Sarsaponin Effects on Ruminal Fermentation and Microbes, Methane Production, Digestibility and Blood Metabolites in Steers

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ABSTRACT : The objective of this study was to evaluate the effects of sarsaponin on methane production, ruminal fermentation, nutrient digestion and blood metabolites using three Holstein steers in a 3×3 Latin Square design. The steers were fed Sudangrass hay plus concentrate mixture at a ratio 1.5:1 twice daily, and sarsaponin (0, 0.5 and 1% of DM), which was given at 09:00 and 17:00 h daily by mixing with concentrate. Rumen samples were collected 0, 2, and 5 h after morning dosing. Ruminal pH was numerically decreased and numbers of protozoa were decreased linearly ($p<0.01$) by treatment. Ruminal ammonia-N was reduced (linear; $p<0.05$) and total VFA was increased (quadratic; $p<0.05$) at 2 and 5 h after sarsaponin dosing. The molar proportion of acetate was decreased (quadratic; $p<0.05$) and propionate was increased (linear; $p<0.01$) at all sampling times. Blood plasma glucose was increased and urea-N was decreased (linear; $p<0.05$) at 2 and 5 h after dosing. Methane was decreased by approximately 12.7% (linear; $p<0.05$). The apparent digestibility of DM and NDF were decreased (quadratic; $p<0.05$) and that of CP remained unchanged due to the sarsaponin. The numbers of cellulolytic bacteria were decreased (quadratic; $p<0.05$), while numbers of total viable bacteria remained unchanged due to the sarsaponin. These results show that sarsaponin can partially inhibit rumen methanogenesis *in vivo* and improve ruminal fermentation, which supports our previous *in vitro* results. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 12 : 1746-1751)***

Key Words : Digestibility, Methane Production, Ruminal Fermentation, Saponin, *Yucca schidigera*

INTRODUCTION

Methane production in the rumen represents a substantial loss of feed energy and has also received attention as a potential contributor to global warming (Moss, 1993). Methane production is affected by a variety of nutritional factors including composition of feed ingredients (Lee et al., 2003), and level of intake, type of carbohydrate, forage processing and changes in the ruminal microflora (Johnson and Johnson, 1995). Many different chemical agents such as bromoethanesulfonic acid, pyromellitic diimide and unsaturated fatty acids (Choi et al., 2004), and bromochloromethane (Chalupa, 1984; McCrabb et al., 1997) are also known to reduce methane production. Methanogenesis may be reduced by the addition of electron acceptors such as nitrate and sulfate (Mathison et al., 1998; Sar et al., 2004), malate (Martin and Streeter, 1995) and fumarate (Asanuma et al., 1999; Bayaru et al., 2001; McGinn et al., 2004). A decrease of methanogenesis in the rumen may be useful due to its increasing the efficiency of energy use in the rumen.

Extracts of the *Yucca schidigera* plant contain sarsaponin, a group of steroidal glycosides that can influence ruminant productivity. Several authors have reported that sarsaponin improves ruminal organic matter digestion (Goetsch and Owens, 1985; Valdez et al., 1986), increases ruminal propionate concentration (Hristov et al., 1999), stimulates the growth of steers (Goodall et al., 1979) and does not affect animal performance (Wu et al., 1994).

Saponins also have antimicrobial properties, particularly in suppressing ciliate protozoa (Wallace et al., 1994; Hristov et al., 1999), peptidase producing bacteria (Wallace et al., 1994; Wang et al., 2000) and cellulolytic bacteria (Wang et al., 2000). Furthermore, methanogenic bacteria were metabolically co-related with ciliate protozoa (Stumm et al., 1982; Finlay et al., 1994; Newbold et al., 1995) and protozoa actively produce hydrogen, which is utilized by methanogenic bacteria (Stumm and Zwart, 1986; Ushida et al., 1997). In recent studies, we have shown that sarsaponin can partially decrease the methane and hydrogen *in vitro* (Lila et al., 2003). It also decreased the molar proportion of acetate, and increased the propionate and butyrate with a corresponding decrease in the acetate:propionate ratio. The total volatile fatty acid (VFA) was increased and ammonia was decreased by sarsaponin. These changes in ruminal fermentation suggest that sarsaponin behaves like ionophores, and the addition of sarsaponin may improve the efficiency of ruminant production.

Keeping the above facts in view, the present paper investigated the effects of sarsaponin on ruminal fermentation patterns and microbes, nutrient digestion, methane production and blood metabolites in steers.

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Table 1. Composition of the experimental diet

Item	Sudangrass hay	Concentrate mixture[1]
Feed composition (% of DM basis)	60.5	39.5
Chemical composition (% of DM basis)		
Organic matter	88.8	93.9
Crude protein	10.2	20.4
Neutral detergent fiber	65.3	20.2
Ash	11.2	6.1

[1] Contained 41.3% corn, 23.4% wheat bran, 8.0% rapeseed oil, 7.4% soybean meal, 6.0% corn germ meal, 5.6% corn gluten feed, 4.8% grains, 0.6% molasses, 1.7% $CaCO_3$, 1.0% NaCl, and 0.2% vitamins (A and D) and trace minerals premix.

MATERIALS AND METHODS

Animals, diet and experimental protocols

Three castrated 10- to 11- mo-old Holstein steers (half-siblings) (mean body weight, 248±27 kg) were housed in digestion stalls. Equal portions of the daily ration (chopped Sudangrass hay:concentrate mixture, 1.5:1) were fed at 09:00 and 17:00 h at the maintenance level. Water and mineral blocks were continuously available. The ingredients composition of and a chemical analysis of the diet are shown in Table 1. Steers were randomly allotted to treatment sequences in 3×3 Latin squares. Each period consisted of 14 d of adaptation and 10 d of measurement. The sarsaponin doses (0, 11.2, or 22.4 g) were given twice daily at 09:00 and 17:00 h daily by mixing with concentrate. Sarsaponin (DK Sarsaponin 35) was supplied by Desert King International, (Chula Vista, CA, USA) and it contained 6% moisture, 2.4% crude protein, 0.8% crude fat, 24.7% crude fiber, 4.9% ash, and 61.1% carbohydrate. The steers were cared for in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Tokyo University of Agriculture and Technology.

Sampling and measurement

From day 15 to 19 of each period, the 4 d digestion trial was carried out by total collection method. During the digestion trial, feces were collected daily, weighed and composited for each steer, dried in a forced air oven at 60°C for 48 h, ground through a 1 mm screen, and then assayed for DM, NDF and CP. Urine was collected in a 20 liter container, containing 300 ml of 3 N H_2SO_4 and a 50 to 100 ml sample was removed and frozen until analyzed for nitrogen. From day 20 to 23 of each period, methane and carbon dioxide production were determined. A head hood collection chamber was used for the measurement of methane and carbon dioxide. The concentration of gases was determined at 1-minute intervals throughout the measurement period by an infrared gas analyzer detector (Fuji Electric, Tokyo, Japan). During the measurement of gases, the chamber was maintained at approximately 20°C. On the final day of each period, samples of rumen fluid and jugular venous blood were collected before feeding, and at 2 and 5 h after morning feeding for ruminal protozoa, ammonia and VFA, and plasma glucose and urea-N, respectively.

Approximately 300 ml of ruminal digesta was collected using a flexible polyvinyl chloride stomach-tube and a sub-sample (100 ml) was taken in a sterilized container for microbiology assay 2 h after feeding. Hungate's method (1969) was used to prepare the media and cultivate the microorganisms. Total viable counts were determined in roll-tubes with the complete medium described by Leedle and Hespell (1980). Cellulolytic medium (Halliwell and Bryant, 1953) was used for the cellulolytic bacterial count. The exact method used for the determination of bacteria has been described previously (Lila et al., 2004b).

The remaining rumen fluid was separated from particulate matter by straining the digesta through four layers of surgical gauze. The pH of the fluid was immediately measured using a portable pH meter. For analysis of VFA and ammonia-N, samples were acidified with 25% meta-phosphoric acid and stored at -30°C until analyzed. After thawing, the rumen fluid was centrifuged at 10,000×*g* for 10 min at 4°C, and the VFA was analyzed with a gas chromatograph (Model GC-14B, Shimadzu Co. Ltd., Kyoto) using a Thermon-3000 5% Shincarbon A column (1.6 m×3.2 mm I.D., 60-80 mesh, Shinwakako, Kyoto). Ammonia-N was determined with the micro-diffusion method. One mL of the rumen fluid was diluted with 4 ml of methylgreen-formalin-saline to count ciliate protozoa (Bayaru et al., 2001). Blood samples were collected into heparinized tubes, immediately placed on ice and centrifuged at 11,000×*g* for 15 min at 4°C. Plasma was removed and frozen until analyzed for glucose and urea-N. The details of the analyses have been described previously (Mohammed et al., 2004).

Statistical analyses were carried out by a three-way ANOVA using the cow, period, and treatment as the variables, and computation was performed using SAS (SAS, 1994). Significance was declared at $p<0.05$. The effect of cow and period were not significant; therefore, the main effects of saponin are presented in Table 2, 3 and 4.

RESULTS AND DISCUSSION

The steers were judged to be in excellent health throughout the study. In this study, the steers consumed 2.25 kg DM plus 11.2 g and 22.4 g of sarsaponin twice daily. The effects of sarsaponin on pH, ammonia, protozoa and the molar proportions of VFA in the ruminal fluid are shown in Table 2. The pH value was numerically decreased by sarsaponin supplementation. This result was consistent

Table 2. Effect of sarsaponin on pH, protozoal number, ammonia-N and volatile fatty acid (VFA) in the rumen fluid of steers

Item	Hr. after feeding	Control	Sarsaponin		SEM	Statistical effect
			0.5% of DM	1% of DM		
pH	0	6.83	6.81	6.72	0.24	NS[1]
	2	6.86	6.76	6.71	0.12	NS
	5	6.83	6.76	6.72	0.28	NS
Protozoa ($\times 10^5$/ml)	0	14.4	5.3	2.4	0.12	L**
	2	6.5	1.9	1.7	0.14	L**
	5	7.3	2.4	2.1	0.12	L**
Ammonia-N (mg/dl)	0	6.9	6.3	6.1	0.29	NS
	2	9.8	9.1	8.3	0.09	L*
	5	8.8	8.3	7.9	0.21	L*
Total VFA (mM)	0	80.7	80.9	81.2	4.29	NS
	2	81.7	82.6	83.3	1.32	Q*
	5	81.1	82.6	82.5	1.56	Q*
VFA composition (mol %)						
Acetate (A)	0	64.0	62.1	60.9	1.32	Q*
	2	62.6	60.6	59.8	0.09	Q*
	5	63.3	61.7	60.9	1.08	Q*
Propionate (P)	0	21.2	22.8	23.5	0.08	L*
	2	22.8	24.0	24.3	0.06	L**
	5	22.1	23.0	23.6	0.09	L**
iso-butyrate	0	0.94	0.69	0.81	0.15	NS
	2	0.59	0.60	0.60	0.13	NS
	5	0.62	0.61	0.59	0.16	NS
Butyrate	0	10.9	11.5	11.8	0.18	NS
	2	11.4	12.2	12.5	0.12	L*
	5	11.0	11.8	12.1	0.09	L*
iso-valerate	0	1.22	1.22	1.15	0.04	NS
	2	1.06	1.07	1.00	0.03	NS
	5	1.29	1.26	1.19	0.02	NS
Valerate	0	1.56	1.53	1.37	0.05	NS
	2	1.34	1.29	1.24	0.10	NS
	5	1.58	1.56	1.50	0.03	NS
A/P	0	3.01	2.72	2.61	0.09	L*
	2	2.74	2.52	2.47	0.07	L*
	5	2.86	2.66	2.59	1.11	L*

[1]NS = Not significant: * $p<0.05$: ** $p<0.01$.
L = Linear effect due to dose of sarsaponin. Q = Quadratic effect due to dose of sarsaponin.

with the previous *in vitro* (Lila et al., 2003) and *in vivo* (Wallace et al., 1994; Hristov et al., 1999) studies. The ciliate protozoa were composed of *Entodinium* spp., *Dasytricha* sp. and *Isotricha* sp. in this study. Headon et al. (1991) explained that the saponin fraction of *Y. schidigera* inhibited the growth of ruminal ciliate protozoa. The total numbers of protozoa decreased linearly ($p<0.01$) due to the treatment. These results were consistent with the previous report (Wallace et al., 1994; Hristov et al., 1999; Lila et al., 2003).

Supplementation with sarsaponin linearly decreased the ruminal ammonia concentration. Reductions in the ruminal ammonia with saponin have been observed *in vitro* (Grobner et al., 1982; Wallace et al., 1994; Lila et al., 2003) and *in vivo* (Hussain and Cheeke, 1995). The *in vivo* responses have been varied, and are apparently related to the dosing levels and the time of sampling. The effects of sarsaponin on ruminal ammonia likely result from decreased bacterial lysis (as a consequence of inhibited protozoal growth) and, to a lesser extent, from an inhibited deaminative activity and direct binding of ammonia in the rumen (supported by the significantly lower concentration of ammonia 2 h after sarsaponin treatment).

The total VFA was increased (quadratic effect; $p<0.05$) at 2 and 5 h after dosing. The molar proportion of acetate was decreased ($p<0.05$) at all sampling times. The molar proportion of propionate ($p<0.01$) and butyrate ($p<0.05$) were increased at 2 and 5 h after dosing. Consequently, the acetate:propionate ratio was decreased ($p<0.05$). The test compound had no effect on *iso*-butyrate, valerate and *iso*-

Table 3. Effect of sarsaponin on concentration of glucose and urea-N in blood plasma of steers

Item	Hr. after feeding	Control	Sarsaponin		SEM	Statistical effect
			0.5% of DM	1% of DM		
Glucose (mg/100 ml)	0	97.0	98.3	96.3	3.54	NS[1]
	2	105.3	108.6	112.0	3.98	L*
	5	87.6	88.6	88.3	3.38	L*
Urea-N (mg/100 ml)	0	6.1	6.1	6.1	1.25	NS
	2	8.4	7.4	6.3	1.28	L*
	5	7.2	6.4	5.2	1.22	L*

[1] NS: Not significant; * $p<0.05$. L = Linear effect due to dose of sarsaponin.

Table 4. Effect of sarsaponin on methane, feed digestibility, nitrogen metabolism, and ruminal total viable counts and cellulolytic bacteria of steers

Item	Control	Sarsaponin		SEM	Statistical effect
		0.5% of DM	1% of DM		
Dry matter intake (kg/d)	4.49	4.49	4.49	0.00	NS[1]
CH_4, L/(d.kg of DMI[a])	29.8	27.3	26.0	1.12	L*
CO_2, L/(d.kg of DMI[a])	563.2	557.9	554.1	24.35	NS
Apparent digestibility (%)					
DM	66.2	64.0	63.9	1.23	Q*
NDF	61.8	59.2	58.9	1.01	Q*
CP	64.2	65.3	65.0	0.87	NS
Nitrogen metabolism (g/d)					
Intake	64.8	64.8	64.8	0.00	NS
Loss					
Feces	23.2	22.5	22.7	3.45	NS
Urine	34.4	30.1	29.7	0.88	L*
Retention	7.2	12.2	12.4	0.24	L*
Total viable counts (log no./ml)	9.4	9.1	9.2	2.53	NS
Cellulolytic bacteria (log no./ml)	8.3	7.9	8.0	0.78	Q*

[a] Dry matter intake. [1] NS: Not significant; * $p<0.05$.
L = linear effect due to dose of sarsaponin, Q = quadratic effect due to dose of sarsaponin.

valerate. The reported effect of saponin on ruminal propionate has been varied. Sarsaponin increased the propionate both *in vitro* (Grobner et al., 1982; Lila et al., 2003) and *in vivo* (Valdez et al., 1986). The decrease in the molar proportion of acetate and the increase in butyrate were consistent with the results of the *in vivo* study (Christopher and Neal, 1987).

The blood biochemical measurements after sarsaponin dosing are shown in Table 3. The post-feeding glucose concentration increased ($p<0.05$) following the treatment. This may be due to the increased gluconeogenesis from propionate, since the ruminal propionate was increased in this study. In contrast, urea-N was decreased ($p<0.05$), reflecting the decreased ruminal ammonia concentration. These results were consistent with fumaric acid (Bayaru et al., 2001) and cyclodextrin-diallyl maleate (Lila et al., 2004a) reported previously from our laboratory, but dissimilar to the results of Hristov et al. (1999).

Feed intake, gas production, digestibility coefficients, N-metabolism and bacterial counts are shown in Table 4. Feeding of sarsaponin to the steer had no effects on DM intake; however, the digestibility of DM and NDF were decreased (quadratic effect; $p<0.05$) and CP was unchanged. The effects of sarsaponin on fiber digestibility have been varied. Goetsch and Owens (1985) observed that cattle fed 44 ppm of yucca saponin had an apparent increased digestibility of OM, but a depressed ruminal degradability of ADF. Hristov et al. (1999) reported that the extent of ruminal degradability of DM was not affected by saponin treatment, although the rate of degradation of insoluble DM was increased. Current data also showed that sarsaponin decreased the numbers of cellulolytic bacteria ($p<0.05$). Similar results were observed with monensin, which also inhibited the cellulolytic bacteria (Chen and Wolin, 1979; Helaszek and White, 1991).

The daily methane production (L/d) and rate of production (L/d.kg-DMI) were 148-182 and 26-32, respectively, throughout the experiment. This is in good agreement with the findings of Shibata et al. (1992), who fed animals at various proportions with hay plus concentrate. Approximately 12.7% of methane was inhibited ($p<0.05$) and carbon dioxide production was unchanged as a result of the treatment. In the *in vitro* experiment (Lila et al., 2003), we observed that sarsaponin

reduced the methane and hydrogen concentration in the ruminal gases, which supported the present results, although we did not measure hydrogen concentration in this experiment. Takahashi et al. (2000) reported that *Yucca shidigera* extract appears to be a promising agent to control rumen methanogenesis *in vitro*. In the present study, the inhibition of methane production by sarsaponin remained for the 23 d of treatment; therefore, further research is necessary as to whether ruminal adaptation to sarsaponin has occurred or not. The main reason for the methane suppressing effects of sarsaponin may be due to the inhibition of H_2-producing bacteria such as cellulolytic bacteria (Wang et al., 2000), as well as other bacteria that use pyruvate-ferredoxin oxidoreductase to metabolize pyruvate to acetyl-SCoA. Rumen protozoa also provide hydrogen as a substrate for methanogens (Stumm and Zwart, 1986; Ushida et al., 1997) and a decreased number of rumen protozoa caused by saponin partially contributed to reducing the methane production in this study. Nitrogen retention was increased ($p<0.05$) by the treatment due to the decrease in urinary N loss.

In conclusion, the present *in vivo* studies suggest that sarsaponin is a potent inhibitor of rumen methanogenesis because it was effective at a low concentration and had no influence on feed intake. However, further research is necessary to investigate the effect on fiber digestion as well as the ruminal adaptation to sarsaponin.

IMPLICATIONS

Lately, sarsaponin is commercially used to remove the ammonia odor from animal excreta and to improve ruminal fermentation. Furthermore, sarsaponin inhibited methane in the present study. Therefore, it may be possible to inhibit methane production with a combination of direct fed microbials (probiotics) or an exogenous fibrolytic enzyme plus sarsaponin to stimulate digestibility without a detrimental effect on the ruminants.

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REFERENCES

Asanuma, N., M. Iwamoto and T. Hino. 1999. Effect of the addition of fumarate on methane production by ruminal microorganisms *in vitro*. J. Dairy Sci. 82:780-787.

Bayaru, E., S. Kanda, T. Kamada, H. Itabashi, S. Andoh, T. Nishida, M. Ishida, T. Itoh, K. Nagara and Y. Isobe. 2001. Effect of fumaric acid on methane production, rumen fermentation and digestibility of cattle fed roughage alone. Anim. Sci. J. 72:139-146.

Chalupa, W. 1984. Manipulation of rumen fermentation. In: Recent Advances in Animal Nutrition (Ed. W. Haresign and D. J. A. Cole). Butterworths, London, pp. 143-160.

Chen, M. and M. J. Wolin. 1979. Effect of monensin and lasalocid on the growth of methanogenic and rumen saccharolytic bacteria. Appl. Environ. Microbiol. 38:72-77.

Choi, N. J., S. Y. Lee, H. G. Sung, S. C. Lee and J. K. Ha. 2004. Effects of halogenated compounds, organic acids and unsaturated fatty acids on *in vitro* methane production and fermentation characteristics. Asian-Aust. J. Anim. Sci. 17:1255-1259.

Christopher, D. L. U. and A. J. Neal. 1987. Alfalfa saponins affect site and extent of nutrient digestion in ruminants. J. Nutr. 117:919-927.

Finlay, B. J., G. Esteban, K. J. Clarke, A. G. Williams, T. M. Embley and R. P. Hirt. 1994. Some rumen ciliates have endosymbiotic methanogens. FEMS Microbiol. Lett. 117:157-161.

Goetsch, A. L. and F. N. Owens. 1985. Effects of sarsaponin on digestion and passage rates in cattle fed medium to low concentrate. J. Dairy Sci. 68:2377-2384.

Goodall, S. R., J. D. Eichenbaum and J. K. Matsushima. 1979. Sarsaponin and monensin effects upon *in vitro* VFA concentration, gas production and feedlot performance. J. Anim. Sci. 49:370-376.

Grobner, M. A., D. E. Johnson, S. R. Goodall and D. A. Benz. 1982. Sarsaponin effects on *in vitro* continuous flow fermentation of a high grain diet. J. Anim. Sci. 55:491-497.

Halliwell, M. E. and M. P. Bryant. 1953. The cellulolytic activity of pure strains of bacteria from the rumen of cattle. J. Gen. Microbiol. 32:441-448.

Headon, D. R., K. Buggle, A. Nelson and G. Killeen. 1991. Glycofractions of the yucca plant and their role in ammonia control. In: Biotechnology in the Feed Industry (Ed. T. P. Lyons). Alltech, Inc., Nicholasville, KY, pp. 95-108.

Helaszek, C. T. and B. A. White. 1991. Cellobiose uptake and metabolism by *Ruminococcus flavefaciens*. Appl. Environ. Microbiol. 57:64-68.

Hristov, A. N., T. A. McAllister, F. H. Van Herk, K. -J. Cheng, C. J. Newbold and P. R. Cheeke. 1999. Effect of *Yucca schidigera* on ruminal fermentation and nutrient digestion in heifers. J. Anim. Sci. 77:2554-2563.

Hungate, R. E. 1969. A roll-tube method for cultivation of strict anaerobes. Methods in Microbiol. 3B:117-132.

Hussain, I. and P. R. Cheeke. 1995. Effect of dietary *Yucca schidigera* extract on rumen and blood profiles of steers fed concentrate or roughage-based diets. Anim. Feed Sci. Technol. 51:231-242.

Johnson, K. A. and D. E. Johnson. 1995. Methane emissions from cattle. J. Anim. Sci. 73:2483-2492.

Leedle, J. A. Z. and R. C. Hespell. 1980. Differential carbohydrate media and anaerobic replica plating techniques in delineating carbohydrate-utilizing subgroups in rumen bacteria populations. Appl. Environ. Microbiol. 34:709-719.

Lee, H. J., S. C. Lee, J. D. Kim, Y. C. Oh, B. K. Kim, C. W. Kim,

and K. J. Kim. 2003. Methane production potential of feed ingredients as measured by *in vitro* gas test. Asian-Aust. J. Anim. Sci. 16:1143-1150.

Lila, Z. A., N. Mohammed, S. Kanda, T. Kamada and H. Itabashi. 2003. Effect of sarsaponin on ruminal fermentation with particular reference to methane production *in vitro*. J. Dairy Sci. 86:3330-3336.

Lila, Z. A., N. Mohammed, N. Ajisaka, S. Kanda, Y. Kurokawa, and H. Itabashi. 2004a. Effect of cyclodextrin diallyl maleate complex on methane production, ruminal fermentation and microbes, *in vitro* and *in vivo*. Anim. Sci. J. 75(1):15-22.

Lila, Z. A., N. Mohammed, T. Yasui, Y. Kurokawa, S. Kanda and H. Itabashi. 2004b. Effects of twin-strain of *Saccharomyces cerevisiae* live cells on mixed ruminal microorganism fermentation *in vitro*. J. Anim. Sci. 82:1847-1854.

Martin, S. A. and M. N. Streeter. 1995. Effect of malate on *in vitro* mixed ruminal microorganism fermentation. J. Anim. Sci. 73:2141-2145.

Mathison, G. W., E. K. Okine, T. A. McAllister, Y. Dong, J. Galbraith and O. I. N. Dmytruk. 1998. Reducing methane emissions from ruminant animals. J. Appl. Anim. Res. 14:1-28.

McCrabb, G. J., K. T. Berger, T. Magner, C. May and R. A. Hunter. 1997. Inhibiting methane production in Brahman cattle by dietary supplementation with a novel compound and the effects on growth. Aust. J. Agric. Res. 48:323-329.

McGinn, S. M., K. A. Beauchemin, T. Coates and D. Colombatto. 2004. Methane emissions from beef cattle: Effects of monensin, sunflower oil, enzymes, yeast and fumaric acid. J. Anim. Sci. 82:3346-3356.

Mohammed, N., N. Ajisaka, Z. A. Lila, K. Hara, K. Mikuni, K. Hara, S. Kanda and H. Itabashi. 2004. Effect of Japanese horseradish oil on methane production and ruminal fermentation *in vitro* and in steers. J. Anim. Sci. 82:1839-1846.

Moss, A. R. 1993. Methane: Global Warming and Production by Animals. Chalcombe Publications, Kingston, UK.

Newbold, C. J., B. Lassalas and J. P. Jouany. 1995. The importance of methanogens associated with ciliate protozoa in ruminal methane production *in vitro*. Lett. Appl. Microbiol. 21:230-234.

Sar, C., B. Santoso, Y. Gamo, T. Kobayashi, S. Shiozaki, K. Kimura, H. Mizukoshi, I. Arai and J. Takahashi. 2004. Effects of combination of nitrate with 1-4 galacto-oligosaccharides and yeast (Candida Kefyr) on methane emission from sheep. Asian-Aust. J. Anim. Sci. 17:73-79.

SAS/STAT® 1994. User's Guide: statistics, Version 6. 4th edn, SAS Institute. Inc., North Carolina.

Shibata, M., F. Terada, K. Iwasaki, M. Kurihara and T. Nishida. 1992. Methane production in heifers, sheep and goats consuming diets of various hay-concentrate ratios. Anim. Sci. and Technol. 63:1221-1227.

Stumm, C. K., H. J. Gijzen and G. D. Vogels. 1982. Association of methanogenic bacteria with ovine rumen ciliates. Br. J. Nutr. 47:95-99.

Stumm, C. K. and K. B. Zwart. 1986. Symbiosis of protozoa with hydrogen utilizing methanogens. Microbiol. Sci. 3:100-105.

Takahashi, J., T. Miyagawa, Y. Kojima and K. Umetsu. 2000. Effects of *Yucca schidigera* extract, probiotics, monensin and L-cysteine on rumen methanogenesis. Asian-Aust. J. Anim. Sci. 13(Suppl.):499-501.

Ushida, K., M. Tokura, A. Takenaka and H. Itabashi. 1997. Ciliate protozoa and ruminal methanogenesis. In: Rumen Microbes and Digestive Physiology in Ruminants (Ed. R. Onodera, H. Itabashi, K. Ushida, H. Yano and Y. Sasaki). Japan Scientific Society Press, Tokyo/S. Karger, Basel, pp. 209-220.

Valdez, F. R., L. J. Bush, A. L. Goetsch and F. N. Owens. 1986. Effect of steroidal sapogenins on ruminal fermentation and on production of lactating dairy cows. J. Dairy. Sci. 69:1568-1575.

Wallace, R. J., L. Arthaud and C. J. Newbold. 1994. Influence of *Yucca shidigera* extract on ruminal ammonia concentrations and ruminal microorganisms. Appl. Environ. Microbiol. 60:1762-1767.

Wang, Y., T. A. McAllister, L. J. Yanke and P. R. Cheeke. 2000. Effect of steroidal saponin from *Yucca schidigera* extract on ruminal microbes. J. Appl. Microbiol. 88:887-896.

Wu, Z., M. Sadik, F. T. Sleiman, J. M. Simas, M. Pessarakli and J. T. Huber. 1994. Influence of *Yucca* extract on ruminal metabolism in cows. J. Anim. Sci. 72:1038-1042.