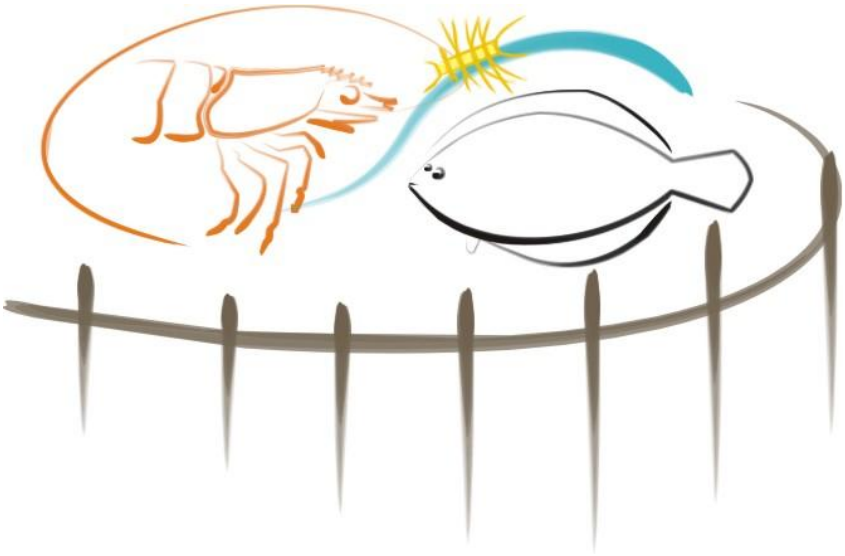
UNIVERSIDADE FEDERAL DO RIO GRANDE

INSTITUTO DE OCEANOGRAFIA

PROGRAMA DE PÓS-GRADUAÇÃO EM AQUICULTURA



Utilização de probióticos e fertilização orgânica com melaço durante a fase de berçário do camarão-rosa *Farfantepenaeus brasiliensis* em sistema super intensivo sem renovação de água.

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Resumo Geral

O cultivo de camarões marinhos é uma das principais atividades econômicas desenvolvidas no ramo da aquicultura. Embora a carcinicultura brasileira seja desenvolvida com a espécie exótica *Litopenaeus vannamei*, espécies nativas como o *Farfantepenaeus brasiliensis* já demonstraram potencial para o cultivo. A aqüicultura expandiu-se o suficiente para ter implicações significativas sobre o meio ambiente e recursos naturais. Dentre os impactos negativos podemos citar a disseminação de doenças e contaminação da água por efluentes. Estratégias para minimizar esses impactos já estão sendo utilizadas, como os sistemas de berçário (intermediário entre a larvicultura e a engorda), sistemas com troca zero de água e utilização de probióticos. O objetivo deste trabalho foi avaliar o efeito da utilização de probióticos e adição de melaço como fonte de carbono sobre o desempenho zootécnico, parâmetros imunológicos e concentração de *Vibrio* spp. durante a produção de juvenis do camarão-rosa *F. brasiliensis* na fase de berçário em sistema BFT (Bio-floc tecnology). O primeiro experimento consistiu de quatro tratamentos utilizando probióticos em sistema BFT i) *Bacillus cereus* var. *toyoi*, ii) Pro-W Inve® (*Bacillus* spp.), iii) Biomin® START grow (*Bacillus* spp., *Enterococcus* spp., *Lactobacillus* spp.) e iv) tratamento controle (sem adição de probióticos). O segundo experimento consistiu de dois tratamentos: Melaço (com adição de melaço) e controle (sem adição de melaço). Durante os experimentos não foi realizada renovação de água. Os seguintes parâmetros de qualidade de água foram monitorados diariamente: temperatura, salinidade, pH e oxigênio dissolvido. Além disso, foi realizado o monitoramento bacteriológico do gênero *Vibrio* spp. durante o período experimental (30 dias) e foi realizada extração de hemolinfa nos dias 0, 15 e 30 para analisar parâmetros imunológicos. Os resultados foram submetidos à análise estatística com nível de significância de 5% para determinar diferenças entre os tratamentos. No experimento 1, os probióticos foram adicionados diariamente conforme indicação do fabricante, para o *Bacillus cereus* var. *toyoi* foi utilizada dose equivalente a de outros produtos a base de *Bacillus* spp. No experimento 2 o melaço foi adicionado sempre que os níveis de amônia apresentavam valores ≥ 1mg/L em uma relação equivalente a 6g de carbono para 1g de amônia. No primeiro experimento, os resultados demonstraram que os camarões produzidos nos tratamentos com probiótico tiveram desempenho zootécnico significativamente superior que o grupo controle. Além disso, a utilização de probióticos foi eficiente para reduzir

significativamente a multiplicação de bactérias do gênero *Vibrio*. No segundo experimento, a adição de melaço contribuiu para melhoria do desempenho zootécnico do camarão apresentando resultados significativamente superiores comparados com o grupo controle, além de manter a concentração de *Vibrio* spp. estável durante o período experimental. Com relação aos parâmetros imunológicos, a concentração de hemócitos granulosos diferiu estatisticamente somente no dia 15, onde o tratamento Biomin apresentou valor superior aos demais tratamentos, enquanto os dados de proteínas totais não apresentaram diferença estatística. No segundo experimento não houve diferença estatística nos parâmetros imunológicos analisados. O uso de probióticos e a adição de melaço foram considerados eficientes e são recomendados para a produção do camarão rosa *F. brasiliensis* em sistema BFT durante a fase de berçário.

Abstract

Marine shrimp culture is the main economic activity developed in the field of aquaculture. Although shrimp culture in Brazil is performed with the exotic species *L. vannamei*, some native species like *Farfantepenaeus brasiliensis* have shown potential for culture. The expansion of aquaculture has been criticized for damage to the environment and natural resources. Among the negative impacts are the spread of diseases and contamination of water bodies by effluent discharge. Strategies to minimize these impacts are already being used, such as nursery systems (transitional system between the hatchery and growout), systems with zero water exchange and use of probiotics. Therefore the proposal of the present work was to evaluate the use of probiotics and the carbon addition (in the molasses form) on performance, immunological parameters and concentration of *Vibrio* spp. during the nursery rearing of the pink shrimp *Farfantepenaeus brasiliensis* in a BFT culture system. In the first experiment the following treatments were evaluated: 1.) Commercial *Bacillus* spp. mixture (Pro-W Inve®), 2.) Commercial *Bacillus* spp., *Enterococcus* spp., *Lactobacillus* spp. mixture (Biomin START-grow®), 3.) *Bacillus cereus* var. *toyoi* and 4.) Control treatment (without probiotic addition). The second experiment consisted of two treatments: 1) Molasses (with molasses addition) and 2.) Control (without molasses addition). No water exchange was carried out during the experiment (30 days). Throughout the experimental period, water temperature, salinity, pH and dissolved oxygen were measured every day and the concentration of presumptive *Vibrio* spp. was followed. For the immunological analysis, the hemolymph was collected in days 0, 15 and 30th. The results were analyzed statistically with a significance level of 5% to determine differences between treatments. In the first experiment the commercial probiotics were added daily following manufacturers' recommendation. For *Bacillus cereus* var. *toyoi* the dose used was equivalent to other probiotics based of *Bacillus* spp. In the second experiment, molasses was added when ammonia levels presented values ≥ 1 mg/L in a ratio equivalent to 6g of carbon to 1g of ammonia. In the first experiment, the results showed that shrimp reared in the presence of probiotics achieved significantly higher performance. Additionally, probiotics significantly reduced the concentration of *Vibrio* spp. In the second experiment, the molasses addition contributed to improve shrimp growth and survival presenting results significantly higher compared with control group, in addition also contributed to the maintenance of

stable concentration of *Vibrio* spp. during the experimental period. According to immunological parameters, the concentration of granular haemocytes was different only on day 15th, where Biomin treatment presented a higher value than the other treatments, while the data of total protein showed no statistical difference. Therefore, the use of probiotics and the addition of molasses were effective and are recommended to *F. brasiliensis* culture during the nursery phase in heterotrophic culture systems.

1. Introdução Geral

1.1 Aquicultura no Brasil e no Mundo

A aqüicultura, definida como o "cultivo de organismos aquáticos, incluindo peixes, moluscos, crustáceos, anfíbios e plantas aquáticas" (Bardach *et al*. 1972), é atualmente a atividade de maior crescimento entre os setores de produção animal (FAO 2007). A intensificação da aqüicultura e globalização do comércio de frutos do mar levaram a notável evolução nesta indústria (Yan-Bo *et al*. 2008). A aqüicultura é responsável por 40% da produção aquícola mundial e esta produção é avaliada em aproximadamente 78 bilhões de dólares (Kesarcodi-Watson *et al.* 2008). Em 2006, a produção de crustáceos representou 9% do total da produção aquícola mundial sendo responsável por 23% do valor total comercializado (FAO 2008).

1.2 Carcinicultura

O cultivo de camarões marinhos é uma das principais atividades econômicas desenvolvidas neste ramo (FAO 2007). Atualmente no Brasil a carcinicultura (cultivo de camarões) é responsável pela produção anual de cerca de 72.500 toneladas de camarão (FAO 2010) sendo desenvolvida com a espécie exótica *Litopenaeus vannamei*, entretanto, espécies nativas de camarão marinho como o *Farfantepenaeus brasiliensis* já demonstraram potencial para o cultivo (Lopes *et al*. 2009).

O desenvolvimento do cultivo de espécies de camarão nativo permite a realização desta atividade em estruturas de baixo custo, como gaiolas e cercados, os quais podem ser instalados em corpos de águas naturais, possibilitando a inserção de comunidades de baixa renda nesta atividade (Wasielesky 2000). Outra possibilidade é o desenvolvimento de programas de repovoamento de camarão, principalmente em áreas aonde o declínio das capturas vem sendo historicamente registrado, como é o caso do estuário da Lagoa dos Patos, Rio Grande, RS, afetando diretamente as comunidades de pescadores artesanais daquela região (D'Incao *et al*. 2002).

Além da sua utilização para consumo, o cultivo de camarões nativos propicia a produção de isca viva para a pesca esportiva, mercado crescente principalmente no litoral dos estados do Rio de Janeiro, São Paulo, Paraná e Santa Catarina. Na região de Cananéia, SP, estima-se uma demanda mensal de cerca de 150.000 unidades de

camarão, onde animais de aproximadamente 5 g têm sido comercializados por valores entre R$ 0,15 e R$ 0,50 a unidade (Preto 2005; Beccato 2009).

1.3 Desafios da aqüicultura

Globalmente, a aquicultura está se expandindo em novas direções, intensificação e diversificação (Qi *et al*. 2009). Com o aumento da intensificação e comercialização da produção aquícola, problemas com doenças surgiram inevitavelmente. As doenças são uma das principais restrições para o cultivo de muitas espécies aquáticas, impedindo tanto o desenvolvimento econômico quanto o social em muitos países (Bondad-Reantaso *et al*. 2005). Doenças infecciosas podem surgir dentro de um país de várias maneiras, por exemplo, a introdução de doenças exóticas, por mudanças bruscas dos fluxos existentes de doenças endêmicas, ou pelo aparecimento de doenças previamente não reconhecidas (Qi *et al*. 2009).

Rosenberry (2001) estimou que devido a WSSV (white spot syndrome vírus) as perdas da carcinocultura no ano de 2000 foram de aproximadamente 200.000MT acarretando um prejuízo de mais de US$ 1 bilhão. As pandemias de doenças virais causadas pelo WSSV e vírus da síndrome de taura, que começou em 1992 e causou perdas de bilhões de dólares mudou a indústria camaroneira (Lightner 2003).

A carcinicultura mundial vem experimentando perdas significantes na produção provocadas por patógenos bacterianos do gênero *Vibrio*, especialmente na larvicultura e na produção de camarões na fase de juvenil (Aguirre-Guzmán *et al*. 2004). Estes micro-organismos oportunistas fazem parte da microbiota natural dos peneídeos, porém provocam doenças quando condições ambientais desfavoráveis se estabelecem nos sistemas de cultivo. Nos peneídeos observa-se efeitos específicos incluindo mortalidade, lesões nos tecidos ou necrose, retardo no crescimento, degradação de tecidos, comprometimento das metamorfoses larvais, entre outros. O impacto da vibriose é variável, mas em alguns casos pode alcançar até 70% da população cultivada. Na vibriose crônica, camarões mortos ou moribundos podem sofrer canibalismo rapidamente contaminando outros indivíduos na população (Nunes & Martins 2002).

Algumas espécies e cepas de *Vibrio* como o *V.harveyi*, causam o fenômeno de luminescência e mortalidade nos camarões, que varia de insignificante até 100% do cultivo de peneídeos, principalmente nas fases de pós-larva e juvenil (Aguirre-Guzmán & Valle 2000).

Outro obstáculo enfrentado para a produção de organismos aquáticos é a utilização de rações artificiais, pois seus custos perfazem cerca de 50–60% dos custos totais da produção (Shiau 1998; Epp 2002). Além disso, no cultivo de camarões apenas de 15-30% do alimento fornecido é convertido em biomassa dos organismos cultivados (Tacon 1999), o restante acaba sendo perdido para os sedimentos, efluentes e a atmosfera (Boyd 2003). Este fato muitas vezes pode causar severos danos ao meio ambiente, através da eutrofização dos corpos de água naturais, que recebem os efluentes e também degradando o sedimento do próprio local do cultivo. (Boyd 2003).

A aqüicultura expandiu-se o suficiente para ter implicações significativas sobre o meio ambiente e recursos naturais, e uma série de preocupações têm sido expressas por ativistas ambientais e cientistas (Dierberg & Kiattisimukul 1996; Naylor *et al*. 1998, 2000). Dentre os impactos negativos, a poluição da água por efluentes é provavelmente a mais comum, e esta preocupação tem atraído maior atenção na maioria dos países (Boyd 2003).

A maioria da produção de camarões e peixes é realizada em viveiros, e estes extravasam efluentes após chuvas fortes, quando são drenados para a despesca e quando há renovação de água. Os efluentes, muitas vezes contêm nutrientes que podem causar a eutrofização dos corpos de água, além de representarem uma importante via de disseminação de doenças (Boyd 2003).

1.4 Estratégias para minimizar impactos da atividade

1.4.1 Sistemas de Berçário e Tecnologia de Bioflocos (BFT-Bio-floc tecnology)

Uma estratégia que tem sido utilizada para aumentar a biosegurança, proporcionar maior controle de estocagem, maior uniformidade de tamanho na despesca e melhorar o desempenho de engorda é o sistema de berçários (um sistema intermediário entre a larvicultura e a engorda) (Yta *et al*. 2004). Outra estratégia utilizada para minimizar os impactos negativos dos efluentes e prevenir a disseminação de doenças é a utilização de sistemas super-intensivos de cultivo de camarão sem renovação de água (Wasielesky *et al*. 2006). Neste tipo de sistema, chamado de BFT (Bio-floc Tecnology) é adicionado de uma fonte de carbono para equilibrar a relação carbono/nitrogênio e estimular o desenvolvimento de bactérias heterotróficas, juntamente com forte aeração e mistura contínua da água, contribuindo para a formação de flocos e estabelecimento de comunidades microbianas (Burford *et al*. 2004). Este

sistema tem demonstrado benefícios de absorção bacteriana de compostos nitrogenados, melhoria da qualidade de água, e conversão de amônia em proteína celular microbiana, a qual também fornece uma fonte suplementar de nutrição (Burford *et al*. 2004; Wasielesky *et al*. 2006).

1.4.2 Fertilização orgânica

Com o objetivo de aumentar a disponibilidade de alimento natural, a fertilização é largamente utilizada, pois estimula a produção de organismos autotróficos e heterotróficos incrementando a cadeia alimentar nas unidades de produção e, conseqüentemente, aumentando a produção dos organismos cultivados. Um exemplo clássico da contribuição dos microorganismos e detritos associados para a alimentação dos camarões é apresentado por Moss (2002). Segundo este autor, o crescimento de camarões cultivados em água rica em matéria orgânica, proveniente de viveiros de cultivo, foi 53% maior do que de camarões alimentados com o mesmo tipo de ração, porém cultivados em água clara.

Muitos autores têm demonstrado resultados satisfatórios em termos de produção e eficácia de retenção do nitrogênio, ao adicionarem fontes de carbono orgânico (açúcar, melaço, amido de mandioca etc.) com manutenção de um sistema de aeração para estimular o desenvolvimento de bactérias heterotróficas em viveiros de camarão (Avnimelech 1999; Burford *et al*. 2003; Hari *et al*. 2004).

O melaço, subproduto do processo de refinamento do açúcar, possui geralmente de 17 a 25% de água e teor de açúcar (sacarose, glicose e frutose) de 45 a 50% (Najafpour & Shan 2003). Esse subproduto vem sendo utilizado na preparação de meios heterotróficos (Emerenciano *et al.* 2007) visando a redução de compostos nitrogenados em berçários de camarão marinho (Samocha *et al.* 2007). O desenvolvimento dos flocos microbianos promove a diminuição da matéria orgânica reduzindo o nitrogênio inorgânico e a necessidade de ração a partir da produção de proteína microbiana (Avnimelech *et al*. 1994; Avnimelech 1999; Browdy *et al*. 2001; Silva *et al*. 2009).

Os flocos fornecem uma fonte suplementar de alimento extremamente importante, pois o requerimento protéico de camarões peneídeos é um fator nutricional importante para o crescimento (Kureshy & Davis 2002) além de ser um componente caro das rações de camarão (Shiau 1998). A utilização de rações com níveis protéicos mais baixos é mais rentável e ambientalmente correta, porque o componente de farinha

de peixe é reduzido. Sabe-se que a produção natural pode suplementar a alimentação do camarão, como observado por Moss (2002) e Decamp *et al*. (2002).

Alguns estudos já demonstraram que, mesmo em sistemas de cultivo semi-intensivos e intensivos, o alimento natural contribui significativamente para o crescimento dos organismos cultivados (Hennig & Andreatta 1998; Moriarty 1997). Anderson *et al.* (1987) determinaram que de 53 a 77% do crescimento de *Litopenaeus vannamei* cultivado em viveiros foi devido à ingestão do alimento natural disponível.

Segundo Lawrence & Lee (1997), quanto maior a intensificação do cultivo, menor deve ser a contribuição do alimento natural, no entanto esta contribuição não deve ficar abaixo dos 25%. Para exemplificar a importância do alimento natural, He & Lawrence (1993) obtiveram baixas taxas de sobrevivência (<50%) cultivando camarões em condições laboratoriais com uma ração sem vitamina C, enquanto que utilizando o mesmo tipo de ração para o cultivo em viveiros, onde havia disponibilidade de alimento natural, foram produzidas mais de 6 ton./ha com uma sobrevivência acima de 80%.

1.4.3 Utilização de probióticos

O desenvolvimento de soluções para melhorar a resistência e a sobrevivência dos camarões em cultivo onde as condições ambientais são variáveis e com possíveis infecções por patógenos é fundamental para sustentar o crescimento da indústria camaroneira (Castex *et al*. 2010). Entre as soluções propostas, o uso de probióticos tem mostrado resultados promissores e agora é amplamente aceito como uma ferramenta complementar para o manejo alternativo de doenças e para melhorar a nutrição dos animais aquáticos (Wang *et al*. 2008).

Muito antes de sua descoberta, os micróbios foram usados para preservar alimentos, e esses métodos empíricos contribuíram para melhorar a saúde humana (Bengmark 1998). No início do século, Metchnikoff (1907, 1908) propôs a implantação bactérias lácticas no intestino humano, com o intuito de suprimir a atividade de outros micróbios prejudiciais (Gatesoupe 1999; Tauber 2003).

A palavra probiótico é construída a partir da palavra oriunda do Latin *pro* (para) e do grego *bios* (vida) (Zivkovic 1999) sendo o oposto de antibiótico, o que significa "contra a vida" (Coppola & Gil-Turnes 2004). Este termo foi usado pela primeira vez por Lilly & Stillwel em 1965 para se referir a uma substância secretada por um micro-organismo que estimulava o crescimento do outro. Segundo Parker (1974) em sua definição original, os probióticos são "organismos e substâncias que contribuem para o

equilíbrio microbiano intestinal". Mais tarde, em 1989, Fuller modificou o conceito para "... suplemento alimentar microbiano vivo que afeta beneficamente o animal hospedeiro, melhorando o equilíbrio microbiano intestinal". No entanto, atualmente a definição internacionalmente aceita é que são microorganismos vivos que conferem benefícios à saúde do hospedeiro quando administrados em quantidades adequadas (Food & Agriculture Organization das Nações Unidas, Organização Mundial da Saúde 2001, Sanders *et al.* 2003).

Os primeiros probióticos utilizados em aqüicultura foram preparações designadas para animais terrestres. Esporos de *Bacillus cereus* var. *toyoi* isolados do solo reduziram a mortalidade das enguias japonesas que foram infectadas por *Edwardsiella* sp. (Kozasa 1986; Gatesoupe 1999). O mesmo micro-organismo aumentou a taxa de crescimento de seriola (Kozasa 1986).

O uso de micro-organismos como *Bacillus* tem como principal vantagem na elaboração de probióticos sua capacidade de esporular, o que lhes confere maior resistência às adversidades ambientais (Hoa *et al*. 2000), durante a elaboração, transporte e armazenamento das rações (Gil-Turnes *et al*. 1999). Outras vantagens da utilização de esporos do gênero *Bacillus* incluem a resistência a componentes tóxicos, extremos de temperatura, dessecação e radiação (Wolken *et al*. 2003), permitindo a formação de produtos estáveis (Hong *et al*. 2005; Ugoji *et al*. 2006). Muitos esporos bacterianos, como *B. coagulans*, *B. subtilis*, *B. clausii*, *B. cereus* var. *toyoi* já são explorados como componentes de produtos para uso humano e em animais (Sanders *et al*. 2003).

Entre os numerosos efeitos benéficos dos probióticos, a modulação do sistema imune é um dos benefícios mais comuns dos probióticos. O papel dos probióticos na modulação do sistema imune tem sido amplamente investigado e analisado em humanos e animais (Fooks *et al*. 1999; Galdeano & Perdigon *et al*. 2006; Herich & Levkut 2002; Sartor 2004).

1.4.4 Sistema imunológico de Crustáceos

Com relação ao sistema imunológico de crustáceos, esse apresenta apenas um sistema imune inato, diferentemente dos vertebrados que possuem, além desse, um sistema imune adaptativo. O sistema adaptativo caracteriza-se pela presença de uma infinidade de receptores e anticorpos altamente específicos e pela indução de células de

memória, que garantem uma resposta de defesa altamente eficiente e específica para os mais diversos patógenos. Esta resposta decorre da presença de uma linhagem celular linfocítica, presente apenas nos vertebrados, e em quem se apóiam todos os mecanismos de especificidade e de memória imunológica. Desta forma, sua ausência nos invertebrados inviabiliza qualquer tentativa de desenvolvimento de vacinas, na concepção clássica da palavra, diminuindo assim de forma substancial, a possibilidade de se prevenir e controlar infecções em crustáceos (Barracco *et al*. 2008).

Condições de estresse geralmente resultam em uma diminuição da resistência imunológica. A hemolinfa contém células circulantes ou hemócitos e uma variedade de fatores humorais dissolvidos no plasma. Os hemócitos são envolvidos principalmente com reações imune celulares, tais como fagocitose e encapsulação de patógenos e sua posterior destruição pela produção de moléculas citotóxicas e microbicidas. Os fatores humorais incluem moléculas de não reconhecimento e auto-imunes efetores tais como lectinas, fatores de coagulação e os componentes do sistema profenoloxidase (proPO) (Millar & Ratcliffe 1994; Söderhäll & Cerenius 1992; Roch 1999; Sritunyalucksana & Söderhäll 2000).

Os parâmetros hemato-imunológicos, como o hemograma, atividade da PO, o índice fagocitário e produção de espécies reativas de oxigênio (ROS), têm sido utilizados para monitorar as condições de saúde de crustáceos (Hauton *et al*. 1997; Le Moullac *et al*. 1997; Hennig *et al*. 1998; Sritunyalucksana *et al*. 1999; Muñoz *et al*. 2000; Sànchez *et al*. 2001; Perazzolo *et al*. 2002).

No presente trabalho foi avaliada a utilização de probióticos na etapa de berçário do camarão-rosa *F. brasiliensis* em sistema BFT para melhoria do desempenho zootécnico, controle de *Vibrio* spp., estimulação do sistema imunológico e melhoria da qualidade de água. Também foram estudados os benefícios da utilização uma fonte de carbono sobre a qualidade da água, o desempenho zootécnico, o controle de *Vibrio* spp. efeito sobre o sistema imunológico no berçário do camarão rosa no sistema BFT.

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The use of probiotics during the nursery rearing of the pink shrimp *Farfantepenaeus brasiliensis* in a zero exchange system

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1. Abstract

The present work evaluated the use of probiotics during the nursery rearing of the pink shrimp *Farfantepenaeus brasiliensis* in a zero exchange aerobic heterotrophic culture system during 30 days. Three replicate tanks were randomly assigned to the following treatments: 1) *Bacillus* spp. mixture (Pro-W Inve®), 2) *Bacillus* sp., *Enterococcus* spp., *Lactobacillus* spp. mixture (Biomin START-grow®), 3) *Bacillus cereus* var. *toyoi* and 4) control treatment (without probiotic addition). Bacteriological analysis monitored the abundance of presumptive *Vibrio spp.* in water of experimental tanks. For the immunological analysis, shrimp hemolymph was collected to determine the granular hemocyte count and total protein concentration. Results showed that mean final weight and specific growth rate of shrimp in the probiotic treatments were significantly higher. Furthermore, shrimp reared in the probiotic treatments showed higher levels of total protein and granular hemocyte. The bacteriological analysis showed that the concentration of *Vibrio* spp. measured in probiotic treatment tanks was lower than that recorded in the control tanks.

2. Introduction

Aquaculture is currently the fastest growing activity among all sectors of animal production and marine shrimp culture is the main economic activity developed in the field of aquaculture (FAO 2007). Although shrimp culture in Brazil is done with the exotic species *L. vannamei*, some native species of shrimp as *Farfantepenaeus brasiliensis* have shown potential for culture (Lopes 2009).

The pink-shrimp *Farfantepenaeus brasiliensis* is an endogenous species at Southeast and Southern Brazil. Its distribution ranges from North Carolina (EUA) until the coastal of Rio Grande do Sul (Brazil) (D'Incao 1999). The fast development of shrimp culture requires strategies to improve production systems, enhance bio-security and reduce environmental impacts (Avella, Gioacchini, Decamp, Makridis, Bracciatelli & Carnevali 2010; Qi, Zhang, Boon & Bossier *in press*).

Penaeid shrimp culture has been threatened by diseases (including those caused by *Vibrio* bacteria) compromising the expansion of this activity (Aguirre-Guzmán, Vazquez-juarez & Ascencio 2001). These opportunistic microorganisms are part of the flora of penaeid shrimp, and may cause illnesses under unfavorable environmental conditions. Specific effects such as mortality, tissue damage or necrosis and growth retardation are reported. Vibriosis has been also implicated as the cause of high mortalities in juvenile penaeid shrimp worldwide (Lightner & Redman, 1998; Castex, Lemaire, Wabete & Chim 2010).

The abuse of antimicrobial drugs, pesticides, and disinfectants in aquaculture has caused the evolution of resistant strains of bacteria and brought concern to the society (Esiobu, Armenta & Ike 2002; Boyd & Massaaut, 1999). Thus, defining alternative strategies to support aquaculture productivity are extremely necessary (Avella *et al*., 2010).

Among the alternatives proposed, the use of probiotics has shown promising results and is now widely accepted as a complementary tool for the management of disease and for improving nutrition of aquatic animals (Wang, Li & Lin 2008). Probiotics are also cited as an alternative to antimicrobial drugs, enhancing the growth and disease-resistance of cultured shrimp (Cutting *in press*), improving the immunosystem response (Erickson & Hubbard 2000; Picchietti, Mazzini, Taddei, Renna, Fausto, Mulero, Carnevali, Cresci & Abelli 2007), and general welfare (Balcázar, de Blas, Ruiz-Zarzuela, Cunningham, Vendrell & Múzquiz 2006; Silvi, Nardi, Sulpizio, Orpianesi, Caggiano, Carnevali & Cresci 2008).

Additionally, there are several studies reporting the development of intensive shrimp culture systems without water exchange as a way to improve biosecurity and reduce environmental impacts (Burford, Thompson, McIntosh, Bauman & Pearson 2003; Wasielesky, Atwood, Atokes & Browdy 2006; Ballester, Abreu, Cavalli, Emerenciano, Abreu & Wasielesky 2010). However, little information is available regarding the use of probiotics in this type of systems. Therefore the proposal of the present work was to evaluate the use o two commercial probiotics and one potential probiotic bacteria *Bacillus cereus* var *toyoi* during the nursery rearing of the pink shrimp *Farfantepenaeus brasiliensis* in a zero exchange aerobic heterotrophic culture system.

3. Material and methods

The experiment was conducted at the Marine Aquaculture Station (EMA/FURG) during 30 days. The experimental system consisted of 12 rectangular plastic tanks (40 L) with a bottom area of 0.20 m^2. *F. brasiliensis* early juveniles (0,46g ± 0,13) were stocked in the tanks at a density equivalent to 150 shrimp.m^{-2} (30 shrimp/tank).

Three replicate tanks were randomly assigned to the following probiotic treatments: 1) Commercial *Bacillus* spp. mixture (Pro-W Inve®), 2) Commercial *Bacillus* spp., *Enterococcus* spp., *Lactobacillus* spp. mixture (Biomin START-grow®), 3) *Bacillus cereus* var. *toyoi* (Bacteriology Laboratory - Biotechnology Center, Federal University of Pelotas, UFPel) and iv) control treatment (without probiotic addition).

To promote the development of the microbial flocs all experimental tanks received an inoculum (500 mL) from a heterotrophic shrimp culture system. Organic fertilization in molasses form was calculated based on Ebeling, Timmons & Bisogni (2006) and Avnimelech (1999) assuming that 6 g of carbon are needed to convert 1 g of TAN, generated from feed, into bacterial biomass.

Commercial probiotics were added daily to the treatment tanks to maintain a concentration of 5.10^4 cfu.ml^{-1} (Treatment 1) and 6.10^3 cfu.ml^{-1} (Treatment 2), following manufacturers' recommendation. Previous analyses assure that the dose applied achieved the recommended concentration. For treatment 3 (*Bacillus cereus* var. *toyoi*) the probiotic dose used was equivalent to the *Bacillus* spp. commercial mixture (T1).

Shrimp were fed twice daily with a diet containing 38% crude protein (Guabi®) via a specially designed feeding tray (Wasielesky *et al*. 2006). The initial feeding rate was 15 % of tank biomass and was adjusted daily according to shrimp consumption. At the end of the trial shrimp remaining in each tank were individually counted to determine survival and weighted to the nearest 0.01 g to determine mean final weight and specific growth rate (SGR). The SGR was calculated as:

$$SGR = (\ln(W_f) - \ln(W_i)) \times 100/t$$

Where: W_f = final weight; W_i = initial weight; t = time.

Feed conversion ratio was determined based on weight gain, survival, total feed consumed, and initial feed moisture content. No water exchange was carried out during the experimental period; only dechlorinated freshwater was added to compensate for evaporation losses.

Throughout the experimental period, water temperature (mercury thermometer, precision ± 0.5^0C), salinity (optical refractometer model RTS – 101, Atago® US, Bellevue, WA, USA, ±1 g L^{-1}), pH (digital pH meter model Handylab 2 BNC, ± 0.01 precision, Schott®, Hattenbergstr, Germany) and dissolved oxygen (dissolved oxygen meter model Handylab/ OXI/set ± 0.01 mg L^{-1} precision, Schott® Cambridge, UK) were measured every day.

Water samples were collected every two days to determine concentrations of total ammonia nitrogen (TAN) (NH3 + NH4 +-N; UNESCO 1983), and nitrite (NO_2; Bendschneider & Robinson 1952). Chlorophyll α concentration were measured twice weekly according to Strickland & Parsons 1972; reactive phosphorus was determined three times during the experimental period (PO_4; Aminot & Chaussepied 1983) and alkalinity and total suspended solids (TSS) once a week (Eaton, Cleserci & Greenberg 1995).

The concentration of presumptive *Vibrio* spp. in water was monitored in days 0, 10, 15, 22 and 30 according to the spread plate technique using thiosulfate citrate bile salt sucrose (TCBS) Difco® (Lennete, Spaulding & Truant 1974).

For the immunological analysis, the hemolymph was collected in days 0, 15 and 30^{th} by inserting a Hamilton syringe (50 µl), into the shrimp ventral sinus, transferred to a tube and left to coagulate for 24 h at 4^0C. The clot was then centrifuged at 2000 x g for 10 min to obtain the serum, which was either immediately used, or aliquoted and stored at -20^0C (Maggioni, Andreatta, Hermes & Barracco 2004). Total protein

concentration (TPC) in shrimp serum (six animals per treatment) was determined according to Bradford method (1976) using bovine serum albumin (BSA) as standard and the result read with UV-VISIBLE 2100 UNICOM OPTICS spectrophotometer (Maggioni *et al.* 2004).

Granular hemocyte count (GHC) was determined using a Neubauer chamber, after collecting the hemolymph (six animals per treatment) directly into an anticoagulant solution (1:4) (modified Alsever solution or MAS: 27 mM sodium citrate, 336 mM sodium chloride, 115 mM glucose, 9 mM EDTA, pH 7.0) (Maggioni *et al.* 2004).

One-way ANOVA was used to determine significant differences ($P < 0.05$) on shrimp performance. A two-way analysis of variance (ANOVA, $\alpha=0.05$) (time×treatment) was used to detect differences of bacteriological and immunological parameters between treatments and the control point. Tukey test was applied when significant differences were detected. All tests were conducted after the confirmation of homogeneity of variances (Cochran test) and normality distribution of data (Kolmogorov–Smirnov's test).

4. Results

Mean final weight and specific growth rate of shrimp were significantly higher in the probiotic treatments (Table 1). The water quality parameters monitored during the experimental period remained at concentrations suitable for shrimp culture and no significant differences were observed among treatments ($p>0.05$) (Table 2).

The probiotic treatments presented higher levels of granular hemocytes compared with control group, although significant difference was observed in Biomin

treatment only in day 15th (Table 3). Biomin® showed the highest levels of granular hemocyte count and total protein (Tables 3 and 4).

The bacteriological analysis showed that probiotic treatments maintained the concentration of *Vibrio* spp. lower than the control group, except for treatment Biomin® which presented the highest concentration of this microorganism (Figure 1).

5. Discussion

Probiotic application to marine organisms aims to increase seafood supply and safety, to control the proliferation of harmful microorganisms, and to develop new drugs. Managing microflora in aquaculture to enhance animal welfare, using beneficial bacterial strains, can be considered a biotechnological tool for the development of sustainable and environmental friendly aquaculture (Avella *et al.* 2010).

Bacillus species have been used as probiotics for at least 50 years. The scientific interest in *Bacillus* species as probiotics though, has only occurred in the last 15 years (Hong, Duc & Cutting 2005; Mazza 1994; Sanders, Morelli & Tompkins 2003). The safety of *Bacillus* species has been extensively reviewed elsewhere (Scan 2000a; Ishibashi & Yamazaki 2001; Logan 2004; Sanders *et al.* 2003).

The manipulation of the gut microbiota through dietary supplementation of beneficial microbe(s) is a novel approach not only from a nutritional point of view but also as an alternate viable therapeutic modality to overcome the adverse effects of antibiotics and drugs.

Chemicals used in aquaculture can cause pollution in the environment. These chemicals can come from antibiotics, pesticides, herbicides, hormones, anesthetics, pigments, minerals, and vitamins (Goldberg, Elliott & Naylor 2001; JSA 2007). This may have been a reason why some pathogens have become resistant to many drugs that

are indiscriminate used in aquaculture (Dixon 2000). As published by Durborow (1999), many pathogens are known to be contagious to humans, including several species of the genera Mycobacterium and Vibrio (especially *M. marinum*, *V. vulnificus*, and *V. parahemolyticus*) (Cole, Cole, Gaydos, Gray, Hyland, Jacques, Powell-Dunford, Sawhney & Au 2009).

The beneficial microorganisms are usually referred as probiotic which after administration are able to colonize and multiply in the gut of host animals and execute numerous beneficial effects by modulating biological systems in host (Cross 2002; Nayak *in press*).

The results of growth and survival (Table I) were similar with those reported by Vita (2008) whose found that the final weight and growth rate of white shrimp *Litopenaeus vannamei* were significantly higher in the tanks treated with probiotic than in the control tanks. Wang, Xu & Xia (2005) determined that shrimp reared in ponds treated with probiotics showed significantly higher survival rate, feed conversion ratio and final production compared with control tanks. This indicates that the addition of the commercial probiotics had a noticeable influence on shrimp production and survival rate.

Additionally, Wang *et al.* (2008) observed that the probiotics application significantly decreased the amount of TN (Total Nitrogen) and TOC (total organic carbon) in pond sediment. TP (total phosphorus), and TIP (total inorganic phosphorus) in sediment were also reduced during the culture. Zhou, Wang & Li (2009) working with shrimp larvae (*Penaeus vannamei*) obtained significantly increased survival rate in all treatments over the controls with the application of the probiotic, *B. coagulans* SC8168.

In the present study the decreased amount of nitrogenous was also observed, similar results were found by Wang *et al*. (2008). Moreover, the specific growth rate and final weight were significantly higher in probiotic treatments, similar with the findings of Zhou *et al*. (2009).

A potential alternative to intensive shrimp production are zero water-exchange systems with microbial flocs (BFT), which has the benefits of bacterial uptake of nitrogen, including ammonia (Burford *et al.* 2003), and conversion of ammonia into cellular protein, which also provides a supplemental source of nutrition (McIntosh 2000a,b; Burford, Sellars, Arnold, Keys, Crocos & Preston 2004b; Wasielesky *et al.* 2006).

Several studies have demonstrated that shrimp achieve better growth when cultured in biofloc-based ponds. This enhanced growth has been attributed to the nutrients contributed from floc materials present in pond water (Moss & Pruder 1995; Otoshi, Montgomery, Look, & Moss 2001; Tacon, Cody, Conquest, Divakaran, Forster & Decamp 2002; Burford *et al.* 2004a;b). The floc is composed of aggregated, suspended particles formed in shrimp culture water and contains phytoplankton, bacteria, zooplankton and detrital materials. It is possible, however, that some components of the floc biomass positively influence shrimp growth through activity not associated with specific nutrients (Ju, Forster, Conquest & Dominy 2008).

The microbial community formed in this system is able to rapidly utilize dissolved nitrogen and convert it into microbial protein (McIntosh 2000a; Burford & Williams 2001). This system offers the possibility to simultaneously maintain a good water quality within aquaculture systems and produce additional food for the aquaculture organisms (de Schryver, Crab, Defoirdt, Boon & Verstraete 2008). Juvenile *L. vannamei* grown in a microbial floc-based system have demonstrated higher growth

rates and survival compared to juveniles grown in a clearwater systems (Wasielesky *et al.* 2006).

The floc biomass could provide a complete source of cellular nutrition as well as various bioactive compounds (Akiyama, Dominy & Lawrence 1992; Fast & Menasveta 2000; Tacon *et al.* 2002; Truus, Vaher, Koel, Mahar & Taure. 2004; Singh Kate & Banerjee 2005) and may contain an as yet undiscovered growth factor (Ju *et al.* 2008). Floc carotenoids have been reported to provide essential nutritional and many bioactive physiological functions in animal tissues, including stimulating animal immune systems. Ju *et al.* (2008) concluded that these results suggest that floc materials that develop in low-water exchange shrimp culture systems could be added to diets to obtain better shrimp growth in clear water systems.

These naturally occurring organisms contribute nutritionally and serve as a pre-/probiotic and/or unknown growth promoter. For these reasons, it was hypothesized that production of microbial flocs could produce a viable alternative ingredient for shrimp feed (Kuhn, Boardman, Lawrence, Marsh & Flick 2009). In the present study, even in the presence of a heterotrophic microbial community, probiotics have shown promising results improving the performance of *F. brasiliensis* juveniles.

A wide range of probiotics, containing either monospecies or multispecies of microorganisms are commercially available. Recently, a number of studies have confirmed the beneficial effects of both forms of probiotics under in vitro and in vivo conditions. However, it is postulated that multispecies/multistrain probiotics are more effective and consistent than their monospecific counter parts since mixed cultures may exert synergistic probiotic properties (Timmerman, Koning, Mulder, Rombout & Beynen 2004; Nayak *in press*).

The probiotic Pro-W Inve® is a mix of *Bacillus subtilis* and *B.licheniformis*. *B. subtilis* is known to produce proteases and other enzymes that enable it to contribute to the natural digestion activity of the host (Ziaei-Nejad, Rezaeib, Takamic, Lovettd, Mirvaghefia & Shakourie 2006) and *B. licheniformis* has been shown to act as an antiviral (Arena, Maugeri, Pavone, Iannello, Gugliandolo & Bisignano 2006). For these reasons it is plausible to expect positive results from the application of a *Bacillus* mix to larviculture of commercially relevant species.

Bacteria belonging to both spore former and non-spore formers are used as probiotics. Several spore forming bacteria, which produce a wide range of antagonistic compounds can be valuable as probiotics (Moriarty 2003). Among spore formers, *Bacillus* spores as *B.toyoi* are routinely being used as probiotics in human and animal practices due to their immunostimulatory properties (Casula & Cutting 2002; Hong *et al.* 2005; Souza, Suita, Wasielesky, Leite, Romano & Ballester 2011).

Spores being heat-stable have a number of advantages over other non-spore-formers such as *Lactobacillus* spp., namely, that the product can be stored at room temperature in a desiccated form without any deleterious effect on viability. In addition, survive extreme environmental conditions enabling long-term survival in conditions that could otherwise kill vegetative bacteria (Nicholson, Munakata, Horneck, Melosh & Setlow 2000 & Cutting *in press*; Souza *et al*. 2011). Another advantage is the ability of survive the low pH of the gastric barrier (Barbosa, Serra, La Ragione, Woodward & Henriques 2005; Spinosa, Braccini, Ricca, De Felice, Morelli, Pozzi & Oggioni 2000) which is not the case for most species of non-spore formers (Tuohy, Pinart-Gilberga, Jones, Hoyles, McCartney & Gibson 2007). There is sufficient evidence of the benefits associated with the use of spore-forming bacteria, such as *Bacillus* spp., as biological

agents for improving water quality and reducing disease in aquaculture (Hong *et al.* 2005; Lalloo, Ramchuran, Ramduth, Gorgens & Gardiner 2007).

The *B.cereus* var. *toyoi* is a spore-forming bacteria that was tested with potential use as probiotic for aquaculture. Spores of *Bacillus toyoi* isolated from soil reduced the mortality of Japanese eel which were infected by *Edwardsiella* sp., (Kozasa, 1986). The same feed additive increased the growth rate of yellowtail (Kozasa, 1986). Gatesoupe (1991 & 1994) observed that spores of *Bacillus toyoi* and other *Bacillus* species when used as feed additive increased the growth of *S. maximus* and *Centropomus undecimalis* (Irianto & Austin 2002). At the present study *B.cereus* var. *toyoi* showed similar results to commercial multispecies probiotics.

Performance results of shrimp reared in the probiotic treatments during this study were significantly higher compared with the control group. The probiotics treatments did not differ significantly among each one on the parameters analyzed, showing that probiotic based of *Bacillus cereus* was effective to improved *F. brasiliensis* culture during the nursery phase in a zero water exchange culture system. Moreover, shrimp reared in the probiotics treatments showed higher levels of granular hemocytes. Also, it was observed that despite the lower concentration of probiotic bacteria in the Biomin® treatment (6.10^3 cfu.ml^{-1} vs 5.10^4 cfu.ml^{-1} in other probiotic treatments) results between probiotic application were similar.

The probiotic effect is more evident when the environmental conditions are unfavorable. Biomin® treatment showed the highest concentration of *Vibrio* spp., probably because of the lower concentration of probiotic bacteria applied to the tanks of this treatment, due to the recommended dose. However, this treatment presented a more pronounced probiotic effect with the increase of granular hemocytes that seem to be involved in phagocytosis of microorganisms, formation of capsules and nodules and

also in the production of toxic molecules and microbicides extremely important to the immune response of the animals, increasing the capacity to resist a unfavorable conditions (Hose, Martin & Gerard 1990; Gargioni & Barracco 1998; van de Braak, Botterblom, Taverne, Van Muiswinkel, Rombout & van der Knaap 2002b).

These results confirm the findings of Krummenauer, Abreu, Lara, Poersch, Encarnação & Wasielesky (2009) in which, the application of the Biomin® probiotic in *Vibrio parahemolitycus* contaminated shrimp under bio-floc conditions resulted in significant higher survival when compare to the control where no probiotics were added. This factor probably reflects the characteristics of some of the strains present in the Biomin® treatment like *Enterococcus* sp. which is a typical intestinal probiotic, known to be able to colonize shrimp gut and hepatopancreas and induce a positive impact on bacterial ecology of the gut by inhibit *Vibrio* spp., throughout competitive exclusion and enhance the immune response (Supamataya Bundit, Boonyarapatlin & Schatzmayr 2006; Swain, Singh & Arul 2009).

The bacteriological analysis showed that the concentration of *Vibrio* spp. measured in probiotic treatment (except Biomin) tanks was lower than that recorded in the control tanks, confirming the successful of the probiotic as an alternative to use of antibiotic. The results showed that the probiotics are effective in the nursery stage of pink shrimp *F. brasiliensis* increasing its performance even in a heterotrophic environmental. Results also demonstrated that *Bacillus cereus* var. *toyoi* is a potentially probiotic microorganism for aquaculture use. Future prospects include tests during other stages of pink shrimp farming as well as their use for the pacific white shrimp *Litopenaeus vannamei*.

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8. Figure Legends

Figure 1 - Abundance of presumptive *Vibrio* in the water of tanks receiving probiotics compared with control (without probiotic).

9. Tables

Table 1 – Mean (±SD) of survival, final weight and specific growth rate of *F. brasiliensis* reared during the nursery phase with probiotics addition in a BFT (Bio-floc tecnology) culture system

Treatament	Survival (%)	Final Weight (g)	SGR (%day)
Inve	91.65 ± 11.02^{a}	1.42 ± 0.40^{a}	0.036 ± 0.007^{a}
Biomin	81.92 ± 2.40^{a}	1.39 ± 0.8^{a}	0.035 ± 0.009^{a}
Toyoi	81.90 ± 13.4^{a}	1.34 ± 0.36^{a}	0.034 ± 0.004^{a}
Control	88.86 ± 6.36^{a}	1.22 ± 0.38^{b}	0.030 ± 0.003^{b}

* Different superscript letters indicate significant differences (p,0.05)

Table 2 - Mean (±SD) of water quality parameters of *F. brasiliensis* reared during the nursery phase with probiotics addition in a BFT (Bio-floc tecnology) culture system

	Treatments			
Parameter	**Inve**	**Biomin**	**Toyoi**	**Control**
Temperature (^{0}C)	26.4 ± 0.25	26.7 ± 0.15	26.5 ± 0.1	26.7 ± 0.25
pH	8.1 ± 0.02	8.1 ± 0.01	8,08 ± 0.03	8.1 ± 0.006
Salinity (g L^{-1})	31.58 ± 0.12	32.32 ± 0.47	32.34 ± 0.4	31.47 ± 0.23
DO (mg L^{-1})	6.19 ± 0.07	6.13 ± 0.04	6.14 ± 0.02	6.11 ± 0.01
TSS (mg L^{-1})	618.56 ± 453.4	592.31 ± 502.61	635.66 ± 485.29	538.09 ± 444.7
Alkalinity (mg L^{-1})	172.08 ± 15.73	186.25 ± 15.53	183.33 ± 28.15	184.16 ± 12.4
TAN (mg L^{-1})	0.91 ± 1.55	1.02 ± 1.47	1.25 ± 1.89	0.92 ± 1.48
Nitrite (MG L^{-1})	4.31 ± 3.54	4.73 ± 3.88	4.11 ± 3.21	3.65 ± 3.11
Fosfate (MG L^{-1})	3.6 ± 1.8	4.29 ± 1.79	4.78 ± 2.19	3.27 ± 1.32
Clorophyll α (µg L^{-1})	61.41 ± 60.75	26.9 ± 19.16	48.56 ± 39.78	49.78 ± 36.51

TSS, Total suspended solids; DO, Dissolved oxygen

Table 3 – Granular hemocyte count during the experimental period

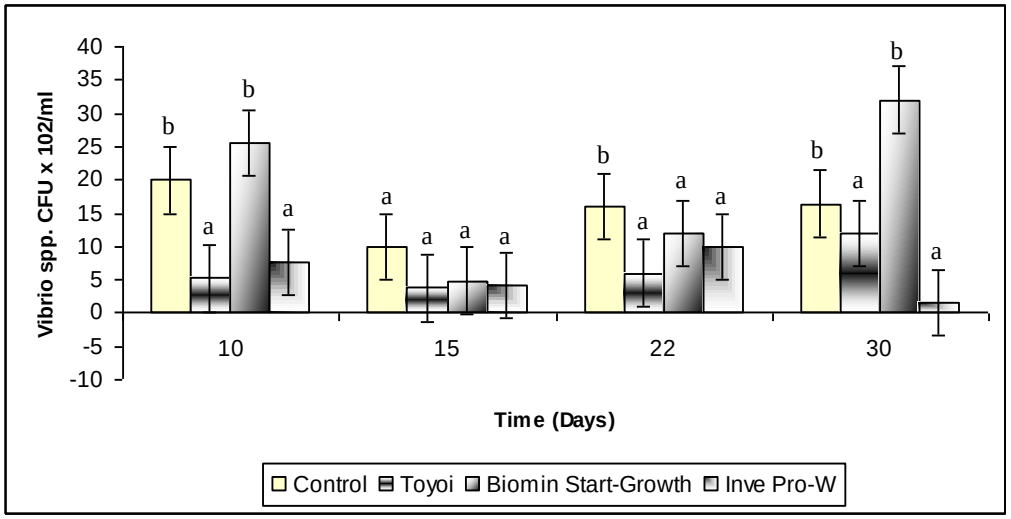
	Granular hemocytes (%HG) Days		
	0	15	30
Control	72.16 ± 2.92[a]	69 ± 2.36[a]	70.83 ± 2.31[a]
Biomin	72.16 ± 2.92[a]	76.66 ± 1.86[b]	75.5 ± 2.58[a]
Toyoi	72.16 ± 2.92[a]	74 ± 2.19[a]	73.66 ± 3.98[a]
Inve	72.16 ± 2.92[a]	72.83 ± 3.65[a]	72.33 ± 4.13[a]

*Different superscript letters indicate significant differences

Table 4 – Total protein count during the experimental period

	Total protein (mg/ml) Days		
	0	15	30
Control	121.16 ± 1.72	121.66 ± 1.96	120.66 ± 3.07
Biomin	121.16 ± 1.72	124.5 ± 2.42	124.5 ± 1.87
Toyoi	121.16 ± 1.72	120.66 ± 2.42	120.5 ± 1.87
Inve	121.16 ± 1.72	120.83 ± 2.31	120.33 ± 2.33

Figure 1


Vibrio spp. CFU x 102/ml
40
35
30
25
20
15
10
5
0
-5
-10
b
a
b
a
a
a
a
a
b
a
a
b
b
a
b
a
10
15
22
30
Time (Days)
Control
Toyoi
Biomin Start-Growth
Inve Pro-W

Use of molasses as a carbon source during the nursery rearing of *Farfantepenaeus brasiliensis* in a zero exchange system

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Running title: Molasses addition as a carbon source in BFT system

Keywords: Molasses, BFT, zero exchange, shrimp culture, *Farfantepenaeus brasiliensis*

1. Abstract

The present work evaluated the use of molasses as a carbon source during the nursery rearing of *Farfantepenaeus brasiliensis* in a zero exchange system. During a 30 days trial three replicate tanks were randomly assigned to the following treatments: 1.) Molasses (with molasses addition) and 2.) Control (without molasses addition). Bacteriological analysis monitored the abundance of presumptive *Vibrio spp.* in the experimental tanks. For the immunological analysis, shrimp hemolymph was collected to determine the total hemocyte counts and total protein concentration. Results showed that survival, mean final weight and specific growth rate of shrimp in the Molasses treatment were significantly higher. Moreover, molasses addition contribute to

maintenance of water quality and the formation of microbial floc and significantly reduced the abundance of presumptive *Vibrio* spp.

2. Introduction

Forty percent of world aquatic production (including capture fisheries) derives from aquaculture, being valued at US$ 78 billion (FAO 2007). In 2006, the crustacean production represented 9% of total aquaculture production being responsible for 23% of the total value commercialized (FAO 2008). Although shrimp culture in Brazil is done with the exotic species *L. vannamei*, some native species of shrimp as *Farfantepenaeus brasiliensis* have shown potential for culture (Lopes 2009).

The pink-shrimp *Farfantepenaeus brasiliensis* is an endogenous species at Southeast and Southern Brazil. Its distribution ranges from North Carolina (EUA) until the coastal of Rio Grande do Sul (Brazil) (D'Incao 1999). The expansion of shrimp farming have been criticized for damage to ocean and coastal resources, destruction of surrounding ecosystems, effluent discharge, invasion of exotic species and spread of pathogens (Naylor, Goldburg, Primavera, Kautsky, Beveridge, Clay, Folke, Lubchenco, Mooney & Troell 2000; Boyd 2003).

Due to the fast development of aquaculture, researchers around the world were faced with the need to develop environmental friendly culture systems. Thus, the development of farming systems called BFT (Bio-floc technology) with zero water exchange, prize for the least water use, streamlining the issuance of effluent into the environment, thus acting to lessen the risk of environmental damage (Burford, Thompson, Bauman & Pearson 2003).

This system is a successful alternative to increase the annual crop in high intensive production, whereby the addition of organic material to balance the carbon to nitrogen ratio, constant aeration and agitation of the water column allow aerobic decomposition and maintain high levels of microbial floc in suspension in fed and/or fertilized ponds (Avnimelech, Weber, Millstien, Hepher & Zoran 1986; Hargreaves 2006).

The basic principle of BFT system is the retention of waste and its conversion to biofloc as a natural food within the culture system (Azim & Little 2008). One of the benefits of these system is the bacterial uptake of nitrogen, including ammonia (Burford *et al.* 2003), and conversion of ammonia into cellular protein, which also provides a supplemental source of nutrition (McIntosh 2000b; Burford, Thompson, McIntosh, Bauman & Pearson 2004b; Wasielesky, Atwood, Stokes & Browdy 2006). Thus it is possible to reduce the demand for protein used in feed contributing to the maintenance of water quality (Burford *et al.* 2003; Hari, Kurup, Varghese, Schrama & Verdegem 2004; Crab, Avnimelech, Defoirdt, Bossier, & Verstraete 2007; Ballester, Abreu, Cavalli, Emerenciano, De Abreu & Wasielesky 2010).

Another successfully strategy utilized is the nursery system (a transitional system between the hatchery and growout) that have been used to produce larger size shrimp during growout while maintaining or increasing harvest yields (Sandifer, Hopkins & Stokes 1987; Samocha, Blacher, Cordova & De Wind 2000; Samocha, Hamper, Emberson, Davis, McIntosh, Lawrence & Van Wyk 2002; Arnold, Coman, Jackson & Groves 2009). Culturing postlarvae in nurseries to a more robust size before stocking into grow-out ponds is suggested to increase the initial survival (Samocha, Lawrence & Bray 1993), which eliminates the need to overstock in anticipation of high mortality, a practice that can lead to the production of small prawns. In addition,

nurseries can potentially increase the number of annual crops through reduced growout duration and head start production in the cooler months in controlled temperature systems (Samocha *et al.* 1993, 2000; Peterson & Griffith 1999; Arnold *et al.* 2009).

Therefore the proposal of the present work was to evaluate the carbon addition, in the molasses form, and its effect on the water quality, immunological parameters and performance of *F.brasiliensis* reared in a zero water exchange microbial floc based nursery system.

3. Material and methods

A 30 days trial was conducted at the Marine Aquaculture Station (EMA/FURG). The experimental system consisted of 6 rectangular plastic tanks (40 L) with a bottom area of 0.20 m^2. *F. brasiliensis* early juveniles (0.46g ± 0.13) were stocked in the tanks at a density equivalent to 150 shrimp.m^{-2} (30 shrimp/tank). Three replicate tanks were randomly assigned to the following treatments: 1.) Molasses (with molasses addition) and 2.) Control (without molasses addition).

To promote the development of the microbial flocs all experimental tanks received an inoculum (500 mL) from a heterotrophic shrimp culture system. Organic fertilization in molasses form was calculated based on Ebeling, Timmons & Bisogni (2006) and Avnimelech (1999) assuming that 6 g of carbon are needed to convert 1 g of TAN, generated from feed, into bacterial biomass.

Shrimp were fed twice daily with a diet containing 38% crude protein (Guabi®) via a specially designed feeding tray (Wasielesky *et al.* 2006). The initial feeding rate was 15 % of total tank biomass and was adjusted daily according to shrimp consumption. At the end of the trial shrimp remaining in each tank were counted to determine survival and weighted to the nearest 0.01g to determine mean final weight

and specific growth rate (SGR). The SGR was calculated as described by Bagenal (1978):

$$SGR = (\ln(W_f) - \ln(W_i)) \times 100/t$$

Where: W_f = final weight; W_i = initial weight; t = time

Feed conversion ratio (FCR) was determined based on weight gain, survival, total feed consumed, and initial feed moisture content. No water exchange was carried out during the experimental period; only dechlorinated freshwater was added to compensate for evaporation losses.

Throughout the experimental period, water temperature (mercury thermometer, precision ± 0.5°C), salinity (optical refractometer model RTS – 101, Atago® US, Bellevue, WA, US, ±1 g L^{-1}), pH (digital pH meter model Handylab 2 BNC, ± 0.01 precision, Schott®, Hattenbergstr, Germany) and dissolved oxygen (dissolved oxygen meter model Handylab/ OXI/set ± 0.01 mg L^{-1} precision, Schott® Cambridge, UK) were measured every day.

Water samples were collected every two days to determine concentrations of total ammonia nitrogen (TAN) (NH3 + NH4 +-N; UNESCO 1983), and nitrite (NO_2; Bendschneider & Robinson 1952). Chlorophyll α concentration were measured twice weekly according to (Strickland & Parsons 1972); reactive phosphorus was determined three times during the experimental period (PO_4; Aminot & Chaussepied 1983) and alkalinity and total suspended solids (TSS) once a week (Eaton, Cleserci & Greenberg 1995).

The concentration of presumptive *Vibrio* spp. in water was monitored in days 0, 10, 15, 22 and 30 according to the spread plate technique using thiosulfate citrate bile salt sucrose (TCBS) Difco® (Lennette, Spaulding & Truant 1974).

For the immunological analysis, the hemolymph was collected in days 0, 15 and 30th by inserting a Hamilton syringe (50 µl), into the shrimp ventral sinus, transferred to a tube and left to coagulate for 24 h at 4°C. The clot was then centrifuged at 2000 x g for 10 min to obtain the serum, which was either immediately used, or aliquoted and stored at -20°C (Maggioni, Andreatta, Hermes & Barracco 2004).

Granular and hialine hemocyte count was determined using a Neubauer chamber, after collecting the hemolymph (six animals per treatment) directly into an anticoagulant solution (1:4) (modified Alsever solution or MAS: 27 mM sodium citrate, 336 mM sodium chloride, 115 mM glucose, 9 mM EDTA, pH 7.0) (Maggioni *et al.* 2004).

The total protein concentration (TPC) in shrimp serum (six animals per treatment) was determined according to the Bradford method (1976) using bovine serum albumin (BSA) as standard (Maggioni *et al.* 2004).

T-test was used to determine significant differences ($P < 0.05$) on shrimp performance. A two-way analysis of variance (ANOVA, $\alpha=0.05$) (time×treatment) was used to detect differences of bacteriological, immunological and water quality parameters between treatments. Tukey test was applied when significant differences were detected. All tests were conducted after the confirmation of homogeneity of variances (Cochran test) and normality distribution of data (Kolmogorov–Smirnov's test).

4. Results

The mean final weight, survival and SGR of shrimp reared in the Molasses treatment were significantly higher than Control (Table 1).

According to bacteriological analysis, the Molasses treatment presented significantly lower and stable concentration of *Vibrio* spp. (Figure 1).

The water quality parameters monitored presented statistical difference during the experimental period. Control treatment presented higher levels of Nitrite and Ammonia as shown in figure 2 and 3. Chlorophyll presented difference only in the 30th day when Molasses treatment presented higher values compared with control. After the second week, the microbial heterotrophic community was established and due to the nitrification, ammonia concentration was reduced in environment (Figure 2).

The immunological analysis presented no statistical differences between treatments, although the Molasses treatment presented higher level of total protein compared with control (Table 4).

5. Discussion

In aquaculture systems phytoplankton and bacteria play a crucial role in the processing of nitrogenous wastes (Shilo & Rimon 1982; Diab & Shilo 1988). In a heterotrophic microbial based production system, bacterial flocs provide more stable water quality than does a phytoplankton-based production system (Boyd & Clay 2002).

The fundamental difference between this culture system and the traditional 'open' or running-water pond-based shrimp culture system is that the culture target is changed from a single-stomached animal, where micro-organisms generally play an important role in digestion and nutrient supply, to the equivalent of a multistomached animal through the provision of an in situ microbial aerobic digester or bioreactor (the microcosm), where microorganisms play a major role in digestion and nutrient supply, as they do in ruminants (Tacon, Conklin & Pruder 1999).

The most promising features of BFT systems (zero-water exchange) are that they offer both increased biosecurity (Bullis & Pruder 1999) and reduced feed costs and water use (Chamberlain & Hopkins 1994; Boyd 2000), and by doing so increase the possibility of moving the shrimp culture industry along a path of greater sustainability and environmental compatibility. Clearly, closed zero-water exchange culture systems can only biologically support a certain level of nutrient input and shrimp biomass without the system 'crash' and compromise shrimp growth and survival (Tacon Cody, Conquest, Divakaran, Forster & Decamp 2002).

This study demonstrated that molasses can be used as a tool to prevent TAN and nitrite increase during the nursery phase of the *F.brasiliensis*, under limited water discharge. Limited water exchange allows a better control of flocculated material in the water. Moss & Pruder (1985) and Otoshi, Montgomery, Look & Moss (2001) documented a reduced growth rate of *L. vannamei* when raised in water with low or without flocculated matter compare to rearing water with high load of particulate matter.

Studies using stable isotopes have shown that the natural biota can contribute to shrimp nutrition in less intensive ponds (Cam, Rollet, Mariotti & Guillaume 1991; Parker, Anderson & Lawrence 1991; Burford, Preston, Glibert & Dennison 2002; Abreu, Ballester, Odebrecht, Wasielesky, Cavalli, Granéli & Anésio 2007). Other reports (Burford *et al.* 2003, 2004) suggest that *L. vannamei* is capable of ingesting and retaining nitrogen derived from natural biota. Guillaume (1997), while working in high density zero exchange ponds, found that by increasing the proportion of flocculated particles in the culture medium, protein requirements of this species can be reduced to 22%. Furthermore, studies by Teichert-Coddington & Rodriguez (1995) and Hopkins, Sandifer & Browdy (1995) also showed that reducing the dietary protein level from

40% to 22% under high stocking densities using a limited discharge management have resulted in acceptable levels of shrimp production (Ballester *et al.* 2010).

Manipulation of C/N ratio by addition of carbohydrate significantly reduced inorganic N concentrations in the water column and total nitrogen in the sediment (Azim & Little 2008). At high carbon to nitrogen ratios (C:N) heterotrophic microorganisms would dominate over autotrophic microorganisms and would assimilate total ammonia nitrogen, nitrite and nitrate, to produce cellular proteins that can serve as supplemental feed source for the shrimp (Avnimelech, Troeger & Reed 1982; Avnimelech, Kochva & Diab 1994; Avnimelech 1999; Moss, Pruder & Samocha 1999; Browdy, Bratvold, Stokes & McIntosh 2001; Burford & Lorenzen 2004). This community probably provided the stable concentration of *Vibrio* spp. contributing to the maintenance of water quality.

The results of water quality parameters analyzed in this study presented acceptable levels to shrimp culture. These results are in the agreement with Boyd & Clay (2002) who observed that bacterial flocs provide more stable water quality and Avnimelech *et al.* (1989, 1999) who reported that the addition of carbohydrate to the production systems reduce the TAN concentration through immobilization by bacterial biomass. In this study, the development of heterotrophic bacteria probably resulted in the positive effect on growth performance as supplemental nutrition source. In the end of the experiment, the water quality parameters do not show significant differences probably because the flocs were already formed in the two treatments. This result reinforces the importance of molasses use to promote the formation of bioflocs, water quality control, in addition the positive effect on growth performance and maintenance of the *Vibrio* spp. concentration.

Moreover, apart from serving as a direct source of nutrients to shrimp, there is evidence that these organisms also exert a positive effect on the shrimp digestive enzyme activity and gut microflora (Moss, Divakaran & Kim 2001b). An important factor contributing to the very high growth rates of shrimp within these zero-exchange culture systems was likely the endogenous production and availability of microbial food organisms ('floc') for the resident shrimp. This 'floc' also has important functions in removing and harnessing potentially toxic fecal wastes and metabolites (e.g. by nitrification) from the shrimp within the culture system (Tacon *et al.* 2002).

In this study the shrimp cultured in a zero-exchange system with molasses addition presented results of survival, final weight and SGR significantly higher compared with control. Our results are agreeing with Krummenauer (2008) who comprovate the efficacy of BFT culture system in high intensive shrimp culture with a production above 2,5kg/m^2. Other authors authors (McAbee, Browdy, Rhodes & Stokes 2003; Otoshi, Tang, Dagdaban, Holl, Tallamy, Moss, Arce & Moss 2006; Otoshi, Scott, Naguwa & Moss 2007a) registered a production around 4,5 – 10kg/m^2, confirming the successful of this system to shrimp production.

According to the immunological analysis, no statistical difference was found among treatments, although the Molasses treatment presented higher level of hyaline hemocyte in day 15^{th} and higher concentration of total protein. Pascual *et al.* (2003) asserted "that haemolymph protein levels of shrimp are affected by nutritional stress.

The results of total protein of this study can be explained by the more pronounced microbial floc in Molasses treatment. Furthermore, these supplemental carbon source is extremely important not only to shrimp performance, but also for maintenance of water quality and has been extensively reviewed.

Crustaceans have only the innate immune system and the immunological components (total proteins and hemocytes) play a fundamental role for the immune defense of animals. Some authors believe, that the hialine hemocytes are the main phagocytic cells (Johansson, Keyser, Sritunyalucksana & Söderhäll 2000; Barraco *et al.*, 2008) and this cells is an intermediate form of granular hemocyte lineage (van de Braak, Botterblom, Liu, van der Knaap & Rombout 2002a). Based on final growth indicators additional molasses had no effect on the shrimp using the limited water discharge (Samocha, Patnaik, Speed, Ali, Burger, Almeida, Ayub, Harisanto, Horowitz & Brock 2007).

These finding prove that this system has no prejudicial effect to shrimp. The bacteriological analysis showed stable concentration of presumptive *Vibrio* bacteria during the experimental period in molasses treatment. This result could be explained because the maintenance of water quality and favorable conditions to the shrimp culture. Finally, we can conclude that the molasses use is efficient to control the C/N ratio and maintenance of a healthy culture environment. Moreover, the BFT system improves the survival, final weight and SGR of pink shrimp *F. brasiliensis*. Future prospects include test of probiotics to enhance the culture of this specie.

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7. Figure Legends

Figure 1 - Comparative abundance of presumptive *Vibrio* spp. in the water during the nursery rearing of *Farfantepenaeus brasilienis* at different treatments

Figure 2 – Fluctuations of Ammonia during the nursery rearing of *Farfantepenaeus brasilienis* at different treatments

Figure 3 - Fluctuations of Nitrite during the experimental period of *Farfantepenaeus brasilienis* at different treatments

8. Tables

Table 1 – Mean (±SD) of survival, final weight and specific growth rate of *F. brasiliensis* reared during the nursery phase with and without molasses addition in a BFT (Bio-floc tecnology) culture system

Treatment	Survival (%)	Final Weight (g)	Specific growth rate (%day)
Molasses	88.87±6.36[a]	1.22±0.38[a]	0.0309 ± 1.06[a]
Control	80.5±2.42 [b]	1.03±0.13 [b]	0.0256 ± 0.97[b]

* Different superscript letters indicate significant differences

Table 2 - Mean (±SD) of water quality parameters of *F. brasiliensis* reared during the nursery phase with and without molasses addition in a BFT (Bio-floc tecnology) culture system

	Treatments	
Parameter	**Molasses**	**Control**
Temperature (^{0}C)	26.7 ± 0.25	26.63 ± 0.11
pH	8.1 ± 0.006	7.99 ± 0.02
Salinity (g L^{-1})	31.47 ± 0.23	31.73 ± 0.56
DO (mg L^{-1})	6.11 ± 0.01	6.13 ± 0.03[a]
TSS (mg L^{-1})	538.09 ± 444.7	522.61 ± 375.81
Alkalinity (mg L^{-1})	184.16 ± 12.4	172.08 ± 21.58
Ammonia (mg L^{-1})	0.92 ± 1.48[a]	1.69 ± 2.41[b]
Nitrite (mg L^{-1})	3.65 ± 3.11[a]	8.28 ± 6.37[b]
Fosfate (mg L^{-1})	3.27 ± 1.32	3.94 ± 1.11
Clorophyll α (µg L^{-1})	49.78 ± 36.51[a]	16.07 ± 24.89[b]

* Different superscript letters indicate significant differences (p,0.05)

Table 3- Granular and hyaline hemocyte count, total protein count in hemolymph of *F. brasiliensis* reared during the nursery phase with and without molasses addition

Treatment	Days	Granular hemocytes (%HG)	Hialine hemocytes (%HH)	Total protein (mg/ml)
Molasses	0	72.16 ± 2.92	27.83 ± 2.92	121.16 ± 1.72
Molasses	15	69 ± 2.36	31 ± 2.36	121.66 ± 1.96
Molasses	30	70.83 ± 2.31	29.16 ± 2.31	120.66 ± 3.07
Control	0	72.16 ± 2.92	27.83 ± 2.92	121.16 ± 1.72
Control	15	71 ± 3.28	29 ± 3.28	119.16 ± 1.94
Control	30	70.83 ± 3.86	29.5 ± 3.39	118.83 ± 2.13

Figure 1

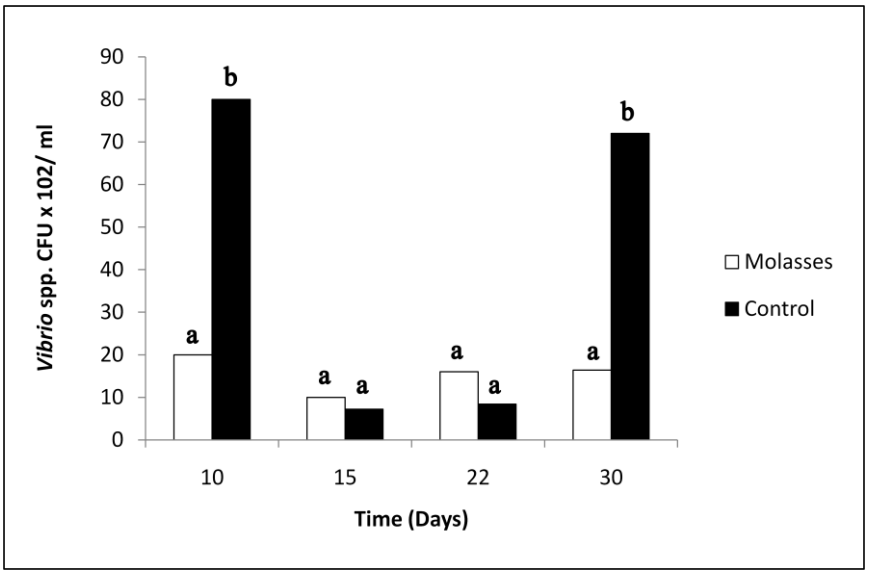


Figure 2

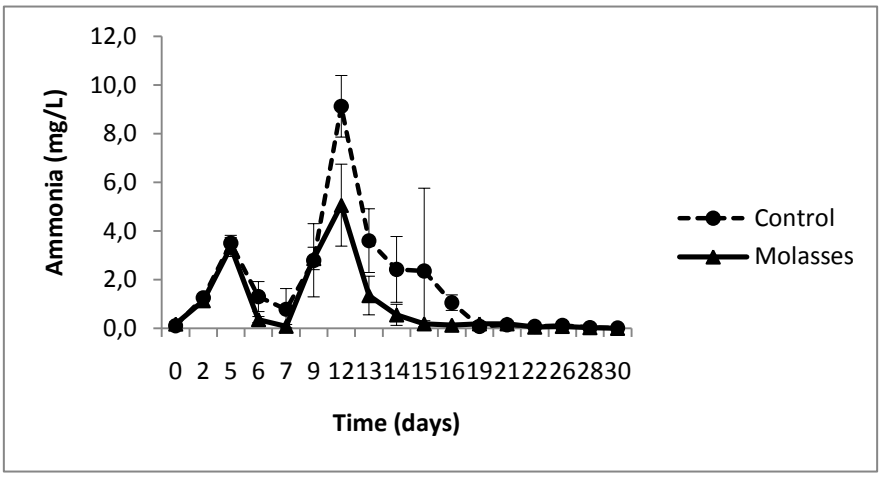
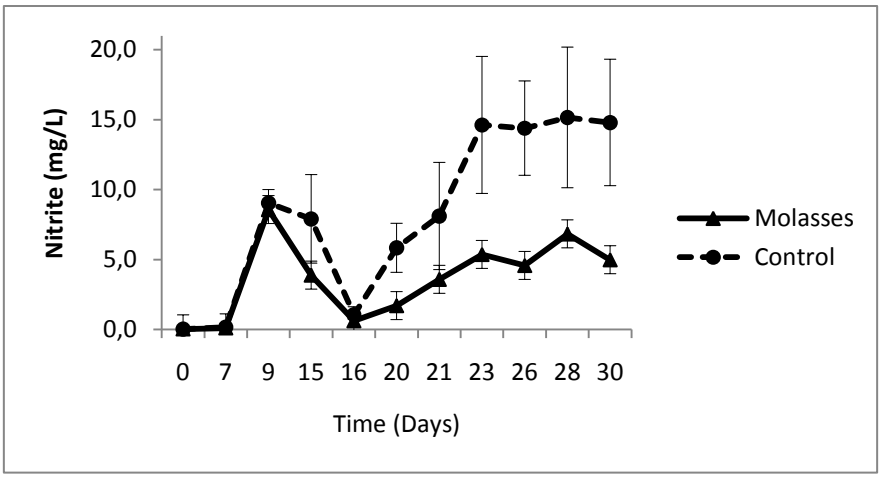


Figure 3



Considerações finais

Utilização de probióticos

- ✓ Os probióticos da inve e toyoi foram eficientes para a manutenção estável da concentração de *Vibrio* spp.
- ✓ O *B.toyoi* é um probiótico potencial para aqüicultura.
- ✓ Os probióticos proporcionaram um desempenho zootécnico superior ao grupo controle.

Adição de melaço como fonte de carbono

- ✓ O tratamento com adição de melaço apresentou resultados zootécnicos significativamente superiores comparado com o grupo controle (sem adição de melaço).
- ✓ O tratamento melaço foi eficaz para a manutenção da concentração de *Vibrio* spp. e qualidade de água.