J. Dairy Sci. 107:7769–7785
https://doi.org/10.3168/jds.2024-24673

The effect of feeding and visiting behavior on methane and hydrogen emissions of dairy cattle measured with the GreenFeed system under different dietary conditions

Rudi de Mol,[1] André Bannink,[1] Jan Dijkstra,[2] Nicola Walker,[3] and Sanne van Gastelen[1]*
[1]Wageningen Livestock Research, Wageningen University & Research, 6700 AH Wageningen, the Netherlands
[2]Animal Nutrition Group, Wageningen University & Research, 6700 AH, Wageningen, the Netherlands
[3]DSM Nutritional Products, Animal Nutrition & Health, 4002 Basel, Switzerland

ABSTRACT

The objectives were to investigate the effect of feeding and visiting behavior of dairy cattle on CH_4 and H_2 production measured with voluntary visits to the GreenFeed system (GF) and to determine whether these effects depended on basal diet (BD) and 3-nitrooxypropanol (3-NOP) supplementation. The experiment involved 64 lactating dairy cattle (146 ± 45 DIM at the start of trial; mean ± SD) in 2 overlapping crossover trials, each consisting of 2 measurement periods. Cows within block were randomly allocated to 1 of 3 types of BD: a grass silage-based diet consisting of 30% concentrates and 70% grass silage (DM basis); a grass silage and corn silage mixed diet consisting of 30% concentrates, 42% grass silage, and 28% corn silage (DM basis); or a corn silage-based diet consisting of 30% concentrates, 14% grass silage, and 56% corn silage (DM basis). Each type of BD was subsequently supplemented with 0 and 60 mg 3-NOP/kg of DM in one crossover or 0 and 80 mg 3-NOP/kg of DM in the other crossover. Diets were provided in feed bins that automatically recorded feed intake and feeding behavior, with additional concentrate fed in the GF. All visits to the GF that resulted in a spot measurement of both CH_4 and H_2 emission were analyzed in relation to feeding behavior (e.g., meal size and time interval to preceding meal) as well as GF visiting behavior (e.g., duration of visit). Feeding and GF visiting behavior were related to CH_4 and H_2 production measured with the GF, in particular the meal size before a GF measurement and the time interval between a GF measurement and the preceding meal. Relationships between gas production and both feeding and GF visiting behavior were affected by type of BD as well as 3-NOP supplementation. With an increase of the time interval between a GF measurement and the preceding meal, CH_4 production decreased with 0 mg 3-NOP/kg of DM but increased with 60 and 80 mg 3-NOP/kg of DM, whereas type of BD did not affect these relationships. In contrast, CH_4 production increased with 0 mg 3-NOP/kg of DM but decreased with 60 and 80 mg 3-NOP/kg of DM upon an increase in the size of the meal preceding a GF measurement. With an increase of the time interval between a GF measurement and the preceding meal, or with a decrease of the size of the meal preceding a GF measurement, H_2 production decreased for all treatments, although the effect was generally somewhat stronger for 60 and 80 mg 3-NOP/kg of DM than for 0 mg 3-NOP/kg of DM. Hence, the timing of GF measurements next to feeding and GF visiting behavior are essential when assessing the effect of dietary treatment on the production of CH_4 and H_2 in a setting where a spot-sampling device such as a GF is used and where the measurements depend on voluntary visits from the cows.

Key words: dairy cattle, feed intake behavior, visiting behavior, spot sampling

INTRODUCTION

Reducing enteric CH_4 production in cattle may significantly contribute to greenhouse gas mitigation and reduce the effect on climate change. Potential mitigation strategies include production intensification, dietary management, rumen manipulation, and selection of low-CH_4-producing animals (Arndt et al., 2022; Beauchemin et al., 2022). The need for high throughput measurements of enteric CH_4 production has led to the development of a variety of measurement techniques (Hammond et al., 2016), including the GreenFeed system (**GF**; C-Lock Inc., Rapid City, SD). The GF can be used in a variety of environments, including in freestall facilities and in grazing conditions. It is a spot-sampling device that measures CH_4, H_2, and CO_2 production from individual dairy cows with low disturbance of the cow's natural behavior, by

Received January 12, 2024.
Accepted April 11, 2024.
*Corresponding author: sanne.vangastelen@wur.nl

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

integrating measurements of airflow, gas concentrations, and detection of head position during each animal's visit (Zimmerman et al., 2011; Hegarty, 2013).

Many studies have used the GF to quantify CH_4 production of dairy cows as well as the CH_4 mitigating potential of dietary strategies. Diurnal patterns of CH_4 (and H_2) emission are related to feed intake patterns (van Lingen et al., 2017), which indicates that spot sampling of gaseous emission should be distributed over 24 h. Collection procedures of spot breath samples in the GF technique differ across studies. Where some studies (e.g., Hristov et al., 2015; Lopes et al., 2016) used a fixed sampling scheme to obtain a representative gas production value of a 24-h feeding cycle, in other studies (e.g., van Wesemael et al., 2019; van Gastelen et al., 2022) data collection was achieved by voluntary visits of dairy cows to the GF. van Gastelen et al. (2022) illustrated that these voluntary visits were well-spread over a 24-h period, and thereby likely captured the full diurnal pattern of CH_4 production. However, it remains unclear how measured CH_4 (and H_2) production during these voluntary visits to the GF relate to the combination of feeding behavior (e.g., meal size) and GF visiting behavior (e.g., interval between preceding meal and GF measurement) of dairy cows, and whether these relationships are affected by composition of the basal diet (**BD**) and high-efficacy CH_4 inhibition strategies such as supplementation with 3-nitrooxypropanol (**3-NOP**).

The objectives of the present study were therefore to (1) investigate the effect of feeding and GF visiting behavior of dairy cattle on CH_4 and H_2 production as measured by voluntary visits to the GF and (2) determine whether these effects depend on BD and 3-NOP supplementation. We hypothesized that feeding behavior, but not GF visiting behavior, would be affected by BD and 3-NOP supplementation. Furthermore, we hypothesized that production of CH_4 and H_2 measured with the GF relates to feeding behavior and GF visiting behavior, and that these relationships thus depend on BD composition and 3-NOP supplementation.

MATERIALS AND METHODS

Experimental Design

This study was part of the experiment described by van Gastelen et al. (2022), where they investigated whether the CH_4 mitigation potential of 3-NOP in dairy cattle was affected by BD composition. The experiment was conducted from November 2019 to February 2020 at the research facilities of Wageningen Livestock Research (Dairy Campus, Leeuwarden, the Netherlands), under the Dutch law on Animal Experiments in accordance with European Union Directive 2010/63. The use of 3-NOP (DSM Nutritional Products Ltd.) in animal feed was preapproved by the Veterinary Drugs Directorate Division (Utrecht, the Netherlands). In short, the experiment involved 64 Holstein-Friesian dairy cows (146 ± 45 DIM at start of trial; mean ± SD) in 2 overlapping crossover trials, each consisting of 2 measurement periods. Cows were blocked according to parity, DIM, and milk yield, which resulted in 11 blocks in total (i.e., 10 blocks of 6 cows and 1 block of 4 cows). Within each block, cows were randomly allocated to 1 of 3 diets (all on a DM basis): a grass silage-based diet (**GS**; 30% concentrates and 70% grass silage), a grass silage and corn silage mixed diet (**GSCS**; 30% concentrates, 42% grass silage, and 28% corn silage), or a corn silage-based diet (**CS**; 30% concentrates, 14% grass silage, and 56% corn silage). Each type of BD was subsequently supplemented with 3-NOP in a crossover design: either 60 mg of 3-NOP/kg of DM (**NOP60**) and a placebo with 0 mg of 3-NOP/kg of DM (**NOP0**) in one crossover, and 80 mg 3-NOP/kg of DM (**NOP80**) and NOP0 in the other crossover (Supplemental Figure S1; see Notes). The experiment started with a covariate period of 2 wk in which baseline measurements took place on a common basal diet, followed by a week for adaptation to the BD the cows were allocated to. After this BD adaptation week, the treatment periods of the crossover trials started; these were composed of adaptation periods and measurement periods (see Supplemental Figure S1). Only data from the covariate period and 4 measurement weeks (i.e., wk 6, 8, 11, and 13 of the trial) were used, corresponding to the data used for statistical analysis by van Gastelen et al. (2022).

Gaseous Exchange

Production of CH_4, H_2, and CO_2 was measured on an individual-cow level using 3 GF systems (C-Lock Inc.; Zimmerman et al., 2011). The dairy cows were loose-housed as 1 group and each GF could be visited by any cow. A dairy cow could visit the GF every 2 h (with a maximum of 12 times/d), where data collection was dependent on voluntary visits to the GF. A bait was offered at the GF for enticement and to encourage the cow to maintain a suitable head position for accurate measurements, with a maximum of 8 so-called cup drops per visit, 1 cup drop per 30 s, and 35.7 ± 2.22 g of feed per cup drop.

Data from the GF systems included: visits where cows were identified with radio frequency identification (**RFID** visits), visits where gas production was measured (emission visits), and visits where GF bait was supplied (bait visits). These 3 types of visits do not necessarily overlap. All emission visits and bait visits overlap with RFID visits, otherwise the visit cannot be linked to a cow. But not every RFID visit is coupled with a bait visit or emission visit, because cows occasionally visit the GF without bait supply and measurements of gas production (i.e., a previous GF visit was less than 2 h ago and 2 h was the interval setting for measurement). Additionally, a bait visit might not be coupled with an emission visit if the criteria (i.e., head position or duration of visit) were considered to be inappropriate. Furthermore, an emission visit might not be coupled with a bait visit if no bait was supplied, despite cows remaining for sufficient time and with a correct head position in the GF system. For the present study, only emission visits were used to investigate the effects on CH_4 and H_2 production, also when those emission visits did not overlap with bait visits. All bait visits were used to calculate the interval between GF visits, also when those did not overlap with emission visits. The interval between 2 successive GF visits was arbitrary maximized at 36 h, which resulted in excluding 2 values (0.03% of the dataset). Please note that this approach deviates from van Gastelen et al. (2022), where both emission visits and bait visits were included in the analyses.

Feed Intake

Feed was supplied 4 times daily as partial mixed ration (**PMR**; excluding GF bait) by using an automated feeding system consisting of the Trioliet feed mixing robot (Triomatic HP 2 300, Trioliet) for mixing the experimental diets, and the Insentec feed bin (**FB**; Hokofarm Group B.V.) to measure feed intake for each individual cow. The dairy cows were housed as 1 group with 32 FB for feeding (i.e., 2 cows per FB), with the cows having access to all FB with their assigned diet. The assignment of the cows to the FB was established at the start of trial and remained the same throughout the trial. Hence, upon dietary changes, the diets in the FB changed and the cows remained assigned to the same FB (for further details, see van Gastelen et al., 2022).

For every visit of a cow to a FB, the start and end times of the visit as well as the start and end weights of the FB content were recorded. The FB visits without intake (16.9% of total visits) were removed from the dataset. Feeding behavior was subsequently analyzed according to Tolkamp and Kyriazakis (1999) and Yeates et al. (2001). The interval length between 2 consecutive FB visits was transformed with a natural logarithm. For every of the 9 treatments (3 BD and 3 levels of 3-NOP), a 3-population model based on 2 Gaussian functions and 1 Weibull function was fitted through the individual transformed time intervals using the fittype option in the fit function in MATLAB (MATLAB version: 9.13.0 R2022b, The MathWorks Inc., Natick, MA). The 2 Gaussian functions describe within-meal intervals and the Weibull distribution describes the between-meal interval. The meal criterion was determined as the intersection between the second Gaussian and the Weibull curve, which was subsequently used to describe the longest interval between 2 consecutive FB visits that are still part of the same meal. The meal criterion differed per treatment (Figure 1), varying between 18.1 min (GSCS_NOP80) and 33.5 min (GS_NOP80). The meal criteria were subsequently used to determine feeding behavior, where visits were clustered into meals per day and daily total meal duration was calculated. According to Abrahamse et al. (2008b), we calculated the number of visits, meal duration, meal size (kg of DMI), and eating rate (kg of DMI per meal divided by eating time in minutes per meal) for each identified meal, where eating time was meal duration minus intervals within meals. Meals with an eating rate larger than 0.5 kg of DM/min (less than 0.1% of the meals, and those also expected to be caused by spilling of feed) were excluded from the analysis.

Sample Collection and Chemical Analysis

Samples of all ration components and the GF bait were taken once weekly and stored at −20°C pending analysis. At the end of the experiment, all feed samples were thawed at room temperature, freeze-dried until constant weight, and ground to pass a 1-mm screen. The samples were subsequently analyzed for chemical composition including DM using wet chemistry as described by Abrahamse et al. (2008a). For the intake of the PMR, DMI was calculated based on the DM content of the individual ration components, the ration composition as mixed by the Trioliet feed mixing robot, and the kilograms of wet weight intake recorded in the FB.

Preprocessing of Collected Data

The feeding behavior (e.g., size of the preceding meal at the FB) as well as the GF visiting behavior (e.g., time interval between preceding meal at a FB and a GF measurement) were used to investigate the effect of feeding and GF visiting behavior of dairy cattle on CH_4 and H_2 production and to determine whether these effects were BD and 3-NOP supplementation dependent. To do so, the data of both systems (i.e., FB and GF) needed to be linked. Although the GF and FB systems have the same time on their time clock in the barn, the time registrations of these systems did not match in the recorded data and we therefore used a time correction of +42 s for the GF data. Without time correction, 6.8% of the GF visits (in the complete experimental period of 13 wk; van Gastelen et al., 2022) showed an overlap with a FB visit, whereas this proportion decreased to a minimum of 1.3% with the time correction. This minimum was achieved by increas-

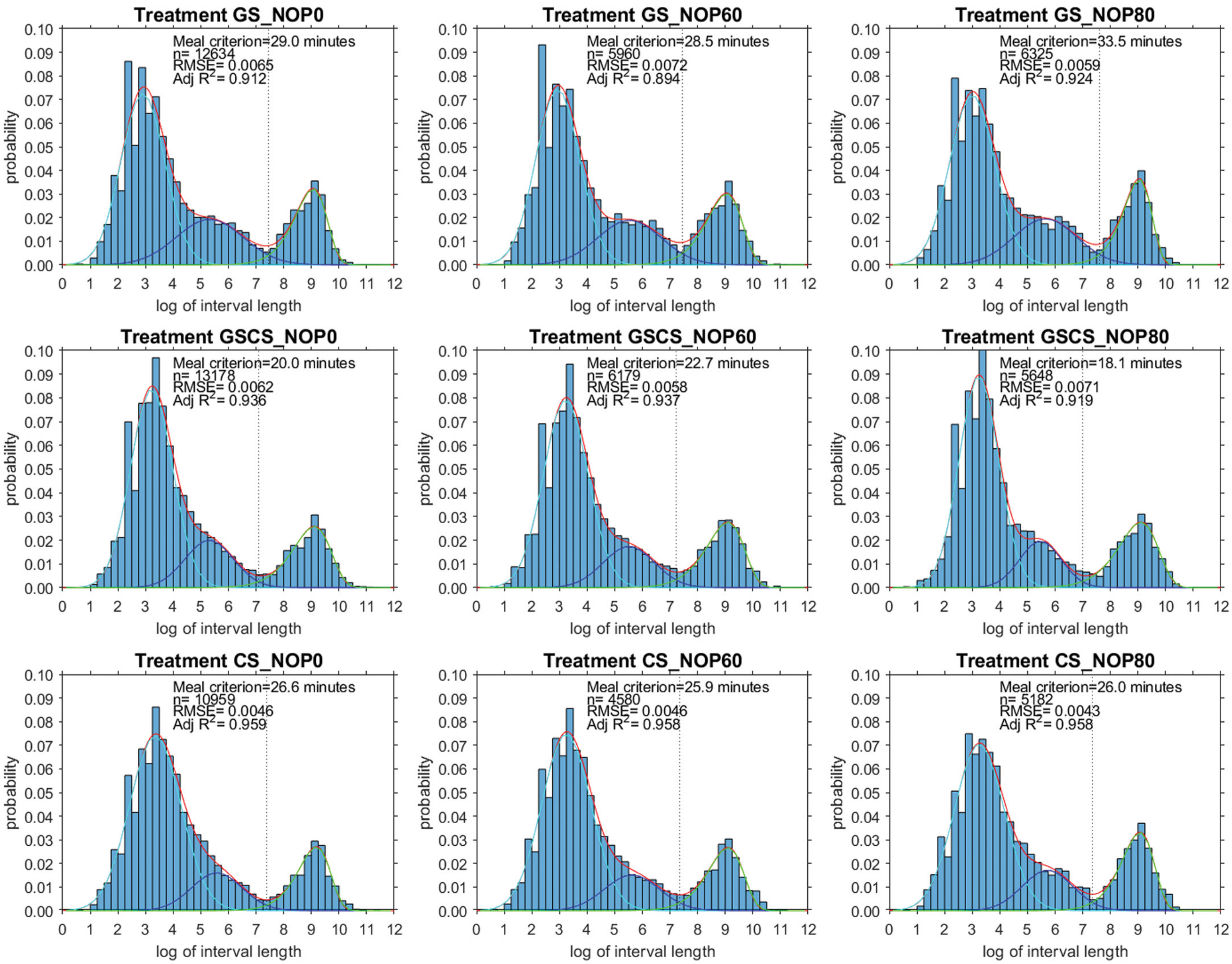

Figure 1. Histogram per treatment of the log-transformed lengths of intervals between successive FB visits; the red curve is a fitted 3-fold density function based on 2 Gaussian functions (light/dark blue lines) and 1 Weibull function (green line); the dotted vertical line marks the intersection of the second Gaussian and the Weibull curve that is used to determine the meal criterion. Each treatment is a combination of diet type: grass silage-based (GS), grass silage and corn silage mixed (GSCS), or corn silage-based (CS) and dose of 3-NOP (NOP0, NOP60, and NOP80 for 0, 60, or 80 mg of 3-NOP/kg of DM). Adj R^2 = adjusted R^2; RMSE = root mean square error.

ing the time correction providing the overlap decreased. Nevertheless, 382 GF visits, despite the time correction, still showed an overlap with a FB visit, which is physically not possible, and were excluded from data analysis.

Statistical Analysis

The final dataset included 63 cows, because 1 cow was excluded due to persistent stealing behavior (stealing on average 9% of her daily DMI from FB she was not assigned to). All parameters related to feed and GF visiting behavior were averaged per cow per period (i.e., covariate period and 4 measurement periods). In line with van Gastelen et al. (2022), the feeding and GF visiting behavior data were subsequently split into 2 datasets, one dataset with the data of the crossover with 0 and 60 mg of 3-NOP/kg of DM and one dataset with the data of the crossover with 0 and 80 mg of 3-NOP/kg of DM, where both datasets included the covariate period as well as baseline measurement for BD. Per crossover, data were subjected to ANOVA in a crossover with a 2 period × 2 treatment design by using the MIXED procedure in SAS (edition 9.4, SAS Institute Inc., Cary, NC). The BD (GS, GSCS, and CS), the 3-NOP dose (0 and 60 mg/kg of DM for the first crossover and 0 and 80 mg/kg of DM for the second crossover), the BD × 3-NOP dose interaction, treatment sequence (i.e., order in which the 3-NOP dosages were supplemented within crossover), period, as well as the baseline measurement from the covariate period were considered fixed effects. The model included block and cow as random factors. The covariance structure variance components provided the best fit with the lowest overall Akaike's information criterion values. The Kenward-Roger option was used to estimate the denominator degrees of freedom. Values are presented (in Supplemental Tables S1 and S2; see Notes) as LSM ± pooled SEM. All *P*-values of pair-wise comparisons of LSM were corrected with a Tukey adjustment. Significance was declared at $P < 0.05$ and trends at $0.05 \leq P \leq 0.10$.

Additionally, all feeding and GF visiting behavior data were combined into 1 dataset to obtain descriptive statistics by using the MEANS procedure in SAS. Subsequently, the Pearson correlation (CORR procedure in SAS) was used to test, for each diet separately (BD × 3-NOP) as well as for all diets together and for the covariate period, the relationship between the measured CH_4 and H_2 production and feeding and GF visiting behavior of dairy cattle. We subsequently used the REG procedure in SAS, according to Sawant (2012), to compare the regression coefficient between the models of individual diets.

RESULTS

Feeding and GF Visiting Behavior

The descriptive statistics of the feeding and GF visiting behavior are presented in Table 1. The effect of BD, 3-NOP, and BD × 3-NOP on feeding and GF visiting behavior are presented in Supplemental Tables S1 and S2, respectively. These results were not the objective of the present study and thus will not be described and discussed in detail. These results did serve, however, as underlying data for our objective, which was to determine whether the relationship between feeding and GF visiting behavior and the CH_4 and H_2 production measurements were BD and 3-NOP supplementation dependent.

Relationship Between Cow Behavior and CH_4 Production

The relationship between the measured CH_4 production and cow behavior per dietary treatment (BD × 3-NOP) is presented in Table 2. In the current study, a relationship is considered to be weak when $|r| \leq 0.20$, moderate when $0.20 < |r| \leq 0.50$, and strong when $|r| > 0.50$. For most treatments, weak relationships were found between measured CH_4 production and duration of the GF visits (usually positive), between measured CH_4 production and GF bait intake during the GF visit (all positive), and between measured CH_4 production and interval length with the previous GF visit (some positive, some negative).

Most of the relationships between measured CH_4 production and interval length with the preceding meal consumed in the FB (Figure 2) and between measured CH_4 production and DMI consumed during the preceding FB meal (Figure 3) were moderate. Overall, however, across all BD and 3-NOP treatments, the relationships appeared weak due to the large differences in relationship (positive and negative ones) for individual dietary treatments. The relationship between measured CH_4 production and interval length with the preceding meal consumed in a FB appeared to be affected by 3-NOP, but not by BD. This relationship was negative for NOP0, but positive for NOP60 and NOP80, irrespective of BD. In other words, with an increase of the time interval between the preceding FB meal and the GF measurement CH_4 production decreased for NOP0, but increased for NOP60 and NOP80. Conversely, the opposite holds for the relationship between measured CH_4 production and DMI during the preceding FB meal, which was, irrespective of BD, positive for NOP0 and negative for NOP60 and NOP80. These relationships indicate that with an increase in the size of the preceding FB meal, CH_4 production increased for NOP0, but decreased for NOP60 and NOP80.

The results of the regression coefficient comparison between the models for individual diets are shown in Table 3 for the relationships between measured CH_4 production and the interval of the preceding FB meal (CH_4 interval models), and in Table 4 for the relationships between measured CH_4 production and the preceding FB meal size (CH_4 meal-size models). Most regression coefficients of the CH_4 interval models differed between the individual diets ($P \leq 0.046$), indicating either a different slope direction (i.e., negative vs. positive) or a smaller or larger slope, with some exceptions. The slope of GS_NOP80 and CS_NOP60 for example only tended to differ from that of CS_NOP80 ($P = 0.056$ and 0.060, respectively). The slope of both GSCS_NOP0 and CS_NOP0 did not differ from that of both covariate and GS_NOP0. The slope of GSCS_NOP80 did not differ from that of both GSCS_NOP60 and CS_NOP80, the slope of GS_NOP80 did not differ from that of CS_NOP60, and the slope of GSCS_NOP0 did not differ from that of CS_NOP0. Most of the regression coefficients of the CH_4 meal-size models differed between the individual diets (*P* in most cases <0.001, maximum $P = 0.043$), but again with some exceptions; the slope of the covariate only tended to differ from that of GS_NOP0 and CS_NOP0 ($P = 0.056$ and 0.067, respectively), and the slope of GSCS_NOP60 did not differ from that of GSCS_NOP80, CS_NOP60, and CS_NOP80. The slope of GSCS_NOP80 did not differ from that of both CS_NOP60 and CS_NOP80, the slope of the covariate did not differ from that of GSCS_NOP0, the slope of GS_NOP0 did not differ from that of CS_NOP0, the slope of GS_NOP60 did not differ from that

Table 1. Descriptive statistics of feeding and GF[1] visiting behavior from lactating dairy cows fed different BD with or without 3-NOP supplementation[2]

Variable	Mean	Median	SE	Minimum	Maximum
Feeding behavior					
Total duration of meals (min/d)	243	243	1.3	85	446
Meal duration (min/meal)	26.5	23.2	0.14	0.1	146.8
Total eating time (min/d)	198	199	1.1	55	347
Eating time (min/meal)	21.6	20.0	0.11	0.1	102.4
Meals (number)					
Full 24-h period	9.2	9.0	0.05	2.0	17.0
Between 0000 h and morning milking	1.8	2.0	0.02	0.0	6.0
Between morning and afternoon milking	4.1	4.0	0.03	1.0	10.0
Between afternoon milking and 0000 h	3.3	3.0	0.03	0.0	7.0
Meal size (kg of DM/meal)	2.33	2.11	0.012	0.04	14.46
Visits per meal	4.53	4.00	0.027	1.00	41.00
Eating rate (g of DM/min)	111	105	0.3	3	486
GF visiting behavior					
GF visits (number)					
Full 24-h period	4.3	4.0	0.05	1.0	9.0
Between 0000 h and morning milking	1.2	1.0	0.02	0.0	4.0
Between morning and afternoon milking	1.8	2.0	0.03	0.0	5.0
Between afternoon milking and 0000 h	1.4	1.0	0.02	0.0	4.0
GF visit duration (min)	4.47	4.78	0.011	2.00	9.82
GF bait intake					
kg of DM/d	0.99	1.00	0.010	0.11	2.05
g of DM/GF visit	230	245	0.6	0	270
Gas production (g/d)					
CH_4	357	351	1.4	48	804
H_2	5.18	3.16	0.056	0.00	20.81
Interval (min)					
Between GF visits[3]	257	217	1.9	0[4]	2113
Between preceding meal FB[5] and GF visit	72.8	5.1	1.85	0.0	1735.9
Between GF visit and next FB meal	86.0	48.9	1.25	0.0	950.3
Preceding meal size FB[6] (kg of DM)	2.42	2.21	0.017	0.04	10.71

[1]GreenFeed system to record gaseous emissions with cow identification (results in table restricted to visits where gas emission was measured).

[2]Based on data collecting during the 4 measurement weeks (i.e., wk 6, 8, 11, and 13 of the trial) only, excluding the covariate period.

[3]Interval between a GF visit with gas emission measurement and a previous GF visit where bait was consumed, irrespective of whether this GF visit resulted in an emission measurement.

[4]The interval between 2 successive GF visits can be zero around midnight: the GF visit is split into 2 visits, 1 starting just before midnight and 1 starting just after midnight.

[5]Feed intake bins with automatic recording and cow identification.

[6]Preceding meal in a feed intake bin before a gas emission measurement in the GreenFeed system.

of GS_NOP80, and the slope of CS_NOP60 did not differ from that of CS_NOP80.

Relationship Between Cow Behavior and H_2 Production

The relationships between the measured H_2 production and cow feeding and GF visiting behavior per dietary treatment (BD × 3-NOP) are shown in Table 5. Only weak relationships were found between the measured H_2 production and duration of GF visits, between the measured H_2 production and GF bait intake during GF visits, and between the measured H_2 production and the interval length with the previous GF visit. For all dietary treatments, the measured H_2 production was negatively related to the interval length of the preceding meal consumed in a FB (Figure 4). These moderate-to strong-relationships indicate that the measured H_2 production decreased when the time interval between the preceding FB meal and a GF measurement increased.

For all dietary treatments, the measured H_2 production was positively related to the DMI consumed during the preceding meal (see also Figure 5). This relationship was clearly affected by 3-NOP, but not by BD. The relationship was weak for NOP0, but moderate for NOP60 and NOP80. These results indicate that upon an increase in the size of the preceding FB meal, the measured H_2 production was somewhat increased for NOP0, but more strongly for NOP60 and NOP80.

The results of the regression coefficient comparison between the models of individual diets are shown in Table 6 for the relationship between measured H_2 production

Table 2. Pearson correlations between the measured CH_4 production (g/d) and GF[1] visit behavior and feeding behavior

BD and 3-NOP dose[2]	Correlation with CH_4 production (g/d)	Duration of GF visit (min)	GF bait intake during visit (g of DM)	Interval preceding GF visit (min)	Interval preceding FB[3] meal (min)	Intake preceding FB meal (kg of DM)
Covariate						
NA	r	0.06	0.08	0.01	−0.25	0.27
	P-value	<0.001	<0.001	0.588	<0.001	<0.001
GS						
0	r	0.05	0.10	0.04	−0.26	0.21
	P-value	0.079	<0.001	0.093	<0.001	<0.001
60	r	0.05	0.11	0.03	0.11	−0.07
	P-value	0.214	0.002	0.467	0.003	0.056
80	r	0.10	0.15	0.00	0.30	−0.12
	P-value	0.007	<0.001	0.959	<0.001	<0.001
GSCS						
0	r	0.05	0.11	0.07	−0.28	0.37
	P-value	0.079	<0.001	0.032	<0.001	<0.001
60	r	0.00	0.07	−0.10	0.21	−0.33
	P-value	1.000	0.112	0.014	<0.001	<0.001
80	r	0.06	0.16	−0.13	0.22	−0.35
	P-value	0.190	<0.001	0.003	<0.001	<0.001
CS						
0	r	0.06	0.07	0.08	−0.27	0.31
	P-value	0.039	0.027	0.013	<0.001	<0.001
60	r	−0.02	0.04	−0.14	0.30	−0.37
	P-value	0.602	0.362	0.002	<0.001	<0.001
80	r	0.01	0.12	−0.10	0.26	−0.32
	P-value	0.823	0.006	0.023	<0.001	<0.001
Overall[4]	r	0.03	0.08	0.00	−0.03	0.05
	P-value	0.006	<0.001	0.891	0.021	<0.001

[1]GreenFeed system recording gaseous emissions with cow identification.

[2]Basal diets were as follows: GS = grass silage-based, GSCS = grass silage and corn silage mixed, CS = corn silage-based with 0, 60, or 80 mg of 3-NOP/kg of DM supplementation, NA = not applicable (no placebo or 3-NOP supplementation).

[3]Feed intake bins with automatic recording and cow identification.

[4]Representing all experimental diets (GS, GSCS, and CS as well as 0, 60, and 80 mg 3-nitrooxypropanol/kg of DM supplementation).

and the interval of the preceding FB meal (H_2 interval models), and in Table 7 for the relationship between measured H_2 production and the preceding FB meal size (H_2 meal-size models). Most of the H_2 interval models differed between the individual diets (*P* in most cases <0.001, maximum $P = 0.028$), indicating either a different slope direction or a different slope size. There are only a few exceptions; the slope of GSCS_NOP0 only tended to differ from that of the covariate period ($P = 0.100$) and did not differ from that of GS_NOP0. Furthermore, the slopes did not differ between GSCS_NOP60 and GS_NOP60 and between CS_NOP80 and GSCS_NOP80. In addition, regression coefficients of most H_2 meal-size models differed between the individual diets (*P* in most cases <0.001, maximum $P = 0.044$), although the number of exceptions (n = 15) was considerable (Table 7). For example, the slope of GS_NOP60 did not differ from the slopes of GS_NOP80, GSCS_NOP60, GSCS_NOP80, CS_NOP60, and CS_NOP80.

DISCUSSION

The data analysis for the current study was restricted to GF visits where CH_4 and H_2 production was successfully determined (emission visits; 7,137 in total), even when those visits were not overlapping with bait visits (76 visits out of the 7,137 emission visits in total). The average CH_4 and H_2 production of all emission visits, regardless of whether they overlapped with bait visits, was respectively 357 and 5.18 g/d, the average CH_4 and H_2 production of the emission visits overlapping with bait visits was respectively 357 and 5.18 g/d, and the average CH_4 and H_2 production of the emission visits that did not overlap with bait visits was 299 and 5.14 g/d, respectively. Despite this large difference in emissions between emission visits with and without bait supply, the effect of the emission visits that do not overlap with bait visits on gas emissions was negligible, likely because these visits only represent 1% of all emission visits.

Relationship Between Cow Behavior and CH_4 Production

For a broad range of diets, the quantity of CH_4 emitted is closely related to the quantity of digestible OM consumed, with further variation moderated by the nutrient composition of the diet (Blaxter and Clapperton, 1965; Mills et al., 2001). In addition, there is a strong relationship between the quantity of CH_4 emitted and the time after feeding and feeding frequency (e.g., Crompton et

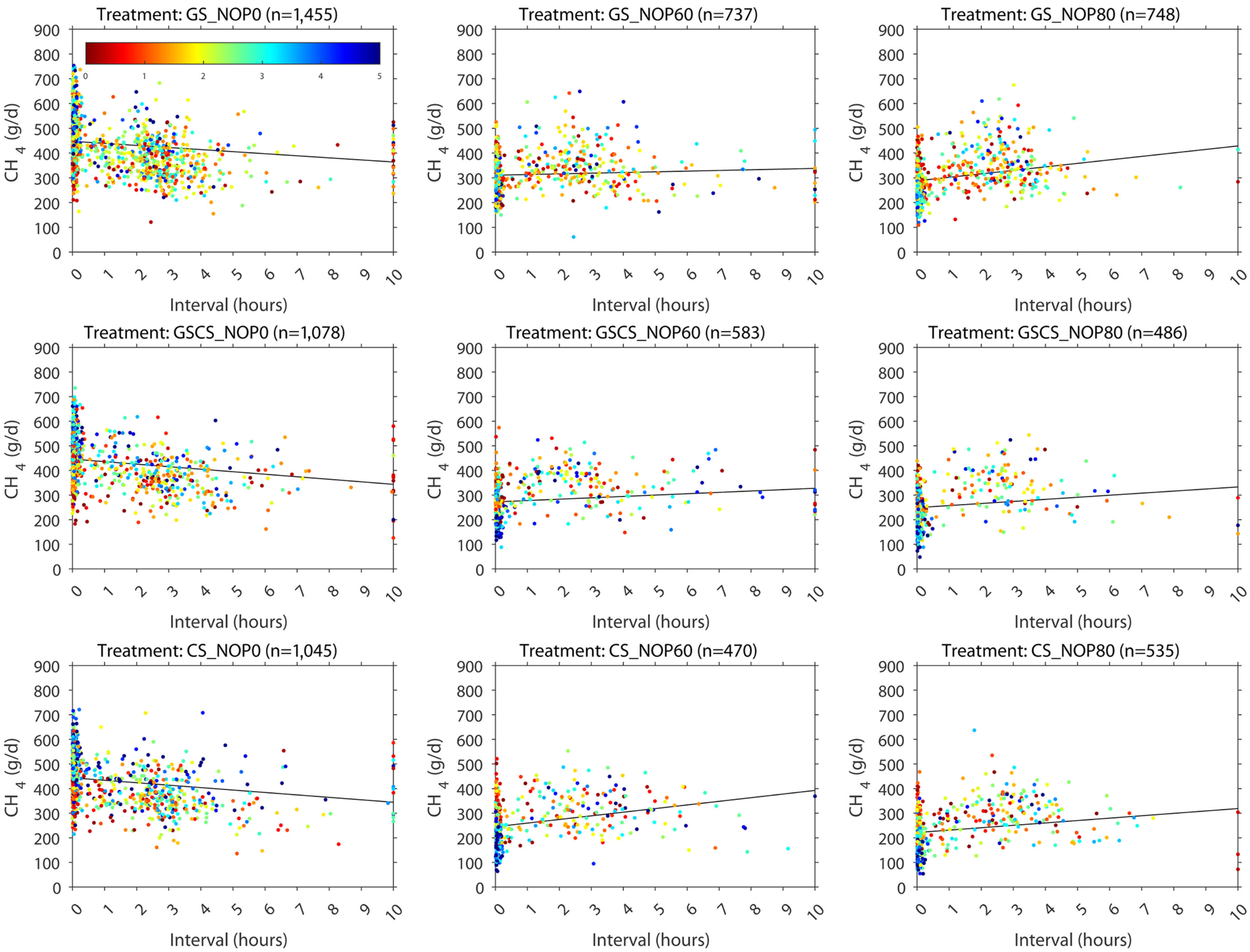

Figure 2. Scatter plots per treatment of CH_4 production (g/d) versus the time interval (h) between a GF visit and the preceding FB meal. The colors of the dots represent DMI during the preceding FB meal (in kg of DM; legend in upper left panel). The black linear regression line is fitted over all visits. GS = grass silage-based diet, GSCS = grass silage and corn silage mixed diet, CS = corn silage-based diet, supplemented with 0 (NOP0), 60 (NOP60), or 80 (NOP80) mg of 3-NOP/kg of DM.

al., 2011; Jonker et al., 2014; van Lingen et al., 2017). Furthermore, headspace accumulation of CH_4 in the rumen is affected by feed supply, rumination, and animal movement, and this accumulation is subsequently associated with the eructation frequency (McCauley and Dziuk, 1965). Hence, the rate of CH_4 production is not constant over 24 h and at any given time depends on (1) time relative to feeding, feed allowance (level), and feed intake patterns, (2) dietary composition, and (3) short-term sporadic variation in gas release from the rumen with specific eructation patterns (Hegarty, 2013). The circadian variation in CH_4 production is best accounted for by using measurement techniques that attempt to sample continuously over 24 h, but can also be captured by using the GF at specific and a sufficient number of moments during that 24-h period. Hammond et al. (2016) recommended sampling schemes for gas collection to distribute the spot sampling of CH_4 production over 24 h to account for diurnal and postprandial variation in CH_4 production and that cows should have continuous access to feed with multiple feedings per day.

Lee et al. (2022) used respiration chamber data to simulate spot sampling to evaluate the accuracy of CH_4 production estimates. They concluded that at least 8 spot samples (i.e., every 3 h within a 24-h cycle) are necessary to accurately estimate daily CH_4 production and detect dietary effects on CH_4 emissions. van Lingen et al. (2023) also used respiration chamber data to simulate spot sampling and concluded that 3 spot samples (i.e., 8 h interval, starting 2 h after feeding)

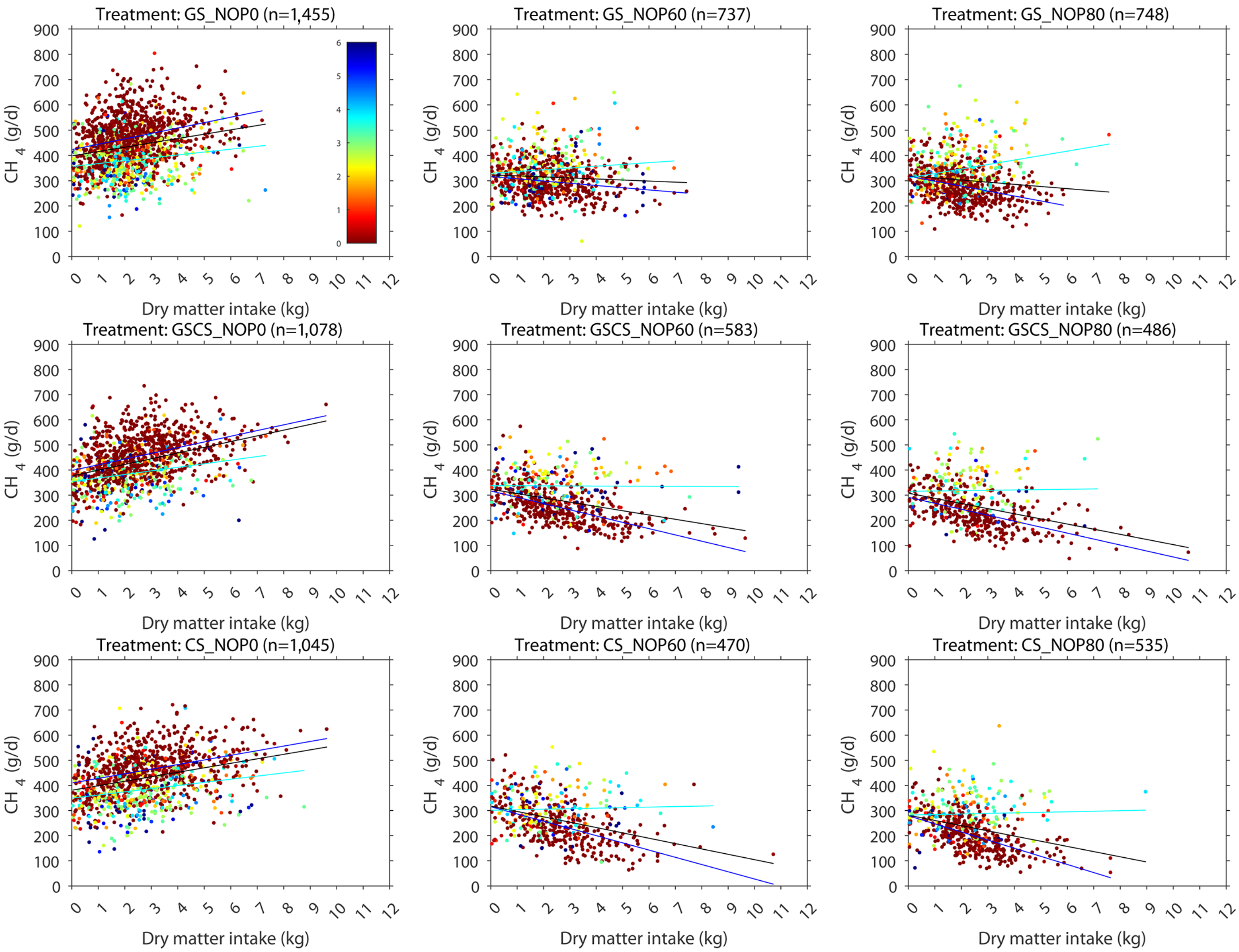

Figure 3. Scatter plots per treatment of CH_4 production (g/d) versus DMI during the preceding FB meal (in kg of DM). The black linear regression line is fitted over all visits. The blue regression lines are fitted on visits with interval lengths between the GF visit and the preceding FB meal lasting less (dark blue) or more (light blue) than 15 min. The colors of the dots represent the interval length between the GF visit and the preceding FB meal (h; legend in upper left panel). GS = grass silage-based diet, GSCS = grass silage and corn silage mixed diet, CS = corn silage-based diet, supplemented with 0 (NOP0), 60 (NOP60), or 80 (NOP80) mg of 3-NOP/kg of DM.

were sufficient when applying twice daily ad libitum feeding, whereas hourly measurements were needed for restricted feeding (van Lingen et al., 2023). In the present study, the average number of daily GF visits resulting in successful CH_4 measurement was 4.31, with an average time interval between consecutive GF visits of 257 min (4.28 h). As illustrated in Supplemental Figure S2 (see Notes), these GF visits were well-spread over the 24-h period. This, in combination with the feeding management applied (i.e., ad libitum feeding, 4 times daily filling of the FB) and in agreement with van Lingen et al. (2023), suggests that we obtained accurate daily CH_4 production values for the different dietary treatments.

Differences in cow feeding behavior (e.g., number and size of meals) can cause substantial measurement error when using spot-sampling techniques (Hammond et al., 2016). Velazco et al. (2014) observed that nitrate-fed cattle consumed a larger number of smaller sized meals, which caused a shorter interval between a feeding event and CH_4 measurement. This resulted in skewed estimates of daily CH_4 production and contributed to the measured difference in CH_4 production between nitrate-fed cattle and urea-fed cattle. Cottle et al. (2015) subsequently determined how much of the variation in daily CH_4 production could be explained by the timing and size of the preceding meal (relative to spot-sampling measurement of CH_4) and showed that only 16.9% of the variance in

Table 3. Comparison of Pearson correlation coefficients of the relationship between GF[1] measured CH_4 production and the time interval with the last FB[2] meal (in min) for combinations of BD type and 3-NOP supplementation[3]

			95% confidence limits			GS			GSCS			CS		
BD[4]	3-NOP[5]	Slope	Lower	Upper	Covariate	0	60	80	0	60	80	0	60	80
Covariate	NA	−0.190	−0.210	−0.169	—	0.007	<0.001	<0.001	0.421	<0.001	<0.001	0.303	<0.001	<0.001
GS	0	−0.139	−0.166	−0.112		—	<0.001	<0.001	0.170	<0.001	<0.001	0.265	<0.001	<0.001
	60	0.046	0.015	0.077			—	<0.001	<0.001	0.046	0.003	<0.001	<0.001	<0.001
	80	0.235	0.181	0.289				—	<0.001	<0.001	0.016	<0.001	0.806	0.056
GSCS	0	−0.171	−0.206	−0.136					—	<0.001	<0.001	0.831	<0.001	<0.001
	60	0.092	0.058	0.127						—	0.145	<0.001	<0.001	0.025
	80	0.140	0.085	0.196							—	<0.001	0.021	0.566
CS	0	−0.165	−0.202	−0.129								—	<0.001	<0.001
	60	0.246	0.175	0.316									—	0.060
	80	0.162	0.111	0.214										—

[1]GreenFeed system recording gaseous emissions with cow identification.
[2]Feed intake bins with automatic recording and cow identification.
[3]The regression coefficients were compared between the diets according to Sawant (2012).
[4]Basal diets were as follows: GS = grass silage-based diet, GSCS = grass silage and corn silage mixed diet, CS = corn silage-based diet.
[5]0, 60, or 80 mg of 3-NOP/kg of DM supplementation. NA = not applicable; no placebo or 3-NOP supplementation.

Table 4. Comparison of Pearson correlation coefficients of the relationship between GF[1] measured CH_4 production and size of the last FB[2] meal (in kg of DM) for combinations of BD type and 3-NOP supplementation[3]

			95% confidence limits			GS			GSCS			CS		
BD[4]	3-NOP[5]	Slope	Lower	Upper	Covariate	0	60	80	0	60	80	0	60	80
Covariate	NA	21.97	19.68	24.27	—	0.056	<0.001	<0.001	0.746	<0.001	<0.001	0.067	<0.001	<0.001
GS	0	17.16	13.14	21.18		—	<0.001	<0.001	0.038	<0.001	<0.001	0.793	<0.001	<0.001
	60	−4.13	−8.36	0.10			—	0.183	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	80	−8.52	−13.44	−3.60				—	<0.001	0.008	<0.001	<0.001	<0.001	0.001
GSCS	0	22.74	19.35	26.14					—	<0.001	<0.001	0.043	<0.001	<0.001
	60	−17.05	−20.96	−13.14						—	0.278	<0.001	0.177	0.276
	80	−20.47	−25.33	−15.61							—	<0.001	0.811	0.956
CS	0	17.85	14.56	21.14								—	<0.001	<0.001
	60	−21.31	−26.18	−16.43									—	0.862
	80	−20.67	−25.97	−15.37										—

[1]GreenFeed system recording gaseous emissions with cow identification.
[2]Feed intake bins with automatic recording and cow identification.
[3]The regression coefficients were compared between the diets according to Sawant (2012).
[4]Basal diets were as follows: GS = grass silage-based diet, GSCS = grass silage and corn silage mixed diet, CS = corn silage-based diet.
[5]0, 60, or 80 mg of 3-NOP/kg of DM supplementation. NA = not applicable; no placebo or 3-NOP supplementation.

Table 5. Pearson correlations between the measured H_2 production (g/d) and GF[1] visit behavior and feeding behavior

BD and 3-NOP dose[2]	Correlation with H_2 production (g/d)	Duration of GF visit (min)	GF bait intake during visit (g of DM)	Interval preceding GF visit (min)	Interval preceding FB[3] meal (min)	Intake preceding FB meal (kg of DM)
Covariate						
NA	r	−0.03	−0.03	−0.02	−0.38	0.12
	P-value	0.020	0.021	0.228	<0.001	<0.001
GS						
0	r	−0.05	−0.01	0.08	−0.34	0.02
	P-value	0.048	0.610	0.002	<0.001	0.244
60	r	0.00	0.02	0.13	−0.41	0.32
	P-value	0.910	0.591	<0.001	<0.001	<0.001
80	r	0.06	0.05	0.12	−0.58	0.33
	P-value	0.100	0.168	0.001	<0.001	<0.001
GSCS						
0	r	−0.03	0.03	0.07	−0.36	0.14
	P-value	0.315	0.298	0.014	<0.001	<0.001
60	r	0.08	0.02	0.07	−0.39	0.31
	P-value	0.067	0.604	0.116	<0.001	<0.001
80	r	0.04	0.00	0.11	−0.46	0.37
	P-value	0.386	0.942	0.017	<0.001	<0.001
CS						
0	r	0.03	0.05	0.04	−0.30	0.10
	P-value	0.264	0.085	0.148	<0.001	0.002
60	r	0.11	0.07	0.02	−0.72	0.40
	P-value	0.015	0.139	0.710	<0.001	<0.001
80	r	0.05	−0.04	0.07	−0.52	0.35
	P-value	0.207	0.353	0.084	<0.001	<0.001
Overall[4]	r	0.03	0.01	0.05	−0.31	0.16
	P-value	0.032	0.251	<0.001	<0.001	<0.001

[1]GreenFeed system recording gaseous emissions with cow identification.

[2]Basal diets were as follows: GS = grass silage-based diet, GSCS = grass silage and corn silage mixed diet, CS = corn silage-based diet with 0, 60, or 80 mg of 3-NOP/kg of DM supplementation, NA = not applicable; no placebo or 3-NOP supplementation.

[3]Feed intake bins with automatic recording and cow identification.

[4]Representing all experimental diets (GS, GSCS, and CS, as well as 0, 60, and 80 mg 3-NOP/kg of DM supplementation).

the CH_4 production measured by the GF was explained by this feeding information. This suggests that the timing of a GF measurement relative to eating or meal size was of relatively little importance, which contrasts to what can be expected from the studies of Crompton et al. (2011), Jonker et al. (2014), and van Lingen et al. (2017).

In the present study, feeding behavior was affected by both BD and 3-NOP, and for BD this also resulted in differences in GF visiting behavior. Cows receiving GS consumed a larger number of smaller sized meals, had a lower eating rate, and visited the GF more often than cows receiving GSCS or CS. This did not result in a shorter time interval between a feeding event and a CH_4 production measurement, but the meal size before a CH_4 measurement was smaller for cows receiving GS. For all 3 BD without 3-NOP (NOP0), a moderate positive relationship between the measured CH_4 production and the DMI of the preceding FB meal was observed. Therefore, measured CH_4 production of cows receiving GS without 3-NOP was lower than that of cows receiving GSCS or CS without 3-NOP, just because those cows consumed smaller meals before the GF measurement of CH_4. This may have resulted in a reduced BD effect on CH_4 production than what could potentially be expected when preceding meal sizes were identical for all BD without 3-NOP. In contrast, for all BD with 3-NOP supplementation (NOP60 and NOP80), a weak-to-moderate negative relationship between the measured CH_4 production and the DMI of the preceding FB meal was observed. The CH_4 production of cows receiving GS with NOP60 or NOP80 was higher because of their smaller sized meal compared with that of cows receiving GSCS or CS with NOP60 or NOP80. This may have resulted in an increased BD effect on CH_4 production than what could potentially be expected when meal sizes were identical for all BD with 3-NOP supplementation.

The effect of 3-NOP on GF visiting behavior was mostly absent, and it can thus be assumed that the difference in CH_4 production between the 3-NOP treatments is caused purely by the effect of 3-NOP on CH_4 production and not by differences in the GF visiting behavior of dairy cattle. Interestingly though, the relationship between cow GF visiting behavior on the measured CH_4 production was clearly 3-NOP dependent, which was made evident by the different regression coefficients for NOP0 (positive relation) compared with those of NOP60 and NOP80 (negative relation). Contrary to the NOP0 treatment, with 3-NOP a positive relationship is expected between

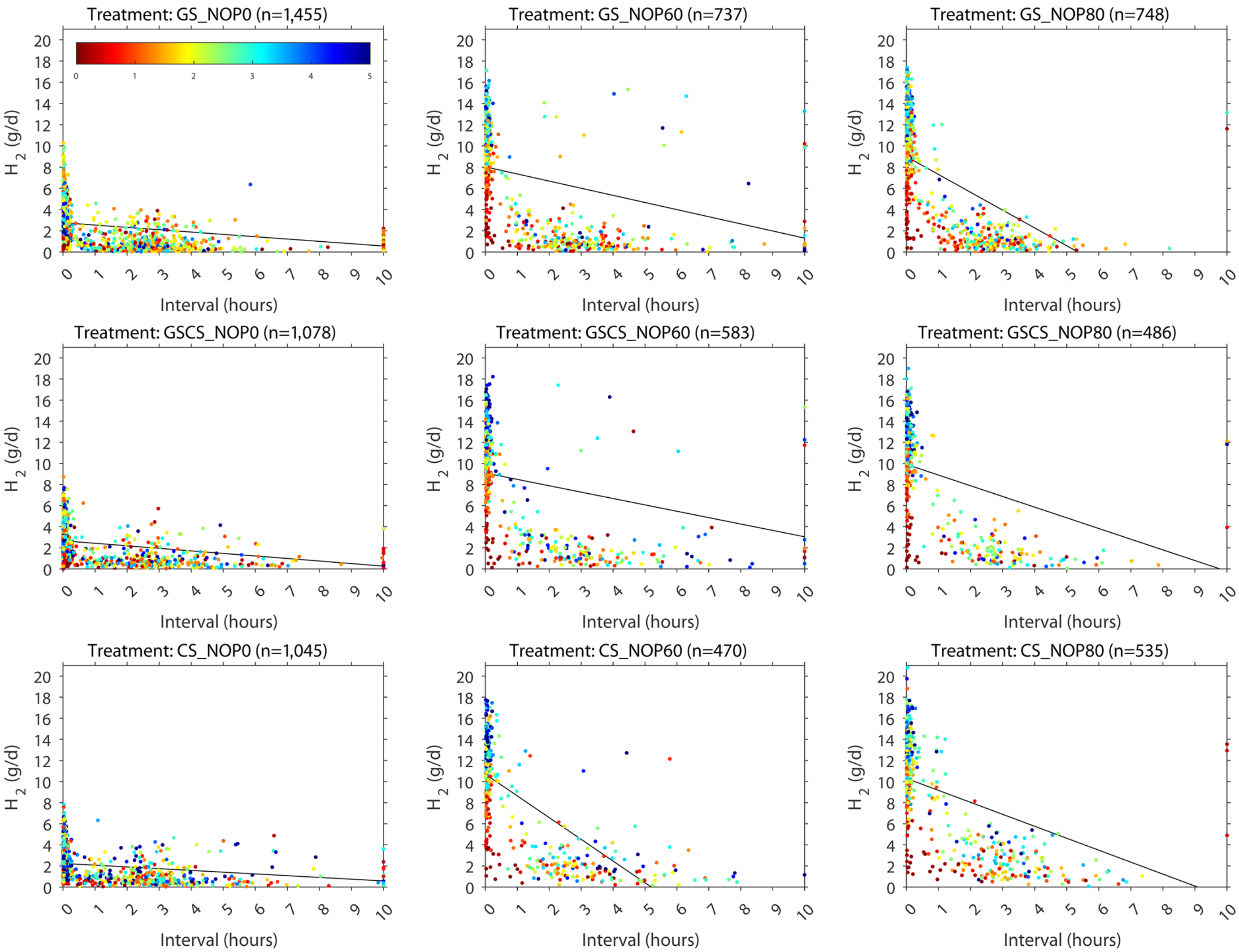

Figure 4. Scatter plots per treatment of H_2 production (g/d) versus the time interval (h) between a GF visit and the preceding FB meal. The colors of the dots represent DMI during the preceding FB meal (in kg of DM; legend in upper left panel). The black linear regression line is fitted over all visits. GS = grass silage-based diet, GSCS = grass silage and corn silage mixed diet, CS = corn silage-based diet, supplemented with 0 (NOP0), 60 (NOP60), or 80 (NOP80) mg of 3-NOP/kg of DM.

measured CH_4 production and the time interval relative to the preceding meal, and a negative relationship with amount of DMI consumed during the preceding meal. Duin et al. (2016) indicated that both archaeal growth and methanogenesis stopped almost immediately upon the addition of 3-NOP in vitro. It has been shown that 3-NOP is most effectively decreasing CH_4 production when added to a PMR, because of the continuous presence in the rumen (e.g., Hristov et al., 2015). Due to its molecular structure, 3-NOP is highly soluble and rapidly metabolized in the rumen, and hence, when 3-NOP is fed in a pulse-dosing manner, its efficacy over 24 h decreases (Muetzel et al., 2019). In the present study, 3-NOP was added to the PMR and therefore continuously present in the rumen considering the observed feeding behavior (>9 meals of 21 min per meal). However, when no new meal with 3-NOP intake is realized, and thus the time interval between the preceding meal and a GF measurement of CH_4 is increasing, more of the originally present 3-NOP in the rumen is metabolized to compounds that do not inhibit methanogenesis (i.e., nitrate, nitrite and 1,3-propanediol). It is thus logical that the measured CH_4 production increases with a larger time interval between the preceding meal and a GF measurement of CH_4 upon 3-NOP supplementation. Additionally, an increased DMI results in more feed being available for enteric CH_4 production, but a larger quantity of 3-NOP will also enter in the rumen and (at least initially) results in a greater 3-NOP efficacy, in line with the dose-effect of 3-NOP reported by Dijkstra et al. (2018), Kebreab et al. (2023),

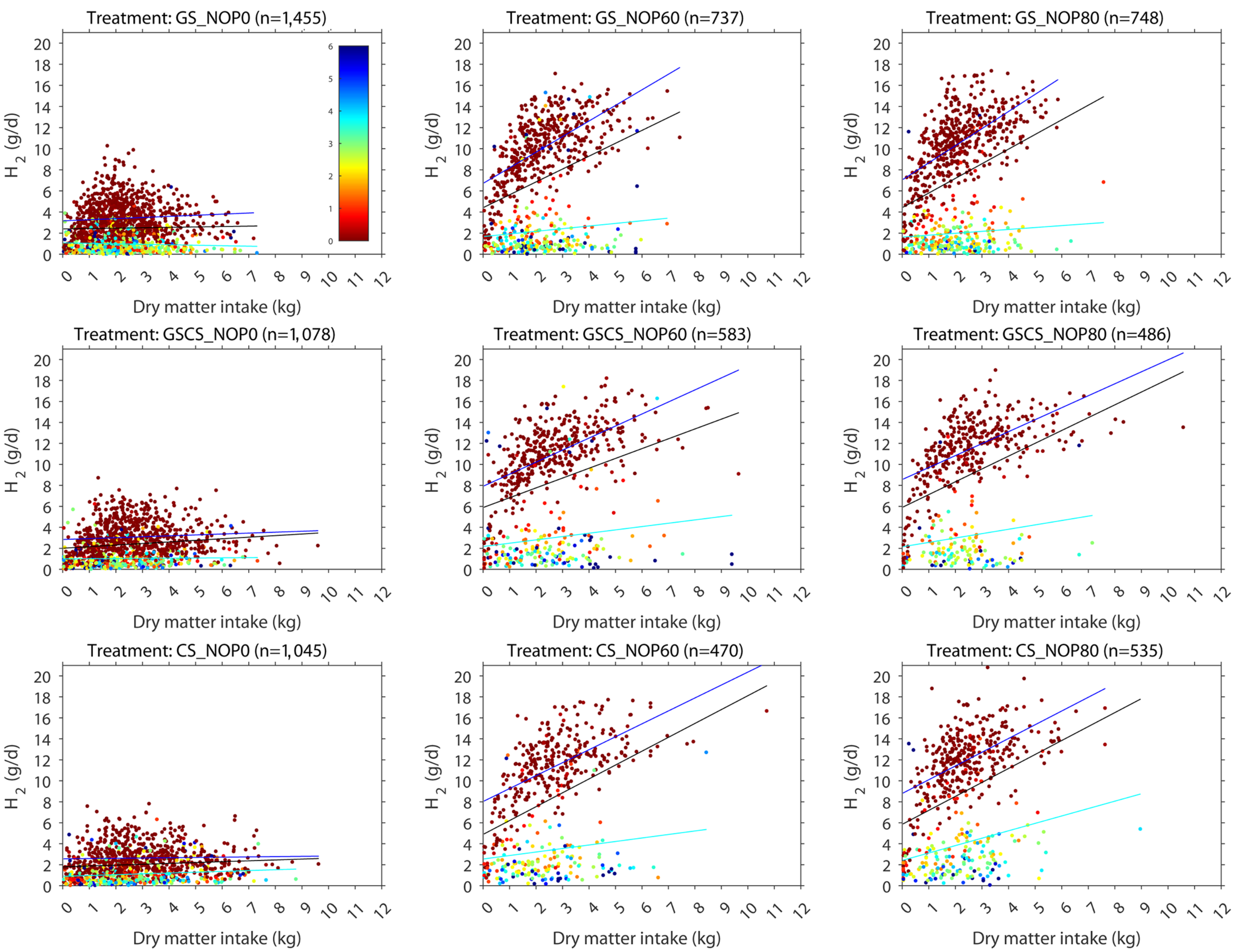

Figure 5. Scatter plots per treatment of H_2 production (g/d) versus DMI during preceding FB meal. The black linear regression line is fitted over all visits. The blue regression lines are fitted on visits with lengths of the time interval between the GF visit and the preceding FB meal lasting less (dark blue) or more (light blue) than 15 min. The colors of the dots represent the interval length between the GF visit and the preceding FB meal (h; legend in upper left panel). GS = grass silage-based diet, GSCS = grass silage and corn silage mixed diet, CS = corn silage-based diet, supplemented with 0 (NOP0), 60 (NOP60), or 80 (NOP80) mg 3-NOP/kg of DM.

and Hristov et al. (2022). Hence, it is sensible that the level of measured CH_4 production decreases as soon as the size of the preceding meal and subsequently the 3-NOP intake increases before a CH_4 measurement.

Relationship Between Cow Behavior and H_2 Production

The measurement of H_2 has gained more interest in recent years because H_2 production measurements contribute to our understanding of rumen fermentation dynamics and microbial activity (e.g., Olijhoek et al., 2016; van Lingen et al., 2017, 2021). Major concerns have been raised about measuring CH_4 production with spot-sampling devices, including the GF, but similar issues are valid for the measurement of H_2 production. Perhaps even more so, because the postprandial variation in H_2 production is far greater than that of CH_4 production (van Lingen et al., 2017). van Gastelen et al. (2017) demonstrated that excluding gas measurements of respiration chambers during feeding and milking of dairy cows (common practice, e.g., Veneman et al., 2015; van Gastelen et al., 2015) resulted in underestimating daily H_2 production by 15.2 ± 6.89%, whereas daily CH_4 production remained unaffected. In a simulation study, van Lingen et al. (2023) demonstrated that daily H_2 production could potentially be inaccurate when not measuring frequently throughout the 24-h period. For cows fed twice

Table 6. Comparison of Pearson correlation coefficients for the relationship between GF[1] measured H_2 production and the time interval with the last FB[2] meal (in min) for combinations of BD type and 3-NOP supplementation[3]

			95% confidence limits			GS			GSCS			CS		
BD[4]	3-NOP[5]	Slope	Lower	Upper	Covariate	0	60	80	0	60	80	0	60	80
Covariate	NA	−0.0046	−0.0049	−0.0043	—	0.001	<0.001	<0.001	0.100	<0.001	<0.001	<0.001	<0.001	<0.001
GS	0	−0.0037	−0.0042	−0.0031		—	<0.001	<0.001	0.407	<0.001	<0.001	0.028	<0.001	<0.001
	60	−0.0112	−0.0130	−0.0094			—	<0.001	<0.001	0.408	0.001	<0.001	<0.001	<0.001
	80	−0.0280	−0.0308	−0.0251				—	<0.001	<0.001	<0.001	<0.001	0.004	<0.001
GSCS	0	−0.0040	−0.0046	−0.0034					—	<0.001	<0.001	0.003	<0.001	<0.001
	60	−0.0101	−0.0120	−0.0081						—	<0.001	<0.001	<0.001	<0.001
	80	−0.0169	−0.0198	−0.0140							—	<0.001	<0.001	0.317
CS	0	−0.0028	−0.0033	−0.0022								—	<0.001	<0.001
	60	−0.0343	−0.0374	−0.0313									—	<0.001
	80	−0.0189	−0.0216	−0.0162										—

[1]GreenFeed system recording gaseous emissions with cow identification.
[2]Feed intake bins with automatic recording and cow identification.
[3]The regression coefficients were compared between the diets according to Sawant (2012).
[4]Basal diets were as follows: GS = grass silage-based diet, GSCS = grass silage and corn silage mixed diet, CS = corn silage-based diet.
[5]0, 60, or 80 mg of 3-nitrooxypropanol/kg of DM supplementation. NA = not applicable; no placebo or 3-NOP supplementation.

Table 7. Comparison of Pearson correlation coefficients for the relationship between GF[1] measured H_2 production and the previous FB[2] meal size (in kg of DM) for combinations of basal diet type and 3-NOP supplementation[3]

			95% confidence limits			GS			GSCS			CS		
BD[4]	3-NOP[5]	Slope	Lower	Upper	Covariate	0	60	80	0	60	80	0	60	80
Covariate	NA	0.159	0.123	0.195	—	0.006	<0.001	<0.001	0.837	<0.001	<0.001	0.040	<0.001	<0.001
GS	0	0.038	−0.041	0.117		—	<0.001	<0.001	0.032	<0.001	<0.001	0.359	<0.001	<0.001
	60	1.220	0.962	1.478			—	0.397	<0.001	0.111	0.992	<0.001	0.597	0.584
	80	1.387	1.099	1.676				—	<0.001	0.018	0.419	<0.001	0.750	0.795
GSCS	0	0.151	0.085	0.216					—	<0.001	<0.001	0.108	<0.001	<0.001
	60	0.936	0.700	1.172						—	0.123	<0.001	0.038	0.044
	80	1.222	0.945	1.500							—	<0.001	0.618	0.604
CS	0	0.082	0.030	0.134								—	<0.001	<0.001
	60	1.322	1.044	1.600									—	0.965
	80	1.332	1.025	1.638										—

[1]GreenFeed system recording gaseous emissions with cow identification.
[2]Feed intake bins with automatic recording and cow identification.
[3]The regression coefficients were compared between the diets according to Sawant (2012).
[4]Basal diets were as follows: GS = grass silage-based diet, GSCS = grass silage and corn silage mixed diet, CS = corn silage-based diet.
[5]0, 60, or 80 mg of 3-nitrooxypropanol/kg of DM supplementation. NA = not applicable; no placebo or 3-NOP supplementation.

daily and restrictedly, a sampling interval as short as 0.5 h was required and for cows fed twice daily ad libitum, a sampling interval of 2.0 h was required to obtain accurate estimates of daily H_2 production.

The present study also shows the importance of the timing of an H_2 measurement by the GF relative to meal consumption and meal size. The level of H_2 measured by the GF was strongly affected by the time interval between the preceding FB meal and the H_2 measurement and moderately by the size of the preceding FB meal. In line with the postprandial response of H_2 as presented by van Lingen et al. (2017), the level of H_2 production in the present study was higher with a shorter time interval relative to the preceding meal. The interval between the preceding FB meal and the measurement of H_2 by the GF was not affected by BD or 3-NOP, suggesting that the effect of BD and 3-NOP on H_2 production was not confounded by differences in cow GF visiting behavior. Despite the agreement in the slope being negative, the regression coefficients of the relationship between H_2 production and the size of the preceding FB meal differed among diets, being considerably steeper for the 3-NOP containing treatments (i.e., NOP60 and NOP80) compared with NOP0. These results suggest that it is of great importance to take GF visiting behavior into account when assessing the effect of treatment on H_2 production measured by a spot-sampling device such as the GF.

The size of a preceding FB meal before a GF H_2 measurement is important because of the positive relation between the preceding FB meal size and H_2 production measured by the GF. This relationship was valid for all BD, but appeared to be more pronounced for NOP60 and NOP80 (i.e., a steeper slope) than for NOP0. The preceding meal consumed before an H_2 measurement was smaller for cows receiving GS. Hence, the measured H_2 production of cows receiving GS might have been lower than that of cows receiving GSCS or CS, just because those cows consumed smaller meals before the GF measurement. This may have resulted in a different BD effect on H_2 production than what could potentially be expected when meal sizes were identical for all BD. The more pronounced relationship between the preceding meal size and the level of H_2 production for NOP60 and NOP80 is in line with the results obtained for CH_4 production and is likely due to the mode of action of 3-NOP. The inhibition of methanogenesis by 3-NOP causes dissolved H_2 to accumulate in the rumen and, if not incorporated into other H_2 sinks (e.g., a shift from acetate to propionate), H_2 will be expelled from the rumen (Alemu et al., 2021; Ungerfeld, 2013). This increased H_2 production upon 3-NOP supplementation is often observed when 3-NOP is given to dairy cattle (Haisan et al., 2014; Hristov et al., 2015; van Gastelen et al., 2020). When the size of the preceding meal before H_2 production measurement increased, intake of 3-NOP also increased, resulting in the measurement of a greater efficacy of 3-NOP and thus a higher H_2 production, although no difference could be observed between NOP60 and NOP80 whereas more 3-NOP was consumed with the latter.

CONCLUSIONS

Feeding and GF visiting behavior of dairy cattle had an effect on both CH_4 and H_2 production measured with the GF using a voluntary visit system, in particular the size of the preceding meal before a GF measurement and the time interval between the last meal and GF measurement. Relationships between the measured gas production and both feeding and GF visiting behavior were affected both by type of BD and 3-NOP supplementation. Hence, the timing of GF measurements as well as feeding behavior is essential, and cannot be ignored, when assessing the effect of dietary treatment on CH_4 and H_2 production in a setting where a spot-sampling device such as a GF is used, and where the measurements depend on voluntary GF visits from dairy cows.

NOTES

This project was financed by DSM Nutritional Products (Basel, Switzerland) and co-financed by the Samenwerkingsverband Noord-Nederland (SNN), Ruimtelijk Economisch Programma (Groningen, the Netherlands). Supplemental material for this article is available at https://data.mendeley.com/datasets/zy82cgvfx6/1 (Figure S1), https://data.mendeley.com/datasets/sfvt7zjdc7/1 (Figure S2), and https://data.mendeley.com/datasets/bbr38jtbdv/1 (Tables S1 and S2). The experiment was conducted under the Dutch law on Animal Experiments in accordance with European Union Directive 2010/63. The use of 3-NOP (DSM Nutritional Products Ltd.) in animal feed was preapproved by the Veterinary Drugs Directorate Division (Utrecht, the Netherlands). The authors have not stated any conflicts of interest.

Nonstandard abbreviations used: 3-NOP = 3-nitrooxypropanol; BD = basal diet; CS = corn silage-based diet; FB = Insentec feed bin used to measure feed intake; GF = GreenFeed system; GS = grass silage-based diet; GSCS = grass silage and corn silage mixed diet; NOP0 = 0 mg of 3-NOP/kg of DM (placebo); NOP60 = 60 mg of 3-NOP/kg of DM; NOP80 = 80 mg of 3-NOP/kg of DM; PMR = partial mixed ration; RFID = radio frequency identification; RMSE = root mean square error.

REFERENCES

Abrahamse, P. A., J. Dijkstra, B. Vlaeminck, and S. Tamminga. 2008a. Frequent allocation of rotationally grazed dairy cows changes grazing behavior and improves productivity. J. Dairy Sci. 91:2033–2045. https://doi.org/10.3168/jds.2007-0579.

Abrahamse, P. A., B. Vlaeminck, S. Tamminga, and J. Dijkstra. 2008b. The effect of silage and concentrate type on intake behavior, rumen function, and milk production in dairy cows in early and late lactation. J. Dairy Sci. 91:4778–4792. https://doi.org/10.3168/jds.2008-1350.

Alemu, A. W., L. K. Pekrul, A. L. Shreck, C. W. Booker, S. M. McGinn, M. Kindermann, and K. A. Beauchemin. 2021. 3-Nitrooxypropanol decreased enteric methane production from growing beef cattle in a commercial feedlot: Implications for sustainable beef cattle production. Front. Anim. Sci. 2:641590. https://doi.org/10.3389/fanim.2021.641590.

Arndt, C., A. N. Hristov, W. J. Price, S. C. McClelland, A. M. Pelaez, S. F. Cueva, J. Oh, J. Dijkstra, A. Bannink, A. R. Bayat, L. A. Crompton, M. A. Eugene, D. Enahoro, E. Kebreab, M. Kreuzer, M. McGee, C. Martin, C. J. Newbold, C. K. Reynolds, A. Schwarm, K. J. Shingfield, J. B. Veneman, D. R. Yanez-Ruiz, and Z. Yu. 2022. Full adoption of the most effective strategies to mitigate methane emissions by ruminants can help meet the 1.5°C target by 2030 but not by 2050. Proc. Natl. Acad. Sci. USA 119:e2111294119. https://doi.org/10.1073/pnas.2111294119.

Beauchemin, K. A., E. M. Ungerfeld, A. L. Abdalla, C. Alvarez, C. Arndt, P. Becquet, C. Benchaar, A. Berndt, R. M. Mauricio, T. A. McAllister, W. Oyhantçabal, S. A. Salami, L. Shalloo, Y. Sun, J. Tricarico, A. Uwizeye, C. De Camillis, M. Bernoux, T. Robinson, and E. Kebreab. 2022. Invited review: Current enteric methane mitigation options. J. Dairy Sci. 105:9297–9326. https://doi.org/10.3168/jds.2022-22091.

Blaxter, K. L., and J. L. Clapperton. 1965. Prediction of the amount of methane produced by ruminants. Br. J. Nutr. 19:511–522. https://doi.org/10.1079/BJN19650046.

Cottle, D. J., J. Velazco, R. S. Hegarty, and D. G. Mayer. 2015. Estimating daily methane production in individual cattle with irregular feed intake patterns from short-term methane emission measurements. Animal 9:1949–1957. https://doi.org/10.1017/S1751731115001676.

Crompton, L. A., J. A. N. Mills, C. K. Reynolds, and J. France. 2011. Fluctuations in methane emission in response to feeding pattern in lactating dairy cows. Pages 176–180 in Modelling Nutrient Digestion and Utilization in Farm Animals. D. Sauvant, J. Milgen, P. Faverdin, and N. Friggens, ed. Wageningen Academic Publishers, Wageningen, the Netherlands.

Dijkstra, J., A. Bannink, J. France, E. Kebreab, and S. van Gastelen. 2018. Short communication: Anti-methanogenic effects of 3-nitrooxypropanol depend on supplementation dose, dietary fiber content, and cattle type. J. Dairy Sci. 101:9041–9047. https://doi.org/10.3168/jds.2018-14456.

Duin, E. C., T. Wagner, S. Shima, D. Prakash, B. Cronin, D. R. Yáñez-Ruiz, S. Duval, R. Rümbeli, R. T. Stemmler, R. K. Thauer, and M. Kindermann. 2016. Mode of action uncovered for the specific reduction of methane emissions from ruminants by the small molecule 3-nitrooxypropanol. Proc. Natl. Acad. Sci. USA 113:6172–6177. https://doi.org/10.1073/pnas.1600298113.

Haisan, J., Y. Sun, L. L. Guan, K. A. Beauchemin, A. Iwaasa, S. Duval, D. R. Barreda, and M. Oba. 2014. The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of Holstein cows in mid lactation. J. Dairy Sci. 97:3110–3119. https://doi.org/10.3168/jds.2013-7834.

Hammond, K. J., L. A. Crompton, A. Bannink, J. Dijkstra, D. R. Yáñez-Ruiz, P. O'Kiely, E. Kebreab, M. A. Eugène, Z. Yu, K. J. Shingfield, A. Schwarm, A. N. Hristov, and C. K. Reynolds. 2016. Review of current in vivo measurement techniques for quantifying enteric methane emission from ruminants. Anim. Feed Sci. Technol. 219:13–30. https://doi.org/10.1016/j.anifeedsci.2016.05.018.

Hegarty, R. S. 2013. Applicability of short-term emission measurements for on-farm quantification of enteric methane. Animal 7:401–408. https://doi.org/10.1017/S1751731113000839.

Hristov, A. N., A. Melgar, D. Wasson, and C. Arndt. 2022. Symposium review: Effective nutritional strategies to mitigate enteric methane in dairy cattle. J. Dairy Sci. 105:8543–8557. https://doi.org/10.3168/jds.2021-21398.

Hristov, A. N., J. Oh, F. Giallongo, T. W. Frederick, M. T. Harper, H. L. Weeks, A. F. Branco, P. J. Moate, M. H. Deighton, S. R. Williams, M. Kindermann, and S. Duval. 2015. An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. Proc. Natl. Acad. Sci. USA 112:10663–10668. https://doi.org/10.1073/pnas.1504124112.

Jonker, A., G. Molano, C. Antwi, and G. Waghorn. 2014. Feeding lucerne silage to beef cattle at three allowances and four feeding frequencies affects circadian patterns of methane emissions, but not emissions per unit of intake. Anim. Prod. Sci. 54:1350–1353. https://doi.org/10.1071/AN14235.

Kebreab, E., A. Bannink, E. M. Pressman, N. Walker, A. Karagiannis, S. van Gastelen, and J. Dijkstra. 2023. A meta-analysis of effects of 3-nitrooxypropanol on methane production, yield, and intensity in dairy cattle. J. Dairy Sci. 106:927–936. https://doi.org/10.3168/jds.2022-22211.

Lee, C., K. A. Beauchemin, J. Dijkstra, D. L. Morris, K. Nichols, P. J. Kononoff, and D. Vyas. 2022. Estimates of daily oxygen consumption, carbon dioxide and methane emissions, and heat production for beef and dairy cattle using spot gas sampling. J. Dairy Sci. 105:9623–9638. https://doi.org/10.3168/jds.2022-22213.

Lopes, J. C., L. F. de Matos, M. T. Harper, F. Giallongo, J. Oh, D. Gruen, S. Ono, M. Kindermann, S. Duval, and A. N. Hristov. 2016. Effect of 3-nitrooxypropanol on methane and hydrogen emissions, methane isotopic signature, and ruminal fermentation in dairy cows. J. Dairy Sci. 99:5335–5344. https://doi.org/10.3168/jds.2015-10832.

McCauley, E. H., and H. E. Dziuk. 1965. Correlation of motility and gas collection from goat rumen. Am. J. Physiol. 209:1152–1154. https://doi.org/10.1152/ajplegacy.1965.209.6.1152.

Mills, J. A. N., J. Dijkstra, A. Bannink, S. B. Cammell, E. Kebreab, and J. France. 2001. A mechanistic model of whole-tract digestion and methanogenesis in the lactating dairy cow: Model development, evaluation, and application. J. Anim. Sci. 79:1584–1597. https://doi.org/10.2527/2001.7961584x.

Muetzel, S., K. Lowe, P. H. Janssen, D. Pacheco, N. Bird, N. Walker, O. Vidoni, L. Schweikert, L. Clasadonte, and M. Kindermann. 2019. Towards the application of 3-nitrooxypropanol in pastoral farming systems. Page 81 in Proceedings of the 7th GGAA—Greenhouse Gas and Animal Agriculture Conference. A. Berndt, L. G. Pereira Ribeiro, and A. L. Abdalla, ed. Local Publishing Committee, Iguassu Falls, Brazil.

Olijhoek, D. W., A. L. F. Hellwing, M. Brask, M. R. Weisbjerg, O. Højberg, M. K. Larsen, J. Dijkstra, E. J. Erlandsen, and P. Lund. 2016. Effect of dietary nitrate level on enteric methane production, hydrogen emission, rumen fermentation, and nutrient digestibility in dairy cows. J. Dairy Sci. 99:6191–6205. https://doi.org/10.3168/jds.2015-10691.

Sawant, S. 2012. Algorithm to compare the slopes (regression coefficients) between the subgroups in simple/multiple regression using PROC REG. PO04 (abstr.) in PharmaSUG 2012 conference proceedings, San Francisco, CA.

Tolkamp, B. J., and I. Kyriazakis. 1999. To split behaviour into bouts, log-transform the intervals. Anim. Behav. 57:807–817. https://doi.org/10.1006/anbe.1998.1022.

Ungerfeld, E. M. 2013. A theoretical comparison between two ruminal electron sinks. Front. Microbiol. 4:319. https://doi.org/10.3389/fmicb.2013.00319.

van Gastelen, S., E. C. Antunes-Fernandes, K. A. Hettinga, G. Klop, S. J. J. Alferink, W. H. Hendriks, and J. Dijkstra. 2015. Enteric methane production, rumen volatile fatty acid concentrations, and milk fatty acid composition in lactating Holstein-Friesian cows fed grass silage- or corn silage-based diets. J. Dairy Sci. 98:1915–1927. https://doi.org/10.3168/jds.2014-8552.

van Gastelen, S., J. Dijkstra, G. Binnendijk, S. M. Duval, J. M. L. Heck, M. Kindermann, T. Zandstra, and A. Bannink. 2020. 3-Nitrooxypropanol decreases methane emissions and increases hydrogen emissions of early lactation dairy cows, with associated changes in nutrient digestibility and energy metabolism. J. Dairy Sci. 103:8074–8093. https://doi.org/10.3168/jds.2019-17936.

van Gastelen, S., J. Dijkstra, J. M. L. Heck, M. Kindermann, A. Klop, R. de Mol, D. Rijnders, N. Walker, and A. Bannink. 2022. Methane mitigation potential of 3-nitrooxypropanol in lactating cows is influenced by basal diet composition. J. Dairy Sci. 105:4064–4082. https://doi.org/10.3168/jds.2021-20782.

van Gastelen, S., M. H. P. W. Visker, J. E. Edwards, E. C. Antunes-Fernandes, K. A. Hettinga, S. J. J. Alferink, W. H. Hendriks, H. Bovenhuis, H. Smidt, and J. Dijkstra. 2017. Linseed oil and DGAT1 K232A polymorphism: Effects on methane emission, energy and nitrogen metabolism, lactation performance, ruminal fermentation, and rumen microbial composition of Holstein-Friesian cows. J. Dairy Sci. 100:8939–8957. https://doi.org/10.3168/jds.2016-12367.

van Lingen, H. J., J. E. Edwards, J. D. Vaidya, S. van Gastelen, E. Saccenti, B. van den Bogert, A. Bannink, H. Smidt, C. M. Plugge, and J. Dijkstra. 2017. Diurnal dynamics of gaseous and dissolved metabolites and microbiota in the bovine rumen. Front. Microbiol. 8:425. https://doi.org/10.3389/fmicb.2017.00425.

van Lingen, H. J., J. G. Fadel, E. Kebreab, A. Bannink, J. Dijkstra, and S. van Gastelen. 2023. Smoothing spline assessment of accuracy of enteric hydrogen and methane production measurements from dairy cattle using various sampling schemes. J. Dairy Sci. 106:6834–6848. https://doi.org/10.3168/jds.2022-23207.

van Lingen, H. J., J. G. Fadel, D. R. Yáñez-Ruiz, M. Kindermann, and E. Kebreab. 2021. Inhibited methanogenesis in the rumen of cattle: Microbial metabolism in response to supplemental 3-nitrooxypropanol and nitrate. Front. Microbiol. 12:705613. https://doi.org/10.3389/fmicb.2021.705613.

Van Wesemael, D., L. Vandaele, B. Ampe, H. Cattrysse, S. Duval, M. Kindermann, V. Fievez, S. De Campeneere, and N. Peiren. 2019. Reducing enteric methane emissions from dairy cattle: Two ways to supplement 3-nitrooxypropanol. J. Dairy Sci. 102:1780–1787. https://doi.org/10.3168/jds.2018-14534.

Velazco, J., D. J. Cottle, and R. Hegarty. 2014. Methane emissions and feeding behaviour of feedlot cattle supplemented with nitrate or urea. Anim. Prod. Sci. 54:1737–1740. https://doi.org/10.1071/AN14345.

Veneman, J. B., S. Muetzel, K. J. Hart, C. L. Faulkner, J. M. Moorby, H. B. Perdok, and C. J. Newbold. 2015. Does dietary mitigation of enteric methane production affect rumen function and animal productivity in dairy cows? PLoS One 10:e0140282. https://doi.org/10.1371/journal.pone.0140282.

Yeates, M. P., B. J. Tolkamp, D. J. Allcroft, and I. Kyriazakis. 2001. The use of mixed distribution models to determine bout criteria for analysis of animal behaviour. J. Theor. Biol. 213:413–425. https://doi.org/10.1006/jtbi.2001.2425.

Zimmerman, P., S. Zimmerman, S. Utsumi, and D. Beede. 2011. Development of a user friendly online system to quantitatively measure metabolic gas fluxes from ruminants. J. Dairy Sci. 94(E-Suppl. 1):759. (Abstr.)

ORCIDS

Rudi de Mol https://orcid.org/0000-0003-4372-401X
André Bannink https://orcid.org/0000-0001-9916-3202
Jan Dijkstra https://orcid.org/0000-0003-3728-6885
Nicola Walker https://orcid.org/0000-0001-6271-3151
Sanne van Gastelen https://orcid.org/0000-0003-4547-8449