Influence of β 1-4 Galacto-oligosaccharides Supplementation on Nitrogen Utilization, Rumen Fermentation, and Microbial Nitrogen Supply in Dairy Cows Fed Silage

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ABSTRACT : In a balanced incomplete block design, two dry Holstein cows were used to investigate the effect of β 1-4 galacto-oligosaccharides (GOS) supplementation on nitrogen (N) utilization, rumen fermentation and microbial N supply in the rumen. During the experiment, cows were fed four diets: orchardgrass (*Dactylis glomerata* L.) silage (OS), OS with GOS supplementation (OSG), OS mixed with alfalfa (*Medicago sativa* L.) silage (MS) and MS with GOS supplementation (MSG). GOS was supplemented at 2% of dry matter intake. Diets were fed at maintenance level of protein and energy. Results showed that N digestion was affected by silage and interaction of silage and GOS supplementation. Cows fed OSG had the highest N digested ($p<0.05$) followed by MS, OS and MSG. Supplementation of GOS to OS or MS diets tended to improve N utilization through reducing the N losses on dairy cows. There was no effect of GOS supplementation on rumen fermentation parameters (i.e. pH, NH_3-N and total VFA) at 1 h and 6 h after feeding. Compared to cows fed MS, cows fed OS silage had higher ($p<0.05$) allantoin excretion (80.8 vs. 67.1 mmol/d) and higher ($p<0.05$) total purine derivatives excretion (92.9 vs. 78.5 mmol/d). The microbial N supply in cows fed OSG was higher ($p<0.05$) than those fed OS, MS and MSG. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 8 : 1137-1142)***

Key Words : Galacto-oligosaccharides, Nitrogen, Microbial Nitrogen Supply, Silage, Dairy Cows

INTRODUCTION

The rumen microbes are able to convert fibrous feeds and low quality protein into valuable nutrients for the ruminant animals. More than half of the amino acids ingested are absorbed by ruminant, and often two-thirds to three-quarters of requirements are derived from microbial protein (Agriculture and Food Research Council, 1992). Hence microbial protein must be considered as an important protein resource. Rumen microbes require nitrogen (N) sources, carbon chains (branched chain volatile fatty acids) and adenosine triphospate (ATP) as the energy source for their protein syntheses. It is known that the process of microbial protein synthesis will be maximized by synchronizing availabilities of both fermentable energy and degradable nitrogen in the rumen.

During the ensiling process of forage, extensive proteolysis occurs resulting in conversion of most protein to non-protein nitrogen (NPN). This nitrogen is present as amino acid, peptides, and only a small proportion of ammonia (Harrison et al., 1994). The rapid rate of degradation of silage NPN and soluble-protein N in the rumen results in a pronounced peak in rumen ammonia concentration following ingestion (Thomas and Thomas, 1985). Excess quantities of ammonia in the rumen is absorbed into the blood stream, converted to urea in liver and subsequently excreted in the urine, contributing to environmental pollution (Tamminga, 1992).

Diets containing a high proportion of silage relative to concentrate result in a low rate of microbial protein synthesis due to the poor ATP yield obtained from silage fermentation (Thomas and Thomas, 1985). Moreover, Chamberlain (1987) demonstrated that over ensiling could reduce the energy for microbial synthesis in the rumen by about 15-20%. The rapid rate of ammonia release from silage relative to the lower levels of fermentable energy suggests that supplementary with a rapidly fermented carbohydrate source may promote microbial protein synthesis.

The β 1-4 galacto-oligosaccharides (GOS) is synthesized enzymatically from lactose by the action of β-D-galactosidase derived from *Bacillus circulans* or *Cryptococcus laurentii* (Tanaka and Matsumoto, 1998). This GOS, which has a β-configuration in the structure indicated that it resists and escape the intestinal digestion and absorption of monogastric animals and reaches the caecum and colon without conformational degradation (Sako et al., 2000). In human or rat, administration of GOS could lead to stimulates colonic bacteria (Bouhnik et al., 1997) and alters in short chain fatty acid concentration in the hindgut (Kikuchi et al., 1996). However, physiological significance of GOS on the rumen ecosystem has not been

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Table 1. Chemical composition of silages and GOS (DM basis)

	OS	MS	GOS
DM (%)	28.2	28.8	96.3
OM (%)	93.0	91.1	99.9
CP (%)	13.1	13.6	0.03
NDF (%)	60.8	56.0	-
ADF (%)	39.2	41.3	-
Hemicellulose (%)	21.6	14.7	-

clearly demonstrated.

The present study was conducted to determine the influence of GOS supplementation on N utilization, rumen fermentation and estimation of microbial yield in Holstein cows fed silage.

MATERIALS AND METHODS

Animals and diets

Two dry Holstein cows (685 and 679 kg) were assigned to a balanced incomplete block design (four diets×two animals×four periods). Cows were fed twice daily in equal amounts at 08:00 h and 16:00 h to meet the maintenance requirements for protein and energy (Agricultural, Forestry and Fisheries Research Council Secretariat, 1994). The four experimental diets were orchardgrass (*Dactylis glomerata* L.) silage (OS), OS with GOS supplementation (OSG), OS mixed with alfalfa (*Medicago sativa* L.) silage (47.07:52.93, DM basis) (MS) and MS with GOS supplementation (MSG). The GOS was obtained from Yakult Central Institute for Microbiological Research (Tokyo, Japan) and was supplemented at 2% of dry matter intake. Structural formula of GOS is gal-$(gal)_n$-glu (n=1-4), consists of monosaccharides 18.3%, disaccharides 38.8%, trisaccharides 23.5%, tetrasaccharides 11.4% and pentasaccharides or other 4.51%. Chemical composition of silages and the GOS used are shown in Table 1. The OS diet and MS diet had similar contents of dry matter (DM) and crude protein (CP), but OS contained higher (neutral detergent fiber) NDF and hemicellulose than MS.

Experimental procedure

Cows were housed in the individual pens for 9 days during the feed adjustment period, followed by the 5 day collection period. During experiments, cows had free access to water and a mineral block. Animals were weighed before and after each collection period. Representative samples of diets were taken for three days in each period. The total fecal and urinary output were collected daily for 5 days. The samples of diet and 10% of daily feces were air dried to a constant weight after oven drying at 60°C for 48 h and ground for analyses. Dry matter, organic matter (OM), CP, NDF, and acid detergent fiber (ADF) of samples were analyzed according to the Agricultural, Forestry and Fisheries Research Council Secretariat (1994). Hemicellulose was estimated by subtracting NDF from ADF. Urine was collected in a container containing 100 ml of 20% H_2SO_4 to reduce pH to below 3 and sampled as much as 300 ml. The samples were stored frozen (-20°C) before analysis of allantoin (Chen and Gomes, 1995), uric acid (Uric Acid C test kit, Wako Pure Chemical Industries, Ltd. Japan) and total N (Kjeldahl).

Collection and analysis of rumen liquor

Samples of rumen liquor were collected at 1 h and 6 h after feeding on the last day of each period. The samples (100 ml) were taken using a flexible stomach tube inserted into rumen via the oesophagus and subsequently strained through four layers of cheesecloth. The pH value was measured immediately by using a digital pH meter (Horiba D-14). Samples were frozen (-20°C) and later analyzed for ammonia nitrogen (NH_3-N) concentration (Conway and O'Malley, 1942) and volatile fatty acid (VFA) concentration by using capillary column gas chromatography (Shimadzu, GC-14A), according to the technique described by Takahashi et al. (1997).

Estimation of microbial nitrogen supply

The amount of microbial purines absorbed (X, mmol per day) corresponding to the purine derivatives excreted (Y, mmol per day) was calculated based on the relationship derived by Chen and Gomes (1995), namely:

$$Y=0.85X+(0.385\ W^{0.75})$$

The supply of microbial N to the animal was estimated as follows:

$$\text{Microbial N (gram/day)} = \frac{70X}{0.116\times0.83\times1,000} = 0.727X$$

where: 0.83 = digestibility of microbial purines; 70=the N content of purines (mg/mmol); and 0.116=ratio of purine-N to total N in mixed rumen microbes (11.6:100).

Statistical analysis

Analysis of variance was carried out using the general linear model procedure of SAS (Statistical Analysis Systems Institute Inc., 1990) in a balance incomplete block design (Chakravarti et al., 1967) with period as a block and each block contained two cows. The rumen liquor sampling time was included as an additional factor in the model for rumen fermentation parameters using the repeated measures analysis. Comparison of means was tested using the Duncan's multiple range test (Steel and Torrie, 1960), when the effect of treatment was significant ($p<0.05$).

Table 2. Apparent digestibility and nitrogen balance of dairy cows fed silage with GOS supplementation

	Diets				S.E.[1]	Main effects (p)		
	OS	OSG	MS	MSG		Silage (S)	GOS (G)	S×G
Intake								
DM (kg/d)	8.53	8.94	8.23	8.32	0.33	NS[2]	NS	NS
OM (kg/d)	7.96	8.30	7.50	7.57	0.32	NS	NS	NS
Apparent Digestibility								
DM (%)	61.9	63.4	58.7	60.1	1.2	*	NS	NS
OM (%)	64.0	65.2	59.7	61.0	1.2	**	NS	NS
Nitrogen balance								
N intake (g/d)	169.2	192.6	186.8	170.7	6.0	NS	NS	NS
N in feces (g/d)	67.1	68.2	74.4	71.2	4.7	NS	NS	NS
N in urine (g/d)	89.4	94.7	106.6	90.6	6.0	NS	NS	NS
Digested (g/d)	102.2^{bc}	124.5^{a}	112.5^{b}	99.5^{c}	2.3	**	NS	***
Retained (g/d)	12.8	29.7	5.9	7.9	6.3	NS	NS	NS
Retained/intake (%)	6.4	14.9	2.9	4.3	3.5	NS	NS	NS
Retained/digested (%)	9.9	22.9	4.8	7.4	5.6	NS	NS	NS
Nitrogen partition								
Fecal N/N intake (%)	40.0	35.6	39.7	41.9	1.5	NS	NS	NS
Urinary N/N intake (%)	53.7	49.6	57.4	53.8	2.5	NS	NS	NS
Total loss/N intake (%)	93.6	85.2	97.1	95.1	3.6	NS	NS	NS

[1] S.E.: standard error of least-square means.
[2] NS: not significant (p>0.1); * p<0.1; ** p <0.05; *** p<0.01.
Means in the same row with different letter are significantly different (p<0.05).

Table 3. Rumen pH, NH_3-N and VFA concentrations 1 h and 6 h after feeding of dairy cows fed silage with GOS supplementation

	Diets								S.E.[1]	Main effects (p)			
	OS		OSG		MS		MSG			Silage (S)	GOS (G)	S×G	Time
	1 h	6 h	1 h	6 h	1 h	6 h	1 h	6 h					
pH	6.79	7.18	6.89	7.31	6.93	7.24	7.03	7.26	0.05	NS[2]	NS	NS	***
NH_3-N, mg/L	193.8	60.3	181.1	71.8	167.6	66.6	160.7	69.7	8.6	NS	NS	NS	***
Total VFA, mM	80.1	43.5	63.5	44.3	60.9	50.2	66.8	47.4	4.7	NS	NS	NS	***
Molar proportion, mol/100mol of total VFA													
Acetate (A)	64.2	70.7	63.4	71.0	67.5	72.7	67.8	71.9	0.8	***	NS	NS	***
Propionate (P)	26.1	19.3	28.0	19.4	24.6	17.2	22.9	18.5	0.8	**	NS	NS	***
Butyrate	9.8	10.1	8.6	9.7	7.6	10.1	8.1	9.7	0.3	*	NS	NS	***
A:P	2.47	3.67	2.28	3.66	2.75	4.24	2.97	3.90	0.12	***	NS	NS	***

[1] S.E.: standard error of least-square means.
[2] NS: not significant (p>0.1); * p<0.1; ** p <0.05; *** p <0.01.

RESULTS AND DISCUSSION

Apparent digestibility and nitrogen balance

There were no refusals during the experiment because diets were offered at maintenance level. The mean DM digestibility in cows fed OS and OSG was higher (p<0.1) than those fed MS and MSG (62.6 vs. 59.4%). Organic matter digestibility in both silage was 64.5 vs. 60.4% (Table 2). Although DM and OM digestibility were not significantly affected by GOS supplementation, cows fed silage with GOS supplementation had slightly higher digestibility than those fed silage alone. The increment of galactose degraded from GOS might induce the activation of *Bifidobacterium* species in the rumen. According to Ogimoto and Imai (1982), *Bifidobacterium pseudolongum* and *Bifidobacterium thermophilum* utilize sugars as substrates and exist in the rumen. Lee et al. (1980) reported that cells of *Bifidobacterium bifidum* contained galactokinase (EC.*2.7.1.6*), hexose 1-phosphate uridylyltransferase (EC.*2.7.7.12*) and UDP-galactose-4-epimerase (EC.*5.1.3.2*), which are enzymes in conventional pathway of galactose metabolism. In rats, supplementation of 5% transgalactooligosaccharide in the diet has been shown to increase β-glucosidase and β-galactosidase activities compared to control diet (Kikuchi et al., 1996).

The N digested in cows fed OS and OSG was higher (p<0.05) than those fed MS and MSG (113.3 vs. 106.0 g/d). However, there was a significant interaction (p<0.01) between silage and GOS supplementation on N digestion. Supplementation of GOS in orchardgrass silage tended to increase N digested. The higher N digestion in this diet could be due to the increased microbial synthesis resulting from higher OM levels. Retained N and retained N/unit of

Table 4. Urinary excretion of purine derivatives and estimation of microbial N supply to the duodenum of dairy cows fed silage with GOS supplementation

	Diets				S.E.[1]	Main effects (p)		
	OS	OSG	MS	MSG		Silage (S)	GOS (G)	S×G
Allantoin (mmol/d)	69.68[b]	91.96[a]	74.11[b]	60.04[b]	3.37	**	NS[2]	**
Uric acid (mmol/d)	11.22	12.93	11.37	11.50	1.15	NS	NS	NS
Total purine derivatives (mmol/d)	80.90[b]	104.89[a]	85.48[b]	71.54[b]	4.01	**	NS	**
Allantoin, % of PD	85.8	87.7	86.3	83.7	1.1	NS	NS	NS
Microbial N supply (g N/d)	25.7[b]	45.6[a]	29.0[b]	17.0[b]	3.2	**	NS	**

[1] S.E.: Standard error of least-square means.
[2] NS:not significant (p>0.1); ** p <0.05.
Means in the same row with different letter are significantly different (p<0.05).

N intake were slightly higher in cows fed silage with supplementation of GOS. Compared to cows fed silage alone, total N loss/unit of N intake of cows fed silage with GOS supplementation was declined 9.0% and 2.1% for OSG and MSG, respectively. The lower total N losses in cows fed silage with GOS supplementation show the higher absorption of N that resulted in a higher retained N/unit of N intake and higher retained N/unit of N digested. These results indicate that supplementation of GOS has improved N utilization through the reduction of N losses in dairy cows fed silage. Additionally, reduced N excretion by dairy cow may contribute to abate environmental pollution.

Rumen fermentation parameters

Concentrations of NH_3-N, VFA and pH in the rumen fluid were used to monitor rumen fermentation pattern (Table 3). The rumen pH was not affected by silage or GOS supplementation. Compared to the values 1 h after feeding, rumen pH values 6 h after feeding was significantly ($p<0.01$) increased in all diets. The cows fed both silages with GOS supplementation had higher rumen pH than those of cows fed silage without GOS supplementation 1 h after feeding. However, the pH values 1 h after feeding were in optimum pH ranges (6.7±0.5) to maintain normal cellulolytic organism (Van Soest, 1994). Russel et al. (1992) reported that net protein synthesis in the rumen declined dramatically when rumen pH declined bellow pH 6.2. In this experiment, rumen pH values 1 h and 6 h after feeding in all diets were relatively higher (averaging 6.9 and 7.3), probably due to rumen liquor contaminated by saliva, when was taken through mouth. As reported by Cassida and Stokes (1986) that ruminant saliva had a pH of 8.45, due to the large amount of bicarbonate.

NH_3-N concentrations 1 h after feeding of both OSG and MSG were slightly lower than those of OS and MS. This decline of NH_3-N concentration might be due to the improved microbial protein synthesis in the presence of GOS supplementation as well as the increased supply of microbial nitrogen particularly in OSG diet (Table 4). NH_3-N concentrations in all diets were above the minimum level of 50 mg/L to support maximum growth rates of rumen bacteria (Satt er and Slyter, 1974). Broudiscou and Jouany (1995) suggested that optimal concentration for bacterial protein production ranged from 80 to 90 mg/L. Rumen NH_3-N concentration was slightly higher for cows fed OS than for cows fed MS, suggesting greater rumen degradation of CP in OS than MS. Ammonia nitrogen concentrations at 6 h after feeding in all diets were significantly ($p<0.01$) lower than those at 1 h after feeding. These results are consistent with those of Martin-Orue et al. (2000) that also found maximum NH_3-N concentration were reached 1 h after feeding and then decreased to minimum values after 5 to 6 h.

Total VFA concentration 6 h after feeding was significantly ($p<0.01$) decreased compared with the values 1 h after feeding. This indicates that the digestion and fermentation in the rumen 6 h after feeding was decreased compared to the results 1 h after feeding. However, total VFA concentration in OSG, MS and MSG diets were slightly lower than the normal concentrations of 70 to 130 mM, suggested by France and Siddons (1993). Molar percentage of the three major VFA 1 h after feeding were not affected by supplementation of GOS, but their concentrations were changed by silage and sampling time ($p<0.01$). The molar percentage acetate in cows fed MS and MSG were significantly ($p<0.01$) higher than those fed OS and OSG. In contrast, molar percentage butyrate in cows fed either OS or OSG were relatively higher ($p<0.1$) than those fed MS or MSG. According to Murphy et al. (1982), ruminal fermentation of structural carbohydrates such as cellulose and hemicellulose in diets exceeding 60% roughage tend to yield high proportions of acetate and butyrate, respectively. The relatively high proportion of acetate and low proportion of butyrate in MS and MSG compared to OS and OSG is due to the high content of ADF and low content of hemicellulose. Moreover, the absorbed acetate and butyrate are used primarily as energy sources in animal tissues through oxidation via the citric acid cycle (France and Siddons, 1993). Ratio of acetate to propionate was significantly ($p<0.01$) different between silage, while the ratio was significantly ($p<0.01$) increased from 1 h after feeding to 6 h after feeding. The lower ratio in cows fed OSG either 1 h or 6 h after feeding suggests suppressed methane production. As suggested by Van Nevel and

Demeyer (1996) that decreased acetate:propionate ratio in the rumen may also have the effect of decreasing methane production. However, Takahashi et al. (1997) found decreased methane production in wethers resulting from supplementation of L-cysteine was not followed by decreased ratio of acetate:propionate.

Urinary excretion of purine derivatives and microbial nitrogen supply

The effect of supplementation of GOS in silage on the purine derivatives excretion and the calculated microbial N supply are summarized in Table 5. There were significant effects ($p<0.05$) of silage and an interaction between silage and GOS supplementation on urinary allantoin excretion, total purine derivatives excretion and microbial nitrogen supply. The microbial nitrogen supply, as calculated from purine derivatives excretion using the equation of Chen and Gomes (1995), ranged from 17 to 45 g/d. The higher microbial nitrogen supply in cows fed OSG may be due to synchronization of the available fermentable energy and degradable nitrogen in the rumen. Structurally, GOS is composed of galactose and glucose molecules joined on β-configuration. Rumen microorganisms are able to breakdown substrates with β-configuration. Thus, GOS could be potentially digested by rumen microorganisms. Gamo et al. (2001) have demonstrated *in vitro* that degradations of β 1-4 galacto-oligosaccharides by mixed rumen microbes added with lactic acid bacteria and yeast were 80.4% and 93.9% after 1 h incubation, respectively. Moreover, the rate of digestion of carbohydrates is a major factor controlling the energy available for microbial growth (Hoover and Stokes, 1991). In comparison with cows fed MS and MSG, cows fed OS and OSG had greater ($p<0.05$) microbial nitrogen supply (23.0 vs. 35.6 g/d). The proportion of urinary allantoin in total purine derivatives was not affected by silage or GOS supplementation and ranged from 83.7 to 87.7%. This proportion was similar to previous experiments of 80 to 85% (Chen and Gomes, 1995) and 86.6% (Vagnoni and Broderik, 1997).

In ruminants, allantoin is the main product of purine catabolism and the principal purine derivatives in urine. Supplementation of GOS in orchardgrass based silage in the present study had highest allantoin, purine derivatives excretion, and microbial N supply to duodenum.

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REFERENCES

Agriculture and Food Research Council. 1992. Nutritive Requirements of Ruminant Animals: Protein. Nutr. Abstr. Rev., (Series B). 62:787-835.

Agriculture, Forestry and Fisheries Research Council Secretariat. 1994. Japanese Feeding Standard for Dairy Cattle, Chuouchikusankai, Tokyo. pp. 19-24.

Bouhnik, Y., B. Flourie, L. D'Agay-Abensour, P. Pochart, G. Gramet, M. Durand and J. Rambaud. 1997. Administration of transgalacto-oligosaccharides increases fecal bifidobacteria and modifies colonic fermentation metabolism in healthy humans. J. Nutr. 127:444-448.

Broudiscou, L. and J. P. Jouany. 1995. Reassessing the manipulation of protein synthesis by rumen microbes. Reprod. Nutr. Dev. 35:517-535.

Cassida, K. A. and M. R. Stokes. 1986. Eating and resting salivation in early lactation dairy cows. J. Dairy Sci. 69:1282-1292.

Chakravarti, I. M., R. G. Laha and J. Roy. 1967. Handbook of Methods of Applied Statistics, Vol. II: Planning of Surveys and Experiments. John Wiley & Sons, Inc., New York. pp. 70-88.

Chamberlain, D. G. 1987. The silage fermentation in relation to the utilization of the nutrients in the rumen. Process Biochem. 22:60-63.

Chen, X. B. and M. J. Gomes. 1995. Estimation of Microbial Protein Supply to Sheep and Cattle Based on Urinary Excretion of Purine Derivatives-An overview of Technical Details. Occasional Publication 1992. International Feed Resources Unit, Rowett Research Institute, Aberdeen, UK.

Conway, E. J. and E. O'Malley. 1942. Microdiffussion methods: ammonia and urea using buffered absorbents (revised methods or ranges greater than 10 μg. N). Biochem. J. 36:655-661.

France, J. and R. C. Siddons. 1993. Volatile fatty acid production. In: Quantitative Aspects of Ruminant Digestion and Metabolism. (Ed. J. M. Forbes and J. France). C.A.B. International, Willingford, UK. pp. 107-122.

Gamo, Y., M. Mii, X. G. Zhou, S. Chetra, B. Santoso, I. Arai, K. Kimura and J. Takahashi. 2001. Effects of lactic acid bacteria, yeasts and galacto-oligosaccharides supplementation on *in vitro* rumen methane production. In: Proceedings of the 1st International Conference on Greenhouse Gases and Animal Agriculture, November 7-11, 2001, Obihiro, Japan. pp. 371-374.

Harrison, J. H. and R. Blauwiekel. 1994. Fermentation and utilization of grass silage. J. Dairy Sci. 77:3209-3235.

Hoover, W. H. and S. R. Stokes. 1991. Balancing carbohydrate and proteins for optimum rumen microbial yield. J. Dairy Sci. 74:3640-3644.

Kikuchi, H., C. Andrieux, M. Riottot, M. Bensaada, F. Popot, P. Beaumatin and O. Szylit. 1996. Effect of two levels of transgalactosylated oligosaccharide intake in rats associated with human faecal microflora on bacterial glycolytic activity, end-products of fermentation and bacterial steroid transformation. J. App. Bacteriol. 80:439-446.

Kim, K. H., Y. G. Oh, J. Choung and D. G. Chamberlain. 1999. Effects of varying degrees of synchrony of energy and nitrogen release in the rumen on the synthesis of microbial protein in cattle consuming grass silage. J. Sci. Food Agric. 79:833-838.

Lee, L., S. Kinoshita, H. Kumagai and T. Tochikura. 1980. Galactokinase of *Bifidobacterium bifidum*. Agric. Biol. Chem. 44:2961-2966.

Martin-Orue, S. M., J. Balcells, F. Vicente and C. Castrillo. 2000. Influence of dietary rumen-degradable protein supply rumen characteristics and carbohydrate fermentation in beef cattle offered high-grain diets. Anim. Feed Sci. Technol. 88:59-77.

Murphy, M. R., L. R. Baldwin and L. J. Koong. 1982. Estimation of stoichiometric parameters for rumen fermentation of roughage and concentrate diets. J. Anim. Sci. 55:411-421.

Ogimoto, K. and S. Imai. 1981. Atlas of Rumen Microbiology. Japan Scientific Societies Press. p. 231.

Russell, J. B., J. D. O'Connor, D. G. Fox, P. J. Van Soest and C. J Sniffen. 1992. A net carbohydrate and protein system for evaluating cattle diets. I. Ruminal fermentation. J. Anim. Sci. 70:3551-3561.

Sako, T., K. Matsumoto and R. Tanaka. 2000. Recent progress on research and application of non-digestible galacto-oligosaccharides. Int. Dairy Sci. 9:69-80.

Satter, L. D. and L. L. Slyter. 1974. Effect of ammonia concentration of rumen microbial protein production *in vitro*. Br. J. Nutr. 32:199-208.

Statistical Analysis Systems Institute. 1990. SAS/STAT® User's Guide Statistic, Version 6, Cary, NC, USA.

Steel, R. G. D. and J. H. Torrie. 1960. Principles and Procedures of Statistics. 2nd edn. McGraw-Hill Book Company, New York. pp. 187-188.

Takahashi, J., A. S. Chaudhry, R. G. Beneke, Suhubdy and B. A. Young. 1997. Modification of methane emission in sheep by cysteine and a microbial preparation. Sci. Total. Environ. 204:117-123.

Tamminga, S. 1992. Nutrition management of dairy cows as a contribution to pollution control. J. Dairy Sci. 75:345-357.

Tanaka, R. and K. Matsumoto. 1998. Recent progress on probiotics in Japan, including galacto-oligosaccharides. Bull. Int. Dairy. Sci. 335:21-27.

Thomas, C. and P. C. Thomas. 1985. Factors affecting the nutritive value of grass silage. In: Recent Advances in Animal Nutrition. (Ed. W. Haresign and D. J. A. Cole). Butterworths, London. pp. 223-256.

Vagnoni, D. B. and G. A. Broderick. 1997. Effects of supplementation of energy or ruminally undegraded protein to lactating cows fed alfalfa hay or silage. J. Dairy Sci. 80:1703-1712.

Van Nevel, C. J. and D. I. Demeyer. 1996. Control of rumen methanogenesis. Environ. Monit. Assest. 42:73-97.

Van Soest, P. J. 1994. Nutritional Ecology of The Ruminant. 2nd edn. Comstock Publishing Associates a Division of Cornell University Press, Ithaca and London. p. 476.