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Studies of Diversity of Rumen Microorganisms and Fermentation in Swamp Buffalo Fed Different Diets

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Abstract: Rumen microorganism community population and diversity in the rumen of four ruminal fistulated swamp buffloes (*Bubalus bulalis*) were assessed by 4 types of libraries: Microscopic; roll-tube; real-time PCR; and PCR-DGGE libraries. All buffaloes were randomly assigned in a 2×2 factorial arrangement in a 4×4 LSD to receive 4 dietary treatments; factor A = 2 sources of roughage (rice straw; RS and 2 urea 2% lime treated rice straw; TRS), factor B = 2 level of urea in concentrate mixture (0 and 4% urea). In addition, rumen fermentation parameters were also estimated to determine the relationship between microorganism changes to rumen fermentation efficiency. It was found that total rumen bacteria, *F. succinogenes, R. albus* cellulolytic bacteria and fungal population were greater by dietary treatment with urea-lime treated rice straw and 4% urea in concentrate mixture while *R. flavefaciens*, protozoal population were significantly reduced ($p<0.05$) and amylolytic, proteolytic bacterial groups, total bacteria were not changed among treatments. The population of rumen methanogenic bacteria was reduced across treatments while its diversity was not changed with 18 different bands in each lane. The ruminal pH, ruminal NH_3-N, BUN concentration, propionic acid and acetic acid were significantly increased ($p<0.05$) when buffaloes consumed both urea-lime treated rice straw and concentrate containing 4% urea. Based on this study, the combination of urea-lime treated rice straw and 4% urea in concentrate supplementation could improve rumen fermentation efficiency, rumen fibrolytic microbes' quantity and rumen ecology for the host buffaloes.

Key words: Rice straw, rumen microorganism, fermentation, real-time PCR, PCR-DGGE, parameters

INTRODUCTION

Rumen microorganisms (bacteria, protozoa and fungi) have a symbiosis relationship with host ruminants they form the key link between the dairy animal and diet degradation and utilization for production of VFA and microbial protein (Weimer, 1998). To improve ruminant productivity, rumen function has been strongly manipulated by supplementing the roughage treated diets with readily fermentable carbohydrate and protein. The relative proportions of various nutrients that are available for absorption by the animal can be expected to change according to the population and activities of individual microbial species and these changes in turn can affect animal production.

Chemical treated rice straw have been worldwide researched and developed in order to enhance the utilization of rice straw as roughage source for ruminants. And the practical methods for treating rice straw with sodium hydroxide (Homb, 1984), ammonium hydroxide (Orden *et al*., 2000) or urea (Wanapat *et al*., 1985) to improve its digestibility and intake has been accepted. However, these approaches have so far been limited mainly to developing countries due to economical, technological constraints and environmental reasons. In an attempt to search for possible alternatives, rice straw treatments using urea plus lime have been studied by Trach *et al*. (2001), Fadel Elseed *et al*. (2003) and Wanapat *et al*. (2009a) who reported that when amount of urea was reduced and combined with calcium hydroxide the improvement in rumen degradability and milk yield were obtained, respectively. In addition, urea has long been known to be a successful replacement for some of degradable true protein in roughage basal diets (Franzolin *et al*., 2010) however the combination of urea-lime treated rice straw and urea level in concentrate has not yet been evaluated. Moreover, to study in diversity of microorganism in the rumen, using only

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conventional methods such as roll-tube technique (Hungate, 1969) expressed only the microbial diversity grossly because of uncultivated of some rumen microbes in the culture medium. Consequently, the modern molecular techniques with the accurate and enumeration of rumen microorganism has been widely applying. Therefore, the objective of this study was to study; the structure of rumen microorganism using real-time PCR and PCR-DGGE technique and rumen fermentation by effect of different level of urea supplementation in swamp buffalo fed urea-lime treated rice straw.

MATERIALS AND METHODS

Animals, diets, experimental design and animal management: About 4 rumen fistulated swamp buffaloes (*Bubalus bubalis*) with an average body weight of 369±28 kg were used in this experiment. All animals were housed in individual pen and given feed twice a day with roughage *ad libitum*, concentrate at 0.3% of body weight. Clean, fresh water and mineral-salt block were offered freely.

The experiment was a 2×2 factorial arrangement in a 4×4 latin square design to examine urea-lime treated rice straw with urea level in concentrate as supplements on ruminal fermentation characteristic and feed intake, digestibility of nutrients. The dietary treatments were as follows: untreated rice straw+0% urea in concentrate (RS0%U as control); Untreated rice straw+4% urea in concentrate (RS4%U); 2% urea plus 2% lime treated rice straw+0% urea in concentrate (TRS0%U); 2% urea plus 2% lime treated rice straw+4% urea in concentrate (TRS4%U). Urea-lime TRS was prepared by using 2 kg of urea plus 2 kg of lime, mixed with 100 kg of water then poured mixture water to 100 kg of RS (5 bales of RS, each bale weighting approximate 20 kg) and then covered with a plastic sheet for a 14 days before feeding to animals (Wanapat *et al*., 1985). Concentrate diets were made from ingredients as shown in Table 1 with different percentage of soybean meal and urea as N sources.

The experiment was conducted for 4 periods; each experimental period lasted for 21 days. During the first 14 days, all animals were fed with their respective diets and during the last 7 days animals were moved to metabolism crates for total collection of feces and urine. Body weights were measured twice at the beginning and at the end of each period.

Data collection, sampling procedures and statistical analysis: Rumen fluid was taken at the last day of each periods for 4 periods at 0, 2, 4 and 6 h post feeding and divided into 4 portions for direct count of total fungi and protozoa by microscope; roll-tube count of 3 bacteria groups, amylolytic, proteolytic and cellulolytic bacteria; extraction DNA for real-time PCR and PCR-DGGE in order to determine the number of total bacteria, 3 dominant cellulolytic bacteria and the number and diversity of methanogenic bacteria; last portion, 50 mL of rumen fluid was add with 5 mL of 1 M H_2SO_4 for NH_3-N analysis (AOAC, 1985) and VFA analysis using HPLC (Samuel *et al*., 1997). Blood sample was also collected to analyze BUN (Crocker, 1967).

Table 1: Ingredients and chemical composition (g kg^{-1} DM) of concentrate, rice straw and urea-lime treated rice straw used in the experiment

	Urea in concentrate (%)			
Items	0	4	Urea-lime RS	RS
Ingredient (%)				
Cassava chip	60.0	74.0	-	-
Rice bran	4.5	8.5	-	-
Brewer's grain	4.0	3.0	-	-
Kapok seed meal	12.0	1.0	-	-
Soy bean meal	16.0	0.0	-	-
Molasses	1.5	6.0	-	-
Urea	0.0	4.0	-	-
Mineral mixture	0.5	0.5	-	-
Salt	0.5	0.5	-	-
Sulfur	0.5	0.5	-	-
Tallow	0.5	1.0	-	-
Chemical composition (DM%)				
DM	90.5	89.6	54.9	90.5
OM	91.1	92.3	87.4	87.2
Ash	8.9	7.7	12.6	12.8
CP	15.0	15.2	5.6	2.0
NDF	9.1	9.2	69.9	77.0
ADF	9.4	9.2	52.5	56.0
TDN	84.3	80.2	53.5	47.0

DM = Dry Matter, CP = Crude Protein, EE = Ether Extract, OM = Organic Matter, NDF = Neutral Detergent Fiber, ADF = Acid Detergent Fiber, TDN = Total Digestible of Nutrients, ME = Metabolizable Energy, TRS = Treated Rice Straw, RS = Rice Straw

Community Deoxyribonucleic Acids (DNA) extraction: Community DNA was extracted from 0.5 g of rumen fluid and digesta by the repeated bead beating plus column (RBB+C) method.

In brief, the RBB+C method employs two rounds of bead beating in the presence of 4% (w/v) Sodium Dodecyl Sulfate (SDS), 500 mM NaCl and 50 mM EDTA, the buffer should also protect the released DNA from degradation by DNAses. After bead-beating, most of the impurities and the SDS were removed by precipitation with ammonium acetate and then the nucleic acids were recovered by precipitation with isopropanol. Genomic DNA was treated with RNase A and Proteinase K and the DNA was purified using columns from the QIAgen DNA Mini Kit (QIAGEN, Valencia, CA).

Real-time Polymerase Chain Reaction (PCR): The targeted bacteria were total bacteria, 3 predominant cellulolytic bacteria (*F. succinogenes*,

R. albus and R. flavefaciens) and methanogenic bacteria. Primer for total bacteria, forward primers (5-CGGCAACGAGCGCAACCC-3'), reverse primers (5'-CCATTGTAGCACGTGTGTAGCC-3'); *F. succinogenes*, Fs219f (5'-GGTATGGGATGAGCTTGC-3') and Fs654r (5'-GCCTGCCCCTGAACTATC-3'); *R. albus* primers, Ra1281f (5'-CCCTAAAAGCAGTCTTAGTTCG-3') and Ra1439r (5'-CCTCCTTGCGGTTAGAACA-3') and for *R. flavefaciens* primers, Rf154f (5'-TCTGGAAACGGATGGTA-3') and Rf425r (5'-CCTTTAAGACAGGAGTTTACAA-3'). Those primers were chosen from previously published sequences that demonstrated species-specific amplication (Koike and Kobayashi, 2001). For methanogenic primers, forward primers (5-TTCGGTGGATCDCARAGRGC-3) and reverse primer (5'-GBARGTCGWAWCCGTAGAATCC-3') were employed as described by Denman *et al.* (2005).

PCR conditions for *F. succinogenes* was 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 of denaturation in the first cycle and 10 min of extension in the last cycle. Amplification of 16 sec rDNA for *R. albus* and *R. flavefaciens* was carried out similarly except an annealing temperature of 55°C. The PCR conditions for methanogenic were as follows: 30 sec at 94°C for denaturing, 30 sec at 58°C for annealing and 90 sec at 72°C for extension (35 cycles) (Wright *et al.*, 2004).

To establish a quantitative assay, the target 16 sec rDNA of each species was amplified using specific primers and PCR conditions as described previously, the purified DNA was quantified by spectrophotometry with multiple dilutions. The target DNA was quantified by using serial 10 fold dilutions from 10^3-10^9 DNA copies of the previously quantified DNA standards. Real-time PCR amplification and detection were performed in a chromo 4™ system (Bio-Rad, USA). In brief, Biostools QuantiMix Easy SYG Kit was used for PCR amplification samples was assayed in duplicate in a 20 μL reaction mixture contained 4-6 mM $MgCl_2$ and SybrGreen, 2 μL of DNA template and 0.8 μL of each primer (10 μM μL^{-1}).

Denaturing Gradient Gel Electrophoresis (DGGE) analyses: The V3 region of archaeal rrs genes was amplified using primers GC-ARC344f, 5'-ACG GGG YGC AGC AGG CGC GA-3'; 519r, 5'-GWA TTA CCG CGG CKG CTG-3' (Bano *et al.*, 2004; Casamayor *et al.*, 2002) and 519r (Lane, 1991) with primer 344f synthesized with a 40-bp GC clamp at its 5' end. The PCR applications were conducted in a total volume of 25 μL containing 0.5 μM of each primer, 80 μM of dNTP mixed, 1.75 mM MgCl2, 1× PCR buffer and 1.25 U of platinum Taq DNA polymerase. The DNA templates were first subjected to an initial denaturation at 95°C for 5 min, 30 sec at 95°C for denaturing, 30 sec at 61°C and decrease 0.5°C per cycle, 1 min at 72°C (10 cycles), 30 sec at 95°C for denaturing, 30 sec at 56°C for annealing, 1 min at 72°C for extension (25 cycles) and 72°C for 30 min. Prior to DGGE, 4 μL of each PCR product were subjected to 1.5% (w/v) agarose gel electrophoresis to confirm successful amplification of the V3 region. Then, 15 μL aliquots were resolved in a 7.5% polyacrylamide gel (37.5:1) containing a 55-65% gradient of denaturants [100% denaturants consisting of 40% (v/v) formamide and 7 M urea]. The DGGE gel was run at 60°C and 85V for 16 h. The DGGE gel was then stained with SYBR® gold. The gel images were captured using photo documentation (Vilber Lourmat, France).

Statistical analysis: The main effects and interactions of treatments on NH_3-N, BUN and VFAs were determined by ANOVA for a 4×4 latin square design with 2×2 factorial arrangements of treatments using the General Linear Models (GLM) procedures of the Statistic Analysis Systems Institute (SAS, 1998). The statistical model included terms for animal, period, urea level, roughage source and the urea level x roughage source interactions. Treatment means were compared by Ducan's New Multiple Range Test (DMRT) (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Chemical composition of feeds: The experimental feed ingredients and there chemical composition during the 4 experimetal periods is shown in Table 1. The percentage composition of the treated and untreated rice straw given to buffaloes were 54.9, 90.5% DM; 87.4, 87.2% OM; 5.6, 2.0% CP; 69.9, 77% NDF and 52.5, 56% ADF. A similar value for urea-lime treated rice straw has been found by Wanapat *et al.* (2009b).

Concentrate ingredients was based on local resources, including cassava chip; rice bran; brewer's grain; kapok seed meal; molasses. Cassava chip was used as main source of energy.

About 4% urea as non-protein nitrogen in concentrated 2 was replaced for percentage of soybean meal to reduce feed cost. But totally, 2 concentrate formulae contained similar Crude Protein (CP) percentage; 15.0 and 15.2%.

Effect on rumen fermentation: Table 2 shows data on rumen fermentation parameters; including rumen pH, NH_3-N total VFA and individual VFAs. In addition, BUN was determined to investigate their relationships with rumen NH_3-N and protein utilization. Rumen pH was significantly different among treatments ($p<0.05$) and ranged from 6.5-6.8. Urea-lime treated rice straw resulted

Table 2: Effect of chemical treated rice straw and urea level in concentrate on rumen fermentation in swamp buffaloes

	Treatments					Contrast		
Items	RS (0%)	RS (4%)	ULRS (0%)	ULRS (4%)	SEM	U	R	U×R
Fermentation parameter								
Ruminal pH	6.5[a]	6.6[a]	6.7[ab]	6.8[b]	0.06	NS	NS	NS
NH_3-N (mg/100 mL)	9.0[a]	12.4[b]	14.4[c]	21.2[d]	0.68	*	*	*
BUN, mg dL^{-1}	8.5[a]	12.0[b]	14.3[c]	19.4[d]	0.77	*	*	NS
Total VFA (mmol L^{-1})	87.6	95.1	102.4	103.1	7.16	NS	NS	NS
Acetate (C2)	67.8[a]	67.2[a]	71.1[b]	69.8[ab]	1.11	NS	*	NS
Propionate (C3)	19.7[a]	20.4[bc]	19.9[ab]	21.0[c]	0.16	0.08	*	NS
Butyrate (C4)	12.5	12.4	9.0	9.2	1.19	NS	NS	NS
C2:C3 ratio	3.4	3.3	3.6	3.3	0.14	NS	NS	NS

[abcd]Value within the row a common superscript are significantly different ($p<0.05$) U = Effect of urea in concentrate, R = Effect of roughage sources, U×R = Interaction effect of urea and roughage sources NS = p>0.05), NS = p>0.05, *p<0.05

in higher pH value than untreated rice straw. This increased is in agreement with the results of Pal and Negi (1978) when ruminants fed with alkali treated straw and resulted in optimal value for fiber, protein digestion and cellulolytic bacteria's activity in the rumen (Hoover, 1986). The net effects from rumen pH increased could; promote rumen bacteria actives especially rumen cellulolytic bacteria which are very sensitive with pH value and improve rice straw digestibility and intake.

An increase of ruminal NH_3-N concentrations (from 9.0-21.2 mg, %) was noted as effect of treatments using urea-lime treated rice straw; 4% urea in concentrate or urea-lime treated rice straw combined with 4% urea in concentrate. The highest NH_3-N concentration (21.2 mg, %) was found in treatment using urea-lime treated rice straw with 4% urea in concentrate. Rumen NH_3-N concentration was linearly correlated with the level of dietary crude protein ($p<0.001$, r^2 = 0.77-0.92), consequently, dietary treatment by urea-lime treated rice straw with 4% urea in concentreate mixture was found highest in NH_3-N concentration. In addition, the increase of ruminal NH_3-N concentration was in optimal level (15-30 mg%, Boniface *et al*., 1986; Wanapat and Pimpa, 1999) for improvement the rumen ecology, microbial protein synthesis, feed digestibility and voluntary intake in ruminant fed low-quality roughage. BUN concentration was increased according to the increase in ruminal NH_3-N concentration and increased linearly with dietary protein levels. It was in normal range. The result in concentration of NH_3-N and BUN indicated that urea-lime treated rice straw can be used as good roughage source and urea can be used at 4% in concentrate for buffaloes without adverse effect on rumen fermentation.

Total VFA concentrations in the rumen were similar among treatments and ranged from 87.6-103.1 mmol L^{-1} which were in normal range from 70-130 mM (France and Siddons, 1993). And an increased in propionic proportion without a reduction of acetic acid proportion ($p<0.05$) was found while butyric acid concentration (mmol L^{-1}) was not changed among treatments and ranged in 9.2-12.5 mmol L^{-1}. The mean value of acetic acid to propionic acid ratio were not significantly different ($p>0.05$) among treatments. The current finding was different with report of Wanapat *et al*. (2009a, b) who shown that 2.2% urea plus, 2.2% lime treated rice straw has resulted in a significantly increased in total VFAs. However, under this study, total VFA concentrations tended to be increased with urea-lime treated rice straw and the most increased in urea-lime treated rice straw with 4% urea in concentrate supplementation. Both proportion of acetate and propionate were increased by treatments using urea-lime rice straw. The increased concentration of propionate or acetate strongly related with the number of ruminal cellulolytic bacterial species. Fibrobacter succinogenes mainly produces primarily succinate, the major precursor of propionate in the rumen while *Ruminococcus albus* is mainly specie produces acetate (Weimer, 1996).

Therefore with an increase in number of *Fibrobacter succinogenes*, *Ruminococcus albus* when buffaloes fed urea-lime treated rice straw+4% urea in concentrate, both propionate and acetate concentration were increased. The net effect would be a maintaining in the ratio of ruminal acetate realtive to ruminal propionate ($p>0.05$).

Effect on rumen microorganism: Rumen protozoa, fungal zoospore and 3 main groups of bacterial population (amylolytic, proteolytic and cellulolytic bacteria) are shown in Table 3. Ruminal anaerobic fungi was affected by the increased population ($p<0.05$) when buffaloes fed urea-lime treated rice straw with 4% urea. The number of protozoa was reduced by both factors, it was in agreement with study of Khampa *et al*. (2005) who reported that the lower number of protozoal count per milliliter was found as level of urea in concentrate mixture increased. Amylolytic and proteolytic bacteria were not altered among treatments while cellulolytic bacterial population was linearly increased by urea-lime treated rice straw.

Table 3: Effect of chemical treated rice straw and urea level in concentrate on rumen microbial population, rumen fermentation in swamp buffaloes

Items	Treatments				SEM	Contrast		
	RS (0%)	RS (4%)	ULRS (0%)	ULRS (4%)		U	R	U×R
Direct count x cell mL^{-1}								
Protozoa (×10^5)	9.6[a]	7.6[ab]	4.1[bc]	3.8[c]	1.28	*	*	*
Fungal zoospores (×10^4)	1.8[a]	3.2[a]	7.6[b]	9.7[b]	1.06	*	*	NS
Roll-tube technique, CFU mL^{-1}								
Amylolytic (×10^7)	8.4	9.2	7.7	10.2	0.84	NS	NS	NS
Proteolytic (×10^7)	6.7	6.5	6.9	6.1	0.27	NS	NS	NS
Cellulolytic (×10^8)	6.7[a]	9.4[c]	8.4[b]	11.6[d]	0.33	*	0.06	NS

[abcd]Value within the row a common superscript are significantly different (p<0.05) U = Effect of urea in concentrate, R = Effect of roughage sources, U x R = Interaction effect of urea and roughage sources NS = p>0.05), NS = p>0.05, *p<0.05

Effect on three predominant cellulolytic bacteria, total bacteria and methanogenic bacteria population: Total bacteria, about 3 predominant cellulolytic species and methanogenic bacteria within the rumen were quantified using a real-time Polymerase Chain Reaction (PCR). 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 were calculated. Logarithms of the DNA concentration (copies mL^{-1}) were plotted against the calculated means (Fig. 1), obtaining a straight line of equations; y = -0.2905x+11.02, y = -0.272x+12.45, y = -0.2771x+12.28, y = -0.2663x+10.85, y = -0.2804x+11.53 (where, y is the log of DNA concentration and x is the C(T)) with a linear correlation coefficient (r2) of 0.998, 0.996, 0.988, 0.944, 0.995 for total bacteria, *F. succinogen*, *R. flavefaciens*, *R. albus* and methanogenic bacteria, respectively.

The equations were used to quantify DNA from rumen fluid of buffaloes fed with different diets. Figure 2 presents the population size of total bacteria, 3 dominant cellulolytic bacteria, methanogenic bacteria in the rumen of swamp buffaloes. Population of bacteria were increased from 0-4 h post feeding. *F. succinogenes*, *R. flavefacience* and *R. albus* were estimated from 4.8×10^{11}-12.7×10^{11}, 0.2×10^{10}-13.9×10^{10}, 3.8×10^{9}-31.7×10^{9} copies mL^{-1} of rumen content, respectively. The population of *F. succinogenes* was highest across dietary treatments as compared to *R. flavefaciens* and *R. albus*. These results were in agreement with studies of Koike and Kobayyashi (2001) and Wanapat and Cherdthong, (2009) who found *F. succinogenes* (3.21×109 copies mL^{-1} rumen fluid) were the most abundant among celulolytic species in the rumen of swamp buffaloes, following by *R. flavefaciens* (4.55×107 copies mL^{-1} rumen fluid) and then *R. albus* (4.44×106 copies mL^{-1} rumen fluid). Apparently because *F. succinogenes* and *R. flavefaciens* can colonize the celluloes more rapidly than can the other 2 species and it can grow more rapidly on cellodextrins (the intermediate products of cellulose hydrolysis) (Shi and Weimer, 1996). *R. albus,* always was less abundant than

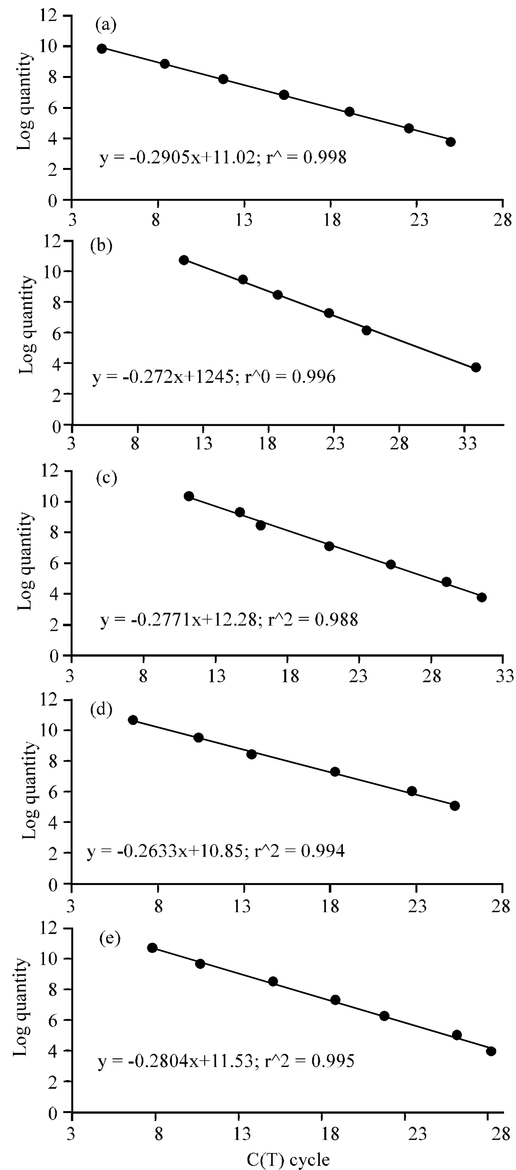

Fig. 1: Standard curve obtained by plotting the logarithm of the DNA concentration for total bacteria a), *F. succinogenes*; b), *R. flavefaciens;* c), *R. albus*; d) methanogenic and e) versus threshold cycle (C (T)) mean values

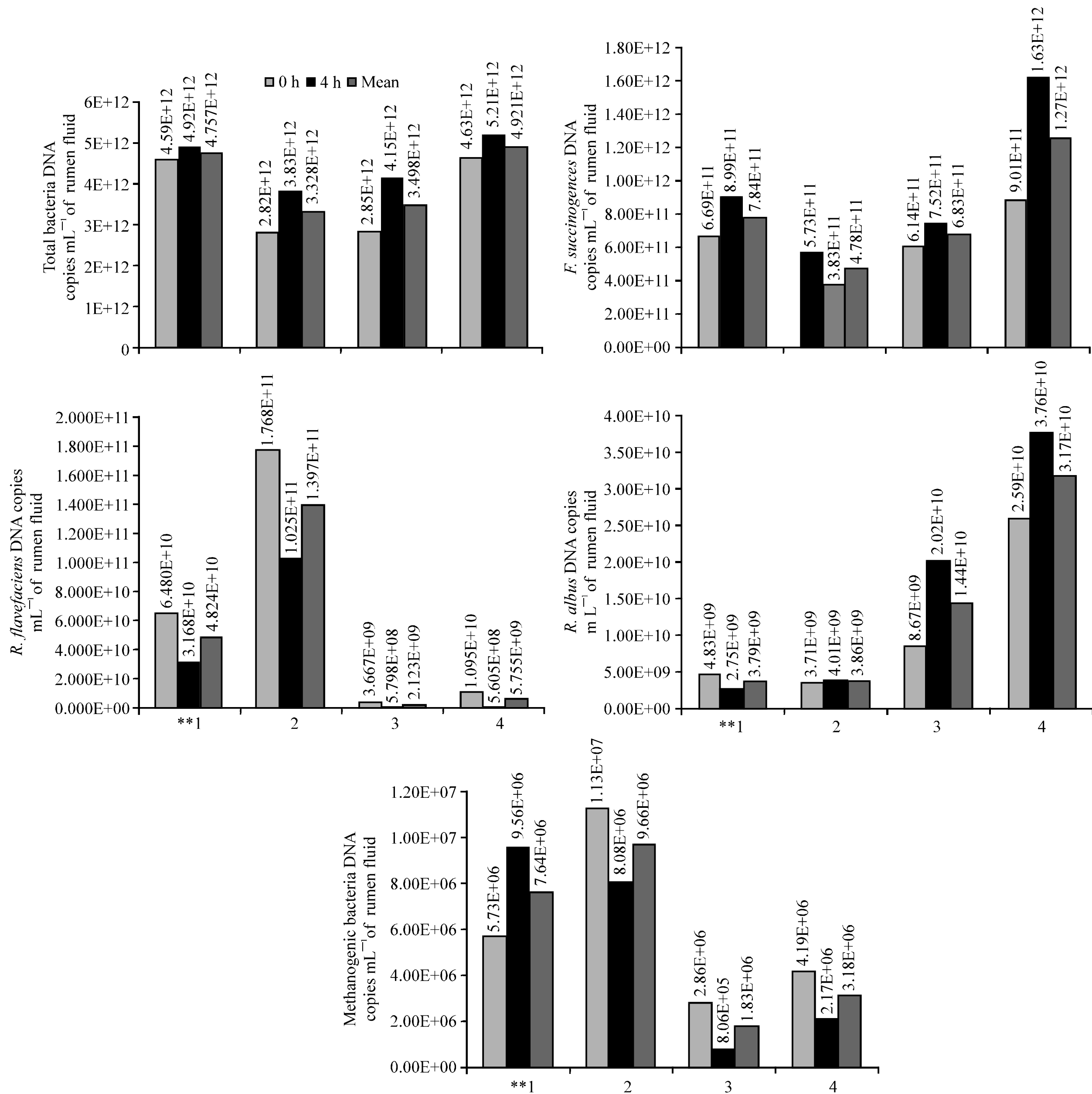

Fig. 2: Effect of roughage source and urea levels on total bacteria, three dominant cellulolutic bacteria and methanogenic population by real-time PCR.) ** T1 = Rice straw+0% urea, T2 = Rice straw+4% urea, T3 = Urea-lime treated rice straw+0% urea, T4 = Urea-lime treated rice straw+4% urea

was *F. succinogenes* and *R. flavefaciens* because it was less effective in colonizing cellulose and was probable reduced to growing on soluble products released by the other species during cellulose hydrolysis (Weimer, 1996). In addition, this study found the increase in population of *F. succinogenes* and *R. albus* as affected by treated rice straw and urea level while *R. flavefacience* population was found highest in treatment using untreated rice straw with 4% urea (13.9×10^{10} copies mL^{-1} rumen content) and were lowest when buffaloes consumed treated rice straw with 0% urea. This increasing was caused of alkali agents that provided a more favorable environment (pH, NH_3-N, break down cell wall) for growth of cellulolytic bacteria. The increase in population of *F. succinogenes*, *R. albus* was promoted to increase rumen fermentation and fiber digestibility.

However, the *R. flaveciences* population was reduced. It could be explain by the reason was found by Chen *et al.* (2004) who reported that an 32-kDa protein (albusin B) which was released from *R. albus* inhibited growth of *Ruminococcus flavefaciens* FD-1. In their research, all seven *R. flavefaciens* strains tested (FD-1, B34b, 17, C94, B1c45, B146 and R13e2) were sensitive to albusin B but other ruminal species tested (*Fibrobacter succinogenes* S85, *Selenomonas ruminantium* D and *Streptococcus bovis* JB-1) and the non-ruminal species *Escherichia coli* ZK126 and *Bacillus subtilis* AD623 were not affected.

Under this study, the quantification of cellulolytic bacteria was affected by diet and sampling time. There are two possible explanations of the increase cell populations at the 4 h post-feeding: cell proliferation after feeding and the additional attachment of new bacteria form liquid phase or other particles (Wanapat and Cherdthong, 2009).

Total bacteria population was similar among treatments. It ranged in 3.3×10^{12}-4.9×10^{12} copies mL^{-1} rumen content. Methanogenic bacterial population was reduced from 1.8×10^{6}-9.7×10^{6} copies mL^{-1} of rumen fluid and reduced from 0-4 h post feeding. Reduction of protozoa had resulted in lowering methanogenic bacteria population by reduced living place of methanogens. In addition, the population density of methanogenic bacteria in the rumen appears to be influenced by diet and particularly by the fiber content of the diet. Moreover, Sahoo *et al.* (2000) have observed that treatment of wheat straw with urea plus calcium hydroxide with a storage time of 21 days have shown to increase the rate of degradation of OM and reduce rumen retention time. The reduced rumen retention time may influence the methanogenic population as methanogens are slow growing and hence proliferate only under conditions of slow rumen particle dilution rate (Preston, 1972).

Effect on methanogenic bacteria diversity: The result analyzed by PCR-DGGE is shown in Fig. 3. It was found that the methanogenic diversity were similar among treatments and had 18 separate bands of DNA morphology in each lane. It indicated that under this study, the predominant methanogenic bacteria in the rumen of swamp buffalo comprised of 18 species. However, other studies on methanogenic in the rumen of swamp buffaloes found 7 different bands (Wanapat *et al.*, 2009a, b) or 13 different bands (Kongmun *et al.*, 2011). This different could be explain by the different response of methanogenic bacteria with diets or the different of percentage range of denaturing gradient used. Under this study, the PCR products were resolved on an 8.0% polyacrylamide gel (37.5:1) with a 55-65% denaturing gradient resulted in 18 different bands. Moreover,

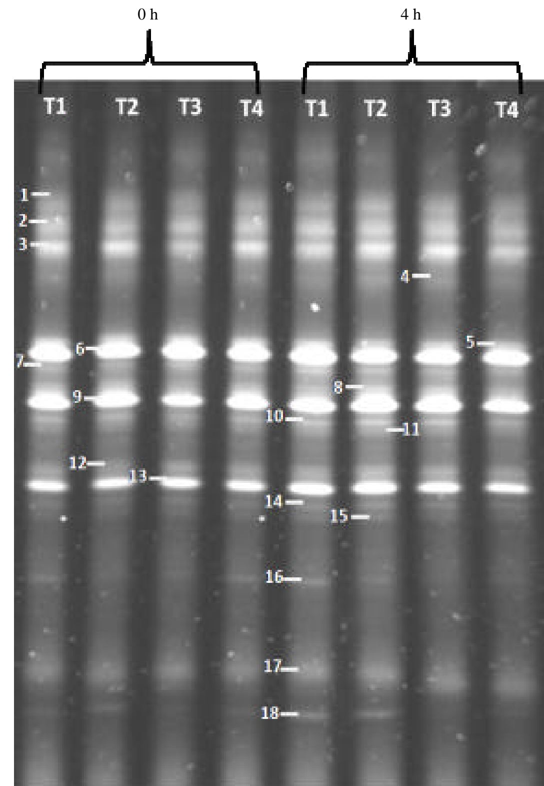

Fig. 3: Photographed gel after DGGE electrophoresis of 16s rDNA fragments from 4 treatments and two times post feeding. Genomic DNA was extracted from rumen fluid, followed by amplification of the 16s rDNA using GC-ARC344f and 519 r primers and separation of the amplicons by gel electrophoresis (T1 = Rice straw+0% urea, T2 = Rice straw+4% urea, T3 = Urea-lime treated rice straw+0% urea, T4 = Urea-lime treated rice straw+4% urea)

identification of bacterial species by gene sequencing technique could provide more information about concerning the bacterial diversity in the rumen.

CONCLUSION

Based on this study, it could be concluded that the diets with 2% urea plus, 2% lime could improve rumen fermentation by increased propionate, acetate production, pH and concentration of ruminal NH_3-N. Diet with 2% urea plus 2% lime with 4% urea in concentrate mixture could increase rumen fibrolytic microbes' quantity, reduce protozoa and methanogenic population. No change in rumen methanogenic bacteria diversity but further research should cut and sequence specific bands to identify methanogenic species under this condition and

also, further experiment should conducted in investigated of cellulolytic bacteria diversity and mechanisms of each fibrolytic microbes related to straw digestion.

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