Dietary *Coleus Amboinicus* Herb Decreases Ruminal Methanogenesis and Biohydrogenation, and Improves Meat Quality and Fatty Acid Composition in *Longissimus Thoracis* of Lambs

Yulianri Rizki Yanza
Poznan University of Life Sciences: Uniwersytet Przyrodniczy w Poznaniu
Malgorzata Szumacher-Strabel
Poznan University of Life Sciences: Uniwersytet Przyrodniczy w Poznaniu
Dorota Lechniak
Poznan University of Life Sciences: Uniwersytet Przyrodniczy w Poznaniu
Sylwester Ślusarczyk
Wroclaw Medical University: Uniwersytet Medyczny im Piastow Slaskich we Wroclawiu
Pawel Kolodziejski
Poznan University of Life Sciences: Uniwersytet Przyrodniczy w Poznaniu
Amlan Kumar Patra
West Bengal University of Animal and Fishery Sciences
Zora Váradyová
Slovak Academy of Sciences: Slovenska akademia vied
Dariusz Lisiak
Instytut Biotechnologii Przemyslu Rolno Spozywczego
Mina Vazirigohar
University of Zanjan
Adam Cieslak (adam.cieslak@up.poznan.pl)
Uniwersytet Przyrodniczy w Poznaniu https://orcid.org/0000-0001-7518-579X

Research

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Abstract

Background: This study aimed to investigate the effect of biologically active compounds (BAC) of *Coleus amboinicus* Lour. (CAL) herb fed to growing lambs on ruminal methane production, ruminal biohydrogenation of unsaturated fatty acids and meat characteristics. An *in vitro* trial (Experiment 1) comprising of control and three experimental diets (CAL constituting 10%, 15%, and 20% of the total diet) was conducted to determine an effective dose for *in vivo* experiments. After the *in vitro* trial, two *in vivo* experiments were conducted on six growing, rumen-cannulated lambs (Experiment 2) and 16 growing lambs (Experiment 3), which were assigned into the control (CON) and one experimental diet (20% of CAL). Several parameters were examined *in vitro* (pH, ammonia and VFA concentrations, protozoa, methanogens and select bacteria populations) and *in vivo* (methane production, digestibility, ruminal microorganism populations, meat quality, fatty acids profiles in rumen fluid and meat, transcript expression of 5 genes in meat).

Results: The CAL lowered *in vitro* methane production by 51%. In the *in vivo* experiments, lambs fed CAL decreased methane production by 20% compared with the CON animals (Experiment 3), which corresponded to the reduced total methanogens counts in all experiments up to 28%, notably *Methanobacteriales*. In Experiment 3, CAL increased or tended to increase the numbers of *Ruminococcus albus, Megasphaeraelsdenii, Butyrivibrioproteoclasticus*, and *Butyrivibriofibrisolvens*. Dietary CAL suppressed the *Holotricha* population, but increased or tended to increase *Entodiniomorpha* population in Experiments 2 and 3. An increase in the polyunsaturated fatty acid (PUFA) proportion in the rumen of lambs was noted in response to the CAL diet, which was mainly attributable to the increase in C18:3 *cis*-9 *cis*-12 *cis*-15 (LNA) proportion. The CAL reduced the mRNA expressions of four investigated genes in meat (*fatty acid synthase, stearoyl-CoA desaturase*, *lipoprotein lipase,* and *fatty acid desaturase 1*).

Conclusions: Summarizing, polyphenols of CAL (20% in diet) origin can mitigate ruminal methane production by inhibiting the methanogens communities. Supplementation of CAL also provides favorable conditions in the rumen by modulating ruminal bacteria involved in fermentation and biohydrogenation of fatty acids. CAL elevated the LNA concentration, which led to improved meat quality through increased deposition of n-3 PUFA.

Highlight

- Inclusion of *Coleus amboinicus* herb (CAH) into sheep diet decreased CH_4 emission.
- CAH did not reduce nutrient digestibility, but inhibited the methanogen community.
- CAH increased ruminal propionate proportion and decreased acetate/propionate ratio.
- CAH elevated n-3 fatty acid concentration in ruminal fluid and meat.
- Supplementation of CAH improved some meat quality traits.

Introduction

Methane (CH_4) is a greenhouse gas mainly produced by anaerobic enteric fermentation in the rumen. It is estimated that farm animals produce 16% of the world's total CH_4 emission, with two-thirds from ruminant origins [1]. This gas is considered as one of the main drivers of climate change and is expected to increase assuming a constant increase in demand of foods for a growing world population[2]. The enteric CH_4 production from ruminants also represents a loss of the total gross dietary energy (2–12%) that perhaps decreases the efficiency of animal production[3]. Enteric CH_4 emission is thus one of the main targets of greenhouse gas mitigation efforts to reduce CH_4 production in the animal sector.

Biologically active compounds (BACs) have been recognized as modulators of rumen microbial fermentation, including methanogenesis[4, 5]. As alternatives to antibiotics, BACs have the potential to decrease CH_4 production in the rumen and can thus help to reduce the negative animal impact on the environment[6, 7]. The use of BACs may also modulate the ruminal biohydrogenation (BH) of unsaturated fatty acids (UFAs), causing changes in the fatty acid (FA) profile of the ruminal fluid and consequently of ruminant products. For instance, some BACs inhibit the microorganisms involved in ruminal BH, and may consequently improve the quality of ruminant-derived products by increasing the content of UFAs [8, 9]. Recent studies have strongly indicated that the reduction of ruminal CH_4 production should be balanced with improvements in rumen performance, and enrichment of animal products with beneficial FAs for sustainable adoption of CH_4 mitigation technologies in the livestock industry [3, 9]. Researchers are still searching for the most effective sources and doses of BACs that could be recommended for long-term application.

One category of BACs are polyphenols, such as phenolic acids, flavonoids, condensed tannins and hydrolysable tannins[5]. Some plants rich in distinct polyphenol fractions exert antibacterial and antimethanogenic effects [5, 10, 11]. *Coleus amboinicus* Lour. (CAL) herb is rich in polyphenolic compounds (mostly phenolic acids and flavonoids), diterpenes, and alkaloids [10–13].*C. amboinicus* grows in tropical regions,

including Asia, Africa and Australia, and is used in human medicine for long time[10, 14]. Our previous short-term *in vitro* study revealed the capacity of CAL to decrease CH_4 production and to modulate ruminal FA composition, mainly n-3 polyunsaturated fatty acids (PUFAs) by altering the microbial activity linked to methanogenesis and FA biohydrogenation [13]. However, no trial involving polyphenol-rich CAL in a long-term *in vivo* experiment focusing on ruminal fermentation has been published to date. Therefore, this study investigated the long-term effects and mode of action of BACs of CAL on rumen methanogenesis and BH in growing lambs. We hypothesized that CAL could 1) affect rumen microbial population (mainly methanogens) and consequently mitigate ruminal CH_4 production, and 2) modulate the BH of UFA, especially n-3 PUFAs, in the rumen and animal tissues, presumably, without any negative effect on rumen parameters or animal performance.

Materials And Methods

Experimental design

The CAL used in this study were purchased from a commercial source (Karya Herbal Nasional, company land-plot at Bogor, Indonesia 6°70'28"S; 106°90'90"E and 6°43'30.1"S; 107°05'09.2"E). The CAL were randomly collected after 2 to 3-month growth period and dried in an oven at 50–60°C for 48 h. The herb leaves were ground and prepared for analyses.

In vitro experiment

The Experiment 1 was carried out using a long-term *in vitro* system employing the rumen simulation technique (RUSITEC) developed by Czerkawski and Breckenridge[15]. The RUSITEC system had eight fermenters of one liter each. The fermenters were placed in a water bath maintained at 39°C throughout the five-day adaptation period and five-day sampling period. The *in vitro* experiment was designed with a completely randomized block design with four diets and two replicates in each run, and repeated three times. The four diets were as follows: diet 1: a control diet (CON, based on grass silage and concentrate at a 45:55 ratio; 11 g of DM); diet 2: 10% CAL (9.9 g DM of CON with 1.1 g DM of CAL); diet 3: 15% CAL (9.35 g DM of CON with 1.65 g DM of CAL diet), and diet 4: 20% CAL (8.8 g DM of CON with 2.2 g DM of CAL). The chemical composition of the grass silage, concentrate, and CAL are presented in Table 1.

Table 1
Chemical composition and fatty acids profile of dietary components and CAL herb.

Item	Grass Silage	Concentrate	CAL
Dry matter content, g/kg	416	889	919
Chemicals composition, g/kg DM			
Ash	86.9	71.9	153
Organicmatter	913	928	847
Crude protein	187	203	214
Etherextract	20.6	38	43.3
aNDF	456	238	405
Fatty acids, g/100 g FA			
C14:0	0.90	0.20	0.45
C16:0	19.9	16.3	18.7
C18:0	5.88	3.71	4.35
C18:1 *cis*-9	8.8	23.7	2.5
C18:2 *cis*-9. *cis*-12	14.4	42.7	10.8
C18:3 *cis*-9 *cis*-12 *cis*-15	36.1	9.20	45.1
∑ Other FA	13.9	4.23	18.1
∑ SFA	30.1	21.4	26
∑ UFA	69.9	78.6	74
∑ MUFA	14.3	26.2	14.8
∑ PUFA	55.5	52.5	59.2
∑ n-6 FA	17.7	43.0	12.3
∑ n-3 FA	37.8	9.42	46.9

CAL, *C. amboinicus* herb; aNDF, NDF analyzed with α-amylase; FA, fatty acids; SFA, saturated fatty acids; UFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 2
Contents of the phenolic acids, flavonoids, and diterpenes identified in CAL.

Compounds	Content (*m*g/g DM)
Siringingacid	0.26
Vanilicacid	0.060
Dihydroxybenzoicacid	0.19
Hydroxybenzoicacid	1.03
Caffeicacid	3.20
Dihydroferulicacid-O-glucuronide	0.25
Luteolin-O-(hexosyl)	0.42
Luteolin-O-glucuronide	4.34
Ferulicacid	0.25
Rosmarinicacidderivative	0.34
Apigenin-O-glucuronide	2.89
Rosmarinicacid	3.36
Luteolin-O-(maloylglycosyl)	1.73
Apigenin derivative	0.88
Carnosciacidglucoside	0.080
Luteolin	0.18
Luteolin-O-(rhamnosyl-hexosyl)	0.15
Apigenin	0.12
3'.4'-Dimethoxyquercetin	0.14
Salvianolicacid C	0.27
Diterpenederivative	0.37
Salvianolicacid C derivative	0.12
5.7-Dihydroxy-4'.6-dimethoxyflavone	0.075
Dihydroxykaurenoicacid	0.045
Trihydroxy-ent-kauranoicacid	0.020
Rosmanol	0.085
Dihydroxykaurenoicacid	0.17
Longikaurin A	0.28
Dihydroxyroyleanone	4.78
Epirosmanol	0.11
Dihydroxy-16-kauren-19-oic acid	0.10
Diterpene	0.22
Acetyldihydroxyroyleanone	13.41
Total phenolicacids	9.30
Total flavonoids	10.94
Total polyphenoliccontent	20.24
Total diterpenes	19.59

Rumen fluid and solid digesta for the *in vitro* experiment were collected before morning feeding from six rumen-cannulated lambs (20 ± 3 kg) for microbial inocula. The lambs, donors of the rumen fluid, were fed the same diet as in the CON treatment. The animals were fed grass silage (300 g/of DM per day) and concentrate (360 g of DM per day). The ruminal contents were collected from the top, bottom, and middle parts of the rumen of each lamb separately, then strained through a four-layer cheesecloth into a prewarmed Schott Duran bottle (Schott, NY, USA), flushed with carbon dioxide (CO_2), and immediately transported to the laboratory in a 39°C-preheated water bath. Before beginning the RUSITEC experiment, samples of ruminal fluid from each lamb were mixed in equal proportions and each fermenter was filled with 500 ml of strained rumen fluid and 400 ml of prewarmed McDougall buffer[16]. Two nylon bags (70 × 140 mm, 100-µm pore size) were put into the fermenters–the first nylon bag contained approximately 11 g DM of rumen solid digesta, and the others contained 11 g DM of the diets (CON, 10% CAL, 15% CAL, or 20% CAL). After 24 h incubation, the nylon bag with rumen digesta was replaced with a new nylon bag containing diets. Finally, each bag with diet was incubated for 48 h. After replacing each bag and closing the fermenter, the fermenter was flushed for 3 min (3 L/min) using N_2 gas to reestablish anaerobic conditions. During the experimental runs, the buffer was supplied continuously into each fermenter by an electronic peristaltic pump (Miniplus 3, Gilson, Middleton, WI, USA) at an average buffer flow rate of 500 ml/day consistently. The fermented fluid was continuously transferred through an overflow tube to the respective effluent vessels containing 10 ml of 6 N HCl (to stop the fermentation process). Samples of fermentation fluid were collected directly from each fermenter 3 h before replacing the bags with the diets. The pH, ammonia concentration, VFA profile, feed degradability, protozoa count, and populations of methanogen, total bacteria, and select bacteria were analyzed. To determine FA profile, samples of the fermenting fluid were directly collected from the effluent vessels while the bags were being replaced. Fermentation gases were collected over 24 h using gas-tight bags (Tecobag 81, Tesseraux Container, Bürstadt, Germany).

In vivo experiment

The Experiment 2 employed six rumen-cannulated lambs allocated into two treatments, i.e., the control diet (CON) and the experimental diet (CAL-containing diet) in a crossover design. The highest level (20%) of CAL was selected based on the *in vitro* results from RUSITEC experiment. The CON and CAL lambs were fed a combination of grass silage and concentrates to meet their nutrient requirements for growth (200 g/day). Lambs on CON diet received 400 g/day of concentrate and *ad libitum* grass silage, with daily intake of silage recorded. Experimental lambs received CAL supplemented concentrate (400 g/day). During the first 14 days of the experiment, the lambs were adapted to gradually increased CAL levels: from 50 g/day to 200 g/day of the dry ground CAL. From the day 15 onwards, the lambs were feed 200 g/d of dry CAL per day. The CAL lambs finally received 400 g/day of concentrate containing 200 g/day of CAL and *ad libitum* grass silage (the daily intake of silage was recorded). In Experiment 2, each period lasted 24 days, with a 21-day adaptation period and a 3-day sampling period. Rumen fluid from each lamb was collected every day for three days of the experimental period, before morning feeding (0 h), and then at 3 h and 6 h after morning feeding [17]. The pH, ammonia concentration, VFA profile, and numbers of protozoa, methanogens, and total bacteria were analyzed. Meanwhile, samples for quantification of total bacteria and methanogens using fluorescence in situ hybridization (FISH) were only collected at the 3 h timepoint.

In Experiment 3, sixteen growing lambs (20 ± 3 kg live weight) were used for the final production performance test. Lambs were randomly allocated into CON or CAL dietary treatments based on their live weight (n = 8 per group). Lambs were kept individually during the whole experiment, except during the period when respiratory chambers were used. In order to reduce stress associated with isolation, two animals were always kept together in each cage placed in the respiratory chamber.The experiment lasted 30 days: a 21-day adaptation stage and an 8-day sampling period, with one day for the slaughtering process. During the adaptation period, the lambs were adapted to the CAL diet, as in Experiment 2. The CON lambs were fed a control diet of grass silage (*ad libitum*, but with recorded intake) and a concentrate mixture (400 g/day). The CAL lambs were also fed grass silage *ad libitum* with recorded intake, and a concentrate (400 g/day) containing CAL (200 g/day). All animals had free access to fresh water. The CON and CAL diets were fed in equal proportion at 8 am and 8 pm daily. Feed intake, feed residue, and amount of feces were recorded daily. Animal weights were recorded weekly. During the sampling period (from day 22 to day 28 of the experiment), each cage was transferred into a respiratory chamber by daily rotation in order to determine the direct CH_4 emission for 24 h consecutively. Two respiratory chambers were used. Each cage was tested twicebut in order to obtain individual lamb's gasses production, obtained results were divided by two.

On the last day of experiment (day 29), the animals were slaughtered 3 h after morning feeding. After slaughtering, the rumen digesta were taken from the top, bottom, and middle of the rumen and squeezed through a four-layer cheese-cloth for analysis of pH, ammonia concentration, and VFA profile in ruminal fluid, FA profile, and populations of protozoa, methanogens, total bacteria, and select bacteria in digesta. Samples of muscle from the right side of each carcass and drawn at the level of the thirteenth thoracic rib was immediately collected. Approximately 5 g of *Longissimus thoracis* (LT) muscle was shock-frozen in liquid nitrogen for gene expression analysis. The LT muscle (ca. 50 g) was cooled and transferred in a cool (4°C) atmosphere to the laboratory for FA analysis. All collected samples were stored at -80°C until analysis.

Meat quality traits

The LT (100 g) from the right-half carcass was used for meat quality analysis that was performed at the laboratory of the Institute of Agricultural and Food Biotechnology (Poland) following the procedures previously described in details by Grochowska et al. [18]. Briefly, the pH was measured in triplicates 24 h post-mortem on samples of LT muscle using a pHmeter with an integrated electrode (pH meter 1140, Mettler-Toledo, USA) following ISO 2917 (2001) instructions. For the instrumental evaluation of meat color, 10 mm thick steaks of LT muscle were cut towards the direction of muscle fibers and exposed to electric light for 15 min. The values of L* (lightness), a* (redness), and b* (yellowness) were determined in triplicates using a Minolta Chroma Meter CR- 400 (Konica-Minolta, Japan). Compositional analysis of LT muscle (water, intramuscular fat (IMF) and total protein content) was performed using minced samples according to the methods described in ISO 1442 (2000) for water, ISO 1444 (2000) for fat (using a Soxtherm device, Gerhardt Analytical System, Germany), and PN-75/A-04018 (2000) for protein (using a Kjeltec System 1002 Distilling Unit, FOSS Analytical, Denmark). The water-holding capacity (WHC) of minced LT muscle samples was determined as described by Grau and Hamm [19], with later modifications introduced by Pohja and Ninivaara[20]. Visual evaluations of meat color and marbling of LT muscle samples were performed by a panel of four assessors using a 1–8 point Soicarni scale for meat color, with 1 being the lightest and 8 the darkest color, and a 1–4 point scale for marbling (developed by the Institute of Agricultural and Food Biotechnology, Poland), with 1 being related to minor and 4 to the greatest marbling. The taste panel of four professional assessors, trained in rating lamb for meat-eating quantity, was used to assess aroma, juiciness, tenderness, and flavor on boiled LT samples. Assessors scored the samples for each trait separately on a 1–5 point scale, where 1 was related to bad and 5 to a very good level of the traits according to the methodology of Barylko-Pikielna[21]. Concerning the abovementioned visual and sensory evaluations of meat, the mean values of the scores given by four assessors were taken for further calculations.

Determination of phenolic acid, flavonoid, and diterpenoid contents

Approximately 100 mg of CAL ground to powder form was extracted three times with 80% methanol (3 ml) for 60 min at 40°C. The extracts were combined and evaporated to dryness, and then 20 mg of this sample was dissolved in 3 ml of Milli-Q water (acidified with 0.2% formic acid) and purified by solid phase extraction (SPE) using Oasis HLB 12cc Vac Cartridge, 500 mg (Waters, Milford, MA). The cartridges were washed with 0.5% methanol (3 ml) to remove carbohydrates and then washed with 80% methanol (3 ml) to elute phenolics and diterpenes. Then obtained fraction (2.5 mg) was re-evaporated and dissolved in 1 mL of 80% methanol (acidified with 0.2% formic acid). The sample was then centrifuged (23,000 × *g* for 5 min) before undergoing spectrometric analysis. All analyses were performed in triplicate for three independent samples that were stored in a freezer at -20°C before analysis.

The CAL compounds were analyzed on a UHRMS DionexUltiMate 3000RS system (Thermo Scientific, Darmstadt, Germany) with a charged aerosol detector interfaced with a high-resolution quadrupole time-of-flight mass spectrometer (HR/QTOF/MS, Compact, Bruker Daltonik, Bremen, Germany). The CAL phenolic acids, flavonoids, and diterpenes were chromatographically analyzed on a Kinetex C18 column (2.1 × 100 mm, 2.6 µm, Phenomenex, USA), with mobile phase A consisting of 0.1% (v/v) FA in water and mobile phase B consisting of 0.1% (v/v) FA in acetonitrile. A linear gradient from 7–50% phase B in phase A over 20 min was used to separate phenolic compounds with a short 0.3 min calibration segment from 0 to 0.5 min. injection 5 µl, a flow rate of 0.3 mL/min, and with the column held at 25°C. Spectra were acquired in negative-ion and positive-ion modes over a mass range from m/z 100 to 1500 at 5 Hz. The operating parameters of the ESI (Electro Spray Ionization) ion source were 3 kV capillary voltage, 6 L/min dry gas flow, 200°C dry gas temperature, 0.7 bar nebulizer pressure, 700.0 V collision radio frequency, 100.0 µs transfer time, and 7.0 µs prepulse storage. Ultrapure nitrogen was used as a drying and nebulizer gas, and argon was used as the collision gas. The collision energy was set automatically in the15 to 75 eVi range, depending on the m/z of the fragmented ion. The readings were calibrated internally with sodium formate introduced to the ion source at the beginning of each separation via a 20 µL loop. The spectra were processed using Bruker Data Analysis 4.3 software. (Bruker Daltonik, Bremen, Germany). The amounts of the individual phenolic acids in the CAL extract were calculated as equivalents of rosmarinic acid (CAS 537-15-5; (R)-rosmarinic acid, Sigma Aldrich) and flavonoids as equivalentsof isoquercetin (CAS 482-35-9; quercetin 3-o-glucopyranoside, Sigma Aldrich). Calibration curves for these two compounds were constructed based on seven concentration points (from 3.9 to 1000 µg/ml). The amount of diterpenes in CAL was calculated as equivalents of carnosic acid (CAS 3650 09 − 7, Sigma Aldrich) at seven concentration points (0.05 to 125µg/ml). All analyses were performed in triplicate. The present analysis procedure on phenolics fraction was following previous published study [14].

Determination of the chemical composition of feeds

Samples of grass silage, concentrate, CAL, and feces were analyzed according to AOAC [22] for 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). The organic matter (OM) content was calculated by subtracting ash concentration from DM content. The aNDFwas determined following the method of Van Soest et al. [23], with the addition of amylase and sodium sulfite without residual ash.

Basic rumen fermentation analysis and CH_4 measurement

The pH of ruminal samples from all the experiments was measured immediately after samples collection using a pH meter (CP-104; Elmetron, Zabrze, Poland). The ammonia concentration was analyzed using the colorimetric Nessler method described earlier by [9]. The VFA profile was determined by gas chromatography (GC Varian CP 3380, Sugarland, TX, USA) following the protocol of Varadyova[24]. The *in vitro* CH_4

concentration was measured using a gas chromatography in SRI PeakSimple model 310 (Alltech, PA, USA) following the procedure described by Kozlowskaet al.[25].

Methane production in the *in vivo* experiment was measured using two respiration chambers (SPA System, Wrocław, Poland). The total chamber volume (8.2 m^3) was ventilated by recirculating fans set at 40 m^3/h giving approximately 5 air changes per hour. The temperature and relative humidity were set at 16°C and 60%, respectively. The concentrations of CH_4 and CO_2weremeasured using two nondispersive infrared spectroscopy detectors operating in the near-infrared spectrum (Servomex 4100, Servomex, UK; 1210 Gfx detector). Measurements were taken at two-second intervals. Two measuring channels were used: the concentration of CO_2 in the range of 0–2.5% (0–48,450 mg/m^3) and the CH_4 concentration in the range of 0-1000 ppm (0–706 mg/m^3). The sample was collected and then ducted to the analyzer via a polyethylene tube with a diameter of 8 mm. The sampling rate was 0.6 L/min. Before starting the experiment, the analyzers were calibrated using as calibration gases (99.999% nitrogen gas by volume, 1210 ppm CH_4 in nitrogen, and 4680 ppm CO_2 in nitrogen). The analyzer was equipped with a 0.17 L cuvette with an optical track of 540 mm for CH_4 and a 0.012 L cuvette with an optical track length of 154 mm for CO_2.

Microbial quantification

The protozoa population was quantified following the method described by Michalowski et al. [26]. Methanogen numbers were quantified by fluorescence in situ hybridization (FISH) technique, following the procedure of Jozefiak et al. [27], with some modifications. Briefly, 50 µL of the rumen fluid was diluted with phosphate-buffered saline (PBS) and pipetted onto 0.22 µm polycarbonate filters (Frisenette K02BP02500) and vacuumed (Vacuum KNF, VacuportNeuberg). After vacuuming, the filters were transferred onto cellulose disks for dehydration in an ethanol series (50%, 80%, and 90%, 3 min each). To allow determination of optimal hybridization, a series of identical filters was prepared in each sample. The 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 of total methanogens (S-D-Arch-0915-a-A-20), r Methanomicrobiales (S-O-Mmic-1200-a-A-21) order and Methanobacteriales (SF-Mbac-0310-a-A-22) order [28]. After hybridization, the filters were washed with washing buffer (20 mM Tris/HCl, pH 7.2; 0.01% SDS; 5 mM EDTA) for 20 min at 48°C. The filters were rinsed gently in distilled water, air-dried, and mounted on object glasses with VectaShield anti-fading agent (Vector laboratories H-1000), which contained 4,6-diamidino-2-phenylindole (DAPI). Filters were maintained at 4°C for one hour in the dark until they were visualized with an Axio Imager M2 microscope (Carl Zeiss Iberia, Madrid, Spain) to distinguish the total count of bacteria (DAPI) from other the methanogens in the rumen fluid.

For analysis of abundances of eight selected species and two genera of rumen bacteria in the *in vivo* experiments, metagenomic DNA was extracted from the ruminal content using a Mini Bead-Beater (BioSpec Products, Bartlesville, OK, USA) for cell lysis and DNA was purified (QIAamp DNA Stool Mini Kit; Qiagen, Hilden, Germany). The DNA concentration was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The DNA used in this experiment had an A260:A280 ratio higher than 1.8. Sequences of primers specific to the particular bacterial species or genera are presented in Table 3[29–36].

Table 3
The sequences of primers specific to the analyzed bacteria species.

Species	Primer sequences	Reference
Streptococcus bovis	F: ”TTCCTAGAGATAGGAAGTTTCTTCGG“	[29]
	R: ”ATGATGGCAACTAACAATAGGGGT”	
Ruminococcusflavefaciens	F:“CGAACGGAGATAATTTGAGTTTACTTAGG“	[30]
	R: “CGGTCTCTGTATGTTATGAGGTATTACC“	
Ruminococcus albus	F: “CCCTAAAAGCAGTCTTAGTTCG“	[31]
	R: “CCTCCTTGCGGTTAGAACA“	
Megasphaeraelsdenii	F: ”AGATGGGGACAACAGCTGGA“	[29]
	R: ”CGAAAGCTCCGAAGAGCCT”	
Prevotella spp.	F: ”GAAGGTCCCCCACATTG “	[29]
	R:”CAATCGGAGTTCTTCGTG”	
Lactobacillus spp.	F: ”TATGGTAATTGTGTGNCAGCMGCCGCGGTAA“	[32]
	R: ”AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT”	
Fibrobacter succinogenes	F: “GTTCGGAATTACTGGGCGTAAA“	[33]
	R: “CGCCTGCCCCTGAACTATC“	
Butyrivibrioproteoclasticus	F: “TCCTAGTGTAGCGGTGAAATG“	[34]
	R: “TTAGCGACGGCACTGAATGCCTA“	
Butyrivibriofibrisolvens	F: “ACACACCGCCCGTCACA“	[35]
	R: “TCCTTACGGTTGGGTCACAGA“	
Anaerovibriolipolytica	F: ”GAAATGGATTCTAGTGGCAAACG“	[36]
	R:”ACATCGGTCATGCGACCAA”	

The specificity of primers (Table 3) was confirmed using the BLAST program in the GenBank Database. The starting DNA concentration was 25 ng/µl and the quantification of each bacterial species DNA and total bacteria in the total rumen DNA was carried out using a QuantStudio 12 Flex PCR system (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). The Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) was used for PCR amplification. The reaction mixture in 10 µl of the final volume contained 4 µl of the 2 × Mastermix, 25 ng of template DNA and 0.5 M of each primer. Amplification involved one cycle at 95°C for 10 min for initial denaturation, 45 cycles of 95°C for 15 s followed by annealing at temperatures depending upon the individual bacteria, and then at 60°C for 62 s. The fluorescent product was detected in the last step of each cycle. To determine the specificity of amplification, an analysis of product melting was performed after a single amplification (0.1°C • s^{-1} increment from 65°C to 95°C with fluorescence collection at 0.1°C intervals). Additionally, product size was verified by gel electrophoresis of samples after the PCR run. Dilution of purified genomic DNA from control strains was used to construct species-specific calibration curves; then further, the calibration curve was used for calculation of the species-specific DNA concentration in total rumen DNA preparations (number of DNA copies). The relative abundances of DNA copy of each bacterial species was then calculated using the formula $2^{-\Delta\Delta Ct}$ (RTA), where the total bacterial DNA level was used as a reference.

Analysis of fatty acid in feed and meat samples

The FA profiles of the grass silage, concentrate, CAL, rumen fluid, and LT muscle were analyzed following the procedure of Cieslak et al.[37], with some modifications. Sample hydrolysis was carried out in a closed system using screw-cap Teflon-stoppered tubes (Pyrex, 15 mL). Three milliliters of 2 M NaOH was added to 100, 2500, 100, 10, and 500 mg of grass silage, concentrate, CAL, rumen fluid, and meat samples, respectively. Hydrolysed samples were incubated on a block heater at 90°C for 40 min. The samples were then extracted and esterified using 0.5 M NaOH in methanol and subsequently converted to fatty acid methyl esters (FAME) using boron trifluoride (Fluka). 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 i.d.) coated with 0.25 µm Agilent HP (Chrompack CP7420) were used. Hydrogen was used as the carrier gas at a flow rate of 1.3 mL/min. The injector and detector temperatures were set at 200°C and 250°C, respectively. The oven was programmed as follows: initially 120°C for 7 min, increasing at 7°C/min to 140°C, holding for 10 min and then increasing at 4°C/min to 240°C. One microliter sample volume was injected into the column. The fatty

acids were identified based on their retention times and expressed as a g/100 g FA. The peaks were identified by comparison of the retention times with FAME standards (37 FAME Mix, Sigma Aldrich, PA, USA).

The conjugated linoleic acid (CLA) peaks were identified via comparison with the retention times of the reference standard (conjugated linoleic acid methyl esters, and a mixture of cis- and trans-9, -11 and − 10,12-octadecadienoic acid methyl esters; Sigma) using Galaxie Work Station 10.1 (Varian, CA, USA). The desaturase index, atherogenic index, and thrombogenic index were calculated as described by Bryszak et al. [9].

Analysis of mRNA expression in meat samples

Transcript analysis of *FADS1, FASN, LPL, SCD*, and *ELOVL5* genes in the meat samples was performed using quantitative PCR (qPCR) analysis. Total RNA was isolated from 100 mg of LT muscle using Extrazol reagent. In brief, the meat samples were homogenized in 0.5 ml of Extrazol reagent using a TissueLyser II (Qiagen, USA). After 10 min incubation, 200 µl of chloroform was added and shaken vigorously for 15 s. The samples were then incubated for 10 min at room temperature and centrifuged for 15 min at 12,000 × *g*. Next, the upper aqueous phase was transferred to a new tube and 0.5 ml of isopropanol was added. The samples were again incubated and centrifuged as in the previous step. The resulting RNA pellet was washed with 1 ml of 75% EtOH and dissolved in RNAse free water (Sigma Aldrich). The quantity and quality of the isolated total RNA was checked using an NP80 NanoPhotometer (Implen, Germany). A reverse transcription reaction (RT) was carried out with 1 µg of total RNA and the Firescript RT cDNA Synthesis MIX with Oligo (dT) and Random primers (Solis BioDyne), following the manufacturer's protocol. The mRNA expression was quantified using QuantStudio 12 Flex PCR system (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) and SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The primer pairs used for RT-qPCR amplification are listed in Table 4[38]. The specificity of reaction products was determined by the melting points (0.1 C/s transition rate). Two genes have been considered as reference, *GAPDH* and *β-actin*. Due to its higher stability, the *β-actin* gene was applied and relative mRNA expression was evaluated by delta-delta CT (ΔΔCT).

Table 4
The sequences of primers specific to the analyzed genes expression in the lamb's LTmuscle.

Gene name	Primer sequence (5′ to 3′)		Reference
	Forward	Reverse	
SCD	GAGTACCGCTGGCACATCAA	CTAAGACGGCAGCCTTGGAT	[38]
ELOVL5	TGCTTCAGTTTGTGCTGACC	TGGTCCTTCTGGTGCTCTCT	[38]
FASN	GGAGGACGCTTTCCGTTACA	TGCTCTTCCTCACGTACCTGAA	[38]
FADS1	CTGCTGTACCTGCTGCACAT	ACGGACAGGTGTCCAAAGTC	[38]
LPL	TCATCGTGGTGGACTGGCT	CATCCGCCATCCAGTTCATA	[38]

Analysed expression of five genes:SCD, *stearoyl-CoA desaturase*;ELOVL5,*fatty acid elongase 5*; FADS1, *fatty acid desaturase 1*;FASN, *fatty acid synthase*, and;LPL, *lipoprotein lipase*.

Statistical analysis

The data of the experiment 1 (RUSITEC) were analyzed using a mixed model procedure (PROC MIXED) in SAS (university edition, version 9.4; SAS Institute, Cary, NC, USA) with repeated measures of day and fermenter treated as the experimental unit. The dietary treatment was considered as the fixed effect, experimental run as the random effect, and the day (6 to 10 days) as the repeated factor. Differences among treatments were further determined using Tukey's post hoc test and linear orthogonal contrast was used to ascertain the tendency of the dose effect of CAL. In experiment 2, data were analyzed using PROC MIXED of SAS with the model containing dietary group, hour, and their interaction (group × hour) as the fixed effects and the animal and hour of sample collection as the random effect with repeated measures. When the significant value of the interaction occurred, Tukey's post hoc test was used to estimate the differences between means. In experiment 3, data were analyzed using PROC TTEST procedure of SAS, and for all parameters each animal was considered as the experimental unit. Significance was accepted at $P < 0.05$ and tended to significance at $0.05 < P < 0.10$. All the values are shown as group means with pooled standard errors of means.

Results

Nutrients and phytochemical composition of CAL

The CAL had higher CP, EE, and ash concentrations than the concentrate and grass silage (Table 1). It also had relatively higher PUFA proportion, especially of n-3 FA, mainly due to the high content of C18:3 *cis*-9, *cis*-12, *cis*-15. The CAL contained 20.24 mg/g DM of total polyphenolic compounds and diterpenes, at 19.6 mg/g DM. Among the various diterpenes present in CAL, acetoxy dihydroxy royleanone had

the highest concentration at 13.4 mg/g DM. The CAL also contained high amounts of dihydroxyroyleanone 4.77 mg/g DM. Among the polyphenols, luteolin-O-glucuronide was present at 4.34 mg/g DM, rosmarinic acid at 3.35 mg/g DM, and caffeic acid at 3.19 mg/g DM (Table 2).

In vitro experiment (Experiment 1)

Increased supplementation with CAL did not alter the basic ruminal fermentation parameters, such as pH and concentrations ofammonia or total VFA (Table 5). However, the molar proportions of acetate, propionate, butyrate, isovalerate, and valerate were dose-dependent. The proportions of acetate and isovalerate were lower in the 15% and 20% CAL treatments ($P \leq 0.05$), but the proportions of butyrate and valerate were higher in the CAL diets than in CON ($P \leq 0.02$). A linearly lower ($P < 0.01$) A/P ratio was observed with increasing CAL. Digestibility of DM, OM, and NDF was unaffected by CAL, but a higher crude protein digestibility was noted for the 10% and 20% CAL supplementation with a linear response ($P = 0.03$). Total gas and CH_4 production (ml or ml/g DN) decreased linearly ($P \leq 0.01$) with increasing levels of CAL in diets. Protozoa counts were unaffected by the inclusion of CAL (Table 6). The 10% CAL addition increased the populations of *Streptococcus bovis, Prevotella*spp.,*Butyrivibrioproteoclasticus*, and*Butyrivibriofibrisolvens*($P \leq 0.02$). The CAL treatments linearly decreased the total *Archaea* and *Methanobacteriales* population ($P < 0.01$).

Table 5
The effect of CAL on *in vitro* ruminal fermentation and methane production (Experiment 1).

Parameters	CON	CAL (% in diet)			SEM	P value	
		10	15	20		Diet	L
Rumenfermentation							
Redoxpotential, mV	-335	-336	-329	-333	2.11	0.35	0.34
pH	6.89	6.89	6.91	6.91	0.001	0.12	0.08
NH_3, mM	9.19	9.19	9.11	9.18	0.25	0.99	0.96
Total VFA, mM	44.7	44.4	44.6	47.1	1.15	0.62	0.36
VFA, molarpercent							
Acetate (A)	61.3^{a}	59.7^{ab}	58.2^{b}	56.9^{c}	0.74	0.01	< 0.01
Propionate (P)	22.9^{bc}	22.7^{c}	23.7^{ab}	24.7^{a}	0.37	< 0.01	< 0.01
Isobutyrate	3.36	3.56	3.57	3.62	0.17	0.78	0.35
Butyrate	8.19^{b}	9.10^{a}	9.36^{a}	9.36^{a}	0.14	0.02	< 0.01
Isovalerate	1.01^{ab}	1.12^{a}	0.91^{b}	0.93^{b}	0.03	0.04	0.13
Valerate	3.01^{c}	3.76^{b}	4.15^{ab}	4.22^{a}	0.14	< 0.01	< 0.01
A/P ratio	2.75^{a}	2.69^{a}	2.51^{b}	2.35^{b}	0.07	< 0.01	< 0.01
Digestibility, g/kg DM							
Drymatter	505	526	499	518	6.40	0.38	0.78
Organicmatter	516	530	504	524	6.36	0.47	0.99
Crude protein	430^{b}	489^{a}	453^{ab}	489^{a}	7.82	0.01	0.03
Neutral detergent fiber	491	506	483	488	7.11	0.66	0.59
Total gas and methane emission							
Gas, mL	2902^{a}	2984^{a}	2920^{a}	2534^{b}	51.0	< 0.01	0.01
Gas, mL/g DM	264^{a}	271^{a}	265^{a}	230^{b}	4.63	< 0.01	0.01
CH_4, mL	92.0^{a}	79.0^{ab}	71.1^{b}	46.6^{c}	3.84	< 0.01	< 0.01
CH_4, mL/g DM	8.64^{a}	7.41^{ab}	6.45^{b}	4.22^{c}	0.34	< 0.01	< 0.01
CH_4, mL/L gas	33.6^{a}	28.3^{ab}	24.2^{b}	18.2^{c}	1.24	< 0.01	< 0.01
CH_4, mL/g DMD	17.1^{a}	14.0^{a}	14.9^{a}	8.61^{b}	0.70	< 0.01	< 0.01

CON: Control diet; CAL: *Coleus amboinicus* Lour. herb diet; SEM: standard error of means; L: linear response; DM: dry matter; NH_3: ammonia; VFA: volatile fatty acid; CH_4: methane; DMD, dry matter digestibility.

Different superscripts (a, b, c) within the same row indicate significant differences ($P < 0.05$).

Table 6
The effect of CAL on *in vitro* ruminal microorganisms (Experiment 1)

Variables	CON	CAL (% of diet)			SEM	P value	
		10	15	20		Diet	L
Total protozoa, 10^3/mL	4.89	4.68	4.68	4.91	0.003	0.40	0.91
Holotricha, 10^3/mL	0.05	0.04	0.04	0.05	0.06	0.72	0.55
Entodinomorpha, 10^3/mL	4.85	4.64	4.64	4.87	0.07	0.39	0.91
Total bacteria, 10^8/mL	4.74	4.31	4.51	4.48	0.06	0.08	0.27
*Streptococcusbovis**	1.00^{ab}	5.26^{a}	0.08^{b}	0.16^{b}	0.70	0.01	0.76
*Ruminococcusflavefaciens**	1.00	0.43	0.88	0.43	0.19	0.63	0.38
*Ruminococcusalbus**	1.00	0.10	3.16	18.5	3.08	0.13	0.09
*Megasphaeraelsdenii**	1.00	4.07	4.63	3.69	0.86	0.47	0.20
Prevotella spp.*	1.00^{b}	8.54^{a}	1.93^{ab}	0.46^{b}	1.06	0.02	0.84
Lactobacillus spp.*	1.00	0.05	0.33	0.11	0.05	0.12	0.22
*Fibrobactersuccinogenes**	1.00	0.57	0.28	0.02	0.10	0.27	0.40
*Butyrivibrioproteoclasticus**	1.00^{b}	5.42^{a}	2.38^{ab}	1.53^{b}	0.54	< 0.01	0.47
*Butyrivibriofibrisolvens**	1.00^{b}	15.0^{a}	4.83^{ab}	3.61^{ab}	1.60	0.01	0.53
*Anaerovibriolipolytica**	1.00	0.23	0.81	0.34	0.13	0.12	0.15
Total methanogens, 10^7/mL	4.39^{a}	3.87^{b}	3.91^{b}	3.32^{c}	0.10	< 0.01	< 0.01
Methanobacteriales, 10^6/mL	3.05^{a}	2.85^{b}	2.81^{bc}	2.67^{c}	0.04	< 0.01	< 0.01
Methanomicrobiales, 10^6/mL	3.06^{a}	2.89^{b}	2.97^{b}	2.67^{ab}	0.04	0.01	0.13
CON: Control diet; CAL: *Coleus amboinicus* Lour. herb diet; SEM: standard error of means; L: linear response;							
*Relative transcript abundance (ΔΔ CT).							
Different superscripts (a, b, c) within the same row indicate significant differences (P < 0.05).							

The FA proportions in the ruminal fluid were altered by CAL supplementation (Table 7). The C18:0, C18:1 *trans*-10, C18:1 *trans*-11, total C18:1 *trans*, C20:1 *trans*, decreased linearly with increasing levels of CALin the diets ($P \leq 0.05$). The C18:1*cis*-9, C18:1*cis*-11, C24:1, C18:2 *cis*-12 *trans*-10 (CLA), C18:2 *cis*-9 *cis*-12 (linoleic acids; LA), PUFA, andn-6 FA proportions in CAL treatments increased linearly ($P \leq 0.05$). The CAL treatments had a lower total SFA and higher total UFA proportion in ruminal fluid, and both were altered in a linear manner ($P < 0.01$). Also, the total BH intermediates, LA-BH and LNA-BH were decreased by CAL with a linear response ($P < 0.01$). The C18:3 *cis*-9 *cis*-12 *cis*-15, n-3 FA, total CLA, PUFA/SFA and LNA/LA proportions were higher at 20% CAL supplementation ($P \leq 0.05$) than the CON.

Table 7
The effect of CALon *in vitro*ruminal FA composition (Experiment 1)

Fatty acid, % of total FA	CON	CAL			SEM	P value	
		10	15	20		Diet	L
Saturated FA							
C10:0	0.26	0.31	0.30	0.28	0.02	0.84	0.77
C12:0	1.33	1.30	1.62	1.42	0.07	0.28	0.29
C14:0	1.53	1.51	1.51	1.47	0.04	0.94	0.56
C15:0	1.14	1.13	1.10	1.09	0.03	0.91	0.49
C16:0	21.2	21.3	21.7	20.9	0.20	0.48	0.89
C17:0	0.76	0.72	0.72	0.72	0.02	0.78	0.39
C18:0	41.0^{a}	36.7^{b}	36.5^{b}	33.8^{b}	0.77	< 0.01	< 0.01
C20:0	0.07	0.09	0.08	0.06	0.01	0.63	0.54
C21:0	0.20	0.21	0.18	0.19	0.02	0.95	0.77
C22:0	0.13	0.16	0.16	0.18	0.01	0.46	0.13
C23:0	0.17	0.16	0.14	0.15	0.01	0.82	0.43
C24:0	0.52	0.46	0.45	0.44	0.02	0.17	0.04
Monounsaturated FA							
C14:1	0.50	0.48	0.37	0.38	0.03	0.12	0.04
C15:1	0.52	0.62	0.55	0.60	0.03	0.69	0.57
C16:1	0.57	0.77	0.61	0.89	0.06	0.16	0.11
C17:1	0.21	0.23	0.25	0.24	0.02	0.90	0.47
C18:1 *trans-10*	1.01^{a}	0.80^{ab}	0.65^{b}	0.57^{b}	0.06	0.05	< 0.01
C18:1 *trans-11* (VA)	2.40^{a}	1.84^{b}	1.86^{b}	1.67^{b}	0.08	< 0.01	< 0.01
C18:1 *cis-9*	10.9^{b}	12.9^{a}	13.8^{a}	13.7^{a}	0.37	0.02	< 0.01
C18:1 *cis-11*	1.14^{b}	1.26^{ab}	1.33^{a}	1.31^{a}	0.03	0.03	< 0.01
C18:1 *cis-12*	0.14	0.14	0.11	0.12	0.01	0.68	0.37
C18:1 *cis-13*	0.20	0.14	0.09	0.14	0.02	0.11	0.09
C18:1 *cis-14*	0.33	0.36	0.37	0.37	0.02	0.85	0.42
C20:1 *trans*	0.95^{a}	0.89^{ab}	0.87^{ab}	0.81^{b}	0.02	0.05	< 0.01
C24:1	0.15^{b}	0.16^{b}	0.27^{a}	0.28^{a}	0.02	< 0.01	0.03
Polyunsaturated FA							
C18:2 *cis-12 trans-10*	0.23^{b}	0.30^{a}	0.23^{b}	0.32^{a}	0.01	< 0.01	0.04
C18:2 *cis-9 trans-11* (RA)	0.09	0.12	0.13	0.09	0.01	0.45	0.99
C18:2 *cis-9 cis-12* (LA)	10.5^{b}	12.6^{a}	12.3^{a}	13.0^{a}	0.35	0.04	0.02

CON: Control diet; CAL: *Coleus amboinicus* Lour. herb diet; SEM: standard error of means; L: linear response; VA, vaccenic acid; RA, rumenic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LA, linoleic acid; MCFA, medium-chain fatty acids; LCFA, long-chain fatty acids;BH int, biohydrogenation intermediates; CLA, conjugated linoleic acids; LA BH, biohydrogenation of linoleic acid; LNA BH, biohydrogenation of linolenic acid.

Different superscripts (a, b, c) within the same row indicate significant differences at $P < 0.05$ and tended to significant at $P < 0.10$.

Fatty acid, % of total FA	CON	CAL			SEM	P value	
		10	15	20		Diet	L
C18:3 *cis-6 cis-9 cis-12*	0.12	0.09	0.10	0.11	0.01	0.43	0.84
C18:3 *cis-9cis-12cis-15* (LNA)	1.25^{b}	1.79^{ab}	1.59^{ab}	4.10^{a}	0.35	0.01	< 0.01
C20:2	0.32	0.31	0.32	0.28	0.03	0.96	0.72
C22:2	0.12	0.09	0.10	0.11	0.01	0.65	0.75
∑ SFA	68.3^{a}	64.1^{b}	64.4^{b}	60.7^{c}	0.79	< 0.01	< 0.01
∑ UFA	31.7^{a}	35.9^{b}	35.6^{b}	39.2^{c}	0.79	< 0.01	< 0.01
∑ MUFA	19.0	20.6	20.8	21.2	0.38	0.15	0.04
∑ PUFA	12.6^{c}	15.3^{b}	14.8^{bc}	17.9^{a}	0.55	< 0.01	< 0.01
∑ n-6	11.1^{b}	13.1^{a}	12.8^{ab}	13.5^{a}	0.35	0.04	0.02
∑ n-3	1.25^{b}	1.79^{b}	1.59^{b}	4.10^{a}	0.35	0.01	< 0.01
n-6/n-3	9.11	8.75	8.98	6.94	0.44	0.26	0.11
PUFA/SFA	0.19^{b}	0.25^{b}	0.23^{b}	0.31^{a}	0.01	< 0.01	< 0.01
LNA/LA	0.12^{b}	0.14^{b}	0.13^{b}	0.31^{a}	0.03	0.01	< 0.01
∑ C18:1	16.0	17.5	17.9	17.9	0.38	0.21	0.06
∑ C18:1 *trans-*	3.33^{a}	2.50^{b}	2.59^{b}	2.28^{b}	0.09	< 0.01	< 0.01
∑ C18:1 *cis-*	12.8^{b}	15.0^{a}	15.3^{a}	15.7^{a}	0.40	0.03	< 0.01
∑ CLA	0.32^{b}	0.42^{a}	0.36^{ab}	0.41^{a}	0.02	0.05	0.11
∑ MCFA	27.0	27.4	27.8	27.1	0.27	0.69	0.83
∑ LCFA	73.0	72.6	72.2	72.8	0.27	0.72	0.69
BH Int	4.31^{a}	3.57^{b}	3.52^{b}	3.31^{b}	0.10	< 0.01	< 0.01
LA BH (%)	64.6^{a}	57.2^{b}	56.1^{b}	53.6^{b}	1.26	< 0.01	< 0.01
LNA BH (%)	94.2^{a}	92.0^{a}	91.5^{a}	78.1^{b}	1.88	< 0.01	< 0.01
RA/VA	0.05	0.08	0.07	0.07	0.01	0.24	0.44

CON: Control diet; CAL: *Coleus amboinicus* Lour. herb diet; SEM: standard error of means; L: linear response; VA, vaccenic acid; RA, rumenic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LA, linoleic acid; MCFA, medium-chain fatty acids; LCFA, long-chain fatty acids;BH int, biohydrogenation intermediates; CLA, conjugated linoleic acids; LA BH, biohydrogenation of linoleic acid; LNA BH, biohydrogenation of linolenic acid.

Different superscripts (a, b, c) within the same row indicate significant differences at $P < 0.05$ and tended to significant at $P < 0.10$.

In vivo experiment

In Experiment 2, ruminal pH and ammonia concentration in cannulated lambs fed the CAL diet was higher than in the CON group ($P < 0.01$), and these variables were post-feeding time dependent ($P < 0.01$; Table 8). The total VFA concentration was similar in both diets and was time-dependent ($P < 0.01$). Butyrate, isovalerate, and valerate proportions decreased when CAL treatment was used ($P < 0.01$). Time-dependent variation was observed in almost all the individual VFA proportions. The ratio of acetate to propionate (A/P) decreased in both diet- and time-dependent manners ($P < 0.01$).

Table 8
The effect of CALon ruminal fermentation in cannulated lambs (Experiment 2).

Parameter	0 h			3 h			6 h			Group		SEM	P value		
	CON	CAL	SEM	CON	CAL	SEM	CON	CAL	SEM	CON	CAL		G	H	GxH
pH	6.87	7.05	0.09	5.95	6.39	0.06	6.34	6.58	0.04	6.39	6.67	0.05	< 0.01	< 0.01	0.20
NH_3, mM	8.75	10.4	0.33	9.34	11.2	0.45	5.85	8.93	0.57	7.98	10.2	0.30	< 0.01	< 0.01	0.27
Total VFA, mM	66.8	67.9	2.77	103.6	101.3	3.83	83.4	86.9	1.50	84.6	85.4	2.36	0.79	< 0.01	0.73
VFA, molarpercent															
Acetate (A)	71.6	69.8	0.39	66.2	66.4	0.41	67.4	67.7	0.44	68.4	67.9	0.32	0.29	< 0.01	0.08
Propionate (P)	15.6 ^{d}	17.5^{c}	0.34	16.3 ^{d}	20.1^{a}	0.49	15.7^{d}	18.9^{b}	0.39	15.9	18.8	0.25	< 0.01	< 0.01	0.04
Isobutyrate	0.42	0.61	0.07	0.52	0.93	0.10	0.46	0.76	0.07	0.47	0.77	0.05	< 0.01	0.15	0.61
Butyrate	10.3^{b}	10.5^{b}	0.11	14.9^{a}	11.1^{b}	0.50	14.4^{a}	11.2^{b}	0.51	13.2	11.0	0.28	< 0.01	< 0.01	< 0.01
Isovalerate	1.00	0.75	0.05	0.53	0.41	0.02	0.62	0.44	0.03	0.72	0.53	0.03	< 0.01	< 0.01	0.31
Valerate	1.11^{c}	0.92^{d}	0.04	1.59^{a}	1.07^{cd}	0.07	1.36^{b}	1.03^{cd}	0.07	1.36	1.01	0.04	< 0.01	< 0.01	0.01
A/P ratio	4.61	4.04	0.11	4.07	3.33	0.10	4.29	3.61	0.09	4.33	3.66	0.06	< 0.01	< 0.01	0.71
CON: Control diet; CAL: *Coleus amboinicus* Lour. herb diet; SEM: standard error of means. D: diet; H: hour; VFA: volatile fatty acids.															
Means of different groups in the same row indicate significant differences at P < 0.05 and tended to significant at P < 0.10.															

Holotricha had a lower population in the CAL group than in the CON group (P = 0.02; Table 9). *Entodiniomorpha* and total protozoa tended to increase (P = 0.07; P = 0.06, respectively) due to CAL supplementation. The CAL diet tended to increase total bacteria abundance (P = 0.09, Table 9). The abundances of total methanogens, *Methanobacteriales* and *Methanomicrobiales* populations were decreased by the CAL diet (P ≤ 0.04).

Table 9
The effect of CALon ruminal microbial populations in cannulated lambs (Experiment 2).

Parameters	Control	CAL	SEM	P value
Total protozoa, 10^5/mL	4.43	5.76	0.42	0.06
Holotricha, 10^5/mL	0.08	0.04	0.01	0.02
Entodiniomorpha, 10^5/mL	4.35	5.72	0.42	0.07
Total bacteria, 10^9/mL	4.12	4.37	0.07	0.09
Total methanogens, 10^8/mL	5.34	4.17	0.18	< 0.01
Methanobacteriales, 10^7/mL	4.20	3.03	0.16	< 0.01
Methanomicrobiales, 10^7/mL	3.35	2.97	0.09	0.04
CON: Control diet; CAL: *Coleus amboinicus* Lour. herb diet; SEM: standard error of means.				

In Experiment 3, the CAL diet did not affect animal performance or digestibility (Table 10); but it significantly lowered the CH_4 production of the lambs, expressed as a L/day, L/kg DMI, L/kg OM (P < 0.01), and CH_4/BW (P = 0.02). The pH value was higher (P = 0.05) and ammonia concentration tended to increase (P = 0.09), when the CAL diet was used. Total VFA concentration did not change, but the propionate proportion

was higher(P = 0.01) in the CAL group than in CON group. The concentrations of isovalerate and valerate as well as the A/P ratio were lower (P ≤ 0.04) in CAL group than the CON group.

Table 10
Impact of CALon performance, methane emission, and ruminal fermentation of lambs (Experiment 3).

Parameters	CON	CAL	SEM	P value
Body weight				
Inital BW, kg	19.3	19.7	0.66	0.70
Final BW, kg	25.1	26.8	0.53	0.13
ADG, g/d	167	196	9.45	0.14
Total tractdigestibility				
Drymatter, g/kg	630	649	8.27	0.26
Organicmatter, g/kg	607	604	6.80	0.82
Crude protein, g/kg	604	601	7.71	0.85
Neutral detergent fiber, g/kg	577	578	13.3	0.97
Methaneemission				
CH_4,L/d	20.0	15.9	0.11	< 0.01
CO_2,L/d	307	274	2.24	< 0.01
CH_4/CO_2,mL/L	63.6	54.4	0.26	< 0.01
CH_4, L/kg DM intake	29.9	21.1	1.44	< 0.01
CH_4, L/kg OM intake	32.4	23.3	1.49	< 0.01
CH_4, L/kg BW	1.02	0.82	0.05	0.02
Rumenfermentation				
pH	6.22	6.35	0.04	0.05
NH_3, mM	9.92	15.5	0.64	0.09
Total VFA, mM	112	118	3.70	0.48
VFA, molarpercent				
Acetate (A)	69.9	68.1	0.51	0.07
Propionate (P)	17.7	19.8	0.46	0.01
Isobutyrate	0.95	0.79	0.05	0.08
Butyrate	9.92	10.2	0.30	0.70
Isovalerate	0.47	0.23	0.05	< 0.01
Valerate	1.06	0.86	0.05	0.04
A/P ratio	3.96	3.46	0.11	0.01

CON: Control diet; CAL: *Coleus amboinicus* Lour. herb diet; SEM: standard error of means; DM: dry matter; OM: organic matter; BW: body weight; ADG, average daily gain; VFA: volatile fatty acids.

The protozoa population was increased (P < 0.01) by the CAL diet (Table 11) as a result of the increased population of *Entodiniomorpha;* but*Holotricha* population decreased (P < 0.01). *M. elsdenii* and *B. proteoclasticus*populations were higher(P ≤ 0.02) in the CAL group than the CON group. Numbers of *R. albus*, *Prevotella* spp., and *B. fibrisolvens*tended to increase (P = 0.06; P = 0.10; P = 0.09, respectively) with the CAL diet. The total methanogens and *Methanobacteriales* populations decreased (P < 0.01 and P = 0.05, respectively) due to CAL supplementation.

Table 11
The effect of CALon ruminal microbial populations in lambs (Experiment 3).

Parameters	CON	CAL	SEM	P value
Total protozoa, 10^5/mL	6.47	8.43	0.45	< 0.01
Holotricha, 10^5/mL	0.05	0.02	0.01	< 0.01
Entodinomorpha, 10^5/mL	6.42	8.41	0.46	< 0.01
Total bacteria, 10^9/mL	4.89	4.77	0.09	0.55
*Streptococcusbovis**	1.01	0.83	0.26	0.75
*Ruminococcusflavefaciens**	1.00	0.94	0.24	0.91
*Ruminococcusalbus**	1.00	4.71	0.92	0.06
*Megasphaeraelsdenii**	1.00	7.68	1.44	0.03
Prevotella spp.*	1.00	3.85	0.86	0.10
Lactobacillus spp.*	1.00	0.10	0.47	0.38
*Fibrobactersuccinogenes**	1.00	5.60	1.89	0.24
*Butyrivibrioproteoclasticus**	1.00	15.9	3.03	0.02
*Butyrivibriofibrisolvens**	1.00	4.55	1.06	0.09
Total methanogens, 10^8/mL	5.08	3.61	0.28	< 0.01
Methanobacteriales, 10^7/mL	3.23	2.73	0.12	0.05
Methanomicrobiales, 10^7/mL	3.08	3.00	0.13	0.75
CON: Control diet; CAL: *Coleus amboinicus* Lour. herb diet; SEM: standard error of means.				
*Relative transcript abundance (ΔΔ CT).				

In Experiment 3, the proportions of C16:0, C23:0, C24:1, LNA, PUFA, the sum of n-6, and the sum of n-3 FA in ruminal fluid were increased by the CAL diet ($P \leq 0.05$, Table 12). The CAL diet significantly decreased the proportions of C18:0 andC18:1 *trans-10*($P \leq 0.05$). Total SFA and PUFA/SFA tended to decrease while the total UFA tended to increase ($P \leq 0.10$). The ruminal biohydrogenation percentage of LA and LNA decreased in the CAL diet ($P \leq 0.05$). Stearic acid (C18:0), sum of SFA, thrombogenicity index (TI), and the atherogenicity index (AI) were decreased ($P \leq 0.05$)in LT muscle by CAL diet. The proportions of C18:3 *cis*-9 *cis*-12 *cis*-15, sum of UFA, PUFA/SFA, total CLA, DΔ9, DΔ9 18:1/18:0, DΔ9 RA/VA increased ($P \leq 0.05$) in the muscle for the CAL diet. The proportions of total PUFA and total n-3 FA in LT muscle tended to increase ($P = 0.07$; $P = 0.07$; $P = 0.08$, respectively) when the CAL diet was used.

Table 12
FA profile in rumen fluid and LT muscle of lambs fed CAL (Experiment 3).

Fatty acid, % of total FA	Rumen fluid				LT muscle			
	CON	CAL	SEM	P value	CON	CAL	SEM	P value
Saturated FA								
C8:0	0.03	0.01	0.01	0.39	1.44	0.60	0.39	0.31
C10:0	0.08	0.49	0.14	0.16	1.85	1.49	0.41	0.68
C12:0	0.64	0.98	0.13	0.19	1.62	1.23	0.26	0.48
C13:0	1.87	1.94	0.17	0.86	1.09	0.82	0.26	0.63
C14:0	1.11	1.29	0.14	0.56	1.77	1.62	0.26	0.79
C15:0	1.46	1.44	0.13	0.92	1.13	1.11	0.22	0.96
C16:0	16.5	18.7	0.49	0.01	14.1	12.5	0.50	0.11
C17:0	0.73	0.62	0.07	0.48	0.87	1.07	0.13	0.48
C18:0	47.3	40.9	1.59	0.03	15.4	12.1	0.73	0.01
C20:0	0.06	0.06	0.02	0.86	0.21	0.20	0.04	0.88
C21:0	0.05	0.11	0.02	0.17	0.26	0.38	0.07	0.41
C22:0	0.11	0.08	0.02	0.37	0.52	0.65	0.08	0.44
C23:0	0.09	0.21	0.03	0.02	0.34	0.71	0.11	0.10
C24:0	0.56	0.55	0.03	0.87	0.18	0.50	0.09	0.09
Monounsaturated FA								
C14:1	0.24	0.28	0.06	0.76	0.69	0.96	0.17	0.45
C15:1	1.11	0.87	0.12	0.32	1.57	1.49	0.20	0.84
C16:1	0.31	0.32	0.05	0.96	1.03	0.99	0.14	0.89
C17:1	0.17	0.17	0.05	0.99	1.24	1.71	0.18	0.20
C18:1 *trans-10*	0.62	0.48	0.04	0.05	0.30	0.21	0.03	0.12
C18:1 *trans-11* (VA)	5.25	4.41	0.30	0.19	1.07	0.75	0.10	0.11
C18:1 *cis-9*	6.98	7.53	0.37	0.49	22.1	22.4	1.11	0.92
C18:1 *cis-11*	1.65	1.68	0.06	0.83	2.30	2.17	0.12	0.62
C18:1 *cis-12*	0.57	0.68	0.06	0.46	0.77	0.62	0.08	0.37
C18:1 *cis-13*	0.12	0.19	0.03	0.26	0.23	0.21	0.05	0.86
C18:1 *cis-14*	1.00	0.81	0.10	0.36	0.63	0.50	0.11	0.57
C20:1 *trans-*	0.84	0.90	0.03	0.36	0.52	0.64	0.11	0.64
C22:1 *n-9*	0.16	0.09	0.03	0.22	0.38	0.60	0.09	0.23

CON: Control diet; CAL: *Coleus amboinicus* Lour. herb diet; SEM: standard error of means; SFA: saturated fatty acids; UFA: unsaturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; BH: biohydrogenation; LNA: linolenic acid; RA: rumenic acid; VA: vaccenic acid; LA: linoleic acid; MCFA: medium-chain fatty acids; LCFA: long-chain fatty acids; nd.:not determined.

Fatty acid, % of total FA	Rumen fluid				LT muscle			
	CON	CAL	SEM	P value	CON	CAL	SEM	P value
C24:1	0.14	0.71	0.11	< 0.01	1.44	1.92	0.14	0.10
Polyunsaturated FA								
C18:2 *cis-12 trans-10* (CLA-*t10*)	0.17	0.66	0.15	0.18	0.51	0.56	0.10	0.82
C18:2 *cis-9 trans-11* (RA)	0.49	0.37	0.05	0.21	0.44	0.69	0.11	0.27
C18:2 *cis-9 cis-12* (LA)	6.42	8.07	0.40	0.02	15.1	17.6	0.93	0.19
C18:3 *cis-6 cis-9 cis-12*	0.15	0.27	0.04	0.11	0.49	0.57	0.09	0.67
C18:3 *cis-9 cis-12 cis-15* (LNA)	1.47	2.28	0.16	< 0.01	1.05	1.73	0.19	0.05
C20:2	0.43	0.37	0.06	0.63	0.35	0.44	0.10	0.68
C20:3 n-6	0.63	0.65	0.07	0.89	4.04	5.11	0.38	0.17
C20:4 n-6	nd.	nd.	nd.	nd.	0.51	0.42	0.05	0.44
C20:5 n-3	nd.	nd.	nd.	nd.	0.74	0.64	0.14	0.73
C22:2	0.08	0.16	0.04	0.28	0.62	0.49	0.15	0.72
C22:5 n-3	nd.	nd.	nd.	nd.	0.64	0.90	0.09	0.17
C22:6 n-3	nd.	nd.	nd.	nd.	0.45	0.79	0.15	0.27
∑ SFA	70.5	67.3	0.96	0.10	40.8	34.9	1.24	0.01
∑ UFA	29.5	32.7	0.96	0.10	59.2	65.1	1.24	0.01
∑ MUFA	18.7	20.1	0.60	0.66	34.3	35.1	0.95	0.70
∑ PUFA	10.8	12.5	0.51	< 0.01	24.9	30.0	1.01	0.07
∑ n-6	8.90	9.72	0.42	0.02	21.6	25.2	1.23	0.17
∑ n-3	1.47	2.28	0.16	< 0.01	2.88	4.06	0.31	0.07
n-6/n-3	6.05	4.37	0.39	0.02	7.86	6.74	0.73	0.47
PUFA/SFA	0.15	0.19	0.01	0.07	0.62	0.86	0.05	0.01
LNA/LA	0.21	0.30	0.02	0.02	0.07	0.10	0.01	0.31
∑ C18:1	15.7	16.8	0.64	0.45	27.4	26.8	1.22	0.81
∑ C18:1 *trans-*	5.48	5.08	0.28	0.52	1.37	0.96	0.11	0.06
∑ C18:1 *cis-*	10.2	11.7	0.58	0.23	26.1	25.8	1.19	0.94
∑ CLA	0.64	0.99	0.19	0.36	0.95	1.61	0.18	0.04
∑ MCFA	23.3	26.3	0.94	0.12	26.3	22.8	1.25	0.17
∑ LCFA	76.7	73.7	0.94	0.12	73.7	77.2	1.25	0.17
BH intermediates	7.81	7.76	0.46	0.95	nd.	nd.	nd.	nd.
LA BH (%)	74.2	67.6	1.58	0.03	nd.	nd.	nd.	nd.
LNA BH (%)	94.4	91.5	0.61	< 0.01	nd.	nd.	nd.	nd.
RA/VA	0.08	0.11	0.02	0.50	nd.	nd.	nd.	nd.
Desaturation index (DI) Δ9	nd.	nd.	nd.	nd.	0.41	0.47	0.01	0.01
DΔ9. C14:1/C14:0	nd.	nd.	nd.	nd.	0.29	0.37	0.06	0.50

CON: Control diet; CAL: *Coleus amboinicus* Lour. herb diet; SEM: standard error of means; SFA: saturated fatty acids; UFA: unsaturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; BH: biohydrogenation; LNA: linolenic acid; RA: rumenic acid; VA: vaccenic acid; LA: linoleic acid; MCFA: medium-chain fatty acids; LCFA: long-chain fatty acids; nd.:not determined.

Fatty acid, % of total FA	Rumen fluid				LT muscle			
	CON	CAL	SEM	P value	CON	CAL	SEM	P value
DΔ9. 16:1/16:0	nd.	nd.	nd.	nd.	0.07	0.07	0.01	0.85
DΔ9. 18:1/18:0	nd.	nd.	nd.	nd.	0.59	0.65	0.01	0.01
DΔ9. RA/VA	nd.	nd.	nd.	nd.	0.27	0.48	0.06	0.04
DΔ9. MUFA/SFA	nd.	nd.	nd.	nd.	0.46	0.50	0.01	0.08
DΔ5. n-6. 20:4n-6/20:3n-6	nd.	nd.	nd.	nd.	0.12	0.08	0.01	0.11
DΔ5. D6. n-6. 20:4n-6/18:3n-6	nd.	nd.	nd.	nd.	0.53	0.43	0.06	0.43
DΔ4. n-3. 22:6n-3/22:5n-3	nd.	nd.	nd.	nd.	0.33	0.41	0.06	0.53
Elongase index	nd.	nd.	nd.	nd.	0.71	0.72	0.01	0.92
Thrombogenic index	nd.	nd.	nd.	nd.	0.85	0.62	0.05	< 0.01
Atherogenicity index	nd.	nd.	nd.	nd.	0.63	0.48	0.03	0.03

CON: Control diet; CAL: *Coleus amboinicus* Lour. herb diet; SEM: standard error of means; SFA: saturated fatty acids; UFA: unsaturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; BH: biohydrogenation; LNA: linolenic acid; RA: rumenic acid; VA: vaccenic acid; LA: linoleic acid; MCFA: medium-chain fatty acids; LCFA: long-chain fatty acids; nd.:not determined.

The CAL diet significantly decreased the mRNA expressions of *FADS1, FASN, LPL*, and *SCD* genes, but not the expression of *ELOVL5* (Fig. 1). The CAL diet also significantly affected some meat characteristics. Lightness color and water-holding capacity of meat were reduced, whereas meat juiciness was increased ($P \leq 0.01$, Table 13) by CAL diet. Redness and flavor values tended to increase ($P = 0.09$; $P = 0.06$, respectively), whereas the ash content tended to decrease due to CAL supplementation ($P = 0.09$).

Table 13
The LT muscle qualityand characteristicsof lamb fed CAL(Experiment 3).

Trait	Unit	Treatments		SEM	*P*-value
		CON (n = 4)	CAL (n = 4)		
Muscle pH 24 h post mortem	pHunits	5.58	5.69	0.05	0.39
L*, lightness	units	41.4	37.0	0.88	0.01
a*, redness	units	12.1	13.2	0.31	0.09
b*, yellowness	units	4.81	4.65	0.36	0.84
Water-holding capacity, %		38.6	29.5	1.69	< 0.01
Watercontent, %		72.9	73.3	0.24	0.40
Intramuscularfatcontent, %		2.58	2.36	0.07	0.13
Total protein content, %		23.3	23.0	0.25	0.58
Ash content, %		1.16	1.13	0.01	0.09
Sensory evaluations					
Aroma	1–5	4.28	4.29	0.05	0.85
Juiciness	1–5	4.19	4.57	0.08	0.01
Tenderness	1–5	4.15	4.36	0.07	0.13
Flavor	1–5	4.07	4.31	0.07	0.06
Subjectivevisualevaluations					
Color	1–8	2.38	2.56	0.10	0.38
Marbling	1–4	1.59	1.53	0.06	0.63

CON: Control diet; CAL: *Coleus amboinicus* Lour. herb diet; SEM: standard error of means.

Discussion

Nutrients and phytochemical composition of Coleus amboinicus Lour.

Several studies confirmed that CAL is rich in nutrients, ash, and BACs [10, 12, 13, 39]. It has been shown that *C. amboinicus* Lour., known also as *Plectranthus amboinicus*,as well as other species of the genus *Plectranthus* (*Lamiaceae*) such as *P. madagascariensis* and *P. ecklonii* have distinct BAC contents [10, 39]. In comparison to our previous study [13], the CAL examined in this experiment was characterized by higher concentrations of protein(214 *vs.* 196 g/kg DM), aNDF (405 *vs.* 363 g/kg DM), *α*-linolenic acid (45.1 *vs.* 35.83 g/100 g FA), and total PUFA (59.2 *vs.* 56.15 g/100 g FA). Regarding BACs, similar concentrations of phenolic acids were detected as in Yanza et al. [13], but the levels of flavonoids and diterpenes were significantly higher (four and nine times, respectively) in this study. The most abundant component in this study was acetyldihydroxyroyleanone (13.4 mg/g DM), but rosmarinic acid dominated in CAL in Yanza et al. [13] study. The content of particular BAC in plants may be attributed to a range of factors, such as geographical area, soil condition, agronomic management, and stage of plant vegetation[40]. The differences between these results and those of Yanza et al. [13] may be attributable to differences in the plant material used, such as in harvesting times (2–3 months and 4–6 months, respectively) and management of plantation (e.g., watering and fertilizer application). The CAL used in this study had higher BAC content than that in Yanza et al. [13], study and thus had more pronounced action in the rumen.

In vitro experiment (10-day RUSITEC fermentation, Experiment 1)

Identification of the concentration of particular BAC in the CAL undoubtedly helped to interpret our results. The gradual replacement of a proportion of the main diet (from 10–20%) by CAL decreased methane production and gradually diminished population of ruminal methanogens (both total methanogens and methanogen species observed). The greatest reduction in total methanogen population (by 24%) as well as the most effective mitigation of CH_4 production (by 49%) was observed for the 20% CAL diet. As in our previous study [13], no negative changes in rumen parameters were noted. Our findings are also supported by other authors[41, 42], who demonstrated CH_4 reduction in response to BAC of plant origin, with no negative consequences such as declined feed degradability, changes in ammonia, total VFA concentration, or microbial population (total protozoa and bacteria population). We have also noted the inhibition of methanogens via a direct toxic antibacterial effect of BAC, but without adverse effects on ruminal fermentation or DM degradability. Despite the toxic action of BAC on methanogens, no effect on protozoa and ruminal bacteria was observed. Only the lowest level of CAL (10%) increased the population of the bacteria *Prevotella*spp., *B. proteoclasticus*, and *B. fibrisolvens.* A distinct effect of the lowest CAL supplementation was also observed in our previous study on *Saponaria officinalis*[43]. We hypothesized that the basic nutrient components of a plant can either interact with BAC or become physically less available for microbiota, resulting in a decreased antibacterial activity.Moreover, the reduction in the methanogen population resulting from the antimethanogenic effect of CAL lowered the acetate:propionate ratio, probably by shifting the free-hydrogen pathways to propionate production. Our findings corroborated with an experiment on *Rosmarinus officinalis*containing diterpenes (such as carnosol, carnosic acid, rosmanol, epirosmanol, isorosmanol, and methyl carnosate) added to a ruminant diet [44]. This supplement affected several groups of rumen microbes involved in protein and fiber degradation as well as CH_4 and ammonia production. *Coleus amboinicus* (Lour.) is a rich source of UFA with the predominance of *α*-linolenic acid (LNA). The higher fat concentration in CAL than in grass silage (43 vs 21 g/kg DM, respectively) may increase the ruminal FA content, and consequently that of LNA. The effect of CAL in the diet on FA profile in the rumen differed from that on bacteria population. For the majority of FA, the significant effect was observed for all CAL (10%, 15%, and 20%) diets.

Dietary CAL modulated the ruminal FA composition by lowering SFA and elevating MUFA and PUFA proportions. The alterations in MUFA and PUFA profiles may suggest the changes in BH process in the rumen. We showed in our short-term study that the polyphenol compounds of CAL origin (flavonoid, phenolic acids, and diterpenes) can alter the action of the ruminal microbiota involved in BH [13]. We also noted higher content of total polyphenols and diterpenes of CAL origin in this long-term study compared with our previous short-term study [13]. The CAL diet decreased C18:1 *trans* concentration due to the BH process of LA. High quantities of polyphenols and diterpenes reduced the final BH step of the C18:1 *cis*-9 and caused a linear decrease in the concentration of stearic acid (C18:0). This was also observed by Vasta et al. [5], who described a negative effect of different polyphenols on C18:0 accumulation in the rumen digesta. The results of the current study thus demonstrate the protective action of CAL on BH of MUFA and PUFA.

In vivo experiments: Experiment 2 with cannulated lambs and Experiment 3 with growing lambs.

The many ruminal fermentation characteristicsin the growing lambs receiving 200 g/day of CAL corroborated with the results of the *in vitro* study. Methane emission (L/d and L/DM intake) was mitigated by 20% and 29%, respectively, and did not interfere with DM, OM, or NDF digestibility. Decreases in CH_4 production are usually associated with adverse effects on fiber digestibility [45]. We suggest that, in this case, CH_4 mitigation was attributed directly to the reduction in total methanogen and *Methanobacteriales*population (in both growing and cannulated lambs), as well asin *Methanomicrobiales* populations (in the cannulated lambs) rather than reduction of carbohydrate digestibility [41]. These

results are supported by our previous short-term *in vitro* studies investigating BAC from two sources (*Sanguisorba officinalis* and CAL), where there were no negative effects on *in vitro* parameters, including DM degradability [13].

The utilization of forages in ruminants requires the coordinatedactivity of bacteria (such as *F. succinogenes, R. albus, R. flavefaciens*, and *B. fibrisolvens*), protozoa, and fungi for proper digestion[5, 46, 47]. Digestibility is also affected by the number and composition of rumen microorganisms. Supplementing the diet with 200 g/day of CAL had a positive effect on the total protozoa population in growing lambs (Experiment 3). The effect was more pronounced for a few particular bacterial species (namely *R. albus, M. elsdenii, B. proteoclasticus*, and *B. fibrisolvens*) than for the total bacteria. In comparison with tannins, BAC of CAL origin exerted either less negative impact on the rumen environment, or else their concentration was more beneficial on the ruminal microorganisms. This is supported by De Nardi et al. [48], who reported an increase in the number of several ruminal bacteria in heifers due to supplementation of flavonoids and essential oils.

Neither digestibility nor total VFA concentration was affected in the present study, although alterations in individual VFAs, such as propionic acid, were noted. The high proportion of propionate was not linked with the abundance of *S. bovis*, the amylolytic bacteria responsible for production of this VFA. The possibility cannot be however excluded that other amylolytic bacteria, not investigated in this study, were not affected. Additionally, higher concentration of propionic acid and lower acetic to propionic ratio can reflect in decreased hydrogen accessible to methanogens[49]. Considering the direct reduction in methanogen count caused by BAC of CAL origin, such a pathway has rather limited importance for mitigation of methanogenesis in this study.

Another aspect of this experiment that is worthy of attention is the changes observed in nitrogen metabolizing bacteria population (*Prevotella* spp., *B. proteoclasticus*, and *B. fibrisolvens)*. CAL is a feed rich in total protein (214 g/kg DM), and is thus the perfect substrate for N metabolism in the rumen. We observed an increase in ruminal ammonia in response to CAL supplementation. It is known that tannins form complexes with proteins, reducing the ammonia level in the rumen fluid. In the present study, protein degradation was not likely limited by CAL. Besides, the higher protein content may influence the ammonia level.

The *in vivo* experiments had higher ruminal pH values. Our observation corroborated the result ofBalcells et al. [50], who observedhigher pH after supplementing dairy cow diet with flavonoids.The extent of rumen fermentation reflected by the total VFA suggests that CAL supplementation did not affect this variable (Experiment 2 and Exp.3). Microbial activity is affected by the properties of the substrate, and higher starch levels in the diet usually reduce the pH of the rumen fluid [51, 52]. The diets in the present study were rich in concentrate (400 g/day) and we expected a reduction in pH. According to Balcells et al. [50], flavonoid supplementation may improve rumen fermentation and reduce the incidence of rumen acidosis. Such activity of some flavonoids may be partially explained by the increased number of lactate-consuming microorganisms (e.g., *M. elsdenii*). Our studies also confirmed this activity of CAL flavonoids by demonstrating increased pH values in the lamb rumen. It should be realized that the CAL contained three types of BAC with distinct activities: flavonoids, diterpenes and phenolic acidsbut the mechanism described above is typical for flavonoids. Another mechanism that may stabilize the pH is the fluctuations of protozoa community. According to Hartinger et al. [53], protozoa incorporate starch granules that are not metabolized to organic acids quickly. Thus, protozoa protect the rumen from large drops in pH, and support stable fermentation conditions. The effect of CAL on rumen protozoa observed in the present study was ambiguous. The *Entodiniomorpha*population significantly increased (Table 11), whereas that of *Holotricha* diminished consistently (Tables 9, 11). Similar trends were described in our short-term study [13], so we can assume that this mode of CAL action is stable for a longer period.

The FA proportion of the rumen fluid was another parameter affected by supplementation with CAL. We noted a positive alteration in the ruminal PUFA proportion in the growing lambs, which can mainly be attributed to the increase in C18:3 *cis*-9 *cis*-12 *cis*-15 FAs in the rumen fluid and meat (Table 12). The CAL polyphenols likely increased the bypass of UFA. Although the majority of bacterial communities involved in BH increased in number, BH of C18 UFA into stearic acid (C18:0) was lower due to CAL supplementation. A similar result was reported by Vasta et al. [5], who, regardless of the type of polyphenol supplementation, observed a reduction in ruminal C18:0 and an increase in C18:1 *trans*-11 (VA) and C18:2 *cis*-9 *trans*-11 (CLA) isomers. Vasta et al.[5] also pointed out a range of actions of polyphenols on bacteria involved in the BH process. The stability of the main BH isomers and the distinct responses of two bacteria species involved in BH process (*B. fibrisolvens* and *B.proteoclasticus*) observed in our study can also demonstrate some other properties of polyphenols. The reduction of BH by CAL despite greater populations of BH bacteria may suggest that CAL may reduce the content of free FA, substrates to BH process due to decreased lipolysis [4]. Besides, a reduction in the numbers of several bacteria species involved in BH, such as *Neisseria weaverii*, *R. amylophilus*, and other unclassified bacteria related to the *Lachnospiraceae*and *Pasteurellaceae*families was observed [54]. In this study, the potential restriction on BH may be supported by the fact that the rumen and meat displayed similar alterations in the FA profile, which likely suggests that the rumen was the locus of the main changes.

Transcript analysis included a panel of five genes of known function in FA metabolism in ruminant muscles [55]. Three of the genes (*FASN, SCD*, and *ELOVL5*) control the *de novo* synthesis and elongation of FA,whereas the *LPL* and *FADS1* genesare involved in FA transport. However, that considering the complexity of the entire gene expression process and the great variation seen in transcript lifespans, any conclusions must

be drawn with caution. Meat from lambs fed the CAL diet was characterized by a significant reduction in the mRNA content of four genes (*FASN, SCD*, *LPL*, and *FADS1*),whereas no changes were observed for the *ELOVL5* transcript. Alterations in the transcript expression of genes regulating lipid metabolism was not reflected in the profile of FA controlled by those genes. For example, the reduced mRNA level of the *SCD* gene was not accompanied by a lower level of C18:1 *cis*-9. According to Garnsworthy et al. [56], the level of C18:1 *cis*-9 in the fat of ruminant products is highly dependent upon the *SCD* gene controlling*de novo* FA synthesis. Besides, the lack of difference in C18:1 *cis*-9 concentration in response to CAL supplementation may suggest that the synthesis of endogenous FA was unaffected. On the other hand, the reduced transcript levels of the *LPL* gene may be associated with the decreased biosynthesis of MUFA. The lack of alteration in the mRNA content of the *ELOVL5* gene regulating FA elongation and the increased n-3 concentration (mainly LNA) in meat may reflect n-3 metabolism in the rumen, rather than that in muscles. Considering the reduced transcript content of another two genes—*FADS1* and *FASN*—this may be linked to the inhibition of the initial stages of the BH process.Pewan et al. [57]suggested that the FASN protein complex controls *de novo* biosynthesis of long-chain FA and affects FA deposition in meat, adipose tissue, and milk. The published evidence on the correlations between n-3 PUFA profile, the activity of lipogenic genes (such as *FASN*), and meat quality, however, is very limited [57].Nevertheless, the n-3 PUFA profile of the meat of lambs fed CAL was improved, which suggests that changes in the FA profile had already occurred in the rumen, leading to more PUFA being available to the tissue. Higher n-3 PUFA levels in meat are beneficial to human health and support cardiovascular, retinal, and brain functions [58, 59]. Positive changes in the FA profile of meat from the experimental lambs increased its quality. However, higher content of PUFAs could decrease the meat shelf life due to a rapid oxidization of FAs ensuing from two or more double bonds in their structure [60].

The oxidative process can shorten the shelf life of fresh meat and negatively affect its consumption by the formation of off-flavors and discoloration [61]. The sensor attributes, likes juiciness and taste, are generally associated with the consumers' preferences[62]. In this study, we observed improved meat characteristics such as juiciness or taste. The better meat sensory quality combined with improved indices of thrombogenicity (TI) and atherogenicity (AI) indexes may help to encourage consumers to select quality meat in the future. Tannin-containing diets sometimes reduce [63] or unchanged [64] tenderness and juiciness of meat depending upon the doses. In this study, juiciness and flavor of lamb meat increased, which would be advantageous from consumer perspectives. It has been suggested that phenolic compounds may increase the activity calpains and accelerating the degradation of myofibrillar proteins during the *postmortem* conditioning of carcass, leading to increased juiciness and tenderness of meat [65, 66]. A study with *Andrographis paniculata*, an annual herb rich in polyphenols,reported that this herb increased the juiciness and tenderness of the LT muscle of goats [66]. Previous study reported that dietary polyphenols from *Mimosa* condensed tannins had no influence on meat lightness and colors of lambs, however the meat metmyoglobin was reduced [67]. Polyphenol inclusion in ruminants' diet might cause different effects on the meat color. Many studies suggested that dietary polyphenols might cause a lighter color of meat [68, 69]. Priolo et al. [70] evaluated the effect of tannin in lambs and stated that the *longissimus* muscle was lighter (lower L*) in tannin-fed group. We assume that the lower lightness and greater redness of color in the present study were influenced by the CAL mineral content, which was not determined in this study. Damanik et al. [12] reported that the CAL contains high concentrationof iron (Fe). Garg et al. [71] stated that the inclusion of tannin in the animal diets do not hamper the utilization of iron for the hemoglobin synthesis. Thus, lambs fed CAL diet might influence its carcass color and lightness due to the high level of Fe affecting the hemoglobin synthesis in the meat. Moreover, polyphenols with high antioxidant activities may reduce the oxidation of myoglobin leading to increased the redness of meat [61]. Increased redness of meat has also been reported due to feeding of polyphenol-rich plants [66, 72].

Conclusions

Polyphenols of CAL origin reduce CH_4 production, which is associated with diminished *Archaea* communities. Polyphenols of CAL modulate the final products of ruminal fermentation and lowers the A/P ratio, with no alteration in ruminal digestibility. CAL also affect ruminal bacteria involved in fermentation and BH by elevating LNA concentration, and ultimately increase deposition of n-3 PUFA. Dietary CAL also improves the meat quality. *Coleus amboinicus* Lour. herb can be used in ruminant nutrition to reduce greenhouse gas emissions and improve meat quality without any negative effects on ruminal fermentation and growth performance.

Abbreviations

ADG: Average daily gain; AI: Atherogenicity; aNDF: Ash free NDF; A/P ratio: Acetate/Propionate ratio; BAC: Biologically active compounds; BH: Biohydrogenation; BW: Body weight; CAL: *Coleus amboinicus* Lour.; CH_4: Methane emission; CO_2: Carbon dioxide emission; CLA: Conjugated linoleic acid;CP: Crude protein; DΔ: Desaturation Δ at -*n*; DAPI: 4,6-diamidino-2-phenylindole; DI: Desaturation index; DM: Dry matter; DMD: Dry matter digestibility; DMI: Dry matter intake; DNA: Deoxyribonucleic acids; EE: Ether extract; EI: Elongase index;ELOVL5: Fatty acid elongase 5; FA: Fatty acids;FADS1: Fatty acid desaturase 1; FASN: Fatty acid synthase;FISH: Fluorescence in situ hybridization; IVDMD: *in vitro* dry matter digestibility; LA: *α*-Linoleic acids; LCFA: Long chain fatty acids; LT muscle: *Longissimus thoracis*muscle;LNA: *α*-Linolenic acids; LPL: Lipoprotein lipase; MCFA: Medium chain fatty acids; mRNA: messenger-RNA;MUFA: Monounsaturated fatty acids; NDFD: Neutral detergent fibre digestibility; NH_3: Ammonia; OM: Organic matter;PBS: Phosphate-buffered saline;PUFA: Polyunsaturated fatty acids;qPCR: Quantitative PCR; RA: rumenic acid; RTA: Relative transcript abundance (ΔΔ CT); RUSITEC: Rumen simulation technique;SCD: Stearoyl-CoAdesaturase; SFA:

Saturated fatty acids;SPE: Solid Phase Extraction;TI: Thrombogenicity; UFA: Unsaturated fatty acids; VA: Vaccenic acid; VFA: Volatile fatty acids;

Declarations

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Authors' contributions

YRY designed the study protocol, provided laboratory analysis, helped with statistical analyses, interpreted the data and wrote first version of the manuscript. MSz interpreted the data and improved the manuscript. DL interpreted the data, reviewed and improved the manuscript. SS provided laboratory analysis of plants' phenolic acid, flavonoid, and diterpenoid contents. PK performed the laboratory analysis. AKP interpreted the data, revised the content substantially and reviewed the manuscript. ZV provided laboratory analysis of rumen fluid.DLi provided laboratory analysis of meat. MV interpreted the data and collected references for discussion. AC designed the study protocol, interpreted the data, wrote the manuscript, and finally reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The experimental datasets of the present study can be obtained from the corresponding author on reasonable request.

Ethics approval of experiments to animal

All experimental procedures were performed in accordance with the guidelines of the National Ethical Commission for Animal Research (Ministry of Science and Higher Education, Poland). The study was approved by the Local Ethical Commission of Poznan University of Life Sciences(license permit no. 35/2019).

Competing interests

The authors declare that they have no competing interests.

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Figures

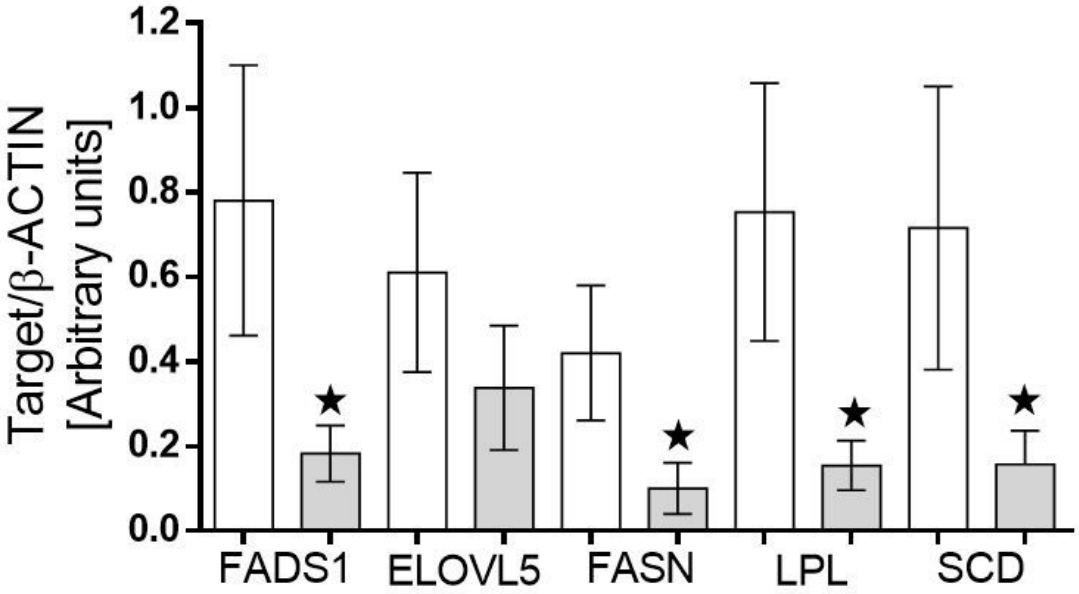

Figure 1

Comparison on LT gene expressions of lambs receiving CON and CAL diet Legends: (CON; white colour); CAL diet (CAL; grey colour); Gene expressions are fatty acid desaturase 1 (FADS1), fatty acid elongase 5 (ELOVL5), fatty acid synthase (FASN), lipoprotein lipase (LPL), andstearoyl-CoAdesaturase (SCD)in the longissimus thoracis muscle of growing lambs; The symbol * indicates significant difference between treatments.

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