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Research Article

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Subtropical Forages Differentially Influenced the Ruminal Fermentation and Microbial Community of Jersey Cow *In Vitro*

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Abstract

Oven dried cassava residues, corn straw silage, elephant grass and sugarcane tail silage were used as substrates to do a 24 h of incubation with a 100 ml glass syringe at 39°C. Gas production was recorded at the end of incubation and ruminal fluid was harvested to determine volatile fatty acids (VFA) using gas chromatograph, quantify microbial populations using real time PCR, and analyze microbial community using high throughput sequencing. Results showed *in vitro* incubation not only decreased population of bacteria, fungi, methanogen and some cellulolytic bacteria ($P<0.05$), but also increased diversity of bacteria, reversed Firmicutes to Bacteroidetes ratio, and decreased abundance of *Prevotella*, *M. gottschalkii* and *Entodinium*. Gas production, acetate/propionate ratio and abundances of *Succiniclasticum*, *Entodinium* and *Diploplastron* were the highest, while total VFA concentration, fungal and cellulolytic bacterial populations, and abundances of Methanomassiliicoccales and *Ostracodinium* were the lowest with cassava residues ($P<0.05$). Influence on fermentation pattern and microbiota of three gramineous substrates was similar, but inoculum incubated with sugarcane tail silage had higher abundance of Methanomassiliicoccales and Diplodinium. In conclusion, cassava residues which is a low neutral detergent fiber forage showed a completely different fermentation pattern and influence on microbe community indicated NDF was the most crucial factor to determine microbial community *in vitro*.

Keywords: Subtropical forages; *In vitro* fermentation; Microbial community; Microbial population

Introduction

Forage usually makes up half or more of the ruminants' diet, influencing dry matter intake and microbial community composition both *in vivo* and *in vitro* [1-3]. On account of distinct nutritive profile, different forages have discrete impacts on ruminal fermentation and microbiota [4,5]. Changes in the ruminal microbial community induced by forage can provide a clear understanding of interaction between forage and microbes [6]. Due to high biodiversity in tropical and subtropical areas, a variety of roughages is available for the ruminants. However, sustainably available roughage sources used for the ruminants on commercial scale are still the by-products of agricultural cultivations. Among those, cassava starch residues, corn straw silage, sugarcane tail silage and elephant grass are the typical representatives and widely used in the southern China. Cassava starch residue is a source of non-forage fiber which has potential to be used as both beef and dairy cattle diet, as its dry matter contains low fiber but high soluble carbohydrates [7]. Corn straw is a by-product of edible corn produced in the subtropical areas and intensively used as forage [8]. Sugarcane is the most productive crop in the tropical areas, and its tail that contains most of leaves is a nutritious forage for the ruminants [9]. Elephant grass is a fast-growing plant and famous for its higher production in the subtropical areas. It is extensively used as a stable forage source for the ruminants [10].

The microbes inhabiting the gut/rumen are known to impose protective effects and nutritional benefits to the host [11] and due to their superior metabolic potentials compared to the host they are rightly considered equivalent to an organ [12,13]. Composition of the microbial community in the rumen and the end products of fermentation depend on the diet fed to the animals [14,15]. To study the impacts of forage source on ruminal fermentation, the *in vitro* techniques are widely used to realize more controlled and reproducible conditions, compared to *in vivo* experiments [16]. On the other hand, the advancement in latest microbial molecular techniques, in particular, high throughput sequencing technology have enabled to explore the rumen microbial consortium with higher precision.

Although, nutritional values and digestibility of these typical subtropical forages have been highly explored previously in both *in vitro* and *in vivo* studies, but their differential impacts on the ruminal microbial community are poorly researched due to limitations of microbial molecular research techniques in the past. Thus, the primary objective of this study was to examine the impacts of four typical subtropical forages on the ruminal fermentation, microbial population and community composition. Secondly, this study also compared the changes in microbial community composition before and after the incubation to elucidate the effect of fiber structure on ruminal microbiota and fermentation.

Materials and Methods

Ethical statement

Ruminal fluid inoculum donors Jersey cows in this study were housed at the Buffalo Research Institute, Chinese Academy of Agricultural Sciences, Nanning, Guangxi province, China. All the experimental protocols regarding animal handling and treatment were approved by the Animal Care Committee, Guangxi University, under

guidance of the International Cooperation Committee of Animal Welfare, China.

Rumen inoculum donors and their rations

Rumen inoculum was collected from three ruminal cannulated dry pregnant Jersey cows (Bos taurus) with similar live weights (~500 kg), before their morning feeding. Animals received 3 kg concentrate per head per day and corn silage for ad libitum, and free access. Composition of the concentrate feed offered to the animals was as follows (based on dry matter): maize 52%, wheat bran 18.5%, soybean meal 8%, cotton seed meal 15%, stone dust 2%, calcium hydrogen phosphate 1.5%, sodium chloride 2% and premix 1%. The premix contained per kilogram: 11.9 g of $MgSO_4 \cdot H_2O$, 2.5 g of $FeSO_4 \cdot 7H_2O$, 0.8 g of $CuSO_4 \cdot 5H_2O$, 3 g of $MnSO_4 \cdot H_2O$, 5 g of $ZnSO_4 \cdot H_2O$, 10 mg of Na_2SeO_3, 40 mg of KI, 30 mg of $CoCl_2 \cdot 6H_2O$, 28.5 g of vitamin A1, 0.44 g of vitamin D, and 16.2 g of vitamin E.

Substrates and their nutritional composition analysis

Cassava residues (*Manihot esculenta*), corn straw silage (*Zea mays*), elephant grass (*Pennisetum purpureum*), and sugarcane tail silage (*Saccharum officinarum*) were used as substrates for *in vitro* fermentation, and taken from the farm of Buffalo Research Institute, Chinese Academy of Agricultural Sciences, Nanning, Guangxi province, China. Forage samples were dried at 65°C, ground through 1 millimeter (mm) screen and stored at -20°C until analysed for nutritional composition and *in vitro* gas production. Forages samples were analysed for dry matter (DM) contents by oven-drying for 8 hours (h) at 105°C, and crude protein (CP) was calculated as N × 6.25 [17]. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents were determined according to the method as described by Van Soest et al. [18]. Nutritional composition of the four substrates is shown in Table 1.

Nutrients	Substrates			
	Cassava residues	Corn straw silage	Elephant grass	Sugarcane tail silage
Dry matter%	95.8	95.3	93.6	95
Protein%	2.26	10.8	13.7	7.68
Neutral detergent fiber %	21.5	52.4	57.9	68.3
Acid detergent fiber%	15.7	30.9	32.5	37.6

Table 1: Nutritive values of substrates used for *in vitro* incubation (Dry matter basis).

In vitro fermentation and gas production

In vitro fermentation system was set up following the procedure as described by Tang et al. [19]. Equal volumes of rumen inoculum taken from the selected three Jersey cows were mixed together. Rumen contents were strained through a four layered cheesecloth into a pre-warmed Erlenmeyer flask. All the laboratory handling of rumen inoculum was performed under the continuous flow of carbon dioxide (CO_2) gas. *In vitro* fermentation process was carried out in glass syringes (100 ml) fitted with plungers [19]. Every glass syringe was anaerobically dispensed with fermentation medium comprising: 10 ml of rumen inoculum, 20 ml of McDougall's buffer solution and 200 mg of dried forage as substrate. For every substrate, eight experimental replicates (n=8) were set devised and resulting 32 in total. In addition, a similar set of four glass syringes containing only fermentation medium was also run to serve as the blank controls to correct the gas production resulting of fermentation of dry matter in the rumen inoculum. Every glass syringe individually containing fermentation medium and substrate was incubated in a shaking water bath at 39°C and gas production was recorded after 24 hr of incubation.

Sampling and volatile fatty acids analysis

After 24 hr of incubation, fermentation process in four of the eight replicates was ceased by placing them into an ice-cold water bath, and samples of fermented rumen inoculum were collected immediately. Collected samples were filtered through a four layered cheese cloth into a 50 ml centrifuge tube. A 2 ml aliquot of the filtrate was instantly subjected to determining the concentrations of volatile fatty acids (VFA) by using a gas chromatograph (GC-2010, Shimadzu, Tokyo, Japan), equipped with a flame ionization detector and a capillary column (HP-INNOWAX, 1909N-133, Agilent Technologies, Santa Clara, CA, USA) as described by Zhang et al. [20]. Another 2 ml aliquot of the filtrate was stored at -20°C for metagenomic DNA extraction.

DNA extraction and real time quantitative PCR (qRT-PCR)

DNA was extracted from 2 ml of the preserved sample following the procedure as reported by Rius et al. [21], and further employed to perform quantitative real-time PCR (qRT-PCR) to quantify the populations of bacteria, methanogen, fungi, protozoa, *Ruminococcus albus, Fibrobacter succinogenes, Selenomonas ruminantium,* and *Prevotella ruminicola [22].* Primers used were the same as described by Jiao et al. (Table 2) [22]. Briefly; standard curves were generated by tenfold serial dilutions of plasmid DNAs containing the extracts of 16s and 18s rRNA gene inserts from every microbial group and bacterial species. qRT-PCR assay was performed with a 10 µL reaction mixture volume using SYBR Green Master Mix (Perfect Real Time Takara, Japan), on a Roche light cycle 480 real time PCR system (Riche, Basel, Switzerland). Reaction mixture contained 5 µL of Fast SYBR Green Master Mix, 0.5 µL of each primer (20 pmol μL^{-1}), 3.5 µL of nuclease-free water and 0.5 µL of DNA template (10 ng μL^{-1}). All standard dilutions and samples were assayed in triplicate with amplification carried out according to the following program: 95°C for 10 min for initial denaturation, then 30 cycles at 95°C for 20 s, annealing for 30 s at 62°C, followed by terminal elongation at 72°C for 5 min. The corresponding qRT-PCR efficiency for every microbial group and bacterial species ranged from 90% to 100%. Total 16S rRNA and 18S rRNA gene copy numbers in samples were determined by relating the threshold cycle values to the standard curves. Copy numbers for the 16S rRNA gene in ml of rumen inoculum were calculated as proposed by Li et al. [23]. Values were converted to log 10 for further statistical analysis.

Primer's name	Sequence (5′-3′)	Size (bp)	Literature cited
Bacteria-F	CGGCAACGAGCGCAACCC		Denman and McSweeney
Bacteria-R	CCATTGTAGCACGTGTGTAGCC	146	

Fungi-F	GAGGAAGTAAAAGTCGTAACAAGGTTTC		
Fungi-R	CAAATTCACAAAGGGTAGGATGATT	120	Denman and McSweeney
Protozoa-F	GCTTTCGWTGGTAGTGTATT		
Protozoa-R	CTTGCCCTCYAATCGTWCT	223	Sylvester
Methanogen-F	TTCGGTGGATCDCARAGRGC		
Methanogen-R	GBARGTCGWAWCCGTAGAATCC	140	Denman
Fibrobacter succinogenes-F	GTTCGGAATTACTGGGCGTAAA		
Fibrobacter succinogenes-R	CGCCTGCCCCTGAACTATC	121	Denman and McSweeney
Selenomonas ruminantium-F	CAATAAGCATTCCGCCTGGG		
Selenomonas ruminantium-R	TTCACTCAATGTCAAGCCCTGG	138	Stevenson and Weimer
Ruminococcus albus-F	CCCTAAAAGCAGTCTTAGTTCG		
Ruminococcus albus-R	CCTCCTTGCGGTTAGAACA	176	Koike and Kobayashi
Prevotella ruminicola-F	GAAAGTCGGATTAATGCTCTATGTTG		
Prevotella ruminicola-R	CATCCTATAGCGGTAAACCTTTGG	74	Stevenson

Table 2: Primers used for qRT-PCR.

High throughput sequencing and bioinformatics analysis

Four DNA samples from every substrate were pooled into one sample to analyze microbial community. Metagenomic DNA samples were sent to the BGI genomic research center in Wuhan, China, for ruminal microbial community composition analysis. High throughput sequencing technique was conducted using illumina Miseq PE 250 platform (Illumina, Santiago, CA, USA). Bacterial and methanogen communities were analyzed using 16S rRNA gene sequencing, while protozoal community was analyzed using 18S rRNA gene sequencing [24]. Primers used for PCR amplifications are shown in Table 3. Sequence data reported in this study have been deposited in the NCBI database (accession no. SRR5930258–SRR5930269). All data processing including sequence quality control, operational taxonomic unit (OTU) based analysis, taxonomy analysis and diversity indices calculations, were performed using the Mothur V 1.31.2 [25]. Bacterial, methanogen and protozoal sequences were grouped into OTUs sharing 97% similarity. Bacterial 16S rRNA genes were blasted against the Green genes database [26] and methanogen 16S rRNA genes against databases provided by Seedorf [27], while protozoal 18S rRNA genes were blasted against databases provided by Kittelmann and Janssen [28]. Bacterial data were summarized at phylum and genus levels and protozoal data were summarized at genus level, while methanogens data were summarized using a mixed taxonomic rank scheme [29]. Microbial taxa those represented >1% of the total community within every microbial group (bacteria, methanogen, and protozoa) were included in the analysis.

Microbes	Primer sequence (5′-3′)	Size (bp)	Literature cited
Bacteria-F	GGCGVACGGGTGAGTAA	427	Hristov
Bacteria-R	CCGCNGCNGCTGGCAC		
Methanogen-F	GCGGTGTGTGCAAGGAGC	472	Jin
Methanogen-R	AGGAATTGGCGGGGGAGCAC		
Protozoa-F	AATTGCAAAGATCTATCCC	511	Kittelmann
Protozoa-R	GACTAGGGATTGGAGTGG		

Table 3: Primers used for microbial community composition analysis.

Statistical analysis of data

All the preliminary data including rumen fermentation parameters, microbial population and microbial relative abundance were sorted by Microsoft excel. Ruminal fermentation and microbial population data were analyzed as a one-way factorial design using the ANOVA procedure of SAS (2005), according to the following statistical model:

$Y_i = \mu + \alpha_i + \varepsilon_i$

Where: Y_i is dependent variable, α_i is effect of substrate (i=1,4) and ε_i is the residual error. Differences among means were tested using Duncan's multiple range tests. Statistical significance was considered if P<0.05.

Results

Effects of substrate on *in vitro* gas production, fermentation parameters and microbial population

After 24 h of incubation, the highest GP, butyrate concentration and acetate/propionate (A/P) ratio, while the lowest total VFA, acetate and propionate concentrations were observed on incubation with cassava residues (P<0.05, Table 4). Second highest GP, the highest propionate concentration and the lowest A/P ratio resulted with corn straw silage among the four substrates (P<0.05). The lowest GP and butyrate (P<0.05), while the highest total VFA and acetate concentrations (P>0.05) were observed with elephant grass. Acetate, propionate as well as total VFA concentrations after incubation with the four substrates were lower than those in fresh rumen inoculum. For the microbial populations; it was found the gene number of bacteria, fungi, *P. ruminicola, R. albus, F. succinogenes, and S. ruminantium* were lower with cassava residues (P<0.05, Table 4), while higher with elephant grass (P<0.05). The highest gene numbers of protozoa and methanogens were observed with cassava residues (P<0.05). Except protozoal population was increased with cassava residues, populations of all the other microbes were decreased as compared with those in fresh rumen inoculum.

Index	Fresh rumen inoculum	Substrate source				SEM	P
		Cassava residues	Corn straw silage	Elephant grass	Sugarcane tail silage		

Gas production, (mL/g)	-	354A	171B	151C	166B	3.88	0.02
TVFA, (mmol/L)	74.7A	60.3C	64.4B	65.0B	62.8B	4.58	0.03
Acetate, (mmol/L)	54.8A	42.0C	45.6B	48.7B	45.0B	3.09	0.01
Propionate, (mmol/L)	10.1A	7.85B	10.6A	9.51A	9.62A	0.93	0.04
Butyrate, (mmol/L)	9.81A	10.4A	8.25B	6.82C	8.26B	0.67	0.01
Acetate/ Propionate	5.46A	5.37A	4.29B	5.12A	4.68A	0.23	0.01
Bacteria, log10(copy/mL)	9.95	9.13	9.36	9.46	9.39	0.08	<0.01
Methanogen, log10(copy/mL)	8.12	7.41	7.06	7.22	7.15	0.24	0.01
Fungi, log10(copy/ml)	5.88	4.4	5.61	6.51	6.36	0.27	<0.01
Protozoa, log10(copy/ml)	7.35	7.65	7.12	6.94	7.13	0.21	0.02
R. albus, log10(copy/ml)	6.7	5.58	6.29	6.46	6	0.15	<0.01
F. succinogenes log10(copy/mL)	6.97	5.47	6.23	6.58	6.44	0.12	<0.01
P. ruminicola, log10(copy/mL)	8.81	7	7.89	8.19	8.13	0.2	0.01
S. ruminantium, log10(copy/mL)	8.71	8.06	8.42	8.52	8.45	0.17	0.01

Table 4: Gas production, ruminal fermentation parameters, and microbial populations in inoculum after 24 hr of *in vitro* incubation with four different substrates. TVFA stands for total volatile fatty acids. Means within a row differ with different superscripts (P<0.05).

Effects of substrates on ruminal bacterial community

After 24 hr of incubation, bacterial Chao1 and Shannon index were increased, but Simpson index was decreased by *in vitro* incubation with the four substrates (Table 5). Comparing the four substrates, the lowest bacterial Chao1 and Shannon index were observed with cassava residues, there was no difference in diversity indices for the other three substrates (Table 5). Community composition analysis showed, at phylum level, abundances of Firmicutes, Proteobacteria and Chloroflexi were increased, but the abundance of Bacteroidetes was decreased (from 60% to <42%) by *in vitro* incubation with the four substrates (Table 6). At genus level, abundance of dominant genus *Prevotella* was substantially decreased (from 57% to <30%), while abundance of unclassified bacterial genera was increased (from 33% to >46%) by *in vitro* incubation with the four substrates (Table 6). Comparing the four substrates, bacterial community of cassava residues hkad lower abundance of phyla Proteobacteria and Synergistetes, but higher abundance of Chloroflexi than that for other three substrates (Table 6). In addition, cassava residues had higher abundances of bacterial genera *Ruminococcus* and *Succiniclasticum*, while lower abundance of unclassified bacterial genera than the other three substrates (Table 6).

Rumen microbes	Index	Substrate source				
		Fresh rumen inoculum	Cassava residues	Corn straw silage	Elephant grass	Sugarcane tail silage
Bacteria	Chao1	706	787	876	920	960
	Shannon	4.94	5.13	5.59	5.68	5.71
	Simpson	0.02	0.02	0.01	0.01	0.01
Methanogen	Chao1	11.5	9	11	16	11
	Shannon	0.53	1.28	1.21	1.36	1.26
	Simpson	0.78	0.32	0.37	0.29	0.33
Protozoa	Chao1	161	162	323	238	315
	Shannon	3.28	2.01	3.44	3.16	3.64
	Simpson	0.07	0.36	0.09	0.12	0.06

Table 5: Ruminal microbial Alpha diversity statistics after 24 hr of *in vitro* incubation with four different substrates.

Taxon	Substrate source				
	Fresh rumen inoculum	Cassava residues	Corn straw silage	Elephant grass	Sugarcane tail silage
Phylum					
Firmicutes	35.3	55.5	55.9	54.3	52.2
Bacteroidetes	60.1	35.4	36.4	37	41.4
Proteobacteria	0.24	0.92	3.67	4.4	2.49
Chloroflexi	1.19	6.68	2.09	1.56	1.87
Fibrobacteres	0.01	0	0.09	0.15	0.12
Spirochaetes	0.83	0.38	0.67	0.95	0.68
Synergistetes	0.01	0.09	0.21	0.2	0.22
Tenericutes	0.31	0.23	0.28	0.44	0.38
TM7	1.11	0.62	0.5	0.74	0.38
Unclassified	0.18	0.07	0.08	0.11	0.13
Genus					
Prevotella	56.5	30	27.5	26.3	29
Ruminococcus	1.15	2.94	0.86	0.93	0.74
Succiniclasticum	1.4	7.86	1.79	1.3	1.79
Coprococcus	1.16	0.25	1.12	2.06	1.29
Comamonas	0	0.06	2.24	3.3	1.29
Butyrivibrio	2.03	1.2	1.59	2.2	1.7
Oscillospira	0.25	0.78	0.73	0.56	0.86
Mogibacterium	0.97	0.55	0.38	0.39	0.5

Clostridium	0.25	0.06	0.28	0.24	0.23
Sphaerochaeta	0.57	0.24	0.18	0.1	0.14
Unclassified	32.7	46.9	56.8	55.9	55.7
Others (<0.5%)	1.67	2.35	3.87	4.33	4

Table 6: Bacterial abundance in Jersey cow inoculum at phylum and genus levels after 24 hr of *in vitro* incubation with four different substrates (percentage of bacteria in total bacteria >0.5%).

Effects of substrates on ruminal methanogen community

After 24 hr of incubation, methanogen Shannon index was increased but Simpson index was decreased by *in vitro* incubation with the four substrates (Table 5). Comparing the four substrates, elephant grass had the highest Shannon index and lowest Simpson index as compared with another substrate (Table 5). Community composition analysis showed, the abundance of *M. gottschalkii* which was the dominant methanogen species, was greatly decreased by *in vitro* incubation with the four substrates (Table 7). The most obvious change observed in methanogen community was increased abundance of Methanomassiliicoccales Group10 sp. substantially after incubation with three gramineous substrates (>21%), especially with Sugarcane tail Silage (>40%, Table 6); while its abundance in the fresh rumen inoculum was very low (0.15%). Comparing the four substrates, methanogen community after incubation with cassava residues appeared with different composition which had higher abundance of *Methanobrevibacter gottschalkii*, but lower abundance of Methanomassiliicoccales Group10 sp. than that of the other three gramineous substrates (Table 7).

Species	Substrate source				
	Fresh rumen inoculum	Cassava residues	Corn straw silage	Elephant grass	Sugarcane tail silage
Methanobrevibacter gottschalkii	88.1	72.3	69.9	59.9	52.5
Methanobrevibacter ruminantium	4.34	19.6	7.02	14.6	5.95
Methanosphaera sp.	0.99	1.04	0.02	0.01	0.02
Methanomassiliicoccales Group10 sp.	0.15	6.67	22	24.2	40.1
Methanomassiliicoccales Group12 sp.	0.02	0.09	0.45	0.52	0.71
Methanobacterium alkaliphilum	0.05	0	0.01	0.01	0.02
Others (<0.5%)	0.25	0.09	0.03	0.03	0.03
Unclassified	3.47	0.19	0.64	0.74	0.73

Table 7: Methanogen abundance in inoculum at genus level after 24 h of *in vitro* incubation with four different substrates. (Percentage of methanogens in total methanogens >0.5%).

Effects of substrates on ruminal protozoal community

After 24 h of incubation, protozoal Chao1 index was greatly increased by *in vitro* incubation with three gramineous substrates (from 161 to >238); however, Shannon index was decreased, and Simpson index was increased by *in vitro* incubation with cassava residues (Table 5). Comparing the four substrates, cassava residues had lower Chao1 and Shannon index, but higher Simpson index than those of the other three gramineous substrates (Table 5). Community composition analysis showed, the abundances of *Entodinium*, Ophryoscolex and Metadinium were decreased, while the abundance of *Ostracodinium* was increased by *in vitro* incubation with the four substrates. In particular, abundance of *Entodinium* was greatly decreased (from 47% to <12%), while abundance of *Ostracodinium* was greatly increased (from 1.5% to >41%) by incubation with three gramineous substrates (Table 8). Besides, abundance of *Diploplastron*, which was a low abundant genus in fresh rumen inoculum and fermented inocula of the three gramineous substrates (<3%), was greatly increased by incubation with cassava residues (from <9.8% to 59%) Comparing of four substrates, cassava residues had much lower abundance of *Ostracodinium*, Polyplastron and Ophryoscolex, and much higher abundance of *Entodinium* and *Diploplastron* than those of other three gramineous substrates. Sugarcane tail silage had lower abundance of *Ostracodinium*, but higher abundance of Diplodinium than those of corn straw silage and elephant grass (Table 8).

Genus	Substrate source				
	Fresh rumen inoculum	Cassava residues	Corn straw silage	Elephant grass	Sugarcane tail silage
Ostracodinium	1.49	2.59	64.2	75.9	41.4
Entodinium	46.7	30.3	10.8	6.76	11.1
Polyplastron	3.58	3.22	11.4	8.24	17.7
Ophryoscolex	19.3	1.68	9.09	5.97	5.43
Anoplodinium.-Diplodinium	4.93	2.04	2.29	1.66	20.1
Metadinium	10.9	0.07	0.01	0.03	0.03
Employer.-*Diploplastron*	9.83	59	0.89	1.12	3.35
Eudiplodinium	1.3	1.07	0.35	0.11	0.49
Epidinium	1.04	0	0.01	0.06	0.03
Dasytricha	0.71	0	0.01	0.08	0.01
Unclassified	0.13	0.01	0.01	0.01	0.01

Table 8: Protozoal abundance in inoculum at genus level after 24 h of *in vitro* incubation with four different substrates (percentage of protozoa in total protozoa >0.5%).

Discussion

Standardized method of *in vitro* batch culture system has been intensively used to evaluate the quality of ruminant's rations recently [30]. Numerous studies have researched the influence of forage source on *in vitro* gas production and rumen fermentation parameters [31,32]. However, studies addressing the changes in ruminal microbial

community composition after *in vitro* incubation with different substrates are limited [33]. In contrast, the current study not only focused the effects of substrate on *in vitro* digestibility of substrates, but it also explored the interaction between forage source and microbial community including bacteria, methanogen and protozoa.

Digestibility of substrates is determined by its *in vitro* gas production. As cassava residues produced highest gas and VFA, indicting it had the highest *in vitro* degradability among all the substrates. The potential reason was higher soluble carbohydrate and the lowest NDF contents of cassava residues among the four substrates; because lower NDF content was always related with higher gas production and digestibility *in vitro* [34,35]. The highest butyrate and the lowest acetate concentrations after incubation with cassava residues were probably due to the increased number of protozoa; because butyrate and acetate are two main VFA produced by protozoa on fermentation of starch and cellulose respectively [36-38].

The batch incubation system is characterized for being unable to keep the microbial growth and population steady for long time due to exhaustion of substrates and accumulation of fermentation products. Besides, response of different microbial groups to *in vitro* incubation was also different which can cause the change in ruminal microbe community profile during *in vitro* incubation [39]. The population of almost every ruminal microbial group in this study including Bacteria, Methanogen, Fungi, *R. albus, F. succinogenes, P. ruminicola* and *S. ruminantium* were decreased after *in vitro* incubation as compared with those in freshly taken ruminal liquor. In particular, fungal population was drastically decreased by incubation with the four substrates. This result was consistent with the findings of Soto et al. [32], who reported that populations of bacteria, fungi, *F. succinogenes, R. flavefaciens* were decreased in fermented inoculum as compared with those in fresh rumen fluid, and this study also reported that bacteria, fungi, methanogens, and *F. succinogenes* were decreased even though a single-flow continuous-cultivation system was used. The rapid disappearance of fermentable substrates can explain the decrease of these microbial groups, especially cellulolytic bacteria and fungi. Whereas, the probable reason behind the drastic decrease of fungal population was the higher sensitivity of anaerobic fungi to *in vitro* incubation system.

Due to different nutritional characteristics of substrates, the response of every microbial group to substrates is also different. This can cause alterations in microbial community profile during *in vitro* incubation [39]. In the present study, *in vitro* incubation with cassava residues resulted in lower populations of bacteria, fungi, *R. albus and F. succinogenes* as compared with those with high fiber containing substrates. These ruminal fiber degrading microbes especially, fungi were decreased in population by incubation with cassava residues which was a low fiber containing substrate. This result indicated low fiber substrate was not beneficial for the growth of fiber degrading microbes during *in vitro* incubation. This result was also consistent with the findings of Saro et al. [40], who reported abundances of ruminal *F. succinogenes, R. flavefaciens* and fungi were higher in high NDF grass hay feeding sheep than in alfalfa hay feeding sheep. Moreover, Huws et al. [41] also reported abundance of ruminal cellulolytic bacteria of steers fed with high NDF grass silage was higher than that fed with low NDF red clover silage.

Firmicutes and Bacteroidetes still stood dominant bacterial phyla after incubation with every substrate. However, abundance of Firmicutes was increased while the abundance of Bcateroidetes was decreased after incubation as compared with those in fresh ruminal fluid; indicating the influence of *in vitro* incubation on bacterial community. At genus level, *Prevotella*, a group of multifunctional key microbe in the rumen was greatly decreased in abundance by *in vitro* incubation with every substrate. *Prevotella* has been reported to be responsible for cellulose, hemicellulose starch and protein degradation in the rumen [42,43]. This suggested that the ability of *in vitro* system to ferment substrate was depressed as compared with that of *in vivo*. This result was consistent with reports addressing that ferment ability of *in vitro* system was lower than that of *in vivo* [44]. Besides, bacterial diversity was increased by *in vitro* incubation which was evidenced by increased Shannon diversity index, but the newly increased bacteria were unclassified bacteria in this study. The possible reason was that when high abundant bacteria such as *Prevotella* were decreased in abundance by *in vitro* incubation, the less abundant species which were fit for *in vitro* environment were increased to take the ecological niche. However, Soto et al. [32] reported bacterial diversity was decreased by *in vitro* batch culture system, which was not consistent with the results of this study. This inconsistent was explained by the difference of method used to explore the diversity. The sequencing method used in this study has higher resolution than terminal-restriction fragment length polymorphism (T-RFLP) which was a method used by Soto et al. [32]; therefore, this study showed higher abundance of unclassified bacterial species than that reported by Soto et al. [32]. Incubation with cassava residues decreased Shannon index, indicating low NDF forages can decrease bacterial diversity. This was consistent with findings of Grilli et al. [45], who reported that goat ruminal bacteria Shannon index was decreased when goat was fed with 60% alfalfa hay as compared to fed with 100% alfalfa hay.

Methanogen population was not greatly influenced by *in vitro* fermentation. However, methanogen community composition was altered, and diversity was increased by incubation with the four substrate. In particular, the abundance of *M. gottschalkii* was decreased, while abundance of Methanomassiliicoccales Group10 sp., previously named as Rumen Cluster C (RCC), was increased after incubation with three gramineous substrates. Methanomassiliicoccales was a group of methanogens that strictly use hydrogen to reduce both methylamines and methanol to methane [46], and degradation products of pectin can promote their growth [47]. Higher abundance of Methanomassiliicoccales in inoculum after incubation with the three gramineous substrates was probably due to higher pectin contents in those substrates, or their fermentation products contained higher methyl compounds; but needs to be further confirmed. RCC are important methane producers in rumen and due to their low abundance, it is hard to get their pure culture. However, in current study we found that *in vitro* incubation of sugarcane tail silage with Jersey cow rumen fluid can elevate the abundance of Methanomassiliicoccales up to more than 40%, indicating forage with high NDF contents was a better source to get Methanomassiliicoccales enriched.

As compared with methanogen and bacterial, protozoal population and diversities were not greatly influenced by *in vitro* incubation, while their community demonstrated variations after incubation. Our study found abundance of low abundant protozoa, such as *Ostracodinium* and *Diploplastron*, and high abundant *Entodinium* in fresh rumen liquor were reversed after *in vitro* incubation with specific substrates. The reason for this change was unclear, but we can predict that this probably be related with inherent variation of protozoal community in ruminants [48], and it has been reported that ruminal protozoal community has a great varation among individual buffalo even though they were feed same diet in same shed. Our study found *Entodinium*

which is a dominant protozoal genus in ruminants, took a larger account of protozoal community (31%) after incubation with cassava residues as compared with the other three gramineous substrates. This result was consistent with findings of Coleman [49], who reported that *Entodinium* has the highest starch uptake rate as compared with another protozoa group. Thus, higher population of *Entodinium* with cassava residues was probably due to high starch contents of this substrate. Whereas, the reason for substantially higher abundance of *Diploplastron* after incubation with cassava residues, and much higher abundance of *Ostracodinium* after incubation with the three gramineous substrates was still unclear. We can predict that NDF contents of these substrates may be the most probable determiners. Though, three gramineous substrates demonstrated similar fermentation patterns and influence on microbial population and community, but incubation with sugarcane tail silage resulted in higher abundances of Methanomassiliicoccales and Diplodinium than those of the other two gramineous substrates; indicated sugarcane tail silage might have special nutritional characteristics to be evaluated further.

Conclusion

The 24 hr *in vitro* batch incubation not only decreased the populations of bacteria, fungi, methanogens and some cellulolytic bacteria, but also changed the microbial community as compared with fresh rumen fluid. The original Firmicutes to Bacteroidetes ratio was reversed and abundance of *Prevotella* was greatly decreased. Cassava residues which is a low NDF contents substrate greatly decreased fungal and cellulolytic bacterial populations, and increased abundances of *Succiniclasticum* and *Diploplastron* as compared with the other three high NDF gramineous substrates. Therefore, it is concluded that NDF contents is important to determine microbial population and community *in vitro*. Besides, Methanomassiliicoccales was greatly enriched after incubation with sugarcane tail silage indicated *in vitro* incubation with sepcial substrate was a potential way to enrich special methanogen.

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Conflict of Interest

We have no conflict of interest.

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