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Effect of the gynosaponin on methane production and microbe numbers in a fungus-methanogen co-culture*

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ABSTRACT

This study investigated the effect of saponins gypenoside (gynosaponins) on methane production and microbe numbers in a co-culture of a fungus, *Piromyces* sp. F1, and a methanogen, *Methanobrevibacter* sp.. Two co-culture systems were used: with methanogen (co-culture I) and without methanogen (co-culture II; methanogen growth inhibited by chloramphenicol). Each co-culture system was treated with 0, 50, 100 or 200 mg gynosaponins/l culture medium. Gas production, methane concentration and volatile fatty acid concentration (VFA) were measured for each treatment group. The numbers of anaerobic fungi and methanogen were quantified by real time PCR. The results showed that, compared with the control, gynosaponin significantly reduced the gas production, methane concentration, methane to TVFA ratio (total volatile fatty acid), TVFA concentration, number of fungi (except for 50 mg dose of gynosaponin in co-culture I) and number of methanogens. Methane was not detected in co-culture II. The individual VFAs proportion of TVFA were not affected by gynosaponins in either of the co-cultures. The pH was higher in both co-cultures than that of the control ($P<0.01$). These data suggest that gynosaponins has the potential for being used as feed additive to modulate the ruminal fermentation, inhibit the methanogen growth and reduce methane production.

KEY WORDS: fungus-methanogen co-culture, gynosaponins, methane, microbial population, *in vitro*

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INTRODUCTION

Methane production from ruminants has been identified as the single largest source of anthropogenic CH_4 (Mathison et al., 1998). Livestock emit methane as part of their natural digestive processes. The rumen is the home to billions of microbes, including bacteria, methanogens, protozoa and fungi. These microbes breakdown feed to produce volatile fatty acids (VFAs), carbon dioxide, ammonia and methane. Rumen anaerobic fungi are the major degrader of plant fibre and produce formate, acetate, lactate, ethanol, CO_2 and H_2. Anaerobic fungi and methanogens can form methane producing co-cultures when grown together in batch culture on recalcitrant cellulosic substrates (Bauchop and Mountfort, 1981; Cheng et al., 2006, 2009). Co-culture of anaerobic fungi and methanogens could enhance fibre degradation and methane production by shifting fungal product formation away from more oxidized end-products, such as lactate and ethanol and towards production of more reduced products such as formate and acetate. Formate is the preferred growth substrate of methanogens in the rumen and in co-culture and although acetate is not used extensively for methane production in the rumen ecosystem, it is also an effective methanogenic substrate (Bauchop and Mountfort, 1981; Teunissen et al., 1992; Nakashimada et al., 2000). Thus, anaerobic fungi and methanogen association plays an important role in methane emission in the rumen.

Plant extracts with high concentrations of secondary metabolites have the potential to reduce methane production (Cheeke, 1996; Staerfl et al., 2010; Szumacher-Strabel et al., 2010). Saponins are high-molecular-weight glycosides in which sugars are linked to a hydrophobic aglycone (sapogenin) which may be triterpenoid or steroidal in nature (Teferedegne et al., 1999; Jung et al., 2004). The effect of saponins on ruminal anaerobic fungi are well documented in mixed microbial fermentation (Kostyukovsky et al., 1991; Osbourn, 1996; Lee et al., 2000; Mao et al., 2010). *In vitro* fungi numbers are reduced by yucca saponin (Wang et al., 2000) and saponin rich *Sesbania pachycarpa* supplementation (Muetzel et al., 2003). No fungi RNA could be detected in the presence of MS (saponin containing a methanol extract of *Sapindus rarak*) higher than 1 mg/ml and methanogen RNA concentration was reduced by a higher concentration of MS (4 mg/ml) (Wina et al., 2005). However, these studies mainly focused on the effect of saponins on fungi numbers and the role of co-culture of fungi and methanogens in methane production was not studied. The co-culturing of anaerobic fungi with methanogen may be a useful way to estimate the bioconversion of cellulose fibre to methane. Thus, the objective of this study was to investigate the effect of gynosaponins, which is derived from *Gynostemma pentaphyllum* Makino

and contains dammarane sapogenins, on methane production, fermentation characteristics and cell numbers in fungus-methanogen co-cultures.

MATERIAL AND METHODS

Inocula

The inoculum in this study was a natural co-culture of an anaerobic fungus associated with methanogen. Firstly, a mixed co-culture of anaerobic fungi and methanogen from goat rumen content was obtained (Cheng et al., 2006, 2009). The mixed co-culture was then purified by rolling-tube to obtain a single anaerobic fungus with methanogen. The anaerobic fungus in the natural co-culture was identified as *Piromyces* sp. F1 and the associated methanogen was identified as *Methanobrevibacter* sp. (Cheng et al., 2009). The isolation procedure involved 5-fold serial dilutions of fresh rumen contents from a sacrificed goat. The dilution series was made in a complex medium under anaerobic conditions, as described by Davies et al. (1993) (medium C: 15% clarified rumen fluid, 2.5 $g \cdot l^{-1}$ yeast extract, 10 $g \cdot l^{-1}$ trypticase peptone, 6 $g \cdot l^{-1}$ $NaHCO_3$, and a basal salts solution) with cellobiose as the energy source (5.0 $g \cdot l^{-1}$) and 1% (v/v) antibiotic mixture containing streptomycin sulphate and penicillin (each at 5 $mg \cdot ml^{-1}$ in the stock solution). These culture solutions were then subcultured anaerobically into fresh tubes of medium C with cellobiose. Axenic cultures of the fungi were picked from roll tube agar using the technique of Joblin (1981) and were maintained in 10% glycerol stored under liquid nitrogen. The roll tube medium contained 1.5% (w/v) agar in addition to the other ingredients used by Orpin (1976). One of symbionts was identified as the monocentric fungi *Piromyces* associated with the methanogen *Methanobrevibacter thaneri* strain CW, which was selected as the inocula in this study. Bacteria in each culture were not detected by PCR with general primers 968f/1401r (Su et al., 2008) and protozoa were not observed by optical invert microscope.

Preparation of saponin and experimental design

The effect of gynosaponins extracted from *Gynostemma pentaphyllum* Makino on fungal fermentation was evaluated in an *in vitro* batch culture under anaerobic conditions. The gynosaponins powder (98% gynosaponin, a group of saponins) was provided by Kangwei Bioengineering Ltd. (China). About 90 kinds of gynosaponin have been also isolated from *G. pentaphyllum* (Cui et al., 1999). Four different doses of gynosaponin were added to each co-culture system: 0, 50, 100 and 200 mg/l of medium (Hu et al., 2005; Wang et al., 2011). The

fermentation was conducted in 160-ml serum bottles containing 90 ml medium (Davies et al., 1993) and 0.8 g rice straw (ground through a 3 mm screen), in triplicate for each treatment (Singh et al., 2001; Hu et al., 2005; Garcia-Gonzalez et al., 2007). Bottles were flushed with CO_2 before sealing with rubber stoppers and aluminum caps, and sterilized at 121ºC for 15 min. Half the bottles were injected with 1 ml chloramphenicol inhibiting the growth of methanogens (Cheng et al., 2009; Fernandes et al., 2010; Ravella et al., 2010) and the other half were injected with 1 ml sterilized medium. The inoculum was cultured in four serum bottles until the straw floating and incorporated four bottles of inocula into one 500 ml vessel under obligated anaerobic and axenic condition. The inocula was uniformly mixed and ten million litre of inocula was draw out and inoculated into the serum bottles. All of serum bottles were incubated at 39ºC for 4 days.

Analysis of fermentation end products

During incubation at 39ºC, the accumulation of the gas produced was measured at 0, 12, 24, 30, 36, 48, 72 and 96 h using the pressure transducer technique of Theodorou et al. (1995). The accumulative gas production approach almost the maximum after 96 h fermentation according to our preliminary study, wherein the fermentation were terminated. From the head-space of each serum bottle, 50 µl of gas sample was drawn out by a gastight syringe to measure methane concentrations according to the method described by Hu et al. (2005).

After 96 h incubation, the experimental culture supernatants were collected and the pH were analysed immediately using a portable pH-meter with a glass electrode (Ecoscan pH 5, Singapore). One milliliter of a solution containing 250 g/l metaphosphoric acid (wt/wt) and 6.4 g/l crotonic acid (wt/wt) was added to 5 ml of fermentation fluid and frozen at -20°C for the VFA analysis by the method of Jouany (1982). Samples were thawed at ambient temperature and centrifuged at 12.000 *g* for 5 min at 4°C in a micro-centrifuge. VFA analysis was carried out on the supernatants (0.6 µl), with crotonic acid included as an internal standard (Cottyn and Boucque, 1968), using a flame ionization detector and a Capillary Column (Supelco, Column No.34292-07 B, 30 m × 0.32 mm × 0.25 µm film thickness, U.S.) with an injector/detector temperature of 180°C/180°C, a column temperature of 140°C and a gas flow rate of 30 ml/min.

Ten milliliters of homogenized fermentation contents were collected for DNA extraction using the modified CTAB method (hexadecyltrimethylammonium bromide) of Doyle and Doyle (1987). The samples were centrifuged at 12.000 *g* for 10 min and the supernatants were discarded. The sediments were resuspended in 800 ml CTAB buffer (0.1 M Tris HCl, pH 8.0, 1.4 M NaCl, 0.02 M EDTA, 2% CTAB), beaten for 4 min using a bead-beater (Biospec Products, Bartlesville, OK, USA) and incubated at 70ºC for 20 min. Chloroform (500 ml) was added and the

samples were vortexed for 1 min and centrifuged at 13.000 *g* for 10 min at ambient temperature. The upper aqueous layers (500 ml) were carefully removed and mixed with 300 ml isopropanol. After centrifuging at 13.000 *g* for 10 min, the resulting DNA pellets were washed with 1 ml iced (-20ºC) 70% ethanol, centrifuged again (13.000 *g* for 20 min) and, after removal of the ethanol, resuspended it in 50 ml TE buffer (pH 8.0) and stored at -20ºC until required.

Real-time PCR assay for quantification of the fungus and methanogen

Real-time PCR was performed on an Applied Biosystems 7300 Real-Time system (Applied Biosystems, CA, USA) using fungal 18S rRNA gene and methanogen mcrA gene-specific primers (Denman et al., 2006, 2007). A reaction mixture (20 l) consisted of 10 l of IQ SYBR Green Supermix (Bio-Rad, CA, USA), 0.2 M of each primer set and 1 l of the template DNA. Measurements were done in triplicate for each run including a negative control (Boechaert et al., 2008). The amount of DNA in each sample was spectrophotometrically determined in triplicate using a GeneQuant pro RNA/DNA calculator (Amersham Biosciences, Bath, UK), and the mean values were calculated. Standard curves for the fungus and methanogen were generated using the serially diluted corresponding gene amplicons obtained from the same co-culture. DNA concentration of the PCR product measured on GeneQuant pro RNA/DNA calculator can be directly related to copy numbers using the following equation:

$$\text{copy number}/\ \mu\text{l} = (C\ /X) \times 0.912 \times 10^{12}$$

where: C - DNA concentration measured (ng/µl); X - PCR fragment or plasmid length (bp/copy), by using the average weight of a bp) (provided by Wageningen Institute of Animal Sciences, the Netherlands).

PCR was performed under the following cycle conditions: one cycle of 50ºC for 2 min and 95ºC for 2 min for the initial denaturation, and 40 cycles at 95ºC for 15 s and 60ºC for 1 min for the primer annealing and product elongation (Denman et al., 2006). Fluorescence detection was performed at the end of each denaturation and extension step. Specificity of the amplified products was confirmed by melting temperatures and dissociation curves after each amplification. Amplification efficiencies for each primer pair were investigated by examining a dilution series of the fungus and methanogen on the same plate in triplicate.

Statistical analysis

Statistical analysis was carried out using one-way ANOVA in the statistical software package SPSS (version11.0; SPSS Inst. Inc. Cary, NC). The dosage of gynosaponins was the source of variation, and the linear or quadratic effects of

increasing dosage of gynosaponins were determined using polynomial contrasts. The statistical model included the effects of treatment and incubation time as the repeated factors. All values were expressed as means of three replicates. Significant differences were declared when $P<0.05$.

RESULTS

Effect of gynosaponins on gas production and methane emission

The effect of gynosaponins on the end products of *in vitro* co-culture fermentation, gas production and methane concentration are shown in Table 1

Table 1. Effect of gynosaponins supplementation on fermentation characteristics of the fungus-methanogen co-culture in 96 h incubation

Item		Gynosaponins dose/mg/l				SEM	P		
		0	50	100	200		T	L	Q
Co-culture I	pH value	6.30^{c}	6.33^{c}	6.48^{b}	6.52^{a}	0.03	<0.01	<0.01	0.43
	Gas production/ml	157.09^{a}	144.54^{a}	69.78^{b}	54.95^{b}	0.04	<0.01	<0.01	0.84
	Methane concentration,%	7.65^{a}	6.73^{a}	2.04^{b}	0.87^{c}	0.94	<0.01	<0.01	0.65
	Methane/TVFA mmol/mM	0.0180^{a}	0.0155^{b}	0.0035^{c}	0.0016^{c}	0.00	<0.001	<0.001	0.69
	Fungus copies x10^{5}/ml	86.59^{b}	127.39^{a}	37.96^{c}	0.84^{d}	16.48	<0.01	<0.01	<0.01
	Methanogen copies x10^{7}/ml	3.90^{a}	2.08^{b}	2.21^{b}	1.14^{b}	0.35	<0.01	<0.01	0.28
	TVFA/mM	29.84^{a}	28.13^{a}	18.38^{b}	12.90^{c}	2.28	<0.01	0.14	<0.01
	VFAs/TVFA%								
	acetate	91.83^{ab}	93.52^{a}	83.85^{b}	89.01^{ab}	5.17	0.126	0.144	0.52
	propionate	6.31^{ab}	5.13^{b}	12.45^{a}	7.65^{ab}	1.20	0.127	0.223	0.39
	butyrate	1.86	1.35	3.70	3.34	0. 42	0.143	0.059	0.92
Co-culture II	pH value	5.98^{c}	5.98^{c}	6.07^{b}	6.33^{a}	0.04	<0.01	<0.01	<0.01
	Gas production/ml	111.86^{a}	107.89^{a}	97.70^{a}	56.55^{b}	6.92	<0.01	<0.01	<0.01
	Fungus copies ×10^{5}/ml	28.99^{a}	17.72^{b}	6.99^{c}	0.27^{c}	3.46	<0.01	<0.01	0.40
	Methanogen copies ×10^{6}/ml	1.90^{a}	1.33^{ab}	1.11^{b}	0.52^{c}	0.16	<0.01	<0.01	0.96
	TVFA/mM	22.77^{a}	24.87^{ab}	21.05^{ab}	19.67^{b}	2.79	0.05	0.22	0.10
	VFAs/TVFA%								
	acetate	92.25^{a}	89.02^{ab}	94.05^{a}	85.86^{b}	1.22	0.054	0.121	0.21
	propionate	5.79^{ab}	8.29^{ab}	4.24^{b}	10.33^{a}	0.99	0.118	0.229	0.31
	butyrate	1.97^{b}	2.70^{ab}	1.71^{b}	3.82^{a}	0.33	0.071	0.079	0.211

P - probability value for linear effect (L), quadratic effect (Q) and treatment effect (T)

and Figure 1. In co-culture I, as gynosaponins level increased, the gas production and methane concentration linearly decreased ($P<0.01$). After incubation for 96 h, the gas production differed significantly between the co-cultures treated with 100 and 200 mg gynosaponins (Figure 1A). In co-culture I, gas production in the 100 and 200 mg gynosaponins groups was lower than that of control. In co-culture I, the gas production was only significantly reduced by the 200 mg gynosaponins dose. There was no significant variation between treatments of the 50 and 100 mg gynosaponins doses ($P>0.05$; Figure 1C). Significant linear and quadratic patterns were observed between gas production and gynosaponin doses. As compared with the control, methane concentrations in culture I were reduced

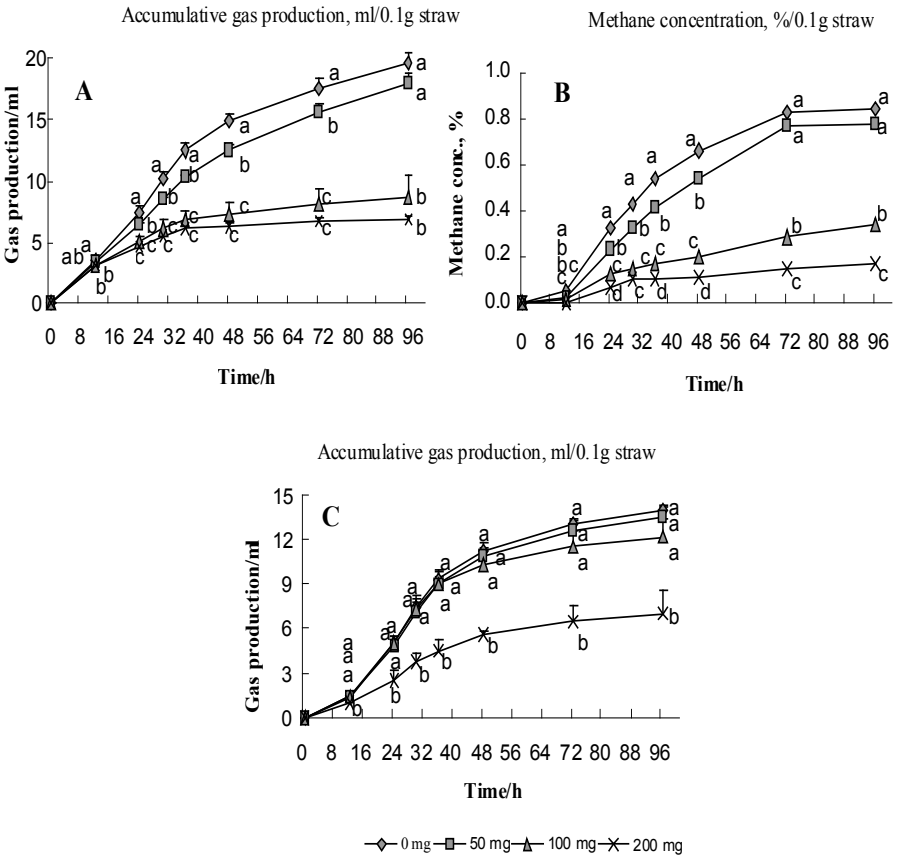

Figure 1. Fitted curves of cumulative gas production (ml) and methane concentration (%). A - Cumulative gas production of co-culture I. B - methane concentration of co-culture I. C - Cumulative gas production of co-culture II; a,b,c in these legends denoted are significantly different at $P<0.05$ level

($P<0.05$ or $P<0.01$) by the addition of gynosaponin except for the 50 mg dose at 72 and 96 h (Figure 1B). The methane to TVFA ratio decreased by gynosaponins addition ($P<0.001$). A linear pattern was observed between the methane to TVFA ratio and gynosaponin dose. Methane was not detected in co-culture II due to the complete inhibition of methanogen by chloramphenicol.

Effect of gynosaponins on volatile fatty acid concentrations and pH

In co-culture I, TVFA concentrations decreased quadratically ($P<0.01$) with increasing gynosaponins concentration ($P<0.01$). In co-culture II, saponin addition caused a significant reduction in TVFA concentration ($P=0.05$). Gynosaponins had no effect on the individual VFAs proportion of TVFA in either of the co-cultures ($P>0.05$). The pH increased in both co-culture I (linear pattern, $P<0.01$) and in co-culture II (linear and quadratic patterns, $P<0.01$) as gynosaponins level increased.

Effects of gynosaponins on numbers of fungi and methanogens

The real-time PCR efficiencies of fungus and methanogen primers were 96.85 and 100.92% ($R^2 = 0.999$ and 0.991), respectively. The copies of methanogenic mcrA genes and fungal 18S rRNA genes in the co-cultures were determined by qPCR (Table 1). In co-culture I, the fungi numbers were highest for 50 mg gynosaponins dose ($P<0.05$) in both co-culture systems and were drastically reduced by the 100 and 200 mg gynosaponins doses ($P<0.01$). The fungi numbers in co-culture II decreased linearly as gynosaponins dose increased ($P<0.01$). The methanogen numbers in both of the co-culture systems were linearly reduced ($P<0.01$) as gynosaponins level increased.

DISCUSSION

Effect on gas and methane production. Theodorou et al. (1995) considered many factors contribute to the generation of gas by fermenting cultures, including the type of microorganisms involved, the rate of growth, the concentration and nature of the growth-limiting nutrient. Thus, gas production was closely related to the microbial growth and the digestion of fermentation substrates. In the current study, the reduction of gas production might be due to the decreased activity or number of fungi, which resulted in decreased digestion of substrates. Wang et al. (2000) also found that steroidal saponins inhibited the digestion of filter paper by

ruminal fungi. In the current study, the gas production pattern in the two co-culture systems occurred differently, with reduced gas production observed with only the 200 mg gynosaponin dose in co-culture II compared to the control. This difference might be caused by the fungus having reduced sensitivity to gynosaponin when co-cultured without methanogens. The inhibition of fungal metabolism resulted in a decrease in hydrogen supply and further reduced methane production. This result was inconsistent with the observation of Staerfl et al. (2010) that yucca saponin have no effect on a methane production. It is deduced that gas production by fungi-methanogen co-cultures differs in the presence or absence of methanogens and gynosaponin can curb methane production in rumen.

Effect on fermentation characteristics. In the present study, the TVFA and acetate concentration were significantly reduced due to a reduction in fungi numbers and activity by gynosaponins. This result agrees with the observations of Lu and Jorgensen (1987), which suggested that lucerne saponin, tested on an *in vitro* fermentation of mixed ruminal microorganisms, resulted in general inhibition of rumen microbial fermentation. However, it is inconsistent with our preliminary observation that gynosaponins had a minor effect on VFA concentration in goats. The individual VFAs proportion of TVFA was unchanged and inconsistent with the observation of Staerfl et al. (2010) that the acetate proportion of TVFA was reduced by yucca saponins and the observation of Dong et al. (2010) that an increase of the propionate concentration was observed by lucerne extract. Due to the different structure of saponins, the saponins displayed different bioactive properties (Hu et al., 2005; Pen et al., 2008). Therein, gynosaponins only affected the TVFA concentration and not the individual VFAs proportion of TVFA in the present study. Higher pH values were observed as gynosaponins dose increased as previously observed in sheep feed yucca saponin (Eryavuz and Dehority, 2004). As the pH is dependent on the TVFA concentration in the co-culture (Mountfort et al., 1982), the increased pH in this study might have been a result of the decreased VFA concentration.

Effect on numbers of the fungi and methanogens. The primary mode of action of saponins on fungi is similar to that of polyene antibiotics, and involves the formation of complexes with membrane sterols (Price et al., 1987). This may result in pore formation and loss of membrane integrity, which further leads to low fungal activity and even fungi death. In the present study, due to a potential synergism between fungi and methanogens, the growth of the fungus was increased in the co-culture with methanogens at low dose of gynosaponins (50 mg/l medium). As observed by Diaz et al. (1993), low levels of *S. rarak* saponins have a positive effect on the fungi *Chytridiomycetes*. The increased fungi numbers in low dose of gynosaponins likely due to the sugar moiety of saponin

as a nutrient for microorganisms. However, the number of fungal cells decreased quickly as gynosaponins level increased. In our preliminary study, we also found similar changes in the growth of the ruminal fungus as the gynosaponins dose increased. The number of ruminal fungi detected in the sample treated with 200 mg of gynosaponins was about 100 times less than the control, showing it was extremely sensitive to gynosaponins. Wang et al. (2000) also reported that *Neocallimastix frontalis* and *Piromyces rhizinflata* were completely inhibited *in vitro* by *Yucca schidigera* saponins.

In the present study, the number of methanogens in both co-culture systems were linearly reduced as gynosaponins level increased. This result was inconsistent with the observation of Guo et al. (2008) and Staerfl et al. (2010) that tea saponins and yucca saponin have no effect on a methanogen population in pure culture. The strong toxic effect of gynosaponins was demonstrated most clearly on the methanogens examined, which agreed with our preliminary observation showing its intensively anti-methanogen characteristics in goat rumen. Methanogen copies were detected in culture II, which DNA concentration of methanogen derived from the original inocula.

CONCLUSIONS

Gynosaponins reduced methane production in the fungus-methanogen co-culture with methanogens, confirming the antimicrobial effect of gynosaponins. It is inferred that a reduction in fungi numbers might also contribute to decreased methane emission. The appropriate level of gynosaponins prompted the growth of the fungus in the co-culture with methanogens, but a high level of gynosaponins inhibited the growth of the fungus and methanogen. It was speculated that gynosaponins might have more effect on fermentation in the co-culture system with methanogens. Thus, data of the present experiment suggest the proper gynosaponins has the potential for being used as feed additives in ruminants.

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