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Adjuvanticidade de *Bacillus toyonensis* e *Saccharomyces boulardii* em vacinas avaliadas em murinos e ovinos

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“O temor do Senhor é o princípio da sabedoria”

Provérbios cap.1 verso 7

Resumo

SANTOS, Francisco Denis Souza. **Adjuvanticidade de *Bacillus toyonensis* e *Saccharomyces boulardii* em vacinas avaliadas em murinos e ovinos.** 2020. 181f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Os probióticos modulam a resposta imune do hospedeiro, podendo aumentar a eficácia de vacinas. O objetivo deste trabalho foi estudar o efeito de *Bacillus toyonensis* e *Saccharomyces boulardii* na resposta imune de murinos e ovinos vacinados com vacinas recombinantes e convencionais. Foram realizados três experimentos em murinos e dois em ovinos. No primeiro experimento, camundongos foram suplementados pela via oral com 1×10^6 esporos viáveis de *B. toyonensis* por grama de ração durante todo o período experimental e outro grupo nos 7 dias que antecederam a primeira vacinação. No segundo experimento, um grupo de camundongos foi suplementado com *B. toyonensis* na mesma concentração, via e período e o outro grupo recebeu a inoculação de 1×10^6 esporos de *B. toyonensis*, como componente da vacina, pela via subcutânea (s.c.) nos dias 0 e 21. Em ambos os experimentos os camundongos foram vacinados pela via s.c. com a glicoproteína D recombinante do herpesvírus bovino tipo 5 (BoHV-5). No terceiro experimento com camundongos, os animais foram suplementados com *B. toyonensis* pela via oral como descrito para os experimentos anteriores, mas vacinados com o fragmento C da toxina tetânica (TTFC) adsorvida aos esporos de *B. subtilis* pela via de mucosa (intranasal). No primeiro experimento com ovelhas, 3×10^8 esporos viáveis de *B. toyonensis* foram administrados pela via oral durante 5 dias e as ovelhas posteriormente vacinadas com a toxina épsilon recombinante (rETX) de *Clostridium perfringens*. No segundo experimento, ovelhas foram suplementados pela via oral com 3×10^8 esporos viáveis de *B. toyonensis* ou *S. boulardii*, durante 5 dias antes das vacinações contra *Clostridium chauvoei*. A resposta imune humoral e celular foi avaliada por ELISA indireto, por ensaios de soroneutralização e pela detecção da expressão de citocinas em esplenócitos e células mononucleares do sangue periférico (PBMCs). Todos os camundongos suplementados com *B. toyonensis*, independentemente do regime, apresentaram elevados níveis séricos de anticorpos (IgG total, IgG1 e IgG2a) com propriedades neutralizantes e níveis esplênicos de transcrição de mRNA de IL-4, IL-12 e IFN-γ superiores quando comparados aos controles. A suplementação com *B. toyonensis* nos camundongos vacinados com a TTFC adsorvida aos esporos de *B. subtilis* modulou a resposta humoral, estimulando níveis superiores de IgA fecal, IgG total e IgG2c quando comparado aos controles, e a resposta celular através da produção de níveis superiores de IL-6, IL-10 e IFN-γ pelos esplenócitos dos animais suplementados. As ovelhas suplementadas com *B. toyonensis* e vacinadas com a rETX, assim como as ovelhas suplementadas com *B. toyonensis* ou *S. boulardii* vacinadas contra *C. chauvoei* apresentaram níveis mais elevados de IgG total, IgG1 e IgG2 quando comparadas com as não suplementadas. PBMCs de todas as ovelhas suplementadas com os probióticos apresentaram níveis de transcrição de mRNA de IL-2, IFN-γ e Bcl6 superiores. A suplementação com *B. toyonensis* e *S. boulardii* durante um período reduzido foi suficiente para modular a resposta imune. Os resultados indicam que probióticos utilizados neste estudo foram capazes de estimular a imunidade e melhorar a resposta às vacinas em murinos e ovinos.

Palavras-chave: Probióticos, Esporos, Adjuvante, Imunomodulação, Vacinas.

Abstract

SANTOS, Francisco Denis Souza. **Adjuvanticity of *Bacillus toyonensis* and *Saccharomyces boulardii* in vaccines evaluated in murine and sheep.** 2020. 181f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Probiotics modulate the immune response and can increase the effectiveness of vaccines The aim of this study was to assess the effects of *Bacillus toyonensis* and *Saccharomyces boulardii* on the immune response of mice and sheep vaccinated with recombinant and conventional vaccines. Three experiments were performed in mice and two experiments in sheep. In the first experiment with mice, one group of animals was supplemented orally with 1×10^6 viable spores of *B. toyonensis* per gram of food throughout the experimental period while other group was submitted to probiotic treatment only 7 days prior to the initial vaccination. In the second experiment, whilst one group followed the same regimen as in the first experiment (every day during the experiment), another group received the inoculation with 1×10^6 viable spores of *B. toyonensis* by subcutaneously on day 0 and day 21. In both experiments, mice were vaccinated Bovine herpesvirus type 5 (BoHV-5) recombinant glycoprotein D. In the third experiment with mice, one group was submitted to oral administration of *B. toyonensis*, as described for the other experiments, however, the animals were vaccinated with the C fragment of the tetanus toxin (TTFC) adsorbed on *B. subtilis* spores by the mucosal (nasal) route. Regarding the experiments with sheep, on the first experimental test, sheep were supplemented by oral route with 3×10^8 viable spores of *B. toyonensis* for 5 days prior to the vaccinations with *Clostridium perfringens* recombinant epsilon toxin (rETX). In the second experiment, one group received the same dose as in the first experiment, but another group was exposed to the same dosage of *S. boulardii.* A third group received a combination of these microorganisms. In all the groups the administration of probiotics was made orally 5 days prior to the vaccinations against *Clostridium chauvoei*. The humoral and cellular immune response was evaluated by indirect ELISA, serum neutralization and by detection of cytokines in splenocytes and peripheral blood mononuclear cells (PBMCs). The groups of mice supplemented with spores of *B. toyonensis* (regardless of the regimen experiment) have presented high serum antibody levels (total IgG, IgG1, and IgG2a) with neutralizing capacity and higher mRNA transcript levels of IL-4, IL-12, and IFN-γ in splenocytes when compared to the controls. The supplementation of *B. toyonensis* in mice vaccinated with TTFC-adsorbed *B. subtilis* spores modulated the humoral immune response of mice, increased fecal IgA, total IgG and IgG2c serum levels and cellular immune response by stimulating the production of high levels of IL-6, IL-10, and IFN-γ in splenocytes. In the experiments with sheep it was observed that groups exposed to *B. toyonensis* and vaccinated with rETX presented higher total IgG, IgG1, and IgG2 levels as well as sheep supplemented either with *B. toyonensis* or *S. boulardii,* in comparison with controls. The PBMCs from supplemented sheep of both experiments presented higher mRNA transcript levels of IL-2, IFN-γ and Bcl6. In conclusion, the short-term probiotic supplementation was sufficient to modulate the immune response. The results indicate these probiotics were able to stimulate immunity and improve the response to vaccines in murine and sheep.

Keywords: Probiotics, Spores, Adjuvant, Immunomodulation, Vaccines.

Sumário

1 INTRODUÇÃO GERAL

Probióticos são microrganismos vivos que, quando ingeridos em quantidades adequadas, conferem efeitos benéficos à saúde do hospedeiro (Hill et al., 2014). Os probióticos podem modificarr a microbiota intestinal, fortalecer e modificar a barreira intestinal, produzir moléculas antimicrobianas, inibir a adesão de patógenos e modular a resposta imune (Forsythe & Bienenstock, 2010). Os microrganismos probióticos modulam a resposta imune do hospedeiro através do aumento na atividade de macrófagos e de células *natural killer*, nos níveis de imunoglobulinas e na produção de citocinas (Maldonado Galdeano et al., 2019; Yousefi et al., 2019). A modulação imune mediada pelos probióticos pode ocorrer tanto localmente, nas mucosas, como em tecidos imunes distantes (de Moreno de LeBlanc et al., 2005; Lemme-Dumit et al., 2018).

As bactérias formadoras de esporos pertencentes ao gênero *Bacillus* são amplamente utilizadas como probióticos para animais e humanos, e sabe-se que um dos seus efeitos benéficos é através da modulação da resposta imune do hospedeiro (Cutting, 2011). Destacam-se neste grupo *B. toyonensis* e *B. subtilis* que são utilizadas há várias décadas como probióticos na alimentação animal, melhorando a eficiência alimentar e provendo benefícios à saúde dos animais (Gil-Turnes, 2007; Hong et al., 2005; Jiménez et al., 2013). *Bacillus toyonensis* exerce efeito imunomodulador sendo capaz de aprimorar a eficácia de vacinas em murinos, ovinos, suínos e aves através da modulação da resposta imune (Gil de los Santos et al., 2012; Roos et al., 2010; 2012; Santos et al., 2018; Schierack et al., 2007). Além disso, os esporos de *B. subtilis* são utilizados como sistema de entrega de antígenos, esporos geneticamente modificados expressam antígenos heterólogos na sua superfície ou antígenos são adsorvidos na capa proteica dos esporos (Duc & Cutting, 2003; Ricca et al., 2014). Os esporos também podem ser utilizados associados aos antígenos como componente da vacina pois induzem um forte efeito adjuvante na resposta imune (Barnes et al., 2007; De Souza et al., 2014; Oliveira-Nascimento et al., 2012).

As leveduras *Saccharomyces cerevisiae* e *S. boulardii* possuem propriedades probióticas e são comumente usadas em humanos e animais (Elghandour et al., 2019; Sen & Mansell, 2020). *Saccharomyces boulardii* é principalmente utilizada no controle de distúrbios gastrointestinais (McFarland, 2010), desenvolve sua ação probiótica

através de vários mecanismos, incluindo a competição com microrganismos patogênicos por nutrientes, produção de peptídeos antimicrobianos, competição por sítios de ligação impedindo a adesão de patógenos na mucosa intestinal e aumento da resposta imune (Naimah et al., 2018; Rodrigues et al., 2000; Sen & Mansell, 2020; Terciolo et al., 2017). A suplementação com *S. boulardii* foi capaz de melhorar a resposta imune de vacinas convencionais e recombinantes em murinos e ovinos (Coppola et al., 2005; Hudson et al., 2016; Roos et al., 2010; 2018; Silveira et al., 2017).

O uso de probióticos pode ser considerado uma alternativa promissora para aprimorar a eficácia de vacinas convencionais e recombinantes (Díaz et al., 2018; Roos et al., 2012; Santos et al., 2018). Porém, o efeito modulador dos probióticos na resposta imune a vacinas depende das características do microrganismo probiótico e do hospedeiro, do período de suplementação (contínua ou curta), do tipo de antígeno vacinal e adjuvante utilizado assim como da via de administração da vacina. O objetivo deste estudo foi avaliar o efeito de *B. toyonensis* e *S. boulardii* na modulação da resposta imune de camundongos e ovinos inoculados com vacinas recombinantes ou convencionais.

2 REVISÃO BIBLIOGRÁFICA

2.1 Probióticos

Os probióticos são definidos como microrganismos vivos que, quando ingeridos em quantidades adequadas, conferem efeitos benéficos à saúde do hospedeiro (FAO/WHO, 2002; Hill et al., 2014). Os microrganismos considerados probióticos devem ser desprovidos de resistência a antibióticos e citotoxicidade, assim como devem ter a capacidade de estimular a imunidade de mucosa do hospedeiro, e fortalecer a resistência à colonização por bactérias patogênicas na mucosa intestinal (Zommiti et al., 2019). Segundo a FAO/WHO (2002) os requisitos mínimos necessários para um microrganismo obter o status de probiótico incluem a avaliação de sua identidade (gênero, espécie e local de onde foi isolado); testes *in vitro* que demonstrem resistência à acidez gástrica, ácidos biliares, enzimas digestivas e que possuam atividade antimicrobiana contra microrganismos potencialmente patogênicos; avaliação de segurança e estudos *in vivo* que comprovem os efeitos benéficos na saúde do hospedeiro.

Entre os microrganismos utilizados como probióticos destacam-se as bactérias ácido lácticas dos gêneros *Lactobacillus* e *Bifidobacterium* que são componentes da microbiota intestinal de humanos e animais (Guarner & Malageada, 2003; Holzapfel & Schillinger, 2002), espécies de leveduras principalmente *Saccharomyces boulardii* e *S. cerevisiae* (Mcfarland, 2006) e de *Bacillus* sp. isolados da natureza como *B. toyonensis*, *B. subtilis*, *B. coagulans*, *B. clausii*, *B. megaterium*, *B. licheniformis* (Cutting, 2011; Hong et al., 2005). Na alimentação animal os probióticos podem ser utilizados como profiláticos, terapêuticos, imunomoduladores e promotores de crescimento, constituindo-se numa alternativa ao uso de antibióticos na dieta animal (Chaucheyras-Durand & Durand, 2010; Coppola & Turnes, 2004; Krehbiel et al., 2003).

O uso de probióticos na nutrição animal melhora a saúde, consequentemente, a produtividade (Musa et al., 2009). A suplementação com probióticos tem efeito significante na absorção e utilização da dieta, aumentando o ganho de peso diário e total em frangos (Samli et al., 2007), perus (Vicente et al., 2007), suínos (Kantas et al., 2015; Liao & Nyachoti, 2017), ovinos, caprinos (Abd El-Tawab et al., 2016; Chiofalo et

al., 2005) e bovinos (Ávila et al., 2000; Uyeno et al., 2015). Além disso, a suplementação com probióticos pode melhorar a quantidade e a qualidade da carne (Antunovic et al., 2005; Musa et al., 2009), do leite (Kritas et al., 2006) e ovos (Peralta-Sánchez et al., 2019) . Os efeitos positivos mediados pelos probióticos na saúde e nos parâmetros produtivos dos animais dependem de vários fatores como: a espécie e a quantidade de microrganismo presente na dieta, a dose (geralmente a recomentada para a maioria dos probióticos é 10^9 UFC por kg de alimento), e a formula do probiótico, a qual pode ser composta por um, dois ou pela associação de vários microrganismos (Simon, 2005). A estocagem dos suplementos contendo probióticos se não realizada corretamente pode alterar o número de microrganismos viáveis, isso ocorre, pois, alguns microrganismos probióticos são sensíveis a exposição a temperaturas, luz e umidade (Markowiak & Śliżewska, 2018).

Os principais mecanismos de ação propostos incluem a modificação da microbiota intestinal, fortalecimento da barreira epitelial do intestino, inibição da adesão de patógenos, exclusão competitiva de microrganismos patogênicos, produção de substancias antimicrobianas e a modulação do sistema imunológico (Bermudez-Brito et al., 2012).

2.1.1 Modulação da resposta imune pelos probióticos

É amplamente discutido que os microrganismos probióticos exercem um efeito modulador na resposta imunológica do hospedeiro, no entanto, os mecanismos de ação dos probióticos não são conhecidos totalmente, por isso são alvos de vários estudos que possuem como objetivo elucidar como ocorre a modulação imune mediada pelos probióticos (Peters et al., 2019). Um dos principais aspectos que devem ser considerados é que os efeitos dos probióticos na modulação da resposta imune dependem das características do microrganismo utilizado e do hospedeiro (Patel et al., 2015). Os efeitos dos probióticos são observados na modulação do perfil e níveis de secreção de citocinas, na influência da população de linfócitos T e no aumento da secreção de anticorpos (Thomas & Versalovic, 2010).

Na mucosa intestinal os probióticos tem a habilidade de interagir com células epiteliais intestinais (IECs), células dendríticas (DCs), macrófagos e linfócitos (Bermudez-Brito et al., 2012). Quando chegam no ambiente intestinal estes microrganismos interagem diretamente com as IECs e com as DCs estimulando a

função intestinal e a resposta imune inata e adaptativa. As IECs e as DCs reconhecem os microrganismos através de seus receptores de reconhecimento padrão (PRRs), principalmente os receptores *toll-like* (TLRs) (Gómez-Llorente et al., 2010). A resposta desencadeada pelas IECs depende do subtipo de células. As células de Paneth produzem defensinas e as células caliciformes aumentam a produção de muco. Os probióticos podem ser processados pelas DCs que respondem a esta interação, com a produção de citocinas, quimiocinas, moléculas do complexo principal de histocompatibilidade (MHC) e moléculas co-estimulatórias que direcionam o desenvolvimento das respostas dos linfócitos T virgens auxiliares em Th1, Th2, Treg e Th17 (Lebeer et al., 2010). Dessa forma as DCs e os linfócitos T ativados migram para os linfonodos mesentéricos, assim como entram na circulação sistêmica estimulando a resposta imune distante do local onde foram ativadas (Chieppa et al., 2006).

Os microrganismos presentes na mucosa intestinal, inclusive os probióticos, podem interagir com as células do sistema imune através das células M que captam o microrganismo e o transportam até as placas de Peyer (Iwasaki, 2007). Huang et al. (2008) estudaram a interação entre os esporos de *Bacillus* sp. e o tecido linfoide associado ao intestino (GALT) de camundongos. Esporos administrados pela via oral na dieta destes animais estimularam o aumento da proliferação celular nos centros germinativos das placas de Peyer, sugerindo que os esporos são capturados pelas células M e interagem com as células apresentadoras de antígenos (APCs) e linfócitos nas placas de Peyer, estimulando a resposta imune humoral e celular. Os esporos estimularam *in vivo* e *in vitro* a expressão de genes de citocinas pró-inflamatórias como TNF-α e IL-6 pelos esplenócitos e nódulos linfáticos mesentéricos dos camundongos. Em cultivos celulares de macrófagos as células vegetativas de *B. subtilis* estimularam a expressão de genes de receptores TLR2 e TLR4 (Huang et al., 2008).

Na lâmina própria do intestino os linfócitos B se diferenciam em células plasmáticas e secretam anticorpos IgA diméricos (Woof & Ken, 2006). Os probióticos estimulam a produção e secreção de IgA pelas células plasmáticas na mucosa intestinal (Galdeano et al., 2007). A interação dos probióticos com as células imunes na mucosa intestinal ou principalmente nas placas de Peyer sinalizam um aumento da produção de citocinas e um aumento no número de células secretoras de IgA na lâmina própria, assim como em superfícies mucosas distantes, como nos brônquios e

nas glândulas mamárias (de Moreno de LeBlanc et al., 2005). A suplementação oral com *B. bifidum* Bb-11 em camundongos aumentou o número de células secretoras de IgA presentes nos linfonodos mesentéricos (MLNs) e no baço, assim como e aumentou os níveis de IgA intestinal e sistêmica (Park et al., 2002). Em leitões vacinados com uma vacina atenuada contra o rotavírus humano, a administração via oral de *L. rhamnosus* GG e *B. animalis lactis* estimulou o aumento da população de células secretoras de IgA (Kandasamy et al., 2015).

2.1.2 *Bacillus toyonensis*

Bacillus toyonensis é uma bactéria Gram positiva formadora de esporos isolada do solo no ano de 1966 no Japão (Kozasa et al., 1977). Foi primeiramente identificada como *B. cereus* var. toyoi, recentemente, foi proposta como uma nova espécie do grupo *B. cereus* com o nome de *B. toyonensis* (Jiménez et al., 2013). *Bacillus toyonensis* não é patogênico, não produz enterotoxinas (Abdulmawjood et al., 2019). Esporos viáveis deste microrganismo são utilizados na preparação do TOYOCERIN® (Rubinum S.A., Barcelona, Espanha) uma apresentação comercial usada como probiótico na alimentação de suínos, bovinos, aves e coelhos em vários países. Na Europa o uso deste probiótico foi autorizado pela primeira vez no ano de 1994, se tornando o primeiro microrganismo utilizado como suplemento na alimentação animal na União Europeia (Williams et al., 2009).

Os efeitos moduladores dos probióticos na resposta imune a vacinas foram estudados através de experimentos com animais suplementados com probióticos e vacinados com antígenos bacterianos e virais. Camundongos foram suplementados com o probiótico *B. toyonensis* e vacinados com uma bacterina de *Escherichia coli* e com uma vacina replicante contra parvovírus canino. Animais que receberam o probiótico apresentaram níveis de anticorpos antígeno específicos superiores quando comparados aos que não receberam a suplementação. Os camundongos vacinados com a bacterina apresentaram aumento nos níveis de IgG cerca de 1.4 vezes maiores, enquanto, os camundongos vacinados com o antígeno viral apresentaram níveis de IgG cerca de 9 vezes maiores contra o parvovírus canino quando comparados aos não suplementados (Coppola et al., 2005).

O probiótico *B. toyonensis* promove efeitos benéficos na saúde de leitões estimulando a imunidade, aumentando a produção intestinal de IgA (Scharek, Guth.

J., et al., 2007). Foi observado uma maior população de linfócitos T e a diminuição da ocorrência de diarreias causadas por *E. coli* patogênica (Scharek, Altherr, et al., 2007). Leitões suplementados com *B. toyonensis* e vacinados com dois antígenos inativados (Influenza e *M. hyopneumoniae*) apresentaram aumento de populações de PBMCs, linfócitos T auxiliares e linfócitos T citotóxicos e níveis de anticorpos superiores contra os dois antígenos (Schierack et al., 2007). Em fêmeas suínas a suplementação com este probiótico reverteu parcialmente à modulação do sistema imune durante a gestação (Schierack et al., 2009).

Camundongos vacinados com uma vacina experimental inativada contra BoHV-5 e suplementados com *B. toyonensis*, apresentaram níveis de IgG anti-BoHV-5 aumentados e níveis de transcrição de RNA mensageiro (RNAm) de IFN-γ, IL-12 e IL-10 superiores quando comparados a controles sem a suplementação probiótica. Após a suspensão da suplementação com probiótico, animais que receberam uma dose extra da vacina apresentaram níveis de IgG anti-BoHV-5 cerca de 1.3 vezes maiores que os não suplementados. A suplementação com este probiótico pode ser um método potencial para aumentar a eficácia de vacinas através da modulação da resposta imune (Roos et al., 2012).

O efeito imunomodulador de *B. toyonensis* também foi observado em ovinos que receberam uma vacina inativada contra BoHV-5 e contra uma bacterina de *E. coli*, a suplementação com o probiótico incrementou a resposta imune humoral aos dois antígenos vacinais nos animais suplementados, comparados aos controles (Roos et al., 2010). Ovinos suplementados com *B. toyonensis* apresentaram títulos de anticorpos neutralizantes contra BoHV-5 cerca de 4 vezes maiores que o grupo controle, e maiores níveis de transcrição de RNAm de IL-4, IL-10, IL-17 e IFN-γ (Roos et al., 2018).

2.1.3 *Saccharomyces boulardii*

Saccharomyces boulardii é uma levedura não patogênica, que possui propriedades probióticas, amplamente utilizada na medicina humana e veterinária no controle e prevenção de distúrbios gastrointestinais (Kalesidis & Pothoulakis, 2012; McFarland & Bernasconi, 1993). A levedura *S. boulardii* foi isolada em 1920 por Henri Boulard, um cientista francês, que observou que certas pessoas na Indochina (atual Vietnam) não foram afetadas por um surto de cólera, pois estavam bebendo um tipo

específico de chá feito a partir do extrato de lichia e mangostão (McFarland, 2010), então, ele isolou uma cepa de *Saccharomyces* das cascas dessas frutas, sendo denominada mais tarde como *Saccharomyces boulardii*. Atualmente, a cepa está disponível em todo o mundo como probiótico em (Kelesidis & Pothoulakis, 2012; Mcfarland & Bernasconi, 1993) várias apresentações comerciais desde 1962 (Sen & Mansell, 2020). Estudos experimentais em humanos e animais mostraram que *S. boulardii* exerce efeito protetor na mucosa intestinal (Hudson et al., 2016) e tem sido usada no tratamento de doenças inflamatórias intestinais como a doença de Crohn (Sivananthan & Petercen, 2018), de diarreias associadas com uso de antibióticos (Doron et al., 2008).

Czerucka & Rampal (2019) revisaram os mecanismos de ação de *S. boulardii* e relataram que existem dois modos de ação em potencial desta levedura no hospedeiro, uma ação contra microrganismos patogênicos (adesão de bactérias e seus componentes), na qual eliminam ou possuem um efeito nos seus fatores de virulência: toxinas, lipopolissacarídeos; e uma ação direta na mucosa intestinal com efeitos tróficos, efeitos na reconstituição epitelial, efeitos anti-secretores, anti-inflamatórios, imunomoduladores. Recentemente ensaios *in vitro* e *in vivo* mostraram a ação direta de *S. boulardii* em células do sistema imune inato, como fagócitos mononucleares (Ibáñez et al., 2019). O efeito de *S. boulardii* na resposta imune do hospedeiro foi primeiramente detectado através do aumento de IgA secretada (sIgA) no fluido intestinal em ratos (Buts et al., 1990). Em camundongos *S. boulardii* estimula a produção de sIgA e a atividade do sistema mononuclear fagocítico de camundongos. A suplementação *S. boulardii* estimulou níveis superiores de IgA anti-toxina A de *Clostridium difficile* intestinal em camundongos suplementados comparado aos controles (Qamar et al., 2001).

*Saccharomyces boular*dii apresenta efeito protetor sendo capaz de diminuir a intensidade de infecções intestinais causadas por bactérias patogenicas (Martins et al., 2010). O efeito protetor de *S. boulardii* a infecção por *Salmonella enterica* serovar Typhimurium (ST) foi demonstrado sendo que suplementação com *S. boulardii* previne a translocação da *Salmonella* pelo fígado e baço (Martins et al., 2013). Pontier-Bres et al. (2014) mostraram em modelo murino, que a adesão de *S. boulardii* a *Salmonella* modifica a migração da *Salmonella* diminuir sua translocação e desencadeando sua a eliminação via fecal.

O efeito terapêutico *S. boulardii* foi demonstrado no tratamento da giardíase em modelo animal. O probiótico diminuiu em cerca de 70% a carga parasitaria, também sendo observado o aumento das vilosidades e criptas intestinais, de muco e número de células caliciformes dos grupos tratados em comparação aos grupos não tratados. Além disso, os animais suplementados com *S. boulardii* também exibiram um aumento significativo na contagem de linfócitos intraepiteliais (Ribeiro et al., 2018).

O probiótico *S. boulardii* apresentou efeitos positivos em camundongos infectados experimentalmente com *Toxocara canis. Saccharomyces boulardii* reduziu a intensidade da infecção em camundongos com toxacaríase (Avila et al., 2012). Em estudo complementar Avila et al. (2013) demonstrou que o efeito protetor não é pela ação direta do microrganismo sobre as larvas do parasito, mas sim pela modulação na expressão das citocinas IL-12 e IFN-γ. O efeito protetor de *S. boulardii* observado sugere que ocorre a estimulação de células que atuam na imunidade inata modulando a expressão principalmente de IL-12 (de Avila et al., 2016).

A modulação da resposta imune a vacinas mediada pela suplementação com *S. boulardii* foi demonstrado em modelo murino e ovino. Camundongos vacinados com uma bacterina de *E. coli* e suplementados com *S. boulardii* apresentaram níveis de anticorpos cerca de 1.8 vezes maiores que os camundongos não suplementados (Coppola et al., 2005). *Saccharomyces boulardii* demonstrou possuir efeito imunomodulador nas vacinas de DNA que codificam os fragmentos de proteínas de *Leptospira* LigAni e LigBrep, ao estimular a resposta humoral (IgG anti-LigAni e -LigBrep (Silveira et al., 2017). Camundongos suplementados com a levedura *S. boulardii* e vacinados com uma vacina inativada contra BoHV-5 apresentaram uma produção de anticorpos IgG , IgG1 e IgG2 anti-BoHV-5 superiores e com desvio para a polarização de uma resposta Th1, além do aumento dos níveis de transcrição de mRNA para as citocinas IFN-γ, IL-12, IL-17 e IL-10 comparada aos animais não suplementados Roos et al. (2018).

Em ovinos vacinados com uma bacterina de *E. coli* e com uma vacina experimental inativada contra o BoHV-5 e suplementados com *S. boulardii*, o probiótico incrementou a resposta imune humoral dos animais que apresentaram altos níveis de anticorpos, anti-BoHV-5 e anti-*E. coli*, quando comparados ao não suplementados (Roos et al., 2010). Recentemente, demonstraram que ovinos suplementados com *S. boulardii* e vacinados com uma vacina inativada contra o BoHV-5 apresentaram níveis superiores de anticorpos neutralizantes contra o vírus,

além de maiores níveis de transcrição para as citocinas IL-4, IL-10, IL-17 e IFN-γ que os controles.

2.1.4 Esporos de *Bacillus* sp. como adjuvantes de vacinas

Os adjuvantes são definidos como compostos que estimulam o sistema imune, aumentam, modulam e prolongam a imunogenicidade dos antígenos co-administrados (Krieg, 2007; Reed et al., 2009). Os adjuvantes representam um componente importante na formulação de vacinas convencionais e recombinantes, pois aumentam a imunogenicidade dos antígenos, dessa forma, a escolha de um determinado adjuvante é essencial para aumentar a eficiência de uma vacina e gerar proteção contra o antígeno vacinal (Lambrecht et al., 2009).

Os adjuvantes são classificados principalmente de acordo com o modo pelo qual interagem com o sistema imunológico. Podem atuar como sistemas de entrega de antígeno, agregando e controlando a liberação do antígeno de forma lenta para serem apresentados pelas APCs, como DCs e macrófagos, estão inclusos nesse grupo os sais minerais, emulsões e lipossomas (Schwendener et al., 2010). Os imunopotenciadores atuam ativando e potencializando a resposta imune inata através dos PRRs, que são reconhecidos principalmente pelos receptores tipo TLR presentes em macrófagos, monócitos, neutrófilos e DCs que tem papel fundamental no reconhecimento de moléculas microbianas. Fazem parte desse grupo as citocinas, saponinas, sais de alumínio e agonistas de receptores TLRs (Apostólico et al., 2016; Iwasaki & Medzhitov, 2004).

Esporos de bactérias do gênero *Bacillus* apresentam potencial como adjuvante de vacinas de mucosas e parenterais. Barnes et al. (2007) demonstraram que esporos *B. subtilis* podem ser usados como adjuvantes de vacinas de mucosa e também administrados pela via parenteral sem a necessidade de conjugação do esporo com o antígeno. Camundongos vacinados com fragmento C da toxina tetânica associados a esporos de *B. subtilis* apresentaram níveis de IgG superiores, além de induzir níveis de IgG1 e IgG2a superiores aos do grupo controle, sugerindo o desenvolvimento de uma resposta balanceada Th1/Th2. Além desta observação, quando a vacina foi administrada pela via intranasal induziu a produção de níveis superiores de IgA anti-fragmento C da toxina tetânica nas mucosas trato respiratório e vaginal

De Souza et al. (2014) demonstraram que a co-administração de esporos de *B. subtilis* com a proteína recombinante p24 gag de HIV aumentou a imunogenicidade do antígeno, promovendo aumento de anticorpos após a administração em camundongos pela via subcutânea. Em um estudo *in vitro* DCs murinas foram estimulados com esporos de *B. subtilis* e promoveram a ativação destas células e aumento de expressão de MHCII, CD40 e da secreção de citocinas pró-inflamatórias como IL-1β, IL-12 e TNF-α. Linhagens de camundongos *knockout* MyD88 e TLR2 quando vacinados com esporos de *B. subtilis* associados com a proteína recombinante p24 gag de HIV apresentaram diminuição significativa nos níveis de anticorpos específicos contra o antígeno, sugerindo que o efeito adjuvante dos esporos é mediado pela via de sinalização que utiliza a molécula adaptadora MyD88, e possivelmente pelo reconhecimento do peptidoglicano via TLR2 pelas APCs, indicando um papel direto da imunidade inata nas propriedades imunomoduladoras mediada pelos dos esporos (De Souza et al., 2014).

Esporos de *B. subtilis* foram utilizados também como adjuvante de vacina de DNA. Camundongos imunizados com uma vacina de DNA que codifica a proteína E7 do papilomavírus humano tipo 16 (HPV-16), quando associada com esporos estimularam maior ativação de linfócitos T CD8+ antígenos específicos *in vivo*. Os camundongos imunizados com a vacina de DNA associada com esporos apresentaram um aumento da proteção às células tumorais previamente implantadas, capazes de expressar oncoproteínas do HPV-16. Estes resultados demonstram pela primeira vez que os esporos de *B. subtilis* também podem conferir efeitos adjuvantes às vacinas de DNA (Aps et al., 2015).

Esporos também podem ser utilizados como sistema de apresentação de antígenos heterólogos para desenvolvimento de vacinas de mucosa. Os antígenos podem ser expressos na superfície do esporo através da fusão de genes da proteína de interesse com genes que codificam para as proteínas presentes na capa do esporo, como CotB, CotC ou CotG, que são sintetizadas durante o processo de esporulação e ficam ancoradas na parte mais externa da capa do esporo, permitindo a apresentação do antígeno as APCs (Tavassoli et al., 2013). Essa técnica foi inicialmente desenvolvida por Isticato et al. (2001) que fusionou a proteína CotB de *B. subtilis* PY79 com o fragmento C da toxina tetânica (TTFC) de *Clostridium tetani*. Camundongos que receberam a quimera CotB-TTFC por via oral ou intranasal apresentaram proteção contra o desafio com a uma dose letal de toxina do tetânica.

Além disso, foi possível verificar que a TTFC expressa na superfície do esporo resiste ao trânsito pelo trato digestivo (Duc et al., 2003). Em outro estudo, a proteína A de *B. anthracis* expressa e apresentada na superfície de esporos induziu imunidade protetora em camundongos (Duc et al., 2007).

Outra abordagem promissora para o uso do sistema de apresentação de antígenos heterólogos na superfície de esporos não recombinantes, que é baseada na adsorção de moléculas heterólogas na superfície do esporo sem a necessidade de manipulações genéticas, evitando assim todas as preocupações sobre o uso e liberação de microrganismos geneticamente modificados (Ricca et al., 2014). A capa proteica da camada superficial dos esporos é carregada negativamente e hidrofóbica, fornecendo uma plataforma adequada para adsorção de antígenos proteicos. A ligação pode ser promovida em condições de baixo pH e requer uma combinação de interações eletrostáticas e hidrofóbicas entre esporo e antígeno (Isticato et al., 2019).

A adsorção de antígenos nos esporos pode ser apropriada para melhorar a imunogenicidade de algumas vacinas principalmente daquelas administradas pela via mucosa (Huang et al., 2010; Ricca et al., 2014). Camundongos imunizados pela via oral e nasal com os antígenos recombinantes TTFC e a com toxina alfa de *C. perfringens* adsorvidos aos esporos de *B. subtilis* apresentaram IgG sérica e sIgA fecal contra os antígenos e tornaram-se protegidos contra o desafio com a toxina do tétano e com a toxina alfa de *C. perfringens* (Huang et al., 2010). Isticato et al. (2013) demonstraram que a subunidade B da enterotoxina termolábil (LTB) de *E. coli* foi adsorvida eficientemente na superfície dos esporos de *B. subtilis*. Quando administrada pela via nasal em camundongos, a LTB adsorvida nos esporos foi capaz de induzir resposta imune humoral e celular com a produção de IgG sérica, sIgA fecal e das citocinas IFN-γ no baço e linfonodos mesentéricos dos animais vacinados.

Esporos de *B. subtilis* inativados pelo calor também tem sido utilizados como sistema de apresentação de antígenos em vacinas de mucosas e também associados aos antígenos como adjuvante vacinas parenterais, essa abordagem também tem se mostrado efetiva na indução de resposta imune humoral e celular (De Souza et al., 2014; Huang et al., 2010; Oliveira-Nascimento et al., 2012; Song et al., 2012). Dessa forma, esporos inativados pelo calor tem se mostrado igualmente eficazes como os esporos vivos na indução de um aumento na resposta imune humoral e celular em camundongos. Além do fato de que os esporos inativados pelo calor são tão eficientes quanto os esporos vivos, é um recurso que facilitaria ainda mais o manuseio e o

armazenamento dessa molécula como adjuvante (De Souza et al., 2014; Song et al., 2012).

2.2 Glicoproteína D de herpesvírus bovino tipo 5

A glicoproteína D (gD) está presente no envelope viral do herpesvírus bovino tipo 1 (BoHV-1) e herpesvírus bovino tipo 5 (BoHV-5), que são importantes patógenos de bovinos (Engels & Ackermann, 1996; Vogel et al., 2003). A gD de BoHV-5 é essencial para a ligação e penetração do vírus nas células permissivas, sendo um dos principais alvos da resposta imune do hospedeiro induzindo uma forte resposta imune humoral e celular durante a infecção (Alves Dummer et al., 2014). Nas células do hospedeiro a gD se liga nos receptores das famílias do fator de necrose tumoral, como o nectin 1 e nectin 2 (Geraghty et al., 2000; Montgomery et al., 1996).

A gD é uma das primeiras proteínas a serem expostas na membrana plasmática da célula infectada, sendo importante na geração da resposta imune pela indução de anticorpos neutralizantes, e, é alvo das células NK (Babiuk et al., 1996). Anticorpos anti-gD podem estimular a citotoxicidade celular mediada por anticorpo (ADCC). Dummer et al. (2009) relataram a expressão da gD recombinante (rgD) de BoHV-5 na levedura *Pichia pastoris* e demonstraram a sua antigenicidade e imunogenicidade. A glicoproteína D recombinante de BoHV-5 quando associada com adjuvantes induziu anticorpos neutralizantes contra o vírus em camundongos (Dummer et al., 2014) e em bovinos (Araujo et al., 2018).

2.3 Clostridioses

As clostridioses são toxinfecções causadas por bactérias do gênero *Clostridium*, bastonetes Gram-positivos, anaeróbios, formadores de esporos. A maioria das bactérias deste gênero são constituintes da microbiota intestinal de animais e seres humanos, no entanto somente algumas espécies são capazes de causar enfermidades dos animais (Lobato et al., 2013). Os clostrídeos que causam doenças atuam basicamente através de dois mecanismos: produção de toxinas e invasão dos tecidos (Aronoff, 2013; Popoff & Bouvet, 2009). Entre os principais microrganismos deste gênero que causam doenças em animais se destacam *Clostridium perfringens*, o qual suas toxinas causam a enteroxemia e gangrena

gasosa, *Clostridium chauvoei*, causador do carbúnculo sintomático em ruminantes e o *Clostridium tetani* caudador do tétano (Bokori-Brown et al., 2011; Collier et al., 1990).

Devido à alta taxa de mortalidade e sua ampla distribuição geográfica as clostridioses representam uma das principais classes de doenças que prejudicam o setor pecuário (Shrestha et al., 2018; Songer, 1996). Somente no Brasil, as clostridioses são a causa de morte de aproximadamente 500.000 bovinos por ano, resultando em uma perda de cerca de US $ 350 milhões (Ferreira et al., 2016). A profilaxia e o controle das clostridioses são realizados mundialmente pela vacinação com preparações contendo bactérias inteiras inativadas e toxóides (Uzal, 2012). A resposta mediada pelos anticorpos induzidos pela vacinação contra as clostridioses pode ser aceita como um parâmetro imunológico de proteção (Cerviño et al., 2011).

2.3.1 Toxina Épsilon de *Clostridium perfringens*

Clostridium perfringens pode ser encontrado no solo e na água, bem como na microbiota de muitos animais. Essa bactéria produz 16 toxinas diferentes, no entato, somente quatro delas: alfa, beta, epsilon e iota são consideradas as mais importantes, pois estão relacionadas à patogênese da maioria das doenças causadas pelo *C. perfringens* (Uzal et al., 2010). Os tipos mais importantes de *C. perfringens* na medicina veterinária agrícola são C e D, pois afetam a maioria dos animais de criação (Uzal et al., 2008). A toxina épsilon (ETX) produzida pelo *C. perfringens* toxinotipos B e D é uma das mais potentes toxinas microbianas, responsável por causar quadros de enterotoxemia em ruminantes domésticos, particularmente em ovinos (Alves et al., 2014; Uzal & Songer, 2008). .

A alternativa mais viável no controle destas enfermidades é a vacinação, realizada com formulações contendo toxinas de *C. perfringens* inativadas com formaldeído (toxóides). As vacinas recombinantes têm surgido como alternativa aos toxóides convencionais, pois apresentam vantagens relacionadas principalmente ao processo de produção (Ferreira et al., 2018; Milach et al., 2012; Moreira et al., 2016; Salvarani et al., 2013). Após a vacinação contra uma toxina bacteriana, a resposta imune desejada deve ser mediada por anticorpos capazes de neutralizar as toxinas impedindo-as de se ligar na célula alvo do hospedeiro (Rees & Steiner, 2018). A capacidade dos anticorpos IgG se difundem facilmente pelos líquidos extracelulares e

sua alta afinidade pelo antígeno torna-os os principais anticorpos que neutralizam as toxinas nos tecidos (Brandtzaeg, 2003).

2.3.2 *Clostridium chauvoei*

Clostridium chauvoei é uma bactéria histolítica, altamente patogênica, que causa o carbúnculo sintomático. O carbúnculo sintomático é uma doença grave de bovinos, ovinos e outros ruminantes domésticos que gera importantes perdas econômicas na criação destes animais (Groseth et al., 2011; Hatheway, 1990; Nagano et al., 2008). A patogênese do carbúnculo sintomático não é totalmente conhecida, mas acredita-se que esporos de *C. chauvoei* do ambiente que são ingeridos, no intestino são fagocitados e então transportados pelos macrófagos para os músculos, onde permanecem em estado latente (Jubb et al., 1991). O acontecimento de trauma ou qualquer outra condição que reduza o oxigênio nesses tecidos pode levar à multiplicação de *C. chauvoei* e consequentemente ao desenvolvimento de doença muscular necrosante e a morte (Uzal et al., 2016).

A profilaxia e o controle do carbúnculo sintomático é realizado pela vacinação (Uzal, 2012). Os antígenos somáticos (parede celular) e principalmente os flagelos de *C. chauvoei* foram os primeiros antígenos estudados para os quais há evidência de envolvimento na indução da imunidade protetora contra o carbúnculo sintomático, além de desempenharem um papel na virulência (Chandler & Gulasekharam, 1974; Stevenson & Stonger, 1980; Tamura et al., 1984; Tamura et al., 1995) . Os anticorpos produzidos na resposta após a vacinação seriam importantes na opsonização do patógeno, protegendo os animais da infecção pelo *C. chauvoei* (Tamura & Tanaka, 1987; Tanaka et al., 1987).

2.3.3 Fragmento C da toxina tetânica

O tétano é uma doença altamente fatal causada pelas toxinas produzidas pelo *C. tetani*, os quais provocam alterações funcionais no sistema nervoso central acarretando rigidez muscular e morte causada por parada respiratória ou convulsões (Linnenbrink & Macmichael, 2006). O homem e todas as espécies animais domésticos são susceptíveis a infecção pelo *C. tetani* (Mallick & Winsle, 2004; Raposo, 2007). A infecção ocorre geralmente quando a bactéria ou os esporos são introduzidos nos tecidos através de ferimentos ou pelas superfícies mucosas, e quando sob

determinadas condições, principalmente a redução de oxigênio local, as bactérias começam a proliferar e a produzir as neurotoxinas (Mallick & Winsle, 2004).

A profilaxia e o controle do tétano são realizados através da vacinação e da administração do soro antitetânico que realiza neutralização da toxina tetânica circulate em casos de infecção (Edlich et al., 2003; Raposo, 2007). A vacina disponível é um toxóide disponível comercialmente é eficaz em estimular repostas mediadas por IgG (Vollman et al., 2014). O toxóide contra o tétano, embora estimule a produção de níveis significativos de proteção, apresenta várias desvantagens no processo de produção. A preparação do toxóide envolve o manuseio de grandes quantidades de toxina tetânica, que é um procedimento perigoso, além disso, há necessidade de repetidas vacinações de reforço. Então, a necessidade de uma nova vacina contra o tétano impulsionou o desenvolvimento de uma série de vacinas alternativas, incluindo derivados da toxina tetânica (Figueiredo et al., 1995; Yu et al., 2016). O fragmento C da toxina tetânica (TTFC) apresentou características bioquímicas semelhantes a toxina nativa de *C. tetani* e tem potencial para ser usado como antígeno de novas vacinas contra o tétano (Calvo et al., 2012; Li et al., 1994). A TTFC, em sua forma recombinante de massa molecular entre 47 e 52 kDa, foi expressa em *E. coli* (Duc et al., 2003; Yu et al., 2011), *Pichia pastoris* (Clare et al., 1991), Salmonella (Roberts et al., 1999), *Lactococcus lactis* (Robinson et al., 1997; Wells et al., 1993) esporos de *B. subtilis* (Isticato et al., 2001) e a imunização com TTFC em modelo murino estimulou altos níveis de IgG, IgA e protegeu significativamente os camundongos ao desafio com a toxina tetânica.

3 HIPÓTESE E OBJETIVOS

3.1 Hipótese

A suplementação com *Bacillus toyonensis* e *Saccharomyces boulardii* estimula a imunidade e melhora a resposta às vacinas contra o herpesvírus bovino tipo 5 e clostridioses em murinos e ovinos.

3.2 Objetivo Geral

Estudar o efeito de *Bacillus toyonensis* e *Saccharomyces boulardii* na modulação da resposta imune em murinos e ovinos inoculados com vacinas recombinantes e convencionais.

3.3 Objetivos Específicos

Avaliar a resposta imune nos camundongos vacinados contra o BoHV-5 e suplementados com *Bacillus toyonensis* em dois períodos diferentes;

Avaliar o efeito imunológico dos esporos de *Bacillus toyonensis* pela via subcutânea, como componente da vacina recombinante contra BoHV-5 em camundongos;

Avaliar a resposta imune de camundongos suplementados com *Bacillus toyonensis* e vacinados pela via mucosa com o fragmento C da toxina tetânica adsorvida em esporos de *Bacillus subtilis*;

Avaliar a resposta imune de ovinos suplementados com *Bacillus toyonensis* durante 5 dias antes da vacinação contra a toxina epsilon de *Clostridium perfringens*;

Avaliar a resposta imune de ovinos suplementados com *Bacillus toyonensis* ou *Saccharomyces boulardii* durante 5 dias antes da vacinação contra *Clostridium chauvoei*.

4 CAPÍTULOS

4.1 Artigo 1 – *Bacillus toyonensis* improves immune response in the mice vaccinated with recombinant antigen of Bovine herpesvirus type 5

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Bacillus toyonensis improves immune response in the mice vaccinated with recombinant antigen of Bovine herpesvirus type 5

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Abstract

Probiotics modulate the immune response and can increase the effectiveness of vaccines. *Bacillus toyonensis* is widely used as a probiotic in animal feed. The aim of this study was to assess the effects of *B. toyonensis* administration on the immune response to an experimental recombinant vaccine against bovine herpesvirus type 5 (BoHV–5) in mice. Mice were vaccinated with BoHV–5 recombinant glycoprotein D and supplemented with the probiotic *B. toyonensis* in two regimes: one group received the probiotic only during seven days prior to the initial vaccination while the second group was given the probiotic throughout the experimental period of seven weeks. Animals supplemented with probiotic *B. toyonensis* in two regimes showed an increase in total IgG, IgG1 and IgG2a levels in serum, in addition to higher titres of antibodies capable of neutralising the BoHV–5 virus than non-supplemented animals ($p<0.05$). Splenocytes from the supplemented mice had higher mRNA transcription levels of cytokines IL–4 and IL–12. These results show that the use of this probiotic may significantly contribute to the response elicited by recombinant vaccines, especially those that rely on increasing antibody and cell-mediated immune responses for efficacy. Further, the data support an immunomodulatory effect for probiotic *B. toyonensis* and imply that enhance effect on the immune response against a BoHV–5 recombinant vaccine in mice.

Keywords: probiotic, immunomodulation, recombinant vaccine

Introduction

Probiotics are live microorganisms, which upon ingesting in adequate amounts, confer beneficial effects to the health of the host (FAO/WHO, 2002). Probiotics produce nutrients and growth factors that modulate the balance and activities of the intestinal microbiota, providing benefits to animal health and nutrition (Chaucheryras-Durand and Durand, 2010). Further, probiotic microorganisms can stimulate both innate and adaptive immune response. Probiotics bind to the receptors expressed on the cell surface of the intestinal lumen and/or to immune cells that activate defence mechanisms, provide protection against pathogens in the intestinal mucosa of the host, and influence immune response through local and systemic immunomodulation (Britti *et al.,* 2006; Forsythe and Bienenstock, 2010).

Microorganisms, including probiotics, present in the intestinal mucosa interact with the cells of the immune system through M cells that capture and transport the microorganism to Peyer's patches (Iwasaki, 2007). The dendritic cells (DCs) resident in the submucosa then capture the antigens of the intestinal lumen and amplify the cytoplasmic processes in epithelial junctions without damaging epithelial integrity (Niess *et al.,* 2005a). Thus, microorganisms present in the intestinal mucosa, even probiotics, are processed by the DCs that subsequently activate naïve T lymphocytes. The DCs and T lymphocytes migrate to the mesenteric lymph

nodes and enter systemic circulation where they stimulate an immune response that is far from the location of their original activation (Chieppa *et al.,* 2006; Leeber *et al.,* 2010).

Bacillus toyonensis is a non-pathogenic Gram-positive bacterium that has been used for the last several decades as a probiotic in animal feed (Gil-Turnes and Conceição, 2007; Williams *et al.,* 2009). The organism was first identified as *B. cereus* var. toyoi (Kozasa *et al.,* 1977), but has recently been proposed as a new species of the group *B. cereus* under the name *B. toyonensis* (Jiménez *et al.,* 2013). Supplementation with the probiotic *B. toyonensis* exerts immunomodulatory effects and is capable of enhancing the effectiveness of conventional vaccines in sheep, pigs and mice (Coppola *et al.,* 2005; Schierack *et al.,* 2007; Roos *et al.,* 2010; 2012).

The use of vaccines is one of the most efficient alternatives for the prophylaxis and the control of infectious diseases (Kaufamman, 2007). The vaccines based on recombinant antigens are considered relatively safer because they result in low adverse reactions such as local irritations and the risk of reversion to virulence, among others (Reed *et al.,* 2009). However, they often show only weak immunogenicity and, therefore, require administration of adjuvants to increase their immunogenicity (Mbow *et al.,* 2010).

Bovine herpesvirus 5 (BoHV-5) is the responsible for outbreaks of meningoencephalitis that can reach a mortality rate of 70–100% (Vogel *et al.,* 2003). The disease affects young cattle and is responsible for economic losses in South America (Campos *et al.,* 2009). Recombinant vaccines against bovine herpesvirus use principal glycoproteins of the viral envelope as antigens. The glycoprotein D of BoHV–5 is essential for the binding and penetration of the virus into the host cells and is also one of the main targets of the host immune response that leads to a strong humoral and cellular immune response during infections (Dummer *et al*., 2014a). One method of assessing vaccine efficacy is the measurement of antigen-specific antibody levels in serum after vaccination as antibody levels correlate directly with protection and are, therefore, used as a reference for determining the influence of probiotics on immune response (MacDonald and Bell, 2010).

Only a few studies have tested the potential of *B. toyonensis* to increase recombinant vaccine efficacy and such previous studies have not identified the immune factor(s) responsible for the *B. toyonensis*-mediated increase in vaccine efficacy (Coppola *et al.,* 2005; Shierack, *et al.,* 2007; Roos *et al.,* 2010; 2012). Thus, the aim of this study was to assess the effect of *B. toyonensis* administration on the immune response of an experimental recombinant vaccine against the BoHV–5 virus in mice. Our results suggest that *B. toyonensis* supplementation could be used to modulate and improve the host response to a recombinant vaccine as it increases both antibody and cell-mediated immune response.

Material and methods

Probiotic

The probiotic *B. toyonensis* used in this study was obtained from the collection of microorganisms of the Microbiology Laboratory, Biotechnology Center, Federal University of Pelotas (UFPel). The bacteria were seeded in Brain Heart Infusion agar (BHI; Neogen, Lansing, MI, USA) and incubated at 37 °C for 24 h. After sufficient colony growth, the individual colonies were inoculated (3–5 colonies) in 500 ml flasks containing 100 ml of Brain Heart Infusion broth BHI (Neogen, Lansing, MI, USA) and incubated overnight (16 to 18 h) in an orbital shaker at 200 rpm. The bacteria in these flasks served as inoculum for the propagation

in a bioreactor (Braun Biotech International STATPLUS ® B; Melsungen, Germany) containing 3.5 L of NYSM medium (Yousten, 1984). Fermentation conditions were controlled and the air supply was maintained between 0.5 and 1.5 (v/v) so that approximately 80% of the dissolved oxygen in the medium was obtained during fermentation. Agitation was maintained at 300 rpm and temperature at 37 °C for 96 h and without pH corrections during the process.

When 90% of the bacteria had sporulated, the culture was centrifuged in a Sorvall centrifuge ® RC–6 plus (Langenselbold, Germany) at 5,000 ×g for 20 min at 4 °C and the sediment suspended in 500 ml of phosphate buffered saline such that the concentration of *B. toyonensis* obtained was approximately 2×10^7 CFU/ml. The final suspension was heated at 80 °C for 15 min in a water bath to eliminate vegetative forms of the bacteria. Purity control was performed at all stages using Gram staining and by inoculation on Brain Heart Infusion agar (Neogen, Lansing, MI, USA).

Supplementation of animals

We used 30 seven-week old female *Swiss* mice and, divided into three groups of 10 animals each and named B.t continuous, B.t 7 days (B.t = *B. toyonensis*) and control. The control group received the commercial ration, free of chemotherapeutic agents (Nuvilab ® CR–1, Nuvital Nutrients S/A, Colombo, Brazil). The groups B.t continuous and B.t 7 days received the same ration but supplemented with 1×10^6 viable spores of *B. toyonensis* per gram of food. All mice began receiving the rations seven days prior to the vaccination for diet adaptation. The group B.t 7 days received supplementation with a probiotic only for seven days prior to the first vaccination, group B.t continuous received supplementation with probiotics throughout the experimental period.

All protocols were reviewed and approved by the Ethics Committee on Animal Experimentation (CEEA No. 1981) of the Federal University of Pelotas (UFPel). The UFPel-CEEA agreement is approved by the Brazilian National Council for Animal Experimentation Control (CONCEA). The mice used in the study were provided by the animal unit at UFPel.

Vaccination

The mice were vaccinated by subcutaneous injection of 0.2 ml recombinant vaccine formulated with recombinant glycoprotein D (rgD) of BoHV–5 and expressed in *Pichia pastoris* (Dummer *et al.,* 2009). In order to formulate the vaccine, we used 40 µg of rgD per dose in phosphate buffered saline (PBS) adsorbed in 10% aluminium hydroxide as an adjuvant. The animals were vaccinated on day 0 and received a booster on day 21 of the experiment. Blood samples were collected by the submandibular puncture on days 0, 14, 28 and 42. After collection, serum was separated, labelled and stored at –20 °C until analysis.

Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA was performed according to Dummer *et al.* (2014b), but with modifications. Microtitre plates (96 well, Corning, Lowell, MA, USA) were coated overnight at 4 °C with 25 ng of rgD per well and subsequently washed thrice with 200 µl/well of phosphate-buffered saline containing 0.05% Tween 20 (PBS-T). Individual serum samples were diluted 1:400 and 100 µl and each was added in triplicate. The plates were incubated at 37 °C for 60 min, washed thrice with PBS-T, 100 µL of HRP-conjugated goat anti-mouse IgG antibody (1:4,000 dilution; Sigma-Aldrich, St. Louis, MO, USA) added and incubated at 37 °C for 90 min. After incubation, the plates were washed five times with PBS-T and 100 µl of

substrate solution was added. Each millilitre of the substrate buffer contained 0.4 mg of ortho-phenylenediamine (Sigma-Aldrich, St. Louis, MO, USA) and 15 µl of H_2O_2. The substrate reaction was allowed to proceed for 15 min in the dark at ambient temperature and then stopped by adding 50 µl of 2 N sulphuric acid per well. Absorbance was measured in a microplate reader (ThermoPlate, Rio de Janeiro, Brazil) with a 492-nm filter and using calibration curve. For IgG isotype analysis, pooled serum samples from each experimental group were diluted 1:2,000 and analysed according to the instruction manual of the Mouse Monoclonal Antibody Isotyping Reagents kit (Sigma-Aldrich, St. Louis, MO, USA), following the same protocol above describe. The absorbance value of each serum sample was divided by the absorbance value from the same animal at day 14 and the results were expressed as fold increase in IgG.

Virus Neutralisation Test

Serum samples collected on day 42 of the experiment were tested for the presence of antibodies against BoHV–5 using the virus neutralisation assay, according to Fischer *et al.* (2007). The serum samples were diluted in minimum essential medium (Gibco, Grand Island, NY, USA) as a 2-fold dilution series from 1:2 until 1:256. Next, 25 µL of each dilution was added into 96 well polystyrene microplates (Corning, Lowell, MA, USA), followed by 25 µl of a BoHV–5 virus suspension containing 100 TCID [50] and incubated for 1 h at 37 °C in an incubator with 5% CO_2. Then 3×10^4 Madin Darby bovine kidney cells (MDBK, originally ATCC CCL22) cells per well were added and the plates incubated for a further 72 h. The presence of the neutralising antibodies was inferred from the absence of a cytopathic effect (CPE). The antibody titres were calculated using the Behrens and Kärben statistical method and expressed as the reciprocal of the highest dilution capable of neutralising 100 TCID [50] of the virus.

Spleen cells cultures and RNA extraction

The mice in the experimental groups were euthanized on day 42 of the experiment, their spleen collected and macerated. The spleen cells (2×10^6) were cultured in 1 mL RPMI 1640 (Gibco, Grand Island, NY, USA) containing 10% foetal bovine serum (Gibco, Grand Island, NY, USA) and antibiotic and antifungal agents (penicillin 10,000 U/ml, streptomycin 10 mg/ml and amphotericin B 25 mg/mL) (Gibco, Grand Island, NY, USA) in 24-well plates (Kasvi, Taiwan, China) and incubated for 24 h at 37 °C in 5% CO_2 atmosphere. Culture medium was replaced after 24 h and the cells were stimulated with 10 µg of rgD, $1 \times 10^{5.3}$ of whole live BoHV–5, 2.5 µg or concanavalin A (ConA; Sigma-Aldrich, St. Louis, MO, USA), and with RPMI 1640, and incubated for approximately 18 h under same conditions. ConA and RPMI were used as a controls, positive and negative respectively, for cell stimuli. After incubation, the supernatant was discarded, the cells collected in TRIzol ® reagent (Life Technologies, Carlsbad, CA, USA) and RNA extracted by the TRIzol method according to the manufacturer's instructions.

cDNA synthesis and qPCR

Approximately 300 ng of RNA was used for cDNA synthesis and the reaction was performed according to the manufacturer's instructions for the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). A quantitative polymerase chain reaction (qPCR) for analysing the relative mRNA transcription of cytokines genes IL–4 and IL–12 was performed on a STRATAGENE M×3005P real-time PCR system (Agilent Technologies, Santa Clara, CA, USA), as described by de Avila *et al.* (2016). Beta-actin and GAPDH genes were used as endogenous reference controls, but subsequently, β-actin was

selected as the internal reference standard based on its efficiency (M-value of 1.8 and 1.98 for GAPDH and β-actin, respectively). The primer sequences used for amplifying the IL–4, IL–12, β-actin and GAPDH genes, as well as qPCR conditions for the latter two have been described previously (Cardona *et al.,* 2003; Dummer *et al.,* 2014). All samples were analysed in triplicate. The comparative threshold cycle ($\Delta\Delta C_t$) method was used to determine the relative amount of mRNA for each gene with β-actin as the reference gene, according to the method as described by Livak and Schmittgen (2001).

Statistical Analysis

The data were analysed using GraphPad Prism version 5.03 (San Diego, CA, USA). The mean absorbance values from each group were subjected to analysis of variance (two-way ANOVA) followed by the Bonferroni test for significant differences at $p<0.05$. The differences in serum neutralisation antibodies and cytokine mRNA transcription were analysed by one-way ANOVA followed by the Dunnett's test. Data from experimental groups were compared among themselves and with controls.

Results

Dynamics of the humoral immune response

The mice supplemented with the probiotic *B. toyonensis* and the control group responded to vaccination with increased levels of total immunoglobulin G (IgG). On day 14 of the experimental period, we observed an approximately 2-fold increase in IgG in the groups supplemented with the probiotic compared to the control group ($p<0.05$). At day 28, compared to controls, animals in the group B.t continuous showed a 4-fold increase ($p<0.05$), whereas those in the group B.t 7 days showed a 2-fold increase ($p<0.05$) in the IgG levels. On the day 42, a similar trend of higher IgG levels in the groups supplemented with probiotics was observed where the group B.t continuous showed a 2-fold increase ($p<0.05$) and group B.t 7 days showed a 1.5-fold increase, compared to controls ($p<0.05$) (Figure 1A. Total IgG fold increase).

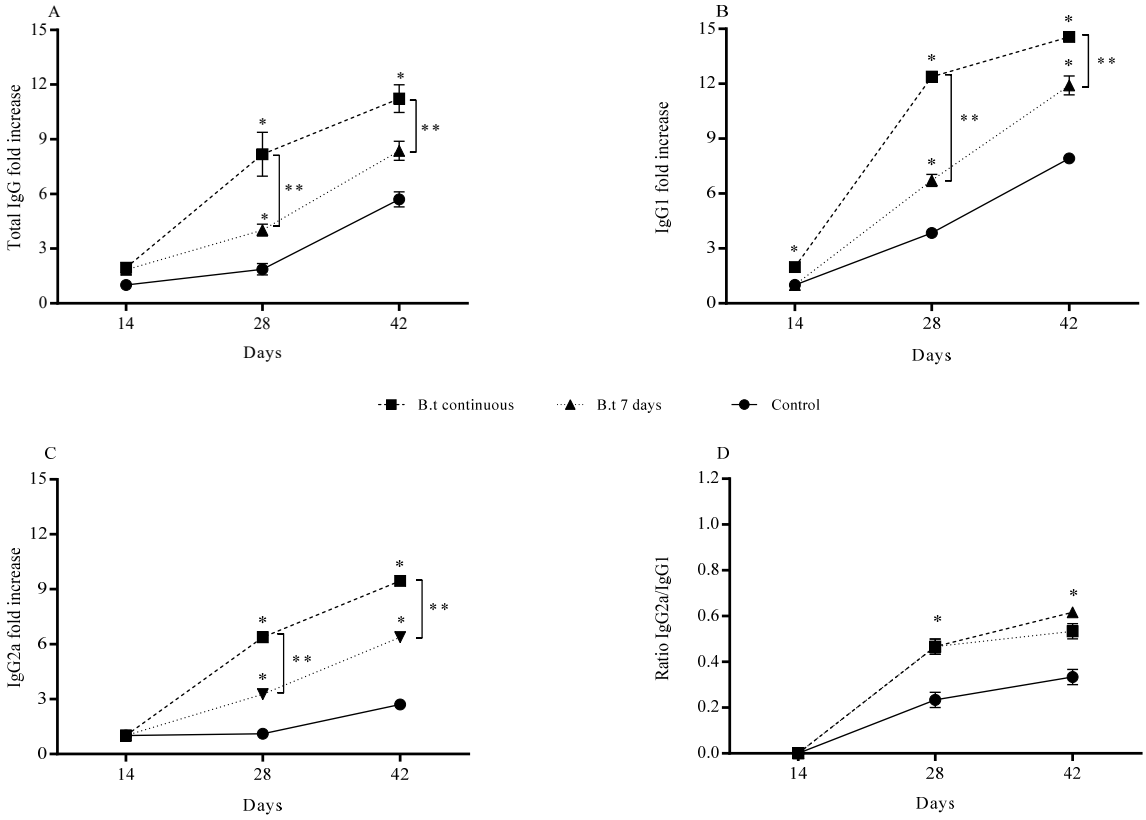

Figure 1. ELISA analysis of sera IgG dynamics. The data represent the mean (± standard error) of IgG fold increase in the mice vaccinated with rgD of BoHV–5 and supplemented with *B. toyonensis*. (A) Total IgG fold increase. (B) IgG1 fold increase. (C) IgG2a fold increase. (D) IgG2a/IgG1 ratio. The statistical analysis was performed by two-way ANOVA followed by the Bonferroni test. Asterisks (*) indicate significant difference ($p<0.05$) between the probiotic supplemented (B.t 7days and B.t continuous) and the control groups on days 14, 28 and 42; (**) means statistically significant difference ($p<0.05$) between the B.t continuous and B.t 7 days groups on days 28 and 42.

The levels of IgG1 and IgG2a isotypes were estimated on experimental days 14, 28 and 42. The sera of mice supplemented with probiotics showed higher IgG1 and IgG2a levels ($p<0.05$) compared to the sera from non-supplemented animals collected on the same day. Compared to the controls, we observed increased levels of IgG1 in the B.t continuous group from day 14 of the experimental period ($p<0.05$), and by day 28, the B.t continuous group showed 3-fold higher levels of IgG1 while the B.t 7 days group showed an approximately 1.8-fold increase ($p<0.05$). The same dynamic was observed on day 42; the B.t continuous group achieved a 2-fold increase and the B.t 7-days group showed a 1.5-fold increase, both compared to controls (Figure 1B. IgG1 fold increase). An increase was also observed in IgG2a levels compared to the controls with the B.t continuous group presenting a 5.8-fold increase ($p<0.05$), and the B.t 7 days group showing a 3-fold increase ($p<0.05$). On day 42, the B.t continuous group showed a 3.5-fold increase compared to the non-supplemented group, while the B.t 7 days group showed a 2.3-fold increase (Figure 1C. IgG2a fold increase). Further, a significantly higher IgG2a/IgG1 ratio was observed in the probiotic supplemented groups, compared to the non-supplemented group (0.5 and 0.6 vs. 0.2 and 0.3 on days 28 and 42, respectively; Figure 1D. IgG2a/IgG1 ratio).

The titres of neutralising antibodies against BoHV–5 were quantified in sera collected on day 42 of the experiment. Both groups, B.t continuous and B.t 7 days, showed higher titres of neutralising antibodies than the control group ($p<0.05$) (Figure 2. Serum neutralisation).

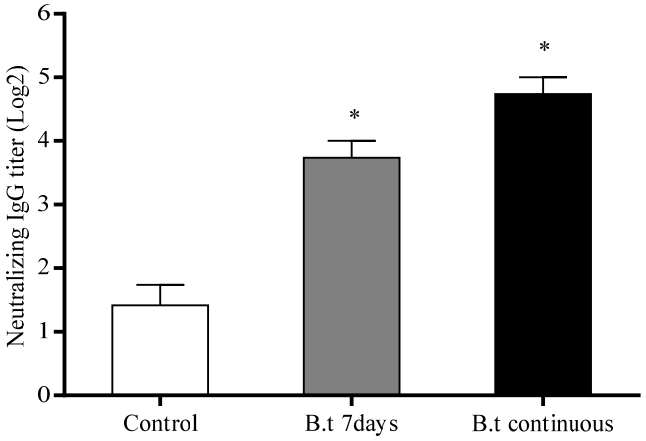

Figure 2. Serum neutralisation. Neutralising antibody titre of mice vaccinated with rgD of BoHV–5 and supplemented with *B. toyonensis*. The titre was determined by viral neutralisation assay on day 42. The data represent the mean (± standard error) of log_2 transformed data expressing the reciprocal of the highest dilution that completely inhibited virus-induced CPE. The statistical analysis was performed by one-way ANOVA followed by Dunnett's test. Asterisks (*) indicate statistically significant difference ($p<0.05$) between the probiotic supplemented (B.t 7days and B.t continuous) and the control groups on day 42.

Cytokine mRNA transcription

The spleen cells of mice in the groups supplemented with probiotic, subjected to an rgD stimulus, showed a distinct mRNA transcription profile for cytokines IL–4 and IL–12 compared to the non-supplemented group (Figure 3. qPCR transcription for IL–4 and IL–12 mRNA). Splenocytes from mice supplemented with the probiotic during the experimental period (B.t continuous) stimulated with rgD showed a 24.4-fold increase in mRNA transcription of IL–4 ($p<0.05$), and a 2.8-fold increase for IL–12 ($p<0.05$) compared to controls. This group, when stimulated with BoHV–5, presented a 4.2-fold increase in mRNA transcription of IL–4 ($p<0.05$) and a 3.4-fold increase in IL–12 mRNA ($p<0.05$) compared to the controls. When the splenocytes of the group supplemented with the probiotic for seven days before the first vaccination (B.t 7 days) were stimulated with rgD, the IL–4 mRNA transcription increased 5.1-fold ($p<0.05$) and that for IL–12 increased 3.5-fold ($p<0.05$), compared to the control group. The B.t 7 days group, when stimulated with BoHV–5, demonstrated a 4.5-fold increase in the transcription of IL-4 ($p<0.05$) and 2.8-fold increase ($p<0.05$) in IL–12 mRNA compared to the controls.

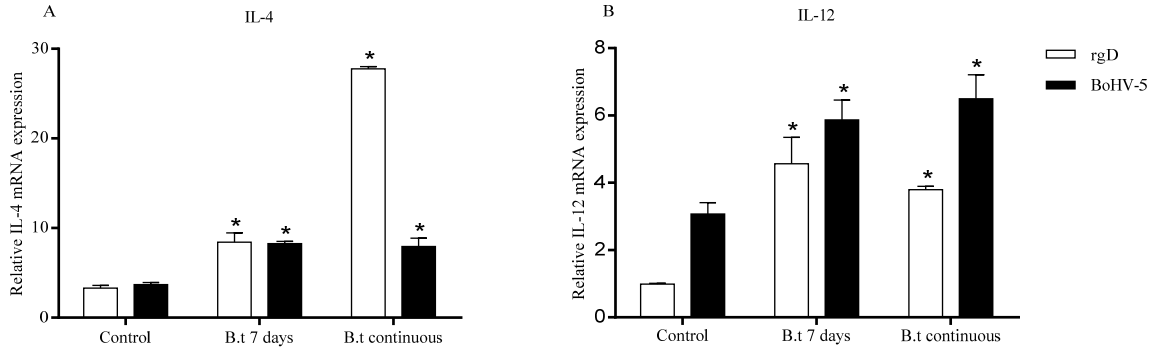

Figure 3. qPCR expression for IL–4 and IL–12 mRNA. The data represent the mean (± standard error) of IL-4 (A) and IL-12 (B) mRNA expression in splenocytes from mice vaccinated with rgD and supplemented with *B. toyonensis*. The relative mRNA expression was determined by the comparative threshold cycle ($\Delta\Delta C_t$). The statistical analysis was performed using one-way ANOVA followed by Dunnett's. Asterisks (*) indicate statistically significant difference ($p<0.05$) between the probiotic supplemented (B.t 7days and B.t continuous) and the control groups.

Discussion

The use of probiotic *B. toyonensis* has been seen as a promising alternative to increase the effectiveness of vaccines (Roos *et al.,* 2012). In piglets vaccinated against *Mycoplasma hyopneumoniae* and *Influenza* virus H1N1 and H3N2 (inactivated vaccines), the supplementation with *B. toyonensis* stimulated an increase in antibody titres and lymphocyte populations (Shierack, *et al.,* 2007). In lambs supplemented with this probiotic before vaccination with an inactivated vaccine against BoHV–5 or *E. coli* F4 demonstrated an increase in humoral immune response and an increase in titres of antibodies against both antigens (Roos *et al.,* 2010). Similarly, in this study, we also observed an immunomodulatory effect in mice supplemented with *B. toyonensis* when vaccinated with a recombinant vaccine against BoHV–5. Mice supplemented with *B. toyonensis* showed significantly higher IgG levels ($p<0.05$) compared to non-supplemented mice, demonstrating a probiotic-mediated modulation of the humoral immune response (Figure 1A). Immune response modulation was observed even in animals that received the probiotic only for the seven prior to the first dose of vaccine (the B.t 7 days group), suggesting that the probiotic is capable of modulating the immune response even when not continually administered.

Immune response modulation mediated by the probiotic was monitored by analysing isotype profiles, namely, IgG1 and IgG2a. We observed that animals supplemented with the probiotic had higher levels of IgG1 (Figure 1B) and a significant increase in IgG2a levels (Figure 1C). We used aluminium hydroxide as an adjuvant in the vaccine and it is known that aluminium-based adjuvants polarise the Th2 response by the activation of inflammasomes through NLRP3. Further, these adjuvants promote secretion of pro-inflammatory cytokines and inhibit polarisation of Th1 response by inhibiting IL–12 from DCs (Coffman *et al.,* 2010; Li *et al.,* 2007; Mori *et al.,* 2012). Our results suggest that *B. toyonensis* modulates the vaccine immune response by increasing total IgG, thus reducing the polarising effect of Th2 responses mediated by aluminium hydroxide.

The immune response to infection by the bovine herpesvirus consists primarily of a strong inflammatory response mediated by the innate immune system; this is followed by an

adaptive immune response, induction of T and B lymphocytes and the production of antibodies responsible for the inhibition of viral particle replication and prevention of secondary infections (Babiuk *et al.,* 1996; Varela *et al.,* 2010). Accordingly, we observed that the supplementation with the probiotic *B. toyonensis* stimulated the production of neutralising antibodies against the BoHV–5 (Figure 2). After vaccination against a viral agent, the neutralising antibodies may be sufficient for either protection or control of future infections (Klasse, 2014). As comparative parameter, the United States Department of Agriculture considers as immunized a herd which has 80% of vaccinated animals with titre equal to or above 8 (USDA, 2005). Animals that received the probiotic showed higher neutralising antibodies titres than non-supplemented animals. The B.t 7 days group (supplemented for seven days prior to the first vaccination) showed antibody titres that were significantly higher ($p<0.05$) than the control group but lower than those of the group supplemented throughout the experimental period, suggesting that the probiotic *B. toyonensis* stimulates the production of neutralising antibodies, even when not continuously administered.

IgG antibodies opsonise viral particles and neutralise them in a mechanism that involves the interaction of the Fc portion of the antibody with the Fc receptor, especially FcγRI, expressed on neutrophils, macrophages and NK cells. This Fc-FcγR interaction amplifies the protective activity of neutralising antibodies *in vivo* (Bournazos *et al.,* 2015; DiLillo *et al.,* 2014). The various IgG subclasses have varied effector activities based on their selective connection with Fc receptors. The subclasses IgG2a and IgG2b bind with higher affinity to FcγRI than other subclasses of IgG (Nimmerjahn and Ravetch, 2005; Ravetch and Kinet, 1991). In mice, IgG2a and IgG2b are the more pro-inflammatory IgG molecules that show greater activity than IgG1 or IgG3 in several *in vivo* models (Nimmerjahn and Ravetch, 2006). In the present study, we observed that supplementation with the probiotic increased IgG2a levels and neutralising antibody titres, compared to non-supplemented controls. This observation is important because the IgG2a subclass is considered the most potent in activating effector mechanisms during a virus-induced immune response (Coutelier *et al.,* 1987; Markine-Goriaynoff and Coutelier, 2002).

One of the probable mechanisms of immune response modulation by probiotics is their ability to stimulate cells to produce cytokines that direct development of the immune response (Fong *et al.,* 2015; Habil *et al.,* 2011; Shida *et al.,* 2011). Thus, the mechanisms of probiotic-mediated immune response modulation may be understood by analysing the cytokine profile produced in response to an interaction with antigens from infectious agents. In the intestinal mucosa, the probiotics are capable of interacting with cells of the intestinal epithelium, lymphocytes, DCs and macrophages (Forsythe and Biennenstrock, 2010). DCs interact with and respond to the bacterial antigens through pattern recognition receptors such as toll-like receptors, which then activate signalling pathways and mediate the innate and/or adaptive immune response (Niess and Reinecker, 2005a, b). The interaction with probiotics leads these cells to initiate the production of cytokines that induce helper T lymphocytes that modulate the type of response (e.g. Th1, Th2, Treg, or Th17) against the activating antigen (Gómez-Llorent *et al.,* 2010; Lebeer *et al.,* 2010).

Studies using splenocytes and peripheral blood mononuclear cell populations (PBMCs) harvested from supplemented animals have been used to demonstrate how the probiotic *B. toyonensis* stimulates the production of cytokines. Splenocytes from mice supplemented with *B. toyonensis* and stimulated with a viral antigen showed high levels of IFN-γ, IL–12 and IL–10 mRNA transcription (Roos *et al.,* 2012). Supplementation with *B. toyonensis* has been shown to modulate the immune response of pigs vaccinated with inactivated antigens against *Mycoplasma* and *Influenza* virus. The PBMCs of piglets supplemented with the probiotic and

stimulated with ConA produced higher concentrations of cytokines IL–4 and IFN-γ compared to the PBMCs of non-supplemented piglets (Shierack, *et al.,* 2007). In the present study, we also found that splenocytes of mice supplemented with *B. toyonensis* stimulated with rgD and BoHV–5 displayed higher levels of IL–4 and IL–12 mRNA transcription compared to the controls (Figure 3). Thus, a limitation of this study is that we did not measured cytokine protein level, but only mRNA transcription at splenocytes.

The cytokine IL–4 is mainly produced by Th2 lymphocytes and promotes B lymphocyte response and the secretion of IgE (Paul and Zhu, 2010). The presence of IL–4 facilitates antigen presentation through increased expression of major histocompatibility complex II molecules, maturation of DCs and proliferation of T lymphocytes, in addition to inducing exchange of the IgG isotype to IgG1 (Finkelman *et al.,* 1990; Luts *et al.,* 1996; Wells *et al.,* 2005). We observed significantly higher levels of IL–4 mRNA transcription splenocytes from probiotic-supplemented animals (both regimens) compared to control group animals ($p<0.05$). This increase in IL–4 mRNA suggests that the transcription of the cytokine may have had a role in probiotic modulation, especially as significantly greater levels of total IgG and IgG1 were detected in the sera of the probiotic-supplemented animals.

The cytokine IL–12 is essential for the activation of the immune response mediated by Th1 cells and is responsible for the induction IFN-γ expression, stimulation of NK cells and differentiation of naïve T lymphocytes into Th1 lymphocytes (Martín-Fontecha *et al*., 2004; Wan and Flavel, 2009). We observed that animal splenocytes supplemented with the probiotic using both regimens had significantly higher expression levels of IL–12 mRNA, suggesting that one of the mechanisms that modulate immune response upon probiotic supplementation may involve IL–12, as observed by others (Ichikawa *et al.,* 2009; Takeda *et al*., 2013; and Matsusaki *et al.,* 2016). We next evaluated the IgG2a/IgG1 ratio (Figure 1D) in the control animals and they remained low at 0.2 and 0.3 at days 28 and 42, respectively. In the supplemented groups, this ratio was significantly higher at 0.5 and 0.6 on days 28 and 42, respectively. The modulation observed in the IgG isotype profile (IgG1 to IgG2a) suggest that IL-12 might play a role in the isotype profile, thus polarisating the vaccinal immune response towards a Th1 response.

The immune modulatory effect observed upon *B. toyonensis* supplementation is very important when considering that aluminium hydroxide was used as the adjuvant in the vaccine, which is known to induce a Th2 response in mice (De Gregorio et al., 2008; Kool *et al.,* 2008). The ineffectiveness of aluminium hydroxide as a Th1 polarisation agent suggests that additional signals (i.e., activation of TLR or IL–12 expression) were presented along with the antigen that modulated the immune response bias toward the Th1/IgG2a cell-mediated immunity (Wang and Singh, 2011). The observation is very important as a cellular immune response is generally required for the control of viral infections (Pinto *et al.,* 2006) and also because it seems possible to shift the aluminium hydroxide modulatory effect from Th2 to Th1 (De Gregorio *et al.,* 2008; Kool *et al.,* 2008; Li *et al.,* 2007). The use of this probiotic may significantly contribute to improving the immune modulation of response elicited by recombinant vaccines, particularly those that rely on increasing antibody and cell-mediated immune responses.

In conclusion, the data obtained in this study indicate that probiotic *B. toyonensis* enhance the immune response in the mice vaccinated with a recombinant vaccine against BoHV–5. The immunomodulatory effect was achieved even upon seven days of supplementation prior to the initial vaccination. However, further studies are needed to better understand the mechanisms involved in probiotic-mediated immunomodulation.

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4.2 Manuscrito 2 – *Bacillus toyonensis* BCT-7112^{T} spores increase the adjuvanticity for recombinant vaccine against BoHV-5

Manuscrito a ser submetido à revista *Research in Veterinary Science*

Bacillus toyonensis BCT-7112^T spores increase the adjuvanticity of recombinant vaccine against BoHV-5

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Abstract

Spore-forming bacteria of the genus *Bacillus*, known to exert their beneficial effects through the modulation of the host immune response, are widely used as probiotics for animals. It has been previously shown that oral supplementation with spores of *B. toyonensis* BCT-7112^T improves the immune response to vaccines. In this study, we used the recombinant glycoprotein D (rgD) from bovine herpesvirus type 5 (BoHV-5) as a model antigen to investigate whether subcutaneously administered spores of *B. toyonensis* could modulate the immune response to the vaccine. Mice were randomly separated into three groups: *B. toyonensis* (B.t) oral, B.t

subcutaneous (s.c.), and control. All the groups were vaccinated with 40 µg of rgD, which was adsorbed in 10% aluminum hydroxide (alumen) as an adjuvant. The group B.t oral received commercial food supplemented with 1×10^6 viable spores of *B. toyonensis* per gram of food, throughout the experimental period. The B.t s.c. group received the same vaccine, with the incorporation of 1×10^6 viable spores/dose in its formulation. The control group received the same feed without probiotics. All the animals were vaccinated subcutaneously on day 0 and received a booster on day 21 of the experiment. Both B.t-supplemented groups, oral and s.c., showed significantly higher titers ($p<0.05$) of total serum IgG, IgG1, IgG2a, and BoHV-5 neutralizing antibodies, compared to the control group. A significantly higher ($p<0.05$) transcription level of cytokines IL-4, IL-12, and IFN-γ was observed in B.t-treated groups. In addition, stimulation of the macrophage-like cell line RAW264.7 with spores of *B. toyonensis* markedly enhanced the proliferation and mRNA transcription levels of IL-4, IL-10, and IL-12 cytokines in these cells. Our findings indicated that the subcutaneous administration of *B. toyonensis* BCT-7112^T spores enhanced the humoral and cellular immune response against BoHV-5, indicating that these spores could increase the vaccine adjuvanticity in mice.

Keywords: Spore, *Bacillus*, probiotic, Adjuvanticity, vaccine

Introduction

Spore-forming bacteria of the genus *Bacillus* are widely used as probiotics for animals and are known to exert their beneficial effects by modulating the host immune response (Cutting, 2011). Adjuvants are compounds that elicit specific immune responses and are essential in the preparation of most vaccine formulations (Lambrecht *et al.*, 2009; Reed *et al.*, 2009). Over the years, although the search for new potential adjuvants for human and animal vaccines has intensified, only a few options are presently licensed for use (Montomoli *et al.*, 2011). Spores of the genus *Bacillus* have been successfully used for antigen delivery, either by

genetically engineering them to express heterologous antigens or associating or adsorbing these antigens to the spore surface coat (Ricca *et al.*, 2014). These approaches have been shown to confer strong adjuvanticity to vaccines that are administered via the mucosal, nasal, and oral routes (Barnes *et al.*, 2007; Huang *et al.*, 2010; De Souza *et al.*, 2014).

Bovine herpesvirus 5 (BoHV-5) is responsible for outbreaks of meningoencephalitis that have a mortality rate of up to 70–100% (Vogel *et al.*, 2003). The disease affects young cattle and is responsible for economic losses in South America (Campos *et al.*, 2011). Recombinant vaccines against bovine herpesvirus use principal glycoproteins of the viral envelope as antigens. The glycoprotein D (gD) of BoHV-5 is essential for the binding and penetration of the virus into the host cells, and is also one of the main targets of the host immune response, resulting in a strong humoral and cellular immune response during infections (Alves Dummer *et al.*, 2014). The immune response to infection by the bovine herpesvirus primarily consists of a strong inflammatory response mediated by the innate immune system, which is followed by an adaptive immune response, induction of T and B lymphocytes, and the production of neutralizing antibodies that inhibit the replication of viral particles and prevent secondary infections (Babiuk *et al.*, 1996; Varela *et al.*, 2010).

Bacillus toyonensis BCT-7112^{T} has long been used as a probiotic in animal nutrition for pigs, poultry, cattle, rabbits, and fish (Williams *et al.*, 2009; Jiménez *et al.*, 2013; Kantas *et al.*, 2015). In piglets vaccinated against *Mycoplasma hyopneumoniae* and influenza virus H1N1 and H3N2 (inactivated vaccines), supplementation with *B. toyonensis* stimulated an increase in antibody titers and lymphocyte populations (Schierack *et al.*, 2007). In lambs that were administered an inactivated vaccine against BoHV-5 or *E. coli* F4, supplementation with this probiotic resulted in an increase in antibody titers against both the antigens (Roos *et al.*, 2010). Santos *et al.* (2018) demonstrated that mice vaccinated with a recombinant viral antigen and orally supplemented with *B. toyonensis* spores had higher levels of serum IgG, IL-4, and IL-

12, compared to vaccinated animals that did not receive the probiotic, confirming that supplementation of *B. toyonensis* enhanced immune response to the vaccine. The aim of this study was to assess the effect of *B. toyonensis* spores administered subcutaneously, as a vaccine component of BoHV-5 vaccine, in a murine model.

Material and methods

Bacterial culture

The bacterium *Bacillus toyonensis* strain BCT-7112^{T} used in this study was obtained from the microbial collection at the Microbiology Laboratory, Biotechnology Center, Federal University of Pelotas (UFPel). The culture of *B. toyonensis* was performed according to Santos *et al.* (2018). Briefly, the bacteria were inoculated on brain heart infusion agar (BHI, Neogen, Lasing, MI, USA) and the plates were incubated at 37 ºC for 24 h. About 3–5 individual colonies were picked and inoculated into BHI broth (Neogen) and placed on an orbital shaker at 37 ºC maintained at 200 rpm for 16 to 18 h. The culture served as an inoculum for propagation in a bioreactor (BIOSTAT® B, Braun Biotech International, Melsungen, Germany) containing 8 liters of Nutrient Yeast Extract Salt medium (NYSM) (Yousten, 1984). The fermentation conditions were controlled and air supply was maintained between 0.5 and 1.5 (v/v), so that approximately 80% of the dissolved oxygen in the medium was utilized during fermentation. The agitation speed was maintained at 300 rpm and temperature at 37 ºC for 96 h. When 90% of the bacterial cells had sporulated, as confirmed by Wirsten-Colink stain, the culture was centrifuged in a refrigerated Sorvall centrifuge® RC-6 plus (Langenselbold, Germany) at 5,000 ×*g* for 20 min at 4 ºC. The supernatant was removed and the spore pellet was suspended in 1 liter of phosphate-buffered saline. Spore counts were determined by serial dilution and plate count method.

Purification of spores

The spores of *B. toyonensis* were purified according to the method described previously by Tavares *et al.* (2013). The spore suspension was centrifuged for 10 min at 10,000 ×*g* at 4 ºC, and the resulting pellet was suspended in 50 mM Tris-HCl (pH 7.2) containing 50 µg/mL of lysozyme and incubated for 1 h at 37 °C. After another round of centrifugation under the same conditions as above, the suspension was washed once with an equivalent volume of distilled water and the pellet was centrifuged and suspended in 0.05% SDS solution using a vortex mixer. The spores were washed thrice with distilled water and suspended in an equivalent volume of distilled water. Following purification, the *B. toyonensis* spore suspension was serially diluted, plated on BHI agar, and incubated at 37 ºC for 24 h. The colonies were then counted and calculated as the titers of colony-forming units (CFU/mL).

Expression of recombinant glycoprotein D

The BoHV-5 gD was cloned into *Pichia pastoris* strain KM71H Mut^{s}, and the recombinant BoHV-5 glycoprotein D (rgD) was expressed, concentrated, and purified as described previously by Dummer *et al.* (2009).

Ethics statement

Mice used in this study were provided by the animal unit at UFPel. All protocols and procedures were reviewed and approved by the Ethics Committee on Animal Experimentation (CEEA No. 1981) of the UFPel. The CEEA of UFPel is accredited by the Brazilian National Council for the Control of Animal Experimentation (CONCEA).

Vaccine preparations and mice immunizations

Thirty female outbred Swiss mice of an age of 4–6 weeks were randomly separated into three groups of 10 animals each: *B. toyonensis* (B.t) oral, B.t subcutaneous (s.c.), and control. Mice in all the groups were vaccinated with 40 µg of rgD adsorbed in 10% aluminum hydroxide (alumen) (Sigma, St. Louis, Missouri, USA) as an adjuvant. The group B.t oral received a commercial feed that was free of chemotherapeutic agents (Nuvilab® CR-1, Nuvital Nutrients S/A, Colombo, Brazil) and supplemented with 1×10^6 viable spores of *B. toyonensis* per gram of food, throughout the experimental period. The B.t s.c. group received the same vaccine with the incorporation of 1×10^6 viable spores/mL in its formulation and received the same feed as above, without the *B. toyonensis* spores. The control group received the same feed without spores. The mice were subcutaneously administered 0.2 mL of the vaccine on day 0 and received a booster on day 21 of the experiment. Blood samples were collected by the submandibular puncture on days 0, 14, 21, 28, and 42. After collection, serum samples were separated, labeled, and stored at –20 ºC until further analysis.

Total serum IgG antibodies against rgD

Indirect ELISA was performed according to Dummer *et al.* (2014). Ninety-six-well microtiter plates were coated with 50 ng of rgD per well and left overnight at 4 ºC. The plates were then washed with PBS-T (phosphate-buffered saline with 0.05% of Tween® 20). Serial 2-fold dilutions were added to each serum sample and the plates were incubated for 1 h at 37 ºC. After three washes, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich, St. Louis, USA) (1:4,000 dilution) was added, followed by incubation at 37 ºC for 1 h. The reactions were visualized with O-Phenylenediamine dihydrochloride (OPD)

(Sigma), stopped using 2N H_2SO_4, and analyzed at an optical density (O.D.) of 492 nm using an EZ Read 400 microplate reader (Biochrom, UK). The results were expressed as the reciprocal of the highest dilution, resulting in a reading of three standard deviations above the value of the negative control serum.

IgG isotyping against rgD

The IgG isotypes (IgG1 and IgG2a) were evaluated by ELISA using pooled serum samples of the experimental groups collected on days 0, 20, and 42. Briefly, the plates were coated with 50 ng of rgD per well as described above. Indirect ELISA was performed according to the instructions of the Mouse Monoclonal Isotyping Reagents kit (Sigma). The results were expressed as absorbance values read at 492 nm using an EZ Read 400 microplate reader (Biochrom, UK).

Virus neutralization test (VNT)

The serum samples collected on day 42 of the experiment were tested for the presence of neutralizing antibodies against BoHV-5 using the VNT, according to Fischer *et al.* (2007). Briefly, each serum sample was serially diluted (2-fold) in quadruplicate, beginning at 1:2 until 1:256 in Minimum Essential Medium (MEM). A suspension of BoHV-5 containing 100 $TCID_{50}$ was added, followed by incubation for 1 h at 37 ºC in a 5% carbon dioxide (CO_2) environment. Approximately 3×10^4 Madin Darby bovine kidney cells (MDBK, originally ATCC CCL22) were then added to each well and the microplates were incubated until 100 $TCID_{50}$ was

observed in the control wells. The absence of cytopathic effect (CPE) resulted from the presence of neutralizing antibodies. Antibody titers were calculated using the statistical method of Behrens and Kärber and expressed as the reciprocal of the highest dilution capable of neutralizing 100 $TCID_{50}$ of the virus.

Spleen cell culture

The mice from all experimental groups were euthanized on day 42 of the experiment, and their spleen was collected and macerated. Spleen cells (2×10^6) were cultured in 1 mL RPMI 1640 medium (Gibco) containing 10% fetal bovine serum (FBS), and antibiotics and antifungal agents (10,000 IU/mL penicillin, 10 mg/mL streptomycin, and 25 mg/mL amphotericin B, Gibco) in 24-well plates (Kasvi, Taiwan, China), and incubated for 24 h at 37 °C in an incubator containing 5% CO_2. After incubation, the medium was replaced and the cells were stimulated with 10 µg rgD, 10 µg concanavalin A (ConA, Sigma), and RPMI 1640 medium, and further incubated for 18 h under the same conditions as above. ConA and medium were used as positive and negative controls, respectively. Following incubation, the supernatant was discarded, cells were collected with TRIzol® reagent (Sigma-Aldrich), and stored at –70 °C until RNA extraction.

RAW264.7 cells

The murine macrophage-like cell line RAW264.7 was cultured as monolayers on Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% (v/v) SFB, in

an atmosphere of 90% humidity containing 5% CO_2 at 37 °C. Four days before use, the cells were detached by gentle scraping and seeded into 24-well plates (Kasvi) in the same medium at a density of approximately 1×10^6 cells per well. The cells were stimulated in quadruplicates with either 1×10^6 viable spores of *B. toyonensis* for 24 h or just the culture medium.

Cell proliferation assay

The sulforhodamine B (SRB) assay was used for the determination of cell density, based on the measurement of the cell protein content. In order to determine whether the spores could affect RAW264.7 macrophage proliferation, the cells were cultured alone or in combination as described above. Briefly, cultures were washed and fixed with 50% trichloroacetic acid (w/v) for 30 min at 4 °C, after which the cells were washed five times with deionized H_2O, stained with 0.4% SRB (w/v) for 45 min at room temperature, and then washed five times with 1% acetic acid (v/v). Finally, the SRB complexes were eluted in 10 mM Tris buffer by shaking for 15 min. Absorbance was measured at 540 nm using a SpectraMAX 190 microplate reader (Molecular Devices, San Jose, CA, USA). The results were expressed as absorbance values read at 540 nm.

RNA isolation, cDNA synthesis, and qPCR

The RNA was extracted using the TRIzol method, according to the manufacturer's instructions. Approximately 400 ng of RNA was used for the synthesis of cDNA, and the reaction was performed using the High Capacity cDNA Reverse Transcription Kit (Applied

Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. A quantitative polymerase chain reaction (qPCR) was performed on a CFX96TM Real-Time System platform (Bio-Rad, Hercules, CA, USA) using specific primers for β-actin, GAPDH, IL-4, IL-10, IL-12, and IFN-γ. The primer sequences used for this reaction have been previously described (Cardona *et al.*, 2003; Dummer *et al.*, 2014). The reaction efficiency for each primer pair was calculated using a two-fold dilution series on a cDNA sample, and the standard curves were represented as the semi-log regression line plot of the C_t value vs. log of the relative input cDNA concentration, according to that described by Bustin *et al.* (2009). A 97.9%–106.9% efficiency was considered acceptable and primers with efficiencies within these limits were included. β-Actin was used as an endogenous reference gene. The qPCR reactions were performed using 1 µL of cDNA (synthesized from 500 ng of RNA), 6.25 µL of GoTaq® qPCR Master Mix (Promega, Madison, WI, USA), 0.25 µM of each primer, and 4.25 µL of RNAse-free water (Sigma), in a total reaction volume of 12.5 µL. The temperature conditions were: denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 60 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 5 min. All the samples were analyzed in triplicate. The comparative threshold cycle ($\Delta\Delta C_t$) method was used to determine the relative amount of mRNA for each gene with β-actin as the reference gene, according to the method described by Livak and Schmittgen (2001).

Statistical Analysis

The obtained data were analyzed using the statistical software GraphPad Prism version 7 (GraphPad, CA, USA). The total serum IgG titers and the mean absorbance for IgG isotypes of each experimental group were compared using a two-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Differences in serum neutralizing antibody titers and relative cytokine mRNA transcription were evaluated using one-way ANOVA, followed by Dunnett's Test. Differences between groups were considered to be statistically significant when $p<0.05$.

Results and discussion

***Bacillus toyonensis* BCT-7112^T spores increase the total serum IgG against rgD and neutralizing antibody titers against BoHV-5 in mice**

In this study, we evaluated the adjuvant effect of *B. toyonensis* spores as a vaccine component administered twice subcutaneously, compared to continuous oral supplementation. It was already known that supplementation of *B. toyonensis* by the oral route increased the immune response to vaccines (Roos *et al.*, 2010; 2012; 2018; Santos *et al.*, 2018; Schierack *et al.*, 2007). However, to the best of our knowledge, their efficacy as an adjuvant of a parenteral vaccine was never tested before.

The IgG response was analyzed on days 0, 14, 21, 28, and 42 of the experiment. On day 14, significantly high IgG levels were detected in the animals that received spores orally and subcutaneously (32-fold and 11-fold increase, respectively, $p<0.05$), compared to the control group. This indicated that spores of *B. toyonensis* stimulated a faster and higher serum IgG response, irrespective of the route of administration and supplementation schedule. The IgG levels continued to increase in all the groups until the 42nd day of the experiment. After the

booster dose, the B.t groups showed significantly higher IgG levels than the control ($p<0.05$) (Fig. 1A), and by day 42, the B.t groups had significantly higher neutralizing antibody titers against BoHV-5 than the control group ($p<0.05$) (Fig.1B).

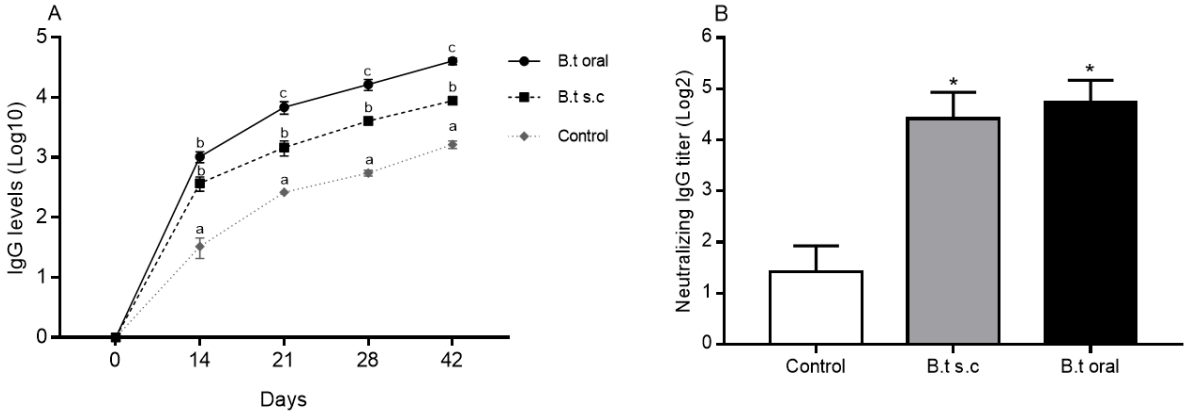

Figure 1. Analysis of serum IgG dynamics. (A) Total IgG levels determined by indirect ELISA of mice vaccinated with a recombinant antigen from BoHV-5 that received *Bacillus toyonensis* spores by oral or subcutaneous administration route. The data represent the mean ± standard error (S.E.M) of Log_{10} transformed data and are expressed as the reciprocal of the highest dilutions. The statistical analysis was performed by two-way ANOVA followed by Turkey's multiple comparisons test. The similar letters indicate no statistical difference ($P > 0.05$) and the different letters indicate a statistical difference ($P < 0.05$) between the experimental groups. (B) Neutralizing antibody titer. The data represent the mean ± S.E.M of log_2 transformed data expressing the reciprocal of the highest dilution that completely inhibited virus-induced CPE. The statistical analysis was performed by one-way ANOVA followed by Dunnett's test. Asterisks (*) indicate a statistically significant difference ($p<0.05$) between the experimental groups on day 42.

The ability of orally administered *Bacillus* spores to elicit an increase in immune response to vaccine could be due to the uptake of spores by M cells in the intestinal mucosa and their entry into Peyer's patches, that are taken up by the antigen-presenting cells (APCs) (Rhee *et al.*, 2004; Hong *et al.*, 2005). These cells can migrate to the mesenteric lymph nodes and enter systemic circulation, where they stimulate an immune response that is far from the location of their original activation (Chieppa *et al.*, 2006; Lebeer *et al.*, 2010).

The spores act by direct activation of APCs, particularly for DCs. In fact, DCs that were stimulated with spores of *B. subtilis* showed upregulation of MHC I, MHC II, and co-stimulatory molecules such as CD40, CD25, and CD86 (Barnes *et al.*, 2007; De Souza *et al.*, 2014; Aps *et al.*, 2015). The possible mechanism for the activation of DCs by spores is the TLR-mediated signaling, particularly the TLR2, TLR4 receptor (Cerovic *et al.*, 2009; De Souza *et al.*, 2014), and through TLR8 (Ugolini et al., 2012). Mice from the B.t s.c. group received the vaccine with the incorporation of 1×10^6 viable spores/dose and presented significantly enhanced immune response to rgD, with high levels of IgG and neutralizing BoHV-5 antibodies. Alumen is known to remain at the site of injection and slowly dissolve over time, which can help sustain the antigen presentation by APCs (Ghimire *et al*., 2012). Thus, it may be suggested that alumen associated with B.t spores might increase the interaction with DCs, thus enhancing the vaccine adjuvanticity. These results suggest that spores of *B. toyonensis* have the potential to be used as a novel adjuvant strategy for parenteral vaccines.

We investigated the titers of neutralizing antibodies against BoHV-5 on day 42 of the experiment (Fig. 1B). It was observed that both groups that received *B. toyonensis* by oral and subcutaneous routes presented a significantly higher titer than the control group. After vaccination against a viral agent, the neutralizing antibodies may be sufficient for either prevention or control of future infections (Klasse, 2014). As a reference, the United States Department of Agriculture considers a herd that has 80% of the vaccinated animals with a titer ≥ 8, to be immunized (USDA, 2005). In this study, even with a model, we detected the titers of 16 and 32 in the mice treated with *B. toyonensis*, whereas the mice from the control group presented the titers of only 2 and 4.

Modulating the IgG isotype profile by *B. toyonensis* BCT-7112^T spores against rgD of BoHV-5 in mice

The profile of the IgG subclass responses was examined after the second dose of the vaccine on days 28 and 42. The animals that received the probiotic by oral route showed high levels of IgG1 at all time-points (Fig. 2A). On the other hand, high levels of IgG2a were detected in the experimental groups that received *B. toyonensis* orally or subcutaneously (Fig. 2B). The IgG2a isotype is considered to be the most potent effector mechanism during a virus-induced immune response (Coutelier *et al.*, 1987; Markine-Goriaynoff and Coutelier, 2002). In this study, we detected higher levels of IgG2a in the treated groups, suggesting that spores might have a role in modulating the IgG isotype. This is an important finding since we used alumen as an adjuvant, which is known to inhibit the production of IL-12, which may result in the suppression of IgG2a (Mori *et al.*, 2012). Also, the analysis of the IgG2a/IgG1 ratio on days 28 and 42 (Fig. 2C) showed significantly higher ratios of 0.7 to 0.8 in the B.t s.c. group. These observations suggest a Th1-type response, which corroborates with previous reports (Huang *et al.*, 2010; Isticato *et al.*, 2013). On the other hand, mice that received *B. toyonensis* spores orally presented lower ratio values of 0.5 to 0.6, suggesting a trend toward a mixed Th1/Th2 immune response.

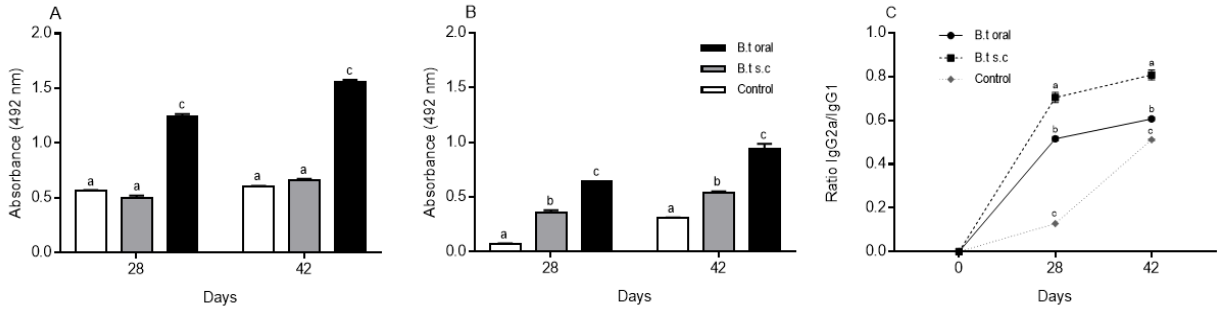

Figure 2. IgG isotype profile. (A) IgG1 levels, (B) IgG2a levels, and (C) IgG2a/IgG1 ratio were determined by indirect ELISA of serum from mice vaccinated with a recombinant antigen of BoHV-5 that received *Bacillus toyonensis* spores by oral or subcutaneous administration route. The data represent the mean ± S.E.M of absorbance values reading 492 nm. The statistical analysis was performed by two-way ANOVA followed by Turkey's multiple

comparisons test. The similar letters indicate no statistical difference ($P > 0.05$) and the different letters indicate a statistical difference ($P < 0.05$) between the experimental groups on the same day.

Bacillus toyonensis BCT-7112^T spores increase the transcription of cytokine mRNA in mice spleen cells

The spleen cells of all the vaccinated animals were cultured, subjected to an rgD stimulus, and analyzed for relative mRNA transcription for cytokines IL-4, IL-12, and IFN-γ. High levels of transcripts of IL-4 mRNA were detected in splenocytes from mice that received the spores orally (Fig. 3A). IL-4 is mainly produced by Th2 lymphocytes and promotes B cell response, secretion of IgE, and induction of the exchange of IgG isotype to IgG1 (Finkelman *et al.*, 1990; Paul and Zhu, 2010). Low levels of IL-12 were detected in the spleen of mice from the control group, whereas spleen from mice that received the spores by oral and subcutaneous routes showed significant transcription levels of IL-12 (Fig. 3B). The differentiation of Th1 cells requires IL-12, which promotes the secretion of IFN-γ by T cells and NK cells (Trinchieri, 2003). High levels of IFN-γ mRNA transcript were detected in spleen cells from mice that received *B. toyonensis* spores; these levels were statistically higher in mice that were treated by spores via oral and subcutaneous routes, compared to the control group (Fig. 3C). IFN-γ increases the expression of MHC class I and class II proteins, activates macrophages, and induces NO production to kill pathogens. This cytokine also directs the differentiation of naïve T lymphocytes into Th1 cells (Bradley et al., 1996; Bastos et al., 2007; Giroux et al., 2003). The modulation of IFN-γ by *Bacillus* spores corroborates with previous observations derived from experiments using spores as a parenteral and mucosal adjuvant (Barnes *et al.*, 2007; Souza *et al.*, 2014; Huang *et al.*, 2010; Isticato *et al.*, 2013).

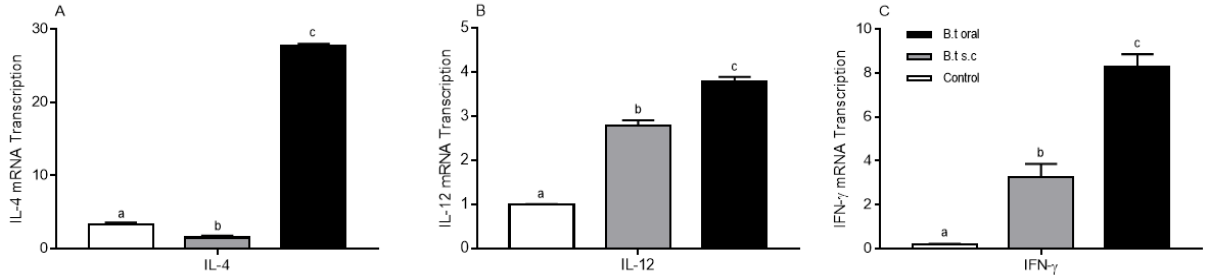

Figure 3. qPCR transcription for IL-4, IL-12, and IFN-γ mRNA. The data represent the mean ± S.E.M of IL-4 (A), IL-12 (B), and IFN-γ (C) mRNA transcription in splenocytes from mice vaccinated with a recombinant antigen from BoHV-5 that received *Bacillus toyonensis* spores by oral or subcutaneous administration route. The relative mRNA transcription was determined by the comparative threshold cycle ($\Delta\Delta C_t$). The statistical analysis was performed using one-way ANOVA followed by Dunnett's. The different letters indicate a statistical difference ($P < 0.05$) between the experimental groups.

Effect of *B. toyonensis* BCT-7112^T spores on RAW264.7 macrophages *in vitro*

In order to better understand the mechanism of modulation of the immune response by *B. toyonensis*, the murine macrophage cell line RAW264.7 was used. The capability of spores to cause macrophage proliferation is important, as they then act as innate and adaptive effector cells playing functions such as the production of cytokines and mediators, phagocytosis, and antigen presentation (Spelman *et al.*, 2014). Thus, the stimulation of macrophages by spores highlights their role in immune functions such as phagocytosis, antigen presentation, and cytokine production (Huang *et al.*, 2008; Xu *et al.*, 2012; Huang *et al.*, 2013). For the cell proliferation assay, the cells were stimulated with 1×10^6 viable spores for 24 h. The exposure of RAW264.7 cells to spores promoted a 3-fold increase ($p<0.05$) in the proliferation of macrophages (Fig. 4A).

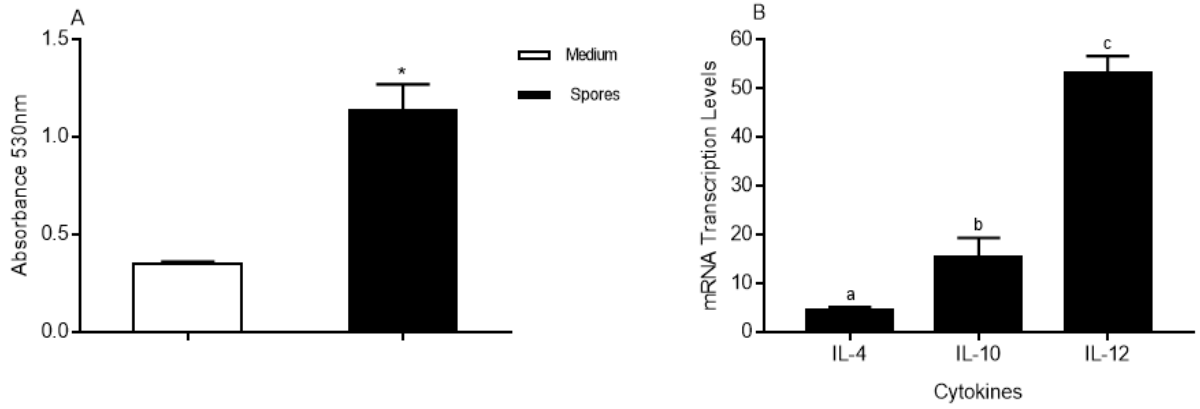

Figure 4. Effect of *Bacillus toyonensis* spores on RAW264.7 cell line macrophages. (A) Cell proliferation assay by sulforhodamine B in RAW264.7 macrophages stimulated for 24 h with *B. toyonensis* spores. The data represent the mean ± S.E.M of absorbance values reading 540 nm. The statistical analysis was performed using one-way ANOVA followed by Student's *t*-test. Asterisks (*) indicate a statistically significant difference ($P < 0.05$) between the treated and untreated cells. (B) qPCR transcription for IL-4, IL-10, and IL-12 mRNA. The data represent the mean ± S.E.M of IL-4, IL-10, and IL-12 mRNA transcription in RAW264.7 macrophages stimulated for 24 h with *B. toyonensis* spores. The relative mRNA transcription was determined by the comparative threshold cycle ($\Delta\Delta C_t$). The statistical analysis was performed using one-way ANOVA. The different letters indicate a statistical difference ($P < 0.05$) between the cytokine mRNA transcription.

Following the same trend, we observed that RAW264.7 cells stimulated with *B. toyonensis* spores showed a distinct level of mRNA transcription for the cytokines IL-4, IL-12, and IL-10, while IFN-γ was not detected (Fig. 4B). Low levels of IL-4 mRNA transcription, although significantly higher than control, were detected ($p<0.05$). IL-4 induces macrophage proliferation and activation and facilitates antigen presentation through increased expression of MHC class II molecules (Gordon and Martinez, 2010; Jenkins *et al.*, 2011). High levels of IL-10 mRNA transcript were produced by these cells. IL-10 can be produced by macrophages and by a number of cell types including T cells and B cells. Its main function is to control the intensity of immune responses, and it also plays a central role in the priming and proliferation of B cells (Ouyang *et al.*, 2011). The level of transcription of IL-12 mRNA was significantly

higher ($p<0.05$) than the other cytokines. The early production of IL-12 by APCs, including macrophages and dendritic cells, is a key event in the initiation of the immune response (Trinchieri, 2003). It is possible that IL-12 signaling in macrophages leads to cellular responses that are associated with improved APC function (Grohmann *et al.*, 2001). Notably, it has been demonstrated that oral administration of probiotics increases macrophage phagocytosis and microbicidal activity at distant sites from the gastrointestinal tract (Maldonado Galdeano *et al.*, 2011). In addition, it was demonstrated that supplementation with probiotics affected the activity of macrophages in the Peyer's patches, as well as in the peritoneum and spleen (Lemme-Dumit *et al.*, 2018).

Conclusions

In summary, our study demonstrated that the subcutaneous administration of *B. toyonensis* BCT-7112^{T} spores as a vaccine component enhanced the immunogenicity of a recombinant vaccine against BoHV-5 in mice. We observed the increased production of serum IgG and neutralizing antibodies against BoHV-5. Importantly, mice that received *B. toyonensis* (orally or subcutaneously) showed significantly higher IgG by day 14, suggesting a faster serum IgG response in vaccinated animals. An increase in the levels of transcription of IL-4, IL-12, and IFN-γ mRNA in spore-treated animals suggests that the spores may use this mechanism to modulate the cellular immune response. Our data showed that spores of *B. toyonensis* stimulated the proliferation of RAW264.7 macrophages, which in combination with increased transcription of IL-4, IL-10, and IL-12 mRNA, suggested that spores may have immunomodulatory effects on macrophages. Thus, the use of *B. toyonensis* spores as a vaccine component by the subcutaneous route enhances the adjuvanticity of vaccine antigen in mice.

Declaration of interest

The authors have no conflicts of interest to declare.

Author contributions

FDSS and FPLL designed the study and wrote the manuscript. FDSS, LRM, VSG, HCF, and PRCR performed the experiments. All authors contributed to and revised the manuscript.

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4.3 Artigo 3 – A probiotic treatment increases the immune response induced by the nasal delivery of spore-adsorbed TTFC

Artigo publicado na revista *Microbial Cell Factories*

A probiotic treatment increases the immune response induced by the nasal delivery of spore-adsorbed TTFC

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Key words: mucosal vaccine; mucosal adjuvant; Bacillus; gut; 16S analysis.

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Abstract

Background: Spore-forming bacteria of the *Bacillus* genus are widely used probiotics known to exert their beneficial effects also through the stimulation of the host immune response. The oral delivery of *B. toyonensis* spores has been shown to improve the immune response to a parenterally administered viral antigen in mice, suggesting that probiotics may increase the efficiency of systemic vaccines. We used the C fragment of the tetanus toxin (TTFC) as a model antigen to evaluate whether a treatment with *B. toyonensis* spores affected the immune response to a mucosally administered antigen.

Results: Purified TTFC was given to mice by the nasal route either as a free protein or adsorbed to *B. subtilis* spores, a mucosal vaccine delivery system proved effective with several antigens, including TTFC. Spore adsorption was extremely efficient and TTFC was shown to be exposed on the spore surface. Spore-adsorbed TTFC was more efficient than the free antigen in inducing an immune response and the probiotic treatment improved the response, increasing the production of TTFC-specific sIgA and causing a faster production of serum IgG. The analysis of the induced cytokines indicated that also the cellular immune response was increased by the probiotic treatment. A 16S RNA-based analysis of the gut microbial composition did not show dramatic differences due to the probiotic treatment. However, the abundance of members of the *Ruminiclostridium* 6 genus was found to correlate with the increased immune response of animals immunized with the spore-adsorbed antigen and treated with the probiotic.

Conclusion: Our results indicate that *B. toyonensis* spores significantly contribute to the humoral and cellular responses elicited by a mucosal immunization with spore-adsorbed TTFC, pointing to the probiotic treatment as an alternative to the use of adjuvants for mucosal vaccinations.

Introduction

Mucosal surfaces are the most common route used by pathogens to enter the human and animal body. For this reason, it is extremely important for a vaccine to induce sIgA antibody

production and elicit immune protection at the mucosal surfaces [1]. While injected vaccines induce specific T cell responses in the bloodstream and serum IgG production but generally fail to induce sIgA, mucosal vaccines administered via the oral or nasal routes induce humoral and cellular immune responses at both the systemic and mucosal sites [2, 3]. Therefore, mucosal, needle-free vaccines are potentially preferable over parenteral vaccinations [4]. However, only few mucosal vaccines are currently licensed for vaccination against viral (Rotavirus, Poliovirus, Influenza type A virus) or bacterial (*Salmonella typhi, Vibrio cholerae*) pathogens [3]. This is mostly due to the low immunogenicity of most mucosal antigens and to the lack of efficient adjuvants and delivery systems [4]. Indeed, adjuvants commonly used in injected vaccines fail to induce sIgA and therefore are not efficient with mucosal antigens, while the lack of appropriate delivery systems does not prevent antigen degradation by enzymes present in the mucosal tissues [3].

Major efforts have been devoted to the development of new mucosal vaccination strategies based on adjuvants able to induce sIgA or on novel delivery systems based on synthetic nanoparticles, viral particles, microbial cells or bacterial spores [5-8].

Increasing interest is also receiving the use of probiotics before and/or during the vaccination period to modulate the immune response [9] and increase the effectiveness of vaccines against bacterial [9, 10] or viral [11, 12] infections. In a recent study, spores of *Bacillus toyonensis* were shown able to increase the immune response to a parenteral vaccine against bovine herpesvirus type 5 (BoHV-5) in mice [13]. *B. toyonensis*, originally defined as *B. cereus* var. toyoi and then identified as a new species by genomic analysis [14], has long been used in animal nutrition for swine, poultry, cattle, rabbits and aquaculture. In 1994 its use has been authorized by the European Community as a feed additive for use in poultry, cattle and rabbits [15]. Animals parenterally immunized with BoHV-5 and orally supplemented with *B. toyonensis* spores had higher serum IgG, IL-4 and IL-12 levels than immunized animals that did not receive the probiotic [13], suggesting this probiotic treatment as a potential alternative to the use of adjuvants.

Aim of this work was to investigate whether the oral treatment with spores of *B. toyonensis* was also effective in inducing the production of specific sIgA thus improving the immune response induced by a mucosal antigen. As a model antigen the C fragment of the tetanus toxin (TTFC), the protective antigen used in evaluations of vaccines against tetanus, was selected [16]. TTFC administered by the oral or nasal route was shown to induce a protective immune response in mice when delivered by *B. subtilis* spores either as a fusion protein exposed on the spore surface [17-19] or as a pure protein adsorbed on the spore surface [20]. The use of *B. subtilis* spores as a mucosal delivery system has been exploited in recent years and tested with several antigens and enzymes [6, 21, 22]. In addition to TTFC, the binding subunit of the heat-labile toxin (LTB) of *Escherichia coli* [23, 24], the protective antigen (PA) of *B. anthracis* [20], the C terminus of toxin A of *Clostridium difficile* [25], the capsid proteins VP26 and VP28 of the White Spot Syndrome virus [26, 27] and the MPT64 antigen of *Mycobacterium tuberculosis* [28] are examples of antigens displayed by *B. subtilis* spores and tested as mucosal vaccines.

Results and Discussion

Spore adsorption of the C fragment of the tetanus toxin (TTFC)

Aliquots (2.0 µg) of TTFC, over-expressed in *E. coli* and purified by affinity chromatography columns (Methods), were incubated in 200 µl of 50 mM sodium citrate buffer at pH 4.0 with 2.0 × 10^9 spores of the *B. subtilis* strain PY79 [29], purified as previously described [30]. After one hour of incubation at 25 °C spores were collected by centrifugation and surface proteins extracted by SDS-DTT treatment [31]. Proteins were then analyzed by Western blotting with anti-TTFC antibody [17] and, as previously reported [20], TTFC was found among the proteins extracted from the spore surface (Fig. 1A). To assess the stability of spore-TTFC interaction, spores adsorbed with TTFC were re-suspended in 200 µl of 50 mM sodium citrate buffer at pH 4.0 and stored one week at 4 °C. Upon centrifugation, spores were used to extract surface proteins as described above while the supernatant was five-fold concentrated by ultra-filtration (3 kDa cut-off) and analyzed by Western blotting. As shown in Figure 1A, TTFC was still

extracted from one-week-old spores (lane 3) and was not present in the supernatant (lane 4), indicating that TTFC was not degraded and or released during the storage at 4 °C.

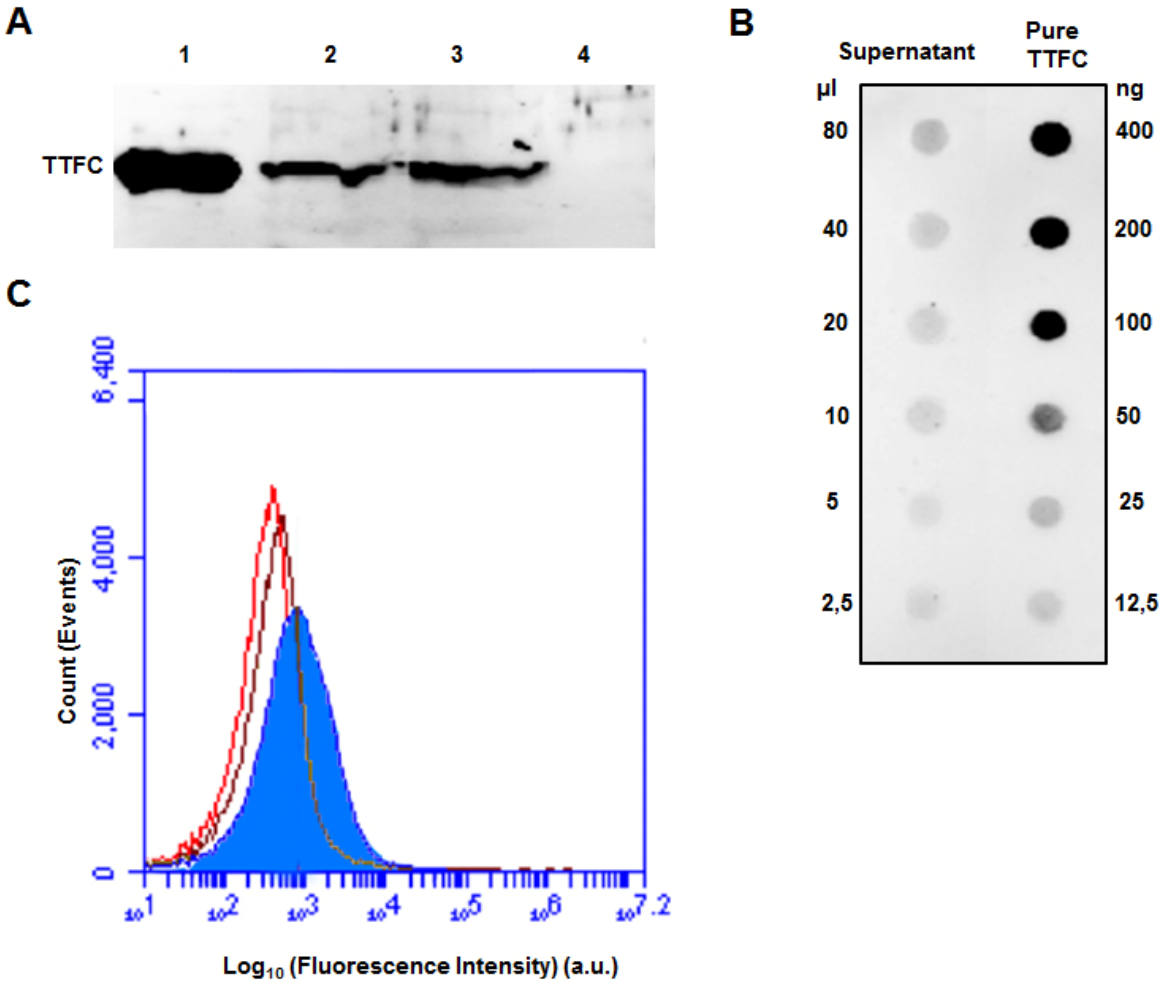

Figure 1. TTFC adsorption on *B. subtilis* spores. (A) Western blotting of spore surface proteins after adsorption with 2.0 µg of purified TTFC. Lanes 1: purified TTFC; 2: proteins extracted from adsorbed spores; 3: proteins extracted from adsorbed spores after one week storage at 4 °C; 4: five-fold concentrated supernatant after one week storage at 4 °C. (B) Dot blotting experiment performed with the serial dilutions of the supernatant (unbound TTFC) fraction of the adsorption reaction. Serial dilutions of purified TTFC were used as a standard. (C) Flow cytometric analysis of: free spores incubated (brown histogram) or not (red histogram) with specific antibodies and TTFC-adsorbed spores incubated with specific antibodies (filled blue histogram). The analysis was performed on the entire spore population

(ungated). Immune-reactions were performed with polyclonal anti-TTFC [17] and anti-rabbit HRP conjugate (panels A and B) or with FITC-conjugated secondary antibodies (C).

To indirectly quantify the amount of TTFC adsorbed on the spore, the adsorption reaction mixture was fractionated by centrifugation and the supernatant, containing the unbound, free TTFC was analyzed by dot blotting with anti-TTFC antibody (Fig. 1B). The intensity of the various spots was then quantified by a densitometry analysis as previously described [22] and indicated that in our experimental conditions less than 3% of TTFC was left free in the supernatant (Table 1). Such a high efficiency of adsorption was not surprising since previous reports have shown that in similar experimental conditions over 90% of reacted proteins were adsorbed to *B. subtilis* spores [22, 24].

Table 1. Densitometric analysis of dot blot experiments of Fig. 1B with the supernatants of the adsorption reaction with wild type spores.

TTFC source	Amount of sample used	Density (OD/mm^2)	Amount of TTFC (ng)	Amount of TTFC µg in 200 µl (% total)
Purified TTFC	50.00 ng	29.053	NA	NA
	25.00 ng	13.121	NA	NA
	12.50 ng	5.294	NA	NA
Free TTFC (supernatant)	80 µl	16.505	23.4	0.05 (2.9)
	40 µl	8.012	11.8	0.05 (2.9)
	20 µl	4.629	4.5	0.04 (2.2)

A flow cytometric approach was used to evaluate the exposure of TTFC on the spore surface. Spores adsorbed with TTFC were reacted with anti-TTFC specific antibody, then with FITC-conjugated secondary antibody and analyzed by flow cytometry (Fig. 1C). In parallel, free spores incubated or not with antibodies (primary and secondary) were analyzed to take into consideration the unspecific fluorescence of spores (Fig. 1C, brown and red histogram, respectively). These controls, overlaid and used as a reference guide in the measurement of the TTFC-specific fluorescence, indicated that when adsorbed with TTFC the majority of the

spore population (64% of the 100,000 counted spores) were specifically fluorescent and, therefore, displayed the antigen (Supplem. Mat. Fig. S1).

A probiotic treatment increases sIgA production induced by a nasal administration of spore-adsorbed TTFC

In a previous study [20], spore-adsorbed TTFC was administered by the nasal route to mice and shown able to induce an antigen-specific mucosal response. We used the same dosage and administration route used before [20] to assess whether a probiotic treatment with *B. toyonensis* was able to influence the mucosal immune response elicited by spore-adsorbed TTFC. To evaluate the effect of the probiotic also on the immune response induced by the pure antigen, parallel groups of animals were also immunized with 2.0 µg of purified TTFC. Fig. 2 schematically shows the experimental plan: three groups of animals received the oral probiotic treatment (1.0×10^6) spores/gram of food from day -7 to day 35), two groups were immunized with 2.0 µg of purified TTFC by the nasal route on day 0, 14 and 28 (blue arrows in Fig. 2) and two groups received 2.0×10^9 spores adsorbed with TTFC by the nasal route on day 0, 14 and 28 (red arrows in Fig. 2). A naive group that did not receive either probiotics or the antigen was also included. Blood samples were collected from all animals at days 14 and 21 and at day 35 all animals were sacrificed for analysis. As calculated in the previous paragraph, 2.0×10^9 spores adsorbed with 2.0 µg of TTFC displayed about 1.9 µg of TTFC (over 90% of the total TTFC), therefore, three doses of spores delivered a total 5.7 µg of TTFC, slightly less than the amount of antigen received by the animals immunized with the purified antigen (6 µg).

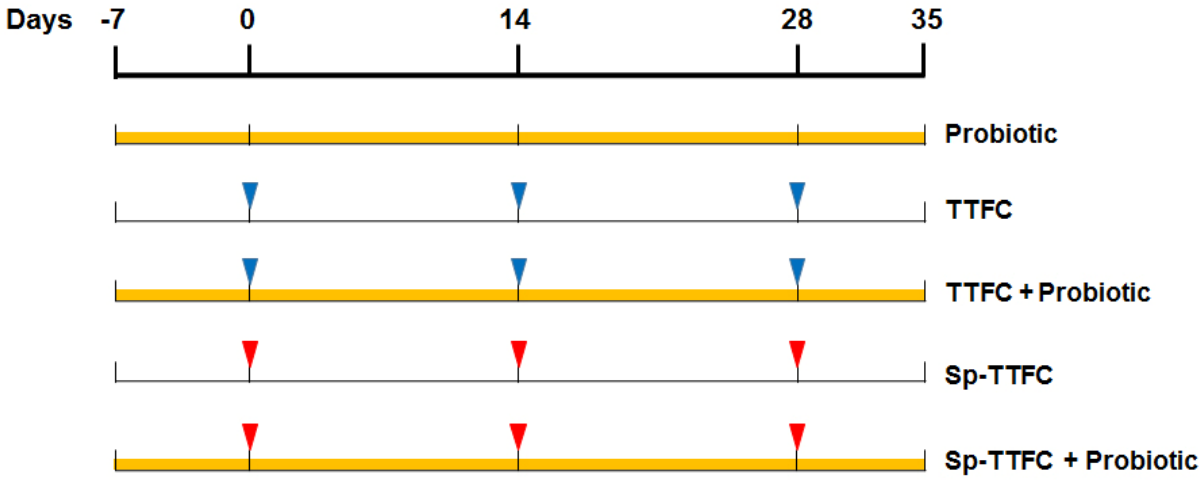

Figure 2. Experimental plan. We used 40 mice that were divided in 6 groups named Naïve (n=4), Probiotic (n=4), TTFC (n=8), TTFC+Probiotic (n=8), Sp-TTFC (n=8), and Sp-TTFC+Probiotic (n=8). Three experimental groups received the oral probiotic treatment (yellow lines) with 1.0×10^6 spores/ gram from day -7 to day 35. One of these groups was immunized with 2.0 µg of purified TTFC (blue arrows) and another one with 2.0×10^9 spores adsorbed with TTFC (Sp-TTFC) (red arrows) on day 0, 14 and 28. Two groups were immunized only with purified TTFC or Sp-TTFC without probiotic treatment. All immunizations were performed by the nasal route. A naive group that did not receive either probiotics or the antigen was also included. Blood samples were collected from all animals at days 0, 14 and 21 and 35, at day 35 all animals were sacrificed for analysis.

High anti-TTFC fecal sIgA levels, indicative of a mucosal immune response, were induced by spore-adsorbed TTFC in animals treated with the probiotic (Fig. 3A). The response was maximal after 14 days and slightly decreased at days 21 and 35. As expected, the free antigen did not induce high levels of sIgA and the treatment with the probiotic cause only a minimal increase (Fig. 3A). The analysis of serum antibodies showed a positive effect of the probiotic on the immune response induced by spore-adsorbed TTFC at day 14 (Fig. 3B). A days 21 and 35 similar levels of IgG were induced by spore-adsorbed TTFC with or without the probiotic treatment (Fig. 3B). Low levels of TTFC-specific IgG were induced by the purified antigen after 14 days, those levels were slightly increased after 21 and 35 days and were not affected by the treatment with the probiotic (white and light grey bars in Fig. 3B, respectively). The ability

of nasally administered spore-adsorbed TTFC to induce a stronger immune response than purified TTFC, at days 21 and 35 (compare white and dark grey bars in Fig. 3B), could be due to an increased antigen uptake by immune cells or, alternatively, to a reduced antigen degradation, as previously suggested for another antigen [24]. Additional experiments are required to fully address this issue. For the aim of this work, it is noteworthy that the probiotic increased the mucosal (sIgA) immune response and accelerated the production of serum IgG induced to spore-adsorbed TTFC.

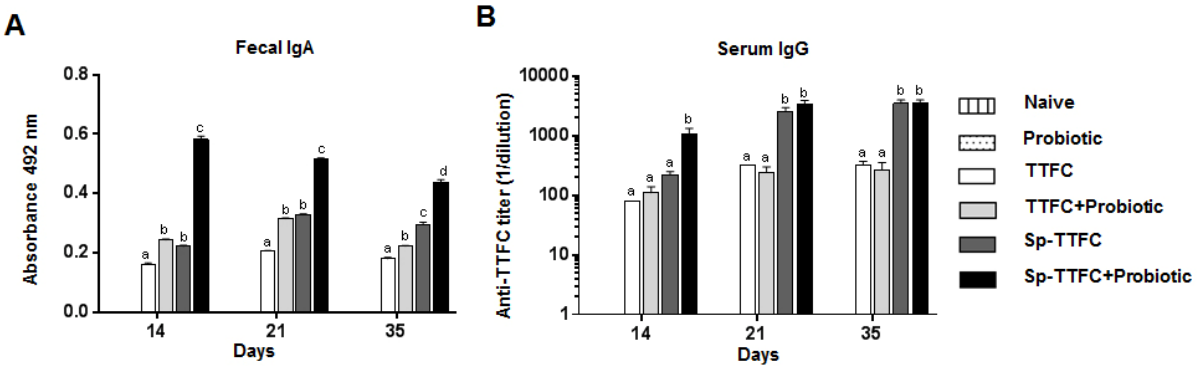

Figure 3. Antibody production. (A) Anti-TTFC specific fecal sIgA detected on days 14, 21 and 35. Data were expressed as the mean (± standard error) of absorbance values at 492nm. (B) Anti-TTFC specific serum IgG detected on days 14, 21, and 35. The data represent the mean (± standard error) of reciprocal endpoint titers. Equal letters mean no statistical difference ($P > 0.05$) and different letters mean a statistical difference ($P < 0.05$) between the experimental groups.

The phenotype of the induced humoral immune response was then examined analyzing IgG subclasses. High levels of IgG1, IgG2b, IgG2c or IgG3 subtypes were induced at all time-points in animals immunized with Sp-TTFC, independently from the probiotic treatment (Fig. 4). Only at day 14 IgG2c was higher in probiotic-treated animals than in those that did not receive B. toyonensis (Fig. 4B). Since in mice, the IgG1 isotype is associated with a Th2 response, whereas IgG2c (analogous to IgG2a in other mouse strains) and IgG2b sometimes associated with IgG3 reflect a Th1 response [32, 33], results of Fig. 4 suggest the induction of

potent and mixed Th1/Th2-type immune responses elicited by spore-adsorbed TTFC independently of the probiotic treatment.

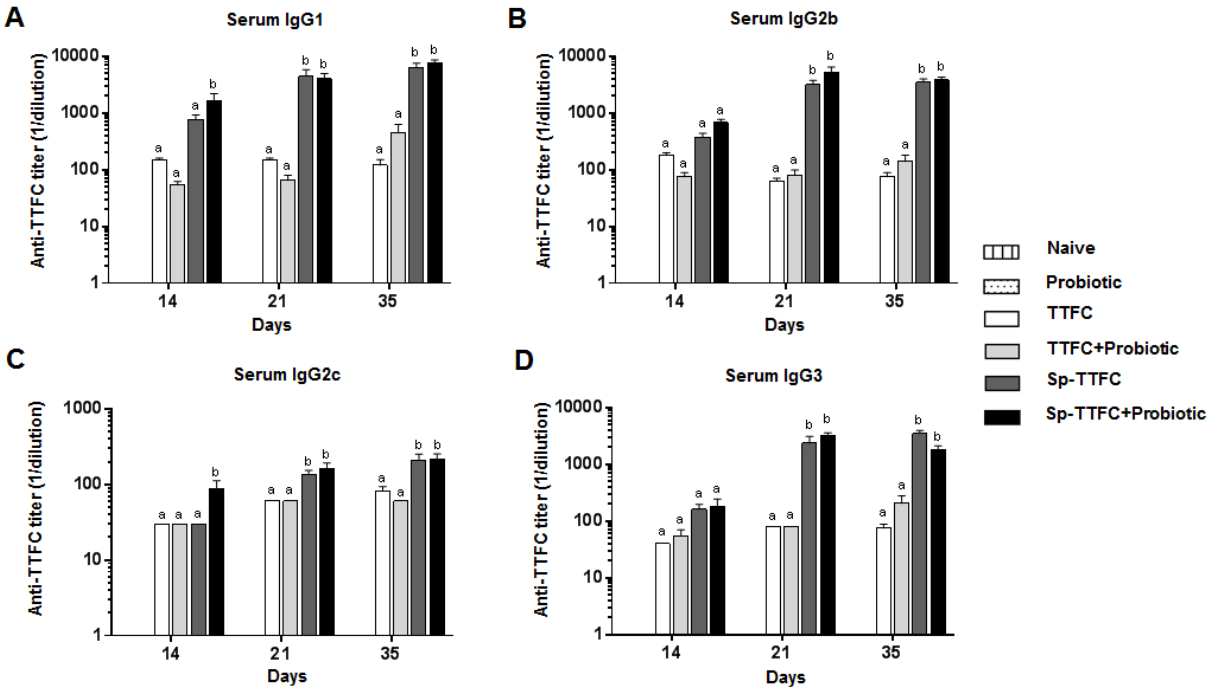

Figure 4. IgG subclasses analysis. The phenotype of the induced humoral immune response. Anti-TTFC IgG1 (A), IgG2b (B), IgG2c (C), and IgG3 (D) levels detected in mice serum on days 14, 21, and 35. The data represent the mean (± standard error) of reciprocal endpoint titers. Equal letters mean no statistical difference ($P > 0.05$) and different letters mean a statistical difference ($P < 0.05$) between the experimental groups.

Altogether, the results of Fig. 3 and 4, indicate that the treatment with *B. toyonensis* spores increases fecal sIgA production in animals nasally immunized with TTFC carried by *B. subtilis* spores while does not affect the level and the phenotype of the serum IgG response.

A probiotic treatment increases the cellular immune response elicited by a nasal administration of spore-adsorbed TTFC

The spleen of all vaccinated animals was analyzed for TTFC-specific production of cytokines IL-4, IL-6, IL-10, IL-12 and IFN-γ. While IL-4 was not produced at detectable levels (not shown),

all other analyzed cytokines were detected in the culture supernatants. High levels of IL-6 were produced by splenocytes from mice that received spore-adsorbed TTFC treated and not treated with the probiotic, however, in probiotic-treated animals IL-6 levels were statistically higher (Fig. 5A). The IL-6 is pro-inflammatory cytokine that plays a central role during the transition from innate to adaptive immunity [34]. Recent studies showed that IL-6 induces the maturation of B cells into antibody-secreting cells and promotes the survival and maintenance of long-lived plasma cells [35].

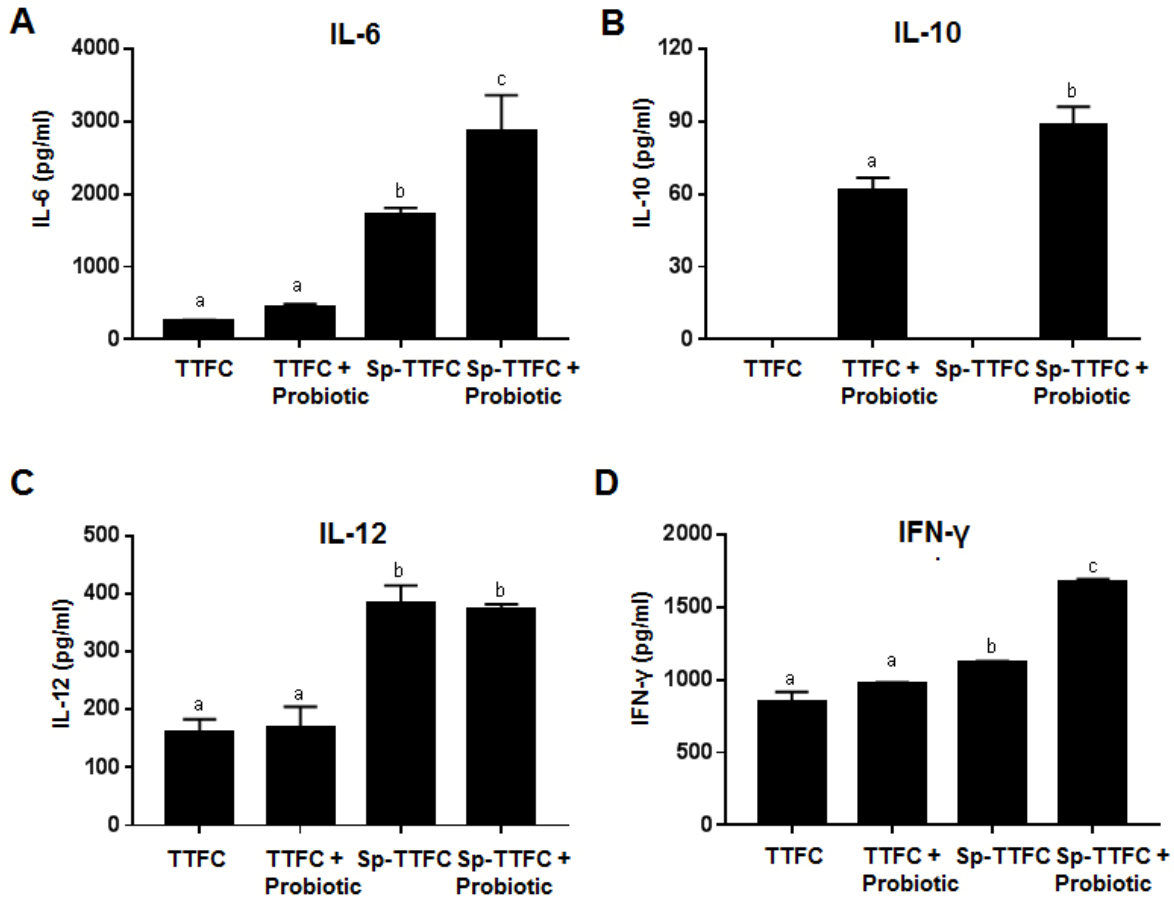

Figure 5. Cytokine production. The cellular immune response elicited by TTFC and spore-adsorbed TTFC treated with probiotic. IL-6 (A), IL-10 (B), IL-12 (C), and IFN-γ (D) levels secreted in vitro from spleen cells. The results were expressed as pg/ml of the mean values (± standard error). Data are reported after subtracting the cytokine values detected in control groups (naive and not immunized mice that received the probiotic). Equal letters mean no statistical difference ($P > 0.05$) and different letters mean a statistical difference ($P < 0.05$) between the experimental groups.

IL-10 was detected only in the spleen of mice immunized with either pure TTFC or spore-bound TTFC that were treated with the probiotic (Fig. 5B). Animals treated with the probiotic but not immunized only showed basal levels of IL-10. Results on IL-10 are consistent with recent reports showing an increase in IL-10 expression in splenocytes of animals supplemented with *B. toyonensis* spores and vaccinated with a parenteral vaccine against bovine herpesvirus type 5 [12, 36]. IL-10 is a cytokine that can be produced by a number of cell types including T cells, B cells and macrophages and acts controlling the intensity of the immune response [37], increasing the survival of B cells, increasing the production of immunoglobulins, and mediating the immune stimulatory effects on T cells [38].

The probiotic treatment did not affect the production of IL-12 that was low in the spleen of mice immunized with TTFC and high in mice immunized with Sp-TTFC, independently from the probiotic treatment (Fig. 5C). Instead, *B. toyonensis* spores were able to increase the IFN-γ levels produced by spleen cells of mice vaccinated with Sp-TTFC (Fig. 5D). IFN-γ directs the differentiation of naïve T lymphocytes into Th1 cells [39], and the induction of a Th1 type of immune response by spores is in agreement with previous reports on spores displaying antigens [20, 24].

Overall, the results of Fig. 5 indicate that the probiotic treatment increases the cellular response to nasally administered TTFC carried by *B. subtilis* spores.

The probiotic treatment did not strongly alter the microbial composition of the animal gut

A 16S DNA-sequencing approach was used to investigate the effect of the probiotic treatment on the gut microbial composition. As reported below, the analysis performed on samples of animals of the control group was in agreement with previous data for mice, with Firmicutes much more abundant than Bacteroidetes [40].

PCoA based on Bray-Curtis distance showed that the gut microbiota of mice of the various groups did not form clear separate clusters, suggesting that the immunizations and/or probiotic treatments did not dramatically alter the microbial composition of the animal gut (Fig. 6). The

OTU representation curves indicated that the microbial diversity of the samples was completely covered while the alpha-diversity analysis showed a higher number of species in two animals of the control (naive) group than in all other groups that did not differ significantly among each other (Suppl. Mat. Fig. S2).

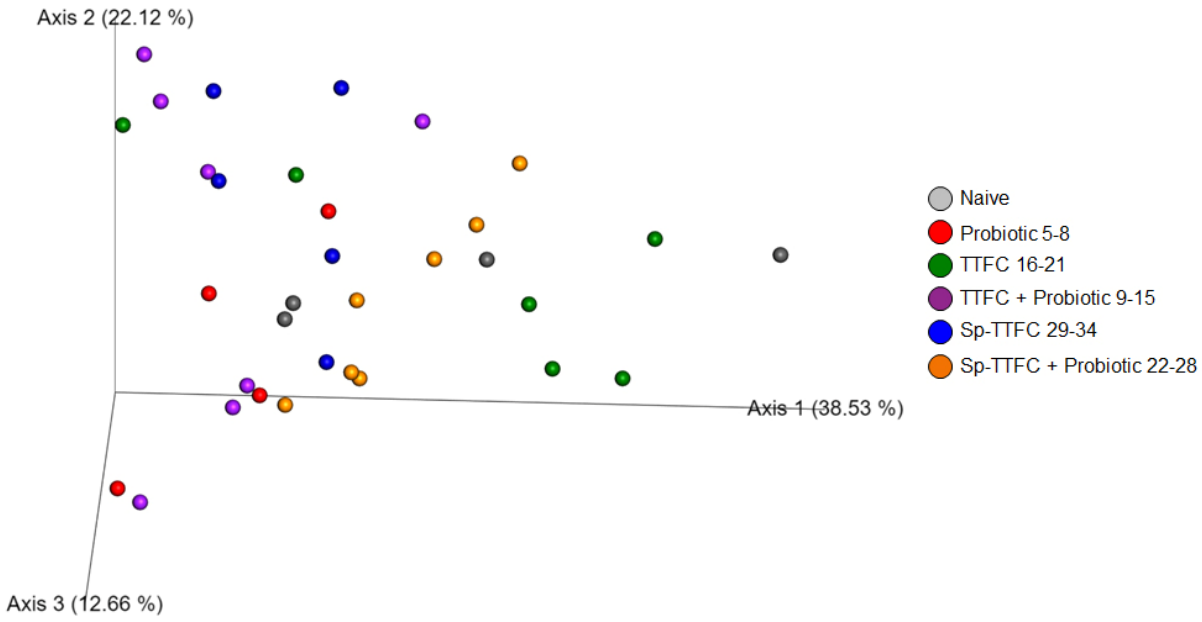

Figure 6. Principal Coordinate Analysis (PCoA). Plots were generated using weighted UniFrac distance matrix.

The analysis of the bacterial composition, reported as the average of the relative abundance of bacterial taxa at the Phylum, Family and Genus level, did not show dramatic differences among the experimental groups. The identified phylotypes showed that Firmicutes were the most abundant bacteria in all groups (54-70%) while Bacteroidetes and Proteobacteria were always less represented (18-33% and 2-14%, respectively) with the latter Phylum that was less represented in all experimental groups with respect to the naive group (Fig. 7).

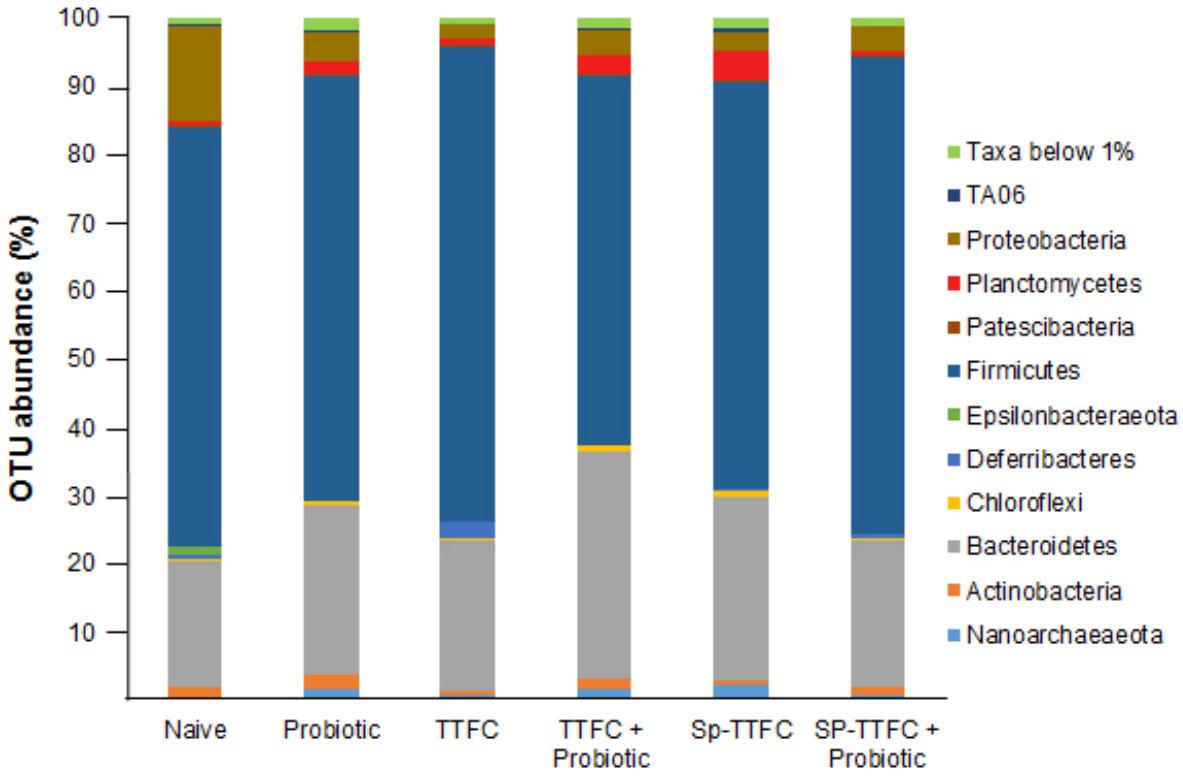

Figure 7. Fecal bacterial composition. Relative Operational Taxonomic Units (OTUs) abundance at the Phylum level in the six experimental groups, reported as mean values within each group. Only Taxa represented by OTUs abundance > 1% have been considered for the analysis.

The analysis at the family and genus level (Suppl. Mat. Fig. S3) was focused on the bacterial taxa of the various groups that showed a statistically significant variation ($P < 0.05$) in their representation with respect to the naive group. By this approach three bacterial genera were found to have a statistically different representation between the probiotic-supplemented and naive groups: *Eubacterium* (Fig. 8A), *Fusobacterium* (Fig. 8B) and *Ruminococcaceae* UCG-014 (Fig. 8C). In addition, also the *Bacillus* genus which includes species used here as the probiotic and the antigen delivery vehicle, was differently represented between probiotic-supplemented and naive groups (Fig. 8D). However, in this case the difference was statistically significant only for two of the three groups (Fig. 8D). Altogether, the results of Fig. 8 indicate that the probiotic treatment did not drastically affect the gut microbial composition but instead altered the abundance of few genera.

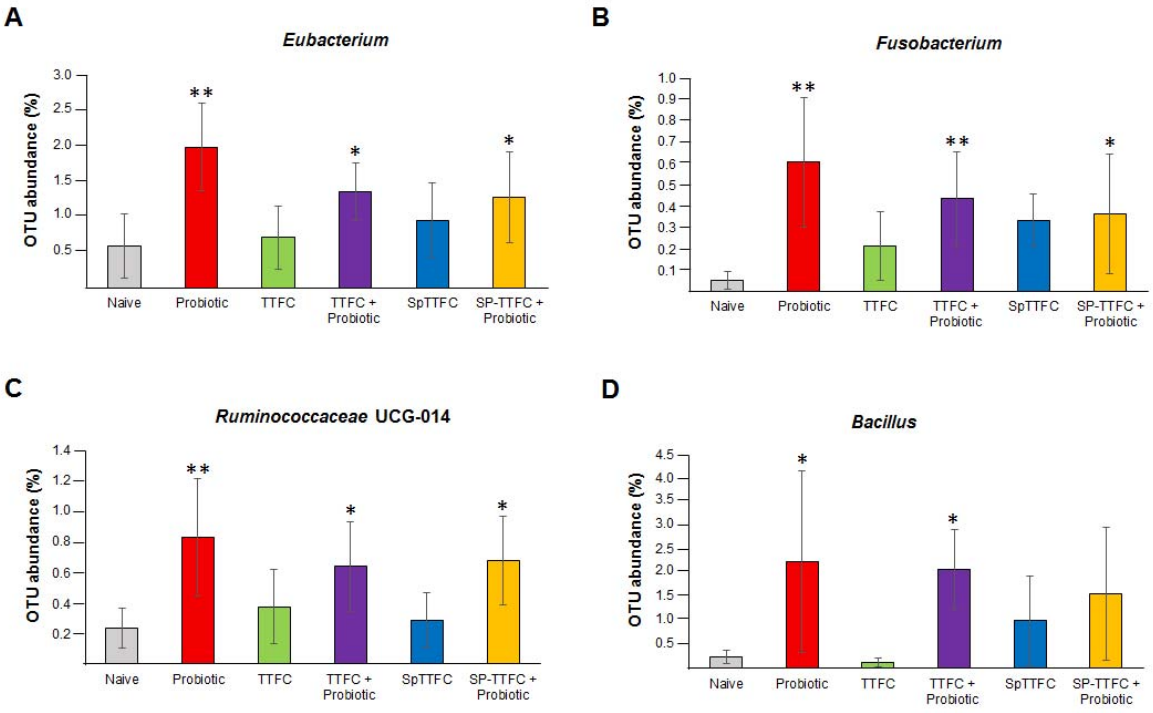

Figure 8. Representativeness of four bacterial genera. The different abundance of four genera between the probiotic treated groups and the control is reported. Statistically significant differences are indicated by asterisks (*P < 0.05; ** P < 0.005).

An additional analysis was performed looking at the statistically relevant differences between genera of the two groups that gave better immune responses (Sp-TTFC and Sp-TTFC+Probiotic) and all other groups. By this approach it was found that members of *Ruminiclostridium* 6 genus were abundant in the gut of animals immunized with spore-displayed TTFC that received the probiotic (Fig. 9). The same genus was also abundant in the gut of animals of the Sp-TTFC group, however, the differences were statistically significant with the naive, probiotic, TTFC groups, and slightly above the threshold (P < 0.05) with the other groups (Fig. 9).

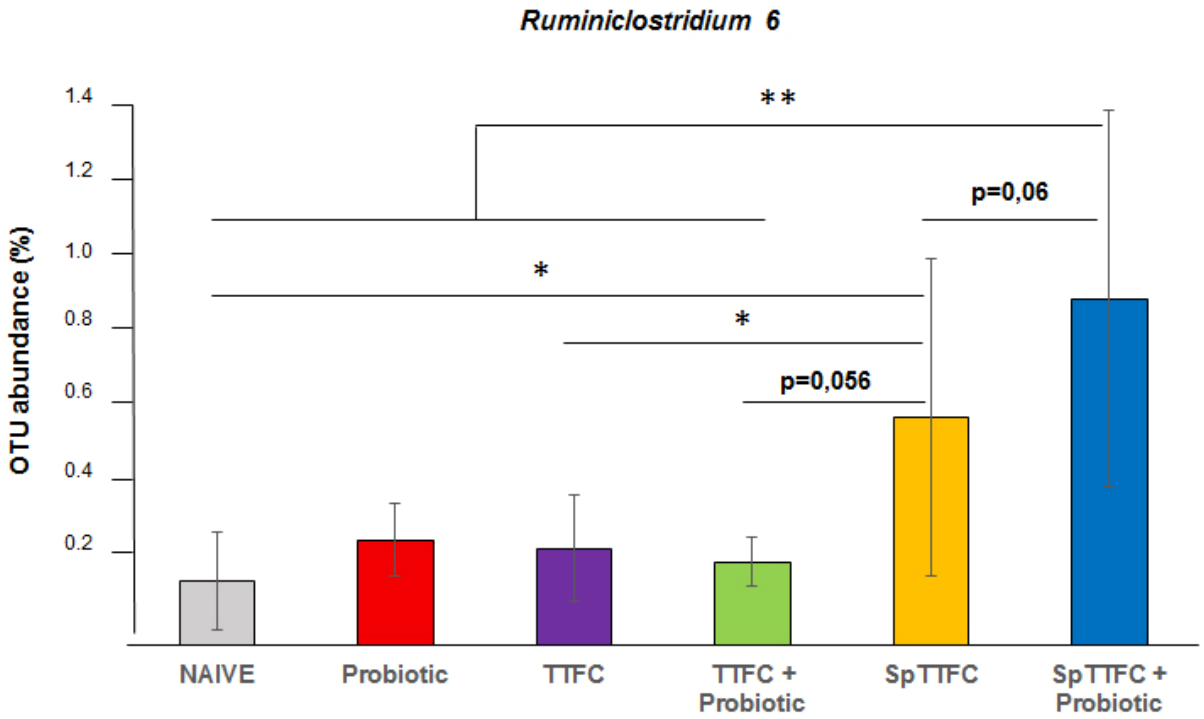

Figure 9. Representativeness of the *Ruminiclostridium* 6 genus. The different abundance of *Ruminiclostridium* 6 between groups immunized with Sp-TTFC and the other groups is reported. Statistically significant differences are indicated by asterisks (*P < 0.05; ** P < 0.005). Differences with P value slightly above the threshold are also shown.

Conclusions

Main conclusion of this manuscript is that a probiotic treatment with *B. toyonensis* spores positively affects a nasal immunization with the C fragment of the tetanus toxin (TTFC) displayed by *B. subtilis* spores. While it was already known that *B. toyonensis* spores increased the immune response to a systemic vaccination [13], their efficacy as adjuvant of a mucosal vaccination was never tested before. The observed increased production of fecal sIgA and of IL-6, IL-10 and IFN-γ in the spleen of immunized animals in response to the probiotic treatment clearly points to the *B. toyonensis* spore as a potential mucosal adjuvant.

B. toyonensis spores also increased the serum IgG production in animals immunized with spore-adsorbed TTFC. However, this effect was only observed at early, day 14, and not at late, day 21 or 35, time points suggesting that the probiotic cause a faster serum IgG response, probably driven by the IgG2c subclass.

The analysis of the gut microbiota did not show dramatic changes in the various experimental groups. Three genera, *Eubacterium*, *Fusobacterium* and *Ruminococcaceae* UCG-014, were found to have statistically significant differences in their representation between the naive group and the groups that received the probiotic treatment. Members of the *Eubacterium* genus belong to the Lachnospiraceae family and are anaerobic, Gram-positive, non-spore-forming rods, previously associated with dietary fiber-induced modulation of the human gut microbiota [41]. Bacteria of the Fusobacterium are obligate anaerobe, Gram-negative rods commonly found as components of the normal flora of the human oropharynx. Some species of the Fusobacterium genus are considered as pathogenic, have been associated with colon cancer or found to increase in response to other infections [42]. The *Ruminococcaceae* UCG-014 genus groups obligate anaerobes belonging to the Ruminococcaceae family, the same family of *Faecalibacterium prausnitzii*, a commensal bacterium of the human gut recently proposed as a probiotic [43]. This analysis then indicates that although the probiotic treatment did not drastically affect the gut microbial composition, it altered the relative abundance of few genera. However, those differences did not correlate with the different immune responses observed.

By comparing the gut microbiota of the two experimental groups that gave better immune responses (Sp-TTFC and Sp-TTFC+Probiotic) vs. all other groups, *Ruminiclostridium* 6 was found statistically more abundant in the Sp-TTFC+Probiotic group. This observation points to a correlation between the abundance of the *Ruminiclostridium* 6 genus and the induction of a strong immune response. Such conclusion is also supported by a recent study [44] in which *Ruminiclostridium* 6 abundance was shown to correlate with high levels of IL-6 in inflamed mice.

Methods

Bacterial Strains, Spore and TTFC Production

The *B. subtilis* strain PY79 [29] was used in this study and sporulation was induced by the exhaustion method [45]. After 30 h of growth in Difco Sporulation (DS) medium at 37 °C with

vigorous shaking, spores were collected, washed three times with distilled water and purified as described before [30]. Spore counts were determined by serial dilution and plating counting. The TTFC (tetanus toxin fragment C) from *C. tetani* was expressed from recombinant plasmid (pET-28b) in the *E. coli* strain BL21 (DE3) (Invitrogen). The plasmid pET-28b-TTFC expressed *C. tetani* TTFC as a 52.6 kDa polypeptide and has been described elsewhere [17]. The expressed protein carried a poly-histidine tag at its 3'-end and following expression was purified using His-Trap column as recommended by the manufacturer (GE Healthcare Life Science).

B. toyonensis BCT-7112^T used in this study was obtained from the collection of microorganisms of the Microbiology Laboratory, Biotechnology Center, Federal University of Pelotas (Brazil). Bacteria were grown in DS medium at 37 °C for 96 h as previously reported [13] and analyzed under the optical microscope for the presence of cells and spores. The cultures containing over 95% of free spores were centrifuged at 5,000×g for 20 min at 4 °C and the pellet suspended in phosphate buffer. Spore counts were determined by serial dilution and plating counting.

Adsorption reaction, Western- and dot-blotting analysis

The adsorption reaction was performed by mixing purified TTFC (2.0 µg) and 2.0 × 10^9 spores in 50mM Sodium Citrate pH 4.0 at 25 °C in a final volume of 200 µl. After 1 hour of incubation, the binding mixture was centrifuged (10 min at 13,000×g) to fractionate pellet and supernatant and stored at 4 °C [31]. The pellet fraction, containing TTFC-adsorbed spores (2.0 × 10^9) was suspended in 20 µl of spore coat extraction buffer [31], incubated at 68 °C for 1 h to solubilize spore coat proteins and loaded onto a 12 % SDS-PAGE gel. The proteins were then electro-transferred to nitrocellulose filters (Amersham Pharmacia Biotech) and used for Western blotting analysis as previously reported [24] using anti-TTFC specific rabbit polyclonal antibodies [17] and Goat Anti-Rabbit (H+L)-HRP Conjugate (Bio-rad). A quantitative determination of the amount of TTFC was obtained by dot blotting experiments analyzing serial dilutions of purified TTFC, and binding assay supernatant. Filters were then visualized by the

ECL-prime (Amersham Pharmacia Biotech) method and subjected to densitometric analysis by Quantity One 1-D Analysis Software (Bio-Rad).

Flow cytometry

A total of 5.0 × 10^5 TTFC-adsorbed spores were blocked with 1xPBS containing 3% of fetal bovine serum for 30 min at 25 °C and subsequently incubated with anti-TTFC specific rabbit polyclonal antibodies diluted at 1:20 for 1 h at 25 °C. After three washes with PBS, fluorescein isothiscyanate (FITC)-conjugated anti-rabbit IgG (1:50 dilution, Invitrogen) was added and incubated for 30 min at 25 °C, followed three washes with PBS. To evaluate the non-specific fluorescence, free spores stained or not with primary and secondary antibodies were analyzed. Samples were then resuspended in 400 µl of PBS and analyzed using by BD AccuriTM C6 Cytometer and BD AccuriTM C6 Software (BD Biosciences, Inc., Milan, Italy) collecting 100,000 events.

Animals, probiotic supplementation and vaccination

Male C57BL/6 mice (Charles River, Italy) 8 weeks old were singularly caged in a temperature-controlled room (23 ± 1 °C) with a 12-h light/dark cycle (6.30 am – 6.30 pm). Treatment, housing, and euthanasia of animals met the guidelines set by the Italian Health Ministry. All experimental procedures were approved by the "Comitato Etico-Scientifico per la Sperimentazione Animale" of the Federico II University of Naples (Italy). We used 40 mice that were divided in 6 groups named Naïve (n=4), Probiotic (n=4), TTFC (n=8), TTFC+Probiotic (n=8), Sp-TTFC (n=8), and Sp-TTFC+ Probiotic (n=8). The Naïve, TTFC, and Sp-TTFC were fed with a commercial feed (Standard chow, Mucedola 4RF21, Italy), free of chemotherapeutic agents; whereas, the Probiotic, TTFC+Probiotic, and Sp-TTFC+ Probiotic groups received the same commercial feed but supplemented with 1.0 × 10^6 spores of *B. toyonensis* per gram of food from 7 days before the first vaccination for diet adaptation.

Mice were vaccinated by the intranasal route on day 0 and received a booster on days 14 and 28 of the experiment. TTFC and TTFC+Probiotic groups were vaccinated with 2.0 µg of purified

TTFC suspended in 50 mM Sodium Citrate buffer. The Sp-TTFC and Sp-TTFC+ Probiotic groups were vaccinated with 2.0 × 10^9 spore-adsorbed with 2.0 µg of TTFC in a volume of 20 µl of 50mM Sodium Citrate buffer. The naïve and probiotic groups were not vaccinated. Blood samples were collected by the submandibular puncture on days 0, 14, 21 and 35. After collection, serum was separated, labelled and stored -20 °C until analysis. Fecal pellets were collected on day 0, 14, 21 and 35 to monitor the induction of the TTFC-specific IgA.

Antibody analysis

Indirect ELISA was performed to evaluated serum levels of total IgG and IgG1, IgG2b, IgG2c, and IgG3 specific against TTFC. Microtitre plates (96 well, Corning) were coated overnight at 4 °C with 0,2 µg of TTFC per well and subsequently washed with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T). Plates were blocked with PBS containing 5% of powder milk. Samples of individual serum samples were serially two-fold diluted starting at 1:2 to 20, 480 and added to the plates in triplicate. After incubation at 37 °C for 1 h, the plates were washed with PBS-T, followed by addition of horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG whole molecule antibodies (1:4,000 dilution, Sigma). Following a further incubation at 37 °C 1 h, the plates were promptly washed again with PBS-T and added developing solution containing 10 ml of substrate buffer, 0.004 g of Ortho-Phenylenediamine (OPD) (Sigma) and 15 µl of H_2O_2 were added, and incubated in the dark at room temperature for 15 min and then stopped by adding 2 N sulphuric acid. Absorbance values were measured in a microplate reader (Thermo Fischer Scientific, MA, USA) with a 492-nm filter. IgG isotype analysis performed according to the instruction manual of the Mouse Monoclonal Antibody Isotyping Reagents kit (Sigma), following the same protocol above describe. For ELISA analysis of fecal IgA, we followed the procedure described by [46], using approximately 0.1 g of fecal pellets that had been suspended in 1% of PBS and 1mM of phenylmethylsulfoyl fluoride (Sigma), incubated at 4 °C overnight, and stored at -20 °C prior to ELISA. The fecal extracts were tested by indirect ELISA for the presence of TTFC-specific IgA using a similar method to

that shown above. Secretory IgA were detected using Goat anti-Mouse IgA alpha chain (HRP) (1:1,000 dilution, Abcam).

Spleen cell cultures and cytokine production

Mice were sacrificed on day 35 and their spleen collected and macerated. Spleen cells (2.0×10^6) were cultured in RPMI 1640 (Gibco) containing 10% foetal bovine serum (Gibco) and antibiotic and antifungal agents (penicillin 10,000 IU ml^{-1}, streptomycin 10 mg ml^{-1} and amphotericin B 25 mg ml^{-1}) (Gibco) in 24-well plates (Corning) and incubated for 24 h at 37 °C in 5% CO_2 atmosphere. Culture medium was replaced after 24 h and the cells were stimulated with 10 µg of TTFC, 10 µg of concanavalin A (ConA; Sigma), and with RPMI 1640, and incubated for 72 h under the same conditions. ConA and RPMI were used as positive and negative control, respectively, for cell stimuli. The supernatants were collected and analyzed for IL-4, IL-6, IL-10, IL-12, and IFN-γ protein levels by ELISA using commercial kits. The assays were performed according to the manufacturers' instructions.

Microbiota identification by 16S rRNA sequencing

Total genomic DNA was extracted from 220 mg of mice fecal samples from all experimental groups using the QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer's instructions.

Partial 16S rRNA gene sequences were amplified from extracted DNA using primer pair Probio_Uni and Probio_Rev, which target the V3 region of the 16S rRNA gene sequence (47). 16S rRNA gene amplification and amplicon checks were carried out as previously described (47). 16S rRNA gene sequencing was performed using a MiSeq (Illumina) at the DNA sequencing facility of GenProbio srl (www.genprobio.com) according to the protocol previously reported (47).

Following sequencing and demultiplexing, the obtained reads of each sample were filtered to remove low quality and polyclonal sequences. All quality-approved, trimmed and filtered data were exported as .fastq files. The .fastq files were processed using a script based on the QIIME

software suite (48). Paired-end reads pairs were assembled to reconstruct the complete Probio_Uni / Probio_Rev amplicons. Quality control retained those sequences with a length between 140 and 400 bp and mean sequence quality score > 20. Sequences with homopolymers > 7 bp and mismatched primers were omitted.

In order to calculate downstream diversity measures (alpha and beta diversity indices, Unifrac analysis), 16S rRNA Operational Taxonomic Units (OTUs) were defined at ≥ 100% sequence homology using DADA2 and OTUs not encompassing at least 2 sequences of the same sample were removed. All reads were classified to the lowest possible taxonomic rank using QIIME2 (48, 49) and the SILVA database v. 132 as reference dataset (50). Biodiversity of the samples (alpha-diversity) was calculated with Chao1 and Shannon indexes. Similarities between samples (beta-diversity) were calculated by weighted uniFrac (51). The range of similarities is calculated between the values 0 and 1. PCoA representations of beta-diversity were performed using QIIME2 (48, 49).

Statistical analysis

The data were analyzed using GraphPad Prism version 7 (San Diego, CA, USA). Differences among the various experimental groups were determined by the one-way or two-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparisons test. The analysis of the fecal microbial composition was performed with SPSS software v. 25 (www.ibm.com/software/it/analytics/spss/). The ANOVA was performed to compare differential abundance of bacterial genera. For multiple comparison the post hoc analysis LSD (least significant difference) was calculated and differences with a P value < 0.05 were considered significant.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

FDSS: performed most of the experiments and contributed to experiment design; AM: contributed to the animal experiments; ARM: contributed to the adsorption reaction, Western and dot-blot analysis; AS: contributed to the treatment of fecal samples and metagenomic analysis; RI: contributed to experiment design and set up of the flow cytometry analysis; FL: contributed to experiment design; SI: contributed to the animal experiments; ER: contributed to experiment design and wrote most of the manuscript; LB: contributed to the metagenomic analysis, experiment design and manuscript writing. All authors read and approved the final manuscript.

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Supplementary Materials

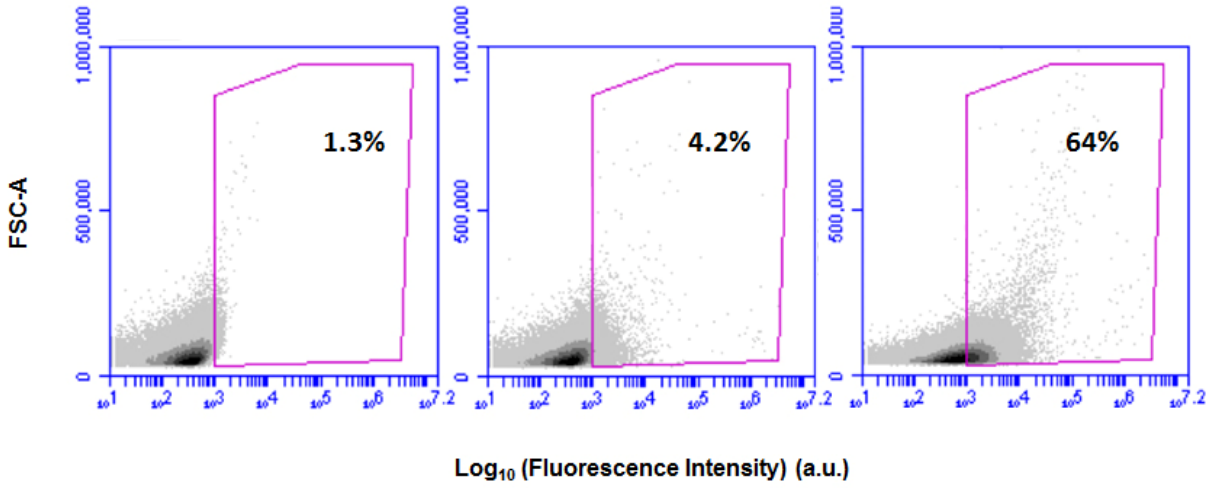

Figure S1. Dot plots of the cytofluorimeter analysis. The dot plots show the forward scatter (FSC-A) vs Fluorescence intensity distribution of free spores without antibodies (Sp), free (Sp (Ab1/Ab2)) and TTFC-adsorbed (Sp-TTFC (Ab1/Ab2)) spores incubated with polyclonal anti-TTFC and FITC-conjugated secondary antibodies. In all cases 100,000 spores were analyzed. Regions boxed in pink contain events (spores) with a fluorescence intensity higher than 1000 a.u.. The percentage of positive events is reported for each graphs.

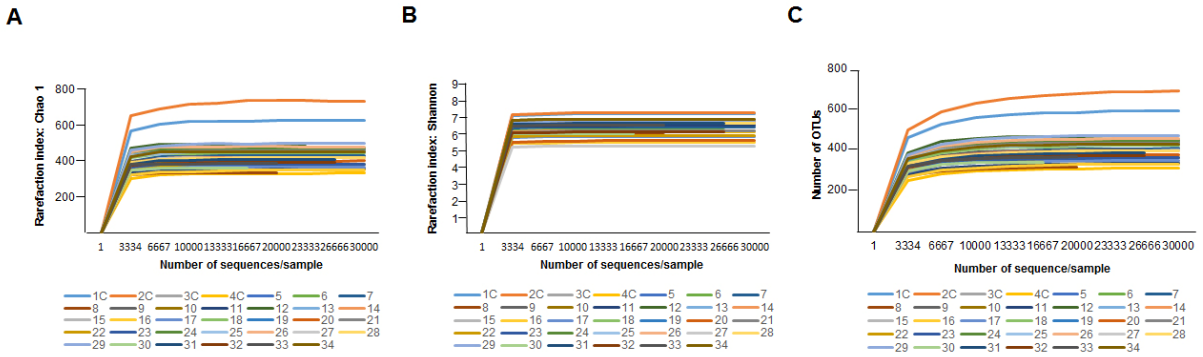

Figure S2. Alpha diversity rarefaction plots. Estimation of the microbial taxa richness and diversity in fecal samples, based on Chao 1 (A) and Shannon (B) indexes. The number of observed OTUs in each sample is also reported (C).

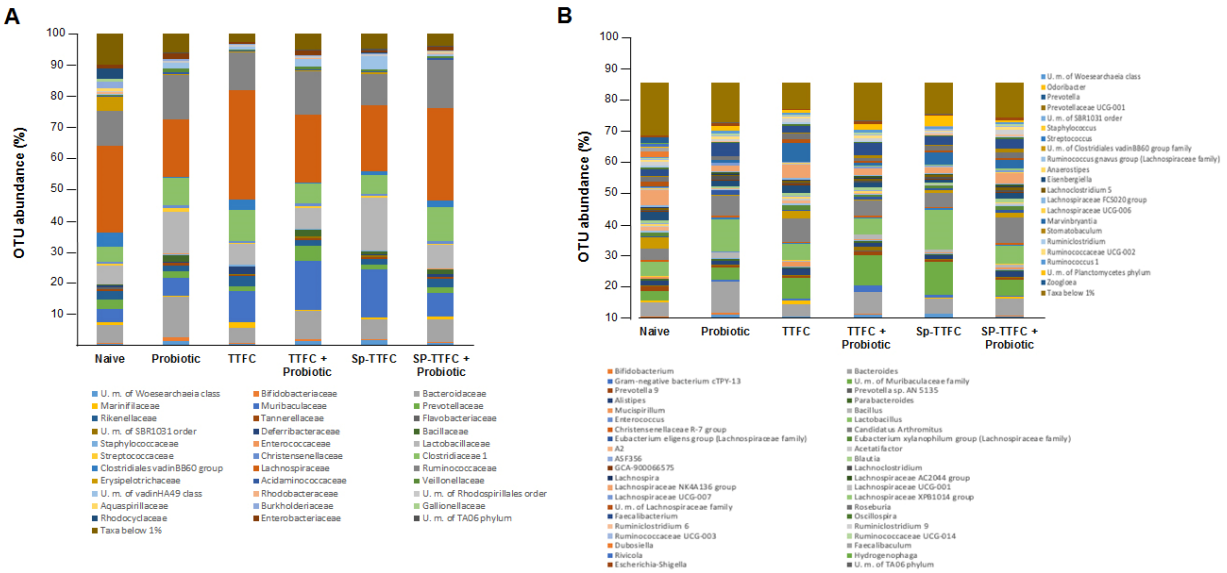

Figure S3. Fecal bacterial composition. Relative Operational Taxonomic Units (OTUs) abundance at the Family (A) and Genus (B) level in the six experimental groups, reported as mean values within each group. Only Taxa represented by OTUs abundance >1% have been considered for the analysis.

4.4 Manuscrito 4 – *Bacillus toyonensis* BCT-7112^{T} transient supplementation improves vaccine efficacy in ewes vaccinated against *Clostridium perfringens* epsilon toxin

Manuscrito submetido à revista *Journal of Applied Microbiology*

Bacillus toyonensis BCT-7112^T transient supplementation improves vaccine efficacy in ewes vaccinated against *Clostridium perfringens* epsilon toxin

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Running headline: Vaccine immunomodulation by *B. toyonensis*

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Abstract

Aim: The aim of the present study was to examine the immune response in ewes to a transient, 5-day supplementation, of the probiotic *Bacillus toyonensis* BCT-7112^T before vaccination against *Clostridium perfringens* epsilon toxin (ETX).

Methods and Results: Ewes were immunized with 200 µg of recombinant ETX adjuvanted with 10% aluminum hydroxide. The treat group was orally supplemented with *B. toyonensis* BCT-7112^T (3×10^8 viable spores) for 5 days prior to the first and second vaccination. Ewes supplemented with *B. toyonensis* BCT-7112^T showed higher neutralizing antibody titers than the non-supplemented ewes ($P < 0.05$), with an increase in serum levels for total IgG anti-ETX

by 3.2-fold ($P < 0.0001$), and for both IgG isotypes IgG1 and IgG2 by 2.1-fold, and 2.3-fold ($P < 0.01$), respectively, compared with the control group. The peripheral blood mononuclear cells of ewes in the supplemented group had a higher ($P < 0.05$) cytokine mRNA transcription levels for IL-2 (6.4-fold increase), IFN-γ (2.9- fold increase) and transcription factor Bcl6 (2.3-fold increase) compared with the control group.

Conclusion: We conclude that a 5 days of supplementation with *B. toyonensis* BCT-7112^{T} prior vaccination is sufficient to significantly improve the immune response of ewes against *C. perfringens* recombinant ETX vaccine.

Significance and Impact of study: These findings open a new perspective in the utilization of *B. toyonensis* BCT-7112^{T} since a short period of supplementation can simplify the probiotic management in livestock, and satisfactorily improve vaccine efficacy.

Keywords: Probiotic, Bacterial Spore, Vaccines, Immunology, Recombinant protein

Introduction

Probiotics are live microorganisms which when administered in adequate amount confer a health benefit on the host (FAO/WHO, 2002; Hill *et al.* 2014). The application of probiotics in ruminant nutrition has been confirmed to improve animal health, productivity, and immunity. Furthermore, probiotics improved growth performance, through enhancing of rumen microbial ecosystem, nutrient digestibility and feed conversion rate (Abd El-Tawab *et al.* 2016). Previous studies have shown that administration of probiotics to sheep significantly improved milk production and protein content, increased weight gain and feed conversion, and reduced the incidence of gastroenteritis and neonatal death of lambs (Lema *et al.* 2001; Antunovic *et al.* 2005; Kritas *et al.* 2006).

Probiotics were able to modulate the host immune response by increasing antibody production, enhancing macrophage phagocytosis and natural killer cell function, and induce

changes in cytokine expression (Forsythe and Bienenstock, 2010; Habil *et al.* 2011; Shida *et al.* 2011). The mechanism of probiotic-mediated immune response modulation is not entirely understood (Bermudez-Brito *et al.* 2012; Fong *et al.* 2015). In the intestinal mucosa, probiotics are capable of interacting with epithelial cells, T lymphocytes, dendritic cells (DCs) and macrophages (Forsythe and Bienenstock, 2010). These probiotics are processed by the DCs that subsequently activate naïve T lymphocytes. The DCs and T lymphocytes migrate to the mesenteric lymph nodes and enter systemic circulation where they stimulate an immune response distant from the site of their original activation (de Moreno de LeBlank *et al.* 2005; Chieppa *et al.* 2006; Leeber *et al.* 2010).

The probiotic *Bacillus toyonensis* BCT-7112^{T} is a Gram-positive, spore-forming bacterium, previously identified as *Bacillus cereus* var. Toyoi, and is the type strain of the species *Bacillus toyonensis*, a novel species of the *B. cereus* group (Jiménez *et al.* 2013). The strain BCT-7112^{T} was isolated in Japan in 1966 and has been used in animal feeds as a probiotic dietary supplement since 1975 (Gil-Turnes *et al.* 2007; Williams *et al.* 2009; Jiménez *et al.* 2013). *B. toyonensis* BCT-7112^{T} exerts immunomodulatory effect and is capable of enhancing the effectiveness of conventional and recombinant vaccines in sheep, pigs, and mice (Schierack *et al.* 2007; Roos *et al.* 2010; 2012; 2018; Santos *et al.* 2018). The epsilon toxin (ETX) produced by *Clostridium perfringens* is one of the most potent known bacterial toxins (Popoff, 2011). ETX is the causative agent of enterotoxaemia, caused by *C. perfringens* toxinotype D, which is a fatal disease responsible for death in sheep worldwide (Popoff, 2011; Popoff *et al.* 2016). The antibodies produced by *C. perfringens* render vaccination against its toxins as a prophylactic measure (Popoff *et al.* 2016; Gonçalves *et al.* 2009). Recombinant vaccines against ETX have emerged as alternatives to conventional toxoids (formaldehyde-inactivated toxins) because they offer advantages in terms of both efficacy and process safety. Moreover, these vaccines are

capable of inducing protective immunity against ETX in livestock (Moreira *et al.* 2016; Ferreira, *et al.* 2018).

Several studies have shown that long-term consumption of probiotics is required to exert their full beneficial effects (Vaughan *et al.* 1999; Galdeano *et al.* 2007; de Moreno de LeBlanc *et al.* 2008). However, a few studies have assessed the relationship between supplementation period and probiotic-mediated immunomodulation. Previous studies have demonstrated that sheep that received probiotics continually showed a better immune response after vaccination (Roos *et al.* 2010; 2018). Santos et al. (2018) showed that supplementation with *B. toyonensis* BCT-7112^T for 7 days prior to the first vaccination could improve the immune response in mice immunized with a recombinant vaccine by inducing both antibody and cell-mediated immune responses, suggesting that the probiotic was capable of modulating the immune response even when not continually administered. Therefore, the aim of the study was to assess the effect of transient supplementation with *B. toyonensis* BCT-7112^T for 5 days prior to the vaccination (first and second dose) on the immune response of a recombinant vaccine against *C. perfringens* ETX in ewes. Our results suggest that the transient *B. toyonensis* BCT-7112^T supplementation modulate and potentiate the vaccine immune response against *C. perfringens* ETX in ewes.

Materials and methods

Probiotic

The probiotic *B. toyonensis* BCT-7112^T was obtained from the collection of microorganisms of the Microbiology Laboratory, Biotechnology Center, Federal University of Pelotas (UFPel). The culturing of *B. toyonensis* BCT-7112^T was performed according to Santos et al. (2018). Briefly, bacteria were seeded on brain heart infusion agar (BHI; Neogen, Lasing, MI, USA) and the plates were incubated at 37 ºC for 24 h. Individual colonies (3–5 colonies) were picked and inoculated into BHI broth (Neogen, Lasing, MI, USA) and kept in an orbital

shaker at 200 rpm for 16 to 18 h. The culture served as inoculum for propagation in a bioreactor (BIOSTAT® B; Braun Biotech International, Melsungen, Germany) containing 8 l of Nutrient Yeast Extract Salt medium (NYSM; 0.5% (w/v) meat peptone, 0.5% (w/v) meat extract, 0.1% (w/v) yeast extract, 0.5 mmol l^{-1} KH_2PO_4, 0.8 mmol l^{-1} $MgSO_4$, 0.06 mmol l^{-1} $MnSO_4$, 0.06 mmol l^{-1} $ZnSO_4$, 0.06 mmol l^{-1} $FeSO_4$, 0.01 mmol l^{-1} $CaCO_3$). Fermentation conditions were controlled and the air supply was maintained between 0.5 and 1.5 (v/v) so that approximately 80% of the dissolved oxygen in the medium was supplied during fermentation. Agitation speed was maintained at 300 rpm and temperature at 37 ºC for 96 h. When 90% of the bacterial cells had sporulated, verified by Wirsten-Colink stain, the culture was centrifuged in a Sorvall centrifuge® RC-6 plus (Langenselbold, Germany) at 5,000 × *g* for 20 min at 4 ºC. The supernatant was removed and the spore pellet were suspended in 1 l of phosphate buffered saline (PBS; 137 mmol l^{-1} sodium chloride, 10 mmol l^{-1} sodium phosphate, 2.7 mmol l^{-1} potassium chloride; pH 7.4). Spore counts were determined by serial dilution and plating counting.

Supplementation and vaccination of ewes

Sixteen adult ewes of the Corriedale sheep breed were divided into two groups, probiotic and control. Each group consisted of 8 animals. The probiotic group received 30 ml of phosphate-buffered saline (PBS) containing 3×10^8 viable spore of *B. toyonensis* orally, once a day for 5 days prior to the first and second vaccination. The control group received only PBS. The ewes were vaccinated subcutaneously with 3 ml recombinant vaccine formulated with *Clostridium perfringens* epsilon toxin recombinant (rETX) (Moreira *et al.* 2016). For vaccine formulation, rETX (200 μg) per dose was mixed with PBS and adsorbed in 10% aluminum hydroxide (alum) (Sigma-Aldrich, St. Louis, MO, USA) as an adjuvant. The animals were vaccinated on day 0 and received a vaccine booster on day 21 of the experiment. The blood

samples were collected from jugular vein puncture on days 0, 21, and 42. The serum samples were separated from the cells by centrifugation and were stored at –20 °C until further analysis.

The animals used in the study were provided by the Animal Teaching Experimentation Center of Palma of the Federal University of Pelotas (UFPel). The animals grazed on natural pastures and were fed on a commercial feed devoid of antimicrobials (Alisul Alimentos S.A., São Leopoldo, RS, Brazil). All protocols were reviewed and approved by the Ethics Committee on Animal Experimentation (CEEA No. 0375) of UFPel. The CEEA of UFPel is accredited by the Brazilian National Council for the Control of Animal Experimentation (CONCEA).

Total IgG, IgG1 and IgG2 isotypes

IgG antibodies were analyzed using indirect enzyme-linked immunosorbent assay (ELISA). Briefly, each well of a 96-well microtiter plate (Corning, Tewksbury, MA, USA) was coated with 100 ng of rETX and kept overnight at 4 °C. The plates were subsequently washed thrice with PBS containing 0.05% Tween-20 (PBS-T). Serum samples (100 µl of serum diluted 1:400 in PBS-T) were added to wells in triplicate, and plates were incubated at 37 °C for 60 min. After incubation, the plates were washed thrice with PBS-T, 100 µl of horseradish peroxidase (HRP)-conjugate goat anti-mouse IgG (1:4000 dilution; Sigma) was added and incubated at 37 °C for 90 min. After incubation, the plates were washed five times with PBS-T and 100 µl of substrate solution was added. The substrate solution contained 97 mmol l^{-1} Na_2HPO_4, 77 mmol l^{-1} $C_6H_8O_7$, 4 mg of ortho-phenylenediamine (OPD; Sigma) and 15 µl of 30% (w/w) H_2O_2. The enzyme reaction was allowed to proceed for 15 min at ambient temperature in the dark and then the reaction was stopped by adding 50 µl of 1 mol l^{-1} H_2SO_4 and the optical density was measured at 492 nm using EZ Read Biochrom 400 microplate reader (Biochrom Ltd., Cambridge, UK). For IgG isotype analysis, the same protocol was followed as described above. IgG1 and IgG2 were detected using mouse anti-sheep IgG1 and IgG2 ovine

(1:2000 dilution; Bio-Rad, Watford, UK). The assay was performed in triplicate. The absorbance value of each serum sample was divided by the absorbance value from that animal's serum from the first collect (day 0). The results were expressed as an IgG fold increase.

Seroneutralization assay

The serum samples collected on day 42 of the experiment were tested for the presence of neutralizing antibodies against rETX using the seroneutralization assay. The procedures were performed in accordance with the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) normative instruction n. 23 (Brasil, 1997). Briefly, 1 ml of standard toxin (NIBSC) was incubated at 37 °C for 1 h with 1 ml of each treatment group pooled serum in serial dilutions from 1:1 to 1:32. Then, ten Swiss Webster mice weighing 18–22 g were intravenously inoculated with 0.2 ml of each sample and subsequently observed for 72 h for survival and then euthanized if necessary. The procedure was repeated with intermediary dilutions of the serum to identify the lower protective dilution. The survival information was used to calculate the IC50 and the results were expressed in international units per ml (IU ml^{-1}) (Reed and Muench, 1938).

Culture of peripheral blood mononuclear cells

On day 42 of the experiment, the blood samples were collected by jugular vein puncture into vacutainer tubes containing 0.38% (v/v) sodium citrate (anticoagulant). The isolation of peripheral blood mononuclear cells (PBMCs) was performed as described by Leite *et al.* (2004). Approximately, 2×10^6 cells were cultured in 1 ml of RPMI 1640 medium (Gibco, Gaithersburg, MD, USA) plus 10% (v/v) fetal bovine serum (Gibco), antibiotic and antifungal (penicillin 10,000 IU ml^{-1}, streptomycin 10 mg ml^{-1} and amphotericin B 25 mg ml^{-1}) (Gibco) in 24-well cell culture plates (Corning). The cells were incubated at 37 °C for 24 h in a humidified atmosphere containing 5% CO_2. After incubation, the culture medium was changed and the

cells were stimulated in quadruplicates with either 10 µg of rETX, or 5 µg of concanavalin A (ConA) (Sigma) or just the cultured medium, and were incubated again for approximately 18 h under the same conditions. ConA and RPMI 1640 medium were used as positive and negative controls, respectively. At the end of the experiment, the supernatant was discarded and the cells were harvested with TRIzol® reagent (Life Technologies, Carlsbad, CA, USA).

RNA extraction, cDNA synthesis, and qPCR

RNA extraction from the PBMCs was performed using TRIzol® (Life Technologies) reagent as per manufacturer's protocol. Approximately, 500 ng of RNA was reverse transcribed using the High capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). A Quantitative polymerase chain reaction (qPCR) was performed on a CFX96TM Real-Time System platform (Bio-Rad, Hercules, CA, USA) using specific primers for amplifying the transcription factor Bcl6 (forward: GTATCCAGTTCACCCGCCAT; reverse: ACATCAGTCAAGATGTCACGGC), interleukin (IL)-2 (forward: CCTCGAGTCCTGCCACAATG; reverse: CCGTAGAGCTTGAAGTAGGTGC), these primers were designed by Vector NTI Software and primer specificity was confirmed by Primer Blast from National Center for Biotechnology Information (NCBI) before commercial synthesis (Sigma). β-actin and IFN-γ primers have been described previously by Puech *et al.* (2015). The reaction efficiency for each primer pair was calculated using a two-fold dilution series on a cDNA sample and the standard curves were represented as the semi-log regression line plot of the C_t value vs. log of the relative input cDNA concentration, conforming described by Bustin et al., 2009. The efficiencies between 97.9% and 106.9% were considered acceptable and primers with efficiencies within these limits were included. β-Actin was used as an endogenous reference gene. The qPCR reactions were performed using 1µl of cDNA (synthesized from 500 ng of RNA), 6.25 µl of GoTaq® qPCR Master Mix (Promega, Madison,

WI, USA), 0.25 mmol l^{-1} of each primer, and 4.25 µl of RNAse-free water (Sigma) in a total volume of 12.5 µl. The temperatures were as follows: denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 60 s and extension at 72 °C for 60 s and a final extension at 72 °C for 5 min. All samples were analyzed in triplicate. The comparative threshold cycle ($\Delta\Delta C_t$) method was used to determine the relative amount of mRNA for each gene with β-actin as the reference gene, according to the method as described by Livac and Schmittgen (2001).

Statistical Analysis

The data were analyzed using GraphPad Prism version 7 (GraphPad Software, La Jolla CA, USA). The mean absorbance values for IgG from each group, obtained by indirect ELISA, were subjected to analysis of variance (two-way ANOVA) followed by the Bonferroni test for significant differences at $P < 0.05$. The mean values of neutralizing antibody titers, obtained by seroneutralization assay, were subjected to Student's *t*-test. The differences in cytokine mRNA transcription were analyzed by one-way ANOVA followed by Student's *t*-test. Data from experimental groups were compared among themselves and with controls.

Results

Humoral immune response against rETX

The ewes in the supplemented *B. toyonensis* BCT-7112^T group and the control group responded to vaccination with increased levels of total IgG anti-rETX. On day 21 of the experiment, the supplemented group showed an approximately 5-fold increase in IgG titer when compared with the control group ($P < 0.0001$). On day 42, a similar trend of higher IgG levels was observed in the supplemented group, it showed a 3.5-fold increase compared with the control group ($P < 0.0001$) (Fig. 1a). The ewes that received *B. toyonensis* BCT-7112^T

supplementation showed higher IgG1 and IgG2 levels compared with non-supplemented ewes. On day 21 and 42, the supplemented group presented approximately 2.1-fold increase in IgG1 levels compared with the control group ($P < 0.001$; $P < 0.01$) (Fig. 1b). The same dynamic was observed in IgG2 levels on day 21 and 42, the supplemented group showed a 2.5-fold increase and 2.2-fold increase, respectively, when compared with the control group ($P < 0.01$; $P < 0.05$) (Fig. 1c).

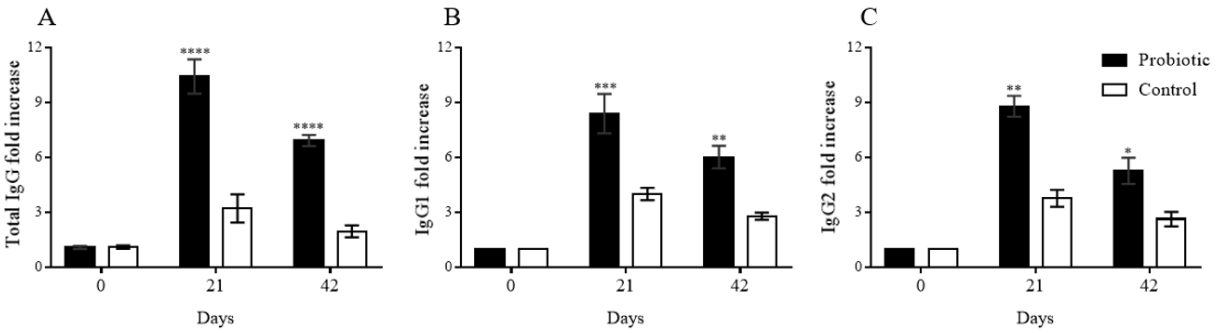

Figure 1 ELISA analysis of sera immunoglobulin IgG levels. The data represent the mean (± standard error) of IgG fold increase in ewes vaccinated with an experimental recombinant vaccine against *C. perfringens* ETX and supplemented with probiotic *B. toyonensis* BCT-7112^T. (A) Total IgG fold increase. (B) IgG1 fold increase. (C) IgG2 fold increase. The statistical analysis was performed by two-way ANOVA followed by the Bonferroni test. Asterisks (*) indicate a significant difference between the probiotic supplemented group and the control group on days 21 and 42. * $P < 0.05$ ** $P < 0.01$; *** $P < 0.001$; and **** $P < 0.0001$.

The sera were collected on day 42 of the experiment, were quantified individually for neutralizing antibodies anti-epsilon toxin. The ewes that received *B. toyonensis* BCT-7112^T supplementation presented significantly higher levels of neutralizing antibodies than the non-supplemented ewes ($P < 0.05$) (Fig. 2).

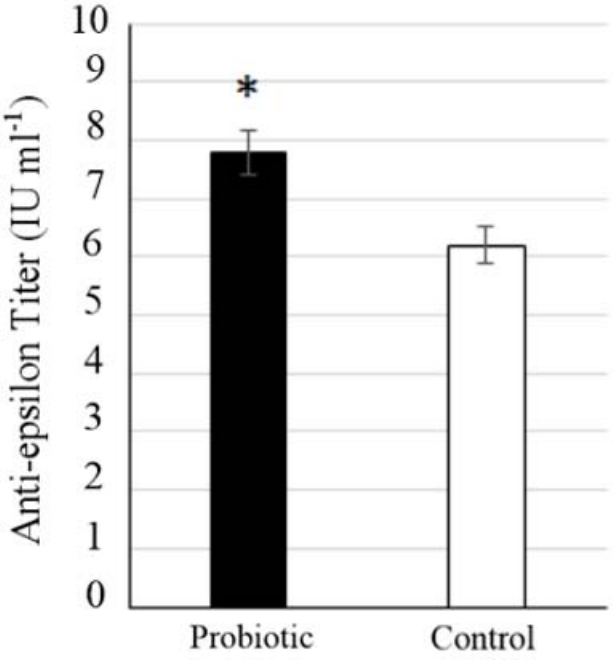

Figure 2 Titers of neutralizing antibodies anti-epsilon toxin in the ewes vaccinated with rETX of *C. perfringens* and supplemented with *B. toyonensis* BCT-7112^{T}. The data represent the mean titers of neutralizing antibodies antitoxins (± standard error) expressed in IU ml^{-1} determined by seroneutralization assay in mice. Asterisk (*) indicate a statistically significant difference ($P < 0.05$) between the probiotic supplemented group and the control group.

IL-2, IFN-γ, and Bcl6 mRNA levels

The PBMCs of ewes in the supplemented *B. toyonensis* BCT-7112^{T} group, subjected to a rETX stimulus, showed a distinct mRNA transcription profile of IL-2, IFN-γ, and Bcl6 compared with the control group (Fig. 3a). The PBMCs of ewes from supplemented group showed a 6.5-fold increase in IL-2 mRNA levels ($P < 0.05$), 3.1-fold increase in IFN-γ ($P < 0.05$), and a 2.4-fold increase in Bcl6 ($P < 0.05$) compared with the control group. When subjected to a ConA stimulus, the PBMCs from the supplemented group presented a similar tendency of a 10-fold increase in IL-2 mRNA levels ($P < 0.05$), 2-fold increase in IFN-γ ($P < 0.05$), and a 4.2-fold increase in Bcl6 ($P < 0.05$) compared with the control group (Fig. 3b).

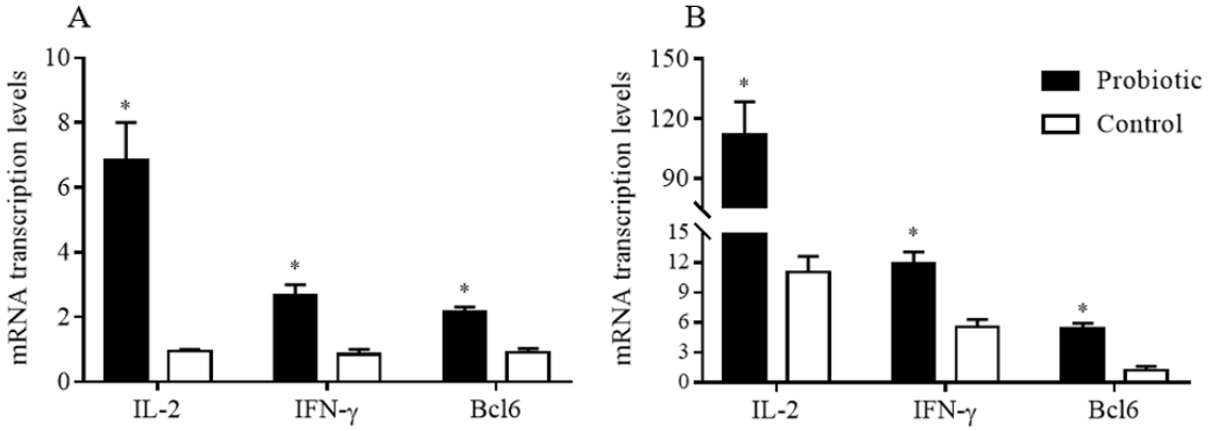

Figure 3 qPCR transcription levels for IL-2, IFN-γ, and Bcl6. Quantitative polymerase chain reaction (qPCR) transcription for IL-2, IFN-γ, and Bcl6 mRNA. The data represent the mean (± standard error) of IL-2, IFN-γ, and Bcl6 mRNA transcription in peripheral blood mononuclear cells (PBMCs) from ewes vaccinated with an experimental recombinant vaccine against *C. perfringens* ETX and supplemented with probiotic *B. toyonensis* BCT-7112^T. The PBMCs were stimulated *in vitro* with recombinant ETX (A) and Concanavalin A (B). The relative mRNA transcription was determined by the comparative threshold cycle ($\Delta\Delta C_t$). The statistical analysis was performed using one-way ANOVA followed by Student's *t*-test. Asterisk (*) indicate a statistically significant difference ($P < 0.05$) between the probiotic supplemented group and the control group.

Discussion

The use of probiotics in small ruminant nutrition has been established to improve animal health, productivity, and immunity (Abd El-Tawab *et al.* 2016). The ability of probiotics to modulate the immune response in animals opens up new prospects for the use of these microorganisms as immunomodulators. Among the strains of *Bacillus* used as animal probiotics, *B. toyonensis* BCT-7112^T is especially suitable because it grows in inexpensive media, has high sporulation rates, resists the temperatures of pelletizing and survives storage under farm conditions (Gil-Turnes *et al.* 2007).

In Brazil alone, there are more than 18 million sheep (Brasil, 2018). The enterotoxaemia, caused by *C. perfringens* ETX, is a major infectious disease, with more than 150 million vaccine doses against clostridiosis are commercialized annually in Brazil (Lobato *et al.* 2010).

Vaccination against ETX represents the best prophylactic measure to inhibit the activity of *C. perfringens* toxins (Popoff *et al.* 2016; Gonçalves *et al.* 2019). After vaccination against a bacterial toxin, as a ETX, the effective immune response is mediated by antibodies capable of neutralizing the toxins and preventing them from binding to the target cell of the host (Rees and Steiner, 2018). The ability of IgG antibodies to diffuse readily through extracellular fluids and their high affinity for the antigen makes them the main antibodies that neutralize toxins in tissues (Brandtzaeg, 2003). The vaccine formulated using a recombinant ETX has been shown to be effective in eliciting levels of neutralizing antibodies higher than required by international standards, as assessed by seroneutralization assay. In addition, the rETX vaccine gave superior results, ~1.5-fold higher, than the commercial vaccine in sheep and ~4.5-fold higher than the minimum recommended by the Brazilian legislation (Moreira *et al.* 2016).

One strategy to evaluate the vaccine efficacy is by quantification of specific antibodies against the antigen after vaccination. These antibody levels may be directly correlated with protection against possible infection and can be used as a reference for determining the influence of probiotics on the vaccine immune response (MacDonald and Bell, 2010). The lambs supplemented with *B. toyonensis* BCT-7112^{T} before vaccination with an inactivated vaccine against bovine herpesvirus 5 (BoHV-5) or *Escherichia coli* F4 demonstrated an increase in antibody titers against both antigens (Roos *et al.* 2010, 2018).

In our study, we observed that ewes vaccinated with a recombinant vaccine against *C. perfringens* ETX and received a transient supplementation with *B. toyonensis* BCT-7112^{T} showed significantly higher IgG, IgG1, and IgG2 levels ($P < 0.0001$; $P < 0.001$; and $P < 0.01$) when compared with non-supplemented ewes. In addition, the ewes from the probiotic group presented a mean titers of neutralizing anti-epsilon antibodies of 7.56 IU ml^{-1}, whereas the Brazilian legislation established the minimum titer of 2.0 IU ml^{-1} (Brasil, 1997). We found that the ewes that received the probiotic showed higher neutralizing antibody titers than the non-

supplemented ewes. It is worth to note the significant ($P < 0.05$) difference of anti-epsilon antibody titers between the probiotic and control group.

While analyzing the levels of IgG isotypes, IgG1 and IgG2, it was observed that ewes in the probiotic group had significantly elevated levels of IgG1 and IgG2. In ruminants as well as human and mouse IgG1 isotype appears to be associated with Th2 response, whereas IgG2 isotype is more related to Th1 response (Estes and Brown, 2002). Our results suggest that the supplementation of *B. toyonensis* BCT-7112^{T} can stimulate a mixed Th1/Th2 immune response. We also found that PBMCs from ewes that received *B. toyonensis* showed significantly higher mRNA expression levels of Th1 cytokines (IFN-γ and IL-2) than the control group. Th1 cells produce IL-2 and IFN-γ to promote IgG2 production (Estes, 1996; Estes and Brown, 2002). The IgG1 and IgG2 isotypes were able to fix complement, IgG2 can initiate the complement activation by the classical pathway in the early phases of an extracellular infection in ruminants (Bastida-Corcuera *et al.* 1999), and has higher opsonizing activity than IgG1 (Mcguire *et al.* 1979; Estes *et al.* 1995).

The probable mechanism by which probiotics modulate immune response is by stimulating cells to produce cytokines that direct progress of the immune response (Habil *et al.* 2011; Shida *et al.* 2011; Fong *et al.* 2015). We observed that the PBMCs from probiotic-supplemented ewes, stimulated with rETX and ConA, showed significantly ($P < 0.05$) higher levels of mRNA for IL-2, IFN-γ, and transcription factor Bcl6 as compared with the control group. In the intestinal mucosa, the probiotics are capable of interacting with the cells of the intestinal epithelium, lymphocytes, DCs, and macrophages (Forsythe and Bienenstock, 2010). DCs interact with and respond to bacterial antigens via pattern recognition receptors such as the Toll-like receptors, which then activate signaling pathways and mediate the innate and/or adaptive immune response (Niess and Reinecker, 2005a, b). The interaction with probiotics leads these cells to initiate the production of cytokines that induce helper T cells that modulate

the type of response (e.g., Th1, Th2, Treg, or Th17) against the activating antigen (Gómez-llorent *et al.* 2010; Lebeer *et al.* 2010).

IL-2 is a key cytokine for the development of adaptive immune response, promoting the proliferation, differentiation, and clonal expansion of T cells (Gaffen and Liu, 2002; Zhou *et al.* 2002). We observed that the PBMCs from probiotic supplemented ewes stimulated with rETX and ConA showed a higher mRNA expression level of IL-2 higher than that of non-supplemented ewes. This significant increase in IL-2 mRNA levels may suggest that the probiotic has the ability to modulate T-cell activation and proliferation. Furthermore, IL-2 in concert with polarizing cytokines facilitates the differentiation of naive Th cells to IFN-γ producing Th1 cells (Boyman and Sprent, 2012). Studies also indicate that IL-2 plays an important role in inducing the generation of memory T cells (Willians *et al.* 2006; Bachmann *et al.* 2007).

We also detected a higher level of IFN-γ mRNA in PBMCs from probiotic supplemented ewes. This finding is very important because the IFN-γ is a cytokine that characterizes a Th1 response and its presence plays an important role in linking innate and adaptive immune responses (Filipe-Santos *et al.* 2006). IFN-γ increases the expression of class I and II MHC proteins on antigen-presenting cells and increases recruitment and activation of lymphocytes in tissues assisting the immune response (Savinov *et al.* 2001; Hill and Sarvetnick, 2002). In addition, the higher IgG2 level detected in ewes that received probiotic suggest that these cytokines might play a role in the vaccine response by stimulating the antibody production. Th1 cells that expressed IL-2 and IFN-γ were able to induce the IgG switch to IgG2 isotype (Estes *et al.* 1996; 2002).

The transcription factor Bcl6 is essential for the development of germinal center B cells and follicular helper T cells (Tfh) (Fazilleau *et al.* 2009). The germinal center is a specialized microstructure formed in the B cell follicles of secondary lymphoid organs for producing long-

term, high-affinity antibody responses (MacLennan, 1994). The Tfh cells are effector CD4 T cells specialized for B cell help function in the selection of B cell clones to produce high-affinity antibody (Crotty, 2011). We observed that PBMCs from ewes supplemented with *B. toyonensis* BCT-7112^{T} had elevated Bcl6 mRNA levels, suggesting that probiotic supplements can activate and regulate Bcl6 expression. Given that Bcl6 is a major transcription factor for Tfh differentiation (Liu *et al.* 2013), an approach for enhancing Tfh differentiation by candidate vaccines would be to modulate the positive and negative signaling pathways to induce Bcl6 expression in CD4 T cells (Choi *et al.* 2013). However, Bcl6 expression is regulated by a complicated signaling pathway that requires further studies to establish the mechanism by which probiotic exert their beneficial effects.

Aluminum salts are commonly used as adjuvants in human and veterinary vaccines (Spickler and Roth, 2003). In our study, aluminum hydroxide (alum) was used as the adjuvant in the formulation of a recombinant vaccine against *C. perfringens* ETX. Alum was traditionally thought to function primarily by forming a long-lasting depot for antigen by promoting their uptake by antigen-presenting cells; however, it is now clear that the innate immune system plays a role in alum adjuvanticity and has also been reported to precipitate and destabilize the phagolysosome leading to the assembly and activation of the NLRP3 inflammasome (Marrack *et al.* 2009; Ghimire *et al.* 2012; Abdrrazak, *et al.* 2015). In mice, alum induces a greatly polarized Th2 cell-dependent antibody isotypes (Germann *et al.* 1995; Brewer *et al.* 1999; Coffman *et al.* 2010). In our study, we observed that probiotic *B. toyonensis* BCT-7112^{T} modulates the vaccine immune response in ewes by increasing IgG2, and IL-2 and IFN-γ mRNA levels. This observation suggests that the probiotic could contribute to the development of a Th1 immune response in ewes. In this context, and as observed earlier in mice that probiotic *B. toyonensis* can modulate the existing Th2 immune responses to promote Th1/IgG2a cell-

mediated immunity, thus reducing the polarizing effect of Th2 responses mediated by alum (Santos *et al.* 2018).

Several studies have demonstrated that probiotics need to be continuously supplied in the diet to exert their beneficial effects (Vaughan *et al.* 1999; Galdeano *et al.* 2007; de Moreno de LeBlanc *et al.* 2008). In our study, we showed that supplementation with *B. toyonensis* BCT-7112^{T} probiotic for five days prior to the first and second dose of vaccine modulates the immune response in ewes vaccinated with rETX vaccine. This is an important finding, considering farm animal management since probiotics did not need to be continuously administered. Several studies have shown that that the immunomodulatory effects of probiotic bacteria occur during the primary vaccine antigen sensitization, and the effect is mediated by stimulating a population of central memory cells that will respond more efficiently upon subsequent encounters with the antigen (Roos *et al.* 2012; Díaz *et al.* 2018).

We conclude that transient supplementation of *B. toyonensis* may significantly improve the immune response against rETX in ewes. Our results suggest that *B. toyonensis* BCT-7112^{T} have an immunomodulatory effect, which opens up new prospects for the use of this probiotic as an alternative to enhance the immune response to vaccines.

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Conflict of interest

The authors report no conflicts of interest.

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4.5 Manuscrito 5 – Immunomodulatory effect of short-term supplementation with *Bacillus toyonensis* BCT-7112^T and *Saccharomyces boulardii* CNCM I-745, in sheep vaccinated with *Clostridium chauvoei*

Manuscrito submetido à revista *Beneficial Microbes*

Immunomodulatory effect of short-term supplementation with *Bacillus toyonensis* BCT-7112^T and *Saccharomyces boulardii* CNCM I-745, in sheep vaccinated with *Clostridium chauvoei*

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Abstract

The bacterium *Clostridium chauvoei* is the causative agent of blackleg in sheep and other ruminants and vaccination is the most effective means of prevention. The current study aims to assess the effect of short-term supplementation with *Bacillus toyonensis* and *Saccharomyces boulardii* on the immune response to *C. chauvoei* vaccine in sheep. Sheep vaccinated with a commercial *C. chauvoei* vaccine and given probiotics orally *B. toyonensis* (3×10^8 cfu), *S. boulardii* (3×10^8 cfu), and a combination of these two microorganisms over five days prior to the first and second doses of the vaccine. Sheep supplemented with *B. toyonensis* and *S. boulardii* showed significantly higher IgG, IgG1, and IgG2 titers ($P<0.05$), when compared with a non-supplemented group. Peripheral blood mononuclear cells from the supplemented group had increased mRNA transcription levels of IFN-γ, IL-2, and Bcl6. Sheep that were given a combination of the two microorganisms displayed no difference in IgG titers and cytokines transcription levels when compared to the control group. These results demonstrate an adjuvant effect of short-term supplementation with *B. toyonensis* and *S. boulardii* on the immune response against *C. chauvoei* vaccine in sheep.

Keywords: probiotics, immunomodulation, clostridial vaccine, antibody, cytokine

Introduction

Probiotics are a range of live microorganisms (e.i. bacteria and fungi), which confer certain health benefits to the host when taken in adequate amounts (FAO/WHO, 2002; Hill *et al.,* 2014). Probiotics have previously been implied to be capable of immunomodulation by instigating proliferation of leukocytes, antibody production, increased phagocyte activity, and changes in cytokine expression (Forsythe and Bienenstock, 2010; Habil *et al.,* 2011; Shida *et al.,* 2011). Although the mechanisms of immune response modulation by probiotics are unclear (Bermudez-Brito *et al.,* 2012; Fong *et al.,* 2015), some studies suggested that the microorganisms are capable of interacting with epithelial cells, T-cells, dendritic cells (DCs), and macrophages in the intestinal mucosa (Forsythe and Bienenstock, 2010). The subsequent processing of these microorganisms in the intestinal mucosa by DCs activates naïve T lymphocytes, which then along with the DCs enter systemic circulation through the mesenteric lymph nodes and illicit a distal immune response away from the activation site (Chieppa *et al.,* 2006; de Moreno de LeBlank *et al.,* 2005; Leeber *et al.,* 2010).

The use of probiotics in ruminant nutrition has been confirmed to improve animal health, productivity, and immunity. Furthermore, these microorganisms improved growth performance, through enhancing of rumen microbial ecosystem, nutrient digestibility and feed conversion rate (Abd El-Tawab *et al.* 2016). Previous studies have shown that administration of probiotics to sheep significantly improved milk production and protein content, increased weight gain and feed conversion, and reduced the incidence of gastroenteritis and neonatal death of lambs (Lema *et al.* 2001; Antunovic *et al.* 2005; Kritas *et al.* 2006). Lambs that received a long-term supplementation with *B. toyonensis and S. boulardii* and were vaccinated with an inactivated vaccine against BoHV–5 or *Escherichia coli* F4, presented an increase in humoral immune response and an increase in titres of antibodies against both antigens. The splenocytes of lambs supplemented with these microorganisms produced higher mRNA transcription levels of cytokines IL-4, IL-10, IL-17, and IFN-γ (Roos *et al.,* 2010; 2018).

One of the most pathogenic *Clostridium* species, *C. chauvoei,* causes considerable losses in livestock production (Uzal *et al.,* 2016) through the manifestation of the blackleg disease, which causes sepsis and high mortality in many ruminant species (Groseth *et al.,* 2011). Oral transmission of the pathogen is most common, although physical transmissions through skin lesions are also possible and the vaccination against the bacterium remains an effective means of controlling blackleg and has been used since the 19th century (Uzal *et al.,*2016). However, the failure of vaccination against blackleg was reported and concerns regarding the efficacy of *C. chauvoei* vaccines in farm ruminants are discussed (Reed and Reynolds, 1977; Uzal *et al.,* 2012). A potential approach for improving vaccine immune response involves modulating the immune system through the use of probiotics in animal feed (Roos *et al.,* 2018; Santos *et al.,* 2018). In this way, the European Food Safety Agency (EFSA) states that vaccination protocols may be employed in order to evaluate the potential role of probiotic microorganisms in improving the humoral immune response against antigen challenges (EFSA, 2011).

Saccharomyces boulardii and *Bacillus toyonensis* are specially interesting in animal production due to their resistance to the stress animal feeds are subjected to and during manipulation and storage (Coppola and Gil-Turnes, 2004; Krehbiel *et al.,* 2003). The bacterium *B. toyonensis* has shown use as an animal feed supplement for the last several decades (Gil-Turnes *et al.,* 2007; Jiménez *et al.,* 2013), as well as exhibited immunomodulatory and adjuvant effects with long-term supplementation, thereby increasing vaccine effectiveness in sheep, pigs and mice (Coppola *et al.,* 2005; Schierack *et al.,* 2007; Roos *et al.,* 2010; 2012; 2018). The yeast *S. boulardii* is widely used both in humans and animals (Zanello *et al.,* 2009). *S. boulardii* exerts its probiotic effects through several mechanisms including competition with pathogens for nutrients, inhibition of pathogen adhesion, and increased immune response to vaccines (Jawhara and Poulain, 2007; Roos *et al.,* 2010; 2018; Zanello *et al.,* 2009).

The current study aims to assess the immunomodulatory effects of short-term supplementation with *B. toyonensis* and *S. boulardii* on *C. chauvoei* vaccine in sheep. The results indicate increased vaccine immune response with a 5-day pre-supplementation with *B. toyonensis* or *S. boulardii* prior to vaccination against *C. chauvoei* vaccine in sheep.

Material and methods

Probiotics

Bacillus toyonensis BCT-7112^{T} and *Saccharomyces boulardii* CNCM I-745 used in the study were sourced from the Microbiology Laboratory, Biotechnology Center, Universidade

Federal de Pelotas (UFPel), Brazil. *B. toyonensis* preparations were made as previously described by Santos et al. (2018); the bacteria were cultured for propagation in a bioreactor (BIOSTAT® B; Braun Biotech International, Melsungen, Germany) containing 3.5 l of Nutrient Yeast Extract Salt medium (NYSM; 0.5% meat peptone, 0.5% meat extract, 0.1% yeast extract, 0.5 mM KH_2PO_4, 0.8 mM $MgSO_4$, 0.06 mM $MnSO_4$, 0.06 mM $ZnSO_4$, 0.06 mM $FeSO_4$, 0.01 mM $CaCO_3$), maintained at 37 °C for 96 h. The resultant culture was spun down in a Sorvall™ RC 6 Plus centrifuge (Thermo Scientific, Langenselbold, Germany) at 5,000×g, for 20 min, at 4 °C and the pellet was resuspended in 500 ml of phosphate buffered saline (PBS; 137 mM sodium chloride, 10 mM sodium phosphate, 2.7 mM potassium chloride; pH 7.4) at a final concentration of approximately 2×10^{10} cfu/ml of *B. toyonensis*. Gram staining and culturing in sheep blood agar were used to ensure the purity of the strains at all stages of culture. *S. boulardii* culture was similarly performed as described in previous reports (Roos *et al*., 2018). Yeasts were cultured in 9 l of Yeast Peptone and Dextrose medium (YPD; BD Difco™, Detroit, MI, USA) in a bioreactor (BIOSTAT® B; Braun Biotech International) maintained at 28 ºC for 72 h. The culture was pelleted down at 4,000×g for 20 min at 4 °C and resuspended in 1 l of PBS. The yield of *S. boulardii* obtained in our experiments was approximately 2×10^9 cfu/ml. Culture purity was ensured at each stage.

Supplementation of the sheep and vaccination

The sheep used in the study, provided by the Palma Animal Teaching Experimentation Center of UFPel, were allowed to graze on natural pasture and fed on an antimicrobial-free commercial feed (Alisul Alimentos S.A., São Leopoldo, RS, Brazil). Thirty-two Corriedale adult ewes were randomly assigned into four groups of eight animals each and labeled *B.t* (*B. toyonensis*), *S.b* (*S. boulardii*), *B.t* + *S.b* (*B. toyonensis* and *S. boulardii*) and control (no supplementation). The *B.t* group was supplemented with 3×10^8 cfu of *B. toyonensis* in 30 mL of PBS, *S.b* group was supplemented with 3×10^8 cfu of *S. boulardii* in 30 mL of PBS, the *B.t* + *S.b* group was supplemented with 3×10^8 cfu viable spores of *B. toyonensis* and 3×10^8 cfu of *S. boulardii* in 30 ml of PBS, while the control group only received PBS supplementation. Probiotic supplementation was done orally once daily with the respective group regimens as described above for five days prior to the first and second vaccination doses. Ewes were vaccinated subcutaneously with 2 ml of a commercial vaccine formulated with inactivated *C. chauvoei* bacterin adsorbed on aluminum hydroxide (adjuvant) (Bio-Vet S/A Laboratory, Brazil). The animals were vaccinated on day 0 and received a booster dose on day 21. The blood samples were collected by jugular vein puncture on days 0, 21, and 42 and stored at −20 °C until analysis.

All study subjects were maintained and handled under veterinary supervision according to protocols reviewed and approved by the institutional Ethics Committee on Animal Experimentation (CEEA No. 0375-2017), with due approval of the UFPel-CEEA from the Brazilian National Council for Animal Experimentation Control (CONCEA).

Culture and preparation of *C. chauvoei* antigen

Antigen from reference *C. chauvoei* strain MT (Brasil, 1997) was prepared according to Crichton *et al.* (1990) with modifications. *C. chauvoei* MT was grown in Reinforced Clostridial Medium (RCM; Oxoid, Basingstoke, UK) in anaerobic conditions at 37 °C for 24 to 48 h to reach a concentration of 4×10^9 cfu/ml. The purity of the culture was confirmed at all stages of the process using Gram staining and culturing in blood agar medium under anaerobic and aerobic conditions. Bacteria were collected by spinning down in a Sorvall™ RCTM centrifuge at 3,000×g for 20 min at 4 °C. The pellet was washed three times in half of

the original volume with PBS and pelleted again. The cells were finally resuspended in 25 mL of sodium phosphate buffer (coating buffer; 50 mM sodium phosphate; pH 8.9), sonicated (1.5 A) for 10 min in a Branson Ultrasonics Sonifier™ SFX150 Cell Disruptor (Fisher Scientific, Hampton, NH, USA) and stored at −20 °C until the analysis.

Dynamics of total serum IgG, IgG1, and IgG2 against *C. chauvoei*

The estimation of total IgG levels was carried out by indirect ELISA following Crichton et al. (1990) with modifications. First, 96-well microplates (Corning®, New York, USA) were coated with 100 µl per well of sonicated *C. chauvoei* antigen suspension diluted in coating buffer and incubated overnight at 4 °C. The next day, plates were washed three times with 200 µl per well of PBS containing 0.05% Tween 20 (PBS-T). Individual serum samples were serially two-fold diluted starting at 1:2 to 1:102,400 and added to the plates in triplicate. After incubation at 37 °C for 60 min, the plates were washed three times with PBS-T, followed by addition of 100 µl of horseradish peroxidase (HRP)-conjugated rabbit anti-sheep IgG whole molecule antibodies (1:10,000 dilution, Sigma-Aldrich, St. Louis, MO, USA). Following a further incubation at 37 °C for 60 min, the plates were promptly washed five times with PBS-T and 100 µl of developing solution containing 10 ml of substrate buffer, 0.004 g of Ortho-Phenylenediamine (OPD) (Sigma-Aldrich) and 15 µl of H_2O_2 were added, and incubated in the dark at room temperature for 15 min. Further, 50 µl of 2N H_2SO_4 was added to each well to stop the reaction. IgG isotype analysis was carried out using the same two-fold serial dilution pattern 1:2 to 1: 102,400 for the serum samples. Mouse anti-sheep IgG1 and IgG2 antibodies (Bio-Rad, Berkeley, CA, USA) were used at 1:2,000 dilution and HRP-conjugated rabbit anti-sheep IgG whole molecule antibodies (Sigma-Aldrich) were used at 1:4,000 dilution. OPD substrate (Sigma-Aldrich) for visualization and 2N H_2SO_4 for reaction termination were used as described above. Absorbance was measured on an EZ Read 400 Microplate Reader (Biochrom, Cambridge, UK) using a 492 nm filter. The results were plotted as $\log_{10}$-transformed mean values against the reciprocals of dilutions, which resulted in values three standard deviations (+3 SD) higher than the absorbance of negative control serum. In order to confirm that by day zero all animals had no antibodies against *Cl. chauvoei*, an ELISA was performed.

Culture of peripheral blood mononuclear cells and RNA extraction

Vacutainer tubes containing 0.38% v/v sodium citrate anticoagulant were used to collect blood samples on Day 42. Peripheral blood mononuclear cells (PBMC) were isolated as previously described (Leite *et al.,* 2004). Approximately 2×10^6 cells were cultured in 24-well plates (Kasvi, China) containing 1 ml of RPMI 1640 medium (Gibco, USA) with 10% fetal bovine serum (FBS; Gibco), 10,000 IU/ml penicillin, 10 mg/ml streptomycin, and 25 mg/ml amphotericin B (Gibco) for 24 h at 37 °C in 5% CO_2 atmosphere. After a change of the medium, PBMCs were activated with a *C. chauvoei* suspension (2×10^7 cfu), 5 µg concanavalin A (ConA) (Sigma-Aldrich) as a positive control, and RPMI 1640 as a negative control, and incubated again for approximately 18 h under the same conditions. The supernatants were then discarded and the cells were suspended in TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA) for RNA extraction following the manufacturer's instructions.

cDNA synthesis and qPCR

Reverse transcription was carried out using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA) using approximately 500 ng of RNA. Relative mRNA expression levels for Bcl6, interleukin (IL)-2, and interferon (IFN)-γ were determined

using quantitative polymerase chain reaction (qPCR) with β-actin and GAPDH used as endogenous reference genes (controls); β-actin was standardized as an internal reference gene based on its efficiency (M-value of 1.8 and 1.98 for GAPDH and β-actin, respectively). qPCR reactions were performed on a Stratagene Mx3005P qPCR system (Agilent Technologies, Santa Clara, USA), as described previously (De Avila *et al.,* 2016). The primers used were as follows: Bcl6 (FOR: GTATCCAGTTCACCCGCCAT; REV: ACATCAGTCAAGATGTCACGGC), IL-2 (FOR: CCTCGAGTCCTGCCACAATG; REV: CCGTAGAGCTTGAAGTAGGTGC). The primers and reaction conditions for β-actin, GAPDH, and IFN-γ were followed as previously described (Puechl *et al.,* 2015). All samples were analyzed in triplicate using the comparative threshold cycle ($\Delta\Delta C_t$) method to determine the relative mRNA expression compared to β-actin as the reference gene, following a previously described method (Livac and Schmittgen, 2001).

Statistical Analysis

The data were analyzed using GraphPad Prism version 7 (USA). The differences in antibody titers between treatment groups were analyzed using $\log_{10}$ transformed titer data. The results were subjected to two-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparisons test. A one-way ANOVA followed by Dunnett's test was used to analyze differences between cytokine mRNA transcription.

Results

Dynamics of total serum IgG, IgG1, and IgG2 antibodies against *C. chauvoei*

All animals of experimental groups had similar antibody levels ($P>0.05$) against *C. chauvoei* on day 0. Total IgG levels were increased in all groups in response to vaccination. Significantly higher IgG titers were observed for *B.t*- and *S.b*-supplemented sheep ($P<0.05$) as compared to the non-supplemented control group. Mean IgG titers as compared to the control group on day 21 of the experiment were as follows: *B.t* group: 4.5 $\log_{10}$ ($P<0.05$); *S.b* group: 4.2 $\log_{10}$ ($P<0.05$); *B.t* + *S.b* group: 3.4 $\log_{10}$ ($P>0.05$); and control group: 3.1 $\log_{10}$. A similar trend of mean IgG titers was observed on day 42: *B.t* group: 3.5 $\log_{10}$ ($P<0.05$); *S.b* group: 3.4 $\log_{10}$ ($P<0.05$); *B.t* + *S.b* group: 3.1 $\log_{10}$ ($P>0.05$); and control group: 2.8 $\log_{10}$ (Figure 1A).

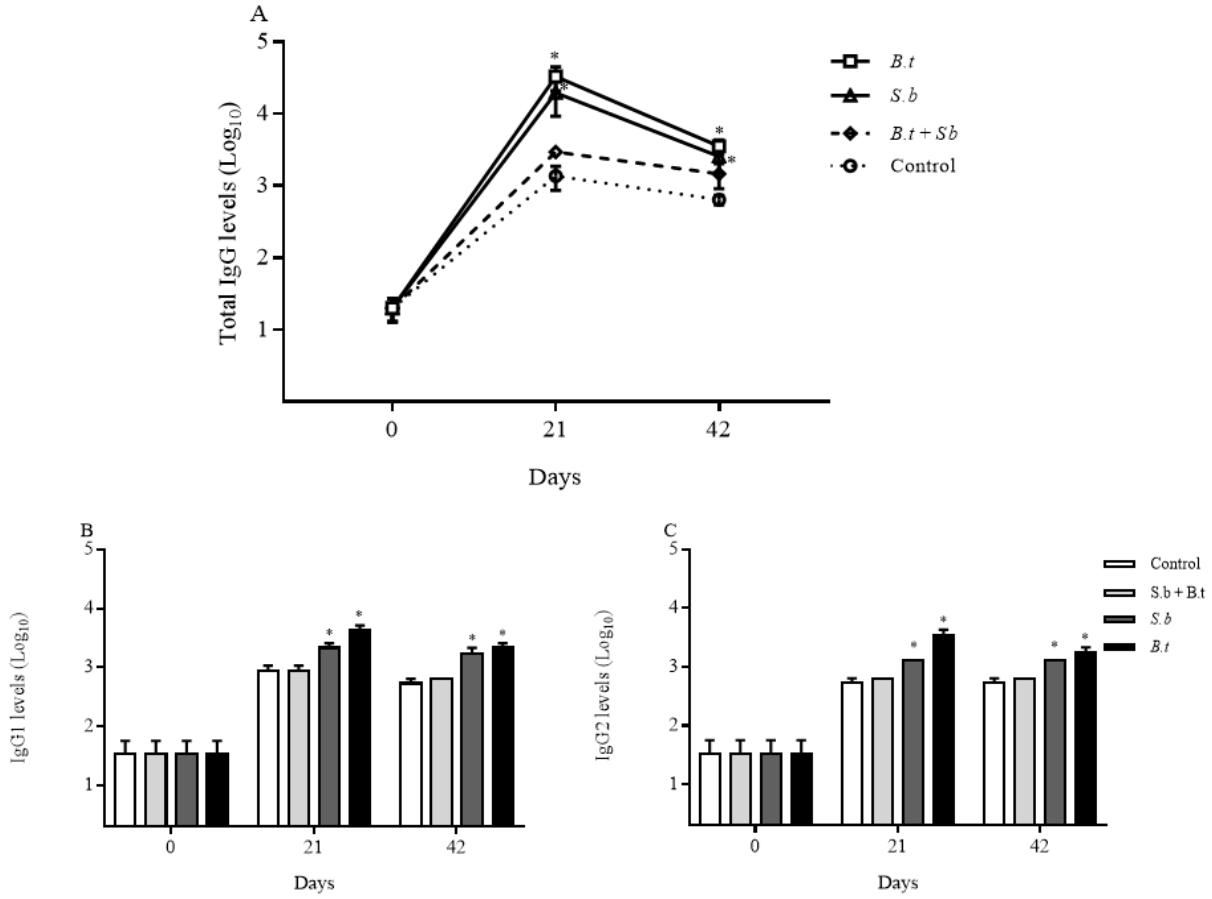

Figure 1. IgG serum dynamics against *Clostridium chauvoei*. Total serum IgG (A), IgG1 (B), and IgG2 (C) levels determined by indirect ELISA of the blood samples collected from the sheep vaccinated with a *C. chauvoei* vaccine and supplemented with probiotics *Bacillus toyonensis* and *Saccharomyces boulardii*. The data represent the mean (±standard error) of Log_{10} transformed data and are expressed as the reciprocal of the highest dilutions. The statistical analysis was performed by two-way ANOVA followed by Turkey's multiple comparisons test. Asterisks (*) indicate a significant difference (P<0.05) between the probiotic-supplemented group (*B.t* and *S.b*) and the control group on the same day.

Higher IgG1 and IgG2 levels (P<0.05) were observed in the *B.t* and *S.b* groups as compared to the control group on the same day. On day 21, the mean IgG1 titers were as follows: *B.t* group: 3.6 log_{10} (P<0.05); *S.b* group: 3.3 log_{10} (P<0.05); and *B.t* + *S.b* group: 2.9 log_{10} (P>0.05) as compared the control group (2.9 log_{10}). A similar trend was observed for the day 42 mean IgG1 titers: *B.t* group: 3.3 log_{10} (P<0.05); *S.b* group: 3.2 log_{10} (P<0.05); *B.t* + *S.b* group: 2.8 log_{10} (P>0.05) as compared to the control group (2.7 log_{10}) (Figure 1B). Significantly higher levels of mean IgG2 titers, as compared to the control group, were also observed, with the *B.t* group having a titer of 3.5 and 3.2 log_{10} (P<0.05) on days 21 and 42, respectively. Interestingly, we observed similar values of mean IgG2 titers on Days 21 and 42 in each of the other groups, with the *S.b* group presenting a titer of 3.1 log_{10} (P<0,05), the *B.t* + *S.b* group presenting a titer of 2.8 log_{10} (P>0.05), as compared to the control group (having a mean IgG2 titer of 2.7 log_{10}) (Figure 1C).

Bcl6, IL-2, and IFN-γ mRNA transcription

PBMCs isolated from sheep in the probiotics-supplemented group showed a distinct mRNA transcription profile for Bcl6, IL-2, and IFN-γ (Figure 2) upon stimulation with *C. chauvoei* antigen and ConA. *B.t* group PBMCs, upon *C. chauvoei* stimulation, showed an

increase of 1.8, 2.3, and 2.6-fold in mRNA transcription (P<0.05) for Bcl6, IL-2, and IFN-γ, respectively. Upon ConA stimulation, this group showed 8, 30, and 141-fold increase in mRNA transcription (P<0.05) for Bcl6, IL-2, and IFN-γ, respectively. PBMCs in *S.b* group upon *C. chauvoei* stimulation presented 1.9 and 2.1-fold increase in mRNA transcription (P<0.05) for Bcl6 and IL-2 and a 1.7-fold increase for IFN-γ (P>0.05) as compared to control. This group, upon ConA stimulation, demonstrated 4.3, 14.5, and 13-fold increases in mRNA transcription (P<0.05) for Bcl6, IL-2, and IFN-γ, respectively. PBMCs from the *B.t + S.b* group sheep exhibited 1.2, 1.4, and 1.2-fold increase in mRNA transcription (P>0.05) for Bcl6, IL-2, and IFN-γ with *C. chauvoei* stimulation, and a similar increase of 2.4 (P<0.05), 1.5 (P>0.05), and 2.1-fold (P<0.05) for Bcl6, IL-2, and IFN-γ was observed with ConA stimulation.

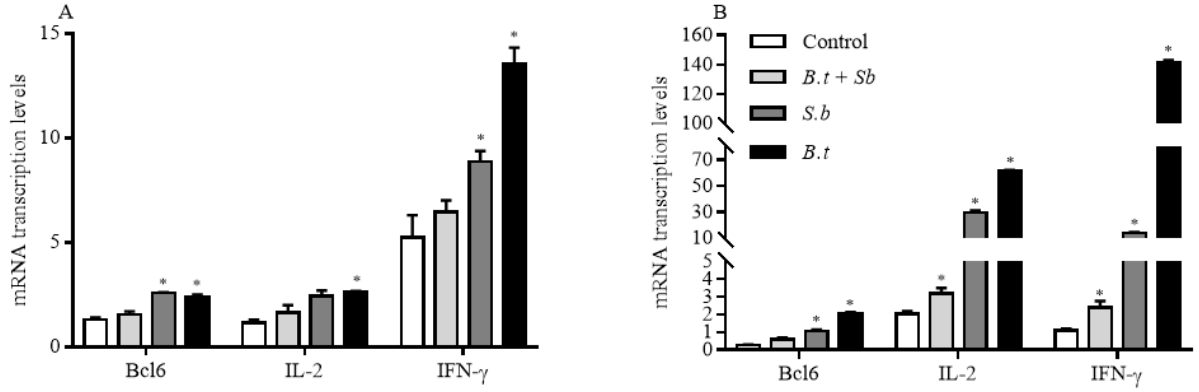

Figure 2. Transcription levels of Bcl6, IL-2, and IFN-γ mRNA determined by the quantitative polymerase chain reaction (qPCR). The data represent the mean (±standard error) of the Bcl6, IL-2, and IFN-γ mRNA relative transcription levels in peripheral blood mononuclear cells (PBMCs) from the sheep vaccinated with a *Clostridium chauvoei* vaccine and supplemented with probiotics *Bacillus toyonensis* and *Saccharomyces boulardii*. The PBMCs were stimulated *in vitro* by *C. chauvoei* (A) and Concanavalin A (B). The relative mRNA transcription was determined by the comparative threshold cycle ($\Delta\Delta C_t$). The statistical analysis was performed using one-way ANOVA followed by Dunnett's test. Asterisks (*) indicate a statistically significant difference (P<0.05) between the probiotic-supplemented groups (*B.t* and *S.b*) and the control group.

Discussion

Probiotics need to be continuously supplied in the diet to exert their beneficial effects (de Moreno de LeBlanc *et al.* 2008; Galdeano *et al.* 2007; Vaughan *et al.* 1999). Antibody response induced by vaccination against clostridiosis can be considered as a protection parameter (Cerviño *et al.,* 2011). ELISA values, has a significant correlation (>90%) with serological response in vaccinated animal (Araujo *et al.,* 2010; Crichton *et al.,* 1990). In this study, we observed that a 5-day supplementation schedule with *B. toyonensis* and *S. boulardii* prior to vaccination in sheep led to a heightened vaccine immune response by increasing total IgG titers with ~100-fold (P<0.05) and ~20-fold (P<0.05), respectively, as compared to non-supplemented sheep (Figure 1A). Also, these probiotic supplementation schedule increased and modulate IgG isotype profile. Sheep that received probiotic had significantly (P<0.05) higher IgG1 and IgG2 titers, comparing with the controls (Figure 1B and C). IgG2 can initiate the classical complement pathway in ruminants, aside from having higher opsonizing activity than IgG1 (Bastida-Corcuera *et al.,* 1999). IFN-γ and IL-2 promote IgG2 production (Estes, 1996; Estes and Brown, 2002), and we observed that PBMCs from probiotics supplemented sheep showed a significant increase in mRNA transcription of IFN-γ, IL-2 and Bcl6 (Figure 2A and B).

One probable mechanism by which probiotics modulate immune response is by stimulating cytokines expression, and by doing so increasing the vaccine effectiveness (Habil *et al.* 2011; Shida *et al.* 2011; Fong *et al.* 2015). The significantly higher ($P<0.05$) levels of IFN-γ mRNA transcription observed in the probiotic-supplemented groups suggest that this cytokine may play a role in modulating vaccine response by the development of a Th1 response and stimulating antibody production. IFN-γ in macrophages induces the expression of Class II MHC molecules on their surface, which subsequently play a critical role in the immune response and are also constitutively expressed on antigen presenting cells like DCs and B cells (Byersdorfer *et al.,* 2004; ten Broeke *et al.,* 2013).

The IL-2 has been previously implicated in the activation of T cells and their proliferation (Boyman *et al.,* 2012), also IL-2, along with others, stimulates the production of IFN-γ that directs the differentiation of naïve T lymphocytes into Th1 cells (Liao *et al.,* 2013). Earlier reports also suggest a putative influence on Th2 cell differentiation for IL-2 (Cote-Sierra *et al.,* 2004), suggesting that driving IL-2 secretion by the probiotics may lead to increased IgG levels and improved vaccine response. Bcl6 is a critical transcription factor regulating B-cell survival, cell cycle control, and somatic hypermutation in the germinal center (Basso *et al.,* 2010; Crotty *et al.,* 2010; Dent *et al.,* 1997). Follicular helper CD4+ T-cells (Tfh), which rely on Bcl6 for their migration and interaction with B-cells, signal B-cells to generate high-affinity antibodies and immunological memory, proving indispensable for the protective immunity granted by vaccines (Choi *et al.,* 2013; Duy *et al.,* 2010). Surprisingly, sheep who received a probiotic association displayed an antagonist effect with regard to vaccine immune response. This observation may be due to the fact that these probiotics (*B. toyonensis* and *S. boulardii*) present different modes of action in the host (Bermudez-Brito *et al.,* 2012), however, we did not evaluate any possible mechanisms mediated by the probiotics.

In conclusion, the data presented in this study show that a 5-day supplementation schedule with *B. toyonensis* or *S. boulardii* prior to vaccination strengthens the immune response against *C. chauvoei* vaccine in sheep. To the best of our knowledge, this is the first report on vaccine response modification following short-term probiotic supplementation in ruminants. Our results suggest prospective novel uses of probiotics as adjuvants to enhance vaccine response in sheep, although, further investigations are necessary to better understand the mechanisms involved in the immunomodulation mediated by these microorganisms in concert with vaccination.

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Declaration of interest statement

The authors have no conflicts of interest to declare.

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5 DISCUSSÃO GERAL E PERSPECTIVAS

É amplamente discutido que os microrganismos probióticos promovem efeitos benéficos a saúde do hospedeiro através da modulação da resposta imunológica (Maldonado Galdeano et al., 2019). Dessa forma, os probióticos melhoram a respostas de vacinas em várias espécies animais, murinos, suínos, ovinos e aves (Schierack et al., 2007; Roos et al., 2010; 2012; 2018; Xu et al., 2017). Neste estudo observamos o efeito modulador dos probióticos *B. toyonensis* e *S. boulardii* na resposta imune de camundongos e ovinos vacinados com antígenos convencionais e recombinantes. A modulação da resposta imune foi demonstrada pelo aumento de anticorpos específicos contra os antígenos vacinais, pela caracterização das classes de anticorpos e pela transcrição de RNAm ou expressão *ex vivo* de citocinas.

Após a vacinação contra um vírus, anticorpos neutralizantes podem ser suficientes para a proteção ou controle de infecções futuras (Klasse, 2014). Nos experimentos realizados utilizando modelo murino, os camundongos foram vacinados pela via parenteral com a glicoproteína D recombinante (rgD) de herpesvírus bovino tipo 5 (BoHV-5) e suplementados com o probiótico *B. toyonensis*. A suplementação com probiótico estimulou de títulos de anticorpos neutralizantes contra o BoHV-5 superiores aos títulos dos animais que não foram suplementados. No experimento realizado com as ovelhas suplementadas com *B. toyonensis* e vacinadas com rETX de *C. perfringens* detectamos maiores títulos de anticorpos antitoxina superiores aos das ovelhas não suplementadas. Neste contexto, esse achado pode ser importante, pois a resposta imune desejada, após a vacinação contra uma toxina bacteriana, deve ser mediada por anticorpos capazes de neutralizar as toxinas impedindo-as de se ligar na célula alvo do hospedeiro (Rees & Steiner, 2018).

Novas estratégias de vacinação através das mucosas tem sido amplamente estudadas, baseadas principalmente em adjuvantes capazes de induzir IgA ou em novos sistemas de administração baseados em nanopartículas sintéticas, partículas virais, células microbianas ou esporos bacterianos (Ding et al., 2018; Hinc et al., 2014; Ricca et al., 2014; Woodrow et al., 2012). *Bacillus toyonensis* foi capaz de modular a resposta imune de camundongos vacinados pela via intranasal com o fragmento da toxina tetânica (TTFC) adsorvido em esporos de *B. subtilis*. A suplementação com este probiótico estimulou a produção de IgA fecal específicas contra TTFC e o rápido

aumento de IgG totais no soro 14 dias após a vacinação, notadamente do isotipo IgG2c. Além disso, esplenócitos dos animais suplementados produziram níveis significativos de IL-6, IL-10 e IFN-γ. Estes resultados são achados novos deste estudo, pois a eficácia de *B. toyonensis* em modular resposta de uma vacina administrada pela via mucosa não havia sido estudada anteriormente.

A microbiota intestinal pode influencias na modulação da resposta imune vacinal (Ciabattini et al., 2019; Zimmermann & Curtis, 2018). Os probióticos modulam a composição da microbiota intestinal pelo aumento e diminuição de microrganismos levando a um equilíbrio ou desequilíbrio da microbiota. Este efeito dá-se principalmente pela interação dos probióticos com barreira epitelial intestinal e com as células imune e através da estimulação da produção de citocinas (Azad et al., 2018). Em nossos estudos nos quais avaliamos o efeito do probiótico *B. toyonensis* na microbiota intestinal, observamos que a suplementação com o probiótico não afetou significativamente a composição da microbiota intestinal. Entretanto, em três gêneros houve uma alteração significativa sendo eles: *Eubacterium*, *Fusobacterium* e *Ruminococcaceae* UCG-014. Quando avaliado o grupo que recebeu o *B. toyonensis* observou-se um aumento significativo nos parâmetros imunológicos avaliados, sendo o gênero *Ruminiclostridium* 6 foi significativamente mais abundante nesse grupo. Essa observação pode sugerir uma correlação entre a abundância deste gênero bacteriano com a modulação da resposta imune. Embora mais estudos são necessários para confirmar essa hipótese.

Neste trabalho o efeito imunomodulador mediado de *B. toyonensis* foi observado quando a suplementação foi realizada durante 7 dias antes da primeira vacinação contra o BoHV-5. O mesmo efeito modulador da resposta imune foi observado em ovelhas suplementadas com *B. toyonensis* durante 5 dias antes da primeira e segunda vacinação com a rETX de *C. perfringens* e em outro experimento com uma vacina comercial contra *C. chauvoei*. A suplementação com *S. boulardii* durante o mesmo período também exerceu efeito imunomodulador nas ovelhas vacinadas. Esses resultados são muito importantes pois é amplamente aceito que para os probióticos exercerem seus efeitos benéficos no hospedeiro é necessário a administração contínua (de Moreno de LeBlanc et al., 2008; Galdeano et al., 2007; Vandenplas et al., 2015). Até onde sabemos, este é o primeiro relato sobre a modulação da resposta a vacinas após a suplementação probiótica durante um período curto em ruminantes. Nossos resultados sugerem que o uso de probióticos

durante 5 dias antes das vacinações melhora a resposta imune vacinal em ovinos, esse achado se torna interessante quando consideramos as práticas de manejo utilizadas na criação destes animais. Embora, sejam necessárias mais investigações para se entender os mecanismos envolvidos na imunomodulação mediada por esses microrganismos na vacinação.

Os microrganismos probióticos modulam o perfil de citocinas que são produzidas pelas células imunes do hospedeiro direcionando assim a polarização a resposta imune (Azad et al., 2018; Barberi et al., 2015). No experimento com os ovinos, as PBMCs das ovelhas suplementadas com os probióticos *B. toyonensis* e *S. boulardii* apresentaram significativos níveis de transcrição de RNAm de IL-2 e IFN-γ, citocinas características de respostas polarizadas pelos linfócitos Th1 (Boyman & Sprent, 2012; Liao et al., 2013). Além disso, as citocinas IL-2 e IFN-γ induzem a troca de classe de IgG para IgG2, um isotipo característicos de repostas Th1 (Estes et al., 1994; 1996; Estes & Brown, 2002) e no soro dos animais suplementados foram detectados significantes níveis de IgG2 antígeno especifica. Um achado novo do presente estudo foi a detecção de significativos níveis de transcrição de RNAm do fator de transcrição Bcl6 nas PBMCs das ovelhas suplementadas. Essa observação é muito interessante e necessita ser mais explorada, já que pode sugerir que um papel dos probióticos é ativar a expressão de Bcl6 e consequentemente na formação de centros germinativos. O centro germinativo é uma microestrutura importante formada no folículo dos órgãos linfoides secundários para a produção de respostas de anticorpos de alta afinidade (Fazilleau et al., 2009; Maclennan, 1994).

O fator de transcrição Bcl6 é essencial na formação centro germinativo para o desenvolvimento de linfócitos T auxiliares foliculares (Tfh) e ativação de linfócitos B (Fazilleau et al., 2009). A ativação de linfócitos B aumenta significativamente a proliferação das células específicas para o antígeno, que é acompanhado com um aumento na geração de células plasmáticas. Além disso, a ativação intrínseca dos linfócitos B promove a expressão do fator de transcrição T-bet, levando preferencialmente a mudança de isotipo para IgG2. Neste estudo observamos uma rápida e elevada produção de IgG e IgG2 antígeno especifica no grupo suplementado, sugerindo que a estimulação do Bcl6, junto com outros fatores, favoreça a formação de centros germinativos, consequentemente na resposta humoral mais rápida. No entanto, a regulação do Bcl6 não é simples e é controlada por uma via de sinalização

complexa (Choi et al., 2013), necessitando de mais estudos para se entender os possíveis mecanismos mediados pelos probióticos na modulação da expressão Bcl6.

Os adjuvantes comumente são utilizados para recrutamento e ativação de células APCS, principalmente DCs e macrófagos, no local da administração do antígeno (Ho et al., 2018). Além disso, eles podem conservar o antígeno e liberá-lo lentamente para fornecer ao sistema imunológico doses de reforço do antígeno por período mais longo (Apostólico et al., 2016). Como foi mostrado nestes experimentos o uso de probióticos concomitante a vacinação estimula a resposta imunológica, assumindo um efeito adjuvante na resposta específica contra o antígeno vacinal.

O efeito dos esporos de *B. toyonensis* foi avaliado, como componente da vacina, administrado pela via subcutânea. Os resultados mostraram que os esporos de *B. toyonensis* possuem um efeito adjuvante aumentando a imunogenicidade da vacina recombinante contra o BoHV-5. Esporos podem estimular diretamente APCs, como macrófagos e DCs a produzirem citocinas e moléculas co-estimulatórias (Barnes et al., 2007; De Souza et al., 2014; Gong et al., 2018). Para se entender melhor o mecanismo dos esporos de *B. toyonensis* em modular a resposta imune foi utilizada a linhagem celular de macrófagos murinos RAW264.7. Nossos dados mostram que os esporos de *B. toyonensis* estimulam a proliferação destes macrófagos e também a transcrição de altos níveis de RNAm das citocinas IL-4, IL-10 e IL-12, corroborando com nossos resultados quando administrado o *B. toyonensis* por via oral. Esporos de *Bacillus* sp. foram inativados pelo calor e utilizados como sistema de apresentação de antígenos em vacinas de mucosas e também associados aos antígenos como adjuvante vacinas parenterais, essa abordagem também tem se mostrado efetiva na indução de resposta imune humoral e celular (Huang et al., 2010; Oliveira-Nascimento et al., 2012; Song et al., 2012). Dessa forma, esporos de *Bacillus toyonensis* podem ser inativados e avaliados quanto ao seu potencial adjuvante em vacinas parenterais e de mucosa.

O alumen foi utilizado nas preparações das vacinas de nossos experimentos em camundongos e ovelhas é extensivamente utilizado em vacinas veterinárias, conhecido por ser um adjuvante relativamente fraco para formulações vacinais com proteínas recombinantes, mas é eficaz para antígenos convencionais como bacterinas e toxóides (McKee et al., 2010). Tradicionalmente, sabe-se que o alumen funciona principalmente formando um depósito de longa duração para antígenos e promovendo sua captação por células apresentadoras de antígeno (Ghimire et al., 2012; Marrack

et al., 2009). A adjuvanticidade do alumen consiste na polarização de respostas Th2 pela ativação do inflamassomo pelo NLRP3, assim como é conhecido que o mesmo inibe a polarização de respostas Th1 pelo bloqueio da secreção de IL-12 pelas DCs (Coffman et al., 2010; Li et al., 2007; Mori et al., 2012). Nossos resultados demonstrados pelos altos níveis de IgG, presença de anticorpos neutralizantes e maiores de níveis de transcrição de IL-4, IL-12 e IFN-γ indicam que os esporos de *B. toyonensis* podem induzir a polarização de respostas Th1, reduzindo o efeito polarizador de respostas Th2 mediado pelo alumen. Essas observações sugerem que os esporos podem potencializar o efeito imunológico do alumen.

Os dados obtidos nesta pesquisa mostram que os probióticos *B. toyonensis* e *S. boulardii* apresentam um efeito modulador na resposta imune dos animais vacinados com antígenos convencionais e recombinantes. Entretanto, estudos mais aprofundados se fazem necessários para um melhor entendimento dos mecanismos envolvidos na imunomodulação mediada por estes microrganismos. Outros experimentos são necessários para se entender principalmente a modulação imune mediada pela levedura *S. boulardii* nos ovinos, embora tenha sido observado seu efeito positivo na resposta da vacina contra *C. chauvoei*, não foi detectado esse efeito na resposta contra a toxina Épsilon de *C. perfringens* (dados não mostrados), além disso sempre foi observado um efeito modulador nos parâmetros imunológicos avaliados nos animais suplementados com *B. toyonensis* em comparação aos suplementados com *S. boulardii*. A suplementação de ovinos com estes probióticos durante um período curto (5 dias) e o efeito positivo na resposta da vacinação, se mostrou um novo achado deste estudo, porém, é necessário a realização de outros experimentos utilizando outros regimes de suplementação como de 3 dias, 1 dia antes das vacinações.

Os mecanismos de ação envolvidos na modulação da resposta imune mediada pelos probióticos ainda não totalmente elucidados e são alvos de várias pesquisas. Neste trabalho foi demonstrado um possível papel dos probióticos estimulando a expressão do Bcl6, entretanto mais experimentos são necessários, como avaliação de centros germinativos nos órgãos linfoides secundários de animais suplementados e avaliação de outros fatore de transcrição e citocinas são necessários para se entender esta via de estimulação da expressão de Bcl6. A partir dos resultados observados nos experimentos com os esporos de *B. toyonensis* se faz necessário mais estudos *in vitro* principalmente com APCs, como as DCs e macrófagos para se

confirmar o potencial dos esporos em ativação e maturação desta população de células. Outro ponto importante a ser explorado e a capacidade dos esporos em potencializar a resposta pelos adjuvantes de efeito conhecido como foi o caso do alumen, utilizado neste trabalho, que teve seu efeito potencializado pelos esporos de *B. toyonensis*.

6 CONCLUSÃO GERAL

Os probióticos estimulam a imunidade do hospedeiro aumentando a eficácia de vacinas. Os resultados deste estudo permitem concluir que *B. toyonensis* apresentou efeito imunomodulador em camundongos vacinados com a glicoproteína D recombinante de BoHV-5. A suplementação com este probiótico modulou a resposta imune humoral e celular mesmo não sendo administrado de forma contínua, mas somente durante 7 dias antes da primeira vacinação. Paralelamente os esporos de *B. toyonensis* presentes na formulação vacinal administrada pela via subcutânea aumentaram a adjuvanticidade da vacina recombinante contra o BoHV-5. A suplementação com *B. toyonensis* modulou a resposta humoral e celular de camundongos vacinados com uma vacina de mucosa contendo o fragmento C da toxina tetânica adsorvidos em esporos de *B. subtilis*.

Os experimentos *in vitro* e *in vivo* em modelo murino e ovino forneceram resultados importantes para um melhor entendimento dos mecanismos de ação envolvidos na modulação da resposta imune mediada pelos probióticos e na adjuvanticidade dos esporos de *B. toyonensis*. A transcrição de RNAm e a expressão de citocinas de esplenócitos e PBMCs de animais suplementados com os probióticos estudados demonstram uma possível via de sinalização mediada pelas citocinas que estimulam e ativação das APCs, linfócitos T e B e a migração destas células a tecidos linfoides distantes modulando assim a resposta imune sistêmica.

A suplementação de ovinos com os probióticos *B. toyonensis* e *S. boulardii* durante 5 dias antes das vacinações é suficiente para modular a resposta imune e aumentar a eficácia das vacinas contra a rETX de *C. perfringens* e contra *C. chauvoei*, demonstrada pelo aumento na produção de anticorpos e na transcrição de RNAm das citocinas nas PBMCs ovinas. Este é um dado importante quando é avaliada a inclusão da suplementação com probióticos na alimentação de ovinos ou de outros ruminantes, pois na maioria das vezes estes animais são criados a extensivamente a campo e apresentam dificuldades no manejo para administração do probiótico diariamente.

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8 ANEXOS

Anexo A – Artigo publicado na revista *Beneficial Microbes*

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Bacillus toyonensis improves immune response in the mice vaccinated with recombinant antigen of bovine herpesvirus type 5

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RESEARCH ARTICLE

Abstract

Probiotics modulate the immune response and can increase the effectiveness of vaccines. *Bacillus toyonensis* is widely used as a probiotic in animal feed. The aim of this study was to assess the effects of *B. toyonensis* administration on the immune response to an experimental recombinant vaccine against bovine herpesvirus type 5 (BoHV-5) in mice. Mice were vaccinated with BoHV-5 recombinant glycoprotein D and supplemented with the probiotic *B. toyonensis* in two regimes: one group received the probiotic only during seven days prior to the initial vaccination while the second group was given the probiotic throughout the experimental period of seven weeks. Animals supplemented with probiotic *B. toyonensis* in two regimes showed an increase in total immunoglobulin (Ig)G, IgG1 and IgG2a levels in serum, in addition to higher titres of antibodies capable of neutralising the BoHV-5 virus than non-supplemented animals ($P<0.05$). Splenocytes from the supplemented mice had higher mRNA transcription levels of cytokines interleukin (IL)-4 and IL-12. These results show that the use of this probiotic may significantly contribute to the response elicited by recombinant vaccines, especially those that rely on increasing antibody and cell-mediated immune responses for efficacy. Further, the data support an immunomodulatory effect for probiotic *B. toyonensis* and imply that enhance effect on the immune response against a BoHV-5 recombinant vaccine in mice.

Keywords: probiotic, immunomodulation, recombinant vaccine

1. Introduction

Probiotics are live microorganisms, which upon ingesting in adequate amounts, confer beneficial effects to the health of the host (FAO/WHO, 2002). Probiotics produce nutrients and growth factors that modulate the balance and activities of the intestinal microbiota, providing benefits to animal health and nutrition (Chaucheryras-Durand and Durand, 2010). Further, probiotic microorganisms can stimulate both innate and adaptive immune response. Probiotics bind to the receptors expressed on the cell surface of the intestinal lumen and/or to immune cells that activate defence mechanisms, provide protection against pathogens in the intestinal mucosa of the host, and influence immune response through local and systemic immunomodulation (Britti *et al.*, 2006; Forsythe and Bienenstock, 2010).

Microorganisms, including probiotics, present in the intestinal mucosa interact with the cells of the immune system through M cells that capture and transport the microorganism to Peyer's patches (Iwasaki, 2007). The dendritic cells (DCs) resident in the submucosa then capture the antigens of the intestinal lumen and amplify the cytoplasmic processes in epithelial junctions without damaging epithelial integrity (Niess *et al.*, 2005a). Thus, microorganisms present in the intestinal mucosa, even probiotics, are processed by the DCs that subsequently activate naïve T lymphocytes. The DCs and T lymphocytes migrate to the mesenteric lymph nodes and enter systemic circulation where they stimulate an immune response that is far from the location of their original activation (Chieppa *et al.*, 2006; Lebeer *et al.*, 2010).

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Bacillus toyonensis is a non-pathogenic Gram-positive bacterium that has been used for the last several decades as a probiotic in animal feed (Gil-Turnes and Conceição, 2007; Williams *et al.*, 2009). The organism was first identified as *B. cereus* var. toyoi (Kozasa *et al.*, 1977), but has recently been proposed as a new species of the group *B. cereus* under the name *B. toyonensis* (Jiménez *et al.*, 2013). Supplementation with the probiotic *B. toyonensis* exerts immunomodulatory effects and is capable of enhancing the effectiveness of conventional vaccines in sheep, pigs and mice (Coppola *et al.*, 2005; Roos *et al.*, 2010, 2012; Schierack *et al.*, 2007).

The use of vaccines is one of the most efficient alternatives for the prophylaxis and the control of infectious diseases (Kaufamman, 2007). The vaccines based on recombinant antigens are considered relatively safer because they result in low adverse reactions such as local irritations and the risk of reversion to virulence, among others (Reed *et al.*, 2009). However, they often show only weak immunogenicity and, therefore, require administration of adjuvants to increase their immunogenicity (Mbow *et al.*, 2010).

Bovine herpesvirus 5 (BoHV-5) is responsible for outbreaks of meningoencephalitis that can reach a mortality rate of 70-100% (Vogel *et al.*, 2003). The disease affects young cattle and is responsible for economic losses in South America (Campos *et al.*, 2009). Recombinant vaccines against bovine herpesvirus use principal glycoproteins of the viral envelope as antigens. The glycoprotein D of BoHV-5 is essential for the binding and penetration of the virus into the host cells and is also one of the main targets of the host immune response that leads to a strong humoral and cellular immune response during infections (Dummer *et al.*, 2014a). One method of assessing vaccine efficacy is the measurement of antigen-specific antibody levels in serum after vaccination as antibody levels correlate directly with protection and are, therefore, used as a reference for determining the influence of probiotics on immune response (MacDonald and Bell, 2010).

Only a few studies have tested the potential of *B. toyonensis* to increase recombinant vaccine efficacy and such previous studies have not identified the immune factor(s) responsible for the *B. toyonensis*-mediated increase in vaccine efficacy (Coppola *et al.*, 2005; Roos *et al.*, 2010, 2012; Schierack *et al.*, 2007). Thus, the aim of this study was to assess the effect of *B. toyonensis* administration on the immune response of an experimental recombinant vaccine against the BoHV-5 virus in mice. Our results suggest that *B. toyonensis* supplementation could be used to modulate and improve the host response to a recombinant vaccine as it increases both antibody and cell-mediated immune response.

2. Material and methods

Probiotic

The probiotic *B. toyonensis* used in this study was obtained from the collection of microorganisms of the Microbiology Laboratory, Biotechnology Center, Federal University of Pelotas (UFPel). The bacteria were seeded in Brain Heart Infusion agar (BHI; Neogen, Lansing, MI, USA) and incubated at 37 °C for 24 h. After sufficient colony growth, the individual colonies were inoculated (3-5 colonies) in 500 ml flasks containing 100 ml of Brain Heart Infusion broth BHI (Neogen) and incubated overnight (16 to 18 h) in an orbital shaker at 200 rpm. The bacteria in these flasks served as inoculum for the propagation in a bioreactor (STATPLUS® B; Braun Biotech International, Melsungen, Germany) containing 3.5 l of NYSM medium (Yousten, 1984). Fermentation conditions were controlled and the air supply was maintained between 0.5 and 1.5 (v/v) so that approximately 80% of the dissolved oxygen in the medium was obtained during fermentation. Agitation was maintained at 300 rpm and temperature at 37 °C for 96 h and without pH corrections during the process.

When 90% of the bacteria had sporulated, the culture was centrifuged in a Sorvall centrifuge® RC-6 plus (Langenselbold, Germany) at 5,000×*g* for 20 min at 4 °C and the sediment suspended in 500 ml of phosphate buffered saline such that the concentration of *B. toyonensis* obtained was approximately 2×10^7 cfu/ml. The final suspension was heated at 80 °C for 15 min in a water bath to eliminate vegetative forms of the bacteria. Purity control was performed at all stages using Gram staining and by inoculation on Brain Heart Infusion agar.

Supplementation of animals

We used 30 seven-week old female *Swiss* mice and, divided into three groups of 10 animals each and named B.t continuous, B.t 7 days (B.t = *B. toyonensis*) and control. The control group received the commercial ration, free of chemotherapeutic agents (Nuvilab® CR-1, Nuvital Nutrients S/A, Colombo, Brazil). The groups B.t continuous and B.t 7 days received the same ration but supplemented with 1×10^6 viable spores of *B. toyonensis* per gram of food. All mice began receiving the rations seven days prior to the vaccination for diet adaptation. The group B.t 7 days received supplementation with a probiotic only for seven days prior to the first vaccination, group B.t continuous received supplementation with probiotics throughout the experimental period.

All protocols were reviewed and approved by the Ethics Committee on Animal Experimentation (CEEA No. 1981) of the Federal University of Pelotas (UFPel). The UFPel-CEEA agreement is approved by the Brazilian National

Council for Animal Experimentation Control (CONCEA). The mice used in the study were provided by the animal unit at UFPel.

Vaccination

The mice were vaccinated by subcutaneous injection of 0.2 ml recombinant vaccine formulated with recombinant glycoprotein D (rgD) of BoHV-5 and expressed in *Pichia pastoris* (Dummer *et al.*, 2009). In order to formulate the vaccine, we used 40 µg of rgD per dose in phosphate buffered saline (PBS) adsorbed in 10% aluminium hydroxide as an adjuvant. The animals were vaccinated on day 0 and received a booster on day 21 of the experiment. Blood samples were collected by the submandibular puncture on days 0, 14, 28 and 42. After collection, serum was separated, labelled and stored at -20 °C until analysis.

Enzyme-linked immunosorbent assay

Indirect ELISA was performed according to Dummer *et al.* (2014b), but with modifications. Microtitre plates (96 well, Corning, Lowell, MA, USA) were coated overnight at 4 °C with 25 ng of rgD per well and subsequently washed thrice with 200 µl/well of phosphate-buffered saline containing 0.05% Tween 20 (PBS-T). Individual serum samples were diluted 1:400 and 100 µl and each was added in triplicate. The plates were incubated at 37 °C for 60 min, washed thrice with PBS-T, 100 µl of horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:4,000 dilution; Sigma-Aldrich, St. Louis, MO, USA) added and incubated at 37 °C for 90 min. After incubation, the plates were washed five times with PBS-T and 100 µl of substrate solution was added. Each ml of the substrate buffer contained 0.4 mg of ortho-phenylenediamine (Sigma-Aldrich) and 15 µl of H_2O_2. The substrate reaction was allowed to proceed for 15 min in the dark at ambient temperature and then stopped by adding 50 µl of 2 N sulphuric acid per well. Absorbance was measured in a microplate reader (ThermoPlate, Rio de Janeiro, Brazil) with a 492-nm filter and using calibration curve. For IgG isotype analysis, pooled serum samples from each experimental group were diluted 1:2,000 and analysed according to the instruction manual of the Mouse Monoclonal Antibody Isotyping Reagents kit (Sigma-Aldrich), following the same protocol above describe. The absorbance value of each serum sample was divided by the absorbance value from the same animal at day 14 and the results were expressed as fold increase in IgG.

Virus neutralisation test

Serum samples collected on day 42 of the experiment were tested for the presence of antibodies against BoHV-5 using the virus neutralisation assay, according to Fischer *et al.* (2007). The serum samples were diluted in minimum essential medium (Gibco, Grand Island, NY, USA) as a 2-fold dilution series from 1:2 until 1:256. Next, 25 µl of each dilution was added into 96 well polystyrene microplates (Corning, Lowell, MA, USA), followed by 25 µl of a BoHV-5 virus suspension containing 100 TCID [50] and incubated for 1 h at 37 °C in an incubator with 5% CO_2. Then 3×10^4 Madin Darby bovine kidney cells (MDBK, originally ATCC CCL22) cells per well were added and the plates incubated for a further 72 h. The presence of the neutralising antibodies was inferred from the absence of a cytopathic effect. The antibody titres were calculated using the Behrens and Kärben statistical method and expressed as the reciprocal of the highest dilution capable of neutralising 100 TCID [50] of the virus.

Spleen cells cultures and RNA extraction

The mice in the experimental groups were euthanised on day 42 of the experiment, their spleen collected and macerated. The spleen cells (2×10^6) were cultured in 1 ml RPMI 1640 (Gibco) containing 10% foetal bovine serum (Gibco) and antibiotic and antifungal agents (penicillin 10,000 U/ml, streptomycin 10 mg/ml and amphotericin B 25 mg/ml) (Gibco) in 24-well plates (Kasvi, Taiwan, China) and incubated for 24 h at 37 °C in 5% CO_2 atmosphere. Culture medium was replaced after 24 h and the cells were stimulated with 10 µg of rgD, $1\times10^{5.3}$ of whole live BoHV-5, 2.5 µg or concanavalin A (ConA; Sigma-Aldrich), and with RPMI 1640, and incubated for approximately 18 h under same conditions. ConA and RPMI were used as a control, positive and negative respectively, for cell stimuli. After incubation, the supernatant was discarded, the cells collected in TRIzol® reagent (Life Technologies, Carlsbad, CA, USA) and RNA extracted by the TRIzol method according to the manufacturer's instructions.

cDNA synthesis and qPCR

Approximately 300 ng of RNA was used for cDNA synthesis and the reaction was performed according to the manufacturer's instructions for the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). A quantitative polymerase chain reaction (qPCR) for analysing the relative mRNA transcription of cytokines genes interleukin (IL)-4 and IL-12 was performed on a STRATAGENE M×3005P real-time PCR system (Agilent Technologies, Santa Clara, CA, USA), as described by de Avila *et al.* (2016). β-actin and GAPDH genes were used as endogenous reference controls, but subsequently, β-actin was selected as the internal reference standard based on its efficiency (M-value of 1.8 and 1.98 for GAPDH and β-actin, respectively). The primer sequences used for amplifying the IL-4, IL-12, β-actin and GAPDH genes, as well as qPCR conditions for the latter two have been described previously (Cardona *et al.*, 2003; Dummer *et al.*, 2014a). All samples were analysed in triplicate. The comparative threshold cycle ($\Delta\Delta C_t$) method was used to determine the

relative amount of mRNA for each gene with β-actin as the reference gene, according to the method as described by Livak and Schmittgen (2001).

Statistical analysis

The data were analysed using GraphPad Prism version 5.03 (San Diego, CA, USA). The mean absorbance values from each group were subjected to analysis of variance (two-way ANOVA) followed by the Bonferroni test for significant differences at $P<0.05$. The differences in serum neutralisation antibodies and cytokine mRNA transcription were analysed by one-way ANOVA followed by the Dunnett's test. Data from experimental groups were compared among themselves and with controls.

3. Results

Dynamics of the humoral immune response

The mice supplemented with the probiotic *B. toyonensis* and the control group responded to vaccination with increased levels of total immunoglobulin G (IgG). On day 14 of the experimental period, we observed an approximately 2-fold increase in IgG in the groups supplemented with the probiotic compared to the control group ($P<0.05$). At day 28, compared to controls, animals in the group B.t continuous showed a 4-fold increase ($P<0.05$), whereas those in the group B.t 7 days showed a 2-fold increase ($P<0.05$) in the IgG levels. On the day 42, a similar trend of higher IgG levels in the groups supplemented with probiotics was observed where the group B.t continuous showed a 2-fold increase ($P<0.05$) and group B.t 7 days showed a 1.5-fold increase, compared to controls ($P<0.05$) (Figure 1A).

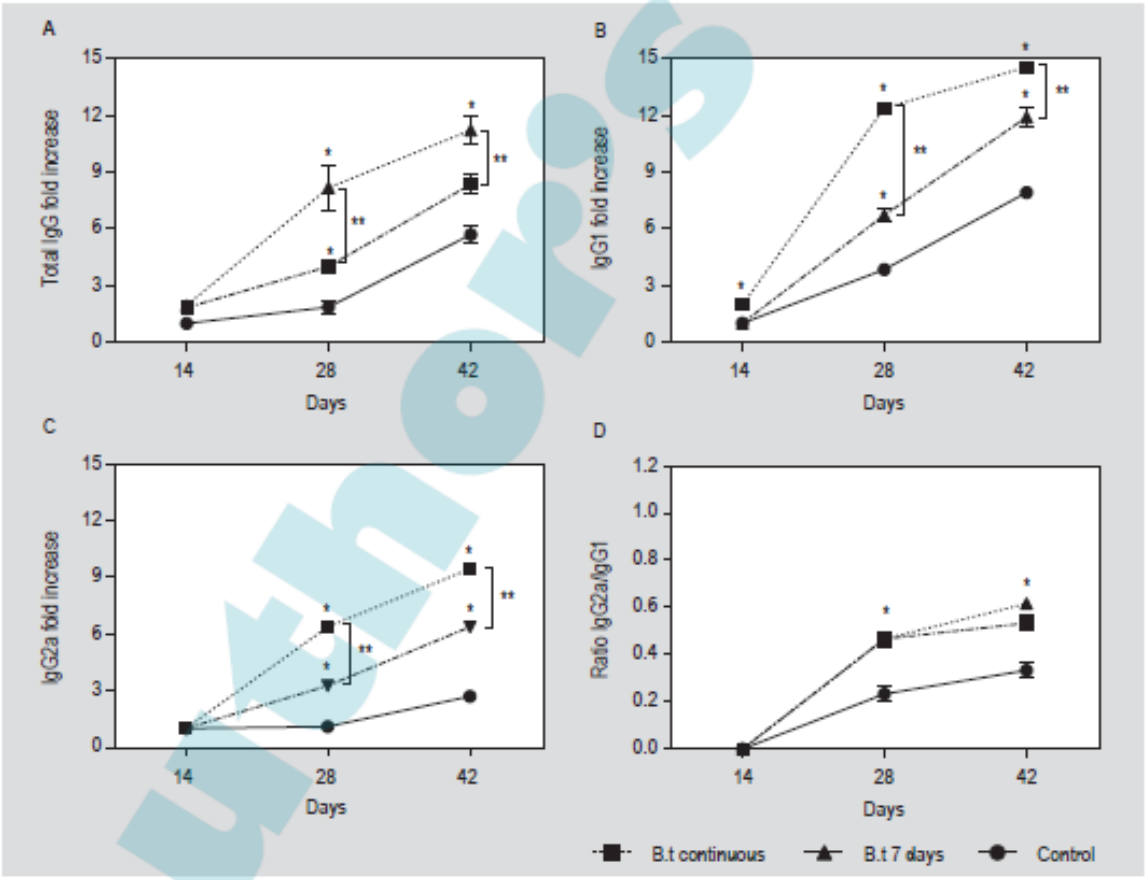

Figure 1. ELISA analysis of sera immunoglobulin (Ig)G dynamics. The data represent the mean (± standard error) of IgG fold increase in the mice vaccinated with recombinant glycoprotein D (rgD) of bovine herpesvirus type 5 and supplemented with *Bacillus toyonensis* (B.t). (A) Total IgG fold increase. (B) IgG1 fold increase. (C) IgG2a fold increase. (D) IgG2a/IgG1 ratio. The statistical analysis was performed by two-way ANOVA followed by the Bonferroni test. Asterisks (*) indicate significant difference ($P<0.05$) between the probiotic supplemented (B.t 7 days and B.t continuous) and the control groups on days 14, 28 and 42; (**) means statistically significant difference ($P<0.05$) between the B.t continuous and B.t 7 days groups on days 28 and 42.

The levels of IgG1 and IgG2a isotypes were estimated on experimental days 14, 28 and 42. The sera of mice supplemented with probiotics showed higher IgG1 and IgG2a levels ($P<0.05$) compared to the sera from non-supplemented animals collected on the same day. Compared to the controls, we observed increased levels of IgG1 in the B.t continuous group from day 14 of the experimental period ($P<0.05$), and by day 28, the B.t continuous group showed 3-fold higher levels of IgG1 while the B.t 7 days group showed an approximately 1.8-fold increase ($P<0.05$). The same dynamic was observed on day 42; the B.t continuous group achieved a 2-fold increase and the B.t 7-days group showed a 1.5-fold increase, both compared to controls (Figure 1B). An increase was also observed in IgG2a levels compared to the controls with the B.t continuous group presenting a 5.8-fold increase ($P<0.05$), and the B.t 7 days group showing a 3-fold increase ($P<0.05$). On day 42, the B.t continuous group showed a 3.5-fold increase compared to the non-supplemented group, while the B.t 7 days group showed a 2.3-fold increase (Figure 1C). Further, a significantly higher IgG2a/IgG1 ratio was observed in the probiotic supplemented groups, compared to the non-supplemented group (0.5 and 0.6 vs 0.2 and 0.3 on days 28 and 42, respectively; Figure 1D).

The titres of neutralising antibodies against BoHV-5 were quantified in sera collected on day 42 of the experiment. Both groups, B.t continuous and B.t 7 days, showed higher titres of neutralising antibodies than the control group ($P<0.05$) (Figure 2).

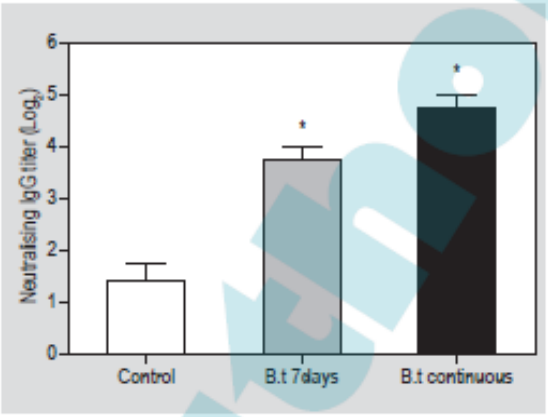

Figure 2. Serum neutralisation. Neutralising antibody titre of mice vaccinated with recombinant glycoprotein D (rgD) of BoHV-5 and supplemented with *Bacillus toyonensis* (B.t). The titre was determined by viral neutralisation assay on day 42. The data represent the mean (± standard error) of log_2 transformed data expressing the reciprocal of the highest dilution that completely inhibited virus-induced CPE. The statistical analysis was performed by one-way ANOVA followed by Dunnett's test. Asterisks (*) indicate statistically significant difference ($P<0.05$) between the probiotic supplemented (B.t 7 days and B.t continuous) and the control groups on day 42.

Cytokine mRNA transcription

The spleen cells of mice in the groups supplemented with probiotic, subjected to an rgD stimulus, showed a distinct mRNA transcription profile for cytokines IL-4 and IL-12 compared to the non-supplemented group (Figure 3). Splenocytes from mice supplemented with the probiotic during the experimental period (B.t continuous) stimulated with rgD showed a 24.4-fold increase in mRNA transcription of IL-4 ($P<0.05$), and a 2.8-fold increase for IL-12 ($P<0.05$) compared to controls. This group, when stimulated with BoHV-5, presented a 4.2-fold increase in mRNA transcription of IL-4 ($P<0.05$) and a 3.4-fold increase in IL-12 mRNA ($P<0.05$) compared to the controls. When the splenocytes of the group supplemented with the probiotic for seven days before the first vaccination (B.t 7 days) were stimulated with rgD, the IL-4 mRNA transcription increased 5.1-fold ($P<0.05$) and that for IL-12 increased 3.5-fold ($P<0.05$), compared to the control group. The B.t 7 days group, when stimulated with BoHV-5, demonstrated a 4.5-fold increase in the transcription of IL-4 ($P<0.05$) and 2.8-fold increase ($P<0.05$) in IL-12 mRNA compared to the controls.

4. Discussion

The use of probiotic *B. toyonensis* has been seen as a promising alternative to increase the effectiveness of vaccines (Roos *et al.*, 2012). In piglets vaccinated against *Mycoplasma hyopneumoniae* and *Influenza* virus H1N1 and H3N2 (inactivated vaccines), the supplementation with *B. toyonensis* stimulated an increase in antibody titres and lymphocyte populations (Schierack *et al.*, 2007). In lambs supplemented with this probiotic before vaccination with an inactivated vaccine against BoHV-5 or *Escherichia coli* F4 demonstrated an increase in humoral immune response and an increase in titres of antibodies against both antigens (Roos *et al.*, 2010). Similarly, in this study, we also observed an immunomodulatory effect in mice supplemented with *B. toyonensis* when vaccinated with a recombinant vaccine against BoHV-5. Mice supplemented with *B. toyonensis* showed significantly higher IgG levels ($P<0.05$) compared to non-supplemented mice, demonstrating a probiotic-mediated modulation of the humoral immune response. Immune response modulation was observed even in animals that received the probiotic only for the seven prior to the first dose of vaccine (the B.t 7 days group), suggesting that the probiotic is capable of modulating the immune response even when not continually administered.

Immune response modulation mediated by the probiotic was monitored by analysing isotype profiles, namely, IgG1 and IgG2a. We observed that animals supplemented with the probiotic had higher levels of IgG1 and a significant increase in IgG2a levels. We used aluminium hydroxide as an adjuvant in the vaccine and it is known that aluminium-

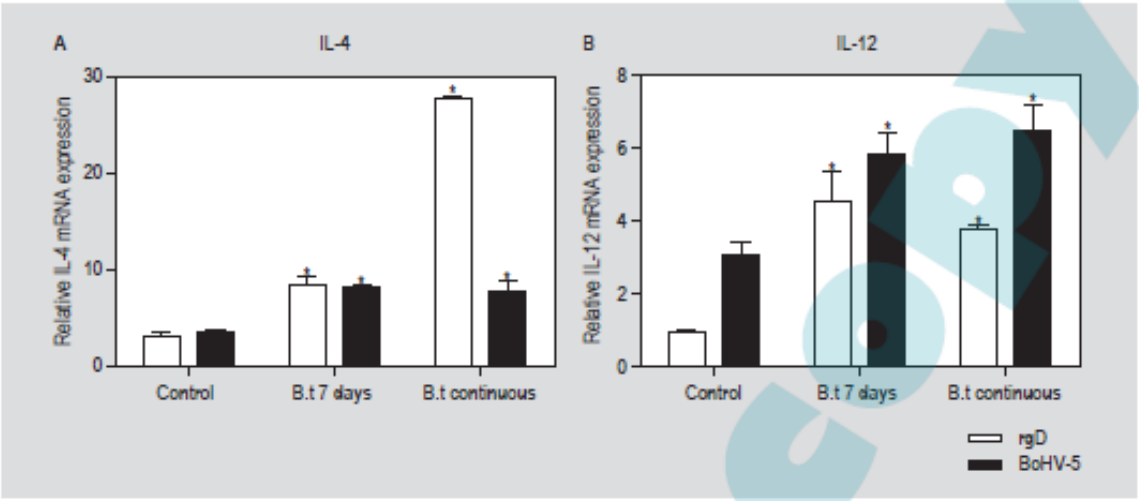

Figure 3. Quantitative polymerase chain reaction (qPCR) expression for interleukin (IL)-4 and IL-12 mRNA. The data represent the mean (± standard error) of IL-4 (A) and IL-12 (B) mRNA expression in splenocytes from mice vaccinated with recombinant glycoprotein D (rgD) and supplemented with *Bacillus toyonensis* (B.t). The relative mRNA expression was determined by the comparative threshold cycle ($\Delta\Delta C_t$). The statistical analysis was performed using one-way ANOVA followed by Dunnett's. Asterisks (*) indicate statistically significant difference ($P<0.05$) between the probiotic supplemented (B.t 7days and B.t continuous) and the control groups. BoHV-5 = bovine herpesvirus type 5.

based adjuvants polarise the Th2 response by the activation of inflammasomes through NLRP3. Further, these adjuvants promote secretion of pro-inflammatory cytokines and inhibit polarisation of Th1 response by inhibiting IL-12 from DCs (Coffman *et al.*, 2010; Li *et al.*, 2007; Mori *et al.*, 2012). Our results suggest that *B. toyonensis* modulates the vaccine immune response by increasing total IgG, thus reducing the polarising effect of Th2 responses mediated by aluminium hydroxide.

The immune response to infection by the bovine herpesvirus consists primarily of a strong inflammatory response mediated by the innate immune system; this is followed by an adaptive immune response, induction of T and B lymphocytes and the production of antibodies responsible for the inhibition of viral particle replication and prevention of secondary infections (Babiuk *et al.*, 1996; Varela *et al.*, 2010). Accordingly, we observed that the supplementation with the probiotic *B. toyonensis* stimulated the production of neutralising antibodies against the BoHV-5. After vaccination against a viral agent, the neutralising antibodies may be sufficient for either protection or control of future infections (Klasse, 2014). As comparative parameter, the United States Department of Agriculture considers as immunised a herd which has 80% of vaccinated animals with titre equal to or above 8 (USDA, 2005). Animals that received the probiotic showed higher neutralising antibodies titres than non-supplemented animals. The B.t 7 days group (supplemented for seven days prior to the first vaccination) showed antibody titres that were significantly higher ($P<0.05$) than the control group but lower than those of the group supplemented throughout the experimental period, suggesting that the probiotic *B. toyonensis* stimulates the production of neutralising antibodies, even when not continuously administered.

IgG antibodies opsonise viral particles and neutralise them in a mechanism that involves the interaction of the Fc portion of the antibody with the Fc receptor, especially FcγRI, expressed on neutrophils, macrophages and natural killer (NK) cells. This Fc-FcγR interaction amplifies the protective activity of neutralising antibodies *in vivo* (Bournazos *et al.*, 2015; DiLillo *et al.*, 2014). The various IgG subclasses have varied effector activities based on their selective connection with Fc receptors. The subclasses IgG2a and IgG2b bind with higher affinity to FcγRI than other subclasses of IgG (Nimmerjahn and Ravetch, 2005; Ravetch and Kinet, 1991). In mice, IgG2a and IgG2b are the more pro-inflammatory IgG molecules that show greater activity than IgG1 or IgG3 in several *in vivo* models (Nimmerjahn and Ravetch, 2006). In the present study, we observed that supplementation with the probiotic increased IgG2a levels and neutralising antibody titres, compared to non-supplemented controls. This observation is important because the IgG2a subclass is considered the most potent in activating effector mechanisms during a virus-induced immune response (Coutelier *et al.*, 1987; Markine-Goriaynoff and Coutelier, 2002).

One of the probable mechanisms of immune response modulation by probiotics is their ability to stimulate cells to produce cytokines that direct development of the immune

response (Fong *et al.*, 2015; Habil *et al.*, 2011; Shida *et al.*, 2011). Thus, the mechanisms of probiotic-mediated immune response modulation may be understood by analysing the cytokine profile produced in response to an interaction with antigens from infectious agents. In the intestinal mucosa, the probiotics are capable of interacting with cells of the intestinal epithelium, lymphocytes, DCs and macrophages (Forsythe and Bienenstrock, 2010). DCs interact with and respond to the bacterial antigens through pattern recognition receptors such as toll-like receptors, which then activate signalling pathways and mediate the innate and/or adaptive immune response (Niess and Reinecker, 2005a,b). The interaction with probiotics leads these cells to initiate the production of cytokines that induce helper T lymphocytes that modulate the type of response (e.g. Th1, Th2, Treg, or Th17) against the activating antigen (Gómez-Llorent *et al.*, 2010; Lebeer *et al.*, 2010).

Studies using splenocytes and peripheral blood mononuclear cell populations (PBMCs) harvested from supplemented animals have been used to demonstrate how the probiotic *B. toyonensis* stimulates the production of cytokines. Splenocytes from mice supplemented with *B. toyonensis* and stimulated with a viral antigen showed high levels of interferon (IFN)-γ, IL-12 and IL-10 mRNA transcription (Roos *et al.*, 2012). Supplementation with *B. toyonensis* has been shown to modulate the immune response of pigs vaccinated with inactivated antigens against *Mycoplasma* and *Influenza* virus. The PBMCs of piglets supplemented with the probiotic and stimulated with ConA produced higher concentrations of cytokines IL-4 and IFN-γ compared to the PBMCs of non-supplemented piglets (Schierack *et al.*, 2007). In the present study, we also found that splenocytes of mice supplemented with *B. toyonensis* stimulated with rgD and BoHV-5 displayed higher levels of IL-4 and IL-12 mRNA transcription compared to the controls. Thus, a limitation of this study is that we did not measured cytokine protein level, but only mRNA transcription at splenocytes.

The cytokine IL-4 is mainly produced by Th2 lymphocytes and promotes B lymphocyte response and the secretion of IgE (Paul and Zhu, 2010). The presence of IL-4 facilitates antigen presentation through increased expression of major histocompatibility complex II molecules, maturation of DCs and proliferation of T lymphocytes, in addition to inducing exchange of the IgG isotype to IgG1 (Finkelman *et al.*, 1990; Luts *et al.*, 1996; Wells *et al.*, 2005). We observed significantly higher levels of IL-4 mRNA transcription splenocytes from probiotic-supplemented animals (both regimens) compared to control group animals ($P<0.05$). This increase in IL-4 mRNA suggests that the transcription of the cytokine may have had a role in probiotic modulation, especially as significantly greater levels of total IgG and IgG1 were detected in the sera of the probiotic-supplemented animals.

The cytokine IL-12 is essential for the activation of the immune response mediated by Th1 cells and is responsible for the induction IFN-γ expression, stimulation of NK cells and differentiation of naïve T lymphocytes into Th1 lymphocytes (Martín-Fontecha *et al.*, 2004; Wan and Flavel, 2009). We observed that animal splenocytes supplemented with the probiotic using both regimens had significantly higher expression levels of IL-12 mRNA, suggesting that one of the mechanisms that modulate immune response upon probiotic supplementation may involve IL-12, as observed by others (Ichikawa *et al.*, 2009; Matsusaki *et al.*, 2016; Takeda *et al.*, 2013). We next evaluated the IgG2a/IgG1 ratio in the control animals and they remained low at 0.2 and 0.3 at days 28 and 42, respectively. In the supplemented groups, this ratio was significantly higher at 0.5 and 0.6 on days 28 and 42, respectively. The modulation observed in the IgG isotype profile (IgG1 to IgG2a) suggest that IL-12 might play a role in the isotype profile, thus polarisating the vaccinal immune response towards a Th1 response.

The immune modulatory effect observed upon *B. toyonensis* supplementation is very important when considering that aluminium hydroxide was used as the adjuvant in the vaccine, which is known to induce a Th2 response in mice (De Gregorio *et al.*, 2008; Kool *et al.*, 2008). The ineffectiveness of aluminium hydroxide as a Th1 polarisation agent suggests that additional signals (i.e. activation of TLR or IL-12 expression) were presented along with the antigen that modulated the immune response bias toward the Th1/IgG2a cell-mediated immunity (Wang and Singh, 2011). The observation is very important as a cellular immune response is generally required for the control of viral infections (Pinto *et al.*, 2006) and also because it seems possible to shift the aluminium hydroxide modulatory effect from Th2 to Th1 (De Gregorio *et al.*, 2008; Kool *et al.*, 2008; Li *et al.*, 2007). The use of this probiotic may significantly contribute to improving the immune modulation of response elicited by recombinant vaccines, particularly those that rely on increasing antibody and cell-mediated immune responses.

In conclusion, the data obtained in this study indicate that probiotic *B. toyonensis* enhance the immune response in the mice vaccinated with a recombinant vaccine against BoHV-5. The immunomodulatory effect was achieved even upon seven days of supplementation prior to the initial vaccination. However, further studies are needed to better understand the mechanisms involved in probiotic-mediated immunomodulation.

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Anexo B – Programa de Doutorado Sanduíche no Exterior - Parecer do Coorientador Estrangeiro

UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II
Scuola Politecnica e delle Scienze di Base
Dipartimento di Biologia

Coordenação de Aperfeiçoamento
de Pessoal de Nível Superior -
CAPES, Brazil

Francisco Denis Santos is a PhD student of the University of Pelotas, Brazil, and visited my laboratory at this Department of the Federico II University of Naples, Italy, from November 23th, 2018 to November 7th, 2019 as a recipient of a CAPES fellowship.

During his stay in my laboratory Denis worked on the project "Spore surface display as a platform for vaccine development" (Spore surface como plataforma para o desenvolvimento de vacinas), which consisted in the display of a model antigen (the C fragment of the tetanus toxin of *Clostridium tetani*, TTFC) on the surface of *Bacillus subtilis* spores and on the use of the spore-displayed antigen to immunize animals by the mucosal route. In parallel, he also evaluated a probiotic as a mucosal vaccine adjuvant, able to increase the efficiency of the immunization. Results of Denis work have been collected in a scientific paper that has been submitted for publication and is currently under evaluation.

Denis had a prominent role in this scientific project performing most of the experiments, contributing to experimental planning, interpretation and discussion of results with other team members and myself, as supervisor of the project.

During his stay in Italy I had the opportunity to know and appreciate Denis. He is an extremely nice person and a brilliant student, highly motivated and interested in expanding his research experience. He spent long hours in the lab and was able to have fruitful interactions with all other lab members. It was a real pleasure have him in the laboratory.

Naples, December 9th, 2019

Ezio Ricca
Professor of Microbiology
Head of the Department of Biology

Anexo C – Parecer da Comissão de Ética em Experimentação Animal

UFPel
CEEA
Comissão de Ética em Experimentação Animal

Pelotas, 17 de junho de 2014

De: Prof. Dr. Éverton Fagonde da Silva

Presidente da Comissão de Ética em Experimentação Animal (CEEA)

Para: Professor Fábio Pereira Leivas Leite

Centro de Desenvolvimento Tecnológico

Senhor Professor:

A CEEA analisou o projeto intitulado: **"Avaliação do efeito imunomodulador de *Bacillus cereus* var. Toyoi em camundongos vacinados com a glicoproteína D recombinante de Herpesvírus bovinos tipo 5",** processo nº23110.001981/2014-74, sendo de parecer **FAVORÁVEL** a sua execução, considerando ser o assunto pertinente e a metodologia compatível com os princípios éticos em experimentação animal e com os objetivos propostos.

Solicitamos, após tomar ciência do parecer e assiná-lo, reenviar o processo à *CEEA*. Salientamos também a necessidade deste projeto ser cadastrado junto ao Departamento de Pesquisa e Iniciação Científica para posterior registro no *COCEPE* (código para cadastro nº **CEEA 1981**).

Sendo o que tínhamos para o momento, subscrevemo-nos.

Atenciosamente,

Prof. Dr. Éverton Fagonde da Silva
Presidente da CEEA

Ciente em: 02 / 07 /2014

Assinatura do Professor Responsável:

Anexo D – Parecer da Comissão de Ética em Experimentação Animal

Pelotas, 08 de agosto de 2017

Certificado

Certificamos que a proposta intitulada **"Efeitos de probióticos na resposta imune de ovinos"**, registrada com o nº 23110.000375/2017-84, sob a responsabilidade de **Fábio Pereira Leivas Leite** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua execução pela Comissão de Ética em Experimentação Animal, em reunião de 10/07/2017.

Finalidade	(X) Pesquisa ( ) Ensino	
Vigência da autorização	Início: 08/2017 Término: 03/2020	
Espécie/linhagem/raça	Ovis aries / Corriedale e Ideal	
Nº de animais	80	60
Idade	1-4 anos	1-4 meses
Sexo	Feminino	Masculino e Feminino
Origem	Fazenda – Centro Agropecuário da Palma / UFPel	

Solicitamos, após tomar ciência do parecer, reenviar o processo à *CEEA*.

Salientamos também a necessidade deste projeto ser cadastrado junto ao *COBALTO* para posterior registro no *COCEPE* (código para cadastro nº **CEEA 0375-2017**).

M.V. Dra. Anelize de Oliveira Campello Felix

Presidente da CEEA

Ciente em: 16/08/2017

Assinatura do Professor Responsável: Fábio Pereira Leivas Leite