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Effects of 3-nitrooxypropanol (Bovaer10) and whole cottonseed on milk production and enteric methane emissions from dairy cows under Swiss management conditions

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ABSTRACT

The objective of this study was to determine the potential effect and interaction of 3-nitrooxypropanol (3-NOP; Bovaer, DSM-Firmenich Nutrition Products Ltd.) and whole cottonseed (WCS) on lactational performance and enteric methane (CH₄) emission of dairy cows. A total of 16 multiparous cows, including 8 Holstein Friesian (HF) and 8 Brown Swiss (BS; 224 ± 36 DIM, 26 ± 3.7 kg milk yield, mean ± SD), were used in a split-plot design, where the main plot was the breed of cows. Within each subplot, cows were randomly assigned to a treatment sequence in a replicated 4 × 4 Latin square design with 2 × 2 factorial arrangements of treatments with four 24-d periods. The experimental treatments were as follows: (1) control (basal TMR), (2) 3-NOP (60 mg/kg TMR DM), (3) WCS (5% TMR DM), and (4) 3-NOP + WCS. The treatment diets were balanced for ether extract, crude protein, and NDF contents (4%, 16%, and 43% of TMR DM, respectively). The basal diets were fed twice daily at 0800 and 1800 h. Dry matter intake and milk yield were measured daily, and enteric gas emissions were measured (using the GreenFeed System, C-Lock Inc.) during the last 3 d of each 24-d experimental period when animals were housed in tiestalls. There was no difference in DMI on treatment level, whereas the WCS treatment increased ECM yield and milk fat yield. No interaction of 3-NOP and WCS occurred for any of the enteric gas emission parameters, but 3-NOP decreased CH₄ production (g/d), CH₄ yield (g/kg DMI), and CH₄ intensity (g/kg ECM) by 13%, 14%, and 13%, respectively. Further, an unexpected interaction of breed by 3-NOP was observed for different enteric CH₄ emission metrics: HF cows had a greater CH₄ mitigation effect compared with BS cows for CH₄ production (g/d; 18% vs. 8%), CH₄ intensity

(g/kg milk yield; 19% vs. 3%), and CH₄ intensity (g/kg ECM; 19% vs. 4%). Hydrogen production was increased by 2.85-fold in HF and 1.53-fold in BS cows receiving 3-NOP. Further, a 3-NOP × time interaction occurred for both breeds. In BS cows, 3-NOP tended to reduce CH₄ production by 18% at approximately 4 h after morning feeding, but no effect was observed at other time points. In HF cows, the greatest mitigation effect of 3-NOP (29.6%) was observed immediately after morning feeding, and it persisted at around 23% to 26% for 10 h until the second feed provision, and 3 h thereafter, in the evening. In conclusion, supplementing 3-NOP at 60 mg/kg DM to a high-fiber diet resulted in 18% to 19% reduction in enteric CH₄ emission in Swiss HF cows. The lower response to 3-NOP by BS cows was unexpected and has not been observed in other studies. These results should be interpreted with caution due to the low number of cows per breed. Finally, supplementing WCS at 5% of DM improved ECM and milk fat yield but did not enhance the CH₄ inhibition effect of 3-NOP of dairy cows.

Key words: enteric methane mitigation, whole cottonseed, 3-NOP, dairy cow

INTRODUCTION

The expanding livestock supply chain, in response to increasing global food demand, is accompanied by the production of approximately 17% of global food system-related greenhouse gas emissions (Jackson et al., 2020), out of which 30% is attributed to methane (CH₄) from ruminants (Jackson et al., 2020; Crippa et al., 2021). Milk production, in turn, contributes around 30% of the emissions from ruminants, of which about 44% is in the form of CH₄ (Opio et al., 2013). Therefore, effort has been put into finding effective ways to reduce this contribution without compromising animal productivity or health and welfare.

Oilseeds, such as whole cottonseed (WCS), can serve as a nutritious feed component with its high energy and

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

protein contents, coupled with a CH₄ mitigation potential due to its high dietary fat content. However, the effect of WCS on milk production has shown discrepancies between studies. Many studies have reported minor or little effects of WCS on milk yield (MY), milk fat yield, and fatty acid (FA) profile when WCS was supplemented up to 30% of DM (Coppock et al., 1987; Pierce et al., 2023). However, Muñoz et al. (2019) used a WCS content of 18.4% of DM and observed improved milk MUFA and PUFA. Similarly, the CH₄ mitigation effect of WCS has been inconsistent: a reduction of up to 42% was reported with a dietary inclusion of 30% of DM of WCS (Nogueira et al., 2020). Conversely, Johnson et al. (2002) reported no effect on CH₄ emission when WCS was supplemented at 4.6% or 9.2% DM with canola seeds (at 2.3% and 4.6% DM). To date, the CH₄ mitigating effect of WCS has been mostly attributed to its high fat content, especially UFA. However, WCS also contains gossypol, a phenolic compound that has recently been linked to reductions in the abundance of protozoa and archaea, as well as acetate concentration in vitro (Castro Veloz, 2023). Ismartoyo et al. (1993) reported in a 12-d in vitro experiment that adding 1 mM (approximately 535 mg/kg) gossypol reduced gas production by 27.4%, but the inhibitory effect was temporary, and the rumen microorganisms adapted to the presence of gossypol. However, data investigating the specific effects of gossypol on methanogenesis in vivo are scarce.

Apart from the plant-derived compounds, 3-nitrooxypropanol (3-NOP) is a reliable CH₄ inhibitor. Its patented product form, Bovaer (DSM-Firmenich Nutrition Products Ltd., Kaiseraugst, Switzerland) has been approved by the European Food Safety Authority for application in dairy cow diets and for all ruminants (including dairy and beef) in many other countries. The mode of action of 3-NOP involves the direct inhibition of methanogenesis by deactivating the enzyme methyl-coenzyme M reductase (Duin et al., 2016). Consequently, this inhibits the growth of methanogenic archaea. The mitigation potential of 3-NOP in dairy cattle has been extensively investigated in the past decade (Kebreab et al., 2023). In dairy cattle, Kebreab et al. (2023) reported an average of 32.7% reduction of enteric CH₄ production with an average dose of 70.5 mg/kg DM 3-NOP. The efficacy of 3-NOP, however, is greatly influenced by other factors such as dosage, type of cattle, and nutrient composition of basal diet, specifically the NDF, fat, and starch contents (Dijkstra et al., 2018; Yu et al., 2021). For example, based on a recent meta-analysis by Kebreab et al. (2023) the mitigation effect of 3-NOP increases by 0.915% for each 1% decrease in dietary NDF content. Hence, the efficacy of 3-NOP is expected to be lower in high-forage and high-fiber diets.

Research has been conducted on the effects of WCS and 3-NOP individually in relation to CH₄ emission and production performance. However, exploration is lacking regarding the possible interactions between 3-NOP and WCS in dairy cow rations, specifically when their methane mitigating potentials have been associated with an inhibition of methanogenic archaea. Our pilot study (Ma et al., 2023) explored the effect of combining 3-NOP with 4 different plant-based compounds, among which WCS was included at 5% and 10% of DM. The results suggested that combining 3-NOP with 5% WCS may enhance the efficacy of 3-NOP in terms of CH₄ intensity due to a numerical increase in MY, whereas WCS fed at 10% of DM did not improve production performance or CH₄ mitigation. However, our pilot study was not designed to test whether WCS alone can decrease absolute CH₄ emission, because the pilot study did not have WCS alone as one of the treatments. Nevertheless, the observed additional decrease in CH₄ intensity when 3-NOP and WCS were combined revealed the potential of WCS to reduce absolute CH₄, which in turn may lead to further mitigation of CH₄ intensity, considering the beneficial effect of WCS on MY. To our knowledge, previous studies have associated the mitigating effects of WCS with its fat (Johnson et al., 2002) or gossypol (Ismartoyo et al., 1993) content. Although the effect of using varying fat levels on enteric CH₄ emission has been extensively studied (Grainger et al., 2008; Muñoz et al., 2019, 2021), the literature focusing specifically on gossypol, especially in vivo studies, is scarce. Therefore, in the current study, we decided to balance for the dietary fat content and to focus on the lesser-known mitigating effects of gossypol on enteric CH₄ emissions.

Hence, the main objective of this experiment was to determine the effects of 3-NOP and gossypol-containing WCS, and their interactions, on CH₄ emissions and lactational performance of dairy cows fed high-fiber diets. Second, we aimed to examine the diurnal variations of gas emissions and feed intake of cows receiving WCS and 3-NOP. We hypothesized that 3-NOP and WCS would exhibit additive mitigating effects on enteric CH₄ emissions. Furthermore, we hypothesize that the mitigating effect from the feed additives observed throughout the day aligns with the pattern of feed intake.

MATERIALS AND METHODS

Experimental Design and Treatments

The experiment was conducted from September to December of 2022 at ETH Zürich, Agrovet-Strickhof Research Station (Lindau, Switzerland). All procedures were approved by the Cantonal Veterinary office of

Zürich, Switzerland (license no. ZH207/2021). The use of Bovaer10 (DSM-Firmenich Nutritional Products Ltd.; Kaiseraugst, Switzerland) in dairy cow feed was authorized in Switzerland in April of 2022.

Sixteen multiparous lactating dairy cows were arranged in a split-plot design, where the main plot was the breed of cows (8 Holstein Friesian [HF] and 8 Brown Swiss [BS] cows). The HF cows averaged (mean \pm SD) 223 \pm 33 DIM, 27.0 \pm 3.99 kg/d MY, and 704 \pm 24 kg BW, and the BS averaged 224 \pm 40 DIM, 25.8 \pm 3.59 kg/d MY, and 751 \pm 53 kg BW at the beginning of the experiment. Within each subplot, cows were randomly assigned to a treatment sequence in a replicated 4 \times 4 Latin square design with a 2 \times 2 factorial arrangement, with 2 supplementation categories of 3-NOP (Yes and No) and 2 supplementation categories of WCS (Yes and No). The experiment had four 24-d periods, each with 19 d of dietary adaptation and 5 d of sampling (d 20–24). The 4 dietary treatments were as follows: (1) the basal control diet (CON), (2) CON with 3-NOP supplementation (3-NOP; 60 mg/kg DM, provided through Bovaer10 at 600 mg/kg DM), (3) basal diet with WCS (WCS; 5% DM, Grigi Cereali e Mangimi Nuovo Molino di Assisi, Bastia Umbra, Italy), (4) a diet containing both 3-NOP and WCS (3-NOP + WCS). Diets without 3-NOP were supplemented with a silicon dioxide and propylene glycol-based placebo containing no 3-NOP.

The ingredients and chemical compositions of the experimental diets are presented in Table 1. All treatments were fed ad libitum at 110% of the previous daily intakes. All cows were fed twice per day at 0800 h and 1800 h at a 60:40 ratio. The 3-NOP or placebo was incorporated in a ground corn-based premix (65% ground corn, 30% Bovaer10, and 5% sunflower oil) before mixing in the TMR at each feeding to reach a target concentration of 60 mg/kg DM. The analyzed concentration of 3-NOP in the premix was 37.3 \pm 2.88 g/kg of DM (n = 12).

During the first 17 d of dietary adaptation period, cows were housed together in a freestall pen with free access to water. Feed intakes were recorded using individual feeding troughs with electronic load cells (Waagen Döhrn GmbH, Wesel, Germany) and an automated identification system (American Calan Inc., Northwood, NH). Feeding troughs were randomly assigned to each cow, and the assignments were maintained throughout the experiment. From d 18 to 24 of each period, cows were housed in a tiestall barn equipped with a feed intake observation system (individual calibrated built-in floor scales; PFA575 Mettler Toledo, Greifensee, Switzerland) with free access to fresh water and feed. The lying cubicle was covered by rubber mats with straw and wood shavings as bedding. Straw and wood shavings were removed during the total collection of feces (d 21 to d 24 of each experimental period). Body weight

was recorded at the end of each period on a cattle scale (Terra ET, Waagen Döhrn).

Data and Sample Collection and Analysis

Cows were milked twice daily at 0530 h and 1530 h throughout the experiment. From d 20 to d 24 of each period, MY were recorded, and milk samples were collected at each milking, preserved with a bronopol pill. The bronopol-conserved milk was analyzed for fat, crude protein, lactose, and urea contents according to International Organization for Standardization (9622; ISO, 2013) using a Fourier-transform infrared spectrophotometer (MilkoScan RM 6 FT6000) at SuisseLab AG (Zollikofen, Switzerland). Milk urea nitrogen was calculated as urea concentration (mg/dL) \times 0.466 and milk true protein as milk crude protein (%) $-$ MUN (mg/dL) \times 6.38/1,000. Milk FA profile was analyzed with a gas chromatograph as described in Birkinshaw et al. (2019). Internal standards (5 mL of n-heptane containing triundecanoic, tetradecanoic methylate, and trivaleranolin) were added to 0.5 mL of milk sample.

Grass and corn silages were sampled once a week and dried at 55°C for 48 h for adjustment of DM content. Alfalfa hay, WCS, concentrate feeds, and pelleted feed were sampled on d 18 and 20 of each experimental period. Total mixed rations and orts were sampled on d 18, 21, and 24 of each period, and samples were stored at -20°C . To determine the apparent total-tract digestibility (ATTD), total collection of feces was conducted separately on d 21 to 24 of each period. Urine was separated using urinals (Birkinshaw et al., 2023) attached to the cow. Feces were collected in a flat steel drawer (119 \times 115 \times 6 cm) placed under an iron grid under each tiestall. The weight of feces was measured twice daily at 0900 h and 2100 h. A subsample of feces was collected after each weighing. Feed ingredients, TMR, orts, pellets, and fecal samples were dried at 55°C for 48 h and milled to pass through a 1-mm screen sieve using a centrifugal mill (ZM 1, Retsch GmbH, Haan, Germany). Total mixed ration samples were composited by treatment and period, and orts of each cow were composited by period, by equal weight on DM basis. Fecal samples were proportionally composited on DM basis for each cow by period based on the fecal outputs from each collection. All samples were analyzed for DM, ash, NDF, and ADF content and feed samples were additionally analyzed for nitrogen (N), starch, ether extract (EE), crude fiber, and gross energy (GE) according to the official methods of analysis (AOAC International, 1997) and performed in duplicate as described by Birkinshaw et al. (2022). Dry matter and total ash were determined using a thermogravimetric device (TGA-701, Leco; AOAC official method 942.5); OM was calculated as the difference

Table 1. Ingredient and nutrient composition of the diets fed in the experiment

Item	Diet ¹			
	CON	3-NOP	WCS	3-NOP + WCS
Feed ingredients, % of DM				
Corn silage ²	37.6	36.9	33.6	32.9
Grass silage ³	16.1	15.8	14.4	14.1
Alfalfa haylage ⁴	16.1	15.8	16.0	15.7
Energy concentrate ⁵	10.7	10.5	16.0	15.7
Protein concentrate ⁶	10.0	9.80	4.50	4.40
Mineralized concentrate ⁷	7.20	7.10	8.60	8.40
Ground corn	1.60	1.60	1.90	1.80
WCS ⁸	—	—	5.00	5.00
Sunflower oil ⁹	0.7	0.7	—	—
Bovaer10 premix ¹⁰	—	2.00	—	2.00
Chemical composition, % of DM				
CP	15.9	15.9	16.0	16.0
NDF	43.2	43.2	42.7	42.7
ADF	24.5	24.5	25.7	25.7
EE	3.98	3.98	3.98	3.98
Total fatty acids (FA)	2.62	2.58	3.03	2.74
Saturated FA	0.005	0.005	0.008	0.007
MUFA	0.007	0.007	0.006	0.006
PUFA	0.014	0.014	0.016	0.016
Starch	22.1	22.1	22.4	22.4
Ash	7.88	7.88	7.96	7.96
Total gossypol, mg/kg	—	—	455	455
Free gossypol, mg/kg	—	—	320	320
GE, MJ/kg of DM	18.7	18.7	18.6	18.6
NE _L , ¹¹ MJ/kg of OM	5.15	5.15	5.11	5.11

¹CON = basal diet; 3-NOP = diet supplemented with 3-nitrooxypropanol (3-NOP concentration = 60 mg/kg DM); WCS = diet supplemented with whole cottonseed (WCS concentration = 50 g/kg DM); 3-NOP + WCS = diet supplemented with 3-NOP and WCS.

²Corn silage was 35.5% DM and contained (DM basis) 6.3% CP and 52.5% NDF.

³Grass silage was 34.8% DM and contained (DM basis) 11.9% CP and 65.2% NDF.

⁴Alfalfa haylage was 90.5% DM and contained (DM basis) 12.3% CP and 53.0% NDF.

⁵UFA-249 (UFA, Sursee, Switzerland) contained (% as-is basis) CP, 39.0; crude fiber, 4.0; crude fat, 5.5; crude ash, 6.5; bypass protein + microbial protein synthesized from ruminal available energy (APDE), 26.5; bypass protein + microbial protein synthesized from ruminal available energy (APDN), 30.0; NE_L, 0.7; calcium, 0.8; phosphate, 0.7; sodium, 0.15; magnesium, 0.4.

⁶UFA-173 (UFA, Sursee, Switzerland) contained (% as-is basis) CP, 24.0; crude fiber, 3.0; crude fat, 7.2; crude ash, 5.0; APDE, 16.0; APDN, 17.0; NE_L, 0.8; calcium, 0.8; phosphate, 0.5; sodium, 0.2; magnesium, 0.3.

⁷Agrovet/Talheim concentrate (no. 50109, Getreidesammelstelle, Thalheim, Switzerland) contained (% as-is basis) CP, 29.0; crude fiber, 2.9; crude fat, 2.6; APDE, 15.9; APDN, 20.6; NE_L, 5.9; calcium, 2.7; magnesium, 10.7; phosphorus, 12.3; sodium 22.2; zinc, 0.32; manganese, 1.0; copper, 0.1; iodine, 0.01; selenium, 0.003; cobalt, 0.003; vitamin A, 1,200,000 IU; vitamin D₃, 200,000 IU; vitamin E, 0.3; biotin, 0.015.

⁸WCS was 92.6% DM and contained (DM basis) CP, 21.9%; NDF, 58.9%; EE, 17.3%; total gossypol, 0.91%; free gossypol, 0.64%; gross energy, 21.1 MJ/kg; SFA, 25.4% total FA; MUFA, 17.8% total FA; PUFA, 56.8% total FA.

⁹Sunflower oil contained (% total FA) SFA, 10.6; MUFA, 34.7; PUFA, 54.7.

¹⁰Bovaer10 premix contained (% as-is basis) Bovaer10 (10% 3-NOP on silicon dioxide + 1,2-propanediol), 30; sunflower oil, 5; ground corn, 65.

¹¹Estimated based on Agroscope (2017) using CP, EE, crude fiber, and nitrogen-free extract (NfE) on OM basis of the feed ingredients sampled throughout the experiment. $NE_{L,OM} = -13.67 + 0.0226 CP + 0.0358 EE + 0.0074$ crude fiber + 0.0222 NfE. $NfE = DM = (EE + CP + ash + crude fiber)$.

between DM and total ash. Bomb calorimetry (C7000, IKA-Werke) was used to determine the GE of the dietary components. Nitrogen in feed components was analyzed by a carbon/nitrogen analyzer (TruMac CN, Leco; AOAC official method 968.06). Crude protein was calculated as $N \times 6.25$. Ether extract was determined using a Soxhlet extractor (Extraction System B-811, Büchi; AOAC official method 963.15), and a Fibertherm FT 12

(Gerhardt GmbH and Co. KG, Königswinter, Germany) to determine ash-corrected detergent fiber fractions. Heat-stable α -amylase (Sigma-Aldrich, St. Louis, MO) was used with NDF (AOAC official method 2002.04) and ADF (AOAC official method 973.18) analysis. Feed FA were analyzed as described in Birkinshaw et al. (2022) and are reported in Supplemental Table S1 (see Notes; Ma et al., 2024). Extraction was performed with

a solvent extractor using hexane and transformed to FA methyl esters.

Feed weight was recorded every 10 s from d 20 to 24 of each period using an automated feed monitoring system (Mettler Toledo GmbH; Greifensee, Switzerland). Daily feed intake (FI) was calculated as the difference between offered and refused feed. Hourly FI measurements were calculated from continuous weight measurements of the feeding plates on as-fed basis. The rate of FI was calculated as the percent of daily FI per hour.

Enteric emissions of CH₄, H₂, and CO₂ were measured using the GreenFeed System (GF; C-Lock Inc., Rapid City, SD) 8 times during the last 3 d of each experimental period to represent a 24-h feeding cycle: 0900, 1500, and 2100 h (sampling d 1); 0300, 1200, and 1900 h (sampling d 2); and 0000 and 0600 h (sampling d 3) based on a method adapted from Hristov et al. (2015). The minimum air flow during measurement period was 32.3 L/s. Cows were trained to use GF 1 wk before the start of the experiment. A pelletized bait feed with a 7-mm diameter (formulated to contain 50% alfalfa hay, 10% soybean meal, 35% ground corn, and 5% molasses) was specifically produced on site for this experiment. The production was carried out using a nonindustrial pellet presser (model RP18, Ekokraft) that operates in the range of 55°C to 75°C. The pellets were used to entice the cow to maintain a suitable head position in the GF during measurements. The pellets were 89% DM and contained (DM basis) 32.6% NDF and 18.7% CP. The GF was configured to dispense 5 drops of pellets at a 30-s interval to ensure 5-min continuous measurement. Each drop of pellet weighs around 36 g. Pellet consumption was included in the daily DMI calculation during sampling days. Each cow was assigned with a radio-frequency identification (RFID) tag attached to a collar for recognition by GF. The GF unit was mounted on a tiestall cart to be moveable between stalls. Upon each measurement time point, the GF unit was calibrated by a standard zero baseline gas and span baseline gas (Linde Gas Benelux BV). Between cows, the GF was moved away from the animal for at least a 2-min wash-out to acclimate to background air concentration. Recovery of CO₂ test was performed before each sampling period. Raw data management was handled by C-Lock Inc. (Rapid City, SD), and the statistical analyses were performed on the validated dataset.

Statistical Analysis

All data analyses were performed in R version 4.4.0 (R Foundation for Statistical Computing). Daily FI, milk production, apparent total-tract nutrient digestibility, feeding and rumination parameters, and gas emissions were analyzed using mixed model with the *lmer* proce-

dure of R (Bates et al., 2015), and denominator degrees of freedom were adjusted by the Kenward-Roger method. The full model was as follows:

$$Y_{ijklmn} = \mu + S_i + P_j + C_k(S_i) + B_l + N_m + W_n + B_l \times N_m + B_l \times W_n + N_m \times W_n + B_l \times N_m \times W_n + e_{ijklmn},$$

where Y_{ijklmn} is variable of interest, μ is the overall mean, S_i is random effect of sequence ($i = 1$ to 4), P_j is the fixed effect of period ($j = 1$ to 4), $C_k(S_i)$ is the random effect of cow nested in sequence ($k = 1$ to 16), B_l is the fixed effect of main plot (breed of cows; $l = 1$ to 2), N_m is the fixed effect of 3-NOP ($m = 1$ to 2), W_n is the fixed effect of WCS ($n = 1$ to 2), $B_l \times N_m$ is the interaction of main plot by 3-NOP, $B_l \times W_n$ is the interaction of main plot by WCS, $N_m \times W_n$ is the interaction of 3-NOP by WCS, $B_l \times N_m \times W_n$ is the 3-way interaction of main plot, 3-NOP, and WCS, and e_{ijklmn} is the random residual.

In the case of enteric CH₄ production, the 3-way interaction was not present, whereas a main plot by 3-NOP interaction was shown. Therefore, the enteric gas production data in 3-h intervals and hourly feeding behavior data were analyzed using a mixed model with repeated measures of time using the *lmer* procedure (Bates et al., 2015). The model was as follows:

$$Y_{ijklmno} = \mu + S_i + P_j + C_k(S_i) + B_l + N_m + W_n + T_o + B_l \times T_o + e_{ijklmno},$$

where $Y_{ijklmno}$ is the intraday gas emission or behavior data, T_o is time of day ($o = 1$ to 8, and 1 to 24 for enteric gas production and behavior data, respectively). Other terms in the model are as previously described.

Data points with studentized residuals outside of ± 3 were considered outlier and were removed from the analysis. Rarely more than 3 entries were removed in all analysis; the final sample sizes for each variable are specified in the footnotes of each table. Milk yield and MY:DMI ratio data from one cow with higher DIM and low milk yield toward the end of experiment was excluded from the dataset. Multiple comparisons among treatment means were conducted by using the Tukey-Kramer method. Statistical differences were declared at $P \leq 0.05$. Tendencies were declared at $0.05 < P \leq 0.10$. The interaction term was excluded from the full model when nonsignificant. Subsequently, the data were fitted to a partial model without interaction terms. Data are presented as least squares means from the final model.

RESULTS

There were no 3-way interactions of 3-NOP \times WCS \times breed for any of the variables except for mixed milk

Table 2. Production performance of lactating dairy cows from 2 breeds¹ receiving dietary treatments²

Item	3-NOP ³			WCS ⁴			Breed ⁵			P-value ⁶						
	No	Yes	SE ⁷	No	Yes	SE	BS	HF	SE	3-NOP	WCS	Breed	3-NOP × WCS	3-NOP × Breed	WCS × Breed	3-NOP × WCS × Breed
Yield, kg/d	24.4	24.4	1.22	24.2	24.5	1.22	23.9	24.8	1.70	0.91	0.52	0.49	0.07	0.61	0.47	0.98
Milk ⁸	28.0	28.3	1.42	27.6 ^b	28.8 ^a	1.42	27.9	28.5	1.98	0.61	0.03	0.82	0.60	0.93	0.07	0.23
ECM ⁹	1.04	1.04	0.057	1.01	1.07	0.057	1.04	1.04	0.079	0.73	0.01	0.98	0.89	0.99	0.04	0.13
Milk fat ¹⁰	0.891	0.877	0.0411	0.872	0.896	0.0441	0.869	0.899	0.0611	0.44	0.19	0.73	0.28	0.77	0.27	0.53
Milk lactose	1.13	1.13	0.065	1.13	1.14	0.065	1.10	1.16	0.091	0.95	0.72	0.67	0.27	0.86	0.54	0.45
Milk composition, %																
Milk fat ¹¹	4.32	4.35	0.139	4.29	4.39	0.139	4.45	4.23	0.189	0.94	0.09	0.49	0.24	0.79	0.10	0.09
Milk true protein	3.67	3.68	0.059	3.68	3.67	0.059	3.71	3.64	0.078	0.82	0.70	0.59	0.98	0.57	0.72	0.87
Milk lactose	4.62	4.65	0.058	4.62	4.65	0.057	4.62	4.65	0.073	0.63	0.50	0.73	0.69	0.43	0.43	0.51
MUN, mg/dL	13.3	13.6	0.48	13.3	13.6	0.48	14.6 ^a	12.2 ^b	0.65	0.29	0.28	0.02	0.95	0.29	0.30	0.62
Milk yield:DMI	0.99	0.99	0.040	0.99	0.99	0.040	1.03	0.96	0.055	0.96	0.70	0.40	0.85	0.36	0.56	0.50
SFA, % FA	68.5	68.3	0.62	68.1 ^b	68.8 ^a	0.62	68.5	68.4	0.841	0.54	0.05	0.93	1.00	<0.01	0.37	0.97
MUFA, % FA	26.7	26.9	0.54	27.1 ^a	26.5 ^b	0.54	26.8	26.8	0.730	0.59	0.05	0.99	0.81	<0.01	0.75	0.75
PUEFA, % FA	4.57	4.64	0.11	4.69	4.52	0.11	4.56	4.65	0.134	0.51	0.12	0.64	0.23	<0.01	0.06	0.08
De novo, % FA	28.2	28.4	0.38	28.8 ^a	27.9 ^b	0.38	28.4	28.3	0.449	0.60	0.04	0.87	0.50	0.03	0.75	0.99
Mixed, % FA	34.6	34.4	0.88	33.9 ^b	35.1 ^a	0.88	33.8	35.2	1.231	0.58	<0.01	0.47	0.42	0.21	0.43	0.04
Preformed, % FA	37.2	37.3	0.91	37.4	37.1	0.91	37.9	36.6	1.250	0.82	0.60	0.45	0.80	<0.01	0.55	0.30
Trans-FA, % FA	3.78	3.76	0.09	3.86	3.68	0.09	3.73	3.82	0.109	0.84	0.06	0.56	0.61	0.28	0.39	0.06
OBCFA, % FA	2.69 ^b	2.89 ^a	0.07	2.77	2.81	0.07	2.73	2.85	0.095	<0.01	0.33	0.41	0.38	<0.01	0.98	0.96
BW, % kg	753	765	6.8	742	775	6.8	747	771	6.8	0.25	<0.01	0.02	0.37	1.00	0.94	0.47
BW change, kg/d	0.64	0.77	0.088	0.61	0.80	0.088	0.65	0.76	0.088	0.42	0.21	0.13	<0.01	0.03	0.37	0.40

^{a,b}In table (within a row) and in footnotes, different superscript letters indicate a significant ($P < 0.05$) difference.

¹BS = Brown Swiss; HF = Holstein Friesian.

²Dietary treatments were CON = basal diet; diet supplemented with 3-nitrooxypropanol (3-NOP, concentration = 60 mg/kg DM); diet supplemented with whole cottonseed (WCS, concentration = 50 g/kg DM); diet supplemented with 3-NOP + WCS. [3-NOP No] is the LSM of CON and WCS diets; [3-NOP Yes] is the LSM of 3-NOP and 3-NOP+WCS diets; [WCS No] is the LSM of CON and 3-NOP diet; [WCS Yes] is the LSM of WCS and 3-NOP+WCS diets.

³Values reported as LSM for the [3-NOP No] and [3-NOP Yes] treatments, average over [No/Yes WCS] and [HF/BS breed].

⁴Values reported as LSM for the [WCS No] and [WCS Yes] treatments, average over [No/Yes 3-NOP] and [HF/BS breed].

⁵Values reported as LSM for the BS and HF breed, average over [No/Yes 3-NOP] and [No/Yes WCS].

⁶P-values obtained from fitting full model.

⁷Sample size of the largest SE published in table: n = 64 for DMI; n = 63 for MY; n = 64 for ECM, fat yield, protein yield, lactose content, MUN content, SFA content, MUFA content, de novo FA content, preformed FA content; n = 63 for milk yield, DMI, PUFA content, mixed FA content, trans-FA content.

⁸MY, 3-NOP × WCS interaction; [3-NOP No WCS No] = 24.7^a, [3-NOP Yes WCS No] = 23.8^a, [3-NOP No WCS Yes] = 24.2^a, [3-NOP Yes WCS Yes] = 24.9^a.

⁹ECM yield (kg/d) = 12.95 × fat (kg/d) + 7.65 × protein (kg/d) + 0.327 × milk yield (kg/d) (DRMS, 2014).

¹⁰Milk fat yield, 3-NOP × WCS × Breed interaction; [BS 3-NOP No WCS No] = 0.95^a, [BS 3-NOP Yes WCS No] = 1.01^a, [BS 3-NOP No WCS Yes] = 1.11^a, [BS 3-NOP Yes WCS Yes] = 1.08^a, [HF 3-NOP No WCS No] = 1.05^a, [HF 3-NOP Yes WCS No] = 1.02^a, [HF 3-NOP No WCS Yes] = 1.03^a, [HF 3-NOP Yes WCS Yes] = 1.07^a, [BS WCS No] = 0.98^b, [BS WCS Yes] = 1.10^a, [HF WCS No] = 1.04^{ab}, [HF WCS Yes] = 1.05^{ab}.

¹¹Milk fat content, 3-NOP × WCS × Breed interaction; [BS 3-NOP No WCS No] = 4.14^a, [BS 3-NOP Yes WCS No] = 4.40^a, [BS 3-NOP No WCS Yes] = 4.65^a, [BS 3-NOP Yes WCS Yes] = 4.44^a, [HF 3-NOP No WCS No] = 4.25^a, [HF 3-NOP Yes WCS No] = 4.19^a, [HF 3-NOP No WCS Yes] = 4.21^a, [HF 3-NOP Yes WCS Yes] = 4.24^a.

¹²SFA content, 3-NOP × Breed interaction; [BS 3-NOP No] = 69.2^a, [BS 3-NOP Yes] = 67.8^a, [HF 3-NOP No] = 67.9^b, [HF 3-NOP Yes] = 69.1^a.

¹³MUFA content, 3-NOP × Breed interaction; [BS 3-NOP No] = 26.3^b, [BS 3-NOP Yes] = 27.3^a, [HF 3-NOP No] = 27.2^a, [HF 3-NOP Yes] = 26.5^b.

¹⁴PUFA content, 3-NOP × WCS × Breed interaction; [BS 3-NOP No WCS No] = 4.32^a, [BS 3-NOP Yes WCS No] = 5.04^a, [BS 3-NOP No WCS Yes] = 4.76^{ab}, [BS 3-NOP Yes WCS Yes] = 4.45^a, [HF 3-NOP No WCS No] = 4.41^{ab}, [HF 3-NOP Yes WCS No] = 4.51^b, [HF 3-NOP No WCS Yes] = 4.75^{ab}, [HF 3-NOP Yes WCS Yes] = 4.42^b, [BS 3-NOP No] = 4.36^b, [BS 3-NOP Yes] = 4.76^a, [HF 3-NOP No] = 4.44^b, [HF 3-NOP Yes] = 4.44^b, WCS × Breed interaction; [BS WCS No] = 4.52, [BS WCS Yes] = 4.59, [HF WCS No] = 4.61, [HF WCS Yes] = 4.69.

¹⁵De novo content (sum of <C16 FA), 3-NOP × Breed interaction; [BS 3-NOP No] = 28.7, [BS 3-NOP Yes] = 28.0, [HF 3-NOP No] = 27.7, [HF 3-NOP Yes] = 28.8.

¹⁶Mixed FA content (sum of C16 FA), 3-NOP × WCS × Breed interaction; [BS 3-NOP No WCS No] = 33.8^{ab}, [BS 3-NOP Yes WCS No] = 34.0^b, [BS 3-NOP No WCS Yes] = 32.9^{ab}, [BS 3-NOP Yes WCS Yes] = 34.9^{ab}, [HF 3-NOP No WCS No] = 34.4^{ab}, [HF 3-NOP Yes WCS No] = 36.2^a, [HF 3-NOP No WCS Yes] = 34.3^{ab}, [HF 3-NOP Yes WCS Yes] = 35.6^{ab}.

¹⁷Preformed FA content (sum of >C16), 3-NOP × Breed interaction; [BS 3-NOP No] = 37.2^{ab}, [BS 3-NOP Yes] = 38.7^a, [HF 3-NOP No] = 37.2^{ab}, [HF 3-NOP Yes] = 35.9^b.

¹⁸Trans-FA content, 3-NOP × WCS × Breed interaction; [BS 3-NOP No WCS No] = 3.65, [BS 3-NOP Yes WCS No] = 4.09, [BS 3-NOP No WCS Yes] = 4.05, [BS 3-NOP Yes WCS Yes] = 3.87, [HF 3-NOP No WCS No] = 3.76, [HF 3-NOP Yes WCS No] = 3.62, [HF 3-NOP No WCS Yes] = 3.65, [HF 3-NOP Yes WCS Yes] = 3.69, 3-NOP × Breed interaction; [BS 3-NOP No] = 3.71, [BS 3-NOP Yes] = 3.86, [HF 3-NOP No] = 3.78, [HF 3-NOP Yes] = 3.73, [BS WCS No] = 3.73, [BS WCS Yes] = 3.71, [HF WCS No] = 3.83, [HF WCS Yes] = 3.81.

¹⁹OBCFA content, 3-NOP × Breed interaction; [BS 3-NOP No] = 2.70^{ab}, [BS 3-NOP Yes] = 2.77^{ab}, [HF 3-NOP No] = 2.68^b, [HF 3-NOP Yes] = 3.02^a.

²⁰BW: body weight recorded at the end of each period.

²¹BW change: difference in BW between 2 experimental periods. 3-NOP × Breed interaction; [BS 3-NOP No] = 0.671, [BS 3-NOP Yes] = 0.624, [HF 3-NOP No] = 0.604, [HF 3-NOP Yes] = 0.922, 3-NOP × WCS interaction; [3-NOP No WCS No] = 0.384^a, [3-NOP Yes WCS No] = 0.844^a, [3-NOP No WCS Yes] = 0.891^a, [3-NOP Yes WCS Yes] = 0.606^{ab}.

FA ($P = 0.04$, Table 2). Therefore, results below will be presented for 2-way interactions or on treatment level for most variables.

Milk Production and Milk FA Profile

We observed a tendency of WCS \times breed interaction for ECM ($P = 0.07$): ECM tended to be greater for BS cows receiving WCS ($P = 0.03$) but was not affected for HF cows. A WCS \times breed interaction ($P = 0.04$, Table 2) occurred in milk fat yield, being greater ($P < 0.01$) for BS cows fed WCS. The yield and composition of milk protein and lactose were not affected by treatment. A breed effect ($P = 0.02$, Table 2) occurred for MUN, being greater ($P = 0.02$) for BS compared with HF regardless of treatment. Feed efficiency (MY:DMI) was not affected by treatment, whereas cows receiving WCS had a greater BW ($P < 0.01$, Table 2) at the end of trial. Body weight change was affected by a 3-NOP and WCS interaction ($P < 0.01$, Table 2), where cows fed the CON diet had lower BW change than other treatment groups. No interactions ($P \geq 0.05$) between 3-NOP and WCS were observed for milk FA profile, indicating that the effects were independent. Cows receiving WCS supplementation had greater SFA content ($P = 0.05$, Table 2) and mixed FA content ($P < 0.01$, Table 2), together with a reduced MUFA content ($P = 0.05$, Table 2), de novo FA content ($P = 0.05$, Table 2), and a tendency in the reduced trans-FA content ($P = 0.06$, Table 2). However, we observed a 3-NOP \times breed interaction for SFA ($P < 0.01$, Table 2): 3-NOP decreased SFA content in the BS milk but increased it in HF milk. In contrast, the 3-NOP \times breed interaction observed for PUFA and MUFA ($P < 0.01$, Table 2) indicated an increase in their content for BS and a decrease for HF cows. Furthermore, a 3-NOP \times breed interaction was detectable in preformed FA content ($P < 0.01$, Table 2), showing that 3-NOP resulted in 7.8% greater preformed FA content in BS milk when compared with HF milk. Additionally, the 3-NOP \times breed interaction observed in odd- and branched-chain FA (OBCFA; $P < 0.01$; Table 2) indicated that 3-NOP only increased OBCFA in HF. The individual milk FA are reported in Supplemental Table S2 (see Notes; Ma et al., 2024).

Nutrient Intake and Apparent Total-Tract Digestibility of Nutrients

A 3-NOP \times WCS interaction ($P = 0.01$, Table 2) occurred for DMI, but no difference in DMI caused by the addition of 3-NOP or WCS, or the combination of both, was observed on the treatment level. A breed effect was detected on the intake of DM, OM, NDF, and ADF ($P < 0.03$, Table 3), with BS having a lower intake than HF. Intakes of total FA, SFA, and PUFA were greater in diets

containing WCS ($P < 0.01$, Table 3). Additionally, the 3-NOP \times WCS interaction ($P = 0.03$, Table 3) indicated that cows had greater MUFA intake when WCS was provided alone.

The ATTD of DM, OM, NDF, and ADF decreased ($P < 0.01$, Table 3) in cows fed WCS. A 3-NOP \times WCS interaction ($P = 0.02$, Table 3) occurred for digestible DM and OM; supplementing 3-NOP together with WCS increased the amount of digestible DM and OM.

Enteric Gas Emissions

Methane production and intensities (g/kg MY and g/kg ECM) were decreased ($P < 0.01$) by 18.3%, 18.7%, and 19.2%, respectively, in HF cows (Figure 1). Methane yield decreased 13.8% (Figure 1) on average by 3-NOP across the 2 breeds. Methane mitigation was affected by a 3-NOP \times breed interaction ($P \leq 0.05$, Table 4).

Further, we detected a 3-NOP \times WCS interaction ($P = 0.03$, Table 4) for H₂ production. Hydrogen production increased ($P < 0.01$) by supplementation of 3-NOP, but it was not different between the diets with or without WCS. Hydrogen production was also affected by a 3-NOP \times breed interaction ($P < 0.01$, Table 4); supplementation of 3-NOP increased H₂ ($P < 0.01$) by 1.53-fold in BS and by 2.86-fold in HF, respectively. No interaction was observed for CO₂ production. The supplementation of 3-NOP and WCS increased ($P < 0.02$) CO₂ production by 3.2% and 2.9%, respectively.

Intraday Pattern of CH₄, H₂ Production, and Feed Intake and Feeding Behavior

The intraday patterns of CH₄, H₂ production, and FI are presented in Figure 2 and LSM in Supplemental Tables S3, S4, and S5 (See Notes; Ma et al., 2024). We found no 3-NOP \times WCS \times time interaction in CH₄ production, H₂ production, or DMI; therefore the intraday pattern was displayed on factor 3-NOP level across time of day. A 3-NOP \times time interaction ($P = 0.05$, Figure 2A) was detected in BS cows, which showed that at 1200 h, supplementing 3-NOP tended to decrease ($P = 0.09$) the CH₄ production. We observed 3-NOP \times time interaction of CH₄ production in HF. The greatest CH₄ reduction of 29.6% was observed at 0900 h ($P < 0.01$, Figure 2B), and the effect was persistent until evening feed provision. The effect of 3-NOP after the second feeding lasted until 2100 h, whereafter the CH₄ was similar between CON and 3-NOP.

Hydrogen production tended to be increased by 3-NOP at 0000 h in BS cows ($P = 0.07$, Figure 2C), but the effect of 3-NOP was not observed at any other time points during the day. A 3-NOP \times time interaction was found in H₂ production for HF cows, where the effect of 3-NOP was

Table 3. Nutrient intake, digestible nutrients,¹ and apparent total-tract digestibility (ATTD) of lactating dairy cows from 2 breeds² receiving dietary treatments³

Item	3-NOP				WCS				Breed				P-value								
	No		Yes		No		Yes		BS		HF		Breed		3-NOP × WCS		WCS × breed		3-NOP × WCS × breed		
	Intake	SE ⁴	Intake	SE ⁴	Intake	SE ⁴	Intake	SE ⁴	Intake	SE ⁴	Intake	SE ⁴	Intake	SE ⁴	Intake	SE ⁴	Intake	SE ⁴	Intake	SE ⁴	
Nutrient intake, kg/d																					
DM ⁵	24.6	0.51	24.7	0.51	24.5	0.51	24.7	0.51	23.6 ^b	0.68	25.8 ^a	0.68	24.7	0.51	24.5	0.51	24.7	0.51	23.6 ^b	0.68	25.8 ^a
OM ⁶	22.8	0.47	23.0	0.47	22.9	0.47	22.9	0.47	21.9 ^b	0.63	24.0 ^a	0.63	22.9	0.47	22.9	0.47	22.9	0.47	21.9 ^b	0.63	24.0 ^a
NDF	10.0	0.23	10.3	0.23	10.1	0.23	10.1	0.23	9.63 ^b	0.31	10.7 ^a	0.31	10.1	0.23	10.1	0.23	10.1	0.23	9.63 ^b	0.31	10.7 ^a
ADF	6.08	0.148	6.24	0.148	6.02 ^b	0.148	6.30 ^a	0.148	5.83 ^b	0.198	6.48 ^a	0.198	6.02 ^b	0.148	6.02 ^b	0.148	6.30 ^a	0.148	5.83 ^b	0.198	6.48 ^a
Total FA	0.84 ^a	0.016	0.78 ^b	0.016	0.75 ^b	0.016	0.87 ^a	0.016	0.78	0.020	0.84	0.020	0.75 ^b	0.016	0.75 ^b	0.016	0.87 ^a	0.016	0.78	0.020	0.84
SFA	0.19	0.042	0.19	0.042	0.16 ^b	0.042	0.22 ^a	0.042	0.18	0.005	0.19	0.005	0.16 ^b	0.042	0.16 ^b	0.042	0.22 ^a	0.042	0.18	0.005	0.19
MUFA ⁷	0.21 ^a	0.019 ^b	0.19 ^b	0.019 ^b	0.19	0.020	0.042	0.020	0.19	0.21	0.49	0.21	0.19	0.020	0.19	0.020	0.042	0.020	0.19	0.21	0.49
PUFA	0.44 ^a	0.085	0.41 ^b	0.085	0.40 ^b	0.085	0.45 ^a	0.085	0.41	0.10	0.44	0.10	0.40 ^b	0.085	0.40 ^b	0.085	0.45 ^a	0.085	0.41	0.10	0.44
ATTD, %																					
DM	71.7	0.61	72.0	0.61	73.4 ^a	0.61	70.3 ^b	0.61	71.8	0.71	71.9	0.71	73.4 ^a	0.61	73.4 ^a	0.61	70.3 ^b	0.61	71.8	0.71	71.9
OM	73.6	0.59	74.0	0.59	75.3 ^a	0.59	72.4 ^b	0.59	73.9	0.69	73.7	0.69	75.3 ^a	0.59	75.3 ^a	0.59	72.4 ^b	0.59	73.9	0.69	73.7
NDF	59.7	0.96	60.9	0.96	62.7 ^a	0.96	57.8 ^b	0.96	60.3	1.10	60.3	1.10	62.7 ^a	0.96	62.7 ^a	0.96	57.8 ^b	0.96	60.3	1.10	60.3
ADF	57.4	1.05	58.4	1.05	60.0 ^a	1.05	55.8 ^b	1.05	57.8	1.20	58.0	1.20	60.0 ^a	1.05	60.0 ^a	1.05	55.8 ^b	1.05	57.8	1.20	58.0
Digestible nutrients, ⁹ kg/d																					
DM ¹⁰	17.6	0.43	17.9	0.43	18.1	0.43	17.4	0.43	16.9	0.57	18.6	0.57	18.1	0.43	18.1	0.43	17.4	0.43	16.9	0.57	18.6
OM ¹¹	16.8	0.41	16.9	0.41	17.2	0.41	16.6	0.41	16.1	0.53	17.7	0.53	17.2	0.41	17.2	0.41	16.6	0.41	16.1	0.53	17.7
NDF	6.00	0.194	6.27	0.194	6.36 ^a	0.194	5.91 ^b	0.194	5.82	0.252	6.44	0.252	6.36 ^a	0.194	6.36 ^a	0.194	5.91 ^b	0.194	5.82	0.252	6.44
ADF	3.50	0.130	3.68	0.130	3.63	0.130	3.55	0.130	3.40	0.169	3.79	0.169	3.63	0.130	3.63	0.130	3.55	0.130	3.40	0.169	3.79

^{a,b}In table and in footnotes, different superscript letters indicate a significant ($P < 0.05$) difference.

¹Digestible nutrient = nutrient intake × ATTD of nutrient.

²BS = Brown Swiss; HF = Holstein Friesian.

³Dietary treatments were CON = basal diet; diet supplemented with 3-nitrooxypropanol (3-NOP, concentration = 60 mg/kg DM); diet supplemented with whole cottonseed (WCS, concentration = 50 g/kg DM); diet supplemented with 3-NOP + WCS. [3-NOP No] is the LSM of CON and WCS diet; [3-NOP Yes] is the LSM of 3-NOP and 3-NOP + WCS diet; [WCS No] is the LSM of CON and 3-NOP diet; [WCS Yes] is the LSM of WCS and 3-NOP + WCS diet.

⁴Sample size of the largest SE published in table: n = 64 for DM, OM, NDF, ADF, total FA, MUFA, PUFA, digestible DM, digestible OM, digestible NDF, digestible ADF, DM digestibility, OM digestibility, NDF digestibility, and ADF digestibility; n = 63 for SFA.

⁵Same as DMI presented in Table 2.

⁶OM intake, 3-NOP × WCS interaction; [3-NOP No WCS No] = 23.1^{ab}, [3-NOP Yes WCS No] = 22.8^{ab}, [3-NOP No WCS Yes] = 22.5^b, [3-NOP Yes WCS Yes] = 23.3^a.

⁷MUFA, 3-NOP × WCS interaction; [3-NOP No WCS No] = 197.8^b, [3-NOP Yes WCS No] = 191.3^b, [3-NOP No WCS Yes] = 214.8^a, [3-NOP Yes WCS Yes] = 187.9^b.

⁸Apparent total-tract digestibility (ATTD; %) = [nutrient intake (kg/d) - fecal loss (kg/d)]/nutrient intake (kg/d).

⁹Digestible nutrient (kg/d) = Nutrient intake (kg/d) × ATTD (%).

¹⁰Digestible DM, 3-NOP × WCS interaction; [3-NOP No WCS No] = 18.3^a, [3-NOP Yes WCS No] = 17.9^{ab}, [3-NOP No WCS Yes] = 16.9^b, [3-NOP Yes WCS Yes] = 17.9^{ab}.

¹¹Digestible OM, 3-NOP × WCS interaction; [3-NOP No WCS No] = 17.5^a, [3-NOP Yes WCS No] = 17.1^{ab}, [3-NOP No WCS Yes] = 16.2^b, [3-NOP Yes WCS Yes] = 17.1^{ab}.

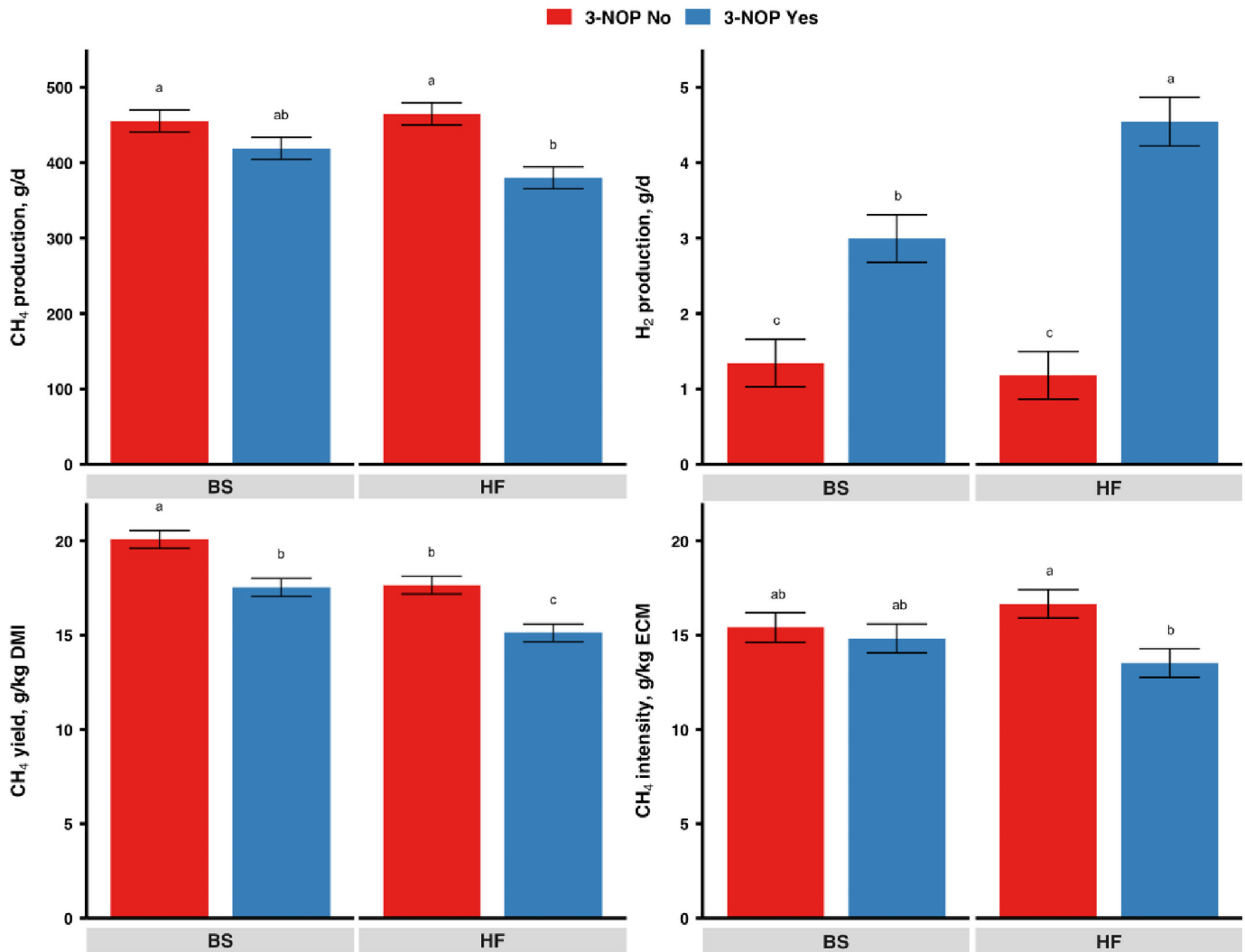


Figure 1. Effect of supplementing 3-nitrooxypropanol (3-NOP) to Brown Swiss cows (BS) and Holstein Friesian cows (HF) on CH₄ production, H₂ production, and CH₄ intensities. Figures are displayed by 3-NOP level and grouped by breed due to breed × 3-NOP interaction. Dietary treatments were CON = basal diet; diet supplemented with 3-NOP at 60 mg/kg DM; diet supplemented with whole cottonseed (WCS, concentration = 10 g/kg DM); and diet supplemented with 3-NOP + WCS. 3-NOP No is the LSM of CON and WCS diet; 3-NOP Yes is the LSM of 3-NOP and 3-NOP+WCS diet. Data are presented as LSM ± SE. a–c: Different letters indicate significant difference ($P < 0.05$).

observed from 0900 h until 2100 h with the greatest H₂ production increase, by 724%, being observed at 0900 h ($P < 0.01$, Figure 2D).

No 3-NOP × time interaction or treatment effect of 3-NOP was observed in the diurnal FI pattern in either breed (Figure 2E and 2F). However, FI (%/h) varied throughout the day ($P < 0.01$). Around 11.3% and 12.5% of daily FI was consumed within the first hour after morning feeding for BS and HF, respectively, whereas 11.7% and 8.9% of daily FI was consumed within the first hour after evening feeding for BS and HF cows, respectively. Feed intake (%/h) of HF cows was greater ($P < 0.01$) in the morning compared with evening, whereas for BS

cows, no significant difference was observed between the 2 feedings.

DISCUSSION

This study evaluated the effects of combining 3-NOP and WCS on milk production, enteric gas emissions, and ATTD of dairy cows. Most studies have investigated the CH₄ mitigating potential of WCS related to its lipid content, whereas in the current study we hypothesized that the antimicrobial property of free gossypol (Wang et al., 2021) in WCS may also contribute to CH₄ reduction independent from lipids. Therefore, the EE content of the

Table 4. Enteric gas emissions of lactating dairy cows from 2 breeds¹ receiving dietary treatments²

Item	3-NOP ³			WCS ⁴			Breed ⁵			P-value ⁶						
	No	Yes	SE ⁷	No	Yes	SE	BS	HF	SE	3-NOP	WCS	Breed	3-NOP × WCS	3-NOP × Breed	WCS × Breed	3-NOP × WCS × Breed
CH ₄ production, ⁸ g/d	460	400	9.1	422	438	9.1	437	422	11.8	<0.01	0.20	0.39	0.11	0.05	0.82	0.30
CH ₄ yield, ⁹ g/kg DMI	18.9	16.3	0.39	17.2	17.9	0.35	18.7	16.4	0.39	<0.01	0.26	<0.01	0.54	0.24	0.75	0.22
CH ₄ intensity, ¹⁰ g/kg MY	18.8	16.6	0.76	17.3	18.1	0.76	18.0	17.5	1.00	<0.01	0.16	0.75	0.66	<0.01	0.66	0.42
CH ₄ intensity, ¹¹ g/kg ECM	16.3	14.1	0.60	14.9	15.6	0.61	15.4	15.1	0.77	<0.01	0.34	0.99	0.39	<0.01	0.97	0.79
H ₂ production, ¹² g/d	1.26	3.75	0.236	2.70	2.32	0.236	2.17	2.85	0.268	<0.01	0.01	0.01	0.03	<0.01	0.16	0.11
CO ₂ production, g/d	13,249 ^b	13,691 ^a	216.0	13,273 ^b	13,667 ^a	210.0	13,135	13,805	279.0	<0.01	0.03	0.10	0.14	0.74	0.33	0.35

^{a,b}In table and in footnotes, different superscript letters indicate a significant ($P < 0.05$) difference.

¹BS = Brown Swiss; HF = Holstein Friesian.

²Dietary treatments were CON = basal diet; diet supplemented with 3-nitrooxypropanol (3-NOP, concentration = 60 mg/kg DM); diet supplemented with whole cottonseed (WCS, concentration = 50 g/kg DM); diet supplemented with 3-NOP + WCS. [3-NOP No] is the LSM of CON and WCS diet; [3-NOP Yes] is the LSM of 3-NOP and 3-NOP+WCS diet; [WCS No] is the LSM of CON and 3-NOP diet; [WCS Yes] is the LSM of WCS and 3-NOP + WCS diet.

³Values reported as LSM for the [3-NOP No] and [3-NOP Yes] treatments, average over [No/Yes WCS] and [HF/BS breed].

⁴Values reported as LSM for the [WCS No] and [WCS Yes] treatments, average over [No/Yes 3-NOP] and [HF/BS breed].

⁵Values reported as LSM for the BS and HF breed, average over [No/Yes 3-NOP] and [No/Yes WCS].

⁶P-values obtained from fitting full model.

⁷Sample size of the largest SE published in table: n = 64 for CH₄ production and CH₄ yield, n = 61 for CH₄ intensities, n = 63 for H₂ production and CO₂ production.

⁸CH₄ production, 3-NOP × Breed interaction; [BS 3-NOP No] = 455^a, [BS 3-NOP Yes] = 419^{ab}, [HF 3-NOP No] = 465^a, [HF 3-NOP Yes] = 380^b.

⁹CH₄ yield [g/kg DMI] = CH₄ production [g/d] / DMI [kg/d].

¹⁰CH₄ intensity (g/kg MY) = CH₄ production (g/d) / MY (kg/d); 3-NOP × Breed interaction; [BS 3-NOP No] = 18.2^{ab}, [BS 3-NOP Yes] = 17.7^{ab}, [HF 3-NOP No] = 19.3^a, [HF 3-NOP Yes] = 15.7.

¹¹CH₄ intensity (g/kg ECM yield) = CH₄ production (g/d) / MY (kg/d); 3-NOP × Breed interaction; [BS 3-NOP No] = 18.2^{ab}, [BS 3-NOP Yes] = 17.7^{ab}, [HF 3-NOP No] = 19.3^b.

¹²H₂ production, 3-NOP × WCS × Breed interaction; [BS 3-NOP No WCS No] = 3.10^b, [BS 3-NOP Yes WCS Yes] = 1.38^d, [BS 3-NOP Yes WCS Yes] = 2.89^{bc}, HF 3-NOP No WCS No = 1.09^d, HF 3-NOP Yes WCS Yes = 1.27^{cd}, HF 3-NOP Yes WCS Yes = 3.68^b; 3-NOP × WCS interaction; [3-NOP No WCS No] = 1.20^b, [3-NOP Yes WCS No] = 4.20^a, [3-NOP No WCS Yes] = 1.33^b, [3-NOP Yes WCS Yes] = 3.28^a; 3-NOP × Breed interaction; [BS 3-NOP No] = 1.18^c, [BS 3-NOP Yes] = 2.99^b, [HF 3-NOP No] = 1.18^a, [HF 3-NOP Yes] = 4.55^a.

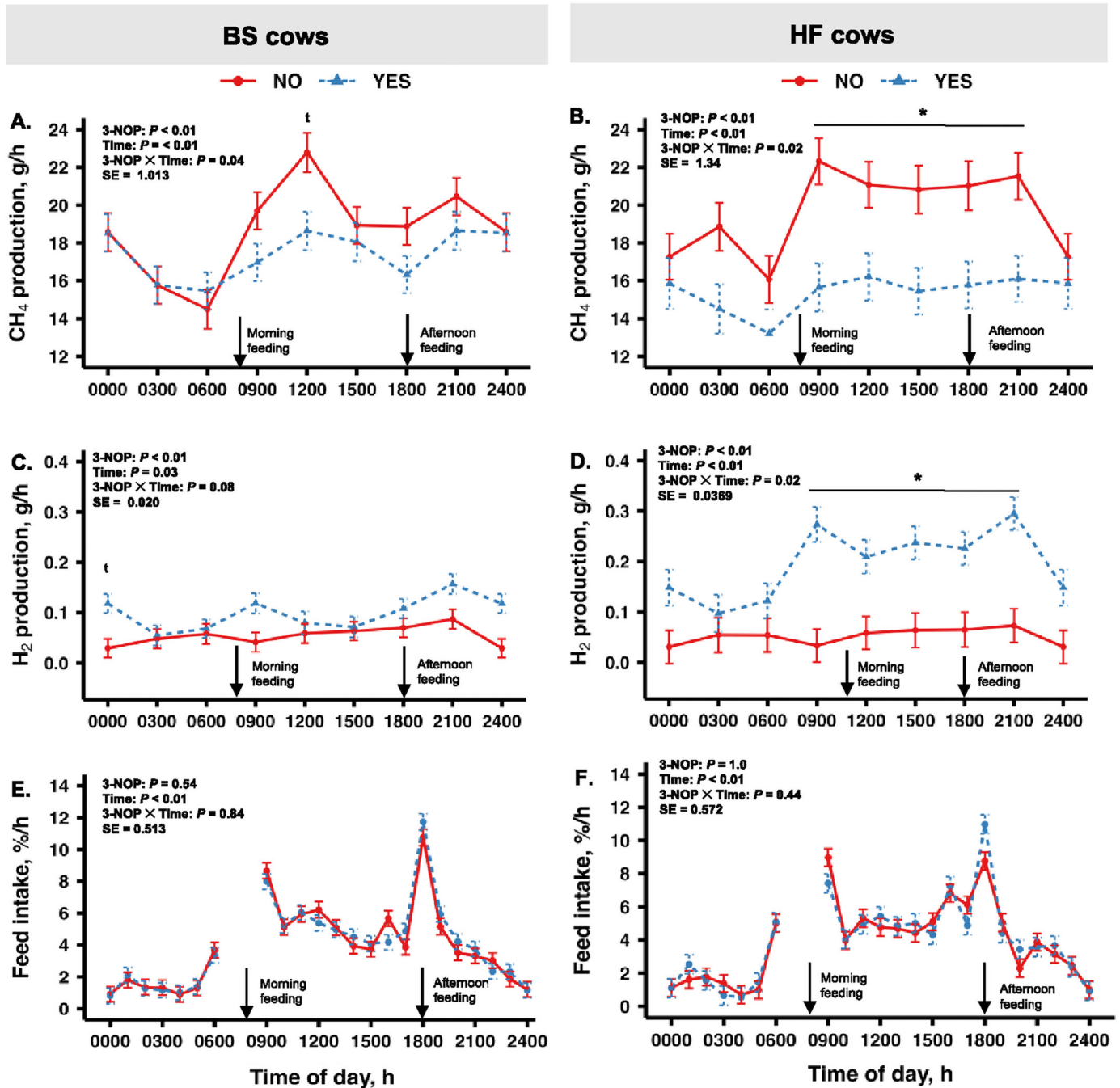


Figure 2. Effect of supplementing 3-NOP to Brown Swiss cows (left-hand column: BS) and Holstein Friesian cows (right-hand column: HF) on the daily pattern of CH₄ production, H₂ production, and feed intake. Cows were fed 1 of 4 treatment diets (CON, 3-NOP, WCS, or 3-NOP + WCS) at 0800 h (a.m.) and 1800 h (p.m.) during the experiment, indicated in the figure by black arrows. Feed intake at 0700 h and 0800 h not displayed due to the cleaning of feed refusals. There was no 3-NOP \times breed \times time interaction. Intraday CH₄ production pattern (A and B) and intraday H₂ production pattern (C and D) are displayed by factor 3-NOP level due to the 3-NOP \times breed effect. Intraday feed intake (expressed as percentage of total daily intake as fed; E and F) is displayed by breed level. Error bars represent SE. Significant difference at a time point between 3-NOP YES and 3-NOP NO denoted as *; tendency denoted as t.

diet without WCS was balanced by including sunflower oil in the diet. Moreover, the widely reported toxicity of free gossypol should be considered when WCS is supple-

mented to dairy cattle, although female ruminants, such as dairy cows, have been reported to be less sensitive to dietary gossypol (Randel et al., 1996). In our study,

the free gossypol levels of the diets with and without WCS were 320 mg/kg and 0 mg/kg, respectively, which was below the reported dosage (535 mg/kg) of observing a reduction in CH₄ emission (Ismartoyo et al., 1993), indicating that a higher inclusion rate would have been optimal. The basal diets in the current experiment were high in fiber content (around 43% NDF of DM), which is higher than the median NDF content (32.5% NDF of DM) of diets in previous studies that have investigated the efficacy of 3-NOP (Kebreab et al., 2023). It is noted that the concentration of methyl-coenzyme M in the rumen might be greater in cows fed high-forage diets, and thereby the overall efficacy of 3-NOP might be lower (Vyas et al., 2018). Finally, it is important to note that this study used an equal number of cows from 2 breeds (HF vs. BS) due to limited availability of multiparous lactating dairy cows from the same breed with a similar MY available at the research facility. Therefore, the breed of cows was considered as the main plot in the statistical model, and the interaction between breed, 3-NOP, and WCS was tested for all variables. Although the study was powered for the main objective (i.e., the effects of 3-NOP and WCS), we unexpectedly observed a 3-NOP and breed interaction for milk FA profiles and enteric gas emissions. Therefore, despite not being the main objective of the study, these interactions are discussed in more detail herein, but should be interpreted with caution due to the low number of cows (n = 8) for each breed.

Milk Production and Nutrient Digestibility

As expected, we found no interactions between 3-NOP and WCS for any of the production parameters, as 3-NOP has rarely altered the production parameters in lactating dairy cows. Indeed, in line with previous research with dairy cows on grass silage (van Gastelen et al., 2022) or corn silage-based diets (Reynolds et al., 2014; Hristov et al., 2015; Melgar et al., 2020a), 3-NOP did not compromise any of the production parameters in the current study. In some experiments, milk fat concentration and yield were reported to increase (Melgar et al., 2020b, 2021), but not in other studies (Hristov et al., 2015; van Gastelen et al., 2022), nor in the current study.

Dry matter intake was not affected by dietary treatments, but a breed effect did occur for DMI, with BS cows having a 2.2 kg/d lower DMI than HF cows. The breed difference in DMI was no longer maintained when converted to per unit of BW, which can be explained by the greater BW of HF BS cows (771 vs. 747 kg). However, the lower DMI did not result in a lower production of milk or milk components in BS cows. Milk yield was not affected by WCS, but ECM yield was increased by 1.2 kg/d, collectively reflecting the numerically higher MY, milk fat, and protein yield. Despite balanced EE content

in the diets, the difference in FA profile between WCS and sunflower oil (supplemented to the No WCS diet) was reflected in the milk FA profile. The SFA content is around 15% higher in WCS, primarily due to greater palmitic acid content, compared with sunflower oil. On the other hand, sunflower oil contains double the amount of MUFA, primarily oleic acid, compared with WCS. As a result, the cows fed WCS Yes diet had greater milk SFA content, together with lower MUFA content, compared with the cows fed WCS No diet.

When 3-NOP was supplemented, we observed an increase of SFA content together with a decrease in PUFA and MUFA in HF milk. This agreed with the results reported by Melgar et al. (2021), who also indicated that accumulated hydrogen, as a consequence of the inhibited methanogenesis, may be incorporated to form SFA by biohydrogenation, and thereby functions as an alternative hydrogen sink. However, it is rather challenging to quantify the amount of H₂ that was redirected to biohydrogenation pathway in the current study. Nevertheless, we calculate the difference in CH₄ production between the CON cows and those treated with 3-NOP, which was 85 g/d for HF and 36 g/d for BS cows, respectively. These figures represent around 21.3 and 9.0 g/d H₂, respectively, not used for methanogenesis and are far greater than the measured H₂ from eructation (2.85 g/d and 2.17 g/d, respectively). As dissolved H₂ in the rumen fluid was not measured, we cannot speculate on the fate of the excess H₂ based on the stoichiometric calculation provided above. Gonzalez-Recio et al. (2018), who investigated the signs of host genetic regulation in dairy cow rumen microbes, reported that the relative abundance of bacteria from the genus *Butyrivibrio* was higher in HF than in BS. This group of bacteria is responsible for producing butyrate. As indicated by Urrutia et al. (2019), dietary supplementation of butyrate decreased milk fat yield. Therefore, in the current study, the observed lower fat yield in HF may partially be attributed to the breed difference in the microbes that are involved in lipid metabolism. Further, the inclusion of 3-NOP led to a decreased preformed FA content in HF milk compared with BS cows supplemented with 3-NOP (35.9 vs. 38.7% of total FA, respectively), in our study. Further, a breed effect, without any interaction with dietary treatments, was observed for MUN with BS cows having 19.7% greater MUN content than HF, which was similar to the findings of Franzoi et al. (2020), who observed a 16.4% greater MUN content in BS than HF cows using a large data set (n = 38,587 and 31,773 for BS and HF, respectively).

In this experiment, no effects of feeding 60 mg/kg of DM of 3-NOP on DMI, nutrient intake, or ATTD were observed, which aligns well with previous studies (Hristov et al., 2015; Lopes et al., 2016; van Gastelen et al., 2020). However, the inclusion of 5% WCS resulted in re-

ductions in the digestibility of DM, OM, NDF, and ADF. Interestingly, fiber digestibility was lower even though the diets were balanced for their forage-to-concentrate ratio and NDF content. It should be noted that in the diet with WCS, a portion of the corn silage and grass silage was replaced with 5% WCS. Therefore, the type of fiber present in the diets may have contributed to the observed variation in digestibility. Indeed, previous studies have reported similar effects of WCS on fiber digestibility. de Souza and Lock (2019) reported a 4.5% decrease in NDF digestibility when WCS was fed at 8.6% of DM, whereas the NDF content in the WCS diet was slightly lower than that of the control diet with soyhulls. This indicates that the type of fiber in WCS is less digestible than that of soyhulls. Indeed, Harvatine et al. (2002) observed that replacing forage NDF with an increasing amount of WCS (5% to 15%) decreased the passage and digestion rate of potentially digestible NDF. Indeed, certain characteristics, such as physically effective NDF content, may contribute to the reduction in fiber digestibility when the same amount of NDF is fed from different sources (Allen, 1997). This may also explain the reduced digestibility with the WCS diets observed in the current study.

Enteric Gas Emissions

One of the main objectives of the present study was to evaluate to what extent WCS can enhance the effect of 3-NOP in decreasing CH₄ emission from dairy cows fed a control diet balanced for NDF and EE contents. In comparison to CON, WCS did not exhibit a CH₄ reducing effect but resulted in a numeric increase of 6% in CH₄ production. With the presence of 3-NOP, WCS led to a numerical increase of 16% in CH₄ production, showing that the effect of WCS counteracted, at least to some extent, the effect of 3-NOP in reducing CH₄ production. The lack of WCS × 3-NOP interaction for CH₄ yield and intensity also indicates that, contrary to our findings in the pilot study (Ma et al., 2023), where we observed a moderate reduction in CH₄ intensity (5.5%) driven by an increase in MY (9.6%), likely through the combined effect of fat and fiber from WCS, this effect did not persist in the current study. We attribute this discrepancy to the balanced CP and EE contents of the diets in the current study, together with the low inclusion rate of WCS. Previous studies have reported a reduction in CH₄ emission when using WCS at inclusion levels higher than those of the current study: Grainger et al. (2008) reported a 12% reduction in CH₄ production (g/d) when WCS was fed at a level of 2.7 kg DM/d with grain supplements (corresponding to ~16% of WCS of total DMI) compared with a control diet. In a long-term follow-up study Grainger et al. (2010) reported a 13% reduction compared with control group in wk 3 and a 23% reduction in wk 12 when

WCS was mixed in TMR at a level of 2.61 kg DM/d (corresponding to ~15% of WCS of total DMI). Muñoz et al. (2019) compared the CH₄ mitigation potential of unprocessed oilseeds, in which the EE of different oilseed diets were balanced within the range of 20.6% to 22.0%. This study reported a 22.4% reduction by WCS compared with rapeseed, and a 15.5% reduction compared with linseed, when WCS was fed at a level of 18.4% of DM. In a subsequent study, Muñoz et al. (2021) supplemented WCS at ~13.6% of DMI to grazing dairy cows and reported a 14% reduction in CH₄ yield compared with the control group fed steam-flaked corn. These studies attributed the mitigating effect of WCS to the fat content and the level of UFA present in WCS. In contrast, Dong et al. (1997) reported that short-chain FA (<C14) were more effective in reducing CH₄ through biohydrogenation, which was later supported by Johnson et al. (2002) linking a lack of effect of WCS on CH₄ emission with the high level of long-chain FA (C18 or greater). However, the current study balanced fat content in the diets with and without WCS; therefore we did not observe an effect on CH₄ emission due to the hydrogenation of UFA.

Further, in an *in vitro* continuous culture system, Castro Veloz (2023) observed a decrease in protozoa count and relative abundance of archaea with an increase in the WCS content from 5% to 15% of DM when NDF and protein, but not fat, content was balanced. It is important to point out that in their study, it cannot be conclusively stated that gossypol was accountable for the reported alteration of rumen microbial relative abundance, as dietary fat can also decrease the number of protozoa and various bacteria, including fibrolytic bacterial populations (Patra, 2013). An *in vitro* study conducted by Ismartoyo et al. (1993) on sheep revealed that adding 1 mM gossypol initially reduced gas production by 27.4%, but the inhibitory effect was temporary, and the rumen microorganisms appeared to adapt to the presence of gossypol during the 12-d experiment.

In cows supplemented with 3-NOP, the average reductions in CH₄ production and yield were 13% and 14%, respectively, in the present study. More specifically, the reduction in HF cows was 18.3% for CH₄ production, which was smaller than the documented average of 30% in HF dairy cows, as reported by Dijkstra et al. (2018) and Kebreab et al. (2023). However, the same studies indicated that the effectiveness of 3-NOP was negatively correlated with dietary NDF content. To assess to what extent the efficacy was affected by the high dietary NDF content of 43% of DM in the current experiment, expected CH₄ reduction was calculated based on a prediction model developed in a recent meta-analysis by Kebreab et al. (2023). Dose of 3-NOP, as well as NDF, fat, and starch contents of the basal diets were included in the calculation. The calculated expected reductions were

21.8%, 21.5%, and 23.5% for CH₄ production, yield, and intensity, respectively, and were slightly greater than the measured values of 18.3%, 17.8%, and 19.2% in HF cows, respectively. Only a few studies have evaluated the efficacy of 3-NOP with a similar dietary NDF content to our study. The median dietary NDF content of studies included in the meta-analysis by Kebreab et al. (2023) was 32.5%, with a maximum of 43.5%. Based on this, the observed CH₄ mitigation effect of 3-NOP is within the expected range.

The unexpected 3-NOP × breed interaction observed for CH₄ production and intensity indicated a lower CH₄ reduction in BS compared with HF cows, being 8% versus 18% and 4% versus 19% for CH₄ production and intensity, respectively. As mentioned previously, the main objective of this experiment was not to test for breed effects, and to the best of our knowledge no prior studies have reported a breed effect for 3-NOP, or other CH₄ inhibitors, in 2 different dairy cattle breeds within the same experiment. It has been shown that host genetics and rumen microbiome jointly associate with CH₄ emissions in dairy cows (Difford et al., 2018), of which both the microbial community structure (Roehe et al., 2016) and their activity (Shi et al., 2014) can contribute to high or low CH₄-emitting phenotype, especially the abundance of methanogens (Wallace et al., 2015; Aguilar-Marin et al., 2020; Arndt et al., 2022). Gonzalez-Recio et al. (2018) compared the relative abundance of organisms from the genus *Methanobrevibacter* and found it higher in HF than in BS cows. Thereby, the observed different response to 3-NOP between breeds may be related to the different relative abundance of methanogens; hence the different concentration of methyl-coenzyme M and the subsequent differences in the efficacy of 3-NOP could be present on breed level. Overall, the observed difference between BS and HF in the current experiment cannot be fully explained with the available data and should be confirmed in future studies with various CH₄ mitigation inhibitors and strategies.

The concomitant increase in H₂ emission that follows the reduction of CH₄ due to 3-NOP has been consistently reported in previous studies (Hristov et al., 2015; Lopes et al., 2016; van Gastelen et al., 2022). In line with the previous findings, the observed absolute increases in H₂ production (g/d) in the current experiment were 1.8 g/d in BS and 3.4 g/d in HF cows, respectively. In previous studies, the effect of 3-NOP fed at a dose of 60 mg/kg of DM on H₂ production has ranged from 1.1, 1.3, and 2.4 g/d in Hristov et al. (2015), Lopes et al. (2016), and Melgar et al. (2021), respectively, to a 5.9 g/d increase observed by van Gastelen et al. (2022). The differences between studies may stem from the degree of CH₄ inhibition and hence accumulation of hydrogen, as well as differences in measurement schemes and techniques (van

Gastelen et al., 2020). Overall, the increase in H₂ emission is a direct consequence of the inhibition of methanogenesis by 3-NOP, as has been described in detail previously Hristov et al. (2015) and Lopes et al. (2016). The differential response to 3-NOP supplementation by breed was also reflected in H₂ emission, as can be expected with more H₂ accumulation in HF cows receiving 3-NOP. The increase in H₂ emission in BS cows does, however, indicate that 3-NOP was inhibiting the last step of methanogenesis also in BS cows even though the effect was not as pronounced, as also evident by the 3-NOP × time interaction for BS cows, discussed below.

Intraday Patterns of CH₄ and H₂ Production, Feed Intake, and Feeding Behavior

The overall mitigation effect of 3-NOP followed a clear daily pattern, and a 3-NOP × time interaction was detected. In BS cows, 3-NOP tended to reduce CH₄ production by 18% at ~4 h after morning feed delivery. Conversely, in HF cows, the mitigation effect was observed immediately after both feeding times, being greatest (29.6%) after morning feed delivery, and this effect persisted at ~23% to 26% for 10 h until the second feed provision, and 3 h thereafter in the evening. From midnight until morning feeding, when the overall CH₄ was at its lowest, no difference in CH₄ production was detected between 3-NOP and CON cows. In agreement with the findings of Reynolds et al. (2014), who dosed 3-NOP (500 mg/d and 2,500 mg/d) twice daily directly into the rumen, the effect of 3-NOP was less sustained when fed in the evening, and the lowest CH₄ concentration was observed 1 to 2 h after the dose was given. The observed variation in mitigation effect between the 2 feeding times in the present study can be attributed to the gradual decrease in feed consumption after evening feeding: less than 5% of the daily FI was consumed per hour during the overnight period. Interestingly, Hristov and Melgar (2020) reported a similar daily pattern with once-a-day feeding, showing the highest mitigation effect of 3-NOP directly after feeding and lowest right before feeding. They further reported that the effect of 3-NOP was sustained for 10 h after morning feed delivery, which agrees with data for HF cows in the current study.

The diurnal pattern of FI with twice-a-day feeding in the current experiment was similar to the studies of Niu et al. (2014) and DeVries et al. (2003), where feed delivery and milking were the greatest stimulators of feed consumption. The FI increased sharply after milking and peaked at the 2 feeding times. A low level of intake during the overnight period (~1.5% from 0000 h to 0500 h) and a moderate level of intake in the afternoon (~5.12% from 1200 h to 1700 h) were observed. This pattern is similar to that reported by Niu et al. (2014). As

expected, the diurnal pattern of CH₄ was clearly related to the FI pattern. The greatest hourly feed consumption (12.5%/h) in HF occurred within 1 h after morning feed delivery. This FI pattern coincided with the peak of CH₄ production for cows receiving the diets without 3-NOP and the greatest CH₄ mitigation effect of 29.6% for cows supplemented with 3-NOP. These findings are consistent with the study by Hristov and Melgar (2020), where the greatest mitigation effect of 3-NOP at 45.2% coincided with a peak FI rate (11.3%/h) within 1 h after morning feed delivery. Interestingly, in contrast to HF cows, in BS cows, we observed a 4-h time lag between morning feed delivery (0800 h) and the greatest CH₄ mitigation effect of 3-NOP (18.4%, 1200 h), which may indicate a difference in fermentation rate and metabolism within the rumen.

CONCLUSIONS

In the conditions of the current study, there was no combined effect of 3-NOP and WCS in terms of CH₄ mitigation. The observed mitigating effect was driven by 3-NOP, showing a reduction of 13%, 14%, and 13% for enteric CH₄ production, yield (g/kg DMI), and intensity (g/kg ECM), respectively, on a high-fiber diet (43% NDF of DM). Unexpected breed by 3-NOP interactions were observed, where the reduction in CH₄ emissions were less pronounced in BS cows. These data are observational due to low numbers of cows per breed and should be interpreted with caution. Whole cottonseed supplemented at 5% of DM increased ECM and milk fat yields but had no effect on CH₄ emissions compared with a control diet balanced for EE content.

NOTES

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Nonstandard abbreviations used: 3-NOP = 3-nitrooxypropanol, or the basal diet with addition of 3-NOP; 3-NOP + WCS = diet containing both 3-NOP and WCS; ATTD = apparent total-tract digestibility; BS = Brown Swiss; CON = basal control diet; EE = ether extract; FA = fatty acid; FI = feed intake; GE = gross energy; GF = GreenFeed System; HF = Holstein Friesian; MY = milk yield; NfE = nitrogen-free extract; OBCFA = odd- and branched-chain FA; WCS = whole cottonseed, or the basal diet with addition of WCS.

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