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Ruminal Methane Production on Simple Phenolic Acids Addition in *in Vitro* Gas Production Method

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ABSTRACT

Methane production from ruminants contributes to total global methane production, which is an important contributor to global warming. In this experiment, six sources of simple phenolic acids (benzoic, cinnamic, phenylacetic, caffeic, p-coumaric and ferulic acids) at two different levels (2 and 5 mM) added to hay diet were evaluated for their potential to reduce enteric methane production using in vitro Hohenheim gas production method. The measured variables were gas production, methane, organic matter digestibility (OMD), and short chain fatty acids (SCFA). The results showed that addition of cinnamic, caffeic, p-coumaric and ferulic acids at 5 mM significantly ($P<0.05$) decreased methane production. Caffeic acid at 5 mM was the most effective out of the simple phenols tested and it decreased methane by 6.3% from the control. The order of simple phenols to decrease methane was: caffeic acid > p-coumaric > ferulic > cinnamic. The addition of simple phenols did not significantly decrease OMD. Addition of simple phenols tends to decrease total SCFA production. It was concluded that methane decrease by addition of phenolic acids was relatively small, and the effect of phenolic acids on methane decrease depended on the source and concentration applied.

Key words: methane, phenolic acids, in vitro, rumen

INTRODUCTION

The most important greenhouse gases in the atmosphere are carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O), and their global atmospheric concentrations have increased significantly in the last 150 years (Monteny *et al*., 2006). These gases have the capacity to raise the earth's temperature through absorption of long wave radiation and contribute to global warming. Agriculture contributes significantly to total greenhouse gas (GHG) emissions. Approximately 20% to 35% of the global GHG emissions originate from agriculture. These figures are 40% and >50% of the anthropogenic emissions of CH4 and N2O respectively (IPCC, 2001). Approximately 70% of methane production arises from anthropogenic sources, of which agriculture accounts for about two-third, with enteric fermentation being responsible for one-third of methane from agriculture (Moss

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et al., 2000). Thus, there is a need to reduce methane emissions from animals, especially from ruminants.

Ruminants can convert herbage that is not of immediate or direct benefit to human beings into high quality foods such as milk and meat. Other non-food products have also been derived from ruminants such as skin and wool. However, ruminants produce methane which is potent as a GHG in the atmosphere. About 73% methane production from livestock is attributed to cattle (Johnson & Johnson, 1995). Methane from enteric fermentation by ruminants is not only an important GHG associated with environmental problems, but it also represents considerable amount of energy losses from the animals. Around 6%–10% of the gross energy of the ruminant diet is lost to methane (Immig, 1996). Therefore, developing feeding strategies to minimize methane emissions is desirable in long-term mitigation of GHG emissions into the atmosphere and for short-term economic benefits.

Many attempts, such as, concentrate (Lovett *et al*., 2005) and lipids supplementation (Van Nevel & Demeyer, 1995; Ungerfeld *et al*., 2005); and use of organic acids (Newbold *et al*., 2005), essential oils (Evans & Martin, 2000; Garcia-Gonzalez *et al*., 2008; Agarwal *et al*., 2009), probiotics and prebiotics (Mwenya *et al.*, 2004; Takahashi *et al*., 2005) have been made to decrease enteric methane production from ruminants, *in vitro* and/or *in vivo*. Antibiotic growth promoters (AGPs) such as monensin and lasalocid have also been shown to decrease methane production (Fuller & Johnson, 1981; Odongo *et al*., 2007; Grainger *et al*., 2008). However, AGPs have been banned in Europe since 2006, and many other countries outside the European Union are also considering banning such products.

It was evident from our previous research that non-tannin phenols play a role in methane reduction due to higher correlation of total phenols (TP) compared to total tannins (TT) with the methane decrease from control (Jayanegara *et al*., 2008a; 2008b). This study aimed at evaluating non-tannin phenols, represented by six sources of simple phenols in the form of phenolic acids, for their potential to reduce methane production *in vitro*. The working hypotheses were that phenolic acids would reduce methane production and that different forms of phenolic acids would elicit different response on methane reduction *in vitro*.

MATERIALS AND METHODS

Six sources of simple phenols (benzoic, cinnamic, phenylacetic, caffeic, p-coumaric and ferulic acids) were evaluated for their potential to reduce methane. Two levels of each phenol (2 and 5 mM) were added to hay diet before *in vitro* incubation. The simple phenols were prepared by solubilizing the phenols in sodium phosphate buffer pH 6.7 to avoid pH>7.5, and adding 130 µl of NaOH (10 M) to completely dissolve the phenols. An appropriate aliquot of solubilized phenolics (≤1 ml) was injected into the syringe from the syringe nozzle before dispensing rumen liquor. The measured variables were gas production, methane, OMD, and short chain fatty acids (SCFA: C_2, C_3, C_4, iso-C_4, C_5, iso-C_5, total SCFA) and ratio of C_2/C_3. This experiment was done in two replicates.

in Vitro Gas Production

Approximately 380 mg of hay substrates (basal diets) were incubated in 100 ml calibrated glass syringes containing 30 ml of medium (10 ml rumen liquor and 20 ml double strength buffer) by following the procedure of Makkar *et al*. (1995), which is a modified protocol from the original method of Menke *et al*. (1979). The rumen fluid and particulate matter were collected before the morning feeding from two cattle fed on roughage and concentrate based diets, mixed, homogenized, strained and filtered through 100 µm nylon net. The glassware used was kept at approximately 39 ^oC and flushed with CO_2 before use. The 30 ml buffered medium containing rumen microbes was dispensed into the syringes and incubation was done at 39 ^oC for 24 h.

Gas Reading and Methane Determination

After 24 h of incubation, the total gas was recorded from the calibrated scale on the syringe. The methane was measured using an infrared (0%–30% range) methane analyser (Pronova Analysentechnik GmbH & Co. KG, Berlin, Germany) calibrated against 10.6% methane (Goel *et al*., 2007). After measuring the total gas volume, the tubing of the syringe outlet was inserted into the inlet of the methane analyser. The display on the methane analyser gives methane as percent of the total gas and this value was used for calculation of methane in total gas volume.

Sampling for SCFA Analysis

Sampling for SCFA analysis was performed according to Hoffmann *et al*. (2008). After 24 h incubation, aliquots of 1 ml volume were pipetted into prepared sampling tubes (1.5 ml Eppendorf cups) kept on ice to immediately stop the fermentation processes. To ensure the withdrawal of homogenous samples, contents were vigorously stirred before pipetting; wide bored tips were used to avoid plugging by feed particles. The samples then were centrifuged (30,000 g, 10 min, 4 ^{o}C) and supernatant were carefully separated from pellet. Aliquot of 630 µl of the supernatant was transferred into a fresh vial and 70 µl of internal standard (methylvaleric acid) was added. These samples were kept at 4 ^{o}C over night to precipitate soluble proteins. They were centrifuged again (30,000 g, 10 min, 4 ^{o}C) to remove the precipitate. Then 500 µl of the acidified, deproteinized supernatant were transferred into 1.5 ml glass vials (VWR/ Merck 548-0003), sealed with serum caps (VWR/Merck 548-0413) for SCFA analysis.

SCFA Analysis

Short chain fatty acids were determined in an acidified, deproteinized rumen fluid sample containing 10% (v/v) of the internal standard. Samples were analysed in a gas chromatograph (GC 14A, Shimazu Corp., Kyoto, Japan) with a stainless steel column packed with 10% SPTM-1000, 1% H3PO4, Chromosorb WAW (Suppelco Inc. Bellafonte, PA, USA). The method used was of Hoeltershinken *et al*. (1997).

To guarantee reliable measurements, internal as well as external standard were used. Methylvaleric acid, which does not naturally occur in rumen liquid, was used as internal standard. This serves as reference for the analytical device and needs to be present in every sample measured. Double distilled water was used to clean the column in regular intervals. Two vials of water were inserted after every 9 vials of samples. An external standard containing known amount of individual SCFA was inserted once per run.

The gas chromatography program detects the individual SCFA-peaks and converts the peak area to concentration (µmol/ml or mM). SCFA in test syringes were corrected for SCFA in the corresponding blanks to obtain net SCFA production. The corresponding blank consisted of buffered medium without the substrate but containing treatments at the similar levels.

Organic Matter Digestibility (OMD)

The contents of the syringes after 24 h of incubation were digested with neutral detergent solution and the undigested feed was recovered on crucibles, washed, dried, and ashed. The OMD of substrate after 24 h was calculated by subtracting this value from the organic matter incubated in the syringe (Blümmel *et al*., 1997a).

Observations and Calculations

The measurements of total gas, methane and OMD were used for calculation of methane decrease and the partitioning of nutrients. Net gas (ml) was calculated from differences of the gas in the test syringe and the corresponding blanks, and similarly net methane produced (ml) was determined by subtracting the methane in the blank syringe from that in

the test syringe. Methane production was expressed as net methane produced in net gas (ml/100 ml).

The effects of treatment, as percent decrease in methane and methane per unit organic matter digested were calculated as (methane in control refers to methane produced from hay incubation):

(1) Percent decrease in methane:

$$\frac{\text{net } CH_4 \text{ in control (ml/100 ml)} - \text{net } CH_4 \text{ in the test (ml/100 ml)}}{\text{net } CH_4 \text{ in control (ml/100 ml)}} \times 100$$

(2) Percent decrease in methane per unit organic matter digested:

$$\frac{\text{net } CH_4/\text{OMD in control (ml/100 mg)} - \text{net } CH_4/\text{OMD in the test (ml/100 mg)}}{\text{net } CH_4/\text{OMD in control (ml/100 mg)}} \times 100$$

The partitioning factor (PF), which is a measure of efficiency microbial protein synthesis (Blümmel *et al*., 1997a) was calculated as:

$$\frac{\text{OMD (mg)}}{\text{Net gas produced (ml)}}$$

Statistical Analysis

The data from experiment were analysed using one-way ANOVA (analysis of variance) option in the Statistica software version 6.0. The differences between means were compared using Tukey's (Honest Significant Different, HSD) test.

RESULTS AND DISCUSSION

General Fermentation Parameters

Methane production in this experiment was expressed as: (1) the net methane produced of the net gas produced (ml/100 ml), and (2) net methane produced per unit organic matter digested. The former variable is useful to know the effect of a treatment on methane concentration. On the other hand, the latter gives the partitioning of the substrate carbon to methane carbon, and the comparison of the values for the control substrate when incubated alone or with the test material gives an insight into the expected *in vivo* effects. This is because in *in vivo*, the proportion of organic matter digested that leads to methane formation is of relevance and the reduction in methane production per unit organic matter digested represents the true efficacy of a manipulation strategy (Goel *et al.*, 2007). The decrease in methane production based on truly degraded substrate could be a result of decrease in methane production or increase in substrate degradation or both.

The partitioning of nutrients to gas, SCFA and microbial mass was evaluated by the PF. It gives the proportion of substrate carbon which goes to the production of gases (consist of fermentative gases plus buffered gas released on buffering of SCFA produced as a result of fermentation, from the bicarbonate buffer present in the incubation medium) and microbial mass. Higher the PF, higher is the partitioning of substrate to microbial mass, i.e. higher is the efficiency of microbial mass synthesis. The PF value should fall in the theoretical range of 2.74 to 4.65 (Blümmel *et al*., 1997b).

Effects of Simple Phenols Addition on Gas, Methane Production, Organic Matter Digestibility and Short Chain Fatty Acids Production

The effects of simple phenols on gas, methane production and OMD are presented in Table 1. Each phenol was added to hay diet at two different levels i.e. 2 and 5 mM.

In general, the addition of simple phenols decreased gas production although most of them were not significantly different and the effects were higher at higher concentration (Table 1). All of the simple phenols at lower concentration (2 mM) and benzoic and phenylacetic acids at 5 mM were not effective in decreasing methane production. Addition of cinnamic, caffeic, p-coumaric and ferulic acids at 5 mM significantly ($P<0.05$) decreased methane production. Caffeic acid at 5 mM was the most effective out of the simple phenols tested and it decreased methane by 6.3% from

Table 1. Gas production, methane production and organic matter digestibility of simple phenols addition

Treatment	Gas (ml/380 mg)	CH_4 (ml/100 ml)	OMD (%)	PF (mg/ml)	CH_4/OMD (ml/100 mg)
Control	76.2^{c}	15.9^{cd}	76.1	3.16	5.05^{bc}
Benzoic					
2 mM	77.0^{c}	16.0^{cd}	75.2	3.09	5.19^{c}
5 mM	74.8^{bc}	16.0^{cd}	75.2	3.18	5.03^{abc}
Cinnamic					
2 mM	75.3^{bc}	15.5^{abc}	75.7	3.18	4.88^{abc}
5 mM	74.5^{bc}	15.4^{abc}	75.5	3.21	4.79^{abc}
Phenylacetic					
2 mM	73.3^{abc}	15.9^{bcd}	73.7	3.18	5.00^{abc}
5 mM	74.3^{bc}	16.4^{d}	75.1	3.20	5.13^{c}
Caffeic					
2 mM	73.3^{abc}	15.6^{abc}	73.2	3.16	4.94^{abc}
5 mM	71.0^{ab}	14.9^{a}	73.4	3.27	4.57^{a}
p-Coumaric					
2 mM	72.5^{abc}	15.5^{abc}	71.8	3.13	4.96^{abc}
5 mM	68.5^{a}	15.1^{a}	71.0	3.28	4.61^{ab}
Ferulic					
2 mM	72.5^{abc}	15.9^{bcd}	75.2	3.28	4.84^{abc}
5 mM	70.8^{ab}	15.2^{ab}	71.4	3.19	4.77^{abc}
SEM	0.49	0.08	0.43	0.014	0.039

OMD= organic matter digestibility; PF=partitioning factor; SEM=standard error of the mean. Values in the same column with different superscripts are different at $P<0.05$.

the control (Figure 1). The magnitude was higher when expressed as decrease of methane per unit OMD and the decrease was 9.4% from control (Figure 2). The order of simple phenols to decrease methane was: caffeic acid > p-coumaric > ferulic > cinnamic. The addition of simple phenols did not significantly decrease OMD and the partitioning factor (PF).

Similar pattern was obtained for the SCFA (Table 2). Addition of simple phenols tends to decrease total SCFA production, although most of them were not significant. Caffeic and p-coumaric acids at 5 mM decreased total SCFA production ($P<0.05$) from control (43.33 and 42.38 vs 49.79 mM). This response was due to the decrease of SCFA such as acetate (C_2), propionate (C_3), butyrate (C_4) and valerate (C_5). No decrease was observed for iso-SCFA, both iso-butyrate (iso-C_4) and iso-valerate (iso-C_5). Although the decrease of C_2 and C_3 was evident, no significant change was observed in the ratio of acetate to propionate (C_2/C_3).

Phenolic acids are common constituents of forage fed to ruminants, where they occur most frequently as hydroxycinnamic acids ester-linked to polysaccharide. Ferulic and p-coumaric acids, the major phenolic acids found in this form, may represent up to 2.5% by weight of the cell walls of temperate grasses (Hartley & Jones, 1977). Cellulolytic bacteria such as *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Fibrobacter succinogenes* and *Butyrivibrio fibrisolvens* are found to adhere or associate in close proximity to fragments of plant material, and are largely responsible for fibre degradation in the rumen (Cheng &

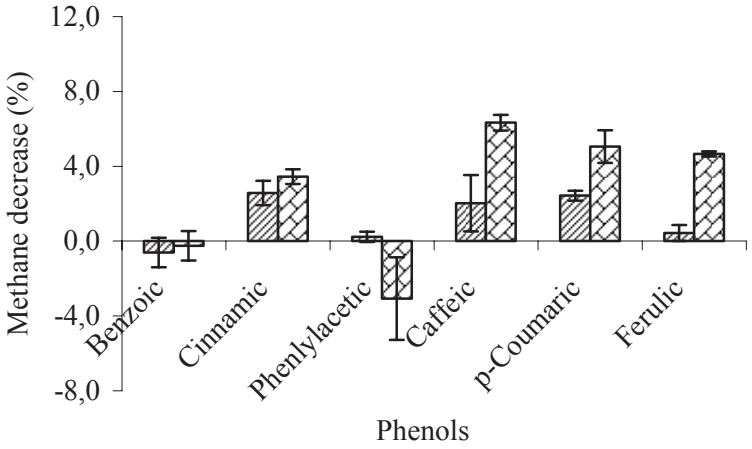

Figure 1. Percentage of methane decrease from control of simple phenols addition (▨=2 mM,▨=5 mM)

Costerton, 1980). In this microenvironment, such organisms would be expected to encounter phenolic acids released during cell wall digestion at concentrations far higher than those suggested by the content in rumen liquor.

Table 2. Short chain fatty acids production (SCFA, in mM) of simple phenols addition

Treatment	C_2	C_3	C_4	i-C_4	C_5	i-C_5	Total SCFA	i-SCFA	C_2/C_3
Control	32.81^{b}	11.91^{bc}	3.94^{b}	0.28	0.59^{bc}	0.24^{ab}	49.79^{b}	0.52^{ab}	2.75
Benzoic									
2 mM	33.40^{b}	12.23^{c}	3.95^{b}	0.32	0.60^{bc}	0.29^{b}	50.79^{b}	0.61^{b}	2.73
5 mM	29.92^{ab}	11.06^{abc}	3.57^{ab}	0.25	0.53^{abc}	0.20^{ab}	45.53^{ab}	0.45^{ab}	2.71
Cinnamic									
2 mM	29.94^{ab}	11.12^{abc}	3.58^{ab}	0.25	0.54^{abc}	0.21^{ab}	45.64^{ab}	0.46^{ab}	2.69
5 mM	30.34^{ab}	11.23^{abc}	3.59^{ab}	0.26	0.55^{abc}	0.21^{ab}	46.17^{ab}	0.47^{ab}	2.70
Phenylacetic									
2 mM	29.27^{ab}	10.96^{abc}	3.52^{ab}	0.28	0.52^{ab}	0.16^{a}	44.70^{ab}	0.43^{ab}	2.67
5 mM	29.69^{ab}	10.79^{ab}	3.44^{ab}	0.24	0.53^{abc}	0.24^{ab}	44.94^{ab}	0.48^{ab}	2.75
Caffeic									
2 mM	30.35^{ab}	11.07^{abc}	3.58^{ab}	0.26	0.55^{abc}	0.25^{ab}	46.06^{ab}	0.51^{ab}	2.74
5 mM	28.54^{a}	10.46^{a}	3.40^{a}	0.24	0.51^{a}	0.19^{ab}	43.33^{a}	0.43^{ab}	2.73
p-Coumaric									
2 mM	31.16^{ab}	11.38^{abc}	3.60^{ab}	0.27	0.55^{abc}	0.25^{ab}	47.21^{ab}	0.52^{ab}	2.74
5 mM	27.90^{a}	10.28^{a}	3.33^{a}	0.21	0.51^{a}	0.15^{a}	42.38^{a}	0.36^{a}	2.71
Ferulic									
2 mM	30.80^{ab}	11.19^{abc}	3.67^{ab}	0.28	0.54^{abc}	0.27^{ab}	46.75^{ab}	0.55^{ab}	2.75
5 mM	29.82^{ab}	10.94^{abc}	3.45^{ab}	0.24	0.51^{a}	0.19^{ab}	45.14^{ab}	0.43^{ab}	2.73
SEM	0.339	0.112	0.042	0.006	0.006	0.009	0.507	0.014	0.008

Values in the same column with different superscripts are different at P<0.05; C_2=acetate, C_3=propionate, C_4=butyrate, C_5=valerate, i-C_4=iso-butyrate, i-C_5=iso-valerate, SCFA=short chain fatty acids, i-SCFA=iso-short chain fatty acids, and C_2/C_3=ratio of acetate to propionate.

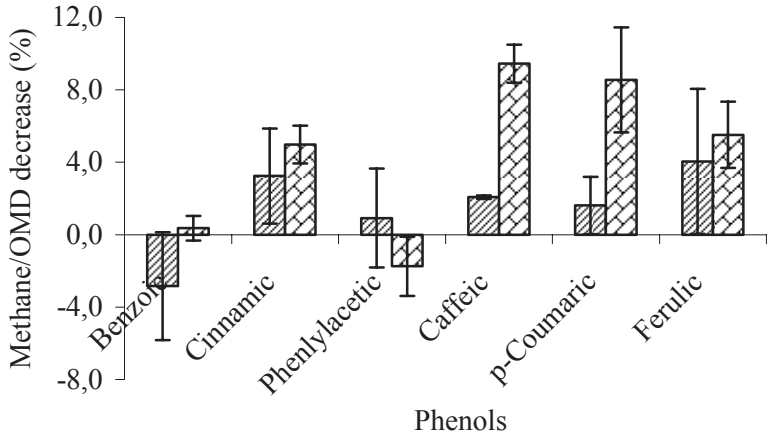

Figure 2. Percentage of methane/OMD decrease from control of simple phenols addition (▨ =2 mM, ▩ = 5 mM)

Natural and related phenolic compounds have shown antimicrobial properties against bacteria and fungi (Borneman *et al*., 1986; Chan *et al*., 2007; Lim *et al*., 2009). In the rumen ecosystem, free phenolic acids have been observed to have a toxic effect on rumen bacteria, especially cellulolytic and fibre degrading bacteria, fungi and protozoa. Phenolic acids similar to those in plant cell walls inhibit the ability of rumen fungi to colonize and degrade plant fibre, although these microbes have the ability to attack certain lignified tissues (Akin, 1982; Chesson *et al*., 1982; Akin & Rigsby, 1985; Rodrigues *et al*., 2007). This might be an argument that the addition of some phenolic acids decreased gas production in this study. However, the mechanism of inhibition of cell wall degrading microbes in rumen fluid by the esters of phenolic acids or by the free acids is not understood in biochemical terms. It is generally believed that inhibition is caused by damage to cell membranes and by inactivation of cell enzymes (Hartley & Akin, 1989).

Since phenolic acids affect activities of rumen microbes, the effect of phenolic acids on methanogenesis could be expected. The decrease in ruminal methane production could be linked to their role in inhibiting fibre degradation and in decreasing protozoa to certain extent. Inhibition of fibre degradation will shift SCFA composition away from acetate and hence less production of hydrogen and less methane formation (Jayanegara, 2008c). On the other hand, anti-protozoal effect of phenolic acids would decrease methane production since a portion of methanogens is attached to protozoa (Vogels *et al*., 1980; Hess *et al*., 2003; Wang *et al*., 2009). These protozoa-associated methanogens have been reported to contribute up to 37% of total rumen methane emissions (Klieve & Hegarty, 1999). Therefore reduction of protozoal counts from the rumen is associated with the decrease in methane production.

The toxic effect of rumen microbes is source and concentration dependent. Benzoic acid in the form of p-hydroxybenzoic acid did not inhibit the growth of *R. flavefaciens* FD-1 when added at 10 mM to cellulose substrate, on contrary, significantly increased the ability of the bacterium to degrade cellulose from the filter paper. Also, this benzoic acid did not decrease in vitro dry matter digestibility of isolated cellulose (Solka-Floc), both at 24 and 48 h (Borneman *et al*., 1986). Varel & Jung (1986) reported that p-coumaric acid addition at 0.1% was the most toxic among the tested phenolic acids on the growth of *R. albus* and *R. flavefaciens*. The order of inhibition of phenolic acids to the growth of *R. albus* and *R. flavefaciens* was p-coumaric acid > vanillin

> ferulic acid > cinnamic acid. Other authors reported the order of toxicity to rumen bacteria and protozoa as p-coumaric > ferulic > sinapic (Akin, 1982; Chesson *et al*., 1982).

Although p-coumaric acid was reported to have the most toxic effect to ruminal micro-organisms, this effect depended on the concentration applied. Addition of p-coumaric acid at 1 mM retained almost 100% of cellulolytic activity of *B. succinogenes*, *R. flavefaciens* and *R. albus*. When the concentration was increased to 5 mM and 10 mM, the average percent cellulolytic activity retained was approximately 80% and 40% for those bacteria, respectively. Similar pattern was obtained for other phenolic acids tested i.e. ferulic, phloretic, 4-hydroxybenzoic and vanilic acids (Chesson *et al*., 1982).

In the present study, addition of all phenolic acids at lower concentration (2 mM) did not significantly decrease methane production and methane production per unit OMD. This suggested that the 2 mM concentration of phenolic acids is below the threshold level at which activity of methanogens was affected. When the concentration was increased to 5 mM, some phenolic acids such as caffeic, p-coumaric and ferulic acids significantly decreased methane production. Cinnamic acid tended to decrease methane but not significantly at 95% confidence interval or α=5%. The order of phenolic acids to decrease methane was the same as their toxicity to cellulolytic bacteria, fungi and protozoa as reported by some authors above i.e. caffeic > p-coumaric > ferulic > cinnamic acids. The order mentioned is related to the chemical structure of each simple phenolic acid. It seems that phenolic acid which has more hydroxyl group attached to its benzene ring is more toxic than the less one. This explains the response of caffeic acid that decreased methane lowest than the other phenolic acids tested, since caffeic acid has two hydroxyl groups attached to its benzene ring and the others have only one or even without hydroxyl group. One the other hand, substitution of the hydroxyl group by methyl group decreases its toxicity as explained by less methane reduction by ferulic acid than caffeic acid addition.

The methane decrease by addition of phenolic acids was relatively small (up to 6.3%). This might be explained by adaptation and defense mechanisms of rumen microbes in the presence of phenolic acids. Hydrogenation of the more toxic phenolic acids to a less toxic form may be one mechanism of defense of organisms active in fibre degradation. Further degradation of the hydrogenated phenolic acids may then occur (Varel & Jung, 1986). The cellulolytic bacteria showed at least a limited ability to modify the more toxic ferulic and p-coumaric acids by hydrogenation of the 2-propenoic side chain, the products proving considerably less toxic to these organisms than the parent acid (Chesson *et al*., 1982). Another possibility is that phenolic acids may be lost from rumen fluid by non-specific absorption to microbial surfaces or by specific uptake and utilisation by some rumen microrganisms (Akin, 1980). Phenolic acids released through cell wall degradation do not appear to decrease substantially the methane production.

CONCLUSIONS

Methane decrease by addition of phenolic acids was relatively small (up to 6.3%), and the effect of phenolic acids on methane decrease depended on the source and concentration applied. Benzoic and phenylacetic acids failed to decrease *in vitro* methane production and this suggested that not all of the phenolic acids are able to reduce methane production. The order of simple phenols to decrease methane was caffeic acid > p-coumaric > ferulic > cinnamic.

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