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Relationships Between *in Vitro* Gas Production and Dry Matter Degradation of Treated Corn Silage by Urea and Formaldehyde

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Abstract: Samples of unfractionated forage, isolated NDF and residue insoluble in 90% Ethanol were fermented *in vitro* and gas production was monitored. The gas volume associated with the ethanol soluble (A fraction) was determined as the difference between the gas from the whole forage and from the ethanol residue.The gas yield associated with the fraction insoluble in 90% ethanol but soluble in neutral detergent soluble (B1 fraction) was determined by subtracting the isolated NDF gas curve from the corresponding ethanol residue curve. This experiment included untreated Corn Silage (CS) and chemically treated corn silage. The rate of gas formation from the A fraction was approximately rather than ($p<0.05$) the B1 fraction. The gas production of A fraction was less than ($p<0.05$) the B1 fraction. The CS was treated with urea (10g kg^{-1}) or formaldehyde (4g kg^{-1}). Cumulative gas production was recorded at 2, 4, 8, 12, 16, 24, 36, 48, 72 and 96 h of incubation and McDonald equation was used to describe the kinetics of gas production. Treatment with urea decreased ($p<0.05$) gas production at 96 h by 217.6 vs 236.7, 240.0, 232.56, 254.0 (mL g^{-1}) for CS, Formaldehyde treated (CSF), urea and formaldehyde treated (CSFU), residue insoluble in 90% ethanol (EIR) and isolated NDF, respectively. The maximum rate of gas production decreased ($p<0.05$) in CS from 0.028 to 0.023, 0.025, 0.027, 0.0235 and 0.0268 for CSU, CSF, CSFU, EIR and isolated NDF, respectively. The gas production of soluble and insoluble fractions (a+b) decreased ($p<0.05$) from 261.8 in EIR to 241.8, 240, 225.0 238.7 and 239.3 mL g^{-1} for CS, CSU, CSF, CSFU and isolated NDF, respectively. The gas production at 96 h in EIR was ($p<0.05$) rather than the other treatments. Associative effects were calculated as the difference between the observed gas production for mixture of urea and formaldehyde and the individual inclusion (urea or formaldehyde). Associative effects generally observed as decreasing of gas production with duration of incubation. The strong correlation between extent of gas production in incubation times and on dry matter *in situ* disappearance was obtained. The poor correlation in initial times between gas production and *in vitro* dry matter and *in situ* dry matter disappearance observed resulting the improved production of *in vitro* and *in situ* dry matter disappearance from gas production in later times of incubation due to all potential components were fermented and produced gas. It is concluded that the associative effects cause decreasing of gas production specially in CSU and also resulted the difference between of the gas formation and the rate of gas production of the A fraction and the B fraction. There is strong positive correlation between gas production with *in vitro* and *in situ* dry matter disappearances.

Key words: Gas, silage, chemical treatments, *In vitro*, *In situ*

INTRODUCTION

The beef NRC[1] including a nutrition model that requires digestion rates of the fiber and soluble carbohydrate fractions to predict animal performance. The carbohydrate portion of each feed is divided into three digestible fractions: the A fraction, containing sugars, short oligosaccharides and organic acids; the B1 fraction containing starch and pectin ; and B2, the digestible fiber fraction[2]. Organic acids are treated as carbohydrates. Performance of ruminants given corn silage diets is low due to low digestibility, which is caused by low nitrogen and high fiber concentration[3]. There is growing interest in the use of supplements for increasing of degradable fiber and bypass protein.

Performance of ruminants given corn silage diets is low due to low quality of protein contents resulting of high fermentation protein in silage processing. There is growing interest in the use of chemical stuffs for improving of poor-quality forage diets.

Supplementation of corn silage with supplements providing digestible fiber and protecting of protein duration of silage processing improved the quality of corn silage[4]. The *in vitro* gas production technique

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has been used as a measure of ruminal degradation of feeds[5, 6] and as an indicator of digestible DMI[7]. This technique also has potential to investigate associative effects between feeds as chemical stuffs[3]. The objective of the present study was to determine digestion characteristics for the A and B1 fraction and to see the relationship of *in situ* and gas production.

MATERIALS AND METHODS

Silage preparation: The ethanol fractionation of silage was examined using Corn silage. Corn silage was treated using 1% urea (CSU), 0.4% formaldehyde (CSF) and treated both of 1% urea and 0.4% formaldehyde. Corn silages were prepared using individual silos made from polyvinyl chloride pipe (15 cm diameter).

Samples were chopped to a 2 cm Length using the paper cutter, wilted to approximately 30% DM and padded to a bulk density of between 0.63 and 0.80 kg L^{-1}. Then the individual silos closed completely. After 60 day, the silage was removed from the silos. A sample was taken for DM, pH and preparation of ethanol residue and isolated NDF.

Chemical analysis: All samples were ground through a 1mm screen in a Wiley mill (model 4, Arthur H. Thomas Co, Philadelphia, PA). Neutral detergent fiber and ADF were measured according to the method of [8]. Determinations of N were conducted using the Kjeldahl method in an automated Kjelfoss apparatus (Foss Electeric, Copenhagen, Denmark). Dry matter was determined by drying the whole sample in a forced air oven at 55°C until a constant weight was achieved.

Preparation of ethanol residue and isolated NDF: Ethanol insoluble residue was prepared the fermentation by stirring 0.5 g of sample in l00 mL of 90% (vol/vol) ethanol for 4 h at room temperature. The sample was filtered through a 37 μm nylon mesh and rinsed three times with acetone using vacuum[9]. The sample then was dried at 50°C overnight to remove residual acetone.

Isolated NDF for each forage was prepared by autoclaving 150 mL serum bottle containing 500 mg of forage and 100 mL of neutral detergent solution for 1 h at 150°C[10]. The isolated NDF from different bottles was combined and rinsed with hot water and 100 mL of ethanol using 37 μm nylon mesh as filter. Residual detergent was removed by soaking the isolated NDF overnight at 39°C in a solution of 1M $(NH4)_2SO4$: 1g of fiber to 100 mL of 1 M $(NH4)_2SO4$, with the fiber weight calculated from the NDF content. The isolated fiber was rinsed again with hot water followed by 100 mL each of ethanol and acetone and allowed to air dry.

***In vitro* gas production:** Three male sheep(37±3.5 kg) were used as donors of ruminal fluid for the preparation of inoculums. The sheep were fed a diet comprising (as fed) 140 g kg^{-1} CP and 2.98 Mcal kg^{-1} ME (containing 430 g kg^{-1} alfalfa hay, 350 g kg^{-1} corn silages, 100 g kg^{-1} barley grain and 120 g kg^{-1} wheat bran plus a mineral/vitamin supplement). Equal volumes of ruminal fluid from each sheep collected 2 h after the morning feeding. Were combined and strained through four layers of buffer prewarmed to 39°C. The inoculum was dispensed (20 mL) per vial in to substrate 35 mL serum vial (containing of 300 mg per vial) which had been warmed to 39°C and flushed with oxygen free CO_2. The vials were sealed immediately after loading and were affixed to a rotary shaker platform (lab-line instruments Inc Melros dark, USA) set at (120 rpm) housed in a incubator. Vials for each time point, as well as blanks (containing no substrate), were prepared in triplicate. Gas production was measured in each vial after 2, 4, 6, 8, 12, 16, 24 and 48 of incubating using a water displacement apparatus[11]. Triplicate vials were removed after 2, 4, 12, 24 and 48 h of incubation and their contents were filtered through monofilament polyester cloth with a pore size of 53 μm. The residues were washed three times by resuspension in phosphate buffer (pH 7.4) followed by centrifugation (2500 rpm, 10 min, 4°C). The pellets were dried at 50°C then ground using a micro-mill. Total N and total dry matter in the residue were determined.

***In situ* procedure:** The procedure of ruminal incubation *in situ* trial followed the method of DeBoer *et al.*[12]. A feedstuff sample of approximately 5 g was packed and sealed in to 612 cm polyester bag (pore size 53 μm). Each feed sample was incubated in 6 replicates (two replicates for each wether) in the rumen for 0, 2, 4, 8, 12, 24, 36, 48, 72 and 96 h. The six 0 h incubation sample bags were directly rinsed with water only. Nylon bags were suspended in the rumen in a polyester mesh bag (25 40 cm pore size 3 μm) on different periods and were removed from the rumen at same time so that they could all be washed simultaneously. The nylon bags were then removed from the mesh bag and placed in a conventional washing machine. Washings were repeated until the rinse water remained clear. Samples were then dried in oven at 55°C until a constant weight was achieved prior to determination of DM disappearance. Replicates within sheep were pooled and ground through a 1mm screen prior to N analyses.

Sample collection: Silage samples were collected by field staff from at least seven different areas within each silo. All seven samples were thoroughly mixed and a composite sample (50 g) was taken the silage samples

Table 1: The percentage of DM disappearance from incubated feedstuffs in *in vitro* trial

	Incubation time (h)					DM disappearance			
Treatments	0	2	12	24	48	a (%)	b (%)	c (%/h)	RSD
CS	5.87	10.11	19.22	55.56	57.7	3.99	66.33	0.00	1.66
CSU	7.35	10.01	18.22	56.22	61.89	4.7	76.24	0.032	1.13
CSF	5.74	9.23	18.11	55.50	57.78	3.5	67.93	0.038	1.05
CSFU	6.63	9.67	18.11	57.85	58.22	3.95	3.95	67.7	2.29
SEM	1.81	1.66	2.18	2.34	3.5	-	-	-	-

Table 2: The *in vitro* disappearance of CP (%)

	Incubation time (h)					CP disappearance			
Treatments	0	4	12	24	48	a (%)	b (%)	c (%/h)	RSD
CS	6.6^{c}	12.66^{a}	20.36^{ab}	49.3b^{c}	54.54^{ab}	6.22	59.87	0.038	1.01
CSU	4.97^{d}	10.24^{b}	19.06^{b}	47.17^{c}	52.07^{b}	7.24	65.38	0.036	1.92
CSF	7.87^{a}	13.63^{a}	22.46^{a}	52.39^{a}	59.04^{a}	4.3	58.78	0.039	1.48
CSFU	7.69^{b}	12.72^{a}	20.7^{ab}	50.69^{ab}	57.15^{a}	6.81	64.75	0.034	1.58
SEM	0.847	0.782	1.28	1.38	2.51	-	-	-	-

were immediately frozen at -20°C until the onset of *in situ* determination. After thawing silage, DM was determined by drying the whole sample in a forced air oven at 55°C until a constant weight was achieved.

Sheep and feeding: Three male sheep (373.5 kg) were fitted with a rumen fistula. All sheep were fed a diet comprising (as fed) 430 g kg^{-1} alfalfa hay, 350 g kg^{-1} corn silage and 220 g kg^{-1} concentrate (containing 100 g kg^{-1} barley grain and 120 g kg^{-1} wheat bran plus a mineral/vitamin supplement).

Chemical analyses: Determinations of N were conducted using Kjeldahl method in an automated Kjelfoss apparatus (Foss Electric, Copenhagen, Denmark). Neutral detergent fiber and ADF were measured according to the method of Goering and Van Soest[8].

Calculations and statistical analyses: Gas production was calculated as mL g^{-1} sample DM and gas production values over time for each sample were fitted to a one-component McDonald model: $y = A(1-e^{-ct})$ that y is the volume of gas production at time t, A the soluble and insoluble gas production (mL g^{-1} DM) and c rate of gas production of insoluble fraction and t is time. Parameters A and c was estimated by an iterative least square method using a nonlinear regression procedure of the statistical analysis system[13]. The actual *in situ* and *in vitro* degradation curves were calculated by iterative least square procedure.

According to[13] this model was followed by[14] as $y = a + b(1-e^{-ct})$ that p is the actual degradation of CP and DM after t, a is the intercept of the degradation curve at time zero, b is the potential degradability of the component of the slowly soluble CP and DM, which will in time be degraded, c represents the constant of degradation rate b at t hours, t is incubation time.

Effective degradability of Crude Protein (EDCP) was calculated from the rumen outflow rate (k) and the constants of a, b and c from the above model. K is usually calculated on 0.02/h, 0.06/h or 0.08/h. The EDCP formula is as EDCP = a+bc/c+k where k is the estimated rate of outflow from the rumen.

The percent disappearance of the DM and CP at the different incubation time was calculated as the difference between the feed and the portion remaining after different incubation times Table 1 and 2. Difference between feedstuffs in rumen disappearance of DM and CP and gas production data were analyzed using the General Linear Model (GLM). Procedure of (SAS institute Inc.,[13], with Duncans multiple range test used for the comparision of means. The test treatments were the only sources of the variation considers analytical variability was included in the error variance.

Gas associated with the A and B1 fractions was estimated using gas curve subtraction[15]. Gas produced from the ethanol soluble fraction (A fraction) was determined by difference between the average gas yields of the whole feed sample and its EIR preparation[15]. Likewise, the gas volume from the soluble fiber and starch (B1 fraction) was determined by subtracting the gas produced by a samples isolated NDF from the gas curve of the EIR preparation.

RESULTS AND DISCUSSION

Chemical composition: The chemical composition of the treatments in this experiment is presented in the Table 3. The DM content of the CSU and CSF was lower than expected Table 3. However the silages in this experiment were well presented, with an expected pH = 4. As expected as corn silage treated with urea the CP contents increased (CSU, CSFU). The chemical treating of corn silage with urea and formaldehyde decreased the concentration

Table 3: Chemical composition of the experimental treatments(%)

Item	CS	CSU	CSF	CSFU
DM	33.21	29.45	29.12	34.28
CP	8.06	12.02	7.89	10.80
N	1.29	1.92	1.26	1.73
NDF	58.35	57.60	57.50	58.50
ADF	38.85	35.85	32.70	37.20
Hemicelluloses*	19.50	21.75	24.80	21.30
pH	3.94	4.07	3.92	4.07

* Hemicelluloses = NDF - ADF

of ADF resulting of high hemicellulose concentrations. These results certified the reports of Liu *et al*.[3]. As urea treated rice straw increased hemicellulose concentrations.

Gas production characteristics: The addition of a new feed to models such as the CNCPS[2] and level 2 of the beef NRC[1] requires a comprehensive chemical analysis and a digestion rate for three distinct carbohydrate fractions. The determination of these digestion rates is time consuming[10,16,17], which restricts the number of samples that can be analyzed. The curve subtraction approach of Pell and Schofield[15] can provide estimates of digestive rates for dynamic models while reducing the labor investment, although the fermentation of three separate forage fractions is still required.

This technique is based on the assumption that the preparation of the forage fractions (EIR and isolated NDF) dose not alter the fermentable carbohydrates[10]. Soluble sugars, short oligosaccharides and organic acids may alter the fermentation of the associated fiber components by changing both the rate and extent[17,18].

Removal of the soluble fractions during extracting eliminates the possibility of interactions that may occur during the fermentation of the whole forage. The effects of soluble carbohydrate and fiber interactions can be assessed by comparing the NDF digestibility of the intact forage with those of the EIR and NDF residues[17]. But this measurement didn't carry out in this experiment. Doane *et al*.[17] showed that the gas yield from isolated NDF fraction of CS was lower than those from whole CS. This result didn't observed in our experiment. During ensiling soluble sugars in the A fraction converted largely to lactate. At the same time, microbial activity and higher acidity clear some of the side chains from the pectic substances and hemicellulose that were originally in the B1 and NDF fractions. These side chains may be further fermented in the A fraction to yield organic acids. This would explain the increased mass of the A fraction in the ensiled forage as material removed from the B1 and NDF accumulated with the A fraction. The amount of gas produced from the A fraction compared to the B1 fraction increased according to Doane *et al*.[17] observations. *In vitro* gas production at 48 h from CSU was lower than to that reported (223.1 mL g^{-1} DM). This difference was attributed to varing particle size, experiment preparation and method differences Table 4.

***In vitro* and *in situ* dry matter and crude protein disappearance:** There was no differences ($p<0.05$) in *in vitro* dry matter disappearance among treatments Table 3. The *in vitro* CP disappearance in CSF at 48 h was more than the other treatments ($p<0.05$). This results is not consistent with the *in situ* CP disappearance that the CSF was lower than the treatments. These differences were attributed to small particle samples attached to vials and absence of *in vitro* fermentation environment in later times as same as initial time.

There was no differences ($p<0.05$) in *in situ* dry matter disappearance among treatments Table 5. The CSF *in situ* CP disappearance at 48h was lower that the on the other treatments ($p<0.05$). These results is more probably due to ruminaly degradation protecting effects of formaldehyde on component of feeds[19]. The pattern *in vitro* and *in situ* DM and CP fermentation (a, b and c parameters) was a little by little different. Generally the variation in fermentation characteristics due to chemical composition differences is predicted Table 6.

Relationship between *in vitro* gas production with dry matter *in situ* and *in vitro* disappearance: The strong correlation between extent of gas production in incubation times and on dry matter *in situ* disappearance is consistent with Nsahlai *et al*.,[6,20]. The gas production of CSU was lower than the other treatments ($p<0.05$) and that is consistent with that negative correlation between high CP and gas production reported for tropical browse species[21] but is not consistent with the results of Getachew *et al*.,[6]. The poor correlation in initial times between gas production and *in vitro* dry matter and *in s itu* dry matter disappearance observed in our study (it isn't shown in Table 7) could be due to feed constituents such as fat and protein, which contribute little or no gas but are degraded *in vitro* and *in situ*.

This was evident from the improved production of *in vitro* and *in situ* dry matter disappearance from gas production in later times of incubation due to all potential components were fermented and produced gas. Since gas production doesn't consider the amount of substrate converted into microbial biomass, gas measurement can be considered an estimate of apparent rumen digestibility[7]. Although gas production reflects the

Table 4: Mean values (n = 3) of cumulative gas produced at different times of incubation of CS, CSU, CSF, CSFU, EIR and NDF fraction and parameters of gas production estimated with

	Cumulative gas (mL g^{-1} DM)				Gas production function	
Feedstuff	12h	24h	48h	96h	A(mL)	C(mL h^{-1})
CS	57.11^{a}	111.56^{a}	186.22^{a}	236.67^{b}	241.8^{b}	0.0287^{a}
CSF	54.67^{ab}	97.78^{a}	168.78^{b}	232.11^{b}	240.0^{c}	0.0231^{c}
CSU	52.22^{ab}	99.33^{a}	167.78^{b}	217.56^{c}	225.0^{f}	0.0256b^{c}
CSFU	53.11^{ab}	105.78^{a}	178.22^{ab}	232.56^{b}	233.8^{e}	0.0279^{ab}
EIR	49.33^{b}	97.67^{a}	179.44^{ab}	258.33^{a}	261.8^{a}	0.0235^{c}
NDF	21.56	63.11^{b}	197.33^{a}	254.0^{a}	239.3^{d}	0.0268^{ab}
SEM	3.74	8.7	9.72	5.6	0.0001	0.00098
A fraction[1]	-	-	-	21.66	20.0	0.0052
B1 fraction[2]	-	-	-	47.33	2.5	0.0019

Gas production of A fraction = difference between the average gas yields of whole feed sample and its EIR preparation, gas production of B1 fraction = difference of gas produced by isolated NDF and EIR preparation

Table 5: The *in situ* disappearance of DM (%)

	Incubation time h						DM disappearance (%)				
Treatments	0	4	12	24	48	96	a (%)	b (%)	c (%/h)	ERDP	RSD
CS	5.87	11.22	21.54^{b}	38.98	60.22^{a}	71.71	3.82	74.99	0.025	45.6	2.75
CSU	7.33	12.56	24.63^{ab}	42.94	54.48^{ab}	65.36	7.79	60.41	0.029	43.2	2.81
CSF	5.74	15.99	26.12^{a}	38.55	52.33^{b}	68.09	8.52	63.4	0.024	49.3	2.8
CSFU	6.62	12.56	25.89^{ab}	40.06	55.74^{ab}	66.55	6.62	63.3	0.029	47.8	1.53
SEM	1.81	5.17	3.47	4.6	4.05	5.64	-	-	-	-	-

Table 6: The *in situ* disappearance of CP (%)

	Incubation time (h)						CP disappearance			
Treatments	0	4	12	24	48	96	a (%)	b (%)	c (%/h)	RSD
CS	6.6^{a}	14.17^{b}	25.87	39.66	56.6^{a}	67.35^{ab}	5.61	66.11	0.03	2.34
CSU	7.87^{a}	17.42^{a}	24.99	35.17	46.31^{b}	69.1^{a}	9.21	67.78	0.023	1.69
CSF	4.97^{b}	14.06^{b}	25.4	39.39	46.45^{b}	61.56^{b}	7.25	55.55	0.027	2.02
CSFU	7.7^{a}	16.37^{ab}	30.29	39.26	56.15^{a}	64.45^{ab}	8.8	58.31	0.03	1.6
SEM	0.847	1.43	4.09	3.28	3.76	1.74	-	-	-	-

Table 7: Correlation (r) between *in vitro* gas production, dry matter disappearance *in situ* (DMIS) and *in vitro* disappearance (DMIV)

DMIS (%disappearance)	DMIV (%disappearance)	GP (mL g^{-1})
CS	0.98	0.99
CSU	0.970	0.99
CSF	0.98	0.98
CSFU	0.96	0.99
DMIV		
CS	-	0.93
CSU	-	0.93
CSF	-	0.93
CSFU	-	0.93

amount of substrate used for VFA production, it has also been shown that gas production is positively related to feed intake[7] and microbial protein synthesis.

CONCLUSION

There was strong positive correlation between gas production, *in vitro* and *in situ* dry matter disappearance so the gas production technique can be suitable replace for *in situ* dry matter and *in vitro* disappearances. As a whole, the wide variation in chemical composition of treatments, gas production, *in situ* and *in vitro* DM and CP disappearances offer users flexibility in formulating ratios according to the productive performance of target animals.

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