Evaluation of the effects of tropical tanniferous plants on rumen microbiota using qRT PCR and DGGE analysis

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ABSTRACT: Tanniferous forages may have bacteriostatic and/or bactericidal effect on different rumen microbial populations. We investigated the influence of the tropical tanniferous plants *Styzolobium aterrimum* (STA), *Styzolobium deeringianum* (STD), *Leucaena leucocephala* (LEU), and *Mimosa caesalpiniaefolia* (MIC) containing 20, 64, 56, and 105 g condensed tannis (CT)/kg dry matter (DM) and *Cynodon* spp. cv. Tifton 85 (CYN) as control on *Fibrobacter succinogenes* and methanogenic microbes in rumen liquor from sheep using the *in vitro* gas production technique (Hohenheim gas test). The relative gene expression of *F. succinogenes* at $t_{½}$ (time point when 50% of the maximal gas production has been reached) analyzed by quantitative PCR was 0.20- and 0.28- fold lower than the control when LEU and STA was applied and 0.91- and 0.85-fold lower with MIC and STD. Methanogenic population was 0.29- and 0.58- fold reduced with STA and LEU compared to the control, but 5.50- and 1.43- fold higher with MIC and STD. At 24 h, *F. succinogenes* was reduced for all legumes as well as methanogenic bacteria, except for MIC which increased 4.15-fold. Denaturing gradient gel electrophoresis (DGGE) of the methanogenic community resulted in different band patterns: CYN presented some strong bands, which became weaker in the analyzed treatments. Some bands appeared weaker, especially in MIC and STD, but not in STA and LEU. MIC seemed to increase the total number of weak bands. Overall, the tannin-rich plants negatively affected the *F. succinogenes* population and caused changes in the structure of the methanogenic community.

Keywords: sheep; condensed tannin; gas production; methanogenic; *Fibrobacter succinogenes*; electrophoresis; molecular techniques

Microorganisms attack organic matter, enter the rumen and break it down gaining adenosine tri-phosphate (ATP) for their own growth, short chain fatty acids (SCFA), which are utilized by the host as energy source, and ammonia (NH_3), which is rapidly utilized for microbial protein synthesis. Fibre degradation in the rumen involves initially fungi, ciliates, and cellulolytic bacteria (Joblin et al., 2002). However, microbial metabolism also generates undesirable compounds for both microorganisms and animals such as nitrate (NO_3), carbonic gas (CO_2), methane (CH_4), and hydrogen (H_2) (Schofield, 2000).

The greenhouse gas methane is produced by methanogenic Archaea reducing on one hand the energy content of the ingested feed but at the same time maintaining the proper ruminal pH. The majority of methanogens use CO_2 as substrate utilizing hydrogen released by other microorganism as electron donor. Consequently some hydrogen producers are influenced by methanogens due to hydrogen interspecies transfer, one of syntrophic relation in the microbiota.

There is a high diversity of methanogens in the rumen (Wright et al., 2004), the most important species being *Methanobrevibacter ruminantium*

and *Methanosarcina barkeri*. The relative proportion of methanogens species is influenced by host animal, diet composition, and geographic area (Ungerfeld et al., 2004).

Fibrobacter succinogenes is the most abundant fibrolytic bacteria in the rumen followed by the genus *Ruminococci* (Koike and Kobayashi, 2001; Joblin et al., 2002). *F. succinogenes* does not produce hydrogen but formate and succinate and does not participate in the hydrogen interspecies transfer. However, it is essential for indirect propionate as it produces succinate that can be used by *Selenomonas* spp. and *Veillonella parvula* (Reilly, 1998; McSweeney et al., 2001a).

Members of *Methanobacteriaceae* family, *Methanobrevibacter ruminantium* being the most important, are dominant in medium with high protozoa (Tokura et al., 1999; Ungerfeld et al., 2004). On the other hand, *Methanomicrobiale mobile* was found to be negatively affected when associated with protozoa (Sharp et al., 1999). The knowledge of relationship between protozoa and methanogens and species involved is important for identification of species resistant or tolerant to certain methanogenic inhibitors and their persistence action.

The alternatives to reduce or divert the formation of enteric methane involve the manipulation of rumen fermentation in one of two possible ways: (*i*) the use of diets, supplements or additives that promote a lower acetate: propionate ratio (C2 : C3) and (*ii*) direct action on the methanogenic microorganisms. This means that simple inhibition of the methanogenesis process does not guarantee improvement in energy efficiency for the animal, since ruminal hydrogen could not be absorbed anymore and alternative sinks like propionate (C3) needed to be employed.

However, tanniferous plants have demonstrated beneficial effects on reducing enteric methane production. Jayanegara et al. (2009) found there was negative relationship between total phenol, total tannins or tannin activity, and methane concentration. Yet, the adsorptive, bacteriostatic, and bactericidal actions of the tannins are not very well known (Getachew et al., 2000). Some studies with tanniferous plants have shown a reduction of the cellulolytic bacterial population with *F. succinogenes* being the most affected species (Bae et al., 1993; McSweeney et al., 2001b).

The use of tanniferous plants in ruminants' diet may either positively influence the low hydrogen-producing bacteria, or direct the hydrogen to other sinks, such as succinate, propionate or butyrate. Results from Tavendale et al. (2005) demonstrated that condensed tannins (CT) action on methanogenesis can be attributed to indirect effects via reduced hydrogen production and via direct inhibitory effects on methanogens. The objective of this study was to evaluate the influence of tanniferous plants on rumen methanogens and *Fibrobacter succinogenes* population using an *in vitro* gas production technique and molecular assessment of *F. succinogenes* and methanogenic Archaea.

MATERIAL AND METHODS

Plant origin and chemical characterization

Four condensed tannin-rich legumes, *Styzolobium aterrimum* L. (STA), *Styzolobium deeringianum* (STD), *Leucaena leucocephala* (LEU), and *Mimosa caesalpiniaefolia* (MIC), commonly named *mucuna preta, mucuna rajada, leucaena*, and *mimosa*, respectively, were obtained from an experimental area of the "Polo Regional Centro-Sul – Apta", SP, Brazil (latitude 22°43'31''S, longitude 47°38'57''W) with no lime and no fertilization. The whole aerial biomass, except for stems thicker than 0.5 cm, was harvested between November 7th, 2004 and January 5th, 2005 when plants were in the late vegetative stage. The collected material was wilted in an open and shaded area for 24 h before being dried up to constant weight in an open-air oven at 40°C, which lasted for about 72 h. *Cynodon* spp. (CYN) grass hay, known as Tifton 85, was obtained from the local market and used as a condensed tannins-free control (CT < 0.1 g/kg DM). All material was ground to pass a 1-mm sieve in a Wiley mill and stored at 4°C before being analyzed.

Dry matter (DM) (AOAC 2005: method 934.01), crude protein (CP) (AOAC 2005: method 954.01), and neutral detergent fibre with amylase and expressed without residual ash (aNDFom) (AOAC 2005: method 2002.04 adapted to Ankom Fibre Analyzer) were determined in the forage samples and fermentation residue. Phenolic compounds were extracted in an ultra-sonic bath with 10 ml of an aqueous acetone solution (700 ml/l) (Makkar et al., 1993). Total phenols (TP) and total tannins (TT) were determined by adding into an aliquot of the supernatant 0.25 ml Folin-Ciocalteau reagent (2*N*)

and 1.25 ml sodium carbonate solution (200 g Na_2CO_3/l) and detected photometrically at 725 nm. To determine TT, a binding tannin agent, insoluble polyvinyl pyrrolidone (PVPP) was added to the extract. A calibration curve was prepared from aliquots of the acid tannic solution (0.1 mg/ml) (Merck GmbH, Darmstadt, Germany). The difference of TP and the PVPP extract readings was an estimate of TT. The concentrations of TP and TT were calculated as tannic acid equivalents (eq) and expressed as g/kg DM.

Condensed tannins were expressed as leucocyanidin equivalent (% of DM) and were determined using Butanol-HCl and concentrations calculated by the formula:

Absorbance at 440 nm × 78.26 × Dilution factor

The dilution factor was equal to 1 if no 70% acetone was added or 0.5 ml per volume of the extract was taken (Porter et al., 1986; Makkar et al., 1993). Chemical composition of the plants is presented in Table 1.

In vitro gas production

An *in vitro* assay (Hohenheim gas test) was carried out in duplicates according to Menke and Steingass (1988) except that the concentration of $NaHCO_3$ was reduced to 33 g/l and that of $(NH_4)HCO_3$ increased to 6 g/l to prevent shortage in N during prolonged incubation times (Liu et al., 2002). Two castrate male adult sheep, Blackface breed, fed mixed diet of 1200 g/kg DM per day but never tanniferous feed, were used as rumen liquid donor animals. The liquid phase of the rumen content was collected before morning feeding, sieved through two layers of cheesecloth, and added into a prewarmed nutritive solution (1 : 2 v/v). The mixture, denoted inoculum, was kept at 39°C saturated with carbonic gas until inoculation.

Each glass syringe (100 ml) was incubated with 375 ± 10 mg air-dry ground substrate (treatment) and 30 ml inoculum. A set of six syringes for each sample and blank (inoculum alone) were prepared and placed in a rotor inside the incubator (39°C). Measurements and samples were taken after 0, $t_{½}$ (time of half maximal gas production), and 24 h in both runs. Triplicates of standard hay and standard concentrate obtained from the Institute of Animal Nutrition, Hohenheim University, Stuttgart, Germany, were included for adjustments. The $t_{½}$ was previously determined in a 96 h *in vitro* assay (Grings et al., 2005) as:

$$t_{½} = (\ln 2/c) + L$$

where:
L = lag phase of the model
c = constant of the model

After measuring gas production and sampling gas for methane analysis, syringes were placed in an ice bath to stop fermentation. The $t_{½}$ for CYN, STA, STD, LEU, and MIC were 17, 6, 6, 7, and 47 h incubation, respectively.

Microbial effects evaluation

A microbiological study was developed at the Research Centre Jülich, Germany, extracting DNA from the content of the syringes at $t_{½}$ and after 24 h. Two samples and two repetitions were included in the analysis. The relative abundance of methanogenic Archaea and *Fibrobacter succinogenes* was estimated by quantitative real time PCR (qRT-PCR) by amplification of specific 16S rDNA gene sequences. Evaluation of the effects on the rumen methanogenic community was performed applying denaturing gradient gel electrophoresis (DGGE). The specific conditions of qRT-PCR and DGGE were determined in preliminary assays.

DNA for qRT-PCR and DGGE were extracted from the *in vitro* contents at $t_{½}$ and 24 h incubation, following the recommendations described in Yu and Foster (2005). Cells were lysed using glass beads and cell lyses buffer, followed by freezing the samples at –80°C and centrifugations. The DNA was concentrated using glassmilk solution and first washed with cold ethanol solution (700 ml/l). The material was concentrated and purified using Wizard DNA Clean-Up System with a vacuum manifold (Promega Co., Madison, USA). DNA concentration and quality was checked by the A260 and A280 absorbance ratio in a spectrophotometer (NanoDrop® ND-1000 UV-Vis, Wilmington, USA).

For DNA amplification qRT-PCR primers *forward* 5'-GTT CGG AAT TAC TGG GCG TAA A-3' and *reverse* 5'-CGC CTG CCC CTG AAC TAT C-3' for *F. succinogenes* and primers *forward* 5'-TTC GGT GGA TCD CAR AGR GC-3' and *reverse* 5'-GBA RGT CGW AWC CGT AGA ATC C-3' for methanogenic Archaea (Denman and McSweeney,

2005, 2006) were used (MWG Biotech, Ebersberg, Germany). Quantitative PCR was performed in an iCycler (Bio-Rad Laboratories, Hercules, USA) using 12.5 µl reaction solution (IQ™ Sybr® Green Supermix, Bio-Rad Laboratories, USA), 1 µl of each primer (10 pmol/µl), 8 µl Milli-Q water, and 2.5 µl template DNA (diluted 1 : 10). Amplification was performed for one cycle at 50°C for 2 min and 95°C for 2 min for initial denaturation, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min for product annealing and elongation. Dissociation analysis of the qRT-PCR products was performed by raising the temperature at 95°C for 2 min followed by 60°C for 15 s (Denman and McSweeney, 2006).

During the exponential phase, the cycle number above which background fluorescence is detectable is defined as cycle threshold (Ct). In this study delta-delta Ct (ΔΔCt) method was applied to estimate *Fibrobacter succinogenes* and Methanogens population changes according to the following equation:

$$\Delta\Delta Ct = \Delta Ct \text{ (control)} - \Delta Ct \text{ (treatment)}$$

where:

ΔCt = difference between $Ct_{\text{(target gene)}} - Ct_{\text{(normalizator gene)}}$

As normalizator gene the 16S rRNA gene of universal bacteria was amplified with the following primer set: *forward* 5'-CGG CAA CGA GCG CAA CCC-3' and *reverse* 5-CCA TTG TAG CAC GTG TGT AGC C-3'. The relative abundance of *Fibrobacter succinogenes* and Methanogens was expressed proportional to the abundance of the total bacterial population and calculated as $2^{-\Delta\Delta Ct}$ (Denman and McSweeney, 2006).

Nested PCR for DGGE of methanogenic Archaea

Aliquots of 1 µl of the extracted DNA from the *in vitro* contents were used as template for the first amplification in a total reaction of 50 µl containing 2.5 µl dNTPs (2mM each), 5.0 µl of primers (10µM) 348aF and 915aR (Watanabe et al., 2004), 5.0 µl Buffer (10x), 5.0 µl $MgCl_2$ (25mM), 2.0 µl BSA (10 mg/ml), 0.6 µl Taq DNA polymerase, and 23.9 µl Milli-Q water. PCR started at 94°C for 10 min and was carried out in 40 cycles each consisting of 1 min at 94°C, 1 min at 61°C, and 2 min at 72°C, followed by a final extension period of 8 min at 72°C. PCR products were analyzed by electrophoresis in 2% (w/v) agarose gels in 1× TAE buffer supplemented with 250 µg/l ethidium bromide.

Gel fragments were excised and processed with the QIAex II agarose gel extraction kit (Qiagen, Hilden, Germany). Aliquots of 2 µl were applied in a second PCR, which contained the same reagent amount as before except for 23.0 µl Milli-Q water and 5 µl of the primers 0357F-GC and 691R (Watanabe et al., 2004). The amplification conditions were 94°C for 10 min, 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min, and a final extension at 72°C for 8 min.

DGGE to study methanogens community

Denaturing gradient gel electrophoresis (DGGE) was performed with a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, USA) using polyacrylamide gels (6% (w/v) acrylamide, 0.1% (w/v) ammonium persulfate, 0.1% (v/v) TEMED (N,N,N',N') tetramethylethylenediamine)), in 1× TAE buffer with gradients from 40 to 60% denaturant at 60°C and a constant voltage of 60 V for 16 h (Liebich et al., 2003). The 100% denaturant corresponds to 7M urea and 40% formamide (w/v) in TAE buffer. The concentration of PCR products was adjusted to 250–300 ng per lane. The DNA bands were analyzed by silver staining with GelCompar II (Applied Maths, Saint-Martens-Latem, Belgium).

Experimental design and statistical analysis

The microbial fingerprints at $t_{½}$ and 24 h were statistically analyzed using Dice coefficients as well as Pearson's correlation. Similarity matrices were clustered in UPGMA (Unweighted Pair Group Method using Arithmetic Averages) dendrograms. Evaluation of the community diversity was performed by calculating the Shannon index (H) (Shannon and Weaver, 1963), species richness (S), and evenness (E) in a completely randomized design**,** using two lanes per treatment. The Shannon index represents the abundance and similarity of the present species being calculated as

$$H = -\sum_{i=1}^{S} p_i \ln p_i$$

where:

p_i = relative abundance of a given i band in the total

profile
S = richness, the number of bands in each lane

The similarity index E was estimated as follows:

$E_h = H/H_{max}$, $H_{max} = \ln S$

Effects of treatments on these diversity parameters were estimated by GLM procedure of SAS (Statistical Analysis System, Version 8.01, 2001). Means were compared by Student's t-test with significant differences accepted when the error probability was lower than 5% ($P < 0.05$). qRT-PCR results of the treatments at $t_{½}$ and 24 h were compared by the $2^{\wedge -\Delta\Delta Ct}$. Median was chosen due to high variability among repetitions.

RESULTS

At both time points, $t_{½}$ and after 24 h of incubation, the leguminous plants reduced the relative gene expression (relative abundance) of the *F. succinogenes* population compared to the control CYN (Figure 1A and 1B, respectively). At $t_{½}$ the highest reduction was observed in treatments LEU and STA being 0.20- and 0.28-fold, respectively. MIC and STD presented lower reduction of *F. succinogenes* population compared to CYN (0.91- and 0.85-fold, respectively). Similar reductions in the abundance of *F. succinogenes*, varying between 0.24 with MIC to 0.74 with STD, were observed in all leguminous treatments at 24 h.

Methanogenic Archaea was as well affected at $t_{½}$ and 24 h (Figure 1C and 1D, respectively). Compared to CYN, the relative abundance of methanogens was reduced 0.29-fold with STA and 0.58-fold with LEU at $t_{½}$. However, MIC and STD treatments resulted in a 5.50- and 1.43-fold increase of the relative abundance of methanogens, respectively. After 24 h, only the MIC treatment resulted in an increased methanogenic population. At the same time point, STD reduced the relative gene expression of methanogenic Archaea 16S rRNA genes.

Figure 2A and 2B illustrates the separation of methanogenic Archaea 16S rRNA gene fragments by DGGE after applying the nested PCR procedure at $t_{½}$ and after 24 h, respectively. The band pattern revealed no obvious differences regarding the treatments either at $t_{½}$ nor after 24 h. This finding was supported by an additionally performed cluster analysis (data

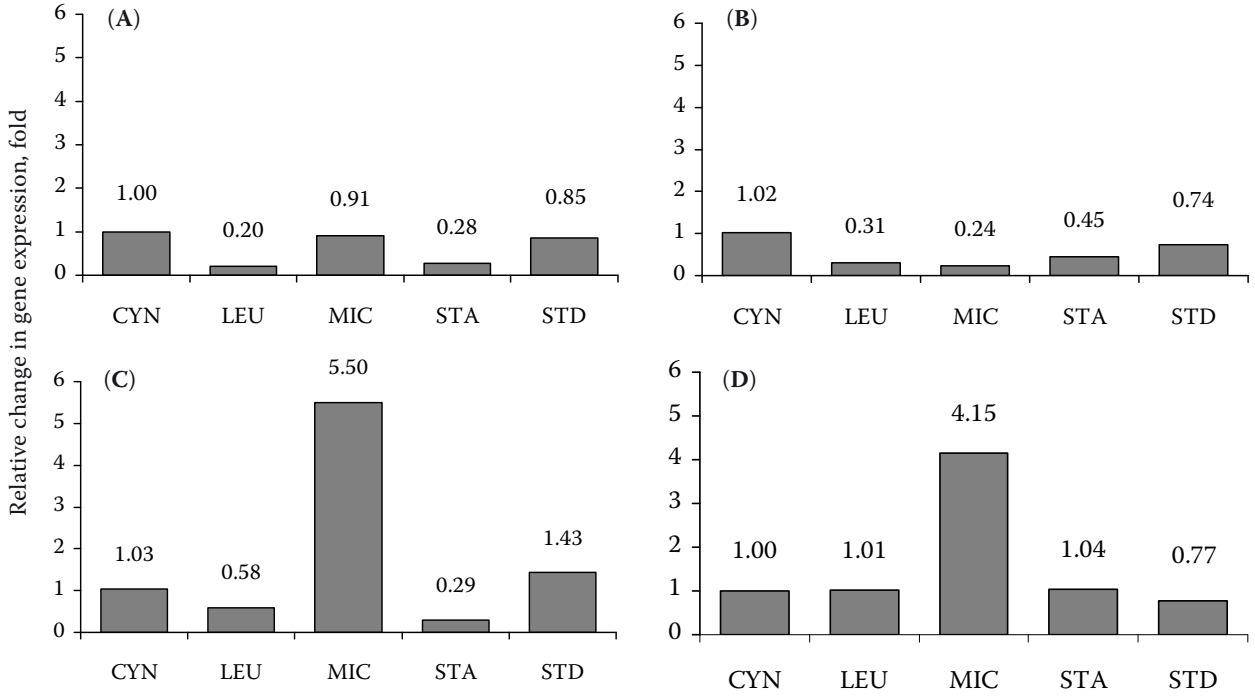

Figure 1. Relative change in gene expression of *Fibrobacter succinogenes* (**A**, **B**) and Methanogens population (**C**, **D**) at $t_{½}$ and 24 h, respectively based on $2^{-\Delta\Delta Ct}$ method where ΔΔCt is the "Cycles Threshold" variation, normalized with general bacterial population

CYN = *Cynodon* spp. (control), STA = *Styzolobium aterrimum* L., STD = *Styzolobium deeringianum,* LEU = *Leucaena leucocephala*, MIC = *Mimosa caesalpiniaefolia*

Table 1. Chemical composition of *Cynodon* spp. grass hay (CYN), *Styzolobium aterrimum* L. (STA), *Styzolobium deeringianum* (STD), *Leucaena leucocephala* (LEU), and *Mimosa caesalpiniaefolia* (MIC)

	CYN	STA	STD	LEU	MIC	SEM
DM	930	923	944	922	932	1
CP	78	241	236	246	191	8
aNDFom	774	637	642	620	706	20
ADFom	393	412	405	275	494	23
TP	6	55	72	114	151	4
TT	3	38	49	98	110	4
CT	0.2	20	64	56	105	3

DM = dry matter (g/kg original matter) at 105°C, CP = crude protein (g/kg original matter), aNDFom = neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash (g/kg original matter), ADFom = acid detergent fibre expressed exclusive of residual ash, TP = total phenols in eq-g tannic acid/kg DM, TT = total tannins in eq-g tannic acid/kg DM, CT = condensed tannin in eq-g leucocyanidin/kg DM, SEM = standard error of means

not shown). Similarly, the diversity indices *H'*, *E*, and *S* did not differ at both measured times ($P > 0.05$, $n = 8$), suggesting that diversity of the methanogenic Archaea was preserved in all treatments (Table 2).

Comparing band distribution between treatments and control (lane 23) at $t_{½}$ (Figure 2A), single bands (bands 3, 4, 8, and 12) in MIC (lanes 4 and 5) were reduced or disappeared while new bands (5a, 5b, and 10a) appeared. Additionally, bands 11 and 13, also present in CYN, became stronger after treatment with MIC in one of the replicates (lane 4). In STD (lanes 6 and 7), bands 3, 4, 5, 10, 11, and 13 became less intense than in the control and bands 8 and 12 disappeared, whereas bands 4b and 5a appeared. Intensity of the bands 3 and 4 was reduced in MIC and STD, but not in LEU and STA. However, bands 10 and 11 became weaker or disappeared in LEU and STA. In general, band 5b was visible in all treatments, being stronger in MIC. The band at position 13 was largely reduced in intensity in treatments with legumes, except in MIC (lane 4) and STA (lane 15). In general, bands at positions 1, 2, 6, and 9 were stable in all treatments.

The result of DGGE after 24 h is presented in Figure 2B, where the lanes 2, 4, 6, 8, and 12 stand for the first incubation and the lanes 3, 5, 7, 9, and 11 for the second. At this time point all legume treatments resulted in the appearance of a new

Table 2. Evaluation of Shannon-Weiner diversity (*H*), richness (*S*), and evenness (*E)* indexes of methanogens community at $t_{½}$ and 24 h *in vitro* incubation of four tanniferous legumes (*Styzolobium aterrimum* L. (STA), *Styzolobium deeringianum* (STD), *Leucaena leucocephala* (LEU), and *Mimosa caesalpiniaefolia*)) compared to *Cynodon* spp. (CYN) grass hay

	Blank	CYN	STA	STD	LEU	MIC	SEM	*P*
Shannon-Weiner (*H*)								
$t_{½}$	1.96	1.88	1.77	1.93	1.93	1.77	0.088	ns
24 h	1.70	1.56	1.78	1.73	1.72	1.73	0.139	ns
Richness (*S*)								
$t_{½}$	14.36	14.63	13.52	15.50	14.63	13.37	1.11	ns
24 h	9.70	9.50	10.75	9.75	10.63	11.00	0.88	ns
Evenness (*E*)								
$t_{½}$	0.75	0.70	0.68	0.70	0.72	0.69	0.024	ns
24 h	0.75	0.70	0.76	0.77	0.74	0.73	0.053	ns

SEM = standard error of means, ns = non significant
means in the same lane followed by the same superscript letter are not significantly different ($P < 0.05$) by Tukey's test

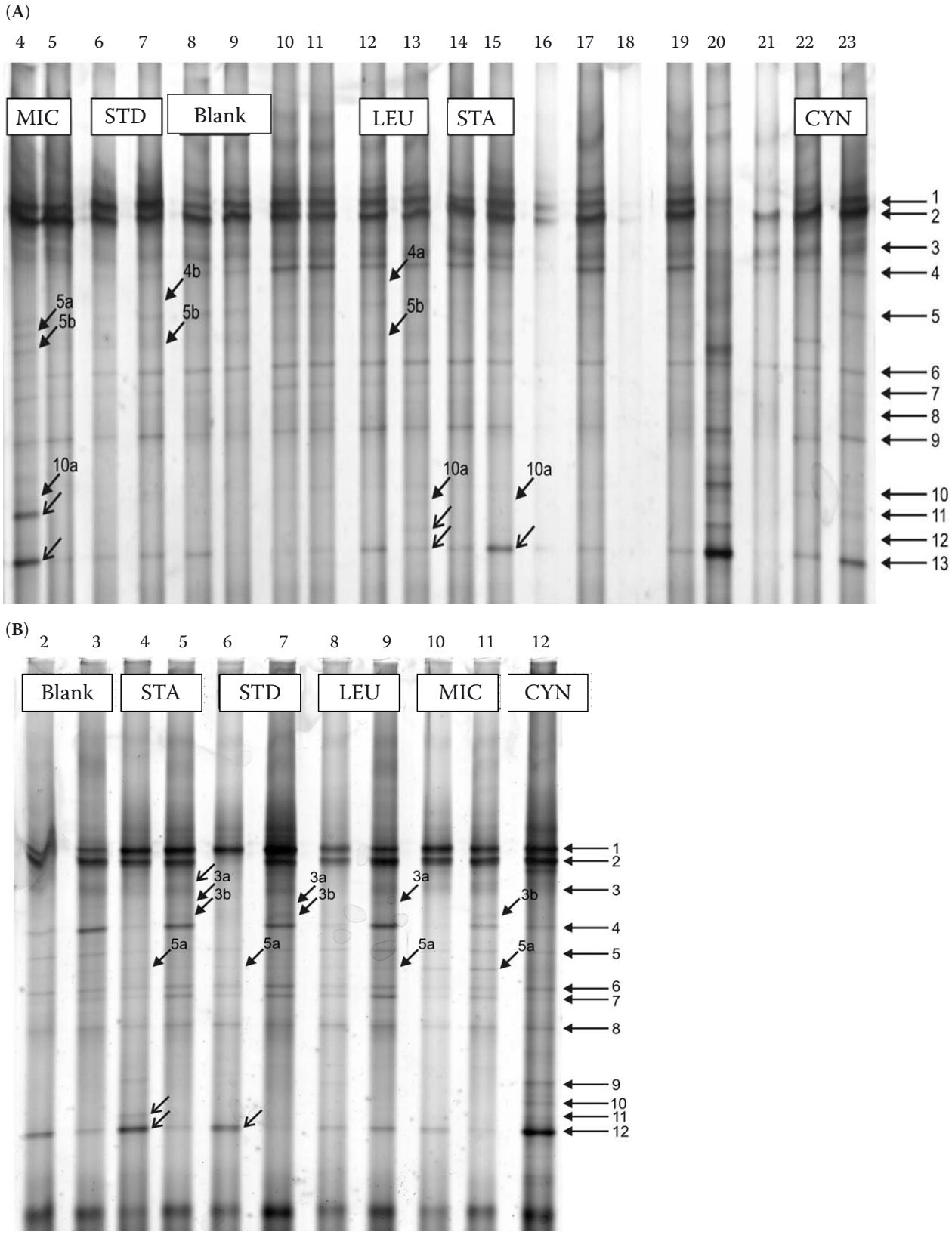

Figure 2. Denaturing gradient gel electrophoresis (DGGE) analysis of 16S rDNA fragments for methanogenic Archaea at $t_{½}$ (**A**) and 24 h (**B**) (lanes not identified are not related to this assay)

CYN = *Cynodon* spp. (control), STA = *Styzolobium aterrimum* L., STD = *Styzolobium deeringianum*, LEU = *Leucaena leucocephala*, MIC = *Mimosa caesalpiniaefolia*

band, 5a. The strongest intensity of the band at this position was observed after MIC treatment. STA and STD treatments led to the appearance of two additional bands between band positions 3 and 4, designated positions 3a and 3b. LEU treatment resulted only in a band at position 3a and MIC treatment in a band in position 3b. Band 7, although visible in CYN, was stronger in all leguminous treatments. Bands 9, 10, and 11 turned weaker or disappeared in all treatments, especially band 11, except in STA at the first run (lane 4). Band 12 was visible in all plants, however, with lower intensity than the control, except at STA (lane 4) and STD (lane 6). In general, bands 1, 2, 3, 5, 6, and 8 were present in all plants in similar intensity like the control.

DISCUSSION

Reduction of cellulolytic bacteria by tanniferous plants was also observed by many authors (Varel and Jung, 1986; Bae et al., 1993; Nelson et al., 1997; Guimarães-Beelen et al., 2006a, b). Some studies reported that *F. succinogenes* was the main cellulolytic species affected by tannins (Singleton, 1981; Nelson et al., 1997; McSweeney et al., 2001b). Therefore, it is not surprising that in the present study *F. succinogenes* was reduced by all treatments at both time points. This reduction may reflect the action of phenolic compounds through (*i*) difficulties of microorganisms adherence to the plant surface due to tannin action, (*ii*) reduced degradability of the tannin-protein complex, (*iii*) direct action on the microorganism cell wall (Singleton, 1981; Nelson et al., 1997) or (*iv*) bacteriostatic action on microbial enzymes such as endoglucanases (Bae et al., 1993; Makkar et al., 1993; Guimarães-Beelen et al., 2006a).

According to McAllister et al. (1994) free phenolic compounds in the rumen liquor are rapidly metabolized by microorganisms. However, phenolic compounds form cross-linking cell wall polymers, which protect the inner plant tissues from digestion. Thus, bacteria that are attached to plant cell wall are more affected by phenolic compounds (McAllister et al., 1994; McSweeney et al., 2001b; Krause et al., 2003). It is known that *F. succinogenes* is attached more firmly to the cellulose than other cellulolytic bacteria (Krause et al., 2003; Bento et al., 2005; Shinkai and Kobayashi, 2007) which may explain why usually this species is strongly affected by tannins.

In contrast to LEU and STA, *F. succinogenes* reduction at $t_{½}$ was less marked in MIC and STD treatment. STD was characterized by lower degradability of dry matter and neutral detergent, which suggests a reduced accessibility of the microorganisms to nutrients and consequently low tannins exposition at $t_{½}$ that persisted up to 24 h. In MIC, as $t_{½}$ was reached only after 47 h, the events at 24 h happened before its $t_{½}$ and should be analyzed first. *F. succinogenes* population recovered at $t_{½}$ (47 h) in MIC (Figure 1A) probably due to reduced tannin effect and/or any adaptation process.

Concerning methanogenic Archaea, different results among legumes were observed at $t_{½}$ (Figure 1C). LEU and STA resulted in the highest decreases (0.58 and 0.29, respectively) in the relative gene expression of methanogenic Archaea. *F. succinogenes* markedly decreased with these two legumes as well, suggesting an interdependent relationship between both species in a syntrophic relationship. However, *F. succinogenes* is not known for syntrophic relations with methanogens as known for other fumarate-reducing bacteria (Moss, 1993; Reilly, 1998). Fumarate-reducing bacteria compete with methanogens for hydrogen and formate as electron donors (Asanuma et al., 1999). It is possible that in LEU and STA treatments other species of fumarate-reducing bacteria may have competed more successfully and displaced reducing the methanogenic Archaea population at $t_{½}$.

STA showed stronger reduction of methanogens than LEU at $t_{½}$. In our concomittant *in vitro* study using the same plant material and methods (in press), STA showed at $t_{½}$ by 60% lower *n*-butyrate than the control CYN, which has been related to protozoa metabolism (Dohme et al., 1999). It is possible that the protozoa was more affected by STA. Interaction between methanogens and protozoa is well documented in studies with tanniferous plants (Wang et al., 1994; Hess et al., 2003; Abdalla et al., 2012), saponins (Hess et al., 2003), and oil (Dohme et al., 1999; Krause et al., 2003).

Relative abundance of methanogens in LEU and STA recovered at 24 h (Figure 1D) after the reduction at $t_{½}$ (Figure 1C). Several studies have demonstrated that on prolonged tannin exposition microorganisms develop ways to survive with its presence (Nelson et al., 1998; McSweeney et al., 1999; Joblin et al., 2002; Mlambo et al., 2007). These mechanisms are related to microbial extracellular secretions that reduce tannin effect and/or tannin-degrading enzymes. More studies

about tannin resistance, tolerance or adaptation to methanogens have to be carried out.

Although the influence of the host cannot be reproduced in the *in vitro* systems, Boguhn et al. (2008) demonstrated that fluctuations in the microbial community structure due to changes in the composition of the feed can be detected *in vitro*. In this study, DGGE analysis showed differences regarding treatments, time, and repetition (Figures 2A and 2B). Christophersen et al. (2004) applying DGGE reported that methanogenic diversity was influenced by diet, passage rate, and the interaction to protozoa species. Boguhn et al. (2008) testing four diets using PCR-SSCP and comparing reference microbes isolated from liquid and solid-associate microbes, found strong differences with primers targeting bacteria, fewer differences for Archaea and clostridia, and no clearly distinguishable differences with primers for *Fibrobacter* sp.

Although in our study the diversity indices did not differ in the methanogenic community, the different band distribution among treatments suggests that the composition of the methanogenic community was indeed affected by the tannin-rich plants. New bands appeared in all treatments. Possibly, the organisms represented in bands 7 and mainly 4, were more affected in MIC and STD treatments due to their lower quality and higher tannin effect.

Tannins could affect some microorganism by attaching its specific enzyme or its growth factor. Coenzyme M, for example, is specific for *Methanobrevibacter ruminantium* but not for the other strain (Miller et al., 1986). *M. ruminantium* could be more directly affected in the presence of tannin than the other strains. *Methanobrevibacter smithii* is formate dependent and could be reduced if tannins favoured other formate dependent microorganism (Rea et al., 2007).

At $t_{½}$, the DGGE pattern of the MIC treatment is characterized by high intensity of new bands (5a, 5b, 10a), increased intensity of the existing ones (8, 9, 10, 11, and 13), and no changes in the remaining bands. This corroborates with the increase in methanogenic relative abundance measured in qRT-PCR with this feed at $t_{½}$. The stability of bands 1, 2, 6, and 9 with all legumes indicates presence of dominant and no sensible tannin-rich forages methanogens in the rumen liquor. Some cellulolytic bacteria (Brooker et al., 1994; Nelson et al., 1998), proteolytics (McSweeney et al., 1999), and fungi (Joblin et al., 2002) have already been identified as tannin tolerant.

After 24 h, band distribution in the control did not change. At the same time, there were fewer differences to the legume treatments compared to $t_{½}$ among legumes. The bands 5b and 5a appeared in all legumes at $t_{½}$ and 24 h, respectively, but not in the control. This suggested that the methanogens present in these bands are related to general leguminous characteristics such as high protein concentration or some adaptation effect to tannins as suggested by Mlambo et al. (2007). Similarly, at 24 h, new bands appeared in MIC at $t_{½}$ or presented higher band intensity (5a). This is an indication of the increase of the methanogenic population in the qRT-PCR. Band 4 in MIC at $t_{½}$ and 24 h exhibited less intensity compared to the other legumes, suggesting direct effect of this plant on the microorganisms in this band.

CONCLUSION

Tanniferous forage negatively affected the *Fibrobacter succinogenes* population regardless of the concentration or type of tannin. Methanogens were differently affected based on the plant material added. Either the best nutritional quality (LEU) or the lowest tannin contents (STA) could have been responsible for the methanogens reduction. On the other hand, high condensed tannin concentration associated with low quality plants (STD and MIC) might have led to an increase of methanogenic Archaea. Changes in the community composition of the methanogenic archaeal community are expected with tanniferous plant.

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Received: 2012–01–13
Accepted after corrections: 2012–10–10

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