of *in situ* accumulated waste. In addition, attention was given to

working with mature biofloc. This leads to the following sub-chapters: labour force quality and demand, carbohydrate input, biofloc microbiota, circularity and mature biofloc. The chapter ends highlighting the societal relevance of the research outcomes for the farmer, the feed producer and the environment.

1. Labour force quality and demand

Changing from a conventional pond setup to a biofloc system involves three main practical steps: introducing an external carbohydrate into the rearing tank, ensuring continuous aeration and water mixing, and reducing water exchange. In biofloc systems, each day the shrimp are fed pelleted feed and carbohydrate is added to the water in the rearing tank (Avnimelech, 2015). While these steps seem straightforward, they demand an in-depth understanding of the biological processes involved to be able to balance the feed and carbohydrate inputs correctly, so that the water quality is maintained appropriate for farming. Large and resourceful companies can hire sufficient qualified staff to manage and operate biofloc systems, raising the overall operational cost. Even if, for example, an industrial-based company can afford it, the risks of system collapse or human errors are always present and could compromise shrimp production. For smaller, less resourceful companies and for small-scale farmers, biofloc system management and operation is even more challenging. Farmers need to accurately calculate the required carbohydrate input to counter nitrogen waste, which varies with feeding level, feed composition, type of carbohydrate, culture intensity and system maturity (Oliveira et al., 2022). Nitrogen waste level fluctuation can also be associated with sudden changes in water quality, for example due to heavy rain. Sometimes this leads to peaks in ammonia or nitrite concentrations that might cause shrimp mortality (Crockett and Lawrence, 2017). Overall, biofloc technology is undoubtedly more laborious and challenging than simply exchanging water, as practiced in conventional pond rearing systems.

Biofloc system management primarily involves providing sufficient carbohydrate to fuel heterotrophic bacterial respiration. This can be done in two ways: directly via the water or indirectly via the formulated shrimp diet (López-Elías et al., 2014; Avnimelech, 2015; Braga et al., 2016). The latter is not common practice, as it requires the carbohydrate to pass through the shrimp's gastro-intestinal tract (GIT) in order to reach the biofloc tank. The success of this approach depends on the resistance of the carbohydrate to digestive enzymes and microbial fermentation in the shrimp GIT. The majority of carbohydrates fulfilling these criteria are non-starch polysaccharides (NSP). NSP is a term grouping non-digestible fiber including β -glucans, pectins & gums, hemicelluloses, cellulose, and lignin. NSP is built of long chains of monosaccharides held together by β -glycosidic bonds. Higher animals, including Pacific white shrimp, lack the enzymes to digest NSP (Kaushik et al., 2022). However, this promising approach does not come

without potential drawbacks, one of which is poor digestion leading to reduced growth in culture animals exposed to a high amount of NSPs (Amirkolaie et al., 2005). Although this is important, the general discussion regarding the viability of this approach will primarily centre around its impact on the overall shrimp, biofloc performance and water quality at system level and less on individual shrimp growth.

In **Chapters 2, 4 and 5**, we observed that a high dietary carbohydrate content reduced the overall nutrient digestibility. In absence of biofloc as additional food, this reduction in nutrient digestibility in the feed will reduce shrimp growth and shrimp performance (Pavasovic et al., 2006). Indeed, in **Chapter 5**, a reduction in individual weight gain when feeding NSP-rich diets compared to a starch-rich diet was observed. However, in the latter treatment, the reason could be more related to a higher mortality of shrimp fed the starch-rich diet, due to a too high ammonia concentration in the rearing tanks during the first week of the experiment. Hence, more feed per individual was fed to the surviving shrimps from the second week of the experiment onwards, explaining higher individual growth, but not higher production at system level. That the way the carbohydrate is administered either via the diet or via the water did not result in differences in shrimp performance, both at the individual level and system level, was shown in **Chapter 4**. In addition, in **Chapters 2, 4, 5**, survival of shrimp fed a NSP-rich diet was consistently high, fluctuating between 86- 95%, which is excellent for biofloc systems (Okomoda et al., 2022; Padeniya et al., 2022). Often, in biofloc systems the survival is much lower, fluctuating between 50 and 65%, as realized for instance by Mansour et al. (2022).

Chapter 4 showed that both carbohydrate addition methods, via diet or via water, are equally effective in maintaining water quality throughout a 42-day culture period. Feeding shrimp with a diet containing a high fraction of NSP-rich ingredients kept the concentrations of ammonia and nitrate low throughout the experiment. This concurs with Tinh et al. (2021c) who showed that inclusion of corn starch in the diet maintained the inorganic nitrogen species concentration equally well as when applying the corn starch via the water. Based on these study results, we conclude that applying carbohydrate via the feed is safe in for a Pacific white shrimp biofloc system. This section implied that administering NSP-rich diets simplifies management and reduces labour demand, all while maintaining shrimp performance and water quality. However, when looking at shrimp performance, applying corn starch via the water resulted in higher production than applying corn starch via the feed (Tinh et al., 2021). This was, however, not the case when applying a diet containing a high amount of NSP-rich ingredients (**Chapter 5**). This discrepancy could be attributed to variations in carbohydrate complexity and fermentability, which will be discussed in the next subchapter.

2. Carbohydrate input

2.1. Carbohydrate type

Carbohydrates are by far the largest fraction of the organic matter present in the biosphere. They are best known as structural components for plants and invertebrates, and are found in cereals, vegetables, legumes, and many fruits that contribute to human and animal nutrition. On average, carbohydrate molecules contain on a weight basis 40% carbon (C). Numerous types of carbohydrates exist, but for many types, higher animals do not produce enzymes able to digest them (**Figure 6.1**).

Only organic acids, starch, sugars and some oligosaccharides can be digested by shrimp. The other types of carbohydrate, shrimp cannot digest. For non-starch polysaccharides (NSP), which includes polysaccharides, β -glucans, pectins & gums, hemicellulose, cellulose and lignin, NSP-degrading enzymes are lacking in shrimp, although some chitinase activity has been reported in shrimp (Clark et al., 1993) and cellulose activity in prawn (González-Peña et al., 2002). It is not clear if the enzyme is provided by microbiota in the gut or by the shrimp themselves. Also for other NSPs, it is not known how much endogenous digestive enzymes and fermentation by microbiota contribute to NSP breakdown in the gut (González-Peña et al., 2002).

In addition, the way food ingredients are processed and the pellets are made influences carbohydrate digestibility in the animal. Maas et al. (2020) showed that the ADC of starch in Nile tilapia (*Oreochromis niloticus*) increased from 91% in steam pelleted feed to 99% in extruded feed. This is because a higher fraction of starch is gelatinized, and thus better digestible, when extruded. In general, the digestibility decreases, going from gelatinized starch to native starch, and from there to NSP in the following order: pectin > hemicellulose > cellulose > lignin (Kaushik et al., 2022).

In vegetated terrestrial areas, the particulate organic carbon influx from land is three times larger than the production by (micro)algae. About 80 to 90% of the organic carbon influx in inland waters is lignocellulose, which is highly resistant to enzymatic breakdown by animal consumers. In addition, the food quality of lignocellulotic litter and heterotrophic bacteria is very low, missing compounds that are essential to fish, shrimps and other invertebrates for growth and reproduction. Not the quantity and origin of food resources, but its biochemical quality determines how fast the nutrients are transferred to higher trophic levels (Brett et al., 2017).

Post-feeding waste from commercial pelleted feed in a shrimp biofloc system is a rich nutrient (e.g., nitrogen, phosphorous) source and a poor energy (e.g., carbon) source. When energy is lacking, the microorganisms in the biofloc cannot utilize all the

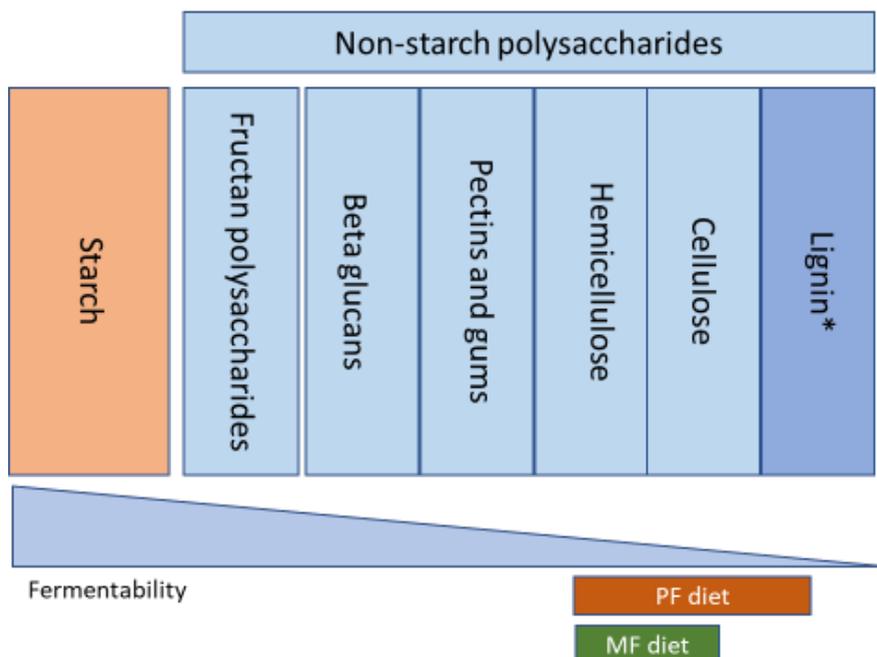


Figure 6.1. Classes of dietary carbohydrates from terrestrial plants. PF - poorly fermentable; MF - moderately fermentable.

nutrients entering the system, causing waste to accumulate and water quality to deteriorate. Therefore, farmers add carbohydrates to the biofloc system so that heterotrophic bacteria can mineralize the waste and immobilize the nutrients within bacterial biomass (Tinh et al., 2023). Contrary to fish and higher invertebrates, microorganisms in aquatic systems can break down lignin, cellulose, and hemicellulose compounds into monomers, which are then either mineralized, releasing CO₂, broken down into dissolved organic matter (DOM), or up cycled through the food web, supporting animal production. For example, estimates of the turn-over rate of NSP-compounds in the watershed of the Amazone river are in the order of 2 to 3 weeks (Ward et al., 2013). Therefore, by introducing NSPs through the water or through the diet into the biofloc system, one can expect that a part of the energy in the NSP will be liberated by the microbiota in the biofloc, stimulating the transfer of nutrients through the food web towards animal production.

That NSP-rich ingredients are effective carbohydrate sources when applied via the water in biofloc systems has been well studied. For example, higher Pacific white shrimp production was reported in systems receiving rice bran, which is a NSP-rich carbohydrate, than in systems receiving molasses (Serra et al., 2015; Rajkumar et al., 2016; Vilani et al., 2016). The fact that Pacific white shrimp production was already enhanced when substituting 40%

of the molasses with a mixture of corn flour and wheat bran (Wang et al. (2016) shows the energy provided by NSP-rich ingredients matters. On the other hand, some studies found no significant differences between the effects of starches and NSPs on shrimp production (Ekasari et al., 2014b; Serra et al., 2015).

In treatments that fed an NSP-rich diet or providing wheat bran via the water, the water quality was maintained conducive to shrimp production (**Chapter 2** and **4**), even when putting a high amount of moderately or poorly fermentable NSP-rich ingredients in the diet. The moderately fermentable NSP was relatively richer in hemicellulose and the poorly fermentable NSP was relatively richer in cellulose (**Figure 6.1**). The type of NSP in the diet had also limited effect on fish performance. Kabir et al. (2020) working with Nile tilapia, in freshwater ponds, and comparing two types of NSP, one containing mainly lignin and cellulose, the other mainly hemicellulose and pectin, observed similar water quality and better growth and fish performance with the diet that was richer in lignin and cellulose. What causes the difference between the tilapia pond and shrimp biofloc system is presently not known. Many factors affect how NSPs are processed in the rearing system, including salinity, species diversity in the food web, the culture species, the feed composition, and how well the rearing system is connected to the wider environment. Much more research is needed on the effect of roles of the types of NSP included in the diet, across different types of rearing systems (e.g., pond or biofloc tank, freshwater or brackish/marine water, shrimp, or fish), culture intensities, feed composition, NSP types, etc. As shown in **Figure 6.1**, the range of NSP types is huge, as is the range of culture systems and culture species.

2.2. Carbohydrate quantity

In our experiments, the C:N ratio of the nutrient input was always below 10, both in biofloc systems primed with mature biofloc (**Chapter 2** and **Chapter 4**) or starting with slightly fertilized clean water (**Chapter 5**). When comparing the outcomes of all treatments studied, a picture emerges that the amount of biofloc produced increases with the amount of nutrients applied to the biofloc rearing system. Experiments with a longer duration would be necessary, preferentially covering a full production cycle, to establish if the amount of carbon applied at one point in time would become limiting to biofloc formation. Because the amount of nutrients fed to the biofloc system increases proportionally to the shrimp biomass present, the probability that this happens is small. Nevertheless, before recommending this technique to farmers as reliable, this should be tested.

Most animals incorporate a limited amount carbohydrate into their body mass. The amount of carbohydrate put in formulated feed is higher, and the majority of the

carbohydrates will be metabolized for energy, releasing CO₂. Biofloc systems have been reported to generate a 2 to 5 times higher CO₂ emission than a conventional culture system (Hu et al., 2014; Tinh et al., 2021b). Such a high CO₂ loss is an environmental concern considering global warming (Willett et al., 2019). Reducing the carbon input, by lowering the C:N ratio of the nutrient input to the rearing system, as well as the reuse of mature biofloc helps to reduce carbon loss and overproduction of biofloc (Peiro-Alcantar et al., 2022). Excess biofloc accumulation, especially in (super)-intensive systems must be managed. Unfortunately, shrimp consume only a small fraction of the produced biofloc in a biofloc system (Krummenauer et al., 2020). Excess biofloc is often discharged to surface waters surrounding the farm. However, biofloc can be turned into a nutritious dietary supplement (Khanjani et al., 2023). By removing excess biofloc and turning it into a valuable supplement, the farmer can prevent the TSS concentration becomes too high in the biofloc rearing tank to keep a healthy culture environment.

Overall, the PhD research indicated that biofloc systems can be managed by reducing the carbon input and avoiding excessive and unnecessary biofloc formation. This applies regardless of factors such as carbohydrate addition method (**Chapter 4**) and the complexity of carbohydrate sources (NSP-rich versus starch-rich ingredients, **Chapter 5**).

3. Microbiota

Microbial communities are key drivers in the productivity and sustainability of shrimp culture systems. They offer ecosystem services including nutrient cycling, organic matter decomposition, and serve as natural food sources for shrimp. In biofloc systems, the microbial communities are purposely shaped to promote growth of heterotrophic bacteria (Avnimelech, 2015; Emerenciano et al., 2017). The consideration for choosing this bacteria group include its ability to mineralize organic matter, to rapidly immobilize ammonia and organic nitrogen into bacterial protein (Ebeling et al., 2006; Hargreaves, 2006), and to produce extracellular polymeric substances (EPS) that bind microbes into a conglomerate (More et al., 2014; Huang et al., 2022b). Besides heterotrophic bacteria, of great importance for biofloc functionality are autotrophic bacteria, due to their effectivity at converting ammonia and nitrite into less harmful nitrogen substances (Ebeling et al., 2006). The fast reactivity of both heterotrophic and autotrophic bacteria to allochthonous nutrient input and human interventions make it possible to maintain a healthy production environment.

Various technical measures such as adjusting the level and type of nutrient input (carbon and nitrogen), aeration, light exposure, and oxygen concentration, can directionally affect bacterial community structure and taxonomic composition (Xu et al., 2016; Jiang

et al., 2020; Tinh et al., 2021c; Mansour et al., 2022). Especially, the level of carbon input and the type of carbohydrates serving as carbon source are important, shown by variations in the prokaryotic communities due to the amount of carbohydrate applied (with vs. without dietary carbohydrate addition in **Chapter 3**, and isonitrogenous vs. *as-is* feeding in **Chapter 5**) and due to differences in carbohydrate types (poorly vs. moderately fermentable NSP, and starch vs. NSP, **Chapter 5**). Differences in the biofloc microbial composition are more pronounced between NSP-rich and starch-rich diets, rather than between poorly and moderately fermentable diets.

In my study, changes in the microbial composition were observed in both mature (**Chapter 3**) and young biofloc (**Chapter 5**), demonstrating the dynamic nature of these communities over time. However, despite differences in relative abundance between experiments, the most predominant microbial taxa observed were in agreement with the commonly reported biofloc core groups in other studies, suggesting a significant role of these groups on biofloc functionality. At phylum level, these groups belong to the *Proteobacteria*, *Cyanobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Verrucomicrobia*, *Firmicutes*, *Acidobacteria*, *Gemmatimonadetes*, *Planctomycetes*; *Cyanobacteria* (Deng et al., 2019; Tinh et al., 2021c; Xu et al., 2022). These phyla were already mentioned in **Chapter 1 (Table 1.1.)**, emphasizing their consistent presence within biofloc systems. Additionally, the major eukaryotic phyla included *Chlorophyta*, *Nematoda* and *Rotifera* (Ray et al., 2010; Maicá et al., 2012; Manan et al., 2016; Chakrapani et al., 2021). Within the fungal community, the predominant phyla identified were *Ascomycota* and *Basidiomycota* (Gao et al., 2019a; Yun et al., 2022).

An important aspect of the biofloc functionality is the ability to efficiently utilize the nutrients present in the water column to maintain water quality. In my study, I evaluated how mature biofloc communities shift over time and adjust to changes in nutrient input. I also looked at fungi, as their role in biofloc systems has been relatively underexplored. Thus, in my study, I provide some novel insights into the community dynamics of the eukaryotic/fungal communities. I noticed significant changes in both prokaryotic and eukaryotic communities over time, with the most notable changes observed in the prokaryotic communities in response to varying levels of NSP input to the biofloc system. This suggests that the prokaryotic community adapts faster to changes in nutrient input, as reflected in their diversity dynamics analyzed in **Chapter 3**. This was also supported by the functionality predictions, which showed that the prokaryotic communities were gradually shifting to carbohydrate metabolism. It is important to note that the ability to adapt rapidly supports nutrient recycling within the food web. However, it is also a potential risk by causing sudden changes in water quality within the system. On the other hand, the eukaryotic community seems to be

more stable in a mature biofloc system, which could be a benefit of reducing the carbon input to the biofloc system (**Chapter 3**).

Although the balance between prokaryotic and eukaryotic groups in a biofloc system warrants further exploration, their coexistence plays a vital role in stabilizing the system. For instance, microalgae, when present in the biofloc system, immobilize inorganic nitrogen waste and provide extra carbon to the system (Hargreaves, 2006; Xu et al., 2016). Meanwhile fungi can utilize organic carbon and produce EPS, akin to heterotrophic bacteria (Mahapatra and Banerjee, 2013; Osemwegie et al., 2020b; Luft et al., 2021). Until today, only a limited number of studies explored the functionalities of eukaryotes, including fungi, in biofloc systems. A possible reason could be the lack of fully annotated reference databases for aquatic eukaryotes. Thus, the upgrading of existing databases is important to develop a more in-depth understanding of the roles of these microbial groups in biofloc systems, and aquatic systems in general.

It should be highlighted that microbial system management should not only focus on water quality but should also consider ecosystem services, for example health benefits delivered by gut microbiota and biofloc (Cardona et al., 2016; Huang et al., 2020; Xu et al., 2022). While this topic was not within the scope of this thesis, the exploration of methods for microbiota control within both the system and the shrimp holds promise for reducing disease outbreaks and antibiotic use. In combination with effective carbon input management, such a holistic approach to shrimp farming merits increased attention, because it can substantially enhance the performance, resilience, and environmental responsibility of biofloc technology.

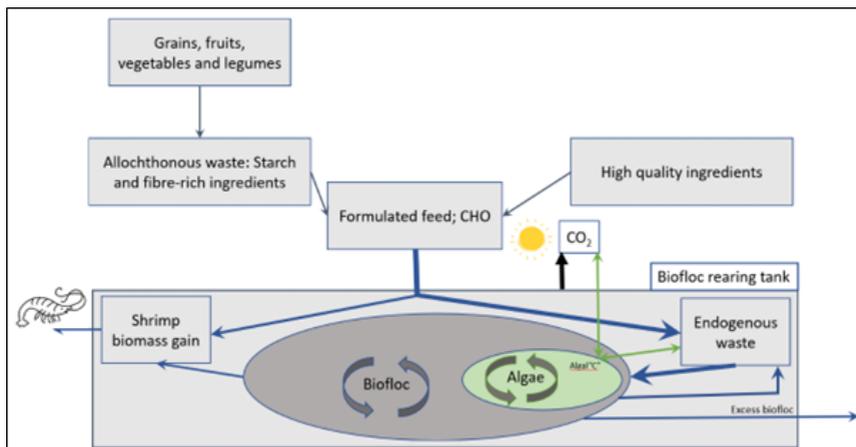


Figure 6.2. Conceptual drawing of the flow of nutrients within a shrimp biofloc system. In the biofloc tank, the shrimp, biofloc and endogenous waste are homogeneously distributed, but they are shown separately in the drawing for clarity.

4. Circularity

The flow of nutrients and carbon through a biofloc system is depicted in **Figure 6.2**. In a shrimp biofloc system receiving formulated feed and/or carbohydrate, the post-feeding waste provides energy (carbon) and nutrients, the latter including nitrogen and phosphorous. This waste is referred to as endogenous waste. Today, low quality products generated during the production and processing of grains, fruits, legumes and vegetables are included in the ingredient mixture of formulated shrimp feeds, to reduce the use of fish meal and other animal products in the feed. This waste can be referred to as allochthonous waste. Once in the biofloc system, the allochthonous waste not retained in new shrimp biomass becomes part of the endogenous waste. In addition, when microalgae are part of the biofloc, they take up nitrogen and phosphorous from the pool of endogenous waste nutrients and introduce new algal carbon. Fresh biofloc is continuously formed while old microbes in the biofloc die and are mineralized. A part of the recycled nutrients in the biofloc are eaten by the shrimp and contribute to the shrimp biomass gain. Nutrients released during mineralization return to the endogenous waste pool while a large percentage of the carbon is respired and leaves the biofloc rearing tank as CO₂. When the carbohydrate input to the biofloc system is high more biofloc is produced than needed. When the concentration of biofloc becomes too high, the excess biofloc is removed.

The nutritional quality of biofloc is high, containing between 18 to 49% crude protein and between 18 to 36% carbohydrate (Khanjani et al., 2023). The protein content in biofloc reflects the protein content in the feed; the higher the protein content in the feed, the higher the protein content in the biofloc. However, the lipid content in biofloc is very low, ranging from 0.5 to 2.0%, exceptionally reaching 4% (Maicá et al., 2012). The nutritional quality of biofloc makes it a good supplemental feed, although the ash content can be high, fluctuating between 13 and 52%, with old biofloc containing more ash than young biofloc (Khanjani and Sharifinia, 2020). It should be noted that the high ash content might be partially due to contamination by salt from brackish water during sample processing.

One advantage of growing shrimp in a biofloc system, is the contribution of biofloc to the nutrition of the shrimp. This allows Pacific white shrimp farmers to use low protein feeds, with 30-32% protein (Jatobá et al., 2014). In **Chapter 5**, the dietary protein content in the NSP-rich diets was even lower (24-25%). Another option is to lower the administration of pelleted feed up to 30%, as suggested by Khanjani et al. (2020). How much biofloc intake contributes to Pacific white shrimp production is highly variable. One of the first reports using stable isotopes to estimate the contribution of biofloc to shrimp production was by Cardona et al. (2015), reporting a 36-40% contribution

of biofloc to production. Since then, reported contributions of biofloc to shrimp production were either higher, reaching 63 to 100% for carbon and 35 to 86% for nitrogen (Krummennauer et al., 2020) or lower, reaching 8% (Castro et al., 2021). The variation in the nutritional quality and in the contribution of biofloc to production is large, and the underlying causes are still not fully understood. This makes it difficult to predict the economic benefits of raising shrimp in biofloc systems, and the contribution of biofloc technology to the re-use of nutrients and circularity (Khanjani et al., 2022).

This also hold for our research. In **Chapter 4**, the FCR at system level was 1.22 and similar between treatments. On average, 49% of the nitrogen input was retained in shrimp biomass gain. In **Chapter 2**, administrating a similar diet as in **Chapter 4** at a higher feeding rate, the FCR was 1.70 with a nitrogen retention efficiency of 38%. Similar values were also observed in **Chapter 5**, with an average FCR of 1.82 and 34% of the nitrogen input retain in shrimp biomass gain. These are good results, but not better than for non-biofloc systems, such as semi-intensive ponds or recirculating aquaculture systems (Verdegem et al., 2023).

Low quality plant-based ingredients are already often included in aquaculture diets (Boyd et al., 2020), and used throughout the aquaculture industry. In our experiments, the inclusion level was higher than recommended, which reduced the feed quality for Pacific white shrimp (lower ADC). This, however, did not lower the survival and growth as normally observed in Pacific white shrimp culture. Hence, more exogenous low-quality plant-based waste was recycled. We did not measure the direct contribution of biofloc to total shrimp production, but most likely biofloc consumption compensated for the lower feed quality. In consequence, the economic feasibility of using NSP-rich diets in biofloc systems, will ultimately depend on the price of the feed, and the ability of feed companies to produce in sufficient quantity these diets to fulfil the demand.

5. Working with mature biofloc

The biofloc systems in **Chapter 2** and **4** were started by adding mature biofloc before stocking shrimp to the biofloc tanks. In doing so, it was possible to maintain favourable culture conditions without adding extra carbon to the biofloc systems. In all treatments of **Chapter 2** and **4**, the C:N ratio of the nutrient input was always below 10. This is lower than the advised C:N ratio of 15 to 20. For example, Asaduzzaman et al. (2008) growing freshwater prawn (*Macrobrachium rosenbergii*) in ponds increased the net yield 40% by increasing the C:N ratio to 20 by applying tapioca into the water besides the feed, the latter having a C:N ratio of 10. In contrast, Ferreira et al. (2021) raised Pacific white shrimp maintaining a C:N ratio of the feed+molasses nutrient input of 12, only adding extra molasses when the total ammonia concentration exceeded 1mg

L⁻¹. In another treatment of the same study, 35% of the volume of the biofloc rearing tanks was replaced with mature biofloc water. In tanks primed with mature biofloc the water quality remained good, while in tanks receiving molasses, peaks of ammonia and nitrite occurred regularly, requiring extra molasses input and labour to maintain water quality. With the management applied, however, both approaches maintained the biofloc system operational and productive. Apparently, the turn-over rate between old and new biofloc when starting with mature biofloc, supplied sufficient organic carbon for the duration of the experiment, as was also observed in the experiments of **Chapter 2**. In addition, a system relying on mature biofloc produces less biofloc, resulting in less sludge accumulation (Martins et al., 2020). Proper disposal of sludge from biofloc systems requires planning and sometime additional treatment which can be costly. Reducing sludge accumulation therefore is important.

Besides mature biofloc, algae can also be a source of organic carbon in biofloc systems and contribute to the nutritional quality of biofloc (Khanjani et al., 2023). In the experiments reported in **Chapters 2, 4 and 5**, the chlorophyll-a concentration increased during the culture period. This might also have contributed to maintaining water quality and supporting shrimp performance in all our treatments. Xu et al. (2016) reported that in presence of daylight, a mixed biofloc system in which microalgae and autotrophic bacteria are well established can be more beneficial for shrimp performance than a biofloc community dominated by heterotrophic bacteria. Additional benefits are that a biofloc system dominated by microalgae-autotrophic bacteria reduces operational costs as the inputs of organic carbon and oxygen are smaller.

More research is needed on the benefits of mature biofloc and microalgae as carbon (energy) sources in addition to allochthonous carbohydrates in biofloc systems. A main question is if it will be possible to develop mixotrophic biofloc systems that maintain the biofloc system healthy for an extended period. Presently, farmers prefer working with biofloc systems in which either heterotrophic or autotrophic bacteria dominate for limited periods of time, starting all over with each culture cycle.

6. Societal relevance of the research

With this PhD project I contributed to the vast amount of ongoing research on biofloc technology. New insights were generated on the potential contribution of low-quality plant-based ingredients to the functioning of biofloc systems. Nevertheless, more work is needed to make biofloc technology reliable and resilient from a technical, economic, and environmental perspective. Here I briefly state some of the insights I gained during my study which are relevant to farmers, feed companies and the environment.

6.1. Farmers

Biofloc technology is still too complicated, especially for small-scale farmers. More work is needed on simplifying the management. My research looked if it is possible to provide all necessary inputs with the feed. Although further fine-tuning will be needed, my research showed this might be possible. Relying on one input would:

- Eliminate the need to manage the feed input and carbohydrate input separately, reducing slightly the workload and reducing opportunities for human error. In case of a spike in ammonia or nitrite concentration, supplying carbohydrate via the water remains possible.
- Simplify water quality management and reduce variation over time.
- Reduce the rate of biofloc formation. Hence, discharge of biofloc sludge will be not needed or be less frequent.

A condition is that feed companies are able produce and distribute shrimp feed containing a high fraction of NSP-rich ingredients, and that this feed is less costly than standard shrimp feed.

6.2. Feed companies

For feed manufacturers, implementing this approach can create a new product line, with unique selling points:

- The feed is tailored to the requirements of biofloc technology, which creates a segmented consumer base.
- The feed is cheaper than a standard feed because the inclusion of more NSP-rich ingredients which are less expensive carbon sources than normally used by the industry.
- To the public the company shows its commitment to increase the nutrient use efficiency of its feeds, reduce unnecessary carbon loss and make more and better use of agricultural waste products than is presently the case.

Challenges include:

- Identifying locally available NSP-rich ingredients, that are available in sufficient quantity and quality, and cheaper than presently used ingredients. In addition, pre-treatment cost to prepare the ingredients for including in the diet should not be excessive.
- The number of potential customers and the local production must be large enough to cover the development costs of bringing a new product on the market.

6.3. Environment

The combination of the farm (technical) set-up, the climate and the production environment put limits to the production capacity. Many farmers operate at the frontier of what is possible on their farm. High stocking densities and feed inputs create hyper-intensive production environments that are different and often more stressful and less healthy than the natural environment of the culture species. This makes the culture animals more vulnerable to disease and more sensitive to stress. A whole industry developed to address imbalances in the production environment and make the culture animals more resilient. Farmers can choose from a wide range of products including disinfectants, antioxidants, pesticides, fertilizers, mineral mixes, prebiotics, probiotics, antibiotics, and feed additives and became dependent on some of these products, which raises their production costs.

Aquaculture feeds can be designed considering the limits of the production environment. This is where feed companies can play a role, by selling packages, that when applied by the farmer, makes the production more predictable, maintains the production environment healthy, keeps the production costs low and makes the animals and the production environment more resilient. The NSP-rich diet for biofloc technology, I tried to develop during my thesis project, aims to do exactly that: feed the animal and nourish the environment. In doing so,

- The carbon input is reduced, and the CO₂ emission is reduced.
- Enough biofloc is formed to maintain water quality and to increase the nutrient use efficiency at system level.
- Low quality underused plant-based ingredients that do not compete strongly with human nutrition are integrated in the aquatic feed system.

My thesis results showed the system needs further finetuning. There is more work to do.



The background of the page is a watercolor-style wash. It features a vertical gradient of colors, starting with a light, pale blue on the right side and transitioning into various shades of teal and turquoise on the left. The colors are blended and layered, creating a soft, organic texture with some darker spots and lighter areas, characteristic of watercolor painting.

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Summary

SUMMARY

This PhD project investigated the effect of non-starch polysaccharides (NSP) on the functioning of biofloc systems used to produce shrimp. Shrimp performance, water quality and biofloc development were monitored. The objectives were to make the performance of shrimp-biofloc systems more predictable and to simplify biofloc system management.

Chapter 2 investigates the effect of supplementation of NSP-containing ingredients in pelleted feed on shrimp production and system performance when fed to a biofloc system. Wheat bran (WB), which contains a lot of NSP, was added to the ingredient mixture of a shrimp feed (control; CONdiet) to create a wheat bran rich diet (WBdiet). Both diets were administered isonitrogenously. Addition of wheat bran in the diet reduced feed digestibility and increased the faecal C:N ratio from 12 in the CONdiet to 20 in the WBdiet. The WBdiet did not affect shrimp survival and reduced the specific growth rate by 2% only compared to the CON diet. At the end of the experiment, the amount of biofloc, expressed as volatile suspended solid (VSS), increased faster in WBdiet fed tanks than in CONdiet fed tanks. Feeding the WBdiet resulted lowered the concentrations of dissolved nitrogen species such as $N-(NO_3+NO_2)$ and total organic nitrogen (TON) in the rearing tanks more than when feeding the CON diet. With both diets, at the end of the experiment 97% of the nitrogen input during culture was still present, while the percentage of carbon loss per kg feed was similar between diets.

The evolution of the microbial community present in the biofloc systems studied in **Chapter 2** is reported in **Chapter 3**. Time had a stronger effect than the type of diet on alpha and beta diversity in biofloc. The addition of wheat bran affected the prokaryotic taxonomical composition, with several genera being enriched such as *Muricauda*, *Pirellula*, and *Cyanobacteriaceae*. In eukaryotic communities, overall, only few taxa were significantly affected by the addition of wheat bran, belonging to *Trebouxiophyceae* and *Suillus* groups. The composition dynamic indicated that the prokaryotic microbial community was more adapted to the NSP-rich diet compared to the eukaryotic community, the latter including fungi. Interestingly, when feeding the WBdiet, the microbial communities in biofloc exhibited functionalities that are abundant in carbohydrate metabolism, more specifically in relation to pentose, fructose, mannose, and galactose metabolism. These results provide a basis for the control of microbial communities when fed low quality plant-based ingredients rich in NSP. While shrimp cannot digest NSP, the biofloc microbiota living in the system can convert it into edible natural food for shrimp.

Chapter 4 compared the effect of wheat bran addition via the diet and via the water on shrimp and system performance in a biofloc system. The experimental set-up mirrored that of **Chapter 2**. However, in this Chapter, both treatments received exactly the same amount of nutrients (such as protein, fat, carbohydrate). Supplying wheat bran via the diet decreased the digestibility of the diet for the shrimp and increased the faecal C:N ratio, which was consistent with the results of **Chapter 2**. Both NSP-addition methods, either via feed or via water, were equally effective. No differences were observed in biofloc quantity and quality and nutrient mass balance between the dietary treatments. When compared to the traditional addition of carbohydrate to the water, supplementing NSP-containing carbon sources via the feed is a more efficient approach in terms of farm labour and feeding management practices.

Chapter 5 compared the administration of diets containing either more poorly or more moderately fermentable NSP (PF and MF diet), fed at isonitrogenous or at *as-is* basis. As a control, a starch-rich diet (ST-diet) was used. Pacific white shrimp had a lower nutrient digestibility when fed the PF and MF diets compared to the ST-diet. NSP types did not alter the shrimp and system performances. Overall, shrimp and biofloc performance were comparable between the NSP-rich diets and the starch-rich diet, regardless of the feeding strategy. Only the MFdiet treatment showed lower shrimp production when fed at *as-is* basis than the other treatments. Feeding iso-nitrogenously compared to at *as-is* basis resulted in higher protein deposition in shrimp and biofloc. The dietary incorporation of NSP-rich ingredients affected the prokaryotic community composition, for example by decreasing *Cyanobacteria* and increasing *Actinobacteria* abundance. In conclusion, when fed iso-nitrogenously, the type of NSP did not affect biofloc system performance and worked as effectively as the STdiet in maintaining water quality.

The final chapter (**Chapter 6**) provides a comprehensive overview of the main findings and puts them in the broader context. The following topics are discussed: labour force quality and demand, carbohydrate input, microbiota composition, circularity and biofloc maturity. The thesis ends with a brief overview of the societal relevance of the thesis research.

The background of the page is a watercolor-style wash. It features a vertical gradient of colors, starting with a deep teal on the left side and transitioning through various shades of blue and cyan towards the right, where it fades into a light, almost white background. The texture is soft and painterly, with visible brushstrokes and color blending.

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Menyelesaikan studi di tingkat doktoral ini bukan hanya sebuah penyelesaian suatu pekerjaan bagi saya. Lebih dari itu, hal ini merupakan pencapaian yang semoga membuat keluarga saya ikut senang. Surat di bawah ini, saya tujukan untuk keluarga tercinta.

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Okay, I have probably been talking too much.

I recognise that there are other people who have not been particularly mentioned in this acknowledgement. I apologise for this oversight and believe me my appreciation for you is as great as it is for those mentioned above.

Alhamdulillah Rabbil 'Alamin, tidak ada yang sulit jika Engkau berkenankan.

Wageningen, Autumn 2023

Apriana Vinasyam

The background of the page is a watercolor-style wash. It features a vertical gradient of colors, starting with a deep teal on the left side and transitioning through various shades of blue and cyan towards the right. The texture is soft and painterly, with visible brushstrokes and color blending. The right side of the page is mostly white, with some light blue washes extending from the teal area.

About the author

ABOUT THE AUTHOR

Apriana Vinasyam was born on April 29th 1988 in Serang, Banten Province, Indonesia. She completed his primary education at SD YPWKS II (Yayasan Pendidikan Warga Krakatau Steel) Cilegon, SLTPN I Cilegon, and SMAN I Serang, Banten Province. She earned her BSc in Aquaculture from The State Polytechnic of Agriculture Pangkep and Brawijaya University, Indonesia, in 2011. Afterwards, she completed her MSc in Aquaculture in IPB University (Institut Pertanian Bogor) Indonesia in 2014, including a Master exchange programme



in Ghent University Belgium (2013-2014), using Lotus Grant Erasmus Mundus Scholarship. During BSc and MSc study, she focused on seaweed tissue culture, fish nutrition and reproduction. Later, she worked at The Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University, as technical editor of Jurnal Akuakultur Indonesia and as a lecturer assistant between 2014-2017. After being awarded a doctoral scholarship from LPDP Indonesia, she started PhD at Aquaculture and Fisheries (AFI) Chair Group, Graduate School of Animal Sciences (WIAS), Wageningen University and Research (WUR) at the end of 2017.

During her PhD, Apriana, also known as Vina, studied about aquaculture system management, particularly in the shrimp-biofloc system. Employing a feed approach, she aimed to enhance the system management practices, intending an improvement in the system efficiency while still maintaining optimal shrimp production. She used plant-based residue as feed ingredients, for example wheat bran, to further improve the nutrient circularity. In 2019-2020, when Covid-19 pandemic hit, she conducted an experiment in Indonesia, while she also applied for the permanent lecturer position in The Department of Aquaculture, IPB University. After a long process involving multiple back and forth trips, for almost four times a year, she was finally accepted in December 2020. Since then, she worked both for her PhD and her lecturing job, which was quite challenging due to the 5-6 hour time difference between the two countries.

Apriana presented her PhD studies in some international seminars, receiving awards as best poster presenter at WISE 2019 and The WIAS Science Day 2019. In addition to her academic activities, Apriana held various roles within social and community associations. She was appointed as community relation Coordinator at The Indonesian Student Association in The Netherlands (PPI Belanda) in 2017-2018, and coordinator of The Indonesian PhD Postdoc communities (Kelurahan PhD Postdoc Indonesia) in 2018-2019. She also contributed as member of organizing committee of The AFI-PhD Exchange Trip Programme in China 2019, and Wageningen Indonesian Scientific Exposure (WISE) 2019. Beyond her academic and social endeavours, Apriana enjoys creating social media videos, hunting and collecting antique teacups, and cooking. Apriana can be reached via email at apriana@apps.ipb.ac.id.



GENERAL INFORMATION

Name PhD candidate	Apriana Vinasyiam
Project title	LPDP
Group	Aquaculture and Fisheries
Promotor	Dr. Marc Verdegem
(co) promotor	Dr. Fotini Kokou
(co) promotor	Dr. Julie Ekasari

EDUCATION AND TRAINING (min 30 ECTS)

YEAR
A. The Basic Package

WIAS Introduction Day	2017
WGS Scientific Integrity course or alternative scientific integrity course (mandatory)	2023
WGS Ethics in Animal Sciences course or alternative ethics course (mandatory)	2023
WIAS Course on Essential Skills (Frank Little)	2017

B. Disciplinary Competences

Aquaculture Production Systems (MSc course)	2017
Species Specific Course (fish)	2017
Advanced Statistics Course Design of Experiments	2017
Research proposal	2018
Introduction to Laboratory Animal Science Course (MSc course)	2018
Systematic Review of Animal Science	2018
Estimation of Genetic Parameters and Breeding Values	2018

C. Professional Competences

Stress Identification and Management	2017
Research Data Management	2018
Reviewing Scientific Paper	2018
Poster and Pitching	2018
Committee member in WISE conference (Wageningen Indonesia Scientific Exposure)	2019
Committee member in AFI knowledge exchange program with China	2019
Project and Time Management	2019
Brain Training	2019
Scientific Writing	2021

D. Societal Relevance (max 6 ECTS)	
WIAS Course Societal Impact of your Research	2019
E. Presentation Skills (max 4 ECTS)	
International Symposium in Chinese & European Aquaculture & Fisheries, Shanghai, China (poster)	2019
EAS (European Aquaculture Society) Annual Conference, Madeira, Portugal (oral)	2021
Fish Nutrition and Health Workshop, Wageningen, the Netherlands (poster)	2022
WIAS Science Day, Wageningen, the Netherlands (poster and oral)	2019, 2021, 2022
WISE conference (Wageningen Indonesia Scientific Exposure), Wageningen, the Netherlands (poster)	2019 & 2023
F. Teaching competences (max 6 ECTS)	
Tutoring Master thesis ring	2019
Supervising MSc students (major thesis)	2018-2020
<i>Anne Regtien</i>	
<i>Iris Menger</i>	
<i>Miguel Knudsen Salazar</i>	
<i>Jui-Che Chiang</i>	
<i>Jonas Fuchs</i>	
<i>Bruno Miguel Costa</i>	
Supervising BSc students (major thesis)	2018-2020
<i>Rangga Mulya Rizki</i>	
<i>Fitri Amalia</i>	
<i>Dana Krysticka Monik</i>	

Education and Training Total = 36.5 ECTS

*One ECTS credit equals a studyload of approximately 28 hours



Colophon

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