EGIDIO ALVES DE SOUZA JUNIOR

EFEITOS DE DIFERENTES ESTRATÉGIAS DE APLICAÇÃO DE PROBIÓTICOS NO CULTIVO DO CAMARÃO MARINHO *Litopenaeus vannamei* (BOONE 1931).

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DEPARTAMENTO DE PESCA E AQÜICULTURA

PROGRAMA DE PÓS-GRADUAÇÃO EM RECURSOS PESQUEIROS E AQÜICULTURA

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Efeitos de Diferentes Estratégias de Aplicação de Probióticos do Cultivo do Camarão Marinho *Litopenaeus Vannamei* (Boone 1931).

Por: Egidio Alves de Souza Junior

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RESUMO

No presente estudo foram avaliadas diferentes estratégias de aplicação de probióticos com o objetivo de determinar a metodologia que tem a capacidade de incrementar os parâmetros de crescimento e a qualidade da água do cultivo super intensivo de *Litopenaus vannamei* em meio heterotrófico com troca zero de água. O experimento foi realizado em laboratório durante trinta dias. Consistiu de doze tanques plásticos de 48-L cada, estocados com 40 camarões (2,26 ± 0,02 g, peso inicial). Os tanques receberam água recirculada por escoamento através de quatro caixas de 500-L. Cada três tanques corresponderam a um dos quatro tratamentos. Neste experimento foram utilizados dois probióticos comerciais (*Bacillus* sp.). Estes produtos foram adicionados na água (WP), na ração (FP), água e ração (WFP) e um tratamento não recebeu os probióticos (NP). Os parâmetros de crescimento e as variáveis de qualidade da água foram mensurados durante o período de cultivo e uma análise bacteriológica do hepatopâncreas dos animais foi realizada no final do experimento. De um modo geral os resultados indicaram que os parâmetros de crescimento foram negativamente afetados quando o probiótico foi adicionado na água. Entretanto a qualidade da água foi melhorada significativamente em todos os tratamentos com a utilização de probióticos. As respostas obtidas neste estudo sugerem que todas as estratégias de aplicação utilizadas neste estudo têm a capacidade de interferir de alguma maneira, sobre os parâmetros de crescimento e sobre as variáveis físicas e químicas de qualidade da água no cultivo super intensivo de *L. vannamei* em meio heterotrófico.

Palavras chave: Probióticos, *Litopenaeus vannamei*, Estratégias.

ABSTRACT

In the present study, different probiotics application strategies were evaluated with the objective to show the methodology that have the ability of enhance growth parameters and water quality variables in no water exchange super intensive heterotrophic culture of *Litopenaeus vannamei*. The experiment was realized in laboratory during thirty days. It consisted in twelve 48-L plastic tanks stocked with 40 shrimp (2.26 ± 0.02 g initial weight) each. Tanks received flow-through water coming from one of four 500-L sources corresponding to the treatments with two commercial probiotics (*Bacillus* sp.) added in the water (WP), feed (FP), water and feed (WFP) and without probiotic addition (NP). Shrimp growth and water quality parameters were recorded during the culture period and bacteriological analysis of shrimp hepatopancreas performed at the end of the experiment. Overall results indicated that growth performance was negatively affected when the probiotic was added in the water, but water quality was significantly improved in all probiotic treatments. The results obtained in this study suggest that all probiotics application strategy tested have the ability to interfere in any ways on the growth parameters and the physical and chemical water quality variables in *L. vannamei* super intensive heterotrophic culture.

Key words: *Litopenaeus vannamei*, Probiotics, Strategies.

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INTRODUÇÃO

As primeiras pesquisas acerca da utilização de probióticos foram realizadas em 1901, quando o pesquisador russo Elie Metchnikoff ganhador do prêmio Nobel de Medicina, definiu os primeiros conceitos sobre o assunto (SHORTT, 1999; BOYLE et al., 2006). De acordo com estudiosos do mundo inteiro, alimentos que contém organismos probióticos são consumidos desde épocas primitivas da história da humanidade. Hoje em dia estes produtos são utilizados nas mais diversas áreas da ciência moderna como biologia, medicina, agricultura e também na aqüicultura (HARWOOD et al., 2001; DECAMP et al., 2007). Os primeiros relatos da utilização de probióticos em aqüicultura foram feitos por Yasudo e Taga em 1980. Eles afirmaram que algumas bactérias teriam a capacidade de controlar biologicamente doenças de peixes, porém, somente em 1986 foram publicados os primeiros resultados (VERSCHUERE et al., 2000).

Na atual conjuntura da aqüicultura mundial, a utilização de probióticos como uma forma de se alcançar sustentabilidade sócio-econômica e ambiental nas áreas de cultivo do camarão branco do Pacífico *Litopenaeus vannamei* (BOONE, 1931) tem levado muitos cientistas a estudar as características destes tipos de produtos e suas diferentes formas de aplicação (WATSON *et a*l., 2008). Estudos recentes demonstraram as vantagens obtidas nos cultivos experimentais e comerciais onde foram utilizados probióticos (OCHOA-SOLANO e OLMOS-SOTO, 2006 ZIAEI-NEJAD et al., 2006). Apesar das pesquisas em ambientes controlados sobre a utilização destes produtos na larvicultura, berçário e engorda de camarões, existem opiniões distintas sobre a eficiência destes métodos em comparação com o sistema tradicional de cultivo sobre seus benefícios para a produção e qualidade da água. Dessa forma é de grande importância à definição de estratégias de aplicação de probióticos mais eficientes no cultivo do camarão marinho *Litopenaeus vannamei*.

2. REVISÃO DE LITERATURA

Nos últimos tempos a modificação do ambiente pela ação do homem vem ocasionando problemas relacionados à perpetuação das espécies e à degradação profunda dos recursos naturais. Uma das principais conseqüências desta falta de sustentabilidade, juntamente com o ritmo acelerado de crescimento da população, é a falta de alimentos para suprir as necessidades humanas. Neste contexto, o cultivo de organismos aquáticos aparece como uma forma eficiente

de diminuir esta carência, pois a aquicultura é considerada uma atividade promissora que produz alimento para milhões de pessoas e ainda gera emprego e renda. Aproximadamente um quarto dos frutos do mar consumidos globalmente é oriundo da atividade (NAYLOR et al., 2000).

A produção mundial da aquicultura no ano de 2006 foi de 66 milhões de toneladas e gerou um faturamento de US$ 86 bilhões naquele ano (FAO, 2008). Dentre as várias formas de se cultivar organismos aquícolas, a carcinicultura desempenha um papel importantíssimo sendo um dos ramos da aqüicultura que mais cresce no mundo. No mesmo ano de 2006, a produção mundial de camarões foi de 3.160.000 toneladas (CASCORBI, 2004; FAO, 2008). Com o rápido e significativo aumento de produção da atividade, os impactos dos nutrientes inorgânicos provenientes de efluentes das fazendas de camarão tornaram-se uma ameaça ao meio ambiente (COWEY e CHO, 1991; ROSENBERRY, 2001). A má qualidade da água resultou na propagação de doenças, o que gerou críticas de algumas organizações não governamentais (HOROWITZ e HOROWITZ, 2001). Segundo Samocha et al. (2007), o surto de doenças virais e bacterianas em fazendas de camarão, juntamente com a deterioração dos ecossistemas costeiros, tem resultado em severas perdas de produção em todo o mundo. Entre os principais agentes causadores de enfermidades do camarão cultivado, podem-se incluir as doenças infecciosas causadas por vírus, bactérias, fungos, protistas e metazoários. Um número de doenças não infecciosas são também incluídas neste contexto e são causadas principalmente pelo desequilíbrio ambiental, má nutrição, agentes tóxicos e fatores genéticos (LIGHTNER e REDMAN, 1998).

Na tentativa de controlar a proliferação de patógenos, muitos antibióticos têm sido empregados em larviculturas comerciais e em fazendas de cultivo (GATESOUPE, 1989). Essa prática pode ter dado origem a inúmeros tipos de organismos resistentes aos mais diversos tipos de produtos. As conseqüências epidemiológicas deste problema iniciaram a exploração de alternativas para o gerenciamento de doenças em sistemas aquícolas, na melhoria da qualidade de água e dos parâmetros zootécnicos nos cultivos de camarão (KARUNASAGAR et al., 1994; AMABILE-CUEVAS et al., 1995; WESTON, 1996; HAMEED et al., 2003). No Brasil, além de problemas desta natureza ocorridos nas fazendas de cultivo, barreiras econômicas também dificultaram o escoamento da produção para mercados internacionais (MAIA e NUNES, 2003).

Essas questões criaram uma grande demanda por sistemas de cultivo de camarão mais produtivos, eficientes, sustentáveis e livres de doenças (HOROWITZ e HOROWITZ, 2001; 2003). Dentre estas alternativas, cultivos super intensivos de produção com a troca zero, ou mínima de água, durante o ciclo de cultivo e com a manipulação das comunidades microbiológicas, presentes ou introduzidas no meio vêm sendo estudados (WYBAN e SWEENEY, 1991; GOMEZ-GIL et al., 2000; DECAMP e MORIARTY, 2006b; SAMOCHA et al., 2007). Nestes sistemas, por exemplo, pode-se prevenir a infestação de doenças entre os organismos do ambiente e os animais cultivados, incrementar o alimento natural diminuindo à necessidade de proteína, melhorar a qualidade da água, assim como, evitar a descarga de efluentes poluentes em águas costeiras (EBELING et al., 2006; WASIELESKY et al., 2006; SAMOCHA et al., 2007; DECAMP et al., 2007). Alguns dos mais altos índices de produção foram alcançados com estes tipos de sistemas, que também são conhecidos como cultivos em meio heterotrófico (BROWDY *et al*., 2001; WASIELESKY et al., 2006). De acordo com Collins (2001) cultivos em meio heterotrófico se baseiam no conceito de que o nitrogênio inorgânico livre (amônia e nitrito) pode ser assimilado como biomassa bacteriana consumível. A base para este tipo de produção primária é a disponibilidade de dois nutrientes fundamentais que são o carbono e o nitrogênio (COLLINS, 2001).

A composição das comunidades microbianas presentes nos cultivos em meio heterotrófico pode ser manipulada pela adição de espécies selecionadas (probióticos), em substituição às bactérias presentes no meio, e pela disponibilização de melaço como uma fonte de carbono orgânico (MORIARTY, 1999; BURFORD et al., 2004). Uma das principais fontes de carbono orgânico utilizado em aquicultura é o melaço de cana-de-açúcar. O melaço também serve para o controle e redução temporal de bactérias oportunistas luminosas do gênero Vibrio (SAMOCHA et al., 2007).

Os probióticos são produtos biológicos feitos de cepas selecionadas de bactérias, microalgas, fungos e/ou leveduras, que convertem rapidamente os sedimentos sólidos em substâncias mais simples e utilizáveis, além de diminuir as concentrações de substâncias tóxicas em ambientes de cultivo (TABBU, 1997). Os principais agentes probióticos estudados para uso em carcinicultura são: as microalgas (*Tetraselmis suecica*) (MAEDA, 1999), leveduras

(*Saccharomyces cerevisiae*) (BERGER, 2000), bactérias gram-positivas (*Bacillus* S11*, Bacillus* sp*, Lactobacillus lactis* AR21) (OCHOA-SOLANO e OLMOS-SOTO, 2006), bactérias gram-negativas (*Photorhodobacterium* sp*., Vibrio alginolyticus*) (RENGPIPAT et al., 1998, 2000; IRIANTO e AUSTIN, 2002), entre outros organismos. Dentre estes tipos de agentes, as bactérias do gênero *Bacillus* sp. representam organismos de grande importância e amplamente utilizados como probióticos para diferentes fases de cultivo de peneídeos (MORIARTY, 1998; 1999; DECAMP e MORIARTY, 2006a 2006b).

Diversos mecanismos são sugeridos como modalidades da ação dos organismos benéficos presentes nos probióticos. Estes organismos possuem capacidade de adesão no trato gastrointestinal de camarões, promovem a imunoestimulação nos hospedeiros, possuem peptídeos antimicrobianos que removem organismos hospedeiros através do princípio da exclusão competitiva (antagonismo), e promovem melhoras na qualidade da água, principalmente pela decomposição da matéria orgânica (GATESOUPE, 1991; FULLER, 1992; WATSON et al., 2008). Até seres humanos que consomem alimentos que contém probióticos são beneficiados, pois estes produtos têm efeitos comprovados na medicina de hoje sobre a regulação da flora intestinal, sobre o tratamento de enfermidades do sistema digestivo e do fígado, no controle dos níveis de colesterol além de propriedades especiais de terapia e profilaxia contra alguns tipos de câncer (REDDY et al., 1973; FERNANDES et al. 1987; GILLAND, 1990; O'SULLIVAN et al., 1992).

Além dessas vantagens, muitos organismos dependem de bactérias presentes em seu trato digestivo para sobreviver (AMABIS e MARTHO, 1985). Peixes e camarões digerem biomassa bacteriana, obtendo assim, aminoácidos e outras substâncias de que necessitam para sua nutrição. Em sistemas semi-intensivos, a contribuição do alimento natural na dieta do camarão cultivado é bastante significativa, podendo representar até 85% (NUNES et al., 1997). Além de proteína, os flocos microbianos formados por colônias de bactérias fixadas em substratos suspensos na água contêm uma concentração significativa de macro (cálcio, fósforo, potássio e magnésio) e micro nutrientes (cobre, ferro, manganês e zinco), assim como ácidos graxos (MOSS, 2006). Essas bactérias são componentes importantes na dieta de organismos detritívoros utilizados na aquicultura como camarões, tilápias e carpas (McGRAW, 2002).

Devido a estas contribuições nutricionais dos flocos microbianos, e com a finalidade de minimizar os riscos de acumulação de compostos nitrogenados em cultivos em meio heterotrófico, podem ser utilizadas dietas com baixo nível protéico sem comprometer o ganho de peso semanal do camarão. A utilização destas dietas e o incremento na produtividade natural significam menores custos de produção, além de responsabilidade ambiental por minimizar o uso de farinha de peixe na composição do alimento (WASIELESKY et al., 2006). Alguns estudos relacionaram o incremento do crescimento animal aos benefícios nutritivos destes organismos, tais como a produção de vitaminas, a disponibilidade de minerais e elementos traço, assim como a produção de enzimas digestivas (HOLZAPFEL e SCHILLINGER, 2002; ZIAEI-NEJAD et al., 2006). Por essas características os probióticos são considerados os principais métodos preventivos a serem desenvolvidos na luta contra doenças na carcinicultura (MORIARTY, 1999).

Em níveis altos da relação carbono/nitrogênio (C:N), microorganismos heterotróficos dominam em relação aos autotróficos, assimilando compostos nitrogenados no processo da decomposição da matéria orgânica para produção de proteína celular que por sua vez pode servir como alimento para os camarões (AVNIMELECH et al., 1994; AVNIMELECH, 1999; MOSS et al., 1999; BROWDY et al., 2001). O conhecimento da associação dos probióticos com a relação Carbono/Nitrogênio (C/N) e o manejo adequado e inteligente do sistema heterotrófico, pode resultar em incremento da produtividade e em melhoras na qualidade da água (EBELING et al., 2006; SAMOCHA et al., 2007; DECAMP et al., 2007). O manejo adequado dos agregados microbianos também permite que sejam feitas trocas zero ou mínimas da água de cultivo, possibilitando assim uma maior biossegurança que pode minimizar os efeitos ambientais adversos da carcinicultura (AVNIMELECH, 2002). As trocas limitadas proporcionam a manutenção das comunidades microbianas, a redução ou a eliminação de infecções na água e a redução da carga de nutrientes e a transferência de patógenos para o ambiente, mantendo uma boa qualidade da água nas fazendas de cultivo (HOPKINS et al., 1993; HOROWITZ e HOROWITZ, 2002).

A aplicação dos probióticos na larvicultura, berçário e engorda de camarões, pode ser feita através de rações (ROBERTSON et al., 2000), alimento vivo (Artêmia e Rotíferos)

(GATESOUPE, 1991; HARZEVILLI et al., 1998), ou diretamente na água e no solo de tanques ou viveiros (AUSTIN et al., 1995; MORIARTY, 1999; OCHOA-SOLANO e OLMOS-SOTO, 2006). O incremento da sobrevivência larval e do crescimento de camarões e peixes foi demonstrado quando cultivados em sistemas com os métodos adequados de aplicação de probióticos em comparação com os cultivos tradicionais (MAEDA, 1999; AUSTIN et al., 1995; BURFORD et al., 2003, 2004; VASEEHARAN et al., 2004; DECAMP e MORIARTY, 2006b). Na Ásia existem registros de várias experiências envolvendo a utilização de bactérias na larvicultura de *Penaeus monodon* (FABRICIUS, 1798) e *Penaues penicillatus* (ALCOCK, 1905) com resultados promissores (ANONYMOUS, 1991). Na América Latina, os carcinicultores de países como Equador e Peru estão conseguindo reverter problemas de quebra na produção causados por vírus (Síndrome da Mancha Branca; WSSV) e bactérias patógenas (*Vibrio* sp.) através de sistemas de cultivo com uso de agregados microbianos (GRIFFITH, 1995; MCNEIL, 2002; GULLIAN, 2004).

De acordo com todas estas informações pode-se sugerir que a presença de comunidades controladas de bactérias benéficas no ambiente é capaz de melhorar o crescimento, a conversão alimentar, o consumo de ração, a sobrevivência, a qualidade da água e a resistência a doenças dos camarões cultivados. Entretanto mesmo diante deste cenário favorável, as opiniões sobre a utilização de probióticos em sistemas heterotróficos com trocas mínimas de água ainda são controversas. Dessa forma, o presente estudo apresenta os efeitos de diferentes estratégias de aplicação de probióticos sobre o desempenho zootécnico do camarão branco do Pacífico *L. vannamei* em relação aos parâmetros de crescimento e de qualidade da água em cultivos com o uso de probióticos e em meio heterotrófico.

3. OBJETIVOS

3.1. OBJETIVO GERAL

O objetivo deste trabalho foi de determinar qual a melhor estratégia de aplicação de probióticos no cultivo do camarão branco do Pacífico *L. vannamei* em meio heterotrófico.

3.2. OBJETIVOS ESPECÍFICOS

Para atender ao objetivo geral deste trabalho, os seguintes objetivos específicos foram propostos:

Analisar e definir qual estratégia de aplicação apresenta melhores resultados sobre os parâmetros de crescimento e sobrevivência (%) de *L. vannamei* em meio heterotrófico.

Analisar e definir qual estratégia de aplicação apresenta os melhores resultados sobre as variáveis físico-químicas de qualidade de água em meio heterotrófico.

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4 ARTIGO CIENTÍFICO

Artigo científico a ser submetido para publicação no periódico The Journal of the World Aquaculture Society.

The Effects of Different Probiotic Application Strategies in Super Intensive Culture of Litopenaeus vannamei (Boone 1931).

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Abstract

The effect of probiotics under different application strategies in an experimental super intensive culture of Litopenaeus vannamei was evaluated on growth parameters and water quality variables during thirty days. The experiment consisted in twelve 48-L plastic tanks stocked with 40 shrimp (2.26 ± 0.02 g initial weight) each. Tanks received flow-through water coming from one of four 500-L sources corresponding to the treatments with two commercial probiotics (Bacillus sp.) added in the water (WP), feed (FP), water and feed (WFP) and without probiotic addition. Shrimp growth and water quality parameters were recorded during the culture period and bacteriological analysis of shrimp hepatopancreas performed at the end of the experiment. Overall results indicated that growth performance was negatively affected when the probiotic was added in the water, but water quality was significantly improved in all probiotic treatments. The responses obtained in this study suggest that all probiotics application strategy used in this study have the ability to interfere in any ways on the growth parameters and the physical and chemical water quality variables in L. vannamei super intensive culture.

Key words: Litopenaeus vannamei, Probiotics, Strategies.

In aquaculture systems, especially under stressful conditions, problems caused by diseases often result in serious economic losses (Balcazár et al. 2006). Diseases caused by bacteria (e.g. Vibrio harveyi, Vibrio parahaemolyticus) and virus (e.g. White Spot Syndrome Virus and Infectious Muscular Necrosis Virus), have been associated with a drastic reduction in shrimp production in different locations along the Brazilian coast (Nunes et al. 2004; Lightner 2005).

The development of high intensity grow-out system with limited or no water exchange based on suspended microbial floc communities have been applied to improve shrimp production around the world (Avnimelech and Bezerano 2007; Watson et al. 2008). This heterotrophic system can reduce the introduction and development of diseases, provide the nutritional benefits of natural productivity and improve overall water quality (Moss et al. 2001; Weirich et al. 2002; Burford et al. 2003: Boyd 2004). More recently, probiotic microorganisms and other forms of immune-stimulation have been employed in this culture system (Wyban and Sweeney 1990; Hopkins et al. 1993; Sandifer and Hopkins 1996; Gomez-Gil et al. 2000; McNeil 2000; Samocha 2007).

The field of probiotics has gained considerable attention and several aquaculture commercial products are available to be applied trough artificial feed (Robertson et al. 2000), live feed (Gatesoupe 1991; Harzevilli et al. 1998; Padilha 2005) and culture water (Austin et al. 1995; Douillet 2000). In shrimp culture the application of spore forming Bacillus sp. as probiotic bacteria in the feed or water has led to significant improvements in survival and growth performance of the blue shrimp Litopenaeus stylirostris (Stimpson 1874), the pacific white shrimp Litopenaeus vannamei, the Indian shrimp Fenneropenaeus indicus (De Man 1888) and the

black tiger shrimp Penaeus monodon (Fabricius 1798) among other penaeid species (Meunpol et al. 2003; Ziaeid-Nejad et al. 2006; Ochoa-Solano and Olmos-Soto 2006; Decamp et al. 2007).

Although the assumed advantages of probiotic Bacillus sp., there are conflicting information about its effectiveness and application strategies in shrimp culture. Therefore, the objective of the present study was to evaluate the effect of different probiotic application strategies on shrimp growth performance and water quality in experimental heterotrophic super intensive L. vannamei culture.

Material and methods

Juveniles of L. vannamei (2.26 ± 0.02g initial weight) were obtained from a shrimp farm in Pernambuco state, Brazil (8°05'24"S; 34°53'33"W). Juveniles were randomly transferred to the microcosm experimental units (48-L plastic tanks) and allowed to acclimate for 24h prior to the thirty-day experimental trial. Twelve tanks units were stocked at a density of 200 shrimp/m^2 (40 shrimp per tank) and covered with nylon mesh to prevent shrimp escape. Each three tanks (treatment replicates) received flow-through water (4800% / day) from four 500-L source tanks corresponding to the treatments with two commercial bacterial supplements (Bacillus sp.) added in the water and feed (WFP), only in the water (WP), only in the feed (FP) and with no probiotics (NP). The probiotic products (concentration of 10^6 CFU) were added daily following the manufacturer's instructions according to its specific application in the feed (5g/kg feed) or culture water (0.001g/L). The Cabon/Nitrogen ratio in the treatments was maintained around 23:1 by adding controlled doses of molasses in the water according to Avnimelech (1999) and Ebeling et al. (2006).

Shrimp were fed a daily amount of 7% of the total biomass adjusted every week and divided in four meals (0700, 1100, 1500 and 1900). Feed (commercial, 35% crude protein) was

offered using a specially designed feed tray per tank (10 cm diameter). Every week ten animals were sampled from each experimental unit, weighted using an analytical balance with precision of 0.01g and returned to their original tank.

Ten days after beginning the experiment, food consumption was evaluated in each treatment during four consecutive days. During the test, no significant differences were observed in shrimp weight (3.14 ± 0.62g) and survival (over 95%) among treatments. One day before the food consumption test all shrimp were harvested and weighted to determine the total biomass in each box. During the test, all food remains were collected after four hours from the trays in pre-weighed filters and oven dried (60 C) to constant weight. Mean food consumption was calculated following the method adapted from Nunes and Parsons (2000). The food lixiviation ratio, used to calculate the food consumption, was estimated after the shrimp were harvested and followed the same procedures of food collection.

At the end of the experiment all shrimp were harvested, weighted and counted to calculate the final survival (%), biomass gain (g), growth rate (g/week) and food conversion ratio (FCR) by dividing the total amount of food offered (g) by the final shrimp biomass gain (g) in each treatment. Water temperature (C), pH and dissolved oxygen (mg/L) were measured twice a day (0700 and 1800), whereas salinity (ppt) and transparency (Secchi disc; cm) were measured daily using a Multi Probe System YSI Model 556 (Yellow Springs Instruments Company, Yellow Springs, Ohio, USA). Nitrate (NO_3–N; mg/L), nitrite (NO_2–N; mg/L), total ammonia (TAN; mg/L) and unionized ammonia (NH_3-N; mg/L) were measured three times a week. For chemical water analysis, samples of 250 ml were collected and frozen immediately. The analysis followed the methods described by Mackereth et al. (1978) for nitrate, by Golterman et al. (1978) for nitrite and by Koroleff (1976) for total ammonia.

The total amount of bacteria in shrimp hepatopancreas was estimated at the end of the experiment. The hepatopancreas of five shrimp, from each treatment was dissected, washed in distilled water and macerated for bacterial analysis. Bacterial concentration was expressed as colony forming units (CFU) per ml for free living bacteria concentrations on marine agar medium sterilized by autoclaving. The CFU were estimated using the spread-plate count technique after the incubation period of 24 h in a stove (30C). Plates were set up in duplicate for each dilution and only plates having between 30 and 300 colonies were considered (APHA, 2000).

Data normality and homogeneity were previously verified by Kolgomorov-Smirnov and Cochran, respectively (Zar 1984). Subsequently, data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's post-hoc test to verified differences among means ($P<0.05$). Percentage data were previously arcsine transformed for the analysis, but only untransformed original data are presented.

Results

Shrimp survival varied between 88,33% in WP to 96% in WFP treatment and there were no significant differences among the treatments (Table 1). Final weight and growth rate were significantly higher in FP followed by NP and WP, but the last one did not differ significantly from WFP (Table 1). Food consumption was lower in the treatments WFP (0.45 g/meal) and WP (0.32 g/meal), differing significantly when compared with the values around 0.60 g/meal in FP and NP. Additionally, shrimp biomass gain was higher in FP and NP but not significantly different from the others treatments. Although the FCR did not differ among the treatments and the best result was found where the probiotics was added in the food (1.54 ± 0.19).

There were no significantly differences among the treatments for water temperature and salinity, but all the other variables recorded were significantly higher in NP when compared with

the probiotic treatments (WP, FP and WFP) (Table 2). The differences occurred for nitrite (NO_2–N; mg/L), nitrate (NO_3–N; mg/L), un-ionized ammonia (NH_3-N; mg/L), total ammonia (TAN; mg/L), pH and dissolved oxygen (mg/L) (Figures 1, 2, 3, 4, 5 and 6).

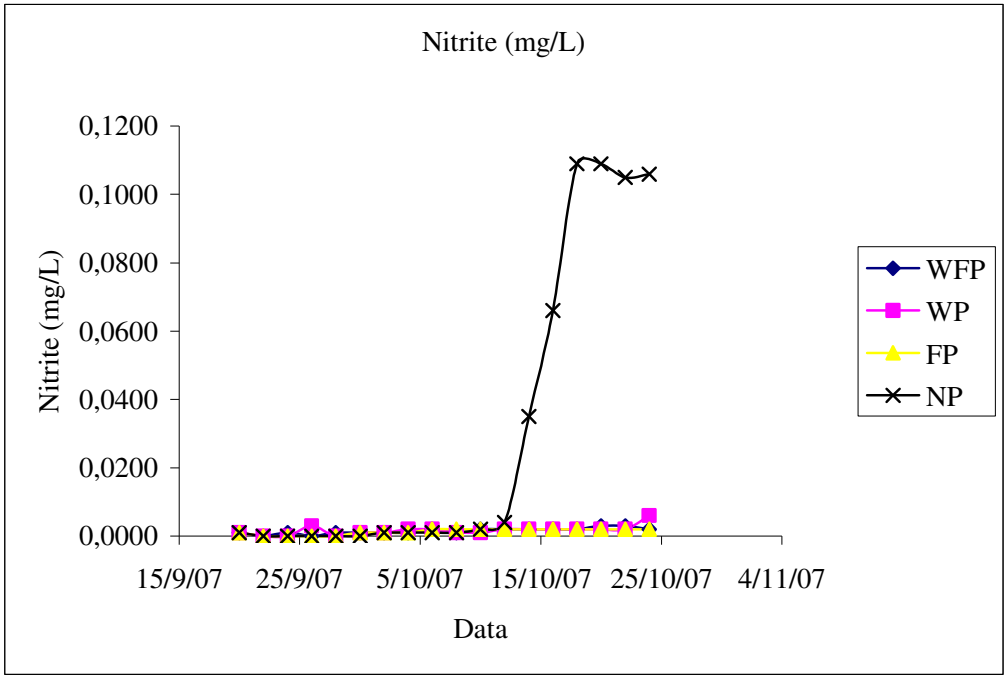

Figure 1. Nitrite (NO_2–N; mg/L) rates during 30 days in L. vannamei super intensive culture under different probiotic application strategies.

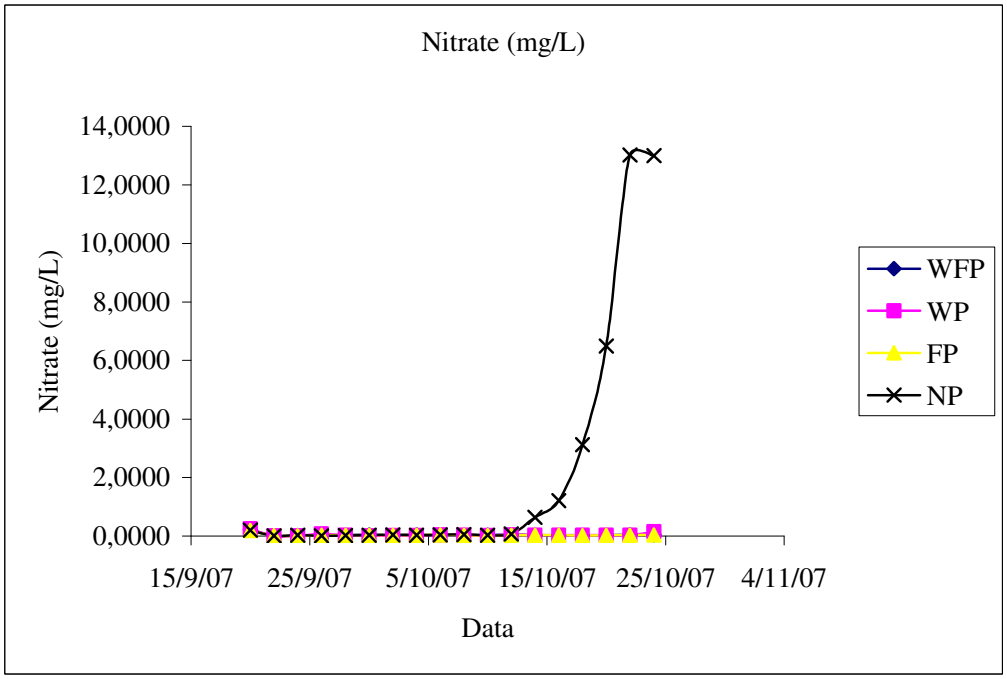

Figure 2. Nitrate (NO_3–N; mg/L) rates during 30 days in L. vannamei super intensive culture under different probiotic application strategies.

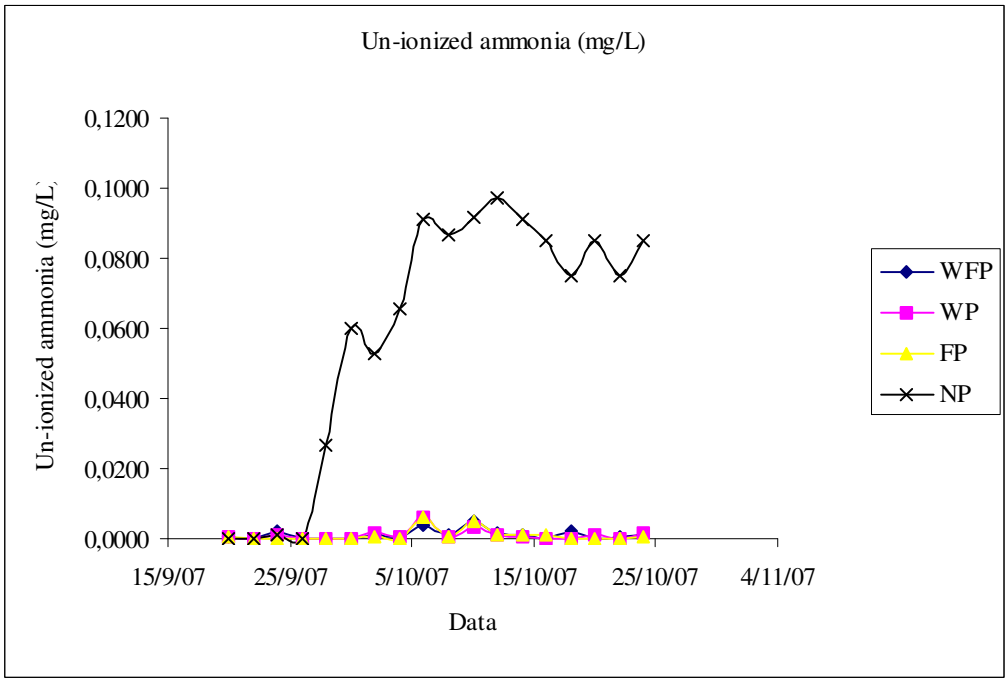

Figure 3. Un-ionized ammonia (NH_3-N; mg/L) rates during 30 days in L. vannamei super intensive culture under different probiotic application strategies.

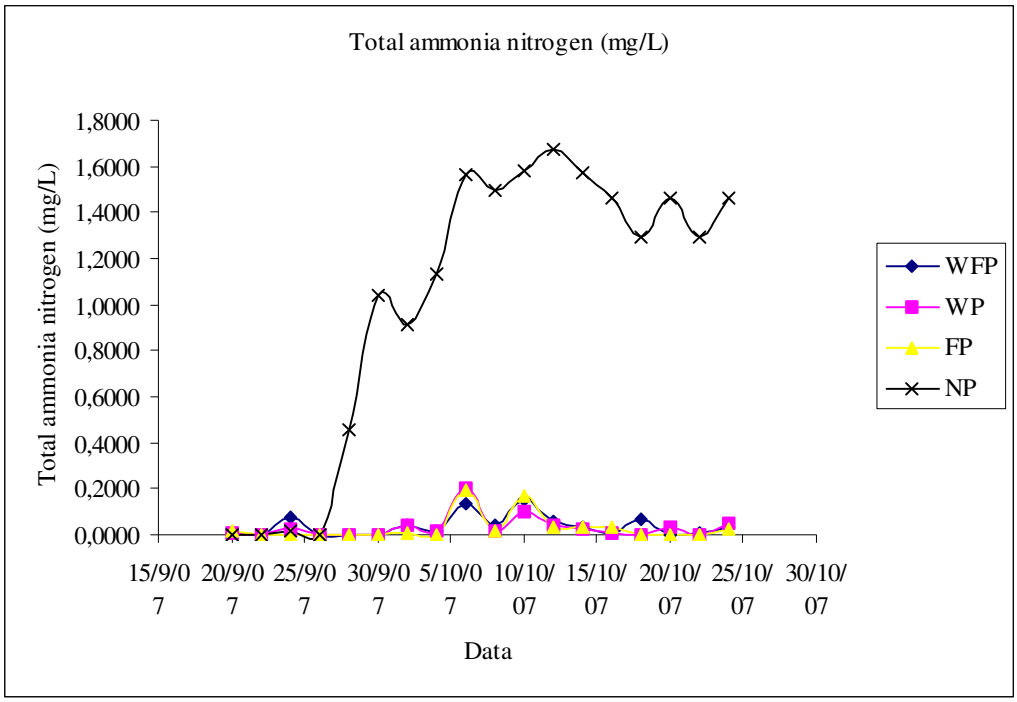

Figure 4. Total ammonia NH_3/ NH4+ (mg/L) rates during 30 days in L. vannamei super intensive culture under different probiotic application strategies.

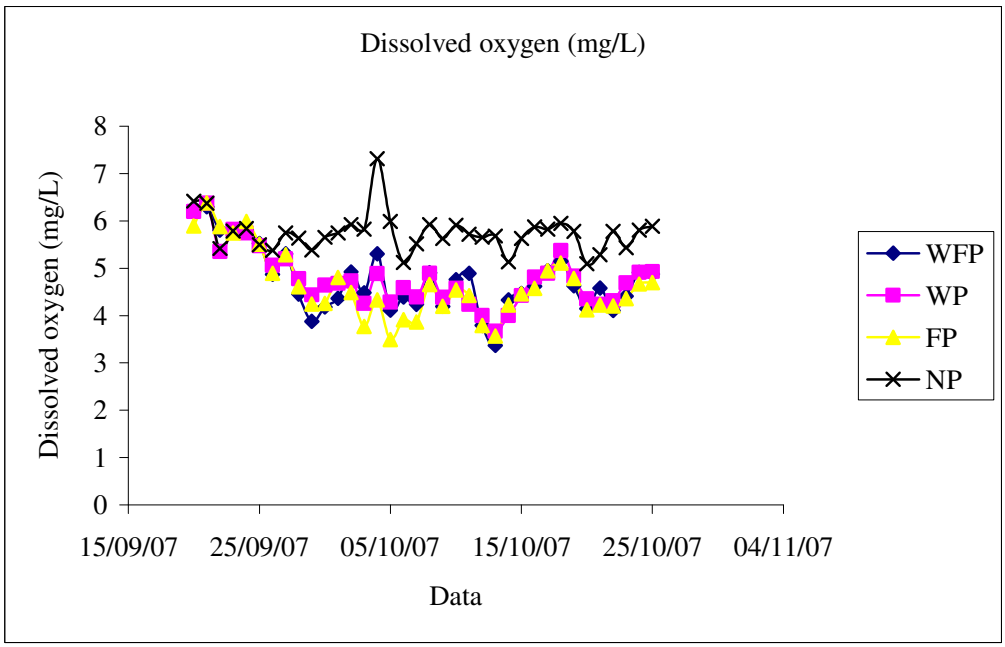

Figure 5. Dissolved oxygen (mg/L) rates during 30 days in L. vannamei super intensive culture under different probiotic application strategies.

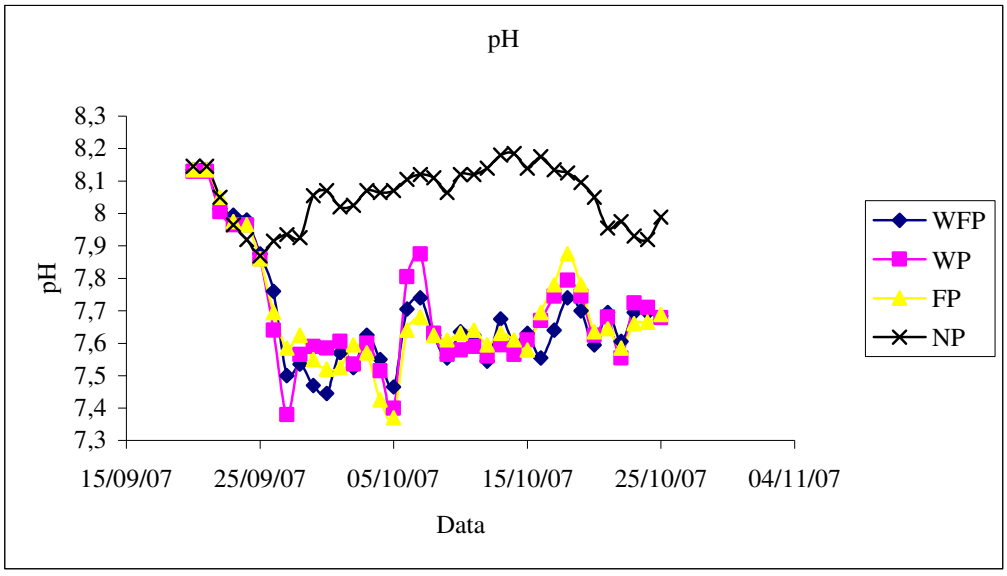

Figure 6. pH rates during 30 days in L. vannamei super intensive culture under different probiotic application strategies.

The bacteriological analysis of shrimp hepatopancreas resulted in 1.8 x 10^4 CFU/ml with no probiotic addition (NP), 4.8 x 10^4 CFU/ml with probiotic only in the food (FP) and uncountable colonies where the product was added in the water (WP and WFP).

Discussion

The microbial floc communities in heterotrophic super intensive systems constitute an important source of food for reared shrimp (Otoshi et al. 2006; Wasielesky et al. 2006). Although it was expected a superior survival and overall growth performance due to the probiotic addition in the experimental heterotrophic system, results showed no significant differences for survival, FCR and biomass gain compared to the control treatment. Accordingly, previous studies did not show the efficiency of these products under different application strategies in penaeid culture (Thoresen 1997; Samocha et al.1998a, b). Patnaik et al. (2007) found no effects on the elimination of pathogenic bacteria of the genus Vibrio using Bacillus sp. probiotics in L. vannamei culture. Similarly, no effects were observed in survival, biomass gain and FCR using Bacillus sp. strains on Penaeus setiferus (Horowitz and Horowitz 2000b) and L. vannamei culture (McIntosh et al. 2000). Taking together this information suggest that application of probiotics are still conflicting in the aquaculture, especially in heterotrophic systems where the maintenance of microbial communities through carbon addition could increase the complexity of the system (Horowitz and Horowitz 2000b; Devaraja et al, 2002).

The results of this experiment for growth rate, final weight and food consumption shrimp treated with probiotics in the water (WP and WFP) indicated inferior performance in heterotrophic system (Table1). This observation could be related to the proportionally large amount of probiotic (10 ppm) bacteria in the treatments where the product was added in the water. It is suggested that the excess of these microorganisms in culture water caused an inverse

effect in shrimp growth performance under our experimental conditions. This is supported by the bacteriological analysis of hepatopancreas that indicated uncountable CFU for individuals held in treatments WP and WFP. Although we are dealing with potentially probiotic microorganisms, it has been suggested for crustaceans that their presence in excess in the host organism can negatively affect their immune system by changing the total concentration of haemocytes (Smith et al. 2003; Ward et al. 2006). The haemocytes represent the first defenses of the invertebrate metabolism against possible invaders, involving an excessive expenditure of energy in its production by the immune system (Robohm 1984; Olivier et al. 1988). Thus, an excessive amount of probiotic bacteria ingested by the shrimp through water and microbial aggregates in treatments WP and WFP could have kept alert their immune system and interfered negatively on their food intake and overall growth performance. Nevertheless, this fact was probably aggravated in the heterotrophic culture without water renewal, molasses addition as carbon source for bacterial growth and low oxygen rates in all probiotics treatments.

The adjustment of the optimal probiotic amount according to the culture practices could be the key to improve shrimp growth performance. Vita (2008) testing the application of probiotic (Bacillus sp.) directly to the water with a inferior concentration (2 ppm) to those used in the present study, found a higher final weight for treated L. vannamei in heterotrophic system. The author attributed this result to the better nutritional quality of the microbial floc (e.g. protein and lipid levels) in the presence of probiotic bacteria. Accordingly, Genc et al. (2007) evaluated the effect of adding different quantities (0, 1.5, 3.0 and 4.5 g/kg diet) of probiotic organisms (Saccharomyces cerevisiae) in the diet and concluded that 3.0 g/kg was the best amount for Penaeus semisulcatus (De Haan 1844) for growth. This observation reinforces the idea that high doses of probiotics in the culture environment can lead to adverse growth results.

Overall water quality parameters recorded during the experiment were within acceptable levels for shrimp culture. The significantly higher water transparency in NP compared to other treatments with the addition of probiotics (WP, FP, and WFP) can be explained by the greater quantity of suspended microbial flocs in these treatments favored by the availability of an organic carbon source (Ebeling et al. 2006). This result was in according to previous study that associated lower water transparency to higher amounts of flocculated material in heterotrophic systems caused by either the addition of probiotic in the water (Vita 2008), or when no probiotics were involved (Weirich et al. 2003; Wasielesky et al. 2006).

The present findings indicated significantly lower levels of total ammonia, unionized ammonia, nitrite, nitrate and pH in the culture water of all probiotic treatments (WP, FP and WFP), while dissolved oxygen was higher without probiotic addition (NP). The interpretation of these results is directly related to the nitrogen cycle in the culture environment coupled with the presence of bacterial communities and C/N ratio in the system.

The nitrogen from shrimp excrement and remains of diet is used by heterotrophic bacteria to their own growth being the excess released in the form of ammonia. As energy source these microorganisms use carbohydrates present in organic matter. Further, the nitrifiers' bacteria (Nitrosomonas and Nitrosococcus) oxidize the ammonia in nitrite. After the ammonia oxidation into nitrite, bacteria of the genus Nitrobacter realize the oxidation into nitrate, which is the best nutrient, absorbed by plants and bacteria (Moriarty 1997; Tsai and Chen 2002; Moriarty et al. 2005; Decamp and Moriarty 2006a, b; Ebeling et al. 2006; Oliveira et al. 2006; Schneider 2006). This nitrification process is optimized by the decomposition of organic matter by heterotrophic bacteria (Moriarty 1998, 1999; Ebeling et al. 2006), which is probably associated with the lower nitrogen compounds levels in our probiotic treatments (WP, FP and WFP). Furthermore, the

similarities found among these treatments indicate that regardless the application strategy the use of Bacillus sp. contributes to the stability in the water quality in this culture system.

During the degradation and oxidation of organic matter and ammonia into nitrite, coupled with the availability of organic carbon, there is the release of carbon dioxide (CO_2) by the bacterial community causing a decrease in pH values (Moriarty 1998; Wasielesky et al. 2006). Nitrification is a source of acidity because it releases hydrogen ions (Boyd 2007). This process was clearly demonstrated in this study where the pH values found in the probiotic treatments were lower when compared with the control, due to the increase in the bacterial community by the addition of Bacillus sp.. Additionally, during the respiration and ammonia oxidation by heterotrophic bacteria a large amount of oxygen is consumed thus resulting the significant lower values of dissolved oxygen found in these probiotic treatments (Van Wyk and Scarpa 1999; Horowitz and Horowitz 2000a; Leonard et al. 2001; Nogueira et al. 2002; Erler 2005).

Conclusion

The responses obtained in this study suggest that all probiotics application strategy used have the ability to interfere in any ways on the growth parameters and the physical and chemical water quality variables in L. vannamei super intensive heterotrophic systems.

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Tables

Table - 1 Mean (± SD) survival (%), FCR (feed conversion ratio), biomass gain (g), food consumption (g), growth rate (g/week) and final weight (g) of Litopenaeus vannamei after 30 days under different probiotic application strategies in super intensive culture.

Treatments				
	WFP	WP	FP	NP
Survival	96,67 (±6,29)a	88,33 (±14,65)a	90,83 (±9,46)a	89,17(±7,64)a
Final weight	3,33 (± 0,72)b	3,51 (± 0,81)b	3,73 (± 0,68)a	3,72 (± 0,72)a
Growth rate	0,27 (± 0,16)b	0,31 (± 0,31)b	0,37 (± 0,39)a	0,37 (± 0,25)a
FCR	1,67 (±0,19)a	1,77 (±0,30)a	1,54 (±0,19)a	1,57(±0,18)a
Biomass gain	51,5 (± 12,70)a	37,3 (± 19,11)a	78,8 (± 13,17)a	71,3 (± 13,74)a
Food consumption	0,45 (± 0,25)b	0,32 (± 0,24)b	0,60 (± 0,28)a	0,59 (± 0,27)a

* Means values followed by different letters in the same row were found to differ at the 0.05 probability level

** WFP = probiotic added in the water and feed; WP = probiotic added in the water; FP = probiotic added in the water and feed and NP = No probiotic.

Table 2 - Water quality variables (mean ± SD) during 30 days of Litopenaeus vannamei culture under different probiotic application strategies in super intensive culture.

Treatments				
Variables	WFP	WP	FP	NP
Temperature (C)	26,18 (± 0,56)a	25,94 (± 0,53)a	26,18 (± 0,59)a	25,85(± 0,43)a
Salinity	33,09 (± 1,66)a	33,35 (± 1,40)a	33,17 (± 1,43)a	33,47 (± 1,68)a
Secchi (cm)	12,42 (± 14,04)b	12,36 (± 14,03)b	12,44 (± 14,02)b	21,86 (± 9,04)a
Oxygen (mg/L)	4,64 (± 0,87)b	4,76 (± 0,81)b	4,79 (± 0,80)b	6,46 (± 6,05)a
pH	7,69 (± 0,21)b	7,68 (± 0,21)b	7,69 (± 0,22)b	8,05 (± 0,09)a
TAN (mg/L)	0,030 (± 0,056)b	0,038 (± 0,046)b	0,033 (± 0,050)b	0,770 (± 0,675)a
NH_3-N (mg/L)	0,000 (± 0,002)b	0,001 (± 0,001)b	0,001 (± 0,001)b	0,042 (± 0,039)a
NO_3–N (mg/l)	0,039 (± 0,038)b	0,042 (± 0,047)b	0,049 (± 0,060)b	2,114 (± 0,060)a
NO_2–N (mg/l)	0,001 (± 0,001)b	0,001 (± 0,001)b	0,002 (± 0,001)b	0,030 (± 0,046)a

* Means values followed by different letters in the same row were found to differ at the 0.05 probability level

** WFP = probiotic added in the water and feed; WP = probiotic added in the water; FP = probiotic added in the water and feed and NP = No probiotic.

*** TAN = total ammonia; NH_3-N = unionized ammonia; NO_3–N = nitrate and NO_2–N = nitrite.

ANEXO -

NORMAS PARA PUBLICAÇÃO NO PERIÓDICO THE JOURNAL OF THE WORLD AQUACULTURE SOCIETY, Vol. 32, No. 1, March, 2001, WORLD AQUACULTURE SOCIETY

Checklist for Manuscript Preparation

(Check items and submit with manuscript)

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Scientific names should accompany common names in the title and when they are first mentioned in the abstract and in the text. Authority for scientific names need not accompany the genus and species unless needed for clarity. - Spell out one to ten unless followed by a unit of measurement (e.g., four fish. 4 kg. 14 fish). Do not begin a sentence with a numeral. Use 1.000 instead of 1000: 0. I3 instead of .13: and 70 instead of percent. Use the 24-hour clock for dial time: 0830, not 8:30 a.m.

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