Journal of Animal and Veterinary Advances 9 (24): 3062-3069, 2010
ISSN: 1680-5593
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Diversity of Rumen Anaerobic Fungi and Methanogenic Archaea in Swamp Buffalo Influenced by Various Diets

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Abstract: The aim of this study was to evaluate the relationship of rumen anaerobic fungi and methanogenic archaea on ruminal fermentation in swamp buffalo. Four rumen fistulated swamp buffaloes with average 369 kg of body weight were used. All animals were randomly assigned according to a 2×2 factorial arrangement in a 4×4 Latin square design to receive four dietary treatments; factor A = two sources of roughage (rice straw and 2% urea+2% lime treated rice straw), factor B = two levels of urea in concentrate mixture (0 and 4%). Roughages were given *ad libitum* together with 3 g kg^{-1} BW of concentrate. It was found that voluntary feed intake, the digestibility of DM, CP, NDF, acetate and propionate concentration were significantly increased ($p<0.05$) by treated rice straw while NH_3-N, BUN and propionic acid concentration were increased by both factors of treated rice straw and 4% urea in concentrate. The real-time PCR quantification of *F. succinogenes* and *R. albus* population and anaerobic fungi were greater ($p<0.05$) but the population of *R. flavefaciens*, protozoa and methanogenic bacteria were reduced ($p>0.05$) as influenced by treated rice straw and urea level. Animal consumed treated rice straw was shown in more diverts in phylogenetic relationship. No change in rumen methanogenic bacteria diversity and relative change with fungi population. In conclusion, the combined use of urea-lime treated rice straw and fed with concentrate (4% urea) could improve rumen ecology, rumen fermentation efficiency and increase anaerobic fungi. The results from this study suggest that feeding with urea-lime treated rice straw more rumen fermentation efficiency though shift fungi and methanogenic population.

Key words: Rumen fungi, methanogenic archaea, real-time PCR, PCR-DGGE, diversity, swamp buffalo

INTRODUCTION

The rumen microbial community is complex and extremely diverse. These microbial rumen symbiotic relationships which highly specific and perform metabolic functions that are important for plant lignocellulosic material digestion of the animal host. Anaerobic fungi belong to the order Neocallimastigales family are part of the complex microbial consortium that have been found in the rumen of ruminant and the gut of herbivorous (Hibbett *et al*., 2007). Ruminal anaerobic fungi are extensively colonizes and degrades plant material in the rumen ecosystem in term of physical and chemical breakdown. It is means of their rhizoid systems and produce potent fibrolytic enzymes such cellulases, xylanases and esterases (Theodorou *et al*., 1989). They are degraded biomass such as cellulose and hemicelluloses and produce acetate, formate, lactate, H_2 and CO_2 and small amount of ethanol. Methanogens, members of domain obligatory archaea are common distributed in the rumen consortium and they produce methane as a major catabolic product. It has been shown that ruminal anaerobic fungi and methanogen can produce methane when grown together in batch culture with cellulosic substrates (Bauchop and Mountfort, 1981). Moreover, methanogenic bacteria enhance anaerobic fungal degradation of fiber in order to increased rates of cellulolytic enzyme activity and dry matter disappearance in co-cultures compared to anaerobic fungal cultures alone (Bauchop and Mountfort, 1981; Mountfort *et al*., 1982; Joblin and Naylor, 1993). In previous studies on the interaction between rumen fungi and methanogens have been used co-cultures from limiting diverts mixed of both species (Bauchop and Mountfort, 1981; Mountfort *et al*., 1982; Teunissen *et al*., 1992; Joblin *et al*., 2002). Although, it is recognized that the anaerobic fungi and methanogen are distribute in the rumen and has potentially on fiber digestion in culture study. However,

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the population and their interrelationships in naturally rumen are more complex than reported involving only *in vitro* and limited research on the effects of chemical treatment of rice straw upon fungal population, particularly those which *in vivo* trial of swamp buffalo and their contribution in the rumen has been reported. Therefore, the aim of this study was conducted to evaluate the structure of rumen anaerobic fungi and methanogenic archaea using real-time PCR and PCR-DGGE technique and rumen fermentation by effect of different level of urea supplementation in swamp buffalo fed on either untreated or urea-lime treated rice straw.

MATERIALS AND METHODS

Animals and experimental design: Four ruminally-fistulated swamp buffaloes (*Bubalus bubalis*) (369±28 kg of BW) were used in 4×4 Latin squares design over four 21 day period (14 day for adaptation and 7 day for collection on metabolism crate). The animals were housed in individual pens (3×5 m). The four dietary treatments were (T1) 0% urea in concentrate and Rice Straw (RS), (T2) 4% urea in concentrate and RS, (T3) 0% urea in concentrate and Urea-Lime Treated Tice Straw (ULTS), (T4) 4% urea in concentrate and ULTS. Concentrate were fed at 0.3% body and fed *ad libitum* of urea-lime treated straw or untreated straw twice daily at 0600 am and 1600 p.m. Water and mineral-salt block were offered on free choice during the experiment. The concentrates are comprised 14% CP and 80% TDN with required levels of minerals (Table 1). ULRS was prepared by using 2 kg of urea and 2 kg lime mixed with 100 kg of water and poured over a stack of 100 kg Rice Straw (RS) then covered with a plastic sheet for a minimum of 10 days before feeding to the ruminants (Wanapat *et al*., 2009b). Animal were sampled though rumen fistula at the end of each period with approximately 20 mL of rumen fluid was taken from the middle part of the rumen by using a 60 mL hand syringe (0 and 4 h post feeding). Rumen fluid was immediately measured for temperature and pH using a portable pH and temperature meter (HANNA instrument HI 8424 microcomputer, Singapore). Rumen fluid samples were collected 10 mL for direct count (Galyean, 1989) and then filtered through 4 layers of cheesecloth and were stored in at -20°C until DNA extraction and analysis of VFA and ammonia nitrogen.

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

	Percentage urea in concentrate			
Items	0	4	Urea-lime RS	RS
Ingredient (%)				
Cassava chip	65.0	65.0	-	-
Rice bran	7.0	9.0	-	-
Brewer's grain	8.0	7.0	-	-
Kapok seed meal	7.0	6.0	-	-
Soy bean meal	8.0	0.0	-	-
Molasses	2.8	5.0	-	-
Urea	0.0	4.0	-	-
Mineral mixture	0.5	1.0	-	-
Salt	0.5	0.5	-	-
Sulfur	0.2	1.5	-	-
Tallow	1.0	1.0	-	-
Chemical composition (Percentage of 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	14.1	14.2	5.6	2.0
NDF	9.1	9.2	69.9	77.0
ADF	9.4	9.2	52.5	56.0
TDN	81.0	80.0	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

Chemical composition, rumen ammonia nitrogen and VFA analysis: Composited dietary samples were analyzed for DM, EE, ash and CP content (AOAC, 1990), NDF and ADF (Van Soest *et al*., 1991). Rumen fluid was measured for pH immediately and for later analysis of NH_3-N was analyzed using the micro Kjeldahl methods (AOAC, 1990) and VFA concentration was determined using HPLC (instruments: controller water model 600 E; water model 484 UV detector; Novapak C_{18} column; column size 4×150 mm; mobile phase 10 mmol L^{-1} H_2PO_4 (pH 2.5) (Samuel *et al*., 1997).

Extraction of genomic DNA and real-time PCR: Rumen digesta and content were separated 0.5 g for DNA extraction by the repeated bead beating plus column (RBB+C) method (Yu and Morrison, 2004). 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). The target for gut fungal-specific primers was employed as described by Denman and McSweeney (2006). The PCR conditions for gut fungi 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). PCR conditions for methanogenic achraea 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).

DGGE conditions and gel analyses: For DGGE, primer MN100 (TCCTACCCTTTGTGAATTTG) and MNGM2C (CTGCGTTCTTCATCGTTGCGCGCCCGCCGCGCGCGG CGGGCGGGGCGGGGGCACGGGGGG) were used as described by Nicholson *et al*. (2010). PCR reactions was adapted according to Nicholson *et al*. (2010) that contained 2.5 mL 10×buffer, 2.5 mL 1/25 dilution genomic

DNA, 0.5 mL dNTP mix (10 mM each), 0.5 mL. Advantage 2 polymerase mix (FastStart Taq, Roche), 10 picomoles of each primer and molecular biology grade water to make a final reaction volume of 25 mL. Thermal cycling consisted of 95°C for 5 min followed by 20 cycles of 95°C for 30 sec, 68°C (-0.5°C each cycle) for 30 sec, 72°C for 30 sec then 12 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec followed by 72°C for 6 min.

Successful amplification was verified by electrophoresis of the reaction mixture on a 1% (w/v) agarose gel. DGGE was performed using a D-Code Universal Mutation Detection System (Bio-Rad Laboratories Ltd., UK) with 16×16 cm glass plates separated by 1 mm spacers. DGGE was performed to separate PCR amplicon, 12 μL of PCR product and dye mix was loaded in each sample well (16-well comb) and using 10% polyacrylamide gel (37.5:1 acrylamide-bisacrylamide ratio) containing a gradient of 15-30% denaturant where 100% denaturant solution contained 7 M urea and 40% (v/v) formamide. Gels were placed in electrophoresis tank using 0.5xTAE (20 mM Tris; pH 7.4, 10 mM sodium acetate, 0.5 M EDTA) running buffer heated to 60°C. Optimized conditions for products amplified with MN100 and MNGM2C were 15-30% denaturant electrophoresed at 200 V for 5 min and 85 V for 8 h in running buffer heated to 60°C. The PCR product for methanogenic archaea was performed, 15 μL aliquots were resolved in a 8% 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. After electrophoresis DGGE gel was stained with SYBR® Gold (Molecular Probes Inc., USA) and then gel images were captured using Photo documentation (Vilber Lourmat, France).

The gel images were then imported into the software package fingerprinting (Bio-Rad UK Ltd) for analysis (Fingerprint Types and Cluster Analysis modules). Cluster analysis was performed using the Dice similarity coefficient with a position tolerance of 0.5% and an optimization parameter of 1% with clusters constructed using the unweighted pair-wise grouping with mathematical averages method.

Statistical analysis: Statistical analyses of similarity indices, ruminal ammonia nitrogen and VFA concentrations were performed using Proc GLM (SAS, 1998 Inst. Inc., Cary, NC). Treatment means were compared by Duncan's New Multiple Range Test (DMRT) (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Ruminal fermentation characteristic: The effect of treated rice straw and urea level in concentrate mixed on rumen fermentation parameters including pH, NH_3-N and ruminal VFAs are shown in Table 2. Rumen pH was significantly increased ($p<0.05$) from 6.5-6.8 as an effected of treated rice straw and urea supplementation. The increased rumen pH in this study is in agreement with the results of Wanapat *et al.* (2009b) when ruminants fed with urea-lime treated straw and resulted in improve nutritive value for fiber, digestion and bacteria's activity in the rumen. An increase of ruminal NH_3-N concentrations (from 9.0-21.2 mg%) was noted as effect of treatments using treated rice straw; 4% urea in concentrate or combination diet. The highest NH_3-N concentration (21.2 mg%) was found in treatment using urea-lime treated rice straw with 4% urea in concentrate. In addition, the increase of ruminal NH_3-N concentration was in optimal level (15-30 mg%) (Boniface *et al.*, 1986; Perdok and Leng, 1990; Wanapat and Pimpa, 1999) and it could be provide ruminal nitrogen source for microbial protein synthesis as described to rumen fiber digestion (Hoover and Stokes, 1991).

Total VFA concentrations in the rumen were similar among treatments while an increased in propionic proportion without changed acetic acid and butyrate proportion ($p<0.05$) was found. However, total VFA tended to be increased when buffaloes offered with

Table 2: Effect of diets on rumen fermentation characteristic in swamp buffalo

	Diets					Contrast		
Item	T1	T2	T3	T4	SEM	U	R	UxR
Ruminal pH	6.5^{a}	6.6^{a}	6.7^{ab}	6.8^{b}	0.06	NS	NS	NS
NH3-N (mg/100 mL)	9.0^{a}	12.4^{b}	14.4^{c}	21.2^{d}	0.68	*	*	*
Total VFA (mmol L^{-1})	87.6	95.1	102.4	103.1	7.16	NS	NS	NS
VFAs, mmol L^{-1}								
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
CH_4, mmol/100 mol[1]	33.4^{a}	31.2^{a}	26.0^{b}	26.2^{b}	1.76	*	*	*

^{abc}Value within the row a common superscript are significantly different ($p<0.05$) NS = $p>0.05$), * = $p<0.05$; [1]Calculated according to Moss *et al.* (2000) CH_4 production = 0.45 (acetate)-0.275 (propionate)+0.4 (butyrate). SEM = Standard Error of the Means

Table 3: Methanogenic archaea, fungi and protozoa in whole rumen contents from swamp buffalo determined by direct count and real-time PCR

	Diets					Contrast		
Items	T1	T2	T3	T4	SEM	U	R	UxR
Real-time PCR (copy g^{-1} content)								
Methanogens ($\times 10^6$)								
h0	5.73	11.30	2.86	4.19	3.80	NS	NS	NS
h4	9.56^a	8.08^a	0.81^b	2.17^b	2.71	*	NS	*
Fungi ($\times 10^7$)								
h0	8.11^a	11.20^a	7.73^a	28.20^b	5.76	*	*	*
h4	8.83	8.35	11.40	12.70	1.53	NS	NS	NS
Direct count (cell mL^{-1})								
Protozoa ($\times 10^5$)	9.60^a	7.60^{ab}	4.10^{bc}	3.80^c	1.28	*	*	*
Fungal zoospores ($\times 10^4$)	1.80^a	3.20^a	7.60^b	9.70^b	1.06	*	*	*

^{bc}Value within the row a common superscript are significantly different ($p<0.05$) NS = $p>0.05$), * = $p<0.05$; SEM = Standard Error of the Means

urea-lime treated rice straw with concentrate containing 4% urea. The results from this study reveal that both proportion of acetate and propionate were increased by treatments using urea-lime rice straw while butyrate proportion and acetate: propionate ratio was similar among treatments. As a normally, acetate proportion was increased when fiber intake was increased (Bach *et al*., 1999). Significant differences in ruminal CH_4 production were found in treated rice straw and urea supplementation. It has been suggested that when cattle fed with high feed efficiencies produce less methane gas than those with low feed efficiencies (Zhou *et al*., 2009).

Rumen microorganism and diversity: Rumen protozoa, fungal zoospore and methanogenic bacterial population are shown in Table 3. Ruminal anaerobic fungi population was higher ($p<0.05$) in buffaloes fed urea-lime treated rice straw with 4% urea while number of protozoa was reduced by both factors. The lower number of protozoal count per milliliter was found as level of urea in concentrate mixture increased as reported by Nour *et al*. (1979). The reduced of protozoal population using urea-lime treated rice straw and urea in concentrate mixed could result in lower methanogens and the calculated methane production. Wanapat *et al*. (2009a) reported protozoa population was not effect by feeding buffalo with corn cop. Moreover, Sahoo *et al*. (2000) have observed that treatment of urea plus lime reduced rumen retention time which may influence the methanogenic population as methanogens are slow rumen particle dilution rate.

Total zoospore fungi were increased as influenced by urea-lime treated rice straw and ranged from 1.8×10^4-9.7×10^4 zoospore mL^{-1}. There is physical form of diet might have effect on increased number of fungi as reported by Chaudhry (2000) and Rezaeian *et al*. (2005) in chemical treatment. The anaerobic fungi and methanogenic bacteria within the rumen were quantified using a real-time Polymerase Chain Reaction (PCR). The standards for real-time PCR were prepared from a simulated rumen matrix and performed as linear regressions derived from the threshold

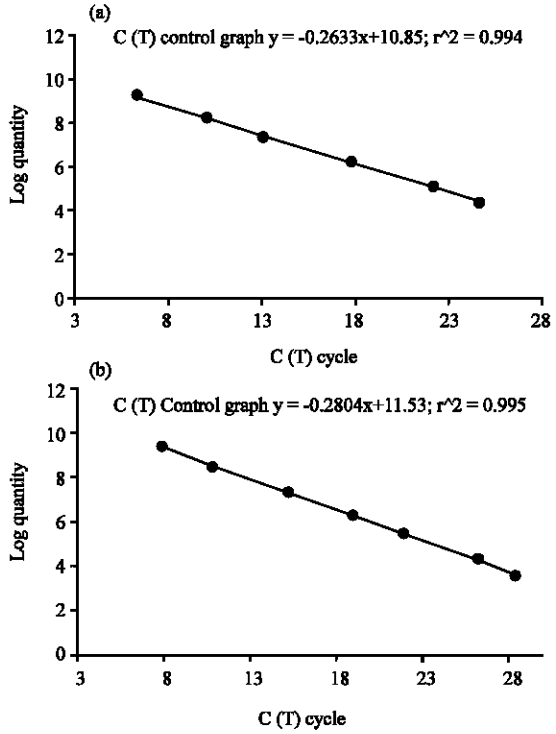

Fig. 1: Standard curve obtained by plotting the logarithm of the DNA concentration for anaerobic fungi (a) and methanogenic archaea (b) versus threshold cycle (C (T)) mean values

cycle [C(T)] of each DNA dilution versus the log quality (Fig. 1a, b) were calculated. Logarithms of the DNA concentration (copies/mL) were plotted against the calculated means (Fig. 1), obtaining a straight line of equations y = -0.2663x+10.85 and y = -0.2804x+11.53; (where y is the log of DNA concentration and x is the C (T) with a linear correlation coefficient (r^2) of 0.944 and 0.995 for fungi and methanogenic bacteria, respectively. The equations were used to quantify DNA from rumen fluid of buffaloes fed with different diets. The result from real-time PCR shown that ruminal anaerobic fungi was higher ($p<0.05$) in buffaloes fed urea-lime treated rice

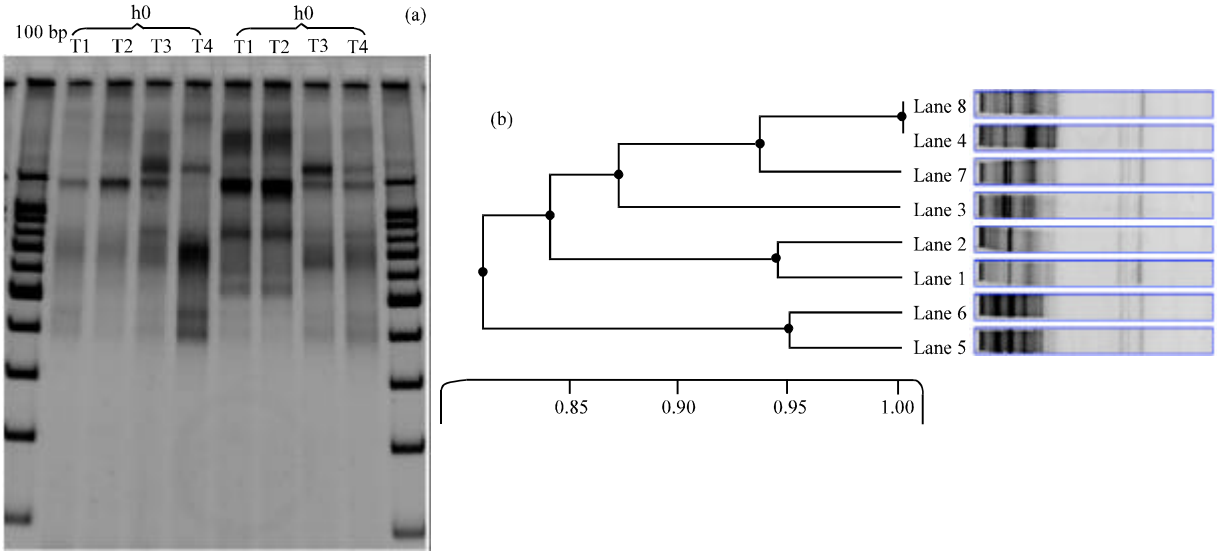

Fig. 2: Negative image of SYBR® Gold stained denaturing gradient gel electrophoresis separation pattern of eight PCR samples in which the rumen anaerobic fungi ITS1 amplicon from rumen content of buffalo (T1 = RS, T2 = RS+4% urea, T3 = ULRS+0% urea and T4 = ULRS+4% urea) are shown (a) along with the corresponding cluster analysis of the sample profiles (b)

straw with 4% urea but recues thereafter. These results were in agreement with studies of Rezaeian *et al.* (2005) who found rumen fungi were significantly higher in the chemical treated straw. However, this study found the decrease in population of fungi after feeding. It is possible that the effects of bacterial fermentation end-products such H_2, formate, lactate and ethanol were strong inhibitors, particularly at high concentrations as reported by Joblin and Naylor (1993). It seems to be methanogenic bacteria enhance anaerobic fungal degradation of fiber as previous report (Bauchop and Mountfort, 1981; Teunissen *et al.*, 1992; Joblin *et al.*, 2002). However, in high efficiently diet could resulted in increase population of cellulolytic bacteria and produce more fermentation end-products which were strong inhibition effect on rumen fungi and beside to methanogenic archaea. Methanogenic archaea was ranged from 1.8×10^6-9.7×10^6 copies mL^{-1} of rumen fluid and it was reduced by both factors ($p<0.05$). This result may explain the relationship of protozoa population decreased after feeding. This result could be relate with the reduction of protozoa population and methane calculated as reported by Ivan *et al.* (2001) methane production can be reduced by reducing rumen ciliated protozoa.

The microbial population diversity monitored by DGGE for rumen anaerobic fungal community profile and total methanogenic bacterial community profile are shown in Fig. 2a, b and 3, respectively. Denaturing gradient gel electrophoresis in its various forms has been used to investigate the ecology of microorganisms in fungal populations in the rumen (Brookman *et al.*, 2000). The rumen anaerobic fungi population of animal after fed rice straw was clearly distinct from the others. There were shifts in rumen fungi and methanogenic bacterial communities composition at different sampling time intervals and the shifts in community composition occurred in different patterns in all of four diets (Fig. 2 and 3). Some animals variation was observed in the rumen methanogens DGGE profiles (Fig. 3a), although T1 and T4 gave similar profiles (Fig. 3b).

The rumen anaerobic fungi population of animal after fed rice straw was clearly distinct from the others. This result suggested that the shifts in microbial population might have been due to different diet and sampling time. Under this study, the anaerobic fungi diversity was slightly different among treatments and had 7 separate bands of DNA morphology across all lanes of the gel and predominant anaerobic fungi in the rumen of swamp buffalo comprised of 7 species. However, Edwards *et al.* (2008) found that six peak in that ITS1 amplicons as individual culture study.

Rumen methanogenic archaea population analysis of the rumen contents from buffalo showed that several different methanogens were likely to be presented. However, the size of methanogens population did not correlate with differences in feed efficiency, diet as reported by Zhou *et al.* (2010). DGGE is well established as a fingerprinting technique in tended for separation of same-sized fragments to approach diversity. However,

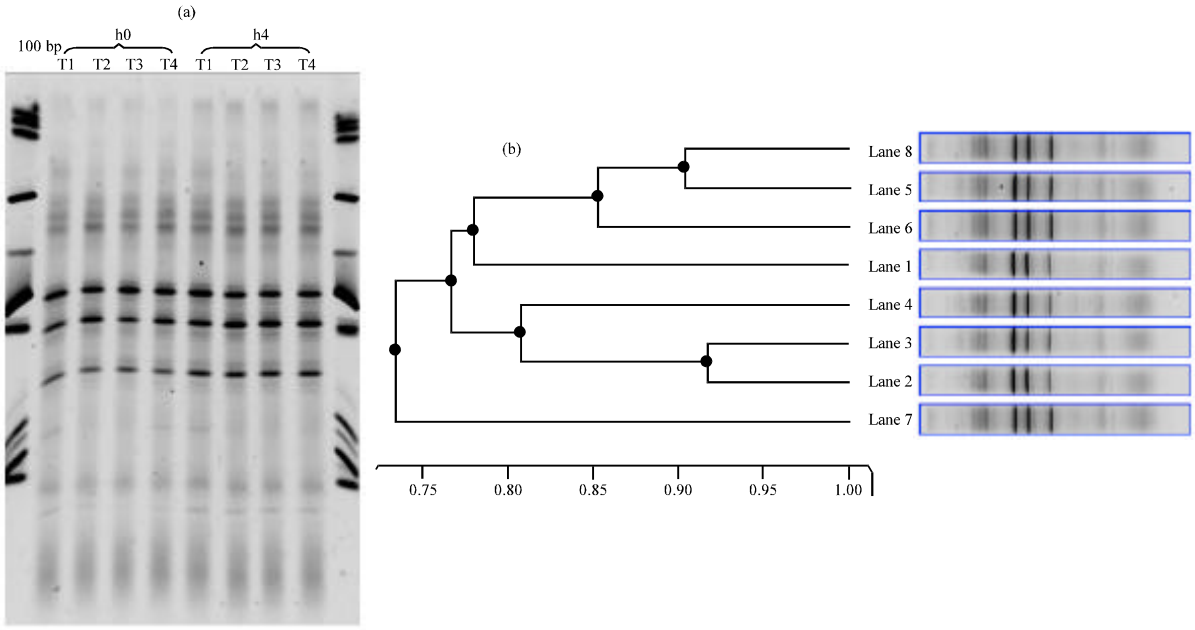

Fig. 3: Negative image of SYBR® Gold stained denaturing gradient gel electrophoresis separation pattern of eight PCR samples in which the rumen methanogenic archaea V3 amplicon using GC-ARC344f and 519r primers (T1 = RS, T2 = RS + 4% urea, T3 = ULRS+0% urea and T4 = ULRS+4% urea) are shown (a) along with the corresponding cluster analysis of the sample profiles (b)

there is still a need for identification of gut fungi species by sequencing technique which would provide more information on anaerobic fungi diversity in the rumen especially in swamp buffalo.

CONCLUSION

In this study, it could be concluded that urea-lime treated rice straw and urea are simple enrichment to obtain rumen fermentation efficiency though contributed of anaerobic fungi and methanogens. No change in rumen methanogenic bacteria diversity. It was suggest that these conditions were sufficiently sustainable fermentation of plant lignocellulosic for ruminant. Further experiment of diverse, fiber degrading of anaerobic fungi and methanogens to management and anaerobic digestion processes rumen ecosystem are required additional attention.

ACKNOWLEDGEMENTS

The researchers would like to express their most sincere gratitude and appreciation to the Commission on Higher Education, Thailand under the Strategic Scholarships for Frontier Research Network program and the Tropical Feed Resources Research and Development Center (TROFREC), Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Thailand for their financial support of research and the use of research facilities. The researchers also acknowledge Agricultural Biotechnology Research Center, Khon Kaen University for real-time PCR analysis and for technical support.

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