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ORIGINAL ARTICLE

# In vitro modulation of rumen microbiota and fermentation by native microorganisms isolated from the rumen of a fed-exclusively-on-pasture bovine

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**Abstract** In order to increase productive yields, modulation of rumen fermentation has been a concern in economically relevant species. The ban of antibiotics has driven attention into alternative strategies to modulate ruminal fermentation. Among these, the use of probiotics appears as an interesting approach. The objective of this work was to assess the potential of native bacteria isolated from the rumen of a fed-on-template-pasture cow to modulate fermentation in vitro and to influence the microbiota structure. Seven native ruminal bacteria strains were used in an in vitro gas production experiment. Fermentation dynamics were evaluated, and volatile fatty acids (VFA) and methane were quantified by high-performance liquid chromatography (HPLC) and gas chromatography (GC), respectively. Microbiota structure was assessed by pyrosequencing and methanogens were quantified by quantitative PCR (qPCR). Added strains modulated fermentation dynamics and VFA synthesis. Neither the general structure of the fermenters microbiota, numbers of methanogenic microorganisms nor methane production were altered by added bacteria. However, addition of two strains reduced the volume of gas produced from soluble carbohydrates, while one of them reduced the ratio of gas production in this phase; this was supported by a VFA concentration diminution (4 h incubation) in almost every treatment. Gas produced by fermentation of non-soluble carbohydrates and its fermentation ratio were enhanced by several strains. Also, the abundances of *Lachnospiraceae*, *Veillonellaceae, Rikenellaceae* and *Succinivibrionaceae* were affected by strain supplementation. Modulation of fermentation by selected ruminal native bacteria was achieved, probably enhancing the fermentation of non-soluble carbohydrates. This study represents a new approach in the knowledge related to the use of probiotics in ruminants.

**Keywords** Native ruminal microorganisms · Probiotics · Fermentation modulation · In vitro gas production · Rumen microbiota

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## Introduction

During their evolution, ruminants have developed a symbiotic microbiota on which their capacity to obtain energy and protein sources from pasture intake relies. Nowadays, production systems can generate misbalances in this ecosystem. Therefore, fermentation of fiber by microorganisms may be affected, and as a consequence, production yields are altered. To overcome these problems, modulation of rumen fermentation has traditionally attracted the interest of dairy and beef cattle breeders, veterinaries and nutritionists. Usually, compounds such as antibiotics or ionophores have been used as feed additives to modulate rumen fermentation and to obtain better productive results, by diminishing methane production or avoiding ruminal acidosis (Nagaraja et al. 1982; Mutsvangwa et al. 2002). Mainly due to the emergence of resistant strains and antibiotic residues in animal products and by-products, their use as feed additives has been restricted. For example, the use of antibiotics has been banned in the European Union since January 2006 (Directive 1831/2003/

CEE, European Commission 2003). To overcome this limitation, different alternatives to the use of antibiotics have been proposed, including the use of probiotics (Chaucheyras-Durand and Durand 2010; Allen et al. 2013). Probiotics can be defined as live microorganisms that, when administered in adequate amounts, exert a beneficial effect on host health (FAO/WHO 2002).

Probiotics for ruminants could be used to modify the rumen microbiota in order to improve feed energy yield. This could be achieved through the modulation of microbial populations to enhance fiber and starch digestion, promoting volatile fatty acids (VFA) synthesis and diminishing or buffering lactate accumulation to avoid acidification of ruminal pH (Calsamiglia et al. 2006; Chaucheyras-Durand and Durand 2010). In order to modify and to modulate ruminal fermentation, it would be necessary to improve the knowledge of ruminal microbial diversity, as well as the existing and potential interrelationships between organisms (Kobayashi 2006). Therefore, isolation and characterization of native ruminal microorganisms is a necessary step that allows the development of efficient and "adapted-to-the-target" probiotics. It is well known that probiotic attributes of different strains, even within the same species, differ, and as a consequence every strain is unique (Soccol et al. 2010). For this reason, the search of new bacterial strains with novel probiotic characteristics is a developing field of research. Current research on probiotics is associated with the characterization of the normal host microbiota in order to understand host–microbe interactions, microbe–microbe interactions within the microbiota, and the combined health effects of these interactions (Soccol et al. 2010).

Following these ideas, Fraga et al. (2013) isolated native bacteria from the normal rumen microbiota of a fed-exclusively-on-template-pasture cow that showed a modulator effect on in vitro ruminal fermentation with oat xylan, microcrystalline cellulose and wheat straw as substrates for fermentation. The effect consisted in a diminution of gas production and an enhanced VFA production, particularly butyr ic acid (Fraga et al. 2014). These effects were observed at the end of the incubation period (96 h incubation) without samples in intermediate times, so AGV dynamics could not be determined. Based on their findings, authors proposed that a shift in bacterial community structure with a probable diminution of methane production occurred. Due to the design of the experiment, these two proposals could not be verified (Fraga et al. 2014). Methane mitigation during fermentations would be a very interesting feature in a probiotic to be used in ruminants, as ruminant-produced-methane has become a concern, since this gas is considered an important greenhouse gas. Also, understanding the microbial group implications in the in vitro fermentations would provide the basis of a further knowledge of how microbial structure modulation impacts the fermentation results.

The objective of this work was to assess the ability of a group of native bacteria isolated from the rumen of a fed-exclusively-on-pasture bovine (Fraga et al. 2013, 2014) to modulate ruminal fermentation dynamics and the fermentation products, and to envisage the changes in microbiota structure using in vitro approaches.

## Material and methods

### Bacterial strains and culture conditions

Strains *Butyrivibrio hungatei* 63C (Bhun 63C), *B. hungatei* 79C (Bhun 79C), *B. hungatei* 58C (Bhun 58C). *Pseudobutyrivibrio ruminis* 50C (Prum 50C), *P. ruminis* 55C (Prum 55C), and two unclassified *Lachnospiraceae* strains, 21C (Lach 21C) and 56C (Lach 56C), previously isolated from the rumen contents of a cow fed exclusively on pasture, were used (Fraga et al. 2013, 2014). These strains were selected for our experiments due to their affiliation to species related to the fibrolytic ruminal function that is part of the so called fibrolytic consortia (Shinkai et al. 2010). Also, these strains showed growth adequate to obtain $10^8$ CFU/mL in less than 24 h culture, an important feature for technical procedures and for the development of probiotic suspensions to be administered. For routine culture of bacteria, a medium after that of Stahl et al. (1988) with modifications was used (Fraga et al. 2014). All manipulations were performed under rigorous anaerobic conditions (Hungate 1950).

### In vitro fermentation experiments

In order to evaluate the effect of the addition of native bacterial strains on ruminal fermentation, microbial community and methane production, an in vitro fermentation assay based on gas production experiments was performed (Theodorou et al. 1994; Mauricio et al. 1999).

Experiments were performed in 125 mL fermenters containing a substrate, buffer solution (Oeztuerk et al. 2005), reductive solution and fresh ruminal fluid. Substrate (0.5 g per fermenter) was an alfalfa hay:corn mix (70:30, percent composition 90 % DM; 93.4 OM; 34.84 % NDF; 21.4 % ADF; 14.6 % CP) ground to pass through a 1 mm sieve, and was allowed to hydrate with the buffer solution (38 mL) and the reductive solution (2 mL) for 18 h at 4 °C (both solutions after Oeztuerk et al. 2005) inside the fermenters. Then, 10 mL of fresh and $CO_2$-gassed ruminal fluid were added to every fermenter, the headspace was saturated with $CO_2$ and fermenters were sealed with rubber caps and aluminum seals. Ruminal fluid was obtained from the rumen of a cannulized Holstein dry cow fed on pasture, grazing on a mixture of grass (*Lolium multiflorum*) and legume (*Trifolium repens* and *Trifolium*

*pratense*, percent composition on a dry matter (DM) basis: forage mass, 2800 kg DM/ha; 50 % NDF; 40 % ADF; 17 % CP) at the Experimental Farm of the Veterinary Faculty (Facultad de Veterinaria. UdelaR, Uruguay). To separate the fluid, the ruminal content was filtered through four layers of cheesecloth. Care and handling of experimental animals were approved by the Bioethics Committee of the Veterinary Faculty, Uruguay.

After the fermenters were sealed, each of the seven strain suspensions was observed under optical microscopy, cell density was calculated by direct counts, and bacterial suspensions were prepared and added to the different fermenters with the aid of sterile and pre-reduced syringes in order to obtain a final suspension of $10^6$ cells/mL. Controls were prepared as described above, but without addition of probiotic. For each strain, three series of fermenters were prepared. Two series were respectively opened at 4 and 8 hours incubation time, to assess pH and VFA concentration, and the rest of the fermenters were kept throughout the experimental time and were opened at the end (96 h). Also, samples from every fermenter were kept at −20 °C to analyze bacterial communities by pyrosequencing, and the presence of methanogenic microorganisms by quantitative PCR (qPCR). All incubations were performed in one batch. The strain was the statistical unit and incubations were performed in triplicate for every strain.

Incubation was performed at 39 °C and internal pressure was measured with a manual manometer D1005PS (Ashcroft®, Stratford, USA) coupled to a 0.6 mm hypodermic needle; measures were done at 2, 4, 8, 10, 12. 18, 24, 48, 72 and 96 h after inoculation, and gas was vented after pressure readings.

When fermenters were opened (4, 8 and 96 h), pH was measured and samples were taken and mixed with perchloric acid (0.1 M, 1:1) in order to analyze VFA concentration by HPLC (Fraga et al. 2014).

Internal gas pressure of the fermenters, expressed in psi (P), was converted to volume of gas (V) by the predictive equation $V=4.40\ P+0.09\ P^2$, determined previously in similar experimental conditions (Fraga et al. 2014). Data for cumulative gas production was fitted by non-linear regression to the mathematical model proposed by Schofield et al. (1994), as follows:

$$V(t) = \frac{V1}{1 + e^{2\ +\ 4kd1(L-t)}} + \frac{V2}{1 + e^{2+4kd2(L-t)}}$$

where $V$ is the volume produced at an experimental time ($t$), $L$ is the lag phase, associated to the time that microorganisms colonize the substrate and adapt their metabolism to its fermentation; $V1$ and $kd1$ are the volume produced from the immediately fermentable fraction (soluble carbohydrates) and the gas production rate, respectively; and $V2$ and $kd2$ are the volume and gas production rate from the insoluble fraction.

## VFA and lactic acid determination

For quantification of lactic acid and major VFA (acetic, propionic and butyric acids), a high-performance liquid chromatography (HPLC) separation was performed after Fraga et al. (2014) with samples obtained at times 0, 4 and 8 and 96 h after the beginning of the in vitro gas production assay. Quantitative analysis was performed against a standard solution containing 5 mg/mL of each acid.

## Methane concentration

Methane from the accumulated gas of the fermenters was determined at 4, 8 and 96 h. Gas was collected with the aid of a device that consisted of a three-way-valve connected at one end to a hypodermic needle, used to drill the fermenter's cap, and at the other to a syringe to measure the volume of gas that was injected to a 10-mL Vaccutainer® tube coupled to the third valve end. Methane concentration was determined using a GC, SRI 8610, (SRI Instruments®) with Argon as the carrier gas and a thermal conductivity detector; these procedures were performed at Cátedra de Microbiología, Facultad de Química, Universidad de la República, Uruguay.

## DNA extraction

For microbial community analysis, DNA was extracted from 10 g of the fermenters' contents using the SDS-based extraction method proposed by Zhou et al. (1996).

## Bacterial community assessment

To assess the bacterial community in each of the fermenters at 4 h of incubation, a massive sequencing procedure was performed. Initial amplification of the V1-V2 region of the bacterial 16S rDNA was done using total DNA from the fermenters' contents. Master mixes for these reactions were prepared with Qiagen Hotstar Hi-Fidelity Polymerase Kit (Qiagen, Valencia CA), forward primer was composed of the Roche Titanium Fusion Primer A (5′-CATCTCATCCCTGCGTGTCTCCGACTCAG-3′), a 10-bp Multiplex Identifier (MID) sequence (Roche, Indianapolis, IN) unique to each of the samples; and the universal bacteria primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′). The reverse primer was composed of the Roche Titanium Primer B (5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG -3′, Edwards et al. 1989), the identical 10-bp MID sequence as the forward primer and the reverse bacterial primer 338R (5′-GCTGCCTCCCGTAGGAGT-3′, Fierer et al. 2008), which spans the V1-V2 hyper variable region of the bacterial 16S rRNA gene. Amplification of each sample was performed as follows. An initial denaturing step at 94 °C for 5 min, followed by a cycling of: denaturing at 94 °C for 45 s, annealing at 50 °C for 30 s and a 90 s extension at

72 °C (35 cycles), then a final 10 min extension at 72 °C. Each sample was gel-purified individually using the Qiaquick Gel Extraction Kit (Qiagen, Valencia CA) (E-Gel Electrophoresis System, Life Technologies, Invitrogen division), and standardized prior to pooling. The 16S rDNA amplicons from the pooled sample were sequenced using a Roche 454 Genome Sequencer FLX Titanium instrument (Microbiome Core Facility, Chapel Hill, NC) using the GS FLX Titanium XLR70 sequencing reagents and protocols. Initial data analysis and base pair calling of each sequence to yield high quality reads, were performed by Research Computing at the University of North Carolina at Chapel Hill (Chapel Hill, NC).

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 (Caporaso et al. 2010). Sequences were split and assigned to the different samples (according to their MID), and primers and MIDs were trimmed and then filtered by length (≥150 bp), quality (≥25 score), 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 (DeSantis et al. 2006) and clustered at 97 % sequence identity using the PyNAST tool (Caporaso et al. 2010) and the UCLUST algorithm (Edgar 2010), respectively. Taxonomic classification was assigned with the basic local alignment search tool (Altschul et al. 1997). Alpha diversity indices were generated with QIIME pipeline and also with Past (Hammer et al. 2001).

### Analysis of potential methanogenic microorganisms by qPCR

The detection and quantification of microorganisms that could potentially produce methane was assessed by quantitative amplification of the methyl coenzyme-M reductase gene (*mcrA*) with primers qmrcA-F and qmrcA-R (Denman et al. 2007). Reactions (25 μL) were performed in a BioRad CFX 96 thermocycler using the SensiMix SYBR No-ROX Kit (Bioline), and reactions conditions were the same as in Denman et al. (2007). Standard curves were performed for efficiency estimation and further sample template quantifications. Template used as a standard was a 474-bp-*mrcA*-sequence inserted in a pGEM®-T Easy Vector (Promega). Three replicates of each DNA sample (20 ng) were used. A no-template (sterile distilled water) negative control was loaded on each plate run.

### Statistical analysis

Data were analyzed using the GLM procedure of SAS software (version 8.2; SAS 185 Institute, Cary, NC. USA). The effects of the addition of the strains were tested using the model:

$$Yik = \mu + Si + \varepsilon ik$$

where Y*ik* is the variable (kinetic parameters of gas production. VFA concentration, A/P, pH or methane concentration) μ is the overall mean, S*i* is the fixed effect of the strain addition (*i*=Control, Bhun 63C, Bhun 79C, Bhun 58C, Prum 50C, Prum 55C, Lach 21C, Lach 56C) measured in *k* replicates (three fermenters) and *εik* is the residual error.

The means among strains were separated using the Dunnett's test as a post hoc analysis. Differences among means with $p<0.05$ were considered statistically significant and $0.05<p<0.10$ were considered tendencies to differences.

Families and genera abundances were compared using the non-parametric Kluskal-Wallis ANOVA test.

## Results

### Gas volume dynamics

The parameters that described the dynamics of gas production in the different treatments and control conditions are depicted in Table 1. Total gas volume per gram of incubated substrate (*Vt, V1+V2*) and lag time (*L*) were not significantly affected by the addition of any of the strains compared to the control fermenters (without added bacteria), but differences were found in other fermentation parameters. The volume of gas produced at the fast fermentation phase (*V1*) was two times lower than the control in fermenters treated with strains Prum 55C and Lach 56C, while the fermentation rate (*kd1*) of this phase was significantly enhanced 2.5 times by the addition of Prum 50C strain (Table 1).

Also, the fermentation rate of the slow phase (*kd2*), was significantly enhanced by strains Lach 56C, Bhun 79C, Bhun 58C and Prum 55C. Moreover, these strains and Prum 50C significantly enhanced the volume of gas produced in this phase (*V2,* Table 1).

### Volatile fatty acids concentration

Concentrations of VFA are shown in Table 2. Lactic acid was not detected in any of the fermenters at any of the time points studied. At 4 h, total VFA concentration of treated fermenters was significantly lower than that of the controls, except in the case of the addition of strain Bhun 58C. A similar situation was observed with acetic acid concentration (Table 2). Also, a lower concentration of butyric acid was found in fermenters treated with Lach 56C, Prum 55C, Lach 21C and Bhun 63C strains. Propionic acid concentration was similar to the control in treated fermenters, except in fermenters treated with the Prum 55C strain, which

**Table 1** Kinetic parameters for fermentation

| Treatment | *V1* (mL/g) | *kd1* ($h^{-1}$) | *L* (h) | *V2* (mL/g) | *kd2* ($h^{-1}$) | *Vt* (mL/g) |
|---|---|---|---|---|---|---|
| Control | 171.8 | 0.0714 | 0.96 | 65.4 | 0.0155 | 237.2 |
| Bhun 63C | 157.9 | 0.0705 | −0.32 | 61.5 | 0.0172 | 219.4 |
| Lach 21C | 166.1 | 0.074 | −0.39 | 78.1 | 0.0215 | 244.2 |
| Prum 50C | 124.1 | 0.179* | 0.87 | 138.7* | 0.0355* | 262.8 |
| Lach 56C | 87.8* | 0.137 | 0.81 | 141.1* | 0.0357* | 229.0 |
| Bhun 79C | 96.0 | 0.147 | 0.74 | 141.1* | 0.0363* | 237.1 |
| Bhun 58C | 141.6 | 0.130 | 1.43 | 139.2* | 0.0365* | 280.9 |
| Prum 55C | 82.8* | 0.151 | 0.64 | 157.9* | 0.0385* | 240.7 |
| P strain effect | 0.0211 | 0.0146 | 0.4283 | 0.0008 | 0.0010 | 0.1183 |

* Significant compared to the control (Dunnett's adjustment) at $p<0.05$ for within-column values

showed a lower concentration of this acid. The Acetic/Propionic ratio (A/P) was significantly lower than the control values in every treatment. At 4 h incubation, pH was significantly higher than the control in fermenters treated with Prum 50C, Lach 56C, Bhun 79C, Bhun58C and Prum 55C (Table 2).

At 8 h of incubation, treated and control fermenters did not show any difference in any of the VFA or in the total VFA concentration, except for Prum 50C-treated fermenters in which butyric acid concentration significantly increased. The A/P ratio as well as pH were, in every treatment case, similar to the control (Table 2).

At the end of the experiment, 96 h incubation, total VFA, acetic and propionic concentrations of treated fermenters were not different compared to the control. Butyric acid concentration tended to increase in every treated fermenter, but was significantly higher in fermenters that had been added with Lach 56C. The A/P and pH of treatments were similar to the control, but Bhun 79C showed a higher A/P ratio than the control, and fermenters treated with Prum 55C had significant higher pH values than the control at the end of the experiment.

## Methane gas production and quantification of potential methanogens by qPCR

Methane concentration in the fermenters' headspace was not significantly altered by the addition of the potential probiotic

**Table 2** VFA, A/P and pH in fermenters at 4, 8 and 96 h incubation times

| | | Incubation (h) | Control | Lach 21C | Lach 56C | Prum 50C | Prum 55C | Bhun 58C | Bhun 63C | Bhun 79C |
|---|---|---|---|---|---|---|---|---|---|---|
| VFA (mM) | Acetic | 4 | 41.5 (2.8) | 28.0 (1.7)* | 31.7 (2.5)* | 29.0 (2.2)* | 24.6 (6.4) | 38.4 (6.2) | 24.6 (1.4)* | 27.8 (3.8)* |
| | | 8 | 37.4 (6.2) | 34.0 (5.5) | 37.6 (9.2)* | 44.1 (1.9) | 30.8 (8.8) | 41.9 (3.1) | 29.4 (3.8) | 32.2 (10.5) |
| | | 96 | 52.5 (15.5) | 49.8 (4.2) | 60.3 (9.3) | 53.2 (8.8) | 60.6 (11.0) | 54.4 (4.9) | 41.3 (8.8) | 61.4 (14.8) |
| | Propionic | 4 | 14.3 (1.0) | 10.9 (0.9) | 12.3 (1.3) | 11.5 (0.6) | 9.8 (2.9)* | 16.1 (2.4) | 11.0 (1.2) | 11.2 (1.5) |
| | | 8 | 15.6 (2.1) | 13.3 (1.7) | 13.7 (3.0) | 15.7 (2.1) | 12.4 (3.5)* | 17.7 (1.2) | 12.5 (0.5) | 12.9 (3.7) |
| | | 96 | 19.4 (5.2) | 18.8 (0.9) | 23.5 (3.9) | 20.0 (2.9) | 24.1 (5.8) | 21.0 (3.3) | 15.8 (3.7) | 25.9 (5.7) |
| | Butyric | 4 | 4.0 (0.2) | 1.1 (0.5)* | 1.8 (1.6)* | 3.6 (0.7) | 1.1 (0.4)* | 3.4 (0.1) | 0.7 (0.02)* | 2.7 (0.3) |
| | | 8 | 3.3 (0.7) | 2.4 (0.5) | 3.9 (1.8) | 6.7 (0.8)* | 1.8 (6.1) | 3.8 (0.3) | 2.2 (0.3) | 3.3 (0.8) |
| | | 96 | 2.5 (0.2) | 7.9 (3.0) | 10.3 (1.7)* | 6.3 (3.8) | 6.3 (2.9) | 8.9 (3.9) | 7.2 (3.4) | 8.0 (1.5) |
| | Total | 4 | 59.4 (3.8) | 39.9 (2.9)* | 45.6 (2.1)* | 44.0 (3.4)* | 35.4 (9.5)* | 57.7 (8.5) | 36.2 (2.2)* | 41.4 (5.5)* |
| | | 8 | 56.0 (8.8) | 50.8 (7.5) | 54.9 (13.9) | 66.2 (4.5) | 44.8 (12.4) | 63.0 (4.3) | 44.8 (12.4) | 48.1 (14.9) |
| | | 96 | 74.0 (20.7) | 76.1 (7.9) | 93.6 (15.0) | 79.4 (15.4) | 90.6 (19.5) | 84.8 (9.2) | 64.1 (15.6) | 94.8 (21.9) |
| | A/P | 4 | 2.4 (0.1) | 2.1 (0.1)* | 2.1 (0.1)* | 2.04 (0.04)* | 2.1 (0.1)* | 1.93 (0.04)* | 1.8 (0.1)* | 2.02 (0.02)* |
| | | 8 | 1.9 (0.2) | 2.1 (0.1) | 2.2 (0.1) | 2.3 (0.3) | 2.0 (0.3) | 1.9 (0.1) | 1.9 (0.2) | 2.0 (0.1) |
| | | 96 | 2.2 (0.1) | 2.1 (0.1) | 2.08 (0.03) | 2.2 (0.1) | 2.1 (0.1) | 2.0 (0.2) | 2.12 (0.04) | 1.91 (0.05)* |
| | pH | 4 | 6.34 (0.05) | 6.40 (0.07) | 6.48 (0.05)* | 6.54 (0.02)* | 6.50 (0.03)* | 6.51 (0.01)* | 6.39 (0.05) | 6.48 (0.04)* |
| | | 8 | 6.40 (0.05) | 6.43 (0.02) | 6.44 (0.03) | 6.44 (0.04) | 6.40 (0.02) | 6.39 (0.06) | 6.44 (0.03) | 6.43 (0.02) |
| | | 96 | 6.42 (0.02) | 6.43 (0.03) | 6.43 (0.03) | 6.42 (0.01) | 6.56 (0.03)* | 6.41 (0.02) | 6.40 (0.02) | 6.42 (0.04) |

VFA concentration, pH and Acetic/Propionic ratio (A/P) at 4, 8 and 96 h incubation times in fermenters. *In the same row, values with asterisks are significantly different compared to the control (Dunnett adjustment, $p<0.05$). Standard deviation is between brackets

strains at any of the analyzed times (4 and 8 h), reaching an average concentration of 1.97 mM after 8 h of incubation. Also, quantification of the *mcrA* gene was assessed to estimate the number of total methanogenic microorganisms in fermenters at the same experimental times. There were no significant differences in the number of copies per ng of the *mcrA* gene at either experimental time in the treatment fermenters compared to the control. At 4 h of incubation, there was an average of $2.7 \times 10^3$ copies of *mcrA* per ng of total DNA, while at 8 h, an almost tenfold increase was observed reaching an average of $1.5 \times 10^4$ copies per ng of total DNA.

### Microbiota composition

An average of 2335 (±650) sequences per sample were analyzed. Diversity indices did not differ between the control and treated fermenters (data not shown). OTUs were affiliated to different taxa levels; the most abundant phylum represented in all treatments was *Bacteroidetes*, with abundances that ranged from 78 % in Bhung79C treatment to 86 % in Prum50C treatment, *Firmicutes* was the second-most abundant phylum, ranging from 5.5 to 12.3 % in Prum50C and Bhung79C treatments, respectively (Fig. 1). Samples had an average of 7.8 % of sequences that could not be classified. Other phyla present in the fermenters, like *Spirochaetes, Fibrobacteres*, *Tenericutes, Proteobacteria* and *Cyanobacteria*, among others (Fig. 1) showed abundances below 1 %. Within *Bacteroidetes*, *Prevotellacea* was the most abundant family (68 to 80 %), and *Prevotella* was the most abundant genera, with more than 80 % of all the sequences.

There were no significant differences in the general composition of the microbiota between the controls and the treated fermenters contents, but significant differences were found when the abundances at the family and genera levels were analyzed. Families *Lachnospiraceae*, *Veillonellaceae,*

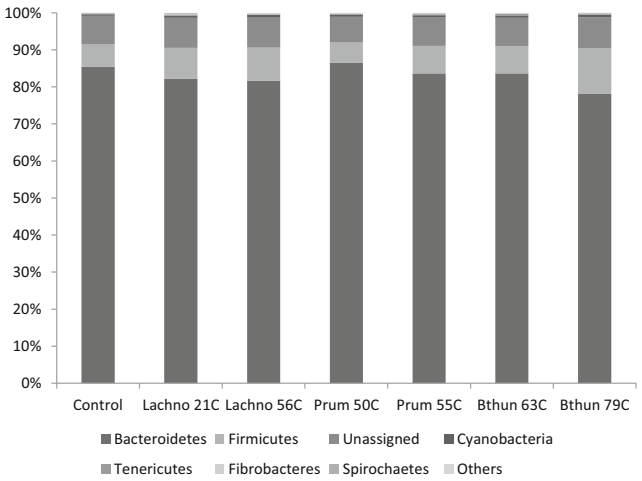


**Fig. 1** Mean percentage contribution of sequences at the phylum level for every treatment

*Rikenellaceae* and *Succinivibrionaceae* were significantly more abundant compared to the control. The family *Lachnospiraceae* was more abundant in fermenters treated with Lach 21C, Prum 55C, Lach 56C, Bhun 58C and Bhun 79C. *Veillonaceae* representatives showed an increased abundance in fermenters treated with Prum 55C, Lach 56C, Bhun 58C and Bhun 79C compared to the untreated control. *Succinivibrionaceae* was absent in the controls, and its presence and abundance in fermenters supplemented with Bhun 63C and Prum 50C was significant, while te presence of *Rikenellaceae* was higher than the control in fermenters where Lach 21C, Prum 50C or Lach 56C was added.

## Discussion

The need for chemicals-free animal products has driven research in exploring new alternatives to successfully modulate ruminal microbial fermentation. Probiotics, also called direct-fed microbials (DFM), represent a natural-non-chemical and secure alternative for this purpose, and in the last years, several efforts have been made in this research (Kobayashi 2006; Seo et al. 2010; Chiquette et al. 2012). In this work, the potential of seven native ruminal bacteria to modulate ruminal and fermentation dynamics and microbiota structure was evaluated using in vitro gas production assays. These strains were originally isolated from the rumen of a cow fed exclusively on pasture (Fraga et al. 2013), and three of them (Prum 55C, Prum 55C and Lach 21C) had been previously analyzed by a similar approach using microcrystalline cellulose, xilan, and wheat straw as substrates for fermentation (Fraga et al. 2014). In the former approach, the authors observed a general increase in AGV concentration after 96 h incubation and a concomitant diminution in the volume of gas produced, but they did not analyze the composition of gas, microbiota structure or the dynamics of fermentation products that are addressed in the present work.

This in vitro methodology was originally developed for the study and prediction of feed fermentation by ruminants (Rymer et al. 2005), but it has been modified and used to analyze the influence of different factors affecting this kind of fermentation (Kung and Hession 1995; Cherdthong and Wanapat 2013; Fraga et al. 2014; Lavrenčič et al. 2014).

Overall, the addition of the different strains to the fermenters resulted in differences in fermentations dynamics compared to the control. Five of the seven strains showed a higher gas production rate at the slow phase of fermentation (*kd2*) and a higher volume produced in this phase (*V2*). This slow phase of fermentation is associated with the fermentation of non-soluble carbohydrates like cellulose and hemicelluloses. These changes in slow phase parameters did not involve a higher production of total gas in any case.

Volumes of gas produced at the fast phase of fermentation were always lower than the control, and this difference was significant for Lach 56C and Prum 55C treatments. These effects together would mean that the addition of the strains induced a modulator effect in fermentation dynamics, particularly at the beginning of the incubation, without affecting total gas production. The last observations are supported by the fact that most of the differences found in VFA production were detected at 4 h incubation, corresponding to the beginning of fermentation. Addition of the different potential probiotic strains (except Bhun 58C) resulted in a lower concentration of total VFA at 4 h incubation,, and these differences were correlative with a lower concentration of acetic acid in all these cases, and a lower concentration of butyric acid in the case of the fermenter treated with Bhun 63C, Lach 21C, Lach 56C and Prum 55C at this experimental time. Interestingly, the A/P ratio was positively affected, as there was more propionic acid in relation to acetic acid.

All together, the addition of the strains affected the first phase of fermentation, lowering gas production (*V1*) and gas production rate (*kd1*). This was supported by a diminished VFA production without changes in global methane production, and showing a better A/P relation.

Differences observed at 4 h incubations or in the fermentations dynamics could not be explained by a general shift of the general structure of microbial community, as there were no differences among treated and control communities.

Although it is known that batch cultures inoculated with rumen fluid like the ones used in this approach had lower diversity indexes than the original inoculums, they support the growth of members of the original microbiota, and particularly fibrolytic bacteria (Soto et al. 2013). In general, the community structure of the fermenters was similar to that reported in in vivo analyses with *Bacteroidetes* and *Firmicutes* as the major phyla of the microbiota (Jami and Mizrahi 2012; Zened et al. 2013; Castro-Carrera et al. 2014; Jami et al. 2014). The slight differences in abundances at a family level between control and treatments that we report could be partially related to the detection of the probiotics themselves, as in some treatments *Lachnospiraceae* members were more abundant. However, significant differences in *Veillonellaceae. Succinivibrionaceae* and *Rikenellaceae* could reflect the probiotic addition effect in the community, as none of the added strains belonged to these families. Interestingly, these families had been previously reported as inhabitants of the rumen of dairy cows and beef calves, and it was shown that their abundance was affected by variations in diet and by the addition of supplements (Wu et al. 2012; Zened et al. 2013; Castro-Carrera et al. 2014). In this study, these families were affected by the addition of a potential modulator.

The potential to modulate the ruminal microbiota in order to obtain better productive results now begins to be a reality (Jami et al. 2014), and this work represents an approach to understand possible ways to use native microorganisms for that purpose. The microorganisms used in this study were able to modulate fermentation, and although not conclusively, these organisms could be used in other approaches to obtain better productive results. Effects on modulation consisted of a shift in fermentation dynamics with less gas produced at the first s tages of fermentation (degradation of fast fermentable carbohydrates), and a consequent increase of the fermentation rates and volume of gas produced at the slow phase of fermentation, which could be associated with a better degradation of non-soluble carbohydrates. Further in vitro approaches in batch culture with different open times or continuous cultures should be performed, and are justified in order to understand the mechanisms of ruminal fermentation modulation by these native bacteria, and to predict possible behavior in the rumen of animals. Understanding the metabolic pathways involved during probiotic modulation would also help to design better strategies of modulation. In vivo trials would shade light in the potential of these strategies to obtain better productive results.

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