Veterinary Microbiology

The use of *Prevotella bryantii* 3C5 for modulation of the ruminal environment in an ovine model

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ARTICLE INFO

ABSTRACT

In the Southern Hemisphere, ruminants are mostly raised in grazing systems where animals consume forage and are supplemented with low amounts of concentrates. Concentrates are usually given separately and are rapidly ingested. This practice leads to changing rumen environment conditions during the day, may alter the rumen microbial metabolism and could affect host performance. The native ruminal *Prevotella bryantii* strain 3C5 was administered every 48 h to wethers under experimental conditions simulating Southern-Hemisphere feeding to evaluate its potential as a rumen fermentation modulator. The inoculum potential was assessed on day 17. The ammonia nitrogen (NH_3-N), volatile fatty acids and ruminal pH were monitored on a 24-h basis 19 days after the beginning of the experiment, and the microbial community structure was assessed by pyrosequencing. The administration of *P. bryantii* modified the fermentation products and daily pH values compared to the control. The NH_3-N concentration in the rumen of treated animals was significantly higher than that of the untreated animals. Modification of the ruminal environment and fermentation pathways was achieved without altering the general structure of the microbial community or the potential methane production. *P. bryantii* 3C5 could be considered in potential probiotic formulations for ruminants in semi-intensive systems.

Introduction

Ruminants are raised in intensive and semi-intensive production systems that include the supplementation with protein-energy concentrates. These practices can generate a misbalance of rumen microorganisms' metabolism, and therefore, production yields are usually affected. To overcome these limitations, feed additives such as antibiotics or ionophores have been extensively used.[1] Due to the emergence risks of resistant strains and antibiotic residues in animal products and byproducts, the use of antibiotics as feed

additives has been progressively restricted. The use of antibiotics has been banned in the European Union since January 2006 (Directive 1831/2003/CEE, European Commission 2003). Therefore, alternatives to the use of antibiotics have been proposed, including the use of probiotics.[2,3]

Probiotics, live microorganisms that confer benefits to the host,[4] could be designed to modulate the rumen microbiota, improving feed utilization. This goal could be achieved by enhancing fiber and starch digestion, promoting volatile fatty acid (VFA) synthesis and diminishing or buffering lactate accumulation to avoid acidification of ruminal contents.[2,5] Some of these issues have been assessed in previous studies using different native and exogenous microorganisms with diverse results.[6–8]

Prevotella is the most represented bacterial genus in the rumen,[9] and in particular, *Prevotella bryantii* strains have been tested on high-producing dairy cows to evaluate the potential of the strains to prevent subacute acidosis.[10,11] Although *P. bryantii* was not effective at preventing ruminal acidosis, the authors observed that its administration could improve the ruminal environment.

In the Southern Hemisphere, ruminants are mostly raised in grazing systems. Animals usually consume forage all day long and are supplemented with low amounts of concentrates generally provided separately from forage, once or twice a day. This practice leads to daily variations of rumen environment conditions.[12,13] Therefore, in these conditions, the use of probiotics could help to stabilize the rumen environment and feed digestion.

The objective of this work was to evaluate the ability of a native *P. bryantii* strain to modulate the ruminal environment and fermentation and the bacterial microbiota structure in a model based on sheep consuming forage and supplemented twice a day with a high-starch concentrate.

Materials and methods

Bacterial cultures and inoculum preparation

P. bryantii 3C5, used in the administration trial, was previously isolated from the rumen of an only pasture-consuming cow at the Experimental Station at Libertad (San José, Uruguay), Faculty of Veterinary, University of Uruguay, and identified by sequencing of the 16S ribosomal RNA gene (JQ674698.1). This strain grew in a culture medium containing lactate as the sole carbon source and exhibited antimicrobial activity against *Escherichia coli* and *Streptococcus bovis*. Bacterial cultures were grown using a modified rumen-fluid-free broth according to Caldwell and Bryant[14] (Table 1). For inoculum preparation, Pb3C5 was inoculated in CO_2-gassed culture bottles containing 50 mL of broth and incubated at $39\,°C$ until a density of 1×10^8 cells/mL was achieved.

Animals, feeding and preparation of samples

Animals were cared for and handled according to the procedures approved by the Honorary Commission of Animal Experimentation (CHEA) of the University of Uruguay and by the National Commission of Animal Experimentation (CNEA,

Table 1 – Medium composition.	
Component	% (m/v)
Yeast extract	0.05
Trypticase	0.2
Hemin	0.0001
Glucose	0.05
Cellobiose	0.05
Soluble starch	0.05
Mineral solution[a] (mL)	3.75
VFA mixture[b] (mL)	0.31
$Na_2S \cdot 9H_2O$	0.025
Cysteine hydrochloride	0.025
Na_2CO_3	0.4

[a] Mineral solution composition (g/L): NaCl, 5.4; KH_2PO_4, 2.7; $CaCl_2 \cdot 2H_2O$, 0.159; $MgCl_2 \cdot 6H_2O$, 0.12; $MnCl_2 \cdot 4H_2O$, 0.06; $CoCl_2 \cdot 6H_2O$, 0.06; $(NH_4)_2SO_4$, 5.4.
[b] VFA mixture: acetic acid, 5.80 mM; propionic acid, 1.60 mM; butyric acid, 0.86 mM; n-valeric acid, 0.18 mM; and isovaleric acid, 0.18 mM.

Uruguay). Ten eleven-month-old Corriedale wethers (*Ovis aries*; $33.8 \pm 4.3\,kg$) with a rumen cannula were individually housed in metabolic cages at the Experimental Station at Libertad (San José, Uruguay), Faculty of Veterinary, University of Uruguay. Animals had access to water and were fed *ad libitum* on alfalfa hay. They were supplemented with cracked corn grain (1% metabolic weight basis) in two supplements, at 10 AM and 4 PM. The average intake was 1347 g per day, and the forage/concentrate ratio was 4.4/1 on a dry matter basis. Animals were randomly assigned to the Control or Pb3C5 group. Every 48 h, wethers in the Pb3C5 group received an intra-ruminal dose of 1×10^9 Pb3C5 cells in 100 mL of culture medium, while animals in the Control group received 100 mL of sterile medium. This experimental procedure was designed after Chiquette et al.[15]

Wethers were adapted to housing, diet and treatments for 16 days. On day 17, samples were taken for inoculum potential experiments. On days 19 and 20, ruminal fluid samples were taken to measure pH, ammonia nitrogen (NH_3-N) and VFA for a 24 h period. Additionally, on day 19, ruminal fluid was taken from each wether to evaluate the microbial community.

Inoculum potential assay and methane production

The effect of *P. bryantii* 3C5 on the fermentative potential of ruminal fluid was assessed using an *in vitro* fermentation assay based on gas production experiments.[16] Experiments were performed in 125 mL fermenters containing a substrate, buffer solution,[17] reductive solution and fresh ruminal fluid from each wether. The substrate (0.5 g per fermenter) was a mix of alfalfa hay (70%) and corn (30%), ground to pass a 1 mm sieve, with 90% dry matter, 93.4% organic matter, 34.8% neutral detergent fiber, 21.4% acid detergent fiber, and 14.6% crude protein. This substrate was allowed to hydrate with the buffer solution (38 mL) and the reductive solution (2 mL) for 18 h at $4\,°C$ (solutions from Oeztuerk et al.[17]) inside the fermenters. Then, 10 mL of fresh ruminal fluid, obtained from both groups of animals, was added to every fermenter. The headspace was saturated with CO_2, and the fermenters were sealed. All incubations were performed in individual

batches. To deduct the gas production of the rumen fluid of the donors, two were incubated without substrate addition. Incubation was performed at 39 °C, and the internal pressure was measured with a manual manometer D1005PS (Ashcroft®, Stratford, USA) coupled to a 0.6 mm needle. Measurements were taken at 2, 4, 8, 10, 12, 18, 24, 48, 72 and 96 h after inoculation, and gas was vented after pressure readings. At times of 4, 8 and 24 h, headspace gas samples from the fermenters were obtained for measuring methane concentration by GC as described before.[18]

Ruminal environment measures

On day 19, ruminal fluid samples were taken every 2 h, covering the first 12 h of the day and then every 4 h, completing the whole 24 h period. The pH was measured immediately. For VFA determination, the samples were mixed 1:1 with 0.1 M perchloric acid. For NH_3-N analysis, rumen fluid samples were preserved with H_2SO_4, 50% (v/v) in a 100:1 relation. Samples were stored at −20 °C until they were analyzed.

Lactic acid and VFA (acetic, propionic and butyric acids) were quantified by HPLC separation.[19] The chromatogram peaks were integrated at 210 nm.[20] The concentration of NH_3-N was determined using a selective electrode (Thermo Scientific Orion) according to manufacturer's instructions.

Structure of the ruminal bacterial microbiota

Total DNA was extracted from 10 g of contents from each animal using the method proposed by Zhou et al.[21] to assess the ruminal bacterial community of wethers by massive sequencing. Extracted DNA was used to amplify and sequence the 16S rDNA V1-V2 region in a GS FLX Titanium XLR70 as in Allai et al.[22] The 16S rDNA sequences generated by pyrosequencing were subsequently analyzed running the Quantitative Insights into Microbial Ecology (QIIME, version 1.8.0) per scripted modules and workflow scripts.[23] The sequences were trimmed and then filtered by length (≥150 bp), quality (≥25 score), and the content of either one or more ambiguous bases or a long homopolymer (>6). Operational taxonomic units (OTU) were generated by aligning the reads to the GreenGenes database[24] and clustered at 97% sequence identity using the PyNAST tool[23] and the UCLUST algorithm,[25] respectively. An analysis of similarity was performed with QIIME using ANOSIM and ADONIS analysis.

Statistics

Cumulated gas volume along time was compared among treatments (control and Pb3C5). For this purpose, the gas produced at a specific time was considered to be dependent on the preceding time. Consequently, this variable was analyzed as a repeated measure over the fermenter substrate, according to the model:

$$Y_{ijk} = \mu + SI + T_j + (S * T)_{ij} + \varepsilon_{ijk}$$

where Y_{ijk} is the volume of gas produced, μ is the overall mean, SI is the effect of Pb3C5 treatment (I = Control, Pb3C5) in k replicates (2 fermenters per wether), T_j is the fixed effect of time

(j = 2, 4, 6, 8, 10, 18, 26, 48, 72 and 96 h), $(S*T)_{ij}$ is the interaction between the strain and the time and ε_{ijk} is the residual error.

All data sets of pH, NH_3-N and VFA in ruminal contents were tested before statistical analysis to ensure that all the assumptions of analysis of variance (additive model, independence of errors, data normality and homoscedasticity) were met. After, they were analyzed using the MIXED procedure as repeated measures, using the wether as the subject for the repeated measurement, according to the following model:

$$Y_{ijk} = \mu + S_i + h_j + (S * h)_{ij} + \varepsilon_{ik}$$

where Y_{ijk} is the variable, μ is the general mean, S_i the fixed effect of the treatment, h_j is the fixed effect of the time of measurement, $(S*h)_{ij}$ is the interaction between treatment and time and ε_{ik} is the residual error.

The data were analyzed using SAS software (version 8.2; SAS 185 Institute, Cary, NC, USA), and differences among means with $p < 0.05$ were considered statistically significant.

Family and genus abundance values were compared using the non-parametric Kruskal–Wallis ANOVA test.

Results

Inoculum activity and potential methane production

No significant differences were observed between the groups in *in vitro* fermentation gas production associated with the addition of ruminal fluid of treated and untreated animals ($p_{treatment} = 0.0622$). The methane concentrations in the fermenters were similar in both groups ($p > 0.05$, data not shown).

### Influence of added bacteria on the ruminal parameters (VFA, pH, NH_3-N)

Total VFA levels (considering acetic, propionic and butyric acids) tended to be higher ($p = 0.0622$) in the ruminal fluid of the treated animals. Acetic and butyric acid concentrations were significantly higher in the Pb3C5-treated animals than in the control group ($p = 0.0187$ and $p = 0.0199$, respectively; Fig. 1). No differences were observed when propionic acid levels were compared between the two groups ($p > 0.05$). Lactic acid was not detected in any sample.

The ruminal pH of the treated animals was significantly lower than that of the controls during most of the day ($p_{treatment} = 0.006$), and the treatment*time interaction was not significant ($p > 0.05$; Fig. 2).

The N-NH_3 concentration in the rumen of the animals treated with Pb3C5 was also significantly higher than recorded values in the ruminal fluid of the control animals ($p = 0.05$); the treatment*time interaction was not significant ($p > 0.05$, Fig. 3).

Influence of added bacteria on the structure of ruminal bacterial microbiota

The microbial community structure, assessed eight hours after the first feed offer on day 19, was similar in both groups of animals ($p_{ADONIS} = 0.837$; $p_{ANOSIM} = 0.712$), and when comparing the structure at different phylogenetic levels, no differences were found ($p > 0.05$). An average 2588.6 (±645.9)
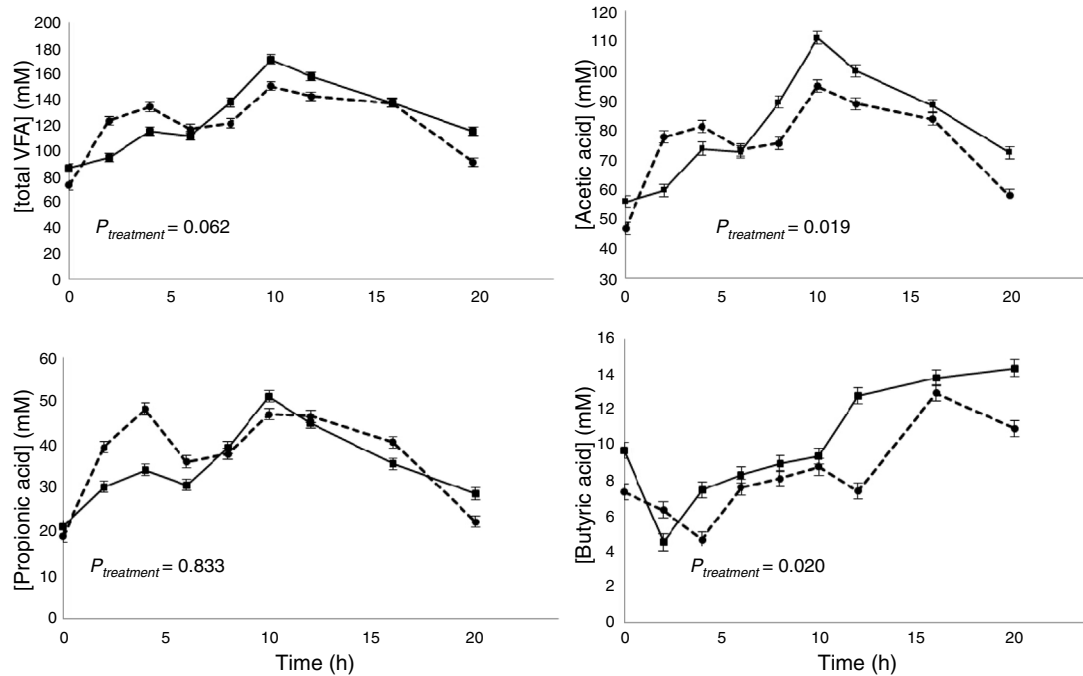
Fig. 1 – Total VFA, acetic, propionic and butyric acid concentration. Means from 5 animals in Control (dash line) and Pb3C5 (full line) treatments for each time are presented. Time 0 corresponds to the first feed intake (8 AM). *p* for treatment is inserted in each graphic.
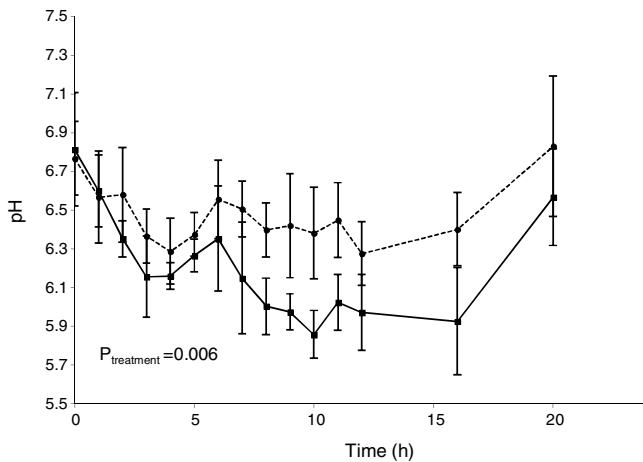
Fig. 2 – Daily ruminal pH. The means of five animals at every time point is presented for the control group (dashed line) and the Pb3C5-treated group (solid line). Time 0 corresponds to the first feed intake, 8 AM; *p* for treatment is inserted in the graphic.
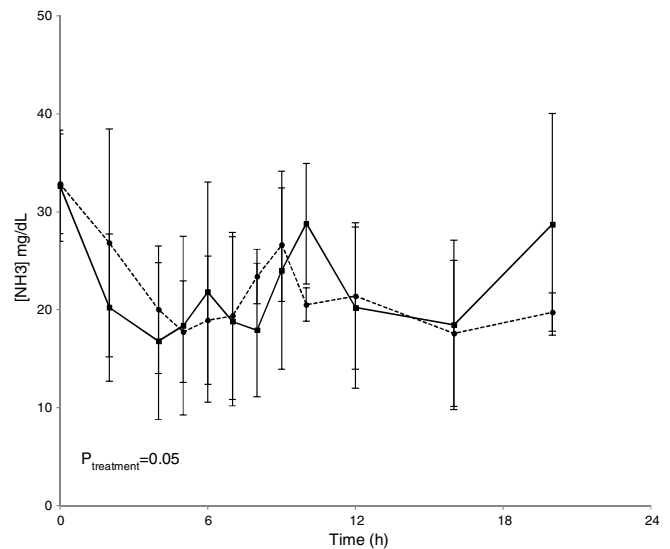
Fig. 3 – Evolution of ruminal ammonia concentration during the day. The means of five animals are presented for each group. Time 0 corresponds to the first feed intake, 8 AM; *p* for treatment is inserted in the graphic.

sequences per animal were analyzed and were designated to 1987 OTUs. The microbiota of every animal was dominated by *Bacteroidetes*, representing more than 65% of all sequences. *Firmicutes* was the second most represented phylum in both groups of animals, while 15.8% and 18.8% of sequences in the control and treated groups, respectively, could not be classified. *Prevotellacea* was the most represented family in the rumen of animals in both treatment and control groups, with 56% and 50% of all the sequences, respectively.

Discussion

The rumen bears a complex ecosystem and, the modulation of ruminal fermentation and the microbiota by probiotic bacteria is a considerable challenge. However, in this work, the ruminal environment was modulated by adding repeated low

doses of the native *P. bryantii* 3C5 strain (Pb3C5). Administration of this strain effectively modified the VFA profile in the treated animals compared to the non-treated controls. The modulation of total VFA in the ruminal contents of treated animals could reflect a fermentative shift associated with the influence of the administered bacteria. This effect was evidenced by a significantly higher concentration of acetic and butyric acids in the Pb3C5-treated animals. It is important to note that butyric acid, which significantly increased in the treated animals, plays an important role in the rumen, being the most energetic VFA along with propionic acid.[5] Butyric acid is also metabolized in the rumen mucosae and intestinal epithelium[7,26,27] as an energy source and exerts mitotic and trophic effects on the host gut epithelia.[28,29] These effects may enhance digestion and absorption efficiency, which may contribute to a better nutrient absorption.[30]

Animals that belonged to the Pb3C5-treated group showed lower ruminal pH values than those in the control group. This observation could be associated with the increase in VFA concentration. Although ruminal pH values were lower than in the control group, these values were never dangerous or harmful to the animals. Ruminants can show subclinical acidosis signs when pH values are between 5.2 and 5.6 for a long time,[31] and they show acute acidosis signs when the ruminal pH reaches values lower than 5.2,[1] but in our conditions, the lowest mean ruminal pH observed was 5.9.

Modifications of the rumen environment were also seen in the daily ruminal NH_3-N concentration, with a higher daily concentration in the Pb3C5-treated animals than in the control group. These results are similar to those obtained when the probiotic potential of *Bacillus subtilis natto* was assessed using *in vitro* and *in vivo* approaches.[6,32] NH_3-N is a microbial protein precursor in the rumen; it is necessary for good microbial growth and microbial protein synthesis and is considered the most important source of nitrogen for protein synthesis, especially for fibrolytic bacteria.[33] Generally, the NH_3-N level is high when protein feedstuffs or good quality young forage are given.[34] The modulation induced by Pb3C5 administration could be associated with a better digestibility of the feed and an influence on host nitrogen and protein metabolism.

These ruminal environment modifications were obtained without altering the potential methane production nor the potential based on *in vitro* gas production experiments.

The structure of the ruminal microbial communities in both groups of animals was similar after the administration of Pb3C5.

The microbiota of all animals was dominated by Bacteroidetes and Firmicutes phyla, and *Prevotella* was the most abundant genus, which is in concordance with several analyses of the ruminal microbiota performed by other authors.[9,35,36]

This result could be observed as a positive trait since the diversity of the rumen microbiota was not affected by the treatment. Considering these results, it is not possible to associate the predominance or the absence of certain groups of microorganisms with the changes observed in the rumen environment induced by Pb3C5 administration. In similar studies performed with human beings, researchers observed significant changes at the community transcriptome level, but they did not find any difference in the community structure.[37]

Conclusion

In this work, the modulation of ruminal fermentation could be achieved by the addition of repeated doses of a native bacterial strain. These doses were small when compared with the whole ruminal microbial community, but a differential fermentation pattern could be observed. Pb3C5 administration modified fermentation products, acetic and butyric acids, which could explain the differences of the daily pH values in the treated animals. Additionally, a differential pattern of NH_3-N concentration in the rumen was observed. The modification of ruminal fermentation and environment was achieved by providing repeated low doses of Pb3C5. Although in this approach, the causes of the ruminal fermentation modulation could not be associated with changes in the microbiota structure, the effect may be associated with changes in ruminal microbial metabolism Additional studies should be performed to shed light on the specific activity of Pb3C5 and its possible use in probiotic formulations for ruminants in semi-intensive systems.

Authors' contribution

MF, CC and PZ initiated and designed the study. MF, SF, NP performed the experiments. KP contributed with strains. MF, SF, CC, analyzed the data. MF, CC, PZ wrote the paper. All authors read, contributed and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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