Short communication: Using diurnal patterns of ^{13}C enrichment of CO_2 to evaluate the effects of nitrate and docosahexaenoic acid on fiber degradation in the rumen of lactating dairy cows

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ABSTRACT

Nitrate decreases enteric CH_4 production in ruminants, but may also negatively affect fiber degradation. In this experiment, 28 lactating Holstein dairy cows were grouped into 7 blocks. Within blocks, cows were randomly assigned to 1 of 4 isonitrogenous treatments in a 2×2 factorial arrangement: control (CON); NO_3 [21 g of nitrate/kg of dry matter (DM)]; DHA [3 g of docosahexaenoic acid (DHA)/kg of DM]; or NO_3 +DHA (21 g of nitrate/kg of DM and 3 g of DHA/kg of DM). Cows were fed a total mixed ration consisting of 21% grass silage, 49% corn silage, and 30% concentrates on a DM basis. Based on the difference in natural ^{13}C enrichment and neutral detergent fiber and starch content between grass silage and corn silage, we investigated whether a negative effect on rumen fiber degradation could be detected by evaluating diurnal patterns of ^{13}C enrichment of exhaled carbon dioxide. A significant nitrate $\times$ DHA interaction was found for neutral detergent fiber digestibility, which was reduced on the NO_3 treatment to an average of 55%, as compared with 61, 64, and 65% on treatments CON, DHA, and NO_3 +DHA, respectively. Feeding nitrate, but not DHA, resulted in a pronounced increase in ^{13}C enrichment of CO_2 in the first 3 to 4 h after feeding only. Results support the hypothesis that effects of a feed additive on the rate of fiber degradation in the rumen can be detected by evaluating diurnal patterns of ^{13}C enrichment of CO_2 . To be able to detect this, the main ration components have to differ considerably in fiber and nonfiber carbohydrate content as well as in natural ^{13}C enrichment.

Key words: feed additives, methane, fiber degradation, ^{13}C enrichment

Short Communication

Nitrate is among the relatively few feed additives that have been shown to effectively mitigate enteric CH_4 production in ruminants (reviewed by Hristov et al., 2013). However, CH_4 -mitigating additives should not adversely affect animal health and performance or quality of animal products to be applicable in practice. Methemoglobinemia is a health risk of feeding nitrate to ruminants, but a gradual introduction of nitrate in the diet alleviates this risk (van Zijderveld et al., 2011). A growing body of literature also documents the effects of nitrate feeding on animal performance. As discussed by Newbold et al. (2014), nitrate might reduce feed intake and the magnitude of the decrease seems larger on diets that contain more NDF. At least 2 factors may be associated with this decrease in feed intake. First, nitrite, as an intermediate in the reduction of nitrate to ammonia, is toxic for fibrolytic bacteria in vitro (Marais et al., 1988). Second, nitrate feeding increases hydrogen production in the rumen (van Zijderveld et al., 2011; Lund et al., 2014; Guyader et al., 2015), which may indicate increased aqueous hydrogen concentrations (Guyader et al., 2015). Increased hydrogen concentration inhibits the regeneration of NAD^+ from NADH (Hegarty and Gerdes, 1999), which may impair metabolism of fibrolytic bacteria. An increased ratio of NADH to NAD^+ will cause a shift from acetate toward propionate formation, partly via the lactic acid pathway. The latter pathway yields less ATP for the fibrolytic microbes. Impaired fibrolytic activity increases retention time of fiber in the rumen and therefore may reduce feed intake.

The effects of nitrate and docosahexaenoic acid (C22:6n-3; **DHA**) on total enteric CH_4 production and milk fatty acid composition in lactating dairy cows in climate respiration chambers (**CRC**) were recently reported by Klop et al. (2016). A negative effect of nitrate on DMI was observed, as well as reduced total-tract digestibility of fiber on the NO_3 treatment. However, no adverse effect of DHA on total-tract fiber digestion

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was observed. Digestibility of NDF was not reported in other studies in which DHA was supplemented to dairy cows (Boeckaert et al., 2008; Moate et al., 2013). It was hypothesized that the between-treatment differences in fiber digestion would cause variation in diurnal pattern of ^{13}C enrichment of CO_2 . When nitrate is supplemented to a diet of which the main components differ considerably in starch (or another non-NDF carbohydrate source) and NDF content, as well as in natural ^{13}C enrichment, an adverse effect on fiber degradation may be detectable from an increased ^{13}C enrichment of exhaled CO_2 . In comparison with corn, grass has a lower natural ^{13}C enrichment (Knobbe et al., 2006) and does not contain starch. When fed as a TMR, changes in the ^{13}C enrichment of CO_2 after a meal hence likely reflect changes in the degradation rate of starch or fiber by the rumen bacteria. The aim of the current study was to investigate if a negative effect on ruminal fiber degradation upon feeding nitrate to dairy cattle can be detected by evaluating diurnal patterns of ^{13}C enrichment in exhaled CO_2 .

The experimental procedures were approved by the Animal Care and Use Committee of Wageningen University (Wageningen, the Netherlands). Detailed information regarding experimental design, diets, feeding, and measurements was reported by Klop et al. (2016). Briefly, 8 primiparous and 20 multiparous (125 ± 16 DIM at the start of the experimental period; mean $\pm$ SD) lactating Holstein dairy cows were divided over 7 blocks based on parity, lactation stage, milk production, and presence or absence of a rumen cannula. Within blocks, cows were randomly assigned to 1 of 4 experimental diets: control (**CON**; no nitrate or DHA added and urea as NPN source), nitrate (**NO₃**; 21 g of nitrate/kg of DM), DHA (3 g of DHA/kg of DM and urea as NPN source), or nitrate and DHA (**NO₃+DHA**; 21 g of nitrate/kg of DM and 3 g of DHA/kg of DM). The sources of nitrate and DHA were Bolifor CNF (Yara, Norway), containing 75% nitrate, and DHAgold (DSM Nutritional Products, Columbia, MD), a whole-cell algae product containing 25.4% DHA, respectively. Feed additives were included in the concentrates and chromium oxide (Cr_2O_3 ; 1.7 g/kg of DM) was used as external marker to determine apparent total-tract digestibility of nutrients.

Diets were isonitrogenous, offered as a TMR, and consisted of 49% corn silage, 21% grass silage, and 30% concentrate on a DM basis. Dietary DM, CP, NDF, and starch contents were on average 454, 165, 355, and 240 g/kg of DM, respectively. Due to lameness, 1 of 8 rumen-cannulated cows was replaced by a noncannulated cow already adapted to the same experimental diet (**NO₃**).

The experimental period lasted 17 d. During the experimental periods, cows were fed individually with equal portions offered twice daily (at 0600 and 1600 h). Until d 9, each block of cows had free access to feed. Thereafter, DMI within a block was restricted to 95% of that of the animal with the lowest voluntary DMI between d 5 and 8, but none of the animals in the block was restricted to less than 80% of its voluntary DMI. Cows were housed in tiestalls until the afternoon of d 13 and in CRC for the remainder of the experimental period. In the CRC, gaseous exchange (CH_4 , O_2 , and CO_2) was measured as described by van Gastelen et al. (2015) and $^{13}\text{CO}_2$ production was determined as described by Gerrits et al. (2012). Data relate to the last 72 h in the CRC, except for digestibility values calculated over the complete period the cows were in the CRC. Natural ^{13}C enrichment of the TMR components was determined by means of combustion isotope ratio MS (Gerrits et al., 2012).

Data were analyzed using PROC MIXED (SAS Institute Inc., Cary, NC). Two cows (receiving the **CON** or **NO₃** treatment) were excluded from analysis because of a feeding error in the CRC. Gaseous exchange data, heat production (**HP**), respiration quotient (**RQ**), and ^{13}C enrichment of CO_2 were averaged per hour and analyzed using repeated measures ANOVA. Main and interaction effects of nitrate, DHA, and time were included as fixed effects in the model. Period (which equals block) was included as random factor in the model and average values for each hour of the day were treated as repeated measures per cow $\times$ treatment combination using a first-order autoregressive covariance structure. Multiple comparisons between treatment least squares means for each hour were made using a SLICE statement in the model. Effects were considered significant if $P \leq 0.05$, and trends if $0.05 < P < 0.10$.

Cows receiving nitrate consumed less DM and a tendency for a **NO₃** $\times$ DHA interaction was observed, because the **NO₃** cows consumed less DM than the **NO₃+DHA** cows. Daily fat- and protein-corrected milk production was not affected by nitrate, and tended to be reduced by DHA (Klop et al., 2016). Distinct responses to meals were observed for HP, RQ, and CH_4 and for ^{13}C enrichment of the CO_2 produced (Figure 1). Both HP and RQ changed over time within day, but no interactions between treatment factors and time were found. The numerically higher RQ and HP peak values for cows on the **CON** treatment shortly after feeding (Figure 1B) can be explained by our visual observations that cows on the **CON** treatment consumed their meals faster than cows on the other treatments. In line with van Zijderveld et al. (2011), the CH_4 -mitigating effect of nitrate was largest during the first hours postfeeding

Figure 1. Diurnal patterns of heat production (A), respiration quotient (B), methane production (C), and ¹³C enrichment of CO₂ (D) of cows receiving different dietary treatments: control (CON); NO₃ (21 g of nitrate/kg of DM); DHA [3 g of docosahexaenoic acid (DHA)/kg of DM]; or NO₃+DHA (21 g of nitrate/kg of DM and 3 g of DHA/kg of DM). For CON and NO₃ treatments n = 6, for DHA and NO₃+DHA treatments n = 7. Arrows indicate feeding times. Significance of main effects and interaction effects is indicated in the figure. Symbols (NS = not significant; † 0.05 < P < 0.10; * P < 0.05; ** P < 0.01; *** P < 0.001) in (C) indicate hourly comparison of each of the additive treatments with the CON treatment. Symbols in (D) indicate effect of nitrate at different time points. The dashed line indicates the overall average background ¹³C enrichment of the TMR (1.0844). Values for atom % ¹³C were 1.0924 and 1.0722 for corn silage and grass silage, respectively, and 1.0794, 1.0791, 1.0808, and 1.0806 for concentrates of the CON, NO₃, DHA, and NO₃+DHA treatment, respectively. The pooled SEM values were 1.4, 0.024, 0.05, and 0.0007 for A, B, C, and D, respectively.

(Figure 1C). The restricted feeding regimen imposed during the measurement period and the more rapid degradation of starch compared with fiber may explain the overall increase in ^{13}C enrichment shortly after feeding. The shorter interval between morning and afternoon feeding (10 h) as compared with the interval between afternoon and morning feeding (14 h) may explain the numerically lower ^{13}C enrichment of CO_2 in the morning (Figure 1D). Feeding nitrate, but not DHA, resulted in a pronounced increase in ^{13}C enrichment of CO_2 in the first 3 to 4 h after feeding only, which resulted in a significant nitrate $\times$ time interaction.

For cows receiving nitrate, the timing of the increased ^{13}C enrichment of CO_2 coincided with that of a marked decrease in CH_4 emission. Based on previous findings (van Zijderveld et al., 2011; Lund et al., 2014; Guyader et al., 2015), it is likely that the period of decreased CH_4 production coincided with an increased concentration of aqueous H_2 in the rumen, which may have impaired the functioning of fibrolytic bacteria. However, only Guyader et al. (2015) measured aqueous H_2 in the rumen, whereas Lund et al. (2014) and van Zijderveld et al. (2011) measured in vivo H_2 emissions. An increase in aqueous H_2 is likely associated with increased H_2 emissions (Hegarty and Gerdes, 1999), but it can also be speculated that supersaturation in the liquid phase may occur, which may give rise to poor relationships between concentrations of dissolved H_2 and gaseous H_2 .

Neither aqueous H_2 concentrations, nor H_2 emissions were measured in the present experiment. Therefore, it cannot be excluded that other mechanisms were involved in the adverse effect of nitrate on ruminal fiber digestion and methane production. As reviewed by Latham et al. (2016), the reduction intermediate nitrite can be particularly toxic to certain fibrolytic microbes, as well as to methanogens. Also, a changed intraruminal reduction potential may disturb reactions involved in electron transfer by microorganisms (Latham et al., 2016).

A nitrate $\times$ DHA interaction was found for apparent total-tract digestibility of NDF ($P = 0.011$; Klop et al., 2016), with NDF digestibility only being reduced on the NO_3 treatment where it averaged 55% compared with 61, 64, and 65% on treatments CON, DHA, and NO_3 +DHA, respectively. In line with reduced feed intake resulting from nitrate feeding (Newbold et al., 2014), based on visual observations, cows on the NO_3 +DHA treatment appeared to have a more gradual intake pattern than cows on the NO_3 treatment. This may explain why values for ^{13}C enrichment of CO_2 of these cows returned to baseline values at a later time point than for the NO_3 treatment (Figure 1D). The more gradual feed intake pattern may also have resulted in a higher overall NDF degradation in the rumen of

NO_3 +DHA cows as compared with NO_3 cows because of a longer retention time of feed. It would also provide an explanation for the numerically smaller decrease in CH_4 per kilogram of DMI observed for cows on the NO_3 +DHA treatment compared with the NO_3 treatment (CH_4 production was 1.37, 1.05, 1.40, and 1.13 mol/kg of DMI on treatments CON, NO_3 , DHA, and NO_3 +DHA, respectively; Klop et al., 2016). Total-tract starch digestibility ($>99\%$) was not affected by feeding nitrate (Klop et al., 2016).

The significantly lower NDF digestibility, absence of changes in starch digestibility, and the significantly higher ^{13}C enrichment of CO_2 for cows on the NO_3 treatment provides strong evidence that effects of feed additives on fiber degradation in the rumen can be detected by evaluating diurnal patterns of ^{13}C enrichment of CO_2 . The difference required to detect effects depends on accuracy of the measurements, number of measurements and repeats, frequency of feeding and meal size, and the expected size of the effect. The concept of evaluating ^{13}C enrichment of CO_2 to evaluate dietary effects on fiber degradation in the rumen might have a broader application potential than the study of effects of feed additives only. For example, fiber degradation may also be impaired in cows with a low rumen pH, and measuring ^{13}C enrichment of CO_2 in repeated spot samples of breath could then be a tool to detect individuals with suboptimal conditions for fiber fermentation.

Results presented here indicate that effects of a CH_4 -mitigating feed additive on fiber degradation in the rumen can be detected by evaluating the change in the diurnal pattern of ^{13}C enrichment of CO_2 . A prerequisite is that the main ration components differ in natural ^{13}C enrichment (e.g., C_3 and C_4 plants; Sudekum et al., 1995) and in content of the nutrients that are expected to be involved in a shift in fermentation (e.g., starch and fiber) or in degradability of a nutrient.

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