Coleus amboinicus (Lour.) leaves as a modulator of ruminal methanogenesis and biohydrogenation in vitro¹

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ABSTRACT: Active plant metabolites (APM) are recognized as modifiers of ruminal microbial fermentation including methanogenesis and biohydrogenation of fatty acids (FA). *Coleus amboinicus* Lour. leaves (CAL) are rich in several APM, which could serve as ruminal fermentation modulators. A phytochemical analysis showed that CAL contain phenolic acids (10.4 mg·g⁻¹ dry matter [DM]; high in rosmarinic acid), flavonoids (2.6 mg·g⁻¹ DM), diterpenes (2 mg·g⁻¹ DM), and linolenic acid (35.4 g (100 g)⁻¹ FA). This study aimed to investigate the effect of CAL on ruminal methanogenesis and biohydrogenation as well as basic fermentation characteristics and microbial populations. The in vitro experiment was carried out using Hohenheim gas test system with 40 mL of buffered ruminal fluid incubated for 24 h at 39 °C in anaerobic conditions. Approximately 400 mg (DM basis) of total mixed ration (TMR) was used as a control substrate and

the CAL substrate was used at doses of 10, 20, 40, and 80 mg DM replacing equal amounts of TMR. Addition of CAL decreased methane production up to 30% linearly as the amount of CAL increased ($P < 0.05$). In vitro dry matter digestibility and ammonia tended to increase with increasing doses of CAL. Concentration of total volatile fatty acids was not affected by the CAL although there appeared to be a minor positive linear trend; however, acetate, butyrate, and isobutyrate proportion increased quadratically ($P < 0.001$). CAL tended to linearly increase α -linolenic acid and conjugated linoleic acid as well as increased stearic acid concentration in buffered ruminal fluid. CAL particularly increased total protozoa and bacterial populations during fermentation, but inhibited methanogens. It is concluded that the CAL may be promising to be used as a feed additive to decrease methanogenesis as well as biohydrogenation of FA in the rumen.

Key words: biohydrogenation, *Coleus amboinicus* Lour., fatty acid, methanogenesis, phenolic acid, rumen

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INTRODUCTION

Ruminants are substantial contributors of anthropogenic and enteric methane emissions in the world (FAO, 2017). Agriculture is responsible for approximately 10% to 12% of global anthropogenic greenhouse gas emissions and enteric methane fermentation contributes 32% to 39% of total methane emission arising from agricultural sources (Moss et al., 2000; Broucek, 2014). However, enteric methane from ruminants accounted for approximately 44% of the total global enteric methane emission from all animals (FAO, 2017). In ruminants, methane is also responsible for 2% to 12% loss of dietary gross energy (Johnson et al., 1991; Moss et al., 2000). The effective strategy to reduce methane emission in ruminants using feed additives is greatly needed (Broucek, 2014; Szczechowiak et al., 2016). Many studies exploited plants rich in bioactive compounds to inhibit methanogenesis in ruminants (Patra and Saxena, 2010; Cieslak et al., 2013). Several studies confirmed that biologically active plant metabolites ($_{APM}$) such as phenolics, saponins, tannins, flavonoids, and essential oils reduce methane emissions from ruminants while simultaneously improving livestock productivity (Moss et al., 2000; Patra and Saxena, 2010). Moreover, APM including phenolics can influence fatty acid ($_{FA}$) concentrations of milk due to modulation of the ruminal microbial FA biohydrogenation process (Cappucci et al., 2018). The research on APM that may mitigate ruminal methane production simultaneously modulating the ruminal biohydrogenation process toward increased health beneficial FA proportions is greatly required.

Coleus amboinicus Lour. leaves ($_{CAL}$) is recognized as a plant rich in several APM (Damanik, 2009) such as total phenolics, flavonoids, alkaloids, tannins, and saponins (Arumugam et al., 2016). It has been confirmed that mode of action of CAL covers cytotoxic, antioxidant, antimicrobial, antifungal, and antiparasitic effects (Hullatti et al., 2011; El-hawary et al., 2012; Selvakumar et al., 2012). CAL is also responsible for anti-inflammatory and anti-tumor activities in rats due to the presence of alkaloids, flavonoids, and saponins fractions (Chen et al., 2014; Ramalakshmi et al., 2014). Dietary polyphenolics are associated with reduced ruminal biohydrogenation rate and the changes of ruminal microbial populations related to biohydrogenation and methanogens (Patra and Saxena, 2010; Cappucci et al., 2018), and consequently the APM content in CAL can be considered as a modulator of ruminal methanogenesis and biohydrogenation.

Although CAL has a high concentration of biologically beneficial APM, there is no report on the effect of CAL on ruminal methanogenesis and biohydrogenation processes. We hypothesized that CAL used in the diet, due to its nutrient and APM content, may modulate ruminal fermentation mitigating methane production and altering FA composition positively. Therefore, the aim of this study was to investigate the effect of CAL on ruminal methanogenesis and biohydrogenation as well as basic ruminal fermentation parameters and microbial populations.

MATERIALS AND METHODS

Preparation of CAL Powder

Fresh CAL were collected in the experimental fields of Bogor Agricultural University, Indonesia (6°33'12.4"S and 106°42'58.3"E). The location is in the temperate tropical climates (from 22 to 31 °C) with high humidity due to intensive rainfall. The CAL were collected from several plants (from 4 to 6 mo of growth stage) of various locations, dried for 3 d under the sun, and kept at 50 °C oven for 2 d. Dried CAL were then packaged and shipped to the Department of Animal Nutrition at the Poznan University of Life Sciences, Poland.

Chemical Analysis of Substrate

The chemical composition of CAL and total mixed ration (TMR) was analyzed following the methods of AOAC (2007) for determination of dry matter (DM; method no. 934.01), ash (method no. 942.05), crude protein (CP; using a Kjel-Foss Automatic 16210 analyzer; method no. 976.05), and ether extract (EE; using a Soxhlet System HT analyzer; method no. 973.18) concentrations. Organic matter (OM) content was calculated subtracting ash concentration from DM content. Ash-free neutral detergent fiber (aNDFom) was determined following the Van Soest et al. (1991) method with addition of amylase and sodium sulfite without residual ash. The FA concentrations in feed samples were determined using a gas chromatography method described by Szczechowiak et al. (2016). Briefly, FA hydrolysis was performed in a closed system using screw-cap teflon-stoppered tubes (Pyrex, 15 mL). A solution of 2 M NaOH (3 mL) was added to 100 mg of feed samples. Hydrolyzed samples were incubated in a block heater at 90 °C for 40 min. Samples were then extracted and

subsequently esterified to FA methyl esters (**FAME**) using borontrifluoride (Fluka-Sigma Aldrich, St. Louis, MO). A gas chromatograph (GC Bruker 456-GC, USA) fitted with a flame ionization detector and a 100-m fused-silica capillary column (0.25 mm ID) coated with 0.25 μ m Agilent HP (Chrompack CP7420) was used for FA analysis. A sample volume of 1 μ L was injected into the column. Identifications of FA including conjugated linoleic acids (**CLA**) were performed based on their retention times with suitable FAME standards (37 FAME Mix, CLA methyl esters, and a mixture of *cis*-9 and *trans*-11 and 10,12-octadecadienoic acid methyl esters; Sigma Aldrich, PA) using Galaxie Work Station 10.1 (Varian, CA), and were expressed as g (100 g)⁻¹ FA. The chemical components and FA proportion of both substrates are presented in [Table 1](#).

Phytochemical Content Analysis of Leaves

The phytochemical analysis of CAL was performed using a method described previously ([Pérez et al., 2016](#)). CAL powder (100 mg) were extracted with 80% of methanol using an extractor (Dionex ASE Accelerated Solvent Extractor, Thermo Fisher, Sunnyvale, CA) for 15 min and this process was repeated three times. The extract was evaporated till the sample content was solid. Subsequently, the solid extract was dissolved in a small volume of acidified water containing 0.2% formic acid, and purified on a solid phase extraction using C18 Sep-Pak cartridge (1 cm³, 360 mg, Waters Corp., Milford, MA). First cartridge was conditioned with 5 mL methanol followed by washing with 5 mL distilled water. After conditioning, sample was washed with 0.5% methanol to remove carbohydrates, and then washed with 80% methanol to elute phenolics. The phenolics fraction was re-evaporated and dissolved in 1 mL of 80% methanol followed by centrifugation (23,000 $\times$ g, 5 min). The phytochemicals were determined using an ultrahigh resolution mass spectrometer system (Dionex UltiMate 3000RS, Thermo Scientific, Darmstadt, Germany) equipped with ultra-performance liquid chromatography (UPLC) and a charged aerosol detector interfaced with a high-resolution quadrupole time-of-flight (TOF) mass spectrometer (Impact II, Bruker Daltonik GmbH, Bremen, Germany). Chromatographic separation of CAL metabolome was performed on an acquit UPLC BEH C18 column (1.7 μ m, 100 mm $\times$ 2.1 mm; Waters, Manchester, UK) at 50 °C. The mobile phase had mixture of 0.1% formic acid (v/v)

in Milli-Q water (A) and 0.1% formic acid (v/v) in acetonitrile (B) at a flow rate of 0.4 mL·min⁻¹. The gradient elution was as follows: 1) 5% of solution B from 0 to 0.5 min with a short 0.3-min calibration segment, 2) the concentration of solution B was increased to 80% from 0.5 to 27 min, 3) the concentration of solution B was increased to 98% from 27 to 27.1 min, 4) the column was eluted with this concentration of solvent B for 2 min, and 5) re-equilibrated for 0.2 min with a flow rate of 0.5 mL·min⁻¹ at 50 °C. Samples were kept at 15 °C in the autosampler. The injection volume was 5.0 μ L. The mass spectrometer was operated in the positive and negative atmospheric pressure chemical ionization (APCI) ionization mode. For calibration, APCI-tuning mix Pos. (Positive ionization mode), quadratic mode and locked mass were used. The following conditions were employed: capillary voltage was set at 2.8 kV, nebulizer at 0.7 bar, dry gas 6.0 liter·min⁻¹, dry temperature at 200 °C and mass to charge number ratio (m/z) scan range at 50 to 1870. Tandem (MS/MS) spectra were acquired in a data-dependent manner, where ions (maximum 2) from each scan were subjected to collision-induced fragmentation if their absolute intensity was over 1,800 counts. Depending on the m/z, variable collision energy in the range from 15 to 35 eV was used. Internal calibration was achieved with Tuning Mix APCI-TOF solution introduced to the ion source via a 20- μ L loop at the start or end of each analysis using a six-port valve. Data were collected and processed by the software Data Analysis 4.3 (Bruker Daltonik GmbH, Germany). Stock solutions of rosmarinic acid, quercetin, and cryptotanshinone were prepared in methanol at a concentration of 5.6, 4.2, and 4.1 mg·mL⁻¹ respectively. Calibration curves were prepared using six concentrations ranging from 6 to 500 μ g·mL⁻¹ of three standard compounds. The amount of phenolic compounds in the CAL sample was determined as an equivalent of rosmarinic acid or quercetin. Cryptotanshinone was used to compute the weights (mg·g⁻¹) of 8 to 11 APM compounds that were identified in the extract.

Experimental Design

The Hohenheim gas test technique ([Menke and Steingass, 1988](#)) was employed to study the effect of partially replacing TMR with CAL powder on the ruminal fermentation characteristics, methane production, microbial communities, and ruminal biohydrogenation with some modification (400 mg of substrate and 40 mL of buffered ruminal fluid)

Table 1. Ingredients, chemical composition, and fatty acid proportion of total mixed ration (TMR) and *C. amboinicus* leaves (CAL)

Item	TMR	CAL
Dry matter, g kg ⁻¹	432	929
Ingredients, g kg ⁻¹ of DM		
Maize silage	352	-
Lucerne silage	172	
Grass silage	75	
Beet pulp	101	
Brewer's grain	63	
Extracted rapeseed meal	38	
Concentrate	189	
Mineral–vitamins mixture ¹	10	
² Chemical composition, g kg ⁻¹ DM		
Organic matter	880	859
Ash	120	141
Crude protein	172	196
Ether extract	25	50
aNDF	336	363
³ Fatty acid proportion, g (100 g) ⁻¹ FA		
Saturated FA		
C8:0	0.17	0.05
C10:0	0.07	0.04
C12:0	0.22	0.28
C14:0	0.79	0.51
C15:0	0.24	0.11
C16:0	32.82	24.35
C17:0	0.21	0.32
C18:0	6.11	7.80
C20:0	0.14	0.38
C21:0	0.06	0.08
C22:0	0.04	0.06
C23:0	0.94	0.16
C24:0	0.31	1.47
Monounsaturated FA		
C14:1	0.28	0.02
C16:1	0.10	0.08
C18:1c9	19.43	5.02
C18:1c11	3.29	0.53
C18:1c12	0.38	0.00
C20:1t	0.69	1.85
C22:1n9	0.03	0.09
C24:1	0.24	0.66
Polyunsaturated FA		
C18:2c9c12	25.18	12.31
C18:3c9c12c15	4.61	35.83
C18:3n6	0.56	0.01
C20:2	0.01	0.08
C20:3n6	0.25	0.17
C20:4n6	0.28	0.08
C20:5n3	0.12	0.07
C22:2	1.43	0.51
C22:5n3	0.45	6.63
C22:6n3	0.55	0.47
SFA	42.11	35.61
UFA	57.89	64.39

Table 1. (Continued)

Item	TMR	CAL
MUFA	24.45	8.24
PUFA	33.44	56.15
n6/n3	4.91	0.30
n6PUFA	27.7	13.08
n3PUFA	5.73	42.99
PUFA/SFA	0.79	1.58
LNA/LA	0.18	2.91
Total C18:1	23.1	5.55
MCFA	34.45	25.35
LCFA	65.31	74.57

¹Declared to contain (g/kg of DM): Ca (190), P organic (20), Mg (110), Na (110), Fe (8), Cu (6), Zn (80), Mn (6), I (0.1), Co (0.2), Se (0.03), and antioxidant (0.5); (IU/g), retinyl acetate (600), cholecalciferol (120), and d-α tocopherol acetate (1.6).

²Chemical composition: DM = dry matter; OM = organic matter; CP = crude protein; EE = ether extract; aNDF = amylase-treated neutral detergent fiber.

³Fatty acid proportion: SFA = saturated fatty acid; UFA = unsaturated fatty acid = MUFA: monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; n6 = omega-6; n3 = omega-3; LNA = linolenic acid; LA = linoleic acid; MCFA = medium-chain fatty acid; LCFA = long-chain fatty acid.

described in Cieslak et al. (2016). The control substrate consisted of 400 mg of TMR on DM basis, whereas in the experimental groups, TMR was replaced by 10, 20, 40, or 80 mg (DM basis) of CAL powder. The study was performed in a 5 × 6 (treatment × glass syringe) experimental design and was completed in three runs that were conducted in 3 consecutive days.

Preparation of Inoculum and Incubation

Fresh ruminal fluid was collected at the slaughter house from nine Polish Holstein-Friesian dairy cows (three cows in each run). All cows were fed a TMR diet (Table 1), similar to the diet used in the in vitro experiment. Ruminal fluid was filtered through a four-layered cheesecloth into two Schott Duran bottles maintained at 39 °C with anaerobic condition and immediately transported to the laboratory, put into the water bath at 39 °C, and mixed in equal volume basis from each cow in a beaker before being diluted with an artificial buffer solution according to a modified protocol of Cieslak et al. (2016). Four hundred milliliters of ruminal fluid were diluted with 1600 mL of buffer (480 mg KH₂PO₄, 480 mg (NH₄)₂SO₄, 480 mg NaCl, 100 mg MgSO₄ • 7H₂O, 64 mg CaCl₂ • 2H₂O, 50 mL of 8% Na₂CO₃, and 600 mg cysteine hydrochloride per liter buffer). The mixed buffered ruminal fluid was continuously bubbled with CO₂ gas

for several minutes before being transferred to the calibrated 100-mL Hohenheim glass syringes containing 400 mg substrates. The syringes were incubated for 24 h at 39 °C in anaerobic conditions.

Total gas production, pH, and methane analysis

The total gas production was recorded every 2 h during 24 h incubation and methane concentration was analyzed after 10 and 24 h of incubation. The methane concentration of headspace gas samples was quantified by gas chromatography (GC; SRI Peak Simple model 310 Alltech, PA) as described by [Szczechowiak et al. \(2016\)](#), with modifications. The GC was equipped with a thermal conductivity detector and Carboxen-1000 column (mesh side 60/80, 15 FT×1.8 INS.S, Supelco, Bellefonte, USA). Nitrogen was used as a carrier gas at a constant flow of 30 mL/min. The oven temperature was initially programmed at 180 °C for 1.5 min, and then was increased to 220 °C at 20 °C min⁻¹. Gas (approximately 500 µL) was taken from the headspace of incubated syringe. The observed peaks were identified by comparison of the retention times with the appropriate gas standards (mix gases of 5.63% CO₂, 5.56% CH₄, 5.10% H₂, and N₂ rest; Multa S.C. Poland) using Peak Simple ver. 3.29. Finally, both total gas and methane gas production after 10 and 24 h were summed up and presented as a one volume/concentration after 24 h of incubation. The pH value was measured (CP-104; Elmetron, Zabrze, Poland) directly after incubation. Ammonia and volatile fatty acid (VFA) concentration, FA profile, microbial population (methanogens and protozoa) in buffered ruminal fluid, and in vitro DM and OM digestibility were analyzed after 24 h of incubation.

Ammonia, VFA Concentration, and FA Profile Analysis

Ammonia concentration was analyzed spectrophotometrically by the modified calorimetric Nessler method according to the protocol of [Szumacher-Strabel et al. \(2002\)](#). Concentrations of VFA were determined on a gas chromatograph (GC Varian CP 3380, Sugarland, TX) with a flame ionization detector and a capillary column (30 m × 0.25 mm; Agilent HP-Innowax, 19091N-133) following the modified Tangerman and Nangengast (1996) method. VFA peaks were performed in MS Work Station 5.0 using an internal standard prepared by mixing individual VFA purchased from Fluka (Sigma Aldrich, MO) followed by measuring the quantitative composition of VFA. The FA

proportion in rumi fluid samples after incubation was analyzed according to [Szczechowiak et al. \(2016\)](#) as was described above, but initially 3 mL of 2 M NaOH was added to 2.5 mL of rumi fluid.

In vitro Feed Degradability Measurement

The in vitro DM digestibility (IVDMD) was measured by transferring the fermented content of a syringe into a 50-mL falcon tube, which was previously weighted and washed with 50 mL of deionized water. The falcon tube was centrifuged for 10 min for 3,500 × *g* at 4 °C and the supernatant was discarded. The falcon was then dried at 50 °C for 4 d, transferred into a previously weighted glass crucible, and dried at 103 °C for 1 d. Three bottles in each run containing buffered ruminal fluid only were also included as a blank control. The percentage loss in weight of the incubated substrate DM after correction of DM residue in the blank control was measured as IVDMD.

Microbial Quantification

The protozoa numbers were counted under a light microscope (Zeiss, type Primo Star no. 5, Jena, Germany) using a drop of a mixed buffered ruminal fluid (1 mL) and 4% formaline (6 mL). The protozoa was counted with the defined volumes: 10 µL for *Entodiniomorphida* and 100 µL for *Holotricha* according to protocol described by [Michalowski et al. \(1986\)](#). For bacterial quantification, total DNA from the buffered ruminal fluid was extracted using QIAamp DNA Stool mini kit (Qiagen GmbH, Hilden, Germany). DNA concentration was measured with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA). *Ruminococcus albus*, *Fibrobacter succinogenes* ([Koike and Kobayashi, 2001](#)), *Butyrivibrio fibrisolvens* ([Li et al., 2009](#)), and total bacteria ([Maeda et al., 2003](#)) were quantified using primers from the literature. The specificity of primers was confirmed using the primer—BLAST program in the GenBank Database. The quantification of each bacterial species and total bacteria was performed with a QuantStudio 12 Flex PCR system (Life Technologies, Grand Island, NY). The reaction mixture in 10 µL of the final volume contained 5 µL of the 2 × Power SYBR Green PCR Master mix (Applied Biosystems, Warrington, Cheshire, UK), 10 ng of template DNA, and 0.5 µM of each primer. Amplification involved one cycle at 95 °C for 10 min for initial denaturation, 45 cycles at 95 °C for 15 s followed by annealing at the temperatures

(dependent on the analyzed bacteria) for 5 s, and finally at 62 °C for 67 s. Detection of the fluorescent product was set as the last step of each cycle. To determine the specificity of amplification, analysis of the melting product was performed after each amplification (0.1 °C/s increment from 65 to 95 °C with fluorescence collection at 0.1 °C intervals). Additionally, product size verification by gel electrophoresis of samples after the PCR run was also included. Dilution of purified genomic DNA from control strains was used to construct species-specific calibration curves (Szczechowiak et al., 2016). The calibration curves were used for the calculation of species-specific DNA concentration using a specific number of DNA copies of the total DNA preparations. The relative level of the DNA copy for each bacterium to total bacteria species was calculated using the formula $2^{-\Delta Ct}$.

Methanogens were quantified using the fluorescence in situ hybridization (FISH) technique as described by Szczechowiak et al. (2016). Approximately 100 µL of fermented ruminal fluid was diluted twice in phosphate-buffered saline and transferred into 0.22 µm polycarbonate filters (Merck Millipore) and was vacuumed (KNF Vaccum, Vacuport-Neuberg, Darmstadt, Germany). The vacuumed filter was washed with three ethanol concentrations (i.e., 50%, 80%, and 90%, respectively) sequentially for 3 min followed by washing with lysozyme (100 mg dissolved in 10 mL water) for 3 min, and the enzyme mix (2 mL of lysozyme and 200 µL of lipase; 25 mg of lipase dissolved in 25 mL water) for 6 min prior to being washed for the second time with previously mentioned ethanol concentrations. For each sample, a series of identical filters were prepared to allow the determination of optimal hybridization. Hybridizations were carried out in 50 µL of hybridization buffer (0.9 M NaCl; 20 mM tris/HCl, pH 7.2; 0.01% SDS) containing oligonucleotide probes for all methanogens (i.e., Archaea, S-D-Arch-0915-a-A-20) and two order-specific probes (i.e., S-O-Mmic-1200-a-A-21 for *Methanomicrobiales* and S-F-Mbac-0310-a-A-22 for *Methanobacteriales*) (Soliva et al., 2004). All samples were subsequently put into a water bath for 24 h at 37 °C. After hybridization, the filters were washed with washing buffer (20 mM Tris/HCl, pH 7.2; 0.01% SDS; 5 mM EDTA) for 10 min at 50 °C. The filters were then rinsed gently in distilled water, air-dried, and mounted on object glasses with VectaShield (Vector laboratories nr. H-1000) anti-fading agent containing DAPI (4',6-diamidino-2-phenylindole). Images of each sample were captured using a fluorescence microscope (Zeiss Axiovert 2000) with AxioImage Observer Software

and filters specific for DAPI and Rhodamine (magnification 100×) installed with a video camera (AxioCamMRm Rev. 3 Fire Wire) and the numbers of methanogens were counted manually.

Calculation and Statistical Analysis

All data, except bacterial population, were analyzed using one-way ANOVA where levels of the CAL powder used in the diet were dependent factors. Inoculum source was included in the model, but it was not significant and was excluded from the final model. Tukey's post hoc test was used for determining the significant differences among groups ($P < 0.05$) and the polynomial contrasts to determine the linear and quadratic responses of different levels of CAL included in the diet. Effects of treatment on bacterial population were analyzed using one-way ANOVA and Tukey's post hoc analysis. Statistical significances were assumed when $P < 0.05$. Statistical significances between groups were marked * $P < 0.05$ and ** $P < 0.01$. All recorded data were analyzed using SAS (version 9.1) statistical software.

RESULTS

Nutrients and Phytochemical Composition of CAL

The concentrations of CP in CAL are higher than in the TMR (172 vs. 196 g.kg⁻¹ of DM, respectively). Meanwhile, the aNDFom concentration in CAL was comparable to the TMR, but EE and polyunsaturated fatty acid (PUFA) content in CAL was relatively greater than in TMR, which was mainly due to high concentration of C18:3c9c12c15 and low concentrations of c16:0 and C18:1c9 in CAL (Table 1). Among the various APM present in CAL, rosmarinic acid content was highest with rich in phenolic acid (Table 2; Figure 1, position 5).

Ruminal Fermentation Characteristics

The pH status of CAL groups was similar compared with the control, but the pH values of 10% and 20% CAL groups were higher compared with the 2.5% CAL group ($P = 0.004$) and pH increased linearly ($P = 0.001$) with increasing doses of CAL (Table 3). Ammonia concentration tended to be different among the groups ($P = 0.056$) with higher concentration for 20% CAL compared with the control. The total gas production, expressed as mL.g⁻¹ DM, was lower in 20% CAL group compared with the control ($P < 0.001$) and showed a dose-dependent linear ($P < 0.001$), and quadratic ($P = 0.010$)

decrease due to CLA powder supplementation. Furthermore, the CAL groups were effective in mitigating methane production ($P < 0.001$). The methane production was dose-dependent, and decreased linearly and quadratically with increasing amount of CAL. Methane production lowered by more than 30% at the highest proportion of CAL used.

Relative to the control group, the IVDMD ($P = 0.099$) was similar among the treatments; however, the IVDMD tended to increase showing the quadratic ($P = 0.017$) response of CAL with greater values for 2.5% to 10% levels. Moreover, methane/IVDMD ($P < 0.001$) ratio decreased with a linear response of CAL supplementation.

Table 2. The bioactive compounds present in of *C. amboinicus* leaves (CAL)

Compound	
¹ Partial phytochemical content, mg g ⁻¹ DM	
Caffeic acid	0.637
Luteolin-O-glucuronide	0.601
Apigenin-glucuronide	0.651
Apigenin-O-hexoside	0.098
Rosmarinic acid	9.761
Luteolin-O-hexoside	0.684
Diterpene-hexoside	0.225
Sesquiterpene	0.512
Diterpene	0.584
Dihydroxyroyleanone	0.691
Acetyl royleanone	0.587
² Phytochemical compound groups, mg g ⁻¹ DM	
Phenolic acid	10.40
Diterpenes	2.03
Flavonoids	2.60

¹The partial phytochemical components of CAL.

²The phytochemical compound groups.

Total VFA concentration tended ($P = 0.055$) to increase linearly, though no significant differences were noted compared with the control group. Molar proportion of acetate, propionate, and isobutyrate increased with quadratic responses ($P < 0.001$) when CAL was used to partially substitute the incubated substrate (control). The butyrate proportion decreased quadratically and linearly ($P < 0.001$), and both valerate and isovalerate proportions were altered in a CAL dose-dependent manner decreasing linearly ($P < 0.001$). The ratio of acetate : propionate ratio (A/P) was lower in the 10% and 20% CAL groups showing a quadratic response ($P = 0.008$).

Ruminal Microbial Populations

The protozoa population increased linearly with increasing amount of CAL ($P < 0.001$; Table 4), which was a result of linear increases in number of *Entodiniomorphida*. However, *Holotricha* population decreased linearly and quadratically ($P < 0.001$; $P = 0.003$, respectively). Methanogens of all CAL groups generally reduced compared with the control except for the *Methanobacteriales* that was not different for 2.5% CAL. The total methanogen populations significantly decreased with linear ($P = 0.005$) and quadratic responses ($P = 0.002$). The *Methanomicrobiales* decreased with a linear and quadratic response ($P < 0.001$; $P = 0.005$, respectively) due to CAL supplementation. *Methanobacteriales* populations tended to decrease linearly ($P = 0.054$) and quadratically ($P = 0.056$) with increasing doses of CAL. The total bacterial abundance in the diet with the highest dose of CAL powder (20%) increased compared with the control (Figure 2). The abundance of *B. fibrisolvens* population of the 20% CAL group was significantly higher compared with the control and the 10%

Figure 1. UV chromatogram (190 to 600 nm) of CAL in concentration 100 mg mL⁻¹. Peak numbers represent main compounds, according to numbering in Table 1. ¹Caffeic acid; ²Luteolin-O-glucuronide; ³Apigenin-glucuronide; ⁴Apigenin-O-hexoside; ⁵Rosmarinic acid; ⁶Luteolin-O-hexoside; ⁷Diterpene-hexoside; ⁸Sesquiterpene; ⁹Diterpene; ¹⁰Dihydroxyroyleanone; ¹¹Acetyl royleanone.

Table 3. The effect of *C. amboinicus* leaves (CAL) supplementation on rumen pH, ammonia, total gas production, methane, VFA, dry matter, and organic matter digestibility after 24 h incubation *in vitro*

¹ Parameters	Control	² CAL				³ SEM	<i>P</i> -value	⁴ Contrast	
		2.5%	5%	10%	20%			Linear	Quadratic
pH	6.58 ^{ab}	6.57 ^b	6.60 ^{ab}	6.64 ^a	6.64 ^a	0.008	0.004	0.001	0.191
NH ₃ , mM	9.36 ^b	9.82 ^{ab}	10.22 ^{ab}	10.04 ^{ab}	10.41 ^a	0.14	0.056	0.148	0.118
Total gas, mL g ⁻¹ of DM	278.7 ^a	242.4 ^{ab}	259.2 ^a	235.5 ^{ab}	201.9 ^b	4.50	<0.001	<0.001	0.010
CH ₄ (mM/g DM)	2.18 ^a	1.69 ^b	1.90 ^{ab}	1.70 ^b	1.25 ^c	0.07	<0.001	0.010	0.006
IVDMD, %	58.26	61.47	61.01	61.59	59.35	0.48	0.099	0.956	0.017
CH ₄ /total gas, mM mL ⁻¹	0.008	0.007	0.007	0.007	0.007	0.001	0.053	0.069	0.410
CH ₄ /IVDMD, mM g ⁻¹	3.76 ^a	2.75 ^{bc}	3.11 ^{ab}	2.77 ^{bc}	2.11 ^c	0.12	<0.001	<0.001	0.073
Total VFA, mM	82.86	82.87	86.95	90.53	88.37	0.91	0.253	0.055	0.354
Acetate, %	63.57 ^b	64.43 ^b	64.38 ^b	67.69 ^a	67.27 ^a	0.33	<0.001	<0.001	<0.001
Propionate, %	20.34 ^c	21.16 ^b	21.26 ^b	22.67 ^a	22.37 ^a	0.18	<0.001	<0.001	<0.001
Isobutyrate, %	1.25 ^b	1.19 ^b	1.12 ^b	1.12 ^b	2.44 ^a	0.10	<0.001	<0.001	<0.001
Butyrate, %	12.28 ^a	11.01 ^a	11.03 ^a	6.56 ^b	7.41 ^b	0.45	<0.001	<0.001	<0.001
Isovalerate, %	1.17 ^a	1.05 ^a	1.04 ^a	0.95 ^a	0.21 ^b	0.08	<0.001	<0.001	0.086
Valerate, %	1.37 ^a	1.16 ^{ab}	1.17 ^{ab}	1.01 ^b	0.30 ^c	0.08	<0.001	<0.001	0.250
A/P ratio	3.13 ^a	3.05 ^{ab}	3.03 ^{ab}	2.99 ^b	3.01 ^b	0.01	0.007	0.006	0.008

^{a-c}The different superscripts in the same row refer to significant difference between treatments (*P* < 0.05).

¹Parameters: NH₃ = ammonia; CH₄ = methane emission; IVDMD = *in vitro* dry matter digestibility; VFA = volatile fatty acid; A/P ratio = acetate : propionate ratio.

²CAL: the level of CAL used in the incubated substrate (control).

³SEM: standard error mean.

⁴Contrast: polynomial contrast results due to the level of CAL used in the diet.

Table 4. The effect of *C. amboinicus* leaves (CAL) supplementation on ruminal microbial populations *in vitro* after 24 h incubation

Parameters	Control	¹ CAL				² SEM	<i>P</i> -value	³ Contrast	
		2.5%	5%	10%	20%			Linear	Quadratic
Total protozoa, 10 ⁴ mL ⁻¹	9.81 ^d	11.53 ^{cd}	14.00 ^b	13.01 ^{bc}	16.45 ^a	0.47	<0.001	<0.001	0.161
<i>Holotricha</i> , 10 ⁴ mL ⁻¹	0.24 ^a	0.21 ^a	0.15 ^b	0.15 ^b	0.12 ^b	0.01	<0.001	<0.001	0.003
<i>Entodiniomorphida</i> , 10 ⁴ mL ⁻¹	9.57 ^d	11.32 ^{cd}	13.85 ^b	12.86 ^b	16.33 ^a	0.48	<0.001	<0.001	0.139
Total methanogens, 10 ⁸ mL ⁻¹	16.74 ^a	6.75 ^b	5.70 ^b	5.84 ^b	4.87 ^b	1.19	0.002	0.005	0.002
<i>Methanobacteriales</i> , 10 ⁷ mL ⁻¹	10.46 ^a	5.53 ^{ab}	5.20 ^b	5.21 ^b	4.55 ^b	0.66	0.006	0.054	0.056
<i>Methanomicrobiales</i> , 10 ⁷ mL ⁻¹	12.73 ^a	9.68 ^a	6.98 ^b	5.64 ^b	5.57 ^b	0.68	0.001	<0.001	0.005

^{a-d}The different superscripts in the same row refer to significant difference between treatments (*P* < 0.05).

¹CAL: the level of CAL used in the incubated substrate (control).

²SEM: standard error mean.

³Contrast: polynomial contrast results due to the level of CAL used in the diet.

CAL group; however, the 5% CAL group had the lowest *B. fibrisolvens* population (Figure 3). In contrary, the abundance of *R. albus* and *F. succinogenes* at the 5% CAL group was higher compared with the control group and all other experimental CAL groups, with the exception of 20% CAL group in the case of *R. albus* (Figure 3).

Ruminal FA Profile

The stearic acid (C18:0) increased linearly when CAL was included in the diet (*P* = 0.021; Table 5).

The CAL powder increased α -linolenic acid (ALA; C18:3 *c9c12c15*; *P* < 0.001), vacenic acid (C18:1 *t11*; *P* = 0.019), and CLA (C18:2*c9t11*; *P* = 0.002) content linearly in the buffered ruminal fluid. The concentrations of C16:0 (*P* = 0.058), C16:1 (*P* = 0.073), and C22:6 n3 (*P* = 0.078) tended to decrease with increasing doses of CAL according to linear responses. Total PUFA (*P* = 0.032) and n-6 (*P* = 0.030) content decreased quadratically with lowest concentrations at 5% CAL dose. Total n-3 FA (*P* = 0.022) and total C18:1 *t* (*P* = 0.019) concentrations increased linearly in a dose-dependent manner.

Figure 2. The effect of CAL on the relative 16S rRNA gene copy abundance (expressed as an arbitrary unit relative the total bacterial gene copy abundance of the control) of rumen total bacteria population in vitro after 24 h incubation. **Significantly different between treatments at $P < 0.01$.

ALA/CLA content increased ($P = 0.001$) when the highest CAL dose (20%) was used and also increased linearly ($P < 0.001$) with increasing doses of CAL.

DISCUSSION

Nutrients and Phytochemical Composition of CAL

CAL is known as a medicinal plant rich in nutrients and mineral components as well as a rich source of APM (Damanik et al., 2001; Arumugam et al., 2016). To our knowledge, this is the first study presenting a detailed composition of the phytochemical contents of CAL. The phenolic acid concentrations were the highest followed by flavonoid and diterpens in CAL. The phenolic compound was dominant due to high accumulation of rosmarinic acid (approximately 9.8 mg·g DM) that has the antimicrobial and antioxidant properties (Cobellis et al., 2016) and hence CAL can potentially affect microbial populations in the rumen. This is also the first in vitro trial to evaluate the effects of CAL on ruminal methanogenesis and biohydrogenation as well as basic ruminal fermentation parameters and microbial population. Results of basic chemical analyses of CAL showed substantial amounts of crude protein and crude fat with rich sources of PUFA.

Effect of CAL on Ruminal Fermentation and Methanogenesis

The CAL modulated ruminal fermentation and effectively decreased methane production. The decreased methanogenesis in the rumen related to

Figure 3. The effect of CAL on the relative 16S rRNA gene copy abundances (expressed as gene copy abundances of a target bacterium with respect to the total bacteria and then assigned to an arbitrary unit relative to the control values) of *R. albus*, *F. succinogenes*, and *B. fibrisolvans* numbers in the rumen in vitro after 24 h incubation. **Significantly different between treatments at $P < 0.01$. *Significantly different between treatments at $P < 0.05$.

the linear decrease of total gas production; however, lower total gas production did not correlate with IVDMD. In the present experiment, IVDMD tended to increase quadratically. This is contrary to the expected direct relationship of decreased gas production and decreased in vitro digestibility.

Table 5. The effect of *C. amboinicus* leaves (CAL) supplementation on ruminal fatty acid proportion after 24 h incubation in vitro

¹ Fatty acid	Control	2.5%	² CAL			³ SEM	<i>P</i> -value	⁴ Contrast	
			5%	10%	20%			Linear	Quadratic
Saturated fatty acid, g (100 g) ⁻¹									
C10:0	0.11	0.10	0.07	0.04	0.09	0.01	0.596	0.618	0.146
C12:0	0.87	0.75	0.85	0.88	0.79	0.06	0.957	0.911	0.774
C14:0	2.08	2.08	1.46	2.01	1.85	0.15	0.663	0.900	0.670
C15:0	0.99	1.14	1.15	0.84	1.01	0.06	0.578	0.641	0.749
C16:0	33.08	30.23	31.41	30.33	28.05	0.67	0.292	0.058	0.965
C17:0	0.38	0.44	0.51	0.43	0.59	0.04	0.455	0.130	0.994
C18:0	28.55 ^b	35.26 ^{ab}	33.22 ^a	34.39 ^a	34.56 ^a	0.76	0.021	0.049	0.056
C20:0	0.35	0.50	0.14	0.48	0.34	0.07	0.582	0.968	0.941
C21:0	0.27	0.20	0.17	0.21	0.26	0.02	0.759	0.795	0.285
C22:0	0.40	0.37	0.16	0.69	0.56	0.08	0.270	0.215	0.795
C24:0	0.45	0.34	0.18	0.67	0.57	0.09	0.434	0.289	0.944
Monounsaturated fatty acid									
C14:1	0.53	0.63	0.64	0.46	0.65	0.03	0.344	0.671	0.540
C15:1	0.39	0.38	0.27	0.42	0.55	0.05	0.645	0.267	0.460
C16:1	1.43	0.90	0.97	1.11	0.76	0.09	0.144	0.073	0.544
C17:1	0.16	0.14	0.15	0.10	0.18	0.02	0.856	0.806	0.348
C18:1 <i>t</i> 11	1.10	1.66	1.82	1.75	2.22	0.14	0.106	0.019	0.443
C18:1 <i>c</i> 9	14.27	10.69	13.48	10.37	12.06	0.59	0.117	0.376	0.162
C18:1 <i>c</i> 11	1.60	1.33	1.56	1.75	1.82	0.08	0.426	0.140	0.999
C20:1 <i>t</i>	0.44	0.54	0.47	0.43	0.42	0.04	0.919	0.607	0.857
C22:1 <i>n</i> 9	0.25	0.36	0.26	0.32	0.25	0.03	0.622	0.830	0.464
C24:1	0.35	0.26	0.12	0.26	0.15	0.03	0.127	0.135	0.272
Polyunsaturated fatty acid									
C18:2 <i>c</i> 9 <i>c</i> 12	8.79	8.16	8.47	8.59	7.81	0.52	0.991	0.756	0.845
C18:2 <i>c</i> 9 <i>t</i> 11	0.14 ^b	0.15 ^{ab}	0.16 ^{ab}	0.17 ^{ab}	0.20 ^a	0.007	0.027	0.002	0.945
C18:3 <i>c</i> 9 <i>c</i> 12 <i>c</i> 15	0.86 ^b	0.84 ^b	1.03 ^b	1.33 ^{ab}	1.70 ^a	0.08	<0.001	<0.001	0.801
C18:2 <i>t</i> 10 <i>c</i> 12	0.21	0.18	0.14	0.23	0.20	0.02	0.487	0.658	0.750
C18:3 <i>n</i> 6	0.07	0.12	0.14	0.10	0.12	0.02	0.673	0.581	0.556
C20:3 <i>n</i> 6	1.02	1.52	0.71	1.13	1.81	0.21	0.519	0.284	0.413
C20:5 <i>n</i> 3	0.31	0.22	0.05	0.23	0.15	0.04	0.235	0.346	0.285
C22:2	0.10	0.11	0.08	0.12	0.15	0.01	0.763	0.287	0.699
C22:6 <i>n</i> 3	0.44	0.41	0.17	0.19	0.13	0.06	0.248	0.078	0.269
SFA	67.53	71.40	69.31	70.96	68.66	4.47	0.504	0.828	0.120
UFA	32.47	28.59	30.69	29.04	31.34	1.72	0.724	0.345	0.380
MUFA	20.54	16.87	19.73	16.96	19.06	1.22	0.713	0.200	0.798
PUFA	11.93	11.72	10.96	12.08	12.28	0.86	0.285	0.945	0.032
n-6	10.09	9.98	9.46	10.05	9.95	0.84	0.260	0.749	0.030
n-3	1.61	1.48	1.25	1.75	1.99	0.10	0.232	0.055	0.719
n6/n3	6.27	6.74	7.57	5.74	5.00	2.47	0.241	0.217	0.121
PUFA/SFA	0.18	0.16	0.16	0.17	0.18	0.12	0.776	0.582	0.985
ALA/CLA	6.23 ^b	5.51 ^b	6.43 ^b	7.84 ^b	8.46 ^a	0.01	0.001	<0.001	0.468
Total C18:1	16.98	13.68	16.85	13.86	16.10	1.15	0.783	0.265	0.873
Total C18:1 <i>t</i>	1.10	1.66	1.82	1.75	2.22	0.14	0.106	0.019	0.443
Total C18:1 <i>c</i>	15.87	12.02	15.04	12.12	13.88	1.14	0.756	0.228	0.875
MCFA	37.13	34.29	34.94	34.09	31.79	2.19	0.147	0.663	0.036
LCFA	30.40	37.11	34.37	36.87	36.87	3.21	0.851	0.899	0.411

¹Fatty acid: SFA = saturated fatty acid; UFA = unsaturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; n6: omega-6; n3 = omega-3; LNA = linolenic acid; LN = linoleic acid; MCFA: medium-chain fatty acid; LCFA: long chain fatty acid.

²CAL: the level of CAL used in the incubated substrate (control).

³SEM: standard error mean.

⁴Contrast: polynomial response according to the level of CAL in the diet.

^{a,b}The different superscripts in the same row refers to significant different between treatments ($P < 0.05$).

Organic matter degraded in the rumen is partitioned into VFA, gas, and microbial biomass (Getachew et al., 2004). It appeared that degraded OM was more partitioned to microbial biomass synthesis as evidenced from greater abundance of total bacteria and protozoa rather than to gas production in this study. A decrease in methane production with antimethanogenic substances is usually associated with adverse influence on ruminal VFA production and in vitro digestibility (Patra and Yu, 2013). Some plants containing the specific bioactive compounds have no adverse effects on feed in vitro digestibility, total gas, and VFA production although they can mitigate the methane emission (Patra and Saxena, 2010; Bodas et al., 2012). Some studies indicated that even higher APM supplementations (*Sanguisorba officinalis* tannin extract at 51.2 g·kg⁻¹ DM) had no negative influence on in vitro parameters including IVDMD, microbial population, or VFA concentrations (Cieslak et al., 2016). In this study, CAL decreased total gas production, it did not affect ruminal fermentation and feed degradability, but effectively decreased methane emission, which could be a practical option to mitigate methane emission in ruminants.

The highest amount of CAL (20%) decreased total gas production by 17% compared with the control without CAL supplementation. Usually, the differences in total gas production may result from the fermentation of carbohydrates. In this study, aNDF content in TMR and CAL differed to a small extent, 336 vs. 363 g·kg⁻¹ of DM, respectively. Therefore, it appears that aNDF content in CAL unlikely reduced total gas production.

Another possible explanation of the reduction of gas volume that was detected in this study could be due to the higher amount of CP incubated with CAL doses (172 vs. 196 g·kg⁻¹ of DM in TMR and CAL, respectively). Cone and van Gelder (1999) stated that gas produced from fermentation of casein is only a third of total gas production from fermentation of carbohydrates, on an equal weight basis, and for every 10 g of CP per kg of substrate, gas production is reduced by 2.48 mL·g⁻¹ of OM incubated. Also a recently published paper underlined this aspect and the effect of CP concentration of the substrate in a gas production experiment (i.e., depressed effect of ammonia on the total gas production; Vanegas et al., 2017).

Based on the obtained results, we hypothesize that methane production was decreased primarily due to inhibition of total methanogen numbers affected by the main bioactive component of CAL, rosmarinic acid. Our findings can be strongly

supported by a recent report where rosemary leaves (a source of rosmarinic acid) have been recognized as a factor inhibiting methanogenic archaea population and some microbial metabolic processes contributing to lowered methane production (Cobellis et al., 2016). The reduction of methanogens may involve decreased protozoa numbers; however, some of APM can have a direct toxic effect on methanogenic cells (Cieslak et al., 2013) as we observed in this study where *Entodiniomorphida* populations increased and total methanogen counts decreased. Oh et al. (2017) suggested that the decreased abundances of methanogens, protozoa, and fungi indicate the direct and indirect inhibition of methanogenesis by the phenolic compound. Polyphenolic mode of action in CAL on microorganisms involves disrupting and leakage of cellular membrane, after polyphenolic adsorption to bacterial membranes (Arumugam et al., 2016). Phenolics and terpenoid may also cause disturbances of ion transport (electrons) through the cell membrane, protein translocation, phosphorylation, and enzyme-dependent reactions in the microorganism's membrane (Cieslak et al., 2013). In this study, the ruminal bacteria and *Entodiniomorphida* populations were not negatively influenced by CAL. The reason may be the fact that the rosmarinic acid as a main source of CAL polyphenols is believed to be less effective against selected ruminal bacteria and protozoa in comparison to methanogens. This is confirmed by research carried out by Cobellis et al. (2016), who suggested that rosmarinic acid compared with other APM (carnosic acid or carnosol) has less antimicrobial activity against selected bacteria and protozoa, where no differences in total bacteria, protozoa, or *R. flavefaciens* were observed, whereas archaea abundance was decreased. Individual group of bacteria may be induced by the CAL phenolic acid content that trigger metabolic enzyme activity of certain bacteria for example, *Lactobacillus planatrum* (Shubha and Bhatt, 2015; Arumugam et al., 2016).

The CAL supplementation reduced *Holotricha*, but increased *Entodiniomorphida* group. *Entodiniomorphida* population may be stimulated by the CAL fatty acids than *Holotricha*. The relatively high α -linolenic acid content in CAL probably has a direct positive effect on the *Entodiniomorphida* protozoa growth, but gives an adverse effect on the *Holotricha* group. In a previous study linolenic and linoleic acids decreased the *Holotricha* population, but the linoleic acid stimulated growth of *Entodiniomorphida* in vitro (Kisidayova et al., 2006). On the other hand, it should be also taken into account that incubation time in batch culture

system is short (24 h) for long generation time of most protozoa groups (18 h). The average initial protozoa population in buffered ruminal fluid in this study was $39.4 \times 10^4 \text{ mL}^{-1}$, whereas after 24 h we observed protozoa count in the range from 9.57 to $16.5 \times 10^4 \text{ mL}^{-1}$. This can also suggest the possible effect of α -linolenic acid not only as a stimulator of the growth of protozoa population, but as a substance that can reduce the negative effects of the in vitro condition on the protozoa population.

In this study, the VFA production showed a tendency to increase linearly when CAL was included in the incubated substrate (control). The increased total VFA was the effect of higher concentration of acetate, propionate, and isobutyrate. Increased propionate may be associated with the abundance of high fermentable sugar alcohols present in CAL, polyols (Zheng et al., 2016). Polyols in CAL may increase microbial activity of microorganisms degrading the substrate yielding in a higher propionic rather than acetic acid content (Biebl et al., 2000). Increased propionate concentration may be attributed to the rechanneling of greater amount of hydrogen produced due to decreased methanogenesis and vice versa (Patra et al., 2017). On the other hand, increased propionate resulted in a lower acetate : propionate ratio. Previous studies showed that phenolic extracts did not affect the VFA accumulation in the rumen, but resulted in a lower acetate proportion than propionate (Oh et al., 2017). Isobutyrate proportion increased due to the CAL included in the diet. The higher protein content of CAL (172 vs. $196 \text{ g} \cdot \text{kg}^{-1} \text{ DM}$, respectively in TMR and CAL) increased the nitrogen and amino acid content in diet that may be degraded by ruminal bacteria to branch short-chain FA (Bentley et al., 1955; Wu, 2017). The higher isobutyrate concentration results from increased N-synthesis involving metabolism of β -aminoisobutyrate to produce more ammonia, what was observed in this study. The isobutyrate and other branched short-chain VFAs (BSCVFA) are known to be important for the growth of bacteria that degrade structural carbohydrates (i.e. *R. albus* and *F. succinogenes*) and utilize urea-N. Increased BSCVFA may also relate to increased cellulolytic bacterial populations in the rumen, the modulation of iso-BSCVFA and ammonia content as well as ruminal in vitro digestibility by CAL inclusion in the diet.

The Effect of CAL on Ruminal FA Proportions

In this study, the CAL was a rich source of ALA (approximately $36 \text{ g} (100 \text{ g})^{-1} \text{ FA}$) compared with

TMR ($4.6 \text{ g} (100 \text{ g})^{-1} \text{ FA}$) along with high amount total FA, which was attributed to increased ALA proportion in the ruminal fluid after incubation. The proportion of ALA increased linearly after 24 h of incubation. Moreover, CAL treatment positively influenced the proportion of C18:1 *t*11, C18:2 *c*9*t*11, n-3, ALA/CLA ratio, and total PUFA and n-6. It is well known that higher content of fat ($30 \text{ g} \cdot \text{kg}^{-1} \text{ DM}$ in TMR vs. $47 \text{ g} \cdot \text{kg}^{-1} \text{ DM}$ in CAL, respectively) and thereby FA content in substrates can modulate the FA proportion in the rumen (Szczechowiak et al., 2016). Also, the content of phenolic compounds in CAL (such as rosmarinic and caffeic acids, tannins, flavonoids, and phenols), due to the antibacterial properties, can modulate populations of ruminal biohydrogenating bacteria and biohydrogenation (Jayanegara et al., 2011; Cappucci et al., 2018). As expected, proportion of C18:0 increased after supplementation of CAL as a consequence of PUFA disappearances in the rumen during biohydrogenation process. This result strengthens our hypothesis that the mode of action of CAL phytochemicals can affect FA metabolism during ruminal biohydrogenation process. Also, data from the in vitro study of Pallara et al. (2014) suggested that olive polyphenols reduced ruminal biohydrogenation by acting on the conversion of vaccenic acid (C18:1 *t*11) to saturated stearic acid (C18:0), the last step of the process. However, results of Cappucci et al. (2018) with the in vivo study suggested that the first step of ruminal linoleic acid (C18:2 *c*9*c*12) biohydrogenation to CLA (C18:2 *c*9*t*11) was probably negatively affected by olive polyphenols. Authors observed the lack of consistency between in vitro and in vivo studies, and among studies in general. They stated that it might be due to several factors such as the ruminal passage rate, interaction with incubated substrate, different amount of lipids in the diet, and the specific composition of phenolic substances.

In the present study, biohydrogenation did not significantly decrease the proportions of C18:2. However, an increase in C18:2 *c*9*t*11 may be the result of numerical decrease of C18:2 since C18:2 *c*9*t*11 is produced mostly as the effect of C18:2 biohydrogenation (Harfoot and Hazlewood, 1997). The increase in C18:3 n-3 was dose-dependent, but significant differences were found only when 20% of CAL was used. The increased proportion of C18:3 resulted also in increased C18:1 *t*11 proportion that is one of the intermediary products of C18:3 biohydrogenation. Results of the relative abundance of *B. fibrisolvens* partly confirmed the potential effect of CAL on ruminal biohydrogenating bacteria. Similarly, Buccioni et al. (2015) pointed out the stimulatory effect of phytofactors

(tannins) on the growth of *B. fibrisolvens*, and consequently ruminal biohydrogenation was reflected by increased C18:2 *c9t11* and C18:1 *t11* proportion. Further studies are required to better characterize the effect of tannins and oils on specific bacterial species involved in the biohydrogenation process.

CONCLUSIONS

The CAL may mitigate the ruminal methane production up to 30% at the dose tested in this study and increase other ruminal fermentation products such as ammonia, VFA production, as well as the feed in vitro digestibility in the rumen. Decreased methane production was related to lowered methanogen number. The CAL also positively modulated the ruminal ecology, affecting biohydrogenation with an increased proportion of few unsaturated FAs and their isomers beneficial for human health.

Conflict of Interest. There is no conflict of interest.

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