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A previous study showed the additive methane (CH_4)-mitigating effect of nitrate and linseed fed to non-lactating cows. Before practical application, the use of this new strategy in dairy cows requires further investigation in terms of persistency of methanogenesis reduction and absence of residuals in milk products. The objective of this experiment was to study the long-term effect of linseed plus nitrate on enteric CH_4 emission and performance in dairy cows. We also assessed the effect of this feeding strategy on the presence of nitrate residuals in milk products, total tract digestibility, nitrogen (N) balance and rumen fermentation. A total of 16 lactating Holstein cows were allocated to two groups in a randomised design conducted in parallel for 17 weeks. Diets were on a dry matter (DM) basis: (1) control (54% maize silage, 6% hay and 40% concentrate; CON) or (2) control plus 3.5% added fat from linseed and 1.8% nitrate (LIN + NIT). Diets were equivalent in terms of CP (16%), starch (28%) and NDF (33%), and were offered twice daily. Cows were fed ad libitum*, except during weeks 5, 16 and 17 in which feed was restricted to 95% of dry matter intake (DMI) to ensure complete consumption of meals during measurement periods. Milk production and DMI were measured weekly. Nitrate and nitrite concentrations in milk and milk products were determined monthly. Daily CH_4 emission was quantified in open circuit respiration chambers (weeks 5 and 16). Total tract apparent digestibility, N balance and rumen fermentation parameters were determined in week 17. Daily DMI tended to be lower with LIN + NIT from week 4 to 16 (−5.1 kg/day on average). The LIN + NIT diet decreased milk production during 6 non-consecutive weeks (−2.5 kg/day on average). Nitrate or nitrite residuals were not detected in milk and associated products. The LIN + NIT diet reduced CH_4 emission to a similar extent at the beginning and end of the trial (−47%, g/day; −30%, g/kg DMI; −33%, g/kg fat- and protein-corrected milk, on average). Diets did not affect N efficiency and nutrients digestibility. In the rumen, LIN + NIT did not affect protozoa number but reduced total volatile fatty acid (−12%) and propionate (−31%) concentrations. We concluded that linseed plus nitrate may have a long-term CH_4-mitigating effect in dairy cows and that consuming milk products from cows fed nitrate may be safe in terms of nitrate and nitrite residuals. Further work is required to optimise the doses of linseed plus nitrate to avoid reduced cows performance.*

Keywords: linseed, methane, milk product, nitrate, ruminant

Implications

Linseed plus nitrate supplemented to dairy cows persistently reduced methane emission for up to 4 months without affecting diet apparent digestibility and animal health. Intake and milk production tended to be lower for cows fed linseed plus nitrate compared with cows fed control diet, but feed efficiency was similar between diets. Nitrate and nitrite were not detected in milk and processed milk products, suggesting that milk from cows fed nitrate is safe for human consumption. These results show that linseed plus nitrate is a viable long-term methane-mitigating feeding strategy for dairy cows.

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Introduction

Linseed and nitrate are both proven dietary treatments for reducing enteric methane (CH_4) emission in ruminants (Doreau *et al*., 2014) and their combination as a CH_4-mitigating strategy appears promising. In a short-term experiment on non-lactating cows, linseed oil (4% of dry matter; DM) plus nitrate (2.25% of DM) reduced methanogenesis by 32% without affecting apparent diet digestibility (Guyader *et al*., 2015). Compared to linseed oil and nitrate fed individually, the effect of this combination on CH_4 production was additive because these two strategies share different modes of action in the rumen: polyunsaturated lipids from linseed are thought to act as inhibitors of H_2 producers such as protozoa, whereas nitrate is thought to act as a H_2 sink, competing with methanogenesis. Nitrate and nitrite are also toxic to methanogens (Guyader *et al.*, 2014).

Before practical application at the farm scale, the long-term effect of dietary linseed plus nitrate on CH_4 emission in dairy cows requires further investigation. Another issue to evaluate is the potential adverse effects of nitrate supplementation on human and animal health. To our knowledge, the effect of dietary nitrate on milk quality, including the absence of nitrate and nitrite residuals in milk, has never been tested, whereas excess nitrite from nitrate reduction in the mouth may promote gastric irritation in humans (Weitzberg and Lundberg, 2013). Nitrate may also alter animal health by increasing the concentration of blood methemoglobin (metHb; Lewis, 1951). Without adaptation, nitrite from nitrate reduction can accumulate in the rumen, passing through the blood and leading to subclinical methemoglobinemia (30% to 40% of metHb; Lee and Beauchemin, 2014).

The main objective of this experiment was to investigate the long-term effect of linseed plus nitrate on CH_4 emission and lactating performance in dairy cows. As a secondary objective, nitrate metabolism was assessed by measuring metHb levels in blood and nitrate and nitrite levels in milk and processed milk products. We also evaluated the effect of linseed plus nitrate on total tract apparent digestibility, nitrogen (N) balance and rumen fermentation parameters.

Material and methods

The experiment was conducted at the dairy cows' experimental facilities at the INRA's Saint-Genès-Champanelle research centre in France from January to May 2014. All procedures involving animals were performed in accordance with French Ministry of Agriculture and European guidelines and regulations for animal research and experimentation.

Animals, diets and feeding

A total of 16 lactating (including seven primiparous) Holstein cows accustomed to handling were used. At the start of the experiment, cows were 61 ± 23 days in milk, with an average milk yield of 33.4 ± 7.1 kg/day and BW of 706 ± 67 kg. The experiment was conducted for 17 weeks as a randomised block design where cows were separated into two groups balanced for calving date and milk production. Cows were housed in a free stall barn in which they had an individual trough accessed via an electronic tag. Cows were adapted for 4 days to a tie stall barn before the start of the two measurement periods (week 5 and weeks 16 to 17) that required tying cows.

The first group of cows (n = 8 of which four primiparous) was fed the control diet (CON), and the second group of cows (n = 8 of which three primiparous) was fed CON with 9.8% extruded linseed and 2.4% calcium ammonium nitrate (75% nitrate in DM) on a DM basis (LIN + NIT). The doses of extruded linseed and nitrate were estimated to reduce CH_4 emission by 10% to 15% when fed alone (Doreau *et al.*, 2014) and by 20% to 30% when fed together. Diets were formulated to meet the requirements of lactating dairy cows (30 kg daily milk production without BW change) and to be equivalent in terms of CP, gross energy (GE) and starch proportions (INRA, 2010; Table 1). On a DM basis, diets were composed of 54% maize silage, 6% natural grassland hay and 40% concentrate given as pellets (InVivo NSA, Longué Jumelles, France).

Two weeks before starting the experiment, all cows were fed CON diet *ad libitum*. Then, animals fed LIN + NIT were

Table 1 *Ingredients and chemical composition of the experimental diets*

Item	Diet: CON	Diet: LIN + NIT
Ingredients (% of DM)		
Maize silage[1]	54.0	54.0
Hay	6.0	6.0
Pelleted concentrate		
Maize	11.9	12.0
Barley	3.36	2.52
Soybean meal	5.24	1.28
Rapeseed meal	2.00	3.12
Sunflower meal	0.00	0.80
Extruded linseed[2]	0.00	9.80
Soybean hulls	6.60	2.00
Wheat bran	6.00	4.20
Dehydrated beet pulp	0.94	0.00
Calcium ammonium nitrate[3]	0.00	2.40
Urea	0.80	0.00
Calcium carbonate	1.13	0.00
Dicalcium phosphate	0.44	0.26
Beet molasses	1.20	1.20
Mineral-vitamin premix	0.20	0.20
Sodium chloride	0.17	0.18
Fungicide	0.02	0.02
Flavouring[4]	0.02	0.02
Chemical composition (% of DM)[5]		
OM	93.1	93.5
CP	15.8	15.6
NDF	34.7	31.9
ADF	18.2	16.6
Starch	28.0	28.8
Ether extract	3.23	6.75
Total FA	2.54	5.86
Gross energy (MJ/kg of DM)	17.6	18.4
FA profile (% of total FA)		
C16:0	16.9	13.9
C18:0	2.40	2.74
C18:1n-9	25.1	23.3
C18:2n-6	43.2	31.6
C18:3n-3	9.1	25.1

CON = diet control; LIN + NIT = diet control containing 10% extruded linseed and 1.8% nitrate on a DM basis; DM = dry matter; OM = organic matter; FA = fatty acid.
[1]Fermentation characteristics of fresh silage juice: pH = 3.57; acetic acid = 0.74 g/100 g; lactic acid = 3.01 g/100 g; $N\text{-}NH_3$ = 0.02 g/100 g.
[2]Extruded linseed (InVivo NSA, Longué Jumelles, France).
[3]Calcium ammonium nitrate ($5Ca(NO_3)_2.NH_4NO_3.10H_2O$; Phytosem, Pont-du-Château, France) contained 75% nitrate on a DM basis.
[4]Gusti, Nutriad, Chester, England.
[5]Average chemical composition from samples (n = 3) taken in weeks 5, 16 and 17.

progressively adapted by replacing CON concentrate with LIN + NIT concentrate over a 2-week period to achieve the dose of 2.4% calcium ammonium nitrate at the beginning of week 3. Hay was offered once daily (0800 h) and before other feeds to ensure ingestion of fibre and prevent ruminal acidosis. Maize silage mixed with concentrates was offered twice daily (66% at 0930 h and 34% at 1600 h). All cows were fed *ad libitum* except during measurement weeks in which feed offered was restricted to 95% of individual voluntary feed intake to ensure complete consumption of the diet. Forage-to-concentrate ratio was kept as close as possible to the target ratio by adjusting the amounts of feed offered every week based on quantity and composition of the refusals of the previous week. Cows had free access to water throughout the experiment.

Measurements and analyses

Liveweight and blood metHb. Animals were weighed the week before starting the experiment (week 0) then in weeks 5, 10, 14 and 20. Blood metHb levels were measured 3.5 h after morning feeding on cows fed LIN + NIT and compared with levels of control samples taken on these same animals in week 0. Blood was then sampled twice a week from week 1 to 3 (adaptation to nitrate) and once a week from week 4 to the end of the experiment (week 17). Blood (10 ml) was sampled from the tail vein into K2-EDTA collection tubes and stored on ice (Venosafe; Terumo, Guyancourt, France) until measurement of metHb concentrations by spectrophotometry (UV-160; Shimadzu, Marne-La-Vallée, France; Kaplan, 1965) within 1 h (CHU Gabriel Montpied, Clermont-Ferrand, France).

Intake. Offered feed and refusals were weighed and recorded daily throughout the experiment. During the two measurement periods (week 5 and weeks 16 to 17), samples (200 g) of hay and concentrates were taken once a week, and samples (200 g) of maize silage were taken twice a week. For each feed and refusals sample, one aliquot was used to determine DM content (103°C for 24 h) and the other aliquot was stored at 4°C (hay and concentrates) or −20°C (maize silage) until further analyses. Chemical composition analyses (ash, N, NDF, ADF, starch, GE, ether extract and fatty acid) were carried out on fresh (hay, concentrates) or freeze-dried (maize silage) samples after grinding (1 mm) (InVivo Labs, Chierry, France) and as previously described (Guyader *et al*., 2015). Juice from fresh maize silage was obtained by maceration to analyse pH, ammonia (N-NH_3; Kjeldahl method 2001.11; AOAC, 2005), acetic and lactic acid (gas chromatography with a flame ionization detector) concentrations (InVivo Labs).

Methane emission. Daily enteric CH_4 emission of each animal was continuously measured for 2 consecutive days using open circuit respiration chambers (one animal per chamber) after 5 and 16 weeks of distribution of dietary treatments (Guyader *et al*., 2015). During measurement periods, chamber rear doors were opened in the morning for milking and to remove faeces and urine, and in the afternoon for milking. Chamber front doors were opened three times a day for feeding. In total, the doors of each chamber were opened for 30 min/24 h. Data collected while doors were open were deleted. To recover 24-h CH_4 emission, missing data were estimated as being similar to the last measurement data before chamber disturbance. Real-time gas emissions in a chamber were calculated by the difference between chamber and ambient gas concentrations multiplied by the airflow corrected for temperature, relative humidity and pressure (Pinares-Patiño *et al*., 2012).

Diet apparent digestibility and N balance. Total tract apparent digestibility and N balance were determined from total and separate collection of faeces and urine for 5 days during week 17 (Guyader *et al*., 2015). At the end of week 16, cows were moved from CH_4 chambers to individual digestibility crates to give animals a 3-day adaptation period to new housing conditions before the first collection. To separate urine from faeces, cows were fitted with a urine collection device connected by a flexible tube to a 30-l flask containing 500 ml of 3 M sulphuric acid to keep a urine pH lower than 3 and thereby avoid N volatilisation. Faeces and urine were removed once daily. Every day, after weighing and mixing of faeces, a 1% fresh aliquot was used to determine DM (103°C for 24 h), and another 1% fresh aliquot was pooled across days for each animal and frozen (−20°C). At the end of the experiment, pooled samples were thawed, freeze-dried and ground (1 mm) to determine organic matter (OM), N, NDF and ADF content as for feed (InVivo Labs). For urine, every day after weighing, a 1% fresh aliquot was pooled across days for each animal and frozen (−20°C). At the end of the experiment, after thawing, the N concentration of faeces and urine was, respectively, determined by the Dumas (method 968.06; AOAC, 2005) and the Kjeldahl (method 2001.11; AOAC, 2005) methods (InVivo Labs), as the Dumas analyser was not adapted to handle liquid samples. The Kjeldahl method does not take into account N-nitrate, but it was assumed that the influence of N-nitrate in urine on the overall N balance was minimal: Lee *et al.* (2015) reported that beef heifers fed 2% nitrate lost 0.17 g/day of N-nitrate in urine (0.39% of total N excretion in urine). Therefore, the use of the Kjeldahl method did not mask the potential effect of treatments on N balance.

Milk yield and composition. Throughout the experiment, milk yield was determined daily. For determination of milk composition (fat, protein, lactose and urea concentration), individual milk samples (30 ml) mixed with potassium bichromate (Merck, Fontenay-Sous-Bois, France) were taken and stored at 4°C before analysis within 2 days (Galilait, Theix, France). Samples were taken at morning and afternoon milking 2 days/week when animals were in the CH_4 chambers (weeks 5 and 16). Milk fat, protein and lactose concentrations were analysed by IR spectrometry with a 3-channel spectrophotometer (MilkoScan; Foss Electric, Hillerod, Denmark; method 972.16; AOAC, 2005). Milk urea concentration was analysed by the dimethylamino-4-benzaldehyde colorimetric method (Potts, 1967).

From data collected in weeks 5 and 16, milk production was converted to fat- and protein-corrected milk (FPCM, kg/day) with 4.0% fat and 3.3% protein (Gerber *et al.*, 2011) and feed efficiency was calculated as the ratio between FPCM and dry matter intake (DMI).

For analysis of nitrate and nitrite residuals in individual milk, samples (300 ml) from the morning milking were taken once a week in weeks 5, 9, 13 and 17. For analysis of nitrate and nitrite residuals in pooled milk and milk products, the morning milk of all animals was pooled by diet in weeks 9 and 17. Pooled milk was sampled (100 ml) and local farmhouse-style products were made (yoghurts, whey, curd and 6-week ripened Saint-Nectaire cheese). All samples were stored at 4°C before analysis within 2 days (Eurofins Analytics, Nantes, France). Nitrate and nitrite residuals in individual milk samples were analysed by ion chromatography (method 993.30; AOAC, 2005) with a limit of quantification of 10 mg/kg for nitrate and 5 mg/kg for nitrite. In pooled milk samples and processed milk products, nitrate and nitrite residuals were analysed by spectrometry after nitrate reduction with cadmium (ISO 14673; ISO, 2004) with a limit of quantification of 5 mg/kg for nitrate and 0.5 mg/kg for nitrite.

Rumen fermentation parameters. On the last day of week 17, rumen samples were collected 3.5 h after the morning feeding by stomach tubing (Shen *et al.*, 2012). Samples were strained through a polyester monofilament fabric (250 μm pore size) and the filtrate was subsampled for volatile fatty acids (VFA; 0.8 ml of filtrate in 0.5 ml of a 0.5 M HCl solution containing 2% (w/v) metaphosphoric acid and 0.4% (w/v) crotonic acid) and NH_3 (1 ml of filtrate in 0.1 ml of 5% orthophosphoric acid) concentration analyses. These samples were stored at −20°C until analysis. For protozoa counting, 2 ml of filtrate was mixed with 2 ml of methyl green-formalin saline solution, and stored at room temperature in the dark until counting.

Concentrations of VFA and NH_3 were analysed by gas chromatography with a flame ionization detector and colorimetry, respectively (Morgavi *et al.*, 2008). Protozoa were counted by microscopy and data were log_{10}-transformed before statistical analyses.

Statistical analyses
Data were analysed using the MIXED procedure of SAS (Version 9.4; SAS Institute, 2009). All statistical models included the animal nested within diet as random effect. Data collected throughout the experiment (intake, milk production and composition) or on two occasions (CH_4 emission) were averaged per individual cow and per week as there was no statistical difference between days within a week. The statistical model included diet ($n = 2$), week ($n = 17$ for intake and milk and $n = 2$ for CH_4) and diet × week interaction as fixed effects. Week was treated as a repeated measurement. For intake, milk production and composition (except for urea), data collected in week 0 were used as covariates, as these parameters were different between groups at the start of the experiment. For continuous measures of CH_4 emission, the model included diet ($n = 2$), week ($n = 2$), hour ($n = 24$), diet × week and diet × hour interactions as fixed effects. Hour was treated as a repeated measurement. As the interaction diet × week was not statistically significant, averaged data of the 2 weeks are presented in the last figure. For the repeated measurements, several covariance structures were tested (variance component, autoregressive, compound symmetry, unstructured and toeplitz) and structure with the lowest Akaike's information criteria was chosen. Then, variance component was always used as covariance structure, except for daily CH_4 emission where compound symmetry was used. Data collected at the end of the experiment (apparent digestibility, N balance, rumen fermentation and microbial parameters) were analysed with diet ($n = 2$) as fixed factor. Differences between diets were considered significant at $P \leqslant 0.05$, and trends were discussed at $0.05 < P \leqslant 0.1$. Least squares means are reported throughout.

Results

Liveweight and blood metHb
During the 17-week experiment, cows fed CON or LIN + NIT lost on average 32 and 22 kg BW to reach a final BW of 697 ± 62 and 662 ± 67 kg, respectively. During the 3-week period of adaptation to nitrate, the maximum metHb level was 13.0% (Figure 1). From week 4 to 17, average metHb level was 1.2%. Maximum metHb level peaked at 30.8% for one cow in week 17, whereas average metHb level for all other cows in that week averaged 4.4%.

Intake and milk yield
Daily DMI was similar between diets in weeks 1, 2, 3 and 17 and tended to be lower with LIN + NIT from week 4 to 16 (−5.1 kg/day on average; $P \leqslant 0.10$; Figure 2). This tendency between diets was also observed for DM and OM intake ($P = 0.070$ and $P = 0.078$, respectively) when cows were in

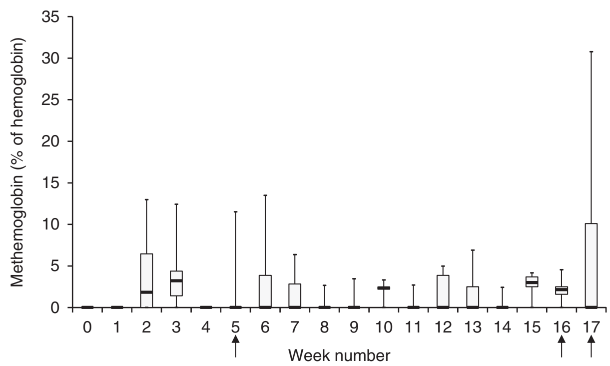

Figure 1 Boxplot of blood methemoglobin levels of lactating cows fed 10% extruded linseed plus 1.8% nitrate ($n = 8$) during 17 weeks. In week 0, animals were fed a control diet. Linseed and nitrate were first incorporated in week 1. Blood was analysed in week 0 and then twice a week during weeks 1, 2 and 3 and once a week from weeks 4 to 17. The box represents the quartiles with the median within the box, and the vertical lines represent the maximum and minimum value, respectively. Arrows indicate measurement weeks.

chambers (Table 2). Fibre intakes were lower with LIN + NIT ($P = 0.008$ for NDF and $P = 0.007$ for ADF) whereas dietary treatments did not affect GE intake.

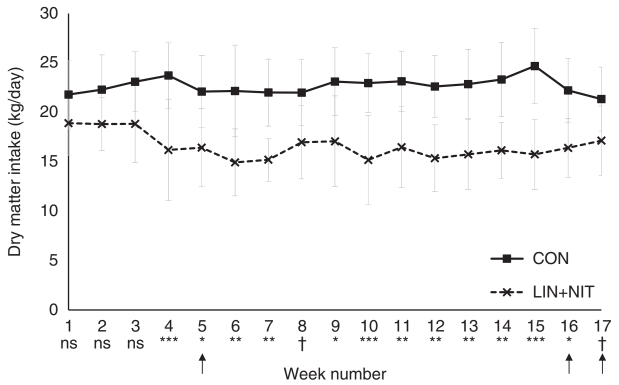

Figure 2 Dry matter intake of lactating cows fed a control diet (CON; $n = 8$) or CON supplemented with 10% extruded linseed plus 1.8% nitrate (LIN + NIT; $n = 8$) during 17 weeks (averages of 4 days/week). Error bars indicate SD. Symbols indicate weekly statistical comparison between CON and LIN + NIT (†$P \leqslant 0.10$; *$P \leqslant 0.05$; **$P \leqslant 0.01$; ***$P \leqslant 0.001$). Arrows indicate measurement weeks.

We found no difference between diets in milk production over two-thirds of the experiment (11 weeks out of 17), whereas in weeks 4, 5, 7, 9, 10 and 17, milk production was lower with LIN + NIT (−2.5 kg/day on average; $P < 0.05$; Figure 3). In chambers, cows fed LIN + NIT produced less milk (−2.8 kg milk/day on average, $P = 0.078$; −4.7 kg FPCM/day on average, $P = 0.045$; Table 2). Feed efficiency was similar between diets in week 5 and tended to be higher for LIN + NIT in week 16 (diet × week, $P = 0.061$).

In chambers, milk fat and lactose concentrations were similar between diets, whereas LIN + NIT reduced milk protein ($P = 0.045$) and urea ($P < 0.001$) concentrations by 6.8% and 60.6%, respectively. For both diets, nitrate and nitrite concentrations in individual milk samples, pooled milk samples and milk products were lower than the limit of quantification, except for curd from CON in week 17 and cheese from CON and LIN + NIT in week 9 in which low nitrite concentrations were detected (1.5 mg/kg).

Methane emission

Diet LIN + NIT decreased daily CH_4 emission by 47% (g/day; $P < 0.001$), 30% (g/kg DMI; $P = 0.002$) and 33% (g/kg FPCM;

Table 2 *Daily nutrient intake, milk yield and composition, and methane emission of lactating cows fed a control diet (*n = 8*) or a diet supplemented with a combination of linseed and nitrate (*n = 8*)*

Item[1]	Diet					*P*-value		
	CON		LIN + NIT					
Week number[2]	5	16	5	16	SEM	Diet	Week	Diet × week
Nutrient intake								
DM (kg/day)	20.8	20.7	18.8	17.3	1.00	0.070	0.182	0.293
OM (kg/day)	19.4	19.2	17.6	16.2	0.93	0.078	0.183	0.292
NDF (kg/day)	7.25	7.19	6.03	5.55	0.338	0.008	0.204	0.326
ADF (kg/day)	3.80	3.80	3.13	2.88	0.177	0.007	0.205	0.319
GE (MJ/day)	368	364	345	318	17.8	0.183	0.172	0.276
Milk yield and composition								
Milk yield (kg/day)	32.6	29.9	28.9	28.1	1.05	0.078	0.001	0.052
FPCM (kg/day)[3]	33.3	30.0	26.8	27.1	1.55	0.045	0.036	0.020
Feed efficiency (kg FPCM/kg DMI)[4]	1.58	1.44	1.48	1.62	0.077	0.692	0.972	0.061
Fat (kg/day)	1.39	1.21	1.03	1.08	0.092	0.060	0.198	0.045
Protein (kg/day)	1.03	1.00	0.85	0.87	0.045	0.026	0.615	0.243
Lactose (kg/day)	1.65	1.50	1.49	1.37	0.055	0.060	<0.001	0.608
Urea (g/day)	7.50	6.08	2.36	2.04	0.551	<0.001	0.061	0.223
Fat (g/kg)	41.9	39.1	36.5	39.1	2.23	0.298	0.961	0.185
Protein (g/kg)	31.5	33.2	29.4	30.9	0.78	0.045	0.009	0.902
Lactose (g/kg)	50.7	50.1	51.9	48.8	0.72	0.948	0.002	0.027
Urea (mg/dl)	22.2	19.4	8.7	7.7	1.51	<0.001	0.216	0.524
Methane emission								
g CH_4/day	414	409	226	211	30.0	<0.001	0.579	0.784
g CH_4/kg DMI	18.9	18.5	13.0	13.1	1.13	0.002	0.834	0.677
g CH_4/kg FPCM	12.1	13.2	8.9	8.1	0.83	0.001	0.781	0.153
% of GE intake	5.34	5.23	3.52	3.56	0.313	0.001	0.825	0.677

CON = diet control; LIN + NIT = diet control containing 10% extruded linseed and 1.8% nitrate on a DM basis; DM = dry matter; OM = organic matter; GE = gross energy; FPCM = fat- and protein-corrected milk; DMI = dry matter intake.
[1]Average of 2 days in chambers in weeks 5 and 16. For intake, milk yield and composition, a covariate (data obtained in week 0) was included in the statistical model.
[2]Number of weeks of distribution of dietary treatment.
[3]FPCM = milk yield (kg/day) × [0.337 + 0.116 × fat (%) + 0.06 × protein (%)] (Gerber *et al.*, 2011).
[4]Feed efficiency = FPCM/DMI.

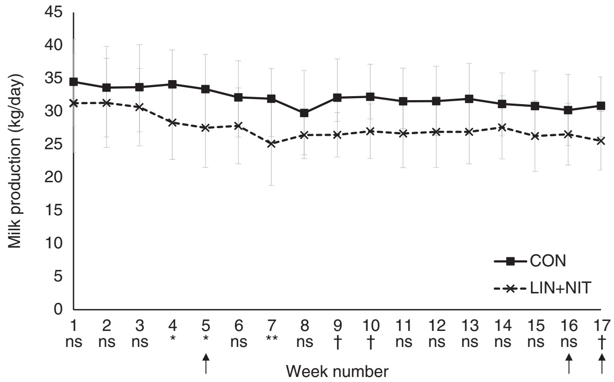

Figure 3 Milk yield of lactating cows fed a control diet (CON; $n = 8$) or CON supplemented with 10% extruded linseed plus 1.8% nitrate (LIN + NIT; $n = 8$) during 17 weeks (averages of 4 days/week). Error bars indicate SD. Symbols indicate weekly statistical comparison between CON and LIN + NIT (†$P \leqslant 0.10$; *$P \leqslant 0.05$; **$P \leqslant 0.01$; ***$P \leqslant 0.001$). Arrows indicate measurement weeks.

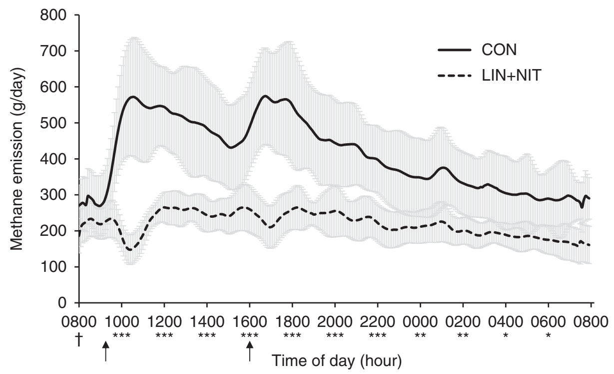

Figure 4 Daily CH_4 production pattern of lactating cows fed a control diet (CON; $n = 8$) or CON supplemented with 10% extruded linseed plus 1.8% nitrate (LIN + NIT; $n = 8$) during 17 weeks (averages of 2 days and 2 weeks of CH_4 measurement; weeks 5 and 16). Error bars indicate SD. Symbols indicate hourly statistical comparison between CON and LIN + NIT (†$P \leqslant 0.10$; *$P \leqslant 0.05$; **$P \leqslant 0.01$; ***$P \leqslant 0.001$). Arrows indicate feeding time.

$P = 0.001$) on average for weeks 5 and 16, without significant effect of week or diet × week interaction (Table 2). This shows that CH_4 emissions after 5 and 16 weeks were similar for cows fed CON and also for cows fed LIN + NIT. Except for a 2-h period before morning feeding, LIN + NIT decreased CH_4 emission all along the day ($P \leqslant 0.05$; Figure 4).

Diet apparent digestibility and N balance

Apparent digestibility of DM, OM and NDF was similar between diets, and averaged 67.5%, 69.4%, and 50.6%, respectively (Table 3). The LIN + NIT diet tended to reduce ADF (−3.8%; $P = 0.070$) and CP (−2.9%; $P = 0.074$) apparent digestibility. N intake was 22% lower with LIN + NIT ($P = 0.001$). Consequently, LIN + NIT led to lower faecal N losses, urinary N losses and N retained in milk ($P = 0.016$, $P < 0.001$ and $P = 0.003$, respectively). However, N distribution was unaffected by diet. On average for both diets, 35.7% ($P = 0.074$), 24.1% ($P = 0.071$) and 29.9% ($P = 0.937$) of N intake was directed towards faeces, urine

Table 3 *Total tract apparent digestibility and nitrogen balance of lactating cows after 17 weeks feeding a control diet (n = 8) or a diet supplemented with a combination of linseed and nitrate (n = 8)*

	Diet			
Item[1]	CON	LIN + NIT	SEM	*P*-value
Total tract apparent digestibility (%)				
DM	67.8	67.2	0.74	0.531
OM	69.8	69.0	0.73	0.458
NDF	51.3	49.9	1.11	0.393
ADF	47.5	43.7	1.35	0.070
CP	65.8	62.9	1.05	0.074
Starch	98.5	97.9	0.24	0.109
N intake (g/day)	548	425	21.6	0.001
Faecal N losses				
g/day	187	157	7.9	0.016
% of N intake	34.2	37.1	1.05	0.074
Urinary N losses				
g/day	138	96	5.7	<0.001
% of N intake	25.4	22.7	0.98	0.071
Milk N output				
g/day[2]	164	126	7.2	0.003
% of N intake	29.9	29.8	1.05	0.937
N balance[3]				
g/day	59.1	46.1	9.87	0.365
% of N intake	10.5	10.5	1.73	0.990

CON = diet control; LIN + NIT = diet CON containing 10% extruded linseed and 1.8% nitrate on a DM basis; DM = dry matter; OM = organic matter; N = nitrogen.
[1]Average of 5 days of total tract apparent digestibility and N balance measurement in week 17.
[2]Milk N output = (milk yield × milk protein concentration)/average N concentration in milk (6.38 g N/g milk protein).
[3]N balance = N intake – faecal and urinary N losses – milk N output.

and milk, respectively. Finally, N balance was positive and similar between diets and averaged 52.6 g/day or 10.5% of N intake.

Rumen fermentation and microbial parameters

Concentration of NH_3 in the rumen did not change with diets (Table 4). Diet LIN + NIT reduced total VFA (−12 mM; $P = 0.020$) and propionate concentrations (−8 mM; $P = 0.003$) without affecting acetate and butyrate concentrations. These differences in VFA profile induced an increase in C2/C3 and (C2 + C4)/C3 ratios ($P = 0.003$) with LIN + NIT. Total concentration of protozoa in the rumen tended to increase with LIN + NIT (+53%; $P = 0.052$).

Discussion

Intake, milk production and N balance

Throughout the experiment, intake and milk production tended to be lower for dairy cows supplemented with LIN + NIT. As feed efficiency (kilograms of FPCM per kilograms of feed) was similar between diets, the lower intake may explain the lower milk production. The lower intake with LIN + NIT is difficult to explain because diets had similar net

Table 4 *Fermentation parameters and protozoa concentration in the rumen of lactating cows after 17 weeks feeding a control diet (*n *= 8) or a diet supplemented with a combination of linseed and nitrate (*n *=8)*

	Diet			
Item[1]	CON	LIN + NIT	SEM	*P*-value
NH_3 (mM)	10.1	11.0	1.65	0.736
VFA concentration (mM)				
Total VFA	104.1	91.7	3.35	0.020
Acetate (C2)	58.6	56.9	1.95	0.561
Propionate (C3)	25.6	17.6	1.65	0.003
Butyrate (C4)	15.2	14.1	1.61	0.635
Minor VFA[2]	4.71	3.08	0.577	0.055
C2/C3	2.36	3.27	0.170	0.003
(C2 + C4)/C3	2.99	4.08	0.213	0.003
Total protozoa (log_{10}/ml)	5.03	5.32	0.095	0.052

CON = diet control; LIN + NIT = diet CON containing 10% extruded linseed and 1.8% nitrate on a DM basis; VFA = volatile fatty acid.
[1]Data from rumen samples taken the last day of week 17.
[2]Minor VFA = sum of isobutyrate, isovalerate, valerate and caproate.

energy content. Individual nitrate supplementation at higher doses than here (1.8%) did not reduce intake of restricted-fed dairy cows (2.1%, Van Zijderveld *et al.*, 2011b; 2.0%, Veneman *et al.*, 2014), but tended to reduce DMI of dairy cows (2.0%, Veneman *et al.*, 2014) and steers (2.3%, Hulshof *et al.*, 2012) fed *ad libitum*. Linseed applied at doses higher than here (3.5% added fat) did not have a negative effect on the intake or milk production of dairy cows (5.1% added fat, Ferlay *et al.*, 2013; 4% added fat, Veneman *et al.*, 2014) fed *ad libitum* or restricted. One study reported a lower DMI (−7%) by lactating cows fed a grass silage-based diet supplemented with linseed (3% added fat, Martin *et al.*, 2011). The only study that simultaneously used linseed plus nitrate (4% added fat plus 2.3% nitrate) on cows did not result in intake changes, but the cows were non-lactating and restricted-fed (Guyader *et al.*, 2015). We hypothesise that LIN + NIT fed together *ad libitum* may have an inhibitory effect on voluntary intake linked to a tendency for lower ADF digestibility. Allen (1996) highlighted the negative correlation between fibre digestibility and voluntary intake through a lower passage rate of particles from the rumen and greater rumen filling.

The LIN + NIT diet had no effect on concentration and production of fat and lactose in milk. This result confirms previous experiments with dairy cows supplemented with nitrate (2.1%, Van Zijderveld *et al.*, 2011b) or extruded linseed (up to 5.1% added fat, Ferlay *et al.*, 2013). The LIN + NIT diet reduced milk protein concentration and production by 7% and 15%, respectively. In dairy cows fed 2.1% nitrate, Van Zijderveld *et al.* (2011b) also reported reduced milk protein concentrations (−5%) but no effect on milk protein production whereas milk yield was stable. The reduced milk protein concentration may not be linked to linseed supplementation, as it was not affected by 3.5% added fat from extruded linseed in hay- or maize silage-based diets (Ferlay *et al.*, 2013).

N balance was positive and similar between diets with the same N distribution between milk, faeces and urine. In addition, average N efficiency (N in milk/N intake) was similar between CON and LIN + NIT (30%) and close to the data given in the literature (25%, with a range between 15% and 40%, Calsamiglia *et al.*, 2010). This result shows that dairy cows use nitrate in the same way as they use other N sources. The marked decrease in milk urea from cows fed LIN + NIT was surprising and in contradiction with previous experiments on dairy cows fed extruded linseed (1.1% added fat, Pezzi *et al.*, 2007) or nitrate (2.1% nitrate, Van Zijderveld *et al.*, 2011b). We assume that this difference comes from the lower N intake of animals fed LIN + NIT, as N intake is known to correlate positively with milk urea (Spek *et al.*, 2013).

The main concern when using nitrate in animal nutrition is its potential negative effect on animal and human health. To avoid increase of blood metHb in animals, progressive adaptation to nitrate is essential (Lee and Beauchemin, 2014). In this study, we did not observe rises in metHb levels in animals fed LIN + NIT, similarly to a previous experiment on dairy cows fed 2.1% nitrate (Van Zijderveld *et al.*, 2011b). However, we cannot explain the greater metHb level observed in the last week of the experiment. Analyses were carried out by an external lab and could not be repeated as metHb needs to be analysed quickly after sampling. In terms of human health, nitrate and nitrite are common food additives used for their anti-bacterial properties against lethal pathogens (European Food Safety Authority, 2009). However, excessive consumption of nitrate from several food sources may promote gastric inflammation linked to the production of nitrite from nitrate reduction in the mouth (Weitzberg and Lundberg, 2013). Regulations have been adopted to keep concentrations of nitrate and nitrite residuals within recommended daily allowances for nitrate and nitrite intake (3.75 and 0.13 mg/kg BW per day, respectively; European Food Safety Authority, 2009), and Europe has limited nitrate concentration in drinking water (50 mg/l, Benjamin, 2000). Here, nitrate and nitrite residuals in milk products were lower than the limit of quantification of the technique (5 mg/kg for nitrate and 0.5 mg/kg for nitrite), except in cheese from CON and LIN + NIT (1.5 mg/kg nitrite). These novel data confirm previous work on lamb meat (El-Zaiat *et al.*, 2013), and show that animals can metabolise nitrate and nitrite without transferring residuals into animal products. Consequently, long-term supplementation with nitrate (4 months) can be safely proposed in ruminant nutrition without risks for human health and as a source of non-protein N.

Methane emission and associated digestive mechanisms

Methane emission (% of GE intake) observed in cows fed CON was low compared to the literature for dairy cows fed similar diets. Nonetheless, methanogenesis was decreased by 30% (g/kg DMI) when dairy cows were supplemented with 1.8% nitrate plus 3.5% added fat from extruded linseed, corresponding to our expected theoretical CH_4 reduction. This confirms our previous results (32%)

obtained on non-lactating cows fed a hay-based diet supplemented with 2.2% nitrate plus 4% added fat from linseed oil (Guyader *et al.*, 2015) and shows that LIN + NIT can efficiently reduce CH_4 emission under various physiological animal conditions and for different diets. However, LIN + NIT may fail to reduce methanogenesis under some specific conditions. Indeed, nitrate (2.2%) did not reduce CH_4 emissions in finishing beef cattle fed a high concentrate-based diet as these animals were already low CH_4-emitters (Troy *et al.*, 2015). The CH_4-mitigating effect of linseed was also not observed in studies in which the level of FA in diets was low (1% to 2% of DM; Livingstone *et al.*, 2015) or not different from their control diet, which contained a rumen inert fat source (Van Zijderveld *et al.*, 2011a; Veneman *et al.*, 2014).

We observed a severe CH_4-mitigating effect of LIN + NIT just after feeding, which was most probably linked to the effect of quickly metabolised nitrate in the rumen. This result agrees with Van Zijderveld *et al.* (2010) and Guyader *et al.* (2015). Methane reduction with LIN + NIT corresponds to a saving of 1.8% of GE intake, without positive responses on apparent digestibility, BW gain or condition score (data not shown). The absence of relationship between CH_4 reductions and dairy cows performance has also been reported previously (Van Zijderveld *et al.*, 2011b).

The CH_4-mitigating effect of LIN + NIT was maintained throughout the 4 months of the experiment, indicating that this dietary strategy could be applied on farms. The long-term CH_4-mitigating effect of nitrate (2.1%) and extruded linseed (up to 3% added fat) fed individually to dairy cows was also maintained during 3 months (Van Zijderveld *et al.*, 2011b) and 1 year (Martin *et al.*, 2011), respectively.

The LIN + NIT diet did not change rumen protozoa concentration as previously observed with non-lactating cows (Guyader *et al.*, 2015). Diet LIN + NIT increased the acetate/propionate and (acetate + butyrate)/propionate ratios due to a decrease in ruminal propionate, which is normally a competitive pathway of methanogenesis. This contrasts with our previous work in which LIN + NIT did not change rumen fermentation parameters (Guyader *et al.*, 2015). However, in the present work, the relationship between CH_4 production and rumen fermentation and microbial parameters should be interpreted with caution given the large differences in time scale between CH_4 measurement periods and rumen samplings through stomach tubing. Consequently, the CH_4-mitigating effect of LIN + NIT would not be explained by a reduction in acetate and butyrate synthesis, or by a reduction in number of protozoa, which are important H_2 producers. Instead, both supplements may act as H_2 sinks. Based on stoichiometric calculation and assuming complete reduction of nitrate to nitrite and ammonia, and complete biohydrogenation of polyunsaturated fatty acids, 325.8 g/day of nitrate and 600.9 g/day of fatty acid (23%, 32% and 25% of C18:1, C18:2 and C18:3, respectively) ingested by dairy cows could have reduced CH_4 by 90.1 and 14.9 g/day, respectively. In total, H_2 consumption by LIN + NIT could have reduced CH_4 emission by 105.0 g/day, explaining 54% of the observed CH_4 reduction. The remaining decrease must therefore be explained by non-stoichiometric processes as LIN + NIT may also act on rumen microbiota: nitrate reduced both quantity (2.6% nitrate to sheep, Van Zijderveld *et al.*, 2010) and activity (2.3% nitrate to non-lactating cows, Guyader *et al.*, 2014) of methanogens. The anti-methanogenic effect of polyunsaturated fatty acids from linseed has also been demonstrated in cattle (2.6% added fat from linseed oil, Guyader *et al.*, 2014; 3.5% added fat from extruded linseed, C. Martin, unpublished results). In addition, H_2 production must have been lowered with LIN + NIT owing to a lower quantity of fermentable substrates in the rumen (lower DMI, quantity of carbohydrates due to lipids substitution and fibre digestibility), which directly reduced CH_4 emission.

Linseed plus nitrate is an efficient feeding strategy to reduce CH_4 emission in the long-term without altering diet apparent digestibility, N efficiency or animal health. However, to make this dietary strategy acceptable by farmers, further work is required to optimise the doses of linseed plus nitrate in an effort to avoid concomitant reduction in intake and milk production. Additional data is needed on changes in rumen microbiota in order to fully understand the CH_4-mitigating effect of the association of linseed plus nitrate. A life cycle assessment will also be needed to evaluate the environmental benefit and economic cost of this dietary strategy in order to raise the prospects of using this strategy at farm level.

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