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Association of fibre degradation with ruminal dissolved hydrogen in growing beef bulls fed with two types of forages

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Abstract

The present study investigated the association between fibre degradation and the concentration of dissolved molecular hydrogen (H_2) in the rumen. Napier grass (NG) silage and corn stover (CS) silage were compared as forages with contrasting structures and degradation patterns. In the first experiment, CS silage had greater 48-h DM, neutral-detergent fibre (NDF) and acid-detergent fibre degradation, and total gas and methane (CH_4) volumes, and lower 48-h H_2 volume than NG silage in 48-h *in vitro* incubations. In the second experiment, twenty-four growing beef bulls were fed diets including 55 % (DM basis) NG or CS silages. Bulls fed the CS diet had greater DM intake (DMI), average daily gain, total-tract digestibility of OM and NDF, ruminal dissolved methane (dCH_4) concentration and gene copies of protozoa, methanogens, *Ruminococcus albus* and *R. flavefaciens*, and had lower ruminal dH_2 concentration, and molar proportions of valerate and isovalerate, in comparison with those fed the NG diet. There was a negative correlation between dH_2 concentration and NDF digestibility in bulls fed the CS diet, and a lack of relationship between dH_2 concentration and NDF digestibility with the NG diet. In summary, the fibre of CS silage was more easily degraded by rumen microorganisms than that of NG silage. Increased dCH_4 concentration with the CS diet presumably led to the decreased ruminal dH_2 concentration, which may be helpful for fibre degradation and growth of fibrolytic micro-organisms in the rumen.

Key words: Fibre degradation: Rumen fermentation: Corn stover silage: Napier grass silage: Dissolved hydrogen: Dissolved methane

Dietary fibre is important for promoting rumination and maintaining rumen health and also provides energy to the host animal for maintenance and production[1,2]. Fibre degradation in the rumen is a complex process that needs a wide range of microorganisms to work as a consortium, including fungi, protozoa, bacteria and methanogens[3,4]. Molecular hydrogen (H_2) is formed during fermentation of carbohydrates to volatile fatty acids (VFA) and transferred to methanogens, which produce methane (CH_4) as the end product[5]. Methanogenesis plays an important role in maintaining a low H_2 partial pressure in the rumen, as elevated ruminal H_2 is understood to inhibit fibre degradation in the rumen[6,7]. A previous study indicates that ruminal dissolved H_2 (dH_2) concentration can be closely associated with rumen fermentation and microbiota in dairy cows fed different types of carbohydrates[8]. However, few studies have been conducted to investigate the association between ruminal dH_2 concentration with fibre digestibility in ruminants.

Both Napier grass (NG, *Pennisetum purpureum*) and corn stover (CS, *Zea mays*) are important forage sources for ruminant production in subtropical areas. NG has a flat, smooth, rigid,

Abbreviations: CS, corn stover; dCH_4, dissolved CH_4; dH_2, dissolved H_2; DMI, DM intake; NDF, neutral-detergent fibre; NG, Napier grass; OM, organic matter; SEM, scanning electron microscopy; VFA, volatile fatty acids.

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regular and compact surface structure. The cell wall of NG is formed of crystalline cellulose[9], which is highly resistant to chemical and biological hydrolysis[10]. CS contains 30–46 % fermentable carbohydrates. The fibre of CS silage has a coarse surface with fibre delamination, which increases the ratio of external:internal-specific surface area, which benefits microbial colonisation and enzymatic attack[11].

We hypothesised that NG and CS silage would have different ruminal fibre degradation extents and patterns, and replacing the less digestible fibre source by the more digestible one would alter rumen fermentation and microbiota, which might be associated with ruminal dH_2 concentration. *In vitro* batch cultures were firstly performed to compare ruminal fibre degradation, H_2 and CH_4 production of these two selected forages. Once established which fibre source was more digestible, an *in vivo* study was then performed to compare fibre digestibility, rumen fermentation and the abundance of microbial groups in growing beef bulls fed NG or CS diets.

Experimental methods

All procedures involving animals were approved by the Animal Care Committee (approval number ISA-W-201801), Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, China.

Forages

CS was harvested 90 d after sowing and immediately after grain harvest. CS was chopped with a chaff cutter (Guangzhou State Machinery Equipment Co. Ltd) to 4 cm, ensiled in polyethylene bags (Wenzhou Quanyu Packing Co. Ltd) and compressed to remove the air. NG (*Pennisetum purpureum*) was harvested after 90 d of regrowth, ensiled in polyethylene bags and compressed to remove the air. After 2 months, samples of NG and CS silage were randomly collected from ten bags and dried at 65°C for 24 h. Samples of both silages were analysed for chemical composition (Table 1), used as a substrate in *in vitro* ruminal batch cultures and scanned with an electron microscope.

In vitro *ruminal batch incubation*

Approximately 200 g samples of both dried forages were collected for *in vitro* ruminal batch incubations. One 100 g subsample was ground with a multi-functional pulverizer and then screened through a 1 mm stainless steel flour sieve. Approximately 1·0 g was weighed into each of 150 ml serum bottles for the investigation of *in vitro* fibre degradation and gas production. The other 100 g subsample was carefully cut to a length of 2 cm, and about 1·0 g was weighed into 150 ml serum bottles for the investigation of changes in the fibre structure before and after incubation by using scanning electron microscopy (SEM). *In vitro* ruminal incubations were performed in a fully automatic *in vitro* batch culture system as described by Wang *et al.*[12], with artificial saliva prepared according to Menke *et al.*[13]. The incubation was stopped at 48 h. Apparent *in vitro* degradation of chemical fractions was determined based on the contents of each fraction in the substrates incubated and the undigested residues after the 48-h *in vitro* incubation. The samples for obtaining SEM images were firstly filtered into Gooch filter crucibles to exclude small particles, dried at 105°C for 24 h and then stored in 50 ml sterile centrifuge tubes for further processing. Three runs of incubation were conducted. Two replicates per substrate were included in each incubation run.

Table 1. Chemical composition (expressed in g/kg of DM unless otherwise indicated) of Napier grass (NG) and corn stover (CS) silage

Item	NG silage	CS silage
DM (g/kg)	212	238
OM	937	933
CP	70·5	102
NDF	709	577
NDIN	67·0	58·8
ADF	434	300
ADIN	41·4	49·2
Starch	67·9	71·5

OM, organic matter; CP, crude protein; NDF, neutral-detergent fibre; NDIN, neutral-detergent insoluble N; ADF, acid-detergent fibre; ADIN, acid-detergent insoluble N.

Animal trial

Animals and diets. The experiment was conducted at the beef cattle farm of Guangxi Huisheng animal husbandry development Co. Ltd. Twenty-four healthy Limousin (sire) × (Aberdeen Angus (sire) × Simmental (dam)) crossbred growing beef bulls with an average body weight of 320 (SD 16·1) kg and 1·5 (SD 0·18) years old at the beginning of the experiment were used. Animals were allocated to twelve blocks according to body weight and DM intake (DMI) measured in a preliminary experiment. Each block contained two animals, and one animal within each block was randomly assigned to one of the two diets. The CS diet was formulated by replacing 55 % NG silage with 55 % CS silage (DM basis). The diets were formulated to meet appropriately 1·2–1·3 times the digestible energy and crude protein requirements of growing beef bulls according to Zhang & Zhang[14] (Table 2).

Bulls were housed in a tie-stall barn and fed individually two equal meals at 09.00 and 16.00 hours. Free access to fresh water was provided throughout the experiment. The experimental period lasted for 70 d, with 60 d of adaptation to the diets and the last 10 d for sampling and data collection, including 5 d for total faeces collection, 2 d for collecting rumen samples and 4 d for measuring body weight. Feed was offered *ad libitum* targeting 5 % refusals during the initial 10 d of the adaption period to estimate DMI. The amount of feed offered daily thereafter was set at 105 % of the previously measured DMI to minimise diet selection. The refusals were collected and analysed to calculate nutrient intake. All of the experimental bulls' health conditions and DMI were recorded daily throughout the experiment to ensure that there was no clinical signs of possible metabolic disorders.

Nutrient digestibility. Feeds, refusals and faecal samples were collected daily from d 61 to 65. Total faeces were collected, weighed and mixed daily. A subsample (1 %, w/w) was acidified with H_2SO_4 (10 %, w/w) to prevent N loss and frozen immediately at −20°C. Another non-acidified subsample (1 %, w/w)

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Table 2. Ingredients and chemical composition of the Napier grass (NG) and corn stover (CS) diets (g/kg DM)

Item	Diets	
	NG	CS
Dietary ingredient (g/kg DM)		
Corn stover silage	238	530
Napier grass silage	292	0
Maize	304	304
Wheat bran	56·0	56·0
Soyabean meal	85·0	85·0
Limestone powder	5·0	5·0
NaCl	5·0	5·0
Rice wine yeast	10·0	10·0
Premix*	5·0	5·0
Chemical composition (g/kg DM)†		
OM	922	922
CP	114	121
NDF	414	387
ADF	198	172
Starch	210	210
NE_m (Mcal/kg of DM)‡	1·55	1·62
NE_f (Mcal/kg of DM)‡	0·94	1·00

OM, organic matter; CP, crude protein; NDF, neutral-detergent fibre; ADF, acid-detergent fibre; NE_m, net energy for maintenance; NE_f, net energy for fattening.
* The premix (vitamins and microelements) was formulated to provide (per kg of DM) 1 000 000 IU of vitamin A, 200 000 IU of vitamin D, 1250 IU vitamin E, 8000 mg of Zn, 80 mg of Se, 120 mg of I, 2000 mg of Fe, 40 mg of Co, 2500 mg of Mn, and 2000 mg of Cu.
† Mean values from the analysis of three samples.
‡ NE_m and NE_f were calculated according to Feng[44]. To convert Mcal to MJ, multiply by 4·184.

was frozen immediately at –20°C. All the samples were dried at 65°C for 24 h in a forced-air oven and ground through a 1 mm screen for subsequent analysis of chemical composition. The acidified samples were used for total N analysis, and non-acidified samples were used for the rest of the chemical analyses.

Rumen sampling. The collection of rumen contents was performed on d 66 and 67 at 2·5 h after the morning feeding. The first 150 ml of rumen contents was discarded to avoid saliva contamination[15]. Then, about 300 ml of rumen contents was taken from the central rumen using the oral stomach tubing technique. About 20 ml of sampled rumen contents was used for immediately measuring pH with a portable pH meter (Starter 300; Ohaus Instruments Co. Ltd). Three 50 ml subsamples were immediately frozen in liquid N_2 and stored at –80°C for DNA extraction for microbial abundance analyses. Two 35 ml rumen content subsamples were immediately transferred to 50 ml plastic syringes for measuring dH_2 and dissolved CH_4 (dCH_4) concentrations[16]. Three more 5 ml subsamples of rumen contents samples were collected and centrifuged at 12 000 ***g*** for 10 min at 4°C, and 1·5 ml of supernatant was acidified with 0·15 ml of metaphosphoric acid (25 %, w/v) and stored at –20°C for subsequent measurement of VFA and ammonium concentrations.

Proximate analyses

Contents of DM (method 945.15), OM (method 942.05) and crude protein (method 945.01, total N × 6·25) were analysed using the methods of AOAC[17]. Neutral-detergent fibre and acid-detergent fibre contents were determined according to Van Soest *et al.*[18] with inclusion of a heat stable *α*-amylase and sodium sulphite and were expressed with residual ash. Gross energy was determined with the use of an isothermal automatic calorimeter (5E-AC8018; Changsha Kaiyuan Instruments Co.). Starch content was measured using amyloglucosidase as described by Karthner & Theurer[19]. Neutral-detergent insoluble N and acid-detergent insoluble N content were measured as described by Licitra *et al.*[20].

The images of fibre structure were obtained by field emission using SEM (model SU8010), according to the manufacturer's instructions. Briefly, the same site of the stem in each forage was selected and coated with gold before scanning by SEM[21].

Dissolved gases were extracted from the liquid phase of rumen contents into the gas phase using the procedure by Wang *et al.*[16]. Gas samples in the gas phase were collected in evacuated tubes for measuring H_2 and CH_4 concentrations using a gas chromatograph (Agilent 7890A, Agilent Inc.)[15]. Concentrations of dH_2 and dCH_4 in the original liquid fraction were calculated using the equations by Wang *et al.*[15].

Individual VFA concentrations were analysed by GC (Agilent 7890A, Agilent Inc.) according to Wang *et al.*[16]. Ammonium concentration was analysed according to Weatherburn[22].

DNA extraction and quantification of microbial groups. Genomic DNA was extracted from 1 ml of rumen contents by physical disruption using a bead beater, according to the protocol described by Yu & Morrison[23], with the exception that DNA was purified with phenol–chloroform–isopentyl alcohol (25:24:1 v/v, Solarbio Co.). Isopentyl alcohol was removed with vacuum. DNA was then dissolved into TE buffer (TRIS 10 mM, EDTA 1 mM, pH = 8·0) after being washed with 70 % ethanol twice. The quality of the DNA extracts was assessed using agarose gel (0·8 %) electrophoresis. Total DNA extracted was quantified with Nano Drop ND1000 (NanoDrop Technologies, Inc.) and then stored at –80°C until further analyses[24].

Total DNA was then diluted to 10 ng/μl to quantify selected groups of micro-organisms, including total bacteria, protozoa, fungi and methanogens, *Ruminococcus albus, Ruminococcus flavefaciens, Fibrobacter succinogenes, Selenomonas ruminantium*, *Prevotella ruminicola* and the genus *Prevotella* spp. (online Supplementary Table S1). Quantitative PCR was performed according to Jiao *et al.*[24] and expressed as $\log_{10}$ copies of 16S or 18S rRNA genes per ml rumen contents.

Statistical analyses. The effects of substrate or diet on response variables *in vitro* and *in vivo* were analysed by using the SPSS 21.0 software (SPSS Inc.). The *in vitro* data were analysed using the general linear model procedure with the following model:

$$Y_{ijk} = \mu + S_i + R_j + e_{ijk}$$

where μ is the overall mean, S_i is the fixed effect of substrates ($i = 2$, NG or CS silage), R_j is the random effect of the incubation run ($j = 3$) and e_{ijk} is the random error term.

The *in vivo* data were also analysed using the procedure of general linear model with the following model:

$$Y_{ijk} = \mu + D_i + B_j + e_{ijk}$$

where μ is the overall mean, D_i is the fixed effect of diets ($i = 2$, NS and CS diets), B_j is the random effect of the block ($j = 12$) and e_{ijk} is the random error term.

The associations of dH_2 concentration and diet with various fermentation and microbiological variables were studied using the RStudio software (R × 64 3.5.0) with the following model[25]:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1 X_2 + \varepsilon$$

where Y is the neutral-detergent fibre (NDF) digestibility, dCH_4 is the concentration or selected microbial groups, β_0 is the intercept, X_1 and X_2 are the main effects of dH_2 concentration and diet, respectively, β_1 to β_2 are the regression coefficients of dH_2 concentration and diet, respectively, X_1X_2 is the interaction between dH_2 concentration and diet, β_3 is the regression coefficient of the interaction term and ε is the random error. The point belonging to the CS diet with '$dH_2 = 0{\cdot}83$ μM' was identified as an outlier by the Dixon Q test of the Data Processing System (DPS) software (version 15.10) and was excluded from the analyses but it is still depicted in graphs[26].

Linear regression was performed by using RStudio software (R × 64 3.5.0) to obtain the correlation coefficients (R^2) and their statistical significance. Statistical significance of effects was declared at $P \leq 0{\cdot}05$ and a tendency towards significance at $0{\cdot}05 < P \leq 0{\cdot}10$.

Results

CS silage had greater *in vitro* digestibility ($P < 0{\cdot}001$) of DM, NDF and acid-detergent fibre, total gas ($P = 0{\cdot}004$) and CH_4 volume ($P = 0{\cdot}03$), and lower H_2 volume ($P < 0{\cdot}001$), in comparison with NG silage (Table 3). The SEM images showed that the CS silage had greater density of hairy appendages than the NG silage before the *in vitro* ruminal incubation, with the surface of both NG and CS silages appearing smooth, intact and contiguous (Fig. 1(a) and (b)). After 48-h *in vitro* rumen incubation, the hairy appendages disappeared from the CS silage and the CS silage seemed to have more small holes than the NG silage (Fig. 1(c) and (d)).

Table 3. Substrate degradation and gas production of Napier grass (NG) or corn stover (CS) silage during 48-h *in vitro* ruminal batch incubation (Mean values with their standard errors)

	Substrates			
Item	NG	CS	SEM	*P*
Substrate degradation (g/kg)				
DM	511	574	8·4	<0·001
NDF	484	555	8·3	<0·001
ADF	436	507	4·7	<0·001
Gas production				
Total gas (ml/g DM)	197	220	5·4	0·004
Hydrogen (μl/g DM)	69·2	50·8	2·24	< 0·001
Methane (ml/g DM)	25·7	28·8	0·98	0·03

NDF, neutral-detergent fibre; ADF, acid-detergent fibre.

Bulls fed the CS diet had greater OM ($P = 0{\cdot}03$), crude protein ($P < 0{\cdot}001$) and gross energy ($P = 0{\cdot}002$) intake, lower NDF ($P = 0{\cdot}03$) and acid-detergent fibre intake ($P < 0{\cdot}001$), and higher daily weight gain ($P = 0{\cdot}03$), than those fed the NG diet (Table 4). Bulls fed the CS diet had greater digestibility of OM ($P = 0{\cdot}002$), NDF ($P = 0{\cdot}048$) and starch ($P = 0{\cdot}02$), and lower digestibility of crude protein ($P = 0{\cdot}004$) than those fed the NG diet.

The Dixon Q test identified a point with '$dH_2 = 0{\cdot}83$ μM' as an outlier for the CS diet. The outlier was removed from all of the analyses. Bulls fed the CS diet had lower ruminal dH_2 concentration ($P < 0{\cdot}001$), molar proportion of valerate ($P = 0{\cdot}02$) and isovalerate ($P = 0{\cdot}02$), and greater dCH_4 concentration ($P < 0{\cdot}001$), and tended to increased molar proportion of butyrate ($P = 0{\cdot}07$), compared with those fed the NG diet (Table 5). There was an interaction between dH_2 and diet on NDF digestibility ($P = 0{\cdot}006$, Table 6), with a negative correlation between dH_2 concentration and NDF digestibility in bulls fed the CS diet (R^2 0·48, $P = 0{\cdot}02$) and no relationship in their counterparts fed the NG diet ($R^2 < 0{\cdot}01$, $P = 0{\cdot}90$; Fig. 2). Ruminal dH_2 concentration did not have an association with dCH_4 concentration in each diet separately (Fig. 3).

Bulls fed the CS diet had greater log 18S rRNA gene copies of protozoa ($P = 0{\cdot}001$) and log 16S rRNA gene copies of *S. ruminantium* ($P = 0{\cdot}05$), *R. albus* ($P = 0{\cdot}001$), *R. flavefaciens* ($P = 0{\cdot}005$) and methanogens ($P = 0{\cdot}003$) and tended to increased total bacteria ($P = 0{\cdot}06$), fungi ($P = 0{\cdot}08$) and *Prevotella* spp. ($P = 0{\cdot}07$) per ml of rumen fluid (Table 7). There was an interaction between dH_2 and diet on 18S rRNA protozoal gene copies ($P = 0{\cdot}03$, Table 6), with no association at the relatively lower dH_2 concentration of the NG diet (R^2 = 0·08, $P = 0{\cdot}37$) and a positive association with dH_2 at the higher dH_2 concentration of the CS diet (R^2 0·45, $P = 0{\cdot}02$; Fig. 4). Ruminal dH_2 concentration did not correlate ($P > 0{\cdot}10$) with the 16S gene copies of methanogens, fungi, *R. flavefaciens* or *R. albus* per ml of rumen fluid in each diet separately (Fig. 4).

Discussion

DMI of the CS diet was greater than that of the NG diet, which is in agreement with a previous study by Ruiz *et al.*[27]. Increased DMI is associated with increased passage rate, which can result in less rumen degradation if not compensated by greater rumen degradation rate or lower tract digestion[28]. However, feeding the CS diet resulted in greater total-tract DM digestibility compared with feeding the NG diet, which makes us think that greater digestion rates with the CS diet might have allowed greater DMI. The CS diet had lower NDF content and greater *in vitro* NDF degradation than the NG diet. Lower fibre content and greater fibre degradation decrease rumen fill with increased outflow of ruminal digesta[28], which might explain the greater DMI and daily weight gain in bulls fed the CS diet.

The fibre structure of CS silage differed from that of NG silage. Differences in fibre structure can be related to a vast range of biochemical compounds[29,30]. From a structural point of view, CS silage had a greater density of hairy appendages than NG silage, which might provide more sites for microbial attachment and

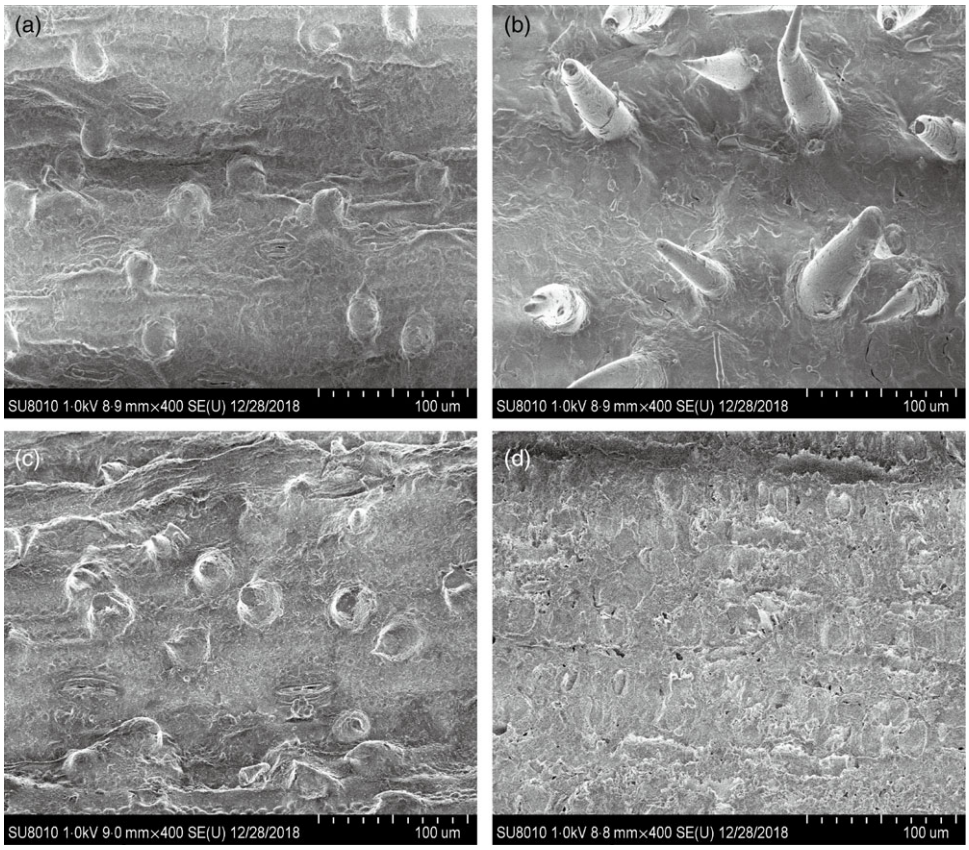

Fig. 1. Scanning electron microscopy images (scale bar = 100 μm) of Napier grass (a and c) or corn stover (b and d) silage before (a and b) and after (c and d) 48-h *in vitro* ruminal incubation.

Table 4. Feed intake and total-tract apparent digestibility and growth performance in bulls fed the Napier grass (NG) or corn stover (CS) diets (Mean values with their standard errors)

	Diet			
Item	NG	CS	SEM	*P*
Intake (kg/d)				
DM	7·30	7·68	0·111	0·03
OM	6·74	7·08	0·102	0·03
CP	0·80	0·93	0·012	<0·001
NDF	3·18	3·02	0·049	0·03
ADF	1·60	1·35	0·026	<0·001
Starch	1·52	1·57	0·030	0·23
GE (MJ/d)	121	129	1·8	0·002
Total-tract apparent digestibility (g/kg)				
DM	607	651	7·8	0·001
OM	639	679	7·1	0·002
CP	689	629	13·1	0·004
NDF	527	559	10·8	0·048
ADF	481	492	16·5	0·63
Starch	866	875	2·5	0·02
Daily weight gain (kg/d)	1·19	1·37	0·057	0·03

OM, organic matter; CP, crude protein; NDF, neutral-detergent fibre; ADF, acid-detergent fibre; GE, gross energy.

colonisation. Both *in vitro* and *in vivo* experiments indicated that the CS silage had greater NDF degradability than the NG silage. It seems that the fibre of the CS silage was easier to degrade by the mixed ruminal microbiota. In agreement, the SEM images showed that the CS silage had greater disruption and physical changes than the NG silage after 48-h *in vitro* rumen incubation.

Hydrogen is generated during fermentation of carbohydrates to VFA and mostly utilised by ruminal methanogens to produce CH_4[7,31]. Maintenance of a low ruminal dH_2 concentration facilitates carbohydrate degradation[32]. Ruminal dH_2 concentration can be affected by many factors, such as the rate of H_2 generation, the saturation factor of dH_2 and the rate of H_2 utilisation for methanogenesis. When the rate of H_2 generation is greater than H_2 utilisation, H_2 accumulates. In our *in vitro* study, CS silage was more degraded and fermented than NG silage, which would be thought to lead to increased H_2 production[7]. However, CS silage had lower *in vitro* H_2 gas volume with greater *in vitro* CH_4 gas volume in comparison with NG silage. Furthermore, feeding the CS diet resulted in lower ruminal dH_2 concentration compared with feeding the NG diet. This might be due to the enhanced capacity of ruminal H_2 utilisation by methanogens in bulls fed the CS diet[7], which is consistent with the greater dCH_4 concentration and 16S rRNA gene copies of methanogens observed in bulls fed the CS diet. Enhancement of methanogenesis results in lower ruminal dH_2 concentration[7] and thus facilitates the re-oxidation of reduced electron carriers, like NADH, leading to an enhancement of microbial ATP generation and fibre degradation in the rumen[33,34]. This proposed mechanism agrees with the negative association of NDF digestibility with dH_2 observed for the CS diet and suggests that the low dH_2

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Table 5. Concentrations of dissolved gases and fermentation end products in the rumens of bulls fed the Napier grass (NG) or corn stover (CS) diets
(Mean values with their standard errors)

	Diet			
Item	NG	CS	SEM	*P*
Dissolved gases				
dH_2 (μM)	0·76	0·24	0·058	<0·001
dCH_4 (mM)	0·55	0·68	0·020	<0·001
pH	6·82	6·78	0·020	0·20
Ammonia (mM)	12·80	10·77	0·831	0·09
Total VFA (mM)	69·0	71·9	3·02	0·50
Acetate:propionate ratio	2·96	3·02	0·071	0·57
Molar percentage of individual VFA (mol/100 mol)				
Acetate	63·1	63·0	0·44	0·88
Propionate	21·4	21·0	0·38	0·48
Butyrate	10·5	11·4	0·33	0·07
Valerate	1·7	1·6	0·03	0·02
Isobutyrate	1·3	1·2	0·02	0·07
Isovalerate	2·0	1·8	0·07	0·02

dCH_4, dissolved methane; dH_2, dissolved hydrogen; VFA, volatile fatty acids.

Table 6. Initial regression equations depicting the associations of diet and ruminal dissolved hydrogen (dH_2) concentration, and their interaction, with neutral-detergent fibre (NDF) digestibility, dissolved methane (dCH_4) concentration and selected microbial groups in growing beef bulls fed the Napier grass (NG) or corn stover (CS) diets

Response	Equations*	R^2†	*P*‡
NDF digestibility	$y = 647{\cdot}80$ (SE 26·53; $P < 0{\cdot}001$) − 118·27 (SE 37·82; $P = 0{\cdot}006$) (if diet = NG)§ − 344·31 (SE 104·50; $P = 0{\cdot}004$) dH_2 + 340·45 (SE 109·84; $P = 0{\cdot}006$) dH_2 (if diet = NG)‖	0·52	0·002
dCH_4	$y = 0{\cdot}66$ (SE 0·07; $P < 0{\cdot}001$) − 0·11 (SE 0·10; $P = 0{\cdot}25$) (if diet = NG) + 0·09 (SE 0·26; $P = 0{\cdot}73$) dH_2 − 0·08 (SE 0·28; $P = 0{\cdot}77$) dH_2 (if diet = NG)	0·47	0·006
Protozoa	$y = 10{\cdot}93$ (SE 0·21; $P < 0{\cdot}001$) − 1·02 (SE 0·30; $P = 0{\cdot}003$) (if diet = NG) − 1·73 (SE 0·82; $P = 0{\cdot}049$) dH_2 + 2·02 (SE 0·86; $P = 0{\cdot}03$) dH_2 (if diet = NG)	0·53	0·002
Methanogens	$y = 8{\cdot}86$ (SE 0·14; $P < 0{\cdot}001$) − 0·46 (SE 0·21; $P = 0{\cdot}04$) (if diet = NG) − 0·94 (SE 0·57; $P = 0{\cdot}11$) dH_2 + 0·95 (SE 0·60; $P = 0{\cdot}13$) dH_2 (if diet = NG)	0·44	0·01
Fungi	$y = 8{\cdot}18$ (SE 0·36; $P < 0{\cdot}001$) − 0·82 (SE 0·51; $P = 0{\cdot}13$) (if diet = NG) − 1·80 (SE 1·41; $P = 0{\cdot}22$) dH_2 + 1·87 (SE 1·48; $P = 0{\cdot}22$) dH_2 (if diet = NG)	0·23	0·16
Ruminococcus albus	$y = 9{\cdot}20$ (SE 0·27; $P < 0{\cdot}001$) − 0·99 (SE 0·39; $P = 0{\cdot}02$) (if diet = NG) − 1·42 (SE 1·07; $P = 0{\cdot}20$) dH_2 + 1·64 (SE 1·12; $P = 0{\cdot}16$) dH_2 (if diet = NG)	0·48	0·005
Ruminococcus flavefaciens	$y = 8{\cdot}72$ (SE 0·20; $P < 0{\cdot}001$) − 0·40 (SE 0·29; $P = 0{\cdot}19$) (if diet = NG) − 0·38 (SE 0·80; $P = 0{\cdot}65$) dH_2 + 0·37 (SE 0·84; $P = 0{\cdot}66$) dH_2 (if diet = NG)	0·37	0·03

* Terms in parentheses after the regression coefficient estimates are standard errors and regression coefficient *P* values. The outlier ($dH_2 = 0{\cdot}83$ μM) identified by the Dixon Q test was excluded from all of the analyses, *n* 23.
† R^2, coefficient of determination of the multiple regression models.
‡ *P* value, statistical significance of the overall models.
§ Main effect of the NG diet.
‖ Regression coefficients for interaction terms are equal to zero for the CS diet.

concentration may have facilitated ruminal fibre degradation in bulls fed the CS diet. On the other hand, even though the range of dH_2 concentration observed with NG was higher compared with CS, dH_2 concentration did not correlate to NDF digestibility with the NG diet, and thus an inhibitory effect of dH_2 on fibre digestion was not apparent with the NG diet. It is possible that in the less digestible NG diet, other aspects related to the forage structure (Fig. 1) limited NDF digestibility, rather than dH_2 concentration. Lower dH_2 concentration with the CS diet would have been the result of enhanced methanogenesis[7], although the mechanisms by which feeding the CS diet could have affected methanogens are unknown.

Although the CS diet had greater fibre digestibility than the NG diet, both diets resulted in similar ruminal pH and VFA concentration. It is well known that VFA concentration is not only determined by VFA production but also by VFA absorption, passage, incorporation into microbial biomass and changes in rumen volume[35–37]. Furthermore, feeding the CS diet resulted in greater butyrate molar percentage in the rumen. As protozoa are butyrate producers[38], it is possible that the increased protozoa population in bulls fed the CS diet could have contributed to the greater butyrate percentage.

Ruminal fibre degradation requires a complex consortium of micro-organisms. Anaerobic fungi are primary colonisers of

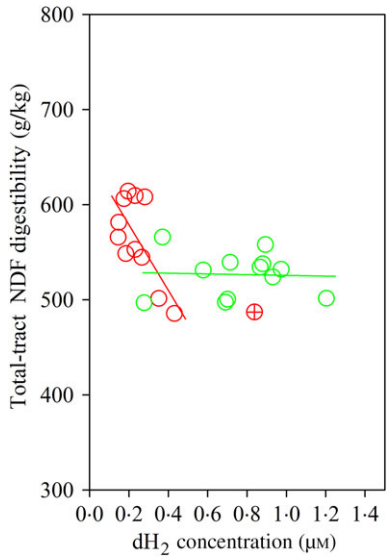

Fig. 2. Association of neutral-detergent fibre (NDF) digestibility with ruminal dissolved hydrogen (dH_2) in bulls fed the Napier grass (NG) or corn stover (CS) diets. The green and red points and solid lines represent the best regression lines fitted using observations each diet separately. NG (green): $y = 529{\cdot}54$ (±22·61; $P < 0{\cdot}001$) − 3·86 (±28·39; $P = 0{\cdot}90$) dH_2; $R^2 < 0{\cdot}01$, n 12; CS (red): $y = 647{\cdot}80$ (±30·59; $P < 0{\cdot}001$) − 344·31 (±120·48; $P = 0{\cdot}02$) dH_2; R^2 0·48, n 11. Equations contain regression coefficients, standard errors and P values and are expressed as regression coefficients with their standard errors. Each point represents an individual animal, with ⊕ being an outlier ($dH_2 = 0{\cdot}83$ μM) identified by the Dixon Q test and excluded from the analysis. Note the effect of diet by dH_2 interaction is shown in Table 6.

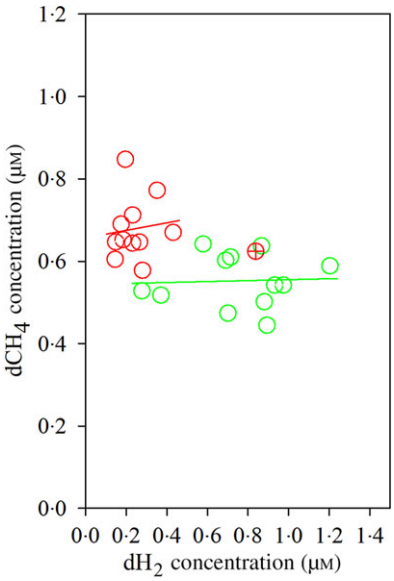

Fig. 3. Association of ruminal dissolved methane (dCH_4) with ruminal dissolved hydrogen (dH_2) in bulls fed the Napier grass (NG) or corn stover (CS) diets. The green and red points and solid lines represent the best regression lines fitted using observations from each diet separately. NG (green): $y = 0{\cdot}54$ (±0·06; $P < 0{\cdot}001$) + 0·01 (±0·08; $P = 0{\cdot}89$) dH_2; $R^2 < 0{\cdot}01$, n 12; CS (red): $y = 0{\cdot}66$ (±0·07; $P < 0{\cdot}001$) + 0·09 (±0·29; $P = 0{\cdot}76$) dH_2; $R^2 = 0{\cdot}01$, n 11. Equations contain regression coefficients, standard errors and P values, and are expressed as regression coefficients with their standard errors. Each point represents an individual animal, with ⊕ being an outlier ($dH_2 = 0{\cdot}83$ μM) identified by the Dixon Q test and excluded from the analysis. Note the effect of diet by dH_2 interaction is shown in Table 6.

Table 7. Selected microbial groups (log_{10} gene copies per ml rumen content) in the rumens of bulls fed the Napier grass (NG) or corn stover (CS) diets
(Mean values with their standard errors)

	Diet			
Item	NG	CS	SEM	*P*
Bacteria	11·91	12·01	0·036	0·06
Protozoa	10·13	10·53	0·071	0·001
Methanogens	8·40	8·63	0·046	0·003
Fungi	7·42	7·71	0·113	0·08
Selected groups of bacteria				
Prevotella spp.	11·60	11·72	0·044	0·07
Prevotella ruminicola	9·14	9·20	0·051	0·45
Selenomonas ruminantium	10·37	10·59	0·075	0·050
Ruminococcus albus	8·37	8·85	0·085	0·001
Ruminococcus flavefaciens	8·33	8·61	0·064	0·005
Fibrobacter succinogenes	9·54	9·61	0·047	0·28
Ruminococcus amylophilus	8·30	8·33	0·066	0·70

plant fibre that physically open plant tissues for subsequent colonisation by fibrolytic bacteria[39,40]. Feeding the CS diet resulted in greater numbers of bacteria, fungi, protozoa and *R. albus* and *R. flavefaciens* 16S and 18 rRNA gene copies, in comparison with feeding the NG diet. It seems that feeding the CS diet favoured the colonisation and growth of fibre degraders, such as fungi, *R. albus* and *R. flavefaciens*, and possibly some protozoa[41]. Fibre degradation generally increases H_2 production. Greater removal of H_2 by methanogens, resulting in lower dH_2 concentration, can favour the growth of H_2 producers[7]. However, we did not find relationships between dH_2 concentration and the abundance of fibre degraders; thus, the negative association of dH_2 concentration with fibre degradation found for the CS diet may be related to other fibrolytic micro-organisms not determined in this study.

Methanogens are the most important H_2 utilisers in the rumen, maintaining a low H_2 partial pressure. We observed a significant effect of the diet, which was not associated with dH_2, on methanogens 16S rRNA gene copies. These results indicate that fibre source was the main factor to influence growth of methanogens, and dH_2 concentration was probably a result of the rate of methanogenesis[7]. Some methanogens are symbiotic of protozoa[38,42,43], which were more abundant with the CS diet. It is possible that fibre in the CS diet provided more sites for the colonisation and growth of protozoa, which could have favoured the growth of methanogens.

Conclusion

Bulls fed the CS diet had greater DMI, NDF digestion and average daily gain than those fed the NG diet. Fibre in CS silage provided more sites for colonisation by rumen micro-organisms, which probably favoured the growth of fibre degraders and resulted in greater ruminal fibre degradation and increased dCH_4 concentration compared with NG silage. Such enhancement of methanogenesis with CS probably decreased ruminal dH_2 concentration, which might have contributed to increased fibre degradation and growth of fibrolytic micro-organisms. Fibre degradation of the NG diet was unrelated to dH_2

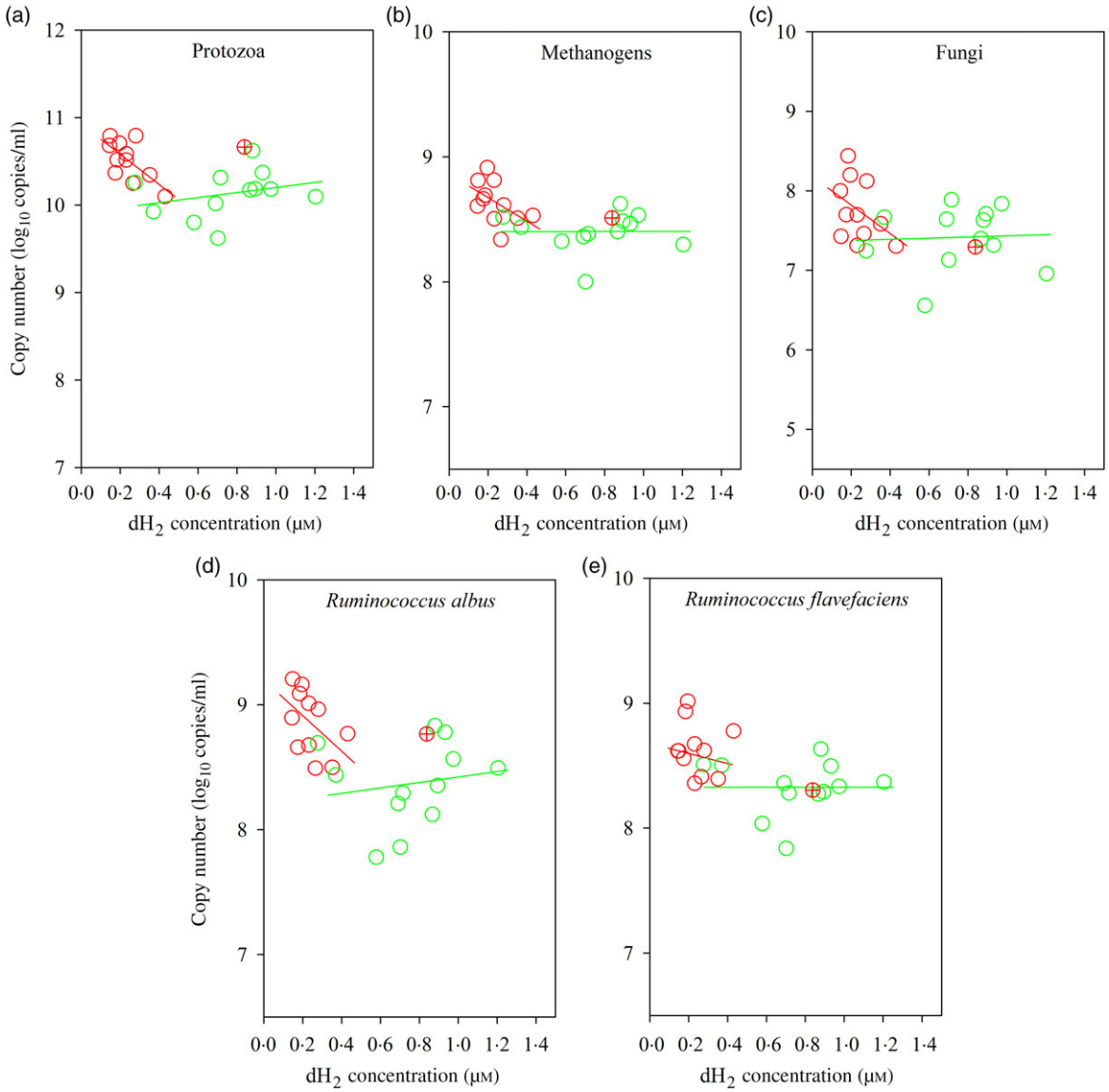

Fig. 4. Associations of ruminal 16S rRNA gene copies of selected microbial groups with ruminal dissolved hydrogen (dH_2) in bulls fed the Napier grass (NG) or corn stover (CS) diets. The green and red points and solid lines represent the best regression lines fitted using observations from the NG (*n* 12) and the CS (*n* 11) diets fitted separately. (a) NG (green): $y = 9{\cdot}91$ (±0·25; $P < 0{\cdot}001$) + 0·29 (±0·31; $P = 0{\cdot}37$) dH_2, R^2 0·08, *n* 12; CS (red): $y = 10{\cdot}93$ (±0·16; $P < 0{\cdot}001$) – 1·73 (±0·64; $P = 0{\cdot}02$) dH_2, R^2 0·45, *n* 11. (B) NG (green), $y = 8{\cdot}40$ (±0·15; $P < 0{\cdot}001$) + 0·003 (±0·19; $P = 0{\cdot}99$) dH_2, $R^2 < 0{\cdot}01$, *n* 12; CS (red): $y = 8{\cdot}86$ (±0·14; $P < 0{\cdot}001$) – 0·94 (±0·54; $P = 0{\cdot}12$) dH_2, $R^2 = 0{\cdot}25$, *n* 11. (C) NG (green) $y = 7{\cdot}36$ (±0·38; $P < 0{\cdot}001$) + 0·07 (±0·48; $P = 0{\cdot}89$) dH_2, $R^2 < 0{\cdot}01$, *n* 12; CS (red) $y = 8{\cdot}18$ (±0·34; $P < 0{\cdot}001$) – 1·80 (±1·33; $P = 0{\cdot}21$) dH_2, $R^2 = 0{\cdot}17$, *n* 11. (D) NG (green), $y = 8{\cdot}20$ (±0·32; $P < 0{\cdot}001$) + 0·22 (±0·40; $P = 0{\cdot}60$) dH_2, $R^2 = 0{\cdot}03$, *n* 12; CS (red): $y = 9{\cdot}20$ (±0·21; $P < 0{\cdot}001$) – 1·42 (±0·83; $P = 0{\cdot}12$) dH_2, $R^2 = 0{\cdot}25$, *n* 11. (E) NG (green): $y = 8{\cdot}33$ (±0·21; $P < 0{\cdot}001$) – 0·001 (±0·26; $P = 0{\cdot}99$) dH_2, $R^2 < 0{\cdot}01$, *n* 12; CS (red): $y = 8{\cdot}72$ (±0·20; $P < 0{\cdot}001$) – 0·38 (±0·79; $P = 0{\cdot}65$) dH_2, $R^2 = 0{\cdot}02$, *n* 11. Equations contain regression coefficients, standard errors and *P* values, and are expressed as regression coefficients with their standard errors. Each point represents an individual animal, with ⊕ being an outlier ($dH_2 = 0{\cdot}83$ μM) identified by the Dixon Q test and excluded from the analysis. Note the effect of diet by dH_2 interaction is shown in Table 6.

concentration and may have been influenced more by structural factors.

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the paper; R. W. and M. W. designed the research and had primary responsibility for the final content. All authors read and approved the final manuscript.

The authors declare that they have no conflicts of interest.

Supplementary material

For supplementary material referred to in this article, please visit https://doi.org/10.1017/S0007114520002962

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