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RESEARCH ARTICLE

Effects of secondary compounds from cactus and acacias trees on rumen microbial profile changes performed by Real- Time PCR

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Abstract

Plant rich secondary compounds had antimicrobial effects by acting against different rumen microbial populations. The current study investigated the influence of spineless cactus (*Opuntia ficus indica f. inermis*), *Acacia nilotica* and A. *saligna* on rumen microbial fermentation, using in vitro gas production technique, and microbial population profile changes, using a molecular-based technique (Real-Time PCR). The acacias and Opuntia reduced significantly total gas production ($p<0.01$), rumen CH_4 production ($p<0.01$) and ammonia concentration ($p<0.001$). At 24h of incubation, Fungi population was 0.30- and 0.03 -fold reduced with *A.nilotica* and *Opuntia* as compared to 0h, but 2-and 1.24- fold higher with *A.cyanophylla* .Increases in the abundance of *F.succinogenes* were observed in all substrates; however, the tanniferous plants and Opuntia reduced the relative abundance of *R.flavefaciens*. Methanogenic population was increased with all substrates, except for *Opuntia* (0. 90- fold lower than the control). There was a significant reduction ($p<0.05$) in rumen protozoa count with *A.cyanophylla*, *Opuntia* and *A.nilotica* (3.68; 5.59 and 5.34 times, respectively). Results suggested that tannin sources from *A.nilotica* and *A.cyanophylla* had an indirect effect on methanogenesis. This study showed an antimicrobial activity of oxalates content of *O. ficus indica*.

Introduction

An interesting challenge for scientists in the field of animal nutrition is the introduction of alternative feedstuffs that could overcome the problems of environmental harshness and production costs. At the same time, the preservation of animal health, production yield and product quality is essential (Vasta et al., 2008). Some indigenous browse species are useful sources of animal feeds, they provide green forage for animals at times when grass and herbaceous species are of low nutritional value (Aregawi et al., 2008). One potential way for increasing the quality and availability of feed resources for livestock in the arid areas and to preserve the rangelands may be through the use of fodder trees and shrubs. These plants have also a potential to prevent desertification, mitigating the effects of droughts, allowing soil fixation and enhancing the restoration of the vegetation and the recuperation of rangelands (Robles et al., 2008; Mulas and Mulas, 2009).

Acacia saligna, *Acacia nilotica* and *Opuntia ficus indica f.inermis* (spineless cactus) are present in large areas of Algeria and are generally considered to be valuable as fodder reserve during drought. These species are adapted to a low moisture environment, thus they can offer a reliable source of feedstuff in the form of leaves and fruits (Mlambo et al., 2009; Ben Salem and Nefzaoui, 2002). The high water level in cactus represents an important alternative to supply water requirement of animals in arid and semi-arid regions, where water may be a limiting factor for animal production (Costa et al., 2009). On the other hand, it is an excellent energy source, rich in non fibrous carbohydrates and presents high dry matter digestibility coefficient (Wanderley et al., 2002). Under African conditions, A.saligna and A.nilotica, leguminous trees are present in sufficient quantities to contribute to ruminant diets (Degen et al., 1995, Mlambo et al., 2008). Their foliage may be used as a protein and energy supplement when animals are given low quality roughage (Krebs, 2007). Interest devoted to this species is partly related to their high protein content, abundant biomass and evergreen habit (Aganga et al., 1998).

However, the presence of anti-nutritional secondary compounds (e.g. tannins, oxalates…), with potential adverse effects on rumen microbial fermentation, feed digestibility and animal performance, could restrict nutrient utilization of shrubby vegetation (Waghorn and McNabb, 2003; Mueller-Harvey, 2006). Plant secondary compounds had antimicrobial effects by acting against bacteria, protozoa and fungi. Phenolic compounds are the main active components (Burt, 2004). The antimicrobial mode of action, either microcidal or microstatic, is considered to come mainly from the potential of their intruding into the bacterial cell membrane to disintegrate membrane structures which causes ion leakage (Bodas et al., 2012). The objective of this study was to evaluate the influence of secondary compound on rumen fermentation, using in vitro gas production technique, and microbial population profile changes using a molecular-based technique (Real- Time PCR).

Material and Methods

Plant material

Cladodes of wild spineless cactus (Opuntia ficus indica f. inermis) growing on marginal lands were randomly harvested in winter from an area of Constantine. This area is located at 36.17 North and 6.37 East and at an elevation of about 693 meters above sea level in the North Eastern part of Algeria. It is characterized by a semi-arid climate with irregular and low precipitations of 350-700 mm pear year. The mean temperature of the coldest period ranges between -0.3°C and 3.2 C while the hottest one is ranging between 33.3 and 37.3 °C.

The *Opuntia* cladodes were cut by hand with a single-machete into small cubes of approximately 50 mm x 30 mm and dried at 50 ◦C (Makkar, 2003) in a ventilated oven. The vetch-oat hay was collected from the Technical Institute of Great crops (I.T.G.C. of Constantine), and dried in the same conditions as for Opuntia pads. Foliage from two browses species (Acacia nilotica (L.) Willd. ex Delile and Acacia saligna (Labill.) Wendl. f. (formerly Acacia cyanophylla Lindl.) was also collected from a semi-arid area of Constantine, in the early autumn. Between six and ten specimens of each plant species were sampled to obtain a representative aliquot of the edible biomass. Leaves were clipped with scissors from the aerial part of the plants, and taken immediately to the laboratory where the samples from the different specimens of the same species were mixed and air dried in the shadow. All dried plant material were then ground in a laboratory mill to pass through a 1mm sieve and stored in tightly sealed plastic bottles for later analysis.

Chemical analysis

Dry matter (DM), Organic matter (OM), Ether extract (EE) (AOAC, 1990) and crude protein (AOAC, 2000) were determined in the forage samples. Neutral and acid detergent fiber (NDF and ADF, respectively) and acid detergent lignin (ADL) were performed by the method of Van Soest et al. (1991). Non-fiber carbohydrates (NFC) were calculated by difference whereby the sum of NDF, CP, EE and ash in percentage subtracted from 100 (Mertens, 1997). Phenolic compounds were extracted following the procedures described by Makkar (2003). Total extractable phenols (TEP) were determined using the Folin-Ciolateau reagent and tannic acid as the standard. The concentration of total condensed tannins (TCT) was measured in the extract using the butanol-HCl assay, with the modifications of Makkar (2003). Feeds were also analyzed for total oxalates (AOAC, 1990). All chemical analyses were performed in triplicate.

Rumen inocula

Ruminal content was supplied from "The experimental center of Agro Evolution- Euro nutrition, Saint Symphorien-France". It was recovered before the morning feeding from two fistulated dried Holstein, housed according to European guidelines for animal welfare and fed, at maintenance on a roughage-concentrate diet (70-30 w/w, 9.8% crude protein on DM basis) twice daily at 8.00A.M and 4.30 P.M (7kg DM/day), with free access to clean drinking water. Rumen fluid was withdrawn through a four layer of muslin cloth into pre-warmed thermos flasks previously flushed with CO_2 gas and taken immediately to the laboratory, where sample of rumen content was kept at 39°C under a constant flow of CO2.

In vitro gas production measurements

In vitro gas production test was done according to Menke et al. (1979). A culture medium containing macro- and micro-mineral solutions, a bicarbonate buffer solution, resazurin and reducing solution was prepared as described by

Menke and Steingass (1988). The medium was maintained at 39°C and saturated with CO_2. Rumen fluid was then added to the buffered mineral solution in the proportion 1:2 (v/v). Each sample of plant material (200 mg) was weighed and placed into a 100ml glass syringe (Fortuna, Poulten and Graf GmbH, Wertheim, Germany) equipped with a luer lock valve. The pistons were pushed until 10 ml of gas remained. The headspace of the syringes was flushed 3 times with CO_2. 30 ml of incubation medium was dispensed trough the valve of the preheated (39°C) and anaerobic syringe with the help of a peristaltic pump. All syringes were placed for 24h at 39°C in an incubator shaker (KS 4000i control, IKA Werke, Staufen, Germany) at 50 rpm. Each substrate was run in duplicate, with two syringes without substrate (blanks).
The carbon dioxide and methane productions(CO_2 and CH_4, respectively) were evaluated at the end of incubation by injection in each syringe, 4ml of sodium hydroxide (NaOH,10N) (Jouany,1994).
The metabolizable energy (ME) content and the organic matter digestibility (OMD) of samples were estimated according to Menke et al. (1979).

Fermentation parameters (rumen pH, ammonia nitrogen and VFA analysis)
At the end of fermentation, the pH was determined immediately in culture fluid with a pH meter. For ammonia-N ($N\text{-}NH_3$) determination; a 5ml sample of the incubated fluid was preserved with 0.5 ml 5% orthophosphoric acid at -20°C. Samples were centrifuged at 12,000xg for 20min, and the supernatant was analyzed by spectrophotometry, for N-NH3 content according to the watherburn technique (1967). For analysis of Volatile fatty acid (VFA) composition, 1ml of 10% hydroxide sodium was added to 10 ml of the incubated fluid and stored at – 20°C until analyzed. the mixture was centrifuged at 5000×g for 10 min at 4°C, after filtration, supernatant was analyzed by gas chromatography (BP21, SGE, Europe Ldt., Buckinghamshire, UK) with a capillary column (length: 30m, inner diameter: 530 µm film :1 µm) and flame-ionization detection. The temperatures of the injector and detector were 280°C, 240°C, respectively.

Enumeration of rumen Protozoa
Samples from the incubated fluid were homogenized and mixed with a methyhl green-formalin-saline solution (50-50, V/V) for protozoa enumeration according to Ogimoto and Imai (1981). The mixture was pipetted into a 10 microlitre Agass Lafont counting chamber (Preciss, France) before and after incubation. Protozoa were counted microscopically under 10 x magnifications. Each sample was counted twice, and if the average of duplicates differed by more than 10%, the counts were repeated.

Analysis of microbial population
Extraction of genomic DNA. For quantification of microbial populations, an aliquot (1.8ml) of the incubated liquid from each syringe was sampled at 0h and after 24h of incubation, and then stored at -20°C immediately. The procedure of total DNA extraction was performed according to the QIAamp ® DNA Stool Mini Kit (purchased from QIAGEN France S.A.S) and used according to the manufacturer's instructions (Singh and al., 2011). Briefly, rumen samples were homogenized in buffer ASL and heated at 95 °C for 5min to lyse microbial cells. After removal of potential inhibitors by incubation with an InhibitEx tablet, the lysates were treated with proteinase K and buffer AL at 70 °C for 10 min to remove protein and polysaccharides. DNA was precipitated by ethanol, applied to a column provided in the kit followed by washes with buffers AW1 and AW2, and then dissolved in buffer AE. The concentration and quality of DNA were determined at A260 nm and A280 nm using a Nanodrop ND-1000 (NanoDropTechnologies, Wilmington, DE, USA).
Real-Time PCR technique. The relative quantification of different microbial groups: total bacteria, total anaerobic fungi, two predominant cellulolytic bacteria (Ruminococus flavefaciens, Fibrobacter succinogenes) and total methanogens, were determined in the samples.
Real-time PCR assays were performed on a Rotorgene-6000 Thermocycler (Corbett Research, Sydney, Australia). Assays were set up using the Power SYBR® Green PCR Master Mix (2X) (Life technologies). The specific primers targeting different microbial groups, used in this study are listed in table 1. These primers were chosen from previously published sequences that demonstrated species-specific amplification. The microbial sample DNA was diluted to 5.3 ng/ul prior to use in the quantitative PCR assays to reduce inhibition. The assay was conducted under the following cycle conditions: 95°C for 15 s for denaturation, followed by 45 cycles of 60 sec each for annealing at 60°C and extension at 72°C. Fluorescence detection was performed at the end of each denaturation and extension steps. Specifity of amplified products was confirmed by melting temperatures and dissociation curves after each amplification. Amplification efficiencies for each primer pair were investigated by examining dilution series of total rumen microbial DNA template on the same plate in triplicate. A negative blank (without the DNA template) was also run for each primer pair. The relative abundance of different groups was quantified using the relative

quantification Δ C_T (Livak and schmittgen, 2001). The results were presented as changes in microbial population relative to control (0h), taken as one.

Table 1. Primers for Real- time PCR assay.

Target species /genes		Forward primer/Reverse primer Sequence (5' to 3')	References
General bacteria	16S rRNA	TCCTACGGGAGGCAGCAGT GGACTACCAGGGTATCTAATCCTGTT	Nadkarni et al. (2002)
Anaerobic fungi	*MAF(18S & ITS1)	GAGGAAGTAAAAGTCGTAACAAGGTTTC CAAATTCACAAAGGGTAGGATGATT	
F.succinogenes	16S rRNA	GTTCGGAATTACTGGGCGTAAA CGCCTGCCCCTGAACTATC	Denman &McSweeney (2006)
R. flavefaciens	16S rRNA	CGAACGGAGATAATTTGAGTTTACTTAGG CGGTCTCTGTATGTTATGAGGTATTACC	
Methanogens	*mcrA*	TTCGGTGGATCDCARAGRGC GBARGTCGWAWCCGTAGAATCC	Vinh et al. (2011)

*MAF (18S & ITS1): Multiple alignments of fungal 18S ribosomal and ITS1 gene sequences

Statistical analysis

One-way analysis of variance was performed on gas production fermentation parameters and real time PCR data, with browse species as the only source of variation (fixed effect) and source of inoculum (random effect) as a blocking factor. Tukey's multiple comparison test was used to determine which means differed from the rest ($P<0.05$). Pearson linear correlation coeffcients (r) were determined pairwise between the variables studied. Analysis of variance (PROC GLM) and correlation (PROC CORR) were performed using the SAS software package (SAS, 2000).

RESULTS

Spineless cactus cladodes were relatively high in oxalates (148.27 g/kg DM) and had the highest ash content (296.413 g/kg DM). However, *A. nilotica* and to a less extent *A. saligna* exhibited a high content of CP (243 and 157 g/kg DM, respectively). The NDF and ADF contents ranged respectively from 290 to 585 g /kg DM and from 120 to 317 g /kg DM, with the highest values for vetch- oat hay. CT was highest in *A.nilotica* and *A.saligna* with 726.3 and 631. 23 g/kgDM, respectively, whereas, not detected in *Opuntia* pads (table 2).

pH, N-NH_3, Total VFA concentrations (millimolar/l) and molar percentages of individual VFA are presented in Table 3. There was no significant difference ($p>0.05$) in the rumen liquor pH between the studied plants. Concentration of NH3-N was lower ($p<0.05$) in all substrates, relatively to vetch-oat hay. The production of VFA was statistically different among feedstuffs ($p<0.05$). The lowest value was noted in *Opuntia* (59.8mmol/l). *A.saligna* had the highest molar proportion of acetate but the highest value of propionate was obtained in *A.nilotica* ($p<0.05$), (72.084 and 22.24 mol/100ml, respectively). However, molar proportion of butyrate was significantly higher ($p<0.05$) in vetch oat- hay and to a less extent in *Opuntia*.

Table 4 shows the gas production data for the investigated plants incubated in vitro for 24 h. Significant ($P<0.05$) differences across all substrates were recorded in total gas, CH_4 productions and in the predicted parameters. The gas production, ME and OMD were highest ($P<0.05$) for vetch- oat hay and to a less extent for cactus, while the *A. nilotica* and *A.saligna* leaves recorded the lowest productions. The same trend was observed for methane emission.

The microbial population determined by Real- Time PCR showed significant changes ($p<0.05$) in the population number of total bacteria, fungi, *R. flavefaciens*, *F. succinogenes* and methanogens (Table 5). At 24h of incubation, the tanniferous plants and *Opuntia* reduced the relative abundance of *R.flavefaciens*, compared to the control 0h, with the highest reduction for *Opuntia* (0.085- fold). Increases in the abundance of *F.succinogenes* varying between 3.07 for *A.nilotica* to 19. 9 for vetch- oat hay were observed in all substrates. Compared to 0h, the relative abundance of fungal population was reduced 0.30- fold with A.nilotica and 0.031- fold with *Opuntia*, at 24h of incubation. However, *A.saligna* and vetch-oat hay resulted in a 2-and 1.24- fold increases of the relative abundance of fungi, respectively. After 24h of incubation, only *Opuntia* inhibited methanogenic population by 0.90-fold. However, *A.nilotica* resulted in the highest increase (6.48- fold), *A.saligna* and vetch- oat hay presented lower increase of methanogens population compared to 0h (1. 73 –and 1.34- fold, respectively). There was a significant reduction ($p<0.05$) in rumen protozoa count with *A.saligna*, *Opuntia* and *A.nilotica* (3. 68; 5. 59 and 5.34 times, respectively).

Table 2. Chemical composition (g/kg dry matter) of experimental substrates.

	Plant species			
	O. ficus indica	**Vetch-oat hay**	***A.nilotica***	***A.saligna***
Dry matter (DM) (g / Kg)	961.18	935.09	900	913.36
Organic matter (OM)	703.58	941.85	920.47	899
ASH	296.41	58.14	80	101
Crude protein (CP)	77.84	108.3	243	157
Neutral detergent fiber (NDF)	325.25	585.19	290	447
Acid detergent fiber (ADF)	119.60	316.82	198	255.13
Lignin (ADL)	19.11	45.20	126.41	148
Ether extracts (EE)	18.59	17.53	17.08	15.88
Non-fiber carbohydrates (NFC)	281.88	235.32	369.92	279.11
Total oxalates	148.27	48.62	nd	nd
TEP	8.59	5.06	213.4	204.8
TCT	nd	57.99	726.3	631. 2

nd =no detected
TEP= Total Extractable Phenols; TCT= Total Condensed Tannins (g /kg DM, standard equivalent).

Table 3. Effect of substrates on rumen fermentation characteristic in Holstein cow, after 24h of incubation

Item	***O. ficus indica***	**Vetch-Oat hay**	***A.nilotica***	***A.saligna***	**S.E.M**
Ruminal pH	7.29a	7.075a	7.01a	6.970a	0.054
NH3-N (mg/100ml)	25b	26.5a	14.41c	10.76d	0.619
Total VFAs (mmol/l)	59.8d	120.9a	73.84b	70.44c	0.422
VFAs, mol/100ml					
Acetate,C2	70.17c	70d	71.45b	72.08a	0.330
Propionate,C3	21.78b	20.04d	22.24a	20.24c	0.359
Butyrate,C4	8.05b	9.96a	6.30d	7.675c	0.493
C2 :C3 ratio	3.22c	3.49b	3.21d	3.561a	0.0592

VFAs: Volatile fatty acids; a, b, c, d: means in a row with different superscripts are significantly different ($p < 0.05$); SEM=Standard error of the mean; P=Probability.

Table4. Effect of substrates on total gas production, methane emission (ml/200mgDM), estimated metabolisable energy and organic matter digestibility, after 24 h of incubation

Item	***O. ficus indica***	**Vetch-Oat hay**	***A.nilotica***	***A.cyanophylla***	**S.E.M**
GT ,ml	25.50b	29.00a	15. 00c	13.00d	2.561
CH_4,ml	5.00b	8.00a	3.50c	3.00d	0.736
ME(MJ/KgDM)	6.11b	6.73a	5.62c	4.86d	0.259
OMD%	41.05b	45.33a	39.15c	33.50d	1.606

GT= gas total; OMD (%) = 14, 88+0,889*Gv+0,45*CP ; ME (MJ/KgDM) =2,20+0,136*Gv+0,057*CP Where, OMD is organic matter digestibility (%); ME: metabolizable energy; CP: crude protein in percent; and Gv: the net gas production in ml from 200mg dry sample, after 24 h incubation (Menke et al., 1979); a, b, c, d: means in a row with different superscripts are significantly different ($p < 0.05$); SEM=Standard error of the mean; P=Probability.

Table 5. Effect of substrates on relative quantification of different microbial groups by Real- Time PCR and on protozoa by direct count, after 24h against 0h (control) of incubation.

Species	**Control**	***O. ficus indica***	**Vetch-Oat hay**	***A.nilotica***	***A.cyanophylla***	**S.E.M**
Total bacteria	1.0a	0.03e	0.03d	0.10b	0.06c	0.126
Methanogens	1.0d	0.90e	1.34c	6.48a	1,73b	0.705
Anaerobic fungi	1.0c	0.03e	1.24b	0.30d	2.0a	0.232
R. flavefaciens	1.0b	0.08e	1. 17a	0.38c	0.33d	0.139
F.succinogenes	1.0e	4.86c	19.79a	3.07d	8.5b	2.215
Direct count (*10^4Cell /ml)						
Protozoa	1.94e	5.59b	12.12a	5.34c	3.68d	1.15

a, b, c, d, e : means in a row with different superscripts are significantly different ($p < 0.05$); SEM=Standard error of the mean; P=Probability

Discussion

In vitro gas production has been widely used to assess the nutritive value of diverse classes of feeds (Bakhashwain et al., 2010; Allam et al., 2012). In the current study, the higher gas production of vetch- oat hay as compared with *Opuntia* could be due to its highest content in OM. Besides, oxalates in cactus might have hampered microbial activity in the rumen as suggested by the reduced gas production (Ben salem et al., 2002). The low gas production of Acacia species might be attributed to their highest levels in lignin and phenolic compounds, particularly CT (Mohammadabadi et al., 2010; Edwards et al., 2012). The CT form complexes with carbohydrates, rendering them undegradable, and then inhibits the microbial enzymes or microorganisms, complexing with lignocellulose, thus preventing the microbial digestion (Griffiths, 1986). Moreover, the low gas production from fermentation of Acacia species was also observed in other studies (Bakhashwain et al., 2010 and Allam et al., 2012). The OMD and ME were negatively correlated to CT content ($r=-0.39$; $p <0.01$ and $r=-0.42$; $p <0.01$, respectively). Adverse effects of CT on ME and OMD of Acacia spp are consistent with in vitro and in sacco studies (Hervás et al., 2003; Getachew et al., 2008). The low ME content of *O. ficus indica* as compared to that of vetch-oat hay, could probably be due to the higher ash content of cactus (Ben salem et al., 2010).

Rumen digestion of feed components by the microbiota (bacteria, archaea, protozoa and fungi) under anaerobic conditions results in the production of VFA, CO_2 and CH_4 (Martin et al., 2010). The highest total CH_4 generated after 24h of incubation by vetch -oat hay might be due to its higher digestible fiber content (Agarwal et al., 2008). The lower methane emission from degradation of Acacia species could be due to their high tannin content. These results are consistent with soltan et al. (2012), who found a pronounced methane reduction with *A. saligna*, associated to their high CT content. Many others, in vitro and in vivo, studies have demonstrated the antimethanogenic activity of tannins (Tavendale et al., 2005; Hess et al., 2006; Goel and Makkar, 2012). However, the mode of action of tannins has not been completely described (Bodas et al., 2012). In general, inhibition of CH_4 production entails an alteration in VFA profile because of alternative electron sinks to dispose of reducing power (Cieslak et al., 2012). In our study, an increase in the concentration of propionic acid occurred with *A.nilotica* and relatively with *Opuntia*, explained probably by their chemical composition. Our results are consistent with Cieslak et al. (2012), who stated that propionate production and methanogenesis are competitive. During fermentation, the conversion of starch to propionic acid, may disturb horizontal hydrogen transfer and thus leading to limitation of methanogenesis process (Szumacher-Strabel and Cieslak, 2012), According to Tavendale et al. (2005), the strong inverse relationship between the molar proportion of propionate and CH_4 may depend on interactions among rumen microbial population, and compounds that promote higher production of C3 in the rumen. Our results are similar to those obtained by Wang et al. (2012); The relative increase in propionate and decrease in butyrate proportions may be also associated to the reduction in protozoa population .Variations in protozoa numbers in the rumen could lead to changes in VFA production and composition (Wang et al., 2012).

The changes in microbial profile in response to the substrates, studied herein were revealed by real time PCR. Tanniferous plants appeared to affect some microbial community. An antiprotozoal effect occurred with acacias tannins, which is consistent with Cieslak et al., (2012), who demonstrated that tannins from *Vaccinium vitis idaea* reduced protozoa numbers in Holstein-Friesian dairy cows' rumen. Tannins can have diverse effects on ruminal protozoa: results obtained *in vitro* and in vivo showed that they generally depress ruminal protozoa populations (Monforte-Briceño et al., 2005; Animut et al., 2008) .In other instances, some studies report unclear effects (Sliwinski et al., 2002), whilst others report a clear defaunating effect (Bhatta et al., 2009; Monforte-Briceño et al., 2005). Bodas et al., (2012) stated that although the mode of action of tannins on protozoa is not clear, it might be similar to that observed on bacteria. This reduction in protozoal number is associated with the observed decrease in ammonia-N concentration, in acacias and to a less extent in Opuntia relatively to vetch-oat hay. This could be due to a reduction in the proteolytic activity of the protozoa (Doreau and Ferlay, 1995) and to deamination processes by CT for acacias (Soltan et al., 2012, Goel and Makkar, 2012).

The increase in the total number of methanogens in the rumen from degradation of acacias was not expected, as tannins generally inhibit methanogens populations (Kamra et al., 2006). A possible explanation may be based on a potential resistance of this population to acacias tannins. This mechanism could be 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 (Longo et al., 2013). In addition, the relative abundance of total methanogens in the rumen could be related to the origin of tannins or their nature (e.g., hydrolysable versus condensed). The CT appear to decrease CH_4 production more through a reduction in fiber digestion (indirect effect), while hydrolysable tannins (HT) appear to act more through inhibition of the growth and/or activity of methanogens and/or hydrogen producing microbes (direct effect) (Jayanegara et al., 2010). Moreover, Some of the ruminal methanogens can be associated intracellularly or extracellularly with ciliates protozoa (Tokura, et al., 1999).This symbiotic relationship results in 40% of methanogenesis in rumen fluid (Hegarty ,1999).Generally, Defaunation is combined with reduced methane production in the rumen (Nagaraja et al., 1997) as methanogens lose their symbiotic partner, resulting in their reduced biological activity (Kamra et al., 2006).However, in the present study, it appears that methanogenesis is not so influenced by the association between protozoa and methanogenic bacteria. This is in agreement with Machmuller et al. (2003) and Cieslak et al. (2012), who stated that ruminal methanogenesis may not always be correlated with number of methanogens in the rumen. Such results might be related to the finding of Sharp et al., (1998); using a group-specific 16S rRNA probes, he found that most of rumen methanogens are being essentially free living in rumen fluid , since only a negligible hybridization signal was detected with the ruminal protozoal fraction.

Among the quantified cellulolytic bacteria, the population of *F.succinogenes* was most abundant in all substrates. This result supports the previous finding that *F.succinogenes* is one of the most common cellulolytic bacteria in the rumen, contributing ca. 5 to 6% of total prokaryotic 16S r RNA in the rumen contents of cattle (Jun et al., 2007). Some studies reported that *F.succinogenes* was the main cellulolytic species affected by tannins

(McSweeney et al., 2001; Longo et al., 2013). However, in the present study reduction of *F.succinogenes* population size was not observed, but a selective effect of tannins on R.flavefaciens occurred. Tannins can be particularly toxic to fibrolytic bacteria (Bhatta et al., 2009), through: (i) bacteriostatic action on microbial enzymes such as endoglucanases (Guimarães-Beelen et al., 2006) (ii) direct effect (Koike and Kobayashi, 2009) or (iii) by reducing nutrient availability (Sallam et al., 2010).

The anaerobic fungi population was relatively increased after 24h of incubation for vetch-oat hay and *A.saligna* as influenced by their high fiber content. In contrast, *O. ficus indica* and *A.nilotica* decreased relatively the fungi population after 24h of fermentation, probably due to their high content in NFC. This finding are in accordance with other study, suggesting that diet can have a significant effect on fungal populations, with high-fiber diets promoting larger fungal population than high-concentrate diets (Bauchop, 1979). Little studies have been carried out on the effect of plant extracts on rumen fungi (Patra and Saxena, 2009). McSweeney et al. (2001) stated that effects of tannins on fungi are more subtle than those on bacteria, and vary with the chemical structure of the tannins and their differing cell-surface receptors.

Utilization of *O. ficus indica* as substrate decreased significantly ($P<0.05$) the relative quantification of methanogens, anaerobic fungi, *R.flavefaciens* and protozoa. This could be related to the high content of *Opuntia* cladodes in Oxalates. Using cultivation-independent molecular techniques, Belenguer et al., (2013) have studied the impact of oxalic acid (OA) on rumen bacterial community in sheep, and found rapid variations in the ruminal microbiota, occuring with OA administration in diets. The administration of OA altered the rumen environment, including the bacterial community. The oxalate has the potential to form a strong chelate with dietary calcium in the form of calcium oxalate crystal (Mcconn and Nakata, 2004; Contreras-Padilla et al., 2011), making this anion less available to animals (Nefzaoui et Ben Salem, 2001) .In ruminants, some essential minerals such as calcium and phosphore have been proven to be important modulators of microbial fermentation (Younes et al., 1993; Durand and Komisarczuk, 1998).

The calcium ($Ca2+$) is the most universal carrier of biological signals: it modulates cell life (carafoli, 2007).This signaling molecule regulates a number of essential processes in eukaryotes (Clapham, 2007). In prokaryotes, Various physiological processes such as spore formation, motility, cell differentiation, transport, virulence and bacterial gene expression are modulated by Ca2+ (Domníguez et al., 2011 ; Guragain et al., 2013). It was recognized that the signaling function of Ca2+ had a number of unique properties. A distinctive property of the Ca2+ signal is autoregulation; it occurs at the transcriptional and post translational levels (Carafoli, 2007).

The consumption of oxalate-rich feed resources, in sufficient quantity may cause mainly hypocalcemia and renal failure, leading to poisoning in livestock (James, 1972; Cheek, 1995). However, the adaptation of the ruminal microbiota to oxalate can prevent animal poisoning, by microbial detoxification (Allison et al., 1981). Belenguer et al., (2013) observed a rapid adaptation of the ruminal microbiota to the consumption of OA, linked to the rapid estimated increase in the abundance of Oxalobacter formigenes (from 0.002 to 0.007% of oxc gene in relation to the total bacteria 16S rDNA: $p< 0.01$), which is assumed to be responsible for oxalate breakdown.

Conclusion

O.ficus indica and acacias affected negatively rumen microbial fermentation in terms of gas production and total VFA, but reduced methane production, and therefore an increase in efficiency of energy utilization would be expected in the animals. An important shift in microbial profile occurred, but *R.flavefaciens* and protozoa seemed to be sensitive to tannins from Acacia species. Furthermore, tannins had antimethanogenic activities but without, apparently, direct effects on relative abundance of methanogens population. This study showed also an antimicrobial activity of oxalates content of *O.ficus indcia*. This mechanism needs evaluation especially, the catalytic potential of *Oxalobacter forminogen* to reduce Oxalates.

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