World J Microbiol Biotechnol (2013) 29:301–307
DOI 10.1007/s11274-012-1183-2

ORIGINAL PAPER

Evaluation of DNA extraction methods of rumen microbial populations

Gabriela Villegas-Rivera · Yevani Vargas-Cabrera · Napoleón González-Silva · Florentino Aguilera-García · Ernestina Gutiérrez-Vázquez · Alejandro Bravo-Patiño · Marcos Cajero-Juárez · Víctor Manuel Baizabal-Aguirre · Juan José Valdez-Alarcón

Received: 24 April 2012 / Accepted: 26 September 2012 / Published online: 5 October 2012

Abstract The dynamism of microbial populations in the rumen has been studied with molecular methods that analyze single nucleotide polymorphisms of ribosomal RNA gene fragments (rDNA). Therefore DNA of good quality is needed for this kind of analysis. In this work we report the evaluation of four DNA extraction protocols (mechanical lysis or chemical lysis with CTAB, ethylxanthogenate or DNAzol®) from ruminal fluid. The suitability of two of these protocols (mechanical lysis and DNAzol®) was tested on single-strand conformation polymorphism (SSCP) of rDNA of rumen microbial populations. DNAzol® was a simple method that rendered good integrity, yield and purity. With this method, subtle changes in protozoan populations were detected in young bulls fed with slightly different formulations of a supplement of multinutritional blocks of molasses and urea. Sequences related to *Eudiplodinium maggi* and a non-cultured Entodiniomorphid similar to *Entodinium caudatum*, were related to major fluctuating populations in an SSCP assay.

Keywords *Bos taurus* · Rumen · PCR-SSCP · rDNA · MBMU · DNA extraction

Gabriela Villegas-Rivera, Yevani Vargas-Cabrera, Napoleón González-Silva, Florentino Aguilera-García are the authors contributed equally to this work.

G. Villegas-Rivera · Y. Vargas-Cabrera · N. González-Silva · F. Aguilera-García · A. Bravo-Patiño · M. Cajero-Juárez · V. M. Baizabal-Aguirre · J. J. Valdez-Alarcón (✉)
Centro Multidisciplinario de Estudios en Biotecnología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Michoacana de San Nicolás de Hidalgo, Km 9.5 carretera Morelia-Zinapécuaro, La Palma, C.P. 58893 Tarímbaro, Michoacán, Mexico
e-mail: jjvaldez_a@hotmail.com; jjvaldez@umich.mx

E. Gutiérrez-Vázquez
Instituto de Investigaciones Agropecuarias y Forestales, Universidad Michoacana de San Nicolás de Hidalgo, Km 9.5 carretera Morelia-Zinapécuaro, La Palma, C.P. 58893 Tarímbaro, Michoacán, Mexico

Introduction

Molecular methods to study rumen microbial communities are contributing to understand how strategies to improve fiber digestion and treat illnesses such as tympany and ruminal acidosis affect ruminal microbiome. One of the most common DNA extraction methods from ruminal content that have been used to date has been reported by Krause et al. (2001a, b) and is based on mechanical lysis (bead–beating) of the cells, which usually results in good DNA yields but with low integrity. This method has been preferred because its low cost and simplicity. The aim of this work was to compare the mechanical lysis-based method with other low cost, simple and commonly used DNA extraction methods based on chemical lysis to obtain DNA from ruminal fluid and test its suitability for microbiome analysis with PCR-SSCP.

Based on the integrity, yield and purity of the DNA obtained, and on the simplicity of the method, we selected DNAzol® to extract ruminal fluid samples that were analyzed by PCR-SSCP of 16S or 18S rDNA's for the content in the microbial populations of animals fed with subtle differences in its diet formulation. Sequence analysis of representative conformomers revealed sequences related to microorganisms commonly found in the rumen. Analysis of samples taken after an adjustment in the formulation of Multinutritional Blocks of Molasses and Urea (MBMU) used as a diet supplement, revealed that sequences related

to *Eudiplodinium maggi* and to a non-cultured Entodiniomorphid related to *Entodinium furca dilobum* change their relative abundance with an opposite pattern in a kinetic analysis.

Materials and methods

Feeding of young bulls and collection of the rumen content samples

Three crossed young Zebu bulls (c.a. 225 kg), were fed ad libitum with maize stubble as basal ration and three different formulations of the MBMU. All formulations contained: 6 % water, 15 % cement, 5 % common salt, 2 % orthophosphate, 0.25 % micromineral mixture, 22.75 % wheat bran. Molasses-urea content in each formula was 47–8 % (formula 1), 45–10 % (formula 2) and 43–12 % (formula 3). To evaluate the effect of the exchange of MBMU formulations, young bulls were fed for 15 days with maize stubble and a formulation of the MBMU and then the formulation of the block was changed. Fifty ml samples of rumen fluid content were taken through a canula at 0, 6, 12, 24, 48, 72, 96, 120, 240 and 336 h after changing formulation of the MBMU. The samples were frozen at −80 °C until extracted.

DNA extraction by mechanical lysis

DNA extraction was as described in Krause et al. (2001a, b). 0.5 g of acid-washed glass beads (150–212 μm; Sigma) were mixed with 1 ml of ruminal fluid in a 2 ml eppendorf tube. The mixture was centrifuged at 10,000 rpm in an Eppendorf 5415 C microfuge and the supernatant was discarded. One ml of ethanol was added and the tube was vortex-mixed for 1 min, centrifuged at 10,000 rpm for 5 min and the supernatant was discarded. The pellet was added with 300 μl of phenol:chlorophorm (1:1 v/v) previously equilibrated with 100 mmol l^{-1} Tris–HCl pH 7.0, 200 μl of ADEM buffer (50 mmol l^{-1} sodium acetate, 10 mmol l^{-1} sodium EDTA, 1 % DMSO, 1 % methanol, pH 7.0) and 100 μl of 35 % PEG 4000 (in 100 mmol l^{-1} Tris–HCl pH 7.0) followed by 2 min of vortex-mixing. The lysate was centrifuged for 5 min at 10,000 rpm and the aqueous phase was recovered, extracted with chlorophorm:isoamyl alcohol (24:1 v/v) and the supernatant was recovered and added with 1/5 volume of 5 mol l^{-1} NaCl and 1/10 volume of 10 % CTAB, incubated at 65 °C for 5–10 min. The polysaccharide was pelleted by centrifugation at 10,000 rpm for 5 min. DNA was precipitated from the supernatant by the addition of 1/10 volume of 3 mol l^{-1} sodium acetate and one volume of 100 % isopropanol. DNA pellet was resuspended in TE buffer (10 mmol l^{-1} Tris–HCl, 1 mmol l^{-1} EDTA, pH 7.0).

DNA extraction by chemical lysis with CTAB

DNA extraction with CTAB was as describe by Ausubel et al. (1999). One ml of ruminal fluid was added with CTAB/ME (100 mmol l^{-1} Tris–HCl, 20 mmol l^{-1} EDTA, 1.4 mol l^{-1} NaCl, 2 % CTAB, 2 % β-mercaptoethanol, pH 8.0) extraction solution in a 30 ml centrifuge tube, incubated at 65 °C for 30 min and extracted with chlorophorm:isoamylic alcohol (24:1 v/v). The aqueous phase was recovered and added with 0.1 volumes of CTAB/NaCl solution (10 % CTAB, 0.7 mol l^{-1} NaCl) and extracted with chlorophorm:isoamyl alcohol. The aqueous phase was added with CTAB precipitation solution (50 mmol l^{-1} Tris–HCl, 10 mmol l^{-1} EDTA, 1 % CTAB, pH 8.0) and incubated at 65 °C for 10 min. The precipitate was collected by centrifugation at 2,700 rpm in a Centra GP8R table top centrifuge (IEC, USA) at 4 °C. The pellet was resuspended in 1 ml of TE-saline buffer (10 mmol l^{-1} Tris–HCl, 1 mmol l^{-1} EDTA, 1 mol l^{-1} NaCl, pH 8.0) added with 0.6 volumes of isopropanol and the DNA precipitated by centrifugation. DNA pellet was washed with 70 % ethanol and resuspended in TE buffer.

DNA extraction by chemical lysis with PEXG

The DNA was extracted as described in Jinghan (1992). One ml of ruminal fluid was added with 2 ml of PEXG extraction buffer (100 mmol l^{-1} Tris–HCl, 50 mmol l^{-1} NaCl, 50 mmol l^{-1} EDTA, 20 mmol l^{-1} PEXG, pH 8.0) in a 30 ml centrifuge tube. The mixture was vortex-mixed for 1 min, incubated for 30 min to 1 h at 65 °C and extracted with 2 ml of equilibrated phenol (pH 8.0). Aqueous phase was recovered and precipitated with one volume of isopropanol. DNA pellet was collected by centrifugation at 12,000 rpm and resuspended in 200 μl of TE buffer.

DNA extraction by chemical lysis with DNAzol®

One ml of rumen fluid content was mixed with 4 ml of DNAzol® (Invitrogen) reagent (Chomczynski 1993), vortexed for at least 1 min, and let stand at room temperature for 15 min, and the mixture was centrifuged at 10,000 rpm for 10 min in an Avanti J-25 centrifuge (Beckman–Coulter). Supernatant was recovered, added with 2 ml of absolute ethanol and incubated in ice for 15 min. DNA was recovered by centrifugation at 10,000 rpm for 15 min at 4 °C. The pellet was washed with 70 % ethanol, dried at room temperature and resuspended in 200 μl of TE (Tris–HCl 10 mmol l^{-1}, EDTA 1 mmol l^{-1}, pH 8.0) buffer.

PCR amplification of 16S or 18S rDNAs

For PCR amplification of 16S rDNA fragments from bacterial species, primer pair SRV3-1 (5′P-CGG YCC AGA CTC CTA CGG G 3′) and SRV3-2 (5′-TTA CCG CGG CTG CTG GCA C-3′), comprising variable region 3, were used (Lee et al. 1996). For 18S rDNA fragment amplification from eukaryotes, primer pair NS7 (5′P-GAG GCA ATA ACA GGT CTG TGA TGC-3′) and NS8 (5′-TCC GCA GGT TCA CCT ACG GA-3′), comprising variable regions 8 and 9 (White et al. 1990), were used. 100 ng of total DNA were always used for PCR reactions. PCR conditions for bacterial and eukaryotic rDNAs were as described. Upstream primers were phosphorylated at the 5′ end, to ensure specific digestion by a λ bacteriophage exonuclease for SSCP analysis.

SSCP analysis

One μg of each PCR product was incubated with 0.5 units of λ bacteriophage exonuclease (Invitrogen, USA) at 37 °C for 2 h (Schwieger and Tebbe 1998). The reaction mixture was extracted with phenol:chloroform:isoamyl alcohol 25:24:1 (v:v:v) and ethanol precipitated at −80 °C for 30 min. Single-stranded DNA was collected by centrifugation at 10,000 rpm in the microfuge for 15 min, the pellet was washed twice with 70 % ethanol, resuspended in 10 μl of TE buffer, and dialyzed by floating on a VSWP 02500 amphiphylic filter (Millipore, USA) on deionized water. Conformomers were generated by heating the single-stranded DNA samples at 95 °C for 5 min in loading buffer (95 % formamide, 10 mmol l^{-1} NaOH, 20 mmol l^{-1} EDTA, 0.02 % bromophenol blue, 0.02 % xylene cyanol), and immediately cooled by rapid transferring to a water–ice bath, and loaded in a 6 % polyacrylamide gel (29:1 %) containing 10 % glycerol. Electrophoresis was run at constant 3 watts for 16–24 h in a DCode Universal Mutation Detection System (Bio-Rad, USA), cooled at 5 °C with a refrigerated circulator (ThermoNESLAB, USA). Gels were immersed in fixation solution (5 % acetic acid, 10 % ethanol) for 15 min with gentle shaking, rinsed twice with deionized water, stained with 2 % silver nitrate solution for 15 min with gentle shaking, rinsed again twice with deionized water and incubated in developing solution (3 % NaOH, 0.09 % formaldehyde) until DNA bands appeared. Stained gels were photographed with an Eagle Eye II photodocumentation system (Stratagene, USA). The images were subject to densitometry analysis with the RFLPScan software (Scanalytics, USA).

For sequence analysis, DNA bands from SSCP gels were cut out, grinded in 50 μl of TE buffer, and 1 μl was used for reamplification in the same conditions. PCR products were either directly sequenced, or cloned into pGEM-T vector (Promega, USA), and transformed in XL1-Blue cells (Strategene, USA). Plasmidic DNA was obtained according to standard protocols (Ausubel et al. 1999). Sequencing reactions were performed in an ABI Prism 310 Genetic Analyzer with the Big Dye Terminator Cycle Sequencing Ready Reaction v 2.0 kit (Applied Biosystems, USA) according to manufacturer instructions. Analysis of the sequences was done with the Sequencing Analysis v 3.0 software (Applied Biosystems, USA). Search of similar sequences was done with the BLAST program (Altschul et al. 1990) at the National Center for Biotechnology Information (NCBI) using Refseq database set for 16S rDNA sequences and nucleotide (nr) database set for 18S rDNA sequences. Sequence comparisons were done with the MegAlign program of DNAStar software (DNAStar Inc., USA). Sequences were registered in the GenBank database under accession numbers JQ288925 to JQ288936.

Results

Comparison of DNA extraction methods

Yield, purity and integrity were compared among the four DNA extraction methods. Similar yields were observed for lysis with CTAB, EXGP and DNAzol®, while mechanical lysis showed the lowest yield of all (Table 1). Mechanical lysis and DNAzol® rendered the best purity (Table 1), while CTAB, EXGP and DNAzol® rendered a high molecular weight DNA with low-intensity smears typical of degraded DNA (Fig. 1a); mechanical lysis always generated smeared signals in agarose gel electrophoresis (Fig. 1b).

Suitability of DNA for PCR and SSCP

Extraction with DNAzol® was the method selected on the basis of purity and integrity of the DNA obtained, for its comparison against Krause's method in a PCR-SSCP assay. We amplified the variable region 3 of the

Table 1 Comparison of DNA extraction methods

	Mechanical	CTAB	EXGP	DNAzol
Yield[a]	50.6 ± 14.9	183.3 ± 33.4	183.33 ± 16.7	141.6 ± 52.9
Purity[b]	1.92 ± 0.55	3.00 ± 1.16	1.00 ± 0.00	1.69 ± 0.18

Values are mean ± standard error of three replicates

[a] Yield is indicated as μg DNA ml^{-1} of ruminal fluid

[b] Purity is indicated as the $As_{260\ nm}/As_{280\ nm}$ ratio

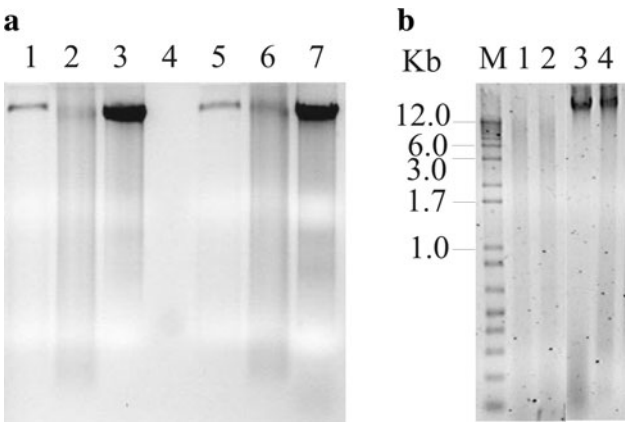

Fig. 1 Integrity of DNA extracted with different methods. **a** DNA extracted with CTAB (*lanes* 1 and 5), EXGP (*lanes* 2 and 6), and DNAzol® (*lanes* 3 and 7). **b** Comparison of DNA extracted with the mechanical-lysis method (*lanes* 1 and 2) and DNAzol® (*lanes* 3 and 4)

prokaryotic 16S rDNA. Four different rumen fluid samples were used for DNA extraction with both methods. An approximately 200 pb fragment was observed for all samples as expected (Fig. 2a). A non-specific PCR product with an approximate molecular size of 850 bp was observed for DNA samples extracted with the mechanical-lysis method. This fragment was not observed when DNA samples obtained with DNAzol® were used as template for the PCR reaction. Long term storage of DNA is required when kinetic analysis are performed during the ruminal digestion process. So samples from the earlier times need to be stored until the experiment is finished prior to PCR-SSCP analysis. Additionally, recovery of some DNA samples is sometimes needed to re-analyze the SSCP pattern. When DNA samples obtained with both methods were stored at 4 °C for 1 month, no amplification products were observed for DNA obtained with the mechanical lysis method, but amplification was still possible with DNA obtained with DNAzol® (Fig. 2b). The presence of contaminants was discarded by cleaning the samples by adsorption to glass powder (Attal et al. 1995). There was not a clear difference in PCR product accumulation for glass powder-cleaned DNA obtained with DNAzol used as template. Cleaning of DNA obtained by the mechanical-lysis method and stored for 1 month did not improve amplification (data not shown). However, for some samples for which PCR amplification was not easily achieved, inclusion of a phenol:chloroform extraction step improved performance of PCR and SSCP electrophoretic pattern (data not shown).

PCR products obtained from DNA extracted with mechanical-lysis method or DNAzol® were heat-denaturated and run in a non-denaturating polyacrilamide gel for SSCP analysis. Similar conformomer patterns were observed (Fig. 3).

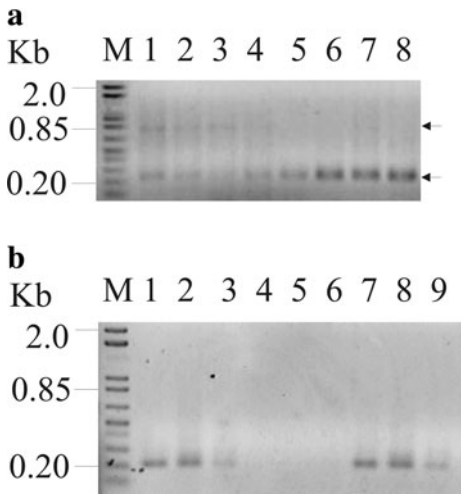

Fig. 2 Suitability of extracted DNA for PCR of 16S rDNA. **a** Presence of an unspecific amplification product (*upper arrow*) of c.a. 850 bp in PCR reactions with DNA obtained by the mechanical lysis method (*lanes* 1–4). The expected fragment of 200 bp (*lower arrow*) was the only PCR product from the DNAzol®-extracted DNA. **b** Stability of DNA. PCR products obtained from DNA extracted with DNAzol® (*lanes* 1–3) immediately after sample collection, or after 1 month of storage at 4 °C after extraction with the mechanical-lysis method (*lanes* 4–6), or DNAzol® (*lanes* 7–9). M, 1 Kb plus (Invitrogen) molecular size marker

To evaluate the accuracy of amplification with DNAzol®, ten conformomer bands were isolated from the gel, reamplified and cloned for sequencing. Results of sequence comparison of the isolated conformomers are shown in Table 2. Similar sequences were obtained from the Refseq database set in GenBank for 16S rDNA sequences. Sequences UMRB 1–10 were similar to previously reported rDNA sequences from uncultured rumen bacteria or to other uncultured bacteria associated with digestive tracts, most of them obtained from rDNA libraries.

Suitability of DNA for PCR-SSCP analysis

DNA was obtained with DNAzol® from ruminal fluid from young bulls fed with maize stubble supplemented with molasses-urea blocks with subtle differences in their formulation (Fig. 4). PCR-SSCP analysis with the NS7/NS8 primer pair that amplified a region of 18S rDNA revealed specific conformomers for samples from bulls supplemented with formulas 1 and 2. Bulls were then fed with formula 2 for 15 days and MBMU composition was shifted to formula 3. Densitometric analysis of the PCR-SSCP gel is shown in Fig. 2. Two conformomers, identified as bands 3 and 4 in Figs. 4 and 5 fluctuated during the course of the experiment following an opposite pattern of relative abundance. These two bands were isolated, reamplified and directly sequenced from the PCR product. For the similarity search, Nucleotide (nr) database set from GenBank

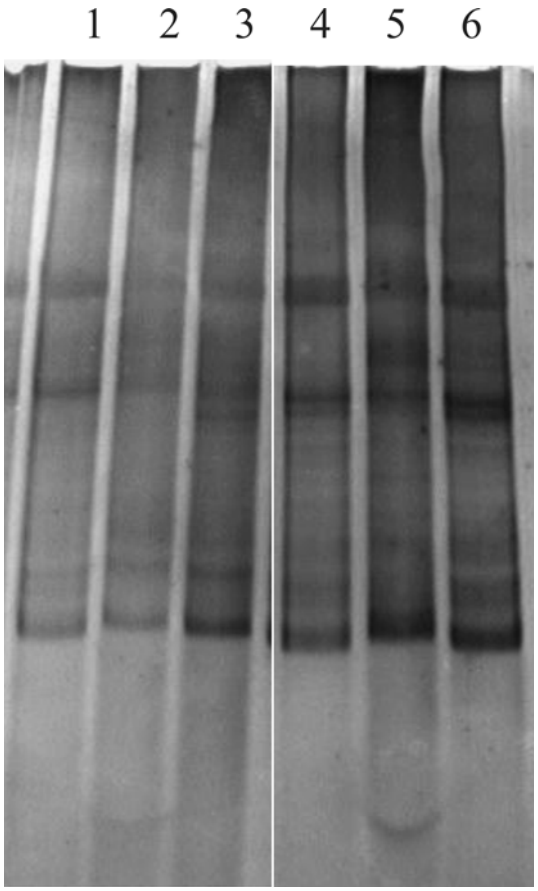

Fig. 3 Suitability of DNA for PCR-SSCP of 16S rDNA. Samples extracted either with mechanical-lysis method (*lanes* 1–3) or with DNAzol® (*lanes* 4–6). Samples from *lanes* 1 and 4, 2 and 5, and 3 and 6, were obtained from the same animal

was used because of the lack of reference sequences for protozoa in the Refseq database set. Both of the sequences (UMRPz5 and UMRPz6) were similar to rDNA sequences from entodiniomorphid protozoa from the rumen (Table 2). UMRPz5 was closely related to *Eudiplodinium magii*, a well known entodiniomorphid from rumen. UMRPz6 sequence resulted similar to an rDNA sequence from an uncultured rumen entodiniomorphid protozoa related to *Entodinium caudatum*.

Discussion

Analysis of 16S rDNA libraries from rumen content has led to the identification of sequences from ruminal microorganisms that had not been previously described (Tajima et al. 2001b; Krause et al. 2003; Edwards et al. 2004). Construction of representative rDNA libraries is skillful and time-consuming, limiting the possibility to analyze multiple samples. PCR-based monitoring of *Ruminococcus* spp., *Prevotella* spp., *Fibrobacter* spp. with specific primers has been addressed in kinetic studies for the evaluation of microbial establishment in immature lambs (Krause et al. 2000), in the evaluation of *Ruminococcus* spp. establishment after repeated administration of cultures containing *Ruminococcus* strains (Krause et al. 2001a, b), and in diets that shifts from hay to grain (Tajima et al. 2001a). In these works, DNA was extracted with the mechanical lysis-based method (Krause et al. 2001a, b). In vitro studies of ruminal fermentations had been accompanied with microbial community analysis by PCR-SSCP (Koch et al. 2006; Strobel et al. 2008) or DGGE profiling (Yu and

Table 2 Sequence identity of selected bands from SSCP gel

Secuence ID	Primer pair	Similar sequence	GenBank accession no.	Identity (%)
Bacteria				
UMRB1	SRV3-1/SRV3-2	*Robinsoniella peoriensis*	AF445285.2	98.9
UMRB2	SRV3-1/SRV3-2	*Erysipelothrix tonsillarumn*	NR040871.1	89.3
UMRB3	SRV3-1/SRV3-2	*Capnocytophaga leadbetteri*	DQ009623.1	90.5
UMRB4	SRV3-1/SRV3-2	*Prevotella amnii*	AM422125.1	95.0
UMRB5	SRV3-1/SRV3-2	*Prosthecobacter fusiformis*	U60015.1	80.9
UMRB6	SRV3-1/SRV3-2	*Oxobacter pfennigii*	NR029268.1	86.7
UMRB7	SRV3-1/SRV3-2	*Dethiobacter alkaliphilus*	EF4224.12	86.7
UMRB8	SRV3-1/SRV3-2	*Clostridium cellobioparum*	NR026104.1	89.0
UMRB9	SRV3-1/SRV3-2	*Eubacterium desmolans*	L34618.1	96.1
UMRB10	SRV3-1/SRV3-2	*Bacteroides capillosus*	AY136666.1	96.2
Protozoa				
UMRPz5	NS7/NS8	*Eudiplodinium maggi*	AM158452.1	98.8
UMRPz6	NS7/NS8	*Entodinium furca dilobum*	AM158442.1	99.0

Sequences in this table were registered in GenBank database under accession numbers JQ288925 to JQ288936

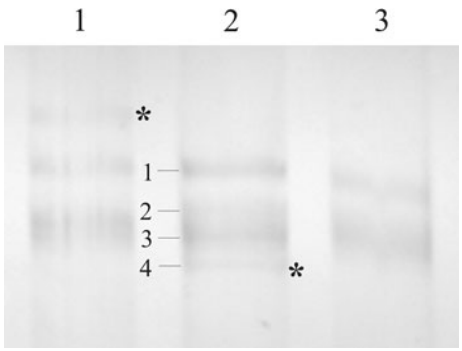

Fig. 4 PCR-SSCP patterns of 18S rDNA from animals fed with different formulations in MUB. Animals were fed as described in materials and methods with formula 1 (*lane* 1), formula 2 (*lane* 2) and formula 3 (*lane* 3). *Asterisks* indicate differentially detected conformomers. Number at the left of *lane* 2 identify the conformomer analyzed in Fig. 5

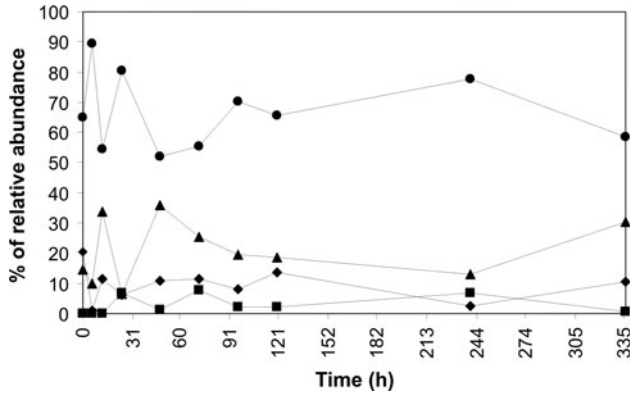

Fig. 5 Kinetic analysis of PCR-SSCP patterns of 18S rDNA in a transition from formula 2 to formula 3. Data represent the mean of two experiments. Each line in the graphic was generated by densitometric analysis of bands 1 (*filled diamond*), 2 (*filled square*), 3 (*filled triangle*) and 4 (*filled circle*), as designated in Fig. 4

Morrison 2004). In these works, DNA was extracted with the Fast DNA kit (Qbiogene, Germany) or with an improved method that also use mechanical lysis in a high SDS-salt-EDTA buffer. Both methods were followed by DNA purification in commercial columns. Recently a method that combines bead beating with phenol:chlorophorm extraction was reported for the extraction of both DNA and RNA from ruminal samples (Popova et al. 2010).

DNAzol®-based extraction of DNA from ruminal fluid samples rendered a better combination of yield, integrity and DNA purity than other chemical-based methods that use CTAB and EXGP for cell lysis. Recently, Schloos et al. (2011) have analyzed the sources of errors in microbiome sequencing approaches that analyze 16S rDNA. Besides ambiguous base calling and mismatching of PCR primers, chimeric PCR products may represent up to 8 % of raw sequence reads. Chimeric PCR products are often generated by aborted extension, mispriming of the aborted DNA and extension over the misprimed sequence (Haas et al. 2011). Extraction of DNA using a mechanical lysis-based method rendered unspecific PCR products which could be originated as a chimeric PCR product. It also resulted in low stability of the stored DNA samples which may generate incomplete DNA sequences that, when used as templates, may generate partial 16S rDNA products that favor chimeric PCR product formation (Wang and Wang 1997). Insufficient quality of DNA extracted by the mechanical-lysis based method has also been noticed by other authors (Krause et al. 2004). In contrast, non unspecific PCR products were observed when DNA was extracted with DNAzol®, and it proved to be stable for at least 1 month. Despite these differences, similar PCR-SSCP conformomer patterns were generated with mechanical lysis or DNAzol®. PCR-SSCP analysis was performed with DNAzol®-extracted samples from bulls fed with different formulations of a diet. No differences were observed in fermentation parameters or food intake when these formulas were used (Gutiérrez and González 2003). We were able to detect changes in the microbial populations of eukaryotes during a kinetic analysis after changing the formulas. The most evident changes were related to sequences similar to entodiniomorphid protozoa. The fluctuating and complementary pattern of relative abundance distribution of these conformomers suggests that the respective populations are competing for the same metabolic niche.

We recommend the use of DNAzol® as the extraction method of choice, because it rendered the better combination of yield integrity and purity. DNAzol® was also better in: (1) the DNA did not generated unspecific amplification products, (2) DNA remained stable when stored at 4 °C, (3) it is performed in shorter times than mechanical lysis-based methods and (4) it does not require further column purification.

Acknowledgments This work was supported in part by grants from the Coordinación de la Investigación Científica-Universidad Michoacana de San Nicolás de Hidalgo (to J.J.V.A. and E.G.V), and Programa de Mejoramiento al Profesorado—Secretaría de Educación Pública (to J.J.V.A.). The authors are grateful to J.C. González for helpful technical assistance, G. Vázquez-Marrufo for suggesting EXGP protocol and J.E. López-Meza for technical support on sequencing.

Conflict of interest None of the authors has any financial or personal relationships with people or organizations that could inappropriately influence or bias the content of this paper.

References

Altschul SF, GishW Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Attal J, Cajero-Juárez M, Houdebine LM (1995) A simple method of DNA extraction from whole cell tissues and blood for detection of transgenic animals by PCR. Transgenic Res 4:149–150

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1999) Short protocols in molecular biology, 4th edn. Wiley, USA

Chomczynski P (1993) A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques 15:532–537

Edwards JE, McEwan NR, Travis AJ, Wallace RJ (2004) 16S rDNA-based analysis of ruminal bacterial diversity. Antonie Van Leeuwenhoek 86:263–281

Gutierrez E, González JC (2003) Intake and rumen fermentation in young bulls fed maize stubble supplemented with multinutritional blocks of molasses-urea. Trop Subtrop Agroecosyst 3:377–380

Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, Giannoukos G, Ciulla D, Tabbaa D, Highlander SK, Sodergren E, Methé B, DeSantis TZ, The Human Microbiome Consortium, Petrosino JF, Knight R, Birren BW (2011) Chimeric 16S rDNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Res 21:494–504

Jinghan AK (1992) A novel technology for DNA isolation. Methods Mol Cell Biol 3:15–22

Koch M, Strobel E, Tebbe CC, Heritage J, Breves G, Huber K (2006) Transgenic maize in the presence of ampicillin modifies the metabolic profile and the microbial population structure of bovine rumen fluid in vitro. Br J Nutr 96:820–829

Krause DO, Smith WJM, Ryan FME, Mackie RI, McSweeney CS (2000) Use of 16S-rRNA based techniques to investigate the ecological succession of microbial populations in the immature lamb rumen: tracking of a specific strain of inoculated *Ruminococcus* and interactions with other microbial populations in vivo. Microb Ecol 38:365–376

Krause DO, Bunch RJ, Conlan LL, Kennedy PM, Smith WJ, Mackie RI, McSweeney CS (2001a) Repeated ruminal dosing of *Ruminococcus* spp. does not result in persistence, but changes in other microbial populations occur that can be measured with quantitative 16S-rRNA-based probes. Microbiology 147:1719–1729

Krause DO, Smith WJ, McSweeny CS (2001b) Extraction of microbial DNA from rumen contents containing plant tannins. Biotechniques 31:294–298

Krause DO, Denman SE, Mackie RI, Morrison M, Rae AL, Attwood GT, McSweeney CS (2003) Opportunities to improve fiber degradation in the rumen: microbiology, ecology and genomics. FEMS Microbiol Rev 27:663–693

Krause DO, Smith WJ, McSweeny CS (2004) Use of community genome arrays (CGAs) to assess the effects of *Acacia angustissima* on rumen ecology. Microbiology 150:2899–2909

Lee D-H, Zo Y-G, Kim S-J (1996) Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single strand conformation polymorphism. Appl Environ Microbiol 62:3112–3120

Popova M, Martin C, Morgavi DP (2010) Improved protocol for high quality co-extraction of DNA and RNA from rumen digesta. Folia Microbiol (Praha) 55:368–372

Schloos PD, Gevers D, Westcott SL (2011) Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. PLoS ONE 6:e27310

Schwieger F, Tebbe CC (1998) A new approach to utilize single-strand-conformation-polymorphism for 16S rRNA gene-based microbial community analysis. Appl Environ Microbiol 64:4870–4876

Strobel E, Seeling K, Tebbe CC (2008) Diversity responses of rumen microbial communities to *Fusarium*-contaminated feed, evaluated with rumen simulating technology. Environ Microbiol 10:483–496

Tajima K, Aminov RI, Nagamine T, Matsui H, Nakamura M, Benno Y (2001a) Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. Appl Environ Microbiol 67:2766–2774

Tajima K, Nagamine T, Matsui H, Nakamura M, Benno Y (2001b) Phylogenetic analysis of archaeal 16S rRNA libraries from the rumen suggests the existence of a novel group of archaea not associated with known rumen methanogens. FEMS Microbiol Lett 200:67–72

Wang GC-Y, Wang Y (1997) Frequency of formation of chimeric molecules as a consequence of PCR coamplification of 16S rRNA genes from mixed bacterial genomes. Appl Environ Microbiol 63:4645–4650

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, pp 315–322

Yu Z, Morrison R (2004) Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques 36:808–812