EFFECT OF FEED AND WATER ADDITIVES ON BACTERIAL COMPOSITION IN A SHALLOW-WATER SHRIMP (*Litopenaeus vannamei*) RESEARCH SYSTEM USING BIOFLOC TECHNOLOGY

A Dissertation

by

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ABSTRACT

The effects different prebiotics, organic acid salts and an essential oil blend have on the bacterial community of biofloc particles (formed of bacteria, leftover feed, and feces) and shrimp gills tissue, hepatopancreas and intestinal contents, as well as on total hemocyte count (THC), were determined for the Pacific white shrimp, *Litopenaeus vannamei*.

Shrimp were stocked in research tanks with a capacity to hold 41-L of artificial 28 g/L salinity. Final weight, weight gain and survival were determined based on termination data. Also, biofloc and shrimp muscle samples were collected to determine ash, protein and lipid composition. In addition, denaturing gradient gel electrophoresis of biofloc and shrimp hepatopancreas, gills and intestine was conducted.

For Trial I, prebiotics, fructooligosaccharide, galactooligosaccharide, mannan-oligosaccharide and inulin, as well as non-prebiotic carbohydrates, wheat starch and sucrose, were added directly to the water. For Trial II, the same additives of Trial I and an essential oil blend were included in the feed with a 3% dietary inclusion level. For Trial III, four diets were prepared with fructooligosaccharide and galactooligosaccharide at 1.5 and 3.0% dietary inclusion level each. Also, 6 diets were prepared with sodium acetate, sodium lactate and sodium propionate each at 0.75 and 1.5% dietary inclusion levels and a control diet with no additive inclusion.

For the present experimental conditions, it can be concluded that the evaluated additives have a significant effect on the bacterial communities of the biofloc and those present in shrimp gills, hepatopancreas and intestinal contents when added to the water or

feed. Significant increase in the THC was observed when the essential oil blend and the organic acid salts were included in feed of Trials II and III. Also, significant effects on water quality and biofloc levels were observed only when wheat starch was added to the culture water.

The effects of feed and water additives on the bacterial populations of biofloc and shrimp gills, hepatopancreas and intestinal contents as well as on THC of shrimp when cultured in a biofloc technology system is a significant contribution to knowledge and to the shrimp aquaculture industry.

DEDICATION

To my parents, Alma and Eligio, and brothers, Jaasiel and Daniel.

To my grandparents, Delia, Carlos, Ma. de los Ángeles and Eligio.

To the love of my life, my wife, Fernanda, and to our sweet nightmare, Santino (AKA “il-ni”).

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Contributors

This work was supervised by a dissertation committee consisting of Professors Dr. Addison L. Lawrence [chair] and Dr. Delbert M. Gatlin III [co-chair] of the Department of Wildlife and Fisheries Sciences, Professor Dr. Christopher A. Bailey [committee member] of the Department of Poultry Science and Dr. Michael E. Hume [committee member] of USDA-ARS.

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NOMENCLATURE

BFT	Biofloc Technology
DGGE	Denaturing Gradient Gel Electrophoresis
ATr	Autotrophic
INL	Initial
CTL	Control
BFP	Biofloc particles
GT	Gills tissue
THC	Total hemocyte count
HP	Hepatopancreas
IC	Intestinal contents
FOS	Fructooligosaccharide
FOS1.5	Fructooligosaccharide 1.5% inclusion level
FOS3.0	Fructooligosaccharide 3% inclusion level
GOS	Galactooligosaccharide
GOS1.5	Galactooligosaccharide 1.5% inclusion level
GOS3.0	Galactooligosaccharide 3% inclusion level
MOS	Manan-oligosaccharide
INU	Inulin
SUC	Sucrose
WSt	Wheat starch
SA0.75	Sodium acetate 0.75% inclusion level

SA1.5	Sodium acetate 1.5% inclusion level
SL0.75	Sodium lactate 0.75% inclusion level
SL1.5	Sodium lactate 1.5% inclusion level
SP0.75	Sodium propionate 0.75% inclusion level
SP1.5	Sodium propionate 1.5% inclusion level

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CHAPTER I

INTRODUCTION

I.1 Shrimp aquaculture

Shrimp is the largest single aquaculture commodity in terms of value, accounting for about 14% of the total value of aquaculture products in 2014 (FAO, 2016; Robalino et al., 2016). According to FAO (2016), there was a global production by aquaculture of 4,679,368 tons of shrimp in 2014 with a value of US$ 24,022,856,000 among which 2,209 tons of shrimp were produced in the USA with a value of US$ 10,316,000.

Traditionally, shrimp have been commercially farmed in earthen ponds using large land areas that are also highly demanded for other purposes like agriculture, wetland conservation, residential, industrial, and tourism activities. Shrimp harvested from commercial shrimp aquaculture contribute more than 55% of the world's total supply, among which Pacific white shrimp *Litopenaeus vannamei* was responsible for about 76% of the total global shrimp yield in 2011 (Yu et al., 2014). However, in recent years, severe economic losses in the shrimp aquaculture industry have resulted from reduced production due to diseases caused by viruses such as the White Spot Syndrome Virus (WSSV) and the Taura Syndrome Virus (TSV), or bacterial outbreaks caused mainly by pathogenic *Vibrio* species (Tsai et al., 2014; Thitamadee et al., 2016). Most diseases generally occur as a result of stress and environmental deterioration in association with the intensification of shrimp farming (Tseng and Chen, 2004). Also, traditional shrimp farming practices in outdoor ponds with high water exchange have

caused environmental degradation and significant crop losses due to disease outbreaks (Cohen et al., 2005; Balcázar et al., 2007; Samocha et al., 2007; Ekasari et al., 2014).

In order to reduce or eliminate water exchange, systems, such as the Biofloc Technology (BFT) system, have been developed in which water quality is controlled without the need to replace the existing water in the system with fresh or seawater. BFT is a system that facilitates intensive cultures and keeps investment and ongoing maintenance costs low as well as incorporating the potential to recycle feed nutrients (Avnimelech, 2012). Due to these advantages, BFT has gained attention in recent years as a desired system for commercial shrimp production.

I.2 Biofloc Technology Systems: An Overview

Biofloc technology (BFT) was initially developed in the early 1970s at Ifremer-COP, French Polynesia (Emerenciano et al., 2013). Presently, the BFT culture system has become an emerging option for the development of eco-sustainable aquaculture (Dantas et al., 2016). BFT is an aquaculture strategy applied to a variety of system types and is currently most commonly used for the culture of shrimp and tilapia (Avnimelech, 2012). When using BFT as the culture strategy, cultured animals need be stocked in tanks or ponds at a high density and water exchange needs to be restricted for the large amount of nutrients from feeds that enters the water and accumulates in the system, contributing to the proliferation of a community of microorganisms including bacteria, algae, protists, and zooplankton (AES, 2016). A significant portion of these organisms are contained on and within biofloc particles which can reach a diameter of up to a few millimeters. Biofloc particles are primarily made up of microorganisms, feces, detritus, and

exopolymeric substances. The latter is a complex mixture of biopolymers comprising polysaccharides, proteins, nucleic acids, uronic acids, humic substances, lipids, etc., resulting from bacterial secretions, shedding of cell surface materials, cell lysates and/or adsorption of organic constituents (Pal and Paul, 2008). The microorganisms present in BFT systems create a nutrient recycling system, which reduces feed costs and improves water quality and shrimp immunity (AES, 2016). The different beneficial effects of using BFT as a shrimp culture strategy and the use of additives to enhance BFT as a food source, to enhance shrimp immunity and to control water quality have been documented (Avnimelech, 2012; Cardona et al., 2016; Ray et al., 2017; Xu et al., 2013; Xu and Pan, 2014a).

The recycling of feed nutrients carried out by bacteria present in biofloc makes it a valuable food source, contributing to the reduction in production costs (Tacon et al., 2002; Wasielesky et al., 2006; Crab et al., 2010). Studies have confirmed that biofloc can be rich in proteins, vitamins and minerals, and can also provide amino acids (Tacon et al., 2002; Kuhn et al., 2009, 2010; Crab et al., 2010). A bacterial community change represents a challenge to the operation of BFT systems because no information can be found in the literature concerning changes in bacterial communities that may promote more nutritious biofloc for shrimp, and how the bacterial community of biofloc particles is related to shrimp gills, hepatopancreas and intestinal bacteria flora. Furthermore, Crab et al. (2012) stated that optimization of the nutritional quality of biofloc is a challenge for further research.

Biofloc technology is considered a practical solution to maintain water quality at optimum levels because this culture system is based on the capacity of autotrophic and

heterotrophic bacteria to utilize the nitrogen present in the water and convert it into new bacterial biomass (Avnimelech, 2009; De Schryver et al., 2008). The limited or zero-water exchange of intensive biofloc shrimp production systems allows for accumulation of inorganic nitrogen, which is controlled via nitrification by chemoautotrophic bacteria (which in this dissertation will be called autotrophic phase) or assimilation by heterotrophic bacteria (which in this dissertation will be called heterotrophic phase). Both, nitrification and accumulation of inorganic carbon, occur simultaneously, but levels of intensity depend on extent of respective bacterial populations.

Nitrification will increase in the presence of high culture densities of chemoautotrophic bacteria. Autotrophic nitrification is a two-step process in which ammonia is biologically oxidized into nitrite (catalyzed by ammonia-oxidizing bacteria such as *Nitrosomonas* spp. and *Nitrosococcus* spp.) and then to nitrate (catalyzed by nitrite-oxidizing bacteria such as *Nitrobacter* spp. and *Nitrospira* spp.) with oxygen as terminal electron acceptor (Rittman and McCarty, 2001). This process reduces alkalinity in the form of carbonates and bicarbonates (Chen and Blancheton, 2006).

In order for the heterotrophic bacteria to utilize organic and inorganic nitrogen in the culture water, it is necessary to control the carbon:nitrogen ratio by reducing the feed protein content and/or by the addition of carbon into the culture water (De Schyver et al., 2008). Uptake of carbon-based substrates and immobilization of nitrogen increase as levels of heterotrophic bacteria also increase.

The close relationship between bacteria present in BFT and water quality makes bacterial community changes an important area of study due to their potential effect on water quality.

One of the downsides of the BFT system is that biofloc is a dynamic growth medium, which potentially results in an increase in populations of pathogenic bacterial species (mainly *Vibrio* spp.) because of high concentrations of organic matter in the culture water (Ferreira et al., 2011). Increases in potential pathogen populations may result in reduced shrimp production due to impaired survival and/or growth rate.

Application of antibiotics in aquaculture for prophylactic and therapeutic purposes has been criticized due to the potential development of antibiotic-resistant bacteria, the presence of antibiotic residues in seafood, undesired modification of the bacterial population in the aquatic environment and suppression of the animal's immune system (Ng et al., 2009). For those reasons, alternatives to antibiotics that improve shrimp immune defense and resistance to pathogens as well as improving growth and survival are desired. Targeted modifications of bacterial populations in the gastrointestinal tract of shrimp have been suggested as alternatives to antibiotics that may hold the key to optimize weight gain and improve health status of farmed animals (Anuta et al., 2016). Such targeted modifications of bacterial populations could be achieved with the use of different additives as reviewed by Anuta et al. (2016).

It is widely known that microorganisms, their cellular components or their metabolites may act as immunostimulants to enhance the shrimp's innate immune system and provide improved protection against pathogens (Smith et al., 2003; Vazquez et al., 2009). However, very few studies (de Jesús Becerra-Dorame et al., 2014; Xu and Pan, 2013, 2014b; Kim et al., 2014) have investigated the immunological potential of microorganisms found in BFT. Furthermore, there were no studies found in the literature that evaluated the effect of different compounds with potential effect on bacterial flora of

biofloc and shrimp gills, hepatopancreas and intestinal contents and on shrimp production and health.

I.3 Additives

A few additives have been studied with the aim to improve water quality and health and growth of the animals when cultured under BFT conditions. Antibiotics and disinfectants are additives commonly used in aquaculture to counter disease outbreaks, however, they are known to depress ecological health and environmental safety (Deng et al., 2013). Also, numerous studies have confirmed that the improper use of antibiotics and disinfectants would increase the risk of drug resistance of pathogenic microbes, threaten food safety and human health and disturb or destroy the normal bacterial populations in any aquaculture environment (Lalumera et al., 2004; Balcazar et al., 2006; Defoirdt et al., 2007) which make them unfeasible to use in BFT.

Positive effects on water quality, health and growth of fish (and other livestock species), due to different additives, such as prebiotics, essential oils and organic acids, have been established when such additives are incorporated in the feed or in the culture water (Anuta et al., 2016; Azhar et al., 2016; Gracia-Valenzuela et al., 2014; Kim et al., 2011; Adams and Boopathy, 2013; da Silva et al., 2013). However, the mode of action has yet to be determined for each additive to achieve higher shrimp production and improve water quality when added to the culture water or to the feed used in a BFT system. A major deterrent to using additives in the culture water is the amount required per unit of production. However, with the demonstration that shrimp can be cultured using shallow water depths of less than 30 cm, resulting in much greater production

levels of up to 25 kg/m^2 per crop (Lawrence et al., 2015), the use of additives in shallow-water culture systems may be commercially feasible. Potential changes in bacterial communities present in biofloc, and their effects on shrimp intestine, gills and hepatopancreas, have not yet been documented when different additives such as prebiotics, organic acids and their salts and/or essential oils are added to the BFT system.

The usage of prebiotics, organic acids and essential oils in different animal industries to increase growth or enhance animal health will be described next as well as different future perspectives on their use to increase shrimp growth and enhance its health.

I.3.1 Prebiotics

Although the definition of prebiotics has passed through several reviews and modifications, it is well accepted that a genuine prebiotic needs to fulfil three criteria: (1) resist gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption, (2) undergo fermentation by microbiota, and (3) selectively stimulate the growth and/or activity of bacteria associated with health and well-being (Venema and do Carmo, 2015). Studies have been conducted to evaluate the efficacy of some prebiotics in shrimp culture, finding improvements in weight gain and feed efficiency ratio (FE) (Zhou et al., 2007; Genc et al., 2007). Zhou et al. (2007) also observed a displacement of potentially pathogenic bacteria in the gastrointestinal tract by mostly *Lactobacillus* sp., known to be beneficial to the health of shrimp. Very little information has been found on the use of prebiotics in a BFT system for shrimp culture and how they could improve production parameters and water quality (Crockett and Lawrence, 2017).

I.3.2 Organic acids and their salts

Organic acids and their salts are generally regarded as safe diet additives and are receiving increasing attention due to their strong antibacterial and prophylactic properties against various pathogenic bacteria. These acids also have been shown to trigger beneficial effects on mineral absorption, nutrient digestibility and growth performance of various organisms. It is believed that the primary antibacterial action of organic acids is by altering the cell cytoplasm pH of bacteria and those that are sensitive to such changes are inhibited or killed, thus reducing the numbers of harmful bacteria within the gastrointestinal tract of the host animal (Booth and Stratford, 2003). Some organic acids are known to inhibit various *Vibrio* strains *in vitro,* but are highly dependent on type and level (da Silva et al., 2013; Defoirdt et al., 2006; Ng and Koh, 2011) when tested *in vivo* with various aquatic species.

Da Silva et al. (2013) concluded that the use of organic acid salts could improve marine shrimp nutrition and health and that propionate has the greatest potential for use as a diet supplement for *L. vannamei*. Meanwhile, Romano et al. (2015) found that an organic acid blend can substantially improve productivity and resistance to pathogenic bacteria and may be a viable alternative to the use of antibiotics in the shrimp industry. There was no information found in the literature on the use of organic acids in a BFT system for shrimp culture.

I.3.3 Essential oils

Essential oils are environmentally friendly alternatives to antibiotics for the control of disease vectors, bacteria and parasites. Essential oils have been used in poultry

and fish nutrition as feed additives to improve performance indices and feed utilization via activation of digestive system structure and function, enhancing absorption and metabolism of nutrients, altering the gut microbiota, and reducing hazardous compounds and free radicals from interacting with cellular compounds (Ezzat Abd El-Hack et al., 2016). There is no information on the effect and mode of action of essential oils when added to feed on water quality and shrimp health and growth when cultured under biofloc conditions.

Three trials were conducted to test the effect those various additives may have on bacterial flora which form biofloc particles (BFP) and which may be present in shrimp gill tissue (GT), hepatopancreas (HP) and intestinal contents (IC).

CHAPTER II

EFFECT OF PREBIOTICS ADDED TO THE CULTURE WATER ON BACTERIAL COMPOSITION OF BIOFLOC AND PACIFIC WHITE SHRIMP *Litopenaeus vannamai* INTESTINE, GILLS AND HEPATOPANCREAS

II.1 Introduction

Antibiotics and disinfectants are additives commonly used in aquaculture to counter disease outbreaks; however, they are known to depress ecological health and environmental safety (Deng et al., 2013). Also, numerous studies have confirmed that the improper use of antibiotics and disinfectants would increase the risk of drug resistance of pathogenic microbes, threaten food safety and human health and disturb or destroy the normal bacterial populations in any aquaculture environment (Lalumera et al., 2004; Balcazar et al., 2006; Defoirdt et al., 2007) which make them unfeasible to use in Biofloc Technology (BFT) systems, a system in which bacterial populations control water quality and provide nutrients to the culture organisms.

Prebiotics are composed of natural, fermentable oligosaccharides that are not digested by the host but provide a source of metabolizable energy to some genera of bacteria that confer beneficial properties to the host (Gibson et al., 2004). Prebiotics have been proposed as an alternative to the use of antibiotics and disinfectants. Although the definition of prebiotics has passed through several reviews and modifications, it is well accepted that a genuine prebiotic needs to fulfil three criteria: (1) resist gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption, (2) undergo

fermentation by microbiota, and (3) selectively stimulate the growth and/or activity of bacteria associated with health and well-being (Venema and do Carmo, 2015).

The effect of prebiotics have been studied to a limited extent for crustacean aquaculture (Daniels and Hoseinifar, 2014; Merrifield and Ringø, 2014). Studies have been conducted to evaluate the efficacy of some prebiotics in shrimp culture, finding improvements in weight gain and feed efficiency ratio (FE) (Zhou et al., 2007; Genc et al., 2007).

Normally, prebiotics are provided to the cultured organisms through their feed, however, it has been proven that shrimp consume biofloc particles as a supplemental source of nutrients (Ray et al., 2017). For this reason, it can be assumed that, with the addition of prebiotics to the culture water, the effect that prebiotics have on bacteria associated with biofloc particles can ultimately have an effect on the shrimp internal bacterial composition. However, to my knowledge, no study on the effect of prebiotics added to the culture water on shrimp production and microbiology has been developed on shrimp cultured under biofloc conditions. Therefore, the objective of this study was to evaluate the effect of prebiotics and non-prebiotic carbohydrates on bacteria profiles in biofloc particles and Pacific white shrimp, *Litopenaeus vannamei*, hepatopancreas, intestine and gills when added directly to the culture water of a biofloc-based shallow-water research system.

The prebiotics fructooligossacharide (FOS), galactooligosaccharide (GOS), mannan-oligosaccharide (MOS) and inulin (INU), as well as the non-prebiotic soluble carbohydrates wheat starch (WSt) and sucrose (SUC), were added to the culture water of

a BFT research system for the culture of *L. vannamei* to evaluate their effect on biofloc and shrimp bacterial communities, shrimp production and health and on water quality.

II.2 Materials and methods

II.2.1 Experimental conditions

A 26-day trial (Trial I) was conducted in the trū® Shrimp Company experimental station located in Balaton, MN, USA to evaluate the effect of different prebiotics on shrimp production and water quality, and on bacterial composition of biofloc and shrimp hepatopancreas, gills and intestinal contents. Thirty-six tanks (0.457 m × 0.457 m × 0.280 m) containing an independent heater, an automatic 48-h feeder, and two air stones were used in this study. Tanks were filled to 20-cm depth with artificial seawater of 28 g/L salinity and maintained at 30.0 ± 1.0ºC. To maintain buffering capacity, sodium bicarbonate ($NaHCO_3$) was added if alkalinity levels fell below 180 mg/L. Application levels were determined using the following formula:

$NaHCO_3$ needed per tank (g) = ((deficiency in alkalinity (mg/L) / concentration of HCO_3 in $NaHCO_3$ (72.646 (%); 0.72646)) × tank volume (L)) / 1,000 (mg/g)

Water lost due to evaporation was replaced weekly with reverse osmosis water to maintain salinity at 28 ± 1 g/L. All tanks were operated as a zero-water exchange BFT system. During the autotrophic phase (ATr; see Chapter I, subheading I.2), autotrophic bacteria were promoted and maintained from the initial day until Imhoff cone readings

reached 3 mL/L. Only during the autotrophic phase, a nitrifying bacteria inoculum (Turbo Start 900, FritzZyme, Mezquite, TX, USA) was added to the culture water according to manufacturer recommendations, and shrimp were given feed pellets and fines formulated to contain 35% crude protein (CP) (as-fed basis) at a mean of 3.33 g/day per tank and 0.037 g/L, respectively. Heterotrophic phase (see Chapter I, subheading I.2) was promoted when Imhoff cone readings reached 3 mL/L. Only during heterotrophic phase, total ammonia nitrogen (TAN; NH_3/NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) levels were maintained at <3, <5 and <100 mg/L, respectively, by feeding a 23% crude protein feed and by adding the prebiotics fructooligosaccharide (FOS), galactooligosaccharide (GOS), inulin (INU) and mannan-oligosaccharide (MOS) and the non-prebiotic soluble carbohydrates wheat starch (WSt) and sucrose (SUC) into the culture water of each tank according to its treatment assignment. The prebiotics and non-prebiotics soluble carbohydrates were added to each tank on feeding days, at a rate of 3% of the feed weight provided.

II.2.2 Shrimp

Post-larvae weighing 0.003 g arrived at experimental station from a commercial hatchery (Shrimp Improvement Systems, SIS, Inc., Islamorada, Florida, USA) and were acclimated in a nursery tank filled with artificial seawater of 28.0 ± 0.5 g/L salinity, at 29.0 ± 1.0ºC. Shrimp in the nursery tank were fed daily with a commercial feed (Ziegler Bros., Inc., Gardners, PA, USA) until reaching a mean individual weight of 6 g. Shrimp with no visual signs of disease or stress were collected from the nursery tank and individually weighed. Shrimp weighing 6.0 ± 0.5 g were stocked into each tank at a

density of 15 shrimp/tank. Only group weight was recorded and mean individual weight was calculated as well as initial biomass based on water volume of the experimental tanks (g/m^3) (Table II.1). Mortality counts and weights were recorded and replaced with the same size shrimp only during a 3-day acclimation period. When the trial started, a small net was used to carefully check for mortalities and leftover feed without disturbing the living organisms. Mortalities were recorded and discarded.

Table II.1. Initial shrimp group weight, biomass and individual shrimp weight for Trial I.[1]

Treatment[2]	Group weight (g)	Biomass (g/m^3)	Individual mean weight (g)
FOS	89.6	2,144.5	6.0
GOS	90.3	2,159.9	6.0
INU	89.9	2,150.9	6.0
MOS	91.1	2,180.2	6.1
WSt	89.2	2,133.3	5.9
SUC	91.3	2,184.5	6.1
PSE[3]	0.41	9.81	0.03

[1] Values are expressed as means per treatment.
[2] FOS = fructooligosaccharide; GOS = galactooligosaccharide; INU = inulin; MOS = mannan-oligosaccharide; WSt = wheat starch; SUC = sucrose.
[3] PSE = pooled standard error of treatment means (n = 6).

II.2.3 Feed and feeding management

Shrimp in Trial I were fed the same reference diet (Table II.2). Two diets were prepared 8 days before shrimp were stocked in the experimental tanks. Each diet was formulated to contain different protein concentrations. To prepare the diets, all dry ingredients were weighed and mixed in an industrial mixer for 15 min until a completely homogenized mixture was achieved. Next, the dry mixture was blended with sodium

hexametaphosphate and alginate (Table II.2) previously mixed with 150 mL of deionized water per kg of dry feed using a hand mixer (Sunbeam Products Inc., Milford, MA) until an appropriate mash consistency was obtained for extrusion. Fish and soybean oil were also added during this step. Extrusion was made using a meat chopper attachment (Model A-800, Hobart Corporation, Troy, OH, USA) fitted with a 3-mm die. Moist feed strands were dried on wire racks in a forced air oven at 35^{o}C to a moisture content of 8-10%. After a 24-h drying period, feed was milled and sifted into the appropriate size for shrimp consumption, bagged, and stored at 4^{o}C until used to feed the shrimp or for proximate analysis composition according to the AOAC (1990) procedures for dry matter, lipid, and ash contents and according to the Dumas method (AOAC, 2005) for crude protein composition. Proximate composition analyses of feed samples were performed in duplicate.

Formulation as well as ash, protein and lipid composition of each diet can be found in Table II.2. Fines were obtained by grinding the higher protein content diet and sieving to obtain particles between 0.595-0.420 mm. During the autotrophic phase (day 0-4), the higher protein content diet was used to feed shrimp and fines were added directly into the culture water. Feed and fines were calculated and actual amounts supplied can be found in Table II.3. The lower protein content diet was used during the heterotrophic phase (day 5 until termination) in order to increase the carbon:nitrogen (C:N) ratio and to promote the dominance of heterotrophic bacteria (Table II.3). Feed efficiency (FE) was adjusted based on biofloc concentration in the tanks, as can be noted in Table II.3, because, as demonstrated, shrimp can supplement their nutritional requirements with biofloc particles (Ray et al., 2017). The analyzed composition of the

diets fed to shrimp during Trial I were slightly higher than the target protein content (Table II.2) possibly due to some fiber that escaped while mixing.

Based on previous experience, it was expected that, for this size, shrimp were going to grow linearly. A feed curve based on number of shrimp, expected shrimp daily growth and FE was used to determine the expected feed regimen offered to each tank (Expected feed regimen = (Shrimp count × Expected weight increase (g)) × FE) (Table II.3). Each 48-h feeder was loaded every other day based on the expected feed regimen of 2 days with adjustments based on leftover feed, water quality and mortalities.

Table II.2. Formulation (%) of the two diets used for Trial I with determined ash, protein and lipid proximate composition (g/kg).

	Protein content	
Ingredient (%)	35%	23%
Squid muscle meal[a]	30.00	21.70
Wheat starch[b]	28.75	41.75
Fish meal[c]	8.00	8.00
Soy protein isolated[d]	5.70	0.00
Dicalcium phosphate[b]	4.20	4.60
Lecithin, dry, 95%[c]	4.00	4.00
Diatomaceous earth[e]	3.80	3.70
Cellulose[f]	3.20	3.20
Calcium carbonate[f]	2.50	2.20
Alginate (Manucol DM)[g]	2.00	2.00
Potassium chloride[f]	1.90	2.00
Magnesium oxide[h]	1.60	1.60
Sodium hexametaphosphate[f]	1.00	1.00
Sodium chloride[f]	0.70	0.90
Menhaden fish oil[c]	0.60	1.10
Soybean oil[i]	0.60	0.70
Vit/Min premix[k]	1.25	1.25
Cholesterol[a]	0.20	0.20
$_{DL}$-Methionine[j]	0.00	0.10
Proximate composition (g/kg, dry weight)		
Crude Protein	385.5	271.6
Crude Lipids	82.5	81.6
Ash	131.4	189.5

[a] Zeigler Bros., Inc. Gardners, PA, USA.
[b] MP Biomedicals Santa Ana, CA, USA.
[c] ADM Co. Chicago, IL, USA.
[d] Solae LLC, St. Louis, MO, USA.
[e] Absorbent Products LTD.
[f] Fisher Scientific.
[g] FMC BioPolymer.
[h] Prince Agri Products.
[i] Consumer's Supply.
[j] Evonik Degussa Corporation.
[k] Composition given in Appendix.

Table II.3. Expected feed curve based on shrimp count, expected weight increase and feed efficiency with actual fines and feed provided for Trial I.

Day	Shrimp count	Expected weight (g)	Expected weight increase (g)	FE	Expected feed regimen (g)	35% CP fines (g)	Feed protein %	Actual feed provided (g)
ACN	15	6.00	-	1.30	2.925	-	35	5.850
ACN	15	6.15	0.15	1.30	2.925	-	-	-
ACN	15	6.30	0.15	1.20	2.700	-	35	5.400
0	15	6.45	0.15	1.20	2.700	3.0	-	-
1	15	6.60	0.15	1.00	2.250	3.0	35	5.175
2	15	6.75	0.15	1.10	2.925	3.0	-	-
3	15	6.90	0.15	1.10	2.925	3.0	35	5.625
4	15	7.05	0.15	1.10	2.700	3.0	-	-
5	15	7.20	0.15	1.10	2.700	-	23	5.175
6	15	7.35	0.15	1.10	2.475	-	-	-
7	15	7.50	0.15	1.10	2.475	-	23	4.725
8	15	7.65	0.15	1.00	2.250	-	-	-
9	15	7.80	0.15	1.00	2.250	-	23	4.500
10	15	7.95	0.15	1.00	2.250	-	-	-
11	15	8.10	0.15	0.90	2.025	-	23	4.050
12	15	8.25	0.15	0.90	2.025	-	-	-
13	15	8.40	0.15	0.90	2.025	-	23	4.050
14	15	8.55	0.15	0.90	2.025	-	-	-
15	15	8.70	0.15	0.90	2.025	-	23	3.825
16	15	8.85	0.15	0.80	1.800	-	-	-
17	15	9.00	0.15	0.80	1.800	-	23	3.600
18	15	9.15	0.15	0.80	1.800	-	-	-
19	15	9.30	0.15	0.80	1.800	-	23	3.600
20	15	9.45	0.15	0.80	1.800	-	-	-
21	15	9.60	0.15	0.80	1.800	-	23	3.600
22	15	9.75	0.15	0.80	1.800	-	-	-
23	15	9.90	0.15	0.80	1.800	-	23	3.600
24	15	10.05	0.15	0.80	1.800	-	-	-
25	15	10.20	0.15	0.80	1.800	-	23	1.800
26	15	10.35	0.15	0.80	0.000	-	-	TMN

ACN = acclimation period; FE = feed efficiency; TMN = termination.
Expected feed regimen = (shrimp count × expected weight increase) × FE.
Feed provided = actual feed loaded on 48-h feeders with the amount calculated for 2 days

II.2.4 Experimental treatments

Non-prebiotic carbohydrates (wheat starch [WSt; MP Biomedicals Santa Ana, CA, USA], and sucrose [SUC; table sugar; Nash Finch Company, Minneapolis, MN, USA]) were used in this study as controls to evaluate the effect of soluble prebiotics. Prebiotics used in this study were short-chain fructooligosaccharide (sc-FOS [FOS in this manuscript]; Ingredion Incorporated, Indianapolis, IN, USA), galactooligosaccharide (GOS; Ingredion Incorporated, Indianapolis, IN, USA), mannan-oligosaccharide (Bio-MOS [MOS in this manuscript]; Alltech, Nicolasville, KY, USA) and inulin (INU; JeTsu Technology Limited, London, UK). Each treatment was randomly assigned to six replicate tanks for a total of 36 tanks in Trial I. Only during the heterotrophic phase, each additive was mixed every other day directly into the culture water of each experimental tank according to its treatment assignment to increase the C:N ratio and to promote heterotrophic bacteria dominance. The amount of carbohydrate added was based on feed rate (carbohydrate added (g) = feed provided (g) × 0.03). A carbohydrate addition of 3% of the feed provided was found to be enough to increase C:N to promote heterotrophic dominance and control water quality.

II.2.5 Data acquisition and analyses

II.2.5.1 Water quality

Dissolved oxygen (DO), temperature and salinity of the culture water were measured daily using a YSI 85 oxygen/conductivity instrument (YSI, Yellow Springs, Ohio, USA). pH was monitored daily using a YSI pH 100 (YSI). Total ammonia-

nitrogen, nitrites, nitrates and alkalinity were recorded weekly from each tank using a Hach DR6000 spectrophotometer (Hach, Loveland, Colorado, USA) according to manufacturer instructions.

II.2.5.2 Biofloc volume and proximate composition

To measure biofloc level in the water, water samples were collected from each tank weekly using a glass beaker and poured into Imhoff cones. After 30 min, water in the Imhoff cones was gently stirred using a glass stirring rod. Fifteen minutes later, the settled biofloc volume was recorded from the bottom of the cone in mL/L. All tools used were thoroughly disinfected with Virkon Aquatic (Syndel USA, Ferndale, WA, USA) before they were in contact with the culture water to avoid cross-contamination.

Proximate composition analyses was performed in samples collected immediately before switching to heterotrophic phase (during autotrophic phase; ATr) and on termination day from each experimental treatment. One liter of culture water was collected from each tank and poured into Imhoff cones for 30 min that allowed biofloc to completely settle to the bottom. Carefully, biofloc samples were collected from the bottom of the Imhoff cones by siphoning the upper phase of the water and removing the bottom cap of to imhoff cone to collect the biofloc into 5 mL tubes. Biofloc was then analyzed for proximate composition according to the AOAC (1990) procedures for dry matter, lipid, and ash contents and according to the Dumas method (AOAC, 2005) for crude protein composition. Proximate composition of biofloc was performed in duplicate per sample unless specified otherwise.

II.2.5.3 Hemolymph

On the morning of day 26, 24-hr after loading feeders and 1-hr before termination, hemolymph was withdraw from the ventral sinus of one shrimp per tank of three randomly chosen tanks per treatment using tuberculin syringes (1 mL 22G x 32 mm) to determine total hemocyte counts. Before hemolymph was withdrawn, syringes were loaded with 300 uL of anticoagulant 1 (Table II.4). Next, the needle was carefully inserted into the ventral sinus to reach the hematopoietic tissue without breaking the ventral nerve. Slowly, a 50-100 uL of hemolymph were extracted. The needle was removed and the exact amount collected was recorded. The sample was carefully poured in a 5-mL microcentrifuge tube. Microcentrifuge tubes contents were gently homogenized using a vortex until hemolymph and anticoagulant 1 were completely mixed. Ten microliters of the mixture were mixed with 90 uL of anticoagulant 2. Samples were gently homogenized with a vortex and total hemocyte counts per milliliter were determined using a Neubauer hemocytometer.

Table II.4. Anticoagulant formula.

Anticoagulant 1	
Reagent	For 1 L:
Trisodium citrate (294.1 g/mol)	7.94 g
NaCl (54.88 g/mol)	22.49 g
Glucose (Anyhdrose dextrose) (180 g/mol)	20.71 g
Distilled water	1 L
Anticoagulant 2	
Reagent	For 500 mL:
Anticoagulant 1	400 mL
Formaldehyde	100 mL

II.2.5.4 Sample collection for denaturing gradient gel electrophoresis

PCR-Denaturing gradient gel electrophoresis (DGGE), a genetic fingerprinting technique that examines bacterial diversity based upon electrophoresis of PCR-amplified 16S rDNA fragments using polyacrylamide gels (Muyzer et al., 1993), was selected to compare bacterial communities among treatments.

For DGGE, biofloc was collected right before switching to the lower protein content diet (during the autotrophic phase) and during termination from each treatment. Approximately, 50-mL of water from three randomly chosen tanks per treatment were poured into sterile Stericup® vacuum filtration systems (Millipore Corporation, Billerica, MA, USA). Biofloc that remained on the membrane was collected, using a sterile dissecting spatula, into sterilized 1.5-mL microcentrifuge tubes. Samples were stored at -80^{o}C until analyzed.

Shrimp intestines, hepatopancreas and gills from each treatment were aseptically collected for DGGE analysis on stocking day (initial sample; INL), immediately before switching to the lower protein content diet (autotrophic phase; ATr) and during termination from each treatment. The weights of the shrimp collected for DGGE were recorded for final production data.

II.2.5.5 Termination and harvest

Shrimp weight, length and hepatopancreas weight of one shrimp per tank from three randomly chosen tanks per treatment were recorded to calculate a condition factor (K; according to Chow and Sandifer, 1991) and the hepatosomatic index.

During termination, water levels in all tanks were reduced and all shrimp were harvested one tank at a time. The final number of shrimp and shrimp group weight were recorded per tank to calculate mean final individual weight, weight gain and survival. Also, on termination day one shrimp per tank from three randomly chosen tanks per treatment was stored for proximate composition according to the AOAC (1990) procedures for dry matter, lipid, and ash contents and to the Dumas method (AOAC, 2005) for crude protein composition. Proximate composition of shrimp muscle was performed in duplicate samples unless specified otherwise.

II.2.6 DNA isolation and PCR

Triplicate samples of biofloc particles (BFP), intestinal contents (IC), hepatopancreas (HP) and gills tissue (GT) from each treatment were thawed and pelleted by centrifugation at 5,000 × *g* for 5 min. Approximately 0.5 g of pelleted samples in each replicate was placed in a sterile microcentrifuge tube for genomic DNA isolation. The pellet was suspended in 180 µL of lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1.2% Triton-100, 20 mg lysozyme mL-1 (Sigma Chemical Company, Sant Louis, MO, USA)) and incubated for 30 min at 37°C. Genomic DNA isolation was conducted using a QIAamp® DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Once isolated, DNA was amplified through PCR as described by Hume et al. (2003), using bacteria-specific PCR primers to conserved regions of the variable V3 region of 16S rDNA.

II.2.7 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis was performed as described by Hume et al. (2003) using polyacrylamide gels (8% v/v, acrylamide-bisacrylamide (BioRad Laboratories, Richmond, CA) ratio 37.5:1). Electrophoresis was performed for 17 h at 60 V using a DCode Universal Mutation Detection System (BioRad). Subsequently, gels were stained with SYBR Green I (1:10,000 dilution; Sigma) and digitalized for analysis.

The analysis of DGGE band pattern relatedness was determined using GelCompare II, v6.6 11 (Applied Maths, Austin, TX) based on the Dice similarity coefficient (SC) and the unweighted pair group method using arithmetic averages for clustering. Comparisons between sample band patterns are expressed as a similarity coefficient (SC) and results were translated as ≥95 = likely the same or identical, 90-94 = very similar, 85-89 = similar, 80-84 = somewhat similar, and ≤79 = not similar.

II.2.8 Calculations and statistical analyses

The responses utilized to compare production parameters among treatments in this study were calculated as follow:

- Percent weight gain, % = [(final weight (g) – initial weight (g)) / (initial weight (g))] × 100
- Feed efficiency ratio (FE) = [weight gain (g) / dry feed offered (g)]
- Percent survival, % = [(final shrimp number – initial shrimp number) / (initial shrimp number)] × 100
- Condition factor (K) = [body weight (g) / (body length (mm))^3)] × 10^5

- Hepatosomatic index (HSI) = [hepatopancreas weight (g) / body weight (g)] × 100
- Total hemocyte count (THC) = (cells counted × dilution factor × 1000) / volume of grid (0.1 mm^3)

Data were analyzed for normality and homogeneity of variances assumptions (Shapiro-Wilk test and Levene's test, respectively). Data were then subjected to one-way analysis of variance (ANOVA) to detect significant differences among treatments ($p \leq 0.05$). Significant one-way ANOVA was followed by a post hoc multiple comparison test (Student's LSD). The analysis was conducted using the SAS 9.2 statistical package (SAS Institute Inc., Cary, NC, USA).

II.3 Results

Mean dissolved oxygen, water temperature, salinity and pH are shown by treatment in Table II.5. Final weight, weight gain, FE, survival, HSI and THC or condition factor score showed no significant differences among treatments (Table II.6). Mean biofloc concentration of the WSt treatment was lower ($p \leq 0.05$) than the rest of the treatments (Figure II.1). Significant differences in water quality were only observed in day 23 when water of tanks to which INU was added had a higher nitrate concentration than the water of tanks containing WSt (Tables II.7 and II.8).

Table II.5. Dissolved oxygen (mg/L), salinity (g/L), temperature (°C) and pH mean results from daily observations during Trial I.[1]

Treatment[2]	Dissolved Oxygen (mg/L)	Salinity (g/L)	Temperature (°C)	pH
FOS	5.61	28.49	30.03	7.95
GOS	5.58	28.52	30.13	7.96
INU	5.59	28.50	30.13	8.02
MOS	5.60	28.51	29.95	7.98
WSt	5.66	28.63	30.03	8.02
SUC	5.63	28.54	30.18	8.02
PSE[3]	0.02	0.03	0.08	0.01

[1] Values represent mean per treatment.
[2] FOS = fructooligosaccharide; GOS = galactooligosaccharide; INU = inulin; MOS = mannan-oligosaccharide; WSt = wheat starch; SUC = sucrose.
[2] PSE = pooled standard error of treatment means (n = 6; mean value of six tanks per treatment).

Table II.6. Final weight, weight gain, feed efficiency and survival as well as K, HSI and THC of shrimp cultured with prebiotics fructooligosaccharide, galactooligosaccharide, inulin and mannan-oligosacchaide and non-prebiotics wheat starch and sucrose added into the culture water for Trial I.[1]

Treatment[2]	Final weight (g)	Weight gain (%)	FE	Survival (%)	K	HSI	THC
FOS	9.7	163	1.3	96.7	0.85	0.04	1.78
GOS	9.7	161	1.3	96.7	1.10	0.04	1.49
INU	9.5	158	1.3	97.8	0.90	0.04	1.19
MOS	9.6	158	1.3	98.9	0.92	0.04	1.36
WSt	9.7	162	1.2	97.8	0.91	0.03	1.70
SUC	9.6	157	1.4	97.8	0.90	0.04	1.43
PSE[3]	0.09	1.47	0.03	0.60	0.03	0.00	0.17
Anova (Pr > F)	0.972	0.813	0.847	0.916	0.082	0.428	0.945

[1] Values represent mean per treatment.

[2] FOS = Fructooligosaccharide; GOS = Galactooligosaccharide; INU = inulin; MOS = mannan-oligosaccharide; WSt = wheat starch; SUC = sucrose. FE = feed efficiency; K = condition factor; HSI = hepatosomatic index; THC = total hemocyte count (10^7 cells/mL).

[3] PSE = pooled standard error of treatment means (final weight, weight gain, FE and survival: n=6; K, HSI and THC: n = 3).

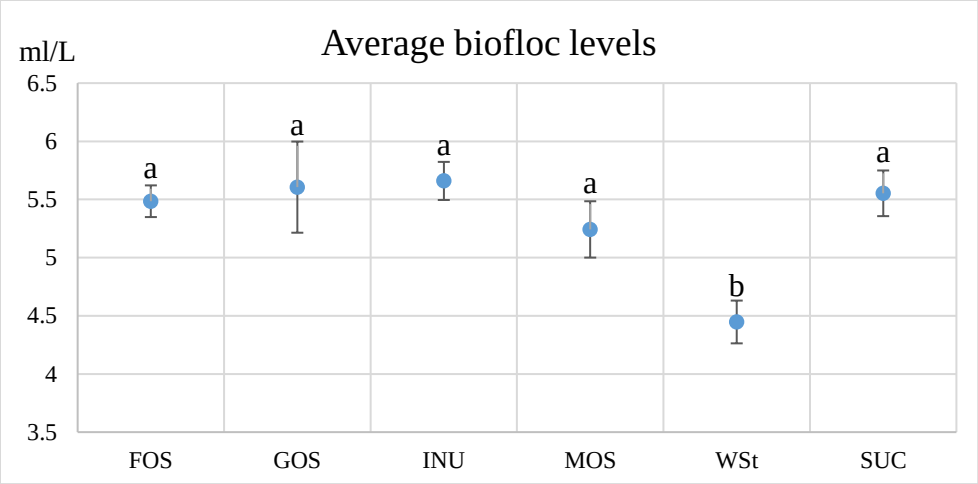

Figure II.1. Mean biofloc levels (mL/L) of tanks subjected to prebiotics fructooligosaccharide, galactooligosaccharide, inulin or mannan-oligosaccharide or non-prebiotic carbohydrates wheat starch or sucrose added directly to the water during Trial I.[1,2]

[1] Values are expressed as means ± PSE (n = 6) of the weekly biofloc readings obtained from Imhoff cones. Different superscript letter means significantly different ($p \leq 0.05$).

[2] FOS = fructooligosaccharide; GOS = galactooligosaccharide; INU = inulin; MOS = mannan-oligosaccharide; WSt = wheat starch; SUC = sucrose.

Table II.7. Mean values over time for total-ammonia nitrogen (TAN) and nitrites of tanks with prebiotics fructooligosaccharide, galactooligosaccharide, inulin or mannan-oligosaccharide or non-prebiotic carbohydrates wheat starch or sucrose added into the culture water for Trial I.[1]

	Total Ammonia-Nitrogen				Nitrites			
Treatment[2]	Day 2	Day 9	Day 16	Day 23	Day 2	Day 9	Day 16	Day 23
FOS	1.34	0.07	0.07	0.05	0.70	0.13	0.12	0.08
GOS	1.75	0.06	0.06	0.06	0.80	0.15	0.17	0.07
INU	1.61	0.07	0.05	0.06	1.01	0.10	0.10	0.10
MOS	1.51	0.09	0.06	0.05	0.72	0.18	0.09	0.10
WSt	1.84	0.07	0.06	0.05	0.80	0.10	0.12	0.14
SUC	1.68	0.06	0.06	0.05	0.88	0.13	0.12	0.07
PSE[3]	0.22	0.00	0.00	0.00	0.05	0.02	0.01	0.01
Anova (Pr>F)	0.991	0.703	0.554	0.580	0.563	0.712	0.121	0.144

[1] Values represent treatment means of the weekly observations from six replicate tanks per treatment.

[2] FOS = fructooligosaccharide; GOS = galactooligosaccharide; INU = inulin; MOS = mannan-oligosaccharide; WSt = wheat starch; SUC = sucrose.

[3] PSE = pooled standard error of treatment means (n = 6).

Table II.8. Mean values over time for nitrates and alkalinity of tanks with prebiotics fructooligosaccharide, galactooligosaccharide, inulin or mannan-oligosaccharide or non-prebiotic carbohydrates wheat starch or sucrose added into the culture water for Trial I.[1]

	Nitrates				Alkalinity			
Treatment[2]	Day 2	Day 9	Day 16	Day 23	Day 2	Day 9	Day 16	Day 23
FOS	5.4	18.2	28.8	41.1[ab]	185.3	163.2	176.2	165.5
GOS	4.9	16.8	25.9	36.8[ab]	194.0	164.3	175.5	165.5
INU	5.1	18.2	26.9	43.4[a]	182.0	184.2	174.5	166.2
MOS	4.4	14.5	27.3	35.2[ab]	199.5	177.2	176.5	164.2
WSt	5.5	17.9	26.8	33.4[b]	185.7	209.2	192.8	165.8
SUC	5.4	16.7	28.8	41.5[ab]	180.7	161.3	174.2	161.7
PSE[3]	0.22	0.71	1.44	1.24	4.20	5.56	3.98	2.29
Anova (Pr>F)	0.700	0.669	0.993	0.016	0.703	0.094	0.771	0.995

[1] Values represent treatment means of the weekly observations from six replicate tanks per treatment.
[2] FOS = fructooligosaccharide; GOS = galactooligosaccharide; INU = inulin; MOS = mannan-oligosaccharide; WSt = wheat starch; SUC = sucrose.
[3] PSE = pooled standard error of treatment means (n = 6). Means in columns with different superscript are significantly different ($p \leq 0.05$).

Proximate analyses of biofloc samples showed that, during the autotrophic phase (ATr), a higher ($p \leq 0.05$) protein content was found in biofloc than the rest of the treatments (Table II.9). No significant differences were found in any of the treatments for lipids or ash composition. Proximate composition showed that muscle of shrimp given MOS had a higher ($p \leq 0.05$) lipid content than shrimp muscle of the GOS, INU and WSt treatments. Also, shrimp muscle of the WSt treatment had higher ($p \leq 0.05$) lipid concentration than the GOS and INU treatment. No significant differences were observed for ash or protein contents of shrimp muscle (Table II.9).

In terms of bacterial community present in the biofloc particles (Figure II.2), although there was little similarity at 61.5%SC between bacterial communities from ATr and WSt, these two communities were more similar to each other than to bacterial

communities from the other treatments. There was some similarity between bacterial communities from the GOS and MOS treatments, while bacterial communities of the FOS, INU and SUC treatments were very similar. However, these two groups shared only a 71%SC.

Table II.9. Ash, protein and lipid contents of biofloc and muscle of shrimp subjected to prebiotics fructooligosaccharide, galactooligosaccharide, inulin and mannan-oligosaccharide and non-prebiotic carbohydrates wheat starch and sucrose added into the culture water for Trial I.[1]

	Biofloc			Muscle		
Treatment[2]	Ash	Protein	Lipids	Ash	Protein	Lipids
ATr	72.9	13.6[a]	7.7			
FOS	72.3	11.2[b]	6.4	6.3	85.1	6.0[ab]
GOS	72.3	10.2[b]	5.6	6.5	84.9	5.4[b]
INU	73.7	10.3[b]	6.1	6.4	87.9	5.5[ab]
MOS	73.2	10.4[b]	6.0	6.3	84.4	6.7[a]
WSt	71.1	10.7[b]	6.8	5.7	86.0	6.4[ab]
SUC	73.6	10.7[b]	5.9	6.5	83.8	6.0[ab]
PSE[3]	0.30	0.25	0.34	0.21	0.35	0.03
Anova (Pr>F)	0.218	0.0001	0.829	0.916	0.155	0.027

[1] Values represent means of three replicate tanks.
[2] ATr = Autotrophic phase; FOS = Fructooligosaccharide; GOS = Galactooligosaccharide; INU = inulin; MOS = mannan-oligosaccharide; WSt = wheat starch; SUC = sucrose.
[3] PSE = pooled standard error of treatment means (n = 3).

Bacterial community profiles of bacteria present in gill tissue are shown in Figure II.3. Profiles from gills with FOS and INU addition were very similar to each other and these were similar to the bacterial community of the GOS treatment. Bacterial communities of the FOS, INU and GOS treatments were somewhat similar to the bacterial community of the MOS treatment. Communities of the FOS, INU, GOS and

MOS treatments were not similar to the bacterial communities collected during the ATr phase and from the INL sample as well as the bacterial communities of the SUC and WSt treatments. Bacterial communities collected during the ATr phase and from the INL sample were somewhat similar to the bacterial communities of the SUC and WSt treatments. Communities collected during the ATr phase and from the INL sample were very similar to each other, while communities of the SUC and WSt were very similar to each other.

In terms of bacterial community present in the hepatopancreas (Figure II.4), bacterial communities collected during the ATr phase and from the INL samples were not similar to each other and each of them was also not similar to the bacterial communities of the rest of the treatments collected on termination. Communities of the MOS and WSt treatments were somewhat similar to each other but they were not similar to the bacterial communities of the FOS, GOS and SUC treatments. Bacterial communities of the FOS and GOS treatments were similar to each other but they were only somewhat similar to the bacterial community of the SUC treatment. The bacterial community of the INU treatment was not similar to any other bacterial community.

Bacterial communities present in the intestinal contents (Figure II.5) in the FOS, GOS and INU treatments were very similar to each other and they were similar to the bacterial communities of the MOS, SUC and WSt treatments. Bacterial communities collected during the ATr phase and from the INL sample were similar to each other but they were somewhat similar to the rest of the bacterial communities collected during termination.

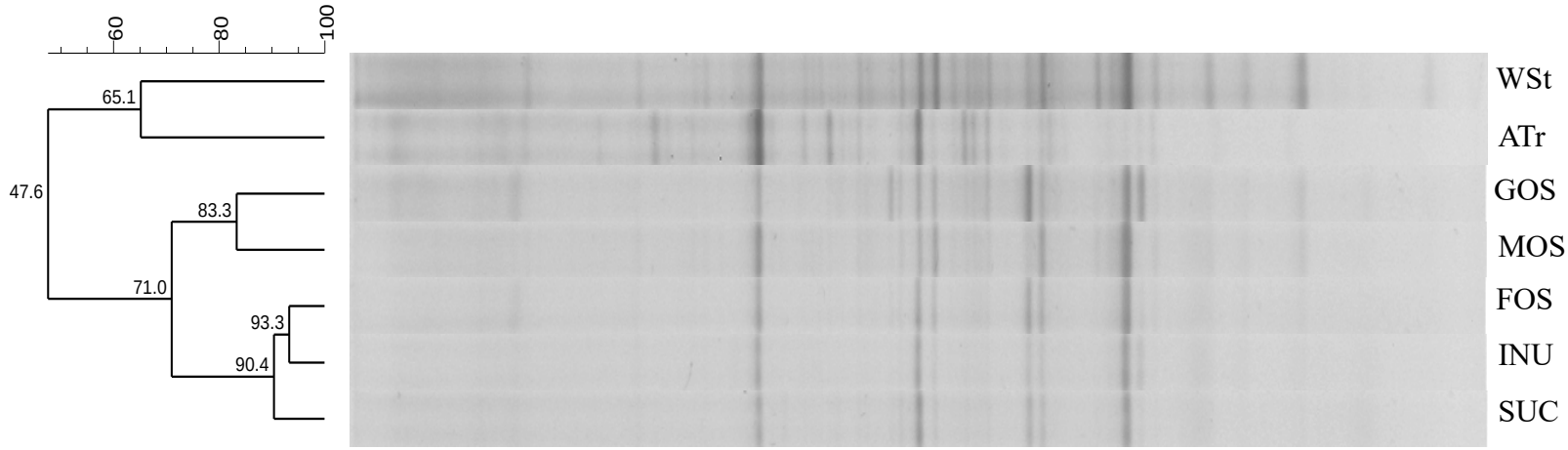

Figure II.2. Dendrogram of the biofloc particles (BFP) bacterial communities of tanks during the autotrophic phase (ATr) or during termination on tanks in which fructooligosaccharide (FOS), galactooligosaccharide (GOS), inulin (INU), mannan-oligosaccharide (MOS), wheat starch (WSt) or sucrose (SUC) was added into the culture water of Trial I.

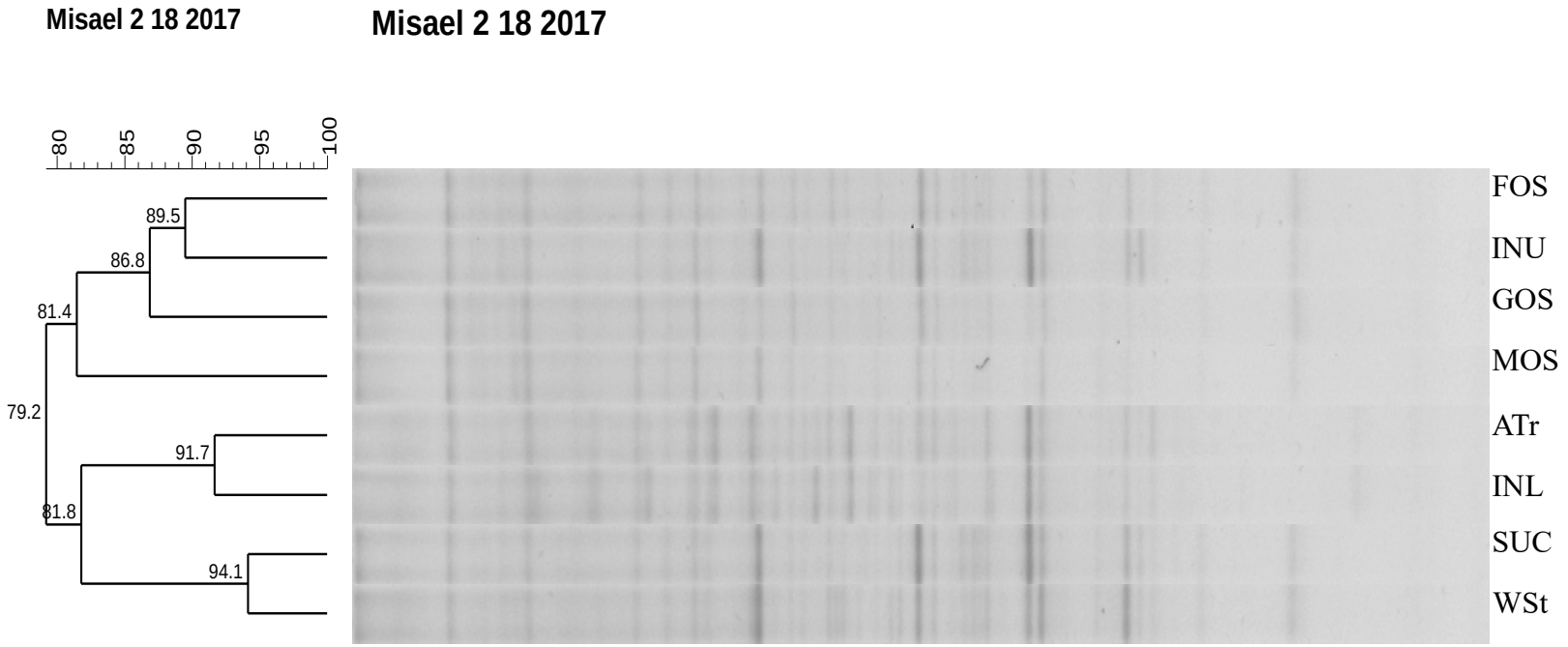

Figure II.3. Dendrogram of the gills tissue (GT) bacterial communities of shrimp collected during stocking day (initial sample; INL) or shrimp collected during the autotrophic phase (ATr) or during termination of tanks in which fructooligosaccharide (FOS), galactooligosaccharide (GOS), inulin (INU),mannan-oligosaccharide (MOS), wheat starch (WSt) or sucrose (SUC) was added to the culture water of Trial I.

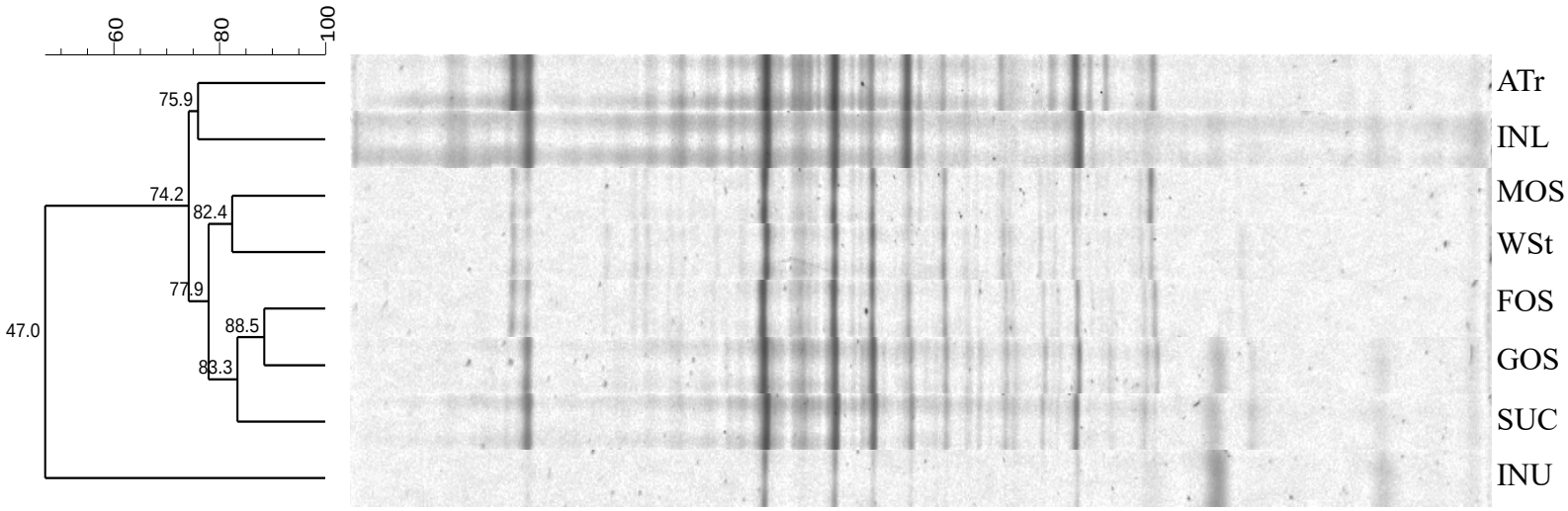

Figure II.4. Dendrogram of the hepatopancreas (HP) bacterial communities of shrimp collected during stocking day (initial sample; INL) or shrimp collected during the autotrophic phase (ATr) or during termination of tanks in which fructooligosaccharide (FOS), galactooligosaccharide (GOS), INU (inulin), mannan-oligosaccharide (MOS), wheat starch (WSt) or sucrose (SUC) was added to the culture water of Trial I.

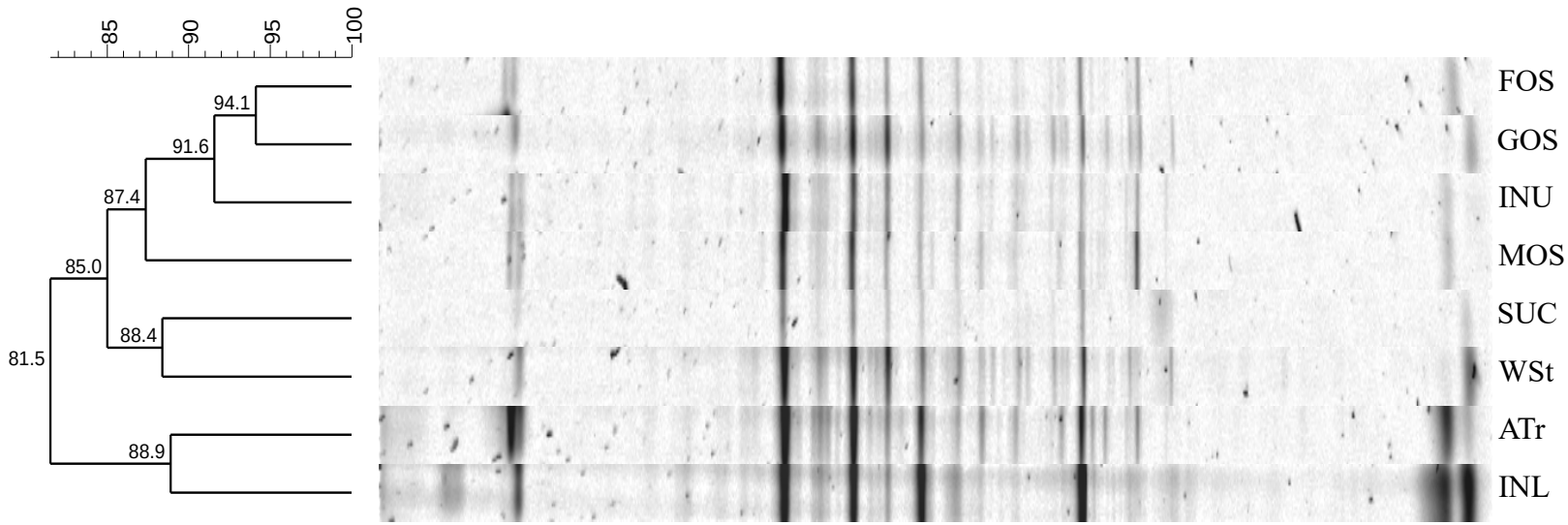

Figure II.5. Dendrogram of the intestinal contents (IC) bacterial communities of shrimp collected during stocking day (initial sample; INL) or shrimp collected during the autotrophic phase (ATr) or during termination of tanks in which fructooligosaccharide (FOS), galactooligosaccharide (GOS), inulin (INU), mannan-oligosaccharide (MOS), wheat starch (WSt) or sucrose (SUC) was added into the culture water of Trial I.

II.4 Discussion

Normally, prebiotics are provided to cultured organisms through their feed, however, it has been proven that shrimp consume biofloc particles as a supplemental source of nutrients (Ray et al., 2017). For this reason, it can be assumed that, with the addition of prebiotics to the culture water, the effect of prebiotics on bacteria associated with biofloc particles can also have an effect on the shrimp internal bacterial composition.

All water quality parameters associated with all treatments in the current feeding trial were within safe limits for shrimp culture, including alkalinity which was lower than targeted (Ebeling et al. 2006; Wasielesky et al., 2006). During nitrification, chemoautotrophs consume carbon (CO_2 or HCO_3) for energy and produce hydrogen ions (H^+) which reduces the alkalinity in the water (Ebeling et al., 2006). The reduction in alkalinity is an indicator of the nitrification process that continuously occurred in all tanks due to the bacteria nitrogen assimilation.

Final weight, weight gain, FE, survival, HIS, THC or K showed no significant differences among treatments. Condition factor (K) is often used to quantify an animal's physical wellbeing, and is considered to be an important parameter for the management of aquaculture systems as it is a useful complement to estimate the growth of crustaceans (Rochet 2000; Araneda et al. 2008). The lack of an effect due to prebiotic addition on any of the production parameters evaluated in the present study is in agreement with results from prebiotic studies on western king prawn *Penaeus latisulcatus* juveniles (Van Hai et al., 2009), Pacific white shrimp *L. vannamei* (Li et al. 2007) and Indian white shrimp *Fenneropenaeus indicus* larvae and postlarvae (Hoseinifar et al., 2010). However, other

studies have shown production enhancement due to the use of prebiotics on *P. semisulcatus* (Genc et al., 2007) and *L. vannamei* (Zhou et al., 2007). For the conditions in which this study was conducted, the lack of effect of the prebiotics on production data could be possibly related to the lack of a source of stress or a pathogen during the experiment. Also, it has to be noted that shrimp in the studies where prebiotics had a positive effect on production performance were stocked at a much smaller size, had a much higher growth rate and stayed under experimental conditions for a longer period of time. Thus, the effects of prebiotics on production performance could possibly be easier to detect in shrimp with higher growth rate, subjected to stressful conditions or exposed to prebiotics for a longer period of time.

During the experiment, no leftover feed was found during the routine checks so the low growth rate could be related to underfeeding. The amount of feed supplied to the organisms was not evaluated in this experiment as all shrimp were fed the same amount of feed regardless their experimental treatment.

The mean biofloc concentration of the WSt treatment was significantly lower than the rest of the treatments. It is possible that, because wheat starch is a polysaccharide with a complex structure, it is more difficult to assimilate by microorganisms, leading to a lower bacterial replication rate and bacterial load in the system. In fact, bacterial community on biofloc of the WSt treatment was significantly different to all other treatments. In contrast, all prebiotics evaluated in Trial I were oligosaccharides, and sucrose a disaccharide, that can be easily digested by microorganisms, so this could lead to a higher replication rate of microorganisms which leads to a higher microorganism biomass and biofloc concentration

Due to the addition of carbohydrate sources, heterotrophic bacteria had a substrate for obtaining carbon and subsequently metabolizing ammonia (Avnimelech 1999; Samocha et al. 2007). For this reason, total ammonia-nitrogen concentrations were reduced to levels tolerable to *L. vannamei* (Lin and Chen 2001) throughout the trial. During weeks 1-3 no significant differences were observed in any water quality parameter measured. However, on week 4, water of tanks in which INU was added had a higher nitrate concentration than the water of tanks were WSt was added. According to Silva et al. (2013), the route of nitrification occurs with successive conversions of ammonia to nitrite and nitrate prevalence at the end of the crop cycle. The lower nitrate concentration in the WSt treatment is an indicator of a lower nitrification process which could be explained by the lower biofloc level and the different bacterial community encountered in this treatment.

A biofloc sample was collected during the autotrophic phase for proximate composition analysis and this turned out to have a significantly higher protein content than the rest of the treatments which can be explained by a higher protein content diet and fines supplied during the first days of the culture as biofloc particles are formed in part on leftover feed (Avnimelech, 2012). No significant differences were found in any of the prebiotic or WSt or SUC treatments collected at the end of the trial for ash, protein or lipids composition of biofloc.

No information in the literature was found for nutritional composition of shrimp muscle when prebiotics were added to the culture water of a BFT system. However, studies have shown that prebiotics are involved in digestion, absorption and metabolism of various nutrients in terrestrial organisms (Swanson et al., 2002a; Swanson et al.,

2002b; Steer et al., 2003). In fact, some prebiotics have been shown to influence protein digestion and intestinal morphology (Teitelbaum and Walker, 2002; Swanson et al., 2002). Also, a key mechanism for which prebiotics confer health to the host is the production of short-chain fatty acids, which have antibacterial activity because of a reduction in the intestinal pH (Gibson and Roberfroid, 1995; Bindels et al., 2013). These studies could lead to an assumption of a possible effect of prebiotics on nutrient retention by the organism. Proximate composition of muscle showed that muscle of shrimp subjected to the MOS treatment had a higher lipid content than shrimp muscle of the GOS, INU and WSt treatments. Also, shrimp muscle of the WSt treatment had higher lipid concentration than the GOS and INU treatment. However, no significant differences were observed for ash or protein contents of shrimp muscle, which could be related to the low growth rate resulting from this experiment.

For this study, heterotrophic bacteria dominance was promoted by providing a lower protein feed content and by the addition of prebiotics or other carbon sources. The fact that the bacterial community of biofloc particles collected during the ATr phase was not similar to the bacterial community of any of the biofloc particles collected during termination is a good indicator that the dominance of different bacteria were successfully promoted due to the increase of the C:N ratio by switching to a lower protein content diet and/or by the addition of a carbon source in the form of oligosaccharides (prebiotics) or non-prebiotic soluble carbohydrates (wheat starch or sucrose).

Bacterial communities from the different samples collected showed differences among treatments that can be related to the prebiotic addition to the culture water. Intestinal content and hepatopancreas samples also showed significantly different

bacterial communities among treatments. Three explanations can be provided as to the effect of prebiotics added to the culture water on intestinal content and hepatopancreas bacteria: (1) prebiotics could be consumed by shrimp when biofloc particles were consumed or (2) prebiotics had an effect on biofloc particle bacteria which entered the shrimp through the consumption of biofloc particles, or (3) a combination of the two previous reasons.

The bacterial community of the WSt treatment was not similar to the bacterial community of the other treatments, which can be attributed to the fact that WSt is a complex carbohydrate that is not as available to microorganisms as the oligosaccharides FOS, GOS, INU and MOS or the disaccharide SUC. Interestingly, although still different, with a 65.1% SC, the bacterial community of the biofloc particles with WSt addition were more closely related to the bacterial community of the biofloc particles collected during the ATr phase than to the rest of the treatments by a 47.6% SC, which supports the hypothesis of a lower carbon availability from WSt to bacteria than oligosaccharide prebiotics. Differences in bacterial communities of biofloc particles collected from the prebiotics treatments could be attributed to different chemical compositions of the prebiotics used in this study.

The gills of crustaceans, such as crabs, shrimp and lobsters, play a critical role in respiration (Mangum, 1985), as well as in osmotic and ion regulation (Henry and Cameron, 1983; Henry, 1987). Bacteria injected into crabs and shrimp can be trapped and subsequently inactivated or degraded in the gills (Martin et al., 1993; Alday-Sanz et al., 2002; Burgents et al., 2005) or externalized at the next molt (Martin et al., 2000). Thus, the crustacean gill is also important for immune defense against bacterial pathogens.

Interestingly, a prebiotic effect was observed on the bacterial composition of the gills of shrimp where prebiotics were added to the culture water as they have a bacterial composition not similar to the bacterial composition of shrimp gills sampled from the initial shrimp, during the ATr phase or from the SUC and WSt treatments.

Similar results were obtained from bacterial communities present in the hepatopancreas samples of shrimp subjected to the different prebiotic treatments vs. WSt and INL or ATr samples. Even though a study has shown that bacterial richness and diversity of the hepatopancreas tissue remains relatively more stable than the bacterial community of the gut during gonadal development of *Neocardina denticulata* (Cheung et al., 2015), this current study showed that the bacteria community of shrimp hepatopancreas can be altered rather soon when prebiotics were added to the culture water. The discrepancy in results could be attributed to the particular conditions of this trial, i.e., the bacterial load in this study was expected to be higher due to the promotion of bacteria to control water quality and due to the zero-water exchange maintained in this study. The high bacterial load was more easily altered by the addition of prebiotics to the water.

In conclusion, results from this study showed that:

(1) Carbon from WSt was not as available to bacteria as oligosaccharides or the disaccharide used in this study and this was reflected in biofloc concentration and its bacteria profile.

(2) Prebiotics and sucrose addition into the culture water altered the bacterial composition of biofloc particles and shrimp gills, hepatopancreas and intestinal contents.

(3) WSt did not have as much of an effect as prebiotics and sucrose on the bacterial composition of biofloc, shrimp gills, hepatopancreas and intestinal contents when it was added to the culture water.

(4) No effect on shrimp production data or water quality was found in this study, which could be related to an external parameter such as underfeeding.

CHAPTER III

EFFECT OF PREBIOTICS AND AN ESSENTIAL OIL BLEND ADDED TO THE FEEDS OF PACIFIC WHITE SHRIMP *Litopenaeus vannamei* ON BACTERIAL COMPOSITION OF BIOFLOC AND SHRIMP INTESTINE, GILLS AND HEPATOPANCREAS

III.1 Introduction

Fisheries and aquaculture are important sources of food, nutrition, income and livelihoods for hundreds of millions of people around the world (FAO, 2016). In 2014, world per capita fish supply reached a new record of 20 kg mainly because of the dramatic growth in aquaculture production; whereas, capture fishery production has remained relatively static since the late 1980s. Global total aquaculture production in 2014 were 73.8 million tons, of which 47.1 million tons were from inland waters and 26.7 million tons were from marine waters (FAO, 2016). Crustacean production in 2014 amounted to a total of 6.9 million tons with a value of US$ 36.2 billion, making them the second most valuable commodity of aquaculture, surpassed only by finfish (FAO, 2016). However, the success of shrimp aquaculture can be compromised due to reduced shrimp health, which is regulated by environment, and pathogen and host interactions (Gainza and Romero et al., 2017). The alteration of any of these factors or interactions can cause disease outbreaks, which lead to significant losses in the shrimp aquaculture industry (Luna et al., 2013).

Due to the detrimental effects of antibiotics and disinfectants on the environment and their risks to global public health (La Para et al., 2011; Wright, 2010; Su et al., 2017), prebiotics and essential oils have been proposed as alternatives to improve shrimp health and reduce the risk of disease outbreaks. In fish species, some studies have documented improvements in feed efficiency (FE) and nonspecific immune responses due to a dietary regimen of commercially available prebiotics of fish such as hybrid striped bass, *Morone chrysops* x *M. saxatilis*, with Grobiotic-A (Li and Gatlin 2004), rainbow trout, *Oncorhynchus mykiss*, with mannan-oligosaccharide (MOS) (Staykov et al. 2007) and Atlantic salmon, *Salmo salar*, with MOS, fructooligosaccharide (FOS) and galactooligosaccharide (GOS) (Grisdale-Helland et al. 2008). The effects of prebiotics also have been studied to a limited extent for crustacean aquaculture (Daniels and Hoseinifar, 2014; Merrifield and Ringø, 2014) with improvements in weight gain and feed efficiency ratio (FE) documented in shrimp (Zhou et al., 2007; Genc et al., 2007).

Plant products present another alternative to antibiotics and disinfectants in aquaculture. The antibacterial efficacy of plant products such as essential oils, as well as their effect on nutrient utilization, growth and survival also have been evaluated with promising results in the aquaculture sector with fish and shrimp (Citarasu et al., 2006; Luo, 1997; Immanuel et al., 2004; Galina et al., 2009).

A variety of prebiotics and essential oils have been evaluated as feed additives included in the feed of different cultured organism. However, no study on the effects that prebiotics or essential oils may have on shrimp production and on the bacterial community have been conducted on shrimp cultured under biofloc conditions. Therefore, the objective of this study was to evaluate the effects of prebiotics (fructooligosaccharide

(FOS), galactooligosaccharide (GOS), inulin (INU) and mannan-oligosaccharide (MOS)) and a commercially available essential oil blend (EOB) on bacterial profiles present in biofloc particles and *Litopenaeus vannamei* hepatopancreas, intestine and gills when these additives are provided to the shrimp through the diet. In addition, the effects of these additives on shrimp health and production as well as on water quality were assessed.

III.2 Materials and methods

III.2.1 Experimental conditions

A 31-day trial (Trial II) was conducted at the trū® Shrimp Company experimental station located in Balaton, MN, USA to evaluate the effect of different prebiotics on shrimp production and bacterial composition of biofloc and shrimp hepatopancreas, gills and intestinal contents. Each tank (0.457 m x 0.457 m x 0.280 m) contained an independent heater, an automatic 48-h feeder, and two air stones. Tanks were filled to 20-cm depth with artificial seawater of 28 g/L salinity, at 30.0 ± 1.0ºC. Water lost due to evaporation was replaced weekly with reverse osmosis water. To maintain buffering capacity, sodium bicarbonate ($NaHCO_3$) was added if alkalinity levels fell below 180 mg/L. Application levels were determined using the following formula:

$NaHCO_3$ needed per tank (g) = ((deficiency in alkalinity (mg/L) / concentration of HCO_3 in $NaHCO_3$ (72.646 (%); 0.72646)) × tank volume (L)) / 1000 (mg/g)

All tanks were operated as zero-exchange Biofloc Technology (BFT) system. During the autotrophic phase (ATr; see Chapter I, subheading I.2), autotrophic bacteria were promoted and maintained from the initial day until Imhoff cone readings reached 3 mL/L. Only during the ATr phase, all shrimp were fed with the same feed and fines formulated to contain 35% crude protein (CP) (as-fed basis) at a mean of 3.4 g/day per tank and 0.037 g/L, respectively. Heterotrophic phase (see Chapter I, subheading I.2) was promoted when Imhoff cone readings reached 3 mL/L. Only during heterotrophic phase, the total ammonia-nitrogen (TAN; NH_3/NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) levels were maintained at <3, <5 and <100 mg/L, respectively, by giving the experimental and control diets formulated to contain 23% CP according to treatment-tank assignment. No source of carbon was added to the culture water other than that provided through the feed.

III.2.2 Shrimp

Post-larvae weighing 0.003 g arrived at the experimental station from a commercial hatchery (Shrimp Improvement Systems, SIS, Inc, Islamorada, Florida, USA) and were acclimated to nursery tank conditions filled with artificial seawater of 28.0 ± 0.5 g/L salinity, at 29.0 ± 1.0^{o}C. Shrimp in nursery tanks were maintained as described for Trial I (Chapter II) and remained there until the experiment stocking date. Shrimp with no visual signs of disease or stress were collected and weighed. Shrimp weighing 2.08 ± 0.5 g, were stocked into each tank at a density of 20 shrimp/tank. Initial group weight was recorded and mean individual weight was calculated as well as initial biomass based on water volume of the experimental tanks (Table III.1). Mortality counts and weights were recorded and replaced with same size shrimp only during a 2-day

acclimation period. When the trial started, a small net was used to carefully check for mortalities and leftover feed without disturbing the living organisms. Mortalities were recorded and discarded.

Table III.1. Initial shrimp group weight, biomass and individual shrimp weight for Trial II.[1]

Treatment[2]	Group weight (g/tank)	Biomass (g/m^3)	Individual mean weight (g)
CTL	41.8	1,001.2	2.1
FOS	43.2	1,029.9	2.2
GOS	42.9	1,025.7	2.1
INU	42.0	1,004.4	2.1
MOS	38.2	913.5	2.0
EOB	41.6	996.2	2.1
WSt	41.4	992.0	2.1
SUC	42.2	1,010.2	2.1
PSE[3]	0.49	11.64	0.02

[1] Values are expressed as means per treatment.
[2] CTL = control; EOB = essential oil blend; FOS = fructooligosaccharide; GOS = galactooligosaccharide; INU = inulin; MOS = mannan-oligosaccharide; WSt = wheat starch; SUC = sucrose.
[3] PSE = pooled standard error of treatment means (n = 4).

III.2.3 Feed and feeding management

Eight diets were used in Trial II and each diet was provided to shrimp according to its treatment. Higher protein content diet and fines were obtained from Trial I. To prepare the experimental diets, all dry ingredients of the reference diet were weighed and mixed in an industrial mixer for 15 min until achieving a completely homogenized mixture. Then, the dry mixture was divided into eight equal parts and each was mixed

with the experimental additives. Each batch was posteriorly blended with sodium hexametaphosphate and alginate (Table III.2) previously mixed with 150 mL of deionized water per kg of dry feed using a hand mixer (Sunbeam Products Inc., Milford, MA) until an appropriate mash consistency for extrusion was obtained. Fish and soybean oil were also added during this step. Extrusion was made using a meat chopper attachment (Model A-800, Hobart Corporation, Troy, OH, USA) fitted with a 3-mm die. Moist feed strands were dried on wire racks in a forced air oven at 35^{o}C to a moisture content of 8-10%. After a 24-h drying period, feed was milled and sifted into the appropriate size for shrimp consumption, bagged, and stored at 4^{o}C until used to feed the shrimp. Samples were also taken for analysis of proximate composition according to the AOAC (1990) procedures for dry matter, lipid, and ash contents and to the Dumas method (AOAC, 2005) for crude protein composition. Proximate composition of feed was performed in duplicate samples per diet.

Formulation as well as ash, protein and lipid composition of each diet can be found in Table III.2. Fines were obtained by grinding the higher protein content diet and sieving to get particles between 0.595-0.420 mm. During the heterotrophic phase (day 0-7), the higher protein content diet was loaded into the feeders and fines were added directly to the culture water as described in Table III.3. The lower protein content reference and experimental diets were loaded on the automatic feeders from day 5 until termination and no additional fines were added in order to increase the C:N ratio and to promote the dominance of heterotrophic bacteria. Based on previous experience, it was expected that, for this size, shrimp were going to grow linearly. A feed curve based on the number of shrimp, expected shrimp growth and FE was used to determine the

expected feed regimen offered to each tank (Expected feed regimen = shrimp count × Expected weight increase (g) × FE) (Table III.3). Each 48-h feeder was loaded every other day based on the expected feed regimen of 2 days with adjustments based on leftover feed and water quality.

Table III.2. Formulation (%) of the experimental diets used for Trial II with determined ash, protein and lipid proximate composition (g/kg).

Ingredient (%)	35% CP	CTL	Experimental diets						
			FOS	GOS	INU	MOS	EOB	WSt	SUC
Squid muscle meal[a]	30.0	21.7	21.7	21.7	21.7	21.7	21.7	21.7	21.7
Wheat starch[b]	28.8	41.8	41.8	41.8	41.8	41.8	41.8	41.8	41.8
Fish meal[c]	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Soy protein isolated[d]	5.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dicalcium phosphate[b]	4.2	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6
Lecithin, dry, 95%[c]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Diatomaceous earth[e]	3.8	3.7	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Cellulose[f]	3.2	3.2	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Calcium carbonate[f]	2.5	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2
Alginate (Manucol DM)[g]	2.0	2	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Potassium chloride[f]	1.9	2	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Magnesium oxide[h]	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6
Sodium hexametaphosphate[f]	1.0	1	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Sodium chloride[f]	0.7	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Menhaden fish oil[c]	0.6	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
Soybean oil[i]	0.6	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Vit/Min premix [r]	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3
Cholesterol[a]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
DL-Methionine[k]	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Table III.2. Continued.

Ingredient (%)	35% CP	CTL	Experimental diets						
			FOS	GOS	INU	MOS	EOB	WSt	SUC
Fructooligosaccharide[l]			3.0						
Galactooligosaccharide[m]				3.0					
Inulin[n]					3.0				
Mannanoligossacharide[o]						3.0			
Essential oil blend[p]							3.0		
Wheat Starch[b]								3.0	
Sucrose[q]									3.0
Proximate composition (g/kg, dry weight)									
Ash	131.4	173.8	150.0	143.6	150.7	151.6	168.6	148.8	147.4
Protein	385.5	233.9	225.8	222.6	230.8	232.3	232.4	226.9	230.1
Lipids	82.5	78.4	78.5	79.6	76.1	81.8	81.4	81.1	79.6

[a] Zeigler Bros., Inc. Gardners, PA, USA
[b] MP Biomedicals Santa Ana, CA, USA
[c] ADM Co. Chicago, IL, USA
[d] Solae LLC, St. Louis, MO, USA
[e] Absorbent Products LTD
[f] Fisher Scientific
[g] FMC BioPolymer
[h] Prince Agri Products
[i] Consumer's Supply
[k] Evonik Degussa Corporation
[l] sc-FOS; Ingredion Incorporated, Indianapolis, IN, USA
[m] GOS; Ingredion Incorporated, Indianapolis, IN, USA
[n] INU; JeTsu Technology Limited, London, UK
[o] Bio-MOS; Alltech, Nicolasville, KY, USA
[p] Regano EX, Ralco Nutrition, Marshall, MN, USA
[q] Nash Finch Company, Minneapolis, MN, USA
[r] Composition given in Appendix

Table III.3. Expected feed curve based on shrimp count, expected weight increase and feed efficiency with actual fines and feed provided for Trial II.

Day	Shrimp count	Expected weight (g)	Expected weight increase (g)	FE	Expected feed regimen (g)	35% CP Fines (g)	Feed protein (%)	Actual feed provided (g)
ACN	20	2.08	-	1.3	3.40	-	35.00	6.74
ACN	20	2.21	0.13	1.2	3.34	-	-	-
0	20	2.35	0.14	1.1	3.25	3.00	35.00	6.39
1	20	2.50	0.15	1.0	3.14	-	-	-
2	20	2.65	0.16	1.0	3.34	3.00	35.00	6.53
3	20	2.82	0.17	0.9	3.19	-	-	-
4	20	3.00	0.18	0.9	3.78	3.00	35.00	7.56
5	20	3.21	0.21	0.9	3.78	-	-	-
6	20	3.42	0.21	0.8	3.36	3.00	35.00	6.72
7	20	3.63	0.21	0.8	3.36	-	-	-
8	20	3.84	0.21	0.8	3.36	-	23.00	6.72
9	20	4.05	0.21	0.8	3.36	-	-	-
10	20	4.26	0.21	0.8	3.36	-	23.00	6.72
11	20	4.47	0.21	0.8	3.36	-	-	-
12	20	4.68	0.21	0.8	3.36	-	23.00	6.72
13	20	4.89	0.21	0.8	3.36	-	-	-
14	20	5.10	0.21	0.8	3.36	-	23.00	6.72
15	20	5.31	0.21	0.8	3.36	-	-	-
16	20	5.52	0.21	0.8	3.36	-	23.00	6.72
17	20	5.73	0.21	0.8	3.36	-	-	-
18	20	5.94	0.21	0.8	3.36	-	23.00	6.72
19	20	6.15	0.21	0.8	3.36	-	-	-
20	20	6.36	0.21	0.8	3.36	-	23.00	6.72
21	20	6.57	0.21	0.8	3.36	-	-	-
22	20	6.78	0.21	0.8	3.36	-	23.00	6.72
23	20	6.99	0.21	0.8	3.36	-	-	-
24	20	7.20	0.21	0.8	3.36	-	23.00	6.72

Table III.3. Continued.

Day	Shrimp count	Expected weight (g)	Expected weight increase (g)	FE	Expected feed regimen (g)	Fines (g)	Feed protein (%)	Actual feed provided (g)
25	20	7.41	0.21	0.8	3.36	-	-	-
26	20	7.62	0.21	0.8	3.36	-	23.00	6.72
27	20	7.83	0.21	0.8	3.36	-	-	-
28	20	8.04	0.21	0.8	3.36	-	23.00	6.72
29	20	8.25	0.21	0.8	3.36	-	-	-
30	20	8.46	0.21	0.8	3.36	-	23.00	2.00
31	20	8.67	0.21	0.8	0.00	-	-	TMN

Expected feed regimen = shrimp count × expected weight increment × FE
Feed provided = actual feed loaded on 48-h feeders with amount calculated for 2 days
TMN = Termination
ACN = Acclimation

III.2.4 Experimental treatments

Prebiotics used in this study were short-chain fructooligosaccharide (sc-FOS (FOS in this manuscript); Ingredion Incorporated, Indianapolis, IN, USA), galactooligosaccharide (GOS; Ingredion Incorporated, Indianapolis, IN, USA), mannan-oligosaccharide (Bio-MOS (MOS in this manuscript); Alltech, Nicolasville, KY, USA), Inulin (INU; JeTsu Technology Limited, London, UK). In addition to prebiotics, non-prebiotic carbohydrates used in this study included wheat starch (WSt; MP Biomedicals Santa Ana, CA, USA), and sucrose (SUC; Table sugar). In addition, a commercially available essential oil blend (EOB; Regano® EX, Ralco Nutrition, Marshall, MN, USA), containing calcium carbonate, diatomaceous earth (flow agent), hemicellulose extract, a proprietary essential oil bled mixture and mineral oil, was evaluated. A 3% inclusion level of each additive was mixed into the diets prepared for Trial II, replacing 0.2%

cellulose and 2.8% diatomaceous earth (Table III.2). A low protein content diet with no additive inclusion was used as a reference diet for Trial II. Each treatment was randomly assigned to four tanks for a total of 32 tanks used in Trial II.

III.2.5 Data acquisition and analyses

All data was acquired and analyzed as described in Chapter II, Section II.2.5 for Trial I with the following exceptions:

(1) An initial hemolymph sample (INL) was collected on stocking day.

(2) No biofloc sample collected from the autotrophic phase was available for proximate composition.

(3) No initial sample or sample collected during the autotrophic phase was available for denaturing gradient gel electrophoresis of shrimp GT, HP or IC.

III.2.6 DNA isolation and PCR

DNA was isolated and PCR was conducted as previously described in Chapter II, section II.2.6 for Trial I.

III.2.7 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was performed as described for Trial I in Chapter II, Section II.2.7.

III.2.8 Calculations and statistical analyses

The responses utilized to compare treatments in this study as well as the statistical analyses were performed as described for Trial I in Chapter II, Section III.2.8.

III.3 Results

Mean dissolved oxygen, temperature, salinity and pH values from daily observations are shown in Table III.4 per treatment. Final weight, weight gain, FE, survival, HSI or K score showed no significant differences among treatments (Table III.5). Total hemocyte count (THC) showed significant differences among treatments, being higher in hemolymph of shrimp fed diets containing EOB or the prebiotics MOS, INU and GOS, and lower in hemolymph of shrimp collected during the initial sampling. No significant differences were observed in mean biofloc concentration among any of the treatments evaluated (Figure III.1). Also, no significant differences were observed in total ammonia-nitrogen, nitrite, nitrate or alkalinity in the culture systems due to the various treatments (Tables III.6 and III.7).

Table III.4. Dissolved oxygen (mg/L), salinity (g/L), temperature (°C) and pH means results from daily observations during Trial II.

Treatment	Dissolved Oxygen (mg/L)	Salinity (g/L)	Temperature (°C)	pH
CTL	5.4	28.7	30.0	8.1
FOS	5.3	28.8	29.9	8.2
GOS	5.3	29.3	30.4	8.2
INU	5.3	28.9	30.5	8.2
MOS	5.2	28.9	30.8	8.1
EOB	5.3	29.2	30.5	8.2
WSt	5.4	28.9	29.8	8.1
SUC	5.4	29.0	29.8	8.2
PSE	0.02	0.05	0.11	0.02

[1] CTL = control reference; EOB = essential oil blend; FOS = fructooligosaccharide; GOS = galactooligosaccharide; INU = inulin; MOS = mannan-oligosaccharide; WSt = wheat starch; SUC = sucrose.

[2] PSE = pooled standard error of treatment means (n = 4).

Table III.5. Final weight, weight gain, feed efficiency and survival as well as K, HSI and THC of shrimp fed the control diet with no additive inclusion and the experimental diets containing prebiotics fructooligosaccharide, galactooligosaccharide, inulin or mannan-oligosaccharide or non-prebiotic carbohydrates wheat starch or sucrose or the essential oil blend at 3% dietary inclusion level used during Trial II.[1]

Treatment[2]	Final weight (g)	Weight gain (%)	FE	Survival (%)	K	HSI	THC
INL							1.33^{b}
CTL	6.6	215.8	1.3	95.0	0.9	0.03	2.06^{ab}
FOS	6.5	202.2	1.3	96.3	0.8	0.03	1.84^{ab}
GOS	6.1	186.6	1.5	97.5	0.8	0.04	2.25^{ab}
INU	6.3	199.9	1.4	98.8	0.8	0.03	2.81^{ab}
MOS	6.4	242.2	1.2	100.0	0.8	0.02	3.05^{a}
EOB	6.6	216.7	1.3	96.3	0.8	0.04	3.10^{a}
WSt	6.2	197.2	1.4	98.8	0.8	0.03	1.77^{ab}
SUC	6.7	217.6	1.3	96.3	0.9	0.04	1.79^{ab}
PSE[3]	0.06	4.97	0.02	0.59	0.01	0.002	0.15
Anova (Pr > F)	0.100	0.201	0.070	0.242	0.307	0.07	0.017

[1] Values represent means per treatment.
[2] INL = initial; CTL = control reference; FOS = fructooligosaccharide; GOS = galactooligosaccharide; INU = inulin; MOS = mannan-oligosaccharide; WSt = wheat starch; SUC = sucrose; EOB = essential oils blend.
FE = feed efficiency; K = condition factor; HSI = hepatosomatic index; THC = total hemocyte count (10^7 cells/mL).
[3] PSE = pooled standard error of treatment means (final weight, weight gain, FE and survival: n=4; K, HSI and THC: n = 3).

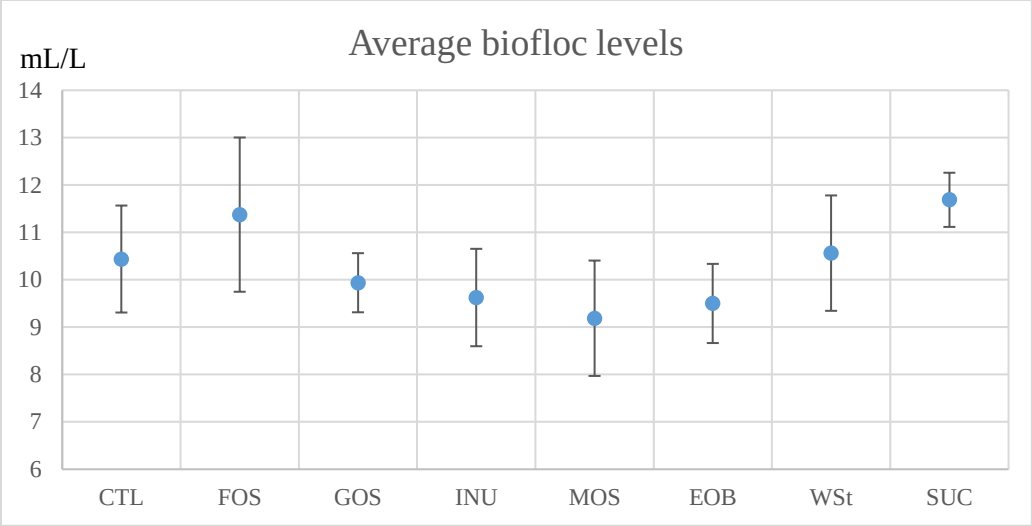

Figure III.1. Mean biofloc levels (mL/L) of tanks subjected to dietary treatments with 3% inclusion of the prebiotics fructooligosaccharide, galactooligosaccharide, inulin or mannan-oligosaccharide or the non-prebiotic carbohydrates wheat starch or sucrose or the essential oil blend during Trial II.[1,2]

[1] Values are expressed as means with no significant differences ($p \leq 0.05$) ± PSE ($n = 4$) of the weekly biofloc readings obtained from Imhoff cones.

[2] CTL = control reference; FOS = fructooligosaccharide; GOS = galactooligosaccharide; INU = inulin; MOS = mannan-oligosaccharide; EOB = essential oil blend; WSt = wheat starch; SUC = sucrose.

Table III.6. Mean values over time for total ammonia-nitrogen (TAN) and nitrite of tanks fed the control diet or the experimental diets containing prebiotics fructooligosaccharide, galactooligosaccharide, inulin or mannan-oligosaccharide or the non-prebiotic carbohydrates wheat starch or sucrose or the essential oil blend at 3% dietary inclusion level used during Trial II.[1]

	Total Ammonia-Nitrogen				Nitrites			
Treatment[2]	Day 3	Day 10	Day 17	Day 24	Day 3	Day 10	Day 17	Day 24
CTL	1.33	1.25	0.17	1.29	0.20	0.22	0.10	0.13
FOS	1.33	1.09	0.07	1.20	0.21	0.23	0.08	0.15
GOS	1.43	1.10	0.10	0.96	0.23	0.24	0.10	0.12
INU	1.30	0.81	0.10	1.14	0.16	0.17	0.10	0.15
MOS	1.97	1.05	0.10	1.32	0.20	0.24	0.14	0.19
EOB	1.10	0.72	0.07	1.33	0.15	0.15	0.09	0.17
WSt	1.63	1.00	0.12	1.45	0.15	0.16	0.09	0.15
SUC	1.10	0.72	0.09	1.70	0.11	0.15	0.08	0.18
PSE[3]	0.10	0.01	0.01	0.08	0.01	0.01	0.01	0.01
Anova (Pr>F)	0.442	0.890	0.563	0.488	0.591	0.455	0.374	0.841

[1] Values represent treatment means of the weekly observations.

[2] CTL = control reference; FOS = fructooligosaccharide; GOS = galactooligosaccharide; INU = inulin; MOS = mannan-oligosaccharide; WSt = wheat starch; SUC = sucrose; EOB = essential oil blend.

[3] PSE = pooled standard error of treatment means (n = 4).

Table III.7. Mean values over time for total nitrate and alkalinity of tanks fed the control diet or the experimental diets containing prebiotics fructooligosaccharide, galactooligosaccharide, inulin or mannan-oligosaccharide or the non-prebiotic carbohydrates wheat starch or sucrose or the essential oil blend at 3% dietary inclusion level used during Trial II.[1]

	Nitrates				Alkalinity			
Treatment[2]	Day 3	Day 10	Week 17	Week 24	Day 3	Day 10	Week 17	Week 24
CTL	35.40	14.21	25.19	22.17	164.67	144.50	162.75	193.50
FOS	27.96	14.07	23.61	20.25	162.33	148.75	172.00	178.00
GOS	30.94	15.42	23.19	23.80	172.00	156.50	170.50	178.25
INU	54.06	11.43	19.15	17.98	159.33	147.50	174.50	188.25
MOS	39.47	13.36	23.00	21.86	175.00	162.25	162.50	173.75
EOB	48.79	13.36	19.08	18.75	164.67	150.25	158.50	173.50
WSt	36.04	13.32	21.83	21.76	167.00	147.25	157.25	181.50
SUC	51.43	14.56	19.30	21.62	164.00	154.25	174.00	183.00
PSE[3]	7.09	0.51	0.82	0.99	2.65	2.73	2.81	2.79
Anova (Pr>F)	0.986	0.743	0.432	0.890	0.901	0.816	0.643	0.650

[1] Values represent treatment means of the weekly observations.
[2] CTL = control reference; FOS = fructooligosaccharide; GOS = galactooligosaccharide; INU = inulin; MOS = mannan-oligosaccharide; WSt = wheat starch; SUC = sucrose; EOB = essential oil blend.
[3] PSE = pooled standard error of treatment means (n = 4).

Significant ($p \leq 0.05$) differences were observed in ash and protein composition of biofloc samples collected at the end of Trial II (Table III.8). Ash content was higher ($p \leq 0.05$) in biofloc of treatments where shrimp were fed diets containing INU or SUC and lower ($p \leq 0.05$) in biofloc of treatments where shrimp were fed diets containing GOS, MOS or the CTL diet. Protein content was higher ($p \leq 0.05$) in biofloc of the CTL, GOS, and MOS treatments and lower ($p \leq 0.05$) in the INU and SUC treatments. No differences ($p \leq 0.05$) were observed for lipid composition of biofloc collected during Trial II (Table III.8).

Differences ($p \leq 0.05$) were observed in ash contents of shrimp muscle having the higher ash content in the GOS treatment and the lowest ash content in the SUC, CTL and FOS treatments (Table III.8). No significant differences were observed in protein or lipid content of shrimp muscle in Trial II (Table III.8).

Table III.8. Ash, protein and lipid content of biofloc and muscle of shrimp fed the control diet with no additive inclusion and the experimental diets with prebiotics fructooligosaccharide, galactooligosaccharide, inulin or mannan-oligosaccharide or non-prebiotic carbohydrates wheat starch or sucrose or the essential oil blend at 3% dietary inclusion level used during Trial II.

	Biofloc			Muscle		
Treatment[1,3]	Ash	Protein	Lipids	Ash	Protein	Lipids
CTL	78.5^{bc}	8.0^{ab}	5.4	6.7^{b}	92.9	15.1
FOS	79.4^{bc}	7.4^{ab}	3.8	6.4^{b}	90.6	15.0
GOS	76.7^{c}	9.5^{a}	5.8	8.0^{a}	97.4	14.6
INU	82.5^{a}	5.5^{b}	4.1	7.1^{ba}	84.9	14.0
MOS	77.5^{c}	9.3^{a}	6.5	6.9^{ba}	94.6	14.0
EOB	78.6^{bc}	7.6^{ab}	4.7	7.1^{ba}	97.8	15.1
WSt	79.3^{bc}	7.8^{ab}	3.9	7.2^{ba}	97.4	14.0
SUC	81.2^{ab}	6.5^{b}	5.7	6.1^{b}	93.3	14.6
PSE[2]	0.41	0.30	0.41	0.13	1.27	0.18
Anova (Pr>F)	0.0001	0.001	0.708	0.002	0.159	0.473

[1] CTL = control reference; FOS = fructooligosaccharide; GOS = galactooligosaccharide; INU = inulin; MOS = mannan-oligosaccharide; WSt = wheat starch; SUC = sucrose; EOB = essential oil blend.
[2] PSE = pooled standard error of treatment means (n = 4). Mean in columns and with the same superscript letter are not significantly different ($p \leq 0.05$).
[3] Proximate composition analyses of ash and lipids were performed with no duplicate sample because not enough sample was available.

In terms of bacterial community present in the biofloc particles (Figure III.2), the bacterial community present during the ATr phase was not similar to the rest of the bacterial communities sampled. The bacterial communities present in the CTL and WSt treatments were very similar to each other and not similar to the rest of the bacterial communities sampled. The bacterial community present in the SUC treatment was not similar to the rest of the bacterial communities sampled; whereas those of the treatments where MOS and FOS were added were very similar to each other and to the GOS and INU treatments and somewhat similar to that of the EOB treatment.

Gills tissue bacterial communities present in the WSt and SUC treatments were very similar to each other; whereas they were determined to be similar to the CTL treatment (Figure III.3). Communities in the WSt, SUC and CTL treatments were somewhat similar to the rest of the treatments. Bacterial communities present in the FOS, GOS, INU and MOS treatments were very similar to each other, and all of them together were only similar to the bacterial community with the EOB addition.

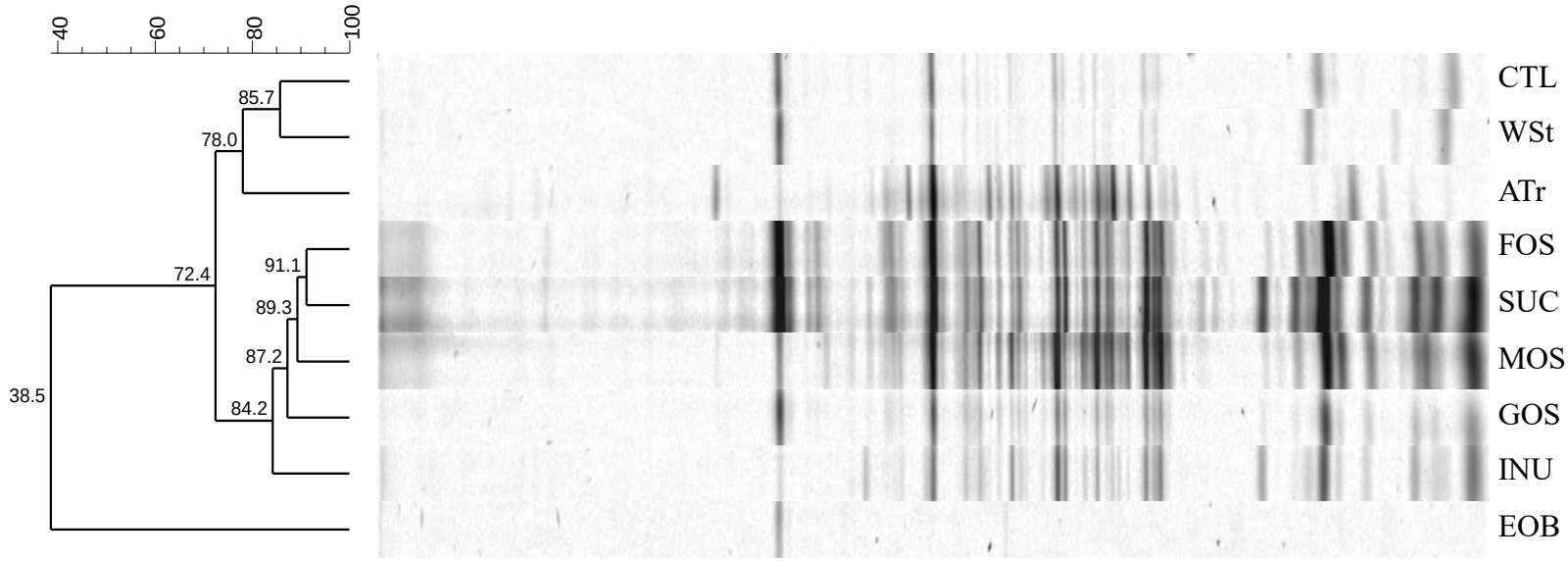

Figure III.2. Dendrogram of the biofloc particles (BFP) bacterial communities collected during the autotrophic phase (ATr) or during termination from tanks in which shrimp were fed diets with no additive inclusion (CTL; control) or diets containing fructooligosaccharide (FOS), galactooligosaccharide (GOS), inulin (INU), mannan-oligosaccharide (MOS), wheat starch (WSt), sucrose (SUC) or essential oil blend (EOB) of Trial II.

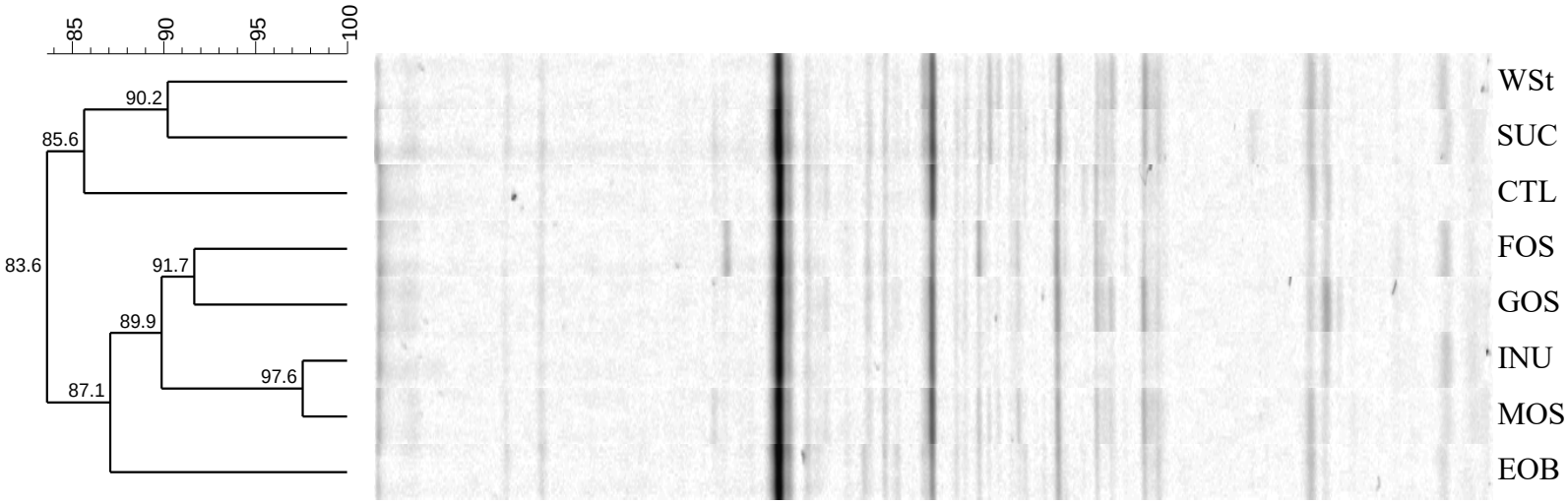

Figure III.3. Dendrogram of the gills tissue (GT) bacterial communities sampled during the autotrophic phase (ATr) or during termination from shrimp fed diet with no additive inclusion (CTL; control) or diets containing fructooligosaccharide (FOS), galactooligosaccharide (GOS), inulin (INU), mannan-oligosaccharide (MOS), wheat starch (WSt), sucrose (SUC) or essential oil blend (EOB) of Trial II.

Hepatopancreas bacterial communities in the CTL and FOS treatments were likely the same or identical to each other and both bacterial communities were not similar to the bacterial communities present in the rest of the samples (Figure III.4). Additionally, the bacterial community of the GOS treatment was not similar to the bacterial communities of the other samples. Bacterial communities of the MOS and EOB treatments were likely the same or identical to each other and they were very similar to the WSt treatment; whereas those of the MOS, EOB and WSt treatments were only similar to the INU and SUC treatments. Also, the bacterial communities of the SUC and INU treatments were likely the same or identical to each other and similar to the bacterial communities of the WSt, EOB and MOS treatments.

Intestinal contents bacterial communities present in the INU, MOS, FOS WSt and SUC treatments were very similar to each other, similar to the GOS treatment and somewhat similar to the CTL and EOB treatments (Figure III.5). In addition, bacterial communities in the CTL and EOB treatments were very similar to each other and somewhat similar to the rest of the samples.

A comparison of different samples collected from the CTL treatment was performed and is shown in Figure III.6. Bacterial communities of the BFP and HP were somewhat similar to each other but not similar to the bacterial communities of the GT and IC. Bacterial communities of the GT and IC were not similar to each other.

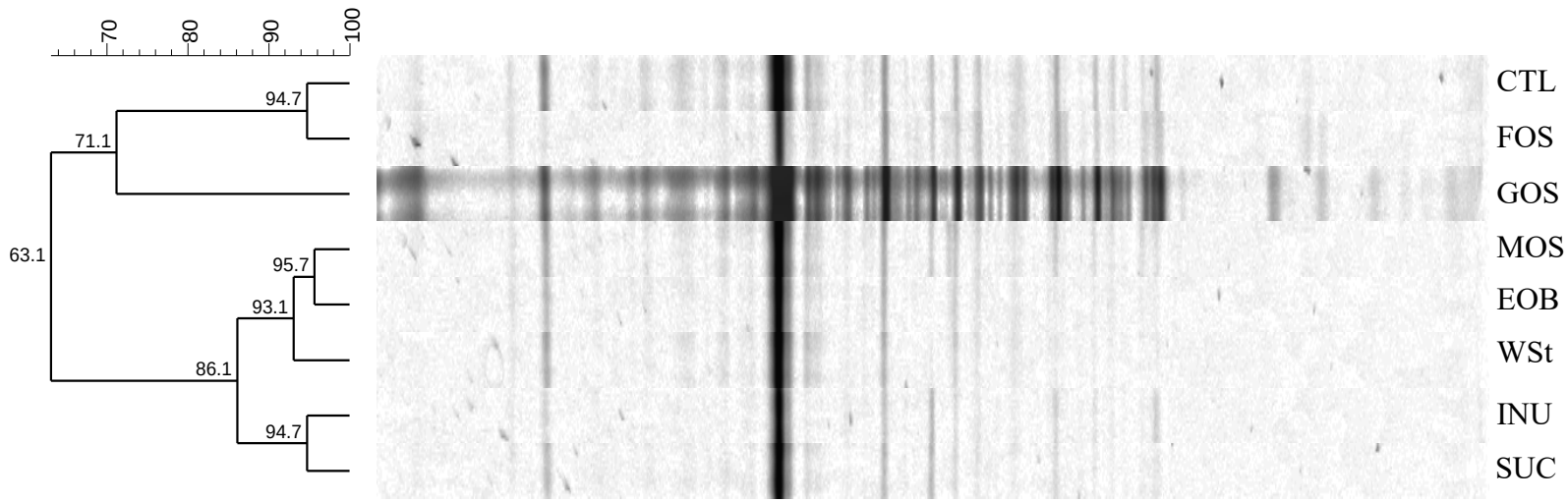

Figure III.4. Dendrogram of the hepatopancreas (HP) bacterial communities sampled during the autotrophic phase (ATr) or during termination from shrimp fed diet with no additive inclusion (CTL; control) or diets containing fructooligosaccharide (FOS), galactooligosaccharide (GOS), inulin (INU), mannan-oligosaccharide (MOS), wheat starch (WSt), sucrose (SUC) or essential oil blend (EOB) of Trial II.

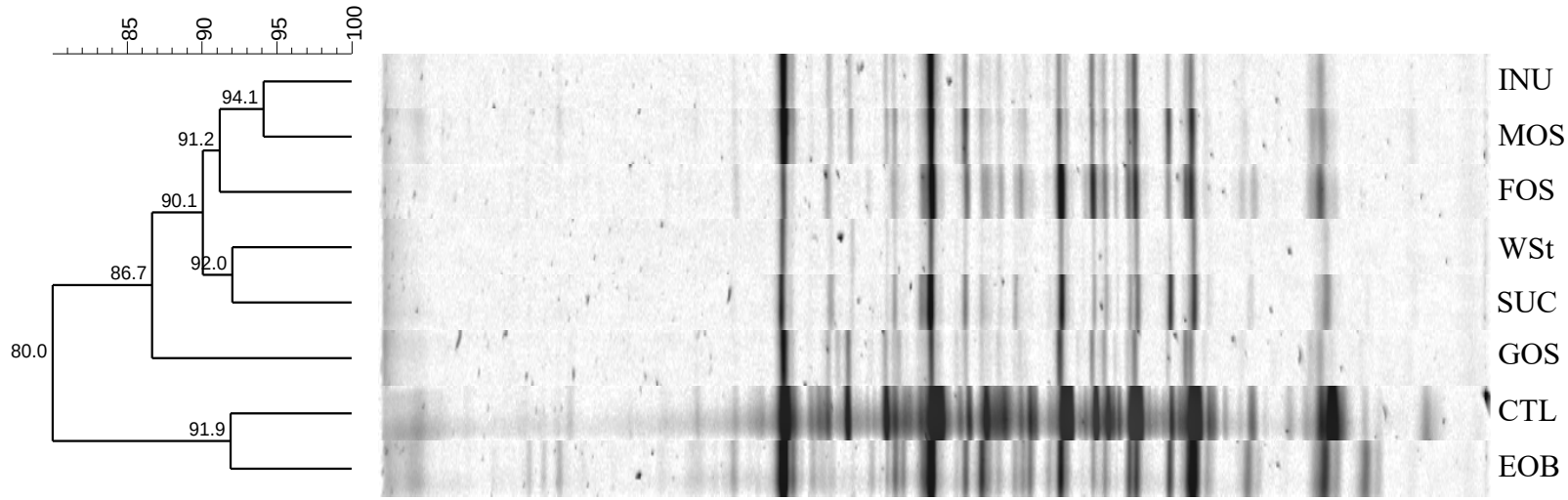

Figure III.5. Dendrogram of the intestinal contents (IC) bacterial communities sampled during the autotrophic phase (ATr) or during termination from shrimp fed diet with no additive inclusion (CTL; control) or diets containing fructooligosaccharide (FOS), galactooligosaccharide (GOS), inulin (INU), mannan-oligosaccharide (MOS), wheat starch (WSt), sucrose (SUC) or essential oil blend (EOB) of Trial II.

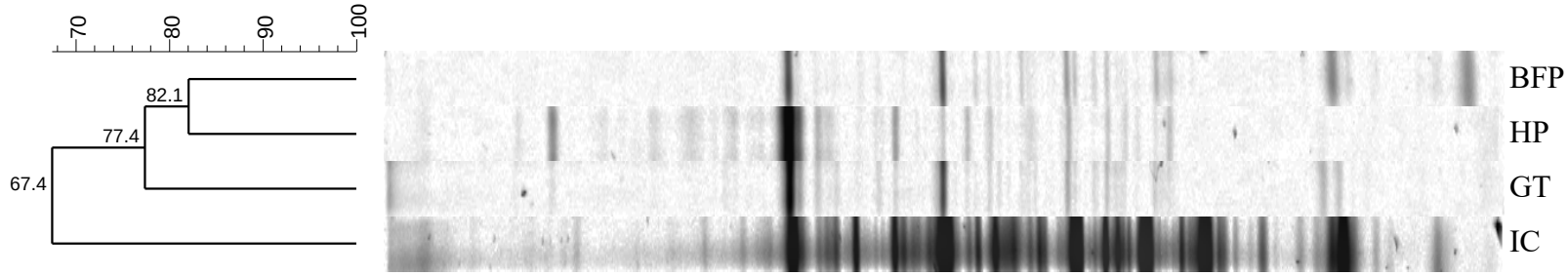

Figure III.6. Dendrogram of the bacterial communities sampled from the control (CTL) treatment of biofloc particles (BFP), gills tissue (GT), hepatopancreas (HP) and intestinal contents (IC) of Trial II.

III.4 Discussion

Experimental feed additives were included to the diets of *L. vannamei* to examine their efficacy on shrimp health and production parameters, water quality and bacterial content of biofloc and shrimp gills, hepatopancreas, and intestinal contents. Diets were prepared replacing cellulose and diatomaceous earth at levels that did not affect shrimp digestibility and growth or survival (Borrer, 1989).

Final weight, weight gain, FE, survival, HSI or K showed no significant differences among treatments. These findings are in agreement with other crustacean studies, e.g., Bio-MOS® in the diet of western king prawn *Penaeus latisulcatus* juveniles (Van Hai et al., 2009), sc-FOS in the diet of Pacific white shrimp *L. vannamei* (Li et al., 2007) and inulin in the diet of Indian white, *Fenneropenaeus indicus* shrimp larvae and postlarvae (Hoseinifar et al., 2010). Other studies have shown production enhancement due to the use of prebiotics, e.g., the addition of MOS in the diet of *P. semisulcatus* (Genc et al., 2007) and sc-FOS in the diet for *L. vannamei* (Zhou et al., 2007). The lack of effect of the prebiotics on production data in Trial II could be related to the lack of a source of stress or a pathogen during the experiment and to the higher growth rate of smaller shrimp used in the studies where an effect on production performances was observed (Genc et al., 2007; Zhou et al., 2007).

Immunostimulants have been proposed as a suitable alternative to the use of antibiotics or growth promoters (Reikel et al., 2007). In fact, it has been proven that diets supplemented with immunostimulants confer considerable benefits to shrimp by boosting their immune system (Reid, 2008). Hemocytes play an important role in antibacterial activity of crustaceans (Chisholm and Smith, 1995). Although the hemocyte count varies

among crustacean species and is known to be affected by a variety of factors such as infection and environmental stress, the THC of circulating hemocytes in crustaceans correlates well with the health condition of the shrimp and its ability to resist pathogens (Le Moullac et al., 1998; Le Moullac and Hanner, 2000). In Trial II, THC showed significant differences, with the highest concentration of hemocytes in hemolymph of shrimp fed diets containing EOB, MOS, INU and GOS and the lower hemocyte count was found in hemolymph of shrimp collected during the initial sampling. An increase in the THC has been observed in shrimp fed herbs (Wu et al, 2017; Bindhu et al., 2014) and other immunostimulants such as β-glucan for 3 days (Thanardkit et al., 2002) and 28 days (Chotikachinda et al., 2008; Srithunyalucksana et al., 2005). The increase in THC caused by these additives is a promising result that needs to be evaluated in shrimp subjected to a stressor or a disease challenge to confirm an enhancement in shrimp production performance due to improvement in health and disease resistance.

Unlike in Trial I, no significant differences were observed in mean biofloc concentration among any of the treatments evaluated. This lack of difference is likely because shrimp were readily consuming the feed provided which reduced the loss of additives to the water. However, an effect in the bacterial composition was observed in biofloc particles related to treatments and will be discussed later.

No significant differences were observed in total ammonia-nitrogen, nitrite, nitrate or alkalinity of water from the various treatments. Previous studies of prebiotics added to the diet of shrimp had much smaller inclusion levels than those used in the current study. The reason for the high dietary inclusion level of prebiotic in Trial II was to check for a possible effect on water quality parameters as affected by bacteria present

in biofloc particles that could possibly be enhanced by dietary prebiotics. The drastic reduction of ammonia and nitrites throughout the experiment could be related to the high inclusion of carbohydrate in the experimental diets. A smaller dietary inclusion level of carbohydrate sources should be evaluated to confirm an effect on water quality when prebiotics are supplemented in the shrimp diet.

Lipid and protein contents were rather low in all bioflocs while ash was consistently high in all treatments. Although shrimp might utilized to some extent the nutrients provided by biofloc, it is obvious that the majority of their nutrient requirements were supplied by the artificial feed. Significant differences were observed in ash and protein composition of biofloc samples collected at the end of Trial II. Ash content was higher in biofloc of treatments containing INU or SUC and significantly lower for diets containing GOS, MOS and the CTL reference. In contrast, protein content was higher in biofloc of the CTL, GOS, and MOS. The effect of the dietary treatments on biofloc proximate composition could be related to the differences in biofloc bacterial composition. Bacterial identification in biofloc could provide further information to relate bacterial composition to diet nutrient availability of biofloc particles.

Significantly higher ash content was found in shrimp muscle of the GOS treatment and lower ash content was found in the shrimp muscle of the SUC, CTL and FOS treatments. No significant differences were observed in protein or lipid content of shrimp muscle, which is in agreement with other studies analyzing muscle composition of shrimp fed diets with prebiotics (Aktaş et al., 2014). However, others have reported significant differences in shrimp whole-body protein composition with the addition of

prebiotics to the diet (Genc et al., 2007). More studies need to be developed to further examine the effect that prebiotics may have on nutrient composition of shrimp.

Bacterial communities showed differences among treatments related to the prebiotic dietary inclusion and were very similar to results obtained in Trial I. Most studies have evaluated the effect that prebiotics have on the gastrointestinal tract of the animal being investigated, however, this current study showed that an effect of prebiotics and the EOB was also be observed in bacterial communities present in other shrimp tissues and organs as well as in biofloc particles of the shrimp culture water. The close relation between biofloc, shrimp and nutrient recycling that exists in a zero-exchange, BFT shrimp culture could explain the effect that different prebiotics have on different tissues of the organisms and in biofloc particles even when the additives are introduced to the culture system through the shrimp diet.

In addition, a comparison of the bacterial community of the different samples collected for the CTL treatment was performed. Interestingly, bacterial communities of the BFP and HP were somewhat similar to each other and not similar to the bacterial communities of the GT and IC. Bacterial communities of the GT and IC were not similar to each other. The differences in communities of different samples from the same treatment could be related to the different functions and environments of the tissues sampled. Differences in bacterial communities of different shrimp tissues are in agreement with previous studies. For example, a comparison between the hepatopancreas and gut microbiota of *Neocaridina denticulate* revealed that bacteria from the phylum Bacteroidetes were more represented in the hepatopancreas, while bacteria from the phylum Firmicutes were more represented in the foregut and intestine samples (Cheung

et al., 2015). The authors explained these differences in relation to the different activities carried out by these organs, i.e., higher cellulolytic activity in the hepatopancreas, while a higher proteolytic activity in the intestine.

In summary, results obtained from this study show the following:

(1) Prebiotics and the essential oil blend changed the bacterial composition of biofloc particles and shrimp gills, hepatopancreas and intestinal contents when they were included in the shrimp diet.

(2) The essential oil blend appeared to improve shrimp health and promoted a potentially higher resistance to pathogens as observed by the higher THC.

(3) Bacterial flora differences among hepatopancreas, intestinal contents, gills and biofloc particles could be related to their different functions and environments.

(4) Even though a higher growth was observed in Trial II than Trial I, no effect of the additives was observed on water quality or shrimp growth.

(5) Identification of bacteria present in biofloc particles, shrimp gills, hepatopancreas, and intestine will provide further insights regarding the effects of the different additives tested.

CHAPTER IV

EFFECT OF DIETARY INCLUSIONS OF PREBIOTICS AND ORGANIC ACID SALTS ADDED TO THE FEEDS OF PACIFIC WHITE SHRIMP *Litopenaeus vannamei* ON BACTERIAL COMPOSITION OF BIOFLOC AND SHRIMP INTESTINE, GILLS AND HEPATOPANCREAS

IV.1 Introduction

Disease outbreaks, among other things, have heavily impacted shrimp production; most recently with early mortality syndrome in Asia and America (TWB, 2013). These outbreaks provide a warning to rapidly expanding aquaculture sectors, such as shrimp aquaculture, of the importance of disease management and adoption of best practices (Bondad-Reantaso et al., 2005).

Prebiotics have been proposed as an alternative to the use of antibiotics and disinfectants in aquaculture. The effect of different prebiotics have been studied to a limited extent in crustacean aquaculture (Daniels and Hoseinifar, 2014; Merrifield and Ringø, 2014). Studies conducted to evaluate the efficacy of some prebiotics in shrimp culture found improvements in weight gain and feed efficiency (FE) (Zhou et al., 2007; Genc et al., 2007).

Another potential alternative to the detrimental use of antibiotics is the group of additives known as organic acids which have been receiving increasing attention due to their strong antibacterial and prophylactic properties against various pathogenic bacteria (da Silva et al., 2013; Defoirdt et al., 2006; Ng and Koh, 2011). Currently, there is an increasing tendency towards using organic acids in commercial aquafeeds both for

controlling disease and enhancing growth performance. Additionally, organic acids are commonly known as safe compounds that regularly contain one or more carboxyl groups (–COOH) and exhibit antibacterial properties (Defoirdt et al., 2009) and are generally composed of short-chain fatty acids (C1-C7), volatile fatty acids or weak carboxylic acids. Research has been done using organic acids in aquaculture organisms with promising results on fish and shrimp production performance and as immune enhancers. For example, it has been reported that the use of organic acids, their salts or mixtures can improve growth, feed utilization and disease resistance of several fish and shrimp species (Castillo et al., 2014; Ng and Koh, 2011, Ringø, 1991; da Silva et al., 2013; Romano et al., 2015; Lückstädt, 2008; Baruah et al., 2007; Hossain et al., 2007).

Organic acids have been shown to have a direct bactericidal effect resulting from a pH decrease within bacterial cells (Ng et al., 2009b; Malicki et al., 2004; Freitag, 2007). These acids also may reduce the gastrointestinal pH thereby inhibiting the growth of pathogenic gram-negative bacteria (Luckstadt, 2008). The main antibacterial activity of organic acids is attributed to altering the cell cytoplasm pH of bacteria thereby inhibiting bacteria sensitive to such changes (Booth and Stratford, 2003). The antibacterial properties of organic acid salts, alone or in combination with organic acids or other food additives, has been examined and reported (Ukuku et al., 2005; Buchanan et al., 1993; Shelef and Addala, 1994; Stekelenburg and Kant-Muermans, 2001). However, no study has been developed on the effect prebiotics or organic acid salts added to the shrimp diet have on shrimp production and microbiology of biofloc, shrimp gills, hepatopancreas, and intestinal contents when cultured using a Biofloc Technology system. Therefore, the objective of this study was to evaluate the effects of the prebiotics fructooligosaccharide

(FOS) and galactooligosaccharide (GOS) at two dietary inclusion levels (1.5 and 3%) as well as the effects of the organic acids sodium acetate (SA), sodium lactate (SL) and sodium propionate (SP) at two dietary inclusion levels (0.75 and 1.5%) on bacterial profiles in biofloc particles and *Litopenaeus vannamei* hepatopancreas, intestine and gills. In addition, effects of these additives on shrimp production and water quality were assessed.

IV.2 Materials and methods

IV.2.1 Experimental conditions

A 26-day trial (Trial III) was conducted in the trū® Shrimp Company experimental station located in Balaton, MN, USA to evaluate the effect of different organic acid salts and prebiotics on shrimp production and bacterial composition of biofloc and shrimp hepatopancreas, gills and intestinal contents. Thirty-six tanks (0.457 m × 0.457 m × 0.280 m) containing an independent heater, an automatic 48-h feeder, and two air stones were used in this trial. Tanks were filled to 20-cm depth with artificial seawater of 28 g/L salinity and maintained at 30.0 ± 1.0°C. Sodium bicarbonate ($NaHCO_3$) was added to maintain buffering capacity, if alkalinity levels fell below 180 mg/L. Application levels were determined using the following formula:

$NaHCO_3$ needed per tank (g) = ((deficiency in alkalinity (mg/L) / concentration of HCO_3 in $NaHCO_3$ (72.646 (%); 0.72646)) × tank volume (L)) / 1000 (mg/g)

Water lost due to evaporation was replaced weekly with reverse osmosis water to maintain salinity at 28 g/L. All tanks were operated as zero-exchange BFT system. During the autotrophic phase (ATr; see Chapter I, subheading I.2), autotrophic bacteria were promoted and maintained from the initial day until Imhoff cone readings reached 3 mL/L. Only during the autotrophic phase, a nitrifying bacteria inoculum (Turbo Start 900, FritzZyme, Mezquite, TX, USA) was added to the culture water according to manufacturer recommendations and shrimp were fed with pellets and fines formulated to containing 35% crude protein (CP) (as-fed basis) at a mean of 3.33 g/day per tank and 0.037 g/L, respectively. Heterotrophic phase (see Chapter I, subheading I.2) was promoted when Imhoff cone readings reached 3 mL/L. Only during heterotrophic phase, total ammonia-nitrogen (TAN; NH_3/NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) levels were maintained at <3, <5 and <100 mg/L, respectively, by feeding the experimental diets formulated to contain a lower protein content (23% CP) to each tank according to its treatment.

IV.2.2 Shrimp

Post-larvae weighing 0.003 g arrived at the experimental station from a commercial hatchery (Shrimp Improvement Systems, SIS, Inc, Islamorada, Florida, USA) and were acclimated to nursery tanks conditions filled with artificial seawater of 28.0 ± 0.5 g/L salinity, at 29.0 ± 1.0ºC. Shrimp remained there until the experiment stocking date and were maintained as defined for Trial I (Chapter II). Shrimp with no visual signs of disease or stress were collected from the nursery tanks and were individually weighed. Shrimp weighing 3.5 ± 0.5 g were stocked into each tank at a

density of 325 shrimp/m^3. Only the initial group weight was recorded and mean individual weight was calculated as well as the initial biomass based on the water volume of the experimental tanks (g/m^3) (Table IV.1). Mortality counts and weights were recorded and replaced with the same size shrimp only during a 2-day acclimation period. When the trial was started, a small net was used to carefully check for mortalities and leftover feed without disturbing the living organisms. Mortalities were recorded and discarded.

Table IV.1. Initial shrimp group weight, biomass, and individual shrimp weight for Trial III.[1]

Treatment[2]	Initial group weight (g/tank)	Initial biomass (g/m^3)	Initial individual weight (g)
CTL	68.2	1631.7	3.6
FOS1.5	67.7	1620.7	3.6
FOS3.0	65.9	1577.4	3.5
GOS1.5	64.3	1540.3	3.4
GOS3.0	64.6	1547.6	3.4
SA0.75	67.2	1607.6	3.5
SA1.5	65.0	1555.3	3.4
SL0.75	65.6	1569.3	3.5
SL1.5	66.6	1593.3	3.5
SP0.75	64.3	1540.0	3.4
SP1.5	65.1	1558.8	3.4
PSE[3]	0.45	10.85	0.02

[1] Values represent treatment means.
[2] CTL = control reference; FOS1.5 = 1.5% fructooligosaccharide; FOS3.0 = 3% fructooligosaccharide; GOS1.5 = 1.5% galactooligosaccharide; GOS3.0 = 3% galactooligosaccharide; SA0.75 = 0.75% sodium acetate; SA1.5 = 1.5% sodium acetate; SL0.75 = 0.75% sodium lactate; SL1.5 = 1.5% sodium lactate; SP0.75 = 0.75% sodium propionate; SP1.5 = 1.5% sodium propionate.
[3] PSE = pooled standard error of treatment means (n = 3).

IV.2.3 Feed and feeding management

Shrimp in Trial III were fed a high-protein diet during acclimation and the first 5 days of the trial. Following this period, experimental diets formulated to contain a lower protein content were fed to promote heterotrophic bacteria dominance. The higher protein content diet and fines were the same as used for Trial I. Experimental diets containing the prebiotics fructooligosaccharide (FOS) and galactooligosaccharide (GOS) at 3% dietary inclusion level were the same used in Trial II. The remaining diets used were prepared 2 weeks before shrimp were stocked in the experimental tanks for Trial III. To prepare the diets, all dry ingredients of the reference diet were weighed and mixed in an industrial mixer for 15 min until a completely homogenized mixture was achieved. Next, the dry mixture was divided into eight equal parts and each was mixed with the experimental additives. Each batch was posteriorly blended with sodium hexametaphosphate and alginate (Table IV.2) previously mixed with 150 mL of deionized water per kg of dry feed using a hand mixer (Sunbeam Products Inc., Milford, MA) until an appropriate mash consistency was obtained for extrusion. Fish and soybean oil were also added during this step. Extrusion was made using a meat chopper attachment (Model A-800, Hobart Corporation, Troy, OH, USA) fitted with a 3-mm die. Moist feed strands were dried on wire racks in a forced air oven at 35^{o}C to a moisture content of 8-10%. After a 24-h drying period, feed was milled and sifted into the appropriate size for shrimp consumption, bagged, and stored at 4^{o}C until used. Proximate composition of the diets was analyzed according to the AOAC (1990) procedures for dry matter, lipid, and ash contents and the Dumas method (AOAC, 2005) for crude protein composition. Proximate composition of feed was performed in duplicate samples.

Formulation of the high protein diet, as well as the experimental diets containing the additives can be found in Table IV.2. Fines were obtained by grinding the higher protein diet. From day 0-5 of the experiment, the high protein diet was provided along with fines obtained from the same diet as shown in Table IV.3. The experimental diets formulated to have a lower protein content were fed from day 6 until termination in order to increase the C:N ratio and promote the dominance of heterotrophic bacteria. Based on previous experience, it was expected that, for this size, shrimp were going to grow linearly. A feed curve based on number of shrimp, expected shrimp growth and FE was used to determine the expected feed regimen offered to each tank (Expected feed regimen = shrimp count × Expected weight increment (g) × FE) (Table IV.3). Each 48-h feeder was loaded every other day based on the expected feed regimen of 2 days with adjustments based on leftover feed, water quality and mortalities.

Table IV.2. Formulation (%) of the experimental diets used for Trial III with determined ash, protein and lipid composition (g/kg).

			Experimental diets									
Ingredient	35% CP	CTL	FOS 1.5	FOS 3.0	GOS 1.5	GOS 3.0	SA 0.75	SA 1.5	SL 0.75	SL 1.5	SP 0.75	SP 1.5
Squid muscle meal[a]	30.0	21.7	21.7	21.7	21.7	21.7	21.7	21.7	21.7	21.7	21.7	21.7
Wheat starch[b]	28.75	43.58	43.58	43.58	43.58	43.58	43.58	43.58	43.58	43.58	43.58	43.58
Fish meal[c]	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Soy protein isolate[d]	5.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dicalcium phosphate[b]	4.2	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6	3.6
Lecithin, dry, 95%[c]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Diatomaceous earth[e]	3.8	3.7	2.2	0.9	2.2	0.9	3.0	2.2	3.0	2.2	3.0	2.2
Cellulose[f]	3.2	3.2	3.2	3.0	3.2	3.0	3.2	3.2	3.2	3.2	3.2	3.2
Calcium carbonate[f]	2.5	1.37	1.37	1.37	1.37	1.37	1.37	1.37	1.37	1.37	1.37	1.37
Alginate (Manucol DM)[g]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Potassium chloride[f]	1.9	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Magnesium oxide[h]	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6
Sodium hexametaphosphate[f]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Sodium chloride[f]	0.7	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Menhaden fish oil[c]	0.6	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
Soybean oil[i]	0.6	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Vit/Min premix[r]	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Cholesterol[a]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

Table IV.2. Continued.

Ingredient	35% CP	CTL	Experimental diets									
			FOS 1.5	FOS 3.0	GOS 1.5	GOS 3.0	SA 0.75	SA 1.5	SL 0.75	SL 1.5	SP 0.75	SP 1.5
DL-Methionine[k]	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Fructo-oligosaccharide[l]			1.5	3.0								
Galacto-oligosaccharide[m]					1.5	3.0						
Sodium acetate[s]							0.75	1.5				
Sodium lactate[t]									0.75	1.5		
Sodium propionate[u]											0.75	1.5
Ash	131.4	173.8	164.3	150.0	159.1	143.6	175.8	172.3	172.7	170.7	172.7	163.5
Protein	385.5	233.9	235.7	225.8	230.9	222.6	235.8	232.2	228.5	235.2	229.4	234.1
Lipids	82.5	78.4	85.7	78.5	82.1	79.6	82.0	85.4	79.8	87.6	90.7	83.4

[a] Zeigler Bros., Inc. Gardners, PA, USA
[b] MP Biomedicals Santa Ana, CA, USA
[c] ADM Co. Chicago, IL, USA
[d] Solae LLC, St. Louis, MO, USA
[e] Absorbent Products LTD
[f] Fisher Scientific
[g] FMC BioPolymer
[h] Prince Agri Products
[i] Consumer's Supply
[k] Evonik Degussa Corporation
[l] sc-FOS; Ingredion Incorporated, Indianapolis, IN, USA
[m] GOS; Ingredion Incorporated, Indianapolis, IN, USA
[n] INU; JeTsu Technology Limited, London, UK
[o] Bio-MOS; Alltech, Nicolasville, KY, USA
[p] Regano EX, Ralco Nutrition, Marshall, MN, USA
[q] Nash Finch Company, Minneapolis, MN, USA
[r] Composition given in Appendix
[s] BeanTown Chemical, Hudson, NH, USA
[t] ACROS Organics, New Jersey, USA
[u] Alfa Aesar, Tewksbury, MA, USA

Table IV.3. Expected feed curve based on shrimp count, expected weight increase and feed efficiency with actual fines and feed provided for Trial III.

Day	Shrimp count	Expected weight (g)	Expected weight increment (g)	FE	Expected feed regimen (g)	35% CP Fines (g)	Feed protein %	Actual feed provided (g)
ACN	19	3.00	-	0.8	3.80	-	35	5
ACN	19	3.25	0.25	0.8	3.80	-	-	-
0	19	3.50	0.25	1.3	6.18	3.0	35	12
1	19	3.75	0.25	1.3	6.18	3.0	-	-
2	19	4.00	0.25	1.2	5.70	3.0	35	11
3	19	4.25	0.25	1.2	5.70	3.0	-	-
4	19	4.50	0.25	1.1	5.23	3.0	35	10
5	19	4.75	0.25	1.1	5.23	-	-	-
6	19	5.00	0.25	1.0	4.75	-	23	9
7	19	5.25	0.25	1.0	4.75	-	-	-
8	19	5.50	0.25	0.9	4.23	-	23	8
9	19	5.75	0.25	0.9	4.23	-	-	-
10	19	6.00	0.25	0.8	3.80	-	23	7
11	19	6.25	0.25	0.8	3.80	-	-	-
12	19	6.50	0.25	0.8	3.80	-	23	7
13	19	6.75	0.25	0.8	3.80	-	-	-
14	19	7.00	0.25	0.8	3.80	-	23	7
15	19	7.25	0.25	0.8	3.80	-	-	-
16	19	7.50	0.25	0.8	3.80	-	23	7
17	19	7.75	0.25	0.8	3.80	-	-	-
18	19	8.00	0.25	0.8	3.80	-	23	7
19	19	8.25	0.25	0.8	3.80	-	-	-
20	19	8.50	0.25	0.8	3.80	-	23	7
21	19	8.75	0.25	0.8	3.80	-	-	-
22	19	9.00	0.25	0.8	3.80	-	23	6
23	19	9.25	0.25	0.8	3.80	-	-	-
24	19	9.50	0.25	0.8	3.80	-	23	6
25	19	9.75	0.25	0.8	3.80	-	23	2
26	19	10	0.25	1.8	0.00	-	-	TMN

ACN = acclimation period; FE = feed efficiency; TMN = termination
Expected feed regimen = shrimp count × expected weight increment × FE
Feed provided = actual feed loaded on 48-h feeders with amount calculated for 2 days

IV.2.4 Experimental treatments

A control diet was used in which no additive was included in the diet. Prebiotics used in this study were fructooligosaccharide (sc-FOS; Ingredion Incorporated, Indianapolis, IN, USA) and galactooligosaccharide (GOS; Ingredion Incorporated, Indianapolis, IN, USA). Feed was prepared to contain 1.5 or 3% dietary inclusion levels of FOS or GOS replacing cellulose (Borrer, 1989).

The effect of different organic acids at two dietary inclusion level also was evaluated. Sodium acetate (SA; sodium acetate Anhydrous, 99% $H_3CCOONa$, BeanTown Chemical, Hudson, NH, USA), sodium lactate (SL; Sodium lactate 60 wt% in water $C_3H_5NaO_3$, ACROS Organics, New Jersey, USA) and sodium propionate (SP; Sodium propionate 99% $C_3H_5NaO_2$, Alfa Aesar, Tewksbury, MA, USA) were included in the diets for *Litopenaeus vannamei* at two different dietary inclusion levels (0.75 and 1.5%) replacing cellulose (Borrer, 1989).

IV.2.5 Data acquisition and analyses

All data was acquired and analyzed as described in Chapter II, Section II.2.5 for Trial I with the following exceptions:

(1) No initial hemolymph sample was collected.

(2) No biofloc sample was collected during the autotrophic phase for proximate composition.

(3) Shrimp muscle was collected during the autotrophic phase for proximate composition.

(4) Condition factor and hepatosomatic index were not evaluated.

(5) No initial sample or sample collected during the autotrophic phase was available for denaturing gradient gel electrophoresis of shrimp GT, HP or IC.

IV.2.6 DNA isolation and PCR

DNA was isolated and PCR was conducted as previously described in Chapter II, section II.2.6 for Trial I.

IV.2.7 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was performed as described for Trial I in Chapter II, Section II.2.7.

IV.2.8 Calculations and statistical analyses

The responses utilized to compare treatments in this study as well as the statistical analyses were performed as described for Trial I in Chapter II, Section III.2.8.

IV.3 Results

Mean dissolved oxygen, water temperature, salinity and pH of the culture water are shown by treatment in Table IV.4. No differences ($p \leq 0.05$) were observed in regard to shrimp final weight, weight gain, FE or survival when the prebiotics or the organic acid salts were added to the dietary treatments at any of the inclusion levels evaluated (Tables IV.5 and IV.6). A positive effect was observed on THC when organic acid salts were added to the feed of *L vannamei* (Table IV.6). Mean biofloc concentrations remained comparable with no differences ($p \leq 0.05$) among any of the treatments evaluated in Trial III (Figures IV.1 and IV.2).

Table IV.4. Dissolved oxygen (mg/L), salinity (g/L), temperature (°C) and pH means from daily observations during Trial III.[1]

Treatment[2]	Dissolved Oxygen (g/mL)	Salinity (g/L)	Temperature (°C)	pH
CTL	5.29	28.64	30.01	8.21
FOS1.5	5.12	28.92	30.43	8.23
FOS3.0	5.09	28.44	30.06	8.17
GOS1.5	5.07	28.69	30.34	8.18
GOS3.0	5.08	28.62	29.90	8.19
SA0.75	5.09	28.87	30.41	8.21
SA1.5	5.18	28.48	29.48	8.20
SL0.75	5.28	28.62	29.79	8.20
SL1.5	5.14	28.73	30.39	8.21
SP0.75	5.15	28.23	29.96	8.19
SP1.5	4.93	28.64	31.44	8.18
PSE[3]	0.03	0.06	0.12	0.01

[1] Values represent treatment means.

[2] CTL = control reference; FOS1.5 = 1.5% fructooligosaccharide; FOS3.0 = 3% fructooligosaccharide; GOS1.5 = 1.5% galactooligosaccharide; GOS3.0 = 3% galactooligosaccharide; SA0.75 = 0.75% sodium acetate; SA1.5 = 1.5% sodium acetate; SL0.75 = 0.75% sodium lactate; SL1.5 = 1.5% sodium lactate; SP0.75 = 0.75% sodium propionate; SP1.5 = 1.5% sodium propionate.

[3] PSE = pooled standard error of treatment means (n = 3).

Table IV.5. Final weight, weight gain, feed efficiency and survival as well as THC of shrimp fed the control diet with no additive inclusion or the experimental diets with 1.5 or 3% dietary inclusion level of prebiotics fructooligosaccharide or galactooligosaccharide used for Trial III.[1]

Treatment[2]	Final weight (g)	Weight gain (%)	FE	Survival (%)	THC
CTL	8.4	212.9	1.47	91.23	1.43
FOS1.5	8.0	204.7	1.57	91.23	1.90
FOS3.0	7.6	209.2	1.81	94.74	1.97
GOS1.5	8.0	224.5	1.39	94.74	1.89
GOS3.0	9.1	229.1	1.34	87.72	2.17
PSE[3]	0.29	5.76	0.11	1.84	0.14
Anova (Pr > F)	0.174	0.528	0.616	0.742	0.652

[1] Values represent treatment means.

[2] CTL = control reference; FOS1.5 = 1.5% fructooligosaccharide; FOS3.0 = 3% fructooligosaccharide; GOS1.5 = 1.5% galactooligosaccharide; GOS3.0 = 3% galactooligosaccharide; THC = total hemocyte count (10^6 cell/mL)

[3] PSE = pooled standard error of treatment means (n = 3).

Table IV.6. Final weight, weight gain, feed efficiency and survival as well as THC of shrimp fed the control diet with no additive inclusion or the experimental diets with 0.75 or 1.5% dietary inclusion level of organic acid salts sodium acetate, sodium lactate or sodium propionate used for Trial III.[1]

Treatment[2]	Final weight g	Weight gain %	FE	Survival %	THC
CTL	8.9	212.9	1.47	91.23	1.43^{b}
SA0.75	7.4	199.5	1.70	94.74	2.18^{ab}
SA1.5	8.3	217.9	1.49	89.47	2.18^{ab}
SL0.75	8.1	222.3	1.40	94.74	2.50^{a}
SL1.5	7.7	220.9	1.39	100.00	2.54^{a}
SP0.75	7.7	225.3	1.40	98.24	1.80^{ab}
SP1.5	7.9	221.9	1.40	96.49	1.87^{ab}
PSE[3]	0.11	3.87	0.05	1.12	0.11
Anova (Pr > F)	0.175	0.687	0.597	0.120	0.033

[1] Values represent treatment means.

[2] CTL= control referenceSA0.75 = 0.75% sodium acetate; SA1.5 = 1.5% sodium acetate; SL0.75 = 0.75% sodium lactate; SL1.5 = 1.5% sodium lactate; SP0.75 = 0.75% sodium propionate; SP1.5 = 1.5% sodium propionate; THC = total hemocyte count (10^7 cells/mL).

[3] PSE = pooled standard error of treatment means (n = 3).

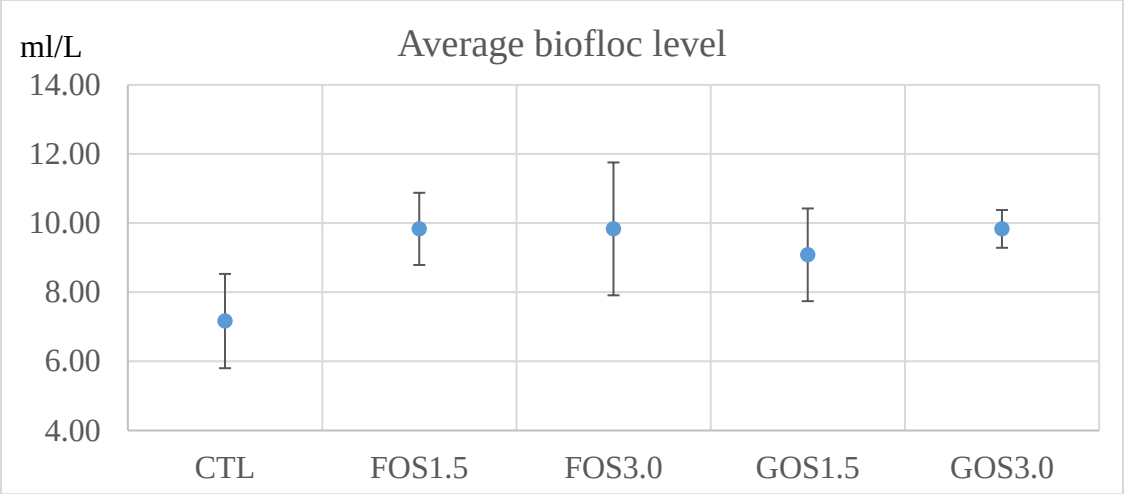

Figure IV.1. Mean biofloc levels (mL/L) of tanks subjected to the dietary control treatment with no additive inclusion or the dietary experimental treatments with 1.5 or 3.0% inclusion of prebiotics fructooligosaccharide or galactooligosaccharide during Trial III.[1,2]

[1] Values are expressed as means with no significant differences ($p \leq 0.05$) ± PSE ($n = 3$) of the weekly biofloc readings obtained from Imhoff cones.

[2] CTL = control reference; FOS1.5 = fructooligosaccharide at 1.5% dietary inclusion; FOS3.0 = fructooligosaccharide at 3% dietary inclusion; GOS1.5 = galactooligosaccharide at 1.5% dietary inclusion; GOS3.0 = galactooligosaccharide at 3.0% dietary inclusion.

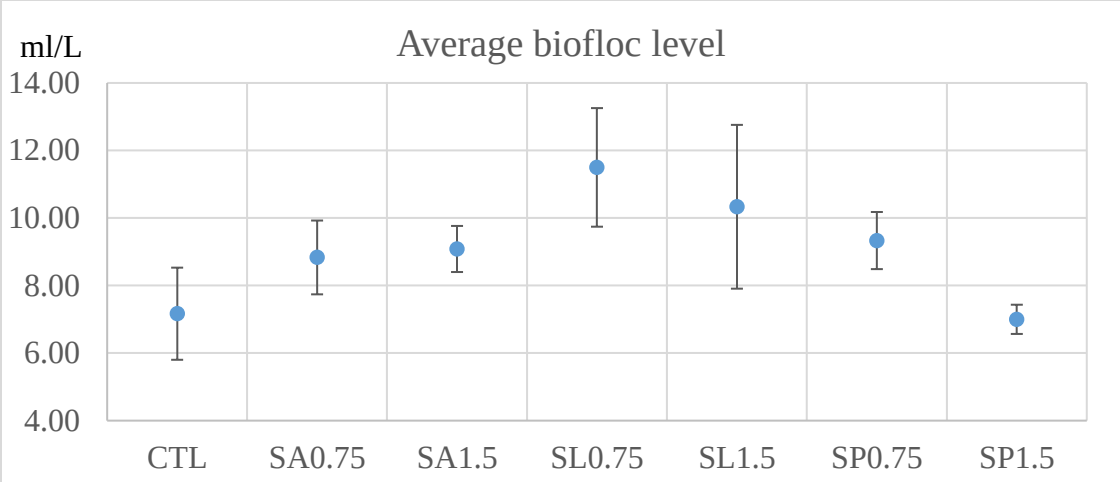

Figure IV.2. Mean biofloc levels (mL/L) of tanks subjected to the dietary control treatment with no additive inclusion or the dietary experimental treatments with 0.75 or 1.5% inclusion of organic acid salts sodium acetate, sodium propionate or sodium lactate during Trial III.[1,2]

[1] Values are expressed as means with no significant differences ($p \leq 0.05$) ± PSE ($n = 3$) of the weekly biofloc readings obtained from Imhoff cones.

[2] CTL = control reference; SA0.75 = sodium acetate at 0.75% dietary inclusion; SA1.5 = sodium acetate at 1.5% dietary inclusion; SL0.75 = sodium lactate at 0.75% dietary inclusion; SL1.5 = sodium lactate at 1.5% dietary inclusion; SP0.75 = sodium propionate at 0.75% dietary inclusion; SP1.5 = sodium propionate at 1.5% dietary inclusion.

Total ammonia-nitrogen, nitrites, nitrates and alkalinity values showed no differences ($p \leq 0.05$) among treatments in any of the observed responses for the treatments with prebiotics or organic acid salts included in the diet (Tables IV.7, IV.8, IV.9 and IV.10). No differences ($p \leq 0.05$) were observed in the nutrient composition of biofloc or shrimp muscle for any of the treatments evaluated in Trial III (Tables IV.11 and IV.12).

Table IV.7. Mean values over time for total-ammonia nitrogen (TAN) and nitrites of the dietary control treatment with no additive inclusion or the dietary experimental treatments with fructooligosaccharide or galactooligosaccharide at 1.5 or 3% inclusion level used for Trial III.[1]

	Total Ammonia-Nitrogen				Nitrites			
Treatment[2]	Day 1	Day 8	Day 15	Day 22	Day 1	Day 8	Day 15	Day 22
CTL	1.85	0.09	0.10	0.07	0.69	0.20	0.22	0.13
FOS1.5	1.63	0.10	0.10	0.09	0.61	0.15	0.20	0.23
FOS3.0	2.17	0.07	0.07	0.08	0.85	0.08	0.10	0.13
GOS1.5	2.02	0.09	0.10	0.09	0.91	0.13	0.16	0.09
GOS3.0	2.23	0.05	0.11	0.10	0.63	0.06	0.17	0.14
PSE[3]	0.14	0.01	0.01	0.00	0.05	0.02	0.02	0.02
Anova (Pr>F)	0.637	0.372	0.360	0.201	0.139	0.401	0.643	0.355

[1] Values represent treatment means of the weekly observations.
[2] CTL = control reference; FOS1.5 = fructooligosaccharide at 1.5% dietary inclusion; FOS3.0 = fructooligosaccharide at 3% dietary inclusion; GOS1.5 = galactooligosaccharide at 1.5% dietary inclusion; GOS3.0 = galactooligosaccharide at 3.0% dietary inclusion.
[3] PSE = pooled standard error of treatment means (n = 3).

Table IV.8. Mean values over time for nitrate and alkalinity of the dietary control treatment with no additive inclusion or the dietary experimental treatments with fructooligosaccharide or galactooligosaccharide at 1.5 or 3% inclusion level used for Trial III.[1]

	Nitrates				Alkalinity			
Treatment[2]	Day 1	Day 8	Day 15	Day 22	Day 1	Day 8	Day 15	Day 22
CTL	5.57	18.78	22.41	29.49	167.0	153.3	156.3	180.0
FOS1.5	5.75	18.16	28.28	25.52	176.7	154.0	143.0	232.0
FOS3.0	6.43	14.93	17.98	23.10	188.3	157.3	147.7	193.7
GOS1.5	4.48	15.33	17.35	23.71	170.0	151.0	150.3	180.7
GOS3.0	3.74	13.72	16.81	20.37	175.3	144.0	144.0	166.3
PSE[3]	0.56	0.88	1.57	1.90	3.37	2.82	3.15	11.75
Anova (Pr>F)	0.677	0.312	0.070	0.704	0.346	0.709	0.737	0.508

[1] Values represent treatment means of the weekly observations.
[2] CTL = control reference; FOS1.5 = fructooligosaccharide at 1.5% dietary inclusion; FOS3.0 = fructooligosaccharide at 3% dietary inclusion; GOS1.5 = galactooligosaccharide at 1.5% dietary inclusion; GOS3.0 = galactooligosaccharide at 3.0% dietary inclusion.
[3] PSE = pooled standard error of treatment means (n = 3).

Table IV.9. Mean values over time for total-ammonia nitrogen (TAN) and nitrites of the dietary control treatment with no additive inclusion or the dietary experimental treatments with sodium acetate, sodium lactate or sodium propionate at 0.75 or 1.5% inclusion level used for Trial III.[1]

	Total Ammonia-Nitrogen				Nitrites			
Treatment[2]	Day 1	Day 8	Day 15	Day 22	Day 1	Day 8	Day 15	Day 22
CTL	1.85	0.09	0.10	0.07	0.69	0.20	0.22	0.13
SA0.75	1.92	0.08	0.11	0.15	0.70	0.08	0.12	0.44
SA1.5	2.23	0.08	0.11	0.12	0.70	0.07	0.17	0.15
SL0.75	2.27	0.10	0.09	0.09	0.94	0.10	0.16	0.29
SL1.5	1.69	0.08	0.11	0.10	0.64	0.12	0.20	0.22
SP0.75	1.77	0.08	0.10	0.14	0.46	0.14	0.18	0.24
SP1.5	1.61	0.11	0.12	0.07	0.76	0.21	0.27	0.16
PSE[3]	0.10	0.01	0.01	0.01	0.06	0.02	0.02	0.03
Anova (Pr>F)	0.449	0.532	0.964	0.578	0.532	0.325	0.653	0.093

[1] Values represent treatment means of the weekly observations.
[2] CTL = control reference; SA0.75 = sodium acetate at 0.75% dietary inclusion; SA1.5 = sodium acetate at 1.5% dietary inclusion; SL0.75 = sodium lactate at 0.75% dietary inclusion; SL1.5 = sodium lactate at 1.5% dietary inclusion; SP0.75 = sodium propionate at 0.75% dietary inclusion; SP1.5 = sodium propionate at 1.5% dietary inclusion.
[3] PSE = pooled standard error of treatment means (n = 3).

Table IV.10. Mean values over time for nitrates and alkalinity of the dietary control treatment with no additive inclusion or the dietary experimental treatments with sodium acetate, sodium lactate or sodium propionate at 0.75 or 1.5% inclusion level used for Trial III.[1]

	Nitrates				Alkalinity			
Treatment[2]	Day 1	Day 8	Day 15	Day 22	Day 1	Day 8	Day 15	Day 22
CTL	5.57	18.78	22.41	29.49	167.0	153.3	156.3	180.0
SA0.75	5.06	17.88	32.10	22.76	170.3	145.7	156.0	189.0
SA1.5	4.11	15.29	22.71	23.07	169.3	149.0	156.3	168.3
SL0.75	8.76	17.20	20.31	25.56	165.7	154.3	155.3	152.0
SL1.5	5.66	15.91	20.31	27.28	197.7	148.0	153.7	169.0
SP0.75	4.52	15.64	16.71	19.43	174.0	144.3	147.0	170.7
SP1.5	4.97	16.99	16.82	27.48	176.0	155.7	164.7	212.0
SPE[3]	0.67	0.83	1.88	1.56	3.56	2.09	3.60	8.16
Anova (Pr>F)	0.677	0.947	0.389	0.714	0.224	0.759	0.961	0.646

[1] Values represent treatment means of the weekly observations.
[2] CTL = control reference; SA0.75 = sodium acetate at 0.75% dietary inclusion; SA1.5 = sodium acetate at 1.5% dietary inclusion; SL0.75 = sodium lactate at 0.75% dietary inclusion; SL1.5 = sodium lactate at 1.5% dietary inclusion; SP0.75 = sodium propionate at 0.75% dietary inclusion; SP1.5 = sodium propionate at 1.5% dietary inclusion.
[3] PSE = pooled standard error of treatment means (n = 3).

Table IV.11. Ash, protein and lipid content of biofloc and muscle of shrimp fed the control diet with no additive inclusion or the experimental diets with prebiotics fructooligosaccharide or galactooligosaccharide at 1.5 or 3.0% dietary inclusion levels used for Trial III.[1,4]

	Biofloc			Muscle		
Treatment[2]	Ash	Protein	Lipids	Ash	Protein	Lipids
ATr				5.8	82.8	14.5
CTL	80.0	5.0	3.2	6.1	85.4	15.4
FOS1.5	80.2	4.0	6.1	6.2	85.2	14.5
FOS3.0	82.0	4.3	5.0	6.1	82.1	14.7
GOS1.5	81.7	3.6	2.9	6.1	85.5	15.4
GOS3.0	80.0	4.9	6.7	6.2	84.4	15.8
PSE[3]	0.55	0.26	0.56	0.11	0.62	0.29
Anova (Pr>F)	0.706	0.449	0.101	0.854	0.535	0.741

[1] Values represent treatment means.
[2] CTL = control reference; FOS1.5 = fructooligosaccharide at 1.5% dietary inclusion; FOS3.0 = fructooligosaccharide at 3% dietary inclusion; GOS1.5 = galactooligosaccharide at 1.5% dietary inclusion; GOS3.0 = galactooligosaccharide at 3.0% dietary inclusion.
[3] PSE = pooled standard error of treatment means (n = 3).
[4] Proximate composition analyses of ash and lipids were performed with no duplicate sample because not enough sample was available.

Table IV.12. Ash, protein and lipid content of biofloc and muscle of shrimp fed the control diet with no additive inclusion or the experimental diets with sodium acetate, sodium lactate or sodium propionate at 0.75 or 1.5% dietary inclusion levels used for Trial III.[1,4]

	Biofloc			Muscle		
Treatment[2]	Ash	Protein	Lipids	Ash	Protein	Lipids
ATr				5.8	82.8	14.5
CTL	80.0	5.0	3.3	6.1	85.4	15.4
SA0.75	78.9	4.3	4.3	6.7	86.4	16.2
SA1.5	78.5	5.1	5.4	6.0	84.1	15.5
SL0.75	82.2	4.0	5.5	6.7	84.1	15.5
SL1.5	82.2	3.5	5.0	6.9	87.0	12.9
SP0.75	82.8	3.4	4.5	6.2	85.0	15.7
SP1.5	81.3	3.8	4.7	6.3	84.2	15.1
PSE[3]	0.57	0.23	0.39	0.12	0.46	0.29
Anova (Pr>F)	0.260	0.265	0.846	0.174	0.318	0.182

[1] Values represent treatment means.
[2] CTL = control reference; SA0.75 = sodium acetate at 0.75% dietary inclusion; SA1.5 = sodium acetate at 1.5% dietary inclusion; SL0.75 = sodium lactate at 0.75% dietary inclusion; SL1.5 = sodium lactate at 1.5% dietary inclusion; SP0.75 = sodium propionate at 0.75% dietary inclusion; SP1.5 = sodium propionate at 1.5% dietary inclusion.
[3] PSE = pooled standard error of treatment means (n = 3).
[4] Proximate composition analyses of ash and lipids were performed with no duplicate sample because not enough sample was available.

Bacterial communities present in biofloc (Figure IV.3) for the GOS1.5, GOS3.0, SA0.75, FOS1.5, FOS1.5 and SL0.75 treatments were likely the same or identical and they were very similar to SL0.75, SL0.75, SA1.5 and CTL. The bacterial community present in the SP1.5 is similar to communities for all other treatments. Bacterial communities present in ATr and SP0.75 were likely the same or identical and they were similar to those in biofloc from the other treatments.

Bacterial communities present in the gills tissue (Figure IV.4) for the SL1.5 and SP1.5 treatments were somewhat similar to each other but not similar to those for the remaining treatments. Communities of the CTL, SL0.75 and FOS1.5 treatments were very similar to each other and only similar to the ATr, SA0.75, SP0.75, SA1.5, FOS3.0, GOS1.5 and GOS3.0 treatments. The bacterial community of the ATr phase was likely the same or identical to the bacterial community of the SA0.75 and both were very similar to the bacterial community of the SP0.75 treatment. Bacterial communities of the ATr, SA0.75 and SP0.75 were similar to the bacterial communities present in the SA1.5, FOS3.0, GOS1.5 and GOS3.0 treatments. In addition, the bacterial communities of the SA1.5 and FOS3.0 treatments were very similar to each other and to the bacterial communities of GOS1.5 and GOS3.0. Bacterial communities present in the GOS1.5 and GOS3.0 treatments were likely the same or identical.

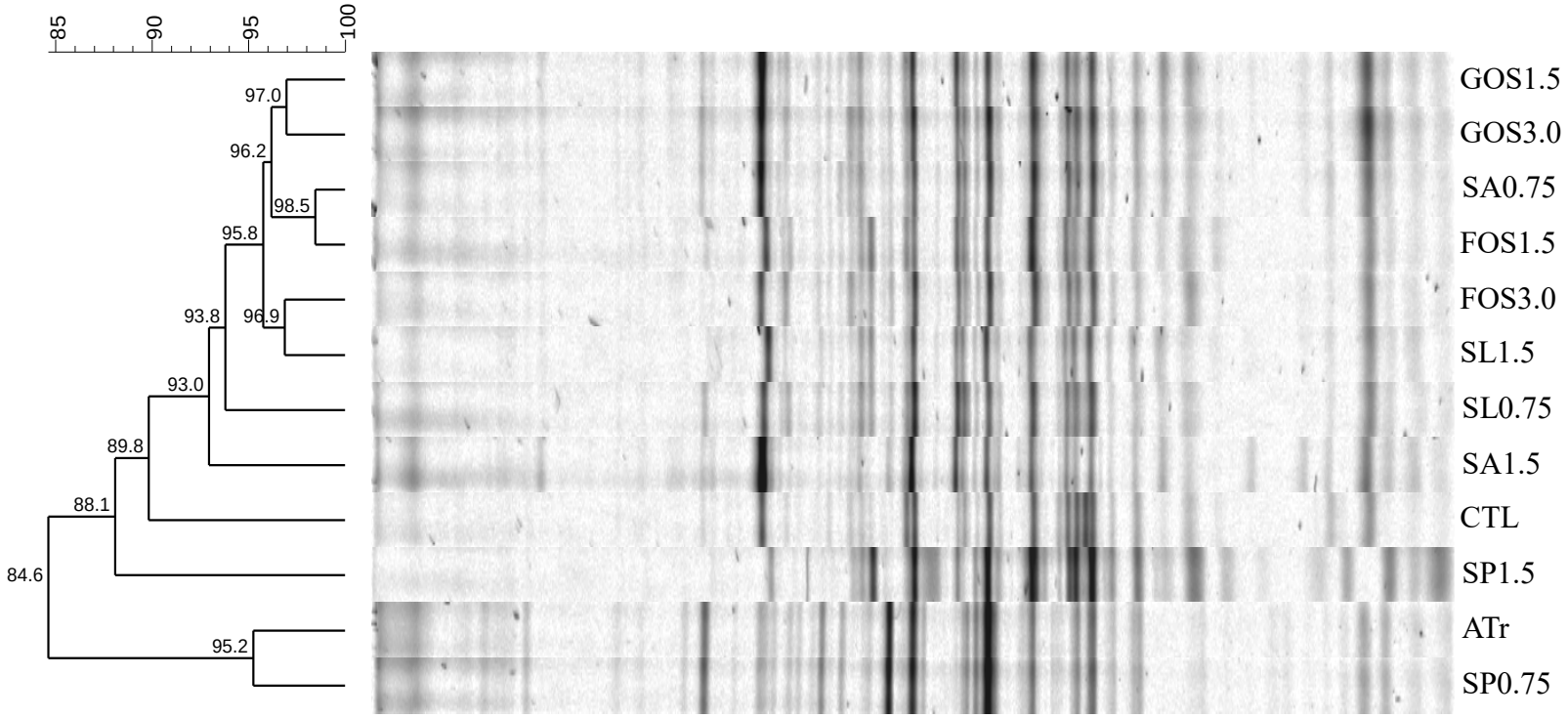

Figure IV.3. Dendrogram of the biofloc particles (BFP) bacterial communities collected during the autotrophic phase (ATr) or during termination from the tanks subjected to the control dietary treatment with no additive inclusion (CTL) or to the experimental treatments with 1.5 or 3.0% dietary inclusion of fructooligosaccharide (FOS) or galactooligosaccharide (GOS) or 0.75 or 1.5% dietary inclusion of sodium acetate (SA), sodium lactate (SL) or sodium propionate (SP) of Trial III.

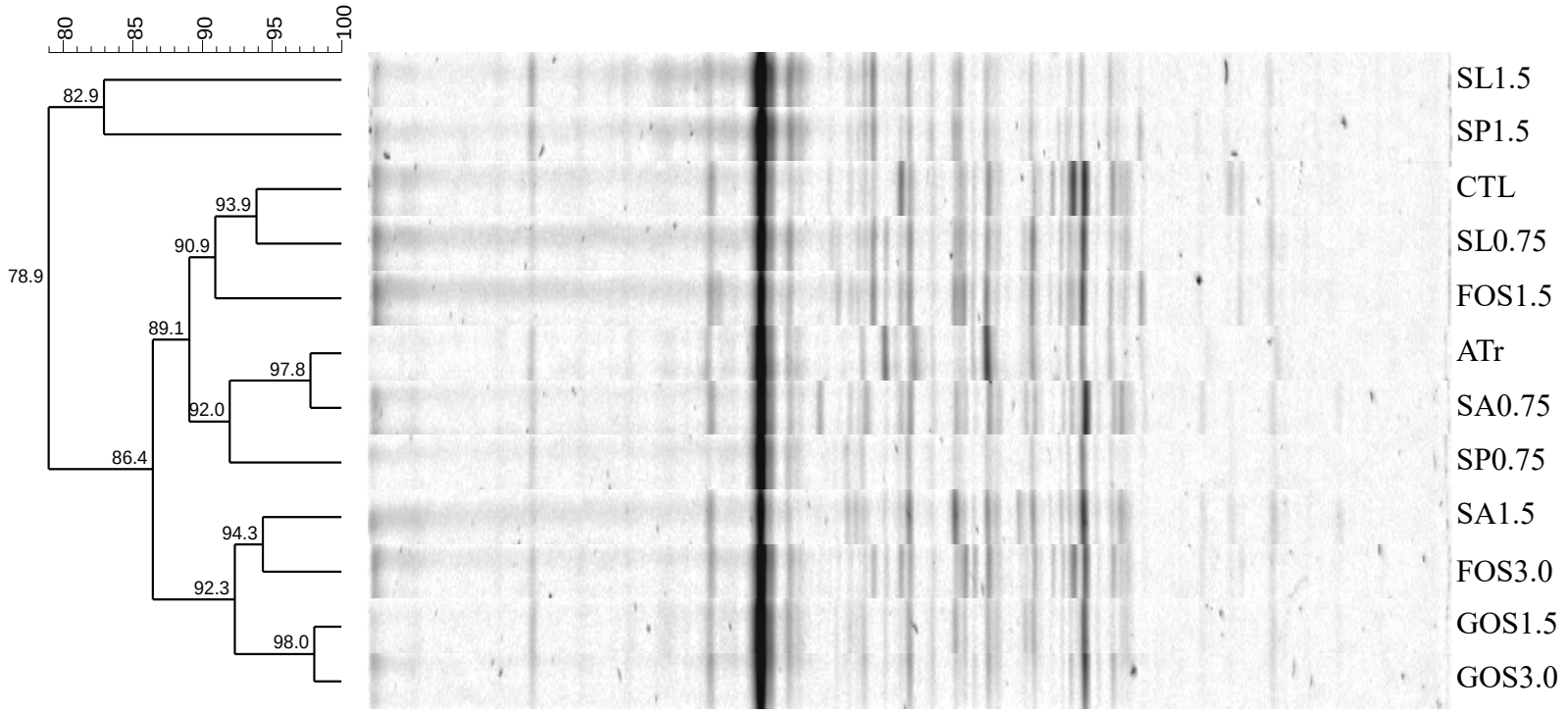

Figure IV.4. Dendrogram of the gills tissue (GT) bacterial communities collected during the autotrophic phase (ATr) or during termination from the tanks subjected to the control dietary treatment with no additive inclusion (CTL) or to the experimental treatments with 1.5 or 3.0% dietary inclusion of fructooligosaccharide (FOS) or galactooligosaccharide (GOS) or 0.75 or 1.5% dietary inclusion of sodium acetate (SA), sodium lactate (SL) or sodium propionate (SP) of Trial III.

Hepatopancreas bacterial communities (Figure IV.5) for the SP0.75, SL1.5 and SA1.5 treatments were not similar to each other and to the other treatments. The bacterial community present during the ATr phase was somewhat similar to the bacterial communities present in the SA0.75, SL0.75, GOS1.5, CTL, FOS1.5, GOS3.0, FOS3.0 and SP0.75 treatments. In addition, those communities for the SA0.75 and SL0.75 treatments were similar to each other and they were similar to the GOS1.5, CTL, FOS1.5, GOS3.0, FOS3.0 and SP1.5 treatments. Bacterial communities present in the GOS1.5, CTL, FOS1.5, GOS3.0 and FOS3.0 treatments are very similar.

Intestinal contents bacterial communities (Figure IV.6) for the FOS1.5 and SL0.75 treatments were very similar to each other, somewhat similar to the CTL treatment and not similar to those of the remaining treatments. Bacterial community of the FOS3.0 treatment was not similar to communities of other treatments. In addition, bacterial communities of the SP0.75, GOS3.0, SA1.5, SA0.75 and GOS1.5 treatments were very similar to each other, similar to SP0.75, and somewhat similar to ATr and SL0.75. The SP1.5 treatment community was somewhat similar to the bacterial communities of the ATr and SL0.75 treatments. Also, the bacterial communities of the SL0.75 and ATr treatments were somewhat similar.

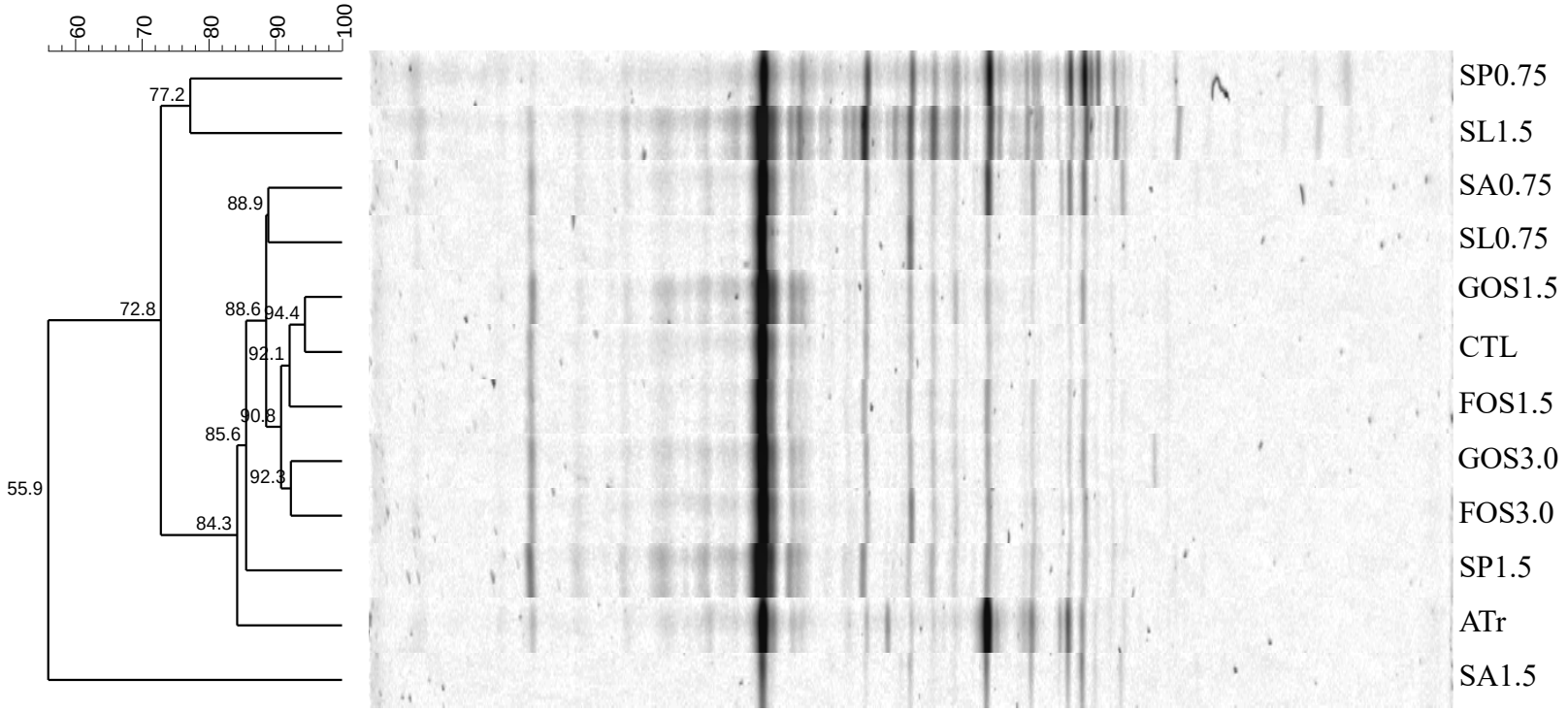

Figure IV.5. Dendrogram of the hepatopancreas (HP) bacterial communities collected during the autotrophic phase (ATr) or during termination from the tanks subjected to the control dietary treatment with no additive inclusion (CTL) or to the experimental treatments with 1.5 or 3.0% dietary inclusion of fructooligosaccharide (FOS) or galactooligosaccharide (GOS) or 0.75 or 1.5% dietary inclusion of sodium acetate (SA), sodium lactate (SL) or sodium propionate (SP) of Trial III.

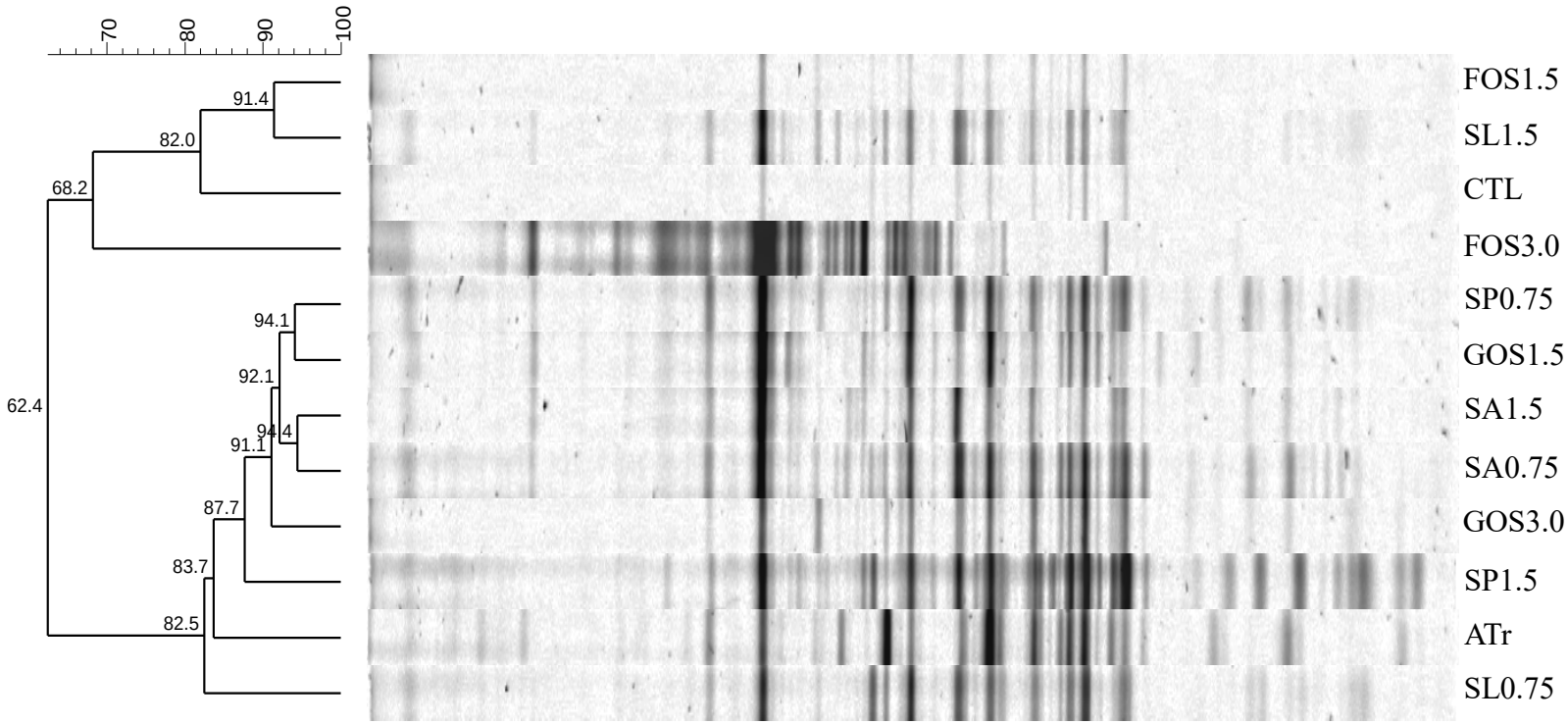

Figure IV.6. Dendrogram of the intestinal contents (IC) bacterial communities collected during the autotrophic phase (ATr) or during termination from the tanks subjected to the control dietary treatment with no additive inclusion (CTL) or to the experimental treatments with 1.5 or 3.0% dietary inclusion of fructooligosaccharide (FOS) or galactooligosaccharide (GOS) or 0.75 or 1.5% dietary inclusion of sodium acetate (SA), sodium lactate (SL) or sodium propionate (SP) of Trial III.

Analysis of the bacterial communities present in the different tissues for the ATr treatment (Figure IV.7) showed that communities present in the GT and IC were similar but not similar to the communities in the BFP and HP. The bacterial community in the BFP was not similar to the bacterial community of the HP.

Analysis of the bacterial communities present in the different tissues for the CTL treatment (Figure IV.8) showed that the HP community was somewhat similar to the IC community but they were not similar to the communities present in the BFP and GT. Bacterial communities present in the BFP were not similar to the bacterial communities present in the GT.

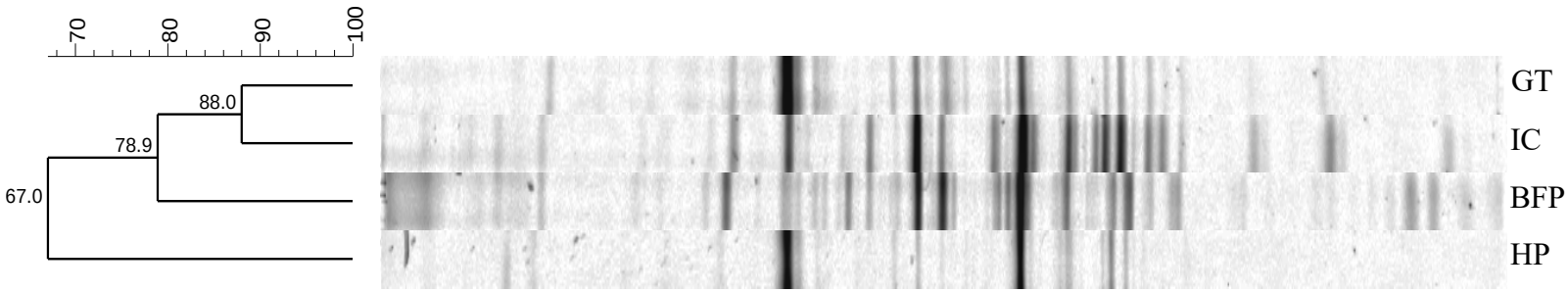

Figure IV.7. Dendrogram of the bacterial communities present in the biofloc particles (BFP), and shrimp gills tissue (GT), hepatopancreas (HP) and intestinal contents (IC) collected during the autotrophic phase (ATr) of Trial III.

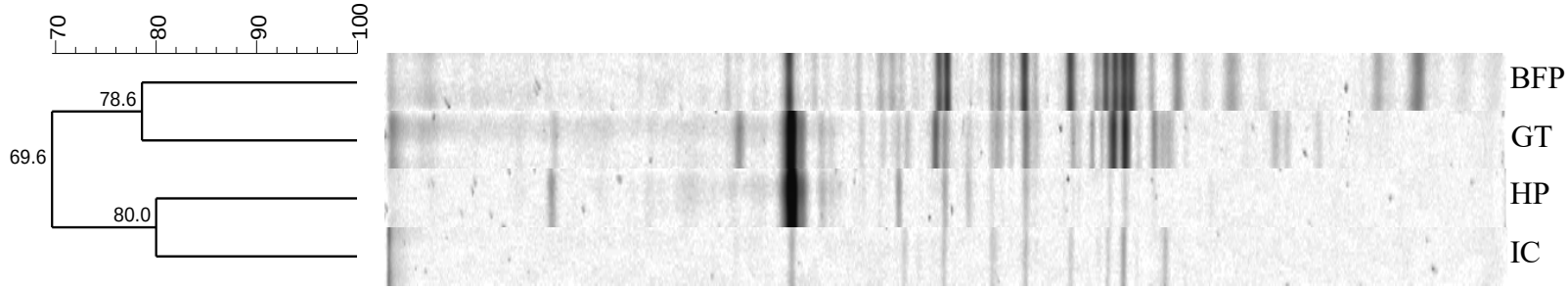

Figure IV.8. Dendrogram of the bacterial communities present in the biofloc particles (BFP), and shrimp gills tissue (GT), hepatopancreas (HP) and intestinal contents (IC) collected during termination from tanks subjected to the control dietary treatment with no additive inclusion (CTL) of Trial III.

IV.4 Discussion

The effects of the prebiotics FOS and GOS included in the diet of *L. vannamei* at 3 or 1.5% levels replacing cellulose and/or diatomaceous earth were evaluated in Trial III. In addition, the effect of the organic acid salts SA, SL and SP included in the experimental diets for *L. vannamei* at 0.75 and 1.5% dietary inclusion levels replacing diatomaceous earth was also evaluated. The variation in fiber was not expected to produce an effect on nutrient digestibility, growth or survival of *L. vannamei* (Borrer, 1989).

In spite there being no water exchange throughout the experiment, all water quality parameters were within safe limits for shrimp culture (Ebeling et al., 2006; Wasielesky et al., 2006). In particular, alkalinity was lower than the target and a dramatic reduction in ammonia and nitrites occurred during second and third week. This indicates that the nitrification process occurred in all tanks at a high rate because, during nitrification, chemoautotrophs consume carbon (CO_2 or HCO_3) for energy and produce hydrogen ions (H^+) which reduces the alkalinity in the water (Ebeling et al., 2006). Total ammonia-nitrogen, nitrites, nitrates and alkalinity readings had no significant differences among treatments in any of the observations for treatments with prebiotics or organic acid salts inclusion in the diet. This result showed that the switch to a lower protein content diet increased the C:N ratio enough to promote the heterotrophic bacteria dominance and drastically reduced the toxic nitrogen waste compounds.

No significant differences ($p \leq 0.05$) were observed in nutrient composition of biofloc or shrimp muscle from any of the treatments evaluated in Trial III. Although some studies have reported that the reduction in protein content of diets does not affect

growth in shrimp cultured under biofloc conditions, in part because shrimp supplement their protein requirements with the protein provided by biofloc (Wasielesky et al. 2006; Ballester et al. 2010; Xu et al., 2012). For this particular experiment, the protein contribution from biofloc was lower than expected. No higher protein content diet was fed during the heterotrophic phase of the experiment. Possibly, the results found here indicate that, for the particular conditions in which this experiment was performed, the system could tolerate a higher protein content diet to promote a higher shrimp growth rate without compromising water quality and shrimp survival.

No significant differences ($p \leq 0.05$) were observed in final weight, weight gain, FE or survival of any of the treatments evaluated on Trial III, which is in agreement with other studies using prebiotics without an effect on growth or survival of *L. vannamei* (Li et al., 2007; Luna-Gonzalez et al., 2012). However, a positive effect ($p \leq 0.05$) was observed on THC when organic acid salts were added to the feed. The important role hemocytes play in antibacterial activity of crustaceans has been well described (Chisholm and Smith, 1995). Although the THC varies among different crustacean species and is known to be affected by a variety of factors, such as culture conditions, infection and environmental stress, the THC of circulating hemocytes correlates well with the health condition of shrimp and its ability to resist pathogens (Le Moullac et al., 1998; Le Moullac and Hanner, 2000). The increase in THC of shrimp fed diets containing organic acid salts is an encouraging result that needs to be evaluated in shrimp subjected to a stressor or a disease challenge to confirm an enhancement in shrimp production performance.

Mean biofloc concentrations remained with no differences among any of the treatments evaluated in Trial III (Figures 12 and 13). This result was also observed in Trial II. These is the first study that evaluated the biofloc level of a shrimp culture when prebiotics or organic acid salts were included in the diet. Apparently, modifications in the diet and, more specifically, prebiotics or organic acid salts dietary inclusion, does not affect biofloc concentration in the culture tanks as much as the inclusion of additives directly to the culture water (Figure 1). However, these results need to be confirmed with more evaluations in order to determine the best route of entry of additives to maintain BFT system under optimal conditions.

Some effects on the bacterial communities of BP, GT, HP and IC were observed in all the dietary treatments evaluated in this study. Similar effects were observed in Trial I and II and are in agreement with other studies evaluating bacterial community changes when additives are included in the diet of different aquatic species (Ringo et al., 2006; Mahious et al., 2006; Bakke-Mckellep et al., 2007).

Further studies are needed to identify bacterial species and to determine if disease resistance and growth is increased in challenged organisms.

In conclusion, data presented in this study confirms the following:

(1) An effect on bacterial communities of biofloc particles and shrimp gills, hepatopancreas and intestinal contents were observed when prebiotics were added to the feed.

(2) The inclusion of organic acid salts into the shrimp diet also altered the bacterial composition of biofloc particles and shrimp gills, hepatopancreas and intestinal contents.

(3) A positive effect on the total hemocyte counts of shrimp fed the diets containing different organic acid salts indicates an improvement in shrimp health and a potential higher resistance to pathogens.

CHAPTER V

CONCLUSIONS

This dissertation compiled results from three trials performed to determine the effect of prebiotics, organic acid salts and a commercially available essential oil blend on bacterial composition of biofloc particles and shrimp gills, hepatopancreas and intestinal contents as well as on shrimp health and production performance of shrimp cultured under Biofloc Technology (BFT) conditions.

Denaturing gradient gel electrophoresis (DGGE) results obtained in the three trials demonstrated that the bacterial flora of biofloc and the cultured organisms may be affected by the type of additive used, regardless of if it is added to the feed or to the culture water. These results also demonstrate that the bacterial community composition present during the autotrophic phase was modified when a heterotrophic dominance was promoted. Shrimp and biofloc bacterial flora did not change much from the autotrophic (ATr) phase with the addition of wheat starch (WSt) because it is a complex carbohydrate that is not easily utilized by microbes.

In general, a difference in bacterial population was observed when prebiotics, organic acid salts or the essential oil blend were included in the diet or added directly to the culture water. Additives included in the feed have the capability to change bacterial communities present in biofloc particles and cultured organism's tissues and organs. Bacterial communities present in the WSt and control (CTL) treatment and collected during the ATr phase were generally the most similar compared to each other but they differ to the rest of the treatments, indicating that prebiotics, organic acid salts and the

essential oil blend (EOB) promoted the proliferation of different bacterial species in the culture system and in cultured organism's internal and external tissues and organs.

Special interest for shrimp culture is the analysis of the bacterial populations of different tissues. In Trial II and III, differences were observed in bacterial composition of different tissues and organs which means that shrimp are able to maintain certain species of bacteria in some locations and avoid others to enter. The difference in bacterial composition could be related to the different functions and environments of the organs or tissues sampled. Also, the relation between the bacterial epiflora present in gills and their nutrient uptake from dissolved organic matter could explain the difference in bacterial populations of the gills when compared to those of the biofloc, hepatopancreas and intestinal contents.

The health parameter evaluated in these studies was the total hemocyte count (THC). A positive effect was observed on the THC when shrimp were fed diets containing the EOB and the organic acid salts. However, this positive effect was not reflected in production data. It has to be noted that no health challenge (viral,, environmental, bacterial or chemical) was performed in any of the trials so, possibly, the difference in bacterial composition along with the increase in the THC, could have a positive effect on shrimp performance parameters when shrimp health is compromised.

The lack of an effect on weight gain or survivability was likely because shrimp had no external source of stress and shrimp had a low growth rate possibly related to underfeeding. However, this must not overshadow the importance of observing changes in bacterial communities and the increase on the THC promoted by the additives used in these studies.

Further research is warranted on the positive effect that the prebiotics, organic acid salts and the EOB may confer to shrimp production by altering the bacterial communities and increasing the THC when a source of stress is induced to the culture organisms and with organisms with a higher growth rate. Furthermore, identification of the bacterial flora present in biofloc and shrimp gills, hepatopancreas and intestinal contents when prebiotics, organic acid salts and essential oils are incorporated into the diet or added directly into the culture water, should yield more information on the advantages of using these additives in shrimp aquaculture.

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APPENDIX

Vitamin/Mineral Premix Ingredient Levels

Ingredient	Level	Ingredient	Level
Calcium (%)	0.16	D-Pantothenic (mg/kg)	2,975.06
Total Phosphorus (%)	0.43	Riboflavin (mg/kg)	1,342.40
Av. Phosphorus (%)	18.48	Thiamine (mg/kg)	1,668.93
Ca:Pav	0.01	Vitamin B-6 (mg/kg)	1,995.46
Salt (%)	0.22	Vitamin B-12 (mcg/kg)	834.47
Sodium (%)	0.11	Vitamin C (mg/kg)	8,272.11
Chloride (%)	0.11	Copper (mg/kg)	2,185.00
Potassium (%)	0.44	Iron (mg/kg)	138.30
Magnesium (%)	0.18	Manganese (mg/kg)	1,013.00
Vitamin A (KIU/kg)	199.55	Zinc (mg/kg)	2,185.00
Vitamin D (KIU/kg)	83.45	Cobalt (mg/kg)	1.30
Vitamin E (IU/kg)	4,970.52	Sulfur (%)	0.19
Vitamin K (mg/kg)	1,015.87	Copper (organic) (mg/kg)	2,185.00
Biotin (mcg/kg)	32,979.60	Manganese (organic) (mg/kg)	1,010.00
Folic acid (mg/kg)	330.16	Zinc (organic) (mg/kg)	2,185.00
Niacin (mg/kg)	4,136.05		