Fermentation Characteristics and Microbial Protein Synthesis in an *In Vitro* System Using Cassava, Rice Straw and Dried Ruzi Grass as Substrates

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ABSTRACT : An *in vitro* gas production system was used to investigate the influence of various substrate mixtures on a natural mix of rumen microbes by measurement of fermentation end-products. The treatments were combinations of cassava (15.0, 30.0 and 45.0%) with different roughage sources (ruzi grass, rice straw or urea treated rice straw). Microbial biomass, net ^{15}N incorporation into cells, volatile fatty acid production, gas volume and rate of gas production increased linearly with increasing levels of cassava inclusion. There was also an effect of roughage source, with rice straw being associated with the lowest values for most parameters whilst similar values were obtained for ruzi grass and urea treated rice straw. The results suggest that microbial growth and fermentation rate increase as a function of readily available carbohydrate in the substrate mixture. A strong linear relationship between ^{15}N enrichment, total volatile fatty acid production and gas production kinetics support the suggestion of the use of the *in vitro* gas production system as a tool for screening feedstuffs as an initial stage of feed evaluation. (***Asian-Aus. J. Anim. Sci. 2000. Vol. 13, No. 8 : 1084-1093***)

Key Words : *In Vitro* Gas Production, Fermentation, Microbial Protein Synthesis, Rumen

INTRODUCTION

The digestion of feedstuffs by rumen microbes produces VFAs, CO_2, CH_4 and traces of H_2 as a by-product of cell growth. The microbes and VFAs are metabolized to meet the energy and protein need of the host animal, but gases are eructated. Measurement of gas evolution *in vitro* can provide a valuable quantitative information simulating the kinetics of rumen digestion (Menke et al., 1979; Menke and Steingass, 1988; Khazaal et al., 1993). The technique is well known as being simple to use, reliable and relatively fast and cheap. Dietary inclusion of cassava has been shown to improve animal weight gain and milk production without any adverse effects on rumen function or milk fat output (Brigstocke et al., 1981; Etman et al., 1993; Sommart et al., 1997a, b). However, direct experimental on the quantitative relationships between microbial protein synthesis and fermentation end-products from highly ruminal degradable starch sources such as cassava accompanied with low quality roughage (rice straw) is lacking.

Therefore, the objective of this experiment was to study the potential for using cassava as a source of nonstructural carbohydrate and its effects on rumen microbial fermentation efficiency when accompanied by different sources of roughage in an *in vitro* gas production system. ^{15}N urea was used as the method of assessing microbial protein synthesis.

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Received May 20, 1999; Accepted November 30, 1999

MATERIALS AND METHODS

Dietary substrate treatments

Substrates were formulated to contain three sources of roughage [rice straw; 5% urea treated rice straw (w/w) and dried ruzi grass (*Brachiria ruziziensis*). The urea treated rice straw was made by the method of according to Wanapat et al. (1996) and the dried ruzi grass was from a crop 45 d old, cut and oven dried at 60℃. The roughages were fed in combination with 3 levels of cassava. All treatments had an intended final crude protein content of 15.0% DM, the treatments being made iso-nitrogenous by the addition of urea. The details of feed ingredient and inclusions and chemical composition (DM, Ash, CP, EE, NDF; AOAC, 1980; Goering and Van Soest, 1970; Van Soest et al., 1991) are shown in table 1.

Experimental design

The experimental design was a 3×3 factorial arrangement in a randomised complete block design using duplicate sets of 3 different incubations as blocks (6 replicates per treatment). The main treatment factors were three levels of cassava (150, 300, 450 g/kg DM) and three types of roughage (rice straw, urea treated straw, dried ruzi grass).

Animals

Two fistulated non-lactating Jersey cows (weighing about 500 kg) at The University of Newcastle upon Tyne, Cockle Park Farm were used as the source of rumen inoculum. The animals were maintained on rice

Table 1. Feed formulation and feed raw ingredients (% DM) used in the *in vitro* experiment

Fibre source	Rice straw			Urea treated rice straw			Dried ruzi grass		
Cassava levels	15.0%	30.0%	45.0%	15.0%	30.0%	45.0%	15.0%	30.0%	45.0%
CSM[1]	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00
Cassava	15.00	30.00	45.00	15.00	30.00	45.00	15.00	30.00	45.00
Urea	2.08	2.08	2.08	0.92	1.18	1.43	0.77	1.05	1.35
Rice straw	67.92	52.92	37.92	-	-	-	-	-	-
UTS[2]	-	-	-	69.08	53.82	38.57	-	-	-
Dried ruzi grass	-	-	-	-	-	-	69.23	53.95	38.65
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Chemical analysis									
DM, %	93.3	92.7	92.5	94.3	93.9	92.7	93.2	92.8	92.0
Ash, % DM	11.2	9.4	7.6	12.0	9.9	8.2	8.1	6.8	5.8
NDF, % DM	62.7	51.0	41.1	63.7	53.7	44.3	63.2	49.1	39.9
EE, % DM	1.5	1.6	1.7	1.8	1.6	2.1	2.0	2.1	1.8
CP, % DM	11.5	12.0	10.3	10.5	9.5	11.3	11.8	12.4	11.5
NSC[3], % DM	13.1	25.9	39.3	11.9	25.3	34.1	14.9	29.5	40.9
ME, MJ/kg[4]	8.50	9.40	10.30	8.60	9.40	10.30	9.40	10.10	10.80

[1] CSM=Cotton seed meal; [2] UTS=Urea treated rice straw; [3] NSC=100-(Ash+CP+NDF+EE); [4] Calculated values.

straw (3 kg/d), Meadow hay (2 kg/d) and groundnut meal (3 kg/d). The animals were fed twice a day with water and a vitamin-mineral block lick being available ad libitum for at least 20 days before sampling of the rumen contents.

Rumen inoculum

Strict anaerobic techniques were used in all steps during the rumen fluid transfer and incubation periods. Rumen fluid samples were removed before morning feeding under vacuum via the rumen fistula into a 2 litre plastic flask and transferred into 2 pre-warmed 1 litre thermos flasks which were then transported to the laboratory.

Medium solution preparation

The medium preparation was as described by Makkar et al. (1995). The reduced medium consisting of 1095 ml H_2O, 730 ml buffer solution (35.0 g $NaHCO_3$ and 4 g NH_4HCO_3 made up to 1 litre with distilled water), 365 ml macro mineral solution (6.2 g KH_2PO_4, 5.7 g Na_2HPO_4, 2.22 g NaCl and 0.6 g $MgSO_4 \cdot 7H_2O$ made up to 1 litre with distilled water), 0.23 ml micromineral solution (10.0 g $MnCl_2 \cdot 4H_2O$, 13.2 g $CaCl_2 \cdot 2H_2O$, 1 g $CoCl_2 \cdot 6H_2O$, 8.0g $FeCl_3 \cdot 6H_2O$ and made up to 100 ml with distilled water), 1 ml resazurine (0.1 g made up to 100 ml with distilled water), 60 ml freshly prepared reduction solution containing 580 mg $Na_2S \cdot 9H_2O$ and 3.7 ml 1 M NaOH and 1.4647 g $^{15}N_2$-urea (99.4 Atom % excess $^{15}N_2$, ISOTEC INC., 3858 Bemner Rd., Miamisburg, OH, USA, Cat No. 85-62005). 660 ml of rumen fluid were mixed with reduced medium (39℃). During transfer, the rumen medium was incubated under CO_2 at 39℃ and stirred using a magnetic stirrer fitted with a hot plate.

Substrate incubation

The mixture of substrates was ground through a 1-mm screen using a Cyclotech mill (Tecator, Sweden). Duplicate sets of sample incubations for the determination of fermentation end-products and gas production were prepared each time. The substrate sample of approximately 500 mg on a fresh weight basis was transferred into a 50 ml serum bottle (Makkar et al., 1995). The bottles were pre-warmed in a water bath at 39℃ for about 1 hour prior to injection of 40 ml of rumen medium (using a 60 ml syringe) to each bottle. The bottles were stoppered with rubber stoppers, crimp sealed and incubated in a water bath set at 39℃. The bottles were gently shaken 30 min after the start of incubation and then at three hour intervals for 12 h.

Sampling and analysis

1) Fermentation characteristics

The measurements of pH, NH_3, ^{15}N incorporation and VFA production were performed after 12, 24, 36 and 48 h incubation periods. pH values of each incubation were measured immediately after each sampling time. One part of the fluid was pipetted into one part of 0.2 M HCl for further analysis for ammonia concentration. The volatile fatty acids were determined in the supernatant obtained from each incubation after being deproteinised (4 part of fluid+1 part of 20% H_3PO_4/50 mM 3-methyl valerate). The clear supernatants, after centrifugation at 15000×g,

were analysed for VFA concentration as detailed in Seal et al. (1992).

2) Gas production kinetics

The rate of gas production was measured by reading and recording the amount of gas volume after incubation using a 100 ml glass syringe connected to the incubation bottle with a 23 gauge, 1.5 inch needle. Readings of gas production were recorded at 12, 24, 36, 48, 60 and 72 h after incubation periods. The kinetics of gas production were described using the equation $y=a+b(1-e^{-ct})$ (Ørskov and McDonald, 1979; Makkar et al., 1995; Blummel and Becker, 1997) where a=the intercept, which ideally reflects the fermentation of the soluble fraction, b=the fermentation of the insoluble fraction (which is with time fermentable), c=rate of gas production, (a+b)=potential extent of gas production, y=gas produced at time t.

Digestibility: True digestibility was estimated after the last gas measurement by treating the bottle contents with neutral detergent solution by the method of Goering and Van Soest (1970) and Van Soest et al. (1991) to obtain NDF residues as described by Pell and Schofield (1993) and Makkar et al. (1995). Digestibility estimate were calculated by the following equation:

% true digestibility=
(true digested substrate) × 100/
weight of sample taken for incubation
% NDF digestibility=
(NDF digested) × 100/NDF initial incubation

3) Efficiency of microbial protein synthesis (EMPS)

mg N incorporated per m mole VFA produced, was measured using the stable isotope ^{15}N as a tracer. The ^{15}N incorporation was measured using mass spectrometer analysis of the microbial pellet obtained following differential centrifugation of the incubation mixture sample which had been treated with 5 ml 1% NaCl (w/vol.) and 0.5% formaldehyde (wt/vol.) at the time of sampling and frozen prior to analysis (Craig et al., 1986; Cecava et al., 1990). Thawed samples were centrifuged at 500 × g at 4℃ for 20 min to separate the feed particles and protozoa (samples were reconstituted with distilled water and re-centrifuged twice). The clear supernatant was centrifuged at 30,000 xg at 4℃ for 30 min and the residue washed three times with distilled water. The microbial pellet obtained was freeze dried and re-weighed for cell dry mater calculation and ^{15}N enrichment analysis.

^{15}N incorporation (mg)=
(Biomass yield, mg) × (%N/100) × (%^{15}N/100)
EMPS=^{15}N incorporation (mg/0.5 g substrate)/
Total VFA produced (mmole/0.5 g substrate)

Statistical analyses

All data obtained from the trials were subjected to the General Linear Models procedure of SAS/STAT (SAS, 1990) according to a 3 × 3 factorial arrangement in a randomised complete block design using sets of incubations as blocks. The model included blocks, cassava levels; type of roughage and interaction effects. The model was as follows: $Y_{ij}=\mu+Bl_i+A_j+Bk+AB_{jk}+E_{ijk}$, Where Y_{ijk}=observation, μ=overall mean, Bl_i=block effect (l=1-6), A_j=cassava levels effect (j=1-3), B_k=type of roughage effect (k=1-3), AB=interaction effect, and E_{ij}=residual. Type III sums of squares were used to determine whether treatment effects were significant. Least square means are presented for all parameters. Contrasts were measured for cassava levels using polynomial orthogonal linear or quadratic comparisons. For type of roughage significance was examined by orthogonal comparisons. Significance is shown at $p<0.001$, $p<0.01$ and $p<0.05$ unless otherwise noted. Multiple regression analysis was performed by PROC REG of SAS (1990) to assess the relationship between parameters.

RESULTS

Fermentation characteristics

1) Inoculum pH

The pH values obtained are given in table 2. The pH value of the inoculum was significantly affected by the proportion of cassava in the substrate and also by the type of roughage. It decreased rapidly during the fermentation period (data not shown) (Sommart, 1998) when compared with the initial pH of the medium solution. The decrease in pH was more pronounced for the substrates containing 450 g/kg cassava and dried ruzi grass and fell to its lowest value of 6.70 on this treatment. However, these values did not fall below the normal range for rumen microbe growth especially for cellulolytic bacteria (pH range 6.5-7.0). These data indicate that the buffering capacity of this *in vitro* system was sufficient to maintain conditions within the expected range.

2) Ammonia concentration

Values for ammonia concentration in the inoculum are reported in table 2. Ammonia concentrations were substantially higher than from the medium solution alone (2.39-2.46 mg NH_3-N/100 ml), indicating that the dietary protein and urea hydrolysis were complete within 12 h of incubation. The concentration of ammonia in the inoculum over the 48 h incubation period ranged between 3.47 to 4.44 mg NH_3-N/100 ml, indicating values in the range for the optimal

Table 2. Effect of cassava levels (15.0, 30.0 and 45.0%) and roughage type on ruminal pH, ammonia concentration (mg NH_3-N/100 ml), total volatile fatty acid concentration (mM TVFA) and molar proportions (mole/100 mole) of acetate, propionate and butyrate and acetate : propionate ratio from *in vitro* incubations with rumen fluid during 12-48 h

	Ruminal						
	PH	NH_3-N	TVFA	Acetate	Propionate	Butyrate	C_2:C_3
Cassava levels							
15.0%	6.88	3.92	53.18	63.97	27.35	6.85	2.36
30.0%	6.83	3.72	59.50	62.69	28.70	6.97	2.22
45.0%	6.79	3.82	64.71	61.55	29.39	7.38	2.11
Roughage type[1]							
RG	6.79	3.58	61.79	62.01	28.97	7.25	2.15
RS	6.88	4.14	56.38	63.33	27.98	6.95	2.28
UTS	6.83	3.74	59.23	62.84	28.48	7.02	2.56
SE	0.06	0.57	13.00	3.12	2.66	0.83	0.24
Comparison							
Cassava	***	NS	***	***	***	***	***
RS vs. RG	***	***	***	***	***	***	***
UTS vs. RG	***	NS	NS	*	NS	*	***
UTS vs. RS	***	***	0.07	NS	NS	NS	NS
Interaction	NS	*	NS	NS	NS	*	NS

*** p<0.001; ** p<0.01; * p<0.05; NS=Non-significant.

[1] RG=Dried ruzi grass, RS=Rice straw, UTS=Urea treated rice straw.

concentration reported in the literature. Generally, there was a trend for concentration to decrease with the higher levels of cassava inclusion, but this was not statistically significant. However, roughage source did affect ammonia concentration. Ammonia concentrations were greater in rice straw than untreated straw or dried ruzi grass. Dried ruzi grass had the lowest concentration. These data imply that rate and amount of ammonia utilisation by mixed rumen microbes were different between the roughage sources. The inclusion of untreated rice straw in the substrate mix seemed to result in the slowest ammonia utilisation. Dried ruzi grass had the lowest ammonia concentration at each level of cassava inclusion and incubation time when compared to either untreated rice straw or urea treated straw, indicating that ammonia incorporation may be greater with this roughage source.

3) Volatile fatty acids

The influence of cassava level and type of roughage on total VFA concentration, production of total VFA, acetic acid proportion, propionic acid proportion and acetic: propionic ratio are shown in table 2. Mean total VFA concentration and proportion of propionic acid increased linearly (p<0.001), but the proportion of acetic acid and the acetic to propionic acid (C_2:C_3) ratio decreased with higher cassava inclusion. There was a substantial decrease in the ratio at the highest levels of cassava. At each incubation time, levels of cassava in the substrate had a significant effect on the proportions of acetic and propionic acid, subsequently affecting this VFA ratio. Molar percentages and production of acetic acid were decreased by cassava inclusion as the incubation time increased (data not shown) (Sommart, 1998). The decrease in proportions was higher when dried ruzi grass was included in the substrate, confirming literature reports of changes in VFA proportions with increases in the roughage: concentrate ratio. The present study also showed a change in molar proportions of VFA production with the rice straw treatment and also between roughage sources.

Total VFA production was highly influenced by levels of cassava and roughage sources in the substrate. Mean total VFA concentration ranged from 48.17 to 64.36 m mole per litre. These values are similar to concentrations in rumen fluid, suggesting no acidic inhibition of microbial growth which can occur *in vitro* systems. Total VFA production (mmole/0.5 g substrate) increased with time of incubation (data not shown) (Sommart, 1998). Increased inclusion of cassava resulted in a substantial increase in total volatile fatty acid production and these increases were simultaneous with a decrease in innoculum pH. Roughage source in the substrate also affected volatile fatty acid production. Although, there was no statistical difference in production between dried ruzi grass and rice straw during the first 24 h incubation, a

Table 3. Effects of cassava levels (15.0, 30.0, 45.0%) and roughage type on rate of gas production (c, %/h), gas production (ml/0.5 g substrate) and digestibility (at 72 h incubation) using an *in vitro* gas production technique

	Gas kinetics[1]			Digestibility, %		Gas, ml
	c	a	b	True	NDF	72 h
Cassava levels						
15.0%	0.041	-35.2	137.1	80.44	66.95	62.6
30.0%	0.044	-25.7	132.8	84.31	67.11	76.0
45.0%	0.049	-16.1	133.8	87.12	66.54	88.0
Roughage type[2]						
RG	0.052	-28.3	136.7	85.64	69.25	105.6
RS	0.035	-17.5	122.7	81.07	60.51	91.3
UTS	0.047	-31.2	144.2	85.14	70.85	104.7
SE	0.004	12.4	6.2	1.2	2.7	7.1
Orthogonal comparison						
Cassava	***	***	NS	***	NS	***
RS vs. UTS	***	***	***	***	***	***
RS vs. RG	***	***	***	***	***	***
UTS vs. RG	***	NS	***	NS	NS	NS
Interaction	***	NS	NS	*	**	NS

*** p<0.001; ** p<0.01; * p<0.05; NS=Non-significant; [1] a, b, c are constants in the exponential equation, $y=a+b(1-e^{-ct})$ where a=the intercept and ideally reflects the fermentation of the soluble fraction, b=the fermentation of the insoluble (but with time fermentable), c=rate of gas production, (a+b)=potential of extent of gas production, y=gas produced at time 't'.; [2] RG=Dried ruzi grass, RS=Rice straw, UTS=Urea treated rice straw.

significant increase was observed at the 36 and 48 h incubation times. The difference between urea treated and non-treated rice straw also showed similar trends. There was a small increase with dried ruzi grass when compared with urea treated rice straw, but this was not significant. Inclusion of rice straw resulted in the lowest total VFA production. There was no difference in total VFA production or the proportion of propionate when urea treated rice straw was compared to dried ruzi grass, but the molar percentage of acetate was higher resulting in a higher C_2:C_3 ratio.

Gas production

Gas production during the fermentation of the inoculum is given in table 3. Gas produced after 72 h incubation ranged between 88.8 and 111.8 ml per 0.5 g of substrate. The volume of gas produced increased ($p<0.001$) with increasing level of cassava inclusion. The volume of gas produced increased linearly ($p<0.001$) with the proportion of cassava in the substrate at each incubation time. In addition, type of roughage also influenced gas production (table 3). Based on these observations the roughage feedstuffs could be ranked as : dried ruzi grass > urea treated rice straw>rice straw with respect to fermentation rate by the rumen microbes *in vitro*. However, the volume of gas produced was not statistical different between dried ruzi grass and urea treated rice straw after 24 h incubation, suggesting that urea treated rice straw improved nutrient utilisation by the rumen microflora used in this experiment. There were no interactions between the main factors on gas production, indicating that there were no changes in the potential extent of digestion of roughage type for any of the cassava inclusion treatments.

Kinetic of gas production analysis

Values for the estimated parameters obtained from the kinetics of gas production models for the substrates studied are reported in table 3. A comparison of rate constants of different treatments indicated significant differences between them. Rate of gas production (c) increased linearly ($p<0.001$) as cassava inclusion in the substrate increased indicating that cassava starch was readily available to the microbial population. Type of roughage in the substrate also had a significant effect on rate of gas production with dried ruzi grass>urea treated rice straw>rice straw. However, the potential of gas production was similar for dried ruzi grass and urea treated rice straw, suggesting improved nutrient utilisation when compare to untreated rice straw. Potential gas production (a+b) values were also affected by cassava levels in the substrate.

Microbial biomass yield and microbial protein synthesis

The microbial biomass, nitrogen content, ^{15}N incorporation and EMPS during the period of incubation are presented in table 4. There were no

Table 4. Effects of cassava levels (15.0, 30.0, 45.0%) and roughage source on microbial nitrogen content (% N), Atom % excess, microbial biomass (mg/0.5 g substrate), ^{15}N incorporation(mg/0.5 g substrate) and efficiency of microbial protein synthesis (EMPS, mg ^{15}N incorporation per mmole TVFA production) of bacteria isolated from *in vitro* incubation

	Microbial				
	% N	Atom %	Biomass, mg	^{15}N incoporation	EMPS
Cassava levels					
15.0 %	7.58	16.98	53.94	0.73	0.34
30.0 %	7.66	18.96	61.28	0.91	0.38
45.0 %	7.75	19.20	67.66	1.02	0.41
Roughage type[1]					
RG	7.73	19.34	66.14	1.01	0.42
RS	7.51	17.10	54.36	0.72	0.33
UTS	7.76	18.70	62.38	0.93	0.39
SE	0.92	2.28	9.33	0.26	0.12
Orthogonal comparison					
Cassava	NS	***	***	***	***
RG vs. RS	NS	***	***	***	***
RG vs. UTS	NS	NS	*	NS	NS
RS vs. UTS	NS	***	***	***	***
Interaction	NS	NS	NS	NS	NS

*** p<0.001; ** p<0.01; * p<0.05; NS=Non-significant.

[1] RG=Dried ruzi grass, RS=Rice straw, UTS=Urea treated rice straw.

interactions between the main factors for any of parameters. The nitrogen content of the dry microbial pellets obtained from differential centrifugation showed no difference between substrates at any of the sampling times and ranged between 7.36 to 8.00 percent with estimated net microbial protein values (N multiplied by 6.25) of 45.0-50.0% CP. These data suggest that the proportion of N in the microbial cells was constant, although contamination by feed particles may have occurred in these systems. However, nitrogen isotope (^{15}N) enrichment was affected by higher levels of cassava inclusion in the substrate. In addition, there were also differences in ^{15}N incorporation between the roughage sources.

There were no differences between dried ruzi grass and urea treated straw, but rice straw had the lowest ^{15}N incorporation (table 4). In the present experiment, Atom % access ranged between 17-20 percent and reached a plateau after 24 h incubation. It is also interesting to note that Atom % excess in the microbial pellets increased with increasing level of cassava in the substrate. The results imply that a higher amount of cassava may favour a higher rate and extent of NPN incorporation. Different responses in Atom % excess to different substrates may also be due to differences in fermentation pathways or to changes in energetic efficiency associated with the proportions of structural and nonstructural carbohydrate available to the microbial population.

Microbial biomass yield and net ^{15}N incorporation are presented in table 4. There were highly significant ($p<0.0001$) increases in microbial biomass yield, microbial N and the incorporation of ^{15}N into cells with the increased inclusion rate of cassava in the substrate. These were also affected by roughage source used in the inoculum, being lower with rice straw when compared to dried ruzi grass and urea treated rice straw. These data indicated that rice straw treatment improved fermentability, microbial growth and microbial protein synthesis to levels similar to dried ruzi grass, but untreated rice straw resulted in lower utilisation. Higher microbial nitrogen yields appeared to be due to an increase in N incorporation, and therefore a higher rate of microbial protein synthesis which was a function of the availability of fermentable carbohydrate to the microbial population.

The efficiency of microbial protein synthesis (EMPS) increased linearly with the inclusion of cassava levels for the first 12 h of the incubation (data not shown) (Sommart, 1998). When either dried ruzi grass or urea treated rice straw were included in the substrate, EMPS was higher than for rice straw after 12 h incubation, indicating that the utilisation of nutrients was significantly improved. Decreases in EMPS were observed after 24 h which may reflect slower microbial growth as either the supply of substrate decreased or microbial lysis occured. These changes were simultaneous with the increased inoculum ammonia concentration after 36 h incubation.

Digestibility

True digestibility estimated at 72 h incubation

Table 5. Equations developed to maximise regression of determination (R^2) for ^{15}N incorporation and microbial biomass by gas kinetics and production together with VFAs production in an *in vitro* system

Equation	Relationship	R^2	p
1	Gas (ml)=-229.9+3.5 digestibility(%)	0.96	0.0001
2	Microbial biomass, mg=24.59+0.57 [Gas volume, ml]	0.98	0.0001
3	Microbial biomass, mg=-15.94+32.60 [TVFA, mmole]	0.87	0.0002
4	Microbial biomass, mg=22.32+43.72 [^{15}N incoporation, mg]	0.98	0.0001
5	^{15}N incoporation, mg=-0.83+0.73 [TVFA, mmole]	0.84	0.0005
6	^{15}N incoporation, mg=-0.41+1.90 [Propionante, mmole]	0.85	0.0004
7	^{15}N incoporation, mg=-1.21+1.42 [Acetate, mmole]	0.83	0.0006
8	^{15}N incoporation, mg=0.07+0.95 [Gas volume, ml]	0.95	0.0001
9	[TVFA, mmole]=1.36+0.016 [Gas volume, ml]	0.90	0.0001
10*	^{15}N incoporation, mg=-0.005+0.009 [Gas volume, ml]+6.19 [c]	0.98	-

* [c]=Rate of gas production.

increased in a linear response to cassava level in the substrate, but cassava level had no affect on NDF digestibility (table 3). There were also differences ($p<0.001$) between rice straw and urea treated rice straw or dried ruzi grass, but no difference between urea treated rice straw and dried ruzi grass was detected in this study. Inclusion of cassava at rates of 150 to 450 g/kg had no effect on NDF digestibility. These results imply that the levels of starch in the substrate did not influence fibre digesting micro-organism in an *in vitro* system. There were interactions between cassava levels and roughage sources on NDF digestibility. NDF digestibility was lowest when rice straw was used as a roughage source with 400 g/kg cassava when compared to dried ruzi grass and urea treated straw.

Stoichiometric relationships

Microbial protein synthesis and VFAs obtained from fermentation are a major indicator as to whether or not substrate digestion provides essential nutrients for the host animal. The quantitative relationship between rumen fermentation end-products provides useful information on fermentation balance and subsequent prediction of animal production. Also, the measurement of gas production is an easy, cheap and rapid method which can be linked to the prediction of net microbial protein yield and VFAs production. To obtain a general perspective on the fermentation balance, simple and multiple regression analysis were conducted to evaluate the relationships between fermentation parameters and microbial yield. The data base used was constructed from various incubation times (12, 24, 36 and 48 h incubation) for each of the 9 substrate treatments with 6 replicates, therefore, 216 observations were used.

1) Gas production and *in vitro* digestibility

The relationship between gas volume and substrate digestibility *in vitro* is presented in table 5. The data shows a highly significant coefficient of determination, suggesting that gas volume is a good parameter from which to predict digestibility of the substrate by rumen microbes in this *in vitro* system. Although, the regression of determination ($R^2=0.96$) was high, it also shows variation due to other factors. The regression coefficient indicated that an increase of 1 unit of digestibility increased innoculum release of gas by 3.5 ml.

2) Gas production and microbial growth

There was a significant relationship ($R^2=0.98$, $p<0.0001$) between gas production and microbial biomass (table 5), suggesting that microbial biomass could be predicted by gas production data. Biomass yield ranged from 53.94 to 67.66 mg/0.5 g substrate, and the gas volumes varied from 88.8 to 111.8 ml/0.5 g substrate. These data also indicated that for each 1 ml of gas released 0.57 mg of biomass was produced. Net microbial protein synthesis, measured as ^{15}N incorporation, also exhibited a significant relationship ($R^2=0.95$, $p<0.0001$) with gas production. Multiple regression analysis also indicated that gas information is sufficient to predict ^{15}N incorporation. In general, gas production was shown to be a good predictor of microbial growth on the substrates used in this study.

3) VFA production and microbial growth or gas production

To quantify the relationship between VFA production and microbial protein synthesis in a batch culture *in vitro* system, it is necessary to have a direct assessment of microbial biomass and net microbial protein synthesis (total N or ^{15}N) together with total VFA production. The data indicate a strong coefficient of determination between total VFA production, acetic acid and propionic acid with either VFAs or microbial biomass yield and ^{15}N incorporation as an index of

microbial protein synthesis (table 5).

DISCUSSION

The results obtained from this study suggest that fermentation in an *in vitro* gas production system is similar to conditions in the rumen and supports the use of these methods to quantify information on rumen digestion and fermentation (Menke et al., 1979; Menke and Steingass, 1988). Additional information on the relationship between gas production, VFA production and microbial protein synthesis were also demonstrated.

A recent report, Gizzi et al. (1998) has confirmed that most species of rumen bacteria (cellulolytic, amylolytic, proteolytic and methanogenic) and protozoa are present in *in vitro* systems. The pH and ammonia concentration of the inoculum were observed to decrease with increased incubation time. A more pronounced decline was shown for incubations which had the highest proportion of cassava in the substrate. These results are consistent with the higher rate of gas production and the consequently higher production of volatile fatty acids (table 2) with this substrate. The increase of ammonia concentration after longer incubation times may also be due to cell lysis and/or engulfment of rumen protozoa with later excreted ammonia.

The VFA production was associated with increased microbial growth (table 5) and it was also apparent that different rates of fermentation were associated with the different roughages used as substrates. Rice straw was the least fermentable while dried ruzi grass and urea treated straw were more rapidly fermented. The optimal concentration of ammonia nitrogen for microbial growth *in vitro* has been reported to range between 2-5 mg N/100 ml (Satter and Slyter, 1974) and it can therefore be assumed that in the current experiment sufficient ammonia was available for microbial growth during the period of highest fermentation rate which was shown to be between 24 and 48 h. In addition, analysis of ^{15}N incorporation into harvested bacteria indicated that of the 9.74 mg ^{15}N provided in the medium in the form of urea only 0.88 mg ^{15}N (SD=0.31) was utilised over the incubation period.

The increase in gas production and volatile fatty acid concentration with specific treatments reflects the increase in the fermentability of substrates in the inoculum. There was a strong coefficient of determination between the volume of gas produced and total volatile fatty acid production during the 48 h incubation [Total VFA (mmole)=1.36+0.016 Gas volume (ml); R^2=0.90]. This relationship has been documented previously (Blummel et al., 1993; Makkar et al., 1995; Doane et al., 1997) and the release of CO_2 during microbial metabolism shown to reflect the production of acetate and butyrate during fermentation (Wolin, 1960; Blummel and Ørskov, 1993; Doane et al., 1997). The rate and extent of fermentation, however, will be determined by substrate availability.

Cumulative gas volume at each sampling time was affected by both levels of cassava in the substrate and roughage source. These findings suggest that the inclusion of cassava as a substrate results in an increased rate and extent of fermentation of the inoculum. Although the overall extent of digestion of dried ruzi grass and urea treated rice straw were the same, in the first 24 h of incubation the rate of fermentation were higher for dried ruzi grass. These findings are in line with the work of Krishnamoorthy et al. (1991a) who found that the type of carbohydrate and the rate of carbohydrate fermentation influenced microbial protein synthesis and the volumes of gas produced. The rate of gas production of this experiment were in the same ranges as those reported by Markkar et al. (1995) who used temperate grass hay as the control substrate.

The values for a, intercept, were negative in all the incubations in this study (table 3). These data suggested that a lag phase due to delays in microbial colonisation of the substrate may occur in the early state of incubation. Several authors (Khazaal et al., 1993; Blummel and Becker, 1997) have also reported negative values with various substrates when using mathamatical models to fit gas production kinetics. This is due to either a deviation from the exponential course of fermentation or delays in the onset of fermentation due to microbial colonisation.

The relationship between gas production and digestibility at 72 h incubation is shown in table 5. Previous research (Khazaal et al., 1993) has demonstrated that the kinetics of gas production are highly correlated with digestibility and feed intake when compared to data using the nylon bag technique. The strong relationship between either the rate of NDF disappearance or true digestibility and gas production has been documented previously (Blummel et al., 1993; Pell and Schofield, 1993; Doane et al., 1997). The results in the current experiment are consistent with these reports.

Microbial biomass isolated from the incubation contained 7.66% N (SE=0.91 range 7.5 to 7.7). These values are in agreement with N composition of mixed rumen microbes reported previously (Craig et al., 1986; Cecava et al., 1990). Clark et al. (1992) have reviewed the literature on the N fraction composition of rumen bacteria and reported total N content and amino acid as a percentage of total N ranged from 4.83 to 10.58 percent and 54.9 to 86.7 percent, respectively. In addition, they also pointed out the large variation in N fraction associated with different

rumen microbial populations and time of sampling. These differences included variation in total nitrogen content and amino acid composition.

In the present studies, the relatively constant nitrogen content observed between incubation times, indicated that the differential centrifugation techniques used provided good separation with little contamination from feed particles. These methods have been used to separate microbial cells from feed particles in rumen fluid (Craig et al., 1987; Cecava et al., 1990; Blummel et al., 1997). High coefficient of determination (R^2=0.98, $p<0.0001$) between ^{15}N incorporation and microbial biomass, confirm a good agreement between these two determinants of microbial growth in this study. However, differential centrifugation techniques do exclude some microbial populations during separation such as protozoa, fungi and bacteria attached to feed particles. These microbial populations may also play an important role in rumen fermentation.

The ^{15}N:total N ratio in the microbial pellets was influenced by the substrate used in the innoculum, indicating that the rate and extent of incorporation of N was different between levels of cassava and the three roughage sources used (table 4). The Atom percent excess increased with the level of cassava in the substrate and the addition of untreated rice straw appeared to lower the enrichment obtained when compared to dried ruzi grass or urea treated rice straw. These results indicate that the availability of carbohydrate is a major factor that governs microbial growth and microbial protein synthesis. In this study, increased inclusion of cassava in the substrate, caused an increase in NSC from 11.9 to 40.9% (table 1). These results support previous work (Krishnamoorthy et al., 1991b; Guzzon et al., 1997; Archimede et al., 1997) in confirming that microbial protein synthesis and microbial amino acid yield are higher when starch rather than cellulose is provided as a substrate in an *in vitro* system.

The data also showed that urea treatment of rice straw improved nutrient availability for microbial growth and microbial protein synthesis resulting in a similar rate to that of dried ruzi grass. In the present studies, it was assumed that ^{15}N-urea would be hydrolysed to ^{15}N-ammonia before incorporation by the microbes. Also, the assumption is made that other factors such as ammonia, vitamins and mineral levels would not limit activity in this *in vitro* system.

CONCLUSION

The use of cassava as a source of readily fermentable carbohydrate together with different sources of roughage were studied using an *in vitro* gas production system to quantify microbial fermentation end products from a simulated mixed rumen population. The data from this study found that microbial protein synthesis and fermentation end products increased with the level of cassava inclusion in the substrate. These were also affected by roughage source, being lower with rice straw, but a substantial improvment in the use of this substrate was observed when rice straw was treated with urea. Data from these incubations indicated characteristics similar to dried ruzi grass. There were no interactions between the main factors on microbial protein synthesis, VFA production or gas volume. The results suggest that microbial growth and fermentation rate increase as a function of readily available carbohydrate in the substrate mixture. The relationships between microbial protein synthesis using ^{15}N incorporation and VFA production and/or cumulative gas production were linear.

ACKNOWLEDGEMENTS

The authors acknowledge the skilled technical of Mr. Kriengsak Sa-aarruk, and personel at Dairy Promotion Organization of Thailand and Khon Kaen University. This work was conducted under the Dairy Nutrition Research Project that is funded by Thailand Research Fund. Particular thanks are due to The British Council, National Center for Genetic Engineering and Biotechnology of Thailand, Khon Kaen University and The University of Newcastle for their generous contributions towards the funding of the Ph.D. programme which made this research possible.

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