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ORIGINAL ARTICLE

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

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Keywords
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Abstract

Aim: The aim of the present study was to examine the vaccine immune response in ewes supplemented with *Bacillus toyonensis* BCT-7112^T during a period of 5-day supplementation before vaccination against a recombinant *Clostridium perfringens* epsilon toxin (rETX).

Methods and Results: Ewes were vaccinated with 200 μg of rETX adjuvanted with 10% aluminium 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 titres than the non-supplemented ewes ($P < 0{\cdot}05$), with an increase in serum levels for total IgG anti-rETX by 3·2-fold ($P < 0{\cdot}0001$), and for both IgG isotypes IgG1 and IgG2 by 2·1-fold and 2·3-fold ($P < 0{\cdot}01$), respectively, compared with the control group. The peripheral blood mononuclear cells of ewes in the supplemented group had a higher ($P < 0{\cdot}05$) 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 humoral immune response of ewes against *C. perfringens* recombinant ETX vaccine.

Significance and Impact of the study: These findings open a new perspective in the utilization of *B. toyonensis* BCT-7112^T as an immunomodulator since a 5 days period of probiotic supplementation is sufficient to improve the vaccine immune response.

Introduction

Probiotics are live micro-organisms which when administered in adequate amount confer a health benefit to the host (Food and Agriculture Organization of the United Nations / World Health Organization (FAO/WHO) 2002; Hill *et al.* 2014). The application of probiotics in ruminant nutrition has been confirmed to improve animal health, productivity and immunity (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 inducing 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 and macrophages. These cells 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 LeBlanc *et al.* 2005; Chieppa *et al.* 2006; Forsythe and Bienenstock 2010; Lebeer *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 *B. 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). *Bacillus 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, 2020).

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). Antibodies produced against *C. perfringens* render vaccination against its toxins as a prophylactic measure (Gonçalves *et al.* 2009; Popoff *et al.* 2016). 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; Maldonado Galdeano *et al.* 2007; de Moreno de LeBlanc *et al.* 2008). However, 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 doses) on the immune response against rETX vaccine in ewes. Our results demonstrated that the transient *B. toyonensis* BCT-7112^T supplementation modulates and potentiates 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 micro-organisms 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) and the plates were incubated at 37°C for 24 h. Individual colonies (3–5 colonies) were picked and inoculated into BHI broth (Neogen, Lasing, MI) and kept in an orbital shaker at 200 rev min^{-1} for 16–18 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 rev min^{-1} and temperature at 37°C for 96 h. When 90% of the bacterial cells had sporulated, verified by Wirtz-Conklin stain, the culture was centrifuged in a Sorvall centrifuge® RC-6 plus (Langenselbold, Germany) at 5000 ***g*** for 20 min at 4°C. The supernatant was removed and the spore pellet was 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

In all, 16 adult ewes of the Corriedale sheep breed were divided into two groups, probiotic and control. Each group consisted of eight animals. The probiotic group received 30 ml of 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. All the animals were vaccinated on day 0 and received a vaccine booster on day 21 of the experiment. The ewes were vaccinated subcutaneously with 3 ml of recombinant vaccine formulated with *C. 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% aluminium hydroxide (alum) (Sigma-Aldrich, St. Louis, MO, USA) as an adjuvant. 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 analysed using indirect enzyme-linked immunosorbent assay (ELISA). Briefly, each well of a 96-well microtiter plate (Corning, Tewksbury, MA) 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). Individual 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 rabbit anti-sheep IgG (1: 5000 dilution; Sigma-Aldrich, St. Louis, MO, USA) 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 (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 individual serum samples collected on day 42 of the experiment were tested for the presence of neutralizing antibodies against ETX 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. Ministério da Agricultura, 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, 10 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) of 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) plus 10% (v/v) foetal 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).

RNA isolation, 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). A quantitative polymerase chain reaction (qPCR) was performed on a CFX96TM Real-Time System platform (Bio-Rad, Hercules, CA) using specific primers for amplifying the transcription factor Bcl6 (forward: GTATCCAGTTCACCCGCCAT; reverse: ACATCAGTCAAGATGTC ACGGC), interleukin (IL)-2 (forward: CCTCGAGTCCT GCCACAATG; reverse: CCGTAGAGCTTGAAGTAGG TGC), 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 twofold 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, as 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 1 μl of cDNA (synthesized from 500 ng of RNA), 6·25 μl of GoTaq® qPCR Master Mix (Promega, Madison, WI), 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 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 described by Livak and Schmittgen (2001).

Statistical Analysis

The data were analysed using GraphPad Prism version 7 (GraphPad Software, La Jolla CA). The Student's *t* test for a significant differences at $P < 0{\cdot}05$ was used to compare the data from probiotic-supplemented group with control group.

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 fivefold increase in IgG titre when compared with the control group ($P < 0{\cdot}0001$). On day 42, a similar trend of higher IgG levels was observed in the supplemented group, showing a 3·5-fold increase compared with the control group ($P < 0{\cdot}0001$) (Fig. 1a). The ewes that received *B. toyonensis* BCT-7112^T supplementation showed higher IgG1 and IgG2 levels compared with non-supplemented ewes. On days 21 and 42, the supplemented group presented approximately 2·1-fold increase in IgG1 levels compared with the control group ($P < 0{\cdot}001$; $P < 0{\cdot}01$) (Fig. 1b). The same dynamic was observed in IgG2 levels on days 21 and 42: the supplemented group showed a 2·5-fold increase and 2·2-fold increase, respectively, when compared with the control group ($P < 0{\cdot}01$; $P < 0{\cdot}05$) (Fig. 1c). The ewes that received *B. toyonensis* BCT-7112^T supplementation

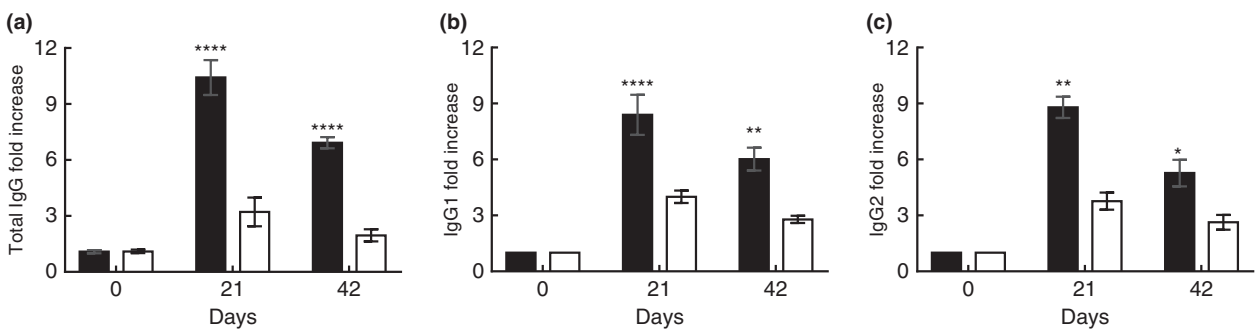

Figure 1 ELISA analysis of sera immunoglobulin IgG levels in ewes vaccinated with an experimental recombinant vaccine against *Clostridium perfringens* ETX and supplemented with probiotic *Bacillus toyonensis* BCT-7112^T. (a) Total IgG fold increase. (b) IgG1 fold increase. (c) IgG2 fold increase. The data represent the mean (±SE) of IgG fold increase obtained from individual serum samples tested in triplicate, from two independent experiments. The statistical analysis was performed by Student's *t* test. Asterisks indicate a significant difference between the (■) probiotic supplemented group and the (□) control group on days 21 and 42. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$ and ****$P < 0{\cdot}0001$.

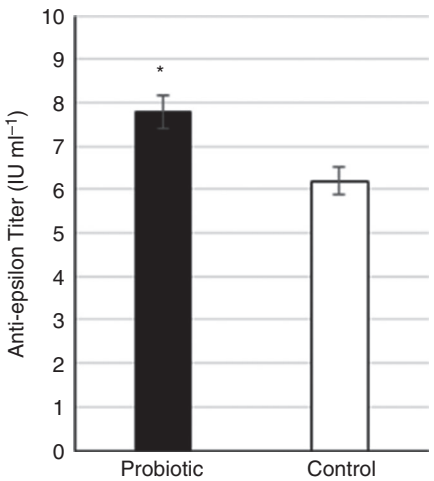

Figure 2 Titres of neutralizing antibodies anti-epsilon toxin in the ewes vaccinated with rETX of *Clostridium perfringens* and supplemented with *Bacillus toyonensis* BCT-7112^T. The data represent the mean titres of neutralizing antibodies antitoxins (±SE) expressed in IU ml^{-1} determined by seroneutralization assay in mice. The statistical analysis was performed by Student's *t* test. Asterisk indicates a statistically significant difference ($P < 0{\cdot}05$) between the probiotic supplemented group and the control group.

presented significantly higher levels of neutralizing antibodies anti-epsilon toxin than the non-supplemented ewes ($P < 0{\cdot}05$) (Fig. 2).

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 ($P < 0{\cdot}05$), 3·1-fold increase in IFN-γ ($P < 0{\cdot}05$) and a 2·4-fold increase in Bcl6 ($P < 0{\cdot}05$) 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 ($P < 0{\cdot}05$), twofold increase in IFN-γ ($P < 0{\cdot}05$) and a 4·2-fold increase in Bcl6 ($P < 0{\cdot}05$) compared with the control group (Fig. 3b).

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). As reported by other authors, probiotics need to be continuously supplied in the diet to exert their beneficial effects (Vaughan *et al.* 1999; Maldonado Galdeano *et al.* 2007; de Moreno de LeBlanc *et al.* 2008). Recently, our group demonstrated that supplementation of *B. toyonensis* for 7 days was sufficient to improve the immune response to an experimental recombinant BoHV-5 gD vaccine in mice model (Santos *et al.* 2018). In the present study, we reduced the supplementation period and evaluated a different experimental vaccine (recombinant *C. perfringens* epsilon toxin), but principally a different animal model (production ruminant *vs* experimental animal). It is worth note that probiotic supplementation for 5 days will facilitate the management as well as the costs for the farmer.

In this study, we observed that ewes that received probiotic supplementation showed significantly higher specific rETX IgG levels and higher neutralizing ETX antibody titres when compared with non-supplemented ewes. Also, we detected an increase in the levels of both IgG1 and IgG2 in probiotic-supplemented ewes, indicating a trend

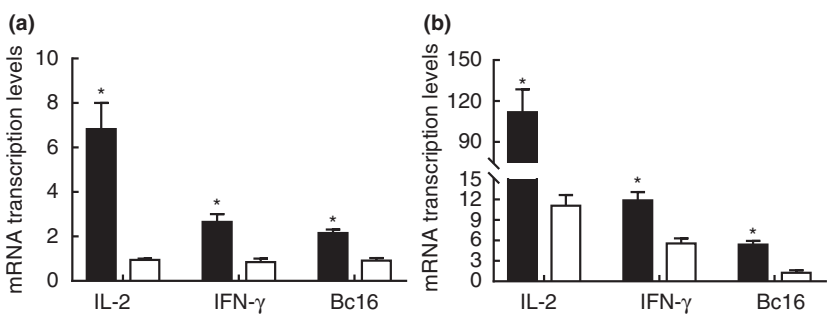

Figure 3 qPCR transcription levels for IL-2, IFN-γ and Bcl6. Quantitative polymerase chain reaction (qPCR) transcription for IL-2, IFN-γ and Bcl6 mRNA transcription in peripheral blood mononuclear cells (PBMCs) from ewes vaccinated with an experimental recombinant vaccine against *Clostridium perfringens* ETX and supplemented with probiotic *Bacillus toyonensis* BCT-7112^T. The PBMCs were stimulated *in vitro* with recombinant ETX (a) and Concanavalin A (b). The data represent the mean (±SE) of IL-2, IFN-γ and Bcl6 relative mRNA transcription determined by the comparative threshold cycle ($\Delta\Delta C_t$). The statistical analysis was performed using Student's *t* test. Asterisk indicates a statistically significant difference ($P < 0{\cdot}05$) between the (■) probiotic supplemented group and the (□) control group.

towards a mixed Th1/Th2 immune response. The alum used as the adjuvant is known to induce polarized Th2 cell-dependent IgG1 antibody isotypes (Germann *et al.* 1995; Brewer *et al.* 1999; Coffman *et al.* 2010). Interestingly, we observed an increase in serum IgG2, which may indicate that the probiotic supplementation contributes to reducing the alum polarizing effect, and by doing so, it promotes a balanced Th1/Th2 vaccine response.

The mechanisms responsible for this immunomodulatory effect need to be characterized; however, there are some evidences that the cytokines might play an important role (Barberi *et al.* 2015; Azad *et al.* 2018). We can speculate that the significant increase in IL-2 mRNA transcription observed in PBMCs from probiotic-supplemented ewes indicates that the probiotic has the ability to modulate T-cell activation and proliferation. Since, IL-2 is a key cytokine for the development of adaptive immune response, promoting the proliferation, differentiation and clonal expansion of T cells (Zhou *et al.* 2002; Gaffen and Liu 2004). The presence of IFN-γ mRNA in probiotic-treated ewes cells may be related to the modulation of a response towards Th1, and it is well known that IFN-γ directs the differentiation of naïve T cells into Th1 cells (Filipe-Santos *et al.* 2006). The modulatory effect of *B. toyonensis* supplementation to stimulate the expression of IFN-γ in PBMCs and spleen cells were previously reported in ovine and swine (Shierack, *et al.* 2007; Roos *et al.* 2018). Furthermore, Th1 cells produce IL-2 and IFN-γ to promote IgG2 isotype switching (Estes, 1996; Estes and Brown 2002), which corroborates with the results described here.

The higher transcription levels of Bcl6 mRNA in PBMCs from supplemented ewes suggest that probiotic is capable of activating and regulating Bcl6 expression, which is an observation not reported before. The transcription factor Bcl6 is essential for the development of germinal centre B cells and follicular helper T cells (Tfh) (Fazilleau *et al.* 2009). The germinal centre is a specialized microstructure formed in the B-cell follicles of secondary lymphoid organs for producing long-term and high-affinity antibody responses (MacLennan 1994). Modulation of Bcl6 activity in formation of Tfh cells may have an important impact for vaccine responses (Choi *et al.* 2013), resulting in enhance of high-affinity antibody responses. Notably, neutralizing antibody anti-epsilon toxin levels are higher in probiotic-supplemented ewes. It has been proposed that microbial stimulation induces the Bcl6 expression and development of Tfh cells (Crotty 2014). However, Bcl6 expression is regulated by a complex signalling pathway that requires further studies to establish the mechanism by which probiotics exert their beneficial effects.

In summary, our results showed that the supplementation with *B. toyonensis* BCT-7112^T for 5 days prior to the first and second doses of vaccine modulates the immune response in ewes vaccinated with rETX vaccine. This fact is an advantage in animal farm management, since the probiotic does not need to be continuously administered, 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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Authors' contribution

F.D.S. Santos: contributed to experiment design, performed most of the experiments and wrote most of the manuscript; M.R.A. Ferreira: contributed to perform most of the experiments; L.R. Maubrigades: contributed to the ELISA and qPCR reactions; V.S. Gonçalves: contributed to the ELISA and cells culture; A.P.S. de Lara: contributed to the probiotic production and animal experiments; C.M. Júnior: contributed to the vaccine production and animal experiments; F.M. Salvarani: contributed to the seroneutralization assay; F.R. Conceição: contributed to the experiment design and analyses of results; F.P. Leivas Leite: contributed to experiment design, analyses of results and manuscript writing. All authors read and approved the final manuscript.

Conflict of Interest

The authors report no conflicts of interest.

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