UNIVERSIDADE FEDERAL DO RIO GRANDE
PROGRAMA DE PÓS-GRADUAÇÃO EM AQUICULTURA

ENGENHARIA GENÉTICA EM PROBIÓTICOS PARA ATIVAÇÃO DO SISTEMA DE RNA INTERFERENTE (RNAi) EM CAMARÕES: UMA POTENCIAL APLICAÇÃO NO CONTROLE DE DOENÇAS VIRAIS

JOÃO COSTA FILHO

RIO GRANDE, RS
2019

UNIVERSIDADE FEDERAL DO RIO GRANDE
PROGRAMA DE PÓS-GRADUAÇÃO EM AQUICULTURA

ENGENHARIA GENÉTICA EM PROBIÓTICOS PARA ATIVAÇÃO DO SISTEMA DE RNA INTERFERENTE (RNAi) EM CAMARÕES: UMA POTENCIAL APLICAÇÃO NO CONTROLE DE DOENÇAS VIRAIS

JOÃO COSTA FILHO

Tese apresentada como parte dos requisitos para obtenção do grau de Doutor em Aquicultura no Programa de Pós-graduação em Aquicultura da Universidade Federal do Rio Grande - FURG.

Orientador: Dr. Luis Fernando Marins
Coorientador: Dr. Josef Altenbuchner

RIO GRANDE, RS
2019

Pós Graduação em
AQUICULTURA
Universidade Federal do Rio Grande - FURG

ATA 01/2019

No dia dezoito de fevereiro de dois mil e dezenove, às oito horas e trinta minutos, no Auditório da Estação Marinha de Aquacultura da FURG, reuniu-se a Banca Examinadora de Tese de Doutorado em Aquicultura, do **JOÃO COSTA FILHO**, orientado pelo Prof. Dr. Luis Fernando Marins, composta pelos seguintes membros: Prof. Dr. Luis Fernando Marins (Orientador – ICB/FURG), Prof. Dr. Dariano Krummenaeur, Prof. Dr. Carlos Eduardo da Rosa (ICB/FURG) e Prof. Dr. Vinícius Farias Campos (UFPel), Título da Tese: "**Engenharia genética em probióticos para ativação do sistema de RNA interferente (RNAi) em camarões: Uma potencial aplicação no controle de doenças virais**". Dando início à defesa, o Coordenador do PPGAq, Prof. Dr. Luis Henrique da Silva Poersch passou a presidência da sessão ao Prof. Dr. Luis Fernando Marins, que na qualidade de orientador, passou a palavra para o candidato apresentar a Tese. Após ampla discussão entre os membros da Banca e o candidato, a Banca se reuniu sob a presidência do Coordenador. Durante esse encontro ficou estabelecido que as sugestões dos membros da Banca Examinadora devem ser incorporadas na versão final, ficando a cargo do Orientador o cumprimento desta decisão. O candidato **JOÃO COSTA FILHO** foi considerado **APROVADO**, devendo a versão definitiva de a Tese ser entregue na Secretaria do PPGAq, no prazo estabelecido nas Normas Complementares do Programa. Nada mais havendo a tratar, foi lavrada a presente ata, que após lida e aprovada, será assinada pela Banca Examinadora, pelo candidato e pelo Coordenador do PPGAq.

PROF. DR. LUIS FERNANDO MARINS (ORIENTADOR – ICB/FURG)

PROF. DR. DARIANO KRUMMENAUER (IO/FURG)

PROF. DR. CARLOS EDUARDO ROSA (ICB/FURG)

PROF. DR. VINÍCIUS FARIAS CAMPOS (UFPel)

JOÃO COSTA FILHO

PROF. DR. LUIS HENRIQUE DA SILVA POERSCH (Coordenador do PPGAq)

SUMÁRIO

INTRODUÇÃO GERAL 1
1. Sanidade no cultivo do camarão *Litopenaeus vannamei* 1
1.1. Vírus da Síndrome da Mancha Branca (WSSV) 1
1.2. Vírus da Mionecrose Infecciosa (IMNV) 3
1.3. O mecanismo do RNA de interferência no combate das viroses 4
2. Probióticos na carcinicultura 6
2.1. Engenharia genética em probióticos 7
3. Edição genômica com o sistema CRISPR/Cas9 9
OBJETIVOS 12
Geral 12
Específicos 12
CAPÍTULO I: Complete Genome Sequence of Native *Bacillus cereus* Strains Isolated from Intestinal Tract of the Crab *Ucides* sp. 13
ABSTRACT 13
VALUE OF THE DATA 15
DATA 15
EXPERIMENTAL DESIGN, MATERIALS AND METHODS 16
Isolation of native *Bacillus* strains 16
Isolation of genomic DNA 17
Library preparation and sequencing 17
Genome assembly 17
ACKNOWLEDGEMENTS 18
REFERENCES 18
CAPÍTULO II: Genetic Manipulation of a Native *Bacillus cereus* Strain with Probiotic Potential in Shrimp Farming 20
ABSTRACT 21
1. INTRODUCTION 21
2. MATERIAL AND METHODS 22
2.1 *Bacillus cereus* #25 22
2.2 Genetic manipulation 23
2.3 Feed supplemented with genetically modified *B. cereus* #25 23

2.4 Bioassays with *Litopenaeus vannamei* 24
2.5 BC25GM colonization in the *L. vannamei* midgut 24
3. RESULTS 25
3.1 Genetic manipulation of *B. cereus* #25 25
3.2 Bioassays with *Litopenaeus vannamei* 26
3.3 BC25GM midgut colonization in *L. vannamei* 27
4. DISCUSSION 29
FUNDING 31
REFERENCES 31
CAPÍTULO III: CRISPR/Cas9 in the Production of *Bacillus subtilis* Strains as Bioreactors of Double-Stranded RNAs (dsRNAs) Against Shrimp Viruses 34
Abstract 34
Introduction 35
Materials and methods 36
Bacterial strains and culture conditions 36
Construction of pJJ2 vector for *rnc* deletion 37
Construction of the vectors pJJ4 and pJJ10 for integration and dsRNA expression 38
Antibiotic resistance removal of the strains 39
Production of GFPmut1 and dsRNA 39
Confirmation of dsRNA production using Reverse Transcriptase PCR (RT-PCR) 40
Results 41
Plasmids 41
Deletion of *rnc* gene 42
Integration of GFPmut1 and dsRNAs 42
Antibiotic resistance removal from JJBs3 and JJBs6 strains 44
Production of GFPmut1, dsRNAs, and detection of dsRNA using RT-PCR 44
Discussion 44
Acknowledgments and Funding Information 46
Conflict of interest 46
References 46
DISCUSSÃO GERAL 52
CONCLUSÕES DA TESE 54

PERSPECTIVAS 55
REFERÊNCIAS DA INTRODUÇÃO E DISCUSSÃO GERAL 56

AGRADECIMENTOS

Ao meu orientador Dr. Luis Fernando Marins e coorientador e Dr. Josef Altenbuchner (IIG - Universität Stuttgart), por compartilhar conhecimentos e experiências. A todos os Professores do programa de Pós-Graduação em Aquicultura (EMA). Em especial ao Dr. Wilson Wasielesky Jr. e seu grupo de pesquisa que sempre esteveram disponíveis oferecendo auxílio, suporte e excelentes conselhos.

Ao Conselho de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa de doutorado e ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pela concessão da bolsa de doutorado sanduíche no exterior e apoio financeiro (N° proc. 454918/2014-3).

A todos os participantes dos grupos de pesquisa dos laboratórios que estive presente durante o desenvolvimento dessa tese:

→ Laboratório de Biologia Molecular do ICB-FURG, em especial a MSc. Loraine Moraes que sempre foi uma grande amiga e colega de bancada.

→ Centro de Microscopia Eletrônica do Sul (CEME-SUL) da Universidade Federal do Rio Grande (FURG), em especial a Dra. Ana Cristina Kalb que nos ofereceu grande auxílio.

→ Laboratórios de Vacinologia; Genômica Funcional, Bioinformática e Proteômica. Ambos do Núcleo de Biotecnologia da Universidade Federal de Pelotas. Em especial agradecimento ao Dr. Vinícius Farias Campos pela disponibilidade em criar boas parcerias de pesquisa.

→ Laboratório de Biologia Molecular vinculado ao Centro de Diagnóstico para Enfermidades de Organismos Aquáticos (CEDECAM-UFC, LABOMAR), em especial a Dr. Rodrigo Maggioni e MSc. Maria das Graças Lima Coelho, um exemplo de excelência profissional e uma grande amiga.

→ Laboratório de Microbiologia Ambiental e do Pescado (LAMAP-UFC, LABOMAR), em especial a Dra. Oscarina Viana de Sousa que inúmeras vezes contribuiu para o desenvolvimento desse trabalho.

→ Aos grupos de pesquisa do Institut für Industrielle Genetik - Universität Stuttgart, em especial Michaela Moses-Endewardt uma grande amiga que vou levar pra vida toda.

RESUMO

As doenças infecciosas, principalmente de etiologia viral, tem sido o principal desafio da produção de camarão. A ativação do mecanismo RNAi (RNA de interferência) pode permitir o controle da replicação de viroses importantes na carcinicultura o WSSV (Vírus da Síndrome da Mancha Branca) e o IMNV (Vírus da Mionecrose Infecciosa). Os trabalhos descritos nessa Tese estão relacionados com o método de produção e entrega de dsRNA (RNA dupla fita) para o camarão branco do *Litopenaeus vannamei*, com o intuito de ativar o mecanismo do RNAi. Na tentativa de produzir dsRNA utilizando bactérias foi realizado sequenciamento genômico e manipulação genética em cepas nativas de *Bacillus*, bem como realizadas edições genômicas em *B. subtilis* através da técnica CRISPR/Cas9. No capítulo I foi sequenciado o genoma de duas cepas nativas de *B. cereus*, isoladas do trato gastrointestinal do caranguejo *Ucides* sp. coletado no Rio Pacoti em Fortaleza (CE). Esses resultados moleculares fornecem novas sobre essas cepas, bem como subsídios para manipulação genética. No capítulo II, uma cepa nativa de *B. cereus* foi manipulada geneticamente para produzir fluorescência (GFPmut1). Além disso, foi promovido seu fornecimento via ração e avaliação da colonização na mucosa do trato intestinal do *L. vannamei*. No capítulo III, foram criados plasmídeos para edição genômica em *B. subtilis* usando o sistema CRISPR/Cas9. Primeiramente o gene *rnc* foi nocauteado e, na sequência, foram integrados genes para produção de GFPmut1, dsRNA relacionadas com as viroses WSSV e IMNV, bem como dsRNA não relacionada. No final, foram produzidas duas cepas *B. subtilis* (JJBs5 e JJBs8) livres de resistência a antibióticos que estão prontas para serem testadas em bioensaios com desafio viral. Em conclusão, os resultados demonstrados nesse trabalho podem representar novas perspectivas na tentativa de mitigar os problemas com as doenças nos cultivos de camarão.

Palavras-chave: RNAi, dsRNA, WSSV, IMNV, *B. subtilis*

ABSTRACT

The infectious diseases, major viral ethology, has been the main challenge in the shrimp production. The activation of the RNAi (interference RNA) mechanism can promote the control of the viral replication, such as (WSSV viruses White Spot Syndrome Virus) and IMNV (Infectious Myonecrosis Virus). Os trabalhos descritos nessa Tese estão relacionados com o método de produção e entrega de dsRNA (RNA dupla fita) para o camarão branco do *Litopenaeus vannamei*, com o intuito de ativar o mecanismo do RNAi. The studies described in this thesis are related to the production and delivery of dsRNA (double-stranded RNA) for *Litopenaeus vannamei*, in order to activate the mechanism of RNAi. On the possibility to produce dsRNA using bacteria was made genomic sequencing and genetic manipulation in native *Bacillus* strains, as well-made genomics editions in *B. subtilis* using the CRISPR/Cas9 technology. In the chapter I was sequenced the genome of the two native *B. cereus* strains isolated from the gastrointestinal tract of crab *Ucides* sp. from the Pacoti River in Fortaleza - CE. These molecular findings can improve information of this strain and give support for genetic manipulation. In the chapter II, a native *B. cereus* strain was manipulated to fluorescence production (GFPmut1). In addition, it was delivered in feed and evaluated the colonization in the intestinal tract of the *L. vannamei*. In the chapter III, plasmids were developed for genomic editing of *B. subtilis* using the CRISPR/Cas9. In the first moment, *rnc* gene was deleted and, in the second step, genes coding for GFPmut1, dsRNA against WSSV and IMNV viruses, and unrelated dsRNA were integrated into *B. subtilis* genome. At the end, two *B. subtilis* strains (JJBs5 and JJBs8) were produced, both of them free of the antibiotic resistance that could be tested in shrimp bioassays with viral challenge. In conclusion, the findings presented in this work could represent new perspectives to mitigate the problems with shrimp disease in commercial farms.

Keywords: RNAi, dsRNA, WSSV, IMNV, *B. subtilis*

INTRODUÇÃO GERAL

1. Sanidade no cultivo do camarão *Litopenaeus vannamei*

O cultivo de organismos aquáticos apresentou em 2016 um crescimento de 46% em comparação ao ano de 2000, sobretudo em relação a crustáceos, os quais ocupam o segundo lugar mundial em valor econômico (FAO, 2018). A espécie mais cultivada no mundo é o camarão *L. vannamei* (3,6 milhões de toneladas em 2016), o qual apresenta boas características zootécnicas, como rusticidade a variações ambientais, bom crescimento e adaptação ao consumo de rações comerciais. No entanto, a ocorrência de enfermidades, principalmente de etiologia viral, tem afetado significativamente a produção, sobretudo na Ásia e América Latina (FAO, 2018). No Brasil ocorreram grandes perdas econômicas em 2004 e 2005, principalmente devido a problemas sanitários (Santos et al., 2013) e recentemente continuam ocorrendo (Globo Rural, 2017).

O surgimento de doenças infecciosas, com seus reflexos econômicos e sociais, tem sido o principal desafio para a carcinicultura mundial (Anderson et al., 2014). Normalmente, a ocorrência dessas enfermidades está associada a alterações nas condições do cultivo, principalmente o aumento das densidades de estocagem (Vieira-Girão et al., 2015). Atualmente, são conhecidos muitos agentes virais capazes de infectar camarões (Reshi et al., 2014). De acordo com Seibert e Pinto (2012), as principais doenças são: Necrose Hipodérmica Hematopoiética Infecciosa (IHHNV), Vírus da cabeça amarela (YHV), Vírus da Síndrome de Taura (TSV), Vírus da Síndrome da Mancha Branca (WSSV) e Vírus da Mionecrose Infecciosa (IMNV). Especialmente no Brasil, o WSSV e o IMNV vêm promovendo constantes prejuízos, sendo necessário o desenvolvimento de pesquisas que promovam a diminuição desses problemas sanitários.

1.1. Vírus da Síndrome da Mancha Branca (WSSV)

A doença causada pelo WSSV é caracterizada por provocar grandes mortalidades nos camarões cultivados, entre 90 e 100% durante um período de 3 a 10

dias do início da infecção (Pantoja & Lightner, 2014). Os danos econômicos causados na carcinicultura mundial são crescentes e até 2012 foram estimados em torno de 8 a 15 bilhões de dólares (Lightner, 2012). Essa enfermidade foi detectada no Brasil em 2004, onde primeiramente causou grandes perdas econômicas na carcinicultura do Sul do país (Feijó et al., 2013). Recentemente, surtos foram identificados no Nordeste, causando impacto econômico e social na região. A mancha branca apareceu no Ceará em 2016 e em seis meses 30 mil toneladas de camarão foram perdidas. Esse valor é o equivalente a 60% da produção do período (Globo Rural, 2017).

O agente causador da WSSV pertence ao gênero *Whispovirus* e família *Nimaviridae* (Mayo, 2002). Seu genoma é composto por dsDNA circular com ~300 kb (Van Hulten et al., 2001a), o vírus é envelopado e apresenta forma cilíndrica (Chen et al., 2002), como demonstrado na Figura 1. O ciclo do vírus pode ser dividido em três etapas: entrada na célula do hospedeiro, replicação do material genético e, finalmente, a montagem e liberação de partículas (Verbruggen et al., 2016). O primeiro obstáculo para o vírus é a entrada na célula. Nessa etapa, esse agente conta com a VP28 (codificada pelo gene Vp28), que é a principal proteína de envelope responsável pelas interações entre o vírus e o hospedeiro (Van Hulten et al., 2001b).

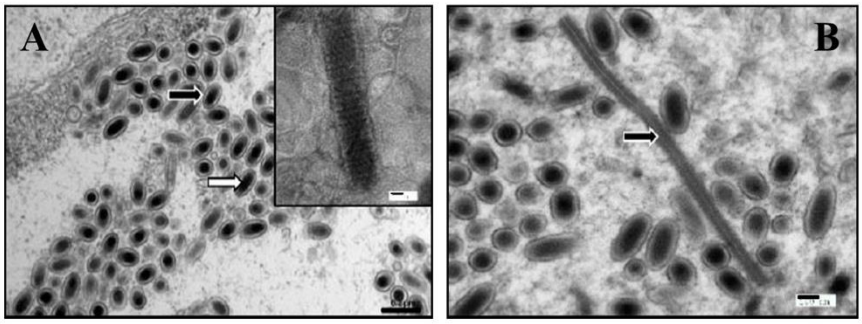

Figura 1 - Características morfológicas do WSSV (Verbruggen et al., 2016). (A) Vírions em forma ovóide contendo nucleocapsídeo (seta branca) dentro de um envelope trilaminar (seta preta). Escala da barra = 0,2 μm. Figura menor: Nucleocapsídeo WSSV corado negativamente com a presença de material estriado, estruturado como uma série de anéis de subunidades. Barra de escala = 20 nm. (B) Nucleocapsídeo antes do envoltório e incorporação da formação de partículas maduras de WSSV. Escala da barra = 100 nm.

Os camarões infectados apresentam deposição de sais cálcio na cutícula da carapaça (0,5 a 2 mm de diâmetro), assim como seu desprendimento, redução no consumo de alimento, anorexia, letargia, coloração avermelhada nos apêndices ou em todo corpo (Pantoja & Lightner, 2014), formação de corpos de inclusão e necrose (Verbruggen et al., 2016). Os sintomas comuns da doença são apresentados na Figura 2. De modo geral o WSSV é a principal virose que afeta o crescimento econômico da carcinicultura (FAO, 2018).

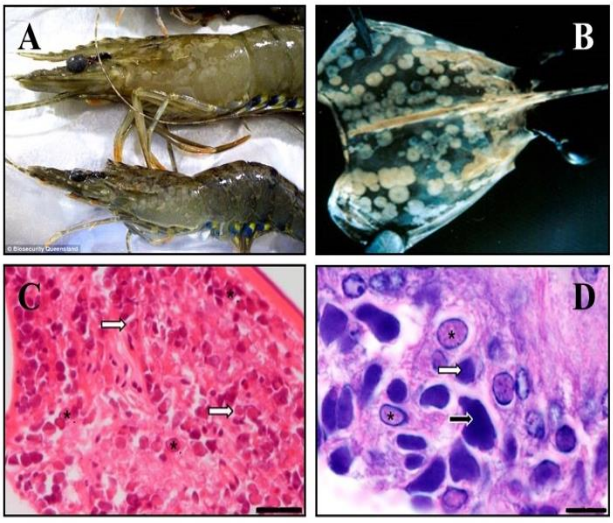

Figura 2 - Sintomas do WSSV. (A) Camarão com deposição de sais cálcio na cutícula da carapaça (Mail Online News, 2017). (B) Desprendimento da carapaça (Aquatic Animal Diseases, 2018). (C) Estágio inicial de células infectadas com formação de inclusão intranuclear eosinofílica (seta branca). Escala da barra = 25 μm. (D) A inclusão geralmente se expande para preencher o núcleo (*) torna-se basofílica e de coloração mais densa a medida que a infecção progride (seta branca). Os núcleos então se desintegram e o conteúdo se funde com o citoplasma (seta preta). Escala da barra de escala = 10 μm (Verbruggen et al., 2016).

1.2. Vírus da Mionecrose Infecciosa (IMNV)

A doença causada pelo IMNV é responsável por provocar mortalidades de 40 a 70% nos camarões cultivados e, consequentemente, grandes prejuízos econômicos. Foi diagnosticada pela primeira vez no ano de 2002 em cultivos de camarões localizados no

Nordeste do Brasil (Andrade et al., 2007). Posteriormente, a doença ocorreu na Indonésia e no Sudeste asiático (Senapin et al., 2011). Nos camarões, os sintomas mais comuns são necrose dos músculos estriados do abdômen, apêndices e cefalotórax, apresentando áreas com aspecto leitoso durante a fase inicial e, em estádios mais avançados, uma coloração avermelhada no abdômen (Nunes et al., 2004), como apresentado na Figura 3. Além disso, pode causar lesões nas brânquias e órgão linfóide, segundo Lightner et al. (2004).

O agente causador da doença do IMNV faz parte da família *Totiviridae.* Seu genoma é composto de uma única molécula de dsRNA com ~7.5 kb, apresenta simetria icosaédrica (Figura 3), não é envelopado e possui 40 nm de diâmetro (Pantoja e Lightner, 2014). O genoma compreende duas sequências de leitura aberta (ORFs) que se sobrepõem em 199 pb. A região ORF1 codifica para principal proteína de capsídeo (MCP) e a ORF2 codifica para uma RNA-dependente RNA polimerase (Tang et al., 2008).

Figura 3 - Características morfológicas e sintomas do IMNV. (A) Morfologia do vírus IMNV codificada por cores (Tang et al., 2008). (B) Camarão apresentando áreas com aspecto leitoso (O autor, 2017). (C) Necroses dos músculos estriados do abdômen. Escala da barra = 100 μm (Feijó et al., 2015).

1.3. O mecanismo do RNA de interferência no combate das viroses

Os crustáceos possuem sistema imune inato com ausência de resposta adaptativa, fato que inviabiliza a tentativa de produzir vacinas para combater patógenos, principalmente de etiologia viral (Barroco et al., 2014). Todavia, segundo esses mesmos autores a descoberta do mecanismo de silenciamento gênico pós-transcricional ou RNA de interferência (RNAi) tem gerado uma nova perspectiva para o tratamento das

doenças infecciosas virais nos camarões cultivados. Essa tecnologia é uma poderosa ferramenta capaz de silenciar a expressão gênica e replicação viral, onde fitas duplas de RNA (dsRNA) conduzem um complexo proteico para degradação do RNA mensageiro (RNAm) homólogo a sua própria sequência (Reshi et al., 2014).

O mecanismo RNAi consiste no processamento de dsRNA (endógenas ou exógenas) em moléculas menores ou siRNAs (small interference RNAs, 20-28 pb) por reações catalisadas por um complexo proteico contendo a enzima Dicer, que é uma endoribonuclease do tipo III. Os siRNAs resultantes são incorporados em um grupo proteico chamado RISC (RNA-induced Silencing Complex), que contém uma proteína da família Argonauta, guiando a degradação específica ou repressão da tradução do RNAm com regiões complementares à sequência do dsRNA desencadeante (Robalino et al., 2007). Mais detalhes do mecanismo estão descritos na Figura 4.

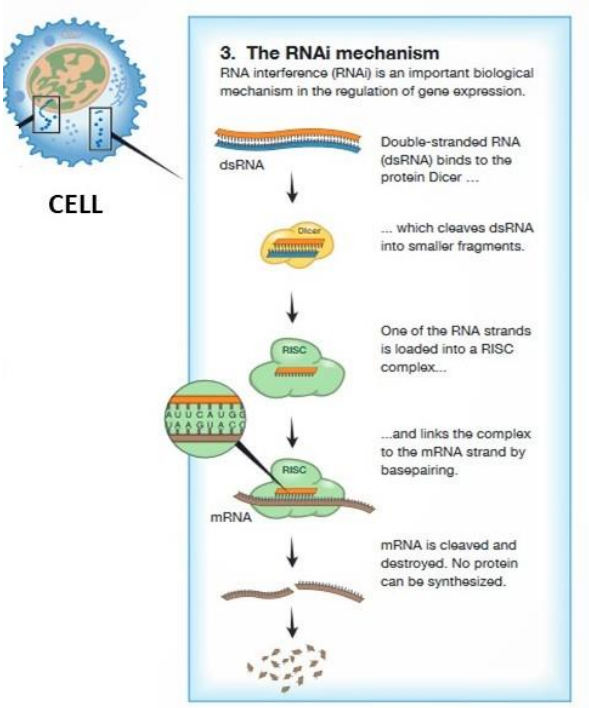

Figura 4 - Processos celulares relacionados ao mecanismo do RNA interferente (RNAi), onde a enzima Dicer e o complexo proteico RISC desempenham um papel fundamental na degradação do RNA endógeno ou exógeno (Fire e Mello, 2006).

Em relação ao camarão *L. vannamei*, o mecanismo de RNAi já foi utilizado em nível experimental como tratamento antiviral. Os estudos demonstraram que a injeção

de dsRNA exógenas relacionadas com sequências específicas para o WSSV (Kumar et al., 2015) e IMNV (Feijó et al., 2015) foram eficazes, promovendo limitação na infecção viral e melhorias na sobrevivência aos camarões. Entretanto, a utilização das dsRNAs antivirais ainda não é viável em escala comercial uma vez que a síntese destas moléculas em laboratório é extremamente cara e trabalhosa, bem como a injeção direta nos camarões é impossível do ponto de vista do manejo. Assim, para que esse tecnologia possa chegar aos produtores e mitigar os problemas causados pelas doenças virais, é necessário o desenvolvimento de novas tecnologias que possam viabilizar sua aplicação nos cultivos comerciais de grande escala.

2. Probióticos na carcinicultura

A utilização de probióticos comerciais na aquicultura apresentou crescimento nos últimos anos, sobretudo pelos benefícios proporcionados no controle de doenças (Akhter et al., 2015). De acordo com a FAO (2001), probióticos são microrganismos que administrados na quantidade adequada promovem benefícios a saúde do hospedeiro. Muitos tipos de microrganismos têm sido reportados por apresentarem potencial probiótico, classificados em Gram-positivos ou Gram-negativos, sendo utilizados na forma simples ou combinada (Hai, et al., 2015). Neste sentido, algumas espécies de *Bacillus* recebem uma atenção especial, apresentando atividades antagônicas sobre bactérias patogênicas (produção natural de antibióticos), produção de enzimas extracelulares e tolerância ao meio ambiente (Banerjee e Ray, 2017). A espécie *B. subtilis* é a mais utilizada como probiótico na carcinicultura. Sua morfologia, formação de biofilme e estrutura desenvolvida para proteção estão demonstradas na Figura 5.

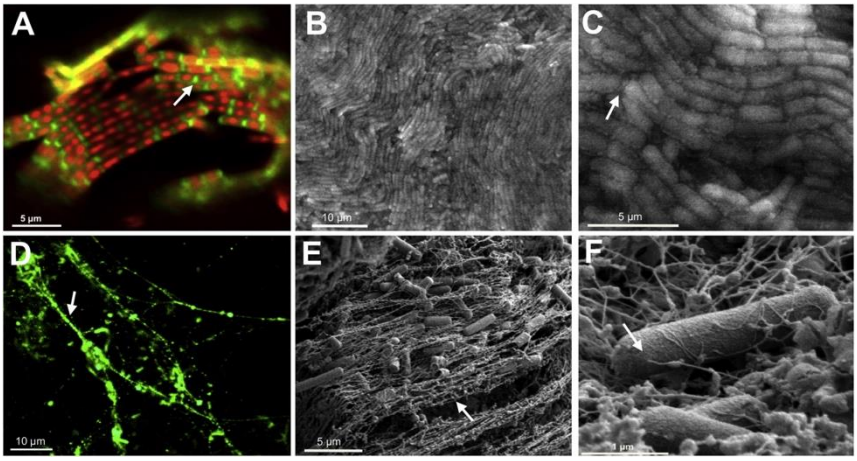

Figura 5 - Estrutura do biofilme de *B. subtilis* utilizando diferentes técnicas de microscopia (Bridier et al., 2013). (A) Visualização das células com marcador fluorescente (seta), através de microscopia de epifluorescência. (B e C) Células em diferentes ampliações (seta). (D) Observação de estruturas fibrilares no biofilme com marcador fluorescente. (E e F) Morfologia do biofilme em diferentes ampliações, com células aderidas (setas brancas), por microscopia de varredura.

Os benefícios dos probióticos para os organismos aquáticos podem ser classificados como: melhorias no crescimento, aumento da taxa de sobrevivência, resistência a doenças, auxílios nos processos digestivos, melhorias na reprodução e qualidade da água no cultivo (Hai et al., 2115; Banerjee e Ray, 2017). Em relação aos camarões cultivados, também foi demonstrado que a utilização de probióticos pode promover proteção contra patógenos e atividade antiviral (Lakshmi et al., 2013). No entanto, a engenharia genética aplicada aos probióticos ou bactérias do gênero *Bacillus* pode ampliar esses benefícios, bem como a sua utilização como veículo para entrega via oral de moléculas que podem fornecer melhorias aos sistemas de cultivo.

2.1. Engenharia genética em probióticos

As espécies de *Bacillus* representam um importante modelo para produção de vários tipos de biomoléculas recombinantes, como enzimas. Nesse sentido, estratégias de engenharia genética foram desenvolvidas para ampliar a possibilidade de edição genômica desses microorganismos (Dong e Zhang, 2014). De acordo com esses mesmos autores normalmente é realizada a recombinação homóloga de genes de

interesse em regiões específicas do genoma. Entretanto, a obtenção de cepas geneticamente modificadas é relativamente difícil e laboriosa. Nos procedimentos é necessário a construção de plasmídeos para transformação genética, otimização de protocolos, bem como o conhecimento do genoma da espécie em questão. Na busca por melhorias na eficiência da edição genômica, novas metodologias vêm sendo desenvolvidas, como exemplo a utilização do CRISPR-Cas9 (Altenbuchner, 2016), que será descrito na próxima seção.

Como evidenciado anteriormente, o mecanismo do RNAi é considerado um importante método de intervenção terapêutica aplicado a carcinicultura. No entanto, a sua utilização em larga escala no cultivo dos camarões está limitada pela quantidade de moléculas de dsRNA a serem sintetizadas e a forma de aplicação (Sarathi et al., 2010). A manipulação genética de microorganismos tem criado a possibilidade para produção de moléculas capazes de promover benefícios principalmente em relação à imunologia e nutrição dos crustáceos (Attasart et al., 2013). Contudo, até o presente momento são escassos os trabalhos que demonstram a produção e aplicação desse tipo pesquisa na aquicultura, em especial na carcinicultura. Além disso, quando desenvolvidos podem estar em condição de pedido de proteção intelectual, o que retarda ainda mais uma aplicação prática.

Em relação ao tratamento de infecções virais em camarões, foi demonstrado que é possível a produção de dsRNA específicas através da manipulação genética de cepas de *Escherichia coli* (Attasart et al., 2013; Taju et al., 2015). Porém, a forma de aplicação no cultivo ainda necessita de mais estudos, uma vez que essa espécie de bactéria não apresenta potencial probiótico. Além disso, é classificada como Gram-negativa e pode ser patogênica para o camarão e para o consumidor. Outro exemplo interessante foi demonstrado por Cheng et al. (2013) utilizando *E. coli* HMS 174 para produzir a enzima fitase, auxiliando no aproveitamento do fósforo da dieta, promovendo melhorias no crescimento e eficiência alimentar para o *L. vannamei*.

Fu et al. (2011) e Nguyen et al. (2014) demonstraram que camarões *L. vannamei* desafiados com WSSV podem ser alimentados com cepas recombinantes de *Bacillus subtilis* que produzem molécula antigênicas da proteína VP28, promovendo aumento da sobrevivência. Desta forma, a manipulação genética aplicada em *Bacillus* pode ser uma importante ferramenta no tratamento de viroses que afetam a carcinicultura, uma vez

que possuem características probióticas e não promovem riscos à saúde humana (Liu et al., 2010). Os probióticos podem ser utilizados na tentativa de produzir dsRNA contra as viroses que afetam a carcinicultura. Contudo, o gene *rnc* precisa ser deletado devido ao fato de estar associado com a produção da Ribonuclease III, uma enzima responsável pela degradação de dsRNA (Durand et al., 2012). De acordo com esses mesmos autores o gene *rnc* é essencial para cepas que possuem os profagos Skin and SPβ. Esse assunto será abordado na discussão do Capítulo III.

3. Edição genômica com o sistema CRISPR/Cas9

Entre as técnicas utilizadas para promover a edição genômica em procariontes o sistema CRISPR/Cas9 (Repetições Palindrômicas Curtas Agrupadas e Regularmente Interespaçadas) é uma recente e inovadora ferramenta de engenharia genética. O mecanismo foi descrito por Jinek et al. (2012) e está relacionado com a imunidade adaptativa de algumas espécies de bactérias. Possui tipos I, II e III, sendo o segundo mais comum. De modo geral, consiste na incorporação de sequências nucleotídicas exógenas no cromossoma bacteriano, obtidas de invasores malsucedidos, servindo como memória de defesa contra um novo ataque desses mesmos elementos.

O sistema do CRISPR tipo II funciona da seguinte forma: através do DNA exógeno incorporado no lócus CRISPR são transcritos CRISPR RNAs (crRNAs) que são conjugados com CRISPR RNA trans-ativados (tracrRNAs), também transcritos pelo mesmo lócus, formando o RNA guia (gRNA). Este último forma um complexo com a enzima Cas9, conduzindo a ruptura no DNA homólogo ao crRNA (Figura 6). No entanto, esse processo só ocorre se adjacente a sequência complementar existir o chamado Motivo Adjacente ao Protoespaçador (PAM), que é um domínio guia constituído de três nucleotídeos (5´-NGG-3´).

Como relatado por Hille e Charpentier (2016), após a quebra do DNA ocorre ativação do sistema de reparo podendo ser através da via de união de extremidades não homólogas (NHEJ), que permite o surgimento de mutações promovendo inativação gênica (nocaute), bem como o reparo dirigido por homologia (HDR), onde um fragmento de DNA com homologia ao local alvo é usado para reparar o DNA através de recombinação homóloga. O mecanismo de reparo dirigido por homologia é utilizado por

procariontes e permite a inserção de qualquer fragmento de DNA desejável (Figura 6). Dessa forma, a aplicação dessa técnica permite a edição genômica e pode ser aplicada em modelos como os *Bacillus*, como demonstrado por Altenbuchner (2016) e Westbrook et al. (2016).

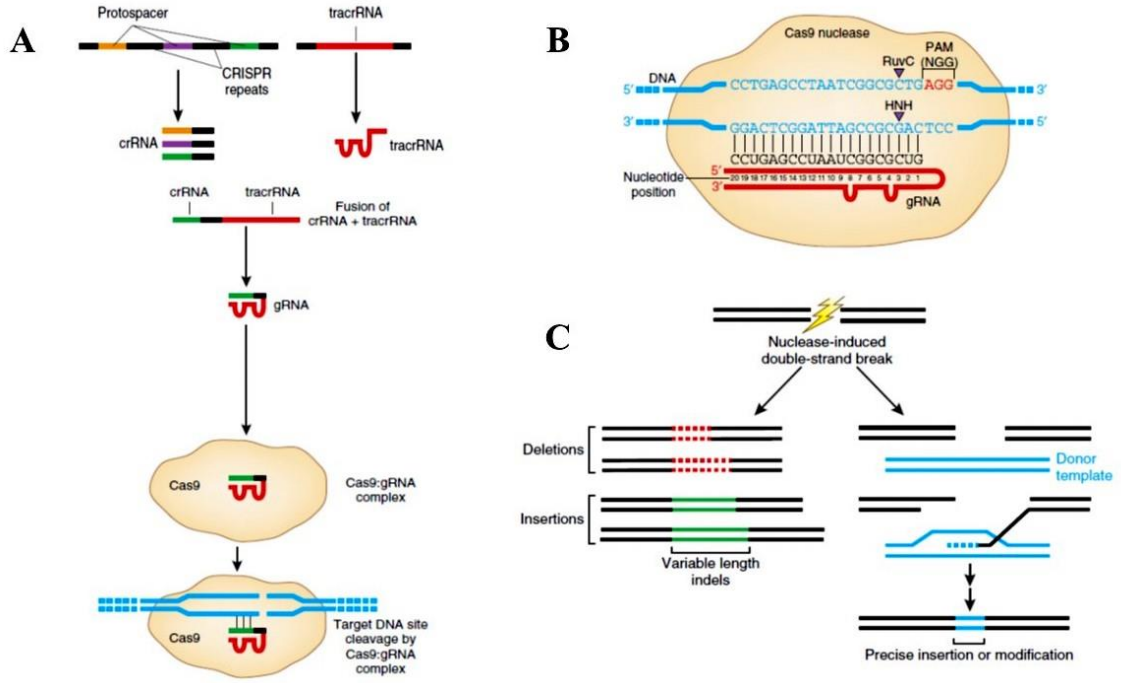

Figura 6 - Mecanismo do CRISPR/Cas9 para edição genômica (Sander e Joung, 2014). (A) Introdução de DNA exógeno no locus CRISPR e transcrição das moléculas crRNA e tracrRNA. Essas são combinadas dando origem ao gRNA que forma o complexo com a enzima Cas9, conduzindo a ruptura no DNA homólogo ao crRNA. (B) Domínio PAM constituído de três nucleotídeos (5´-NGG-3´) identificado pela enzima Cas9, bem como domínios RuvC e HNH, que exibem atividade nucleásica da Cas9. (C) A ruptura no DNA pode ser corrigida por reparo não homólogo, produzindo mutações por inserção ou deleção com comprimento variável, bem como o reparo dirigido por homologia, introduzindo uma precisa inserção ou recombinação utilizando DNA molde.

Contudo, os problemas com as doenças infecciosas dos camarões podem ser mitigados com a utilização de dsRNAs produzidas a partir de *Bacillus* recombinantes obtidos pela técnica CRISPR/Cas9. Nesse procedimento, é necessário o nocaute do gene *rnc* envolvido na produção da enzima ribonuclease III, que atua na degradação das

dsRNA e recombinação homóloga de sequências para produção de dsRNA relacionadas com as viroses WSSV e IMNV. A realização do presente trabalho pode contribuir para produção dessas moléculas, representando uma novidade com a aplicação de uma recente e inovadora técnica de biologia molecular.

OBJETIVOS

Geral

Transformar, através de técnicas de engenharia genética, bactérias probióticas nativas e comerciais, em biorreatores para produção de dsRNAs capazes de ativar o sistema RNA interferente (RNAi) contra duas das principais viroses que acometem os camarões cultivados: o vírus da macha branca (WSSV) e o vírus da mionecrose infecciosa (IMNV).

Específicos

- Realizar sequenciamento genômico e manipulação genética em cepas nativas de *Bacillus*.
- Produzir vetores de expressão com o mecanismo CRISPR/Cas9.
- Executar o nocaute do gene *rnc* (produz Ribonuclease III, que degrada dsRNA) em *B. subtilis* utilizando a técnica CRISPR/Cas9.
- Realizar recombinação homóloga com a sistema CRISPR/Cas9 para produção de dsRNA relacionadas com as viroses WSSV e IMNV.

CAPÍTULO I: Complete Genome Sequence of Native *Bacillus cereus* Strains Isolated from Intestinal Tract of the Crab *Ucides* sp.

Publicado no periódico Data in Brief (http://dx.doi.org/10.1016/j.dib.2017.11.049)

Complete Genome Sequence of Native *Bacillus cereus* Strains Isolated from Intestinal Tract of the Crab *Ucides* sp.

João Costa Filho [a], Sérgio Jorge [b], Frederico Schmitt Kremer [b], Natasha Rodrigues de Oliveira [b], Vinicius Farias Campos [b], Luciano da Silva Pinto [b], Odir Antônio Dellagostin [b], Rubens Galdino Feijó [c], Francisca Gleire Rodrigues de Menezes [d], Oscarina Vianade Sousa [d], Rodrigo Maggioni [d], Luis Fernando Marins [a]

[a] Federal University of Rio Grande, Institute of Biological Sciences, Rio Grande, RS, Brazil.
[b] Federal University of Pelotas, Center for Technology Development, Biotechnology Unity, Pelotas, RS, Brazil.
[c] Federal Institute of Education, Science and Technology of Ceará, Aracaú, CE, Brazil.
[d] Federal University of Ceará, Institute of Marine Sciences, Fortaleza, CE, Brazil.

ABSTRACT

Bacillus cereus is a gram positive bacterium with sporulation capacity. Here, we report the complete genome sequence of two native *B. cereus* strains (#25 and #29) isolated from intestinal tract of the crab *Ucides* sp. from Pacoti River in the State of Ceará, Brazil. The findings of this study might increase the molecular information for *Bacillus* strains. The data can be used in comparative analyses, origin and distribution, as well support for genetic engineering.

Specifications Table	
Subject area	Biology
More specific subject area	Microbiology and genomics.
Type of data	Table, text file, image and figure.
How data was acquired	Scanning Electron Microscope (JEOL JSM - 6610 LV), Ion Torrent PGM system and bioinformatics applications.
Data format	Analyzed
Experimental factors	Isolation and characterization of native strains #25 and #29. Genomic DNA extraction and sequencing procedures.
Experimental features	Sequencing using Ion Torrent PGM (Life Technologies, Saint Aubin, France), FASTQ (bamToFastq program from the BedTools package), Phred score (Q) using the fastq_quality_filter script from the fastx-toolkit package (http://hannonlab.cshl.edu/fastx_toolkit/). *De novo* genome assemblies using MIRA (http://mira-assembler.sourceforge.net/), SPAdes and Newbler (http://www.roche.com/), consensus assembly was generated using CISA. Contigs was ordered with the program CAR, using the genome of *Bacillus cereus* strain 03BB102 (GenBank: CP009318.1) as reference. Annotation was performed using Genix and Artemis. A BLAST search was performed (https://www.ncbi.nlm.nih.gov/BLAST/). Chromosome features were drawing using DNAPlotter.
Data source location	The native strains #25 and #29 were isolated from intestinal tract of the crab *Ucides* sp., from Pacoti River in Fortaleza, State of Ceará, Brazil. Latitude: 03º 43' 02" S and Longitude: 38º 32' 35" W.
Data accessibility	The complete genome sequence of native *B. cereus* strains #25 and #29 were deposited in GenBank - NCBI under the accession number CP020803.1 and CP020804.1, respectively.

VALUE OF THE DATA

- The data of the present study might increase the molecular information for *B. cereus* strains, isolated from crab in saline environment. The genomic data can be used in development of other researchers such as comparative analyses using genes and genomes, origin and distribution of strains.
- Our data can give support for genetic engineering of *B. cereus* strains.
- This work can offer contributions for the molecular diversity of the *B. cereus* strains improving the pathogenicity assessment or probiotic application in the production of marine organisms.

DATA

In the present work, we describe the whole-genome shotgun (WGS) analysis of two strains of *B. cereus* (Fig. 1) from the collection of bacterial morphotypes from Laboratory of Environmental and Fish Microbiology at the Institute of Marine Sciences (LABOMAR/UFC - Brazil). The strains presented 99% of identity with the *Bacillus cereus* ATCC 14579 (accession number NC_004722). Genomic features of *Bacillus cereus* strains #25 and # 29 (Table 1) presented similar size, noncoding sequences, transfer RNA and ribosomal RNA sequences, respectively. Chromosome features of *Bacillus cereus* strains were drawing, as showed in Fig. 2.

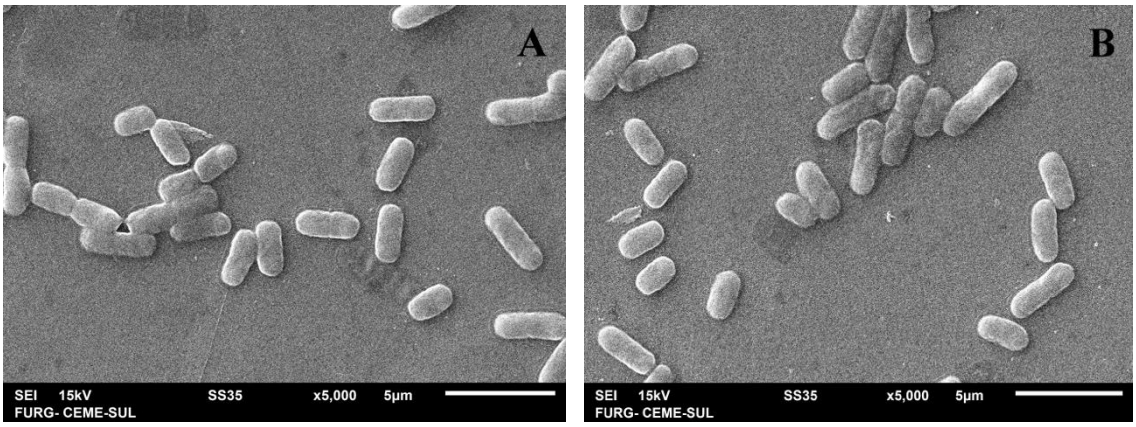

Fig. 1. Scanning Electron Microscope of *Bacillus cereus* native strains #25 (A) and #29 (B).

Table 1. Genomic features of native *Bacillus cereus* strains #25 and # 29.

Strain	Genomic size	Gaps	C+G (%)	CDS	tRNAs	rRNAs
#25	4.82 Mb	66	35.11	5132	21	2
#29	4.98 Mb	161	35.32	5443	77	5

C+G (%): guanine and cytosine content; CDS: protein coding genes; tRNAs: transfer RNA; rRNAs: ribosomal RNA.

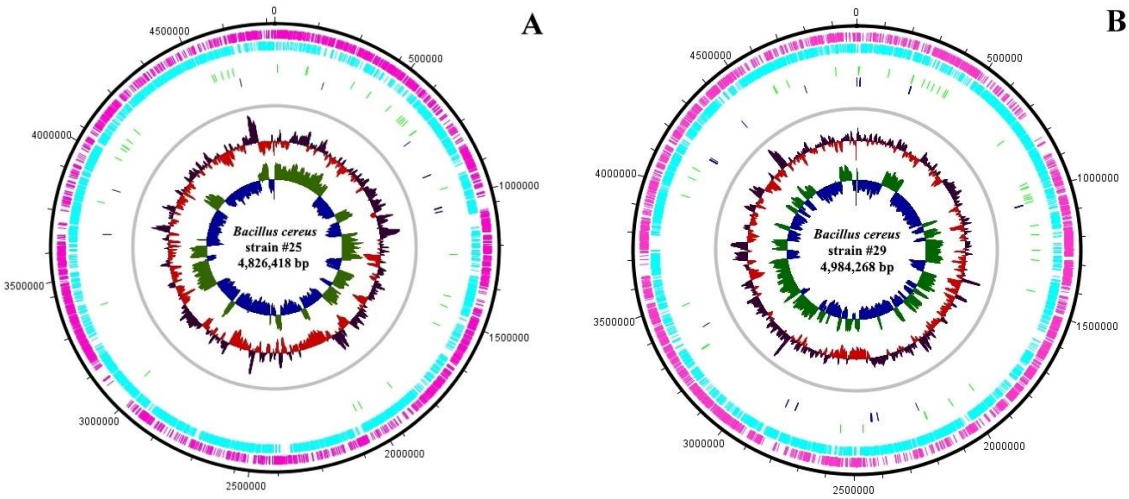

Fig. 2. Chromosome features of native *Bacillus cereus* strains #25 (A) and #29 (B). Track 1, genome size. Tracks 2 and 3, coding sequence (CDS - forward and reverse). Tracks 4 and 5, genes and tRNA on forward and reverse strands. Track 6, chromosomic sequence. Tracks 7 and 8, GC content and GC skew [(G-C)/(G+C)].

EXPERIMENTAL DESIGN, MATERIALS AND METHODS

Isolation of native *Bacillus* strains

The native strains #25 and #29 were isolated from intestinal tract of the crab *Ucides* sp., from Pacoti River in the State of Ceará, Brazil. The intestinal tracts were homogenized with saline solution (NaCl 0.85%) and incubated at 70ºC for 1 hour. An inoculum was plated on solid medium (NaCl 1.5%) and maintained at 30ºC for 48 hours. The colonies similar with *Bacillus* were inoculated in Tryptone Soy Agar (TSA)

and the culture integrity was evaluated with Gram Staining by microscopy. The gram positive *Bacillus* with sporulation capacity were destined to the Laboratory of Structural Genomics of the Federal University of Pelotas (UFPel, Brazil), for whole genome sequencing.

Isolation of genomic DNA

The strains were cultivated in Luria-Bertani (LB) medium at 37°C with vigorous shaking (250 rpm) for 12 hours. The integrity was accessed by microscopy using Gram Staining method. Scanning Electron Microscope (JEOL JSM - 6610 LV) was performed using the pellet from culture washed twice with ultrapure water and quickly fixed in Bunsen flame. Genomic DNA was obtained using *Illustra Bacteria GenomicPrep Mini Spin* kit (GE Healthcare, USA), according the manufacturers guidelines.

Library preparation and sequencing

Bacterial genome sequencing was performed using the Ion Torrent PGM (Life Technologies, Saint Aubin, France) on 100 ng of DNA. The DNA library was constructed using enzymatic fragmentation and adaptor ligation with the Ion Xpress Plus fragment library kit (Life Technologies). Fragment size selection was performed using E-Gel® SizeSelect 2% (Invitrogen). After diluting the library at 100 pM, template preparation, emulsion PCR, and ion sphere particle (ISP) enrichment were performed using the Ion One Touch template kit (Life Technologies). The ISPs were loaded and sequenced on a 318 chip (Life Technologies).

Genome assembly

Raw sequence reads were obtained from the Ion Torrent server in BAM format and converted to FASTQ using the bamToFastq program from the BedTools package [1]. Reads with more than 20% of the bases with Phred score (Q) smaller than 20 where removed using the fastq_quality_filter script from the fastx-toolkit package (http://hannonlab.cshl.edu/fastx_toolkit/). *De novo* genome assemblies were generated

for each strain using MIRA (http://mira-assembler.sourceforge.net/), SPAdes [2] and Newbler (http://www.roche.com/), and for each strain a consensus assembly was generated using CISA [3]. The final set of contigs from each strain was ordered with the program CAR [4], which is able to identify genome inversions and transpositions, using the genome of *Bacillus cereus* strain 03BB102 (GenBank: CP009318.1) as reference. The genome annotation was performed using Genix [5], and manually revised using Artemis [6].

A BLAST search [7] was performed against the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/BLAST/) to identify the most similar strain. Chromosome features of *Bacillus cereus* strains were drawing using DNAPlotter [8].

ACKNOWLEDGEMENTS

This work was supported by the National Council for Scientific and Technological Development (CNPq - 454918/2014-3), Financial Assistance for Educational Project or Research / Coordination of Improvement of Higher Education Personnel (AUXPE/CAPES - 2900/2014) and Center of Electron Microscopy of South (CEME-SUL) of the Federal University of Rio Grande (FURG).

REFERENCES

[1] A.R. Quinlan, I.M. Hall, BEDTools: a flexible suite of utilities for comparing genomic features, Bioinform. 26 (2010) 841-842.

[2] A. Bankevich, S. Nurk, D. Antipov, A.A. Gurevich, M. Dvorkin, A.S. Kulikov, V.M. Lesin, S.I. Nikolenko, S. Pham, A.D. Prjibelski, A.V. Pyshkin, A.V. Sirotkin, N. Vyahhi, G. Tesler, M.A. Alekseyev, P.A. Pevzner, SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J. Comput. Biol. 19 (2012) 455-477.

[3] S-H. Lin, Y-C. Liao, CISA: contig integrator for sequence assembly of bacterial genomes. PLoS One. 8 (2013).

[4] C. Lu, K-T. Chen, S-Y. Huang, H-T. Chiu, CAR: contig assembly of prokaryotic draft genomes using rearrangements. BMC Bioinformatics. 15 (2014) 381.

[5] F.S. Kremer, M.R. Eslabão, O.A. Dellagostin, L da S. Pinto L, Genix: A New Online Automated Pipeline for Bacterial Genome Annotation. FEMS Microbiol. Lett. 11 (2016).

[6] K. Rutherford, J. Parkhill, J. Crook, T. Horsnell, P. Rice, M-A. Rajandream, B. Barrell, Artemis: sequence visualization and annotation. Bioinform. 16 (2000) 944-945.

[7] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool. J. Mol. Biol. 215 (1990) 403-410.

[8] T. Carver, N. Thomson, A. Bleasby, M. Berriman, J. Parkhill, DNAPlotter: circular and linear interactive genome visualization. Bioinform (Oxford, England). 25 (2009) 119-20.

CAPÍTULO II: Genetic Manipulation of a Native *Bacillus cereus* Strain with Probiotic Potential in Shrimp Farming

Submetido ao periódico Aquaculture

'Declarations of interest: none'

Genetic Manipulation of a Native *Bacillus cereus* Strain with Probiotic Potential in Shrimp Farming

João Costa Filho [a,b,*], Jade Riet [a,b], Oscarina Viana de Sousa [c], Rodrigo Maggioni [c], Rubens Galdino Feijó [d], Wilson Wasielesky Jr. [b], Luis Fernando Marins [a]

[a] Federal University of Rio Grande, Institute of Biological Sciences. Campus Carreiros - Av. Itália, km 8, CEP 96203-900, Rio Grande, Rio Grande do Sul, Brazil. E-mails: dqmluf@furg.br; jaderiet@hotmail.com

[b] Federal University of Rio Grande, Marine Aquaculture Station. Rua do Hotel, 2, Querência, CEP 96210-030, Rio Grande, Rio Grande do Sul, Brazil. E-mail: manow@mikrus.com.br

[c] Federal University of Ceará, Institute of Marine Sciences. Meireles, CEP 60165-080, Fortaleza, Ceará, Brazil. E-mails: oscarinasousa@yahoo.com.br; maggioni@ufc.br; albertojpn@uol.com.br

[d] Federal Institute of Education, Science and Technology of Ceará. Av. Des. Armando de Souza, R. Estevão Louzada - Buriti, CEP 62580-000, Acaraú, Ceará, Brazil. E-mail: rubensfeijo2005@yahoo.com.br

* Corresponding author: Campus Carreiros - Av. Itália, km 8, CEP 96203-900, Rio Grande, Rio Grande do Sul, Brazil. Phone: +55 53 999214810. E-mail: jo_filho@hotmail.com

ABSTRACT

The objective of this work was to perform the genetic manipulation of the native *B. cereus* #25 strain and to evaluate the colonization in the intestinal tract of *Litopenaeus vannamei* under culture conditions. The genetic manipulation was confirmed by expression of the GFPmut1, absence of amylase activity, and growth in spectinomycin. In the shrimp bioassay the addiction of BC25GM in the feed did not bring difference for water quality and growth performance. It was observed the presence of cells expressing GFPmut1 in the midgut of the animals under epifluorescence microscopy. The relative fluorescence was 35% higher in the midgut of the shrimps fed with BC25GM in the feed. In conclusion, the present work showed that it was possible to manipulate a native *Bacillus* strain with a non-specific plasmid. It seems that an identity of 50% is sufficient for homologue recombination between the endogenous gene and the integrative plasmid. The BC25GM has genes that could be toxic to humans, which could be deleted with new of molecular biology techniques. The use of the native strains could be a new biotechnology tool applied in aquaculture.

Keywords: *Bacillus cereus*, GFPmut1, probiotics, *Litopenaeus vannamei*, carciniculture

1. INTRODUCTION

The culture of aquatic organisms showed a growth in the last years, mainly in relation to crustaceans, where the most produced species in the world is the shrimp *Litopenaeus vannamei* (FAO, 2018). However, one of the main challenges of the carciniculture has been the occurrence of infectious diseases, mainly of viral etiology, which often cause serious economic and social repercussions (Lafferty et al., 2015). Thus, it is necessary to develop new technologies that promote actions to reduce the health problems observed in shrimp farming, minimizing economical losses. Faced with this issue, it has been reported on the benefits of probiotic bacteria and their application in aquaculture by bringing sanitary improvements in culture systems (Lakshmi et al., 2013).

Genetic manipulation in microorganisms has created the possibility to produce molecules capable of promoting benefits mainly in relation to the immunology and nutrition of crustaceans. Some studies involving the treatment of viral infections in shrimp have demonstrated that it is possible to produce antiviral molecules through the genetic manipulation of strains of bacteria such as *Escherichia coli* (Attasart et al., 2013; Taju et al., 2015) and *Bacillus subtilis* (Nguyen et al., 2014). As examples of this we can mention the double-stranded RNA (dsRNA) that act on the activation of the interference RNA mechanism (Taju et al., 2015) and antiviral proteins (Fu et al., 2011, Nguyen et al., 2014). In this sense, the use of gram-positive bacteria as bioreactors of beneficial molecules to the immunology of shrimp can be an interesting tool to mitigate some of the problems faced in the development of the important sector of food production.

Although there are many strains of *Bacillus* already commercially used even in aquaculture, and several of them with established genetic manipulation protocols, there are no studies (to our knowledge) that address the development for native *Bacillus* species isolated from crustaceans. Thus, the objective of this work was to perform the genetic manipulation of the native strain *B. cereus* #25 isolated from the crab *Ucides* sp. and to evaluate its presence and colonization in the intestinal tract of *Litopenaeus vannamei* under culture conditions.

2. MATERIAL AND METHODS

2.1 *Bacillus cereus* #25

Bacillus cereus #25 was obtained from the collection of bacterial morphotypes of the Laboratory of Environmental Microbiology and Fish at the Institute of Marine Sciences - LABOMAR/UFC (Brazil). The strain was isolated from crabs (*Ucides* sp.) collected at Pacoti River - Fortaleza, Ceará, Brazil. The genome was already sequenced by Costa Filho et al. (2018).

2.2 Genetic manipulation

The integration vector pSG1154, obtained from the Bacillus Genetic Stock Center (BGSC), was used for genetic manipulation of *B. cereus* #25. The vector has two recombination sites for amylase gene (*amyE*), spectinomycin resistance gene, green fluorescent protein (GFPmut1) controlled by the P_{xyl} promoter, which is induced by xylose. *Escherichia coli* One Shot TOP10 Electrocomp (Invitrogen, Brazil) was used for cloning and the isolation of the plasmids was performed with the Plasmid prep Mini Spin kit (GE HealthCare, Brazil), following the manufacturer's protocol. The transformation of *B. cereus* #25 was performed according to the methodology described by Xue et al. (1999). Transformed strain was inoculated into LB medium containing spectinomycin (250 $\mu g.mL^{-1}$) and xylose (0.2%). Aliquots were used for visualization of *B. cereus* #25 expressing GFPmut1 on the Olympus IX81 epifluorescence microscope.

In order to test the occurrence of homologous recombination of pSG1154 plasmid on the amylase gene, transformed *B. cereus* #25 colonies were incubated for 48h at 37ºC in LB agar (1% starch). Colonies were covered with lugol solution (iodine crystals 5%; potassium iodate 10%). The absence of the transparent halo indicated no amylase activity and confirmation of the genome integration.

2.3 Feed supplemented with genetically modified *B. cereus* #25

A genetically modified *B. cereus* #25 (hereafter named BC25GM) inoculum was incubated in LB containing xylose and spectinomycin. The culture remained at 37°C and agitation at 250 rpm until the optical density of 1.0 (600 nm) was reached. After centrifugation at 5,000 g for 5 minutes, the pellet was resuspended in sterile water from the experimental bioassay (10:1), and then sprayed on the feed (40% PB and 7.5% EE), where for the control group was used only water. The feed were incubated for 1 hour and 30 minutes at 37°C and stored at 4°C for up to two days. The final concentration of BC25GM was 10^8 $bacteria.g^{-1}$ of the feed, determined by spectrophotometry (600 nm). The amount of feed supplied to the shrimps was standardized to 8% of the shrimp biomass and divided into three times daily, beginning in the morning (08:30h), early afternoon (12:30h) and late afternoon (16:30h).

2.4 Bioassays with *Litopenaeus vannamei*

Juveniles of *Litopenaeus vannamei* (1.7 ± 0.1 g) were obtained from the Marine Aquaculture Station (EMA/FURG, Brazil) and transported to the Institute of Biological Sciences (ICB/FURG, Brazil). The first experimental culture was conducted after 10 days of acclimatization and lasted for 15 days. Two treatments were tested in triplicate: one group received only the regular feed (control) and another with BC25GM supplementation. A closed system with polyethylene tanks of 0.33 m^2 and volume of 50 L was used. Each triplicate contained 20 shrimps, totaling 60 per treatment. Water renewal was carried out daily to control nitrogen compounds. Dissolved oxygen, temperature, salinity, total ammonia, non-ionized ammonia and pH were monitored daily.

Biometry were performed during the experiment to adjust feed quantity. The growth parameters evaluated were weight gain (mean final weight - mean initial weight), specific growth rate ([ln final weight - ln initial weight / days of cultivation] * 100), apparent feed conversion (feed offered / weight gain) and survival ([initial number of animals - final number of animals / initial number of animals)] * 100). The data obtained from the performance and water quality were analyzed with the SAS program (version 9.0), using the t test ($P < 0.05$).

2.5 BC25GM colonization in the *L. vannamei* midgut

Colonization of shrimp midgut by BC25GM was assessed by fluorescence of GFPmut1 expression. At the end of the experimental period ten shrimps per group were fasted during 24 hours for analysis of relative fluorescence. Subsequently, the midguts were dissected (Figure 1), sonicated with 300 µl of phosphate buffered saline, for 30/10 seconds on/off, 50% amplitude, for 3 minutes and 30 seconds, using the Qsonica Sonicators. The fluorescence of GFPmut1 was measured on the FilterMax F5 fluorimeter (485 nm excitation and 535 nm emission). The proteins obtained from the extract were quantified with the Qubit® Protein Assay kit (Invitrogen, Brazil). Relative

fluorescence was estimated by the ratio between the reading obtained on the fluorimeter and the proteins observed ($\mu g.mL^{-1}$).

For ensuring the presence of BC25GM in the midgut of the shrimp, histological slides were prepared for analysis by epifluorescence microscopy. The tissues were fixed in paraformaldehyde 4%, included in paraplast and cut into slices with 6 µm of thickness. No coloring method was used.

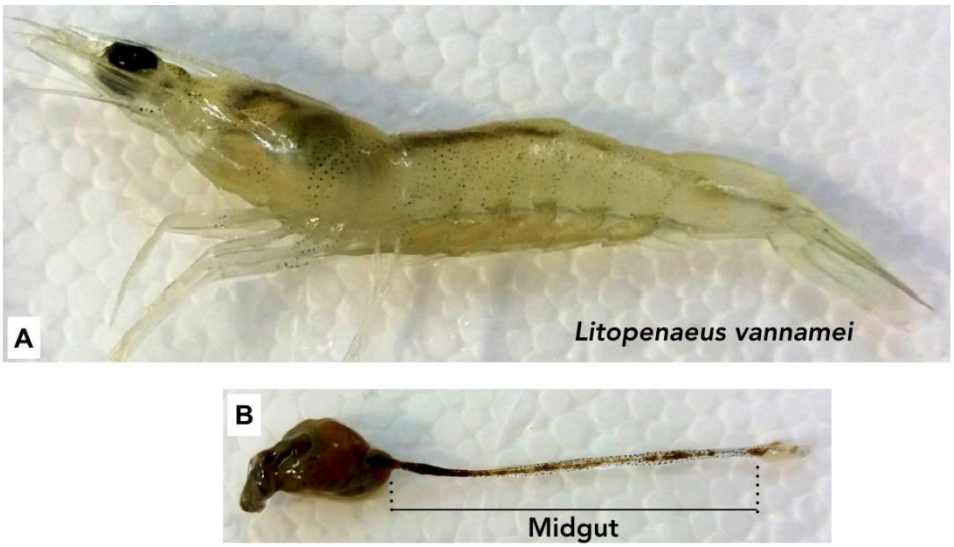

Figure 1. A) Juvenile of *Litopenaeus vannamei* used in the present study. B) Dissected shrimp intestinal tract, evidencing the midgut region used for histological and fluorescence analyses.

3. RESULTS

3.1 Genetic manipulation of *B. cereus* #25

The genetic manipulation of *B. cereus* #25 was confirmed by three distinct characteristics. Expression of the GFPmut1 fluorescent protein, whose gene present in the plasmid pSG1154 is activated by xylose 0.2% (Figure 2A-B), absence of amylase activity in the transformed strains (Figure 2C-D), and growth in medium containing spectinomycin, since pSG1154 confers resistance to this antibiotic.

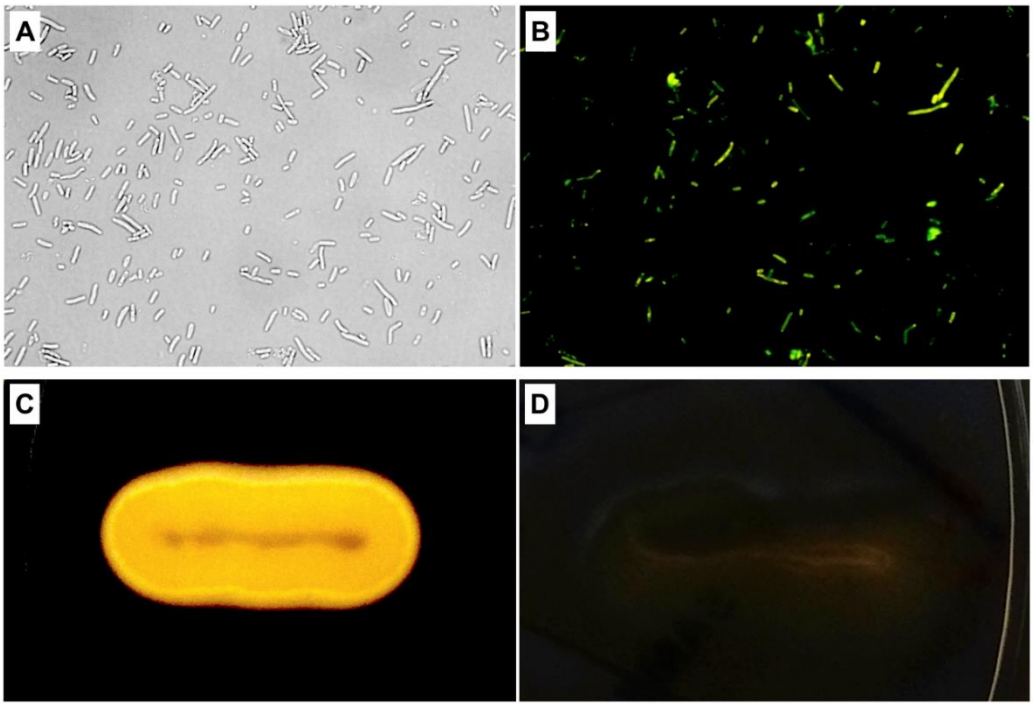

Figure 2. Phenotypic characteristics of genetically modified *B. cereus* #25. BC25GM under white light (A) and epifluorescence (B) microscopy, evincing the expression of GFPmut1. Amylase activity evidenced by the presence of a clear halo around the wild type *B. cereus* #25 colony (C), which was not observed in the BC25GM (D).

3.2 Bioassays with *Litopenaeus vannamei*

Water quality indicators and growth parameters determined during the bioassay showed no difference between the treatments (Tables 1 and 2).

Table 1 - Values (mean ± standard deviation) of the water quality indicators during 15 days of experiment.

Parameters	Treatments		*P values
	Feed with BC25GM	Regular feed	
DO ($mg.L^{-1}$)	5.6 ± 0.3	5.4 ± 0.1	0.311
Temperature (°C)	27.8 ± 0.2	27.9 ± 0.1	0.398
Salinity ($g.L^{-1}$)	20.2 ± 0.05	20.3 ± 0	0.116
Total ammonia ($mg.L^{-1}$)	0.41 ± 0.04	0.41 ± 0.05	0.942
NH_3 ($mg.L^{-1}$)	0.02 ± 0	0.01 ± 0.005	0.373
pH	7.6 ± 0	7.5 ± 0.05	0.116
Daily renewals (%)	85.6	85.6	-

* No difference between the treatments by the *t* test ($P < 0.05$).

Table 2 - Values (mean ± standard deviation) of the growth parameters during 15 days of experiment.

Parameters	Treatments		* P vales
	Feed with BC25GM	Regular feed	
Initial weight (g)	1.8 ± 0.1	1.7 ± 0.1	0.496
Final weight (g)	3.1 ± 0.3	3.1 ± 0.2	1.000
Weight gain (g)	1.3 ± 0.2	1.4 ± 0.1	0.565
SGR	3.5 ± 0.2	3.9 ± 0.1	0.088
AFC	2.1 ± 0.3	2.1 ± 0.3	0.970
Survival (%)	100	100	-

SGR - specific growth rate; AFC - apparent feed conversion; * No difference between the treatments by the *t* test ($P < 0.05$).

3.3 BC25GM midgut colonization in *L. vannamei*

Figure 3 shows the BC25GM presence in the midgut of the shrimps under epifluorescence microscopy. In all images was not observed bacterial aggregates just disperse cells. GFPmut1 was detected showing that cells were viable and expressing this gene. Figure 4 shows the relative fluorescence in the midguts of the shrimps. This result

shows that GFPmut1 could be measured in the intestinal tract of the shrimps fed with the feed supplemented with BC25GM, with the increase of 35% ($P < 0.05$) in relation to the basal fluorescence observed in the midgut of the shrimps fed only with the regular feed.

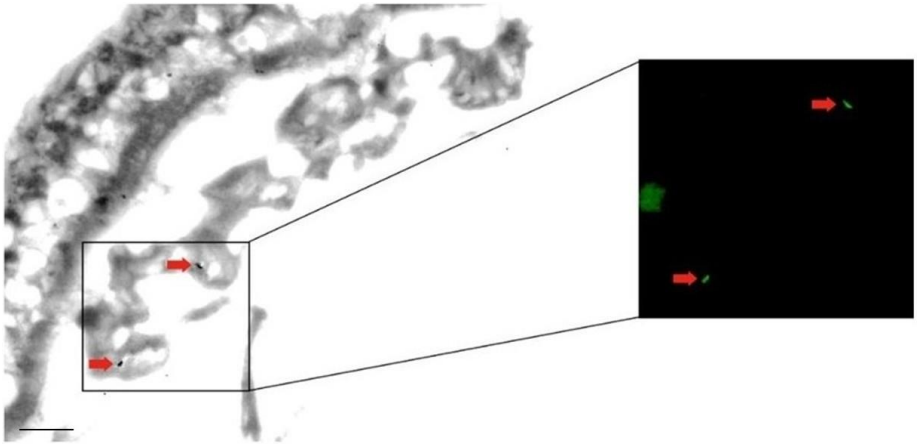

Figure 3. (A) The absence of the GFP cells in the midgut. (B) White light and epifluorescence microscopy evincing the presence of transgenic *B. cereus* #25 (red arrows) expressing GFPmut1 in the midgut of the *L. vannamei*. Scale bar 10 µm.

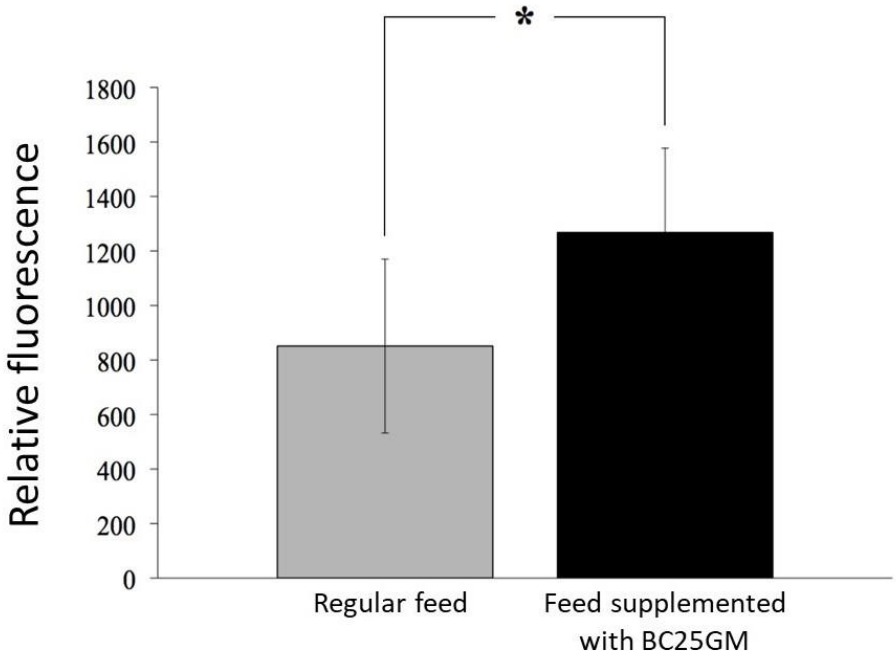

Figure 4. Relative fluorescence in the midgut of the shrimps fed with regular feed and BC25GM supplementation for 15 days.

4. DISCUSSION

In the present work, a native strain *B. cereus* #25 was employed for genetic manipulation. This strain was isolated from the intestinal tract of the crab from the Pacoti River (Ceará, Brazil) and characterized as a potential probiotic (Souza, O.V., personal communication). The characterization involved the identification of catalase activity and antagonism to the *Vibrio harveyi*. The genome of this strain was previously sequenced (Costa Filho et al., 2018).

The plasmid pSG1154, obtained from the Bacillus Genetic Stock Center (BGSC), was used for genetic manipulation. This vector was developed for integration into the amylase gene of *B. subtilis*. Two amylase genes were identified in the genome of *B. cereus* #25. The first one has 1,584 bp and encodes for a protein with 527 amino acids. The other one is shorter, with 1,356 bp and the protein has 451 amino acids. However, in contrast with the amylase gene from the *B. subtilis, B. cereus* amylase genes are shorter, lacking the starch binding domain (CBM26). Even with a more accurate investigation throughout *B. cereus* #25 genome, the CBM26 domain was not encountered. It seems that the native strain has a functional amylase system even without the CBM domain (Figure 2C).

One relevant point is the use of the pSG1154 vector for integration in another species, as applied in this work. The question is: what is the minimal homology necessary for homologue recombination? The alignment of the amylase genes from the *B. cereus* #25 and *B. subtilis* revealed only 39.6% of identical sites and 51.5% of identity. The comparison between the *B. subtilis* amylase gene and the smaller form of *B. cereus* resulted in a lower percentage of identical sites (19,5%). Thus, we hypothesized that this smaller amylase form could be not functional since it is unlikable that homologous recombination has occurred in both endogenous amylase genes. Considering that BC25GM expresses GFP (Figure 2B) and the absence of the amylase activity (Figure 2D), the homology was sufficient for recombination between the

plasmid and the endogenous gene. In the same sense, Olubajo and Bacon (2008) also performed genetic manipulation in *B. mojavensis* using the same pSG1154 plasmid.

BC25GM was also evaluated regarding its colonization capacity in the intestinal tract of *L. vannamei*. GFPmut1 expression was identified using histological analyses (Figure 3). Only isolated cells were observed, suggesting that BC25GM is not proliferating. In this case, cells aggregates should be observed. The relative fluorescence was also detected using the protein extract of the shrimp intestinal tract (Figure 4), showing that this strategy allows detecting of the probiotic presence. However, even without proliferation, BC25GM was detected until 17 days after the last administration (data not shown). Similar to our findings, Duc et al. (2004) reported that a strain of *B. cereus* (used for humans) also colonized for 18 days the intestinal tract of the mice after restriction of supply. In that report, the authors did not recommend the use of *B. cereus* as a probiotic for humans due the presence of toxin-encoding genes. Regarding this question, we performed an investigation to identify those genes in the *B. cereus* #25 genome. The results pointed for 12 toxin genes related with intestinal infections and health risks in humans (Duc et al., 2004; Zhu et al., 2016). Among them are genes coding for cytotoxin K (*cytK*), non-hemolytic enterotoxin (*NheI*), and hemolysin BL (*Hbl*).

Although the BC25GM presented genes encoding for enterotoxins, its administration did not indicate toxic effects for *L. vannamei*. The growth and water quality results revealed no differences in relation to the control group. Therefore, the native strain has potential application in carciniculture, once it was obtained from a crustacean intestinal tract. This fact indicates that the microorganism is adapted to the Pacoti River environment, where strong salinity variations in a daily basis are common. Maybe other *Bacillus* species could not be able to colonize crustaceans living there. However, the viability of the *B. cereus* #25 as probiotic depends on the deletions of the enterotoxin genes. Recent technologies for genome editing such as CRISPR/Cas9 could be employed for this. Thus, native strains could be used as bioreactors for production and delivery of benefits molecules. One example is the production and delivery of dsRNA working for the activation of the RNA interference system, promoting advantages for immunology of crustaceans against viruses (Feijó et al., 2015). Another substantial way could employ for molecules, such as phytases, that according with

Cheng et al. (2013) promote better use of the phosphorus in the feed, benefiting the nutrition and reducing environmental impacts by the fecal excretion.

In conclusion, the present work showed that it was possible to manipulate a native *Bacillus* strain with a non-specific plasmid. It seems that an identity of 50% is sufficient for homologue recombination between the endogenous gene and the integrative plasmid. The BC25GM has genes that could be toxic to humans, which should be deleted with molecular biology techniques. The use of the native strains could be a new biotechnology tool applied in aquaculture.

FUNDING

This work was supported by the National Council for Scientific and Technological Development (CNPq, Proc.No. 454918/2014-3). This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) - Finance Code 001. Costa Filho, João is a student at Post-Graduation Program in Aquaculure. L.F. Marins is a research fellow from CNPq/Brazil (Proc. number 305928/2015-5).

REFERENCES

Attasart, P., Namramoon, O., Kongphom, U., chimwai, C., Panyim, S., 2013. Ingestion of bacteria expressing dsRNA triggers specific RNA silencing in shrimp. Virus Research. 171, 252-256. https://doi.org/10.1016/j.virusres.2012.11.012

Cheng, W., Chiu, C.S., Guu, Y.K., Tsai, S.T., Liu, C.H., 2013. Expression of recombinant phytase of *Bacillus subtilis* E20 in *Escherichia coli* HMS 174 and improving the growth performance of white shrimp, *Litopenaeus vannamei*, juveniles by using phytase-pretreated soybean meal containing diet. Aquaculture Nutrition. 19, 117-127. https://doi.org/10.1111/j.1365-2095.2012.00946.x

Costa Filho, J., Jorge, S., Kremer, F.S., Oliveira, N.R., Campos, V.F., Pinto, L.S., Dellagostin, O.A., Feijó, R.G., Menezes, F.G.R., Sousa, O.V., Maggioni, R., Marins,

L.F., 2018. Complete genome sequence of native *Bacillus cereus* strains isolated from intestinal tract of the crab *Ucides* sppp. Data in Brief. 16, 381-385. https://doi.org/10.1016/j.dib.2017.11.049

Duc, L.H., Hong, H.A., Barbosa, T.M., Henriques, A.O., Cutting, M., 2004. Characterization of *Bacillus* Probiotics Available for Human Use. Appl. Environ. Microbiol. 70, 2161-2171. https://10.1128/AEM.70.4.2161-2171.2004

Fao. 2018. The State of World Fisheries and Aquaculture 2018. Rome. 227p.

Feijó, R.G., Maggioni, R., Martins, P.C.C., Abreu, K.L., Oliveira-Neto, J.M., Guertler, C., Justino, E.B., Perazzolo, L.M., Marins, L.F., 2015. RNAi-based inhibition of infectious myonecrosis virus replication in Pacific white shrimp *Litopenaeus vannamei.* Dis. Aquat. Organ. 114, 89-98. https://10.3354/dao02853

Fu, L.L., Wang, Y., Wu, Z.C., Li, W.F., 2011. In vivo assessment for oral delivery of *Bacillus subtilis* harboring a viral protein (VP28) against white spot syndrome virus in *Litopenaeus vannamei*. Aquaculture. 322, 33-38. https://doi.org/10.1016/j.aquaculture.2011.09.036

Lafferty, K.D., Harvell, C.D., Conrad, J.M., Friedman, C.S., Kent, M.L., Kuris, A.M., Powell, E.N., Rondeau, D., Saksida, S.M., 2015. Infectious Diseases Affect Marine Fisheries and Aquaculture Economics. Annual Review of Marine Science. 7, 471-496. https://doi.org/10.1146/annurev-marine-010814-015646

Lakshmi, B., Viswanath, B., Gopal, D.V.R., 2013. Probiotics as Antiviral Agents in Shrimp Aquaculture. Journal of Pathogens. 1-13. http://dx.doi.org/10.1155/2013/424123

Nguyen, A.T.V., Pham, C.K., Pham, H.T.T., Pham, H.L., Nguyen, A.H., Dang, L.T., Huynh, H.A., Cutting, S.M., Phan, T.N., 2014. *Bacillus subtilis* spores expressing the VP28 antigen: a potential oral treatment to protect *Litopenaeus vannamei* against White

spot syndrome. FEMS Microbiology Letters. 358, 202-208. http://10.1111/1574-6968.12546

Olubajo, B., Bacon, C.W., 2008. Electrotransformation of *Bacillus mojavensis* with fluorescent protein markers. Journal of Microbiological Methods. 74, 102-105. http://10.1016/j.mimet.2008.03.011

Taju, G., Madan, N., Majeed, S.A., Kumar, T.R., Thamizhvanan, S., Otta, S.K., Hameed, A.S.S., 2015. Immune responses of whiteleg shrimp, *Litopenaeus vannamei* (Boone, 1931), to bacterially expressed dsRNA specific to VP28 gene of white spot syndrome virus. J. Fish Dis. 38, 451-465. http://10.1111/jfd.12256

Xue, G.P., Johnson, J.S., Dalrymple, B.P., 1999. High osmolarity improves the electro transformation efficiency of the gram-positive bacteria *Bacillus subtilis* and *Bacillus licheniformis*. J. Micro. Methods. 34, 183-191. https://doi.org/10.1016/S0167-7012(98)00087-6

Zhu, K., Hölzel, C.S., Cui, Y., Mayer, R., Wang, Y., Dietrich, R., Didier, A., Bassitta, R., Märtlbauer, E., Ding, S., 2016. Probiotic *Bacillus cereus* Strains, a Potential Risk for Public Health in China. Front. Microbiol. 718. http:// 10.3389/fmicb.2016.00718

CAPÍTULO III: CRISPR/Cas9 in the Production of *Bacillus subtilis* Strains as Bioreactors of Double-Stranded RNAs (dsRNAs) Against Shrimp Viruses

Capítulo submetido ao periódico Marine Biotechnology

João Costa Filho [1,2,*], Luis Fernando Marins [1] and Josef Altenbuchner [2]

CRISPR/Cas9 in the Production of Bacillus subtilis Strains as Bioreactors of Double-Stranded RNAs (dsRNAs) Against Shrimp Viruses

[1] Federal University of Rio Grande, Institute of Biological Sciences, Laboratory of Molecular Biology. Campus Carreiros - Av. Itália, km 8, CEP 96203-900, Rio Grande, Rio Grande do Sul, Brazil.

[2] University of Stuttgart, Institute of Industrial Genetics. Allmandring 31, 70569 Stuttgart, Germany.

* Corresponding author: joc_filho@hotmail.com / Telephone number: +55 53999214810

Abstract

Culture of shrimps, especially *Litopenaeus vannamei*, has showed an increasing in the last years. However, the incidents of infectious diseases have been the most challenge for world shrimp production. The activation of the RNA interference mechanism (RNAi) by antiviral double-strand RNAs (dsRNAs) has bringing new perspectives on the control of shrimp virus diseases. However, the use of this technology in shrimp culture is limited by the synthesis of a large amount of dsRNA molecules, application by individual muscle injection, and the production costs. Genetic manipulation of probiotics *Bacillus* could be applied to produce dsRNA. In this way, plasmids were created for genome editing of *B. subtilis* using the recent CRISPR/Cas9 technology. In the first step, the *rnc* gene (coding for a type III RNAse) was deleted in order to prevent endogenous dsRNA degradation. Later, sequences to produce dsRNA related to viruses that affect shrimp culture were integrated into *B. subtilis* genome.

Finally, we produced transformed strains free of antibiotic resistance that could be used in shrimp bioassays with viral challenge. These strains could represent alternative tools for minimizing the problems caused by diseases in shrimp farming.

Keywords: WSSV, IMNV, RNAi, probiotics, *Litopenaeus vannamei*

Introduction

Culture of aquatic organisms including shrimps (*Litopenaeus vannamei*) showed an increasing in the prior years. However, the problems with viruses have affected the world production of shrimp farming (FAO 2018). The occurrences of infectious diseases and economic losses have been the biggest challenge for world shrimp production (Anderson et al. 2014). Especially two viruses have promoted constant problems: White Spot Syndrome Virus (WSSV) and Infectious Myonecrosis Virus (IMNV). In this context, it is necessary the development of new technologies to mitigate the problems with diseases in shrimp farming.

RNA interference mechanism (RNAi) or Post-Transcriptional Gene Silencing has bringing new perspectives on the control of shrimp virus diseases (Barroco et al. 2014). This technology is employed to silence viral gene expression, where double strand RNA (dsRNA) leads to the degradation of homologous messenger RNA (Reshi et al. 2014). This technology has been used in *L. vannamei* by injection of specific dsRNA against IMNV (Feijó et al. 2015) and WSSV (Kumar et al. 2015). The obtained results indicated a promising technique against virus infection, promoting improvements in shrimp survival and health.

The use of RNAi in shrimp culture is limited for the synthesis of large amount of the dsRNA molecules, application by injection (Sarathi et al. 2010) and, production costs. The possibility to produce dsRNA using genetic manipulation of bacteria such as *Escherichia coli* (Taju et al. 2015) has been demonstrated. However, *E. coli* is a gram-negative bacterium with toxic potential to human health. Gram-positive bacteria from *Bacillus* genus could be a better option. In this way, Nguyen et al. (2014) showed that recombinant *B. subtilis* could be used in the diet of *L. vannamei* for delivery of molecules such as VP28 antigen, which has antiviral properties against WSSV.

However, to produce dsRNA it is necessary to knockout of *rnc* gene. This gene codes for a type III RNAse, a key enzyme for dsRNA processing reactions and ribosomal RNA maturation in bacteria (Durand et al. 2012).

Some technologies have been adopted to promote genetic manipulation in prokaryotes, such as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats), a recent molecular biology tool. This system was described by Jinek et al. (2012) as a type of prokaryotic adaptive immune system. It is based on the incorporation of nucleotide sequence into the bacterial chromosome derived from unsuccessful mobile intruders, serving as defense memory against eventual attack by the corresponding elements. Transcription of those clusters generates guides RNA (sgRNA), which are associated to specific nucleases to form protein complexes (Cas) capable to break double strand target DNA. Hille and Charpentier (2016) reported that after this rupture occurs the activation of repair system conducting random mutations in target site. In these process fragments with homologue terminals in the interrupted segment can be inserted promoting recombination of desirable sequence.

The aim of this work was, in the first moment, to knockout the *rnc* gene in *B. subtilis*. Second, we developed plasmids for genome editing of *B. subtilis* using CRISPR/Cas9 system. These plasmids were designed for expression of a fluorescent protein (GFPmut1) and dsRNA against WSSV (VP28 gene) and IMNV (ORF1a gene). Third, we produced new strains free of antibiotic resistance that could be used in shrimp bioassays with viral challenge.

Materials and methods

Bacterial strains and culture conditions

E. coli strain JM109 was used for cloning following the protocol suggested by Chung et al. (1989). *B. subtilis* 168 IIG-Bs2 strain was transformed according to the conventional two-step procedure with Spizizen's minimal medium (Anagnostopoulos and Spizizen 1961) and, when necessary, 2% cellobiose was used. The IIG-Bs2 genotype is *ΔmanPA::erm, Δpks*::*CmR, Δ(SPβ, Skin, PBSX, proΦ1, proΦ2, proΦ3,*

ICE-Bs1) (Wenzel and Altenbuchner 2015). This strain has resistance to erythromycin and chloramphenicol, and several prophage genes deleted.

The strains were propagated in LB liquid medium and agar plates at 37°C, if not otherwise indicated. To select plasmids in *E. coli*, ampicillin was used at a final concentration of 100 µg.mL^{-1} and kanamycin at 50 µg.mL^{-1}. For *B. subtilis*, kanamycin was used at a final concentration of 5 µg.mL^{-1}, erythromycin 5 µg.mL^{-1}, chloramphenicol 5 µg.mL^{-1}, and tetracycline 15 µg.mL^{-1}.

For the CRISPR/Cas9 system application the plasmid cured cells was carried out plating transformed strains on LB agar containing kanamycin followed of incubation at 30°C. In the subsequent day, the transformed colonies were placed on LB agar with kanamycin and 0.2% mannose followed of incubation at 30°C. Colonies were placed on LB agar without antibiotics and incubated at 50°C. In the next day were peaked to get single colonies at 42°C. For testing the plasmid loss, isolated colonies were placed in LB agar with and without kanamycin, incubated at 30°C. The plasmid-cured cells were checked by colony PCR to confirm the successful deletion. All the procedures were carried out according with Altenbuchner (2016).

Construction of pJJ2 vector for *rnc* deletion

For deletion of the *rnc* gene the plasmid pJJ2 was produced. In the first step, the pJOE8999 plasmid (Altenbuchner 2016) was cut with *Bsa*I and *lacZ α* was replaced by the sequence for expression of sgRNA (20 bp) complementary to *rnc* gene (checked with sgRNA Designer software) to get pJJ1. In the second step, we identified the *rnc* flanking genes at *B. subtilis* genome: *fabG* upstream and *smc* downstream. The chromosomal DNA of *B. subtilis* 168 was used as template to amplify by PCR (Q5 high-fidelity DNA polymerase) fragments *fabG* (805 bp) and *smc* (1,013 bp), using the oligonucleotides s12154/s12155 and s12156/s12157 (Table 1), which contain the *Sfi*I site at the 5' region. These fragments were cut with *Sfi*I and inserted between two *Sfi*I sites of pJJ1 to get pJJ2. Plasmids were isolated from *E. coli* using innuPrep plasmid minikit (Analytic Jena AG, Jena, Germany). Purification of digested and amplified DNAs was carried out with NucleoSpin gel and PCR cleanup kit (Macherey-Nagel, Düren, Germany).

For constitutive sgRNA expression (*rnc* gene) in pJJ2, the semisynthetic promoter P_{vanP*} was used, followed by the λ oop terminator sequence. Two fragments for the *rnc* gene deletion were inserted, one with homology with *fabG* and other with *smc* genes. The pJJ2 vector contains Cas9 expression under the control of mannose-inducible promoter P_{manP}, pUC18 minimal origin of replication in *E. coli*, kanamycin resistance gene allowing the selection in *B. subtilis* and *E. coli*, and temperature-sensitive replication origin $pE194^{ts}$ for replication in *B. subtilis*.

Construction of the vectors pJJ4 and pJJ10 for integration and dsRNA expression

The pJJ4 plasmid was designed to harbor the sequences of interest and integration into *B. subtilis* genome. For dsRNA expression, we used fragments of VP28 gene from WSSV (613 bp, GenBank: AY422228) and ORF1a gene from IMNV (597 bp, GenBank: AY570982). These fragments were synthesized in tandem (Epoch Life Science Inc.), flanked by two P_{ylb} promoters in a bidirectional way. P_{ylb} has strong expression during the stationary phase of *B. subtilis* growth (Yu et al. 2015). This strategy is based on sense and antisense transcription of the target sequences, generating dsRNAs after hybridization. In addition, this dsRNA gene structure was associated to a reporter gene. We used GFPmut1 gene driven by the xylose-inducible promoter (P_{xyl}) in order to label the transformed *B. subtilis* strain. For subcloning, the complete gene construction was flanked by *Sfi*I sites. This fragment was inserted into *Sfi*I site of the pJOE9620 plasmid (unpublished) to get pJJ4. The pJOE9620 plasmid contains the Cas9 gene under the control of mannose-inducible promoter P_{manP} and sgRNAs for integration between the *yjaZ* and *trpS* genes. This genome region is rich in oligopeptide transporters genes, which are not essential for *B. subtilis* growth. The sgRNA was directed to the *appD* gene, coding for an oligopeptide transporter.

The preparation of the pJJ10 followed the same strategy of the pJJ4 construction. The only one difference was the substitution of the VP28 and ORF1a genes by 600 bp of the IGSF4D gene from zebrafish *Danio rerio*. The preparation of this plasmid was necessary because viral challenge experiments involving interfering RNA required control groups treated with unrelated dsRNAs to eliminate non-specific responses.

Antibiotic resistance removal of the strains

Since the *B. subtilis* IIG-Bs2 strain has resistance to chloramphenicol and erythromycin, it was necessary to remove this resistance to antibiotics. So, the transformed strains can be considered safe for shrimp culture. Gene deletion system based on the mannose phosphoenolpyruvate-dependent phosphotransferase was used to remove the antibiotic resistance of all strains transformed for dsRNA production, according to Wenzel and Altenbuchner (2015). In brief, IIG-Bs2 is resistant to mannose since *manP* and *manA* genes were previously deleted. Plasmids pJOE7644.2 and pJOE6782.1 (Reuβ et al. 2017) contain spectinomycin and *manP* genes, turning cells resistant to spectinomycin and sensitive to mannose at the same time. However, since this method is based on single homologous recombination, four types of cells can be generated: cells resistant to spectinomycin and sensitive to mannose, cells sensitive to both spectinomycin and mannose, cells resistant to spectinomycin and mannose, and cells sensitive to spectinomycin and resistant to mannose. This last type of cells is our objective and they can be obtained by counter-selection. The last step was to verify whether the selected cells lose chloramphenicol and erythromycin resistance after recombination with pJOE6782.1 and pJOE7644.2, respectively.

Production of GFPmut1 and dsRNA

For induction of GFPmut1, transformed strains were grown in LB agar plate (0.2% xylose). After three days, fluorescence was visualized in transilluminator (FastGene/Green LED). The same strains were grown in overnight LB culture (triplicate), diluted to 0.05 OD at 600 nm and grown during 13 hours at 37°C and 150 rpm (10 ml). For dsRNA isolation, it was used 4.8 x 10^8 bacteria of culture treated with lysozyme (20 $mg.ml^{-1}$), using manufacturer's protocol of the Monarch Total RNA Miniprep Kit. The dsRNAs were purified with DNase I ($1U.\mu l^{-1}$) and RNase A (10 $\mu g.ml^{-1}$) at the presence of 0.4 M NaCl to avoid dsRNA degradation. Quantification (ng) was based on the absorbance at 260 nm.

Confirmation of dsRNA production using Reverse Transcriptase PCR (RT-PCR)

RT-PCR method was employed for confirmation of dsRNA production. The dsRNA extracted from the transformed strains were used to synthesize cDNA, according with ProtoScript® II First Strand cDNA Synthesis Kit. cDNA was used as template in PCR reactions with the oligonucleotides s12520/s12521 for detection of dsRNA (VP28 and ORF1a) and s12522/s12523 for unrelated dsRNA (IGSF4D). The PCR was performed under the following conditions: 1 min initial denaturation at 94°C, 30 cycles of denaturation (30 s at 95°C), annealing (30 s at 60°C), and extension (30 s at 72°C) and, a final extension at 72°C for 10 min. Reactions using dsRNA as template were used as negative controls. The fragments were visualized in 1% agarose gel electrophoresis.

Table 1. Oligonucleotides used in this study, obtained by Eurofins MWG Operon (Ebersberg, Germany).

Name	Sequence (5' to 3')	Purpose
s12154	AAGGCCAACGAGGCCTTCAGGCCGCATTATTAACG	*Sfi*I sites in CRISPR vector
s12155	AAGGCCAGTCTGGCCCATAGTAACCTCCATAGGCAC	
s12156	AAGGCCAGACTGGCCTGCTCAGGAAGCTTTAGCT	
s12157	AAGGCCTTATTGGCCTTGCTGGAGCTCATCTAC	
s12154	AAGGCCAACGAGGCCTTCAGGCCGCATTATTAACG	Confirmation of *rnc* deletion
s12157	AAGGCCTTATTGGCCTTGCTGGAGCTCATCTAC	
s12288	ATCACGTTTGCCGGTTAGGT	Confirmation of integration IGSF4D gene
s12289	CCTTGGCCAACACTTGTCAC	
s12290	AAGGCCTTTGTCGGTAGCTC	Confirmation of integration VP28 and ORF1a genes
s12291	ATTCTTGATGAAGGCGCGGA	
s12520	GTGCACGTCACCTACTCTGG	Confirmation of dsRNAs production
s12521	GTCTGGAGGTGGCAGCATAC	
s12522	CCCATGTACTGTGCGGTCTG	
s12523	ATTATAGGTGGCAAGGTGGCG	

Results

Plasmids

The vectors obtained in this study are presented in Fig 1. All plasmids were checked by sequencing (GATC Biotech, Constance, Germany), and the sequences were according to previously designed.

Deletion of *rnc* gene

B. subtilis 168 was transformed for deletion of *rnc* gene. IIG-Bs2 strain was transformed with pJOE9303 (unpublished - CRISPR/Cas9 system) to integrate the competence genes *comK* and *comS* to get the strain JJBs1 (Table 2). This strain was transformed with pJJ2 to induce the CRISPR/Cas9 system and deletion of *rnc* gene (700 pb). Transformed colonies were tested for the plasmid loss and about 90% of all colonies had lost the plasmid. Therefore, the colonies were also checked by PCR using the oligonucleotides s12154/s12157. The *rnc* gene was successfully deleted since the PCR fragment was reduced from 2.5 kb (wild strain JJBs1) to 1.8 kb. At the end, it was possible to get the strain JJBs2 (Table 2).

Integration of GFPmut1 and dsRNAs

JJBs2 strain was transformed with pJJ4 to replace the region of 8.3 kb (between the *yjaZ* and *trpS* genes) by the sequence designed to express GFPmut1 and antiviral dsRNA. The transformed colonies were also tested for the plasmid loss and 90% of all colonies had lost the plasmid. The colonies were checked by PCR using the oligonucleotides s12288/s12289 and s12290/s12291. The region of 8.3 kb was successful replaced by the sequence of interest and the JJBs3 strain was obtained (Table 2). The identical procedure was implemented using pJJ10 to get the JJBs6 strain. In this case, colonies were confirmed by PCR employing the oligonucleotides s12288/s12289.

Table 2. *B. subtilis* strains produced in this study.

Strain	Genotype	Construction
JJBs1	*ΔmanPA::erm, Δpks*::*CmR, Δ(SPβ, Skin, PBSX, proΦ1, proΦ2, proΦ3,* ICE-Bs1*), ΔcomK::(P_{lic}-comK, comS)*	pJOE9303 → IIG-Bs2
JJBs2	*ΔmanPA::erm, Δpks*::*CmR, Δ(SPβ, Skin, PBSX, proΦ1, proΦ2, proΦ3,* ICE-Bs1*), ΔcomK::(P_{lic}-comK, comS), Δrnc*	pJJ2 → JJBs1
JJBs3	*ΔmanPA::erm, Δpks*::*CmR, Δ(SPβ, Skin, PBSX, proΦ1, proΦ2, proΦ3,* ICE-Bs1*), ΔcomK::(P_{lic}-comK, comS), Δrnc, Δ(appD, appF, appB, appC, yjbA)::(GFPmut1, VP28, ORF1a)*	pJJ4 → JJBs2
JJBs4	*ΔmanPA, Δpks*::*CmR, Δ(SPβ, Skin, PBSX, proΦ1, proΦ2, proΦ3,* ICE-Bs1*), ΔcomK::(P_{lic}-comK, comS), Δrnc, Δ(appD, appF, appB, appC, yjbA)::(GFPmut1, VP28, ORF1a)*	pJOE7644.2 → JJBs3
JJBs5	*ΔmanPA, Δpks, Δ(SPβ, Skin, PBSX, proΦ1, proΦ2, proΦ3,* ICE-Bs1*), ΔcomK::(P_{lic}-comK, comS), Δrnc, Δ(appD, appF, appB, appC, yjbA)::(GFPmut1, VP28, ORF1a)*	pJOE6782.1 → JJBs4
JJBs6	*ΔmanPA::erm, Δpks*::*CmR, Δ(SPβ, Skin, PBSX, proΦ1, proΦ2, proΦ3,* ICE-Bs1*), ΔcomK::(P_{lic}-comK, comS), Δrnc, Δ(appD, appF, appB, appC, yjbA)::(GFPmut1, IGSF4D)*	pJJ10 → JJBs2
JJBs7	*ΔmanPA, Δpks*::*CmR, Δ(SPβ, Skin, PBSX, proΦ1, proΦ2, proΦ3,* ICE-Bs1*), ΔcomK::(P_{lic}-comK, comS), Δrnc, Δ(appD, appF, appB, appC, yjbA)::(GFPmut1, IGSF4D)*	pJOE7644.2 → JJBs6
JJBs8	*ΔmanPA, Δpks, Δ(SPβ, Skin, PBSX, proΦ1, proΦ2, proΦ3,* ICE-Bs1*), ΔcomK::(P_{lic}-comK, comS), Δrnc, Δ(appD, appF, appB, appC, yjbA)::(GFPmut1, IGSF4D)*	pJOE6782.1 → JJBs7

Antibiotic resistance removal from JJBs3 and JJBs6 strains

Strains JJBs3 and JJBs6 had both erythromycin and chloramphenicol resistance removed. The counter-selection method generated 20% of all colonies with no resistance. After these procedures, it was possible to obtain the strains JJBs5 and JJBs8 (Table 2), free of resistance and ready to test in shrimp bioassays using viral challenge.

Production of GFPmut1, dsRNAs, and detection of dsRNA using RT-PCR

Strains JJBs5, JJBs8, and JJBs2 were grown in LB agar plate (0.2% xylose) and visualized in transilluminator. Fluorescence related to GFPmut1 expression was observed in JJBs5 and JJBs8 strains (Fig 2).

The dsRNAs amount was higher for the strains JJBs5 (6,667 ng) and JJBs8 (6,933 ng) compared with the more reduced production of JJBs2 (160 ng) as presented at Fig 3. These results indicated the average production of dsRNA utilizing the amount of 4.8 x 10^8 bacteria can be about 6,800 ng. The confirmation of dsRNA presence with the strains JJBs5 (VP28 and ORF1a) and JJBs8 (IGSF4D) was positive using the cDNA as template for PCR reactions (Fig 3), compared with the negative control using dsRNA.

Discussion

B. subtilis genome edition using CRISPR/Cas9 system has been successfully done in current researches (Burby and Simmons 2017). The system applied in this work provides substantial advantages for genome engineering, such as integration by a single cross over and excision of the vector by counter-selection marker, as described by Altenbuchner (2016). The pJJ2 vector was able to knockout the *rnc* gene of the IIG-Bs2 strain, which codes for a RNAse III. Even this enzyme is essential for many gram-positive bacteria, according with Durand et al. (2012) RNAse III has a protective action against prophage genes. In fact, the authors proved that toxin-encoding mRNAs are produced by *txpA/RatA* and *yonT/as-yonT* cassettes, belonging to Skin and SPβ

prophages. However, the *rnc* gene can be inactivated in strains lacking Skin and SPβ prophages, such as the initial IIG-Bs2 strain used in this study.

The pJJ4 and pJJ10 vectors allowed integration of sequences coding for GFPmut1, and dsRNAs related and non-related to shrimp viruses. GFPmut1 expression was employed for initial identification of integration into the genome of the strains. The use of the bidirectional P_{ylb} promoter showed a good strategy for dsRNA expression. P_{ylb} efficiency was described by Yu et al. (2015) as a promoter with high-level expression on target genes in the stationary phase of *B. subtilis* growth. The amount of dsRNA produced is important to establish the quantity required to activate RNAi mechanism and to protect shrimps against viruses. Normally, the amount of 5 μg g^{-1} of shrimp is enough (Feijó et al. 2015). So far, the strain JJBs5 has provided a good production of dsRNA and can be used in viral challenge assays as oral delivery.

Bacterial production of dsRNA against shrimp viruses was already demonstrated. These studies focused in the gram-negative bacteria *E. coli* HT115. Sarathi et al. (2010) showed protection against WSSV infection in shrimps, employing dsRNA injection from *E. coli* HT115. The author's highlighted that the method for administration should be more investigated. In the same way, Attasart et al. (2013) reported that oral delivery of dsRNA via *E. coli* HT115 vehicle is possible to induce RNAi in the farmed shrimp. Even though *E. coli* is a good bioreactor for dsRNA production, its use for human consumption is questionable. Thus, probiotics such as *B. subtilis* are better options for production of antiviral molecules. In fact, *B. subtilis* is already largely used in carciniculture (Akhter et al. 2015). In the present study we developed a way to increase the capacity of this bacteria to protect shrimps against pathogens. In addition, our strains are free of antibiotic resistance, removed using the mannose phosphoenolpyruvate-dependent phosphotransferase system, described by Wenzel and Altenbuchner (2015).

In conclusion, plasmids were created for genome editing of *B. subtilis* using the recent CRISPR/Cas9 system. In the first step, the *rnc* gene was deleted and later sequences were integrated to produce GFPmut1, dsRNA related to viruses WSSV and IMNV, as well non-related dsRNA (IGSF4D). We produced new strains (JJBs5 and JJBs8) free of antibiotic resistance. These strains can be considered as bioreactors for dsRNA production, which could be used in shrimp bioassays with viral challenge.

These findings could represent new perspectives on the tentative to mitigate the problems with diseases in shrimp farming, responsible for critical economic losses and social impacts.

Acknowledgments and Funding Information

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) - Finance Code 001. Costa Filho, João is a student at Post-Graduation Program in Aquaculure. We appreciate the financial support from the Institut für Industrielle Genetik - Universität Stuttgart and National Council for Scientific and Technological Development (CNPq, Brazil). L.F. Marins is a research fellow from CNPq/Brazil (Proc. number 305928/2015-5).

Conflict of interest

None of the authors has any conflict of interest to declare.

References

AKHTER N, WU B, MEMON AM, MOHSIN M (2015) Probiotics and prebiotics associated with aquaculture: A review. Fish Shellfish Immun. https://doi.org/10.1016/j.fsi.2015.05.038

ALTENBUCHNER J (2016) Editing of the *Bacillus subtilis* genome by the CRISPR-Cas9 system. Appl Environ Microbiol. https://doi.org/10.1128/AEM.01453-16

ANDERSON JL, VALDERRAMA D, JORY D (2014) PRODUCTION: Global Shrimp Review. Global Aquaculture Advocate (November/December) 17-6

ANAGNOSTOPOULOS C, SPIZIZEN J (1961) Requirements for transformation in *Bacillus subtilis*. J Bacteriol 81:741-746

ATTASART P, NAMRAMOON O, KONGPHOM U, CHIMWAI C, PANYIM S (2013) Ingestion of bacteria expressing dsRNA triggers specific RNA silencing in shrimp. Virus Res. https://doi.org/10.1016/j.virusres.2012.11.012

BARROCO MA, PERAZZOLO LM, ROSA RD (2014) In: MORALES V, CUÉLLAR-ANJEL J Patología e imunología de camarones penaeidos, OIRSA, Panamá

BURBY PE, SIMMONS LA (2017) CRISPR/Cas9 Editing of the *Bacillus subtilis* Genome. Bio-protocol. https://doi.org/10.21769/BioProtoc.2272

CHUNG CT, NIEMELA SL, MILLER RH (1989) One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc Natl Acad Sci USA 86:2172-2175

DURAND S, GILET L, CONDON C (2012) The Essential Function of *B. subtilis* RNase III Is to Silence Foreign Toxin Genes. PLOS Genetics. https://doi.org/10.1371/journal.pgen.1003181

FAO (2018) The State of World Fisheries and Aquaculture 2018. Rome

FEIJÓ RG, MAGGIONI R, MARTINS PCC, ABREU KL, OLIVEIRA NETO JM, GUERTLER C, JUSTINO EB, PERAZZOLO LM, MARINS LF (2015) RNAi-based inhibition of Infectious myonecrosis virus replication in the Pacific white shrimp (*Litopenaeus vannamei*) by dsRNAs targeted to different genome regions. Dis Aquat Organ. https://doi.org/10.3354/dao02853

HILLE F, CHARPENTIER E (2016) CRISPR-Cas: biology, mechanisms and relevance. Philos Trans R Soc Lond B Biol Sci. https://doi.org/10.1098/rstb.2015.0496

JINEK M, CHYLINSKI K, FONFARA I, HAUER M, DOUDNA JA, CHARPENTIER E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. https://doi.org/10.1126/science.1225829

KUMAR A, LARAMORE S, ALEXANDER P, THOMAS ALLNUTT FC, SAYRE RT (2015) Double Stranded RNA Simultaneously Targeting Four White Spot Syndrome Virus (WSSV) genes Provides Protection against WSSV in *Litopenaeus Vannamei*. IJMO 2:5-10.

NGUYEN ATV, PHAM CK, PHAM HTT, PHAM HL, NGUYEN AH, DANG LT, HUYNH HA, CUTTING SM, PHAN TN (2014) *Bacillus subtilis* spores expressing the VP28 antigen: a potential oral treatment to protect *Litopenaeus vannamei* against white spot syndrome. FEMS Microbiol Lett. https://doi.org/10.1111/1574-6968.12546

RESHI ML, WU JL, WANG HV, WONG JR (2014) RNA interference technology used for the study of aquatic virus infections. Fish Shellfish Immun. https://doi.org/10.1016/j.fsi.2014.06.008

REUß DR, ALTENBUCHNER J, MÄDER U, RATH H, ISCHEBECK T, SAPPA PK, THÜRMER A, GUÉRIN C, NICOLAS P, STEIL L, ZHU B, FEUSSNER I, KLUMPP S, DANIEL R, COMMICHAU FM, VÖLKER U, STÜLKE J (2017) Large-scale reduction of the *Bacillus subtilis* genome: consequences for the transcriptional network, resource allocation, and metabolism. Genome Res. https://doi.org/10.1101/gr.215293.116

SARATHI M, SIMON MC, VENKATESAN C, THOMAS J, RAVI M, MADAN N, THIYAGARAJAN S, HAMEED ASS (2010) Efficacy of bacterially expressed dsRNA specific to different structural genes of white spot syndrome virus (WSSV) in protection of shrimp from WSSV infection. J Fish Dis. https://doi.org/10.1111/j.1365-2761.2010.01157.x

TAJU G, MADAN N, MAJEED SA, KUMAR TR, THAMIZHVANAN S, OTTA SK, HAMEED ASS (2015) Immune responses of whiteleg shrimp, *Litopenaeus vannamei* (Boone, 1931), to bacterially expressed dsRNA specific to VP28 gene of white spot syndrome virus. J Fish Dis. https://doi.org/10.1111/jfd.12256

WENZEL M, ALTENBUCHNER J (2015) Development of a markerless gene deletion system for *Bacillus subtilis* based on the mannose phosphoenolpyruvate-dependent phosphotransferase system. Microbiology. https://doi.org/10.1099/mic.0.000150

YU X, XU J, LIU X, CHU X, WANG P, TIAN J, WU N, FAN Y (2015) Identification of a highly efficient stationary phase promoter in *Bacillus subtilis.* Scientific Reports. https://doi.org/10.1038/srep18405

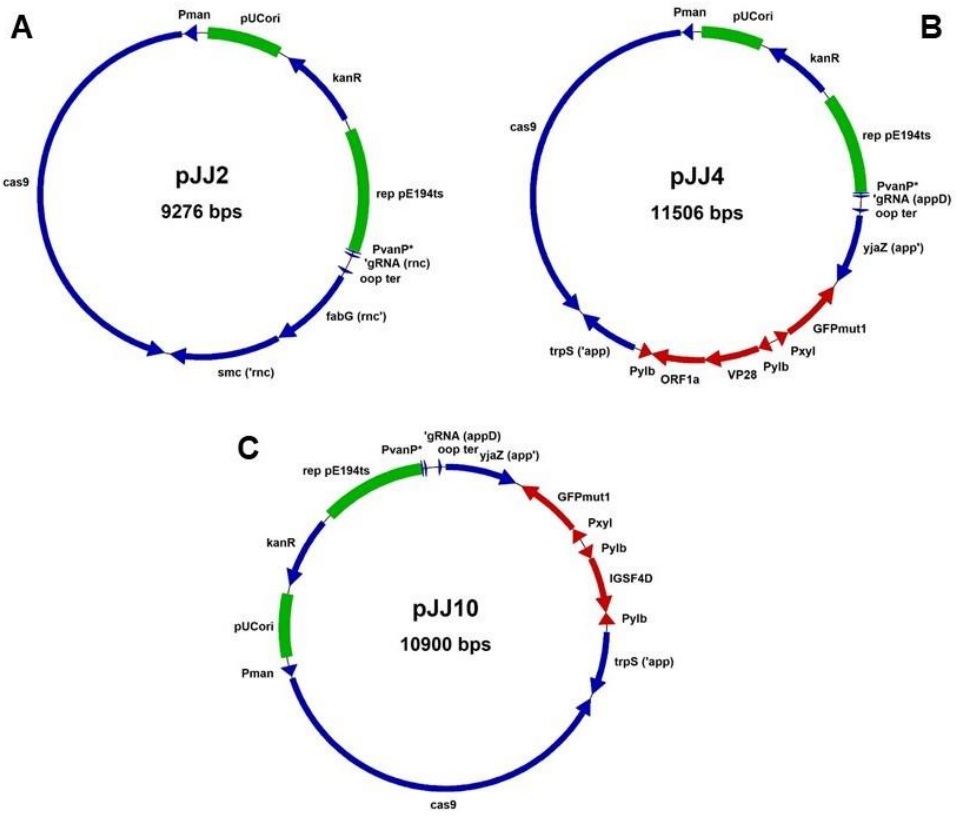

Fig 1 Physical map of the pJJ2, pJJ4, and pJJ10 vectors containing the Cas9 gene under control of the mannose-inducible promoter (P_{manP}), pUC18 minimal origin, kanamycin resistance gene (kanR), temperature-sensitive replication origin $pE194^{ts}$, and λ oop terminator. (A) sgRNA for *rnc* gene transcribed by semisynthetic promoter P_{vanP*}; sequence of *fabG* and *smc* genes used for deletion containing *rnc* gene. (B) sgRNA for *appD* gene transcribed by P_{vanP*}; sequence of *yjaZ* and *trpS* genes used for deletion; GFPmut1 transcribed by xylose-inducible promoter P_{xyl} and sequences for dsRNA production (VP28 from WSSV and ORF1a from IMNV) transcribed by two bidirectional promoters P_{ylb}. (C) Same as pJJ4, but with the sequence to produce virus-unrelated dsRNA (IGSF4D gene)

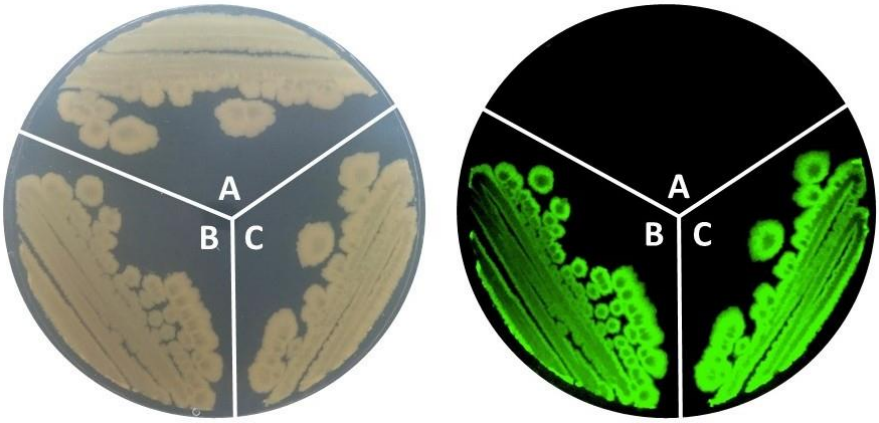

Fig 2 Expression of GFPmut1 in *B. subtilis* strains: (A) JJBs2 (negative control), (B) JJBs5 (expressing antiviral dsRNA and free of antibiotic resistance), and (C) JJBs8 (expressing non-related dsRNA and free of antibiotic resistance)

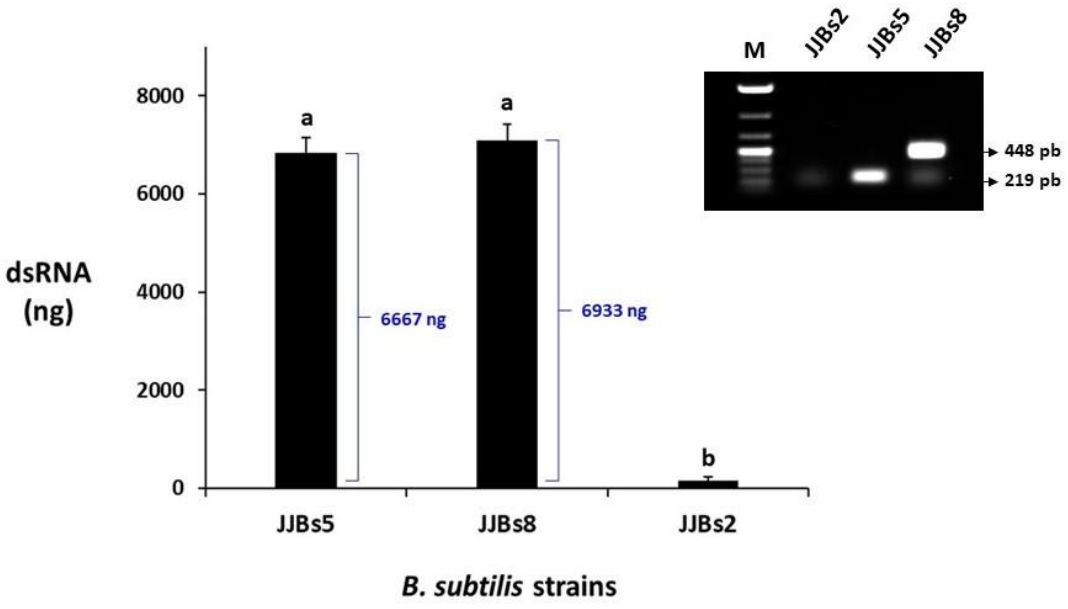

Fig 3 Production of dsRNA using the amount of 4.8 x 10^8 bacteria for the JJBs5 (dsRNA-VP28ORF1a - 6667 ng) and JJBs8 (IGSF4D - 6933 ng) strains, compared with the strain JJBs2 as negative control. Identification of dsRNA production with the strains JJBs5 (VP28 and ORF1a) and JJBs8 (IGSF4D) using the cDNA as template in PCR reactions. M: DNA marker 1Kb Plus DNA Ladder

DISCUSSÃO GERAL

O setor da carcinicultura é competitivo e busca cada vez mais a eficiência em produção. As doenças infecciosas promovem constantes prejuízos socioeconômicos e são o principal entrave para o crescimento da atividade (Anderson et al., 2014). O desenvolvimento de pesquisas visando a prevenção e tratamento de enfermidades em sistemas de cultivo se tornou uma prioridade nessa área de estudo. Desta forma, é essencial a aplicação de um método de intervenção terapêutica que possa ser utilizado no cultivo comercial dos camarões. Estudos realizados mostraram que o mecanismo RNAi foi capaz de promover proteção contra viroses em nível experimental (Feijó et al., 2015). Contudo, a indução do RNAi por injeção intramuscular é uma prática que dificulta sua aplicação nos cultivos de camarões. Esse fato é especialmente justificado pelas dificuldades de manejo e o alto custo na síntese *in vitro* de grandes quantidades de moléculas de dsRNA.

Com o desenvolvimento do presente trabalho demonstramos que foi possível manipular geneticamente uma cepa nativa de *Bacillus cereus* (Capítulo II). Entretanto, para a sua utilização é necessário o sequenciamento completo do seu genoma (Capítulo I), não só para a identificação da espécie, mas também para detecção da presença de genes que codificam para metabólitos como toxinas. Comprovamos, também, que é possível produzir dsRNA utilizando o probiótico *B. subtilis* com genoma editado através da recente e inovadora técnica CRISPR/Cas9 (Capítulo III). Os resultados desses pesquisas podem representar um alternativa para atenuar os problemas sanitários da carcinicultura. Desta forma, o Brasil pode ser o primeiro país capaz de apresentar uma solução contra as viroses, sendo essa com condições de aplicação a nível comercial via ração.

Contudo, este trabalho abriu caminho para alguns fatores que necessitam de mais investigação. As cepas produtoras de dsRNAs precisam ser avaliadas quando a possibilidade de exportação dessas moléculas para fora das células, assim como, é necessário ser verificado se os camarões possuem a capacidade de absorvê-las em nível gastrointestinal, sem algum tipo de degradação. Após uma melhor compreensão desses fatores as cepas podem ser testadas em experimentos com desafio viral, avaliando a efetividade no aumento da sobreviência dos camarões, ativação do sistema RNAi e

expressão de genes relacionados a esse mecanismo. Desta forma, esse trabalho criou a possibilidade de desenvolvimento de outros pesquisas que podem ser relizadas para responder essas perguntas.

CONCLUSÕES DA TESE

- Esse trabalho acrescentou informações moleculares em cepas nativas de *Bacillus* isoladas de crustáceos. Esses dados podem ser usados no desenvolvimento de novas pesquisas relacionadas com diversidade molecular e engenharia genética.

- A cepa de *B. cereus* #25 foi manipulada geneticamente para a produção de GFPmut1 com um plasmídeo específico para *B. subtilis*, indicando que uma identidade de aproximadamente 50% foi suficiente para ocorrência de recombinação homóloga. A cepa BC25GM possui genes relacionados com a produção de endotoxinas, porém não afetou o crescimento dos camarões. Esses genes podem ser deletados com a técnicas de engenharia genética e a cepa pode apresentar potencial aplicação na aquicultura, podendo ser um veículo para entrega de moléculas benéficas a produção.

- Plasmídeos com o mecanismo CRISPR/Cas9 foram produzidos para edição genômica em *B. subtilis.* As cepas transformadas (JJBs5 e JJBs8) tiveram o gene *rnc* deletado e sequências para produzir dsRNAs (VP28, ORF1a e IGDF4D) foram recombinadas. Assim como foram removidas resistências antimicrobianas. Esses resultados representam uma alternativa para mitigar os problemas com a ocorrência de viroses na carcinicultura.

PERSPECTIVAS

A possibilidade de utilização das cepas geneticamente modificadas na alimentação dos camarões em nível comercial ainda necessita superar alguns desafios. De acordo com a Instrução Normativa Interministerial MAPA/MPA Nº 28 de 08/06/2011 é permitido o uso de probióticos na dieta de organismos aquáticos desde que composto por microorganismos que não sejam patogênicos ou geneticamente modificados. Frente essa questão, cabe ressaltar que estudos em nível experimental são necessários para o estímulo das mudanças. Como exemplo, temos salmão transgênico com maior capacidade de crescimento, que foi liberado para produção e consumo em 2018 pela FDA (Food and Drug Administration). Além disso, pesquisas estão sendo realizadas com manipulação de probióticos com aplicação na prevenção de doenças humanas, fato que demonstra que mudanças futuras serão adotadas em relação a esse tema.

Em síntese, nosso grupo de pesquisa é pioneiro no Brasil frente a utilização da tecnologia CRISPR/Cas9 dedicada a edição genômica de probióticos como *B. subtilis*. Nossos esforços geraram abertura para novas pesquisas voltados a produção de moléculas específicas que podem trazer benefícios para a aquicultura. A edição genômica através da técnica CRISPR/Cas9 é considerada uma ferramente revolucionário na biologia e a implementação dessa tecnologia em território nacional pode ser considerada um salto científico, representando a aplicação de conhecimentos obtidos no exterior para melhorias na brasileira. Sendo assim, a engenharia genética aplicada em probióticos pode ser o início de uma nova e consistente linha de pesquisa nacional, com ampla aplicação na aquicultura e também em outras áreas relacionadas.

REFERÊNCIAS DA INTRODUÇÃO E DISCUSSÃO GERAL

AKHTER, N.; W.U.B.; MEMON, A.M.; MOHSIN, M. 2015. Probiotics and prebiotics associated with aquaculture: A review. Fish & Shellfish Immunology. 45, 733-741.

ALTENBUCHNER, J. 2016. Editing of the Bacillus subtilis Genome by the CRISPR-Cas9 System. Applied and Environmental Microbiology. 82:5421-5427.

ANDERSON, J.L.; VALDERRAMA, D.; JORY, D. 2014. PRODUCTION: Global Shrimp Review. Global Aquaculture Advocate (November/Dezember). 17-6.

ANDRADE, T.P.D.; SRISUVAN, T.; TANG, K.F.J.; LIGHTNER, D.V. 2007. Real-time reverse transcription polymerase chain reaction assay using TaqMan probe for detection and quantification of Infectious myonecrosis virus (IMNV). Aquaculture. 264: 9-15.

AQUATIC ANIMAL DISEASES. 2018. Diseases of crustaceans. Disponível em: http://library.enaca.org/Health/FieldGuide/html/cv020wsd.htm#. Acesso em: 10 Mai. 2018.

ATTASART, P.; NAMRAMOON, O.; KONGPHOM, U.; CHIMWAI, C.; PANYIM, S. 2013. Ingestion of bacteria expressing dsRNA triggers specific RNA silencing in shrimp. Virus Research. 171: 252-256.

BRIDIER, A.; MEYLHEUC, T.; BRIANDET, R. 2013. Realistic representation of *Bacillus subtilis* biofilms architecture using combined microscopy (CLSM, ESEM and FESEM). Micron. 48, 65-69.

BANERJEE, G.; RAY, A.K. 2017. The advancement of probiotics research and its application in fish farming industries. Research in Veterinary Science. 115, 66-77.

BARROCO, M.A.; PERAZZOLO, L.M.; ROSA, R.D. 2014. Avances em la imunoloía del camarón. In: MORALES, V.; CUÉLLAR-ANJEL, J. Patología e imunología de camarones penaeidos. OIRSA, Panamá, Chap. 6: 99-166.

CHEN, L.L.; WANG, H.C.; HUANG, C.J.; PENG, S.E.; CHEN, Y.G.; LINS, J.; CHEN, W.Y.; DAI, C.F.; YU, H.T.; WANG, C.H. 2002. Transcriptional analysis of the DNApolymerase gene of shrimp white spot syndrome virus. Virology. 30: 136-147.

CHENG, W.; CHIU, C.S.; GUU, Y.K.; TSAI, S.T.; LIU, C.H. 2013. Expression of recombinant phytase of *Bacillus subtilis* E20 in *Escherichia coli* HMS 174 and improving the growth performance of white shrimp, *Litopenaeus vannamei*, juveniles by using phytase-pretreated soybean meal containing diet. Aquaculture Nutrition. 19, 117-127.

DONG, H.; ZHANG, D. 2014. Current development in genetic engineering strategies of *Bacillus* species. Microbial Cell Factories. 13:63.

DURAND, S.; GILET, L.; CONDON, C. 2012. The Essential Function of *B. subtilis* RNase III Is to Silence Foreign Toxin Genes. PLOS Genetics. 8(12).

FAO. 2001. Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria.

FAO. 2018. The State of World Fisheries and Aquaculture 2018. Rome. 227p.

FEIJÓ, R.G.; KAMIMURA, M.T.; OLIVEIRA-NETO, J.M.; CÂNDIDA, M.V.N; GOMES, A.C.S; COELHO, M.G.L.; VASCONCELOS, R.F.; GESTEIRA, T.C.V.; MARINS, L.F.; MAGGIONI, R. 2013. Infectious myonecrosis virus and white spot syndrome virus coinfection in Pacific white shrimp (*Litopenaeus vannamei*) farmed in Brazil. Aquaculture (Amsterdam). 380-383: 1-5.

FEIJÓ, R.G.; MAGGIONI, R.; MARTINS, P.C.C.; ABREU, K.L.; OLIVEIRA NETO, J. M.; GUERTLER, C.; JUSTINO, E.B.; PERAZZOLO, L.M.; MARINS, L.F. 2015. RNAi-based inhibition of Infectious myonecrosis virus replication in the Pacific white shrimp (*Litopenaeus vannamei)* by dsRNAs targeted to different genome regions. Diseases of Aquatic Organisms. 114, 89-98.

FIRE, A.Z.; MELLO, C.G. 2006. RNA interference. The Nobel Prize in Physiology or Medicine 2006.

FU, L.L.; WANG, Y.; WU, Z.C.; LI, W. F. 2011. In vivo assessment for oral delivery of *Bacillus subtilis* harboring a viral protein (VP28) against white spot syndrome virus in *Litopenaeus vannamei*. Aquaculture. 322:323, 33-38.

GLOBO RURAL. 2017. Doença derruba produção de camarões no Ceará. Disponível em: http://g1.globo.com/economia/agronegocios/globo-rural/noticia/2017/02/doenca-derruba-producao-de-camaroes-no-ceara.html. Acesso em: 19/02/2019.

HAI, N.V. The use of probiotics in aquaculture. Journal of Applied Microbiology. 2015. 119, 917-935.

HILLE, F.; CHARPENTIER, E. 2016. CRISPR-Cas: biology, mechanisms and relevance. Philosophical Transactions of the Royal Society B. 371.

JINEK, M.; CHYLINSKI, K.; FONFARA, I.; HAUER, M.; DOUDNA, J.A.; CHARPENTIER E. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 337:816-821.

KUMAR, A.; LARAMORE, S.; ALEXANDER, P.; THOMAS ALLNUTT, F.C.; SAYRE, R.T. 2015. Double Stranded RNA Simultaneously Targeting Four White Spot Syndrome Virus (WSSV) genes Provides Protection against WSSV in *Litopenaeus Vannamei.* International Journal of Marine Science and Ocean Technology. 2: 5-10.

LAKSHMI, B.; VISWANATH, B.; GOPAL, D.V.R. 2013. Probiotics as Antiviral Agents in Shrimp Aquaculture. Journal of Pathogens.

LIGHTNER, D.V. 2012. Global transboundary disease politics: The OIE perspective. Journal of Invertebrate Pathology. 110, 184-187.

LIGHTNER, D.V.; PANTOJA, C.R.; POULOS, B.T.; TANG, K.F.J.; REDMAN, R.M.; ANDRADE, T.P.D.; BONAMI, J.R. 2004. Infectious Myonecrosis: New Disease in Pacific White Shrimp. The Advocate Global Aquaculture Alliance. 17-22.

LIU, K.F.; CHIU, C.H.; SHIU, Y.L.I.; CHENG, W.; LIU, C.H. 2010. Effects of the probiotic, *Bacillus subtilis* E20, on the survival, development, stress tolerance, and immune status of white shrimp, *Litopenaeus vannamei* larvae. Fish & Shellfish Immunology. 28: 837-844.

MAIL ONLINE NEWS. 2017. Australia's prawn industry faces 'RUIN' after a foreign disease wipes out $25 million of stock on farms. Disponível em: http://www.dailymail.co.uk/news/article-4154644/Australian-s-prawn-industry-WIPED-OUT.html. Acesso em: 10 Mai. 2018.

MAYO, M.A. 2002. A summary of taxonomic changes recently approved by ICTV. Archives of Virology. 147: 1655-1663.

NUNES, A.J.P.; MARTINS, P.C.; GESTEIRA, T.C. 2004. Carcinicultura Ameaçada. Panorama da Aqüicultura. 14: 37-57.

NGUYEN, A.T.V.; PHAM, C.K.; PHAM, H.T.T.; PHAM, H.L.; NGUYEN, A.H.; DANG, L.T.; HUYNH, H.A.; CUTTING, S.M.; PHAN, T.N. 2014. *Bacillus subtilis* spores expressing the VP28 antigen: a potential oral treatment to protect *Litopenaeus vannamei* against White spot syndrome. FEMS Microbiology Letters. 358:202-208.

PANTOJA, C.R.; LIGHTNER, D.V. 2014. Enfermidades virales del camarón. In: MORALES, V.; CUÉLLAR-ANJEL, J.Q. Patología e imunología de camarones penaeidos. OIRSA, Panamá, Chap. 2: 99-166.

RESHI, M.L.; WU, J.L.; WANG, H.V.; WONG, J.R. 2014. RNA interference technology used for the study of aquatic virus infections. Fish & Shellfish Immunology. 40: 14-23.

ROBALINO, J.; BARTLETT, T.; CHAPMAN, R.W.; GROSS, P.; BROWDY, C.; WARR, G. 2007. Double-stranded RNA and antiviral immunity in a marine shrimp: inducible host mechanism and evidence for the evolution of viral counter-responses. Developmental Comparative Immunology. 31: 539-547.

SANDER, J.D.; JOUNG, J.K. 2014. CRI SPR-Cas systems for editing, regulating and targeting genomes. Nature Biotechnology. 32,4.

SANTOS, R.N.; VARELA, A.P.M.; CIBULSKI, S.P.; LIMA, F.E.S.; SPILK, F.R.; HEINZELMANN, L.S.; LUZ, R.B.; ABREU, P.C.; ROEHE, P.M.; CAVALLI, L.S. 2013. A brief history of White Spot Syndrome Virus and its epidemiology in Brazil. Virus Reviews and Research. 1-7.

SEIBERT, C.H.; PINTO, A.R. 2012. Challenges in shrimp aquaculture due to viral diseases: distribution and biology of the five major penaeid viruses and interventions to avoid viral incidence and dispersion. Brazilian Journal of Microbiology. 857-864.

SENAPIN, S.; PHIWSALYA, K.; GANGNONNGIW, W.; FLEGEL, T.W. 2011. False rumours of disease outbreaks caused by infectious myonecrosis virus (IMNV) in the whiteleg shrimp in Asia. Journal of Negative Results in Biomedicine. 10:10.

TAJU, G.; MADAN, N.; MAJEED, S.A.; KUMAR, T.R.; THAMIZHVANAN, S.; OTTA, S.K.; HAMEED, A.S.S. 2015. Immune responses of whiteleg shrimp,

Litopenaeus vannamei (Boone, 1931), to bacterially expressed dsRNA specific to VP28 gene of white spot syndrome virus. Journal of Fish Diseases. 38: 451-465.

TANG, J.; OCHOA, W.F.; SINKOVITS, R.S.; POULOS, B.T.; GHABRIAL, S.A.; LIGHTNER, D.V.; BAKER, T.S.; NIBERT, M.L. 2008. Infectious myonecrosis virus has a totivirus-like, 120-subunit capsid, but with fiber complexes at the fivefold axes. Proceedings of the National Academy of Sciences of the United States of America. 105, 17526-17531.

VAN HULTEN, M.C.W; WITTEVELDT, J.; PETERS, S.; KLOOSTERBOER, N.; TARCHINI, R.; FIERS, M.; SANDBRINK, H.; KLEIN LANKHORST, R.; VLAK, J.M. 2001a. The white spot syndrome virus DNA genome sequence. Virology. 286: 7-22.

VAN HULTEN, M.C.W.; WITTEVELDT, J.; SNIPPE, M.; VLAK, J.M. 2001b. White spot syndrome virus envelope protein VP28 is involved in the systemic infection of shrimp. Virology. 285, 228-233.

VERBRUGGEN, B.; BICKLEY, L.K.; AERLE, R.V.; BATEMAN, K.S.; STENTIFORD, G.D.; SANTOS, E.M.; TYLER, C.R. 2016. Molecular Mechanisms of White Spot Syndrome Virus Infection and Perspectives on Treatments. Viruses, 8:23.

VIEIRA-GIRÃO, P.R.N.; ROCHA, I.R.C.B.; GAZZIENO, M.; VIEIRA, P.R.N.; LUCENA, H.M.R.; COSTA, F.H.F.; RÁDIS-BAPTISTA, G. 2015. Low Salinity Facilitates the Replication of Infectious Myonecrosis Virus and Viral Co-Infection in the Shrimp *Litopenaeus Vannamei*. Journal Aquaculture Research & Development. 6:302.

SARATHI, M.; SIMON, M.C.; VENKATESAN, C.; THOMAS, J.; RAVI, M.; MADAN, N.; THIYAGARAJAN, S.; HAMEED, A.S.S. 2010. Efficacy of bacterially expressed dsRNA specific to different structural genes of white spot syndrome virus

(WSSV) in protection of shrimp from WSSV infection. Journal of Fish Diseases. 33: 603-607.

WESTBROOK, A.Y.; MOO-YOUNG, M.; CHOU, C.P. 2016. Development of a CRISPR-Cas9 Tool Kit for Comprehensive Engineering of *Bacillus subtilis.* Applied and Environmental Microbiology. 82, 16.