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SHORT COMMUNICATION

Rumen degradability and gas production as influenced by different strains of *Bacillus thuringiensis*

F.C. Campos, A.L. Abdalla Filho, P.S. Corrêa, C. Nazato, R.G. Monnerat, C.M. McManus, S.M. Tsai, A.L. Abdalla, and H. Louvandini

Abstract: The effect of six *Bacillus thuringiensis* (*Bt*) strains on diet degradability was evaluated using an in vitro gas production technique. Spores (5.7×10^6 spores) of different *Bt* strains (907, 1192, 2036, 2493, 2496, and S1185) plus a control (no spores) were used as treatments with four replicates (inocula) in duplicate. Fermentation processes were evaluated and ruminal microorganisms were quantified. Compared with the control, the *Bt*907 strain decreased dry matter (DM) and organic matter (OM) degradability without affecting the *Fibrobacter succinogenes* population, whereas the other strains reduced this population without altering DM and OM degradability.

Key words: fermentation (ruminal), methane (enteric), spores (bacterial).

Résumé : Les effets de six souches de *Bacillus thuringiensis* (*Bt*) sur la dégradabilité des diètes ont été évalués au moyen d'une technique de production de gaz in vitro. Les spores ($5{,}7 \times 10^6$ spores) des différentes souches de *Bt* (907, 1192, 2036, 2493, 2496 et S1185) ainsi qu'un témoin (aucune spore) ont été utilisés comme traitement avec quatre réplicats (inoculations) en double. Les processus de fermentation ont été évalués et les micro-organismes du rumen quantifiés. Par rapport au témoin, la souche *Bt*907 a diminué la dégradabilité des matières sèches (DM — « dry matter ») et matières organiques (OM — « organic matter ») sans avoir d'effet sur la population de *Fibrobacter succinogenes*, tandis que les autres souches ont réduit cette population sans modifier la dégradabilité des DM et OM. [Traduit par la Rédaction]

Mots-clés : fermentation (dans le rumen), méthane (entérique), spores (bactériennes).

Introduction

Helminth infection is a major limiting factor for sheep production worldwide and the search for alternative treatments against gastrointestinal parasites is important due to the increasing spread of anthelmintic resistance. *Bacillus thuringiensis* Berliner 1915 (*Bt*) gained prominence as an alternative to treat these parasites by showing in vitro nematicidal activity against free and adult worms. This was due to the presence of toxic (δ-endotoxin or cry protein) crystals, which bind at specific sites in the gut of endoparasites, causing cell lysis and death (Lara et al. 2016).

The focus of most mammalian studies concerning cry toxins has been on monogastric species, where the acidic stomach environment inactives these toxins. In contrast, the ruminant gastrointestinal tract is characterized by an extensive forestomach system where ruminal fermentation by enzymes and abundant flora of microorganisms occurs at a neutral pH. Reports on the safety of cry protein in rumen epithelial cells in vitro tests have been documented (Bondzio et al. 2008); however, the interaction of *Bt* with the microbial population is still unclear and may interfere in the proper functioning of the ruminal fermentation and, consequently affect the nutritional status of the animals. We used in vitro gas production to evaluate the effects of adding six different *Bt* strains (907, 1192, 2036, 2493, 2496, and S1185) on the ruminal fermentation process by measuring diet

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F.C. Campos, A.L. Abdalla Filho, C. Nazato, A.L. Abdalla, and H. Louvandini. Laboratory of Animal Nutrition, Centre for Nuclear Energy in Agriculture, University of São Paulo, Piracicaba, 13400-970, Brazil.

P.S. Corrêa and S.M. Tsai. Laboratory of Molecular Cell Biology, Centre for Nuclear Energy in Agriculture, University of São Paulo, Piracicaba, 13400-970, Brazil.

R.G. Monnerat. Embrapa Genetic Resources and Biotechnology (Cenargen), Brasília, 70770-900, Brazil.

C.M. McManus. Institute of Biological Sciences, University of Brasília, Brasília, 70910-900, Brazil.

Corresponding author: H. Louvandini (email: louvandini@cena.usp.br).

degradability, total gas, methane (CH_4), and short-chain fatty acid (SCFA) productions. In addition, real-time quantitative polymerase chain reaction (qPCR) technique was used to assess the relative abundance of *Fibrobacter succinogenes* (Hungate 1950) Montgomery et al. 1988, *Ruminococcus flavefaciens* (Sijpesteijn 1948), anaerobic fungi, and methanogenic archaea.

Material and Methods

Spores from six different *Bt* strains (907, 1192, 2036, 2493, 2496, and S1185) supplied by Genetic Resources and Biotechnology (Cenargen, Brasília, Brazil) were used. The number of spores used in this study was based on the findings of Freire et al. (2014) that suggested toxicity in mammals at doses higher than 10^8 colony forming units (CFU) for rats and 10^{11} CFU for humans. Due to the lack of studies with ruminants, a dose lower than 10^8 CFU per animal was adopted. To estimate the ruminal volume of an adult sheep weighing 40 kg of body weight (BW), we followed the equation proposed by Blunnell and Gillingham (1985) [sheep reticulorumen $(L) = (0.77 \times BW^{0,57}) - 3.49$] resulting in a volume of 2.81 L. Considering the volume (160 mL) of the glass bottles used in the in vitro assay, we calculated the required amount of spores, resulting in 5.7×10^6 CFU for each strain which were diluted in 100 μL (distilled water).

All procedures involving animals were approved by the Ethics Committee on the Use of Animals (Protocol No. 2013-23) of Escola Superior de Agricultura "Luiz de Queiroz" — University of São Paulo (ESALQ-USP, Piracicaba, Brazil). Four adult rumen-cannulated Santa Inês male sheep (60 ± 2.5 kg BW) were used as inocula donors. The inocula were obtained as described by Lima et al. (2017). To reduce the individual animal effect, the four different inocula were prepared by mixing the ruminal contents of two animals (animal A + animal B, animal C + animal D, animal A + animal D, and animal B + animal C). Seven bottles were prepared for each inoculum, base diet (BD) without *Bt* (control), BD + *Bt*907, BD + *Bt*1192, BD + *Bt*2036, BD + *Bt*2493, BD + *Bt*2496, and BD + *Bt*S1185), defined as treatments. The same procedure was used for the blank bottles (without BD and without *Bt*), which were used to correct the total gas production (TGP) values, and for the internal standard, composed of Tifton-85 grass (*Cynodon* spp.) instead of BD. The BD was prepared by mixing 30% concentrate (70% corn and 30% soybean meal) and 70% hay (*Cynodon* spp.). All feeds were ground in a Wiley mill through a 1 mm sieve. The dietary ingredients were analyzed based on the dry matter (DM) content. Organic matter (OM; ID number 934.01), crude protein (CP; ID number 2001.11), neutral detergent fiber (NDF; ID number 2002.04), acid detergent fiber (ADF; ID number 973.18), and acid detergent lignin (ADL; ID number 973.18) concentrations were determined according to the Association of Official Analytical Chemists (AOAC 2011). Tifton 85 hay chemical composition (g kg^{-1} DM) was OM = 912, CP = 142, NDF = 696, ADF = 438, ADL = 97.8; and concentrate mixture was OM = 961, CP = 171, NDF = 502, ADF = 62.2, ADL = 6.06.

In the in vitro gas production assay (Abdalla et al. 2012), 0.5 g of BD was weighed in filter bags (Ankom F57, ANKOM Technology, Macedon, NY, USA) and incubated in glass bottles (160 mL) with 50 mL of incubation medium (Menke's buffered medium), 25 mL of rumen inoculum, and 100 μL of spores solution to complete 75 mL of final volume and 85 mL of head space. The bottles were immediately sealed with butyl septum stoppers, then manually mixed and incubated at 39 °C in a forced-air oven for 24 h. Gas pressure was measured using a pressure transducer and data logger at intervals of 4, 8, 12, and 24 h, to determine the TGP, using the equation V (mL) = 7.365 × P (psi), in which V is the gas volume and P is the measured pressure. During each of the pressure measurements, 2.5 mL of gas was sampled from the bottles using a 5 mL syringe, and stored in a 12 mL vacuum tube for CH_4 measurement using a gas chromatograph system (Lima et al. 2017). Methane gas production (CH_4GP) and TGP were calculated relative to DM. The conversion efficiency of methane (CH_4effic) in relation to the TGP volume was obtained by the equation: CH_4effic (%) = (CH_4GP/TGP) × 100.

At the end of the 24 h incubation, all filter bags were removed from the bottles and immediately immersed in cold water (−4 °C), so that the microbial fermentation process was interrupted. All bags were washed with neutral detergent solution to remove the soluble fraction, without α-amylase, for 1 h at 90 °C, washed with hot water and then acetone. The DM (24 h at 105 °C) and ash (4 h at 400 °C) contents of the residual were determined. The difference between the incubated DM sample and the nondegraded residual DM was the DM degradability (DMD). The same calculation was performed with the incubated and nondegraded residual OM to determine the OM degradability (OMD).

The content of each glass bottle was used for pH measurement and aliquots were taken to characterize the fermentation profile and ruminal microbial community evaluation. Ammonia nitrogen (N-NH_3) values were analyzed by the micro-Kjeldahl method. The concentrations of SCFA were determined using a gas chromatograph as described by Abdalla Filho et al. (2017). The procedure described by Dehority et al. (1983) was used for microscopic protozoa counting.

For microbial community evaluation, DNA extraction of the ruminal samples was carried out using the PowerLyzer™ PowerSoil kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) and the concentration and purity were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, DE, USA). The relative abundance of *F. succinogenes*, *R. flavefaciens*, total anaerobic fungi, and methanogenic archaea was assessed by real-time qPCR and calculated using the $2^{-\Delta\Delta Ct}$ method. The shifts in microbial communities

Table 1. Dry matter degradability (DMD), organic matter degradability (OMD), total gas production (TGP), methane gas production (CH_4GP), and conversion efficiency of methane (CH_4effic) of different *Bacillus thuringiensis* (*Bt*) strains on ruminal fermentation in vitro.

	Treatments								
	Control	*Bt*907	*Bt*1192	*Bt*2036	*Bt*2493	*Bt*2496	*Bt*S1185	SE	*P*
DMD (g kg^{-1})	601a	557b	574ab	568ab	578ab	574ab	581ab	13.7	0.036
OMD (g kg^{-1})	577a	530b	548ab	541ab	552ab	547ab	556ab	14.1	0.034
TGP (mL g^{-1} DM)	102	95.9	99.9	97.2	101	96.7	104	3.25	0.219
CH_4GP (mL g^{-1} DM)	5.70ab	4.84ab	5.59ab	4.73b	5.95a	5.08ab	4.84ab	0.42	0.059
CH_4effic (%)	5.54ab	5.00ab	5.40ab	4.72b	5.83a	5.24ab	4.52b	0.31	0.022

Note: Means followed by different letters in the same row indicate statistical difference ($P < 0.05$). SE, standard error of the difference between means.

Table 2. In vitro ruminal fermentation variables, number of protozoa, and relative abundance of the rumen microbial community at 24 h of incubation in response to the addition of different strains of *Bacillus thuringiensis* (*Bt*).

	Treatments								
	Control	*Bt*907	*Bt*1192	*Bt*2036	*Bt*2493	*Bt*2496	*Bt*S1185	SE	*P*
pH	6.89	6.90	6.90	6.90	6.88	6.91	6.89	0.01	0.816
N-NH_3 (mg 100 mL^{-1})	40.3	40.2	38.5	40.9	40.4	40.5	38.2	1.14	0.681
Total SCFA (mmol L^{-1})	74.7	76.1	74.9	75.2	76.9	75.2	77.5	0.71	0.283
SCFA (%)									
Acetate	60.93	62.02	62.03	62.25	61.40	62.06	61.16	0.43	0.217
Propionate	20.01	19.23	19.30	19.16	19.62	19.34	19.73	0.29	0.286
Isobutyrate	0.92	0.92	0.92	0.90	0.94	0.92	0.92	0.01	0.679
Butyrate	14.31	14.02	13.95	13.93	14.20	13.91	14.46	0.13	0.140
Isovalerate	2.66	2.66	2.65	2.59	2.68	2.63	2.63	0.03	0.220
Valerate	1.16	1.16	1.15	1.17	1.17	1.14	1.11	0.02	0.743
A:P ratio	3.05	3.25	3.24	3.28	3.14	3.22	3.12	0.06	0.230
Protozoa (10^5 mL^{-1})	5.23ab	5.11ab	5.64b	5.36ab	5.64b	4.63a	5.44ab	0.29	0.028
Fibrobacter succinogenes	1a	0.87ab	0.31c	0.52bc	0.37c	0.28c	0.28c	0.13	0.003
Ruminococcus flavefaciens	1	0.96	1.94	2.30	1.11	2.29	2.10	0.74	0.672
Anaerobic fungi	1	3.81	3.07	2.78	2.01	4.46	0.48	1.72	0.776
Methanogenic archaea	1	0.91	1.89	2.11	2.08	1.73	0.40	0.94	0.872

Note: Means followed by different letters in the same row indicate statistical difference ($P < 0.05$). SE, standard error of the difference between means; N-NH_3, ammonia nitrogen; SCFA, short-chain fatty acids; A:P, acetate:propionate.

due to spore inclusion in relation to the control were determined by considering the relative abundance of target population in control as 1.

A complete randomized design with seven treatments and four repetitions (inocula) in duplicate was used in this study. The sources of variation were controlled by analysis of variance using the GLM procedure from SAS version 9.2 (Statistical Analysis System Institute, Cary, NC, USA) and means were compared by PDIFF procedure adopting a 5% significance level.

Results

The inclusion of *Bt* spores did not affect the TGP ($P > 0.05$), whereas CH_4GP of *Bt*2036 and *Bt*2493 treatments differed, with a decrease of 20.5% for *Bt*2036 ($P < 0.05$) (Table 1). However, no *Bt*-treated group differed from the control for this variable ($P > 0.05$). *Bt*2036 and *Bt*S1185 reduced CH_4effic by 1.11% and 1.31%, respectively, compared with *Bt*2493 ($P < 0.05$). None of the strains studied differed from control ($P > 0.05$). *Bt*907 decreased DMD by 7.32% and OMD by 8.00% compared with the control ($P < 0.05$), while the other treatments did not differ for these variables ($P > 0.05$).

The percentage of each SCFA and acetate:propionate ratio (A:P) was not affected by *Bt* spore inclusion ($P > 0.05$) (Table 2). Protozoa count showed no differences between *Bt* strains and the control group ($P > 0.05$). There was only a reduction in the number of protozoa in *Bt*2496 when compared with *Bt*1192 and *Bt*2493 ($P < 0.05$). Lower relative abundance of the *F. succinogenes* population was found for *Bt*1192, *Bt*2036, *Bt*2493, *Bt*2496, and *Bt*S1185 when compared with the control group

($P < 0.05$). The relative abundance of *R. flavefaciens*, anaerobic fungi, and methanogenic archaea were not affected by the different treatments ($P > 0.05$).

Discussion

Factors that decrease the digestibility of foods are related to the extent of lignification of the diet or interference in the ruminal microbial population. Since all treatments received the same BD composition, the possible diet effect in this situation can be excluded.

Many microbial species are found in the rumen with major groups including degraders of structural carbohydrates, nonstructural carbohydrates, pectin, and protein, as well as methanogenic archaea. Except for *Bt*907, the inclusion of *Bt* strains decreased the relative abundance of *F. succinogenes*, which is indicative of *Bt* toxins acting on this ruminal microbial population, since the ideal ruminal environment conditions found in our results were pH between 6.0 and 7, N-NH_3, and SCFA availability.

One possible explanation for our findings is that *Bt* toxins interfered with the adhesion process of this microbial population in the substrate, which may have caused death by "starvation" of *F. succinogenes*. It is important to notice that, differently from observed with *Bt*907, DMD, and OMD were not affected by the *Bt* strain inclusion, suggesting a compensation of other cellulolytic microbial organisms related to diet degradation. One species that may have benefited in this situation was *R. flavefaciens*, although no statistical significance was found microbial population increased along with a decrease in *F. succinogenes* in these *Bt* treatments. Another population which may have taken advantage of this situation was the anaerobic fungi, due to its high fibrolytic capacity.

We also have to consider that it may be possible that the toxic proteins or the dose of *Bt*907 used in this study interfered in the populations of *R. flavefaciens*, *F. succinogenes*, anaerobic fungi, methanogenic archaea, and protozoa without resulting in a lethal action on these microorganisms. This could explain the decrease in DMD and OMD, without reducing these microbial populations when compared with the control.

Based on the results of this study, we concluded that the inclusion of *Bt907* strain in ruminant production systems does not seem to be an interesting alternative for gastrointestinal helminth control due to the impairing of diet degradability. The other strains decreased the population of *F. succinogenes* without impairing diet degradability. However, further in vivo studies are needed to determine how these strains could affect hematological traits, apparent digestibility of nutrients, and animal performance.

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References

Abdalla, A.L., Louvandini, H., Sallam, S.M.A., Bueno, I.C.S., Tsai, S.M., and Figueira, A.V.O. 2012. In vitro evaluation, in vivo quantification, and microbial diversity studies of nutritional strategies for reducing enteric methane production. Trop. Anim. Health Prod. **44**: 953–964. doi:10.1007/s11250-011-9992-0. PMID:22083272.

Abdalla Filho, A.L., Corrêa, P.S., Lemos, L.N., Dineshkumar, D., Issakowicz, J., Ieda, E.H., et al. 2017. Diets based on plants from Brazilian Caatinga altering ruminal parameters, microbial community and meat fatty acids of Santa Inês lambs. Small Ruminant Res. **154**: 70–77. doi:10.1016/j.smallrumres.2017.07.005.

Association of Official Analytical Chemists (AOAC). 2011. Official Methods of Analysis of AOAC International. 18th ed. AOAC International, Gaithersburg, MD, USA.

Bondzio, A., Stumpff, F., Schön, J., Martens, H., and Einspanier, R. 2008. Impact of *Bacillus thuringiensis* toxin Cry1Ab on rumen epithelial cells (REC) — a new *in vitro* model for safety assessment of recombinant food compounds. Food Chem. Toxicol. **46**: 1976–1984. doi:10.1016/j.fct.2008.01.038. PMID:18325653.

Blunnell, F.L., and Gillingham, M.P. 1985. Foraging behavior: dynamics of dining out. Pages 5–79 in R.J. Hudson and R.G. White, eds. Bioenergetics of wild herbivores. CRC Press, Boca Raton, FL, USA.

Dehority, B.A., Damron, W.S., and McLaren, J.B. 1983. Occurrence of the rumen ciliate *Oligoisotricha bubali* in domestic cattle (*Bos taurus*). Appl. Environ. Microbiol. **45**: 1394–1397. PMID:16346277.

Freire, I.S., Miranda-Vilela, A.L., Barbosa, L.C., Martins, E.S., Monnerat, R.G., and Grisolia, C.K. 2014. Evaluation of cytotoxicity, genotoxicity and hematotoxicity of the recombinant spore-crystal complexes Cry1Ia, Cry10Aa and Cry1Ba6 from *Bacillus thuringiensis* in Swiss mice. Toxins (Basel), **6**: 2872–2885. doi:10.3390/toxins6102872.

Lara, A.P.S.S., Lorenzon, L.B., Vianna, A.M., Santos, F.D.S., Pinto, L.S., Berne, M.E.A., and Leite, F.P.L. 2016. Larvicidal activity of *Bacillus thuringiensis* var. *israelensis* Cry11Aa toxin against *Haemonchus contortus*. Parasitology, **143**: 1665–1671. doi:10.1017/S0031182016001451.

Lima, P.M.T., Moreira, G.D., Sakita, G., Natel, A.S., Mattos, W.T., Gimenes, F.M.A., et al. 2017. Nutritional evaluation of the legume *Macrotyloma axillare* using in vitro and in vivo bioassays in sheep. J. Anim. Physiol. Anim. Nutr. **102**: e669–e676. doi:10.1111/jpn.12810.