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Enteric methane emission and digestion in dairy cows fed wheat or molasses

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ABSTRACT

The aim of this experiment was to measure enteric methane (CH_4) emission and its relation with rumen digestion in dairy cows fed diets rich in 1 of the 2 carbohydrate sources, starch or sugar. The rations were based on late first-cut grass–clover silage supplemented with wheat (Wh), NaOH-treated wheat (Wh+NaOH), sugar beet molasses (Mo), or sugar beet molasses with addition of sodium bicarbonate (Mo+Bic). Wheat and molasses made up 35% of dry matter in the 2 diets with molasses and wheat, respectively. Four cows fitted with ruminal, duodenal, and ileal canulae were used in a 4 × 4 Latin square design. Nutrient digestibility was measured using chromium oxide and titanium oxide as flow markers, and emissions of CH_4 and hydrogen were measured via open-circuit indirect calorimetry on 4 consecutive days. Data were analyzed using PROC MIXED of SAS (version 9.4; SAS Institute Inc., Cary, NC) with treatment and period as fixed effects and cow as random effect. Furthermore, orthogonal contrasts were calculated. The cows produced 32.5, 33.6, 36.2, and 35.1 L of CH_4/kg of dry matter intake (DMI) on diets Wh, Wh+NaOH, Mo, and Mo+Bic, respectively. The emission of CH_4 per day, per kilogram of DMI, and per kilogram of energy-corrected milk as well as daily hydrogen emission were higher on the Mo diet compared with the Wh diet. With the present inclusion of wheat and molasses in the diet, no effects of NaOH treatment of wheat or of sodium bicarbonate supplementation to the Mo diet could be demonstrated on CH_4 emission expressed per kilogram of DMI or per kilogram of energy-corrected milk. The duodenal flow of starch was higher when wheat was treated with NaOH. Under the conditions in the present experiment, ruminal NDF digestibility was not affected by carbohydrate source, NaOH treatment of wheat, or bicarbonate supplementation. Total volatile fatty acid concentration in the rumen and the proportions of acetate and propionate were not affected by carbohydrate source, NaOH treatment of wheat, or bicarbonate supplementation. Likewise, we could not show any influence of diet on microbial protein synthesis or efficiency of microbial protein synthesis expressed as grams of microbial protein synthesis per kilogram of true rumen-digested organic matter. We concluded that CH_4 emission was increased when wheat was replaced by molasses, whereas no effect of manipulating rumen fermentation by NaOH treatment of wheat or addition of bicarbonate to molasses could be found with a level of approximately 25% of dry matter from starch and sugar, respectively.
Key words: enteric methane, buffer, bicarbonate, mitigation strategy

INTRODUCTION

Enteric methane (CH_4) constitutes a significant part of the carbon footprint of ruminant products. It is well established that dietary carbohydrate (**CHO**) composition influences the rumen fermentation pattern and enteric CH_4 production (Jentsch et al., 2007). Starch and sugar are both highly degradable in the rumen, but their rumen fermentation pathways differ, resulting in different VFA profiles and thereby different CH_4 production. Starch and fiber are the quantitatively most important CHO fractions in most diets. Therefore, there has recently been an intense focus on the difference between starch and fiber in CH_4 production and the effect of fiber source. Brask et al. (2013a) showed that replacing maize silage with grass silage as well as replacing low-fiber grass silage with high-fiber grass silage resulted in higher CH_4 production per kilogram of OM fermented in the rumen, a higher proportion of acetic acid, and a lower proportion of propionic acid. According to Boadi et al. (2004), the higher propionic acid production on maize silage diets acts as an alternative hydrogen sink, reducing the amount of hydrogen transformed into CH_4, whereas the higher acetate production on grass silage diets enhances CH_4 production (Johnson and Johnson, 1995). Contrary to the multiple comparisons of starch and fiber in the diet, few studies have evaluated the effect of replacing starch with sugar. Hindrichsen et al. (2004) and Jentsch et al. (2007) showed that starch and sugar influence rumen fermentation, and therefore CH_4 production, differently. Methane release in a Rusitec in

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vitro system was 1.04 mmol/g of degraded OM from wheat, whereas it was 1.37 mmol/g of degraded OM from molasses (Hindrichsen et al., 2004). High dietary starch content favors the proportion of propionate, whereas dietary sugar increases the proportion of butyrate (e.g., Jentsch et al., 2007), resulting in hydrogen (H_2) surplus and increased CH_4 production (Ungerfeld and Kohn, 2006). However, the effect of sugar on CH_4 production is pH dependent, as sugar increases CH_4 production only when rumen pH is maintained at a high level (Hindrichsen and Kreuzer, 2009). Rumen pH also affects the fermentation pattern, as a decrease in pH favors propionate production and thereby reduces hydrogen available for CH_4 production (Murphy et al., 1982). Rumen acidosis can be prevented by reducing the ruminal degradability of starch in the ration by chemical treatment of grains (Nozière et al., 2010), which moves some of the starch digestion to the small intestine (Larsen et al., 2009). Furthermore, a buffer (e.g., sodium bicarbonate) can be added to the diet to stabilize pH and in this way prevent rumen acidosis.

At intensive fermentation rates of quickly degradable nutrients, such as starch and sugar, rumen pH decreases, leading to a lower CH_4 production due to inhibition of fibrolytic bacteria and methanogens as well as enhanced propionate production (Beauchemin et al., 2008). It is therefore unclear whether the methanogenic properties of starch and sugar can be characterized merely by their content in a given diet or whether this effect interacts with the overall rumen pH. Elucidating this interaction between rumen pH and CHO source with respect to enteric CH_4 is essential, as a wide range of prediction equations based merely on chemical composition of the diets have been published recently. These equations seem to have problems accounting for the variation between diets if only production level and dietary chemical composition are taken into account.

We hypothesized that a diet rich in starch from wheat would lead to lower CH_4 emissions than a diet with an equivalent amount of sugar from molasses due to a higher production of propionate and a lower production of butyrate in the rumen. Furthermore, we expected to achieve a higher ruminal pH when wheat was treated with NaOH and when bicarbonate was added to the molasses diet.

The first aim of this experiment was to examine the effect of starch and sugar on ruminal and intestinal metabolism and enteric CH_4 production. Furthermore, the effects on these parameters were examined when we attempted to modify the rumen environment either by feeding NaOH-treated whole-kernel wheat to affect the ruminal starch degradation and pH or by adding sodium bicarbonate to a diet with sugar from molasses to manipulate the rumen pH in a different way.

MATERIALS AND METHODS

Animals and Diets

The experiment complied with the guidelines of the Danish Ministry of Justice with respect to animal experimentation and care of animals under study. Four lactating Danish Holstein dairy cows (3 primiparous and 1 multiparous) were assigned to 1 of 4 rations over 4 periods according to a balanced 4 × 4 Latin square design. Each period consisted of 4 wk.

On average, the cows were 176 DIM (SD = 158 d) and had a milk yield of 27.2 kg (SD = 8.3 kg) and a BW of 548 kg (SD = 38 kg) at the beginning of the experiment. All animals were fitted with a ruminal cannula (no. 1C; Bar Diamond Inc., Parma, ID), a duodenal cannula (open T-piece placed 60 cm caudal to the pylorus), and an ileal cannula (open T-piece placed 20 cm cranial to the cecum). The cows were housed in a tiestall with rubber mats and had free access to water. They were milked twice daily at 0600 and 1700 h. Total mixed rations were prepared once a day and fed to the cows on an ad libitum basis at 0630 and 1630 h. The feed intake was recorded on a daily basis, and orts were removed in the morning just before feeding. Twice daily, throughout the experiment, 10 g of chromium oxide (Cr_2O_3) and 13 g of titanium oxide (TiO_2) were placed in the rumen via the ruminal cannula in connection with feeding, except when the cows were in the respiration chambers.

The rations were formulated according to the Norfor feed evaluation system (Volden, 2011), and they contained concentrates based on rolled wheat (**Wh**), NaOH-treated whole-wheat kernels (**Wh+NaOH**), beet molasses (**Mo**), or beet molasses plus sodium bicarbonate (**Mo+Bic**). Other dietary ingredients were soybean meal, mineral and vitamin supplements, and late first-cut grass–clover silage with a low (<10% of DM) clover proportion [chemical composition: 511, 80, and 123 g/kg DM of NDF, indigestible NDF (**iNDF**), and protein, respectively, and an OM digestibility of 69.9% calculated in vivo, based on in vitro OM digestibility of 68.6%; Åkerlind et al., 2011]. The dietary composition of the 4 diets is given in Table 1. Sodium hydroxide treatment of wheat was performed by adding 3.0 kg of NaOH and 8.0 kg of water/100 kg of wheat, followed by mixing. The rations were mixed once daily and fed as TMR with a forage:concentrate ratio of 49:51 on a DM basis.

Sampling and Recordings

Milk yield was measured daily, and composition was measured once a week during the morning and evening

Table 1. Dietary and chemical composition (g/kg of DM unless otherwise noted) of the 4 experimental diets[1]

Item	Wh	Wh+NaOH	Mo	Mo+Bic
Composition of rations				
Grass-clover silage[2]	494	490	494	490
Wheat	353			
NaOH-treated wheat		359		
Sugar beet molasses			353	350
$NaHCO_3$				9.3
Soybean meal	141	140	141	140
Minerals and vitamins	12	12	12	12
DM in diets, g/kg	473	471	450	453
Chemical composition				
Ash	60.6	74.6	97.5	103
CP	175	176	178	180
Crude fat	25.8	24.4	16.5	16.7
Fatty acids	17.0	17.0	11.0	11.0
Starch	227	249	14.0	12.0
Sugar	34.2	30.0	241	238
NDF	318	290	280	277
Sodium[3]	1.9	7.0	3.2	5.7
Potassium[3]	19.2	19.1	27.0	26.8
Chloride[3]	8.3	8.2	9.4	9.3
Sulfur[3]	2.4	2.4	3.4	3.3
CAB,[3,4] mEq/kg of DM	175	399	340	446
Gross energy, MJ/kg of DM	18.2	18.0	17.4	17.3

[1]The rations were based on late first-cut grass–clover silage supplemented with wheat (Wh), NaOH-treated wheat (Wh+NaOH), sugar beet molasses (Mo), or sugar beet molasses with addition of sodium bicarbonate (Mo+Bic).

[2]DM: 325 g/kg; NDF: 511 g/kg of DM; CP: 123 g/kg of DM.

[3]Feed table values (Møller et al., 2005).

[4]CAB (cation-anion balance): (mEq/kg of DM) = [(Na/23.0 + K/39.1) − (Cl/35.5 + S/16.0)] × 1,000, where Na, K, Cl, and S are given as g/kg of DM.

milkings. Milk data are given for only the last week of the 4 experimental weeks in each period. The animals were weighed at the beginning of the experiment as well as just before and after the respiration chamber measurements. Samples of the mixed diets and refusals were taken daily in connection with the morning feeding from d 15 to 20 in each period, and DM was measured daily for both diets and refusals from each cow. Samples of diets were stored at −20°C and pooled within each period before chemical analyses.

Twelve samples, representing every second hour of the day, were taken of duodenal chyme (600 mL), ileal chyme (300 mL), and feces (350 mL) from d 15 to 19 at 1000 and 1800 h on d 15; 0200, 1200, and 2000 h on d 16; 0400, 1400, and 2200 h on d 17; 0600, 1600, and 2400 h on d 18; and 0800 h on d 19. Samples from the duodenum and ileum were taken in tube-formed plastic bags mounted to the cannulas. At each sampling time, duodenal, ileal, and fecal samples were added to the frozen pooled sample from previous samplings during the same period. At the end of the period, representative subsamples from thawed material were taken and freeze-dried before chemical analyses. At the 12 sampling times, rumen liquid was sampled from the ventral ruminal sac with a collection tube (no. RT, Bar Diamond Inc.). The rumen liquid pH was measured immediately, and 2 samples of 8 mL were taken and frozen (−20°C) immediately for VFA and ammonia (NH_3) analysis.

Total rumen evacuations were performed approximately 5 h after feeding at 1200 h on the last day (d 28) in each period. Rumen content was divided in mat and free fluid fractions using a sieve basket, and the weight of each fraction was recorded before subsampling and compositing a pooled sample by weight for DM determination (60°C). Rumen liquid was collected from the liquid phase using a cup, filtered over 2 layers of cheesecloth, and transferred to prewarmed vacuum-insulated bottles. Samples were taken directly to the laboratory and handled according to the standard procedure of our laboratory (Brask et al., 2015). Feed particles and protozoa were removed by double centrifugation at 500 × g for 5 min at 3°C. The supernatant was collected and centrifuged at 17,300 × g for 20 min at 3°C. Then, the pellet was collected and resuspended in 200 mL of NaCl (9%) and centrifuged at 17,300 × g for 20 min at 3°C. Next, the pellet (microbial matter) was stored at −20°C. Finally, the microbial matter was freeze-dried and analyzed for ash, nitrogen, and purine content to calculate microbial CP synthesis and efficiency.

Chemical Analyses

Dry matter of feeds and rumen samples was determined after 48 h at 60°C. Ash was determined by combustion at 525°C for 6 h. Nitrogen was determined using the Dumas principle (Hansen, 1989), and the CP was calculated as N × 6.25. Crude fat was analyzed as Soxhlet extraction with petroleum ether (Soxtec 2050, Foss Analytical, Hillerød, Denmark) after hydrolyzing with HCl (Stoldt, 1952).

Starch content in feed, digesta, and feces was determined enzymatically (Kristensen et al., 2007). Total sugar was determined using the Luff-Schoorl method (European Community, 2012).

The NDF content was analyzed using neutral detergent extraction according to Mertens (2002) with a Fibertec M6 System (Foss Analytical) using heat-stable amylase and corrected for ash. The iNDF in freeze-dried ground (1.5 mm) feed samples was determined as residual NDF after 288 h (12 d) of Dacron bag (12-µm pore size; TP Filter, Upplands Väsby, Sweden) incubation in the rumen of 3 heifers fed a standard ration (Åkerlind et al., 2011). Gross energy was determined using an adiabatic bomb calorimeter (Parr 6300 Oxygen Bomb Calorimeter, Parr Instrument Co., Moline, IL).

The concentrations of VFA were analyzed according to the method described by Canibe et al. (2007) using a gas chromatograph (model 6890; Hewlett Packard, Palo Alto, CA) equipped with a flame ionization detector and a 30-m SGE BP1 column (Scientific Instrument Services, Ringoes, NJ). For determination of NH_3-N, the rumen fluid was made alkaline with KOH, and NH_3-N was determined by titration after distillation.

Chromium oxide was determined by spectrophotometry after oxidation to chromate (Schürch et al., 1950). Titanium oxide was analyzed according to Myers et al. (2004) with the exception that 15 mL of 30% H_2O_2 was added instead of 10 mL, and 5 drops of 30% H_2O_2 were added to the solution just before measuring the absorbance. The OM digestibility was determined in vitro for grass silage as described by Tilley and Terry (1963). Milk concentrations of fat, protein, and lactose monohydrate were analyzed by a Milkoscan Msc4000 infrared analyzer (Foss Analytical).

Methane Measurements

During the fourth week of each period, CH_4 production was measured for 2 × 48 h in 4 open-circuit polycarbonate respiration chambers with a volume of 17 m^3 (Hellwing et al., 2012). The animals were housed individually. The chambers were located in the barn where the cows were usually housed to minimize changes in the environment, and the daily routines during the CH_4 measurements were identical to the period outside the chambers. The mean ambient temperature in the chambers was 21.1°C, ranging from 15.6 to 29.5°C.

The cows changed chambers diagonally after the first 48 h to balance out any differences in background levels of CH_4 and CO_2. Cow and chamber were confounded over periods, and therefore every ration was tested in every chamber. Chambers were opened twice daily at 0600 and 1700 h for about 20 min during milking and subsequent feeding. Methane was measured as the accumulated amount in liters over 24 h and is reported under standard conditions (0°C, 101.325 kPa), where 1 mol of CH_4 is equal to 22.41 L (Brouwer, 1965). The measurements during the openings of the chambers for milking and feeding were deleted (about 60 min/d). The CH_4 production during these periods was replaced by values corresponding to the mean value for the rest of the day. Airflow was measured using a HFM-200 flow meter with a laminar flow element from Teledyne Hastings Instruments (Hampton, VA). The background (inlet air) as well as the chamber outlet air concentration of CH_4 was measured for 30 s every 12.5 min in each chamber. Before each measurement, the measuring system was flushed with sample air either from background or chamber for 2 min before the gas sample was measured for 30 s. Methane and CO_2 were measured with an infrared analyzer. All instruments were from Columbus Instruments (Columbus, OH). The airflow was adjusted individually for each animal depending on their BW and milk yield to obtain a CO_2 concentration in the chamber below but close to 9,000 ppm. The instruments were calibrated every second day with zero gas (nitrogen) and a span gas with nitrogen and 20.55% O_2, 5,000 ppm of CO_2, and 800 ppm of CH_4 (Yara Praxair AS, Oslo, Norway). Recovery of CH_4 was measured 6 times during the experiment and was on average 97%. Data were not corrected for recovery due to the high recovery.

Calculations and Statistical Analyses

Dry matter flow was calculated as the average flow determined using the 2 external markers independently. For each of the 2 markers, DM flow was calculated from marker dosage and marker concentrations in duodenal and ileal digesta and in feces, assuming 100% recovery of the markers in digesta and feces. Flow of nutrients was subsequently calculated based on dry matter flow and nutrient concentration. Content of digestible NDF (**dNDF**) was calculated as NDF − iNDF. Rumen fractional rate of passage of iNDF was calculated as intake of iNDF (kg/h; assuming that intake of iNDF equals rumen output) divided by rumen iNDF pool

(kg). Rumen fractional rate of digestion of dNDF was calculated as duodenal flow of dNDF (kg/h) divided by rumen dNDF pool (kg; Robinson et al., 1987). Rumen pool was determined based on total rumen evacuation and chemical analysis of a representative subsample.

Microbial synthesis of DM and CP was calculated from the DM, N, and purine concentration in rumen-isolated bacteria, purine concentration in duodenal DM content, DM flow at the duodenum, and ratio between purine and the given nutrient in the isolated microbial pellet, assuming that all purines flowing at the duodenum originate from rumen microbes (Zinn and Owens, 1986). Microbial efficiency was calculated as microbial CP synthesized divided by true rumen-digested OM, where true rumen-digested OM was calculated as OM intake with feed minus duodenal OM flow (corrected for microbial OM flow at the duodenum; Madsen and Hvelplund, 1988).

Apparent ruminal digestibility of each nutrient was calculated as nutrient intake minus duodenal nutrient flow divided by nutrient intake. Apparent total-tract digestibility of each nutrient was calculated as the nutrient intake minus fecal nutrient flow divided by nutrient intake.

Average ECM (defined as 3.14 MJ/kg) yield for each cow per period was calculated according to Sjaunja et al. (1991) as

$$\text{ECM} = \text{milk yield} \times (383 \times \text{fat \%} + 242 \times \text{protein \%} + 783.2)/3{,}140.$$

For data on feed intake, milk yield, milk composition, digestibility, microbial protein synthesis, passage rate, and gas exchange per day, data were analyzed in SAS (version 9.4; SAS Institute Inc., Cary, NC) using PROC MIXED with treatment and period as fixed effects and cow as random effect.

For data on rumen pH, rumen VFA composition, NH_3-N concentration, and gas exchange data based on hourly values, a mixed model with repeated measurement in SAS was also used with treatment, period, time, and treatment × time as fixed effects. Time was repeated effect per cow per period. For all variables, compound symmetry and autoregressive 1 were tested as covariance structure, and the covariance structure giving the lowest Akaike information criterion and Bayesian information criterion values was chosen. For all reported values, the lowest Akaike information criterion and Bayesian information criterion values were found for compound symmetry.

The default estimation method for PROC MIXED in SAS, which is restricted maximum likelihood, was used to estimate the covariance parameters. The denominator's degree of freedom was estimated with the Satterthwaite approximation.

The results are reported as LSM and SEM values for each treatment. Besides the mean treatment effect, the significance of orthogonal contrasts was calculated for Wh versus Mo (effect of CHO source), Wh versus Wh+NaOH (effect of NaOH treatment), and Mo versus Mo+Bic (effect of addition of sodium bicarbonate). P-values <0.05 were regarded as significant, and P-values of $0.05 < P < 0.10$ were regarded as a tendency.

RESULTS

Feed Intake and Digestibility

Dry matter intake was significantly lower for the Wh diet (16.6 kg) compared with the Mo (17.9 kg; $P = 0.04$) and Wh+NaOH (18.1 kg; $P = 0.03$) diets, whereas DMI was equal for the 2 molasses diets, Mo and Mo+Bic (17.7 kg; $P = 0.70$; Table 2). Water intake was lower for the Wh diet (67.8 kg) than for the Wh+NaOH diet (81.4 kg; $P = 0.005$) and for the Mo diet (88.2 kg; $P = 0.001$), whereas adding bicarbonate to the molasses diet did not affect water intake.

Organic matter intake was lower in the Wh diet (15.6 kg) than in the Wh+NaOH diet (16.7 kg; $P = 0.05$). Organic matter digestibility was not affected by diet in any part of the digestive tract, and OM total-tract digestibility was on average 75.8%.

Intake of starch was highest on the 2 wheat-based diets (3.77 and 4.10 kg) compared with the 2 diets with molasses (0.26 and 0.21 kg). Rumen digestibility of starch was significantly reduced ($P = 0.001$) from 87.8% in the rolled wheat diet (Wh) to 69.7% in the diet with NaOH-treated wheat, resulting in a significantly higher ($P < 0.001$) duodenal flow of starch of 1.12 kg in the Wh+NaOH diet compared with 0.43 kg in the Wh diet. Rumen digestibility of the low intake of starch in the Mo diet was significantly (32.3%; $P < 0.001$) lower than in the Wh diet (87.8%). There was no effect of diet on the starch digestibility of the small intestine. Despite a numerically higher total-tract starch digestibility in the Wh diet (98.3%) than in the Mo diet (93.3%), the difference was not significant ($P = 0.14$). Sugar intake was higher for molasses diets (4.33 and 4.23 kg) compared with wheat-based diets (0.57 and 0.54 kg).

The intake of NDF was slightly higher for the Wh diet than for the Wh+NaOH diet despite a lower DMI for the wheat diet because the NaOH treatment of wheat markedly decreased the NDF content from 140 to 80 g/kg of DM in wheat. Total-tract digestibility of NDF was significantly lower in the Wh diet (64.6%) than in the Wh+NaOH (67.6%; $P = 0.03$) and Mo (67.9%; $P = 0.02$) diets. There were no significant effects of NaOH

treatment of wheat and of adding bicarbonate to the molasses diet on the rumen NDF digestibility.

The small intestinal digestibility of protein was higher in the Wh diet (73.8%) than in the Mo diet (65.0%; $P = 0.005$). However, there was no effect of treatment on total-tract digestibility of protein.

Rumen Fermentation

All measurements of total and individual VFA were significantly affected by the time of sampling ($P < 0.001$). Total VFA concentration and acetate and propionate proportions were unaffected by treatment (Table 3). The average diurnal butyrate proportion was numerically higher on the Mo diet (19.0%) compared with the Wh diet (15.0%). Butyrate proportion was higher for the Mo diet than for the Wh diet at all sampling times (Figure 1). Despite this, the mean proportion of butyrate was not significantly higher for the Mo diet than for the Wh diet ($P = 0.14$). There was no effect of CHO source on acetate plus butyrate: propionate ratio ($P = 0.31$). Isobutyrate was the only VFA showing a significant ($P < 0.001$) difference between the Mo (0.52%) and Wh (0.91%) diets. There was no effect of NaOH treatment of wheat or of bicarbonate supplementation to molasses on concentration of any of the measured VFA or the ratio between VFA, as shown in Table 3.

There was no difference between the Wh and Mo diets for average and minimum pH in the ventral rumen. The NaOH treatment of wheat did not significantly affect average and minimum pH in the ventral rumen. Replacing wheat with molasses tended ($P = 0.06$) to reduce the number of the 12 observations per cow having a pH lower than 6.0. There was a tendency ($P = 0.08$) for NaOH treatment of wheat to reduce the number of observations with a pH lower than 6.0. Bicarbonate supplementation of the molasses diet did not affect average or minimum pH in the ventral rumen. The highest pH values were reached before the morning feeding at 0630 h (Figure 1). There was no effect of treatment ($P = 0.63$) on the ammonia-N (NH_3-N) concentration,

Table 2. Intake and apparent digestibility of nutrients

	Diet[1]						Contrast P-value[2]		
Item	Wh	Wh+NaOH	Mo	Mo+Bic	SEM	Treatment P-value[2]	Wh vs. Mo	Wh vs. Wh+NaOH	Mo vs. Mo+Bic
DMI, kg/d	16.6	18.1	17.9	17.7	0.7	0.10	0.04	0.03	0.70
Water intake, L/d	67.8	81.4	88.2	89.5	6.5	0.002	0.001	0.005	0.70
OM									
Intake, kg/d	15.6	16.7	16.2	15.9	0.7	0.20	0.26	0.05	0.55
Duodenal flow, kg/d	8.76	9.54	8.71	8.19	0.62	0.15	0.92	0.16	0.33
Rumen digestibility, %	43.7	42.8	46.5	48.4	3.3	0.49	0.49	0.83	0.63
Small intestine digestibility, %	50.9	50.4	46.5	44.4	2.3	0.22	0.21	0.88	0.53
Total-tract digestibility, %	75.4	75.9	76.1	75.6	0.6	0.74	0.33	0.47	0.51
Starch									
Intake, kg/d	3.77	4.10	0.25	0.21	0.12	<0.001	<0.001	0.07	0.81
Duodenal flow, kg/d	0.45	1.24	0.17	0.16	0.08	<0.001	0.02	<0.001	0.92
Rumen digestibility, %	87.8	69.7	32.3	23.1	4.10	<0.001	<0.001	0.001	0.02
Small intestine digestibility, %	71.2	76.2	66.8	72.1	10.2	0.92	0.76	0.73	0.72
Total-tract digestibility, %	98.3	96.6	93.3	92.7	2.4	0.27	0.14	0.60	0.84
Sugar									
Intake, kg/d	0.57	0.54	4.33	4.23	0.18	<0.001	<0.001	0.89	0.63
NDF									
Intake, kg/d	5.28	5.24	5.03	4.92	0.22	0.22	0.19	0.83	0.55
Duodenal flow, kg/d	1.81	1.58	1.72	1.55	0.16	0.43	0.60	0.22	0.35
Rumen digestibility, %	65.5	69.4	65.9	68.8	3.0	0.61	0.91	0.31	0.44
Small intestine digestibility, %	−19.1	−22.3	−27.0	−31.4	11.4	0.76	0.54	0.80	0.73
Total-tract digestibility, %	64.6	67.6	67.9	66.8	1.2	0.08	0.02	0.03	0.33
CP									
Intake, kg/d	2.92	3.18	3.18	3.19	0.16	0.40	0.19	0.18	0.96
Duodenal flow, kg/d	3.45	3.57	3.64	3.48	0.23	0.68	0.30	0.49	0.38
Rumen digestibility, %	−19.6	−12.4	−14.7	−10.0	9.1	0.85	0.68	0.55	0.69
Small intestine digestibility, %	73.8	70.3	65.0	63.3	1.9	0.007	0.005	0.14	0.43
Total-tract digestibility, %	70.4	70.6	66.0	66.2	2.1	0.31	0.19	0.95	0.96

[1]The rations were based on late first-cut grass–clover silage supplemented with wheat (Wh), NaOH-treated wheat (Wh+NaOH), sugar beet molasses (Mo), or sugar beet molasses with addition of sodium bicarbonate (Mo+Bic).

[2]P-values are given for the overall treatment effects and for orthogonal contrasts for Wh versus Mo (effect of carbohydrate source), Wh versus Wh+NaOH (effect of NaOH treatment), and Mo versus Mo+Bic (effect of addition of sodium bicarbonate).

Table 3. Ruminal distribution of VFA, pH values, and ammonia (NH_3-N) concentration

Item	Diet[1]				SEM	Treatment *P*-value[2]	Contrast *P*-value[2]		
	Wh	Wh+NaOH	Mo	Mo+Bic			Wh vs. Mo	Wh vs. Wh+NaOH	Mo vs. Mo+Bic
VFA total, mmol/L	110	110	108	114	5.9	0.92	0.83	0.98	0.51
Distribution, mol/100 mol									
Acetate (A)	59.7	60.8	57.9	57.9	1.4	0.45	0.41	0.58	0.99
Propionate (P)	22.6	20.8	20.8	21.3	1.2	0.67	0.29	0.31	0.77
Butyrate (B)	15.0	15.8	19.0	18.4	1.7	0.35	0.14	0.77	0.81
Isobutyrate	0.91	0.91	0.52	0.54	0.04	<0.001	<0.001	0.99	0.81
Valerate	1.81	1.66	1.77	1.86	0.15	0.80	0.85	0.49	0.66
A:P	2.71	2.96	2.83	2.76	0.16	0.73	0.63	0.31	0.77
A+B:P	3.40	3.72	3.78	3.63	0.25	0.72	0.31	0.38	0.68
Rumen pH, ventral	6.40	6.45	6.45	6.47	0.09	0.95	0.69	0.71	0.88
Minimum pH, ventral	5.98	6.04	6.08	6.18	0.12	0.14	0.25	0.47	0.19
No. of observations pH <6, ventral	2.25	0.50	0.25	1.00	0.93	0.18	0.06	0.08	0.41
NH_3-N, mg/100 g	14.9	12.8	16.1	16.8	2.27	0.63	0.72	0.53	0.82

[1]The rations were based on late first-cut grass–clover silage supplemented with wheat (Wh), NaOH-treated wheat (Wh+NaOH), sugar beet molasses (Mo), or sugar beet molasses with addition of sodium bicarbonate (Mo+Bic).

[2]*P*-values are given for the overall treatment effects and for orthogonal contrasts for Wh versus Mo (effect of carbohydrate source), Wh versus Wh+NaOH (effect of NaOH treatment), and Mo versus Mo+Bic (effect of addition of sodium bicarbonate).

but it was affected by sampling time ($P < 0.001$), and there was an interaction between treatment and time ($P < 0.03$).

Rumen Microbial Synthesis and Turnover

There were no effects of diet on the microbial synthesis of DM and protein and no effects on efficiency of the microbial protein synthesis. Rumen evacuation data showed a difference in some of the rumen pool characteristics between the Wh and Mo diets. The pool of free fluid was significantly higher ($P = 0.04$) for the Mo diet (14.9 kg) than for the Wh diet (11.9 kg). In accordance with this, the content of DM in the rumen pool was lower for the Mo diet (117 g/kg) than for the Wh diet (124 g/kg). However, the total rumen pool (kg) was unaffected by diet. There was a tendency toward a higher free fluid pool when wheat was treated with NaOH ($P = 0.07$) or when bicarbonate was added to the molasses diet ($P = 0.10$). Treatment of wheat with NaOH caused a higher DM concentration in the rumen pool (131 vs. 124 g/kg; $P = 0.04$) and a larger rumen protein pool (2.04 vs. 1.82 kg; $P = 0.01$). For the 3 pools of NDF (Table 4), there were no significant effects of CHO source, NaOH treatment, or addition of bicarbonate.

There was a tendency ($P = 0.09$) toward higher passage rate of iNDF due to NaOH treatment of wheat (2.63% vs. 2.37%/h), whereas there was no difference between Wh and Mo diets in this regard. The degradation rate of dNDF was not significantly affected by CHO source, NaOH treatment, or addition of bicarbonate.

Methane Production

Dry matter intake during the 4 d of CH_4 measurements in the metabolism chambers (Table 5) was not different ($P = 0.49$, Student's *t*-test) from DMI during the period of digesta sampling (Table 2). However, the lower intake for the Wh diet compared with the other 3 diets was less pronounced during CH_4 measurements than during digesta sampling.

Replacing wheat with molasses consistently increased methane production irrespective of the unit for expression of CH_4 production and increased the production of H_2 from only 5.8 for the Wh diet to 27.1 L/d for the Mo diet ($P < 0.001$).

Treatment of wheat with NaOH increased the production of CH_4 from 547 to 640 L/d ($P = 0.04$) and from 0.091 to 0.101 L of CH_4/L of CO_2 ($P = 0.04$). However, when CH_4 production was related to DMI or kilograms of ECM, it was not significantly affected by the NaOH treatment. Production of CH_4 per kilogram of true digested OM in the rumen was numerically higher for the Wh+NaOH diet than for the Wh diet ($P = 0.12$). There was no effect of bicarbonate addition to the molasses diet regardless of whether the CH_4 production was expressed as liters per day or related to feed intake, digested OM, or milk yield.

Methane production for the Wh diet was significantly lower than for the Mo diet during 1400 to 1700 h, which was 7 to 10 h after the morning feeding at 0630 (Figure 2). Likewise, the Wh diet gave less CH_4 production from 2300 to 0200 h, which was 6 to 9 h after the afternoon feeding at 1630 h. Hence, the lower diurnal CH_4 production on the Wh diet was mainly due

to these periods, whereas the difference in CH_4 production between the 2 diets was much smaller in the first hours after feeding and in the hours before the morning feeding.

Milk Production

The Wh diet led to a higher production of milk (22.2 vs. 19.3 kg/d; $P = 0.03$) and ECM (23.7 vs. 22.1 kg/d; $P = 0.08$) than the Mo diet (Table 6). Treatment of wheat with NaOH increased the ECM production by approximately 3 kg/d ($P = 0.01$). The concentration of fat (48.1 vs. 44.4 g/kg; $P = 0.06$) and protein (41.5 vs. 39.9 g/kg; $P = 0.02$) was higher in the Mo diet than in the Wh diet, whereas lactose was significantly lower in the Mo diet than in the Wh diet (46.1 vs. 47.0; $P = 0.05$). There was no effect of bicarbonate supplementation of the molasses diet on milk yield, ECM yield, or content of fat, protein, or lactose.

DISCUSSION

Carbohydrate Source

Methane Production. Methane production per day and per kilogram of DMI was high in the present experiment compared with earlier studies (Brask et al., 2013a,b), where cows usually produced less than 600 L of CH_4/d and less than 30 L/kg of DMI. This may be related to the rather mature grass silage used in this study, as we have previously shown (Brask et al., 2013a) that cows eating more mature grass silage also produce more CH_4. The lower daily CH_4 production (L/d) for the Wh diet compared with the Mo diet could partly be

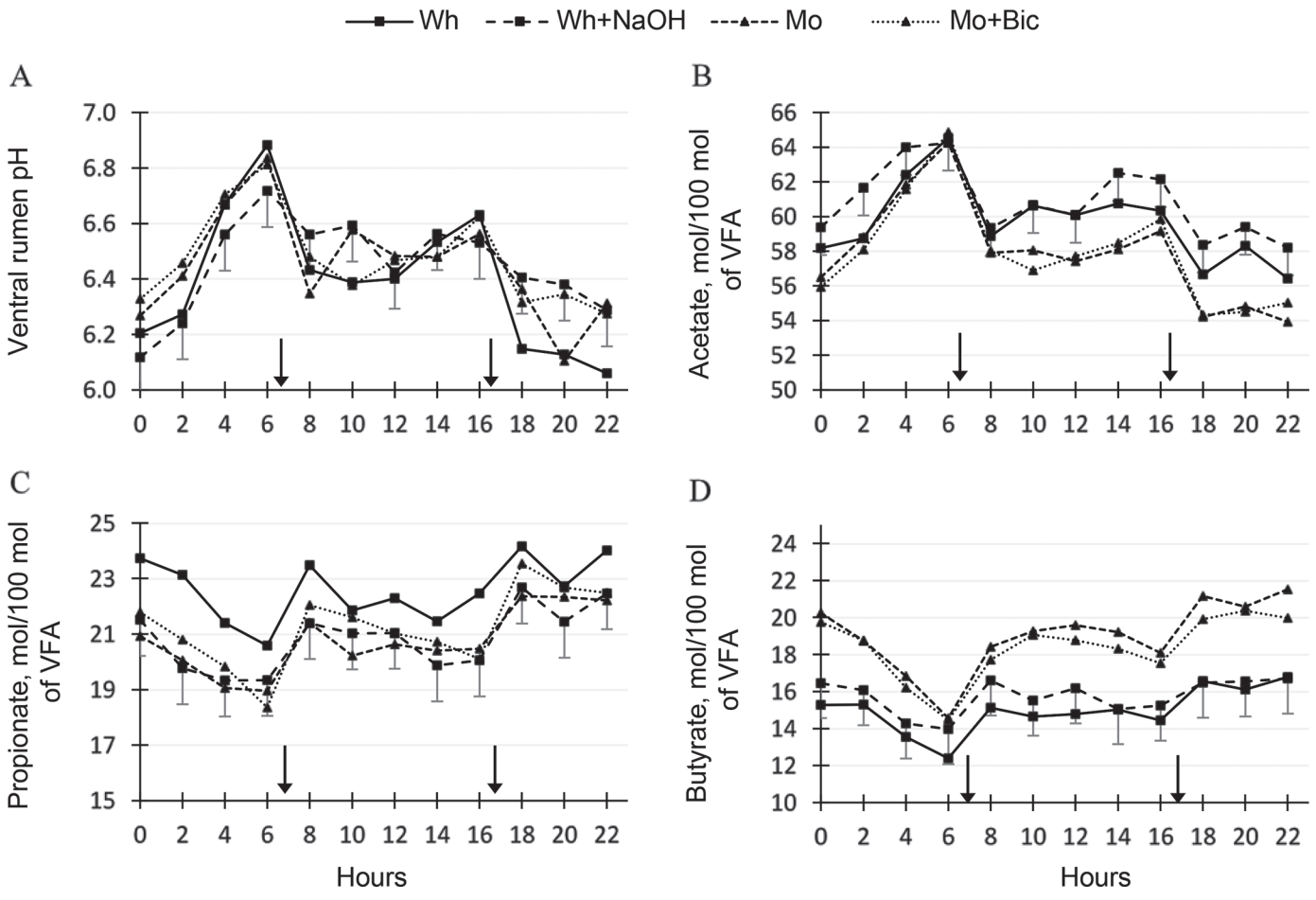

Figure 1. Diurnal pH profile in the ventral rumen (A), acetate proportion of total VFA (B), propionate proportion of total VFA (C), and butyrate proportion of total VFA (D). Arrows indicate feeding times (0630 and 1630 h). The rations were based on late first-cut grass–clover silage supplemented with wheat (Wh), NaOH-treated wheat (Wh+NaOH), sugar beet molasses (Mo), or sugar beet molasses with addition of sodium bicarbonate (Mo+Bic). The SEM from the statistical model, including all sampling times, was added on the Wh+NaOH graph (error bars) and was 0.13, 1.6, 1.3, and 1.9 units for panels A, B, C, and D, respectively.

Table 4. Microbial synthesis, microbial efficiency, rumen pool sizes, rumen passage rate of indigestible NDF (iNDF), and degradation rate of digestible NDF (dNDF)

Item	Diet[1]				SEM	Treatment *P*-value[2]	Contrast *P*-value[2]		
	Wh	Wh+NaOH	Mo	Mo+Bic			Wh vs. Mo	Wh vs. Wh+NaOH	Mo vs. Mo+Bic
Microbial synthesis									
DM, kg/d	2.91	2.68	3.04	2.81	0.22	0.29	0.49	0.23	0.24
Protein, kg/d	1.62	1.47	1.69	1.60	0.12	0.21	0.48	0.15	0.35
Efficiency, g of protein/kg of true digested OM in rumen	178	160	169	161	17	0.74	0.64	0.36	0.67
Efficiency, g of protein/kg of digested carbohydrates in rumen	215	192	207	196	19	0.69	0.69	0.31	0.62
Rumen pools									
Total, kg	77.6	78.1	77.4	77.7	7.6	0.99	0.95	0.83	0.91
Free fluid, kg	11.9	14.5	14.9	17.2	2.4	0.02	0.04	0.07	0.10
DM, g/kg	124	131	117	115	3	0.004	0.04	0.04	0.50
DM, kg	9.67	10.21	9.06	8.95	0.94	0.09	0.21	0.26	0.82
iNDF, g/kg of NDF	289	290	290	279	13	0.91	0.95	0.96	0.56
OM, kg	8.89	9.37	8.19	8.11	0.88	0.06	0.14	0.29	0.85
Protein, kg	1.82	2.04	1.90	1.95	0.16	0.04	0.22	0.01	0.44
NDF, kg	5.14	4.94	4.75	4.74	0.55	0.44	0.18	0.47	0.98
iNDF, kg	1.47	1.41	1.37	1.32	0.13	0.64	0.41	0.61	0.69
dNDF, kg	3.67	3.53	3.38	3.42	0.44	0.50	0.19	0.50	0.84
Rates									
iNDF passage, %/h	2.37	2.63	2.22	2.25	0.13	0.06	0.27	0.09	0.80
dNDF degradation, %/h	4.11	4.47	4.21	4.15	0.44	0.65	0.75	0.28	0.85

[1]The rations were based on late first-cut grass–clover silage supplemented with wheat (Wh), NaOH-treated wheat (Wh+NaOH), sugar beet molasses (Mo), or sugar beet molasses with addition of sodium bicarbonate (Mo+Bic).

[2]*P*-values are given for the overall treatment effects and for orthogonal contrasts for Wh versus Mo (effect of carbohydrate source), Wh versus Wh+NaOH (effect of NaOH treatment), and Mo versus Mo+Bic (effect of addition of sodium bicarbonate).

explained with lower feed intake. The higher intake of the Mo diet could be expected due to increased palatability when molasses is added to the diet (Primdal et al., 2014). However, also CH_4 per kilogram of DMI differed between Wh and Mo diets. When CH_4 production was related to the amount of true digested OM in the rumen, the numerical difference was rather high, with a CH_4 production of 65.3 L/kg of true rumen-digested OM for the Mo diet versus only 58.5 L/kg of true rumen-digested OM for the Wh diet. This indicates that CH_4 production per kilogram of true digested OM in the rumen in diets with molasses is higher than that

Table 5. Methane and hydrogen production

Item	Diet[1]				SEM	Treatment *P*-value[2]	Contrast *P*-value[2]		
	Wh	Wh+NaOH	Mo	Mo+Bic			Wh vs. Mo	Wh vs. Wh+NaOH	Mo vs. Mo+Bic
DMI, kg/d	16.9	18.4	18.5	18.2	0.9	0.26	0.09	0.11	0.73
CH_4, L/d	547	616	671	640	36	0.02	0.004	0.04	0.30
CH_4, L/kg of DMI	32.5	33.6	36.2	35.1	1.4	0.16	0.05	0.51	0.48
CH_4, L/kg of ECM	24.4	24.3	31.5	29.3	1.6	0.01	0.003	0.96	0.20
CH_4,[3] L/kg of true digested OM in rumen	58.5	65.3	65.3	62.4	2.8	0.31	0.12	0.12	0.48
CH_4, L/kg of total digested OM	45.9	47.7	52.7	51.8	1.7	0.02	0.01	0.33	0.60
CH_4, % of GEI[4]	7.10	7.39	8.24	8.03	0.30	0.04	0.01	0.41	0.53
CH_4:CO_2, L/L	0.091	0.101	0.112	0.108	0.004	0.01	0.001	0.04	0.36
H_2, L/d	5.8	4.7	27.1	27.3	3.7	0.001	0.001	0.76	0.96

[1]The rations were based on late first-cut grass–clover silage supplemented with wheat (Wh), NaOH-treated wheat (Wh+NaOH), sugar beet molasses (Mo), or sugar beet molasses with addition of sodium bicarbonate (Mo+Bic).

[2]*P*-values are given for the overall treatment effects and for orthogonal contrasts for Wh versus Mo (effect of carbohydrate source), Wh versus Wh+NaOH (effect of NaOH treatment), and Mo versus Mo+Bic (effect of addition of sodium bicarbonate).

[3]True OM digestibility determined for each cow and diet in the previous week was used together with the actual OM intake in the respiration chambers to calculate kilograms of true digested OM in the rumen.

[4]GEI = gross energy intake (kg/d).

Table 6. Milk production measured during the 4 d in the respiration chambers

Item	Diet[1]				SEM	Treatment *P*-value[2]	Contrast *P*-value[2]		
	Wh	Wh+NaOH	Mo	Mo+Bic			Wh vs. Mo	Wh vs. Wh+NaOH	Mo vs. Mo+Bic
Milk yield, kg/d	22.2	24.6	19.3	19.7	2.9	0.01	0.03	0.06	0.73
ECM, kg/d	23.7	26.5	22.1	22.0	2.2	0.004	0.08	0.01	0.91
Fat, g/kg of milk	44.4	44.9	48.1	46.5	3.1	0.20	0.06	0.75	0.37
Protein, g/kg of milk	39.9	40.2	41.5	41.9	1.8	0.02	0.02	0.53	0.54
Lactose, g/kg of milk	47.0	48.5	46.1	46.5	0.4	0.003	0.05	0.01	0.32

[1]The rations were based on late first-cut grass–clover silage supplemented with wheat (Wh), NaOH-treated wheat (Wh+NaOH), sugar beet molasses (Mo), or sugar beet molasses with addition of sodium bicarbonate (Mo+Bic).

[2]*P*-values are given for the overall treatment effects and for orthogonal contrasts for Wh versus Mo (effect of carbohydrate source), Wh versus Wh+NaOH (effect of NaOH treatment), and Mo versus Mo+Bic (effect of addition of sodium bicarbonate).

in diets with wheat. This is in accordance with results from Hatew et al. (2015), who found a decrease in both daily CH_4 production and CH_4 production per kilogram of rumen-fermentable OM when the starch level was increased from 11.5% to 21.2% in the diet. They also found that replacing native corn grain with more rapid fermentable gelatinized corn grain further decreased the CH_4 production per kilogram of rumen-fermentable OM. The reducing effect on the CH_4 production of both more starch and making the starch more rapidly fermentable occurred even if there were no differences in the rumen pH or proportion of butyric acid proportion during the first 8 h after feeding, and there was only a slight increase in the proportion of propionic acid due to the gelatinized grain.

The loss of gross energy as CH_4 was 8.2% for the Mo diet, which was significantly more than the 7.1% on the wheat diet. Kirchgessner et al. (1994) found an increase in the proportion of GE intake lost as CH_4 from 6.1% to 6.8% when 6.5 kg of DM from a starch-rich concentrate was replaced with 4.5 kg of DM from sucrose and 1.7 kg of DM from soybean meal. Müller et al. (1994) reported

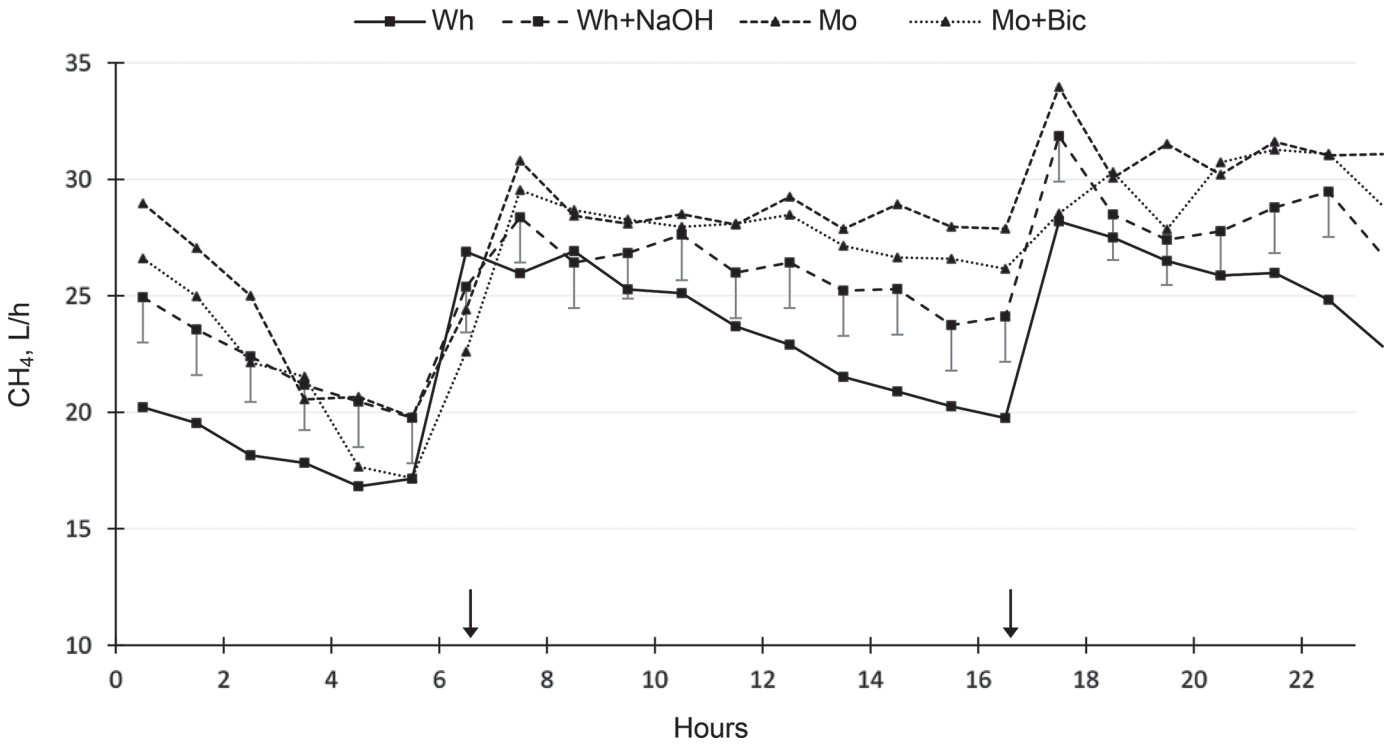

Figure 2. Diurnal methane (CH_4) production. Arrows indicate feeding times (0630 and 1630 h). The rations were based on late first-cut grass–clover silage supplemented with wheat (Wh), NaOH-treated wheat (Wh+NaOH), sugar beet molasses (Mo), or sugar beet molasses with addition of sodium bicarbonate (Mo+Bic). Error bars show SEM (1.95).

an increase in the proportion of GE intake lost as CH_4 from 6.2% to 7.2% when 7.5 kg of DM from fodder beets and 2.0 kg of DM from soybean meal replaced 5.8 kg of DM from a starch-rich concentrate and 4.6 kg of DM from grass silage.

The higher CH_4 production per kilogram of DMI on the Mo diet than on the Wh diet is also in line with the findings of Murphy et al. (1982) as well as Hindrichsen and Kreuzer (2009), who found a higher CH_4 production from sugar than from starch in an in vitro system. However, in an in vivo experiment with dairy cows, a diet with molasses did not result in more enteric CH_4 than a diet with wheat (Hindrichsen et al., 2005). However, in that study, sugar replaced only 60% of the amount of starch omitted, whereas in the present study 213 g of starch was replaced by an equal amount of sugar (i.e., 207 g).

On the molasses diet, 27 L of H_2/d escaped the rumen, whereas only 5 L of H_2/d was emitted on the wheat diet. Peak emission of hydrogen was observed just after feeding.

During both day and night, the butyric acid proportion of VFA in the rumen was higher for the Mo diet than for the Wh diet (Figure 1D), and this pattern coincided with the diurnal pattern of methane (Figure 2). The diurnal mean proportion of butyric acid was 19.0 versus 15.0 for the Mo and Wh diets, respectively. The diurnal mean proportion of propionate was 20.8 for the Mo diet versus 22.6 for the Wh diet. According to stoichiometry (Ungerfeld and Kohn, 2006), ruminal fermentation of 1 mol of glucose can yield 1 mol of butyrate and 2 mol of H_2 or alternatively yield 2 mol of propionate while utilizing 2 mol of H_2. Most H_2 is used for methane production, when 4 mol of H_2 reacts with 1 mol of CO_2 to form 1 mol of CH_4 and 2 mol of H_2O. Despite the lack of significance between the diets regarding average diurnal butyrate and propionate proportion in the rumen, the joint changes of butyrate and propionate are probably the reason for the effect on H_2 and methane.

Contrarily, Hindrichsen and Kreuzer (2009) did not find differences in emitted hydrogen between sugar and starch diets in vitro. Pinares-Patiño et al. (2011) found higher emissions of hydrogen when sheep were fed concentrate instead of grass, indicating that hydrogen production in some situations may exceed the utilization capacity of the rumen methanogens.

Rumen Metabolism. For the 2 molasses diets, low rumen digestibility of starch was found. Larsen et al. (2009) reported microbial polysaccharide synthesis in the rumen, especially in diets rich in starch or sugar. Microbial polysaccharides analyzed as starch could explain the low rumen starch digestibility in diets with molasses in the present trial, as starch intake (0.25 and 0.21 kg/d for Mo and Mo+Bic, respectively) was very low. No sugar analyses were made in digesta and feces because previous work has shown that sugars are rapidly fermented in the rumen with a degradation rate of 400 to 700%/h and, therefore, none of the sugar in feed can be found in duodenal or fecal material (Weisbjerg et al., 1998). Rumen NDF digestibility is often reduced in diets with a high starch content. Khalili and Huhtanen (1991) reported that the depression of fiber digestion can be divided into an effect of the CHO per se and the decrease in rumen pH due to rapidly fermentable CHO. However, these effects are difficult to distinguish. Hatew et al. (2015) found no effect on NDF total-tract digestibility of either level of starch (212 vs. 115 g/kg of DM) or fermentation rate of the starch source (5.5%/h for native corn grain vs. 15.5%/h for gelatinized corn grain). Surprisingly, there was no effect of starch source and no effect of amount of starch on rumen pH during the first 8 h after morning feeding, implying that the lack of effect on rumen NDF digestibility might be due to lack of effect on pH.

Rumen NDF digestibility in the present study with diets based on late first-cut grass–clover silage was on average 67%. This is in line with an earlier study where rumen NDF digestibility was 66% for diets based on late first-cut grass–clover silage and 70% for diets based on early first-cut grass silage, whereas it was only 51% for diets based on maize silage (Brask et al., 2013a). In the present experiment, there was no effect on rumen NDF digestibility of CHO source, and there were no significant differences between treatments on rumen pH. In a study with a rumen fermenter with varying pH and varying levels of easily fermentable CHO (Weisbjerg et al., 1999), pH did not affect NDF digestibility. However, increasing amounts of starch decreased the NDF digestibility by 23 percentage units ($P = 0.004$), whereas similar increases in the amount of molasses decreased the NDF digestibility by only 13 percentage units ($P = 0.1$). This indicates that the negative effect of easily digestible CHO on NDF degradation is due to an effect of substrate more than to an effect of pH.

In the present experiment, there was no significant effect of the CHO source on iNDF passage rate. Stensig et al. (1998) found a higher passage rate of iNDF in diets with 20 to 30% sucrose in DM compared with diets with 20 to 30% wheat flour. They also found a lower rate of degradation of NDF for sucrose diets, and they measured a decreased rate of degradation of NDF for 30% inclusion compared with 20% inclusion for both sources of CHO. In combination, this led to a lower total-tract digestibility of NDF when high amounts of sucrose were fed. This is contradictory to the present experiment, where the total-tract digestibility of NDF was lowest for the Wh diet due to a slightly higher rate

of passage of iNDF and a slightly lower degradation rate of dNDF compared with the Mo diet. One possible explanation for the different effects of sugar and starch found in the present study compared with the study of Stensig et al. (1998) could be the sources of sugar and starch. The wheat flour and the sucrose used by Stensig et al. (1998) may have been more readily available in the rumen than starch from wheat and sugar from molasses in the present experiment. Stensig et al. (1998) used early-lactation cows, which may have been more vulnerable to high amounts of readily available CHO than the mid-lactation cows in the present study.

The duodenal material is heterogeneous, and therefore the standard error of the mean value of the rumen digestibility of NDF was higher: 3.0 units compared with only 1.2 units for total-tract NDF digestibility. This means that the difference of 3.3 units found between Wh and Mo in the total-tract digestibility is probably a real difference, whereas the lack of difference in rumen digestibility may be due to the larger variation. A biological reason to believe that the lower total-tract NDF digestibility in the Wh diet is in fact real is that in the Wh diet, 16% of the dietary NDF came from wheat, whereas all NDF came from grass–clover silage in the Mo diet because NDF from wheat kernels is less digestible (3.5%/h) than NDF from grass–clover silage (4–5%/h; Norfor, 2019).

Compared with the Wh diet, the Mo diet increased diurnal water intake and water intake per kilogram of DM (4.06 kg of water/kg of DM for the Wh diet vs. 4.44 kg for the Mo diet; $P < 0.001$), which may be due to the osmotic effect of a high content of cations in the Mo diet (Araba et al., 2002). In theory, the higher water intake would dilute the rumen fluid and increase liquid outflow from the rumen. This would be seen as a lower total VFA concentration and a higher rumen pH. In line with this, there were more observations with pH below 6.0 and lower water intake in the Wh diet, which was also in agreement with earlier studies (Owens et al., 2008; Ramin and Huhtanen, 2013).

Rumen fermentation of sugar from molasses increased the butyrate proportion compared with the Wh diet at all sampling times, and for 8 of the 12 sampling times butyrate tended to be significantly higher for the Mo diet ($P < 0.10$). Higher butyrate proportions for the Mo diet were most evident after feeding, which illustrates that sugar is quickly fermented. Vallimont et al. (2004) reported no significant difference between starch and sucrose in the daily average VFA pattern but found more butyrate with sucrose feeding in the first 5 h post-feeding in an in vitro system, indicating rapid degradation of sugar. Stensig et al. (1998) found that the mean diurnal butyrate proportion was 2.6 percentage units higher with 20% of DM from sucrose compared with 20% from pure wheat starch. When the 30% sucrose and 30% pure wheat starch were compared, the butyrate proportion was 5.3 percentage units higher for the sucrose diet in accordance with the 4 percentage units higher butyrate for the Mo diet than for the Wh diet in the present experiment. Dijkstra et al. (2012) reported that butyrate is absorbed faster than other VFA and that increased production of butyrate due to sugar feeding is not necessarily fully reflected in the measured butyrate concentrations in the rumen.

The larger pool of free fluid for the Mo diet than the Wh diet and the lower concentration of DM in the rumen pool for the Mo diet indicate that there were more osmotically active products in the rumen of Mo-fed cows during rumen evacuation approximately 5 h after feeding than in the Wh-fed cows. Also, water intake was higher for the Mo diet than for the Wh diet.

NaOH Treatment of Wheat

The amount of OM fermented in the rumen is the most important driver for the production of CH_4 (Brask et al., 2015). Rumen digestibility of starch was lower for the diet with NaOH-treated wheat compared with rolled wheat in agreement with earlier findings (Phipps et al., 2001; Larsen et al., 2009). Despite the lower starch digestibility of 69.7% for NaOH-treated wheat versus 87.8% for the rolled wheat, the difference in the amount of starch digested in the rumen was only 0.46 kg/d between rolled wheat and NaOH-treated wheat due to a higher feed intake in the diet with treated wheat. This may have counteracted the expected effect of the NaOH treatment of wheat on the rumen fermentation pattern, and therefore no effect on the production of CH_4 per kilogram of DMI could be expected.

The inclusion of 3 kg of NaOH/100 kg of wheat was based on findings by Mortensen et al. (1993) to obtain a significant decrease in starch degradation in the rumen without supplying extreme amounts of NaOH. Furthermore, Hymøller et al. (2014) showed a total-tract starch digestibility of 99.2% of wheat kernels treated with 3 kg of NaOH/100 kg of wheat. When NaOH reacts with wheat kernels, only a limited amount of OH ions are left in the product because a surplus will react with atmospheric CO_2 to form water and Na_2CO_3. Therefore, NaOH cannot be expected to have a direct pH effect in the rumen but only an indirect effect due to Na_2CO_3 and to the lower rumen digestibility of starch. When no direct effect of NaOH can be expected on pH, no effect on NDF digestibility can be expected. As discussed elsewhere, the extra dietary Na can be the reason for

the higher water intake due to the NaOH treatment of wheat. In fact, the NaOH treatment in the present trial did not significantly affect the average rumen pH in the medial or ventral rumen compared with the rolled wheat kernels, which is in agreement with the findings of Phipps et al. (2001).

Apparently, rumen pH was affected only by NaOH treatment or bicarbonate supplementation shortly after feeding. In general, rumen pH did not reach a critical level at any point of time in this trial. In this trial, 13 out of 16 observations with pH <6 were from 1 multiparous early-lactation cow, which compared with the 3 primiparous cows had a higher milk yield and feed intake, which might indicate a larger effect of NaOH treatment and of bicarbonate supplementation in cows with higher yield and higher feed intake.

In the present experiment, feed intake tended to be lower for the Wh diet than for the Wh+NaOH diet. The lower rumen starch digestibility of NaOH-treated wheat might have increased feed intake and, therefore, passage rate as indicated by the tendency to the increased iNDF passage rate.

No effect of NaOH treatment of wheat was found on the rumen fermentation pattern by O'Mara et al. (1997). Khalili and Huhtanen (1991) concluded that the depression in fiber digestion with sugar feeding is mainly due to a decreased pH, as the negative effect of sugar is alleviated by feeding sodium bicarbonate. There was no significant increase in the rumen NDF digestibility in the present trial when wheat was treated with NaOH treated or when the molasses diet was supplemented with bicarbonate. The lack of effect on NDF digestibility can probably be explained by the high rumen pH, as pH did not reach a critically low level for fiber digestion, which is between 6.0 and 6.3 (Dijkstra et al., 2012). In fact, only 1 cow had a few samplings with a pH below 6.0.

Stensig et al. (1998) found that the mean diurnal acetate proportion was 60.1% and the propionate proportion was 22.3% in cows fed 20% of DM from wheat starch compared with 55.9% acetate and 26.6% propionate when 30% of the DM was from pure wheat starch. Similarly, they found a diurnal acetate proportion of 57.4% and a propionate proportion of 22.9% in cows fed 20% of DM from sucrose compared with 53.9% acetate and 24.5% propionate when 30% of the DM was from sucrose.

This means that the level of starch and sugar is very important for the rumen VFA distribution. Hence, the level of approximately 25% starch and sugar in the Wh and Mo diets, respectively, in the present study was probably not high enough to cause negative effects on the rumen pH, the rumen VFA distribution, or the rumen NDF digestibility. Therefore, no clear effects of NaOH treatment of wheat or bicarbonate supplementation to the molasses diet could be expected.

In accordance with our findings, Bougouin et al. (2018) found no effect on apparent total-tract NDF digestibility when bicarbonate was added to a starch-rich diet (231 g/kg of DM) despite the fact that they found a significant increase in both mean and minimum pH in the rumen. This indicates that lower pH is not per se the major reason if NDF digestibility is negatively affected by increased dietary concentration of fermentable CHO.

The higher DM concentration in the rumen pool for the Wh+NaOH diet could be due to more intact starch and less osmotically active starch degradation products in this diet compared with the Wh diet because the NaOH treatment of wheat reduced the rumen degradation of starch from 88% to 70%. The higher amount of free fluid in the Wh+NaOH and Mo+Bic diets could be due to the osmotic effect of the higher intake of Na in these 2 diets followed by a higher water uptake. There were no effects of NaOH treatment on microbial protein efficiency (g of protein/kg of rumen-digested CHO), which means that the amount of rumen fluid did not influence the microbial protein efficiency.

Bicarbonate Supplementation

Surprisingly, bicarbonate supplementation of the molasses diet affected neither the average nor the minimum pH in the ventral rumen. As discussed above, the lack of effect on pH could probably be ascribed to the fact that the sugar level was not high enough to cause adverse effects on the pH or VFA distribution in the rumen. Furthermore, the lack of effect of bicarbonate on CH_4 emission was probably due to the lack of effect on pH and NDF digestibility in the rumen. High NDF content in forage and relatively high forage:concentrate ratio resulted in pH values in the rumen that were far from critical pH levels. Therefore, an effect on fiber digestion and methanogenic bacteria could not be expected because NDF digestibility is unaffected above a critical pH level but gets seriously affected below this level (Huhtanen et al., 2006).

In the present experiment, none of the 2 molasses-based diets gave very low minimum pH. The higher CH_4 production for the molasses diet compared with the wheat diet supports Hindrichsen et al. (2005), who hypothesized that sucrose diets are more methanogenic than isoenergetic diets with starch at high pH, whereas the difference at low pH is minimal. Hindrichsen and Kreuzer (2009) found that the increased CH_4 production at high ruminal pH was due to an increase in fiber digestibility. However, rumen fiber digestibility was not different between the Wh and the Mo diets in the pres-

ent trial; therefore, differences in fiber digestibility cannot explain the difference in CH_4 production between the Wh and Mo diets.

CONCLUSIONS

Replacing wheat with molasses and thereby increasing the sugar:starch ratio in the diet increases the CH_4 production per kilogram of DMI from dairy cows, presumably by increasing the proportion of butyrate in the rumen VFA. Treatment of wheat with NaOH did not influence the CH_4 production per kilogram of DMI under the present conditions. When rumen pH does not reach a critical low value, there is no effect on CH_4 production of bicarbonate supplementation to diets with molasses.

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