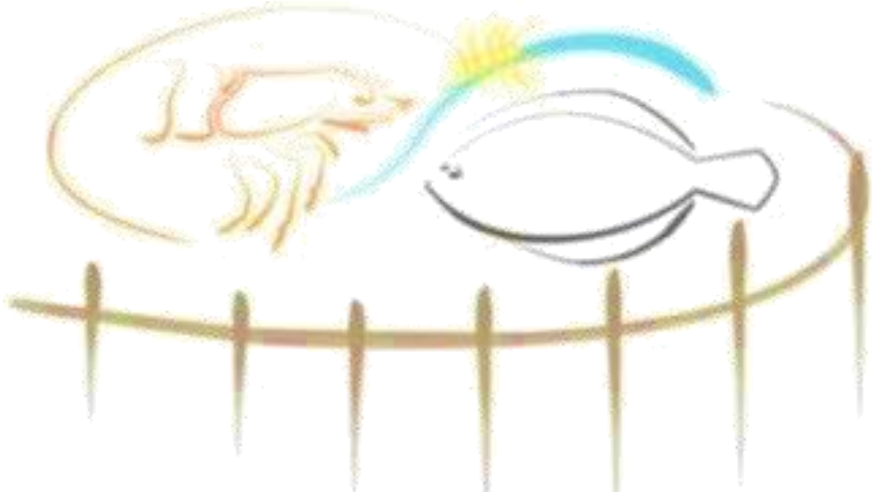
Universidade Federal do Rio grande - FURG

Instituto de Oceanografia

Programa de Pós-Graduação em Aquicultura



Engenharia genética no probiótico *Bacillus subtilis*: expressão de fitase fúngica melhora parâmetros zootécnicos, sistema imune, resposta inflamatória e defesa antioxidante em zebrafish (*Danio rerio*) alimentado com dieta rica em matéria vegetal

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Dedicatória

À minha família.

A vocês o meu eterno amor e admiração.

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SUMÁRIO

RESUMO GERAL

A enzima fitase catalisa a hidrólise de fitato, o qual representa a forma de armazenamento do fósforo nos vegetais. O fitato é um fator antinutricional para animais monogástricos, pois estes são ineficientes na produção de fitases intestinais. Portanto, o incremento de ingredientes vegetais na ração em sistemas de produção animal é limitado. Com isso, o objetivo da presente tese foi obter um *Bacillus subtilis* geneticamente modificado capaz de expressar uma fitase fúngica e avaliar a eficácia desta enzima na dieta do zebrafish (*Danio rerio*). No capítulo I desenvolvemos uma construção genética baseada em plasmídeo replicativo contendo o gene da fitase do fungo *Aspergillus fumigatus* (Phy-Af) fusionado ao sinal de secreção do gene da levansacarase (SacB) do próprio *B. subtilis*. A atividade da fitase produzida pelo *B. subtilis* transgênico no sobrenadante da cultura foi 2,7 vezes superior do que os controles. Após determinar a atividade da fitase, realizamos um experimento de alimentação do zebrafish, durante 30 dias, com uma dieta rica em vegetais. O experimento foi constituído por dois grupos: Phy-Af (com dieta suplementada com *B. subtilis* expressando a fitase) e o grupo controle (dieta com *B. subtilis* não transgênico). Foi realizada a biometria inicial dos animais e final para avaliar o desempenho zootécnico e foram analisados genes relacionados com o transporte de peptídeos (*slc15a1b* e *slc15a2*), apetite (*ghrl*), fatores de crescimento muscular (*igf1*, *myod* e *myog*) e metabolismo de minerais (*bglap*). Os peixes alimentados com o *B. subtilis* transgênico apresentam melhores índices de desempenho quando comparados ao controle. Neste mesmo sentido, todos os genes analisados foram superexpressados no grupo tratado com o probiótico expressando a fitase fúngica. No capítulo II, o objetivo foi avaliar o efeito do *B. subtilis* transgênico sobre o sistema imune e a resposta inflamatória no zebrafish alimentado com a ração rica em matéria vegetal. Neste caso, foram analisados genes relacionados com a produção de linfócitos (*cd4* e *ikzf1*), resposta inflamatória (*ifnphi1*, *ifng*, *tnf-α* e *il1b*) e a resposta ao estresse oxidativo (*sod1*). Novamente, todos os genes analisados foram induzidos nos zebrafish alimentados com dieta suplementada com o probiótico transgênico. Considerando que plasmídeo replicativo pode ser instável e levar a perda da característica ao longo das gerações bacterianas, no capítulo III abordamos a aplicação da tecnologia CRISPR-Cas9 para edição do genoma do *B. subtilis* usando a construção da fitase fúngica desenvolvida no capítulo I. Como resultado, obtivemos a cepa BsJJ14 com o gene da

fitase fúngica (oriunda de *A. fumigatus*) integrada ao genoma do *B. subtilis* e comparamos capacidade de manutenção da resistência ao antibiótico com a cepa Phy-Af, que no capítulo III denominamos de BsJJ5. Os resultados mostraram que após 65 gerações, apenas 11% das células (BsJJ5) mantiveram o fenótipo de resistência a antibióticos, e a linhagem transformada pelo método CRISPR-Cas9 não perdeu o fenótipo no final do experimento. O perfil de atividade da cepa BsJJ14 foi 20% maior do que na cepa BsJJ5 transformada com o plasmídeo replicativo. Em conclusão, a presente tese demostrou que as técnicas usadas para a transformação de *Bacillus* tanto via episssomal quanto integrada ao genoma foram eficientes e, além disso, a fitase recombinante apresentou atividade enzimática e promoveu efeitos benéficos à saúde do zebrafish alimentado com uma dieta rica em ingrediente vegetal.

Palavras-chave: Probiótico; proteína vegetal; fitato; transformação bacteriana; CRISPR-Cas9.

ABSTRACT

The phytase enzyme catalyzes the phytate hydrolysis, which represents the form of storage of phosphorus in vegetables. Phytate is an antinutritional factor for monogastric animals, since they are inefficient in the production of intestinal phytases. Therefore, the increment of plant ingredients in the feed in animal production systems is limited. The objective of the present thesis was to obtain a genetically modified *Bacillus subtilis* capable of expressing a fungal phytase and to evaluate the efficacy of this enzyme in the diet of zebrafish (*Danio rerio*). In chapter I we developed a replicative plasmid-based genetic construct containing the phytase gene from the fungus *Aspergillus fumigatus* (Phy-Af) fused to the secretory signal of the *B. subtilis* levansacarase (SacB) gene. The phytase activity produced by transgenic *B. subtilis* in the culture supernatant was 2.7 fold higher than the controls. After determining phytase activity, we performed a zebrafish feeding experiment for 30 days on a plant-rich diet. The experiment consisted of two groups: Phy-Af (with diet supplemented with *B. subtilis* expressing phytase) and the control group (diet with non-transgenic *B. subtilis*). The initial and final biometrics were used to evaluate the performance of the animals and to analyze the genes related to peptide transport (*slc15a1b* and *slc15a2*), appetite (*ghrl*), muscle growth factors (*igf1*, *myod* and *myog*) and mineral metabolism (*bglap*). The fish fed with transgenic *B. subtilis* presented better performance indices when compared to the control. In the same sense, all the genes analyzed were induced in the group treated with the probiotic expressing fungal phytase. In chapter II, the objective was to evaluate the effect of transgenic *B. subtilis* on the immune system and the inflammatory response in zebrafish fed with the feed rich in vegetable matter. In this case, genes related to the production of lymphocytes (*cd4* and *ikzf1*), inflammatory response (*ifn1*, *ifng*, *tnf-alpha* and *il1b*) and the response to oxidative stress (sod1) were analyzed. Again, all genes analyzed were induced in zebrafish fed a diet supplemented with the transgenic probiotic. Considering that the replicative plasmid may be unstable and lead to loss of trait throughout the bacterial generations, in chapter III we approached the application of CRISPR-Cas9 technology for editing the genome of *B. subtilis* using the fungal phytase construct developed in Chapter I. As a result, we obtained the strain BsJJ14 with fungal phytase integrated into the genome of *B. subtilis* and compared maintenance capacity of antibiotic resistance with the strain Phy-Af, which in chapter III we call BsJJ5. The results showed that after 65 generations, only 11% of the BsJJ5 cells maintained the

antibiotic resistance phenotype, and the strain transformed by the CRISPR-Cas9 method did not lose the phenotype at the end of the experiment. The activity profile of the BsJJ14 strain was 20% greater than in the BsJJ5 strain transformed with the replicative plasmid. In conclusion, the present thesis demonstrated that the techniques used for the transformation of *B. subtilis* both episomal and integrated into the genome were efficient and, in addition, recombinant phytase showed enzymatic activity and promoted beneficial health effects of zebrafish fed on a diet rich in vegetable ingredient.

Keywords: Probiotics; vegetable protein; phytate; bacterial transformation; CRISPR-Cas9.

INTRODUÇÃO GERAL

Aquicultura

A aquicultura é o sistema de produção de alimentos que mais cresce mundialmente, contribuindo substancialmente para o suprimento global de peixes. De acordo com a Organização das Nações Unidas para Alimentação e Agricultura (FAO 2018), em 2016 a aquicultura respondeu por cerca de 110,2 milhões de toneladas de pescados (incluindo plantas aquáticas). A taxa de crescimento entre 2001-2016 foi de 5,8%, os maiores índices de produção de pescados em 2016 foram liderados por países asiáticos como a China, por exemplo. O continente asiático respondeu por cerca de 71,5 milhões de toneladas, seguido pelos países da América com a produção de cerca de 3, 3 milhões de toneladas. A previsão é que a produção mundial de pescados, consumo e comércio continuem aumentando, porém em um ritmo mais lento. Enquanto a tendência para os próximos anos mostra que haverá uma desaceleração no crescimento da produção asiática, outras regiões como a África e América Latina manterão o ritmo de crescimento (FAO, 2018). A perspectiva é que a produção na região latino-americana cresça aproximadamente 49,2%. Além disso, essa região recebe as maiores projeções de consumo de pescado, com um crescimento acima de 18% até 2030 (FAO, 2018).

Dentre os países latinos, a produção na aquicultura brasileira ganhou força nas últimas décadas. Em 2016, o Brasil ocupava a oitava posição entre os principais produtores de pescado em águas interiores, e também vinha crescendo no cenário da produção de crustáceos marinhos (FAO, 2018). Segundo a FAO (2018), muitos mercados emergentes e exportadores, como o Brasil, Índia e Indonésia, ganharam importância e aumentaram a produção, em parte, graças a várias medidas que foram tomadas, como melhoramento nos sistemas de produção, no manejo adequado, na sanidade e no uso de alimentos com maior qualidade nutricional (Lebel et al., 2018; Santos & Ramos, 2018). Entre esses fatores que viabilizaram o crescimento da aquicultura, a alimentação é um fator chave para alcançar melhores índices de produção para a maioria das espécies.

Alimentação na aquicultura

Os alimentos usados na aquicultura são considerados completos por conter todos nutrientes essenciais para atender a demanda fisiológica, bioquímica, nutricional, de

crescimento, reprodução e saúde dos animais que vivem em sistemas de confinamento (Jobling, 2016; Hoseinifar et al., 2017). Segundo Tacon & Metian (2015) mais de 70% da produção total da aquicultura mundial depende do fornecimento de alimentação externa. A farinha e o óleo de peixes, ainda são as principais fontes de proteínas e lipídeos das rações aquícolas (Tacon & Metian, 2008; Olsen & Hasan, 2012; FAO, 2018). Esses ingredientes são capazes de promover o aumento da eficiência alimentar e o crescimento através de uma melhor palatabilidade, aumento da absorção de nutrientes e melhora na digestão (Tacon & Metian, 2008; Jobling, 2016; Hoseinifar et al., 2017). Embora esses ingredientes sejam de alta qualidade nutricional, eles são responsáveis por elevar o custo da produção animal (Olsen & Hasan, 2012, Asche & Oglend, 2016). Grande parte da composição proteica e lipídica das rações aquícolas depende de recursos naturais, como os peixes forrageiros (Pahlow et al., 2015). Isso é um fator limitante, pois a produção desses ingredientes flutuam de acordo com as variações das capturas dessas espécies, que é influenciada, principalmente, pela ocorrência de fenômenos climáticos como o El Niño, por exemplo (FAO, 2018). Espera-se que o uso de rações na aquicultura aumente à medida que a produção aquícola se expanda para atender às demandas do crescimento populacional. No entanto, a oscilação da oferta nas capturas e a elevada demanda pela aquicultura têm tornado esses insumos ainda mais caros e isto vem conduzindo ao uso de alimentos alternativos na composição das dietas aquícolas (Olsen & Hasan, 2012; FAO, 2018, Turchini et al., 2019). Embora o uso de peixes oriundos dos estoques naturais tenha reduzido nos últimos anos, esta ainda é a principal fonte para a produção de farinha de peixe (FAO 2018). Entre os setores de produção animal, a aquicultura é o maior usuário deste insumo (68%) (Tacon & Metian, 2015).

Como o setor de produção animal que mais cresce no mundo, a demanda da aquicultura por alimento não deverá ultrapassar a oferta ecológica e, além disso, a aquicultura assim como qualquer outro sistema de produção deve ser economicamente viável, socialmente justa e ambientalmente sustentável (Olsen & Hasan, 2012; Froehlich et al., 2018). Um desafio na aquicultura a é explorar o uso de produtos alimentares alternativos para melhorar o desempenho animal.

Ingredientes alternativos para rações na aquicultura

Desenvolver um recurso proteico seguro e sustentável a partir de ingredientes alternativos representa uma oportunidade para diminuir a dependência de recursos oriundos dos estoques nativos. Alguns estudos indicam que a inclusão de novas fontes proteicas como insetos, subprodutos de aves, biomassa microbiana como fonte de proteína e lipídeos, farinhas de carne e ossos podem ser uma alternativa à farinha de peixe em rações aquáticas (Jobling, 2016; Gamboa-Delgado & Márquez-Reyes, 2018). Entretanto, a inclusão de muitas destas fontes é limitada a técnicas de produção ineficientes, baixa oferta, instabilidade de mercado, custo de aquisição da biomassa, impactos ambientais, qualidade nutricional e digestibilidade (Tacon & Metian, 2015; FAO, 2018). Subprodutos de peixe processados também são alternativas de substituição por terem um perfil nutricional bastante semelhante ao da farinha e do óleo de peixe (Silva et al., 2018; Stevens et al., 2018). A incorporação de material vegetal na dieta é mais uma alternativa à substituição de proteína animal na aquicultura (Kokou & Fountoulaki, 2018). A inclusão de proteínas vegetais nas dietas pode fornecer cerca da metade da proteína dietética necessária para algumas espécies. Os concentrados proteicos produzidos a partir de soja, trigo e outros grãos ou oleaginosas são as fontes de proteínas alternativas mais promissoras para uso em alimentos aquáticos (Alhazzaa et al., 2018).

O aumento do preço da farinha de peixe tem tornado as proteínas vegetais mais competitivas (Asche & Oglend, 2016; Turchini et al., 2019). A proteína da soja é amplamente investigada como um substituto alternativo à proteina derivada da farinha de peixe, e isso é favorecido principalmente pela ampla oferta da soja nos últimos anos, ao custo razoável, oferta constante e suas atrativas características nutricionais como, elevado teor de proteína, digestibilidade e posssui alguns aminoácidos nutrionalmente importantes (Zhou et al., 2018).

Problemas dos ingredientes vegetais para rações na aquicultura

Os alimentos de origem vegetal, no seu conjunto, tem um conteúdo proteico inferior quando comparados com os de origem animal. Além disso, a presença de grandes quantidades de carboidratos, fibras e outras moléculas orgânicas, como fitatos e saponinas, apresentam ao nutricionista aquícola problemas que geralmente não são encontrados em fontes de origem animal (Makkar et al., 2007; Alhazzaa et al., 2018; Kokou & Fountoulaki, 2018). Apesar desses problemas, os alimentos comercializados

usam até certa medida, ingredientes vegetais em suas dietas na aquicultura. Porém, de acordo com Ghosh et al., (2019) a inclusão de matéria vegetal como única fonte de proteína resultou em perdas de produção e é uma preocupação para os aquicultores. Estes problemas estão fortemente atrelados à presença de antinutrientes que podem interferir na aceitação da ração e no desempenho animal (Kokou & Fountoulaki, 2018).

Alguns desses antinutrientes podem ser reduzidos, destruídos ou inativados pelo calor, fermentação ou processos mecânicos como trituração (Gupta et al., 2015; Nikmaram et al., 2017). Entretanto, componentes como a saponina e o fitato resistem a esses processos, apresentando baixos níveis de redução (Nikmaram et al., 2017). A soja, por exemplo, que é o principal ingrediente vegetal presente nas rações, tem níveis de inclusão limitada para as diversas espécies de peixes, pois é constituída por vários antinutrientes como os compostos fenólicos, saponinas, glucosinolatos, oligossacarídeos, proteínas antigênicas (por exemplo, glicinina e β-conglicinina) e fitatos (ácido fítico) (Li et al., 2017; Zhou et al., 2018). Estudos indicam que a inclusão do farelo de soja na alimentação influencia negativamente a saúde dos peixes, desencadeando distúrbios na função imunológica, processo inflamatório e retardo no crescimento (Ulloa et al., 2016; Li et al., 2017; Miao et al., 2018, Zhang et al., 2018). Em robalo-japonês (*Lateolabrax japonicus*), por exemplo, a substituição de farinha de peixe por farelo de soja na dieta resultou em expressão elevada de genes pró-inflamatórios intestinais (Zhang et al., 2018). Já em larvas de zebrafish (*Danio rerio*), Hedrera et al. (2013) indicaram que antinutrientes presentes no farelo de soja utilizado na suplementação da dieta causaram inflamação intestinal.

Ácido fítico

O ácido fítico, conhecido como hexafosfato de inositol ou fitato, possui de quatro a seis grupos fosfato ligados. O fitato é amplamente presente em vegetais, servindo principalmente, como um composto de armazenamento para íons de fósforo, inositol e fosfato inorgânico, que são usados no metabolismo energético da planta durante os processos, como a germinação (Dersjant-Li et al., 2015) (Figura 1). Em geral, o fitato representa entre 50-80% do fósforo total nos diferentes cereais, porém a quantidade de fitato em grãos e sementes é altamente variável.

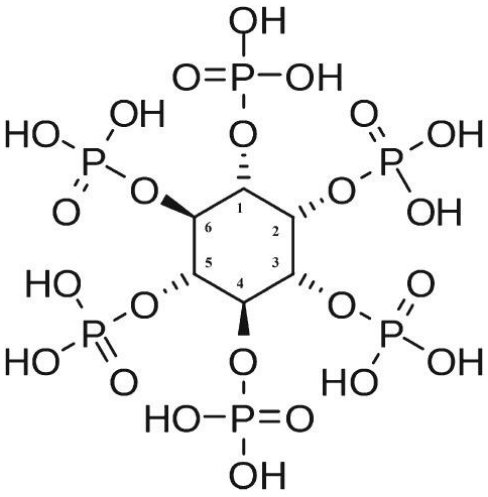

Figura 1- Estrutura química do ácido fítico (mio-inositol, 1, 2, 3, 4, 5, 6 - hexaquisfosfato). Fonte: Dersjant-Li et al. (2015).

Em sementes monocotiledôneas como trigo, cevada e arroz, o fitato se acumula na camada de aleurona (Coulibaly et al., 2011). O milho difere de outros cereais, pois mais de 80% do ácido fítico é concentrado no germe (Coulibaly et al., 2011; Kumar et al., 2012). Na soja, o teor de fitatos não é uniformemente distribuído na semente (Bartalné-Berceli et al., 2016), mas possui grandes quantidades de ácido fítico, que fornece mio-inositol, fósforo e cátions minerais necessários, durante a germinação da semente e estabelecimento de mudas. O ácido fítico é então depositado em corpos de proteína de soja, como fitina, por exemplo (Wang et al., 2015). A quantidade de fitato presente nas rações depende do tipo de vegetal incluído (tabela 1).

Tabela 1 - Conteúdo de fitato em ingredientes de rações comumente usadas (tabela reduzida a partir de Dersjant-Li et al., 2015; Humer & Zebeli, 2015).

Ingredientes	**Fitato P (g kg^{-1})**
Milho	1,7–2,05
Soja	3,88–4,53
Trigo	1,6–2,2
Cevada	1,69–1,96
Farinha de Canola	6,45–7,4
Centeio	2,2
Farinha de Girassol	7,48–7,7
Sorgo	1,8

O fósforo presente no ácido fítico é amplamente indisponível como um fator nutricional para os animais monogástricos, porque a capacidade de degradação

insuficiente no trato gastrointestinal impede que o fósforo esteja biologicamente disponível, por isso a alimentação com dietas à base de grãos leva à excreção do fitato por animais como aves, porcos e peixes (Dersjant-Li et al., 2015; Humer & Zebeli, 2015). A presença dos grupos fosfato faz do fitato uma molécula altamente carregada, ou seja, gera um campo eletronegativo possibilitando ligações com cátions de minerais divalentes formando um composto salino misto chamado fitato (Dersjant-Li et al., 2015; Humer & Zebeli, 2015). Essa eletronegatividade formada pelos grupos fostatos é capaz de quelar e diminuir a disponibilidade minerais como cálcio, magnésio, zinco, manganês, ferro e cobre (Humer & Zebeli, 2015). O fitato também pode formar complexos com proteínas e desta forma reduzir a quantidade de proteína disponível, a estabilidade destas e a sua digestibilidade. Também, reduz a disponibilidade de carboidratos e aminoácidos, interage com enzimas digestivas e forma complexos com lipideos e seus derivados (Kumar et al., 2012; Dersjant-Li et al., 2015). Assim, o fitato torna o fósforo indisponível para a maioria dos animais monogástricos como peixes, que são impossibilitados de utilizar de forma eficaz esse tipo de fósforo. Isso ocorre porque em muitas espécies há ineficiência ou mesmo ausência da produção de fitases intestinais que atuam na hidrólise do fitato durante a digestão (Kumar et al., 2012; Dersjant-Li et al., 2015). Dessa forma, a maioria do fósforo ligado ao fitato acaba sendo excretado pelos animais aquáticos, propiciando a proliferação de microalgas e, consequentemente, prejudicando o ambiente aquático (Kumar et al., 2012).

Sendo o fósforo um elemento indispensável para o crescimento de todos os organismos vivos, as dietas normalmente são completadas com fontes de fósforo inorgânico a fim de satisfazer as necessidades diárias (Kumar et al., 2012). Alguns processos químicos e mecânicos como a germinação, imersão, ebulição, autoclavagem podem ajudar na redução dos compostos antinutricionais como os fitatos presentes nos ingredientes vegetais. Porém, esses processos podem levar à perda parcial ou total de alguns nutrientes essenciais como proteínas e minerais e reduzir a qualidade do produto final (Gupta et al., 2015). Algumas abordagens levam a um campo moderno e eficaz como a adição de enzimas que hidrolisam o fitato ou no uso de ingredientes geneticamente modificados que contenham o teor de fitato reduzido (Herman & Schmidt, 2016; Alhazzaa et al., 2018). A redução enzimática dos fitatos na indústria de produção animal vem sendo a rota preferida para o alcance da eficiência alimentar, sem a perda da qualidade nutricional e sem a presença de reações indesejáveis. Neste

contexto, as fitases ganham considerável destaque devido sua capacidade de degradar o fitato ao serem adicionadas às rações comerciais.

Fitases

Fitase, ou mioinositol-hexafosfato fosfohidrolase, é uma fosfatase fitato-específica que catalisa a hidrólise do ácido fítico formando inositol e fosfatos (Bhavsar & Khire, 2014). De acordo com a classificação filogenética e as características bioquímicas, as fitases são divididas em três grupos diferentes: 3-fitases (EC 3.1.3.8), 6-fitases (3.1.3.26) e 5-fitases (3.1.3.72) (Bhavsara & Khire, 2014). Conforme o pH de atividade, as fitases são classificadas como ácidas ou alcalinas. Quanto às suas características estruturais e catalíticas, as fitases são divididas em três subgrupos: histidina fosfatase ácida (HFA), ß- hélice (FBH) e fosfatase ácida "purple" (FAP) (Bhavsara & Khire, 2014). A fitase é amplamente encontrada em vegetais e em micro-organismos (bactérias e fungos) (Bhavsara & Khire, 2014; Dersjant-Li et al., 2015).

As fitases microbianas podem ser produzidas intracelularmente ou secretadas para o meio extracelular, sendo funcionais em aplicação nos alimentos para processos de fermentação (Bhavsara & Khire, 2014; Dersjant-Li et al., 2015). Os fungos filamentosos também produzem fitases, sendo que a maioria das fitases comercialmente disponíveis são derivadas das espécies pertencentes ao gênero *Aspergillus*. Com o uso das técnicas de engenharia molecular, as propriedades das fitases de fungos estão sendo constantemente melhoradas (Bhavsara & Khire, 2014; Dersjant-Li et al., 2015). As fitases de *Aspergilus fumigatus* estão classificadas no grupo das histidinas fosfatase ácida (HFA), e se destacam por exibir termoestabilidade superior em comparação com outras fitases fúngicas, chegando a apresentar 70% de atividade relativa a 90°C por 20 minutos (Rodriquez et al., 2000; Rebello et al., 2017). Além disso, são fitases com atividade mínima de 80% em pHs variando de 4.0-7.3 (Wyss et al., 1999; Singh et al., 2016). As fitases comercialmente disponíveis são, em geral, do tipo histidinas ácidas de *Aspergillus* e *Escherichia coli* (Mukhametzyanova et al., 2012; Dersjant-Li et al.; 2015).

A adição de fitases na alimentação de suínos e aves é uma prática comum e seus benefícios já estão muito bem documentados. Porém, seu uso na área da aquicultura ainda é insipiente. Entretanto, alguns estudos já têm demonstrado que os benefícios incluem maior digestibilidade e biodisponibilidade de fósforo, nitrogênio e outros

minerais, redução na quantidade necessária de fosfóro inorgânico, melhora na mineralização dos ossos; diminuição da quantidade de fósforo excretado para o ambiente aquático e estímulo do sistema imunológico (Kumar et al., 2012; Bhavsar & Khire, 2014). Zebrafish alimentados com ração rica em vegetais e suplementados com uma fitase purificada apresentaram aumento no teor lipídico, ganho de peso, crescimento específico e melhora da qualidade da água de cultivo (Liu et al., 2013). Em nível de expressão gênica, alguns relatos indicam que as fitases participam ativamente na melhoria dos níveis de expressão dos transportadores de peptídeos *slc15a1b* e *Slc15a2* (Rønnestad et al., 2014; Lo Cascio, 2018). Ainda, como visto por Bano & Muhammad (2017), a fitase foi capaz de melhorar a mineralização óssea em *Labeo rohita*.

Vários estudos têm focado seus esforços na identificação e na caracterização de fitases em diferentes fontes. Entretanto, a engenharia de proteínas é a principal ferramenta utilizada para promover melhorias nas enzimas e a engenharia genética pode auxiliar no desenvolvimento de novos organismos biorreatores mais eficientes (Bhavsar & Khire, 2014). Muitas proteínas recombinantes são produzidas em bactérias, fungos, células animais e vegetais (Liu et al., 2013). Cada um desses sistemas tem vantagens e desvantagens relacionadas a fatores como custos, segurança na produção, facilidade na extração, purificação e complexidade na produção de moléculas (Liu et al., 2013).

A suplementação de fitases na aquicultura é efetuada na forma purificada. Isto representa um obstáculo, pois o isolamento e a purificação de enzimas é um processo, em sua maioria, difícil e de alto custo (Dudley et al., 2014; Singh et al., 2016). Uma alternativa ao uso de fitases purificadas é a utilização de micro-organismos potencialmente produtores destas enzimas. Várias espécies de *Bacillus* são amplamente utilizadas na alimentação aquática como probióticos. *Bacillus subtilis*, em particular, é um dos principais produtores industriais de enzimas e um ótimo hospedeiro microbiano GRAS (geralmente reconhecido como seguro) para a expressão heteróloga de proteínas (Syngai et al., 2015).

Probióticos

Há diversas versões para o conceito de probióticos, mas em 1989 Fuller deu uma definição mais precisa para este termo e historicamente probióticos são reconhecidos

como sendo um suplemento alimentar microbiano vivo que afeta beneficamente o animal hospedeiro por melhorar seu equilíbrio intestinal (Banerjee & Ray, 2017). Ao longo das décadas muitos estudos vêm trazendo a luz muitas informações que corroboram constantemente ao endendimento sobre tipos, aplicações, efeitos e benefícios dos probióticos.

Segundo Zhao et al. (2019) existem 1.730 probióticos conhecidos (768 de produção, 264 de pesquisa, 22 de proteção ambiental, 17 de controle de doenças e 659 outros), os quais estão sendo usados em humanos, animais e plantas. Na aquicultura já foram classificadas diversas espécies de micro-organismos com atuação probiótica como, por exemplo, *Lactobacillus rhamnosus*, *Lactobacillus casei* BL23, *Staphylococcus* sp., *Enterococcus feacium*, espécies do gênero *Bacillus*, entre outros. Atua desempenhando melhorias na reprodução, qualidade da água, proteção contra vírus, contra bactérias patogênicas e produção de enzimas (Banerjee & Ray, 2017).

Dentre os grupos de probióticos já identificados para uso na aquicultura, as bactérias do gênero *Bacillus* são classificadas como promotoras de diversos benefícios (Banerjee & Ray, 2017; Kuebutornye et al., 2019). Segundo Fritze (2004), este gênero está constituído de 88 espécies, dentre as quais *Bacillus subtilis* é a espécie mais relevante deste grupo. *Bacillus subtilis* é uma bactéria gram-positiva, capaz de esporular (Hong et al., 2005; Banerjee & Ray, 2017; Kuebutornye et al., 2019). *Bacillus subtilis* é altamente resistente ao calor e produtos químicos e é capaz de sobreviver ao pH do fluido gástrico devido à formação de esporos, tornando-a espécie ideal como aditivo alimentar para uso humano e animal (Chen et al., 2016). É uma espécie amplamente utilizada na alimentação de organismos aquaticos como probiótico, pois é capaz de melhorar o desempenho no crescimento, aumentar as respostas imunológicas, fornecer nutrientes, controlar infecções bacterianas e melhorar a qualidade da água (Kang et al., 2014; Ÿztürk et al., 2016). Além desses benefícios, este probiótico é um candidato promissor em aplicações biotecnológicas com protocolos bem estabelecidos para a modificação genética (Kang et al., 2014; Yang et al., 2016). É considerado como um hospedeiro de expressão alternativa para produção de proteínas heterólogas. Esta bactéria gram-positiva não produz endotoxinas e secreta proteínas para o meio de cultura, simplificando a purificação de proteínas recombinantes (Chen et al., 2016; Ÿztürk et al., 2016).

Proteínas recombinantes

Dada à importância das proteínas em aplicações industriais, nutricionais e médicas, a produção de proteínas atraves da tecnologia do DNA recombinante cresceu rapidamente. A tecnologia do DNA recombinante envolve o uso de recombinação genética para reunir material genético de múltiplas fontes, criando sequências de DNA que não são naturalmente encontradas no genoma. Proteínas produzidas com a tecnologia de DNA recombinante são chamadas de proteínas recombinantes (Wingfield, 2015). As proteínas recombinantes proporcionaram avanços importantes na biotecnologia. Os primeiros avanços ocorreram a partir da produção da insulina humana recombinante em 1982, e daí em diante a tecnologia de proteínas recombinantes se expandiu largamente para as mais diversas áreas, incluindo os setores de produção animal como a aquicultura (Wingfield, 2015).

Gerar uma proteína recombinante requer o uso de um sistema de expressão eficiente, e a seleção de um sistema de expressão apropriado depende das características e da aplicação pretendida da proteína recombinante (Celie et al., 2016; Freudl, 2018). Ao longo de décadas, *B. subtilis* vem sendo testado como uma plataforma eficiente para a expressão de diversas proteínas heterólogas (Chen et al., 2016; Zhang et al., 2017). Os avanços para a geração de proteínas recombinantes estão diretamente relacionados à descoberta dos plasmídeos por volta de 1950 (Summers, 2015). Os plasmídeos constituem a base para a clonagem de DNA e de expressão gênica na biotecnologia atual. Entretanto, há uma preocupação recorrente quanto ao uso de plasmídeos em relação à possibilidade da transferência horizontal de genes para espécies selvagens (Nielsen et al., 2014). Em alguns casos como, por exemplo, os plasmídeos replicativos com transferência episomal, pode haver uma instabilidade estrutural, afetando a manutenção do plasmídeo ao longo das gerações bacterianas (Yang et al., 2016). Portanto, é indispensável à mantutenção uma população de bactérias que contêm o plasmídeo de interesse para o sistema continuar funcional. Além disso, grande parte dos plasmídeos é dependente de marcadores de seleção com resistência a antibióticos, o que gera forte resistência para a inclusão de microrganismos com este tipo de engenharia no processo de produção de proteínas em sistema industrial e para suplementação na alimentação (Ho, 2014).

Estas questões, no entanto, podem ser contornadas através do desenvolvimento de diversas tecnologias, dentre elas os vetores bifuncionais, que são tanto segregacional quanto estruturalmente estáveis e pela recombinação homóloga (Goyal et al., 2018). A integração de elementos genéticos no cromossomo dos hospedeiros possibilita melhorar os processos metabólicos e aumentar a atratividade para a produção de proteínas em escala industrial (Palomares et al., 2004). Visto que a instabilidade de plasmídeos é problemática e o uso de antibióticos para seleção é indesejável, a tecnologia CRISPR-Cas9 tornou-se uma abordagem alternativa para mediar essas questões limitantes para a produção de proteínas recombinantes (Altenbuchner, 2016; Yang et al., 2016).

Sistema CRISPR-Cas9 para edição do genoma

A engenharia genética moderniza constantemente seus procedimentos moleculares a fim de atender a demanda por processos seguros, rápidos e consistentes. Por isso, uns dos sistemas mais modernos que podem ser utilizados para obtenção de probióticos transgênicos é o sistema CRISPR-Cas9 (Altenbuchner, 2016; Wang et al., 2016). Ocorrendo naturalmente em bactérias e arqueas, o sistema CRISPR-Cas do tipo II atua como um sistema imune adaptativo formando uma barreira de proteção contra vírus e plasmídeos invasores. O lócus CRISPR-Cas é constituído primeiramente por um conjunto de repetições palindrômicas curtas, interespaçadas e regularmente agrupadas (*do inglês, Clustered Regularly Interspaced Short Palindromic Repeats, CRISPR*) (Hsu et al., 2014; Altenbuchner, 2016; Wang et al., 2016). Esse conjunto de repetições são intrecaladas com pequenos fragmentos de DNA do invasor, como um vírus, por exemplo. Outro componente do lócus CRISPR-Cas é o operon de genes codificadores das proteínas Cas (*CRISPR-Associated proteins*), entre as quais a Cas9 é a endonuclease de DNA orientada por RNA de especificidade facilmente personalizável. A Cas9 é usada como uma ferramenta para edição programada do genoma e controle transcricional, tanto em células eucarióticas quanto procarióticas (Hsu et al., 2014; Jakutyte-Giraitiene & Gasiunas, 2016).

A enzima Cas9 foi originada de *Streptococcus pyogenes* e possui dois domínios nucleases amplamente utilizados para engenharia do genoma mediada por CRISPR. Após transcrito, esse sistema forma um complexo composto por enzima Cas e uma estrutura de RNA duplo formado por RNA CRISPR (crRNA) e crRNA transativador (tracrRNA) (Altenbuchner, 2016). O método de engenharia génetica mediado por

CRISPR-Cas9 superou as limitações que os métodos anteriores tinham, e começou a gerar mutantes livres de DNA estranho confiáveis em muitas cepas bacterianas do tipo selvagem, incluindo *B. subtilis* (Hsu et al., 2014; Altenbuchner, 2016; Wang et al., 2016). A introdução deste sistema não só eliminou a necessidade de usar marcadores de seleção para edição de genoma, como marcadores a base de antibióticos, por exemplo, mas também aumentou drasticamente a eficiência de edição (Altenbuchner, 2016). Para aplicações industriais e de grau alimentício, traços de DNA estranho nas células devem ser removidos por cura de plasmídeo e isso torna difícil o rastreamento dos clones com os genes alvos integrados ao genoma. Por isso, o uso de genes de proteínas fluorescentes como mecanismo de rastreio de transformantes positivos é uma ferramenta alternativa (Wong et al., 2010; Jeong et al., 2018)

A proteína verde fluorescente (GFP) oferece maneiras eficientes de visualizar a expressão gênica e o direcionamento de proteínas (Mullineaux, 2016). A GFP pode ser facilmente detectada por contagem de colônias fluorescentes sob luz UV de comprimento de onda na faixa de 490 nm, microscopia de epifluorescência, microscopia confocal a laser e citometria de fluxo para células individuais ou medição direta de fluorescência. Além disso, os fenótipos GFP são detectáveis em todas as fases de crescimento das bactérias, mesmo sob condições de estresse nutricional (Ma et al., 2011; Trip et al., 2011).

Zebrafish (*Danio rerio*)

A vasta literatura demonstra que *Danio rerio*, também conhecido como zebrafish ou paulistinha, tem sido cada vez mais utilizado como uma ferramenta de pesquisa. Isso se justifica pelo fato de ser uma espécie de pequeno porte, sendo possível sua criação em pequenas áreas, de fácil manutenção, alta taxa reprodutiva, além de possuir seu genoma sequenciado (Ribas & Piferrer, 2014; Simonetti et al., 2015). Esta espécie tem sido considerada como modelo em estudos de toxicologia, desenvolvimento, neurobiologia, estudos genéticos, sendo possivel avaliar os efeitos fisiológicos, celulares, e processos moleculares usando metodologias inovadoras (Simonetti et al., 2015; Leigh et al., 2018).

Nos últimos anos, o zebrafish tornou-se um modelo popular em muitos campos de pesquisa da aquicultura (Ribas & Piferrer, 2014), sendo considerado um importante

organismo para o entendimento dos mecanismos nutricionais (Leigh et al., 2018). No contexto nutricional, em comum com muitas espécies de produção na aquicultura, o zebrafish demanda um teor elevado de proteína na composição de suas dietas, tendo a farinha peixe como sua principal fonte proteica (Ulloa et al., 2014; Fernandes et al., 2016). Assim, nos últimos anos muitas pesquisas buscam melhor compreender o padrão de substituição da farinha de peixe por ingredientes vegetais, como os derivados da soja, por exemplo, usando o zebrafish como modelo e avaliando os efeitos sobre o crescimento, resposta imune, respostas antioxidantes e repostas inflamatórias. Desta forma, entendemos que esta é uma espécie modelo, capaz de fornecer uma ampla contribuição em termos de nutrição no desafio de substituir as fontes proteicas nas rações e analisar o efeito de enzimas digestivas.

OBJETIVO

Objetivo Geral

Produzir uma linhagem de *Bacillus subtilis* geneticamente modificado para a expressão de uma fitase fúngica e avaliar os efeitos na dieta de zebrafish (*Danio rerio*) alimentado com dieta rica em matéria vegetal.

Objetivos específicos

- Produzir uma construção genética para o gene da fitase de *Aspergillus fumigatus*, otimizando para expressão e secreção da proteína recombinate em *Bacillus subtilis*;

- Transformar a cepa de *B. subtilis* KM0 com a construção da fitase;

- Determinar a atividade enzimática da fitase recombinante;

- Testar a eficácia da fitase expressa pela cepa *B. subtilis* KM0 como suplemento em uma ração rica em vegetal para *Danio rerio*, avaliando o desempenho zootécnico*;*

- Avaliar o perfil de expresão gênica de fatores nutricionais, fatores de crescimento, mineralização óssea, resposta imune, resposta inflamatória, resposta oxidativa e fator de condição de *D. rerio* alimentados com dieta a base de ingredientes vegetais suplementada com *B. subtilis* KM0 transgênico para o gene da fitase de *Aspergilus fumigatus.*

- Desenvolver uma cepa de *B. subtilis* através do sistema CRISPR-Cas9 para integração da construção da fitase, avaliar a atividade enzimática e a estabilidade do plasmídeo.

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CAPÍTULO I

Bacillus subtilis secreting fungal phytase expressively improves the zootechnical parameters of zebrafish (*Danio rerio*) fed with high content of vegetable matter

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Bacillus subtilis secreting fungal phytase expressively improves the zootechnical parameters of zebrafish (*Danio rerio*) fed with high content of vegetable matter

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Resumo

A aquicultura é um importante setor da produção mundial de alimentos, para o qual a fonte de proteína para dietas comerciais é um enorme gargalo. As proteínas vegetais são mais baratas, mas são acompanhadas por compostos antinutricionais como o fitato. As fitases são enzimas produzidas em microrganismos capazes de degradar o fitato. A adição de fitases purificadas a rações comerciais melhora a absorção de nutrientes da matéria vegetal. No entanto, a produção e purificação de fitases têm um alto custo. Uma alternativa é manipular geneticamente uma bactéria probiótica para produzir e secretar fitase diretamente no trato digestivo do hospedeiro. No presente estudo, uma cepa de *Bacillus subtilis* foi projetada para produzir e secretar uma fitase fúngica. Os peixes alimentados com alto teor de vegetais suplementados com probiótico transgênico apresentaram expressiva melhora nos parâmetros zootécnicos analisados. Estes resultados foram corroborados com a expressão de genes relacionados ao transporte de peptídeos (*slc15a1b* e *slc15a2*), apetite (*ghrl*), crescimento somático (*igf1*, *myod* e *miod*) e metabolismo ósseo (*bglap*). Todos os genes analisados foram fortemente induzidos no grupo de peixes tratados com o probiótico transgênico, corroborando com os resultados observados para os parâmetros zootécnicos. Este estudo mostra, pela primeira vez, que uma fitase recombinante expressando probiótico transgênico é capaz de minimizar os efeitos antinutricionais do alto conteúdo de matéria vegetal incluída na dieta dos peixes. Os resultados aqui apresentados abrem a possibilidade de os probióticos transgênicos serem utilizados como biorreatores para a produção de moléculas capazes de interferir em outros sistemas fisiológicos nos peixes.

Palavras-chave: Fitato; probiótico transgênico; transporte peptídico; apetite; crescimento somático; metabolismo ósseo.

Abstract

Aquaculture is an important sector of world food production for which the source of protein for commercial diets is a huge bottleneck. Vegetable proteins are cheaper, but are accompanied by antinutritional compounds like phytate. Phytases are enzymes produced in microorganisms capable of degrading phytate. The addition of purified phytases to commercial feed improves the nutrient absorption of vegetable matter. However, the production and purification of phytases has a high cost. An alternative is to genetically manipulate a probiotic bacterium to produce and secrete phytase directly into the host's digestive tract. In the present study, a strain of *Bacillus subtilis* was engineered to produce and secrete a fungal phytase. The fish fed with a high vegetable content supplemented with transgenic probiotic showed an expressive improvement in the zootechnical parameters analysed. These results were corroborated with the expression of genes related to peptide transport (*slc15a1b* and *slc15a2*), appetite (*ghrl*), somatic growth (*igf1*, *myod* and *myog*) and bone metabolism (*bglap*). All the genes analysed were strongly induced in the group of fish treated with the transgenic probiotic, corroborating with the results observed for the zootechnical parameters. This study shows, for the first time, that a transgenic probiotic expressing recombinant phytase is able to minimize the antinutritional effects of the high content of vegetal matter included in the fish diet. The results presented herein open the possibility of transgenic probiotics being used as bioreactors for the production of molecules capable of interfering in other other physiological systems in fish.

Key-words: Phytate; transgenic probiotic; peptide transport; appetite; somatic growth; bone metabolism.

Introduction

Aquaculture is one of the fastest growing industrial sectors in the world. However, the high demand for fishmeal in feed composition is still a concern regarding economic and environmental impacts. Although the use of fish from natural stocks has declined in recent years, it is still the main source for fishmeal production (FAO 2018); accounting for about 50% of protein sources in commercial diets (Montoya-Camacho et al. 2018). Among the animal production sectors, aquaculture is the largest user of this input (68%) (Tacon and Metian 2015). World demand is expected to raise the price of fishmeal between 16% and 20% by 2030 (FAO 2018), further contributing to the increase in the cost of production in aquaculture. With this, the aquaculture industry is encouraged to promote the inclusion of alternative sources of fishmeal. Plant ingredients are promising candidates for substitutes due to the wide supply and nutritional quality (Hardy 2010, Herman and Schmidt 2016; Han et al. 2018). However, the antinutritional complexes formed by phytates and the large undigested phosphorus load limit the supplementation of plant ingredients in fish diets (Kokou and Fountoulaki 2018). The enzymatic reduction of phytates in the animal production industry has been the preferred route to achieve food efficiency (Kirk et al. 2002; Lemos and Tacon 2017). Phytase (myo-inositol-hexaquisphosphate phosphohydrolase) is an enzyme of the phosphatase class and catalyzes the phytate hydrolysis, resulting in the formation of myo-inositol pentaqui-, tetrakis-, tris-, bis- and monophosphate isomers as well as the release of minerals, proteins, amino acids and enzymes complexed to it (Greiner and Konietzny 2010; Lv et al. 2015). Histidine acid phosphatases (HAPs) include the largest number of representatives already studied, and among these, the phytases produced by the fungus *Aspergilus fumigatus* stand out for their high thermostability (Wyss et al. 1999; Rebello et al 2017; Singh et al. 2016), in addition to high activity at pHs between 4.0-7.3 (Wyss et al. 1999, Singh et al. 2016).

The addition of phytase to the diet of fish and crustaceans has contributed to the bioavailability of phosphorus, as well as providing a positive effect on growth and body composition (Dersjant-Li et al. 2015, Abo Norag et al. 2018, Biswas et al. 2019; Li et al. 2019). Its effects and effectiveness are known, but the availability of phytases that are widely effective for aquaculture is still limited. Also, the phytases used in feed supplementation for aquaculture species are purified enzymes of fungal origin. This is

an obstacle, since the isolation and purification of enzymes is a difficult and costly process (Dudley et al. 2014; Singh et al. 2016). An alternative is the use of genetically modified probiotic microorganisms for the expression of heterologous phytases. Several species of *Bacillus* are widely used as probiotics in feeding aquatic organisms. *Bacillus subtilis*, in particular, is a microorganism generally recognized as safe (GRAS) and an excellent bioreactor for the production of heterologous proteins (Syngai et al. 2015). Some strains of *B. subtilis* have already been genetically engineered to facilitate the production of recombinant proteins. An example is the KM0 strain developed by Rahmer et al. (2015). This strain is characterized for being highly competent for genetic transformation, and has great potential for the expression of heterologous proteins.

In recent years, zebrafish (*Danio rerio*) has become a popular fish model in many aquaculture research fields (Ulloa et al. 2011; Leigh et al. 2018). Using this model, Liu et al. (2013) observed that the supplementation of high-dose purified phytase in the zebrafish diet resulted in increased lipid content, weight gain, specific growth and improved water quality. The literature is still scarce regarding the contribution of probiotic microorganisms producing phytases in fish diets. According to Gioacchini et al. (2014), probiotics in zebrafish diet positively influences growth, larval development and improves reproductive performance. Thus, this fish model seems to be adequate for the study of the nutritional effects resulting from the supplementation of genetically modified probiotics. In this context, the objective of the present study was to supplement a plant-rich diet with a genetically modified strain of *B. subtilis* that expresses a fungal phytase and to evaluate its effect in zootechnical parameters of zebrafish and in the expression of genes related to appetite, transport of intestinal peptides, somatic growth and bone mineralization.

Material and Methods

Experimental diet

The formulated diet contained 40.84% protein, with 21.6% of the total proteins being of vegetal origin. Analysis of the proximal feed composition (Table 1) followed AOAC protocols (1999). The diet was pelleted, oven dried and stored at -20^{o}C.

Bacterial strains

Escherichia coli BL21 (DE3) (Invitrogen, Brazil) was used as host for DNA manipulations. *Bacillus subtilis* KM0 was kindly provided by Dr. Josef Altenbuchner of the Universitat de Stuttgart (Germany).

Genetic construction for phytase expression in *B. subtilis*

Previously, the phytase nucleotide sequence of *Aspergillus fumigatus* (GenBank: AHZ62778.1) and the signal peptide (SacB) of levansucrose from *B. subtilis* (GenBank: CAA26513.1) were selected. The fused gene was codon-optimized for expression in *B. subtilis* through OPTIMIZER software (http://gnemos.urv.es/OPTIMIZER). The stationary phase promoter of *B. subtilis* P_{ylb} (Yu et al. 2015) was used to drive SacB-Phytase expression. The complete genetic construct (1,544 bp) was synthesized by the GeneArt service (Life Technologies, Brazil). Primers Pylb (*Sca*I)-FOR (5'-AGTACTACTTCTCAAAGATCCATGT-3') and Phy (*XmaI*)-REV (5'-CCCGGGTTAAGAGAGCATTCGCCC-3') were used to amplify, by PCR, the entire construct. Both primer included sites for restriction enzymes *Sca*I and *Xma*I at 5' region for future subcloning. PCR conditions were: 30 cycles of 60 sec at 94°C, 30 sec at 60^{o}C, 90 sec at 72°C, followed by a final step of 10 min at 72°C. The PCR product was purified (Illustra GFX™ PCR DNA and Gel Band Purification Kit; GE HealthCare, Brazil), digested with *Sca*I and *Xma*I (New England Biolabs, Brazil), and subcloned at the corresponding sites of the pJJ5 plasmid (kindly provided by Dr. Josef Altenbuchner from Universitat of Stuttgart, Germany). Replicative plasmid pJJ5 contains two origins of replication: one for gram-negative bacteria derived from the pUC18 plasmid, and the other for gram-positive bacteria derived from plasmid pUB110. In addition, pJJ5 also contains a spectinomycin resistance gene. The resultant plasmid (pJJ5-SacBPhy; Fig. 1) was confirmed by sequencing.

Transformation of *B. subtilis* KM0

Bacillus subtilis KM0 was transformed with the replicative pJJ5-SacBPhy plasmid according to the standard two-step procedure with minimal Spizizen medium (Anagnostopoulos and Spizizen, 1961). Transformed colonies were selected in solid LB (Luria-Bertani) medium containing spectinomycin (100 μg.mL^{-1}) after overnight incubation at 37°C.

Phytase activity

Colonies of *B. subtilis* transformed with the plasmid pJJ5-SacBPhy were used as *inocula* for culture in liquid LB medium containing spectinomycin (100 μg.mL^{-1}). Optical density (OD = 600 nm) of the culture was monitored until the stationary phase of growth (approximately 16 h). After, the culture was centrifuged at 8,000 x g for 30 min at 4°C. The supernatant was centrifuged again under the same conditions and then used for enzymatic analysis. Phytase activity was determined according to the method proposed by Heinonen and Lahti (1981), with minor modifications for assays in 96-well plates. The activity was determined in the supernatant having as substrate 1 mM sodium phytate ($C_6H_6O_{24}P_6Na_{12}$; Sigma-Aldrich, Brazil) buffered with 2 M sodium acetate (pH 5.0). The reaction occurred for 30 min at 40°C and was determined by the addition of the color reaction prepared with acetone, 5 M sulfuric acid and 10 mM ammonium molybdate (2:1:1, v/v). After 30 sec, the addiction 1 M citric acid stopped the reaction. The available inorganic orthophosphate was measured. Five replicates were used for each assay. The absorbance was read at 405 nm. One unit of phytase activity was defined as the amount of enzyme required to release 1 μmol of phosphate per minute, under assay conditions. All procedures to evaluate phytase activity were also performed on untransformed *B. subtilis* colonies, which were considered as controls.

Zebrafish diet supplemented with *B. subtilis* KM0

Bacillus subtilis was grown in medium LB containing spectinomycin (100 μg.mL^{-1}) for approximately 16 hs (overnight) at 37°C, under agitation at 250 rpm. Then, the cells were collected by centrifugation at 8,000 x g for 10 min, at 4°C. The supernatant was discarded. The bacterial pellet was washed with sterile saline, resuspended with sterile water, and sprinkled on feed. The density measurement of the cells in suspension was performed with a spectrophotometer (OD = 600 nm) and related to the colony forming units (CFU). Following the recommendations of Xu et al. (2015), the dose of *B. subtilis* KM0 was 10^{10} CFU/feed g. According to the authors, this dosage administered orally is able to survive and colonize the intestinal tract of the zebrafish. The feed was daily supplemented with the probiotic, which according to Merrifield et al. (2010), is an effective and viable method for administration of *Bacillus* species. All procedures were performed for both transformed and non-transformed strains.

Zebrafish assay

A total of 120 adults of the zebrafish (*Danio rerio*) with mean initial weight of 215. 6 (± 6.03) mg were used, with no difference in initial weight between treatments. The fish were distributed in a completely randomized design in glass aquaria with capacity for 5 L of water at the density of 2 fish/L, as recommended by Schneider et al. (2009). Before the start of the experiment, the fish were acclimated for 15 days. The experimental group consisted of two experimental groups with six replicates. One group received feed supplemented with non-transformed *B. subtilis* (Control), while the other group received the transgenic phytase-expressing probiotic (phy-Af). During the acclimation period, the fish were fed with commercial feed (Tetra Colorbits, Germany). From the beginning of the experiment, the commercial feed was replaced with the experimental feed (Table 1) for 30 days. The experiment was carried out in a static and biofiltered system. The tanks were cleaned daily and about 70% of the total volume of water was removed. Water treatment was carried out with hypochlorite to eliminate genetically modified probiotics (DeQueiroz and Day 2008). The quality of the water was monitored regularly twice a week, and the temperature, pH, oxygen, nitrogen compounds and photoperiod were kept at the optimum levels recommended for zebrafish (Siccardi et al. 2009; Avdesh et al. 2012). For the weighting procedure, the animals were anesthetized in tricaine buffered solution (100 $mg.L^{-1}$) and euthanized with lethal tricaine dose (500 $mg.L^{-1}$). At the end of the 30-day period, the fish were dissected and tissues were collected for gene expression analyzes.

From the biometric data were calculated: Weight Gain (%): $WG = [(W_f-W_i) / W_i] \times 100$, where W_f = final weight e W_i = initial weight; Specific Growth Rate: $SGR = [(\ln W_f - \ln W_i) \times 100] / T$, where T = experimental time (30 days); Survival: $S = 100 \times$ (final fish number / initial fish number); Total Feed Consumption: $TFC = \sum DFI$, where DFI = daily feed intake; Apparent Feed Conversion: AFC = TFC / WG. The daily feed intake (DFI) was calculated as follows: a certain amount of feed was weighed at the beginning of the day. This feed was used for the daily feeding of each tank until the satiety, that is, when the fish stopped to search for food. At the end of the day, the leftover feed was weighted and the difference between the initial weight and the leftover was considered the daily consumption of each tank.

RNA extraction and cDNA synthesis

Total RNA was isolated from the intestine, liver, muscle, and bone of ten individuals per treatment using TRIZOL reagent (Life technologies, USA), following the manufacturer's recommendations. Total RNA was treated with the RNAse free DNAse I kit (Invitrogen, Brazil), and quantified spectrophotometrically (BioDrop, Isogen Life Science, B.V, Veldzigt, Netherlands). The quality and integrity of total RNA was analysed by 1% agarose gel electrophoresis. The cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Brazil), following the manufacturer's instructions.

Gene expression

Target genes were previously selected at GenBank (https://www.ncbi.nlm.nih.gov), with the specific primers being designed using GenBank Primer-Blast tool (Table 2). Quantitative analysis of genes was performed by real-time quantitative reverse transcriptase PCR (qRT-PCR). For each reaction, cDNA was diluted 5X and used following the recommendations of the PowerUP SYBR Green Master Mix kit (Applied Biosystems, Brazil), using the 7500 Real-time platform PCR system (Applied Biosystems, Brazil). The efficiency of all primers was measured by serial dilutions of the cDNA (Table 2). The conditions of the qPCR reactions were as follows: an initial step of 50°C / 2 min and 95°C / 2 min, followed by 40 cycles of 95°C / 15 sec and 60°C / 15 sec. The dissociation curve was performed at 95°C / 15 sec, 60°C / 1 min and 95°C / 30 sec. The target genes analysed in the gut were peptide transporters *slc15a1b* and *slc15a2* (PepT1 and PepT2, respectively) and ghrelin (*ghrl*). In the muscle were analysed genes related to myogenic differentiation (*myod* and *myog*), and muscle hypertrophy as *igf1* (insulin-like growth factor I). This gene was also analysed in the liver. Osteocalcin (*bglap*) was used as the gene related to bone metabolism. For the normalization of expression data, *ef1α* (elongation factor 1 alpha), *actb1* (β-actin), *rpl13a* (ribosomal protein L13 alpha) and *β2m* (β-2-microglobulin) genes were used (Table 2). Normalizing genes were evaluated by the geNorm software (Vandesompele et al. 2002).

Statistical analyzes

The data were previously tested for their normality and homogeneity. Significant differences between treatments were determined by Student's t test. Results are

expressed as mean ± standard error of the mean (SEM). For all analyzes, the significant difference was considered when $p < 0.05$.

Ethics statement

Zebrafish cultivation and experimental procedures were approved by the Ethics Commission on Animal Use (CEUA) of the Federal University of Rio Grande (FURG), under approval number P002/2019.

Results

Bacillus subtilis KM0 transformation and phytase activity

The transformation protocol produced dozens of *B. subtilis* colonies resistant to spectinomycin. Of these, at least 15 were cultured in liquid LB medium in the presence of the antibiotic. After centrifugation, the supernatant was recovered and the phytase activity was evaluated. In all transformed colonies tested, phytase activity was detected at levels much higher than that detected in the supernatant of untransformed colonies, which were sensitive to spectinomycin. In this way, we could verify that the plasmid pJJ5-SacBPhy was efficient in the transformation and that the SacB secretion peptide fused to the phytase gene allowed the export of the enzyme to the culture medium. The results of the phytase activity detected in the medium was: Phy-Af = 92.66 (± 2.38) U. mL^{-1}; Control = 34.38 U. mL^{-1} (± 2.74) (Fig. 2).

Zebrafish zootechnical parameters

Zebrafish survival (S), during the period of experiment in the control group was 98%, whereas in the Phy-Af group it was 90%, with no significant difference between the treatments. There was also no significant difference in Total Feed Consumption (TFC) between the experimental groups (Control = 58 ± 0.02 mg, Phy-Af = 56 ± 0.05 mg; Fig. 3A). The Apparent Feed Conversion (AFC) was significantly lower in the Phy-Af group (2.3 ± 0.5) than in the control (12.1 ± 2.7) (Fig. 3 B). The Weight Gain (WG) in the Phy-Af group was significantly higher (32.6 ± 8.2%) than in the control (4.5 ± 1.4%) (Fig. 3C). The specific growth rate (SGR) was also significantly higher in the Phy-Af group (0.33 ± 0.24) than in the control group (0.06 ± 0.01) (Fig. 3D).

Gene expression

In relation to the effect of phytase produced by transgenic probiotics, the results of the expression of the peptide transporter genes showed statistically significant differences between the experimental groups. Both analysed transporters (*slc15a1b* and *slc15a2*) had their expression increased in the Phy-Af group (0.22 ± 0.06 and 0.21 ± 0.04, respectively, Fig. 4A and 4B) when compared to the control (0.01 ± 0.00 e 0.05 ± 0.04). Concerning the gene coding for the orexigenic ghrelin hormone, a significant induction was also observed in the Phy-Af group (0.30 ± 0.07) compared to the control (0.09 ± 0.02) (Fig. 4C).

Among the genes that regulate somatic growth, insulin-like growth factor (*igf1*) is one of the most important. Expression of this gene was analysed both in the liver and in the zebrafish muscle. Hepatic *igf1* showed a significant induction in the Phy-Af group (0.25 ± 0.1) relative to the control (0.04 ± 0.03) (Fig. 5A). In the same sense, the muscular *igf1* was also induced in the Phy-Af group (0.46 ± 0.09) in relation to the control (0.06 ± 0.02) (Fig. 5B). Among the genes related to muscle growth, myogenic factors *myog* and *myod* (Fig. 5C-D) have outstanding action. The analysis of these two genes showed that both are quite induced in the Phy-Af group (*myod* = 0.32 ± 0.07, *myog* = 0.61 ± 0.22) when compared to the control group (*myod* = 0.14 ± 0.03, *myog* = 0.04 ± 0.01). The effect of treatments on bone mineralization was assessed by expression of the osteocalcin gene (*bglap*). Expression of this gene also significantly increased in the Phy-Af group (0.18 ± 0.05), while in the control the expression was 0.04 ± 0.01 (Fig. 6).

Discussion

The results of this study show that *B. subtilis* KM0 expressing a fungal phytase may contribute to the metabolism of phytate present in fish diets supplemented with high content of vegetable ingredients. In general, results presented here for zebrafish showed a significant improvement in the zootechnical parameters analysed, corroborated by the expression of the genes related to intestinal transport of peptides, appetite, somatic growth and bone metabolism.

It is not new that diets supplemented with phytase may improve zootechnical parameters. Liu et al. (2013) observed that the use of a purified phytase in zebrafish feeding was also able to improve the indices of body development, besides promoting

the increase of the lipid content and to improve the water quality of the culture. However, enzymatic purification processes have as the main disadvantages of the cost, the extraction difficulty and the production complexity (Wingfield 2015). The novelty here is the genetic manipulation of a probiotic bacterium as a bioreactor for expression of a fungal phytase already recognized for its efficiency (Brugger et al. 2004), which was fused to a levansucrose (SacB) secretion peptide from *B. subtilis* itself. In fact, the SacB signal peptide has been shown to be efficient in targeting alpha-amylase secretion in *B. subtilis*, as demonstrated by Heng et al. (2005). The strategy used here increased the ability of *B. subtilis* to export the enzyme to the medium and may have been the main reason for the changes observed in zebrafish fed with this transgenic probiotic.

Analysis of phytase activity in the culture medium of transgenic and non-transgenic probiotics is shown in Fig. 2. A 2.7-fold increase in phytase activity was observed in the Phy-Af group when compared to the control. In this case, the phytase activity observed in the controls may be from an endogenous phytase whose gene is located between nucleotides 2,151,256 - 2,150,108 (GeneID: 940065) from the genome of *B. subtilis* 168. Although *B. subtilis* strain 168 has no phytase activity (Idriss et al. 2002), and some other strains have demonstrated this activity in the extracellular medium (Shimizu, 1992). Apparently, the KM0 strain used here belongs to the latter group.

The use of a phytase-expressing probiotic did not alter feed intake, as shown in Fig. 3A. There was no difference in the amount of diet ingested between treatments. On the other hand, the transgenic probiotic expressing fungal phytase drastically decreased the apparent feed conversion (Fig. 3B) by approximately 80% relative to the controls. This extreme improvement in feed conversion probably caused the increase in weight gain (7-fold, Fig. 3C) and the specific growth rate (5.5-fold, Fig. 3D) observed in the Phy-Af group when compared to controls. These expressive results may be related to the fact that probiotics constantly persist in the digestive tract of zebrafish, functioning as true enzymatic bioreactors. In this case, unlike what happens when a certain amount of purified phytase is added to the feed, the microorganism continually secretes the enzyme, increasing the efficiency of the phytate degradation and allowing the bioavailability of the inorganic phosphorus.

The hydrolysis of the phytate molecule provides the nutrients and minerals bound to it, such as amino acids and peptides. Expression analyzes of genes encoding the peptide transporters PepT1 and PepT2 (encoded by the *slc15a1b* and *slc15a2* genes, respectively) showed fairly significant increases in fish treated with phytase-expressing *B. subtilis*. Figures 4A and 4B show 22-fold increase for *slc15a1b* and 2.3-fold increase for *slc15a2*. The induction detected in the expression of these peptide transporters may be indicative of increased availability of free nutrients, including the peptides active in the digestion as a result of the phytate hydrolysis present in the plant ingredients (Romano et al. 2013). The expression of PepT1 and PeT2 transporters occurs predominantly in the proximal intestine (Verri 2003), and PepT1 modulation can be performed even in the presence of different protein sources, as occurred in zebrafish using *Spirulina* as a protein source during the fasting and feedback period (Lo Cascio et al. 2018). In this same sense, common carp fed diets composed of wheat proteins were supplemented with Lys-Gly dipeptide, being these the main promoters of the increase of PepT1 expression (Ostaszewska et al. 2010).

Ghrelin is an orexigenic hormone and acts by stimulating food intake and weight gain (Napolitano et al., 2018). The Fig. 4C shows a significant 3.3-fold increase in expression of this gene compared to control. The results observed in the weight gain in the present study may be related to the ghrelin effect of the fish fed with the probiotic expressing recombinant phytase. A similar effect was observed in chickens fed a diet containing phytase (Liu et al., 2009). Since we used a diet with a higher amount of vegetable matter than the commercial zebrafish fed with a diet, it was clear that the growth observed in the 30 days of experiment was below that which is commonly observed when the fish is fed with protein rich feed of animal origin. Thus, the increased availability of nutrients caused by the action of phytase produced by the probiotic may have an effect on appetite control to trigger the compensatory growth system. In fact, ghrelin is found in a wide range of cells and tissues, including the brain and digestive tract, is a peripheral growth hormone (GH) secretion modulator, and can also act directly in the pituitary gland by modulating GH releasing factors (Won and Borski, 2013).

Although GH has not been analysed in the present study, we analysed a gene closely related to the somatotrophic axis: insulin-like growth factor 1 (*igf1*). One of the

major organs affected by circulating GH is the liver. Liver tissue responds to GH stimulation producing several growth factors, especially IGF1. IGFs are associated with neuroendocrine growth control but can have a wide range of other functions such as osmoregulation (in fish), cartilage sulfation, reproduction, cell proliferation and protein synthesis (Wood et al. 2005). In addition to the liver, skeletal muscle also responds to circulating GH expressing IGFs. In this case, IGFs may act more locally inducing the expression of myogenic factors that will promote muscle hypertrophy and hyperplasia (for review see Johnston et al. 2011). Expression of *igf1* was evaluated in both liver and skeletal muscle of zebrafish fed with the probiotic expressing phytase. The results shown in Fig. 5A and 5B show a significant increase in the expression of this gene in the order of 6.3 fold in the liver and 7.7 fold in the muscle comparing to controls. Thus, the expression of *igf1* corroborates with the results observed for the weight gain and the specific growth rate of the fish fed with the transgenic probiotic. The genes coding for the myogenic factors *myod* and *myog* behaved in the same way as *igf1*. The observed increases for these two genes in the muscle were on the order of 2.3 fold for *myod* and 15.2 fold for *myog*.

Zootechnical parameters and growth-related genes all pointed in the direction that probiotic by expressing fungal phytase made more nutrients available to zebrafish due to phytate degradation. However, the most direct evidence that this is a cause of observed growth arises from the expression of the gene coding for osteocalcin (*bglap*). Osteocalcin is an important protein in bone metabolism and one of its functions is related to the absorption of calcium by the bone matrix (Gavaia et al. 2006; Cancela et al. 2014). Although the role of this protein is not widely elucidated in the whole process of bone formation in fish, it is unanimous that the increase in its expression is a strong indicator of the ability of the animal organism to metabolize minerals. In this sense, the 4.5-fold increase in osteocalcin gene expression (Fig. 6) observed in the Phy-Af group reinforces the hypothesis that the transgenic probiotic is actually producing and secreting an active phytase and the recombinant enzyme efficiently degrades the phytate present in the feed supplied to the zebrafish.

In conclusion, this is the first study, to our knowledge, that uses a probiotic bacterium to produce and secrete an active phytase which proved extremely efficient to improve the zootechnical parameters of zebrafish fed with a feed with high vegetable

matter content. Significant improvement of the zootechnical parameters was corroborated by the expression of genes related to appetite, peptide transport, somatic growth and bone metabolism. The use of probiotics genetically modified as biofactories for the production of molecules that impact the metabolism and physiology of the host organism opens up enormous possibilities of applications not only in the field of nutrition but also in other physiological processes, in addition, probiotics can offer alternatives to the antimicrobial compounds used in aquaculture.

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Conflict of Interest

The authors declare no conflict of interest.

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Table 1 Experimental diet used to feed zebrafish (*Danio rerio*)

Constituents	**%**
Fish meal	2.0
Casein [a]	10.0
Soybean	50.0
Maize starch	5.0
Wheat bran	8.0
Fish oil	6.0
Mineral and vitamin blend [b]	2.0
Meat and bone meal	4.0
Corn gluten (prothenosis) [c]	13.0
Total	100
Centesimal composition	
Crude protein	40.8
Gross lipid	8.7
Non-nitrogenous extract	44.8
Ashes	5.7

[a] LabSynth, SP, Brazil

[b]Premix M. Cassab, SP, Brazil. Vitamin A (500.000 Ul/kg), Vit. D3 (250.000 Ul/kg), Vit. E (5000 mg/kg), Vit. K3 (500 mg/kg), Vit. B1 (1000 mg/kg), Vit. B2 (1000 mg/kg), Vit. B6 (1000 mg/kg), Vit. B12 (2000 mcg/kg), Niacin (2500 mg/kg), Calcium pantothenate (4000 mg/kg), Folic acid (500 mg/kg), Biotin (10mg/kg), Vit. C (10000 mg/kg), Choline (100.000 mg/kg), Inositol (1000 mg/kg), Selenium (30 mg/kg), Iron (5000 mg/kg), Copper (1000 mg/kg), Manganese (5000 mg/kg), Zinc (9000 mg/kg), Cobalt (50 mg/kg), Iodine (200 mg/kg).

[c]Ingredion, SP, Brazil

Table 2 Genes and primers used for qRT-PCR analyzes.

Gene symbol	Sequence (5' - 3')	Efficiency (%)	GenBank accession
Normalizing genes			
ef1α	F: 5'-CAAAATTGGAGGTATTGGAACTGTAC-3' R: 5'-TCAACAGACTTGACCTCAGTGGTT-3'	88.7	NM131263
β2m	F: 5'-GCCTTCACCCCAGAGAAAGG-3' R: 5'-GCGGTTGGGATTTACATGTTG-3'	101.0	NM131163
rpl13a	F: 5'-TCTGGAGGACTGTAAGAGGTATGC-3' R: 5'-AGACGCACAATCTTGAGAGCAG-3'	89.2	NM212784
actb1	F: 5'-GCTGTTTTCCCCTCCATTGTT-3' R: 5'-TCCCATGCCAACCATCACT -3'	98.5	NM131031
Target genes			
igf1	F: 5'-ACTTTGTGGGCACATGCAAA-3 ' R: 5'-CATGATCTCATTGCGAATTCCTT-3'	90.8	NM131825
myod	F: 5'-GGAGCGAATTTCCACAGAGACT-3' R: 5'-GTGCCCCTCCGGTACTGA-3'	97.9	NM131262
myog	F: 5'-GGCCGCTACCTTGAGAGAGA-3' R: 5'-GAGCCTCAAAGGCCTCGTT-3'	92,4	NM131006
slc15a1b	F: 5'-GCATCTACGCAAAGCAGAGC-3' R: 5'-ATGAGGGCAACCACCATGAG-3'	100.2	NM198064
slc15a2	F: 5'- CACAGCCGGAGAAGTCATGT -3' R: 5'- GAACGGATTTCATGCTCGCC -3'	90.4	NM001039828
grhl	F: 5'- GTGGCACCAGCTTCCTCAGT-3' R: 5'- CACTCTTGGTGGCCTTCGA-3'	87.1	NM001083872
bglap	F: 5'- GTTTGTGAAGCGTGACGTGG -3' R: 5'- ATAGGCGGCGATGATTCCAG -3'	92.3	NM001083857

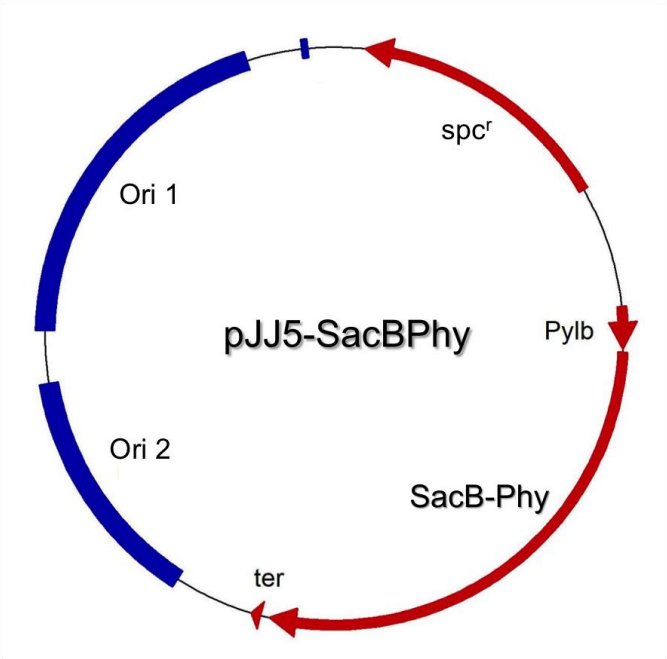

Figure 1 Replicative expression plasmid used for *Bacillus subtilis* KM0 transformation. SacB-Phy: SacB (levansucrose) signal peptide from *B. subtilis* fused to phytase from *Aspergillus fumigatus*. Pylb: Phase stationary *B. subtilis* promoter. ter: gene terminator. Ori 1: replication origin for *Escherichia coli*. Ori 2: replication origin for *B. subtilis*. spcr: spectinomycin resistance gene.

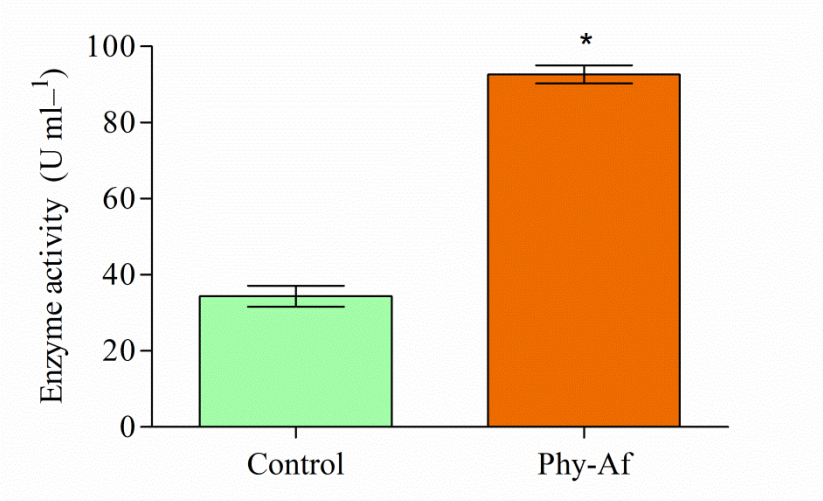

Figure 2 Phytase activity measured in the collected supernatant of transgenic (phy-Af) and non-transgenic (Control) *Bacillus subtilis* KM0 cultures. Asterisk denotes statistically significant difference (Student's t test; $p < 0.05$).

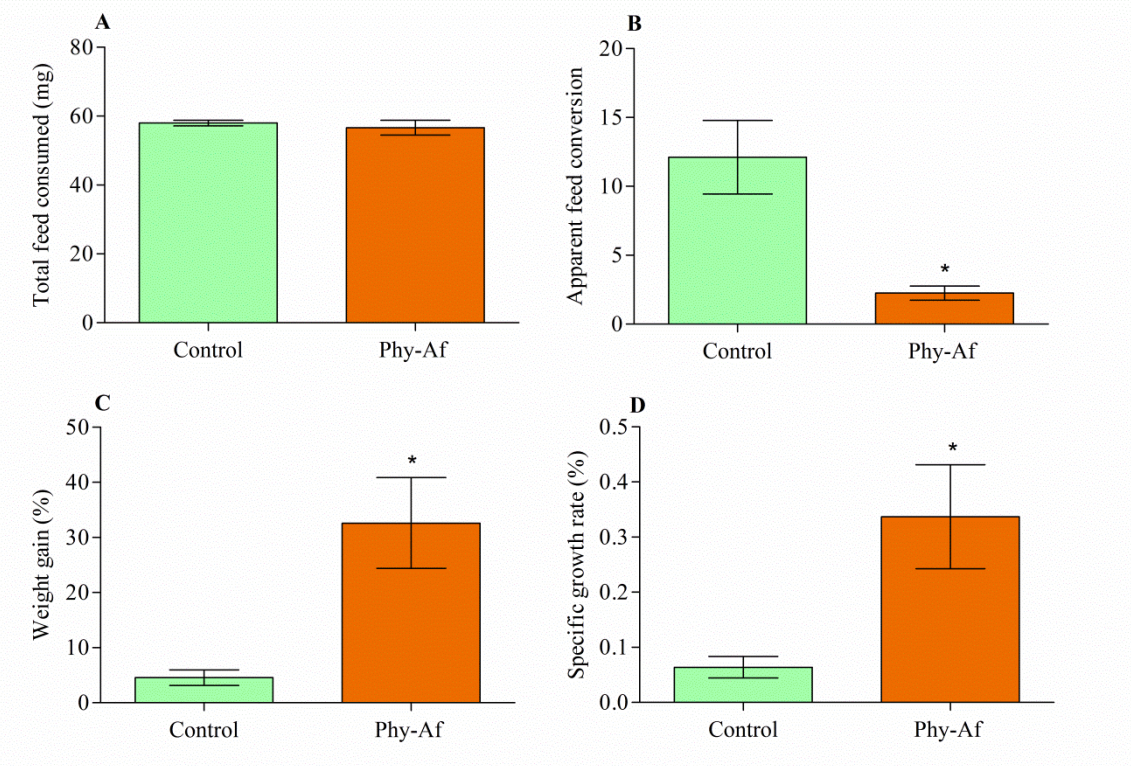

Figure 3 Average values (± SEM) of zootechnical parameters of zebrafish (*Danio rerio*) fed for 30 days with transgenic *Bacillus subtilis* KM0 expressing phytase (phy-Af) and non-transgenic (Control). (A) Total feed cosumption, (B) apparent feed conversion, (C) weight gain, and (D) specific growth rate. Asterisks denote statistically significant difference (Student's t test; $p < 0.05$).

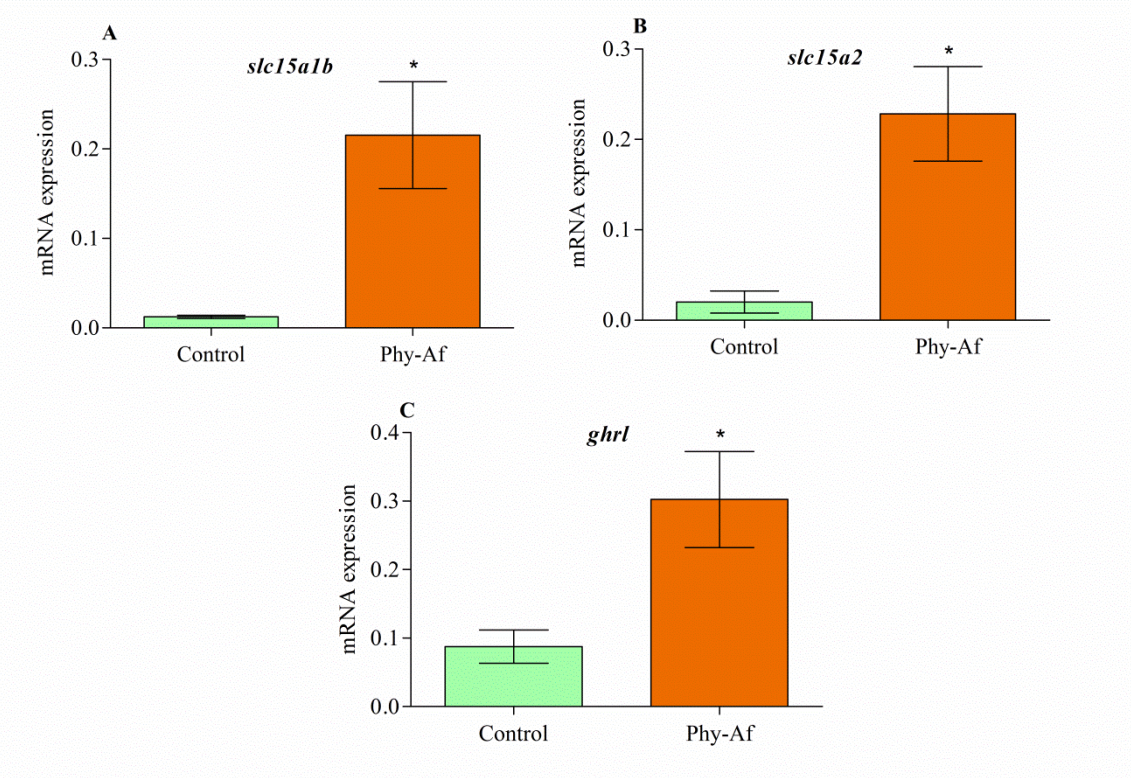

Figure 4 Expression of genes related to peptides transport (A: *slc15a1b*, B: *slc15a2*) and appetite (C: Ghrelin) in the intestine of zebrafish (*Danio rerio*) fed for 30 days with transgenic *Bacillus subtilis* KM0 expressing phytase (phy-Af) and non-transgenic (Control). Data are mean ± SEM (*n*= 10 individuals/treatment). Asterisks denote statistically significant difference (Student's t test; $p < 0.05$).

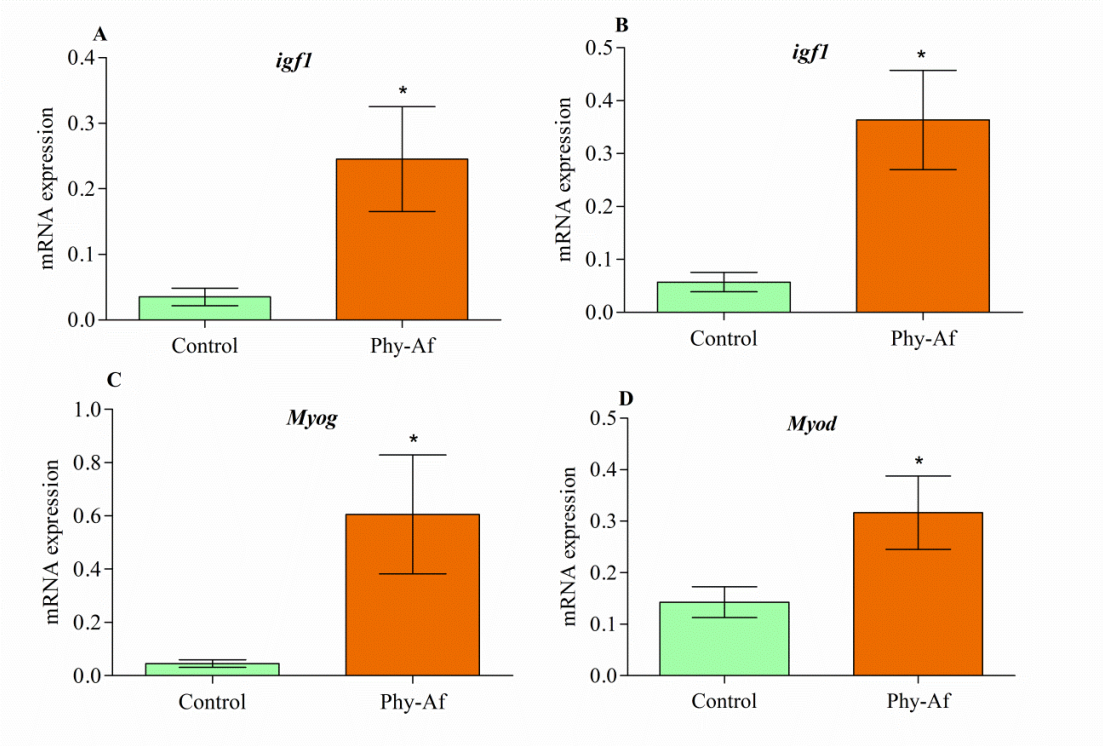

Figure 5 Expression of genes related to somatic growth in liver (A: *igf1*) and skeletal muscle (B: *igf1*, C: *myog*, and D: *myod*) of zebrafish (*Danio rerio*) fed for 30 days with transgenic *Bacillus subtilis* KM0 expressing phytase (phy-Af) and non-transgenic (Control). Data are mean ± SEM (*n*= 10 individuals/treatment). Asterisks denote statistically significant difference (Student's t test; $p < 0.05$).

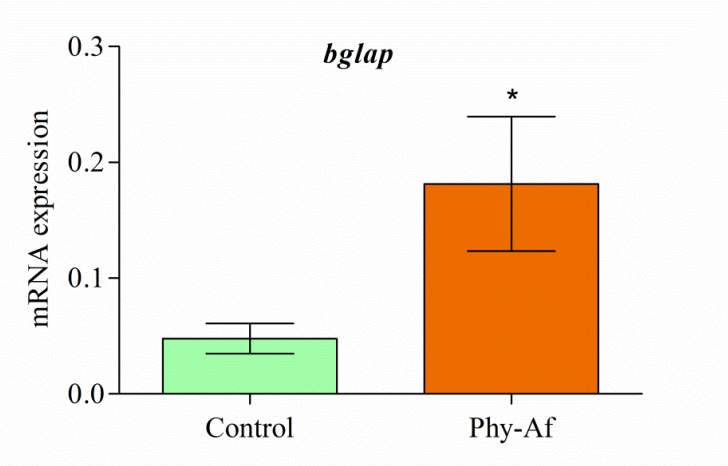

Figure 6 Expression of osteocalcin (*bglap*) gene in bone of zebrafish (*Danio rerio*) fed for 30 days with transgenic *Bacillus subtilis* KM0 expressing phytase (phy-Af) and non-transgenic (Control). Data are mean ± SEM (*n*= 10 individuals/treatment). Asterisks denote statistically significant difference (Student's t test; $p < 0.05$).

CAPÍTULO II

Probiotic expressing heterologous phytase improves the immune system and attenuates inflammatory response in zebrafish fed with a diet rich in soybean meal

Manuscrtito submetido à revista Fish Physiology and Biochemistry

Probiotic expressing heterologous phytase improves the immune system and attenuates inflammatory response in zebrafish fed with a diet rich in soybean meal

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Resumo

Embora a aquicultura esteja entre os setores de produção de alimentos que mais crescem no mundo, um dos gargalos para a continuidade de sua expansão é a dependência de proteína animal em formulações comerciais de rações. Proteínas vegetais são uma alternativa devido ao baixo custo e alta disponibilidade. No entanto, esta fonte de proteína é acompanhada por uma série de compostos antinutricionais e pró-inflamatórios, incluindo o fitato. As fitases podem ser adicionadas na alimentação para a degradação do fitato e aumentar a disponibilidade de nutrientes. No entanto, o uso de fitases purificadas aumenta significativamente o custo da produção. Uma alternativa interessante é usar probióticos geneticamente modificados como biorreatores para a produção de fitase. No presente trabalho, uma cepa de *Bacillus subtilis* capaz de secretar uma fitase fúngica foi utilizada para avaliar o efeito de uma ração com alto teor de farelo de soja sobre zebrafish (*Danio rerio*). Analisamos o fator de condição (K) dos peixes e a expressão de genes relacionados ao sistema imune, resposta inflamatória e estresse oxidativo. Os resultados obtidos demonstram que o probiótico transgênico foi eficiente em melhorar o fator de condição dos peixes, estimulando o sistema imunológico, reduzindo a resposta inflamatória e o estresse oxidativo. Assim, os probióticos que atuam como biorreatores de fitase podem ser considerados uma ferramenta interessante não só para a adaptação de espécies comerciais ao alimento de menor custo, mas também abrem possibilidade para o tratamento de doenças relacionadas à nutrição, inclusive em humanos.

Palavras-chave: Fitato; probiótico transgênico; fator de condição; sistema imunológico; resposta inflamatória; estresse oxidativo.

Abstract

Although aquaculture is among the fastest growing food production sectors in the world, one of the bottlenecks for the continuity of its expansion is the dependence of animal protein on commercial feed formulations. Vegetable proteins are an alternative due to the low cost and high availability. However, this protein source is accompanied by a series of antinutritional and pro-inflammatory compounds, including phytate. Phytases can be added in feed for phytate degradation and increase nutrient availability. However, the use of purified phytases significantly increases the production costs. An interesting alternative is to use probiotics genetically modified as bioreactors for phytase production. In the present study, a strain of *Bacillus subtilis* secreting a fungal phytase was used to evaluate the effect of a feed with high content of soybean meal on zebrafish (*Danio rerio*). We analysed the condition factor (K) of fish, and the expression of genes related to the immune system, inflammatory response and oxidative stress. The results obtained demonstrate that the transgenic probiotic was efficient in improving the fish condition factor, stimulating the immune system, reducing inflammatory response and oxidative stress. Thus, probiotics acting as phytase bioreactors can be considered an interesting tool not only for the adaptation of commercial species to feed of lower cost, but also opens possibility for the treatment of diseases related to nutrition, including in humans.

Key-words: Phytate; transgenic probiotic; condition factor; immune system; inflammation response; oxidative stress.

Introduction

Fishmeal is the main source of protein in aquaculture feeds and the increase in demand from the rapid expansion of this sector of food production has made this input expensive and limited. Thus, reducing the use of fishmeal has become a crucial issue for the development of aquaculture production (Pahlow et al. 2015; FAO 2018). Soy protein is widely investigated as an alternative substitute for fishmeal, and this is favoured mainly by a wide range of soybeans in recent years, tied to its reasonable reasonable cost, the constant supply and its attractive nutritional characteristics such as high protein content, digestibility and balanced amino acid levels (Zhou et al. 2018). However, the inclusion and substitution levels of soy-based ingredients is limited for the various species of fish because soy is composed of several antinutrients such as phenolic compounds, saponins, glucosinolates, oligosaccharides, antigenic proteins (for example, glycine and β-conglycinin) and phytates (Li et al. 2017; Zhou et al. 2018).

Studies indicate that the inclusion of soybean meal in feed adversely influences fish health, triggering disturbances in immune function, inflammatory process and decreased growth (Ulloa et al. 2016; Li et al. 2017; Zhang et al. 2018; Miao et al. 2018). In Japanese sea bass (*Lateolabrax japonicus*), for example, the replacement of fishmeal with soybean meal in the diet resulted in high expression of intestinal pro-inflammatory genes (Zhang et al. 2018). In zebrafish larvae (*Danio rerio*), Hedrera et al. (2013) indicated that antinutrients present in soybean meal supplemented the diet developed intestinal inflammation. In some cases, supplementation of microorganisms such as microalgae or substances such as lactoferrin, butyrate and taurine in feed can minimize the inflammatory effects in fish fed with soy-based ingredients (Ulloa et al. 2016; Rimoldi et al. 2016; Bravo- Tello et al. 2017).

Phytate is a known antinutrient in diets with soy-derived ingredients. It is resistant to heat and even after application of various processing methods in soybean, it is still possible to observe phytate levels ranging from 2-18 g/kg (Zhou et al. 2018). This antinutrient is able to chelate minerals and also affect the availability of proteins, amino acids and digestive enzymes in the animal organism (Gupta et al. 2015). One way to minimize the phytate negative effects is to add the phytase enzyme in the diets, which catalyzes the hydrolysis of phytic acid and its salts (phytates), generally producing inositol, monophosphate and inorganic phosphate (Vohra and Satyanarayana

2003; Jain and Singh 2017). Due to these characteristics, phytase represents a product widely used in animal feed (Dersjant-Li et al. 2014; Singh et al. 2016).

Fungal phytases are widely used commercially because of their excellent biochemical and catalytic characteristics. In addition to the phytate hydrolyzing function, phytase appears to have indirect effects on important physiological systems in fish. Lazado et al. (2010) showed, through an *in vitro* assay, that phytase was able to boost the immune response in renal leukocytes of *Gadus morhua*. Hu et al. (2016) observed that phytase induced anti-inflammatory responses in tilapia. These evidences indicate that phytase also has a potential action on the immune system and anti-inflammatory response in fish. However, the relationship between these immune responses and feeding with high soybean diet is to must be analysed.

The production and purification of phytases is an expensive and laborious process. An alternative is the production of recombinant phytases using an expression system in bioreactors such as *Escherichia coli*, for example. However, as the enzyme's destination is food, gram-negative bacteria such as *E. coli* are not the best models of heterologous protein expression since they can produce toxins that can contaminate the feed and render commercial production unfeasible. The use of gram-positive bacteria with probiotic characteristics such as *Bacillus subtilis* seems to be the best option because this type of bioreactor can be incorporated into the feed and produce the enzyme directly in the lumen of the host digestive tract, saving the production and purification steps that represent a very high cost. *Bacillus subtilis* compose a group of probiotics commonly used in aquaculture as a source of protein or in the fermentation of plant ingredients (Bairagi et al. 2004; Zhang et al. 2011; Ringø and Song 2016). On the other hand, genetic engineering of *B. subtilis* has advanced considerably and made this probiotic a potential bioreactor for heterologous expression of proteins (Zhang et al. 2017).

Zebrafish (*Danio rerio*) is an interesting model for physiological studies, which may encompass several molecular and cellular mechanisms, including immunological and anti-inflammatory responses (Spence et al. 2008; Simonetti et al. 2015; Brugman 2016). Zebrafish demands high protein content in the composition of their diets, with fishmeal as the main source of protein (Ulloa et al. 2014; Fernandes et al. 2016). In this context, the objective of this study was to evaluate the effect of a diet rich in soybean

meal supplemented with the probiotic *Bacillus subtilis* KM0 expressing a fungal phytase in the zebrafish immune, anti-inflammatory and antioxidant responses.

Material and methods

Preparation of probiotic strains

The strain of *B. subtilis* KM0/Phy-Af from the collection of the Laboratory of Molecular Biology of the Federal University of Rio Grande (FURG, Brazil) was used as a probiotic phytase producer. This strain was previously transformed with a replicative plasmid for gram-positive bacteria kindly provided by Dr. Josef Altenbuchner (University of Stuttgart, Germany). The plasmid has an origin of replication for *Bacillus* and expresses the phytase gene from *Aspergillus fumigatus* fused to the signal peptide of the SacB gene (levansucrose) from *B. subtilis*. A *B. subtilis* stationary phase promoter (P_{ylb}) was used for the expression of the target gene (Yu et al. 2015). For selection of the transformants, the plasmid contains the spectinomycin resistance gene.

A single colony of strain KM0/Phy-Af was inoculated into 10 mL of Luria-Bertani medium (LB) under constant stirring at 250 rpm at 37°C for 16 hours. After, the culture was centrifuged at 8,000 x g for 10 min at 4°C. The supernatant was discarded and the bacterial pellet washed with sterile saline (0.85% NaCl), and re-centrifuged under the above conditions. The pellet was resuspended with sterile water and sprinkled on the feed. Then, the feed was oven dried at 37°C for one hour. Bacterial counts were performed to determine the colony forming units (CFU) per gram of feed. The same procedure was performed for the non-transgenic KM0 strain, which served as the experimental control.

Feed formulation

A basal diet was formulated to contain soybean meal as the main protein source. The diet consisted of 40.84% of protein, of this total 73.5% was derived from vegetable ingredients, soybean meal being the most abundant vegetable ingredient (Table 1). All dry ingredients were mixture. The oil and water were added further. The diet were forced through a meat grinder and dried at 60^{o}C. The diet was crushed into the desired size to feed the fish. The diet proximal composition (protein, lipid and ashes) was assayed according to AOAC (1999).

Table 1 Basal diet used to fed zebrafish (*Danio rerio*)

Ingredients	**%**
Fish meal	2.0
Casein[a]	10.0
Soybean meal	50.0
Maize starch	5.0
Wheat bran	8.0
Fish oil	6.0
Mineral and vitamin blend[b]	2.0
Meat and bone meal	4.0
Corn gluten (prothenosis)[c]	13.0
Total	100.0
Proximate composition	
Crude protein	40.8
Crude fat	8.7
Non-nitrogenous extract (NNE)d	44.8
Crude ash	5.7

[a]LabSynth, SP, Brazil

[b]Premix M. Cassab, SP, Brazil. Vitamin A (500.000 Ul/kg), Vit. D3 (250.000 Ul/kg), Vit. E (5000 mg/kg), Vit. K3 (500 mg/kg), Vit. B1 (1000 mg/kg), Vit. B2 (1000 mg/kg), Vit. B6 (1000 mg/kg), Vit. B12 (2000 mcg/kg), Niacin (2500 mg/kg), Calcium pantothenate (4000 mg/kg), Folic acid (500 mg/kg), Biotin (10mg/kg), Vit. C (10000 mg/kg), Choline (100.000 mg/kg), Inositol (1000 mg/kg), Selenium (30 mg/kg), Iron (5000 mg/kg), Copper (1000 mg/kg), Manganese (5000 mg/kg), Zinc (9000 mg/kg), Cobalt (50 mg/kg), Iodine (200 mg/kg).

[c]Ingredion, SP, Brazil

[d]Calculated. NNE = 100 – (crude protein + crude fat + crude ash)

Zebrafish assay

We used 120 fish with a final weight of 246.91 ± 7.60 mg and a final length of 2.56 ± 0.04 cm (data are presented as mean values ± SEM), distributed in 12 glass aquaria (5 L) at the density of 2 fish/L, as recommended by Schneider et al. (2009). The fish were acclimated in the experimental environment for 15 days, the fisheres were fed the commercial Tetra Color Bits (47.5% protein) and were fasted for 24 hours before the start with the experimental diet. The experimental design was completely randomized, consisting of 2 groups. The groups were designated control (basal diet

supplemented with the non-transgenic strain *B. subtilis* KM0) and Phy-Af group (basal diet supplemented with *B. subtilis* KM0/Phy-Af). Following the recommendations of Xu et al. (2015), the dose of *B. subtilis* was 10^{10} CFU/g of feed. According to the authors, this dosage administered orally is sufficient to colonize the intestinal tract of the zebrafish. The feed was supplemented daily with the probiotic and administered to the fish twice daily.

The temperature of the aquaria was maintained at 28°C, and the aquariums were equipped with a biofilter system. About 50% of the aquaria water was changed every two days. The experiment lasted 30 days. To calculate the condition factor (K), we used the Fulton equation $K = 100 (W/L^3)$, where W = weight and L = standard length. At the end of the experiment the weight (mg) and length (mm) of all fish were determined. All fish were photographed for length determination by image analysis (Jones et al. 1999; Siccardi et al. 2009) using ImageJ software (Image processing and analysis in Java, http://rsb.info.nih.gov/ij). The condition factor was statistically analysed by the Student's t test for independent samples.

Tissue collection, RNA extraction and cDNA synthesis

At the end of the experimental period, the fish were fasted for 24 h. For dissection of the tissues (kidney and intestine), ten fish per treatment were randomly selected from each replica and euthanized with tricaine methanesulfonate lethal dose (500 mg/L). Total RNA was isolated from tissues shortly after dissection using the TRIZOL reagent (Life technologies, Brazil), following the manufacturer's recommendations. Total RNA was treated with the RNAse free DNAse I kit (Invitrogen, Brazil) and spectrophotometrically quantified (BioDrop, Isogen Life Science, B.V, Veldzigt, Netherlands). The quality and integrity of total RNA was analysed by 1% agarose gel electrophoresis. The cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Brazil), following the manufacturer's instructions.

Gene expression

The analysed genes were previously selected in GenBank (https://www.ncbi.nlm.nih.gov), with the specific primers designed by GenBank Primer-Blast tool (Table 2). Quantitative analysis of the genes was performed by

quantitative real-time PCR (qRT- PCR). Each sample (*n*=10) was analysed in duplicate. The 7500 Real-time PCR system (Applied Biosystems, Brazil) was used for qRT-PCR reactions. The cDNA was diluted 5x and used according to the recommendations of the PowerUP SYBR Green Master Mix kit (Applied Biosystems, Brazil). The efficiency of the primers was measured by qRT- PCRs with serial dilutions of the cDNA. The conditions of the reactions used in the qRT- PCR assays were: 50^{o}C/2 min, 95^{o}C/2 min, followed by 40 cycles at 95^{o}C/15 sec and 60^{o}C/15 min. The dissociation curve was performed at 95^{o}C/15 sec, 60^{o}C/1 min and 95^{o}C/30 sec. The genes analysed in the kidney were those related to the immune response: *cd4* and *ikzf1*. Genes related to the inflammatory response (*ifnphi1*, *ifng*, *tnf-α* and *il1b*) and the antioxidant response (*sod1*) were analysed in the intestine. For the normalization of the expression data, the *ef1α* (elongation factor 1 alpha), *actb1* (β-actin) and *rpl13a* (ribosomal protein L13 alpha) (Table 2) genes were used. All normalizing genes were evaluated by the geNorm VBA applet for Microsoft Excel software (Vandesompele et al. 2002).

Table 2 Genes and primers used in qRT- PCR analyses.

Gene symbol	Sequence (5'-3')	Efficiency (%)	GenBank accession
Normalizing genes			
ef1α	F: 5'-CAAAATTGGAGGTATTGGAACTGTAC-3' R: 5'-TCAACAGACTTGACCTCAGTGGTT-3'	89	NM131263
rpl13a	F: 5'-TCTGGAGGACTGTAAGAGGTATGC-3' R: 5'-AGACGCACAATCTTGAGAGCAG-3'	89	NM212784
actb1	F: 5'GCTGTTTTCCCCTCCATTGTT-3' R: 5'-TCCCATGCCAACCATCACT -3'	99	NM131031
Target genes			
ikzf1	F: 5'-GCCGACATGGTGGTCAGCCC-3' R: 5'-GTGCTCTGCGGCGCTGTCTT-3'	84	NM130986
cd4	F: 5'-GGTTCTGGTGCCACTGATCATTGG-3' R: 5'- AGAGGCTGCCGCATGGATCTCA-3'	84	NM001366061
il1b	F: 5'-CCACGTATGCGTCGCCCAGT-3' R: 5'-GGGCAACAGGCCAGGTACAGG-3'	93	NM212844
tnf-α	F: 5'-TGAGCCATGCAGTGATGCGCT-3' R: 5'-TCCTGCGTGCAGATTGAGCGG -3'	97	NM212859
ifnphi1	F: 5'- AGCATGGGAGCAGATCCGGA-3' R: 5'- TGACCCTTGCGTTGCTTGCGA-3'	98	NM207640
ifng	F: 5'- ACGCTTGCAAAGGATTGGGTTGG-3' R: 5'-ACACAGCCTGGCAAGTGCAGG -3'	91	NM001020793
sod1	F: 5'-CACCGTCTATTTCAATCAAGAGG-3' F: 5'-AGAATGTTGGCCTGACAAAGTTA-3'	82	NM131294

Ethics statement

Zebrafish cultivation and experimental procedures were approved by the Ethics Commission on Animal Use (CEUA) of the Federal University of Rio Grande (FURG), under approval number P002/2019.

Results

Figure 1 shows the changes in the condition factor (K) related to the different feeds used and the treatments with the two types of probiotics supplemented (transgenic and non-transgenic). The fish in the control group showed a significant decrease in K when comparing the beginning (1.93 ± 0.07) with the end of the experiment (1.43 ± 0.13). The K of the fish of the Phy-Af group showed no significant difference when comparing the initial condition (1.73 ± 0.07) to the final period (1.55 ± 0.14).

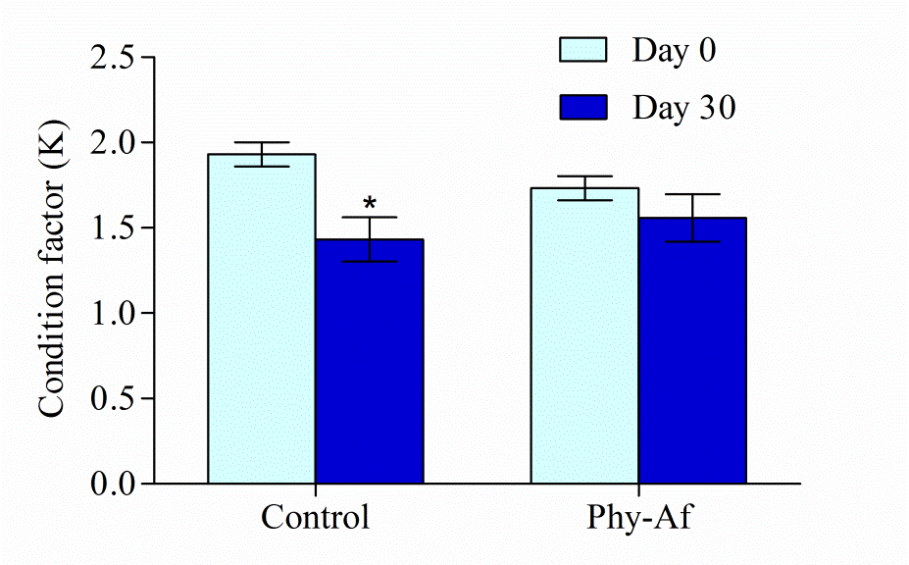

Fig. 1 Condition factor (K) of the zebrafish (*Danio rerio*) fed a diet rich in soybean meal. In the control group the feed was supplemented with *B. subtilis*, and in the phy-Af group the feed was supplemented with *B. subtilis* expressing *A. fumigatus* phytase (Phy-Af). Day 0 refers to the analysis of biometrics data (weight and length) shortly after the end of the acclimation period with commercial feed, and day 30 refers to the analysis of the biometrics data (weight and length) shortly after 30 days of feeding with the experimental diet. Data are expressed as means ± SEM. Significant differences are indicated by an asterisk (*) (Student's t-test, $p < 0.05$).

The expression results for *cd4* and *ikzf1* revealed that *B. subtilis* expressing phytase induced a significant increase in immune system responses over control. Figure 2 (A and B) shows that the expression of *cd4* and *ikzf1* in controls was 0.17 (±0.04) and 0.16 (±0.04), respectively. In the Phy-Af group the expression of *cd4* was 0.71 (±0.27) and *ikzf1* 1.02 (±0.37), significantly higher than in controls.

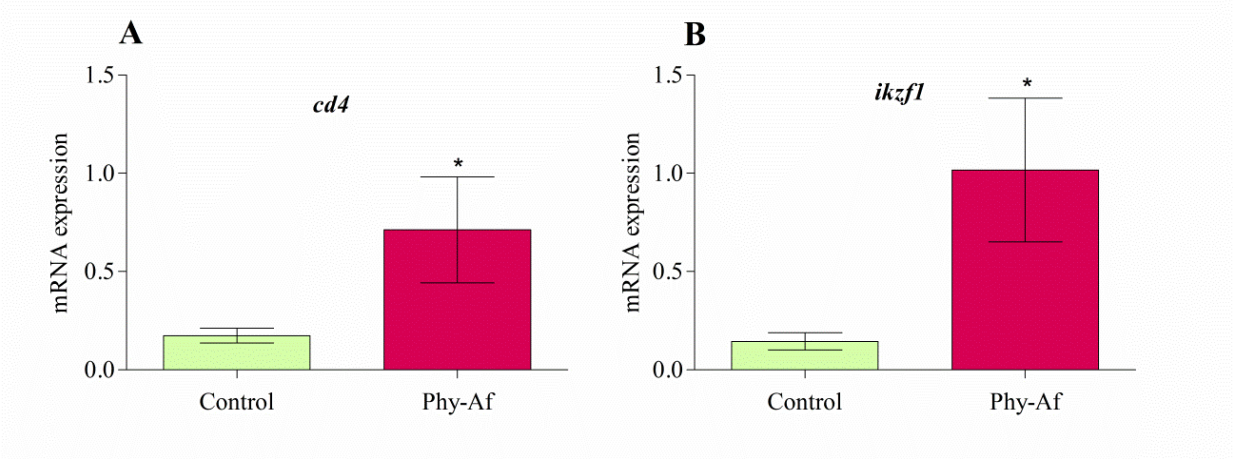

Fig. 2 Expression of genes related to the immune system (A: *cd4*; B: *ikzf1*) in the zebrafish kidney (*Danio rerio*). Control: fish fed with a diet supplemented with non-transgenic *Bacillus subtilis*. Phy-Af: fish fed a diet supplemented with *B. subtilis* transgenic expressing *Aspergillus fumigatus* phytase. Values are expressed as means ± SEM (*n*= 10 individuals/treatment). Significant differences are indicated by an asterisk (*) (Student's t test, p <0.05).

Expression of genes related to viral response is not a zebrafish problem based on the soybean diet but was significantly decreased ($p < 0.05$) in the Phy-Af group, where the diet was supplemented with fungal phytase expressing probiotic transgenic (Figure 3). More specifically, the expression of *ifnphi1* in the controls was 0.64 (±0.03), while in the Phy-Af group it was 0.03 (±0.00) (Fig. 3A). For the *ifng* gene, control = 0.64 (±0.23); Phy-Af = 0.03 (±0.00) (Fig. 3B). For *tnf-α*, control = 0.30 (±0.10); Phy-Af = 0.03 (±0.01) (Fig. 3C). For *il1b*, control = 0.68 (±0.06); Phy-Af = 0.05 (±0.01) (Fig. 3D).

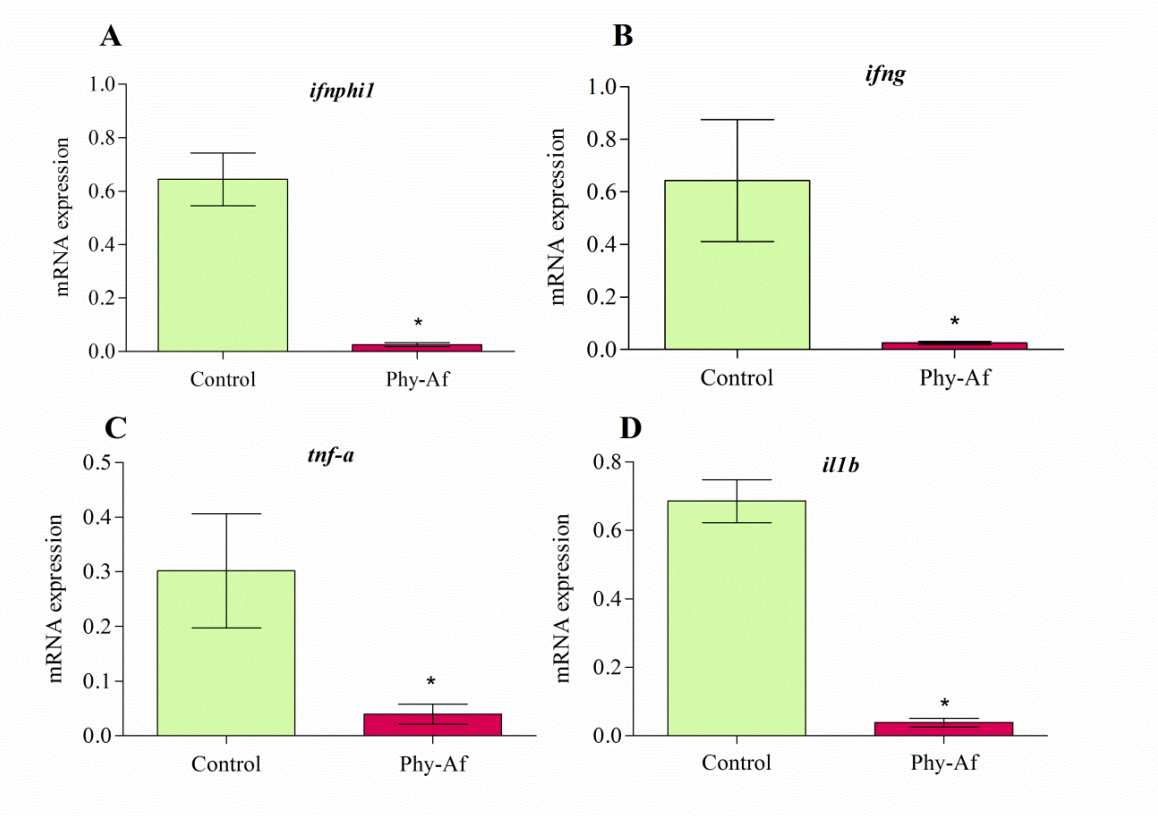

Fig. 3 Expression of the genes related to the inflammatory response (A: *ifnphi1*; B: *infg*; C: *tnf-α*; D: *il1b*) in the zebrafish intestine (*Danio rerio*). Control: fish fed with a diet supplemented with non-transgenic *B. subtilis*. Phy-Af: fish fed a diet supplemented with *B. subtilis* transgenic expressing *A. fumigatus* phytase. Values are expressed as means ± SEM (*n*= 10 individuals/treatment). Significant differences are indicated by an asterisk (*) (Student's t test, $p < 0.05$).

Regarding the antioxidant defenses, the gene coding for superoxide dismutase (*sod1*) in the zebrafish intestine was analysed. Figure 4 shows the results of expression analysis, where *sod1* presented values of 0.23 (±0.02) for the control group, and 0.16 (±0.02) for the Phy-Af group.

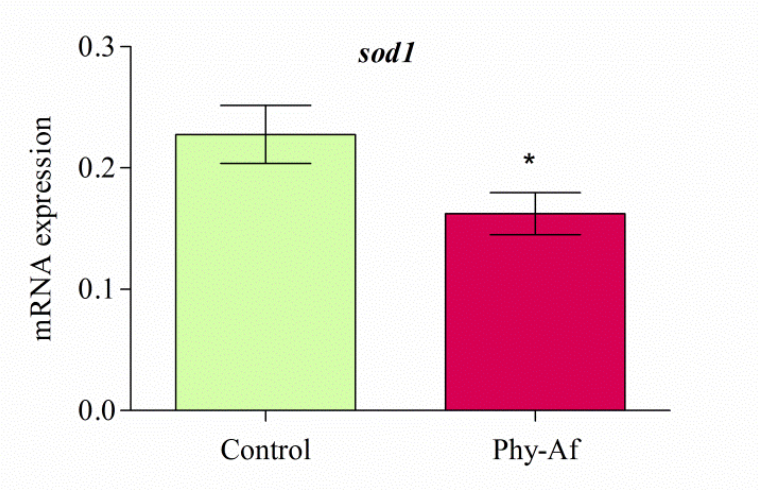

Fig. 4 Expression of the gene related to the antioxidant defenses (*sod1*) in the zebrafish intestine (*Danio rerio*). Control: fish fed with a diet supplemented with non-transgenic *B. subtilis*. Phy-Af: fish fed a diet supplemented with *B. subtilis* transgenic expressing *A. fumigatus* phytase. Values are expressed as means ± SEM (*n*= 10 individuals/treatment). Significant differences are indicated by an asterisk (*) (Student's t test, $p < 0.05$).

Discussion

In the present study, *B. subtilis* genetically modified to express a fungal phytase significantly influenced the overall health status of zebrafish fed with asoybean meal rich diet. Figure 1 shows the evolution of the condition factor (K) of the fish fed with the commercial diet and the subsequent drop in this parameter from the substitution by the diet rich in soybean meal. The diet change impacted the zebrafish differently, where the control group presented a decrease in K of 26%, which was statistically significant ($p < 0.05$). On the other hand, the group treated with the transgenic probiotic had a K loss of approximately 10%, which was not statistically significant. These results demonstrate that the phytase produced by the probiotic mitigates the negative impact of replacing a commercial feed rich in animal protein with a high vegetable diet.

The K result is related, in part, to the immune responses observed in fish treated with *B. subtilis* expressing heterologous phytase. Among the analysed genes *cd4*, is expressed in the T-helper lymphocytes ($CD4^+$) performing important function in activating and stimulating other leukocytes to multiply and attack antigens, and coordinating the immune response by the release of cytokines (Ashfaq et al. 2019).

Figure 2A shows that the probiotic expressing phytase induced a 4.4 fold increase in *cd4* expression relative to the control group. Another gene related to the immune system was *ikzf1*. This gene encodes for a zinc finger protein, which is involved not only in the regulation of lymphopoiesis (Mayer et al. 2002; Lam et al. 2004; John and Ward 2011; Schwickert et al. 2014) but also in a wide range of processes such as apoptosis, cell cycle arrest, proliferation and differentiation (Fan and Lu 2016). Figure 2B shows that this gene was strongly upregulated (6.4 times) in the zebrafish group treated with the transgenic probiotic. The increase in expression of genes like *cd4* and *ikzf1* is a strong indication that the phytase produced by *B. subtilis* is causing a major effect on the immune system of zebrafish, through the production of lymphocytes. The results observed here are corroborated by Lazado et al. (2010), which showed that phytase was able to boost the immune response in renal leukocytes of Atlantic cod *Gadus morhua*. Likewise, Liu et al. (2009) showed that the percentages of $CD4^+$ and $CD8^+$ T cells were increased by the addition of phytase in the broiler feed.

The second group of genes analysed here involves the inflammatory response. These genes were selected by the fact that soybean meal has a number of antinutrients that can cause inflammation in the intestine. Fuentes-Appelgren et al. (2014) have shown that the inclusion of soy-derived components at high levels in the diet of zebrafish larvae not only stimulates the immune system but also produce inflammatory signals that lead to the development of intestinal enteritis. The authors attributed this inflammatory process not to phytate, but to the presence of saponins. This compounds are amphipathic glycosidic molecules, which contain a hydrophobic aglycone to which one or more hydrophilic sugar chains are attached. The presence of saponins in commercial diets has also been linked to intestinal inflammation in cultured fish such as salmon, carp and flounder (Gonçalves et al. 2008; Sørensen et al., 2011; Chen et al. 2011).

The genes related to the inflammatory response analysed here were *inf1*, *infg*, *tnf-α* and *il1b*. Interferons (*inf1* and *infg*), interleukin (*il1b*), and tumor necrosis factor (*tnf-α*) are proinflammatory cytokines that play a critical role in host resistance predisposed to infection, acting as immunomodulators and mediators of inflammatory responses (Huising 2004; Zou and Secombes 2011). Figure 3 (A, B, C and D) shows that the expression of these four genes had a drastic decrease in the group of zebrafish

treated with the diet supplemented with the transgenic probiotic. A 95% reduction for *inf1*, 95% for *infg*, 90% for *tnf-α* and 93% for *il1b* was observed. These results indicate that the phytase produced by the probiotic drastically reduced the inflammatory process induced by the diet with high soybean meal content. Taking into account the findings of Fuentes-Appelgren et al. (2014), the question that arises is: how would phytase be reducing the pro-inflammatory effect of saponins? Although phytases are not enzymes with high substrate specificity, it is virtually impossible to hypothesize that phytase would be promoting the direct degradation of saponins. The most reasonable explanation may come from the study of Liu et al. (2009), which analysed the effects of phytate and phytase on chicken immune functions. These authors suggest that phytate complexed with amino acids and proteins are refractory to digestion and lead to increased secretion of mucins by the gastrointestinal epithelium. These mucins are high molecular weight proteins that serve to protect the epithelium against injury and are strongly related to the onset of cancer and inflammatory diseases (Hollingsworth and Swanson 2004; Kufe 2009). Thus, phytase hydrolyzes phytate, releases the complexed nutrients to it and decreases the production of mucins. Thus, the epithelium returns to its natural state, enhancing host immunity and perhaps reducing the pro-inflammatory effects of soybean meal compounds such as saponins. Ramos et al. (2017) observed that the addition of an enzymatic complex consisting of phytase and carbohydrases prevented enteritis in *Mugil liza* fed a diet rich in soybean meal.

It is known that inflammatory processes are related to the production of reactive oxygen species (ROS). In fact, according to Lugrin et al. (2013), inflammation and oxidative stress are two processes that feed one another, establishing a vicious cycle that perpetuates and propagates the inflammatory response. Thus, in relation to the experiment that was carried out in the present study, the genes related to zebrafish's antioxidant defense system should respond in the same way as the genes related to the anti-inflammatory responses. The gene coding for superoxide dismutase (*sod1*) was analysed and the result is shown in figure 4. Expression of this gene had a significant reduction of 30% in the zebrafish group that had the feed supplemented with the transgenic probiotic. This result corroborates with the results observed for genes related to the inflammatory response, showing that both systems are directly related and also that phytase plays an important role not only in the reduction of inflammation but also in the reduction of oxidative stress in zebrafish intestine.

In conclusion, the results obtained in the present study prove the efficiency of phytase in the activation of the zebrafish immune system fed with soybean meal rich diet. This source of vegetable protein is made up of a number of antinutritional and pro-inflammatory factors such as phytate and saponins. The use of a genetically modified probiotic to express a fungal phytase has been shown to be an interesting strategy to overcome the presence of antinutritional factors in vegetal feed. In addition, this probiotic produced an active enzyme in sufficient quantity to influence the immune system of zebrafish and attenuate the inflammatory responses from the diet rich in vegetable matter. In addition, oxidative stress associated with inflammatory processes was also attenuated, resulting in an improvement in general health and in the condition factor of the fish treated with the transgenic probiotic. Thus, genetically modified probiotics seem to be an interesting tool for adapting commercially important species to aquaculture at lower cost diets, which proteins can come from cheaper and more abundant sources. This technology also opens possibilities for the treatment of diseases related to nutrition not only for aquaculture species, but also for human digestive diseases, can contribute to reduction of glucose and glycated hemoglobin, reduction of total cholesterol and LDL cholesterol, significant improvement of the eradication rate of bacteria, prevention of diarrhea, decrease in liver aminotransferase levels and improving insulin resistance, decrease overt hepatic encephalopathy in patients with liver cirrhosis.

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Conflict of Interest

The authors declare no conflict of interest.

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CAPÍTULO III

Comparing methods of genetic manipulation in *Bacillus subtilis* for expression of recombinant enzyme: replicative or integrative (CRISPR-CAS9) plasmid?

Short Communication submetido ao Journal of Biotechnology

Comparing methods of genetic manipulation in *Bacillus subtilis* for expression of recombinant enzyme: replicative or integrative (CRISPR-Cas9) plasmid?

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Resumo

No presente trabalho avaliamos a estabilidade de linhagens de *Bacillus subtilis* transformadas com plasmídeo replicativo ou integrativo (via CRISPR-Cas9) para expressar uma fitase recombinante. Esta enzima tem importantes aplicações para o aumento da digestibilidade da matéria vegetal adicionada às rações de animais cultivados. Ambos os modos de transformação não afetaram o crescimento de *B. subtilis*, mas a estabilidade da construção gênica foi grandemente reduzida nas estirpes transformadas com o plasmeo replicativo. Após 65 gerações, apenas 11% das células mantiveram o fenótipo de resistência a antibióticos associado ao construto. A linhagem transformada pelo método CRISPR-Cas9 não perdeu o fenótipo ao final do experimento. A análise da atividade da fitase no final de 65 gerações mostrou que a cepa com a construção genética integrada no genoma de *B. subtilis* foi 20% maior do que na cepa transformada com o plasmídeo replicativo. Os resultados obtidos demonstram que a tecnologia CRISPR-Cas9 é eficiente em *B. subtilis* e que as cepas produzidas apresentam menor risco de perder a característica e transferir os genes heterólogos para outras bactérias através da transferência horizontal de genes.

Palavras-chave: Probiótico; fitase; transformação bacteriana; estabilidade do plasmídeo; transferência horizontal de genes.

Abstract

In the present study we evaluated the stability of *Bacillus subtilis* strains transformed with replicative or integrative plasmid (via CRISPR-Cas9) to express a recombinant phytase. This enzyme has important applications for the increase of the digestibility of vegetal matter added to the feeds of cultivated animals. Both transformation methods did not affect the growth of *B. subtilis*, but the stability of the genetic construct was very reduced in the strains transformed with the replicative plasmid. After 65 generations, only 11% of the cells maintained the antibiotic resistance phenotype associated with the construct. The strain transformed by the CRISPR-Cas9 method did not lose the phenotype at the end of the experiment. Analysis of phytase activity at the end of 65 generations showed that the strain with the genetic construct integrated into the genome of *B. subtilis* was 20% higher than in the strain transformed with the replicative plasmid. The results obtained here demonstrate that CRISPR-Cas9 technology is efficient in *B. subtilis* and that the strains produced have a lower risk of losing the characteristic and transfer the heterologous genes to other bacteria through horizontal gene transfer.

Keywords: Probiotic; phytase; bacterial transformation; plasmid stability; horizontal gene transfer.

Although there are a wide variety of microorganisms with potential for the production of recombinant proteins, *Bacillus* species have been noted not only for their probiotic characteristics, but also for their superior ability to secrete proteins (Illing, 2002; Zweers, 2008). Among these gram-positive bacteria, *B. subtilis* is generally recognized as safe (GRAS) and can be used as a food supplement even for humans. Its ability to secrete proteins along with the possibility of being added to food makes this bacterium a potential bioreactor for recombinant proteins directly in the intestinal lumen of the host.

Bacillus subtilis can be easily manipulated with replicative plasmids, and there is a common sense that the higher the number of plasmid copies in the cell, the greater the amount of recombinant protein produced. However, episomal plasmids can cause problems since they can be transferred by conjugation to other bacteria, including to different species. This process may also result in loss of the plasmid by the original strain along with its ability to produce the recombinant protein. It is already well established that, depending on the type of bacteria, a very large number of plasmid molecules can lead to a very high energy cost to the cell and compromise the production of the heterologous protein (Gill et al., 2009; Kroll et al., 2010). On the other hand, the homologous recombination can provide a greater stability of the genetic construct that integrates the genome of the host cell. However, this process generally results in the integration of only one copy of the genetic construct, which in theory reduces the ability of the cell to synthesize the recombinant protein. Recently, the innovative CRISPR-Cas9 genomic editing technology has been successfully applied in *B. subtilis* (Altenbuchner, 2016).

In the present study, we manipulated *B. subtilis* to express and secrete a recombinant phytase of fungal origin. Phytases are enzymes that degrade phytic acid (phytate), which is the main form of phosphorus storage in plants, and monogastric animals do not assimilate this form of phosphorus. Thus, this enzyme has great importance as an additive in feed used in animal production, since it allows the inclusion of vegetable ingredients in higher amounts in the diets, reducing the dependence of animal protein.

Our objective was to evaluate if phytase production would be altered in *B. subtilis* transformed with replicative plasmid or with integrative plasmid based on

CRISPR-Cas9 technology. For this purpose, the KM0 strain was first manipulated with the replicative plasmid pJJ5 (Figure 1A), which contains two origins of replication: one for gram-negative bacteria derived from the pUC18 plasmid, and the other for gram-positive bacteria derived from pUB110 plasmid. In addition, this plasmid has a spectinomycin resistance gene and a genetic unit consisting of a promoter that is activated during the stationary phase of the *B. subtilis* (Pylb; Yu et al., 2015) culture, which controls the expression of phytase from the fungus *Aspergillus fumigatus* (GenBank accession number AHZ62778). The phytase gene was optimized for expression in *B. subtilis* through OPTIMIZER software (http://gnemos.urv.es/OPTIMIZER). In addition, the secretion signal of levansucrase (SacB; GenBank accession number CAA26513) from *B. subtilis* was added to the 5'-region of the phytase gene to produce a fusion protein to facilitate secretion. The *B. subtilis* strain transformed with the replicative pJJ5 plasmid was named BsJJ5. Spectinomycin resistance was used to evaluate the stability of the pJJ5 plasmid.

For the genomic edition of *B. subtilis*, the plasmid pJOE9620 (kindly provided by Dr. Josef Altenbuchner, University of Stuttgart, Germany) was used as a basis for cloning. This plasmid contains the GFPmut1 gene driven by the xylose-inducible promoter (Pxyl) in order to label the transformed *B. subtilis* strain. It also contains the Cas9 gene under the control of mannose-inducible promoter PmanP and sgRNAs for integration between the *yjaZ* and *trpS* genes. This genome region is rich in oligopeptide transporters genes, which are not essential for *B. subtilis* growth. The sgRNA was directed to the *appD* gene, coding for an oligopeptide transporter. The same genetic construct used to express phytase in pJJ5 plasmid was cloned into the *Sfi*I site of plasmid pJOE9620. The resulting plasmid was named pJJ14 (Figure 1B). The *B. subtilis* strain transformed with the integrative pJJ14 plasmid was named BsJJ14, which also carries a chloramphenicol resistance gene integrated by CRISPR-Cas9 technology as well (data not shown). Chloramphenicol resistance was used to evaluate the stability of the integrated genetic construct.

The cepa *B. subtilis* KM0 was transformed with pJJ5 or pJJ14 plasmid according to the standard two-step procedure with minimal Spizizen medium (Anagnostopoulos and Spizizen, 1961). Transformed colonies were selected in solid LB (Luria-Bertani) medium containing spectinomycin (100 µg/mL) or chloramphenicol (10 µg/mL) after

overnight incubation at 37°C. Bacterial growth was evaluated by optical density (OD600) spectrophotometer (BioMate3, ThermoScientific, USA). A single colony of each strain was individually inoculated into 10 mL LB medium and grown overnight, shaking at 250 rpm at 37°C. Thereafter, the culture was diluted to an optical density OD600 = 0.05 nm, and cultured under the conditions cited above. Periodically, a 1 mL aliquot was used to measure optical density. The stability of the genetic constructs present in the BsJJ5 and BsJJ14 strains was determined according to De Gelder et al. (2007), with modifications. A pre-inoculum was incubated in LB medium for 16 hours, shaken at 250 rpm at 37°C, with the selection antibiotic (spectinomycin or chloramphenicol). After reaching the stationary growth stage, the culture was centrifuged at 8,000 x g for 10 min at 4°C. For removal of the antibiotic, the pelett was washed with sterile saline (0.85% NaCl). Then, an aliquot was diluted to an optical density (OD600) = 0.05 nm and cultured in non-selective LB medium until the culture reached a (OD600) = 0.1 nm. This procedure was repeated 13 times, and in each stage aliquots of the cultures were plated in medium with or without antibiotic. Stability was determined by the ratio between the colony forming units (CFU) of the selective and non-selective plaques.

After approximately 65 generations, a single colony of each strain was inoculated into 5 ml of selective LB medium at 37°C, and grown overnight. Subsequently, the cultures were centrifuged at 8,000 x g for 10 min at 4°C for removal of the antibiotic and the peletts were washed with sterile saline (0.85% NaCl). Optical density (OD600) was measured, the culture was diluted in 10 mL of non-selective LB medium and a new growth was established with initial OD600 = 0.05 nm. The growth was continued for 16 hours for the culture to reach the stationary phase and activate the Pylb promoter for phytase expression. After, the culture was centrifuged at 8000 x g for 30 min at 4°C. The supernatant was collected for the determination of phytase activity using the method of Heinonen and Lahti (1981), with slight adaptations for assays in 96-well plates. The activity was determined in the supernatant having 1 M sodium phytate ($C_6H_6O_{24}P_6Na_{12}$; Sigma-Aldrich) as a substrate buffered with 2 M sodium acetate (pH 5.0). The reaction occurred for 30 min at 40°C and determined by the addition of the color reaction (AAM) prepared with acetone, 5 M sulfuric acid and 10 mM ammonium molybdate (2:1:1, v/v). After 30 sec, the adding of 1 M citric acid quenched the reaction. The released inorganic orthophosphate was measured. The assay

was performed in five-fold. Absorbance was read at 405 nm. One unit of phytase activity was defined as the amount of enzyme needed to release 1 μmol of phosphate per minute under the assay conditions.

The transformation protocol of *B. subtilis* with the plasmid pJJ14 showed to be efficient since the results of Figure 2 presents a significant number of colonies expressing the GFPmut1 used as the reporter gene. Figure 3 shows that the genetic manipulation method used did not affect the growth of both BsJJ5 and BsJJ14 strains. However, the stability test of the genetic construct over the generations shown in Figure 4 pointed to a significant loss of the antibiotic resistance characteristic in the strain transformed with the replicative plasmid (BsJJ5). In this case, only about 11% of the cells maintained resistance to spectinomycin at the end of the 65 generations. On the other hand, the strain BsJJ14 remained without loss of the resistance phenotype throughout the experiment. Finally, Figure 5 shows that phytase activity was even higher in the BsJJ14 strain than in the BsJJ5 strain at the end of the 65 generations. This approximately 20% of difference shows that the replicative plasmid, although present in a less number of cells, still manages to produce a reasonable amount of enzyme probably due to the high copy number maintained in each cell. However, loss of the plasmid has important implications that concern not only the short short-life of the strain from a commercial point of view, but also the possibility of horizontal transfer. Sharing characteristics such as resistance to antibiotics with other bacteria can generate multi-resistant strains with unpredictable consequences for organisms and the environment. In conclusion, the use of integrative plasmid via CRISPR-Cas9 technology seems to be a viable alternative for the expression of recombinant enzymes in *B. subtilis*, generating very stable strains and with low risk of horizontal transfer of the manipulated characteristics.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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Figure legends

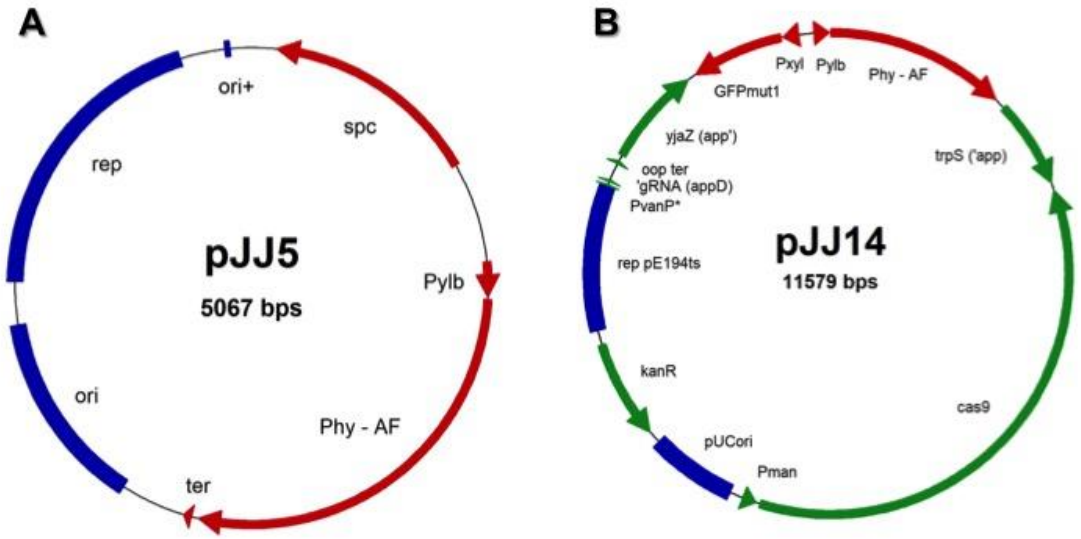

Figure 1. Constructions of plasmids used for *B. subtilis* transformation. A) replicative plasmid pJJ5. spc: spectinomycin resistance gene; Pylb: stationary phase promoter of *B. subtilis*; Phy-AF: fungal phytase *Aspergillus fumigatus*; Ori: origin of replication for gram-negative bacteria; rep: origin of replication for gram-positive bacteria; ter; termination region. B) integrative plasmid pJJ14. Pxyl: promoter activated by xylose; GFPmut1: gene coding for green fluorescent protein; Pman: promoter activated by mannose; gRNA: RNA guide; YjaZ: gene used for homologous recombination.

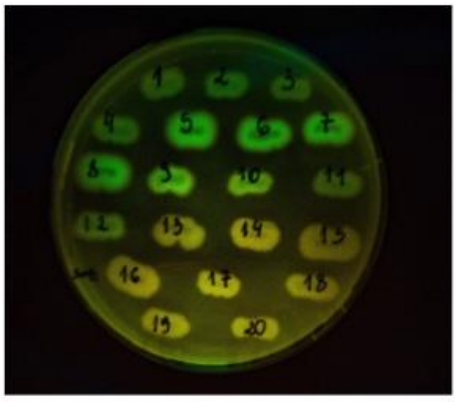

Figure 2. Fluorescent plate imaging of *Bacillus subtilis* strain pJJ14 stably expressing GFPmut1 (Colonies 1-12) after 3 days of xylose induction.

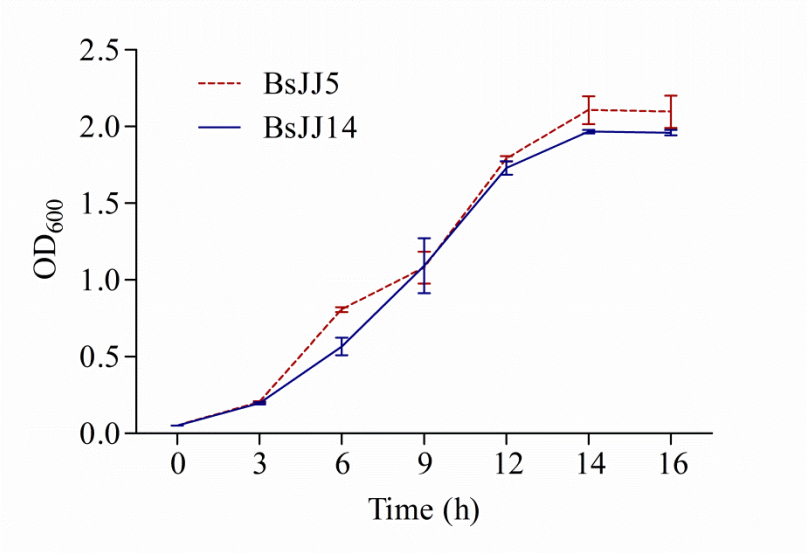

Figure 3. Growth curve of *B. subtilis* BsJJ5 and BsJJ14 strains. Cells were cultured in Luria bertania (LB) medium at 37°C at 250 rpm, OD_{600} optical density was measured every 3 hours for up to 16 hours. The assay was performed in triplicate (the data are presented at the mean ± standard deviation)

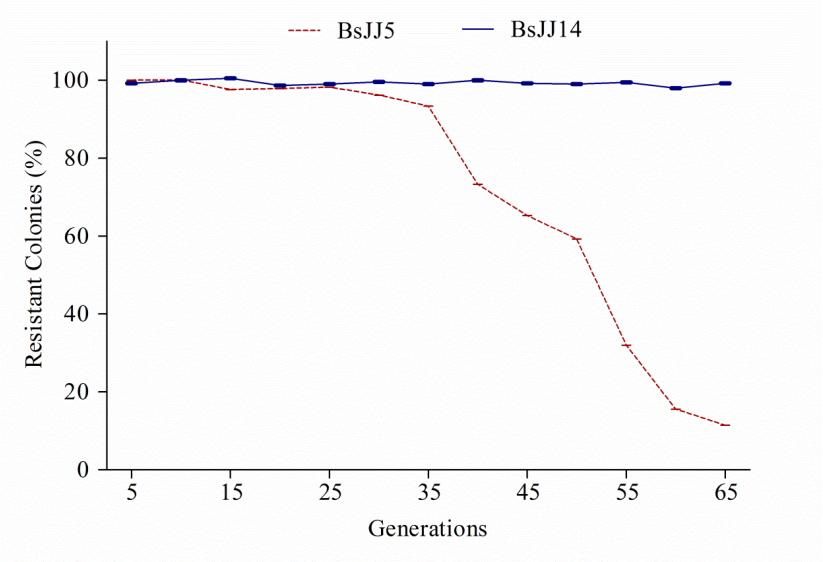

Figure 4. Stability analysis of BsJJ5 and BsJJ14 *B. subtilis* strains grown for 65 generations. Resistance to antibiotics spectinomycin (BsJJ5) and chloramphenicol (BsJJ14) were the phenotypic characteristics evaluated. The experiment was carried out in triplicate and the data represent the mean with standard deviation of the results of the colonies count

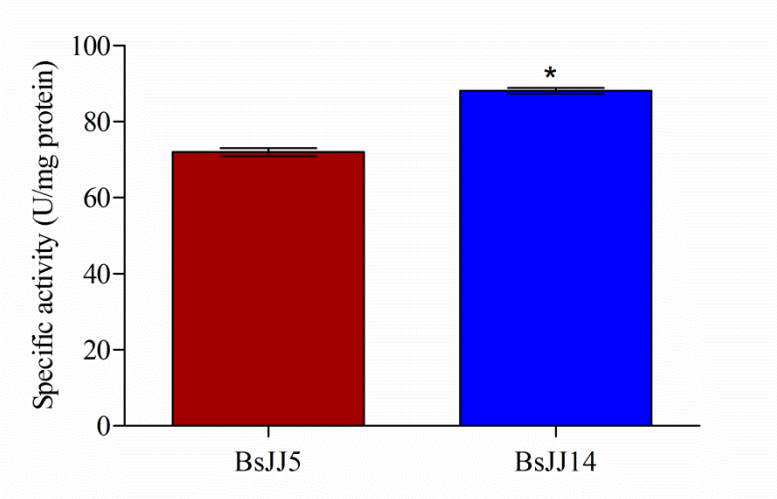

Figure 5. Analysis of phytase activity at the end of 65 generations in BsJJ5 and BsJJ14 strains. The asterisk means statistical difference (P <0.05, Student's t-test).

DISCUSSÃO GERAL

A fitase tem um potencial considerável na tecnologia de alimentação animal e na proteção ambiental. Atua reduzindo os efeitos antinutrientes do fitato nas dietas dos animais, aumentando assim a disponibilidade de energia, minerais e aminoácidos. A suplementação de fitases na aquicultura é amplamente efetuada na forma purificada e isto representa um obstáculo, pois o isolamento e a purificação de enzimas é um processo, em sua maioria, difícil e de alto custo (Dudley et al., 2014; Singh et al., 2016). Uma alternativa ao uso de fitases purificadas é a utilização de micro-organismos potencialmente produtores dessas enzimas. Várias espécies de *Bacillus* são amplamente usadas para a expressão heteróloga de proteínas. *Bacillus* também um dos principais produtores industriais de enzimas e um ótimo hospedeiro microbiano GRAS (geralmente reconhecido como seguro). Já na alimentação aquática, *Bacillus subtilis* é amplamente usado como o probiótico (Syngai et al., 2015). Para o desenvolvimento da presente tese selecionamos a sequência de uma fitase de *Aspergillus fumigatus* que possui uma plasticidade térmica (Rodriquez et al., 2000) e elevada atividade em diferentes pHs (Wyss *et al.* 1999, Singh *et al.* 2016). Uma construção genética constituída pela sequência da fitase fúngica, por um sinal de secreção e pelo promotor de expressão de fase estacionária (Pylb), foi clonado no vetor pJJ5 e usado para transformação episomal em *Bacillus subtilis*. Com isso obtivemos clones positivos (Phy-Af) para o gene da fitase que apresentaram atividade enzimática 2,7 vezes maiores que o controle não transgênico. Após constatar a atividade da fitase (Phy-Af), desenvolvemos um experimento de alimentação de zebrafish durante 30 dias com uma dieta rica em ingredientes vegetais suplementada com Phy-Af e com o controle não transgênico. Os resultados do experimento mostraram que a suplementação do *B. subtilis* KM0 expressando uma fitase fúngica não afetou a ingestão de alimento e promoveu melhoras significativas no ganho de peso, no crescimento específico e melhorou a conversão alimentar de zebrafish. A expressão de genes que codificam os transportadores de peptídeos PepT1 e PepT2 (codificados pelos genes *slc15a1b* e *slc15a2*, respectivamente) aumentaram 22 vezes para o *slc15a1b* e 2,3 vezes para o *slc15a2* no grupo alimentado com Phy-Af em relação ao controle. Nossos resultados se somam aos observados em suínos (Vigors et al., 2014) e em frangos de corte (Cowieson et al., 2018). Nesses animais, a fitase também contribuiu para o aumento da expressão do mRNA dos transportadores peptídicos. Sincronicamente, os peixes do grupo Phy-Af

apresentaram aumento na expressão do gene do fator de crescimento semelhante à insulina 1 (*igf1*) no fígado e na musculatura esquelética. Os resultados mostraram um aumento significativo na expressão desse gene na ordem de 6,3 vezes no fígado e 7,7 vezes no músculo em comparação com os controles. Assim, a expressão de *igf1* corrobora com os resultados observados para o ganho de peso e a taxa de crescimento específico dos peixes alimentados com o probiótico transgênico. Seguindo o perfil de expressão dos genes já citados, os fatores miogênicos *myod* e *myog* também aumentaram na proporção de 2,3 vezes para *myod* e 15,2 vezes para *myog* no músculo. Considerando que o fósforo é um elemento indispensável para o crescimento de todos os organismos vivos, e as dietas baseadas em ingredientes de origem vegetal normalmente são completadas com fontes de fósforo inorgânico a fim de satisfazer as necessidades diárias (Kumar et al., 2012), avaliamos se a Phy-Af poderia influenciar na capacidade de metabolizar minerais como o fósforo e cálcio. Para isso analisamos os níveis de expressão do gene da osteocacina que é uma proteína importante no metabolismo ósseo e uma de suas funções está relacionada à absorção de cálcio pela matriz óssea (Gavaia et al., 2006; Cancela et al., 2014). Nossos resultados mostraram que houve um aumento de 4,5 vezes na expressão gênica da osteocalcina no grupo alimentando com Phy-Af quando comparado ao controle. A ração experimental impactou negativamente no fator de condição (K) dos peixes em ambos os grupos (Phy-Af e controle). Porém a mudança de dieta impactou os zebrafish de forma diferente, onde o grupo controle apresentou uma diminuição no K em 26%, o que foi estatisticamente significativo ($p < 0,05$). Por outro lado, o grupo tratado com o probiótico transgênico (Phy-Af) apresentou uma perda de K de aproximadamente 10%, o que não foi estatisticamente significativo. Em nosso estudo observamos o aumento na expressão dos genes *cd4* e *ikzf1* que estão relacionados à resposta imune em teleósteos. Esse é um forte indício de que a fitase produzida por *B. subtilis* foi capaz de influenciar no sistema imunológico do zebrafish, através da produção de linfócitos. Os genes relacionados à resposta inflamatória apresentaram uma redução de 95% para *ifnphi1* e para *infg*, 90% para *tnf-α* e 93% para *il1b* no grupo tratado com Phy-Af. Estes resultados indicam que a fitase produzida pelo probiótico reduziu drasticamente os possíveis processos inflamatórios induzidos pela dieta com alto teor de ingredientes vegetais. O mesmo perfil de redução foi observado para o gene que codifica a resposta antioxidante de superóxido dismutase (*sod1*) no grupo (Phy-Af). Em nosso trabalho de alimentação de zebrafish com o *B. subtilis* expressando a fitase de *A. fumigatus*, a cepa

transgênica para o gene da fitase foi obtida através da transformação com um plasmídeo de transformação episssomal. Porém, um problema de grande importância para sistemas que usam plasmídeos episssômicos é a capacidade de manutenção e estabilidade do plasmídeo. Portanto, para a produção de proteínas heterólogas de forma eficiente é necessário garantir que, na divisão celular, as células filhas transportem pelo menos uma cópia do plasmídeo (De Gelder et al., 2007; Yang *et al.* 2016). Por outro lado, a recombinação homóloga pode proporcionar uma maior estabilidade da construção genética, que integra o genoma da célula hospedeira, e uma técnica moderna para a recombinação homóloga é o uso da edição genômica pelo sistema CRISPR-Cas9 (Altenbuchner, 2016). Usando o sistema CRISPR-Cas9 para transformação de *Bacillus*, obtivemos a cepa BsJJ14 com o gene da fitase fúngica integrada ao seu genoma, observamos que esta cepa permaneceu sem perda do fenótipo de resistência ao longo de um ensaio de manutenção da resistência ao antibiótico, enquanto que a cepa BsJJ5 (Phy-Af usada no experimento de alimentação) ao fim das 65 gerações apenas cerca de 11% das células mantiveram a resistência à espectinomicina. A atividade fitásica para a cepa BsJJ14 foi ainda maior do que para linhagem BsJJ5 após 65 gerações.

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CONSIDERAÇÕES FINAIS

- ✓ O sistema de expressão de *B. subtilis* tem alguns fatores limitantes como a transcrição, o dobramento de proteínas, a translocação através da membrana, o processamento de péptido sinal e de proteólise. Embora a produção de proteínas recombinantes eucarióticas em bactérias possua algumas limitações, em nosso estudo realizamos a otimização do códon como uma estratégia para viabilizar a expressão da fitase fúngica em *B. subtilus*. Também usamos um peptídeo sinal (SacB - Levansucrase de *Bacillus subtilis*), para promover a secreção da fitase para o meio extracelular e observamos um considerável rendimento no total de proteínas excretadas a partir do sobrenadante.
- ✓ O nosso sistema de expressão foi constituído pelo promotor autoindutível Pylb. A natureza autoindutível deste promotor permite a expressão de proteínas sem indutores como os açúcares β-D-1-tiogalactopiranósido isopropílico (IPTG), manose, xilose ou glicose, o que pode viabilizar a redução dos custos de produção em grande escala geradas pela aquisição destes indutores. Os promotores autoindutíveis são estimulados por diversos fatores ambientais e o promotor Pylb, por exemplo, promove a expressão do gene alvo apenas na fase estacionária de crescimento, que foi constatado pela atividade da fitase após esse período. Também usamos uma cepa supercompetente e facilmente transformada com DNA exógeno, o que contribuiu para o sucesso na obtenção dos transgênicos para o gene da fitase.
- ✓ Constatamos que a fitase de *Aspergillus fumigatus* expressa pela cepa KM0 foi capaz de melhorar significativamente o desempenho de zebrafish alimentados com uma dieta rica em proteína vegetal. Ainda, os efeitos benéficos dessa fitase, além de estarem relacionados a melhorias no crescimento, também relacionam-se à ativação transcricional de genes transportadores de aminoácidos, de genes promotores de crescimento, imunidade, resposta inflamatória e resposta antioxidante.
- ✓ O ácido fítico é uma molécula natural, abundante em vegetais. Dentre outras funções, o fitato inibe a absorção de minerais essenciais para a formação óssea. Em nosso estudo observamos que a fitase foi capaz melhorar a expressão do gene da osteocalcina que é uma proteína que influencia as propriedades ósseas dos peixes e é considerada uma proteína específica do tecido ósseo estando diretamente relacionada à concentração de cálcio e fósforo.

- ✓ Outra constatação relevante observada na presente tese, é que a transição de uma ração comercial balanceada para uma ração rica em ingredientes vegetais afetou o fator de condição dos peixes. Isso leva a considerar que a transição para dietas com maior quantidade de ingredientes vegetais deve ser repensada e realizada cautelosamente, ainda que a fitase testada tenha minimizado a redução no fator de condição.
- ✓ Até onde sabemos, esta é a primeira vez que uma linhagem bacteriana transgênica do gênero *Bacillus* transgênico com a capacidade de excretar uma fitase recombinante foi suplementado à ração. Os resultados nos levam a uma visão mais profunda sobre a importância da incorporação de fitase na alimentação através de um propagador biológico. Isto possibilita uma economia frente às fitases purificadas disponíveis no mercado, além de criar uma possibilidade para contribuir mais amplamente com estudos que explorem fontes alternativas de proteínas adicionais as novas rações. Considerando isto, é notório que o estudo será relevante no campo da nutrição de peixes, pois o zebrafish é tido como um organismo modelo, com extensa literatura sobre sua morfologia intestinal e desenvolvimento do trato digestório.
- ✓ A integração de DNA exógeno no cromossomo de uma célula é um processo oneroso, entretanto o surgimento da tecnologia CRISPR-Cas9 simplifica os procedimentos de transformação microbiana por promover o direcionamento da inserção gênica. Nos trabalhos aqui apresentados, esta tecnologia permitiu a obtenção de uma cepa expressando gene da fitase de *A. fumigatus* e forma estável e duradoura.

PERSPECTIVAS

- ✓ Probióticos geneticamente modificados para expressar e secretar enzimas podem ser utilizados para os mais variados propósitos, incluindo aspectos nutricionais, imunológicos, reprodução, combate a infecções bactérias e a viroses, por exemplo.
- ✓ No campo nutricional, probióticos geneticamente modificados podem ser modelos para a expressão de diversas enzimas, como a tripsina e quiotripisinas, que são importantes na digestão de proteínas, ou expressão de carboidrases que atuem na hidrólise de polissacarídeos não amiláceos presentes em rações vegetais.
- ✓ A presença de glicosídeos em ingredientes de origem vegetal, como as saponinas, por exemplo, é um tópico importante na alimentação aquática. Neste sentido a expressão de glicosidases que possam atuar na hidrólise desses antinutriente parece ser uma via alternativa.
- ✓ Considerando nossos resultados relacionados à resposta imune e inflamatória, sugere-se um teste desafio para melhor avaliar as respostas imunes dos peixes alimentados com fitase.
- ✓ Esta tecnologia também abre possibilidades para o tratamento de doenças extrapolando, inclusive, para doenças crônicas humanas.