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Effect of phytosterols on rumen fermentation *in vitro*

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ABSTRACT. We investigated the effect of phytosterols on rumen fermentation *in vitro* using gas syringes as incubators. Phytosterols were dissolved in ethyl acetate (8.3%) and added at various concentrations to the common diet in rumen fluid. *In vitro* gas production (GP) was recorded after 3, 6, 12, 18, and 24 h incubation. Incubation was stopped at 6, 12, and 24 h and the inoculants were then tested for pH, dry matter digestibility (DMD), microbial protein yield (MCP), lactic acid, NH3-N, and volatile fatty acids (VFAs). GP was consistently higher than the control; particularly, treatments at 12, 18, and 24 h reached extremely significant levels ($P < 0.01$). Compared to the control group, the pH of ruminal fluid was slightly lower after incubation, and DMD and MCP increased with increasing phytosterol level except for the content of MCP at 6 h, which changed only minimally. Lactate was significantly lower after treatment compared to the control at 12 h ($P < 0.01$) and 24 h ($P < 0.05$), while NH_3-N at 12 h ($P < 0.05$) and 24 h ($P < 0.01$) after treatment decreased significantly. Acetate, propionate, butyrate, and total VFA for all treatments were higher than those of the control, particularly for butyrate at 6 h ($P < 0.01$). These results suggest that phytosterols modify rumen fermentation by inhibiting released harmful products and promoting the release of beneficial product, which may be

useful for improving nutrient utilization and animal health.

Key words: Phytosterols; Rumen fermentation; Cow

INTRODUCTION

Phytosterols, also known as plant sterols, belong to the triterpene family, which includes more than 200 different sterols among the more than 4000 types of triterpenes. These compounds cannot be synthesized by animals or humans, but are naturally present in vegetable products, principally oils, pulses, and dried fruits (Goad et al., 1991). Phytosterols play an important role in reducing blood cholesterol levels, controlling certain illnesses such as cancers of the colon, breast, and prostate (Awad and Fink, 2000), and preventing prostatic hyperplasia (Wilt et al., 1999). Their function as immune modulators and their anti-inflammatory properties have been described previously (Bouic, 2001). Several studies have also shown that these compounds can be used as antioxidants for plants or animals (Lampi et al., 1999). Recently, products enriched in phytosterols have become commonly used in North America, Europe, and Asia. Phytosterols, phytostanols, and phytosterol esters have been considered generally recognized as safe (GRAS) for a range of food applications by the U.S. Food and Drug Administration, and were approved as new feed additives by the Ministry of Agriculture of China in 2008. However, few studies have examined the use of phytosterols as a feed additive for ruminants because the complicated rumen is considerably different from the system of mono-gastric animals. We found that oral supplementation of plant sterols could improve milk yield by 0.44-1.71 kg/day and could decrease ketone and milk urea nitrogen (MUN) contents in the milk (Jin, 2010). The objective of the present study was to evaluate the effect of phytosterols on rumen fermentation *in vitro* and to understand the mechanism by which phytosterols improve dairy cow performance.

MATERIAL AND METHODS

Materials

The substrate used was a common total mixed ration diet (with a 5:5 forage:concentrate diet, 54.6% dry matter, 17.15% crude protein, and 34.72% neutral detergent fiber), which was dried at 65°C for 48 h and broken up by passing it through a 1-mm screen. Phytosterols at 91% purity (including 40.95% β-sitosterol, 24.57% campesterol, 21.84% stigmasterol, and 3.64% brassicasterol) were obtained from Jiangsu Chunzhigu Development Co., Ltd. (Jiangsu, China). Phytosterols appeared as white powder and were insoluble in water, but dissolved in some organic solvents.

Experimental design

According to Menke and Steingass (1988), 100-mL glass syringes were used as incubators. Four groups (control, treatment I, treatment II, and treatment III) dissolved in 6 mL 8.3% ethyl acetate with phytosterols at 0, 1, 3, and 6 μg, respectively, were incubated with 200 mg TMR (total mixed ration). Syringes were filled with 30 mL medium consisting of 10 mL

rumen fluid and 20 mL buffer solution as described by Menke and Steingass (1988). Rumen fluid was collected from 3 healthy dairy cows, which were killed after a 7-day adaptation to the diet. The syringes were placed in a water bath at 39°C. A total of 48 syringes were incubated. *In vitro* gas production (GP) was recorded at 3, 6, 12, 18, and 24 h incubation. Four syringes from each group were stopped at 6, 12, and 24 h after inoculation. The medium was discharged into a 50-mL weighted centrifuge tube and then immersed in ice water to stop fermentation. After measuring pH, the tubes were centrifuged at 150 *g* for 15 min to separate the deposits and supernatant for the determination of dry matter digestibility (DMD), microbial protein yield (MCP), lactic acid, NH3-N, and volatile fatty acids (VFAs).

Measurement of *in vitro* fermentation parameters

The pH of the rumen liquor was determined immediately after removal using a pH meter (model pH211; Hanna; Smithfield, RI, USA). For determining the *in vitro* digestibility of dry matter, tubes were centrifuged at 150 *g* for 15 min and the residues were washed twice with distilled water, centrifuged again, and dried to a constant weight at 65°C. The index was calculated based on dispersion. NH3-N concentration was determined by colorimetry (Searle, 1984). Concentrations of MCP were determined based on Lowry et al. (1951), with modifications described in Makkar et al. (1982), and bovine serum albumin was used as a standard. Lactic acid was determined using the method described by Barker and Summerson (1941), and calcium lactate was used as a standard. To determine VFAs, 1 mL fermentation medium was placed in centrifuge tubes, mixed uniformly with 0.2 mL 25% metaphosphoric acid using crotonic acid as an internal standard, and then centrifuged at 12,000 *g* for 10 min. The supernatant was decanted into another test tube, capped, and stored in a refrigerator at 4°C until analysis by gas chromatography (GC-14B; Shimadzu). Subsamples were injected into a 30 m x 0.32 mm x 0.25 μm capillary. The temperatures of the detector, column, and vaporization were 220°, 130°, and 180°C, respectively. Nitrogen was used as a carrier.

Statistical analysis

The effect of different concentrations of plant sterols on the various parameters was evaluated by using the one-way analysis of variance (ANOVA) protocol in SPSS13.0 version 13.0, 2004 (SPSS, Inc.; Chicago, IL, USA). One-way ANOVA was again used to determine whether the differences were significant. This was followed by multiple comparisons testing using least significant difference (LSD) to identify differences. Means were compared by employing the LSD multiple range test at a significance level of $P < 0.05$. All data are reported as means ± standard error (SE).

RESULTS

GP was consistently higher than the control at 41.13, 39.83, and 44.5 mL at 24 h, for treatments I, II, and III, respectively (Table 1). GP for treatment III was significantly higher than that of the control, except at 6 h ($P < 0.01$ and $P < 0.05$ at 3 h). GP for treatment II at 12 h and treatment I at 18 h and 24 h were also significantly higher than that of the control ($P < 0.05$).

Table 1. Influence of phytosterols on *in vitro* gas production.

Time	Control (mL)	Treatment I (mL)	Treatment II (mL)	Treatment III (mL)
3 h	2.77 ± 0.21^{a}	3.00 ± 0.27^{ab}	3.15 ± 0.25^{ab}	3.72 ± 0.35^{b}
6 h	10.54 ± 0.39^{a}	10.95 ± 0.51^{a}	11.20 ± 0.66^{a}	11.67 ± 0.26^{a}
12 h	22.25 ± 0.56^{Aa}	23.08 ± 0.71^{ABab}	24.08 ± 0.70^{ABbc}	25.43 ± 0.37^{Bc}
18 h	30.86 ± 1.07^{Aa}	34.50 ± 0.54^{ABbc}	33.00 ± 0.89^{ABab}	36.13 ± 0.77^{Bc}
24 h	37.37 ± 1.34^{Aa}	41.13 ± 0.43^{ABb}	39.83 ± 0.96^{ABab}	44.50 ± 0.61^{Bc}

Within the same row different lowercase letters mean significant at $P<0.05$ and different capital letters mean significant at $P < 0.01$.

The rumen fermentation parameters are shown in Tables 2 and 3. The presence of phytosterols decreased pH, lactate, and NH_3-N levels, but increased DMD, MCP, and VFA levels. The pH of treatment III was significantly lower than that of the control at 24 h ($P < 0.05$), but all values were within a normal range (from 5.5-6.8). Lactate from treatment III was significantly lower than the control at 12 h ($P < 0.01$) and 24 h ($P < 0.05$). NH_3-N from treatment III at 12 h ($P < 0.05$) and 24 h ($P < 0.01$), and from treatment II at 24 h ($P < 0.05$) was significantly lower than that of the control. DMD was significantly higher than the control at 24 h ($P < 0.01$), except that DMD for treatment III over all times measured and for treatment II at 6 h was significantly higher than the control ($P < 0.01$). MCP content was higher than the control except for treatment I at 6 h, which was slightly lower than the control, and treatment III, which was significantly higher than the control at 24 h ($P < 0.01$).

Table 2. Influence of phytosterols on rumen fermentation parameters.

Item	Time (h)	Control	Treatment I	Treatment II	Treatment III
pH	6	6.87 ± 0.03^{a}	6.85 ± 0.02^{a}	6.86 ± 0.02^{a}	6.83 ± 0.03^{a}
	12	6.84 ± 0.01^{a}	6.80 ± 0.01^{a}	6.82 ± 0.01^{a}	6.83 ± 0.02^{a}
	24	6.77 ± 0.01^{a}	6.74 ± 0.01^{ab}	6.75 ± 0.01^{ab}	6.73 ± 0.01^{b}
DMD (%)	6	18.19 ± 2.41^{Aa}	19.40 ± 0.82^{Aa}	23.84 ± 0.89^{Bb}	24.41 ± 0.32^{Bb}
	12	25.83 ± 0.49^{Aa}	8.10 ± 0.17^{ab}	27.23 ± 0.68^{a}	30.02 ± 1.33^{Bb}
	24	34.08 ± 21.64^{Aa}	37.45 ± 0.37^{ABb}	41.60 ± 1.24^{BCc}	45.62 ± 0.51^{Cd}
MCP (mg/dL)	6	11.34 ± 0.44^{a}	11.18 ± 0.47^{a}	11.55 ± 0.73^{a}	11.47 ± 0.14^{a}
	12	13.91 ± 0.34^{a}	13.86 ± 0.33^{a}	14.10 ± 0.51^{a}	14.31 ± 0.25^{a}
	24	13.97 ± 0.14^{Aa}	$1\ 4.01 \pm 0.49^{Aa}$	14.77 ± 0.43^{ABa}	16.19 ± 0.35^{Bb}
Lactate (mM)	6	1.34 ± 0.10^{a}	1.34 ± 0.07^{a}	1.28 ± 0.19^{a}	0.98 ± 0.09^{a}
	12	1.63 ± 0.06^{Aa}	1.47 ± 0.10^{ABa}	1.32 ± 0.17^{ABab}	1.00 ± 0.08^{Bb}
	24	1.39 ± 0.06^{a}	1.16 ± 0.16^{ab}	1.13 ± 0.15^{ab}	0.85 ± 0.05^{b}
NH_3-N (mg/dL)	6	8.93 ± 0.15^{a}	9.00 ± 0.20^{a}	8.50 ± 0.13^{a}	8.49 ± 0.25^{a}
	12	10.07 ± 0.26^{a}	9.16 ± 0.07^{ab}	8.57 ± 0.18^{ab}	7.65 ± 0.12^{b}
	24	9.88 ± 0.14^{Aa}	9.80 ± 0.29^{Aa}	8.78 ± 0.17^{ABb}	8.10 ± 0.49^{Bb}

DMD = dry matter digestibility; MCP = microbial protein yield. Within the same row different lowercase letters mean significant at $P < 0.05$ and different capital letters mean significant at $P < 0.01$.

VFAs were higher in treatments than the control for most times except for 6 h, and for butyrate at 24 h, which was slightly lower than that of control. Acetate and total VFA in all treatments were significantly higher than the control ($P < 0.05$) at 6 h. The amount of butyrate in all treatments ($P < 0.01$) and other VFAs in treatment III ($P < 0.05$) were significantly higher than in the control. The amount of propionate from treatment II ($P < 0.05$) and treatment III ($P < 0.01$), butyrate from treatment I ($P < 0.05$) and treatment III ($P < 0.05$), and TVFA from treatment II ($P < 0.05$) and treatment III ($P < 0.05$) were significantly higher than in the control

at 12 h. Acetate levels in treatment III ($P < 0.01$), propionate from treatment II and treatment III ($P < 0.05$), other VFAs from treatment I and treatment III ($P < 0.05$), and TVFA from treatment III ($P < 0.05$) were significantly higher compared to the control at 24 h.

Table 3. Influence of phytosterols on the production of volatile fatty acids (VFAs).

Time	Item	Control	Treatment I	Treatment II	Treatment III
6 h	Acetate	18.01 ± 2.48^{a}	24.59 ± 1.40^{b}	24.03 ± 0.58^{b}	24.20 ± 1.08^{b}
	Propionate	4.61 ± 0.39^{a}	4.80 ± 0.11^{a}	5.42 ± 0.31^{a}	5.16 ± 0.25^{a}
	Butyrate	1.04 ± 0.09^{A}	1.31 ± 0.02^{B}	1.33 ± 0.03^{B}	1.29 ± 0.03^{B}
	Other VFA[1]	0.56 ± 0.03^{A}	0.53 ± 0.01^{A}	0.61 ± 0.05^{A}	0.71 ± 0.05^{B}
	TVFA	24.21 ± 2.67^{a}	31.24 ± 1.51^{b}	31.27 ± 1.20^{b}	31.48 ± 0.89^{b}
12 h	Acetate	28.59 ± 1.64^{a}	31.26 ± 0.89^{a}	31.40 ± 0.69^{a}	31.61 ± 1.11^{a}
	Propionate	6.34 ± 0.23^{Aa}	6.82 ± 0.28^{ab}	7.62 ± 0.38^{bc}	7.98 ± 0.35^{BC}
	Butyrate	1.64 ± 0.06^{a}	1.90 ± 0.07^{a}	1.83 ± 0.04^{ab}	1.88 ± 0.02^{b}
	Other VFA	0.61 ± 0.05^{a}	0.74 ± 0.05^{a}	0.71 ± 0.04^{a}	0.74 ± 0.01^{a}
	TVFA	37.43 ± 126^{a}	40.71 ± 1.21^{ab}	41.95 ± 1.08^{b}	41.60 ± 0.77^{b}
24 h	Acetate	41.14 ± 1.49^{Aa}	42.43 ± 0.93^{a}	43.03 ± 1.00^{a}	46.37 ± 0.40^{Bb}
	Propionate	8.29 ± 0.19^{a}	9.08 ± 0.29^{a}	9.47 ± 0.42^{b}	9.36 ± 0.23^{b}
	Butyrate	2.23 ± 0.05^{a}	2.24 ± 0.06^{a}	2.28 ± 0.08^{a}	2.32 ± 0.02^{a}
	Other VFA	0.91 ± 0.06^{Aa}	1.26 ± 0.08^{Bb}	1.00 ± 0.05^{Aa}	1.26 ± 0.10^{Bb}
	TVFA	52.56 ± 1.65^{Aa}	54.01 ± 0.81^{ABa}	55.94 ± 1.12^{ABab}	59.05 ± 0.61^{Bb}

Within the same row different lowercase letters mean significant at $P < 0.05$ and different capital letters mean significant at $P < 0.01$. Other VFA1 = the sum of isobutyrate, isovalerate and valerate.

DISCUSSION

As a new type of feed additive, phytosterols are widely used in animal husbandry to reduce blood cholesterol levels and enhance animal growth. However, few studies have examined the impact of phytosterols on ruminants owing to the complicated rumen. One of the goals of ruminal nutritionists and microbiologists is to improve ruminant nutritional efficiency by manipulating the ruminal microbial ecosystem. The metabolism of phytosterols is generally associated with lipids (Albrecht et al., 2002). In rumen fermentation, many unsaturated fatty acids, such as triglyceride and galactose-esters are hydrolyzed to free fatty acids by microbial lipases. Microbes can also hydrogenate unsaturated fatty acids through the action of isomerase and reductase (Bauchart, 1993). Most phytosterols have 1 or 2 carbon-carbon double bonds, which may be converted to phytostanols by chemical hydrogenation or as conjugated forms in which the 3β-OH group is esterified to a fatty acid or a hydroxycinnamic acid, or glycosylated with a glucose or a 6-fatty-acyl hexose (Moreau et al., 2002). During rumen fermentation, phytosterols can be converted into phytostanols through the reduction of the double bond at position 5, forming a subgroup of "sterols" or phytosterol fatty acid esters (PSEs). However, *in vivo* studies showed that phytostanols and PSEs may produce a more marked effect. Heinemann et al. (1988) reported that sitotanol at a level of 1.5 g/day was required to lower serum cholesterol significantly in humans, whereas up to 20 g/day sitosterol was required to achieve the same effect in humans. Commercial cholesterol-lowering spreads and supplements enriched with sitotanol and sterol esters are recommended at a dose of 300 mg/day (Hallikainen et al., 2000). These results may explain why a lower level is being used on ruminants. We previously found that dairy cows that received a diet mixed with 200 mg/day or 800 mg/day of phytosterols showed increased milk production by 1.71 kg/day and 0.44 kg/day, respectively (Jin, 2010). Milk ketones and MUN in treated cows were significantly lower

than in the control. This result indicates that a low level of phytosterols may decrease plasma cholesterol, improve oxidation enzyme activities, and decrease oxidative products in dairy cows compared with high dosages (up to 25-50 g/day) in monogastric animals (Jin, 2010).

pH is an important parameter that reflects the extent and pattern of diet fermentation. In our study, the pH of ruminal fluid following the treatments was slightly decreased compared to the control value, which may be due to the increased production of VFAs. However, all values were in the normal range (pH 5.5-6.8). NH_3-N is the degradation product of exogenous protein and is the nitrogen-containing material for MCP synthesis. However, excessive NH_3-N can increase urea concentration in fluids in the urogenital tract, impairing reproductive performance (Ferguson and Chalupa, 1989). Consumption of excessive CP increases energy requirements by 13.3 kcal of digestible energy/g of excessive N, which also has a negative impact on the environment (National Research Council, 1989). In our study, adding a low level of phytosterols decreased NH_3-N, but increased the production of MCP, which may be attributed to a decreased number of protozoa. Jouany (1996) hypothesized that ciliate protozoa contribute significantly to intraruminal cycling of microbial N and the efficiency of MCP synthesis; thus, a reduced protozoal population may improve dietary N utilization and increase MCP content in the intestine.

In this study, GP was increased, while lactate was decreased. More efficient lactate removal may help prevent acidosis and indirectly stimulate fiber digestion. Phillipson and McAnally (1942) noted that an increase in fatty acids was associated with a decrease in lactic acid. Improvement in GP may function together with the removal of lactate to buffer ruminal fermentation.

Previous studies have suggested that phytosterols inhibit the activity of some lipases, esterase, and other enzymes involved in the sterol absorption process (Trautwein et al., 2003). We also found that phytosterols could inhibit the activities of lipoproteinesterase, hepatic lipase, and total lipase in the blood (Xi YM, Jin ZH, Lin LJ and Han ZY, unpublished results). Inhibition of any of these enzymes can result in lipid degradation in the rumen, which would have been utilized in the intestine for better dairy performance. Jenkins (1993) reported that lipid degradation might promote a decrease in cellulose degradation. VFAs are the end products of rumen microbial fermentation and represent the main supply of metabolizable energy for ruminants (Van Soest, 1982). Typically, propionate has a higher percentage in non-structural carbohydrate-fermented products, while fermentative fibrous material mainly produces acetic acid. Our study indicated that DMD and the production of VFAs increased quickly, which may be rooted in the improved degradation of cellulose and protein. This indicates that phytosterols can improve rumen metabolism and increase production capacity.

CONCLUSION

The addition of phytosterols influenced rumen fermentation *in vitro*, reduced the production of NH_3-N and lactate concentration, and increased microbial mass yield, *in vitro* DMD, and VFAs. Further studies are required to understand the mechanism of ruminal fermentation in ruminants.

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