Methanogenic potential of tropical feeds rich in hydrolyzable tannins^{1,2}

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ABSTRACT: The present study was carried out to determine the effect of *Acacia nilotica*, a tropical plant rich in hydrolyzable tannins (HT), on rumen fermentation and methane (CH₄) production in vitro. We used leaves and pods from *A. nilotica* alone and combined. The combination of HT from *A. nilotica* leaves and pods and condensed tannins (CT) from *Calliandra calothyrsus* and *Leucaena leucocephala* were also evaluated to assess potential differences in biological activity between HT and CT. Four series of 24-h incubations were performed using rumen contents of 4 sheep fed a tropical grass (natural grassland based on *Dichanthium* spp.). A first experiment tested different levels of replacement of this tropical forage (control [CTL] without tannins) by *A. nilotica* leaves or pods: 0:100, 25:75, 50:50, 75:25 and 100:0. A second experiment tested the mixture of *A. nilotica* leaves and pods in different proportions: 100:0, 75:25, 50:50, 25:75, and 0:100. A third experiment tested the 50:50 combination of *A. nilotica* leaves or pods with *C. calothyrsus* and *L. leucocephala*. *Acacia nilotica* pods and leaves had a high content of HT (350 and 178 g/

kg DM, respectively), whereas *C. calothyrsus* and *L. leucocephala* had a high content of CT (361 and 180 g/kg DM, respectively). The inclusion of HT from *A. nilotica* leaves and pods decreased CH₄ production dose-dependently ($P < 0.01$). Total replacement of the CTL by *A. nilotica* decreased CH₄ production by 64 and 55% with leaves and pods, respectively. Pods were richer in HT than leaves, but their antimethanogenic effect did not differ ($P > 0.05$). Although *A. nilotica* leaves and pods inhibited fermentation, as indicated by the lower gas production and VFA production ($P < 0.01$), this effect was less pronounced than for CH₄. Volatile fatty acid production decreased by 12% in leaves and by 30% in pods when compared with the CTL alone. Positive associative effect was reported for VFA, when HT-rich sources and CT-rich sources were mixed. Combining the 2 sources of HT did not show associative effects on fermentation or CH₄ production ($P > 0.05$). Hydrolyzable tannin-rich sources were more effective in suppressing methanogenesis than CT-rich sources. Our results show that HT-rich *A. nilotica* leaves and pods have the potential to reduce ruminal CH₄ production.

Key Words: *Acacia nilotica*, methane, hydrolyzable tannins, rumen fermentation, ruminant

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J. Anim. Sci. 2019.97:2700–2710

doi: 10.1093/jas/skz199

¹This work was funded by the AnimalChange project, which received funding from the European Commission FP7 Food, Agriculture and Fisheries, Biotechnology. We thank Harry Archimède (INRA, Guadeloupe) for the supply of *Leucaena leucocephala* and control forage; Philippe Lecomte and Bérénice Bois (CIRAD, Senegal) for the supply of *Acacia nilotica* and Emanuel Tillard (CIRAD, Réunion island) for the supply of *Calliandra calothyrsus*; the staff of UE 1414

Herbipôle (INRA, Theix), especially André Guittard, for animal care and management; and Angélique Torrent for determination of condensed tannin contents.

²The authors declared no conflict of interest.

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Received January 7, 2019.

Accepted March 20, 2019.

INTRODUCTION

Enteric methane (CH_4) accounts for 39% of global greenhouse gas emissions from the livestock sector (Gerber et al., 2013). A strategy to reduce CH_4 production is the use of tannin-rich feeds. Tannins are classified into hydrolyzable tannins (HT) and condensed tannins (CT). The decrease in CH_4 yield (g CH_4 per kg DMI) using tannins has been ascribed to the lower degradability of feeds due to reduced digestibility of feeds induced by the formation of complexes, mainly with proteins, and to a direct negative impact on microbial populations (Jayanegara et al., 2015; Pineiro-Vazquez et al., 2015). Condensed tannins are recognized for their antimethanogenic potential, which particularly depends on the source, type, and level of CT in the plant (Rira et al., 2015). Few studies have addressed the ability of HT to reduce CH_4 production. A major factor of low animal productivity in tropical regions is the inadequate quantity and quality of forage available in the dry season. Leaves of leguminous trees are a valuable source of forage for ruminants (Goodchild and McMeniman, 1994). They are often rich in CT, which may limit their digestibility in the rumen; they also contain other polyphenolic compounds such as HT, which have not yet been investigated. We hypothesized that HT contained in *Acacia nilotica* would inhibit methanogenesis without impairing rumen fermentation. Hence, we studied the effect of HT contained in *A. nilotica* leaves and pods used at different doses to establish whether a potential reduction in CH_4 production is dissociated from total gas and VFA production. A second objective was to study whether there is any associative effect on CH_4 production and rumen fermentation when using different mixtures of *A. nilotica* leaves and pods and mixtures of *A. nilotica* with CT-rich plants (*Calliandra calothyrsus* and *Leucaena leucocephala*).

MATERIALS AND METHODS

Origin and Chemical Characterization of Plants

Three legume shrubs were collected from tropical areas. *Acacia nilotica* ssp. *nilotica* was chosen because of its high HT content. Two morphological parts were studied: leaves and pods, which are both consumed by animals during the dry season. *Acacia* samples were collected from the Ferlo region of Senegal in January 2017 at mid dry season. The Ferlo region has a mean annual rainfall of 200 to 400 mm and mean temperature of 28 to 30 °C.

Calliandra calothyrsus and *L. leucocephala*, which are rich in CT, were chosen because they are among the most widely used forage trees in humid tropics. *Calliandra calothyrsus* was collected in the Réunion Island from ~2 m high native shrubs; crown leaves were harvested in December 2016 at a late vegetative stage. *Leucaena leucocephala* was collected in Guadeloupe, Grande-Terre Island, from natural shrubs with regrowth of less than 6 mo. The forage used as control (CTL) was hay made from a 75-d regrowth of natural grassland based on *Dichanthium* spp. harvested in December 2016 in Guadeloupe, Grande-Terre Island. Fresh material was air-dried at a maximum of 40 °C, ground to pass through a 1-mm screen, placed in airtight plastic containers, and stored until use.

Gross composition of plants was determined according to the Association of Official Analytical Chemists (AOAC, 1990). Organic matter was determined by ashing at 550 °C for 6 h (AOAC, 1990; method 923.03). Crude protein was determined by the Dumas method ($\text{N} \times 6.25$; AOAC, 1990; method 992.15). Cell wall components (NDF and ADF) were determined using sodium sulfite, with heat-stable amylase and including residual ash (AOAC, 1990; methods 200.04 and 973.18). Dry matter digestibility was predicted following a sequential hydrolysis with pepsin in 0.1 N HCl and then with fungal cellulase (Aufrère and Michalet-Doreau, 1988). Different fractions of CT in plants were extracted as described by Terrill et al. (1992). Total CT was the sum of free-extractable, protein-bound, and fiber-bound fractions. Free CT were extracted using a mixture of acetone:water:diethyl ether (4.7:2.0:3.3), followed by extraction of protein-bound CT using boiling sodium dodecyl sulfate containing 2-mercaptoethanol. Fiber-bound CT was determined on the residue remaining from the extraction of protein-bound CT. Condensed tannin concentrations in all 3 fractions were determined spectrophotometrically at 550 nm (model Lambda 25, Perkin Elmer, Courtaboeuf, France) by a modified butanol-HCl procedure (Porter et al., 1986, modified by Terrill et al., 1992), and total CT was the sum of 3 fractions. Quebracho extract was used as standard.

For HT, the rhodanine method was used for determination of gallotannins (Inoue and Hagerman, 1988). The potassium iodate method was used to estimate total HT (gallotannins and ellagitannins; Hartzfeld et al., 2002). The rhodanine assay detects only free gallic acid, so esters must be hydrolyzed before determination. The assay uses oxygen-free acid hydrolysis followed by specific staining of gallic

acid by rhodanine (Sigma–Aldrich). Hydrolyzable tannins were extracted with a 70% aqueous acetone solution in an ultrasonic bath, for 20 min. The hydrolysis was performed in 2 N H₂SO₄ at 100 °C for 26 h. Absorbance was measured at 520 nm with spectrophotometer (model 6715, Jenway, Villepinte, France). The gallotannin content was calculated as the difference between values from nonhydrolyzed and hydrolyzed samples. The potassium iodate method consists of the conversion of HT to methyl gallate (Sigma–Aldrich) via methanolysis, followed by oxidation with potassium iodate. Oxidation of methyl gallate by potassium iodate forms a chromophore, which is determined spectrophotometrically at 525 nm. The methyl gallate concentration was calculated from a standard methyl gallate calibration curve. Ellagitannins were calculated using the difference between total HT and gallotannins.

Procedure for In Vitro Fermentation and Measurements

Fermentations were performed using a batch technique (Rira et al., 2015). The donor animals were 4 Texel wethers fitted with a ruminal cannula and weighing 80.7 ± 6.9 kg. Management of experimental animals followed the guidelines for animal research of the French Ministry of Agriculture and other applicable guidelines and regulations for animal experimentation in the European Union (European Commission, 2010). Approval number for ethical evaluation was APAFIS#8218-2016121517182412 v1. Wethers were daily fed 900 g hay (natural grassland based on *Dichanthium* spp.) divided into equal amounts at 0700 and 1900 h. Wethers were adapted to the diet for 2 wk before being used as donors. Four series of 24-h incubations were performed; for each series, rumen fluid from one wether was used. Samples of ruminal contents were obtained through the cannula before morning feeding. Contents were strained through a polyester monofilament cloth (250-µm mesh aperture). A thermos flask that was saturated with CO₂ just before taking rumen fluid was used to transport the samples to the laboratory. The flask was filled to the top to reduce contact with air. The liquid phase was sent to the laboratory within 10 min after rumen sampling.

Incubations were performed in 100-mL vials containing 400 mg of feed sample, 25 mL of anaerobic buffer (Goering and Van Soest, 1970, modified by Mould et al., 2005), and 15 mL of strained rumen fluid. Vials were gassed with CO₂, sealed to ensure anaerobic conditions, and then placed in a water bath

at 39 °C for 24 h. Three experiments were performed simultaneously in each incubation series. Experiment 1 was designed to determine the effects of replacement of CTL forage by *A. nilotica* leaves or pods on CH₄ and VFA production. Mixtures of CTL and *A. nilotica* leaves and of CTL and *A. nilotica* pods were incubated in ratios of 0:100, 25:75, 50:50, 75:25, and 100:0. Experiment 2 was used to study additivity or associativity, that is, synergy or antagonism, between *A. nilotica* leaves and pods. Combinations of *A. nilotica* leaves and pods were incubated at ratios of 0:100, 25:75, 50:50, 75:25, and 100:0. Experiment 3 examined additivity or associativity between CT and HT. Hydrolyzable tannin sources (*A. nilotica* leaves and pods) and CT sources (*C. calothyrsus* and *L. leucocephala*) were used alone and as combinations of CT- and HT-rich sources in a 50:50 ratio (4 combinations: *A. nilotica* leaves:*C. calothyrsus*, *A. nilotica* leaves:*L. leucocephala*, *A. nilotica* pods:*C. calothyrsus*, *A. nilotica* pods:*L. leucocephala*).

Sample Collection and Analyses

Gas production was measured at 24 h using a pressure transducer. After recording pressure, a gas sample (5 mL) was taken for CH₄ analysis. Gas composition was determined by gas–liquid chromatography (Micro GC 3000A; Agilent Technologies, Les Ulis, France) within 2 h after sampling. Gas molar concentration was calibrated using a certified standard. For VFA determination, 0.8 mL of filtrate was mixed with 0.5 mL of 4 mg/mL crotonic acid and 20 mg/mL metaphosphoric acid in 0.5 M HCl and frozen at –20 °C until analysis. Volatile fatty acids were analyzed by gas chromatography using crotonic acid as internal standard on a Perkin Elmer Clarus 580 GC (Perkin Elmer, Courtaboeuf, France) equipped with a Stabilwax-DA column (30 m by 0.53 mm i.d.; Morgavi et al., 2013).

Statistical Analysis

Data were analyzed using the PROC MIXED procedure in SAS 9.4 (SAS Inst. Inc., Cary, NC). The model included as fixed effects 1) HT source (Exp. 1, *n* = 2) or CT source:HT source combination (Exp. 3, *n* = 4); 2) CTL:HT ratio (Exp. 1, *n* = 5), *A. nilotica* pod:leaf ratio (Exp. 2, *n* = 5), or HT source:CT source ratio (Exp. 3, *n* = 3); and 3) interactions between ratio and source (Exp. 1) and between ratio and source combination (Exp. 3). Animal was taken as a random effect for all experiments. Differences between ratios were also analyzed using linear and quadratic contrasts. Effects were declared significant at *P* < 0.05.

Table 1. Gross chemical composition, tannin content (g/kg DM), and in vitro digestibility of *Acacia nilotica*, *Calliandra calothyrsus*, and *Leucaena leucocephala*

Item	Control	<i>Acacia nilotica</i> leaves	<i>Acacia nilotica</i> pods	<i>Calliandra calothyrsus</i>	<i>Leucaena leucocephala</i>
OM	927	921	956	956	897
CP	84	115	140	217	336
NDF	701	159	227	333	223
ADF	326	106	152	228	125
CT ¹	ND	80	157	361	180
HT ¹					
Gallotannins	ND ¹	31	84	6	Trace
Ellagitannins	ND	147	266	27	13
HT:(HT + CT)	ND	0.69	0.69	0.08	0.07
In vitro DM digestibility,%	52.9	83.0	72.0	40.6	74.5

¹HT = hydrolyzable tannins; CT = condensed tannins; ND = not detected.

Table 2. Effect of increasing level of replacement of control forage by *Acacia nilotica* leaves and pods on total gas, methane, and VFA production in vitro

Item	Control				% of <i>Acacia nilotica</i> leaves				% of <i>Acacia nilotica</i> pods				<i>P</i> -value			
		25	50	75	100	25	50	75	100	SEM	Source	Ratio ¹		Source × ratio		
												Linear	Quadratic			
Total gas, mL/24 h	34.41	37.10	34.28	32.25	25.61	36.77	31.00	28.07	23.26	1.502	0.027	<0.001	<0.001	0.48		
Methane, mL/24 h	3.92	4.24	3.34	2.69	1.41	4.08	2.86	2.44	1.76	0.263	0.42	<0.001	0.002	0.40		
Total VFA, mM/24 h	45.61	45.77	44.78	41.47	40.17	44.66	40.55	37.07	31.98	2.342	0.001	<0.001	0.13	0.10		
VFA composition, %																
Acetate	54.15	62.98	65.11	68.75	70.44	61.02	61.93	63.99	68.05	2.221	0.022	<0.001	0.051	0.67		
Propionate	19.08	20.43	19.07	16.45	16.31	22.81	22.25	20.58	18.08	1.232	<0.001	0.002	<0.001	0.21		
Butyrate	19.92	9.66	9.13	8.85	8.47	8.98	8.23	7.19	6.28	0.847	0.050	<0.001	<0.001	0.72		
Isobutyrate	1.40	1.51	1.11	0.67	0.38	1.40	1.33	1.29	0.92	0.322	0.010	<0.001	0.056	0.07		
Isovalerate	2.30	2.61	2.62	2.05	1.20	2.49	2.65	2.83	2.26	0.705	0.040	0.039	0.003	0.10		
Valerate	2.91	2.65	2.77	3.08	3.11	3.07	3.40	3.97	4.28	0.456	<0.001	<0.001	0.31	0.17		
Caproate	0.25	0.16	0.20	0.15	0.09	0.22	0.21	0.15	0.13	0.045	0.42	0.002	0.77	0.92		
Acetate:propionate	2.88	3.12	3.49	4.27	4.39	2.72	2.84	3.13	3.90	0.332	0.002	<0.001	0.01	0.24		

¹Ratio between control forage and *Acacia nilotica* leaves or pods.

RESULTS

The chemical composition and tannin content of plants are presented in Table 1. High concentrations in HT were observed only in *A. nilotica* pods and leaves, with a high proportion of ellagitannins (76% and 82%, respectively). All tested plants were rich in total CT. The highest content of total CT was detected in *C. calothyrsus* (361 g/kg DM). The lowest CT content was recorded for *A. nilotica* leaves (80 g/kg DM). In vitro digestibility of plants ranged from 83.0% to 40.6%.

Experiment 1: Dose-Response of HT Sources

Table 2 shows total gas, CH₄, and VFA production, after 24 h of fermentation. Replacing CTL with *A. nilotica* leaves and pods resulted in a linear and quadratic reduction of total gas and CH₄ production ($P < 0.001$). The decrease in gas production was

greater for pods than for leaves ($P = 0.027$), but there was no source effect on CH₄ production. Total VFA production decreased linearly with increasing levels of *A. nilotica* ($P < 0.001$), the effect being greater with pods than with leaves ($P = 0.001$). Acetate and valerate proportions and the acetate:propionate ratio increased with the level of replacement of CTL by *A. nilotica* leaves and pods, whereas propionate, butyrate, isobutyrate, and isovalerate decreased. Acetate and butyrate proportions and the acetate:propionate ratio were lower for *A. nilotica* pods than for leaves, whereas propionate, isobutyrate, isovalerate, and valerate proportions were higher for *A. nilotica* pods than for leaves. Source × dose interaction was not significant for all parameters.

Experiment 2: Mixture of HT Sources

Effects of HT mixture on gas, CH₄, and total VFA production are presented in Table 3. The

Table 3. Effect of *Acacia nilotica* leaf:pod ratio on total gas, methane, and VFA production in vitro

Item	Homogeneous: leaf:pod ratio					SEM	P-value of ratio	
	0:100	25:75	50:50	75:25	100:0		Linear contrast	Quadratic contrast
Total gas, mL/24 h	23.26	26.55	26.88	18.16	25.61	2.506	0.64	0.93
Methane, mL/24 h	1.76	1.87	1.29	1.07	1.41	0.237	0.062	0.36
Total VFA, mM/24 h	31.98	35.03	36.30	38.52	40.17	2.263	<0.001	0.71
VFA composition, %								
Acetate	68.05	71.80	70.68	69.77	70.44	2.599	0.73	0.52
Propionate	18.08	15.80	17.05	16.90	16.31	1.398	0.59	0.71
Butyrate	6.28	6.08	7.05	8.13	8.47	0.966	0.004	0.59
Isobutyrate	0.92	0.67	0.51	0.49	0.38	0.310	0.005	0.34
Isovalerate	2.26	2.02	1.52	1.46	1.20	0.773	0.008	0.68
Valerate	4.28	3.50	3.13	3.15	3.11	0.587	0.011	0.10
Caproate	0.13	0.13	0.08	0.10	0.09	0.034	0.16	0.63
Acetate:propionate	3.90	4.99	4.19	4.18	4.39	0.608	0.93	0.67

Table 4. Effect of combination of condensed tannin-rich and hydrolyzable tannin-rich sources on total gas, methane, and VFA production in vitro

Item	HT:CT ratio ¹				P-value			
	100:0	50:50	0:100	SEM	Ratio linear	Ratio quadratic	Combination	Ratio × combination
Total gas, mL/24 h	24.43	24.95	20.27	0.854	<0.001	<0.001	<0.001	<0.001
Methane, mL/24 h	1.58	1.95	2.13	0.142	<0.001	0.19	<0.001	<0.001
Total VFA, mM/24 h	36.08	38.67	34.34	3.830	0.238	0.001	<0.001	<0.001
VFA composition, %								
Acetate	69.24	66.69	63.11	1.464	<0.001	0.61	0.13	0.69
Propionate	17.19	18.90	21.55	0.694	<0.001	0.44	0.002	0.09
Butyrate	7.38	6.05	5.91	0.742	0.002	0.13	0.018	0.24
Isobutyrate	0.65	1.48	2.01	0.281	<0.001	0.23	0.036	0.37
Isovalerate	1.73	3.16	3.69	0.683	<0.001	0.053	0.026	0.18
Valerate	3.69	3.48	3.49	0.501	0.32	0.52	0.005	0.22
Caproate	0.11	0.24	0.25	0.035	<0.001	0.014	0.32	0.58
Acetate:propionate	4.14	3.57	3.00	0.191	<0.001	0.98	0.027	0.44

¹Ratio between HT source and CT source; HT sources: *Acacia nilotica* leaves and pods; CT sources: *Leucaena leucocephala* and *Calliandra calothyrsus*. Values are means of the 4 combinations between the 2 sources of CT and the 2 sources of HT.

pod:leaf ratio had no effect on gas and CH₄ production. However, total VFA increased linearly with increased proportions of leaves. Acetate and propionate proportions and the acetate:propionate ratio were unaffected by the pod:leaf ratio; butyrate increased linearly and minor VFA (isobutyrate, valerate, isovalerate) decreased linearly when the pod:leaf ratio increased ($P < 0.01$).

Experiment 3: Combination of HT and CT Sources

The effects of combining HT sources (*A. nilotica* leaves and pods) and CT sources (*L. leucocephala* and *C. calothyrsus*) on total gas, CH₄, and total VFA production are shown in Table 4. The presence of CT sources increased CH₄ production but decreased total gas produced ($P < 0.001$), whereas VFA was greater in the HT:CT (50:50) ratio than when each tannin source was used alone ($P < 0.001$, quadratic

effect). There was a significant ratio × combination interaction for total gas, CH₄, and VFA production ($P < 0.001$). Figure 1 shows the nature of this interaction for CH₄ production. In particular, the 50:50 mixture of CT-rich *C. calothyrsus* with *A. nilotica* leaves results in a negative associative effect as more CH₄ than expected was produced. In contrast, the 50:50 mixture of *C. calothyrsus* and *A. nilotica* pods produced less CH₄ than expected. For VFA production (Fig. 2) and total gas production (Supplementary Fig. 1), a positive associative effect occurred, when HT-rich plants were mixed with CT-rich plants in a 50:50 ratio. The total VFA and gas production observed for all 4 HT:CT mixtures were higher than predicted.

Individual VFA displayed linear responses to the variation in HT:CT ratio ($P < 0.001$). Acetate and butyrate proportions decreased and propionate, isobutyrate, and isovalerate increased

Figure 1. Effect of combination of hydrolyzable tannin sources and condensed tannin sources on methane production. ANI = *Acacia nilotica* leaves, ANp = *Acacia nilotica* pods, LL = *Leucaena leucocephala*, CC = *Calliandra calothyrsus*. HT:CT ratio is between brackets.

Figure 2. Effect of combination of hydrolyzable tannin and condensed tannin sources on VFA production. ANI = *Acacia nilotica* leaves, ANp = *Acacia nilotica* pods, LL = *Leucaena leucocephala*, CC = *Calliandra calothyrsus*. HT:CT ratio is between brackets.

with increasing proportions of CT. The effect of mixing HT and CT sources was significant for all individual VFA except for acetate. The ratio $\times$ combination interaction was not significant for any individual VFA ($P > 0.05$).

DISCUSSION

In this study, we tested whether the use of tropical tannin-rich plants reduces CH_4 production in vitro. We used plants instead of extracts because it is the prevailing and most practical way

of providing these secondary plant compounds to animals in the tropics. The effect of plants is discussed considering their HT and CT content, but it is acknowledged that the influence of other plant constituents cannot be entirely dissociated from that of tannins.

Variability of Tannin Content of Plants

Accurate information on the HT content of plants is scarce. Hydrolyzable tannins are often calculated by difference between total phenols or total tannins measured by the Folin–Ciocalteu method and CT measured by the butanol–HCl method. In our study, total HT content was determined by methods, which specifically measured the content in total HT and gallotannins. A more accurate method using HPLC showed a wide variety of phenolic compounds in *A. nilotica*, including HT and CT that were identified but not quantified (Mueller-Harvey et al., 1987; Al-Wakeel et al., 2007); among them caffeic acid is one of the most important. The concentration of HT that we found in *A. nilotica* leaves is in the range of values reported in other studies using nonspecific methods that estimated HT by the difference between total tannins and CT (Rubanza et al., 2005; Alam et al., 2007). Note that some authors have found in *A. nilotica* leaves lower HT contents than in the present study (Gemed and Hassen, 2015; Pal et al., 2015) or no HT (Zabré et al., 2018). The concentration of HT in *Acacia* pods was high in our experiment, but Goel et al. (2015) also reported high values (186 g/kg DM, estimated by the difference between total tannins and CT). In our leaf and pod samples, ellagitannins were predominant compared with gallotannins, as reported in plants containing HT (Hassanat and Benchaar, 2013). A similar ratio between these fractions was obtained by Alam et al. (2007) in *Acacia* leaves. The amount of CT in *A. nilotica* is controversial. Some studies have reported 5 g/kg DM (Nsahlai et al., 2011; Pal et al., 2015) others 146 g/kg DM (Phale and Madibela, 2006). In our samples, pods were richer in CT than leaves; the same trend was reported by Nsahlai et al. (2011). For *L. leucocephala* used in this work, the amount of CT was higher than that previously found in plants of the same origin but using the vanillin-HCl method for CT detection (Rira et al., 2015). The CT content of *C. calothyrsus* was also relatively high, but similar to the maximum CT content reported in the literature (368 g/kg DM, Tuwei et al., 2003). Between-study discrepancies in values of HT and CT contents depend on

the methodology used for tannin determination (Makkar, 2000 for CT and HT; Schofield et al., 2001 for CT). We hypothesize that the method of analysis that we used for CT, which involves a double-extraction procedure, extracts more tannins than most methods reported in the literature. In addition, many factors such as type of subspecies (e.g., Mahdi et al., 2006, for *A. nilotica* pods), plant stage of maturity, location, climatic conditions (e.g., Tuwei et al., 2003 for *C. calothyrsus*), and soil characteristics are determinants of tannin concentration in plants.

Ruminants in tropical areas voluntarily browse leaves and pods of *A. nilotica* during the dry season, especially at the end when grasses became scarce. In addition, breeders often cut leaves and pods to feed their animals, especially small ruminants. Hydrolyzable tannins are potentially toxic to ruminants because their degradation by ruminal microbes generates pyrogallol, which has hepatotoxic and nephrotoxic effects (Reed, 1995). Although some HT-rich plants such as *Terminalia oblongata* and *Clidemia hirta* have been reported to produce acute toxicity, the supplementation of goats with *A. nilotica* leaves (25% of the diet; Rubanza et al., 2007) or pods (31% of the diet; Hidosa and Gemiyo, 2017) improved weight gain and increased feed intake without any adverse effect on digestibility. In addition, detannification of *A. nilotica* pods using different methods did not improve intake and digestibility by goats suggesting the nontoxic character of the plant (Tshabalala et al., 2013).

Effects of Increasing Level of HT Sources on Rumen Fermentation and CH₄ Production

The effects of HT on rumen fermentation and CH₄ production are less documented than those of CT. In addition, most studies have tested HT extracts and there is little information on HT-rich forages, which are the easiest way of supplying desirable secondary plant compounds to ruminants. The present study used as source of HT *A. nilotica*, a plant widely distributed in dry tropical and subtropical areas in Africa and Asia (Heuzé et al., 2016). We considered 2 fractions eaten by animals: leaves and pods. Despite the presence of CT in *A. nilotica* leaves and pods, it is assumed that effects on CH₄ production are mainly due to HT because 1) they represent 70% of total tannins and 2) direct comparisons between HT and CT extracts have shown that HT are as effective as (Hassanat and Benchaar, 2013) or more effective than CT (Jayanegara et al., 2015; Min et al., 2015).

in reducing ruminal CH₄ production. However, it is possible that some effects observed with *A. nilotica* could be due to compounds other than tannins such as saponins (Solomon-Wisdom and Shittu, 2010) that are known for their antimethanogenic properties (Martin et al., 2010).

The decrease in CH₄ production that we observed was consistent with data obtained by Pal et al. (2015) who used *A. nilotica* leaves (37.7 g/kg DM of HT, 4.6 g/kg DM of CT) and found a greater inhibition of CH₄ production with *A. nilotica* than with other tannin-containing plants. Compared with a control, Zabré et al. (2018) did not find a decrease in CH₄ production with a subspecies of *A. nilotica* rich in CT but poor in HT, whereas a strong decrease was observed with a sample of *Acacia raddiana* rich in HT. Among HT, ellagitannins are probably the major CH₄ inhibitor (Baert et al., 2016). In our study, increasing proportions of *Acacia* in the diet linearly decreased both CH₄ and total VFA, but the decrease in CH₄ was up to 3- to 6-fold higher than the decrease in total VFA. For instance, pure *A. nilotica* leaves decreased CH₄ by 64% and total VFA by 11% when compared with CTL, whereas pure *A. nilotica* pods decreased CH₄ by 55% and total VFA by 29%. A similar result was found by Gemedu (2015) who observed a 33% reduction in CH₄ production when lucerne was replaced by 30% of *A. nilotica* in a sheep diet. In contrast, the concentration of VFA and organic matter digestibility were not affected. Also, in accordance with our results, HT extracts of chestnut (*Castanea sativa*) and valonea (*Quercus aegilops*) induced a stronger decrease in CH₄ than in VFA production in vitro (Hassanat and Benchaar, 2013; Min et al., 2015). The effect of HT on the VFA profile is more inconsistent. In our study, the acetate:propionate ratio increased, which is not in line with the CH₄ decrease; this could be explained by the ruminal degradation of HT by tannin-degrading bacteria into acetate and butyrate (Goel et al., 2005). Electron acceptors, such as caffeic acid, which is present in *A. nilotica*, may have favored the production of acetate (Cord-Ruwisch et al., 1988). Other studies have reported the opposite trend with chestnut extract (Jayanegara et al., 2015) or no effect on acetate:propionate ratio with chestnut and valonea extracts (Hassanat and Benchaar, 2013; Min et al., 2015). Few studies have tested the effect of HT on rumen fermentation and CH₄ production in vivo. High amounts of HT from chestnut extract (9% of the diet) markedly decreased digestibility in goats and sheep (Zimmer and Cordesse, 1996), whereas a lower dose of chestnut extract (1.5% of the diet)

failed to affect CH₄ production and rumen concentration of VFA in steers (Krueger et al., 2010).

Acacia nilotica pods were more detrimental for fermentation than *A. nilotica* leaves because they generate less gas and total VFA, whereas the effect on CH₄ production did not differ. On the contrary, Nsahlai et al. (2011) did not show a difference in gas production and DM degradability between leaves and pods of *A. nilotica* but, although they reported a much higher CT content in pods than in leaves, HT content and CH₄ production were not measured. Our results are consistent with the HT content in leaves and pods for gas and VFA production, but the absence of difference in CH₄ production is difficult to explain. Several hypotheses can be proposed but each of them needs support from experimental data: 1) the nature of HT differs between leaves and pods, as suggested by Salminen and Karonen (2011), and 2) the presence in *A. nilotica* of other antinutritional compounds, such as saponins and alkaloids, which have been found in leaves by Solomon-Wisdom and Shittu (2010), may be involved in the decrease in CH₄ production. It is also possible that CH₄ production cannot decrease beyond a certain threshold of HT concentration.

Based on data mainly obtained using CT, it is recognized that the decrease in CH₄ production is due to 1) the decrease in carbohydrate fermentation and 2) a specific action of tannins on microbes involved in CH₄ production. The impairment in carbohydrate fermentation when HT are fed can be due to the inhibition of fiber-degrading microbes and to the decrease in carbohydrate hydrolases involved in feed degradation processes (McSweeney et al., 2001; Nsahlai et al., 2011). Results from this study, however, suggest that the main mechanism by which HT reduced CH₄ production was by affecting microbes. The negative effect of HT on protozoa, which are hydrogen producers, and methanogens, which convert hydrogen into CH₄, has been demonstrated with purified extracts of HT from chestnut and sumach (*Rhus typhina*; Jayanegara et al., 2015) or with tannic acid (Yang et al., 2017). The difference of action between CT and HT is illustrated by 2 results. First, Jayanegara et al. (2015) comparing HT and CT extracts found that the decrease in in vitro digestibility was higher for CT than for HT, but that the opposite was observed for CH₄ production; second, Zabré et al. (2018) reported that an HT-rich plant (*A. raddiana*) decreased the population of methanogens, but that a sample of *A. nilotica* rich in CT but poor in HT significantly decreased the concentration of anaerobic fungi and fiber-degrading bacteria. In addition, Bhatta et al. (2009) reported a greater

decrease of CH₄ with a mixture of HT and CT than with HT alone; this was attributed to a greater antimethanogen and antiprotozoal activity of the combination of HT and CT than of HT alone. All these results suggest that HT might be more effective than CT for CH₄ mitigation.

Associative Effects of Combination of HT and CT Sources

We hypothesized first that combining the 2 sources of HT may result in associative effects on CH₄ inhibition without noticeable effects on the extent of fermentation. The HT composition varies between organs (Baert et al., 2017) and according to the season for a given plant organ (Hatano et al., 1986). The leaves and pods that we tested were harvested at different periods of the year. In our study, CH₄ and VFA production varied linearly with changes of the leaf:pod ratio. This does not allow conclusions to be drawn about differences in HT composition between leaves and pods. However, the results suggest that there is no advantage in storing pods and leaves of *A. nilotica* to give them at the same time to animals.

Associative effects may be more likely between HT- and CT-rich feeds than between 2 HT-rich feed because of major differences between these 2 tannin classes. However, the literature is scarce on this topic. Jayanegara et al. (2013) reported that combining a tannin-poor plant with HT- or CT-rich plants resulted in an associative effect on CH₄ production that was reduced more than expected from the sum of individual plants. These authors suggested that associative effects of combining feeds on CH₄ production are observed when combining plants that differ greatly in their CH₄ production potential, which is not the case for the HT- and CT-rich plants used in this study. It can be hypothesized that differences between combinations are explained by the HT:CT ratio. It is noted that both HT-rich sources had similar ratios (2.22 vs. 2.23 for *A. nilotica* leaves and pods, respectively). Likewise, both CT sources were similar (0.07 vs. 0.09 for *L. leucocephala* and *C. calothyrsus*, respectively). This suggests that the HT:CT ratio is not the main driver of the differences between combinations. These differences could be explained by other plant secondary compounds. Saponins, flavonoids, terpenoids, and alkaloids that may interfere with tannins were shown in *A. nilotica* leaves (Solomon-Wisdom and Shittu 2010).

Our experiment showed that the effect of combining HT and CT plants produced more VFA than

expected from an additive response. This effect is moderate but statistically significant. This may suggest that fiber degradation is less affected when the 2 classes of tannins are mixed, perhaps because of a less negative effect on bacterial activity. If the associative response of VFA to the mixture of HT and CT is confirmed in vivo, it could be interesting to develop mixed cultures of HT- and CT-rich trees in the same area.

CONCLUSION

Few studies have assessed the effects of HT on CH₄ production, particularly using a plant that grows in the tropics. This work shows that *A. nilotica* strongly inhibits CH₄ production without marked adverse effects on rumen fermentation in vitro. The effect of *A. nilotica* on CH₄ production was more marked than that of CT-rich plants such as *C. calothyrsus* and *L. leucocephala*. This may reveal that HT are more suitable for CH₄ mitigation than CT. Indeed, the use of CT for mitigation is often questioned because of their negative effect on diet digestibility. The interest of HT should be confirmed in vivo; long-term trials are needed to conclude about the possible adaptation of rumen microbes to HT. It is also necessary to determine the optimal level of HT to avoid their possible toxicity. If the use of *A. nilotica* is developed in the future, both leaves and pods can contribute to CH₄ decrease, taking advantage of consumption at different periods of the year.

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