PAPER

The use of quantitative real time polymerase chain reaction to quantify some rumen bacterial strains in an *in vitro* rumen system

Lucy Onime,[1] Cristina Zanfi,[1] Chiara Agostinis,[2] Roberta Bulla,[3] Mauro Spanghero[1]

[1]Dipartimento di Scienze Agrarie ed Ambientali, Università di Udine, Italy
[2]Dipartimento Materno-neonatale, Istituto di Ricovero e Cura a Carattere Scientifico Burlo Garofolo, Trieste, Italy
[3]Dipartimento di Scienze della Vita, Università di Trieste, Italy

Abstract

The aim of this work was to quantify four rumen bacterial strains (*Butyrivibrio fibrisolvens, Ruminococcus albus, Streptococcus bovis, Megasphaera elsdenii*) in an *in vitro* batch rumen fermentative system by quantitative real time polymerase chain reaction (qPCR). The experiment was a 2×2 factorial arrangement with two types of liquid rumen, collected from dairy cows (DC) and fattening bulls (FB) and two types of fermentation substrate (forage:concentrate ratios, 75:25 and 25:75) and was replicated in two fermentation runs. Fermentation fluids from FB compared to those from DC had lower pH, higher total VFA concentrations (averages of 0 and 24 h samplings, 6.70 *vs* 7.04 and 72.6 *vs* 42.7 mmol/l P<0.001) and contained less acetic (P=0.014) and more propionic (P<0.01) and butyric (P=0.029) acids. The two types of substrates incubated produced very small differences in the end fermentation products.

B. fibrosolvens concentrations were higher (P<0.001) in the DC fermentation fluids compared to that from bulls (averages of 0 and 24 h sampling times, 3.47 *vs* 1.38 x10^9 copies /mL), while *M. elsdenii* was detected only in FB fermentation fluids. *R. albus* and *S. bovis* concentrations were not different between the two types of rumen liquid. With the only exception for *B. fibrosolvens*, bacteria strains considered in this study increased their concentrations in the fermentation fluid during the 24 h of *in vitro* incubation.

Introduction

The *in vitro* rumen fermentation in batch culture systems are the simplest simulations of the rumen conditions. These techniques make use of uncomplicated apparatus (*e.g.* jars, flasks, tubes, stopped serum vials, glass syringes, *etc.*), utilise buffered rumen fluid without liquid turnover but have a limited duration of fermentation (*e.g.* 24 and/or 48 h). Despite this high simplification of rumen conditions, these systems have been largely utilised to rank the nutritive value of feeds (Getachew *et al.,* 2002; Spanghero *et al*., 2010) or to study the effect of different additives in modifying rumen fermentation (Cardozo *et al*., 2005; Speight and Harmon, 2010).

Recent progress in molecular techniques allows direct quantification of different microbial strains in rumen fluid by quantitative real time polymerase chain reaction (qPCR) and some recent papers have applied these procedures to samples collected *in vivo* or from *in vitro* continuous systems (Martínez *et al.*, 2010, Palmonari *et al*., 2010; Popova *et al*., 2011). Moreover, the application of molecular techniques to fermentation fluid from batch *in vitro* systems would represent a possible improvement in their investigative potential.

The aim of this experiment was to use the qPCR to monitor variations in four rumen bacterial strains (*Butyrivibrio fibrisolvens, Ruminococcus albus, Streptococcus bovis, Megasphaera elsdenii*) in an *in vitro* batch system. Different fermentative conditions were created using two types of rumen inoculums, collected from dairy cows (DC) and fattening bulls (FB) and two types of substrate (with different proportions of forages and concentrates).

Materials and methods

In vitro rumen fermentation

The experiment was a 2×2 factorial arrangement with two types of liquid rumen (DC and FB, respectively) and two types of fermentation substrate (forage:concentrate ratios, 75:25 and 25:75, designated F and C, respectively) and was replicated in two fermentation runs. The apparatus used (Ankom, Tech. Co., Fairport, NY, USA) is composed of four digestion jars (2 L capacity), which have to be filled with pre-warmed buffer solutions (39°C, 1660 mL), with filtered rumen fluid (400 mL) and with 24 filter bags (size 5*3 cm,

250 mg dried substrate/bag, Ankom 57). In each fermentation run, two jars were filled with rumen fluid collected at slaughter house from 3 culled dairy cows and the other two with rumen fluid from 3 fattening bulls. One of the two jars with the same rumen fluid was filled with 18 bags containing forages (two types of meadow hay), 4 bags of extracted soya bean and 2 bags of corn meal (forage:concentrate ratios, 75:25, F substrate); the other jar was filled with 6, 4 and 14 bags containing corn, extracted soya bean and forages, respectively (forage:concentrate ratios, 25:75, C substrate).

At the beginning of fermentation and after 24 h, the jars were opened under a CO_2 flow to allow duplicate 20 mL rumen fluid collection. The pH of each sample was measured and the sample was divided into two parts and frozen until needed with one part allotted for DNA extraction and the other for volatile fatty acid (VFA) analysis. At the end of incubation (48 h) the bags were removed from the jars, carefully rinsed with tap water and dried (60°C oven for 48 h). Samples of feed substrates were analysed for dry matter and for CP (nitrogen x 6.25) contents (AOAC, 2000, methods 930.15 and 976.05, respectively). The neutral detergent fiber (NDF) content (Van Soest *et al.,* 1991) of feed samples and fermentation bag residues was analysed by Ankom[II] Fiber Analyser (Ankom) and were used to calculate the NDF degradability (NDFD).

Volatile fatty acid analysis by gas-liquid chromatography

Duplicate 10 mL fermentation fluid samples were thawed, centrifuged at 13,400 g (30 min

at 10°C), filtered through a 0.45 μm filter and 1.5 mL of the filtrate was added with 3 mL of 2-ethylbutyrate acid solution (Sigma-Aldrich, CR, 99% pure, code 109959). Samples were analyzed for VFA by gas-liquid chromatography (Carlo Erba, 5300 Mega series GC) equipped with a Nukol glass column (length: 30 m; internal diameter: 0,25 mm; filter thickness: 0,25 μm; code 24107, Supelco Inc., Bellefonte, PA, USA). The analysis was run with a program temperature ranging from 100 to 200°C with an increment of 10°C/min and split ratio 1:30. The FID and injector temperatures were maintained at 200°C and pressure of carrier gas (He) was 200 kPa. All the detected peaks were resolved in 12 min, taking into account the 2 min standby at the end of each race. The Standard Acid Volatile Mix (Supelco Inc., code 46795-U) was chosen as the external standard for calculating the response factor and evaluation of retention time. Several runs were made using the external standard and the internal standard at comparable concentrations to obtain the response factor for each individual volatile fatty acid.

DNA extraction procedures

Ten mL of fermentation fluid sample was thawed, centrifuged at 500 g for 5 min to sediment plant debris and the resulting supernatant centrifuged at 13,500 g for 15 min. The pellet was washed and subjected to extraction using the QIAamp® DNA Stool Mini Kit (QIAGEN, Düsseldorf, Germany, code 51504) following manufacturer's instructions. The exception being that reaction volumes were scaled up proportionally. The extracted DNA was run on 0.8% agarose gel and ultraviolet (UV) absorbance at 280, 260 and 230 nm were measured using a spectrophotometer (NanoDrop ND-1000, Nanodrop Technologies, Wilmington, DE, USA) to determine DNA concentration and purity.

Quantitative qPCR

The following microorganisms were chosen as target to be enumerated using qPCR: *B. fibrisolvens, R. albus S. bovis, M. elsdenii.* Specific primers that would amplify 16S rRNA gene sequences were obtained from literature and their annealing temperatures are shown in Table 1. Quantitative PCR was performed using a RotorGene6000 QPCR thermal cycler (Explera, Qiagen, Milano, Italy) in a 10 μL reaction mixture consisting of 0.5 μL of DNA template (prediluted 1:50), 2× DyNAmo Flash SYBR green qPCR (ThermoFisher Scientific Finnzymes) and 300 nM of each primer.

Standards were generated using dilutions of purified genomic DNA (purchased from DSMZ, Braunschweig, Germany) or extracted from pure cultures with known concentration. The 16S rRNA gene copy numbers were calculated using the copy number calculator at the URI Genomics and Sequencing center web site (http://www.uri.edu/research/gsc/resources/cndna.html). Dilution series of the standards ranging from 10^1 to 10^6 copies of the 16S rRNA gene were used. The efficiency and functionality of the primer used in each PCR assay were checked using positive and negative controls. PCR amplifications were performed in triplicate for all standards with a 10 min denaturing step at 95°C, followed by 45 cycles of 94°C for 30 s, annealing temperature for 30 s (Table 1), and 72°C for 40 s. Melt curve analysis was performed between 55°C and 95°C. Each run included a calibration curve and a negative control. Fluorescence of the sample spectrum was acquired using 470 nm excitation filter and detected at 510 nm during each elongation stage; qPCR followed by melting curve analysis allowed differentiation of amplicons and identification of false positives. The concentration of the amplified DNA was calculated using the Cycling feature in the RotorGene 6000 software (Rotor-Gene ScreenClust HRM Software). The data obtained were expressed as copies per mL.

Statistical analysis

Data of pH, VFA content, VFA proportions and qPCR bacterial counts were analysed with the following four factors model:

$$y = \mu + \alpha_i + \beta_j + \gamma_k + \delta_l + (\alpha\beta)_{ij} + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + (\alpha\beta\gamma)_{ijk} + \varepsilon_{ijkl}$$

where
μ=overall mean;
α=fixed effect of origin of rumen fluid (i=1,2);
β=fixed effect of type of fermentation substrate (j=1,2);
γ=fixed effect of sampling time (k=1,2);
δ=fixed effect of fermentation run (block, l=1,2).

NDFD were analysed with the same model without the effect of sampling time.

Results and discussion

The two rumen inoculums were collected from animals fed very different diets in terms of starch and fibre contents. The dairy cows culled for low milk yield or for fertility problems are usually at the end of their lactation and therefore receive diets with high fibre levels, while fattening bulls are generally fed with diets rich in concentrates in the last period of fattening cycle. According to the different rumen liquor source utilised, there were several differences such as pH, total yield and composition of VFA of the fermentation fluids (Table 2). Fermentation was intense in fermenters with rumen fluid from FB, given their lower pH values (P<0.001) and higher total VFA concentrations (P<0.001) compared to DC fluids (averages of both samplings, 6.70 *vs* 7.04 and 72.6 *vs* 42.7 mmol/l). Moreover, FB fermentation liquids contained less acetic (P=0.014) and more propionic (P<0.01) and butyric (P=0.029) acids than that of cows. Finally, the fiber degradation of both hay samples was higher (P<0.001) when bags were incubated

Table 1. PCR primers used for quantification of selected rumen bacteria by real-time PCR.

Target	Primer sequences (5' to 3')	References	Annealing temperature	Product size
Butyrivibrio fibrisolvens	F:ACACACCGCCCGTCACA R:TCCTTACGGTTGGGTCACAGA	Klieve *et al.*, 2003	60°C	64bp
Ruminococcus albus	F:CCCTAAAAGCAGTCTTAGTTCG R:CCTCCTTGCGGTTAGAACA	Koike and Kobayashi, 2001	55°C	175bp
Streptococcus bovis	F:ATGTTAGATGCTTGAAAGGAGCAA R:CGCCTTGGTGAGCCGTTA	Klieve e*t al.*, 2003	60°C	90bp
Megasphaera elsdenii	F:AGATGGGGACAACAGCTGGA R:CGAAAGCTCCGAAGAGCCT	Stevenson and Weimer, 2007	54°C	95bp

F, forward primer; R, reverse primer.

in fermentation fluid obtained from DC than FB (48% *vs* 43% and 67% *vs* 56%).

The type of substrate added with the incubated bags in jars C and F differed. In fact, CP and NDF contents of hays (sample 1, 12.0 and 57.4% DM; sample 2, 11.0 and 62.2% DM), corn meal (9.8% and 9.7% DM) and extracted soybean meal (52.9% and 18.4 % DM) allowed to calculate that the substrates incubated in the F and C jars differed greatly in terms of the overall NDF contents (48.6 and 23.6 % DM, respectively), while were similar for the CP contents (18.2% and 17.4% DM, respectively). However, the two types of substrates incubated produced very small differences in the end fermentation products and there was only a tendency to have higher proportion of propionic acid after 24 h of fermentation in the C jars (P=0.137 for the interaction type of rumen inoculums origin and sampling time). The different fermentative substrates affected the NDFD of soya bean meal, which was higher (P=0.021) in the C fermenters (63% *vs* 58%).

Overall both the degradability and fermentation data indicate that the two factors included in the experiment (*e.g.* type of rumen liquor and substrate) showed a very different capacity to influence fermentation. While both the fermentation end products profile and the NDFD of hays support the hypothesis of a more favourable environment for fibrolytic fermentation in the DC fermentation fluids, the rotating jar system appears to be slightly insensitive to modifications of substrate based on different proportions of bags containing forages and concentrates. The lack of substrate effect could be due to a very low ratio between DM substrate and rumen fermentative liquor in fermentative jars of the Daisy apparatus (*i.e.* 3-4 g DM incubated/L of rumen fermentation fluid) when compared with other batch *in vitro* systems (7 and 10 g/L, in the Menke and

Table 2. Effect of rumen inoculum origin, type of substrate and sampling time on the pH, volatile fatty acids and neutral detergent fibre digestibility (measured at only 48 h of fermentation) in an *in vitro* system.

		Rumen inoculum				Significance						RSE
		Cows		Bulls								
Forage:concentrate		75:25	25:75	75:25	25:75	L	S	T	LxS	LxT	SxT	
Sampling time, h												
pH	0	7.18	7.24	6.88	6.92	<0.001	0.766	<0.001	0.699	0.383	0.158	0.08
	24	6.93	6.82	6.51	6.47							
Total VFA, mmol/L	0	29.60	32.10	61.90	71.40	<0.001	0.365	0.003	0.054	0.129	0.730	9.08
	24	57.80	51.14	68.84	88.70							
Acetic acid, mmol/100 mmol	0	76.90	76.30	63.90	65.00	0.014	0.318	0.044	0.873	0.671	0.256	4.32
	24	72.70	67.91	62.80	57.73							
Propionic acid, mmol/100 mmol	0	15.50	15.50	23.80	23.20	<0.001	0.231	0.179	0.852	0.073	0.137	2.04
	24	18.02	20.51	21.00	24.60							
Butyric acid, mmol/100 mmol	0	7.50	8.20	12.40	11.80	0.029	0.440	0.017	0.658	0.354	0.453	2.41
	24	9.12	11.57	16.20	17.66							
NDFD, %												
Meadow hay (forage 1)	48	48.8	47.1	43.4	42.5	<0.001	0.097	-	0.607	-	-	1.81
Meadow hay (forage 2)	48	66.3	66.8	57.4	56.6	<0.001	0.586	-	0.298	-	-	2.05
Soya bean meal, extract	48	55.4	64.5	60.0	62.0	0.628	0.021	-	0.123	-	-	5.37
Corn meal	48	90.5	89.9	88.1	89.4	0.018	0.493	-	0.117	-	-	1.36

L, inoculum origin; S, type of substrate; T, sampling time; interaction LxSxT, P>0.10; RSE, residual standard error; VFA, volatile fatty acids; NDFD, neutral detergent fibre digestibility.

Table 3. Effect of rumen inoculum origin, type of substrate and sampling time on the absolute abundance of target rumen bacteria determined by real-time PCR (expressed as 16S rRNA copies/mL) in fermentation fluid from an *in vitro* system.

		Rumen inoculum				Significance						RSE
		Cows		Bulls								
Forage:concentrate		75:25	25:75	75:25	25:75	L	S	T	LxS	LxT	SxT	
Sampling time, h												
Butyrivibrio fibrisolvens, ×10⁹	0	4.43	3.96	1.48	1.21	<0.001	0.223	0.110	0.351	0.082	0.734	0.76
	24	3.39	2.09	1.41	1.43							
Ruminococcus albus, ×10⁷	0	0.77	0.16	0.06	0.91	0.265	0.067	0.004	0.017	0.293	0.028	0.40
	24	1.86	0.33	1.80	1.34							
Streptococcus bovis, ×10⁷	0	0.12	0.09	0.08	0.07	0.034	0.187	0.003	0.026	0.362	0.615	0.04
	24	0.27	0.14	0.11	0.17							
Megasphaera elsdenii, ×10⁴	0	-	-	0.86	1.15	-	0.972	0.150	-	-	0.684	0.83
	24	-	-	1.98	1.74							

L, inoculum origin; S, type of substrate; T, sampling time; interaction LxSxT, P>0.10; RSE, residual standard error.

Steingass (1988) and Tilley and Terry (1963) systems, respectively). A shortage of incubated substrate with respect to fermentation fluid causes an excess in fermentability and this probably diminishes any possible impact on the fermentation process, whatever the substrate may be. A further limitation could be the utilisation of bags to fed the fermentation jar, because their porosity limit the access to substrate of rumen microbiota.

In this trial, the bacteria species were chosen as representative of the groups of organisms which play a significant role in rumen function. *B. fibrisolvens* and *R. albus*, were selected to represent hemicellulolytic and cellulose degrading rumen bacteria (Church, 1988; Koike and Kobayashi, 2009). Within the non fiber utilisers we selected *S. bovis* as an amylolytic and lactate-producing bacteria and *M. elsdenii* as a propionate producing and lactate utilizing rumen bacteria. Results of qPCR quantification of the target rumen bacterial are presented in Table 3. *B. fibrosolvens* concentrations were higher (P<0.001) in the fermentation fluid from cows compared to that from bulls (averages of two sampling times, 3.47 *vs* 1.38×10^9 copies /mL). This is in agreement with what observed in the rumen liquor of animals fed a high forage diets compared to a high concentrate diets in earlier culture-based studies, (Dehority and Orpin, 1988; Latham *et al.*, 1972) and more recently using molecular techniques (Gudla *et al.*, 2011; Klieve *et al.*, 2003; Tajima *et al.*, 2001). *B. fibrosolvens* appeared quite stable during the fermentation in the FB fermentation fluid with the tendency (P=0.082) to a reduction in the DC fermentation fluid (interaction rumen liquor x sampling time). A decrease of concentration of this bacteria during *in vitro* fermentation was also found by Weimer *et al.* (2011).

In this experiment *R. albus* concentration did not change with respect to rumen liquid origin and there was a significant interaction (P=0.017) among the type of rumen liquor and the substrate, which is difficult to interpret. However, the interaction between substrate and sampling time (P=0.028) indicated, during the 24 h of fermentation, a clear increase of concentrations of *R. albus*, which doubled for the F substrate and increased of about 50% for the C substrate. The aptitude of *R. albus* to increase its population density in the *in vitro* fermentation fluids was also found by Weimer *et al.* (2011) in batch culture systems and by Muetzel *et al.* (2009) in a continuous fermentation systems. The *S. bovis* concentration was not different between the two types of rumen liquid, which is surprising given its starch-utilising aptitude (Klieve *et al.*, 2003). However, Klieve *et al.*

(2003) did not found *in vivo* an increase of this bacteria after a great increment of dietary starch and hypothesized that *S. bovis* is not one of the major starch-utilizing bacteria in the rumen. Finally, we observed a significant increment of concentration (P=0.003) during the *in vitro* fermentation, which was not found for this strain by Weimer *et al.* (2011). The fourth bacteria considered, *M. elsdenii,* utilises lactate which helps it to adapt favourably to concentrate diets and to mitigate the acidic conditions of the rumen (Henning *et al.*, 2010a; 2010b). In the first fermentation run this bacteria was not detected in rumen inoculum from cows, while in second run the concentrations were negible or very low ($0.14 \pm 0.07 \times 10^4$ copies /mL). Also Huws *et al.* (2010) did not detect either *M. elsdenii* among the bacteria in the rumen from samples taken *in vivo* from animals fed forage diets, while Klieve *et al.* (2003) was able to demonstrate *in vivo* a rapid growth of this strain after the increase of grain in the diets of animals. In accordance with Weimer (2011) and similarly to what described for *S. bovis*, M. *elsdenii* had the tendency (P=0.150) to increase its population density from 0 to 24 h of fermentation (average of both substrates, from 1.01 to 1.86 $\times 10^4$ copies /mL). With the only exception for *B. fibrosolvens*, bacteria strains considered in this study increased their concentrations in the fermentation fluid during the 24 h of *in vitro* incubation. This could be attributed to the suitable environmental conditions, to a low levels of competition and also to a lesser predation by protozoa, which disappear quickly in the jar rotating fermenter (our unpublished data) as usually found *in vitro* systems (Muetzel *et al.*, 2009).

Conclusions

The origin of rumen inoculums had a clear impact on the concentration of *B. fibrosolvens* and *M. elsdenii* in the fermentation fluids after 24 h of *in vitro* rumen fermentation. On the contrary, in the specific experimental condition adopted, the substrate type failed to influence the concentration of the bacterial strains studied. Given the relevant increment of populations density during fermentation for *R. Albus, S.bovis* and *M. elsdeni* it can be concluded that *in vitro* conditions did not depress the bacterial growth of these strains.

References

AOAC, 2000. Official Methods of Analysis, 17th ed., Association of Official Analytical Chemists, Gaithersburg, MD, USA.

Cardozo, P.W., Calsamiglia, S., Ferret, A., Kamel, C., 2005. Screening for the effects of natural plant extracts at two pH level on in vitro rumen microbial fermentation of a high-concentrate diet for beef cattle. J. Anim. Sci. 83:2572-2579.

Church, D.C., 1988. The ruminant animal: digestive physiology and nutrition. Prentice-Hall Ed., Englewood Cliffs, NJ, USA.

Dehority, B.A., Orpin, C.G., 1988. Development of, and natural fluctuations in, rumen microbial populations. In: P.N. Hobson (ed.) The rumen microbial ecosystem. Elsevier Publ., London, UK, pp 151-183.

Getachew, G., Crovetto, G.M., Fondevila, M., Krishnamoorthy, U., Singh, B., Spanghero, M., Steingass, H., Robinson, P.H., 2002. Laboratory variation of in vitro gas production and estimated metabolizable energy values of ruminant feeds. Anim. Feed Sci. Tech. 102:169-180.

Gudla, P., AbuGhazaleh, A.A., Ishlak, A., Jones, K., 2011. The effect of level of forage and oil supplement on biohydrogenation intermediates and bacteria in continuous cultures. Anim. Feed. Sci. Tech. 171:108-116.

Henning, P.H., Horn, C.H., Leeuw, K.J., Meissner, H.H., Hagg, F.M., 2010a. Effect of ruminal administration of the lactate-utilizing strain Megasphaera elsdenii (Me) NCIMB 41125 on abrupt or gradual transition from forage to concentrate diets. Anim. Feed Sci. Tech. 157:20-29.

Henning, P.H., Horn, C.H., Steyn, D.G., Meissner, H.H., Hagg, F.M., 2010b. The potential of Megasphaera elsdenii isolates to control ruminal acidosis. Anim. Feed Sci. Tech. 157:13-19.

Huws, S.A., Lee, M.R., Muetzel, S.M., Scott, M.B., Wallace, R.J., Scollan, N.D., 2010. Forage type and fish oil cause shifts in rumen bacterial diversity. FEMS Microbiol. Ecol. 73:396-407.

Klieve, A.V., Hennessey, D., Ouwerkerk, D., Forster, R.J., Mackie, R.I., Attwood, G.T., 2003. Establishing populations of Megasphaera elsdenii YE 34 and Butyrivibrio fibrisolvens YE 44 in the rumen of cattle fed high-grain diets. J. Appl. Microbiol. 95:621-630.

Koike, S., Kobayashi, Y., 2001. Development and use of competitive PCR assays for the rumen cellulolytic bacteria: Fibrobacter succinogenes, Ruminococcus albus and Ruminococcus flavefaciens. FEMS Microbiol. Lett. 204:361-366.

Koike, S., Kobayashi, Y., 2009. Fibrolytic rumen

bacteria: their ecology and functions. Asian Austral. J. Anim. 22:131-138.

Latham, M.J., Storry, J.E., Sharpe, M.E., 1972. Effect of low-roughage diets on the microflora and lipid metabolism in the rumen. J. Appl. Microbiol. 24:871-877.

Martínez, M.E., Ranilla, M.J., Tejido, M.L., Ramos S., Carro, M.D., 2010. Comparison of fermentation of diets of variable composition and microbial populations in the rumen of sheep and Rusitec fermenters. I. Digestibility, fermentation parameters, and microbial growth. J. Dairy Sci. 93: 3684-3698.

Menke, K.H., Steingass, 1988. Estimation of the energetic feed value obtained from chemical analysis and gas production using rumen fluid. Anim. Res. Dev. 28:7-55.

Muetzel, S., Lawrence, P., Hoffmann, E. M., Becker, K. 2009. Evaluation of a stratified continuous rumen incubation system. Anim. Feed. Sci. Tech. 151: 32-43.

Palmonari, A., Stevenson, D.M., Mertens, D.R., Cruywagen, C.W., Weimer, P.J., 2010. pH dynamics and bacterial community composition in the rumen of lactating dairy cows. J. Dairy Sci. 93:279-287.

Popova, M., Martin, C., Eugène, M., Mialon, M.M., Doreau, M., Morgavi, D.P., 2011. Effect of fibre and starch-rich finishing diets on methanogenic Archaea diversity and activity in the rumen of feedlot bulls. Anim. Feed. Sci. Tech. 166-167:113-121.

Spanghero, M., Berzaghi, P., Fortina, R., Masoero, F., Rapetti, L., Zanfi, C., Tassone, S., Gallo, A., 2010. Technical note: precision and accuracy of in vitro digestion of neutral detergent fiber and predicted net energy of lactation content of fibrous feeds. J. Dairy Sci. 93:4855-4859.

Speight, S.M., Harmon, D.L., 2010. Batch culture evaluation of carbohydrate inhibitors to moderate rumen fermentation. Anim. Feed. Sci. Tech. 155:156-162.

Stevenson, D.M., Weimer, P.J., 2007. Dominance of Prevotella and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. Appl. Microbiol. Biotechnol. 75:165-174.

Tajima, K., Aminov, R.I., Nagamine, T., Matsui, H., Nakamura, M., Benno, Y., 2001. Diet-dependent shifts in the bacterial population of the rumen revealed with Real-Time PCR. Appl. Environ. Microb. 67:2766-2774.

Tilley, J.M.A., Terry, R.A., 1963. A two-stage method for the in vitro digestion of forage crops. J. Brit. Grassland Soc. 18:104-111.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Method for dietary fibre, neutral detergent fibre and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583-3597.

Weimer, P.J., Stevenson, D.M., Mertens, D.R., Hall, M.B., 2011. Fiber digestion, VFA production, and microbial population changes during in vitro ruminal fermentations of mixed rations by monensin-adapted and unadapted microbes. Anim. Feed. Sci. Tech. 160:68-78.