**Ph. D. Thesis**

# EFFECTS OF "PHLOROGLUCINOL", AN ANTI-METHANOGENIC AGENT, ON *IN VITRO* RUMEN METHANOGENESIS

(インビトロでのルーメンメタン生成に対する抗メタン生成剤フロログルシノールの影響)

**GRADUATE SCHOOL OF BIORESOURCES**

**MIE UNIVERSITY**

**KI AGENG SARWONO**

**May, 2019**

## List of Contents

## List of Tables

## List of Figures

## General Introduction

Domesticated ruminant is of significant importance to human life as it is a vital source of food, clothing, housing, and transport. Domesticated ruminant products, such as meat and milk are essential to fulfill high quality protein, minerals, vitamins, and micronutrients needed by humans. Ruminant importance lies on its ability to convert plant structural polysaccharides, which are abundant in numbers and indigestible by humans and other animals, to edible food such as meat and milk. Ruminant developed a mutual symbiosis with microorganisms which produces the required enzymes for digesting plant structural polysaccharides. Ruminant provides the required environment and plant structural polysaccharides for the microorganisms in exchange for short-chain fatty acid (SCFA) which is absorbed and converted into energy needed by the ruminant.

However, the anaerobic fermentation of plant structural polysaccharides possesses an adverse effect for the environment in the form of methane ($CH_4$). It is widely known that ruminant is considered as one of the major contributors for global $CH_4$ emission. Rumen fermentation in the livestock sector contributes 2.12 Gt $CO_2$ eq/year of $CH_4$, which is equal to 6.3% of the total global anthropogenic emission of greenhouse gases (Smith et al., 2014). $CH_4$ also represents an economical loss from rumen digestion. $CH_4$ represents a loss of dietary energy by 2 – 12% depending on the feed. Therefore, it is of great importance to reduce the amount of $CH_4$ emission from rumen processes.

$CH_4$ is produced in the rumen by methanogen mainly by undergoing hydrogenotrophic pathway using $CO_2$ as a main carbon source and $H_2$ as a main electron donor. $CO_2$ and $H_2$ are abundant in rumen because these compounds are the main product of plant structural

polysaccharides digestion by fibrolytic bacteria and protozoa among other products. Utilization of this metabolic hydrogen in the rumen by methanogenesis helps rumen to maintain a low partial pressure of $H_2$. Partial pressure of $H_2$ plays a vital role in rumen fermentation as it influences fermentation of important microorganisms, enzyme metabolism, production of $H_2$ and other products. Failure in keeping a low partial pressure in the rumen will lead to inhibition of normal rumen functioning.

*Ruminococcus albus*, for example, produces different product based on $H_2$ concentration. *R. albus* is a rumen bacteria which considered as being among the most active bacteria involved in plant fibre degradation in the rumen (Stewart et al., 1997). R. *albus* produces ethanol, acetate, $H_2$, $CO_2$, and formate in pure culture where there is no organisms to utilize $H_2$ produced. When *R. albus* is co-cultured with methanogen, ethanol is not produced and the production of acetate increases (Moomaw and Hungate, 1963). The production of ethanol by *R. albus* is regulated by a feedback mechanism. $H_2$ is produced by *R. albus* by oxidizing NADH to $NAD^+$ and $H_2$. This reaction is limited if $H_2$ accumulates. In this condition, NADH and acetyl-coA are used to produce ethanol. When there is a $H_2$ utilizer to maintain $H_2$ low pressure, the oxidation of NADH to $NAD^+$ and $H_2$ continues and acetyl-coA is used for acetate production (Chesson and Forsberg, 1997).

Considering the importance and natural function of methanogenesis, redirection of $H_2$ utilization is proposed as a $CH_4$ inhibition strategy (McAllister and Newbold, 2008). The strategy is to create $H_2$ starvation for methanogen in rumen by providing an alternative sink for $H_2$ disposal in the rumen and redirect $H_2$ utilization from $CH_4$ production to SCFA production. Newbold et al. (2007) demonstrated that addition of fumarate reduces $CH_4$ production by 17% and increases propionate production. The $CH_4$ reduction was caused by the redirection of $H_2$

utilization by 44% from $CH_4$ production to propionate production. Fumarate is a dicarboxylic acid which is naturally occurs in the rumen as one of the products of fibre degradation. In the rumen, fumarate is reduced to propionate by using $H_2$. This finding showed that adding an alternative $H_2$ sink agent can reduce $CH_4$ production from the rumen and increase energy for ruminant in the form of SCFA.

In order to reduce $CH_4$ emission, many strategies were proposed including dietary strategies. Many compounds were used as a $CH_4$ mitigation agent including antibiotic growth promotor (AGP) such as monensin and lasalocid. Since 2006, use of AGP was banned in Europe and many countries soon followed. It had raised awareness about food safety and shift the preference from synthetic compound to natural compounds originated from plants for mitigating $CH_4$ emission from ruminant (Makkar et al., 2007). The use of polyphenolic compounds, such as hydrolysable tannin (HT), condensed tannin (CT), and simple phenolic such as cinnamic, cafeic, ferulic acid, p-coumaric has attracted many attentions from scientists. The reduction of $CH_4$ production depends on the type and dose of phenolic compounds supplemented. McSweeney et al. (2001) suggested that both of HT and CT were metabolized by rumen microorganisms resulting in phloroglucinol.

Phloroglucinol (1,3,5-trihydroxybenzene) is a simple phenolic which naturally occurs in the rumen as a result of rumen metabolism of tannin. Phloroglucinol can be degraded by several rumen microorganisms into acetate by using $H_2$ in a pure culture experiment (Krumholz and Bryant, 1986). Martinez-Fernandez et al. (2017) also demonstrated that phloroglucinol can redirect $H_2$ utilization to acetate production when methanogenesis is inhibited. This finding offers a possibility to use phloroglucinol as a $CH_4$ mitigation agent by redirection of rumen fermentation. However, very little is known of the effect of phloroglucinol on $CH_4$ production, rumen fermentation, and microbial population when supplemented.

Diet is also one of the factors that can affect $CH_4$ production in the rumen (Beauchemin et al., 2008). $CH_4$ production in the rumen is directly related to the characteristic of the dietary substrate including the amount of $H_2$ produced from its digestion, change in ruminal pH, and microorganisms present (Johnson and Johnson, 1995, Janssen, 2010). Different ratios of forage and concentrate in the diet can change the amount of $CH_4$ produced (Aguerre et al., 2011). A low forage: concentrate ratio may reduce $CH_4$ production because concentrate degradation in the rumen favor formation of propionate which consume $H_2$. On the contrary, high forage: concentrate ratio may increase $CH_4$ production because it prefers formation of acetate which in the process liberate $H_2$ (Valadares et al., 1999). Therefore, it is important to understand the effect of phloroglucinol addition in different forage: concentrate ratios on $CH_4$ production. However, the effect of phloroglucinol in different forage: concentrate ratio on $CH_4$ production is little to known.

Therefore, the objectives of this study are to investigate the inhibitory effect of phloroglucinol on $CH_4$ production, rumen fermentation, and rumen microbial population when it is supplemented alone and in different forage: concentrate ratio.

Chapter 1 investigates the effect of phloroglucinol alone on $CH_4$ emission, rumen fermentation profiles, and rumen microbial population density *in vitro*.

Chapter 2 investigates the effect of phloroglucinol and forage to concentrate (F:C) ratio on $CH_4$ emission, rumen fermentation profiles, and microbial population density.

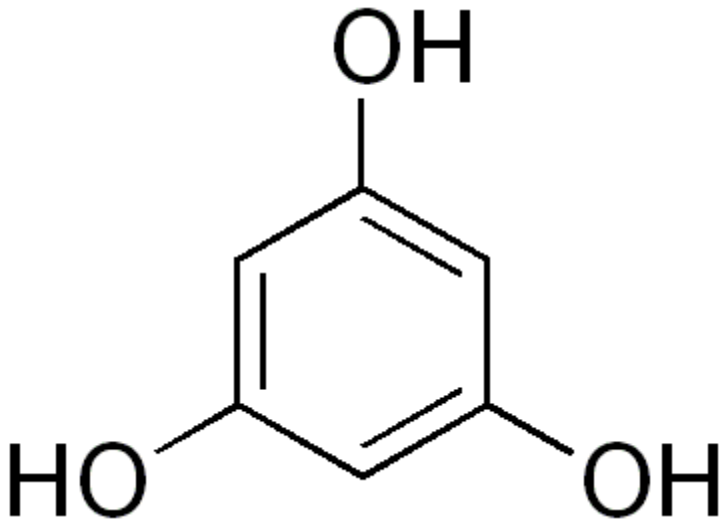



Figure 1-1. Chemical structure of phloroglucinol.

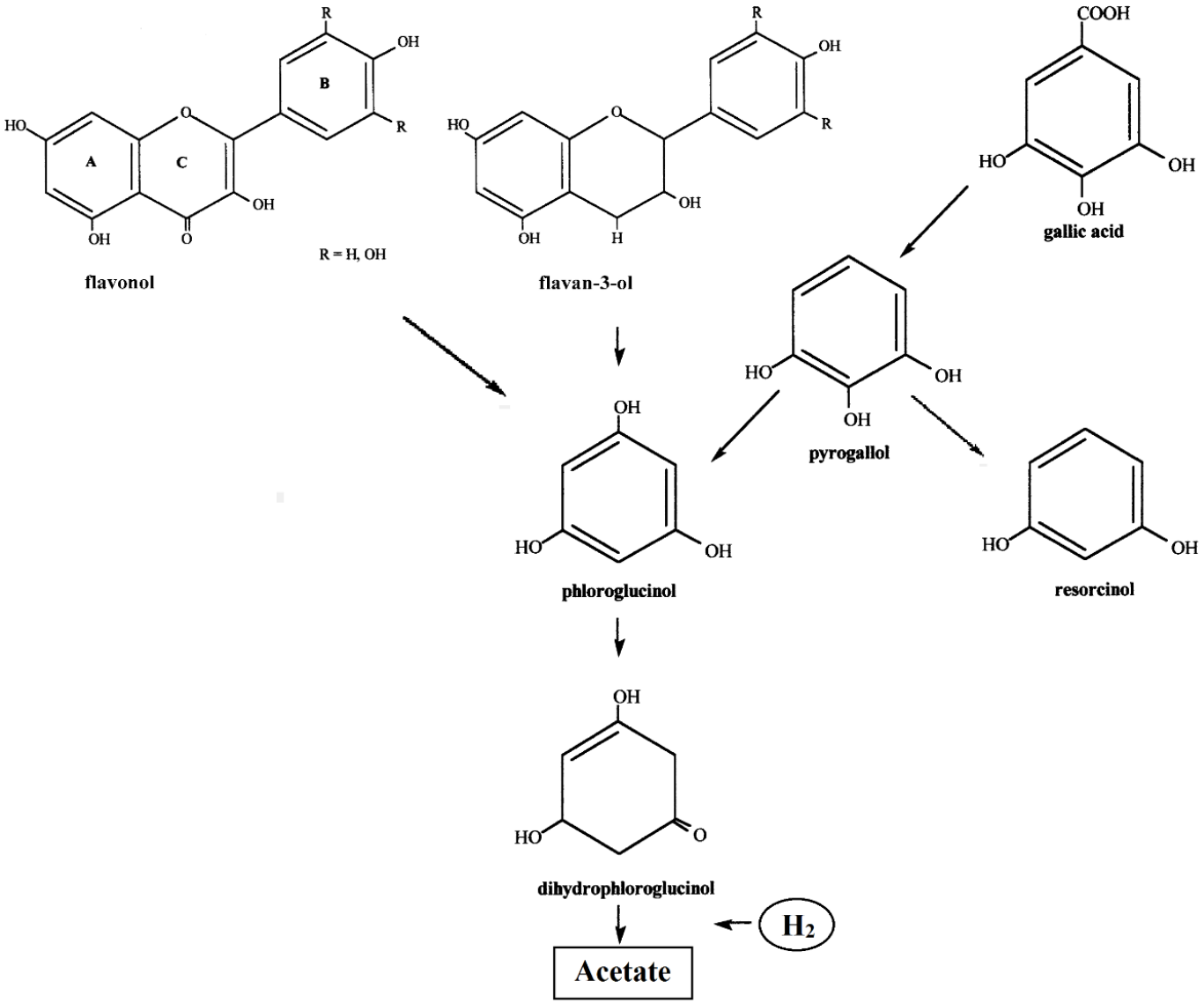



Figure 1-2. Pathway of degradation of phloroglucinol in the rumen (McSweeney et al. 2001).

# Chapter 1

# Effect of phloroglucinol supplementation on methanogenesis, *in vitro* rumen fermentation, and microbial population density

## 2.1. Introduction

Ruminants are one of the greatest sources of $CH_4$, a major greenhouse gas that poses a serious environmental threat, with 23-fold more global warming potential (GWP) than $CO_2$ (Steinfeld et al., 2006). Rumen fermentation in the livestock sector contributes 2.12 Gt $CO_2$ eq/year of $CH_4$, which is equal to 6.3% of the total global anthropogenic emission of greenhouse gases (Smith et al., 2014). Incremental increases in the atmospheric $CH_4$ 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, $CH_4$ 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 $CH_4$ (Johnson and Johnson, 1995). Therefore, it is important to reduce $CH_4$ emission from ruminants for both environmental and economic benefits.

$CH_4$ is a natural by-product of anaerobic fermentation in the rumen. $CH_4$ 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. $CH_4$ production serves as an electron sink to help rumens 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 (Moomaw and Hungate, 1963, Knapp et al., 2014). Considering the nature and function of methanogenesis,

McAllister and Newbold (2008) proposed a strategy to reduce $CH_4$ production by redirecting rumen fermentation to reduce methanogenesis. One of the strategies proposed is the supplementation of an alternative $H_2$ sink in an effort to redirect rumen fermentation away from methanogenesis. The use of dicarboxylic acids, such as fumarate, malate, and aspartate to reduce $CH_4$ production in the rumen has been evaluated (Newbold et al., 2007). These compounds were found to reduce $CH_4$ production but increase short-chain fatty acid (SCFA) production, which is the main energy source of ruminant.

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 hydrogen (Tsai et al., 1976, Krumholz and Bryant, 1986). Previous study showed that phloroglucinol can redirect $H_2$ utilization to acetate when methanogenesis is completely inhibited (Martinez-Fernandez et al. 2017). This showed that phloroglucinol may act as an alternative $H_2$ sink agent, and might be able to reduce $CH_4$ production. Supplementation of phloroglucinol can create competition for hydrogen between methanogen and phloroglucinol-degrading bacteria, which will reduce the amount of hydrogen available for methanogens to produce $CH_4$. However, the effect of phloroglucinol supplemented alone on ruminal mixed cultures remains unknown. Therefore, the objective of this study was to investigate the effect of phloroglucinol on $CH_4$ production, fermentation profiles, and microbial population during *in vitro* rumen fermentation.

## 2.2. Materials and Methods

### *2.2.1. Animal and Sampling*

Animal handling was performed according to the Mie University guidelines. 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.

### *2.2.2. In vitro incubation*

Pooled ruminal fluid was strained through four layers of surgical gauze. The filtered fluid was mixed with McDougall buffer (McDougall, 1948) 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 2-1. 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 glass syringe. The composition of the headspace gas 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 digestibility. 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 (Weatherburn, 1967). Molar amounts of SCFA and $CH_4$ were used to calculate $CH_4$ per total SCFA ($CH_4$/SCFA). Molar amounts of $CH_4$ were calculated by assuming an ideal gas constant of 0.082 at air pressure 1 atm and 20°C. A complete hydrogen balance was calculated in accordance to method by Ungerfeld (2015).

### *2.2.3. 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.

### *2.2.4. Quantitation of the microbial population by quantitative real-time PCR*

Primers for real-time PCR are listed at Table 2-2. Real-time PCR was conducted using a StepOnePlus® Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Real-time PCR was used to determine the relative abundance of following 11 microbial populations using previously validated primers (Table 2-2), methanogen, *F. succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Streptococcus bovis*, *Selomonas ruminantium*, *Butyrivibrio fibrisolvens*, genus *Prevotella*, genus *Bacteroides*, *Eubacterium ruminantium*, and genus *Coprococcus*, conducted by using comparative $C_T$ method. Total bacteria was used as an endogenous control.

$C_T$ of 16S ribosomal RNA gene (16S rDNA) of total bacteria was quantified according to the method described by Abrar et al. (2016). The reaction mixture consisted of 10 µL of Thunderbird SYBR qPCR master mix (Toyobo Co. LTD., Osaka, Japan), 6.2 µL of sterile Milli-Q water, 0.6 µL of each specific forward and reverse primer, 0.4 µL 50x ROX, and 1 µL of template DNA. The PCR condition 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.

$C_T$ of *R. albus* was quantified according to method described by Koike and Kobayashi (2001) with some modification. The reaction mixture consisted of 10 µL of Thunderbird SYBR qPCR master mix, 7.6 µL of sterile Milli-Q water, 0.5 µL of each specific forward and reverse primer, 0.4 µL 50x ROX, and 1 µL of template DNA. The PCR conditions 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.

$C_T$ of *R. flavefaciens* was quantified according to method described by Tajima et al. (2001), with some modification. The reaction mixture consisted of 10 µL of Thunderbird SYBR qPCR master mix, 6.8 µL of sterile Milli-Q water, 0.9 µL of each specific forward and reverse

primer, 0.4 µL 50x ROX, and 1 µL of template DNA. The PCR condition included one cycle of polymerase activation 95°C for 1 min, 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.

$C_T$ of *F. succinogenes* was quantified according to the method described by Tajima et al. (2001) with some modification. The reaction mixture consisted of 10 µL of Thunderbird SYBR qPCR master mix, 6.8 µL of sterile Milli-Q water, 0.9 µL of each specific forward and reverse primer, 0.4 µL 50x ROX, and 1 µL of template DNA. The PCR condition 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.

$C_T$ of methanogens were quantified using a methanogen-specific gene, mcrA, following the method described by Abrar et al. (2016). The reaction mixture consisted of 10 µL of Thunderbird SYBR qPCR master mix, 6.2 µL of sterile Milli-Q water, 1.2 µL of each specific forward and reverse primer, 0.4 µL 50x ROX, and 1 µL of template DNA. The PCR condition 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 30 s.

$C_T$ of 16S rDNAs of *S. bovis, S. ruminantium, B. fibrisolvens, Prevotella, Eubacterium ruminantium,* and genus *Coprococcus,* was quantified according to the method described by Mullins et al. (2013).

$C_T$ of 16S rDNA of genus *Bacteroides* was quantified according to the method described by Layton et al. (2006).

$C_T$ of 16S rDNA of *Coprococcus* was quantified according to the method described by Da Silva et al. (2018).

*2.2.5. 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, where appropriate. Polynomial contrasts were conducted to test the data for linear and quadratic trends. 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. Statistical analysis was conducted using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA).

## 2.3 Results

*2.3.1. Effect of phloroglucinol on in vitro rumen fermentation.*

Total gas and $CH_4$ production were significantly reduced 24 h after the addition of phloroglucinol ($P<0.001$; Figure 2-1 and 2-2). Compared with the control, gas production was reduced 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 reduction ($P<0.001$). Simultaneously, $CH_4$ production was significantly reduced by 8.1, 12.0, and 15.0% compared with the control, following supplementation with 6, 8, and 10 mmol/L phloroglucinol, respectively.

Supplementing phloroglucinol significantly reduced dry matter digestibility (DMD) ($P<0.001$; Figure 2-3). Higher doses of phloroglucinol reduced DMD in a linear and concentration-dependent manner ($P<0.001$). DMD was reduced 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.

Supplementation of phloroglucinol had no significant effect on $CH_4$ production when $CH_4$ was presented as $CH_4$ per digested dry matter ($CH_4$/DDM; Figure 2-8).

Phloroglucinol did not affect ruminal pH after 24 h incubation (Figure 2-5); 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; Figure 2-4). $NH_3$-N decreased with increasing doses of phloroglucinol ($P$<0.001). Supplementation of 8 and 10 mmol/L of phloroglucinol reduced $NH_3$-N by 62 and 70%, respectively.

Total SCFA concentration was not significantly affected by phloroglucinol supplementation. (Figure 2-7). Phloroglucinol had no linear or quadratic effect on total SCFA was not found. When $CH_4$ was presented as $CH_4$ per total SCFA ($CH_4$/Total SCFA), a significant reduction was observed ($P$<0.001; Figure 2-9). Addition of 6, 8, and 10 mmol/L phloroglucinol reduced $CH_4$/total SCFA by 8, 12, and 16%, respectively. The effect was linear ($P$<0.001), rather than quadratic.

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 reduced ($P$<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 (Figure 2-10).

*2.3.2. Effects of phloroglucinol on microbial population density.*

Phloroglucinol supplementation did not significantly affect the relative quantity of all target with the exception for genus *Coprococcus* ($P$=0.006; Figure 2-21). 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; Figure 2-19) and *Eubacterium ruminantium* ($P$=0.018; Figure 2-20).

*2.3.3. Effects of phloroglucinol on hydrogen balance.*

Phloroglucinol supplementation did not have any significant effect on $[2H]_{produced}$ ($P$=0.061; Figure 2-22). Phloroglucinol significantly reduced $[2H]_{incorporated}$ and $[2H]_{recovery.}$ A significant linear and quadratic effect of phloroglucinol on $[2H]_{incorporated}$ was observed (Figure 2-23). A linear effect was also observed on $[2H]_{recovery}$ (Figure 2-24).

## 2.4. Discussion

Phloroglucinol supplementation at 6 mmol/L or higher significantly reduced $CH_4$ production *in vitro* in a dose-dependent manner. This study is the first to document the inhibitory effect of phloroglucinol alone on $CH_4$ emission in mixed ruminal culture *in vitro*. Our results confirmed previous findings suggesting that simple phenolics such as cinnamic, caffeic, ferulic acid, *p*-coumaric, and benzoic acid can reduce $CH_4$ production (Jayanegara, 2010, Ushida et al., 1989, Asiegbu et al., 1995).

Phenolic compounds might reduce $CH_4$ production from the rumen through inhibition of methanogen growth and/or through inhibition of carbohydrate digestion, thus reducing $H_2$ production (Tavendale et al., 2005, Jayanegara et al., 2015). In the present study, phloroglucinol had no effect on the methanogen population density. This showed that $CH_4$ reduction by phloroglucinol was not directly related to the inhibition of methanogen growth. $CH_4$ reduction by phloroglucinol was presumably related to inhibition of nutrient digestibility by ruminal microbes, particularly carbohydrate digestion, thus reducing the amount of $H_2$ available for $CH_4$ 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. This is complemented by the changes in the proportion of SCFA. Propionate production was reduced by phloroglucinol addition. This finding indicate that phloroglucinol might inhibit starch digestion. In this study, corn was used as a substrate, along with wheat bran and Italian-rye grass hay. Corn starch is easily fermented in the rumen by amylolytic bacteria, resulting in higher levels of gas and propionate compared with fibrous diets (Kaufmann et al., 1980). Therefore, any reduction in starch fermentation could reduce total gas production and DMD.

The effect of phloroglucinol on gas production and DMD in the present study, showed that $CH_4$ inhibition by phloroglucinol might be partly caused by reduced carbohydrate digestion. Phenolic compounds and derivatives, such as hydrolysable tannin, condensed tannin, and phloroglucinol, contain a functional group, which is a free hydroxyl group (−OH) attached to a benzene ring. Free hydroxyl groups allow interaction between phenolic compounds and nutrients, such as carbohydrate, fibre, and protein through strong hydrogen bond formation resulting in complexes (Silanikove et al., 2001). Tannins inhibit fibre digestion by creating complexes with

lignocellulose, preventing microbial digestion of fibre (McSweeney et al., 2001). Inhibition of carbohydrate digestion could lead to reduced $H_2$ and $CO_2$ gas production, which are the main precursors for $CH_4$ production. Therefore, any reduction in fibre digestibility by phenolic compounds and their derivatives could indirectly reduce $CH_4$ production.

Phloroglucinol supplementation also suppressed $NH_3$-N production after 24 h. High levels of phloroglucinol (10 mmol/L) reduced $NH_3$-N by 72%. This finding further demonstrated that phloroglucinol might exert an inhibitory effect on nutrient digestion, and is partly related to reduced DMD. Phenolic compounds have an inhibitory effect on the ruminal digestion of proteins (Makkar, 2003, Mueller-Harvey, 2006, Jayanegara et al., 2012a, Kondo et al., 2014, Getachew et al., 2008). Tannin creates complexes with proteins by forming hydrogen bonds between its free hydroxyl groups and the carbonyl groups of peptides in the protein (McSweeney et al., 2001, Silanikove et al., 2001), it may inhibit the ruminal digestion of proteins through suppression of protease activity, suppression of microbial growth or activity, or by protection of the substrate protein. High levels of $NH_3$-N reduction might indicate that phloroglucinol has a higher affinity for protein than fibre. This is consistent with the findings of McSweeney et al. (2001), suggesting that the effects of phenolics on fibre digestion are secondary anti-nutritional effects compared with protein digestion.

Interestingly, although phloroglucinol was likely to reduce carbohydrate digestion due to the lower level of total gas production and propionate, phloroglucinol supplementation did not affect total SCFA production, which is the main fermentation by-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 act as an alternative $H_2$ sink agent, and redirect $H_2$ utilization from $CH_4$ production to acetate. This finding is complemented with increasing relative quantity of *Coprococcus.* This finding showed that *Coprococcus* is a significant contributor to rumen metabolism of phloroglucinol. 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 OTUs assigned to *Coprococcus* spp. were when phloroglucinol was added when methanogenesis was inhibited. Tsai and Jones (1975) found that eight strains of ruminal bacteria from *Streptococcus* and *Coprococcus* were able to metabolize phloroglucinol. One strain of *Coprococcus* was found to degrade one molecule of phloroglucinol into two molecules of acetate and two molecules of $CO_2$ (Tsai et al., 1976). A further study found that *Eubacterium oxidoreducens* utilizes hydrogen to reduce phloroglucinol to acetate (Krumholz and Bryant, 1986). By redirecting $H_2$ utilization from $CH_4$ production to acetate production, phloroglucinol might be able to substitute the lost SCFA from inhibited carbohydrate digestion and simultaneously reducing $CH_4$ production from the rumen.

Phloroglucinol supplementation was also found to significantly reduce $CH_4$/Total SCFA in a dose-dependent manner. This suggests that the level of $CH_4$ production can be reduced by phloroglucinol without lowering the amount of energy available for the ruminant in the form of SCFA. Previous studies have shown similar effect of phenolic compounds on $CH_4$/total SCFA (Jayanegara et al., 2012b). Those authors also stated that reducing $CH_4$/total SCFA showed fermentative productivity, which might occur when an alternative $H_2$ sink is available. Our findings suggest that phloroglucinol is most probably act as an $H_2$ sink, redirecting $H_2$ utilization and reducing $CH_4$ production.

A complete hydrogen balance showed that phloroglucinol did not have any significant effect on [2H] produced but significantly reduced $[2H]_{incorporated}$ and $[2H]_{recovery}$ although there was a possible redirection of $H_2$ to acetate by phloroglucinol addition. This finding was similar to the previous study by Ungerfeld (2015) that found a significant reduction of $[2H]_{incorporated}$ and $[2H]_{recovery}$ when methanogenesis is inhibited. The author further suggested that the formula used to calculate the hydrogen balance did not include a major [H] sink when methanogenesis is inhibited. The potential major [H] sinks not accounted by the formula are fermentation product other than propionate, butyrate, and $H_2$, microbial biomass, and reductive acetogenesis.

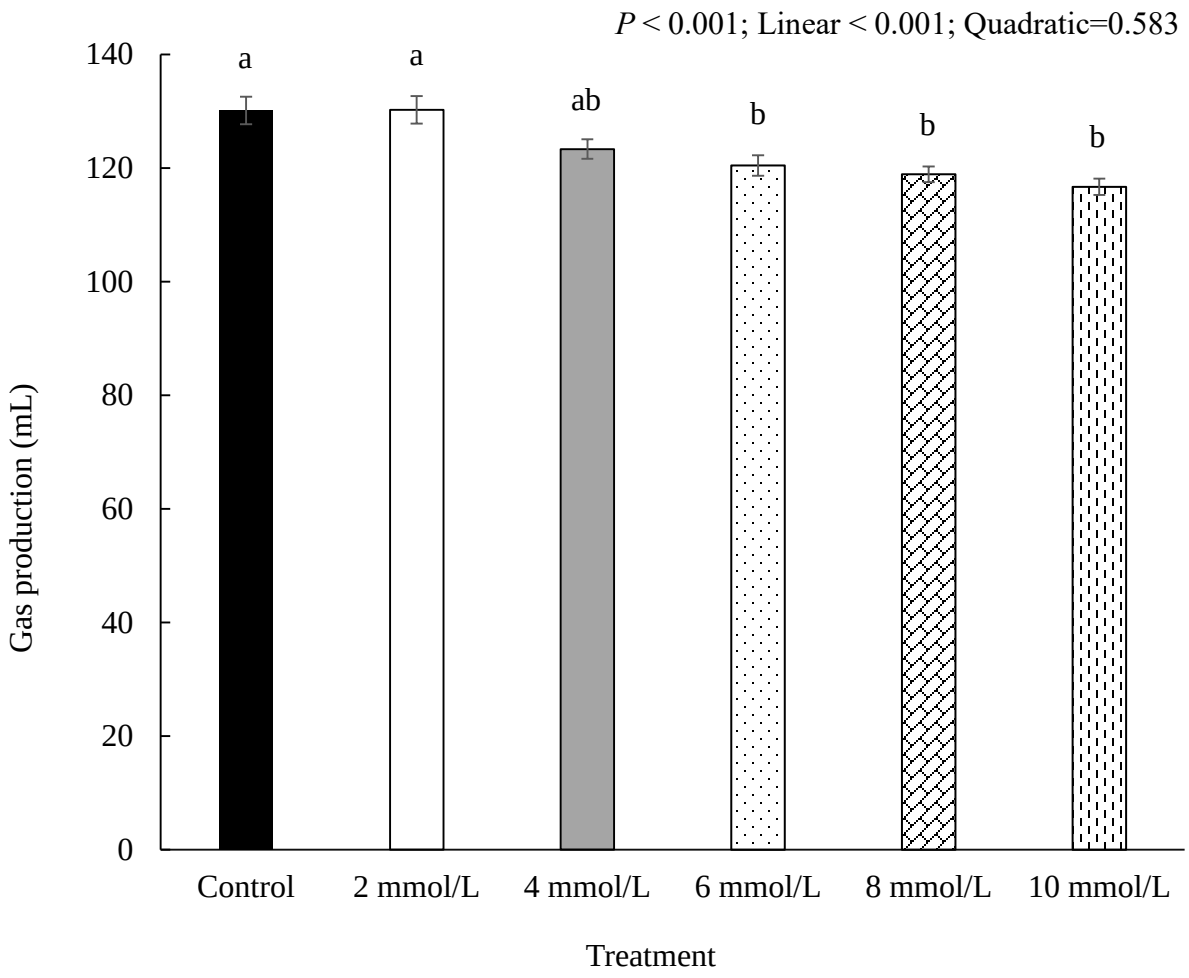
In conclusion, Phloroglucinol was able to significantly reduce $CH_4$ production. The effect was dose-dependent following supplementation at 6 mmol/L or higher. Phloroglucinol reduced $CH_4$ indirectly as it did not affect the number of methanogens. Phloroglucinol supplementation did not affect total SCFA production, but reduced $NH_3$-N production significantly. Phloroglucinol might reduce $CH_4$ production by redirecting the $H_2$ utilization of acetate and partially by inhibiting nutrient digestibility.

Table 2-1. 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 |

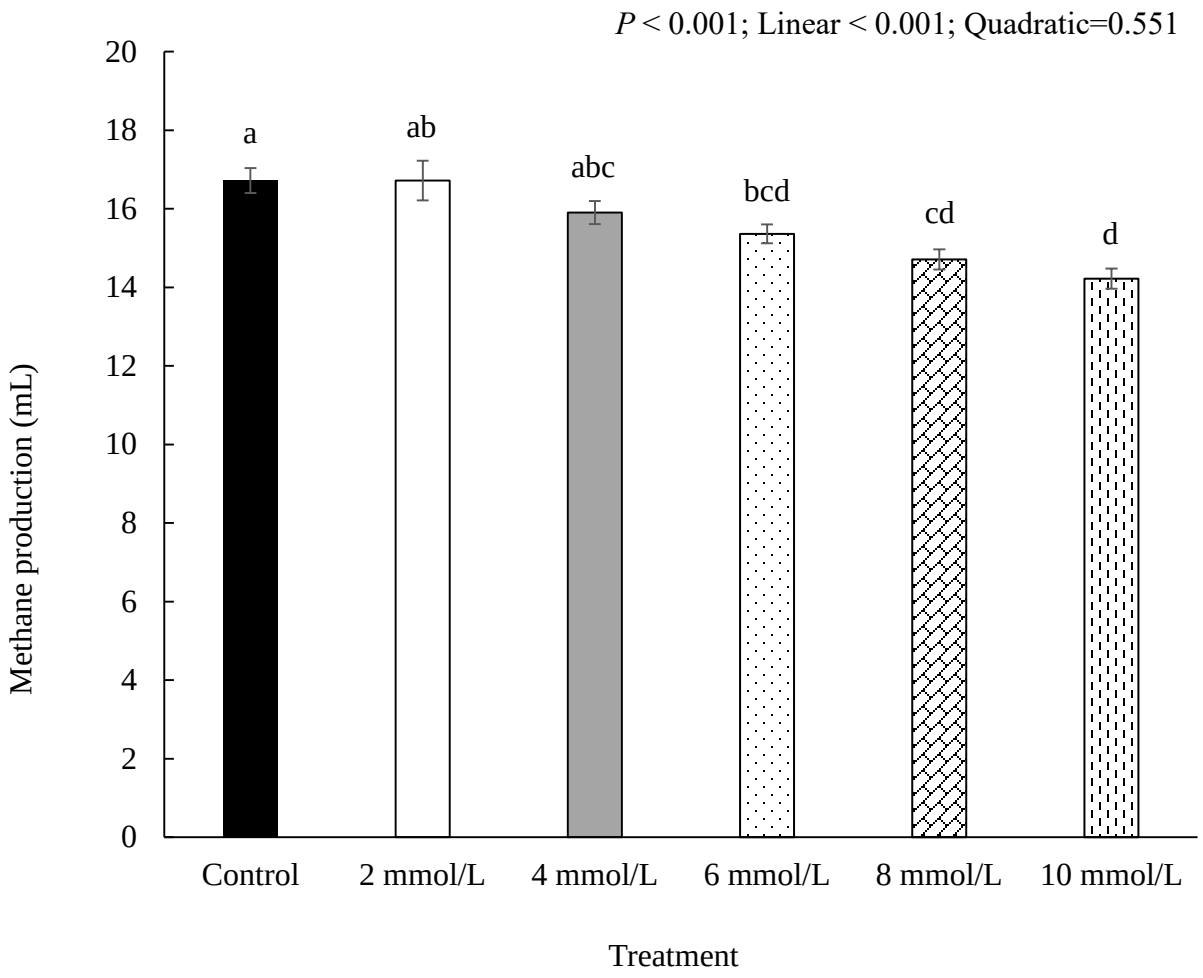
Table 2-2. Primers used in recent study.

| Target | Name | Sequence (5′ → 3′ ) | Reference |
|---|---|---|---|
| Total bacteria | 1114-f | CGGCAACGAGCGCAACCC | (Denman and McSweeney, 2006) |
| | 1275-r | CCATTGTAGCACGTGTGTAGCC | |
| *Ruminococcus albus* | Ra1281f | CCCTAAAAGCAGTCTTAGTTCG | (Koike and Kobayashi, 2001) |
| | Ra1439r | CCTCCTTGCGGTTAGAACA | |
| *Ruminococcus flavefaciens* | Rflf | GGACGATAATGACGGTACTT | (Tajima et al., 2001) |
| | Rflr | GCAATCYGAACTGGGACAAT | |
| *Fibrobacter succinogenes* | Fsf | GGTATGGGATGAGCTTGC | (Tajima et al., 2001) |
| | Fsr | GCCTGCCCCTGAACTATC | |
| Methanogens | q-mcrA-f | TTCGGTGGATCDCARAGRGC | (Denman et al., 2007) |
| | q-mcrA-r | GBARGTCGWAWCCGTAGAATCC | |
| *Streptococcus bovis* | StrBov 2F | TTCCTAGAGATAGGAAGTTTCTTCGG | Mullins et al. 2013 |
| | StrBov 2R | ATGATGGCAACTAACAATAGGGGT | |
| *Selomonas ruminantium* | SelRum 2F | CAATAAGCATTCCGCCTGGG | Mullins et al. 2013 |
| | SelRum 2R | TTCACTCAATGTCAAGCCCTGG | |
| *Butyrivibrio fibrisolvens* | ButFib 2F | ACCGCATAAGCGCACGGA | Mullins et al. 2013 |
| | ButFib 2R | CGGGTCCATCTTGTACCGATAAAT | |
| *Prevotella* | PrevGen 4F | GGTTCTGAGAGGAAGGTCCCC | Mullins et al. 2013 |
| | PrevGen 4R | TCCTGCACGCTACTTGGCTG | |
| *Bacteroides* | AllBac 296f | GAGAGGAAGGTCCCCCAC | Layton et al. 2006 |
| | AllBac 412r | CGCTACTTGGCTGGTTCAG | |
| *Eubacterium ruminantium* | EubRum 2F | CTCCCGAGACTGAGGAAGCTTG | Mullins et al. 2013 |
| | EubRum 2R | GTCCATCTCACACCACCGGA | |
| *Coprococcus* | CopGenF | CATCCTGATGACGGTTTCTTAACC | Da Silva et al. 2018 |
| | CopGenR | GTTGCGGGACTTAACCCA | |



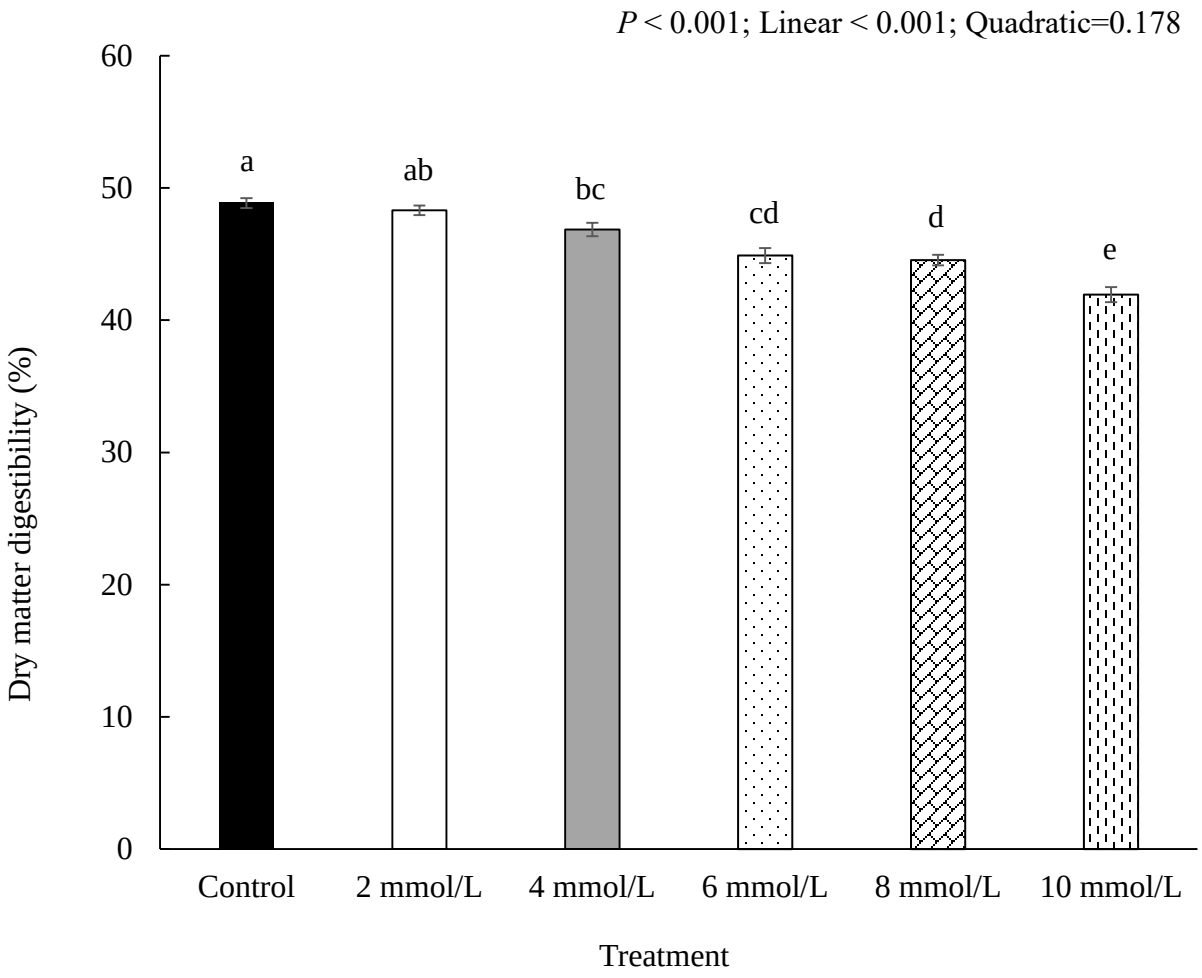

Error bar shows standard error of mean

Figure 2-1. Effect of phloroglucinol on total gas production.



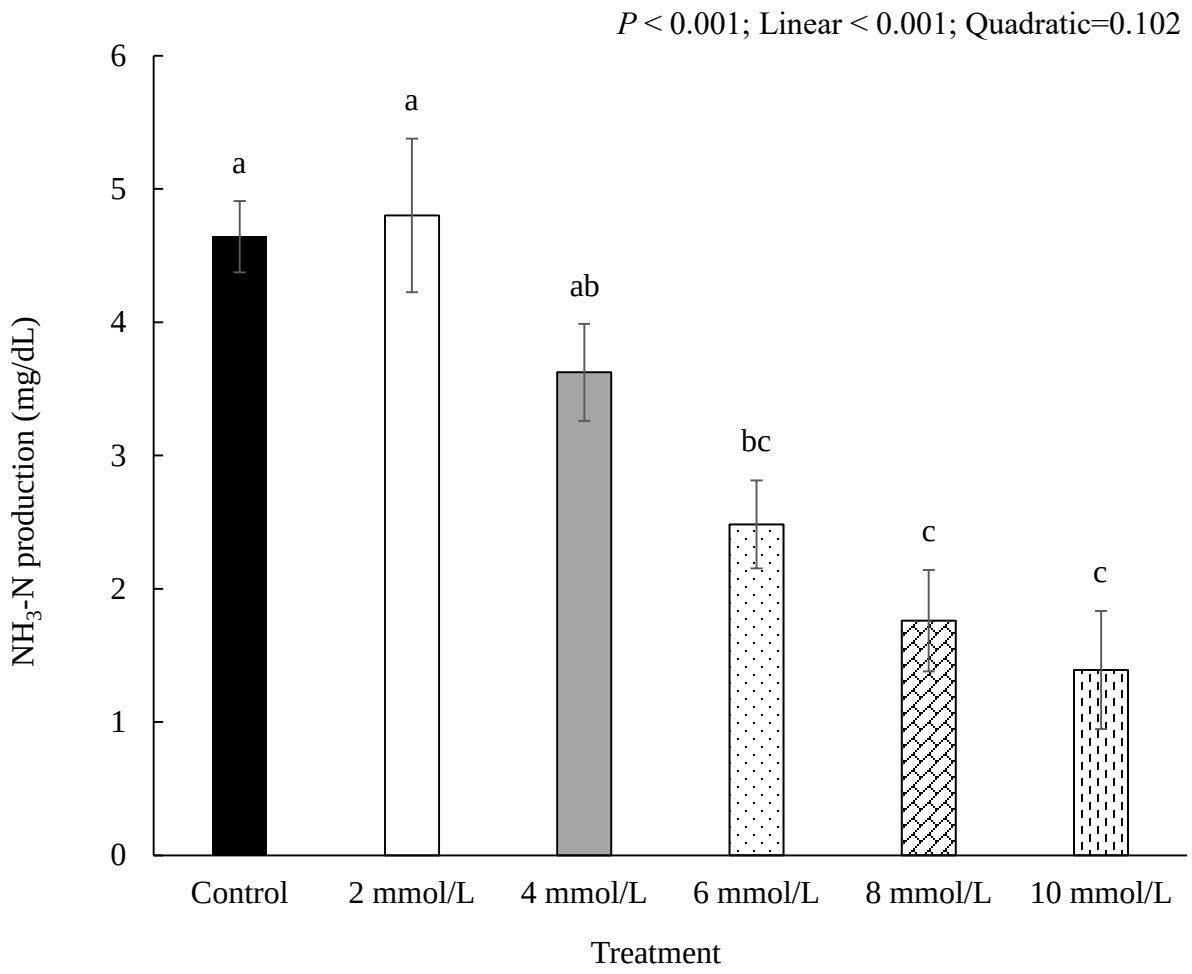

Error bar shows standard error of mean

Figure 2-2. Effect of phloroglucinol on methane production.



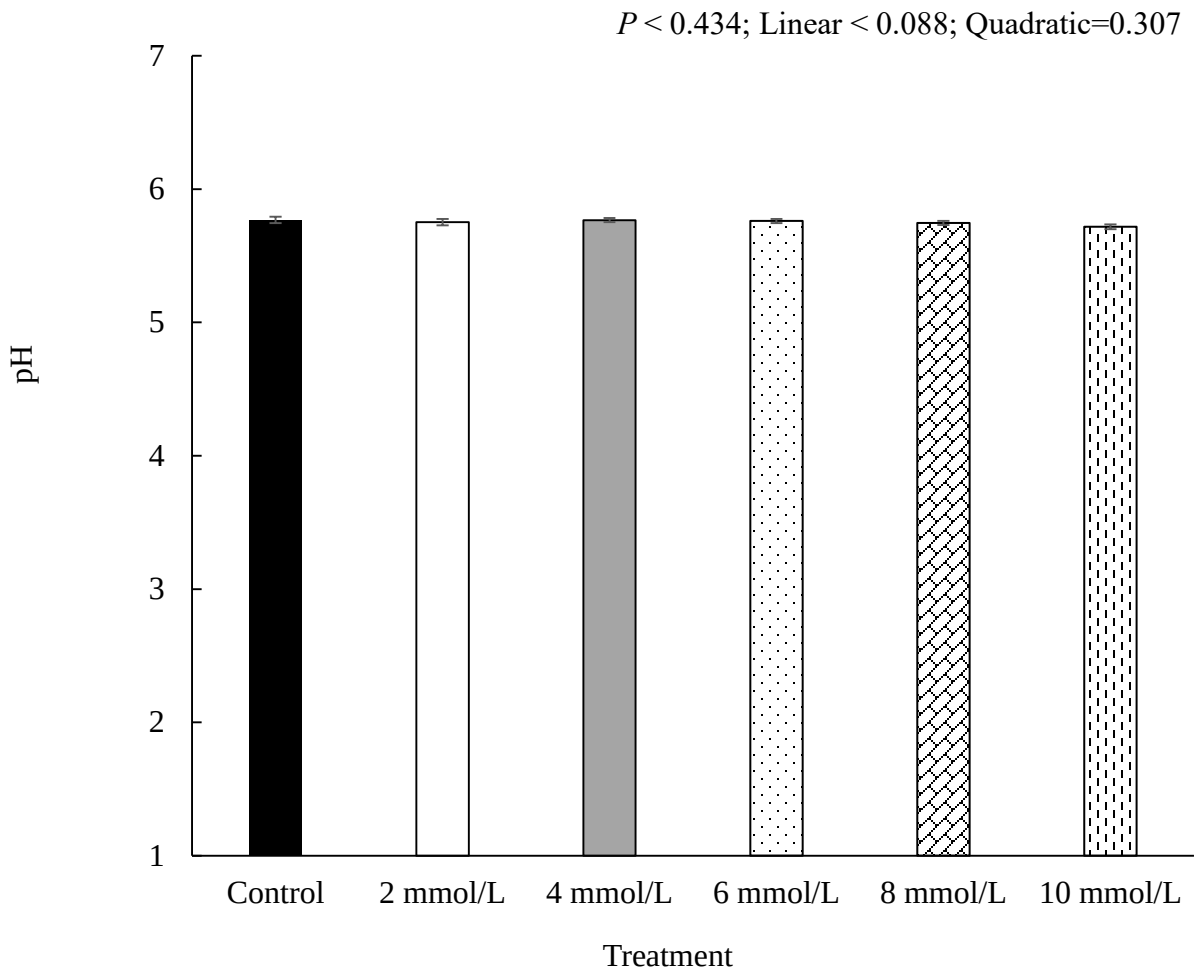

Error bar shows standard error of mean

Figure 2- 3. Effect of phloroglucinol on dry matter digestibility (DMD).



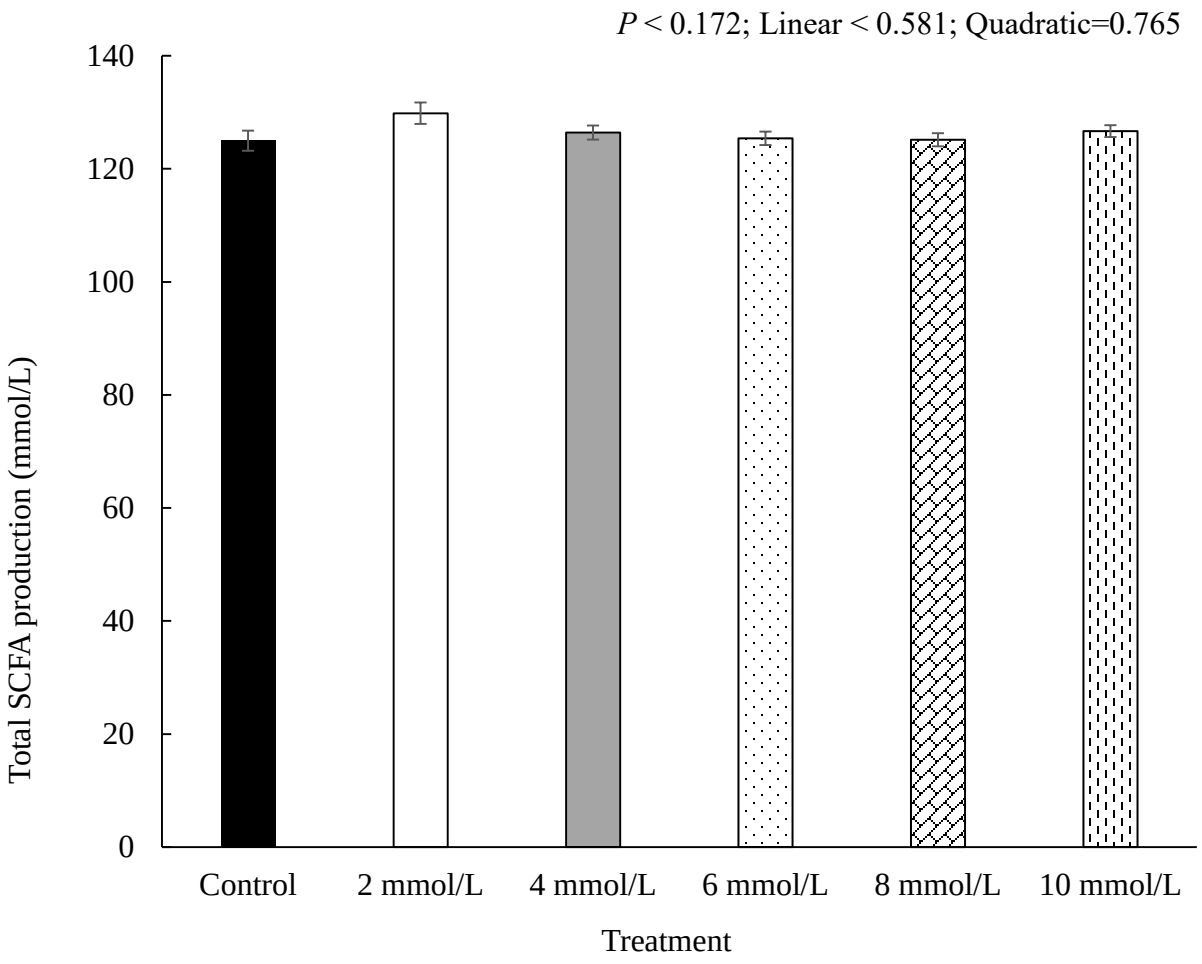

Error bar shows standard error of mean

Figure 2- 4. Effect of phloroglucinol on $NH_3$-N.




Error bar shows standard error of mean

Figure 2- 5. Effect of phloroglucinol on pH.




Error bar shows standard error of mean

Figure 2- 6. Effect of phloroglucinol on total SCFA production.

Acetate $P < 0.001$; Linear < 0.001; Quadratic=0.989
Propionate $P < 0.001$; Linear < 0.001; Quadratic=0.987
Butyrate $P < 0.859$; Linear < 0.724; Quadratic=0.992

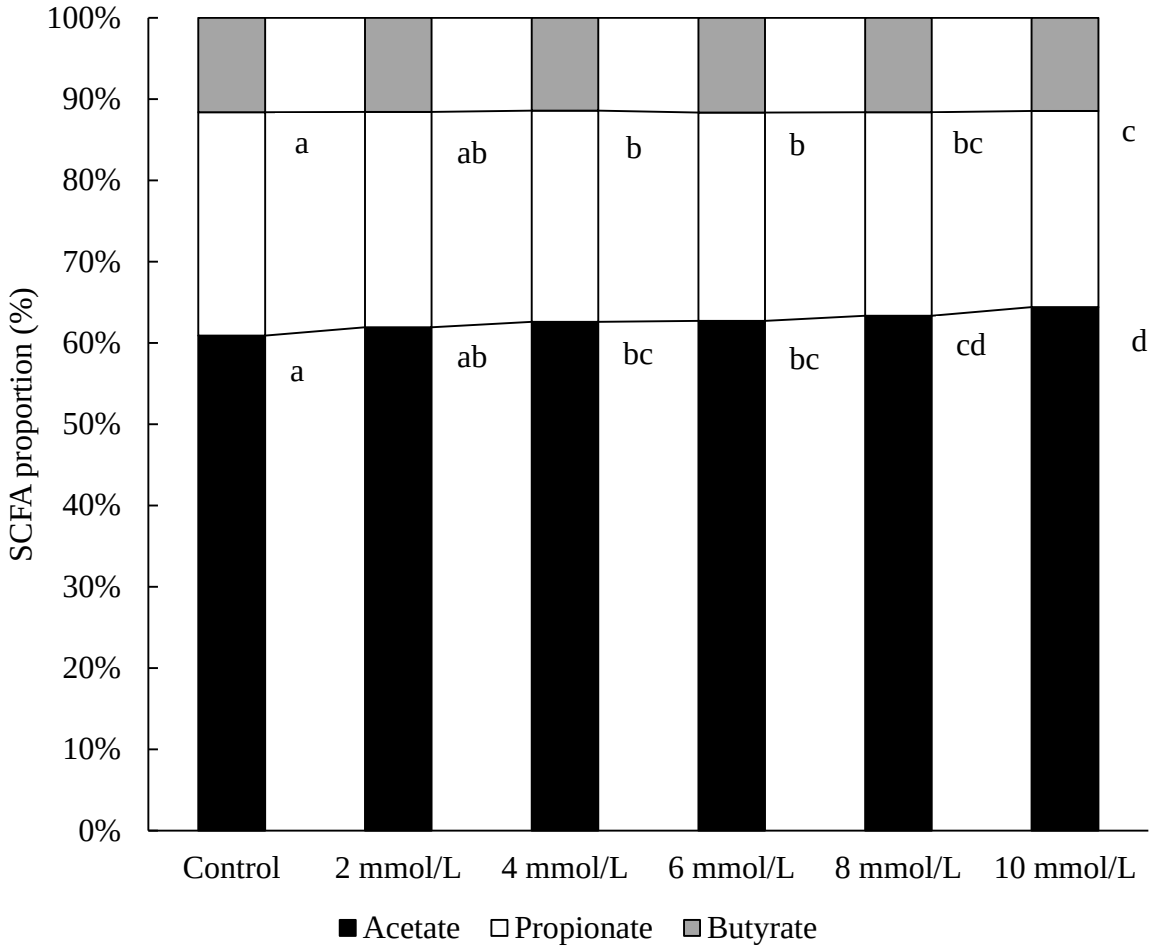
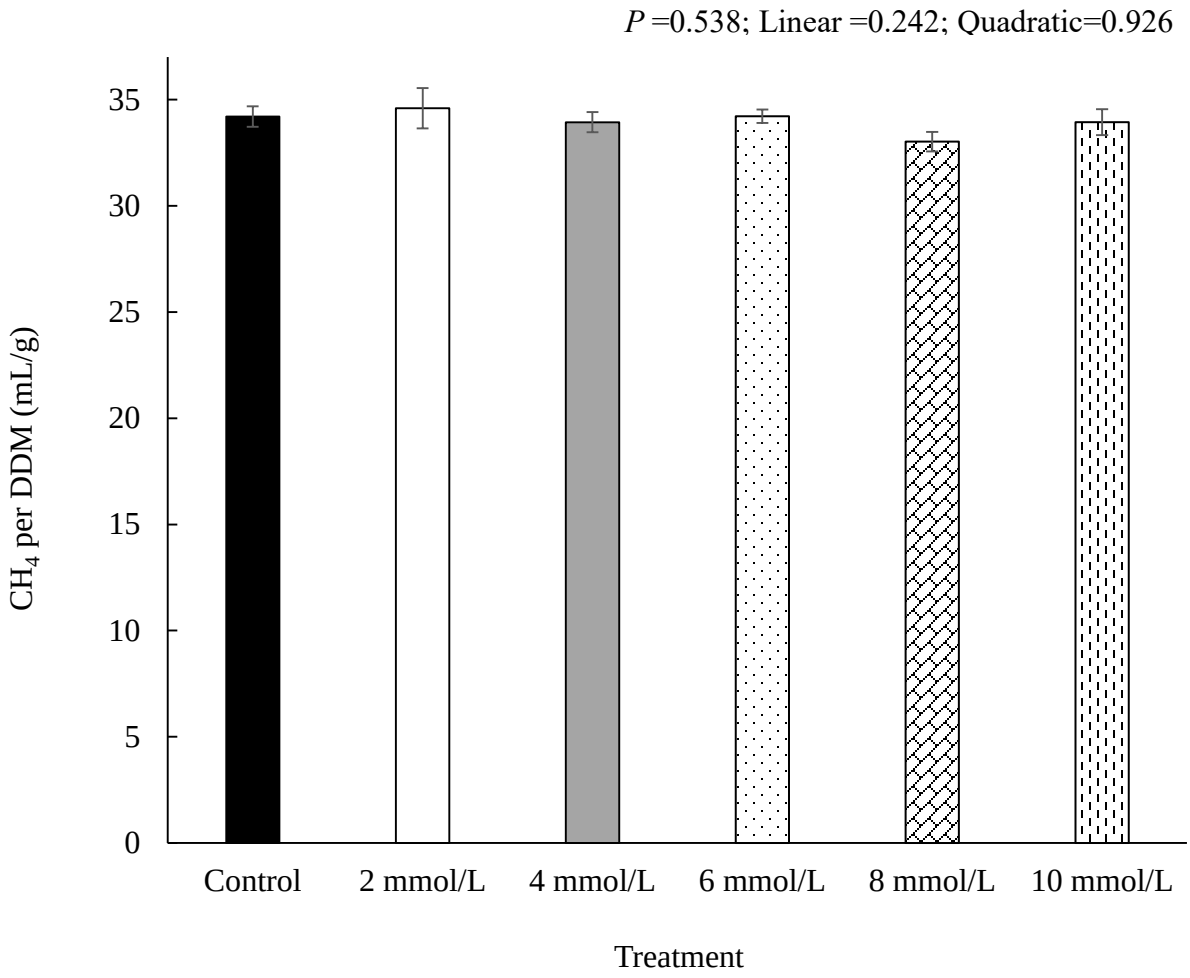
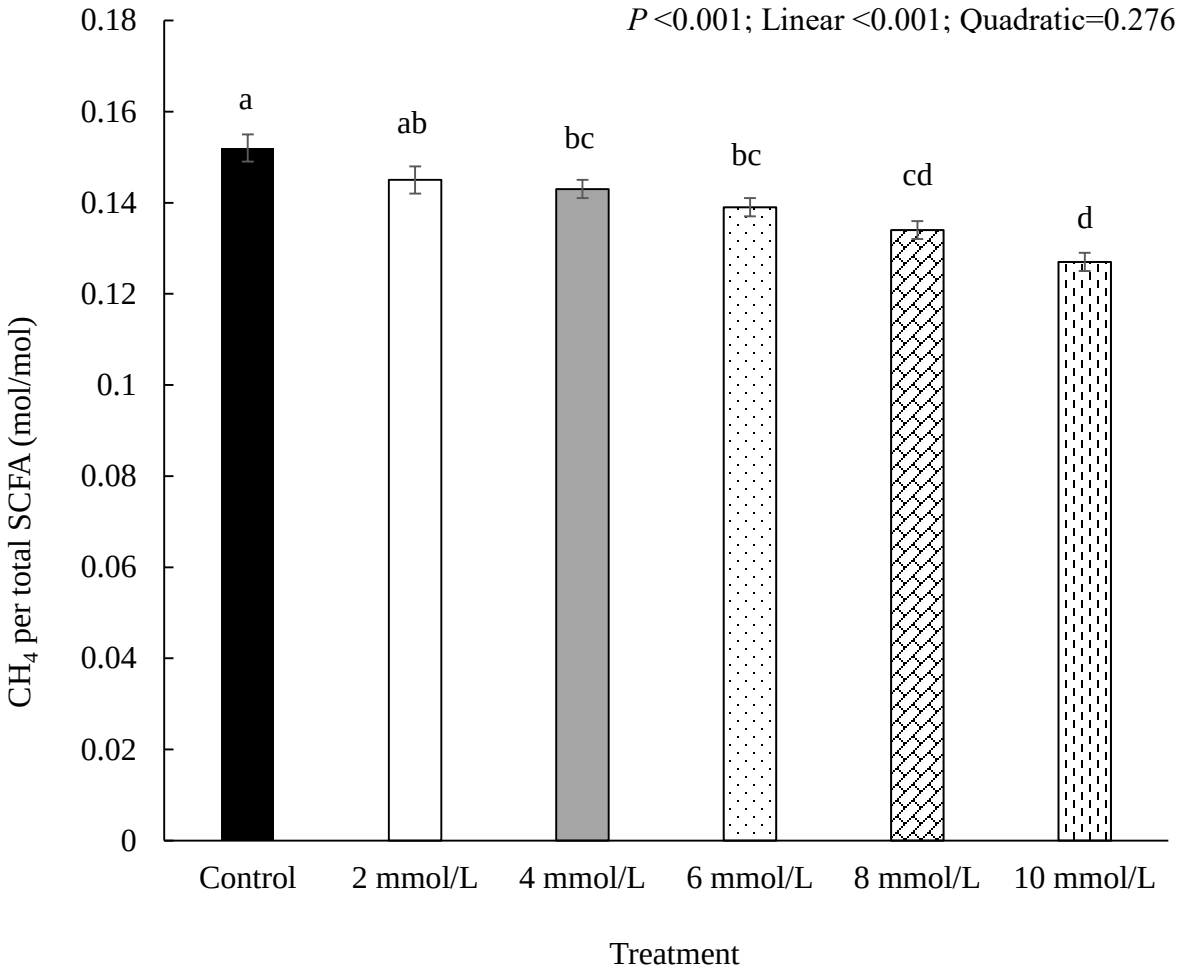



Figure 2- 7. Effect of phloroglucinol on proportion of SCFA.




Error bar shows standard error of mean

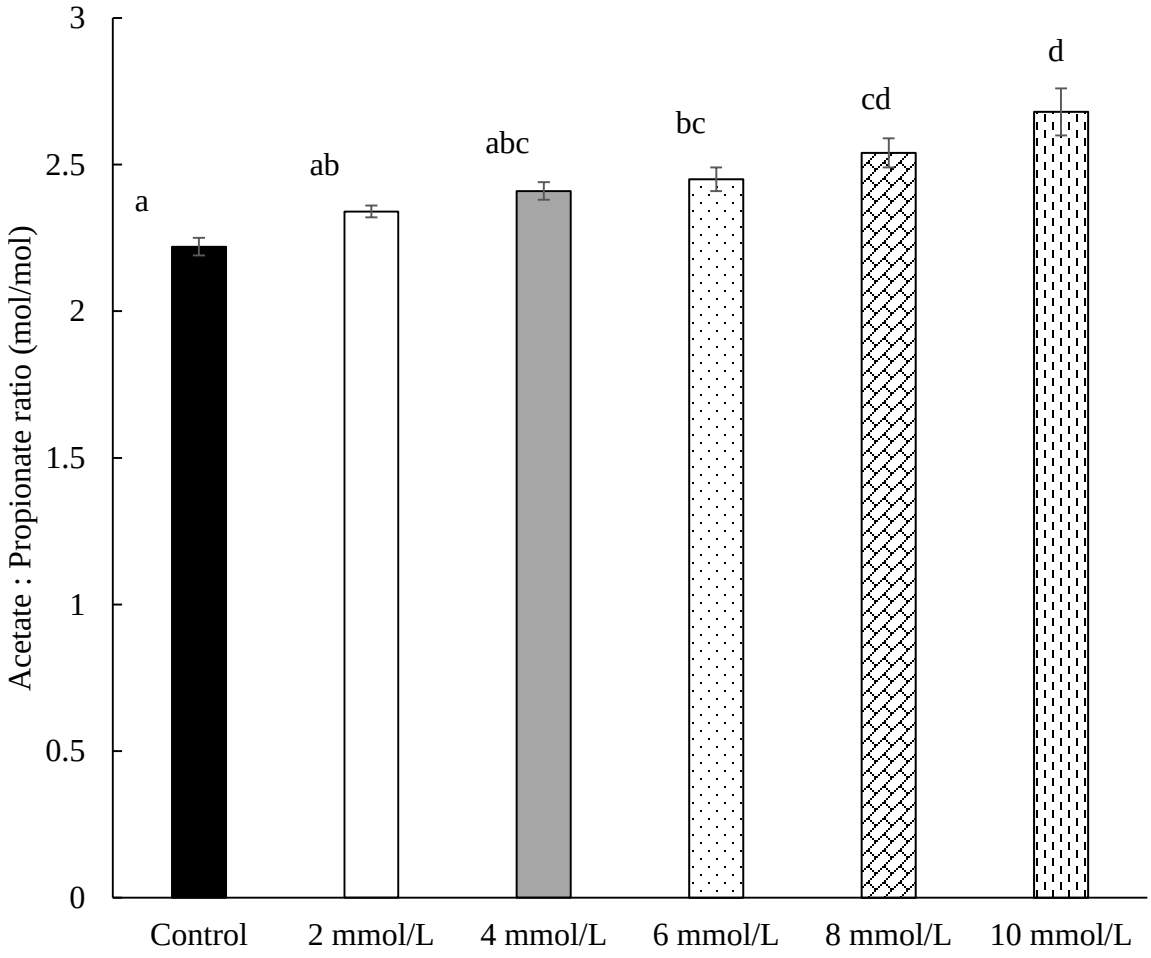
Figure 2- 8. Effect of phloroglucinol on $CH_4$ per digested dry matter (DDM).




Error bar shows standard error of mean

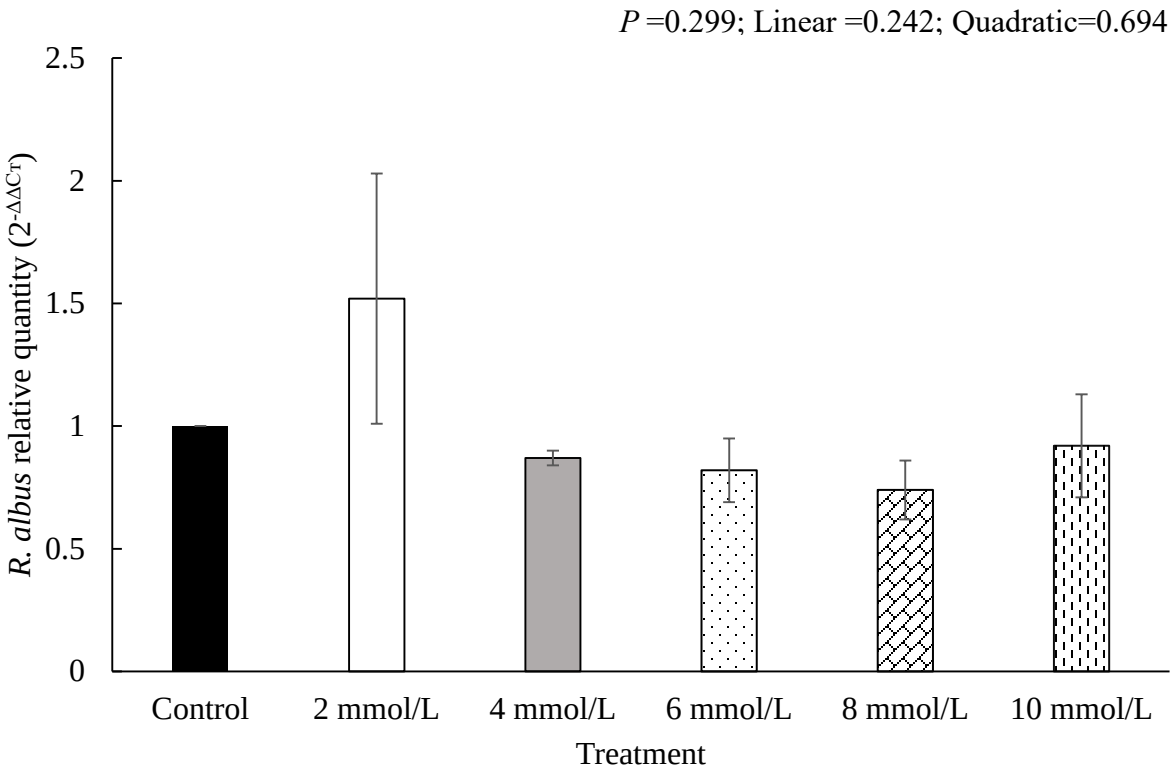
Figure 2- 9. Effect of phloroglucinol on $CH_4$ per total SCFA.

*P* <0.001; Linear <0.001; Quadratic=0.665



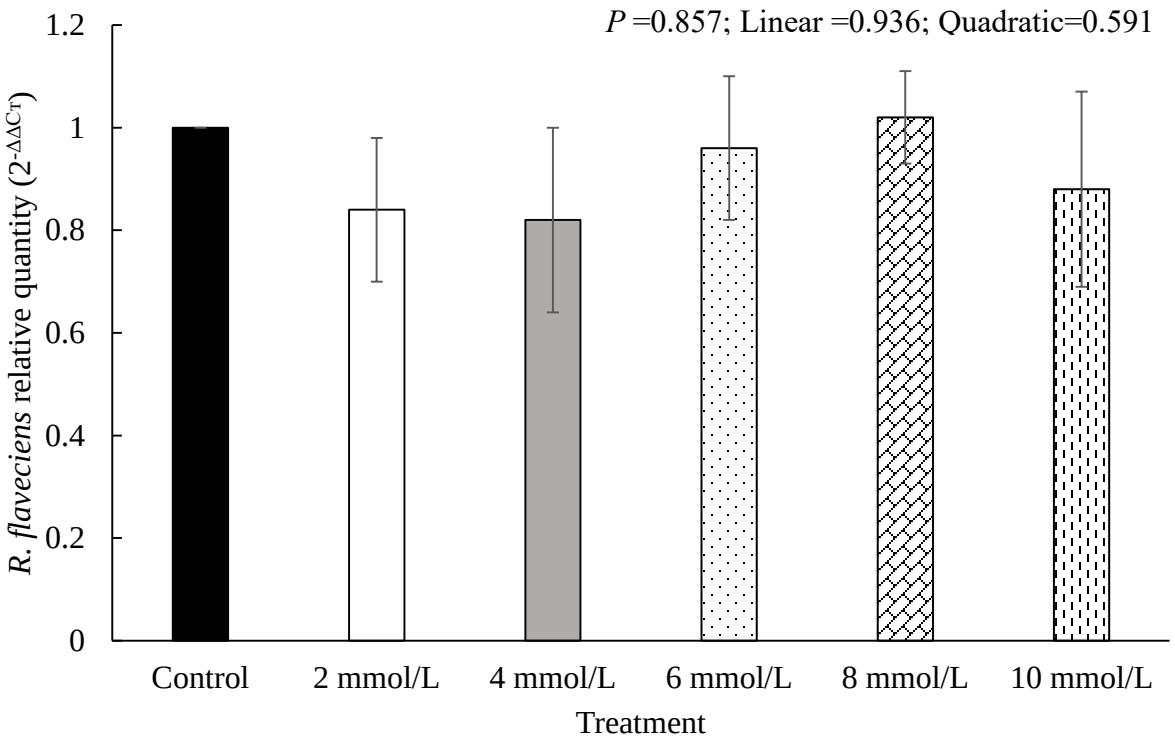

Error bar shows standard error of mean

Figure 2- 10. Effect of phloroglucinol on acetate per propionate ratio.



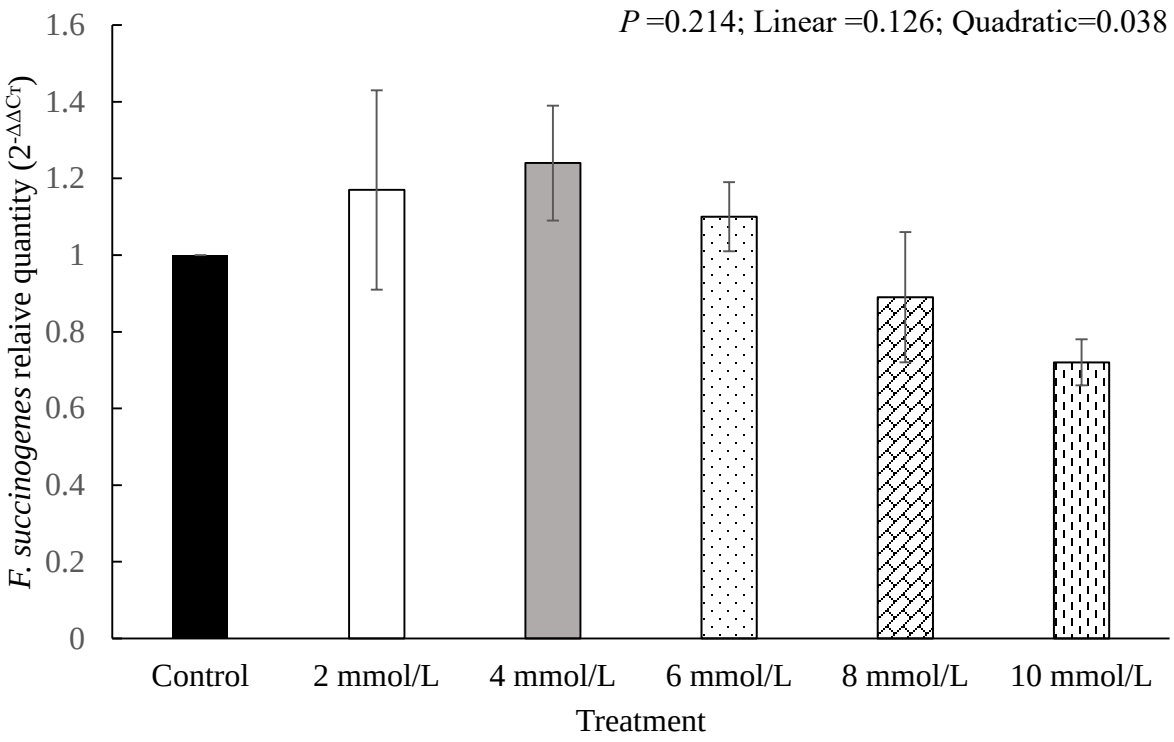

Error bar shows standard error of mean

Figure 2- 11. Effect of phloroglucinol on relative quantity of *Ruminococcus albus*.



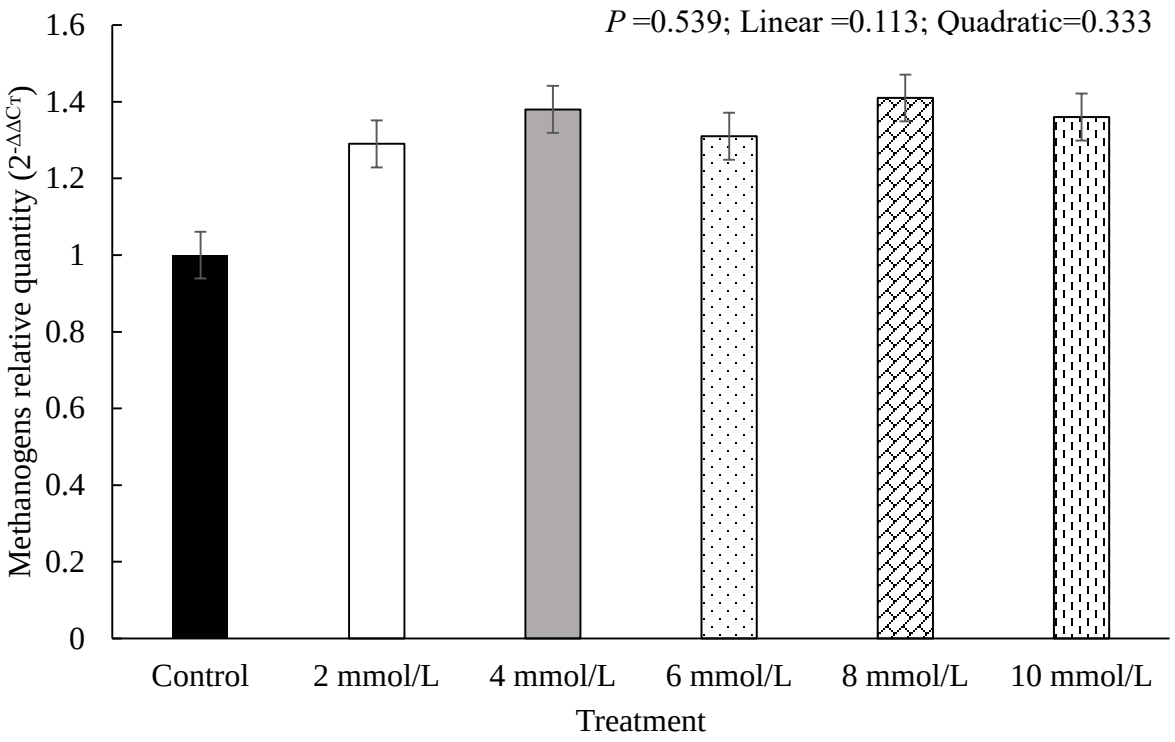

Error bar shows standard error of mean

Figure 2- 12. Effect of phloroglucinol on relative quantity of *Ruminococcus flaveciens*



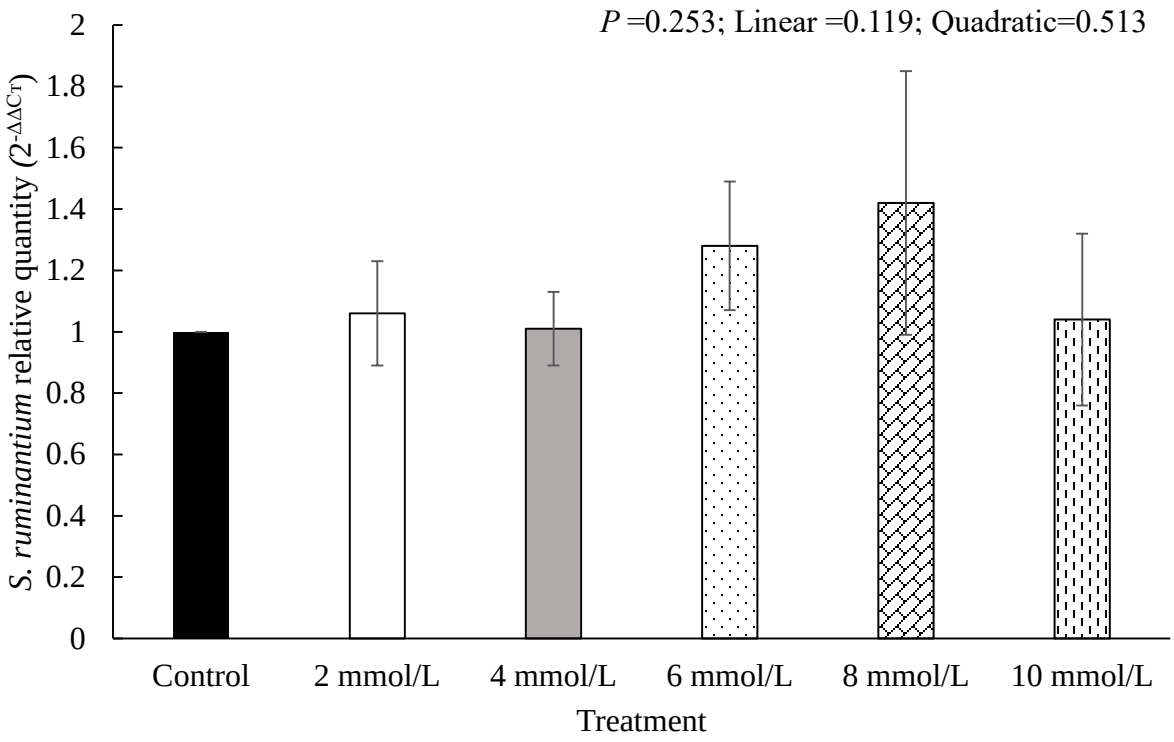

Error bar shows standard error of mean

Figure 2- 13. Effect of phloroglucinol on relative quantity of *Fibrobacter succinogenes.*



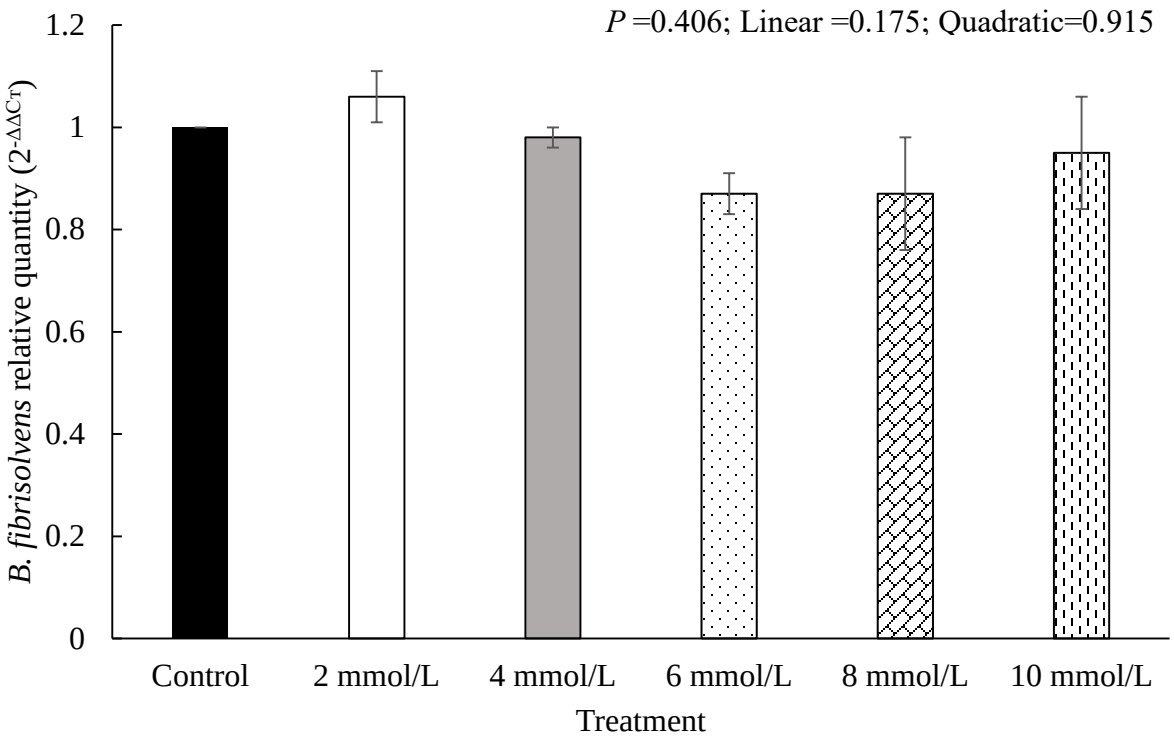

Error bar shows standard error of mean

Figure 2- 14. Effect of phloroglucinol on relative quantity of methanogens.



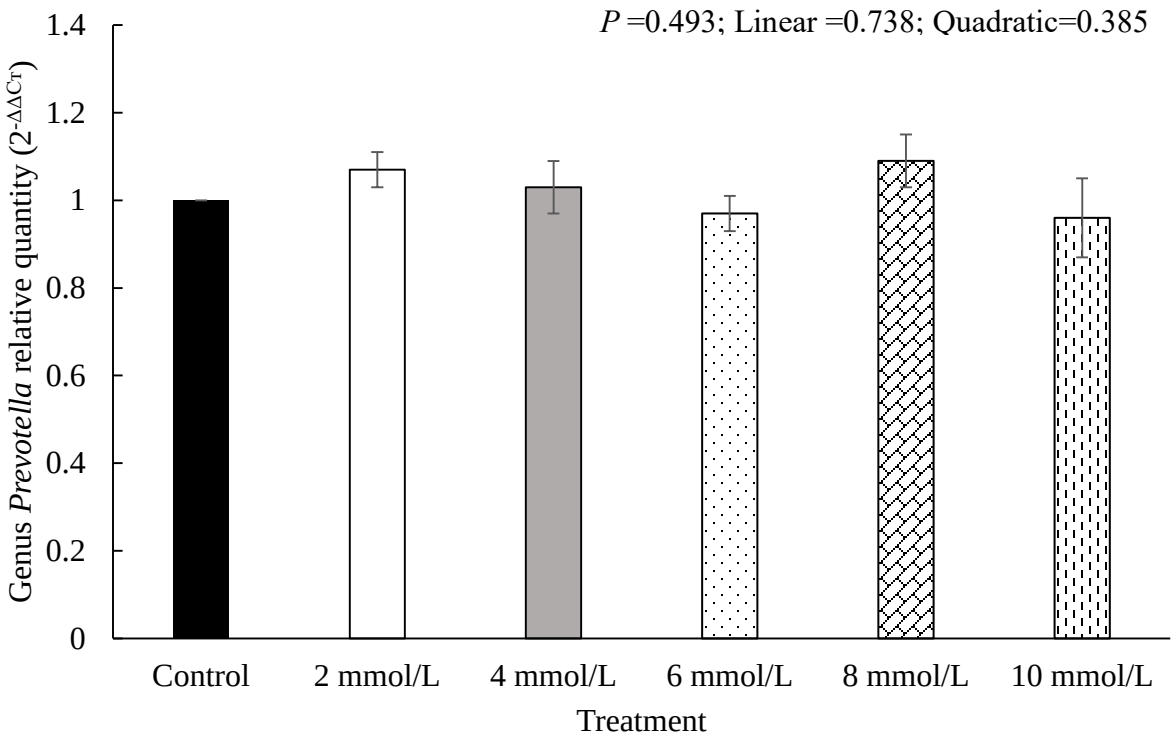

Error bar shows standard error of mean

Figure 2- 15. Effect of phloroglucinol on relative quantity of *Selomonas ruminantium.*



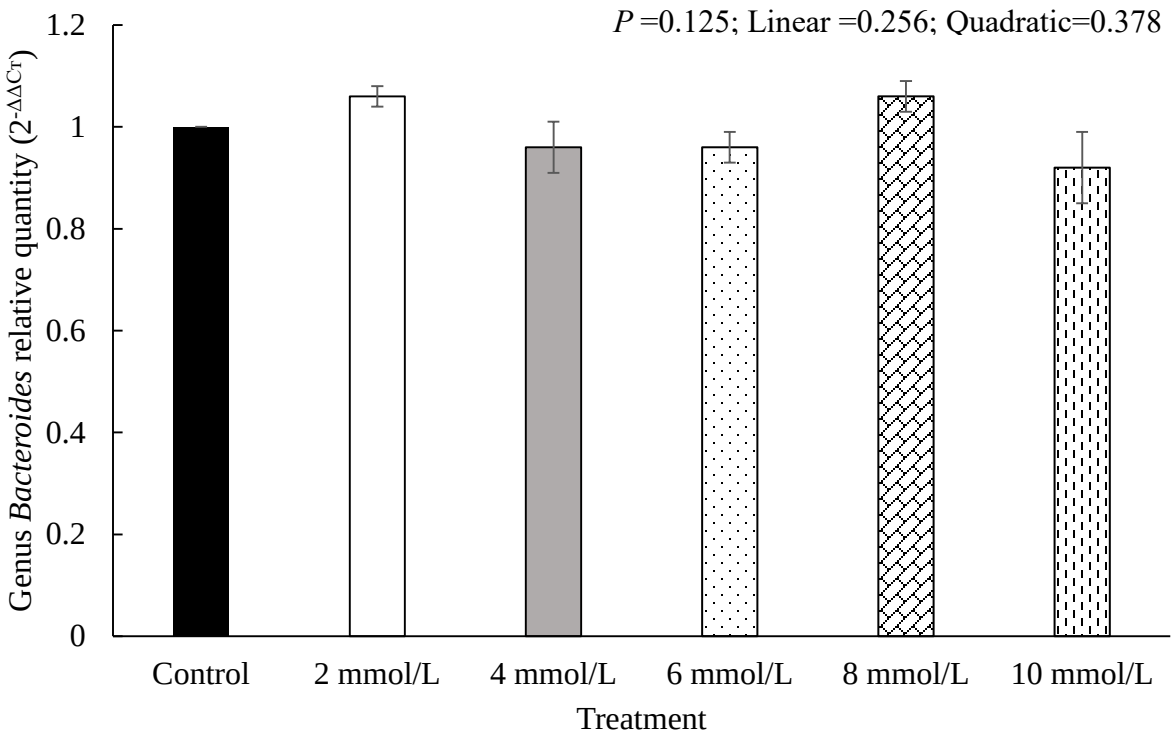

Error bar shows standard error of mean

Figure 2- 16. Effect of phloroglucinol on relative quantity of *Butyrivibrio fibrisolvens.*



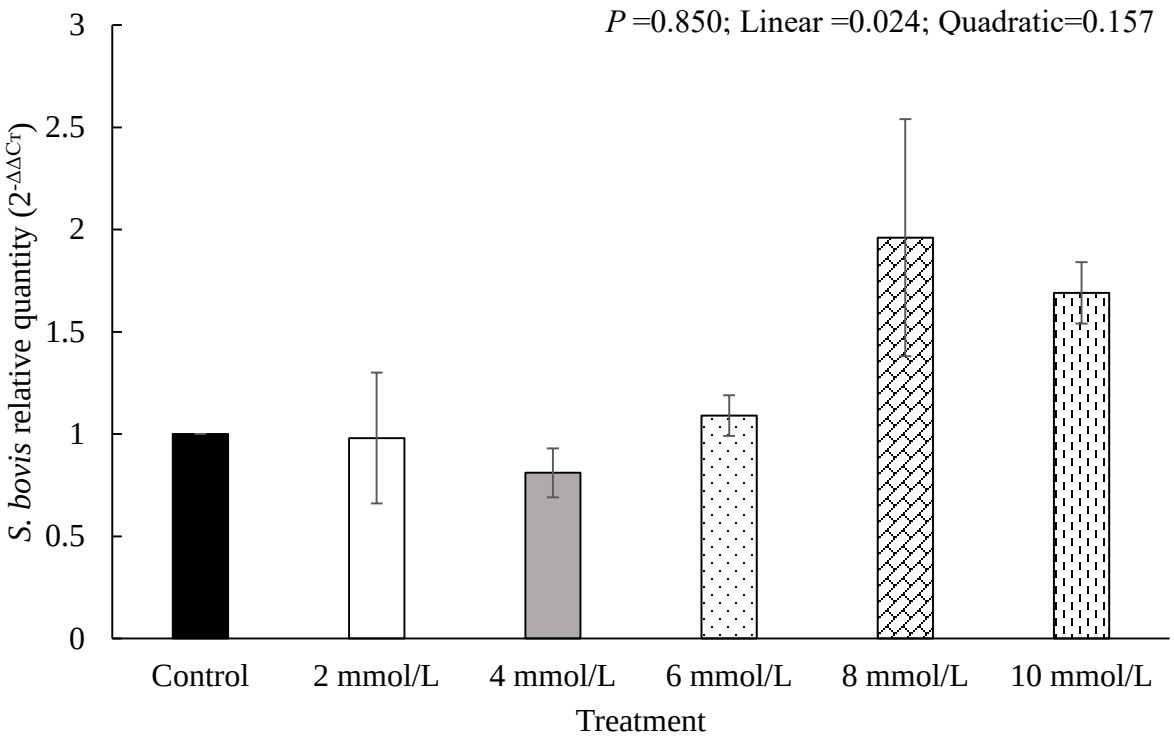

Error bar shows standard error of mean

Figure 2- 17. Effect of phloroglucinol on relative quantity of genus *Prevotella.*



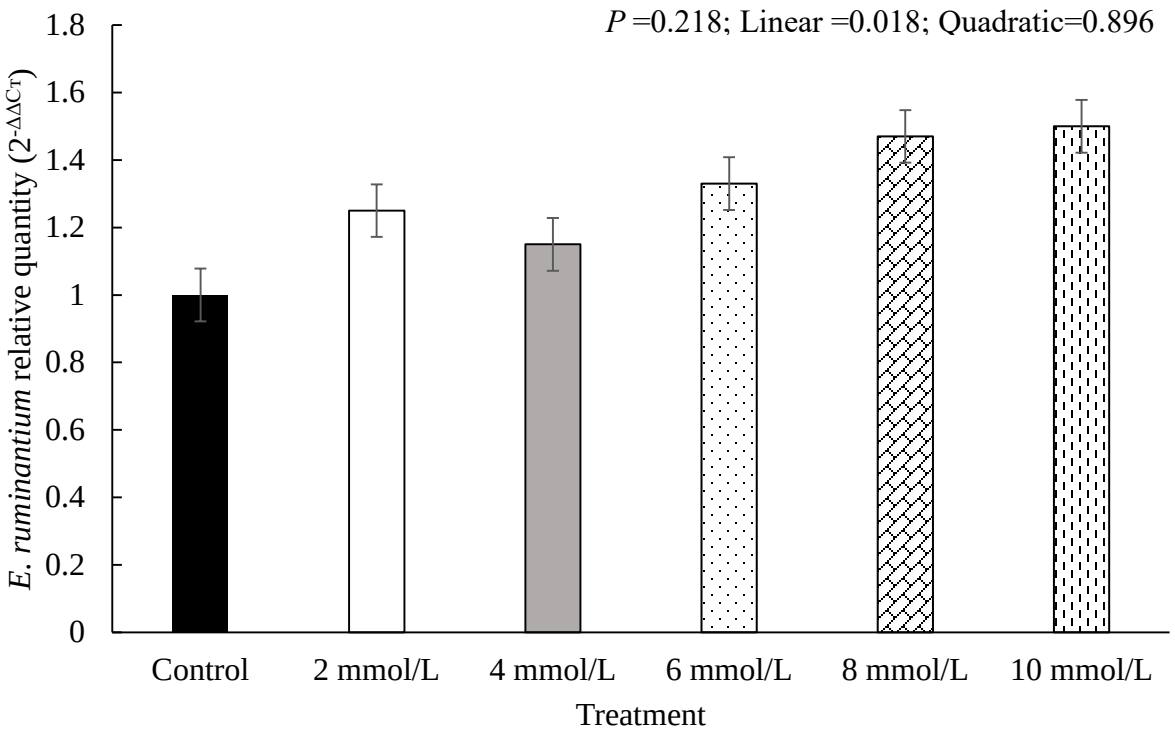

Error bar shows standard error of mean

Figure 2- 18. Effect of phloroglucinol on relative quantity of genus *Bacteroides.*



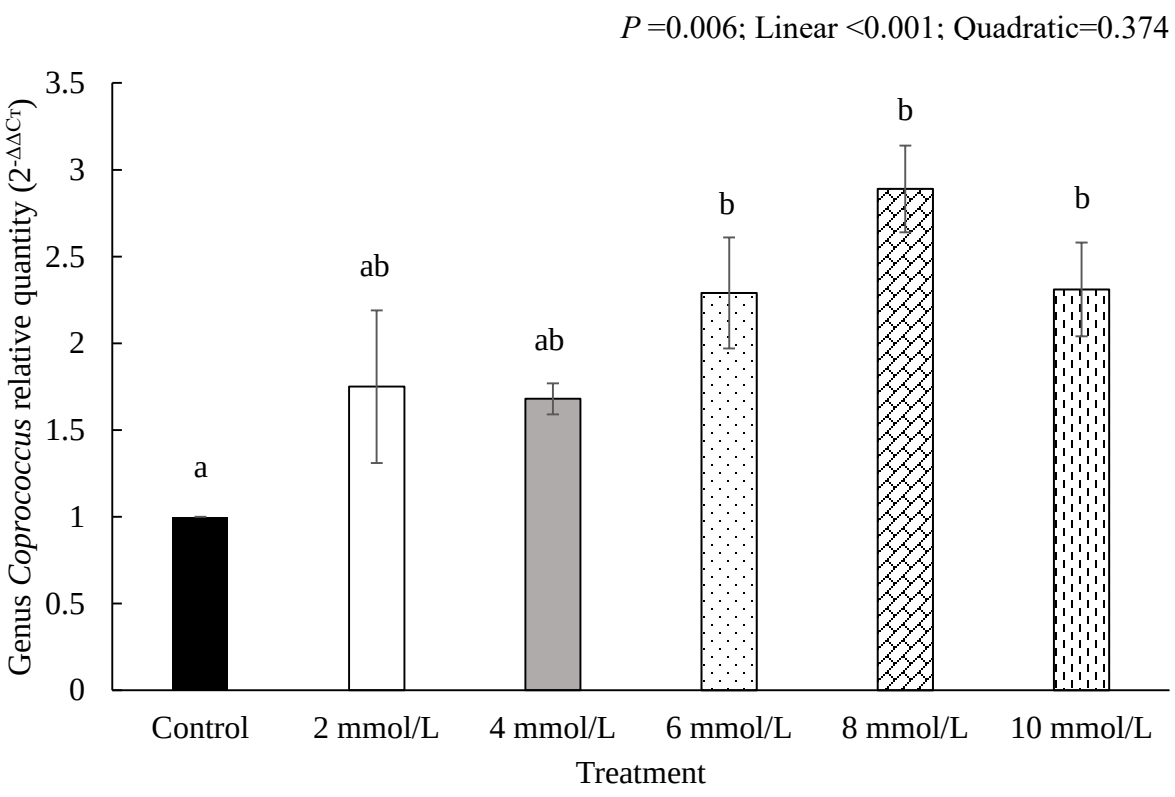

Error bar shows standard error of mean

Figure 2- 19. Effect of phloroglucinol on relative quantity of *Streptococcus bovis.*



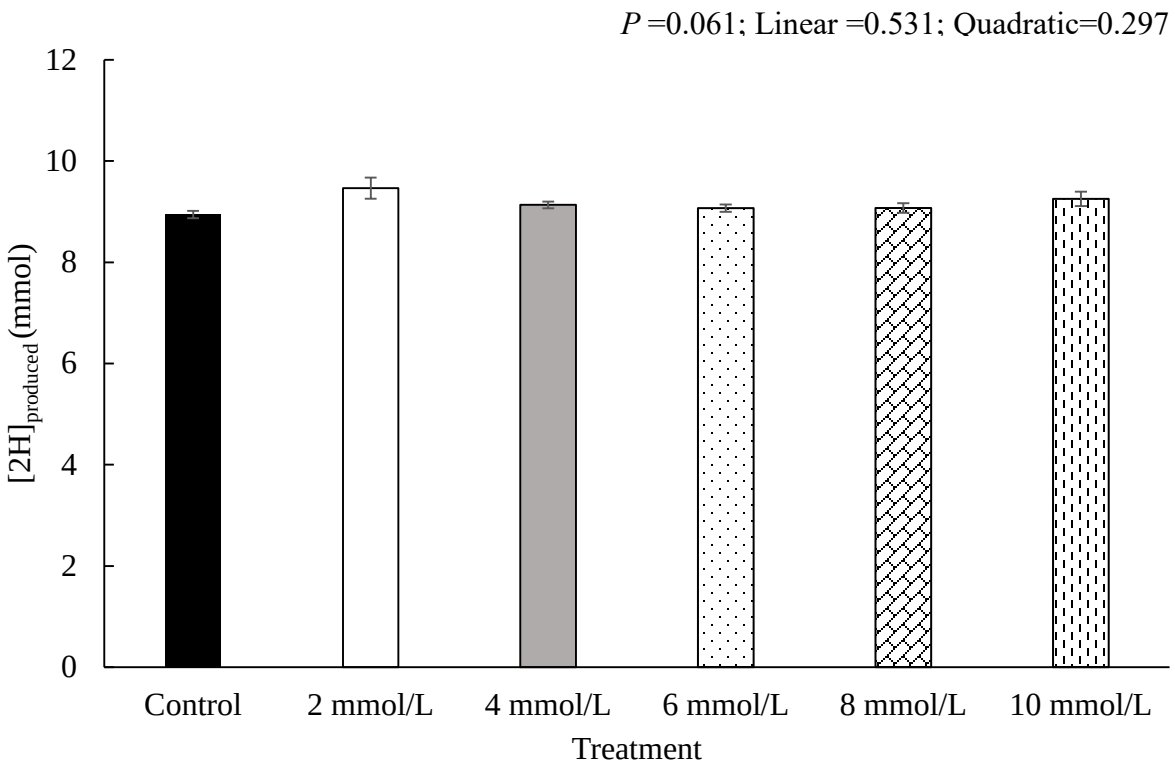

Error bar shows standard error of mean

Figure 2- 20. Effect of phloroglucinol on relative quantity of *Eubacterium ruminantium.*




Error bar shows standard error of mean

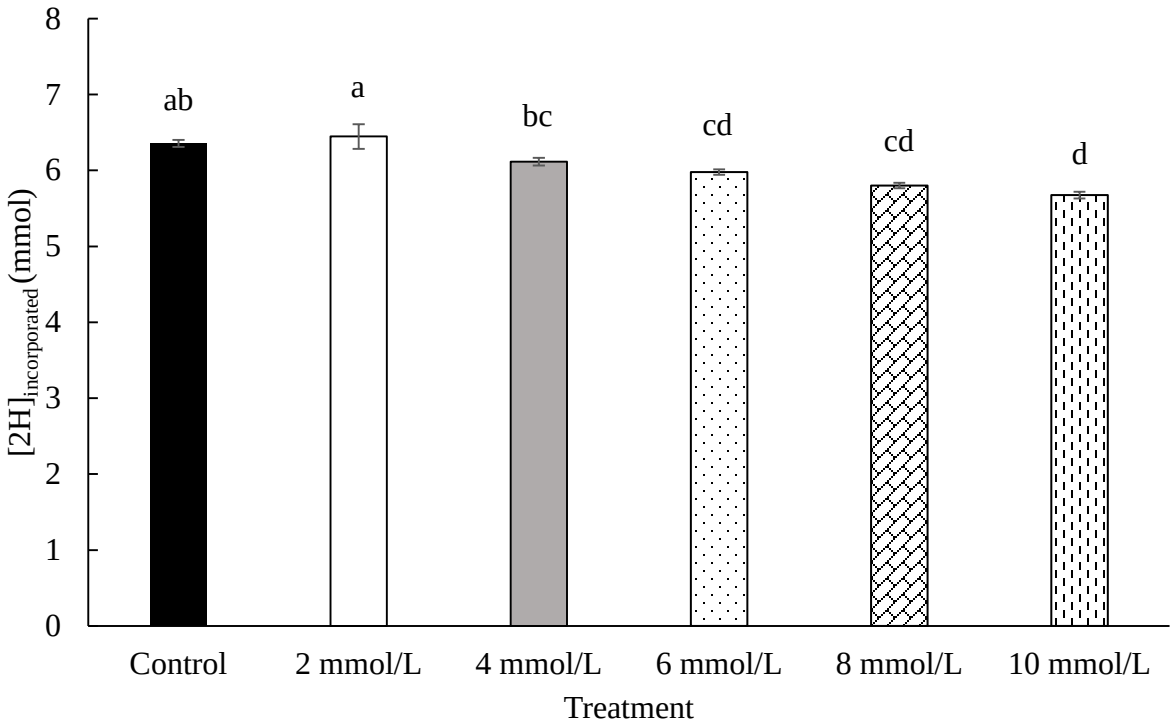
Figure 2- 21. Effect of phloroglucinol on relative quantity of genus *Coprococcus*.




Error bar shows standard error of mean

Figure 2- 22. Effect of phloroglucinol on $[2H]_{produced}$

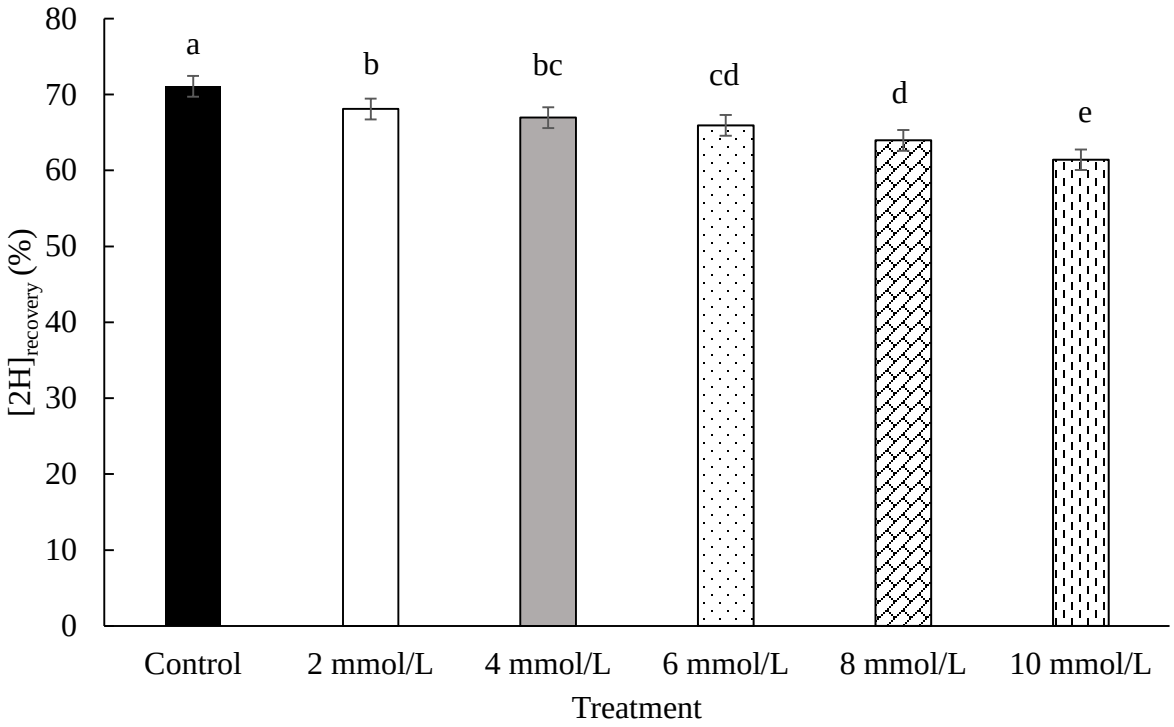
*P* <0.001; Linear <0.001; Quadratic=0.030




Error bar shows standard error of mean

Figure 2- 23. Effect of phloroglucinol on $[2H]_{incorporated}$.

*P* <0.001; Linear <0.001; Quadratic=0.113




Error bar shows standard error of mean

Figure 2- 24. Effect of phloroglucinol on $[2H]_{recovery}$.

# Chapter 2

## The effect of phloroglucinol and forage:concentrate ratio on methanogenesis, *in vitro* rumen fermentation, and microbial population density

### 3.1. Introduction

$CH_4$ emission from ruminant is widely known as a threat for environment and an economical loss. $CH_4$ is the second biggest greenhouse gas in the atmosphere and also a potent greenhouse gases. $CH_4$ also represent inefficiency of ruminant digestibility as its represent 2-12% loss of dietary energy depending on the feed.

McAllister and Newbold (2008) proposed a dietary strategy to mitigate $CH_4$ emission from ruminant by redirecting of $H_2$ utilization to short-chain fatty acid (SCFA) production. The strategy is create a competition of $H_2$ utilization by adding a substrate that may act as an alternative $H_2$ sink agent, such as fumarate and aspartate. Those compound are a precursor of propionate that naturally occur in rumen from carbohydrate digestion in the rumen. Phloroglucinol is a phenolic compound which naturally occur in the rumen as a product of rumen metabolism of tannin. Several rumen bacteria have been known to be able to degrade phloroglucinol by using formate or $H_2$ to acetate. This finding showed an opportunity to use phloroglucinol as an alternative $H_2$ sink agent. Previous *in vivo* study had showed that phloroglucinol was able to redirect utilization of $H_2$ to acetate production when $CH_4$ production was completely inhibited (Martinez-Fernandez et al., 2017). However, the effect of phloroglucinol on $CH_4$ production, rumen fermentation, and microbial population is not known.

$CH_4$ formation in the rumen is directly related to feed characteristic including the amount of $H_2$ produced from its digestion (Janssen, 2010). Diet containing high concentrate tends to

produce less $CH_4$ because starch digestion favour propionate production. Propionate formation is a $H_2$ consuming reaction which will lead to less $H_2$ available for $CH_4$ production. In the other hand, diet containing high structural carbohydrate produce more acetate which in the reaction is liberating $H_2$. (Valadares et al., 1999). Meanwhile, $H_2$ is required by rumen bacteria especially from genus *Coprococcus* to reduce phloroglucinol to acetate. Therefore, it is important to understand the effect of phloroglucinol and different forage:concentrate (F:C) ratio on $CH_4$ production, rumen fermentation, and microbial population *in vitro*.

## 3.2. Materials and Methods

### *3.2.1. Animal and Sampling*

Rumen fluid was collected from three male Friesland sheep. The animal were given 800 gram of Italian rye grass hay and 500 gram of concentrate every day. The concentrate were consisted of wheat bran, corn, soy bean, and alfalfa, in a ratio of 1:1:1:1. The ration was divided into a same portion and given twice a day at 10:00 and 17:00. The animal was placed in individual cage. Water and mineral block were offered ad libitum. Animal handling was performed according to the Mie University guidelines. The rumen fluid was collected by using a stomach tube just before morning feeding. Collected rumen fluid was kept at 39°C during transportation to the laboratory.

### *3.2.2. In vitro incubation*

Pooled rumen fluid was filtered through four layers of surgical gauze. The filtered rumen fluid was diluted with McDougall buffer (McDougall, 1948) which was pre-flushed with $N_2$ gas and pre-warmed at 39°C in a ratio of 2:1 (buffer : rumen fluid). The composition of each

treatment is described at Table 3-1. The composition of the substrate was divided into two dietary treatment groups: low F:C diet and high F:C diet. The low F:C diet consisted of 20% rye grass hay and 80% concentrate to create an F:C ratio of 20:80. The high F:C diet consisted of 80% rye grass hay and 20% concentrate to create an F:C ratio of 80:20. Each substrate was finely ground by using Wiley mill to pass through a 1 mm sieve. Phloroglucinol was prepared by dissolving it in pore ethanol to 1 M. Three different doses of phloroglucinol (0, 6, and 10 mmol/L media) were added to empty serum bottles and were dried at 40°C overnight. After ethanol were evaporated, substrates were weighed into the bottle accordingly. 50 mL of media were added under $N_2$ gas. The serum bottle then tightly capped by using rubber septum and aluminium cap. The media was incubated for 24 h at 39°C by using shaking water bath at 180 rpm. Each treatment consisted of three bottles, three bottles of substrate only which were used as control group and three non-substrate bottles which were used as blank group were also included in the incubation. The incubation was repeated three time on three separate days.

After 24 h, all bottles was put on ice to stop the fermentation. Total gas production were analysed by measuring cumulative headspace gas using glass syringe. The composition of the headspace gas 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. Some part of culture fluid was also transferred to 5 mL centrifugation tube and was kept at -30°C for quantification of microbial population. After centrifugation, the culture fluid was transferred into a 2-mL tube and kept at -30°C until analysis of SCFA, and ammonia-N ($NH_3$-N). Meanwhile, the constant weight of the residue was measured to determine dry matter digestibility. SCFAs in culture fluid were analysed by high-

performance liquid chromatography (HPLC). $NH_3$-N in the fluid was determined using the phenol-hypochlorite method (Weatherburn, 1967). Molar amounts of SCFA and $CH_4$ were used to calculate $CH_4$ per total SCFA ($CH_4$/SCFA). Molar amounts of $CH_4$ were calculated by assuming an ideal gas constant of 0.082 at air pressure 1 atm and 20°C. [2H] balance was calculated as described by Ungerfeld (2015).

### *3.2.3. 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.

### *3.2.4. Quantitation of the microbial population by quantitative real-time PCR*

Primers (specific for *Ruminococcus albus*, *Ruminococcus flaveciens*, *Fibrobacter succinogenes*, methanogens, and protozoa) for real-time PCR are listed at Table 3-2. Real-time PCR was conducted using a StepOnePlus® Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Relative quantification of *Ruminococcus albus*, *Ruminococcus flaveciens*, *Fibrobacter succinogenes*, and methanogens were performed by using comparative $C_T$ method. Absolute quantification of protozoa was performed by using standard curve method. The standard DNA used to construct the standard curve was prepared as described by Lwin et al. (2012).

The DNA copy number of 16S ribosomal RNA gene (16S rDNA) of total bacteria was quantified according to the method described by Abrar et al. (2016). The reaction mixture consisted of 10 µL of Thunderbird SYBR qPCR master mix (Toyobo Co. LTD., Osaka, Japan),

6.2 µL of sterile Milli-Q water, 0.6 µL of each specific forward and reverse primer, 0.4 µL 50x ROX, and 1 µL of template DNA. The PCR condition 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.

The DNA copy number of 16S rDNA of *R. albus* was quantified according to method described by Koike and Kobayashi (2001) with some modification. The reaction mixture consisted of 10 µL of Thunderbird SYBR qPCR master mix, 7.6 µL of sterile Milli-Q water, 0.5 µL of each specific forward and reverse primer, 0.4 µL 50x ROX, and 1 µL of template DNA. The PCR conditions 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.

The DNA copy number of 16S rDNA of *R. flavefaciens* was quantified according to method described by Tajima et al. (2001), with some modification. The reaction mixture consisted of 10 µL of Thunderbird SYBR qPCR master mix, 6.8 µL of sterile Milli-Q water, 0.9 µL of each specific forward and reverse primer, 0.4 µL 50x ROX, and 1 µL of template DNA. The PCR condition included one cycle of polymerase activation 95°C for 1 min, 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.

The DNA copy number of 16S rDNA of *F. succinogenes* was quantified according to the method described by Tajima et al. (2001) with some modification. The reaction mixture consisted of 10 µL of Thunderbird SYBR qPCR master mix, 6.8 µL of sterile Milli-Q water, 0.9 µL of each specific forward and reverse primer, 0.4 µL 50x ROX, and 1 µL of template DNA. The PCR condition 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.

Methanogens were quantified using a methanogen-specific gene, mcrA, following the method described by Abrar et al. (2016). The reaction mixture consisted of 10 µL of Thunderbird SYBR qPCR master mix, 6.2 µL of sterile Milli-Q water, 1.2 µL of each specific forward and reverse primer, 0.4 µL 50x ROX, and 1 µL of template DNA. The PCR condition 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 30 s.

The DNA copy number of 18S rRNA of protozoa was quantified following the method described by Sylvester et al. (2004). The reaction mixture consisted of 10 µL of Thunderbird SYBR qPCR master mix, 6.6 µL of sterile Milli-Q water, 1.0 µL of each specific forward and reverse primer, 0.4 µL 50x ROX, and 1 µL of template DNA. The PCR condition included one cycle of polymerase activation at 95°C for 1 min, 40 cycles of denaturing at 95°C for 15 s, and annealing 55 for 20 sec and extension at 72°C for 45 s.

### *3.2.5. Statistical Analysis*

Data were subjected to a factorial analysis of variance (ANOVA) based on a randomized complete block design. The first factor was diets with different forage-to-concentrate ratios (low and high) and the second factor was doses of PHLO addition (0, 6, and 10 mmol/L). Different *in vitro* runs served as blocks in the ANOVA statistical model due to the variation of rumen microbial activity in each sampling period. An effect was considered significant at the probability level of $P<0.05$. Tukey's test was conducted to compare differences among the treatments, either for the main effects or their interaction. Statistical analysis was conducted using IBM SPSS Statistics version 25.0 (SPSS Inc., Chicago, IL, USA).

### 3.3. Result

The data revealed that PHLO addition significantly lowered $CH_4$ production in both diets ($P$<0.001; Figure 3-2) in a dose-dependent manner. In the low F:C diet, $CH_4$ was lowered by 18% by the addition of 10 mmol/L PHLO, meanwhile in the high F:C diet, $CH_4$ was lowered by 32%. However, there was no significant effect of dietary treatments on $CH_4$ production ($P$=0.070). The decrease of $CH_4$ by PHLO was accompanied by the simultaneous decrease of total gas production, DMD, and $NH_3$-N (Figure 3-1, Figure 3-3, Figure 3-6). Total gas production was lowered by 8% and 3% in low and high F:C diet with 10 mmol/L PHLO, respectively. Meanwhile, supplementation of 10 mmol/L PHLO lowered DMD by 5% in the low F:C diet and 14% in the high F:C diet. Addition of 10 mmol/L lowered $NH_3$-N by 52% and 75% in low and high F:C diet, respectively. A low F:C diet resulted in significantly higher total gas production, DMD, and $NH_3$-N than a high F:C diet (total gas production $P$<0.001; DMD $P$<0.001; $NH_3$-N $P$<0.001). There was a significant effect of the dietary treatment on pH ($P$<0.001; Figure 3-5); pH of low F:C diet was lower than that of high F:C diet. The pH of high F:C diet was significantly lowered by addition of PHLO ($P$<0.001).

When $CH_4$ was presented as $CH_4$/DDM, the data showed that PHLO lowered $CH_4$/DDM significantly along with a higher PHLO level ($P$<0.001; Figure 3-4). In low F:C diet, addition of 10 mmol/L PHLO lowered $CH_4$/DDM by 13% meanwhile in the high F:C diet, $CH_4$/DDM was lowered by 20%. The effect of dietary treatments was observed where $CH_4$/DDM of high F:C diet was higher than that of low F:C diet ($P$<0.001).

PHLO addition significantly affected total SCFA production of both dietary treatments but in contrasting manner ($P$=0.031; Figure 3-7). PHLO addition lowered total SCFA production of

low F:C diet, and meanwhile, for high F:C diet, total SCFA production increased. Addition of 10 mmol/L PHLO lowered total SCFA production of low F:C diet by 9%, on the other hand, total SCFA production of high F:C diet increased by 17%. There was significant effect of dietary treatment on total SCFA production ($P$=0.001). Low F:C diet had higher total SCFA production than high F:C diet on all PHLO dose except for dose of 10 mmol/L PHLO where high F:C diet had higher total SCFA production than low F:C diet.

Changes in the production of individual SCFAs were also observed. PHLO addition significantly increased acetate production of high F:C diet but lowered the acetate production of low F:C diet ($P$=0.001; Figure 3-8). The addition of 10 mmol/L PHLO lowered acetate of low F:C diet by 7% and increased acetate production of high F:C diet by 23%. PHLO had a trend toward significant effect on propionate and butyrate production (propionate $P$=0.101; Figure 3-9 & butyrate $P$=0.061; Figure 3-10). Propionate production of low F:C diet was lowered by PHLO meanwhile in case of the high F:C diet, propionate production increased. Propionate was lowered by 10% in low F:C diet but increased by 12% in high F:C diet by addition of 10 mmol/L PHLO. Meanwhile, addition of 10 mmol/L PHLO lowered butyrate production by 15% and 6% on low and high F:C diet, significantly. A significant effect of dietary treatment was observed where the proportion of acetate for the low F:C diet was lower than that of the high F:C diet ($P$<0.001); meanwhile, the proportions of propionate and butyrate of the low F:C diet were higher than those of the high F:C diet. PHLO addition significantly increased acetate:propionate ratio ($P$=0.004). Acetate:propionate ratio of low F:C diet were lower than that of high F:C diet ($P$<0.001; Figure 3-11). When $CH_4$ was presented as $CH_4$/total SCFA, A significant effect of PHLO addition was observed on $CH_4$/total SCFA ($P$<0.001; Figure 3-12). Addition of 10 mmol/PHLO lowered

$CH_4$/total SCFA of low F:C diet by 3% and by 49% on high F:C diet, but there was no significant effect of dietary treatments on $CH_4$/total SCFA

There was a significant interaction effect of F:C + PHLO on $[2H]_{produced}$ ($P$<0.001; Figure 3-13). PHLO did not have any significant effects on $[2H]_{produced}$ ($P$=0.302). There was a significant effect of dietary treatments on $[2H]_{produced}$ ($P$=0.003). $[2H]_{produced}$ of low F:C diet were higher than that of the high F:C diet except at a PHLO dose of 10 mmol/L, where the $[2H]_{produced}$ of high F:C diet wasere higher than that of the low F:C diet.

There was a significant effect of PHLO addition on $[2H]_{incorporated}$ ($P$<0.001; Figure 3-14). 10 mmol/L PHLO lowered $[2H]_{incorporated}$ by 14% and 5% on low F:C diet and high F:C diet, respectively. The effects of dietary treatment were also observed where the $[2H]_{incorporated}$ of low F:C diet was higher than that of the high F:C diet. $[2H]_{recovery}$ of both diets was lowered with the supplementation of PHLO ($P$<0.001; Figure 3-15). 10 mmol/L PHLO lowered $[2H]_{recovery}$ by 2% and 26% on low F:C diet and high F:C diet, respectively. Dietary treatments significantly affected $[2H]_{recovery}$ ($P$<0.001) where $[2H]_{recovery}$ of low F:C diet were higher than that of high F:C diet.

Real-time PCR data showed that PHLO addition significantly lowered relative quantity of *R. albus* and methanogens (Figure 3-16, Figure 3-19). However, there was no significant effect of PHLO on *R. flaveciens*, *F. succinogenes*, and protozoa (Figure 3-17, Figure 3-18, Figure 3-20). Dietary treatments significantly affected relative quantity of all measured microbial population. Relative quantity of *R. flaveciens*, *F. succinogenes*, methanogens, and protozoa on low F:C diet were lower than that of high F:C diet. On the other hand, relative quantity of *R. albus* on low F:C diet were higher than that of high F:C diet.

### 3.4. Discussion

Phloroglucinol addition in both diet significantly reduced $CH_4$ production. This finding showed that phloroglucinol has similar effectivity on $CH_4$ reduction regardless the F:C ratio in the diet. Simple phenolic compound such as cinnamic, caffeic, ferulic acid, *p*-coumaric, and benzoic acid can reduce $CH_4$ production in the rumen (Jayanegara, 2010, Ushida et al., 1989, Asiegbu et al., 1995). Our finding is in agreement to the report of Martinez-Fernandez et al. (2017) that showed phloroglucinol can reduce more $CH_4$ when supplemented in combination with chloroform than when chloroform was supplemented alone. Our study demonstrated that phloroglucinol was able to reduce $CH_4$ production when supplemented alone and have similar effect on different F:C ratio.

However, there was not any effect of F:C ratio on $CH_4$ production. $CH_4$ production is related to feed characteristics including $H_2$ produced from its degradation (Johnson and Johnson, 1995, Janssen, 2010). Forage was degraded in the rumen into acetate which liberated $H_2$ in the reaction meanwhile high concentrate diet produce more propionate which consume $H_2$ in its formation (Valadares et al., 1999). Our finding was not in agreement with previous study that suggest high F:C ratio could increase $CH_4$ production (Lovett et al., 2003, Aguerre et al., 2011, Agle et al., 2010, García-Martínez et al., 2007).

Phenolic compound such as tannins might reduce $CH_4$ production by inhibiting methanogen growth and reducing feed digestibility (Tavendale et al., 2005, Jayanegara et al., 2015). Both mechanisms were reflected in this study. Phloroglucinol significantly reduced methanogen relative quantity. It has been known that phenolic compounds is toxic to methanogen (Field and Lettinga, 1992). Tavendale et al. (2005) demonstrated that tannin could inhibit methanogen growth. Our finding showed that phloroglucinol also can inhibit methanogen

growth. Methanogen is the main actor of $CH_4$ production in the rumen. Therefore, any reduction of methanogen relative quantity might partly explain $CH_4$ reduction by phloroglucinol.

Phloroglucinol might also indirectly reduce $CH_4$ production by reducing DMD. The reduction on DMD was presumably related to reduction on total gas production and $NH_3$-N. It seems that phloroglucinol may inhibit carbohydrate digestion as indicated by reduction on total gas production in both diet. The inhibition of carbohydrate digestion might be related to reduction relative quantity of *R. albus*. This finding showed that phloroglucinol may reduce carbohydrate digestion by inhibiting cellulolytic bacteria growth. Less digestion of cellulose resulting less total gas production that may lead to less $CH_4$ production. Phenolic compound, such as phloroglucinol, contain free hydroxyl group (-OH) attached to a benzene ring. Free hydroxyl group allow feed and phenolic compound to form a complexes through strong hydrogen bond (Silanikove et al., 2001) and inhibit microbial digestion of fibre (McSweeney et al., 2001). Phenolic compound also might inhibit $NH_3$-N through formation of complexes between its free hydroxyl group and the carbonyl groups of peptides in the protein (Silanikove et al., 2001). The complexes may inhibit the ruminal digestion of proteins through suppression of protease activity, suppression of microbial growth or activity, or by protection of the substrate protein (McSweeney et al., 2001).

However, total SCFA production, another indicator of carbohydrate digestion, showed contradicting response to addition of phloroglucinol. Total SCFA production of low F:C ratio was reduced by addition of phloroglucinol, but in high F:C ratio, phloroglucinol increased total SCFA production. This finding might be related to $H_2$ produced from feed digestion as indicated by $[2H]_{produced}$ and propionate proportion. Starch digestion in the rumen produce more propionate in compare to fibre (Valadares et al., 1999) thus resulting in less $H_2$ production. This finding

showed that when $H_2$ is available, phloroglucinol may redirect its utilization to SCFA production and may substitute SCFA loss from inhibited carbohydrate digestion. The change on total SFCA production also might explain the effect of phloroglucinol on pH where phloroglucinol supplementation reduced pH of high F:C ratio but increased pH of low F:C ratio.

Increasing acetate production also might indicate that part of $CH_4$ reduction was attributed to redirection of $H_2$ utilization for SCFA production. This finding was complemented with simultaneous reduction of $CH_4$/Total SCFA. Reduction of $CH_4$/Total SCFA suggests that the level of $CH_4$ production can be reduced by phloroglucinol without lowering the amount of energy available for the ruminant in the form of SCFA. Our finding was in agreement with the effect of phenolic compounds on $CH_4$/total SCFA (Jayanegara et al., 2012b). Reducing $CH_4$/total SCFA showed fermentative productivity, which might occur when an alternative $H_2$ sink is available. Our findings suggest that phloroglucinol is most probably act as an $H_2$ sink, redirecting $H_2$ utilization and reducing $CH_4$ production. Phloroglucinol can be degraded by several rumen bacteria, mainly from genus *Coprococcus* and *Eubacterium* by using $H_2$ or formate resulting acetate (Tsai et al., 1976, Krumholz and Bryant, 1986). Supplementation of phloroglucinol to the rumen may stimulate an increase number of *Coprococus* (Martinez-Fernandez et al., 2017) that can use of $H_2$ for phloroglucinol degradation resulting more acetate production and lead to $H_2$ scarcity for methanogen to produce $CH_4$.

Increasing F:C ratio reduced DMD, SCFA, and $NH_3$-N but increased ruminal pH. Diet containing high forage has higher content of structural carbohydrate such as fibre than diet containing high concentrate. Fibre is less digestible than starch which might explain less digestibility of high F:C diet than low F:C diet (Moe and Tyrrell, 1979). Lower DMD by increasing F:C ratio might reduce total SCFA production. Then, lower total SCFA of high F:C

diet might explain higher pH of high F:C diet than low F:C diet. Concentrate diets had been known to reduce ruminal pH (Van Kessel and Russell, 2006).

The calculation of metabolic hydrogen balance showed that $[2H]_{incorporated}$ and $[2H]_{recovery}$ were reduced by phloroglucinol addition despite there is a significant increase of acetate that may indicate a possible redirection of $H_2$ to SCFA. The possible explanation to this finding is the formula used to calculate the hydrogen balance does not account for acetate produced through the reductive processes such as reduction of phloroglucinol. The finding was in agreement to study of Ungerfeld (2015) which found that the $[2H]_{recovery}$ was reduced when methanogenesis is inhibited. The author furthermore stated that the formula does not account for a major [H] sink when $CH_4$ was inhibited. The potential unaccounted major [H] sinks are fermentation product other than propionate, butyrate, and $H_2$, microbial biomass, and reductive acetogenesis.

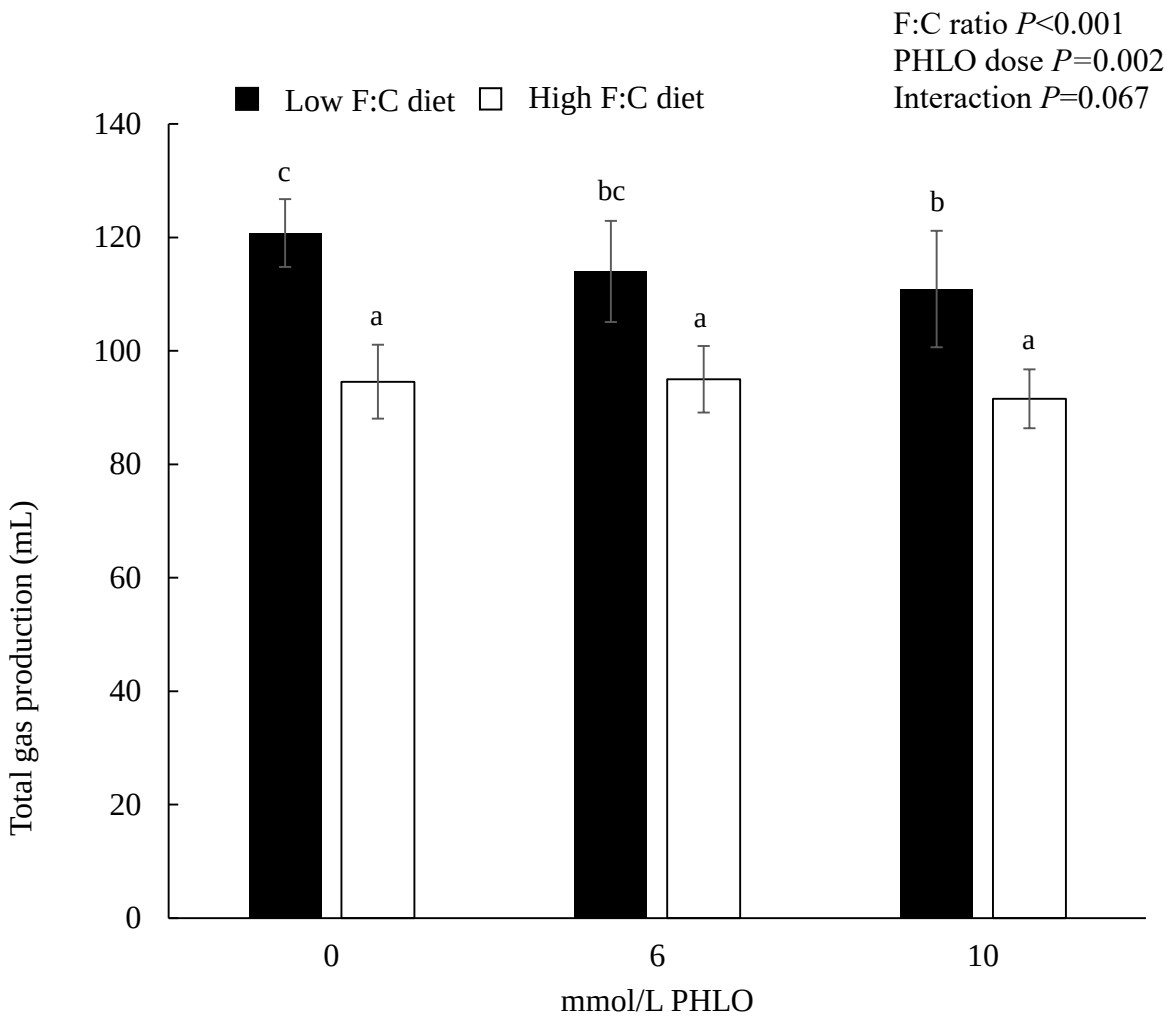
In conclusion, phloroglucinol is able to reduce $CH_4$ production in different F:C ratio when supplemented alone. The reduction was related to reduction of methanogen number and nutrient digestibility. Phloroglucinol also might be able to reduce $CH_4$ by redirect $H_2$ utilization form $CH_4$ to SCFA production.

Table 3-1. Composition of experimental *in vitro* batch cultures

| Material | Low F:C diet | | | High F:C diet | | |
|---|---|---|---|---|---|---|
| | Control | 6 mmol/L | 10 mmol/L | Control | 6 mmol/L | 10 mmol/L |
| Inoculum (mL) | 50 | 50 | 50 | 50 | 50 | 50 |
| Rye grass hay (g) | 0.2 | 0.2 | 0.2 | 0.8 | 0.8 | 0.8 |
| Corn starch (g) | 0.45 | 0.45 | 0.45 | 0.15 | 0.15 | 0.15 |
| Wheat bran (g) | 0.35 | 0.35 | 0.35 | 0.05 | 0.05 | 0.05 |
| 1 M Phloroglucinol (mL) | - | 0.3 | 0.5 | - | 0.3 | 0.5 |

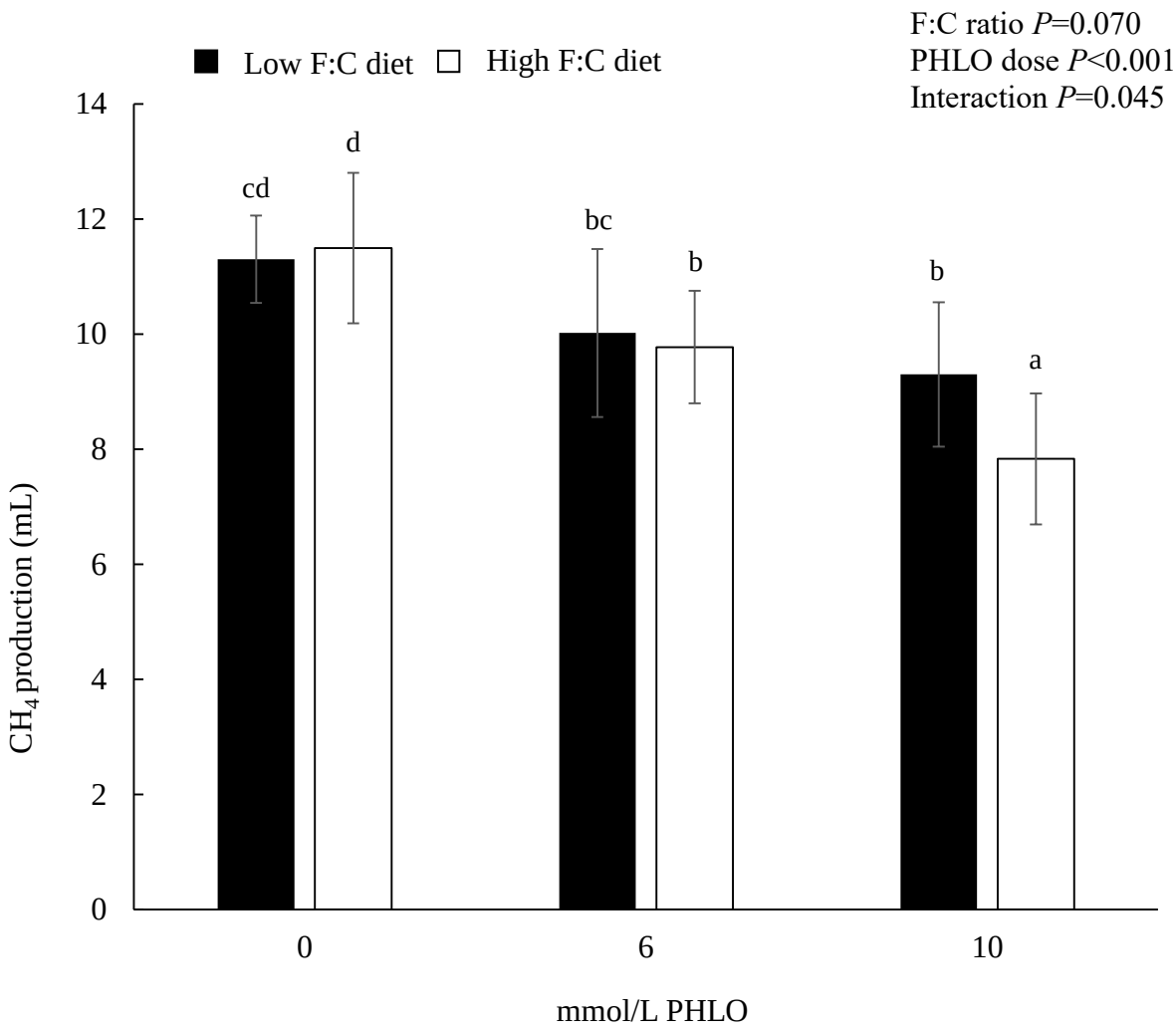
Table 3-2. Primers used in this study.

| Target | Name | Sequence (5′ → 3′) | Product size (bp) | Reference |
|---|---|---|---|---|
| Total bacteria | 1114-f | CGGCAACGAGCGCAACCC | 130 | (Denman and McSweeney, 2006) |
| | 1275-r | CCATTGTAGCACGTGTGTAGCC | | |
| *Ruminococcus albus* | Ra1281f | CCCTAAAAGCAGTCTTAGTTCG | 175 | (Koike and Kobayashi, 2001) |
| | Ra1439r | CCTCCTTGCGGTTAGAACA | | |
| *Ruminococcus flavefaciens* | Rflf | GGACGATAATGACGGTACTT | 835 | (Tajima et al., 2001) |
| | Rflr | GCAATCYGAACTGGGACAAT | | |
| *Fibrobacter succinogenes* | Fsf | GGTATGGGATGAGCTTGC | 445 | (Tajima et al., 2001) |
| | Fsr | GCCTGCCCCTGAACTATC | | |
| Methanogens | q-mcrA-f | TTCGGTGGATCDCARAGRGC | 140 | (Denman et al., 2007) |
| | q-mcrA-r | GBARGTCGWAWCCGTAGAATCC | | |
| Protozoa | 316f | GCTTTCGWTGGTAGTGTATT | 223 | (Sylvester et al., 2004) |
| | 539r | CTTGCCCTCYAATCGTWCT | | |



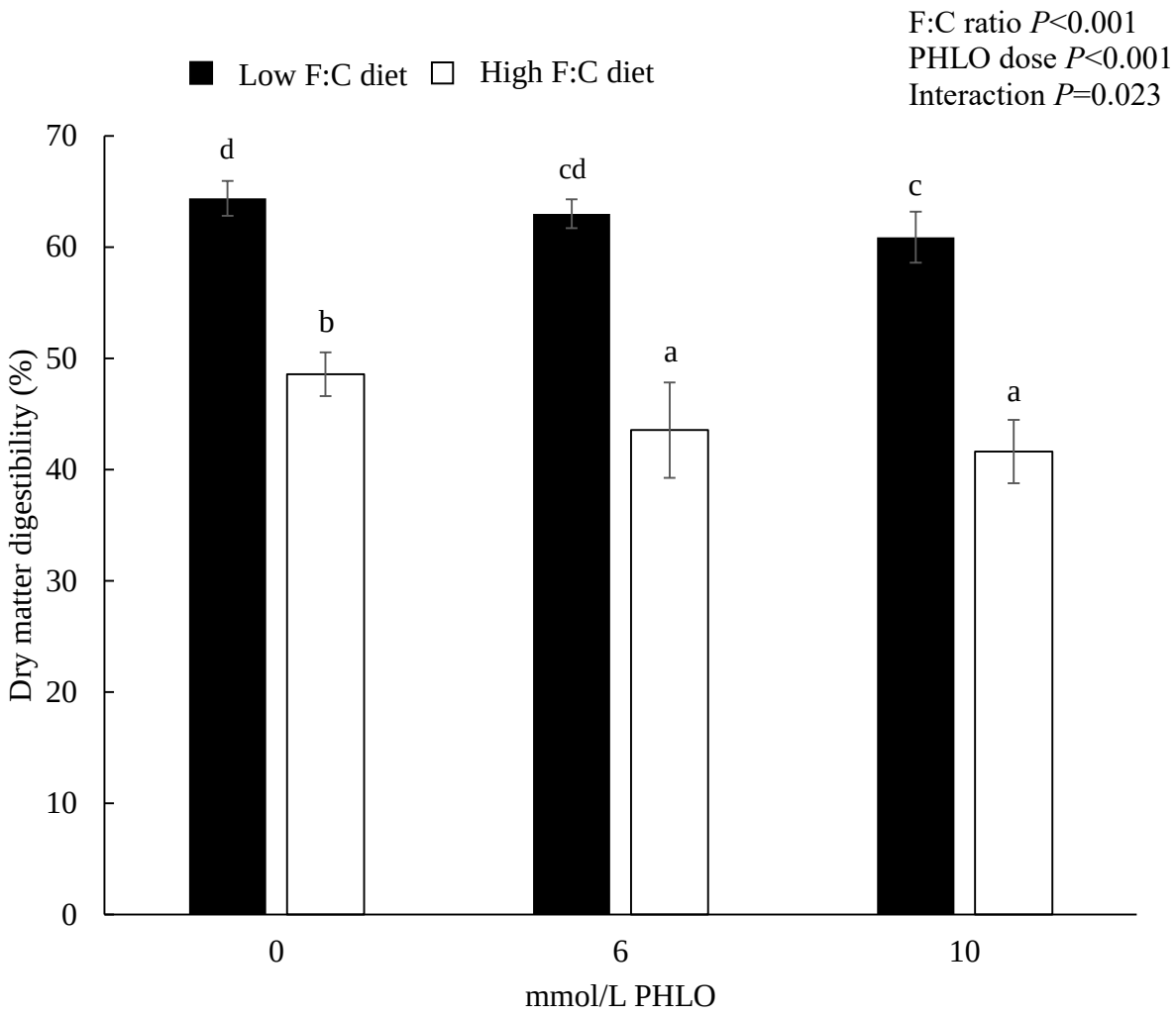

Error bar shows standard error of mean

Figure 3- 1. Effect of phloroglucinol supplementation and F:C ratio on total gas production



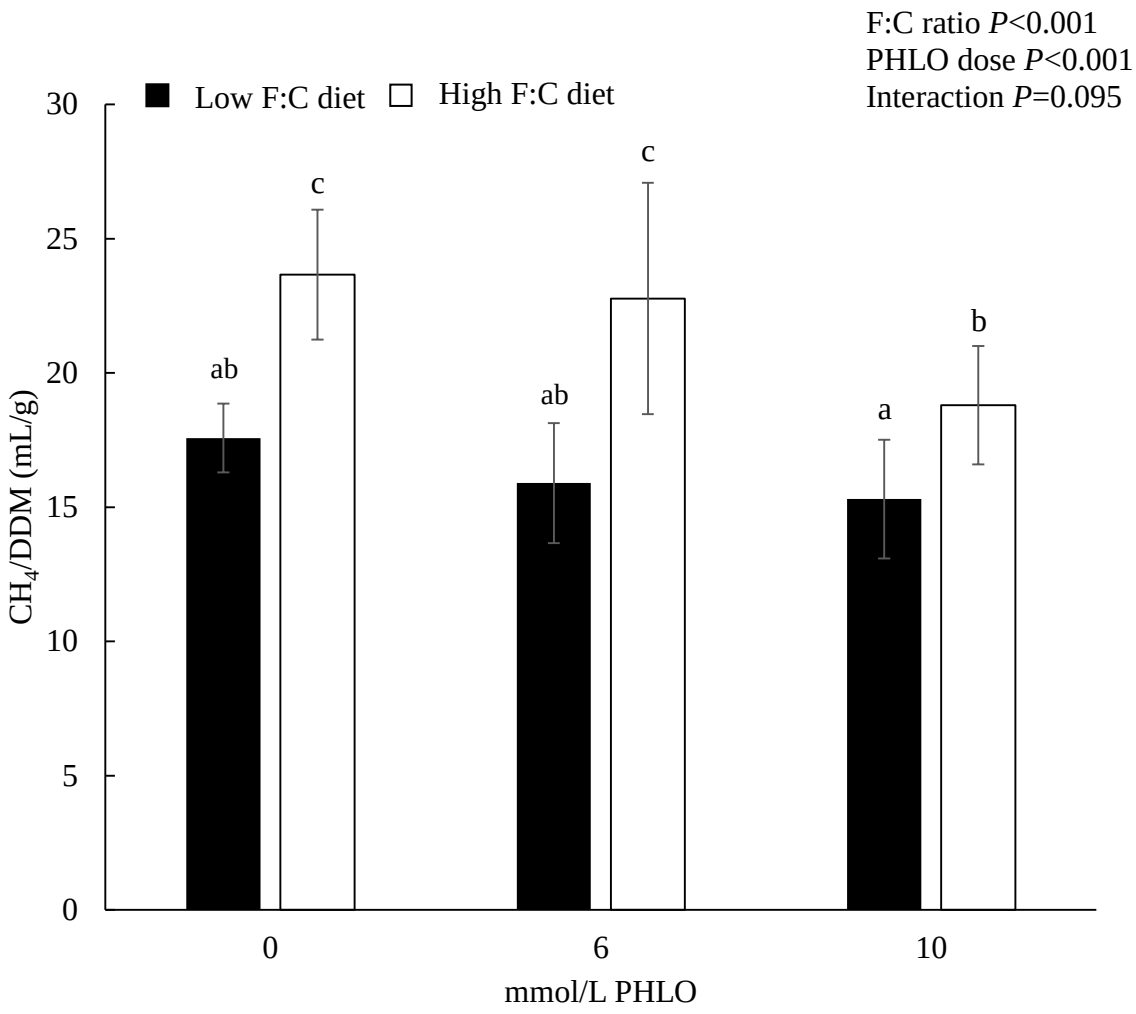

Error bar shows standard error of mean

Figure 3- 2. Effect of phloroglucinol supplementation and F:C ratio on $CH_4$ production



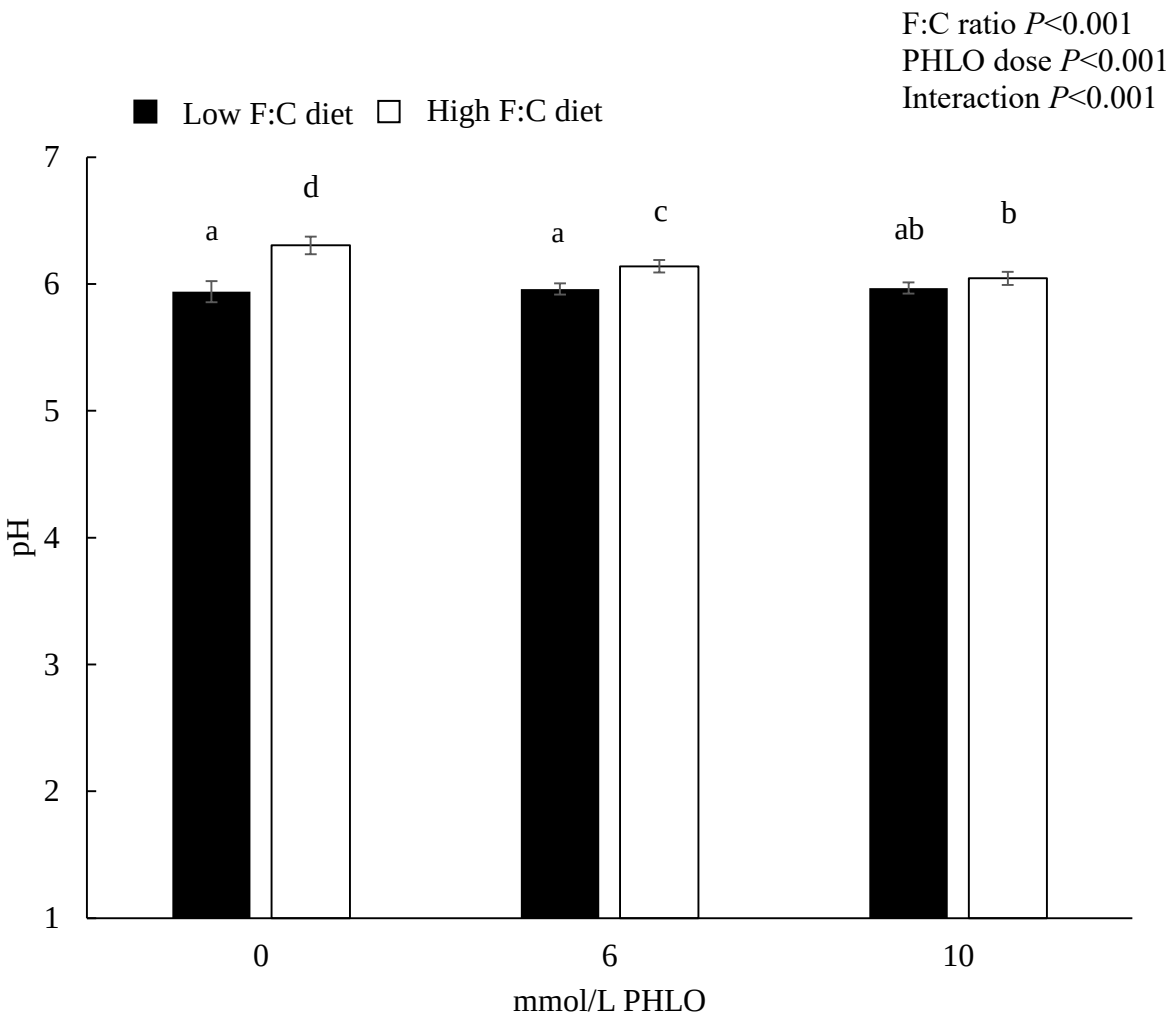

Error bar shows standard error of mean

Figure 3- 3. Effect of phloroglucinol supplementation and F:C ratio on dry matter digestibility



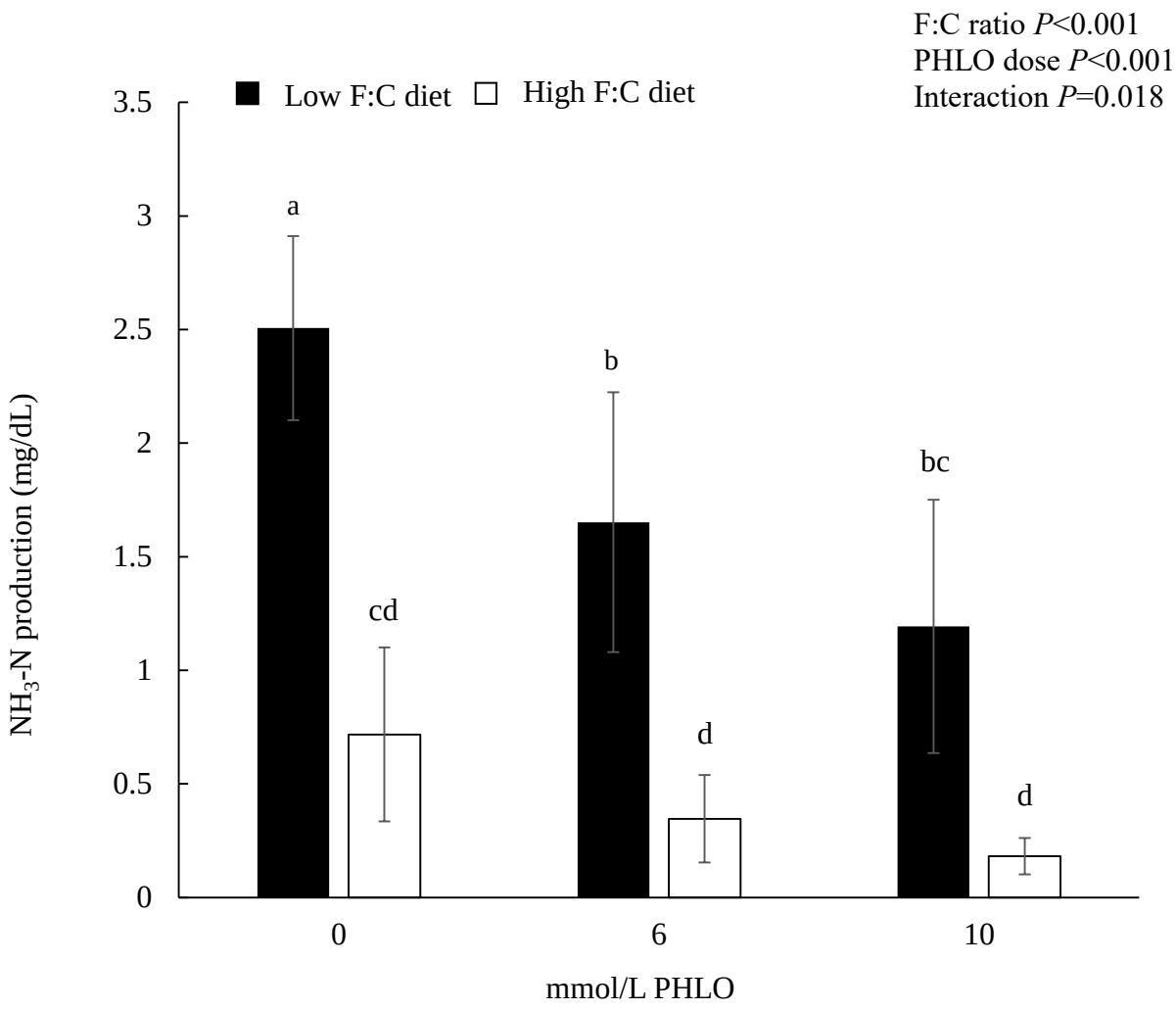

Error bar shows standard error of mean

Figure 3- 4. Effect of phloroglucinol supplementation and F:C ratio on $CH_4$/DDM



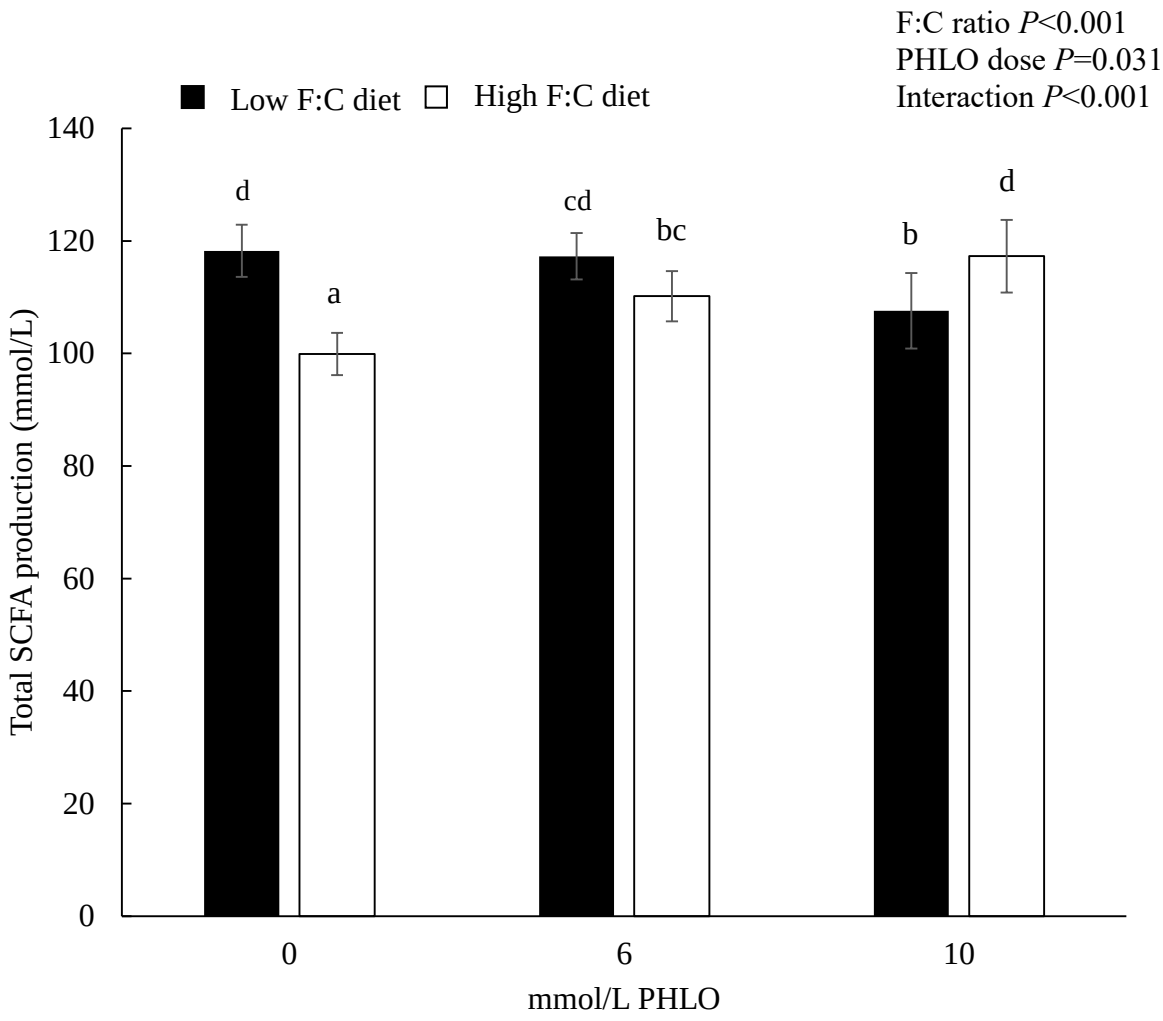

Error bar shows standard error of mean

Figure 3- 5. Effect of phloroglucinol supplementation and F:C ratio on pH.



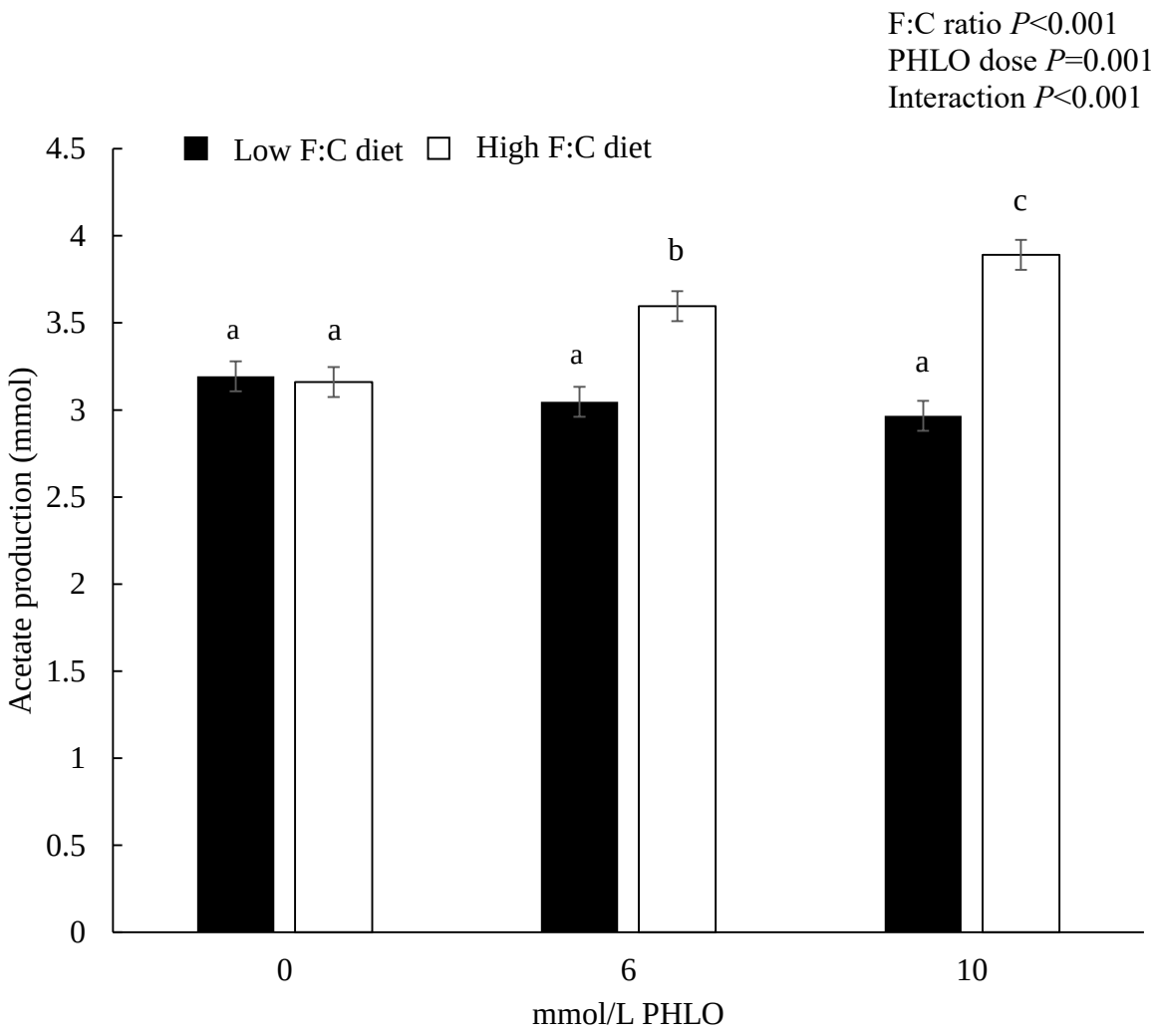

Error bar shows standard error of mean

Figure 3- 6. Effect of phloroglucinol supplementation and F:C ratio on $NH_3$-N.



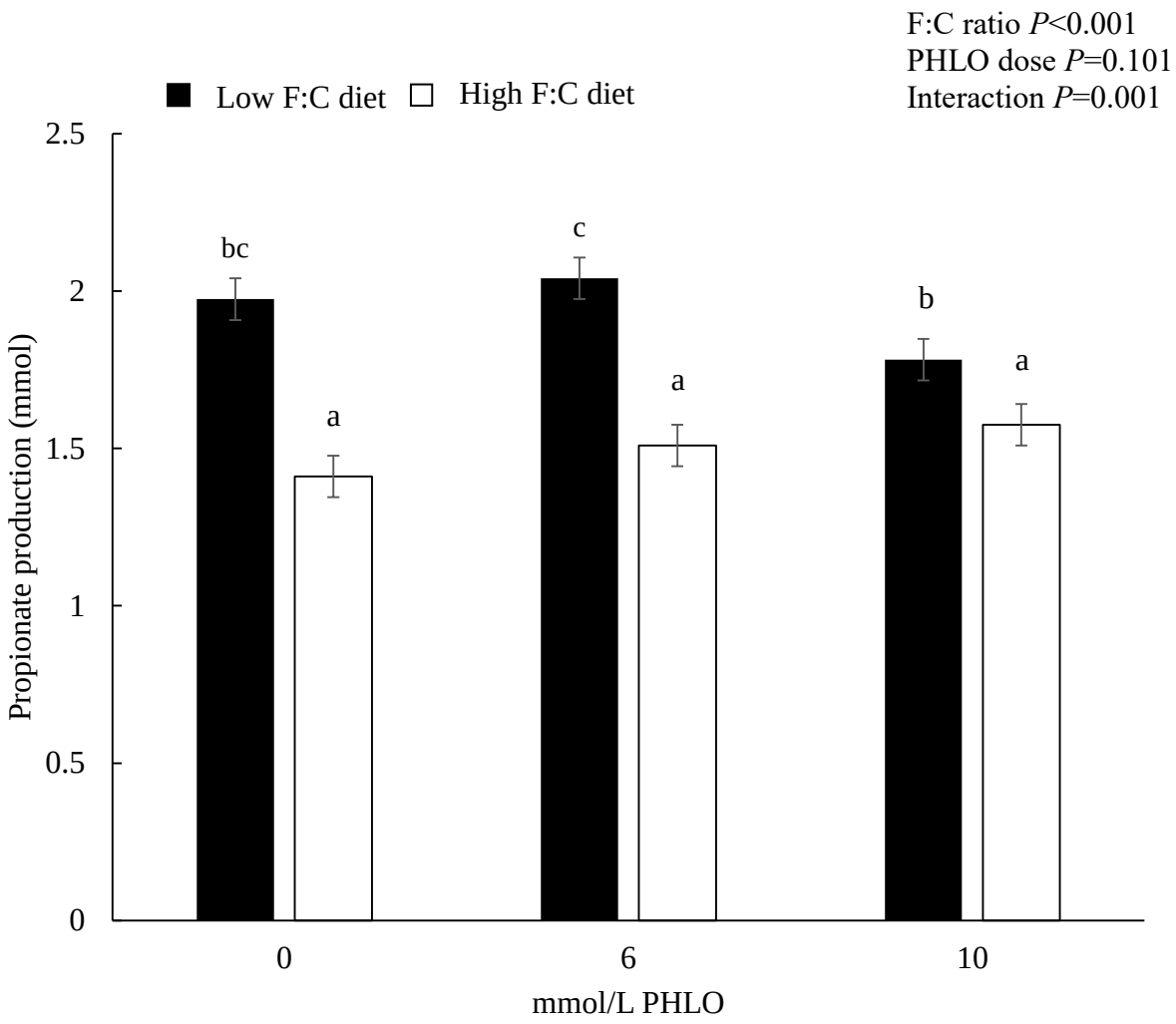

Error bar shows standard error of mean

Figure 3- 7. Effect of phloroglucinol supplementation and F:C ratio on total SCFA.



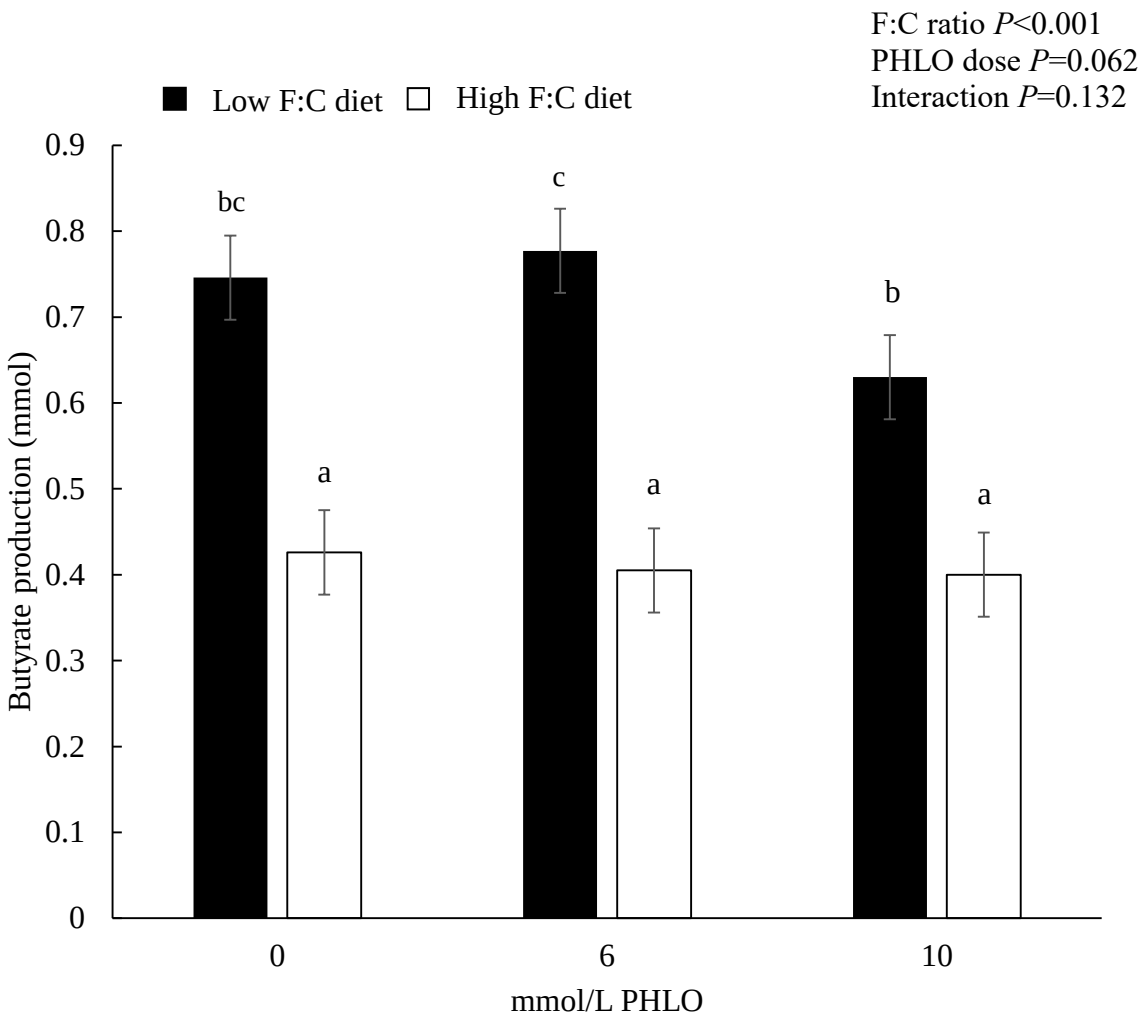

Error bar shows standard error of mean

Figure 3- 8. Effect of phloroglucinol supplementation and F:C ratio on acetate production.



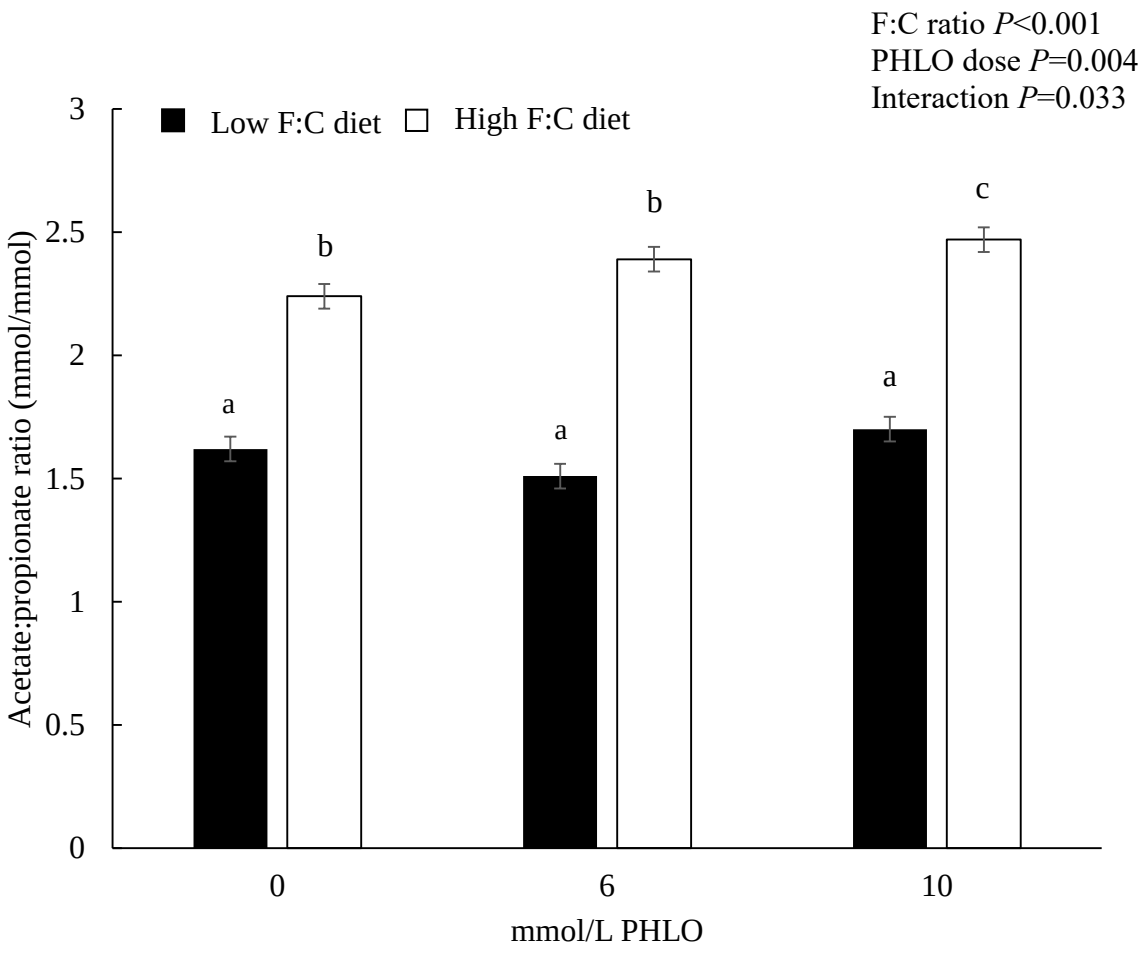

Error bar shows standard error of mean

Figure 3- 9. Effect of phloroglucinol supplementation and F:C ratio on propionate production.



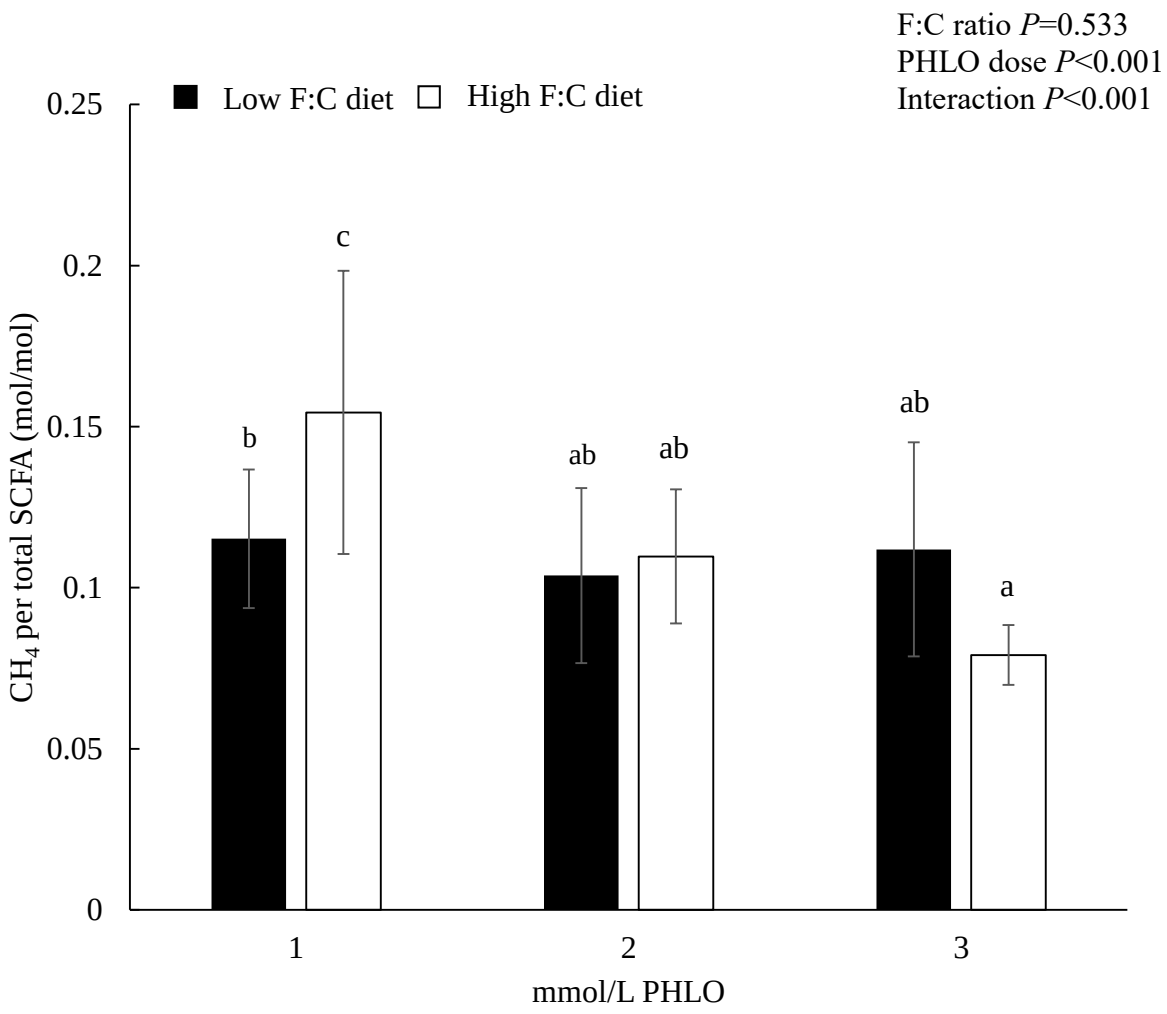

Error bar shows standard error of mean

Figure 3- 10. Effect of phloroglucinol supplementation and F:C ratio on butyrate production.



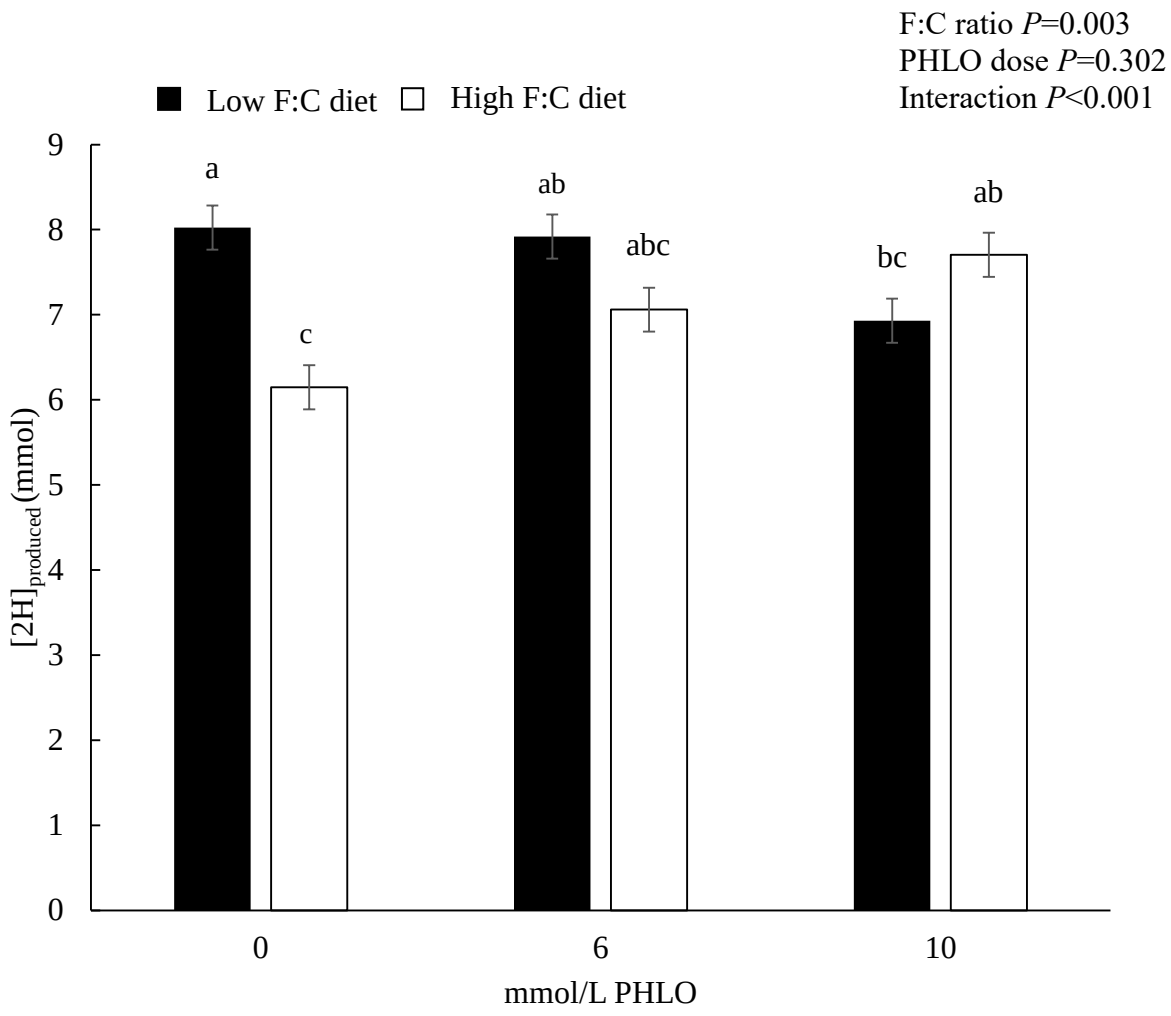

Error bar shows standard error of mean

Figure 3- 11. Effect of phloroglucinol supplementation and F:C ratio on acetate:propionate ratio.



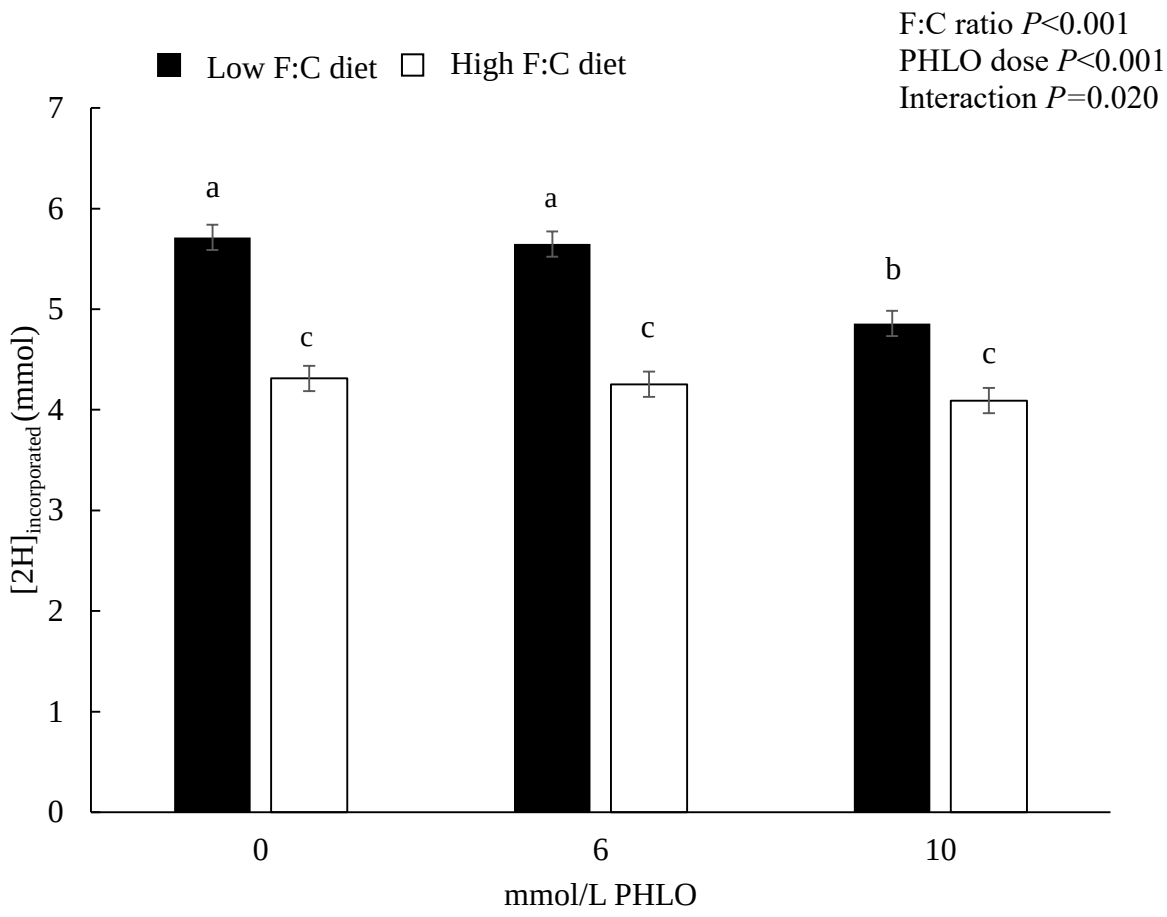

Error bar shows standard error of mean

Figure 3- 12. Effect of phloroglucinol supplementation and F:C ratio on $CH_4$ per total SCFA.



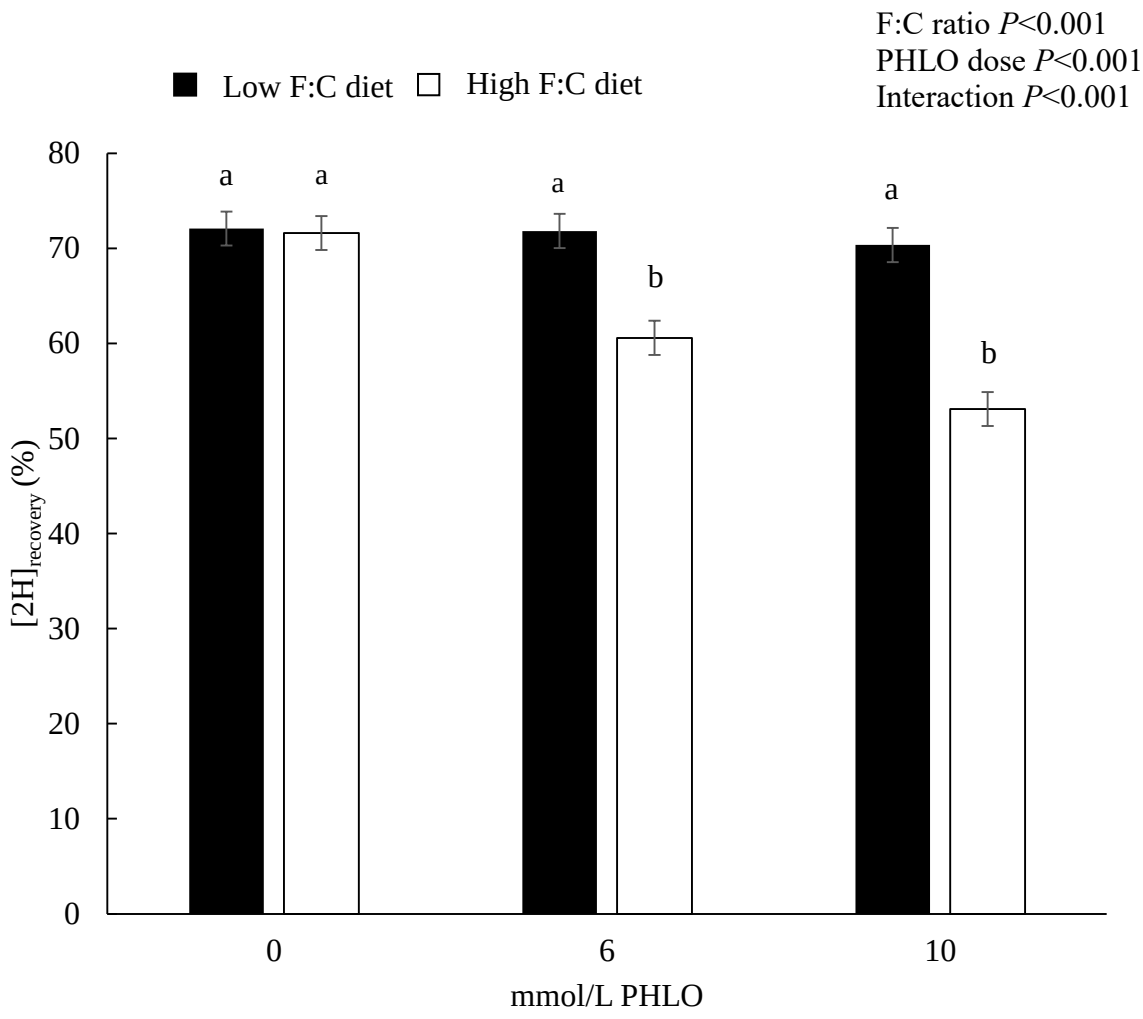

Error bar shows standard error of mean

Figure 3- 13. Effect of phloroglucinol supplementation and F:C ratio on $[2H]_{produced}$.



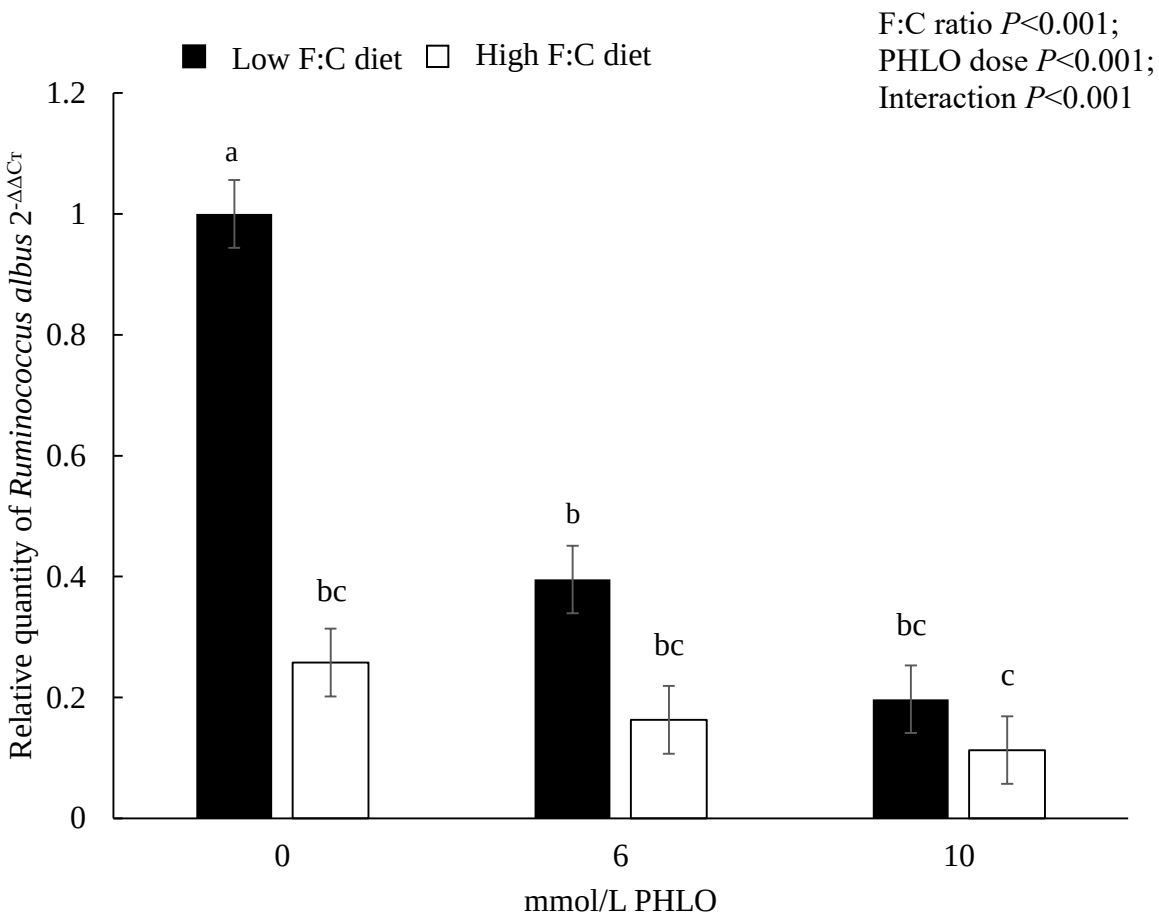

Error bar shows standard error of mean

Figure 3- 14. Effect of phloroglucinol supplementation and F:C ratio on $[2H]_{incorporated}$.



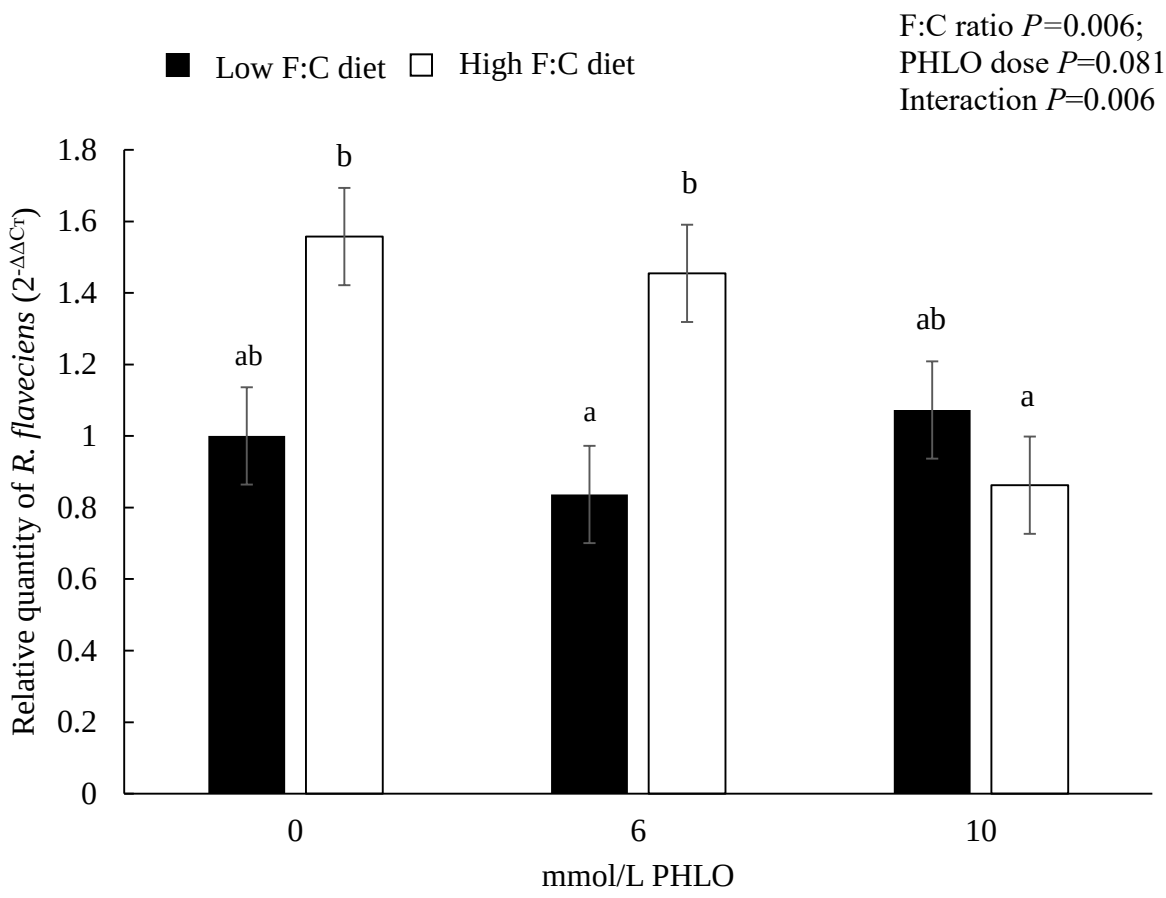

Error bar shows standard error of mean

Figure 3- 15. Effect of phloroglucinol supplementation and F:C ratio on $[2H]_{recovery}$.



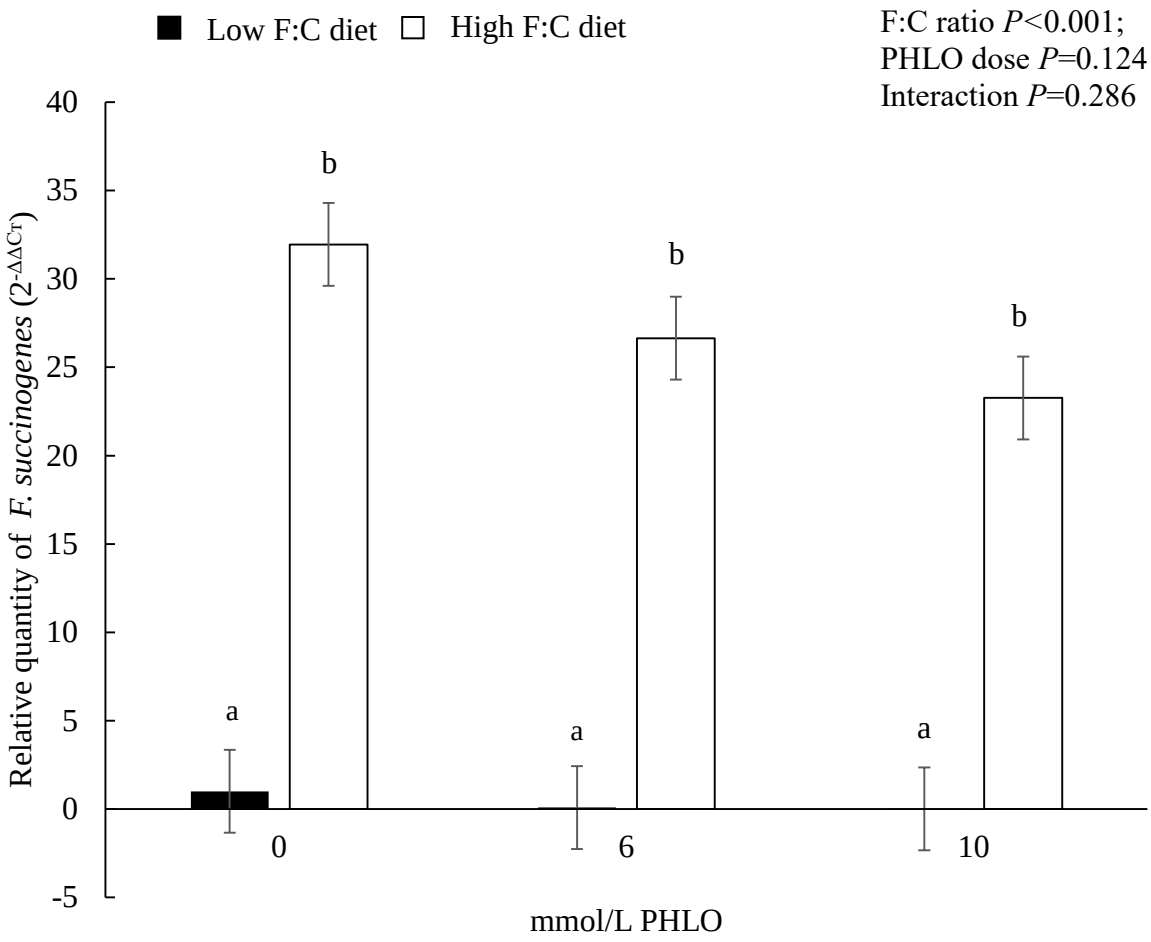

Error bar shows standard error of mean

Figure 3- 16. Effect of phloroglucinol supplementation and F:C ratio on relative quantity of *Ruminococcus albus*



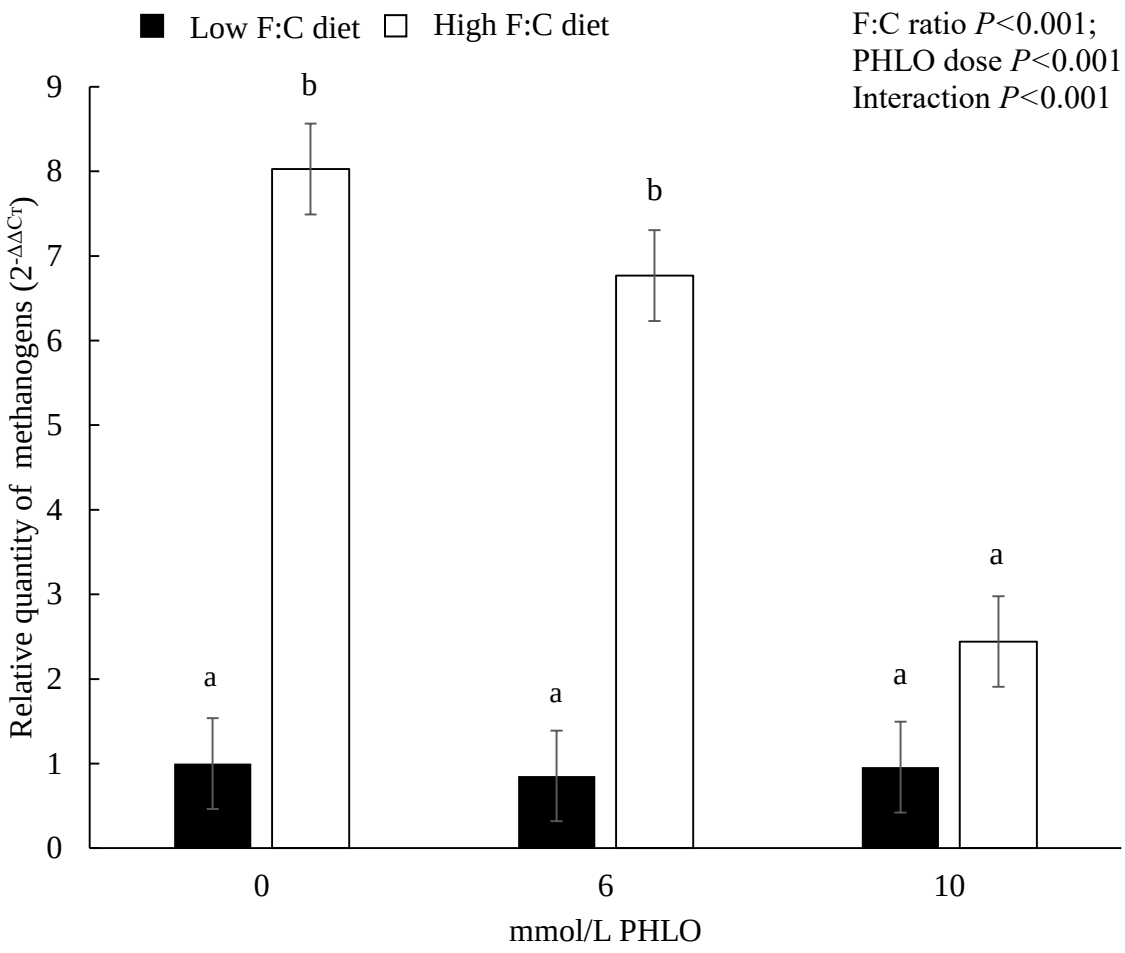

Error bar shows standard error of mean

Figure 3- 17. Effect of phloroglucinol supplementation and F:C ratio on relative quantity of *R. flaveciens*



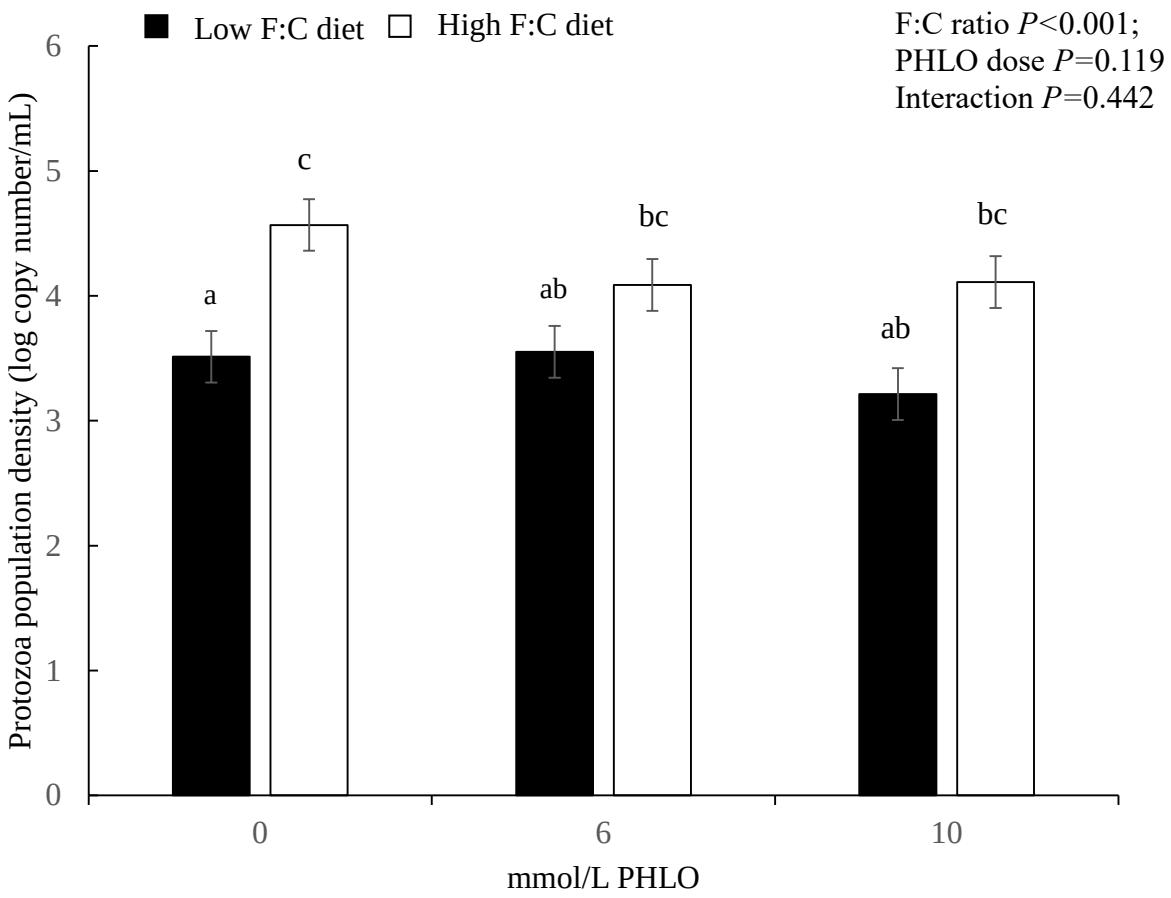

Error bar shows standard error of mean

Figure 3- 18. Effect of phloroglucinol supplementation and F:C ratio on relative quantity of *F. succinogenes*




Error bar shows standard error of mean

Figure 3- 19. Effect of phloroglucinol supplementation and F:C ratio on relative quantity of methanogens.




Error bar shows standard error of mean

Figure 3- 20. Effect of phloroglucinol supplementation and F:C ratio on relative quantity of protozoa.

## General Discussion

Phloroglucinol have been known to be able to redirect $H_2$ utilization to acetate production when methanogenesis is inhibited (Martinez-Fernandez et al., 2017). This finding showed that phloroglucinol is an alternative $H_2$ sink agent and might be able to reduce $CH_4$ production when it is supplemented alone. However, no study had been conducted to investigate the effect of phloroglucinol alone on $CH_4$ production, rumen fermentation profiles, and microbial population.

Results from the current study confirm that phloroglucinol can reduce $CH_4$ production from the rumen. In the Chapter 1, $CH_4$ emission was reduced by up to 15% by an addition of 10 mmol/L phloroglucinol. In the Chapter 2, phloroglucinol reduced $CH_4$ production in all of the diets. $CH_4$ was reduced by 18% in low F:C ratio and 32% in high F:C ratio by addition of 10 mmol/L phloroglucinol. Our results also suggest that the inhibitory effect of phloroglucinol was in a dose-dependent manner where a higher dose of phloroglucinol reduced more $CH_4$ linearly. A previous study showed that the combination of chloroform and phloroglucinol reduced $CH_4$ production by 46% (Martinez-Fernandez et al., 2017). However, the majority of reduction in the previous finding is attributable to chloroform rather than phloroglucinol. This study is the first to show the effect of phloroglucinol on $CH_4$ reduction when it is supplemented alone.

The reduction of $CH_4$ by phloroglucinol was accompanied with reduction on digestibility. In Chapter 1, an addition of 10 mmol/L phloroglucinol reduced DMD by 13%, total gas production by 10%, and $NH_3$-N production by 70%. This result was consistent with the findings in Chapter 2 which showed that 10 mmol/L phloroglucinol reduced DMD by 5% and 32%, total gas production was reduced by 8% and 3%, and $NH_3$-N was reduced by 52% and 74% in low and high F:C ratio, respectively. This finding showed that phloroglucinol suppressed digestion of

carbohydrate and protein. Our results showed that phloroglucinol possess anti-nutrient traits which were presumably related to its chemical structure. Phloroglucinol is a simple phenolic compound which contain free hydroxyl groups that allow interaction with feed nutrients especially carbohydrate and protein. Free hydroxyl group may create complexes with lignocellulose and protein through strong hydrogen bounds (Silanikove et al., 2001). McSweeney et al. (2001) suggested that the mechanisms of polyphenolics on inhibiting nutrient digestion are suppression of enzyme activity, suppression of microbial growth or activity, or by protection of substrates. In this study, phloroglucinol did not have any effect on the relative quantity of dominant bacterial group in the rumen. The current findings showed that addition of phloroglucinol did not suppress microbial growth in the rumen. Therefore, the reduction of digestibility by phloroglucinol is not due to inhibition of microbial growth. However, other mechanisms were not investigated in this study. Therefore, it would be interesting to investigate the mechanisms of phloroglucinol on inhibition of nutrient digestion in future studies.

Simultaneous reduction of DMD and total gas production indicate that some part of $CH_4$ reduction in the rumen by phloroglucinol is related to retardation of nutrient digestibility, particularly, carbohydrate digestibility. In the rumen, $CH_4$ was mainly produced by using hydrogenotrophic pathway, using $H_2$ and $CO_2$ produced from fibre and starch digestion. Therefore, any reduction on fibre and starch digestion presumably will lead to reduction in $CH_4$ production. Our finding is in agreement to previous studies which demonstrated that polyphenolics compound might reduce $CH_4$ production indirectly through inhibition of carbohydrate digestion (Tavendale et al., 2005, Jayanegara et al., 2015).

Effect of phloroglucinol on SCFA production varied. In Chapter 1, phloroglucinol did not have any effect on the total SCFA production. In chapter 2, phloroglucinol reduced the total

SCFA production of low F:C ratio but in high F:C ratio, total SCFA production increased. These results showed that the effect of phloroglucinol on total SCFA production depends on the characteristic of the diets. In Chapter 1, the diet contains 35% forage meanwhile in chapter 2, low F:C ratio contains 20% forage and high F:C ratio contains 80% forage. Our results showed that the higher content of forage in the diet increased the total SCFA production. It might be related to the $H_2$ produced from forage digestion. Forage digestion in the rumen favour production of acetate which the reaction will liberate $H_2$ (Valadares et al., 1999). On the other hand, rumen microorganisms need $H_2$ to reduce phloroglucinol to acetate (Krumholz and Bryant, 1986). Therefore, the more $H_2$ that is available, the more phloroglucinol is degraded to acetate. Thus, it will increase total SCFA production. These findings are supported by the changes on SCFA proportion by phloroglucinol addition.

Our study showed that addition of phloroglucinol increased acetate production. In chapter 1, acetate was increased in expense of propionate. In chapter 2, acetate increment was not accompanied with simultaneous reduction on propionate. In both chapters, phloroglucinol did not affect butyrate production. This finding showed that phloroglucinol might redirect $H_2$ utilization in the rumen to acetate production. Therefore, some part or the $CH_4$ reduction by phloroglucinol is attributable to redirected $H_2$ utilization. Previous studies confirm that phloroglucinol can be degraded by rumen microorganisms to acetate (Krumholz and Bryant, 1986, Krumholz et al., 1986, Martinez-Fernandez et al., 2017). Redirection of fermentation in the rumen by phloroglucinol serve two function. First function is to reduce $CH_4$ emission from the rumen. The second function is to provide energy for ruminant in the form of SCFA. In previous studies, a combination of chloroform and phloroglucinol increased daily weight gain of treated animals. The results must be interpreted carefully because it was a short period test, but the

results showed that phloroglucinol increase utilization of a substrate which previously was not considered as an energy yielding nutrient for host animals (Martinez-Fernandez et al., 2017).

In relation to redirection of $H_2$ utilization of phloroglucinol to acetate, our results showed that an increase in relative quantity of genus *Coprococcus* suggests that *Coprococcus* is a major contributor for degradation of phloroglucinol. *Coprococcus spp.* were able to degrade 1 molecule of phloroglucinol to 2 molecule of acetic acid and 2 molecules of $CO_2$ (Tsai et al., 1976). Patel et al. (1981) showed that *Coprococcus* reduced phloroglucinol to dihydrophloroglucinol by using NADPH as an electron donor. Recent findings also showed several increases of OTUs assigned to *Coprococcus spp*.

The effect of phloroglucinol on methanogen relative quantity was not consistent. In Chapter 1, there was no significant effect of phloroglucinol on methanogen relative quantity. Although, in Chapter 2, phloroglucinol addition reduced methanogen severely in high F:C ratio but not in low F:C ratio. This finding might indicate that $CH_4$ reduction in high forage diet by phloroglucinol is partly related to inhibition of methanogen growth. Phenolic compound may reduce $CH_4$ emission directly by inhibiting methanogen growth (Tavendale et al., 2005).

Based on the result, the conclusions are as follows:

1. Phloroglucinol can reduce $CH_4$ production and the inhibitory effect was in a dose-dependent manner.
2. $CH_4$ was reduced by phloroglucinol in doses ranging from 6 mmol/L to 10 mmol/L.
3. Phloroglucinol might reduce $CH_4$ production by inhibiting digestibility of nutrients, redirecting $H_2$ utilization, and partially by inhibiting methanogen growth.
4. Genus *Coprococcus* is a major contributor of phloroglucinol degradation in the rumen.

Finally, future studies are required to have a comprehensive understanding on the anti methanogenesis effect of phloroglucinol on *in vivo* environment. Studies investigating addition of natural product rich in phloroglucinol, such as *Ecklonia cava* are needed to increase understandings of phloroglucinol in practical conditions.

## Summary

Ruminants are major contributors of methane ($CH_4$) emission. Ruminants mainly emit $CH_4$ as a result from digestion of feed and rumen microorganisms digest structural carbohydrate in the feed resulting in the production $H_2$ and $CO_2$. These compounds are used by methanogens to produce $CH_4$.

One of the strategies to reduce $CH_4$ emission from rumen is to add a compound that can be an alternative $H_2$ sink agent and redirect $H_2$ utilization from $CH_4$ to SCFA production. Addition of the compound may create $H_2$ starvation for methanogen and may lead to less $CH_4$ production. Phloroglucinol is a natural product of rumen metabolism of tannin and several rumen bacteria reportedly can reduce phloroglucinol to acetate by using $H_2$. This result showed that phloroglucinol may be able to be used as an alternative $H_2$ sink agent. However, the effect of manipulation on rumen fermentation *in vitro* by using phloroglucinol is not known. Therefore, we aimed to study the effect of phloroglucinol in $CH_4$ mitigation strategy.

In the first study, the experiment was designed to determine the effect of phloroglucinol alone on $CH_4$ emission, rumen fermentation profiles, and rumen microbial population density. The incubation was conducted by using a substrate only as control and 5 different doses of phloroglucinol. The results showed that 10 mmol/L phloroglucinol reduced $CH_4$ production by 15.0%. Total gas production, dry matter digestibility (DMD), $CH_4$/total SCFA, and $NH_3$-N were simultaneously reduced by phloroglucinol, but it had no effect on the total SCFA concentration. Acetate increased at the expense of propionate. This might indicate the redirection of $H_2$ utilization from $CH_4$ to acetate, and might be related to $CH_4$ reduction. Phloroglucinol had no effect on methanogen, but it reduced the population density of *R. albus.*

In the second study, the effect of phloroglucinol and forage to concentrate (F:C) ratio on $CH_4$ emission, rumen fermentation profiles, and microbial population density were investigated. The treatments were 0, 6, 10 mmol/L phloroglucinol, and two different F:C ratio, low and high F:C ratios. The results showed that phloroglucinol reduced $CH_4$ significantly in both diets. However, there were no effect of F:C ratio and F:C + phloroglucinol on $CH_4$ production. Acetate proportion in both diet increased by addition of phloroglucinol. DMD, $NH_3$-N, and methanogen relative quantity were reduced by phloroglucinol. Phloroglucinol significantly affected total SCFA production in both diets. The reduction of $CH_4$ by phloroglucinol was probably related to simultaneous reduction on methanogen relative quantity and DMD.

In conclusion, phloroglucinol is able to reduce $CH_4$ production in the rumen *in vitro*. It might change the fermentation of the rumen by redirecting $H_2$ utilization to SCFA production. However, the reduction of $CH_4$ was followed by simultaneous reduction of digestibility.

## References


Abrar A, Kondo M, Kitamura T, Ban-Tokuda T, Matsui H. 2016. Effect of supplementation of rice bran and fumarate alone or in combination on *in vitro* rumen fermentation, methanogenesis and methanogens. *Animal Science Journal* **87,** 398–404.

Agle M, Hristov AN, Zaman S, Schneider C, Ndegwa PM, Vaddella VK. 2010. Effect of dietary concentrate on rumen fermentation, digestibility, and nitrogen losses in dairy cows. *Journal of Dairy Science* **93,** 4211–4222.

Aguerre MJ, Wattiaux MA, Powell JM, Broderick GA, Arndt C. 2011. Effect of forage-to-concentrate ratio in dairy cow diets on emission of methane, carbon dioxide, and ammonia, lactation performance, and manure excretion. *Journal of Dairy Science* **94,** 3081–3093.

Asiegbu FO, Paterson A, Morrison IM, Smith JE. 1995. Effects of cell wall phenolics and fungal metabolites on methane and acetate production under in vitro rumen conditions. *The Journal of General and Applied Microbiology* **41,** 475–485.

Beauchemin KA, Kreuzer M, O'Mara F, Mcallister TA. 2008. Nutritional management for enteric methane abatement: a review. *Australian Journal of Experimental Agriculture,* **48,** 21–27.

Chesson A, Forsberg CW. 1997. Polysaccharide degradation by rumen microorganisms. *In:* Hobson PN, Stewart CS. (eds.) *The Rumen Microbial Ecosystem.* Dordrecht: Springer Netherlands.

Denman SE, McSweeney CS. 2006. Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiology Ecology* **58,** 572–582.

Denman SE, Tomkins N, McSweeney CS. 2007. Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. *FEMS Microbiology Ecology* **62,** 313–322.

Field JA, Lettinga G. 1992. Toxicity of Tannic Compounds to Microorganisms. *In:* Hemingway RW, Laks PE. (eds.) Plant Polyphenols: Synthesis, Properties, Significance. Boston, MA: Springer US.

García-Martínez R, Ranilla MJ, Tejido ML, Carro MD. 2007. Effects of disodium fumarate on in vitro rumen microbial growth, methane production and fermentation of diets differing in their forage:concentrate ratio. *British Journal of Nutrition* **94**, 71.

Getachew G, Pittroff W, Putnam DH, Dandekar A, Goyal S, DePeters EJ. 2008. The influence of addition of gallic acid, tannic acid, or quebracho tannins to alfalfa hay on *in vitro* rumen fermentation and microbial protein synthesis. *Animal Feed Science and Technology* **140,** 444–461.

Janssen PH. 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Animal Feed Science and Technology* **160,** 1–22.

Jayanegara A. 2010. Ruminal methane production on simple phenolic acids addition in *in vitro* gas production method. *Media Peternakan,* **32**.

Jayanegara A, Goel G, Makkar HPS, Becker K. 2015. Divergence between purified hydrolysable and condensed tannin effects on methane emission, rumen fermentation and microbial population *in vitro*. *Animal Feed Science and Technology* **209,** 60–68.

Jayanegara A, Leiber F, Kreuzer M. 2012a. Meta-analysis of the relationship between dietary tannin level and methane formation in ruminants from *in vivo* and *in* vitro experiments. *Journal of Animal Physiology and Animal Nutrition* **96,** 365–375.

Jayanegara A, Marquardt S, Wina E, Kreuzer M, Leiber F. 2012b. *In vitro* indications for favourable non-additive effects on ruminal methane mitigation between high-phenolic and high-quality forages. *British Journal of Nutrition* **109,** 615–622.

Johnson KA, Johnson DE. 1995. Methane emissions from cattle. *Journal of Animal Science* **73,** 2483–2492.

Kaufmann W, Hagemeister H, Dirksen G. 1980. Adaptation to changes in dietary composition, level and frequency of feeding. *In:* Ruckebusch Y, Thivend P. (eds.) Digestive Physiology and Metabolism in Ruminants: Proceedings of the 5th International Symposium on Ruminant Physiology, held at Clermont — Ferrand, on 3rd–7th *September, 1979.* Dordrecht: Springer Netherlands.

Knapp JR, Laur GL, Vadas PA, Weiss WP, Tricarico JM. 2014. Enteric methane in dairy cattle production: Quantifying the opportunities and impact of reducing emissions. *Journal of Dairy Science* **97,** 3231–3261.

Koike S, Kobayashi Y. 2001. Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. *FEMS Microbiology Letters* **204,** 361–366.

Kondo M, Hirano Y, Ikai N, Kita K, Jayanegara A, Yokota H-O. 2014. Assessment of anti-nutritive activity of tannins in tea by-products based on *in vitro* rumen fermentation. *Asian-Australasian Journal of Animal Sciences* **27,** 1571–1576.

Krumholz LR, Bryant MP. 1986. *Eubacterium oxidoreducens* sp. nov. requiring H2 or formate to degrade gallate, pyrogallol, phloroglucinol and quercetin. *Archives of Microbiology* **144,** 8–14.

Krumholz LR, Crawford RL, Hemling ME, Bryant MP. 1986. A rumen bacterium degrading quercetin and trihydroxybenzenoids with concurrent use of formate or $H_2$. *Progress in clinical and biological research* **213,** 211–214.

Lovett D, Lovell S, Stack L, Callan J, Finlay M, Conolly J, O'Mara FP. 2003. Effect of forage/concentrate ratio and dietary coconut oil level on methane output and performance of finishing beef heifers. *Livestock Production Science* **84,** 135–146.

Lwin KO, Kondo M, Ban-Tokuda T, Lapitan RM, Del-Barrio AN, Fujihara T, Matsui H. 2012. Ruminal fermentation and microbial ecology of buffaloes and cattle fed the same diet. *Animal Science Journal* **83,** 767–776.

Makkar HPS. 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. *Small Ruminant Research* **49,** 241–256.

Makkar HPS, Francis G, Becker K. 2007. Bioactivity of phytochemicals in some lesser-known plants and their effects and potential applications in livestock and aquaculture production systems. *Animal* **1,** 1371–1391.

Martinez-Fernandez G, Denman SE, Cheung J, McSweeney CS. 2017. Phloroglucinol degradation in the rumen promotes the capture of excess hydrogen generated from methanogenesis inhibition. *Frontiers in Microbiology* **8,** 1871.

Matsui H, Wakabayashi H, Fukushima N, Ito K, Nishikawa A, Yoshimi R, Ogawa Y, Yoneda S, Ban-Tokuda T, Wakita M. 2013. Effect of raw rice bran supplementation on rumen methanogen population density and *in vitro* rumen fermentation. *Grassland Science* **59,** 129–134.

McAllister TA, Newbold CJ. 2008. Redirecting rumen fermentation to reduce methanogenesis. *Australian Journal of Experimental Agriculture* **48,** 7–13.

McDougall EI. 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. *Biochemical Journal* **43,** 99–109.

McSweeney CS, Palmer B, McNeill DM, Krause DO. 2001. Microbial interactions with tannins: nutritional consequences for ruminants. *Animal Feed Science and Technology* **91,** 83–93.

Moe PW, Tyrrell HF. 1979. Methane production in dairy cows. *Journal of Dairy Science* **62,** 1583–1586.

Moomaw CR, Hungate RE. 1963. Ethanol conversion in the bovine rumen. *Journal of Bacteriology* **85,** 721–722.

Mueller-Harvey I. 2006. Unravelling the conundrum of tannins in animal nutrition and health. *Journal of the Science of Food and Agriculture* **86,** 2010–2037.

Newbold CJ, López S, Nelson N, Ouda JO, Wallace RJ, Moss AR. 2007. Propionate precursors and other metabolic intermediates as possible alternative electron acceptors to methanogenesis in ruminal fermentation *in vitro*. *British Journal of Nutrition* **94,** 27–35.

Patel TR, Jure KG, Jones GA. 1981. Catabolism of phloroglucinol by the rumen anaerobe *Coprococcus*. *Applied and Environmental Microbiology* **42,** 1010–1017.

Silanikove N, Perevolotsky A, Provenza FD. 2001. Use of tannin-binding chemicals to assay for tannins and their negative postingestive effects in ruminants. *Animal Feed Science and Technology* **91,** 69–81.

Smith P, Clark H, Dong H, Elsiddig EA, Haberl H, Harper R, House J, Jafari M, Masera O, Mbow C, Ravindranath NH, Rice CW, Roble Do Abad C, Romanovskaya A, Sperling F, Tubiello F. 2014. Chapter 11 - Agriculture, forestry and other land use (AFOLU). *Climate Change 2014: Mitigation of Climate Change. IPCC Working Group III Contribution to AR5.* Cambridge University Press.

Steinfeld H, Gerber P, Wassenaar T, Castel V, Rosales M, Haan CD. 2006. Livestock's long shadow: environmental issues and options, Rome, Food and Agriculture Organization of the United Nations (FAO).

Stewart CS, Flint HJ, Bryant MP. 1997. The rumen bacteria. *In:* Hobson PN, Stewart CS. (eds.) *The Rumen Microbial Ecosystem.* Dordrecht: Springer Netherlands.

Sylvester JT, Karnati SKR, Yu Z, Morrison M, Firkins JL. 2004. Development of an assay to quantify rumen ciliate protozoal biomass in cows using Real-Time PCR. *The Journal of Nutrition* **134,** 3378–3384.

Tajima K, Aminov RI, Nagamine T, Matsui H, Nakamura M, Benno Y. 2001. Diet-dependent shifts in the bacterial population of the rumen revealed with Real-Time PCR. *Applied and Environmental Microbiology* **67,** 2766–2774.

Tavendale MH, Meagher LP, Pacheco D, Walker N, Attwood GT, Sivakumaran S. 2005. Methane production from *in vitro* rumen incubations with *Lotus pedunculatus* and

*Medicago sativa*, and effects of extractable condensed tannin fractions on methanogenesis. *Animal Feed Science and Technology* **123,** 403–419.

Tsai C-G, Jones GA. 1975. Isolation and identification of rumen bacteria capable of anaerobic phloroglucinol degradation. *Canadian Journal of Microbiology* **21,** 794–801.

Tsai CG, Gates DM, Ingledew WM, Jones GA. 1976. Products of anaerobic phloroglucinol degradation by *Coprococcus* sp. Pe15. *Canadian Journal of Microbiology* **22,** 159–164.

Uddin MK, Kondo M, Kita J, Matsui H, Karita S, Goto M. 2010. Effect of supplementation of soy sauce cake and vinegar brewer's cake with total mixed ration silage-based diet on nutrient utilization by Holstein steers. *Journal of Food Agriculture & Environment* **8,** 282–287.

Ungerfeld EM. 2015. Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: A meta-analysis. *Frontiers in Microbiology* **6,** 37.

Ushida K, Tanaka H, Kojima Y. 1989. Effect of phenolic acids on gas and volatile fatty acid production by mixed rumen population with or without protozoa. *Nihon Chikusan Gakkaiho* **60,** 1135–1142.

Valadares RFD, Broderick GA, Filho SCV, Clayton MK. 1999. Effect of replacing alfalfa silage with high moisture corn on ruminal protein synthesis estimated from excretion of total purine derivatives. *Journal of Dairy Science,* 82**,** 2686–2696.

Van Kessel JAS, Russell JB. 2006. The effect of pH on ruminal methanogenesis. *FEMS Microbiology Ecology* **20,** 205–210.

Weatherburn MW. 1967. Phenol-hypochlorite reaction for determination of ammonia. *Analytical Chemistry* **39,** 971–974.

**Acknowledgements**

First of all, praise and gratitude due to Allah SWT with His compassion and mercifulness has allowed me finalizing this Ph.D. Thesis.

I would like to express my profound gratitude to Professor Dr. Hiroki Matsui, Head of Laboratory of Animal Production, Graduate School of Bioresources, Mie University as my supervisor who has given me suggestion, encouragement and a full support not only for finishing my experiment and thesis, but also during my stay in Mie University.

I also would like to express the gratefulness to Associate Professor Dr. Tomomi Ban-Tokuda, Laboratory of Animal Production, Graduate School of Bioresources, Mie University, Associate Professor Dr. Makoto Kondo, Laboratory of Grassland and Animal Feed Production, Graduate School of Bioresources, Mie University and Dr. Anuraga Jayanegara, Faculty of Animal Science, Bogor Agricultural University for their limitless kind support and advices.

Besides my advisor, I would like to thank the rest of my thesis committee: Professor Dr. Yoshihiro Yamada, Head of Laboratory of Insect Ecology, Graduate School of Bioresources, Mie University, and Prof. Teruhisa Umezaki, Head of Laboratory of Crop Science, Graduate School of Bioresources, Mie University, for their insightful comments and advice.

I would like to express my thankfulness to The Life Science Research Center (Center for Molecular Biology and Genetics), Mie University for the permission to using facilities to perform real-time PCR assay.

My special gratitude goes to Indonesian community in Mie University for sharing all experience during my stay and treat me as one of their family. My special thanks also goes to Budi Wardiman for animal handling during experiment.

Last but not least, a huge thank is addressed to my wife, Hani Sukmawati, my daughter, Miyuki Siti Tsabita, and all of my family in Indonesia for their endless love, care, and encouragement for the whole of my life.

Ki Ageng Sarwono