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Effects of Phloroglucinol on *In Vitro* Methanogenesis, Rumen Fermentation, and Microbial Population Density

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ABSTRACT

This study investigated the effect of phloroglucinol (1,3,5-trihydroxybenzene) supplementation alone on methane production, rumen fermentation profiles, and microbial population structure of mixed *in vitro* cultures. Treatments included a control group containing a substrate with no supplement, and substrates supplemented with 2, 4, 6, 8, or 10 mmol/L of phloroglucinol. The results revealed that phloroglucinol was able to decrease methane production in a dose-dependent manner. The highest decrease was observed with 8 and 10 mmol/L supplementations. The relative quantity of methanogen was not affected by phloroglucinol, whereas genus *Coprococcus* was increased with increasing concentrations of phloroglucinol ($p<0.05$). Total gas production, dry matter digestibility (DMD), and NH_3-N were significantly lowered by phloroglucinol ($p<0.001$). Total short-chain fatty acid (SCFA) concentration was not affected by phloroglucinol. Acetate proportion increased with the addition of phloroglucinol at the expense of propionate ($p<0.001$). This might indicate the redirection of [H] from methane to acetate, and might be related to methane inhibition. Our study concluded that supplementation of phloroglucinol alone could decrease methane production by inhibiting nutrient digestibility in the rumen and by possible redirection of rumen fermentation to acetate production. Genus *Coprococcus* could be an important actor for phloroglucinol metabolism in the rumen.

Keywords: methane, methanogen, phloroglucinol, rumen

INTRODUCTION

Ruminants are one of the greatest sources of methane, a major greenhouse gas that poses a serious environmental threat, with 28- to 34-fold more global warming potential (GWP) than CO_2 (Myhre *et al*., 2013). Rumen fermentation in the livestock sector contributes 2.12 Gt CO_2 eq/year of methane, which is equal to 6.3% of the total global anthropogenic emission of greenhouse gases (Smith *et al*., 2014). Incremental increases in the atmospheric methane concentration enhance the greenhouse effect by trapping heat energy received from the sun in the Earth's atmosphere, resulting in global warming and leading to climate change. Besides its threat to the environment, methane also represents a loss of dietary energy in ruminants. Depending on the type of feed, ruminants loose between 2 and 12% of the gross energy intake from feed as methane (Johnson & Johnson, 1995). Therefore, it is important to decrease methane emission from ruminants for both environmental and economic benefits.

Methane is a natural by-product of anaerobic fermentation in the rumen. Methane in the rumen is produced mainly through the hydrogenotrophic pathway using CO_2 as the main carbon source and H_2 as the main electron donor. Methane production serves as an electron sink to help rumen maintain low partial pressure of H_2. Partial pressure of H_2 plays an important role in rumen fermentation. High partial H_2 pressure in the rumen inhibits ruminal fermentation of carbohydrates, the rate of microbial growth, and the synthesis of microbial protein (Knapp *et al*., 2014). A strategy to mitigate methane production by supplementing an alternative [H] sink in an effort to redirect rumen fermentation away from methanogenesis has been proposed (Broucek, 2018, Lan & Yang, 2019). The use of dicarboxylic acids, such as fumarate and malate to decrease methane production in the rumen has been evaluated (Martin *et al*., 2010, Song *et al*., 2011, Li *et al*., 2018). These compounds were found to decrease methane production but increase short-chain fatty acid (SCFA) production, which is the main energy source of ruminant. Other compounds also have been found to lower methane production by redirecting [H] utilization, such as nitrate and sulfate (Zijderveld *et al*., 2011, Lund *et al*., 2014, Patra & Yu, 2014, Klop *et al*., 2016).

Phloroglucinol (1,3,5-trihydroxybenzene) is a simple phenolic compound that can be found naturally

in the rumen as an intermediate product of polyphenol degradation, such as condensed tannin (CT) and hydrolysable tannin (HT) (McSweeney *et al*., 2001). Previous studies have shown that pure cultures of ruminal microorganisms are capable of degrading phloroglucinol to acetate with the use of H_2 (Tsai *et al*., 1976, Patel *et al*., 1981 Krumholz & Bryant, 1986). Supplementation of phloroglucinol can create competition for H_2 between methanogen and phloroglucinol-degrading bacteria, which will decrease the amount of hydrogen available for methanogens to produce methane.

Previous *in vivo* study showed that phloroglucinol can redirect [H] toward acetate when methanogenesis is completely inhibited by addition of chloroform (Martinez-Fernandez *et al*., 2017). This showed that in the absence of methanogenesis which is a natural [H] sink and H_2 pressure was high, phloroglucinol was able to redirect [H] toward acetate production. But, there was no effect found on methane production between chloroform only treatment and combination of chloroform and phloroglucinol treatment. Another study by Hierholtzer *et al*. (2012) found that phloroglucinol was able to decrease methane production from sludge collected from wastewater treatment. Sarwono *et al.* (2019) found that phloroglucinol could decrease methane production in different F:C ratio, but there was little information regarding the role of phloroglucinol-degrading bacteria on [H] utilization. Therefore, the objective of this study was to investigate the effect of supplementation of phloroglucinol alone on methane production, fermentation profiles, and microbial population during *in vitro* rumen fermentation.

MATERIALS AND METHODS

Animal and Sampling

Animal handling was performed according to the Mie University guidelines for animal handling. Three male Friesland sheep were used as ruminal fluid donors. The animals were offered a daily ration consisting of 1 kg Italian rye grass hay and 360 g concentrate. The concentrate consisted of wheat bran, soy bean, and corn, in a 1:1:1 ratio on dry matter basis. The ration was divided into the same portion and offered twice daily at 10:00 and 17:00. Each animal was placed in an individual pen. Water and mineral block were available *ad libitum*. Rumen fluid was orally collected through a stomach tube just before the morning feeding and kept at 39°C during transportation to the laboratory.

In Vitro Incubation

Pooled ruminal fluid was strained through four layers of surgical gauze. The filtered fluid was mixed with McDougall buffer with ratio 1:2 (rumen fluid : buffer). The buffer was pre-warmed to 39°C and flushed with N_2 gas. The diluted rumen fluid was used as inoculum. The composition of each treatment is described in Table 1. The substrate used consisted of 0.35 g of Italian rye grass hay, 0.30 g of wheat bran, and 0.35 g of corn. Each substrate was finely ground to pass through a 1 mm sieve using a Wiley mill. Phloroglucinol was dissolved in pure ethanol to 1 M. Five different doses of phloroglucinol (2, 4, 6, 8, and 10 mmol/L media) were added to empty 120 mL serum bottles and dried overnight at 40°C. After ethanol was evaporated, substrates were weighed into the bottle, and then 50 mL of media was dispensed under N_2 gas. The serum bottle was tightly capped with a butyl rubber septum and aluminium cap. Then, it was incubated for 24 h at 39°C with shaking at 180 rpm in a water bath. Each treatment consisted of three bottles, and three non-substrate bottles were also included in the incubation. The incubation was repeated three times on three separate days.

After 24 h, all of the serum bottles were placed on ice to stop fermentation. The cumulative headspace gas was measured using a 100 mL glass syringe to determine total gas production. Methane production (mL) was analysed using a gas chromatograph (GC-8A; Shimadzu Corporation, Kyoto, Japan) as described by Matsui *et al*. (2013). After gas analysis was completed, the culture fluid was transferred to a 50-mL centrifugation tube and was centrifuged at 1000 *g* for 5 min at 4°C to separate the residue and the culture fluid. The culture fluid was transferred into a 2-mL tube and kept at -30°C until analysis of SCFA, ammonia-N (NH_3-N), and quantification of the microbial population. Meanwhile, the constant weight of the residue was measured to determine dry matter degradability. SCFAs in culture fluid were analysed by high-performance liquid chromatography (HPLC) as described by Uddin *et al*. (2010). NH_3-N in the fluid was determined using the phenol-hypochlorite method as described by Abrar *et al*. (2016).

A complete metabolic hydrogen ([H]) balance was estimated in accordance to Ungerfeld (2015). $[2H]_{produced}$ is total amount of reducing equivalent pairs produced. $[2H]_{incorporated}$ showed total amount of metabolic hydrogen incorporated into H_2, CH_4, propionate, and butyrate. $[2H]_{recovery}$ is percentage of hydrogen produced recovered in CH_4, propionate, butyrate, and H_2 divided by [2H]produced. Molar amounts of methane for calculation [H] balance were calculated by using the following equation :

$$pV = nRT$$

where p is the pressure of the gas assumed at 1 atm, V is the volume of the gas, n is the number of moles of the gas, R is ideal gas constant of 0.082, and T is the temperature assumed at 20°C.

DNA Extraction from the Rumen Fluid

Microbial DNA was extracted from ruminal fluid using QIAamp DNA Stool Mini Kit according to the manufacture's instruction (Qiagen, Hilden, Germany). The extracted DNA was stored at -30°C until analysis.

Quantitation of the Microbial Population by Quantitative Real-Time PCR

Cycle threshold (C_T) of following 11 microbial populations, methanogen, *F. succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Selonomonas ruminan-*

tium, *Butyrivibrio fibrisolvens*, genus *Prevotella*, genus *Bacteroides*, *Streptococcus bovis*, *Eubacterium ruminantium*, and genus *Coprococcus* were quantified by using real-time PCR to determine its relative abundance. Real-time PCR was conducted by using comparative $\Delta\Delta C_T$ method, as described by Schmittgen & Livak (2008). Total bacteria was used as an endogenous control. Real-time PCR was conducted using a StepOnePlus® Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Specific primers for target group and the amount used in this study are listed in Table 2. The reaction mixture (20 μL) for real-time PCR consisted of 1 μL of DNA template, 10 μL of Thunderbird SYBR qPCR master mix (Toyobo Co. LTD., Osaka, Japan), 0.4 μL 50x ROX, specific forward and reverse primer, and sterile Milli-Q water. The PCR condition of all target groups included one cycle of polymerase activation at 95°C for 1 min, 40 cycles of denaturing at 95°C for 15 s, and annealing and extension at 60°C for 1 min except for *R. flavefaciens* which include 40 cycles of denaturing at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 60°C for 30 s, and methanogen which include denaturing at 95°C for 15 s, and annealing and extension at 60°C for 30 s. Data of relative quantity of target groups were expressed by $2^{-\Delta\Delta C_T}$.

Statistical Analysis

Data were subjected to analysis of variance (ANOVA) according to a randomized complete block design. Different *in vitro* runs served as blocks in the ANOVA statistical model. Tukey's test was used to identify differences between treatment means when the ANOVA result showed a significant different. An effect was considered significant at the probability level of $P<0.05$, and $P<0.10$ was considered as a tendency to be significant. Polynomial contrasts were conducted to test the data for linear and quadratic trends. Statistical analysis was conducted using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA).

Table 1. Composition of experimental *in vitro* batch cultures

Material	Control	Phloroglucinol				
		2 mmol/L	4 mmol/L	6 mmol/L	8 mmol/L	10 mmol/L
Rye grass hay (g)	0.35	0.35	0.35	0.35	0.35	0.35
Corn starch (g)	0.35	0.35	0.35	0.35	0.35	0.35
Wheat bran (g)	0.30	0.30	0.30	0.30	0.30	0.30
Inoculum (mL)	49.00	49.00	49.00	49.00	49.00	49.00
Phloroglucinol 1 M solution (mL)	-	0.10	0.20	0.30	0.40	0.50
Distilled water (mL)	1.00	1.00	1.00	1.00	1.00	1.00

Table 2. Primers used in recent study

Target	Name	Sequence (5′→ 3′)	Amount added (μL)	Reference
Total bacteria	1114-f	CGGCAACGAGCGCAACCC	0.6	Abrar *et al*. (2016)
	1275-r	CCATTGTAGCACGTGTGTAGCC	0.6	
Ruminococcus albus	Ra1281f	CCCTAAAAGCAGTCTTAGTTCG	0.5	Lwin *et al*. (2012)
	Ra1439r	CCTCCTTGCGGTTAGAACA	0.5	
Ruminococcus flavefaciens	Rflf	GGACGATAATGACGGTACTT	0.9	Lwin *et al*. (2012)
	Rflr	GCAATCYGAACTGGGACAAT	0.9	
Fibrobacter succinogenes	Fsf	GGTATGGGATGAGCTTGC	0.9	Lwin *et al*. (2012)
	Fsr	GCCTGCCCCTGAACTATC	0.9	
Methanogens	q-mcrA-f	TTCGGTGGATCDCARAGRGC	1.2	Abrar *et al*. (2016)
	q-mcrA-r	GBARGTCGWAWCCGTAGAATCC	1.2	
Selonomonas ruminantium	SelRum 2F	CAATAAGCATTCCGCCTGGG	0.45	Mullins *et al*. (2013)
	SelRum 2R	TTCACTCAATGTCAAGCCCTGG	0.45	
Butyrivibrio fibrisolvens	ButFib 2F	ACCGCATAAGCGCACGGA	0.2	Mullins *et al*. (2013)
	ButFib 2R	CGGGTCCATCTTGTACCGATAAAT	0.2	
Genus Prevotella	PrevGen 4F	GGTTCTGAGAGGAAGGTCCCC	0.2	Mullins *et al*. (2013)
	PrevGen 4R	TCCTGCACGCTACTTGGCTG	0.2	
Genus Bacteroides	AllBac 296f	GAGAGGAAGGTCCCCCAC	0.2	Ban-Tokuda *et al*. (2017)
	AllBac 412r	CGCTACTTGGCTGGTTCAG	3.6	
Streptococcus bovis	StrBov 2F	TTCCTAGAGATAGGAAGTTTCTTCGG	8.8	Mullins *et al*. (2013)
	StrBov 2R	ATGATGGCAACTAACAATAGGGGT	8.8	
Eubacterium ruminantium	EubRum 2F	CTCCCGAGACTGAGGAAGCTTG	0.2	Mullins *et al*. (2013)
	EubRum 2R	GTCCATCTCACACCACCGGA	0.2	
Genus Coprococcus	CopGenF	CATCCTGATGACGGTTTCTTAACC	0.5	Da Silva *et al*. (2018)
	CopGenR	GTTGCGGGACTTAACCCA	0.5	

RESULTS

In Vitro Rumen Fermentation

Total gas production were significantly lowered 24 h after the addition of phloroglucinol (p<0.001; Table 3). Compared with the control, gas production was decreased by 7.5%, 8.6%, and 10.3% with the inclusion of 6, 8, and 10 mmol/L of phloroglucinol in the culture, respectively. There was a concentration-dependent and linear decrease (p<0.001). Simultaneously, methane production (mL) was significantly decreased (p<0.001) by 8.1%, 12.0%, and 15.0% compared with the control, following supplementation with 6, 8, and 10 mmol/L phloroglucinol, respectively. However, supplementation of phloroglucinol had no significant effect on methane production when methane was presented as methane per digested dry matter (CH_4/DDM).

Supplementing phloroglucinol significantly decreased dry matter degradability (DMD) (p<0.001). Higher doses of phloroglucinol decreased DMD in a linear and concentration-dependent manner (p<0.001). DMD was decreased by 7.3%, 8.9%, and 13.4% compared with the control by the addition of 6, 8, and 10 mmol/L of phloroglucinol, respectively.

Phloroglucinol did not affect ruminal pH after 24 h incubation; pH was between 5.72 and 5.77 after 24 h. Phloroglucinol supplementation had neither a linear nor a quadratic effect on pH. NH_3-N was significantly abated by phloroglucinol supplementation (p<0.001). NH_3-N decreased with increasing doses of phloroglucinol (p<0.001). Supplementation of 8 and 10 mmol/L of phloroglucinol decreased NH_3-N by 62% and 70%, respectively.

Total SCFA concentration was not significantly affected by phloroglucinol supplementation. Phloroglucinol had no linear or quadratic effect on total SCFA. Acetate proportion was increased significantly by phloroglucinol addition (p<0.001). The proportion of acetate to total SCFA increased concomitantly with increasing level of phloroglucinol (p<0.001), in a linear rather than a quadratic manner. The addition of 6, 8, or 10 mmol/L phloroglucinol increased the proportion of acetate by 8%, 12%, and 16%, respectively. The proportion of propionate was significantly decreased (p<0.001). A linear decrease in propionate was observed (p<0.001) with increasing doses of phloroglucinol. Additionally, the level of butyrate was not significantly affected by phloroglucinol supplementation.

Simultaneously, the acetate to propionate ratio increased significantly (p<0.001) following phloroglucinol supplementation in a linear (p<0.001) rather than a quadratic manner. The acetate to propionate ratio was significantly affected when phloroglucinol was supplemented at 6 mmol/L or higher.

Micobial Population Density

Phloroglucinol supplementation did not significantly affect the relative quantity of all target with the exception for *Coprococcus* (p=0.006; Table 4). Relative quantity of genus *Coprococcus* was linearly increased by increasing dose of phloroglucinol. A significant linear trend was also observed for *Streptococcus bovis* (p=0.024) and *Eubacterium ruminantium* (p=0.018).

Hydrogen Balance

Phloroglucinol supplementation did not have any significant effect on $[2H]_{produced}$ (*P*=0.061; Table 5). Phloroglucinol significantly decreased $[2H]_{incorporated}$ and $[2H]_{recovery.}$ A significant linear and quadratic effect of phloroglucinol on $[2H]_{incorporated}$ was observed. A linear effect was also observed on $[2H]_{recovery}$.

DISCUSSION

Phloroglucinol supplementation at 6 mmol/L or higher significantly lowered methane production *in vitro* in a dose-dependent manner. Our finding showed that

Table 3. *In vitro* methanogenesis and rumen fermentation supplemented by phloroglucinol

Variables	Control	Phloroglucinol					p-value		
		2 mmol/L	4 mmol/L	6 mmol/L	8 mmol/L	10 mmol/L	Treatment	Linear	Quadratic
Total gas production (mL)	130.11 ± 2.41^{a}	130.22 ± 2.41^{a}	123.33 ± 1.74^{ab}	120.44 ± 1.77^{b}	118.89 ± 1.40^{b}	116.67 ± 1.42^{b}	<0.001	<0.001	0.583
CH_4 production (mL)	16.72 ± 0.32^{a}	16.72 ± 0.50^{ab}	15.90 ± 0.29^{abc}	15.36 ± 0.24^{bcd}	14.71 ± 0.25^{cd}	14.22 ± 0.26^{d}	<0.001	<0.001	0.551
CH_4 per digested dry matter (mL/g)	34.21 ± 0.48	34.60 ± 0.95	33.935 ± 0.47	34.21 ±0.31	33.02 ± 0.46	33.94 ± 0.61	0.538	0.242	0.926
Dry matter degradability (%)	48.86 ± 0.37^{a}	48.31 ± 0.37^{ab}	46.86 ± 0.50^{bc}	44.90 ± 0.57^{cd}	44.54 ± 0.39^{d}	41.94 ± 0.57^{e}	<0.001	<0.001	0.178
pH	5.77 ± 0.23	5.75 ± 0.25	5.77 ± 0.15	5.76 ± 0.15	5.75 ± 0.16	5.72 ± 0.18	0.434	0.088	0.307
NH_3-N (mg/dL)	4.64 ± 0.27^{a}	4.80 ± 0.58^{a}	3.62 ± 0.36^{ab}	2.48 ± 0.33^{bc}	1.76 ± 0.38^{c}	1.39 ± 0.44^{c}	<0.001	<0.001	0.102
Total SCFA (mmol/L)	124.98 ± 1.78	129.84 ± 1.89	126.40 ± 1.25	125.39 ± 1.21	125.14 ± 1.15	126.67 ± 1.07	0.172	0.581	0.765
Percentage to total SCFA									
Acetate (%)	60.91 ± 0.23^{a}	61.93 ± 0.18^{ab}	62.61 ± 0.25^{bc}	62.71 ± 0.33^{bc}	63.36 ± 0.31^{cd}	64.41 ± 0.46^{d}	<0.001	<0.001	0.989
Propionate (%)	27.47 ± 0.24^{a}	26.48 ± 0.21^{ab}	25.97 ± 0.28^{b}	25.65 ± 0.34^{b}	25.02 ± 0.41^{bc}	24.11 ± 0.55^{c}	<0.001	<0.001	0.987
Butyrate (%)	11.63 ± 0.08	11.59 ± 0.19	11.43 ± 0.18	11.65 ± 0.10	11.62 ± 0.13	11.48 ± 0.15	0.859	0.724	0.992
Acetate : Propionate	2.22 ± 0.03^{a}	2.34 ± 0.02^{ab}	2.41 ± 0.03^{abc}	2.45 ± 0.04^{bc}	2.54 ± 0.05^{cd}	2.68 ± 0.08^{d}	<0.001	<0.001	0.665

Note: Values presented are mean ± standard error of mean (n= 9). Means in the same row with different superscripts differ significantly (p≤0.05).

Table 4. Relative microbial population density as expressed by $2^{-\Delta\Delta C_T}$ supplemented by phloroglucinol

Species	Control	Phloroglucinol					p-value		
		2 mmol/L	4 mmol/L	6 mmol/L	8 mmol/L	10 mmol/L	Treatment	Linear	Quadratic
Ruminococcus albus	1.00 ± 0.00	1.52 ± 0.51	0.87 ± 0.03	0.82 ± 0.13	0.74 ± 0.12	0.92 ± 0.21	0.299	0.242	0.694
Ruminococcus flaveciens	1.00 ± 0.00	0.84 ± 0.14	0.82 ± 0.18	0.96 ± 0.14	1.02 ± 0.09	0.88 ± 0.19	0.857	0.936	0.591
Fibrobacter succinogenes	1.00 ± 0.00	1.17 ± 0.26	1.24 ± 0.15	1.10 ± 0.09	0.89 ± 0.17	0.72 ± 0.06	0.214	0.126	0.038
Methanogens	1.00 ± 0.00	1.29 ± 0.14	1.38 ± 0.11	1.31 ± 0.19	1.41 ± 0.26	1.36 ± 0.14	0.539	0.113	0.333
Selonomonas ruminantium	1.00 ± 0.00	1.06 ± 0.17	1.01 ± 0.12	1.28 ± 0.21	1.42 ± 0.43	1.04 ± 0.28	0.253	0.119	0.513
Butyrivibrio fibrisolvens	1.00 ± 0.00	1.06 ± 0.05	0.98 ± 0.02	0.87 ± 0.04	0.87 ± 0.11	0.95 ± 0.11	0.406	0.175	0.915
Genus Prevotella	1.00 ± 0.00	1.07 ± 0.04	1.03 ± 0.06	0.97 ± 0.04	1.09 ± 0.06	0.96 ± 0.09	0.493	0.738	0.385
Genus Bacteroides	1.00 ± 0.00	1.06 ± 0.02	0.96 ± 0.05	0.96 ± 0.03	1.06 ± 0.03	0.92 ± 0.07	0.125	0.256	0.378
Streptococcus bovis	1.00 ± 0.00	0.98 ± 0.32	0.81 ± 0.12	1.09 ± 0.10	1.96 ± 0.58	1.69 ± 0.15	0.850	0.024	0.157
Eubacterium ruminantium	1.00 ± 0.00	1.25 ± 0.07	1.15 ± 0.04	1.33 ± 0.21	1.47 ± 0.21	1.50 ± 0.21	0.218	0.018	0.896
Genus *Coprococcus*	1.00 ± 0.00^{a}	1.75 ± 0.44^{ab}	1.68 ± 0.09^{ab}	2.29 ± 0.32^{b}	2.89 ± 0.25^{b}	2.31 ± 0.27^{b}	0.006	<0.001	0.374

Note: Values presented are mean ± standard error of mean (n= 9). Means in the same row with different superscripts differ significantly (p≤0.05).

Table 5. Metabolic hydrogen balance

Variables	Control	Phloroglucinol					p-value		
		2 mmol/L	4 mmol/L	6 mmol/L	8 mmol/L	10 mmol/L	Treatment	Linear	Quadratic
$[2H]_{produced}$ (mmol)	12.232 ± 0.158	12.768 ± 0.183	12.441 ± 0.102	12.390 ± 0.098	12.400 ± 0.088	12.591 ± 0.089	0.070	0.361	0.334
$[2H]_{incorporated}$ (mmol)	7.671± 0.060^{ab}	7.729 ± 0.143^{a}	7.380± 0.058^{bc}	7.236± 0.054^{cd}	7.036 ± 0.118^{de}	6.878± 0.061^{e}	<0.001	<0.001	0.023
$[2H]_{recovery}$ (%)	62.758± 0.600^{a}	60.519 ± 0.539^{b}	59.295± 0.338^{bc}	58.410± 0.262^{cd}	56.755 ± 0.331^{d}	54.634± 0.430^{e}	<0.001	<0.001	0.084

Note: Values presented are mean ± standard error of mean (n= 9). Means in the same row with different superscripts differ significantly (p≤0.05).

phloroglucinol can inhibit methane production in the presence of methanogenesis. Our finding is in agreement to previous study that found methane decrease by phloroglucinol supplementation (Sarwono *et al*., 2019). Our results confirmed previous findings suggesting that simple phenolics such as cinnamic, caffeic, ferulic acid, *p*-coumaric, and benzoic acid can decrease methane production (Jayanegara, 2010).

Phenolic compounds might decrease methane production from the rumen through inhibition of methanogen growth and/or through inhibition of carbohydrate digestion, thus decreasing H_2 production (Jayanegara *et al*., 2015). In the present study, phloroglucinol had no effect on the methanogen population density. This showed that the decrease of methane production by phloroglucinol was not directly related to the inhibition of methanogen growth. The decrease of methane production by phloroglucinol was presumably related to the inhibition of nutrient digestibility by ruminal microbes, particularly carbohydrate digestion, thus decreasing the amount of H_2 available for methane production. The latter mechanism was reflected in our study.

Phloroglucinol supplementation significantly suppressed total gas production and DMD, indicating that it might suppress carbohydrate fermentation. Phloroglucinol supplementation also suppressed NH_3-N production after 24 h. High levels of phloroglucinol (10 mmol/L) decreased NH_3-N by 72%. This finding showed that phloroglucinol might inhibit nutrient degradation. This finding is in agreement with Kisworo *et al*. (2017) that found lowered gas production, methane, and NH_3-N by phenolic compound from solid herbal waste. The possible explanation is that phloroglucinol contain three free hydroxyl groups in its chemical structure which allow interaction with nutrients, such as carbohydrate, fibre, and protein through strong hydrogen bond formation resulting in complexes (Dobreva et al., 2011). Our finding is in agreement with previous study which suggest that phenolic compound might inhibit fibre and protein digestion (McSweeney *et al*., 2001, Jayanegara & Palupi, 2010). Of particular interest, inhibition of fibre digestion could lead to lower H_2 and CO_2 gas production, which are the main precursors for methane production. Therefore, any decrease in carbohydrate and protein digestibility by phloroglucinol could indirectly decrease methane production.

Interestingly, although phloroglucinol was likely to decrease carbohydrate digestion, phloroglucinol supplementation did not affect total SCFA production, which is the main fermentation product of carbohydrate digestion in the rumen. This might be related to changes in the level of SCFA proportion following phloroglucinol supplementation. In this study, The SCFA concentration shifted to acetate at the expense of propionate; however, butyrate was not affected by phloroglucinol. This showed that phloroglucinol might redirect rumen fermentation from methane production to acetate production. Our finding showed that treatment of 10 mmol/L phloroglucinol produced 0.274 mmol of acetate more than control, which is lower than predicted moles of acetate (1 mmol) produced from phloroglucinol

degradation in rumen (1 molecule of phloroglucinol= 2 molecules of acetate + 2 molecules of CO_2 (Tsai *et al*., 1976). This finding showed that phloroglucinol could redirect [H] toward acetate production under functioning methanogenesis *in vitro*. Meanwhile, lower amount of acetate production than prediction might showed that phloroglucinol was not only used as a hydrogen sink but also was used to inhibit nutrient digestion.

The change of rumen fermentation by phloroglucinol to acetate production was accompanied by simultaneous increment of genus *Coprococcus* relative quantity. This finding showed that *Coprococcus* plays an important role on phloroglucinol metabolism in the rumen. Increasing genus *Coprococcus* relative quantity might partly related to reduction of methane production as it increases the competition for [H] utilization with methanogen. This finding was in agreement with the study of Martinez-Fernandez *et al.* (2017). An increase of acetate production was observed simultaneously with an increase of several operational taxonomic units (OTUs) assigned to *Coprococcus* spp. when phloroglucinol was added under methanogenesis inhibited. Tsai & Jones (1975) found that genus *Coprococcus* were able to metabolize phloroglucinol.

A complete metabolic hydrogen balance showed that phloroglucinol did not have any significant effect on $[2H]_{produced}$ but significantly lowered $[2H]_{incorporated}$ and $[2H]_{recovery}$ although there was a possible redirection of [H] toward acetate by phloroglucinol addition. This finding was similar to the previous study by Ungerfeld (2015) that found a significant decrease of $[2H]_{incorporated}$ and $[2H]_{recovery}$ when methanogenesis is inhibited. This might be related to the formula used to calculate the hydrogen balance which did not include [H] sink other than main fermentation products such as propionate, butyrate, and H_2. This include redirection of [H] to acetate by phloroglucinol supplementation.

CONCLUSION

Phloroglucinol was able to significantly lower methane production by inhibiting nutrient digestibility and by possible redirection of rumen fermentation to acetate production in the rumen. Genus *Coprococcus* could be an important actor for phloroglucinol metabolism in the rumen

CONFLICT OF INTEREST

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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