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Effect of combining the methanogenesis inhibitor 3-nitrooxypropanol and cottonseeds on methane emissions, feed intake, and milk production of grazing dairy cows

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ABSTRACT

No single enteric CH_4 mitigating strategy has been consistently effective or is readily applicable to ruminants in grassland systems. When CH_4 mitigating strategies are effective under grazing conditions, mitigation is mild to moderate at best. A study was conducted to evaluate the potential of combining two CH_4 mitigation strategies deemed feasible to apply in grazing dairy cows, the methanogenesis inhibitor 3-nitrooxypropanol additive (**3-NOP**) and cottonseed supplementation (**CTS**), seeking to enhance their individual CH_4 mitigating potential. Forty-eight dairy cows were evaluated in a continuous grazing study and supplemented with either a starch-based concentrate (**STA**) or one that contained cottonseeds (1.75 kg DM/d; CTS), and with either 19 g/d of 10% 3-NOP (Bovaer®) or the additive's carrier (placebo), in a 2 × 2 factorial arrangement of treatments. Treatments were supplied mixed with a concentrate supplement (5 kg/d as fed) and offered in two equal rations at milking. Methane emissions were measured on weeks 4 and 8 using the sulphur hexafluoride tracer gas technique over a 5-d period. The 3-NOP and CTS treatments tended to interact on absolute CH_4 such that 3-NOP decreased CH_4 by 13.4% with STA, but there was no mitigation with 3-NOP and CTS. Treatment interactions were also obtained for CH_4 yield, where 3-NOP tended to decrease CH_4 when supplied with STA, and tended to increase it with CTS. The increase in CH_4 yield with the CTS diet was driven by a numerical decrease in DM intake. Methane intensity was not affected by the 3-NOP or CTS treatments. Total volatile fatty acids in ruminal fluid were not affected by 3-NOP supplementation, but a reduction in acetate and an increase in propionate proportion occurred, resulting in decreased acetate: propionate. The 3-NOP additive decreased grass intake; however, energy–corrected milk yield and milk composition were largely unaffected. Milk urea increased with 3-NOP supplementation. Combining twice daily supplementation of 3-NOP and CTS did not enhance their CH_4 mitigation potential when fed to grazing dairy cows. The relatively low inhibition of CH_4 production by 3-NOP compared to studies with total mixed rations may result from the mode of delivery (pulse dosed twice daily) and time gap caused by experimental handling and moving of animals to pasture after 3-NOP supplementation in the milking parlour, which could have impaired the synchrony between the additive presence in the rumen and grass intake in paddocks.

Implications

No methane mitigating strategy is currently applicable to grazing dairy cows. We proposed combining twice-daily supplementation with 3-nitrooxypropanol and cottonseeds to enhance their effectiveness. We learned that 3-nitrooxypropanol tended to mildly decrease methane emissions with a starch-based concentrate, but not with cottonseeds. The additive's effectiveness was likely reduced due to the time gap between its administration in the milking parlour and the cows' grass ingestion in the paddocks, allowing 3-nitrooxypropanol metabolism in the rumen, and the higher lipid and fibre content of cottonseeds. Continuous delivery alternatives for 3-nitrooxypropanol into the rumen are crucial to boost effectiveness in grazing systems.

Introduction

In the last decade, several enteric CH_4 mitigation strategies for ruminants have been evaluated globally with varying degrees of

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success (see meta-analysis by Arndt et al., 2022; and review by Beauchemin et al., 2022). Most of the work with dairy cows has been carried out in intensive confined production systems with high genetic merit cows and total mixed rations (**TMR**) with relatively high concentrate input (i.e. about 30–50% DM). Less work has taken place on pasture-based systems with dairy cattle of lower production levels maintained mostly outdoors throughout the year and fed diets based on grazed grass, conserved forages, and lower concentrate supply levels. Since the 1950 's, only 15% of the published *in vivo* experimental work on CH_4 mitigation has been conducted under grazing conditions (Vargas et al., 2022). In addition, effective CH_4 mitigation strategies that are relevant and have been validated for pasture systems provide only mild to moderate mitigation, e.g. −17% CH_4 intensity with increasing feeding level, −13% CH_4 intensity with decreasing grass maturity and −12% absolute CH_4 when including tanniferous forages (Arndt et al., 2022). And, they often decrease CH_4 intensity but increase absolute CH_4 production. Two CH_4 abatement strategies that appear feasible to apply under grazing conditions and are closest to practical application by farmers are the CH_4 mitigating additive 3-nitrooxypropanol (**3-NOP**) and cottonseed supplementation (Arndt et al., 2022; Hristov et al., 2022).

The additive 3-NOP is a compound that targets the enzyme methyl coenzyme-M reductase, key for CH_4 formation in the rumen, inhibiting methanogenesis. Meta-analyses indicate that this compound is effective at decreasing the CH_4 yield of dairy cows compared to diets non-supplemented with 3-NOP in confined-total mixed ration-based systems (−38.8%; Dijkstra et al., 2018; −30.9%; Kebreab et al., 2023), and its efficacy is both dose- and diet-dependent (Dijkstra et al., 2018; Jayanegara et al., 2018; Kebreab et al., 2023). Almost all 3-NOP studies have been undertaken in intensive systems (tie stalls, free stalls or feedlots), with a few reporting CH_4 reductions with high forage diets fed to confined animals (Miller et al., 2023). In confinement systems, 3-NOP continuous supply to the rumen relies on the additive being well mixed with forages and concentrate in TMR and delivered continuously in every mouthful of feed, thus promoting a consistent fermentation and CH_4 mitigating pattern. In grazing systems, supplements are delivered usually once or twice daily, or even once every few days in more extensive systems where animal management is less frequent. Few studies with 3-NOP have been conducted providing the additive in a supplement that the animal may consume only once or twice a day. Reynolds et al. (2014) dosed cows fed TMR twice daily with 3-NOP administered through the rumen cannula, and Muetzel et al. (2019) dosed cows fed grass in confinement twice daily with 3-NOP administered mixed into a supplement. To our knowledge, no published studies at the time of writing have evaluated the use of 3-NOP under grazing conditions.

Supplementation of dietary lipids is an effective CH_4 mitigation strategy (Beauchemin et al., 2020; Arndt et al., 2022; Beauchemin et al., 2022) that acts through lowering the amount of fermentable organic matter in the rumen, having toxic effects on methanogens and protozoa, inhibiting H_2 producing bacteria, and by offering an alternative ruminal electron sink via biohydrogenation of unsaturated fatty acids (Beauchemin et al., 2009). In the grazing systems of southern Chile, cottonseed is a feed ingredient that is reasonably accessible to dairy farmers. Compared to whole linseed and rapeseed, cottonseed as a source of lipids was more effective in decreasing the CH_4 yield of dairy cows fed TMR (Muñoz et al., 2019) and grazed grass (Muñoz et al., 2021). Others have also reported moderate decreases in CH_4 yield and intensity with cottonseed (Grainger et al., 2008b; Grainger et al., 2008a; Grainger et al., 2010).

One alternative to increase the effectiveness of 3-NOP and cottonseed supplemented separately to mitigate CH_4 is to evaluate the effects of their combination and identify possible interactions between them – be those synergistic, antagonistic, or wholly additive (i.e., lack of interaction). Based on their differing mechanisms for inhibiting CH_4 production, we hypothesised that the combination of 3-NOP and cottonseed supplemented twice daily to grazing dairy cows would have an additive effect at decreasing CH_4 yield compared to those not given 3-NOP or cottonseed. The aim of this study was to evaluate the effects of combining the supplementation of the methanogenesis inhibitor 3-NOP and cottonseeds supplemented twice daily on CH_4 emissions, feed intake, and milk production of grazing dairy cows.

Material and methods

The experiment was conducted in spring between October 25th and December 19th of 2021, at INIA Remehue experimental farm (40°31′S; 73°03′W, Osorno, Chile).

Animals

Forty-eight Holstein Friesian cows, 12 primiparous and 36 multiparous, were used in this grazing study. Cows were blocked into 12 groups balanced according to parity (three groups of primiparous cows, and nine groups of multiparous cows with 2.7 ± 1.16 lactations; mean ± SD), lactation stage (73.0 ± 8.7 DIM), milk production (25.3 ± 3.2 kg) and BW (418 ± 29 kg for the primiparous and 467 ± 48 kg for the multiparous cows) at the beginning of the experiment, and within blocks, randomly allocated to one of four treatments.

Experimental design and diet

The study was conducted over 8 continuous weeks and had a 2 × 2 factorial arrangement of treatments under a completely randomised block design.

The concentrate treatments were as follows: (i) a starch−based supplement without cottonseed and with a 3-NOP placebo (**STA, PBO**); (ii) a STA supplement with 19 g/d of the 3-NOP-containing additive Bovaer® 10 (DSM Nutritional Products AG, Kaiseraugst, Switzerland) (**STA, 3-NOP**); (iii) a supplement with 1.75 kg DM/d of whole cottonseed with lint and PBO (**CTS, PBO**); and (iv) a CTS supplement with 3-NOP (**CTS, 3-NOP**). The 3-NOP additive contained a minimum of 10% (w/w) 3-NOP carried in propylene glycol and silicon dioxide (so cows were supplied 1.9 g 3-NOP daily) to achieve a 3-NOP target dose rate of 95 mg/kg DM calculated considering an estimated intake of 20 kg DM/cow d^{-1}. A relatively higher dose than the widely reported 3-NOP inclusion target of 60–80 mg/kg DM with TMR was chosen in this study to account for the additive being fed only twice daily instead of continuously. The 3-NOP dose targeted was also based on previous reports of decreased CH_4 from cattle that were fed high-forage diets (36.4% NDF; Vyas et al., 2016). The placebo contained the 3-NOP carrier at the same amount as the 3-NOP treatment.

Diets consisted of *ad libitum* grazed grass supplemented with 5 kg/d (as fed basis) of a concentrate according to treatments offered in two equal rations in the milking parlour (Table 1). Both the STA and CTS supplements were based on ground corn. In the STA supplement (which was used as a control for the CTS treatment), ground barley and rapeseed meal replaced cottonseeds to obtain at formulation isoenergetic (13.4 MJ estimated metabolisable energy/kg DM) and isonitrogenous (14% CP) supplements. Both STA and CTS concentrates included a vitamin and mineral premix. Prior to the beginning of the study, the four concentrate supplements were manufactured by homogenously mixing all ingredients (including the 3-NOP additive and the placebo) and stored in 25-kg feed bags. One exception to this was the inclusion

Table 1
Ingredient and chemical composition of pasture and treatment supplements[1] offered to grazing dairy cows.

	Pasture		STA concentrate		CTS concentrate	
Items	Week 4	Week 8	PBO	3-NOP	PBO	3-NOP
Ingredient, % DM						
Ground corn	–	–	56.4	56.4	55.7	55.7
Whole cottonseed with lint	–	–	–	–	39.1	39.1
Rapeseed meal	–	–	15.2	15.2	–	–
Ground barley	–	–	23.1	23.2	–	–
Placebo[2]	–	–	0.39	–	0.39	–
Formulated 3-NOP product[3]	–	–	–	0.39	–	0.39
Mineral and vitamin premix[4]	–	–	4.88	4.88	4.82	4.82
Chemical composition						
DM, %	14.1	16.8	88.7	88.9	90.7	90.7
Ash, % DM	11.5	10.9	7.05	7.22	6.22	5.60
CP, % DM	21.3	21.4	13.5	13.3	14.5	15.5
Starch, % DM	–	–	49.3	50.2	28.8	25.4
Ether extract, % DM	3.26	2.98	1.44	1.33	14.6	13.7
NDF, % DM	40.7	40.6	16.2	16.5	24.1	25.1
ADF, % DM	25.4	26.6	–	–	–	–
In vitro digestibility, % DM	81.4	76.6	89.4	89.3	74.6	72.6

[1] Concentrates without cottonseed (STA), with whole cottonseed with lint (CTS), and with a placebo (PBO) or the methanogenesis inhibitor 3-nitrooxypropanol (3-NOP). Mean values from composite samples of concentrates from weeks 1–4 and 6–8 of the study.
[2] 3-NOP carrier: SiO2 and propylene glycol.
[3] Bovaer® 10 (DSM Nutritional Products AG, Kaiseraugst, Switzerland) composition: 10% (w/w) 3-NOP carried in SiO2 and propylene glycol.
[4] Containing per kg: Ca 200 g, P 50 g, Mg 50 g, Cl 90 g, Na 60 g, S 20 g, Cu 1 200 mg, Mn 2 000 mg, Zn 5 500 mg, I 100 mg, inorganic Se 25 mg, Co 10 mg, organic Se 10 mg, vitamin A 100 000 IU, vitamin D 50 000 IU, vitamin E 1 000 IU (Vetersal Lechería Alta Producción, Veterquímica, Osorno, Chile).

of the whole cottonseed with lint in the CTS supplements which could not be homogenously mixed at the manufacturing plant and was added to rations daily using a cement mixer.

Cows grazed perennial ryegrass-based pasture as their main diet component. A grazing area of 16 ha divided into three main paddocks was used in the study. Each paddock was subdivided into four equal sub-paddocks and assigned to treatment groups which strip-grazed separately. Daily, following the morning milking, each group was offered a grazing area with a fresh strip of grass. Electric fences in front and behind each group limited access to the area grazed the previous day. There were 2.5 grazing rotations during the study i.e. cows went back to plots previously grazed. The distance covered by the cows between the milking parlour, management chute and furthest grazing paddock was 1.1 km. Cows had access to fresh clean water, supplied by mobile troughs in each grazing strip throughout the study. Cows were milked twice daily at 0630 and 1600 h.

Sampling and measurements

Methane measurements were done in weeks 4 and 8 of the study using the sulphur hexafluoride (**SF_6**) tracer gas technique as described by Muñoz et al. (2021). Permeation tubes were filled by National Institute of Water and Atmospheric Research (Auckland, New Zealand) in October 2020 with an initial SF_6 charge of 2733 ± 73 mg. The SF_6 release rates after 8 weeks of calibration at 39 °C were 4.2 ± 0.86 mg/d. The permeation tubes were dosed orally to cows in week 3 of the study. On the first measurement day, after the morning milking and treatment supplementation, the CH_4 measurement equipment was placed on each cow. Each sampling unit consisted of one evacuated V-shaped PVC canister positioned on the cows' neck connected to a sampling line continually sampling the eructed and exhaled gases from near to the cow's muzzle. The air flow to the canister was restricted by a crimped capillary tube fitted within an in-line air filter to a sampling rate of ~0.36 SCCM. In addition, four sampling units were placed outside the grazing area to measure ambient SF_6 and CH_4 gases. All canisters were replaced after 24 h, and this was repeated daily for 5 days. The samples in canisters were pressurised to above atmospheric pressure (around 120 kPa) with N_2, left to rest for at least 1 h, prior to sub-sampling into four evacuated chromatography vials. Daily samples were analysed in duplicate by gas chromatography with auto-sampler (Perkin Elmer Clarus 600; Waltham, MA, USA). Methane was separated using a Carboxen-1010 plot column, 15 m × 0.32-mm ID (Supelco, Sigma-Aldrich, St. Louis, MO, USA) and a flame ionisation detector operating at 250 °C. The SF_6 gas was separated using an Elite GS Molesieve column, 30 m × 0.53-mm ID × 50-µm film thickness (Perkin Elmer, Waltham, MA, USA) and an electron captor detector operating a 300 °C. Methane emissions were calculated as the product of the SF_6 permeation tube release rate and the ratio of CH_4:SF_6 concentration in samples, adjusted for background gas concentrations (Muñoz et al., 2015). Individual daily CH_4 emission was averaged over the 5 days of measurement, prior to statistical analysis.

The CH_4 conversion factor was calculated as: [Ym; CH_4 production (g/d) × 1.396 (g/L) × 0.03954 (MJ/L) ÷ gross energy intake (MJ/d) × 100)].

Grass intake was measured on the same 5 days as CH_4 production (weeks 4 and 8), using the double n-alkane technique (Mayes et al., 1986). Twice daily (after milking) cows were orally dosed using a balling gun with cellulose bungs containing the synthetic alkane C_{32}. An exception to this was that, on the first day of dosing, cows received two boluses in the morning and one bolus in the afternoon, and on the last day of dosing, cows received only the morning bolus. Cellulose bungs contained on average 414 ± 36.9 and 431 ± 40.3 mg of alkane C_{32} in periods 1 and 2, respectively. Alkane C_{32} was dosed for 12 d, 7 d to reach a steady state and 5 d for measurement where twice daily faeces collection was carried out after milking by faeces voiding or rectal grab. At the end of the collection period, the refrigerated faeces samples were combined per cow and period, dried at 60 °C for 48 h, and ground through a 1-mm sieve prior to alkane concentration determination. On faecal collection days, samples of the concentrate and grass per treatment were collected daily and combined per treatment, dried, and ground as described for faeces. N-alkanes C_{31}, C_{32}, C_{33} and C_{35} concentration in feed and faeces samples were determined using gas chromatography as by Martínez et al. (2017). Briefly, samples were ground to 10 µm, and the n-alkanes were extracted and quantified

using GC (GC 2010 Plus; Shimadzu, Kyoto, Japan) fitted to a flame ionisation detector using helium as the carrier gas. The column was a 30 m × 0.53 mm (i.d.) capillary RTX-1 (Restek®, Bellefonte, Pennsylvania, USA) with 1.50 μm film thickness. Grass intake was calculated according to equation by Mayes et al. (1986):

$$Grass\ intake\ (kg\ DM/d) = \frac{\frac{F_i}{F_j}(D_j + I_c \times C_j) - I_c \times C_j}{G_i - (\frac{F_i}{F_j} \times G_j)}$$

where G_i and F_i are the respective concentrations (mg/kg DM) of the alkane C_{33} in grass and faeces; G_j and F_j are the respective concentrations of the alkane C_{32} in grass and faeces; D_j is the amount of alkane j dosed (mg/d).

Using alkanes C_{31}, C_{33} and C_{35} as internal markers, apparent DM digestibility estimates were calculated by subtracting to one the alkane concentration in feed over the alkane concentration in faeces. Faecal alkane concentrations were corrected for incomplete recovery using the recovery rates published by Dillon (1993) which were 0.777 (C_{31}), 0.844 (C_{33}) and 0.891 (C_{35}). The digestibility estimates were obtained as an average of the three alkanes.

Twice daily individual milk yield was automatically recorded at each milking and cow BW was measured using an automatic scale when leaving the milking parlour. These individual measurements were averaged weekly per cow throughout the study (DeLaval Alpro MM15; DeLaval International, Tumba, Sweden). On weeks 1, 3, 4, 6 and 8 of the study, individual milk samples were collected at two consecutive milkings, combined for each cow according to yield, and stored refrigerated using bronopol as a preservative until analyses. Milk fat, milk protein, and milk urea concentrations were determined using IR spectroscopy (Milkoscan FT6000, Foss Electric, Hillerød, Denmark). To estimate energy−corrected milk (**ECM**) yield, milk production was adjusted for energy using the equation by Tyrrell and Reid (1965).

In week 6 of the study, rumen fluid was collected from all cows after the morning and evening milkings at 0830 and 1730 h using a stomach tube (Ruminator; profs-products.com (accessed on 06 March 2022)) and the first 150 mL discarded to avoid saliva contamination. The individual collection time was recorded. Rumen samples were filtered, and rumen pH was immediately measured (ExStik pH Meter, Extech Instruments, Boston, U.S.). Samples were frozen (−20 °C) until analysed. Volatile fatty acid concentration was determined from a 1 mL sample aliquot preserved with 0.2 mL of 20% (m/v) meta-phosphoric acid by gas chromatography (Perkin Elmer Clarus 580, PerkinElmer, Waltham, US) using a capillary column (Elite-FFAP; PerkinElmer, Shelton, CT, USA) and flame ionisation detector (Ungerfeld et al., 2019).

All pasture characteristics were assessed by measurements conducted in samples cut at 3 cm from the ground (Pérez-Prieto et al., 2012). Pregrazing herbage mass was measured per treatment twice weekly by cutting a 1 × 5 m strip of grass with a motor scythe in the area due to be grazed, collecting, and weighing all mown pasture, and determining its DM (subsample dried at 60 °C for 48 h). Four measurements of grass height were made per strip with a rising platemeter (F200 Farmworks, Feilding, New Zealand) before and after cutting. Pasture density was determined by dividing the pregrazing mass by the cutting depth. Estimates of forage mass pregrazing and postgrazing were carried out daily by multiplying the grass height (measured with a rising platemeter over 60 times crisscrossing the area) by the grass density. The daily grazing area of each treatment group was calculated based on the daily estimate of available forage adjusted according to an initial predetermined allowance of 22 kg DM/cow. As the study progressed, the grazing areas were adjusted to maintain a target postgrazing height of around 6 cm compressed herbage. Botanical composition was determined on measurement weeks through samples of pasture obtained by cutting at ground level 15 handfuls of grass and manually separating them into ryegrass, clover, dead material, and other species, on a DM basis (60 °C for 48 h).

Grass and concentrate samples for chemical analysis were collected daily in CH_4 measurement weeks, pooled weekly per treatment, dried at 60 °C for 48 h, and ground through a 1-mm sieve using a Wiley mill, prior to chemical analysis. The nutrient composition of the samples was analysed based on the following methods: nitrogen (**N**) analysed in duplicate and CP calculated as N × 6.25 (Method 2001.11; AOAC International, 2016); NDF expressed including residual ash as by Van Soest et al. (1991); ADF expressed including residual ash as by Goering and Van Soest (1970); ash as by Bateman (1970); ether extract determined using a Soxtec system HT6 following the manufacturer manual; starch based on AOAC Official Method 996.11; AOAC International (2016), and *in vitro* digestibly (**IVD**) as by Goering and Van Soest (1970). Gross energy was calculated based on chemical composition as per FAO (2003).

Statistical analyses

In week 4, one cow from the STA 3-NOP treatment died suddenly at pasture without apparent clinical signs and, as determined by necropsy, by causes unrelated to treatment, and was removed from the dataset. Also, a cow from the CTS PBO treatment developed and received treatment for mastitis in weeks 7 and 8 of the study and her records for those weeks were excluded. Animal data from the first 2 weeks of the experiment were omitted from the analysis as considered for adaptation to treatments.

Repeated measurements (CH_4, DM intake, DM digestibility, milk yield and composition, rumen fermentation and BW) were initially analysed using a mixed model including the main effects of supplementation with 3-NOP and cottonseed, their interaction, the main effects of week and parity (primiparous or multiparous) and their double and triple interactions with 3-NOP and cottonseed supplementation:

$$\begin{aligned} response = {} & overall\ mean + 3NOP + cottonseed + (3NOP \\ & \times cottonseed) + week + (week \times 3NOP) + (week \\ & \times cottonseed) + (week \times 3NOP \times cottonseed) + parity \\ & + (parity \times 3NOP) + (parity \times cottonseed) + (parity \\ & \times 3NOP \times cottonseed) + cow\ (random) + error \end{aligned}$$

For each treatment factor, n = 12. Because parity only interacted with 3-NOP or cottonseed on total volatile fatty acid (**VFA**) concentration ($P = 0.003$), parity was left in the model for rumen variables only, which also included time of sampling (am or pm), and the double and triple interactions between 3-NOP and cottonseed with time of the day and with parity:

$$\begin{aligned} response = {} & overall\ mean + 3NOP + cottonseed + time + parity \\ & + (3NOP \times cottonseed) + (3NOP \times time) + (3NOP \\ & \times parity) + (cottonseed \times time) + (cottonseed \times parity) \\ & + (3NOP \times cottonseed \times time) + (3NOP \times cottonseed \\ & \times parity) + cow\ (random) + error \end{aligned}$$

For all other animal response variables, a reduced model was used including the fixed main effects and double and triple interactions of 3-NOP, cottonseed, and week, and the random effect of cow:

$$\begin{aligned} response = {} & overall\ mean + 3NOP + cottonseed + week \\ & + double\ interactions + triple\ interaction \\ & + cow\ (random) + error \end{aligned}$$

Milk yield and BW in the 2-wk period immediately prior to the beginning of the trial were used as covariables of milk yield and BW change, respectively. The effect of treatments on pasture characteristics was determined including the fixed main effects and interactions of 3-NOP, cottonseed and measurement date. For all variables, significance for fixed effects and interactions was declared at $P < 0.05$ and tendencies at $0.05 \leq P < 0.10$. When interactions were significant or tendencies identified, the PBO and 3-NOP treatments were separately compared within the STA and CTS treatments.

Outliers were identified as those observations falling outside of the 99% distribution of studentised residuals. Outliers so identified were examined for obvious typos or mistakes, and non-physiological values. The DM intake of two cows in week 8 of the study was unusually low (6.75 kg/d) or high (30.3 kg/d), and those observations were discarded for the DM intake, digestibility, and CH_4 variables analyses. Because the reason for the unusually high or low DM intake was clearly related to DM intake estimation but not to those two animals themselves, which had a DM intake within the main range in week 4, those observations were retained for the analyses of all other response variables. For all other response variables where outliers with physiological values were identified, each analysis was run again excluding them. As the conclusions of the analysis did not change substantially when outliers within physiological values were removed, the results are presented including those observations. All data were analysed using JMP 17.2.1 (SAS Institute Inc., Cary, NC) software.

Results

Diet chemical composition

The chemical composition of pasture and concentrates are presented in Table 1. As the trial progressed from week 4 to week 8, the pasture had a similar composition of CP and NDF, increased DM and ADF, and decreased ether extract and IVD. Compared to STA, the CTS concentrate had higher proportions of CP, ether extract and NDF, and lower starch and IVD. The concentrates containing PBO or 3-NOP were very similar to each other. The calculated chemical composition of the experimental diets is presented in Table 2. In weeks 4 and 8 of the study, ingested diets had numerically similar CP contents and CTS diets had higher ether extract content. The NDF content of the CTS diet was numerically somewhat higher than the STA diet.

Feed intake and diet DM digestibility

Supplementing 3-NOP decreased pasture intake ($P = 0.003$) and total DM intake ($P = 0.003$), and increased diet concentrate proportion ($P = 0.005$; Table 3). Supplementing CTS had no effects on pasture intake, total DM intake or concentrate proportion (all $P \geq 0.39$). There was an interaction between 3-NOP and CTS on diet DM digestibility ($P < 0.001$), where with the CTS concentrate, 3-NOP decreased DM digestibility ($P < 0.001$), but not with the STA concentrate ($P = 0.11$).

Methane emissions

Treatments 3-NOP and CTS tended to interact on absolute CH_4 ($P = 0.050$), where with the STA concentrate, 3-NOP numerically decreased CH_4 by 13.4%, but not with the CTS concentrate (Table 4). There was an interaction between 3-NOP and CTS on CH_4 yield ($P = 0.011$), where 3-NOP tended to decrease CH_4 yield by 9.7% compared to PBO with the STA concentrate ($P = 0.067$) and tended to increase it by 10.2% with the CTS concentrate ($P = 0.066$). A similar response was observed on CH_4 as a proportion of ingested gross energy (Ym; interaction $P = 0.011$). For treatment interactions on CH_4 yield and Y_m, additional contrasts, conducted between CTS and STA within the PBO treatment, showed no effects of CTS supplementation ($P = 0.23$; contrast not shown). Supplementing 3-NOP had no effects on CH_4 intensity ($P = 0.20$) or CH_4 over ECM yield ($P = 0.56$). Supplementing CTS had no effects on CH_4 intensity ($P = 0.10$) or CH_4 expressed over ECM yield ($P = 0.40$).

Rumen fermentation

Rumen pH tended to be lower with 3-NOP than with PBO only in the afternoon sampling and with the STA supplement (interaction 3-NOP × CTS × Time, $P = 0.028$; Table 5). There were no other significant 3-way interactions between treatments and time on rumen fermentation. There tended to be an interaction between 3-NOP and CTS on isobutyrate molar percentage with 3-NOP numerically increasing isobutyrate with the CTS supplement, but not with the STA supplement ($P = 0.097$). There tended to be an interaction between 3-NOP and CTS on valerate molar percentage with 3-NOP numerically decreasing valerate with the STA supplement, and numerically increasing it with the CTS supplement ($P = 0.088$). Supplementing 3-NOP decreased acetate ($P < 0.001$) and increased propionate ($P = 0.002$) molar percentage, resulting in a decreased acetate to propionate ratio ($P < 0.001$). Molar percentage of butyrate ($P = 0.008$) and 2- and 3-methylbutyrate ($P = 0.005$) were increased by 3-NOP, and 3-NOP had no effects on total VFA concentration ($P = 0.21$) or caproate ($P = 0.34$) molar percentage. Supplementing CTS decreased total VFA concentration ($P < 0.001$), increased acetate ($P < 0.001$), and decreased propionate ($P = 0.015$), thus increasing the acetate to propionate ratio ($P = 0.002$). The CTS supplement had no effects on molar percentages of butyrate ($P = 0.11$), 2- and 3-methylbutyrate ($P = 0.30$) or caproate ($P = 0.14$).

Milk production, milk composition and performance

The 3-NOP and CTS treatments tended to interact on milk yield ($P = 0.078$) with 3-NOP numerically decreasing milk yield with the

Table 2
Chemical composition of the experimental diets ingested by dairy cows offered grazed grass supplemented with concentrates without cottonseed (STA) or with whole cottonseed with lint (CTS) on weeks 4 and 8 of the study[1,2].

	Week 4		Week 8	
Items	STA	CTS	STA	CTS
DM, %	35.3	38.3	37.8	39.2
CP, % DM	19.1	19.5	19.0	19.5
Ether extract, % DM	2.73	6.35	2.50	6.35
NDF, % DM	33.8	35.8	33.3	35.8
Ash, % DM	10.3	9.39	9.76	9.39

[1] Calculated as ingestion of nutrients in dietary components divided by DM intake of components.
[2] Placebo and 3-Nitrooxypropanol additives were assumed not to alter chemical composition of diets.

Table 3
Diet intake and digestibility of grazing dairy cows supplemented concentrates without cottonseed (STA), with whole cottonseed with lint (CTS), and with a placebo (PBO) or the methanogenesis inhibitor 3-nitrooxypropanol (3-NOP).

	STA		CTS			*P*-value[1,2]		
Items	PBO	3-NOP	PBO	3-NOP	SEM	INH	CNT	INH × CNT
Pasture intake, kg DM/d	11.1	10.3	11.5	9.87	0.37	0.003	0.95	0.26
Concentrate offered, kg DM/d	4.44	4.45	4.54	4.54	–	–	–	–
Total estimated intake, kg DM/d	15.5	14.8	16.0	14.4	0.37	0.003	0.84	0.25
Concentrate proportion, kg DM/kg DM	0.290	0.303	0.288	0.318	0.007	0.005	0.39	0.27
Diet DM digestibility	0.684[a]	0.715[a]	0.722[y]	0.654[x]	0.013	0.16	0.38	<0.001

Abbreviations: INH = CH_4 inhibitor effect; CNT = concentrate type effect; INH × CNT = CH_4 inhibitor and concentrate type interaction.
[1] Main effects of week for all variables: $P < 0.001$. CNT × week interaction for all variables: $P \geq 0.45$, except diet digestibility ($P < 0.001$). INH × week interaction for all variables: $P \leq 0.026$, except diet digestibility ($P = 0.41$). CNT × INH × week interaction for all variables: $P \geq 0.22$, except diet digestibility ($P = 0.004$).
[2] Diet DM digestibility contrast between PBO and 3-NOP within the STA concentrate ($P = 0.11$), and within the CTS concentrate ($P < 0.001$).
[a] Within the STA concentrate, values within a row with different superscripts differ ($P < 0.05$) due to INH × CNT interaction.
[x,y] Within the CTS concentrate, values within a row with different superscripts differ ($P < 0.05$) due to INH × CNT interaction.

Table 4
Methane emissions of grazing dairy cows supplemented concentrates without cottonseed (STA), with whole cottonseed with lint (CTS), and with a placebo (PBO) or the methanogenesis inhibitor 3-nitrooxypropanol (3-NOP).

	STA		CTS			*P*-value[1,2]		
Items	PBO	3-NOP	PBO	3-NOP	SEM	INH	CNT	INH × CNT
CH_4, g/d	319	276	307	307	10.7	0.054	0.39	0.050
CH_4 yield, g/kg DM intake	20.8[a]	18.8[a]	19.5[x]	21.5[x]	0.75	0.99	0.35	0.011
CH_4 intensity, g/kg milk yield	13.6	13.0	14.7	13.8	0.57	0.20	0.10	0.83
CH_4, g/kg energy-corrected milk	13.7	13.4	14.4	13.9	0.68	0.56	0.40	0.90
Y_M, MJ CH_4/100 MJ GE intake[3]	6.68[a]	6.04[a]	6.27[x]	6.92[x]	0.24	0.99	0.35	0.011

Abbreviations: INH = CH_4 inhibitor effect; CNT = concentrate type effect; INH × CNT = CH_4 inhibitor and concentrate type interaction; Ym = CH_4 conversion factor.
[1] Main effects of week for all variables: $P \leq 0.011$, except absolute CH_4 ($P = 0.43$). CNT × week interaction for all variables: $P \geq 0.63$, except for CH_4 intensity ($P = 0.074$) and CH_4 intensity of energy–corrected milk yield ($P = 0.074$). INH × week interaction for all variables: $P \geq 0.22$. CNT × INH × week interaction for all variables: $P \geq 0.27$.
[2] CH_4 yield contrast between PBO and 3-NOP within the STA concentrate ($P = 0.067$), and within the CTS concentrate ($P = 0.066$). YM contrast between PBO and 3-NOP within the STA concentrate ($P = 0.070$), and within the CTS concentrate ($P = 0.064$).
[3] Ym calculated as [CH_4 production (g/d) × 1.396 (g/L) × 0.03954 (MJ/L) ÷ gross energy intake (MJ/d) × 100)].
[a] Within the STA concentrate, values within a row with different superscripts differ ($P < 0.05$) due to INH × CNT interaction.
[x] Within the CTS concentrate, values within a row with different superscripts differ ($P < 0.05$) due to INH × CNT interaction.

Table 5
Rumen fermentation characteristics of grazing dairy cows supplemented concentrates without cottonseed (STA), with whole cottonseed with lint (CTS), and with a placebo (PBO) or the methanogenesis inhibitor 3-nitrooxypropanol (3-NOP).

	STA		CTS			*P*-value[1,2,3]			
Items	PBO	3-NOP	PBO	3-NOP	SEM	INH	CNT	INH × CNT	INH × CNT × time
Rumen pH									
a.m.	7.30[m]	7.27[m]	7.51[n]	7.29[n]	0.91	0.32	0.99	0.74	0.028
p.m.	7.12[o]	6.77[o]	6.85[p]	6.80[p]					
Total volatile fatty acids, mM	118	109	90.4	87.5	4.52	0.21	<0.001	0.52	0.20
Acetate, mol/100 mol	64.5	60.8	66.7	63.5	0.61	<0.001	<0.001	0.68	0.21
Propionate, mol/100 mol	15.2	17.0	13.7	15.7	0.53	0.002	0.015	0.82	0.59
Butyrate, mol/100 mol	16.9	19.0	16.8	17.5	0.47	0.008	0.11	0.15	0.10
Isobutyrate, mol/100 mol	0.89	0.87	0.85	0.95	0.034	0.25	0.52	0.097	0.92
2- and 3-methylbutyrate, mol/100 mol	0.97	1.09	0.96	1.23	0.062	0.005	0.30	0.25	0.52
Valerate, mol/100 mol	1.33	1.04	0.79	0.84	0.093	0.22	<0.001	0.088	0.13
Caproate, mol/100 mol	0.23	0.23	0.17	0.22	0.026	0.34	0.14	0.33	0.71
Acetate/Propionate, mM/mM	4.29	3.66	5.02	4.20	0.18	<0.001	0.002	0.60	0.58

Abbreviations: INH = CH_4 inhibitor effect; CNT = concentrate type effect; INH × CNT = CH_4 inhibitor and concentrate type interaction.
[1] Main effects of time (a.m. or p.m. sampling) for all variables: $P < 0.001$, except for pH ($P = 0.80$) and butyrate ($P = 0.55$). INH × time (a.m. or p.m. sampling) interaction for all variables: $P \geq 0.10$, except for 2- and 3-methylbutyrate ($P = 0.050$). CNT × time (a.m. or p.m. sampling) interaction for all variables: $P \geq 0.12$, except for pH ($P = 0.037$), total volatile fatty acids ($P = 0.076$), acetate ($P = 0.018$), caproate ($P = 0.014$) and acetate/propionate ($P = 0.016$).
[2] Main effects of parity (primiparous or multiparous) for all variables $P \geq 0.16$, except for butyrate ($P = 0.065$). INH × parity interaction for all variables: $P \geq 0.23$, except rumen pH ($P = 0.057$). CNT × parity interaction for all variables: $P \geq 0.10$, except for total VFA ($P = 0.003$). CNT × INH × parity interaction for all variables: $P \geq 0.24$, except for total VFA ($P = 0.078$).
[3] pH contrast between PBO and 3-NOP within the STA concentrate in a.m. sampling ($P = 0.90$), and in p.m. sampling ($P = 0.066$), and within the CTS concentrate in both a.m. and p.m. sampling ($P \geq 0.22$).
[m,n,o,p] Within concentrates at a set sampling time (a.m. or p.m.), values within a row with different superscripts differ ($P \leq 0.10$) due to INH × CNT × time interaction.

STA concentrate, but not with the CTS concentrate (Table 6). Supplementing 3-NOP had no effects on the yield of milk fat ($P = 0.30$), protein ($P = 0.70$) or ECM ($P = 0.36$), nor did it affect the concentration of milk fat ($P = 0.40$) or protein ($P = 0.46$). Supplementing 3-NOP increased milk urea by 6.6% ($P = 0.046$). BW change ($P = 0.35$) and milk somatic cell count ($P = 0.68$) were not

Table 6
Milk production and composition of grazing dairy cows supplemented concentrates without cottonseed (STA), with whole cottonseed with lint (CTS), and with a placebo (PBO) or the methanogenesis inhibitor 3-nitrooxypropanol (3-NOP).

	STA		CTS			*P* value[1,2]		
Items	PBO	3-NOP	PBO	3-NOP	SEM	INH	CNT	INH × CNT
Milk and component yield, kg/d								
Whole milk	23.3	21.9	21.7	22.1	0.52	0.37	0.18	0.078
Fat	0.93	0.89	0.92	0.88	0.040	0.30	0.80	0.87
Protein	0.84	0.80	0.79	0.81	0.026	0.70	0.35	0.27
Energy-corrected milk	24.2	23.1	23.4	23.0	0.82	0.36	0.58	0.62
Milk composition								
Fat, %	3.81	3.88	4.02	3.75	0.12	0.40	0.77	0.17
Protein, %	3.43	3.50	3.45	3.45	0.047	0.46	0.67	0.48
Milk urea, mg/L	341	357	320	350	11.3	0.046	0.22	0.54
BW change, kg/d	0.45	0.44	0.21	0.42	0.10	0.35	0.22	0.31
Milk Log SCC[5]	1.61	1.74	1.80	1.77	0.11	0.68	0.30	0.45

Abbreviations: INH = CH_4 inhibitor effect; CNT = concentrate type effect; INH × CNT = CH_4 inhibitor and concentrate type interaction; SCC = somatic cell count.
[1] Main effects of week for all variables: $P < 0.001$, except milk log SCC ($P = 0.025$). CNT × week interaction for all variables: $P \leq 0.053$, except milk log SCC ($P = 0.11$). INH × week interaction for all variables: $P \geq 0.27$, except milk protein %, milk urea and milk log SCC (all $P \leq 0.094$). CNT × INH × week interaction for all variables: $P \leq 0.095$, except milk fat, milk Log SCC, and milk protein yield (all $P \geq 0.17$).
[2] Milk yield contrast between PBO and 3-NOP within the STA concentrate ($P = 0.065$), and within the CTS concentrate ($P = 0.52$).

affected by 3-NOP. Supplementing CTS had no effects on the yield of milk fat ($P = 0.80$), protein ($P = 0.35$) or ECM ($P = 0.58$), nor did it affect the concentration of milk fat ($P = 0.77$), protein ($P = 0.67$) or urea ($P = 0.22$). The CTS concentrate had no effects on BW change ($P = 0.22$) or milk somatic cell count ($P = 0.30$).

Pasture characteristics

There was an interaction between 3-NOP and CTS on pasture allowance ($P = 0.002$) with 3-NOP increasing pasture allowance with the STA concentrate ($P = 0.019$) and decreasing it with the CTS concentrate ($P = 0.035$; Table 7). Supplementing 3-NOP had no effects on pasture pregrazing mass ($P = 0.19$), pregrazing height ($P = 0.68$), density ($P = 0.60$) or the grazing area offered to adjust for equal pasture availability ($P = 0.45$). Pasture postgrazing height was lower with 3-NOP ($P = 0.009$). Supplementing CTS had no effects on pasture pregrazing mass ($P = 0.55$), pregrazing height ($P = 0.19$), density ($P = 0.51$), grazing area offered ($P = 0.38$), and postgrazing height ($P = 0.43$).

Discussion

Effects on rumen fermentation and CH_4 emissions

In the present study, the interactions found between the 3-NOP and CTS treatments on CH_4 emissions disproved our hypothesis. That is, there were no additive effects on CH_4 mitigation between the two strategies evaluated when supplemented twice daily to grazing dairy cows. A previous study evaluated the combined effects of 3-NOP and lipids on CH_4 emission. The authors reported effective methanogenesis inhibition by both 3-NOP (32% decrease) and canola oil (25% decrease) on their own, and their combination resulted in additive (52% decrease) CH_4 emission mitigation (Zhang et al., 2021). In that study, there were no interactions between the strategies evaluated and both strategies were independently effective in decreasing CH_4. This contrasts with our study where the individual main effects of each strategy on CH_4 yield were not significant, and the mitigation strategies did not act independently. Recently, (Maigaard et al., 2024) reported decreases in CH_4 yield

Table 7
Pasture characteristics and botanical composition as affected by grazing dairy cows supplemented concentrates without cottonseed (STA), with whole cottonseed with lint (CTS), and with a placebo (PBO) or the methanogenesis inhibitor 3-nitrooxypropanol (3-NOP).

Items[3]	STA		CTS			*P* value[1,2]		
	PBO	3-NOP	PBO	3-NOP	SEM	INH	CNT	INH × CNT
Pregrazing mass, kg of DM/ha	1 994	2 316	1 995	2 119	158.7	0.19	0.55	0.54
Pregrazing height, cm	11.7	11.9	11.6	11.1	0.32	0.68	0.19	0.28
Density, kg DM/ha per cm	282	288	292	318	29.1	0.60	0.51	0.74
Area offered, m^2/cow per day	75.6	80.3	74.3	75.1	3.69	0.45	0.38	0.60
Pasture allowance4, kg DM/cow per day	17.2[b]	19.6[a]	18.3[x]	16.2[y]	0.71	0.87	0.11	0.002
Postgrazing height, cm	6.3	5.6	6.3	6.0	0.20	0.009	0.43	0.30
Botanical composition[4]								
Lolium perenne	0.69	0.56	0.64	0.64	–	–	–	–
Trifolium repens	0.11	0.15	0.13	0.14	–	–	–	–
Other species	0.02	0.05	0.03	0.03	–	–	–	–
Dead material	0.09	0.10	0.11	0.13	–	–	–	–

Abbreviations: INH = CH_4 inhibitor effect; CNT = concentrate type effect; INH × CNT = CH_4 inhibitor and concentrate type interaction.
[1] Main effects of week for all variables: $P < 0.001$, except pregrazing pasture mass ($P = 0.68$) and density ($P = 0.086$). CNT × week interaction for all variables: $P \geq 0.18$. INH × week interaction for all variables: $P \geq 0.59$. CNT × INH × week interaction for all variables: $P \geq 0.12$, except pregrazing mass ($P = 0.007$).
[2] Pasture allowance contrast between PBO and 3-NOP within the STA concentrate ($P = 0.019$), and within the CTS concentrate ($P = 0.035$).
[3] Measured > 3 cm.
[4] Mean values from 2 composite samples per treatment in CH_4 measurement weeks.
[a,b] Within the STA concentrate, values within a row with different superscripts differ ($P < 0.05$) due to INH × CNT interaction.
[x,y] Within the CTS concentrate, values within a row with different superscripts differ ($P < 0.05$) due to INH × CNT interaction.

of 6–7% with supplementation of cracked rapeseeds, 12–13% with nitrate and 18–23% with 3-NOP supplementation. Similarly to our study, their combinations did not enhance their individual effects (absence of additivity between strategies).

In our study, 3-NOP significantly increased propionate concentration with both supplements with no interaction and decreased the acetate−to−propionate molar ratio from 4.72 to 3.90 mM/mM. So, despite the mild inhibition caused by 3-NOP on CH_4 production, 3-NOP did act on ruminal VFA concentrations shifting fermentation from acetate towards propionate. This shift is consistent with the beef and all ruminant meta-regressions reported by Kim et al. (2020), but not entirely with the dairy cattle meta-regressions, where 3-NOP decreased acetate, but did not affect propionate concentrations. Mild CH_4 inhibition (10%), yet with noticeable reductions in the acetate−to−propionate ratio (6.5%), have been previously reported when 3-NOP was dosed twice daily through the rumen cannula (Reynolds et al., 2014). The authors speculated that greater inhibition was not observed due to the 3-NOP compound rapidly washing out of the rumen with liquid outflow.

The level of methanogenesis inhibition by 3-NOP observed with the STA supplement in our study is more modest than previously reported results (−13.4% absolute CH_4 and −9.7% CH_4 yield). Based on a meta-analysis of 14 experiments with a range of 3-NOP doses, supplemental methods, diet compositions, and animal species, 3-NOP consistently reduced CH_4 emissions (all three metrics) by > 30% (Kebreab et al., 2023). Also using meta-analyses, others (Dijkstra et al., 2018; Jayanegara et al., 2018; Kim et al., 2020; Almeida et al., 2021) have reported similar efficacy evidence to Kebreab et al (2023).

The lower effectiveness of 3-NOP to decrease CH_4 obtained in the present study cannot be explained by the dose rate used. Methane inhibition by 3-NOP has been reported to be dose dependant i.e., increasing with increased 3-NOP dose (Kim et al., 2020; Kebreab et al., 2023). A range of 3-NOP doses have been reported in the literature (40–340 mg/kg DM), but a practical dose of 60 mg 3-NOP/kg DM for dairy cows has been recommended (Hegarty et al., 2021). The 3-NOP dose rate of our study (130 mg/kg DM intake) was higher than the practical dose recommended and that of other experiments with confined dairy cows (Haisan et al., 2014; Hristov et al., 2015; Lopes et al., 2016; Haisan et al., 2017).

Another possibility to consider is that the 3-NOP dose targeted in this study could have induced some level of supplement rejections or palatability issues of the 3-NOP supplements. Palatability studies with doses of 3-NOP of up to 120 mg/kg DM intake in dairy cows fed TMR diets reported no observed palatability issues (Melgar et al., 2018), and beef cattle fed 3-NOP at 100 mg/kg diet DM rapidly adjusted to 3-NOP showing no eating preferences within 7 days (Lee et al., 2020). Also, studies with dairy (Melgar et al., 2020b) and beef (Vyas et al., 2016) cattle using 3-NOP doses of up to 200 mg 3-NOP/kg DM did not notice effects on DM intake. That said, the daily 3-NOP dose supplied in two portions of concentrate in our study still had a considerably higher concentration in feed (380 mg 3-NOP/kg concentrate) compared to the studies by Vyas et al. (2016), Melgar et al. (2018), and Lee et al. (2020). The animals did not seem to reject the supplements. Although, unfortunately, and due to a lack of appropriate infrastructure, supplement refusals in the milking parlour, if they took place, were not measured. It is recommended that further studies evaluate whether supplying 3-NOP in the concentrate at concentrations like the one we used, as required by pulse-dose supplementation, may impair concentrate palatability.

One major factor that could have compromised the effectiveness of 3-NOP in the present study is its mode of supply. In all studies used in meta-regressions (Dijkstra et al., 2018; Jayanegara et al., 2018; Kim et al., 2020; Almeida et al., 2021), 3-NOP was supplied mixed in either partial mixed rations or TMR leading to a continuous supply of the additive. In our study, the 3-NOP supply pattern restricts the delivery of the compound into the rumen to twice daily when pulse-dosed in the milking parlour with concentrate supplementation. Thus, synchrony between the CH_4-inhibiting activity of 3-NOP and the presence of grass from main grazing bouts was limited, causing a mismatch between peak 3-NOP inhibitory activity and CH_4 production. A limitation of the SF_6 technique for CH_4 measurement, while suitable for grazing animals, is that it collects a 24-h gas sample, not allowing for analysis of intraday CH_4 emissions. There have been previous reports that CH_4 production inhibition by 3-NOP can be limited when not supplemented continuously. When 3-NOP was dosed in a supplement during milking but access to non-grazed pasture rations was withheld for 1 h (to simulate the delay in time between milking and grazing), no significant effects on CH_4 production were detected (Muetzel et al., 2019). Reynolds et al. (2014) reported transitory CH_4 inhibitory effects of 3-NOP lasting for only 2–3 h, and smaller residual effects were maintained throughout the day. In addition to passage out of the rumen (Reynolds et al., 2014), 3-NOP is rapidly metabolised in the rumen and disappears through its own mode of action (Yu et al., 2021). *In vitro*, Duin et al., 2016 reported almost all 3-NOP to be broken down after 12 h. Thus, because 3-NOP inhibitory activity on methanogenesis is relatively short-lived in the rumen, in systems where 3-NOP cannot be supplied continuously with feed, shortening the interval between additive delivery and digestion and fermentation of grass, which is the largest portion of daily ingested feed, is critical. Slow−release formulations of 3-NOP especially intended for pasture−fed cows are underway (Muetzel et al., 2019).

In addition to the 3-NOP delivery restricted to twice daily in our study, on CH_4 measurement weeks, the time gap between 3-NOP dosing in the milking parlour and pasture grazing in the paddocks was augmented because of the experimental handling of the animals. We estimate this time gap to be around 3.5 h in the morning and 1.5 h in the afternoon. In this time frame, the cows were moved from the milking parlour to the management chute where experimental procedures associated with the SF_6 and n-alkane techniques were carried out, and then back to the grazing paddocks; a greater morning gap was due to the SF_6 canister replacement being done in the mornings. This may help explain why our rumen fermentation results showed shifts consistent with studies where more substantial decreases in CH_4 production were obtained (Kim et al., 2020). That is, because rumen fermentation (assessed in week 6 and not on CH_4 measurement weeks) was measured closer in time to 3-NOP supplementation. It has been reported that the diurnal pattern of CH_4 production follows closely the diurnal pattern of rumen fermentation (Brask et al., 2015). Outside CH_4 measurement weeks, the time spent between 3-NOP dosing and feeding in paddocks was estimated to be around 60 min. It is not uncommon for dairy cows to daily spend varying amounts of time away from grazing paddocks due to prolonged milking times and/or long walking distances towards the milking parlour, especially in large pasture-based dairy herds. For example, a survey of Australian dairy farmers reported that herds of over 300 cows spent between 1.18 and 3.45 h away from the paddock between the end of milking and the beginning of grazing (Beggs et al., 2015).

The possible impact of the basal diet (of higher roughage levels than with TMR) on the observed lack of efficacy of 3-NOP in this study is unknown. The increased 3-NOP dose compared to most studies with dairy cows might have helped improving 3-NOP efficacy with diets that were also somewhat high in NDF, especially with CTS supplementation (Dijkstra et al., 2018; Kebreab et al., 2023). The tendency towards a lack of effect of 3-NOP with the CTS supplement on absolute CH_4 emissions obtained in the present

study can be partly explained by the higher NDF, and also ether extract concentrations of the CTS supplement. Diet nutrient composition has been reported to be influential over 3-NOP efficacy, with lower additive efficacy when diet NDF and crude fat concentrations increase (Kebreab et al., 2023). Lower 3-NOP efficacy with high forage diets of higher NDF concentration has been speculated to be a consequence of greater CH_4 formation with forage than grain diets (Vyas et al., 2018). Greater CH_4 production with higher methanogen abundance and/or higher concentrations of methyl coenzyme M (3-NOP's structural analogue) imply that there is more competition for 3-NOP to bind with methyl coenzyme M reductase and inhibit CH_4 production. In addition, the CH_4 mitigating efficacy of 3-NOP at a given dose is enhanced by 3.08% for each 1% DM decrease in dietary crude fat concentration for TMR diets between 3 and 6% crude fat levels (Kebreab et al., 2023). Our results are consistent with this meta-analysis. It is noted that the tendency to increase CH_4 yield with 3-NOP and CTS has a mathematical explanation driven by a numerical decrease in DM intake. Additional factors to consider for evaluating 3-NOP effectiveness with grass diets are pasture's varying composition with season progression and grazing management, and its digestibility, NDF, and water contents compared to TMR diets.

The lack of a consistent effect of CTS on decreasing CH_4 emission in our study was unexpected, and disagrees with what others have reported. Total mixed rations with whole CTS (3.28 kg/d) were more effective in decreasing CH_4 emission (−14% CH_4 yield) than TMR diets with whole linseed or rapeseed (Muñoz et al., 2019), and the authors attributed the results to a higher oil concentration and a lower intake of digestible fibre with CTS. A concentrate supplement including whole CTS (2.61 kg DM/d) decreased CH_4 yield up to 15% in lactating dairy cows fed forage-based diets (Grainger et al., 2010). Muñoz et al. (2021) offered grazing dairy cows 2.3 kg DM/d of whole CTS as part of a concentrate supplement and found a 14% decrease in CH_4 yield, although the effects were transient. These discrepancies may be influenced by the lower level of CTS used in our study compared to others.

In our study, there were no interactions of 3-NOP or CTS with parity for CH_4 variables. A recent study by Maigaard et al. (2024) evaluated the effects of combined supplementation of primiparous and multiparous cows with 3-NOP, rapeseed, and nitrate. The authors found 2-way interactions between treatments and parity on DM intake, with treatments exerting a more pronounced decrease in DM intake in multiparous than in primiparous cows. The authors attributed the 2-way interactions of parity with 3-NOP and rapeseed on DM intake as responsible for the 3-way interaction they observed between fat, 3-NOP, and parity on CH_4 yield, with primiparous but not multiparous cows supplemented with rapeseed responding to 3-NOP. Based on the differential responses of DM intake and CH_4 yield of primiparous and multiparous cows to treatments, the authors deemed that the efficiency of additives was in fact similar across parities, which agrees with our study.

Effects on diet intake, milk production and performance

In the present study, 3-NOP supplementation decreased pasture intake by 10.5% across both concentrate supplements. Reports on the effect of 3-NOP on DM intake are not consistent. A meta-analysis reported up to 4.5% decrease in DM intake with 3-NOP inclusion (Almeida et al., 2021); another meta-analysis reported a tendency towards decreased DM intake in beef, but not in dairy cattle (Kim et al., 2020), and others have reported no effect of 3-NOP on DM intake (Jayanegara et al., 2018; Arndt et al., 2022). The mechanism involved in lower intake with 3-NOP may be related to increased portal concentrations of propionate affecting satiety (Allen, 2000), which would be consistent with our findings. Furthermore, we expected that the lower DM intake with 3-NOP would also increase pasture postgrazing height indicating that less forage was removed by the cows, but the opposite was observed. How this result was affected by the interaction found between 3-NOP and CTS on pasture allowance is difficult to interpret. This illustrates that the relationship between 3-NOP supply and grass diets (as affected by composition and grazing management) and its effects on DM intake are unknown and merit further study.

The present study found that in the afternoon rumen sampling, 3-NOP supplementation tended to decrease rumen pH with the STA concentrate. This contrasts with previous results of meta-analyses that have shown increased rumen pH with 3-NOP supplementation (Jayanegara et al., 2018; Kim et al., 2020). Also, in our study, supplementing 3-NOP decreased DM digestibility with the CTS supplement. Yet, meta-analyses have reported no effects of 3-NOP on nutrient digestibility (Jayanegara et al., 2018; Kim et al., 2020). This was most likely related to lipid content, as CTS can decrease DM digestibility, particularly fibre digestibility (Muñoz et al., 2019). The discrepancies between our findings and that of others are difficult to explain and may be related to differences in basal diet composition (Kebreab et al., 2023), and/or the mode of supply of the additive and its higher 3-NOP concentration in feed.

In the present study, 3-NOP tended to decrease milk yield with the STA supplement, but not with the CTS supplement. Importantly, ECM yield was unaffected by treatments and this result was mainly driven by the lack of effect of treatments on milk composition. Meta-analyses based on TMR diets have reported that 3-NOP does not compromise milk yield (Jayanegara et al., 2018; Arndt et al., 2022). However, a tendency to decrease milk yield with increasing 3-NOP supplementation has been reported by another meta-analysis (Kim et al., 2020). In our study, the tendency towards decreased milk yield agreed with the negative effects of 3-NOP on DM intake. The higher milk urea concentration as a result of 3-NOP supplementation found in our study agrees with a meta-analysis of 5 studies by Hristov et al., 2022, but not with Jayanegara et al., 2018 meta-analysis. The MUN increase with 3-NOP has been explained as a consequence of increased butyrate absorption from the rumen, which could stimulate blood flow and NH_3 absorption from the rumen (Hristov et al., 2022). In agreement, 3-NOP increased rumen butyrate concentration in our study, which may be an indication of greater butyrate absorption.

In our study, 3-NOP supplementation had no effect on BW change. Results of 3-NOP on BW of dairy cows have been inconsistent with some studies reporting increased BW (Haisan et al., 2014; Hristov et al., 2015) and others not (Reynolds et al., 2014; Melgar et al., 2020a). Similarly, CTS supplementation also did not affect BW change or milk SCC. Few studies have evaluated the effects of whole CTS on variables associated with lactation cow's health. Our results agree with Sun et al. (2022) that reported no effects of CTS on MUN or milk SCC, and Muñoz et al. (2021) who reported no effects of CTS supplementation on health or reproduction performance.

We are unaware of any published study reporting 3-NOP supplementation twice daily to dairy cows at milking under grazing condition. The forage quality of the present study was representative of temperate grasslands in spring, typical of pasture-based dairy systems in the south of Chile (Muñoz et al., 2016), and the diet concentrate proportion supplemented (30%) is on the high end of concentrate spring supplementation levels (Muñoz et al., 2015).

Conclusion

Twice daily supplementation of grazing dairy cows with supplements that provided a combination of 3-NOP and CTS did not

increase the effectiveness of each CH_4 mitigation strategy supplemented separately, disproving our hypothesis. The 3-NOP additive modestly decreased or tended to decrease CH_4 emissions (absolute and yield) only with the STA concentrate, but not with the CTS concentrate. The lesser CH_4 inhibitory effects of 3-NOP than previously reported with TMR were most likely related to the mode of 3-NOP supply (twice daily dosing at milking) being uncoupled with the pasture grazing bouts. Extended time gaps between additive delivery and access to pasture due to experimental handling likely further impaired 3-NOP effectiveness. The 3-NOP additive decreased grass intake; however, ECM yield and milk composition were largely unaffected. Supplementing grazing dairy cows with CTS did not decrease CH_4 production and this may be related to insufficient levels of supplementation. There is a need for effective and pronounced CH_4 mitigation strategies that can be applicable to grazing dairy systems by pulse-dosing at milking. Alternatives for continuous delivery of 3-NOP into the rumen are required for boosting its CH_4 mitigation effectiveness in grazing systems.

Ethics approval

Animal care and experimental procedures were performed in accordance with the requirements of the Chilean Law 20.380 on Animal Protection and with the approval of INIA's Institutional Animal Care and Use Committee (Certificate 01/2019).

Data and model availability statement

None of the data were deposited in an official repository. The data that support the study findings are available from the authors upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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Declaration of interest

The authors declare no conflicts of interest.

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