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Gas exchange, rumen hydrogen sinks, and nutrient digestibility and metabolism in lactating dairy cows fed 3-nitrooxypropanol and cracked rapeseed

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ABSTRACT

Fat in the form of cracked rapeseed and 3-nitrooxypropanol (3-NOP, market as Bovaer) were fed alone or in combination to 4 Danish Holstein multicannulated dairy cows, with the objective to investigate effects on gas exchange, dry matter intake (DMI), nutrient digestion, and nutrient metabolism. The study design was a 4 × 4 Latin square with a 2 × 2 factorial treatment arrangement with 2 levels of fat supplementation; 33 g of crude fat per kg of dry matter (DM) or 64 g of crude fat per kg of DM for low and high fat diets, respectively, and 2 levels of 3-NOP; 0 mg/kg DM or 80 mg/kg DM. In total, 4 diets were formulated: low fat (LF), high fat (HF), 3-NOP and low fat (3LF), and 3-NOP and high fat (3HF). Cows were fed ad libitum and milked twice daily. The adaptation period lasted 11 d, followed by 5 d with 12 diurnal sampling times of digesta and ruminal fluid. Thereafter, gas exchange was measured for 5 d in respiration chambers. Chromic oxide and titanium dioxide were used as external flow markers to determine intestinal nutrient flow. No interactions between fat supplementation and 3-NOP were observed for methane yield (g/kg DM), total-tract digestibility of nutrients or total volatile fatty acid (VFA) concentration in the rumen. Methane yield (g/kg DMI) was decreased by 24% when cows were fed 3-NOP. In addition, 3-NOP increased carbon dioxide and hydrogen yield (g/kg DM) by 6% and 3,500%, respectively. However, carbon dioxide production was decreased when expressed on a daily basis. Fat supplementation did not affect methane yield but tended to reduce methane in percent of gross energy intake. A decrease (11%) in DMI was observed, when cows were fed 3-NOP. Likely, the lower DMI mediated a lower passage rate causing the tendency to higher rumen and total-tract neutral detergent fiber digestibility, when the cows were fed 3-NOP. Total VFA concentrations in the rumen were negatively affected both by 3-NOP and fat supplementation. Furthermore, 3-NOP caused a shift in the VFA fermentation profile, with decreased acetate proportion and increased butyrate proportion, whereas propionate proportion was unaffected. Increased concentrations of the alcohols methanol, ethanol, propanol, butanol, and 2-butanol were observed in the ruminal fluid when cows were fed 3-NOP. These changes in rumen metabolites indicate partial re-direction of hydrogen into other hydrogen sinks, when methanogenesis is inhibited by 3-NOP. In conclusion, fat supplementation did not reduce methane yield, whereas 3-NOP reduced methane yield, irrespective of fat level. However, the concentration of 3-NOP and diet composition and resulting desired mitigation effect must be considered before implementation. The observed reduction in DMI with 80 mg 3-NOP/kg DM was intriguing and may indicate that a lower dose should be applied in a Northern European context; however, the mechanism behind needs further investigation.

Key words: Bovaer, cattle, feed additive, fat

INTRODUCTION

To fulfill the Paris Agreement goals of limiting the increase in global temperature to 1.5°C, anthropogenic greenhouse gas emissions need to be reduced, including enteric methane from dairy cows (Clark et al., 2020). Methanogenesis is considered the major hydrogen sink in the anaerobic rumen environment (Ungerfeld et al., 2003), and manipulating methanogens may therefore pose a thermodynamic challenge for the fermentation processes due to hydrogen accumulation (Janssen, 2010). The compound 3-nitrooxypropanol (**3-NOP**) inhibits the enzyme methyl-coenzyme M reductase (**MCR**), the last step in methanogenesis, due to a reversible oxidation of the nickel atom (from Ni^{+} to Ni^{2+}) in the MCR enzyme (Duin et al., 2016). Studies

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have reported reductions in methane yield (g/kg DMI) of 26% to 54%, depending on the diet composition and concentration of 3-NOP (Garcia et al., 2022; van Gastelen et al., 2022; Kebreab et al., 2023). Adding extra fat to the diet has a different mode of action for reducing methane, and the methane mitigation potential depends on the fat source, inclusion level, and fatty acid (**FA**) composition (Patra, 2013). The effect of fat can be divided into 3 different modes of action. The primary mode of action is the dilution effect caused by replacing fermentable OM with nonfermentable FA (Beauchemin et al., 2022). In addition, FA have a direct inhibiting effect on fibrolytic bacteria (Ivan et al., 2013) and methanogens (Giger-Reverdin et al., 2003). Finally, biohydrogenation constitutes a minor hydrogen sink when unsaturated FA are hydrogenated to saturated FA (Czerkawski and Clapperton, 1984). Studies using either dietary fat (Beauchemin et al., 2009; Brask et al., 2013b) or 3-NOP (Melgar et al., 2020a,c) as methane mitigating strategy have been performed with promising results. However, limited literature is available, addressing the effect of combining the 2 strategies, especially regarding nutrient digestion and metabolism in dairy cows. Furthermore, the aspect of alternative hydrogen sink pathways being induced, when methanogenesis is inhibited, have mostly been investigated in in vitro systems (Ungerfeld et al., 2003; Ungerfeld et al., 2019). In the present study, lactating dairy cows were fed 3-NOP in combination with fat, supplemented as cracked rapeseed, with the objective to investigate (1) gas exchange, (2) hydrogen sink pathways other than methane formation, and (3) metabolism and digestion of nutrients in different digestive tract sections, especially focusing on the rumen and effects on microbial matter synthesis. To fulfill the objectives, multicannulated cows were used to obtain high quality data on nutrient digestion and metabolism.

Based on results from previous studies it was hypothesized that inclusion of both 3-NOP and cracked rapeseed would reduce enteric methane production without compromising DMI and rumen nutrient digestibility. In addition, based on a meta-analysis, where fat was found to decrease the reduction potential of 3-NOP (Kebreab et al., 2023), it was hypothesized that 3-NOP and fat could interact with respect to possible methane reduction capacity, even though 3-NOP and fat have 2 different modes of action.

MATERIALS AND METHODS

The experiment was performed at Aarhus University, AU Viborg–Research Centre Foulum, Denmark, from April to July 2020, and complied with the guidelines of Danish Ministry of Environment and Food (2014), law no. 474 (May 15, 2014, and executive order 2028 of December 14, 2020) concerning animal experimentation and care of animals under experiments and under consideration of the ARRIVE Guidelines (Percie du Sert et al., 2020). A license was obtained from the Danish Animal Experiments Inspectorate.

Animals and Experimental Design

Four lactating multiparous Danish Holstein cows (2 second- and 2 third-parity cows) were used in a balanced 4 × 4 Latin square design. The cows were on average (±SD) 52 ± 10 DIM at the beginning of the experiment. The BW and BCS of cows were on average 633 ± 48 kg and 3.13 ± 0.13, respectively, at the beginning of the experiment. The cows were weighed at the beginning of the experiment and in each experimental period. All 4 cows were multicannulated with a rumen cannula (Bar Diamond Inc., ID), and duodenum and ileum simple T-cannulas. The animals were housed in tiestalls bedded with rubber mats and saw-dust and were washed daily. The cows had free access to drinking water. Each experimental period lasted for 21 d, with 11 d of adaptation (d 1–11), 5 d of digesta sampling (d 12–16) and 5 d in respiration chamber (d 16–21). The phases within a period will afterward be referred to as the adaptation, sampling, and chamber period, respectively. In total, the experiment lasted for 12 wk.

Diets and Feeding

Energy density and nutrient composition of the diets, including FA content, were aligned with the NorFor system (Volden, 2011). The diets were arranged 2 × 2 factorial with 2 levels of fat aiming for a crude fat level of 33 g/kg DM and 64 g/kg DM for low- and high-fat diets, respectively. The concentrations of 3-NOP were either 0 mg/kg DM or 80 mg/kg DM. The diets were termed low fat (**LF**), high fat (**HF**), 3-NOP and low fat (**3LF**), and 3-NOP and high fat (**3HF**). The 3-NOP dose of 80 mg/kg DM was chosen, based on previously used doses in published studies with lactating dairy cows. The proportion of corn silage, grass-clover silage, barley, and soybean meal were the same across diets, leading to a similar CP and NDF content across diets (Table 1). Rapeseed was included in the ration by replacing a given amount of nonfat DM from the rapeseed meal with the same amount of nonfat DM from rapeseed. The whole rapeseeds were cracked daily using a roller mill (roller gap <0.15 mm; Skiold A/S). The forage proportion was 61.2% for the 2 LF diets (LF and 3LF) and 58.8% for the 2 HF diets (HF and 3HF).

The cows were fed TMR ad libitum in the morning (0615 h) and in the afternoon (1710 h). The TMR

Table 1. Formulated TMR ingredients and analyzed chemical composition in diets: low fat (LF), high fat (HF), 3-nitrooxypropanol (3-NOP) and low fat (3LF), and 3-NOP and high fat (3HF)[1,2]

	Diet			
Item	LF	HF	3LF	3HF
Barley	170	164	170	164
Soybean meal	38.8	37.4	38.8	37.4
Rapeseed meal	165	124	165	124
Rapeseed, cracked	—	71.8	—	71.8
Grass-clover silage	306	294	306	294
Corn silage	306	294	306	294
Mineral and vitamin mix with placebo[3]	14.8	14.8	—	—
Mineral and vitamin mix with 3-NOP[3]	—	—	14.8	14.8
DM, % of fresh matter	42.7 ± 1.67	43.3 ± 1.31	42.4 ± 1.38	43.7 ± 1.44
Ash	66.8 ± 9.63	64.1 ± 8.81	66.3 ± 9.58	64.3 ± 8.54
NDF	292 ± 5.67	283 ± 4.15	295 ± 2.46	282 ± 2.94
ADF	18.2 ± 0.38	17.9 ± 0.39	18.3 ± 0.32	17.8 ± 0.20
ADL	2.54 ± 0.22	2.58 ± 0.15	2.32 ± 0.18	2.58 ± 0.12
Crude fat	31.3 ± 2.06	62.0 ± 1.41	34.5 ± 2.38	64.8 ± 3.77
Fatty acids[4]	18.0	46.9	18.0	46.9
CP	166 ± 8.85	157 ± 10.9	164 ± 10.6	161 ± 11.3
Starch	204 ± 4.04	198 ± 7.65	202 ± 8.57	198 ± 6.49
NE_{L20},[5] MJ/kg DM	6.74	7.15	6.74	7.15

[1]Dose of 3-NOP: 80 mg/kg DM.

[2]Means ± SD; g/kg DM unless otherwise stated, n = 4.

[3]The 2 mineral mixes contained (on DM basis) 52.4% VM2 Green Mineral Mix (analytical substances: 16.8% calcium, 5.0 phosphorus, 6.5% magnesium, 9.0% sodium, 0.6% sulfur; also (g/kg DM): vitamin A = 600,000 IU, vitamin D = 190,000 IU, vitamin E = 4,000 IU, Mn = 4,000 mg, Cu = 1,500 mg, Zn = 4,500 mg, I = 225, Co = 25 mg, Se = 50 mg; Vilofoss, Denmark), 16.4% ADE vitamin (Vilofoss), 9.80% limestone, 16.4% salt, and either 5.10% placebo or 5.10% 3-NOP supplement.

[4]Fatty acid concentration is calculated based on analyzed fatty acids in each feedstuff and TMR composition.

[5]Net energy for lactation at 20 kg DMI/d, calculated according to NorFor (Akerlind and Volden, 2011).

refusals were collected just before the afternoon feeding in the sampling- and chamber period, and the daily feed intake (kg) was recorded. The feed and the feed refusals for the sampling days were dried at 60°C for 48 h to determine the DM concentration, and thereby calculate the DMI per cow per day. Drinking water intake was recorded in the sampling period.

The company Royal DSM delivered a placebo and a 3-NOP supplement; the latter contained 10.6% active 3-NOP mixed into a carrier substance of silicon dioxide + 1,2 propanediol (Bovaer; DSM Nutritional Products). The placebo contained 100% carrier substance. To avoid contamination, the LF and HF diets were always handled before the 3LF and 3HF diets in the mixer, followed by a cleaning procedure. The placebo and the 3-NOP supplement were first mixed with minerals, vitamins, salt, and limestone, giving 2 identical mixtures except for their 3-NOP content. An auger mixer was used for mixing the basic feedstuffs in all 4 diets: grass-clover silage, corn silage, rapeseed meal, and soybean meal. Thereafter, the basic mix was split in 4 and mixed with the specific mineral premix and cracked rapeseed or corresponding rapeseed meal in a paddle mixer.

Homogeneity of 3-NOP Concentration

To monitor the concentration of 3-NOP in the TMR and mineral mixes, pooled samples of the TMR from d 12–16 and a spot sample of the mineral mix were sent to DSM (Kaiseraugst, Switzerland) at d 16 in each period. Samples from d 12 and 13 were frozen and thawed before pooling. Furthermore, a set of samples from the first day of the experiment were sent for analysis.

Rumen, Digesta, and Feces Sampling

During the sampling period 12 collections of feces (approximately 350 g was collected when the cows defecated during around 2 h of sampling), ruminal liquid, duodenal and ileum content were performed with time intervals of 8–10 h, covering every second hour of a 24-h period, starting 1000 h on d 12 and ending 0800 h on d 16. Chromic oxide (Cr_2O_3) and titanium dioxide (TiO_2) were used as external flow markers to determine nutrient flow. The markers were dosed in the rumen at every milking, every day of the experiment starting at d 1 of adaptation period. The quantity of markers were 20 g of Cr_2O_3 (2 × 10 g) and 26 g of TiO_2 (2 × 13 g)

per cow per day. Ruminal liquid was sampled from the ventral part of the rumen with a 90-cm steel rumen sampler (Bar Diamond Inc.), with an attached 50 mL syringe. Immediately after sampling, pH was measured using a glass electrode connected to a digital pH meter (Meterlab PHM 220, Radiometer). The tubes with ruminal liquid were put on ice, and then stored at −20°C until analysis. Samples from the duodenum (0.5 L) and the ileum (0.2 L) were collected in plastic bags attached to the cannulas. Feces and digesta samples were pooled within cow and period.

Redox Potential

The redox potential in the rumen content was measured simultaneously with the rumen sampling, which resulted in 12 observations per cow per period. A redox meter with a platinum electrode was used (Intellical MTC101 ORP/redox electrode). When opening the rumen cannula, the electrode was placed directly into the rumen approximately at the depth of 15 cm from the surface of the rumen material closest to the cannula. After 2 min the redox potential was registered. The values were not corrected to a hydrogen electrode standard.

Milking

Cows were milked twice daily (0530 and 1630 h), and the milk yield was recorded using a continuous flowmeter. On d 6 (evening), 7 (morning), 20 (evening), and 21 (morning), milk samples were collected from the continuous flow sample and analyzed for protein, lactose and fat content by an infrared analyzer (Milcoscan 7RM FT+, Foss Analytical) at Eurofins Steins Laboratories (Vejen, Denmark).

Blood

On d 12 (1100 h), blood was sampled from the tail (vein or artery) by venipuncture into 9-mL sodium heparin vacuettes. The vacuettes were placed on ice, and afterward centrifuged at 3,000 × *g* for 20 min at 4°C. The plasma fraction was transferred to another tube and stored at −20°C. The blood samples were analyzed for nonesterified fatty acids (**NEFA**) and BHB.

Gas Exchange

Gas exchange was measured for 5 consecutive days per period (d 16 to 21) except for period 3, where data of 2 d were discarded due to a technical issue. The measurements were done in 4 transparent polycarbonate respiration chambers by open-circuit indirect calorimetry as described by Hellwing et al. (2012). To balance out any possible differences in inlet air composition, each cow stayed in one of the 4 chambers for 3 d and shifted to the chamber in the diagonal for the last 2 d (Hellwing et al., 2012). Flow and concentrations of the gases carbon dioxide, methane, oxygen, hydrogen, and hydrogen sulfide were measured in the background and outlet air (Columbus Instruments), as well the temperature, pressure and humidity (Veng Systems). The chambers were opened 2 times daily when the cows were milked and fed. Measurements from these time slots (approximately 60 min per d) were omitted, and the level of gas exchanges during the timeslots were expected to be equal with the mean of the day. The flow from the chambers were measured by mass flow meter (Teledyne Hasting) at standard temperature and pressure (0°C and 101.325 kPa). The measured gas production/use was converted from liter to gram by multiplying by 0.716, 1.96, 1.43, 0.0899, and 1.52 for methane, carbon dioxide, oxygen, hydrogen, and hydrogen sulfide, respectively, based on the ideal gas law. Gas exchange data were corrected with the results from recovery tests performed for methane and carbon dioxide. A recovery test was performed before and after the experiment. The average recovery was 98.5% ± 0.39% and 99.5% ± 0.55% for methane and carbon dioxide, respectively. Other gases than methane and carbon dioxide were corrected by an average recovery of the 2 gases.

Microbial Biomass Sampling

For analyses of microbial biomass, OM, and CP, approximately 2.5 L of ruminal fluid was collected on d 16 at 1000 h for 2 cows, and at 1100 h for the other 2 cows. The fluid was collected through the rumen fistula with a vacuum pump. The fluid was filtered through 2 layers of cheese cloths into prewarmed thermos flasks and transported to the laboratory immediately afterward. The laboratory procedure to isolate the microbial biomass was performed according to the description in Olijhoek et al. (2016). In short, the samples were centrifuged twice (5 min of 500 × *g* at 3°C), thereafter the supernatant was centrifuged (20 min of 17,300 × *g* at 3°C). The precipitate was transferred into 200 mL of saline solution and centrifuged like the supernatant. The isolated microbial matter was stored in the freezer (−20°C).

Laboratory Analyses

Samples of feces, digesta, TMR, feedstuffs, blood, and ruminal fluid were stored at −20°C and used for later analysis. Before analysis, samples were freeze-

dried (except for blood and ruminal fluid), and ground through a 1.00-mm or 0.5-mm screen, the latter for starch analysis. Samples were analyzed for ash content by combustion at 525°C for 6 h. For determining NDF, ADF, and ADL, the Fibertech M6 System (Foss Analytical) was used as described by Mertens (2002). Heat-stable amylase and sodium sulfite was used in the analysis of NDF. Samples analyzed for ADF and ADL were analyzed sequentially. The content of NDF, ADF, and ADL was reported as ash-free, where ADF was corrected with residual ash in the ADL residue. Organic matter digestibility (**OMD**) was determined in vitro for the grass and corn silage and enzymatically for barley, rapeseed meal, and cracked rapeseed as described in Hansen et al. (2022). Conversion of in vitro OMD to in vivo OMD was done according to (Åkerlind et al., 2011). Nitrogen and carbon content was determined by the Dumas method (Hansen, 1989) using a Vario MAX CN (Elementar Analysesysteme GmbH). The nitrogen content was multiplied with 6.25 to get the CP content. Crude fat was analyzed using Soxhlet extraction with petroleum ether (Soxtec 2050, Foss Analytical) where the first step is to hydrolyze the sample with HCl (Stoldt, 1952). The FA content in the individual feedstuffs were analyzed by GC as described in Lashkari et al. (2021), with the modification that C19:0 was used as internal standard instead of C17:0. Furthermore, a 60 m × 0.25 mm i.d., 0.20 μm thick column was used (Zebron ZB-FAME, Phenomenex). Starch content was enzymatically determined (Kristensen et al., 2007) using the glucose oxidase electrode technique (YSI 2900D, YSI Inc.).

Purines in microbial matter and duodenum chyme were analyzed by the method of Zinn and Owens (1986) and modified by Thode (1999) by hydrolyzing the nucleotides by adding perchloric acid to the freeze-dried sample. Thereafter, a buffer (containing ammonium dihydrogen phosphate) was transferred to the sample, followed by transfer of silver nitrate to the solution, since the purines, adenine and guanine, precipitate in reaction with silver nitrate. The external digestive marker cromic oxide was oxidized to chromate and determined colorimetrically (Schürch et al., 1950). Titanium dioxide was determined after digestion with sulfuric acid and thereafter hydrogen peroxide. The absorbance was measured spectrophotometrically according to Myers et al. (2004), with the only modification that 15 instead of 10 mL of 30% hydrogen peroxide was added, and additionally 5 last drops of hydrogen peroxide was added just before measuring. Blood plasma glucose was determined enzymatically according to standard procedures (Siemens Diagnostics Clinical Methods for ADVIA 1800). In short, the ADVIA system was programmed to run the analysis automatically: 2 solutions from the Glucose-Hexokinase-3 enzyme kit (Siemens Diagnostics Clinical Methods for ADVIA 1800) were transferred to 2 subsamples of the blood plasma sample, and the absorbance was measured for each subsample. The glucose concentration was equal to the difference in absorbance between solution-mix 1 and 2. The NEFA were determined using the Walko, NEFA C ACS-ACOD assay method, whereas BHB was determined as an increase in absorbance at 340 nm due to the production of NADH at slightly alkaline pH in the presence of β-hydroxybutyrate dehydrogenase as proposed by Harano et al. (1985). Analyses of NEFA and BHB were performed using an autoanalyzer, ADVIA 1800 Chemistry System (Siemens Medical Solutions).

VFA in Silage Extracts and VFA, Formate, Succinate, Alcohol, and Ammonia in Rumen Samples

Extracts of the silages were prepared by blending 100 g of fresh silage with 1 L of deionized water. After blending, pH was measured. Volatile fatty acids and L-lactate were analyzed in the metaphosphoric acid stabilized supernatant. Concentrations of VFA were determined in stabilized fermented liquid after methanol-chloroform extraction, using 2-ethylbutyrate as internal standard. Gas chromatography was used, and the oven was set to increase from 100°C to 200°C at 10°C/min. The GC had a split/splitless injector at 225°C and a flame ionization detector at 250°C. The column was a 30 m × 0.53 m × 1 μm HP-FFAP column (Agilent Technologies Inc.), and the carrier gas was He at 0.3405 atm. Formate and succinate in rumen samples were analyzed in 4 out of the 12 possible samples per cow collected during the sampling week (0800, 1200, 1600, and 2400 h). The samples were analyzed as described in Jensen et al. (1995) and Canibe et al. (2007) by a Hewlett Packard gas chromatograph (model 6890, Agilent Technologies Inc.) with a flame ionization detector and a 30-m SGE BP1 column (Scientific Instrument Services). Ammonia was determined in phosphate buffered samples using a Cobas Mira Plus (Roche Diagnostics Systems) with a Randox Ammonia Kit-AM1015.

Concentrations of alcohols in the ruminal liquid were determined by headspace GC-MS according to the method to determine alcohols in plasma in Kristensen et al. (2007).

Calculations and Statistical Analyses

The DMI in both periods (sampling and chamber) were averaged per cow per period. Digestibility was calculated based on data for DMI in the sampling period (d 12–16), and gas yield was calculated based on DMI in the chamber period (d 16–21). The 2 external

markers were used to determine the DM flows in and out of each section of the digestive tract. An average of the 2 flows (calculated with each of the markers) was calculated and used for further calculations. Production of microbial DM, OM, and CP (kg/d) were calculated as described in Brask et al. (2015). The true rumen digestibility of OM was calculated as [OM intake (kg/d) − (duodenal flow of OM (kg/d) − duodenal flow of microbial OM (kg/d))]/OM intake (kg/d) × 100. The true rumen digestibility of CP was calculated in a similar way. The microbial efficiency was calculated as: microbial CP (kg/d)/kg OM truly digested in the rumen. Nitrogen efficiency was calculated as nitrogen output in milk (kg/d)/nitrogen intake (kg/d), where factor 6.38 × N was used to calculate the CP content in milk. Carbon balance in the rumen was calculated as carbon intake (kg/d) − duodenal flow of carbon (kg/d). Contributions of carbon in terms of endogenous sources or bicarbonate were not included, as well as an assumption of even absorption rate across VFA was made. Knowing the proportions of VFA, combined with glucose fermentation stoichiometry (Brask et al., 2015), we calculated the expected carbon dioxide production from rumen fermentation. Yield of ECM was calculated from fat, protein, and lactose monohydrate content by the equation in Sjaunja et al. (1991).

The statistical analyses were performed in R (version 4. 0. 4) using a linear mixed effect model, through the lmer function in the lme4 package (Bates et al., 2015). The model was fitted with restricted maximum likelihood. Rumen VFA, alcohol concentration, and pH were averaged per cow within period. The following model was used for gas data, DMI, digestibility data, microbial flows, rumen VFA and alcohol concentrations, rumen pH, milk data, and blood metabolites:

$$Y_{ijpc} = \mu + FAT_i + NOP_j + (FAT \times NOP)_{ij} + P_p + \delta_c + e_{ijpc}, \quad [1]$$

where Y_{ijpc} is the response variable, μ is the overall mean, FAT_i is the fixed effect of fat level (i = LF, HF), NOP_j is the fixed effect of 3-NOP (j = 0 mg/kg DM, 80 mg/kg DM), $(FAT \times NOP)_{ij}$ is the interaction, P_p is the fixed effect of period (p = 1, 2, 3, 4), δ_c is the random effect of cow (c = 1, 2, 3, 4), and e_{ijpc} is the random residual error assumed to be independent and $\sim N\left(0, \sigma_e^2\right)$. No observations were discarded, leading to 16 observations to be included in the statistical model. Estimated marginal means and standard error of means (**SEM**) were reported using the emmeans package (Lenth, 2020). *P*-values were computed using an ANOVA type-2 Satterthwaite approximation for denominator degrees of freedom. Statistical significances were declared at $P \leq 0.05$ and tendencies were considered when $0.05 < P \leq 0.10$. Normality of residuals and homogeneity of variance was tested using qq- and residual plots, respectively.

RESULTS

Diet Composition

The chemical composition of the 4 diets is reported in Table 1. The crude fat content varied from 32.9 g/kg DM in average for the 2 LF diets, to 63.4 g/kg DM in average for the 2 HF diets. The FA content (Table 2) varied likewise from 18.0 to 46.9 g/kg DM for low and HF diets, respectively. The total FA content of the rapeseed was 419 g/kg DM, and the major FA was oleic acid (C18:1n-9) with 57.0 g/100 g FA (Table 2). Due to the fat addition in the high fat diets, the concentration of NDF, CP, starch, and ash were slightly lower for HF and 3HF diets. According to the NorFor system (Volden, 2011), the net energy for lactation at 20 kg of DMI per day (NE_{L20}; calculated according to NorFor; Åkerlind and Volden, 2011) content was 6.74 MJ/kg DM for the LF diets, and 7.15 MJ/kg DM for HF diets. Average recovery of 3-NOP was 101% ± 6.5% causing the actual average level of 3-NOP in 3LF and 3HF diets to be 81 ± 5.2 mg/kg DM. No traces of 3-NOP were found in the LF and HF diets.

DMI and Digestibility of Nutrients

There was no interaction between fat supplementation and 3-NOP regarding DMI, but DMI was reduced when cows were fed 3-NOP ($P = 0.01$; Table 3). The DMI was not affected by the rapeseed supplementation. No interactions of fat supplementation and 3-NOP were observed with respect to total-tract digestibility of OM, NDF, CP, and crude fat. Furthermore, the true rumen digestibility of OM and CP was not affected by 3-NOP or rapeseed supplementation ($P > 0.1$). There were a tendency for a higher rumen NDF digestibility ($P = 0.09$) as well as a tendency to higher total-tract NDF digestibility ($P = 0.06$), when cows were fed 3-NOP. The digestibility of fat in the small intestine was lower when cows were fed HF diets ($P = 0.04$), whereas the total-tract fat digestibility was not affected by any of the diets.

Gas Exchange

No interactions were observed between fat supplementation and 3-NOP with respect to production of methane, carbon dioxide, hydrogen, or consumption of oxygen (g/d) or yield (g/kg DM; Table 4). Methane

Table 2. Chemical composition of feedstuffs (± SD)

Item	Grass-clover silage (n = 2)	Corn silage (n = 2)	Barley (n = 3)	Soybean meal (n = 3)	Rapeseed meal (n = 1)	Rapeseed, cracked (n = 1)
DM, % of fresh matter	31.7 ± 1.22	34.5 ± 0.90	87.4 ± 0.21	93.1 ± 1.82	92.4	94.7
Nutrient, g/kg DM						
OM	934 ± 5.34	970 ± 0.14	980 ± 0.29	929 ± 8.28	911	961
NDF	370 ± 0.18	365 ± 3.85	199 ± 12.5	84.6 ± 4.14	250	194
ADF	23.5 ± 0.33	20.7 ± 0.21	5.94 ± 0.67	5.73 ± 0.38	18.5	15.4
ADL	1.59 ± 0.20	2.09 ± 0.68	1.20 ± 0.44	0.36 ± 0.37	7.96	6.96
CP	144 ± 10.2	83.8 ± 2.65	105 ± 9.12	502 ± 2.73	393	195
Crude fat	40.0 ± 8.49	29.0 ± 5.66	29.7 ± 0.58	21.3 ± 1.53	40.0	487
Digestibility of OM,[1] %	80.4 ± 0.14	76.6 ± 0.24	85.0 ± 1.17	91.2 ± 0.27	79.9	84.5
Fatty acid (FA), g/100 g of FA						
C16:0	17.8 ± 0.046	18.9 ± 0.22	25.0 ± 0.97	18.3 ± 0.36	9.00	4.84
C18:0	2.40 ± 0.11	2.11 ± 0.005	1.50 ± 0.021	4.61 ± 0.065	1.93	1.49
C18:1n-9	2.17 ± 0.12	20.2 ± 0.26	10.8 ± 0.18	11.5 ± 1.73	43.3	57.0
C18:1n-7	0.37 ± 0.002	1.12 ± 0.12	0.84 ± 0.002	1.49 ± 0.050	10.0	3.45
C18:2n-6	14.0 ± 0.26	45.3 ± 0.53	53.9 ± 0.61	53.9 ± 1.00	26.2	20.7
C18:3n-3	54.6 ± 0.010	9.01 ± 0.033	5.51 ± 0.12	8.01 ± 0.39	5.39	9.68
Total fatty acids,[2] g/kg DM	12.5 ± 1.71	17.8 ± 0.83	19.4 ± 1.38	11.6 ± 0.94	28.2	419
Extract,[3] g/kg DM			—	—	—	—
Acetate	18.5 ± 0.73	16.5 ± 1.23	—	—	—	—
Propionate	0.42 ± 0.038	ND[4]	—	—	—	—
Butyrate	ND	ND	—	—	—	—
Caproate	0.022 ± 0.00	0.015 ± 0.0012				
L-Lactate, g/kg DM	36.3 ± 0.12	38.0 ± 1.78	—	—	—	—
NH_3, g/kg DM	2.19 ± 0.15	1.56 ± 0.13	—	—	—	—
pH	4.03 ± 0.014	3.63 ± 0.03	—	—	—	—

[1]The in vivo OM digestibility (%) was calculated based on in vitro OM digestibility (%), for (1) concentrates: 5.38 + 0.867 × enzymatic in vitro OM digestibility, (2) grass silage: 4.10 + 0.959 × in vitro OM digestibility, and (3) corn silage: 6.73 + 0.950 × in vitro OM digestibility (Åkerlind et al., 2011).

[2]Total fatty acids are the sum of those shown and C14:0, C20:0, C20:2n-6, C22:0, and C24:0.

[3]Concentrations of valerate and isovalerate were below the limit of detection, and therefore not shown.

[4]ND = not detected.

Table 3. Intake and apparent and true digestibility of nutrients in different compartments of the digestive tract when cows were fed diets: low fat (LF), high fat (HF), 3-nitrooxypropanol (3-NOP) and low fat (3LF), and 3-NOP and high fat (3HF)[1]

	Diet					*P*-value[2]		
Component	LF	HF	3LF	3HF	SEM	Fat	3-NOP	Fat × 3-NOP
DMI, kg/d	23.1	23.2	21.1	19.9	1.27	0.51	0.01	0.44
Drinking water, L/d	89.8	90.7	80.5	75.6	5.73	0.60	0.01	0.42
OM								
Intake, kg/d	21.6	21.7	19.7	18.6	1.19	0.56	0.01	0.43
Duodenal flow, kg/d	13.2	13.5	11.7	11.5	0.71	0.92	0.01	0.56
Rumen digestibility, %	38.8	37.5	40.3	38.5	1.55	0.36	0.43	0.88
True rumen digestibility, %	54.9	54.2	56.6	55.6	1.56	0.51	0.23	0.87
Small intestine digestibility, %	50.8	49.2	48.5	53.1	1.53	0.29	0.54	0.053
Hindgut digestibility, %	6.17	7.54	8.32	4.96	2.01	0.63	0.92	0.27
Total-tract digestibility, %	71.8	70.7	71.9	72.6	0.70	0.78	0.18	0.24
NDF								
Intake, kg/d	6.74	6.56	6.20	5.62	0.36	0.11	0.01	0.35
Duodenal flow, kg/d	3.03	2.98	2.62	2.28	0.23	0.21	<0.01	0.33
Rumen digestibility, %	55.2	54.5	57.7	59.7	2.01	0.73	0.09	0.51
Small intestine digestibility, %	−12.1	−15.8	−23.8	−18.3	6.89	0.89	0.29	0.49
Hindgut digestibility, %	9.50	10.1	15.9	7.97	1.56	0.04	0.20	0.022
Total-tract digestibility, %	54.6	52.7	56.1	56.9	1.35	0.67	0.06	0.34
Crude fat								
Intake, kg/d	0.73	1.44	0.73	1.29	0.07	<0.001	0.30	0.30
Duodenal flow, kg/d	1.01	1.74	0.98	1.47	0.11	<0.001	0.14	0.22
Rumen digestibility, %	−40.2	−20.7	−36.1	−14.4	9.37	0.06	0.59	0.91
Small intestine digestibility, %	75.4	70.9	75.6	71.7	2.06	0.04	0.75	0.87
Hindgut digestibility, %	0.0747	2.10	6.30	−1.27	3.00	0.38	0.65	0.14
Total-tract digestibility, %	65.7	65.6	69.0	68.4	2.49	0.90	0.25	0.92
CP								
Intake, kg/d	3.84	3.67	3.46	3.21	0.23	0.22	0.03	0.81
Duodenal flow, kg/d	4.97	4.82	4.47	4.05	0.26	0.20	0.02	0.51
Rumen digestibility, %	−30.3	−32.7	−30.6	−26.4	4.53	0.85	0.53	0.49
True rumen digestibility, %	27.1	30.7	28.3	34.6	3.47	0.12	0.38	0.64
Small intestine digestibility, %	72.9	70.8	71.9	71.8	0.10	0.24	0.97	0.29
Hindgut digestibility, %	2.61	6.07	5.18	4.06	2.73	0.40	0.84	0.13
Total-tract digestibility, %	65.6	63.8	65.4	65.8	1.09	0.29	0.18	0.11
Rumen carbon balance,[3] kg/d	3.67	3.68	3.06	2.52	0.31	0.42	0.03	0.41

[1]Dose of 3-NOP: 80 mg/kg DM; crude fat content was 31.3, 62.0, 34.5, and 64.8 g/kg DM for LF, HF, 3LF, and 3HF diets, respectively.
[2]Statistical significances = $P \leq 0.05$ and tendencies = $0.05 < P \leq 0.10$.
[3]Rumen carbon balance is a calculated value based on carbon intake (kg/d) minus carbon flow to duodenum (kg/d).

and carbon dioxide production (g/d) were reduced ($P < 0.001$ and $P = 0.019$, respectively) by 3-NOP and hydrogen production and oxygen consumption (g/d) increased ($P < 0.001$ and $P = 0.021$, respectively) by 3-NOP supplementation, whereas fat supplementation had no effect. Methane yield (g/kg DM) was reduced by 24% when cows were fed 3-NOP ($P < 0.001$). Further, 3-NOP reduced the methane as percentage of gross energy intake (**GEI**) and methane intensity (g/kg ECM) ($P < 0.001$). Oxygen consumption, and carbon dioxide and hydrogen yield increased significantly; the latter was on average increased by 3,553% ($P < 0.001$) when the cows were fed 3-NOP. Carbon dioxide production from fermentation (in percent of measured carbon dioxide production), tended to increase when cows were fed 3-NOP ($P = 0.100$). Methane yield was unaffected by fat supplementation ($P = 0.27$). However, fat supplementation tended to decrease methane in percentage of GEI ($P = 0.06$), and also methane intensity (g/kg ECM; $P = 0.06$). Lastly, fat supplementation decreased the RQ value ($P = 0.02$), whereas 3-NOP had no effect ($P = 0.24$).

Diurnal production of methane and hydrogen (g/h) is shown in supplementary material (Supplemental Figures S1 and S2; https://erda.au.dk/archives/6137766792c486797212bca095e4e31f/published-archive.html). An interaction was observed between 3-NOP and time of the day ($P < 0.001$) with respect to methane production, where the methane production decreased right after the cows were fed newly mixed feed in the afternoon. Despite the interaction, the main effect of 3-NOP was clear as 3-NOP supplementation on average reduced methane production (g/h) by 45%, 35%, and 28% in the time slots; 1700–2300 h, 0700–1600 h, and 2400–0600 h, respectively. Likewise, an interaction between 3-NOP and time ($P < 0.001$) was observed with

Table 4. Gas exchange (g/d), yield (g/kg DMI),[1] and intensity (g/kg ECM) measured in respiration chambers when cows were fed low fat (LF), high fat (HF), 3-nitrooxypropanol (3-NOP) and low fat (3LF), and 3-NOP and high fat (3HF) diets[2]

	Diet					*P*-value[3]		
Item	LF	HF	3LF	3HF	SEM	Fat	3-NOP	Fat × 3-NOP
Gas exchange, g/d								
Methane	406	406	277	233	14.9	0.12	<0.001	0.12
Carbon dioxide	15,147	15,033	14,111	12,661	763	0.19	0.019	0.26
Oxygen	9,858	9,939	9,112	8,736	444	0.66	0.021	0.49
Hydrogen	0.45	0.73	18.5	17.3	1.18	0.58	<0.001	0.40
Hydrogen sulfide	0.28	0.27	0.41	0.31	0.07	0.38	0.19	0.51
Gas yield, g/kg DMI								
Methane	17.0	16.8	13.5	12.1	0.69	0.27	<0.001	0.45
Carbon dioxide	634	620	677	658	14.4	0.13	<0.01	0.78
Oxygen	413	410	439	453	12.6	0.54	0.01	0.39
Hydrogen	0.019	0.030	0.89	0.90	0.05	0.84	<0.001	0.99
Hydrogen sulfide	0.012	0.011	0.019	0.016	0.0023	0.41	0.02	0.71
Methane/ECM,[4] g/kg	11.7	11.1	8.27	6.64	0.83	0.06	<0.001	0.29
Methane/carbon dioxide, g/g	0.027	0.027	0.020	0.019	0.00093	0.54	<0.001	0.42
Hydrogen/methane, g/g	0.0011	0.0017	0.068	0.074	0.0048	0.46	<0.001	0.54
Methane, g/kg total-tract digested OM	26.4	26.8	19.8	17.2	0.92	0.20	<0.001	0.09
Methane, % of GE intake	5.18	5.04	3.89	3.20	0.19	0.06	<0.001	0.19
RQ value[5]	1.12	1.10	1.12	1.06	0.014	0.02	0.24	0.13
Carbon dioxide from fermentation, g/d	366	339	444	419	58.9	0.64	0.19	0.98
Carbon dioxide from fermentation, % of measured carbon dioxide	11.3	10.7	13.2	13.8	1.34	0.98	0.100	0.67

[1]DMI is an average of DMI on animal level during the chamber period, though DMI during the chamber period was not different from DMI in the sampling period.

[2]Dose of 3-NOP: 80 mg/kg DM; crude fat content was 31.3, 62.0, 34.5, and 64.8 g/kg DM for LF, HF, 3LF, and 3HF diets, respectively.

[3]Statistical significances = $P \leq 0.05$ and tendencies = $0.05 < P \leq 0.10$.

[4]ECM is calculated from fat, protein, and lactose monohydrate content by the equation in Sjaunja et al. (1991).

[5]RQ value = carbon dioxide production (L/d)/oxygen consumption (L/d).

respect to hydrogen production (g/h), and opposite to methane production, the hydrogen production peaked right after the cows were fed newly mixed feed in the afternoon. The main effect of 3-NOP supplementation was clearly on the hydrogen production (g/h), since hydrogen production was increased 4,268%, 2,164%, and 2,617% in the time slots; 1700–2300 h, 0700–1600 h, and 2400–0600 h, respectively (Supplemental Figure S2).

Microbial Production and Efficiency

Feeding 3-NOP decreased the flow of microbial CP to the duodenum ($P = 0.04$) and there was a tendency to a lower microbial OM production ($P = 0.08$; Table 5). However, the negative effect of 3-NOP on microbial CP flow leveled out when corrected for DMI ($P = 0.95$). Fat supplementation increased the microbial CP flow in terms of percent of total CP flow to the duodenum ($P = 0.01$). In contrast, there was no effect of any of the diets on microbial efficiency in grams of CP per kilogram of truly rumen digested OM. Furthermore, negative values were observed for apparent rumen digestibility of CP (Table 3).

Ruminal Variables

Fat supplementation and 3-NOP both reduced the total VFA concentration in the rumen ($P < 0.01$; Table 6). Fat supplementation did not affect the VFA proportions, except for interacting with 3-NOP with respect to valerate proportion. However, 3-NOP affected the VFA profile more distinctly, as acetate proportion decreased when cows were fed 3-NOP ($P < 0.001$), and butyrate and valerate proportion increased (both $P < 0.001$). Propionate proportion was not affected by any of the treatments. The ratio between acetate and propionate was unaffected by the diets. Rumen pH was higher, when cows were fed 3-NOP ($P < 0.01$), whereas the redox potential was lower ($P < 0.01$). Rumen pH and redox potential were not affected by fat supplementation. Lactate concentration in the ruminal fluid was lower, when cows were fed 3-NOP ($P = 0.01$), whereas concentration of ethanol, methanol, propanol, butanol, 2-butanol ($P < 0.001$), formate, and succinate ($P < 0.01$) in the ruminal fluid all increased.

Milk Production

No interactions were found between 3-NOP and fat supplementation with respect to milk data (Table 7). A tendency to decreased daily milk production both in terms of kilograms (−7.2%) and kilograms of ECM (−6.1%) was observed, when cows were fed 3-NOP ($P = 0.06$ and $P = 0.07$, respectively). Providing the cows with 3-NOP increased the milk fat percent ($P = 0.03$), whereas fat supplementation caused increased milk lactose percent ($P < 0.01$). Furthermore, both fat supplementation and 3-NOP increased the nitrogen efficiency ($P < 0.01$).

Blood Metabolites and Weight Changes

The concentration of NEFA in the blood increased for cows receiving the diets with HF content ($P = 0.04$; Table 8). A tendency for interaction between fat supplementation and 3-NOP was observed for the blood concentration of BHB ($P = 0.08$), as the decrease in concentration of BHB when fat level was increased, was more pronounced when fat supplementation was combined with 3-NOP. Feeding 3-NOP caused an average reduction in BW from d 1 to d 21 of 36 kg ($P < 0.01$; data not shown). No effect of fat supplementation was observed for BW changes (data not shown). No significant effect on BCS was detected (data not shown).

Table 5. Microbial composition, microbial flow to duodenum (kg/d), microbial CP synthesis (g/kg DM), microbial flow out of total flow (%), and microbial efficiency for cows fed low fat (LF), high fat (HF), 3-nitrooxypropanol (3-NOP) and low fat (3LF), and 3-NOP and high fat (3HF) diets[1]

	Diet					*P*-value[2]		
Item	LF	HF	3LF	3HF	SEM	Fat	3-NOP	Fat × 3-NOP
OM, g/kg DM	785	771	767	767	14.7	0.51	0.33	0.53
CP, g/kg DM	498	489	485	473	17.5	0.43	0.28	0.91
C, g/kg DM	419	418	417	425	5.80	0.51	0.54	0.31
OM flow, kg/d	3.46	3.63	3.17	3.21	0.26	0.54	0.08	0.71
CP flow, kg/d	2.19	2.31	2.00	1.96	0.15	0.71	0.04	0.48
CP synthesis, g/kg DMI	94.9	99.4	95.6	98.0	4.30	0.42	0.95	0.80
CP flow out of total CP flow to duodenum, %	44.1	47.8	45.0	48.3	1.63	0.01	0.50	0.85
Efficiency, g of CP/kg OM truly digested in the rumen	186	197	181	188	9.76	0.37	0.50	0.85

[1]Dose of 3-NOP: 80 mg/kg DM, crude fat content was 31.3, 62.0, 34.5, and 64.8 g/kg DM for LF, HF, 3LF, and 3HF diets, respectively.
[2]Statistical significances = $P \leq 0.05$ and tendencies = $0.05 < P \leq 0.10$.

Table 6. Rumen VFA, pH, ammonia, and other hydrogen sinks for cows fed low fat (LF), high fat (HF), 3-nitrooxypropanol (3-NOP) and low fat (3LF), and 3-NOP and high fat (3HF) diets[1] (all values are means of 12 diurnal samples)[2]

	Diet					*P*-value[3]		
Item	LF	HF	3LF	3HF	SEM	Fat	3-NOP	Fat × 3-NOP
Total VFA, m*M*	130	121	119	115	2.78	<0.01	<0.01	0.22
VFA, mol/100 mol VFA								
Acetate	55.2	55.7	50.1	51.5	0.84	0.25	<0.001	0.58
Propionate	25.3	25.3	25.4	24.0	1.33	0.52	0.62	0.55
Isobutyrate	0.67	0.73	0.73	0.78	0.05	0.11	0.13	0.92
Butyrate	14.8	14.9	19.2	18.0	0.68	0.43	<0.001	0.37
Valerate	1.81	1.85	2.35	1.99	0.08	0.03	<0.001	0.01
Caproate	0.62	0.68	0.84	0.61	0.12	0.49	0.56	0.26
Acetate/propionate, m*M*/m*M*	2.20	2.26	1.98	2.15	0.15	0.40	0.25	0.68
Formate, m*M*	0.25	0.07	1.90	1.52	0.39	0.49	<0.01	0.81
Succinate, m*M*	0.03	0.03	0.13	0.15	0.03	0.78	<0.01	0.83
Ammonium, m*M*	5.35	5.41	5.32	5.12	0.47	0.81	0.59	0.67
Rumen pH	6.12	6.19	6.28	6.39	0.07	0.10	<0.01	0.67
Redox potential, mV	−185	−183	−224	−235	11.7	0.65	<0.01	0.51
Glucose, m*M*	0.67	0.63	0.50	0.46	0.10	0.69	0.10	0.96
L-Lactate, m*M*	1.30	1.44	1.24	0.59	0.14	0.11	0.01	0.02
Ethanol, m*M*	1.31	1.52	2.98	2.39	0.18	0.33	<0.001	0.07
Methanol, m*M*	0.10	0.12	0.38	0.37	0.052	0.97	<0.001	0.74
Propanol, m*M*	0.20	0.21	0.41	0.35	0.020	0.28	<0.001	0.13
Butanol, m*M*	0.023	0.020	0.060	0.061	0.003	0.81	<0.001	0.34
2-Butanol, m*M*	0.029	0.026	0.039	0.034	0.0014	<0.01	<0.001	0.69

[1]Dose of 3-NOP: 80 mg/kg DM; crude fat content was 31.3, 62.0, 34.5, and 64.8 g/kg DM for LF, HF, 3LF, and 3HF diets, respectively.
[2]Except for formate and succinate, where the analysis was based on 4 rumen samples per cow per period collected at 0800, 1200, 1600, and 2400 h.
[3]Statistical significances = $P \leq 0.05$ and tendencies = $0.05 < P \leq 0.10$.

DISCUSSION

The study evaluated 4 diets, varying in level of fat supplementation (cracked rapeseed) and 3-NOP, fed to 4 dairy cows in a Latin square design. The aim was to investigate consequences, among others on gas exchange and DMI, when lactating dairy cows were fed 3-NOP alone or in combination with cracked rapeseed. If not otherwise mentioned, interactions between 3-NOP and fat supplementation were not observed. Multicannulated animals were used in the study to combine gas data from respiration chambers, with a comprehensive set of response parameters from different parts of the digestive tract, thereby quantifying effects in each section of the digestive tract.

Dry Matter Intake

In the present study, DMI was reduced on average by 11% when 3-NOP was fed to the cows. The same dose as used in the present study has previously been used for high yielding dairy cows, without negative effects on DMI (Hristov et al., 2015). In addition, Kim et al. (2020) reported in a meta-analysis that 3-NOP did not affect DMI of dairy cows, whereas beef cows tended to reduce their DMI with increasing concentration of

Table 7. Milk production and composition when cows were fed low fat (LF), high fat (HF), 3-nitrooxypropanol (3-NOP) and low fat (3LF), and 3-NOP and high fat (3HF) diets[1]

	Diet					*P*-value[2]		
Item	LF	HF	3LF	3HF	SEM	Fat	3-NOP	Fat × 3-NOP
Milk, kg/d	37.5	39.0	34.6	36.4	3.32	0.22	0.06	0.91
Milk fat, %	3.70	3.68	3.82	3.83	0.15	0.86	0.03	0.73
Milk protein, %	3.41	3.42	3.45	3.28	0.09	0.18	0.34	0.14
Milk lactose, %	4.84	4.91	4.78	4.92	0.03	<0.01	0.40	0.25
ECM,[3] kg/d	36.0	37.6	33.9	35.2	3.26	0.20	0.07	0.91
Nitrogen efficiency, kg N output in milk/kg N intake	0.34	0.37	0.36	0.38	0.018	<0.01	<0.01	0.68

[1]Dose of 3-NOP: 80 mg/kg DM; crude fat content was 31.3, 62.0, 34.5, and 64.8 g/kg DM for LF, HF, 3LF, and 3HF diets, respectively.
[2]Statistical significances = $P \leq 0.05$ and tendencies = $0.05 < P \leq 0.10$.
[3]ECM is calculated from fat, protein, and lactose monohydrate content by the equation in Sjaunja et al. (1991).

Table 8. Plasma concentrations (m*M*) of nonesterified fatty acids (NEFA), BHB, and glucose for cows fed low fat (LF), high fat (HF), 3-nitrooxypropanol (3-NOP) and low fat (3LF), and 3-NOP and high fat (3HF) diets[1]

	Diet					*P*-value[2]		
Item	LF	HF	3LF	3HF	SEM	Fat	3-NOP	Fat × 3-NOP
NEFA	0.07	0.08	0.05	0.15	0.02	0.04	0.33	0.11
BHB	1.01	0.96	1.27	1.02	0.07	0.02	0.01	0.08
Glucose	3.70	3.65	3.62	3.79	0.13	0.43	0.66	0.19

[1]Dose of 3-NOP: 80 mg/kg DM; crude fat content was 31.3, 62.0, 34.5, and 64.8 g/kg DM for LF, HF, 3LF, and 3HF diets, respectively.
[2]Statistical significances = $P \leq 0.05$ and tendencies = $0.05 < P \leq 0.10$.

3-NOP. Melgar et al. (2020c) reported a tendency to a linear decrease in DMI when 3-NOP concentration was increased from 0 to 200 mg of 3-NOP/kg DM to high yielding dairy cows. However, the relationship between 3-NOP and DMI is complex, as Melgar et al. (2020b) actually reported an increase in DMI for 10 h after supplying lactating dairy cows with 3-NOP. A recent study, where 3-NOP was included in similar Northern European diets, reported reductions in DMI of 2.6% and 5.6% for 3-NOP concentrations of 60 and 80 mg/kg DM, respectively (van Gastelen et al., 2022); a concentration of 51 mg of 3-NOP/kg of DM did not affect DMI (van Gastelen et al., 2020). The decreased DMI has previously been suggested to be caused by propionate formation in the rumen, affecting satiety of the cows (Zhang et al., 2021; van Gastelen et al., 2022). However, the present study provided no support for this mechanism, since 3-NOP was observed to reduce rumen total VFA concentration without affecting the propionate proportion. In addition, propionate levels of 29 mol/100 mol of total VFA (50 mmol/L) have previously been reported not to affect DMI (Hernández-Castellano et al., 2021).

Nutrient Digestibility

In the present study, no interactions were observed between 3-NOP and fat supplementation with respect to digestibility of nutrients, except for hindgut NDF digestibility. Rumen and total-tract NDF digestibility tended to be increased by 3-NOP (3.85 and 2.85 percentage points, respectively), which likely was caused by the lower DMI (Illius and Allen, 1994). In a meta-analysis by Jayanegara et al. (2018), total-tract OM, NDF, and CP digestibility in ruminants were increased numerically by 3-NOP supplementation. Garcia et al. (2022) reported increased total-tract NDF digestibility (~24 percentage points across nitrogen sources; rapeseed meal or urea) and CP digestibility (only significant for the diets with rapeseed meal; 12 percentage points) when they fed 100 mg of 3-NOP/kg of DM, but as in the present study, Garcia et al. (2022) also observed a lower DMI (~18.4% across nitrogen sources). However, the reduced DMI observed in Garcia et al. (2022) for cows fed 3-NOP, might be caused by variation in diet quality, as illustrated by a higher dietary ADF content in the 3-NOP period compared with the control period.

The rapeseed supplementation (~6.3% crude fat) did not affect the total-tract digestibility of nutrients (OM, NDF, crude fat, or CP) in the present study, and neither did Brask et al. (2013b) observe an effect on nutrient digestibility, when they fed different types of rapeseed as fat source (up to 6.5% crude fat). However, feedstuffs with a high crude fat content are diverse, and studies, using other high fat feedstuffs than rapeseed, have reported reduced DMI and total-tract NDF digestibility (Beauchemin et al., 2007; Ramin et al., 2021) or reduced GEI (Giagnoni et al., 2022a).

Methane Emission

In the present study, supplying 80 mg of 3-NOP/kg of DM reduced methane yield by 24%. The reducing effect of 3-NOP was persistent over the day, but the highest reduction was observed right after the cows were provided fresh feed in the afternoon (Supplemental Figure S1). However, the reduction in methane yield was less than the 33% predicted using the equation of Kebreab et al. (2023), taking 3-NOP dose and nutrient composition of the basal diet into account. The equation by Kebreab et al. (2023) includes content of crude fat, NDF and starch as variables, and the equation is based on a dataset with mean DMI at 22.8 kg/d, similar to the intake in the present study (23.1 kg DMI/d for LF diet). A study by van Gastelen et al. (2022), which is included in the meta-analysis by Kebreab et al. (2023), reported methane yield reductions of 28% and 42% for grass and corn silage based diets, respectively. The reported concentrations of 3-NOP were 75 and 87 mg/kg of DM for the grass- and corn-based diets, respectively (van Gastelen et al., 2022). The NDF contents, reported by van Gastelen et al. (2022), ranged from 323 to 329 g/kg DM, which is higher than in the present study (282 to 295 g/kg DM). In comparison, Hristov et al.

(2015) also fed a dose of 80 mg/kg of DM to lactating dairy cows and observed a 29% reduction in methane yield, which is more similar to the reductions obtained in the present study, however the actual concentration of 3-NOP was not reported. The 2 diets used by Hristov et al. (2015) were based on corn silage with an average NDF content across diets of 276 g/kg DM. Kebreab et al. (2023) reported the reduction potential of 3-NOP to be impaired by fat, however in the present study, this was not observed, and the methane yield for the 3HF diet was numerically 10% lower than for the 3LF diet. Thus, it remains unclear if, and how, the reduced DMI affected the interaction between fat supply and 3-NOP.

An effect of fat supplementation on methane yield was expected, since the rapeseed contained 419 g of FA/kg of DM, which is a normal level, and the majority of the FA were unsaturated (Table 2). The rapeseed constituted 72 g/kg DM of the HF diets in the present study, causing the calculated FA content (based on FA analyses of each feedstuff and diet composition) to be 47 g/kg DM. In comparison, Brask et al. (2013b) used 69 g of rapeseed/kg DM in a diet, where the FA content of the rapeseed was 385 g/kg DM, causing the FA content of the diet to be 50 g/kg DM, and they observed a 13% reduction in methane yield. However, other studies, using similar crude fat levels, have reported unaffected methane yields when a high concentration of rapeseed was included in the diet (Brask et al., 2013a; Hellwing et al., 2014). The reduction in methane production due to the included fat level in the present study was expected to be ~11%, equivalent to 3.8% reduction per 10 g added FA per kg of DM as calculated according to Børsting et al. (2020). According to the NorFor equation (Nielsen et al. (2013): Methane (MJ/cow per day) = 1.23 × DMI – 0.145 × FA + 0.012 × NDF), the expected reduction in methane production in the present study should have been 15%, whereas using the equations of Grainger and Beauchemin (2011) a reduction of ~20% should have been obtained. The lack of effect of fat supplementation in the present study was due to one cow emitting even more methane (18.0 vs. 16.7 g/kg DM; data not shown) when fed the HF compared with the LF diet irrespective of 3-NOP inclusion. Excluding this cow, the obtained average reduction in methane yield was 10% for fat supplementation (data not shown). According to Giagnoni et al. (2022b), variation between animals regarding response to a given methane mitigation strategy may be expected. However, in the present study there was a tendency to a reducing effect of rapeseed supplementation on methane as percentage of GEI, which align with results from Brask et al. (2013a) and Hellwing et al. (2014), where the effects of fat on methane as percentage of GEI were reported to be significant. Likewise, there was a tendency to a reducing effect of fat supplementation on methane intensity (g/kg ECM) in the present study ($P = 0.06$), whereas Beauchemin et al. (2009) reported no effect of inclusion of rapeseed on the methane intensity (g/kg FCM), but significant reductions in methane yield (16.0%) and methane as percentage of GEI (18.4%).

Hydrogen Emission and Rumen Hydrogen Sinks

When methanogenesis is inhibited, enteric hydrogen emission increases (Ungerfeld et al., 2003; Lopes et al., 2016), which was also observed in the present study, where hydrogen production and yield increased by 2,900% and 3,500%, respectively, when cows were fed 3-NOP. It is often overlooked that hydrogen is considered as a greenhouse gas, due to its GWP being only 5 in a 100-yr horizon (Derwent et al., 2020). However, the elevated emission of hydrogen (g/d) only reduces the positive effect of 3-NOP on total greenhouse gases emitted by 1.5% (data not shown).

Using a lower 3-NOP concentration (51 mg/kg DM) than in the present study, van Gastelen et al. (2020) reported the increase in hydrogen production (g/d), as measured in respiration chambers, to be only 1,000%. Several other studies have measured gas emissions from dairy cows fed 3-NOP by use of GreenFeed systems, but due to the discrete spot sampling in GreenFeed units and hydrogen being a very rapidly emitted gas in connection to feed intake, direct comparison of hydrogen measurements from respiration chambers and GreenFeed systems is critical (van Gastelen et al., 2020). Based on stoichiometry, the currently observed increase in hydrogen emission, when 3-NOP was fed to the cows, accounted for only 28% and 19% for the low and high fat diets, respectively (data not shown), of the expected based on the decrease in hydrogenotrophic methane formation (4 mol of hydrogen to 1 mol of methane). This phenomenon has been observed for other methanogenesis inhibitors as well (Ungerfeld et al., 2003; Martinez-Fernandez et al., 2016). This suggests that the predominant part of hydrogen is redirected into other sinks, when methanogenesis is inhibited.

Hydrogen accumulation may affect the thermodynamics of rumen processes, which consequently may cause a decreased DMI. Less carbon dioxide being reduced to methane may be coupled to a lower re-oxidation of NADH to NAD^+, which subsequently negatively affects rumen fermentation (Russell and Wallace, 1997). Hydrogen is a strong electron donor, and inhibiting the methanogenesis causes the major terminal electron accepting process in the rumen (respiratory reduction of carbon dioxide to methane) to be limited (Ungerfeld, 2015). Alternative electron acceptors are thus required, as indicated in the present study by the decreased ru-

men redox potential and increased concentrations of reduced compounds, such as valerate, formate, succinate, and C1 to C4 alcohols (methanol, ethanol, propanol, butanol, 2-butanol). Particularly the rumen concentration of ethanol was increased from 1.4 to 2.7 m*M* when 3-NOP was supplemented. The major part of ethanol is expected to be metabolized into acetate (Czerkawski and Breckenridge, 1972) through the reaction: $CH_3CH_2OH + H_2O \rightarrow CH_3COOH + 2H_2$ (Berg et al., 2012). However, in a situation where methanogenesis is inhibited and the hydrogen pressure is high, the acetate forming pathway does likely not proceed, which is also indicated in the present study by lower rumen acetate concentration, when 3-NOP was provided to the cows. Ethanol formation in the rumen may be a quantitative important hydrogen sink, but it was not quantified in the present study. Raun and Kristensen (2011) measured the net portal appearance of ethanol in lactating cows fed 2 diets (with 14 g of ethanol or propanol per kg DM), giving similar differences in ruminal ethanol concentrations as observed in the present study. Assuming that the similar difference in ruminal ethanol concentrations resulted in a similar difference in net portal appearance of ethanol, the observed increase in rumen ethanol concentration could indicate that ethanol constitutes a hydrogen sink equivalent to 4.5 g of hydrogen per day. Indeed, the 4.5 g of hydrogen per day rely on the assumptions that ethanol is formed at the expense of acetate, and that 1 mol of ethanol takes up 1 mol of hydrogen (H_2, with a molar weight of 2.016 g/mol) based on the stoichiometry. Thus, the observed increase in ruminal ethanol concentration indicates that ethanol formation could be a quantitatively important hydrogen sink. Furthermore, this could explain why the recovery of hydrogen not used for methane formation was below 30%, based on respiration chamber data.

Carbon Dioxide Formation

The carbon dioxide yield was higher than expected for cows with reduced methane production due to 3-NOP, based on stoichiometric calculations (assuming 1 mol more carbon dioxide, with 1 mol less methane). Thus, carbon dioxide yield (g/kg DM) increased 6% where only 1.8% was expected. Carbon dioxide yield was also increased 4.4% at a dose of 80 mg of 3-NOP/kg of DM in van Gastelen et al. (2022). Exhaled carbon dioxide originates from tissue aerobic metabolism of nutrients (Madsen et al., 2010; Patel et al., 2022). Furthermore, ruminal fermentation of glucose to acetate and butyrate produces 1 and 1.5 mol of carbon dioxide and 1 and 0.5 mol of methane per mol glucose fermented, respectively (Ungerfeld and Kohn, 2006). Therefore, any change in VFA profile induced by 3-NOP is also reflected in carbon dioxide recovery. In the present study, a carbon balance in the rumen was calculated, which together with VFA proportions was basis for quantifying the amount of carbon dioxide from fermentation as g/d and as proportion of measured carbon dioxide (%). There was no significant difference in carbon dioxide production from rumen fermentation (431 and 352 g/d for cows fed with or without 3-NOP, respectively). However, there was a tendency for the proportion of carbon dioxide from rumen fermentation out of measured carbon dioxide production, where 13% and 11% of the measured carbon dioxide originated from rumen fermentation for diets with or without 3-NOP, respectively. This result indicates that more carbon dioxide was eructed from the rumen as a result of altered ruminal fermentation and altered ruminal VFA concentrations, when 3-NOP diets were fed. This overall greater carbon loss in carbon dioxide result in a greater loss of dietary gross energy with 3-NOP.

Microbial Production, Efficiency, and Rumen Fermentation Products

Despite the lowered redox potential in the rumen, when cows were fed 3-NOP, and the fact that microbial biomass is an alternative hydrogen sink (Czerkawski, 1986), it is striking that 3-NOP both tended to reduce microbial OM production (kg/d) and decreased microbial CP production (kg/d). However, when related to DMI there was no effect of 3-NOP on microbial CP efficiency (g/kg DMI). Increased FA content in the feed has previously been reported to increase microbial AA synthesis (g/d) (Weisbjerg et al., 1992), despite the fact that fat supplementation inhibits the fibrolytic bacteria in the rumen, and biohydrogenation of FA redirects hydrogen into the saturation of unsaturated FA. In the present study, microbial efficiency (g CP/kg OM truly digested in the rumen) was unaffected of the fat supplementation, despite the values of microbial efficiency were numerically increased when the cows were fed the high fat diets.

Jayanegara et al. (2018) reported in their meta-analysis that rumen concentration of total VFA was reduced when cows were fed 3-NOP, as also observed in the present study. The decreased total VFA concentration was likely caused by the lower DMI, and thereby lower fermentation of OM to VFA. Acetate is the main driver for rumen pH (Allen, 1997), and a reduction in acetate proportion and an concurrent increase in rumen pH was observed in the present study for cows fed 3-NOP; findings that have also been reported previously (Jayanegara et al., 2018). Fat supplementation also had a reducing effect on rumen concentration of total VFA in the present study, which was expected

since fat supplementation inhibits the rumen microbes (Giger-Reverdin et al., 2003) and, furthermore, fat is not fermented in the rumen. However, rumen total VFA concentrations were not reduced in Brask et al. (2013b) and Beauchemin et al. (2009), as well as no effects on rumen pH were observed. In contrast, although the concentration of total VFA was reduced in the present study, rumen pH was not affected by fat supplementation.

Fermentation of 1 mol glucose to 2 mol of acetate yields more energy to the microbes in terms of Gibbs free energy change and liberated ATP [ΔG (kJ/mol) = −305, ATP = 4] compared with fermentation of 1 mol glucose to 1 mol of butyrate [ΔG (kJ/mol) = −301, ATP = 3] (Wolin et al., 1997; Kristensen et al., 2003). However, in a situation where hydrogen pressure is increased, and there is a greater need for alternative electron acceptors, fermentation with butyrate as the end product is more favorable, as it liberates less H^+ and e^- per mol of glucose (0.66 electron pair compared with 1 electron pair for the acetate pathway) (Kristensen et al., 2003). This might be the reason why higher butyrate proportion and lower acetate proportion were observed in the present study when 3-NOP was fed to the cows. The same principle applies for lactate concentration (Dijkstra, 1994), but probably due to the fact that lactate is an intermediate product in the acrylate pathway (Cummings and Macfarlane, 1991), rumen concentration of L-lactate was lower for cows fed 3-NOP, compared with cows fed no 3-NOP in the present study. An interaction was observed between fat supplementation and 3-NOP with respect to valerate proportion, and a meta-analysis have shown that inhibition of methanogenesis is associated with increased rumen valerate concentration (Ungerfeld, 2018). Based on stoichiometry, production of valerate does not cause a production of carbon dioxide or hydrogen (Brask et al., 2015). However, valerate can be a result of fermentation of both amino acids and carbohydrates (Nagaraja et al., 1997), therefore the reasons for our results are not clear. No effect of 3-NOP or fat supplementation was found with respect to caproate proportion; however, both valeric and caproate are reduced compounds, therefore it is important to report these in studies where methanogenesis is inhibited (Ungerfeld, 2015). Rumen propionate concentration was surprisingly not affected by the diets in the present study (see discussion about DMI).

Milk Production and Plasma Metabolites

Data from milk production from Latin square designs with 4 cows and periods of 3 weeks, should be interpreted with care, however tendencies with respect to milk data were observed in the present study. Higher energy intake and likewise higher intake of metabolizable energy drives milk production up (Coulon and Rémond, 1991; Yan et al., 2006), and consequently the reduced DMI in the present study tended to decrease milk production (kg/d) and ECM (kg/d), when cows were fed 3-NOP. The milk fat percentage was increased 3.7% when cows were fed 3-NOP. Previously it has been reported that milk fat content would decrease, if energy intake increased (Coulon and Rémond, 1991), and milk fat yield explained from 49% to 66% of the variation of cow's energy balances in Xu et al. (2018). In the present study, the increased fat yield when cows were fed 3-NOP must be seen in a context with the reduced DMI and a tendency to decreased milk yield. However, the study was performed with 4 cows, and studies with more cows receiving 3-NOP for longer periods are needed to clarify the effect of 3-NOP on milk yield (van Gastelen et al., 2022).

Lactose is the main component driving osmolarity in milk (Rigout et al., 2002). However, in the present study milk production was unaffected when cows were supplied with high levels of fat, but lactose percentage was increased. Previous studies report no effect of fat on lactose percentage (Martin et al., 2008; Brask et al., 2013b).

Increased plasma concentrations of NEFA and BHB are indicators of negative energy balance (Adewuyi et al., 2005). The blood concentration of NEFA in the present study was unaffected by 3-NOP, whereas 3-NOP and fat supplementation tended to interact with respect to BHB concentration in the blood ($P = 0.08$), where fat supplementation reduced, and 3-NOP increased the concentration of BHB. With respect to fat supplementation, the effect of BHB concentration was opposite to that expected, as increased fat supply previously has been reported to increase the BHB concentration (Moallem et al., 2007). Furthermore, increased absorption of FA can also increase concentration of NEFA (Grummer and Carroll, 1991), which can explain the increased NEFA concentration in plasma when cows received fat supplementation. The measured levels for NEFA and BHB are both below the threshold for developing subclinical ketosis (Raboisson et al., 2014).

CONCLUSIONS

No interaction between fat supplementation and 3-NOP was observed for methane production, yield, or intensity, neither for DMI or total-tract digestibility of nutrients. Fat supplementation only tended to reduce methane as a percentage of GEI by 10%, whereas methane production, yield, and intensity were decreased by 3-NOP. A decrease in DMI was observed

when 3-NOP was fed to the cows, and tendencies to increased rumen NDF digestibility and total-tract NDF digestibility were also observed. Hydrogen emission increased when 3-NOP was fed; however, the data indicate that inhibition of methanogenesis lead to an upregulation of alternative hydrogen-utilizing pathways with other electron acceptors than carbon dioxide (e.g., ethanol and formate formation). Clarifying the quantitative importance of rumen hydrogen sinks other than methanogenesis, as highlighted in the present study, is highly relevant in future studies on methane mitigating strategies. Moreover, the reduced DMI with 3-NOP was intriguing but the underlying mechanism is unclear and warrants further investigations.

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