Journal of Animal and Veterinary Advances 9 (11): 1667-1675, 2010
ISSN: 1680-5593

Rumen Fermentation, Microbial Protein Synthesis and Cellulolytic Bacterial Population of Swamp Buffaloes as Affected By Roughage to Concentrate Ratio

Anusorn Cherdthong, Metha Wanapat, Phongthorn Kongmun,
Ruangyote Pilajun and Pichad Khejornsart
Tropical Feed Resources Research and Development Center (TROFREC),
Department of Animal Science, Faculty of Agriculture, Khon Kaen University,
Khon Kaen 40002, Thailand

Abstract: Four rumen-fistulated, male swamp buffalo were randomly assigned according to a 4×4 Latin square design to evaluate the effect of the urea-treated rice straw to concentrate ratio (R:C) on rumen fermentation, nutrient digestibilities, microbial protein synthesis and cellulolytic bacterial population. Animals were fed R:C of 100:0, 75:25, 50:50 and 25:75, respectively. Results showed that digestibility of nutrients were significantly affected by R:C especially those of OM and fiber. However, digestibility of CP, ruminal NH_3-N and plasma urea N were similar among treatments ($p>0.05$) whereas ruminal pH was decreased significantly ($p<0.01$) when concentrate ratio was increased. Total VFA concentrations and C3 were significantly different among treatments and were greatest at 50:50 of R:C supplementation ($p<0.01$). Total viable bacteria, proteolytic bacteria and bacteria cell count were not altered among treatments ($p>0.05$) whereas amylolytic bacteria, cellulolytic bacteria and fungal zoospore were significantly different ($p<0.01$), responding to a change in proportion of R:C. Moreover, using of real-time PCR technique provided that feeding of a 100% roughage remarkably increased these three cellulolytic bacteria numbers up to 3.54×10^9 copies mL^{-1} for *F. succinogenes*, 7.38×10^7 copies mL^{-1} for *R. Flavefaciens* and 5.80×10^6 copies mL^{-1} for *R. albus* in rumen digesta, respectively. It is most notable that *F. succinogenes* were the highest in population in the rumen of swamp buffalo. In addition, efficiency of rumen microbial N synthesis were enriched by R:C supplementation, especially at the ratio of 50:50 ($p<0.05$). Based on this study, it could be concluded that supplementation of R:C at 50:50 improved digestibilities of nutrients, ruminal ecology and microbial protein synthesis efficiency.

Key words: Swamp buffalo, roughage to concentrate ratio, digestibility, rumen fermentation, real-time PCR technique, cellulolytic bacteria

INTRODUCTION

In the tropics, likewise in Thailand, buffaloes and cattle are raised as an integral part of the crop production system especially where rice is the main commodity. Swamp buffalo (*Bubalus bubalis*) are able to utilize feed more efficiently than beef cattle where the feed supply is of low quantity and/or quality. Wanapat (2010) reported that buffalo had different rumen microorganisms than those in beef cattle, particularly the rumen bacteria which belong to >500 different species (Collado and Sanz, 2006) and have the ability to recycle nitrogen to the rumen. Thus, any variations between cattle and swamp buffalo in the proportions and number of rumen bacteria, protozoa and fungal zoospores might attribute to the explanation of the differences in digestive capability due to fermentation end products available for the absorption and utilization by ruminants (Wanapat and Cherdthong, 2009). Feeding the bugs, feeding the cows has been commonly referred to in ruminant production systems since the rumen is an essential fermentation vat to initiate anaerobic fermentation by prevailing microorganisms (bacteria, protozoa and fungi) to produce end-products for animal uses (Dehority and Tirabasso, 2000; Wora-Anu *et al*., 2000).

Bacteria are the most numerous of these microorganisms and play a major role in the biological degradation of dietary fiber. *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens* are presently recognized as the major cellulolytic bacterial species found in the rumen (Koike and Kobayashi, 2001; Koike *et al*., 2003). Recent advances in molecular biology techniques allow the analysis of such bacteria without cultivation, thereby identifying many functional but

Corresponding Author: Metha Wanapat, Tropical Feed Resources Research and Development Center (TROFREC), Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand

uncultured, bacteria as new targets for basic and applied research (Russell *et al*., 2009). Real-time Polymerase Chain Reaction (PCR) has been successfully used for quantifying protozoa, cellulolytic fungi and cellulolytic bacterial species (Wanapat and Cherdthong, 2009; Wanapat *et al*., 2009a; Kongmun *et al*., 2010). This technique is both reliable and simple to perform. Increased knowledge concerning the rumen cellulolytic bacterial population will allow insight into the fiber-digestion capabilities of ruminant animals. However, very limited research has been conducted in swamp buffalo with regard to the ruminal bacterial population using molecular techniques. A number of dietary factors could influence rumen fermentation and microbial population dynamics especially the basal roughage sources, their physical form and fermentation end-products (Wanapat, 2008). Local feed resources particularly low-quality roughages and agricultural residues are of prime importance for ruminants raised in the tropics (Orskov, 1999).

These feeds exhibit close relationships with rumen ecology, microbes and rumen fermentation patterns. Moreover, concentrate diets are generally provided because DMI and VFA production are higher than with diets based only on roughage (Nocek and Tamminga, 1991; Suarez *et al*., 2007). The results show that both roughage and concentrate are important to the efficiency of swamp buffaloes on rumen fermentation or rumen microorganisms (Van Soest *et al*., 1991). Therefore, this study was conducted to determine effects of roughage to concentrate ratio on nutrient digestibility, rumen fermentation, microbial protein synthesis and cellulolytic bacterial population in swamp buffaloes.

MATERIALS AND METHODS

All procedures involving animals were approved by the Ethical Principles for the use of animals for scientific purposes of the National Research Council of Thailand.

Animals, treatments and experimental design: Four rumen-fistulated, 3 years old male swamp buffalo were randomly assigned to receive four ratios of roughage-to-concentrate ratios (R:C) of 100:0, 75:25, 50:50 and 25: 75 in a 4×4 Latin square design. All animals received feed according to respective R:C ratios at 2.2% body weight and urea-treated rice straw (UTS; 5% urea, after method of Wanapat *et al*., 2009b) was used as roughage source on rumen ecology, rumen microorganisms, microbial protein synthesis and digestibility of nutrients. Concentrates are high quality, low fiber feeds such as cereals and milling byproducts that contain a high concentration of digestible energy per unit weight and volume. All animals were kept in individual pens and water was available for *ad libitum* consumption. The experiment was conducted for 4 periods and each period lasted 21days. During the first 14 days, all animals were fed respective diets for *ad libitum* intake whereas during the last 7 days, the animals were moved to metabolism crates for total collection. Chemical composition of concentrate and UTS are shown in Table 1.

Data collection and sampling procedures: Feeds were sampled and fecal samples were collected from the total collection of each individual animal on each treatment during the last 7 days of each period at morning and afternoon feeding. Composited samples were dried at 60°C, ground (1 mm screen using Cyclotech Mill, Tecator) and then analyzed for DM, ether extract, ash, CP content (AOAC, 1990) and NDF and ADF (Van Soest *et al*., 1991). At the end of each period, rumen fluid and jugular blood samples were collected at 0, 2, 4 and 6 h after feeding. At the time of sampling, 10 mL of blood was drawn into each of the tubes. Each tube contained 12 mg of EDTA. Approximately 200 mL of rumen fluid was taken at each time from the middle part of the rumen using a 60 mL hand syringe.

Temperature and pH of rumen fluid were measured using a portable pH and temperature meter (Hanna Instruments HI 8424 microcomputer, Singapore). Rumen fluid samples were then filtered through 4 layers of cheesecloth. Samples were divided into 4 portions; 1 portion was used for NH_3-N analysis with 5 mL of 1 M HSO2 4 added to 50 mL of rumen fluid. The mixture was centrifuged at 16,000× g for 15 min and the supernatant

Table 1: Ingredients and chemical compositions of concentrate and Urea-Treated rice Straw (UTS)

	Ratio	
Item	Concentrate (%)	UTS (%)
Ingredient		
Cassava ship	80.0	-
Rice brand	6.0	-
Coconut meal	3.0	-
Palm kernel	3.0	-
Sulfur	1.0	-
Premix mineral	1.0	-
Molasses	2.0	-
Urea	3.0	-
Salt	1.0	-
Chemical composition (Percentage of DM)		
DM	94.0	55.0
OM	90.6	91.8
CP	14.0	8.2
NDF	13.6	72.5
ADF	7.1	54.4
Ash	9.4	8.2
ME[1] (Mcal Kg^{-1})	2.8	1.9
TDN[1]	83.3	55.1

Calculated values

was stored at -20°C before NH_3-N analysis using the micro-Kjeldahl methods (AOAC, 1990) and VFA analysis using HPLC (Samuel *et al*., 1997). A second portion was fixed with 10% formalin solution in sterilized 0.9% saline solution. The total direct count of bacteria, protozoa and fungal zoospores were made by the methods of Galyean (1989) based on the use of a hemocytometer (Boeco, Hamburg, Germany). A third portion was cultured for groups of bacteria using a rolltube technique (Hungate, 1969) for identifying bacteria groups (cellulolytic, proteolytic, amylolytic and total viable count bacteria). The final portion was stored at -20°C for microbes population analysis using real-time PCR techniques. Community DNA was extracted from 0.25 mL aliquots of each sample by the RBB+C method (Yu and Morrison, 2004) which was shown to substantially increase DNA yields. The quality and quantity of these DNA samples were also determined by agarose gel electrophoresis and spectrophotometry. The primers used for the real time PCR are as follows: primers for *Fibrobactor succinogenes*, Fs219f (5'GGT ATG GGA TGA GCT TGC-3') and Fs654r (5'-GCC TGC CCC TGA ACT ATC- 3') were selected to allow amplification (446-bp product) of all 10 *F. succinogenes* strains deposited in GenBank. For *Ruminococcus albus* primers, Ra1281f (5'-CCC TAA AAG CAG TCT TAG TTC G-3') and Ral 439r (5' CCT CCT TGC GGT TAG AAC A- 3') (175-bp product). *Ruminococcus flavefaciens* primers, Rf154f (5'-TCT GGA AAC GGA TGG TA-3') and Rf425r (5'- CCT TTA AGA CAG GAG TTT ACA A-3') were also selected to allow species-species amplification (295 bp) of all seven *R. flavefaciens* strains deposited in GenBank. All these primer sets were previously published by Koike and Kobayashi (2001). Regular PCR conditions for *F. succinogenes* were as follows: 30 sec at 94°C for denaturing, 30 sec at 60°C for annealing and 30 sec at 72°C for extension (48 cycles) except for 9 min denaturation in the first cycle and 10 min extension in the last cycle. Amplification of 16S rRNA for the other two species was carried out similarly except an annealing temperature of 55°C was used. Quantification of anaerobic fungal population, primer and condition was previously published by Denman and McSweeney (2006). Four sample derived standards were prepared from treatment pool set of community DNA. The regular PCR was used to generate sample derived DNA standards for each real-time PCR assay. Then the PCR product was purified using a QIA quick PCR purification kit (QIAGEN, Inc., Valencia, CA) and quantified using a spectrophotometer. For each sample derived standard, copy number concentration was calculated based on the length of the PCR product and the mass concentration. Tenfold serial dilution wasmade in Tri-EDTA prior to real-time PCR (Yu *et al*., 2005). In total, 4 real-time PCR standards were prepared. The conditions of the real-time PCR assays of target genes were the same as those of the regular PCR described above. Biotools QuantiMix EASY SYG KIT (B and M Labs, S. A., Spain) was used for real-time PCR amplification. All PCRs were performed in duplicate.

A blood sample (about 10 mL) was collected from a jugular vein (at the same time as rumen fluid sampling) into tubes containing 12 mg of EDTA and plasma was separated by centrifugation at 500× g for 10 min and stored at -20°C until analysis of plasma urea N according to the method of Crocker (1967). Urine samples were analyzed for total N (IAEA, 1997) and allantoin in urine was determined by HPLC as described by Chen and Gomes (1995). The amount of microbial purines absorbed was calculated from purine derivative excretion based on the relationship derived by Chen and Gomes (1995).

Statistical analysis: Statistical analyses were performed using the GLM procedure (SAS Inst. Inc., Cary, NC). Data were analyzed using the model

$$Y_{ijk} = \mu + M_i + A_j + P_k + \epsilon_{ijk}$$

Where:

Y_{ijk} = Observation from animal j, receiving diet i in period k
μ = The overall of mean
M_i = The mean effect of different ratio of dietary (i = 1, 2, 3, 4)
A_j = The effect of animal (j = 1, 2, 3, 4)
P_k = The effect of period (k = 1, 2, 3, 4)
ϵ_{ijk} = The residual error

Treatment means were statistically compared by the new multiple range test of Duncan (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Chemical composition of feeds: The chemical compositions of concentrate and UTS are shown in Table 1. Concentrate diets contained 14.0% CP and 83.3% TDN on a DM basis. The concentrate contained 80% cassava chip and therefore, low NDF. The UTS contained 55.0% DM and 8.2% CP on a DM basis and was similar to that reported by Wanapat *et al*. (2009b).

Effect on feed intake and digestibility: The effects of R:C ratio on feed intake of swamp buffalo are shown in Table 2. Initial BW and BW change were not altered among treatments and the values were stable at 422.3-433.3 kg and 0.3-0.6 kg day^{-1}, respectively. Total

Table 2: Feed intakes and apparent digestibility of buffaloes fed different ratio of dietary

	Roughage		Concentrate ratio			Contrast[1]		
Item	100:0	75:25	50:50	25:75	SEM	L	Q	C
Initial BW (kg)	433.3	425.0	423.8	422.3	4.81	NS	NS	NS
BW change (kg day^{-1})	0.3	0.6	0.5	0.3	2.22	NS	NS	NS
DM intake/day/kg	9.4	9.1	9.2	9.0	0.16	NS	NS	NS
BW%	2.2	2.2	2.2	2.2	0.02	NS	NS	NS
g/kgW$^{0.75}$	98.7	96.2	98.9	98.7	1.10	NS	NS	NS
Apparent digestibility (%)								
DM	67.6	69.4	70.8	68.2	3.54	NS	NS	NS
OM	71.9^{a}	73.1^{b}	77.2^{c}	72.9^{b}	1.99	NS	*	NS
CP	53.0	54.8	56.5	52.4	3.95	NS	NS	NS
NDF	58.7^{a}	55.9^{b}	50.4^{c}	45.5^{d}	0.80	*	NS	NS
ADF	50.8^{a}	45.8^{b}	42.4^{c}	40.2^{d}	0.54	**	NS	*

$^{a-c}$Values within the same row not bearing a common superscript differ ($p<0.05$), [1]Linear (L), quadratic (Q) and cubic (C) effects of different ratio of dietary. * = $p<0.05$, ** = $p<0.01$ and NS = $p>0.05$

Table 3: Rumen temperature, NH_3- N, BUN and Volatile Fatty acid (VFA) concentration as affected by feeding different ratio of dietary

	Roughage		Concentrate ratio			Contrast[1]		
Item	100:0	75:25	50:50	25:75	SEM	L	Q	C
Ruminal pH	7.0^{a}	6.6^{b}	6.4^{c}	6.2^{d}	0.05	**	NS	NS
Ruminal temperature (°C)	39.1	39.1	39.0	38.6	0.56	NS	NS	NS
NH_3- N (mg %)	20.3	18.0	19.2	18.6	3.60	NS	NS	NS
Plasma urea N (mg %)	16.3	14.6	15.6	12.9	5.29	NS	NS	NS
Total VFA (mM L^{-1})								
VFAs, mol/100 mol	107.5^{a}	118.4^{b}	120.7^{b}	112.0^{c}	1.01	**	**	NS
Acetate (C2)	77.5^{a}	74.5^{b}	66.0^{c}	65.8^{c}	0.47	**	NS	**
Propionate (C3)	17.8^{a}	19.3^{ab}	23.5^{ab}	19.4^{c}	0.45	NS	*	NS
Butyrate (C4)	4.8^{a}	6.3^{a}	10.6^{b}	13.8^{b}	0.65	**	NS	NS
C2: C3 ration	4.4^{a}	3.9^{b}	2.8^{c}	3.4^{d}	0.09	**	NS	NS
C2 + C4: C3 ration	4.6^{a}	4.2^{b}	3.3^{c}	4.1^{b}	0.12	NS	*	NS

$^{a-c}$Values within the same row not bearing a common superscript differ ($p<0.05$), [1]Linear (L), quadratic (Q) and cubic (C) effects of different ratio of dietary, * = $p<0.05$, ** = $p<0.01$ and NS = $p>0.05$

DMI was not significantly affected ($p<0.05$) by R:C ratio. Digestibilities of OM, NDF and ADF were significantly different ($p<0.05$) among treatments. Digestibility of OM was highest in R:C ratio at 50:50 while NDF, ADF were highest in R:C ratio at 100:0. Digestion coefficients of OM were enhance appreciably by increasing level of concentrate regardless of type of straw. Readily available energy provide by concentrate could have attributed largely to the increase. However, the value for NDF and ADF were slightly depressed particularly the highest level of concentrate. It has been suggested that high concentrate ratio with readily degradable carbohydrate (cassava chip) in rumen could decrease ruminal pH and depressed fiber digestion as similar result was reported by Sung *et al.* (2007).

Characteristics of ruminal fermentation and blood metabolites in cattle: Measured rumen variables included temperature, pH, NH_3-N and VFA. Plasma urea N was also determined to investigate the relationship with rumen NH_3-N and protein utilization. The pattern of ruminal fermentation and overall means are shown in Table 3. Significant differences in rumen fluid pH were found in current study ($p<0.05$). Increasing the levels of concentrate in the ration effected a reduction in the rumen pH. As a general rule, the rumen pH is higher in animals fed a roughage-type diet than those fed concentrates. Under this study, fiber digestion decreases at low rumen pH, especially below pH 6.0 as observed previously in studies using continuous culture of mixed ruminal microorganisms *in vitro* rumen culture (Hu *et al*., 2005), *in sacco* disappearance. Rumen fluid temperature were not altered among treatments and the values were stable at temperature of 38.6-39.1°C. Ruminal NH_3-N is a major source of N for microbial protein synthesis (Bryant, 1974; Wanapat *et al*., 2008). Ruminal NH_3-N concentrations were 18.0-20.3 mg dL^{-1} and were close to those previously reported by Church and Santos (1981). Concentrations of plasma urea N are highly correlated to the concentration of NH_3 production in the rumen (Preston *et al*., 1965). There were also no significant differences ($p>0.05$) in plasma urea N concentrations.

The production of total VFA, acetate acid, propionic acid and butyric acid proportions, acetic:propionic ratio and acetic plus butyric:propionic ratio are shown in Table 3. The influence of R:C ratio on total VFA concentrate, production of total VFA, C2, C3, C4, C2: C3 and C2 + C4: C3 were significantly different ($p<0.01$).

Table 4: Effect of levels of roughage to concentrate ratio on ruminal microbes and variable bacteria in swamp buffaloes

	Roughage		Concentrate ratio			Contrast[1]		
Item	100:0	75:25	50:50	25:75	SEM	L	Q	C
Ruminal microbes (cell/g)								
Bacteria ($\times10^{11}$)	6.9	7.3	7.1	6.8	2.05	NS	NS	NS
Protozoa ($\times10^{6}$)	1.7[a]	3.1[b]	3.5[b]	5.8[c]	1.54	**	NS	NS
Fungal zoospore ($\times10^{4}$)	2.1[a]	2.2[a]	1.9[b]	1.7[b]	0.87	*	NS	NS
Variable bacteria (CFU mL^{-1})								
Total ($\times10^{9}$)	11.1	11.2	11.0	10.8	1.68	NS	NS	NS
Amylolytic ($\times10^{8}$)	1.4[a]	1.7[a]	4.4[b]	5.6[c]	0.17	**	*	**
Proteolytic ($\times10^{7}$)	5.5	4.5	3.5	5.0	2.03	NS	NS	NS
Cellulolytic ($\times10^{9}$)	10.9[a]	10.9[a]	10.4[b]	10.3[b]	0.07	**	NS	**

[a-c]Values within the same row not bearing a common superscript differ ($p<0.05$), [1]Linear (L), quadratic (Q) and cubic (C) effects of different ratio of dietary. * = $p<0.05$, ** = $p<0.01$ and NS = $p>0.05$

High-concentrate diets tend to ferment toward propionate and low rumen pH also results in greater ruminal propionate molar proportions (Calsamiglia *et al*., 2008). Mean total VFAs and C3 concentrations were highest and C2: C3 and were lowest in R:C ratio at 50:50. Such increase indicated an improvement of ruminal fermentation by the microbes. Total VFA concentrations in all treatments ranged from 107.5-120.7 mM and were similar to those reported by France and Siddons (1993).

Rumen microorganism population: Table 4 shows the rumen microorganism population data. Ruminal microbial counts and variable bacteria were significantly different ($p<0.01$) among treatments; protozoa and amylolytic bacteria were greatest when supplemented R:C ratio at 25:75 (5.8×10^{6} and 5.6×10^{8} cell g^{-1}, respectively). Moreover, cellulolytic bacteria were increased when supplemented R:C ratio at 100:0 and 75:25 (10.9×10^{9} cell g^{-1}) which correlates with the greatest NDF and ADF digestibility.

These results showed effects of R:C ratio which changed diversity of rumen microorganism. Increasing concentrate ratio affected to decline of cellulose degradation efficiency at lower pH was attributed to the inhibition of cellulolytic bacteria, since most ruminal cellulolytic bacteria are pH-sensitive (Russell and Wilson, 1996). The pH sensitivity can be explained by intracellular pH regulation of cellulolytic bacteria. When the extracellular pH of acid-sensitive bacteria declines, the intracellular pH is relatively stable but the increase in the transmembrane pH gradient cause a logarithmic accumulation of intracellular fermentation acid anions and hence leads to anion toxicity and product inhibition (Russell and Wilson, 1996).

On the other hand, Wora-Anu *et al.* (2000) reported that R:C ratios of 100:0, 60:40 and 40:60 could decrease the cellulolytic bacterial population in swamp buffalo (5.62×10^{10}, 4.06×10^{10} and 4.57×10^{10} CFU mL^{-1}), respectively. Under the current study were also found that supplementation of R:C ratio at 25:75 could reduced cellulolytic bacterial.

Population of *R. albus*, *F. succinogenes*, *R. flavefaciens*, fungal and protozoa in the rumen incubation: External standards for real-time PCR were prepared from a simulated rumen matrix. For each standard, linear regressions derived from the threshold cycle [C(T)] of each DNA dilution versus the log quality (Fig. 1) were calculated. Logarithms of the DNA concentration (copies mL^{-1}) were plotted against the calculated means (Fig. 1) obtaining astraight line of equations $y = -0.3718x + 11.72$, $y = -0.1337x + 9.57$, $y = -0.4956x + 14.09$ and $y = -0.3139x + 12.7$ (where y is the log of DNA concentration and x is the Ct) with a linear correlation coefficient (r^2) of 0.993, 0.995, 0.997 and 0.991 for *F. succinogenes* (Fig. 1a), *R. flavefaciens* (Fig. 1b), *R. albus* (Fig. 1c) and anaerobic fungi (Fig. 1d), respectively.

The equations were used to quantify DNA from rumen digesta samples. The accuracy of each real-time PCR was validated by quantifying known numbers of target species templates (*F. succinogenes, R. flavefaciens, R. albus* and anaerobic fungi) are shown in Table 5. All supplemented treatments had effects on *F. succinogenes*, *R. flavefaciens*, *R. albus* and anaerobic fungi population ($p<0.01$). *F. succinogenes* was most dominant (10^{9} copies mL^{-1} of rumen digesta) among the three species followed by *R. flavefaciens* (10^{7} copies mL^{-1} of rumen digesta) and *R. albus* (10^{6} copies mL^{-1} of rumen digesta).

Similarly, Koike and Kobayashi (2001) and Russell and Rychlik (2001) reported that *F. succinogenes* was the major cellulolytic bacteria of rumen digesta in sheep and were present at only 0.1% of total population and that ruminococci were relatively minor. The dynamics of cellulolytic bacteria were in good correlation with the response to diet shift, particularly the changes of concentrate (Kobayashi, 2006; Mosoni *et al*., 2007). In this study, feeding of a 100% urea-treated rice straw remarkably increased these three cellulolytic bacteria numbers up to 3.54×10^{9} copies mL^{-1} for *F. succinogenes*, 7.38×10^{7} copies mL^{-1} for *R. Flavefaciens* and 5.80×10^{6} copies mL^{-1} for *R. albus* in rumen digesta, respectively.

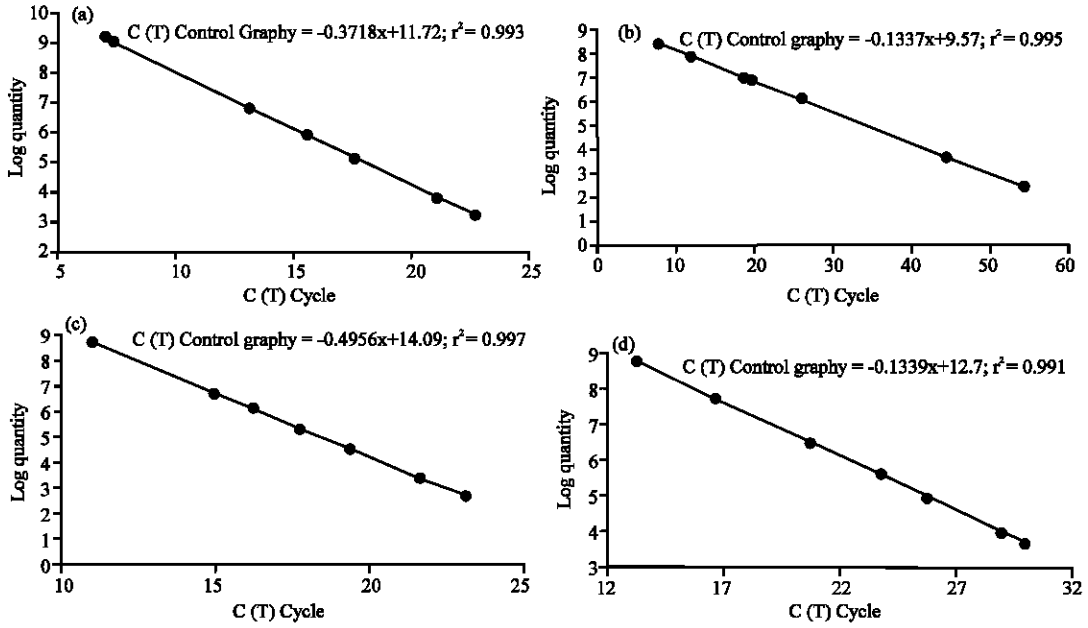

Fig. 1: Standard curve obtained by plotting the logarithm of the DNA concentration for *F. succinogene* (a), *R. favefaciens* (b), *R. albus* (c) and anaerobic fungi vs. threshold cycle (Ct) mean values by using real- time PCR

Table 5: Comparative quantity of predominant cellulolytic bacterial and anaerobic fungal population using real-time PCR techniques

	Roughage		Concentrate ratio			Contrast[1]		
Item	100:0	75:25	50:50	25:75	SEM	L	Q	C
Real-time PCR technique, copies mL^{-1} of incubation								
F. succinogenes ($\times 10^9$)	3.54^a	3.34^a	3.03^b	2.93^b	0.12	*	NS	NS
R. flavefaciens ($\times 10^7$)	7.38^a	5.55^b	2.70^c	2.58^c	0.50	**	NS	NS
R. albus ($\times 10^6$)	5.80^a	5.06^a	3.46^b	3.64^b	0.39	**	NS	*
Anaerobic fungal ($\times 10^7$)	2.30^a	2.00^a	1.50^b	1.50^b	0.22	*	NS	NS

$^{a-c}$Values within the same row not bearing a common superscript differ ($p<0.05$), [1]Linear (L), quadratic (Q) and cubic (C) effects of different ratio of dietary, * = $p<0.05$, ** = $p<0.01$ and NS = $p>0.05$

The proportion of roughage in the diet might influence the population size or the proportion of cellulolytic bacterial numbers in the rumen. In addition, the three cellulolytic bacteria numbers examined in the present study were significantly different, responding to a change in proportion of urea-treated rice straw and concentrate. As the results show, the lowest numbers of the three cellulolytic bacteria were found when increasing the level of concentrate.

It is possible that dietary conditions might have influenced on reduced numbers of cellulolytic bacteria. Moreover, rumen pH together with microbial population, nature of substrates, environmental factors such as temperature and the existence of cations and soluble carbohydrates have been suggested as factors governing bacterial attachment (Miron *et al*., 2001). Ruminal pH is one of most important of these factors because the cellulolytic bacteria numbers are very sensitive to the pH change (Sung *et al*., 2007). When ruminants are fed fiber-deficient rations, ruminal pH declines, microbial ecology is altered and the animals become more susceptible to metabolic disorders. As Koike *et al*. (2003) quantified the cell numbers of *F. succinogenes*, *R. flavefaciens* and *R. albus*, attached to straw and they were analyzed by competitive PCR showing that the numbers of all the three species increased gradually with increased neutral-detergent fiber disappearance. In addition, Tajima *et al*. (2001) reported that the quantity of *F. succinogenes* DNA predominant in animals on the hay diet fell 20-fold on the third day of the switch to a grain diet and further declined on day 28 with a 57-fold reduction in DNA.

Therefore, in this experiment, the quantification of bacterial DNA demonstrated the decreases of the three cellulolytic bacteria numbers (*F. succinogenes, R. flavefaciens and R. albus*) as being influenced by higher concentrate feeds. Moreover, a pattern for the anaerobic

Table 6: Effect of different ratio of dietary on nitrogen balance, excretion of urine derivatives (PD) and microbial nitrogen supply in swamp buffaloes

	Roughage		Concentrate ratio			Contrast[1]		
Item	100:0	75:25	50:50	25:75	SEM	L	Q	C
Nitrogen balance (g day^{-1})								
N intake	106.2	102.8	104.5	101.6	3.55	NS	NS	NS
Faecal N	25.8^{a}	22.6^{b}	17.1^{c}	25.2^{a}	0.61	NS	NS	**
Urinary N	35.1	36.5	37.9	37.4	4.01	NS	NS	NS
N absorption	80.4^{a}	80.2^{a}	87.4^{b}	76.4^{c}	0.08	**	*	NS
N retention	45.3^{a}	43.7^{a}	49.5^{b}	39.0^{c}	10.40	*	*	NS
PD (mmol day^{-1})								
Allantoin excretion	37.3^{a}	36.7^{a}	45.8^{b}	39.3^{c}	1.43	NS	**	NS
Allantoin absorption	25.6	24.7	35.7^{b}	28.7^{c}	1.12	NS	NS	NS
Microbial protein supply[2] (g N day^{-1})	48.6^{a}	47.6^{a}	57.5^{b}	50.4^{c}	2.01	NS	*	NS
EMNS[3], gN/ kg OMDR	30.1^{a}	30.4^{a}	34.1^{b}	32.1^{a}	1.54	NS	*	NS

$^{a-c}$Values within the same row not bearing a common superscript differ ($p<0.05$), [1]Linear (L), quadratic (Q) and cubic (C) effects of different ratio of dietary, [2]Microbial N (g of N day^{-1}) = 0.727×total absorption of PD, [3]EMNS = efficiency of microbial N synthesis [g of N kg^{-1} of OM Digested in the Rumen (OMDR)], assuming that rumen digestion = 65% of digestion in total tract (ARC, 1990)

fungal population was revealed with a significant increase from 1.5×10^{7}-2.3×10^{7} copies mL^{-1} when roughage ratio was increased ($p<0.01$).

N balance and eficiency of microbial protein synthesis: As shown in Table 6, N intake and Urinary N were not affected by treatments. However, faecal N, N absorption and N retention were affected by supplementation of R:C ratio at 50:50. With regards to N utilization, Owens and Zinn (1988) stated that N excretion and N retention should reflect differences in N metabolism because N retention was the most important index of the protein nutrition status of ruminants. Supplementation of R:C ratio at 50:50 resulted in the greatest allantoin excretion and allantoin absorption suggesting that any increase in microbial N synthesis was related to increases in diet digestion. Microbial N supplies from the rumen are shown in Table 6. The microbial N supply as calculated from purine derivative excretion using the equation of Chen and Gomes (1995) ranged from 48.6-57.5 g of N day^{-1}. The efficiency of rumen microbial protein synthesis was also increased as R:C ratio at 50:50.

CONCLUSION

Ratio of roughage to concentrate at 50:50 was significantly effected on ruminal pH, OM, fiber digestion, VFA production as well as microbial nitrogen synthesis. Higher ruminal pH and higher cellulolytic bacteria were obtained as result of higher ratio of roughage. Moreover, applicability of real-time PCR techniques for the quantification of cellulolytic bacterial numbers (*F. succinogenes, R. albus* and *R. flavefaciens*) in the rumen digesta of swamp buffalo have provided additionally useful data. *F. succinogenes* was found to be the most predominant of the three species and influenced by the roughage to concentrate ratio. Results obtained here in could be used in manipulating feeding regimes for swamp buffalo.

ACKNOWLEDGEMENTS

The researchers wish to express sincere thanks to the Tropical Feed Resources Research and Development Center (TROFREC), Khon Kaen University, Thailand and to the Thailand Research Fund (TRF) through the Royal Golden Jubilee Ph.D. Program for providing financial support of research and research facilities and experimental animals. Laboratory equipment provided by the Agricultural Biotechnology Research Center, Khon Kaen University, Thailand was greatly appreciated.

REFERENCES

AOAC, 1990. Official Methods of Analysis. 15th Edn., Association of Official Analytical Chemists, Washington, DC., USA., pp: 200-210.

CARC, 1990. The nutrient requirements of ruminant livestock. Technical Review by an Agricultural Research Council Working Party, UK.

Bryant, M.P., 1974. Nutritional features and ecology of predominant anaerobic bacteria of the intestinal tract. Am. J. Clin. Nutr., 27: 1313-1319.

Calsamiglia, S., P.W. Cardozo, A. Ferret and A. Bach, 2008. Changes in rumen microbial fermentation are due to a combined effect of type of diet and pH. J. Anim. Sci., 86: 702-711.

Chen, X.B. and M.J. Gomes, 1995. Estimation of Microbial Protein Supply to Sheep and Cattle Based on Urinary Excretion of Purine Derivative-an Overview of the Technique Details. IFRU, RRI, Aberdeen, UK.

Church, D.C. and A. Santos, 1981. Effect of graded levels of soybean meal of non-protein nitrogen molasses supplement on consumption and digestibility of wheat straw. J. Anim. Sci., 53: 1609-1615.

Collado, M.C. and Y. Sanz, 2006. Quantification of mucosa-adhered microbiota of lambs and calves by the use of culture methods and fluorescent *in situ* hybridization coupled with flow cytometry techniques. Vet. Microbiol., 121: 299-306.

Crocker, C.L., 1967. Rapid determination of urea nitrogen in serum or plasma without deproteinization. Am. J. Med. Technol., 33: 361-365.

Dehority, B.A. and P.A. Tirabasso, 2000. Antibiosis between ruminal bacteria and ruminal fungi. Appl. Environ. Microbiol., 66: 2921-2927.

Denman, S.E. and C.S. McSweeney, 2006. Development of a real time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. FEMS Microbiol. Ecol., 58: 572-582.

France, J. and R.C. Siddons, 1993. Volatile Fatty Acid Production. In: Quantitative Aspects of Ruminant Digestion and Metabolism, Forbes, J.M. and J. France (Eds.). CAB International, Willingford, UK., pp: 107-122.

Galyean, M., 1989. Laboratory Procedure in Animal Nutrition Research. 1st Edn., Department of Animal and Life Science, New Mexico States University, USA., pp: 162-167.

Hu, Z.H., H.Q. Tu and R.F. Zhu 2005. Influence of particle size and pH on anaerobic degradation of cellulose by rumen microbes. Int. Biodeter. Biodegr., 55: 233-238.

Hungate, R.E., 1969. A Roll Tube Method for Cultivation of Strict Anaerobes. In: Methods in Microbiology, Norris, J.R. and D.W. Ribbons (Eds.). Academic Press, Inc., New York, pp: 117-132.

IAEA, 1997. Estimation of the Rumen Microbial Protein Production from Purine Derivatives in Rumen. Animal International Atomic Energy Agency, Vienna, Austria, pp: 31–37.

Kobayashi, Y., 2006. Inclusion of novel bacteria in rumen microbiology: Need for basic and applied science. Anim. Sci. J., 77: 375-385.

Koike, S. and Y. Kobayashi, 2001. Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobactor succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. FEMS Microbiol. Lett., 204: 361-366.

Koike, S., J. Pan, Y. Kobayashiand and K. Tanaka, 2003. Kinetics of *in sacco* fiber-attachment of representative ruminal cellulolytic bacteria monitored by competitive PCR. J. Dairy Sci., 86: 1429-1435.

Kongmun, P., M. Wanapat, P. Pakdee and C. Navanukraw, 2010. Effect of coconut oil and garlic powder on *in vitro* fermentation using gas production technique. Livest. Sci., 127: 38-44.

Miron, J., D. Ben-Ghedalia and M. Morrison, 2001. Invited review: Adhesion mechanisms of rumen cellulolytic bacteria. J. Dairy Sci., 84: 1294-1309.

Mosoni, P., F.C. Durand, C.B. Maillet and E. Forano, 2007. Quantification by Real-time PCR of cellulolytic bacteria in the rumen of sheep after supplementation of a forage diet with readily fermentable carbohydrates: Effect of a yeast additive. J. Applied Microbiol., 103: 2676-2685.

Nocek, J.E. and S. Tamminga, 1991. Site of digestion of starch in the gastrointestinal tract of dairy cows and its effect on milk yield and composition. J. Dairy Sci., 74: 3598-3629.

Orskov, E.R., 1999. Supplement strategies for ruminants and management of feeding to maximize utilization of roughages. Prev. Vet. Med., 38: 179-185.

Owens, F.N. and R. Zinn, 1988. Protein Metabolism of Ruminant Animals. In: The Ruminant Animal Digestive Physiology and Nutrition, Church, D.C. (Eds.). Waveland Press, Illinois, pp: 227-249.

Preston, R.L., D.D. Schnakanberg and W.H. Pfander, 1965. Protein utilization in ruminants: I. Blood urea nitrogen as affected by protein intake. J. Nutr., 86: 281-287.

Russell, J.B. and D.B. Wilson, 1996. Why are ruminal cellulolytic bacteria unable to digest at low pH. J. Dairy Sci., 79: 1503-1509.

Russell, J.B. and J.L. Rychlik, 2001. Factors that alter rumen microbial ecology. Science, 292: 1119-1122.

Russell, J.B., R.E. Muck and P.J. Weimer, 2009. Quantitative analysis of cellulose degradationand growth of cellulolytic bacteria in therumen. FEMS Microbiol. Ecol., 67: 183-197.

Samuel, M., S. Sagathewan, J. Thomas and G. Mathen, 1997. An HPLC method for estimation of volatile fatty acids of ruminal fluid. Indian J. Anim. Sci., 69: 805-807.

Steel, R.G.D. and J.H. Torrie, 1980. Principles and Procedures of Statistics. 1st Edn., McGaw Hill Book Co. Inc., New York, USA.

Suarez, B.J., C.G. van Reenen, N. Stockhofe, J. Dijkstra and W.J.J. Gerrits, 2007. Effect of roughage source and roughage to concentrate ratio on animal performance and rumen development in veal calves. J. Dairy Sci., 90: 2390-2403.

Sung, H.G., Y. Kobayashi, J. Chang, A. Ha, H. Hwang and J.K. Ha, 2007. Low ruminal pH reduces dietary fiber digestion via reduced microbial attachment. Asian-Aust. J. Anim. Sci., 20: 200-207.

Tajima, K., T. Nagamine, H. Matsui, M. Nakamura and R.I. Aminov, 2001. Phylogenetic analysis of archaeal 16S rRNA libraries from the rumen suggests the existence of a novel group of archaea not associated with known methanogens. FEMS Microbiol. Lett., 200: 67-72.

Van Soest, P.J., J.B. Robertson and B.A. Lewis, 1991. Methods for dietary fiber, neutral detergent fiber and *Nonstarch polysaccharides* in relation to animal nutrition. J. Dairy Sci., 74: 3583-3597.

Wanapat, M. and A. Cherdthong, 2009. Use of real-time PCR technique in studying rumen cellulolytic bacteria population as affected by level of roughage in Swamp buffalo. Curr. Microbiol., 58: 294-299.

Wanapat, M., 2008. Potential uses of local feed resources for ruminants. Trop. Anim. Health. Prod., 41: 1035-1049.

Wanapat, M., 2010. Current researches towards rumen fermentation and microbial ecology of swamp buffaloes. Proceedings of the International Conference on Biochemistry and Medical Chemistry, Feb. 23-25, Cambridge, UK., pp: 431-435.

Wanapat, M., A. Cherdthong, P. Pakdee and S. Wanapat, 2008. Manipulation of rumen ecology by dietary lemongrass (*Cymbopogon citratus* Stapf.) powder supplementation. J. Anim. Sci., 86: 3497-3503.

Wanapat, M., R. Pilajun and P. Kongmun, 2009a. Ruminal ecology of swamp buffalo as influenced by dietary sources. Anim. Feed Sci. Tech., 151: 205-214.

Wanapat, M., S. Polyorach, K. Boonnop, C. Mapato and A. Cherdthong, 2009b. Effects of treating rice straw with urea or urea and calcium hydroxide upon intake, digestibility, rumen fermentation and milk yield of dairy cows. Livest. Sci., 125: 238-243.

Wora-Anu, S., M. Wanapat, C. Wachirapakorn and N. Nuntaso, 2000. Effects of roughage to concentrate ratio on ruminal ecology and voluntary feed intake in cattle and swamp buffaloes fed on urea-treated rice straw. Asian Aust. J. Anim. Sci., 13: 236-236.

Yu, Z. and M. Morrison, 2004. Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques, 36: 808-812.

Yu, Z., F.C. Jr. Michel, G. Hansen, T. Wittum and M. Morrison, 2005. Development and application of real-time PCR assays for quantification of genes encoding tetracycline resistance. Applied Environ. Microbiol., 71: 6926-6933.