

Effect of Legume (*Phaseolus calcaratus*) Hay Supplementation on Rumen Cellulolytic Bacterial Populations in Swamp Buffaloes Investigated by the Real-Time PCR Technique

^{1,2}Vongpasith Chanthakhoun and ¹Metha Wanapat

¹Tropical Feed Resources Research and Development Center (TROFREC),
Department of Animal Science, Faculty of Agriculture, Khon Kaen University,
Khon Kaen 40002, Thailand

²Department of Animal Science, Faculty of Agriculture and Forest Resource,
Souphanouvong University, Luangprabang, LAO PDR, Thailand

Abstract: A quantitative real-time PCR approach was used to determine the population densities of major ruminal cellulolytic bacterial species (*Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*) in rumen fluid and digesta of swamp buffalo (*Bubalus bubalis*). Four rumen-fistulated, male swamp buffalo were randomly assigned according to a 4×4 Latin square design to evaluate the effect of *Phaseolus calcaratus* Hay (PCH) supplementation. PCH contained 18.3% Crude Protein (CP) and 2.8% condensed tannins. Animals were given 0, 300, 600 and 900 g day⁻¹ as supplements. All animals were given *ad libitum* access to rice straw while additional concentrate (12.6% CP) was given at 0.3% body mass and each period lasted for 21 days. At the end of each period, rumen fluid and digesta was collected at 0, 4 h post morning feeding. It was found that PCH supplementation increased these three cellulolytic bacteria *F. succinogenes* between 2.5 and 5.5×10⁹, *R. flavefaciens* between 3.6 and 9.1×10⁹, *R. albus* between 5.7 and 17.9×10⁸ copies mL⁻¹ at 0, 300, 600 and 900 g day⁻¹ of supplementation, respectively. Moreover, at 4 h post-morning feeding, the populations of the two cellulolytic bacteria were higher than those found at 0 h post-morning feeding. It is most notable that *R. flavefaciens* and *R. albus* were the highest in population in the rumen of swamp buffalo, hence indicating high ability in utilizing high fibrous feeds.

Key words: Cellulolytic bacteria, swamp buffalo, fed legumes, supplementation, evaluate, determinate, Thailand

INTRODUCTION

Swamp buffaloes (*Bubalus bubalis*) are important contributors to meat, draft power, fuel and leather production in many developing countries and able to utilize feed efficiently than cattle where the feed supply is of low quality and the ability to recycle nitrogen to the rumen. Thus, variations between cattle and swamp buffalo in the proportions and number of rumen bacteria, protozoa and fungal zoospores could attribute to the explanation of differences in digestive capability due to fermentation end-products available for absorption and utilization by ruminants (Wanapat and Rowlinson, 2007). There are abundantly available local feed resources for ruminants especially those raised in the tropics (Wanapat, 2008). The complex symbiotic microbiota of the rumen is responsible for breakdown of plant fiber, ability the ruminal host animal's lack, This microbiota is highly responsive to changes in diet, age, antibiotic use and the

health of the host animal and varies according to geographical location, season and feeding regimen (Bryant, 1959; Hungate, 1966). However, tropical legumes and browse also contain phenolics other than lignin which limit the digestibility of cell wall carbohydrates and proteins. The presence of tannins in legumes and browse species may also result in binding of proteins and thus inhibit microbial attack and make them an alternative source of by-pass protein to be evaluated as a supplement for ruminant production systems in the tropics. Therefore, anaerobic rumen fibrolytic bacteria, protozoa and fungi degrade fibrous materials allowing ruminants to utilize plant fiber for nutrition. 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 (Forster *et al.*, 1997; Shinkai and Kobayashi, 2007).

Recent advances in molecular biology techniques allow the analysis of such bacteria without cultivation, thereby identifying many functional but uncultured bacteria as new targets for basic and applied research (Kobayashi, 2006). Moreover, DNA-based methods offer the option of storing samples until their analysis which could be an important advantage under field conditions (Castillo *et al.*, 2006). The recent development of Real-time PCR has been successfully used for quantifying protozoa (Skillman *et al.*, 2006; Sylvester *et al.*, 2004), cellulolytic fungi Denman and McSweeney (2006) and cellulolytic bacterial species (Koike and Kobayashi, 2001; Koike *et al.*, 2003; McSweeney and Denman, 2007). Real-time PCR is an approach that allows continuous monitoring of PCR product formation and techniques vary according to the method of fluorescence generation. Real-time PCR has the ability to enumerate targeted bacteria with high sensitivity Zimmermann and Mannhalter (1996) and has been used to analyze various environmental samples such as water (Leser *et al.*, 1995) and rumen digesta (Reilly and Attwood, 1998). 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. Real-time PCR assay development for the cellulolytic rumen bacterial species *F. succinogenes*, *R. albus* and *R. flavefaciens* was reported by Koike and Kobayashi (2001). However, very limited researches have been conducted in swamp buffaloes with regards to ruminal bacterial population using molecular techniques. The project was conducted to study the effects of *Phaseolus calcaratus* Hay (PCH) on cellulolytic ruminal bacterial populations in swamp buffaloes using the real-time PCR technique.

MATERIALS AND METHODS

Animals and sample collection: Four, rumen fistulated, Thai-native swamp buffaloes (*Bubalus bubalis*) about 2.5 years old with 260±10 kg body mass were randomly assigned to receive four ratio of *Phaseolus Calcaratus* Hay (PCH) supplementation (0, 300, 600 and 900 g day⁻¹) according to a 4×4 Latin square design. All animals were given *ad libitum* access to rice straw while additional concentrate (12.6% CP) was given at 0.3% body mass (Table 1). All animals were kept in individual pens and received free choice of water and mineral lick-blocks. The experiment was conducted for four periods each period lasted 21 days. At the end of each period, rumen fluid and digesta was collected at 0, 4 h post morning feeding by hands through rumen fistulated buffaloes from the middle part of the rumen and was stored at -20 for DNA

Table 1: Chemical composition of Rice Straw (RS), legume (PCH) and concentrate mixture

Item	Concentrate	RS	PCH
Ingredients, dry mater (%)			
Cassava chip	63.0	-	-
Rice bran	15.0	-	-
Palm kernel meal	15.0	-	-
Molasses	3.0	-	-
Urea (46 %N)	1.0	-	-
Salt	1.0	-	-
Sulphur	1.0	-	-
Mineral mixture	1.0	-	-
Total	100.0	-	-
Chemical composition			
DM (%)	88.6	93.0	89.6
In % of dry matter			
Ash	5.7	14.6	10.6
CP	12.6	2.7	18.3
NDF	20.8	78.4	51.2
ADF	8.7	46.2	30.7
CT	-	-	2.8

RS = Rice Straw, PCH = *Phaseolus calcaratus* Hay, DM = Dry Matter, OM = Organic Matter; CP = Crude Protein, NDF = Neutral-Detergent Fiber, ADF = Acid-Detergent Fiber, CT = Condensed Tannins

extraction according to Yu and Morrison (2004) technique. Feed chemical composition samples were dried at 60°C and ground (1 mm screen using Cyclotech Mill, Tecator, Sweden) and then analyzed for DM, ether extract, ash and CP content (AOAC, 1990), NDF and ADF were determined according to Goering and Van Soest (1970).

DNA extraction: Community DNA was extracted from 0.5 mL aliquots of rumen digesta by the RBB+C method as described by Yu and Morrison (2004). In brief, the cell lysis is achieved by 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 which are very active in the rumen and gastrointestinal sample. After bead beating most of the impurities and the SDS are removed by precipitation with ammonium acetate and then the nucleic acids are removed by precipitation with isopropanol. Genomic DNA can then purified via sequential digestion with RNase A and proteinase K and the DNA was purified using columns from QIAGEN DNA Mini Stool Kit (QIAGEN, Valencia, CA).

Real-time PCR: The primers used for the real-time PCR are as follows: primers for *Fibrobacter 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 Ra1439r (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). Real-time PCR amplification and detection were performed using a Choromo4 detection system (Bio-Rad, Hercules, CA). The reaction was conducted in a final volume of 10 µL containing the following: 5.1 µL Quatimix EASY SYG Kit (BIOTOOLS B and M Labs, S.A.), 0.408 µL as a forward primer, 0.408 µL as a reverse primer, 2.244 µL distilled water and 2 µL of DNA solution of unknown concentration. 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 of denaturation in the first cycle and 10 min of extension in the last cycle. Amplification of 16S rRNA genes for *R. flavefaciens* and *R. albus* was carried out similarly except at an annealing temperature of 55°C. To determine the specificity of amplification, an analysis of the product melting curve was performed after the last each cycle of amplification.

A sample-derived standard was prepared from the treatment pool set of community DNA instead of amplifying the target genes from individual community DNA samples and then pooling the PCR products. Then the PCR product was purified using a QIA quick PCR purification kit (QIAGEN, Inc., Valencia, CA) and quantified using spectrophotometry. For each sample derived standard, the copy number concentration was calculated based on the length of the PCR product and the mass concentration. Ten-fold serial dilutions were made in Tri-EDTA prior to real-time PCR. In total, three 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 earlier. The 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.

Statistical analysis: Statistical analyses were performed using Proc GLM (Statistical Analysis Systems, 1996) mean separations with a significant ($p < 0.05$) for treatments were statistically compared using the Duncan's New Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

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

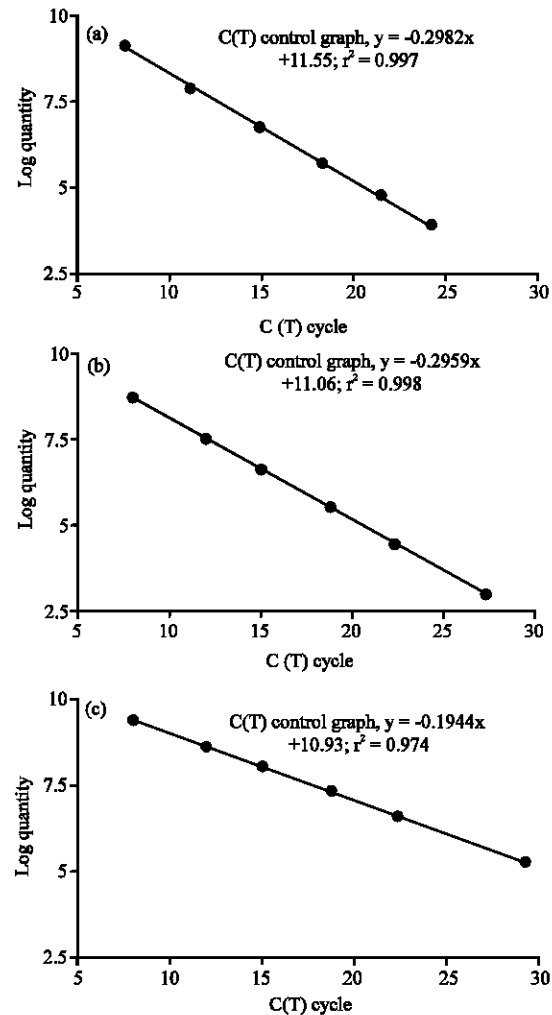


Fig. 1: Standard curve obtained by plotting the logarithm of the DNA concentration for *F. succinogenes* (a), *R. flavefaciens* (b) and *R. albus* (c) vs. threshold cycle (Ct) mean values. The curve was constructed using data from all the eight triplicate standards amplifications

calculated. Logarithms of the DNA concentration (copies mL⁻¹) were plotted against the calculated means (Fig. 1) obtaining a straight line of equations $y = -0.2982x + 11.55$, $y = -0.2959x + 11.06$ and $y = -0.1944x + 10.93$ (where y is the log of DNA concentration and x is the Ct) with a linear correlation coefficient (r^2) of 0.997, 0.998 and 0.974 for *F. succinogenes* (Fig. 1a), *R. flavefaciens* (Fig. 1b) and *R. albus* (Fig. 1c), respectively. The equations were used to quantify DNA from rumen digesta samples. Table 1 and 2 shows data on chemical composition of experimental feeds and cellulolytic bacterial population obtained by real-time PCR technique. Concentrate mixture

Table 2: Quantification of cellulolytic bacterial DNA from rumen sampling hours using Real-time PCR technique as influenced by the PCH supplementation in swamp buffalo

Item	Treatments				SEM	Contrasts		
	0	300	600	900		L	Q	C
<i>F. succinogenes</i> ×10⁹ copies mL⁻¹								
0 h-post feeding	2.6	3.9	6.7	2.6	1.4	NS	NS	NS
4	2.4	3.4	4.3	3.3	0.4	NS	*	NS
Mean	2.5 ^a	3.7 ^{ab}	5.5 ^b	3.0 ^a	0.7	NS	*	NS
<i>R. flavefaciens</i> ×10⁸ copies mL⁻¹								
0 h-post feeding	3.9	3.7	6.1	8.5	1.6	0.06	NS	NS
4	6.1 ^a	3.6 ^a	7.6 ^b	9.6 ^b	1.3	*	NS	NS
Mean	5.0 ^a	3.6 ^{ab}	6.9 ^a	9.1 ^c	0.9	*	NS	NS
<i>R. albus</i> ×10⁸ copies mL⁻¹								
0 h-post feeding	2.7	3.8	5.6	12.3	3.6	NS	NS	NS
4	8.8	13.8	7.4	23.5	5.4	NS	NS	NS
Mean	5.7 ^a	8.8 ^a	6.5 ^a	17.9 ^b	2.4	*	NS	NS

^{a,b,c}Means in the same row with different superscripts differ (p<0.05); T1: 0 (Control), T2: 300 g, T3: 600 g, T4: 900g/hd/day, PCH, SEM = Standard Error of Mean. L: Linear, Q: Quadratic, C: Cubic

was formulated to contain carbohydrate and protein sources using cassava chip and oil-crop meals resulting in 12.6% CP and 20.8% NDF, respectively. Meanwhile, rice straw and *Phaseolus calcaratus* Hay (PCH) contained 2.7 and 18.3% CP, 51.2% NDF, respectively. In addition, PCH had 2.8% condensed tannins. Three predominant cellulolytic bacterial population namely, *F. succinogenes*, *R. flavefaciens* and *R. albus* were quantified by real-time PCR technique using DNA extracted from rumen fluid and digesta sampled at 0 and 4 h post morning feeding.

There was an increasing trend for the two species except for one species after feeding times especially for *R. flavefaciens*. Supplementation level of PCH resulted in increased population of *F. succinogenes* quadratically while in *R. flavefaciens* and *R. albus* were increased linearly. These findings suggest that PCH had provided addition of both physical and chemical sources enabling these bacteria to grow favourably. Significant increases were found in *R. flavefaciens* and *R. albus* when PCH were supplemented at 600 and 900 g day⁻¹. However, when determined by total means there were increasing trends for all bacteria species after feeding times.

High level of PCH increased cellulolytic bacteria numbers in the rumen fluid and digesta (Table 2) which could be related to the effect of condensed tannin (2.8% in the DM). However, Hart and Wanapat (1992) who compared Untreated Rice Straw (URS) and Urea Treated (5%) Rice Straw (UTRS) in swamp buffaloes, it was revealed that Organic Matter (OM) digestibility and voluntary OM intake were significantly improved by 17 and 25%, respectively. In addition, rumen retention time was decreased by 34% indicating pronounced activities of rumen cellulolytic bacteria. In another trial by Chanjula *et al.* (2004) increasing level of cassava hay in rice straw based-diet dramatically

decreased rumen protozoa (6.2-2.1×10⁵ cell g⁻¹ rumen) while cellulolytic bacteria exhibited an increasing trend from 5.3-7.2×10⁷ CFU g⁻¹ rumen. The effects were attributed by cassava hay containing higher protein (25%) and condensed tannins (4%). On the other hand, the studies conducted to investigate effect of urea level on energy sources by Wanapat *et al.* (2009) and effect of roughage to concentrate ratio by Wanapat and Cherdthong (2009), reported that in swamp buffalo using PCR-DGGE and Real-time PCR techniques, it was found that population of predominant cellulolytic bacteria were higher in population of *F. succinogenes*, *R. flavefaciens* and *R. albus* in both rumen fluid and digesta, respectively.

The dynamics of truly cellulolytic rumen bacteria were in good correlation with response to diet shift particularly increasing level of concentrate and the application of Real-time PCR technique allowed an investigation on possible changes in the number of cellulolytic bacterial species *in vivo* (Mosoni *et al.*, 2007). In another experiment, it was found that reduced rumen protozoal numbers, increased bacterial numbers, significantly reduced ammonia nitrogen, higher daily body weight gain and improved feed conversion efficiency (Russell and Rychlik, 2001). Currently, Khiaosa-Ard *et al.* (2009) have shown effective result of condensed tannins on reducing rumen protozoa and increasing bacterial population. Moreover, condensed tannins has been shown to bind to the terminal end of linoleic acid and hence, reducing bihydrogenation and elevating levels of conjugated linoleic acid in lower-gut. Moreover, rumen pH, together with microbial population, nature of substrates, environmental factors such as temperature and existence of cations and soluble carbohydrates have been suggested as factors governing bacterial attachment of feed particles (Miron *et al.*, 2001). Ruminant pH is one of the most important of these factors because cellulolytic bacteria are very sensitive to pH changes (Sung *et al.*, 2007). When ruminants fed with fiber-deficient rations, ruminal pH declines, microbial ecology is altered and the animals become more susceptible to metabolic disorders (Russell and Rychlik, 2001). As Koike *et al.* (2003) quantified cell numbers of *F. succinogenes*, *R. flavefaciens* and *R. albus* which attached to straw by competitive PCR and illustrated that numbers of all three species increased gradually with increased NDF disappearance. On the other hand, Wora-Anu *et al.* (2000) reported that roughage to concentrate ratio at 100: 0, 60: 40 and 40: 60 could decrease cellulolytic bacterial population in swamp buffalo (5.62, 4.06 and 4.57×10¹⁰ CFU mL⁻¹), respectively. Furthermore, when urea-treated rice straw was solely fed the three cellulolytic bacteria numbers were increased up to 2.65×10⁹ and 3.54×10⁹ copies per milliliter for *F. succinogenes*, 5.10×10⁷ and

7.40×10^7 copies mL^{-1} for *R. flavefaciens* and 4.00×10^6 and 6.00×10^6 copies mL^{-1} for *R. albus* in rumen fluid and digesta, respectively. The distribution of the three cellulolytic bacteria species in digesta were highest at 3.21×10^9 , 4.55×10^7 and 4.56×10^6 copies mL^{-1} for *F. succinogenes*, *R. flavefaciens* and *R. albus*, respectively as reported by Wanapat and Cherdthong (2009). In the study by Koike *et al.* (2003) who suggested that the increase in attached cell numbers observed could be mostly attributed to cell proliferation on the straw after 6 h, the numbers of attached cells of the three species gradually increased and peaked at 24 h (10^9 g^{-1} DM for *F. succinogenes* and 10^7 g^{-1} DM for *R. flavefaciens*) or 48 h (10^6 g^{-1} DM for *R. albus*). There are two possible explanations of the increased cell populations on 4 h post-feeding; one is cell proliferation after feeding and additional attachment of new bacteria from the liquid phase or from other particles. More data found under this study are shown in Table 2.

CONCLUSION

Based on this study, it was found that PCH supplementation between 600-900 g/hd/day could improve higher cellulolytic bacterial population in swamp buffaloes. Moreover, applicability of Real-time PCR technique for quantification of cellulolytic bacterial species (*F. succinogenes*, *R. albus* and *R. flavefaciens*) in the rumen fluid and digesta of swamp buffaloes could offer useful information. As found, *R. flavefaciens* and *R. albus* were the most predominant of the three species as influenced by PCH supplementation in the rumen of swamp buffalo.

RECOMMENDATIONS

Therefore, legume supplementations such as those of PCH were useful sources in enhancing rumen ecology and subsequent fermentation.

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 Master Degree Research Grants: TRF-MAG and Khon Kaen University for providing financial support of research, research facilities and experimental animals. Laboratory equipment provided by the Agricultural Biotechnology Research Center, Faculty of Agriculture, 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.
- Bryant, M.P., 1959. Bacterial species of the rumen. *Bacteriol. Rev.*, 23: 125-153.
- Castillo, M., M.S. Martin-Orue, E.G. Manzanilla, I. Badiola and M.J. Gasa, 2006. Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR. *Vet. Microbiol.*, 114: 165-170.
- Chanjula, P., M. Wanapat, C. Wachirapakorn and P. Rowlinson, 2004. Effect of various levels of cassava hay on rumen ecology and digestibility in Swamp buffaloes. *Asian-Aust. J. Anim. Sci.*, 17: 663-669.
- 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.
- Forster, R.J., J. Gong and R.M. Teather, 1997. Group-specific 16S rRNA hybridization probes for determinative and community structure studies of *Butyrivibrio fibrisolvens* in the rumen. *Applied Environ. Microbiol.*, 63: 1256-1260.
- Goering, H.K. and P.J. van Soest, 1970. Forage Fiber Analysis (Apparatus, Reagents, Procedures and Some Applications). *Agricultural Handbook 379*, Agricultural Research Service, United States Department of Agriculture, Washington, DC., pp: 1-20.
- Hart, F.J. and M. Wanapat, 1992. Physiology of digestion of urea-treated rice straw in swamp buffalo. *Asian-Aust. J. Anim. Sci.*, 5: 617-622.
- Hungate, R.E., 1966. *The Rumen and its Microbes*. Academic Press, New York.
- Khiaosa-Ard, R., S.F. Bryner, M.R.L. Scheeder, H.R. Wettstein, F. Leiber, M. Kreuzer and C.R. Soliva, 2009. Evidence for the inhibition of the terminal step of ruminal α -linolenic acid biohydrogenation by condensed tannins. *J. Dairy Sci.*, 92: 177-188.
- 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: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. *FEMS Microbiol. Lett.*, 204: 361-366.

- Koike, S., J. Pan, Y. Kobayashi 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.
- Leser, T.D., M. Boye and N.B. Hendriksen, 1995. Survival and activity of a *Pseudomonas* sp. strain B13 (FR1) in a marine microcosm determined by quantitative PCR and an rRNA-targeting probe and its effect on the indigenous bacterioplankton. *Applied Environ. Microbiol.*, 61: 1201-1207.
- McSweeney, C.S. and E. Denman, 2007. Effect of sulfur supplements on cellulolytic rumen micro-organisms and microbial protein synthesis in cattle fed a high fibre diet. *J. Applied Microbiol.*, 103: 1757-1765.
- 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.
- Reilly, K. and G.T. Attwood, 1998. Detection of clostridium proteoclasticum and closely related strains in the rumen by competitive PCR. *Applied Environ. Microbiol.*, 64: 907-913.
- Russell, J.B. and J.L. Rychlik, 2001. Factors that alter rumen microbial ecology. *Science*, 292: 1119-1122.
- Shinkai, T. and Y. Kobayashi, 2007. Localization of ruminal cellulolytic bacteria on plant fibrous materials as determined by fluorescence *in situ* hybridization and real-time PCR. *Applied Environ. Microbiol.*, 73: 1646-1652.
- Skillman, L.C., A.F. Toovey, A.J. Williams and A.D. Wright, 2006. Development and validation of a real-time PCR method to quantify rumen protozoa and examination of variability between entodinium populations in sheep offered a hay-based diet. *Applied Environ. Microbiol.*, 72: 200-206.
- Statistical Analysis Systems, 1996. SAS Users Guide: Statistics. 5th Edn., SAS Institute for Statistical Analysis, Cary, USA.
- 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.
- Sylvester, J.T., S.K. Karnati, Z. Yu, M. Morrison and J.L. Firkins, 2004. Development of an assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR. *J. Nutr.*, 134: 3378-3384.
- 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. and P. Rowlinson, 2007. Nutrition and feeding of swamp buffalo: Feed resources and rumen approach. *Proceedings of the 8th World Buffalo Congress*, Oct. 19-22, Caserta, Italy, pp: 67-73.
- Wanapat, M., 2008. Potential uses of local feed resources for ruminants. *Trop. Anim. Health. Prod.*, 41: 1035-1049.
- Wanapat, M., R. Pilajun and P. Kongmun, 2009. Ruminant ecology of swamp buffalo as influenced by dietary sources. *Anim. Feed Sci. Tech.*, 151: 205-214.
- Wora-Anu, S., M. Wanapat, C. Wachirapakorn and N. Nuntasu, 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. *Bio Technol.*, 36: 808-812.
- Zimmermann, K. and J.W. Mannhalter, 1996. Technical aspects of quantitative competitive PCR. *Biotechniques*, 21: 268-279.