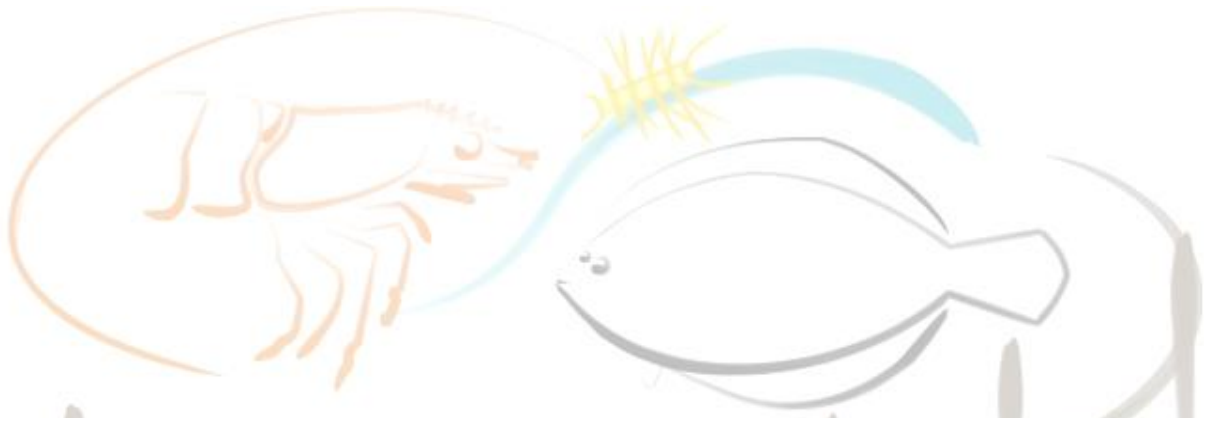**UNIVERSIDADE FEDERAL DO RIO GRANDE**

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PROGRAMA DE PÓS-GRADUAÇÃO EM AQUICULTURA

APLICAÇÃO DE PROBIÓTICOS COMO UMA FERRAMENTA DE MANEJO DA COMUNIDADE MICROBIANA E DO CULTIVO DE *Litopenaeus vannamei* **EM SISTEMA DE BIOFLOCOS**

Bárbara Linhares Hostins

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Aplicação de probióticos como uma ferramenta de manejo da comunidade microbiana e do cultivo de *Litopenaeus vannamei* em sistema de bioflocos

Aluna: Bárbara Linhares Hostins

Orientador: Prof. Dr. Wilson Wasielesky Jr.

Co-Orientador: Profª. Drª. Dionéia Evangelista Cesar

Co-Orientador no exterior: Prof. Dr. Ir. Peter Bossier

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RESUMO GERAL

A presente tese teve como objetivo avaliar influência da adição de probióticos na densidade bacteriana, desempenho zootécnico e resistência a doenças ao longo da produção de *Litopenaeus vannamei*. O primeiro capítulo objetivou definir qual é o tempo adequado de aplicação do probiótico pós-cloração/decloração da água simulando a fase de preparação que precede a estocagem dos camarões; Além disso, a influência de *Bacillus* sp. sobre a densidade de *Vibrio* sp. Recomenda-se a aplicação de probióticos imediatamente após a desinfecção da água, e que a fertilização com carbono orgânico inicie 24 horas pós cloração/decloração da água. Através da adoção de tais procedimentos, torna-se possível obter-se uma redução de *Vibrio* sp. O segundo capítulo avaliou a influência do modo operacional do sistema de bioflocos na proteção contra Necrose Aguda do Hepatopâncreas (AHPND). Pós-larvas de *L. vannamei* foram cultivadas em 2 tipos de bioflocos, Autotrófico e Heterotrófico, com e sem probióticos. Após os 21 dias, os camarões foram desafiados com patógeno de AHPND em água clara e em bioflocos correspondentes a cada tratamento. Após 96 horas, observou-se um efeito protetor dos bioflocos heterotróficos, e autotróficos com probióticos, evidenciado pelas taxas de sobrevivência. O terceiro capítulo avaliou os efeitos da suplementação com probióticos na redução de lesões histopatológicas no hepatopâncreas (HP) ocasionadas por surto de vibriose, e compará-los em bioflocos e água clara. Após 30 dias, observou-se redução nas lesões do HP nos tratamentos com probióticos além de melhor desempenho zootécnico e sobrevivência dos camarões. O quarto capítulo comparou os efeitos dos probióticos sobre a densidade de *Bacillus* e *Vibrio* spp. no trato intestinal dos camarões cultivados em bioflocos e água clara. Verificou-se que o uso de probióticos reduziu a densidade de *Vibrio* sp., enquanto a abundância de *Bacillus* aumentou. O quinto capítulo objetivou identificar os efeitos de dose-resposta da inclusão de probióticos na ração dos camarões na fase de engorda. Sugere-se que aplicando 3 g de probiótico por kg de ração, em duas alimentações diárias contribuiu para melhor sobrevivência e redução na densidade de *Vibrio*. Além disso, os bioflocos desempenham função probiótica, considerando a presença de *B. subtilis* na água e no trato intestinal no início do experimento. Os resultados obtidos no presente estudo contribuem para o entendimento da ecologia microbiana no sistema de bioflocos, a qual é um ponto chave para o desenvolvimento deste sistema de cultivo.

ABSTRACT

The present study aimed to evaluate the influence of probiotics supplementation on bacterial density, growth performance and disease resistance throughout the production cycle of *L. vannamei*. The first chapter simulated the common disinfection practice prior stocking the shrimp and to evaluate the bacterial abundance of a probiotic (*Bacillus* sp) added post water chlorination/dechlorination in different times. Moreover, the influence of the probiotics on the density of *Vibrio* sp. It is recommended the application immediately after disinfection, and that the addition of organic carbon sources starts 24 hours post chlorination/dechlorination. By adopting such procedures, it is observed a decrease in *Vibrio* sp. density. The second chapter verified the influence of different operational modes of bioflocs on the protection against Acute Hepatopancreatic Necrosis Disease (AHPND). L. vannamei post larvae were reared in 2 different types of bioflocs, Autotrophic and Heterotrophic, with and without probiotics. After 21 days, the shrimp were challenged with AHPND in new sea water and in biofloc suspensions corresponding to each treatment. Followed 96 hours, it was observed a protective effect of heterotrophic bioflocs, and autotrophic bioflocs with probiotic application. The third chapter investigated the effects of probiotics on the reduction of histopatological lesions on shrimp hepatopancreas (HP) after a vibriosis outbreak. It was verified a reduction on HP lesions in the treatments with probiotic supplementation, with an increase in growth performance and survival rates. On the fourth chapter, variation on *Bacillus* e *Vibrio* spp. density were evaluated in the gut of shrimp cultured in clar water and in bioflocs. It was verified that the use of probiotics reduced the density of *Vibrio* sp., while the abundance of *Bacillus* increased. The fifth chapter aimed to identify dose-dependent effects of probiotics included to the daily feed of shrimp during grow-out phase. It is suggested that the application of 3 g of probiotics per kg of feed, twice a day contributed to increase survival rates and the reduction of *Vibrio*. Beside, the bioflocs may have a probiotic role as well, considering the presence of *B. subtilis* in the water and in the gut in the initial phase of the experiment. The results obtained in the present phD thesis are relevant for understanding the microbial ecology in biofloc system, which is a key issue to the sustainable development of this culture system.

INTRODUÇÃO GERAL:

A expansão das atividades de aquicultura acompanha a intensificação dos cultivos nas ultimas décadas (FAO, 2016). Essa intensificação, associada a práticas de manejo inadequadas, resulta na deterioração das condições ambientais, bem como no surgimento de doenças e bactérias patogênicas resistentes (Mohapatra et al. 2013).

Como forma de prevenir e/ou mitigar os impactos negativos causados pela prevalência de enfermidades, produtores muitas vezes adotam o uso de antibióticos. No entanto, o uso indiscriminado de antibióticos, especialmente quando não há sintoma aparente de doença pode resultar no desenvolvimento de bactérias patogênicas altamente resistentes (Defoirdt et al., 2007; Crab et al., 2010; Mohapatra et al., 2013). Além disso, o uso inadequado de antibactericidas e desinfetantes tem se provado ineficiente no tratamento de algumas doenças bacterianas, e ate mesmo contribuído para aumentar o risco de doenças. (Attramadal et al.,2012; De Schryver et al., 2014).

A definição do termo probiótico foi proposta por diversos autores. Por exemplo, Fuller (1989) os define como microrganismos vivos suplementados ao alimento que afetam beneficamente o hospedeiro melhorando seu equilíbrio intestinal. Já Gatesoupe (1999), por sua vez, definiu-os como sendo células microbianas administradas de modo que possam se fixar no trato intestinal e promover a saúde dos organismos produzidos. Verschuere et al. (2000) propuseram uma modificação na definição do termo "probiótico" como "um microrganismo vivo no qual promove efeitos benéficos por meio da modificação da comunidade microbiana associada ao hospedeiro ou ao ambiente de cultivo". Tal modificação pode promover melhor aproveitamento do alimento, aumentando a capacidade de resistência a doenças e atuando na qualidade da água do ambiente de cultivo. A organização das Nações Unidas para agricultura e

alimento (FAO) juntamente com a organização mundial da saúde (WHO) definiram probióticos como "microrganismos vivos que, quando administrados em dosagens adequadas, conferem benefícios à saúde do hospedeiro" (FAO, 2002; WHO, 2002).

Deste modo, o uso de probióticos na produção de organismos aquáticos surge a partir da crescente demanda por alternativas que sejam ambientalmente amigáveis e contribuam para o incremento do processo produtivo (Verschuere et al. 2000), o que requer também a incorporação e aprimoramento de novas técnicas de monitoramento (Cardoso 2007) .

A aplicação de bactérias probióticas como profilaxia e tratamento contra patógenos é usualmente utilizada na indústria animal como uma alternativa à administração de antibióticos (Gatesoupe 2016). Na aquicultura, quando adicionados aos tanques e viveiros de cultivo, ou incluídos no alimento, os probióticos são capazes de modificar a composição bacteriana da água, sedimento e do trato intestinal dos organismos cultivados (Moriarty 1998). Especialmente no ambiente aquático, a relação entre a microbiota presente no trato intestinal e no ambiente é muito próxima (Del'Duca et al. 2015; De Schryver & Vadstein 2014), enfatizando a possível interação que pode ocorrer entre a comunidade microbiana do ambiente de cultivo e as bactérias probióticas adicionadas à água ou à ração.

Estudos abordam a utilização de microrganismos de diferentes gêneros com propriedades probióticas no cultivo de organismos aquáticos. Entre eles, estão: *Phaeobacter sp, Pediococcus sp, Lactobacillus sp, Aeromonas sp, Pseudomonas sp, Alteromonas sp, Enterococcus faecium* (Moriarty, 2003; Hai et al., 2007; D'alvise et al., 2013; Castex et al., 2008; Sha et al., 2016). No entanto, bactérias do gênero *Bacillus* sp (Gram-positivas formadoras de esporos) têm sido amplamente utilizadas na formulação

de probióticos comerciais em diferentes fases de produção de peneídeos. Essas bactérias podem ser introduzidas no ambiente de cultivo por serem na maior parte das vezes não patogênicas e não tóxicas aos organismos (Farzanfar, 2006). Além disso, produzem esporos altamente resistentes, os quais conferem inúmeras vantagens, como: podem ser armazenados em temperatura ambiente; e são capazes de sobreviver ao baixo pH das barreiras gástricas (Cutting, 2011). Bactérias do gênero *Bacillus* também são capazes de secretar polipeptídeos que agem contra uma vasta gama de bactérias Gram positivas e negativas, além de vibrios patogênicos (Decamp & Moriarty 2006; Villaseñor et al. 2013; Sapcharoen & Rengpipat 2013). Por exemplo, doses de incorporação de *Bacillus subtilis* entre 10^7 e 10^8 CFU/g de alimento pode aumentar a resposta imune e a sobrevivência do hospedeiro em um sistema convencional de cultivo em água clara (Kiron et al., 2012).

Os fatores acima citados estão entre os principais mecanismos de atuação dos probióticos, de acordo com Balcázar et al. (2007). Além disso, os polipeptídeos produzidos são compostos que restringem a atividade de patógenos e melhoram o crescimento e a sobrevivência dos organismos produzidos (Mohapatra et al. 2013). O incremento do desempenho zootécnico pode ser atribuído à contribuição enzimática ao processo digestivo, estimulada pela presença de probióticos (Wang et al. 2007). Alguns autores relatam melhores resultados de crescimento e sobrevivência como efeito da utilização de probióticos (Balcazar et al. 2007; Zhou et al. 2009; Nimrat et al. 2012; Krummenauer et al. 2014; Zokaeifar et al. 2014).

Outro mecanismo de atuação dos probióticos é a exclusão competitiva (antagonismo) com os organismos potencialmente patogênicos presentes no sistema de aquicultura. Neste sentido, o trato intestinal é considerado a rota de transmissão

primária no processo de infecção por patógenos (De Schryver and Vadstein, 2014), e as bactérias probióticas podem competir com potenciais patógenos por nutrientes (Gatesoupe 1999) ou por sítios de adesão no trato intestinal (Joborn et al. 1997; Mohapatra et al., 2013; Hamza et al., 2015). Essa capacidade de adesão à mucosa gastro-intestinal auxilia na resistência a organismos patogênicos, reduzindo sua densidade no hospedeiro (Villaseñor et al., 2011; Akhter et al., 2015; Lazado et al., 2015). Sendo assim, ressalta-se a importância de se monitorar a microbiota do trato intestinal e do ambiente de cultivo a fim de se determinar a eficiência de colonização e as alterações na comunidade microbiana ocasionadas pela adição de microrganismos probióticos (Merrifield et al, 2010; Verscheure et al., 2000).

Tais mecanismos contribuem para a modulação positiva da resposta imune dos organismos, ativando a produção de células e substâncias que atuam nos processos de defesa celular e humoral. Neste sentido, Rengpipat et al. (2000) e Gullian et al. (2004) verificaram aumento na resposta imune de *L. vannamei* e *Penaeus monodon* contra bactérias do gênero *Vibrio*. Da mesma forma, Silva et al. (2013) verificaram redução nas concentrações de *Vibrio* no hepatopâncreas de larvas e pós-larvas de *L. vannamei* após aplicação de probióticos do gênero *Bacillus* sp.

As bactérias do gênero *Vibrio* spp. são consideradas patógenos oportunistas ou secundários. Tais bactérias estão naturalmente presentes no ambiente de cultivo, e são altamente eficientes em se beneficiar de mudanças e ocupar nichos ecológicos relacionados ao uso da água como ambiente de cultivo em sistemas de aquicultura (Skjermos & Vadstein, 1999). Normalmente, desencadeiam enfermidade em decorrência de condições de estresse, tais como baixas concentrações de oxigênio e

altas densidades de estocagem (Nunes & Martins 2002; Brown et al., 2012). Os surtos de vibriose são os exemplos mais conhecidos (Kimes et al., 2012; Phippen et al., 2016), sendo uma das enfermidades responsáveis por mortalidades massivas de camarões produzidos em diversas localidades (Baticados et al. 1990; Soto-Rodriguez et al. 2012). Entre as espécies de *Vibrio* que desencadeiam enfermidades, atenção especial tem sido dada a *Vibrio parahemolyticus*. Tal espécie foi descrita como o principal agente etiológico causador da necrose aguda do hepatopâncreas (AHPND) ou "Early Mortality Syndrome (EMS) (Tran et al., 2013), e é causa de mortalidades massivas de camarões peneídeos em países como China, Vietnam, Malásia, Tailândia, México e Filipinas (Hirono et al., 2017). AHPND surge tipicamente durante os primeiros 20 a 30 dias de produção, geralmente na fase de berçário. Neste contexto, a eficácia da aplicação de probióticos contra a infecção por *V. parahemolyticus* pode ser confirmada por diversas pesquisas (Moriarty 1998; Gatesouppe 1999; Vallamil et al. 2003; Li 2008; Souza et al. 2013; Krummenauer et al. 2013; Aguilera-Rivera et al. 2014).

Os probióticos também podem exercer efeito sobre os microrganismos presentes no ambiente aquático (Verschuere et al. 2000). A administração destes manteve melhores concentrações de oxigênio dissolvido, amônia e ácido sulfídricoda água de cultivo de camarões no estudo realizado por Banerjee et al. (2010). Silva et al (2013) verificaram diminuição na densidade de Vibrios presente na água com a aplicação de probióticos. Também atuam auxiliando na decomposição da matéria orgânica (Gatesoupe 1991; Fullet 1992; Watson et al. 2008; Wang & He 2009), justificando assim sua aplicação durante a preparação da água de cultivo que precede a estocagem dos camarões.

Sanz (2014) verificou que a metodologia convencional de cloração no preparo da água não foi eficiente no combate a *Vibrio parahemolyticus*, e esta ineficiência foi atribuída ao rápido tempo de replicação da espécie (8-12 min), e ao fato de que o cloro diminui a diversidade da comunidade microbiana. De Schryver et al. (2014) também argumentam que os métodos de desinfecção da água pré estocagem pode contribuir para a dispersão de *V. parahemolyticus* ao invés de combater o patógeno. Por outro lado, Mohapatra et al. (2013) e Hu et al. (2016) observaram que a aplicação de probióticos com *Bacillus* em sua composição contribuiu para o aumento na composição e diversidade de espécies microbianas responsáveis pela manutenção na qualidade da água.

Neste sentido, Defoirdt et al. (2011) e Defoirdt (2016) sugerem uma abordagem que inclua o patógeno, hospedeiro e o ambiente como uma solução de prevenção a doenças que seja efetiva a longo termo. De Schryver et al. (2014) e De Schryver & Vadstein (2014) inferem que o manejo da comunidade microbiana dos sistemas de cultivo pode ser um ponto chave para mitigar os riscos de doenças bacterianas, especialmente surtos de vibriose.

Tratando-se de comunidade microbiana presente no ambiente de cultivo, pode-se sugerir a administração de probióticos em sistemas que utilizam a tecnologia dos bioflocos, visto que este é um sistema de produção que estimula o aparecimento de uma comunidade microbiana. Esses bioflocos são agregados compostos por fitoplâncton, zooplâncton, protozoários, bactérias e detritos (Burford et al. 2004). A biota bacteriana é responsável pela reciclagem de resíduos, aumentando a eficiência alimentar, crescimento, sobrevivência e resistência a doenças dos organismos cultivados (McIntosh 2001; Wasielesky et al. 2006; Avnimelech 2007)

A comunidade microbiana no sistema de bioflocos pode ser operada de diferentes maneiras, de modo que este manejo influenciará diretamente na composição e densidade bacteriana e, consequentemente, nos meios de remoção de compostos nitrogenados do sistema. Um sistema quimioautotrófico é baseado na atuação predominante de bactérias nitrificantes, as quais oxidam amônia a nitrito e, nitrito a nitrato utilizando carbono inorgânico. Este modo autotrófico (i.e. quimioautotrófico) de operar o sistema de bioflocos pode ser resultado a partir de condições que possibilitem o crescimento de bactérias nitrificantes. Por não necessitarem de carbono orgânico em seus processos, ambientes com a predominância de bactérias autotróficas apresentam baixas relações C/N (menores que 10), proporcionando uma eficiente conversão de nitrogênio pela comunidade bacteriana predominantemente autotrófica (Ebeling, 2006; Ray & Lotz, 2014).

A remoção do nitrogênio a partir da assimilação do mesmo ocorre em um sistema que é operado heterotroficamente. Um sistema de bioflocs heterotrófico é alcançado a partir da adição diária de fontes de carbono, de modo que a relação C/N seja de pelo menos 10. Neste sistema, bactérias heterotróficas assimilam o nitrogênio amoniacal para produzir biomassa bacteriana, utilizando para isto, carbono orgânico como fonte de energia. (De Schryver et al., 2008). A produção de biomassa neste processo é relativamente mais alta quando comparada à conversão autotrófica (Ebeling et al., 2006).

No sistema de bioflocos, outros componentes benéficos aos camarões podem ser mantidos. Por exemplo, diversos microrganismos constituintes dos bioflocos produzem substâncias que apresentam ação preventiva e protetora contra infecções por *Vibrio* sp quando ingerida pelos organismos cultivados (De Schryver et al., 2008), oferecendo,

vantagem probiótica na prevenção e combate a enfermidades. Por exemplo, Ferreira et al. (2015) isolaram, a partir de um sistema de bioflocos, uma espécie do gênero *Bacillus* sp., a qual apresentou atividade probiótica e promoveu a redução da densidade de *Vibrio* na água.

É crescente o número de estudos que abordam os efeitos da adição de probióticos sobre os camarões cultivados em sistema de bioflocos. Krummenauer et al. (2014) e Aguilera-Rivera et al. (2014) obtiveram redução de lesões no hepatopâncreas de *L. vannamei* com a aplicação de probióticos após surto de vibriose em sistema heterotrófico durante a fase de engorda. Resultados semelhantes foram observados por Vogeley et al. (2010) e Santos et al. (2013) em sistema BFT durante a fase de berçário e com a aplicação de probiótico. Sugere-se que a aplicação de probióticos pode maximizar seu potencial benéfico quando associados aos microrganismos constituintes no sistema BFT, produzindo um efeito sinérgico entre o probiótico e os bioflocos e criando uma vantagem deste sistema na prevenção de doenças, e na manutenção dos parâmetros de qualidade da água em níveis adequados (Ziaei-Nejad et al. 2006; Chiu et al. 2007; Aguilera-Rivera et al. 2014).

No entanto, os mecanismos de atuação, bem como os efeitos da adição de bactérias externas em diferentes dosagens e frequências em um meio relativamente rico em microrganismos ainda não são claros. Para isto faz-se necessária à aplicação de técnicas que possibilitem a identificação e quantificação desses microrganismos, a fim de que se possam criar medidas de manejo aplicáveis e efetivas a todas as fases que envolvem o processo produtivo.

A maior parte das técnicas de biologia molecular que são cultivo-independente são baseadas na amplificação de ácidos nucleicos, e apresentam resultados qualitativos ou semi-quantitativos. Nestes casos, não é possível visualizar células individualmente e/ou avaliar a distribuição especial de organismos agregados ou não. No entanto, a hibrização *in situ* fluorescente (FISH), a qual é uma técnica molecular que permite a identificação, visualização e contagem direta e individual de bactérias sem a necessidade de cultivo prévio. Essa técnica é baseada na utilização de sondas complementares ao RNA ribossomal das bactérias. As sondas podem ser desenhadas para serem específicas em diferentes níveis taxonômicos, isto é, para reconhecerem apenas uma espécie, ou grandes grupos bacterianos como domínios. Alguns estudos abordam sua utilização na aquicultura em diferentes aspectos (Cytryn et al. 2006; Garcia & Olmos 2007; Pereira et al. 2011 Balcázar et al. 2010;), com destaque para o estudo realizado por Del'Duca et al (2013) que utilizaram a técnica de FISH para avaliar a presença e a eficiência de uma bactéria probiótica na água e no trato intestinal de *Oreochromis niloticus*. Os resultados indicaram que a técnica FISH é uma ferramenta potencial na caracterização da dinâmica de bactérias probióticas, bem como sua eficiência no controle de bactérias patogênicas.

Levando em conta todos os benefícios já confirmados e descritos em relação à utilização dos probióticos, e considerando a carência de informações sore a interação e os mecanismos de ação dessas bactérias com os microrganismos componentes do sistema BFT, o presente trabalho objetiva avaliar o efeito da aplicação de probióticos em diferentes fases no processo de produção de *L. vannamei* em sistema de bioflocos. Para tanto, a tese foi dividida em 5 capítulos que objetivaram responder cinco perguntas específicas, de acordo com os objetivos descritos.

OBJETIVOS:

Objetivo Geral:

A presente tese tem como objetivo utilizar diferentes abordagens da aplicação de probióticos, a fim de se avaliar a influência da adição destas bactérias na densidade bacteriana, na melhoria do desempenho zootécnico e na resistência a doenças do camarão *L. vannamei* cultivado em sistema de bioflocos.

Objetivos Específicos:

- Definir qual é o tempo adequado de aplicação do probiótico pós-cloração/decloração da água simulando a fase de preparação que precede a estocagem dos camarões, e verificar a densidade bacteriana de *Bacillus* e *Vibrio* neste processo;

- Avaliar a influência de diferentes modos operacionais do sistema de bioflocos com e sem aplicação de probióticos na capacidade de controle contra infecção experimental com a cepa de *Vibrio parahemolyticus* como agente etiológico de AHPND na fase de berçário em bioflocos e água clara;

- Comparar o efeito da aplicação de probióticos no desempenho zootécnico e na redução de lesões histopatológicas em *L. vannamei* produzido em sistema de bioflocos e em água clara com surto de vibriose na fase de engorda;

- Comparar a eficácia de um probiótico comercial e avaliar a abundância de bactérias do gênero *Vibrio* e *Bacillus* no trato intestinal de *Litopenaeus vannamei* e cultivado em sistema de bioflocos e em água clara;

- Comparar o efeito dose-resposta de diferentes concentrações de aplicação de probiótico comercial no desempenho zootécnico, sobrevivência e densidade bacteriana de *Bacillus subtilis*, *B. subtilis* – complex e *Vibrio* sp. na água e no trato intestinal de *L. vannamei* cultivado em sistema de bioflocos;

REFERÊNCIAS BIBLIOGRÁFICAS:

Alday-Sanz, V. 2014. APHNS: Observations and doubts. In: Browdy, C.L., Hargreaves, J., Tung H. Avnimelech, Y .2014. Proceedings of the Biofloc Technology and Shrimp Disease Workshop. December 9-10, 2013, Ho Chi Minh City, Vietnam. The Aquaculture Engineering Society, Copper Hill, VA USA.

Aguilera-Rivera, D, Prieto-Davó, A., Escalante, K., Chavez C., Cuzón G., Gaxiola, G. 2014. Probiotic effect of FLOC on *Vibrios* in the Pacific White shrimp *Litopenaeus vannamei*. Aquaculture, 424-425, 415-419.

Akhter, N., Wu, B., Memon, A.M., Mohsin, M., 2015. Probiotics and prebiotics associated with aquaculture: A review. Fish and Shellfish Immunology, 45, 733-741.

Attramadal, K.J.K., Salvesen, I., Xue, R.Y., Øie, G., Storseth, T.R. 2012. Recirculation as a possible microbial control strategy in the production of marine larvae. Aquac Eng 46, 27–39.

Avnimelech, Y. 1999. Carbon/nitrogen ratio as a control element in aquaculture systems. Aquaculture, 176, 227-235.

Avnimelech, Y. 2007. Feeding with microbial flocs by tilapia in minimal discharge bio-flocs technology ponds. Aquaculture, 264, 140-147.

Azim, M.E., Little, D.C. 2008. The biofloc technology (BFT) in indoor tanks: Water quality, biofloc composition, and growth and welfare of Nile tilapia (*Oreochromis niloticus*). Aquaculture, 283, 29-35.

Balcázar, J., Lee, N., Pintado, J., Planas, M. 2010. Phylogenetic characterization and in situ detection of bacterial communities associated with seahorses (*Hippocampus guttulatus*) in captivity. Systematic and Applied Microbiology, 33, 71-77.

Balcazar, J.L., Rojas-Luna, T., Cunningham, D.P. 2007. Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (*Litopenaeus vannamei*) following immersion challenge with *Vibrio parahaemolyticus*. Journal of Invertebrate Pathology, 96,147-150.

Banerjee, S., Khatoon, H., Shariff, M., Yusoff, F. 2010. Enhancement of *Penaeus monodon* shrimp postlarvae growth and survival without water exchange using marine *Bacillus pumilus* and periphytic microalgae. Fish Science, 76, 481–487.

Baticados, M., Cruz-Lacerda, M.C., Durendez-Fernandez, RC; Gacutan, R.Q., Lavilla-Pitogo, C.R., Liopo, G.D. 1990. "Disease of Penaeid Shrimps of the Philippines." Aquaculture Extension Manual No. 16. SEAFDC, The Philippines.

Burford, M.A., Thompson, P.J., McIntosh, R.P., Bauman, R.H, Pearson, D.C. 2004. The contribution of flocculated material to shrimp (*Litopenaeus vannamei*) nutrition in a high-intensity, zero-exchange system. Aquaculture, 232(1-4), 525–537.

Cardoso, E. 2007. Panorama da pisicultura mineira e perspectivas para o setor. Anais do III Seminário de aqüicultura, maricultura e pesca aquicultura. Belo Horizonte, MG, 39-52.

Castex, M., Chim, L., Pham, D., Lemaire P., Wabete, N., Nicolas, J.L, Schimidely P., C Mariojouls. 2008. Probiotic *P. acidilactici* application in shrimp *Litopenaeus stylirostris* culture subject to vibriosis in New Caledonia. Aquaculture, 275,182-193.

Chiu, C., Guu., Y., Liu, C., Pan, T., Cheng, W. 2007. Immune response and gene expression in white shrimp *Litopenaeus vannamei*, induced by *Lactobacillus plantarum*. Fish and Shellfish Imunology, 23, 364-377.

Crab, R., Lambert, A., Defoirdt, T., Bossier, P., Verstraete, W., 2010. The application of bioflocs technology to protect brine shrimp (*Artemia franciscana*) from pathogenic *Vibrio harveyi.* Journal of Applied Microbiology 109, 1643-1649.

Cutting, S.M. 2011. Bacillus probiotics. Food Microbiology, 28, 214–220.

Cytryn, E., Minz, D., Gieseke, A., Rijn, J. 2006. Transient development of filamentous Thiothrix species in a marine sulfide oxidizing, denitrifying fluidized bed reactor. FEMS Microbiology Letters, 256, 22-29.

D'alvise, P.W., Lillebo, S., Wergeland, H.I., Gram, L., Bergh, O. 2013. Protection of cod larvae from vibriosis by *Phaeobacter spp.*: A comparison of strains and introduction times. Aquaculture, 384-387, 82-86.

Defoirdt T., Boon N., Sorgeloos P., Verstraete W., Bossier P. (2007) Alternatives to antibiotics to control bacterial infections- luminescent vibriosis in aquaculture as an example. *Trends Biotechnol* **25**, 472-479.

Defoirdt T., Sorgeloos P., Bossier P. (2011) Alternatives to antibiotics for the control of bacterial disease in aquaculture. *Current Opinion in Microbiology* **14**, 251-258.

Defoirdt T. (2016) Implications of Ecological Niche Differentiation in Marine Bacteria for Microbial Management in Aquaculture to Prevent Bacterial Disease. *PLOS Pathogens*, **http://dx.doi.org/10.1371/journal.ppat.1005843**.

De Schryver, P., Crab, R., Defoirdt, T., Boon, N., Verstraete, W. 2008. The basics of bio-flocs technology: The added value for aquaculture. Aquaculture, 277(3-4). 125–137.

De Schryver, P., Vadstein, O., 2014. Ecological theory as a foundation to control pathogenic invasion in aquaculture. The ISME Journal, 1-9.

De Schryver, P., Defoirdt, T., Sorgeloos, P. 2014. Early Mortality Syndrome Outbreaks: A Microbial Management Issue in Shrimp Farming? PLoS Pathog, 10(4), 1-2.

Del'duca, A., Cesar, D.E., Diniz, C.G., Abreu, P.C. 2013. Evaluation of the presence and efficiency of potential probiotic bacteria in the gut of tilapia (*Oreochromis niloticus*) using the fluorescent in situ hybridization technique. Aquaculture, 388-391, 115-121.

Del`Duca, A., Cesar, D.E., Abreu, P.C., 2015. Bacterial community of pond's water, sediment and in the guts of tilapia (*Oreochromis niloticus*) juveniles characterized by fluorescent *in situ* hybridization technique. Aquaculture Research 46(3), 707-715.

Ebeling, J.M., Timmons, M.B., Bisogni, J.J. 2006. Engineering analysis of the stoichiometry of hotoautotrophic, autotrophic, and heterotrophic control of ammonia-nitrogen in aquaculture production systems. Aquaculture, 257, 346–358.

FAO/WHO. 2002. Guidelines for the Evaluation of Probiotics. in Food. Report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food, London Ontario, Canada, 1–11.

Farzanfar, A. 2006. The use of probiotics in shrimp aquaculture. FEMS Immunology and Medical Microbiology, 48, 149–158.

Ferreira, G.S., Bolívar, N.C., Pereira, S.A., Guertler, C., Vieira, F.N., Mouriño, J.L.P., Seiffert, W.Q., 2015. Microbial biofloc as a source of probiotic bacteria for the culture of *Litopenaus vannamei*. Aquaculture 448, 273-279.

Food Agriculture Organization. 2016. The State of World Fisheries and Aquaculture. Roma: FAO.

Fuller, R., 1989. A review: probiotic in man and animals. Journal Applied Environmental Microbiology, 63, 1034-1039.

Fuller, R. History and development of Probiotics. 1992. In: Fuller, R. (Ed.). Probiotics: the scientific basis. New York: R. Chapman & Hall, 1:1-8.

Garcia, A., OLMOS, J. 2007. Quantification by fluorescent in situ hybridization of bacteria associated with *Litopenaeus vannamei* larvae in Mexican shrimp hatchery. Aquaculture, 262, 211-218.

Gatesoupe, F.J. 1991. The effect of three strains of lactic bacteria on the production rate of rotifers, *Brachionus plicatilis*, and their dietary value for larval turbot, *Scophthalmus maximus*. Aquaculture, 96, 335–342.

Gatesoupe, F.J. 1999. The use of probiotics in aquaculture. Aquaculture,180, 147-165.

Gullian, M., Thompson, F., Rodriguez, J. 2004. Selection of probiotic bacteria and study of their immunostimulatory effect in *Penaeus vannamei*. Aquaculture, 233, 1-14.

Hai, N.G., Fotedar, R., Buller, N. 2007. Selection of probiotics by various inhibition test methods for use in the culture of western king prawns, *Penaeus latisulcatus* (Kishinouye). Aquaculture, 272, 231-239.

Hamza, F., Kumar, A.R., Zinjarde, S., 2016. Antibiofilm potential of a tropical marine *Bacillus licheniformis* isolate: role in disruption of aquaculture associated biofilms. Aquaculture Research 47(8), 2661-2669.

Hirono, I., Tinwongger, S., Nochiri, Y., & Kondo, H. (2016). Latest research on acute hepatopancreatic necrosis disease (AHPND) of penaeid shrimps. In R. V. Pakingking Jr., E. G. T. de Jesus-Ayson, & B. O. Acosta (Eds.), Addressing Acute Hepatopancreatic Necrosis Disease (AHPND) and Other Transboundary Diseases

for Improved Aquatic Animal Health in Southeast Asia: Proceedings of the ASEAN Regional Technical Consultation on EMS/AHPND and Other Transboundary Diseases for Improved Aquatic Animal Health in Southeast Asia, 22-24 February 2016, Makati City, Philippines (pp. 3-10). Tigbauan, Iloilo, Philippines: Aquaculture Department, Southeast Asian Fisheries Development Center.

Hu, X., Cao, Y., Wen, G., Zhang, X., Xu, Y., Xu, W., Xu, Y., Li, Z., 2016. Effect of combined use of *Bacillus* and molasses on microbial communities in shrimp cultural enclosure systems. doi:10.1111/are.13101

Joborn, A., Olsson, J., Westerdahl, A., Conway, P.L., Kjelleberg, S. 1997. Colonization in the fish intestinal tract and production of inhibitory substances in intestinal mucus and faecal extracts by *Carnobacterium sp*.

Kimes N.E., Grim C.J., Johnson W.R., Hasan N.A., Tall B.D., Kothary M.H., Kiss H., Munk, A.C., Tapia R., Green L., Detter C., Bruce D.C., Brettin T.S., Colwell R.R., Morris P.J. (2012) Temperature regulation of virulence factors in the pathogen *Vibrio coralliilyticus*. *ISME* **6**, 835-846.

Kiron, V. 2012. Fish immune system and its nutritional modulation for preventive health care. Animal Feed Science and Technology, 173, 111–133.

Kozasa, M. 1986. Toyocerin *Bacillus toyoi* as growth promotor for animal feeding. Microbiol. Aliment. Nutr. 4, 121–135.

Krummenauer, D., Samocha, T., Poersch, L., Lara, G., Wasielesky, W. 2014. The Reuse of Water on the Culture of Pacific White Shrimp, *Litopenaeus vannamei*, in BFT System. Journal of the Aquaculture Society, 45, 3-14.

Krummenauer, D., Poersch, L., Romano, L.A., Lara, G.R., Encarnação, P., Wasielesky, W., 2014.The Effect of Probiotics in a *Litopenaeus vannamei* Biofloc Culture System Infected with *Vibrio parahaemolyticus.* Journal of applied Aquaculture 26, 370-379.

Lazado, C.C., Caipang, C.M.A., Estante E.G., 2015. Prospects of host-associated microorganisms in fish and penaeids as probiotics with immunomodulatory functions. Fish and Shellfish Immunology 45, 2-12.

McIntosh, R.P. 2001. Changing paradigms in shrimp farming: establishment of heterotrophic bacterial communities. Global Aquaculture Advocate, February: 53–58.

Mohapatra, S., Chakraborty, T., Kumar, V., Deboeck, G., Mohanta, K.N. 2013. Aquaculture and stress management: a review of probiotic intervention. Journal of animal physiology and animal nutrition, 97, 405-430.

Moriarty, D.J.W. Control of luminous *Vibrio* species in penaeid aquaculture ponds. 1998. Aquaculture, 164, 351-358.

Nimrat, S., Suksawat, S., Boonthai, T., Vuthiphandchai, V. 2012. Potential *Bacillus* probiotics enhance bacterial numbers, water quality and growth during early development of white shrimp (*Litopenaeus vannamei*). Veterinary Microbiology, 159, 443-450.

Nunes, A.J.P., Martins P.C. 2002. Avaliando o estado de saúde de camarões marinhos na engorda. Revista Panorâma da aquicultura, 72, 23-33.

Pereira, C., Salvador, S., Arrojado, C., Silva, Y., Santos, A., Cunha, A., N Gomes, A Almeida. 2011. Evaluating seasonal dynamics of bacterial communities in

marine fish aquaculture: a preliminary study before applying phage therapy. Journal of Environmental Monitoring, 13, 1053-1058.

Phippen B.L., Ivanina A.V., Sokolova I.M., Oliver J.D. (2016) *Vibrio coralliilyticus* causes rapid immune response and mortality in the Eastern oyster, *Crassostrea virginica*, after exposure to anoxia. Oral Presentation, Vibrio Conference, Roscoff, France.

Ray, A.J., Lotz, J.M. 2014. Comparing a chemoautotrophic-based biofloc system and threeheterotrophic-based systems receiving different carbohydrate sources. Aquacultural Engineering, 63, 54–61.

Rengpipat, S., Rukpratanporn, S., Piyatiratitivorakul S., Menasaveta, P. 2000. Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus* S11). Aquaculture, 191, 271-288.

Sapcharoen, P., Rengpipat, S. 2013. Effects of the probiotic *Bacillus subtilis* (BP11 and BS11) on the growth and survival of Pacific white shrimp, *Litopenaeus vannamei*. 2013. Aquaculture Nutrition, 19, 946-954.

Sha, Y., Wang, L., Liu, M., Jiang, K., Xin, F., Wang, B., 2016. Effects of lactic acid bacteria and the corresponding supernatant on the survival, growth performance, immune response and disease resistance of *Litopenaeus vannamei*.Aquaculture 452, 28-36.

Silva, E.F., Soares M.A., Calazans N.F., Vogeley J.L., Valle B.C., Soares, R., Peixoto, S. 2013. Effect of probiotic (*Bacillus spp*.) addition during larvae and postlarvae culture of the white shrimp *Litopenaeus vannamei*. Aquaculture Research, 44, 13-21.

Skjermo, J., Vadstein, O., 1999. Techniques for microbial control in the intensive rearing of marine larvae. Aquaculture 177, 333–343.

Soto-Rodriguez, S.A., Gomez-Gil, B., Lozano, R., Rio-Rodriguez, R., Dieguez, A.L., Romalde, J.L. 2012. Virulence of *Vibrio harveyi r*esponsible for the ''Bright-red'' Syndrome in the Pacific white shrimp *Litopenaeus vannamei*. Journal of Invertebrate Pathology, 109, 307-317.

Souza, D.M., Suita, S.M., Leite, F.P.L., Romano L.A., Wasielesky W., BALLESTER E.L.C., 2012. The use of probiotics during the nursery rearing of the pink shrimp *Farfantepenaeus brasiliensis* (Latreille, 1817) in a zero exchange system. Aquaculture Research, 43(12), 1828–1837.

Tran L., Nunan L., Redman R.M., Mohney L.L., Pantoja C.R., Fitzsimmons K., Lightner D.V. (2013) Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. *Diseases of Aquatic Organisms* **105**, 45-55.

Unesco, 1983. Chemical methods for use in marine environmental monitoring. Manual and Guides 12, Intergovernamental Oceanographic Commissiony. Paris, France.

Verschuere, L., Rombaut, G., Sorgeloos, P. 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiology and Molecular Biology Reviews 64, 655-671.

Villaseñor, L., Castellano-Cervantes T., Gomez-Gil, B., Carrillo-Garcia A.E., CAMPA-Cordova A.I, Ascencio F. 2013. Probiotics in the intestinal tract of juvenile whiteleg shrimp *Litopenaeus vannamei*: modulation of the bacterial community. World journal of microbiol. Biotechnol, 29, 257-265.

Vogeley, F. 2010. Berçário experimental de camarões marinhos em sistema heterotrófico com o uso de probiótico. Dissertação (Mestrado em Recursos Pesqueiros e Aquicultura). Universidade Federal Rural de Pernambuco.

Wang, Y., Zhenli, H. 2009. Effect of probiotics on alkaline phosphatase activity and nutrient level in sediment of shrimp, *Penaeus vannamei*, ponds. Aquaculture, 287, 94-97.

Wang, C., Pan, L.Q., Zhang, K.Q., Xu, W.J., Zhao, D.H., Mei, L., 2016. Effects of different carbon sources addition on nutrition composition and extracellular enzymes activity of bioflocs, and digestive enzymes activity and growth performance of *Litopenaeus vannamei* in zero-exchange culture tanks. Aquaculture Research, 47(10), 3307-3318.

Wasielesky, W., Atwood, H., Stokes, A., Browdy, C.L. 2006. Effect of natural production in a zero exchange suspended microbial floc based super-intensive culture system for white shrimp *Litopenaeus vannamei*. Aquaculture, 258, 396-403.

Zhou, X., Wang, Y., Li, W. 2009. Effect of probiotic on larvae shrimp (*Penaeus vannamei*) based on water quality, survival rate and digestive enzyme activities. Aquaculture, 287, 349-353.

Ziaei-Nejad, S., Rezaei, M.H., Takami G.A., Lovett, D.L., Mirvaghefi, A.R., Shakouori, M. 2006. The effect of *Bacillus spp*. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. Aquaculture, 252, 516-524.

Zokaeifar, H., Babaei, N., Saad, C.R., Kamarudin, M.S., Sijam, K., Balcazar. J.L. 2014. Administration of *Bacillus subtilis* strains in the rearing water enhances the water

quality, growth performance, immune response, and resistance against *Vibrio harveyi* infection in juvenile white shrimp, *Litopenaeus vannamei*. Fish & Shellfish Immunology, 36, 68-74.

CAPÍTULO 1

Water disinfection prior shrimp stocking: Variations in bacterial density and effectiveness of probiotic application

Running head: Probiotic application after water disinfection

*Artigo a ser submetido à revista Aquaculture International

Observação: Tabelas e figuras foram incluídas no corpo do texto para facilitar a leitura da tese, além de um resumo em português.

Desinfecção da água que precede a estocagem dos camarões: Variações na densitade bacteriana e eficácia da apliação de probióticos

Resumo

O presente experimento (96 horas) foi realizado com o objetivo de avaliar o processo de desinfecção da água que precede a estocagem dos camarões no início de um ciclo de produção. Adicionalmente, de avaliar a abundância bacteriana de um probiótico comercial composto por *Bacillus* adicionado à água após a cloração/decloração da água. Além disso, verificar se as bactérias probióticas que colonizam a água são capazes de ocupar nichos "vazios" criados pós desinfecção através da influência que podem exercer sobre a densidade de *Vibrio* sp. O experimento foi composto de 4 tratamentos, 3 réplicas cada: -**T1** No ProW (sem probiotico); -**T2** ProW 0 – adição do probiótico imediatamente após cloração/decloração da água; -**T3** Pro W 12 – adição do probiótico 12 horas após cloração/decloração da água; and - **T4** ProW 24- adição do probiótico 24 horas após cloração/decloração da água. Densidades microbianas e bacterianas foram quantificadas em 0, 12, 24, 48, 72 e 96 horas após a cloração/ decloração da água. A densidade microbiana total foi significativamente mais elevada nos tratamentos com aplicação de probiótico, em 24 horas pós a cloração. Da mesma forma, a densidade bacteriana de *Bacillus* sp. foi maior em 24 horas pós cloração da água. A porcentagem de *Bacillus* sp., *B. subtilis* e *B. subtilis* – complex foi significativamente maior em T1 (sem ProW), indicando a presença natural destas bactérias na água. Não foram detectadas diferenças significativas na densidade de *Vibrio* sp., as quais foram mantidas menores que *Bacillus* ao longo do experimento. Desta forma, a administração dos probióticos é recomendada imediatamente após a desinfecção da água. Recomenda-se também que a fertilização com fontes de carbon orgânico iniciem-se 24 horas pós cloração/decloração da água. Através da adoção de tais procedimentos, torna-se possível obter-se um suplemento de nutrientes para o estabelecimento de uma comunidade microbiana estável ao longo do ciclo de cultivo.

Abstract

This 96-hours study aimed to evaluate the disinfection prior stocking shrimp in the beginning of a production cycle. Additionally, to evaluate bacterial abundance of a *Bacillus* probiotic mixture after water chlorination. Moreover, to verify if the probiotic colonization of the water is able to occupy the "empty" niches by the disinfection by influencing on *Vibrio* sp. abundance. The experiment was composed of 4 treatments, 3 replicates each: -**T1** No ProW (without probiotic); -**T2** ProW 0 – probiotic application immediately after water disinfection; -**T3** Pro W 12 – probiotic application 12 hours after water disinfection; and - **T4** ProW 24- probiotic application 24 hours after water disinfection. Microbial and bacterial density was assessed at 0, 12, 24, 48, 72 and 96 hours post water chlorination. Microbial density was significantly higher in the treatments with probiotic application, at 24 hours after disinfection. Likewise, bacterial density of *Bacillus* sp. was higher at 24 hours post chlorination. The percentage of Bacillus sp, B. subtilis and B. subtilis – complex was higher in T1 (without ProW), indicating the natural presence of these bacteria in the water. No differences were found in the density of Vibrio, which was maintained low throughout the trial. It is recommended the administration of the probiotic immediately after water disinfection. Organic carbon fertilization starts 24 hours after disinfection practice. By adopting such procedures, it would be possible to have a reduction in *Vibrio* sp proliferation, as well as a supplement for the establishment of a stable microbial community along the culturing cycle.

Key words: *Bacillus;* chlorination; microbial density; probiotics; *Vibrio;* water disinfection

Introduction

The intensification of aquaculture industry has been associated, in most of the cases, to the high incidence of infectious diseases during the last decades. Besides, it is also one of the factors that can negatively affect the future prospects for this sector due to the high mortality rates (FAO 2016). One of the main routes of pathogen proliferation is through the water. They can be transmitted by water, fish to fish, by food and also by vectors (agent for transferring a gene from one organism to another) (Yanong 2003).

In this context, since aquaculture water has been recognized as the main carrier of microbial pathogens, water quality is one of the most important parameters to be managed during the production cycle, in order to prevent the risk of disease (Vaz-Moreira et al. 2014). Such risks can be reduced by disinfection of initial fill water, prior stocking the organisms. Water treatment methods such as UV-C radiation (direct photolysis), ozonation (O3) and chlorination (Cl2) are commonly applied (Jorquera et al. 2002) as disinfection practice. For instance, Garrido-Pereira et al. (2013) observed a decrease in probiotic bacteria (Bacillus sp.) when submitted culturing water to UV raditation treatment. Chlorination has been widely used for microbiological control in seawater in some intensive culturing systems. It is reported to have a high efficiency as a disinfectant against virus and bacteria, and is readily available at low cost (White 1992; Pascho et al. 1995). Considering that, disinfection practice has a particular relevance to avoid or minimize the spreading of pathogens (Leal et al. 2016), including bacteria from genus *Vibrio* sp.

Vibrio sp. are gram negative bacteria that can occur naturally in marine and estuarine environments. They can also be part of the intestinal bacterial community of *L. vannamei* and even some species are probiotics (Verschuere et al. 2000; Lakshmi et

al. 2013; Liu et al. 2015; Huang et al. 2016). However, most species are considered an opportunistic bacteria and a potential pathogen, causing vibriosis outbreaks and huge production losses (Saulnier et al. 2000). Vibriosis is also considered one of the most prevalent diseases that affect cultured aquaculture organisms worldwide (Chatterjee and Haldar 2012).

Although disinfection practices are reported to be efficient on controlling potential pathogen bacteria, it is important to consider that this process removes the naturally occurring microbial community that traditionally evolves into an essential component for optimal shrimp production (Anderson et al. 1987). De Schryver et al. (2014) suggest that the ecosystem disturbance caused by the current practice of disinfecting ponds to remove potential pathogens or their carriers prior to stocking shrimp post larvae most probably does more harm than good. The authors affirm that after disinfection, the increase in nutrient availability combined with a destabilized and impoverished microbial community (and a consequent lack of competition) favors fast-growing bacteria (such as many pathogenic *Vibrio* spp.) in recolonizing the environment (Attramadal et al. 2012). Moreover, disinfection practices decrease the bacterial density (Defoirdt 2016). In this sense, the use of probiotics during this initial period in the water can represent one hypothesis to immediately colonize the disinfected water, and possibly control or minimize growth of potential harmful pathogens.

Probiotics are live microorganisms that provide health benefits to the host when administered in adequate levels (Verschuere et al 2000). They can be applied via water or feed (Moriarty 1998; Skjermo and Vadstein 1999) either single or as a combination of strains (Villaseñor et al. 2014). Among the different bacterial species with probiotic properties, the genus *Bacillus* has been most widely applied in penaeid culture (Decamp

and Moriarty 2006; Villaseñor et al. 2013; Sapcharoen and Rengpipat 2013; Fyzul et al. 2014). Several authors report *Bacillus* sp. to have positive effects towards the culture of *L. vannamei.* Nimrat et al. (2012) observed that the use of Bacillus as a potential probiotic enhanced the number of beneficial bacteria in the water. Similarly, Venkateswara (2007) reported that probiotic bacteria can improve water quality and inhibit the pathogens in water. Kumar et al. (2016) reinforce that probiotics added to the culturing water are able to outgrow pathogenic organisms present in the environment.

De Schryver and Vadstein (2014) and De Schryver et al. (2014) suggest that microbial management strategies may be the key to minimizing the risk of vibriosis outbreaks. For this reason, strategies for disease control should focus on the entire community rather than on a single or a few pathogens (Attramadal et al. 2012). The authors also suggest stocking shrimp postlarvae in systems with a mature microbiota, as environments primarily colonized by slow-growing harmless bacteria might best guarantee the prevention of disease.

Microbially matured water systems have been developed to minimize the presence of pathogens that are able to grow fast and are consequently capable of quickly invading ''empty'' niches (De Schryver et al. 2014), created post disinfection, for instance. In this context, the application of probiotics after disinfection, followed by the organic carbon fertilizations, may contribute to create a microbially mature water represented, in this case, by the biofloc culturing system.

The Biofloc Technology (BFT) is based on the stimulation and establishment of a microbial community, which is responsible for maintaining the water quality, improve growth and survival of produced aquatic organisms (McIntosh et al. 2001; Wasielesky et al. 2006; Avnimelech 2007). It is also reported the capacity of this microbial

community to inhibit the proliferation of pathogens by competitive exclusion for food and space in the water or in the gut (Crab et al. 2010). Regarding the combination of probiotic application and organic fertilization, Hu et al. (2016) verified that the combined use of *Bacillus* and molasses as a carbon source helped to increase abundance of the microbial community. Besides, the combination was responsible for effectively inhibit pathogens, and promote the formation and development of a beneficial microbial community structure in biofloc-rich water. Ferreira et al. (2015) investigated the probiotic properties of Bacillus *sp.* isolated from biofloc-rich water and supplemented to *L. vannamei*. They verified lower number of vibrios in the water in the experimental units treated with *Bacillus*, which showed antagonism against *Vibrio harveyi*. The authors observed in vitro inhibitory characteristics of *Bacillus* against the putative pathogen *Vibrio alginolyticus* and a reduction of *Vibrio sp.* prevalence in the water when supplemented with the probiotic.

Thus, the present study aimed to simulate the phase in which preparation and disinfection of water is done, prior the stocking of shrimp to start a production cycle of *Litopenaeus vannamei* in biofloc system. Additionally, it was aimed to evaluate bacterial abundance of a commercial probiotic mixture composed by *Bacillus* species after water disinfection with chlorine.

2. Material and Methods

2.1. Probiotic Mixture and application

The commercial probiotic mixture (Sanolife-ProW® - INVE Aquaculture) used in this study is composed of *Bacillus subtilis* and *Bacillus licheniformes* strains, in a bacterial concentration of $5x10^{10}$ CFU g^{-1}. The product was added to the culture water

every 48 hours, at a concentration of 0.5 ppm or 0.5 mg L^{-1} and activated according to the manufacturer's recommendations

2.2. Experimental design

The experiment lasted 96 hours, and was carried out in 12 cylindrical tanks with 2 L effective volume, allocated to a temperature controlled room. Temperature in all tanks was maintained in the range of 27±1 °C during the experimental period. Salinity and dissolved oxygen were maintained between 30±0.5 and 6.02±0.5 mg L^{-1}. Aeration was constantly provided in each tank through an airstone by using an air blower and the light regime was set at 12 h light/12 h dark.

Each treatment was composed by three replicates, as follows:

-**T1** – No ProW (without probiotic);

-**T2** – ProW 0 – probiotic application immediately after water disinfection

-**T3** – Pro W 12 – probiotic application 12 hours after water disinfection

- **T4** – ProW 24- probiotic application 24 hours after water disinfection

The experiment intended to simulate the disinfection process prior shrimp stocking and biofloc formation. Hence, the experimental units were filled with 2 L of filtered (sand filter) natural seawater disinfected with a chlorine solution (10 ppm measured immediately after chlorination) and dechlorinated using ascorbic acid powder (1 ppm). The probiotic application followed this disinfection process. Dechlorinated municipal freshwater was used to compensate for evaporative losses and to maintain salinity throughout the experiment. Water samples were collected at 0, 12, 24, 48, 72 and 96 hours post disinfection and fixed with paraformaldehyde 2% (final concentration).

2.3. Analysis of the presence and efficiency of the commercial probiotic mixture and putative pathogenic bacteria by fluorescence in situ hybridization (FISH)

Aliquots of water samples were filtered on polycarbonate filters (Nuclepore® - 0.2 μm) and refrigerated until hybridization. *Bacillus subtillis* and *Bacillus subtillis-*complex (covers *B. licheniformes)*, components of the probiotic mixture added to the water, and putative pathogenic bacteria (*Vibrio* sp.) were identified with rRNA-targeted oligonucleotide probes (Table 1) by a fluorescence *in situ* hybridization (FISH) protocol (Del'Duca et al 2013). A negative control made with a probe with no specificity for bacteria, and a positive control with the probe for eubacteria group were used to evaluate the hybridization efficiency. All probes were labeled with the Cy3 fluorochrome. In addition to each specific probe, DAPI was used to determine the total bacterial abundance. The bacterial abundance was determined by direct counting at 1000× magnification using an epifluorescence microscope (Olympus® BX-60) equipped with the Chroma U-N41007, U-MWU2 and U-MWG2 optical filter set.

Table 1: rRNA-targeted oligonucleotide probes of different bacterial species used in this study. All probes were labelled with fluorochrome Cy3.

| Probe	Specificity	Sequence 5 – 3'	%FA*	Reference
NON	Negative Control	TAGTGACGCCGTCGA	30	Yokokawa and Nagata 2005
Bacil1	*Bacillus*	GCCGCCTTTCAATTTCGAAC	35	Ichijo et al.2010
BsubC	*B. subtilis-* complex	AAGCCACCTTTTATGTTTGA	35	Present study
Bsub	*B. subtilis*	CGTTCAAACAACCATCCGG	35	Present study
Vib519a	*Vibrio*	ACCACCTGCATGCGCTT	40	Hugget et al. 2008

*Percentage of Formamide (FA) in *in situ* Hybridization buffer

2.4. Statistical Analyses

One-way analysis of variance (ANOVA) was used to identify significant differences in bacterial abundance among treatments. The ANOVA was followed by Tukey's post hoc comparison when significant differences were found. Statistical significance was taken as P< 0.05.

4. Results

Total bacterial density in the water post disinfection with chlorine did not differ among treatments for each sampling period. However, T2 (ProW 0: applied immediately after chlorination/dechlorination) and T3 (ProW 12: applied 12 hours after chlorination/dechlorination) in 24 hours showed higher values than T1 (No ProW: without probiotic application), T2 and T3 in 12 hours (p <0.05). T3 in 24 hours was also significantly higher than T3 in 48 hours (p<0.05). In 72 hours post disinfection, bacterial abundance in T4 (ProW 24: applied 24 hours after chlorination/dechlorination) was significantly higher than T2 and T3 in 12 hours (Figure 1).
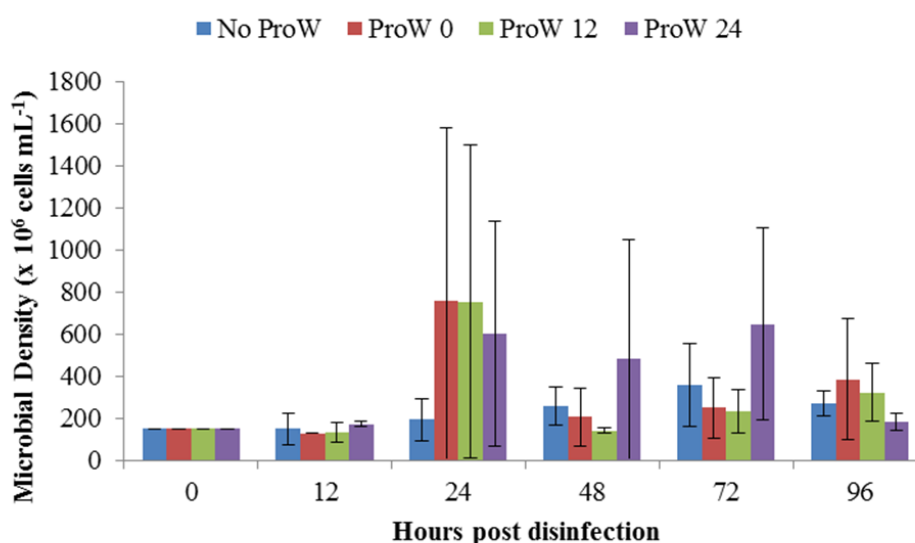
Figure 1: Total microorganisms density present in culturing water post disinfection and prior shrimp stocking with different timing of probiotic application (– **T1** – No ProW (without probiotic); -**T2** – ProW 0 – probiotic application immediately after water disinfection; -**T3** – Pro W 12 – probiotic application 12 hours after water disinfection and – **T4** – ProW 24- probiotic application 24 hours after water disinfection.

The bacterial abundance of genus *Bacillus* sp. was significantly higher at ProW 0 in 12 hours. At the same sampling timing, ProW 24 showed the lowest bacterial abundance ($P < 0.05$). When comparing bacterial densities among treatments and different sampling times, T2 and T3 were significantly higher than all treatments in 48, t2 and 96 hours post disinfection. Additionally, these treatments presented higher abundance than T1 in 24 hours ($p < 0.05$). Significantly higher bacterial density was observed in T4 in 24 hours, comparing to all treatments in 72 and 96 hours (Figure 2A).

Regarding the percentage of *Bacillus* sp. relative on the total microbial density, is was possible to observe that in 12 hours post disinfection, the treatment in which the probiotic was added immediately after bleaching (T1) presented the highest values. On the other hand, T1 showed higher percentage at 24 hours. In 48 hours post disinfection, T1, T2 and T3 presented the higher values among treatments. At 0, 72 and 96 hours no differences were detected among treatments (Figure 2B).
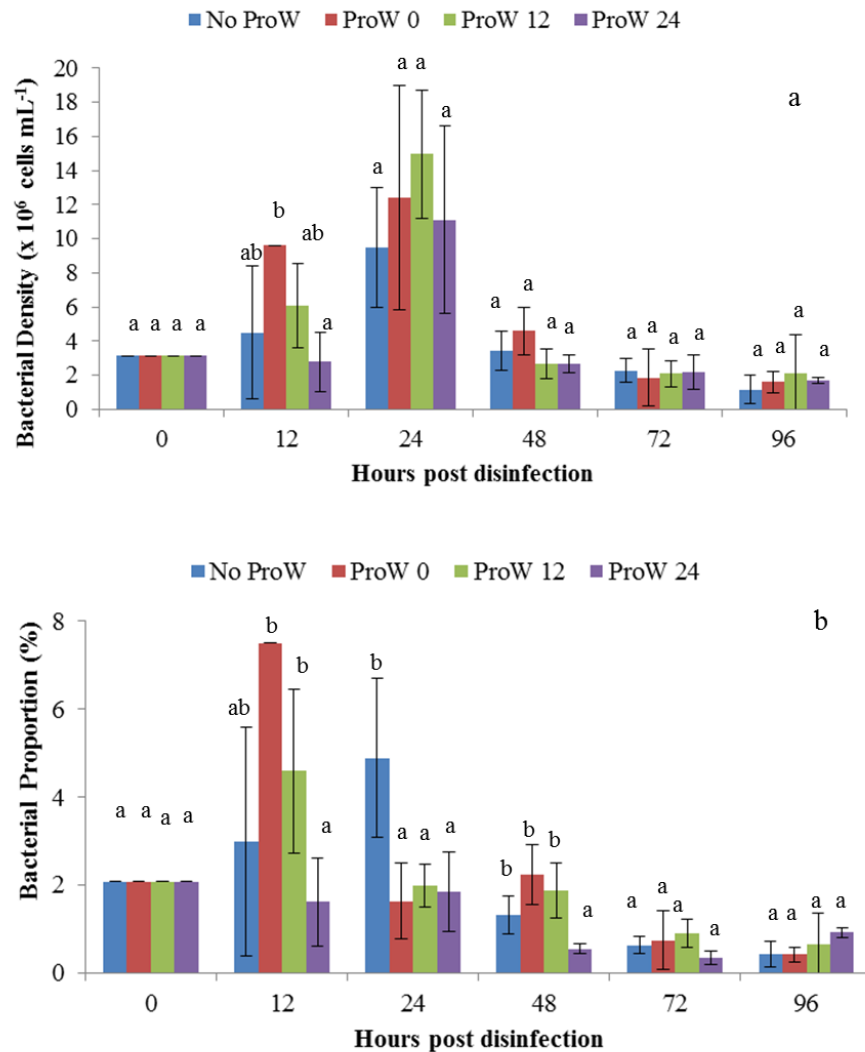
Figure 2: (a) Bacterial density and **(b)** percentage of *Bacillus* sp. on the total bacterial abundance present in culturing water post disinfection and prior shrimp stocking with different timing of probiotic application (–**T1** – No ProW (without probiotic); -**T2** – ProW 0 – probiotic application immediately after water disinfection; -**T3** – Pro W 12 – probiotic application 12 hours after water disinfection and – **T4** – ProW 24- probiotic application 24 hours after water disinfection.

B. subtilis and *B. subtilis*- complex (covers *B. licheniformis*) were quantified as compounds of the probiotic mixture. Although no differences were observed among treatments along the 96 hours of experiment, the bacterial density slightly increased in

24 hours post disinfection (Figure 3A). The highest percentage of these bacteria was verified in T1 in 24 hours ($p < 0.05$) (Figure 3B).
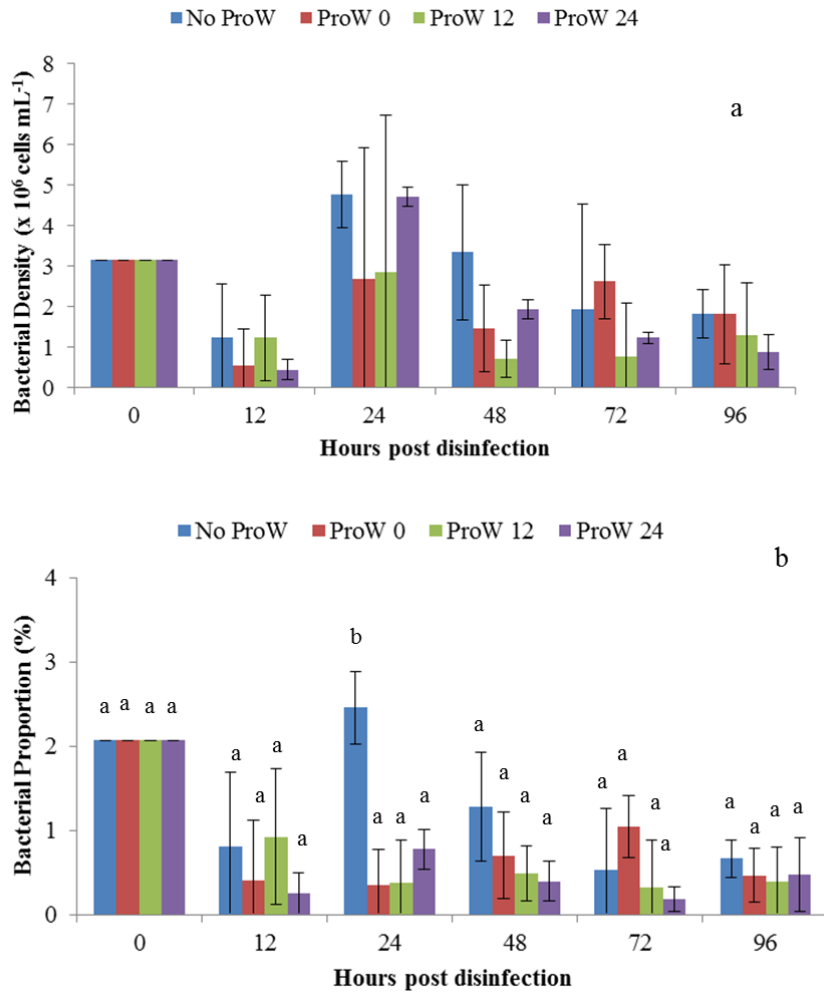
Figure 3: (a) Bacterial density and **(b)** percentage of *B. subtilis and B. subtilis –* complex sp. on the total bacterial abundance present in culturing water post disinfection and prior shrimp stocking with different timing of probiotic application (−**T1 –** No ProW (without probiotic); -**T2** – ProW 0 – probiotic application immediately after water disinfection; -**T3** – Pro W 12 – probiotic application 12 hours after water disinfection and – **T4** – ProW 24- probiotic application 24 hours after water disinfection.

For the density of *Vibrio,* no significant differences (p>0.05) were found among the treatments along the experimental period in 0, 12, 24 and 48 hours. (Figure 4A and B). Bacterial density in T2 was significantly higher than T1, T3 and T4 at 72 hours (Figure 4A). The percentage of Vibrio in T2 at 72 hours was significantly higher than T4. Treatment 1 (without probiotic T1) showed higher percentage than T2, T3 and T4 at 24 hours (Figure 4B).
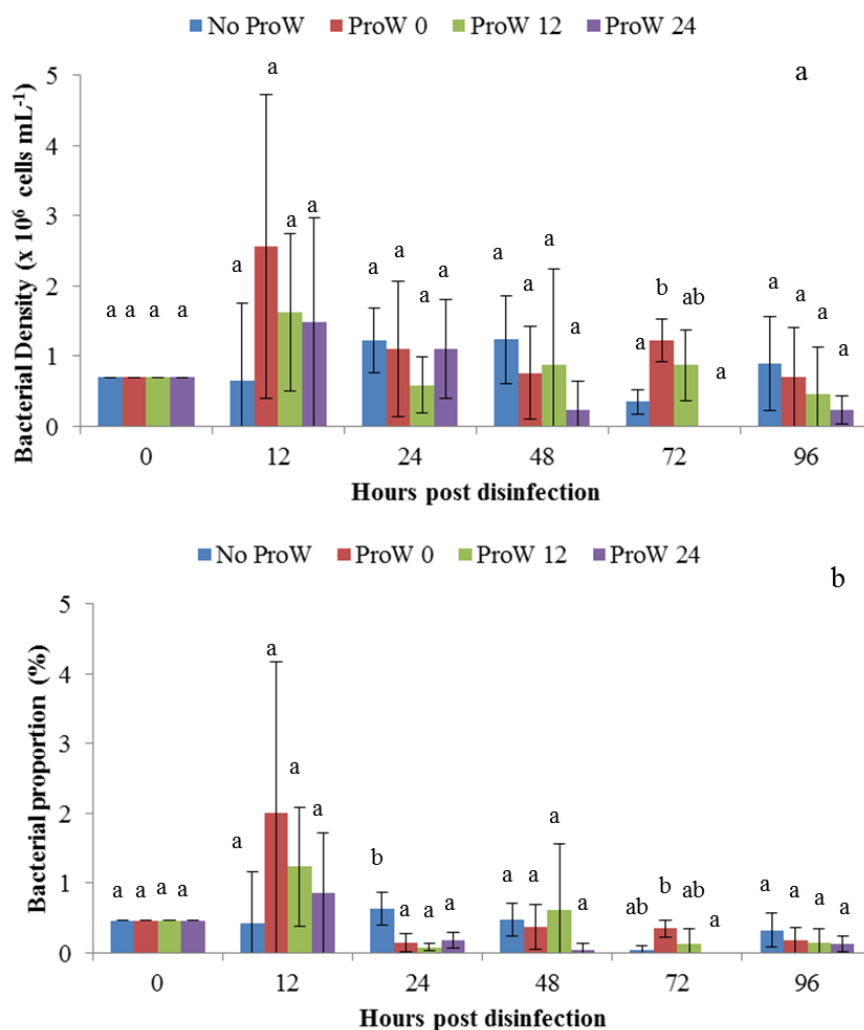
Figure 4: (a) Bacterial density and **(b)** percentage of *Vibrio* sp.. on the total bacterial abundance present in culturing water post disinfection and prior shrimp stocking with different timing of probiotic application (**T1** – No ProW (without

probiotic); -**T2** – ProW 0 – probiotic application immediately after water disinfection; -**T3** – Pro W 12 – probiotic application 12 hours after water disinfection and – **T4** – ProW 24- probiotic application 24 hours after water disinfection.

4. Discussion

Water treatment and disinfection through chlorination has been proposed in aquaculture due to the well documented high biocidal efficiency of chlorine against viruses and bacterial pathogens (Sako et al. 1988; Inouye et al. 1990; Frerichs 1990). However, some authors have recently argued that this practice contributes to decrease bacterial diversity and abundance, causing more harm than positive effects to the culture in terms of pathogen proliferation (Attramadal et al. 2012; De Schryver et al. 2014).

In the present study, despite there was no significant difference between 0 and 12 hours post disinfection, the total bacterial abundance was approximately 150×10^6 cells mL^{-1}. Chang et al. (1998) investigated the effects of chlorination as a chemical disinfectant against white spot baculovirus, and verified that chlorine concentrations of 10 ppm did not have any virucidal and bactericidal effects, concentration used in this experiment. Lechevalier et al. (1988) reported bacterial resistance to disinfection, and attributes it to the attachment or association to surfaces or other microrganisms. Likewise, Zheng et al. (2017) verified that the proportion of antibiotic resistant bacteria increased after chlorination of water. On the other hand, total bacterial abundance significantly increased in 24 hours, especially in the treatments where the probiotic was applied. Mathieu et al. (2016) investigated the bacterial repopulation on surfaces after chlorination and verified that the bacterial community recovers immediately after water

resupply. The presence of the probiotic possibly contributed to recover and increase the total bacterial abundance in 24 hours in this case. The presence of probiotics can modify the bacterial composition of the water (Ashraf 2000). Venkateswara (2007) and Wang et al. (2005) reported that probiotic water probiotics and can improve water quality of aquaculture and inhibit the pathogens in water. Such fact can be confirmed by the increasing density of *Bacillus* sp. at the same time.

The doubling time of *Bacillus* is approximately 120 minutes (Burdet et al. 1986). At the treatment ProW 0, the percentage of *Bacillus* sp. on the total bacterial density increased significantly, evidencing its presence 12 hours after the disinfection. It is important to highlight that the product is administered in the form of cysts or spores, and it is reported that the production of this extracellular capsule helps to protect bacteria from chlorine (Lechevalier et al, 1988). Such fact possibly caused the late increasing of *Bacillus* and total microbial abundance as well, observed in the present study. However, further studies may be needed in order to verify the bacterial density in shorter sampling intervals.

The proportion of *Bacillus* on the total bacterial abundance was higher at the treatment without probiotic application in 24 hours. Additionally, the probes used to quantify *B. subtilis*-complex in the present experiment covers *B. licheniformis* as a compound of the probiotic mixture. The microarray probes (Kyselkova et al. 2009) were adapted to FISH in the present study by adding Cy3. However, by comparing the bacterial density of *Bacillus* genus with the density of *B. subtilis* and *B. subtilis*-complex it is observed that there are more *Bacillus* species besides the exogenous probiotics present in the water post disinfection.

The presence of this bacterium in the treatments in which the probiotic was not added is due to the fact that *Bacillus* genus are among the most widespread microorganisms in nature (Surokulova 2013). They have also been isolated frequently from sea water (Ivanova et al. 1999). These authors found that most of the bacilli (11 strains) of marine origin belonged to the species *Bacillus subtilis*, which was also found in higher density in treatment without probiotics at 24 hours and in the initial samples in the present study. Likewise, Ferreira et al. (2015) isolated *Bacillus sp*. bacteria from a biofloc-rich water of *L. vannamei* culture and investigated the antagonistic *in vitro* activity and the total number of *Vibrio* and *Bacillus* in the water by platting technique. These results can complement the data obtained in the present study.

Probiotics can also have a direct effect on other microorganisms, commensal and/or pathogenic ones (Soccol et al. 2010). In this sense, Moriarty (1998) claimed that probiotics could be used in aquaculture not only as feed supplements, but also as water additives, since there is a high association between the cultured organism and the environmental microbiota (De Schryver et al. 2014).

The density of *Vibrio* sp. was slightly higher in 12 hours but decreased in 24 hours, same time as the density of Bacillus increased. In this sense, one of the main modes of action of probiotic bacteria is antagonistic activity (Kumar et al. 2016). Besides, such phenomenon can occur in the cultured system (Kesarcodi-Watson et al., 2008) through the production of substances which have antagonistic properties, because of the formation of organic acids and bacteriocins (Ringø, et al. 2010; 2012). Additionally, the ability of Bacillus species of auto-aggregating may also allow probiotic organisms to create a barrier, which could effectively prevent colonization by pathogens (Kos et al. 2003; Algburi et al. 2016).

In general, bacteria of genus *Vibrio* sp. have a short generation time, of 11 to 13 minutes (Ulitzur 1974). Besides that, these bacteria that are capable to quickly increase in density can be considered opportunistic pathogens, leading to vibriosis outbreaks in penaeid culture (Kimes et al. 2012; Phipen et al. 2016). Due to these reasons, several authors affirm that the disinfection practice can do more harm than good in terms of vibriosis outbreaks (De Schryver et al. 2014). For instance, Defoirdt (2016) suggests that the disinfection practice is not effective and may not result in a complete eradication of all incoming bacteria, as it was observed in the present study.

Practices such as disinfection or cleaning of ponds or tanks prior to stocking, do not provide appropriate environments for the establishment of stable microbial communities (Verschuere et al. 2000). De Schryver and Vadstein (2014) and De Schryver et al. (2014) argue that the increase in nutrient availability and empty ecological niches after disinfection combined with a destabilized and impoverished microbial community (and a consequent lack of competition) favors fast-growing bacteria (such as many pathogenic *Vibrio* spp.) in recolonizing the environment (Attramadal et al. 2012).

In the present study, the water disinfection leads to a low bacterial abundance, nutrients availability. Normally, this condition can favor the proliferation of fast growing bacteria, such as *Vibrio*. However, the addition of the probiotic immediately after the disinfection may possibly simulate the process of maturation of the water, in which the probiotic bacteria start occupying the empty niches by increasing the density and compete with *Vibrio*.

Furthermore, the results observed in the present study corroborates with the affirmations of Verschuere et al. (2000), suggesting that instead of allowing spontaneous primary colonization of the rearing water by bacteria accidentally present, the water could be preemptively colonized by the addition of probiotic bacteria, since it is generally recognized that preemptive colonization may extend the reign of pioneer organisms.

Following the peak of relatively high densities in 24 hours, bacterial abundance decreased until the end of the 96-hours experiment. This is possibly due to the lack of nutrients in the water, which is a limiting factor for bacterial growth. Considering that, it is suggested the procedures of organic carbon fertilization could start, in order to obtain the biofloc formation to guarantee the stocking of shrimp in a microbially stable environment. In this context, Hu et al. (2016) reported an increase on bacterial abundance in the water when combined *Bacillus* with molasses as a carbon source added daily to the system. In fact, carbon sources stimulate the biomass production of heterotrophic bacteria (Avnimelech 1999), increasing the diversity.

In conclusion, based on the results of bacterial density during the initial process of a shrimp culturing cycle, it is recommended that the probiotic could be administered to the water immediately after chlorination and dechlorinating of water. By adopting such procedures, it would be possible to have a reduction in *Vibrio* sp. proliferation, as well as a supplement for the establishment of a stable microbial community along the culturing cycle.

References

Algburi A, Zehm S, Netrebov V, Bren AB, Chistyakov V, Chikindas ML (2016) Subtilosin Prevents Biofilm Formation by Inhibiting Bacterial Quorum Sensing doi:10.1007/s12602-016-9242-x

Anderson RK, Parker PL, Lawrence A (1987) A $^{13}C/^{12}C$ tracer study of the utilization of presented feed by a commercially important shrimp Penaeus vannamei in a pond growout system. Journal of the world aquaculture society 18(3):148-155

Ashraf A. (2000) Probiotics in fish farming evaluation of a candidate bacterial mixture Vattenbruksinstitutionen. Report 19, Umea, Ph Licentiate thesis, 1–18

Attramadal, K.J.K., Salvesen, I., Xue, R.Y., Øie, G., Storseth, T.R. 2012. Recirculation as a possible microbial control strategy in the production of marine larvae. Aquac Eng 46, 27–39

Avinimelech Y (1999) Carbon/nitrogen ratio as a control element in aquaculture systems. Aquaculture 176:227-235

Avnimelech, Y (2007) Feeding with microbial flocs by tilapia in minimal discharge bio-flocs technology ponds. Aquaculture 264: 140–147

Chang PS, Chen LJ, Wang YG (1998) The effect of ultraviolet irradiation, heat, pH, ozone, salinity and chemical disinfectants on the infectivity of white spot syndrome baculovirus. 166(1-2): 1-17

Chatterjee S., Haldar S. 2012. Vibrio related diseases in aquaculture and development of rapid and accurate identification methods. J. Mar. Sci. Res. Dev. doi:10.4172/2155-9910.S1-002.

Crab R, Lambert A, Defoirdt T, Bossier P, Verstraete W (2010) The application of bioflocs technology to protect brine shrimp (*Artemia franciscana*) from pathogenic *Vibrio harveyi.* Journal of Applied Microbiology 109: 1643-1649.

De Schryver P, Vadstein O (2014) Ecological theory as a foundation to control pathogenic invasion in aquaculture. The ISME Journal 1-9.

De Schryver P., Defoirdt T., Sorgeloos, P. 2014. Early Mortality Syndrome Outbreaks: A Microbial Management Issue in Shrimp Farming? PLOS Pathogens, 10(4), 1-2.

Decamp O, Moriarty DJW (2006) Probiotics as alternative to antimicrobials: Limitations and potential. J World Aquac 37(4): 60–62.

Defoirdt T. 2016 Implications of Ecological Niche Differentiation in Marine Bacteria for Microbial Management in Aquaculture to Prevent Bacterial Disease. PLOS Pathogens, http://dx.doi.org/10.1371/journal.ppat.1005843.

Del'Duca A, Cesar DE, Diniz CG, Abreu PC (2013) Evaluation of the presence and efficiency of potential probiotic bacteria in the gut of tilapia (*Oreochromis niloticus*) using the fluorescent in situ hybridization technique. Aquaculture 388-391: 115-121.

Epstein S, Rossel J (1995) Enumeration of sandy sediment bacteria: search for optimal protocol. Marine Ecology Progress Series 117:289–298.

FAO 2016. The state of world fisheries and aquaculture. FAO, Rome.

Ferreira GS, Bolívar NC, Pereira SA, Guertler C, Vieira FN, Mouriño JLP, Seiffert WQ (2015) Microbial biofloc as a source of probiotic bacteria for the culture of *Litopenaus vannamei*. Aquaculture 448:273-279.

Frerichs, G.N., 1990. Efficacy of chemical disinfectants against snakehead rhadovirus. J. Appl. Ichthyol. 6, 117– 123.

Fyzul A, N., Harbi A.H., Austin B. 2014. Review: Developments in the use of probiotics for disease control in aquaculture. Aquaculture 431, 1-11.

Hu X, Cao Y, Wen G, Zhang X, Xu Y, Xu W, Xu Y, Li Z (2016) Effect of combined use of *Bacillus* and molasses on microbial communities in shrimp cultural enclosure systems. doi:10.1111/are.13101

Huang Z, Li X, Wang L, Shao Z (2016) Changes in the intestinal bacterial community during the growth of white shrimp, *Litopenaeus vannamei.* Aquaculture Research 47:1737-1746

Huggett M, Crocetti G, Kjelleberg S, Steinberg P (2008) Recruitment of the sea 15 urchin *Heliocidaris erythrogramma* and the distribution and abundance of inducing 16 bacteria in the field. Aquatic Microbial Ecology 53: 161-171.

Ichijo T, Yamaguchi N, Tani K, Nasu M (2010) Oligonucleotide probes for phylogenetic detection of waterborne bacteria. Journal of Health Science 56: 321– 325.

Inouye, K., Ikeya, F., Yamazaki, T., Hara, T., 1990. Virucidal activities of various germicidal to infectious pancreatic necrosis virus (IPNV). Fish Pathol. 25, 81–86.

Ivanova EP, Vysotskii MV, Svetashev VI, Nedashkovskaya OI, Gorshkova NM, Mikhailov VV, Yumoto N, Shigeri Y, Taguchi T, Yoshikawa S (1999) Characterization of Bacillus strains of marine origin. Int Microbiol 2(4): 267-271

Jorquera MA, Valencia G, Eguchi M, Katayose M, Riquelme C (2002) Disinfection of seawater for hatchery aquaculture systems using electrolytic water treatment. Aquaculture 207:213-224

Kesakordi-Watson A, Kaspar H, Lategan MJ, Gibson L (2008) Probiotics in Aquaculture: The need, principles and mechanisms of action and screening processes. Aquaculture 274:1-14.

Kimes, N.E., Grim, C.J., Johnson, W.R., Hasan, N.A., Tall, B.D., Kothary, M.H., Kiss, H., Munk, A.C., Tapia, R., Green, L., Detter, C., Bruce, D.C., Brettin, T.S., Colwell, R.R., Morris, P.J. 2012. Temperature regulation of virulence factors in the pathogen *Vibrio coralliilyticus*. ISME 6, 835-846.

Kos B, Suskovic J, Vukovic S, Simpraga M, Frece J, Matosic S (2003) Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. Journal of applied Microbiology 94: 981-987.

Kumar V, Roy S, Meena DK, Sarkar UK (2016) Application of Probiotics in Shrimp Aquaculture: Importance, Mechanisms of Action, and Methods of Administration. Reviews in Fisheries Sciences and Aquaculture 24(4): 342-368.

Kyselková M., Kopecký J., Frapolli M., Défago G., Ságová-Marecková M., Grundmann G.L., Moënne-Loccoz Y. (2009) Comparison of rhizobacterial community composition in soil suppressive or conducive to tobacco black root rot disease. *ISME J* **10 (3)**, 1127-1138.

Lakshimi B, Viswanath B, Gopal DVRS (2013) Probiotics as Antiviral Agents in Shrimp Aquaculture. Journal of Pathogens, http://dx.doi.org/10.1155/2013/424123.

Lechevalier MW, Cawthon CD, Lee RG (1988) Factors Promoting Survival of Bacteria in Chlorinated Water Supplies. Applied and environmental microbiology 54(3):649-654

Liu XF, Li Y, Li JR, Cai LY, Li XX, Chen JR, Lyu SX (2015) Isolation and characterisation of *Bacillus* spp. antagonistic to *Vibrio parahemolyticus* for use as probiotics in aquaculture. World J Micobriol Biotechnol 31: 795-803.

Mathieu L, Francius G, El Zein R, Angel E, Block JC (2016) Bacterial repopulation of drinking water pipe walls after chlorination. The Journal of Bioadhesion and Biofilm Research 32(8):925-934

McIntosh D, Samocha TM, Jones ER, Lawrence AL, Horowitz S, Horowitz A (2001) Effects of two commercially available low-protein diets (21% and 31%) on water and sediment quality, and on the production of *Litopenaeus vannamei* in an outdoor tank system with limited water discharge. Aquacultural Engineering 25: 69-82.

Moriarty DJW (1998) Control of luminous *Vibrio* species in penaeid aquaculture ponds. Aquaculture 164: 351-358

Nimrat S, Suksawat S, Boonthai T, Vuthiphandchai V (2012) Potential *Bacillus* probiotics enhance bacterial numbers, water quality and growth during early development of white shrimp (*Litopenaeus vannamei*). Veterinary Microbiology 159:443-450.

Pascho, R.J., Landolt, M.L., Ongerth, J.E., 1995. Inactivation of Renibacterium salmoninarum by free chlorine. Aquaculture 131, 165– 175.

Phippen, B.L., Ivanina, A.V., Sokolova, I.M., Oliver, J.D. 2016 *Vibrio coralliilyticus* causes rapid immune response and mortality in the Eastern oyster, *Crassostrea*

virginica, after exposure to anoxia. Oral Presentation, Vibrio Conference, Roscoff, France.

Ringø E., L. Lovmo M., Kristiansen Y., Bakken I., Salinas R., Myklebust R., E. Olsen T. M. Mayhew. (2010). Lactic acid bacteria vs. pathogens in the gastro-intestine of fish (a review). *Aquacult. Res* **41**, 451–467

Ringø, E., R. E. Olsen, J. L. G. Vecino, S. Wadsworth, and S. K. Song. (2012).Use of immunostimulants and nucleotides in aquaculture (a review). *J. Marine Sci :Res. Develop* **1**, 104.

Sako, H., Yshida, N., Maeno, Y., Sorimachi, M., 1988. Bactericidal activities of five disinfectants on Aeromonas salmonicida, Vibrio anguillarum, and V. ordalli. Fish Pathol. 23, 219–229.

Sapcharoen P, Rengpipat S (2013) Effects of probiotic *Bacillus subtilis* (BP11 and BS11) on the growth and survival of Pacific white shrimp, Litopenaeus vannamei. Aquaculture Nutrition 19 : 946-954.

Saulnier D, Haffner P, Goarant C, Levy P, Ansquer D (2000) Experimental infection models for shrimp vibriosis studies: a review. Aquaculture 191:133-143.

Skjermo J, Vadstein O (1999) Techniques for microbial control in the intensive rearing of marine larvae. Aquaculture 177 :333–343.

Soccol CR, Vandenberghe LPS, Spier MR, Medeiros ABP, Yamaguishi CT, Lindner JD, Pandey A, Thomaz-Soccol, V (2010) The Potential of Probiotics: A Review. Food Technol. Biotechnol 48 (4) :413–434

Sorokulova I (2013) Modern Status and Perspectives of Bacillus Bacteria as Probiotics. J. Prob. Health 1:1-5.

Ulitzur S (1974) *Vibrio parahaemolyticus* and *Vibrio alginolyticus*: Short Generation-Tilne Marine Bacteria. Microbial Ecology 1: 127-135

Vaz-Moreira I, Nunes OC, Manaia CM (2014) Bacterial diversity and antibiotic resistance in water habitats: searching the links with the human microbiome. FEMS 38:761-778

Venkateswara, A. R. Bioremediation to restore the health of aquaculture. Pond Ecosystem Hyderabad 500 082, India, pp 1–12 (2007).

Verschuere, L., Rombaut, G., Sorgeloos, P., 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiology and Molecular Biology Reviews 64, 655-671.

Villaseñor IEL, Castellano-Cervantes T, Gomez-Gil B, Carillo-Garcia ÁE, Campa-Cordova ÁI, Ascencio F (2013) Probiotics in the intestinal tract of juvenile whiteleg shrimp *Litopenaeus vannamei*: modulation of the bacterial community. World J Microbiol Biotechnol 29: 257-265.

Wang Y, He Z (2009) Effect of probiotics on alkaline phosphatase activity and nutrient level in sediment of shrimp *Penaeus vannamei*, ponds. Aquaculture 287:94-97.

Wasielesky W, Atwood H, Stokes A, Browdy CL (2006) Effect of natural production in a zero exchange suspended microbial floc based super-intensive culture system for white shrimp *Litopenaeus vannamei*. Aquaculture 258: 396–408.

White, G.C., 1992. The Handbook of Chlorination and Alternative Disifectants, 3rd edn. Van Nostrand-Reinhold, New York.

Yanong RPE (2003) Fish Health Management Considerations in Recirculating Aquaculture Systems – Part 2: Pathogens1 UF/IFAS Circular 121:1-9

Yokokawa T, Nagata T, (2005) Growth and grazing mortality rates of phylogenetic groups of bacterioplankton in coastal marine environments. Applied and Environmental Microbiology 71: 6799–6807.

Zheng J, Su C, Zhou J, Xu L, Qian Y, Chen H (2017) Effects and mechanisms of ultraviolet, chlorination, and ozone disinfection on antibiotic resistance genes in secondary effluents of municipal wastewater treatment plants. Chemical Engineering Journal 317 : 309–316

CAPÍTULO 2

Managing bioflocs operational parameters minimizes the risk for Acute Hepatopancreatic Necrosis Disease (AHPND) outbreaks – a laboratory study

Artigo a ser submetido à revista Aquaculture

Observação: Tabelas e figuras foram incluídas no corpo do texto para facilitar a leitura da tese, além de um resumo em português.

O Manejo operacional do sistema de bioflocos pode minimizar o risco de surtos de Necrose Hepatopancreática Aguda (AHPND) – Um estudo laboratorial

Resumo

O presente estudo hipotetizou que modo no qual manejo da comunidade microbiana dos bioflocos é operado pode resultar em diferentes níveis de resistência contra doenças, com sobrevivência como parâmetro avaliado. Para tanto, pós-larvas de *Litopenaeus vannamei* foram cultivadas durante 21 dias em 2 modos operacionais do sistema de bioflocos com ou sem probióticos. O experimento consistiu em 5 tratamentos: Bioflocos Autotróficos sem probióticos; Bioflocos Autotróficos com probióticos; Bioflocos Heterotróficos sem probióticos; Bioflocos Heterotróficos com probióticos e água clara com renovações. Os bioflocos heterotróficos foram operados a uma relação C/N diária de aproximadamente 18/1. Nos bioflocos autotróficos, a adição de carbono foi feita somente na formação dos bioflocos . Apartir daí, a adição de carbono foi interrompida nestes tratamentos. No fim do período experimental, os camarões foram submetidos a um teste de desafio (96 horas) com a cepa de Vibrio parahemolyticus (ag. et. de Necrose Hepatopancreática aguda). O desafio foi realizado em 3 diferentes formas: 1- Camarões cultivados em bioflocos, desafiados em água clara 2- Camarões cultivados em bioflocos, desafiados em bioflocos; e 3- Camarões não experimentais, desafiados em bioflocos. Após 96 horas, as baixas taxas de sobrevivência dos camarões provenientes do sistema de bioflocos e desafiados em água clara sugerem que o efeito protetor está na água com bioflocos. Quando os camarões foram desafiados em bioflocos, verificou-se que os tratamentos heterotróficos com e sem probióticos, e autotrófico com probióticos apresentaram altas taxas de sobrevivência ($p < 0.05$). O mesmo ocorreu quando camarões

não experimentais foram desafiados em bioflocos, confirmando que o modo operacional do sistema de bioflocos pode influenciar na proteção contra infecções por AHPND.

Abstract

We hypothesized for this study that biofloc microbial communities that are managed in different ways result in different levels of disease protection, using survival rates as a parameter of evaluation. In order to investigate such hypothesis, *Litopenaeus vannamei* post-larvae were cultured during 21 days in two different ways of operating the biofloc system. The experimental setup was composed of five treatments: Autotrophic bioflocs without probiotics application; Autotrophic bioflocs with probiotics application; Heterotrophic bioflocs without probiotics application; Heterotrophic bioflocs with probiotics application; and Water flow through system. In the autotrophic bioflocs, the input of carbon was managed only to start-up the system. Once the bioflocs had appeared, carbon dosing was stopped in the autotrophic biofloc tanks. In the heterotrophic biofloc tanks, the glucose suspension was kept being added on a daily basis. At the end of the culture period (21 days), a 96 hours EMS/AHPND challenge test was performed in 3 different approaches: 1- Shrimp from bioflocs, challenged in new sea water; 2-Shrimp from bioflocs, challenged in biofloc suspension corresponding to each treatment; and 3-Non-experimental shrimp, randomly selected from a recirculation (RAS) system challenged in biofloc suspension corresponding to each treatment. After 96 hours, no differences were observed in shrimp from BFT challenged in sea water, and low survival rates were detected, suggesting that the protection is in the culturing water. When shrimp from BFT were challenged in BFT suspension, Heterotrophic bioflocs with and without probiotic application and Autotrophic bioflocs with probiotics showed the higher survival rates. Autotrophic bioflocs with probiotic application and Heterotrophic treatments showed higher survival rates when non-experimental shrimp from RAS were challenged in biofloc suspension, confirming that

the operational mode of the biofloc system can influence on the protection against EMS/AHPND. Such informations reinforce the importance of the microbial management in aquaculture.

1.Introduction

The high incidence of infectious diseases, resulting in mortalities, has become a real challenge for the aquaculture industry, and the main cause of production and economic losses during the last decades. It is the primary factor that negatively affects the future of this sector (FAO, 2016).

In many cases, mortalities caused by pathogenic bacteria are not attributed to specific obligate pathogens, but rather to the proliferation of opportunistic pathogenic bacteria (Defoirdt, 2016). These species are inherently present in the culture water and are highly effective in taking advantage of ecological changes related to the aquaculture-based use of water as growth environment in aquaculture systems (Skjermos and Vadstein, 1999). They are typically characterized as organisms that can become pathogenic following a perturbation in the environment (Brown et al., 2012). Suboptimal rearing conditions such as extremely high stocking densities, associated with environmental parameters like low oxygen concentrations and variations in temperatures can lead to opportunistic bacterial disease outbreaks. Vibriosis is the most widely known example (Kimes et al., 2012; Phippen et al., 2016).

Vibriosis is caused by Gram negative bacteria of the genus *Vibrio*, and can be considered as one of the most prevalent diseases that affect cultured aquaculture organisms worldwide (Chatterjee and Haldar, 2012). Among the *Vibrio* spp. that are able to cause disease special attention has recently been given to *Vibrio*

parahaemolyticus, which has been reported to be the main ethiological agent that causes the Acute Hepatopancreatic Necrosis Disease (AHPND) or Early Mortality Syndrome (EMS) (Tran et al., 2013). This disease has caused mass mortality of penaeid shrimp in China, Vietnam, Malaysia, Thailand, Mexico and the Philippines (Hirono et al., 2017), and typically appears during the first 20 to 30 days of culture. In the terminal stage, the hepatopancreas shows extensive intertubular, hemocytic aggregations, which causes hepatopancreas dysfunction and severe secondary *Vibrio* infections (Lightner et al., 2012; Aranguren et al., 2017).

Considering the substantial impact resulting from the prevalence of AHPND causing *V. parahemolyticus*, producers in many cases have adopted the use of antibiotics and/or disinfectants to control this bacterial disease. The indiscriminate use of antibiotics, especially in situations when there is no apparent disease, evidently results in a major problem of resistance development by bacterial aquaculture pathogens (Defoirdt et al., 2007; Crab et al., 2010; Mohapatra et al., 2013). Moreover, the random use of desinfectants has also proven ineffective in treating diseases caused by vibrio species and in some cases has even been suggested to contribute to disease risk (Attramadal et al.,2012; De Schryver et al., 2014).

In this context, there is an urgent need to find non-antibiotic based and environmentally friendly alternatives to prevent AHPND from occurring (Sha et al., 2016). Defoirdt et al. (2011) and Defoirdt (2016) suggest that an approach which considers simultaneously pathogen, host and environment will probably be the most effective solution in the long term to prevent pathogenic disease. De Schryver et al. (2014) and De Schryver and Vadstein (2014) affirm that manipulations of man-based aquaculture environments based on ecological selection principles allow to manage the

microbial community and as such are key in minimizing the risk of vibriosis outbreaks and thus also AHPND.

Biofloc Technology (BFT) is a fish and shrimp culture technique based on the stimulation and establishment of a microbial community to achieve recycling of nutrients, mainly nitrogen waste, and maintaining water quality (McIntosh, 2001; Wasielesky et al., 2006; Avnimelech, 2007). In addition, it has been shown that other benefits such as improved growth, survival and disease resistance can also be expected (Wang et al., 2016; Xu et al., 2016; Crab et al., 2010).

Parameters influencing biofloc production will determine nitrogen conversion processes in the system. A chemoautotrophic system is based on nitrifying bacteria that oxidize ammonium to nitrite and nitrite to nitrate, which is the least toxic nitrogen form, fixing inorganic carbon in the process (Kuhn et al., 2013). Such autotrophic (i.e. chemoautotrophic) way of operating a biofloc, provides the conditions to favor the predominance of autotrophic bacteria. These groups do not utilize organic carbon to perform nitrification, hence, when predominant, the C/N ratios are low (considerably lower than 10) (Ebeling, 2006; Ray & Lotz, 2014).

Chemoautotrophic systems are characterized by a slow accumulation of suspended solids (Ebeling et al., 2006; Ray and Lotz, 2014), because of the low biomass yield per mol of ammonium nitrogen oxidized. For instance, for every g of total ammonium nitrogen (TAN) converted to nitrate nitrogen (NO_3-N), 4.18g of oxygen is consumed and 0.20g of microbial biomass is produced (Ebeling et al., 2006).

Another common type of biofloc system is achieved by operating it in a heterotrophic way through the addition of carbon to a C/N ratio of at least 10. Heterotrophic bacteria assimilate the ammonium nitrogen into their bacterial biomass

by utilizing organic carbon as a source of energy (De Schryver et al., 2008). In this way, the nitrogen is completely removed from the water phase but it also leads to a higher accumulation of solids in the water (Ebeling et al., 2006). The biomass production in this process is much higher than in the autotrophic conversion. For each g of TAN assimilated by the heterotrophic bacteria 8.07g of microbial biomass is produced and 4.71g of oxygen is consumed (Ebeling et al., 2006)

It has also been reported that biofloc have the capacity to control disease. Bioflocs have been suggested to protect aquatic animals from bacterial disease in several cases. Ekasari et al. (2014) verified that biofloc-grown *L. vannamei* survived better when challenged with myonecrosis virus (IMNV). Crab et al. (2010) used *Artemia franciscana* as a model system to verify the effects of bioflocs, and found higher survival when the brine shrimp were challenged with *V. harveyi*. The authors suggested that bioflocs have a protective effect against bacterial diseases. However, only little is known about the reason for this protective activity of the bioflocs.

The application of probiotic bacteria has become another successful alternative for the control of diseases (Gram et al., 1999; Gildberg et al., 1997). Probiotics are live microorganisms that can promote health and growth benefits to the host when administered in adequate levels. They can be administered via water or feed (Moriarty, 1998; Skjermo and Vadstein, 1999) with one or more strains (Villaseñor et al., 2014). Several authors have reported the positive effects of *Bacillus* sp. in *L. vannamei* culture. Nimrat et al. (2012) observed that the use of *Bacillus* as a potential probiotic enhanced the number of beneficial bacteria in shrimp and in the sea water. Similarly, Sha et al. (2016) verified antibacterial activity and higher stimulation of immune-related genes when probiotics were administered. Li et al. (2009) reported that *Bacillus* had positive

effects on shrimp immune responses and disease resistance, with reduction in the number of *Vibrio*.

The above-mentioned results were obtained in flow-through conventional systems. However, positive effects of probiotic application are also reported in biofloc system. For instance, Krummenaeuer et al. (2014) observed higher survival of shrimp reared in bioflocs, but naturally infected with *V. parahaemolyticus* and treated with a multistrain probiotic, including *Bacillus*. Ferreira et al. (2015) isolated *Bacillus licheniformis* strain from biofloc suspension and verified antagonistic activity against *V. alginolyticus*. Aguilera-Rivera et al. (2014) suggested that the addition of a commercial probiotic mixture contributed to the prevention of outbreaks of opportunistic pathogenic bacteria in BFT system and clear water. Zokaeifar et al. (2014) observed a significantly higher survival of *L. vannamei* supplemented with *Bacillus subtilis* after a challenge with *V. harveyi*.

Based on the theory of De Schryver and Vadstein (2014) and De Schryver et al. (2014), we hypothesized for this study that biofloc operational parameters (more particularly C/N ratio) will probably influence microbial community composition, in a way that it can result in bioflocs with differential capacity to control disease. Here, this hypothesis was verified using a *V. parahaemolyticus* strain that causes AHPND as a pathogen. In addition, it was verified if Bacillus-based probiotics would allow to control AHPND independently from the imposed operational parameters .

2. Material and Methods

2.1. Experimental shrimp

Experimental post-larvae of whiteleg shrimp *L. vannamei* were obtained from Shrimp Import Services (SIS, Miami, USA) at the age of 10 days (PL_{10}) and maintained in the larval raceway system of the Laboratory of Aquaculture & *Artemia* Reference Center (Ghent University, Belgium) until use.

2.2. Probiotic Mixture

The commercial probiotic mixture (Sanolife-ProW® - INVE Aquaculture) used in this study is composed of *Bacillus subtilis* and *Bacillus licheniformes* strains, in a bacterial concentration of $5x10^{10}$ CFU g^{-1}.

2.3 Preparation of biofloc suspensions

In order to grow the bioflocs to be used in the study, *L. vannamei* juveniles were stocked in 12 tanks of 40 L (35 L effective seawater volume) at a density of 100 g $tank^{-1}$. Seawater was maintained at a salinity of 32 ppt and a temperature of 27.89 ± 0.56 °C by use of a climate controlled room. The shrimp were fed a commercial diet (Crevetec grower® 2x4mm, 40% CP) initially at 8% on wet body weight day^{-1}.

Six tanks were operated as autotrophic biofloc systems with or without application of probiotics and six tanks were operated as heterotrophic biofloc systems with or without probiotics. In order to promote the growth and establishment of bioflocs in the 8 biofloc tanks, a glucose (D+ glucose - VWR®) solution (50% of C) was added continuously to each tank as a carbon source by means of a peristaltic pump (Multi channel cassette pump 205 CA – Watson-Marlon®) to an estimated total daily C:N ratio of 18. The amount of glucose added to the water was calculated based on theoretical daily nitrogen excretion by the shrimp, following the methods described by Ebeling et

al. (2006) and Avnimelech (2009). Once the bioflocs had appeared (TSS > 100 mg L^{-1}) and total ammonium nitrogen (TA-N) concentrations dropped below 0.05 mg L^{-1}, carbon dosing was stopped in the autotrophic biofloc tanks to allow a shift towards a nitrifying community. In the heterotrophic biofloc tanks, the glucose supplemention was kept on a daily basis.

The bioflocs were prepared over a period of 70 days and the total suspended solids content were kept under 500 mg L^{-1}. During this period, the probiotic (Sanolife ProW®; INVE Aquaculture, Belgium was added to the culture water every 48 hours, in a concentration of 0.5 ppm or 0.5mg L^{-1} according to the manufacturer recommendations.

2.4. Experimental trial

2.4.1 Preparation of experimental pathogens

Pathogenic *Vibrio parahaemolyticus* strain pV1 was originally isolated from diseased shrimp in Thailand. Presence of toxic genes in the bacterial strains was previously confirmed using specific primers AP2 and AP3 (Kumari, 2015). The strain was preserved at -80°C in Marine Broth (40.1 g L^{-1}, Carl Roth) containing 20% sterile glycerol.

Prior to use, the bacterial strain was grown overnight at 28°C on agar bacteriological grade (20 g L^{-1}, Biokar diagnostics) and then subcultured to log phase in Marine Broth (40.1 g L^{-1}, Carl Roth) at 28°C during continuous shaking. Bacterial cell numbers were subsequently determined spectrophotometrically at 550 nm according to the McFarland standard (BioMerieux, Marcy L'Etoile, France), and set to an optical density of 1.0 approximately corresponding to 1.2×10^9 cells mL^{-1}.

This suspension of *V. parahaemolyticus* was then used in the AHPND challenge as mentioned below (section 2.4.3), and added to the water 24h prior to stocking the shrimp in the challenge test experimental units at a final concentration of 10^7 CFU mL^{-1}.

2.4.2 Culture of shrimp in biofloc systems with different operational parameters

The culture of shrimp during 21 days was carried out in 18 rectangular transparent acrylic tanks with a bottom area of 5 cm^2 and 10 L effective volume, allocated to a temperature controlled room. Temperature in all tanks was maintained in the range of 27.8 and 28.8 °C during the experimental period. Salinity was maintained between 34.30 and 35.20 ppt. Aeration was provided in each aquarium through an airstone by using an air blower and the light regime was set at 12 h light/12 h dark.

L. vannamei post-larvae (PL22) of 0.025 ± 0.01 g were stocked at a density of 30 shrimp per tank. The experimental setup was composed of five treatments with three replicate tanks each:

- Autotrophic bioflocs without probiotics application and without glucose supplemenation (**A-BFT**).

-Autotrophic bioflocs with probiotics application but without glucose supplementation (**A-BFT+ProW**)

-Hetetotrophic bioflocs without probiotics application but with glucose supplementation to an estimated C/N ratio of 18 (see below) (**H-BFT**)

− Hetetotrophic bioflocs with probiotics application and with glucose supplementation to an estimated C/N ratio of 18 (**H-BFT+ProW**)

- Water flow through system (**FT**)

The bioflocs experimental units were filled with 5L of biofloc suspension previously prepared in an autotrophic or heterotrophic way (see section 2.3), suplemented with 5L natural seawater.

For the control tanks operated as flow-through system, the tanks were filled with 10L natural sea water and a water exchange of 80% was performed every 48 hours. Natural pre-heated sea water was used for the renewals procedures. In all tanks, municipal freshwater was used to compensate for evaporative losses and to maintain salinity throughout the experimental period.

The shrimp were fed a 40% crude protein (CP) commercial diet (Crevetec grower[®]) twice a day (0900 and 1800 h) during 21 days. Feeding rate was adapted according the methodology proposed by Jory et al. (2001).

Dissolved oxygen (DO) and pH were measured daily (0900 and 1800 h) using a portable DO meter (Field LabOxi – Oxical SL - WTW[®]), and a pH meter (pH Control-JBL[®]), respectively. Dissolved inorganic nitrogen (total ammonium nitrogen, NO_2-N, and NO_3-N), and total suspended solids (TSS) were determined weekly following the procedures in the Standard Methods for the Examination of the Water and Wastewater (1999).

2.4.3 EMS/AHPND challenge

At the end of the shrimp culture period (21 days), an EMS/AHPND challenge test was performed. The post larvae (PL43) were randomly selected and redistributed over 90 tanks of 1L volume containing new seawater, or suspension from an experimental tank, as described below. The test consisted of a negative (non-

challenged) control and challenges for the different treatments (n=5 each), in which the shrimp were immersed in a *V. parahaemolyticus* (pV1) suspension.

The challenge test aimed to investigate the differences in protective effects against AHPND by Autotrophic and Heterotrophic-based biofloc and the presence or not of the probiotic mixture. Besides, it was designed to verify whether the protection was related to the suspension of the bioflocs as such (hypothesis 1, H1), or whether it is related to the influence that bioflocs have on the shrimp upon long term exposure (through eg. Immunomodulation) (hypothesis 2, H2). For that, the challenge was performed in three (3) different approaches, as follows: 1- Experimental shrimp were grown in different bioflocs, then removed from the biofloc suspension before challenging them in new sea water (**BFT to SW; H2**); 2- Experimental shrimp were grown in differentbioflocs, and then challenged in their respective biofloc suspensions (**BFT to BFT, H1**); and 3- Non-experimental shrimp were randomly selected from a recirculation (RAS) system (and thus not hadw any previous contact with bioflocs), and challenged in one of the different types of biofloc suspensions (**SW to BFT, H1**). The challenge test was run for 96 hours, and shrimp mortality was determined daily.

2.5. Statistical Analyses

Levene's and Kolmogorov-Smirnov tests were used to assess homoscedasticity and normality of all data, respectively. As all data was normally distributed and the variances of the variables were equal, the data were analyzed using one-way ANOVA. All survival data was transformed in arcsin. However, it was found that the variances of this variable was not equal, therefore a non-parametric test (Kruskal–Wallis test) was applied, followed by a Mann–Whitney U test ($P > 0.05$).

3. Results

3.1. Water Quality during 21 days of culture

The water quality parameters are expressed at Tables 1 and 2. No differences were observed among the treatments in DO, pH and salinity. (Table 1).

Table 1: Mean (± standard deviation) values of water quality parameters during 21 days of culture of *L. vannamei* culture in Autotrophic (A-BFT) and Heterotrophic-based (H-BFT) bioflocs with and without application of probiotic and in a flow-through system.

Parameters	A-BFT	A. BFT+ProW	H-BFT	H- BFT+ProW	Flow through
DO (mg L^{-1})	5.88 ± 0.47^a	5.90 ± 0.41^a	5.97 ± 0.39^a	6.01 ± 0.34^a	5.98 ± 0.37^a
pH	7.73 ± 0.09^a	7.78 ± 0.08^a	7.80 ± 0.04^a	7.86 ± 0.06^a	7.88 ± 0.09^a
Salinity.	34.40 ± 2.06^a	35.10 ± 2.03^a	35.20 ± 1.38^a	34.30 ± 1.35^a	35.20 ± 1.03^a

The levels of Total Ammonium Nitrogen (TAN) and Nitrite (NO_2 -N) were equal among the treatments (Table 2). Nitrate (NO_3-N) concentrations were significantly higher in the A-BFT treatments (A-BFT and A-BFT+ProW). Total suspended solids measurements was performed only in the BFT treatments, since in the Sea water systems there was no accumulation of significant amounts of particulate organic matter or suspended solids. This parameter was significantly higher in the H-BFT treatments (H-BFT and H-BFT + ProW).

Table 2: Mean (± standard deviation) values of nitrogen compounds (Total Ammonium Nitrogen –TAN, Nitrite – NO_2^--N and Nitrate NO_3^-- N) and total suspended solids (TSS) during 21 days of culture of *L. vannamei* culture in Autotrophic (A-BFT) and Heterotrophic-based (H-BFT) bioflocs with and without application of probiotic and in a flow-through system.

Parameters	A- BFT	A.BFT+ProW	H- BFT	H. BFT+ProW	Flow through
TA-N (mg L^{-1})	0.07 ± 0.03^a	0.05 ± 0.01^a	0.02 ± 0.04^a	0.01 ± 0.01^a	0.02 ± 0.01^a
NO$_2$-N (mg L^{-1})	0.28 ± 0.07^a	0.32 ± 0.04^a	0.20 ± 0.05^a	0.26 ± 0.05^a	0.22 ± 0.05^a
NO$_3$- N (mg L^{-1})	$23,53 \pm 2,47^b$	$23,63 \pm 4,08^b$	0.20 ± 0.25^a	0.22 ± 0.10^a	0.20 ± 0.02^a
TSS (mg L^{-1})	117.88 ± 38.29^a	109.77 ± 11.45^a	334 ± 113.81^b	408.33 ± 72.19^b	-

3.2 Survival in EMS/AHPND challenge test

Survival was monitored daily during the 96 hours of challenge tests as performed in the three different approaches.

3.2.1 Experimental shrimp cultured in bioflocs, challenged in new sea water (BFT to SW)

No mortality was observed in the negative control during the 96 hours of experiment, where no pathogen was added to the water (Figure 1). One day (24 hours) post immersion in *V. parahaemolyticus* suspension, survival did not differ between the negative control and the experimental treatments, except for the treatment H-BFT which showed a significantly lower survival (33±11%). After 48h, 72h and 96h, however, , survival were significantly lower in all experimental treatments as compared to the negative control. At every time point, survival did not show significant differences among treatments.
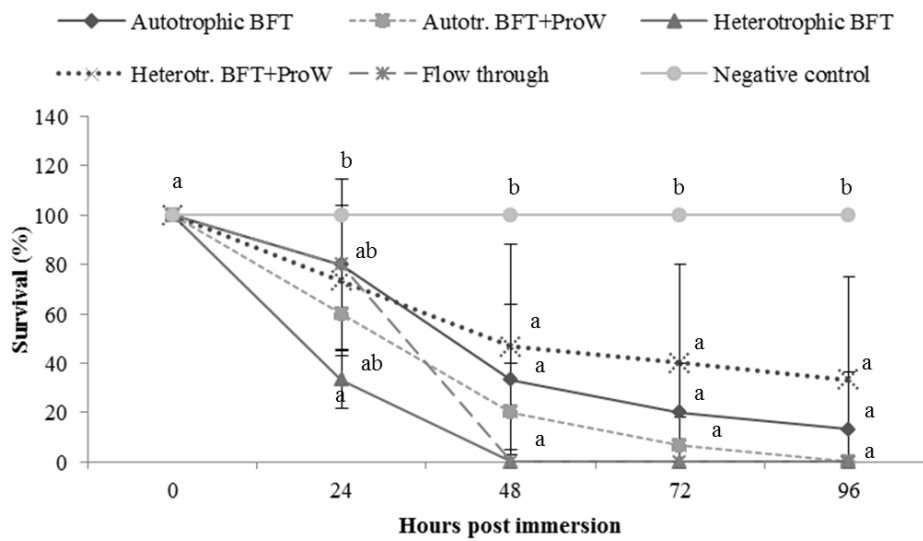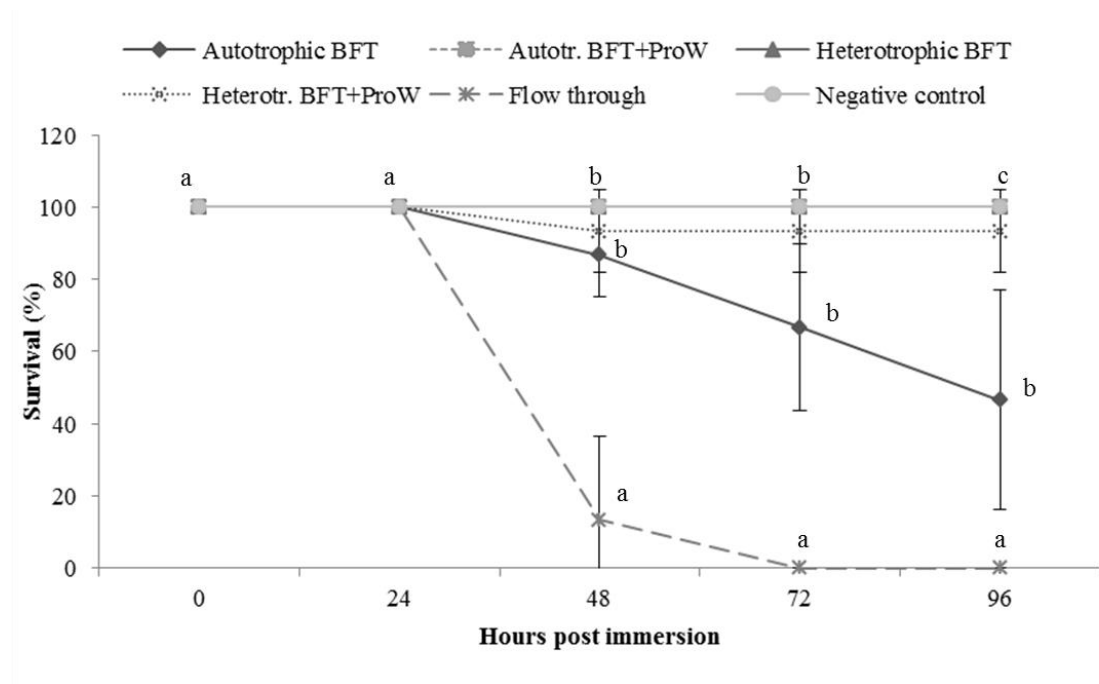
Figure 1: Mean values ± standard deviation of survival (%) of Pacific white shrimp culture for 21 days in Autotrophic or Heterotrophic bioflocs with or without probiotic application, or in a flow-through system and then challenged with acute hepatopancreatic necrosis disease (AHPND) causing bacterial strain of *V. parahemolyticus* in fresh sea water (BFT to SW). Different letters indicate statistical differences at each time point (P<0.05).

3.2.1 Experimental shrimp cultured in bioflocs, challenged in biofloc suspension (**BFT to BFT**)

No mortality was observed in the negative control during the 96 hours of experiment (Figure 2). No significant differences were observed among treatments and the negative control at 24 hours post challenge, and the survival was 100% in all treatments. At 48h and 72h post challenge, the shrimp challenged in water from the flow through system showed the lowest survival (13±23%), which was significantly lower as compared to other treatments. No significant differences were observed among the different biofloc treatments. At 96h after immersion, 100% mortality was found in the

flow through treatment, which was significantly different from the A-BFT treatment (46±30%). This treatment, then again, also was significantly lower than the A-BFT+ProW (100±0.00%) and the Heterotrophic bioflocs with (93±11%) and without probiotics (100±0%).
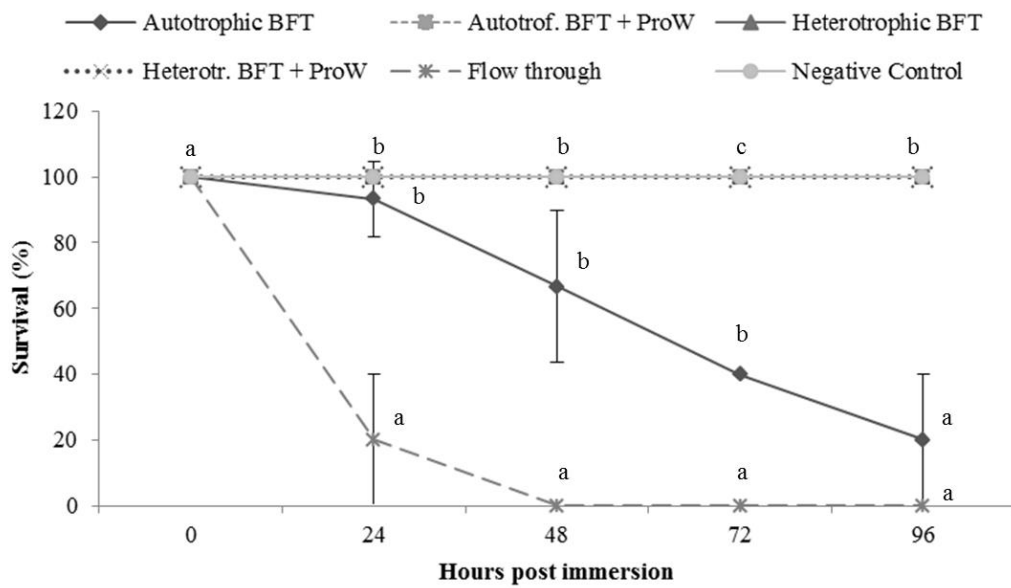

Figure 2: : Mean values ± standard deviation of survival (%) of Pacific white shrimp culture for 21 days in Autotrophic or Heterotrophic bioflocs with or without probiotic application, or in a flow-through system and then challenged with acute hepatopancreatic necrosis disease (AHPND) causing bacterial strain of *V. parahemolyticus* in fresh sea water (BFT to BFT). Different letters indicate statistical differences at each time point (P<0.05).

*3.2.2 Non-experimental shrimp challenged in biofloc suspension (**RAS to BFT**).*

No mortalities were detected in non-challenged shrimp (Figure 3). However, at 24h post immersion, shrimp taken from a RAS system and challenged in water from the flow through system showed a significantly lower survival (20±20%) than all other

treatments. The same treatment also showed the significantly lowest survival at 48 hours after challenge. At 48h, the shrimp challenged in water from the A-BFT treatment showed an intermediate survival. The shrimp challenged in biofloc suspension from treatmentA-BFT+ProW remained all alive during the 96 hours of challenge with *V. parahaemolyticus.* The same results were observed for H-BFT with and without probiotic addition.

Figure 3: Mean values ± standard deviation of survival (%) of Pacific white shrimp culture for 21 days in Autotrophic or Heterotrophic bioflocs with or without probiotic application, or in a flow-through system and then challenged with acute hepatopancreatic necrosis disease (AHPND) causing bacterial strain of *V. parahemolyticus* in fresh sea water (RAS to BFT). Different letters indicate statistical differences at each time point (P<0.05).

4. Discussion

The levels of total ammonium nitrogen and nitrite-N did not show significant differences among the treatments in the present study. In addition, nitrate-N levels were significantly higher in Autotrophic-based bioflocs (A-BFT), which is indicative for nitrification. Ray and Lotz (2014) observed similar results when monitoring inorganic nitrogen levels in a chemoautotrophic-based and three heterotrophic-based biofloc (H-BFT) systems. The removal of ammonium nitrogen in A-BFT occurs mainly by the nitrification process, with nitrate as a final product (Hargreaves 2006). This process with no carbon addition as for instance demonstrated by Zhu and Chen (2001). Nitrifying bacteria are known to have lower growth rates (relative to heterotrophs and to be more sensitive to variations in water quality parameters such as pH and temperature (Crab et al., 2007; Ebeling et al., 2006). The occurrence of this process is evidenced in the present study by the accumulation of NO_3-N when no carbon source was supplemented (except for the start-up period, see M&M). These results are similar to those reported by Krummenauer et al. (2014) on the culture of *L. vannamei* in a biofloc system in with carbon dosing was limited to a short period, from which it can be concluded that the autotrophic biofloc systems in the current study performed adequately. The continuous addition of glucose as an organic carbon source increases the C:N ratio and stimulates the production of microbial biomass in the presence of oxygen, while assimilating the nitrogen (Avnimelech, 1999; Ebeling et al., 2006). Investigating the effects of different C:N ratios in a *L. vannamei* biofloc culturing system, Xu et al (2016) affirm that the microbial community can shift from chemoautotrophic bacteria (nitrification) to heterotrophic bacteria when carbon input increases or vice versa. These observations are in accordance with the results obtained

in this study, possibly evidencing the presence of a pure heterotrophic system, where there is no nitrite or nitrate-N production (Ebeling et al., 2006).

The stimulation of heterotrophic growth leads to a concomitant increase in the total suspended solids levels (TSS). This parameter is an important quantitative indicator of the bioflocs (De Schryver et al., 2008) as it reflects the development of the biofloc in the water (Xu et al., 2016). TSS was significantly higher in heterotrophic treatments during the experimental period, which corroborates with the observations of Ray and Lotz (2014) who compared chemoautotrophic and heterotrophic- based bioflocs. This observation is also in agreement with the statement that biomass production in a heterotrophic system is 40 times greater than the biomass generated in autotrophic nitrification (Ebeling et al., 2006; Hargreaves 2006).

Despite the difference in operational parameters between the treatments, the water quality levels were kept within the recommended range for *L. vannamei* (Lin and Chen, 2001; Lin and Chen, 2003; Kuhn et al., 2010).

The experimental period of 21 days shrimp production was followed by a *V. parahaemolyticus* challenge test to induce AHPND. Hargreaves (2013) emphasized the need to understand the role of the bioflocs in controlling or encouraging pathogenic bacteria, especially vibrios. In the present study, the presence of bioflocs clearly affected the resistance of *L. vannamei* to the AHPND causing pathogen, although this effect clearly depended on the type of bioflocs used.

When shrimp originating from the autotrophic or heterotrophic biofloc systems were challenged with the pathogen in new seawater, there was no significantly increased survival as compared to shrimp that originated from the flow-through system. Thus, despite the fact that immunostimulatory effects have been attributed to bioflocs in

earlier studies (Ekasari et al., 2014; Bossier et al., 2016; Ahmad et al., 2017), this kind of activity could not provide a significant protection for the shrimp that were challenged with AHPND causing *V. parahaemolyticus* in the current study. This suggests that bioflocs do not work by means of the hypothesis 2 set forward in this study, and that the protective effect induced by the bioflocs should have another mode of action.

When non-experimental shrimp (thus not been in contact with bioflocs before) were challenged in the different types of suspensions from the 21 days culturing period, clear differences in survival could be observed. This confirms hypothesis 1 that the protection is related to the biofloc suspension as such. The suspension from the flow-through system did not provide a substantial protection as all shrimp died within 48h after challenge, whereas the shrimp housed in the autotrophic bioflocs showed intermediate survival and the shrimp housed in the heterotrophic bioflocs showed the same survival as the negative control. For the shrimp that were cultured in the different systems for 21 days and then challenged in their respective suspension a completely similar survival pattern was observed.

An explanation for the differences in survival may be found in the differences in microbial ecology according to the type of suspension. De Schryver and Vadstein (2014) suggested the use of the ecological theory of r/K selection to manage microbial communities in aquaculture. According to this concept, an environment rich in nutrients per microbial cell, low in competition and with frequent perturbations selects for microorganisms with a high capacity to exploit nutrients and increase in population size, termed fast-growing opportunistic r-strategists (De Schryver and Vadstein, 2014). Since a flow through system cannot sustain high levels of micro-organisms due to continuous wash-out, this typically represents an environment that houses fast growing r-strategists

and as such – in theory - provides a low level of competition (Attramadal et al. 2012) upon introduction of a fast growing pathogen such as the *V. parahaemolyticus* used in this study. In correspondence to this theory, the water from the flow-through system did not have a controlling effect upon addition of the pathogen to the water and hence a high mortality of the shrimp was observed.

Biofloc suspensions, then again, theoretically represent a microbial environment with a lower amount of available nutrients per microbial cell and with a more stable community composition. Such an environment would act controlling for the proliferation of an opportunistic fast-growing micro-organisms upon its introduction, simulating a microbially mature water (Skjermo and Vadstein 1999). The controlling effect of bioflocs towards the opportunistic *V. parahaemolyticus* pathogen used in this study could indeed be observed from the higher survival of the shrimp in these treatments. Nonetheless, there was a difference in the level of protection that the autotrophic and the heterotrophic bioflocs could provide, which again may be explained by the differences in microbial ecology between the two types of bioflocs. Such differences were observed in terms of nitrogen dynamics by Ray and Lotz (2014). Additionally, the authors suggest that that differences in management and can lead to substantial disparity in system function and shrimp production (Ray and Lotz 2014).

The heterotrophic bioflocs were continuously supplied of carbon resulting in the presence of a highly abundant, yet stable, microbial community mainly dominated by heterotrophic micro-organisms (likely of the K-strategist type) according to the theory of De Schryver and Vadstein (2014). Attramadal et al., (2014) suggest that a stable microbial community can be achieved in a an environment colonized by K-strategists When a fast-growing heterotrophic pathogen – like the *V. parahaemolyticus* in this

study that was introduced in the biofloc suspensions 24h prior to exposure to the shrimp – is introduced in such an environment it is faced by a highly competitive environment resulting in low chance for proliferation. In addition, the pathogen may attach to the microbial aggregates present in the system. De Schryver et al (2008), suggest that the extracellular polymeric substances (EPS) that can be found whithin the bioflocs contribute to encapsulate microbial cells, binding components to the floc. More et al. (2014) affirm that EPS have an important role in floc formation and aggregation of different organic/inorganic compounds, showing adsorption abilities. Such adsorption of the pathogen by the bioflocs in combination with the highly competitive environment is likely the explanation for the inactivation and/or reduction of the infective pressure of the *V. parahaemolyticus* as observed in this study.

The autotrophic bioflocs also represent a stable microbial ecosystem that theoretically is dominated by K-strategists, be it of a different ecology as these systems do not thrive on carbon added continuously to the system. As a result, the level of competition upon introduction of a heterotrophic fast-growing pathogen is expected to be lower, which was confirmed by a higher survival of the shrimp in the challenge test as compared to the flow through system treatment. However, to compensate for the lower protective characteristics of the autotrophic bioflocs, it was tried to use probiotics to increase the protection of the shrimp against AHPND as some researchers observed that the use of probiotics, most of them *Bacillus* spp, promoted resistance against *Vibrio* infections (Balcazar et al., 2007; Villaseñor et al., 2015; Sha et al., 2016).

From the results, it is clear that the use of probiotic was indeed able to increase to the protective capacity of the autotrophic biofloc system in the case of AHPND infection. Similarly, Krummenauer et al. (2014) verified, after a vibriosis outbreak, that

the use of a multistrain probiotic contributed to increase the survival rates, in biofloc system. Aguillera-Rivera et al. (2014) also obtained higher survival rates in a biofloc system supplemented with probiotics, with low level lesions on shrimp tissues for these treatments. As protection was already high in case of the heterotrophic bioflocs in the present study, the additional protection provided by the probiotics was logically limited.

In conclusion, the results of the present study illustrate the clear protective effects of biofloc systems in case an AHNPD causing pathogen is introduced in the water, evidenced by the enhanced survival rates. Further studies which include an analysis of the microbial community composition in the bioflocs and the changes therein during shrimp culture should be performed to confirm the explanation on the protective effects as described above. In addition, this study clearly showed the potential of using probiotics in case the bioflocs would not be able to provide full protection. Overall, the observations from this study clearly show the importance of microbial management in aquaculture systems, and more specifically the influence of operational parameters of biofloc systems in order to minimize disease risk.

5.References

Aguillera-Rivera, D., Prieto-Davo, A., Escalante, K., Chávez, C., Cuzon, G., Gaxiola, G. 2014. Probiotic effect of FLOC on *Vibrios* in the Pacific White shrimp *Litopenaeus vannamei*. Aquaculture 424-425, 215-219.

Ahmad, I., Babitha Rani, A.M., Verma, A.K. 2017. Biofloc technology: an emerging avenue in aquatic animal healthcare and nutrition Aquacult Int. doi:10.1007/s10499-016-0108-8

Aminot A. Chaussepied M., 1983.Manuel des analyses chimiques en milieu marin. In: CNEXO, pp 395. Brest.

Andrews, J.H., Harris, J.F. 1986. r- and K- selection in microbial ecology. Adv Microb Ecol 9: 99–147.

APHA (American Public Health Association), 1999. Standard Methods for the Examination of Water and Wastewater, 20st edition, pp 1193. Washington, DC.

Aranguren, L.F., Han, J.E., Thang, K.F.J. 2016. Enterocytozoon hepatopenaei (EHP) is a risk factor for acute hepatopancreatic necrosis disease (AHPND) and septic hepatopancreatic necrosis (SHPN) in the Pacific white shrimp *Penaeus vannamei*. Aquaculture, 10.1016/j.aquaculture.2016.12.038.

Attramadal, K.J.K., Salvesen, I., Xue, R.Y., Øie, G., Storseth, T.R. 2012. Recirculation as a possible microbial control strategy in the production of marine larvae. Aquac Eng 46, 27–39

Attramadal, K.J.K., Truong, T.M., Bakke, I., Skjermo, J., Olsen, Y., Vadstein, O. 2014. RAS and microbial maturation as tools for K-selection of microbial communities improve survival in cod larvae. Aquaculture, 432, 483, 490.

Avinimelech Y. 1999. Carbon/nitrogen ratio as a control element in aquaculture systems. Aquaculture 176, 227-235.

Avnimelech Y. (2007) Feeding with microbial flocs by tilapia in minimal discharge bio-flocs technology ponds. *Aquaculture* **264**, 140–147.

Balcazár J.L., Rojas-Luna T., Cunningham D.P. 2007. Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (*Litopenaeus vannamei*) following immersion challenge with *Vibrio parahaemolyticus.* Journal of Invertebrate Pathology 96, 147-150.

Bossier, P., De Schryver, P., Defoirdt, T., Ruwandeepika, H.A.D., Natrah, F., Ekasari, J., Toi, H., Nhan, D., Tinh, N., Pande, G., Karunasagar I., Van Stappen, G. 2016. Microbial community management in aquaculture. Procedia Food Science, 6, 37-39.

Brown S.P., Cornforth D.M., Mideo N. 2012. Evolution of virulence in opportunistic pathogens: generalism, plasticity, and control. Trends in Microbiology, 20 (7), 336-342.

Cardona, E., Gueguen, Y., Magré, K., Lorgeoux, B., Piquemal, D., Pierrat, F., Noguier, F., Saulnier, D., 2016. Bacterial community characterization of water and intestine of the shrimp Litopenaeus stylirostris in a biofloc system. BMC Microbiology, DOI 10.1186/s12866-016-0770-z.

Chatterjee S., Haldar S. 2012. Vibrio related diseases in aquaculture and development of rapid and accurate identification methods. J. Mar. Sci. Res. Dev. doi:10.4172/2155-9910.S1-002.

Crab R., Avnimelech Y., Defoirdt T., Bossier P., Verstraete W. 2007. Nitrogen removal techniques in aquaculture for a sustainable production. Aquaculture 270, 1–14.

Crab R., Lambert A., Defoirdt T., Bossier P., Verstraete W. 2010. The application of bioflocs technology to protect brine shrimp (*Artemia franciscana*) from pathogenic *Vibrio harveyi.* Journal of Applied Microbiology 109, 1643-1649.

De Schryver P., Defoirdt T., Sorgeloos, P. 2014. Early Mortality Syndrome Outbreaks: A Microbial Management Issue in Shrimp Farming? PLOS Pathogens, 10(4), 1-2.

De Schryver, P., Vadstein, O., 2014. Ecological theory as a foundation to control pathogenic invasion in aquaculture. The ISME Journal, 1-9.

Decamp O., Moriarty D.J.W. 2006. Probiotics as alternative to antimicrobials: Limitations and potential. World Aquac 37(4), 60–62.

Defoirdt T., Boon N., Sorgeloos P., Verstraete W., Bossier P. 2007. Alternatives to antibiotics to control bacterial infections- luminescent vibriosis in aquaculture as an example. Trends Biotechnol 25, 472-479.

Defoirdt T., Sorgeloos P., Bossier P. 2011. Alternatives to antibiotics for the control of bacterial disease in aquaculture. Current Opinion in Microbiology 14, 251-258.

Defoirdt T. 2016 Implications of Ecological Niche Differentiation in Marine Bacteria for Microbial Management in Aquaculture to Prevent Bacterial Disease. PLOS Pathogens, http://dx.doi.org/10.1371/journal.ppat.1005843.

Ebeling J.M., Timmons M.B., Bisogni J.J. 2006. Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic control of ammonia-nitrogen in aquaculture production systems. Aquaculture, 257, 346–358.

Ekasari J., Azhar M.H., Surawidjaja E.H., Nuryati S., De Schryver P., Bossier P. 2014. Immune response and disease resistance of shrimp fed biofloc grown on different carbon sources. Fish and Shellfish Immunology, 41, 332-339.

FAO (2016). The state of world fisheries and aquaculture. FAO, Rome.

Ferreira G.S., Bolívar N.C., Pereira S.A., Guertler C., Vieira F.N., Mouriño J.L.P., Seiffert W.Q. 2015. Microbial biofloc as a source of probiotic bacteria for the culture of *Litopenaus vannamei*. Aquaculture, 448, 273-279.

Furtado P.S., Poersch L.H., Wasielesky W. 2011. Effect of calcium hydroxide, carbonate and sodium bicarbonate on water quality and zootechnical performance of shrimp *Litopenaeus vannamei* reared in bio-flocs technology (BFT) systems. Aquaculture 321(1-2), 30–135.

Fyzul A, N., Harbi A.H., Austin B. 2014. Review: Developments in the use of probiotics for disease control in aquaculture. Aquaculture 431, 1-11.

Gildberg A., Mikkelsen H., Sandaker E. 1997. Probiotic effect of latic acid bacteria in the feed on growth and survival of *Gadus morhua*. Hydrobiologia 352, 279-285.

Gram L., Melchiorsen J., Spanggard B. 1999. Inhibition of *Vibrio anguillarum* by *Pseudomonas flurescens* AH2, a possible probiotic treatment of fish. Applied and Environmental Microbiology 65, 969-973.

Gutierrez-Wing, M.T., Malone, R.F., 2006. Biological filters in aquaculture: trends and

research directions for freshwater and marine applications. Aquac. Eng. 34 (3), 163-171.

Hajek, A. 2004. Natural Enemies: An Introduction to Biological Control. Cambridge University Press: Cambridge.

Hamza, F., Kumar, A.R., Zinjarde, S. 2016. Antibiofilm potential of a tropical marine *Bacillus licheniformis* isolate: role in disruption of aquaculture associated biofilms. Aquaculture Research 47(8), 2661-2669.

Hargreaves, J.A. 2006. Photosynthetic suspended growth systems in aquaculture. Aquacultural Engineering 34, 344-363.

Hargreaves, J.A., 2013. Biofloc production systems for aquaculture. SRAC Publication 4503, 1-12.

Hirono, I., Tinwongger, S., Nochiri, Y., Kondo, H. 2016. Latest research on acute hepatopancreatic necrosis disease (AHPND) of penaeid shrimps. In R. V. Pakingking Jr., E. G. T. de Jesus-Ayson, & B. O. Acosta (Eds.), Addressing Acute Hepatopancreatic Necrosis Disease (AHPND) and Other Transboundary Diseases for Improved Aquatic Animal Health in Southeast Asia: Proceedings of the ASEAN Regional Technical Consultation on EMS/AHPND and Other Transboundary Diseases for Improved Aquatic Animal Health in Southeast Asia, 22-24 February 2016, Makati City, Philippines (pp. 3-10). Tigbauan, Iloilo, Philippines: Aquaculture Department, Southeast Asian Fisheries Development Center.

Jory, D.E., Cabrera, T.R., Dugger, D.M., Fegan, D., Lee, P.G., Lawrence, A.L., Jackson, C.J., Mcintosh, R.P., Castañeda, J. 2001. A global review of shrimp feed manage-ment: status and perspectives. In: The New Wave, Proceedings of the Special Session on Sustainable Shrimp Culture (ed by Browdy C.L., Jory D.E.) pp. 104–152. The World Aquaculture Society, Baton Rouge, LA,.

Kimes, N.E., Grim, C.J., Johnson, W.R., Hasan, N.A., Tall, B.D., Kothary, M.H., Kiss, H., Munk, A.C., Tapia, R., Green, L., Detter, C., Bruce, D.C., Brettin, T.S., Colwell, R.R., Morris, P.J. 2012. Temperature regulation of virulence factors in the pathogen *Vibrio coralliilyticus*. ISME 6, 835-846.

Krummenauer, D., Poersch, L., Romano, L.A., Lara, G.R., Encarnação, P., Wasielesky, W., 2014. The Effect of Probiotics in a *Litopenaeus vannamei* Biofloc Culture System Infected with *Vibrio parahaemolyticus.* Journal of applied Aquaculture 26, 370-379.

Kuhn, D.D., Smith S.A., Boardman, G.D., Angier, M.W., Marsh, L., Flick, J.R. 2010. Chronic toxicity of nitrate to Pacific white shrimp, *Litopenaeus vannamei*: Impacts on survival, growth, antennae length, and pathology. Aquaculture 306, 329-333.

Li, J., Tan, B., Mai, K. 2009. Dietary probiotic *Bacillus* OJ and isomaltooligosaccharides influence the intestine microbial populations, immune responses and resistance to white spot syndrome virus in shrimp (*Litopenaeus vannamei*). Aquaculture 291, 35-40.

Lightner, D.V., Redman, R.M., Pantoja, C.R., Noble, B.L., Tran, L.H. 2012. Early mortality syndrome affects shrimp in Asia. Glob Aquacult Advocate, Jan/Feb 2012: 40.

Lin, Y.C., Chen, J.C. 2001. Acute toxicity of ammonia on *Litopenaeus vannamei* (Boone) juveniles at different salinity levels. J. Exp. Mar. Biol. Ecol. 259,109-119.

Lin, Y.C., Chen, J.C. 2003. Acute toxicity of nitrite on *Litopenaeus vannamei* (Boone) juveniles at different salinity levels. Aquaculture 224, 193-201.

McIntosh, D., Samocha, T.M., Jones, E.R., Lawrence, A.L., Horowitz, S., Horowitz, A. 2001. Effects of two commercially available low-protein diets (21% and 31%) on water and sediment quality, and on the production of *Litopenaeus vannamei* in an outdoor tank system with limited water discharge. Aquacultural Engineering 25, 69-82.

Mohapatra, S., Chakraborty, T., Kumar, V., DeBoeck, G., Mohanta, K.N. 2013. Aquaculture and stress management: a review of probiotic intervention. Animal Physiology and Animal Nutrition 97, 405-430.

Moriarty D.J.W. 1998. Control of luminous *Vibrio* species in penaeid aquaculture ponds. Aquaculture 164, 351-358.

Nimrat, S., Suksawat, S., Boonthai, T., Vuthiphandchai, V. 2012. Potential *Bacillus* probiotics enhance bacterial numbers, water quality and growth during early development of white shrimp (*Litopenaeus vannamei*). Veterinary Microbiology 159, 443-450.

Niu, Y., Defoirdt, T., Baruah, K., Wiele, T.V., Dong, S., Bossier, B. Bacillus sp. LT3 improves the survival of gnotobiotic brine shrimp (*Artemia franciscana*) larvae challenged with Vibrio campbellii by enhancing the innate immune response and by decreasing the activity of shrimp-associated vibrios. Veterinary Microbiology, 173, 279-288.

Phippen, B.L., Ivanina, A.V., Sokolova, I.M., Oliver, J.D. 2016 *Vibrio coralliilyticus* causes rapid immune response and mortality in the Eastern oyster, *Crassostrea virginica*, after exposure to anoxia. Oral Presentation, Vibrio Conference, Roscoff, France.

Rajkumar, M., Pandey, P.K., Aravind, R., Vennila, A., Bharti, V., Sekharan, C., Purushothaman, C.S. 2015. Effect of different biofloc system on water quality, biofloc composition and growth performance in Litopenaeus vannamei (Boone, 1931). Aquaculture Research, 47(11), 3432-3444.

Ray, A.J., Lotz, J.M. 2014. Comparing a chemoautotrophic-based biofloc system and threeheterotrophic-based systems receiving different carbohydrate sources. Aquacultural Engineering, 63, 54–61.

Rungrassamee, W., Klanchui, A., Maibunkaeuw, S., Karoonuthaisiri, N. 2016. Bacterial dynamics in intestines of the black tiger shrimp and the Pacific white shrimp during *Vibrio harveyi* exposure. J Invertebr Pathol. 133, 12-9.

Samocha, T.M., Patnaik, S., Speed, M., Ali, A.M., Burger, J.M., Almeida, R.V., Ayub, Z. 2007. Use of molasses as carbon source in limited discharge nursery and grow-out systems for *Litopenaeus vannamei*. Aquacultural Engineering 36(2), 184–191.

Sapcharoen, P., Rengpipat, S. 2013. Effects of probiotic *Bacillus subtilis* (BP11 and BS11) on the growth and survival of Pacific white shrimp, *Litopenaeus vannamei*. Aquaculture Nutrition 19, 946-954.

Sha, Y., Wang, L., Liu, M., Jiang, K., Xin, F., Wang, B. 2016. Effects of lactic acid bacteria and the corresponding supernatant on the survival, growth performance, immune response and disease resistance of *Litopenaeus vannamei*. Aquaculture 452, 28-36.

Silva, E.F., Soares M.A., Calazans N.F., Vogeley J.L., Valle B.C., Soares, R., Peixoto, S. 2013. Effect of probiotic (*Bacillus spp*.) addition during larvae and postlarvae culture of the white shrimp *Litopenaeus vannamei*. Aquaculture Research, 44, 13-21.

Skjermo, J., Vadstein, O. 1999. Techniques for microbial control in the intensive rearing of marine larvae. Aquaculture 177, 333–343.

Strickland, J.D.H., Parsons, T.R., 1972. A Practical Handbook of Seawater Analysis. Ottawa: Fishery Research Board Canada. 310 pp.

Suita, S.M., Ballester, E.L.C., Abreu, P.C., Wasielesky, W. 2015. Dextrose as a carbono source in the culture of Litopenaeus vannamei (Boone, 1931) in a zero exchange system. Lat. Am. J. Res. 43(3), 526-533.

Tran, L., Nunan, L., Redman, R.M., Mohney, L.L., Pantoja, C.R., Fitzsimmons, K., Lightner, D.V. 2013. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Diseases of Aquatic Organisms 105, 45-55.

UNESCO, 1983. Chemical methods for use in marine environmental monitoring. Manual and Guides 12, Intergovernamental Oceanographic Commissiony. Paris, France.

Vanwyk, P, Scarpa, J. 1999. Water Quality and Management. In*: Farming Marine Shrimp in Recirculating Freshwater Systems* (ed by Van Wyk, P.), pp. 128–138. Florida Department of Agriculture and Consumer Services, Tallahassee.

Villaseñor, I.E.L., Rodríguez, M.E.M., Gomez-Gil, B., Valle, F.A., Córdova, Á.I.C. 2011. Beneficial effects of four *Bacillus* strains on the larva cultivation of *Litopenaeus vannamei.* Aquaculture 321, 136-144.

Villaseñor, I.E.L., Castellano-Cervantes, T., Gomez-Gil, B., Carillo-Garcia, Á.E., Campa-Cordova, Á.I., Ascencio, F. 2013. Probiotics in the intestinal tract of juvenile whiteleg shrimp *Litopenaeus vannamei*: modulation of the bacterial community. World J Microbiol Biotechnol 29, 257-265.

Villaseñor, I.E.L., Voltolina, D., Gomez-Gil, B., Ascencio, F., Campa-Córdova, Á.I., Audelo-Naranjo, J.M., Zamudio-Armenta, O.O. 2015. Probiotic modulation of the

gut bacterial community of juvenile *Litopenaeus vannamei* challenged with *Vibrio parahemolyticus* CAIM 170. Lat. Am. J. Aquat. Res. 43(4), 766-775.

Wang, Y.B. 2007. Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei*. Aquaculture 269, 259-264.

Wasielesky, W., Atwood, H., Stokes, A., Browdy, C.L. 2006. Effect of natural production in a zero exchange suspended microbial floc based super-intensive culture system for white shrimp *Litopenaeus vannamei*. Aquaculture 258, 396–408.

Xu, W.J., Morris, T.C., Samocha, T.M. 2016. Effects of C/N ratio on biofloc development, water quality, and performance of *Litopenaeus vannamei* juveniles in a biofloc-based, high-density, zero-exchange, outdoor tank system. Aquaculture, 453, 169-175.

Zhou, X., Wang, Y., Li, W. 2009. Effect of probiotic on larvae shrimp (*Pennaeus vannamei*) based on water quality, survival rate and digestive enzyme activities. Aquaculture 287, 349-353.

Zhu, S., Chen, S., 2001. Effects of organic carbon on nitrification rate in fixed film biofilters. Aquacultural Engineering 25, 1–11.

Zokaeifar, A., Babaei, N., Saad, C.R., Kamarudin, M.S., Sijam, K., Balcazar, J.L. 2014. Administration of *Bacillus subtilis* strains in the rearing water enhances the water quality, growth performance, immune response, and resistance against *Vibrio harveyi* infection in juvenile white shrimp *Litopenaeus vannamei*. Fish and Shellfish Immunology 36, 68-74.

CAPÍTULO 3

Efficacy and variations in bacterial density in the gut of *Litopenaeus vannamei* reared in a BFT system and in clear water supplemented with a commercial probiotic mixture.

*Artigo submetido à revista Aquaculture

Observação: Tabelas e figuras foram incluídas no corpo do texto para facilitar a leitura da tese, além de um resumo em português.

Eficácia e variações na densidade bacteriana presente no trato intestinal de *Litopenaeus vannamei* cultivado em bioflocos e em água clara, suplementado com probiótico comercial.

Resumo

O objetivo do presente estudo foi avaliar a capacidade de colonização de um probiótico comercial no trato intestinal de *Litopenaeus vannamei* cultivado em BFT e água clara, além de verificar os efeitos do probiótico na densidade bacteriana de *Vibrio* sp. e *Bacillus* sp. adicionado à água. O experimento teve duração de 45 dias e foi composto por 4 tratamentos: Bioflocos sem aplicação de probiótico (**BFT**); Bioflocos com aplicação de probiótico (**BFT+ProW**); água clara sem aplicação de probiótico (**CW**); e água clara com aplicação de probiótico (**CW+ProW**). O probiótico comercial utilizado (Sanolife PRO-W® - INVE Aquaculture) é composto por cepas de *Bacillus subtilis*-complex e *Bacillus licheniformis* e foi adicionado à agua a cada 48 horas. A densidade bacteriana no trato intestinal dos camarões foi verificada utilizando-se a técnica de Hibridização in situ Fluorescente (FISH) na qual sondas de oligonucleotídeos foram utilizadas para identificar e quantificar *Bacillus* sp. e *Vibrio* sp. A densidade microbiana total (cells.10^8 mL^{-1}) foi significativamente maior nos tratamentos com probiótico (BFT+P e CW+P). No entanto, a porcentagem de *Bacillus* sp. relativa à densidade microbiana total foi maior em água clara quando o probiótico foi aplicado. A porcentagem de *Vibrio* sp. foi maior nos tratamentos sem aplicação de probióticos.   O uso de PRO-W® reduziu a densidade bacteriana de *Vibrio* sp. em bioflocos e água clara, enquanto a abundancia de *Bacillus* aumentou. Os resultados obtidos no presente estudo contribuem para o entendimento da ecologia microbiana no sistema de bioflocos, a qual é um ponto chave para o desenvolvimento deste sistema de cultivo.

Abstract

The purpose of this study was to evaluate the colonization ability of a commercial probiotic mixture in the gut of *Litopenaeus vannamei* reared in BFT and a clear water culture and to verify the effects of the probiotic on the gut bacterial abundance of *Vibrio* sp. and *Bacillus* sp. when added to the biofloc or clear water culture system. The 45-day experiment contained four treatments: Biofloc with no Probiotic application (BFT); Biofloc with Probiotic application (BFT+ProW); Clear Water with no Probiotic application (CW); and Clear Water with Probiotic application (CW+ProW). The commercial probiotic mixture was composed of *Bacillus subtilis*-complex and *Bacillus licheniformes* strains and was added to the water every 48 hours. The bacterial abundance in the gut of shrimp was assessed using fluorescence in situ hybridization (FISH) in which rRNA-targeted oligonucleotide probes were used to identify and quantify *Bacillus* sp. and *Vibrio* sp. Total microbial abundance (cells.10^8 mL^{-1}) was higher in the probiotic application treatments (BFT+P and CW+P). However, the percentage of *Bacillus* sp. of the total bacterial abundance was higher in clear water than in BFT when the probiotic mixture was added. The percentage of *Vibrio* sp. was higher in the treatments without probiotic addition. The use of Sanolife PRO-W$^®$ decreased the bacterial abundance of *Vibrio* sp. in both the BFT and clear water culture systems, while the abundance of *Bacillus* increased. The results obtained in this study can contribute to our understanding of the microbial ecology of biofloc, which is currently considered a key issue of this culture system.

Keywords: *Litopenaeus vannamei*; Probiotics; Biofloc Technology; Bacterial density; *Vibrio sp.*; Fluorescence *in situ* hybridization.

1. Introduction

The use of probiotics has been widely explored in aquaculture practice. Therefore, its supplementation is a result of the current investigation to find antibiotic-free and environmental friendly alternatives in order to promote the healthy development of aquaculture (Sha et al., 2016).

By definition, probiotics are live microorganisms that provide health benefits to the host when administered at appropriate levels (Moriarty, 1998). These microorganisms can colonize the intestinal tract and multiply, promoting the efficient modulation of various biological systems in the aquatic host (Gatesoupe, 1999; Mohapatra et al., 2013). They can be administered via the water or as feed additives (Kumar et al., 2016; Moriarty 1998; Skjermo and Vadstein, 1999) with either single or a combination of probiotics in a mixture (Villaseñor et al., 2014).

Different bacterial species are used as probiotics in penaeid cultures, especially bacteria of the genus *Bacillus* (Ninawe and Selvin, 2009; Sapcharoen and Rengpipat, 2013). The ability to form spores, providing protection against acid and bile salts and other environmental conditions, make *Bacilli* efficient probiotics. In addition, species of this genus have a high capacity to produce compounds of biotechnological importance such as antibiotics, enzymes, amino acids, and vitamins. They are also capable of degrading cholesterol, influencing the immune status of the host and have antiviral activity (Sorokulova, 2013).

Bacillus species have been used as a dietary supplement in humans and animals for years. Probiotic *Bacillus* has a higher growth and survival rate (Balcazar et al., 2007; Zhou et al., 2009; Nimrat et al., 2012; Krummenauer et al., 2014b; Zokaeifar et al., 2014), improves the activities of digestive enzymes (Wang, 2007; Zhou et al., 2009), enhances the immune response and confers disease resistance against pathogenic *Vibrio*

species (Gullian et al., 2004; NavinChandram et al., 2014; Sha et al., 2016) in shrimp farming.

Vibrio sp. occur naturally in marine and estuarine environments. They can also be part of the intestinal bacterial community of *L. vannamei*, and some species are even probiotics (Verschuere et al., 2000; Lakshmi et al., 2013; Liu et al., 2015; Huang et al., 2016). However, most species are considered opportunistic bacteria and potential pathogens that cause vibriosis outbreaks and huge production losses (Saulnier et al., 2000).

The gut is considered as the primary pathogen transmission route (De Schryver and Vadstein, 2014). In this context, the probiotic bacteria compete with pathogenic *Vibrio* for adhesion sites, nutrients and chemical compounds in the water and in the gut of the host (Gatesoupe, 1999; Mohapatra et al., 2013; Hamza et al., 2015). Hence, they can stick to the mucosal epithelium of the gastrointestinal tract and help to resist pathogens, by decreasing their numbers in the host (Villaseñor et al., 2011; Akhter et al., 2015; Lazado et al., 2015). It is important to monitor the microbiota during probiotic addition to determine the efficiency of gut colonization and the alterations that occur in the bacterial community due to the administration of these microorganisms (Merrifield et al, 2010; Verscheure et al., 2000).

Positive effects of *Bacillus* on *L. vannamei* have been discovered by observing the antagonistic activity, modulation of the microbiota, a decrease in the number, and an increase in the resistance against Vibrio in conventional - clear water (Li et al., 2009; Nimrat et al., 2011; Zokaeifar et al., 2014; Sha et al., 2016) and in Biofloc Technology culture systems (Aguilera-Rivera et al., 2014; Ferreira et al., 2015).

Biofloc Technology (BFT) is based on the establishment of a microbial community that is responsible for maintaining water quality and improving growth and survival rates (McIntosh, 2001; Wasielesky et al., 2006; Avnimelech, 2007). In addition, this microbial community can inhibit the proliferation of pathogens by competitive exclusion for food and space in the water or in the gut (Crab et al., 2010). Hu and colleagues (2016) verified that the combined use of *Bacillus* and molasses as a carbon source helped to increase the diversity of the microbial community. In addition, this treatment effectively inhibited pathogens and promoted the formation and development of a beneficial microbial community structure in biofloc-rich water.

The interaction between the microbial community present in biofloc-rich water as well as the associated effects of the addition of external probiotic bacteria as a commercial product is still unclear, as is the modulation of the microbial community in terms of the presence, bacterial abundance and composition under these conditions. Knowledge about such interactions is important for establishing strategies for disease control, water quality and microbial community management in biofloc culture systems.

The primary objective of this study was to compare the efficiency of colonization of a commercial probiotic mixture in the gut of *L. vannamei* reared in BFT and clear water culture and to verify the effects of this probiotic *Bacillus* mixture on the abundance of the putative pathogenic *Vibrio* in the presence of a Biofloc Technology Culture System.

2. Material and Methods

2.1. Animal origin

Post-larval *L. vannamei* were obtained from the commercial hatchery Aquatec Ltda (Canguaretama, Rio Grande do Norte, Brazil) and were transferred to the hatchery sector of the Marine Station of Aquaculture, Institute of Oceanography, Federal University of Rio Grande. In the laboratory, shrimp were cultured until they reached an average weight of 0.6 ± 0.02 g before the experimental units were stocked.

2.2 Probiotic Mixture

The commercial probiotic mixture (Sanolife –PRO-W® - INVE Aquaculture) used in this study was composed of *Bacillus subtilis*-complex and *Bacillus licheniformis* strains, at a bacterial concentration of $5x10^{10}$ CFU g^{-1}. The product was added to the culture water every 48 hours, at a concentration of 0.5 ppm or 0.5 mg L^{-1} and activated according to the manufacturer's recommendations

2.3. Experimental design

The trial was carried out in 12 rectangular polyethylene tanks with a bottom area of 0.20 m^2 and an effective volume of 50 L, allocated to an acclimatized room. Shrimp (0.64 ± 0.02 g) were transferred and stocked at 150 shrimp m^{-2} (30 shrimp per tank). The experimental setup contained four treatments with three replicates each: Biofloc with no Probiotics application (BFT); Biofloc with Probiotics application (BFT+ProW); Clear Water with no Probiotics application (CW); and Clear Water with Probiotics application (CW+ProW).

The biofloc experimental units were filled with 5 L of biofloc-rich water (10% reuse water) obtained from an *L. vannamei* grow-out study in nine 35-m^3 tanks, plus 45 L of filtered (sand filter) natural seawater treated with a chlorine solution (10 ppm measured immediately after chlorination) and dechlorinated using ascorbic acid powder (1 ppm). For the clear water treatments, the tanks were filled with 50 L filtered (sand filter) and pre-treated sea water. Dechlorinated municipal freshwater was used to compensate for evaporative losses and to maintain salinity throughout the experiment.

Sugar cane molasses (C:H:N=30.05:2.78:0.79) was added as an organic carbon source when the total ammonia level reached 1.0 mg L^{-1} to foster the development of microbial aggregates in the biofloc treatments. Organic carbon supplementation was based on the addition of 6 g of carbon for each 1 g of total ammonium nitrogen (TA-N) in the water. This procedure followed the methods described by Avnimelech (2009), Ebeling et al. (2006) and Samocha et al. (2007).

A water exchange of 80% was performed every 48 hours in the clear water treatments. Hence, natural, filtered and pre-treated sea water at the same temperature as in the experimental units was used for the renewal procedure.

The shrimp were fed a 40% crude protein (CP) commercial diet (Potimar 40 J, Guabi®, Brazil) twice a day (0800 and 1700 h) for 45 days. The feeding rate was adapted according to the methodology proposed by Jory et al (2001) and was initially 10% of the total biomass.

2.4. *Water quality and sampling*

Dissolved oxygen (DO), temperature and pH were measured twice daily (0800 and 1700 h) using a YSI 556 MPS meter (YSI Inc., Yellow Springs, United States).

Total ammonium nitrogen (UNESCO, 1983) and nitrite-nitrogen (NO_2-N) (Aminot and Chaussepied, 1983) were measured twice a week. Salinity (using a manual optical refractometer), nitrate-nitrogen (NO_3-N) (Aminot and Chaussepied, 1983), total suspended solids (TSS) (Strickland and Parson, 1972) and alkalinity (APHA, 1989) were measured weekly.

Shrimp (5) from each tank (15 shrimp per treatment) were killed by thermal shock in an ice bath and aseptically necropsied to remove the intestinal tract at the end of the experimental period (45 days). The intestinal tract samples were fixed in a final concentration of 2% paraformaldehyde and individually weighed for further analysis.

2.5. Analysis of the presence and efficiency of the commercial probiotic mixture and putative pathogenic bacteria by fluorescence in situ hybridization (FISH)

The intestine samples were processed according to a protocol adapted from Epstein and Rossel (1995). A Tween solution (0.0001%) was added to each sample, which was then sonicated (Vibra Cell VCX 130PB, Sonics & Materials ®) three times (range 110.7 μm per 60 s). After sonication, the samples were centrifuged three times at 500G for five minutes. The supernatant was removed, and the remaining contents were washed twice with ultrapure water. Three supernatant fractions were placed in the same bottle and shaken vigorously. The material was then centrifuged as previously described. Aliquots of each sample were filtered on polycarbonate filters (Nuclepore® - 0.2 μm) and refrigerated until hybridization.

Bacillus genus components of the probiotic mixture added to the biofloc-rich water and putative pathogenic bacteria (*Vibrio* sp.) were identified with rRNA-targeted oligonucleotide probes (Table 1) by a fluorescence *in situ* hybridization (FISH) protocol (Del'Duca et al 2013). A negative control made with a probe with no specificity for

bacteria was used to evaluate the hybridization efficiency. All probes were labeled with the Cy3 fluorochrome. In addition to each specific probe, DAPI was used to determine the total bacterial abundance. The bacterial abundance was determined by direct counting at 1000× magnification using an epifluorescence microscope (Olympus® BX-60) equipped with the Chroma U-N41007, U-MWU2 and U-MWG2 optical filter set.

Table 1: rRNA-targeted oligonucleotide probes of different bacterial species used in this study. All probes were labelled with fluorochrome Cy3.

Probe	Specificity	Sequence 5 – 3'	%FA*	Reference
NON	Negative Control	TAGTGACGCCGTCGA	30	Yokokawa and Nagata 2005
Bacil1	*Bacillus*	GCCGCCTTTCAATTTCGAAC	35	Ichijo et al.2010
Vib519a	*Vibrio*	ACCACCTGCATGCGCTT	40	Hugget et al. 2008

*Percentage of Formamide (FA) in *in situ* Hybridization buffer

2.6. Statistical Analyses

One-way analysis of variance (ANOVA) was used to identify significant differences in bacterial abundance among treatments. The ANOVA was followed by Tukey's post hoc comparison when significant differences were found. Statistical significance was taken as $P< 0.05$.

3. Results

3.1. Water Quality

The water quality parameters are shown in Table 2, and they were divided according to the biofloc (BFT) and clear water (CW) groups. Temperature, dissolved oxygen, pH, salinity and alkalinity did not differ significantly among treatments (Table 2).

Table 2: Mean (± standard deviation) values of water quality parameters throughout the experiment for *L. vannamei* culture in biofloc (BFT) and in clear water (CW) with and without probiotic application. Different letters indicate statistical differences (P<0.05).

Treatment	Temp. (°C)	$D.O_2$ (mg L^{-1})	pH	Salinity	Alkalinity (mg L^{-1})
BFT	27.77 ± 0.87^a	5.72 ± 0.31^a	7.83 ± 0.12^a	32.50 ± 1.95^a	181.25 ± 18.74^a
BFT+P	27.74 ± 0.92^a	5.72 ± 0.34^a	7.82 ± 0.11^a	32.47 ± 2.00^a	175.00 ± 19.71^a
CW	27.63 ± 0.85^a	5.86 ± 0.40^a	7.93 ± 0.07^a	29.22 ± 2.55^a	181.25 ± 15.30^a
CW+P	27.66 ± 0.91^a	5.88 ± 0.42^a	7.94 ± 0.07^a	29.08 ± 2.44^a	175 ± 13.69^a

The levels of nitrogen compounds, total ammonia nitrogen (TAN) and nitrite (NO_2- N) were significantly higher in the CW and CW+P treatments. On the other hand, the nitrate (NO_3-N) concentrations were significantly higher in the BFT and BFT+P treatments. A total suspended solids measurement was performed only for the BFT and BFT+P treatments (Table 3) because no accumulation of significant amounts of particulate organic matter or suspended solids occurred in the clear water systems.

Table 3: Mean (± standard deviation) values of nitrogen compounds (total ammonia nitrogen – TAN, nitrite – NO_2-N and nitrate – NO_3-N) and total suspended solids (TSS) of *L. vannamei* culture in biofloc (BFT) and clear water (CW) with and without probiotic application. Different letters indicate statistical differences (P<0.05).

| Treatment	TAN (mg L^{-1})	NO_2-N (mg L^{-1})	NO_3-N (mg L^{-1})	TSS (mg L^{-1})
BFT	0.04 ± 0.007^a	0.18 ± 0.09^a	50.36 ± 12.41^b	420.50 ± 89.44
BFT+P	0.04 ± 0.005^a	0.16 ± 0.09^a	53.06 ± 12.45^b	425.50 ± 102.41
CW	0.38 ± 0.16^b	0.43 ± 0.21^b	0.10 ± 0.22^a	-
CW+P	0.40 ± 0.13^b	0.42 ± 0.25^b	0.12 ± 0.23^a	-

3.2 Bacterial density

The total bacterial density in the gut of *L. vannamei* cultured in the BFT+ProW treatment ($3.67\pm0.18\cdot10^8$ cells g^{-1}) was significantly higher, followed by the CW+ProW ($2.12\pm0.3\cdot10^{-8}$ cells g^{-1}) treatment. The treatments with no ProW® supplementation (BFT – $1.65\pm0.9\cdot10^8$ cells g^{-1}; CW – $1.53\pm0.04\cdot10^8$ cells g^{-1}) did not differ between each other and had the lowest bacterial abundance (Figure 1).
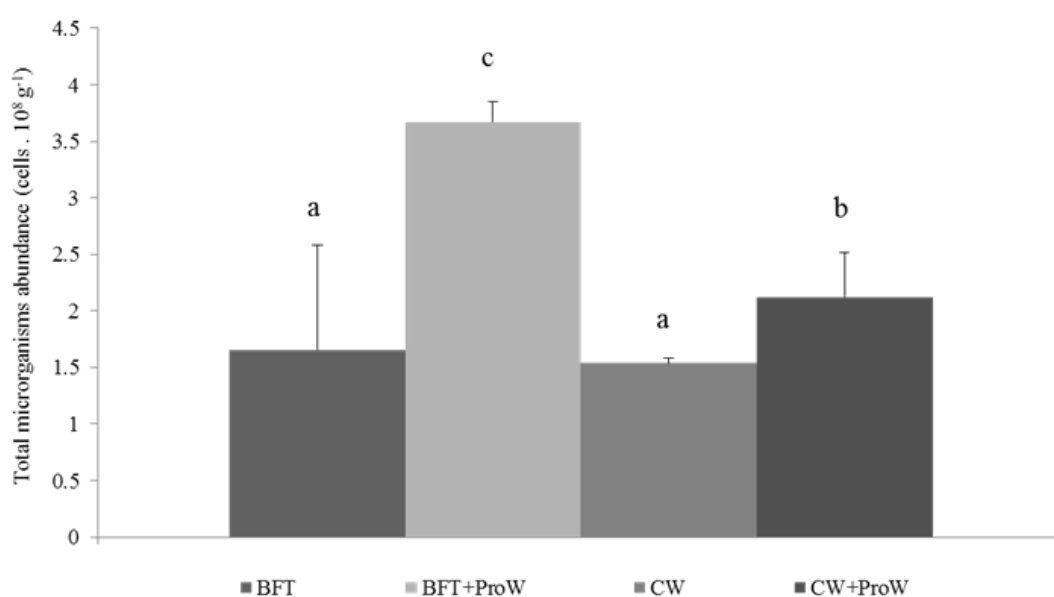
Figure 1: Total abundance of microorganisms ± Standard Deviation (cells $\cdot10^8$ g^{-1}) in the *L. vannamei* gut in the BFT, BFT+ProW, CW and CW+ProW treatments. Different letters indicate statistical differences (P<0.05).

Regarding the bacterial density of *Bacillus* as the component of the probiotic mixture, a higher number of cells was detected in the treatments containing ProW (BFT+ProW – $0.63\pm0.06\cdot10^8$ cells g^{-1}; CW+ProW – $0.69\pm0.09\cdot10^8$ cells g^{-1}; BFT- $0.18\pm0.18\cdot10^8$ cells g^{-1}; CW- $0.07\pm0.07\cdot10^8$ cells g^{-1}).

On the other hand, the density of *Vibrio* was higher in both treatments without the probiotic (BFT- $0.23\pm0.04\cdot10^8$ cells g^{-1}; CW- $0.27\pm0.04\cdot10^8$ cells g^{-1}; BFT+ProW – $0.06\pm0.01\cdot10^8$ cells g^{-1}; CW+ProW – $0.07\pm0.02\cdot10^8$ cells g^{-1}) (Figure 2).
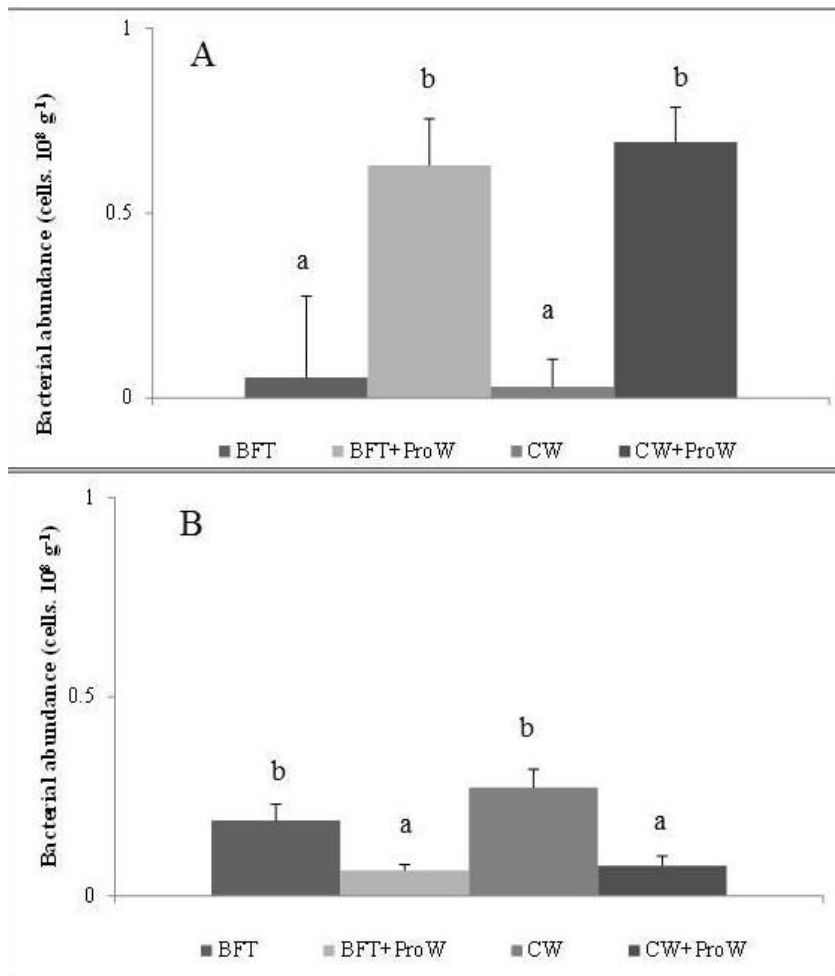
Figure 2: Specific bacterial abundance $\pm$ Standard Deviation (cells $\cdot 10^8 \ g^{-1}$) of *Bacillus sp.* (A) and *Vibrio sp.* (B) in the *L. vannamei* gut in the BFT, BFT+ProW, CW and CW+ProW treatments. Different letters indicate statistical differences (P<0.05).

Regarding the percentage of *Bacillus* and *Vibrio* to the total bacterial abundance, the most significant contribution of *Bacillus* was in the CW+P treatment, followed by the BFT+P treatment. The BFT and CW treatments showed the lowest contribution of *Bacillus* sp. to the total bacterial abundance.

On the other hand, *Vibrio sp.* percentage was significantly higher in the treatments with no probiotic application (BFT and CW) and lower in the treatments in which the probiotic mixture was administered (Table 4).

Table 4: Percentage (Mean ± Standard Deviation) of *Bacillus sp.* and *Vibrio sp.* in the total bacterial abundance observed in BFT and clear water with and without probiotic application. Different letters indicate statistical differences (P<0.05).

	BFT	BFT+P	CW	CW+P
Bacillus sp	3.58±3.08[a]	17.26±4.14[b]	1.90±1.08[a]	32.85±2.46[c]
Vibrio sp	17.77±9.80[b]	1.75±0.49[a]	17.74±3.24[b]	3.54±0.53[a]

4. Discussion

In addition to promoting direct host benefits, a probiotic can control water quality and modify the aquatic community structure by interacting with other microorganisms (Verschuere et al., 2000; Neway-Fyzul et al., 2014; Dawood and Koshio, 2016). Nimrat et al. (2012) investigated the effectiveness of a mixed *Bacillus* probiotic during the rearing of *L. vannamei* in the larval and post-larval stages. The authors found positive results for water quality, especially for pH and the ammonia level, and observed that the number of beneficial bacteria was significantly higher in the culture water.

The application of a probiotic mixture did not change the water quality parameters analyzed in this study. Water temperature, dissolved oxygen, pH, salinity and alkalinity were similar between treatments with and without probiotic in the clear water and BFT systems. These values remained within the proper range for rearing *L. vannamei* (Van Wyk and Scarpa, 1999; Furtado et al., 2011). However, the mean values of total suspended solids (TSS) in the biofloc treatments were below the range recommended by Gaona et al. (2016) for *L. vannamei*.

No differences were observed in the concentration of nitrogen compounds (TAN, NO_2-N and NO_3-N) between treatments with and without probiotic. However, in the BFT treatments, lower concentrations of ammoniacal nitrogen and higher nitrate levels were observed, indicating that nitrification occurred, but it did not occur in the clear water treatments. However, the nitrogen concentrations observed in the clear water treatments were maintained in the optimal range for *L. vannamei* (Lin and Chen, 2001; Lin and Chen, 2003; Kuhn et al., 2010) via water exchanges performed every 48 hours.

Total nitrogen (TN) and total organic carbon (TOC) decreased significantly in *L. vannamei* culture ponds treated with a commercial probiotic mixture containing *Bacillus* sp., *Lactobacillus* sp., *Nitrosomonas* sp. and *Nitrobacter* sp. (Wang et al., 2009). The nitrifying bacteria should have been primarily responsible for these reductions. However, Gram-positive bacteria, including *Bacillus*, can perform heterotrophic nitrification and aerobic denitrification using organic matter substrates. These capacities could represent an advantage over the traditional removal of nitrogen by autotrophic bacteria in conventional pond culture systems (Song et al., 2011; Yang et al., 2011; Zhang et al., 2012).

The nitrification observed in the BFT treatments could possibly be performed by other microorganisms present in the floc, as a diversity of photoautotrophic, chemoautotrophic and heterotrophic microorganisms is found in this culture system (Ebeling et al., 2006). Some heterotrophic microorganisms have been reported to nitrify and/or transform many types of nitrogen compounds (Yang et al., 2011). These mechanisms occur by converting the nitrogen in the microbial biomass in the presence of oxygen and an organic carbon source, which is the main principle of biofloc formation (Avnimelech, 1999; Ebeling et al., 2006). Moreover, the autotrophic

ammonia nitrogen oxidizing reaction carried out by the nitrifying bacteria can occur simultaneously in the water (Hargreaves 2006), even though these bacteria are known to have a slower growth rate and are more sensitive to variations in water quality parameters such as pH and temperature (Crab et al., 2007; Ebeling et al., 2006). The occurrence of this process is evidenced in the present study by the accumulation of NO_3-N, which has similarly been reported by Krummenauer et al. (2014) for the culture of *L. vannamei* in a Biofloc system.

Normally, the colonization of the mucosal membrane by *Bacillus* is not permanent. The density of these bacteria can be controlled by varying the dose of probiotic. When added to the water, the probiotic can be absorbed via host osmoregulation processes and through the feed (Kesarcodi-Watson et al., 2008). The establishment of the probiotic in the gastrointestinal tract of the host is directly related to the dosage and ingestion time. The higher the concentration of probiotic in the water, the shorter is the ingestion time for the shrimp (Riquelme et al., 2001). In the present study, the probiotic mixture was added to the water at a concentration of $12.5.10^5$ CFU L^{-1} every 48 hours. The intestine of the shrimp was colonized by the probiotic *Bacillus* mixture added to both the BFT and CW systems at the end of the 45-day experiment.

Hence, the addition of the probiotic mixture to the water can facilitate the colonization of *Bacillus* in the gut by increasing its numbers (Nimrat et al., 2012) and can decrease the presence of *Vibrio* (Silva et al., 2013). De Schryver and Vadstein, (2014) suggested that aquatic organisms have a closer association between the host intestinal microbiota and the environmental microbiota. Likewise, Del`Duca et al., (2015) verified that a strong similarity in the bacterial community exists among the gut, the water and the pond sediment in cultured tilapia. Thus, a probiotic can be used as a

strategy for manipulating the microbial diversity and controlling pathogenic infections in the gut of the host when applied on a rational basis.

The use of an experimental or commercial probiotic, predominantly *Bacillus* spp., promoted resistance against *Vibrio* infections in previous studies. These results were confirmed by challenge tests (Balcazar et al., 2007; Villaseñor et al., 2015), antagonistic activity *in vitro* (Liu et al., 2015) or presumptive *Vibrio* counts (Silva et al, 2013). These confirmations are in concordance with the present study in which similar results for the bacterial abundance of *Vibrio* and *Bacillus* in the gut of *L. vannamei* reared in BFT and clear water were obtained.

As previously mentioned, the quantification of specific bacteria of the *Bacillus* and *Vibrio* genera in the gut of *L. vannamei* showed an antagonist tendency. Higher numbers of *Bacillus* (BFT+ProW and CW+ProW) were associated with a lower abundance of *Vibrio* (BFT and CW) and vice versa. One of the principal modes of action of a probiotic is competition for adhesion sites and colonization of the intestine, as well as competition for chemicals or available energy, avoiding the establishment of harmful pathogens (Gatesoupe, 1999; Mohapatra et al., 2013), and also exerting anti-adhesive activity against *Vibrio harveyi* (Hamza et al., 2015).

The presence and the number of probiotic organisms in this study induced a higher total bacterial abundance in the intestinal tract of *L. vannamei* in a BFT system than in clear water (CW), which would probably affect the bacterial diversity and the composition of the microbiota, which can be affected by the contact with the surrounding environment and the feed intake (Tzuc et al., 2014). The addition of a commercial probiotic mixture containing *Bacillus* was able to modify the gut bacterial community in *L. vannamei*, enhancing the bacterial diversity and decreasing the number

of *Vibrio* sp. in clear water (Villaseñor et al., 2014; Li et al., 2009). The probiotic seemed to have greater ability to colonize the intestinal tract in this study, and it represented greater than 30% of the total bacteria in the shrimp gut.

The use of BFT as a culture system contributed to an increase in the bacterial abundance in the gut of *L. vannamei*, with or without the addition of the probiotic mixture. Similarly, Hu et al. (2016) assessed the effect of the combined use of a probiotic and molasses as a carbon source on the microbial community structure and verified that this combination promoted the formation and development of a beneficial microbial community structure in a high-density intensive aquaculture of *L. vannamei*. These results corroborate those of the present study in which a combination of molasses and a probiotic enhanced the total bacterial abundance in the BFT treatments.

Bacillus species such as *B. subtilis* and *B. amyloliquefaciens* can auto-aggregate and co-aggregate (Algburi et al., 2016). Auto-aggregation of probiotic strains may be required for their adhesion to host epithelial cells in the gut. As in a biofloc, co-aggregation may facilitate the integration of exogenous bacteria, allowing the development of a multispecific biofilm. Additionally, this ability may also allow probiotic organisms to create a barrier, which could effectively prevent colonization by pathogens (Kos et al., 2003; Algburi et al., 2016)

The use of a commercial probiotic mixture can contribute to homeostasis and prevent outbreaks of opportunistic pathogenic bacteria in a BFT system and clear water. A synergistic effect is possible between the probiotic and the heterotrophic bacterial community in the biofloc for *L. vannamei*, which could prevent the development of lesions in the hepatopancreas caused by pathogenic *Vibrio* bacteria in the water (Aguillera-Rivera et al., 2014).

Ferreira et al., (2015) investigated the probiotic properties of *Bacillus sp.* isolated from biofloc-rich water and supplemented to *L. vannamei*. They verified a lower number of *Vibrio* in the water in the experimental units treated with *Bacillus*, which showed antagonism against *Vibrio harveyi*. Isolated *Bacillus sp.* bacteria from the biofloc-rich water of an *L. vannamei* culture were assessed for antagonistic *in vitro* activity and the total number of *Vibrio* and *Bacillus* in the water by a plating technique. The authors observed in vitro inhibitory characteristics of *Bacillus* against the putative pathogen *Vibrio alginolyticus* and a reduction of the prevalence of *Vibrio sp.* in the water when cultures were supplemented with the probiotic.

Different factors affect the effectiveness of probiotic bacteria. For example, *Bacillus* enzymes are active in living and dead cells (Tan and Qian, 1996). In this context, most of the viability analyses and antagonism tests are performed with bacteria grown in culture medium. However, this technique may present some methodological difficulties, since the assessment of *Bacillus* and *Vibrio* growth is affected by the culture medium and can cause false negative results (Letchumanan et al., 2014).

Many culture-independent molecular biology techniques are based on the amplification of nucleic acids with qualitative or semi-quantitative results. In these cases, it is not possible to visualize each individual and to evaluate the spatial distribution of aggregated or non-aggregated microorganisms. However, the FISH technique allows the quantification of individual microorganism cells. It is an efficient tool to visualize and quantify the specifically labeled bacteria in this study. This technique has been applied in aquaculture to characterize the microbiota and bacterial diversity of water and waste water (Garcia and Olmos, 2007; Paungfoo et al., 2007; Payne et al., 2007; Pereira et al., 2011) and the intestinal tract of fish (Asfie et al., 2003;

Balcázar et al., 2010; Huber et al., 2004) and oysters (Hernandez-Zarate and Olmos, 2006). Additionally, Del'Duca et al. (2013; 2015) used the FISH technique to evaluate the efficiency of *Bacillus sp.* as a probiotic and its ability to control putative pathogenic bacteria in the gut of tilapia. The authors suggest that the FISH technique can quantify and follow changes in the number of probiotic and pathogenic microorganisms. Thus, the microbial community structure (i.e., the taxa and number of each taxa of bacteria) allows an assessment of the probiotic efficiency (Del'Duca et al., 2013).

The total bacterial abundance quantified in the present study was higher in the BFT system in the presence of the probiotic mixture. However, the results also indicate that *Bacillus sp.* can colonize the gut more effectively when *L. vannamei* is cultured in clear water than in a BFT system, which can possibly be attributed to the composition of the biofloc system in which a large diversity of microorganisms take part (De Schryver et al., 2008). On the other hand, the proportion of *Vibrio* related to the total microorganisms was lower in the treatment containing the probiotic mixture, which can be associated with the role of the biofloc in controlling or encouraging potential pathogenic bacteria, especially *Vibrio* (Hargreaves, 2013).

Hence, these observations indicate that adding exogenous bacteria to an existing bacterial community can affect the dynamics and interactions between the various groups of organisms that compose the biofloc system. These indications can be further reflected in different approaches to managing the biofloc or the probiotic application. Thus, it is possible to reinforce the importance of probiotic supplementation, especially in conventional clear water culture systems.

5. Conclusions

The commercial probiotic mixture composed of *Bacillus subtilis* and *B. licheniformes* colonized the intestinal tract of *L. vannamei* and controlled the putative pathogenic populations of *Vibrio sp.*

The difference in efficiency of ProW® between the two culture systems was in proportion of *Bacillus* to the total microorganisms in the gut of the shrimp. Higher probiotic colonization occurred in CW. The intestinal tract of the shrimp farmed in BFT had a greater number of microorganisms in the presence of the probiotic.

The addition of the probiotic did not modify the water quality in either system. Likewise, it promoted a decrease in the density of *Vibrio* and an increase of *Bacillus* in the shrimp gut in both systems.

The number of pathogenic and probiotic individual cells as well as the proportion are important in shrimp farming. The FISH technique is a tool that allows for the quantification and monitoring of a probiotic along with the detection of putative pathogens or any other type of bacteria present in the digestive tract of shrimp and in the water.

Additionally, the results obtained in this study can contribute to our understanding of the microbial ecology of a biofloc. It is currently considered a key issue of this culture system.

Aquaculture for the support in the experimental use of the probiotic mixture (ProW®).

W.J. Wasielesky Jr. and D.E Cesar are research fellows at CNPq.

6. References

Aguillera-Rivera, D., Prieto-Davo, A., Escalante, K., Chávez, C., Cuzon, G., Gaxiola, G., 2014. Probiotic effect of FLOC on *Vibrios* in the Pacific White shrimp *Litopenaeus vannamei*. Aquaculture, 424-425, 215-219.

Akhter, N., Wu, B., Memon, A.M., Mohsin, M., 2015. Probiotics and prebiotics associated with aquaculture: A review. Fish and Shellfish Immunology, 45, 733-741.

Algburi, A., Zehm, S., Netrebov, V., Bren, AB., Chistyakov, V., Chikindas, M.L., 2016. Subtilosin Prevents Biofilm Formation by Inhibiting Bacterial Quorum Sensing, doi:10.1007/s12602-016-9242-x.

Aminot, A., Chaussepied, M., 1983. Manuel des analyses chimiques en milieu marin. CNEXO, Brest. 395 pp.

APHA (American Public Health Association), 1998. Standard Methods for the Examination of Water and Wastewater, 20st edition. . Washington, DC. 1193 pp.

Asfie, M., Yoshijima, T., Sugita, H., 2003. Characterization of the goldfish fecal microflora by the fluorescent in situ hybridization method. Fisheries Science 69, 21–26.

Avinimelech, Y., 1999. Carbon/nitrogen ratio as a control element in aquaculture systems. Aquaculture 176, p. 227-235.

Avnimelech, Y., 2007. Feeding with microbial flocs by tilapia in minimal discharge bio-flocs technology ponds. Aquaculture 264, 140–147.

Avnimelech, Y., 2009. Biofloc Technology: A practical Hand Book. The World Aquaculture Society, Baton Rouge, Louisiana, United States.

Balcázar, J., Lee, N., Pintado, J., Planas, M., 2010. Phylogenetic characterization and in situ detection of bacterial communities associated with seahorses (*Hippocampus guttulatus*) in captivity. Systematic and Applied Microbiology 33, 71–77.

Balcazár, J.L., Rojas-Luna, T., Cunningham D.P., 2007. Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (*Litopenaeus vannamei*) following immersion challenge with *Vibrio parahaemolyticus*. Journal of Invertebrate Pathology 96, 147-150.

Castex, M., Chim, L., Pham, D., Lemaire, P., Wabete, N., Nicolas J.L., Schmidely, P., Mariojouls, C., 2008. Probiotic *P. acidilactici* application in shrimp *Litopenaeus stylirostris* culture subject to vibriosis in New Caledonia. Aquaculture 275, 182-193.

Crab, R., Avnimelech, Y., Defoirdt, T., Bossier, P. and Verstraete, W. 2007 Nitrogen removal techniques in aquaculture for a sustainable production. Aquaculture 270, 1–14.

Crab, R., Lambert, A., Defoirdt, T., Bossier, P., Verstraete, W., 2010. The application of bioflocs technology to protect brine shrimp (*Artemia franciscana*) from pathogenic *Vibrio harveyi*. Journal of Applied Microbiology 109, 1643-1649.

D'Alvise, P.W., Lillebo, S., Wergeland, H.I., Gram, L., Bergh, O., 2013. Protection of cod larvae from vibriosis by *Phaeobacter* spp.: A comparision of strains and introduction times. Aquaculture 384-387, 82-86.

Dawood, M.A.O., Koshio, S., 2016. Recent advances in the role of probiotics and prebiotics in carp aquaculture: A review. Aquaculture 454, 243-251.

De Schryver, P., Vadstein, O., 2014. Ecological theory as a foundation to control pathogenic invasion in aquaculture. The ISME Journal, 1-9.

De Schryver, P; Crab, R., Defoirdt, T., Boon, N., Verstraete, W., 2008. The basics of bio-flocs technology: The added value for aquaculture. Aquaculture 277(3-4): 125–137.

Decamp, O., Moriarty, D.J.W., 2006. Probiotics as alternative to antimicrobials: Limitations and potential. World Aquac 37(4), 60–62.

Del`Duca, A., Cesar, D.E., Abreu, P.C., 2015. Bacterial community of pond's water, sediment and in the guts of tilapia (*Oreochromis niloticus*) juveniles characterized by fluorescent *in situ* hybridization technique. Aquaculture Research 46(3), 707-715.

Del'Duca A., Cesar, D.E., Diniz, C.G., Abreu, P.C., 2013. Evaluation of the presence and efficiency of potential probiotic bacteria in the gut of tilapia (*Oreochromis niloticus*) using the fluorescent in situ hybridization technique. Aquaculture 388-391, 115-121.

Ebeling, J.M., Timmons, M.B., Bisogni, J.J., 2006. Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic control of ammonia-nitrogen in aquaculture production systems. Aquaculture 257, 346–358.

Epstein, S., Rossel, J., 1995. Enumeration of sandy sediment bacteria: search for optimal protocol. Marine Ecology Progress Series 117, 289–298.

FAO, 2016. The state of world fisheries and aquaculture. FAO, Rome.

Ferreira, G.S., Bolívar, N.C., Pereira, S.A., Guertler, C., Vieira, F.N., Mouriño, J.L.P., Seiffert, W.Q., 2015. Microbial biofloc as a source of probiotic bacteria for the culture of *Litopenaus vannamei*. Aquaculture 448, 273-279.

Furtado, P.S., Campos, B.R., Serra, F.P., Klosterhoff, M., Romano, L.A., Wasielesky, W., 2014. Effects of nitrate toxicity in the Pacific white shrimp Litopenaeus vannamei, reared with biofloc technology (BFT). Aquacult. Int. 23, 315.

Furtado, P.S., Poersch, L.H., Wasielesky, W. 2011. Effect of calcium hydroxide, carbonate and sodium bicarbonate on water quality and zootechnical performance of shrimp *Litopenaeus vannamei* reared in bio-flocs technology (BFT) systems. Aquaculture 321(1-2), 130–135.

Garcia, A.T., Olmos, J.S., 2007. Quantification by fluorescent in situ hybridization of bacteria associated with *Litopenaeus vannamei* larvae in Mexican shrimp hatchery. Aquaculture 262, 211-218.

Gatesoupe, F.J., 1999. The use of probiotics in aquaculture. Aquaculture 180, 147-165.

Gullian, M., Thompson, F., Rodriguez, J., 2004. Selection of probiotic bacteria and study of their immunostimulatory effect in *Penaeus vannamei*. Aquaculture, 233, 1-14.

Hai, N.V., Fotedar, R., Buller, N., 2007. Selection of probiotics by various inhibition test methods for use in the culture of western king prawns, *Penaeus latisulcatus* (Kishinouye). Aquaculture 272, 231-239.

Hamza, F., Kumar, A.R., Zinjarde, S., 2016. Antibiofilm potential of a tropical marine *Bacillus licheniformis* isolate: role in disruption of aquaculture associated biofilms. Aquaculture Research 47(8), 2661-2669.

Hargreaves, J.A. 2006. Photosynthetic suspended growth systems in aquaculture. Aquacultural Engineering 34, 344-363.

Hargreaves, J.A., 2013. Biofloc production systems for aquaculture. SRAC Publication 4503, 1-12.

Hernandez-Zarate, G., Olmos-Soto, J., 2006. Identification of bacterial diversity in the oyster *Crassostrea gigas* by fluorescent in situ hybridization and polymerase chain reaction. J Appl Microbiol 100, 664–672.

Hu, X., Cao, Y., Wen, G., Zhang, X., Xu, Y., Xu, W., Xu, Y., Li, Z., 2016. Effect of combined use of *Bacillus* and molasses on microbial communities in shrimp cultural enclosure systems. doi:10.1111/are.13101

Huang, Z., Li, X., Wang, L., Shao, Z., 2016.Changes in the intestinal bacterial community during the growth of white shrimp, *Litopenaeus vannamei.* Aquaculture Research 47, 1737-1746.

Huber, I., Spangaard, K., Appel, K., Rossen, L., Nielsen, T., Gram, L., 2004. Phylogenetic analysis and in situ identification of the intestinal microbial community of rainbow trout (*Oncorhynchus mykiss*, Walbaum). Journal of Applied Microbiology 96, 117–132.

Jory, D.E., Cabrera, T.R., Dugger, D.M., Fegan, D., Lee, P.G., Lawrence, A.L., Jackson,C.J., Mcintosh, R.P., Casta˜neda, J., 2001. A global review of shrimp feed manage-ment: status and perspectives. In: Browdy, C.L., Jory, D.E. (Eds.), The New Wave,Proceedings of the Special Session on Sustainable Shrimp Culture, Aquaculture2001. The World Aquaculture Society, Baton Rouge, LA, pp. 104–152.

Kesakordi-Watson, A., Kaspar, H., Lategan, M.J., Gibson, L., 2008. Probiotics in Aquaculture: The need, principles and mechanisms of action and screening processes. Aquaculture, Oxford, 274, 1-14.

Kos, B., Suskovic, J., Vukovic, S., Simpraga, M., Frece, J., Matosic, S., 2003. Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. Journal of applied Microbiology, 94, 981-987.

Krummenaeur, D., Samocha, T., Poersch, L., Lara, G.R., Wasielesky, W., 2014 The Reuse of Water on the Culture of Pacific White Shrimp, *Litopenaeus vannamei*, in BFT System. Journal of the World Aquaculture Society 45 (1), 3-14.

Krummenauer, D., Poersch, L., Romano, L.A., Lara, G.R., Encarnação, P., Wasielesky, W., 2014.The Effect of Probiotics in a *Litopenaeus vannamei* Biofloc Culture System Infected with *Vibrio parahaemolyticus.* Journal of applied Aquaculture 26, 370-379.

Kuhn, D.D., Smith, S.A., Boardman, G.D., Angier, M.W., Marsh, L., Flick J.R., 2010. Chronic toxicity of nitrate to Pacific white shrimp, *Litopenaeus vannamei*: Impacts on survival, growth, antennae length, and pathology. Aquaculture 306, 329-333.

Kumar, V., Roy, S., Meena, D.K., Sarkar, U.K., 2016. Application of Probiotics in Shrimp Aquaculture: Importance, Mechanisms of Action, and Methods of Administration. Reviews in Fisheries Sciences and Aquaculture, 24(4), 342-368.

Lakshimi, B., Viswanath, B., Gopal, D.V.R.S., 2013. Probiotics as Antiviral Agents in Shrimp Aquaculture. Journal of Pathogens, http://dx.doi.org/10.1155/2013/424123.

Lazado, C.C., Caipang, C.M.A., Estante E.G., 2015. Prospects of host-associated microorganisms in fish and penaeids as probiotics with immunomodulatory functions. Fish and Shellfish Immunology 45, 2-12.

Letchumanan V., Chan K.G., Lee L.H., 2014. *Vibrio parahaemolyticus*: a review on the pathogenesis, prevalence, and advance molecular identification techniques. Front. Microbiol., 5,705, 10.3389/fmicb.2014.00705.

Li, J., Tan, B., Mai, K., 2009. Dietary probiotic *Bacillus* OJ and isomaltooligosaccharides influence the intestine microbial populations, immune responses and resistance to white spot syndrome virus in shrimp (*Litopenaeus vannamei*). Aquaculture 291, 35-40.

Lightner, D.V., 1996. "A Handbook of Shrimp Pathology and Diagnostic Procedures for Disease of Cultured Penaeid Shrimp." World Aquacult. Soc., Baton Rouge, LA.

Lightner, D.V., Tran, L., Nunan, L., Redman, R.M., Mohney, L.L., Pantoja, C.R., Fitzsimmons, K., 2013. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Dis Aquat Org., 105, 45–55.

Lin, Y.C., Chen, J.C., 2001. Acute toxicity of ammonia on *Litopenaeus vannamei* (Boone) juveniles at different salinity levels. J. Exp. Mar. Biol. Ecol. 259,109-119.

Lin, Y.C., Chen, J.C., 2003. Acute toxicity of nitrite on *Litopenaeus vannamei* (Boone) juveniles at different salinity levels. Aquaculture 224, 193-201.

Liu, X.F., Li, Y., Li, J.R., Cai, L.Y., Li, X.X., Chen, J.R., Lyu, S.X., 2015. Isolation and characterisation of *Bacillus* spp. antagonistic to *Vibrio parahemolyticus* for use as probiotics in aquaculture. World J Micobriol Biotechnol 31, 795-803.

McIntosh, D., Samocha, T.M., Jones, E.R., Lawrence, A.L., Horowitz, S., Horowitz, A., 2001. Effects of two commercially available low-protein diets (21% and 31%) on water and sediment quality, and on the production of *Litopenaeus vannamei* in an outdoor tank system with limited water discharge. Aquacultural Engineering 25, 69-82.

Merrifield, D., Dimitroglou, A., Foey, A., Davies, J., Baker, R., Bøgwald, J., Castex, M., Ringø, E., 2010. The current status and future focus of probiotic and prebiotic applications for salmonids. Aquaculture 302, 1–18.

Mohapatra, S., Chakraborty, T., Kumar, V., DeBoeck, G., Mohanta, K.N., 2013. Aquaculture and stress management: a review of probiotic intervention. Animal Physiology and Animal Nutrition 97, 405-430.

Mohney, L.L., Lightner, D.V., BELL, T.A., 1994. An epizootic of vibriosis in Ecuadorian pond reared *Penaeus vannamei* Boone (Crustacea: Decapoda) J. World Aquacult. Soc., 25, 116–125.

Moriarty, D., 2003. Probiotics in Aquaculture. Microbiology Australia 3, 15-17.

Moriarty, D.J.W., Control of luminous *Vibrio* species in penaeid aquaculture ponds. Aquaculture 164, 351-358, 1998.

Navinchandran, M., Iyapparaj P., Moovendhan, S., Ramasubburayan, R., Prakash, S., Immanuel, G., Palavesam, A., 2014. Influence of probiotic bacterium *Bacillus cereus* isolated from the gut of wild shrimp *Penaeus monodon* in turn as a potent growth promoter and immune enhancer in *P. monodon*. Fish and Shellfish Immunology 36, 38-45.

Nimrat, S., Boonthai, T., Vuthiphandchai V., 2011. Effects of probiotic forms, compositions and mode of probiotic administration on rearing of Pacific white shrimp (*Litopenaeus vannamei*) larvae and postlarvae. Animal Feed Science and Technology 169, 244-258.

Nimrat, S., Suksawat, S., Boonthai, T., Vuthiphandchai, V., 2012. Potential *Bacillus* probiotics enhance bacterial numbers, water quality and growth during early development of white shrimp (*Litopenaeus vannamei*). Veterinary Microbiology 159, 443-450.

Newaj-Fyzul, A., Harbi, A.H., Austin, B., 2014. Review: Developments in the use of probiotics for disease control in aquaculture. Aquaculture 431, 1-11.

Ninawe, A.S., Selvin, J., 2009. Probiotics in shrimp aquaculture: avenues and challenges. Crit. Rev. Microbiol., 35 (1), 43-66.

Paungfoo, C., Prasertsan, C., Burrell, P., 2007. Nitrifying bacterial communities in an aquaculture waste water treatment system using fluorescence in situ hybridization (FISH), 16S rRNA gene cloning, and phylogenetic analysis. Biotechnology and Bioengineering 97, 985–990.

Payne, M., Hall, M., Sly, L., Bourne, D., 2007. Microbial diversity within early-stage cultured *Panulirus ornatus* Phyllosomas. Applied and Environmental Microbiology 73, 1940–1951.

Pereira, C., Salvador, S., Arrojado, C., Silva, Y., Santos, A., Cunha, A., Newton Gomesand, N., Almeida, A., 2011. Evaluating seasonal dynamics of bacterial communities in marine fish aquaculture: a preliminary study before applying phage therapy. Journal of Environmental Monitoring 13, 1053–1058.

Riquelme, C.E., Jorquera, M.A., Rojas A.I., Avendaño, R.E., Reyes, N., Addition of inhibitor-producing bacteria to mass cultures of *Argopecten purpuratus* larvae (Lamarck, 1819). Aquaculture, 192 (2-4), 111-119.

Samocha, T.M., Patnaik, S., Speed, M., Ali, A.M., Burger, J.M., Almeida, R.V., Ayub, Z., 2007. Use of molasses as carbon source in limited discharge nursery and grow-out systems for *Litopenaeus vannamei*. Aquacultural Engineering 36(2), 184–191.

Sapcharoen, P., Rengpipat, S., 2013. Effects of probiotic *Bacillus subtilis* (BP11 and BS11) on the growth and survival of Pacific white shrimp, Litopenaeus vannamei. Aquaculture Nutrition, 19, 946-954.

Saulnier, D., Haffner, P., Goarant, C., Levy, P., Ansquer, D., 2000. Experimental infection models for shrimp vibriosis studies: a review. Aquaculture 191, 133-143.

Sha, Y., Wang, L., Liu, M., Jiang, K., Xin, F., Wang, B., 2016. Effects of lactic acid bacteria and the corresponding supernatant on the survival, growth performance, immune response and disease resistance of *Litopenaeus vannamei*.Aquaculture 452, 28-36.

Silva, E.F., Soares, M.A., Calazans, N.F., Vogeley J.L., Valle, B.C., Soares, R., Peixoto, S., 2013. Effect of probiotic (*Bacillus* spp.) addition during larvae and postlarvae culture of the white shrimp *Litopenaeus vannamei*. Aquaculture Research 44, 13-21.

Skjermo, J., Vadstein, O., 1999. Techniques for microbial control in the intensive rearing of marine larvae. Aquaculture 177, 333–343.

Song, Z.F., An, J., Fu, G.H., Yang, X.L., 2011. Isolation and characterization of an aerobic denitrifying *Bacillus* sp. YX-6 from shrimp culture ponds. Aquaculture 319, 188-193.

Sorokulova, I., 2013. Modern Status and Perspectives of Bacillus Bacteria as Probiotics. J. Prob. Health, 1, 1-5.

Souza, D.M., Suita, S.M., Leite, F.P.L., Romano, L.A., Wasielesky, W., Ballester, E.L.C., 2012. The use of probiotics during the nursery rearing of the pink shrimp *Farfantepenaeus brasiliensis* (Latreille, 1817) in a zero exchange system. Aquaculture Research 43(12), 1828–1837.

Strickland, J.D.H., Parsons, T.R., 1972. A Practical Handbook of Seawater Analysis. Ottawa: Fishery Research Board Canada. 310 pp.

Tan, T.C., Qian, Z.R., 1996. Activity of the enzyme system in thermally killed *Bacillus* cells. Enzyme Microb Technol., 19, 150-156.

Tzuc, T.J., Escalante, D.R., Herrera, R.R., Cortés, G.G., Ortiz, M.L.A., 2014. Microbiota form *Litopenaeus vannamei*: digestive tract microbial community of Pacific White shrimp (*Litopenaeus vannamei*). SpringerPlus 3, 280.

UNESCO, 1983. Chemical methods for use in marine environmental monitoring. Manual and Guides 12, Intergovernamental Oceanographic Commissiony. Paris, France.

Vanwyk, P, Scarpa, J., 1999. Water Quality and Management. In: Van Wyk, P., et al. (Eds.), Farming Marine Shrimp in Recirculating Freshwater Systems. Florida Department of Agriculture and Consumer Services, Tallahassee, pp. 128–138.

Verschuere, L., Rombaut, G., Sorgeloos, P., 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiology and Molecular Biology Reviews 64, 655-671.

Villaseñor, I.E.L., Castellano-Cervantes, T., Gomez-Gil, B., Carillo-Garcia, Á.E., Campa-Cordova, Á.I., Ascencio, F., 2013. Probiotics in the intestinal tract of juvenile whiteleg shrimp *Litopenaeus vannamei*: modulation of the bacterial community. World J Microbiol Biotechnol 29, 257-265.

Villaseñor, I.E.L., Rodríguez, M.E.M., Gomez-Gil, B., Valle, F.A., Córdova, Á.I.C., 2011. Beneficial effects of four *Bacillus* strains on the larva cultivation of *Litopenaeus vannamei.* Aquaculture 321, 136-144.

Villaseñor, I.E.L., Voltolina, D., Gomez-Gil, B., Ascencio, F., Campa-Córdova, Á.I., Audelo-Naranjo, J.M., Zamudio-Armenta, O.O., 2015. Probiotic modulation of the gut bacterial community of juvenile *Litopenaeus vannamei* challenged with *Vibrio parahemolyticus* CAIM 170. Lat. Am. J. Aquat. Res. 43(4), 766-775.

Wang, Y., He, Z., 2009. Effect of probiotics on alkaline phosphatase activity and nutrient level in sediment of shrimp *Penaeus vannamei*, ponds. Aquaculture 287, 94-97.

Wang, Y.B., 2007. Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei.* Aquaculture 269, 259-264.

Wasielesky, W., Atwood, H., Stokes, A., Browdy, C.L., 2006. Effect of natural production in a zero exchange suspended microbial floc based super-intensive culture system for white shrimp Litopenaeus vannamei. Aquaculture, 258, 396–408.

Yang, X.P., Wang, S.M., Zhang, D.W., Zhou, L.X., 2011. Isolations and nitrogen removal of an aerobic heterotrophic nitrifying-denitrifying bacterium, *Bacillus subtilis* A1. Bioresource Technology 102, 854-862.

Zhang, Q.L., Liu, Y., Ai, G.M., Miao, L.L., Zheng, H.Y., Liu, Z.P., 2012. The characteristics of a novel heterotrophic nitrification-aerobic denitrification bacterium, *Bacillus methylotrophicus* strain L7. Bioresource Technology 108, 35-44.

Zhou, X., Wang, Y., Li, W., 2009. Effect of probiotic on larvae shrimp (*Pennaeus vannamei*) based on water quality, survival rate and digestive enzyme activities. Aquaculture 287, 349-353.

Zokaeifar, A., Babaei, N., Saad C.R., Kamarudin M.S., Sijam, K., Balcazar, J.L., 2014. Administration of *Bacillus subtilis* strains in the rearing water enhances the water quality, growth performance, immune response, and resistance against *Vibrio harveyi* infection in juvenile white shrimp *Litopenaeus vannamei*. Fish and Shellfish Immunology, 36, 68-74.

CAPÍTULO 4

Effect of probiotic mixture supplementation on hepatopancreatic lesions caused by a vibriosis outbreak in *Litopenaeus vannamei* reared in BFT and clear water system

Running title: Effect of probiotics in BFT and clear water

Keywords: *Litopenaeus vannamei*; Vibriosis; hepatopancreatic lesions; probiotics; bioflocs.

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Observação: Tabelas e figuras foram incluídas no corpo do texto para facilitar a leitura da tese, além de um resumo em português.

Efeitos da suplementação com probiótico em lesões hepatopancreáticas causadas por surtos de vibriose em *Litopenaeus vannamei* cultivados em sistema de bioflocos e água clara.

Resumo

O presente estudo objetivou avaliar os efeitos da suplementação com probiótico em lesões hepatopancreáticas causadas por surto de vibriose em *Litopenaeus vannamei,* e comparar tais efeitos em BFT e água clara. Juvenis de *L. vannamei* provenientes de um cultivo na fase de engorda foram naturalmente infectados por *V. parahemolyticus.* Os camarões foram transferidos e estocados nas unidades experimentais, sendo que o experimento foi compost pelos seguintes tratamentos em triplicata: -Bioflocos sem aplicação de probióticos (**BFT**); - Bioflocos com aplicação de probióticos (**BFT+ProW**); - Água clara com aplicação de probióticos (**CW**); e – Água clara com aplicação de probióticos (**CW+ProW**). O probiótico comercial (Sanolife-ProW® - INVE Aquaculture) foi adicionado à água a cada 48 horas (0.5 ppm). A análise histopatológica do hepatopancreas (HP) foi realizada no período inicial e final (30 dias) de experimento. A presença do probiótico contribuiu para o aumento da biomassa, ganho de peso e sobrevivência final, os quais foram significativamente maiores em BFT e água clara. Os sinais clínicos de infecção por *Vibrio* spp. foram confirmados por analises histológicas das amostras iniciais, as quais evidenciaram necrose epitelial dos túbulos, presença de células mortas e colônias bacterianas. Após 30 dias, o HP dos camarões cultivados em BFT e água clara com aplicação de probióticos apresentou redução nas lesões. Por outro lado, os cortes histológicos de HP proveniente de água clara sem aplicação de probióticos evidenciaram alto número de células mortas, desprendimento celular e atrofia dos túbulos hepatopancreáticos. A combinação do uso de probióticos no sistema de bioflocos pode maximizar os efeitos positivos da comunidade microbiana nos bioflocos, garantindo assim mmelhor desempenho e redução dos impactos negativos causados por surtos de vibriose.

1. Abstract

This study aimed to evaluate the effect of probiotic supplementation on hepatopancreatic lesions caused by a vibriosis outbreak in *Litopenaeus vannamei,* comparing it in BFT and clear water. *L. vannamei* from a grow-out culture were naturally infected with *V. parahemolyticus*. Shrimp were transferred and stocked at the experimental units, which setup was composed of four treatments, three replicates each: - Bioflocs without Probiotics application (**BFT**); - Bioflocs with Probiotics (**BFT+ProW**); - Clear Water without Probiotics (CW); and − Clear Water with Probiotics (**CW+ProW**). The probiotic mixture (Sanolife-ProW® - INVE Aquaculture) was added to the water each 48 hours (0.5 ppm). Histopathologic analysis of the hepatopancreas (HP) was performed in the beginning and in the end (30 days) of the experiment. The presence of probiotics contributed to increase biomass, weight gain and survival rates, which were significantly higher in BFT and Clear water. The signs of *Vibrio* spp. infection were evidenced by the histological analysis from the initial samples, with tubular epithelium necrosis, the presence of dead cells and bacterial colonies. After 30 days, HP of shrimp from BFT and clear water with probiotic application showed reduction of HP lesions. On the other hand, final samples of clear water with no probiotic addition evidence high level of dead cells, cellular detachment and tubular atrophy. The results show that combining the use of probiotics and BFT system can maximize the positive effects from the microbial community in the bioflocs, assuring better growth performance and reduction of the negative impacts caused by Vibrio outbreaks.

2. Introduction

The high incidence of infectious diseases has become a real challenge for aquaculture industry, and the main cause of production and economic losses during the last decades. Besides, it is also one of the factors that can negatively affect the future prospects for this sector due to the high mortality rates (FAO, 2016).

In most of the cases, the mortalities are not attributed to specific obligate pathogenic bacteria, but rather the proliferation of opportunistic pathogenic bacteria (Defoirdt 2016). These species commonly occur in the sea water and take advantages of ecological changes introduced when the water is used in aquaculture (Skjermos & Vadstein 1999). They are typically characterized as organisms that can become pathogenic following a perturbation to their host or in the environment (Brown, Cornforth & Mideo 2012). Suboptimal rearing conditions such as extremely high stocking densities, associated to environmental parameters like low oxygen concentrations and variations in temperatures can lead to opportunistic bacterial disease outbreaks, including vibriosis (Kimes, Grim, Johnson, Hasan, Tall, Kothary, Kiss, Munk, Tapia, Green, Detter, Bruce, Brettin, Colwell & Morris 2012; Phippen, Ivanina, Sokolova & Oliver 2016).

Vibriosis outbreaks are caused by gram negative bacteria from the genus *Vibrio* spp., and can be considered one of the most prevalent diseases that affect cultured aquaculture organisms worldwide (Chatterjee & Haldar, 2012). This disease can be local or systemic, affecting all the organs and tissues (Roque, Molina-Aja, Bolan-Mejia & Gomez-Gil 2001). Besides, it has been reported that shrimp can be mainly infected through feed, gill and hepatopancreas (Esteve & Herrera, 2000; Chatterjee & Haldar, 2012).

Among the major species of *Vibrio* spp. that are able to cause disease, special attention has been given recently to *Vibrio parahemolyticus*, which is reported to be the ethiological agent that causes the Early Mortality Sindrome/Accute Hepatopancreathic Necrosis Disease (Tran, Nunan, Redman, Mohney, Pantoja, Fitzsimmons & Lightner 2013). It is estimated that the losses to the Asian shrimp industry, due to EMS/AHPND, have already reached 1 USD billion (Global Aquaculture Alliance 2013).

Considering all the negative impacts caused by the incidence of vibriosis in the shrimp culture, producers most of the time adopt the use of antibiotics in order to control bacterial diseases. However, the indiscriminate use of antibiotics, especially in situations that there is no apparent disease, can result in a major problem of resistance development of the aquaculture pathogens (Defoirdt, Boon, Sorgeloos & Bossier 2007; Crab, Lambert, Defoirdt, Bossier & Verstraete 2010; Mohapatra, Chakraborty, Kumar, DeBoeck & Mohanta 2013). Hence, the resistance determinants can be transmitted to bacteria of the terrestrial environment, including animal and human pathogens, as has been reported for *Salmonella enterica* serotype Typhimurium and *Vibrio cholera* (Cabello 2006).

In this context, there is an urgent need to find non antibiotics and environmental friendly alternatives in order to promote healthy development of aquaculture (Sha, Wang, Liu, Jiang, Xin, Wang 2016). Defoirdt, Sorgeloos & Bossier (2011) and Defoirdt (2016) suggest that an approach which includes pathogen, host and environment, will probably be most effective solution in the long term to prevent and control pathogens. De Schryver, Defoirdt & Sorgeloos (2014) affirm that adopting strategies that includes favoring the growth and management of microbial community may be the key to minimizing the risk of vibriosis outbreaks.

The Biofloc Technology (BFT) is based on the stimulation and establishment of a microbial community, which is responsible for maintaining the water quality, improve growth and survival (McIntosh, Samocha, Jones, Lawrence, Horowitz & Horowitz 2001; Wasielesky, Atwood, Stokes & Browdy 2006; Avnimelech 2007). It is also reported the capacity of this microbial community to inhibit the proliferation of pathogens by competitive exclusion for food and space in the water or in the gut (Crab et al. 2010). Ekasari, Azhar, Surawidjaja, Nuryati, De Schryver & Bossier (2014) verified that the use of the BFT system contributed to increase the activity of immunoparameters such as phenoloxidase (PO) activity, respiratory burst and survival rates of *L. vannamei* following a challenge test with myonecrosis virus (IMNV). These data presented above can indicate the potential of the BFT system to be a strategy to control and minimize the risk of diseases.

The application of probiotic bacteria has become a successful alternative to the use of antibiotic as well (Gram, Melchiorsen & Spanggard 1999; Gildberg, Mikkelsen & Sandaker 1997). By definition, probiotics are live microorganisms that provide health benefits to the host when administered in adequate levels. In the host, these microorganisms are able to colonize the intestinal tract and multiply, promoting the efficient modulation of various biological systems (Gatesoupe 1999; Mohapatra et al. 2013). They can be administered via water routine or feed additives (Moriarty 1998; Skjermo & Vadstein, 1999) with either single or a combination of probiotics, in a mixture (Villaseñor, Castellano-Cervantes, Gomez-Gil, Carillo-Garcia, Campa-Cordova & Ascencio 2013).

Among the use of different bacterial species with probiotic properties addressed by a number of authors (Moriarty 2003; Hai, Fotedar & Buller 2007; D'alvise, Lillebo,

Wergeland, Gram & Berg 2013; Castex, Chim, Pham, Lemaire, Wabete, Nicolas, Schimidely & Mariojouls 2008), bacteria of genus *Bacillus* sp. have been widely applied in penaeid cultures (Decamp & Moriarty 2006; Villaseñor et al. 2013; Sapcharoen & Rengpipat 2013; Fyzul, Harbi & Austin 2014). The investigations report the benefits provided by *Bacillus* concerning higher growth and survival rates (Balcazar, Rojas-Lunas & Cunningham 2007; Zhou, Wang & Li 2009; Nimrat, Suksawat, Boonthai & Vuthiphandchai 2012; Krummenauer, Poersch, Romano, Lara, Encarnação & Wasielesky 2014; Zokaeifar, Babaei, Saad, Kamarudin, Sijam & Balcazar 2014), improvement the digestive enzymes activities (Wang 2007; Zhou *et al*. 2009), enhancing immune response and disease resistance against pathogenic Vibrio (Gullian, Thompson & Rodriguez 2004; NavinChandram, Iyapparaj, Moovendhan, Ramasubburayan, Prakash, Immanuel & Palavesam 2014; Sha, Wang, Liu, Jiang, Xin & Wang 2016).

Several authors report *Bacillus* positive effects towards *L. vannamei* by observing antagonistic activity, modulation of the microbiota, decrease in the number, and increasing the resistance against Vibrio in conventional - clear water (Li, Tan & Mai 2009; Nimrat *et al*. 2011; Sha *et al*. 2016) and in Bioflocs culture systems (Krummenauer *et al*. 2014b).

Ferreira, Bolíva, Pereira, Guertler, Vieira, Mouriño & Seiffert (2015) investigated the probiotic properties of *Bacillus* sp. isolated from biofloc-rich water and supplemented to *L. vannamei*. The authors verified lower number of Vibrio in the water in the experimental units treated with *Bacillus*, which showed antagonism against *Vibrio harveyi*. Aguilera-Rivera, Prieto-Davo, Escalante, Chávez, Cuzón & Gaxiola (2014) suggested that the addition of a commercial probiotic mixture contributed to

homeostasis and prevented outbreaks of opportunistic pathogenic bacteria in BFT system and clear water. The authors also affirm that there is a synergistic effect between the probiotics and the heterotrophic bacterial community present in the bioflocs for *L. vannamei*, which prevented the progressions of lesions in hepatopancreas cause by pathogenic Vibrios present in the water.

Additionally, Hu, Cao, Wen, Zhang, Xu, Xu, Xu, Li (2016) verified that the combined use of *Bacillus* and molasses as a carbon source helped to increase diversity of the microbial community, effectively inhibited pathogens, and promoted the formation and development of a beneficial microbial community structure in biofloc-rich water.

Hence, understanding the interaction between the microbiota present in the biofloc-rich water and in the animal farmed, associated to the addition of a probiotic bacteria is important to establish sustainable and alternative strategies for diseases control, water quality and microbial community management.

The main objective of this study was to evaluate the effect of probiotic mixture supplementation on hepatopancreatic lesions caused by a vibriosis outbreak in *Litopenaeus vannamei* reared in BFT and clear water system.

3. Material and Methods

3.1. Location and origin of animals

The present study was conducted at the Marine Station of Aquaculture of the Federal University of Rio Grande, located at Cassino Beach, Rio Grande do Sul Southern Brazil (32°10'S).

L. vannamei juveniles used in the experiment were transferred from a grow-out culture in a greenhouse with $35m^2$ tanks lined with high-density polyethylene (HDPE) and a water depth of 1.0m, 300 shrimp m^{-2} of stocking density. These juveniles were all infected naturally by *V. parahaemolyticus*, identified by clinical symptoms and confirmed through monoclonal antibody conjugated with Rhodamine B in combination with fluorescein to *V. parahaemolyticus* (Chemetron Dako, Argentina) (1:200 in PBS), according to Lightner (1996) and Krummenauer *et al*. (2014).

3.2 Probiotic Mixture

The commercial probiotic mixture (Sanolife-ProW® - INVE Aquaculture) used in this study is composed by *Bacillus subtilis* and *Bacillus Licheniformes* strains, in a bacterial concentration of 5.10^{10} cfug^{-1}. The product was added to the culture water each 48 hours, in a concentration of 0.5ppm or 0.5 mgL^{-1} and activated according to the manufacturer recommendations.

3.3. Experimental design

The trial was carried out in twelve rectangular polyethylene tanks with 400L of effective volume. Shrimp (13.20 ± 3.62g) naturally infected with *V. parahemolyticus* were transferred and stocked at 300 shrimp m^{-2}. The experimental setup was composed of four treatments with three replicates each: - Bioflocs without Probiotics application (**BFT**); - Bioflocs with Probiotics application (**BFT+ProW**); - Clear Water without Probiotics application (CW); and – Clear Water with Probiotics application (**CW+ProW**).

The Bioflocs experimental units were filled with 400L of biofloc-rich water (100% of reuse water) obtained from the same grow-out tanks that *Litopenaeus vannamei* juveniles have been previously cultured before being transferred to the

present experimental units. For the treatments in Clear Water, the tanks were filled with 400L of filtered (sand filter) natural seawater treated with a chlorine solution (10ppm measured immediately after chlorination) and dechlorinated using ascorbic acid powder (1ppm). Dechlorinated municipal freshwater was used to compensate for evaporative losses and to maintain salinity throughout the 30 days of experimental period.

Sugar cane molasses was added as organic carbon source in order to maintain the microbial aggregates in the treatments with Bioflocs. The supplementation of organic carbon was based on the addition of 6g of carbon for each 1g of total ammonium nitrogen (TA-N) in the water for the conversion of nitrogen in biomass by the heterotrophic bacteria. This procedure followed the methods described by Avnimelech (2009), Ebeling, Timmons & Bisogni (2006) and Samocha, Patnaik, Speed, Ali, Burger, Almeida & Ayub (2007).

Water exchange of 80% was performed every 48 hours in the treatments with Clear Water. Hence, natural, filtered and pre-treated sea water in the same temperature as the experimental units was used for the renewals procedures.

The shrimp were fed a 40% crude protein (CP) commercial diet (Potimar 40 J, Guabi®, Brazil) twice a day (0800 and 1700 h) during 30 days. Feeding rates was adapted according the methodology proposed by Jory, Cabrera, Dugger, Fegan, Lee, Lawrence, Jackson, McIntosh & Castañeda (2001).

3.4. Water quality and sampling

Dissolved oxygen (DO), temperature and pH were measured twice daily (0800 and 1700 h) using a YSI 556 MPS meter (YSI Inc., Yellow Springs, United States). Total ammonium nitrogen (UNESCO 1983) and nitrite-nitrogen (NO_2-N) (Aminot & Chaussepied 1983) were measured twice a week. Salinity (using a manual optical

refratometer), nitrate-nitrogen (NO_3-N) (Aminot & Chaussepied 1983), total suspended solids (TSS) (Strickland & Parson, 1972) and alkalinity (APHA 1989) were measured weekly.

3.5. Growth performance and survival

Shrimp were randomly sampled weekly from each tank and individually weighed to the nearest 0.01g. At the end of each trial, the zootechnical performance of *L. vannamei* was evaluated by the final weight (g), weight gain (g), Biomass (g) and survival rate (%).

3.6. Histology

A total of nine shrimp were sampled from each treatment at days 0 (before stocking), 15 and 30, with three shrimps being collected per tank. The shrimp were anesthetized with ice after being collected and fixed with Davidson's fixative for 24 h, after which they were transferred to 70% alcohol. The samples were processed in an LUPE PT 05 automatic tissue processor using Paraplast- Plus (ParaplastTissue Embedding Media, Microsystems Inc., Bannockburn, IL, USA).

Following embedding, blocks were sectioned at a thickness of 4 lm using a LUPETEC MRPO3 microtome and stained with hematoxylin–eosin. Histological slides were analyzed under a microscope (Zeizz Primo Star) with an attached digital camera (Zeizz AxioCam ERc5s) to evaluate the lesions and possible reduction of it when shrimp were treated with probiotics.

3.7. Statistical Analyses

For statistical analysis of the data, the software STATISTICA 7.0 (StatSoft Inc. 2004, Tulsa, Oklahoma, USA) was used. The data were verified for the homoscedasticity and normality. One-way analyses of variances (ANOVA) were used to identify significant differences of the values of bacterial abundance among treatments. The ANOVA was followed by a Duncan's post-hoc comparison test when significant differences were found. All statistical analyses were examined at $P < 0.05$.

4. Results

4.1. Water Quality

The water quality parameters verified in the different treatments (BFT and Clear Water with and without Probiotic application) and are expressed at Tables 1. No significant differences were observed for temperature (°C), dissolved oxygen (mgL^{-1}), pH and salinity (Table 1). The levels of nitrogen compounds, Total Ammonia Nitrogen (TAN), Nitrite ($N-NO_2$) and Nitrate ($N-NO_3$) were significantly higher in the Biofloc culture treatments. Total suspended solids measurements was performed only in the BFT treatments (Table 1), since in the Clear Water systems there was no accumulation of significant amounts of particulate organic matter or suspended solids.

Table 1: Mean (± standard deviation; SD) values of water quality parameters during the 30 days of *L. vannamei* culture in Bioflocs with addition of ProW[®] (BFT+P); Bioflocs without ProW[®] (BFT); Clear Water with ProW[®] (CW+P); and Clear Water without ProW[®] (CW). Different letters indicate statistical differences (P<0.05).

| Parameters	BFT+P	BFT	CW + P	CW
T (°C)	$26,08 \pm 1,53$	$26,73 \pm 1,35$	$25,94 \pm 1,62$	$25,96 \pm 1,39$
DO_2 (mg L^{-1})	$6,01 \pm 0,47$	$5,90 \pm 0,41$	$6,17 \pm 0,39$	$6,15 \pm 0,34$
pH	$7,81 \pm 0,28$	$7,87 \pm 0,23$	$8,14 \pm 0,15$	$8,14 \pm 0,16$
Salinity.	$32,4 \pm 2,06$	$32,1 \pm 2,03$	$31,2 \pm 1,38$	$31,3 \pm 1,35$
TAN (mg L^{-1})	$0,19 \pm 0,64^a$	$0,13 \pm 0,47^a$	$1,67 \pm 1,05^b$	$1,69 \pm 0,98^b$
NO_2-N (mg L^{-1})	$2,28 \pm 3,37^a$	$1,87 \pm 3,27^a$	$0,10 \pm 0,05^b$	$0,16 \pm 0,09^b$
NO_3- N (mg L^{-1})	$53,80 \pm 20,5^a$	$54,00 \pm 17,38^a$	$0,78 \pm 1,42^b$	$0,50 \pm 0,94^b$
TSS (mg L^{-1})	493.88 ± 24.57	474.44 ± 75.13	-	-

4.2 Growth performance and survival

Final Weight (g) was significantly higher in the treatment in Bioflocs with Probiotic application. In the same treatment were observed the highest weight gain. The lowest values for this parameters were obtained in Clear Water treatment. Clear Water with Probiotic application did not differ from the other treatments. On the other hand, Biomass and survival rate were higher in the treatments with the use of probiotic, both BFT and clear water (Table 2).

Table 2: Mean (± standard deviation; SD) values of growth and survival during the 30 days of *L. vannamei* culture in Bioflocs with addition of ProW[®] (BFT+P); Bioflocs without ProW[®] (BFT); Clear Water with ProW[®] (CW+P); and Clear Water without ProW[®] (CW). Different letters indicate statistical differences (P<0.05)

.

Parameters	BFT + P	BFT	CW + P	CW
Final Weight (g)	17,75 ± 3,83 [a]	16,17 ± 3,73 [b]	16,71±3,59 [b]	16,23±3,68 [b]
Weight Gain(g)	5,24±0,53 [a]	3,67±0,18 [bc]	4,35±0,97 [ab]	2,80±0,44 [c]
Biomass (g)	1947,9±89,2 [a]	1479,7±101,8 [b]	1827,4±140,4 [a]	1444,5±44,2 [b]
Survival rate (%)	88 ± 4,19 [a]	73 ± 4,27 [b]	86±2,2 [a]	75 ± 3,93 [b]

4.3 Histology

The histopathological analysis allowed comparing the hepatopancreatic lesions on BFT and CW with the presence or not of the probiotic mixture. Shrimp from the initial samples evidence the histological signs of *Vibrio* spp. infection, with tubular epithelium necrosis, the presence of dead cells inside the lumen and bacterial colonies (Figure 1).
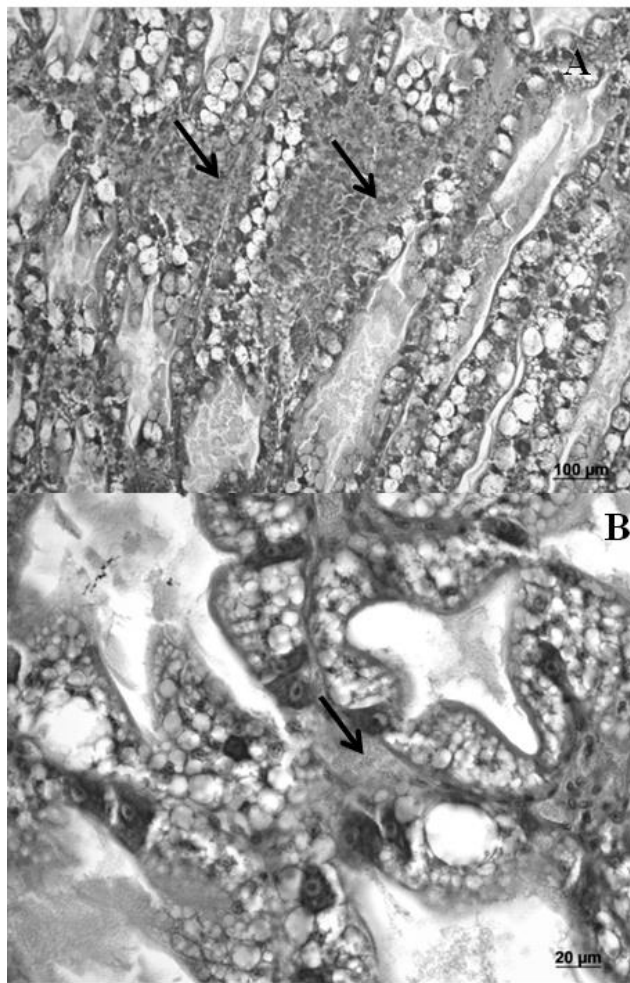
Figure 1: Initial samples. Hepatopancreas showing typical vibriosis signs. A: HP showing a tubular epithelium necrosis and dead cells inside the lumen (arrows). B: Extracellular bacterial colonies surrounding the HP tubules (arrow).

After 30 days of experimental period, the HP of shrimp cultured in BFT with probiotic application showed reduction of HP lesions, presenting a healthy aspect with well-formed tubules and no signs of degeneration or tubular atrophy (Figure 2).
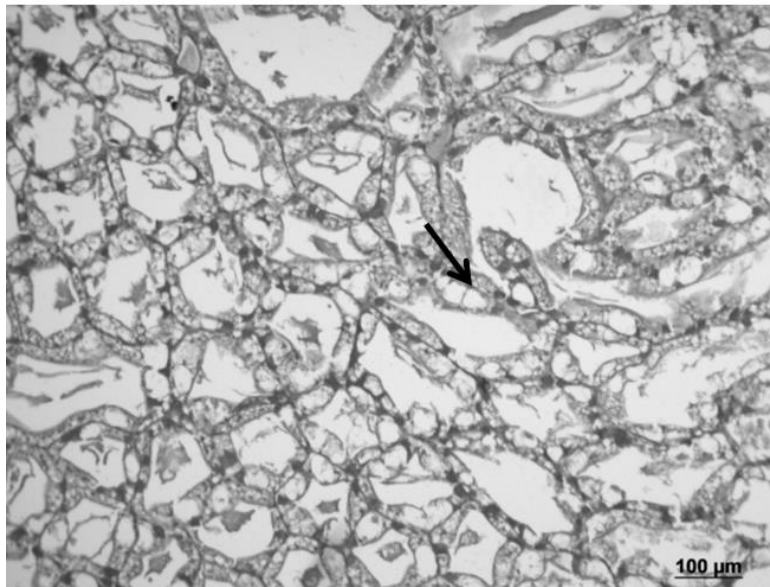
Figure 2: HP histology of shrimp sampled from BFT + P treatment. Bar 100µm. HP tubules with no necrosis or degeneration, arrows for lipidic vacuoles.

In the treatment in clear water with probiotic addition, hemocitic nodules and infiltrations were observed around the tubules, with no necrosis observed (Figure 3).
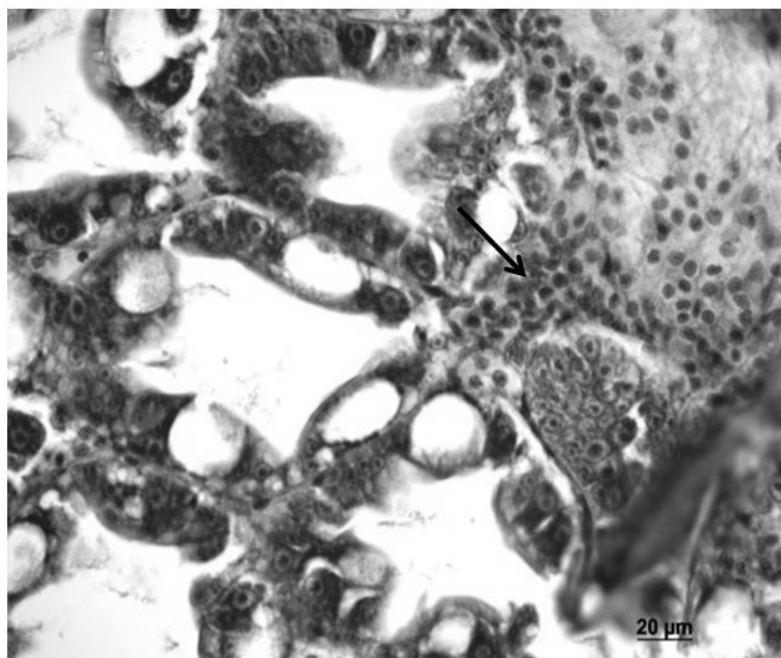
Figure 3: HP histology of shrimp sampled from CW+P treatment. Bar 20µm. Hemocitic infiltration around the tubules (arrow).

On the other hand, final samples of clear water treatments with no probiotic addition evidence high level of dead cells and cellular detachment and tubular atrophy (Figure 4).
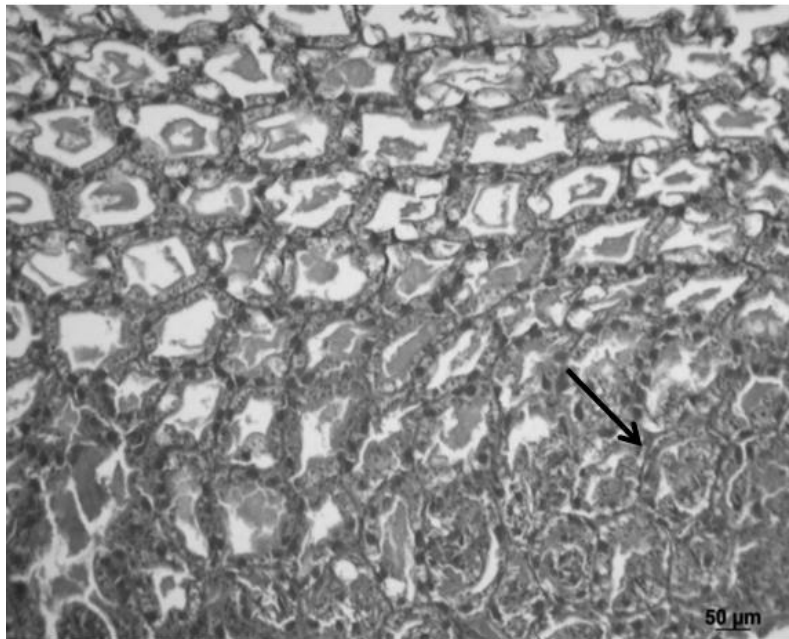
Figure 4: HP histology of shrimp sampled from CW with no probiotic application. Bar 50µm. Atrophied and degenerated tubule epithelium, arrow for cellular detachment.

5. Discussion

The use of probiotic in aquaculture systems and its positive effects in different hosts has been well reported by several authors (Gatesoupe 1999; Verschuere, Rombaut & Sorgeloos 2000; Castex *et al.* 2008; D'alvise *et al.* 2013; Navin Chandran *et al.* 2014; Fyzul *et al.* 2014; Dawood & Koshio 2016).

In this context, *Bacillus sp*. have been successfully applied for the improvement of penaeids growth performance, immune response and water quality (Villaseñor,

Rodriguez, Gomez-Gil, Valle & Campa-Cordova 2011; Nimrat, Suksawat, Boonthai & Vuthiphandchai 2012; Souza, Suita, Leite, Romano, Wasielesky & Ballester 2012; Krummenauer *et al*. 2014). For example, Wang & He (2009) verified a significantly decrease on nitrogen levels in *L. vannamei* culture ponds treated with a commercial probiotic mixture containing *Bacillus sp*. Some authors affirm that Gram-positive bacteria including *Bacillus* are able to perform heterotrophic nitrification and aerobic denitrification from organic matter substrates, which could represent an advantage over the traditional removal of nitrogen by the autotrophic bacteria in conventional pond culture systems (Song, An, Fu & Yang 2011; Zhang, Liu, Ai, Miao, Zheng & Liu 2012). However, in the present study, the presence of the probiotic mixture did not have an influence in the levels of nitrogen compounds for the different treatments. The same results were observed by Liu, Li, Li, Cai, Li, Chen & Lyu (2005) and Krummenauer *et al*. (2014).

By comparing the concentration of nitrogen compounds between BFT and clear water system, differences were verified. TA-N concentrations were higher in clear water treatments than in BFT. It can be attributed to the fact that the BFT system consists of a predominant heterotrophic community, and according to Yang, Wang, Zhang & Zhou (2011), a number of heterotrophic microorganisms have been reported to nitrify and/or transform many types of nitrogen compounds by converting it in microbial biomass in the presence of oxygen and the addition of an organic carbon source, which is the main principle of the bioflocs formation (Avnimelech 1999; Ebeling *et al.* 2006). Hence, the accumulation of TA-N in clear water treatments can be justified by the water exchanges performed every 48 hours, not allowing a complete nitrification process.

Nitrite (NO_2-N) and Nitrate (NO_3-N) concentrations were higher in BFT treatments. Nitrate is the end product of the nitrification process, in which autotrophic ammonia nitrogen oxidizing reaction by the nitrifying bacteria can occur simultaneously in the water (Hargreaves 2006), even though these bacteria are known to have slower growth rates and to be more sensitive to variations in water quality parameters such as pH and temperature (Crab *et al.* 2007; Ebeling *et al.* 2006). The occurrence of this process is evidenced in the present study by the accumulation of NO_3-N concentrations, which were similarly reported by Krummenauer *et al.* (2014) on the culture of *L. vannamei* in Biofloc system.

Despite the differences verified, the levels of nitrogen compounds remained under the recommended range for *L. vannamei* (Lin & Chen, 2001; Lin & Chen, 2003; Kuhn, Smith, Boardman, Angier, Marsh & Flick 2010; Furtado, Campos, Serra, Klosterhoff, Romano & Wasielesky 2014).

Additionally, no statistical differences were observed in water temperature, dissolved oxygen, pH, salinity and alkalinity between the treatments and the values remained within the proper range for rearing *L. vannamei* (Van Wyk & Scarpa, 1999; Furtado, Poersch & Wasielesky 2011).

Regarding to growth performance parameters, the treatments with probiotic supplementation showed higher weight gain, biomass and survival rates in the present study. Likewise, the efficiency of probiotic supplementation, whether or not commercial, has been well documented. For example, Zhou *et al.* (2009) and Bernal, Marrero, Campa-Cordova & Mazón-Suástegui (2016) verified higher growth and survival rates when evaluated the efficiency of different *Bacillus* sp. strains in larvae and juveniles of *L. vannamei* respectively. Additionally, Ziaei-Nejad, Rezaei, Takami,

Lovett, Mirvaghefi & Shakouori (2006) and Wang *et al.* (2009) evaluated growth parameters of *Fenneropenaeus indicus* and *L. vannamei* with commercial probiotic mixture application and similarly obtained higher results of growth and survival rates.

One of the main modes of action of probiotic bacteria described, is concerning its role in digestive processes in shrimp. The probiotics can stimulate the production of digestive enzymes (proteases and lipases) and antimicrobial substances, reflecting on higher growth and survival (Ziaei Nejad *et al.* 2006; Zokaeifar *et al*. 2014). The results discussed above were performed in conventional flow-through system with water exchange, and highlight the positive effects of applying probiotics in the shrimp culture.

Although the role of probiotic bacteria added in the microbial community of the BFT system is still not clear, the present study approach the positive effects that it can cause to the growth performance of *L. vannamei*. Similarly, Aguillera-Rivera *et al.* (2014) and Krummenauer *et al*. (2014) obtained higher survival e growth rates when added commercial probiotics in L. *vannamei* reared in BFT system. However, Ferreira *et al.* (2015) did not verify any significant effect of an isolated *Bacillus* sp. strain on growth performance of *L. vannamei* in BFT system. Additionally, Hu *et al.* (2016) evaluated the combination of the use of *Bacillus* sp. as probiotic and the use of molasses as a carbon source. The authors concluded that it can increase diversity of the microbial community, inhibit pathogens, and promote the formation and development of a beneficial microbial community structure in *L. vannamei* culture. By increasing the diversity and having a well-developed benefic microbial community in the BFT system, it can reflect on better growth and survival rates. It is confirmed that the bioflocs can serve as a high quality food source for *L. vannamei*, improving growth performance parameters (Wasielesky *et al*. 2006). This interaction between the probiotic bacteria

added to biofloc environment regarding to growth performance could be verified in the present study. Nevertheless, further studies are needed in order to understand the mecanisms behind this synergic effect (Aguillera-Rivera *et al.* 2014).

The effect of the probiotics towards *V. parahemolyticus* outbreak in the present study were investigated through histopathological analysis of the hepatopancreas (HP). Esteve & Herrera (2000) affirm that HP is an ideal indicator of the physiological condition of shrimp, since it reflects the state of metabolic level, ecdysis phase and disease.

In the present study, the presence of vibriosis was first identified by the clinical syptoms of the disease (alternating disoriented swimming and lethargy, reddening of the pleural borders, the antennal scales, and the uropods and telson, and opacity of abdominal muscle) and histopathological analysis of HP tissues before stocking the shrimp (initial sample). The results obtained by the microscopical observation of the initial sample corroborates with the description made by Morales-Covarrubias (2004). The author describes presence of bacterial colonies, necrosis and hemocytes infiltrates in HP affected by vibriosis, since this organ is part of the infection route of *Vibrio* sp (Martin, Rubin & Swanson 2004).

In the treatments in which the probiotic mixture was not applied, changes in the hepatopancreatic architecture were observed, with severe levels of necrosis and hemocytes infiltration. Dettached cells were also observed, with the hepatopancreatic tubuli without the cellular lining because of cytolisis. In the same way, Soto-Rodriguez, Gomez-Gil, Lozano, Rio-Rodriguez, Dieguez & Romalde (2012) verified the presence of severe necrosis in *L. vannamei* cultured in clear water with no probiotic application, affected by *Vibrio harveyi*. The results of the present study are in concordance with

other investigations describing *Vibrio* sp. infections (Mohney, Lightner, & Bell 1994; Lightner 1996; Peña-Navarro & Varela-Mejias 2015).

On the other hand, the use of probiotics in this trial possibly helped to reduce the negative effect caused by the vibriosis, as observed in previous studies (Gatesouppe 1999; Vallamil, Figueras, Planas & Novoa 2003; Li, Yeh & Chen 2008). Villaseñor, Voltolina, Gomez-Gil, Ascencio, Campa-Córdova, Audelo-Naranjo & Zamudio-Armenta (2015) reported higher performance and survival of *L. vannamei* towards Vibrio infections with probiotic application in clear water. Ferreira *et al.* (2015) observed a decrease in the number of Vibrio in the culture water of *L. vannamei* with the use of *Bacillus* sp. isolated from a biofloc system. In the same way, Krummenauer *et al.* (2014) verified a reduction in HP lesions of *L. vannamei* when added probiotics in BFT system. The reduction of lesions and the healthy aspect of the HP in the treatments with probiotic can be an indicative of its effectiveness, especially in the BFT system, where there is possibly a synergistic effect between the microbial community present in the bioflocs and the probiotic added to the culture (Aguillera-Rivera *et al.* 2014).

Moreover, the probiotic bacteria is able compete with pathogenic *Vibrio* for adhesion sites, nutrients and chemical compounds in the water and in the gut of the host (Mohapatra *et al.* 2013; Hamza, Kumar & Zinjarde 2015). Hence, they are able to stick with the mucosal epithelium of gastrointestinal tract and help to resist pathogens, by decreasing its number in the host (Villaseñor *et al.* 2011; Akhter, Wu, Memon & Mohsin 2015; Lazado, Caipang & Estante 2015). The process described above is one important mode of action of probiotics, which can explain the recovery of tissues, as well as better growth performance of *L. vannamei*.

In conclusion, the commercial probiotic mixture composed by *Bacillus subtilis* and *B. licheniformes* contributed to the improvement of growth performance parameters and survival rates of *L. vannamei* juveniles. The higher growth and survival can be confirmed by the reduction of HP lesions caused by a vibriosis outbreak, analyzed with histopathological technique.

Nevertheless, the combination of the use of probiotics in BFT culture system can maximize the positive effects from the microbial community present in the bioflocs, assuring better results in water quality parameters, growth performance and disease resistance.

6. Aknowledgments

7. References

Aguillera-Rivera D., Prieto-Davo A., Escalante K., Chávez C., Cuzon G., Gaxiola G. (2014) Probiotic effect of FLOC on *Vibrios* in the Pacific White shrimp *Litopenaeus vannamei*. *Aquaculture* **424-425**, 215-219.

Akhter N., Wu B., Memon A.M., Mohsin M. (2015) Probiotics and prebiotics associated with aquaculture: A review. *Fish and Shellfish Immunology* **45**, 733-741.

Aminot A. Chaussepied M., (1983) Manuel des analyses chimiques en milieu marin. In: *CNEXO*, pp 395. Brest.

APHA (American Public Health Association), 1998. Standard Methods for the Examination of Water and Wastewater, 20st edition, pp 1193. Washington, DC.

Avinimelech Y. (1999) Carbon/nitrogen ratio as a control element in aquaculture systems. *Aquaculture* **176**, 227-235.

Avnimelech Y. (2007) Feeding with microbial flocs by tilapia in minimal discharge bio-flocs technology ponds. *Aquaculture* **264**, 140–147.

Avnimelech Y. (2009) Biofloc Technology: A practical Hand Book. The World Aquaculture Society, Baton Rouge, Louisiana, United States.

Balcazár J.L., Rojas-Luna T., Cunningham D.P. (2007) Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (*Litopenaeus vannamei*) following immersion challenge with *Vibrio parahaemolyticus. Journal of Invertebrate Pathology* **96**, 147-150.

Bernal M.G., Marrero R.M., Campa-Córdova A.I, Mazón-Suástegui J.M. (2016) Probiotic effect of *Streptomyces* strains alone or ir combination with *Bacillus* and *Lactobacillus* in juveniles of the white shrimp *Litopenaeus vannamei*. *Aquacult. Int.,* **DOI 10.1007/s10499-016-0085-y**.

Brown S.P., Cornforth D.M., Mideo N. (2012) Evolution of virulence in opportunistic pathogens: generalism, plasticity, and control. *Trends in Microbiology*, **20 (7)**, 336-342.

Cabello F.C. (2006) Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environ Microbiol*., **8**, 1137-1144.

Castex M., Chim L., Pham D., Lemaire P., Wabete N., Nicolas J.L., Schmidely P., Mariojouls C. (2008). Probiotic *P. acidilactici* application in shrimp *Litopenaeus stylirostris* culture subject to vibriosis in New Caledonia. *Aquaculture* **275,** 182-193.

Chatterjee S., Haldar S. (2012) Vibrio related diseases in aquaculture and development of rapid and accurate identification methods. *J. Mar. Sci. Res. Dev.* **doi:10.4172/2155-9910.S1-002**.

Crab R., Avnimelech Y., Defoirdt T., Bossier P., Verstraete W. (2007) Nitrogen removal techniques in aquaculture for a sustainable production. *Aquaculture* **270**, 1–14.

Crab R., Lambert A., Defoirdt T., Bossier P., Verstraete W. (2010) The application of bioflocs technology to protect brine shrimp (*Artemia franciscana*) from pathogenic *Vibrio harveyi. Journal of Applied Microbiology* **109**, 1643-1649.

D'Alvise P.W., Lillebo S., Wergeland H.I., Gram L., Bergh O. (2013) Protection of cod larvae from vibriosis by *Phaeobacter* spp.: A comparision of strains and introduction times. *Aquaculture* **384-387**, 82-86.

Dawood M.A.O., Koshio S. (2016) Recent advances in the role of probiotics and prebiotics in carp aquaculture: A review. *Aquaculture* **454**, 243-251.

De Schryver P., Defoirdt T., Sorgeloos, P. (2014) Early Mortality Syndrome Outbreaks: A Microbial Management Issue in Shrimp Farming? *PLOS Pathogens*, **10(4)**, 1-2.

Decamp O., Moriarty D.J.W. (2006) Probiotics as alternative to antimicrobials: Limitations and potential. *World Aquac* **37(4)**, 60–62.

Defoirdt T., Boon N., Sorgeloos P., Verstraete W., Bossier P. (2007) Alternatives to antibiotics to control bacterial infections- luminescent vibriosis in aquaculture as an example. *Trends Biotechnol* **25**, 472-479.

Defoirdt T., Sorgeloos P., Bossier P. (2011) Alternatives to antibiotics for the control of bacterial disease in aquaculture. *Current Opinion in Microbiology* **14**, 251-258.

Defoirdt T. (2016) Implications of Ecological Niche Differentiation in Marine Bacteria for Microbial Management in Aquaculture to Prevent Bacterial Disease. *PLOS Pathogens*, **http://dx.doi.org/10.1371/journal.ppat.1005843**.

Ebeling J.M., Timmons M.B., Bisogni J.J. (2006) Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic control of ammonia-nitrogen in aquaculture production systems. *Aquaculture* **257**, 346–358.

Ekasari J., Azhar M.H., Surawidjaja E.H., Nuryati S., De Schryver P., Bossier P. (2014) Immune response and disease resistance of shrimp fed biofloc grown on different carbon sources. *Fish and Shellfish Immunology* **41**, 332-339.

Esteve M., Herrera F.C. (2000) Hepatopancreatic alterations in *Litopenaeus vannamei* (Boone, 1931) (Crustacea: Decapoda: *Penaeidae*) experimentally infected with a *Vibrio algynolyticus* strain. *Journal of invertebrates Pathology* **76**, 1-5.

FAO (2016). The state of world fisheries and aquaculture. *FAO*, Rome.

Ferreira G.S., Bolívar N.C., Pereira S.A., Guertler C., Vieira F.N., Mouriño J.L.P., Seiffert W.Q. (2015) Microbial biofloc as a source of probiotic bacteria for the culture of *Litopenaus vannamei*. *Aquaculture* **448**, 273-279.

Furtado P.S., Campos B.R., Serra F.P., Klosterhoff M., Romano L.A., Wasielesky W., (2014) Effects of nitrate toxicity in the Pacific white shrimp *Litopenaeus vannamei*, reared with biofloc technology (BFT). *Aquacult. Int*. **23**, 315-328.

Furtado P.S., Poersch L.H., Wasielesky W. (2011) Effect of calcium hydroxide, carbonate and sodium bicarbonate on water quality and zootechnical performance of shrimp *Litopenaeus vannamei* reared in bio-flocs technology (BFT) systems. *Aquaculture* **321(1-2)**, 130–135.

Fyzul A, N., Harbi A.H., Austin B. (2014) Review: Developments in the use of probiotics for disease control in aquaculture. *Aquaculture* **431**, 1-11.

GAA (2013) Cause of EMS shrimp disease identified. *GAA News Releases*. Available: http://www.gaalliance.org/newsroom. Accessed in 16 March, 2017.

Gatesoupe F.J. (1999) The use of probiotics in aquaculture. *Aquaculture* **180**, 147-165.

Gildberg A., Mikkelsen H., Sandaker E. (1997). Probiotic effect of latic acid bacteria in the feed on growth and survival of *Gadus morhua*. *Hydrobiologia* **352**, 279-285.

Gram L., Melchiorsen J., Spanggard B. (1999) Inhibition of *Vibrio anguillarum* by *Pseudomonas flurescens* AH2, a possible probiotic treatment of fish. *Applied and Environmental Microbiology* **65**, 969-973.

Gullian M., Thompson F., Rodriguez J. (2004) Selection of probiotic bacteria and study of their immunostimulatory effect in *Penaeus vannamei*. *Aquaculture* **233**, 1-14.

Hai N.V., Fotedar R., Buller N. (2007) Selection of probiotics by various inhibition test methods for use in the culture of western king prawns, *Penaeus latisulcatus* (Kishinouye). *Aquaculture* **272**, 231-239.

Hamza F., Kumar A.R., Zinjarde S. (2016) Antibiofilm potential of a tropical marine *Bacillus licheniformis* isolate: role in disruption of aquaculture associated biofilms. *Aquaculture Research* **47(8),** 2661-2669.

Hargreaves J.A. (2006) Photosynthetic suspended growth systems in aquaculture. *Aquacultural Engineering* **34**, 344-363.

Hu X., Cao Y., Wen G., Zhang X., Xu Y., Xu W., Xu Y., Li Z. (2016) Effect of combined use of *Bacillus* and molasses on microbial communities in shrimp cultural enclosure systems. *Aquaculture Research*, **doi:10.1111/are.13101.**

Jory D.E., Cabrera T.R., Dugger D.M., Fegan D., Lee P.G., Lawrence A.L., Jackson C.J., Mcintosh R.P., Castañeda J. (2001) A global review of shrimp feed management: status and perspectives. In: *The New Wave, Proceedings of the Special Session on Sustainable Shrimp Culture* (ed by Browdy C.L., Jory D.E.) pp. 104–152. The World Aquaculture Society, Baton Rouge, LA,.

Kimes N.E., Grim C.J., Johnson W.R., Hasan N.A., Tall B.D., Kothary M.H., Kiss H., Munk, A.C., Tapia R., Green L., Detter C., Bruce D.C., Brettin T.S., Colwell R.R., Morris P.J. (2012) Temperature regulation of virulence factors in the pathogen *Vibrio coralliilyticus. ISME* **6**, 835-846.

Krummenauer D., Poersch L., Romano L.A., Lara G.R., Encarnação P., Wasielesky W., (2014) The Effect of Probiotics in a *Litopenaeus vannamei* Biofloc Culture System Infected with *Vibrio parahaemolyticus. Journal of applied Aquaculture* **26**, 370-379.

Kuhn D.D., Smith S.A., Boardman G.D., Angier M.W., Marsh L., Flick J.R. (2010) Chronic toxicity of nitrate to Pacific white shrimp, *Litopenaeus vannamei*: Impacts on survival, growth, antennae length, and pathology. *Aquaculture* **306**, 329-333.

Lazado C.C., Caipang C.M.A., Estante E.G. (2015) Prospects of host-associated microorganisms in fish and penaeids as probiotics with immunomodulatory functions. *Fish and Shellfish Immunology* **45**, 2-12.

Li C.C., Yeh, S.T., Chen, J.C. 2008. The immune response of white shrimp *Litopenaeus vannamei* following *Vibrio alginolyticus* injection. Fish and Shellfish Immunology. 25, 853-860.

Li J., Tan B., Mai K. (2009) Dietary probiotic *Bacillus* OJ and isomaltooligosaccharides influence the intestine microbial populations, immune responses and resistance to white spot syndrome virus in shrimp (*Litopenaeus vannamei*). *Aquaculture* **291**, 35-40.

Lightner D.V. (1996) A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp. World Aquaculture Society, Baton Rouge, LA

Lin Y.C., Chen J.C. (2001) Acute toxicity of ammonia on *Litopenaeus vannamei* (Boone) juveniles at different salinity levels. *J. Exp. Mar. Biol. Ecol*. **259**,109-119.

LinY.C., Chen J.C. (2003) Acute toxicity of nitrite on *Litopenaeus vannamei* (Boone) juveniles at different salinity levels. *Aquaculture* **224**, 193-201.

Liu X.F., Li Y., Li J.R., Cai L.Y., Li X.X., Chen J.R., Lyu S.X. (2015) Isolation and characterisation of *Bacillus* spp. antagonistic to *Vibrio parahemolyticus* for use as probiotics in aquaculture. *World J Micobriol Biotechnol* **31**, 795-803.

Martin G.G., Rubin N., Swanson E. (2004) *Vibrio parahaemolyticus* and *V. harveyi* cause detachment of the epithelium from the midgut trunk of the penaeid shrimp *Sicyonia ingentis*. *Dis. Aquat. Org*. **60**, 21-29.

McIntosh D., Samocha T.M., Jones E.R., Lawrence A.L., Horowitz, S., Horowitz A. (2001) Effects of two commercially available low-protein diets (21% and 31%) on water and sediment quality, and on the production of *Litopenaeus vannamei* in an outdoor tank system with limited water discharge. *Aquacultural Engineering* **25**, 69-82.

Mohapatra S., Chakraborty T., Kumar, V., DeBoeck G., Mohanta K.N. (2013) Aquaculture and stress management: a review of probiotic intervention. *Animal Physiology and Animal Nutrition* **97**, 405-430.

Mohney L.L., Lightner D.V., Bell T.A. (1994) An epizootic of vibriosis in Ecuadorian pond reared *Penaeus vannamei* Boone (Crustacea: Decapoda) *J. World Aquacult. Soc.* **25**, 116–125.

Morales-Covarrubias M.S. (2004) Enfermedades del camarón: detección mediante análisis en fresco e histopatología. Editorial Trillas, México, D.F.

Moriarty D.J.W. (2003) Probiotics in Aquaculture. *Microbiology Australia* **3**, 15-17.

Moriarty D.J.W. (1998) Control of luminous *Vibrio* species in penaeid aquaculture ponds. *Aquaculture* **164**, 351-358.

Navinchandran M., Iyapparaj P., Moovendhan S., Ramasubburayan R., Prakash S., Immanuel G., Palavesam A. (2014) Influence of probiotic bacterium *Bacillus cereus* isolated from the gut of wild shrimp *Penaeus monodon* in turn as a potent growth promoter and immune enhancer in *P. monodon*. *Fish and Shellfish Immunology* **36**, 38-45.

Nimrat S., Boonthai T., Vuthiphandchai V. (2011) Effects of probiotic forms, compositions and mode of probiotic administration on rearing of Pacific white shrimp (*Litopenaeus vannamei*) larvae and postlarvae. *Animal Feed Science and Technology* **169**, 244-258.

Nimrat S., Suksawat S., Boonthai T., Vuthiphandchai V. (2012) Potential *Bacillus* probiotics enhance bacterial numbers, water quality and growth during early development of white shrimp (*Litopenaeus vannamei*). *Veterinary Microbiology* **159**, 443-450.

Peña-Navarro N., Varela-Mejias A. (2015) Análisis histopatológico en *Litopenaeus vannamei* infectado con *Vibrio parahaemolyticus*. *Agron. Mesoam.* **26(1)**, 43-53.

Phippen B.L., Ivanina A.V., Sokolova I.M., Oliver J.D. (2016) *Vibrio coralliilyticus* causes rapid immune response and mortality in the Eastern oyster, *Crassostrea virginica*, after exposure to anoxia. Oral Presentation, Vibrio Conference, Roscoff, France.

Roque A., Molina-Aja A., Bolan-Mejia C., Gomes-Gil B. (2001) In vitro susceptibility to 15 antibiotics of vibrios isolated from penaeid shrimps in Northwestern Mexico. *International journal of Antimicrobial Agents* **17**, 383-387.

Samocha T.M., Patnaik S., Speed M., Ali A.M., Burger J.M., Almeida R.V., Ayub Z. (2007). Use of molasses as carbon source in limited discharge nursery and grow-out systems for *Litopenaeus vannamei*. *Aquacultural Engineering* **36(2)**, 184–191.

Sapcharoen P., Rengpipat S. (2013) Effects of probiotic *Bacillus subtilis* (BP11 and BS11) on the growth and survival of Pacific white shrimp, *Litopenaeus vannamei*. *Aquaculture Nutrition* **19**, 946-954.

Sha Y., Wang L., Liu M., Jiang K., Xin F., Wang B. (2016) Effects of lactic acid bacteria and the corresponding supernatant on the survival, growth performance, immune response and disease resistance of *Litopenaeus vannamei. Aquaculture* **452**, 28-36.

Skjermo J., Vadstein O. (1999) Techniques for microbial control in the intensive rearing of marine larvae. *Aquaculture* **177**, 333–343.

Song Z.F., An J., Fu G.H., Yang X.L. (2011) Isolation and characterization of an aerobic denitrifying *Bacillus* sp. YX-6 from shrimp culture ponds. *Aquaculture* **319**, 188-193.

Soto-Rodriguez S.A., Gomez-Gil B., Lozano R., Rio-Rodriguez R., Dieguez A.L., Romalde J.L. (2012) Virulence of *Vibrio harveyi* responsible for the ''Bright-red'' Syndrome in the Pacific white shrimp *Litopenaeus vannamei*. *Journal of Invertebrate Pathology,* **109**, 307-317.

Souza D.M., Suita S.M., Leite F.P.L., Romano L.A., Wasielesky W., Ballester E.L.C., (2012) The use of probiotics during the nursery rearing of the pink shrimp *Farfantepenaeus brasiliensis* (Latreille, 1817) in a zero exchange system. *Aquaculture Research* **43(12)**, 1828–1837.

Strickland J.D.H., Parsons T.R., (1972) A Practical Handbook of Seawater Analysis. Ottawa: Fishery Research Board Canada. 310 pp.

Tran L., Nunan L., Redman R.M., Mohney L.L., Pantoja C.R., Fitzsimmons K., Lightner D.V. (2013) Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. *Diseases of Aquatic Organisms* **105**, 45-55.

UNESCO, 1983. Chemical methods for use in marine environmental monitoring. Manual and Guides 12, Intergovernamental Oceanographic Commissiony. Paris, France.

Vanwyk P, Scarpa J. (1999) Water Quality and Management. In*: Farming Marine Shrimp in Recirculating Freshwater Systems* (ed by Van Wyk, P.), pp. 128–138. Florida Department of Agriculture and Consumer Services, Tallahassee.

Verschuere L., Rombaut G., Sorgeloos P. (2000) Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews* **64**, 655-671.

Villamil L., Figueras A., Planas M., Novoa B. (2003) Control of *Vibrio alginolyticus* in Artemia culture by treatment with bacterial probiotics. *Aquaculture* **219**, 43-56.

Villaseñor I.E.L., Rodríguez M.E.M., Gomez-Gil B., Valle F.A., Córdova Á.I.C. (2011) Beneficial effects of four *Bacillus* strains on the larva cultivation of *Litopenaeus vannamei. Aquaculture* **321**, 136-144.

Villaseñor I.E.L., Castellano-Cervantes T., Gomez-Gil B., Carillo-Garcia Á.E., Campa-Cordova Á.I., Ascencio F. (2013) Probiotics in the intestinal tract of juvenile whiteleg shrimp *Litopenaeus vannamei*: modulation of the bacterial community. *World J Microbiol Biotechnol* **29**, 257-265.

Villaseñor I.E.L., Voltolina D., Gomez-Gil B., Ascencio F., Campa-Córdova Á.I., Audelo-Naranjo J.M., Zamudio-Armenta O.O. (2015) Probiotic modulation of the gut bacterial community of juvenile *Litopenaeus vannamei* challenged with *Vibrio parahemolyticus* CAIM 170. *Lat. Am. J. Aquat. Res*. **43(4)**, 766-775.

Wang Y., He Z. (2009) Effect of probiotics on alkaline phosphatase activity and nutrient level in sediment of shrimp *Penaeus vannamei*, ponds. *Aquaculture* **287**, 94-97.

Wang Y.B. (2007) Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei. Aquaculture* **269**, 259-264.

Wasielesky W., Atwood H., Stokes A., Browdy C.L. (2006) Effect of natural production in a zero exchange suspended microbial floc based super-intensive culture system for white shrimp *Litopenaeus vannamei*. *Aquaculture* **258**, 396–408.

Yang X.P., Wang S.M., Zhang D.W., Zhou L.X. (2011) Isolations and nitrogen removal of an aerobic heterotrophic nitrifying-denitrifying bacterium, *Bacillus subtilis* A1. *Bioresource Technology* **102**, 854-862.

Zhang Q.L., Liu Y., Ai G.M., Miao L.L., Zheng H.Y., Liu Z.P. (2012) The characteristics of a novel heterotrophic nitrification-aerobic denitrification bacterium, *Bacillus methylotrophicus* strain L7. *Bioresource Technology* **108**, 35-44.

Zhou X., Wang Y., Li W. (2009) Effect of probiotic on larvae shrimp (*Pennaeus vannamei*) based on water quality, survival rate and digestive enzyme activities. *Aquaculture* **287**, 349-353.

Ziaei-Nejad S., Rezaei M.H., Takami G.A., Lovett D.L., Mirvaghefi A.R., Shakouori M. (2006) The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. *Aquaculture* **252**, 516-524.

Zokaeifar A., Babaei N., Saad C.R., Kamarudin M.S., Sijam K., Balcazar J.L. (2014) Administration of *Bacillus subtilis* strains in the rearing water enhances the water quality, growth performance, immune response, and resistance against *Vibrio harveyi* infection in juvenile white shrimp *Litopenaeus vannamei*. *Fish and Shellfish Immunology* **36**, 68-74.

CAPÍTULO 5

Dose-dependent effects of a commercial probiotic mixture on the bacterial density of *Litopenaeus vannamei* reared in a biofloc system

Running title: Dose-effect of probiotics in BFT system

Keywords: Dose-effect; probiotics; bacterial density; *Litopenaeus vannamei*; bioflocs.

Dose-resposta de probiótico comercial na variação da densidade bacteriana de

Litopenaeus vannamei **cultivado em sistema de bioflocos**

Resumo

O presente estudo objetivou comparer os efeitos de cinco diferentes dosagens de um probiótico comercial sobre a abundância microbiana total e abundância de *Bacillus subtilis*, *B. subtilis*-complex e *Vibrio* spp. no trato intestinal e na água de cultivo de *L. vannamei* em sistema de bioflocos. O experimento consistiu em 5 tratamentos, nos quais o probiótico foi incluído na ração: -**T0 -** 0 g de Pro2 kg $^{-1}$ de ração (sem probiótico); -**T1** - 1 g de Pro2 kg $^{-1}$ de ração; -**T2** - 2 g de Pro2 kg $^{-1}$ de ração; -**T3** - 3 g de Pro2 kg $^{-1}$ de ração; - **T4** - 1 g dePro2 kg $^{-1}$ de ração (100% das alimentações diárias) e -**T5** - 3 g de Pro2 kg $^{-1}$ de ração (100% das alimentações diárias). Os tratamentos T2, T3 e T4 apresentaram sobrevivências significativamente mais elevadas que T0 e T1. A densidade microbiana total presente na água aumentou significativamente do dia 0 ao dia 15. A densidade bacteriana das espécies de *Bacillus* no trato intestinal e na água não apresentou diferenças significativas entre os tratamentos. Em T0 a densidade de *Vibrio* foi significativamente mais elevada na água, onde o probiótico não foi adicionado. De modo geral, a densidade bacteriana de *Bacillus* foi maior nas amostras iniciais, indicando que a comunidade microbiana dos bioflocos pode desempenhar atividade probiótica. Em determinadas situações, a adição de bactérias exógenas a um meio com abuntante comunidade microbiana pode não exercer influência na abundância dos demais grupos de organismos que compõem o sistema de bioflocos.

1.Abstract

This study aimed to compare the effects of five doses of a commercial probiotic on total microbial abundance and on the abundance of *Bacillus subtilis*, *B. subtilis*-complex and *Vibrio* spp. in the gut and in the biofloc suspension of *L. vannamei* reared in a biofloc system. The experimental setup contained 5 treatments, in which the probiotic was added to the feed as follows: -**T0 -** 0 g of Pro2 kg $^{-1}$ of feed (without probiotic); -**T1** - 1 g of Pro2 kg $^{-1}$ of feed; -**T2** - 2 g of Pro2 kg $^{-1}$ of feed; -**T3** - 3 g of Pro2 kg $^{-1}$ of feed; - **T4** - 1 g of Pro2 kg $^{-1}$ of feed (100% daily feeding) and -**T5** - 3 g of Pro2 kg $^{-1}$ of feed (100% daily feeding). Survival rates were significantly higher in T2, T3 and T4 than in T0 and T1. Total microbial density of the biofloc suspension increased significantly from day 0 to day 15. No differences were found in *Bacillus* species density among the treatments in the gut and in the bioflocs. In T0, the density of *Vibrio* was higher in the water, where the probiotic was not applied. In general, the bacterial density of *Bacillus* was higher in the initial samples, indicating that the bioflocs themselves may have a probiotic role. In some situations, adding exogenous bacteria to an abundant bacterial community may not affect the abundance of various groups of organisms that compose the biofloc system.

2. Introduction

The use of probiotics has been widely explored in aquaculture practices. They are defined as live and non-pathogenic microorganisms in single or mixed culture that provide health benefits to the host. These benefits can be potentialized when these probiotics are administered in appropriate dosages (FAO/WHO 2001; Kumar, Roy, Meena & Sarkar 2016). For instance, they can be administered via water or as feed additives to serve as growth promoters and competitive exclusion agents (Moriarty 1998; Skjermo & Vadstein 1999; Dong, Van, & Cutting 2009).

Different bacterial species are used as probiotics in penaeid cultures, especially bacteria of the genus *Bacillus* (Ninawe & Selvin 2009; Sapcharoen & Rengpipat 2013). The ability to form spores, providing protection against acid and bile salts and other environmental conditions, make *Bacillus* spp. efficient probiotics. In addition, species of this genus have a high capacity to produce compounds of biotechnological importance such as antibiotics, enzymes, amino acids, and vitamins. They are also capable of degrading cholesterol, influencing the immune status of the host and displaying antiviral and antibacterial activity (Sorokulova 2013).

Bacillus subtilis is one of the most applied spore probiotic species. This bacterium grows efficiently with low-cost carbon and nitrogen sources, because its enzymes are very efficient at breaking down a great variety of proteins, carbohydrates and lipids of animal and vegetable origin into their constituent units (Soneenschein 1993). For this reason, it has been widely applied as a feed supplement in conventional clearwater shrimp culture. It can be directly incorporated into feed pellets at a suitable temperature (Diaz-Rosales et al 2009). For instance, Seenivasan, Radhakrishnan, Muralisankar & Bhavan (2012) found that *B. subtilis* can be used at 30 g of probiotic

kg^{-1} in the experimental diet of *Macrobrachium rosenbergii* to enhance the survival, growth, nutritional indices, tissue biochemical components and energy utilization performance of the animals. Pham, Tran, Doan, Le, Nguyen, Nguyen, Hong, Cutting & Pham (2017) observed that the oral administration of 1×10^9 cfu g^{-1} of *B. subtilis* protected *Penaeus monodon* against white spot viral disease. Sapcharoen & Rengpipat (2013) supplemented the diet of *L. vannamei* with two strains of *B. subtilis* at 25 g of bacteria per kg of feed and verified higher growth, immunostimulation and resistance against experimental *Vibrio* infection.

Vibrio spp. occur naturally in marine and estuarine environments. They can also be part of the intestinal bacterial community of *L. vannamei*, and some species are even probiotics (Verschuere, Rombaut & Sorgeloos 2000; Lakshmi, Viswanath & Gopal 2013; Liu, Li, Li, Cai, Li, Chen & Lyu 2015; Huang, Li, Wang & Shao 2016). However, most species are considered opportunistic bacteria and potential pathogens that cause vibriosis outbreaks and huge production losses (Saulnier, Haffner, Goarant, & Ansquer 2000).

The gut is considered the primary pathogen transmission route and has a close relation with the aquatic environment (De Schryver & Vadstein 2014). In this context, the probiotic bacteria compete with pathogenic *Vibrio* for adhesion sites, nutrients and chemical compounds in the water and in the gut of the host (Gatesoupe 1999; Mohapatra, Chakraborty, Kumar, DeBoeck & Mohanta 2013; Hamza, Kumar & Zinjarde 2015). Hence, they can stick to the mucosal epithelium of the gastrointestinal tract and help to resist pathogens, by decreasing their numbers in the host (Villaseñor, Rodríguez, Gomez-Gil, Valle & Córdova 2011; Akhter, Wu, Memon & Mohsin 2015; Lazado, Caipang & Estante 2015). It is important to monitor the water microbiota

during probiotic addition to determine the efficiency of gut colonization and the alterations that occur in the bacterial community due to the administration of these microorganisms (Merrifield, Dimitroglou, Foey, Davies, Baker, Bøgwald, Castex & Ringø 2010; Verscheure *et al*. 2000). Del`Duca, Cesar & Abreu (2015) verified that a strong similarity in the bacterial community exists between the gut and the water for tilapia cultured in clear water. On the other hand, Cardona, Gueguen, Magré, Lorgeoux, Piquemal, Pierrat, Noguier & Saulnier (2016) found a high similarity between the bacteria in the gut of shrimp cultures in clear water, but low similarity was found between the water and intestine samples of shrimp cultured in a BFT system.

Biofloc technology (BFT) is a fish and shrimp culture technique based on the stimulation and establishment of a microbial community to achieve recycling of nutrients, mainly nitrogen waste, and to maintain water quality (McIntosh, Samocha, Jones, Lawrence, Horowitz & Horowitz 2001; Wasielesky, Atwood, Stokes & Browdy 2006; Avnimelech 2007). In addition, it has been shown that other benefits such as improved growth, survival and disease resistance can also be expected (Wang, Pan, Zhang, Xu, Zhao & Mei 2016; Xu, Morris & Samocha 2016; Crab, Lambert, Defoirdt, Bossier & Verstraete 2010). In this sense, this microbial community can inhibit the proliferation of pathogens by competitive exclusion for food and space in the water or in the gut (Crab *et al*. 2010).

Concerning probiotic supplementation and adequate dosing, most reported results are from conventional clearwater culturing systems (Nimrat, Suksawat, Boonthai & Vuthiphandchai 2012; Sha, Wang, Liu, Jiang, Xin & Wang 2016), as previously mentioned. Shen, Fu, Li & Zhu (2010) observed higher growth and survival rates upon

adding 1×10^{4} and 5×10^{4} CFU g^{-1} feed of *B. subtilis* to the diet of *L. vannamei* reared in clear water.

In addition, positive effects of probiotic application are reported in biofloc systems. For instance, Krummenauer, Poersch, Romano, Lara, Encarnação & Wasielesky (2014) observed higher survival rates in shrimp reared in bioflocs, but naturally infected with *V. parahaemolyticus* and treated with a multistrain probiotic containing *Bacillus subtilis*. Ferreira, Bolívar, Pereira, Guertler, Vieira, Mouriño & Seiffert (2015) isolated a *Bacillus licheniformis* strain from biofloc suspension and verified its antagonistic activity against *V. alginolyticus*. Aguilera-Rivera, Prieto-Davo, Escalante, Chávez, Cuzón & Gaxiola (2014) suggested that the addition of a commercial probiotic mixture contributed to preventing outbreaks of opportunistic pathogenic bacteria in BFT systems.

Feeding represents 40-60% of the total production costs in shrimp/fish farming, and global spending on probiotics increased from \$2.7 bn in 2011 to \$4 bn in 2016 (Olmos & Paniagua-Michel 2014). Thus, finding the right dosage of the probiotic to be applied to a biofloc culture can represent an advantage from an economic point of view, since lower dosages can be incorporated to the feed during the production cycles. Little is known about the dosages and bacterial concentrations of probiotics that must be added to the daily feed and promote the interaction between the microbial communities present in biofloc-rich water.

Furthermore, the associated effects of adding different doses of external probiotic bacteria to the feed as a commercial product are still unclear, as is the modulation of the microbial community in terms of presence, bacterial abundance and composition under these conditions. Knowledge about such interactions is important for

establishing strategies for disease control, feed management and microbial community management in biofloc culture systems.

The primary objective of this study was to compare five different doses of a commercial probiotic mixture in the gut and in the culturing biofloc suspension of *L. vannamei* reared in a BFT system. Another goal is to verify the effects of this probiotic mixture on the abundance of *Bacillus subtilis* and the putative pathogen *Vibrio* in a biofloc technology culture system.

3. Material and Methods

3.1. Animal origin

Post-larval *L. vannamei* were obtained from the commercial hatchery Aquatec Ltda (Canguaretama, Rio Grande do Norte, Brazil) and were transferred to the hatchery sector of the Marine Station of Aquaculture, Institute of Oceanography, Federal University of Rio Grande. In the laboratory, shrimp were cultured in biofloc system until they reached an average weight of 5.14 ± 0.30 g before the experimental units were stocked.

3.2 Probiotic Mixture

The commercial probiotic mixture (Sanolife – PRO2[®] - INVE Aquaculture) used in this study was composed of *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus licheniformis* strains at a bacterial concentration of 2×10^{10} CFU g^{-1}.

3.3. Experimental design

Experimental design

The trial was carried out in 18 rectangular polyethylene tanks with a bottom area of 0.20 m^2 and an effective volume of 50 L, located in a climatically stabilized room. Shrimp (5.14±0.30 g) were transferred and stocked at 150 shrimp m^{-2} (30 shrimp per tank). The experimental setup contained five (5) treatments, each one corresponding to a different dose of the probiotic mixture, and one control, without the probiotic application. Each treatment included three replicates. The treatments were as follows:

-**T0 -** 0 g of Pro2 kg $^{-1}$ of feed (without probiotic)

-**T1** - 1 g of Pro2 kg $^{-1}$ of feed (2×10^{10} CFU kg^{-1})

-**T2** - 2 g of Pro2 kg $^{-1}$ of feed (4×10^{10} CFU kg^{-1})

-**T3** - 3 g of Pro2 kg $^{-1}$ of feed; (6×10^{10} CFU kg^{-1})

- **T4** - 1 g of Pro2 kg $^{-1}$ of feed (100% daily feeding) (4×10^{10} CFU kg^{-1} twice a day)

-**T5** - 3 g of Pro2 kg $^{-1}$ of feed (100% daily feeding) (6×10^{10} CFU kg^{-1} twice a day)

In all treatments, the shrimp were fed a 40% crude protein (CP) commercial diet (Potimar 40 J, Guabi®, Brazil) twice a day (0800 and 1700 h) for 30 days. For the treatments T1, T2 and T3, the probiotic was administered only in the first feeding of the day. For T4 and T5, the probiotic was added to the feed twice a day (100% daily feeding). The feeding rate was adapted according to the methodology proposed by Jory et al. (2001) and was initially 4% of the total biomass. The amount of daily feed offered varied in a range between approximately 4 and 6 g feed day^{-1}.

The biofloc experimental units were filled with 5 L of biofloc-rich water (10% reused water) obtained from an *L. vannamei* grow-out study in nine 35 m^3 tanks, plus 45 L of filtered (sand filter) natural seawater treated with a chlorine solution (10 ppm

measured immediately after chlorination) and dechlorinated using ascorbic acid powder (1 ppm). Dechlorinated municipal freshwater was used to compensate for evaporative losses and to maintain salinity throughout the experiment.

Sugar cane molasses (C:H:N=30.05:2.78:0.79) was added as an organic carbon source when the total ammonia level reached 1.0 mg L^{-1} to foster the development of microbial aggregates in the biofloc treatments. Organic carbon supplementation was based on the addition of 6 g of carbon for each 1 g of total ammonium nitrogen (TA-N) in the water. This procedure followed the methods described by Avnimelech (2009); Ebeling, Timmons & Bisogni (2006) and Samocha, Patnaik, Speed, Ali, Burger, Almeida & Ayub (2007).

3.4. Water quality and sampling

Dissolved oxygen (DO), temperature and pH were measured twice daily (0800 and 1700 h) using a YSI 556 MPS meter (YSI Inc., Yellow Springs, United States). Total ammonium nitrogen (UNESCO, 1983) and nitrite nitrogen (NO_2-N) (Aminot and Chaussepied, 1983) were measured twice a week. Salinity (measured with a manual optical refractometer), nitrate nitrogen (NO_3-N) (Aminot and Chaussepied, 1983), total suspended solids (TSS) (Strickland and Parson, 1972) and alkalinity (APHA, 1989) were measured weekly.

Biofloc suspension and shrimp samples were collected at days 0 (initial sampling), 15 and 30 (final sampling). Shrimp (3) from each tank (9 shrimp per treatment) were killed by thermal shock in an ice bath and aseptically necropsied to remove the intestinal tract at the end of the experimental period (30 days). The intestinal tract and water samples were fixed in a final concentration of 2% and 20%

paraformaldehyde solution, respectively. The gut samples were individually weighed for further analysis.

3.5. Growth performance and survival

Shrimp were randomly sampled weekly from each tank and individually weighed to the nearest 0.01g. At the end of each trial, the zootechnical performance of *L. vannamei* was evaluated by the final weight (g), weight gain (g) and survival rate (%).

3.6. Analysis of the presence and efficiency of the commercial probiotic mixture and putative pathogenic bacteria by fluorescence in situ hybridization (FISH)

Intestine samples were processed according to a protocol adapted from Epstein & Rossel (1995). A TWEEN solution (0.0001%) was added to each sample, which was then sonicated (Vibra Cell VCX 130PB, Sonics & Materials ®) three times (range 110.7 μm per 60 s). After sonication, the samples were centrifuged three times at $500{\times}g$ for five minutes. The supernatant was removed, and the remaining contents were washed twice with ultrapure water. Three supernatant fractions were placed in the same bottle and shaken vigorously. The material was then centrifuged as previously described. Biofloc suspension samples were also sonicated in order to detach bacterial cells from the microbial aggregates. Aliquots of each sample (water and intestinal tract) were filtered on polycarbonate filters (Nuclepore® - 0.2 μm) and refrigerated until hybridization.

Bacillus subtillis and the *Bacillus subtillis* complex (covers *B. pumilus* and *B. licheniformis)*, components of the probiotic mixture added to the biofloc-rich water, and putative pathogenic bacteria (*Vibrio* sp.) were identified with rRNA-targeted

oligonucleotide probes (Table 1) by a fluorescence *in situ* hybridization (FISH) protocol (Del'Duca Cesar, Diniz & Abreu 2013). A negative control made with a probe with no specificity for bacteria was used to evaluate the hybridization efficiency. All probes were labeled with the Cy3 fluorochrome. In addition to each specific probe, DAPI was used to determine the total bacterial abundance. The bacterial abundance was determined by direct counting at 1000× magnification using an epifluorescence microscope (Olympus® BX-60) equipped with the Chroma U-N41007, U-MWU2 and U-MWG2 optical filter set.

Table 1: rRNA-targeted oligonucleotide probes of different bacterial species used in this study. All probes were labelled with fluorochrome Cy3.

Probe	Specificity	Sequence 5 – 3'	%FA*	Reference
NON	Negative Control	TAGTGACGCCGTCGA	30	Yokokawa and Nagata 2005
BsubC	*B. subtilis*- complex	AAGCCACCTTTTATGTTTGA	35	Kyselková *et al*. 2009
Bsub	*B. subtilis*	CGTTCAAACAACCATCCGG	35	Kyselková *et al*. 2009
Vib519a	*Vibrio*	ACCACCTGCATGCGCTT	40	Hugget et al. 2008

*Percentage of Formamide (FA) in *in situ* Hybridization buffer

3.7. Statistical Analyses

One-way analysis of variance (ANOVA) was used to identify significant differences in bacterial abundance among treatments. The ANOVA was followed by Tukey's post hoc comparison when significant differences were found. Statistical significance was taken as $P< 0.05$.

4. Results

4.1. Water Quality

The water quality parameters are shown in Table 2. Temperature, dissolved oxygen, pH, salinity and nitrogen compounds (total ammonium nitrogen – TAN, nitrite nitrogen – NO_2-N, nitrate nitrogen – NO_3-N) and total suspended solids (TSS) did not differ significantly among treatments (Table 2).

Table 2: Mean (± standard deviation) values of water quality parameters throughout the experiment with different dosing of a commercial probiotic mixture in *L. vannamei* cultured in biofloc (BFT) system. Different letters indicate statistical differences (P<0.05).

Parameters	0g kg^{-1}	1g kg^{-1}	2g kg^{-1}	3g kg^{-1}	1g kg^{-1}(100%)	3g kg^{-1}(100%)
T (°C)	27.08 ± 1.33	27.03 ± 1.45	27.04 ± 0.62	27.06 ± 0.39	27.10±0.52	27.08±1.02
DO_2 (mg L^{-1})	5.53 ± 0.47	5.92 ± 0.51	5.67 ± 0.39	5.85 ± 0.34	5.97±0.63	5.59±0.72
pH	7.81 ± 0.38	7.77 ± 0.23	7.74 ± 0.15	7.84 ± 0.26	7.82±0.22	7.82±0.16
Salinity.	30.40 ± 2.06	30.52 ± 1.93	30.20 ± 1.38	30.3 ± 1.95	30.24±1.43	30.22±1.52
TAN (mg L^{-1})	0.09 ± 0.002	0.07 ± 0.007	0.12 ± 0.01	0.05 ± 0.008	0.07±0.002	0.05±0.003
NO_2-N (mg L^{-1})	0.28 ± 0.02	0.27± 0.07	0.10 ± 0.05	0.12 ± 0.08	0.21±0.04	0.19±0.01
NO_3-N (mg L^{-1})	63.80 ± 23.50	65.03 ±27.38	59.78±22.42	60.50±30.94	62.23±20.45	58.72±19.75
TSS (mg L^{-1})	533.19±41.23	530.83±47.58	517.50±33.70	499.87±32.45	502.30±29.54	537.35±42.81

4.2 Growth performance and survival

Final weight (g) and weight gain (g) did not differ significantly among treatments. Survival rates were significantly higher in treatments T2, T3 and T4 than in

T0 and T1. The probiotic dose of 1 g kg^{-1} (T1) did not differ significantly from the other treatments (Table 3).

Table 3: Mean (± standard deviation) values of growth performance parameters throughout the experiment with different dosing of a commercial probiotic mixture in *L. vannamei* cultured in biofloc (BFT) system. Different letters indicate statistical differences (P<0.05).

Parameters	T0	T1	T2	T3	T4	T5
Final W. (g)	9.21 ± 2.30	9.21 ± 2.18	7.93±1.36	8.56±2.01	8.64±1.61	9.05±2.59
W. Gain(g)	3.51±0.59	3.82±0.30	2.72±0.96	3.64±0.47	3.52±0.33	4.25±1.77
Surv.rate (%)	63.33±7.63[a]	83.33 ± 20.20[ab]	91.66±2.88[b]	96.66 ±5.77[b]	78.33±5.77[a]	98.33±2.88[b]

4.3 Bacterial density

4.3.1 Bacterial density in the biofloc suspension and in the gut of L. vannamei

The total density of bacteria present in the biofloc suspension of each treatment increased significantly (p<0.05) from day 0 (initial sample) (852.23±0.00) until day 15 (**T0**- 1356.50±5412.19; **T1**- 12308.80±4094.30; **T2**- 7265.10±5980.02; **T3**- 7879.99±7051.97; **T4**- 10442.00±5421.85; **T5**- 7896.84±3149.35 x 10^8 cells mL^{-1}). Although no significant differences were detected between days 15 and 30, it was verified that the bacterial abundance decreased during this sampling period. On day 30, this parameter was higher in **T2** (1079.83±1151.92 x 10^8 cells mL^{-1}) than in **T4** (3838.90±3588.96 x 10^8 cells mL^{-1}). The other treatments did not differ among each other (**T0**- 3020.34±2049.25; **T1**- 2418.05±2756.96; **T3**- 4458.47±398.98; **T5**- 1056.39±352.00 × 10^8 cells mL^{-1}) (Figure 1A).

No significant differences were detected in total microorganism abundance in the gut of *L. vannamei* under different probiotic doses at day 0 ($55.34 \pm 23.86 \cdot 10^8$ cells mL^{-1}), day 15 (**T0**- 38.32 ± 16.15; **T1**- 28.29 ± 16.18; **T2**- 22.28 ± 16.14; **T3**- 68.70 ± 67.95; **T4**- 49.87 ± 35.24; **T5**- $28.54 \pm 30.65 \times 10^8$ cells mL^{-1}) and day 30 (**T0**- 73.92 ± 14.50; **T1**- 106.59 ± 116.98; **T2**- 222.32 ± 270.36; **T3**- 191.45 ± 105.79; **T4**- 47.81 ± 29.76; **T5**- $210.72 \pm 141.21 \times 10^8$ cells mL^{-1}). However, there was a tendency toward increase at day 30, especially in treatments T2, T3 and T4 (Figure 1B).
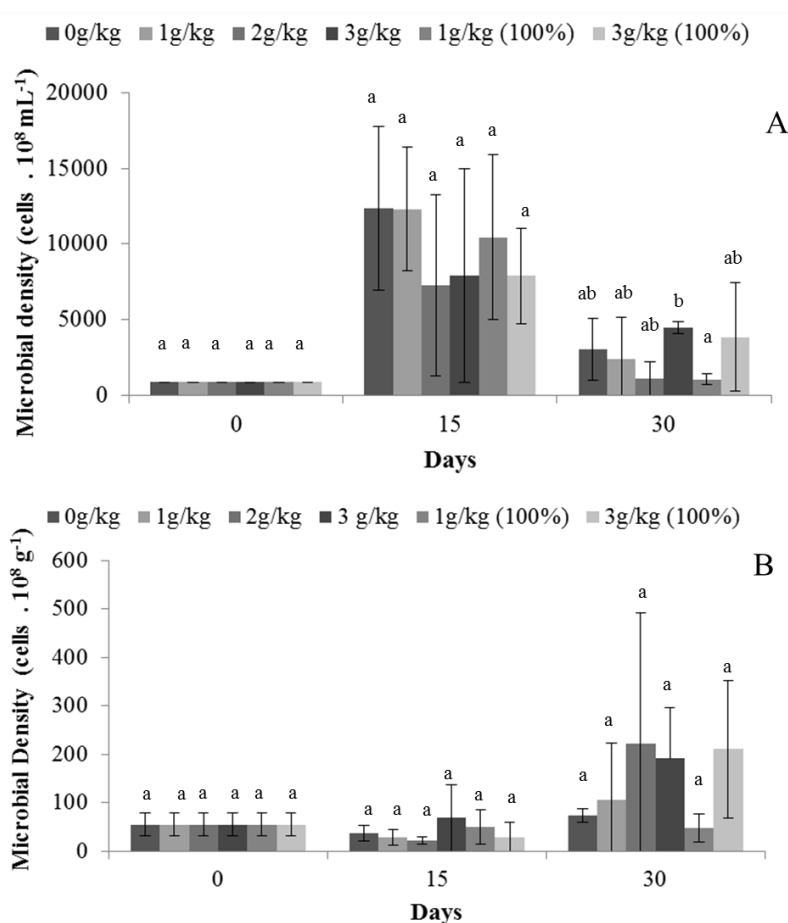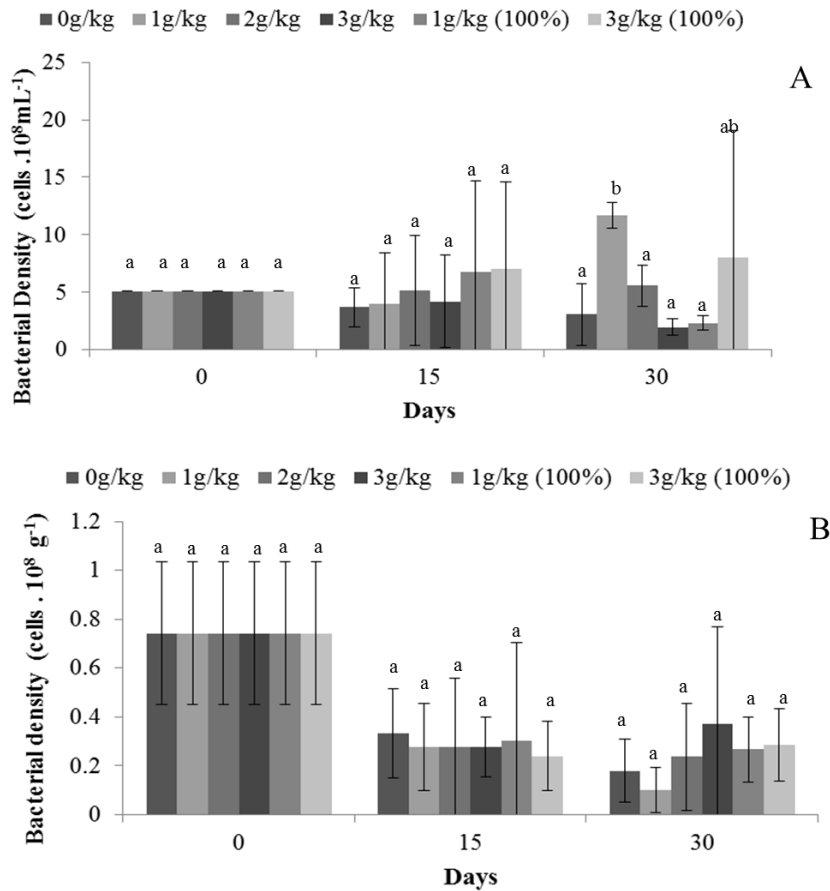

Figure 1: (A) Total microorganisms density present in culturing biofloc suspension; and (B) Total microorganisms density present in the gut of *L. vannamei* reared in biofloc system under different dosing (**T0** 0 g of Pro2 kg $^{-1}$ of feed (without probiotic); **T1** 1 g of Pro2 kg $^{-1}$ of feed ; **T2** 2 g of Pro2 kg $^{-1}$ of feed ; **T3** 3 g of Pro2 kg

$^{-1}$ of feed; **T4** 1 g of Pro2 kg $^{-1}$ of feed (100% daily feeding) and **T5** 3 g of Pro2 kg $^{-1}$ of feed (100% daily feeding)) of probiotics.

Regarding the bacterial density of *Bacillus subtilis* as a component of the probiotic mixture, no significant differences were observed on Day 0 (initial sample) ($5.06\pm0.00 \times 10^8$ cells mL^{-1}) or day 15 (**T0**- 3.66 ± 1.66; **T1**- 3.98 ± 4.43; **T2**- 5.11 ± 4.80; **T3**- 4.18 ± 4.02; **T4**- 6.74 ± 7.91; **T5**- $6.97\pm7.60\times10^8$ cells mL^{-1}). On day 30, a higher number of cells was detected in T1 (11.66 ± 1.12 x 10^8 cells mL^{-1}) compared with T0 ($3.04\pm2.68\times10^8$ cells mL^{-1}), T2 ($5.56\pm1.80\times10^8$ cells mL^{-1}), T3 ($1.92\pm0.71\times10^8$ cells mL^{-1}) and T4 ($2.29\pm0.61\times10^8$ cells mL^{-1}). The dosing of T3 did not differ significantly from the other treatments, with a bacterial abundance of $7.96\pm11.10\times10^8$ cells mL^{-1} (Figure 2A).

The density of *B. subtilis* present in the intestinal tract of *L. vannamei* was significantly higher at day 0 (initial sample) ($0.74\pm0.29.$ 10^8 cells mL^{-1}) than at days 15 (**T0**- 0.33 ± 0.18; **T1**- 0.27 ± 0.17; **T2** - 0.27 ± 0.28; **T3** - 0.27 ± 0.12; **T4**- 0.30 ± 0.40; **T5** - 0.23 ± 0.14 x 10^8 cells mL^{-1}) and 30 (**T0**- 0.17 ± 0.12; **T1**- 0.10 ± 0.09; **T2**- 0.23 ± 0.22; 3 g kg^{-1}- 0.37 ± 0.39; **T4**- 0.26 ± 0.13; **T5**- 0.28 ± 0.14 x 10^8 cells mL^{-1}), except for treatment T3, which was not significantly different. Likewise, no differences were observed among treatments during the experimental period (Figure 2B).

Figure 2: (A): Bacterial density of *Bacillus subtilis* present in culturing biofloc suspension; and (B) Bacterial density of *Bacillus subtilis* present in the gut of *L. vannamei* reared in biofloc system under different dosing (**T0** 0 g of Pro2 kg^{-1} of feed (without probiotic); **T1** 1 g of Pro2 kg^{-1} of feed ; **T2** 2 g of Pro2 kg^{-1} of feed ;**T3** 3 g of Pro2 kg^{-1} of feed; **T4** 1 g of Pro2 kg^{-1} of feed (100% daily feeding) and **T5** 3 g of Pro2 kg^{-1} of feed (100% daily feeding)) of probiotics.

B. subtilis complex abundance did not differ among treatments in the biofloc suspension on day 15 (**T0**- 0.58±0.40; **T1**- 1.25±1.27; **T2**- 2.73±3.82; **T3** - 0.64±0.96; **T4**- 1.39±1.98; **T5**- 1.39±0.98 ×10^8 cells mL^{-1}) or day 30 (**T0**- 7.47±6.18; **T1**- 7.15±6.32; **T2**- 2.63±2.12; **T3**- 1.31±0.62; **T4**- 0.46±0.25; **T5**- 3.60±3.18 ×10^8 cells mL^{-1}). The same was true in the gut on day 15 (**T0**- 0.05±0.05; **T1**- 0.03±0.03; **T2** -

0.06±0.10; **T3** - 0.06±0.05; **T4**- 0.06±0.10; **T5** - 0.04±0.07×10^8 cells mL^{-1}) and day 30 (**T0**- 0.02±0.02; **T1**- 0.19±0.30; **T2**- 0.05±0.04; **T3**- 0.04±0.07; **T4**- 0.04±0.04; **T5**- 0.07±0.07×10^8 cells mL^{-1}). Nonetheless, a higher density was observed in the beginning of the experiment (7.85±0.00×10^8 cells mL^{-1} in the water; 1.09±1.79. 10^8 cells mL^{-1} in the gut) (Figure 3A and B).
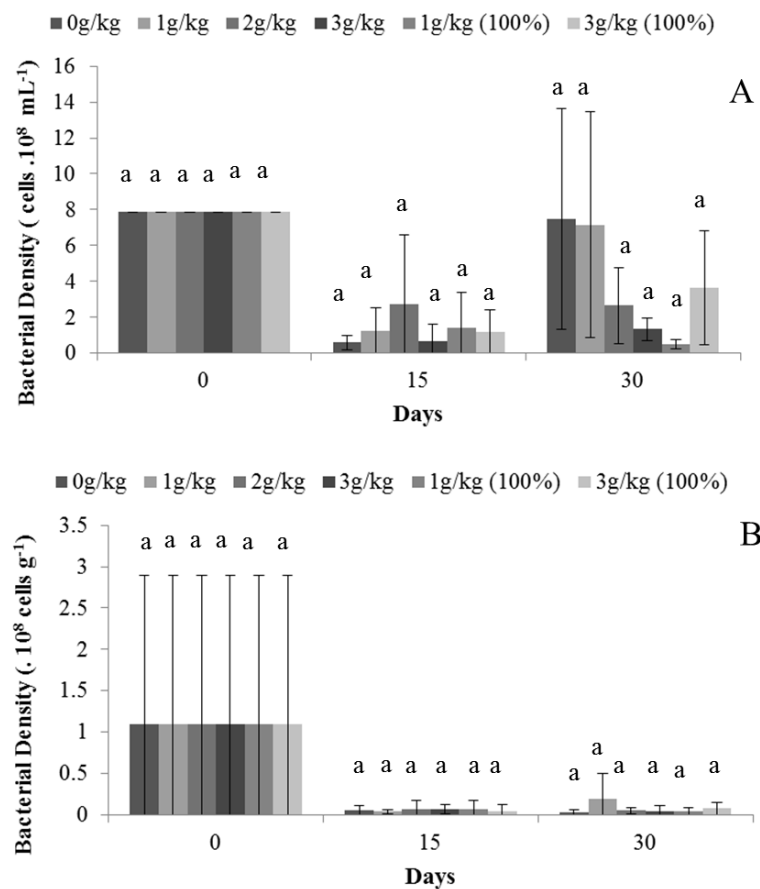
Figure 3: (A): Bacterial density of *Bacillus subtilis*-complex present in culturing biofloc suspension; and (B) Bacterial density of *Bacillus subtilis*- complex present in the gut of *L. vannamei* reared in biofloc system under different dosing (**T0** 0 g of Pro2 kg $^{-1}$ of feed (without probiotic); **T1** 1 g of Pro2 kg $^{-1}$ of feed ; **T2** 2 g of Pro2 kg $^{-1}$ of feed ;**T3** 3 g of Pro2 kg $^{-1}$ of feed; **T4** 1 g of Pro2 kg $^{-1}$ of feed (100% daily feeding) and **T5** 3 g of Pro2 kg $^{-1}$ of feed (100% daily feeding)) of probiotics.

The abundance of *Vibrio* spp. in the biofloc suspensions from each treatment is shown on Figure 4A. No differences were verified among treatments at days 0 and 15. Bacterial abundance was significantly higher on day 0 ($1.57 \pm 0.00 \times 10^8$ cells mL^{-1}) than on day 15 (**T0**- 0.05 ± 0.10; **T1**- 0.11 ± 0.20; **T2**- 0.49 ± 0.71; **T3**- 0.00 ± 0.00; **T4**- 0.05 ± 0.10; **T5**- $0.05 \pm 0.10 \times 10^8$ cells mL^{-1}) ($p < 0.05$), and there were no differences observed between day 0 and day 30 (**T0**- 8.09 ± 1.42; **T1**- 1.03 ± 0.91; **T2**- 0.96 ± 0.67; **T3**- 0.89 ± 0.14; **T4**- 1.89 ± 1.29; **T5**- $1.19 \pm 1.07 \times 10^8$ cells mL^{-1}), except for the control (**T0**), without probiotic supplementation, which was significantly higher. The same treatment showed the highest density of *Vibrio* among the different probiotic doses on the 30th day.

On the other hand, the abundance of *Vibrio* in the intestinal tract did not differ among treatments on day 0 ($0.03 \pm 0.05 \times 10^8$ cells mL^{-1}), day 15 (**T0**- 0.02 ± 0.03; **T1**- 0.01 ± 0.02; **T2**- 0.00 ± 0.00; **T3**- 0.06 ± 0.11; **T4** – 0.09 ± 0.16; **T5**- $0.02 \pm 0.02 \times 10^8$ cells mL^{-1}) or day 30 (**T0**- 0.01 ± 0.01; **T1**- 0.02 ± 0.04; **T2**- 0.07 ± 0.13; **T3**- 0.006 ± 0.01; **T4**- 0.006 ± 0.01; **T5**- $0.00 \pm 0.00 \times 10^8$ cells mL^{-1}) (Figure 4B).
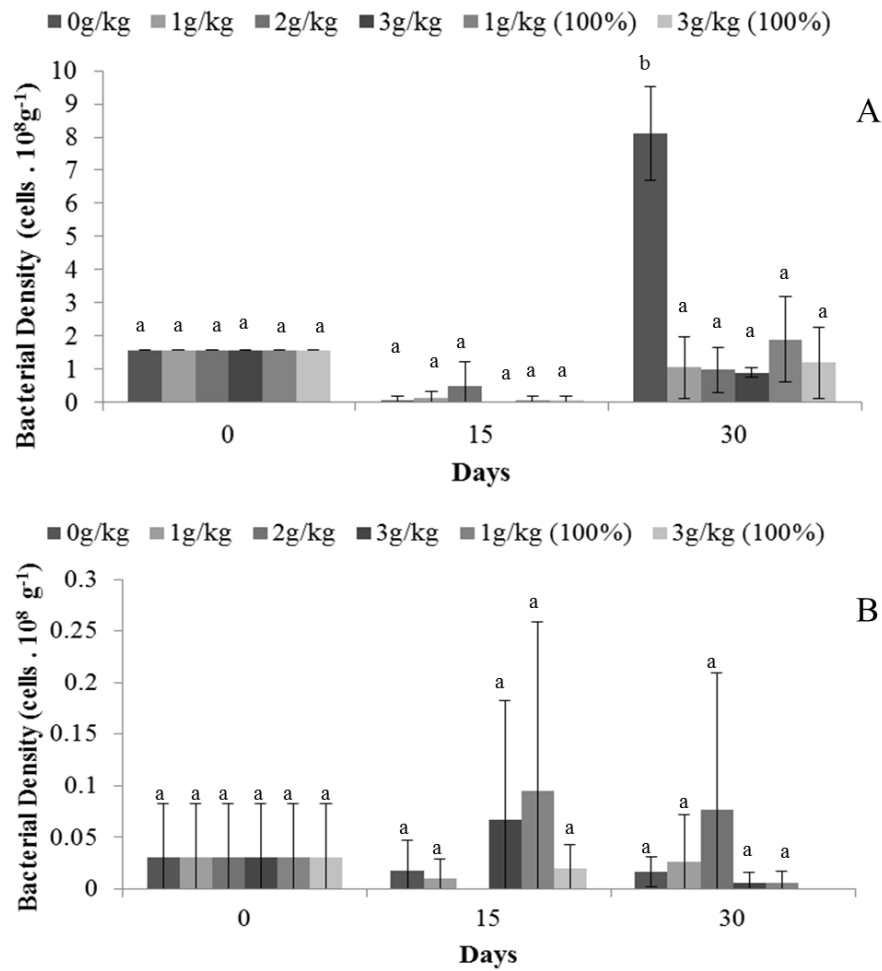
Figure 4: (A): Bacterial density of *Vibrio* spp. as a putative pathogenic bacteria present in the culturing biofloc suspension; and (B) Bacterial density of *Vibrio* spp. as a putative pathogenic bacteria present in the gut of *L. vannamei* reared in biofloc system under different dosing (**T0** 0 g of Pro2 kg $^{-1}$ of feed (without probiotic); **T1** 1 g of Pro2 kg $^{-1}$ of feed; **T2** 2 g of Pro2 kg $^{-1}$ of feed; **T3** 3 g of Pro2 kg $^{-1}$ of feed; **T4** 1 g of Pro2 kg $^{-1}$ of feed (100% daily feeding) and **T5** 3 g of Pro2 kg $^{-1}$ of feed (100% daily feeding)) of probiotics.

4.3.2 Percentages of B. subtilis and Vibrio spp.

Regarding the percentages of *B. subtilis,* the *B. subtilis* complex and *Vibrio* spp. among all bacteria present in the biofloc suspensions, the most significant contribution

of *B. subtilis* was in the initial sample (Day 0), followed by T4. The same treatment did not present significant differences among T0, T1, T2, T3 and T5 (Table 4). The same pattern was observed for the *B. subtilis* complex, in which the initial sample showed a higher contribution of these bacteria to the total microbial abundance. The percentage of this sample was significantly higher than the percentages of T3, T4 and T5.

Table 4: Percentage (Mean ± Standard Deviation) of *Bacillus subtilis* and *Vibrio sp.* in the total bacterial abundance observed in the culturing water of *L. vannamei* reared in BFT under different dosing of probiotic supplementation. Different letters indicate statistical differences (P<0.05).

	Initial sample	T0	T1	T2	T3	T4	T5
B. subtilis	0.59±0.00[ab]	0.07±0.04[a]	1.02±0.75[b]	1.09±0.88[ab]	0.04±0.01[a]	0.24±0.11[ab]	0.15±0.05[ab]
B.subtilis complex	- 1.37±2.12[a]	0.03±0.03[a]	0.50±0.77[a]	0.09±0.09[a]	0.06±0.10[a]	0.18±0.29[a]	0.07±0.09[a]
Vibrio spp.	0.18±0.00[ab]	0.53±0.60[b]	0.12±0.11[ab]	0.11±0.74[ab]	0.02±0.004[a]	0.20±0.16[ab]	0.07±0.07[a]

In the shrimp gut, the 1 g kg^{-1} treatment yielded the highest percentage of *B. subtilis*, followed by T2. The lowest percentage of *B. subtilis* in the total bacterial density was observed in T3. No differences were observed in the percentage of the *B. subtilis* complex in the gut. The highest percentage of *Vibrio* sp. in the intestinal tract was measured in T0, in which the probiotic was not applied. On the other hand, the lowest contribution of *Vibrio* to the total bacterial density was found in probiotic treatment T3, followed by T5 (Table 5).

Table 5: Percentage (Mean ± Standard Deviation) of *Bacillus subtilis* and *Vibrio sp.* in the total bacterial abundance observed in the gut of *L. vannamei* reared in BFT under

different dosing of probiotic supplementation. Different letters indicate statistical differences (P<0.05).

	Initial sample	T0	T1	T2	T3	T4	T5
B. subtilis	1.60±0.95[b]	0.25±0.19[a]	0.12±0.15[a]	0.16±0.24[a]	0.42±0.62[a]	0.77±0.68[ab]	0.15±0.05[a]
B.subtilis - complex	0.92±0.00[b]	0.53±0.73[ab]	0.36±0.18[ab]	0.35±0.44[ab]	0.02±0.01[a]	0.05±0.03[a]	0.07±0.09[a]
Vibrio spp.	0.07±0.13[a]	0.02±0.01[a]	0.01±0.01[a]	0.07±0.13[a]	0.00±0.01[a]	0.00±0.01[a]	0.00±0.00[a]

5. Discussion

The use of biofloc technology as a shrimp culturing system offers the possibility of maintaining good water quality with minimal or zero water exchange (De Schryver, Crab, Defoirdt, Boon & Verstraete 2008). This can be attributed to the fact that the BFT system consists of a predominantly heterotrophic community, and according to Yang, Wang, Zhang & Zhou (2011), a number of heterotrophic microorganisms have been reported to nitrify and/or transform many types of nitrogen compounds by converting them to microbial biomass in the presence of oxygen and the addition of an organic carbon source, which is the main principle of biofloc formation (Avnimelech 1999; Ebeling *et al.* 2006). The levels of nitrogen compounds remained whithin the recommended range for *L. vannamei* (Lin & Chen., 2001; Lin & Chen., 2003; Kuhn, Smith, Boardman, Angier, Marsh & Flick; Furtado, Poersch & Wasielesky 2014) and did not differ among treatments.

The accumulation of nitrate-N in all treatments is evidence of the nitrification process, which has nitrate accumulation as a final product (Hargreaves 2006). In a biofloc system, nitrification could possibly be performed by other microorganisms present in the floc, as a diversity of photoautotrophic, chemoautotrophic and

heterotrophic microorganisms is found in this culture system (Ebeling *et al*. 2006). Moreover, the autotrophic ammonia nitrogen oxidizing reaction carried out by the nitrifying bacteria can occur simultaneously in the water (Hargreaves 2006), even though these bacteria are known to have a slower growth rate and are more sensitive to variations in water quality parameters such as pH and temperature (Crab, Avnimelech, Defoirdt, Bossier & Verstraete 2007; Ebeling *et al*. 2006). The occurrence of this process is evidenced in the present study by the accumulation of NO_3-N, which has similarly been reported by Krummenauer *et al.* (2014) for the culture of *L. vannamei* in a Biofloc system.

Water temperature, dissolved oxygen, pH, salinity and total suspended solids (TSS) were similar among the treatments. These values remained within the proper range for rearing *L. vannamei* (Van Wyk & Scarpa, 1999; Furtado, Poersch & Wasielesky 2011; Samocha *et al*. 2007).

The application of the probiotic mixture did not interfere in the water quality parameters analyzed during this study. Aguilera-Rivera *et al*. (2014) and Krummenauer *et al.* (2014) obtained similar results, with no significant differences in ammonia, nitrite or nitrate between biofloc treatments with and without probiotic application. On the other hand, Wu, Zhao, Peng, Cu, Wang & Liang (2016) verified that application of a commercial *B. subtilis* probiotic improved the water quality by reducing the levels of pH, nitrite and soluble reactive phosphorus. Likewise, Nimrat *et al*. (2012) investigated the effectiveness of a mixed *Bacillus* probiotic during the rearing of *L. vannamei* in the larval and post-larval stages. The authors found positive results for water quality, especially for pH and ammonia levels. It seems that if the probiotic is added to feed or to the water in a relatively rich environment such as the biofloc system, it has a lower

influence on water quality parameters. In this sense, the probiotic is more effective in terms of water quality maintenance when added to an environment with lower bacterial abundance, such as clear water.

Regarding growth performance parameters, no differences were found in final weight or weight gain among the different probiotic doses. Similarly, Ferreira *et al.* (2015) did not verify any significant effect of a *Bacillus* sp. strain on the growth performance of *L. vannamei* in a BFT system. The strain was included in the feed at a concentration of 1.15×10^4 CFU g^{-1}. Wu *et al.* (2016) also did not observe differences in the growth performance of *L. vannamei* when *B. subtilis* was added to the water to the water at a concentration of 5×10^4 CFU mL^{-1}. These results, in combination with the present study, suggest that the presence of the probiotic does not have an influence on the growth performance of shrimp cultured in a biofloc system. For instance, Aguilera-Rivera *et al.* (2014) did not observe any influence of probiotic application on *L. vannamei* growth. However, the authors found a significant difference in growth when comparing BFT with clear water. It is confirmed that the bioflocs can serve as a high-quality food source for *L. vannamei*, improving growth performance parameters (Wasielesky *et al*. 2006).

On the other hand, the highest survival rates were verified when 2 and 3 g of probiotic was added per kg of feed offered once a day. Additionally, higher survival rates were verified at the treatment in which 3 g of probiotic was added per kg of feed and offered twice a day. These dosing regimens mentioned above correspond to 4×10^{10} CFU kg^{-1}, 6×10^{10} CFU kg^{-1} and 1.2×10^{11} CFU kg^{-1} of feed, respectively. Considering that during the experimental period, a range of 4 to 6 g of feed was offered daily to the shrimp based on the biomass, the final bacterial concentration added to each tank was

approximately 10^7 to 10^8 CFU feeding^{-1}. Relevantly, it is reported elsewhere that a level between 10^7 and 10^8 CFU g^{-1} of *Bacillus* incorporation in the feed enhances immune response and survival rates. Shen *et al.* (2010) also observed higher survival rates when *B. subtilis* was added at 1×10^4 and 5×10^4 CFU g^{-1} feed to the diet of *L. vannamei* reared in clear water. Li et al. (2009) and Zokaeifar, Babaei, Saad, Kamarudin, Sijam, Balcazar, (2014) verified higher survival rates than the control (without probiotics) with 10^8 and 10^{10} *B. subtilis* incorporated in the feed. These results are in concordance with the present study, in which the lowest survival rates were observed in the treatment without probiotics.

However, it is important to highlight that, similarly to T0, T4, in which the probiotic was applied twice a day, also showed lower survival rates. This fact indicates that, in some situations, the application of probiotics may not have a strong influence on survival rates. Such information can represent an economic advantage to farmers, taking in account the high costs of probiotics and feed (Olmos & Paniagua-Michel 2014). Further study is needed to investigate in which situations, microbial community operational aspects and culturing systems this information is relevant.

One of the main described modes of action of probiotic bacteria in shrimp concerns its role in digestive processes. The probiotics, when added to the feed, can stimulate the production of digestive enzymes (proteases and lipases) and antimicrobial substances, reflecting on higher survival (Ziaei-Nejad, Rezaei, Takami, Lovett, Mirvaghefi & Shakouor 2006; Zokaeifar *et al*. 2014). The results discussed above were performed in a conventional flow-through system with water exchange and highlight the positive effects of applying probiotics in the shrimp culture.

The intestinal community of aquatic animals is highly dynamic (Stephens, Burns, Stagaman, Wong, Rawls, Guillemin & Bohannan 2016). In the present study, the total microbial density present in the water confirmed this dynamic nature by increasing in the first 15 days and decreasing again by day 30 of the experiment. The same pattern was observed by Wu *et al.* (2016) when adding 5×10^4 CFU mL^{-1} of *B. subtilis* to *L. vannamei* cultured in clear water. The authors verified an increase in microbial diversity in the middle phase of the experiment (16 days) and a decrease in the final phase. Additionally, they did not observe any differences between treatment and control (without probiotics).

On the other hand, Hu *et al.* (2016) reported an increase in bacterial diversity in the water when combining *Bacillus* with molasses as a carbon source added daily to the system. In fact, carbon sources stimulate the biomass production of heterotrophic bacteria (Avnimelech 1999), increasing the diversity. In the present study, molasses as a carbon source was added only to start up the biofloc system, and not daily. This fact may have influenced the bacterial abundance over the course of the experiment, since the only available source of carbon is through the feed, and this limited carbon is being consumed by the probiotic bacteria and the microbial community present in the bioflocs. It is an indication that the way that biofloc systems are operated directly influences the profile and composition of the microbial community in the bioflocs.

Del'Duca *et al.* (2015) and De Schryver, Defoirdt & Sorgeloos (2014) suggest that there is a high similarity between the microbiota present in the water and in the gut of aquatic organisms. Although there were no significant differences, the microbial abundance in the gut of shrimp slightly increased in the gut on day 30 in the treatments 2 g kg^{-1} (T2), 3 g kg^{-1} (T3) and 3 g kg^{-1} (100%) (T5), while abundance decreased over

the same period in the water. However, by comparing the results of microbial abundance between the biofloc suspension and the shrimp gut, it is possible to observe that the enhancement in the water was higher by two orders of magnitude. This prominent difference may indicate that the consumption of bioflocs by the shrimp is not directly influenced by the abundance in the biofloc-rich water and instead remains constant. When in the water, the probiotic can be absorbed via host osmoregulation processes and through the feed (Kesakordi-Watson, Kaspar, Lategan & Gibson 2008).

The density of *B. subtilis* as a probiotic in the water was higher when the probiotic was added to the feed at concentrations of 2×10^{10} (T2) and 1.2×10^{11} CFU kg^{-1} (T5). The *B. subtilis* complex showed a tendency to be higher in T0, T1 and T2 in 30 days, although no differences were found. Shen *et al.* (2010) suggested that the effect of probiotic bacteria is not dose dependent, and extremely high dosing can cause an imbalance of microbial flora in the intestinal tract. For the *B. subtilis* complex, the bacterial abundance was higher at day 0 than in the middle and end of the experimental period. In the beginning of the experiment, no probiotic had yet been added to the water; the bacterial abundance was due to the microbial community present in the bioflocs. The presence of *Bacillus* sp. in the water during this period provides evidence that these bacteria are naturally present in the bioflocs, as was already stated by Ferreira *et al.* (2015). In fact, bacteria of the genus *Bacillus* are among the most widespread microorganisms in nature; they can be found in soil, water and air (Olmos & Paniagua-Michel 2014).

Regarding *B. subtilis* and the B. *subtilis* complex in the intestinal tract, it was verified that the density decreased in 15 and 30 days, comparing to the initial sample. Wu *et al.* (2016) suggest that the high organic load in the water promotes a growing

abundance of indigenous organisms, decreasing the abundance of *B. subtilis*. Additionally, the bioflocs may have a probiotic function in this case. Ferreira *et al*. (2015) isolated *Bacillus sp*. bacteria from the biofloc-rich water of an *L. vannamei* culture and plated them to investigate the antagonistic *in vitro* activity and the total number of *Vibrio* and *Bacillus* in the water. These results complement the data obtained in the present study.

When the probiotic was not included in the feed, the density of *Vibrio* was significantly higher in the water on day 30. Ferreira *et al.* (2015) observed a reduction of *Vibrio* sp. prevalence in the water when a probiotic supplement was used. Otherwise, species of the genus *Vibrio* are naturally present in the water as well (Lakshimi *et al*. 2013). Furthermore, no *Vibrio* was detected in the gut of *L. vannamei* with the higher dose of probiotic in the feed, even when the probiotic compounds were not abundant in the gut. Riquelme *et al*. (2001) affirm that the higher the concentration of probiotic in the water, the shorter is the ingestion time for the shrimp. In this case, the addition of the probiotic in a proportion of 3 g kg^{-1} associated with the higher frequency of 100% of the daily feed may possibly contribute to the reduction of *Vibrio* density in the gut of shrimp. In addition to the bacterial density, another important property of probiotics is the production of substances that have antagonistic properties, such as organic acids and bacteriocins (Ringø, Lovmo, Kristiansen, Bakken, Salinas, Myklebust, Olsen & Mayhew 2010; Ringø, Olsen, Vecino, Wadsworth & Song 2012). These substances can alter the metabolism of microbiota to produce short-chain fatty acids, increase sodium and water absorption, decrease colonic motility (Sakata, Kojima, Fujieda, Miyakozawa, Takahashi, & Ushida 1999), and support the health of the host, providing protection

against infections by stimulating the immune system (Lara-Flores & Aguirre-Guzman, 2009; Lara-Flores, 2011).

Regarding the percentages of *B. subtilis* and the *B. subtilis* complex relative to the total microbial density in the intestinal tract of *L. vannamei*, there was a tendency toward increasing colonization. However, this is possibly due to colonization by other bacterial groups present in the bioflocs, not the probiotic bacteria themselves. BFT systems contain a high microbial diversity and abundance (Manan, Moh, Kazan, Suratman, & Khwanuddin 2016), and the addition of exogenous bacteria at a relatively low concentration probably did not allow the high growth and establishment of these bacteria in the gut. This fact, associated with the low percentage of *Vibrio*, can explain the lower percentage of probiotic bacteria and highlight the importance of BFT as a culturing system.

In the present study, the high standard deviations obtained are possible evidence of a methodological difficulty in collecting the samples. Since each area of the microbial aggregate has a different profile and characteristics (De Schryver *et al.* 2008), further studies are needed to investigate the differences in the microbial community in different regions of the bioflocs.

The addition of a commercial probiotic mixture containing *Bacillus* was able to modify the gut bacterial community in *L. vannamei*, enhancing the bacterial abundance and decreasing the number of *Vibrio sp*. (Villaseñor *et al*. 2014; Li *et al.* 2009). In addition, Aguillera-Rivera et al. (2014) suggest that there is a synergistic effect between the microbial community in the bioflocs and the probiotic bacteria added to the feed. This interactive effect of the probiotic bacteria added to the biofloc environment regarding growth performance could be verified in the present study. Nevertheless,

further studies are needed in order to understand the mechanisms behind this synergistic effect (Aguillera-Rivera *et al.* 2014).

In conclusion, the results of the present study suggest that the probiotic mixture added at a dose of 3 g kg^{-1} at a frequency of 100% of the daily feed contributed to enhanced survival rates and decreased the number of *Vibrio* in the gut of *L. vannamei*. Moreover, the results also indicate that the bioflocs can have a probiotic role due to the significant presence of *B. subtilis* in the water and in the gut of shrimp prior the beginning of the experiment.

The total microbial density, associated with the low percentage of *Bacillus* in the water, shows the potential of the bioflocs to have a high bacterial abundance, which can compete with the exogenous probiotic bacteria and decrease their growth and presence. However, the microbial community naturally present in the bioflocs increases resistance against potential pathogens, representing an economic advantage to farmers.

These observations indicate that, in some situations, adding exogenous bacteria to an abundant bacterial community may not affect the dynamics and interactions between the various groups of organisms that compose the biofloc system. Such results can be brought to bear on different approaches to managing and operating the biofloc system or the application of probiotic.

7. References

Aguillera-Rivera D., Prieto-Davo A., Escalante K., Chávez C., Cuzon G., Gaxiola G. (2014) Probiotic effect of FLOC on *Vibrios* in the Pacific White shrimp *Litopenaeus vannamei*. *Aquaculture* **424-425**, 215-219.

Akhter N., Wu B., Memon A.M., Mohsin M. (2015) Probiotics and prebiotics associated with aquaculture: A review. *Fish and Shellfish Immunology* **45**, 733-741.

Aminot A., Chaussepied M. (1983) Manuel des analyses chimiques en milieu marin. CNEXO, Brest. 395 pp.

APHA (American Public Health Association) (1998) Standard Methods for the Examination of Water and Wastewater, 20st edition. . Washington, DC. 1193 pp.

Avinimelech, Y., 1999. Carbon/nitrogen ratio as a control element in aquaculture systems. *Aquaculture* **176**, p. 227-235.

Avnimelech Y.(2007) Feeding with microbial flocs by tilapia in minimal discharge bio-flocs technology ponds. *Aquaculture* **264**, 140–147.

Avnimelech Y. (2009) Biofloc Technology: A practical Hand Book. The World Aquaculture Society, Baton Rouge, Louisiana, United States.

Cardona E., Gueguen Y., Magré K., Lorgeoux B., Piquemal D., Pierrat F., Noguier F., Saulnier D.(2016) Bacterial community characterization of water and intestine of the shrimp *Litopenaeus stylirostris* in a biofloc system. *BMC Microbiology*, **DOI 10.1186/s12866-016-0770-z.**

Crab R., Avnimelech Y., Defoirdt T., Bossier P. Verstraete W. (2007) Nitrogen removal techniques in aquaculture for a sustainable production. *Aquaculture* **270**, 1–14.

Crab R., Lambert A., Defoirdt T., Bossier P., Verstraete W. (2010) The application of bioflocs technology to protect brine shrimp (*Artemia franciscana*) from pathogenic *Vibrio harveyi. Journal of Applied Microbiology* **109,** 1643-1649.

De Schryver P., Vadstein O. (2014) Ecological theory as a foundation to control pathogenic invasion in aquaculture. *The ISME Journal*, 1-9.

De Schryver P., Defoirdt T., Sorgeloos, P. (2014) Early Mortality Syndrome Outbreaks: A Microbial Management Issue in Shrimp Farming? *PLOS Pathogens*, **10(4)**, 1-2.

De Schryver P; Crab R., Defoirdt T., Boon N., Verstraete W. (2008) The basics of bio-flocs technology: The added value for aquaculture. *Aquaculture* **277**(3-4): 125–137.

Del`Duca A., Cesar D.E., Abreu P.C. (2015) Bacterial community of pond's water, sediment and in the guts of tilapia (*Oreochromis niloticus*) juveniles characterized by fluorescent *in situ* hybridization technique. *Aquaculture Research* **46**(3), 707-715.

Del'Duca A., Cesar D.E., Diniz C.G., Abreu P.C. (2013) Evaluation of the presence and efficiency of potential probiotic bacteria in the gut of tilapia (*Oreochromis niloticus*) using the fluorescent in situ hybridization technique. *Aquaculture* **388-391**, 115-121.

Dong T.C., Van P.H., Cutting S.M. 2009. Bacillus Probiotics. *Nutra Foods*, **8**, 7–14.

Ebeling J.M., Timmons M.B., Bisogni J.J. (2006) Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic control of ammonia-nitrogen in aquaculture production systems. *Aquaculture* **257**, 346–358.

Epstein S., Rossel J. (1995) Enumeration of sandy sediment bacteria: search for optimal protocol. *Marine Ecology Progress Series* **117,** 289–298.

FAO/WHO. 2002. Guidelines for the Evaluation of Probiotics. in Food. Report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food, London Ontario, Canada, 1–11.

Ferreira G.S., Bolívar N.C., Pereira S.A., Guertler C., Vieira F.N., Mouriño J.L.P., Seiffert W.Q. (2015) Microbial biofloc as a source of probiotic bacteria for the culture of *Litopenaus vannamei*. *Aquaculture* **448**, 273-279.

Furtado P.S., Poersch L.H., Wasielesky W. (2011) Effect of calcium hydroxide, carbonate and sodium bicarbonate on water quality and zootechnical performance of shrimp *Litopenaeus vannamei* reared in bio-flocs technology (BFT) systems. *Aquaculture* **321**(1-2), 130–135.

Gatesoupe F.J. (1999) The use of probiotics in aquaculture. *Aquaculture* **180**, 147-165.

Hamza F., Kumar A.R., Zinjarde S.(2016) Antibiofilm potential of a tropical marine *Bacillus licheniformis* isolate: role in disruption of aquaculture associated biofilms. *Aquaculture Research* **47(8)**, 2661-2669.

Hargreaves J.A. 2006. Photosynthetic suspended growth systems in aquaculture. *Aquacultural Engineering* **34**, 344-363.

Hu X., Cao Y., Wen G., Zhang X., Xu Y., Xu W., Xu Y., Li Z. (2016) Effect of combined use of *Bacillus* and molasses on microbial communities in shrimp cultural enclosure systems. doi:10.1111/are.1310

Huang, Z., Li, X., Wang, L., Shao, Z., 2016.Changes in the intestinal bacterial community during the growth of white shrimp, *Litopenaeus vannamei.* Aquaculture Research 47, 1737-1746.

Huber I., Spangaard K., Appel K., Rossen L., Nielsen T., Gram L. (2004) Phylogenetic analysis and in situ identification of the intestinal microbial community of rainbow trout (*Oncorhynchus mykiss*, Walbaum). *Journal of Applied Microbiology* **96**, 117–132.

Jory D.E., Cabrera T.R., Dugger D.M., Fegan D., Lee P.G., Lawrence A.L., Jackson C.J., Mcintosh R.P., Castañeda J. (2001) A global review of shrimp feed management: status and perspectives. In: *The New Wave, Proceedings of the Special Session on Sustainable Shrimp Culture* (ed by Browdy C.L., Jory D.E.) pp. 104–152. The World Aquaculture Society, Baton Rouge, LA,.

Kesakordi-Watson A., Kaspar H., Lategan M.J., Gibson L. (2008) Probiotics in Aquaculture: The need, principles and mechanisms of action and screening processes. *Aquaculture* **274**, 1-14.

Kos B., Suskovic J., Vukovic S., Simpraga M., Frece J., Matosic S. (2003) Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *Journal of applied Microbiology* **94**, 981-987.

Krummenauer D., Poersch L., Romano L.A., Lara G.R., Encarnação P., Wasielesky W. (2014) The Effect of Probiotics in a *Litopenaeus vannamei* Biofloc Culture System

Infected with *Vibrio parahaemolyticus. Journal of applied Aquaculture* **26**, 370-379.

Kuhn D.D., Smith S.A., Boardman G.D., Angier M.W., Marsh L., Flick J.R. (2010) Chronic toxicity of nitrate to Pacific white shrimp, *Litopenaeus vannamei*: Impacts on survival, growth, antennae length, and pathology. *Aquaculture* **306**, 329-333.

Kumar V., Roy S., Meena D.K., Sarkar U.K .(2016) Application of Probiotics in Shrimp Aquaculture: Importance, Mechanisms of Action, and Methods of Administration. *Reviews in Fisheries Sciences and Aquaculture*, **24(4)**, 342-368.

Kyselková M., Kopecký J., Frapolli M., Défago G., Ságová-Marecková M., Grundmann G.L., Moënne-Loccoz Y. (2009) Comparison of rhizobacterial community composition in soil suppressive or conducive to tobacco black root rot disease. *ISME J* **10 (3)**, 1127-1138.

Lakshimi B., Viswanath B., Gopal D.V.R.S. (2013) Probiotics as Antiviral Agents in Shrimp Aquaculture. *Journal of Pathogens* **ttp://dx.doi.org/10.1155/2013/424123**.

Lara-Flores M., Aguirre-Guzman G. (2009). The use of probiotic in fish and shrimp aquaculture (a review). In: Perez-Guerra N, Pastrana-Castro L (eds) Probiotics: production, evaluation and uses in animal feed. **Research Signpost**, Kerala, 75–89.

Lara-Flores M. (2011).The use of probiotic in aquaculture: an overview. *Int. J. Microbiol. Res* **2**, 471–478.

Lazado C.C., Caipang C.M.A., Estante E.G. (2015) Prospects of host-associated microorganisms in fish and penaeids as probiotics with immunomodulatory functions. *Fish and Shellfish Immunology* **45**, 2-12.

Li J., Tan B., Mai K. (2009) Dietary probiotic *Bacillus* OJ and isomaltooligosaccharides influence the intestine microbial populations, immune responses and resistance to white spot syndrome virus in shrimp (*Litopenaeus vannamei*). *Aquaculture* **291**, 35-40.

Lin Y.C., Chen J.C. (2001) Acute toxicity of ammonia on *Litopenaeus vannamei* (Boone) juveniles at different salinity levels. J. *Exp. Mar. Biol. Ecol*. **259**,109-119.

Lin Y.C., Chen J.C. (2003) Acute toxicity of nitrite on *Litopenaeus vannamei* (Boone) juveniles at different salinity levels. *Aquaculture* **224**, 193-201.

Liu X.F., Li Y., Li J.R., Cai L.Y., Li X.X., Chen J.R., Lyu S.X. (2015) Isolation and characterisation of *Bacillus* spp. antagonistic to *Vibrio parahemolyticus* for use as probiotics in aquaculture. *World J Micobriol Biotechnol* **31**, 795-803.

Manan H., Moh, JHZ., Kazan NA., Suratman S., Ikhwanuddin M. (2016). Identification of biofloc microscopic composition as the natural bioremediation in zero water exchange of Pacific white shrimp, *Penaeus vannamei*, culture in closed hatchery system. *Appl Water Sci* DOI 10.1007/s13201-016-0421-4.

McIntosh D., Samocha, T.M., Jones, E.R., Lawrence, A.L., Horowitz, S., Horowitz, A., 2001. Effects of two commercially available low-protein diets (21% and 31%) on water and sediment quality, and on the production of *Litopenaeus vannamei* in an outdoor tank system with limited water discharge. Aquacultural Engineering 25, 69-82.

Merrifield, D., Dimitroglou A., Foey A., Davies J., Baker R., Bøgwald J., Castex M., Ringø E. (2010) The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture* **302**, 1–18.

Mohapatra S., Chakraborty T., Kumar V., DeBoeck G., Mohanta K.N. (2013) Aquaculture and stress management: a review of probiotic intervention. *Animal Physiology and Animal Nutrition* **97**, 405-430.

Moriarty D.J.W. (1998) Control of luminous *Vibrio* species in penaeid aquaculture ponds. *Aquaculture* **164**, 351-358,.

Nimrat S., Suksawat S., Boonthai T., Vuthiphandchai V.(2012) Potential *Bacillus* probiotics enhance bacterial numbers, water quality and growth during early development of white shrimp (*Litopenaeus vannamei*). *Veterinary Microbiology* **159**, 443-450.

Ninawe A.S., Selvin J. (2009) Probiotics in shrimp aquaculture: avenues and challenges. *Crit. Rev. Microbiol*. **35 (1),** 43-66.

Olmos J., Paniagua-Michel J. (2014). *Bacillus subtilis* A Potential Probiotic Bacterium to Formulate Functional Feeds for Aquaculture. *Microbial & Biochemical Technology* **6(7),** 361-367.

Pham K.C., Tran H.T.T., Doan C.V., Le P.H., Nguyen A.T.V., Nguyen H.A., Hong H.A., Cutting S.M., Pham T.N. (2017) Protection of Penaeus monodon Against White Spot Syndrome by Continuous Oral Administration of a Low Concentration of *Bacillus subtilis* Spores Expressing the VP28 Antigen. *Letters in Applied Microbiology*, **64(3),** 184-191.

Ringø E., L. Lovmo M., Kristiansen Y., Bakken I., Salinas R., Myklebust R., E. Olsen T. M. Mayhew. (2010). Lactic acid bacteria vs. pathogens in the gastro-intestine of fish (a review). *Aquacult. Res* **41**, 451–467

Ringø, E., R. E. Olsen, J. L. G. Vecino, S. Wadsworth, and S. K. Song. (2012).Use of immunostimulants and nucleotides in aquaculture (a review). *J. Marine Sci :Res. Develop* **1**, 104.

Sakata, T., T. Kojima, M. Fujieda, M. Miyakozawa, M. Takahashi, and K. Ushida. (1999).Probiotic preparation dose-dependently increase net production rates of organic acids and decrease that of ammonia by pig caecal bacteria in batch culture. *Digest. Dis. Sci* **44**, 1485–1493.

Samocha T.M., Patnaik S., Speed M., Ali A.M., Burger J.M., Almeida R.V., Ayub Z. (2007) Use of molasses as carbon source in limited discharge nursery and grow-out systems for *Litopenaeus vannamei. Aquacultural Engineering* **36(2)**, 184–191.

Sapcharoen P., Rengpipat S. (2013) Effects of probiotic *Bacillus subtilis* (BP11 and BS11) on the growth and survival of Pacific white shrimp, *Litopenaeus vannamei. Aquaculture Nutrition* **19**, 946-954.

Saulnier D., Haffner P., Goarant C., Levy P., Ansquer D. (2000) Experimental infection models for shrimp vibriosis studies: a review. *Aquaculture* **191**, 133-143.

Sha Y., Wang L., Liu M., Jiang K., Xin F., Wang B. (2016) Effects of lactic acid bacteria and the corresponding supernatant on the survival, growth performance, immune response and disease resistance of *Litopenaeus vannamei.Aquaculture* **452**, 28-36.

Shen W.Y., Fu L.L., Li W.F., Zhu Y.R. (2010) Effect of dietary supplementation with Bacillus subtilis on the growth, performance, immune response and antioxidant activities of the shrimp (*Litopenaeus vannamei*). *Aquaculture Research* **41**, 1691-1698.

Skjermo J., Vadstein O. (1999) Techniques for microbial control in the intensive rearing of marine larvae. *Aquaculture* **177**, 333–343.

Seenivasan C., Radhakrishnan S., Muralisankar T., Bhavan P.S. (2012) *Bacillus subtilis* on survival, growth, biochemical constituents and energy utilization of the freshwater prawn *Macrobrachium rosenbergii* post larvae. *Egyptian Journal of Aquatic Research* **38**, 195–203.

Sorokulova I. (2013) Modern Status and Perspectives of Bacillus Bacteria as Probiotics. *J. Prob. Health* **1**, 1-5.

Stephens W.Z., Burns A.R., Stagaman K., Wong S., Rawls J.F., Guillemin K., Bohannan B. J. (2016) The composition of the zebrafish intestinal microbial community varies across development. *The ISME Journal* **10**, 644-654.

Strickland J.D.H., Parsons T.R. (1972) A Practical Handbook of Seawater Analysis. Ottawa: Fishery Research Board Canada. 310 pp.

UNESCO (1983) Chemical methods for use in marine environmental monitoring. Manual and Guides 12, Intergovernamental Oceanographic Commissiony. Paris, France.

Vanwyk P, Scarpa J. (1999) Water Quality and Management. In: Van Wyk, P., et al. (Eds.), *Farming Marine Shrimp in Recirculating Freshwater Systems*. Florida Department of Agriculture and Consumer Services, Tallahassee, pp. 128–138.

Verschuere L., Rombaut G., Sorgeloos P. (2000) Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews* **64**, 655-671.

Villaseñor I.E.L., Castellano-Cervantes T., Gomez-Gil B., Carillo-Garcia Á.E., Campa-Cordova Á.I., Ascencio F.(2013) Probiotics in the intestinal tract of juvenile whiteleg shrimp *Litopenaeus vannamei*: modulation of the bacterial community. *World J Microbiol Biotechnol* **29**, 257-265.

Villaseñor I.E.L., Castellanos-Cervantes T., Gomez-Gil B., Carrillo-García A.E., CampaCórdova A.I., Ascencio F. (2013) Probiotics in the intestinal tract of juvenile whiteleg shrimp *Litopenaeus vannamei*: modulation of the bacterial community. *World J. Microbiol. Biotechnol*. **29,** 257- 265.

Villaseñor I.E.L., Rodríguez M.E.M., Gomez-Gil B., Valle, F.A., Córdova Á.I.C. (2011) Beneficial effects of four *Bacillus* strains on the larva cultivation of *Litopenaeus vannamei. Aquaculture* **321**, 136-144.

Villaseñor I.E.L., Voltolina D., Gomez-Gil B., Ascencio F., Campa-Córdova Á.I., Audelo-Naranjo J.M., Zamudio-Armenta O.O. (2015) Probiotic modulation of the gut bacterial community of juvenile *Litopenaeus vannamei* challenged with *Vibrio parahemolyticus* CAIM 170. *Lat. Am. J. Aquat. Res*. **43(4),** 766-775.

Wang C., Pan L.Q., Zhang K.Q., Xu W.J., Zhao D.H., Mei L. (2016) Effects of different carbon sources addition on nutrition composition and extracellular enzymes activity of bioflocs, and digestive enzymes activity and growth performance of *Litopenaeus vannamei* in zero-exchange culture tanks. *Aquaculture Research*, **47(10)**, 3307-3318.

Wasielesky W., Atwood H., Stokes A., Browdy C.L. (2006) Effect of natural production in a zero exchange suspended microbial floc based super-intensive culture system for white shrimp *Litopenaeus vannamei. Aquaculture* **258**, 396–408.

Wu D.X., Zhao S. M., Peng N., Cu C.P., Wang J., Liang Y.X. (2016) Effects of a probiotic (Bacillus subtilis FY99-01) on the bacterial community structure and composition of shrimp (*Litopenaeus vannamei*, Boone) culture water assessed by denaturing gradient gel electrophoresis and high-throughput sequencing. *Aquaculture Research*, **47**, 857-869.

Xu W.J., Morris T.C., Samocha T.M. (2016) Effects of C/N ratio on biofloc development, water quality, and performance of *Litopenaeus vannamei* juveniles in a biofloc-based, high-density, zero-exchange, outdoor tank system. *Aquaculture*, **453**, 169-175.

Yang X.P., Wang S.M., Zhang D.W., Zhou L.X. (2011) Isolations and nitrogen removal of an aerobic heterotrophic nitrifying-denitrifying bacterium, *Bacillus subtilis* A1. *Bioresource Technology* **102**, 854-862.

Ziaei-Nejad S., Rezaei M.H., Takami G.A., Lovett D.L., Mirvaghefi A.R., Shakouori M. (2006) The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. *Aquaculture* **252**, 516-524.

Zokaeifar A., Babaei N., Saad C.R., Kamarudin M.S., Sijam K., Balcazar J.L.(2014) Administration of *Bacillus subtilis* strains in the rearing water enhances the water quality, growth performance, immune response, and resistance against *Vibrio harveyi* infection in juvenile white shrimp *Litopenaeus vannamei*. *Fish and Shellfish Immunology* **36**, 68-74.

CONCLUSÕES GERAIS:

Os estudos da presente tese foram realizados com o objetivo de avaliar a utilização de um probiótico comercial como uma ferramenta de manejo da comunidade microbiana ao longo do ciclo de produção de *L. vannamei* em sistema de bioflocos. A partir dos resultados obtidos, é possível afirmar os benefícios da utilização de probióticos podem ser ressaltados em determinadas situações.

O processo de preparação da água inicial, o qual precede a estocagem dos camarões nas unidades de cultivo, é de suma importância principalmente para garantir a qualidade da água nas fases iniciais da produção. O processo de desinfecção da água ocasiona a redução da densidade bacteriana nas primeiras horas e, consequentemente, aumenta a disponibilidade de nutrientes que pode favorecer a proliferação de Vibrios patogênicos. Deste modo, recomenda-se que a aplicação de probióticos seja realizada imediatamente após o processo de cloração/decloração da água. Tal procedimento contribui para aumentar a densidade microbiana total e a densidade bacteriana de bactérias do gênero *Bacillus* da água em até 24 horas após cloração/decloração da água. Além disso, a aplicação de probiótico contribuiu para manter baixa a abundância e a porcentagem bacteriana de Vibrio na água. Considerando tais informações, recomenda-se ainda que as fertilizações com carbono orgânico sejam iniciadas a partir de 24 horas após a aplicação do probiótico, permitindo assim a manutenção de bactérias benéficas na formação dos bioflocos e estocagem dos camarões nas unidades de cultivo.

Após o estabelecimento da comunidade microbiana no sistema de bioflocos, constata-se que o modo operacional desse sistema em termos de fertilização orgânica e com ou sem aplicação de probióticos pode influenciar na resistência a doenças. A enfermidade conhecida como necrose hepatopancreática aguda (AHPND) vem

causando altas taxas de mortalidade no mundo inteiro, principalmente nos primeiros dias de cultivo, na fase de berçário (20 a 30 dias) e tem *V. parahemolyticus* como principal agente etiológico. É possível concluir que a resistência a pós-larvas de *L. vannamei* contra esta infecção está presente na comunidade microbiana presente no sistema de bioflocos, mesmo que o camarão nunca tenha sido cultivado em sistema de bioflocos previamente.

Quando a comunidade microbiana é manipulada com o objetivo de se obter um sistema puramente heterotrófico (altas razões C/N) com adição diária de fontes de carbono, foi possível concluir que os bioflocos podem exercer uma função probiótica nestes casos, conferindo resistência a AHPND e não sendo necessária a aplicação de bactérias probióticas externas. A presença de probióticos é essencial na resistência contra AHPND quando o sistema de bioflocos é operado de maneira autotrófica (aplicando-se fontes de carbono somente nas primeiras semanas), com menor razão C/N e consequentemente menor produção de biomassa bacteriana heterotrófica.

Passada a fase de berçário, os camarões (aproximadamente 1 g) são estocados nas estruturas de engorda. Durante esta fase, os riscos de se ocorrerem surtos de vibriose também são iminentes. Neste sentido, concluiu-se também que a presença do probiótico contribuiu para colonizar o trato intestinal de *L. vannamei* e controlar a densidade de Vibrio como potencial patógeno tanto em bioflocos como em água clara. No entanto, é importante também ressaltar que a proporção de probióticos é maior no trato intestinal dos camarões no sistema de água clara, onde há uma menor abundância microbiana em geral, salientando a importância da aplicação de probióticos neste sistema.

Adicionalmente, a colonização e a densidade bacteriana dos bioflocos podem ser refletidas também em outros órgãos dos camarões, sendo responsável pela redução de lesões no hepatopâncreas causado por surtos de vibriose durante a fase de engorda em bioflocos e água clara. Tal processo pode ocorrer devido à estimulação do sistema imune dos camarões, ocasionada pela presença dos probióticos, fato que já é documentado na literatura.

Finalmente, sugere-se que a aplicação de probiótico na fase final de engorda de *L. vannamei* em sistema de bioflocos seja feita na dosagem de 3 g de probióticos por cada kg de ração adicionados nas duas alimentações diárias. Com esta dosagem é possível obter melhores taxas de sobrevivência e menor densidade de Vibrio no trato intestinal dos camarões.

No entanto, faz-se de suma importância ressaltar o potencial probiótico da comunidade microbiana naturalmente presente no sistema de bioflocos, que apresenta uma alta abundância de microrganismos com potencial benéfico para os camarões, incluindo de conferir resistência a doenças. Esta alta abundância bacteriana pode também competir com o probiótico exógeno adicionado ao alimento, consequentemente diminuindo sua densidade na água ou no trato intestinal. Neste sentido, conclui-se que em algumas situações, a adição de probióticos a um meio com uma comunidade bacteriana naturalmente abundante pode não afetar a dinâmica e a interação entre os microrganismos que compõem o sistema de bioflocos.

Finalmente, a partir das constatações obtidas na presente tese, conclui-se que a técnica de hibridização in situ (FISH) permitiu a quantificação e o monitoramento da densidade bacteriana do probiótico. Além disso, permite a detecção de potenciais

patógenos ou qualquer tipo de bactéria presente do trato intestinal ou na água durante o cultivo de *L. vannamei* em água clara ou em sistema de bioflocos.