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Full Length Research Paper

Laboratory cultured faecal inoculum as a substitute for fresh rumen inoculum for *in vitro* feed evaluation

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This study was carried out to: (1) Preserve whole rumen fluid (RF) or faecal fluid (FF) by culturing using simple techniques; and (2) Study the effect of substituting fresh RF with fresh or laboratory cultured FF as an alternative inoculum source for *in vitro* fermentation. Faeces and RF were collected from fistulated Jersey cows and cultured (RF or FF with salivary buffer containing maize stover (MS) and lucerne (1:1) at 39°C for three days). For fresh incubation or cultured incubation systems, RF or FF were mixed with salivary buffer containing MS (1 g) and incubated at 39°C for 72 h. True degradability (TD), total gas produced and gas kinetic parameters were determined after incubation. Exocellulase, endocellulase and hemicellulase specific activities (µg reducing sugar/mg crude protein) were assayed. Inocula were alive for 42 days but most active in the first 2 weeks. Cultured FF is a better substitute to fresh RF as shown by its exocellulase activity and TD, compared to differences (P<0.05) observed between fresh RF and FF for exocellulase activity (35 µg glucose/mg protein) and TD (56 g/kg). This study suggests that cultured FF could reduce the cost of experimentation without compromising the reliability of results.

Key words: Faecal inoculum, cellulases, *in vitro* fermentation, rumen fluid.

INTRODUCTION

Most *in vitro* digestibility studies rely on the fermentation of feed using buffered rumen fluid as an inoculum (De Boever et al. 2005; Menke et al., 1979; Mirzaei-Aghsaghali et al., 2007; Prates et al., 2010; Stern et al., 1997; Tilley and Terry, 1963). This is because rumen inoculum has been found to yield results which mimic those from *in vivo* studies (Brown et al., 2002; Gizzi et al., 1998). Despite the reliability of results obtained from using rumen fluid, there are still some major drawbacks which render it difficult to be exploited by researchers especially those in developing countries. These drawbacks include; the requirement of animals and surgical operation (fistula), constant care to avoid infection, long term maintenance (expensive), difficulties of travelling when animals are far from the laboratory and ethical issues of using these fistulated animals (Mauricio et al., 1999, 2001; Stern et al., 1997).Finding alternative inoculum (faecal fluid) which does not involve surgical procedures is necessary. Numerous studies have shown positive correlations between rumen and faecal inocula in *in vitro* digestibility studies from either bovine or sheep (Cone et al. 2002; El-Meadaway et al. 1998; Gonçalves and Borba, 1996; Mauricio et al. 2001; Mould et al. 2005). While many studies engage in comparing rumen and faecal inoculum incubations of the same animals (El-Meadaway et al., 1998; Váradyová et al., 2007), others compared faecal inocula from ruminants and hindgut fermenters (Denek and Can, 2007; Denek et al., 2008). Denek and Can (2007) reported that buffered faecal

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Abbreviations: RF, Rumen fluid; **FF,** faecal inoculums; **FI,** fresh incubations; **CI,** cultured incubations.

inoculum from hindgut fermenters had the highest potential for predicting *in vitro* dry matter degradability than faecal inocula from cattle and sheep.

Campbell et al. (2002) and Váradyová et al. (2007) estimated the fermentative capacity among lemur species using faecal inoculum. El-Meadaway et al. (1998) showed that there were minimal differences observed in *in vitro* dry matter degradability between rumen inoculum and 3% fresh faecal samples but values were lower when 6 to 15% fresh faecal inoculum was used. The results were similar to those obtained by El Shaer et al. (1987) Nsahlai and Umunna (1996). Collecting fresh samples every day for *in vitro* incubations can be expensive (Hervás et al., 2005) especially when the experimental animals are far from the laboratory. Studies showing the management of whole rumen or faecal inoculum in the laboratory for a few days using simpler techniques are not many. Different storage methods that have been investigated include: storage at low temperature or freezing over 24 h interval (Hervás et al., 2005; Prates et al., 2010), liquid nitrogen freezing (Prates et al., 2010) and cryoprotection (glycerol or dimethyl sulphur oxide) (Denek et al., 2010; Prates et al., 2010). These studies demonstrated that maximum fermentation was not greatly affected but the fermentation rate and initiation delay was affected.

Continuous *in vitro* culture systems mimicking rumen fermentation have demonstrated the importance of these systems in elucidating knowledge of the rumen ecosystem. However, the extensive use of these systems is limited by inherent problems in the technique as well as difficulties and complexities specific to each system (Vatthauer et al., 1970a, b). The cost of the system as well as running and maintenance cost is a major limitation especially for developing countries. Therefore managing faecal inoculum in the laboratory using simple and affordable techniques would reduce the cost of experimentation.

This study investigated: (1) The preservation of whole rumen fluid (RF) or faecal fluid (FF) in the laboratory by using a simple culturing technique; and (2) the possibilities of substituting fresh RF with fresh or cultured FF as an inoculum source. It was hypothesized that culturing rumen or faecal inoculum in the laboratory will not affect fibre fermentation. This potentially would reduce the cost incurred for daily collection of inocula.

MATERIALS AND METHODS

Chemical composition of maize stover

Maize (white maize hybrid PAN6479) was cultivated in spring at Ukulinga, University of KwaZulu-Natal South Africa. Maize stovers were harvested at grain dry stage, dried and milled through a 1-mm screen. Dry matter (DM) was determined by drying milled samples in a fan oven at 90°C overnight. Nitrogen was determined by micro-Kjeldahl method and crude protein calculated as N × 6.25 according to AOAC (1990). Neutral detergent fibre (NDF), acid detergent fibre and acid (ADF) detergent lignin (ADL) were determined as described by Van Soest et al. (1991). Hemicellulose was calculated by subtracting ADF from NDF and cellulose by subtracting ADL from ADF.

Experimental animals

Two rumen fistulated Jersey cows (400 kg ± 20 and 3.8 year old) were obtained from the Ukulinga Research Farm, University of KwaZulu Natal, SA. Fistulated cows (non-lactating) were fed *ad libitum* on hay (veld) in the morning before allowing them to graze on an open field where *Pennisetum clandestinum* (Kikuyu grass) and other grass hay were dominant in Autumn.

Inoculum collection and preparation

For enzyme assays, a modified procedure previously described by Smith et al. (1974) was employed when collecting rumen samples from fistulated cows. Rumen fluid (200 ml) was collected from both cows at the same time through a fistula before feeding in the morning, strained through four layers of cheese cloth (pre-warmed and flushed with CO_2) and treated immediately with 1500 µl of phyenylmethylsulfonyl fluoride (0.1 mM PMSF 1: 100 RF) to inhibit proteases from lysing enzymes of interest (Tilley and Terry, 1963). Faecal suspensions (faecal fluid) were made by diluting 150 g of faeces (collected before feeding from the rectum) into 150 ml of homogenization buffer (50 mM sodium-acetate buffer, 0.02% (m/v) NaN3, and 0.1 mM EDTA at pH 5.0) before straining through four layers of cheese cloth. It was treated with 150 µl PMSF before being transported in an airtight thermo flask maintained at 38°C to the laboratory. For *in vitro* digestibility studies, rumen fluid (RF) and faecal fluid (FF) for fresh incubations (FI) were collected as described for enzyme in the previous paragraph but for the exclusion of PMSF as well as replacing homogenization buffer with salivary buffer (buffer C) for faecal inoculum. Buffer C was made by titrating 2 L of warmed solution A (solution A, $NaHCO_3$ (19.60 g), Na_2HPO_4 (7.40 g), KCl(1.14 g), NaCl (0.94 g) and $MgCl.6H_2O$ (0.26 g), in 2 L distilled water) with 2 ml of Solution B (5.3 g $CaCl_2.2H_2O$ in 100 ml distilled water) just before use while continuously stirring to form a complete buffer solution. Ammonium sulphate (5.8 g) was dissolved in buffer C as a nitrogen supplier (Tilley and Terry, 1963). Buffer C was flushed with CO_2 till the whitish solution became clear. Carbon dioxide was generated in the airtight thermo flask while in the field by reacting calcium carbonate with sulphuric acid to maintain anaerobiosis.

Rumen fluid and faecal fluid culturing

Rumen fluid or FF for laboratory culturing was collected as described in the previous paragraph. Rumen fluid or faecal filtrate (198 ml) was pipetted into 402 ml of salivary buffer (flushed with CO_2 and placed in an incubator to equilibrate to 39°C for 1 h) containing 3 g lucerne and 3 g maize stover, which had been milled through a 1-mm sieve. Culture samples were flushed with CO_2 and incubated for 72 h at 38.5°C in airtight Duran® bottles (Duran Group, Germany) (4 L). After 72 h, cultured RF or FF were used as inocula for *in vitro* cultured incubations (CI) as well as analysed for cellulase activities.

Protein isolation was done using a slightly modified procedure described by Henry et al. (1975). Both RF and FF (100 ml) were used for protein isolation. Sample solutions in sealed centrifuge tubes were placed on a shaker for 30 min to facilitate bacteria detachment from fibres before centrifuging (6500 x*g*, 30min at 4°C) to settle particulate matter (Figure 1). The supernatant was centrifuged (30 000 x*g*, 15 min at 4°C) to settle bacteria cells.

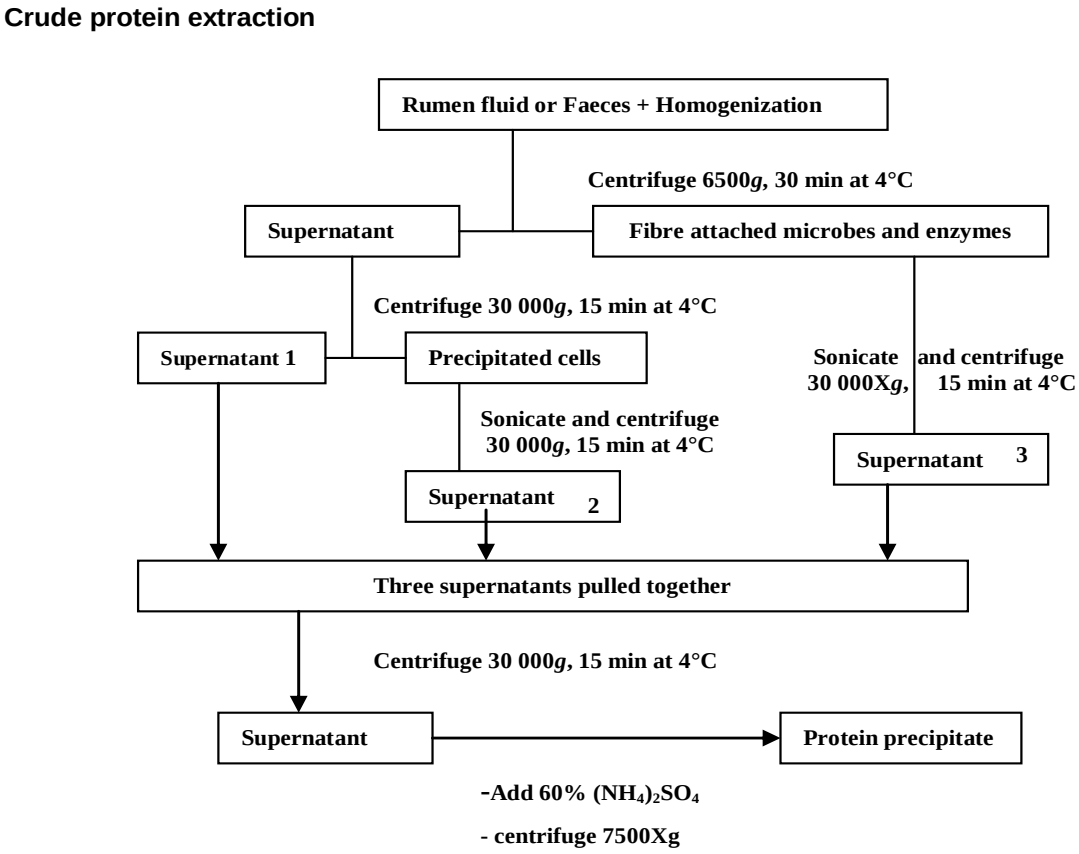

Figure 1. A flow chart for the precipitation of cellulase and hemicellulase from rumen fluid and faeces.

Settled particles and bacterial cells were dissolved in 10 ml and 5 ml of homogenization buffer respectively, sonicated (to lyse bacteria cells) and centrifuged (30 000x*g*, 15 min at 4°C). Supernatants of the three different steps were pooled (Figure 1) and centrifuged (30 000 x*g*, 15 min at 4°C) to settle any unlysed cells. Ammonium sulphate (60% (m/v) $(NH_4)_2SO_4$) was dissolved in the sample solution to facilitate protein precipitation before centrifuging (7000 x*g*, 15 min at 4°C). The precipitate was dissolved in 10 ml of storage buffer (20 mM sodium acetate, 0.02 % (m/v) NaN_3, and 0.1 mM EDTA at pH 5.0) before dialyzing. Each sample solution was pipetted into a dialysis membrane and immersed in a 2 L storage buffer solution overnight (12 h). Dialyzed sample solutions were concentrated using polyethylene glycol 20 000.

Determination of protein concentrations

Bradford (1976) dye-binding assay was used to determine crude protein concentrations. A standard curve for a micro assay was prepared with bovine serum albumin (BSA) concentrations of 0, 5, 10, 20, 30 and 40 µg/100 µl of reaction buffer (20 mM sodium acetate, 0.02% (m/v) NaN_3 and 0.1 mM EDTA at pH 5.0). 900 µl of Bradford reagent (600 mg Coomassie Brilliant Blue G-250 dissolved in 1 L of 2% perchloric acid and filtered through Whatman number 1 filter paper) was pipetted into the standard solution (100 µl) and absorbance read at 595 nm after 3 min. For the unknown protein sample solutions, 5 µl was diluted into 100 µl of the reaction buffer before adding Bradford reagent (900 µl) and allowed to stand for 3 min. Each assay was performed in triplicates and absorbance was measured at 595 nm. Unknown protein concentrations were determined from the standard curve.

Enzyme assays

Exocellulase activity (crystalline cellulase)

Exocellulase activity (crystalline cellulase) was assayed following a slightly modified version of the method described by Gerrit et al. (1984) to suit the crude protein assays. Crystalline cellulase activity was measured by pipetting 0.5 ml of 1% (m/v) crystalline cellulose in the reaction buffer into 0.5 ml of crude protein solution obtained from rumen fluid or faeces and incubated for 72 h at 39°C (standard assay condition). The enzyme reaction was stopped by boiling at 100°C, following which the reaction mixture was centrifuged (6000xg, 5 min at 4°C) and 400 µl samples analysed for reducing sugars using 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959).

Endocellulase (carboxymethyl cellulase) activity

Endocellulase (carboxymethyl cellulase) activity was investigated as described by Gerrit et al. (1984). The reaction mixture contained 0.5 ml of 0.5% (m/v) carboxymethyl cellulose (CMC) in the reaction buffer at pH 5.5 and 0.5 ml of crude enzyme. The enzyme reaction was stopped by boiling at 100°C after incubating at 39°C for 2 h. Samples were centrifuged at 6000xg at RT for 5 min, then 400 µl of the reaction mixture was analysed for reducing sugars using DNS method. Each system was represented by three samples each of which was analysed in triplicates. Specific enzyme activity was

measured in µg of glucose/µg protein.

Hemicellulase (xylanase) activity

A modified procedure described by Seyis and Aksoz (2005) to suit crude protein assays was used to assay xylanase activity. The assay was carried out by pipetting 0.6 ml of 0.1% (m/v) xylan solution in the reaction buffer (pH 5.0) into 0.4 ml of crude protein solution and incubating at 39°C for 1 h. The reaction was stopped with 100 µl of 200 mM NaHCO3, centrifuged (at 6000xg for 5 min) and analysed for reducing sugars using the DNS method. Each ecosystem was represented by three samples each of which was analysed in triplicates. Specific activities of the above enzymes were defined as µg of xylose/ mg crude protein.

Quantification of reducing sugars

A standard curve of micrograms of reducing sugars (glucose or xylose) against absorbance at 540 nm was plotted as described by Wood and Bhat (1988). Dinitrosalicylic acid reagent, 600 µl (0.001 M sodium metabisulphate, 0.708 M potassium sodium tatrate, 0.25 M sodium hydroxide, and 0.021 M phenol) was pipetted into 400 µl of the sample solution and boiled for 5 min. The reaction mixture was then cooled under running tap water and the absorbance measured at 540 nm. The absorbance values were translated into reducing sugars using the standard curve.

***In vitro* digestibility**

In vitro gas production

The automated gas production method applied in this study was described by Pell and Schofield for the computerized pressure transducer system. Maize stover (milled through a 1-mm sieve) was weighed (1 g) and transferred into a 250 ml Duran® bottles containing 67 ml of salivary buffer (buffer C) which had been flushed with CO_2 (to avoid the consumption of initial CO_2 produced from fermentation by the buffer). The substrate-buffer mixture was placed in the incubator to equilibrate to 39°C (for 1 h) before inoculation. Rumen fluid or FF (33 ml) was transferred into Duran® bottles under a stream of CO_2. For FI incubations fresh RF and FF were used while cultured RF and FF were used for CI. After inoculation, samples were transferred into an incubator and pressure logging was done every 20 min for 72 h at 39°C. The negative control was the same mixture as above with no substrate but the positive control was lucerne and grass hay (1:1) incubated with rumen fluid (to correct for variations observed in repeats if any). Substrate incubation with each inoculum type was replicated three times with five pseudo repeats. The pH of all incubation systems were monitored before and after every experiment. The terminal pressure data was converted to gas volumes (ml) using a predetermined calibration equation (Schofield et al., 1994). *In vitro* gas values were fitted into the exponential equation:

$$GP = \frac{Maxp}{1 + e^{[2 + 4C(lt - t)]}} \quad (1)$$

(Campos et al., 2004)

$$GP = \frac{A}{1 + \exp[2 + 4a(lt - t)]} + \frac{B}{1 + \exp[2 + 4b(lt - t)]} \quad (2)$$

$$PF = \frac{TD}{Maxp} \quad (3)$$

$$DEF = \frac{TD}{(T_{1/2} \times V_{1/2})} = \frac{2PF}{T_{1/2}} \quad (4)$$

(Ouda and Nsahlai, 2009)

µ = Maxp*C

where GP is the total gas volume (ml) at time t, maxp is maximum gas production, C is the overall degradable rate, A and B are the gas volume (ml) from fast (soluble sugars and starch) and slowly (cellulose and hemicellulose) degradable fractions, respectively, a_1 and b_1 are the degradation rates (h^{-1}) for fast and slowly degradable digestion fractions, respectively, and it is the bacteria colonization or lag time (h). PF= partitioning factor, TD= truly degradable fraction of feed, DEF = degradability efficiency factor, $V_{1/2}$ = half GP, µ = gas produced at the point of inflection and $T_{1/2}$ = time taken to produce half maximum gas.

Monitoring whole faecal inoculum activity over time

The procedure was the same as described for gas production with a few modifications. The incubation time was changed to 7 days. After every 7 days, the gas was stopped and 33 ml was used to inoculate another system containing fresh feed. This procedure continued at 7 days intervals until day 42.

***In vitro* degradability and volatile fatty acids**

True degradability was determined according to Tilley and Terry (1963), and modified by Cottyn and Boucque (1968), in which the second stage (pepsin digestion) was substituted with neutral detergent extraction. After 72 h incubation as described for gas production above, samples were centrifuged (8000xg, 15 min, 4°C) and residues dried for 72 h in a fan oven at 70°C. The difference in mass between the incubated material and the residue equals the apparent degraded fraction (APD) of MS. The residue was refluxed in neutral detergent solution and the residue (NDF) dried (fan oven, 70°C for 72 h). Truly degraded fraction (TD) was calculated by subtracting the mass of NDF from that of the incubated material. The difference between APD and TD was recorded as microbial yield.

Five millilitres filtrate was transferred onto 1 ml of 25% metaphosphoric acid solution and allowed to stand in ice for 30 min before centrifuging (8000xg, 20 min at 4°C) (Cottyn and Boucque, 1968). The filtrate (2 ml) was filtered through a syringe filter (0.45 µm pore from SUPELCO, USA) into a 2 ml clear ABC Screw Top Vial from SUPELCO, USA. Vials were then transferred into an automatic sampler (HT280 from Brescia, Italy) linked to a Gas Chromatograph (YL6100GC, Young Lin, Korea) for analysis. A temperature-programmed cycle from 90 to 120°C rising by 5°C was applied in the column (BPX70x30x0.25x0.25). The injector block temperature was maintained at 260°C with nitrogen as the carrier gas (60 ml/min). Hydrogen and air flow rate to the detector (flame ionization detector) were 60 and 300 ml/min respectively. The injection volume was 1 µl/split 1:80. The theoretical calculations of CO_2 and methane (CH4) were estimated as described by Groot et al. (1998) based on the stoichiometric balance of the fermentation of glucose to propionic acid (PP), acetic acid (Acet), butyric acid (nB), iso-butyric acid (iB), CO_2 and methane (Czerkawski 1978;

Table 1. The specific activities of exocellulase, endocellulase and hemicellulase of crude protein extracts from fresh and cultured systems of rumen fluid and faecal suspension.

Enzyme source	Hemicellulase specific activity[1]	Endocellulase specific activity[2]	Exocellulase specific activity[2]
Fresh			
Rumen fluid	42.91[a]	41.60[a]	23.52[a]
Faecal fluid	6.98[c]	6.13[c]	15.11[b]
Cultured			
Rumen fluid	8.96[c]	18.65[b]	12.33[b]
Faecal fluid	18.83[b]	5.65[c]	23.47[a]
SEM	2.014	3.123	5.381
P- value	**	**	**

[1]= µg xylose /mg crude protein, [2] = µg glucose/mg crude protein, **P< 0.001, [a, b, c] Numbers with different superscripts in the same column were different (P<0.05).

Van Soest, 1994).

$$CO_2 = \frac{Acet}{2} + \frac{PP3}{4} + \frac{(nB+iB)}{2} \quad (5)$$

$$CH_4 = Acet + 2(nB+iB) - CO_2 \quad (6)$$

Statistical analysis

In vitro enzyme and digestibility estimates derived from RF and FF inoculum were subjected to analyses of Variance (ANOVA) using the general linear model of SAS (2002) based on the statistical model:

$Y_{ijk} = \mu + E_i + Sj + (ES)_{ij} + e_{ij}$;

where Y is the individual observation, µ is the overall mean, E is the effect of the inoculum source, S is the effect of pre-incubated inoculum, (ES) is the interaction effect of inoculum and pre-incubated inoculum, and e is the random variation. Student Newman- Keuls' test was used to compare means.

RESULTS

Chemical composition of whole maize stover

Chemical analyses of maize stover showed 92.5, 89.9, 87.8, 54.3, 7.3, 33.5, 47.0 and 5.5% for dry matter, organic matter, NDF, ADF, ADL, cellulose, hemicellulose and crude proteins, respectively.

Enzyme assays

Exocellulase specific activity differed (P<0.001) between fresh RF and FF. Fresh RF specific activity was higher than that observed in FF (Table 1). Cultured RF and FF differed in their exocellulase specific activity (*P*<0.001). Faecal fluid exocellulase specific activity was higher than that of RF in cultured systems.

Endocellulase specific activity differed (P<0.001) in both FI and CI. Endocellulase specific activity of RF was higher than that of FF in both FI and IC. Fresh RF and FF differed (P<0.001) in their hemicellulase specific activities. Rumen fluid hemicellulytic activity was higher than that of FF (Table 1). Cultured RF and FF differed (P<0.001) in their hemicellulase specific activity. FF hemicellulase specific activity was higher than that of RF in cultured systems.

In vitro digestibility

Fermentation systems were all active as indicated by a small drop in pH after incubations for both FI and CI systems. In the FI system, the pH drop in RF (7.38 to 6.62) was slightly higher than that of FF (pH 7.21 to 6.97) while pH changes between the cultured systems were not significant. The APD of MS did not differ between RF and FF in both FI and CI (Table 2).

True degradability was slightly higher (P<0.001) in RF than FF in both FI and CI systems. Microbial yield was significantly different (*P*<0.001) in both FI and CI systems. The highest microbial yield was observed in RF cultured medium. The total gas production (GP) from MS differed (P<0.001) between RF and FF for both FI and CI systems (Table 2). Total gas production from MS was higher in RF than FF for both FI and CI incubations. For the 24 h gas measurement, there was no significant difference between RF and FF in the CI systems. There was no difference in the degradation rate of MS between RF and FF for both FI and CI systems. The time taken to produce half GP ($T_{1/2}$) and the gas produced at the point (hour) of inflection (U) were also not different between RF and FF for both incubation systems. The partitioning factor (PF) differed (P<0.01) between RF and FF. Partitioning factor was highest in cultured FF followed by fresh FF. The lowest PF was observed in cultured RF. Degradability efficiency factor did not differ between RF and FF for both FI and CI systems. Total SCFA differed

Table 2. *In vitro* digestibility of Maize stover by fresh and cultured rumen or faecal inoculums.

Digestibility parameter	Fresh inoculum		Cultured inoculum		SEM	P-value
	Rumen fluid	Faecal fluid	Rumen fluid	Faecal fluid		
APD (g/kg)	524	519	549	549	20.6	ns
TDeg (g/kg)	719^{b}	663^{c}	745^{a}	681^{b}	6.3	**
MY (g/kg)	195	144	196	132	29.2	**
GP						
(ml/g)^{24}	100^{c}	82^{b}	60^{a}	57^{a}	5.1	**
(ml/g)^{72}	150^{a}	136^{a}	144^{a}	116^{b}	4.2	**
C (h^{-1})	0.027	0.024	0.024	0.028	0.0010	ns
$T_{1/2}$ (h)	21.43	26.59	26.31	24.03	0.720	ns
µ (ml)	4.15	3.15	3.51	3.29	1.213	ns
PF	4.8^{b}	4.9^{b}	4.2^{ab}	5.9^{a}	0.11	**
DEF	0.46	0.37	0.44	0.50	0.032	ns

*= P< 0.05, ** = P<0.01, ns = non-significant, $^{24 \text{ or } 72}$ = Total gas volume (GP) produced after 24 and 72 h of incubation respectively, nm= not measurable, PF= partitioning factor, DEF = degradability efficiency, $T_{1/2}$= time taken to produced half the total gas, µ = gas at the point of inflection, APD=apparent degradability, TD= True degradability, C= rate of maize stover fermentation, MY = microbial yield. $^{a, b, c}$ Numbers with different superscripts in the same row were different (P<0.05).

(P <0.01) between RF and FF. Total SCFA was highest in FI for RF, followed by FF in CI then RF in CI and lastly FF in FI (Table 3). The Molar proportions of acetic acid (Acet), propionic acid (PP) and n-butyric (nB) acid were different (P<0.01) between RF and FF. Generally, Acet had the highest molar proportions followed by PP and nB in both FI and CI systems. The highest molar proportion of Acet was observed in RF of FI. Propionic acid was highest in FF of CI. Methane and CO_2 productions were higher (P<0.01) in FI than CI systems. The highest methane and CO_2 production were observed in Fresh RF and the lowest in cultured FF.

Gas production kinetics

Gas volumes produced from rapidly degradable fraction (A) of MS did not differed (P<0.01) between RF and FF for both FI and CI systems as well as the gas produced from slowly degradable fraction (B) (Table 4). The gas produced from B was generally higher than that of A in all incubations. The fermentation rates of A (a_1) were similar between RF and FF for both FI and CI systems. The rate of fermentation of B (b_1) differed (P<0.01) between RF and FF for both FI and CI systems. The fastest b_1 was observed in fresh RF followed by cultured FF and thirdly fresh FF and cultured RF. The lag time (lt) was generally longer (*P*<0.01) for CI than FI systems. The longest lt was observed in FF for FI and the shortest in RF for FI systems.

Faecal fluid activity over time

Total gas production decreased (P<0.001) with time (Table 5). The highest GP was observed after 7 days (191ml) and the least after 21 days. Interestingly, GP at day 42 was higher than that of day 35. The second highest value was recorded on day 14. The overall rate of gas production (C) from MS was highest on day 21 and lowest on day 7. The results showed that the overall rate of MS fermentation increases with time. Apart from day 21, a_1 and b_1 were not different for the different incubation days. Results also showed that lt decreases (P<0.001) with time. The gas produced at the point of inflection (µ) also increased with time. Both A and B from MS fermentation generally decreased over time (P<0.001).

DISCUSSION

Many studies have shown that FF is a possible substitute for RF but insists on more research to improve on its value (Dixon et al., 1982; Holden, 1999; Mabjeesh et al., 2000; Mauricio et al., 2001; Mould et al., 2005; Omed et al., 2000; Tufarelli et al., 2010). The percentage difference of digestibility parameters obtained when fresh RF was used as an inoculum compared to fresh FF is still a limiting factor. The time spent on collecting RF or FF for daily incubations is not cost effective. That is why one of the objectives of this study was to preserve whole rumen or faecal inoculum in the laboratory for extended periods using a very simple laboratory technique. The second objective was to investigate the possibilities of substituting fresh RF with fresh or cultured FF for *in vitro* evaluation of forages. Activities of exocellulase, endocellulase and hemicellulase were higher in FI for RF than FF. This was probably due to higher microbial population in the liquid phase of RF than in the strained

Table 3. Short chain fatty acid and gas production calculated from VFA.

Digestibility parameter	Fresh inoculum		Cultured inoculum		SEM	P-value
	Rumen fluid	Faecal fluid	Rumen fluid	Faecal fluid		
SCFA						
Total (µM)	982[a]	541[c]	570[c]	612[b]	47.7	**
Molar proportion						
Acetic acid	0.732[a]	0.605[b]	0.625[b]	0.609[b]	0.0321	**
Propionic acid	0.204[c]	0.337[b]	0.329[b]	0.37[a]	0.0143	**
n-Butyric acid	0.054	0.056	0.044	0.021	0.0051	**
iso-Butyric acid	0.001	nm	nm	nm	0.0020	**
Iso-valeric acid	0.003	0.003	0.001	ns	0.0001	ns
Carbon dioxide	0.497[a]	0.470[b]	0.461[b]	0.428[c]	0.0071	**
Methane	0.342[a]	0.245[b]	0.252[b]	0.222[c]	0.0101	**

** = P<0.01, ns = non significant, SCFA = short chain fatty acids, nm = not measured (signal was too low), [a, b, c] Numbers with different superscripts in the same row were different (P<0.05).

Table 4. Gas parameters of maize stover when ncubated with fresh or cultured inocula.

Inoculum	A (ml)	a_1 (h^{-1})	B (ml)	b_1 (h^{-1})	lt (h)
Fresh					
Rumen fluid	55.6	0.107	94.7	0.028	2.3
Faecal fluid	45.8	0.095	91.1	0.023	4.6
Cultured					
Rumen fluid	41.6	0.076	99.4	0.023	4.0
Faecal fluid	31.1	0.085	85.1	0.027	4.3
SEM	0.00	0.0001	3.41	0.0001	0.00
P-value	ns	ns	ns	**	***

**=P<0.01, ***=P<0.001, ns= non-significant, a and b are the gas volumes (ml) from fast (soluble sugars and starch) and slowly (cellulose and hemicellulose) degradable fractions, respectively, a1 and b1 are the degradation rates (h−1) for fast and slowly degradable fractions, respectively, and lt is the bacteria colonization or lag time (h).

faecal inoculum (Mauricio et al., 2001). Although the quantity of crude protein extracted from equal volumes was higher for FF than that of RF, cellulolytic activity remained lower in FI incubations. The higher crude protein in FF was associated with endogenous protein contamination. After culturing FF for 72 h, its cellulolytic (exocellulase and endocellulase) activity was relatively higher than that of cultured RF. This implies that culturing FF with MS might have increased the initial microbial population, decreases the amount of endogenous contaminants, selects mostly microbes that can survive on MS and allowed for the expression of more active fibrolytic enzymes relative to the type of substrate available. A shorter retention time in the hindgut of cows has always been associated with lower fermentation (Hidayat et al., 1993). However, the rate at which fermentation occurs in the hindgut is unresolved although a comparative study on bovine rumen fluid and faeces by Mauricio et al. (2001) showed higher rates of gas production in RF than FF. If the retention time is very short in the hindgut of ruminants, the microbes that inhabit this chamber must be able to harness energy from the fast moving fibrous feed as fast as possible in order to survive. Therefore, it is possible that hindgut cellulolytic microbes have a higher fibrolytic potential but limited by their numbers as demonstrated by our results when FI was compared with CI systems. Therefore, cultured FF can be a better substitute to fresh RF compared to fresh FF as demonstrated by its exocellulase activity. The difference in exocellulase specific activity between fresh RF and cultured FF (0.4%) was smaller compared to the difference between fresh RF and fresh FF (33%).

Apparent degradability did not differ between RF and FF incubations while TD differed. True degradability correlation between RF and FF was relatively high (r^2 = 0.71) for FI incubations. Culturing both inoculum increased TD for both RF and FF by 2.6 and 1.6%, respectively. This increment in TD was associated to higher fibrolytic microbial population influenced by the culturing conditions (high fibre and lucerne providing proteins to boost microbial growth) (Hobson, 1971; Kern et al., 1974; Mauricio et al., 2001). Interestingly, culturing slightly decreased the disparity in TD observed between RF and FF in FI and CI from 4.5 to 4.0%. However, this was contrary to the results obtained by Akhter et al. (1995) who reported a 40% deviation of TD between RF and FF. The difference in TD between fresh RF and cultured FF (7%) is much smaller when compared to the difference between fresh RF and fresh FF (14%), suggesting that, laboratory culturing of FF may reduce the error margin when using FF as a substitute for fresh RF than fresh FF.

Total gas production was generally higher in RF than FF in both FI (Kern et al., 1974) and CI incubations. Although less microbial diversity and absence of protozoa in FF has been associated with low and slow rate of gas

Table 5. Gas production from maize stover over a period using faecal inoculum from cow.

Days	GP	C	A	a1	B	b1	lt	T1/2	μ
7	191.0	0.020	81.0	0.059	110.0	0.013	10.0	45.0	2.7
14	169.8	0.015	72.5	0.070	97.3	0.013	9.8	43.6	2.5
21	108.2	0.045	25.3	0.124	82.8	0.028	5.4	16.5	4.8
28	122.3	0.025	74.3	0.055	48.0	0.016	7.8	28.2	3.0
35	109.1	0.024	65.0	0.061	44.1	0.016	7.0	28.2	2.5
42	130.0	0.026	77.3	0.063	52.6	0.018	8.5	27.7	3.3
SEM	19.80	0.0103	13.71	0.0102	6.34	0.0111	1.21	7.123	0.60
P-value	**	ns	**	ns	**	ns	**	**	ns

** = P < 0.01, GP= total gas produced over time, ns= non-significant, a and b are the gas volumes (ml) from fast (soluble sugars and starch) and slowly (cellulose and hemicellulose) degradable fractions, respectively, C= overall rate of maize stover breakdown, a_1 and b_1 are the degradation rates (h^{-1}) for fast and slowly degradable fractions, respectively, $T_{1/2}$= time taken to produced half the total gas, μ = gas at the point of inflection and lt is the bacteria colonization or lag time (h).

production (Hidayat et al., 1993), it is likely that lower fibrolytic microbial population initially also plays a major role (Cone et al., 2002). The GP produced from MS fermentation by RF or FF was higher in the FI systems than CI systems unlike TD which increased in CI systems. The decrease in GP and an increase in TD imply conversion of energy lost in the form of gas into other metabolic substrates such as PP, nB or Acet as shown by the results. The difference in GP produced between RF and FF in the FI incubations after 24 and 74 h were 10.4 and 3.7%, respectively. This implies that fermentation was much slower in the FF system initially but gradually increases as the population of microbes increases with time (Váradyová et al., 2005). These results were similar to those obtained in studies comparing rumen and faecal inoculum from sheep (Grings et al., 2005; Kiran and Krishnamoorthy, 2007). For the CI systems, the difference in GP production between RF and FF at 24 h was much smaller (2.5%) than observed in the FI systems (10.4%). This was linked to an increase in fibrolytic microbial population after culturing, hence increasing the rate of fermentation. This result was similar to that obtained by Rymer and Givens (2000) where FF was pre-incubated overnight before incubation to enrich initial microbial number. They concluded that GP production profile parameters produced by RF can be extrapolated from FF gas production profile. Therefore, the lower fermentation in the hindgut may be associated to low microbial populations, poorer feed quality, shorter retention time and the absence of protozoa rather than the fibrolytic efficiency of the microbes present .Although the overall rate of fermentation of MS was not statistically different between RF and FF for both FI and CI system, a small increase in C for FF in CI was noticeable. This implies that C was enhanced by culturing. The overall rate from FF in CI was 5% higher than that of RF in FI , hence more evidence for substituting RF with cultured FF than fresh FF (C for FF was 12% less than that of RF in FI). The gas produced at the point of inflection (U) showed no differences upon analysis. This suggested their similarities in microbial activity after establishment. Therefore, culturing may improve microbial population as well as eliminates dietary differences often observed between the rumen and hindgut. Partitioning factor and degradability efficiency factor (DEF) were highest for FF in CI systems compared to all the other inocula (RF in CI or RF and FF in FI). This is an indication of a higher efficiency of microbial protein synthesis as demonstrated by other researchers (Jackson et al., 2010; Krishnamoorthy and Robinson, 2010). High PF and DEF showed that FF can generate data that are good enough in formulating ruminant feeds (Váradyová et al., 2005). The high PF for FF in CI shows that culturing improves the fibrolytic competence of cultured inoculum, by concentrating microbes that can digest MS hence increasing fibrolytic microbial population as well as the overall fermentation rate. High PF has also been associated with high feed intake although other studies (Mauricio et al., 2001) have shown that it is not a good parameter to be used solely in feed formulation. The total SCFA difference between fresh RF and cultured FF (37%) is much smaller when compared to the deference observed between fresh RF and fresh FF (48%). This suggests that culturing FF improves MS fermentation. The highest molar proportion of propionic acid was observed in CI systems for FF. This was probably due to its low GP (CO_2 and methane) and high TD. This implies that acetate: propionate was higher than in all other systems.

There were no differences in the gas produced from A or B between RF and FF in the FI incubations. Therefore, using gas parameters from FF to estimate digestibility is plausible. Váradyová et al. (2005) were not able to establish strong relationship between RF and FF gas parameters as shown in this study. However, the rate at which A or B was fermented was higher for RF than FF in FI incubations. These results were similar to those carried on sheep by Mauricio et al. (2001). Culturing the inocula saw the production of higher gas from B by RF

than FF which surprisingly had a higher fermentation rate. This implies that MS was converted to other metabolic substrates apart from gas. This is an indication that culturing faecal microbes under suitable anaerobic conditions before application improves initial microbial population, microbial specificity and fermentation rate. It is positive to think that microbes in the hindgut can be very effective in fermenting high roughage feeds at a higher rate but are limited by their initial microbial numbers during *in vitro* incubation. This is because passage rate at the hindgut might be relatively high; therefore microbes have a very limited time to extract nutrients so as to survive in that ecosystem. The lag time (lt) for FF was longer than that of RF in both FI and CI incubations. However, lt for RF was highly correlated (r^2 = 0.8) to that of FF in cultured incubations. This implies that culturing decreases microbial colonization time.

Monitoring FF microbial activities in the laboratory over a period showed that FF inoculum was active for 42 days as demonstrated by GP measurements. Generally, GP decreases over time. Total gas production was highest after week 1 but decreased by 11, 43, 36, 43 and 31% for week 2, 3, 4 and 5 respectively. Decreased GP was associated to lower microbial activity which implies that FF fermentation potential decreases over time. However, this is not conclusive as TD increases with decreased GP as seen in CI systems after 72 h. This suggests that the microbial systems may still be efficient in hydrolyzing carbohydrates into metabolites (SCFA) other than gases. The gas parameters, C, a1, b1 and lt were not significantly different between the different weeks. Therefore, culturing of faecal inoculum under suitable condition would minimize the large error margin often observed between fresh FF and RF. Apart from reducing the disparity of the results obtained between FF and RF, the cost spent on transportation for daily collection of faecal inoculum (especially when experimental animals are many miles away from the laboratory), will be reduced as cultured FF were still very active in week 1. Cost reduction will be a great relief especially for the developing countries where funds are limited for buying and management of sophisticated *in vitro* Continues Culture Systems.

Conclusion

Faecal inoculum is a potential substitute of rumen inoculum for *in vitro* feed evaluation as demonstrated by the small differences observed in their true digestibility and gas production. Faecal inoculum is a better substitute for fresh RF when cultured before application as demonstrated by the smaller differences observed in their degradability parameters. Culturing whole rumen fluid or faecal fluid was successful for three days with an increase in fermentation rate and less than 5% loss in activity. Monitoring inoculum activities over time shows that whole FF was active for six weeks with week 1 being the best inoculum source for feed evaluation. However, the lag time still remains a major problem to resolve as it did increase slightly with culturing. The drop in GP and an increased in TD observed in cultured ecosystems demands for more research on the microbial variety which can possible play a vital role as a feed additive in animal production.

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