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CONTRIBUTION OF BOTH SOLUBLE AND INSOLUBLE FRACTIONS OF UNTREATED AND TREATED *ACACIA SALIGNA* AND *LEUCAENA LEUCOCEPHALA* WITH DIFFERENT LEVELS OF UREA TO RUMEN FERMENTATION, *IN VITRO*

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Abstract

The objectives of this work are to characterise the in vitro fermentation contribution of both soluble and insoluble fractions, and the effects of ensiling of acacia (Acacia saligna, AC) and leucaena (Leucaena leucocephala, LE) leaves with different levels of urea (U, 0, 1, 3 or 5%) on gas production, energy value and organic matter digestibility (OMD%) of AC and LE. The acacia and leucaena were ensiled for 35 days. Ground samples (200 mg DM) of the ensiled materials from the eight treatments were incubated in glass syringes with rumen fluid obtained from fistulated sheep fed berseem hay and commercial concentrate mixture twice a day. Cumulative gas production was recorded at 3, 6, 9, 12, 24, 48, 72 and 96 h of incubation and the kinetics of gas production was described by using the equation: Gas (Y) = a + b (1-exp^{-ct}).Ensiling of AC and LE leaves with U increased crude protein and ash, while the contents of tested samples of total phenol (TP), total tannins (TT) and condensed tannins (CT) were decreased. Also, ensiling of AC and LE leaves with U significantly (P <0.05) decreased gas production. All washed samples showed losses of soluble material. The gas production was, in general, significantly (P<0.05) higher for the unwashed substrates. Leucaena gave the highest values of gas production compared with acacia. The gas production volume was significantly (P <0.05) higher for ensiled AC and LE leaves without U than ensiled AC with 5%U or ensiled LE with 3%U. The maximum rate of gas production increased after ensiling AC and LE leaves with 5% and 3% U, respectively. The calculated values of metabolizable energy (ME) and net energy (NE) were significantly (P<0.05) increased for ensiled AC with 3 and 5%U, while ensiled LE with U was not significantly affected. The organic matter digestibility (OMD %) and microbial protein production were significantly (P<0.05) higher for ensiled AC and LE with U, while short chain fatty acids (SCFA) were significantly (P<0.05) decreased. The concentrations of TP, TT and CT were strongly correlated (p<0.01). The TP, TT and CT were negatively related (p<0.01) with neutral detergent fiber (NDF) and acid detergent fiber (ADF), but not with hemicelluloses (HEMI). The crude protein was strongly correlated (p<0.01) with NDF, ADF and CT and negatively related (p<0.01) with TF and TT, but not with HEMI. In conclusion, there were negative effects on the in vitro gas production occurring more consistently when AC and LE were ensiled with different levels of U, while OMD% and microbial proteins were significantly (P<0.05) increased. The in vitro digestibility and gas production parameters were significantly correlated with chemical composition of shrubs. Finally, it is generally more appropriate to measure the degradation of organic matter as usual dry matter can give problems of interpretation of the significance of the soluble fraction.

Key words**:** *Acacia saligna*, energy value, gas production*, in vitro,* insoluble residual*, Leucaena leucocephala*.

MATERIAL AND METHOD

Samples and site description

Two tropical plants used in this study: *Acacia saligna* and *Leucaena leucocephala* leaves (whole leaves: rachis plus leaflets) have been sampled from the Experimental Farm, Faculty of Agriculture, Alexandria University, Alexandria, Egypt. Duplicate 100 g samples of AC and LE leaves were treated with water (20 ml/100 g fresh leaves) or with urea solutions at increasing levels of 1, 3 or 5%. Each sample of treated leaves was stored in a plastic bag which was closed with adhesive rubber to create anaerobic conditions. Storage time was 35 days. Bags containing treated leaves were stored in a big black plastic bag which was also closed with adhesive rubber. After storage, treated leaves were dried at 65 ^{o}C for 48 h and then ground to pass through a 1 mm screen.

Procedure for removal of soluble compounds

All forages were analysed in duplicate in three different runs according to the procedure described by Pedraza (1998). Samples of

known dry matter content (0.5 g, 1-mm screen) were soaked in 150 ml distilled water at room temperature during 1 h and 45 min while shaken intermittently. Samples were then filtered through a Whatman No.1 filter paper and washed with water until approximately 500 mL of filtrate was recovered. The filter paper containing the insoluble residue was dried at 60°C until constant weight.

The filter papers were transferred to previously tared plastic bags and sealed. Weighing was carried out after 1h, to calculate the filter paper insoluble residue. The soluble fraction was calculated by difference between initial weight of feedstuff and insoluble residue.

Chemical Analyses

Representative samples of ensiled AC and LE with different levels of urea (0, 1, 3 or 5%) were subjected to dry matter (DM), organic matter (OM), ether extract (EE), crude fiber (CF) and ash determinations following the procedure of AOAC (1990). Nitrogen (N) content was measured by the Kjeldahl method (AOAC 1990). Crude protein (CP) was calculated as N X 6.25. The neutral detergent fiber (NDF), acid detergent fiber (ADF) and hemicelluloses (HEMI) were determined according to Van Soest et al. (1991). Total phenols (TP), total tannins (TT) and condensed tannins (CT) were determined according to Makkar (1999). All chemical analyses were carried out in duplicate samples.

Measurement of in vitro gas production

In vitro gas production was undertaken according to Menke and Steingass (1988). Rumen fluid was collected before morning feeding from fistulated sheep fed berseem hay and commercial concentrate mixture twice a day. The rumen fluid was filtered through four layers of cheese–cloth and flushed with CO_2. The CO_2-flushed rumen fluid was added (1:2, v/v) to the buffered mineral solution (Onodera and Henderson, 1980), which was maintained in a water bath at 39^{o}C, and combined.

All laboratory handling of rumen fluid was carried out under a continuous flow of CO_2. Samples (200 mg) of ensiled AC and LE and insoluble residue were accurately weighed into glass syringes fitted with plungers. The syringes were pre-warmed at 39^{o}C before injecting 30 ml rumen fluid-buffer mixture into each syringe and excess gas was released. The syringes were incubated in a water bath at 39 ^{o}C. Two blank syringes containing 30 ml of the medium only were also included.

All the syringes were gently shaken 30 min after the start of incubation and every one hour for the first 12 h of incubation, thereafter five times daily. The gas production was recorded at 3, 6, 9, 12, 24, 48, 72 and 96 hours of incubation. Total gas values were corrected for blank incubation which contained only rumen fluid. Cumulative gas production (Y) at time (t) was fitted to the exponential model of Ørskov and McDonald (1979) as follows: Gas (Y) = a + b (1-exp-ct), where; a = gas production from the immediately soluble fraction, b = gas production from the insoluble fraction, c = gas production rate constant for the insoluble fraction (b), t = incubation time.

Determination of degradation of dry matter in situ

Degradation of dry matter in situ at 24 h of incubation was undertaken according to osuji et al (1993).

Estimation of energy values, organic matter digestibility, short chain fatty acids and microbial proteins

The energy values and the percentages of organic matter digestibility of forages can be calculated from the gas produced on incubation of 200 mg feed dry matter after 24 h of incubation with the levels of crude protein, ash and crude fat (Menke et al., 1979 and Menke and Steingass, 1988) as follows:

$$ME\ (MJ/kg\ DM) = 2.20 + 0.136\ GP + 0.057\ CP + 0.0029\ CP^2$$

$$OMD\ (\%) = 14.88 + 0.889\ GP + 0.45\ CP + 0.0651A$$

Where: ME is the metabolizable energy, OMD (%) is the percentage of organic matter digestibility, GP is the 24 h net gas production (ml/200 mg DM) after 24 h of incubation. CP, crude protein (%) and A, ash content (%).

$$NE\ (Mcal/Ib) = [2.2 + (0.0272 \times Gas) + (0.057 \times CP) + (0.149 \times CF)] / 14.64$$

Where: NE is the net energy; Gas, the net gas production in ml from one-gram dry sample after 24 h of incubation; CP, crude protein (%); CF, crude fat (%) then, net energy unit converted to be MJ/kg DM.
Short chain fatty acids (SCFA) were calculated according to Getachew et al. (2005) as follows:

$$SCFA = (-0.00425 + 0.0222\ GP) \times 100$$

Where: GP is 24 h net gas production (ml/200 mg DM).
Microbial protein was calculated as 19.3 g microbial nitrogen per kg OMD according to Czerkawski (1986).

Statistical Analysis
Data were subjected to analysis of variance (ANOVA) using the General Linear Model (GLM). Significant differences between individual means were identified using least significance difference (LSD) multiple range test (SAS, 2000). A simple correlation analysis was used to establish the relationship between chemical compositions and polyphenolic concentrations and *in vitro* gas production according to Steel and Torrie (1980).

RESULTS AND DISCUSSIONS

Chemical Composition
The chemical composition of ensiled AC and LE leaves with different levels of U (0, 1, 3 and 5% U) for 35 days are presented in Table 1. Results indicated great variations between the tested samples in their contents of CP, NDF, ADF, TF, TT and CT contents. The CP values ranged from 181 to 369.4 g/kg DM and were higher in LE compared with AC. These results are in agreement with those of Kumar and D'Mello (1995). The present data show that U treatments of AC and LE increased their CP contents. Also, NDF and ADF content were increased after ensiling with U for 35 days. El-Serafy et al. (1983) reported that urea addition changed the chemical composition of water hyacinth, total nitrogen increased and crude fiber (CF), NDF and ADF decreased as the period of ensiling increased. For all the samples the ADF fraction was a large proportion of the NDF, indicating high content of cellulose and lignin and low levels of hemicellulose. These results are in agreement with those of Abdulrazak et al. (2000). The values of TP, TT and CT concentration ranged between 12.3-112.2, 11.9-96.6 (eq. g tannic acid/kg DM) and 00.8-24.0 eq. g leucocyanidin/kg DM), respectively. Ensiling of AC and LE leaves without U had the highest values of all of them, while AC and LE with 5% of urea had the lowest values. The results of AC are consistent with values reported in the literature (Akkari et al., 2008). Ash content in the samples ranged from 84.7-134.7 g/kg DM and was the lowest in ensiled AC and LE without U.

In Vitro *Gas Production*
Gas production for the means of the ensiled LE and AC with different levels of urea (0, 1, 3, 5 %) is presented in Fig.1 and 2 and Tables 2 and 3. The cumulative volume of gas production increased with increasing time of incubation, and the differences in gas production occurring during the early hours indicated little differences in total gas produced at 24 h. There were significant ($P<0.05$) differences among the tested samples in terms of total gas production and parameters.
The produced gas at 96 h ranged from 25 -36.7 ml/200 mg DM. Total gas produced at 96 h of incubation was significantly ($P < 0.05$) higher for the ensiled AC and LE without U than in AC with 5% U. The highest GAS24, GAS96 and b were observed for the ensiled AC and LE leaves without U. Hadi et al. (2003) suggested that interactions between NDF, CP, ADL and ash contents influenced the kinetics of gas production. Kamalak et al. (2005) noted considerable variations among alfalfa varieties in terms of gas production at all incubation times resulting to the differences in the chemical composition of the varieties of alfalfa. The present data showed that all washed samples showed losses of soluble material. In general, leucaena leaves had the greatest washing loss compared with acacia leaves (Table 2).
The gas production was, in general, higher for the unwashed treated or non-treated acacia leaves, while the results of leucaena were on the opposite side may be leucaena contains some rumen microflora antagonistic substances which are removed by washing.

The results are agreement with Arhab et al, 2007 who showed that the gas production of the insoluble fraction of vetch-oat hay was higher than that of the *Aristida pungens* and date palm leaves.

Estimated gas production rate (c) varied from 0.028 ml/h in insoluble fraction of untreated acacia to 0.053 ml/h in unwashed treated LE with 3% of urea. The values of (c) increased after ensiling with urea. The rate degradation of the all washed samples is lower than its unwashed ones.

The results are agreement with Arhab et al, 2007. The intake of a feed is mostly explained by the rate of gas production (c) which affects the passage rate of feed through the rumen, whereas the potential gas production (a +b), is associated with degradability of feed (Khazaal et al. 1995). Although the present study showed that the ensiling AC and LE leaves with urea decreased the values of produced gas, (a) and (b), the value of (c) was significantly ($P<0.05$) increased compared with ensiled samples without U.

Determination of degradation of dry matter in situ

Degradation of dry matter *in situ* in untreated and treated leuceana and acacia with different levels of urea and unsoluble fractions (0, 1, 3, 5%) are presented in Table 4.

The present data showed that the range values of undegradable DM were from 39.37 to 91.11. The lowest value was 39.37 for unwashed and untreated leucaena with U, while the highest value was 91.11 for washed and treated acacia with 5% U. Treatment of leucaena and acacia with urea caused increase undegradable DM. This indicates that this feedstuff contains some rumen microflora antagonistic substances, tannins which are decreased by urea treatment (Table 1).

The same trand of values were obsorbed by washing of leuceana and acacia (Table 4).

Energy contents, organic matter digestibility, short chain fatty acids and microbial protein

The predicted metabolizable energy (ME, MJ/kg DM), net energy (NE, MJ/kg DM), organic matter digestibility (OMD, %), short chain fatty acids (SCFA, mM) and microbial protein (MP, mg/kg DM) of ensiled AC and LE leaves with 0, 1, 3 and 5% U are presented in Table 3. The present data show that the ME and NE were higher ($P<0.05$) for ensiled AC leaves with 3% U than for ensiled AC leaves without U, while no significant differences were detected between LE samples. Khazaal et al. (1993) correlated the chemical composition (i.e. CP, NDF, ADF or ADL) with the in vitro two-stage digestibility, in sacco degradability and gas production with voluntary intake.

The calculated organic matter digestibility from gas production values at 24 h was subsequently highest in ensiled AC and LE leaves with 3% U (489.2 and 536.9 g/kg DM, respectively) and lowest in ensiled AC and LE leaves without U (455 and 517 g/kg DM, respectively) (Table 3). Condensed tannin concentrations were significantly correlated with the in vitro dry matter digestibility ($r=0.77$, $P=0.043$), extent of degradation ($r=0.829$, $P=0.021$) and cumulative gas production at 24 h ($r=0.798$, $P=0.032$) (Khazaal et al., 1993).

Microbial proteins and SCFA ranged from 54.89-64.77 g/kg DOM and 40.15-57.91 mM, respectively. Microbial proteins were significantly ($P<0.05$) increased after ensiling with urea, while SCFA were decreased. Blümmel et al. (1997) noted an inverse relationship between in vitro gas production and microbial biomass yield.

The relationship between the concentration of phenolic compounds, crude protein and cell-wall component of the untreated and urea treated of AC and LE

Table 1. Proximate analysis (g/kg DM) of ensiled acacia and leucaena leaves with different levels of urea (0, 1, 3, or 5% U) for 35 days

Items	CP	EE	Ash	NDF	ADF	HEMI	TF	TT	CT
AC (0%)	181.0	18.1	84.7	410.9	318.4	92.5	112.2	96.6	27.6
AC (1% U)	222.1	16.6	98.9	419.5	324.5	95.0	69.3	68.8	6.3
AC (3% U)	268.4	16.1	96.7	472.0	347.5	124.5	46.1	45.7	1.4
AC (5% U)	322.0	15.2	91.8	486.5	374.5	112	17.6	17.2	1.0
LE (0%)	251.6	53.1	93.2	428.8	361.2	67.6	88.3	59.9	4.9
LE (1% U)	350.4	53.8	134.7	473.9	412.4	61.5	23.1	22.7	1.4
LE (3% U)	369.4	45.7	131.3	489.1	418.6	70.5	13.4	13.0	1.1
LE (5% U)	368.9	34.9	108.2	559.5	457.4	102.1	12.3	11.9	0.8

AC, acacia; LE, leucaena; AC(0%, 1%U, 3%U, 5%U), acacia with 0%, 1%, 3% and 5% of urea; LE (0%, 1%U,3%U, 5%U), leuceana with 0%, 1%, 3%, 5% of urea; CP, crude protein; EE, ether extract; CF, crude fiber; NDF, nutrant detergent fiber; ADF, acid detergent fiber; HEMI, hemicellulose; TF, total phenol (eq-g tannic acid/kg DM); TT, total tannins (eq-g tannic acid/kg DM); CT, condensed tannin (eq-g leucocyanidin/kg DM).

Table 2. Cumulative gas production (ml/200 mg DM) after 12, 24, 48, 72, 96 h of incubation and gas production parameters in ensiled leuceana leaves with different levels of urea (0, 1, 3, or 5% U) for 35 days

	12	24	48	72	96	a	b	c
Leuceana (raw)	18.0	28.0	34.7	36.0	36.7	6.156	31.522	0.044
Leuceana (raw), Insoluble	12.0	24.0	32.7	32.7	35.3	0.000	37.183	0.044
Leuceana (1%)	15.0	24.0	30.0	30.0	30.0	3.459	27.517	0.052
Leuceana (1%), Insoluble	10.0	23.0	30.0	32.0	33.0	0.000	35.264	0.041
Leuceana (3%)	14.0	24.0	27.3	28.0	29.3	3.590	25.735	0.053
Leuceana (3%), Insoluble	9.0	22.0	30.0	30.0	32.0	0.000	34.542	0.042
Leuceana (5%)	15.0	24.0	31.0	31.0	31.0	2.854	29.352	0.049
Leuceana (5%), Insoluble	10.0	20.0	28.0	30.0	32.0	0.000	35.259	0.041

Table 3. Cumulative gas production (ml/200 mg DM) after 12, 24, 48, 72, 96 h of incubation and gas production parameters in ensiled acacia leaves with different levels of urea (0, 1, 3, or 5% U) for 35 days

Items	12	24	48	72	96	a	b	c
Acacia (raw)	16	25	33	34	34	3.532	31.746	0.046
Acacia (raw), insoluble	9	17	29	29	31	0.852	33.034	0.028
Acacia (1% U)	16	24	32	32	33	3.639	30.147	0.046
Acacia (1%U),insoluble	11	20	30	32	32	1.329	33.374	0.034
Acacia (3%U)	15	24	31	32	33	3.026	30.808	0.046
Acacia (3%U),insoluble	9	18	26	26	28	0.0	29.148	0.037
Acacia (5%U)	12	20	26	26	27	0.749	26.651	0.051
Acacia (5%U),insoluble	8	16	23	24	25	0.0	26.435	0.037

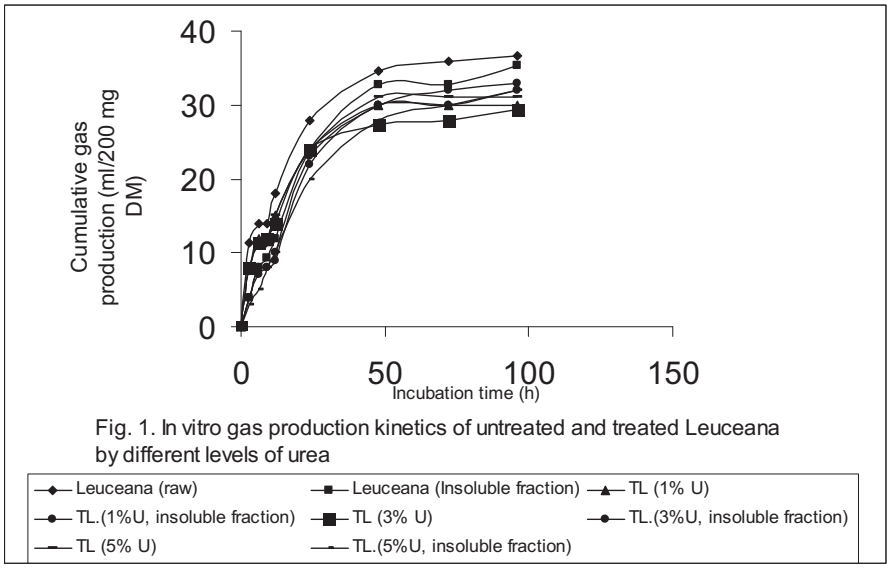

Fig. 1. In vitro gas production kinetics of untreated and treated Leuceana by different levels of urea

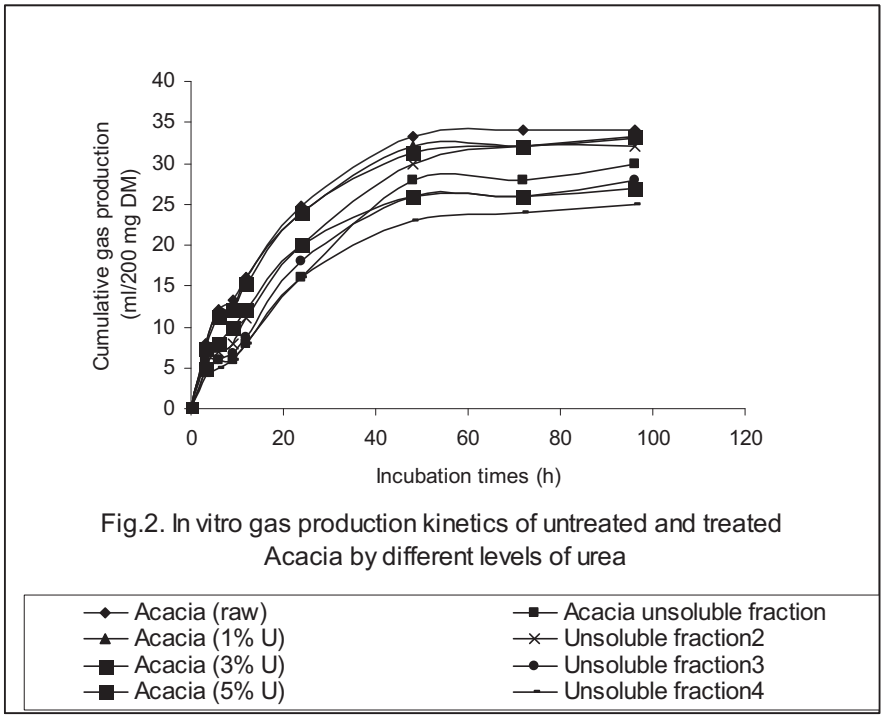

Fig.2. In vitro gas production kinetics of untreated and treated Acacia by different levels of urea

Table 4. Undegradable dry matter and disappearance materials in untreated and treated leuceana and acacia with different levels of urea and unsoluble fractions

Sample type (S)	Undegradable DM (%)	Disappearance %
Leucaena, raw	39.37	60.63
Unsoluble Leu, raw	64.68	35.32
Leucaena,1% U	48.28	51.72
Unsoluble Leu, 1% U	72.25	27.75
Leucaena, 3% U	53.46	46.54
Unsoluble Leu, 3% U	68.94	31.06
leucaena, 5% U	58.88	41.12
Unsoluble Leu, 5% U	73.64	26.36
Acacia, raw	48.24	51.76
Unsoluble Ac, raw	94.73	5.27
Acacia, 1% U	62.00	38.00
Unsoluble Ac, 1%U	86.49	13.51
Acacia, 3% U	69.72	30.28
Unsoluble Ac, 3%U	79.66	20.34
Acacia, 5% U	71.98	28.02
Unsoluble Ac, 5%U	91.11	8.89

Table 5. Metabolizable energy (ME), net energy (NE), organic matter digestibility (OMD), short chain fatty acids (SCFA) and microbial protein (MP) synthesis prediction in untreated and treated acacia and leuceana with different levels of urea

	ME (MJ/kg DM)	OMD (%)	SCFA	MP
Acacia	6.60^{c}	45.50^{e}	50.51^{b}	54.89^{e}
Acacia 1%	6.74^{c}	46.85^{d}	49.03^{b}	56.52^{d}
Acacia 3%	7.00^{b}	48.92^{c}	49.03^{b}	59.02^{c}
Acacia 5%	6.76^{c}	47.75^{cd}	40.15^{c}	57.60^{cd}
leucaena	7.52^{a}	51.70^{b}	57.91^{a}	62.36^{b}
Leucaena 1%	7.55^{a}	52.86^{ab}	49.03^{b}	63.76^{ab}
Leucaena 3%	7.63^{a}	53.69^{a}	49.03^{b}	64.77^{a}
Leucaena 5%	7.60^{a}	53.52^{a}	49.03^{b}	64.56^{a}

ME, metabolizable energy (MJ/kg DM); NE, net energy (MJ/kg DM); OMD, organic matter digestibility (%); SCFA, short chain fatty acids (mM), MP, microbial protein (g/kg DOM).
a,b,c,d,e means within the same column with differing superscript are significantly different.

CONCLUSIONS

Ensiling of AC and LE leaves with U increased crude protein and ash, while the contents of tested samples of total phenol (TP), total tannins (TT) and condensed tannins (CT) were decreased. Also, ensiling of AC and LE leaves with U significantly ($P < 0.05$) decreased gas production. All washed samples showed losses of soluble material. The gas production was, in general, significantly ($P<0.05$) higher for the unwashed substrates. Leucaena gave the highest values of gas production compared with acacia. The gas production volume was significantly ($P < 0.05$) higher for ensiled AC and LE leaves without U than ensiled AC with 5%U or ensiled LE with 3%U. The maximum rate of gas production increased after ensiling AC and LE leaves with 5% and 3% U, respectively.

The calculated values of metabolizable energy (ME) and net energy (NE) were significantly ($P<0.05$) increased for ensiled AC with 3 and 5%U, while ensiled LE with U was not significantly affected. The organic matter digestibility (OMD %) and microbial protein production were significantly ($P<0.05$) higher for ensiled AC and LE with U, while short chain fatty acids (SCFA) were significantly ($P<0.05$) decreased.

The concentrations of TP, TT and CT were strongly correlated ($p<0.01$). The TP, TT and CT were negatively related ($p<0.01$) with neutral detergent fiber (NDF) and acid detergent fiber (ADF), but not with hemicelluloses (HEMI). The crude protein was strongly correlated ($p<0.01$) with NDF, ADF and CT and negatively related ($p<0.01$) with TF and TT, but not with HEMI. In conclusion, there were negative effects on the *in vitro* gas production occurring more consistently when AC and LE were ensiled with different levels of U, while OMD% and microbial proteins were significantly ($P<0.05$) increased. The *in vitro* digestibility and gas production parameters were significantly correlated with chemical composition of shrubs.

Finally, it is generally more appropriate to measure the degradation of organic matter as usual dry matter can give problems of interpretation of the significance of the soluble fraction.

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