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Effect of Chinese herbal medicines on rumen fermentation, methanogenesis and microbial flora *in vitro*

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Abstract

This study was carried out to evaluate the effects of three Chinese herbal medicines (CHMs) on rumen fermentation, methane emission and population of ruminal microbes using an *in vitro* gas production technique. Three healthy wethers of Dazu Black goats with similar bodyweights and permanent rumen fistulae were utilized as donors of ruminal fluid. The three botanical medicines were cablin patchouli herb (CPH), atractylodes rhizome (AR) and Amur cork tree (AC). Each CHM was added at a level of 25 g/kg to the substrate dry matter. *In vitro* gas production was recorded, and methane concentration was determined at 12 and 24 hours of incubation. After 24 hours, the incubation was stopped, and the inoculants were measured for pH, ammonia nitrogen and volatile fatty acids (VFAs) concentrations. Total deoxyribonucleic acid of ruminal microbes was extracted from the inocula, and populations were determined by a real-time quantitative polymerase chain reaction. Populations of total rumen methanogens, protozoa, total fungi, *Ruminococcus albus*, *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* were expressed as a proportion of total rumen bacterial 16S ribosomal deoxyribonucleic acid. Compared to the control, CPH decreased gas production and methane production at 12 and 24 hours of incubation, and inhibited methanogens and total fungi growth. AR decreased acetate to propionate ratio, and methanogens and total fungi populations, but increased propionate molar proportion. AC decreased total VFA concentration, acetate to propionate ratio, gas production at 12 and 24 hours of incubation, methane production at 12 and 24 hours of incubation, and methanogens and total fungi growth, but increased the propionate molar proportion. In conclusion, CPH and AC both suppressed methanogenesis significantly, and the suppression was mediated primarily via the direct action against the rumen microbes involved in methane formation. AC also indirectly abates methane release by occupying the hydrogen (H_2) normally utilized for methanogenesis.

Keywords: *In vitro* gas production, methane, rumen fermentation parameter, rumen microbes

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Introduction

In many developing countries, a significant proportion of ruminants survives predominantly on roughage-based diets of low quality, which results in the production of much larger amounts of methane than from concentrate-based diets of high quality. Methane generation represents a substantial portion of energy being wasted. Besides, methane is one of the greenhouse gasses and has 21 times more global warming potential than carbon dioxide (Moss *et al.*, 2000). Hence, there has been growing alertness over the inhibition of methanogenesis from the perspectives of nutrition improvement and greenhouse gas abatement. The utilization of chemical feed additives to inhibit ruminal methanogenesis has been developed. However, these chemical additives have negative effects on host animals or possess a short-term duration of effectiveness (Kumar *et al*., 2014). Nowadays an urgent appeal for 'clean, green and ethical' animal production forces scientists to divert research to feed-additive technology to exploit 'natural products' as feed additives (Durmic & Blache, 2012).

Chinese herbal medicine (CHM) remains the most ancient yet living tradition with sound philosophical, experiential and experimental bases. In China, records of medicinal use of CHM dates back as far as 3000 BC, and today CHM is still in wide use as part of ethnoveterinary practices for the prevention and treatment of animal diseases as well as the maintenance of animal health because of the easy and cheap availability

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and validity with few side effects (Lu, 2011). CHM is a kind of natural drug. It has a natural structure and biological activity, and the double characteristics of nutrient and drug, which endues it with the function of adjusting the physiological status of the body from a holistic perspective (ChPC, 2010; Liu *et al*., 2011). According to the theories of modern pharmacology and nutriology, CHM contains a variety of biologically active substances, such as antibacterial materials, alkaloids, polysaccharides, glycosides, essential oils, tannins and organic acids, as well as a certain amount of amino acids, minerals, vitamins, pigments and unknown growth-regulatory factors (Liu & Xu, 2011; Lu, 2011; Wang & Wang, 2016). Therefore, the possible mechanisms of CHM would be associated with exerting nutrition supplements, ameliorating nonspecific immunity, inhibiting or killing bacteria, producing hormone-like or vitamin-like effects, resisting stress and oxidation, protecting feed from oxidation and mildew, and so on (Liu & Xu, 2011; Lu, 2011; Liu *et al*., 2011; Wang & Wang, 2016).

Wang *et al*. (2010) found that two traditional Chinese medicine compounds, composed of cablin patchouli herb (CPH), atractylodes rhizome (AR), Amur cork tree (AC) and cypsum with weight ratios of 1:1:1:0.5 and 1:1:1:1, could alleviate stress from the intense heat of summer and improve fattening performance in beef cattle. Subsequently, Wang *et al.* (2012) and Wang *et al*. (2013) suggested that these two compounds had positive actions on the profile of volatile fatty acids (VFA), the activity of cellulolytic enzymes, and the degradability of dietary nutrients in the rumens of goats when these compounds are added to diets which composed of 50% dried rice straw and 50% mixed concentrate. Further, Wang & Zhang (2011) observed the effect of these two compounds in suppressing methanogenesis *in vitro*. CPH, AR and AC are botanical medicines that contain an array of plant secondary metabolites, which probably exert major influences on ruminal microbial metabolism. Thus, the aim of the present study was to evaluate the effects of these three CHMs on rumen fermentation, methanogenesis and microbial flora *in vitro*.

Materials and Methods

Three CHMs were used in this study. The dried aerial part of CPH is commonly known as *Pogostemon cablin* in Latin. The dried rhizome of AR is commonly known as *Atractylodes lancea* in Latin and the dried bark of AC as *Phellodendron chinensis* in Latin. All of these were usually easily available on the traditional Chinese medicine market in China and were purchased from Rongchang County Hospital of Traditional Chinese Medicine in Chongqing City, southwestern China. All medicines were naturally and gradually air-dried in the shade in summer and finely ground to powder through a 1-mm screen. Then they were preserved in tightly closed plastic jars stored in a dry, dark and cool place. A sample of each CHM was taken for the analysis of gross energy (GE), dry matter (DM), organic matter (OM), crude protein (CP), amylase-treated ash-free neutral detergent fibre (aNDFom), ash-free acid detergent fibre (ADFom), ether extract (EE), calcium (Ca) and phosphorus (P) (Table 1).

Table 1 Chemical compositions of the Chinese herb medicines and the substrate used (dry matter basis, g/kg)

	CPH[1]	AR[2]	AC[3]	Substrate[4]
Gross energy (MJ/kg)	19.52	18.65	21.27	19.92
Organic matter	918.1	844.8	925.0	854.6
Crude protein	67.7	48.4	105.1	116.0
aNDFom[5]	691.4	171.0	436.0	469.9
ADFom[6]	520.8	104.8	242.3	334.6
Ether extract	16.7	69.3	26.6	25.5
Calcium	33.4	17.9	18.8	10.1
Phosphorus	14.0	7.8	11.1	7.4

[1] CPH: cablin patchouli herb
[2] AR: atractylodes rhizome
[3] AC: Amur cork tree
[4] The substrate included 500 g rice straw, 260 g corn, 60 g wheat bran, 120 g soybean meal, 35 g rapeseed meal, 10 g calcium carbonate, 5 g sodium chloride and 10 g vitamin and trace mineral premix per kg dry matter
[5] aNDFom: amylase-treated ash-free neutral detergent fibre
[6] ADFom: ash-free acid detergent fibre

An *in vitro* gas production (GP) test was conducted with a semi-automated Reading Pressure Technical (RPT) (Mauricio *et al*., 1999). The three CHMs were added at a level of 25 g/kg of DM of the substrate. Meanwhile, a control treatment was set up with the substitution of substrate for the CHMs at an equivalent amount. The substrate had the same ingredients as the basal diet used in the study by Wang *et al.* (2012) and Wang *et al*. (2013) (Table 1). The incubation procedures were performed in 180-mL serum bottles. Every bottle contained 750 mg of the substrate (Theodorou *et al*., 1994). Subsequently, the designated amounts of the CHMs or the equal amount of substrate were added to the bottles. Before weighting, both the substrate and the CHMs were finely ground using a 1-mm screen and dried at 65 °C for 4 hours in an oven. After that, 90 mL of artificial saliva, prepared by the method of Menke & Steingass (1988), was injected into the bottles using a syringe. Finally, the bottles with the substrate, CHMs and artificial saliva were placed overnight in an incubator at 39 °C after sealing with butyl rubber stoppers and aluminium caps. At the same time, four bottles that contained the incubation medium without substrate and CHMs were incubated as the blanks to correct the gas production resulting from the activity of the rumen fluid. Furthermore, the *in vitro* GP test with the same treatment sequence was carried out twice to provide replication, and each treatment of two separate runs had four repeats.

Three healthy wethers of Dazu Black goat (25.2 ± 1.2 kg), fitted with permanent rumen fistula, were used as donors of rumen fluid. They were fed twice daily at 07:00 and 19:00 on 600 g/day of a mixed diet (the ingredients and chemical composition of which were identical to those of the substrate) and had free access to water. In the morning of the experimental day, mixed rumen contents were obtained in equal proportions before the morning feeding from the three wethers, and transported to the laboratory, where they were quickly filtered through four layers of cheesecloth into a flask under CO_2 in a water bath at 39 °C until use. A total of 10 mL filtered rumen fluid was injected into the incubation bottles through the stopper using a syringe. Shortly afterwards, the bottles were shaken to mix the contents completely and put into the incubator at 39 °C. According to methods described by Zhang *et al*. (2008), gas pressure was recorded at 12 and 24 hours of incubation using a pressure transducer to calculate total GP (mL/g DM incubated). Then 20 µL of gas was drawn out through the stopper with a needle to determine methane concentration by gas chromatography (GC), thus methane production (mmol/g DM incubated) was estimated. After termination of incubation at 24 hours, the incubation fluids were sampled. A portion of the samples were stored at -20 °C for later analysis of end-products. The other samples were stored at -80 °C immediately for analysis of microbe communities by real-time polymerase chain reaction (PCR).

The experimental procedures were approved by and conformed to the requirements of the Animal Care and Use Committee of Southwest University in Chongqing City, southwestern China.

Gross energy was determined by an isoperibol bomb calorimeter (model 1281, Parr Instrument Co., Moline, IL) with benzoic acid used as a standard. DM was determined by loss of weight after drying a 2 g aliquot of each sample for 24 hours at 105 °C. OM was calculated as weight loss on ignition at 600 °C for 18 hours in a muffle furnace (AOAC, 2005). CP was measured by multiplying nitrogen (N) from a Leco model FP-2000 N analyser (Leco Corp., St. Joseph, MI) according to the Dumas combustion method using EDTA as a standard with a factor of 6.25 (AOAC, 2005). EE was quantified using diethyl ether as an extraction fluid in a Soxhlet apparatus (AOAC, 2005). αNDFom and ADFom were analysed with a fibre analyser (FIWE6, VELP, Italy) using reagents described by Van Soest *et al*. (1991). Sodium sulphite and heat-stable α-amylase were used in the αNDFom determination. Ca and P were determined by inductively coupled plasma atomic emission spectroscopy after dry ashing at 550 °C to prepare the homogenized samples (AOAC, 2005). All assays were conducted in triplicate.

The methane concentration in the headspace gas at 12 and 24 hours of incubation was determined by GC (GC-2010, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a capillary column (HP-INNOWAX, 19091N-133) of 30 m × 0.25 mm × 0.25 µmol in size (Hu *et al*., 2005). Fermentation parameters of the incubation fluids at the end of 24 hours, such as ammonia N and VFA, were determined using methods described by Hu *et al*. (2005). The pH value was assayed using a pH meter (model PB-10/C, Sartorius, Germany). The concentration of ammonia N was measured by colorimetry with a 721 spectrophotometer (Shanghai, China). VFA concentration was analysed by GC (GC-2010, Shimadzu, Kyoto, Japan).

The genomic deoxyribonucleic acid (DNA) of rumen microbes from the incubation fluids was extracted by the bead-beating method with a mini-bead beater (Biospec Products, Bartlesville, OK, USA), as described by Zoetendal *et al*. (1998). Quantitative PCR was conducted with a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using the SYBR Premix Ex Taq II perfect real time (TaKaRa Bio, Dalian, China). The PCR mixture incorporated 2 µL template DNA, 0.2 mmol dNTP, 0.3 µmol primer, 1.5 mmol magnesium chloride and 1.25 U Taq in a total 20-µL volume. The real-time PCR assays for microorganisms were completed as one cycle at 95 °C for 10 seconds for initial denaturation, followed by 40 cycles of denaturation at 95 °C for 5 seconds, and annealing at 60 °C for 34 seconds. Melting curve analysis was

performed after amplification to verify the specificity of the real-time PCR. The amplification efficiencies for each primer pair were investigated by examining the dilution series of the total rumen microbial DNA template on the same plate in triplicate. The primer sequences used to determine total bacteria, total fungi, *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* were cited from Denman & McSweeney (2006). The primer sequences to determine methanogens and protozoa were cited from Denman *et al.* (2007), and the primer sequences used to determine *Ruminococcus albus* cited by Koike & Kobayashi (2001).

Quantification for methanogen, protozoa, fungi, *R. albus*, *F. succinogenes* and *R. flavefaciens* were expressed as a proportion relative to total rumen bacterial 16S ribosomal DNA (rDNA) according to the following equation:

$$\text{relative quantification} = 2^{-(\text{ct target-ct total bacteria})}$$

where: Ct represents threshold cycle.

Data were subject to a one-way analysis of variance using the PROC GLM procedure of SAS (SAS Institute, 2005). Results were expressed as mean ± standard deviation (M ± SD). Comparisons between average values for each CHM and those observed in the control were conducted by Duncan's multiple range tests. Degree of significance was defined as $P \leqslant 0.05$ is significant.

Results

No differences ($P > 0.05$) were observed in each fermentation parameter between the control and CPH treatment (Table 2). The addition of AR decreased the acetate to propionate ratio ($P = 0.029$), but increased the propionate molar proportion ($P = 0.023$). The addition of AC lowered total VFA concentration ($P = 0.004$) and acetate to propionate ratio ($P = 0.018$), but heightened propionate molar proportion ($P = 0.014$).

Table 2 Effects of the Chinese herb medicines on rumen fermentation parameters *in vitro*

Items	Control	CPH[1]	AR[2]	AC[3]	SEM[4]
pH	6.71 ± 0.03	6.69 ± 0.02	6.68 ± 0.02	6.70 ± 0.02	0.00
NH_3-N[5] (mg/dL)	19.96 ± 1.02	19.99 ± 1.11	20.01 ± 1.07	19.91 ± 1.49	0.20
Total VFAs[6] (mmol/L)	5.56 ± 0.06	5.52 ± 0.10	5.51 ± 0.31	4.50* ± 0.15	0.08
VFAs[6], (mol/100 mol)					
Acetate	81.06 ± 0.99	81.09 ± 1.40	81.05 ± 1.41	80.64 ± 0.73	0.17
Propionate	15.21 ± 0.28	15.19 ± 0.26	16.52* ± 0.20	17.07* ± 0.14	0.15
Butyrate	3.72 ± 0.76	3.66 ± 0.56	3.70 ± 0.54	3.70 ± 0.64	0.07
Acetate/propionate	5.32 ± 0.15	5.33 ± 0.10	4.90* ± 0.06	4.72* ± 0.07	0.05

* within each row these means significantly different from the control at $P \leqslant 0.05$
[1] CPH: cablin patchouli herb
[2] AR: atractylodes rhizome
[3] AC: Amur cork tree
[4] SEM: standard error of mean
[5] NH_3-N: ammonia nitrogen
[6] VFAs: volatile fatty acids

No differences ($P > 0.05$) existed for GP and methane production at 12 and 24 hours of incubation between the control and AR treatment (Table 3). The addition of CPH reduced GP at 12 ($P = 0.042$) and 24 hours ($P = 0.039$) of incubation and methane production at 12 ($P = 0.007$) and 24 hours ($P = 0.032$) of incubation. The addition of AC also lessened GP at 12 ($P = 0.043$) and 24 hours ($P = 0.044$) of incubation and methane production at 12 ($P = 0.009$) and 24 hours ($P = 0.018$) of incubation.

No differences existed ($P > 0.05$) for the quantities of protozoa, *R. albus*, *F. succinogenes* and *R. flavefaciens* relative to total bacterial 16S rDNA between the control and all CHM (Table 4). CPH, AR and AC inhibited the growth of methanogens (*P*-values 0.000, 0.000 and 0.000, respectively) and total fungi (*P*-values 0.038, 0.043 and 0.030, respectively).

Table 3 Effect of the Chinese herb medicines on gas and methane production *in vitro*

Items	Control	CPH[1]	AR[2]	AC[3]	SEM[4]
Gas production (mL/g)					
12 h	148.4 ± 1.4	130.9* ± 1.3	144.3 ± 1.6	133.3* ± 1.9	1.3
24 h	251.1 ± 5.4	217.9* ± 1.0	247.2 ± 8.6	228.8* ± 1.5	2.5
Methane production (mmol/g)					
12 h	0.84 ± 0.07	0.63* ± 0.03	0.88 ± 0.04	0.64* ± 0.05	0.02
24 h	1.79 ± 0.17	1.31* ± 0.04	1.71 ± 0.02	1.41* ± 0.03	0.03

* Within each row these means significantly different from the control at $P \leqslant 0.05$
[1] CPH: cablin patchouli herb
[2] AR: atractylodes rhizome
[3] AC: Amur cork tree
[4] SEM: standard error of mean

Table 4 Effect of the Chinese herb medicines on rumen microbial populations (% of total bacterial 16S rDNA, $\times 10^{-2}$) *in vitro*

Items	Control	CPH[1]	AR[2]	AC[3]	SEM[4]
Methanogens	191.52 ± 11.01	39.83* ± 5.07	25.89* ± 2.38	81.88* ± 6.56	9.77
Protozoa	204.5 ± 9.9	204.4 ± 20.7	205.4 ± 21.0	205.6 ± 11.3	2.3
Total fungi	44.57 ± 3.05	19.96* ± 2.81	20.33* ± 2.02	16.18* ± 4.23	1.99
Ruminococcus albus	309.0 ± 25.9	310.5 ± 11.0	310.8 ± 12.6	309.4 ± 15.9	2.5
Fibrobacter succinogenes	20.66 ± 3.51	20.55 ± 3.07	20.72 ± 3.47	20.85 ± 3.84	0.51
Ruminococcus flavefaciens	379.3 ± 21.4	379.2 ± 26.0	380.0 ± 42.2	380.4 ± 22.7	4.3

* within each row these means significantly different from the control at $P \leqslant 0.05$
[1] CPH: cablin patchouli herb
[2] AR: atractylodes rhizome
[3] AC: Amur cork tree
[4] SEM: standard error of mean

Discussion

The products tested, CPH, AR and AC, fall into the category of natural plant products, which produce an enormous variety of secondary metabolites to provide protection against microbial and insect attack (Hart *et al.*, 2008). Nowadays, there is increasing interest in the use of plants that contain phytochemicals and their extracts to manipulate the function of the gastrointestinal tract in both ruminant and non-ruminant livestock (Greathead, 2003; Wang & Wang, 2016). Generally, these bioactive substances can be structured into terpenes and isoprenoids, phenols and alkaloids. The four major classes with current potential use in ruminant nutrition are saponins, tannins, organosulphur compounds and essential oils (Bodas *et al.*, 2012). Indeed, except for some nutrient substances, three raw plant medicinal drugs that were used in the current study contain a quantity of one or more classes of these bioactive compounds. For example, CPH includes essential oils, tannins and amaroids, AR, alkaloids such as berberine, N-free crystalline substances, erapressed oils, mucoid substances, steroids and closely related sterols and AC, essential oils, carotene or carotenoid and hiamine (ChPC, 2010; Liu & Xu, 2011; Wang & Wang, 2016). Because the concentrations of secondary compounds in each of these raw plant materials might be low, a considerable amount (25 mg CHM per g of total DM incubated) was added to supply enough amounts of active compounds. Addition of each CHM would not be economically feasible under practical feeding conditions, but the situation would be different if the active compounds could be extracted or synthesized artificially to be utilized as feed additives. In addition, because more than four fifths of DM of every CHM was OM, which can be degraded and fermented to some extent, 768.75 mg of substrate were added to the control cultures so that the amount of

total DM incubated was the same in all cases.

Methane produced by enteric fermentation represents a loss of energy to ruminants and results in a detrimental impact on the environment because of its greenhouse effect. As a result, the exploration of alternatives for the mitigation of this gas has won considerable attention in the last decade (Moss *et al.*, 2000). Since numerous chemical additives and antibiotics have been withdrawn in most countries, new alternatives are needed to manage efficient animal production systems and public concern about animal welfare and environmental protection. More recently, bioactive plants and plant products rich in secondary metabolites have been studied as alternative natural feed additives to replace antibiotics (Durmic & Blache, 2012). Some bioactive plant metabolites show potential to alter rumen fermentation and decrease methane production (Patra, 2012). The ruminal antimethanogenic activity of saponins, tannins and essential oils extracted from an array of plant materials has been extensively demonstrated in many *in vitro* and *in vivo* studies with variable efficacy, depending on the chemical nature and ruminal concentration, though their modes of action are so different and have not been completely elucidated (Benchaar & Greathead, 2011; Goel & Makkar, 2012). There is still a long way to go before the phytochemical fractions involved in the reduction of methane production are extracted and isolated and artificially synthesized, and then used as feed additives. However, feeding the plants that contain these bioactive compounds directly could be an alternative approach to motivate similar changes in rumen fermentation because these anti-methanogenic plant materials might be grown in large areas like any field crops, especially for the developing world (Newbold & Rode, 2006).

Gas production (GP) during incubation *in vitro* was strongly correlated with the degradation of OM of feedstuffs (Menke *et al.*, 1979). Higher GP meant more violent fermentation in the rumen of feedstuffs. In the present study, GP at 12 and 24 hours of incubation of the control were higher than those of CPH treatment and AC treatment, indicating that both CPH and AC inhibited fermentation in the rumen liquor *in vitro*. However, only AC influenced the degradability of the OM of the substrate, thus resulting in a reduction of total VFA concentrations. The addition of AR had no impact on GP at 12 and 24 hours of incubation and total VFA concentration. VFAs were regarded as one of rumen fermentation indexes, and typified rumen fermentation pattern and nutriment degradation efficiency (Pitt *et al.*, 1996). The VFA profile in the rumen was affected mainly by the compositions of diet fed to ruminants. In the present study, acetate molar proportion of every group was relatively high, since 50% of substrate was dried rice straw, which is categorized as roughage of low quality. The addition of CPH did not influence the VFA profile, suggesting that *in vitro* rumen fermentation was not altered by CPH. Compared with the control, AR treatment had an increased propionate molar proportion, decreased acetate to propionate ratio, and an unchanged total VFAs concentration, implying that AR improved the VFA profile and promoted functioning *in vitro* rumen fermentation. With the addition of AC, an increased propionate molar proportion and a decreased acetate to propionate ratio were observed, along with a decreased total VFA concentration, showing that AC was harmful to *in vitro* rumen fermentation, but was beneficial to the shift of fermentation pattern.

Both CPH and AC reduced methanogen population relative to total bacterial 16S rDNA, and mitigated methane production at 12 hours and 24 hours of incubation. The elimination of ruminal methanogenic microorganisms was an intrinsic and leading cause of the reduction of methane production of CPH and AC treatment in comparison with the control. However, there was another reason for the action of AC against *in vitro* ruminal methanogenesis. The abatement of methane emission and the modification of VFA profile in response to the addition of AC seemed to be concomitant, and the reduction in total VFAs production was a consequence of impaired methanogenesis. Methanogens survive by utilizing hydrogen (H) in the rumen and try to compete with propionate-producing microbes that also utilize H to form propionate (McAllister & Newbold, 2008). As a result, AC led to a lower availability of H for methanogens followed by a mitigation of methane emission *in vitro* by channelling H_2 utilized for methanogenesis to synthesis of propionate. Therefore, AC indirectly reduced methane release via the interference of H uptake by methanogenic bacteria. Although AR had anti-methanogen characteristics, which was demonstrated by the present result about methanogen population relative to total bacterial 16S rDNA, ruminal methanogenesis was not suppressed by AR. This finding suggested that methanogenesis might not always be correlated with the number of methanogens in the rumen. Kamra *et al.* (2006) also indicated that while methanogenesis in the presence of 5 mmol bromoethanesulphonic acid was completely inhibited on two substrates (lucerne and maize forage), the numbers of methanogens, which were estimated using specific primers by real-time PCR, were not eliminated completely in the *in vitro* gas production test. The study on the effect of *Salvia officinalis* on methane synthesis showed that these two parameters were not correlated either (Broudiscou *et al.*, 2000).

Defaunation is usually connected with lowered methane production in the rumen because a symbiotic relationship exists between some methanogens and ciliate protozoa, and methanogens lose their symbiotic partner, incurring their reduced biological activity (Sharp *et al.*, 1998). However, the extermination of protozoa from the rumen in the practical production is controversial because it may bring about a risk to the

health of animals and humans. Methane production does not always reduce with the absence of protozoa from the rumen microbiota, because most methanogens survive in the liquid portion of rumen content (Morgavi *et al.*, 2012). In the present study, the protozoa population, relative to total bacterial 16S rDNA, was not affected with the addition of each CHM, suggesting that the CHMs were not poisonous to ruminal microecology and animal health. Fungi and cellulolytic bacteria play important roles in keeping a stable intra-ruminal environment for structural carbohydrate digestion. Feed intake and digestibility commonly decrease with the inhibition of their activities (Patra & Saxena, 2009). In the present study, the CHMs seemed to have anti-fungi property, suggesting that they might have a negative impact on ruminal fibre degradation. However, the responses of three fibrolytic microbes to the CHMs were not the same with fungi, illustrating that further research would be needed to clarify the relationship between three CHMs and ruminal fibre fermentability.

Conclusions

Three CHMs inhibited methanogens proliferation, but only CPH and AC abated methane release. CPH had no impact on rumen fermentation parameters. AR enhanced propionate production to improve rumen fermentation. AC evoked a shift of fermentation pattern towards decreased acetate to propionate ratios by channelling H_2 utilized for methanogenesis to synthesis of propionate. Three CHMs suppressed total fungi growth, but had no effects on the reproduction of protozoa, *R. albus*, *F. succinogenes* and *R. flavefaciens*. More studies are required to evaluate the persistence of the antimethanogenic effects *in vivo* to specify the chemical nature of the active compounds that are responsible for such effects, and to testify the usefulness and applicability under diverse practical conditions.

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Authors' Contributions

W.J. Wang and S.P. Wang contributed equally to this work.

Conflict of Interest Declaration

There is no conflict of interest.

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