JOANA ANGELICA LYRA VOGELEY DE CARVALHO

EFEITO DO USO DE BACTÉRIAS PROBIÓTICAS NA SOBREVIVÊNCIA DE LARVAS DE *Litopenaeus vannamei* EXPOSTAS À INFECÇÃO EXPERIMENTAL POR *Vibrio* spp.

RECIFE,

2011

UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM RECURSOS PESQUEIROS E AQUICULTURA

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Dissertação apresentada ao Programa de Pós-Graduação em Recursos Pesqueiros e Aquicultura da Universidade Federal Rural de Pernambuco como exigência para obtenção do título de Mestre.

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Recife,
Fevereiro, 2011

Ficha catalográfica

C331e Carvalho, Joana Angelica Lyra Vogeley de
Efeito do uso de bactérias probióticas na sobrevivência de larvas de *Litopenaeus vannamei* expostas à infecção experimental por *Vibrio* spp. / Joana Angelica Lyra Vogeley de Carvalho. -- 2011.
xxf.: il.

Orientador: Sílvio Ricardo Maurano Peixoto.
Dissertação (Mestrado em Recursos Pesqueiros e Aquicultura) – Universidade Federal Rural de Pernambuco, Departamento de Pesca e Aquicultura, Recife, 2011.
Referências.

1. *Bacillus* 2. *Vibrio harveyi* 3. *Vibrio alginolyticus* 4. Camarão marinho I. Peixoto, Sílvio Ricardo Maurano, Orientador II. Título

CDD 639.3

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Dedicatória

Aos grandes homens da minha vida: meu pai João e meu esposo Freddy... Fontes de amor e inspiração.
Sem vocês nada disso seria possível!
A busca continua! Vamos juntos?

Agradecimentos

- ✓ A Deus pela oportunidade de realizar mais esta etapa da minha vida;
- ✓ Ao meu pai pela vida, amor, exemplo, dedicação e empenho para me proporcionar sempre as melhores oportunidades;
- ✓ A minha mãe Ilzenete Bezerra pela vida, amor e alegria sempre;
- ✓ A Alexandre pela imprescindível presença e amor paterno;
- ✓ Ao meu esposo Freddy... a quem eu tenho uma intensa admiração. Pelo amor de cada dia, pelo incentivo e apoio em mais essa jornada e por me apresentar ao mundo da Engenharia de Pesca;
- ✓ Ao meu sogro Antônio Cardim e sogra Mary Vogeley pelo inestimável carinho e apoio, por me receber como uma filha e pela presença em todos os momentos;
- ✓ Aos meus irmãos Amanda e Bruno pelos quais tenho um amor profundo e me esforço para ser um bom exemplo;
- ✓ A minha tia Solange pelo exemplo de dedicação e conquistas;
- ✓ Aos meus familiares: tios, tias, primos, com quem divido vários bons momentos e que são fundamentais na minha vida;
- ✓ Aos professores Dr. Sílvio Peixoto e Drª Roberta Soares pela indispensável atenção, presença, orientação, ricos ensinamentos e confiança;
- ✓ Ao Prof. Dr. José Vitor pela fundamental paciência e atenção sempre;
- ✓ Ao Prof. Alfredo Gálvez pelos indispensáveis ensinamentos e atenção e aos que fazem parte do Laboratório de Produção de Alimento Vivo (LAPAVI);
- ✓ A Roberta Nery e Bruna do Valle. Por onde eu começo? Por encarar esse desafio junto comigo desde o começo, pela inigualável presença e empenho, pelos finais de semana e madrugada de animado trabalho, por iluminar os meus dias, pelo entusiasmo, pelo carinho... pela eterna amizade. Nada disso seria possível sem vocês!

- ✓ A Emanuell Felipe... o que seria de mim sem você? Pelo inestimável apoio, força e dicas sempre, pelas madrugadas de escrita, pelo eterno bom humor e alegria... pela amizade construída;
- ✓ A toda equipe do laboratório que alegra e enriquece os meus dias, e me faz querer estar na Rural todos os dias (rsrsrs): Roberta Nery, Bruna do Valle, Emanuell Felipe, Nathalia Calazans, Kennya Addam, Juliana Interaminense, Camila Brito, Marcelo Soares, Amanda Monteiro, Phelps (o nosso guardião e companheiro canino) e o agregado Luciano Santos ("Amor") pela força e apoio logístico sempre (rsrs).
- ✓ À Adriana Lima e Ebenézer Oliveira. Vocês são uma eterna fonte de inspiração na minha vida!
- ✓ Aos amigos de turma Adriana Lima, Willy Vila Nova, Natália Lacerda, Diogo Nunes, Ilka Branco, João Luiz Farias, Jones Santander, Marcus Vinícius, Vanessa Andrade, César Calzavara e Leônidas Oliveira pela compainha e momentos de descontração;
- ✓ À Universidade Federal Rural de Pernambuco e ao Programa de Pós-Graduação em Recursos Pesqueiros e Aquicultura;
- ✓ À Fundação de Amparo a Ciência e Tecnologia do Estado de Pernambuco (FACEPE), pela concessão da bolsa de mestrado;
- ✓ Aos professores do Programa de Pós-graduação em Recursos Pesqueiros e Aquicultura pela contribuição para minha formação pessoal e profissional;
- ✓ À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa de mestrado sanduíche, realizado na Universidade Federal do Rio Grande (FURG/RS) através do programa PROCAD;
- ✓ À empresa Netuno pela doação dos animais utilizados nos experimentos;
- ✓ À empresa INVE na pessoa do Dr. Marcos Santos pela doação do probiótico e rações;
- ✓ Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pelo apoio financeiro;

Resumo

O presente estudo teve como objetivo avaliar a sobrevivência de larvas de *Litopenaeus vannamei* tratadas com bactérias probióticas e posteriormente infectadas por *Vibrio harveyi* e *Vibrio alginolyticus*. Os camarões foram mantidos em dois tanques de 80L nas mesmas condições, mas apenas um deles foi previamente tratado com probiótico comercial (*Bacillus* spp.) adicionado diariamente na água na concentração de 10^5 UFC/ml. Este protocolo foi mantido até os animais alcançarem o estágio de desenvolvimento larval necessário para os experimentos de infecção por *Vibrio* spp. Foram realizados três experimentos independentes de infecção: $Zoea_1$ a $Zoea_3$, $Mísis_1$ a $Mísis_3$ e Pós-larvas $(PL)_{10}$ a PL_{14} com os seguintes tratamentos com quatro repetições cada: somente *V. harveyi*; probiótico comercial + *V. harveyi*; somente *V. alginolyticus*; probiótico comercial + *V. alginolyticus*; sem *Vibrio* spp. e probiótico (controle). No experimento com PL, somente os tratamentos com *V. harveyi;* probiótico comercial + *V. harveyi* e controle foram utilizados. As espécies de *Vibrio* foram inoculadas apenas no início de cada experimento na concentração de 10^7 UFC/ml. Ao final dos experimentos, uma amostra de água e camarão de cada parcela experimental foi submetida à quantificação de *Vibrio* spp. Adicionalmente foi realizado um teste de antagonismo *in vitro* de *Bacillus* spp. contra *Vibrio* spp. Nos experimentos com zoea e mísis, as larvas cultivadas no tratamento *V. harveyi* apresentaram uma sobrevivência significativamente inferior quando comparadas com as dos tratamentos *V. alginolyticus* e controle. Houve um aumento significativo na sobrevivência de larvas zoea e mísis tratadas com probiótico + *V. harveyi* com 81,07 e 90,13%, em comparação com as larvas infectadas apenas com *V. harveyi* com 12,80 e 69,13%, respectivamente. Não foram observadas diferenças significativas na sobrevivência das pós-larvas entre tratamentos. A quantidade total de *Vibrio* spp. na água e camarões diminuiu nos tratamentos com o uso de probiótico. O probiótico (*Bacillus* spp.) apresentou atividade inibitória contra *Vibrio* spp. *in vitro.* Os resultados indicaram um aumento na sobrevivência dos estágios larvais iniciais (zoea e mísis) infectadas por *V. harveyi* após tratamento com *Bacillus* spp. A administração de bactérias probióticas é uma alternativa promissora para a prevenção de *Vibrio* spp. na larvicultura de *L. vannamei*.

Palavras-chave: *Bacillus, Vibrio harveyi, Vibrio alginolyticus,* camarão marinho

Abstract

This study aimed to evaluate the survival of *Litopenaeus vannamei* shrimp larvae treated with probiotic bacteria and subsequently infected by *Vibrio harveyi* and *Vibrio alginolyticus*. Shrimp were kept in two 80L tanks under similar culture conditions, but only one tank was previously treated with commercial probiotic (*Bacillus* spp.) added daily in the water at a concentration of 10^5 CFU/ml. This protocol was maintained until the animals reach the stage of development needed for the infection experiments by *Vibrio* spp. Three independent infection experiments were performed: $Zoea_1$ to $Zoea_3$, $Mysis_1$ to $Mysis_3$ and Postlarvae $(PL)_{10}$ to PL_{14} with the following treatments with four replicates each: only *V. harveyi*; commercial probiotic + *V. harveyi*; only *V. alginolyticus*; commercial probiotic + *V. alginolyticus*; and without *Vibrio* spp. and probiotic (Control). In the experiment with PL, only the treatments with *V. harveyi*; commercial probiotic + *V. harveyi* and control were used. *Vibrio* species were inoculated only at the beginning of each experiment at a concentration of 10^7 CFU/ml. At the end of experiments, a sample of water and shrimp from each experimental unit was submitted to quantification of *Vibrio* spp. Additionally, was realized an antagonism test of *Bacillus* spp. against *Vibrio* spp. In the experiments with zoea and mysis, larvae cultured in treatment *V. harveyi* had a significantly lower survival when compared with treatments *V. alginolyticus* and control. There was a significant increase in survival of zoea and mysis larvae treated with probiotic + *V. harveyi* with 81.07 and 90.13%, differentiating the larvae infected only with *V. harveyi* with 12.80 and 69.13%, respectively. There were no significant differences in survival of postlarvae among treatments. Total *Vibrio* spp. counts decreased in the water and shrimp of treatments with the use of probiotic. The probiotic (*Bacillus* spp.) showed inhibitory activity against *Vibrio* spp. *in vitro*. The results indicated an increase in the survival of early larval stages (zoea and mysis) infected by *V. harveyi* after treatment with *Bacillus* spp. The administration of probiotic bacteria is a promising alternative for the prevention of *Vibrio* spp. in larviculture of *L. vannamei*.

Keywords: *Bacillus, Vibrio harveyi, Vibrio alginolyticus,* marine shrimp

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1. Introdução

O crescimento mundial da produção de *Litopenaeus vannamei* deve-se ao avanço de novas tecnologias de reprodução em cativeiro e larvicultura, além da crescente demanda do produto no mercado internacional, da boa rentabilidade do agronegócio e da capacidade de gerar renda, empregos e divisas (ROCHA e RODRIGUES, 2004). Porém, a intensificação da carcinicultura vem sendo acompanhada pela ocorrência de doenças, destacando-se as causadas por bactérias patógenas oportunistas (SHARIFF et al., 2001).

Entre as bactérias potencialmente patógenas, as do gênero *Vibrio* são frequentemente associadas com baixas taxas de sobrevivência em larviculturas ou sistemas de engorda (SAULNIER et al., 2000). Espécies como *Vibrio harveyi* e *Vibrio alginolyticus* podem infectar larvas, juvenis e adultos de camarões peneídeos, comprometendo a cadeia produtiva (LAVILLA PITOGO et al., 1998). Essas bactérias podem sem encontradas fixadas em microalgas, no zooplâncton, na comunidade bacteriana do trato digestório dos camarões e no biofilme da parede dos tanques de cultivo (THOMPSON et al., 2002). Dessa forma, a abundância natural de *Vibrio* spp., assim como sua ubiquidade, taxa de multiplicação e habilidade para adaptar-se à mudanças ambientais em sistemas aquícolas, ressaltam a importância da avaliação de seus efeitos patogênicos nos camarões e do seu controle nos sistemas de cultivo (SAULNIER et al., 2000).

A tentativa de controle de bactérias potencialmente patógenas tornou-se uma prática comum entre os administradores de larviculturas (GOMEZ-GIL et al., 2000). No entanto, o uso abusivo de antibióticos pode causar maior resistência nas linhagens bacterianas (GULLIAN et al., 2004). Nesse sentido, um método alternativo, em detrimento do uso de antibióticos em sistemas de aquicultura, é a aplicação de probióticos que são definidos como um conjunto de microrganismos vivos que, quando consumidos em quantidades adequadas, conferem benefícios a saúde do hospedeiro (REID et al., 2003).

Entre as bactérias probióticas utilizadas na aqüicultura, o gênero *Bacillus* tem demonstrado atividade inibitória contra várias espécies de *Vibrio* em testes realizados *in vitro* e *in vivo*. Nesse sentido, o objetivo da presente pesquisa foi avaliar o efeito do uso de bactérias probióticas na sobrevivência de larvas de *L. vannamei* expostas à infecção por *V. harveyi* e *V. alginolyticus.*

2. Revisão de literatura

O cultivo de camarões marinhos tornou-se uma importante atividade em muitos países tropicais. No Brasil, a partir da década de 90, com a introdução da espécie *L. vannamei*, essa indústria experimentou um período de rápido crescimento alcançando uma produção de 90.190 t em 2003, com destaque para a região Nordeste, a qual foi responsável por 95,2% dessa produção (ROCHA, 2007). No ano seguinte, houve uma queda de 16% na produção de camarão devido a alguns fatores como a desvalorização do dólar e a ocorrência de enfermidades, as quais são consideradas como uma das principais causas de variação na produção de camarões (MOSS, 2002; MADRID, 2005).

Em vários países, a intensificação das atividades de carcinicultura tem resultado em um aumentado na incidência de doenças causadas por bactérias patógenas oportunistas como as do gênero *Vibrio*, as quais são frequentemente associadas com altas taxas de mortalidade no cultivo de camarões peneídeos (SAULNIER et al., 2000). Mortalidades associadas à presença de *Vibrio* sp. têm sido relatadas para *Penaeus monodon* e *L. vannamei* na Indonésia (SUNARYANTO e MARIAM, 1986), Tailândia, India (KARUNASAGAR et al., 1994), Filipinas (BATICADOS et al., 1990; LAVILLA-PITOGO et al., 1990), Austrália (PIZZUTTO e HIRST, 1995), Taiwan (SONG e LEE, 1993; LIU et al., 1996) e Equador (ROBERTSON et al., 1998).

Espécies como *V. vulnificus, V. alginolyticus, V. campbellii, V. splendidus, V. damsela, V. parahaemolyticus, V. penaeicida* e *Vibrio harveyi* são descritas como as principais

bactérias patogênicas para camarões peneídeos (LIGHTNER, 1996). Assim, algumas pesquisas têm sido realizadas para avaliar a suscetibilidade de camarões a diferentes linhagens dessas espécies. Soto-Rodríguez et al. (2006) ao submeterem diferentes fases larvais de *L. vannamei* a infecção por *V. campbellii* observaram um significativo aumento na taxa de mortalidade desses animais. Resultados similares foram encontrados por Aguirre-Guzmán et al. (2001) para *V. penaeicida, V. harveyi* e *V. parahaemolyticus.* Em outro estudo, Lee et al. (1996) obtiveram 50% de mortalidade em *P. monodon* após sete dias de infecção dos animais com *V. alginolyticus* através de injeção intra-muscular.

Alguns produtos extracelulares como proteases, fosfolipases e hemolisinas são considerados como um dos mecanismos de patogenicidade de *Vibrio* spp. Pesquisas foram realizadas com a exposição de diferentes espécies de camarões a esses produtos, resultando em um significativo aumento na taxa de mortalidade dos animais (AUSTIN e ZHANG, 2006). Além disso, órgãos como hepatopâncreas e brânquias quando infectados por *Vibrio* spp. podem ter suas funções comprometidas, levando o animal à uma consequente morte (ESTEVE e HERRERA, 2000).

Segundo Saulnier et al. (2000), *Vibrio* spp. podem atuar como agente primário ou secundário de infecção. Essas bactérias podem agir de forma oportunista, quando os camarões estão imunologicamente comprometidos devido a causas primárias como a presença de outros agentes infecciosos, deficiência nutricional, práticas de manejo e estresse ambiental em decorrência de bruscas alterações nos parâmetros físico-químicos da água de cultivo. Em estudo realizado por Wang e Chen et al. (2005), juvenis de *L. vannamei* submetidos a estresse por salinidade tiveram uma diminuição na resposta imune resultando numa menor resistência a *V. alginolyticus*. Phuoc et. al (2008) concluíram que em ausência de estresse por exposição a concentrações de amônia ou salinidade, as pós-larvas de *L. vannamei* não foram suscetíveis a infecção por diferentes linhagens de *V. campbellii*, *V. harveyi* e *V. penaeicida.*

Antibióticos vêm sendo utilizados na tentativa de minimizar os problemas causados por *Vibrio* spp. na aquicultura (GULLIAN et al., 2004). No entanto, o emprego abusivo dessas substâncias tem causado um aumento na resistência de linhagens de bactérias patógenas e preocupação da sociedade que consome produtos aquícolas (ESIOBU et al., 2002). Nesse sentido, o uso de bactérias probióticas vem surgindo como uma alternativa para o uso de antibiótico. Verschuere et al. (2000) definem probióticos como "suplementos microbianos vivos que têm efeito benéfico ao hospedeiro, através da modificação da comunidade microbiana no hospedeiro ou no ambiente, o que proporciona melhorias na utilização dos alimentos, maior resposta imunológica do hospedeiro a doenças, e melhor qualidade de água".

De acordo com Decamp e Moriarty (2006), probióticos têm proporcionado um incremento na produção de camarão similar aquele encontrado quando substâncias antimicrobianas são utilizadas. Vários benefícios têm sido relatados para utilização de bactérias probióticas em sistemas de aquicultura, como a criação de um ambiente hostil para patógenos pela produção de compostos inibitórios (bacteriocinas, lisozimas, proteases e peróxido de hidrogênio) e competição por nutrientes e locais de adesão; fornecimento de nutrientes essenciais e enzimas que resultam em uma melhor nutrição dos animais cultivados, conversão do material orgânico dissolvido na água de cultivo e melhora da resposta imune (GATESOUPE, 1999; GOMEZ-GIL et al., 2000; IRIANTO e AUSTIN, 2002; BALCAZAR et al., 2006).

Espécies do gênero *Bacillus* são facilmente encontradas em sedimentos marinhos, brânquias, cutícula e trato intestinal dos camarões (SHARMILA et al., 1996). Essas bactérias são amplamente utilizadas na aquicultura, podem ser administradas tanto através do alimento como diretamente na água de cultivo (MORIARTY, 1998), e têm demonstrado atividade inibitória contra várias espécies de *Vibrio*. Vaseeharan e Ramasamy (2003) ao avaliarem o antagonismo *in vitro* de *Bacillus subtilis* BT 23 contra o *V. harveyi* em *P. monodon,* encontraram zonas inibitórias ao redor do crescimento do *Bacillus* de 3 a 6 mm e redução da

mortalidade dos camarões em condições *in vivo*, demonstrando que o crescimento dessa espécie patogênica foi controlado. Resultados similares foram encontrados por Balcazar et al. (2007), onde a mortalidade de *L. vannamei* infectados por *V. parahaemolyticus* foi reduzida após adição de *B. subtilis* na ração.

Rengpipat et al. (2000) demonstraram que é possível aumentar a produção de camarões pela adição de *Bacillus* S11 no cultivo de *P. monodon*, pois o crescimento e a sobrevivência das pós-larvas foram significativamente superiores comparados ao grupo controle. Ao administrar *B. subtilis* E20 na água de cultivo de larvas e pós-larvas de *L. vannamei*, Liu et al. (2010) encontraram melhor taxa de sobrevivência e desenvolvimento larval, maior inibição do crescimento de espécies de *Vibrio* na água e uma significativa diminuição da mortalidade dos camarões submetidos a estresse de salinidade e exposição a concentrações de nitrito.

A composição natural da flora bacteriana intestinal de camarões marinhos pode ser modificada pelo fornecimento de bactérias probióticas diretamente na alimentação (ZIAEI-NEJAD et. al., 2006). Ao utilizar *B. subtilis* UTM 126 no controle de *Vibrio* spp. Balcazar et. al. (2007b), observaram que o mecanismo de ação do probiótico pode ter sido a exclusão competitiva, pois detectaram ao final do estudo a presença de *B. subtilis* no hepatopâncreas de juvenis de *L. vannamei*. Ke Li et al. (2007) ao administrar *Bacillus licheniformis* a juvenis de *L. vannamei* encontraram uma quantidade significativamente mais baixa de *Vibrio* spp. no trato intestinal desses animais.

A adição de bactérias probióticas em sistemas de aquicultura pode estimular a resposta imunológica do hospedeiro contra a infecção por bactérias patogênicas. A utilização de *Bacillus* sp. proporcionou uma proteção contra doenças através da ativação das defesas imunológicas humoral e celular no camarão *P. monodon* (RENGPAT et al., 2000). Tseng et al. (2009) ao administrar *B. subtilis* para juvenis de *L. vannamei* observaram um incremento da atividade da fagocitose e um aumento na resistência dos camarões contra *V. alginolyticus*.

Algumas bactérias podem participar do processo digestório dos camarões pela produção de enzimas extracelulares como proteases e lipases (OCHOA e OLMOS, 2006). Zhou et al. (2009), encontraram um aumento na atividade da amilase, lipase e protease em larvas de *L. vannamei* tratadas com *Bacillus coagulans* e sugeriu que o consequente aumento da digestão e absorção do alimento contribuiu para o incremento na sobrevivência. A administração de *B. subtilis* E20 para larvas de *L. vannamei* resultou em um significativo aumento na sobrevivência dos camarões devido a uma possível melhora no estado nutricional dos animais, a qual pode ter sido causada pelo incremento da atividade enzimática e consequente maior absorção de nutrientes (LIU et al., 2010).

A biotecnologia avançou ao ponto em que probióticos comerciais estejam disponíveis para utilização na aquicultura. O emprego de um probiótico comercial (BACILLOGEN-ZINC) e culturas de *Bacillus pumilus* no cultivo de larvas de *Marsupenaeus japonicus* resultaram em um aumento de 97 e 95%, respectivamente, na sobrevivência dos animais comparados com o grupo controle (EL-SERSY et al., 2006). Em outro estudo, a adição de um probiótico comercial composto por cepas de *Bacillus* spp. no cultivo de diferentes estágios de larvas do camarão *Fenneropenaeus indicus* proporcionou uma maior sobrevivência dos animais (ZIAEI-NEJAD et al. 2006).

Diante do exposto, a utilização de bactérias probióticas parece ser uma alternativa promissora para o cultivo de camarões marinhos, sobretudo na produção de larvas. Considerando a importância da carcinicultura para o Nordeste do Brasil, as perdas na produção devido à ocorrência de doenças e a disponibilidade de probióticos comerciais para utilização nas larviculturas regionais, estudos que avaliem o desempenho desses produtos frente a infecções bacterianas por diferentes espécies e linhagens de *Vibrio* spp. se fazem necessários. Dessa forma, o presente estudo avalia o efeito do uso de bactérias probióticas na sobrevivência de larvas de *L. vannamei* expostas a infecção experimental por *V. harveyi* e *V. alginolyticus.*

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4. Artigo científico

Artigo científico a ser submetido para publicação no periódico *Aquaculture*.

Effect of the addition of probiotic bacteria on the survival of marine shrimp *Litopenaeus vannamei* larvae submitted to infection by *Vibrio* spp.

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Abstract

This study aimed to evaluate the survival of *Litopenaeus vannamei* shrimp larvae treated with probiotic bacteria and subsequently infected by *Vibrio harveyi* and *Vibrio alginolyticus*. Shrimp were kept in two 80L tanks under similar culture conditions, but only one tank was previously treated with commercial probiotic (*Bacillus* spp.) added daily in the water at a concentration of 10^5 CFU/ml. This protocol was maintained until the animals reach the stage of development needed for the infection experiments by *Vibrio* spp. Three independent infection experiments were performed: $Zoea_1$ to $Zoea_3$, $Mysis_1$ to $Mysis_3$ and Postlarvae $(PL)_{10}$ to PL_{14} with the following treatments with four replicates each: only *V. harveyi*; commercial probiotic + *V. harveyi*; only *V. alginolyticus*; commercial probiotic + *V. alginolyticus*; and without *Vibrio* spp. and probiotic (Control). In the experiment with PL, only the treatments with *V. harveyi*; commercial probiotic + *V. harveyi* and control were used. *Vibrio* species were inoculated only at the beginning of each experiment at a concentration of 10^7 CFU/ml. At the end of experiments, a sample of water and shrimp from each experimental unit was submitted to quantification of *Vibrio* spp. Additionally, was realized an antagonism test of *Bacillus* spp. against *Vibrio* spp. In the experiments with zoea and mysis, larvae cultured in treatment *V. harveyi* had a significantly lower survival when compared with treatments *V. alginolyticus* and control. There was a significant increase in survival of zoea and mysis larvae treated with probiotic + *V. harveyi* with 81.07 and 90.13%, differentiating the larvae infected only with *V. harveyi* with 12.80 and 69.13%, respectively. There were no significant differences in survival of postlarvae among treatments. Total *Vibrio* spp. counts decreased in the water and shrimp of treatments with the use of probiotic. The probiotic (*Bacillus* spp.) showed inhibitory activity against *Vibrio* spp. *in vitro*. The results indicated an increase in the survival of early larval stages (zoea and mysis) infected by *V. harveyi* after treatment with *Bacillus* spp. The administration of probiotic bacteria is a promising alternative for the prevention of *Vibrio* spp. in larviculture of *L. vannamei*.

Keywords: *Bacillus, Vibrio harveyi, Vibrio alginolyticus,* marine shrimp

1. Introduction

Farming of white shrimp *Litopenaeus vannamei* has become a significant economic activity in several developing countries, such as Brazil. However, this industry has been suffering with an increased incidence of infectious pathogens. Bacterial diseases are often associated with low survival rates during larvae production, and the genus *Vibrio* has been closely related with this problem (Saulnier et al., 2000, Decamp et al., 2008). Furthermore, *Vibrio* species can affect shrimp at juvenile and adult stages (Gomez Gil et al., 1998; Lavilla Pitogo et al., 1998), compromising the whole production cycle.

In order to overcome bacterial diseases and improve larvae production, many hatcheries have been relying on the use of antibiotics. However, this procedure has resulted in the presence of resistant strains of bacteria in shrimp culture (Gullian et al., 2004). In this context, the use of probiotics has been increasingly reported as an alternative to antibiotic treatment (Balcazar et al., 2006). Probiotics are defined as ‘‘live microorganisms, which when are consumed in adequate amounts, confer a health benefit for the host’’ (Reid et al., 2003). The benefits of probiotics in aquaculture may include the competitive exclusion of pathogenic bacteria, supply of essential nutrients and enzymes resulting in enhanced nutrition of the cultured animal, improvement of water quality and lower incidence of diseases by enhancement immune response of shrimp (Verschuere et al., 2000).

The genus *Bacillus* is known to antagonize potential pathogens in the aquatic environment (Irianto and Austin, 2002). These microorganisms compete with other bacteria for nutrients and space and can exclude them through the production of different antibiotic compounds (Moriarty, 1998). Therefore, the genus *Bacillus* contains most of the bacteria species used as probiotics by the aquaculture industry (Ninawe and Selvin, 2009). The effect of these probiotic bacteria in reducing shrimp mortality against *Vibrio* infection has been described in

previous papers (Vaseeharan and Ramasamy, 2003; Balcazar et al., 2007ab; Das et al., 2010), but there are few studies covering all larval stages. Thus, the objective of this study was to evaluate the effect of *Bacillus* spp. addition on the survival of *L. vannamei* larvae submitted to infection by *Vibrio* spp.

2. Materials and Methods

2.1 Source of bacterial strains and inoculum preparation

Three *Bacillus* strains were obtained from a commercial probiotic (INVE Sanolife® MIC) containing spores of *B. subtilis*, *B. licheniformis* and *B. pumilus*. Before application of the commercial probiotic in the water, spores were rehydrated according to manufacturer's instructions and a sample was plated onto marine agar (MA; Himedia) by the spread plate technique to confirm the density of probiotic bacteria.

The strains of *Vibrio harveyi* and *Vibrio alginolyticus* used to infection experiments were obtained from the collection of Laboratório de Camarões Marinhos at the Federal University of Santa Catarina, Brazil. The strains were stored in cryovials (-80 °C) containing tryptic soy broth (TSB; Himedia) supplemented with 2.0% NaCl and sterile glycerol (30% v/v) prior to use.

For preparing *Vibrio* inoculum, the strains were recovered from cryopreservation, plated in tryptic soy agar (TSA; Himedia) enriched with 2.0% NaCl and incubated overnight at 30 °C. The bacterial colonies were grown in 12 ml of TSB supplemented with 2.0% NaCl at 30 °C for 24 h. After incubation, the cells were harvested by centrifugation (4000 rpm for 15 min), washed twice with sterile saline solution (2.5% NaCl) and re-suspended in the same solution. This suspension was serially diluted and the dilutions was both inoculated onto thiosulfate citrate bile salts sucrose agar (TCBS; Himedia) and measured using a spectrophotometer at the optical density of 600 nm. This procedure aimed to verify the relationship between absorbance and Colony Forming Units (CFU) per milliliter. Then, the

absorbance was adjusted to an optical density at 600 nm, corresponding to a cell density of approximately 10^9 CFU/ml. The bacterial suspension of *V. harveyi* and *V. alginolyticus* was serially diluted to achieve the density of 10^7 CFU/ml, used for shrimp infection bioassays.

2.2 Antagonism assays

Antagonism assays were performed by the agar well diffusion plate method, adapted from Vaseeharan and Ramasamy (2003). All assays were carried out in triplicate. The strains of *V. harveyi*, *V. alginolyticus* and commercial probiotic (*Bacillus* spp.) were grown in TSB supplemented with 2.0% NaCl at 30 °C. After 24 h of incubation, samples of culture *Vibrio* species were plated in TSA enriched with 2.0% NaCl and incubated overnight at 30 °C. Colonies were suspended in sterile saline solution (2.5% NaCl) and centrifuged (4000 rpm for 15 min). The bacterial suspension was adjusted to an optical density of 0.5 MacFarland Standard, corresponding to 10^8 CFU/ml. *V. harveyi* and *V. alginolyticus* were inoculated evenly into the separate TSA plates enriched with 2.0% NaCl containing four punched wells of 3 mm diameter, using *swabs* soaked in the bacterial suspension. The commercial probiotic culture with 2 days old was centrifuged (4000 rpm for 15 min) and supernatant was discarded. The bacterial cells were suspended in sterile TSB enriched with 2.0% NaCl. Thereafter, 20 µl of *Bacillus* spp. suspension was introduced into the three wells of the agar medium and incubated. One well served as a control and was inoculated only with sterile TSB with 2.0% NaCl. After incubation at 30 °C for 24h, the diameter of the clear zone around each well was measured using a dial caliper.

2.3 Probiotic treatment and experimental design

L. vannamei larvae used in this study were obtained from a commercial hatchery (Maricultura Netuno S/A) located in Pernambuco, Brazil, and transferred to our laboratory in the previous development stage needed for each experiment. Three independent infection

experiments were performed: $Zoea_1$ to $Zoea_3$ (Experiment Z), $Mysis_1$ to $Mysis_3$ (Experiment M) and PL_{10} to PL_{14} (Experiment PL). Before infection, shrimp were maintained in two fiberglass tank (80 L) under constant aeration, temperature of 32 ± 1 °C and salinity 34-35‰. In a tank, the animals were treated with commercial probiotic (Sanolife® MIC, INVE) added daily in the water until they reached the development stage needed for each infection experiment, as follows: $Nauplius_{1-2}$ to $Zoea_1$, $Zoea_1$ to $Mysis_1$ and PL_1 to PL_{10} for experiments Z, M and PL, respectively. The concentration of probiotic used in this study followed manufacturer's recommendations for shrimp larviculture, corresponding to a cell density of 10^5 CFU/ml. The second tank was maintained at the same conditions, but it was not treated with commercial probiotic.

The water of the two tanks was supplemented with the microalgae *Chaetoceros calcitrans* at a concentration of 10.0x104 cells/ml. Specific commercial diets (Frippak-INVE) for *L. vannamei* Zoea (1 CAR), Mysis (2CD), PL_{1-4} (3CD) and PL_{5-10} (PL+300) were offered every 2 hours in all experiments. Moreover, in mysis and PL phases, frozen and live *Artemia* nauplii were offered *ad libitum* once a day, respectively.

2.3.1 Shrimp infection bioassays

After treatment with probiotic, shrimp larvae were transferred to experimental units with 5 L capacity. All experimental units remained submerged in a water table equipped with immersion heater to maintain the same conditions of the previous probiotic treatment. The animals were infected with bacterial suspension of *V. harveyi* and *V. alginolyticus* added directly into the water only at the beginning of the experiments at a concentration of 10^7 CFU/ml. The infection bioassays lasted 96 hours and there was no water renewal during this period. The experiments Z and M consisted of five treatments with four replicates each at a density of 100 larvae/L, where: VH = only *V. harveyi*; PVH = commercial probiotic + *V. harveyi*; VA = only *V. alginolyticus*; PVA = commercial probiotic + *V. alginolyticus*; C =

without inoculum of probiotic and *Vibrio*. In experiment PL, only treatments with *V. harveyi* were used (VH, PVH and C), and shrimp were stocked at a density of 40 PL/L. In all the experiments, the shrimps of treatment VH, VA and C was not treated with probiotic previously. After the infection of experimental units, water samples were inoculated onto TCBS agar plates to confirm the concentration of *Vibrio* sp. required in the trials. During the infection bioassays, the experimental units were continuously inoculated with commercial probiotic at the same concentration (10^5 CFU/ml). At the end of each experiment, survival was determined by counting the animals.

2.4 Bacterial quantification

Samples of water were collected from each experimental unit at the end of all bioassays to *Vibrio* spp. quantification. The same was performed for shrimp larvae, by maceration. All samples were serially diluted (1/10) in sterile saline solution (2.5% NaCl) followed by plating on medium agar TCBS to determine the presumptive *Vibrio* spp. counts. After the incubation period of 24 hours at 30 ºC, the number of bacterial colonies was counted and the amount of bacteria was calculated as CFU.

2.5 Statistical analysis

The values of survival and concentration of *Vibrio* spp. in the water and shrimp were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test to determine differences among treatments. Results are presented as means ± standard error (SE).

3. Results

3.1 Antagonism assay

The commercial probiotic composed of *Bacillus* spp. showed inhibitory activity against *V. harveyi* and *V. alginolyticus*. The diameter of the inhibitory zones around the wells was 12 mm for both *Vibrio* species. No inhibitory zone was found around the control well (Figure 1).

Insert Figure 1

3.2 Shrimp infection bioassay

In the experiment Z, there was no significant difference in the zoea survival of treatments VA (62.7 ± 9.9%), PVA (66.4 ± 6.8%) and C (66.0 ± 1.7%). A similar pattern was observed for mysis (experiment M) with values of 95.0 ± 1.2% (VA), 98.5 ± 1.0% (PVA) and 98.8 ± 0.8% (C). On the other hand, larvae exposed to *V. harvey*i (VH) presented a significantly lower survival than the control (C). This difference in larvae survival related to *V. harveyi* was more pronounced for zoea than mysis stage (Figure 2).

In the experiment Z and M, larvae infected by *V. harveyi* (VH) showed a significant increase in their survival when the probiotic was used (PVH) (Figure 2). In addition, zoea larvae reared in the treatment PVH obtained a higher survival (81.1 ± 2.2%) when compared to control (66.0 ± 1.7%), but there was no significant difference. However, postlarvae survival (experiment PL) was not affected by the presence of *V. harveyi* (VH) nor probiotic (PVH) in comparison to control treatment (Figure 2).

Insert Figure 2

3.3 Bacterial quantification

After the infection, all treatments with *V. harveyi* and *V. alginolyticus* were at the required concentration of *Vibrio* (10^7 CFU/ml). At the end of the trials, the concentration of

198 *Vibrio* spp. in the water showed a significant increase in the treatments with *V. harveyi* (VH)
199 in the experiments Z and M, when compared with the control (Table 1). However, for *V.*
200 *alginolyticus* (VA), only the final concentration of *Vibrio* spp. in the experiment M differed
201 significantly from the control. In the experiment PL, there was a significant difference
202 between treatments, showing a higher concentration in the treatment VH ($17.0\pm1.1x10^3$
203 CFU/ml), followed by treatments PVH ($7.2\pm0.5x10^3$ CFU/ml) and C ($2.6\pm1.0x10^3$ CFU/ml).

204 At the end of the experimental period, $Zoea_3$ infected by *V. harveyi* showed a
205 significantly higher concentration of *Vibrio* spp. when compared with shrimp treated with
206 probiotic and control. However, the same was not observed for *V. alginolyticus*. $Mysis_3$ and
207 PL_{14} in the treatments infected by *Vibrio* species showed a higher concentration of these
208 bacteria than in shrimp treated with probiotic and control, but no significant differences were
209 found (Table 1).

210

211 Insert Table 1

212

213 **4. Discussion**

214 In the present study, *Bacillus* spp. probiotic promoted a clearing zone with a diameter
215 of 12 mm against *V. harveyi* and *V. alginolyticus*. Balcazar et al. (2007ab) tested *in vitro B.*
216 *subtilis* UTM 126 and other bacterial species against *V. parahaemolyticus* and encountered
217 clear zones of 8-12 mm. In another study, the same authors observed clear zones of 10-15 mm
218 against different *V. harveyi* strains and *V. alginolyticus*. The inhibitory mechanism between
219 *Bacillus* spp. and *Vibrio* spp. was not characterized in our study. Nevertheless, previous
220 studies reported that the antibacterial mechanism of bacteria can be due several factors, either
221 singly or in combination, such as production of antibiotics, bacteriocins, lysozymes,
222 proteases, hydrogen peroxide and the alteration of pH values by the production of organic
223 acids (Verschuere et al., 2000). Ma et al. (2009) observed antimicrobial activity of the

Lactobacillus spp. against *V. parahaemolyticus* and *V. harveyi*. These authors suggested that the antimicrobial activity was caused by bacteriocin-like substance. Nakayama and Nomura (2009) investigated *in vitro* the effect of *Bacillus* species against *V. harveyi* and observed that *B. subtilis* inhibited *Vibrio* growth while *B. licheniformis* and *Bacillus megaterium* suppressed the *Vibrio* haemolysins, which is considered virulence factor in *V. harveyi*.

The infection of zoea and mysis with *V. alginolyticus* did not influence overall survival of animals in the present study. Some *V. alginolyticus* strains have been reported as probiotic bacteria (Austin et al., 1995; Vandenberghe et al., 1998; Gomez-Gil et. al., 2002), while others as pathogenic agents to shrimp (Lightner, 1996; Lee et al., 1996; Chiu et al., 2007). Different authors suggested that survival of shrimp larvae depends on the source of bacterial isolates (Muroga et al., 1994; Karunasagar et al., 1994; Austin and Zhang, 2006). As an example, a *V. harveyi* strain at a concentration of 10^2 CFU/ml caused mortality in penaeid shrimp larvae (Prayitno and Latchford, 1995), while other strains of the same species did not affect survival at concentration of 10^6 CFU/ml (Abraham et al., 1997).

In this study, differences in the shrimp survival were observed between *Vibrio* species, where *V. harveyi* infections resulted in higher mortality of larvae. Moreover, the zoea stage was more susceptible to *V. harveyi* than mysis stage. Similar results were obtained by Aguirre-Guzmán et al. (2001) when submitted *L. vannamei* larvae to *V. harveyi* and *V. alginolyticus* at the concentration of 10^7 CFU/ml. These authors concluded that bacterial species and strains influenced larval survival, and that early larval stages were less resistant than advanced ones, corroborating with our results. In another study, Soto-Rodriguez et al. (2006) infected larvae of *L. vannamei* with *V. harveyi* and *Vibrio campbelli* and also observed differences in the survival of shrimp between *Vibrio* species, being zoea stage more susceptible to infection by *V. campbellii* than mysis.

V. harveyi-related isolates have been associated with different diseases and high larval mortality of several penaeid species, including *L. vannamei* (Karunasagar et al., 1994;

Praytino and Latchford, 1995; Robertson et al., 1998; Vandenbergue et al., 1999). The pathogenicity mechanisms of *V. harveyi* were not investigated in this study, however, extracellular products (toxins) such as proteases and haemolysins, and the ability to attach to host cells have been considered to be determinants of virulence (Austin and Zhang, 2006). According to Esteve and Herrera (2000), shrimp exposed to *Vibrio* spp. will die when a sufficiently large number of hepatopancreatic cells are damaged by the pathogen, rendering the organ nonfunctional.

In our experiment with *L. vannamei* postlarvae, only *V. harveyi* was tested as this bacterium was the most pathogenic to zoea and mysis stages. However, postlarvae survival was not affected by the presence of *V. harveyi*, which appeared to be more virulent to early larval stages (Lavilla-Pitogo et al., 1990; Prayitno and Latchford, 1995). Although *Vibrio* spp. act as primary pathogen, these bacteria are also considered as a secondary pathogen. Therefore, disease outbreaks and shrimp mortality could occur when the animals are submitted to stress situations, such as abrupt changes in environmental conditions, nutritional deficiencies or poor management practices (Saulnier et al., 2000; Liu et al., 2010). Nevertheless, in our study the animals were submitted to controlled and stable conditions, which may have contributed to the high survival in PL stage.

Probiotic bacteria have been used to enhance the survival, growth, immunity, and disease resistance in aquaculture species (Farzanfar, 2006). The present findings suggested that the addition of *Bacillus* spp. probiotic significantly improved survival of zoea and mysis larvae infected by *V. harveyi*. Our results are in agreement with those found by Vaseeharan and Ramasamy (2003) showing 90% reduction in cumulative mortality of *P. monodon* juveniles treated with *B. subtilis* BT23 and infected with *V. harveyi* (*in vivo*). These authors also observed a greater inhibitory effect of *B. subtilis* BT23 against the growth of *V. harveyi* (*in vitro*). In addition, Balcazar et al. (2007) found a reduction in mortality of juveniles of *L. vannamei* infected with *V. parahaemolyticus* after treatment with *B. subtilis* UTM 126.

Furthermore, the same commercial probiotic used in our study (Sanolife® MIC, INVE) improved survival of *P. monodon* postlarvae after infection with *V. harveyi* added in the water (Das et. al. 2010).

Previous studies reported that probiotic bacteria may stimulate the immune response and, consequently, improve resistance of shrimp to infectious agents (Rengpipat et al., 2000; Tseng et al., 2009). Juveniles of *L. vannamei* treated with *Lactobacillus plantarum* had an increase in their immune response and disease resistance against *V. alginolyticus* (Chiu et al., 2007). Similarly, the administration of *B. subtilis* E20 to larvae of *L. vannamei* increased phenoloxidase and phagocytic activity, resulting in higher survival after challenged with *V. alginolyticus* (Liu et al., 2010). These results encourage us to suppose that probiotic bacteria may have contributed to immune response and increased larval survival of *L. vannamei* in the present study.

Probiotics compete with bacterial pathogens for nutrients and adhesion sites and inhibit the growth of pathogens (Moriarty, 1999; Irianto and Austin, 2002). Kennedy et al. (1998) suggested that the inoculation of *B. subtilis* in the water resulted in the apparent reduction of *Vibrio* spp. in fish larvae. In our study, we observed a tendency of reduction in the *Vibrio* spp. counts for shrimp treated with probiotic. Balcazar et al. (2007) suggested that the main action mechanism of *B. subtilis* was the competitive exclusion of the pathogen, as they found the presence of *B. subtilis* UTM 126 in the *L. vannamei* hepatopancreas infected by *V. harveyi*. In addition, *P. monodon* treated with *Bacillus* S11 and exposed to *V. harveyi* had a greater survival compared with the control group, which was associated by the authors with the ability of *Bacillus* spp. to colonize both the culture water and *P. monodon* digestive tract, replacing the *Vibrio* spp. (Rengpipat et al., 2000).

At the end of all trials, the concentration of *Vibrio* spp. in the culture water infected only by *V. harveyi* was higher than control. However, a decrease in *Vibrio* spp. concentration was observed when the probiotic was added in the water. Similarly, Decamp et al. (2008)

tested the same commercial probiotic used in the present study in the larviculture of *L. vannamei* during 12 months and showed that these probiotic bacteria were able to inhibit the growth of *Vibrio* spp. and improve larvae survival. Das et al. (2010) used *Bacillus* sp. as probiotic for *P. monodon* postlarvae before the infection by *V. harveyi*, resulting in lower mortality of shrimp and *Vibrio* spp. counts in the water.

In summary, the probiotic evaluated in this study increased the survival of larvae infected with *V. harveyi* and decreased *Vibrio* spp. counts in the water and larvae. The results also indicated a positive effect of *Bacillus* spp. against *Vibrio* spp. under *in vivo* and *in vitro* conditions. Our findings support the view that the administration of probiotic bacteria is a promising alternative to prevention of *Vibrio* spp. during the larviculture of *L. vannamei*.

Acknowledgements

The authors are grateful to the company INVE for providing the probiotic used in the experiments. This study was supported by the Pernambuco State Council of Science and Technology (FACEPE) and the Brazilian Council for Scientific and Technological Development (CNPq). S. Peixoto is researcher fellow of CNPq.

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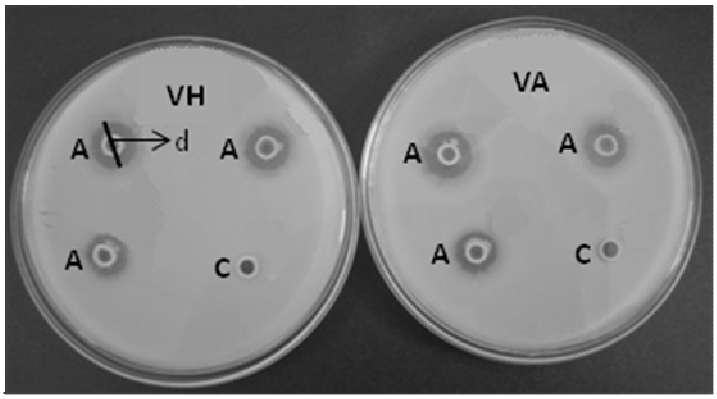
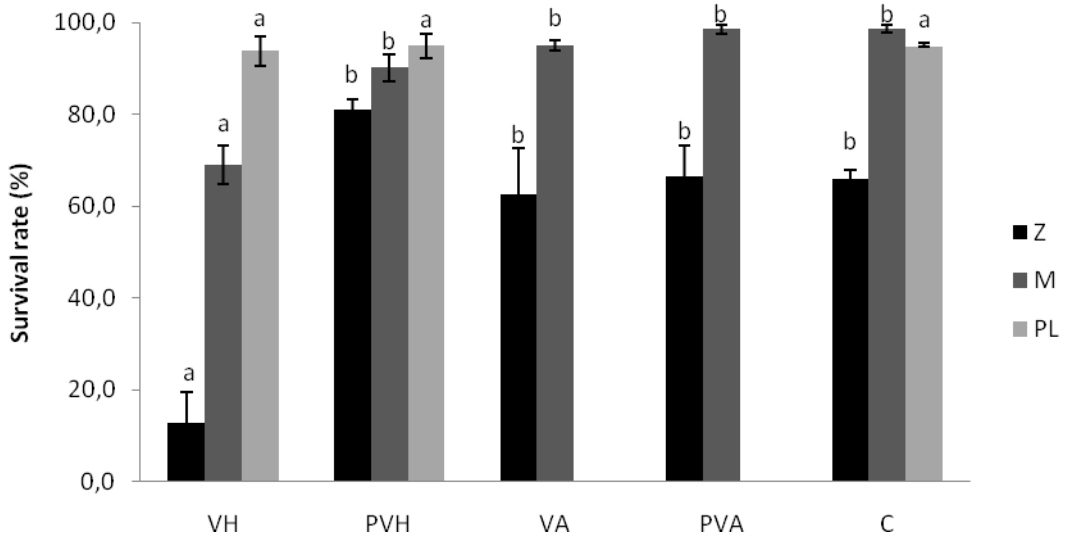


Figure 1 – Petri dishes containing *Bacillus* spp. probiotic in the wells (A) showing inhibitory zones (d = diameter) against the growth of *V. harveyi* (VH), *V. alginolyticus* (VA) and control (C) with no inhibitory zone.



425

426 Figure 2 – Survival of *Litopenaeus vannamei* zoea (Z), mysis (M) and postlarvae (PL) treated
427 with commercial probiotic (*Bacillus* spp.) and exposed to experimental infection by *Vibrio*
428 sp.
429 VH = only *V. harveyi*; VA = only *V. alginolyticus*; PVH = *V. harveyi* + commercial probiotic;
430 PVA = *V. alginolyticus* + commercial probiotic; C = control, without inoculum of probiotic
431 and *Vibrio* sp. Means with different letters were significantlly different (P<0,05).

Table1. Mean (± SE) values of total *Vibrio* spp. count in the water and shrimp *Litopenaeus vannamei* (stages $Zoea_3$, $Mysis_3$ and PL_{14}) treated with commercial probiotic (*Bacillus* spp.) and exposed to experimental infection by *Vibrio* sp.

	Stages	Treatments	*Vibrio* spp. counts	
			Water (10^3 CFU/ml)	Shrimp (10^2 CFU/shrimp)
Experiment Z	$Zoea_3$	VH	4.5 ± 2.3^{a}	13.0 ± 3.7^{a}
		PVH	0.6 ± 0.2^{ab}	1.5 ± 0.3^{b}
		VA	0.6 ± 0.0^{ab}	6.5 ± 2.3^{ab}
		PVA	0.8 ± 0.3^{ab}	1.0 ± 0.0^{b}
		C	0.2 ± 0.0^{b}	1.0 ± 0.0^{b}
Experiment M	$Mysis_3$	VH	30.0 ± 3.8^{a}	119.0 ± 110.0^{a}
		PVH	6.2 ± 1.1^{ab}	9.6 ± 2.6^{a}
		VA	26.0 ± 3.1^{a}	24.9 ± 9.9^{a}
		PVA	32.0 ± 23.0^{a}	10.5 ± 5.2^{a}
		C	1.8 ± 0.5^{b}	1.5 ± 0.1^{a}
Experiment PL	PL_{14}	VH	17.0 ± 1.1^{a}	82.0 ± 37.0^{a}
		PVH	7.2 ± 0.5^{b}	29.0 ± 9.8^{a}
		C	2.6 ± 1.0^{c}	28.0 ± 6.1^{a}

Different superscript letters in the same column (within the same ontogenic stage and experiment) indicate significant differences ($p<0.05$).

VH = only *V. harveyi*; PVH = *V. harveyi* + commercial probiotic; VA = only *V. alginolyticus*; PVA = *V. alginolyticus* + commercial probiotic; C = control, without inoculum of probiotic and *Vibrio* sp.

ANEXO I

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