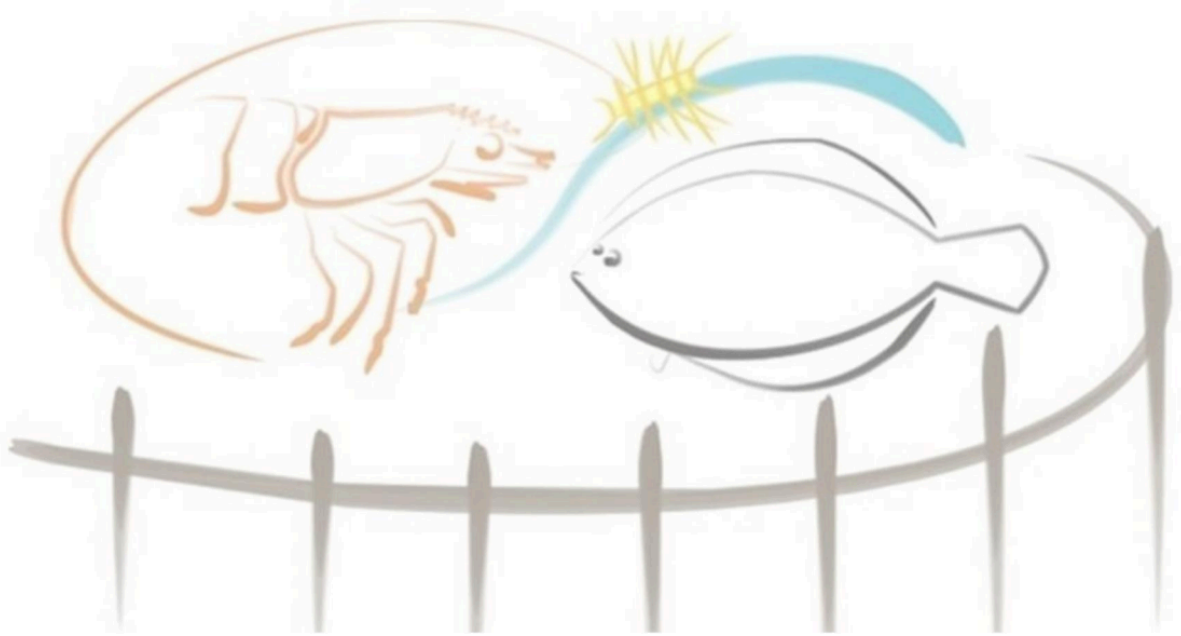
Universidade Federal do Rio grande - FURG
Instituto de Oceanografia
Programa de Pós-Graduação em Aquicultura



Probióticos convencionais e não convencionais no cultivo experimental do camarão branco *Litopenaeus vannamei*

Luiza Morais de Medeiros

Rio Grande - RS
Fevereiro de 2022

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Tese apresentada como parte dos requisitos para obtenção do grau de Doutora em Aquicultura pelo Programa de Pós-Graduação em Aquicultura da Universidade Federal do Rio Grande-FURG.

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Conte-me e eu esqueço.

Mostre-me e eu apenas me lembro.

Envolva-me e eu compreendo.

Confúcio

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Pós Graduação em AQUICULTURA
Universidade Federal do Rio Grande - FURG

ATA 02/2022

No dia vinte e dois de fevereiro de dois mil e vinte e dois, às oito horas e trinta minutos, reuniu-se a Banca Examinadora de Tese de Doutorado em Aquicultura, da **LUIZA MORAIS DE MEDEIROS**, orientada pelo Prof. Dr. Luis Fernando Marins, composta pelos seguintes membros: Prof. Dr. Luis Fernando Marins (Orientador – IO/FURG), Prof. Dr. Wilson Wasielesky Junior (Co orientador - IO/FURG), Prof. Dr. Dariano Krummenuer (IO/FURG), Profª. Dª. Virgínia Fonseca Pedrosa (IO/FURG), Profª. Drª. Lucielen Oliveira dos Santos (EQA/FURG) e o Prof. Dr. Rodrigo Maggioni (UFC). Título da Tese: **"Probióticos convencionais e não convencionais no cultivo experimental do camarão branco *Litopenaeus vannamei*"**. Dando início à defesa, o Coordenador do PPGAq, Prof. Dr. Dariano Krummenauer passou a presidência da sessão ao Prof. Dr. Luis Fernando Marins, que na qualidade de orientador, passou a palavra para a candidata apresentar a Tese. Após ampla discussão entre os membros da Banca e a candidata, 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. A candidata **LUIZA MORAIS DE MEDEIROS** foi considerada **APROVADA**, 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, pela candidata e pelo Coordenador do PPGAq.

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SUMÁRIO

RESUMO

Probióticos exercem inúmeros efeitos benéficos na aquicultura, especialmente no que se refere ao desempenho zootécnico, conversão alimentar e resposta imune. Diversos microrganismos podem ser caracterizados como probióticos e o desenvolvimento da biotecnologia tem permitido que estes microrganismos possam ser utilizados como plataformas de expressão de proteínas heterólogas, as quais que podem potencializar os efeitos benéficos já reconhecidos dos probióticos convencionais. Na presente Tese foram realizados três experimentos de 45 dias, cada um testando um probiótico diferente como aditivo na ração do camarão *Litopenaeus vannamei*. No capítulo I, testou-se o potencial probiótico de uma cepa de *Bacillus subtilis* (cepa E) previamente isolada do trato gastrointestinal dos camarões. Neste estudo foram avaliados parâmetros como desempenho zootécnico, composição centesimal do tecido muscular, número de vacúolos lipídicos no hepatopâncreas e a expressão de genes relacionados à digestão, ao metabolismo de aminoácidos e às defesas antioxidantes. Os resultados mostraram que a suplementação da ração com a cepa E não afetou o desempenho zootécnico dos camarões, mas aumentou a concentração de lipídios no músculo e no hepatopâncreas. Além disso, genes relacionados aos processos digestivos e ao metabolismo de aminoácidos foram fortemente reduzidos, indicando que a suplementação alimentar com a cepa E de *B. subtilis* pode melhorar a absorção de nutrientes da ração e minimizar os efeitos tóxicos dos compostos nitrogenados presentes na água de cultivo. No capítulo II, o objetivo foi avaliar o efeito de uma cepa de *B. subtilis* KM0 geneticamente modificada que expressa e secreta uma fitase fúngica, a qual pode melhorar aspectos nutricionais relacionados com o metabolismo do fósforo de camarões. Quando foi adicionado à ração comercial de *L. vannamei*, esse *B. subtilis* geneticamente modificado não alterou de forma significativa parâmetros de crescimento. No entanto, houve uma diminuição na expressão de genes relacionados com a digestão e um aumento de 39% no teor de fósforo no tecido muscular e, também, um aumento na contagem de vacúolos lipídicos no hepatopâncreas do camarão. No capítulo III, o objetivo foi suplementar a ração comercial com uma cepa geneticamente modificada da cianobactéria *Synechococcus elongatus* expressando uma β-glicosidase heteróloga, a qual pode impactar o metabolismo de carboidratos do camarão. Os resultados demonstraram que a suplementação da ração comercial com as cianobactérias (selvagem ou transgênica) não afetou de forma negativa os índices de crescimento, peso final e taxa de conversão alimentar dos camarões em

comparação com os controles. Entretanto, as cianobactérias alteraram a expressão de genes relacionados à digestão (*amy*, *gdh* e *cathB*) e ao sistema imune (*tgase*), enquanto a cianobactéria transgênica, isoladamente, aumentou significativamente o conteúdo de fósforo e lipídio no músculo dos camarões. Em conclusão, a presente tese demonstrou que o uso tanto de probióticos isolados do trato intestinal do camarão *L. vannamei* assim como probióticos geneticamente modificados podem trazer diversos benefícios aos camarões, o que é interessante do ponto de vista ambiental e econômico, contribuindo para a sustentabilidade da carcinocultura.

Palavras-chave: Probióticos, *Bacillus subtilis*, *Synechococcus elongatus*, fitase, β-glicosidase, carcinocultura.

ABSTRACT

Probiotics exert numerous beneficial effects in aquaculture, especially regarding zootechnical performance, feed conversion and immune response. Several microorganisms can be characterized as probiotics and the development of biotechnology has allowed these microorganisms to be used as platforms for the expression of heterologous proteins, which can enhance the already recognized beneficial effects of conventional probiotics. In the present Thesis, three 45-day experiments were carried out, each one testing a different probiotic as an additive in the diet of *Litopenaeus vannamei* shrimp. In chapter I, the probiotic potential of a strain of *Bacillus subtilis* (strain E) previously isolated from the gastrointestinal tract of shrimp was tested. In this study, parameters such as zootechnical performance, proximate composition of muscle tissue and the expression of genes related to digestion, the immune system and antioxidant defenses were evaluated. The results showed that the supplementation of the diet with strain E did not affect the zootechnical performance of the shrimp but increased the concentration of lipids in the muscle and hepatopancreas. In addition, genes related to digestive processes and amino acid metabolism were strongly reduced, indicating that feed supplementation with the E strain of *B. subtilis* can improve the absorption of nutrients from the feed and minimize the toxic effects of the nitrogen compounds present in the water of animals creation. In chapter II, the objective was to evaluate the effect of a genetically modified strain of *B. subtilis* KM0 that expresses and secretes a fungal phytase, which can improve nutritional aspects related to the phosphorus metabolism of shrimp. When added to commercial feed of *L. vannamei*, this genetically modified *B. subtilis* did not significantly alter growth parameters. However, there was a decrease in the expression of genes related to digestion and an increase of 39% in phosphorus content in muscle and an increase in lipid vacuole count in shrimp hepatopancreas. In chapter III, the objective was to supplement the commercial feed with a genetically modified strain of the cyanobacterium *Synechococcus elongatus* expressing a heterologous β-glucosidase, which can impact the carbohydrate metabolism of shrimp. The results showed that the supplementation of commercial feed with cyanobacteria (wild or transgenic) did not negatively affect the growth rates, final weight and feed conversion rate of shrimp compared to the controls. However, cyanobacteria altered the expression of genes related to digestion (*amy*, *gdh* and *cathB*) and to the immune system (*tgase*), while the transgenic cyanobacterium, alone, significantly increased the phosphorus and lipid content in shrimp

muscle. In conclusion, the present Thesis showed that the use of both probiotics isolated from the intestinal tract of *L. vannamei* as well as genetically modified probiotics can bring several benefits to shrimp, which is interesting from an environmental and economic point of view, contributing to the sustainability of the shrimp farming.

Keywords: Probiotics, *Bacillus subtilis*, *Synechococcus elongatus*, phytase, β-glucosidase, shrimp farming.

INTRODUÇÃO GERAL

Aquicultura

De acordo com as últimas estatísticas mundiais sobre aquicultura compilado pela FAO, a produção atingiu outro recorde histórico de 114,5 milhões de toneladas em peso vivo em 2018. Nesse mesmo ano, os crustáceos corresponderam a 9,4 milhões de toneladas. A aquicultura tem sido o setor de produção de alimentos que se expandiu mais rapidamente em todo o mundo, sendo que a produção aquícola mundial cresceu em média 5,3 % ao ano no período de 2001-2018 (FAO, 2020). A produção da aquicultura também atingiu outro recorde histórico: estima-se que gerou 82 milhões de toneladas, movimentando um valor total de US$ 250 bilhões. Dessa forma, a aquicultura é responsável por 46 % da produção total e 52 % de peixes para consumo humano. Camarões marinhos dominam a produção na aquicultura e são uma importante fonte de ganhos para uma série de países em desenvolvimento na Ásia e na América Latina. O setor de criação de camarões, que agora fornece a maior parte do volume para o mercado global, também sofreu o impacto de surtos de doenças e variações de preços associadas aos ciclos de alta e baixa. Os altos volumes de produção aquícola em 2018 e 2019 empurraram os preços de mercado para níveis baixos, levando a um planejamento conservador por parte dos produtores. Além disso, espera-se que as espécies que requerem maiores proporções de farinha e óleo de peixe em suas dietas tenham um crescimento na produção mais lento devido aos preços mais altos e à disponibilidade reduzida de farinha de peixe. O Brasil ocupa a 10ª posição na produção de aquicultura marinha e costeira de crustáceos. No entanto, desde 2014 não há estatística pesqueira oficial e os dados são obtidos por levantamentos realizados pela FAO e seus parceiros (FAO, 2020).

Alimentação na aquicultura

A farinha e o óleo de peixe são produtos de uso quase exclusivo para fabricação de ração para organismos aquáticos. A farinha de peixe é rica em macro e oligoelementos, contém nutrientes essenciais, bem como muitos compostos que são biologicamente ativos (Hardy, 2010). Quando há um desequilíbrio no nível de aminoácidos, há também uma alteração no metabolismo das proteínas, logo é importante que na alimentação de camarões exista uma fonte de proteína que tenha um equilíbrio adequado dos aminoácidos para promover um desempenho de crescimento satisfatório (Jin et al., 2019). Além da importância

da proteína na ração, outro fator importante é o óleo de peixe, que é considerado a principal fonte de lipídios para aquicultura devido às suas altas proporções de ácidos graxos altamente insaturados de cadeia longa e seus benefícios nutricionais (Zhou et al., 2014).

Um dos desafios da aquicultura é o fornecimento de nutrientes ao sistema, principalmente na forma de rações compostas produzidas industrialmente. À medida que esta indústria continua a se expandir, também aumenta a demanda por ingredientes-chave para rações (Gia Vo et al., 2020). A produção de farinha e óleo de peixe flutua de acordo com as mudanças nas capturas das espécies, o que afeta a abundância dos estoques pesqueiros. A redução progressiva na oferta ocorre ao mesmo tempo em que há demanda crescente impulsionada por uma indústria de aquicultura de rápido crescimento, o que aumentou os preços da farinha e do óleo de peixe. Como consequência, a incorporação de farinha e óleo de peixe nas dietas está diminuindo (FAO, 2020; Zhu et al., 2021). Em virtude disso, a indústria da aquicultura investiga ativamente fontes alternativas de proteína para substituir os subprodutos da pesca na alimentação de animais aquáticos (To e Liou, 2021). Além disso, a otimização da digestão de proteínas é um objetivo fundamental para o alcance do alto desempenho zootécnico na aquicultura (Dai et al., 2018).

O aumento da concorrência global e as mudanças na demanda, os avanços tecnológicos e as novas descobertas por meio de pesquisas estão fazendo com que a indústria de manufatura de ingredientes adote novas tecnologias de processamento e novos ingredientes. Paralelamente, ocorre o crescimento acelerado da produção de rações para a aquicultura, o que resulta em várias fontes alternativas de proteína vegetal testadas e que estão disponíveis no mercado (Galkanda-Arachchige et al., 2021). No entanto, embora as fontes de proteína vegetal terrestre se mostrem promissoras na pesquisa por proteína alimentar alternativa, as proteínas vegetais apresentam conhecidas limitações nutricionais. Um dos principais fatores que contribuem para um menor valor nutricional é a presença de fatores antinutricionais (ANFs) de ocorrência natural, por exemplo, fitato, taninos, inibidores de enzimas e saponinas, entre outros, que diminuem indiretamente a biodisponibilidade dos minerais (Nikmaram et al., 2017). Além disso, a substituição completa da farinha de peixe em rações para espécies marinhas é mais difícil e exigirá mais esforços de pesquisa (Hardy, 2010).

Ácido fítico e fitases

O ácido fítico possui seis grupos fosfato em uma única molécula de seis carbonos, e é a principal forma de armazenamento de fosfato em muitos tecidos vegetais (Hussain et al., 2021). É um composto onipresente (variando de 0,4% a 6,4% em peso), naturalmente presente em cereais, grãos, sementes oleaginosas e nozes (Nikmaram et al., 2017). O fitato diminui a digestibilidade de proteínas e aminoácidos e a eficiência de absorção em peixes e outros animais por ser uma molécula polianiônica que se quela em nutrientes carregados positivamente (íons metálicos como Ca^{2+}, Mg^{2+}, Zn^{2+} e Fe^{2+}). Essa molécula atua como um fator antinutricional, formando complexos com proteínas, afetando a digestão e inibindo as enzimas digestivas, como α-amilase, tripsina, fosfatase ácida e tirosinase (Hussain et al., 2021). Assim, a presença do fitato na matéria vegetal é uma das principais restrições que limitam o uso de proteínas vegetais na alimentação animal (Kumar et al., 2012).

Sabe-se que o fósforo é um elemento essencial para plantas e animais, pois é componente dos ácidos nucléicos e desempenha um papel no metabolismo de lipídios, sacarídeos e proteínas (Truong et al., 2020). Além disso, o fósforo não digerido e excretado na água contribui para o processo de eutrofização (Kumar et al., 2012). Visto que o fitato indisponibiliza o fósforo, a redução enzimática do teor de ácido fítico em alimentos e rações é desejável, pois melhora a qualidade nutricional dos alimentos (Singh e Satyanarayana, 2015). A fitase, quimicamente conhecida como mio inositol (1,2,3,4,5,6) - hexafosfato fosfohidrolase, catalisa a hidrólise do fitato, o que produz uma molécula de inositol e seis moléculas de fosfato inorgânico, tornando o fósforo disponível para absorção. Logo, esta enzima pode reduzir o efeito antinutricional do fitato e melhorar a digestibilidade do fósforo, cálcio, aminoácidos e energia, além de reduzir o impacto negativo da excreção de fósforo inorgânico no meio ambiente (Dersjant-Li et al., 2015).

As fitases de ocorrência natural são abundantes e podem ser obtidas de plantas, animais e microorganismos (bactérias, fungos e leveduras) (Hussain et al., 2021). As fitases produzidas por microrganismos são comumente utilizadas como aditivo comercial para rações. Em 1999, a primeira geração da fitase fúngica obtida a partir de *A. niger* foi disponibilizada comercialmente (Jatuwong et al., 2020). As fitases fúngicas são histidina fosfatases ácidas e são amplamente utilizadas para a produção de fitases em escala comercial. O papel da fitase no desempenho do crescimento dos peixes está bem documentado (Afzal et al., 2019; Li et al., 2019; Shahzad et al., 2018). As fitases fúngicas melhoram o valor nutricional de alimentos e rações, pois aumenta a utilização proteínas, vitaminas e

aminoácidos ao degradar os complexos fitina-proteína, melhorando uso e a biodisponibilidade de diferentes nutrientes, além de melhorar a digestibilidade de minerais (Hussain et al., 2021). Por outro lado, as fitases também mitigam os impactos causados pela descarga excessiva de fósforo em ambientes aquáticos (Singh e Satyanarayana, 2015).

Saponinas e β-glicosidases

As saponinas são fitoquímicos de ocorrência natural, estrutural e funcionalmente diversos, amplamente distribuídos nas plantas. São um grupo complexo e quimicamente variado de compostos que consistem em triterpenóides ou agliconas esteroidais ligadas a porções de oligossacarídeo. A combinação de uma estrutura de aglicona hidrofóbica e moléculas de açúcar hidrofílicas torna as saponinas altamente anfipáticas e confere propriedades de formação de espuma e emulsificação (Moses et al., 2014). Além disso, as saponinas são consideradas um dos fatores antinutricionais (ANFs) presentes em proteínas vegetais que são alternativas na substituição da farinha de peixe. Quando há uma redução da saponina na dieta, há também um melhor desempenho de crescimento (Fadel et al., 2021). Foi reportado que as saponinas da soja agravaram a inflamação intestinal de forma dose dependente concomitante com a expressão regulada positivamente de citocinas inflamatórias, destruíram a estrutura das junções intracelulares, diminuíram as atividades de parâmetros antioxidantes selecionados e aumentaram a apoptose das células epiteliais intestinais em peixes (Gu et al., 2018). Também, foi demonstrado que a saponina de soja na dieta de *Monopterus albus* diminuiu o crescimento, danificou a estrutura intestinal e a barreira intestinal, induzindo inflamação intestinal nos animais (Hu et al., 2021). Em termos gerais, as saponinas podem afetar a utilização de proteínas, minerais e vitaminas (Zhou et al., 2018). A hidrólise enzimática de ANFs, como as saponinas, tem o potencial de aumentar a biodisponibilização de nutrientes (Zhou et al., 2018). O uso de farelo de soja processado tratado com enzimas mostrou melhor desempenho de crescimento e eficiência alimentar do que farelo de soja convencional em dietas de trutas arco-íris, o que demonstra que o bioprocessamento enzimático pode reduzir os ANFs e aumentar o conteúdo de proteína no farelo de soja processado convencional sem comprometer o valor nutricional (Kumar et al., 2020).

As β-glicosidases podem hidrolisar a ligação glicosídica β-1,4 de dissacarídeos, oligossacarídeos e moléculas substituídas por glicose e, também, catalisar as reações sintéticas por meio de reação hidrolítica reversa ou transglicosilação. Por ser um grupo

diversificado de enzimas com ampla distribuição em bactérias, fungos, plantas e animais, tem potencial para ser utilizado em vários processos biotecnológicos, como produção de biocombustíveis, hidrólise de isoflavonas, realce de sabor, síntese de oligossacarídeos e a produção combustível como o etanol a partir de resíduos agrícolas (Ahmed et al., 2017; Singhania et al., 2013). Além da hidrólise da celulose, já está bem documentado que β-glicosidases também podem hidrolisar saponinas (Renchinkhand et al., 2015; Yan et al., 2018).

Probióticos na Aquicultura

Em uma das primeiras revisões que discutiram o que seriam probióticos Ringø e Gatesoupe (1998) mostraram que esses microrganismos são capazes de colonizar o intestino e atuar de forma antagônica contra patógenos gram-negativos. Além disso, são capazes de produzir bacteriocinas inofensivas e podem reduzir a necessidade de uso de antibióticos na aquicultura. Hill et al. (2014) sugeriram como definição que probióticos são "microrganismos vivos que, quando administrados em quantidades adequadas, conferem um benefício à saúde do hospedeiro". Para serem usados como probióticos, é essencial que os microrganismos sejam considerados como GRAS (geralmente reconhecidos como seguros); que é um status usado para resolver o problema da colonização de patógenos em diferentes ecossistemas, afirmados pela FDA Estado-Unidense (*US Food and Drug Administration*) ou determinados independentemente por especialistas qualificados (Bouchard et al. 2013).

Os probióticos surgiram como uma alternativa ao uso de agentes antimicrobianos, com o objetivo de melhorar a saúde dos peixes (Martínez Cruz et al., 2012). Alguns estudos apontam que a primeira aplicação de probióticos na aquicultura foi realizada em 1986 (Gatesoupe, 1999; Ringø et al., 2020). Na década de 1990, Munro et al. (1995) relacionaram bactérias que são benéficas (probióticas) com a estimulação do crescimento de algas unicelulares. Rapidamente, o interesse por tais microrganismos aumentou e um número crescente de trabalhos científicos tem tratado explicitamente de probióticos desde então (por exemplo, Ruiz et al., 1996; Gildberg et al., 1997; Ringø e Gatesoupe, 1998; Hu et al., 2022; Ma et al., 2022; Kolanchinathan et al., 2022).

A introdução dos probióticos na aquicultura substituiu significativamente o uso de antibióticos sintéticos comerciais. Dessa forma, os probióticos têm sido aplicados junto com as rações comerciais para controlar ou prevenir infecções bacterianas ou fúngicas (Ock Kim et al., 2020), ou adicionados aos sistemas de cultivo para melhorar a qualidade da água.

Evidências crescentes sugerem que os antibióticos podem alterar a microbiota intestinal, o que potencialmente afeta a saúde do hospedeiro. Em *Litopenaeus vannamei*, o uso de antibióticos pode alterar a diversidade, composição e função microbiana intestinal (Zeng et al., 2019), enquanto microrganismos probióticos têm a capacidade de melhorar o desenvolvimento e a microflora intestinal, além de produzirem várias substâncias antibacterianas naturais (Aarti et al., 2017; Amoah et al., 2019). Hostins et al. (2017; 2019) observaram que os probióticos podem ser usados para complementar o efeito protetor dos bioflocos, de forma que o manejo da comunidade microbiana pode ser uma ferramenta para reduzir o risco de doenças, estabelecer sistemas altamente biosseguros, além de controlar infecções patogênicas no intestino do hospedeiro quando aplicado de forma racional.

As bactérias mais utilizadas como probióticos na aquicultura são bactérias ácido lácticas e *Bacillus*. Entretanto, também são utilizados vários outros gêneros como *Aeromonas*, *Alteromonas, Arthrobacter, Bifidobacterium, Clostridium, Paenibacillus, Phaeobacter, Pseudoalteromonas, Pseudomonas, Rhodosporidium, Roseobacter, Streptomyces* e, até mesmo, *Vibrio*. Microalgas (*Tetraselmis*) e leveduras (*Debaryomyces, Phaffia e Saccharomyces*) também podem ser consideradas como probióticos (Ringø et al, 2020). No entanto, existem alguns microrganismos que são mais comumente utilizados na aquicultura e incluem bactérias antagônicas (*Bacillus, Pseudomonas, Alteromonas e Flavobacterium odoratum*), os quais são microrganismos funcionais que melhoram a absorção de nutrientes e a atividade digestiva do hospedeiro (bactérias ácido-láticas e leveduras) e microrganismos que melhoram a qualidade da água (denitrificantes e bactérias nitrificantes) (Qi et al, 2009). Além disso, cepas endêmicas, autóctones ou derivadas do hospedeiro também podem ser usadas como probióticos e conferem benefícios à saúde (Luis-Villaseñor et al., 2011; Pham et al., 2014).

Esses microrganismos comumente utilizados podem ser ditos “probióticos convencionais”. No entanto, existem outros que não são tão comuns que podem ser caracterizados como “não convencionais”. Dentre esses, estão as bactérias gram-negativas e gram-positivas, leveduras, bacteriófagos, algas unicelulares e actinobactérias (Butt et al., 2021; Das et al., 2008), além de probióticos geneticamente modificados. Dentro desse cenário, algumas cianobactérias também podem ser consideradas como probióticos não convencionais, pois apresentam potencial probiótico através de sua atividade imunomoduladora (Riccio and Lauritano, 2020) e, também, são capazes de produzir compostos bioativos com diversas aplicações, como a inibição de patógenos (Santhakumari et

al., 2017). Um estudo recente relatou que cianobactérias podem ter um impacto de longo alcance no microbioma intestinal levando ao aumento da riqueza bacteriana, podendo desempenhar um papel importante no desenvolvimento e na saúde de peixes (Rosenau et al., 2021).

Para que um microrganismo seja considerado com potencial probiótico algumas características devem ser atendidas, como a promoção do crescimento, inibição de patógenos, melhoria na digestão de nutrientes, da qualidade da água, aumento da tolerância ao estresse e efeitos na reprodução, aumento na assimilação de macronutrientes e melhora no estado de saúde dos animais através da ativação do sistema imunológico (Olmos et al., 2020; Pham et al., 2014). Essas características se expressam devido à capacidade que os probióticos têm de produzir moléculas que possuem atividade bactericida sobre as bactérias patogênicas intestinais do hospedeiro, proporcionando uma barreira contra a proliferação de patógenos oportunistas (Martínez Cruz et al. 2012).

A administração de probióticos também pode resultar no aumento do consumo e a absorção de nutrientes da ração devido à sua capacidade de liberar uma ampla gama de enzimas digestivas e, também, de nutrientes que podem participar do processo de digestão e utilização da ração, juntamente com a absorção de componentes da dieta, levando a uma melhora no bem-estar e na saúde do hospedeiro. Além disso, os probióticos contribuem para a maturação intestinal, prevenção de distúrbios intestinais, pré-digestão de fatores antinutricionais encontrados nos ingredientes da ração, microbiota intestinal e metabolismo (Olmos et al., 2020; Ringø et al., 2020).

Engenharia genética em probióticos na aquicultura

Microrganismos podem ser reprogramados para produzir moléculas de interesse, através da manipulação do metabolismo, adicionando e excluindo seletivamente genes relacionados a determinadas vias metabólicas (Choudhary e Mahadevan, 2020). As espécies de *Bacillus* são consideradas cepas promissoras com inúmeras vantagens, incluindo: não toxicidade, facilidade para manipulação genética, alto rendimentos de proteínas heterólogas, taxa de crescimento rápida e baixa necessidade de nutrientes (van Dijl e Hecker, 2013). Entre as espécies de *Bacillus*, *B. subtilis* tem sido amplamente utilizada como uma biofábrica para a produção de proteínas recombinantes devido à sua natureza geralmente considerada segura (GRAS), além de um sistema secretor naturalmente eficiente. Além disso, existe um amplo

conhecimento sobre sua genética, fisiologia e processos de fermentação em larga escala (Schallmey et al., 2004, James et al., 2021).

Outros microrganismos com potencial em aplicações biotecnológicas são as microalgas e cianobactérias. A exploração dessa diversidade genética permite o uso eficiente de microrganismos fotossintéticos como biofábricas de enzimas recombinantes que serão úteis e podem ser utilizadas em diferentes indústrias como na produção de alimento para consumo humano, ração animal, aquicultura, cosméticos e biocombustíveis. Além disso, por serem fotoautótrofos, com requisitos nutricionais mínimos, as microalgas apresentam vantagens em comparação com outras células microbianas (Brasil et al., 2017).

A expressão recombinante é um método importante para facilitar a produção de proteínas alvo. Com o desenvolvimento da biotecnologia, novos sistemas de expressão e novas técnicas para a exibição de proteínas heterólogas em células probióticas têm sido desenvolvidos (Yao et al., 2020). A engenharia genética em probióticos tem sido utilizada com diversos objetivos, como aplicações em alimentos, biomédicas (Zuo et al., 2020), produção de biocombustíveis (Hlavova et al., 2015), dentre outros. Whelan et al. (2014) modificaram geneticamente uma bactéria probiótica para secretar imunomodulador de parasitas para tratamento direcionado no local da inflamação intestinal em camundongos e porcos, demonstrando que a eficiência anti-inflamatória de um probiótico pode ser melhorada por um transgene imunorregulador e que esse tratamento pode ter um potencial como opção terapêutica para a doença inflamatória intestinal. Amiri-Jami et al. (2015) também modificaram uma bactéria probiótica para a produção recombinante de ácidos graxos ômega-3, mostrando que probiótico transgênico produtor de EPA/DHA pode ser utilizado como fonte segura, alternativa e econômica para a produção industrial e farmacêutica desses ácidos graxos poli-insaturados de alto valor comercial. Tang et al. (2016) demonstraram que a administração oral de esporos de *B. subtilis* recombinantes em camundongos pode fornecer proteção efetiva contra *Clonorchis sinensis*, uma zoonose de origem alimentar que pode ser ocasionada pela ingestão de peixe cru contaminado por esse trematódeo.

Na aquicultura, alguns estudos recentes já mostram a aplicação da engenharia genética em probióticos. Santos et al. (2020) desenvolveram uma cepa de *B. subtilis* com o objetivo de melhorar a digestibilidade de rações que apresentam altos níveis de proteína vegetal, que foi projetado para produzir e secretar uma fitase fúngica. Riet et al. (2021) desenvolveram uma cepa geneticamente modificada de *B. subtilis* para a produção de dsRNAs antivirais contra a proteína viral 28 (VP28) do vírus da mancha branca (WSSV), que minimiza a replicação viral

e pode se tornar uma importante ferramenta terapêutica para as espécies da aquicultura. Nesse mesmo sentido, Zhuang et al. (2021) construíram uma *Synechococcus elongatus* PCC 7942 que expressa VP28 para proteção de camarões contra o WSSV. Levando em consideração que os probióticos têm um papel na regulação imunológica, o portador de uma vacina oral seria mais propício para interagir com o hospedeiro e poderia estimular uma resposta imunológica eficaz na mucosa intestinal e até mesmo sistêmica (Yao et al., 2020).

HIPÓTESES

Hipótese geral

A administração dietética de probióticos convencionais ou não convencionais é capaz de alterar dois níveis de organização biológica do camarão *Litopenaeus vannamei*: genético-molecular e composição tecidual.

Hipótese específica 1

A administração dietética de uma cepa de *Bacillus subtilis* isolada do intestino do camarão *L. vannamei* é capaz de melhorar o desempenho zootécnico e alterar a composição do tecido muscular e o perfil de expressão gênica relacionado às defesas antioxidantes, digestão e metabolismo de aminoácidos do camarão cultivado experimentalmente.

Hipótese específica 2

A administração dietética de uma cepa de *Bacillus subtilis* geneticamente modificada para expressar uma fitase heteróloga é capaz de melhorar o desempenho zootécnico e alterar a composição do tecido muscular e o perfil de expressão gênica relacionado às defesas antioxidantes, digestão e metabolismo de aminoácidos do camarão cultivado experimentalmente.

Hipótese específica 3

A administração dietética da cianobactéria *Synechococcus elongatus* geneticamente modificada para expressar uma β-glicosidase heteróloga é capaz de melhorar o desempenho zootécnico e alterar a composição do tecido muscular e o perfil de expressão gênica relacionado à resposta imune, defesas antioxidantes, digestão e metabolismo de aminoácidos do camarão cultivado experimentalmente.

OBJETIVOS

Objetivo Geral

Introduzir diferentes probióticos (convencionais e não convencionais) como aditivos dietéticos no cultivo experimental do camarão branco *Litopenaeus vannamei*, avaliando seus efeitos em dois níveis de organização biológica: genético-molecular e composição tecidual.

Objetivos específicos

- Testar os efeitos de uma cepa de *Bacillus subtilis* isolada do intestino do camarão *L. vannamei* como aditivo dietético, avaliando o desempenho zootécnico, composição do tecido muscular, número de vacúolos lipídicos no hepatopâncreas e o perfil de expressão gênica relacionado às defesas antioxidantes, digestão e metabolismo de aminoácidos de *L. vannamei* cultivado experimentalmente;
- Testar os efeitos de uma cepa de *Bacillus subtilis* geneticamente modificada para expressar uma fitase heteróloga como aditivo dietético, avaliando o desempenho zootécnico, composição do tecido muscular, número de vacúolos lipídicos no hepatopâncreas e o perfil de expressão gênica relacionado às defesas antioxidantes, digestão e metabolismo de aminoácidos de *L. vannamei* cultivado experimentalmente;
- Testar os efeitos de uma cianobactéria *Synechococcus elongatus* geneticamente modificada para expressar uma β-glicosidase heteróloga como aditivo dietético, avaliando o desempenho zootécnico, composição do tecido muscular e o perfil de expressão gênica relacionado à resposta imune, defesas antioxidantes, digestão e metabolismo de aminoácidos de *L. vannamei* cultivado experimentalmente.

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CAPÍTULO I

A native strain of *Bacillus subtilis* increases lipid accumulation and modulates expression of genes related to digestion and amino acid metabolism in Pacific white shrimp *Litopenaeus vannamei*

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Marins. A Biol. Laura Dall'Agno está de acordo e é coautora do manuscrito.

A native strain of *Bacillus subtilis* increases lipid accumulation and modulates expression of genes related to digestion and amino acid metabolism in Pacific white shrimp *Litopenaeus vannamei*

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Abstract

The use of probiotics has been an interesting alternative for shrimp farming, as the benefits provided by these bacteria are already recognized. In the present study, a strain of *Bacillus subtilis* (strain E) was isolated from the gastrointestinal tract of the shrimp *Litopenaeus vannamei* and molecularly identified. The probiotic potential of strain E was characterized, and it was used as a food additive in an experiment with *Litopenaeus vannamei* for 45 days. The zootechnical performance, proximate composition of muscle tissue, lipid concentration in hepatopancreas and expression of genes related to digestion, amino acid metabolism and antioxidant defenses in different tissues of shrimp were evaluated. The results showed that the supplementation of the feed with strain E did not affect the zootechnical performance of the shrimp, but it increased the lipid concentration in the muscle and hepatopancreas. Furthermore, genes related to digestion and amino acid metabolism were strongly reduced, indicating that feed supplementation with the strain E of *B. subtilis* may improve the absorption of nutrients from the feed and minimize the toxic effects of the nitrogen compounds present in the rearing water.

Keywords: Probiotic bacteria, shrimp farming, digestion, immune system, antioxidant defenses.

1. INTRODUCTION

Shrimp farming is one of the sectors with the highest economic value within aquaculture activities, with the Pacific white shrimp (*Litopenaeus vannamei*) being the most commercially produced crustacean (FAO, 2020). This shrimp has been widely farmed due to its ability to tolerate different environmental conditions. It also has a good growth rate and can easily adapt to commercial feeds. However, the search for increased productivity has led to inadequate management practices, especially about the high densities of shrimp that can be difficult to support if the system is not adequate. Thus, diseases caused by viruses and bacteria on shrimp farms have become increasingly prominent (Shen et al., 2010). Viral diseases such as White Spot Syndrome (WSS) can cause catastrophic losses on farms in a few days (Wen et al., 2014), as well as bacterial diseases caused by *Vibrio* species can cause equally harmful impacts on shrimp production by causing high mortality rates (Zokaeifar et al., 2012a). Additionally, nutrition is also a factor that needs to be considered, since feed has been reported as the most important component of the production cost of shrimp farms in several countries (Karim et al., 2014; Nisar et al., 2021; Penda et al., 2013; Shang et al., 1998). Fishmeal, the main source of protein in commercial feed, has become a scarce and expensive ingredient. Alternative protein sources such as plant matter have showed negative effects on fish nutrition due to the presence of anti-nutritional compounds (Azeredo et al., 2017; Estruch et al., 2018). One of the possibilities to increase the efficiency in the digestibility of commercial feed is to use probiotic bacteria that can produce and secrete digestive enzymes.

There are numerous bacterial species that can be considered as probiotics. Among these, some *Bacillus* species have gained special attention due to their high antagonistic activity to pathogens, production and availability of extracellular enzymes such as amylases, cellulases, lipases and proteases, and ability to withstand high temperatures (Yu et al., 2009; Banerjee and Ray, 2017). *Bacillus subtilis* is a Gram-positive, non-pathogenic, spore-forming bacterium widely used for oral bacterial therapy, prophylaxis of gastrointestinal disorders, and for its capacity to improved water quality and increased animal survival in aquaculture (Shen et al., 2010; Lee et al., 2017). Although *B. subtilis* is a probiotic widely recognized for its presence in the gastrointestinal tract of animals, this bacterium can be found in the most diverse environments (terrestrial or aquatic) due to the presence of strain-specific genes that enhance its adaptability (Earl et al., 2008). Thus, the objective of the present work was to

isolate *B. subtilis* strains adapted to the gastrointestinal tract of *L. vannamei*, carry out the probiotic characterization of the isolated strains and evaluate their effects when added to the feed regarding zootechnical performance, the proximate composition of muscle, lipid accumulation in hepatopancreas, and expression of genes related to digestion, amino acid metabolism and antioxidant defenses of Pacific white shrimp.

2. MATERIAL AND METHODS

2.1. Isolation of bacteria from the gastrointestinal tract of shrimp

Shrimps of approximately 7 g (n = 30) were kept in 150 L tanks at an average temperature of 29 °C, salinity of 30 $g.L^{-1}$, alkalinity of 150 $mg.L^{-1}$ of $CaCO_3$ and total suspended solids of an average of 500 $mg.L^{-1}$. Before removing the intestines, the shrimp were fasted for two days to clean the gastrointestinal tract. To remove the intestines, the shrimps were finished by hypothermia, sterilized in formaldehyde (50 $mg.L^{-1}$) for five minutes to remove external bacteria and washed with sterile water for one minute to remove the disinfectant (Boonthai et al., 2011). Intestines from four shrimp were pooled to form a sample. In total, four samples were obtained, which were macerated in 0.9 % NaCl. Afterwards, the samples were diluted (1:10; v/v) in 0.9 % NaCl for plating (in duplicate) on LB solid medium (Luria-Bertani). After incubation for 16 h at 35 °C, bacterial colonies were characterized by morphology, and the most frequent morphotypes were isolated for further identification by molecular taxonomy and probiotic characterization.

2.2. Molecular identification

For molecular identification of bacterial colonies isolated from the gastrointestinal tract of shrimp, the gene encoding ribonuclease III (*rnc*) was used, which is considered an essential gene for *B. subtilis* (Herskowitz and Bechhofer, 2000). Although *rnc* is not a gene conventionally used in molecular taxonomy, Condon and Putzer (2002) performed a phylogenetic analysis of several bacterial ribonucleases and concluded that *rnc* is among the most conserved genes in the Eubacteria Kingdom. A previous analysis using the BLAST tool from GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that, although conserved, this gene has variable regions that even allow the taxonomic differentiation of *Bacillus* species (data not shown).

DNA from strains of different morphotypes was extracted by heating, in which bacterial cells were collected from each colony, diluted in 10 µL of ultrapure water and incubated for five minutes at 95 °C. After a brief centrifugation, 1 µL of the supernatant was directly used in a polymerase chain reaction (PCR) to amplify the *rnc* gene, using degenerate primers designed for *Bacillus* species (Table 1). For all PCR reactions, Platinum Taq DNA Polymerase (Invitrogen, Brazil) was used, according to the manufacturer's instructions. The thermocycler was programmed for an initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s (denaturation), 60 °C for 30 s (annealing) and 72 °C for 60 s (extension), with a final extension at 72 °C for 5 min. All PCR products were analyzed in 1 % agarose gel electrophoresis. The amplified fragments were purified using the Quick PCR Purification Kit (Invitrogen, Brazil) and sequenced. The sequences obtained were submitted to GenBank's BLAST tool to obtain the gene identity indexes.

Table 1. Degenerate primers used in PCR reactions to amplify the *rnc* gene from *Bacillus* species.

Primer	Sequences (5'-3')	Amplicon (nt)
RNC3-F1	tgwwtcaagcatttacrcattcatck	573
RNC3-R	attcwcgrttgtgvgcwggkcct	
RNC3-F2	tcatcktatgtgaatgagcatcgraa	548
RNC3-R	attcwcgrttgtgvgcwggkcct	
RNC3-F3	yccgrcwatgagygaaggagakt	414
RNC3-R	attcwcgrttgtgvgcwggkcct	

2.3. Phenotypic characterization of isolated strains

To analyze the phenotypic characteristics, the strains were first cultivated in Tryptone Soy Broth (TSB) for 16 h at 35 °C. Then, the Gram stain technique was used for morphological and structural characterization of the bacterial cell wall, classifying bacteria as Gram-positive or Gram-negative, based on their response to dyes (Tortora et al., 2012).

For analysis of exopolysaccharide (EPS) production, the congo red test was used. For this, the strains were cultivated in solid medium containing 0.8 g of congo red dye for 1 L of Agar Brain Heart Infusion (BHI), with the addition of 36 g of sucrose. The plate was

incubated for a period of 24 h at a temperature of 35 °C and for 48 h at room temperature, following the methodology adapted from Freeman et al. (1989). EPS production is observed when the inoculum turns black on the Congo Red Agar plate.

To test the ability to adhere, the strain was inoculated in TSB medium and kept at a temperature of 35 °C for two days. After the first incubation period, 200 µL of the culture was applied in triplicate in sterile polystyrene microplates containing 96 wells in a "u" shape and incubated again at 35 °C for two days, without shaking. The inocula were then removed and the wells washed with sterile distilled water three times and kept in an oven at 60 °C for one hour. After drying, 200 µL of 1 % crystal violet solution was aliquoted into each well and incubated for one minute, followed by three more washes with distilled water and drying at room temperature, according to the methodology adapted from Christensen et al. (1985). Positive adhesion on polystyrene microplates is observed by the formation of aggregates and purple coloration in the wells after drying.

The bacterial isolate was submitted for screening for extracellular enzymes with potential relevance in digestive processes or virulence factors. Tests were carried out in accordance with established procedures in the literature and inoculated in specific media: caseinase (milk Agar), gelatinase (Tryptone Soy Agar -TSA medium, supplemented with 0.5 % gelatin) and amylase (Nutrient Agar supplemented with 0.1 % soluble starch) (Rodrigues et al., 1993); cellulase (Carboxymethylcellulose Agar-CMC) (Teather and Wood, 1982).

To test the hemolytic activity, the isolated strains were inoculated into a Shrimp Hemolymph Agar medium, composed of 1 mL of *L. vannamei* shrimp hemolymph containing 200 ppm of Rose Bengal, following the methodology proposed by Chang et al. (2000). The hemolytic zone can be seen by the formation of a halo around the inoculum. As a positive control for hemolytic activity, the pathogen *Vibrio parahaemolyticus* IOC 18950 was used.

To assess the response to different antibiotics, the bacterial strain was grown in TSA medium, incubated at 35 °C for 24 h and adjusted to 0.5 McFarland scale in 1 % saline solution using a spectrophotometer, with wavelength reading of 625 nm. One unit on this scale corresponds approximately to a homogeneous suspension of *E. coli* of 1.5 x 10^8 cells per mL. Afterwards, the strain was plated on Muller Hinton Agar (MH) using a sterile swab and commercial discs containing the antibiotics nalidixic acid (30 µg), chloramphenicol (30 µg), florfenicol (30 µg) and oxytetracycline (30 µg) were placed on top of the inoculum. The plate was incubated for 24 h at 35 °C and the diameter of the inhibition halo around the discs was measured with a digital caliper. The results were analyzed according to resistance, moderate

susceptibility or strain susceptibility to antibiotics, according to the methodology proposed by Charteris et al. (1998).

The selected strains were also tested for tolerance to variations in temperature, pH and salinity. As for temperature, the isolated strains were cultivated in LB medium and incubated at 4 °C or 40 °C for a period of up to two days. Tolerance to different temperatures was observed by the capacity of bacterial strains to grow, demonstrated by the turbidity of the medium, according to the protocol adapted from Cai et al. (1999). As for pH, the strains were grown in LB medium adjusted to different pHs (5.0 and 9.0) and incubated for up to two days at 35 °C. Tolerance to different pH ranges was observed by bacterial growth, evidenced by the turbidity of the medium (Cai et al., 1999). For the salinity test, the strains were grown in LB medium adjusted for different salinities (0, 30 and 80 $g.L^{-1}$), and incubated at 35 °C for up to two days. The growth in face of different salinity rates was observed by the turbidity of the culture medium.

The antagonism test against the pathogens *Vibrio harveyi* ATCC 14126 and *V. parahaemolyticus* IOC 18950 was performed using the crossed streak methodology, adapted from Williston et al. (1947). *Vibrio* strains are inoculated in the central streak, while the bacterial strain tested is plated perpendicular to the central streak, with a one-centimeter distance. After incubation for 24 hours at 35 °C, it is evaluated whether there is a positive antagonism (with inhibition of the growth of the central streak) or negative antagonism (with the meeting of the perpendicular streak with the central).

2.4. Feed supplementation with the strain of *B. subtilis* isolated from the gastrointestinal tract of *L. vannamei*

For the supplementation of *L. vannamei* feed, the strain of *B. subtilis* with the best probiotic potential isolated from the gastrointestinal tract of shrimp was chosen. As a control strain, *B. subtilis* KM0 (Altenbuchner, 2016) was used. This strain is derived from the *B. subtilis* 168 strain, widely recognized for its probiotic potential. To prepare the diet containing the probiotics, cultures (3 mL) of the probiotic strains were added to erlenmayers (125 mL) containing 50 mL of minimal salts medium (Anagnostopoulos and Spizizen, 1960) used in the protocols for the transformation of *B. subtilis*, with addition of glucose and casein hydrolyzate as suggested by Riet et al. (2021). Probiotics were grown overnight at 35 °C with orbital shaking at 250 rpm. After cultivation, the strains were standardized to an optical density (OD_{600}) of 1.6. Then, the cultures were centrifuged at 5,000 × g for 10 min at 4 °C. After

centrifugation, the supernatant was discarded and 2 mL of saline solution (NaCl 0.9 %) was added to wash the pellet, repeating this process twice (adapted from Zokaeifar et al., 2012b). Then, the pellets were diluted in saline solution so that each gram of feed received 10^{10} colony forming units (CFU) of probiotics.

2.5. Experiment with *L. vannamei*

Shrimp juveniles were obtained from Aquatec (Rio Grande do Norte, Brazil) and the experiment performed in the Marine Station of Aquaculture from the Federal University of Rio Grande (FURG, Brazil). Shrimps with an average weight of 0.63 ± 0.09 g were stored in tanks with a useful volume of 150 L at a stoking density of 266 shrimps/m^3. The entire experiment was carried out in clear water where the animals were acclimated before starting the experiment, without any addition of commercial probiotics. The physical and chemical parameters of the water were evaluated daily. The temperature was maintained at 26 °C throughout the experimental period. Salinity and dissolved oxygen were maintained at 30 $g.L^{-1}$ and 6 ± 0.5 $mg.L^{-1}$, respectively. Aeration was constantly supplied to each tank through a porous stone using an air blower. In addition, a daily water change (35 %) was performed in order to keep the levels of nitrogen compounds below the minimum levels of tolerance of shrimp.

The experimental design consisted of three treatments (carried out in quadruplicate). The experimental groups were divided into control, which received only commercial feed moistened with a 0.9 % NaCl solution (Feed), treatment with the *B. subtilis* KM0 strain (Feed+KM0) and the group with the isolated shrimp strain (hereinafter referred to as strain E) (Feed+E). Shrimps were fed twice a day with a commercial 38 % crude protein diet (Guabi, Brazil) for 45 days at a rate of 10 % of the estimated biomass at the beginning and reduced to 5 % at the end of the experiment.

The following formulas were used to calculate the zootechnical performance of the shrimp:

- Weight gain (g) = Pf – Pi, where Pf = final weight and Pi = initial weight;
- Biomass gain = (Average Pf x number of individuals at the end) - (Average Pi x initial number of individuals);
- Feed conversion ratio = feed intake/biomass gain;
- Survival (%) = (final shrimp population × 100)/initial population.

2.6. Tissue collection

At the end of the 45-day period, five shrimp were randomly removed from each tank, euthanized and stored at -20 °C to analyze the proximate composition from muscle tissue. In addition, shrimp from each treatment were separated for tissue collection for gene expression analysis. The hepatopancreas and muscle of four shrimps per replica (sixteen per treatment) were dissected and placed individually in 500 μL of Trizol Reagent (Invitrogen, Brazil), according to the manufacturer's protocol. Whole shrimps were also separated for histological analysis.

2.7. Proximate composition analysis

Muscle from five shrimps from each treatment was used for proximate composition analysis. The analysis of the moisture content was made by drying the samples in an oven at 100 °C until constant weight. Protein content was determined by the Kjeldahl method and the total ether extract according to the Soxhlet method (AOAC, 2000). Ashes were obtained by incineration in a muffle for 6 h at 600 °C. Phosphorus analyzes were performed according to Silva and Queiroz (2002).

2.8. Gene expression analysis

RNA extraction was performed with Trizol Reagent (Invitrogen, Brazil) in the proportion of 100 mg of tissue for each 1 mL of reagent. The extracted RNA was treated with DNase I (Invitrogen, Brazil) and the concentration was determined spectrophotometrically and the quality was determined by 1 % agarose gel electrophoresis. For cDNA synthesis, the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Brazil) was used. Gene expression levels were determined by quantitative PCR (qPCR), according to Livak and Schmittgen (2001), with n = 8. Seven genes were analyzed in the hepatopancreas, of which five are related to digestion (amylase, *amy*; lipase, *lip*; trypsin, *tryp*; cathepsin B, *cathB*; chymotrypsin, *chymo*) (Duan et al., 2018; Flores-Miranda et al., 2015), and two related to amino acid metabolism (glutamine synthetase, *gs*; glutamate dehydrogenase, *gdh*) (Lage et al., 2018). In muscle, two genes related to the antioxidant defense system were analyzed (glutathione peroxidase, *gpx*; superoxide dismutase, *sod*) (Sharawy et al., 2020). All primers used in qPCRs are described in Table 2. Prior to gene expression analysis, the efficiency of the primers in serial dilutions was performed. The chosen cDNA dilution was 1:10 and the efficiency and expression reactions were performed with the PowerUP SYBR Green Master

Mix kit (Applied Biosystems, Brazil), following the programming of 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 15 min. The *40S-s24*, *ef1α* and *60S-12*1 reference genes were used for the normalization of gene expression data. To determine the stability of the reference genes, the software geNorm VBA applet for Microsoft Excel (Vandesompele et al., 2002) was used. All reactions were performed in QuantStudio 3 Real-Time PCR (Applied Biosystems, Brazil).

Table 2. Analyzed genes and primers used in qPCR reactions.

Gene	Sense (5'-3')	Antisense (5'-3')	Amplicon (nt)	GenBank
amy	ctctggtagtgctgttggct	tgtcttacgtgggactggaag	116	AJ133526
lip	actgtctcctctgctcgtc	atggtttctggaataggtgttt	131	XM027365317
tryp	cggagagctgccttaccag	tcggggttgttcatgtcctc	141	X86369
chymo	ggctctcttcatcgacg	cgtgagtgaagaagtcgg	182	XM037943862
cathB	ggatgtaacggaggcttc	ctgtatgctttgcctcca	248	XM027359505
gdh	aggttgtggaggaccagttg	ccgtggatcatctcgtaggt	166	EU496492
gs	ttccgtctcctgaaataccg	aggagccttgggaatgaagt	193	JN620540
gpx	agggacttccaccagatg	caacaactccccttcggta	117	AY973252
sod	tggagtgaaaggctctggct	acggaggttcttgtactgaaggt	175	DQ005531
40S-s24	caggccgatcaactgtcc	caatgagagcttgcctttcc	204	XM027373709
60S-l21	gttgacttgaagggcaatg	cttcttggcttcgattctg	246	XM027359925
ef1a	ccaccctggccagattca	gcgaacttgcaggcaatg	75	DQ858921

2.9. Histological analysis

Whole shrimps were injected with Davidson's solution (11.5 % acetic acid, 22 % formalin, 33 % ethanol) and kept in this condition for 48 hours. After this period, hepatopancreas were dissected and transferred to a container containing 70% ethanol. After successive dehydrations in increasing concentrations of ethanol, hepatopancreas were clarified in xylene and embedded in Paraplast at 60 °C. The embedded hepatopancreas were sectioned into 3 μm thick slices. Histological sections were stained with Hematoxylin and Eosin (Bell and Lightner, 1988) to count lipid storage cells. Lipid vacuoles were observed and counted using a compound microscope at 40× magnification.

2.10. Statistical analysis

To test for possible differences between treatments, one-way analysis of variance (ANOVA) was used. Normality and heterogeneity were assessed using the Shapiro-Wilk and Levene tests, respectively. For each case, when significance was detected among treatments, a later comparison of means was performed using Tukey's test. All numerical data were expressed as mean ± standard error. Differences were considered statistically significant when $p < 0.05$.

3. RESULTS

3.1. Isolation and identification of bacterial strains from the gastrointestinal tract of *L. vannamei*

Four strains characterized as Gram-positive were isolated from the gastrointestinal tract of shrimp. The PCR products for the *rnc* gene of these four strains were sequenced and the amplicon obtained from strain E showed 100 % identity with the *B. subtilis rnc* gene sequence available in GenBank (data not shown). Strain E was chosen for the other experiments because it belongs to a species recognized by the GRAS (Generally Recognized as Safe) list of the North American FDA (US Food & Drug Administration; https://www.fda.gov/food/food-ingredients- packaging/generally-recognized-safe-gras).

3.2. Phenotypic characterization of the strain E of *B. subtilis*

Strain E showed positive adherence to the polystyrene microplate but was not able to produce exopolysaccharides (EPS). As for the enzymatic activity, strain E was not able to produce gelatinase and cellulase, but activity was detected for caseinase and amylase. Also, strain E did not show β-hemolysis activity. Stress resistance tests showed that strain E can grow at 40 °C, but not at 4 °C. As for pH, strain E grows at pH 9, but does not grow at pH 5. Regarding salinity, strain E was able to grow in all tested salinities (0, 30 and 80 $g.L^{-1}$). The antibiogram showed that strain E is sensitive to the antibiotics tested (nalidixic acid, chloramphenicol, florfenicol and oxytetracycline). The pathogen antagonism test showed that strain E presents positive antagonism to both *V. harveyi* and *V. parahaemolyticus* (Figure 1).

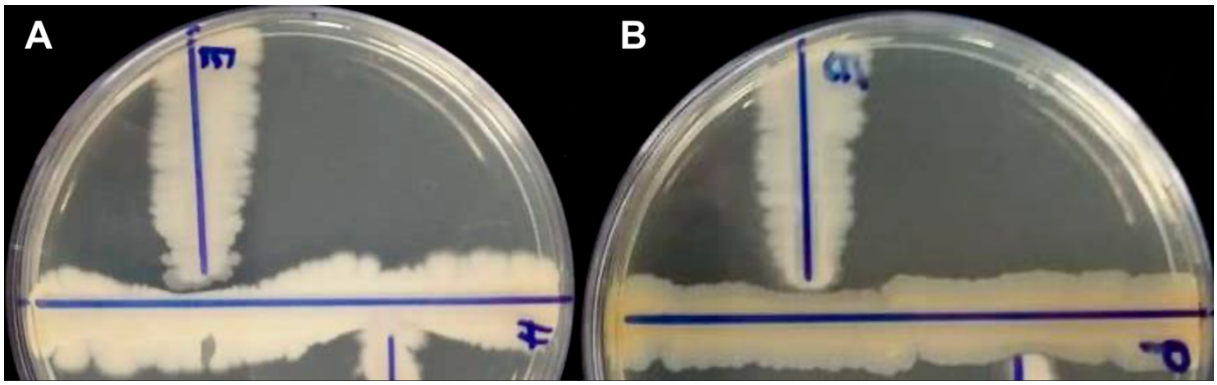

Figure 1. Antagonism test of *Bacillus subtilis* strain E (vertical streak) to *Vibrio harveyi* (horizontal streak in A) and *Vibrio parahaemolyticus* (horizontal streak in B).

3.3. Zootechnical performance and proximate composition of muscle tissue

The results of the zootechnical performance are shown in Table 3. There were no significant differences among the experimental groups for all analyzed variables ($p > 0.05$).

Table 3. Zootechnical performance of *Litopenaeus vannamei* fed with commercial feed or commercial feed supplemented with different *Bacillus subtilis* strains.

	Feed	Feed+KM0	Feed+E
Initial weight (g)	0.61± 0.06	0.62 ± 0.05	0.66 ± 0.14
Final weight (g)	5.44 ± 0.49	5.09 ± 0.45	4.90 ± 0.53
Weight gain (g)	4.84 ± 0.49	4.47 ± 0.42	4.27 ± 0.66
Feed conversion ratio	1.39 ± 0.13	1.50 ± 0.15	1.60 ± 0.24
Survival	100 %	100 %	100 %

As shown in Table 4, no significant differences were detected among the experimental groups regarding body moisture, crude protein, ash and phosphorus ($p > 0.05$). However, shrimp fed the diet supplemented with E strain had significantly higher lipid content compared to shrimp fed the diet supplemented with the KM0 strain ($p < 0.05$).

Table 4. Proximate composition of *Litopenaeus vannamei* muscle fed with commercial feed or commercial feed supplemented with different *Bacillus subtilis* strains.

	Feed	Feed+KM0	Feed+E
Moisture	74.56 ± 0.63	73.97 ± 0.22	74.77 ± 0.82
Ash	1.63 ± 0.27	1.53 ± 0.23	1.54 ± 0.39
Protein	18.97 ± 1.74	19.26 ± 0.44	18,86 ± 0.87
Lipid	0.75 ± 0.65^{ab}	0.47 ± 0.43^{b}	1.22 ± 1.56^{a}
Phosphorus	79.24 ± 9.03	98 ± 1.41	102.86 ± 5.79

Different letters represent statistically significant differences ($p < 0.05$).

3.4. Relative expression of genes related to digestion

The relative expression of *cathB*, *chymo*, *tryp*, *lip* and *amy* genes is shown in Figure 2. Only *amy* was not altered with the addition of *B. subtilis* strains to the diet. The *chymo*, *tryp* and *lip* genes had their expression significantly reduced ($p < 0.05$) in both Feed+KM0 and Feed+E treatments. The *cathB* gene had its expression reduced only in the Feed+E treatment ($p < 0.05$) in comparison with Feed+KM0 treatment.

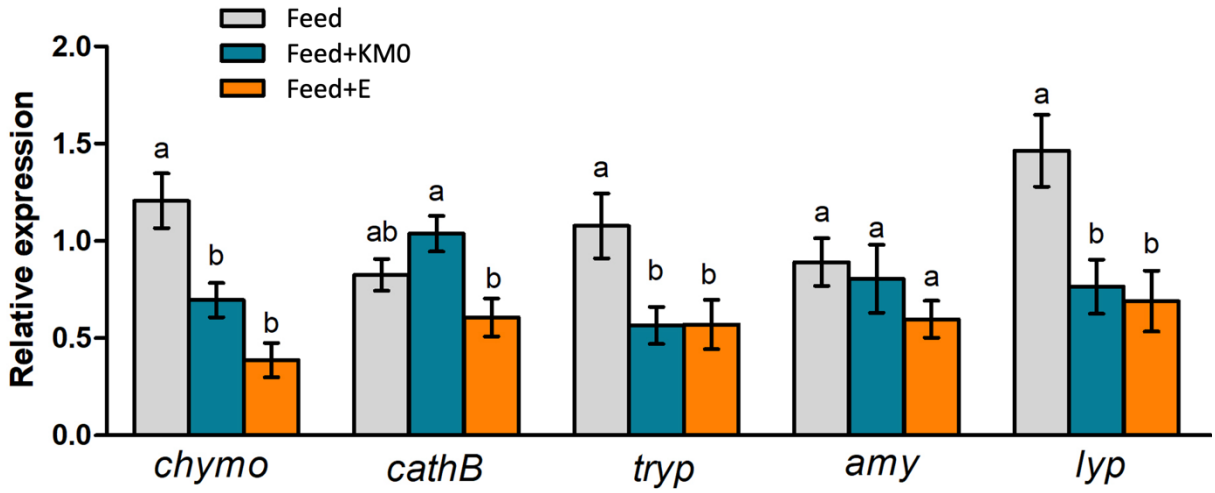

Figure 2. Relative expression of genes related to digestion in *Litopenaeus vannamei* hepatopancreas fed diets supplemented with different strains of *Bacillus subtilis*. Feed: shrimp fed only commercial feed; Feed+KM0: shrimp fed commercial feed with *B. subtilis* KM0; Feed+E: shrimp fed commercial feed with *B. subtilis* strain E. One-way ANOVA was used for each gene separately. Different letters represent statistically significant differences ($p < 0.05$).

3.5. Relative expression of genes related to amino acid metabolism

The expression of *gs* and *gdh* are shown in Figure 3. The *gdh* gene had its expression reduced in both treatments with *B. subtilis* strains ($p < 0.05$), while *gs* was reduced only in the Feed+E treatment ($p < 0.05$).

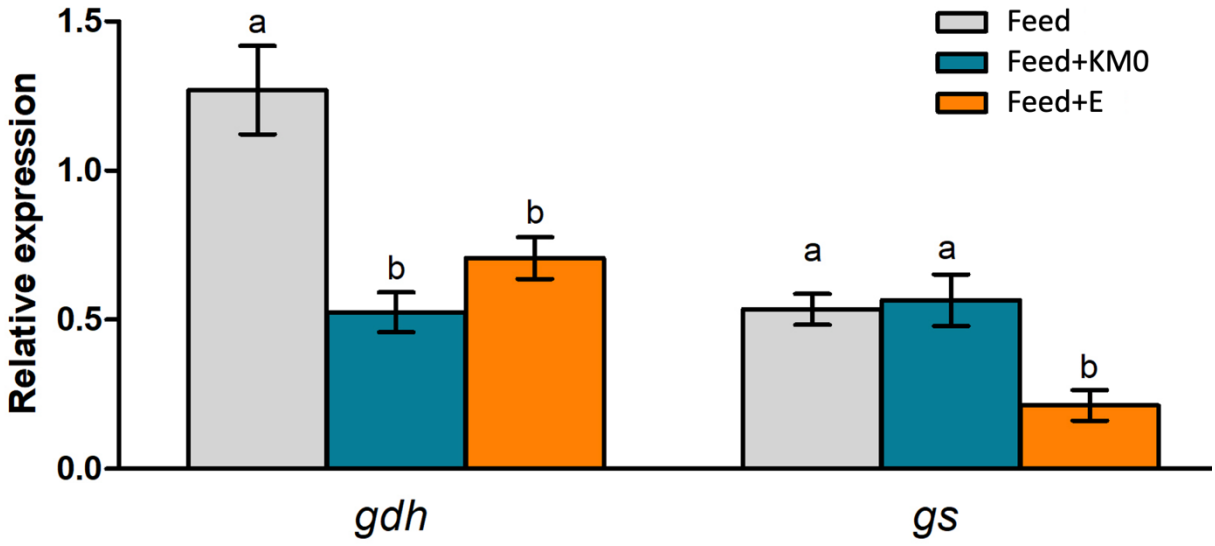

Figure 3. Relative expression of genes related to amino acid metabolism in *Litopenaeus vannamei* hepatopancreas fed diets supplemented with different strains of *Bacillus subtilis*. Feed: shrimp fed only commercial feed; Feed+KM0: shrimp fed commercial feed with *B. subtilis* KM0; Feed+E: shrimp fed commercial feed with *B. subtilis* strain E. One-way ANOVA was used for each gene separately. Different letters represent statistically significant differences ($p < 0.05$).

3.6. Relative expression of genes related to antioxidant defenses

The expression of *gpx* and *sod* are shown in Figure 4. The treatments with the strains of *B. subtilis* did not produce alterations in the two analyzed genes ($p > 0.05$).

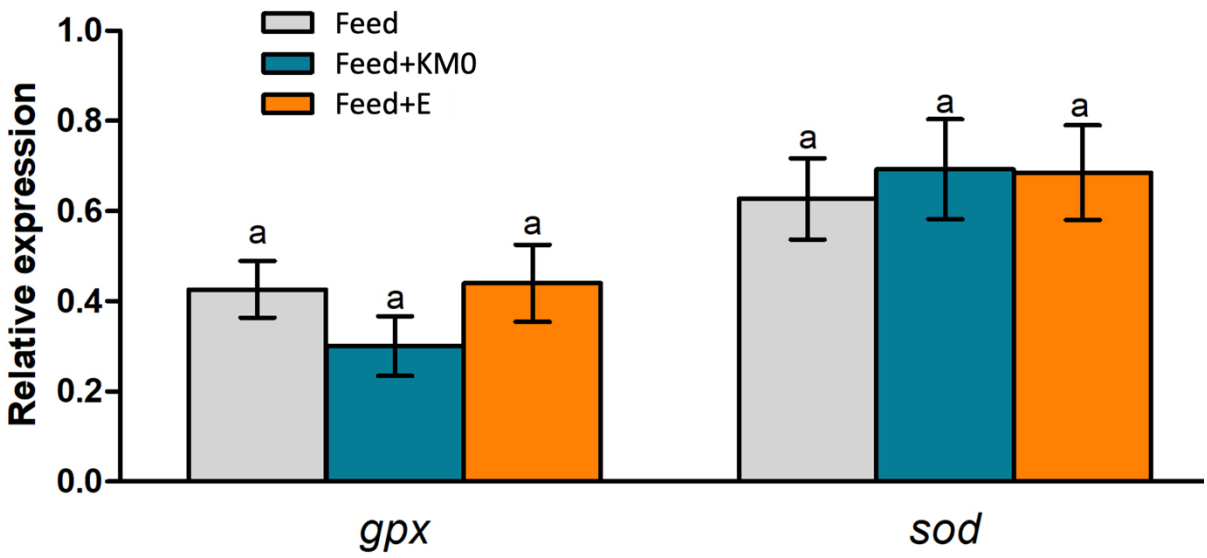

Figure 4. Relative expression of genes related to antioxidant defenses in *Litopenaeus vannamei* muscle fed diets supplemented with different strains of *Bacillus subtilis*. Feed: shrimp fed only commercial feed; Feed+KM0: shrimp fed commercial feed with *B. subtilis* KM0; Feed+E: shrimp fed commercial feed with *B. subtilis* strain E. One-way ANOVA was used for each gene separately. Equal letters represent the absence of statistical differences ($p > 0.05$).

3.7. Histology of the hepatopancreas

The result of lipid vacuole counts in shrimp hepatopancreas is shown in Figure 5. Shrimp fed with diet supplemented with both *B. subtilis* KM0 and *B. subtilis* strain E had a higher number of lipid vacuoles (31.55 ± 0.74 and 32.68 ± 1.89, respectively) compared to the control treatment (Feed; 29.28 ± 1.13).

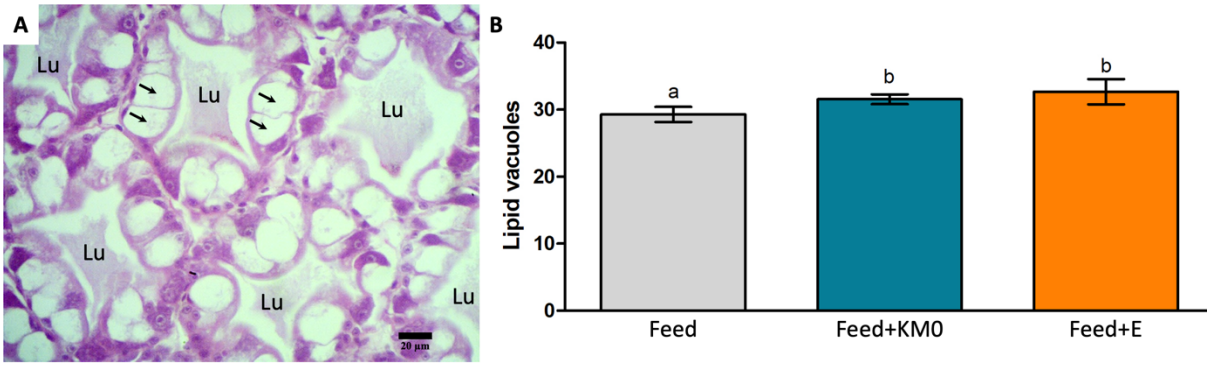

Figure 5. Histological analysis of *Litopenaeus vannamei* hepatopancreas. A) Illustrative photo of the histological sections of shrimp hepatopancreas (40× magnification). Lu: lumen of the hepatopancreatic tubules; arrows indicate some examples of lipid vacuoles; B) Graphic

representation of lipid vacuole counts. Feed: shrimp fed only commercial feed; Feed+KM0: shrimp fed commercial feed with *Bacillus subtilis* KM0; Feed+E: shrimp fed commercial feed with *B. subtilis* strain E. Data are expressed as mean ± standard error from four independent replicates. Different letters represent statistically significant differences (One way ANOVA; $p < 0.05$).

4. DISCUSSION

In the first stage of this study, a bacterial strain with probiotic potential was isolated from the gastrointestinal tract of shrimp *L. vannamei*. This strain, called strain E, was molecularly identified as a strain of *B. subtilis*, a species recognized as GRAS by the US FDA. Considering that probiotic bacteria tend to adapt to specific environments due to the presence of genes related to adaptability, it can be concluded that probiotics applied in shrimp farming isolated from shrimp itself can have a greater impact on the benefits already known to be produced by such bacteria. In fact, the isolation of probiotics from shrimp has been shown to be a valid practice for shrimp farming, enhancing the control or inhibition of pathogenic bacteria, zootechnical performance, digestive enzyme activity and host immune responses against pathogens or physical stress (Li et al., 2021; Kim et al., 2020; Wang et al., 2020; Zuo et al., 2019).

Strain E was phenotypically characterized in terms of its probiotic potential. First, it is necessary to assess the presence of certain virulence factors in candidate probiotic strains. One of these is gelatinase activity. This enzyme is an extracellular endopeptidase that promotes the hydrolysis of bioactive compounds such as gelatin, collagen and hemoglobin, being considered a harmful enzymatic activity in strains intended for use in aquaculture (Muñoz-Atienza et al. 2013). Strain E did not show gelatinase activity, which is a positive characteristic of this strain. Another important virulence factor is the β-hemolysis activity, which makes iron available to the microorganism and can cause anemia and edema in the case of a vertebrate host (Vesterlund et al., 2007). Strain E also did not show β-hemolysis activity, which is another positive factor to be considered. In addition to the virulence factors that need to be absent, the probiotic strain must also have additional characteristics, such as the expression of extracellular enzymes and adhesion to surfaces. The presence of enzymes such as caseinase and amylase, for example, is a positive factor for probiotic candidates, as it makes nutrients bioavailable, aiding in the digestive processes of the hosts (Banerjee and Ray,

2017). Strain E showed both caseinase and amylase activity, making it a candidate for a probiotic that can help in the digestive processes of shrimp.

Although strain E does not produce exopolysaccharides (EPS), it was able to adhere to the polystyrene plate. Adhesiveness is an important feature in probiotic candidates, as it is well established that bacteria capable of colonizing the surface of the intestinal mucosa are more crucial in maintaining nutrition, physiology and animal immunity than are free-living bacteria (Banerjee and Ray, 2017). Bacterial adhesion can occur non-specifically, when based on physicochemical factors, or specifically, involving adhesin molecules on the surface of adherent bacteria and receptor molecules on epithelial cells (Salminen et al., 1996). Furthermore, the adherence to the intestinal tract of hosts by probiotic bacteria creates a competitive exclusion, preventing the establishment of pathogenic bacteria and reducing their harmful effects (Vieira and Pereira, 2016).

The stability of bacterial strains is an important factor for survival in a culture environment and for colonizing the host's gastrointestinal tract. *Bacillus* species are known to withstand high temperatures and drying processes, which makes them widely used as probiotics in shrimp feed (Yu et al., 2009). Stress tolerance tests showed that strain E can grow at high temperature (40 °C), as well as at basic pH and in a wide range of salinity (from 0 to 80 $g.L^{-1}$). Additionally, this strain was sensitive to different antibiotics, which demonstrates the absence of resistance genes. This feature is important because it reduces the possibility of the occurrence of horizontal transfer of genes related to antimicrobial resistance to other microorganisms present in the environment. Also, strain E showed positive antagonism against *V. harveyi* and *V. parahaemolyticus*. *Vibrio* species are widely distributed in marine and estuarine environments and can act as agents of food-borne diseases, impacting public health (Silveira et al., 2016). In aquaculture, *V. harveyi* and *V. parahaemolyticus* have been considered as opportunistic pathogens, which can affect a range of marine species, causing high mortality in crops and may even affect the health of consumers (Cheng et al., 2010; Zokaeifar et al., 2012a; Silveira et al., 2016). Hostins et al. (2017) showed that the addition to water of a mixture of commercial probiotics containing *Bacillus* species decreased the abundance of *Vibrio* sp. in the gastrointestinal tract of *L. vannamei* raised in biofloc (BFT) or clear water. Thus, strain E can be considered an alternative tool for combating and preventing vibriosis that commonly affect shrimp farms.

In the second stage of this study, an experiment was carried out with *L. vannamei*, where parameters such as proximate composition of muscle tissue, zootechnical performance,

lipid accumulation in hepatopancreas and expression of genes related to digestion and antioxidant defenses were evaluated. The experiment evaluated three experimental groups for 45 days. The first group of shrimps was fed only commercial feed. The second group was fed a commercial feed supplemented with the KM0 strain, originated from *B. subtilis* 168 and considered as a positive control. The third group was fed with feed supplemented with strain E. After 45 days, no difference was observed between the experimental groups in terms of zootechnical performance. Thus, it can be inferred that strain E does not affect shrimp growth as strain KM0, already recognized for its probiotic potential.

The analysis of the proximate composition of the muscular tissue of the shrimps in group E showed a significant increase of 2.6 times in the amount of lipids, when compared to the KM0 group. Likewise, the count of lipid vacuoles in the hepatopancreas showed that both strains of *B. subtilis* used in the present study increased the concentration of lipids in that tissue. Although, in the present study, a test of lipolytic activity of strain E was not performed, it is known that *B. subtilis* secretes two types of lipases (*lipA* and *lipB*), whose genes are differentially expressed depending on the growth conditions (Eggert et al., 2003). The observation that shrimp fed the diet supplemented with E strain is favoring the concentration of lipids in both muscle and hepatopancreas may be an interesting feature from a commercial point of view, since shrimps have been considered an important source of functional lipids, especially phospholipids (Sun et al., 2020). Interestingly, Tsai et al. (2019) reported that *Bacillus subtilis* E20 increased apparent digestibility coefficients of *L. vannamei*, showing that supplementation with the probiotic enhanced the absorption of nutrients with consequent increase in growth performance. However, the authors did not observe an increase in the concentration of lipids in the shrimp tissues. This observation shows that each probiotic strain can generate specific effects on its hosts.

To evaluate the response of the shrimp to the addition of *B. subtilis* strains in the diet, the expression of genes related to digestion, amino acid metabolism, and antioxidant defenses were quantified. Relative expression of genes related to antioxidant defenses (*gpx* and *sod*) were not altered by treatments with probiotics. However, the digestion-related genes showed an almost homogeneous expression pattern of decreased transcriptional activity. Except for *amy*, which was not altered by the treatments, all the others had their expression reduced with the addition of probiotics in the shrimp diet. The *chymo*, *tryp* and *lip* genes were significantly downregulated in both probiotic treatments. As previously mentioned, *Bacillus* species are capable of producing and secreting lipases. Likewise, these bacteria can also secrete high

amounts of proteolytic enzymes. According to Contesini et al. (2018), bacteria of the genus *Bacillus* are among those that most produce and secrete proteases with outstanding properties such as high stability in adverse environmental conditions such as extremes of temperature and pH, being resistant to the presence of organic solvents, detergents, and oxidizing agents. In a scenario where probiotic bacteria produce and secrete significant amounts of proteases and lipases, it is expected that the host intestine will decrease the production and secretion of such enzymes, starting with a decrease in the transcription rate of endogenous related genes. An interesting difference between the strain KM0 and the strain E was observed in the expression of the *cathB* gene, which encodes Cathepsin B. This cysteine protease was first identified in *L. vannamei* by Stephens et al. (2012). It is an enzyme that participates not only in the intracellular hydrolysis of proteins, but also in the extracellular hydrolysis of proteins soon after food ingestion, increasing the capacity of amino acid absorption by the intestine. The fact that the *cathB* gene was significantly reduced in shrimp treated with strain E in relation to strain KM0 indicates an interesting difference between the two probiotic strains. Although there is a lot of information about the production of proteases in *Bacillus* species, the opposite occurs when it comes to a more specific group such as cysteine proteases. Recently, Yamazawa et al. (2022) identified the *yabG* gene product of *B. subtilis* as a cysteine peptidase, which is related to spore formation. Thus, *B. subtilis* is also capable of producing and secreting cysteine proteases, and strain E seems to differ from strain KM0 in this specific characteristic.

Regarding genes related to amino acid metabolism, the *gdh* gene had its transcription rate downregulated in shrimp hepatopancreas in both treatments with probiotics. The enzyme glutamate dehydrogenase is present in the mitochondrial matrix of eukaryotic cells and catalyzes the oxidative deamination of glutamate to form α-ketoglutarate which can be used as fuel for the Krebs cycle to drive the electron transport chain and production of ATP by oxidative phosphorylation (Dawson and Storey, 2012). Furthermore, this enzyme is linked to several cellular processes, including ammonia metabolism, acid-base balance, redox homeostasis, lactate production, and lipid biosynthesis via oxidative generation of citrate (Plaitakis et al., 2017). Considering that glutamate dehydrogenase induces the production of Krebs cycle intermediates, and that one of these intermediates (citrate) is used for the synthesis of lipids, it is reasonable to hypothesize that the decrease in the transcription of the *gdh* gene is a response to an already high concentration of lipids in the hepatopancreas of shrimp that were treated with the probiotic strains. Another gene related to amino acid

metabolism analyzed in the present study was *gs*. This gene encodes the enzyme glutamine synthetase, which catalyzes the formation of glutamine from glutamate and NH_4^+. Qiu et al. (2018) considered this enzyme as an important marker of ammonia stress in *L. vannamei*. These authors demonstrated that expression of *gs* is increased in shrimp exposed to high concentrations of ammonium and that hepatopancreas plays a key role in the response to stress caused by excess nitrogen. In the present study a significant decrease in *gs* expression was observed only in the hepatopancreas of shrimp treated with strain E, and this is another interesting difference between the two probiotic strains studied here. It is possible that the strain E has a greater capacity than strain KM0 to reduce the nitrogen compounds present in the rearing water and, thus, reduce the stress in shrimp. In fact, it is well established that the administration of *B. subtilis* strains confers benefits in maintaining the quality of rearing water in aquaculture (for review see Hlordzi et al., 2020).

In conclusion, in the present study, a strain of *B. subtilis* (strain E) was isolated from the gastrointestinal tract of *L. vannamei*, which has several phenotypic characteristics that characterize it as a potential probiotic. In the functional experiment with shrimp, the supplementation of the feed with strain E did not change the zootechnical performance of the shrimp but increased the lipid concentration in muscle and hepatopancreas. Additionally, exposure to the strain E strongly decreased the expression of genes related to digestion and amino acid metabolism, suggesting that this potential probiotic can facilitate digestive processes and nutrient absorption as well as minimize the toxic effects caused by nitrogenous compounds present in rearing water. Thus, strain E can be considered an interesting tool in shrimp farming in terms of better use of nutrients present in the feed, as well as in the maintenance of water quality that can negatively impact shrimp farming.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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CAPÍTULO II

Recombinant *Bacillus subtilis* expressing a fungal phytase as a probiotic additive in the diet of Pacific white shrimp *Litopenaeus vannamei*

Manuscrito submetido ao periódico Aquaculture (FI = 5,135)

Recombinant *Bacillus subtilis* expressing a fungal phytase as a probiotic additive in the diet of Pacific white shrimp *Litopenaeus vannamei*

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Abstract

Phosphorus is an essential mineral for all living beings and one of the most expensive additives in the formulation of feed for aquaculture. This mineral is present in the vegetable matter in the form of phytic acid (phytate), which is considered an antinutritional factor. Phytate can form complexes with proteins, lipids and minerals, decreasing the digestibility of these biomolecules. This compound can be degraded by phytases, which have been used in commercial feeds to mitigate the negative effects of phytate. However, these enzymes undergo costly isolation and purification processes. In the present study, a genetically modified *Bacillus subtilis* strain that expresses a fungal phytase was used as a feed additive. The probiotic was added to the commercial feed of shrimp *Litopenaeus vannamei* and its effects on zootechnical performance, proximate composition of muscle, lipid concentration in hepatopancreas and expression of genes related to digestion, amino acid metabolism and antioxidant defenses were analyzed. Although the genetically modified probiotic had no impact on growth parameters, there was a 39% increase in phosphorus content in muscle. In addition, genes related to digestion were downregulated in shrimp hepatopancreas, as well as an increase in lipids in this tissue. These results demonstrates that the genetically modified probiotic increased the efficiency of the use of plant-derived phosphorus, which may imply a decrease in the addition of this element in the diets, as well as minimizing the impact of shrimp farms on the eutrophication of adjacent ecosystems.

Keywords: Phosphorous, genetically modified probiotic, feed additive, shrimp farming.

Introduction

The growth of the aquaculture industry has generated a significant increase in the demand for fish meal and oil, essential components in the production of animal feed. In 2018, around 12% of world fish production was used to produce these inputs and this growing demand has generated price increases and greater pressure on fish stocks (FAO 2020). In a scenario where extractive fishing is experiencing stagnant growth, alternative sources of protein need to be established to sustain the demand arising from the growth of aquaculture, whether of animal or vegetable origin. In the case of vegetable proteins, there is an additional challenge due to the presence of anti-nutritional compounds that prevent their use at higher concentrations (Hardy 2010; Montoya-Camacho et al. 2019). Even so, vegetables such as rapeseed, lupine and even fermented cotton have been used to replace fish meal in fish and shrimp diets (Kaiser et al. 2021; Sun et al. 2016; Weiss et al. 2020).

Among the antinutritional compounds present in plant matter, phytic acid (or phytate) is one of the most limiting and represents the largest form of phosphorus storage in plants (Dersjant-Li et al. 2015). Its antinutritional characteristic comes from the electronegativity of phosphorus, which allows it to chelate and decrease the availability of minerals such as calcium, magnesium, zinc, manganese, iron and copper (Hardy 2010; Bharadwaj et al. 2014; Humer and Zebeli 2015). Phytate can also interact with proteins, carbohydrates and lipids, forming complexes that reduce the digestibility of these biomolecules with a consequent decrease in nutrient absorption by the gastrointestinal tract (Kumar et al. 2012; Dersjant-Li et al. 2015). The formation of these complexes also makes the phosphorus present in plant matter unavailable to most monogastric animals, since these animals do not produce or produce few enzymes capable of hydrolyzing phytate during the digestive process (Kumar et al. 2012; Dersjant-Li et al. al. 2015). Thus, most of the plant phosphorus is excreted and ends up eutrophicating the environment which, in the case of the aquatic environment, causes the proliferation of microalgae with damaging effects to adjacent ecosystems (Kumar et al. 2012; Hung et al. 2015).

Phytate removal or degradation increases the bioavailability of many cations, and consequently, improve the nutritional value of the feed. Phytase is a phytate-specific phosphatase that catalyzes the hydrolysis of phytic acid generating inositol and phosphates (Bhavsar and Khire 2014; Lemos and Tacon 2017). The objective of phytase supplementation in diets is to hydrolyze dietary phytate into absorbable phosphorus forms. This information

has been well documented in several studies with fish and shrimp, showing that the use of phytase in the diet improves growth, efficiency in absorption of nitrogen and phosphorus, macronutrient digestibility, and amino acid bioavailability (Biswas et al. 2007; Green et al. 2021; Maas et al. 2021; Qiu and Davis 2017; Rachmawati and Samidjan 2016; Shahzad et al. 2021).

Among the commercially available phytases, those belonging to the class of histidine acid phosphatases and produced by the filamentous fungus *Aspergillus fumigatus* are characterized for their high thermostability and activity over a wide pH range (Singh and Satyanarayana 2015; Rebello et al. 2017). Additionally, bacterial phytases produced by *Bacillus* species can be considered interesting alternatives to fungal phytases not only for their thermostability, but also for their resistance to protease action and greater phytate specificity (Kim et al. 1998; Oh et al. 2004). Cheng et al. (2012) expressed *B. subtilis* phytase C in *Escherichia coli* and used the purified recombinant enzyme as an additive in *L. vannamei* feed. The addition of the recombinant enzyme to a diet high in soybean meal (40%) for shrimp increased growth and feed efficiency. However, feed additives with commercial phytases have their cost significantly increased, as these enzymes need to go through costly isolation and purification processes (Dudley et al. 2014).

An interesting alternative to reduce the cost of phytase additives in commercial feeds is to use genetic engineering techniques to transform a probiotic bacterium into a biofactory of heterologous enzymes. In this scenario, the genetically modified probiotic can be added to the feed, and the secreted heterologous phytase can degrade the phytate present in plant matter, increasing the digestibility of the feed. Recently, Santos et al. (2020) evaluated the effect of adding a genetically modified strain of *B. subtilis* capable of secreting a fungal phytase in zebrafish (*Danio rerio*) feed. The authors used a feed rich in soybean meal and observed that the addition of the genetically modified probiotic improved the fish condition factor, as well as stimulating the immune system, decreasing inflammatory responses and oxidative stress in gastrointestinal tract. In the present study, the genetically modified strain of *B. subtilis* developed by Santos et al. (2020) was used as an additive in the feed of shrimp *L. vannamei*, evaluating the zootechnical performance, proximate composition of muscle, lipid vacuoles counting in hepatopancreas, and expression of genes related to digestion, amino acid metabolism and antioxidant defenses.

Material and methods

Preparation of feed supplemented with probiotic bacteria

The strains of *B. subtilis* used in this study are the same ones used by Santos et al. (2020). The KM0 strain of *B. subtilis* was used as a control, which is derived from the *B. subtilis* 168 strain and had its genome modified to generate a super-competent strain for genetic manipulation (Rahmer et al. 2015). The KM0-Phy strain was manipulated by Santos et al. (2020) to express and secrete *A. fumigatus* phytase. Inoculums of the probiotic strains were first cultivated for 16 h at 37 ºC in volumes of 3 mL in minimal salts medium (Anagnostopoulos and Spizizen 1960), in a shaker incubator at 250 rpm. The next day, the cultures were added to erlenmayers (125 ml) containing 50 ml of the culture medium suggested by Riet et al. (2021), which consists of a minimal salts medium with glucose and casein hydrolyzate. The cultures followed the same protocol used for culture of inocula, but the concentration of bacteria was monitored in a spectrophotometer until reaching the stationary phase (OD_{600} = 1.6). According to Santos et al. (2020), the genetic construct used to express fungal phytase has maximum yield in the stationary phase of bacterial growth. Then, cultures were centrifuged at 5,000 × g for 10 min at 4 ºC. After centrifugation, the supernatant was discarded and 2 mL of saline solution (0.9%) NaCl was added to wash the pellet, and this process was repeated twice (adapted from Zokaeifar et al. 2012). Then, the pellet was diluted in saline solution so that each gram of feed received 10^{10} colony-forming units (CFU) of the probiotic strains (Riet et al. 2021). The commercial feed used (Guabi, Brazil) is free of probiotics and contains 35 % crude protein, 9 % lipids, 15 % ash and 1.5 % total phosphorous.

Experimental design

Juveniles of *L. vannamei* with an average weight of 0.6 ± 0.1 g were purchased from Aquatec (Rio Grande do Norte, Brazil) and the experiment was carried out at the Marine Aquaculture Station of the Federal University of Rio Grande (FURG, Brazil). Shrimps were acclimated for a week in 150 L tanks with clear water. Throughout the experiment, salinity was maintained at 30 g/L, temperature at 29 °C, and oxygenation close to saturation (6 ± 0.5 mg/L). Every day, 30% of the water was changed in order to keep ammonia, nitrites and nitrates within tolerance levels for the species. Three treatments, with four replications each, were used, in a total of 12 tanks. Each tank received 40 shrimps, totaling 480 individuals (density of 266 shrimp/m^3). In the control treatment (CON), the shrimps received only

commercial feed moistened with saline solution (NaCl 0.9%). In the second treatment (KM0), the shrimps received commercial feed added with *B. subtilis* KM0. In the third treatment (KM0-Phy), the shrimps received commercial feed supplemented with genetically modified *B. subtilis* expressing fungal phytase. The diets were administered twice a day (early in the morning and early in the afternoon) for 45 days, at a rate of 10% of estimated biomass at the beginning and reduced to 5% at the end of the experiment.

Tissue collection

The experimental period lasted 45 days. At the end of this period, shrimps from each treatment were randomly separated for analysis of proximate composition of muscle tissue (five individuals per treatment, immediately frozen at -20 °C) and gene expression (seven individuals per treatment). For gene expression analysis, hepatopancreas and muscle tissue were individually dissected and placed in 500 μL of Trizol Reagent (Invitrogen, Brazil), according to the manufacturer's protocol. Whole shrimps were also separated for histological analysis.

Zootechnical performance

To calculate weight gain, biomass gain, feed conversion ratio and survival, the following formulas were used:

- Weight gain (g) = Pf - Pi, where Pf = final weight and Pi = initial weight;
- Biomass gain = (Average Pf x number of individuals at the end) - (Average Pi x initial number of individuals);
- Feed conversion rate = feed consumption / biomass gain;
- Survival (%) = (final shrimp population × 100) / initial population.

Proximate composition

Proximate composition analysis was performed from shrimp muscle (n = 5). To assess tissue moisture, the samples were dried in an oven at 100 ^{o}C until constant weight. Protein levels were obtained using the Kjeldahl method and the total ether extract using the Soxhlet method, following the instructions of AOAC (2000). Ashes were quantified by muffle incineration for 6 h at 600 ^{o}C. To determine the phosphorus content, the method proposed by Silva and Queiroz (2002) was used, expressed in mg/100 g of fresh sample.

Gene expression analyses

Gene expression analyses were performed using quantitative PCR (qPCR), according to Livak and Schmittgen (2001). First, total RNA was extracted from tissues using Trizol reagent (Invitrogen, Brazil), as per manufacturer's instructions. The RNA obtained was treated with DNAse I, quantified spectrophotometrically, and the RNA quality was verified in 1% agarose gel electrophoresis. RNA was reverse transcribed into complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Brazil), as per the manufacturer's instructions. For each tissue a different set of genes was analyzed. In the hepatopancreas, genes related to digestion (amylase, *amy*; lipase, *li*p; trypsin, *tryp*; cathepsin B, *cathB*; chymotrypsin, *chymo*) and amino acid metabolism (glutamine synthetase, *gs*; glutamate dehydrogenase, *gdh*) were analyzed. In muscle, two genes related to the antioxidant defense system (glutathione peroxidase, *gpx*; superoxide dismutase, *sod*) were analyzed. The primers used in the qPCR reactions are shown in Table 1. Previously, all primers had their efficiencies calculated from serial dilutions. For all analyses, a 1:10 cDNA dilution was used. All reactions were performed on the 7300 Real-time PCR System platform (Applied Biosystems, Brazil), with the PowerUP SYBR Green Master Mix kit (Applied Biosystems, Brazil). Reactions were performed with the following schedule: 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 15 s. The reference gene *ef1a* was used to normalize results of gene expression data in all tissues analyzed. In addition, *60S-121* and *rps3A* genes were used to normalize expression data in hepatopancreas and muscle, respectively. To determine the stability of the reference genes, the geNorm VBA applet software for Microsoft Excel (Vandesompele et al. 2002) was used.

Table 1. Genes and primer sequences used in qPCR reactions.

Gene	Sense (5'-3')	Antisense (5'-3')	Amplicon (nt)	GenBank
amy	ctctggtagtgctgttggct	tgtcttacgtgggactggaag	116	AJ133526
lip	actgtctcctctgctcgtc	atggtttctggaataggtgttt	131	XM027365317
tryp	cggagagctgccttaccag	tcggggttgttcatgtcctc	141	X86369
chymo	ggctctcttcatcgacg	cgtgagtgaagaagtcgg	182	XM037943862
cathB	ggatgtaacggaggcttc	ctgtatgctttgcctcca	248	XM027359505
gdh	aggttgtggaggaccagttg	ccgtggatcatctcgtaggt	166	EU496492
gs	ttccgtctcctgaaataccg	aggagccttgggaatgaagt	193	JN620540
gpx	agggacttccaccagatg	caacaactccccttcggta	117	AY973252

sod	tggagtgaaaggctctggct	acggaggttcttgtactgaaggt	175	DQ005531
rps3A	ggcttgctatggtgtgctcc	tcatgctcttggctcgctg	101	XM027376915
60S-l21	gttgacttgaagggcaatg	cttcttggcttcgattctg	246	XM027359925
ef1a	ccaccctggccagattca	gcgaacttgcaggcaatg	75	DQ858921

Histological analysis

Whole shrimps were prepared for histological analysis by injection into tissues and complete submersion in Davidson's solution (11.5% acetic acid, 22% formalin, 33% ethanol) for 48 hours. Afterwards, the shrimp were removed from the fixative solution and had the hepatopancreas dissected. The tissue obtained was first placed in 70% ethanol and subjected to successive dehydration in increasing concentrations of ethanol. After this process, the hepatopancreas was clarified in xylene, embedded in Paraplast at 60 ^{o}C and sectioned into 3 µm thick slices. After staining with Hematoxylin and Eosin (Bell and Lightner, 1988), lipid storage cells were counted under a compound microscope at 40x magnification. Lipid vacuoles were counted from 10 random visual fields from each hepatopancreas sample. Samples from four individuals from each treatment were analyzed.

Statistical analysis

To test for possible differences among treatments, one-way analysis of variance (ANOVA) was used. Normality and heterogeneity were assessed using the Shapiro-Wilk and Levene tests, respectively. For each case, when significance was detected among treatments, a subsequent comparison of means was performed using the Tukey test. All numerical data from gene expression were expressed as mean ± standard error. The other data were expressed as mean ± standard deviation. Differences were considered statistically significant when $p < 0.05$.

Results

Zootechnical performance

No significant differences were observed between treatments for any of the analyzed parameters. The results are shown in Table 2.

Table 2. Zootechnical performance of *L. vannamei* fed on commercial feed (CON), commercial feed supplemented with KM0 or KM0-Phy strains of *B. subtilis*.

	Treatments		
	CON	KM0	KM0-Phy
Initial weight (g)	0.61± 0.06	0.62 ± 0.05	0.66 ± 0.06
Final weight (g)	5.44 ± 0.49	5.09 ± 0.45	5.63 ± 0.43
Weight gain (g)	4.84 ± 0.49	4.47 ± 0.42	4.98 ± 0.43
Feed conversion ratio	1.36 ± 0.13	1.46 ± 0.15	1.31 ± 0.11
Survival (%)	100	100	100

Proximate composition of muscle tissue

Among the parameters analyzed for proximate composition of *L. vannamei* muscle, there was a significant decrease in the percentage of ash of shrimp that received commercial feed supplemented with probiotic strains (KM0 or KM0-Phy). Also, the phosphorus retention in shrimp muscle was higher in KM0-Phy than the control treatment (CON). No changes were detected in the other analyzed parameters. The results are shown in Table 3.

Table 3. Proximate composition (%) of *L. vannamei* muscle fed commercial feed (CON), commercial feed supplemented with KM0 or KM0-Phy strains of *B. subtilis*.

	Treatments		
	CON	KM0	KM0-Phy
Moisture (%)	74.56 ± 0.63	73.97 ± 0.22	73.91 ± 0.81
Ash (%)	1.63 ± 0.27[a]	1.53 ± 0.23[b]	1.45 ± 0.27[b]

Protein (%)	18.97 ± 1.74	19.26 ± 0.44	19.28 ± 0.08
Lipid (%)	0.75 ± 0.65	0.47 ± 0.43	0.68 ± 0.55
Phosphorus (mg/100 mg)	79.24 ± 9.03[a]	98.92 ± 1.41[a,b]	110.02 ± 14.13[b]

Different letters represent statistically significant differences ($p < 0.05$).

Expression of genes related to digestion

Of five genes analyzed related to digestion (Figure 1), only *cathB* and *amy* were not significantly altered in shrimp fed with feed supplemented with both KM0 and KM0-Phy strains. The other genes (*chymo*, *tryp* and *lip*) were significatively downregulated by both probiotic treatments, when compared to control group.

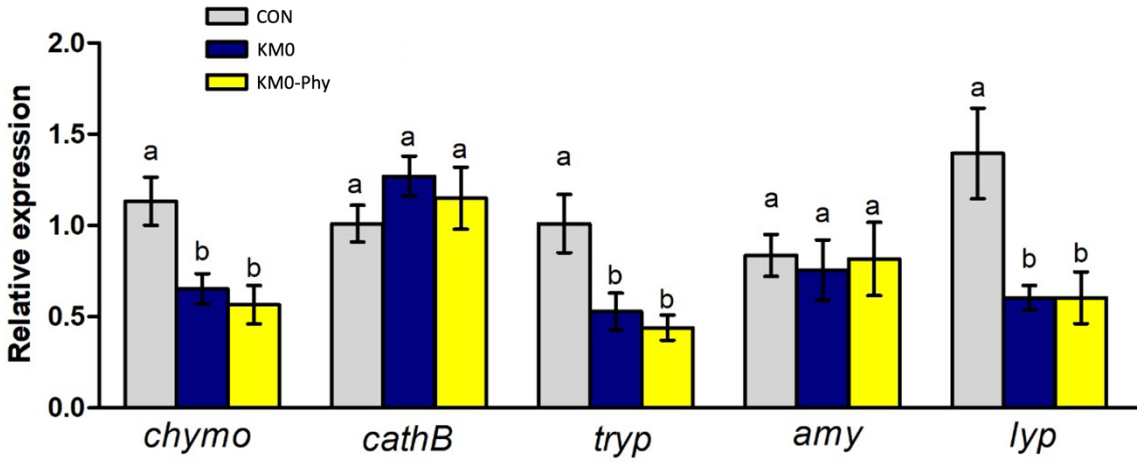

Figure 1. Expression of genes related to digestion in *L. vannamei* hepatopancreas fed with feed supplemented with different strains of *B. subtilis*. CON: shrimp fed only commercial feed; KM0: shrimp fed commercial feed with *B. subtilis* KM0; KM0-Phy: shrimp fed commercial feed with *B. subtilis* KM0-Phy. Different letters represent statistically significant differences ($p < 0.05$).

Expression of genes related to amino acid metabolism

Regarding genes related to amino acid metabolism (*gdh* and *gs*); Figure 2, only *gdh* was downregulated in shrimp fed with feed supplemented with KM0 strain. The *gs* gene was not altered in any treatment.

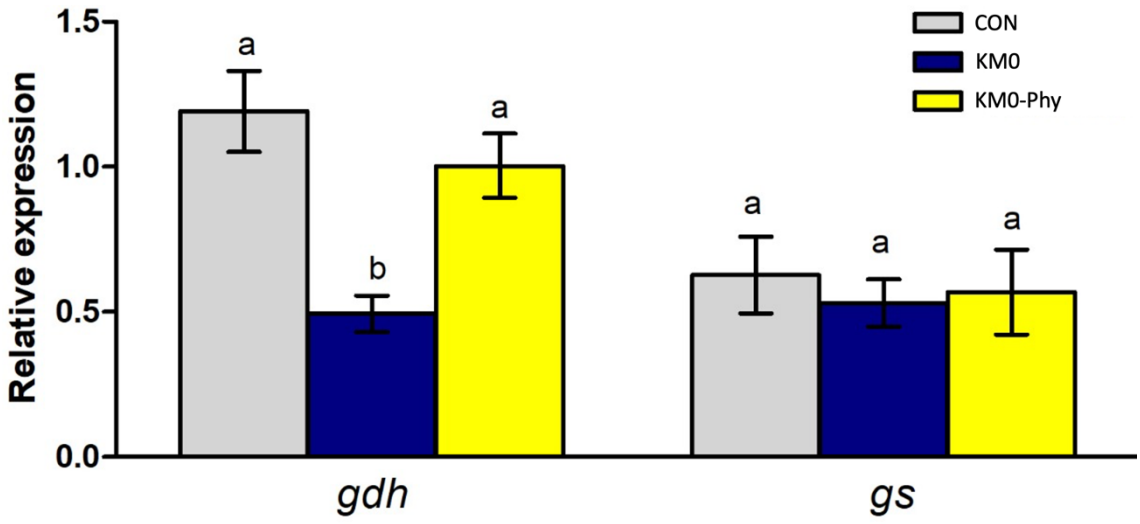

Figure 2. Expression of genes related to amino acid metabolism in *L. vannamei* hepatopancreas fed with feed supplemented with different strains of *B. subtilis*. CON: shrimp fed only commercial feed; KM0: shrimp fed commercial feed with *B. subtilis* KM0; KM0-Phy: shrimp fed commercial feed with *B. subtilis* KM0-Phy. Different letters represent statistically significant differences ($p < 0.05$).

Expression of genes related to antioxidant defense system

Genes related to antioxidant defense system (*gpx* and *sod*; Figure 3) were not altered in any treatment.

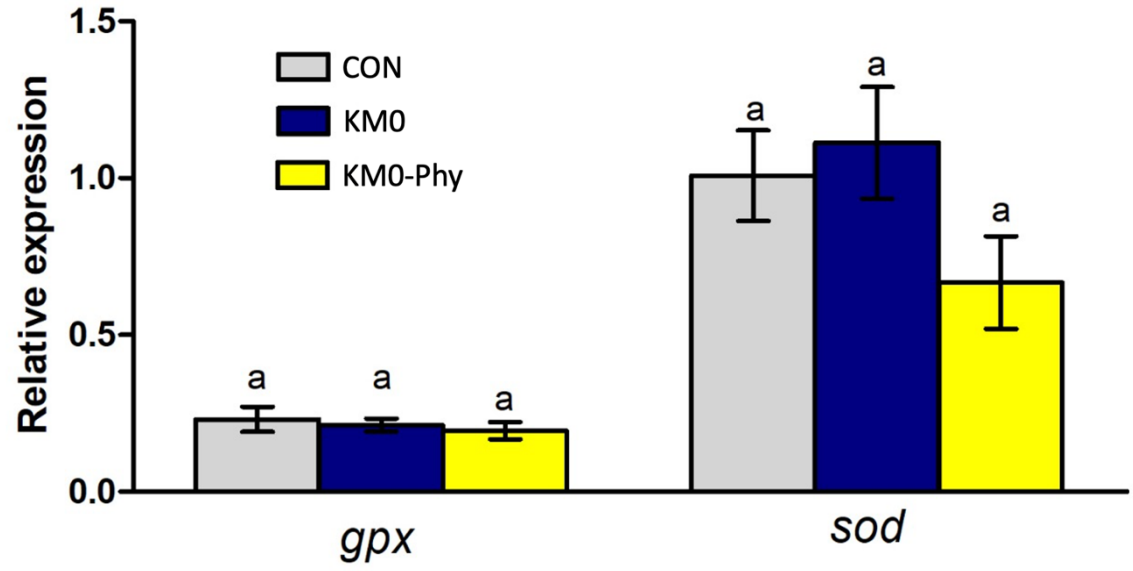

Figure 3. Expression of genes related to the antioxidant defense system in *L. vannamei* muscle fed with feed supplemented with different strains of *B. subtilis*. CON: shrimp fed only

commercial feed; KM0: shrimp fed commercial feed with *B. subtilis* KM0; KM0-Phy: shrimp fed commercial feed with *B. subtilis* KM0-Phy. Different letters represent statistically significant differences ($p < 0.05$).

Histological analysis

Figure 4 shows the result of lipid vacuoles counts in shrimp hepatopancreas. Shrimp fed with diet supplemented with *B. subtilis* KM0-Phy had a higher number of lipid vacuoles (34.47 ± 1.39) compared to the control treatment (29.28 ± 1.13). The counting of lipid vacuoles from shrimp feed with diet supplemented with *B. subtilis* KM0 (31.55 ± 0.74) was not statistically different from the other two treatments.

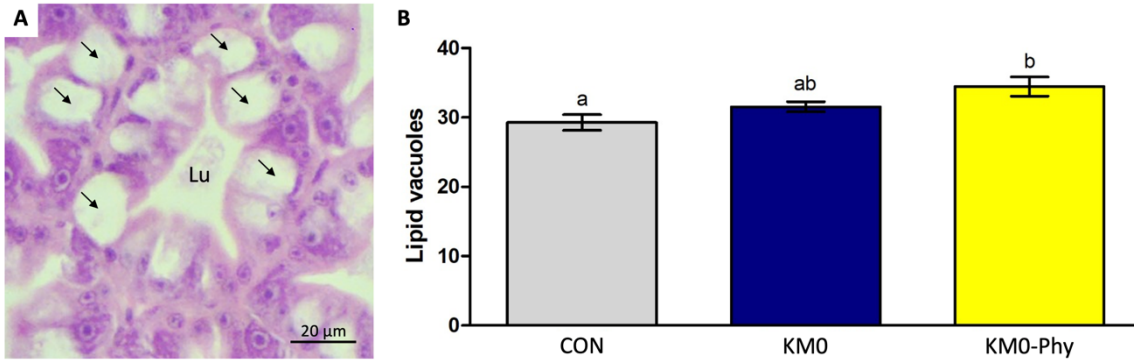

Figure 4. Count of lipid vacuoles in the hepatopancreas of *Litopenaeus vannamei* from histological analysis. A) Histological section showing the structures observed in shrimp hepatopancreas (40x magnification). Lu: lumen of the hepatopancreatic tubules; arrows indicate some examples of lipid vacuoles; B) Graph showing the result of the lipid vacuoles count. CON: shrimp fed only commercial feed; KM0: shrimp fed commercial feed with *B. subtilis* KM0; KM0-Phy: shrimp fed commercial feed with *B. subtilis* KM0-Phy. Different letters represent statistically significant differences ($p < 0.05$).

Discussion

Phosphorus is a key element in the constitution of fundamental molecules for living beings, such as nucleic acids and phospholipids in cell membranes. Furthermore, it is involved in production of energy molecules such as adenosine triphosphate (ATP), which are essential for the entire cell metabolism. Taking these characteristics into account, phosphorus is a limiting mineral for the growth of all living beings. However, each organism has specific requirements regarding phosphorus levels. While phosphorus deficiency can cause serious

metabolic problems related to growth, its excess can be excreted and eutrophicate the adjacent water bodies with negative consequences from an environmental point of view. It is known that only approximately 40 % of the phosphorus available in feed is absorbed by farmed aquatic organisms, while the surplus is secreted and released into the water (Sugiura 2018). Thus, the rational use of phosphorus is a balance point between animal production and environmental protection.

Due to the low concentration of phosphorus in natural waters, aquatic organisms need to obtain this essential element from food (Coloso et al. 2001). In the case of cultivated aquatic organisms, phosphorus must be present in the feed in its bioavailable form. However, this mineral is among the most expensive feed supplements used in aquaculture (Fox et al. 2006), and the phosphorus present in plant matter is not bioavailable to monogastric animals because it is in the form of phytate, which is considered an antinutritional factor. The addition of phytases can alleviate the problem, as these enzymes degrade phytate and release inorganic phosphorus for assimilation by the body. However, the addition of phytase to the feed represents a significant addition to its final cost. In the present study, the hypothesis that the addition of a probiotic strain of *B. subtilis* capable of secreting a fungal phytase (KM0-Phy) in *L. vannamei* feed could degrade the phytate present in plant matter and increase the bioavailability of phosphorus for the shrimp. Additionally, the impact of KM0-Phy strain on growth, survival, proximate muscle composition, lipid vacuoles in hepatopancreas and expression of genes related to digestion, amino acid metabolism and antioxidant defenses were evaluated.

After 45 days of experimentation, it was observed that the addition of the strain KM0-Phy to the shrimp diet did not change any parameters of growth or survival. The lack of growth induction observed here can be explained by the fact that probiotics may have a greater impact on the early stages of shrimp development, as suggested by Toledo et al. (2019). On the other hand, chemical composition analysis showed a significant increase of 39% in phosphorus content of muscle tissue of shrimp that had the probiotic KM0-Phy added to the feed, even if the feed used is already supplemented with 1.5% phosphorus. Interestingly, shrimp fed diet with KM0 strain also had an increase in muscle phosphorus at an intermediate level between CON and KM0-Phy groups. It is known that *Bacillus* species can produce Ca^{2+}-dependent beta-propeller type phytases, which have high thermal stability, optimal catalytic activity at neutral pH and high specificity for the calcium-phytate complex (Fu et al. 2008). Thus, it would be expected that the presence of *B. subtilis* in the feed could

have some effect on the bioavailability of phosphorus for shrimp. However, genetic manipulation for expression and secretion of a fungal phytase performed in KM0-Phy strain significantly enhanced this characteristic.

The increase observed in the phosphorus concentration in muscle tissue of shrimp fed diet with *B. subtilis* KM0-Phy additive suggests that the phytate was degraded by fungal phytase secreted by the genetically modified probiotic. Thus, it is reasonable to expect that the decrease in phytate could make more nutrients available and modulate expression of genes related to digestion in shrimp hepatopancreas. Quantification of digestion-related gene expression demonstrated a significant downregulation of proteases (*chymo* and *tryp*) and lipase (*lip*) transcription in both KM0 and KM0-Phy treatments. It is known that *Bacillus* species are capable of producing and secreting proteases and lipases. Priest (1977) already described numerous digestive exoenzymes produced by *Bacillus* species, especially carbohydrates, proteases and lipases. Some *Bacillus* proteases stand out for their high stability under adverse environmental conditions such as temperature and pH extremes, presence of organic solvents, detergents, and oxidizing agents (Contesini et al. 2018). Also, according to Eggert et al. (2003), *B. subtilis* can produce and secrete two types of lipases (lipA and lipB) from genes that are differentially expressed according to growth conditions. In addition, the counting of lipid vacuoles in the hepatopancreas showed that *B. subtilis* KM0-Phy used in the present study increased the concentration of lipids in that tissue in comparison to control group. The addition of phytases to the fish diet has been linked to an increase in lipids in the body (reviewed by Zheng et al. 2020). However, this relationship has not, to our knowledge, been established in crustaceans. Thus, the presence of a probiotic bacterium in the intestine capable of producing and secreting digestive enzymes decreases the need for the host to produce and secrete its own enzymes. This directly reflects on the regulation of genes as a way of saving the energy that is invested in the processes of absorption of nutrients from food. In fact, it has often been reported that dietary probiotic supplementation increases the activity of digestive enzymes in shrimp intestine. Recently, Wang et al. (2020) showed that probiotics such as *B. subtilis* and *B. licheniformis* can induce the activity of digestive enzymes in the gastrointestinal tract of tiger shrimp *Penaeus monodon*. Also, Zokaeifar et al. (2012) showed that the addition of *B. subtilis* strains to *L. vannamei* feed significantly increased digestive enzyme activity in shrimp. These studies, which only analyze the activity of digestive enzymes in the gastrointestinal tract, cannot differentiate between the action of shrimp enzymes and those secreted by probiotics.

In the present study, genes related to amino acid metabolism (*gdh* and *gs*) were also analyzed. The only difference observed was a downregulation of *gdh* in the group of shrimps treated with the KM0 strain. Apparently, genetic manipulation in the KM0 strain to express a fungal phytase increased the transcription of *gdh* to the same levels observed for the control group. The *gdh* gene encodes the enzyme glutamate dehydrogenase, which catalyzes the oxidative deamination of glutamate to form α-ketoglutarate in the mitochondrial matrix. This chemical reaction results in the production of a Krebs cycle intermediary and can accelerate ATP production by oxidative phosphorylation in the electron transport chain (Dawson and Storey, 2012). It is possible that the greater availability of phosphorus in the shrimp treated with the KM0-Phy strain is favoring a higher glutamate dehydrogenase expression compared to the shrimp treated with the KM0 strain. In the case of genes related to oxidative stress (*gpx* and *sod*) analyzed in muscle tissue, no difference was observed among treatments. This result shows that the increase in phosphorus availability did not imply a change in the shrimp muscle's oxidative status.

In conclusion, the use of a genetically modified strain of *B. subtilis* expressing a fungal phytase was able to increase the availability of phosphorus for shrimp. Although the increased availability of this mineral was not reflected in growth, it was possible to observe a downregulation in expression of genes related to digestion. Also, it is possible that the action of the phytase produced by the probiotic enables a decrease in phosphorus additives in the feed, with an impact on its cost. In addition, the greater efficiency in the use of phosphorus present in the feed will certainly imply a decrease in the excretion of this element by shrimp, with a consequent reduction in the impact of shrimp farms on adjacent ecosystems.

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Conflict of interest

The authors declare no conflict of interests.

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CAPÍTULO III

Dietary supplementation of *Synechococcus elongatus* PCC 7942 expressing a heterologous β-glucosidase on the expression of genes related to digestion, immune system, and antioxidant defenses of the shrimp *Litopenaeus vannamei*

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Effect of supplementation of *Synechococcus elongatus* PCC 7942 expressing a heterologous β-glucosidase in the diet of white shrimp *Litopenaeus vannamei*

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Abstract

Cyanobacteria, in general, are a rich source of nutrients in addition to producing bioactive compounds capable of stimulating the immune system of hosts. Also, they can be manipulated with relative ease to produce heterologous proteins. In the present study, a strain of *Synechococcus elongatus* (PCC7942) was manipulated to produce a prokaryotic β-glucosidase. This strain was added to the diet of *Litopenaeus vannamei* shrimp and parameters such as zootechnical performance, proximate composition of muscle tissue and expression of genes related to digestion, amino acid metabolism, immune system and antioxidant defenses were evaluated. Histology of shrimp hepatopancreas was also performed. The results showed that transgenic cyanobacteria did not produce negative effects on shrimp zootechnical performance. However, an increase in lipid and phosphorus deposition was observed in the muscle tissue of shrimp, as well as lipid in hepatopancreas. Also, an increase in a gene related to the immune system (*tgase*) and another related to both carbohydrate metabolism and amino acid metabolism (*gdh*) was observed. Furthermore, it was observed that both strains of cyanobacteria (wild and transgenic) produced effects on carbohydrate metabolism (increased *amy* expression) and digestive system (decreased *cathB* expression). It is possible that cyanobacteria in the diet may be serving as a source of carbohydrates, reducing the use of proteins as a source of energy. It can be concluded that transgenic cyanobacteria had a beneficial effect on shrimp by increasing lipid deposition in muscle and hepatopancreas as well as phosphorus absorption from feed, which can minimize the environmental impact of shrimp farms.

Keywords: cyanobacteria, probiotic, β-glucosidase, digestion, lipid deposition, phosphorous absorption

Introduction

Probiotics have played an important role in the expansion of aquaculture worldwide, as the use of these microorganisms in farming systems has brought benefits not only to the environment, but also to the health of farmed animals, especially in stimulating the immune system, growth, and feed efficiency (Hai, 2015; Yao et al., 2020). According to Ringø (2020), shrimp farming has benefited, since the 1990s, from species of bacteria from almost twenty genera, with *Lactobacillus* and *Bacillus* being the most represented. However, there are a variety of unconventional microorganisms that have so far been used as probiotics, which include gram-negative and gram-positive bacteria, yeasts, bacteriophages, actinobacteria, and unicellular algae (Das et al., 2008; Akhter et al., 2015). Among these microorganisms are cyanobacteria, a group of gram-negative photoautotrophic prokaryotes that have been suggested as probionts for shrimp post-larvae (Preetha et al., 2007), in addition to being available as supplements to provide a high-protein diet in aquaculture (Singh et al., 2007).

Cyanobacteria are rich sources of vitamins, essential amino acids, minerals (González-Davis et al., 2012), fatty acids (Maltsev and Maltseva, 2021a), and have immunomodulatory activity (Riccio and Lauritano, 2020). Furthermore, they can produce bioactive compounds with different applications, such as the inhibition of pathogens (Santhakumari et al., 2017). According to González-Davis et al. (2012), microalgae and cyanobacteria showed promising antibacterial activity and can be used as a biological control in cultures of *Artemia salina*, in addition to supplying a large part of the energy needs of the shrimp *Litopenaeus vannamei*. Furthermore, when incorporated into the diet, they can improve parameters related to growth in shrimp (Sivakumar et al., 2011a). A recent study reported that cyanobacteria may have an impact on the gut microbiome, leading to increased bacterial richness and playing an important role in fish development and health (Rosenau et al., 2021).

Cyanobacteria can also be genetically manipulated with relative ease and can be considered an interesting platform to produce heterologous proteins due to their photosynthetic efficiency (Dismukes et al., 2008). Recently, Azevedo et al. (2019) manipulated the cyanobacterium *Synechococcus elongatus* for the expression of a β-glucosidase of the GH3 family (AmBGL17). AmBGL17 was isolated from an Amazonian soil microorganism and biochemically characterized by Bergman et al. (2014). β-glucosidases catalyze the hydrolysis of β-glycosidic bonds, being key enzymes for carbohydrate metabolism in many organisms (Chang et al., 2011). In agriculture, these enzymes are

indicators of soil quality, as their catalytic action results in the availability of glucose as a carbon source for the growth and activity of beneficial microorganisms (Merino et al., 2016). However, the application of β-glucosidases in aquaculture has not been evaluated. In this context, the aim of this study was to supplement a commercial feed with a genetically modified strain of *S. elongatus* expressing a heterologous β-glucosidase and to evaluate its effects on the expression of genes related to digestion, immune system, and antioxidant defenses of *L. vannamei* shrimp.

Material and methods

Experimental design

Larvae of *L. vannamei* were purchased from Aquatec (Rio Grande do Norte, Brazil). Juveniles with an average weight of 2.6 ± 1.9 g were produced at the Marine Aquaculture Station from the Federal University of Rio Grande (FURG, Brazil). Shrimps were stored in tanks with a useful volume of 150 liters with clear water. The animals were acclimated for one week. The experiment was carried out in clear water and the physical and chemical parameters of the water were evaluated daily. Temperature, salinity and dissolved oxygen in all tanks were maintained in the range of 26.42 ± 2 °C, 32.2 ± 1 $g.L^{-1}$ and 6.24 ± 0.6 $mg.L^{-1}$ during the experimental period. Aeration was constantly supplied to each tank with air stone using an air blower. Every day, 30% of the water was changed in order to keep the levels of nitrogen compounds within tolerance levels for the species. Three treatments, with four replications each, were used, in a total of 12 tanks. The experimental groups were divided into: (i) control treatment (CON), the shrimps received only commercial feed moistened with saline solution (NaCl 0.9%), (ii) PCC 7942, shrimps received commercial feed added with *S. elongatus* PCC 7942 strain and (iii) AMBGL17, shrimps received commercial feed supplemented with genetically modified *S. elongatus* PCC 7942 (PT7AMBGL17) expressing prokaryotic β-glucosidase. The diets were administered twice a day for 45 days, at a rate of 10% of estimated biomass at the beginning and reduced gradually to 5% at the end of the experiment. The commercial feed used contains 38% crude protein (Guabi, Brazil) and is free of probiotics. Each tank received 40 shrimps, resulting in a culture density of 266 $shrimp.m^{-3}$ in each experimental unit.

Preparation of feed supplemented with strains of *S. elongatus*

Two strains of *S. elongatus* used in this work belong to the Molecular Biology Laboratory of Federal University of Rio Grande (FURG, Brazil) and are the same ones used by Azevedo et al. (2019). As a control strain, *S. elongatus* PCC 7942 (Invitrogen) strain was used. The PT7AMBGL17 strain was manipulated by Azevedo et al. (2019) to express a prokaryotic β-glucosidase. Cultures (50 mL) of *S. elongatus* PCC 7942 and *S. elongatus* PT7AMBGL17 were added to erlenmeyers (2 L) containing 1 L of BG-11 medium (Rippka et al., 1979), and were maintained at 34 °C, with constant lighting of 8,000 lux. *S. elongatus* PT7AMBGL17 cultures were supplemented with spectinomycin (10 $\mu g.mL^{-1}$), a selection agent for genetic manipulation. Expression of the gene of interest was induced by adding 5 μM $NiSO_4$ to the cultures and incubating for 24 h when the absorbance (750 nm) of the cultures reached the value of 1 (Blasi et al., 2012). Optical density (OD) was monitored in a spectrophotometer (Femto, 600s). After induction, cultures were centrifuged at 1,500 × g at 4 °C for 20 min to collect cyanobacteria cells, and the supernatant was discarded (Kiataramgul et al., 2020). For preparation, feed was ground into fine particles and 10% unflavored powdered gelatin, previously dissolved in distilled water at 60 °C was added, homogenized, and added cyanobacteria pellet dissolved in distilled water at 40 °C, containing approximately 10^8 cells/100 g of feed. The control diet was prepared in the same way, but without adding any cyanobacterial strain. The mixtures were repelletized and dried in an oven at 45 ºC overnight. After this period, the feeds were cooled slowly and stored in a clean glass bottle at 4 °C until used. This process was carried out weekly.

Tissue collection

At the end of the 45-day period, shrimps from each treatment were randomly removed and euthanized for analysis of proximate composition of muscle tissue (five individuals per treatment, immediately frozen at -20 °C) and gene expression (seven individuals per treatment). For gene expression analysis, hemolymph (300 μL) of each shrimp was removed from the base of the pleopod of the first abdominal segment with 1 mL sterile syringes containing 300 μL of a pre-cooled (4 °C) solution (450 mM NaCl, 10 mM KCl, HEPES 10 mM, 10 mM EDTA, pH 7.2) used as an anticoagulant. Hepatopancreas and muscle tissue were individually dissected and placed in 500 μL of Trizol Reagent (Invitrogen, Brazil), according to the manufacturer's protocol.

Zootechnical performance

To calculate weight gain, biomass gain, feed conversion ratio and survival, the following formulas were used:

- Weight gain (g) = Pf - Pi, where Pf = final weight and Pi = initial weight;
- Biomass gain = (Average Pf x number of individuals at the end) - (Average Pi x initial number of individuals);
- Feed conversion rate = feed consumption / biomass gain;
- Survival (%) = (final shrimp population × 100) / initial population.

Proximate composition of muscle tissue

The analysis of the moisture content was done by drying the samples in an oven at 100 °C until constant weight. Protein content was determined by the Kjeldahl method and total ether extract by the Soxhlet method (AOAC, 2000). Ashes were obtained by incineration in a muffle for 6 h at 600 °C. Phosphorus analyzes were performed according to Silva and Queiroz (2002), being expressed in mg/100 g of fresh sample.

Gene expression analyses

Total RNA extraction was performed with Trizol reagent (Invitrogen, Brazil). Extracted RNA was treated with DNase I (Invitrogen, Brazil) and the concentration was determined using a Biodrop spectrophotometer (Isogen Life Science). The quality was determined by 1% agarose gel electrophoresis. cDNA was synthesized using HighCapacity cDNA Reverse Transcription kit (Applied Biosystems). All procedures were performed following the manufacturer's protocol. Gene expression analyses were performed using quantitative PCR (qPCR), according to Livak and Schmittgen (2001). For each tissue a different set of genes was analyzed: (i) Hepatopancreas, five genes related to digestion (amylase, *amy*; lipase, *li*p; trypsin, *tryp*; cathepsin B, *cathB*; chymotrypsin, *chymo*) and two genes of amino acid metabolism (glutamine synthetase, *gs*; glutamate dehydrogenase, *gdh*); (ii) muscle, two genes related to the antioxidant defense system (glutathione peroxidase, *gpx*; superoxide dismutase, *sod*) and (iii) hemocytes, four genes related to immune system (penaeidin, *pen*; peroxynectin, *px*; prophenoloxidase, *propo*; transglutaminase, *tgase*). All primers are described in Table 1. Efficiency of all primers was performed in serial dilutions and 1:10 cDNA dilution of cDNA was used. Reactions were performed on the 7300 Real-time PCR System platform (Applied Biosystems, Brazil), with the PowerUP SYBR Green Master Mix kit (Applied Biosystems, Brazil), following the schedule: 50 °C for 2 min, 95 °C for 2

min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 15 s. The reference genes *ef1a*, *60S-121* and *rps3A* were used to normalize the gene expression data. To determine the stability of each reference gene in the different tissues analyzed the geNorm VBA applet software for Microsoft Excel (Vandesompele et al., 2002) was used. Gene expression in hemocytes and muscle was normalized using *ef1a* and *rps3A*. For hepatopancreas, *ef1a* and *60S-121* were used.

Table 1. Analyzed genes and sequence of primers used in qPCR reactions.

Gene	Sense (5'-3')	Antisense (5'-3')	Amplicon (nt)	GenBank
amy	ctctggtagtgctgttggct	tgtcttacgtgggactggaag	116	AJ133526
lip	actgtctcctctgctcgtc	atggtttctggaataggtgttt	131	XM027365317
tryp	cggagagctgccttaccag	tcggggttgttcatgtcctc	141	X86369
chymo	ggctctcttcatcgacg	cgtgagtgaagaagtcgg	182	XM037943862
cathB	ggatgtaacggaggcttc	ctgtatgctttgcctcca	248	XM027359505
gdh	aggttgtggaggaccagttg	ccgtggatcatctcgtaggt	166	EU496492
gs	ttccgtctcctgaaataccg	aggagccttgggaatgaagt	193	JN620540
gpx	agggacttccaccagatg	caacaactccccttcggta	117	AY973252
sod	tggagtgaaaggctctggct	acggaggttcttgtactgaaggt	175	DQ005531
ef1a	ccaccctggccagattca	gcgaacttgcaggcaatg	75	DQ858921
rps3A	ggcttgctatggtgtgctcc	tcatgctcttggctcgctg	101	XM027376915
60S-l21	gttgacttgaagggcaatg	cttcttggcttcgattctg	246	XM027359925

Hepatopancreatic histology

For histology of the hepatopancreas, shrimp were injected with Davidson's solution (11.5% acetic acid, 22% formalin, 33% ethanol and distilled water) for 48 hours, then transferred to 70% alcohol until processing. Tissues were dehydrated through successive immersions in ethanol of increasing concentrations, clarified in xylol and embedded in Paraplast at 60 °C. Regions of the hepatopancreas were selected and sectioned at 3 μm. Histological sections were stained with Hematoxylin and Eosin (Bell and Lightner, 1988) for lipid storage cell count. Histological sections were observed using a compound microscope at 40 × magnification.

Statistical analysis

One-way analysis of variance (ANOVA) was used to assess whether there were statistical differences between treatments. Normality and heterogeneity were assessed using the Shapiro-Wilk and Levene tests, respectively. For each case, when significance was detected among treatments, a subsequent comparison of means was performed using the Tukey test. All numerical data from gene expression were expressed as mean ± standard error. The other data were expressed as mean ± standard deviation. Differences were considered statistically significant when $p < 0.05$.

Results

Zootechnical performance and proximate composition of muscle tissue

Results of zootechnical performance are described in Table 2. No significant differences were observed in weight gain, feed conversion and survival between treatments.

Table 2. Zootechnical performance of *L. vannamei* fed on commercial feed (CON) or commercial feed supplemented with *S. elongatus* PCC 7942 (PCC7942) and *S. elongatus* PT7AMBGL17 (AMBGL17) strains for 45 days.

	Treatments		
	CON	PCC7942	AMBGL17
Initial weight (g)	2.98 ± 0.56	2.48 ± 0.19	2.54 ± 0.29
Final weight (g)	7.48 ± 0.17	7.73 ± 0.53	7.80 ± 0.27
Weight gain (g)	4.49 ± 0.71	5.24 ± 0.55	5.27 ± 0.36
Feed conversion ratio	1.65 ± 0.28	1.39 ± 0.15	1.37 ± 0.09
Survival (%)	98.9 ± 0.57	98.9 ± 0.57	99.3 ± 0.5

As shown in Table 3, shrimps in the treatment supplemented with *S. elongatus* PT7AMBGL17 strain had significantly higher body moisture, lipids and phosphorus values compared to the other groups ($p < 0.05$). There were no significant differences in the amount of ash and crude protein between groups.

Table 3 - Proximate composition (%) of the muscle tissue of *L. vannamei* fed commercial feed (CON) or commercial feed supplemented with *S. elongatus* PCC 7942 (PCC7942) and *S. elongatus* PT7AMBGL17 (AMBGL17) strains for 45 days.

	Treatments		
	CON	PCC7942	AMBGL17
Moisture (%)	76.18 ± 0.87^{a}	77.31 ± 0.64^{a}	74.61 ± 0.63^{b}
Ash (%)	1.26 ± 0.32	1.28 ± 0.28	1.25 ± 0.28
Protein (%)	18.62 ± 2.39	18.06 ± 1.0	19.56 ± 1.26
Lipid (%)	0.82 ± 0.37^{ab}	0.55 ± 0.18^{a}	1.02 ± 0.52^{b}
Phosphorus (mg/100 mg)	65.73 ± 1.95^{a}	65.52 ± 1.0^{a}	73.02 ± 1.86^{b}

Different letters represent statistically significant differences ($p < 0.05$).

Expression of genes related to immune system and antioxidant defense

The expression levels of *pen* (penaeidin), *px* (peroxynectin), *propo* (prophenoloxidase) and *tgase* (transglutaminase) genes are shown in Fig. 1. Analyzing the results of genes related to the immune system, no significant differences ($p > 0.05$) were found between treatments, except for tgase gene. The expression of *tgase* was significantly higher in shrimps from AMBGL17 treatment (1.076 ± 0.120) compared to control (0.369 ± 0.033) PCC7942 (0.528 ± 0.042) treatment ($p < 0.05$). Analyzing the expression data of *gpx* (glutathione peroxidase) and *sod* (superoxide dismutase) genes, related to antioxidant defense (Figure 2), no significant differences ($p > 0.05$) were observed among treatments.

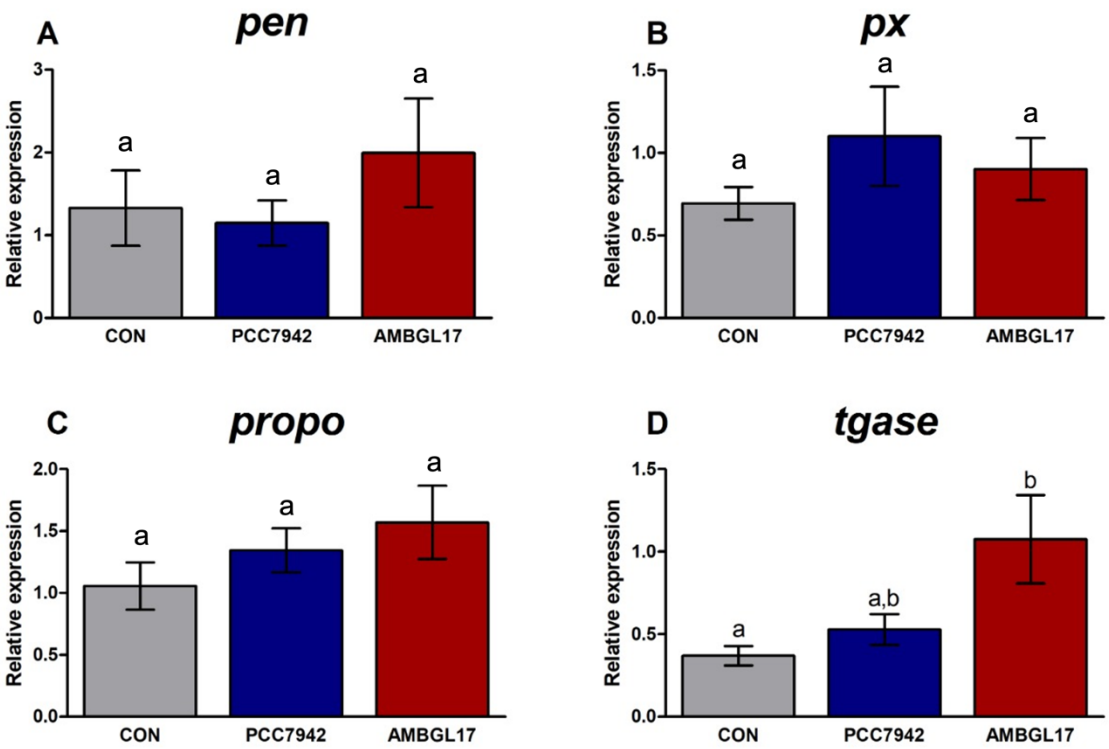

Figure 1. Relative expression of genes related to the immune system (*pen*, *px*, *propo*, *tgase*) in *L. vannamei* hemocytes fed with feed supplemented with different strains of *S. elongatus* PCC 7942. CON: shrimp fed only commercial feed; PCC7942: shrimp fed commercial feed with *S. elongatus* PCC 7942; AMBGL17: shrimp fed commercial feed with *S. elongatus* PT7AMBGL17. Different letters represent statistically significant differences ($p < 0.05$).

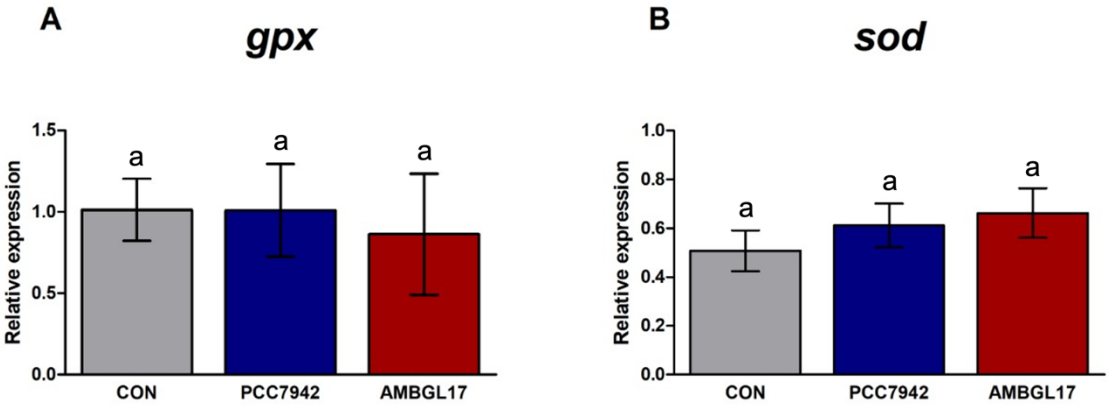

Figure 2. Relative expression of genes related to antioxidant defense system (*gpx* and *sod*) in *L. vannamei* muscle fed with feed supplemented with different strains of *S. elongatus* PCC 7942. CON: shrimp fed only commercial feed; PCC7942: shrimp fed commercial feed with *S.*

elongatus PCC 7942; AMBGL17: shrimp fed commercial feed with *S. elongatus* PT7AMBGL17. Different letters represent statistically significant differences ($p < 0.05$).

Expression of genes related to digestion and amino acid metabolism

The expression levels of digestion-related genes are shown in Fig. 3. The expression of *amy* (amylase) gene was significantly higher in shrimp from PCC 7942 (1.384 ± 0.159) and AMBGL17 (1.521 ± 0.085) treatments compared to control (0.391 ± 0.090) ($p < 0.05$). The opposite was observed for expression of *cathB* gene (cathepsin B) which was significantly lower in shrimp from PCC7942 (1.468 ± 0.137) and AMBGL17 (1.861 ± 0.098) treatments compared to the control group (2.906 ± 0.137). No significant differences ($p > 0.05$) were found between treatments for *chymo* (chymotrypsin), *tryp* (trypsin) and *lip* (lipase) genes.

The results of expression of genes involved in amino acid metabolism are shown in Figure 4. Expression of *gs* (glutamine synthetase) and *gdh* (glutamate dehydrogenase) genes were affected in different ways. While *gs* showed a significantly lower expression in PCC7942 (1.182 ± 0.121) and AMBGL17 (1.185 ± 0.073) treatments compared to the control group (2.353 ± 0.167), *gdh* gene presented a significantly higher expression in shrimp from the AMBGL17 (8.800 ± 0.630) treatment compared to the control (4.360 ± 0.231) and PCC7942 (4.678 ± 0.592) groups ($p < 0.05$).

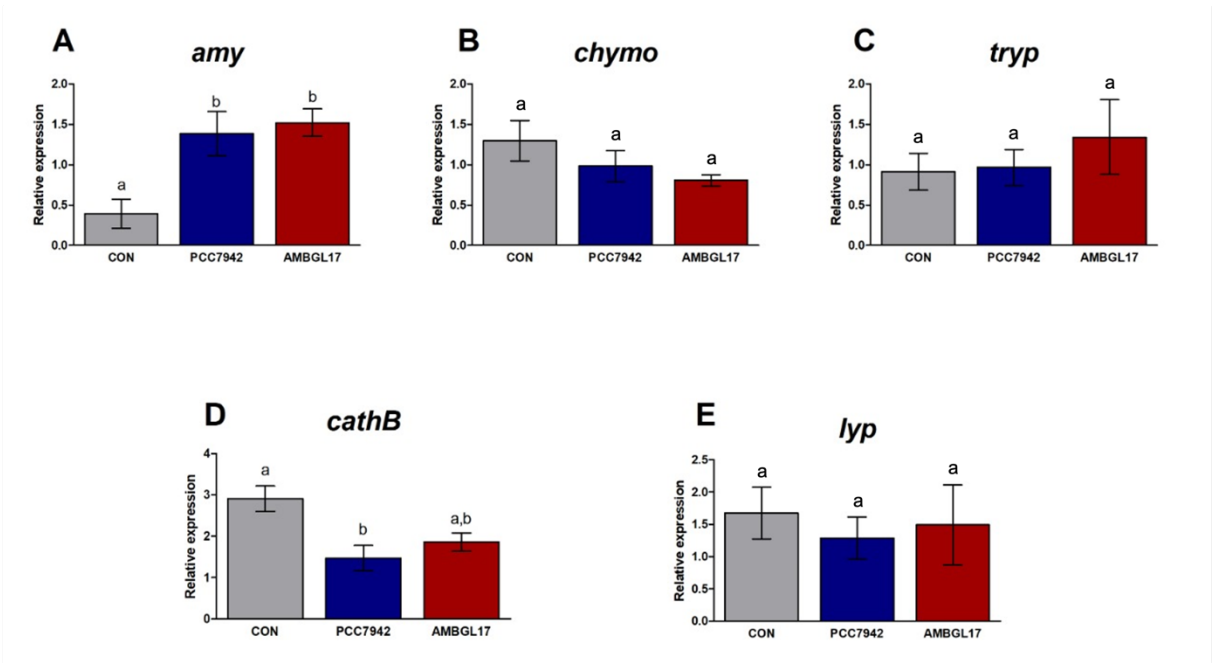

Figure 3. Relative expression of genes related to digestion (*amy*, *chymo*, *tryp*, *cathB* and *lip*) in *L. vannamei* hepatopancreas fed with feed supplemented with different strains of *S.*

elongatus PCC 7942. CON: shrimp fed only commercial feed; PCC7942: shrimp fed commercial feed with *S. elongatus* PCC 7942; AMBGL17: shrimp fed commercial feed with *S. elongatus* PT7AMBGL17. Different letters represent statistically significant differences ($p < 0.05$).

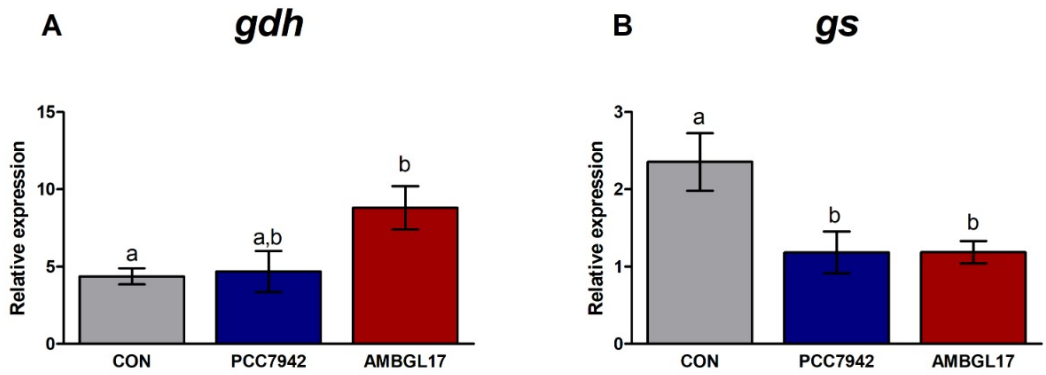

Figure 4. Relative expression of genes related to amino acid metabolism (*gdh* and *gs*) in *L. vannamei* hepatopancreas fed with feed supplemented with different strains of *S. elongatus* PCC7942. CON: shrimp fed only commercial feed; PCC7942: shrimp fed commercial feed with *S. elongatus* PCC7942; AMBGL17: shrimp fed commercial feed with *S. elongatus* PT7AMBGL17. Different letters represent statistically significant differences ($p < 0.05$).

Hepatopancreatic histology

Shrimp hepatopancreas fed with diet *S. elongatus* PT7AMBGL17 had a higher number of lipid vacuoles (43.06 ± 1.08) compared to the control (24.81 ± 2.02) and to the treatment with the *S. elongatus* strain PCC7942 (39.48 ± 0.83) (Fig. 5).

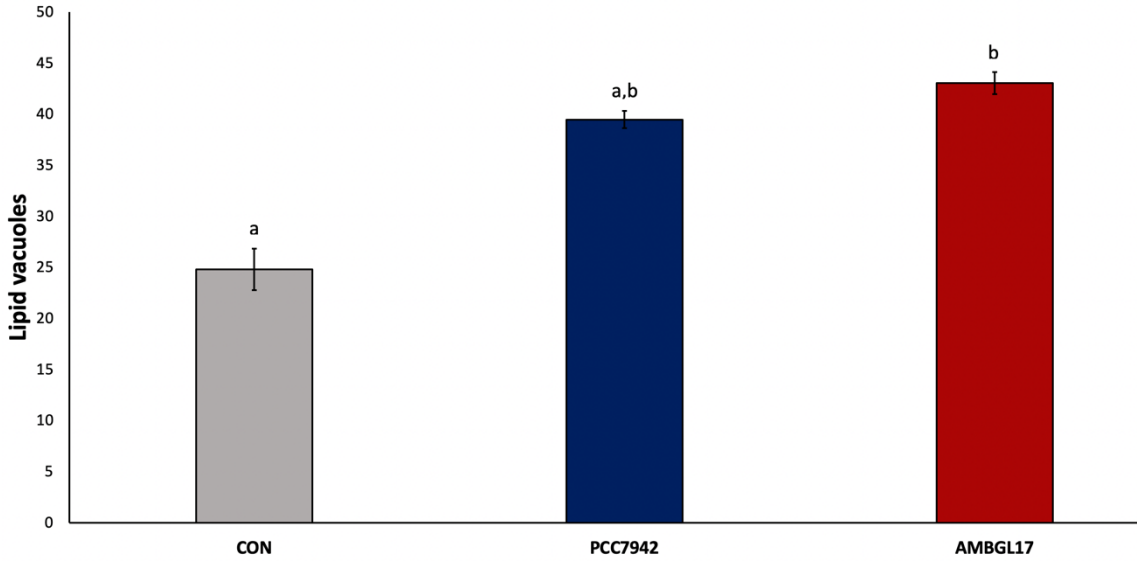

Figure 5. Lipid vacuoles count in *L. vannamei* hepatopancreas. CON: shrimp fed only commercial feed; PCC7942: shrimp fed commercial feed with *S. elongatus* PCC7942; AMBGL17: shrimp fed commercial feed with *S. elongatus* PT7AMBGL17. Different letters represent statistically significant differences ($p < 0.05$).

Discussion

This is the first investigation that evaluated the influence of supplementation of *L. vannamei* shrimp feed with a transgenic *S. elongatus* cyanobacterium expressing a heterologous β-glucosidase. According to the results, the diets supplemented with cyanobacteria did not change the growth, final weight, feed conversion ratio and survival of shrimp compared to the control treatment. The results indicate the zootechnical performance of shrimp was not affected by the ingestion of cyanobacteria (transgenic or wild). Zhuang et al. (2021) also engineered *S. elongatus* PCC 7942 to express the Vp28 protein, which is a structural protein of the White Spot Syndrome virus (WSSV) and plays an important role in the response of *L. vannamei* in the early stages of infection. These authors reported a decrease in the growth of shrimp fed with cyanobacteria compared to shrimp fed with commercial feed. However, it is noteworthy that the cyanobacteria expressing Vp28 was administered as a dry powder, which may have reduced its nutritional value. As in the present study, Sivakumar et al. (2011) administered *Synechococcus* sp. as live food for juveniles of *Penaeus monodon* shrimp and observed that, although the length was not affected at the end of the experimental period, the weight of shrimp fed with *Synechococcus* sp. was lower than the treatments with *Chlorela* sp. and *Phormidium* sp. However, these authors used algal biomass as the only food source for shrimp, and not as a supplement to commercial feed. In contrast, the

cyanobacterium *Arthrospira platensis* replaced up to 75% of the fishmeal in the diet without any reduction in *L. vannamei* survival or growth (Macias-Sancho et al., 2014).

The results of the present study showed that the administration of *S. elongatus* PT7AMBGL17 expressing β-glucosidase significantly affected the body composition of *L. vannamei*. The results revealed that shrimp fed diet supplemented with transgenic *S. elongatus* had higher lipid and phosphorus content in muscle and lower moisture content. Regarding the lipid content, it is known that cyanobacteria, including *S. elongatus* PCC 7942, can synthesize short carbon chain fatty acids (SCFAs) (Gong and Miao, 2019), which have the potential to improve the growth performance in *L. vannamei* (Chen et al., 2020). However, we did not observe differences in the amount of lipids between the PCC7942 group and the control group. This observation suggests that SCFAs eventually produced by cyanobacteria are not impacting the amount of lipids in muscle tissue. However, an increase in lipids was observed in shrimp from the group fed with cyanobacteria expressing β-glucosidase (AMBGL17). Recently, Huang et al. (2020b) demonstrated that β-glucosidase overexpression facilitates glucose release in transgenic tobacco. Thus, it is possible that the β-glucosidase activity produced by transgenic cyanobacteria is making more sugars available to shrimp. This hypothesis can be supported by the increased expression of the gene that codes for amylase (*amy*) in the hepatopancreas of shrimp that had their feed supplemented with transgenic cyanobacteria. It is known that the increase in sugars in the circulatory system can affect the body's homeostasis. Thus, it is possible that these sugars may be being converted to lipids and stored in muscle tissue or other tissues such as the hepatopancreas. In fact, the histological analysis showed a significant increase in lipid vacuoles in the hepatopancreas of shrimp fed with the feed supplemented with transgenic cyanobacteria. Although it is already known that *de novo* lipogenesis can be induced by excess carbohydrates in the diet of humans (Hellerstein, 1999; Kersten, 2001) and fish (Bou et al., 2016; Li et al., 2019; Taj et al., 2020), this direct relationship remains to be proven in crustaceans.

Another observation of the results of the proximate composition is the increase of approximately 11% of phosphorus in the muscle tissue of shrimp fed with feed supplemented with transgenic cyanobacteria. This result indicates that the presence of β-glucosidase increased the phosphorus bioavailability for shrimp. However, this relationship is probably not straightforward, as there is no evidence that this enzyme may be involved in the phosphorus cycle. However, it is certain that β-glucosidase increases the availability of glucose to other microorganisms, which can be the source of enzymes such as phosphatases

and phytases that act in the conversion of organic to inorganic phosphorus, which can be absorbed by shrimp and other organisms. The greater absorption of phosphorus by organisms also has environmental implications, as it reduces the contribution to adjacent ecosystems and its consequent eutrophication.

Romano et al. (2015) reported that the addition of a mixture of organic acids can increase the bioavailability of phosphorus in *L. vannamei*, with a consequent increase in the immune response and resistance to pathogenic bacteria. To assess whether the increase in phosphorus absorption observed in the present study had effects on the immune system of the shrimp, the expression of the genes *pen*, *px*, *propo* and *tgase* was evaluated. Of the genes related to the immune system analyzed, only *tgase* was significantly induced in shrimp fed on the feed supplemented with the transgenic cyanobacteria. Transglutaminase (TGase) is part of the coagulation system of *L. vannamei* and plays an important role in inhibiting the entry of pathogens into the body, which is why it is considered an important immunological factor in shrimp (Zheng et al., 2018b). Furthermore, TGase has been shown to be involved in the regulation of certain genes related to the immune system, particularly antimicrobial peptides (AMPs), suggesting that the release of these may depend on the activation of the coagulation system (Zhu et al., 2016). Therefore, the increase in *tgase* expression observed in this study suggests an increase in the immune responsiveness of *L. vannamei*. Other genes analyzed related to the immune system of shrimp such as *sod* and *gpx* (Pilotto et al., 2019; Zheng et al., 2018), which play a role in the antioxidant defense system, were not altered by treatments with cyanobacteria.

Regarding digestion, the treatments tested in this study showed two different types of impacts on gene expression. The first is related to the presence of cyanobacteria (regardless of whether it is transgenic or wild), whose effect can be observed in the increased expression of the *amy* gene in treatments PCC7942 and AMBGL17, or in the decreased expression of *cathB* and *gs* in these same treatments. In the case of amylase, it can be suggested that the cyanobacteria itself is making more sugars available to the shrimp since these microorganisms are able to produce and store glycogen from the fixed CO_2 through photosynthesis. For example, *Synechococcus* sp. PCC 7002, an euryhaline species, has high glycogen production and is considered a promising source of carbohydrates to produce biofuels (Aikawa et al., 2014). Furthermore, functional genes related to sucrose synthesis have been identified in *S. elongatus* PCC 7942 (Martínez-Noël et al., 2013). Thus, the presence of sugars produced by cyanobacteria would be inducing the expression of shrimp amylase.

For *cathB* and *gs* the effect was the opposite. The presence of cyanobacteria decreased the expression of those genes. Cathepsin B is a cysteine proteinase whose gene was first identified in *L. vannamei* by Stephens et al. (2012). These authors reported the expression of *cathB* in most shrimp tissues, except for pleopods and ocular peduncle. Furthermore, they observed that this enzyme participates not only in the hydrolysis of intracellular proteins during fasting, but also in the hydrolysis of extracellular proteins after food ingestion. Thus, this enzyme plays an important role in the digestive processes that involve the provision of amino acids for the body. The fact that the presence of the cyanobacteria used in the present study decreased the expression of *cathB* in the shrimp hepatopancreas suggests that the shrimp metabolism is using more sugars than proteins as an energy source. If this is the case, the lower use of protein as an energy source implies a lower impact on the shrimp's ammonia metabolism. In fact, the *gs* gene that codes for glutamine synthetase is considered an important marker of ammonia-induced stress, and its decrease observed in groups of shrimps treated with cyanobacteria may be related to the greater use of sugars in relation to protein catabolism. Qiu et al. (2018) demonstrated that the expression of *gs* is increased in *L. vannamei* exposed to higher concentrations of ammonia, concluding that shrimp accelerate the production of glutamine from glutamate and NH_4^+ to reduce the stress caused by excess nitrogen in the body. Finally, an increase in *gdh* was observed in the group of shrimps treated with transgenic cyanobacteria (AMBGL17), when compared to the control group. Glutamate dehydrogenase is a crucial enzyme that links amino acid metabolism to carbohydrate metabolism. This enzyme is present in the mitochondrial matrix of eukaryotic cells and catalyzes the reversible reaction of glutamate to α-ketoglutarate + NH_4^+. Thus, this enzyme works as a modulator in the synthesis of amino acids from carbohydrates, or in the oxidative deamination of glutamate to form α-ketoglutarate which can be used as fuel for the Krebs cycle to drive the electron transport chain and production of ATP by oxidative phosphorylation (Dawson and Storey, 2012). In the scenario where the group of shrimps treated with cyanobacteria expressing a β-glucosidase had greater availability of carbohydrates, it is feasible to assume that *gdh* is acting to form amino acids from carbohydrate derivatives.

In summary, the present study reports the use of cyanobacterial strains (wild or manipulated to express a prokaryotic β-glucosidase) as an additive in *L. vannamei* shrimp feed. The results showed that both cyanobacteria did not produce negative effects on the zootechnical performance of the shrimp. However, an increase in the deposition of lipids and

phosphorus in the muscle tissue of shrimp treated with transgenic cyanobacteria was observed. In this group, an increase in a gene related to the immune system (*tgase*) and another one related to both carbohydrate metabolism and amino acid metabolism (*gdh*) was also observed. In addition, it was observed that both strains of cyanobacteria produced effects on carbohydrate metabolism (increased expression of *amy*) and on the digestive system (decreased expression of *cathB*). Apparently, cyanobacteria in the feed may be serving as a source of carbohydrates, reducing the use of proteins as an energy source. It can be concluded that cyanobacteria (wild or transgenic) had a beneficial effect on shrimp not only by increasing the expression of genes related to the immune system and digestion, but also by the greater absorption of phosphorus from the feed, which can minimize environmental impact of shrimp farms.

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Author contribution

All authors contributed to the conception and design of the study. All authors contributed to the visualization of data, drafting of the article, and revising it. All authors approved the final version to be submitted.

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Competing interests

The authors declare no competing interests

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DISCUSSÃO GERAL

Probióticos são amplamente utilizados na aquicultura e têm proporcionado benefícios à saúde dos animais aquáticos (Yao et al., 2020). Os efeitos benéficos da administração de probióticos foram reportados como fontes de melhora na resposta imunológica e no sistema digestivo, através do estímulo da produção de enzimas endógenas que contribui com a melhora das atividades enzimáticas no intestino (Butt et al., 2021; Ringø et al., 2020).

Dentre as opções de obtenção de probióticos para a carcinocultura, o isolamento de microrganismos a partir do intestino do camarão é uma alternativa interessante com grande potencial de aplicação na aquicultura comercial (Li et al., 2021; Ock Kim et al., 2020; Wang et al., 2020; Zuo et al., 2019). Além disso, uma outra opção é o desenvolvimento e o uso de microrganismos geneticamente modificados que podem ser utilizados como suplemento alimentar com o objetivo de melhorar o fator de condição dos animais, estimulando o sistema imunológico, reduzindo a resposta inflamatória e o estresse oxidativo (Erpel et al., 2016; Santos et al., 2020), como biorreatores para a produção de moléculas capazes de interferir nos sistemas fisiológicos de peixes (Santos et al., 2020), ou como sistema de expressão heteróloga para a produção de vacinas orais para *L. vannamei* (Zhu et al., 2020).

Na presente Tese observou-se que o uso de diferentes cepas (convencionais ou não convencionais) como aditivos dietéticos afetou de forma distinta a composição corporal dos camarões. No entanto, quanto ao ganho de peso e na taxa de conversão alimentar, não houve diferenças significativas nos três experimentos realizados. No capítulo I, a suplementação da dieta com a cepa E de *B. subtilis* promoveu significativo aumento na composição de lipídeos no músculo e na contagem de vacúlos lipídicos nos hepatopâncreas de *L. vannamei*. Outro estudo também relatou que a administração de probióticos aumenta o conteúdo de lipídios no músculo (Sadat Hoseini Madani et al., 2018). Na suplementação da dieta com a cepa transgênica de *B. subtilis* expressando a fitase (capítulo II), observou-se um aumento na concentração de fósforo no tecido muscular dos camarões, sugerindo que o fitato presente na matéria vegetal da ração foi degradado pela fitase fúngica secretada pelo probiótico geneticamente modificado. Camarões alimentados com *S. elongatus* transgênicas suplementada na dieta (capítulo III) apresentaram maior teor de lipídeo e fósforo no músculo e menor teor de umidade. Um estudo recente demonstrou que a superexpressão de um gene multifuncional β-glicosidase facilita a liberação de glicose em tabaco transgênico (Huang et al., 2020). Assim, é possível que a atividade da β-glicosidase produzida por cianobactérias

transgênicas esteja disponibilizando mais açúcares para os camarões. Essa hipótese pode ser corroborada pelo aumento da expressão do gene que codifica a amilase (*amy*) no hepatopâncreas de camarões que tiveram sua alimentação suplementada com cianobactérias transgênicas. Sabe-se que o carboidrato absorvido que é utilizado para fornecer energia pode contribuir para deposição de lipídios e glicogênio após a conversão bioquímica e que a composição lipídica do corpo dos peixes foi influenciada pelos níveis de carboidratos e lipídios da dieta (Gümüş e İkiz, 2009). Além disso, o aumento significativo de fósforo no músculo de animais que tiveram a ração suplementada *S. elongatus* P_{T7}AMBGL17 expressando β-glicosidase pode ser devido ao papel indireto que a β-glucosidase desempenha na aquisição de P (Zacher et al., 2021). Sabe-se que o suplemento de fósforo pode contribuir para maior deposição de zinco (Niu et al., 2008), o que pode promover ma melhora na imunidade do *L. vannamei* (Shi et al., 2021). De fato, quando analisados os genes relacionados ao sistema imunológico, a *tgase* foi induzida significativamente em camarões alimentados com a ração suplementada com a cianobactéria transgênica. Sabe-se que a TGase demonstrou estar envolvida na regulação de certos genes relacionados ao sistema imunológico, particularmente peptídeos antimicrobianos (AMPs), sugerindo que a liberação destes pode depender, dentre outros fatores, da ativação do sistema de coagulação (Zhu et al., 2016).

Com relação à expressão de genes, o uso da cepa E de *B. subtilis* isolada do intestino do camarão (capítulo I) e da cepa KM0 gerou diferenças significativas na expressão dos genes que codificam para enzimas relacionadas à digestão (*chymo, tryp e lip*), que tiveram uma expressão mais baixa comparada ao controle. Além disso, observou-se que a expressão do gene *cathB* foi significativamente reduzida em camarões tratados com a cepa E em relação à cepa KM0, o que pode demonstrar que a cepa E é capaz de produzir e secretar mais cisteína-proteases do que a cepa KM0. Gao et al. (2019) demonstram que enzimas extracelulares da microbiota intestinal também estão envolvidas no metabolismo de substâncias macromoleculares, como aminoácidos e carboidratos, logo sugere-se que intestino hospedeiro pode diminuir a produção e secreção de tais enzimas, começando com uma diminuição na taxa de transcrição desses genes. Sobre o metabolismo de aminoácidos, o gene *gdh* teve uma taxa de transcrição menor em hepatopâncreas de camarão nos tratamentos com probióticos, enquanto o gene *gs* também teve uma menor taxa de transcrição, porém, apenas para os animais alimentados com a cepa E. Possivelmente a menor expressão de *gdh* pode ser uma resposta a uma alta concentração de lipídios no hepatopâncreas dos camarões que foram

tratados com as cepas probióticas, pois a glutamato desidrogenase induz a produção de intermediários do ciclo de Krebs, e um dos intermediários (citrato) é usado para a síntese de lipídios (Dawson e Storey, 2012). Além disso, a menor taxa de transcrição de *gs* pode indicar que a cepa E tem capacidade para reduzir os compostos nitrogenados presentes na água de criação e, assim, reduzir o estresse no camarão.

Com relação ao capítulo II, a quantificação da expressão de genes relacionados à digestão demonstrou uma regulação negativa significativa da transcrição de proteases (*chymo* e *tryp*) e lipase (*lip*), em ambos os tratamentos KM0 e KM0-Phy. Sabe-se que as espécies de *Bacillus* são capazes de produzir e secretar proteases e lipases. Priest (1977) já descreveu inúmeras exoenzimas digestivas produzidas por espécies de *Bacillus*, especialmente carboidratases, proteases e lipases. Algumas proteases de *Bacillus* se destacam por sua alta estabilidade em condições ambientais adversas, como temperaturas e pH extremos, presença de solventes orgânicos, detergentes e agentes oxidantes (Contesini et al., 2018). Além disso, de acordo com Eggert et al. (2003), *B. subtilis* pode produzir e secretar dois tipos de lipases (lipA e lipB) a partir de genes que são diferencialmente expressos de acordo com as condições de crescimento.

A respeito dos genes analisados no capítulo III, o uso de cianobactérias (transgênicas ou selvagens) na dieta apresentou um aumento da expressão de *amy* e diminuição da expressão de *cathB* e *gs*. Esses resultados sugerem que o metabolismo do camarão está utilizando mais açúcares do que proteínas como fonte de energia, o que pode implicar no menor uso de proteína como fonte de energia, com consequente impacto no metabolismo de amônia de camarões. Além disso, o uso de cianobactérias transgênicas apresentou maiores níveis transcricionais do gene *gdh* quando comparado ao grupo controle. No cenário em que o grupo de camarões tratados com cianobactérias expressando uma β–glicosidase apresentou maior disponibilidade de carboidratos, é possível supor que *gdh* esteja atuando na formação de aminoácidos a partir de derivados de carboidratos.

Finalmente, sugere-se que o uso de diferentes probióticos (convencionais ou não convencionais) impactou de forma significativa o sistema imune e os processos digestivos do camarão. O uso de um probiótico endógeno resultou em um aumento da concentração de lípidíos no músculo e no hepatopâncreas dos animaise. Pôde-se observar, também, os benefícios dos probióticos transgênicos, como o *B. subtilis* KM0-Phy expressando uma fitase e de *S. elongatus* P_{T7}AMBGL17 expressando uma β-glicosidase na dieta de *L. vannamei*. Além disso, o uso dos probióticos transgênicos aumentou a deposição de fósforo no músculo

de *L. vannamei*, o que pode contribuir para um ganho econômico e ambiental para a carcinocultura, visto que a adição desse mineral impacta no custo da ração e sua excreção é o principal fator que contribui para a e eutrofização dos ambientes aquáticos.

CONSIDERAÇÕES FINAIS

- ✓ Constatamos que a cepa endógena do *L. vannamei* (cepa E) foi capaz de aumentar significativamente a deposição de lipídios no músculo e no hepatopâncreas do camarão, além de diminuir a expressão de genes relacionados à digestão e ao metabolismo de aminoácidos. Devido aos seus efeitos benéficos para o camarão, este probiótico pode ser uma alternativa para a carcinocultura.
- ✓ Constatamos que a fitase de *Aspergillus fumigatus* expressa pela cepa KM0-Phy foi capaz de aumentar a deposição de fósforo no músculo de *L. vannamei* alimentados com uma dieta comercial.
- ✓ Ao nosso conhecimento, o capítulo II representa o primeiro estudo em *L. vannamei* que utiliza uma linhagem bacteriana transgênica do gênero *Bacillus* capaz de excretar uma fitase recombinante que foi suplementado à uma ração comercial. Os resultados demonstraram que a fitase heteróloga é capaz de disponibilizar mais fósforo da ração, podendo diminuir os custos relacionados à incorporação deste elemento nas rações comerciais.
- ✓ Ao nosso conhecimento, o capítulo III representa o primeiro estudo em que uma cianobactéria (*S. elongatus*) geneticamente programada para expressar uma β-glicosidase heteróloga foi utilizada como suplemento na ração de *L. vannamei*. Esta cianobactéria disponibilizou mais carboidratos para o camarão, resultando numa maior deposição de lipídios no músculo e no hepatopâncreas do camarão, além de melhorar a biodisponibilidade de fósforo para o hospedeiro o que, além de alterar o metabolismo, também pode minizar os impactos ambientais da carcinicultura.

PERSPECTIVAS

- ✓ Sugere-se que a cepa E de *B. subt*ilis, isolada do trato intestinal de *L. vannamei*, tenha seu genoma completamente sequenciado para que se transforme numa plataforma exclusiva para a produção de moléculas de interesse para a carcinocultura.
- ✓ Levando em consideração que a cepa geneticamente modificada de *B. subtilis* que expressa uma fitase heteróloga induziu a um aumento de 39 % no conteúdo de fósforo no

músculo do camarão, sugere-se o desenvolvimento de experimentos com quantidades decrescentes de fósforo na ração para avaliar o quanto esse probiótico poderia economizar deste elemento na alimentação de camarões.

- ✓ Uma vez que a cepa de *S. elongatus* expressando uma β-glicosidase parece estar disponibilizando mais carboidratos para camarões, sugere-se que sejam realizados experimentos com quantidades decrescentes destes elementos na dieta para avaliar o quanto este probiótico não convencional estaria compensando o metabolismo de açúcares do hospedeiro.

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