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JULIANA RANGEL DE AGUIAR INTERAMINENSE

"AVALIAÇÃO DE *Bacillus subtilis* e *Shewanella algae* NO ANTAGONISMO CONTRA *Vibrio* spp., PRODUÇÃO DE ENZIMAS E MODULAÇÃO DA RESPOSTA IMUNE DO CAMARÃO *Litopenaeus vannamei*"

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Tese apresentada ao programa de pós-graduação em ciências biológicas, área de concentração: biotecnologia da Universidade Federal de Pernambuco, como requisito para a obtenção do grau de doutora em ciências biológicas.

Orientador: Prof. Dr. Ranilson de Souza Bezerra.
Co-orientador: Dr. Diego de Souza Buarque.

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Aprovado em 27/06/2017

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A minha filha Lívia, o maior elo de amor que nasceu em minha vida.

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“Renda-se como eu me rendi. Mergulhe no que você não conhece como eu mergulhei, não se preocupe em entender, viver ultrapassa qualquer entendimento.”

Clarisse Linspector

RESUMO

O presente estudo teve como objetivo avaliar o potencial probiótico de isolados bacterianos oriundos do hepatopâncreas/estômago de *Litopenaeus vannamei*. *Bacillus subtilis* (IPA-S.51) e *Shewanella algae* (IPA-S.252 e IPA-S.111) foram inicialmente submetidas a testes de verificação de antagonismo contra *Vibrio alginolyticus* cedido pela UFSC e isolado de camarões doentes. O experimento foi constituído por cinco tratamentos: IPA-S.51, IPA-S.252, IPA-S.111, Probiótico Comercial e Controle, sem adição de bactéria. Um ensaio subsequente de verificação de antagonismo contra *Vibrio parahaemolyticus* ATCC 17802 também foi realizado utilizando as bactérias que obtiveram uma maior frequência de halos inibitórios durante o primeiro ensaio *in vitro* (IPA-S.51 e IPA-S.252) e um Controle. Os isolados bacterianos foram cultivadas em diferentes condições de pH, salinidade e períodos de cultivo (12, 24, 36, 48 e 60h). Por fim, um cultivo de juvenis de *L. vannamei* constituído por três tratamentos: IPA-S.51, IPA-S.252 e Controle foi realizado durante 60 dias. As bactérias foram ofertadas misturadas à ração durante todo o período de cultivo. Amostras de camarões foram coletadas após 15, 30, 45 dias e após desafio com *V. parahaemolyticus* via injeção e água de cultivo. A partir das amostras coletadas foi realizada a quantificação de bactérias heterotróficas totais e *Vibrio* spp. presentes no hepatopancreas, intestino e fezes dos camarões; a verificação da atividade das enzimas digestivas presentes no hepatopâncreas e a quantificação de genes de imunidade presentes na hemolinfa dos camarões. *B. Subtilis* demonstrou ter uma maior freqüência na produção de halos de inibição contra os patógenos testados. A análise de GLM revelou o tipo de probiótico e faixa de pH como as variáveis que mais influenciaram o tamanho dos halos. No experimento *in vivo*, a utilização de IPA- S.51 e IPA- S.252 diminuiu a carga de *Vibrio* no hepatopancreas. A administração de *B. subtilis* e *S. algae* proporcionou um aumento da imunidade de *L. vannamei*, uma vez que a mortalidade acumulada após 48 h de injeção de *Vibrio* foi menor para os camarões do tratamento IPA-S.252 e também menor depois do desafio através de imersão para ambos IPA-S.252 e IPA-S.51. Camarões que receberam *B. Subtilis* em sua alimentação obtiveram maiores níveis de expressão de proPO, LGBP e HEM antes e após desafio com *V. parahaemolyticus* via injeção. As atividades de proteases totais, tripsina e quimotripsina foram melhoradas e pareceram influenciar os parâmetros de crescimento que foram mais elevados para os camarões dos tratamentos IPA-S.252 e IPA-S.51. Além disso, os desafios com *V. parahaemolyticus* influenciaram principalmente os níveis de glicina aminopeptidase semelhante ao obtido para uma infecção por WSSV. Os resultados sugerem o potencial probiótico de *B. subtilis* e *S. algae* como ferramenta controlar a infecção por *V. parahaemolyticus* em juvenis de *L. vannamei* e também aumentar seu desempenho de crescimento.

Palavras-chave: Probiótico. *Litopenaeus vannamei*. *Bacillus subtilis*. *Shewanella algae.*

ABSTRACT

The present study aimed to evaluate the probiotic potential of bacterial isolates from *Litopenaeus vannamei* hepatopancreas / stomach. *Bacillus subtilis* (IPA-S.51) and *Shewanella algae* (IPA-S.252 and IPA-S.111) were submited to an antagonism against *Vibrio alginolyticus* provided by UFSC and isolated from moribund shrimps. The experiment consisted of five treatments: IPA-S.51, IPA-S.252, IPA-S.111, Commercial Probiotic and Control, without addition of bacteria. A subsequent test of antagonism against *Vibrio parahaemolyticus* ATCC 17802 was also carried out using bacteria that obtained a higher frequency of inhibitory halos during the first *in vitro* (IPA-S.51 and IPA-S.252) and a Control . The bacterial isolates were grown at different pH, salinity and culture periods (12 , 24, 36, 48 and 60h). Finally, a *L. vannamei* juveniles rearing consisting of three treatments: IPA-S.51, IPA-S.252 and Control was carried out for 60 days. The bacteria were offered mixed with the feed during the entire rearing period. Samples of shrimp were collected after 15, 30, 45 days and after challenge with *V. parahaemolyticus* via injection and rearing water. From the collected samples, the quantification of total heterotrophic bacteria and *Vibrio* spp. present in hepatopancreas, intestine and shrimp feces; the verification of digestive enzymes activities of hepatopancreas and the quantification of immunity genes present in the shrimp hemolymph were performed. *B. Subtilis* has been shown to have a higher frequency in the production of inhibitory halos against the pathogens tested. The GLM analysis revealed the probiotic type and pH range as the variables that most influenced the halos size. In the *in vivo* experiment, the use of IPA-S.51 and IPA-S.252 decreased the *Vibrio* load on hepatopancreas. The administration of *B. subtilis* and *S. algae* provided an increase in L. vannamei immunity, since the accumulated mortality after 48 h of *Vibrio* injection was lower for the shrimps of IPA-S.252 treatment and also lower after the immersion challenge for both IPA-S.252 and IPA-S.51. Shrimps that received *B. subtilis* in their diet had higher levels of proPO, LGBP and HEM expression before and after challenge with *V. parahaemolyticus* via injection. Total proteases, trypsin and chymotrypsin activities were improved and appeared to influence the highest growth parameters registered for IPA-S.252 and IPA-S.51 shrimp treatments. Besides that, challenges with *V. parahaemolyticus* mainly influenced glycine aminopeptidase levels similar to that obtained from a WSSV infection. The results suggest the probiotic potential of *B. subtilis* and *S. algae* as a tool to control *V. parahaemolyticus* infection in *L. vannamei* juveniles and also to enhance their growth performance.

Keywords: Probiotic. *Litopenaeus vannamei*. *Bacillus subtilis*. *Shewanella algae*.

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LISTA DE ABREVIATURAS E SIGLAS

ATCC – American Type Culture Collection
Coleção de microrganismos Norte Americana

BApNA – Benzoyl-DL-arginine-p-nitroanilide
Benzoil arginina p-nitroanilida

CFU – Colony Forming Units
Unidades Formadoras de Colônias

DWG- Daily Weight Gain
Ganho de Peso Diário

GLM- Generalized Linear Models
Modelos Lineares Generalizados

HEM – Hemocyanin
Hemocianina

IPA- Instituto Agronômico de Pernambuco

IPA-S.51- Isolado bacteriano da espécie *Bacillus subtilis*

IPA-S.111- Isolado bacteriano da espécie *Shewanella algae*

IPA-S.252- Isolado bacteriano da espécie *Shewanella algae*

LGBP – Lipopolysaccharide and β-1,3-glucan-binding protein
Proteína de Ligação a Lipopolissacarídeo e β-1,3-glucano

proPO – Prophenoloxidase
Profenoloxidase

qRT-PCR – Quantitative reverse transcription polymerase chain reaction
Reação em cadeia da polimerase via transcriptase reversa quantitativa

SApNA– Succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide
Succinil-alanina-alanina-prolina-fenilalanina-p-nitroanilida ()

SGR- Specific Growth Rate
Taxa de Crescimento Específico

TCBS- Ágar Tiossulfato Citrato Bile Sacarose
Thiosulfate Citrate bile sucrose Agar

THB- Total heterotrophic bacteria
Bactérias Heterotróficas Totais

TSA- Tryptone Soy Agar
Ágar Triptona de Soja

TSB- Tryptone Soy Broth

Caldo Triptona de Soja

WGR- Weight Gain Rate

Taxa de Ganho de Peso

SUMÁRIO

1 INTRODUÇÃO

No início dos anos 1990, a introdução da espécie *Litopenaeus vannamei* revolucionou a carcinicultura marinha no Brasil (BARBIERI JUNIOR e OSTRENSKY NETO, 2002). A partir dessa década, o cultivo de camarões se tornou uma atividade importante e bastante rentável (BURGOS-HERNÁNDEZ et al., 2005). No entanto, a partir do ano de 2004, houve um declínio na carcinicultura, o que gerou uma queda na produção e na produtividade (ROCHA, 2007). As causas apontadas para justificar esse declínio dizem respeito a condições climáticas e, principalmente, à ocorrência de enfermidades virais e bacterianas em fazendas de cultivo instaladas (RODRIGUES, 2005).

Dentre as doenças bacterianas, destacam-se as vibrioses, as quais são causadas por bactérias patogênicas oportunistas do gênero *Vibrio*, que geram altas taxas de mortalidade em *L. vannamei* (BALASUNDARAM et al., 2012). A abundância natural de *Vibrio spp.*, assim como sua taxa de multiplicação e habilidade para adaptar-se a mudanças ambientais em sistemas aquícolas, ressaltam a importância da avaliação de seus efeitos patogênicos em camarões cultivados (SAULNIER et al., 2000).

Dentre as espécies de bactérias prejudiciais ao cultivo, destaca-se *Vibrio parahaemolyticus*, uma bactéria marinha Gram-negativo que afeta diversos órgãos dos camarões (YANAGIHARA et al., 2010). Uma das doenças causadas por *V. parahaemolyticus* é a Hepatopancreatite Necrosante Aguda (ANPND), a qual afeta algumas espécies de camarão, incluindo *L. vannamei* (AL-OTHRUBI et al., 2014). Essa doença causa nado errático, crescimento reduzido, amolecimento do exoesqueleto, anorexia, forte atrofia e palidez do hepatopâncreas (MEJÍAS e NAVARRO, 2014).

Nos últimos anos, algumas medidas vêm sendo tomadas para tentar controlar a infecção de camarões cultivados através de bactérias patogênicas, incluindo *V. parahaemolyticus*. Dentre estas medidas, os antibióticos têm sido utilizados para a desinfecção prévia do ambiente de cultivo e no tratamento de infecções causadas por patógenos bacterianos (AKINBOWALE et al., 2007). No entanto, esse método não se mostrou eficaz, uma vez que existe o risco dos antibióticos entrarem em contato com o meio ambiente e causar um aumento na resistência de patógenos bacterianos, através da transferência de genes de resistência entre esses microrganismos (YOUSEFIAN e AMIRI, 2009; AL-OTHRUBI et al., 2014). Além disso, os antibióticos podem ser introduzidos na alimentação humana através da ingestão de camarões tratados (CABELLO, 2006). Portanto, alternativas ao uso de antibióticos são de grande importância para o cultivo de camarões e para prevenir danos ao meio ambiente.

Dentre as alternativas utilizadas para minimizar os efeitos das vibrioses, tem sido bastante difundida a substituição de antibióticos por probióticos, que são microrganismos vivos que afetam beneficamente o hospedeiro através da melhoria de diversos aspectos da sua fisiologia, como por

exemplo, melhorar o crescimento e a sua resistência a patógenos (FULLER, 1989; WANG et al., 2007; BUGLIONE et al., 2008).

De uma forma geral, os probióticos têm sido isolados do trato digestivo dos camarões ou da água utilizada para o cultivo desses animais (CRUZ et al., 2012). Em muitos casos, as bactérias isoladas do trato digestivo fazem parte da microbiota dos camarões, as quais representam microrganismos altamente importantes para os processos digestivos e da imunidade de vários animais (LUIS-VILLASEÑOR et al., 2012).

Dentro do contexto abordado, uma compreensão sobre o efeito dos probióticos na digestão e na imunidade do camarão para o melhor controle das doenças e maior desenvolvimento sustentável dos camarões se torna uma importante questão científica nesta área (BACHÈRE, 2000; LIU et al., 2013). Diante do exposto, o presente estudo visa à caracterização de um microrganismo que influencie nos mecanismos envolvidos com a resposta imunológica e digestiva de *L. vannamei* com o objetivo de incrementar a sua resistência e desempenho zootécnico, promovendo uma carcinicultura mais sustentável.

2 REVISÃO DE LITERATURA

2.1 *Litopenaeus vannamei* E A CARCINICULTURA NO BRASIL

O cultivo de camarões marinhos no Brasil teve início na década de 1980 com algumas dificuldades referentes à adaptação climatológica da espécie exótica *Marsupenaeus japonicus***.** Mais tarde, a atenção do setor voltou-se para as espécies nativas (*Farfantepenaeus subtilis, F. paulensis, F. brasiliensis* e *Penaeus schimitti*) (ROCHA et al., 2004). No entanto, a revolução da carcinicultura no nosso país ocorreu com o camarão branco do pacífico *Litopenaeus vannamei*, anteriormente *Penaeus vannamei*, pertencente à família Penaeidae de crustáceos decápodes (PÉREZ-FARFANTE e KENSLEY,1997). Sabe-se que *L. vannamei* converte seu alimento mais rapidamente em ganho de peso e é mais rústico do que as espécies de camarão nativo, fatores importantes para a carcinicultura (WAKIDA-KUSUNOKI et al. 2011).

Com a evolução da produção do *L. vannamei* no Equador, o Brasil adotou o cultivo da espécie nos anos 1990 e, em pouco tempo, o camarão branco do Pacífico se destacou devido a sua capacidade de adaptação às mais variadas condições de cultivo, altas taxas de crescimento e sobrevivência, boa produtividade e grande aceitação no mercado (OSTRENSKY NETO, 2002). Nessa mesma década, os laboratórios nacionais passaram a dominar tecnologias relacionadas à reprodução e produção de pós-larvas, iniciando a distribuição comercial e intensificando as técnicas nas fazendas de camarão (LIMA, 2007). A maior valorização comercial do produto acabou por eleger o camarão branco *L. vannamei* dominante entre as espécies cultivadas em todo o mundo (GHAFFARI et al., 2014).

Segundo os dados da Associação Brasileira de Criadores de Camarão (ABCC), o Brasil tem uma área com potencial para carcinicultura estimada em 1.000.000 de hectares, utilizou apenas 25.000 ha (2,5%) em 2015, cuja produção de 76.000 t contribuiu para a geração de R$ 1,5 a 2,0 bilhões de reais e 50.000 empregos (ABCC, 2016). A ABCC afirma ainda que, regiões com grande potencial para carcinicultura situam-se principalmente em áreas tropicais do Nordeste. Entre os maiores produtores estão o Ceará e o Rio Grande do Norte, mas a atividade cresce também nos estados da Bahia, Pernambuco, Piauí e Sergipe (ABCC, 2016).

Apesar do elevado desenvolvimento da carcinicultura, esta atividade vem experimentando perdas significantes na produção, provocada por patógenos virais ou bacterianos. Dentre as doenças causadas por vírus, os seguintes patógenos em particular têm causado grandes problemas em fazendas de camarão no Brasil: o Vírus da Mancha Branca (WSSV), o Vírus da Infecção Hipodermal e Necrose Hematopoiética (IHHNV) (WICKINS e O'C LEE, 2002) e mais recentemente, o Vírus da Mionecrose Infecciosa (IMNV), mais encontrado em fazendas da Região Nordeste (REIS, 2008).

As doenças bacterianas são causadas principalmente por microrganismos do gênero *Vibrio*, especialmente nas fases de larvicultura e na engorda dos camarões (fase juvenil) (AGUIRRE-GUZMÁN et al., 2004). As bactérias do gênero *Vibrio* pertencem a ecossistemas aquáticos, apresentando origem marinha e/ou estuarina. Estes patógenos estão presentes em camarões de vida livre ou cultivados, sendo os principais responsáveis pela maior parte das patologias bacterianas em camarões. (VANDERZANT et al., 1971; RUANGPON e KITAO, 1991). As principais espécies do gênero *Vibrio* que representam risco para o cultivo dos peneídeos são: *V. alginolyticus, V. anguillarum, V. campbelli, V. carcharie, V. damsela*, *V. harveyi, V. ordalli, V. parahaemolyticus, V. salmonicida, V. splendidus* e *V. vulnificus* (RODRICK, 1991; LIGHTNER, 1996).

2.2 BACTERIOSES NA CARCINICULTURA

2.2.1 *Vibrio alginolyticus*

Vibrio alginolyticus é uma espécie bacteriana Gram-negativo, que possui larga distribuição geográfica e pode ser encontrada em ambientes marinhos e estuarinos (LARSEN et al., 1981; BARBIERI et al., 1999). O *V. alginolyticus* é um dos causadores da vibriose, também conhecida como “síndrome da gaivota”, que pode ser caracterizada como infecção localizada ou sistêmica afetando todos os órgãos e tecidos (NUNES e MARTINS, 2002; MORALES-COVARRUBIAS, 2008; ROQUE et al. 2001). A vibriose pode ser observada como uma infecção localizada, levando a formação de manchas pretas ou marrons em resposta a uma possível erosão na cutícula dos animais e também na forma sistêmica, afetando todos os órgãos e tecidos (ROQUE et al., 2001; GALLI et al., 2004).

De acordo com Bing et al. (1993) e Lee et al (1997 e 1999), *V. alginolyticus* é a espécie de *Vibrio* dominante nos estágios larvais de *L. vannamei* tanto saudáveis como doentes. Dentre os efeitos prejudiciais deste patógeno, as suas proteases secretadas atuam destruindo a cascata enzimática que controla a coagulação da hemolinfa do camarão. Além disso, a virulência de *V. alginolyticus* está correlacionada com a atividade da enzima colagenase, que pode provocar o amolecimento do tecido muscular dos camarões (BRAUER et al., 2003; YISHAN et al., 2011).

Um possível modo de infecção dos camarões por *V. alginolyticus* consiste em três passos básicos: (1) a bactéria penetra em pequenas aberturas ou feridas no exoesqueleto do hospedeiro por meio de motilidade quimiotáctica; (2) dentro dos tecidos do hospedeiro a bactéria implanta sistemas ferro – sequestrantes por meio de sideróforos que "roubam" ferro do hospedeiro e (3) a bactéria eventualmente danifica os organismos por meio de produtos extracelulares como, por exemplo, hemolisinas e proteases (THOMPSON et al., 2004).

2.2.2 Vibrio parahaemolyticus

V. parahaemolyticus é uma bactéria Gram-negativa que tem sido comumente associada a infecções em organismos aquáticos. Esta bactéria é halofílica, pertence naturalmente ao ambiente marinho e pode ser encontrada em todo o mundo. Além disso, representa uma grande preocupação para a saúde humana, pois esta espécie é a principal causadora de gastroenterite bacteriana associada ao consumo de frutos do mar contaminados em todo o mundo (DEPAOLA et al., 2003; GOPAL et al., 2005 ; ZIMMERMAN et al., 2007; TURNER et al 2013; VINOJ et al. 2014) . De acordo com Gomez-Gil et al. (2004), *V . parahaemolyticus* afeta principalmente camarões em fase juvenil e adulta e pode causar lesões intestinais e mortalidade em crustáceos em poucas horas.

Durante vários anos, grandes mortalidades de larvas de camarão também foram relatadas em todo o mundo devido à vibriose provocada pelo *V. parahaemolyticus* (LIGHTNER 1996; VASEEHARAN e RAMASAMY 2003). Embora relatos de mortalidade de grande porte serem atribuídos a doenças virais, doenças bacterianas ganharam recentemente importância após a identificação de certas estirpes de *V. parahaemolyticus* como o agente causador da Síndrome da Mortalidade Precoce (*Early Mortality Sindrome* - EMS), que causou perdas de grande escala na produção de camarão cultivado na China, Vietnã, Tailândia e Malásia (FAO , 2013; . TRAN et al , 2013) .

De acordo com Tran et al. (2013), camarões afetados pela EMS são acometidos por severa atrofia dos hepatopancreas juntamente com descamação massiva das células epiteliais do hepatopâncreas. Nos últimos anos, algumas medidas vêm sendo tomadas para tentar controlar a infecção de camarões cultivados através de bactérias patogênicas, incluindo *V. parahaemolyticus*. Dentre estas medidas está o uso de probióticos.

2.3 MICRORGANISMOS PROBIÓTICOS PARA CARCINICULTURA

O uso de probióticos dentro das fazendas de camarão tem crescido (FERREIRA et al., 2017). Quando administrados em doses adequadas, os probióticos podem fornecer benefícios na saúde do seu hospedeiro (ARAYA et al., 2002), tais como: aumentar os números de bactérias úteis a flora intestinal e também proliferar na água de cultivo e erradicar bactérias patogênicas através da competição pelo consumo de nutrientes (SAHU et al., 2008).

Os microrganismos probióticos são utilizados em todo o mundo e apresentam diversas funções, como a modulação da imunidade inata de organismos ajudando-os a resistir a agentes patogênicos (IBRAHEM, 2015). Na aqüicultura, os probióticos são um dos principais fatores nutricionais que podem influenciar na função gastrointestinal de organismos, realizando a competição por exclusão com bactérias patogênicas e produção de compostos inibitórios, por exemplo, (DIPLOCK et al., 1999). Esses microrganismos benéficos são também úteis para a

reciclagem de nutrientes e para a degradação de matéria orgânica, melhorando o meio ambiente aquático (MORIARTY et al., 1997). Em contrapartida, bactérias patogênicas propiciam a má qualidade da água, estresse e doenças para os organismos aquáticos, podendo agir como patógenos primários ou secundários (MOHNEY et al, 1994).

No intúito de se controlar as doenças causadas por microrganismos patogênicos de etiologia bacteriana, o uso de antibióticos na indústria da aquicultura tornou-se uma abordagem tradicional, apesar de levar a geração de microrganismos resistentes (CABELLO, 2006). Frequentemente, antibióticos são administrados em doses sub-terapêuticas para o incremento da produção animal ocasionando o aparecimento de bactérias resistentes, que colocam em risco o equilíbrio ambiental e a qualidade do produto ao consumidor (ZHOU, 1995; COOK et al., 2003; KIM et al, 2004; RYSZ e ALVAREZ, 2004; AKINBOWALE et al., 2006, 2007). Dessa maneira, as bactérias benéficas denominadas probióticos proporcionam uma abordagem alternativa para a utilização de antibióticos para fins de uma aquicultura sustentável (BALCAZAR et al., 2006; UTISWANNAKUL et al., 2011).

Quando administrados como suplemento em dietas para animais, os probióticos devem ser capazes de sobreviver e passar através do trato intestinal desses organismos. A aplicação de probióticos na dieta proporciona a criação de um ambiente hostil para patógenos pela produção de compostos inibitórios, competição por nutrientes essenciais e locais de adesão, impedindo a colonização dos patógenos no intestino, excluindo-os do trato intestinal. Além disso, é possível a obtenção e o fornecimento de nutrientes essenciais e enzimas que resultam numa maior nutrição dos animais cultivados. (GATESOUPE, 1999; GOMEZ-GIL et al., 2000; ROBERTSON et al. 2000 IRIANTO e AUSTIN, 2002; BALCAZAR et al., 2006; NAVINCHANDRAN et al, 2014).

Vários autores confirmam o uso benéfico de microrganismos probióticos aplicados na aquicultura. Chiu et al. (2007) concluíram que a administração de *Lactobacillus plantarum* na dieta de *L. vannamei* aprimorou capacidade de resposta imunológica aumentando a resistência a infecção por *V. alginolyticus* no camarão. Já Wang e Gu (2010) observaram um aumento do peso final em *L.vannamei* em diferentes tratamentos com adição de três cepas probióticas distintas, *L. acidophilus, Rhodopseudomonas palustris* e *Bacillus coagulans*. Mais recentemente, Kongnum e Hongpattarakere (2012) analisaram a adição de *L. plantarum* em dieta ofertada para *L. vannamei*. Os resultados demonstraram que a alimentação do camarão com a dieta suplementada propiciou uma ótima sobrevivência (98,89%) em comparação a dos grupos controle sem adição de bactérias (68,89%). A carga bacteriana total e de víbrios não fermentadores diminuiu nos grupos que receberam o probiótico. Após a infecção com *V. harveyi* a sobrevivência foi maior para os grupos com dieta suplementada (77%) em comparação com o grupo controle (67%).

2.3.1 *Bacillus subtilis*

Bactérias do gênero *Bacillus*, são Gram-positivas, aeróbias com forma de bastonete e formadoras de esporos. Além disso, são produtoras de antibióticos como metabólitos secundários (KÜMMERER, 2009). Espécies de *Bacillus* têm sido reportadas como produtoras de uma vasta gama de substâncias extracelulares e peptídeos antimicrobianos contra uma variedade de microrganismos (KORENBLUM et al., 2005; MORIKAWA et al., 1992; PEREZ et al., 1993). Os polipeptídeos produzidos por *Bacillus* spp. (bacitracina, gramicidina S, polimixina e tirotricina) são ativos contra uma ampla gama de bactérias Gram-positivo e Gram-negativo, especialmente *Vibrio* spp. (DRABLOS et al., 1999; MORIKAWA et al., 1992; PEREZ et al., 1993). Essas bactérias são comumente encontradas em sedimentos marinhos e são, portanto, facilmente ingeridas pelos camarões (MORIARTY, 1999).

A administração de estirpes de *B. subtilis* pode melhorar o crescimento e resistência a doenças estimulando genes ligados à resposta imunológica de *L. Vannamei*. Neste sentido, **g**enes ligados ao processo de melanização podem ser modulados por *B. subtilis* para uma maior proteção contra patógenos (ZOKAEIFAR et al. 2012).

2.3.2 *Shewanella algae*

Shewanella spp. são bactérias Gram-negativo, bacilos móveis, cuja característica fenotípica mais importante é a produção de sulfeto de hidrogênio (HOLT et al. 2005). Resultados com análise de 16S rRNA levaram a uma proposta de uma nova família, Shewanellaceae (IVANOVA et al., 2004), contendo cerca de 30 espécies para o gênero *Shewanella*, cuja maioria é formada por psicrófilos. Essa característica permite um relacionamento mútuo entre os peixes e bactérias que vivem no intestino e além disso são bactérias de pouco interesse para microbiologistas clínicos (HOLT et al., 2005; HAU e GRALNICK, 2007; SUKOVICH et al., 2010). De acordo com Winn et al. (2008), a maior parte das espécies são encontradas em habitats aquáticos e marinhos, podem crescer a temperaturas de 42°C, são capazes de reduzir nitrato e em geral são sensíveis à maioria dos agentes antimicrobianos efetivos contra bacilos Gram-negativos. Dessa maneira, estirpes de *Shewanella* estão sendo cada vez mais utilizadas como probióticos na aquicultura (JIANG et al. 2013 e LOBO et al. 2014).

Algumas pesquisas têm demonstrado os benefícios de *S. algae* para camarões cultivados. Dentro deste contexto, tem sido demonstrado que ração suplementada com *Shewanella algae* aumentou a sobrevivência de *L. vannamei* submetido à infecção com *Vibrio harveyi* (SUANTIKA et al., 2013). Além disso, camarões alimentados com ração suplementada com *S. algae* também apresentaram resposta antibacteriana em uma ampla faixa de temperatura e pH com níveis condizentes aos da faixa ideal para cultivo de camarões (SHAKIBAZADEH et al. 2010).

2.4 SISTEMA IMUNE DE *L. vannamei*

O sistema imune inato ou natural dos crustáceos está intimamente relacionado à hemolinfa, que consiste de uma fração representada pelas células circulantes ou hemócitos e de uma fração líquida, constituída pelo plasma e os fatores humorais nele dissolvidos (BARRACO, 2008). Entretanto, o camarão também dispõe de barreiras físico-químicas contra microrganismos estranhos, como a quitina, um polímero β-1,4 ligante da N-acetil-D-glucosamina, um dos mais abundantes polissacarídeos na natureza e um componente comum do exoesqueleto dos insetos, carapaça de crustáceos e paredes das células fúngicas (ESTEBAN et al., 2000).

Os hemócitos são geralmente classificados em três tipos: hialino, semi-granular e grandes células granulares (TSING et al., 1989). Depois que patógenos são reconhecidos pelas proteínas de reconhecimento padrão (PRPs) presentes nos hemócitos, estes são ativados e inicia-se o processo de melanização mediada pelo sistema da pró-fenoloxidase e também a estimulação da fagocitose e formação de nódulos que protegem os crustáceos de infecções (SODERHALL et al., 1992; VARGAS-ALBORES e YEPIZ-PLASCENCIA, 2000; YANG et al. 2015). Além disso, outros mecanismos também participam do sistema de imunidade inata dos camarões: aglutinação celular mediada por lectinas; formação de peptídeos antimicrobianos(AMPs) e produção de espécies reativas de oxigênio e de nitrogênio; (IWANAGA e LEE, 2005; YANG et al. 2015).

Como os invertebrados têm um sistema circulatório aberto, após uma lesão, apresentam mecanismos eficientes que evitam a perda de hemolinfa rapidamente e também ajudam no aprisionamento de micróbios, impedindo que os mesmos se espalhem pela hemocele do organismo (JOHANSSON et al., 1999; THEOPOLD et al., 2002). Dois diferentes mecanismos de coagulação são reconhecidos em invertebrados, (THEOPOLD et al., 2004; JIRAVANICHPAISAL et al., 2006). O primeiro é uma cascata proteolítica ativada por componentes microbianos, como LPS e β-1,3-glicanas (KAWABATA et al., 1996) e o segundo é uma reação de coagulação dependente de transglutaminase (TGase), ocorrendo em insetos e crustáceos (BARRACO et al. 2008).

Outro processo importante para a defesa dos camarões é a melanização, a qual é desencadeada pela ativação do sistema da profenoloxidase (proPO). Por sua vez, este sistema é estimulado pelo reconhecimento de moléculas na hemolinfa dos invertebrados (SÖDERHÄLL e CERENIUS, 1998). A enzima ativadora da profenoloxidase é uma serino protease (ppA) (PERAZZOLO e BARRACCO, 1997). A fenoloxidase (PO) é a enzima terminal do sistema proPO, enquanto que ppA é ativada por vários polissacáridos microbianos, incluindo b-1,3-glucano e lipopolissacárido (LPS) das paredes celulares de bactérias e fungos (SMITH et al., 1984). O sistema de proteínas de reconhecimento não específico inclui a proteína de ligação β- 1,3 - glucano e proteína de ligação a LPS,as quais reconhecem e respondem à presença de particulas estranhas e têm sido

relatadas em várias espécies de crustáceos, incluindo camarões peneídeos (VARGAS-ALBORES e YEPIZ-PLASCENCIA, 2000; CHENG et al. 2005).

Outros componentes importantes na supressão da proliferação de microrganismos na hemolinfa dos invertebrados são os peptídeos antimicrobianos e as lectinas (WANG e WANG, 2015). Após reconhecimento dos agentes patogênicos, as lectinas podem funcionar como aglutininas, opsoninas e moduladores da expressão gênica (WANG e WANG, 2015). Por exemplo, uma lectina de *Fenneropenaeus chinensis,* pode ser secretada para a hemolinfa e funcionar como um receptor padrão de reconhecimento e também efetor na imunidade inata do camarão (SUN et al., 2008).

Em relação aos peptídeos antimicrobianos, os mesmos compartilham certas características comuns, tais como tamanho pequeno, caráter catiônico e semelhanças nos padrões estruturais ou motivos (BACHÈRE et al, 2004 ; JENSEN et al, 2006). Esses peptídeos tem uma atividade antimicrobiana contra bactérias Gram positivo e negativo, fungos filamentosos, e em alguns casos, vírus e protozoários (BACHÈRE, 2003). A resposta imune dos camarões também envolve as reações celulares de defesa compreendem essencialmente a fagocitose de microrganismos, a formação de cápsulas e nódulos, a infiltração hemocitária e a formação de armadilhas extracelulares de ácidos nucleicos (BARRACCO et al., 2014). A ativação dessas respostas pode acionar diferentes vias imunológicas de sinalização celular que levam à produção e liberação de moléculas citotóxicas, como espécies reativas de oxigênio e nitrogênio, enzimas hidrolíticas (lisozimas, proteases, lipases, entre outras), etc (BARRACCO et al., 2014).

Além das respostas antimicrobianas, os camarões contam ainda com mecanismos antivirais de defesa. O primeiro relato de uma defesa antiviral em camarões foi descrito por Robalino et al., (2004; 2005) e trabalhos posteriores apontam que esse processo é mediado por três mecanismos: apoptose celular e autofagia, produção de citocinas do tipo interferon, análogas aos interferons de mamíferos e o sistema de RNA de interferência (RNAi) (PERAZZOLO et al., 2012).

2.5 SISTEMA DIGESTÓRIO DE *L. vannamei*

O sistema digestório dos crustáceos possui as funções de ingestão, transporte dos nutrientes, mecânica da digestão, hidrólise, absorção, armazenagem dos nutrientes e transporte das fezes. A sua estrutura compreende a boca, intestino anterior, intestino médio e intestino posterior (CECCALDI, 1997). O trato digestório dos camarões também está relacionado com a resposta imunológica desses animais, uma vez que este é revestido por uma camada quitinosa e repleto de enzimas, capazes de inativar e digerir a maioria dos microrganismos que não fazem parte de sua microbiota natural (BARRACO et al., 2014).

Dentre as enzimas digestivas de organismos aquáticos estudadas, as proteases são as que possuem maior destaque na literatura internacional, principalmente porque as proteínas são nutrientes indispensáveis para a estrutura funcional dos camarões. Uma vez que as proteínas estão sendo continuamente usadas para crescimento e reparo de tecidos, um contínuo suplemento de proteínas ou seus aminoácidos constituintes são necessários (SHIAU, 1998).

As proteases digestivas mais importantes são tripsina, quimotripsina e aminopeptidases. A tripsina e a quimotripsina são endoproteases, ou seja, clivam as ligações peptídicas dentro da proteína (Figura 1), enquanto que aminopeptidases são exoproteases, isto é, clivam resíduos de aminoácidos na posição N-terminal da proteína (GONZALES & ROBERT-BAUDOUY, 1996).

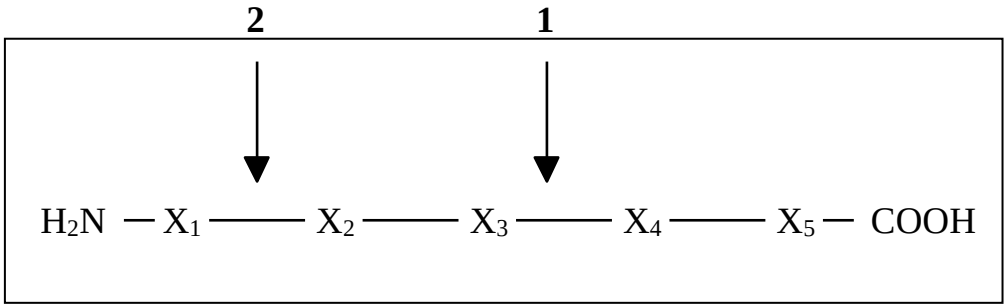

Figura 1: Classificação das proteases: Endoproteases clivam ligações peptídicas dentro da proteína (1). Exoproteases, mais especificamente as aminopeptidases, clivam resíduos localizados na posição N-terminal da proteína (2). Aminoácidos (X). Figura modificada de GONZALES & ROBERT-BAUDOUY (1996).

Internamente, o sistema digestório dos camarões possui um curto esôfago e estômago bastante reduzido (proventrículo). No estômago, observam-se revestimentos quitinosos com elementos calcários, alguns formando dentes que constituem o moinho gástrico. A glândula digestiva (hepatopâncreas) atua na produção de enzimas utilizadas na degradação química do alimento (PURINA, 2000). O hepatopâncreas de crustáceos é constituído por células secretoras de zimogênios que são responsáveis pela digestão extracelular, enquanto as células digestivas estão envolvidas na absorção, digestão intracelular e transporte de nutrientes (CECCALDI, 1997).

O isolamento e seleção de bactérias probióticas do trato digestório evita a introdução de bactérias exóticas no sistema de cultivo e demonstra a capacidade desses micorganismos ingressarem no hepatopâncreas e não serem afetados pela atividade enzimática deste. Além disso, a verdadeira colonização do probiótico acontece quando os microrganismos administrados continuam se multiplicando mesmo depois de muito tempo, passando a fazer parte da flora intestinal do hospedeiro (TORO, 2005; FERREIRA, 2012). Uma seleção criteriosa por bactérias probióticas

deve avaliar os métodos de colonização, a capacidade de competição contra patógenos e crescimento imunoestimulatório eficiente em camarões (GULLIAN et al., 2004).

Dentre os efeitos benéficos dos probióticos no sistema digestório dos camarões, destaca-se participação destas bactérias na produção de enzimas extracelulares como peptidases e lipases (OCHOA-SOLANO e OLMOS-SOTO, 2006). Neste contexto, Zhou et al. (2009) encontraram um aumento na atividade da amilase, lipase e peptidase em larvas de *L. vannamei* tratadas com *Bacillus coagulans* e especulou que um consequente aumento da digestão e absorção do alimento pode ter contribuído para o incremento na sobrevivência. Portanto, mediante os efeitos benéficos dos probióticos, torna-se importante avaliar a eficácia de diferentes espécies de bactérias na melhoria dos processos digestivos e imunes de *L. vannamei*.

3. OBJETIVOS

3.1 GERAL

Avaliar o efeito da adição de *S. algae* e *B. subtilis* na dieta de juvenis de *L. vannamei* com o intuito de melhorar seu crescimento, imunidade e produção de enzimas digestivas e contribuir no conhecimento da interação de camarões com probióticos, no que diz respeito aos aspectos sobre a proteção contra a bactéria patogênica *Vibrio parahaemolyticus.*

3.2 ESPECÍFICOS

1. Avaliação *in vitro* da inibição de *S. algae e B. subtilis* contra cepas patogênicas de camarões (*Vibrio* spp.);
2. Quantificar a carga bacteriana (*Vibrio* spp. e heterotróficas totais) do intestino, hepatopâncreas e fezes de *L. vannamei* cultivados com a adição de *S. algae* e *B. subtilis* em sua alimentação;
3. Analisar a atividade de proteases, peptidases e amilases digestivas antes e após desafio com *Vibrio parahaemolyticus* via injeção e água de cultivo no extrato do hepatopâncreas de *L. vannamei* cultivado com *S. algae* e *B. subtilis* em sua alimentação;
4. Avaliar o desempenho zootécnico de *L. vannamei* cultivado com *S. algae* e *B. subtilis* em sua alimentação;
5. Analisar a modulação da expressão de transcritos relacionados ao sistema imune de *L. vannamei* cultivado com *S. algae* e *B. subtilis* em sua alimentação;
6. Analisar a modulação da expressão de transcritos relacionados ao sistema imune de *L. vannamei* tratados com *S. algae e B. Subtilis* e posteriormente infectados com *Vibrio parahaemolyticus* via injeção e água de cultivo.

ARTIGO 1

***In vitro* and *in vivo* potential probiotic activity of *Bacillus subtilis* and *Shewanella algae* for use in *Litopenaeus vannamei* rearing.**

Submetido ao periódico Aquaculture

In vitro and *in vivo* potential probiotic activity of *Bacillus subtilis* and *Shewanella algae* for use in *Litopenaeus vannamei* rearing

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Abstract

Shrimp farming intensification has led to problems concerning the use of chlorination and antibiotics. An alternative solution is the use of probiotics to control pathogens. In this regard, the aim of this work was to investigate the antimicrobial activity of three candidate probiotic strains: *Bacillus subtilis* (IPA-S.51), *Shewanella algae* (IPA-S.252 and IPA-S.111) isolated from *Litopenaeus vannamei* against the pathogenic bacteria *Vibrio alginolyticus* and *Vibrio parahaemolyticus*. IPA-S.51 and IPA-S.252 were added in the feed and then offered to *L. vannamei* juveniles during 45 days. A commercial probiotic (CP) was used as a positive control in the first *in vitro* assay and *in vivo* experiment. Shrimp hepatopancreas, intestine and feces were collected every week for total heterotrophic bacteria and TCBS quantification. A General Linear Model analysis revealed the probiotic type and pH as the most important variables that influenced candidate inhibitory effect against vibrios. IPA-S.51 produced a greater inhibitory frequency against *V. alginolyticus* and *V. parahaemolyticus*. In the *in vivo* experiment, IPA-S.51 and IPA-S.252 decreased the *Vibrio* load in the hepatopancreas. It is suggested that this load was shifted to the intestine and ultimately eliminated through feces. Moreover, shrimp fed with IPA-S.51 presented greater final weight, weight gain rate and daily weight gain than the Control and IPA-S.252. Overall, our results showed that *B. subtilis* strain administration significantly improved shrimp growth and could develop in shrimp hepatopancreas and intestine. Furthermore, feed supplemented with *S. algae* and *B. subtilis* strains could also control *Vibrio* load in the *L. vannamei* hepatopancreas.

Keywords: *Bacillus subtilis*; *Shewanella algae*; probiotic; inhibition; *Vibrio* spp.; *Litopenaeus vannamei*.

1. Introduction

Shrimp farming is one of the main aquacultural activities in many countries, accounting for about 3,496,000 tons of shrimp and nearly U$15 billion in profits annually (FAO, 2012). In this

industry, the whiteleg shrimp *Litopenaeus vannamei* is the main marine species reared in Brazil, as well as in other developing countries (FAO, 2014). Owing to the global rising production of shrimp from aquaculture and the relative decline in their price, annual per capita availability of crustaceans grew substantially from 0.4 kg in 1961 to 1.8 kg in 2013 (FAO, 2016). In recent years, shrimp farming has decreased (FAO, 2016), mainly due to diseases and deteriorating environmental conditions, which have caused serious economic losses (Balcázar et al., 2006). The shrimp industry has experienced severe set-backs as a result of the frequent outbreaks of epidemic diseases, particularly caused by *Vibrio* spp. and/or viruses (Zheng and Wang, 2016).

Among the approaches used to control pathogens, antibiotics were introduced into shrimp farming to limit microorganism development (Balcázar et al., 2006). However, it has been demonstrated that pathogenic bacteria have developed antimicrobial resistance following antibiotic administration (Cabello, 2006). An alternative treatment is the use of probiotics or beneficial bacteria that control pathogens. Probiotics for aquaculture are generally defined as a set of live microorganisms which, when consumed in adequate amounts, provide a health benefit to the host by competing with pathogenic bacteria through exclusion and modification of the microbial flora associated with the host and its environment. Probiotics also contribute to enzymatic digestion and subsequent improved feed utilization by the animal and/or increased immune response against pathogens (Verschuere et al., 2000; Balcázar et al., 2006).

Among probiotic bacteria, the introduction of *Bacillus subtilis* has shown very promising results in shrimp farming. These bacteria have been used to improve the growth performance, health and disease management of *L. vannamei* (Zokaeifar et al., 2012). *Shewanella algae* is also being increasingly used as a probiotic in aquaculture since it is able to inhibit or reduce the pathogenicity of *Vibrio harveyi* in whiteleg shrimp farming (Jiang et al., 2013).

The ongoing study was carried out to evaluate the *in vitro* antimicrobial activity of *Bacillus subtilis* and *Shewanella algae* and to quantify *Vibrio* spp. load in the gastrointestinal tract of reared juveniles of *L. vannamei* given feed supplemented with *B. subtilis* and *S. algae*. Therefore, this

work aims to understand the potential of the use of specific probiotic bacterial strains to improve conditions in shrimp farming.

2. Materials and methods

2.1. Probiotic candidates and pathogens

The candidates of shrimp probiotic strains were obtained from the Instituto Agronômico de Pernambuco (IPA). *Bacillus subtilis* (IPA-S.51) and two, previously isolated *Shewanella algae* strains: IPA-S.252 from the hepatopancreas/stomach of reared *L. vannamei* and IPA-S.111 from the water used to rear *L. vannamei* with a salinity of 30g/L. These isolates were previously screened for proteolytic and amilolytic activities. Among the strains that showed protease and/or amylase production, IPA-S.51, IPA-S.252 and IPA-S.111 were selected and subsequently their 16S rRNA gene region was sequenced with fD1 (forward) (5'- AGAGTTTGATCCTGGCTCAG-3') and rD1 (reverse) (5'- AAGGAGGTGATCCAGCC-3'). The sequences were compared with GenBank of the National Center for Biotechnology Information (NCBI) through the BLASTn program. A Commercial Probiotic (CP) was also studied as a positive control. CP contained spores of *Bacillus subtilis*, *B. licheniformis* and *B. pumilus* at a concentration of $5x10^{10}$ Colony Forming Units (CFU)·g^{-1}.

The shrimp pathogens, *Vibrio parahaemolyticus* (ATCC 17802), *V. vulnificus* (ATCC 27562) and *V. alginolyticus* strains, were obtained from the Universidade Federal de Santa Catarina (Laboratório de Camarões Marinhos Cultivados- Brazil).

2.2. First *in vitro* assay

V. alginolyticus was cultured overnight at 30°C in Tryptic Soy Agar (TSA) (Himedia Laboratories, Mumbai, India) supplemented with 2% NaCl. Subsequently, the bacteria turbidity was adjusted to 0.5 MacFarland standard (10^8 CFU mL^{-1}) in a saline solution (2% NaCl). Petri dishes with TSA (2% NaCl) was previously prepared with the aid of sterilized Pasteur pipettes before the

saline solution with *Vibrio* was spread over the TSA with a swab. The culture medium and its NaCl concentration were according to Thompson et al. (2004).

B. subtilis, *S. algae* and CP strains were grown in Tryptic Soy Broth (TSB) for different time periods: 12, 24, 36, 48 and 60 hours at 30°C. The CP was added on TSB (2% NaCl) (Himedia Laboratories, Mumbai, India) at a ratio of 2 g L^{-1}, according to the manufacturer's recommendations. One milliliter of the probiotic candidates and CP culture was centrifuged at 4320 x*g* for 15 minutes to obtain a cell-free extract (culture supernatant fluid). The bacterial cell biomass formed was suspended and homogenized in sterile 200µL of TSB medium. A 50 µL of the cell-free extract and the cell biomass were added into different wells of an agar plate previously seeded with *V. alginolyticus*. The same aliquot of TSB medium was added into a well as a control. The experiment was done in triplicate and the plates were incubated at 30ºC for 24h. Pathogen inhibition was determined by the inhibitory halo produced around the well containing probiotic candidates and CP. The diameter of the clear zones around the wells were measured with a digital caliper.

An estimated 100 µL aliquots of serial dilutions (1:10) v/v of probiotic candidates and CP in sterile saline (2% NaCl) were plated onto TSA with 2% NaCl and incubated at 30°C for 24h. After the incubation period, the quantification of colony forming units (CFU) was performed.

2.3. Second *in vitro* assay

The second *in vitro* assay protocol did not use the cell-free-extract, as the IPA-S.51 cell biomass fraction tended to produce the largest clear zones. The antagonism production frequency was also greater with different incubation periods for the cell biomass fraction.

The candidate probiotics were cultured separately in a set of 12 tubes with 40 mL of TSB. A set of three TSB media tubes was supplemented with different NaCl concentrations (0, 10, 20 and 30g L^{-1} of NaCl) in combination with different levels of pH (5.0; 7.0 or 9.0). Each tube of the three sets was incubated for a specific incubation time (12, 24, 26, 48 and 60 h) at 30ºC. For this purpose, 10 mL of culture was centrifuged at 1800 x*g* for 15 min and then suspended in 200 µL of TSB with the same pH and NaCl culture concentration. After that, 30µL of the suspended cell biomass were

added to the wells. Petri plates with TSA media supplemented with 2% NaCl were previously prepared and inoculated with *V. parahaemolyticus* (10^8 CFU mL^{-1}). The assays were performed in triplicate for each bacterial culture condition. Petri plates were incubated for 24h at 30ºC and then the inhibitory zone was measured as describe above. The same well diffusion method was also used for further assessment of candidate probiotics against *V. vulnificus*, however only the optimal culture conditions were used for them. The CFU mL^{-1} number of *B. subtilis* and *S. algae* strains used in the *in vitro* assay was also estimated.

2.4. *In vivo* experiment

L. vannamei post larvae (PL10) were obtained from a commercial hatchery (Aquatec®) and transported to LTA/UFRPE (Laboratório de Tecnologia em Aquicultura/ Universidade Federal Rural de Pernambuco). The post larvae were maintained in a 1000 L tank, with seawater (23g L^{-1}) and continuous aeration until reaching a weight of 1.05 ± 0.25 g and then stocked in (width x length x height = 0.48 m x 0.56 m x 0.89 m) polypropylene tanks containing 50 L of seawater (23g L^{-1}). This seawater had been previously disinfected with sodium hypochlorite (40 ppm) for 24 h. Then, the seawater was dechlorinated with ascorbic acid (1g $1000L^{-1}$). The seawater was also filtered in 50, 25, 5 and 1 micron filters, received an ultraviolet light treatment and then maintained with continuous aeration. The average water temperature was about 28-29ºC.

The stock density was 130 shrimp m^{-2} and 25% of the water was renewed daily. During the entire experiment (45 days), the shrimp were fed four times per day, an amount equivalent to 10% of the biomass of each tank using shrimp feed with 40% crude protein (Camaronina® 40CR2, Purina, Agribrands do Brasil S. A., São Paulo, Brazil).

2.4.1. Bacterial addition in feed and experimental design

The antibacterial activity observed through *in vitro* analysis revealed IPA-S.51 and IPA-S.252 strains as candidates for *in vivo* experiments and, thus, they were added to the feed. CP was also added to the feed and was used as a positive control. Cultures of IPA-S.51, IPA-S.252 and CP (growth in 40 mL of TSB containing 2% NaCl during 48h) were incubated at 30°C. After that, the

cells were harvested by centrifugation (1800 *xg* for 20 min), washed twice with sterilized seawater and re-suspended in 40 mL of seawater for addition in the Camaronina® feed (adapted from Jiang et al., 2013).

At the end of the feed preparation, the bacterial final concentration in the mixture was about 10^6 CFU g^{-1} for IPA-S.51, IPA-S.252 and CP. After preparation, the feed was stored for a period not exceeding 72 hours at 4°C until being used. Finally, the shrimp were fed the Camaronina® feed supplemented with IPA-S.51, IPA-S.252 or CP (four replicates each). The control group consisted of shrimp fed Camaronina® without bacterial addition.

Growth parameters were recorded at the end of 45 days of rearing including survival rate (*final number* of *shrimp*/*initial number* of *shrimp*) x 100), final weight, weight gain rate [WGR = (final weight - initial weight) x 100)], specific growth rate [SGR= (ln final weight - ln initial weight) / rearing days x 100] and daily weight gain [DWG= (weight gain / days of rearing)] of whiteleg shrimp juveniles.

2.4.2. Bacterial quantification of shrimp tissues and feces

Bacterial quantification was performed in triplicate. Four juvenile shrimps were sampled from each tank (n=4) for each group and individually washed in 70% alcohol to eliminate external bacteria. Sixteen hepatopancreas and intestine samples were examined from each shrimp group at different time periods: 7, 14, 21, 28 and 35 days of rearing. For the feces examination, two shrimp of each treatment reared for 7, 14, 21, 28 and 35 days were maintained in 1.5 L of disinfected seawater (in triplicate) and the feces were sampled with an automatic pipette. After that, the tissues and feces were asseptically macerated and separately homogenized in saline solution (2% NaCl) and diluted serially (1:10). Then, 100 µL of each dilution was plated onto TSA (2% NaCl) for total heterotrophic bacteria (THB) quantification and thiosulfate bile sucrose (TCBS, Himedia agar) (Himedia Laboratories, Mumbai, India) for presumptive *Vibrio* spp. quantification. All petri dishes were incubated at 30°C for 24 h. THB and *Vibrio* spp. Colony Forming Units (CFU) were calculated as CFU g^{-1} in the hepatopancreas, intestine and feces separately.

2.5. Data analysis

Data sets with an excessive amount of zero values are often overly dispersed and cannot be modeled with a single conventional probability model (e.g. normal). Hurdle models are an alternative to analyze such data. In this approach, two models could be fitted to the data, one for the positive and null values, and another only for the positive values. Gamma distribution could be used to model the positive values. Because gamma distributions are exponential components, the Generalized Linear Model (GLM) framework is an alternative to analyze the data. In matrix notation the model is $g[E(Y)] = X\beta$, where Y is the vector of the response variable (e.g. diameter of halo), X is the design matrix of the explanatory variables (e.g. time, pH), β is the vector of parameters, $E(\,)$ is the expectation, and $g[\quad]$ is the link function. Logarithmic function was used to model the positive values using gamma density distribution.

The explanatory variables used were the candidate probiotics (IPA-S.51, IPA-S.252 and IPA-S.111), bacterial fractions (cell biomass or cell-free extract), rearing (culture) time, pH, NaCl concentration and CFU (Colony Formirng Units) of bacterial culture. The effects of the explanatory variables on the expectations of diameter of halos of the second *in vitro* assay are shown to use the predictions of diameter of halo means as calculated using the GLM fitted to the data. R software was used in the analyses.

Significant differences between inhibitory zones and CFU quantifications of the probiotic candidates in the first and second *in vitro* assays were tested by Two-way and One-way analysis of variance (ANOVA), respectively. The *post-hoc* Tukey test was used for both experiments. One-way ANOVA followed by Tukey was used to determine significant differences in shrimp survival and growth data (means ± standard error). Significant differences between bacterial quantification in shrimp tissues (means ± standard error) were performed by One-way ANOVA followed by the *post-hoc* Fisher test. These statistical analyses were performed with Origin 8.0 software.

3. Results

3.1. First *in vitro* assay

The inhibitory zones of candidate probiotic and CP strains against *V. alginolyticus* are recorded in Table 1. The antimicrobial activity was observed for all strains tested. However, the clear zones did not appear at all culture periods. The percentages of zeros (absence of inhibition) were 65% and 75% in the experiments with cell biomass and cell-free extracts, respectively.

The inhibitory halos produced by the 12 h cultures of CP (cell-free extract) and IPA-S.252 strains were similar. At 24h, only the IPA-S.111 cell biomass fraction produced an inhibitory halo, whereas the clear zone diameter at 36h was observed only for IPA-S.51and IPA-S.252 cell biomass fractions (17.4 ± 0.2 mm and 10.9 mm, respectively). The inhibitory halo produced by the IPA-S.51 cell biomass fraction was larger than the inhibitory halo produced by CP (cell biomass and cell-free extract) at 60h.

IPA-S.51 produced more clear zones during the final 24h of the total bacterial growth period. On the other hand, IPA-S.252 and IPA-S.111 produced more of these zones during the initial 24h of the bacterial growth period. It was also observed that IPA-S.51 and CP were able to produce inhibitory halos at lower CFU concentrations than IPA-S.252 and IPA-S.111 (Table 2).

The bacterial growth at 12 and 24h was higher in IPA-S.252 than in IPA-S.51 and CP (Table 2). IPA-S.111 CFU mL^{-1} cells were 6.9 x 10^8 at 24h, when it produced an inhibitory halo from the cell-free extract. The largest clear diameters were observed for IPA-S.51 cell-free extract and cell biomass at 48 and 60h (Table 1) with CFU concentrations of 280.6 and 0.5 x 10^8 CFU mL^{-1}, respectively.

According to the GLM analysis, the CFU variable did not converge when it was included as an explanatory covariate nor was the response variable of the proposed model significantly influenced by the CFU data. The probiotic is the most important variable to explain the variability in halo diameters. The multiple determination coefficient (R^2) for the first *in vitro* assay responses was 0.89. This indicates that the model was adequate to describe the influence of the variables studied on the inhibitory zone produced by candidate probiotics against *V. alginolyticus*.

3.2. Second *in vitro* assay

The antimicrobial activity of candidate probiotic strains was also observed against the pathogenic bacteria *Vibrio parahaemolyticus*. In this regard, IPA-S.51 was effective against *V. parahaemolyticus* at the pH range tested. Moreover, at 12h of bacterial growth, IPA-S.51 also produced antagonism towards the pathogenic bacteria in a wide range of salinities: 0 to 30g L^{-1} in acid and neutral pH, 0 to 20 g L^{-1} in basic pH. A similar antagonistic activity was observed at 36h: 0 to 30g L^{-1} only in acid and neutral pH.

In the last periods of IPA-S.51 bacterial growth (48 and 60h), there were no significant differences among the halo sizes, however, larger diameters were observed during the 48 h of growth for all species: 14 to 20 mm for IPA-S.51, 15 to 24 mm for IPA-S.252 and 19 mm for IPA-S.111. IPA-S.252 and IPA-S.111 were effective against *V. parahaemolyticus* only at 48h of growth in neutral pH and in 0 and 30gL^{-1} of NaCl, respectively.

The CFU numbers at 48h of bacterial growth (when the greatest antagonism activity was observed) were 7.1 x 10^7 CFU mL^{-1}, 9.6 x 10^7 CFU mL^{-1} and 1.4 x 10^9 CFU mL^{-1} for IPA-S.51, IPA-S.252 and IPA-S.111, respectively. There were no significant differences among the CFU mL^{-1} means throughout the bacterial growth period (data not shown).

The model did not converge when CFU was included as an explanatory covariate. Both probiotic and pH were the most significative variables to explain the response variable (diameter of halos). The experimental data with R^2 value for the responses of *in vitro* antagonistic effect against *V. parahaemolyticus* was 0.93, thus the model was adequate to describe the influence of variables on the inhibitory zone produced by the candidate probiotic.

The experimental data were used to calculate the predicted values of inhibitory halos produced by candidate probiotics against *V. parahaemolyticus* (Figure 1). The figure indicates that most of the positive responses for IPA-S.51 were observed at the beginning of the bacterial culture (before approximately 25h of cultivation). The larger diameters of halos were registered at 0 and 10 g L^{-1} of NaCl, (14 and 16 mm, respectively). At 20 g L^{-1} and 30 g L^{-1} the largest halos registered

presented 12mm diam. In general, the inhibitory effect tendency was the reduction of halo diameter after 25h until 60h, when the diameter began to be larger. For IPA-S.252 and IPA-S.111 the inhibitory effects were similar. Both strains produced antagonistic activity only at 48h of cultivation (Figure 1) and the inhibitory reactions were detected only in extreme salinities (0 and 30 g L^{-1}). Both *S. algae* strains produced greater inhibition in neutral pH, but IPA-S.252 also exhibited antagonism at pH 5 and a salinity of 30 g L^{-1}.

The best *in vitro* bacterial growth conditions for candidate probiotics that produced the largest inhibitory halos against *V.parahaemolyticus* were also tested in an *in vitro* assay against *V. vulnificus.* Only IPA-S.51 was effective in inhibiting *V. vulnificus* in basic pH and 20g L^{-1} of NaCl. The inhibitory diameter ± Standard Error (SE) was 10 ± 1mm and the CFU number was 3.5 ± 0.3 x 10^7 CFU mL^{-1} (data not shown).

3.3. Bacterial quantification of shrimp tissues and feces

The TCBS counts in juvenile shrimp hepatopancreas was higher for IPA-S.51 in the first and fourth rearing weeks, for IPA-S.252 in the first and third weeks and the Control in the fifth rearing week (Table 3). In the fifth rearing week, the *Vibrio* spp. quantification in hepatopancreas was smaller for IPA-S.51 and IPA-S.252. Therefore, a decrease of TCBS counts was observed in the fifth rearing week compared to the first week in both IPA-S.51 and IPA-S.252, while the TCBS quantification in the Control increased.

IPA-S.252 had an increase in the intestine TCBS counts in the third and fourth weeks, however at 28 rearing days, TCBS counts were similar in all treatments. Overall, the TCBS quantification increased in the fifth rearing week in all treatments. Increasing TCBS counts were also observed in the feces for IPA-S.252 compared to IPA-S.51 and Control in the second rearing week (Table 3). Overall, in the fifth rearing week, the *Vibrio* quantification in feces decreased with IPA-S.51, but increased with the Control and IPA-S.252.

In the third and fourth weeks, the THB number in hepatopancreas with IPA-S.252 was higher than with IPA-S.51 and Control (Table 4). Furthermore, the THB counts in IPA-S.252 were

also higher compared to the TCBS counts. In the fifth rearing week the hepatopancreas THB load was smaller with IPA-S.252 compared to IPA-S.51. The THB load under each treatment increased during the first rearing week.

The THB numbers in the intestine were similar in all treatments except with IPA-S.51 in the fifth week, where THB numbers were higher in comparison to the other treatments and also to the *Vibrio* load in the same rearing period. The THB load in feces was not different among treatments, but both IPA-S.51 and IPA-S.252 THB numbers were higher than TCBS counts in the first rearing week (Table 4). In the fifth rearing week, IPA-S.252 THB load in feces was also higher than the TCBS counts.

These events did not influenced shrimp survival by the end of the experimental rearing period (Table 5). Control and CP survival were the same 86.8 ± 8% and IPA-S.252 survival was 75 ± 15%. IPA-S.51 had excellent survival (100%). The final weight was highest with CP (6.7 ± 0.05g), but IPA-S.51 (6.2 ± 0.04g) obtained the best final weight compared to Control (5.58 ± 0.08g) and IPA-S.252 (5.83 ± 0.1g). The WGR and DWG of CP and IPA-S.51 were also greater than the other treatments (Table 5), however CP had the best parameters. Regarding the SGR values, IPA-S.51 (3.95 ± 0.01%) and IPA-S.252 (3.81 ± 0.04%) had similar rates, but IPA-S.51 SGR was greater than the Control (3.71 ± 0.03%). The positive control (CP) had a greatest SGR compared to the other treatments.

4. Discussion

4.1. First *in vitro* assay

The probiotic *Bacillus subtilis* has been shown to produce a wide range of extracellular substances and antimicrobial peptides against a variety of microorganisms (Abriouel et al., 2011). Regarding this role, a greater antagonistic effect for IPA-S.51 against *V. alginolyticus* appeared at 60h, when the strain produced a smaller CFU number (10^3 cells) than produced at 48h of bacterial growth. This event can be explained by the lack of nutrients in the culture medium that can feature a

stationary phase of bacterial growth. According to Pepper et al. (2015), in bacterial cultures with high initial cell density as was the case with ours, it was observed that waste products can inhibit cell growth. In addition, in this phase it is easier for the cells to synthesize some toxic components against pathogens (Pepper et al., 2015).

Bacillus inhibitory effects are attributed to many causes: alterations of the pH in growth medium, depletion of essential nutrients and production of volatile compounds (Yilmaz et al.,2006). According to Das et al. (2014), the outer membrane of some bacteria also possesses a wide spectrum of inhibitory proteins that are responsible for controlling microbial diseases at different incubation periods, e.g. 24 h, 48 h, 72 h and 96 h. In the first *in vitro* assay, it is possible to affirm that the IPA-S.51 antagonism against *V. alginolyticus* was greater than CP, as the inhibitory halos produced with 48 and 60h of incubation were approximately twice as large as the CP inhibitory halos.

4.2. Second *in vitro* assay

In terms of pH and NaCl concentration, the maximum antibacterial activity frequency in this study was found in accordance with the suitable range for shrimp culture suggested by previous studies (Wyban and Sweeney, 1991; Bray et al., 1994). According to those authors, an appropriate NaCl concentration for shrimp culture (especially *L. vannamei*) is suggested to be within a range of 10–25 g L^{1}. In the present study, the largest halo frequency, with the *B. subtilis* strain, appeared at 10 g L^{-1} of NaCl in acid and neutral pH. In addition, the largest inibitory halo was also observed at basic pH. A wide range of pH tolerance was observed for *B. subtilis* L10 and G1, at pH 4 to 9, as observed in our analysis (Zokaeifar et al., 2012).

According to predicted values for GLM, the reduction of inhibitory halo diameter with IPA-S.51 from 25h until 60h of growth and then the inhibition increase after 60h against *V. parahaemolyticus* were similar to that observed in the first *in vitro* assay. A greater antagonistic effect for IPA-S.51 against *V. alginolyticus* also appeared at 60h. Regarding this inhibitory

characteristic, our study suggests that the lack of nutrients in the culture medium and stationary phase of bacterial growth had an influence as cited above.

IPA-S.252 and IPA-S.111 strains were only effective against *V. parahaemolyticus* in the extremes of salinity (0 and 30g L^{-1}) with 48h of bacterial growth and in neutral pH. A similar antagonism was also observed by Shakiba Zadeh et al. (2010), who reported inhibitory effect characteristics with *S. algae* cultured during 35-40h. The strain exhibited greater antibacterial effect at pH 7.5–8 using a bacterial medium with extreme NaCl concentrations (0 and 50%). In the same work, a decrease in this activity was observed with a NaCl concentration of 25%, corroborating our results. According to those authors, the antagonistic effect may be due to the synergistic effect of production of antimicrobial metabolites and lower NaCl concentration that is critical to species of *Vibrio*. In the present study, 2% NaCl was used in the TSA medium of *in vitro* assays, thus, it is possible that only the antimicrobial metabolites had potential inhibition against the vibrios. Krier et al. (1998) have documented that production of bacteriocin compounds in extreme conditions can be higher than under optimal growth conditions.

Concerning salt concentrations, some mechanisms are important to bacterial survival and growth in high salt environments, such as Na^+ efflux, K^+ uptake, glutamate transport and biosynthesis and the accumulation of compatible solutes, as observed in *S. algae*. In particular, genes involved in peptidoglycan biosynthesis and DNA repair are highly expressed and are accompanied by a rapid and instantaneous enhancement of the transcription of large- and small-ribosome subunits. This suggests that the structural changes in the cell wall and some stress responses occur in *S. algae* (Fu et al., 2014). It is well accepted, for example, that a specific physical or chemical stress (such as extreme salt concentrations) can dramatically improve the gastrointestinal robustness of a strain (Mills et al., 2011).

Indeed, *Shewanella* spp. are being increasingly used as probiotics in aquaculture (Jiang et al., 2013). With regard to beneficial properties, these bacteria produce omega-3 fatty acids and other hydrocarbons, probably to increase the fluidity of the cell membrane in cold water because most of

the *Shewanella* strains are psychrotolerant. Free fatty acid (FFA) production can also be a result of a mutualistic relationship between fish and bacteria living in their intestines (Hau et al., 2007; Sukovich et al., 2010). According to Desbois and Smith (2010), amongst the diverse and potent biological activities of FFAs, these molecules present the ability to kill or inhibit the growth of bacteria. The antibacterial properties of FFAs are used by many organisms to defend against parasitic or pathogenic bacteria, *e.g.* microalgae species (Desbois and Smith, 2010; Kokou et al., 2012).

4.3. Bacterial quantification of shrimp tissues and feces

Studies using adult penaeid shrimp have revealed that the multiplication of pathogenic bacteria begins in the hepatopancreas (De la Peña et al., 1995). The proliferation of the pathogen in the hepatopancreas eventually seizes control of the digestive tract and can replace the normal flora of the gut (Dempsey et al., 1989).

The addition of probiotic bacteria in the aquaculture system can trigger an action mechanism through competitive exclusion in which the probionts proliferate in the environment and compete for existing nutrients resulting in the displacement of pathogenic bacteria (Stavric and D'Aoust, 1993). It is supposed that in the present study, the mechanism of competitive exclusion of *Vibrio* load started in the hepatopancreas then passed to the intestine since higher TCBS quantification was registered in IPA-S.51 in the fourth rearing week and IPA-S.252 in the third and fourth weeks compared to the Control. It is suggested that the hepatopancreas *Vibrio* load could exit the shrimp gut through the feces as a greater number of TCBS colonies were registered with IPA-S.51 during the fourth week and with IPA-S.252 during the fifth week, when the TCBS quantification was highest compared to the other rearing weeks in this treatments.

Competitive exclusion is one of the mechanisms that regulates the immunity of the host (Chabrillon et al., 2005). The increment in numbers of the probiotic strain leads to nutrient and energy consumption that would otherwise be available for pathogenic bacteria (Verschuere et al., 2000; Denev et al., 2009). Due to adaptation to the same ecological niche, indigenous

microorganisms have great potential for competitive exclusion, as is the case with the *B. subtilis* and *S. algae* strains used in the present work (Lalloo et al., 2010).

In previous studies, it has also been reported that *Shewanella* and *Bacillus* strains were used as probiotics for their nutrition, disease resistance and other beneficial activities in fish (Nayak, 2010) and shrimp (Shakiba Zadeh et al., 2010). Hao et al. (2014) reported that the final weight, weight gain, percent weight gain and specific growth rate of shrimp were significantly increased by single or combined use of dietary *Shewanella haliotis* (D4), *B. cereus* (D7) and *Aeromonas bivalvium* (D15) dosed at 1×10^7 cell g^{-1} for 28 days. The reason why the growth of aquatic animals fed with probiotics increased may be attributed to enhanced digestive activity, e.g. protease and amylase that could stimulate and improve feed digestibility in the host (Zhang et al., 2010). Data not yet published have demonstrated that *B.subtilis* and *S. algae* strains have the ability of producing protease.

Ariole and Ekeke (2016) studied an *S. algae* indigenous strain isolated from a healthy shrimp (*Penaeus monodon*). The authors reported protease, amylase, lipase and gelatinase production by the *S. algae* strain that could improve feed digestibility and feed utilization. *Bacillus* spp. are recognized as enzyme producers and according to Ziaei-Nejad et al. (2006), enhance the specific activity of lipase, protease and amylase in the shrimp digestive tract, therefore, *Bacillus* strains could be also responsible for increased weight gain and nutrient absorption and consequently improved digestion (Gullian et al., 2004). In fact, in the present study, IPA-S.51 final weight, WGR, DWG and SGR were greater than in the Control, indicating an ability to improve *L. vannamei* juvenile growth.

Although the CP is constituted of three *Bacillus* strains, shrimp fed with *B. subtilis* (IPA-S.51) reached a very similar final weight as the positive control. The three strains of CP are species closely related to *Bacillus subtilis*, but they differ metabolically and secrete different enzymes. This metabolic and enzyme production difference could impact the final effect of the product and therefore manufacturers should communicate not only the qualitative characteristics of their

product, but also the quantitative characteristics (Priest et al. 1987; Sharifuzzaman and Austin, 2017).

In addition, our results demonstrated that the *B. subtilis* strain was reisolated from shrimp hepatopancreas and intestine during the first rearing week and from intestine in the second week. Probably, the *B. subtilis* load increased from the initial rearing week until the fifth week, contributing to the higher number of THB in comparison with TCBS counts in the IPA-S.51 group in hepatopancreas and intestine in the fifth rearing week. Although some other bacterial species can grow in TCBS, likewise Gram-negative bacteria that could present a pathogenic risk, more recent studies in culture media have affirmed that TCBS is the best selective medium for isolating potentially pathogenic *Vibrio* species (Abbott et al., 1993; Pfeffer and Olive, 2003).

Luis-Villaseñor et al. (2013) observed that a mix of three *Bacillus* strains and a commercial probiotic (Alibio®) similarly increased in the shrimp gut microbial communities over time, compared to the control group. The probiotic treatments modulated the microbiota and reduced the diversity of gut communities and also influenced the health of the host. In this regard, Niu et al. (2014) suggest that the *Bacillus* LT3 strain could also decrease the *in vivo* pathogenic activity of *V. campbellii* rather than inhibiting its growth, and this might be one of the factors contributing to the protective effect of strain LT3.

Besides the isolation of IPA-S.51, THB increment in shrimp gastrointestinal tract and TCBS count reduction in the shrimp hepatopancreas, we could not affirm yet if shrimp bacterial microbiota were modulated by IPA-S.51, since it was not identified the bacterial microbiota of shrimp intestine. However, gastrointestinal microbiota modulation is performed by probiotics that stimulate a beneficial bacterial community and reduce undesirable species in shrimp intestines (Mohapatra et a., 2013).

Studies of *S. algae* application in shrimp culture are scarce, therefore it is necessary to consider strain safety. In the Ariole and Ekeke (2016) study, the strain was susceptible to 15 different antibiotics and this characteristic is considered ideal for a real probiotic strain.

Additionally, in the Shakibazadeh et al. (2011) study an experiment with LD50 in a mammalian model was used to assess the safety of an *S. algae* strain used in shrimp culture for human consumption. The LD50 value was approximately 10^{36} CFU/animal, fairly high and most likely safe for use as a probiotic.

5. Conclusion

Overall, it was observed that the IPA-S.51 and IPA-S.252 probiotic candidates produced an inhibition effect against *Vibrio* pathogens under different bacterial growth periods, pH and NaCl concentrations. Regarding pH and NaCl conditions, the maximum antagonistic levels were in the same range as for optimum shrimp culture. Additionally, IPA-S.51 was able to grow inside the *L. vannamei* digestive system. In the *in vivo* experiment, the candidate probiotics (IPA-S.51 and IPA-S.252) could control the *Vibrio* load in the hepatopancreas. It is suggested that this load was shifted to the intestine and ultimately eliminated through feces in the IPA-S.51 and IPA-S.252 groups. Finally, IPA-S.51 was able to improve *L. vannamei* growth, as the final weight, weight gain rate, daily weight gain and specific growth rate values were greater than those observed in the Control.

Conflicts of interests: none

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Table 1. Average values (± SE) of inhibitory halos produced against *V. alginolyticus* under different bacterial culture fractions (Cell Biomass and Cell-free extract) of IPA-S.51 (*B. subtilis*), IPA-S.252 (*S. algae*), IPA-S.111 (*S. algae*) and CP (commercial probiotic) cultured during 12, 24, 36, 48 and 60h.[1]

Culture fraction	Culture time	Inhibitory halos (mm)			
		IPA-S.51	IPA-S.252	IPA-S.111	CP
Cell Biomass	12h	-	12 ± 2[a]	-	12.5 ± 2.5[a]
Cell-free extract		-	13 ± 1[a]	-	14.5 ± 2.5[a]
Cell Biomass	24h	-	-	16.1 ± 3	-
Cell-free extract		-	-	-	-
Cell Biomass	36h	17.4 ± 0.2[b]	10.9[a]	-	-
Cell-free extract		-	-	-	-
Cell Biomass	48h	-	-	-	-
Cell-free extract		22.4 ± 0.9	-	-	-
Cell Biomass	60h	25.3 ± 0.8[b]	-	-	10.1 ± 0.5[a]
Cell-free extract		12.7 ± 1.4[a]	-	-	8.7 ± 0.4[a]

[1]Different superscript letters within the same culture time indicate significant differences among treatments ($P < 0.05$). The absence of an inhibitory halo is indicated by (-).

Table 2. Average values (± SE) of Colony Forming Units (CFU) mL^{-1} of IPA-S.51 (*B. subtilis*), IPA-S.252 (*S. algae*), IPA-S.111 (*S. algae*) and CP (commercial probiotic) cultured during 12, 24, 36, 48 and 60h present in cell biomass fraction.[1]

	Culture time (10^8 CFU mL^{-1})				
Bacteria	12h	24h	36h	48h	60h
IPA-S.51	2.81 ± 1^a	1.1 ± 0.01^a	30 ± 5.8^a	280.6 ± 259.9^a	0.5 ± 0.3^a
IPA-S.252	246.7 ± 3.3^b	3.5 ± 0.3^b	440 ± 92.4^b	45.5 ± 0.3^a	247.7 ± 116.9^a
IPA-S.111	252 ± 85.4^b	6.9 ± 0.9^b	47.5 ± 15.9^a	30 ± 2.9^a	122 ± 50.8^a
CP	0.08 ± 0.01^a	0.1 ± 0.03^a	0.2 ± 0.05^a	0.04 ± 0.006^a	0.1^a

[1]Different superscript letters in the same column indicate significant differences among treatments ($P < 0.05$).

Table 3. Average values (±SE) of TCBS count in hepatopancreas, intestine and feces samples of *L. vannamei* reared during 07, 14, 21, 28 and 35 days under different treatments: Control, IPA-S.51 (*B. subtilis*), IPA-S.252 (*S. algae*).[1]

		Vibrio count (10^5 CFU g^{-1})				
		Rearing days				
Sample	Treatments	07	14	21	28	35
Hepatopancreas	Control	1 ± 0.2^{Aa}	3 ± 1^{Aab}	2 ± 0.3^{ABa}	6.2 ± 2.6^{Bbc}	10.6 ± 2.4^{Bd}
	IPA-S.51	15^{Bc}	0.2 ± 0.1^{Aa}	0.1 ± 0.02^{Aa}	6.8 ± 1.6^{Bb}	2.54 ± 0.03^{Aab}
	IPA-S.252	18^{Bb}	0.4 ± 0.1^{Aa}	4.2 ± 0.8^{Ba}	1.16 ± 0.04^{Aa}	0.69 ± 0.1^{Aa}
Intestine	Control	5.5 ± 4.4^{Aa}	54 ± 44^{Aa}	285 ± 275^{Aa}	370 ± 290^{Aa}	5380 ± 2620^{Ab}
	IPA-S.51	0.4 ± 0.3^{Aa}	85 ± 20^{Aa}	48 ± 12^{Aa}	1930 ± 1690^{ABab}	4660 ± 3660^{Ab}
	IPA-S.252	0.1 ± 0.03^{Aa}	62 ± 0.6^{Aa}	4940 ± 1867^{Bb}	4995 ± 1573^{Bb}	3690 ± 1270^{Aab}
Feces	Control	1.6 ± 0.5^{Aa}	3 ± 0.9^{Aa}	48 ± 29^{Bb}	62 ± 10^{Bb}	63.4 ± 11^{Ab}
	IPA-S.51	3 ± 1^{Aa}	3.1 ± 1.5^{Aa}	51.4 ± 3.6^{Bc}	78 ± 17^{Bc}	38 ± 17^{Ab}
	IPA-S.252	7.1 ± 1.1^{Aab}	44 ± 15^{Bc}	5.4 ± 1.1^{Aa}	4.7 ± 1.3^{Aa}	41 ± 15^{Abc}

[1]Different superscript capital letters in the same column indicate significant differences among treatments ($P < 0.05$). Different superscript lowercase letters in the same line indicate significant differences among rearing days ($P < 0.05$).

Table 4. Average values (±SE) of total heterotrophic bacteria (THB) count in hepatopancreas, intestine and feces samples of *L. vannamei* reared during 07, 14, 21, 28 and 35 days under different treatments: Control, IPA-S.51 (*B. subtilis*), IPA-S.252 (*S. algae*).[1]

		THB count (10^5 CFU g^{-1})				
		Rearing days				
Sample	Treatments	07	14	21	28	35
Hepatopancreas	Control	23 ± 7.5^{Aa}	99 ± 63^{Aa}	18 ± 3^{Aa}	$0.5 \pm 0{,}1^{Aa}$	$489 \pm 159^{ABb*}$
	IPA-S.51	24 ± 6^{Aa}	23 ± 7^{Aa}	20 ± 2^{Aa}	22 ± 3^{Aa}	$649 \pm 51^{Bb*}$
	IPA-S.252	15 ± 5^{Aa}	$426 \pm 126^{Bb*}$	$870 \pm 30^{Bc*}$	15 ± 1.4^{Aa}	$469 \pm 123^{Ab*}$
Intestine	Control	8 ± 2^{Aa}	1051 ± 599^{Aa}	3100 ± 1860^{Aa}	2173 ± 607^{Aa}	3030 ± 1290^{Aa}
	IPA-S.51	0.7 ± 0.2^{Aa}	486 ± 55^{Aa}	553 ± 57^{Aa}	2833 ± 531^{Aa}	$40250 \pm 16750^{Bb*}$
	IPA-S.252	5 ± 1^{Aa}	375 ± 10^{Aa}	6320 ± 533^{Aa}	3520 ± 281^{Aa}	3840 ± 1720^{Aa}
Feces	Control	10.8	38 ± 21	208 ± 74	6 ± 0.2	78
	IPA-S.51	$280 \pm 135^{*}$	93 ± 2	208 ± 15	192 ± 130	140 ± 32
	IPA-S.252	$306 \pm 38^{*}$	3 ± 0.2	66 ± 11	51 ± 3	$312 \pm 138^{*}$

[1]Different superscript capital letters in the same column indicate significant differences among treatments ($P < 0.05$). Different superscript lowercase letters in the same line indicate significant differences among rearing days ($P < 0.05$). Asterisks indicate significant differences between THB and TCBS counts under the same treatments and at the same rearing day.

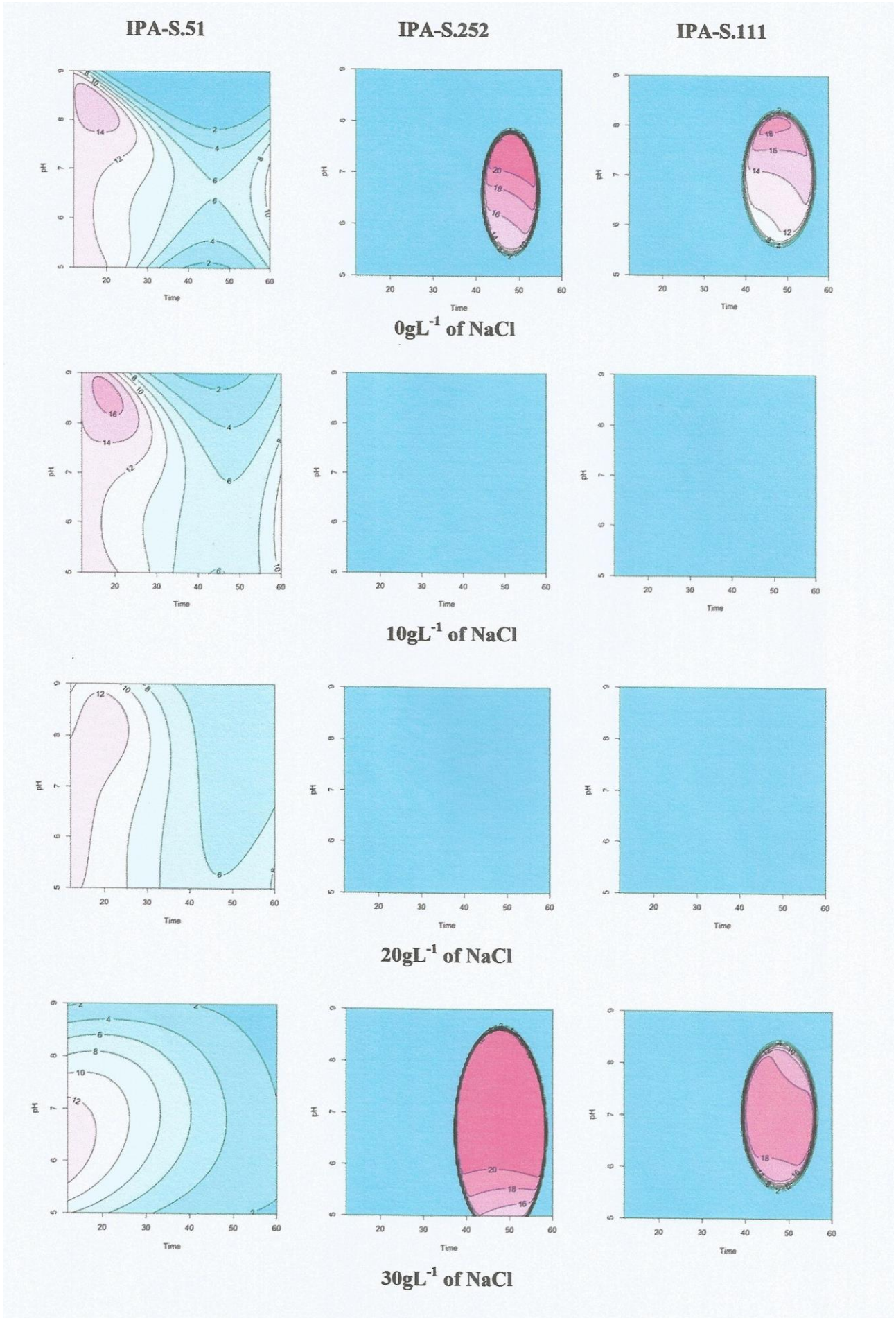
Table 5. Average values ± SE of *L. vannamei* juvenile growth after 45 days of rearing.[1]

Treatment	Survival (%)	Final weight (g)	WGR (%)	DWG ($g.dia^{-1}$)	SGR (%)
Control	86.8 ± 8	5.58 ± 0.08^{a}	431.3 ± 7.4^{a}	0.10 ± 0.001^{a}	3.71 ± 0.03^{a}
CP	86.8 ± 8	6.7 ± 0.05^{c}	538.6 ± 3.4^{c}	0.149 ± 0.001^{c}	4.12 ± 0.03^{c}
IPA-S.51	100	6.2 ± 0.04^{b}	490.5 ± 4.4^{b}	0.114 ± 0.001^{b}	3.95 ± 0.01^{b}
IPA-S.252	75 ± 15	5.83 ± 0.1^{a}	454.8 ± 9.9^{a}	0.106 ± 0.002^{a}	3.81 ± 0.04^{ab}

[1]Different superscript letters in the same column indicate significant differences among treatments ($P < 0.05$).

Figure Captions

Figure 1. Predictions of halo diameter (mm) produced by probiotic candidates against *V. parahaemolyticus*: IPA-S.51 (*B. subtilis*); IPA-S.252 (*S. algae*) and IPA-S.111 (*S. algae*) in 0g L^{-1}, 10g L^{-1}, 20g L^{-1} and 30g $L^{-1}$of NaCl.


IPA-S.51
IPA-S.252
IPA-S.111
0gL-1 of NaCl
10gL-1 of NaCl
20gL-1 of NaCl
30gL-1 of NaCl
pH
Time

ARTIGO 2

Effects of dietary *Bacillus subtilis* and *Shewanella algae* in expression profile of immune-related genes from hemolymph of *Litopenaeus vannamei* challenged with *Vibrio parahaemolyticus*

A ser submetido ao periódico Fish and Shellfish Immunology

Effects of dietary *Bacillus subtilis* and *Shewanella algae* in expression profile of immune-related genes from hemolymph of *Litopenaeus vannamei* challenged with *Vibrio parahaemolyticus*

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Abstract

B. subtilis and *S. algae* effects in growth and innate immunity were assessed on *L. vannamei* juveniles. During 60 days, shrimps were reared in three treatments: Bs, fed with 10^6 CFU of *B. subtilis* per gram of commercial feed, Sa, fed with 10^6 CFU of *S. algae* per gram of commercial feed and Control (without bacterial addition). Then, the animals were subjected to a *V. parahaemolyticus* injective challenge. For this purpose, four treatments were established: Control shrimps challenged (Vibrio), Bs shrimps challenged (Vibrio+Bs), Sa shrimps challenged (Vibrio+Sa) and Control, shrimps not submitted to probiotic treatments and challenged. The shrimps' hemolymph was sampled 45-days after rearing and 24h post-challenge for quantification of prophenoloxidase (proPO), lipopolysaccharide and β-1,3-glucan-binding protein (LGBP) and hemocyanin (HEM) transcripts by qPCR. Moreover, shrimps' final weight and survival were also verified. *B. subtilis* administration enhanced shrimps growth and improved proPO, LGBP and HEM expression levels before and after challenge. After 60-days of feeding, Sa final weight was higher than the Control, whereas Vibrio+Sa cumulative mortality after 48h of *Vibrio* challenge was lower than Vibrio group. These results could be correlated with the proPO and LGBP up regulation in Vibrio + Sa compared to Vibrio group, protecting *L. vannamei* from the bacterial infection. Together, these results suggest the probiotic potential of *B. subtilis* e *S. algae* in the modulation of immune-related genes as a tool to control *V. parahaemolyticus* infection inside shrimps.

Keywords: *Bacillus subtilis*, *Shewanella algae*, probiotic, immunity, hemolymph, *Litopenaeus vannamei.*

2. Introduction

Despite the remarkable growth in world production of cultivated shrimp, there are great mortalities due to diseases [1]. In this concern, Infectious diseases are the most significant cause of economic losses in shrimp farms [2]. Disease outbreaks are particularly caused by the main shrimp pathogens: white spot sindrome virus (WSSV), yellow headed virus (YHV), infectious myonecrosis

virus (IMNV), and *Vibrio* bacteria [3,4]. Some *Vibrio* species can cause vibriosis (e.g. *V. alginolyticus* and *V. harveyi*), as well as it was recently discovered early mortality syndrome (EMS), which is closely related to *V. parahaemolyticus* [5,6].

Once foreign particles enter the haemocoel, shrimp engage an innate immune response that includes cellular and humoral reactions [7]. The main immune reactions occur in hemolymph, which contain three different hemocytes types: hyaline, granular and semigranular [8]. Several immune molecules are produced and stocked in the granules of the hemocytes before they are released into the hemolymph after activation by cell wall components of bacteria and / or fungi, such as peptideoglycan, lipopolysaccharides and β-glucans [9].

Pattern recognition proteins recognize and bind microbial cell wall components and activate different immune response [10,11]. The innate immunity system of shrimps protects from pathogenic infections and mainly consists in prophenoloxidase (proPO) system, coagulation system, phagocytosis, encapsulation and nodule formation, antimicrobial peptides formation and cell agglutination [12,13].

Previous studies reports *Bacillus* species as able to induce the innate immune system of crustaceans [14,15]. Indeed, *Bacillus* spp. is often used as a beneficial microorganism in aquaculture systems once can added to health-promoting functional foods as therapeutic, prophylactic, and growth supplements [16]. The oral administration of *Bacillus* PC465 enhanced the growth performance and survival of *L. vannamei*, improved digestion and nutrient absorption, microbial structures in the gut, immune status and response to viral infection [17]. The probiotic supplementation significantly increased the transcription of prophenoloxidase enzyme (proPO), penaeidin 3a (Pen-3a) an antimicrobial peptide, peroxinectin, a cell adhesion molecule, C-type lectin 3 (Lec-3), a pattern recognition receptor, and thioredoxin (Trx) antioxidant enzyme in *L. vannamei* hemocytes. Likewise, probiotic treatment increased the transcription of hemocyanin in *L. vannamei* hepatopancreas [17].

Shewanella spp., e.g. *Shewanella algae* [18], *Shewanella putrefaciens* e *Shewanella baltica* [19] has also been used as probiotics to increase nutritional and disease resistance [20]. According to Chabrillón et al. [21] and Shakibazadeh et al. [22], different species of the genus *Shewanella* have antagonistic abilities that affect *Vibrio* species and can further modulate immunological genes from animals e.g. fish from rearings [23], however, studies about stimulation of shrimp immune genes by *Shewanella* spp. are still scarce. Taking into account all these previous considerations, the aim of the current study was to compare the dietary effects of *B. subtilis* and *S. algae*, isolated from *L. vannamei* gut reared in Brazil northeast, on *L. vannamei* juveniles growth, innate immune and *V. parahaemolyticus* challenge resistance.

2. Materials and methods

2.1 Candidate probiotics

The probiotic candidate strains *Bacillus subtilis* (IPA-S.51) and *Shewanella algae* (IPA-S.252) were provided by Instituto Agronômico de Pernambuco (IPA). Both strains were previously isolated from the hepatopancreas/stomach of *L. vannamei* grown at 30g.L^{-1} of salinity. Its 16S rRNA gene region was sequenced with fD1 (forward) (5´- AGAGTTTGATCCTGGCTCAG-3´) e rD1 (reverse) (5'- AAGGAGGTGATCCAGCC-3') and then the sequences were compared with GenBank, National Center for Biotechnology Information (NCBI) through BLASTn program. *B. subtilis* and *S. algae* were grown in Tryptic Soy Broth (TSB) at 30°C during 48h. After that, bacterial cells were collected by centrifugation (1800 xg / 20 min) and then suspended in sterile sea water for further addition in shrimp feed.

2.2 *L. vannamei* feed

Experimental feed were prepared adding *B. subtilis* and *S. algae* separately to a commercial pelleted feed with 40% of crude protein (Camaronine 40CR2, Purina, Agribrands do Brasil SA, São Paulo, Brazil) (adapted from Jiang et al. [24]). At the end of feed preparation the

bacteria concentration in the mixture was approximately 10^6 Colony Forming Units (CFU). g^{-1}. After preparation, the feed was stored for a maximum period of 72 h at a temperature of 4°C, until use.

2.3 Shrimp and rearing trials

L. vannamei postlarvae (PL10) were obtained from a commercial larviculture (Aquatec®), located in Canguaretama, Rio Grande do Norte and transferred to LTA / UFRPE (Laboratório de Tecnologia em Aquicultura / Universidade Federal Rural de Pernambuco). The animals were acclimatized in a 1000 L tank, until reach a weight of 1.05 ± 0.25 g. After, shrimps were stocked in (width x length x height = 0.48 m x 0.56 m x 0.89 m) polypropylene tanks containing 50 L of seawater (23g L^{-1}) with constant aeration and maintained at 28 - 29ºC through heaters with thermostat. The shrimps were distributed in three treatments with four replicates each: Bs, fed with *B. subtilis*, Sa, fed with *S. algae* and Control without bacterial addition. Each group included four replicates. The stocking density was 130 shrimps.m^{-2} and 25% of the water was changed daily. During the whole experiment (60 days), shrimps were fed four times a day. The amount of feed supplied was equivalent to 10% of the biomass of each tank.

At the end of 45 rearing days, shrimps final weight and survival rate (*final number* of *shrimp*/*initial number* of *shrimp)* x 100) were evaluated. The shrimps final weight was also measured after 60 rearing days.

2.4 *V. parahaemolyticus* challenge

Vibrio parahaemolyticus (ATCC 17802) was grown in Tryptone Soy Broth (TSB supplemented with 2% NaCl, Difco) for 24 h at 30ºC before being centrifuged at 1800 *g* for 20 min. Fluid supernatants were removed and the pellets re-suspended in sterile saline (2% NaCl) in order to stabilize a stock solution for infection assays in a concentration between $3x10^7$ and 1×10^7 CFU.mL^{-1}.

For the *V. parahaemolyticus* challenge, ten shrimps from each treatment reared for 60 days were distributed in three experimental tanks of polypropylene (the same used for the entire

experiment), the water aeration and temperature were maintained as described for the rearing. The challenge was performed by injecting 100 µl of a *V. parahaemolyticus* suspension in shrimps third abdominal segment. The final concentration of the injected solution was between $3x10^6$ and $1x10^6$ $CFU.mL^{-1}$.

Four treatments were established: animals fed without bacterial addition and challenged with *V. parahaemolyticus* (Vibrio), animals fed with *B. subtilis* and challenged with *V. parahaemolyticus* (Vibrio+Bs), shrimps fed with *S. algae* and challenged with *V. parahaemolyticus* (Vibrio+Sa) and Control, where animals not submitted to treatments with the experimental diets were injected with 100 µl of sterile saline solution to evaluate a possible mortality due to the injection procedure. Shrimps cumulative mortality was recorded after 24, 48, 72 and 96h of *V. parahaemolyticus* challenge.

2.5 Quantitative RT-PCR (qRT-PCR)

The hemolymph of eight animals were sampled at the end of the 45 and 60 rearing days and 24h after *V. parahaemolyticus* challenge via injection for determination of immune related genes expression. The hemolymph samples were collected by sterile syringes of 1 ml.

Quantitative RT-PCR (qRT-PCR) was performed using three biological samples for each group (Controls, with probiotics and infected with *V. parahaemolyticus*), each obtained from a pool of eight shrimps. Total RNA was extracted from the hemolymph using TRIzol reagent (Invitrogen) and purified using the RNeasy mini kit (Qiagen, Germany). After that, it was quantified using NanoVue equipment (GE Healthcare). cDNA synthesis was performed using the QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's guidelines. Quantitative RT-PCR was performed following the methods described by Livak and Schimittgen [25] for delta delta Ct calculations to conduct relative quantification of the transcripts. The calibrator was the control. Hemolymph cDNAs from *L. vannamei* were quantified using SYBR Green PCR Master Mix (Applied Biosystems) in a Step One Plus PCR System (Applied Biosystems). The qRT-PCR

reaction consisted of 1 µL of 10-fold diluted cDNA (5 ng), 12.5 µL of SYBR Green and 0.2 µM of each primer in a 25 µL total volume. β-actin was used as the internal control. The PCR program comprised 40 cycles at 94°C (15 seconds) and 60°C (1 min), followed by melt curve generation. Melt curves were analyzed to check the specificity of amplification.

Gene-specific primers were designed as: prophenoloxidase (proPO), forward primer, 5'-CGGTGACAAAGTTCCTCTTCG-3' and reverse primer, 5'-TGCAGGTCGCCGTAGTAAG-3' [26]; lipopolysaccharide and β-1,3-glucan-binding protein (LGBP), forward primer, 5'-CATGTCCAACTTCGCTTTCAGA-3' and reverse primer, 5'- GCTCCGTAGGGCCAGTTAC-3'[27], β-actin (B-ACT), forward primer, 5'- CCACGAGACCACCTACAAC-3' and reverse primer, 5'-TCCTTCTGCATCCTGTCGG-3' (GenBank no.: AF300705) and hemocyanin (HEM), forward primer, 5'-CTTAGTGGTTCTTGGGCTTGTC-3' and reverse primer, 5'-GGTCTCCGTCCTGAATGTC-3' (GenBank no.: X82502).

2.6 Statistical analysis

Significant differences between Bs, Sa and Control final weight gain and survival data after 45 days of rearing and *V. parahaemolyticus* challenge (means ± Standard Error) were performed by One-way (ANOVA) followed by Fisher post hoc test. Differences in data collected from RTqPCR experiments after 45-day rearing and *V. parahaemolyticus* challenge were also assessed by One-way (ANOVA) followed by Fisher post hoc test. Two sample T test was used to verify significant differences in immune genes expressions before and after *V. parahaemolyticus* challenge. The statistical analyses were performed with Origin 8.0 software.

3. Results

3.1 Immune status analysis

Fig. 1 shows the proPO, LGBP and HEM mRNA expression levels in different shrimp treatments reared for 45 days.

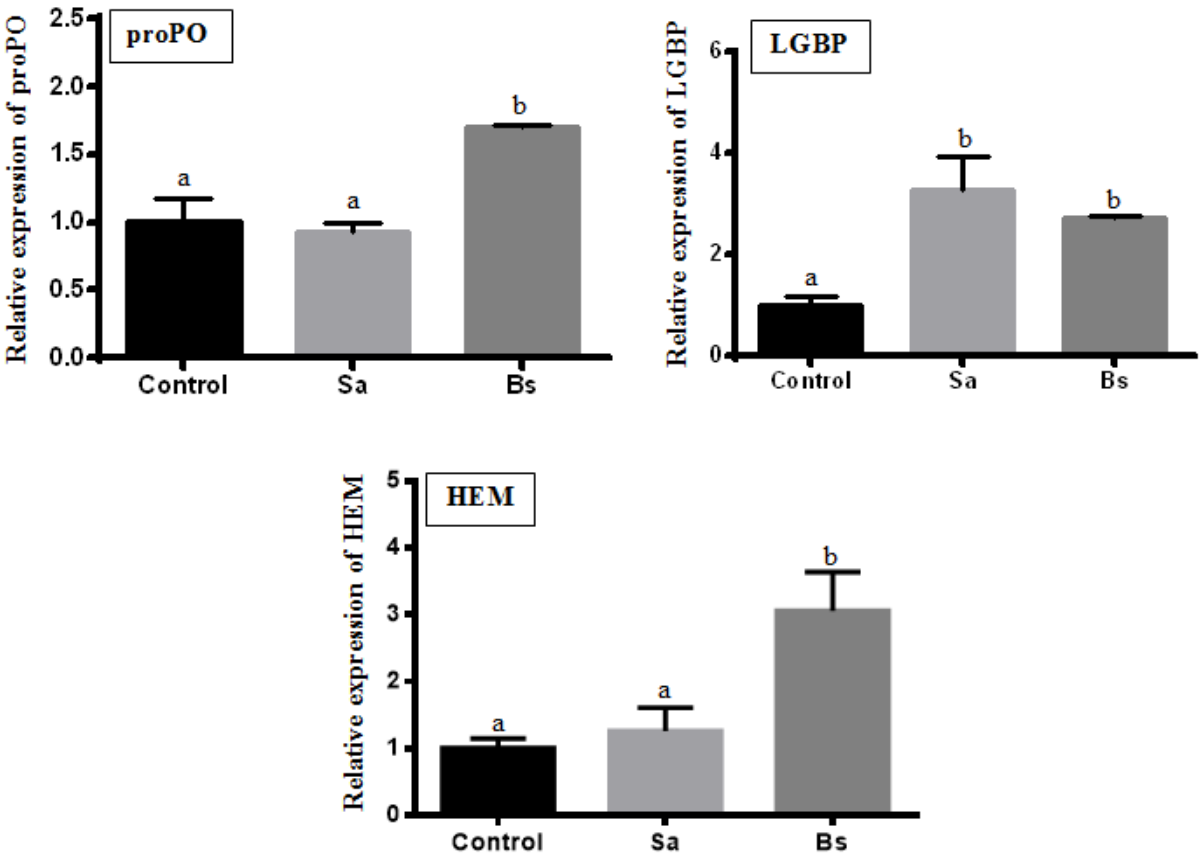

Fig.1. Relative expressions through the real-time PCR analysis of immune-related genes, including prophenoloxidase (proPO), lipopolysaccharide and β-1,3-glucan-binding protein (LGBP) and Hemocyanin (HEM) of *L. vannamei* fed with *S. algae* (Sa), *B. subtilis* (Bs) and without bacteria (Control) for 45 days. Each bar represents the mean fold change relative to the control ± S.D. Significant ($p < 0.05$) differences are indicated by different letters (a and b).

The dietary inclusion of *B. subtilis* IPA-S.51 significantly improved the transcription of proPO and HEM in *L. vannamei* juveniles hemolymph compared to Control and Sa. After 45-day rearing the proPO, LGBP and HEM expressions of Bs were 1.71, 2.73 and 3.06-fold, respectively. The shrimps fed with *S. algae* IPA-S.252 and Control had a similar proPO and HEM expression trend. The proPO and HEM levels of Sa and Control were 0.93 and 1.26-fold; 1 and 1-fold, respectively. The LGBP gene was significantly up-regulated in all shrimp fed with *B. subtilis* (2.73-fold) and *S. algae* (3.28-fold) compared to the Control (1.01-fold).

The mRNA expression levels of proPO, LGBP and HEM in different treatments challenged with *V. parahaemolyticus* via injection are shown in Fig. 2.

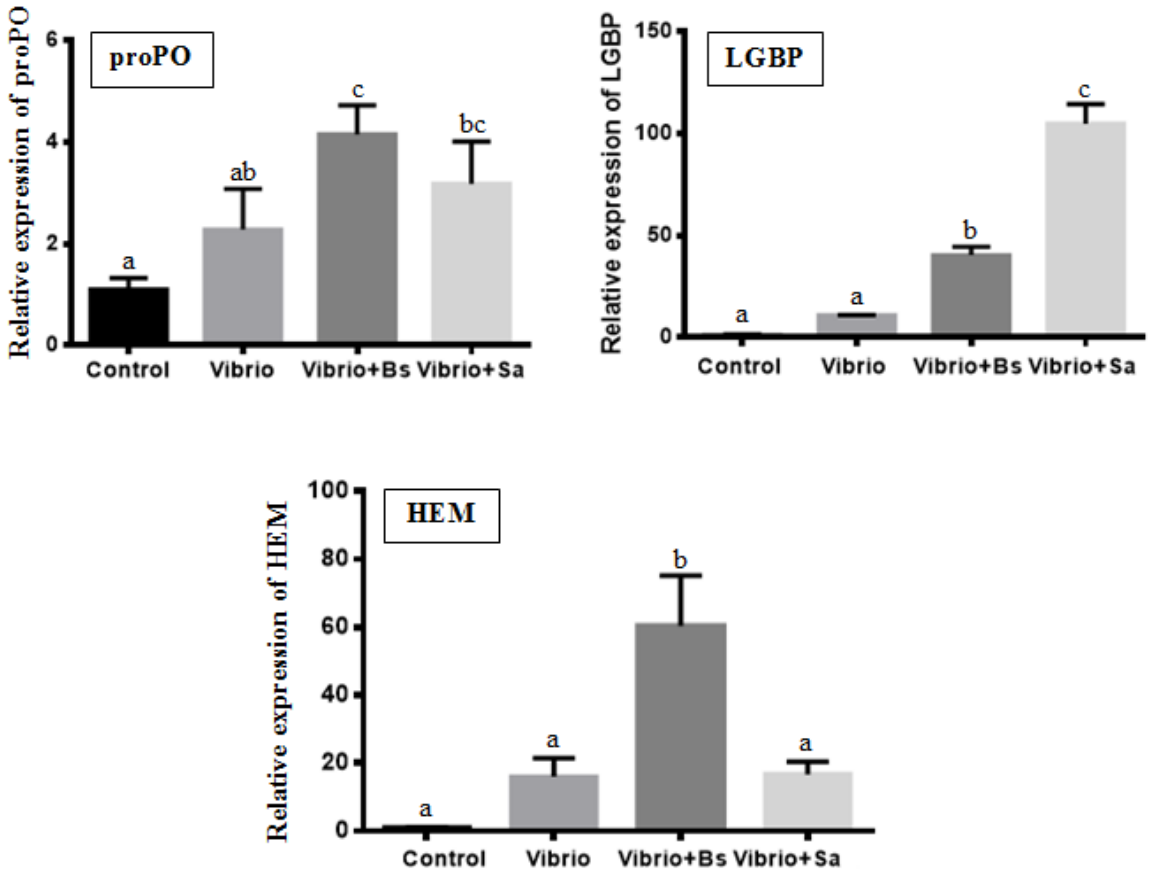

Fig.2. Relative expressions through the real-time PCR analysis of immune-related genes, including prophenoloxidase (proPO), lipopolysaccharide and β-1,3-glucan-binding protein (LGBP) and Hemocyanin (HEM) of *L. vannamei* reared for 60 days distributed in different treatments: animals fed without bacterial addition and challenged with *V. parahaemolyticus* (Vibrio), animals fed with *B. subtilis* and challenged with *V. parahaemolyticus* (Vibrio+Bs), animals fed with *S. algae* and challenged with *V. parahaemolyticus* (Vibrio+Sa) and Control, animals not submitted to treatments with experimental diets injected with sterile saline solution. Each bar represents the mean fold change relative to the control ± S.D. Significant ($p < 0.05$) differences are indicated by different letters (a, b and c).

After 24h of *V. parahaemolyticus* challenge, the expression levels of proPO in Vibrio + Bs (4.15-fold) was higher than Vibrio group (2.28-fold) but similar to Vibrio+Sa (3.18-fold). Vibrio and Vibrio + Sa groups did not shown significant differences in proPO expression.

The LGBP levels were up regulated in Vibrio+Bs (40.40-fold) and Vibrio+Sa (104.90-fold), but Vibrio + Sa showed a higher mRNA expression level compared to Vibrio+Bs and Vibrio (10.91-fold). LGBP expression of Vibrio+Bs was also higher than Vibrio group. The HEM expression was superior for Vibrio + Bs (60.36-fold) compared to the others groups: Vibrio (16.02-fold) and Vibrio + Sa (16.81-fold). Both Vibrio and Vibrio + Sa had similar LGBP levels.

Significant differences in proPO, LGBP and HEM expressions were found in shrimps groups after and before *V. parahaemolyticus* challenge. Bs group had all the immune genes up regulated after challenge. Regarding to Sa group, LGBP and HEM levels after *V. parahaemolyticus* injection were higher, however the proPO levels were similar before and after the challenge. The groups fed with feed without bacterial supplementation (Control and Vibrio) only had a LGBP expression up regulated after challenge, proPO and HEM levels did not have significantly differences before and after *Vibrio* challenge.

3.2 Growth and survival measurements

Bacillus subtilis supplementation was associated with a significant ($p < 0.05$) increase in *L. vannamei* final weight after 45 days of rearing. The Bs final weight (6.2 ± 0.04g) was significantly higher than Control (5.58 ± 0.08g) and Sa (5.83 ± 0.1g), both Control and Sa final weight were significantly similar. Shrimp survival after the 45-day feeding period was not significantly different among shrimp groups. Control and Sa survival was 86.8 ± 8 and 75 ± 15% respectively and Bs had an excellent survival (100%).

Sa had the higher final weight after 60-day feeding period, (9.35 ± 0.1g) and was significant different of Control (7.68 ± 0.1g) and Bs (8.4 ± 0.1g). Bs also had a significantly superior final weight compared to the Control. Shrimp cumulative mortality after 24h of *V. parahaemolyticus* challenge did not have significant differences: Sa (65 ± 5%), Bs (75 ± 5%) and Control (85 ± 5%)

(Figure 3). At 48h of challenge significant differences between Sa (85 ± 5%) and Control (100%) were observed. Bs (92.5 ± 2,5%) cumulative mortality was similar to the other groups and 100% of mortality was observed at 72 and 96h of challenge (Figure 3).

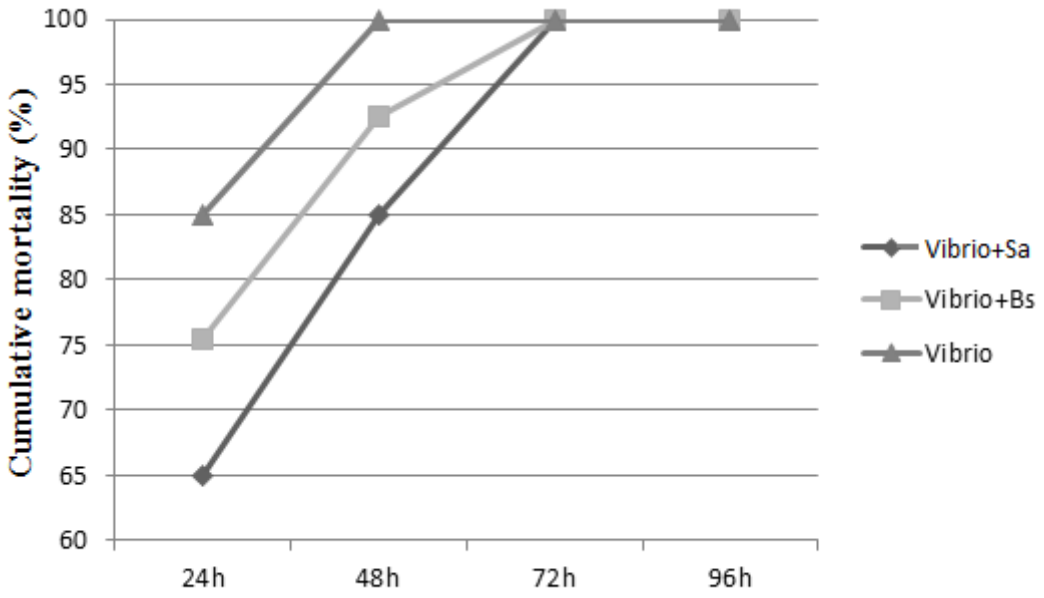

Fig. 3. Cumulative mortality (%) during 96h of *L. vannamei* reared for 60 days distributed in different treatments: animals fed without bacterial addition and challenged with *V. parahaemolyticus* (Vibrio), animals fed with *B. subtilis* and challenged with *V. parahaemolyticus* (Vibrio+Bs), animals fed with *S. algae* and challenged with *V. parahaemolyticus* (Vibrio+Sa).

4. Discussion

The effective performance of a probiotic depends on their adhesion of the gut cells, which in turn improves the host immune system [28]. Once the probiotic adheres to the cell, various biological activities take place, which primarily include the release of cytokines and chemokines, strictly linked to the stimulation of the immune system [29].

In the present study, *B. subtilis* was reisolated from *L. vannamei* hepatopancreas and intestine during the experimental rearing and Bs group had a higher level of the proPO transcription after 45 days of rearing than Sa and Control. Shrimps ProPO system could be activated by several microbial polysaccharides and specific pattern recognition proteins (PRPs), such as LPS- and β-1,

3-glucan-binding protein (LGBP) and peptidoglycan-binding proteins (PGBP) [30]. Our study found that during the 45-day rearing, Bs group had a significantly higher LGBP level than the control group; which may be correlated with the up regulating of proPO gene.

Final weight of Bs group was significantly improved after 45 days of rearing compared to Control and Sa and there was not observed mortality in the group plots. An increase in shrimps final weight of Bs group was also observed after 60 days of rearing compared to Control. Chai et al.[17], reported during a *Bacillus* PC465 feeding trial a significantly up-regulated transcriptions of immune-related genes as peroxinectin and hemocyanin and a "dose effect" was observed in shrimps infected by White Spot Sindrome Virus (WSSV) where in a much higher survival was observed for shrimps fed with 10^9 cells g^{-1} dose compared with 10^7 cells g^{-1} dose. According to the authors, growth may be promoted by an increase in digestive enzyme activities (protease, amilase and lipase) recorded in the study. In our previous work, we found that *B. subtilis* and *S. algae* had the ability of producing protease (unpublished results).

After *V. parahaemolyticus* challenge, proPO and LGBP genes were significantly higher to Bs group than the Control. These findings demonstrate that administration of *B. subtilis* (IPA-S.51) can improve growth performance and immune resistance in shrimps through an enhanced immune genes expression before and after a *V. parahaemolyticus* challenge. The shrimp proPO and LGBP expression in Bs were significantly up-regulated after *V. parahaemolyticus* challenge and these levels were higher compared to the levels observed after a 45-day rearing. This pattern of immune defense was suggested by Hao et al. [31]. The authors report that the probiotic application might result in an enhancement or prolongation of the desirable effects on the host immune response, growth performance and disease resistance.

The HEM expression was also higher to Bs before and after *V. parahaemolyticus* challenge in comparison with the other groups. Hemocyanin has been reported as a novel and important defense molecule in mollusks and arthropods [32]. Besides its primary function as a respiratory protein, it has been suggested that hemocyanin could be functionally converted into

phenoloxidaselike enzyme, antiviral agent, antimicrobial protein [33-35]. In fact, the phenoloxidase expression was higher to Bs group in comparison to Control and Sa and this level maybe could be influenced by the hemocyanin convertion during the offer of *B. subtilis* supplemented feed.

Huang et al. [36] report that hemocyanin concentration decrease to the lowest level in *Vibrio*-resistant and normal shrimps about 3 and 12h after pathogenic infection respectively. According to the authors, the hemocyanin concentration in resistant and normal shrimp decreased about 42.1% and 44.4%, and recovered at 12 h and 48 h after a *Vibrio harveyi* injection, respectively. In the present study, although the HEM level was not detected before 12h of infection, the Control group kept the HEM level similar at 24h post *Vibrio* injection. Vibrio + Bs presented a higher HEM level at 24h post *Vibrio* injection compared to the HEM level of Control group reared for 45 days. Consequently, although the cumulative mortalities after 24h of challenge did not present significant differences, Vibrio group cumulative mortality after 24h of challenge were 10% superior to the rate verified to Vibrio+Bs. In fact, Vibrio and Control groups did not have differences between immune genes expression after *V. parahaemolyticus* challenge.

Positive results were also reported by Luis-Villaseñor et al. [37] after a *L. vannamei* challenge with *V. parahaemolyticus* injection (10^5 CFU g^{-1}). The authors observed a survival increase in shrimps treated with *Bacillus* mix (33 %) compared to the control (9 %). They concluded, after a molecular analysis to investigation, that *Bacillus* mix administration induced modulation of the intestinal microbiota of *L. vannamei* and increased its resistance to *V. parahaemolyticus.*

Hao et al. [31] also observed promising results in shrimp growth, i.e. final weight, weight gain, percent weight gain and specific growth rate were improved after 28-days of feeding regime with *Shewanella haliotis, Bacillus cereus* and *Aeromonas bivalvium*. In modern aquaculture, the ability of *Bacillus* spp. to sporulate, grow fast and tolerate a wide range of physiological conditions has been reported to improve the quality of sea water, reduce the load of harmful bacteria and maximize the host's response without antibiotics [38].

Regarding to immune expressions of shrimps fed with *S. algae*, only the LGBP gene was up regulate compared to Control after 45-day feeding regime. According to Amparyup et al. [39], the differences in LGBP transcript expression could be due to variations between the response to live and dead microrganisms cells or to different microorganisms and so cell wall components, in addition to differences between host species or strains.

Some authors [11,40-43] indicate that the transcript level of LGBP is increased in response to *Vibrio* and LPS challenges after a variable time period of 3-24 h, thus, in the present work it is suggested that the proPO zymogen has not been converted to active PO (phenoloxidase) once that the LGBP could be not induced the activation cascade of serine proteinase (SPs) that leads to a final serine proteinase formation designated as a proPO-activating enzyme (PPAE). PPAE converts the inactive proPO zymogen in active phenoloxidase (PO) to produce the quinones, which can cross-link neighboring molecules to form melanin around invading microorganisms [42].

Similar to prophenoloxidase, according to Shi et al. [43], the hemocyanin can be directly activated by microbial protease and enhanced by pathogen associated molecular patterns and thus, be recognized by LGBP. However, the HEM levels did not be improved in Sa group. Hao et al. [31] report that most probiotics could not affect only one pathway or immunemechanism (a probiotic may play the role of immunosuppressant or immunostimulant on different targets within the immune system). This immune genes expression results did not influenced the Sa final weight, after 45 days of rearing.

Although proPO and HEM genes have not been activated by *S. algae* supplemented feed after 45 days of rearing, an improvement in final weight after 60 days of rearing were observed. According to Zokaeifar et al. [15], beyond the expression of immune genes and a higher survival rate in treated shrimp may be also due to the competitive exclusion, higher digestive enzyme activity and better growth performance as observed in the present study.

However, a decrease in cumulative mortality in Sa group after 48h of *V. parahaemolyticus* challenge were observed compared to the other shrimp groups. This could be correlated with the

proPO and LGBP up regulation compared to Vibrio group and indicates a requirement for a longer period of time to the effective action of *S. algae* probiotic properties in comparison to *B. subtilis.*

Similar results were reported by Díaz-Rosales et al. [20]. The authors studied the effects of the dietary administration of *Shewanella putrefaciens* and *Shewanella baltica*, on the immunological responses of Senegalese sole (*Solea senegalensis*) and its survival after *Photobacterium damselae* sub sp. Piscicida challenge. The cumulative mortality percentage after the challenge was 100% in the groups fed with control diet and mortality rates observed in the groups fed with diets supplemented with *S. putrefaciens* and *S. baltica* ranged from 75 to 100 and 65 to 80%, respectively. The authors also observed the improvement of growth by *Shewanella* supplemented feed offered to fishes compared to those fed with control diet.

Studies about *Shewanella* probiotic application and its effects are still scarce. The utilization of *Shewanella* genus is more widely studied in fish as Senegalesse sole, *Solea senegalensis* [20, 23 44, 45], Abalone, *Haliotis discus* [24], Gilthead sea bream, *Sparus aurata* L. [45, 46], than in shrimps [18,29,47].

In conclusion, *B. subtilis* administration enhanced *L. vannamei* growth performance and improved proPO, LGBP and HEM genes levels before and after *V. parahaemolyticus* challenge in comparison to Control. *S. algae* feed supplementation also conferred greater growth performance after 60-day feeding regime and cumulative mortality decrease after 48h of *V. parahaemolyticus* challenge, once that a quality shrimp feed offer and immune genes up regulation post-challenge consequently leads to higher digestive enzyme activity and stimulate antibacterial responses, respectively. In general, the supplementation of *L.vannamei* juveniles feed with *B.subtilis* and *S. algae* results in positive results to shrimp immune status, growth performance and decrease mortality after *V. parahaemolyticus* challenge. The present study confirms the strains probiotic potential, but pilot scale *in vivo* experiment is necessary to the production of a commercial probiotic product.

Conflicts of interests: none

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ARTIGO 3

Effects of *Bacillus subtilis* and *Shewanella algae* on digestive enzymes of white shrimp, *Litopenaeus vannamei* and its relationship with defense against *Vibrio parahaemolyticus* challenge.

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Effects of *Bacillus subtilis* and *Shewanella algae* on digestive enzymes of white shrimp, *Litopenaeus vannamei* and its relationship with defense against *Vibrio parahaemolyticus* challenge.

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Abstract

The need for increased disease resistance, growth of aquatic organisms and feed efficiency has brought about the use of probiotics in aquaculture practices. Based on this approach, the aim of the present study was measured the digestive enzymes activities of *L. vannamei* juveniles fed with *B. subtilis* (IPA-S.51) and *S. algae* (IPA-S.252) supplemented feed. The treatments were designated as follows: feed + *B. subtilis* (IPA-S.51), feed + *S. algae* (IPA-S.252) and feed without bacteria (Control). Shrimps of all the treatments were subsequently challenged with *V. parahaemolyticus* via injection and immersion. The bacterial supplemented feed was offered to *L. vannamei* during 60 days. Shrimp hepatopancreas were collected after 15, 30, 45 and after *Vibrio* challenges to measure total proteolytic, chymotrypsin, trypsin, amylase and aminopeptidase activities by *in vitro* assays. At the end of 60 days, highest values of final weight, WGR, DWG and SGR were obtained by shrimps fed with *S. algae* and *B. subtilis* when compared with Control. *B. subtilis* and *S. algae* administration provide an enhance in *L. vannamei* immune status, once that shrimp cumulative mortality after 48h of *Vibrio* injective was lower to IPA-S.252 and to both IPA-S.252 and IPA-S.51 after immersion challenge. Total proteolytic, trypsin and chymotrypsin activities were improved and seems to influence the higher growth parameters registered to shrimps fed with *B. subtilis* and *S. algae* supplemented feed. Besides that, *V. parahaemolyticus* challenges influenced the glycine aminopeptidase level in the same mode as previously verified to a WSSV infection, thus, glycine aminopeptidase level can further use as specific parameter for an infection recognize.

Keywords: *Bacillus subtilis*, *Shewanella* algae, probiotic, enzyme activity, hepatopancreas, *Litopenaeus vannamei.*

1.Introduction

The growth of aquaculture as an industry has accelerated over the past decades and resulted in environmental damages and low productivity of crops. The need for increased disease

resistance, growth of aquatic organisms, and feed efficiency has brought about the use of probiotics in aquaculture practices (Cruz et al., 2012). The probiotics intake can improve microbial balance, leading to improve food absorption, digestive enzyme activity and reduction of problems regarding to shrimp gastrointestinal pathogens, e. g. *V. parahaemolyticus*, etiological agent of AHPNS (Acute Hepatopancreatic Necrosis Syndrome) or EMS (Early Mortality Syndrome) (Balcazar et al., 2006; Vine et. al., 2006; Leano et al., 2012; Joshi et al., 2014). Since 2009, EMS has caused a huge decline in the production of farmed white shrimp (*L. vannamei*) (Soonthornchai et al., 2015).

The metabolic activity of the bacterial gastrointestinal community plays an important role in maintaining the health of the host organism since one of its basic functions within the intestinal microbiota is digestion (Ushakovaa et al, 2015). The composition and peculiar characteristics of intestinal microbiota metabolism can be influenced by several factors such as: diversity, composition, physicochemical properties of nutrients provided in the diet, pH, host age, immunity and secretion of specific bacterial metabolites (Gibson and Roberfroid, 1995; Meimandipour et al., 2009; Perfenov and Bondarenko, 2012).

The digestive organs are sensitive to food components that cause immediate changes in the health and growth of animals by increasing the enzymatic activity of the digestive tract which consequently improves the absorption of food (Shan et al., 2008; Mohapatra et al., 2013; Chai et al. 2016). Feed additives can also directly stimulate relevant digestive enzymes, for example, by the production of small peptides that can stimulate specific cellular effectors (Teshima et al, 1993; Espe et al., 1999; Bairagi et al., 2002).

The bioactive peptides production has also been considered as an effective mode of antioxidant activity in diets containing probiotic bacteria (Syngai et al., 2016). Some studies provide evidence that greater amounts of dietary protein levels also improve immune system functionality, increasing shrimp's hemocytes concentration (Pascual et al., 2004).

Bacterial species of *Bacillus* genus are capable of producing a wide variety of extracellular substances and antimicrobial peptides (Perez, et al., 1993; Korenblum et al., 2005).

Zokaeifar et al. (2012) report a high level of digestive enzyme activity in *L. vannamei* fed with a *B. subtilis* supplemented diet. Similar results were published by Xu and Pan (2012). According to the authors, shrimps reared in bioflocs systems exhibited relatively high protease and amylase activities, indicating that relevant extracellular enzymes could be produced by *Bacillus* sp. attaching to the bioflocs.

Other studies also reports the benefits of *Shewanella* spp., *Shewanella algae*, *Shewanella putrefaciens* and *Shewanella baltica* (Díaz-Rosales et al., 2009; Zadeh et al., 2010), in the control of shrimp and fish diseases, including vibriosis. In fact, several species of *Shewanella* have demonstrated antimicrobial activity against different pathogens, despite limited information on their mechanisms (Makridis et al., 2008; Zadeh et al., 2010), and enzyme activity contribution. Ariole and Ekeke (2016), showed that an indigenous probiotic (*S. algae*) previously isolated from healthy shrimp (*Penaeus monodon*) intestine was able to produce extracellular enzymes such as protease, amylase, lipase and gelatinase which could improve feed digestibility and feed utilization. However, this study was performed by *in vitro* tests; therefore, a study about the enzymatic activity of shrimp digestive tract fed with *S. algae* is necessary, as well the shrimp growth analysis in order to confirm the *S. algae* probiotic potential for shrimp farming.

Based on previous studies about *B. subtilis* and *S. algae* as potential probiotics for aquaculture, the objective of the present study was measured an digestive enzymes activity of *L. vannamei* juveniles fed with *B. subtilis* and *S. algae* supplemented diets and investigate its relationship with shrimp growth. In addition, the work verifies an influence of a *V. parahaemolyticus* challenge in shrimps digestive enzymes production.

2. Materials and methods

2.1 *L. vannamei* juveniles rearing

L. vannamei post larvae (PL10) were obtained from a commercial hatchery (Aquatec®), located in Canguaretama, Rio Grande do Norte and transported to LTA / UFRPE (Laboratório de

Tecnologia em Aquicultura/ Universidade Federal Rural de Pernambuco). The post larvae were kept in storage tanks until they had an average weight of 1.05 ± 0.25g. After reaching the ideal weight, juveniles were kept in polypropylene tanks (width x length x height = 0.48 m x 0.56 m x 0.89 m) containing 50 L of sea water (23 g / L) previously disinfected with sodium hypochlorite (40 ppm) during 24 h. Later, the seawater was dechlorinated with ascorbic acid (1g $1000L^{-1}$). The seawater was also filtered in 50, 25, 5 and 1 micron filters, received an ultraviolet light treatment and then maintained with continuous aeration. The water temperature was stabilized at 28 - 29 ° C through thermostatic heaters and 25% of the water was changed daily.

During the whole experiment (60 days), the shrimps were fed four times a day. The amount of feed supplied was equivalent to 10% of the biomass of each tank. The shrimp were distributed in three treatments with four replicates each, according to the bacterial species added in the feed: IPA-S.51 (*Bacillus subtilis*), IPA-S.252 (*Shewanella algae*), and Control, without addition of bacteria.

Growth parameters at the end of 60 days of rearing including survival rate (*final number* of *shrimp*/*initial number* of *shrimp*) x 100), final weight, weight gain rate [WGR = (final weight - initial weight) x 100)], specific growth rate [SGR = (ln final weight-ln initial weight) / rearing days x 100] and daily weight gain [DWG = (weight gain / days of rearing)] of white shrimp juveniles were verified.

2.2 *L. vannamei* juveniles challenge with *V. parahaemolyticus* via injection and water

V. parahaemolyticus (ATCC 17802) was grown in Tryptone Soy Broth (TSB supplemented with 2% NaCl, Difco) for 24 h at 30ºC before being centrifuged at 1800 g for 20 min. Fluid supernatants were removed and the pellets re-suspended in sterile saline (2% NaCl) in order to stabilize a stock solution for challenge assays in a concentration between $3x10^7$ and 1×10^7 $CFU.mL^{-1}$.

For the *V. parahaemolyticus* injective challenge, ten shrimps from each treatment (IPA-S.252, IPA-S.51 and Control), reared for 60 days were distributed in two experimental tanks, the same used for the previous rearing. The challenge was performed by injecting 100 μl of a *V. parahaemolyticus* suspension in shrimps third abdominal segment. During the challenge, the shrimps continued to be fed normally according to their respective treatments. The shrimps cumulative mortality after 24, 48, 72 and 96h of *V. parahaemolyticus* injective challenge was recorded.

For the *V. parahaemolyticus* immersion challenge, *V. parahaemolyticus* was cultivated, centrifuged and re-suspended as described for the *V. parahaemolyticus* injective challenge, to be subsequently added to the *L. vannamei* juveniles rearing water grown for 60 days fed with *B. subtilis* (IPA-S.51), *S. algae* (IPA-S.252) and without bacteria (Control). The challenge test was performed with 18 shrimps from each treatment randomly distributed in nine plots (three replicates for each treatment). The challenge was carried out in experimental plastic tanks with a useful volume of 10 liters filled with 5 liters. Five milliliters of a saline solution containing 10^8 CFU.mL^{-1} of *V. parahaemolyticus* were added to the shrimp rearing water and after 24 hours, the shrimps hepatopancreas was sampled for enzyme assays. The concentration of *V. parahaemolyticus* in water was 10^5 CFU.mL^{-1}. The shrimps continued to be fed normally according to their respective treatments and the cumulative mortality after ten days of *V. parahaemolyticus* immersion challenge was observed.

2.3 *L. vannamei* feed

B. subtilis and *S. algae* pure cells were previously isolated from the hepatopancreas / stomach of *L. vannamei* grown at 30g / L salinity. The strains were assigned by Instituto Agronômico de Pernambuco (IPA). *B. subtilis* and *S. algae* were cultured for 48 h, harvested by centrifugation and suspended in sterile sea water and then additioned in a 40% crude protein pelleted feed (Camaronina 40CR2, Purina, Agribrands do Brasil SA, São Paulo, Brazil) (Adapted from Jiang et al., 2013). At the end of the feed preparation, the bacterial concentration was

approximately 10^6 Colony Forming Units (CFU). g^{-1} of feed. After preparation, the feed was stored for a maximum period of 72 hours at 4 ° C, until use.

2.4 Enzyme extraction and total soluble protein determination

Four shrimps from each treatment (IPA-S.51, IPA-S.252 and Control) were sampled from the tanks after the 15, 30 and 45 days of rearing. Four shrimps challenged with *V. parahaemolyticus* via injection and rearing water from each treatment were also sampled at the end of 24h of challenges period. The hepatopancreas were dissected immediately after the animals were killing by decapitation and then homogenized (40 $mg.mL^{-1}$) in chilled 0.15 M NaCl solution using a Potter-Elvehjen tissue homogenizer (Bodine Electric Company, Chicago, IL , USA) at 30-50 rpm for 3 min at 4 ° C. The homogenates were centrifuged at 10,000 g for 25 min at 4 ° C, to remove tissue debris. The supernatants (crude enzyme extract) were stored at -20 ° C for further use. Total soluble protein was determined following the procedure described by Bradford (1976), using bovine serum albumin as a protein standard.

2.5 Enzyme activity assays

Total proteolytic activity assay were performed using azocasein as a substrate in microcentrifuge tubes. Triplicate samples of each enzyme extract (30 µL) were incubated with 10 gL^{-1} of azocasein (50 µL) dissolved in 0.1 M Tris-HCl pH8 for 60 min at 25 ° C (Bezerra et al. 2005). Then 100 $g.L^{-1}$ of trichloroacetic acid (120 µL) was added to stop the reaction and the mixture was centrifuged at 8000 g for 5 min. The supernatant (70 µL) was mixed with 1 M NaOH (130 µL) and the absorbance was measured on a microplate reader (Bio-Rad 680, Japan) at 450 nm. Blank samples were also tested similarly (with 9 g.L-1 NaCl instead of the crude extract). Protease activity was expressed as units per mg of protein. A unit (U) of enzymatic activity was defined as

the amount of enzyme required to hydrolyze azocasein and produce a change in absorbance of 0.001 mL -1 min -1.

The trypsin and chymotrypsin activities were determined in 96-well microplates using benzoyl-DL-arginine-p-nitroanilide (BApNA) and succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide (SApNA) as specific substrates respectively (Bezerra et al. 2005). Three replicates of enzymatic extracts (30 μL) were incubated in 4 mM BApNA and SApNA (30 μL) dissolved in dimethylsulfoxide (DMSO) and 0.1 M Tris-HCL (140 μL), pH 8.0. Reactions occurred at 25 ° C for 15 min and were recorded at 405 nm using a microplate reader. Activity was expressed as protease mU mg^{-1} of protein. A unit of protease activity is defined as the amount of enzyme required to produce 1 μmol of p-nitroaniline.min-1 from the substrates used.

Aminopeptidase activity was also evaluated using aminoacyl-β-naphthylamide as the substrate. The substrates used were: Ala, Arg, Leu, Ser, Gly, Ile, Tyr and His. The procedure was carried out by incubating 4.2 mM of substrate (50 μL), 50 mM of sodium phosphate buffer, pH 7.0, (600 μL) and H2O (50 μL) at 37ºC. After 120 min, the reaction was stopped by adding Garnet reagent (250 μl) prepared in sodium acetate buffer (0.2 M), pH 4.2, containing 100 ml L^{-1} of Tween 20. The absorbance was measured at 525 Nm and the amount of β-naphthylamide was determined using a standard (β-naphthylamide curve). Activity was expressed as amount of mU protease per mg^{-1} protein. A unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μmol of β-naphthylamide min^{-1} (Oliveira et al., 1999).

The amylase activity was determined using the Bernfeld (1955) method with 2% (w/v) starch solution as the substrate. The reaction consisted of 60 μL of the hepatopancreas crude extract, 375 μL of starch solution and 375 μL of 10 mM phosphate buffer (pH 7.5). After 10 min of incubation at 37 ° C, 100 μL of this mixture was added to 1 mL of 3,5-dinitrosalicylic acid solution (DNSA) and kept in boiling water in a water bath for 10 minutes to stop the reaction. All tests was performed in quadruplicate. One unit of amylase activity was expressed as mg of maltose released at 37 ° C per minute per mg of protein.

2.6 Statistics

The enzymatic activities and growth data were submitted to the normality and homogeneity analysis. Differences between enzymatic activities means were analyzed using One way ANOVA and Tukey or Fisher post hoc test, taking into account the necessary premises. The tests were processed through the program Origin 8.0 (Software, Inc., U.S.).

3. Results

3.1 Growth and survival of *L. vannamei* juveniles.

Shrimps growth performance such as survival rate, final weight, WGR, DWG and SGR after 60 days of rearing are presented in Table 1.

Table 1. Average values ± SE of *L. vannamei* juvenile growth after 60 days of rearing[1]

Treatment	Survival (%)	Final weight (g)	WGR (%)	DWG ($g.dia^{-1}$)	SGR (%)
Control	86.1 ± 8.2	7.68 ± 0.1^{a}	662.6 ± 11.6^{a}	0.11 ± 0.001^{a}	3.31 ± 0.02^{a}
IPA-S.51	97.3 ± 3	8.4 ± 0.1^{b}	734.6 ± 14^{b}	0.12 ± 0.002^{b}	3.46 ± 0.03^{b}
IPA-S.252	73.1 ± 17	9.35 ± 0.1^{c}	830 ± 11^{c}	0.14 ± 0.002^{c}	3.65 ± 0.02^{c}

[1]Different superscript letters in the same column indicate significant differences between treatments ($P < 0.05$).

Survival rate did not presented differences between treatments. Highest values of final weight, WGR, DWG and SGR were obtained by shrimps fed with *S. algae* and commercial feed when compared with the other treatments. Shrimps fed with *B. subtilis* and commercial feed also presented better results to all the growth parameters compared to the Control.

Shrimp cummulative mortality ± SE after 24h of *V. parahaemolyticus* injective challenge did not have differences: IPA-S.252 (65 ± 5%), IPA-S.51 (75 ± 5%) and Control (85 ± 5%). At 48h

post-challenge, differences between IPA-S.252 (85 ± 5%) and Control (100%) were observed. IPA-S.51 (92.5 ± 2.5%) cumulative mortality was similar to the other treatments and 100% of mortality was observed at 72 and 96h of challenge to all treatments. After ten days of *V. parahaemolyticus* immersion challenge, cumulative mortality of IPA-S.252 (33.3%) and IPA-S.51 (16.7%) were lower than the Control (58.3 ± 8.3%). Differences in IPA-S.252 and IPA-S.51cumulative mortality were not found.

3.2 Enzyme activity assays

Figure 1 displays the total proteolytic activity (azocasein as substrate) in *L. vannamei* hepatopancreas fed with *S. algae* (IPA-S.252) and *B. subtilis* (IPA-S.51) supplemented feed and without bacteria (Control) during 15, 30, 45 and 60 days + *V. parahaemolyticus* injective and immersion challenge. After 15 and 30 days of rearing, the proteolytic activity of IPA-S.252 and IPA-S.51 were higher than the Control. After these periods, IPA-S.252 proteolytic activity was also greater than the values verified to IPA-S.51. After 45 days of rearing, similar greatest proteolytic activity values were recorded to IPA-S.252 (6.55 ± 0.32 $mU.mg^{-1}$) and IPA-S.51 (6.66 ± 0.25 $mU.mg^{-1}$) compared to Control (4.82 ± 0.14 $mU.mg^{-1}$).

Proteolytic activities registered after challenges were approximately five times greater than the observed after 15, 30 and 45 days of rearing (Figure 1). IPA-S.252 proteolytic levels after injective and immersion challenges were lower than the registered to the other treatments, the levels verified were 22.58 ± 0.45 and 16.13 ± 0.6 $mU.mg^{-1}$, respectively. After injective challenge, the activity of IPA-S.51 (24.23 ± 1.15 $mU.mg^{-1}$) and Control (24.42 ± 1.47 $mU.mg^{-1}$) were similar. After immersion challenge, the highest value was verified to IPA-S.51 (23.83 ± 0.9 $mU.mg^{-1}$) compared to the other treatments.

Differences between proteolytic activities over time in the treatments were analyzed. IPA-S.252 proteolytic activities decreased since 15 days until 45 days of rearing (Figure 1). However the highest proteolytic level for IPA-S.252 treatment was recorded after injection challenge. IPA-S.51 activities were similar over the time and greater than the activities registered in the other periods.

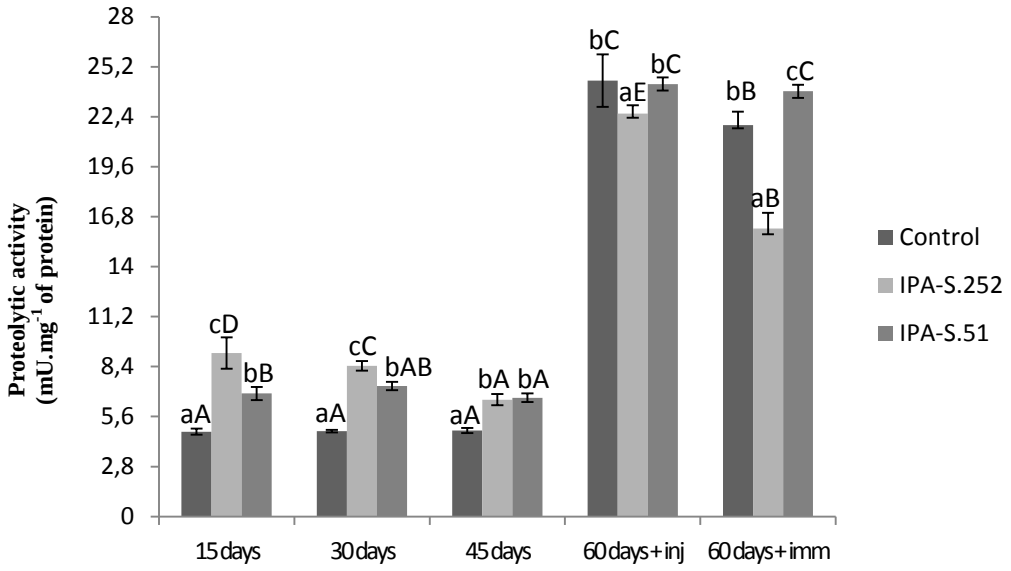

Figure 1. Total proteolytic activity in the hepatopancreas of *L. vannamei* fed with *B.subilis* + commercial feed (IPA-S.51), *S.algae* + commercial feed (IPA-S.252) and comercial feed without bacteria (Control) during 15, 30, 45 days and 60 days + *V. parahaemolyticus* injective and immersion challenge. Different overlapping lower case letters represent statistical differences between treatments ($P < 0.05$). Capital letters indicate significant differences between proteolytic activity in the same treatments and different rearing periods ($P < 0.05$).

Chymotrypsin activity was initially shown to be higher for IPA-S.252 (1.06 mU.mg-^{1}) than to IPA-S.51 (0.06 ± 0.08 mU.mg-^{1}) and Control (0.58 ± 0.07 mU.mg-^{1}) (Figure 2). This activity remained greater after 30 days of rearing (0.9 ± 0.01 mU.mg-^{1}) as observed for total proteolytic activity, IPA-S.51 and Control activities were 0.69 ± 0.05 and 0.36 ± 0.04 mU.mg-^{1}, respectively.

After 45 days of rearing, IPA-S.51 had the highest activity (0.65 ± 0.02 mU.mg-^{1}), IPA-S.252 and Control presented similar activities: 0.46 ± 0.07 and 0.48 ± 0.07 mU.mg-^{1}, respectively. After both challenges, IPA-S.252 and IPA-S.51 had greater chymotrypsin levels than Control (Figure 2). After injective challenge, IPA-S.252 and IPA-S.51 chymotrypsin levels were 10.08 ± 1.22 and 12.84 ± 1.47 mU.mg-^{1}, respectively and Control, 4.22 ± 0.29 mU.mg-^{1}. After immersion challenge, chymotrypsin level of IPA-S.252 (23.04 ± 0.48 mU.mg-^{1}) was greater than the level verified to IPA-S.51 (15.21 ± 0.73 mU.mg-^{1}) and Control (5.71 ± 0.97 mU.mg-^{1}).

Chymotrypsin activities after 15, 30 and 45 days of rearing were expressively lower than the registered after challenges (Figure 2). Initially, IPA-S.252 activities did not presented differences, but after 45 days of rearing, its activity decreased. However, after immersion challenge, IPA-S.252 had the highest activity compared to the obtained after the other periods of rearing. Similarly to IPA-S.252, after challenges, IPA-S.51 also had a greater chymotrypsin activity than the other periods of rearing. IPA-S.51 activity after immersion challenge was higher than the value found after injective challenge. Differences were not detected between values recorded after 15, 30 and 45 days of rearing to IPA-S.51.

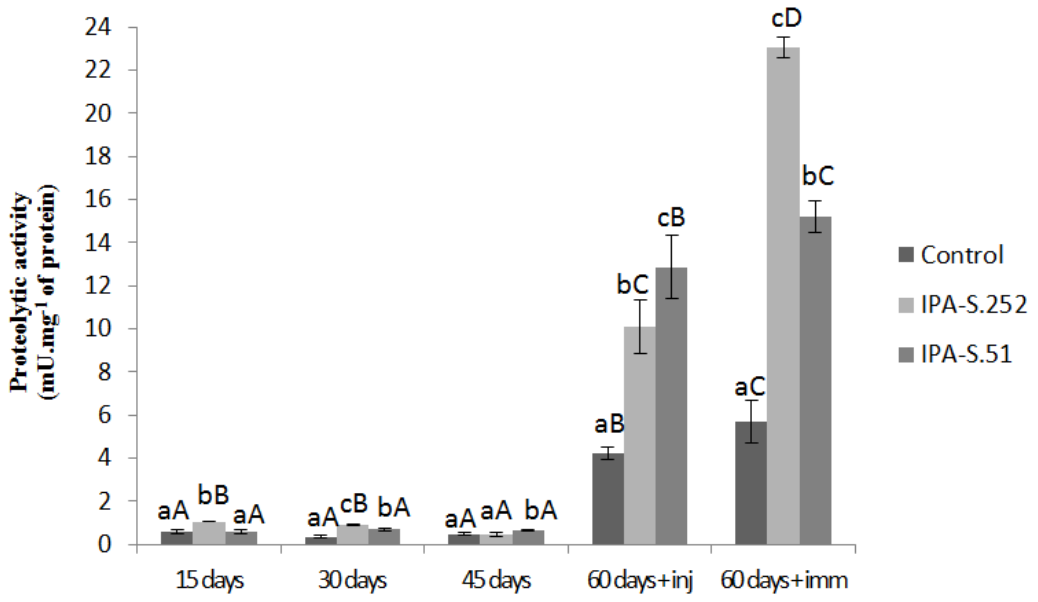

Figure 2. Chymotrypsin activity in the hepatopancreas of *L. vannamei* fed with *B.subilis* + commercial feed (IPA-S.51), *S.algae* + commercial feed (IPA-S.252) and comercial feed without bacteria (Control) during 15, 30, 45 days and 60 days + *V. parahaemolyticus* injective and immersion challenge. Different overlapping lower case letters represent statistical differences between treatments ($P < 0.05$). Capital letters indicate significant differences between proteolytic activity in the same treatments and different rearing periods ($P < 0.05$).

IPA-S.51trypsin activity after 15 days of rearing (9.78 ± 0.45 mU.mg-^{1}) and post injection challenge (70.67 ± 1.96 mU.mg-^{1}) were higher than the levels found to Control after the same periods: 5.48 ± 0.51 mU.mg-^{1}, and 38.68 ± 3.25 mU.mg-^{1}, respectively (Figure 3). IPA-S.252

trypsin activities were the lower observed after 30 (7.36 ± 0.28 mU.mg-^{1}), 45 (4.24 ± 0.19 mU.mg-^{1}), injection and immersion challenge (61.62 ± 5.39 mU.mg-^{1}) except after 15 days of rearing (9.68 ± 0.74 mU.mg-^{1}). Control had greater levels of trypsin activity since 30 days of rearing compared to IPA-S.252. In general, all values of trypsin activity found were higher after the challenges as observed to total proteolytic activity and chymotrypsin activity.

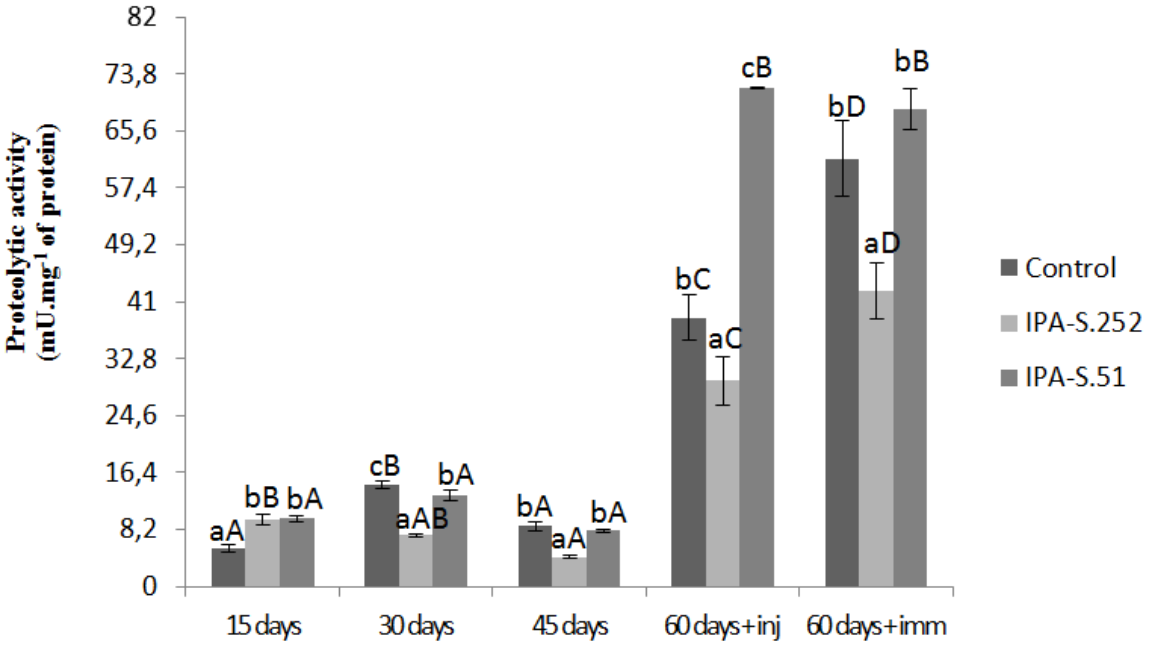

Figure 3. Trypsin activity in the hepatopancreas of *L. vannamei* fed with *B.subilis* + commercial feed (IPA-S.51), *S.algae* + commercial feed (IPA-S.252) and comercial feed without bacteria (Control) during 15, 30, 45 days and 60 days + *V. parahaemolyticus* injective and immersion challenge. Different overlapping lower case letters represent statistical differences between treatments ($P < 0.05$). Capital letters indicate significant differences between proteolytic activity in the same treatments and different rearing periods ($P < 0.05$).

Eight essential amino acids for penaeid shrimp were observed in the *L. vannamei* juveniles hepatopancreas (Table 2). In general, the aminopeptidase activities were greater for shrimps fed with bacterial supplemented feeds, principally for Gly- and Ile-. Over time, the treatments aminopeptidasic activities tended to be improved. The Gly- levels were approximately twice times higher after *V. parahaemolyticus* immersion challenge.

Higher levels of aminopeptidase activities were found for non-polar (Gly-) and aromatic (Tyr-) substrates. Lower levels occurred with hydrophobic (Pro-) and (Ala-) substrates. IPA-S.51 had greater values of Leu- activities than the other groups after 15, 30, 45 days and after injective and immersion challenges. IPA-S.51 also presented a greater Ile activity than Control (Table 2). Using the Gly- and Tyr- substrate, the highest aminopeptidasic activity over time was found after immersion challenge for IPA-S.252 (Table 2). The higher activity of Gly- registered from IPA-S.252 was also greater than IPA-S.51 and Control after the immersion challenge.

IPA-S.252 aminopeptidase activity using Ile- as substrate were improved over time and were higher than Control and IPA-S.51 after 30, 45 and 60 days + injective and immersion challenges. Control group had higher activities using Tyr- as substrate, after 15, 45 and 60 days + injective challenge in comparison to IPA-S.51 and IPA-S.252.

Table 2. Means ± SD of aminopeptidase activity (U mg^{-1} of protein) using aminoacyl b-naphthylamide as substrates (Gly, Ala, Pro, His, Arg, Leu, Ile, Arg and Tyr) in hepatopancreas extract of *L. vannamei* juveniles fed with commercial feed + *S.algae* (IPA-S.252), commercial feed + *B. subtilis* (IPA-S.51) and commercial feed without bacteria during 15, 30, 45 and 60 days + *V. parahaemolyticus* injective and immersion challenge[1].

Substrates	Treatments	15 days	30 days	45 days	60 days +inj	60 days +imm
Gly	Control	77.80 ± 4.16^{aA}	74.86 ± 2.20^{aA}	80.75 ± 0.02^{bA}	101.87 ± 2.16^{aB}	209.90 ± 9.01^{aC}
	IPA-S.252	93.74 ± 8.33^{aA}	94.58 ± 2.84^{bA}	86.47 ± 1.06^{cA}	157.35 ± 12.94^{bB}	344.27 ± 9.36^{cC}
	IPA-S.51	83.68 ± 2.57^{aB}	88.05 ± 3.75^{bB}	66.81 ± 1.75^{aA}	195.24 ± 0.04^{cC}	258.68 ± 0.83^{bD}
Ala	Control	2.21 ± 0.2^{aAB}	0.48 ± 0.004^{aA}	3 ± 0.56^{aB}	9.18 ± 0.69^{aC}	34.63 ± 1.26^{cD}
	IPA-S.252	4.59 ± 0.34^{bB}	3.83 ± 0.3^{bA}	8.95 ± 0.8^{bC}	29.34 ± 0.42^{cE}	17.48 ± 0.1^{bD}
	IPA-S.51	8.49 ± 0.6^{cB}	3.85 ± 0.34^{bA}	17.25 ± 1.89^{cC}	19.04 ± 1.28^{bC}	9.70 ± 0.74^{aB}
Pro	Control	11.33 ± 1.65^{bB}	12.5 ± 0.85^{bB}	7.82 ± 0.77^{bA}	7.56 ± 0.43^{aA}	20.49 ± 1.69^{aC}
	IPA-S.252	26.99 ± 0.03^{cC}	5.84 ± 0.6^{aA}	5.32 ± 0.35^{aA}	16.32 ± 1^{cB}	27.41 ± 2.18^{bC}
	IPA-S.51	4.95 ± 0.49^{aA}	15.02 ± 1^{bC}	10.13 ± 0.67^{cB}	10.17 ± 0.95^{bB}	17.44 ± 0.85^{aD}
His	Control	9.21 ± 0.51^{aA}	9.58 ± 0.18^{aA}	8.85 ± 0.86^{abA}	33.25 ± 0.002^{aC}	23.79 ± 1.28^{bB}
	IPA-S.252	9.80 ± 0.51^{aB}	9.64 ± 0.63^{aB}	7.33 ± 0.69^{aA}	34.20 ± 2.14^{aD}	16.89 ± 0.002^{aC}
	IPA-S.51	12.28 ± 0.70^{bA}	11.91 ± 0.17^{bA}	9.49 ± 0.56^{bA}	30.06 ± 2.77^{aB}	39.40 ± 0.42^{cC}
Leu	Control	5.87 ± 0.52^{aA}	12.37 ± 0.35^{aB}	20.97 ± 0.86^{bC}	33.25 ± 1.71^{bD}	52.70 ± 1.30^{bE}
	IPA-S.252	5.50 ± 0.32^{aA}	14.69 ± 0.68^{bB}	14.34 ± 0.36^{aB}	17.54 ± 0.01^{aC}	37.99 ± 0.86^{aD}
	IPA-S.51	17.34 ± 0.68^{bA}	16.36 ± 0.004^{cA}	20.27 ± 0.68^{bA}	44.43 ± 3^{cC}	33.38 ± 2.11^{aB}
Ile	Control	13.09 ± 1.25^{aA}	12.20 ± 0.36^{aA}	15.75 ± 0.007^{aB}	25.70 ± 0.43^{aC}	39.76 ± 0.61^{aD}
	IPA-S.252	12.55 ± 0.33^{aA}	15.66 ± 0.63^{bB}	18.36 ± 0.33^{bC}	37.80 ± 0.44^{bD}	42.21 ± 0.01^{bE}
	IPA-S.51	15.41 ± 1.02^{aA}	14.20 ± 1.05^{bA}	14.71 ± 0.96^{aA}	25.08 ± 3.84^{aB}	41.80 ± 1.27^{bC}
Arg	Control	17.61 ± 1.02^{bA}	17.47 ± 1.02^{aA}	24 ± 2.41^{aA}	90.07 ± 7.69^{bC}	40.95 ± 2.56^{aB}
	IPA-S.252	17.74 ± 1.20^{bA}	26.37 ± 0.85^{bA}	20.79 ± 1.01^{aA}	52.48 ± 3.19^{aB}	80.82 ± 6.81^{cC}
	IPA-S.51	14.09 ± 0.17^{aA}	30.08 ± 0.47^{cC}	23 ± 0.56^{aB}	78.56 ± 1.70^{bE}	55.93 ± 0.86^{bD}
Tyr	Control	107.74 ± 4.45^{cA}	110.89 ± 6.21^{aA}	104.60 ± 1.57^{cA}	181.67 ± 1.33^{cC}	62.66 ± 0.006^{aB}
	IPA-S.252	23.54 ± 1.64^{aA}	168.24 ± 3.95^{cC}	30.21 ± 0.28^{aB}	169.45 ± 0.11^{bC}	191.43 ± 10.64^{bD}
	IPA-S.51	70.41 ± 5.06^{bAB}	143.66 ± 1.32^{bC}	84.94 ± 3.41^{bB}	57.34 ± 4.59^{aA}	266.81 ± 10.65^{cD}

[1]Different superscript capital letters in the same column indicate significant differences between treatments ($P < 0.05$). Different superscript lowercase letters in the same line indicate significant differences between rearing periods ($P < 0.05$).

Assays about the effect of bacterial supplemented feed in amylase activity of *L. vannamei* hepatopancreas showed a similar pattern as observed in the total proteolytic, chymotrypsin, trypsin and peptidases activities (Figure 4). The higher values were observed after *V. parahaemolyticus* challenges for all the treatments. IPA-S.252 had lower amylase activities after 30, 45 and 60 days + injective and immersion challenges compared to Control and IPA-S.51 and shrimps fed with *S. algae* also presented lower levels of trypsin activities after the same periods of rearing. Likewise, IPA-S.252 had lower values of total proteolytic activity after challenges.

Only after 15 days, the amylolytic activity was greater to IPA-S.252 (8.93 ± 0.22 U mg^{-1}) than IPA-S.51 (7.38 ± 0.04 U mg^{-1}) and similar to Control (8.71 ± 0.31 U mg^{-1}) (Figure 4). Significantly differences of Control amylolytic activities, over time, were observed. The activities were greater after immersion challenge than after injective challenge, the levels were 33 ± 0.8 and 32 ± 0.5 U mg^{-1}, respectively. Similar amylolytic activity pattern was observed to IPA-S.252, its activity level was higher after immersion challenge compared to the obtained after injective challenge, the activity values were 28.34 ± 2.02 and 31.69 ± 1 U mg^{-1}.

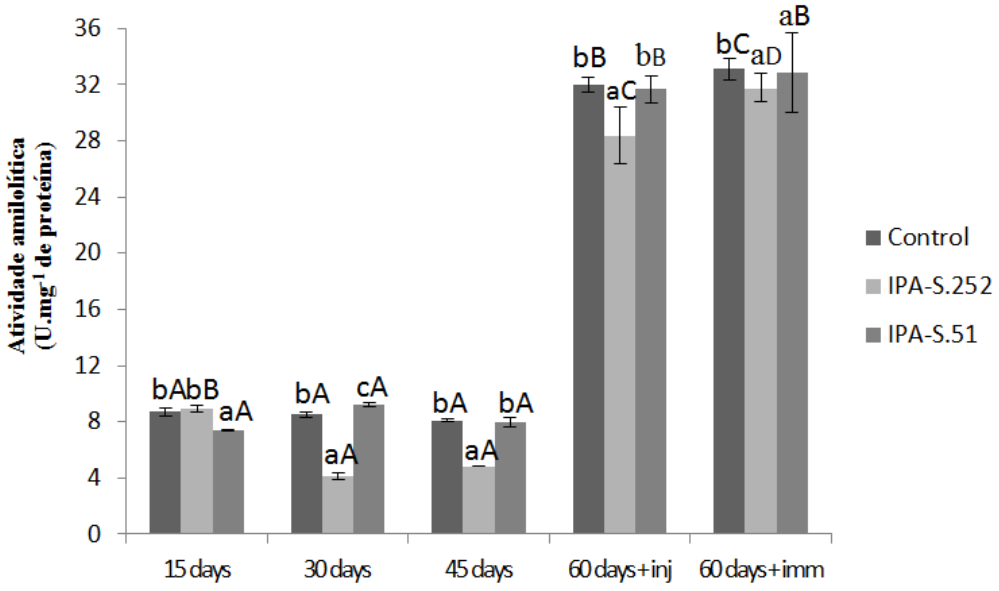

Figure 4 Amylase activity in the hepatopancreas of *L. vannamei* fed with *B.subilis* + commercial feed (IPA-S.51), *S.algae* + commercial feed (IPA-S.252) and comercial feed without bacteria (Control) during 15, 30, 45 days and 60 days + *V. parahaemolyticus* injective and immersion

challenge. Different overlapping lower case letters represent statistical differences between treatments ($P < 0.05$). Capital letters indicate significant differences between proteolytic activity in the same treatments and different rearing periods ($P < 0.05$).

4. Discussion

Bacterial strains isolated from *L. vannamei* digestive tract found to have the capacity for extracellular enzyme production, which may be relevant in the digestive processes and leads to nutrient and energy absorption of this species (Tzuc et al., 2014). In the present study, the feed supplementation of *L. vannamei* juveniles with *B. subtilis* and *S. algae* during 60 days of rearing resulted in a significant increase in final weight, weight gain rate, daily weight gain and specific growth rate of shrimps.

The increase in digestive enzyme activities can improve the feed digestion and growth performance. This effect was in accordance with results obtained in the present study. Total proteolytic activity was higher to IPA-S.252 and IPA-S.51 after 45 days. IPA-S.51 also had a higher proteolytic activity compared to Control and IPA-S.252 after immersion challenge.

Heterotrophic bacteria were linked with the increase of protein in animals cultures once that they were able to produce exogenous enzymes that stimulate the production of endogenous enzymes by shrimp (Ong and Johnston, 2006; Zhou et al, 2009; Ziaei-Nejade et al., 2006; Becerra-Dórame et al., 2012). Trypsin and chymotrypsin are endoproteases, e.g. they cleave the peptide bonds within the protein, whereas aminopeptidases are exoproteases, that is, cleave amino acid residues at the N-terminal position of the protein (Gonzales and Robert-Baudouy, 1996).

In the present study, chymotrypsin activity was produced by IPA-S.252 and had great values than IPA-S.51 and Control after 30 days of rearing and after immersion challenge. Gong et al. (2014) analyzed the chymotrypsin transcript expression of mud crab (*Scylla paramamosain*) in order to determine chymotrypsin influence in immune reactions after bacterial challenge with *V. alginolyticus.* The authors found a significantly increase about 20-fold in chymotrypsin mRNA

expression in crabs injected with *V. alginolyticus* compared to the Control group. Pan et al. (2005) and Zhao et al. (2007) also reports the hepatopancreas role in immune responses after bacterial infection.

Dimopoulos et al. (2000) have identified serine proteases, as digestive enzymes e.g. chymotrypsin and trypsin involved on the prophenoloxidase (ProPO) cascade enzyme activation. Serine proteases have been associated as regulatory proteins in activating the proPO and clotting systems of *Fenneropenaeus chinensis*, *Penaeus monodon* and *L. vannamei* (Supungul et al., 2002; Jimenez-Vega et al., 2005; Dong and Xiang, 2007; Xue et al., 2013). The proPO system triggers the melanization process in response to the identification of foreign agents by hemocytes (Soderhall and Cerenius, 1992).

Trypsin activity of IPA-S.51 was greater than Control after 15 days of rearing and after injective and immersion challenges. Chymotrypsin was observed to be involved in innate immune reactions after bacterial and viral challenges (Shi *et al.*, 2008). This correlation between digestive enzymes and immune functions was reported by Roszer (2014). The author cites the epithelial cells of the midintestinal gland (hepatopancreas) are major sources of immune molecules, such as lectins, hemocyanin, ferritin, antibacterial and antiviral proteins, proteolytic enzymes and nitric oxide.

Wu et al., (2017) observed that during a White spot syndrome virus (WSSV) infection, 14 amino acids activities were up-regulated and glycine level was increased twice. Similarly, in the present work, the glycine aminopeptidase dosage level was approximately twice as high after the challenge by immersion with *V. parahaemolyticus* for all treatments, however the administration of *B. subtilis* and *S. algae* through the diet induced a greater level of glycine aminopeptidase after bacterial challenge via injection compared to Control.

Aminopeptidases have been widely investigated by biochemical studies and their dosage is a diagnostic or preventive measure in some pathologies related to their physiological role. Degradation of peptides by aminopeptidases leads to the formation of amino acids, such as leucine,

valine, phenylalanine or methionine, and volatile compounds such as aldehydes, alcohols and acids that can be observed even at low concentrations (Papamanoli et al., 2003).

The ten essential aminoacids for farmed shrimps are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Tyrosine and cystine are semi-essential and can reduce the requirement for phenylalanine and methionine in diets (Guillaume, 1997). This study recorded the highest tyrosine activities from Control after 15, 45 and 60 days + injective challenge in comparison to IPA-S.51 and IPA-S.252.

The ten essential amino acids for cultured prawns are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Tyrosine and cystine are semi-essential amino acids and can reduce the requirement of phenylalanine and methionine in diets, since cystine and tyrosine can be synthesized by the animal from methionine and phenylalanine, respectively (Tacon, 1989; Guillaume, 1997; Stech, 1999). If tyrosine is included in the diet the amount of phenylalanine required decreases (Wilson, 2003). In the present study, tyrosine aminopeptidase dosage to Control after 15, 45 and 60 days + challenge with *V. parahaemolyticus* was higher than the dosage registered to IPA-S.51 and IPA-S.252.

According to Boone and Schoffeniels (1979) and Waxman (1975), valine, leucine and isoleucine levels are increased in crustaceans when an infection occur and these aminoacids are constituents of hemocyanins. The hemocyanin is not involved solely in oxygen transport within the hemolymph of invertebrates, hemocyanin and hemocyanin-derived peptides have been linked to innate immunity (Coates and Nairn, 2014). Decker and Jaenicke (2004) observed antimicrobial peptides derived from hemocyanin during immune challenge. Hemocyanin derived peptide sequences contain positively charged residues of histidine, indicative of antimicrobial peptides (AMPs) (Coates and Nairn, 2014).

Indeed, IPA-S.252 had greater values compared to Control and IPA-S.51 of leucine activity since 30 days of rearing. A higher Ile level were also recorded to IPA-S.51 after immersion challenge compared to Control. This results suggest an influence of *B. subtilis* and *S. algae* in *L.*

vannamei juveniles immune response, once that shrimp cumulative mortality after 48h post injective were significantly lower to IPA-S.252 than the Control and after ten days of immersion challenge. IPA-S.51 also presented lower cumulative mortality than the Control after immersion challenge.

In the present work, there was also observed an increment of amylase activity after *V. parahaemolyticus* challenge as observed to proteases activities. However, after both challenges, only shrimps fed with *B. subtilis* presented greater amylase activities compared to Control. Chai et al., (2016) recorded a significantly increasing in amylase of shrimps hepatopancreas fed with *Bacillus* PC465 isolated from *Fenneropenaeus chinensis* gut compared the Control group.

Zhou et al. (2009), found an increase of amylase and protease activities of lipase in *L. vannamei* treated with *B. coagulans* SC8168 and linked this events with a consequent increase in digestion and absorption of food and survival improvement. Indeed, *Bacillus* is known as a producer of extracellular protease and amylase (Jamilah et al., 2009).

IPA-S.252 had lower amylase activities after 30, 45 and 60 days + injective and immersion challenges compared to Control and IPA-S.51. Until the present moment, there was not reported effects of *Vibrio* challenges in crustaceans amylase activity. However, this event did not influenced the shrimp growth performance, since the growth parameters were higher to shrimps that received *B. subtilis* and *S. algae* in its feed. Lower trypsin activities of IPA-S.252 after 30, 45 and 60 days + injective and immersion challenges and total proteolytic activity after challenges were recorded and also did not affect the shrimps growth, once that the chymotrypsin levels of IPA-S.252 were higher and could be improve the proteins absorption.

According to Fox et al. (1994) and Shiau, (1998), *L. vannamei* is able to increasing or decreasing trypsin production to digest dietary protein and obtain the amino acids needed for protein synthesis and avoid self-damage to tissues. Trypsin activity in *L. vannamei* hepatopancreas is modulated by internal and external factors linked with shrimp ontogeny, origin and quantity of

feed as well feeding frequency, molting and stress factors (Hernández-Cortés et al., 1999; Le Vay et al., 2001; Sanchez-Paz et al., 2003; Córdova-Murueta et al., 2004; Sainz et al., 2005).

5. Conclusions

B. subtilis and *S. algae* administration provide an enhance in *L. vannamei* juveniles immune status, once that total proteolytic, trypsin, chymotrypsin and amylase activities were improved and cumulative mortality reduced after injective and immersion challenges with *V. parahaemolyticus.* The increase in digestive enzyme activities are suggested to influence the higher growth parameters registered to shrimps fed with *B. subtilis* and *S. algae* supplemented feed. Besides that *V. parahaemolyticus* challenges influenced the glicyne aminopeptidase level as previously verified to a WSSV infection, thus, we suggest that glycine aminopeptidase level can further use as specific parameters for an infection recognize.

Conflicts of interests: none

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7 CONSIDERAÇÕES FINAIS

Neste estudo, observou-se que a utilização de *B. subtilis* e *S. algae* isolados do trato digestório de *L. vannamei* tiveram efeito positivo no antagonismo contra patógenos em diferentes condições físico-químicas e de crescimento e também reduziram a carga de *Vibrio* spp. no hepatopâncreas de camarões da espécie *Litopenaeus vannamei*. A administração de *B. subtilis* aumentou os níveis de expressão de importantes transcritos para a resposta imunológica dos animais como a profenoloxidase (proPO), Proteína de Ligação a Lipopolisacarideo- e β-1,3-glucano (LGBP) e hemocianina (HEM) antes e após desafio com *V. parahaemolyticus*. Apesar da oferta de *S. algae* apenas ter estimulado uma maior expressão de LGBP, a mortalidade acumulada após 48h de desafio dos camarões que receberam *S. algae* em sua alimentação foi menor que a do grupo Controle desafiado.

As atividades enzimáticas de proteases totais, quimotripsina e tripsina do hepatopâncreas dos camarões cultivados com *B. subtilis* e *S. algae* evidenciaram que essas bactérias podem propiciar uma maior performance de crescimento, uma vez que o peso final dos grupos que receberam os isolados em sua alimentação foi superior ao grupo Controle após 60 dias de cultivo. Além disso, o nível de glicina aminopeptidase dos camarões desafiados com o *Vibrio* foi modulado positivamente assim como o nível de glicina encontrado em camarões infectados por WSSV. Os níveis de aminopeptidases fornecem informações importantes que podem ser úteis para uma mais rápida e fácil detecção de infecções por microrganismos. Considerando os resultados obtidos, as cepas estudadas foram consideradas aptas para serem acrescentadas em cultivos em escala-piloto e posteriormente participarem de testes para o desenvolvimento de um produto probiótico para a carcinicultura.

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ANEXO A - NORMAS DO PERIÓDICO AQUACULTURE

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ANEXO B - NORMAS DO PERIÓDICO FISH AND SHELLFISH IMMUNOLOGY

New submissions

Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts your files to a single PDF file, which is used in the peer-review process. As part of the Your Paper Your Way service, you may choose to submit your manuscript as a single file to be used in the refereeing process. This can be a PDF file or a Word document, in any format or layout that can be used by referees to evaluate your manuscript. It should contain high enough quality figures for refereeing. If you prefer to do so, you may still provide all or some of the source files at the initial submission. Please note that individual figure files larger than 10 MB must be uploaded separately.

References

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct.

Formatting requirements

There are no strict formatting requirements but all manuscripts must contain the essential elements needed to convey your manuscript, for example Abstract, Keywords, Introduction, Materials and Methods, Results, Conclusions, Artwork and Tables with Captions. If your article includes any Videos and/or other Supplementary material, this should be included in your initial submission for peer review purposes. Divide the article into clearly defined sections. Please ensure your paper has consecutive line numbering - this is an essential peer review requirement. Figures and tables embedded in text. Please ensure the figures and the tables included in the single file are placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file.

Revised submissions

Use of word processing software Regardless of the file format of the original submission, at revision you must provide us with an editable file of the entire article. Keep the layout of the text as

simple as possible. Most formatting codes will be removed and replaced on processing the article. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: https://www.elsevier.com/guidepublication). See also the section on Electronic artwork. To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor. Subdivision - numbered sections Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

ANEXO C - NORMAS DO PERIÓDICO ANIMAL FEED SCIENCE AND TECHNOLOGY

Submission

Our online submission system guides you stepwise through the process of entering your article details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail. Poorly written and/or presented manuscripts (relative to the journal's guidelines) may be returned to authors for upgrading by the editorial office, prior to a review for scientific merit. Before preparing their manuscript, it is suggested that authors examine the editorial by the Editorsin-Chief in Vol. 134/3-4, which outlines several practices and strategies of manuscript preparation that the Editors-in-Chief have found to be successful. This editorial also outlines practices that can lead to difficulties with reviewers and/or rejection of the manuscript for publication. There is also an example of an Animal Feed Science and Technology manuscript available on the journal website at http://www.elsevier.com/locate/anifeedsci. Submit your article Please submit your article via https://www.evise.com/evise/jrnl/ANIFEE.

Referees

Please submit the names and institutional e-mail addresses of several potential referees. For more details, visit our Support site. Note that the editor retains the sole right to decide whether or not the suggested reviewers are used. PREPARATION AUTHOR INFORMATION PACK 15 Jun 2017 www.elsevier.com/locate/anifeedsci 8 Peer review This journal operates a single blind review process. All contributions will be initially assessed by the editor for suitability for the journal. Papers deemed suitable are then typically sent to a minimum of two independent expert reviewers to assess the scientific quality of the paper. The Editor is responsible for the final decision regarding acceptance or rejection of articles. The Editor's decision is final.

More information on types of peer review.

Use past tense for current findings, and the present tense for "truths" and hypotheses. Article Structure Manuscripts should have numbered lines, with wide margins and double spacing throughout, i.e. also for abstracts, footnotes and references. Every page of the manuscript, including the title page, references, tables, etc., should be numbered continuously. However, in the text no reference should be made to page numbers; if necessary, one may refer to sections. Avoid excessive usage of italics to emphasize part of the text. Introduction State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described. If reference is made to AOAC, ISO or similar analytical procedure(s), the specific procedure identification number(s) must be cited. A number of references for neutral and acid detergent fibre (NDF, ADF) assays exist, and an alternative reference to the now out-of-print USDA Agriculture Handbook 379 must be used. There are many options for NDF and ADF assays (e.g. sodium sulfite, alpha amylase, residual ash), which must be specified in the text. For more details see the editorial in Vol. 118/3-4. The following definitions should be used, as appropriate: a. aNDFom-NDF assayed with a heat stable amylase and expressed exclusive of residual ash. b. NDFom-NDF not assayed with a heat stable amylase and expressed exclusive of residual ash. c. aNDF-NDF assayed with a heat stable amylase and expressed inclusive of residual ash. d. NDF-NDF assayed without a heat stable amylase and expressed inclusive of residual ash. e. ADFom-ADF expressed exclusive of residual ash. f. ADF-ADF expressed inclusive of residual ash. g. Lignin (sa)-Lignin determined by solubilization of cellulose with sulphuric acid. h. Lignin (pm)-Lignin determined by oxidation of lignin with permanganate. While expressions of NDF and ADF inclusive of residual ash will continue to be acceptable (i.e., the terms aNDF, NDF and ADF above), the Editors-in-Chief highly recommend reporting all fibre values, including digestibilities, on an OM basis. Silica is partially soluble in ND, is quantitatively recovered in AD, and so may contribute to the 'fibre' values and to subsequent digestibility coefficients. Reporting 'hemicellulose' values as the difference between NDF and ADF is generally only acceptable if the analyses have been sequential on the same sample. Crude fibre (CF), nitrogen-free extract (NFE) and total digestible nutrients (TDN) are not acceptable terms for describing feeds and should only be referred to in a historical context.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. Avoid extensive citations and discussion of published literature. Combined 'Results and Discussion' sections are only acceptable for 'Short Communications', except under compelling circumstances.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Title

Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

Author names and affiliations.

Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lowercase superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author. • Corresponding author. Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author. • Present/permanent address. If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

The abstract should be clear, descriptive and not longer than 400 words. It should contain the following specific information: purpose of study; experimental treatments used; results obtained, preferably with quantitative data; significance of findings; conclusions; implications of results if appropriate. Graphical abstract Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system.

Image size

Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. You can view Example Graphical

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of all formulations should be likewise identified. SI or SI-derived units should be used throughout (e.g. MJ and not Kcal for energy concentrations). Concentrations should be expressed on a 'per kg' basis (w/w); however, w/v, v/v, mol/mol or M may be accepted depending on the circumstances. In addition, 'units' and 'equivalents' are acceptable. Normality should be avoided, as it may be ambiguous for certain acids. If analytical standards have been used, they should be specified by name (e.g. yeast RNA) and form (e.g. lactose monohydrate). Percents should only be used when describing a relative increase or decrease in a response. Proportions should be maximum 1.0 or ≤1.0. For more details see the editorial in Vol. 118/3-4. Percent is only used to indicate relative changes. For composition, both w/w (often solids composition g/kg) and w/v (e.g. g/L), v/v (e.g. m/L), mol/mol or M can be accepted depending on the circumstances. Specify units (e.g. g/L) and never as percent. Digestibility/metabolisability and degradability should always be expressed as a coefficient (not %), and the content of, for example, the digestible component should be expressed as g/kg: thus, the coefficient of digestibility of dry matter is 0.8, while the content of digestible dry matter is 800g/ kg. A distinction between true and apparent digestibility should be made, as well as between faecal and ileal (e.g. coefficient of total tract apparent digestibility - CTTAD). The terms 'availability' and 'bioavailability' should be avoided without definition in context. In chemical formulae, valence of ions should be given as, e.g. Ca2+, not as Ca++. Isotope numbers should precede the symbols e.g. 18O. The repeated use of chemical formulae in the text is to be avoided where reasonably possible; instead, the name of the compound should be given in full. Exceptions may be made in the case of a very long name occurring very frequently or in the case of a compound being described as the end product of a gravimetric determination (e.g. phosphate as P2O5). Math formulae Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text). If differences between treatments are statistically significant, this should be indicated by adding the actual 'P' value obtained. If 0.10 > P > 0.05, then differences can be considered to suggest a trend, or tendency, to a difference, but the actual 'P' value should be stated. Further information on this issue can be found in Animal Feed Science and Technology Vol. 129/1-2. Spaces should be used between all values and units, except for the following: Between the value and degrees or percent.

Reference formatting

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the

pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following examples: Reference style Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given. Example: '..... as demonstrated [3,6]. Barnaby and Jones [8] obtained a different result ....' List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text. Examples: Reference to a journal publication: [1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59. Reference to a book: [2] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000. Reference to a chapter in an edited book: [3] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304. Reference to a website: [4] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/ aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13.03.03).

ANEXO D - COMPROVAÇÃO DE SUBMISSÃO DE ARTIGO