ROBERTA MARIA CAVALCANTI NERY FERREIRA

EFEITOS DE *Bacillus* spp. **SOBRE ATIVIDADES DE PEPTIDASES DIGESTIVAS EM PÓS-LARVAS DO CAMARÃO BRANCO DO PACÍFICO** *Litopenaeus vannamei*

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PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO

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EFEITOS DE *Bacillus* spp. SOBRE ATIVIDADES DE PEPTIDASES DIGESTIVAS EM PÓS-LARVAS DO CAMARÃO BRANCO DO PACÍFICO *Litopenaeus vannamei*

Roberta Maria Cavalcanti Nery Ferreira

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Prof. Dr. Silvio Ricardo Maurano Peixoto
Orientador

Prof. Dr. Ranilson de Souza Bezerra
Coorientador

Profa. Dra. Roberta Borda Soares
Coorientadora

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Roberta Maria Cavalcanti Nery Ferreira

Dissertação julgada adequada para obtenção do título de Mestre em Recursos Pesqueiros e Aquicultura. Defendida e aprovada em 19/08/2013 pela seguinte Banca Examinadora.

Prof. Dr. Silvio Ricardo Maurano Peixoto
(Orientador)
Departamento de Pesca e Aquicultura
Universidade Federal Rural de Pernambuco

Prof. Dr. Ranilson de Souza Bezerra
Departamento de Bioquímica
Universidade Federal de Pernambuco

Profa. Dra. Roberta Borda Soares
Departamento de Pesca e Aquicultura
Universidade Federal Rural de Pernambuco

Prof. Dr. Eudes de Souza Correia
Departamento de Pesca e Aquicultura
Universidade Federal Rural de Pernambuco

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Resumo

O presente estudo teve como objetivo avaliar os efeitos da aplicação de *Bacillus* sp. isolados de camarões selvagens sobre a atividade de enzimas proteolíticas de pós-larvas do camarão *Litopenaeus vannamei*. Bactérias foram isoladas do trato digestório de indivíduos da espécie *Farfantepenaeus subtilis* capturados no litoral sul de Pernambuco. Nove bactérias foram identificadas como *Bacillus* e selecionadas de acordo com seu perfil de inibição *in vitro* de cepas de *Vibrio.* As linhagens com maior potencial de inibição, *Bacillus circulans* e *Paenibacillus thiaminolyticus,* foram administradas no cultivo experimental de pós-larvas de *L. vannamei,* misturadas à ração. Foram formuladas quatro dietas com emprego de diferentes probióticos: *Bacillus circulans* (B); *Paenibacillus thiaminolyticus* (P), probiótico comercial Sanolife Mic INVE (M) e controle sem a adição de bactérias, com quatro repetições cada. Os animais foram alimentados quatro vezes ao dia, durante o período de 40 dias. A cada dez dias de cultivo, foi realizada a análise de atividade enzimática dos animais. Hepatopâncreas de 20 camarões foram coletados, homogeneizados em tampão Tris-HCl 0,01M, pH 8,0 com adição de NaCl (9%) e centrifugados para obtenção dos extratos enzimáticos. A atividade proteolítica total do extrato foi mensurada utilizando azocaseína 1% como substrato. Para a determinação da atividade proteolítica específica foram utilizados os substratos BApNA, SApNA e Leu-p-Nan. Após 40 dias, os camarões alimentados com probióticos mostraram uma atividade da quimotripsina significativamente maior ($P<0,05$) do que o controle. A atividade da tripsina foi mais elevada e significativamente diferente ($P<0,05$) no M em relação a C. A administração de bactérias através da ração aumentou a atividade de enzimas proteolíticas em pós-larvas do *L. vannamei*, mas não influenciou diretamente os parâmetros zootécnicos de cultivo como peso, sobrevivência e taxa de crescimento específico.

Palavras-chave: camarão marinho, carcinicultura, protease, probiótico.

Abstract

The present study aimed to evaluate the effects of the use of *Bacillus* sp. on the activity of proteolytic enzymes in *Litopenaeus vannamei* postlarvae. Bacteria were isolated from the digestive tract of wild *Farfantepenaeus subtilis* individuals captured in the southern coast of Pernambuco. Nine bacteria were identified as *Bacillus* and used in the *in vitro* antagonism test against various species of *Vibrio*. For enzymatic analyzes, hepatopancreas of 20 postlarvae were weighed, homogenized in 0.01 M Tris-HCl, pH 8.0, with added 0.15M NaCl solution (0.2 mg/ml) and centrifuged at 10,000 g for 25 minutes at 4 °C. The nonspecific proteolytic activity of the crude extract was measured using azocasein 1% as substrate. To determine the specific proteolytic activity it was used BApNA, SApNA and Leu-p-Nan. After 40 days, shrimp fed with probiotics showed activity of chymotrypsin significantly higher ($P < 0.05$) than the control. Trypsin activity was significantly higher in M with respect to C. The administration of bacteria through the diet increased the activity of proteolytic enzymes in post-larvae of *L. vannamei*, but did not directly influence the performance parameters in growing weight, survival and specific growth rate.

Key words: marine shrimp, carciniculture, protease, probiotic.

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1- Introdução

A produção mundial de camarões vem sendo prejudicada por doenças, principalmente aquelas causadas por bactérias patogênicas oportunistas (AUSTIN, 1993; MORIARTY, 1999; GOMEZ-GIL et al., 2000). Este quadro também é observado no Brasil, onde a carcinicultura baseada na espécie *Litopenaeus vannamei* atingiu o recorde de produção em 2003, chegando a 90 mil toneladas. Porém, a partir de 2004, a atividade vem enfrentando problemas com enfermidades que reduziram em 30% os valores produzidos entre 2003 e 2006 (FAO, 2006)

Doenças causadas por bactérias são consideradas como a maior causa de mortalidade nas larviculturas de camarão (Wyban e Sweeney 1991; Wilkenfeld 1992) e prejudicam a produção consistente de larvas (Daniels 1993). Entre estas, *Vibrio* spp. tem sido os principais responsáveis por doenças em camarões cultivados, especialmente a bactéria luminosa *V. harveyi* (Baticados et al. 1990).

Os antibióticos têm sido utilizados como agentes terapêuticos e promotores de crescimento na alimentação animal desde 1950. No entanto, o uso indiscriminado de antibióticos traz alterações importantes na microbiota dos sistemas de aquicultura e ambiente circundante, criando resistência bacteriana aos antimicrobianos comumente utilizados (Resende et al. 2012). A comissão Swann restringiu a utilização de antibióticos como promotores de crescimento, deixando esses antibióticos apenas para uso no tratamento de doença (SWANN, 1969).

Existe um interesse crescente, tanto do setor produtivo como do mercado consumidor, em controlar ou eliminar o uso de antibióticos na produção de larvas de camarões peneídeos. Desta forma, métodos alternativos precisam ser desenvolvidos para manter o ambiente microbiano saudável nos tanques de larvicultura (IRIANTO e AUSTIN, 2002). Entre estes métodos, o uso de bactérias probióticas para controlar organismos potencialmente patogênicos vem se destacando não só por sua eficiência, mas também devido ao conceito de responsabilidade ambiental associado a estes produtos junto ao mercado consumidor.

A seleção de probióticos na aquicultura é geralmente baseada em resultados de testes que mostram antagonismo em relação a agentes patogênicos, a capacidade de sobreviver e colonizar o intestino e uma capacidade para aumentar a resposta imune no hospedeiro (VILLASEÑOR et al., 2011).

Tais agentes probióticos podem ativamente inibir a colonização de potenciais patógenos no trato digestório por ação antibiótica, competição por nutrientes e/ou espaço, alteração do metabolismo microbiano ou através da estimulação da imunidade do hospedeiro (GOMEZ-GIL et al., 2000). Sabe-se que a presença de probióticos pode estimular a produção de enzimas endógenas pelas larvas de camarão (LOVETT e FELDER, 1990; KAMARUDIN et al, 1994). Além disso, as bactérias, particularmente as do gênero *Bacillus* secretam uma vasta gama de exoenzimas (MORIARTY, 1996, 1998). O aumento das atividades de enzimas digestivas foi observado em grupos de pós-larvas e juvenis de camarões marinhos tratados com probiótico, *B. coagulans* SC8168, mesmo nas últimas fases (ZHOU et al., 2009). O sistema digestório do camarão foi ativado em particular nos estágios larval e pós-larval, quando os probióticos têm o maior efeito (LOVETT e FELDER, 1990; KAMARUDIN et al, 1994).

Mesmo com tantos benefícios em potencial, muitas dúvidas ainda permanecem no setor produtivo e comunidade científica sobre o custo-benefício de produtos comerciais disponíveis na larvicultura de camarões.

Com base nessas informações faz-se necessário a investigação das atividades de enzimas proteolíticas inespecífica e específicas em pós-larvas de *L. vannamei* cultivadas com probióticos isolados de camarões selvagens e um probiótico comercial e suas contribuições para o aumento da sobrevivência e ganho de peso.

2- Revisão de literatura

O controle de infestações bacterianas ou presença de bactérias potencialmente patogênicas é uma prática comum entre os administradores de larviculturas. Entretanto, este controle tem sido baseado no uso de produtos químicos, e, mais recentemente, tem sido testado o uso de vacinação, probióticos e outras formas imunoestimulantes para as larvas de camarões peneídeos (GOMEZ-GIL et al., 2000).

As infestações causadas por membros do gênero *Vibrio* são as que mais se destacam, uma vez que estas bactérias fazem parte da flora autóctone dos camarões e da água de cultivo, representando, portanto, uma constante fonte de infecção para os animais, já que em condições adversas podem causar lesões nos tecidos com ou sem necrose, retardo no crescimento e comprometimento das metamorfoses larvais, acompanhados por índices ou taxas de mortalidade variáveis (LIGHTNER, 1996; COSTA, 2009). Nas Filipinas, doenças causadas por *Vibrio* spp. luminescentes afetaram significativamente a sobrevivência dos camarões e diversas fazendas tiveram que ser fechadas em 1996 (MORIATY 1999). Segundo Aguirre-Guzmán et al. (2002), as espécies: *Vibrio parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. harveyi* são reportadas como as principais espécies causadoras de infecções nos camarões de cultivados.

Muitos mecanismos de ação pelos quais os probióticos melhoram a saúde dos organismos hospedeiros têm sido relatados, incluindo, além da criação de um ambiente hostil para patógenos pela produção de compostos inibitórios e competição por nutrientes essenciais e locais de adesão, o fornecimento de nutrientes essenciais e enzimas que resultam numa maior nutrição dos animais cultivados e captação direta de material orgânico dissolvido na água pelas bactérias probióticas (GATESOUPE, 1999; GOMEZ-GIL et al., 2000; IRIANTO e AUSTIN, 2002; BALCAZAR et al., 2006).

Espécies do gênero *Bacillus* são amplamente utilizadas na aquicultura. Essas bactérias são facilmente encontradas em sedimentos marinhos e naturalmente presentes nas brânquias,

cutícula e trato intestinal de organismos bentônicos como os camarões (SHARMILA et al., 1996), e têm demonstrado atividade inibitória contra várias espécies de *Vibrio* em testes realizados *in vitro* e *in vivo*. Vaseeharan e Ramasamy (2003) ao avaliarem o antagonismo *in vitro* de *B.subtilis* BT 23 contra o *V. harveyi* em *Penaeus monodon*, encontraram zonas inibitórias ao redor do crescimento de 3 a 6 mm e redução da mortalidade dos camarões em até 60% em condições *in vivo*, demonstrando que o crescimento dessa espécie patogênica foi controlado.

A ingestão de alimentos e a repartição, assimilação e aproveitamento de nutrientes compõem o processo conhecido como nutrição.

Investigações sobre os processos digestivos em camarões peneídeos têm sido realizadas com o intuito de avaliar a capacidade dos organismos para hidrolisar, absorver a assimilar os principais nutrientes da dieta (GUZMAN et al., 2001). Estudos sobre a atividade das enzimas digestivas do camarão *L. vannamei* vêm se tornando frequentes, pois a indução dessas enzimas sintetizadas e secretadas no hepatopâncreas desses crustáceos tem influência direta na adaptação dos animais às variações na composição dietária (LE MOULLAC et al., 1997).

Vários trabalhos têm enfocado a atuação de enzimas como tripsina, quimotripsina, aminopeptidades, lipases e carboidrases no sistema digestivo do *L. vannamei*, (LE BOULAY et al., 1996; VAN HORMHOUDT e SELLOS, 1996; VAN HORMHOUT et al., 1995) sendo esse estudo essencial para a compreensão do mecanismo de digestão e um melhor conhecimento das necessidades nutricionais (LE MOULLAC et al., 1997). Em conjunto, essas enzimas digestivas presentes nos hepatopâncreas de *L. vannamei* são capazes de hidrolisar uma variedade de substratos e vários fatores estão implicados em sua regulação. Entre esses fatores destacam-se: a dieta (LE MOULLAC et al. 1996; GUZMAN et al., 2001; BRITO et al., 2001), variações ontogênicas (LOVETT e FELDER, 1990; LEMOS e RODRIGUEZ, 1998), tamanho corporal (LEE e LAWRENCE, 1985), ritmo circadiano (GONZALEZ et al., 1995; MOLINA et al., 2000), fases do ciclo de muda (MOLINA et al.,

2000; SANCHEZ-PAZ et al., 2003) e até mesmo um efeito estimulante da água de tanques tem sido relatado (MOSS et al., 2001).

A atividade tríptica em *L. vannamei* foi primeiramente evidenciada por Lee e Lawrence (1982). Em estudos posteriores, extratos enzimáticos da glândula digestiva do camarão branco exibiram três isoformas de tripsina (KLEIN et al., 1996; LE MOULLAC et al., 1996; EZQUERRA et al., 1997; MUHLIA-ALMAZÁN et al., 2003). De acordo com Van Wormhoudt et al. (1996) a eficiência catalítica da tripsina é maior em crustáceos peneídeos comparada aos vertebrados e em *L. vannamei* é a enzima mais ativa de todas as proteases caracterizadas (LEMOS et al., 2000).

A maior parte do conhecimento sobre a enzima quimotripsina baseia-se em fontes de mamíferos, embora a pesquisa sobre as enzimas de outros grupos de vertebrados e invertebrados já esteja disponível. As propriedades catalíticas dessas enzimas, como a hidrólise de substratos sintéticos e os efeitos de alguns inibidores da protease, são semelhantes aos dos mamíferos. Van Wormhoudt et al. (1992) relata a purificação de duas isoformas de quimotripsina nas glândulas do intestino médio de *L. vannamei.* Porém a atividade de quimotripsina não foi detectada por Gates e Travis (1973) em *L. setiferus* e por Lee et al. (1984) em *L. vannamei*, provavelmente devido à falta de substratos sensíveis e altamente específicos. Tsai et al. (1986) evidenciaram atividade de quimotripsina e tripsina nas glândulas intestinais, estômago e intestino de *Penaeus. monodon*, *Panulirus penicillatus*, *M. japonicus*, *Metapenaeus monoceros* e *Macrobrachium rosenbergii.* Estes autores concluíram que a quimotripsina foi tão importante quanto a tripsina nos processos digestivos destes crustáceos decápodes.

Entre as carboidrases dos camarões peneídeos, a α-amilase (Van WORMHOUDT et al., 1995, FERNÁNDEZ et al., 1997), é uma das enzimas digestivas mais estudadas em *L. vannamei*, representando 1% do extrato bruto do hepatopâncreas desses animais (Van WORMHOUDT et al. 1996). Três isoformas da enzima amilase foram determinadas em *L.*

vannamei (Wormhoudt Van et al. 1996). Os estudos sobre a digestão de carboidratos são importantes porque são frequentemente incluídos em rações comerciais para a redução dos custos de alimentação (WIGGLESWORTH e GRIFFTH, 1994).

As enzimas são proteínas que desempenham importante papel como catalisadores em diversas reações bioquímicas. São fundamentais para o metabolismo biológicos dos seres vivos, visto que, sem a catálise, as reações não ocorreriam em uma escala de tempo útil. Agindo em sequências organizadas, as enzimas catalisam centenas de reações sucessivas pelas quais as moléculas de nutrientes são degradadas, aumentando a velocidade das reações, sem afetar o seu equilíbrio. Existe uma correlação entre a estrutura das proteínas e os peptídeos que fazem parte da molécula enzimática e suas propriedades biológicas. Provavelmente, apenas uma fração da molécula denominada sítio ativo é a responsável pela ligação da enzima ao substrato, e essa fração determina a especificidade enzimática (NELSON e COX, 2004).

No hepatopâncreas dos camarões existem 3 tipos de células indispensáveis a digestão, porém uma delas, as células B (Blasenzellen), possuem um grande vacúolo, contendo enzimas digestivas (CECCALDI, 1987).

As secreções produzidas pelo hepatopâncreas são compostas principalmente por enzimas digestivas (CECCALDI, 1987). Dentre elas destacam-se as proteases, carbohidrases e lipases. As proteases podem ser endopeptidases, quebram as ligações peptídicas no interior das cadeias protéicas (tripsina e quimotripsina), ou exopeptidases que quebram as ligações aminoterminais, carboxiterminais e dipeptídios (aminopeptidases, carboxipeptidases A e B e dipeptidases). Dentre as enzimas proteolíticas dos camarões, a tripsina é a mais importante por hidrolisar de 50 a 60% da proteína consumida (DALL, 1992). No grupo das carbohidrases são incluídas as amilases, maltases, sacarases e principalmente a quitinase, que permite a digestão da quitina do próprio exoesqueleto e de microcrustáceos dos quais se alimentam. Além dessas, tem-se encontrado enzimas como a desoxirribonuclease, muclease e fostatases

alcalinas (CECCALDI, 1987; DALL e MORIARTY, 1983; DALL, 1992; CRUZ-SUÁREZ, 2000).

A atividade enzimática é modulada por uma série de fatores, como parâmetros físicos e químicos da água (pH, oxigênio, salinidade e temperatura), idade e tamanho do camarão, ingredientes que compõem a dieta, nível e fonte de proteína, aditivos alimentares, frequência alimentar e pela quantidade de alimento (MOLINA et al., 2000; LLOMITOA, 2000). Dentre os fatores internos, a atividade enzimática é influenciada por mudanças morfológicas relacionadas à ontogenia; taxas metabólicas; ritmo circadiano e processos fisiológicos da muda (DALL, 1992; LEMOS et al., MUHLIA-ALMAZÁN e GARCIA-CARREÑO, 2002).

Córdova-Murueta et al. (2004) sugerem que o estresse alimentar representado pela mudança repentina da composição da dieta (como mudança no teor protéico) pode influenciar a atividade da tripsina e quimotripsina em *L. vannamei*, passando a ter a mesma importância que o ciclo de muda na atividade enzimática. Esse trabalho reforça a conclusão do experimento de Muhlia-Almazán e García-Carreño (2002) que concluíram que o estresse alimentar, representado pela inanição, pode ser tão acentuado quanto o estresse fisiológico causado pelo ciclo da muda no *Penaeus vannamei*.

Variação na atividade enzimática também foi relatada por Molina et al. (2000) trabalhando com *L. vannamei* em condições controladas, os quais detectaram um pico máximo de atividade proteolítica às 14 horas e outro de menor intensidade às 02 horas quando os camarões foram alimentados às 12 horas e 20 horas. Delgado *et al.* (2003) analisaram, em viveiro de cultivo de *L. vannamei*, a atividade das enzimas digestórias de acordo com o peso corpóreo (de 2 a 12 g), tendo evidenciado que a atividade de protease não específica diminuiu em camarões a partir de 6 g.

Algumas bactérias podem participar do processo digestório dos camarões pela produção de enzimas extracelulares como proteases e lipases (OCHOA e OLMOS, 2006). Zhou et al. (2009), encontraram um aumento na atividade da amilase, lipase e protease em larvas de *L.*

vannamei tratadas com *B. coagulans* SC8168 e especulou que um consequente aumento da digestão e absorção do alimento pode ter contribuído para o incremento na sobrevivência.

Para ter um efeito benéfico, as linhagens bacterianas isoladas devem ser toleráveis pelo hospedeiro e capazes de sobreviver e crescer no local onde são aplicadas enquanto exercerem os seus efeitos benéficos, e não devem ser patogênicas (GOMEZ-GIL et. al., 2000; BALCÁZAR et. al., 2007; TINH et. al., 2008). Dessa forma, alguns autores sugerem o isolamento e utilização de bactérias do próprio ambiente ou hospedeiro. Vine et al. (2006) propuseram a seleção de probióticos do organismo que está sendo cultivado ou que se tem interesse de cultivar. Defoirdt et. al. (2007) recomendaram o isolamento de bactérias probióticas do sistema de cultivo, o que pode facilitar seu desenvolvimento e estabelecimento no hospedeiro.

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4- Artigo científico

Effects of *Bacillus* spp. **on activities of digestive peptidases in post-larvae of the Pacific white shrimp** *Litopenaeus vannamei*

Roberta Nery Ferreira[1], Joana Vogeley[1], Juliana Interaminense[2], Ranilson Bezerra[2], Roberta Soares[1], Silvio Peixoto[1]

[1]Laboratório de Tecnologia em Aquicultura, Departamento de Pesca e Aquicultura Universidade Federal Rural de Pernambuco, Brazil, Rua Dom Manoel de Medeiros, s/n Dois Irmãos, Recife, PE, CEP: 52171-900, Brazil

[2]Laboratório de Enzimologia, Departamento de Bioquímica da Universidade Federal de Pernambuco, Brazil, Cidade Universitária, Recife, PE, CEP: 50670-420, Brazil

ABSTRACT. The study investigated the effect of *Bacillus* spp. isolated from the digestive tract of indigenous *Farfantepenaeus subtilis*, on the proteolytic enzymes activity of *Litopenaeus vannamei* post-larvae. Bacteria were isolated from the digestive tract of *F. subtilis* (body weight of 14g) captured in coastal waters off northeastern Brazil. Nine bacteria were initially identified as *Bacillus* and two were selected *in vitro* according to their trait inhibitory to *Vibrio*, identified as *Bacillus circulans* and *Paenibacillus thiaminolyticus.* Four treatments were established according to different diets supplemented with probiotic bacteria: *Bacillus circulans*; *Paenibacillus thiaminolyticus*, commercial probiotic (*Bacillus* spp.) and control diet, without the addition of bacteria. Post-larvae (10 days after the metamorphosis; PL10) were fed four times a day, during a experimental period of 40 days. Analysis of zootechnical performance and enzymatic activity of animals were performed every ten days. Although, final survival and zootechnical performance did not differ significantly among treatments, overall results indicated that the administration of probiotic bacteria through the diet (*B. circulans*, *P. thiaminolyticus* and commercial *Bacillus* spp) increased the total and specific activity of proteolytic enzymes, especially for advanced post-larval stages (PL 40-50) of *L. vannamei*.

INTRODUCTION

Probiotic administration has shown benefic effects on shrimp growth, survival and health (Moriarty, 1998; Skjermo & Vadstein, 1999; Luis-Villaseñor *et al*., 2011; Newaj-Fyzul, et al., 2013). Some of the mechanisms that have been suggested to explain these benefits are the competition with pathogen bacteria for adhesion places and nutrients in the digestive tract, contribution to enzymatic digestion and stimulation of the immunological response by the host (Gomez-Gil *et al.*, 2000; Kumar-Sahu *et al*., 2008).

It has been suggested that the presence of probiotics can stimulate the production of endogen enzymes by the shrimp larvae (Lovett & Felder, 1990; Kamarudin *et al*., 1994). Furthermore, particularly bacteria of the genus *Bacillus*, secrete a vast range of exoenzymes which can offer an increment the natural enzymatic activity of the shrimp (Moriarty, 1997, 1998). Luis-Villaseñor *et. al*., (2011) reported the ability of *Bacillus* spp. to adhere and grow in intestinal mucosa and modulated the intestinal microbiota in juvenile *Litopenaeus vannamei*. The digestive enzyme activity of larvae and juvenile *L. vannamei* was increased when treated with *Bacillus coagulans* SC8168 (Zhou *et al*., 2009). Similarly, two strains of *B. subtilis* were reported to improve digestive enzyme activity of juvenile shrimp (Zokaeifar *et al*., 2012).

Probiotic prospection studies usually select bacteria that are able to produce inhibitory compounds against pathogenic agents, as well as to survive and colonize digestive tract of culture species (Balcázar *et al*. 2006; Leyva-Madrigal *et al*., 2011; Martínez Cruz *et al*., 2012). Therefore, most probiotic strains have been isolated from the digestive tract microbiota of aquatic animals (Luis-Villaseñor *et. al*., 2011). The inhibitory effect on pathogenic *Vibrio* spp. has been performed *in vitro* as the first criteria to select potential probiotic (Nguyen *et al*., 2007). Nevertheless, different research approaches (e.g. *in vitro* and *in vivo*) are necessary to evaluate not only the potencial of probiotic bacteria strains in producing antagonism against pathogens, but also their ability to improve additional desirable features, such as the enzymatic activity of the host.

The present study aimed to investigate the effect of *Bacillus* spp. isolated from the digestive tract of indigenous *Farfantepenaeus subtilis*, which showed trait inhibitory to *Vibrio*, on the proteolytic enzymes activity of *L. vannamei* post-larvae.

MATERIAL AND METHODS

Bacterian isolation

Adults of the *F. subtilis* (mean body weight of 14g) were captured in coastal waters off northeastern Brazil (8º36'S; 35º1'W) and kept alive for approximately 6 hoours under constant aeration and controlled water quality conditions. At the laboratory, individuals were externally disinfected with ethanol (70%) and washed three times with distilled water, before being dissected using sterilized scissors and pincers. The gastrointestinal tracts were removed and washed externally with sterile saline solution (2,5% NaCl). Hepatopancreas and gut samples were collected using a *swab* and inoculated on Petri dishes containing *Bacillus* selective MYP Agar Base (Phenol Red Egg Yolk Polymyxin Agar Base - Himedia) and Marine Agar (Himedia). After 24 - 48 h incubated at 30 °C, the Colony Forming Units (CFU) in the plates were isolated according to their morphological characteristics (morphotypes) as coloration, border format, size and texture (Koneman *et al*., 2011).

The bacteria cultures obtained were subjected to Gram staining technique and a portion of the colonies identified as Gram-positive bacilli were striated in a new Petri dish containing the same culture medium of the original plate, and incubated under similar conditions.

Bacillus Identification

The Gram-positive bacilli were subjected to the catalyze and spores formation tests. The spores formation analisis consisted of the supplementation of culture medium with 5 mg/ml of Manganese Sulfate ($MnSO_4$). Addionally, after 48h incubation at 30 °C, the colonies were subjected to the coloring technique, using malachite green to help the visualization of the spores under the microscope (Koneman *et al*., 2008). The isolates defined Bacteria presumptively defined as *Bacillus* were identified as *Bacillus circulans* and *Paenibacillus thiaminolyticus* through biochemical assays using a commercial kit (API 50CH - Biomerieux).

Experimental design

Bacillus circulans and *Paenibacillus thiaminolyticus* were lyophilized and mixed with the commercial feed for post-larvae (40% PB) in order to obtain a concentration of 10^{7-8} CFU/g. The lyophilized bacteria had its concentration confirmed by spread plating technique a sample in MYP Agar. The diets were sterilized in autoclave during 30 min at 50 °C, crushed, and its was added 5% of Agar Agar and moistened and 45% of sterile distilled water at 45 °C. After homogenization, the diets were dried in an incubator for 18 hours at 45 °C and pelletized again using 0.85 and 1.3 μ sieves. The same procedure was performed to the

commercial probiotic diet consisting of *Bacillus* and the control diet, but in this case no bacteria were added.

Experimental design

Post-larvae of *L. vannamei* (10 days after the metamorphosis) were stocked in the experimental units (30 liters) at density of 25 shrimps/liter. Four treatments were establhed, with four replicates each, corresponding to the experimental diets: *B. circulans* (B); *P. thiaminolyticus* (P), commercial probiotic (M) and control diet -treatment with no addition of bacteria (C). The feed was offered four times a day (at 8:00, 12:00, 14:00 and 20:00 h) and the experiment lasted 40 days.

All the experimental units were subjected to constant aeration and controlled environmental conditions. The water quality parameters (dissolved oxygen, salinity, pH and temperature) were measured daily using a multiparameter (YSI 556). Survival was assessed by counting the individuals at the end of the experimental period. Growth of post-larvae were assented by weighing individuals from each experimental unit in analytical balance (0.001g).

Preparation of raw extracts and total soluble protein determination

Twenty post-larvae were collected from each experimental unit every 10 days. The individuals were thawed and their digestive glands were removed, weighed and homogenized at a concentration of 0.2 mg tissue/ml in a solution of 0.01 M Tris-HCl, pH 8.0, by adding 0.15 M NaCl. This solution was centrifuged at 10,000g during 25 min. at 4 °C to remove tissue remains and lipids. The supernatant (raw extracts) was removed and stored at 25 °C for further analysis. The measurement of total soluble protein in raw extracts was determined as described by (Smith *et al*., 1985), using albumin from bovine serum as a standard protein.

Enzymatic assays

Total proteolytic activity

Total enzymatic activity of the proteases in raw extracts was evaluated using 1% azocasein as a substrate, prepared in 0.1 M Tris-HCl (pH 8.0) solution. Aliquots containing 30 μL of the raw extract were incubated with 50μL of the azocasein substrate for 60 min. at 25 °C. After the reaction time, 240μL of trichloroacetic acid solution (10%) was added to stop the reaction. After 15min, the solution was centrifuged at 8,000 g for 5 min. and 70 μL of the supernatant was withdrawn and mixed at 130μL of 1M sodium hydroxide (revealing solution) in microplates. The absorbance was measured in a microplate reader (Bio-Rad 680) at a wavelength of 450nm. A negative control (blank) was performed by replacing the enzyme

extract for a solution of 0.1 M Tris-HCl, pH 8.0, with addition of 0.15 M NaCl. This analysis were performed in triplicate for each sample, and a unit of enzyme activity (U) was defined as the amount of enzyme required to hydrolyze azocasein and result in a change of 0.001 absorbance units per minute.

Specific proteolytic activities

The enzymatic activities of trypsin, chymotrypsin and leucino aminopeptidase were determined in microplates using specific substrates Nα-benzoyl-DL-arginine-*p*-nitroanilide (BApNA), N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (SApNA), and *p*-nitroanilide-leucino (Leu-p-Nan), respectively (Bezerra et al., 2005). These substrates were used at a concentration of 8mM. All assays were performed in triplicate. The enzyme extract (30μL) were incubated with 140μL tampon Tris-HCl 0.1 M (pH 8.0) and 30μL of the substrate for a period of 15 min. Absorbance readings were measured at 405nm wavelength, using a microplate reader (Bio-Rad 680). A unit (U) of activity was defined as the amount of enzyme required to produce one mole of *p*-nitroaniline per minute. The specific activity was expressed in units per milligram of protein.

Statistical analyses

Analysis of variance (ANOVA) was used to determine significant differences ($P < 0.05$) among treatments in terms of survival, growth and enzymes activities. Where significant differences were found, Tukey poshoc test was used to separate means at 95% of significance level.

RESULTS

Survival of post-larvae did not differ significantly among the treatments, but higher value (69.57%) was observed for *B. circulans* diet (Table 1). Similarly, overall growth performance of post-larvae (final weight, weight gain and SGR) was similar among treatments, with *P. thiaminolyticus* showing higher values (Table 1). Regarding the weight gain of the animals, there was a greater increase on the 30th day of culture (Figure 1).

On the 10th experimental day, a higher total proteolytic activity in shrimp fed with *P. thiaminolyticus* (1.255 ± 0.176 U.mg^{-1}) was observed, differing significantly from the control (0.980 ± 0.050 U.mg^{-1}) and M treatment (0.966 ± 0.096 U.mg^{-1}). Regarding specific proteolytic activities, trypsin showed significant lower activity in treatment M (0.764 ± 0.122 U.mg^{-1}) when compared to control (1.112 ± 0.179 U.mg^{-1}). For leucino aminopeptidase and chymotrypsin there were no significant differences among treatments (Table 1).

Total and trypsin proteolytic activity did not present significant differences among treatments after 20 days. Proteolytic activities of leucino aminopeptidase and chymotrypsin were similar between *B. circulans* and *P. thiaminolyticus* treatments, but did not differ from M and C diets (Table 1).

After thirty days, the total proteolytic activity increased in all treatments, showing significantly higher values for *B. circulans* (1.825 ± 0.146 $U.mg^{-1}$) when compared to commercial probiotic (1.258 ± 0.357 $U.mg^{-1}$). Although the specific proteolytic activities also showed an increase, they did not differ among treatments for this period (Table 1).

At the end of the experiment (40th day), higher values of total proteolytic activity were found for M and B treatments, but they were not significant different from the control group. On the other hand, trypsin activity showed a significant increase in the treatment M (2.643 ± 0.385 $U.mg^{-1}$) when compared to control (1.591 ± 0.61 $U.mg^{-1}$). For chymotrypsin activity, all treatments showed a significant increase when compared to control (Table 1).

Results for total and specific proteolytic activity within each treatment and experimental period are presented in Fig. 2 to 5. Total proteolytic activity showed two distinguished higher peaks at 10^{th} and 30rd days for all treatments. The activity of trypsin and chymotrypsin increased till the 30rd day, but declined significantly at the end of the experiment for all treatments, as indicated in the Fig. 3 and 4, respectively. Overall, leucino aminopeptidases values showed only one significantly higher peak of activity at 30rd day for all tratments (Fig. 5).

DISCUSSION

The management of selected *Bacillus* have been related with an increase in shrimp survival due to, for example, the reinforcement of their immune response (Panigrahi *et al*., 2005). Zhou *et al*., (2009) showed that the cultivation of *L. vannamei* post-larvae with *B. coagulans* at increased concentrations ($1.0x10^5$ UFC. mL^{-1}, $5.0x10^5$ UFC. mL^{-1} e $1.0x10^6$.UFC. mL^{-1}) increased significantly the post-larvae survival from 7% to 13.1%. A similar conclusion was obtained by Ziaei-Nejad *et al*., (2006) for *Fenneropenaeus indicus* larvae cultivated with commercial *Bacillus* compared to control-group. However, in the present study there was no significant increase in *L. vannamei* survival or weight gain due to the probiotic supplementation in the diet when compared to control group.

The increase was observed in total proteolytic activity on the 40th day of culture in treatments B and M. This result indicates that there was a good use of the protein source provided by the feed, which had raw protein content of 40%. This is essential for *L. vannamei* in its post-larval stage, because the shrimp has a higher demand for protein, as long as it needs

to form a large amount of body tissues at this development stage. According to Tacon (1990), even shrimp species with omnivorous habits, such as *L. vannamei*, requires high levels of raw protein (RP) during its initial life stages. Lovett & Felder (1990) reported that ontogenetic development influences the enzymatic activity of the shrimp proteases, as it increased from the nauplii stage, reaching a peak at the final Zoea stage, but decreased again during early post-larval stages. However, these authors argued that total proteolytic activity increases slightly during the rest of the post-larval development, which is in accordance with the present study.

The stimulation of the growth of aquatic organisms by probiotic consisting of *Bacillus* strains has been associated to the increase of the feed conversion and protein efficiency ratio, attributed to an increased activity of digestive enzymes (Bairagi *et al*., 2004; Kesarcodi-Watson *et al*., 2008).

Wang *et al*., (2012) evaluated the effects of dead bacterial cells (non-viable) and viable cells of *B. coagulans* as supplement in diets offered to the *L. vannamei* larvae. These authors observed after 50 days of culture, that the addition of this probiotic in both forms resulted in higher shrimp weight gain and survival compared to the control group. These results suggest that the benefits of probiotics may not depend totally on the success of its colonization and development in the microbial gut communities of the host.

In our study, after 30 days of culture, we observed the highest activities of digestive enzymes for all treatments that correlate to the weight gain of the animals. This result can be explained by changes in the composition of the indigenous microbiota, what generates a disturbance that interferes in the alimental conversion, and thus, in weight gain (Spinosa & Bernardi 2006). In this case, there was a greater production of proteolytic enzymes, which may have contributed to a greater digestion and absorption of food.

Although the exogenous enzymes produced by the probiotic do not contribute significantly to the intestinal enzyme activity, the presence of probiotic may stimulate the production of endogenous enzymes by the shrimp (Ding *et al*., 2004; Ziaei-Nejad *et al*., 2006). This might be related to the increase in protease activities in all treatments at the 30th day of culture.

Bacteria, particularly those of the genus *Bacillus*, secrete a wide range of exoenzymes (MORIARTY, 1996, 1998). We have no consistent results to determine if the activities analyzed are due to enzymes synthesized by the post-larvae or by the probiotic bacteria at the digestive tract of the animals. However, in the present study, total proteolytic activity for larvae reared in all probiotic treatments was higher at 10 and 40 days of cultivation. In

accordance, Zhou et al. (2009) observed a significant increase in the protease activity during the stages of PL_{1-2} and PL_{7-8} when cultured with probiotic ($5,0x10^5$ UFC. mL^{-1}).

In our study, *L. vannamei* post-larvae from all treatments showed a significant increase in the activity of chymotrypsin when compared to control group at the end of the experimental period. The same pattern was observed for trypsin with the addition of commercial probiotic (M) and *P. thiaminolyticus* (P) in the diet. Among the shrimp proteolytic enzymes, trypsin is the most important for hydrolyzing from 50 to 60% of the consumed protein (DALLAS, 1992). The trypsin cleaves peptide bonds on the carboxyl side of positively charged amino acid residues, such as arginine and lysine, and chymotrypsin catalyze more efficiently the hydrolysis of protein peptide bonds in carboxyl part of aromatic amino acids, such as phenylalanine, tyrosine, and tryptophan (KLOMKLAO et al., 2007).

In our study, the higher activities with SApNA and BApNA in *L. vannamei* post-larvae can be explained by the fact that the digestive system of crustaceans presented higher concentrations of trypsin and chymotrypsin, which are the most important digestive enzymes in crustaceans (Fernández *et al*., 1997; Fernández Gimenez *et al*., 2002). The hepatopancreas is the organ responsible for their synthesis, secretion and storage.

According to Guillaume (1997), crustaceans require a balanced supplementation of essential amino acids. Holme *et al*., (2009) reported that the essential amino acids in the crustaceans diet are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. This information emphasizes the importance of the chymotrypsin and trypsin increment in *L. vannamei* post-larvae observed in the present study.

In this work, the leucino aminopeptidase activities were less significant when compared with other specific activities for *L. vannamei* post-larvae. The physiological role of this enzyme has been related to preventive action in some pathologies (Andrade, 2011). The leucino aminopeptidase was also higher on the 40th day in the treatment B and this can be attributed to the presence of the arginine amino acids, originated from trypsin activity. Andrade (2011) noted, in a study of *L. vannamei*, that the presence of this amino acid can stimulate the activity of aminopeptidases.

CONCLUSION

Overall results indicated that the administration of *B. circulans* and *P. thiaminolyticus* through the feed increased the activity of proteolytic enzymes in post-larvae of *L. vannamei*, but it did not reflect directly on their zootechnical performance.

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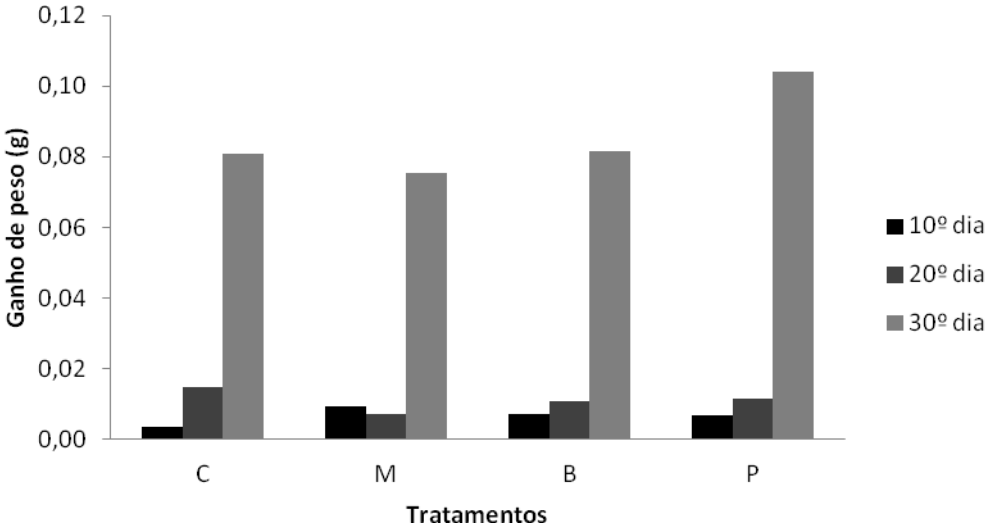

Figure 1. Weight gain (g) of *L. vannamei* fed diets supplemented with probiotics *P. thiaminolyticus* (P), *B. circulans* (B), commercial probiotic (M) and probiotics free (Control) measured on the 10th, 20th and 30th day of cultivation.

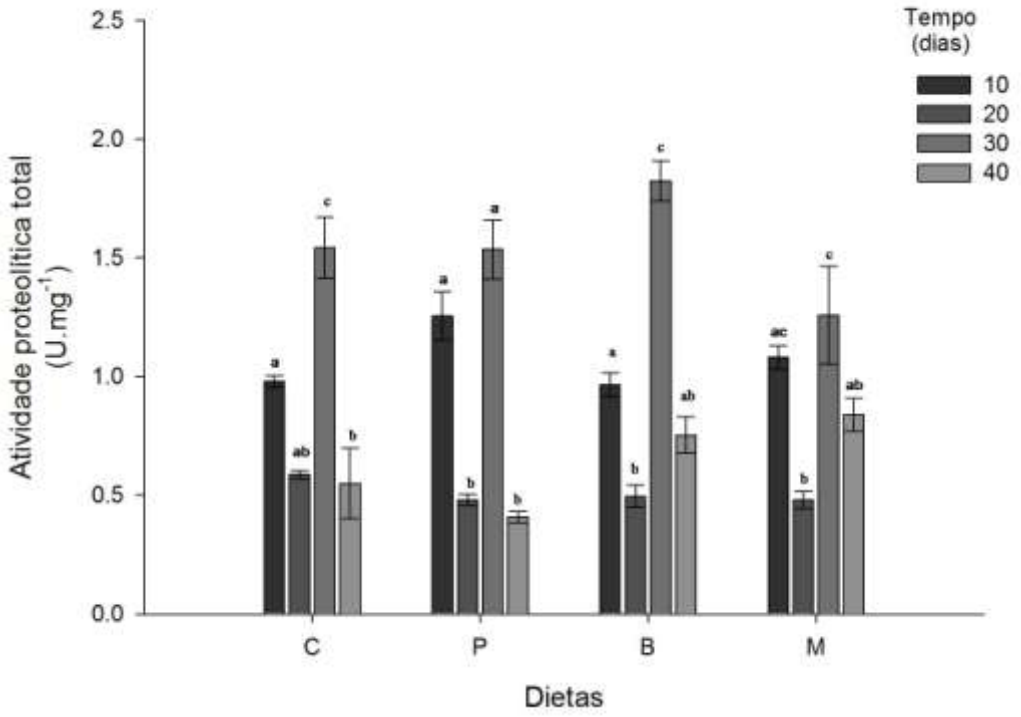

Figure 2. Total proteolytic activity of hepatopancreas (mean ± SEM) of *L. vannamei* fed diets supplemented with probiotics *P. thiaminolyticus* (P), *B. circulans* (B), commercial probiotic (M) and probiotics free (Control). Means within same diet with the same superscript are significantly identical ($p < 0.05$).

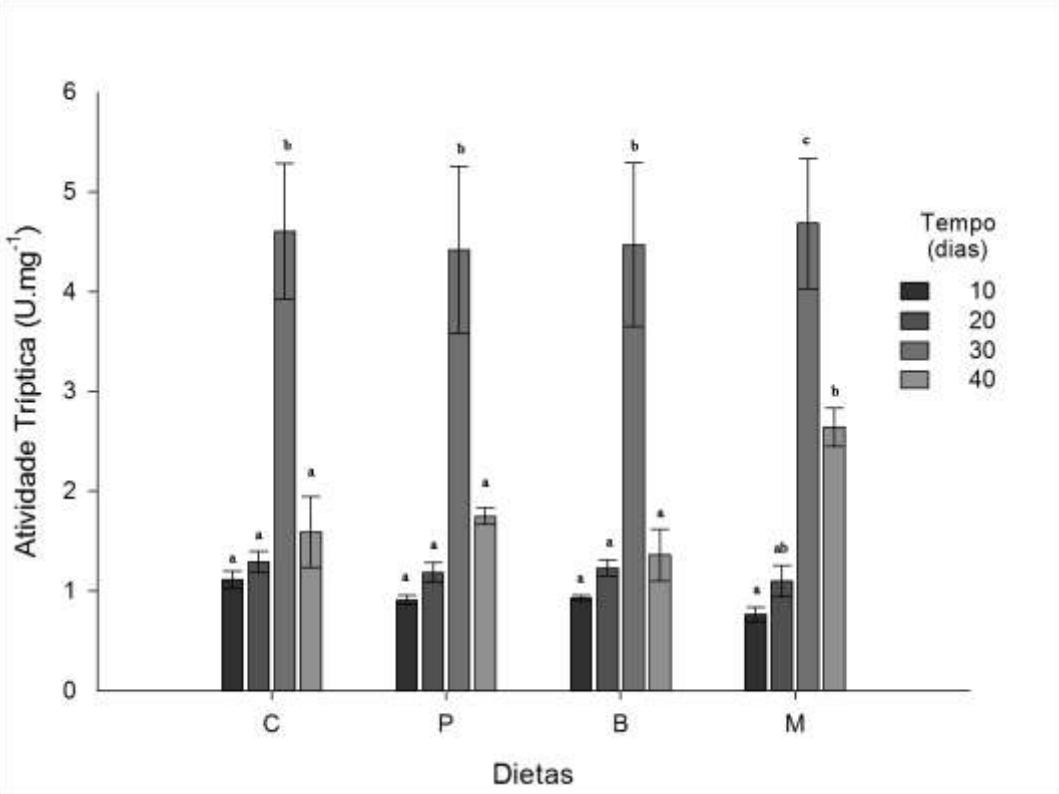

Figure 3. Trypsin activity of hepatopancreas (mean ± SEM) of *L. vannamei* fed diets supplemented with probiotics *P. thiaminolyticus* (P), *B. circulans* (B), commercial probiotic (M) and probiotics free (Control). Means within same diet with the same superscript are significantly identical ($p < 0.05$).

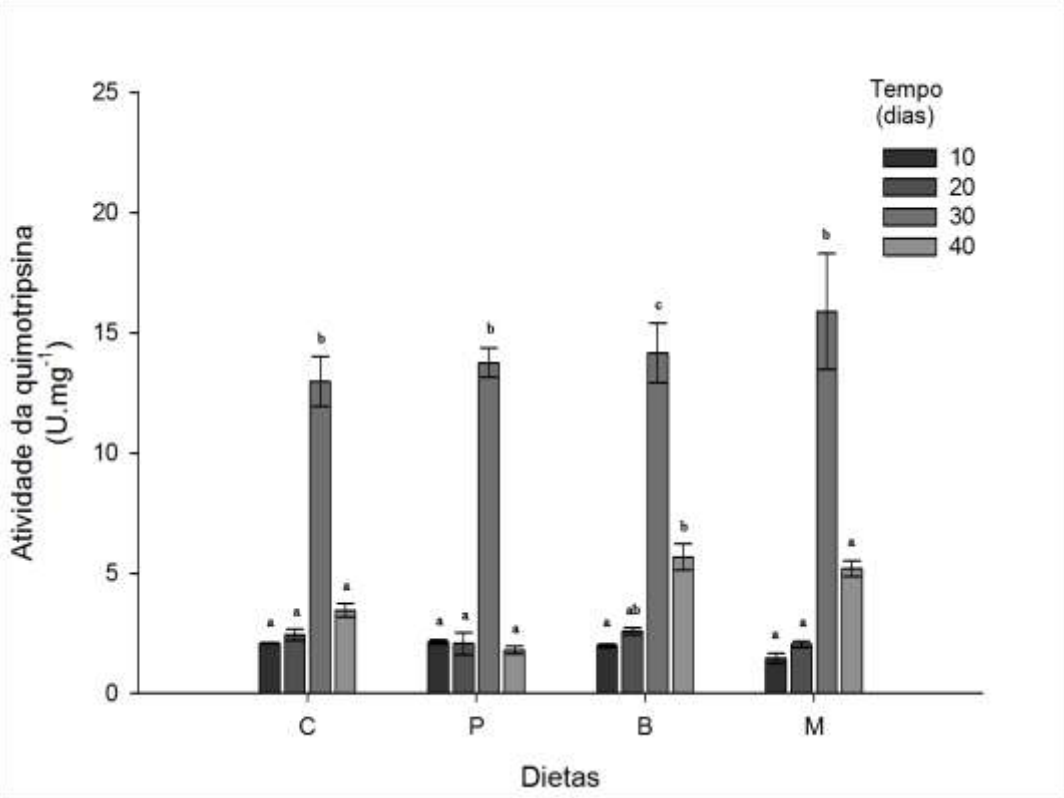

Figure 4. Chymotrypsin activity of hepatopancreas (mean ± SEM) of *L. vannamei* fed diets supplemented with probiotics *P. thiaminolyticus* (P), *B. circulans* (B), commercial probiotic (M) and probiotics free (Control). Means within same diet with the same superscript are significantly identical ($p < 0.05$).

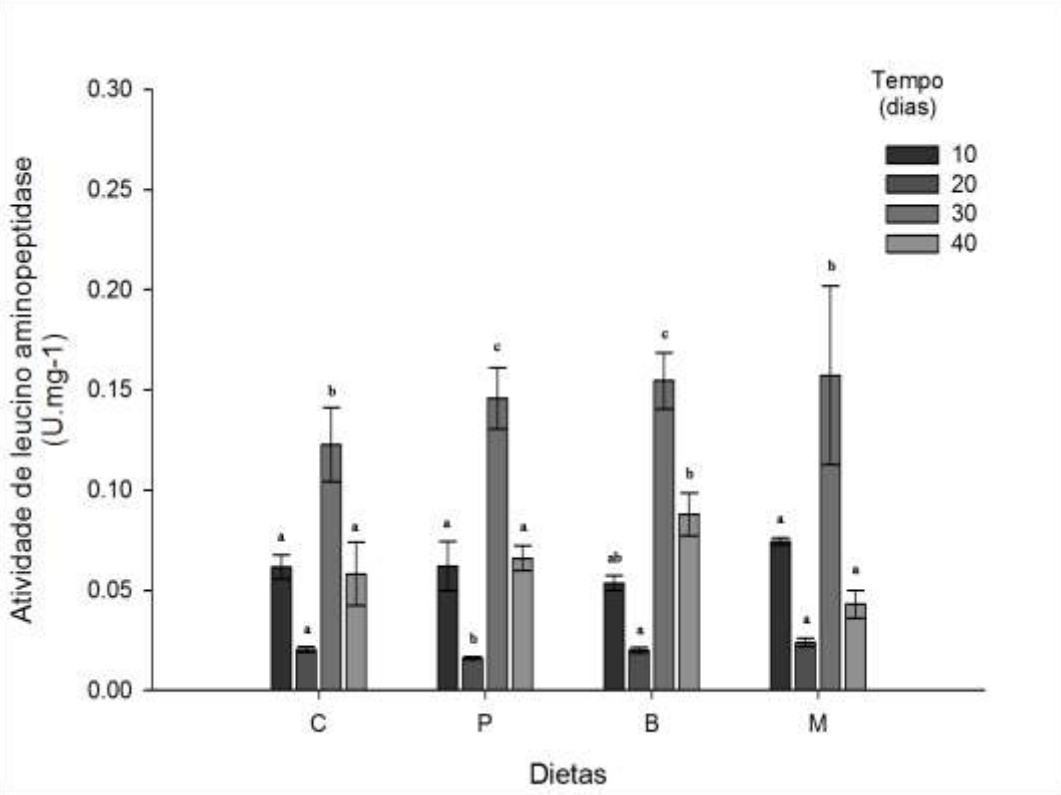

Figure 5. Leucine aminopeptidase activity of hepatopancreas (mean ± SEM) of *L. vannamei* fed diets supplemented with probiotics *P. thiaminolyticus* (P), *B. circulans* (B), commercial probiotic (M) and probiotics free (Control). Means within same diet with the same superscript are significantly identical ($p < 0.05$).

Table 1. Mean values (± SEM) of post-larvae growth indicators of marine shrimp (*Litopenaeus vannamei*) fed diets containing different probiotic bacteria for a period of 40 days.

	C	B	P	M	*p*-valor ANOVA
Final weight (mg)	100.9 ± 8.9	101.2 ± 12.2	123.9 ± 8.5	93.7 ± 13.9	0.303
Weight gain (%)	5283.1 ± 476.8	5303.8 ± 652.9	6510.8 ± 455.5	4901.8± 741.2	0.303
SGR (mg.day^{-1})	13.3 ±0.3	13.2±0.4	13.9 ±0.2	12.9±0.5	0.343
Survival (%)	60.23 ± 1.7	69.57 ± 3.7	61.30 ± 3.9	60.5 ± 4.8	0.261

*No significant differences were found among treatments (p<0,05); SGR-Specific growth rate.
C: control; B: *B. circulans; P: P. thiaminolyticus; M:* commercial probiotic diet with *Bacillus*.

Table 2. Total and specific proteolytic activity of hepatopancreas (mean ± SEM) of *L. vannamei* fed diets supplemented with probiotics *P. thiaminolyticus* (P)*, B. circulans* (B)*,* Sanolife Mic INVE (M) and probiotics free (Control) during 40 days.

		Activity (U.mg^{-1})			
Days		Total	Trypsin	Leucine aminopeptidase	Chymotrypsin
10	**Control**	0.980 ± 0.050^{a}	1.112 ± 0.179^{a}	0.062 ± 0.012^{a}	2.084 ± 0.054^{a}
	P	1.255 ± 0.176^{b}	0.911 ± 0.092^{ab}	0.062 ± 0.024^{a}	2.134 ± 0.191^{a}
	B	0.966 ± 0.096^{a}	0.931 ± 0.053^{ab}	0.054 ± 0.008^{a}	1.989 ± 0.118^{a}
	M	1.080 ± 0.097^{ab}	0.764 ± 0.122^{b}	0.074 ± 0.003^{a}	1.463 ± 0.372^{a}
20	**Control**	0.578 ± 0.035^{a}	1.291 ± 0.217^{a}	0.020 ± 0.002a	2.430 ± 0.395a
	P	0.481 ± 0.047^{a}	1.185 ± 0.201^{a}	0.016 ± 0.001^{ab}	2.067 ± 0.794^{ab}
	B	0.496 ± 0.093^{a}	1.232 ± 0.163^{a}	0.020 ± 0.002^{ab}	2.591 ± 0.258^{ab}
	M	0.480 ± 0.070^{a}	1.098 ± 0.313^{a}	0.024 ± 0.004^{b}	2.037 ± 0.245^{b}
30	**Control**	1,543 ±0,258^{a,b}	4,605 ± 1,359^{a}	0,123 ± 0,032^{a}	12,979 ±1,771^{a}
	P	1,536 ±0,249^{a,b}	4,420 ± 1,680^{a}	0,146 ± 0,026^{a}	13,758 ±1,065^{a}
	B	1,825 ± 0,146^{a}	4,472 ± 1,418^{a}	0,155 ± 0,024^{a}	14,160 ±2,151^{a}
	M	1,258 ± 0,357^{b}	4,685 ± 1,309^{a}	0,157 ± 0,077^{a}	15,896 ±4,162^{a}
40	**Control**	0,550 ± 0,296^{ab}	1,591 ± 0,613^{a}	0,058 ± 0,032^{a}	3,454 ± 0,478^{a}
	P	0,408 ± 0,046^{a}	1,749 ± 0,140^{ab}	0,066 ± 0,012^{a}	5,799 ± 0,274^{b}
	B	0,753 ± 0,153^{ab}	1,364 ± 0,450^{a}	0,088 ± 0,021^{a}	5,678 ± 0,936^{c}
	M	0,840 ± 0,139^{b}	2,643 ± 0,385^{b}	0,043 ± 0,014^{a}	5,196 ± 0,563^{c}

Treatments sharing the same letters in the same column for each experimental period are not significantly different (p<0.05).

4. 1- Normas da Revista Latin American Journal of Aquatic Research

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