



CAPRIC AND LAURIC ACID MIXTURE DECREASED RUMEN METHANE PRODUCTION, WHILE COMBINATION WITH NITRATE HAD NO FURTHER BENEFIT IN METHANE REDUCTION*

Miroslav Joch^{1,2*}, Mariana Vadroňová^{1,2}, Miroslav Čěšpiva³, Petra Zabloudivá³, Alena Výborná¹, Yvona Tyrolová¹, Václav Kudrna¹, Denisa Tichá^{1,2}, Vladimír Plachý², Zuzana Hroncová²

¹Department of Nutrition and Feeding of Farm Animals, Institute of Animal Science, Přátelství 815, 104 00 Prague, Czech Republic

²Department of Microbiology, Nutrition and Dietetics, Faculty of Agrobiological Sciences, Food and Natural Resources, Czech University of Life Sciences, Kamýcká 129, 165 00 Prague, Czech Republic

³Research Institute of Agricultural Engineering, Drnovská 507, 161 01 Prague, Czech Republic

*Corresponding author: joch.miroslav@vuzv.cz

Abstract

This study aimed to evaluate the methane-reducing potential of individual and combined treatments of low levels of nitrate (NIT) and a mixture of capric/lauric acid (CL) in dairy cows. Both *in vitro* and *in vivo* experiments were conducted. In the *in vitro* experiment, the anti-methanogenic effects of NIT (1.825 mmol/l) and CL (250 mg/l; capric acid, 125 mg/l + lauric acid, 125 mg/l) were evaluated in a 2 × 2 factorial design using consecutive batch incubations with rumen fluid. The NIT and CL reduced ($P < 0.05$) methane production by 9.2% and by 21.3%, respectively. However, combining NIT with CL did not show ($P > 0.05$) any benefit in methane reduction compared to the use of CL alone. In the *in vivo* experiment, eight multiparous dry Holstein cows were fed two diets in a crossover design for two 21-day periods (14 days of adaptation and 7 days of sampling). The treatments were: 1) silage-based basal diet + 100 g stearic acid per cow/d (CON) and 2) silage-based basal diet + 50 g capric acid + 50 g lauric acid per cow/d (CL). Gas emissions were measured using open-circuit respiration chambers. Methane production (g/d) was reduced (by 11.5%; $P = 0.012$) when the diet was supplemented with CL. However, supplementation with CL increased ruminal ammonia-N concentration (by 28.5%; $P = 0.015$) and gas ammonia production (g/d; by 37.2%; $P = 0.005$). Ruminal pH, protozoa count, and total and individual volatile fatty acid concentrations (VFA) did not differ ($P > 0.05$) between the treatments. Treatment did not affect the intake and apparent total tract digestibility ($P > 0.05$). In conclusion, our results suggest that low CL levels have anti-methanogenic potential. However, low levels of CL may compromise nitrogen use efficiency.

Key words: rumen fermentation, methane, medium-chain fatty acid, nitrate

Increasing atmospheric concentrations of methane continue to be a global concern (Saunio et al., 2020). About 30% of total anthropogenic methane emissions come from enteric fermentation (mostly from the rumen), together with manure management (Jackson et al., 2020). Apart from its environmental impact, rumen methane production is recognized as a fermentative inefficiency, resulting in the loss of up to 12% of the gross energy consumed by the ruminants (Johnson and Johnson, 1995). Although significant scientific advances have been made and numerous compounds and substances have been evaluated as additives for reducing rumen methane production, there have not yet been breakthroughs that could be universally applied in ruminant feeding practice (Patra et al., 2017; Beauchemin et al., 2020). Most anti-methanogenic additives supplemented at sufficiently high concentrations to achieve substantial methane inhibition exert adverse effects on digestion or are toxic to animals (Patra et al., 2017). In contrast, combined treatment us-

ing additives with a complementary mode of action may overcome these adverse effects because the required concentrations can be reduced (Kim and Rhee, 2016; Patra et al., 2017).

Nitrate is effective in reducing methane production in the rumen (Latham et al., 2016). The competition for dihydrogen is thought to be responsible for the observed inhibition of methane production (Latham et al., 2016). Nitrate provides an alternative mechanism for maintaining the low partial pressures of dihydrogen because its reduction to ammonia is energetically more favorable than the reduction of carbon dioxide to methane (Latham et al., 2016). In addition, the direct toxicity of nitrite, an intermediate of nitrate reduction, to methanogens has been suggested (Zhou et al., 2012). Many studies have shown that medium-chain fatty acids (MCFA) can reduce methane production by ruminants (Yanza et al., 2021). In pure MCFA, lauric acid (C12:0) has been reported to have a particularly high potential for suppressing rumi-

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nal methanogenesis. Direct inhibition of rumen methanogens and antiprotozoal properties has been suggested as the mode of anti-methanogenic effect in lauric acid (Dohme et al., 2001; Soliva et al., 2003). However, the methane reduction inefficiency of pure lauric acid has also been reported (Hristov et al., 2009, 2011). Capric acid (C10:0) decreases methane production during *in vitro* rumen fermentation (Goel et al., 2009). It seems that the anti-methanogenic effects of capric acid are rather due to its antibacterial (Marounek et al., 2002), antifungal (Chadeganipour and Haims, 2001), or antiprotozoal (Dohme et al., 2001) properties than due to direct inhibition of methanogens (Dohme et al., 2001).

Although nitrate, capric acid, and lauric acid supplemented individually demonstrated the ability to inhibit methanogenesis, they may exert adverse effects at concentrations high enough to achieve effective methane inhibition. The addition of nitrate may result in the accumulation of nitrite in the rumen. Nitrite converts hemoglobin to methemoglobin, resulting in toxicosis known as methemoglobinemia (Latham et al., 2016). Concurrently, the increasing dietary concentration of MCFA linearly decreases feed digestibility (Yanza et al., 2021). Moreover, animal intake may be reduced by supplementation with higher doses of MCFA (Hristov et al., 2011). The inefficiency of methane inhibitors at low doses and the adverse effects at high doses may be addressed using a combination of inhibitors with complementary mechanisms of action to decrease methane production additively or synergistically (Patra et al., 2017).

We hypothesized that: (1) individual supplementation of both nitrate and capric/lauric acid mixture would reduce methane production; and (2) combined supplementation of nitrate and capric/lauric acid mixture would have an additive or synergistic effect that leads to lower methane production than when they are used individually. Therefore, our objectives in this study were to evaluate the effects of individual and combined supplementation of two additives (nitrate and a capric/lauric acid mixture) on rumen methane production. The novelty of the present study lies in evaluating the combined effects of additives with a complementary mode of anti-methanogenic action.

Material and methods

Cows were handled following the applicable national and European legislation (Directive 2010/63/EU, on the protection of animals used for scientific purposes; European Union 2010). All animal procedures were approved by the Animal Ethics Committee of the Institute of Animal Science (Prague, Czech Republic).

In vitro experiment

Experimental design and treatments

In vitro consecutive batch culture technique (Theodorou et al., 1987) was used to identify whether the

combination of nitrate and capric/lauric acid mixture has additive/synergistic effects that lead to less rumen methane production than when they are used individually. A 2×2 factorial design was used for the experiment with the following treatments: 1) control (CON; no additive); 2) nitrate (NIT; 1.825 mmol/l of nitrate); 3) capric/lauric acid mixture (CL; 250 mg/l; 125 mg/l of capric acid + 125 mg/l of lauric acid); and 4) nitrate + capric/lauric acid mixture (NIT+CL; 1.825 mmol/l + 250 mg/l, respectively). Sodium nitrate was used as a nitrate source. All tested compounds were commercial products with purity $\geq 98\%$ (Merck, Prague, Czech Republic). The control and treatment bottles were incubated in triplicate during each series. Five series were performed for a total of 120 h of incubation.

Animals, diets, and substrate

Fresh rumen contents were manually collected through a rumen cannula (internal diameter 10 cm) from different sites in the rumen 3 h after morning feeding. Two mid-lactation Holstein cows (647 $\pm$ 9 kg body weight, 33 $\pm$ 8 kg milk/d) were used as donors. The cows were fed a total mixed ration consisting (DM basis) of corn silage (328 g/kg), alfalfa silage (117 g/kg), legume-cereal silage (48 g/kg), ensiled corn cobs with leaves (LKS; 41 g/kg), brewers grain (31 g/kg), wheat straw (20 g/kg), liquid supplement (50:50 mixture of molasses:glycerol; 92 g/kg), and concentrate mixture (323 g/kg). The diet was provided twice daily (04:00 and 16:00 h) *ad libitum*.

After collection, rumen contents were immediately transported to the laboratory in thermos flasks. The interval between sampling and further sample processing was 20–30 min. In the laboratory, rumen contents were strained under continuous CO₂ flushing through a stainless-steel sieve (250 μ m; Retsch, Haan, Germany) to obtain rumen fluid. Rumen fluids from the two donor cows were pooled in equal proportions.

The experimental substrate consisted of (DM basis): corn silage (300 g/kg), alfalfa silage (300 g/kg), and barley (400 g/kg). The dried feeds were ground to pass through a 1-mm screen. The chemical composition of the substrate (DM basis) was as follows: organic matter (OM), 951 g/kg; crude protein (CP), 154 g/kg; crude fat, 25 g/kg; starch, 306 g/kg; neutral detergent fiber (NDF), 354 g/kg; and acid detergent fiber (ADF), 193 g/kg.

In vitro incubation

In the first incubation series, the dried ground substrate (100 mg) was weighed in sterile CO₂-flushed serum bottles (60 ml volume). To each serum bottle, 10 mL of culture fluid was dispensed using a manual pipette (Acura 835, 1–10 mL, Socorex, Switzerland). The culture fluid was prepared by mixing the composite fresh rumen fluid with medium (1:9, v/v). The medium was prepared according to Menke et al. (1979), with the modification that the medium contained 30% of clarified rumen fluid (autoclaved at 121°C, 15 min; centrifuged at 27,000 $\times$ g, 20 min). The culture fluid was im-

mediately gassed with CO₂ and maintained at 39°C for 10 min before filling up the bottles. Nitrate and capric/lauric acid mixture were supplied to bottles via the addition of 100 µl filter-sterilized distilled water solution and 100 µl ethanol stock solution, respectively, to reach the desired concentration in 10 ml of culture fluid. Equivalent amounts of water and ethanol were added to the control and blank bottles to compensate for any possible effects of the solvents on fermentation. The initial headspace gas phase in all the incubations was CO₂. All serum bottles were sealed and placed in a temperature-controlled water bath (SW 22; Julabo, Germany) at 39°C and a shaking frequency of 90 rpm. After an initial 24 h incubation (39°C), 1 mL of each culture was transferred to a new series of bottles with fresh medium (9 ml each), substrate (100 mg), with or without (control) respective test compounds, and was incubated for another 24 h. Overall, five serial cultures were performed for a total of 120 h incubation.

Sampling and chemical analyses

The total volume of produced gas was estimated from the gas pressure of the headspace using Boyle's law equation (Romero-Pérez and Beauchemin, 2018). Headspace gas pressure was measured using a manometer (Traceable, Fisher Scientific, Pittsburgh, USA) after each 24 h of incubation. The headspace gas was then sampled by displacement into a tube (5 mL) pre-filled with distilled water via the insertion of a 23-gauge needle through the stopper of the bottle. Methane concentration in the headspace gas was measured using gas chromatography (Joch et al., 2019). Briefly, 100 µl of gas was injected. The injector temperature was 43°C, the column was 33°C (isothermal), and the flame ionization detector was 180°C. Ultra-high purity H₂ was used as a carrier gas.

The concentration of VFA in the cultures was analyzed by comparison with known standards (Supelco Volatile Fatty Acid Standard Mix; Merck, Missouri, USA) using a gas chromatograph (GC, 82 F, Labio, Czech Republic) equipped with a flame ionization detector and Stabilwax capillary column (15 m × 0.53 mm ID × 0.5 µm; Restek, USA), with H₂ as the carrier gas (Joch et al., 2022).

In vivo experiment

Based on the results of the *in vitro* experiment, a mixture of capric/lauric acid alone was selected for *in vivo* testing because a combination of capric/lauric acid mixture with nitrate had no further benefits in terms of methane reduction. To better manifest the effects on rumen fermentation and methane production, the concentration of capric/lauric acid mixture from the *in vitro* experiment was nearly tripled in the *in vivo* experiment (from 250 mg/l to 694 mg/l), considering an approximate rumen content volume of an adult dry Holstein cow of 60 l, and an average rumen dilution rate of 0.10 h⁻¹ (Rode et al., 1985; Reynolds et al., 2004).

Table 2. Ingredients (%) and chemical composition (g/kg of DM unless otherwise stated) of basal diet fed to dry cows

Item	Amount
Ingredients (%)	
corn silage	17.4
legume-cereal silage	21.7
alfalfa silage	32.5
wheat straw	20.0
brewers grain	6.6
vitamin and mineral premix [†]	1.8
Chemical composition (g/kg of DM)	
dry matter (g/kg as fed)	399.2
crude protein	160.6
crude fat	14.3
organic matter	904.8
starch	66.5
NDF	454.2
ADF	266.5
NFC [‡]	275.7
gross energy (MJ/kg of DM)	18.51

[†]Vitamin and mineral premix, declared contents (per kg): 180 g of Ca, 20 g of P, 75 g of Na, 80 g of Mg, 51 g of S, 114 g of Cl, 300,000 IU of vitamin A, 100,000 IU of vitamin D, 4,000 mg of vitamin E, 24 mg of vitamin B₁, 12 mg of vitamin B₂, 6 mg of vitamin B₆, 60 µg of vitamin B₁₂, 360 mg of niacinamide, 24 mg of d-calcium pantothenate, 5,000 µm of biotin, 12,000 mg of choline chloride, 4,000 mg of Zn, 3,000 mg of Mn, 900 mg of Cu, 93 mg of I, 12 mg of Co, 18 mg of Se, 12 × 10¹⁰ CFU of *Saccharomyces cerevisiae* MUCL 39885 (Mipro Pren 250, Sano-Moderne Tierernährung GmbH, Loiching, Germany).

[‡]NFC (non-fiber carbohydrates) = 1000 – (NDF g/kg + crude protein g/kg + crude fat g/kg + ash g/kg).

Animals, experimental design, and treatments

Eight multiparous dry Holstein cows (means ± standard deviation): 3 ± 1 of parity and 699 ± 78 kg of BW were used in a crossover design with two periods of 21 days. Each experimental period comprised 14 days of treatment adaptation followed by 7 days of data and sample collection. Cows were stratified by parity and weight and randomly assigned to one of the two airflow-controlled chambers constructed to fit four cows each (four free-stall beds to simulate typical free-stall housing on dairy farms). The chambers were then randomly assigned to one of the two dietary treatments. Treatments were: 1) control (CON), silage-based basal diet (Table 2) + 100 g of stearic acid (purity ≥ 95%, Merck, Prague, Czech Republic) per cow/d; 2) capric/lauric acid mixture (CL), silage-based basal diet + 50 g of capric acid (purity ≥ 99.5%, Merck, Prague, Czech Republic) + 50 g of lauric acid (purity ≥ 98%, Merck, Prague, Czech Republic) per cow/d. Stearic acid was used as a control treatment as it has been reported to have minimal effects on rumen fermentation (Hristov et al., 2004, 2011). While stearic and lauric acids were supplied in the form of powder/flakes easily miscible with feed ration, capric acid was supplied in solid form with a melting point of approximately 32°C. Therefore, we decided to feed the daily amount of the respective fatty acids in the form of pel-

lets. Fatty acids were pelleted with wheat bran (100 g of fatty acids and 900 g of wheat bran per kg of pellets as fed). The daily amount of pellets (1 kg) was divided into two equal meals individually administered at 05:00 h and 17:00 h before the morning and evening administration of the basal diet, respectively. The basal diet was fed *ad libitum* as TMR (Table 2). The cows were fed a basal diet three times a day (05:00, 11:00, and 17:00 h).

Gas emission measurements

The gas emissions were measured using two open-circuit respiration chambers. Each chamber ($11.2 \times 5.4 \times 3.0$ m; 181 m³ of air space) contained four free-stall beds elevated from the floor by 10 cm. The cows were bedded on rubber mats with wheat straw as bedding and had free access to fresh water. Each chamber had a separate inlet and outlet equipped with a fan. The airflow from the outlet was set higher than the inlet to ensure a negative pressure in the chambers minimizing errors through air leaking out. An average outlet flow of 828 m³/h resulted in an air exchange in the chambers of 4.6 times per hour. The emissions of methane, ammonia, nitrous oxide, and carbon dioxide were recorded during the last 7 days of each period. Air samples were sequentially drawn from samplers placed in the inlets and outlets. The intervals between samplings from the same sampler were 5 min and 36 s. The concentrations of individual gases were analyzed using a photoacoustic multi-gas monitor (Innova Model 1412i; AirTech Instruments, Ballerup, Denmark). The production of gases was calculated as the product of the concentration in the outlet air corrected for the concentration in the inlet air and volumetric airflow.

Rumen fermentation characteristics and protozoa counting

Rumen fluid samples (250 ml) were collected from all cows on day 18 of each period (3 h after morning feeding) using an oral-rumen tube and a vacuum pump. The tube head was inserted at a depth of about 180 cm to reach the central rumen. Samples were immediately placed on ice and transported to the laboratory, where subsamples of rumen fluid were analyzed for pH (WTW portable pH meter with a WTW SenTix 41 electrode; WTW, Weilheim, Germany). For the analysis of VFA and ammonia-N, rumen liquid samples (0.5 ml) were acidified with 10 µl of 9 M H₂SO₄. VFA concentrations were determined using the same method as that used for the *in vitro* experiment. Ammonia-N levels were analyzed using the phenol-hypochlorite reaction according to Weatherburn (1967), with absorbance measured at 625 nm (Libra S22, Biochrom, Cambridge, UK).

Protozoa were counted manually according to the method described by Dehority (1993). Briefly, a 10-ml aliquot of rumen fluid was transferred into a sterile 50-ml Falcon tube by pipetting with cut-off tips, and 10 ml of a 50% formalin solution (i.e., 18.5% formaldehyde) was added. After mixing, three replicate 1-ml aliquots of the formalized sample were pipetted (using wide-bore

tips; Axygen, Union City, CA, USA) into test tubes, and two drops of brilliant green dye were added to each test tube. The solution was then mixed and allowed to stand overnight. The next day, 9 ml of 30% glycerol solution was added. After mixing, 1 ml of the sample was pipetted (wide-bore tips) to a Sedgewick Rafter counting chamber (S52 glass; Pyser-SGI, Edenbridge, Kent, UK). Protozoa were counted under a microscope (magnification 100×) by counting 2×20 grids in each of the three replicates. The number of protozoa was calculated from the average of the three counts.

Dry matter intake and apparent total tract digestibility

Feed intake was recorded on a chamber basis by weighing the diet offered and refused over the last 7 days of each period. Samples of experimental diets and refusals were collected daily and stored at -20°C. These samples were thawed and composited by a chamber within each period. Samples of individual components of the basal diet were collected once during the last 7 days of each period. All mentioned samples were dried at 60°C (forced-air oven) for 48 h; ground to pass a 1-mm mill screen (Retsch, GmbH, Haan, Germany); and analyzed for DM, ash, CP, NDF, ADF, starch, and gross energy (GE) as described previously (Joch et al., 2022).

Spot fecal samples were collected from each cow on days 17 (before morning feeding) and 18 (6 h after morning feeding) of each experimental period. Fecal samples were dried in a forced-air oven (at 60°C for 72 h) and ground to pass a 1-mm sieve. Dried and ground fecal samples were composited (on an equal DM weight basis) by cow and period and analyzed for DM, ash, CP, NDF, ADF, and GE as described for feed analysis. Acid-insoluble ash (AIA) was used as an internal marker to estimate apparent total tract digestibility (Van Keulen and Young, 1977). Apparent total tract digestibility was calculated based on the concentrations of individual nutrients and AIA in the feed and fecal samples according to the following equation: Nutrient digestibility (%) = $[1 - (AD/AF) \times (NF/ND)]$, where AD and AF are the AIA concentrations in the diet and feces, respectively; and ND and NF are the concentrations of individual nutrients in the diet and feces, respectively.

Statistical analysis

Data from *in vitro* consecutive batch incubations were analyzed as a completely randomized design with a 2×2 factorial arrangement of treatments (Kaps and Lamberson, 2004) using the GLM procedure of SAS (SAS Enterprise Guide version 7.1, SAS Institute Inc., Cary, NC, USA), according to:

$$Y_{ijk} = \mu + N_i + C_j + (NC)_{ij} + e_{ijk}$$

where Y_{ijk} is the dependent variable, μ is the overall mean, N_j is the effect of nitrate (i.e., 0 mmol/l versus 1.825 mmol/l), C_i is the effect of a mixture of capric/

lauric acid (i.e., 0 mg/l versus 250 mg/l), $(NC)_{ij}$ is the effect of the interaction between nitrate and a mixture of capric/lauric acids, and e_{ijk} is the residual error. Before analysis, data from five days of incubation were averaged per bottle. Where a significant interaction was observed, an analysis of simple effects was completed using the SLICE option of SAS to compare the effects of nitrate inclusion within each capric/lauric acid mixture level, and vice versa. The treatment means were compared using Tukey's multiple comparison test. Differences were considered statistically significant at $P < 0.05$.

Data from the *in vivo* experiment were analyzed as a crossover design by analysis of variance using the MIXED procedure of SAS. The chamber was the experimental unit for feed intake and gas emissions. Nevertheless, for the convenience of data interpretation, all results were expressed as per cow per day. Feed intake and gas emissions were analyzed as repeated measures using the following model:

$$Y_{ijkl} = \mu + T_i + D_j + (TD)_{ij} + P_k + C_l + e_{ijkl}$$

where Y_{ijkl} is the dependent variable, μ is the overall mean, T_i is the treatment effect, D_j is the effect of the day, P_k is the period effect, C_l is the chamber effect, and e_{ijkl} is the residual error. All terms were fixed effects, except for the chamber, which was a random effect. The first-order autoregressive covariance structure provided the best fit, according to the Akaike information criterion.

The ruminal (pH, VFA, ammonia-N, and protozoa counts) and digestibility data were analyzed using the PROC MIXED procedure in SAS with a cow as the experimental unit according to the following model:

$$Y_{ijk} = \mu + T_i + P_j + W(C)_{kl} + e_{ijk}$$

where Y_{ijk} is the dependent variable, μ is the overall mean, T_i is the treatment effect, P_j is the period effect, $W(C)_{kl}$ is the cow-within-chamber effect, and e_{ijk} is the residual error. The cow-within-chamber effect was random, and all others were fixed. Differences were considered statistically significant at $P < 0.05$.

Results

In vitro experiment

In the *in vitro* experiment, we focused on the principal effects of NIT, CL, and their combination on rumen fermentation. Therefore, only three main parameters (total gas production (TGP), methane, and VFA) and their respective proportions are presented (Table 1).

The TGP decreased ($P < 0.05$) only with the combination of NIT and CL compared to that of CON. Compared to that of CON, supplementation with CL alone or in

combination with NIT decreased ($P < 0.05$) VFA concentrations by 11.0% and 7.7%, respectively.

All tested compounds and their combinations decreased ($P < 0.05$) methane production parameters. However, there was a strong interaction between NIT and CL supplementation for all methane parameters, that is, methane production per DMI ($P < 0.001$), relative methane production per TGP ($P < 0.001$), and methane production per VFA ($P = 0.003$). When NIT supplementation was compared within each level of CL supplementation (using simple effects analysis), NIT reduced methane production per DMI in the absence of CL ($P < 0.001$), whereas no such effect was observed in the presence of CL ($P = 0.154$). Relative methane production per TGP was reduced by NIT in both the absence ($P < 0.001$) and presence ($P = 0.007$) of CL. Methane production per VFA was reduced by NIT in the absence of CL ($P < 0.001$); however, this effect of NIT disappeared in the presence of CL ($P = 0.169$). Conversely, the anti-methanogenic activity of CL remained significant regardless of the NIT level.

In summary, CL had stronger anti-methanogenic potential than NIT. Moreover, combining CL with NIT did not show any benefit in methane reduction compared to the use of CL alone.

In vivo experiment

Methane production (g/d), compared to that of the CON diet, was reduced ($P = 0.012$) by 11.5% when cows were fed a diet supplemented with CL (Table 3). Similarly, methane yield (g/kg DMI) was reduced ($P < 0.001$) by 11.8% when cows were fed the CL diet. The loss of energy as methane (% GEI) was decreased ($P < 0.001$) by 11.8% (i.e., 1.1 percentage points) when cows were fed the CL diet compared to that of the CON diet. Ammonia production (g/d; $P = 0.005$) and ammonia yield (g/kg DMI; $P < 0.001$) increased by 37.2 and 37.9%, respectively, when cows were fed the CL diet compared to when they were fed the CON diet. Neither the production nor yield of nitrous oxide and carbon dioxide was affected by diet ($P > 0.05$; Table 3).

The rumen concentrations of total ($P = 0.999$) and individual VFA ($P > 0.05$) were not affected by the diet, suggesting that neither the extent nor type of carbohydrate fermentation in the rumen was altered (Table 4). Similarly, the ratio of acetate:propionate in the rumen fluid ($P = 0.331$) and rumen pH ($P = 0.152$) remained unaffected by the diet. Concentrations of ammonia-N in rumen fluid were higher ($P = 0.015$) in cows fed the CL diet than in cows fed the CON diet. The total protozoa count did not differ ($P = 0.746$) between the diets.

The dry matter intake (DMI; $P = 0.855$), OM intake ($P = 0.847$), NDF intake ($P = 0.751$), and GE intake ($P = 0.791$) were not affected by diet (Table 3).

The total tract apparent digestibility of DM, CP, crude fat, OM, gross energy, NDF, and ADF was not altered ($P > 0.05$) by the diet (Table 5).

Table 1. Effects of nitrate (NIT), capric/lauric acid mixture (CL), and their combination (NIT+CL) on *in vitro* rumen fermentation parameters

Item	Treatment [†]				SEM	P-value		
	CON	NIT	CL	NIT+CL		NIT	CL	NIT × CL
TGP [‡] (mL/g DMi [§])	222.0 a	221.4 a	219.1 ab	216.2 b	0.76	0.048	<0.001	0.158
Methane (mL/g DMi)	17.4 a	15.8 b	13.7 c	14.0 c	0.46	0.001	<0.001	<0.001
Methane (% TGP)	7.8 a	7.1 b	6.2 d	6.5 c	0.19	<0.001	<0.001	<0.001
Methane (mmol/mol VFA)	76.0 a	67.6 b	66.7 b	65.0 b	1.33	<0.001	<0.001	0.003
VFA (mmol/L)	91.1 a	89.5 a	80.1 c	84.1 b	1.35	0.008	<0.001	0.172

[†]Treatment: CON (control; no additive); NIT (1.825 mmol/l of nitrate); CL (125 mg/l of capric acid + 125 mg/l of lauric acid); NIT+CL (1.825 mmol/l of nitrate + 125 mg/l of capric acid + 125 mg/l of lauric acid).

[‡]TGP, total gas production.

[§]DMi, dry matter incubated.

a, b, c, d – treatment means with different letters within rows differ significantly (P<0.05).

Table 3. Feed intake and gas emissions of dairy cows fed control (CON) or capric/lauric acid mixture (CL) supplemented diet

Item	Treatment [†]		SEM	P-value
	CON	CL		
Intake				
DMI (kg/d)	12.6	12.5	0.24	0.855
OM intake (kg/d)	11.5	11.5	0.22	0.847
NDF intake (kg/d)	5.6	5.5	0.10	0.751
GEI (MJ/d) [‡]	237	235	4.3	0.791
Gases				
methane (g/d)	382.5	338.6	6.30	0.012
methane (g/kg DMI)	31.4	27.7	0.69	<0.001
methane (% GEI) [§]	9.3	8.2	0.20	<0.001
ammonia (g/d)	35.5	48.7	1.88	0.005
ammonia (g/kg DMI)	2.9	4.0	0.16	<0.001
nitrous oxide (g/d)	3.3	3.5	0.07	0.212
nitrous oxide (g/kg DMI)	0.27	0.29	0.009	0.168
carbon dioxide (g/d)	12979	12382	338.8	0.631
carbon dioxide (g/kg DMI)	1051	1018	24.1	0.207

[†]Treatment: CON, basal diet + 100 g of stearic acid per cow/d; CL, basal diet + 50 g capric acid + 50 g lauric acid per cow/d.

[‡]GEI, gross energy intake.

[§]The amount of energy loss as methane, proportionally to GEI (% GEI), was calculated by accounting for the energy content of methane of 55.65 MJ/kg (Eggleston et al., 2006).

SEM, standard error of the mean.

Table 4. Rumen fermentation parameters of dry cows fed control (CON) or capric/lauric acid mixture (CL) supplemented diet

Item	Treatment [†]		SEM	P-value
	CON	CL		
pH	6.99	6.91	0.045	0.152
Protozoa count (× 10 ⁴ /mL)	3.95	3.76	0.433	0.746
Total VFA (mM)	88.4	88.5	3.44	0.999
Acetate (mM)	65.0	67.2	2.41	0.605
Propionate (mM)	13.8	12.4	0.82	0.431
Butyrate (mM)	6.67	5.90	0.393	0.253
Valerate (mM)	0.84	0.79	0.063	0.751
Isobutyrate (mM)	1.02	1.00	0.037	0.849
Isovalerate (mM)	1.15	1.15	0.064	0.973
BCVFA [‡] (mM)	2.16	2.15	0.099	0.964
Acetate:propionate	4.97	5.48	0.242	0.331
Ammonia-N (mg/dl)	12.2	15.7	0.79	0.015

[†]Treatment: CON, basal diet + 100 g of stearic acid per cow/d; CL, basal diet + 50 g capric acid + 50 g lauric acid per cow/d.

[‡]BCVFA, branched-chain VFA (isobutyrate + isovalerate).

SEM, standard error of the mean.

Table 5. Apparent total tract digestibility (%) of nutrients in dry cows fed control (CON) or capric/lauric acid mixture (CL) supplemented diet

Item	Treatment [†]		SEM	P-value
	CON	CL		
Dry matter	63.7	64.0	1.58	0.883
Crude protein	68.1	68.4	1.45	0.800
Crude fat	69.8	73.1	1.86	0.282
Organic matter	65.9	66.0	1.52	0.964
Gross energy	63.8	64.4	1.66	0.687
NDF	53.9	55.3	2.53	0.473
ADF	49.7	52.3	3.16	0.378

[†]Treatment: CON, basal diet + 100 g of stearic acid per cow/d; CL, basal diet + 50 g capric acid + 50 g lauric acid per cow/d.

SEM, standard error of the mean.

Discussion

Individual supplementation with both NIT and CL reduced methane production *in vitro*, confirming our first hypothesis of the individual anti-methanogenic effects of NIT and CL. The anti-methanogenic effect of CL was validated *in vivo*. In contrast, our data provide little support for the second hypothesis that combined supplementation with NIT and CL has an additive or synergistic anti-methanogenic effect.

In vitro experiment

In the current study, the addition of NIT (1.825 mmol/l) reduced methane production by 9.2% compared with that of the CON. The inhibitory effect of NIT on rumen methanogenesis has been well documented both *in vitro* (Almeida et al., 2022) and *in vivo* (Latham et al., 2016). Our results are consistent with these findings. In addition, we found that methanogenesis was significantly reduced by a very low concentration of NIT (1.825 mmol/l). Previously, *in vitro* batch incubation studies evaluated much higher concentrations of NIT. For example, NIT at 5 mmol/l and 12 mmol/l decreased methane production by 30% (Patra and Yu, 2014) and over 70% (Zhou et al., 2012), respectively. Our results are consistent with this trend in which methane production is reduced by 5–6% for each mmol of NIT. The NIT potential to inhibit methane production is attributed to two mechanisms: 1) the energetically more favorable use of hydrogen in the reduction of NIT to ammonia (compared to the reduction of carbon dioxide to methane) and 2) the direct toxicity of nitrite, an intermediate of nitrate reduction, to methanogens (van Zijderveld et al., 2011; Zhou et al., 2012).

CL (250 mg/l) reduced methane production by 21.3%, as compared to that by the CON. Although no previous study has evaluated the combined effects of capric and lauric acids on methanogenesis, these acids are individually capable of inhibiting rumen methane, particularly at higher concentrations (Božić et al., 2009; Goel et al.,

2009; Yanza et al., 2021). Goel et al. (2009) reported that capric acid decreased rumen methane production *in vitro* by 55 and 88% at 400 and 600 mg/l, respectively, while reducing VFA production. However, in the same study, a lower concentration (200 mg/l) of capric acid had no such effect (Goel et al., 2009). No methane inhibition effect of capric acid at higher concentration (~600 mg/l) was also reported *in vitro* using RUSITEC (Dohme et al., 2001). In lauric acid, high concentrations (600–5,000 mg/l) effectively decreased *in vitro* methane production, but reduced fiber digestion (Dohme et al., 2001) and VFA production (Božić et al., 2009). In general, high levels of capric acid and lauric acid considerably reduce methane production *in vitro*. However, at least part of the mitigation effect is due to the reduced overall fermentation. In keeping with this, the decreased VFA concentrations in our experiment suggest that overall fermentation can be reduced even by a relatively low concentration of CL.

The primary aim of the *in vitro* experiment in our study was to investigate whether the methane inhibition effects of NIT (1.825 mmol/l) and CL (250 mg/l) are additive/synergistic. Theoretically, for fully additive effects of NIT+CL, methane reduction should reach over 30% (9.2% + 21.3%). However, methane was reduced only by about 19.5% and did not reach a greater extent than with CL alone. The non-additive effects of NIT and CL were confirmed by the significant interactions between these two compounds. Moreover, analysis of simple effects showed that under combined supplementation with NIT and CL, the anti-methanogenic effect is driven primarily by CL. Several reasons may explain the loss of NIT activity in the presence of CL. First, CL may inhibit microbes responsible for the dissimilatory reduction of nitrate to ammonia, resulting in lower electron consumption via this route. Second, CL reduced overall fermentation, as suggested by the lower levels of VFA. Therefore, the hydrogen availability for the reduction of NIT may also be reduced. Finally, CL might directly inhibit rumen methanogens (Dohme et al., 2001; Soliva et al., 2003), leaving less room for the direct anti-methanogenic effects of nitrite. Similar results for the combination of NIT with lipid compounds were observed by Patra and Yu (2015 a) using an 18-day consecutive *in vitro* incubation. The authors reported that a combination of NIT (5 mmol/l) and garlic oil (250 mg/l) reduced methane production to a similar extent as when these compounds were supplemented alone (Patra and Yu, 2015 a). Interestingly, the same authors reported additive effects of NIT (5 mmol/l) and garlic oil (270 mg/l) during short-term incubation (24 h) (Patra and Yu, 2015 b). Therefore, it seems that the additive/synergistic effects may be lost over time, likely due to the adaptation of the rumen microbiota.

In vivo experiment

Based on the results obtained from the *in vitro* experiment and following the principle of parsimony, the CL mixture alone was selected to be tested *in vivo* because the combination of CL with NIT did not show any fur-

ther benefit in rumen methane reduction. The translation of *in vitro* results to *in vivo* doses is challenging in rumen methane mitigation studies. At the same concentration (on a dilution basis, mg/l), methane inhibitors are usually more effective *in vitro* than *in vivo* (Mohammed et al., 2004; Martínez-Fernández et al., 2013), but more pronounced *in vivo* effects have also been reported (Castro-Montoya et al., 2015). The discrepancy between *in vitro* and *in vivo* effects of methane inhibitors may be explained by several factors: (1) decreased microbial densities and changed microbial communities as rumen content is usually strained before *in vitro* inoculation; (2) differences in the degradation rate of the compounds *in vitro* and *in vivo*; (3) lower distribution homogeneity across the rumen compartments particularly for the compounds with low water solubility (Soto et al., 2012; Martínez-Fernández et al., 2013), and (4) tendency of some compounds (e.g., fatty acids) to concentrate on feed particles in the rumen (Harfoot and Hazlewood, 1997). Consequently, we expected a lower anti-methanogenic activity of CL *in vivo*. Therefore, the concentration *in vivo* was almost triple that used in the *in vitro* experiment (from 250 mg/l to 694 mg/l), considering an approximate rumen content volume of an adult dry Holstein cow of 60 l and an average rumen dilution rate of 0.10 h^{-1} (Rode et al., 1985; Reynolds et al., 2004). This increase resulted in a daily dose of 100 g CL (0.8% of diet DM), which is still one of the lowest daily doses used in *in vivo* studies of MCFA (Yanza et al., 2021).

Supplying the diet with CL at 100 g/d reduced methane production (g/d) by 11.5% compared to that with the CON diet (with stearic acid at 100 g/d). This result agrees with a study in beef heifers, where supplementation of a diet with a low dose (125 g/d) of coconut oil (containing lauric and capric acids) reduced methane by 13% (Jordan et al., 2006). Conversely, feeding lauric acid alone at a higher dose (240 g/d) did not reduce methane production in dairy cows (Hristov et al., 2009, 2011). These results suggest that capric acid may support the methane inhibitory effect of lauric acid. Previously, another MCFA, myristic acid, was reported to have a supportive effect on the methane-reducing potential of lauric acid (Soliva et al., 2003). Several factors may explain the anti-methanogenic effect of CL: (1) direct inhibition or change of activity in rumen methanogenic archaea (Dohme et al., 2001; Zhou et al., 2013); (2) antiprotozoal properties (Hristov et al., 2011); (3) general inhibition of rumen fermentation due to antibacterial effects (Desbois and Smith, 2010); and (4) coating of feed particles with MCFA (i.e., fat). In the current study, the direct effect of CL on rumen methanogenic archaea was most likely because the number of protozoa and VFA remained unchanged compared to that in the CON. Moreover, the coating effect of fat should have been similar between the treatments as in the CON diet was the same amount of fat (CL was replaced by stearic acid). It is also likely that stearic acid in the CON diet compensated for other nonspecific effects of fat on rumen fermentation, corroborating the specific action of CL against rumen methanogens.

orating the specific action of CL against rumen methanogens.

The unaffected protozoa count by CL in our study contrasts with the general assumption of the potent antiprotozoal activity of MCFA. The lack of an antiprotozoal effect might be due to a low dose of CL. Hristov et al. (2009) reported that a pulse dose of lauric acid (240 g/d) and coconut oil (530 g/d) to the rumen reduced protozoal counts by over 80% and 83%, respectively. In contrast, in our study, the cows consumed only 100 g/d of MCFA (50 g capric acid and 50 g lauric acid). Moreover, the effectiveness of CL as a defaunating agent may have been reduced by the mode of application, as CL was divided into two equal meals in our study. It has been shown that lauric acid administered directly into the rumen at a single pulse dose is more effective than lauric acid applied in the TMR (Hristov et al., 2009). Our findings support the previous report that the methane-suppressing effect of MCFA might be independent of their protozoa-inhibiting effect (Machmüller, 2006).

Supplementation of CL increased the concentration of rumen ammonia-N, which is in contrast with studies showing that the inclusion of capric and lauric acid is usually (Hristov et al., 2004, 2011), but not always (Yabuuchi et al., 2006; Hristov et al., 2009), associated with reduced proteolysis. The reduction in proteolysis and, consequently, ammonia levels, is attributed to the reduction in protozoal digestion of bacterial proteins (Hristov et al., 2004). Conceivably, in our study, the level of CL was too low to inhibit protozoa and possibly ammonia-producing bacteria. However, the CL level might have been high enough to inhibit rumen bacteria that primarily use ammonia as a substrate for protein synthesis, resulting in the accumulation of ammonia in the rumen (Correddu et al., 2015). In line with the increased rumen ammonia, the emission of gaseous ammonia in cows fed the CL diet also increased. The CL diet increased gas ammonia production (g/d) and ammonia yield (g/kg DMI) by 37.2 and 37.9%, respectively, compared with that of the CON diet. Presumably, higher levels of rumen ammonia result in a higher rate of ammonia detoxification by conversion to urea in the liver and higher levels of excreted urea in the urine (Schwab and Broderick, 2017). This may result in a higher rate of ammonia production, which, in addition to other factors, depends on the urea concentration in the urine (Monteny and Erisman, 1998). Taken together, CL supplementation seemed to decrease nitrogen use efficiency.

In the present study, the intake of DM and other nutrients was not affected by CL supplementation. Variable results have been obtained in previous studies. The effect on DMI is likely dose-dependent because higher doses of MCFA ($\geq 1\%$ of DM) decreased DMI (Hristov et al., 2011; Hollmann et al., 2012; Faciola and Broderick, 2013), whereas lower doses did not have such an effect (Burdick et al., 2022). Our results agree with this assumption since we did not observe an effect of CL on DMI at a low supplementation level of CL at 0.8% of

DM. The decrease in DMI observed in previous studies could be because of reduced diet digestibility (Hollmann et al., 2012; Faciola and Broderick, 2013; Burdick et al., 2022). In our study, the lack of CL effects on nutrient digestibility was consistent with the lack of CL effects on DMI.

Conclusion

Our *in vitro* results suggest that even low concentrations of nitrate and capric/lauric acid mixture have significant anti-methanogenic potential. However, the combination of nitrate and capric/lauric acid mixture did not show any benefit in methane reduction compared to the use of capric/lauric acid mixture alone. Our *in vivo* results confirmed the anti-methanogenic potential of capric/lauric acid mixture. However, supplementation of this mixture resulted in increased rumen ammonia-N concentrations and gas emissions of ammonia, suggesting a compromised nitrogen use efficiency.

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