Journal of Animal and Feed Sciences, 16, Suppl. 2, 2007, 95–100

Effect of form of nitrogen on populations of fibre-associated ruminal microbes in pre-treated rice straw *in vitro*[*]

X.L. Chen, J.K. Wang, Y.M. Wu and J.X. Liu[1]

College of Animal Sciences, Institute of Dairy Science, Ministry of Education,
Key Laboratory of Molecular Animal Nutrition, Zhejiang University
Hangzhou 310029, P.R. China

ABSTRACT

The study was conducted to investigate the effect of ammonium bicarbonate and casein on rumen fermentation and relative populations of fibre-associated cellulolytic microbes in untreated and sodium hydroxide treated rice straw *in vitro*. Form of nitrogen had different effect on rumen fermentation and fibre-associated microbial populations. Cumulative gas production significantly increased with addition of nitrogen. Addition of casein significantly improved rumen fermentation and microbial populations. Casein had higher efficiency to supply available nitrogen to microbial growth and degradation of straw than ammonium bicarbonate. It is inferred that a supply of protein nitrogen would be needed for maximum degradation of straw.

KEY WORDS: ammonium bicarbonate, casein, rumen fermentation, fibre-associated microbes, treated rice straw

INTRODUCTION

Straw as a low-nitrogen (N) feed resource is the largest by-products of cereal production. Treatment with ammonia or alkali compounds is now widely accepted and applied as the most successful chemical method to improve the nutritional value of straw (Liu et al., 2002). However, sodium hydroxide (NaOH) does not contribute N to straw. Since N is a limiting nutrient in low-quality forages, it is necessary to supply ammonia N for the rumen microbes in NaOH treated straw.

[*] Supported by the the National Science Funds for Distinguished Young Scholar, Grant No. 30325033, and Trans-Centur y Training Programme Foundation for the Talents by the MOE, P.R. China
[1] Corresponding author: e-mail: liujx@zju.edu.cn

Ruminal cellulolytic microbes such as *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Fibrobacter succinogenes*, *Butyrivibrio fibrisolvens* and fungi, play an important role in fibre digestion. They require ammonia N for their optimum growth when host was fed fibre basal diet (Ranilla et al., 2001). The form of N may affect substrate digestion (Griswold et al., 1996). Casein is readily attacked by rumen microbes (Chalmers et al., 1954), and may be used as a good source of protein N. Fujimaki et al. (1989) found no difference in digestion of cell wall carbohydrate when non-protein N replaced ammonia, while Carro and Miller (1999) assumed that N sources other than ammonia are required not only for maximal microbial growth but also substrate digestion. These contrasting results might be explained by differences in the composition of diet such as source and availability of readily fermentable carbohydrates. Quantification of population sizes of ruminal microbes using real-time PCR would be appropriate method to reveal the effect of N forms on microbial growth. In this study, casein was used in comparison with ammonium bicarbonate (AB) as supplementary N source to untreated and NaOH treated rice straw to investigate their effect on fibre degradation and populations of ruminal microbes.

MATERIAL AND METHODS

Material

The straw (contained 8.8 g/kg N and 727 g/kg neutral detergent fibre, NDF) obtained in Zhejiang Province (China) was treated with NaOH (45 g/kg straw dry matter, DM) by the method of Wang et al. (2007). The content of N and NDF of NaOH treated straw was 9.8 and 644 g/kg DM, respectively.

In vitro gas test

Incubations were carried out using the methods described by Theodorou et al. (1994). One gram of untreated or NaOH treated rice straw was incubated in triplicate with 10 ml rumen fluid collected from three donor sheep before morning feeding and 90 ml buffer medium prepared as described by Menke and Steingass (1988), with exception that no ammonium bicarbonate (AB) was added. Casein or AB was added at a level equivalent to 140 mg/l N. Non-N treatment was used as negative control. In each incubation run, three blanks were included simultaneously to correct the gas production volume for gas release from endogenous substrates.

Sampling and analysis

At the 24 h of incubation time, the fermentation was stopped by swirling the bottle in ice water. About 30 ml of mixed fermentation medium was taken for analysis of ammonia-N, volatile fatty acids (VFAs) and microbial protein (MCP). The remaining contents were filtered through nylon bags (40 μm pore size) and the residues were used to extract total ruminal microbial DNA for quantification of populations of *R. albus*, *R. flavefaciens*, *F. succinogenes*, *B. fibrisolvens* and total fungi attached to straws. Species-specific real-time quantitative PCR was performed using the ABI 7500 real time PCR system (Applied Biosystems) with fluorescence detection of SYBR green dye. Amplification consisted of an initial denature at 95°C for 15 s followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. Specificity of amplified products was confirmed by melting temperatures and dissociation curves after each amplification. Amplification efficiencies for each primer pairs were investigated by examining dilution series of total rumen microbial DNA template on the same plate in triplicate.

Calculations and statistical analysis

Relative population sizes of *R. albus*, *R. flavefaciens*, *F. succinogenes*, *B. fibrisolvens* and total rumen fungi were expressed as a proportion of total rumen bacterial 16S rDNA. The main effects of straw, N form and their interactive effect on rumen fermentation parameters and rumen microbial populations were analysed using the general linear model (GLM) procedure of SAS.

RESULTS

In vitro rumen fermentation variables for untreated and NaOH treated rice straw are shown in Table 1. The cumulative gas production at 24 h incubation increased when N was added compared with non-N treatment. The highest gas production (93 ml/g DM) was observed in NaOH treated straw with supply of protein and the lowest (48 ml/g DM) in untreated straw with non-N addition. Addition of AB and casein increased significantly the concentration of total VFAs in NaOH treated straw. No significant differences were found in total VFAs, molar proportion of acetate, propionate and butyrate between AB and non-N treatment in untreated straw. Concentration of MCP for two straws was increased significantly by either AB or casein.

Population sizes of rumen microbes attached to straws relative to rumen bacterial 16S rDNA *in vitro* with or without N addition are shown in Table 2. In untreated straw, addition of AB and casein increased the populations of *F. succinogenes*, *B. fibrisolvens*

Table 1. Rumen fermentation *in vitro* of untreated and sodium hydroxide (NaOH) treated rice straw supplemented with ammonium bicarbonate (AB) or casein

Items	Untreated straw			NaOH treated straw			SEM	Significance of[1]		
	none	AB	casein	none	AB	casein		S	N	S×N
GP_{24}[2], ml/g DM	44	52	48	77	87	93	0.7	**	**	**
Ammonia nitrogen, mg/dl	7.3	27.2	26.2	1.4	17.0	17.4	0.55	**	**	**
Total volatile fatty acids	35.3	34.2	40.4	47.2	50.1	57.0	0.50	**	**	**
acetate, molar %	73.5	73.7	74.7	74.7	74.5	73.0	0.30	NS	NS	**
propionate, molar %	19.1	18.5	18.5	18.9	18.6	20.3	0.20	**	**	**
butyrate, molar %	7.4	7.7	6.8	6.4	6.9	6.6	0.21	**	*	NS
Microbial protein, mg/ml	0.72	0.89	0.94	0.98	1.15	1.10	0.025	**	**	NS

[1] S - straw, N - N form, S × N - interactive effect of straw and N form, *P<0.05, **P<0.01, NS - P>0.05; [2] GP_{24} - gas production at 24 h incubation *in vitro*

and total fungi, while no significant effect on Ruminococci was observed. In NaOH treated straw, N form had little effect on population of *F. succinogenes*. Addition of AB and casein significantly increased the attached populations of *R. albus* and *B. fibrisolvens,* with higher populations for addition of casein than AB. Highest populations of *R. flavefaciens* and fungi were found in NaOH treated straw with non-N than with addition of N.

Table 2. Population sizes of rumen microbes (% rumen bacterial 16S rDNA) attached to untreated and sodium hydroxide treated rice straw *in vitro* supplemented ammonium bicarbonate (AB) or casein

Items	Untreated straw			NaOH treated straw			SEM	Significance of[1]		
	none	AB	casein	none	AB	casein		S	N	S×N
R. albus	0.16	0.14	0.20	0.79	2.11	2.46	0.032	**	**	**
R. flavefaciens	0.75	0.76	0.90	1.64	1.06	1.17	0.023	**	**	**
F. succinogenes	12.90	14.71	16.03	2.61	2.55	2.99	0.220	**	**	**
B. fibrisolvens	0.004	0.010	0.017	0.034	0.094	0.337	0.0015	**	**	**
Total fungi	0.49	1.52	1.13	4.24	3.69	2.86	0.053	**	**	**

[1] S - treatment, N - N form, S × N - interactive effect of straw and N form, **P<0.01

DISCUSSION

In vitro gas production technique has been widely applied to simulate fermentation kinetics in the rumen and predict *in vivo* digestibility. The main fermentation end products from the *in vitro* gas production technique are gases (CO_2 and CH_4), microbial biomass and VFAs. The rumen fermentation variables *in vitro* reflect digestion of substrate. The cumulative gas production after 24 h incubation increased when AB and

casein were added. Addition of casein significantly increased total VFAs concentration. The production of VFAs may depend on the type of fermentable organic matter, rumen microbes involved and rumen environment. In the present study, the energy and fermentation environment was constant for the same type of straw, thus fermentable organic matter, and then VFAs, varied only with addition of casein and microbes involved. From the result shown in Table 1, rumen MCP concentration was significantly higher after addition of casein, compared with non-N treatment, probably resultant from the improved balance of energy and N for rumen microbial growth. Compared with untreated straw, concentration of ammonia-N in NaOH treated straw is lower. Probably more N was used in NaOH treated straw to support its faster degradation.

Recently, with the advancement of molecular enumeration methods, real-time PCR has been widely accepted to monitor microbes in the environment due to its rapid, sensitive and reproducible advantage, especially for low abundance templates. Real-time PCR using species-specific primers have been successfully developed to monitor bacterial populations in the rumen (Denman and McSweeney, 2006). In the present study, rumen main cellulolytic microbes had been detected using real-time PCR technique to investigate their responses to N form for treated and untreated rice straw.

Form of N and rate of fibre degradation had effects on the rumen microbial populations attached to straw. *R. albus*, *R. flavefaciens* and *F. succinogenes* are considered as the representative cellulolytic species in the rumen. Relative population of the three cellulolytic bacteria is about 5.04 to 17.1% of total rumen bacterial 16S rDNA in the present study with higher value in untreated straw. This result is consistent with other reports that higher populations of cellulolytic bacteria were observed in higher cellulose diets (Weimer et al., 1999). Rumen microbial communities varied when N was added in different straws (Table 2), resulting in improved rumen fermentation characteristics. Relative population of *F. succinogenes* was predominant in untreated straw compared with other microbes, which would have major contribution to rumen fermentation. Addition of AB seems to have slight effect on rumen microbial community and rumen fermentation in the untreated straw due to its lower rate of degradation, but has significant effect on NaOH treated straw. There are more carbohydrate fermented in NaOH treated straw and more available N supplied by AB to meet requirement of microbial growth. The presence of N increased the population of *B. fibrisolvens*, suggesting *B. fibrisolvens* is not only cellulolytic but also proteolytic species. High N content of hay has been observed to stimulate growth of *B. fibrisolvens* (Koike et al., 2003).

CONCLUSIONS

The results of the present study have confirmed the importance of N for microbial growth. Form of N could affect rumen microbial community and fermentation *in vitro*

for different rate of degradation of substrates. Addition of ammonium bicarbonate (AB) would be more effective to increase rumen microbial populations and improve rumen fermentation in NaOH treated than in untreated rice straw. Addition of casein had larger influence on rumen microbial growth than AB. Supplementation of protein N would be needed for maximum degradation of straw *in vitro* by ruminal microbes.

REFERENCES

Carro M.D., Miller E.L., 1999. Effect of supplementing a fibre basal diet with different nitrogen forms on ruminal fermentation and microbial growth in an *in vitro* semi-continuous culture system (RUSITEC). Brit. J. Nutr. 82, 149-157

Chalmers M.I., Cuthbertson D.P., Synge R.L.M., 1954. Ruminal ammonia formation in relation to the protein requirement of sheep. I. Duodenal administration and heat processing as factors influencing fate of casein supplements. J. Agr. Sci. 44, 254-262

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

Fujimaki T., Kobayashi M., Wakita M., Hoshino S., 1989. Influence of amino acid supplement on cellulolysis and microbial yield in sheep rumen. J. Anim. Physiol. Anim. Nutr. 62, 119-124

Griswold K.E., Hoover W.H., Miller T.K., Thayne W.V., 1996. Effect of form of nitrogen on growth of ruminal microbes in continuous culture. J. Anim. Sci. 74, 483-491

Koike S., Yoshitani S., Kobayashi Y., Tanaka K., 2003. Phylogenetic analysis of fiber-associated rumen bacterial community and PCR detection of uncultured bacteria. FEMS Microbiol. Lett. 229, 23-30

Liu J.X., Susenbeth A., Südekum K.-H., 2002. *In vitro* gas production measurements to evaluate interactions between untreated and chemically treated rice straw, grass hay, and mulberry leaves. J. Anim. Sci. 80, 517-524

Menke K.H., Steingass H., 1988. Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. Anim. Res. Develop. 28, 7-55

Ranilla M.J., Carro M.D., López S., Newbold C.J., Wallace R.J., 2001. Influence of nitrogen source on the fermentation of fibre from barley straw and sugar beet pulp by ruminal micro-organisms *in vitro*. Brit. J. Nutr. 86, 717-724

Theodorou M.K., Williams B.A., Dhanoa M.S., McAllan A.B., France J., 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feed. Anim. Feed Sci. Tech. 48, 185-197

Wang J.K., Liu J.X., Li J.Y., Wu Y.M., Ye J.A., 2007. Histological and rumen degradation changes of rice straw stem epidermis as influenced by chemical pretreatment. Anim. Feed Sci. Tech. 136, 51-62

Weimer P.J., Waghorn G.C., Odt C.L., Mertens D.R., 1999. Effect of diet on populations of three species of ruminal cellulolytic bacteria in lactating dairy cows. J. Dairy Sci. 82, 122-134