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Effect of fibre- and starch-rich finishing diets on methanogenic *Archaea* diversity and activity in the rumen of feedlot bulls

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ABSTRACT

Methanogenesis plays an important role in maintaining low hydrogen partial pressure in the rumen and consequently in feed digestion. Diet composition and lipid supplementation are known to influence the extent of methane (CH₄) production in ruminants. However, the impact of diet composition on rumen methanogens is poorly understood. This study examined CH₄ production and methanogen community abundance, diversity and metabolic activity in the rumen of fattening bulls fed a fibrous diet (F; *n* = 10) or a starch rich diet supplemented with extruded linseed (SL; *n* = 10). Net CH₄ emissions from bulls fed SL were lower than emissions from bulls fed F. Methanogenic *Archaea* were studied using culture independent methods targeting the functional *mcrA* gene. Methanogen abundance was similar in the two groups of bulls, but protozoa numbers were 65% lower in the F group. The SL diet decreased methanogen diversity and metabolic activity, which were evaluated by PCR-DGGE and reverse transcriptase qPCR, respectively. In contrast, no changes occurred in bacterial numbers and diversity between diets. Data indicate that reduction in net CH₄ production with the SL diet was associated with changes in rumen microbiota, particularly methanogen activity. Characterization of the activity and structure of the whole rumen microbiota should provide a better prediction of methanogenesis.

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1. Introduction

Methane (CH₄) is produced in the gastrointestinal tract of ruminants by a particular group of microbes, the methanogenic *Archaea* (Baldwin and Allison, 1983). Methanogens use dihydrogen (H₂) to reduce carbon dioxide to CH₄, thus keeping H₂ partial pressure low and favouring fermentation of feeds. However, CH₄ is an undesirable end product of rumen fermentation as it represents an energy loss to the host animal and because it is a greenhouse gas.

Various strategies have been proposed to reduce enteric CH₄ emissions, mainly by dietary manipulation (Martin et al., 2010). CH₄ production/kg product in feedlot cattle decreases with increasing proportion of starch in the diet (Beauchemin and McGinn, 2005; Doreau et al., 2011). Lipid supplementation, within levels that do not affect dietary digestion and animal

Abbreviations: cDNA, complementary DNA; DGGE, denaturing gradient gel electrophoresis; DM, dry matter; qPCR, real time quantitative polymerase chain reaction; SF₆, sulfur hexafluoride; tNA, total nucleic acids.

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Table 1

Chemical composition of the experimental diets.

	Diet ^a		Barley straw
	F	SL	
Organic matter (g/kg DM ^b)	800	824	852
Crude protein (g/kg DM)	159	200	41
aNeutral detergent fibre (g/kg DM)	379	196	601
aAcid detergent fibre (g/kg DM)	206	104	383
Starch (g/kg DM)	75	337	31
Ether extract (g/kg DM)	28	47	–
Gross energy (MJ/kg DM)	18.9	19.7	19.3

^a Diet rich in fibre (F) or rich in starch and supplemented with lipids (SL).^b DM, Dry matter.

performance, has also been shown to generally decrease CH₄ production (Beauchemin et al., 2008; Eugène et al., 2008; Martin et al., 2008, 2010). It was recently shown that the combined effect of a high starch diet supplemented with extruded linseed reduced CH₄ production in feedlot bulls compared to a diet higher in fibre (Eugène et al., in this issue).

Previous findings suggest that there is no link between CH₄ production in ruminants and the numbers of methanogens in faunated and defaunated sheep (Newbold et al., 1995; Popova et al., 2010a) or with a coconut oil supplemented diet (Machmüller et al., 2003). In contrast, there was a shift in methanogen diversity in the rumen of steers following a switch from a low to high concentrate diet (Zhou et al., 2010), but CH₄ production was not measured. The methanogenic communities in ruminants with high and low residual feed intake fed a low energy diet were also reported to differ (Zhou et al., 2009). Understanding the ecology of methanogens in ruminants fed different diets can help to elucidate the microbial mechanisms of CH₄ production in the rumen.

The methanogenic pathway involves a series of co-enzymes, many of them unique and ubiquitous to methanogens (Rouviere and Wolfe, 1988). For instance, methyl coenzyme-M reductase is crucial to the terminal step of methanogenesis and the *mcrA* gene coding for this enzyme can be used as a phylogenetic and functional marker to enumerate methanogens and to assess their diversity in an ecosystem (Luton et al., 2002). Targeting a gene which codes for an enzyme also offers the potential for development of activity based detection methods by analysis of messenger RNA. Thus molecular biology tools that target the *mcrA* gene in DNA and mRNA extracted from the rumen are a reliable method to estimate the abundance, diversity and activity of rumen methanogens.

The aim of our work was to compare the methanogenic community in terms of number, diversity and metabolic activity in the rumen of young bulls fed two feedlot diets differing in the amount of fibre and energy dense starch and lipid components.

2. Materials and methods

2.1. Animals, diet and experimental design

The study was conducted at the animal experimental facilities of INRA's Experimental Unit in Bourges (France) and at the Herbivore Research Unit in Saint-Genès Champanelle (France). Animal procedures were in accordance with the guidelines for animal research of the French Ministry of Agriculture and applicable European guidelines and regulations for animal experimentation (www2.vet-lyon.fr/ens/expa/acc-regl.html).

Animals and treatments are described in Eugène et al. (in this issue). Briefly, bulls were divided in two groups and fed two fattening diets for 16–18 mo until slaughter. The two dietary treatments consisted of a diet rich in fibre (F) and a diet rich in starch supplemented with lipids (SL). Both diets were composed of concentrate and of barley straw offered *ad libitum*. Concentrate of the F diet (on a dry matter (DM) basis) consisted mainly of cereal by-products (403 g/kg, wheat and maize bran), dehydrated lucerne pellets (224), dehydrated beet pulp (212), rapeseed and palm kernel meal (75) and other ingredients (86). The concentrate of the SL diet consisted mainly of cereals (464; barley, maize and oat grains), dehydrated beet pulp (60), rapeseed meal (214) and a linseed mixture (120) containing extruded linseed and other components to achieve a theoretical level of 12 g/kg of DM as omega-3 fatty acids from linseed. Ingredients such as molasses and soya meal (142) completed the diet. Eugène et al. (in this issue) includes details of the analysis methods and the full list of ingredients. The complete chemical composition of the diets is in Table 1. Feed intake was recorded 5 d/wk and bulls were weighed every 2 wk. From the total of 50 bulls used by Eugène et al. (in this issue), 10 bulls were randomly selected from each diet group for our study.

2.2. CH₄ production

CH₄ production was measured at the start, middle and end of the fattening period. Each bull was measured individually during a 5 d period using the sulfur hexafluoride (SF₆) tracer technique (Eugène et al., in this issue). Only results of CH₄ production at the end of fattening from the 20 bulls were used in the data presented here.

2.3. Rumen content sampling for fermentation and microbial parameters

Bulls were slaughtered at INRA-Theix's experimental abattoir at average age of 18 ± 1.3 mo and body weight of 711 ± 55.8 kg. The entire gastrointestinal tract was removed as soon as possible after slaughter and representative samples of homogenised rumen contents were collected and weighed along with whole rumen contents.

The pH was measured with a portable pH-meter (CG840, electrode Ag/AgCl, Schott Geräte, Hofheim, Germany) at 5 locations immediately after opening the rumen. An aliquot of rumen contents (~ 250 g) was strained through polyester monofilament fabric ($250 \mu\text{m}$ mesh aperture) to separate the liquid phase. For volatile fatty acids (VFA), 0.8 ml rumen fluid filtrate was mixed with 0.5 ml of 4 mg/ml (w/v) crotonic acid and 20 mg/ml (w/v) metaphosphoric acid in 0.5 M HCl, kept at 4°C for at least 2 h, centrifuged ($16,500 \times g$ for 10 min at 4°C) and supernatant stored at -20°C until analysis. VFA were analysed by gas chromatography using crotonic acid as the internal standard (Morgavi et al., 2003) on a CP 9002 Gas Chromatography (Chrompack, Middelburg, Germany). One ml of rumen fluid was added to 1 ml MFS solution (35 ml/l formaldehyde, 0.14 M NaCl, 0.92 mM methylgreen) and stored in the dark at room temperature (i.e., 21°C) until protozoa were counted. Rumen fluid/MFS solutions were diluted in an equal volume of Phosphate Buffer Saline (PBS) before protozoa enumeration (Ranilla et al., 2007) under a microscope ($\times 400$) in a Neubauer Chamber.

Another aliquot of total rumen contents (~ 30 g) was diluted with 15 ml ice cold PBS pH 6.8 and homogenised, for three 1 min cycles with 1 min intervals on ice, using a Polytron grinding mill (Kinematica GmbH, Steinhofhalde Switzerland). Approximately 0.5 g were transferred to a 2 ml Eppendorf tube and mixed with 1 ml of RNeasy Lysis Solution (Qiagen, Crawley, UK). Tubes were kept at 4°C overnight to allow the solution to thoroughly penetrate the cells, as suggested by the manufacturer, and stored at -80°C until molecular analyses.

2.4. Total nucleic acids extraction and cDNA synthesis

Total nucleic acids (tNA) were extracted as described by Popova et al. (2010b). Each tNA sample was divided into two equal fractions; DNA in one of the fractions was digested with rDNase, RNase-free (from the Total RNA Isolation Kit, Macherey Nagel, France) using a modified protocol, as described by Popova et al. (2010b).

The yield and purity of extracted tNA and RNA samples were assessed by optical density measurements using a NanoQuant Plate on an Infinity spectrophotometer (TECAN, Switzerland). RNA integrity was estimated with the Agilent RNA 6000 Nano Kit on an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's recommendations. Results were analysed using the Software 2100 Expert, version B.02.07.SI482 (Beta; Agilent Technologies, Waldbronn, Germany). Indicators of RNA integrity were the RNA Integrity Number (RIN, maximum value 10), the 23S rRNA/16S rRNA ratio (optimum value 2) and the absence of degradation (low baseline; Schroeder et al., 2006).

Total RNA ($1 \mu\text{g}$) was reverse transcribed in cDNA using Random Primers (Promega, Madison, WI, USA) $0.5 \mu\text{g}/\mu\text{g}$ RNA. To allow template/primers to anneal, the mixture was brought to 70°C for 5 min followed by 5 min on ice. After annealing, a buffer mixture containing AMV reverse transcriptase (Promega, Madison, WI, USA), dNTP and DEPC-treated water was added to each tube and reverse transcription was performed for 60 min at 37°C using MyCycler thermal Cycler (BioRad, Hercules, CA, USA). In a final volume of $40 \mu\text{l}$, each reaction had 0.625 mM each dNTP, 30 units of AMV reverse transcriptase and $1.25 \times$ reaction buffer supplied with the enzyme.

A negative control with no template was completed for the reaction mixture. In addition, a negative control without addition of reverse transcriptase was completed for each sample. These negative controls were also used in subsequent PCR and qPCR reactions to confirm the amplification of cDNA templates free of DNA contamination.

2.5. Microbial diversity analysis by PCR-DGGE

The *mcrA* and *rrs* genes were used as molecular markers for methanogen and bacterial populations, respectively. For PCR amplification, 50 ng of total nucleic acids samples and $5 \mu\text{l}$ of cDNA samples were used.

PCR amplification of the *mcrA* sequences used the primers previously described to be specific for methanogens (Table 2) with the addition of a GC clamp (40 bp) at the 5'-end of the forward primer (Muyzer et al., 1993). Each reaction contained in

Table 2
Oligonucleotides used for PCR-DGGE and qPCR analysis.

Oligonucleotides	Oligonucleotide Sequences	Target	Amplicon length (bp)	Use in	References
534R – 341F-GC	5'-ATT ACC GCG GCT GCT GG 5'-(GC)-CC TAC GGG AGG CAG CAG	<i>rrs</i> Bacteria	193	PCR-DGGE	Muyzer et al. (1993)
<i>mcrA</i> R – <i>mcrA</i> F-GC	5'-TTC ATT GCR TAG TTW GGR TAG TT 5'-(GC)-GGT GGT GTM GGA TTC ACA CAR TAY GCW ACA GC	<i>mcrA</i> Methanogens	460–490	PCR-DGGE	Luton et al. (2002)
<i>qmcrA</i> -R – <i>qmcrA</i> -F	5'-GBARGTCGAWWCCGTAGAATCC 5'-TTCGGTGGATCDCARAGRC	<i>mcrA</i> Methanogens	140	qPCR	Denman et al. (2007)
799R2 – 520F	5'-AACAGGATTAGATACCCTG 5'-AGCAGCCGCGGTAAT	<i>rrs</i> Bacteria	280	qPCR	Edwards et al. (2007)

a 50 µl final volume, 1.25 units of HotStarTaq DNA Polymerase (Qiagen), 1 × PCR Buffer (supplied with the enzyme), 3 mM MgCl₂, 0.2 mM dNTP mix (Sigma, St. Louis, MO, USA) and 0.25 µM each primer. PCR was performed using MyCycler thermal Cycler (BioRad Laboratories) according to the protocol of Lutton et al. (2002).

The V3 region of the bacterial *rrs* gene was amplified in PCR using primers 341f-GC clamp and 534r (Table 2). Each reaction contained in 50 µl final volume 1.25 units of HotStarTaq DNA Polymerase (Qiagen), 1 × PCR Buffer (supplied with the enzyme), 0.2 mM dNTP mix and 0.25 µM of each primer. PCR was performed using MyCycler thermal Cycler (BioRad Laboratories) as described by Sadet et al. (2007).

PCR amplification products were analysed by agarose gel (20 mg/ml of TAE Buffer 1 ×, 40 mM Tris base, 40 mM glacial acetic acid, 1 mM EDTA) electrophoresis using ethidium bromide staining. Amplicon size and concentration were estimated using a Low DNA Mass Ladder (Invitrogen Corporation, Carlsbad, CA, USA) and an imaging system ChemImager (Alpha Innotech, San Leandro, CA, USA).

PCR products were loaded onto a 6–8 mg/ml (w/v) gradient polyacrylamide gel with different ranges of denaturant concentrations (100% denaturant was defined as 7 mol/l urea and 400 (v/v) formamide (Muyzer et al., 1993)). The *mcrA* PCR products were applied to a gradient of 10–30% denaturant. The denaturant gradient used to separate the bacterial *rrs* PCR products was 30–55%. DGGE was run in 0.5 × TAE Buffer for 5.3 h (for *mcrA*) and 5 h (for *rrs*) at 60 °C using a fixed voltage of 200 mV in DGGE 2001 (CBS Scientific Co, Del Mar, CA, USA). Approximately 100 ng of PCR product for both *mcrA* and *rrs* were loaded per well of the polyacrylamide gel.

Gels were silver stained using a commercial kit (BioRad Laboratories). The large number of samples required several gels to complete the study. To normalize for differences among gels, an outside sample from a wether was used as the standard. The same *mcrA* or *rrs* PCR products of this rumen sample were run on every gel and used as standard for comparison of gels. The similarity of standards between gels was 90.3% for the *mcrA* and 88.6% for bacterial *rrs*.

Gel images were acquired using an optical density calibrated scanner (Epson Scanner, Epson, France) at a spatial resolution of 600 d.p.i. and each band was considered an operational taxonomical unit. Images were analysed with GelCompar II version 4.0 (Applied Maths, Kortrijk, Belgium). Banding profiles were quantified within each profile by determining the total number of bands (S), the peak surface of each band (ni) and the sum of all the peak surfaces of all bands (N; Fromin et al., 2002). This was used to calculate the community biodiversity using three indices: (i) the Shannon index (*H*) calculated as:

$$H = -\sum \left(\frac{ni}{N} \right) \ln \left(\frac{ni}{N} \right)$$

(ii) the dominance index (*c*) calculated as:

$$c = \sum \left(\frac{ni}{N} \right)^2; \text{ and}$$

(iii) the evenness index (*e*) calculated as:

$$e = \frac{H}{\ln S}$$

according to Fromin et al. (2002) and Sadet et al. (2007).

DGGE profiles were normalised and compared using hierarchical clustering to join similar profiles into groups (Fromin et al., 2002). To this end, all images of DGGE gels were matched using the internal control sample and bands were quantified after local background subtraction. A tolerance in the band position of 1% was applied. The similarity among profiles was calculated with the Pearson product moment correlation coefficient and the clustering was done with the unweighted pair-group method using arithmetic averages (UPGMA).

2.6. Microbial communities' quantification and gene expression

Real time quantitative PCR was carried out using a StepOne™ system (Applied Biosystems, Courtabeuf, France). Assays were in triplicate using SYBR Premix Ex Taq™ (Takara Bio Inc, Otsu, Japan) and were used 20 ng of tNA or 20 µl of total cDNA. Negative controls without DNA template were run with every assay to assess overall specificity.

Primers used to quantify *mcrA* DNA or cDNA templates are in Table 2. Reaction volume was set to 25 µl supplemented with additional 1 mM MgCl₂ and 0.3 µM of each primer. The PCR parameters for *mcrA* sequence amplification were an initial denaturation step (50 °C for 2 min and 95 °C for 1 min) and 40 cycles of amplification (95 °C for 15 s, 60 °C for 1 min). Amplicon specificity was checked via dissociation curve analysis of PCR end products by increasing the temperature at a rate of 0.4 °C/min from 60 to 95 °C. Average amplification efficiency, slope and *R*² of qPCR assays targeting *mcrA* gene were of 96%, −3.43 and 0.995, respectively.

Bacterial *rrs* DNA quantification used primers targeting the V3 region of the bacterial *rrs* gene (Table 2). Assays were in a total volume of 20 µl and the final concentration of *rrs* primers was 0.5 µM. The PCR parameters for *rrs* sequence amplification were an initial denaturation step at 95 °C for 10 min and 40 cycles of amplification (95 °C for 15 s, 60 °C for 1 min); the amplified product specificity was determined by dissociation curve analysis obtained by temperature increase from 60 to 95 °C at the rate of 0.6 °C/min. Average amplification efficiency, slope and *R*² of qPCR assays targeting *rrs* gene were of 89%, −3.64 and 0.999, respectively.

Gene copies in rumen content samples were quantified and results expressed as gene copies/g DM of rumen content or total gene copies in whole rumen contents. Absolute quantification involved use of standard curves that had been prepared with PCR products corresponding to a partial sequence of *mcrA* gene of *Methanobrevibacter ruminantium* 1093 (DSM) and almost the entire sequence of *rrs* gene of *Prevotella bryantii* B14 (DSM 11371). Standard curves were created using triplicate fold dilutions series ranging from 2.5×10^2 to 2.5×10^8 copies for *mcrA* gene and from 1×10^2 to 1×10^8 copies for bacterial *rrs* gene.

Expression of the functional *mcrA* gene was assessed using relative quantification by the threshold cycle (C_t) of the qPCR. Statistical analysis of gene expression was on the 2^{-C_t} values as suggested by Schmittgen and Livak (2008).

2.7. Statistical analysis

To determine treatment effects of the diet on CH_4 and VFA production, pH, gene copy numbers and diversity indexes data from each animal were analysed using PROC MIXED of SAS (2003) with Tukey's adjustment. The model included diet as a fixed effect with bull (diet) as a random effect. Effects were declared significant at $P \leq 0.05$ and trends were declared for $P < 0.10$.

3. Results

3.1. Dry matter intake, CH_4 output and fermentation parameters

At the end of the fattening period, bulls fed the fibrous diet consumed more feed and had more rumen contents ($P < 0.01$) than those fed SL (Table 3). Bulls fed F produced 21% more CH_4 (g/d) than those receiving SL ($P < 0.01$). However, there was no difference between groups when emissions were calculated as g CH_4 /kg DM intake or relative to gross energy intake.

At slaughter, total VFA molar concentration were not higher in bulls fed F compared to those fed SL. However the proportion of acetate was lower, whereas proportions of the isoacids (iso-valerate, iso-butyrate) were higher in bulls fed SL (Table 3). There was no difference between diets in propionate and butyrate molar proportions or in the acetate:propionate molar ratio. Ruminal pH was similar between the two groups of bulls.

3.2. Microbial community structure

Total nucleic acids (DNA and RNA) were extracted from whole rumen contents with similar yields between diets. The amount of RNA extracted when expressed as proportion of the tNA sample was equally similar across samples and diets (data not shown). After DNA digestion, RNA integrity was checked using an Agilent RNA 6000 Nano Kit. Electropherograms presented two major peaks corresponding to the 16S and 23S ribosomal RNA (rRNA). Mean RIN was $7.4 (\pm 0.14)$ and mean rRNA 16S/rRNA 23S ratio was 0.97 ± 0.22 .

Results of qPCR quantification of the Archaeal *mcrA* and bacterial *rrs* gene copies are summarized in Table 4. The *mcrA* gene concentration as well as total copy number in the rumen contents were similar between both groups of bulls. The concentration of bacterial *rrs* copies tended ($P = 0.09$) to increase in bulls fed SL, but there was no difference in total *rrs* copies in rumen contents between diets. There was an increase of >65% in protozoal numbers in bulls fed SL as compared to those

Table 3

Dry matter intake, CH_4 output and rumen fermentation parameters in bulls ($n = 20$) fed a fibre or a starch-rich diet supplemented with lipids.

	Diet ^a		SEM	P
	F	SL		
DM intake (kg/d)	10.2	7.9	0.29	<0.01
Total rumen contents (kg DM ^b)	6.4	4.7	0.33	<0.01
pH	6.7	6.9	0.05	0.12
CH_4 output				
CH_4 (g/d)	357	282	16.21	<0.01
CH_4 /GE intake (g/kg) ^c	108	102	0.56	0.47
CH_4 /DM intake (g/kg)	35	35.7	2.63	0.17
Fermentation parameters				
Total VFA (mM)	81.8	68.7	5.67	0.12
Acetate (mmol/mol)	678	647	0.09	0.03
Propionate (mmol/mol)	176	179	0.74	0.80
Butyrate (mmol/mol)	98	107	0.62	0.32
Iso butyrate (mmol/mol)	13	19	0.10	<0.01
Valerate (mmol/mol)	10	14	0.09	0.02
Iso valerate (mmol/mol)	20	30	0.37	<0.01
Acetate/propionate	3.9	3.7	0.19	0.36

^a Diet rich in fibre (F) or rich in starch and supplemented with lipids (SL).

^b DM, dry matter.

^c GE, gross energy.

Table 4Rumen microbial parameters in bulls ($n = 20$) fed a fibre or a starch-rich diet supplemented with lipids.

	Diet ^a		SEM	P
	F	SL		
Microbial numbers				
Bacterial <i>rrs</i> copy number/g DM ^b (log ₁₀)	11.77	12.21	0.17	0.09
Total bacterial <i>rrs</i> copies (log ₁₀)	15.05	15.59	0.19	0.11
<i>mcrA</i> copy number/g DM (log ₁₀)	9.02	9.07	0.16	0.83
Total <i>mcrA</i> copies (log ₁₀)	12.77	12.78	0.17	0.97
Protozoa cell/ml (×10 ⁵)	4.28	13.2	1.42	<0.01
Microbial diversity				
<i>rrs</i> DNA band number	45.8	49.8	1.8	0.13
<i>rrs</i> DNA Shannon diversity index	1.54	1.58	0.02	0.11
<i>rrs</i> DNA evenness diversity index	0.93	0.93	0.004	0.61
<i>rrs</i> DNA dominance diversity index	0.035	0.031	0.002	0.14
<i>mcrA</i> DNA band number	19.6	14.5	0.9	<0.01
<i>mcrA</i> DNA Shannon diversity index	1.17	1.03	0.03	<0.01
<i>mcrA</i> DNA evenness diversity index	0.91	0.9	0.008	0.55
<i>mcrA</i> DNA dominance diversity index	0.08	0.10	0.006	<0.01
Gene expression				
<i>mcrA</i> cDNA band number	12.78	8	0.8	<0.01
<i>mcrA</i> cDNA Shannon diversity index	0.95	0.79	0.04	<0.01
<i>mcrA</i> cDNA evenness diversity index	0.86	0.88	0.02	0.39
<i>mcrA</i> cDNA dominance diversity index	0.14	0.19	0.02	<0.05
<i>mcrA</i> 2 ^{-Ct} (×10 ⁻⁷)	1.72	0.867	0.187	<0.01

^a Diet rich in fibre (F) or rich in starch and supplemented with lipids (SL).^b DM, dry matter.

fed F (Table 4). Small Entodiniomorphs (<100 μm) were predominant and tended ($P=0.09$) to be more abundant in bulls fed SL as compared to those fed F (983 versus 970 cells/1000 cells). *Dasytricha* were more abundant ($P=0.05$) in F, but they represented just 30 cells per 1000 protozoal cells and there was no difference in large Entodiniomorphs and *Isotricha* sp. that represented on average 4.4 and 16.8 cells per 1000 protozoa, respectively.

Diversity indices were calculated from the *mcrA* and *rrs* DNA PCR-DGGE profiles (Table 4). The *mcrA* profiles in both DNA and cDNA samples had higher number of bands and Shannon diversity index ($P<0.01$) and lower dominance index in bulls fed F than in those fed SL, but the evenness index was similar between diets. There was no difference between diets neither in the number of bands nor in the values of the diversity indexes for the *rrs* DNA PCR-DGGE profiles.

Cluster analysis of *mcrA* DNA PCR-DGGE profiles revealed differences in the rumen methanogenic community between bulls fed F and SL (Fig. 1). Most SL samples grouped together and formed a well defined cluster with similarity of 88.6% among samples. The band patterns for bulls fed F, although well separated from those receiving SL, did not generate a distinct cluster. The similarity between samples was 74.9%. Concerning PCR-DGGE profiles for *mcrA* cDNA sequences (Fig. 2), samples from the 20 bulls had a high similarity without an obvious distinction in clusters between diets. Cluster analysis of the *rrs* DNA sequences did not show any difference in the bacterial community structure due to diet (data not shown). The *mcrA* cDNA amplicons were generated during qPCR from all samples. The *mcrA* 2^{-Ct} values were lower in bulls fed SL than in bulls fed F (Table 4).

4. Discussion

DM intake, CH₄ production and fermentation patterns are consistent with those obtained over the fattening period for the 50 bulls used in the companion paper (Eugène et al., in this issue). Net CH₄ emissions (CH₄ g/d) were reduced by 21% in bulls fed SL compared to those fed F. Higher DM intake by bulls fed F resulted in a higher total weight of rumen contents compared to bulls fed SL. As a consequence of the higher DM intake with F, there was no difference in CH₄ emissions between diets when expressed as g CH₄/kg DM intake. These results may be due to the higher starch and lipid content of the SL diet as compared to diet F. However the lipids supplemented by extruded linseed accounted for only 20 g/kg of DM in the SL diet, and previous studies showed that diets rich in starch reduced CH₄ production in feedlot cattle (Beauchemin and McGinn, 2005; Doreau et al., 2011). Moreover, the supply of lipids from linseed decreased the amount of CH₄ emitted by dairy cows (Martin et al., 2008), although Woodward et al. (2006) found no effect of lipid supplementation on methanogenesis in a long term study with dairy cows, suggesting that effects of lipids on CH₄ emissions are transitory. In their review, Martin et al. (2010) reported a decrease in CH₄ production of 4.8% with each supplement of 10 g linseed lipids/kg DM added to the ration, and suggested that effect of lipids on methanogenesis may depend on diet.

There were no large differences in rumen fermentation parameters between diets. As a result of the high starch content of the SL diet, there was a decrease in the proportion of acetate in rumen fluid. However, the acetate:propionate ratio remained constant in the two groups. Lack of differences in the acetate propionate ratio precludes a stoichiometric shift in H₂ utilisation. In addition, rumen pH which is also highly positively correlated to CH₄ production (Lana et al., 1998), was not

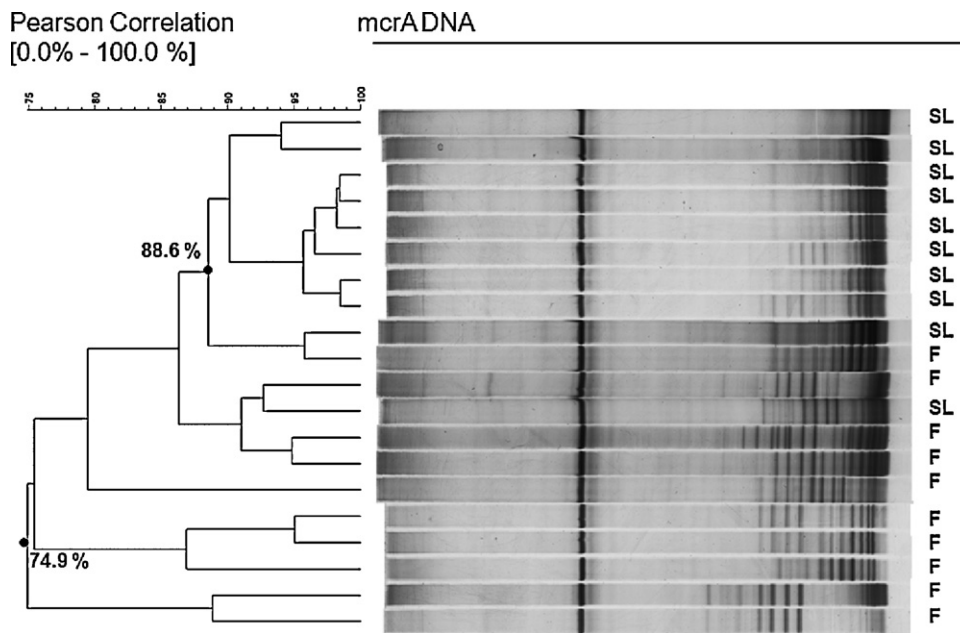


Fig. 1. PCR-DGGE profiles of present rumen methanogens (mcrA DNA) in bulls ($n = 20$) fed a fibre (F) or a starch-rich diet supplemented with lipids (SL). The similarity among profiles was calculated with the Pearson product-moment correlation coefficient and the clustering was done with the unweighted pair-group method using arithmetic averages (UPGMA).

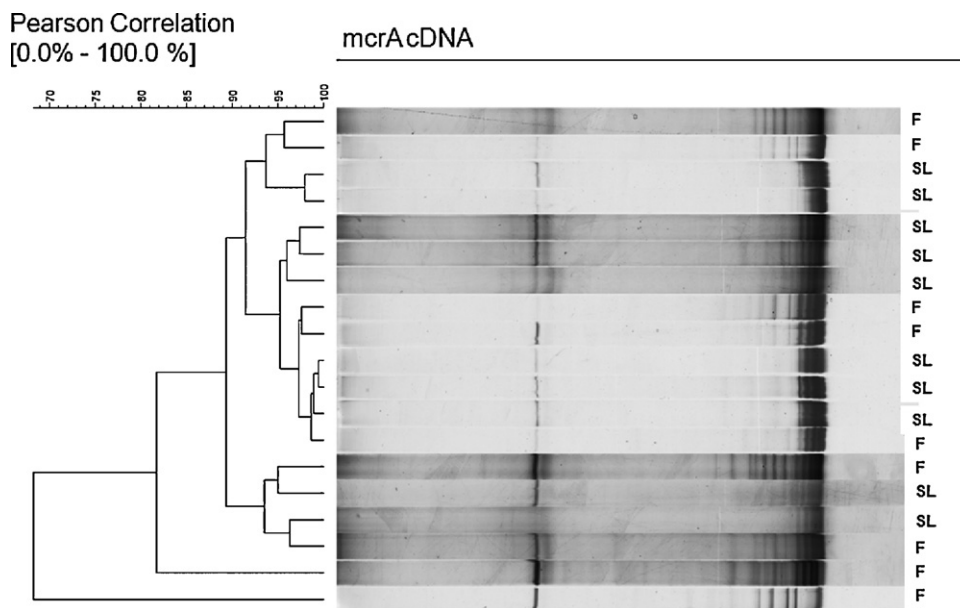


Fig. 2. PCR-DGGE profiles of metabolically active rumen methanogens (mcrA cDNA) in bulls ($n = 20$) fed a fibre (F) or a starch-rich diet supplemented with lipids (SL). The similarity among profiles was calculated with the Pearson product-moment correlation coefficient and the clustering was done with the unweighted pair-group method using arithmetic averages (UPGMA).

affected by the starch rich diet (Table 3 and Eugène et al., in this issue). The absence of ruminal fermentation changes partly explains the lack of differences in CH_4 emission/kg DM intake. Martin et al. (2010) suggested that a low acetate:propionate ratio and depressed CH_4 production may not always be linked in high concentrate feeding in ruminants.

Lipids are known to have toxic effects on rumen microbes. Linolenic acid, abundant in linseed oil, has inhibitory activity against a range of common rumen bacteria in pure culture with cellulolytic bacteria being particularly sensitive (Maia et al., 2007). As discussed above, the concentration of linseed lipids in the rumen of SL fed bulls was low and unlikely to affect rumen microbes as evaluated by the absence of quantitative (qPCR) and qualitative (DGGE) changes in bacteria. However, the tools we used targeted total bacteria and so gave only a global overview of this microbial community. In contrast protozoa

increased in numbers, likely due to the higher proportion of starch in the SL diet (Mackie et al., 1978). Additionally, as mentioned above for bacteria, the concentration of linolenic acid in the SL diet was probably not toxic to protozoa. The increase in isobutyric and isovaleric acids, which results from deamination of valine and leucine respectively, can also be associated with higher protein degradation by protozoa (Eugène et al., 2004). This is consistent with the higher protozoal concentrations in the rumen of the bulls fed diet SL.

That the total number and concentration of *mcrA* copy numbers in the rumen contents of bulls was not influenced by diet is comparable to results reported with high and low RFI steers fed high and low energy diets (Zhou et al., 2010), although in the latter study there were no measures of CH₄ production. Lipid supplementation of diets depressed CH₄ production in sheep by 14% without affecting numbers of methanogen numbers (Machmüller et al., 2003), suggesting that the number of rumen methanogens may not be a key factor affecting CH₄ production in all situations.

Intake of fibrous concentrate enhanced development of a more diverse methanogenic community as shown by the higher number of bands and higher Shannon index of PCR-DGGE profiles from F compared to SL. Also, individual variability among bulls fed diet SL was less marked, as they grouped together in a well defined distinct cluster. As the SL diet was rich in starch and contained oil, it may have had a specific selection pressure on the methanogenic community. However, PCR-DGGE analysis of *mcrA* cDNA sequences did not reveal an impact of diet on metabolically active community members. Changing from a low to a high concentrate diet has been reported to have an impact on the structure of the methanogenic community (Zhou et al., 2010), and these authors suggested that diet composition may specifically select methanogens that differ in their methanogenic pathway and differences at the strain or genotype level may play an important role in CH₄ production (Zhou et al., 2010).

Expression of the functional *mcrA* gene suggested that methanogens in bulls fed SL were less metabolically active than in bulls fed F. Accordingly, CH₄ production by these bulls (g/d) was lower than that from bulls fed F. A decrease in CH₄ production associated with lower methanogenic activity has been described in sheep fed coconut oil (Machmüller et al., 2003). A decrease in CH₄ emissions may be due to a lower CH₄ production rate per single methanogenic cell and could depend on availability of H₂ as a substrate.

Dihydrogen is a major end product of protozoa metabolism, suggesting that the higher CH₄ production/kg DM intake may be due to a higher production of H₂/kg DM intake in bulls fed SL. This is consistent with the higher total tract digestibility in faunated compared to defaunated ruminants (Eugène et al., 2004). The extent of feed digestion in bulls fed SL, compared to those fed F, may have been enhanced by the higher protozoal population and thereby provide abundant H₂ for methanogenesis.

5. Conclusions

High starch diets and/or lipid supplementation are feeding strategies known to mitigate CH₄ emissions by ruminants without affecting their production. Differences in CH₄ production under two diet conditions in our study could be attributed to changes in rumen microbiota, particularly to methanogen activity, whereas other changes, such as the increase in protozoal numbers in the starch rich diet, may have diminished the CH₄ abatement potential of this diet. As to bacteria, further studies are needed to evaluate effects of diet on this microbial community. To better understand the effect of dietary, and other, CH₄ mitigations in ruminants, it seems essential to use a holistic approach in studying the rumen ecosystem. Interactions involving H₂ producers and methanogens and other H₂ users in the rumen should be better explored as they could open new targets for modification of the rumen microbiota for mitigation purposes.

Conflict of interest

None.

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