Livestock Science 127 (2010) 38–44

Contents lists available at ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci

Effect of coconut oil and garlic powder on *in vitro* fermentation using gas production technique

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ARTICLE INFO

Article history:
Received 13 February 2009
Received in revised form 4 August 2009
Accepted 18 August 2009

Keywords:
Coconut oil
Garlic powder
In vitro fermentation
Real-time PCR

ABSTRACT

An *in vitro* gas technique trial was conducted to investigate the effect of coconut oil (Co), garlic powder (G) and their mixtures on *in vitro* fermentation. Incubation was carried out using rumen fluid obtained from swamp buffaloes. The experimental design was a completely randomized design (CRD). The dietary treatments were ratio of Co and G supplementation at 0:0, 16:0, 8:4, 4:8 and 0:16 mg with rice straw as a roughage source. Cumulative gas production was recorded at 0, 2, 4, 6, 8, 12, 18, 24, 36, 48, 60 and 72 h of incubation. *In vitro* true digestibility (IVTD) was determined after 48 h incubation. Cumulative gas production at 72 h was significantly lowest ($P<0.05$) at Co:G, 16:0 mg. Garlic powder supplementation at 16 mg decreased ($P<0.05$) NH_3–N concentration and increased ($P<0.05$) *in vitro* true digestibility (IVTD) while supplemented coconut oil at 16 mg decreased ($P<0.05$) IVTD. Total volatile fatty acids (VFAs) were lowest ($P<0.05$) by garlic powder supplementation at 16 mg. However, supplementation of Co:G, 8:4, 4:8 and 0:16 mg tended to increase the proportion of propionate, decrease C2:C3 ratio and reduce ($P<0.05$) methane (CH_4) production. Protozoal population was significantly lowest ($P<0.05$) at Co:G, 8:4 mg. Moreover, application of quantitative PCR to quantify predominant cellulolytic bacteria (16S rRNA) and fungi (18S rRNA) targets revealed that treatments did not have an effect on *Ruminococcus flavefaciens* and total fungi population. However, it was found that supplementation of Co:G at 8:4 mg increased *Ruminococcus albus* population ($P<0.05$). Based on this study, it suggests that supplementation of Co:G at 8:4 and 0:16 mg could improve ruminal fluid fermentation in terms of volatile fatty acid profile, reduced methane losses and reduced protozoal population.

1. Introduction

Modification of ruminal fermentation using feed additives, such as antibiotics, has proved to be a useful strategy to improve production efficiency in dairy cattle. The use of antibiotics as feed additives has proved to be a useful tool to reduce energy and nitrogen losses from the diet (McGuffey et al., 2001). However, the use of antibiotics as feed additives in dairy cows has been of increasing concern due to the potential appearance of residues in milk. Furthermore, the use of antibiotics as a feed additive has been banned in the European Union (Russell and Houlihan, 2003). For this reason, scientists are interested in evaluating the potential use of natural antimicrobials such as herbs and plant extracts. Currently, the use of plant herbs has resulted in improving rumen ecology (Kamra, 2005; Wanapat et al., 2008a).

Garlic (*Allium sativum*) is an herb or spice plant that has been used by humans as a source of antimicrobial agents for the gastrointestinal. It has a complex mixture of many secondary plant products including allicin ($C_6H_{10}S_2O$), diallyl sulfide ($C_6H_{10}S$), dialyl disulfide ($C_6H_{10}S_2$) and allyl mercaptan (C_3H_6S) among others (Lawson, 1996). These compounds could manipulate rumen fermentation such as decreased in the proportion of acetate and increased in proportion of propionate and butyrate, inhibition of methanogenesis and decreased in the CH_4:VFA ratio (Busquet et al., 2005b).

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1871-1413/$ – see front matter
doi:10.1016/j.livsci.2009.08.008

Moreover, Soliva et al. (2003) and Machmüller et al. (2003), reported the effect of medium chain fatty acid and coconut oil on inhibition of methanogenesis, and significantly reduced daily methane release without negatively affecting the total tract nutrient digestion. Garlic powder and coconut oil supplementation could improve rumen fermentation and end products but coconut oil supplementation could affect on nutrient digestion whereas garlic powder had no affect (Wanapat et al., 2008b). Moreover, using conventional methods such as roll-tube technique (Hungate, 1969) can only determine the microbial diversity grossly due to some microbes that cannot be cultivated with the current technique (Deng et al., 2007). For this reason, there had been increasing interest in the accurate and rapid enumeration of microbial populations especially using modern molecular techniques. Therefore, the objective of this *in vitro* study was to study the effect of coconut oil, garlic powder and their mixtures on improving ruminal fermentation in swamp buffalo rumen fluid using *in vitro* gas and real-time PCR techniques.

2. Materials and methods

2.1. Experimental design and fermentation technique

This study was conducted using an *in vitro* gas technique at various incubation time intervals. The experimental design was a complete randomized design (CRD) with three replications per treatment. The treatments were ratio of coconut oil (Co) and garlic powder (G), (Co:G) 0:0, 16:0, 8:4, 4:8 and 0:16 mg supplemented in the diets. Garlic powder was prepared by grinding fresh garlic (*A. sativa*) then oven-dried at 60 °C while refined coconut oil was used in this experiment. 60:40 roughage (R) and concentrate (C) ratio were used as substrates. Substrates and dried garlic were milled to a 1-mm screen. Substrates were prepared and weighed to 200 mg of DM into 50 ml bottles then supplemented with coconut oil and garlic powder according to the above ratio for various time incubations. Feed and chemical compositions are shown in Table 1. Rice straw was used as a roughage source.

Two, male 5-year-old, rumen fistulated, Thai native swamp buffaloes with an initial BW of 415 ± 15 kg were used as rumen fluid donor. Buffalo rumen fluid was collected from buffalo fed with roughage: concentrate at 60:40 (14.0% CP and 79.8% TDN, dry matter basis). The 1000 ml rumen liquor was obtained from each of the buffalo before the morning feeding. The rumen fluid was filtered through four layers of cheesecloth into pre-warmed thermos flasks. Preparation of artificial saliva was done according to Menke and Steingass (1988). Artificial saliva was prepared and rumen fluid was mixed in a 2:1 ratio to prepare fermentation solution. The serum bottles with the mixture of substrate treatments were pre-warmed in a water bath at 39 °C for 1 h before filling with 30 ml of rumen inoculum's mixture. During the incubation, the gas production was recorded at 0, 2, 4, 6, 8, 10, 12, 18, 24, 36, 48, 60 and 72 h. Cumulative gas production data were fitted to the model of Orskov and McDonal (1979) as follows:

$$y = a + b(1-e^{(-ct)})$$

where a = the gas production from the immediately soluble fraction, b = the gas production from the insoluble fraction, c = the gas production rate constant for the insoluble fraction (b), t = incubation time, $(a+b)$ = the potential extent of gas production and y = gas produced at time "t".

Table 1
Ingredients and chemical composition (g/kg DM) of concentrate, rice straw and garlic powder used in the experiment.

Item	Concentrate	Rice straw	Garlic powder
Ingredient (kg of DM)			
Cassava chip	76.0		
Rice bran	7.3		
Palm kernel meal	10.0		
Urea	3.2		
Molasses	2.0		
Mineral premix	0.5		
Salt	0.5		
Sulfur	0.5		
Chemical composition			
DM, %	89.7	90.1	93.0
		% of DM	
OM, %	90.0	82.5	97.0
CP, %	13.8	2.8	19.2
NDF, %	12.0	77.5	6.5
ADF, %	6.6	61.3	5.1
Ether extract, %	2.8	1.2	0.5
TDN [a]	80.0	–	–

[a] Calculated value.

2.2. Determination of fermentation parameters

Inoculum's ruminal fluid was collected at 0, 4, 8, 12 and 24 h post inoculations. Rumen fluid samples were then filtered through four layers of cheesecloth. Samples were divided into 3 portions; the first portion was used for NH_3–N analysis. The sample was centrifuged at 16,000 ×g for 15 min, and the supernatant was stored at −20 °C before NH_3–N analysis using the micro-Kjeldahl methods (AOAC, 1990) and VFA analysis using HPLC (Samuel et al., 1997). The second portion was fixed with 10% formalin solution in sterilized 0.9% saline solution. The total direct count of protozoa was made by the methods of Galyen (1989) based on the use of a hemocytometer (Boeco, Hamburg, Germany). The final portion was stored at −20 °C for DNA extraction (Yu and Morrison, 2004). At 48 h post inoculation a set of sample was determined *in vitro* true digestibility (IVTD) according to Van Soest and Robertson (1985). In brief, the content of the bottle was transferred quantitatively to a spoutless beaker by repeated washing with 100 ml neutral detergent solution. The content was refluxed for 1 h and filtered through pre-weighed Gooch crucibles. The DM of the residue was weighed and IVTD of feed was calculated as follows:

$$\text{True digestibility (TD)} = \frac{(\text{DM of feed taken for incubation} - \text{NDF residue}) \times 100}{\text{DM of feed taken for incubation}}$$

2.3. Quantitative analysis of microbial populations

Community DNA was extracted from 1.0 ml aliquots of each sample by the RBB+C method (Yu and Morrison, 2004), which was shown to substantially increase DNA yields. The quality and quantity of these DNA samples were also determined by agarose gel electrophoresis and spectrophotometry. In total, 40 samples belonging to five treatments, four incubation times (0, 4, 8 and 12) and 2 replicates were extracted for genomic DNA.

The primers used for the real-time PCR are as follows: primers for *Fibrobactor succinogenes*, Fs219f (5′GGT ATG GGA TGA GCT TGC-3′) and Fs654r (5′-GCC TGC CCC TGA ACT ATC-3′), were selected to allow amplification (446-bp product) of all 10 *F. succinogenes* strains deposited in GenBank. For *Ruminococcus albus* primers, Ra1281f (5′-CCC TAA AAG CAG TCT TAG TTC G-3′) and Ra1439r (5′ CCT CCT TGC GGT TAG AAC A-3′) (175-bp product). *Ruminococcus flavefaciens* primers, Rf154f (5′-TCT GGA AAC GGA TGG TA-3′) and Rf425r (5′-CCT TTA AGA CAG GAG TTT ACA A-3′), were also selected to allow species–species amplification (295 bp) of all seven *R. flavefaciens* strains deposited in GenBank. All these primer sets were previously published by Koike and Kobayashi (2001). Regular PCR conditions for *F. succinogenes* were as follows: 30 s at 94 °C for denaturing, 30 s at 60 °C for annealing and 30 s at 72 °C for extension (48 cycles), except for 9 min denaturation in the first cycle and 10 min extension in the last cycle. Amplification of 16S rRNA for the other two species was carried out similarly except an annealing temperature of 55 °C was used. Quantification of anaerobic fungal population, primer and condition, was previously published by Denman and Mcsweeney (2006).

Four sample-derived standards were prepared from treatment pool set of community DNA. The regular PCR was used to generate sample-derived DNA standards for each real-time PCR assay. Then the PCR product was purified using a QIA quick PCR purification kit (QIAGEN, Inc., Valencia, CA) and quantified using a spectrophotometer. For each sample-derived standard, copy number concentration was calculated based on the length of the PCR product and the mass concentration. Tenfold serial dilution was made in Tri-EDTA prior to real-time PCR (Yu et al., 2005). In total, 4 real-time PCR standards were prepared. The conditions of the real-time PCR assays of target genes were the same as those of the regular PCR described above. Biotools QuantiMix EASY SYG KIT (B&M Labs, S. A., Spain) was used for real-time PCR amplification. All PCRs were performed in duplicate.

2.4. Statistical analysis

Data were analyzed by using the General Linear Models (GLM) procedures (SAS Inst. Inc., Cary, NC). Data were analyzed using the model $Y_{ij} = \mu + T_i + \varepsilon_{ij}$ where Y_{ij}, observation from treatment *i*, *j*, the replication; μ, the overall mean, T_i, the mean of treatment and ε_{ij}, the residual effect. Multiple comparisons among treatment means were performed by Duncan's New Multiple Range Test (DMRT) (Steel and Torrie, 1980). Comparison between control and supplements was tested by orthogonal contrast and trend of ratios responded was performed by orthogonal polynomials.

3. Results and discussions

3.1. Gas production, kinetic analysis of gas production

Cumulative gas production for each of the substrate treatments was presented as gas production curves (Fig. 1) and values for the estimated parameters obtained from the kinetics of gas production models for substrates studied are given in Table 2. The intercept value (*a*) for the different treatments representing gas production from soluble fractions ranged from −0.7 to 0.9 and was not significantly different among treatments ($P > 0.05$). Whereas, gas production from the insoluble fraction (*b*), potential extent of gas production ($a + b$) and gas production rate constants for the insoluble fraction (*c*) were significantly different among treatments ($P < 0.05$). Cumulative gas production at 72 h was significantly different among treatments ($P < 0.05$) and was highly significant ($P < 0.01$) when compared between control and supplemented groups. It was found that cumulative gas was linearly decreasing when the level of coconut oil supplementation was increased.

3.2. Ammonia nitrogen (NH_3–N) and in vitro true digestibility (IVTD)

Ammonia nitrogen concentrations were linearly decreasing ($P = 0.019$) when increasing supplementation with garlic powder particularly at 0:16, (Co:G) group. As shown in Table 2 and Fig. 1, supplementation of 16 mg of garlic powder could decrease NH_3–N concentration during 0–8 h of incubation time. It ranged from 23.5 to 24.1 mg/dl, which were different ($P < 0.05$) from others which ranged from 23.9 to 29.2 (0:0), 24.7 to 28.2 (16:0), 21.6 to 27.6 (8:4) and 25.3 to 27.7 (4:8), respectively. At the last 24 h of incubation time,

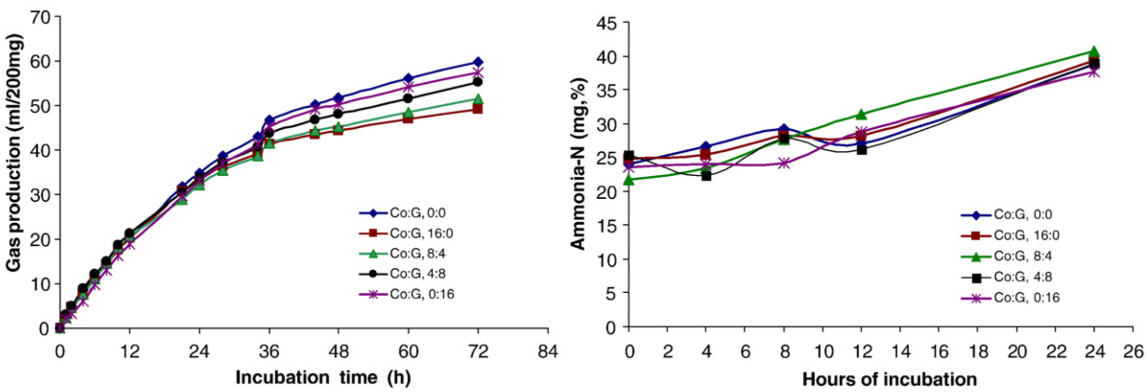

Fig. 1. The effect of level of coconut oil (Co) and garlic powder (G) ratio supplementation on cumulative gas production and ruminal ammonia nitrogen concentration at different times of incubation.

Table 2
Effect of level of coconut oil (Co) and garlic powder (G) ratio supplementation on kinetic of gas production, ruminal ammonia nitrogen, *in vitro* true digestibility, volatile fatty acid production and CH_4 production from *in vitro* fermentation using swamp buffalo rumen fluid.

Items	Co:G ratio, mg					SEM	Contrast [1]			
	0:0	16:0	8:4	4:8	0:16		Control vs Supplements	*L*	*Q*	*C*
Fermentation kinetic values [2]										
a	0.4	0.4	0.4	0.9	−0.7	0.574	0.863	0.264	0.177	0.297
b	67.0^{a}	51.5^{c}	54.7^{bc}	58.6^{b}	66.6^{a}	1.806	0.001	<0.001	0.211	0.679
a+*b*	67.4^{a}	51.4^{c}	55.1^{bc}	59.5^{b}	65.9^{a}	1.699	<0.001	<0.001	0.441	0.868
c	0.030^{a}	0.043^{c}	0.037^{b}	0.034^{ab}	0.030^{a}	0.002	0.016	<0.001	0.648	0.397
Gas (72 h, ml)	59.8^{a}	49.2^{c}	51.6^{bc}	55.3^{ab}	57.4^{a}	1.765	0.009	0.005	0.952	0.710
Ammonia nitrogen (mg/dl)	24.3^{a}	24.3^{a}	24.1^{ab}	23.4^{ab}	23.0^{b}	0.364	0.183	0.019	0.789	0.579
in vitro true digestibility (%)	34.7^{c}	30.5^{d}	36.3^{bc}	38.5^{b}	51.5^{a}	1.036	0.003	<0.001	0.006	0.012
Total VFAs (mM)	182.0^{b}	195.4^{a}	167.6^{cd}	178.5^{bc}	160.9^{d}	3.940	0.176	<0.001	0.226	0.003
VFA (mol/100 mol)										
Acetate (C2)	57.8^{ab}	60.3^{a}	55.1^{bc}	57.0^{b}	54.0^{c}	0.910	0.285	0.002	0.251	0.015
Propionate (C3)	30.4^{bc}	28.8^{c}	32.5^{ab}	30.9^{bc}	33.1^{a}	0.650	0.245	0.003	0.287	0.012
Butyrate (C4)	11.8^{bc}	10.8^{c}	12.4^{ab}	12.0^{ab}	12.9^{a}	0.330	0.455	0.003	0.276	0.051
C2:C3	1.9^{b}	2.1^{a}	1.7^{cd}	1.8^{bc}	1.6^{d}	0.060	0.262	0.001	0.143	0.009
CH_4, mol/100 mol [3]	45.5^{b}	48.4^{a}	36.9^{d}	41.0^{c}	35.5^{d}	0.783	<0.001	<0.001	0.003	<0.001

Values in the same row with different superscripts differ ($P<0.05$).
[1] Linear (*L*), quadratic (*Q*), and cubic (*C*) effects of supplemented treatments.
[2] *a* = gas production from the immediately soluble fraction, *b* = gas production from the insoluble fraction, *c* = gas production rate constant for the insoluble fraction (*b*), (*a* + *b*) = potential extent of gas production.
[3] Calculated according to Moss et al. (2000) CH_4 production = 0.45(acetate) − 0.275(propionate) + 0.4(butyrate).

NH_3–N concentration of all treatments was increased up to 37.6–40.8 mg/dl. These results were similar with Cardozo et al. (2004) who reported that garlic oil in continuous culture reduced ammonia N, suggesting that the deamination was inhibited. This inhibition was explained by Ferme et al. (2004) to be related with *Prevotella* spp., which were mainly responsible for protein degradation and amino acid deamination, suggesting a mechanism of action of garlic oil on protein metabolism. However, it is also possible that the reduction in deamination relates to a reduction in the availability of dehydrogenases. Several studies have been reported that garlic oil has an effect on pure cultures of ruminal bacteria at concentrations of less than 100 mg/kg; *Streptococcus bovis* was the most resistant species, and *Prevotella ruminicola*, *Clostridium ticklandii* and *Peptostreptococcus anaerobius* were the most sensitive species (Busquet et al., 2006). Weimer (1998), suggested that control of these ammonia-hyperproducing species provides dairy producers with the opportunity to reduce both the expensive loss of protein and the discharge of nitrogenous wastes.

Coconut oil and garlic powder supplemented treatment affected on *in vitro* true digestibility (IVTD) when compared with the control group ($P<0.01$). The IVTD was highest when supplemented with 16 mg of garlic powder and lowest in 16 mg of coconut oil supplemented group. As a result, digestibility was relatively decreased with increased amount of coconut oil supplementation, as shown in Table 2. Similarly, Machmüller et al. (2003) found that coconut oil could depress ruminal fiber degradation and linearly decreased digestibility in response to increasing levels of coconut oil (Dong et al., 1997). Harfoot et al. (1974) showed that fatty acids might adsorb either onto rumen microbes or onto feed particles when incubated together and showed that medium chain fatty acids (MCFAs) had interacted with dietary fiber. In contrast with Busquet et al. (2005a) who showed that the supplemented garlic oil and its compounds did not affect on true DM, OM, NDF and ADF digestibilities. Moreover, Yang et al. (2007) observed that supplementation of garlic and berry essential oil did not affect total digestibilities of DM, OM, fiber and starch, while ruminal DM and OM digestibility was increased.

3.3. Volatile fatty acids and methane gas production

Coconut oil and garlic powder supplementation affected on total VFAs and individual VFAs production. The coconut oil supplemented at 16 mg could increase ($P<0.05$) total VFA and proportion of acetate productions, while Jordan et al. (2006), who used 7% refined coconut oil in beef heifers found no changes on acetate and total VFA, as well as Dohme et al. (1999). The molar proportion of propionate was influenced by garlic powder supplementation. In this study, supplementation of garlic powder 16 mg (Co:G, 0:16) resulted in lowest ($P<0.05$) total VFA and highest ($P<0.05$) proportion of propionate. Moreover, the proportion of butyrate tended to increase when supplemented with garlic powder. This result was similar to Wanapat et al. (2008b), who found the supplementation of garlic powder on rumen ecology and found that increasing garlic powder in diets resulted in a reduction of total VFAs and the proportion of acetate, but increased in the proportion of propionate and butyrate. In the present study, the C2:C3 ratio was significantly lowest ($P<0.05$) when supplemented with Co:G, 0:16. Busquet et al. (2005b) using garlic oil and its compounds on *in vitro* fermentation study suggested that diallyl disulfide and allyl mercaptan compounds in garlic were responsible for most of their effects. Calculation of ruminal methane (CH_4) production using VFAs according to Moss et al. (2000) showed that garlic powder supplemented treatment (Co:G; 8:4, 4:8 and 0:16) had significantly lower ($P<0.05$) CH_4 production than those with no garlic powder supplementation (Co:G; 16:0) and control. Moreover, it was significantly lowest ($P<0.05$) at 8:4 and 0:16, (Co:G) supplemented groups. These results were in agreement with those of Van Nevel and Demeyer

(1988), who reported that methane was the main hydrogen sink in the metabolic pathway of rumen fermentation and inhibition of its synthesis of reducing equivalents that need to be disposed of, with propionate and butyrate being the main alternatives. Although, in this study the effect of coconut oil on reduction of methane gas production was not obtained. However, many research studies had confirmed the effect of coconut oil on reducing ruminal methane gas e.g. Soliva et al. (2003), who studied on suppressing ruminal methanogenesis by using non-esterified lauric (C_{12}) and myristic (C_{14}). It was found that C_{14} alone had no effect on methanogenesis but with a 2:1 proportion of C_{12}/C_{14}, the maximum methane suppressing effect (96%) was achieved and was similar to that with C_{12} alone. This ratio (2:1, C_{12}/C_{14}) was almost equivalent to that of coconut oil (2.6:1) and coconut oil was shown to be highly effective in suppressing methane formation in ruminants (Dong et al., 1997; Machmüller and Kreuzer, 1999.)

3.4. Population of R. albus, F. succinogenes, R. flavefaciens, fungal and protozoa in the rumen incubation

The accuracy of each real-time PCR was validated by quantifying known numbers of target species templates (*R. albus, F. succinogenes, R. flavefaciens* and anaerobic fungi). Then, those templates were used for generated standard curve. In this study, those standard curves showed a highly linear relationship between *ct* value and known number template dilution by given highly R^2 value of standard curve in each species are shown in Fig. 2. All supplemented treatments had effects on *R. albus* and *F. succinogenes* population ($P<0.05$), whereas *R. flavefaciens* and anaerobic fungi were unaffected. Quantifying the predominant cellulolytic bacteria in *in vitro* incubation provided an interesting data. It was shown in Table 3, that population of *F. succinogenes* and *R. flavefaciens* were 10^8, while 10^6 copies/ml of incubation for *R. albus*. Similarly, Wanapat and Cherdthong (2008), who studied rumen cellulolytic bacteria population using real-time PCR and found that three cellulolytic bacterial numbers were 3.0×10^9, 3.07×10^7 and 2.93×10^6 copies/ml of rumen fluid for F. *succinogenes*, R. *flavefaciens* and R. *albus*, respectively. Moreover, in this study *R. albus* population was significantly highest ($P<0.05$) in Co:G, 8:4 supplemented group.

In addition, ruminal anaerobic fungi population was not influenced by supplemented treatments, which ranged from 2.6 to 3.5×10^7 copies/ml of incubation. Although, garlic oil had fungicidal properties as reported by Pai and Platt (1995), under this study, anaerobic fungi was not affected by supplemented garlic powder but tended to be lowest with 16 mg coconut oil supplementation. All supplemented treatments had effects on protozoal population ($P<0.01$). As shown in Table 3, protozoal population was lowest in Co:G, 8:4 but was not significantly different in Co:G, 4:8 and 0:16 supplementation ($P>0.05$). Under this study it was found that garlic powder was more effective than coconut oil on

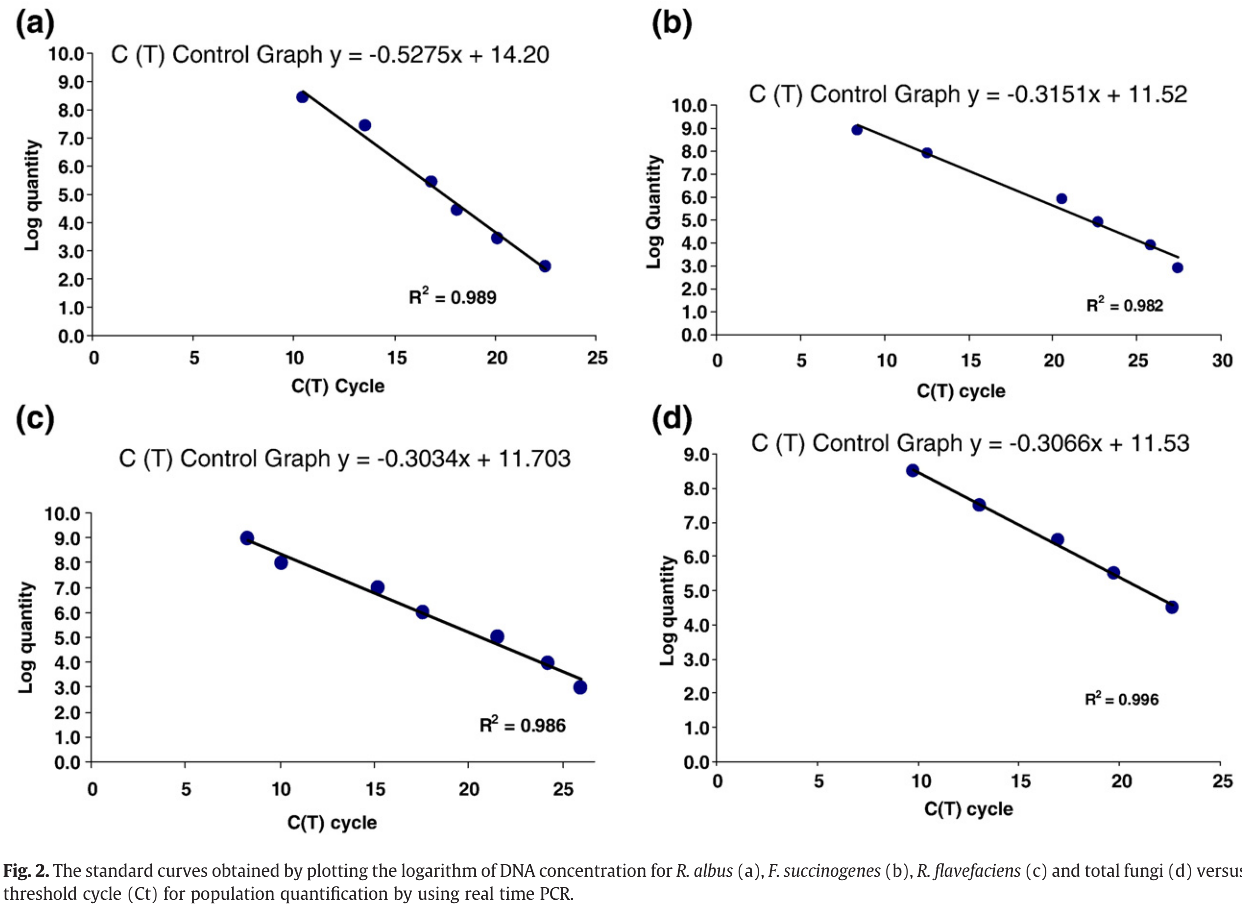

Fig. 2. The standard curves obtained by plotting the logarithm of DNA concentration for *R. albus* (a), *F. succinogenes* (b), *R. flavefaciens* (c) and total fungi (d) versus threshold cycle (Ct) for population quantification by using real time PCR.

Table 3
Effect of level of coconut oil (Co) and garlic powder (G) ratio supplementation on predominant cellulolytic bacterial, fungal and protozoal population from *in vitro* fermentation with swamp buffalo rumen fluid.

Items	Co:G ratio, mg					SEM	Contrast [1]			
	0:0	16:0	8:4	4:8	0:16		Control vs supplements	*L*	*Q*	*C*
Real-time PCR technique, copies/ml of incubation										
R. albus, $\times 10^5$	18.3[a]	14.5[a]	33.8[b]	14.1[a]	10.7[a]	0.338	0.988	0.096	0.020	0.015
F. succinogenes, $\times 10^8$	3.0[a]	1.3[b]	2.8[a]	1.8[b]	2.9[a]	0.246	0.028	0.022	0.389	0.008
R. flavefaciens, $\times 10^8$	7.6	5.5	6.7	7.2	7.8	0.995	0.482	0.150	0.795	0.839
Anaerobic fungal, 10^7	3.2	2.6	3.1	3.2	3.5	0.280	0.714	0.074	0.644	0.573
Direct count protozoa, CFU/ml										
Protozoa, $\times 10^5$	14.3[a]	10.3[b]	5.8[c]	7.3[cb]	6.4[cb]	0.911	0.003	0.117	0.157	0.180

[a–c] Values in the same row with different superscripts differ ($p < 0.05$).
[1] Linear (*L*), quadratic (*Q*), and cubic (*C*) effects of supplemented treatments.

reducing protozoal population. However, it was found that the population of ruminal protozoa in *in vitro* incubation ranged from 10^5 to 10^6 cell/ml of incubation. The present data clearly demonstrates that active ingredients in garlic could impact on growth of protozoa as also earlier described by Busquet et al. (2005a,b). It was stated that, *Archaea* had unique membrane lipids that contained glycerol linked to long chain isoprenoid alcohols essential for the stability of the cell membrane (De Rosa et al., 1986). The synthesis of this membrane is catalyzed by hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, an enzyme that has also been described in the liver and that participates in the synthesis of cholesterol. Garlic and some derived organo-sulfur compounds are strong inhibitors of HMG-CoA reductase (Gebhardt and Beck, 1996), which could have an effect on the synthesis of the isoprenoid unit causing the membrane of *Archaea* to become unstable and the cells die. Moreover, Dohme et al. (1999), who studied on the effect of coconut oil on protozoal population, reported that the number of protozoa per milliliter of rumen fluid was significantly reduced by coconut oil, when using coconut oil instead of protected fat. Furthermore, Dohme et al. (2001) studied in *in vitro* and found that C_{12} (lauric acid) had affected on decreased ciliate protozoal count while C_{14} (myristic acid) did not show any effects.

4. Conclusions

Garlic powder supplementation resulted in more efficiency than coconut oil on ammonia nitrogen concentration, *in vitro* true digestibility, proportion of propionate, C2:C3 ratio and methane gas production. However, based on this study the supplemented mixtures (8:4 and 0:16, Co:G) could be an alternative means as feed enhancers to improve ruminal fluid fermentation end products in terms of reducing fermentation losses, improving VFAs profiles, reducing protozoal population and increasing cellulolytic bacterial population.

Acknowledgements

The authors would like to express their most sincere gratitude and appreciation to the Thailand Research Fund (TRF) via “The Royal Golden Jubilee Ph.D. Program”, Tropical Feed Resources Research and Development Center (TROFREC), and the Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Thailand, for their financial support of research and the use of research facilities.

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