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ORIGINAL ARTICLE

Evaluation of native potential probiotic bacteria using an in vitro ruminal fermentation system

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Abstract Rumen microbiota provides an important source of protein to grazing animals and produces volatile fatty acids (VFA), the main energy source for ruminants generated by fibre fermentation. Probiotics can be used to modulate rumen fermentation, and native microbiota is a source of potentially useful microorganisms. In this work, ruminal bacterial strains were isolated and subsequently identified, and their potential to modify fermentation patterns with wheat straw, microcrystalline cellulose and oat xylan as substrates was assessed by in vitro gas production and VFA fermentation patterns. Four of the isolates were identified as *Pseudobutyrivibrio ruminis* and two corresponded to new members of the *Lachnospiraceae* family. The addition of one *P. ruminis* (strain 50C) and one *Lachnospiraceae* (strain 21C) to the fermentation system which used wheat straw as the substrate significantly increased total VFA concentration without altering the total gas produced in one case and showed a decrease in total gas production in the other. All bacterial strains induced higher butyric acid concentrations with the three substrates (up to 31 mM in the case of *Lachnospiraceae* 21C incubated with oat xylan and 25 mM in microcrystalline cellulose fermenters to which *P. ruminis* 50C had been added) compared to the control, which had concentrations of <1 mM. Analysis of the fermentation products suggested that the addition of probiotics to the fermentation system had the potential to induce metabolic shifts that would result in better energy yields. These results show that native bacteria have promising features as fermentation modulators, thereby justifying further research to assess their use as probiotics for ruminants.

Keywords Rumen · Probiotics · Volatile fatty acids · In vitro gas production · *Pseudobutyrivibrio ruminis* · *Lachnospiraceae*

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Introduction

Rumen microbiota is a complex microbial community in which the domains Bacteria, Archaea and Eukarya coexist. This symbiotic community is responsible for fibre breakdown and allows ruminants to obtain nourishment from grass and forage consumption. Following the anaerobic microbial breakdown of fibre, ruminants use the volatile fatty acids (VFA) as their main energy source, while the microbial biomass is the primary source of good quality protein (Russell et al. 1992). Acetic, butyric and propionic acids are produced as by-products of microbial fermentations and account for most of total VFA in an approximate proportion of 70:20:10 (Lana et al. 1998).

Farmers and nutritionists have focused on the modulation or modification of rumen fermentation with the aim to improve productive yields by reducing energy and protein losses. Consequently, research has involved the development of diet formulations and feed additives that have the potential to modify the ruminal environment and enhance or inhibit specific microbial populations (Calsamiglia et al. 2006). Traditionally, compounds with antimicrobial activity, such as ionophores, have been used to achieve this aim (Nagaraja et al. 1982). Nowadays and mainly due to the emergence of

resistant strains and antibiotic residues in animal products and by-products, the use of such compounds as feed additives has been banned since January 2006 in the European Union (European Union Directive 1831/2003/CEE; Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32003R1831:EN:NOT).

The scientific community has proposed alternatives to the use of antibiotics, including the use of probiotics. Probiotics can be defined as living microorganisms which when administered in adequate amounts have the potential to exert a beneficial effect on the health of the host (Food and Agriculture Organization/World Health Organization 2002).

Probiotics in ruminants could be used to modify the microbiota in order to improve feed energy yield. This could be achieved by manipulating the microbial population to enhance fibre and starch digestion, promoting VFA and diminishing or buffering lactate accumulation and thereby avoiding acidification of ruminal pH (Calsamiglia et al. 2006). However, knowledge of ruminal microbial diversity and of the existing and potential interrelationships between organisms needs to be improved to achieve any modification and modulation of ruminal fermentation (Kobayashi 2006). Therefore, isolation and characterization of native ruminal microorganisms is a first and necessary step that would allow the development of more efficient and "adapted-to-the-target" probiotics.

In the work study reported here, native bacterial isolates were obtained from the rumen of a cow grazing exclusively on grass pasture. Isolates were identified, and the ability of these isolates to modify fermentation dynamics in an in vitro approach was assessed. The ability of these isolates to modulate fermentation product profiles was also studied using three different substrates, namely, wheat straw as a fibrous complex substrate and oat xylan and microcrystalline cellulose as pure major components of insoluble fibre.

Materials and methods

Rumen sampling

Ruminal content was obtained manually from the ruminal sac of a cannulated dry Holstein cow fed only pasture, grazing on a mixture of grass (*Lolium multiflorum*) and legumes (*Trifolium repens* and *T. pratense*) at the Experimental Farm of the Veterinary Faculty (Facultad de Veterinaria, UdelaR, Uruguay). The care and handling of experimental animals were approved by the Bioethics Committee of the Veterinary Faculty, Uruguay.

Culture media, bacterial isolation and identification

A medium containing ruminal fluid (RM) was used for the routine culture of ruminal isolates. The composition of the medium was basically that reported by Stahl et al. (1998) with minor modifications; clarified rumen fluid was included (20 %) and prepared according to Grubb and Dehority (1976), the VFA mixture was acetic acid (5.8 mM), propionic acid (1.6 mM), butyric acid (8.6×10^{-1} mM), *n*-valeric (1.80×10^{-1} mM) and isovaleric ($1{,}80\times10^{-1}$ mM), modified in accordance with Macy et al. (1982).

For the isolation of native bacteria, ruminal content was obtained as described above and immediately gassed with CO_2; proper dilutions were made in sterile phosphate-buffered saline supplemented with Na_2S-Cys (0.25 g/L) that had been previously gassed with CO_2. Dilutions were seeded in 25-mL bottles that contained melted sterile RM, and the roll-tube method was performed (Hungate 1947). After 10 days of incubation at 39 °C native ruminal strains were isolated with the aid of sterile and pre-reduced syringes and needles. Based on the ability to reach high cell concentrations within 24 h, six of the native ruminal strains were randomly chosen for identification and further study on modulation properties.

Genomic DNA was extracted with the GeneElute Bacterial Genomic DNA Extraction kit (Sigma, St. Louis, MO) according to the manufacturer's directions. Identification of isolates was achieved by PCR amplification and sequencing of the almost complete 16S rRNA gene using primers 27F [5′-AGAGTTTGAT(C/T)(A/C)TGGCTCAG-3′] and 1492R (5′-TACCTTGTTACGACTT-3′). The PCR reactions were performed in a T1 thermocycler (Biometra GmbH, Göttingen, Germany) using a cycling programme that included an initial cycle of 94 °C for 3 min, 30 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1.5 min and a final extension at 72 °C for 10 min. Sequences were obtained from the sequencing service of Macrogen Inc. (Seoul, South Korea) with primers 27F, 1492R and internal primer 518F (5′-CCAGCAGCCG CGGTAAT-3′) and compared using the Classifier tool of the Ribosomal Database Project (Wang et al. 2007) and using BLASTn to compare with data in the GenBank database [National Center for Biotechnology Information (NCBI); Altschul et al. 1997].

In vitro gas production

In order to evaluate the potential effect of the probiotic bacteria on ruminal fermentation, we performed an in vitro gas production assay using different substrates following the method of Theodorou et al. (1994) with the modifications of Mauricio et al. (1999). Experiments were performed in 125-mL fermenters containing 0.5 g of each substrate [wheat straw, microcrystalline cellulose (Avicel® PH101; FMC BioPolymer, Philadelphia, PA) or oat xylan (Sigma)]. Wheat straw was ground to pass through a 1-mm sieve [percent composition:dry matter (DM) 87.3; DM:organic matter 96.0; neutral detergent fibre 74.7; acid detergent fibre 45.7; crude

protein 5.63; acid detergent lignin 7.87]. The fermenters were filled with a buffer solution, and Na_2S was included as a reductive solution after Oeztuerk et al. (2005). Substrates were allowed to hydrate at 4 °C for 18 h, at which time 10 ml of fresh, CO_2 gassed ruminal fluid was added to each fermenter, the headspace was saturated with CO_2 and the fermenters were sealed with rubber caps and aluminium seals.

The ruminal fluid used in these experiments was obtained from the ruminal content of the same animal described above and immediately processed. To separate the fluid from the rest, the content was filtered through four layers of cheesecloth.

After the fermenters were sealed, the suspensions of each of the six strains were observed microscopically. Cell density was calculated by direct microscopy counts, and proper suspensions were prepared and added to the different fermenters using sterile and pre-reduced syringes so as to obtain a final suspension of approximately 10^6 cells/mL. Controls were prepared as described above but no potential probiotic strain was added. All incubations were performed in one batch. The strain was the statistical unit, and incubations were performed in triplicate for each strain–substrate combination.

Inoculated fermenters were incubated at 39 °C, and internal pressure was measured with a manual manometer (Cole Palmer Instruments Co., Vernon Hills, IL) coupled to a 0.6-mm hypodermic needle; measurements were made at 2, 4, 6, 8, 10, 18, 26, 48, 72 and 96 h after inoculation, and gas was vented after each pressure reading. When the last pressure measure had been made (t=96 h) the fermenters were opened, the pH was measured and samples were taken and mixed with perchloric acid (0.1 M, 1:1) in order to analyse VFA concentration by high-performance liquid chromatography (HPLC).

Internal gas pressure of the fermenters, expressed in psi (P), was converted to volume of gas (V) by the predictive equation $V=4.40\,P+0.09\,P^2$. This equation had been determined to be valid in previous experiments conducted under similar conditions.

VFA and lactic acid determination

Lactic acid and VFA (acetic, propionic and butyric acids) were quantified by HPLC separation. Samples were first centrifuged (15,000 rpm, 15 min, 5 °C), and the supernatants (100 μL) obtained were injected into a Waters modular HPLC system (Waters Associates, Milford, MA). The HPLC system included a binary HPLC pump (Waters model 1525), an auto sampler (Waters model 717 plus), a reverse-phase C18 column (5-mm particles, 150×34.6 mm; Phenomenex, Torrance, CA) and a photodiode array detector (Waters model 2998) linked to Empower 2 (Waters) chromatography data software. The temperature of the column was set at 30 °C. The mobile phase used was: (A) acetonitrile and (B) phosphoric acid (H_3PO_4) 0.5 %, pH=2, and it was pumped at 1 mL/min by the following gradient mode (min/%B): 2/98, 12/50, 12.25/95, 17.25/95, 17.50/2, 23/2. Quantitative analysis was performed against a standard solution containing 5 mg/mL of each acid.

Statistical analysis

Cumulated gas volume along time was compared among treatments (strains). For this purpose, the gas produced at a specific time was considered to be dependent on the preceding time. Consequently, this variable was analysed as a repeated measure over the flask (fermenter), separately for each substrate, according to the model:

$$\mathrm{Y}ijk = \mu + \mathrm{S}I + \mathrm{T}j + (\mathrm{S}*\mathrm{T})ij + \varepsilon ijk$$

where Yijk is the volume of gas produced, μ is the overall mean, SI is the fixed effect of the strain addition (I = Control, 50C, 55C, 60C, 21C, 22C, 62C) measured in k replicates (3 fermenters), Tj 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 εijk is the residual error. The interaction between treatment (strain) and time was considered to be important, as a significant interaction means that a strain produced gas faster or slower than the other one. Therefore, when S*T was significant, the strains were compared for each time separately, using the same model without the Tj and (S*T)ij terms.

For VFA concentration, pH and the acetic:propionic ratio (A/P) the model was similar, but in this case there was only one final measurement. Therefore, the term time and its interaction with the strain was excluded from the model:

$$\mathrm{Y}ik = \mu + \mathrm{S}I + \varepsilon ik$$

where Yijk is the concentration of acid (acetic, propionic, butyric, total VFA), A/P or pH, μ is the overall mean, SI is the fixed effect of the strain addition (I = Control, 50C, 55C, 60C, 21C, 22C, 62C) measured in k replicates (3 fermenters) and εik is the residual error.

All data were analysed using the MIXED procedure (PROC MIXED) of SAS software (ver. 8.2; SAS Institute, Cary, NC).

The Dunnett test was chosen as a post hoc analysis to compare means among strains, as this test compares each of a number of treatments (strains) with a single control. Differences among means with $P<0.05$ were considered to be statistically significant, and values with $0.05<P<0.10$ were considered to show a tendency to differ.

Results

Identification of ruminal bacterial strains

Six ruminal bacterial strains were randomly selected to evaluate their potential as modulators of ruminal fermentation. Strains 62C, 60C, 50C and 55C are affiliated with *Pseudobutyrivibrio ruminis*. Two isolates, 21C and 22C, are as yet uncharacterized members of the *Lachnospiraceae* family. Sequences were deposited at the Genetic sequence database at the NCBI under GenBank IDs: JF781301, JF781302, JQ316641, JQ316644, JQ316645 and JQ316655.

Modulation of in vitro gas production

The interaction S*T was significant ($P<0.001$) for the three substrates. Consequently, we analysed the effect of adding each strain at each sampling time separately.

Wheat straw

In fermenters containing straw, significant differences in gas volume production were observed at the beginning and end of the experiment. The addition of strains 21C, 22C and 62C led to significantly higher volumes of gas relative to the control at 2 h of incubation ($P=0.02$, 0.002 and 0.005, respectively; Fig. 1). At 96 h, strains 21C and 62C had induced significant lower gas volumes, and strain 22C showed the same tendency ($P=0.050$, 0.049 and 0.091, respectively; Fig. 1).

Oat xylan

Similar to fermenters containing wheat straw, those containing oat xylan showed a higher production of gas during the initial hours of incubation (up to 18 h) following the addition of each of the six strains (Fig. 2). However, at longer incubation times (48–96 h) gas accumulation in oat xylan fermenters supplemented with bacterial strains did not differ significantly from that of the controls.

Microcrystalline cellulose

The addition of ruminal bacterial strains 60C and 21C to microcrystalline cellulose fermenters resulted in the production of relatively higher volumes of gas during the initial stages of fermentation (Fig. 3). The addition of strains 21C, 22C and 62C separately to microcrystalline cellulose-fermenters produced a significantly lower volume of accumulated gas after 48 h of incubation relative to the control (Fig. 3), resembling the results obtained at late experimental times in fermenters that contained straw (Fig. 1).

VFA concentration and pH

When the concentration of VFA was analysed by HPLC we were unable to detect lactic acid in any substrate–strain combination. The concentration of the remaining VFA as well as of the A/P ratio and pH of the fermenters after 96 h incubation are shown in Table 1.

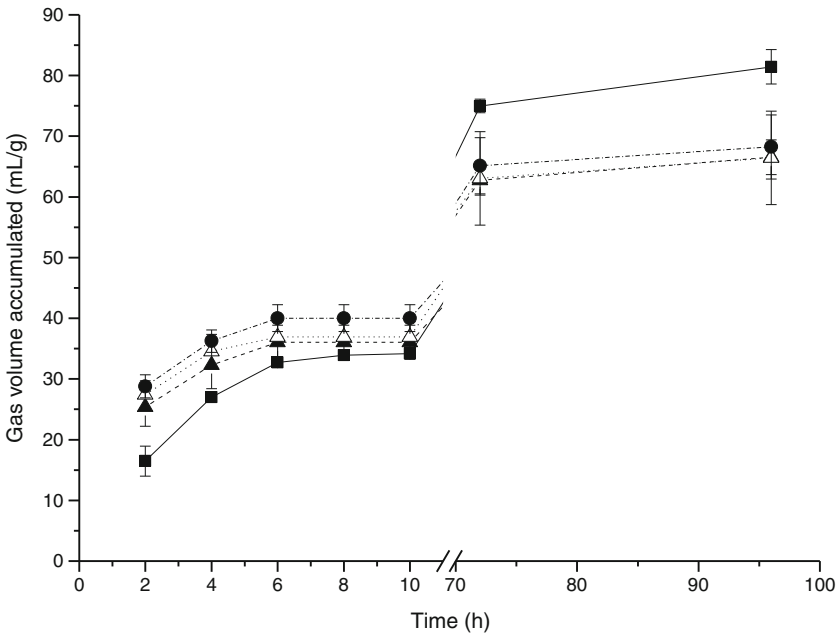

Fig. 1 Accumulated gas volume per gram of incubated substrate over time in fermenters which used wheat straw as substrate. *Filled triangle*, *filled circle*, *open triangle* Fermentation systems to which strains 21C, 22C and 62C, respectively, had been added. *Filled square* Control fermentation system, which differed from experimental systems only by the absence of ruminal strains

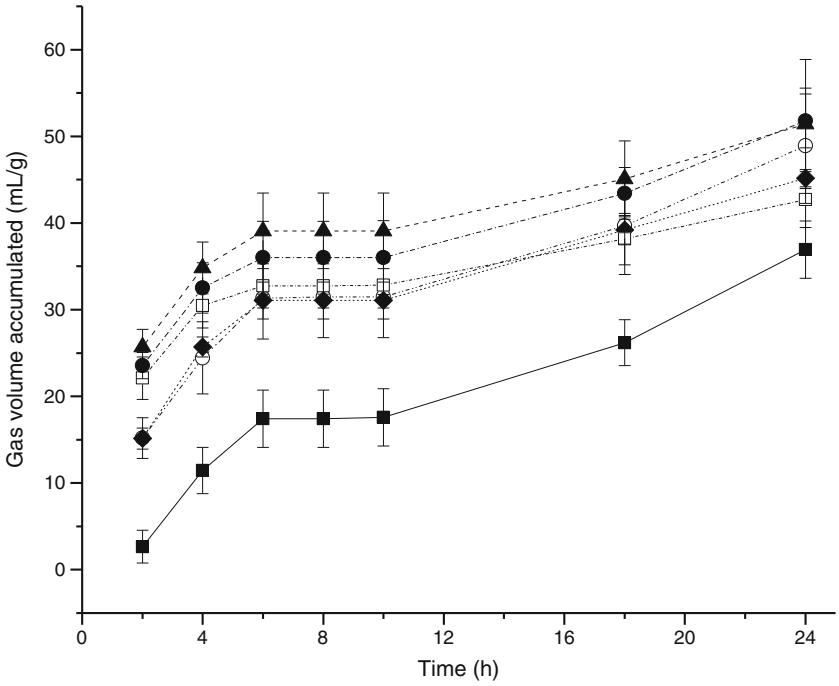

Fig. 2 Accumulated gas volume per gram of incubated substrate over time in fermenters which used oat xylan as the substrate. During the first 24 h of incubation, gas production in all fermentation systems to which ruminal bacterial strains had been added differed from that in the control (*filled square*). *Filled triangle* Strain 21C, *filled circle* strain 22C, *open triangle* strain 62C, *open square* strain 60C, *open circle* strain 50C, *filled diamond* strain 55C

Wheat straw

Fermenters with wheat straw as substrate and inoculated with strains 21C and 50C produced significantly higher concentrations of total VFA than the control (Table 1). The acetic acid concentration significantly decreased only in those fermenters treated with strain 55C, with acetic acid concentrations falling below that of the control. In general, the concentration of propionic acid produced in the different treatments was similar to that of the control, but the addition of strains 60C and 55C to the fermenter produced a relatively lower concentration of this acid. Butyric acid concentration was higher in all treatments than in the control, with concentrations reaching as high as 16.9 mM; in comparison the control produced <1 mM butyric acid (Table 1). The A/P ratio was only affected by the addition of strain 22C and was significantly higher in this treatment than in the control. Fermenters treated with 22C and 62C had a significant higher pH values than the control, while the opposite effect was observed following the addition of strain 50C (Table 1).

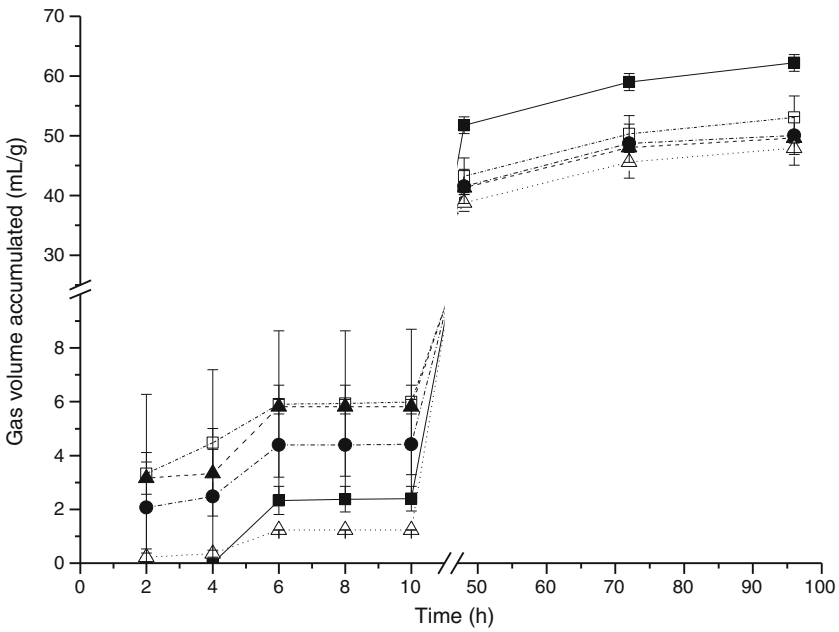

Fig. 3 Accumulated gas volume per gram of incubated substrate over time in fermenters which used microcrystalline cellulose as substrate. Only the results for those ruminal bacterial strains whose addition resulted a significantly different production of gas relative to the control (*filled square*) are shown. *Filled triangle* Strain 21C, *filled circle* strain 22C, *open triangle* strain 62C, *open square* strain 60C

Table 1 Fermentation products produced in fermenters with wheat straw, oat xylan or microcrystalline cellulose as substrate[a]

Variables	Substrate	Control fermentation	Ruminal bacterial strains					
			21C	22C	62C	60C	50C	55C
VFA concentration (mM)								
Acetic acid	Wheat straw	41.2 (3.1)	43.9 (2.8)	41.8 (3.2)	35.9 (5.4)	32.6 (0.6)	44.3 (2.2)	30.6 (1.6)*
	Oat xylan	47.0 (5.5)	49.6 (1.7)	44.3 (3.5)	42.7 (1.1)	48.1 (2.2)	32.4 (2.7)	22.1 (21.0)*
	Cellulose	38.3 (4.5)	39.5 (3.2)	34.5 (3.1)	31.7 (4.9)	31.5 (3.5)	39.8 (0.6)	27.4 (3.2)*
Propionic acid	Wheat straw	13.9 (1.0)	14.7 (0.9)	12.0 (0.5)	12.1 (2.0)	9.6 (0.2)*	15.1 (0.8)	9.2 (0.4)*
	Oat xylan	11.8 (0.9)	11.5 (0.8)	10.6 (0.7)	10.2 (0.2)	11.2 (0.8)	6.2 (0.3)*	4.9 (4.9)*
	Cellulose	8.8 (1.0)	9.5 (0.8)	12.3 (0.4)*	7.7 (1.3)	6.4 (0.9)*	9.3 (0.2)	5.5 (0.8)*
Butyric acid	Wheat straw	0.3 (0.1)	16.9 (0.8)*	13.4 (1.0)*	11.6 (1.57)*	11.9 (0.2)*	16.2 (0.4)*	7.8 (4.7)*
	Oat xylan	0.7 (0.2)	31.0 (0.9)*	22.6 (2.0)*	22.3 (0.6)*	30.4 (2.1)*	11.5 (9.5)	13.8 (13.1*
	Cellulose	0.1 (0.1)	24.8 (2.0)*	17.3 (2.1)*	16.2 (2.1)*	18.8 (2.3)*	25.0 (0.4)*	16.6 (2.1)*
Total VFA	Wheat straw	55.3 (4.0)	75.5 (4.5)*	67.2 (3.7)	59.6 (9.0)	54.1 (0.7)	75.6 (3.3)*	47.8 (5.0)
	Oat xylan	59.5 (6.0)	92.2 (2.2)	77.5 (6.2)	75.2 (0.9)	89.8 (4.8)	50.17 (6.4)	40.8 (39.1)
	Cellulose	47.2 (5.4)	73.8 (6.1)*	64.1 (4.7)*	55.7 (8.3)	56.7 (6.8)	74.1 (0.7)*	49.5 (6.1)
Acetic:propionic ratio	Wheat straw	2.96 (0.02)	2.98 (0.01)	3.49 (0.42)*	2.99 (0.06)	3.40 (0.02)	2.94 (0.02)	3.34 (0.07)
	Oat xylan	3.97 (0.39)	4.31 (0.14)	4.16 (0.10)	4.19 (0.08)	4.30 (0.15)	5.21 (0.16)*	4.68 (0.28)*
	Cellulose	4.37 (0.12)	4.18 (0.05)	2.80 (0.30)*	4.13 (0.10)	4.93 (0.19)*	4.28 (0.01)	5.00 (0.16)*
Final pH	Wheat straw	5.8	5.82	5.88*	5.92*	5.87	5.49*	5.87
	Oat xylan	5.8	5.18*	5.14*	5.16*	5.14*	5.49*	5.14*
	Cellulose	5.8	5.50*	5.51*	5.52*	5.51*	5.49*	5.50*

*Significant from control (Dunnett adjustment) at $P<0.05$ for within-row values

Standard deviation is indicated between brackets

[a] Volatile fatty acid (VFA) concentration, pH and acetic/propionic ratio (A/P) were determined at 96 h of incubation in fermenters with either wheat straw, oat xylan or microcrystalline cellulose as substrate

Oat xylan

No significant differences in total VFA concentration were found in the strain-treated fermenters compared to the control. However, addition of strain 55C induced a lower production of acetic acid. Propionic acid concentration was lower in the fermenters treated with strains 50C and 55C, respectively, with with oat xylan as the substrate (Table 1).

Microcrystalline cellulose

Similar to the results obtained with straw as substrate, the addition of strains 22C and 50C to fermenters with microcrystalline cellulose as substrate resulted in a higher production of total VFA; this enhanced VFA production was also observed in fermenters treated with 21C (Table 1). The concentration of acetic acid was lower in fermenters treated with strain 55C, and propionic acid concentration was enhanced in the fermenter treated with 22C; the opposite was observed with the addition of strains 55C and 60C (Table 1). As observed in the wheat straw experiments, the addition of all strains resulted in increased concentrations of butyric acid. The A/P ratio fell following the addition of 22C but increased with the addition of strains 60C and 55C. In all cases the pH was slightly lower in the treatments than in the control fermenters (Table 1).

Discussion

In this study, we isolated bacterial strains from the native ruminal microbiota of a grazing cow and subsequently evaluated their potential to modulate ruminal fermentation in vitro. We determined that four of the isolates were affiliated with *Pseudobutyrivibrio ruminis*, which is frequently isolated from the rumen (Forster et al. 1996; Nyonyo et al. 2013); this species is referred as *Butyrivibrio*-like and forms an assemblage of species typically associated with hemicellulose degradation. This bacterium can account for 24–30 % of ruminal cultivable bacteria (Shoep and Greeg 2007). *P. ruminis* has been related to fibrolysis based on its detection in fibrolytic consortia (Koike et al. 2003; Shinkai et al. 2010), and its phenotype resembles *Butyrivibrio fibrisolvens* (van Gylswyk et al. 1996).

The 16S DNA sequences of strains 21C and 22C were found to be identical to an as yet uncharacterized group of bacteria isolated from the rumen contents of a fistulized sheep, related to *Lachnobacterium* (Kenters et al. 2011). These

organisms are members of the *Lachnospiraceae* family that includes the *Butyrivibrio*-like species.

In vitro gas production methodology was used to evaluate the possible modification or modulation of fermentation following the addition of potential probiotics to the fermenter with wheat straw, a fibrous substrate, or either of two pure substrates, oat xylan and microcrystalline cellulose, respectively, as substrate. This technique has been used to study the influence of chemicals on fermentation dynamics (García et al. 2007), the influence of the roughaghe:concentrate proportion on fermentation, microbial interactions and methane production (Kumar et al. 2013a, b) and the effect of a *Megasphaera eldesnii* strain in buffering lactic acid concentration (Kung and Hession 1995).

The addition of the different ruminal bacterial isolates to the fermentation system produced differences in the volume of gas produced and in VFA content compared to the control, indicating that these isolates modulated the fermentation patterns for all three substrates . The gas production patterns also varied among the different substrates. The effects of *P. ruminis* 62C and *Lachnospiraceae* 21C and 22C on fermentation were similar with all three strains, gas volume increased during the first stages of straw fermentation and decreased during the later phases of the experiment compared to the control. This modulating effect observed during the early stages of straw degradation (and also in xylan degradation; Fig 2) may be related to the breakdown of hemicelluloses since xylan the second most important structural component of straw (cellulose being the most important) (Joseleau et al. 1992). When the late stages of straw fermentation are compared, the modulating effect resembled the behaviour in the microcrystalline cellulose fermentations.

Our results indicate that *Lachnospiraceae* 21C induced a higher concentration of total VFA compared to the control. This finding and the decreased total gas production suggest that the addition of ruminal bacterial strains to the fermentation system induced some metabolic modifications that resulted in a more efficient fermentation with respect to the conversion of carbon sources into VFA instead of gas. It is likely that the addition of the potential probiotics induced the retention of metabolic hydrogen through VFA production. The release of hydrogen or its transfer to methane is one of the energetic losses of ruminal fermentation as these gases are lost through eructation. About 3–15 % of ingested energy is lost through methane emission (Calsamiglia et al. 2006). If these strains are able to modify fermentation in order to induce hydrogen retention, then the modulating effect would improve energy and carbon utilization by ruminants.

The addition of the ruminal bacterial strains to the fermentation system resulted in significantly higher butyric acid concentrations compared to the control fermenters independently of the substrates. The addition of *P. ruminis* 50C and *Lachnospiraceae* 21C to the fermenters also resulted in significantly higher total VFA concentrations at the end of the experiment when the substrate was straw or microcrystalline cellulose. These results, the absence of any difference in total gas volume with the addition of strain 50C to the fermenter and the decrease in total gas volume following the addition of strain 21C supports the hypothesis of a metabolic shift related to the addition of bacterial probiotics.

The higher levels of butyric acid associated to the addition of the *P. ruminis* strains (50C, 55C, 60C and 62C) could be also explained by the ability of these bacteria to form butyric acid as a product of their own metabolism (Schoep and Gregg 2007). Nevertheless, as the initial bacterial inocula were about 10^6 cells/mL (about 1,000-fold lower than the approximate microbial population initially present in the rumen fluid inocula), this difference should also involve changes in the microbial community.

Butyric acid plays an important role in the rumen, being the most energetic VFA together with propionic acid (Calsamiglia et al. 2006). Butyric acid is the most metabolized acid in the rumen epithelium (Baldwin VI and Jesse 1996; Baldwin VI and McLeod 2000) and has mitotic effects on the epithelium during its development (Menstchel et al. 2001). Our observation that the addition of probiotics to the fermentation system enhanced butyric acid production without altering the A/P ratio or pH should be taken into consideration in the formulation of additives for animal production. In this context, Seymour et al. (2005) demonstrated a strong positive correlation between this acid, DM intake and milk production.

Based on the results of our study, in vitro gas production technique would appear to be a useful tool to evaluate the ability of potential probiotic bacterial strains to modulate ruminal fermentation. This technique is less expensive and easier to implement than other approaches, and it can be used to screen the attributes of microbial strains prior to complex and expensive approaches, such as vivo trials (Sirohi et al. 2013). We observed that the addition of native ruminal bacterial strains in relative low single doses was able to modify ruminal fermentation patterns. The positive effect was shown by the production of higher quantities of VFA compared to the control without increasing the volume of gas produced. This is an interesting issue for the design of new probiotic additives for animal feed. In particular, strains *P. ruminis* 50C and *Lachnospiraceae* 21C are good candidates for such research as they induced a higher production of VFA without altering the gas volume and produced a higher quantity of butyrate, a very important VFA in terms of milk quality and production (Seymour et al. 2005). We hypothesize that these bacteria induce a higher fermentation of xylan at the beginning of fermentation and ultimately induce a higher production of VFA at the end of fermentation through cellulose degradation.

Based on our results, we conclude that small doses of ruminal native bacteria are suitable for modulation of ruminal

fermentation, thereby justifying further research in this area to improve productive yields.

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Conflict of interest None.

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