Animal Nutrition 5 (2019) 185–190

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original Research Article

Effect of traditional Chinese medicine compounds on rumen fermentation, methanogenesis and microbial flora *in vitro*

Shui Ping Wang [a, b, 1], Wen Juan Wang [a, 1], Zhi Liang Tan [b, *], Guo Wei Liu [a, b], Cheng Fu Zhou [a, b], Meng Jie Yin [a, b]

[a] College of Animal Science, Southwest University, Chongqing 402460, China
[b] Key Laboratory of Agro-Ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha 410125, China

ARTICLE INFO

Article history:
Received 28 February 2018
Received in revised form
8 September 2018
Accepted 28 September 2018
Available online 25 October 2018

Keywords:
Traditional Chinese medicine compound
Fermentation characteristics
Methanogenesis
Ruminal microbes
In vitro

ABSTRACT

This study was conducted to investigate the effects of traditional Chinese medicine compounds (TCMC) on rumen fermentation, methane emission and populations of ruminal microbes using an *in vitro* gas production technique. Cablin patchouli herb (CPH), Atractylodes rhizome (AR), Amur Cork-tree (AC) and Cypsum were mixed with the weight ratios of 1:1:1:0.5 and 1:1:1:1 to make up TCMC1 and TCMC2, respectively. Both TCMC were added at level of 25 g/kg of substrate dry matter. *In vitro* gas production was recorded and methane concentration was determined at 12 and 24 h of incubation. After 24 h, the incubation was terminated and the inoculants were measured for pH, ammonia nitrogen, volatile fatty acids (VFA). 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 with the control, the 2 TCMC decreased ($P \leq 0.05$) total VFA concentration, acetate molar proportion, acetate to propionate ratio, gas and methane productions at 12 and 24 h, hydrogen (H) produced and consumed, and methanogens and total fungi populations, while the 2 TCMC increased ($P \leq 0.05$) propionate molar proportion. Traditional Chinese medicine compound 1 also decreased ($P \leq 0.05$) *R. flavefaciens* population. From the present study, it is inferred that there is an effect of the TCMC in suppressing methanogenesis, probably mediated via indirect mode by channeling H_2 utilized for methanogenesis to synthesis of propionate and direct action against the rumen microbes involved in methane formation. In addition, the relative methane reduction potential (RMRP) of TCMC2 was superior to that of TCMC1.

© 2018, Chinese Association of Animal Science and Veterinary Medicine. Production and hosting by Elsevier B.V. on behalf of KeAi Communications Co., Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

In major developing countries, a large proportion of roughages is widely utilized in the diets of ruminants. Modulating rumen microbial ecosystem to improve digestibility of fibrous feeds and mitigate enteric methane emissions from ruminants are some of the most important fields for animal nutritionists. Methane produced during anaerobic fermentation in the rumen results in a 2% to 12% of gross energy lost from the animal, and contributes to emissions of greenhouse gases into the atmosphere which may lead to a damaging impact on the environment. The methane abatement strategies involve utilization of feed additives (chemicals, organic acids, and probiotics) and oils supplementation in diets, alteration of

* Corresponding author.
E-mail address: zltan@isa.ac.cn (Z.L. Tan).
[1] These authors contributed equally to this work.
Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2018.09.004
2405-6545/© 2018, Chinese Association of Animal Science and Veterinary Medicine. Production and hosting by Elsevier B.V. on behalf of KeAi Communications Co., Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

feeding practices, and complementation of low-quality or high-fiber diets with deficient nutrients (Gerber et al., 2013). However, the utilization of above-mentioned strategies by livestock producers has been limited for some reasons, such as decrease in fiber utilization efficiency, short-term duration of effectiveness, controversial research results between *in vitro* and *in vivo*, potential risk to animal health, and high cost of feed or labor (Kumar et al., 2014).

Up to date, traditional Chinese medicine (TCM) has still been remaining the most thriving vitality with philosophical, experiential and experimental bases. In China, TCM has been warmly used to prevent and treat human and animal diseases, and also taken as health care products for human and green feed additives for animal, because TCM is easy and cheap to get, and is effective with fewer side effects. TCM is a kind of natural drugs, and keeps its natural structure and biological activity, and owns double features of nutrient and drug which endues it with the function of regulating physiological status of the body from a holistic perspective (Liu et al., 2011; Wang and Wang, 2016). There is a great variety of TCM in China, and every TCM has complicatedly various components which generate diversely biological functions, let alone TCM compound (TCMC) which is comprised of more than one TCM. The overwhelming majority of TCM is botanical medicines called "Chinese herbal medicine (CHM)" in China. According to the theories of modern pharmacology and nutriology, TCM 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 (Lu, 2011). Therefore, the possible mechanisms of TCM would 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 (Lu, 2011; Liu et al., 2011).

Wang et al. (2010) and Wang et al. (2011) found that the 2 TCMC composed of Cablin patchouli herb (CPH), Atractylodes rhizome (AR), Amur Cork-tree (AC) and Cypsum with the weight ratios of 1:1:1:0.5 and 1:1:1:1 could serve beef cattle via alleviating stress from the intense heat of summer and improving fattening performance. Wang et al. (2012), Wang et al. (2012a, 2012b) and Wang et al. (2013) also observed those above-mentioned phenomena when the TCMC as feed additives were supplemented to the diets composed of 50% of dried rice straw and 50% of mixed concentrate in goats. In addition, they also reported that the TCMC had some positive actions on the ruminal concentrations and proportions of volatile fatty acids (VFA), the activity of celluolytic enzymes, and the ruminal degradability and total apparent digestibility of dietary nutrients, which inferred that the TCMC had the potential of modulating rumen fermentation as well as microbial ecosystem, and further acted on ruminal methanogenesis. Thus, the aim of the present study was to evaluate the effects of the TCMC on rumen fermentation, methanogenesis and microbial flora *in vitro*.

2. Materials and methods

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

2.1. Experimental additives

Four types of TCM were used in this study. The dried aerial part of CPH is commonly known as *Pogostemon cablin* in Latin and guǎng huò xiāng in Chinese pinyin. The dried rhizome of AR is commonly known as *Atractylodes lancea* in Latin and nán cāng zhú in Chinese pinyin. The dried bark of AC is commonly known as *Phellodendron chinensis* in Latin and chuān huáng bò in Chinese pinyin. Cypsum, a natural mineral medicine with hydro calcium sulfate fibriform crystallized polymeric, is commonly known as *Gypsum Fibrosum* in Latin and shēng shí gāo in Chinese pinyin. All of them were usually and easily available on the TCM market in China and were purchased from Rongchang County Hospital of TCM located in Chongqing City, southwestern China. All medicines were naturally and gradually air-dried in the shade in summer and finely ground to powders through a screen of 1 mm. Cablin patchouli herb, AR, AC and Cypsum were mixed according to the weight ratios of 1:1:1:0.5 and 1:1:1:1 to make up TCMC1 and TCMC2, respectively. The difference between TCMC1 and TCMC2 was the dosage of Cypsum, and TCMC2 had 2-fold dosage of Cypsum compared with TCMC1. Then the 2 TCMC were preserved in tightly closed plastic jars and stored in a dry, dark and cool place. After mixing, a sample of each TCMC was taken for the analyses of gross energy (GE), dry matter (DM), organic matter (OM), crude protein (CP), ether extract (EE), amylase-treated ash-free neutral detergent fiber (αNDFom), ash-free acid detergent fiber (ADFom), calcium (Ca) and phosphorous (P). On the basis of DM, TCMC1 and TCMC2 contained 16.30 and 14.24 MJ/kg of GE, 757 and 663 g/kg of OM, 61 and 68 g/kg of CP, 436 and 362 g/kg of αNDFom, 283 and 202 g/kg of ADFom, 38 and 24 g/kg of EE, 59 and 83 g/kg of Ca, 10 and 15 g/kg of P, respectively.

2.2. In vitro fermentations

An *in vitro* gas production (GP) test was conducted with the semi-automated Reading Pressure Technology (RPT; Mauricio et al., 1999). Traditional Chinese medicine compound 1 and TCMC2 were added at the level of 25 g/kg of DM of the substrate, which was the same as the supplementation level in the diets as described in the previous studies of Wang et al. (2010, 2012) when the 2 TCMC were fed to beef cattle and goats. Meanwhile, a control was set up with the substitution of the substrate for the TCMC in an equivalent amount. The substrate had the same ingredients as the basal diet used in the study of Wang et al. (2012), which consisted of 500 g of rice straw, 260 g of corn, 60 g of wheat bran, 120 g of soybean meal, 35 g of rapeseed meal, 10 g of calcium carbonate, 5 g of sodium chloride and 10 g of vitamin and trace mineral premix per kg of DM. According to the measured chemical composition, the substrate per kg could provide 19.92 MJ GE, 854.6 g OM, 116.0 g CP, 469.9 g αNDFom, 334.6 g ADFom, 25.5 g EE, 10.1 g Ca and 7.4 g P based on DM. The incubation procedures were carried out in 180-mL serum bottles. Every bottle contained 750 mg of the substrate (Theodorou et al., 1994). Subsequently, the designated amounts of the TCMC or the equivalent substrate were added into the bottles as the TCMC treatments or the control, respectively. Before weighing, both the TCMC and the substrate were finely milled using a 1-mm screen and dried at 65 °C for 4 h in an oven. After that, 90 mL of artificial saliva prepared by the method of Menke and Steingass (1988) was poured into the bottles using a syringe. Finally, the bottles with the substrate, the TCMC and the buffer medium were placed in an incubator at 39 °C overnight after sealing with butyl rubber stoppers and aluminum caps. At the same time, 4 bottles containing incubation medium without any substrate and TCMC were incubated as the blanks to correct the GP resulting from the activity of the rumen fluid. Furthermore, the *in vitro* GP test with the same treatment sequence was performed twice to provide replication, and each treatment of 2 separate runs had 4 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/d of a mixed diet inwhich ingredients and chemical compositions were identical with the above-mentioned substrate, and had free access to water. In

the morning of the second day, mixed rumen contents were obtained before the morning feeding from the 3 wethers in equal proportion, and transported to the laboratory quickly then filtered through 4 layers of cheesecloth into a flask under CO_2 in the water bath at 39 °C until used. Ten milliliter of filtered rumen fluid was injected through the stopper using a syringe into the incubation bottles. 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), the gas pressure was recorded at 12 and 24 h of incubation using a pressure transducer to calculate total GP (mL/g DM incubated) and then 20 μL of gas was drawn out by a needle through the stopper to determine methane concentration by a gas chromatography (GC) to estimate methane production (mmol/g DM incubated). After termination of incubation at 24 h, the incubation fluids were sampled. A portion of samples was stored at −20 °C for later analysis of end-products, and another was stored at −80 °C immediately for the later analysis of microbe communities by real-time polymerase chain reaction (PCR).

2.3. Chemical analytical procedures

Gross energy was determined by an isoperibol bomb calorimeter (Model number 1281, Parr Instrument Co., Moline, IL) with benzoic acid used as a standard. Dry matter was determined by loss of weight after drying a 2-g aliquot of each sample for 24 h at 105 °C, and OM was calculated as weight loss upon ignition at 600 °C for 18 h in a muffle furnace (AOAC, 2005). Crude protein was measured by multiplying nitrogen (N) obtained from a Leco model FP-2000 N analyser (Leco Corp., St. Joseph, MI) according to the Dumas Combustion Method using ethylenediamine tetra-acetate (EDTA) as a standard with a factor of 6.25 (AOAC, 2005). Ether extract was quantified using diethyl ether as an extraction fluid in a Soxhlet apparatus (AOAC, 2005). Amylase-treated ash-free neutral detergent fiber and ADFom were analyzed with a fiber analyzer (FIWE6, VELP, Italy) using reagents described by van Soest et al. (1991). Sodium sulfite and heat-stable α-amylase were used in the αNDFom determination. Calcium 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 h of incubation were 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 μm in size (Hu et al., 2005). Fermentation parameters of the incubation fluids at the end of 24 h, such as ammonia N and VFA, were determined using methods described by Hu et al. (2005). The pH value was measured 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). The VFA concentration was analyzed by GC (GC-2010, Shimadzu, Kyoto, Japan).

2.4. Analysis of rumen microbial population

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 $MgCl_2$, and 1.25 U Taq in a total 20-μL volume. The real-time PCR assays for microorganisms were completed as follows: one cycle at 95 °C for 10 s for initial denaturation, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing at 60 °C for 34 s. 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 primers of total bacteria, total fungi, *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* were cited from Denman and McSweeney (2006), the primers of methanogens and protozoa were cited from Denman et al. (2007), and the primer *Ruminococcus albus* of was cited from Koike and Kobayashi (2001).

2.5. Data calculation and statistical analysis

According to Demeyer (1991), during 24 h of incubation, hydrogen (H) produced (mmol/g DM incubated) was estimated as (2A + P + 4B), and H consumed (mmol/g DM incubated) was estimated as (4M + 2P + 2B), thus H recovery (%) was calculated as (H consumed/H produced) × 100, where acetate (A), propionate (P), butyrate (B) and methane (M) were expressed as net molar production (mmol). Moreover, H consumed via CH_4/VFA was evaluated as 4M/(2P + 2B).

The reduction of methane production by TCMC was usually followed by a reduction of VFA production. The reductions of methane and VFA were expressed as a proportion of total production in controls, and were calculated as (1 - CH_4 or VFA production in treatment incubations/CH_4 or VFA production in control). Thereby, the relative methane reduction potential (RMRP) of a treatment versus control was estimated as a ratio of reduction in methane production to reduction in VFA production, which was used for the selection of optimal combination, as described by Lin et al. (2012).

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: relative quantification = $2^{-(ct\ \text{target} - ct\ \text{total bacteria})}$, where *ct* represents threshold cycle.

Data were analyzed with an one-way ANOVA analysis of variance using the PROC GLM procedure of SAS (SAS Institute, 2005). Multiple comparisons of means among treatments were conducted by the Duncan's multiple range tests. Degree of significance was defined as follows: $P > 0.05$, not significant and $P \leq 0.05$, significant.

3. Results

3.1. Effects of the TCMC on in vitro rumen fermentation parameters

No differences ($P > 0.05$) were observed in pH value, ammonia N concentration, and butyrate molar proportion among different

Table 1
Effect of traditional Chinese medicine compounds (TCMC) on 24 h of rumen fermentation parameters *in vitro*.

Item	Control	TCMC1	TCMC2	SEM	*P*-value
pH	6.71	6.69	6.70	0.000	0.056
Ammonia nitrogen, mg/dL	19.96	19.70	20.19	0.272	0.055
Total VFA, mmol/L	5.56[a]	4.39[c]	4.73[b]	0.113	0.000
VFA, mol/100 mol					
Acetate	81.06	79.64	78.89	0.305	0.007
Propionate	15.21	17.35[a]	17.29[a]	0.242	0.000
Butyrate	3.72	2.99	3.80	0.168	0.116
Acetate to propionate ratio	5.32	4.58[b]	4.57[b]	0.082	0.000

SEM = standard error of the mean; VFA = volatile fatty acids.
TCMC1 is comprised of Cablin patchouli herb (CPH), Atractylodes rhizome (AR), Amur Cork-tree (AC) and Cypsum with the weight ratio of 1:1:1:0.5.
TCMC2 is comprised of CPH, AR, AC and Cypsum with the weight ratio of 1:1:1:1.
[a, b, c] Within a raw, means with different superscripts differ at $P \leq 0.05$.

treatments (Table 1). The addition of TCMC1 and TCMC2 decreased ($P \leq 0.05$) total VFA concentration, acetate molar proportion, and acetate to propionate ratio, but increased ($P \leq 0.05$) propionate molar proportion. In addition, total VFA concentration of TCMC2 treatment was higher ($P \leq 0.05$) than that of TCMC1 treatment.

3.2. *Effects of the TCMC on* in vitro *GP, methane production and H balance*

The addition of TCMC1 and TCMC2 reduced ($P \leq 0.05$) GP and methane production at 12 and 24 h of incubation, and H produced and consumed, but unaffected ($P > 0.05$) H recovery (Table 2). Hydrogen produced of TCMC2 treatment was higher ($P \leq 0.05$) than that of TCMC1 treatment. With the addition of TCMC2, H consumed via CH_4 to via VFA was lowered ($P \leq 0.05$). In addition, the RMRP of TCMC2 was higher ($P = 0.002$) than that of TCMC1 (Fig. 1).

3.3. *Effects of the TCMC on* in vitro *rumen microbe population*

Both TCMC1 and TCMC2 did not mediate ($P > 0.05$) the evolution of protozoa, *R. albus*, and *F. succinogenes*, but inhibited ($P \leq 0.05$) the growth of methanogens and total fungi (Table 3). Traditional Chinese medicine compound 2 treatment had less quantity of methanogens, relative to total bacterial 16S rDNA, than TCMC1 treatment. The proliferation of *R. flavefaciens* was suppressed ($P \leq 0.05$) with the addition of TCMC1, but was unaffected ($P > 0.05$) with the addition of TCMC2.

4. Discussion

In the present study, the 2 TCMC were composed of CPH, AR, AC and Cypsum. Cablin Patchouli Herb, 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 an increasing interest in the use of plants containing phytochemicals and their extracts to manipulate the function of gastrointestinal tract in both ruminant and non-ruminant livestock (Greathead, 2003; Wang and Wang, 2016). Generally, these bioactive substances can be classified into terpenes or isoprenoids, phenols and alkaloids, and 4 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, the 3 raw plant medicinal drugs used in this study contain a certain

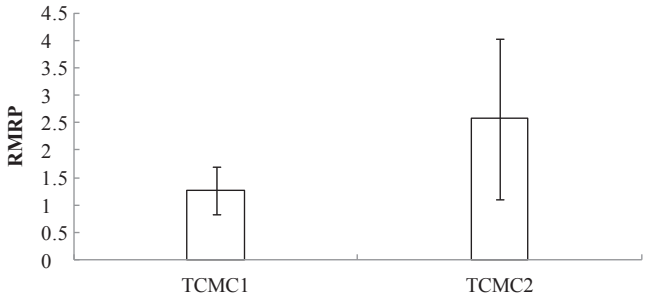

Fig. 1. The relative methane reduction potential (RMRP) by traditional Chinese medicine compounds (TCMC). RMRP is expressing as a ratio of reduced methane production relative to reduced total volatile fatty acids (VFA) production because of the addition of TCMC1 and TCMC2. TCMC1 is comprised of Cablin patchouli herb (CPH), Atractylodes rhizome (AR), Amur Cork-tree (AC) and Cypsum with the weight ratio of 1:1:1:0.5. TCMC2 is comprised of CPH, AR, AC and Cypsum with the weight ratio of 1:1:1:1. Bar is the standard error of the mean.

quantity of one or more classes of bioactive compounds listed above (Wang and Wang, 2016). It has been confirmed that CPH contains essential oils, tannins and amaroids, AR contains various alkaloids like berberine, N free crystalline substances, erapressed oils, mucoid substances, steroids and closely related sterols, as well as AC contains essential oils, carotene or carotenoid and hiamine (Wang and Wang, 2016). Although the definitive categories and actual concentrations of these secondary compounds contained with the 3 raw plant materials above were not determined in the present study, the concentration of each kind of secondary compound might be relatively low. Therefore, a considerable amount of each TCMC (25 mg TCMC per g of total DM incubated) was added into the substrate. Moreover, because more than two-thirds of the 2 TCMC might be composed of OM, which can be degraded and fermented to some extent, an extra substrate (18.75 mg) was added into the control cultures so that the amount of total DM incubated was the same in all cases.

It has been well known that the mitigation of enteric methane in ruminants has significant economical and environmental benefits, which attracts the scientific community to explore various ways to manipulate rumen microbial population to change fermentation pattern. Whilst numerous chemical additives and antibiotics have been studied and utilized for this purpose, the use of 'natural products' to modify rumen fermentation is attracting the closest attention with a concept of 'clean, green and ethical' animal production being promoted (Durmic and Blache, 2012). Based on this background, bioactive plant metabolites become an important contemporary research field for the replacement of chemical feed additives because some of these metabolites show potential to alter rumen fermentation and

Table 2
Effect of traditional Chinese medicine compounds (TCMC) on gas and methane production and hydrogen balance *in vitro*.

Item	Control	TCMC1	TCMC2	SEM	*P*-value
Gas production, mL/g					
12 h	148.48^{a}	112.14^{b}	117.38^{b}	3.565	0.000
24 h	251.11^{a}	203.13^{b}	206.15^{b}	5.152	0.000
Methane production, mmol/g					
12 h	0.84^{a}	0.46^{b}	0.52^{b}	0.033	0.000
24 h	1.79^{a}	1.30^{b}	1.28^{b}	0.056	0.000
Hydrogen balance					
Produced, mmol/g	10.70^{a}	8.28^{b}	9.01^{c}	0.242	0.000
Consumed, mmol/g	9.29^{a}	6.99^{b}	7.15^{b}	0.271	0.000
Recovery, %	86.73	84.22	79.77	1.816	0.303
Consumed via methane to via VFA	3.40^{a}	2.90^{ab}	2.63^{b}	0.121	0.022

SEM = standard error of the mean; VFA = volatile fatty acids.
TCMC1 is comprised of Cablin patchouli herb (CPH), Atractylodes rhizome (AR), Amur Cork-tree (AC) and Cypsum with the weight ratio of 1:1:1:0.5.
TCMC2 is comprised of CPH, AR, AC and Cypsum with the weight ratio of 1:1:1:1.
$^{a, b, c}$ Within a raw, means with different superscripts differ at $P \leq 0.05$.

Table 3
Effect of traditional Chinese medicine compounds (TCMC) on 24 h of rumen microbial populations (% of total bacterial 16S rDNA, $\times 10^{-2}$).

Items	Control	TCMC1	TCMC2	SEM	*P*-value
Methanogens	191.52^{a}	80.98^{b}	53.19^{c}	10.101	0.000
Protozoa	204.58	202.89	193.54	2.234	0.091
Total fungi	44.57^{a}	24.31^{b}	24.99^{b}	1.626	0.000
Ruminococcus albus	309.08	297.91	293.79	3.208	0.131
Fibrobacter succinogenes	20.66	20.65	20.67	0.576	1.000
Ruminococcus flavefaciens	379.31^{a}	192.23^{b}	384.21^{a}	15.512	0.000

SEM = standard error of the mean.
TCMC1 is comprised of Cablin patchouli herb (CPH), Atractylodes rhizome (AR), Amur Cork-tree (AC) and Cypsum with the weight ratio of 1:1:1:0.5.
TCMC2 is comprised of CPH, AR, AC and Cypsum with the weight ratio of 1:1:1:1.
$^{a, b, c}$ Within a raw, means with different superscripts differ at $P \leq 0.05$.

decrease methane production (Patra, 2012). The anti-methanogenic activity of saponins, tannins and essential oils extracted from a diverse array of plant materials has been extensively demonstrated in many *in vitro* and *in vivo* studies with variable efficacy depending on their chemical nature and ruminal concentration though their modes of action are so tremendously different and have not been completely elucidated (Benchaar and Greathead, 2011; Goel and Makkar, 2012). In fact, there is still a long way to go before the phytochemical fractions involved in reduction of methane production are extracted and isolated, or artificially synthesized (once their structure are identified) and then used as feed additives. However, directly feeding the plants containing these bioactive compounds could be an alternative approach to motivate similar changes in rumen fermentation, just as the results demonstrated by the 2 TCMC in the present study. Furthermore, Wang et al. (2010) suggested that the 2 TCMC could give rise to more efficient animal production.

Gas production during incubation *in vitro* was well correlated with OM digestibility of fermented feedstuffs (Menke et al., 1979). Higher GP meant more violent fermentation in the rumen for feedstuffs. The VFA were usually regarded as one of rumen fermentation indexes, and typified rumen fermentation pattern and nutrient digestion efficiency (Pitt et al., 1996). In the present study, the addition of TCMC1 and TCMC2 decreased GP at 12 and 24 h of incubation, indicating that the 2 TCMC could inhibit *in vitro* fermentation of substrate, then decrease degradability of OM of substrate, finally induce a reduction in total VFA production. The inference above could be verified by the detection results of total VFA concentration in the incubation fluids, which further implied that H produced might be declined by the 2 TCMC because of the decrease of total fermentable OM (Jordan et al., 2006). The VFA profile in the rumen was mainly affected by the compositions of diet fed to ruminants (Pitt et al., 1996). In the present study, acetate molar proportion of every group was relatively high since 50% of substrate was dried rice straw which can be categorized into the roughage with low quality. However, the addition of TCMC1 and TCMC2 decreased acetate molar proportion, but increased propionate molar proportion, which suggested that the 2 TCMC could inhibit acetate fermentation with the promotion of propionate fermentation, consequently induce a decrement in the ratio of acetate to propionate as well as a specific shift in the fermentation pattern without any drastic effects on ruminal pH. Higher propionate yield in the rumen always means better performance for ruminants. Thus, the improvement in the efficiency of ruminal fermentation by the 2 TCMC should be one of reasons in the study of Wang et al. (2010) who declared an amelioration in the finishing performance of beef cattle suffering a moderate heat stress. Moreover, it is noteworthy that the decrease of methane production in response to the addition of the 2 TCMC was accompanied by the change of VFA profile. Actually, it has been well established that direct or indirect reduction of methane production implicates a change in VFA profile. Hydrogen accumulation impedes the pathway for carbon (C) 2 synthesis and prefers C_3 generation (van Nevel and Demeyer, 1996), bringing out a reduction of C_2:C_3 ratio, as it was observed upon the addition of the 2 TCMC. Thus, the abatement of methane emission and the modification of VFA profile in response to the addition of the 2 TCMC seemed to be concomitant, and the depression in total VFA production could be a consequence of an impaired methanogenesis. Methanogens survive by consuming H in the rumen and try to compete with propionate producing microbes that also consume H to form propionate (McAllister and Newbold, 2008). As a result, the 2 TCMC could lead to a lower availability of H for methanogens following by a mitigation of methane emission *in vitro* by channeling H_2 utilized for methanogenesis to synthesis of propionate.

Except that ruminal VFA patterns were shifted from acetic to propionic acid by the addition of the 2 TCMC, which indirectly reduced methane release via the interference of H uptake by methanogenic bacteria, a lower methanogen population relative to total bacterial 16S rDNA of TCMC1 and TCMC2 treatments compared with that of the control showed that every TCMC might directly depress rumen methane production by the inhibition on methanogens. In fact, the elimination of ruminal methanogenic microorganisms by the 2 TCMC was an intrinsic and leading cause for the reduction of methane production at 12 and 24 h of incubation of TCMC1 and TCMC2 treatments in comparison with that of the control. Meanwhile, since it might be deduced from the results of VFA and H balance in the incubation fluids that there was no fundamental difference in the capability of reducing methane emissions by limiting H supply for rumen methanogenesis between TCMC1 and TCMC2, the inherent reason for the higher RMRP of TCMC2 than that of TCMC1 was the stronger capability of anti-methanogen of TCMC2 than that of TCMC1. It has long been considered that defaunation of the rumen represents a methane mitigation possibility because protozoa are H_2 producers and shelter a substantial population of associated methanogens (Kamra et al., 2006). However, removal of protozoa from the rumen of farm ruminants is controversial because it may mean a risk to animal health, and methane production is not always reduced with the absence of protozoa from the rumen microbiota (Morgavi et al., 2012). In the present study, protozoa population, relative to total bacterial 16S rDNA, was not influenced by the addition of the 2 TCMC, suggesting that the TCMC were not harmful to ruminal microecology and animal health. Fungi and cellulolytic bacteria play important roles in keeping a stable intra-ruminal environment for structural fibre digestion. Feed intake or digestibility is commonly decreased by the inhibition of their activities (Patra and Saxena, 2009). In the present study, the TCMC seemed to have anti-fungi property, suggesting that the TCMC might have a negative effect on ruminal fibre degradation. However, fibrolytic microbes responding to the TCMC were as various as fungi, and only TCMC1 could inhibit the growth of *R. flavefaciens*, showing that more works would be needed to clarify the relationship between the 2 TCMC and ruminal fiber fermentability *in vivo*.

5. Conclusions

Addition of the 2 TCMC abated methane release along with a shift of fermentation pattern towards a reduced acetate to propionate ratio, while TCMC2 had a greater advantage over TCMC1. Methanogens and total fungi populations, relative to total bacterial 16S rDNA, were decreased by the 2 TCMC, with the proliferation of *R. flavefaciens* being depressed only by TCMC1. Further research is required to elucidate the actions on fibrolytic microorganisms surviving in the rumen, to evaluate the persistence of *in vivo* antimethanogenic effects, to specify the chemical nature of active compounds being responsible for such effects, and to testify the usefulness and applicability under diverse practical conditions.

Conflicts of interest

None.

Acknowledgments

This work was financially supported partly by Fundamental Research Funds for the Central Universities (XDJK2014C154).

References

AOAC. Official methods of analysis. 18th rev. ed. Gaithersburg, MD, USA: Association of Official Analytic Chemists; 2005.

Benchaar C, Greathead H. Essential oils and opportunities to mitigate enteric methane emissions from ruminants. Anim Feed Sci Technol 2011;166–167:338–55.

Bodas R, Prieto N, García-González R, Andrés S, Giráldez FJ, López S. Manipulation of rumen fermentation and methane production with plant secondary metabolites. Anim Feed Sci Technol 2012;176:78–93.

Demeyer DI. Quantitative aspects of microbial metabolism in the rumen and hindgut. Rumen microbial metabolism and ruminant digestion. INRA editions. Paris, France: Jouany JP; 1991.

Denman SE, McSweeney CS. Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. FEMS Microbiol Ecol 2006;58:572–82.

Denman SE, Tomkins NW, McSweeney CS. Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. FEMS Microbiol Ecol 2007;62:313–22.

Durmic Z, Blache D. Bioactive plants and plant products: effects on animal function, health and welfare. Anim Feed Sci Technol 2012;176:150–62.

Gerber PJ, Hristov AN, Henderson B, Makkar H, Oh J, Lee C, et al. Technical options for the mitigation of direct methane and nitrous oxide emissions from livestock: a review. Animal 2013;7:220–34.

Goel G, Makkar HPS. Methane mitigation from ruminants using tannins and saponins. Trop Anim Health Prod 2012;4:729–39.

Greathead H. Plants and plant extracts for improving animal productivity. Proc Nutr Soc 2003;62:279–90.

Hart KJ, Yáñez-Ruiz DR, Duval SM, McEwan NR, Newbold CJ. Plant extracts to manipulate rumen fermentation. Anim Feed Sci Technol 2008;147:8–35.

Hu WL, Liu JX, Ye JA, Wu YM, Guo YQ. Effect of tea saponin on rumen fermentation *in vitro*. Anim Feed Sci Technol 2005;120:333–9.

Jordan E, Kenny D, Hawkins M, Malone R, Lovett DK, O'Mara FP. Effect of refined soy oil or whole soybeans on intake, methane output, and performance of young bulls. J Anim Sci 2006;84:2418–25.

Kamra DN, Agarwal N, Chaudhary LC. Inhibition of ruminal methanogenesis by tropical plants containing secondary compounds. Int Congr Ser 2006;1293:156–63.

Koike S, Kobayashi Y. Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefacies*. FEMS Microbiol Lett 2001;204:361–6.

Kumar S, Choudhury PK, Carro MD, Griffith GW, Dagar SS, Puniya M, et al. New aspects and strategies for methane mitigation from ruminants. Appl Microbiol Biotechnol 2014;98:31–44.

Lin B, Lu Y, Wang JH, Liang Q, Liu JX. The effects of combined essential oils along with fumarate on rumen fermentation and methane production *in vitro*. J Anim Feed Sci 2012;21:198–210.

Liu HW, Tong JM, Zhou DW. Utilization of Chinese herbal feed additives in animal production. Agric Sci China 2011;10:1262–72.

Lu CD. Nutritionally related strategies for organic goat production. Small Rumin Res 2011;98:73–82.

Mauricio RM, Mould FL, Dhanoa MS, Owen E, Channa KS, Theodorou MK. A semi-automated *in vitro* gas production technique for ruminant feedstuff evaluation. Anim Feed Sci Technol 1999;79:321–30.

McAllister TA, Newbold CJ. Redirecting rumen fermentation to reduce methanogenesis. Aust J Exp Agric 2008;48:7–13.

Menke KH, Raab L, Salewski A, Steingass H, Fritz D, Schneider W. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor *in vitro*. J Agric Sci 1979;93:217–22.

Menke KH, Steingass H. Estimation of the energetic feed value obtained from chemical analysis and gas production using rumen fluid. Anim Res Dev 1988;28:7–55.

Morgavi D, Martin C, Jouany JP, Ranilla MJ. Rumen protozoa and methanogenesis: not a simple cause-effect relationship. Br J Nutr 2012;107:388–97.

Patra AK, Saxena J. Dietary phytochemicals as rumen modifiers: a review of the effects on microbial populations. Antonie Leeuwenhoek 2009;96:363–75.

Patra AK. Enteric methane mitigation technologies for ruminant livestock: a synthesis of current research and future directions. Environ Monit Assess 2012;184:1929–52.

Pitt RE, van Kessel JS, Fox DG, Pell AN, Barry MC, van Soest PJ. Prediction of ruminal volatile fatty acids and pH within the net carbohydrate and protein system. J Anim Sci 1996;74:226–44.

SAS. Online doc version 9.1.3. SAS Inst. Inc. Cary, NC, USA: SAS Institute; 2005.

Theodorou MK, Williams BA, Dhanoa MS, McAllan AB, France J. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feed. Anim Feed Sci Technol 1994;48:185–97.

van Nevel CJ, Demeyer DI. Control of rumen methanogenesis. Environ Monit Assess 1996;42:73–97.

van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci 1991;74:3583–97.

Wang SP, Wang WJ, Wu M, Zuo FY, Zhou P, Zhang JH, et al. Effect of Chinese medicine prescription on goat: IV. The internal environment parameters of rumen. Chin J Anim Sci 2012b;23:64–7.

Wang SP, Wang WJ, Wu M, Zuo FY, Zhou P, Zhang JH. Effect of Chinese medicine prescription on goat: III. The activity of ruminal celluolytic enzyme and the serum enzyme. Chin J Anim Sci 2012a;21:56–60.

Wang SP, Wang WJ, Zuo FY, Zhou P, Zhang JH. Effect of Chinese medicine prescription on goat: VI. The ruminal degradation characteristics of nutrients from the diet. Chin J Anim Sci 2013;5:47–52.

Wang SP, Wang WJ, Zuo FY, Zhou P, Zhao JJ, Zhang JH. Effect of Chinese medicine prescription on beef cattle in summer: II. Blood gas analysis, concentrations of metabolites and parameters of immune and antioxidant capability in serum. Acta Vet Zootech Sin 2011;42:734–41.

Wang SP, Wang WJ. Effects of dietary supplementation of Chinese herb medicine mixture on rumen fermentation, nutrient digestion and blood profile in goats. S Afr J Anim Sci 2016;46:247–60.

Wang WJ, Wang SP, Zuo FY, Zhou P, Zhang JH. Effect of Chinese Medicine prescription on goat: I. The apparent digestibility of nutrients and the blood routine parameters. Chin J Anim Sci 2012;5:59–62.

Wang WJ, Wang SP, Zuo FY, Zhou P, Zhao JJ, Zhang JH. Effect of Chinese medicine prescription on beef cattle in summer: I. Finishing performance, physiological parameters, serum hormone level and enzymatic activity. Acta Vet Zootech Sin 2010;41:1260–7.

Zhang CM, Guo YQ, Yuan ZP, Wu YM, Wang JK, Liu JX, et al. Effect of octadeca carbon fatty acids on microbial fermentation, methanogenesis and microbial flora *in vitro*. Anim Feed Sci Technol 2008;146:259–69.

Zoetendal EG, Akkermans ADL, De Vos WM. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl Environ Microbiol 1998;64:3854–9.