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

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ABSTRACT

Complex interactions between rumen microbiota, cow genetics, and diet composition may exist. Therefore, the effect of linseed oil, *DGAT1* K232A polymorphism (*DGAT1*), and the interaction between linseed oil and *DGAT1* on CH₄ and H₂ emission, energy and N metabolism, lactation performance, ruminal fermentation, and rumen bacterial and archaeal composition was investigated. Twenty-four lactating Holstein-Friesian cows (i.e., 12 with *DGAT1* KK genotype and 12 with *DGAT1* AA genotype) were fed 2 diets in a crossover design: a control diet and a linseed oil diet (LSO) with a difference of 22 g/kg of dry matter (DM) in fat content between the 2 diets. Both diets consisted of 40% corn silage, 30% grass silage, and 30% concentrates (DM basis). Apparent digestibility, lactation performance, N and energy balance, and CH₄ emission were measured in climate respiration chambers, and rumen fluid samples were collected using the oral stomach tube technique. No linseed oil by *DGAT1* interactions were observed for digestibility, milk production and composition, energy and N balance, CH₄ and H₂ emissions, and rumen volatile fatty acid concentrations. The *DGAT1* KK genotype was associated with a lower proportion of polyunsaturated fatty acids in milk fat, and with a higher milk fat and protein content, and proportion of saturated fatty acids in milk fat compared with the *DGAT1* AA genotype, whereas the fat- and protein-corrected milk yield was unaffected by *DGAT1*. Also, *DGAT1* did not affect nutrient digestibility, CH₄ or H₂ emission, ruminal fermentation or ruminal archaeal and bacterial concentrations. Rumen

bacterial and archaeal composition was also unaffected in terms of the whole community, whereas at the genus level the relative abundances of some bacterial genera were found to be affected by *DGAT1*. The *DGAT1* KK genotype was associated with a lower metabolizability (i.e., ratio of metabolizable to gross energy intake), and with a tendency for a lower milk N efficiency compared with the *DGAT1* AA genotype. The LSO diet tended to decrease CH₄ production (g/d) by 8%, and significantly decreased CH₄ yield (g/kg of DM intake) by 6% and CH₄ intensity (g/kg of fat- and protein-corrected milk) by 11%, but did not affect H₂ emission. The LSO diet also decreased ruminal acetate molar proportion, the acetate to propionate ratio, and the archaea to bacteria ratio, whereas ruminal propionate molar proportion and milk N efficiency increased. Ruminal bacterial and archaeal composition tended to be affected by diet in terms of the whole community, with several bacterial genera found to be significantly affected by diet. These results indicate that *DGAT1* does not affect enteric CH₄ emission and production pathways, but that it does affect traits other than lactation characteristics, including metabolizability, N efficiency, and the relative abundance of *Bifidobacterium*. Additionally, linseed oil reduces CH₄ emission independent of *DGAT1* and affects the rumen microbiota and its fermentative activity.

Key words: dairy cow, enteric methane production, linseed oil, *DGAT1* K232A polymorphism

INTRODUCTION

Several dietary strategies have been proposed to mitigate enteric CH₄ production, including the use of feed additives and improving forage quality (Beauchemin et al., 2009; Martin et al., 2010). Numerous studies have shown the potential of dietary lipid supplementation to reduce CH₄ emission, many of which have been

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reviewed by Grainger and Beauchemin (2011) and Hristov et al. (2013). To date, linseed is considered to be one of the most effective dietary lipid sources to reduce enteric CH₄ production from dairy cows (Beauchemin et al., 2009; Martin et al., 2010). Relatively few studies have considered the wider consequences of dietary linseed oil on the functioning of the rumen microbial ecosystem. Veneman et al. (2015) reported no effect of linseed oil supplementation on CH₄ emission or the rumen microbiota as a whole. Martin et al. (2016) reported significant decreases in CH₄ emissions upon extruded linseed supplementation for both corn-silage-based and hay-based diets, whereas the abundance of rumen methanogens was not affected by linseed supply in the corn-silage-based or hay-based diets.

Little is known whether host genetics can also influence the responses to dietary linseed oil. The acyl CoA:diacylglycerol acyltransferase 1 gene, located on chromosome 14, mediates the final step in triglyceride synthesis (Schennink et al., 2008). Many studies have investigated associations between the K232A polymorphism of this gene (i.e., a lysine to alanine substitution on the 232nd amino acid; *DGAT1*) and milk production traits of dairy cows. Although *DGAT1* has no effect on fat- and protein-corrected milk (**FPCM**) yield, the *DGAT1* K allele is associated with a higher fat content, protein content, and fat yield, but lower milk production and protein and lactose yield (e.g., Banos et al., 2008; Näslund et al., 2008; Bovenhuis et al., 2015). Additionally, *DGAT1* has a marked effect on milk fatty acid (**MFA**) composition. The *DGAT1* K allele is associated with a larger fraction of C16:0, and smaller fractions of C18 UFA in milk fat (e.g., Schennink et al., 2007; Duchemin et al., 2013). Several of the MFA that have been associated with CH₄ emission (van Gastelen and Dijkstra, 2016) are also affected by *DGAT1*, in particular C18 UFA in both the *cis* and *trans* isomers.

The *DGAT1* gene is expressed in the small intestine, liver, adipose tissue, and the mammary gland (DeVita and Pinto, 2013; Muise et al., 2014). Thus, effects of *DGAT1* on traits other than milk production might be expected. S. van Engelen (Wageningen University & Research; unpublished data) performed a genome-wide association study (**GWAS**) to determine regions of the bovine genome that are associated with predicted CH₄ yield (g/kg of DMI) using the CH₄ prediction equations based on MFA profile published by Dijkstra et al. (2011) and Van Engelen et al. (2015). The association with *DGAT1* was significant in the GWAS for predicted CH₄ yield, suggesting that the *DGAT1* K allele is associated with higher predicted CH₄ yield. The association between *DGAT1* and CH₄ yield has not been studied before and could be of statistical and biological significance. To the best of our knowledge, no

study has investigated if the genetic variation of dairy cows, namely *DGAT1*, affects the rumen bacterial and archaeal composition, one of the potential biological explanations for the relation between *DGAT1* and CH₄ yield. In addition, little information is available on the association of *DGAT1* with nutrient digestion or energy and N balance of dairy cattle.

Therefore, the objectives of the present study were to investigate the effects of dietary linseed oil, *DGAT1*, and the interaction between dietary linseed oil and *DGAT1* on CH₄ and H₂ emission, energy and N metabolism, lactation performance, ruminal fermentation, and rumen bacterial and archaeal composition of dairy cows.

MATERIALS AND METHODS

Experimental Design

The experiment was conducted from January to April 2015, in accordance with Dutch law and approved by the Animal Care and Use Committee of Wageningen University & Research (Wageningen, the Netherlands). The experiment followed a crossover design with 2 dietary treatments and 24 lactating Holstein-Friesian cows (i.e., 12 cows with *DGAT1* KK genotype and 12 cows with *DGAT1* AA genotype; each group had 6 primiparous and 6 multiparous cows). The 12 cows with *DGAT1* KK genotype were sired by 10 bulls, and the 12 cows with *DGAT1* AA genotype were sired by 9 bulls. Additionally, 1 bull sired 2 cows with the *DGAT1* KK genotype and 1 cow with *DGAT1* AA genotype. At the start of the experiment, the cows with the *DGAT1* KK genotype and *DGAT1* AA genotype were, on average, 215 ± 65 and 216 ± 68 DIM (means ± SD) and produced 23.9 ± 5.66 and 26.9 ± 5.87 kg of milk/d, respectively. The cows were blocked in pairs according to their *DGAT1* genotype, parity, DIM, and milk production. Within each block, cows were randomly allocated to a dietary treatment sequence in a crossover design with 2 periods: a control diet (**CON**) and linseed oil diet (**LSO**). Treatment periods lasted 17 d and were composed of a 12-d adaptation period followed by a 5-d measurement period. There was a 14-d wash-out period between the treatment periods of the same block of cows.

Diets, Feeding, and Housing

Both the CON and LSO diet consisted of 40% corn silage, 30% grass silage, and 30% concentrates on a DM basis. The ingredient and chemical composition of both diets are presented in Table 1. Linseed oil (Linagro NV, Lichtervelde, Belgium) was added to the concentrate of

the LSO diet, substituting a part of the CON concentrate ingredients, to achieve a difference of 22 g/kg of DM in fat content between the 2 diets. To determine apparent total-tract feed digestibility, Cr_2O_3 (1.5 g/kg of concentrate DM) was included in the concentrates of both diets as an external marker. Concentrates were produced by Research Diet Services (RDS BV, Wijk bij Duurstede, the Netherlands) in 1 batch and hence were assumed to be of uniform composition throughout the experiment. Diets were formulated to meet the requirements for maintenance and milk production of the lactating dairy cows. The NE_L was calculated with the VEM (feed unit lactation) system according to Van Es (1978), and intestinal digestible protein and rumen degradable protein balance were calculated according to Van Duinkerken et al. (2011).

Cows were fed and milked at 0600 and 1600 h. Just before milking, the feed refusals were weighed and a new portion of the diet provided. The diets were fed as a TMR in 2 equal daily portions. The concentrate

was provided in meal form and manually mixed into the roughage mixture at the time of feeding. Cows had free access to clean drinking water throughout the experiment. Cows were fed individually and feed refusals were collected to determine DMI throughout the experiment. Cows were fed ad libitum during the first 8 d of the adaptation period. From d 9 onward, feed intake was restricted per block to 95% of the ad libitum DMI of the cow within a block consuming the lowest amount of feed during d 5 to d 8 as described previously by van Zijderveld et al. (2011a). The cows were fed restricted amounts of feed to avoid confounding effects of DMI on enteric CH_4 production, similar to van Zijderveld et al. (2011a). At all times, a minimum DMI of at least 82% of the ad libitum intake of the cow with the greatest DMI within each block was ensured.

During the 12-d adaptation period, the cows were individually housed in tiestalls to become accustomed to the diet and restriction in movement. On d 13 (1400 h), after the adaptation period, 4 cows (2 blocks) were

Table 1. Chemical composition (g/kg of DM, unless otherwise stated) of the TMR ingredients (grass silage, corn silage, concentrates; analyzed) and of the complete TMR (calculated) for the control diet (CON) and linseed oil (LSO) diet

Item	Silage		Concentrate		TMR	
	Grass ¹	Corn ²	CON ³	LSO ⁴	CON ⁵	LSO ⁶
DM (g/kg of product)	558	318	878	890	468	469
OM	911	959	880	889	921	924
CP	140	83	394	361	194	184
Crude fat	33	34	33	108	34	56
Gross energy (MJ/kg of DM)	18.3	18.4	18.0	19.7	18.2	18.7
NDF	546	330	203	178	357	349
ADF	303	185	101	91	195	192
ADL	15	9	17	16	13	13
Starch	— ⁷	373	18	14	154	153
Reducing sugars	88	— ⁷	137	124	67	63
Fatty acids						
C16:0	2.7	2.7	2.4	7.1	2.6	4.0
C18:0	0.26	0.44	0.48	2.9	0.40	1.1
C18:1 <i>cis</i> -9	0.30	3.2	3.8	17.9	2.5	6.8
C18:2n-6	2.3	8.3	6.8	19.7	6.1	9.9
C18:3n-3	8.5	1.0	1.0	35.4	3.2	13.5
Total fatty acids	16.2	16.1	14.4	86.8	15.6	37.3

¹ NE_L = 6.2 MJ/kg of DM; intestinal digestible protein (DVE) = 64 g/kg of DM; rumen-degraded protein balance (OEB) = 11 g/kg of DM; ensiling characteristics: acetic acid = 9 g/kg of DM, lactic acid = 13 g/kg of DM, ammonia-N = 6% of total N, and pH = 5.9.

² NE_L = 7.0 MJ/kg of DM; DVE = 56 g/kg of DM; OEB = -8 g/kg of DM; ensiling characteristics: acetic acid = 11 g/kg of DM, lactic acid = 55 g/kg of DM, ammonia-N = 8% of total N, and pH = 3.7.

³Concentrate added to the CON diet contained (g/kg of DM): soybean meal = 400, soybean meal, formaldehyde treated = 200, rapeseed meal = 100, rapeseed meal, formaldehyde treated = 100, sugar beet pulp = 119, sugarcane molasses = 40, CaCO_3 = 15, NaCl = 8.0, NaHCO_3 = 2.0, trace mineral and vitamin mix = 8.0, MgO = 7.0, and Cr_2O_3 = 1.5.

⁴Concentrate added to the LSO diet contained (g/kg of DM): soybean meal = 369, soybean meal, formaldehyde treated = 184, rapeseed meal = 92, rapeseed meal, formaldehyde treated = 92, sugar beet pulp = 109, sugarcane molasses = 37, CaCO_3 = 15, NaCl = 8.0, NaHCO_3 = 2.0, trace mineral and vitamin mix = 8.0, MgO = 7.0, Cr_2O_3 = 1.5, and linseed oil (Linagro NV, Lichtervelde, Belgium) = 76.

⁵TMR contained grass silage, 300 g/kg of DM; corn silage, 400 g/kg of DM; CON concentrate, 300 g/kg of DM.

⁶TMR contained grass silage, 300 g/kg of DM; corn silage, 400 g/kg of DM; LSO concentrate, 300 g/kg of DM.

⁷Not determined.

individually transported to 1 of 4 identical climate respiration chambers (**CRC**), located approximately 200 m from the tiestalls, for a 5-d period to determine gaseous exchange, energy and N balance, and apparent total-tract nutrient digestibility. A detailed description of the CRC design and gas measurements is reported by Heetkamp et al. (2015) and van Gastelen et al. (2015). Briefly, in each CRC (i.e., an area of 11.8 m² and a volume of 34.5 m³), relative humidity was maintained at 65% and temperature at 16°C. The CRC were equipped with thin walls with windows, to allow audio-visual contact to minimize the effect of social isolation on cow behavior and performance. Cows were exposed to 16 h of light per day (from 0530 to 2130 h) and housed in the CRC until d 17 (0900 h).

In addition to van Gastelen et al. (2015), a H₂ analyzer (type MGA 3000 multi gas analyzer, ADC Gas Analysis Ltd., Hoddesdon, UK) was installed in series with the O₂, CO₂, and CH₄ gas analyzers. The H₂ concentrations were measured using an electrochemical cell technique, which has a relatively slow response time compared with the nondispersive infrared method of the CO₂ and CH₄ gas analyzers and the paramagnetic method of the O₂ gas analyzer. Therefore, sampled air from the CRC was flushed through the gas analysis system for 180 s before the analyzer readout was logged. To have as many measurements as possible, inlet air was not sampled in every sequence, but once per hour. Therefore, inlet and exhaust air of each CRC was sampled with an average interval of 12.5 min (i.e., 4 times 12-min intervals for each CRC and 1 interval of 15 min for inlet air) instead of the 10-min interval reported by van Gastelen et al. (2015). Production of CO₂, H₂, and CH₄ and consumption of O₂ was calculated from the difference between inlet and exhaust gas volumes.

The ventilation rate within the CRC was 58 m³/h to ensure that the H₂ peak after feeding was within the detection limit of the H₂ analyzer (i.e., 0–100 ppm). Staff entered the CRC twice daily at 0600 and 1600 h for approximately 30 min for feeding and milking. van Gastelen et al. (2015) did not use the gas concentration data during these feeding and milking times. The H₂ concentration peak occurred directly after feeding when staff was still inside the CRC. Therefore, we calculated the daily CH₄ and H₂ production on 2 data sets: (1) without the gas concentration data during feeding/milking (as was done by van Gastelen et al. (2015), partially missing the H₂ concentration peak after feeding), and (2) with the gas concentration data during feeding/milking (to capture the H₂ concentration peak directly after feeding). Excluding the gas concentration measurements during feeding/milking would have underestimated daily H₂ production by 15.2 ± 6.89%. Daily productions of CH₄ were unaffected when exclud-

ing these gas measurements compared with including these gas measurements (data not shown). Thus, for the present study it was decided to not discard the gas measurements during feeding and milking.

Gas concentrations and ventilation rates were corrected for pressure, temperature, and relative humidity to arrive at standard temperature pressure dew point volumes of inlet and exhaust air. Once a day, calibration gasses were sampled for gas analysis instead of the inlet air, and the analyzed and actual values of these calibration gasses were used to correct the measured gas concentrations from the inlet air and exhaust air of all compartments. Before the present experiment started, CRC were checked by releasing known amounts of CO₂ in each compartment and comparing these values with the data from the gas analysis system to calculate the recovery. The recovered amounts of CO₂ were between 99 and 101%.

Sample Collection and Measurements

Samples of grass silage, corn silage, and both concentrates were obtained when fresh feed was prepared (i.e., twice weekly). These samples were subsequently pooled per treatment period, subsampled, and stored at –20°C pending analyses. During the 5-d period in the CRC, feed residues were collected twice daily (0600 and 1600 h), weighed, and stored at 4°C. At the end of the 5-d period in the CRC, daily orts were pooled per cow, mixed, subsampled, and stored at –20°C pending analyses.

Rumen fluid samples (~1 L) were collected 1 h before and 4 h after morning feeding on d 12 and 4 h after morning feeding on d 17 using the oral stomach tube (**OST**) technique, similar to Shen et al. (2012). Rumen fluid samples could not be collected 1 h before morning feeding on d 17, because the cows were still housed in the CRC and the gas measurements were priority. To collect rumen fluid with the OST method, a probe was inserted in the ventral cranial part of the rumen via the esophagus. The probe was 190 cm long and the head of the probe consisted of small holes allowing only rumen fluid (i.e., no fibrous content) to be collected. Rumen fluid was collected by using a 500-mL suction pump, which was attached to the probe. The first 500 mL of rumen fluid was discarded to limit saliva contamination of the rumen fluid samples. Rumen pH was measured immediately after sampling using a calibrated portable electronic pH meter (pH electrode HI99141, Hanna Instruments, IJsselstein, the Netherlands) and 2 rumen fluid subsamples of 600 µL each were acidified with an equal volume of ortho-phosphoric acid, and directly stored at –20°C to stop microbial fermentation pending VFA analysis. On d 17, 4 h after morning feeding,

100 mL of rumen fluid was sampled, directly stored on dry ice, and transferred to a -80°C freezer pending microbiota analysis.

Milk yield was recorded for each milking, both during the adaptation period in the tiestalls and the measurement period in the CRC. Milk from cows in the CRC was collected twice daily at 0600 and 1600 h. A milk sample (10 mL) of each milking event was collected in a tube containing sodium azide (5 μL) for preservation, stored no longer than 4 d at 4°C , and analyzed for fat, protein, lactose, and urea content. Milk composition was corrected for differences in milk yield between milking events on the same day, and the average milk composition on a daily basis was used for data analysis. An additional milk sample (5 g/kg of milk) was collected at each milking event, pooled per cow for the entire period in the CRC, and stored at -20°C pending milk energy and N analyses. For MFA composition, milk samples were collected according to van Gastelen et al. (2015), pooled per cow per period, and stored at -20°C until analyses.

Measurements of CH_4 , H_2 , and CO_2 production, and O_2 consumption were based on data recorded from d 14 (0800 h) through d 17 (0800 h), whereas energy and N balance, and apparent total-tract feed digestibility were based on manure (mixture of feces and urine) and feces collections from d 13 (1400 h) through d 17 (0900 h). Cows were weighed when entering and leaving of the CRC. The feces and urine produced during the 5-d period in the CRC were quantitatively collected, weighed, mixed, subsampled, and stored at -20°C pending analyses. In addition, samples of condensed water (i.e., collected from the heat exchanger) and 25% sulfuric acid solution wt/wt (i.e., through which the outflowing air was led to trap aerial ammonia) of each CRC were collected, because of N volatilization in the form of ammonia due to the mixing of feces and urine. During the 5 d in the CRC, rectal grab samples (~ 300 g) were collected twice daily at 0600 and 1600 h and stored at -20°C . Prior to analysis, the grab samples were thawed, pooled per cow and period, mixed, and subsampled.

Chemical Analyses

Samples of feed, feed residues, manure, and rectal grab samples were thawed at room temperature, oven-dried at 60°C , ground to pass a 1-mm screen using a Wiley mill (Peppink 100AN, Olst, the Netherlands), and analyzed by wet chemistry [i.e., ash, DM, N, starch, reducing sugars (i.e., all carbohydrates with reducing properties and soluble in 40% ethanol), NDF, ADF, and ADL] as described by Abrahamse et al. (2008). Bomb calorimetry (ISO 9831; International Organiza-

tion for Standardization, 1998) was used to determine gross energy (GE). Crude protein was calculated as $\text{N} \times 6.25$, where N was determined using the Kjeldahl method with CuSO_4 as catalyst (ISO 5983; International Organization for Standardization, 2005). The N concentrations in manure and of roughages were determined in fresh material according to Klop et al. (2016). The FAME of the feed samples were determined as described by Khan et al. (2009) using GC (Carlo Erba 8560 HRGC, Rodano, Italy) with a fused silica capillary column (100 m $\times$ 0.250 mm and 0.2 μm film thickness; SP-2560, Supelco, St. Louis, MO) and helium as the carrier gas. Crude fat content was analyzed according to the gravimetric method NEN-ISO 1735 (ISO 1735; International Organization for Standardization, 2004a) with modifications as described by Klop et al. (2017).

Grass silage, corn silage, and concentrates were analyzed for DM, ash, N, crude fat, starch (except for grass silage), sugars (except for corn silage), NDF, ADF, ADL, GE, and FAME. Feed residues were analyzed for DM, ash, and crude fat. Manure samples were analyzed for DM, N, and GE. The rectal grab samples were analyzed for DM, ash, N, crude fat, starch, NDF, and GE. Chromium oxide was determined in the concentrates and rectal grab samples using an atomic absorption spectrophotometer (AA240FS, Varian, Palo Alto, CA) after oxidation by wet destruction as described in detail by Pellikaan et al. (2013).

The concentration of individual VFA in the rumen fluid samples of d 12 (i.e., 1 h before and 4 h after morning feeding) was determined using GC (Fisons HRGC Mega 2, CE Instruments, Milan, Italy) with a split/splitless injector and helium as carrier gas as described by van Gastelen et al. (2015). Milk samples from individual milking events were analyzed for proximate composition (fat, protein, and lactose content) by mid-infrared spectroscopy (ISO 9622; International Organization for Standardization, 1999), and for MUN using the pH difference technique (ISO 14637; International Organization for Standardization, 2004b) at Qlip (Zutphen, the Netherlands). The MFA composition of the pooled milk samples was determined using GC (Thermo Electron Corporation, Waltham, MA) by Qlip with a split/splitless injector and H_2 as carrier gas as described by van Gastelen et al. (2015). The GE and N content of the pooled milk samples were analyzed as described above.

Microbiota Analysis

Rumen fluid samples taken 4 h after morning feeding on d 17 were analyzed for bacterial and archaeal concentrations and community composition, using quanti-

tative PCR (**qPCR**) and Illumina MiSeq sequencing of PCR-amplified 16S ribosomal RNA (**rRNA**) gene fragments. Total DNA was extracted from the rumen fluid samples using a protocol involving a combination of bead beating, stool transport and recovery buffer (Roche Diagnostics Nederland B.V., Almere, the Netherlands), and the Maxwell 16 Instrument (Promega, Leiden, the Netherlands) as described in detail by van Lingén et al. (2017). For absolute quantification of bacteria and archaea, SYBR green qPCR assays were performed with sample DNA extracts using an iCycler iQ real-time detection system (Bio-Rad Laboratories B.V., Veenendaal, the Netherlands). The qPCR procedure, primers, cycling conditions, and standards used are described by van Lingén et al. (2017). For combined bacterial and archaeal composition profiling, barcoded amplicons from the V4 region of 16S rRNA genes were generated from sample DNA extracts using a 2-step PCR strategy (Caporaso et al., 2012; Tian et al., 2016; van Lingén et al., 2017). As described by Ramiro-García et al. (2016), the 16S rRNA gene sequencing data were analyzed using NG-Tax, an in-house bioinformatics pipeline. Operational taxonomic units (**OTU**) were defined using an open reference approach, and taxonomy was assigned to those OTU using a SILVA 16S rRNA gene reference database (Quast et al., 2013). Preliminary analysis of the samples confirmed the necessity of excluding 3 of the rumen fluid samples from further microbial data analysis (i.e., both sequence and qPCR-based analysis) due to issues associated with salivary contamination of the samples during collection.

Statistical Analysis

All parameters related to feed intake, milk production, and milk composition while cows were housed in the CRC were averaged per cow over a 4-d period. The parameters related to energy and N balance were expressed per kilogram of metabolic BW ($BW^{0.75}$) per day. All univariate data were subjected to ANOVA in a crossover with a 2 period $\times$ 2 treatment design using the MIXED procedure in SAS (edition 9.2, SAS Institute Inc., Cary, NC). Treatments (i.e., diet and *DGAT1*), their interaction, diet sequence, and period were considered fixed effects. The model included block as random factor, and cow within diet $\times$ *DGAT1* was considered as subject. For all analyses, the fixed effect of CRC was initially included in the model, but was removed because it was found to be not significant. The fixed effect of diet sequence was found to be significant twice (i.e., MUN and C22:0) and a tendency was found once (i.e., C22:4n-6), but diet sequence was always kept in the model. The covariance structure compound symmetry provided best fit with the lowest

overall Akaike's information criterion values. Ruminant VFA data were subjected to repeated measures ANOVA to take repeated sampling within the same cow per treatment period into account. Similar to the above described model, this model included treatments, their interactions, sequence, and period as fixed effects, and block as random effect. Again, the fixed effect of diet sequence was found to be significant once (i.e., total VFA concentration) and a tendency was found twice (i.e., molar proportions of acetate and butyrate), but diet sequence was always kept in the model. For both models, pairwise comparisons of means were tested with the Tukey-Kramer method. The Kenward-Roger option was used to estimate the denominator degrees of freedom. All results are reported as least squares means with significance of effects declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$. No multiple testing correction was applied.

Permutational multivariate analysis of variance (**PERMANOVA**; Anderson, 2001) was used to assess the significance of changes in the rumen bacterial and archaeal composition in terms of the microbiota as a whole community (with the OTU summarized to the genus level) with respect to different factors [i.e., diet (LSO and CON), *DGAT1* (*DGAT1* KK and AA genotype), and diet $\times$ *DGAT1*]. The PERMANOVA was applied on the Bray-Curtis distance matrices, and Bonferroni correction for multiple testing was applied on a nominal significance of 0.05. The Matlab Fathom toolbox (Jones, 2015) was used for calculations. The aforementioned ANOVA analysis was also used to determine the effect of diet, *DGAT1*, and diet $\times$ *DGAT1* interaction on the relative abundance of individual bacterial and archaeal genera that were (1) consistent in all animals and (2) were $>0.05\%$ in terms of relative abundance.

RESULTS AND DISCUSSION

Intake and Digestibility of Nutrients

Nutrient intake, DMI, fatty acid (**FA**) intake, and apparent total-tract digestibility of nutrients were not affected by *DGAT1* polymorphism and diet $\times$ *DGAT1* interaction. Feeding the LSO diet resulted in an increased crude fat intake ($P < 0.001$), intake of individual FA ($P < 0.001$), and intake of total FA ($P < 0.001$) compared with the CON diet (Table 2). These results are in line with the difference in chemical composition between the LSO diet and CON diet; linseed oil increased the dietary fat content from 34 to 56 g/kg of DM (Table 1). The LSO diet (2.3% linseed oil on DM basis) increased apparent total-tract digestibility of crude fat compared with the CON diet ($P < 0.001$),

Table 2. The effect of *DGAT1* K232A polymorphism (AA or KK) on intake and apparent total-tract digestibility of nutrients of lactating dairy cows fed diets with (LSO) or without (CON) linseed oil

Item	Diet		<i>DGAT1</i> genotype			<i>P</i> -value		
	CON	LSO	AA	KK	SEM	Diet	<i>DGAT1</i>	Diet × <i>DGAT1</i>
Nutrient intake (kg/d, unless otherwise stated)								
DM	18.0	17.5	17.8	17.6	0.69	0.649	0.825	0.935
OM	16.5	16.2	16.5	16.3	0.63	0.686	0.825	0.934
CP	3.48	3.22	3.37	3.33	0.130	0.165	0.927	0.939
Crude fat	0.60	0.99	0.80	0.79	0.032	<0.001	0.817	0.892
Gross energy (MJ/d)	327.5	328.2	329.8	325.8	12.73	0.971	0.825	0.934
NDF	6.41	6.12	6.30	6.22	0.243	0.404	0.826	0.936
ADF	3.50	3.36	3.45	3.41	0.133	0.450	0.826	0.936
ADL	0.24	0.23	0.24	0.23	0.009	0.379	0.823	0.936
Starch	2.77	2.68	2.75	2.71	0.106	0.557	0.825	0.936
Reducing sugars	1.21	1.11	1.17	1.15	0.045	0.134	0.824	0.939
Fatty acid intake (g/d)								
C16:0	46.7	70.6	59.0	58.3	2.34	<0.001	0.816	0.899
C18:0	7.1	19.7	13.5	13.3	0.59	<0.001	0.816	0.866
C18:1 <i>cis</i> -9	45.2	118.3	82.3	81.2	3.53	<0.001	0.815	0.869
C18:2n-6	108.7	173.7	142.1	140.3	5.68	<0.001	0.816	0.896
C18:3n-3	57.9	236.9	148.5	146.3	6.82	<0.001	0.819	0.854
Total fatty acids	280.4	651.6	469.3	462.7	18.92	<0.001	0.815	0.873
Apparent digestibility (% of intake)								
DM	78.8	79.2	79.4	78.6	0.54	0.626	0.306	0.786
OM	80.3	80.8	80.9	80.2	0.49	0.553	0.344	0.635
CP	77.0	77.0	77.3	76.7	0.58	0.999	0.422	0.687
Crude fat	68.8	77.1	73.8	72.1	0.93	<0.001	0.203	0.975
Gross energy	78.3	79.0	79.0	78.2	0.52	0.373	0.267	0.576
NDF	70.8	71.0	71.6	70.2	0.82	0.858	0.215	0.498
Starch	99.0	99.0	99.0	99.0	0.04	0.999	0.999	0.165

but the apparent total-tract digestibility of the other nutrients was unaffected (Table 2). The increased crude fat digestibility of the LSO diet may have been the result of UFA from linseed having a higher total-tract digestibility than SFA (van Zijderveld et al., 2011b). Several studies also reported the effect of linseed oil on apparent total-tract digestibility of nutrients, but the results reported are variable. Similar to this study, Benchaar et al. (2012) did not find an effect of adding increasing amounts of linseed oil to the diet [i.e., grass silage and corn silage; 50:50 forage-to-concentrate ratio (F:C); DM basis] on apparent total-tract digestibility of DM, OM, CP, NDF, starch, and GE. Martin et al. (2008), however, observed decreased DM, OM, and NDF digestibility when supplementing 5.7% linseed oil to a forage-rich diet (i.e., corn silage and grass hay; 65:35 F:C; DM basis). Ueda et al. (2003) reported an increase in OM and NDF digestibility when 3% linseed oil was added to a forage rich diet (i.e., grass hay; 65:35 F:C; DM basis), whereas the digestibility of these nutrients decreased when 3% linseed oil was added to a concentrate rich diet (35:65 F:C; DM basis). Benchaar et al. (2015) observed a decreased DM, OM, NDF, and GE digestibility when 4% linseed oil was added to a corn-silage-based diet (60:40 F:C; DM basis), whereas DM, OM, and GE digestibility increased and NDF digestibility was unaffected when linseed oil was

supplemented to a red-clover-silage-based diet (60:40 F:C; DM basis). Taken together, these results suggest that the effect of linseed oil on nutrient digestibility may vary with the source of forage in the basal diet, the forage to concentrate ratio, as well as the amount of linseed oil added.

Lactation Performance and Milk Fatty Acid Profile

No diet × *DGAT1* interaction effect on milk production and milk composition was observed. Compared with the *DGAT1* AA genotype, the *DGAT1* KK genotype was associated with a higher milk fat and protein content ($P < 0.001$ and $P = 0.005$, respectively; Table 3), and tended to have a lower milk yield and lactose yield. The FPCM yield did not differ between the *DGAT1* KK and *DGAT1* AA genotype, which is consistent with a similar DMI, nutrient intake, and gross energy intake (GEI) between the 2 *DGAT1* genotypes. The major effect of *DGAT1* on milk production traits has been often observed, with the K allele associated with a higher milk fat and protein content, but lower milk production than the A allele (e.g., Schennink et al., 2007; Bovenhuis et al., 2015), which is in line with the present study. Many studies also reported the K allele to be associated with a higher fat yield and lower protein yield, although the reduced protein yield is not

Table 3. The effect of *DGAT1* K232A polymorphism (AA or KK) on milk production and milk composition of lactating dairy cows fed diets with (LSO) or without (CON) linseed oil

Item	Diet		<i>DGAT1</i> genotype			<i>P</i> -value		
	CON	LSO	AA	KK	SEM	Diet	<i>DGAT1</i>	Diet × <i>DGAT1</i>
Milk production (kg/d)	24.1	26.3	26.7	23.7	1.15	0.202	0.078	0.686
FPCM ¹ (kg/d)	26.8	27.3	27.2	26.9	1.08	0.726	0.816	0.628
Milk fat content (%)	4.93	4.44	4.22	5.16	0.102	0.002	<0.001	0.803
Milk protein content (%)	3.66	3.48	3.44	3.70	0.063	0.045	0.005	0.529
Milk lactose content (%)	4.64	4.68	4.68	4.63	0.033	0.384	0.347	0.991
Fat yield (g/d)	1,156	1,126	1,102	1,180	45.0	0.643	0.230	0.583
Protein yield (g/d)	882	892	900	874	35.4	0.837	0.601	0.702
Lactose yield (g/d)	1,121	1,229	1,251	1,099	55.9	0.171	0.058	0.728
MUN (mg/dL)	13.3	7.8	10.9	10.2	0.39	<0.001	0.195	0.652

¹Fat- and protein-corrected milk = $(0.337 + 0.116 \times \text{fat \%} + 0.06 \times \text{protein \%}) \times \text{milk yield (kg/d)}$.

consistently reported (e.g., Näslund et al., 2008). This is not confirmed by the results of the present study (Table 3). In the present study, compared with the *DGAT1* AA genotype, fat yield of the *DGAT1* KK genotype was only numerically higher (1,180 vs. 1,102 g/d) and protein yield numerically lower (874 vs. 900 g/d).

The LSO diet resulted in a decreased milk fat content, milk protein content, and MUN ($P < 0.045$; Table 3). The decrease in milk protein content was also observed by Benchaar et al. (2012, 2015) and may be the result of a dilution effect rather than a direct negative effect of the increased dietary fat content (Schroeder et al., 2004), as the milk yield numerically increased and protein yield was unaffected by the linseed oil. The decreased MUN content may have been a consequence of the lower CP content of the LSO diet. In general, a decreased MUN content is associated with an improved milk N efficiency (Spek et al., 2013), and the significantly higher milk N efficiency with the LSO diet (27.5%) compared with the CON diet (24.6%; discussed in the Energy and Nitrogen Retention section) is in line with the decrease in MUN. The decrease in milk fat content in the present study is in agreement with Martin et al. (2008), Ferlay et al. (2013), and Benchaar et al. (2015), but in contrast to others (e.g., Benchaar et al., 2012; Livingstone et al., 2015). The response of milk fat content to linseed oil is the result of the balance between a decrease in de novo FA synthesis and an increase in exogenous FA uptake and secretion by the mammary gland (Schroeder et al., 2004). The UFA from linseed oil have been reported to inhibit ruminal fibrolytic activity and subsequently decrease production of acetate and butyrate, which are precursors of de novo synthesized short- and medium-chain MFA (Bauman and Griinari, 2003). Also, these dietary UFA and several *trans* FA, the latter formed from ruminal biohydrogenation of the UFA, are potent inhibitors of de novo milk fat synthesis in the mammary gland (Bauman et al., 2011). In turn, dietary UFA increase the content of long-chain UFA in

milk (Schroeder et al., 2004; Benchaar et al., 2012). In the present study, the inhibitory effects of linseed oil on de novo synthesized FA is greater than the increase in long-chain FA in milk (Table 4).

A diet × *DGAT1* interaction for the individual MFA C14:0 and C15:0 was observed (Table 4). Previously, Van Vuuren et al. (2013) also observed an interaction between *DGAT1* and linseed oil supplementation for certain MFA, although these were different from the ones reported in the present study, namely C18:0 and C20:1n-11. In addition, compared with the *DGAT1* AA genotype, the *DGAT1* KK genotype was associated with a higher C14:1 *cis*-9, C16:0, C16:1 *cis*-9, C22:0, C24:0, and total SFA content ($P < 0.034$), and a lower *iso* C17:0, C18:1 *cis*-9, C18:1 *cis*-12, C18:2n-6, C18:3n-3, and total PUFA content ($P < 0.030$) in the milk (Table 4). These results are largely in agreement with data in the literature. According to Schennink et al. (2007), Van Arendonk et al. (2009), Duchemin et al. (2013), and Bovenhuis et al. (2016), the *DGAT1* K allele is associated with a larger fraction of C16:0, and smaller fractions of C14:0 (also reported by Lu et al., 2015), and C18 UFA in milk. In addition, Duchemin et al. (2013) reported an increase in milk C14:1 *cis*-9, C16:1 *cis*-9, and total SFA, and a decrease in total milk UFA for the *DGAT1* K allele. Furthermore, according to Van Arendonk et al. (2009), Lu et al. (2015), and Bovenhuis et al. (2016), the *DGAT1* K allele is associated with increased contents of C15:0 and C17:0 in milk.

The LSO diet of the present study resulted in a lower content of short- and medium-chain fatty acids (SMCFA) in the milk, with the exceptions of C4:0 and C16:1 *trans*-9 (Table 4). These results are generally in line with Chilliard et al. (2009), Benchaar et al. (2012), and Saliba et al. (2014). As previously stated, dietary UFA and several *trans* FA are potent inhibitors of de novo milk fat synthesis in the mammary gland, and the UFA from the linseed oil potentially inhibit ruminal fibrolytic activity, thereby decreasing the precursors for

Table 4. The effect of *DGAT1* K232A polymorphism (AA or KK) on milk fatty acid (FA) composition of lactating dairy cows fed diets with (LSO) or without (CON) linseed oil

FA (g/100 g of FA)	Diet		<i>DGAT1</i> genotype			<i>P</i> -value		
	Control	Linseed	AA	KK	SEM	Diet	<i>DGAT1</i>	Diet × <i>DGAT1</i>
C4:0	3.40	3.58	3.52	3.46	0.067	0.068	0.505	0.282
C6:0	2.29	2.00	2.12	2.17	0.039	<0.001	0.451	0.198
C8:0	1.32	1.03	1.17	1.18	0.029	<0.001	0.732	0.280
C10:0	3.15	2.15	2.67	2.63	0.086	<0.001	0.738	0.258
C12:0	3.69	2.39	3.06	3.01	0.106	<0.001	0.718	0.440
<i>iso</i> C14:0	0.09	0.07	0.08	0.08	0.003	<0.001	0.854	0.580
C14:0 ¹	11.8	9.3	10.8	10.3	0.17	<0.001	0.026	0.022
C14:1 <i>cis</i> -9	1.03	0.76	0.83	0.96	0.032	<0.001	0.008	0.900
<i>iso</i> C15:0	0.26	0.20	0.23	0.23	0.006	<0.001	0.964	0.893
<i>anteiso</i> C15:0	0.44	0.37	0.41	0.40	0.009	<0.001	0.804	0.901
C15:0 ²	1.06	0.81	0.87	1.01	0.024	<0.001	<0.001	0.037
<i>iso</i> C16:0	0.22	0.18	0.20	0.19	0.007	<0.001	0.543	0.655
C16:0	32.4	22.4	26.0	28.9	0.43	<0.001	<0.001	0.852
C16:1 <i>trans</i> -9	0.18	0.17	0.18	0.17	0.005	0.448	0.448	0.861
C16:1 <i>cis</i> -9	1.59	1.11	1.20	1.50	0.043	<0.001	<0.001	0.409
<i>iso</i> C17:0	0.37	0.34	0.37	0.35	0.006	0.004	0.027	0.635
<i>anteiso</i> C17:0	0.42	0.35	0.39	0.38	0.008	<0.001	0.329	0.689
C17:0	0.61	0.50	0.56	0.55	0.007	<0.001	0.873	0.268
C17:1 <i>cis</i> -9	0.24	0.19	0.21	0.22	0.010	<0.001	0.471	0.840
C18:0	9.0	13.7	11.5	11.2	0.20	<0.001	0.341	0.607
C18:1 <i>trans</i> -6	0.19	0.51	0.37	0.33	0.012	<0.001	0.075	0.483
C18:1 <i>trans</i> -9	0.15	0.30	0.23	0.22	0.007	<0.001	0.194	0.726
C18:1 <i>trans</i> -10	0.19	0.49	0.33	0.35	0.032	<0.001	0.623	0.572
C18:1 <i>trans</i> -11	0.88	2.60	1.77	1.71	0.104	<0.001	0.654	0.754
C18:1 <i>trans</i> -15 + C18:1 <i>cis</i> -11	0.74	1.25	1.02	0.97	0.021	<0.001	0.147	0.794
C18:1 <i>cis</i> -9 ³	18.2	23.7	21.9	20.0	0.54	<0.001	0.016	0.492
C18:1 <i>cis</i> -12 ⁴	0.21	0.57	0.41	0.37	0.012	<0.001	0.012	0.098
C18:1 <i>cis</i> -13	0.10	0.20	0.15	0.15	0.005	<0.001	0.484	0.567
C18:2 <i>cis</i> -9, <i>trans</i> -11	0.42	1.01	0.76	0.68	0.046	<0.001	0.238	0.399
C18:2n-6	1.71	1.67	1.79	1.60	0.053	0.597	0.015	0.620
C18:3n-3	0.47	0.75	0.65	0.57	0.023	<0.001	0.017	0.478
C18:3n-6	0.07	0.06	0.06	0.06	0.002	<0.001	0.067	0.397
C20:0	0.13	0.14	0.14	0.14	0.002	0.002	0.634	0.158
C20:1 <i>cis</i> -11	0.04	0.10	0.07	0.07	0.008	<0.001	0.969	0.419
C20:2n-6	0.04	0.02	0.03	0.03	0.002	<0.001	0.625	0.807
C20:3n-6	0.09	0.05	0.06	0.07	0.003	<0.001	0.103	0.535
C20:4n-3	0.01	0.04	0.02	0.02	0.003	<0.001	0.499	0.821
C20:4n-6	0.11	0.07	0.09	0.09	0.003	<0.001	0.203	0.394
C20:5n-3	0.05	0.05	0.05	0.05	0.001	0.179	0.651	0.651
C22:0	0.05	0.04	0.04	0.05	0.002	<0.001	0.016	0.661
C22:5n-3	0.09	0.07	0.08	0.08	0.003	<0.001	0.483	0.196
C24:0	0.03	0.01	0.01	0.03	0.003	<0.001	0.019	0.576
OBCFA ⁵	3.46	2.83	3.09	3.19	0.056	<0.001	0.090	0.090
SFA ⁶	70.8	59.6	64.1	66.2	0.68	<0.001	0.034	0.353
MUFA ⁷	23.0	30.7	27.6	26.0	0.59	<0.001	0.062	0.451
PUFA ⁸	3.02	3.76	3.56	3.22	0.107	<0.001	0.030	0.844
n-6 to n-3 ratio ⁹	3.20	2.06	2.63	2.63	0.039	<0.001	0.903	0.467

¹Diet × *DGAT1* interaction; CON AA = 12.35, LSO AA = 9.33, CON KK = 11.24, LSO KK = 9.34.²Diet × *DGAT1* interaction; CON AA = 0.96, LSO AA = 0.78, CON KK = 1.17, LSO KK = 0.84.³C18:1 *cis*-9 represents the sum of C18:1 *cis*-9 and C18:1 *trans*-12, as these 2 FA could not be separated in the analysis. The portion of C18:1 *trans*-12 is considered to be negligible, as this FA is always present in small amounts.⁴Diet × *DGAT1* interaction; CON AA = 0.22, LSO AA = 0.60, CON KK = 0.20, LSO KK = 0.53.⁵Sum of all odd- and branched-chain fatty acids (i.e., *iso* C14:0, *iso* C15:0, *anteiso* C15:0, C15:0, *iso* C16:0, *iso* C17:0, *anteiso* C17:0, and C17:0). Diet × *DGAT1* interaction; CON AA = 3.36, LSO AA = 2.83, CON KK = 3.56, LSO KK = 2.83.⁶Sum of all SFA.⁷Sum of all MUFA.⁸Sum of all PUFA.⁹Ratio between the sum of all n-6 FA (i.e., C18:2n-6, C18:3n-6, C20:3n-6, and C20:4n-6) and the sum of all n-3 FA (i.e., C18:3n-3, C20:4n-3, C20:5n-3, and C22:5n-3) reported in this table.

SMCFA synthesis (Bauman and Griinari, 2003). Milk SMCFA are synthesized de novo in the mammary gland primarily from acetate, and ruminal acetate proportion was decreased upon feeding linseed oil (discussed in the Ruminal Fermentation section). Only MFA C4:0 does not require acetate for its production as it can be produced directly from β -hydroxybutyrate derived from the blood (Bernard et al., 2008).

The LSO diet resulted in lower contents of all odd- and branched-chain FA in milk compared the CON diet ($P < 0.004$; Table 4). This is in line with Chilliard et al. (2009) and, in regard to C15:0 and C17:0, also with Benchaar et al. (2012). The odd-chain FA C15:0 and C17:0 are mainly synthesized de novo by ruminal bacteria from propionate. However, despite the increase in propionate proportions (Table 7), this ruminal synthesis of C15:0 and C17:0 may decrease when cows are fed dietary fat. This is because rumen bacteria preferably use preformed FA available in the ruminal environment (Byers and Schelling, 1988).

The intake of C18:3n-3 increased with the LSO diet ($P < 0.001$; Table 2). This increase is associated with an increase of C18:3n-3, as well as the biohydrogenation intermediates (e.g., C18:1 *trans*-11) and end products (i.e., C18:0) in milk upon feeding linseed oil ($P < 0.001$; Table 4), suggesting high levels of biohydrogenation, and is in line with Benchaar et al. (2012), Ferlay et al. (2013), and Livingstone et al. (2015). Despite the increased intake of C18:2n-6 with the LSO diet ($P < 0.001$; Table 2), C18:2n-6 does not increase in milk upon feeding linseed oil, which is in agreement with Ferlay et al. (2013) and Saliba et al. (2014). Milk C18:2 *cis*-9,*trans*-11 did increase with the LSO diet ($P < 0.001$; Table 4), suggesting high levels of biohydrogenation of C18:2n-6 in the rumen as well as increased endogenous production of C18:2 *cis*-9,*trans*-11 in the mammary gland using C18:1 *trans*-11 produced in the rumen as substrate (Griinari et al., 2000).

In the present study, both C18:1 *trans*-10 and C18:1 *trans*-11 increased with the LSO diet ($P < 0.001$; Table 4), which is consistent with Benchaar et al. (2012) and Saliba et al. (2014). The increase in C18:1 *trans*-11 content in milk may be the result of its production during the biohydrogenation of dietary C18:2n-6 and C18:3n-3. An increase in C18:1 *trans*-10 in milk is generally associated with milk fat depression, and occurs when low fiber diets or diets supplemented with PUFA-rich plant oils are fed, resulting in a shift in rumen microbial composition and a changed biohydrogenation pathway (Griinari and Bauman, 1999). In the present study, milk fat content (%) decreased, but intake of NDF and starch, ruminal pH, and milk fat yield (g/d) were unaffected with the LSO diet (Tables 2, 3, and 7). The increase of C18:1 *trans*-11 was greater compared

with C18:1 *trans*-10 as the C18:1 *trans*-11 to C18:1 *trans*-10 ratio increased with the LSO diet (i.e., 4.6 ± 0.19 for the CON diet and 6.0 ± 0.46 for the LSO diet; $P = 0.009$). This indicates a change in the rumen biohydrogenation pathway, potentially the result of the increased rumen UFA load (Lock, 2010), causing changes in microbiota composition or activity, and thus changes in ruminal fermentation characteristics.

Energy and Nitrogen Retention

No diet $\times$ *DGAT1* interaction was observed for the energy balance characteristics of the cows (Table 5). The ME intake (MEI) to GEI ratio was lower for the *DGAT1* KK genotype (66.8%) compared with the *DGAT1* AA genotype (67.9%; $P = 0.023$). To the best of our knowledge, the effects of *DGAT1* on energy balance measured in CRC have not been quantified previously. The energy output in milk was unaffected by *DGAT1* in the present study, which is in agreement with Bovenhuis et al. (2015) who only observed a difference in milk energy output for the *DGAT1* AK genotype compared with *DGAT1* AA and KK genotypes in parity 1. Bovenhuis et al. (2015) suggested that, in view of the absence of differences in energy output in milk, no large differences in energy balance between cows with different *DGAT1* alleles would be expected. In the present study, total energy retention was unaffected by *DGAT1* (Table 5), supporting the hypothesis of Bovenhuis et al. (2015). This is in agreement with Banos et al. (2008), who reported only a small positive effect of the *DGAT1* K allele on cumulative effective energy balance estimated based on live weight and BCS.

The LSO diet resulted in a decreased energy loss in the form of CH₄ ($P = 0.022$; Table 5) and an increased MEI to GEI ratio ($P = 0.006$; Table 5). The decreasing effect of linseed oil on energy loss in the form of CH₄, together with the tendency of reduced GE digestibility, may explain the difference in MEI to GEI ratio, because the GEI was unaffected by feeding linseed oil (Table 5).

The mean N balance was not affected by diet, *DGAT1*, and diet $\times$ *DGAT1* interaction (Table 5). Milk N efficiency tended ($P = 0.076$) to be higher for the *DGAT1* AA genotype (26.6%) compared with the *DGAT1* KK genotype (25.5%). A few studies have reported the effect of dietary linseed oil on N balance and were reviewed by Hristov and Jouany (2005). These authors indicated that the effects of fat supplementation on the N balance of cattle are inconsistent in the literature. In the present study, the LSO diet reduced N excretion in manure ($P = 0.010$; Table 5), which is similar to Benchaar et al. (2015) and might be the result of the tendency observed for a lower N intake of cows fed the LSO diet. The LSO diet resulted in a greater efficiency

Table 5. The effect of *DGAT1* K232A polymorphism (AA or KK) on energy and nitrogen balance of lactating dairy cows fed diets with (LSO) or without (CON) linseed oil

Item	Diet		<i>DGAT1</i> genotype			<i>P</i> -value		
	CON	LSO	AA	KK	SEM	Diet	<i>DGAT1</i>	Diet × <i>DGAT1</i>
Metabolic BW ¹ (kg ^{0.75})	123	123	123	122	2.0	0.951	0.736	0.632
Energy balance (kJ/kg of BW ^{0.75} per day, unless otherwise stated)	2,662	2,665	2,667	2,660	78.0	0.984	0.950	0.964
GEI ²	185	169	178	176	4.7	0.022	0.697	0.591
CH ₄ production	704	684	680	708	23.4	0.549	0.406	0.681
Energy in manure	2,083	2,103	2,108	2,079	61.7	0.824	0.744	0.730
DEI ³	1,773	1,811	1,809	1,776	52.7	0.614	0.668	0.981
MEI ⁴	66.7	68.0	67.9	66.8	0.32	0.006	0.023	0.346
MEI to GEI ratio (%)	982	968	974	976	19.6	0.611	0.939	0.795
Heat production ⁵	678	711	701	688	23.0	0.309	0.688	0.475
Energy in milk	114	132	134	112	29.5	0.657	0.615	0.493
ER total ⁶	96	88	95	89	6.9	0.404	0.518	0.418
ER protein ⁷	17	44	38	23	24.2	0.437	0.667	0.544
ER fat ⁸								
N balance (mg/kg of BW ^{0.75} per day)								
N intake	4,556	4,232	4,409	4,380	134.0	0.095	0.878	0.974
N manure	2,663	2,360	2,477	2,545	79.4	0.010	0.548	0.960
N milk	1,120	1,160	1,168	1,111	37.1	0.455	0.282	0.471
N condense + N acid	121	116	117	120	7.8	0.684	0.817	0.152
N balance	652	597	646	603	46.6	0.404	0.518	0.418
N efficiency ⁹	24.6	27.5	26.6	25.5	0.44	<0.001	0.076	0.262

¹The mean BW per cow per balance period was used to calculate metabolic BW (BW^{0.75}).²GEI = gross energy intake.³Digestible energy intake (DEI) = GEI × apparent total-tract digestibility of GE (% of intake).⁴ME intake (MEI) = GEI – methane production – energy in manure (feces and urine).⁵Heat production (kJ/d) = 16.175 × VO₂ (L/d) + 5.021 × VCO₂ (L/d), where VO₂ = volumes of O₂ consumed, and VCO₂ = volumes of CO₂ produced (Gerrits et al., 2015).⁶Energy retention total = MEI – heat production – energy in milk.⁷Energy retention protein = protein gain (N × 6.25) × 23.6 kJ/g (energetic value of protein).⁸Energy retention fat = energy retention total – energy retention protein.⁹N efficiency = N milk/N feed (%).

Table 6. The effect of *DGAT1* K232A polymorphism (AA or KK) on gaseous consumption and production of lactating dairy cows fed diets with (LSO) or without (CON) linseed oil

Item	Diet		<i>DGAT1</i> genotype		SEM	<i>P</i> -value		
	CON	LSO	AA	KK		Diet	<i>DGAT1</i>	Diet × <i>DGAT1</i>
O ₂ consumption (g/d)	7,987	7,926	7,996	7,917	241.3	0.859	0.819	0.922
CO ₂ production (g/d)	12,351	12,001	12,184	12,168	379.2	0.517	0.975	0.997
Respiratory quotient	1.12	1.10	1.10	1.11	0.005	<0.001	0.178	0.580
H ₂								
g/d	1.79	1.65	1.71	1.73	0.097	0.310	0.906	0.196
g/kg of DMI	0.103	0.096	0.098	0.101	0.0063	0.455	0.748	0.286
g/kg of FPCM ¹	0.069	0.061	0.064	0.066	0.0041	0.211	0.718	0.460
CH ₄								
g/d	409	375	396	388	13.7	0.085	0.658	0.863
g/kg of DMI	22.9	21.5	22.3	22.1	0.32	0.006	0.688	0.572
g/kg of FPCM ¹	15.5	13.8	14.7	14.6	0.35	0.002	0.925	0.430
% of GEI ²	6.96	6.38	6.70	6.64	0.096	<0.001	0.680	0.552
CH ₄ to CO ₂ ratio ³	0.033	0.031	0.032	0.032	0.0004	0.001	0.248	0.552
H ₂ to CH ₄ ratio ⁴	0.0045	0.0045	0.0044	0.0046	0.00028	0.926	0.660	0.352

¹Fat- and protein-corrected milk = (0.337 + 0.116 × fat % + 0.06 × protein %) × milk yield (kg/d).

²Gross energy intake.

³Ratio between CH₄ (g/d) and CO₂ (g/d).

⁴Ratio between H₂ (g/d) and CH₄ (g/d).

of dietary N utilization for milk N production ($P < 0.001$). This is in contrast with Benchaar et al. (2015), who observed no effect of linseed oil supplementation on N efficiency.

Gas Exchange

No diet × *DGAT1* interaction was found for O₂ consumption and CO₂, CH₄, and H₂ production, and gaseous ratios (Table 6). None of the gas exchange characteristics were affected by *DGAT1*. The results indicate that *DGAT1* does not affect CH₄ emission of dairy cows nor the response in CH₄ emission of dairy cows to dietary linseed oil. As mentioned before, S. van Engelen (Wageningen University & Research; unpublished data) performed a GWAS to determine regions of the bovine genome that are associated with predicted CH₄ yield (g/kg of DMI) using the equations published by Dijkstra et al. (2011) and van Engelen et al. (2015). The association with *DGAT1* was significant in the GWAS for predicted CH₄ yield, suggesting that the K allele is associated with higher predicted CH₄ yield. The results of the present study suggest that the proposed relation between *DGAT1* and CH₄ based on predicted CH₄ yield using MFA is not in line with actual observations on CH₄ emission. Presumably, the relationship observed by S. van Engelen (Wageningen University & Research; unpublished data) is due to the association between *DGAT1* and the MFA that were used to predict CH₄ yield. The CH₄ prediction equations, used in the GWAS by S. van Engelen (Wageningen University & Research; unpublished data), included several C18 UFA (both *cis*

and *trans* isomers), which were affected by *DGAT1* in the present study (Table 4) as well as in other studies (e.g., Schennink et al., 2007; Duchemin et al., 2013).

The LSO diet did not affect H₂ production (g/d), yield (g/kg of DMI), and intensity (g/kg of FPCM), which is consistent with Veneman et al. (2015). Similarly, the unaffected O₂ consumption and CO₂ production upon the LSO diet in the present study is consistent with Livingstone et al. (2015). The LSO diet did not affect the H₂ to CH₄ ratio, but decreased the respiration quotient (**RQ**; $P < 0.001$), CH₄ emissions (i.e., g/kg of DMI, g/kg of FPCM, and % of GEI; $P < 0.006$), and CH₄ to CO₂ ratio ($P = 0.001$; Table 6). The RQ is lower or equal to 1.0 when only substrate oxidation occurs (Gerrits et al., 2015). However, de novo FA synthesis and ruminal anaerobic fermentation of dietary carbohydrates can result in a RQ larger than 1.0 (Gerrits et al., 2015). The decreased RQ found for the LSO diet may result from the higher dietary fat intake and fat digestibility (Table 2) because fat is not fermented in the rumen (Beauchemin et al., 2008) and an increased absorption of FA reduces the need to synthesize FA from carbohydrates in the intermediary metabolism.

The decrease in CH₄ emissions with the LSO diet in the present study is in line with previous studies (e.g., Martin et al., 2008; Benchaar et al., 2015). According to Grainger and Beauchemin (2011), a 10 g/kg of DM increase in dietary fat should result in a decreased CH₄ yield by 1 g/kg of DMI in cattle. In the present study, the average increase in dietary fat content (22 g/kg of DM) was associated with a significant decrease of 1.4 g/kg of DMI in CH₄ yield. This is lower than that

reported by Grainger and Beauchemin (2011) in their meta-analysis, but higher than some other studies (e.g., Veneman et al., 2015). Fat analyses of the feed residuals suggest that the cows in the present experiment were not selecting against the concentrate supplemented with linseed oil included in the TMR (results not shown; fat content in residual feed was not different from the fat content in TMR offered). Benchaar et al. (2015) suggested that the forage of the basal diets affected the extent of CH₄ mitigation of linseed oil supplementation. For the red-clover-based diet, an increase in dietary fat content of 27 g/kg of DM decreased CH₄ yield with 1.7 g/kg of DMI, whereas for the corn-silage-based diet, an increase in dietary fat content of 36 g/kg of DM decreased CH₄ yield with 4.0 g/kg of DMI (Benchaar et al., 2015). The forages used in the present experiment (i.e., a mixture of grass silage and corn silage) may have influenced the extent of CH₄ mitigation of dietary linseed oil. In addition, the 2 diets differed in CP content (194 and 184 g/kg of DM for CON and LSO, respectively; Table 1), but the effect of dietary CP on CH₄ emission is reported to be variable in the literature. Ellis et al. (2009) found a positive relationship between dietary CP content and CH₄ emission of beef cattle, whereas Reynolds et al. (2010) did not observe differences in CH₄ yield with different dietary CP contents. The 10 g/kg of DM difference in dietary CP content between the 2 diets in the present study may not be expected to significantly affect CH₄ emission, especially because the dietary CP content (i.e., higher than 180 g/kg of DM; Table 1) was above calculated requirements for the dairy cows involved. Overall, it seems likely that the basal diet may have played an important role in the CH₄ mitigation effect of dietary linseed oil in the present study.

Ruminal Fermentation

Three rumen fluid samples per cow within diet were collected: 1 h before and 4 h after morning feeding on d 12, and 4 h after morning feeding on d 17. None of the ruminal fermentation parameters significantly differed between d 12 and 17 at 4 h after morning feeding, with the exception of ruminal pH ($P < 0.001$, 6.56 and 6.68, respectively). To have a balanced design as well as the ability to distinguish between time and day effects, it was decided to use only the samples of 1 h before and 4 h after morning feeding of d 12 for ruminal fermentation data analysis.

Time of sampling (i.e., 1 h before feeding or 4 h after feeding) affected pH, total VFA concentration, and VFA molar proportions ($P < 0.001$; Table 7), except for isovalerate. As expected, ruminal pH declined after feeding and total VFA concentration was higher 4 h after feeding compared with 1 h before feeding (data not shown). Total VFA concentration was unaffected by diet, *DGAT1*, and diet $\times$ *DGAT1* interaction. The lack of an effect of the LSO diet is in agreement with Ueda et al. (2003), Doreau et al. (2009), and Benchaar et al. (2012), and all these results are consistent with the lack of effect of diet, *DGAT1*, and diet $\times$ *DGAT1* interaction on ruminal pH in the present study.

In general, ruminal pH in the present study seems relatively high compared with the total VFA concentration (Table 7). According to the prediction equation derived by Dijkstra et al. (2012), pH should be around 6.2 with a VFA concentration of 100 mM. In the present study, however, pH was around 6.76 with a VFA concentration of approximately 93 mM, probably as a result of collecting rumen fluid with the OST sampling technique. Duffield et al. (2004) and Wang et al. (2016)

Table 7. The effect of *DGAT1* K232A polymorphism (AA or KK) on ruminal pH, total VFA concentration, and VFA molar proportions of lactating dairy cows fed diets with (LSO) or without (CON) linseed oil¹

Item	Diet		<i>DGAT1</i> genotype		SEM	<i>P</i> -value			
	CON	LSO	AA	KK		Diet	<i>DGAT1</i>	Diet $\times$ <i>DGAT1</i>	Time ²
pH	6.76	6.76	6.75	6.77	0.028	0.929	0.575	0.147	<0.001
Total VFA (mM)	93	93	94	92	2.1	0.913	0.536	0.469	<0.001
VFA (% of total VFA)									
Acetate	67.4	66.6	67.1	66.9	0.18	0.004	0.425	0.502	<0.001
Propionate	16.5	17.2	16.7	17.0	0.18	0.010	0.226	0.654	<0.001
Butyrate	12.6	12.7	12.7	12.6	0.12	0.763	0.647	0.150	<0.001
Isobutyrate	0.88	0.89	0.89	0.88	0.026	0.771	0.444	0.788	<0.001
Valerate	1.19	1.20	1.20	1.19	0.016	0.432	0.559	0.746	<0.001
Isovalerate	1.43	1.47	1.45	1.46	0.056	0.633	0.946	0.570	0.367
Acetate to propionate ratio	4.16	3.93	4.08	4.01	0.051	<0.001	0.236	0.766	<0.001

¹Values shown represent the mean of values on d 12 (i.e., 1 h before and 4 h after morning feeding).

²Time = effect of time of sampling on d 12; 1 h before and 4 h after morning feeding.

reported a higher ruminal pH when sampling rumen fluid using OST compared with rumen cannulation. Using the OST technique in the present study resulted in collection of rumen fluid from the ventral cranial part of the rumen or the reticulum. The pH within these regions of the reticulo-rumen is generally higher compared with other sites (e.g., Duffield et al., 2004; Li et al., 2009) as a result of rumination and the consequent entry of saliva.

The proportions of the individual VFA were unaffected by *DGAT1* and diet $\times$ *DGAT1* interaction (Table 7). Feeding the LSO diet resulted in an increased proportion of propionate ($P = 0.010$), whereas the proportion of acetate and the acetate to propionate ratio decreased compared with the CON diet ($P = 0.004$ and $P < 0.001$, respectively). Several other studies have also reported a shift in VFA pattern toward proportionally more propionate and less acetate when a linseed oil containing diet was fed (e.g., Benchaar et al., 2012, 2015; Ivan et al., 2013; Martin et al., 2016), which is also consistent with the reduction of CH₄ emission observed for the LSO diet (Table 6). This shift in fermentation toward propionate at the expense of acetate supports the key role of the redox state of NAD in rumen fermentation and CH₄ production (van Lingen et al., 2017). As discussed before, dietary linseed oil may reduce fiber degradation in the rumen, whereas degradation of other carbohydrates (e.g., starch) remains unaffected (Doreau and Chilliard, 1997). This results in proportionally more propionate and less acetate. The apparent total-tract digestibility of NDF in the present study was unaffected by the LSO diet, but a decrease in rumen fiber digestion can be partially compensated for by digestion in the large intestine (Martin et al., 2008).

Rumen Microbiota

Bacterial and Archaeal Concentrations. The concentration of archaea and bacterial 16S rRNA genes in the rumen were unaffected by diet, *DGAT1*, and diet $\times$ *DGAT1* interaction (Table 8). An absence of effect of linseed oil on archaeal concentrations is in agreement with Veneman et al. (2015) and Martin et al. (2016). Similar to Martin et al. (2016), despite the unaffected archaeal concentration, CH₄ emission decreased with the LSO diet. The archaea to bacteria ratio decreased ($P = 0.029$, Table 8) with the LSO diet. This reduced ratio suggests that per unit substrate fermented by bacteria, a smaller archaeal concentration is present to form CH₄, helping to explain the observed reduction in CH₄ emission when feeding the LSO diet.

Numerous studies have repeatedly failed to find a correlation between CH₄ emission and archaeal concentration (e.g., Morgavi et al., 2010). In the present

study, archaeal concentration (log₁₀ 16S copies/mL of rumen fluid) was not related to CH₄ production, but was related to CH₄ yield ($r = 0.34$, $P = 0.019$), and tended to be related to CH₄ intensity ($r = 0.28$, $P = 0.055$) without considering effects of linseed oil and *DGAT1*. Additionally, the archaea to bacteria ratio was not related to CH₄ production, but was related to CH₄ yield ($r = 0.43$, $P = 0.002$) and CH₄ intensity ($r = 0.48$, $P = 0.001$) without considering effects of linseed oil and *DGAT1*. For both CH₄ yield and CH₄ intensity, the archaea to bacteria ratio provided the strongest correlation, which for CH₄ yield is in agreement with Wallace et al. (2014).

Bacterial and Archaeal Composition. Bacteria (91.8 $\pm$ 2.1% of the obtained 16S rRNA gene sequences) were represented by 1,077 different OTU, whereas the archaea (7.9 $\pm$ 2.0% of the 16S rRNA sequences) were represented by 16 different OTU. In agreement with previous studies, the total number of bacterial OTU was much higher than archaeal ones (e.g., Kittelman et al., 2013; Veneman et al., 2015). The 1,093 OTU could be summarized to 89 different genus-level phylogenetic groupings (87 for bacteria and 2 for archaea). The rumen bacterial and archaeal community as a whole (i.e., PERMANOVA results) tended to be affected by diet ($P = 0.081$), but was not affected by *DGAT1* ($P = 0.326$) and diet $\times$ *DGAT1* interaction ($P = 0.365$). We also individually analyzed the relative abundances of bacterial and archaeal genera to examine if these were affected by diet, *DGAT1*, and diet $\times$ *DGAT1* interaction (Table 8). This was done because changes in taxa of low relative abundance can be masked when the total rumen microbiota is analyzed as a whole.

Methanobrevibacter was the most abundant archaeal genus, which is in line with other studies (e.g., Janssen and Kirs, 2008; Veneman et al., 2015). Both of the detected archaeal genera in this study were unaffected by diet, *DGAT1*, and diet $\times$ *DGAT1* interaction, with the exception of *Methanobrevibacter* for which a tendency for a diet $\times$ *DGAT1* interaction was observed. The lack of an effect of *DGAT1* and of the interaction between *DGAT1* and dietary linseed oil on archaeal genera is in line with the lack of an effect of these factors on CH₄ emissions in the present study (Table 6). The lack of an effect of linseed oil on both archaeal genera is in agreement with Veneman et al. (2015) and consistent with the unaffected archaeal concentrations in the present study. These results are also in agreement with Mosoni et al. (2008), who demonstrated that the long-chain FA of dietary linseed oil (such as linolenic acid) do not affect archaeal concentrations and relative abundances.

Prevotella was the most abundant bacterial genus in the present study, which was reported by others as well (e.g., Henderson et al., 2013; Veneman et al., 2015).

Table 8. The effect of *DGAT1* K232A polymorphism (AA or KK) on total concentrations and genus level relative abundances of archaea and bacteria in the rumen of lactating dairy cows fed diets with (LSO) or without (CON) linseed oil¹

Microbiota	Diet		<i>DGAT1</i> genotype			<i>P</i> -value		
	CON	LSO	AA	KK	SEM	Diet	<i>DGAT1</i>	Diet × <i>DGAT1</i>
Quantitative PCR analysis								
Ruminal concentration (log ₁₀ 16S copies/ mL of rumen fluid)								
Archaea	8.73	8.69	8.70	8.73	0.030	0.271	0.503	0.947
Bacteria	10.22	10.25	10.21	10.26	0.029	0.545	0.187	0.938
Archaea to bacteria ratio	0.034	0.028	0.032	0.030	0.0017	0.029	0.474	0.936
Universal 16S rRNA gene sequencing								
Relative abundance archaea (%)								
<i>Methanobrevibacter</i> ²	7.7	7.3	7.5	7.5	0.40	0.537	0.965	0.095
<i>Methanosphaera</i>	0.40	0.40	0.42	0.39	0.025	0.919	0.466	0.290
Relative abundance bacteria (%)								
<i>Acetivibrio</i>	2.0	1.8	2.1	1.7	0.15	0.450	0.069	0.596
<i>Bifidobacterium</i> ³	0.28	0.39	0.47	0.19	0.088	0.366	0.039	0.068
<i>Blautia</i>	0.13	0.22	0.16	0.19	0.047	0.206	0.778	0.257
<i>BS11</i> gut group, g-NA ⁴	0.99	0.78	1.05	0.71	0.164	0.419	0.164	0.898
<i>Butyrivibrio</i> ⁵	5.1	4.8	5.1	4.7	0.25	0.449	0.381	0.089
<i>Christensenellaceae</i> , g-NA	4.1	4.1	4.4	3.8	0.39	0.846	0.259	0.744
<i>Desulfovibrio</i>	0.09	0.09	0.09	0.08	0.016	0.901	0.551	0.850
<i>Erysipelotrichaceae</i> , incertae sedis	0.12	0.28	0.21	0.19	0.089	0.209	0.833	0.928
<i>Erysipelotrichaceae</i> , g-NA	0.38	0.24	0.19	0.44	0.120	0.376	0.151	0.986
<i>Fibrobacter</i>	0.65	0.95	0.92	0.69	0.153	0.150	0.254	0.419
<i>Lachnospiraceae</i> , incertae sedis	2.8	2.9	2.7	3.0	0.19	0.638	0.194	0.781
<i>Lachnospiraceae</i> , g-NA	1.7	1.8	1.6	1.9	0.22	0.679	0.236	0.727
<i>Leuconostoc</i>	0.11	0.09	0.08	0.12	0.025	0.472	0.209	0.484
<i>Mogibacterium</i>	1.6	1.5	1.5	1.6	0.11	0.784	0.264	0.381
<i>Moryella</i>	0.09	0.11	0.11	0.09	0.028	0.611	0.657	0.969
<i>Prevotella</i>	28.4	29.3	28.8	28.9	1.04	0.503	0.975	0.811
<i>Prevotellaceae</i> , g-NA	3.7	4.3	4.2	3.8	0.38	0.292	0.496	0.434
<i>Pseudobutyrvibrio</i>	0.72	0.81	0.76	0.77	0.070	0.434	0.935	0.660
<i>RF16</i> , g-NA	1.32	0.58	1.06	0.82	0.129	<0.001	0.269	0.420
<i>Rikenellaceae</i> RC9 gut group	2.9	1.8	2.4	2.3	0.16	<0.001	0.554	0.823
<i>Ruminobacter</i>	0.39	0.37	0.35	0.40	0.086	0.877	0.707	0.400
<i>Ruminococcaceae</i> , incertae sedis	1.4	1.5	1.4	1.6	0.23	0.989	0.526	0.630
<i>Ruminococcaceae</i> , g-NA	4.9	4.8	4.8	4.9	0.25	0.827	0.637	0.743
<i>Ruminococcus</i> ⁶	10.1	9.5	9.0	10.7	0.65	0.443	0.078	0.087
<i>Saccharofermentans</i>	1.4	1.8	1.5	1.7	0.10	0.016	0.240	0.655
<i>Schwartzia</i>	0.07	0.07	0.06	0.08	0.016	0.997	0.373	0.472
<i>Selenomonas</i>	0.54	0.55	0.50	0.59	0.075	0.943	0.435	0.845
<i>Streptococcus</i>	0.34	0.34	0.33	0.34	0.063	0.952	0.860	0.465
<i>Succinilactium</i>	3.5	3.3	3.3	3.5	0.14	0.438	0.240	0.882
<i>Sutterella</i>	0.20	0.13	0.20	0.13	0.032	0.236	0.157	0.814
<i>Treponema</i>	0.13	0.14	0.13	0.15	0.035	0.930	0.705	0.860
<i>Victivallis</i>	0.05	0.09	0.06	0.08	0.017	0.112	0.618	0.598
<i>Xylanibacter</i>	3.3	3.2	3.6	2.9	0.27	0.884	0.097	0.419

¹Values shown represent d 17, 4 h after morning feeding, and that of individual bacterial and archaeal genera that were (1) consistent in all animals and (2) were >0.05% in terms of relative abundance.

²Diet × *DGAT1* interaction; CON AA = 8.1, LSO AA = 6.8, CON KK = 7.2, LSO KK = 7.8.

³Diet × *DGAT1* interaction; CON AA = 0.30, LSO AA = 0.65, CON KK = 0.25, LSO KK = 0.14.

⁴Taxonomic association of the genus-level phylogenetic grouping could not be assigned.

⁵Diet × *DGAT1* interaction; CON AA = 5.5, LSO AA = 4.6, CON KK = 4.5, LSO KK = 4.9.

⁶Diet × *DGAT1* interaction; CON AA = 10.1, LSO AA = 7.8, CON KK = 10.2, LSO KK = 11.0.

The relative abundance of *Bifidobacterium* ($P = 0.039$) was lower for the *DGAT1* KK genotype compared with the *DGAT1* AA genotype (Table 8). *Bifidobacterium* is a sugar fermenting bacteria (Trovatelli and Matteuzzi, 1976) producing acetate. However, intake of reducing sugars (Table 2) as well as the molar proportions of acetate (Table 7) was not affected by *DGAT1*.

With the LSO diet, *Rikenellaceae* RC9 gut group and a nonassigned genus (**g-NA**) within the *RF16* decreased ($P < 0.001$ for both), whereas *Saccharofermentans* increased ($P = 0.016$; Table 8). There is no cultured representative of *RF16*, g-NA, and hence the lack of knowledge with respect to the physiology of this group makes it unclear why their relative abundance

decreased with the LSO diet. The metabolic function and role of the *Rikenellaceae* RC9 gut group in the rumen microbiome remains to be defined, but Zened et al. (2013) already demonstrated that supplementation of sunflower oil decreased its relative abundance. *Saccharofermentans* is a sugar fermenting bacteria (Chen et al., 2010). The reducing sugar content and intake of the LSO diet was lower compared with the CON diet (Table 1, 2), which is not consistent with the increased relative abundance of sugar fermenting bacteria.

The PUFA present in linseed oil are believed to have a toxic effect on cellulolytic bacteria (Nagaraja et al., 1997; Martin et al., 2010). This negative effect of linseed supplementation on cellulolytic bacteria has not been confirmed in vivo in dairy cows by Veneman et al. (2015). Additionally, in the present study, no effect was observed on cellulolytic genera (such as *Fibrobacter*, *Butyrivibrio*, and *Ruminococcus*, $P > 0.150$, Table 8). This indicates that dietary linseed oil at the level used in the present study does not have a toxic effect on cellulolytic bacteria, and thus does not affect their relative abundance, and that this is therefore not the mode of action of dietary linseed oil to decrease CH_4 production. The results of the present study, however, do not reject the potential toxic effect of dietary linseed oil on the metabolic activity of cellulolytic bacteria.

Overall, the results indicate that several rumen bacterial genera were affected by dietary linseed oil, which could be linked to changes in dietary composition, differences in ruminal fermentation characteristics, and gaseous exchange. Further, several rumen bacterial genera were affected by *DGAT1*, but could not be linked with ruminal fermentation characteristics and gaseous exchange because the latter 2 were not affected by *DGAT1*. As a consequence, the biological implications of their change appear to be limited. Despite some bacterial genera being affected by diet, *DGAT1*, and diet $\times$ *DGAT1* interaction (i.e., the latter only tendencies), the bacterial and archaeal community as a whole was not significantly affected. This is perhaps because more than 75% of the bacterial genera analyzed were unaffected by diet, *DGAT1*, and diet $\times$ *DGAT1* interaction, and because the quantities of the affected bacterial genera were relatively small, therefore representing only a minor part of the rumen microbiota.

Implications

We acknowledge that the results of this study could have been different if the cows would have been fed ad libitum. We restricted feed intake to ensure similar feed intake between treatments, thus avoiding confounding effects of DMI on CH_4 production. Feed intake restriction ranged from 82 to 95% with an average of $92 \pm$

0.7%, with all cows being in positive energy balance and no difference in milk yield (25.5 ± 1.08 when fed ad libitum and 25.3 ± 1.16 when feed intake was restricted). We, therefore, consider the overall effect of restricted feeding to be minimal. However, when considering the individual treatments, this may be different. The LSO receiving cow was in 67% of the cases the one with the lowest ad libitum feed intake within a block, and thus the CON receiving cow was in 67% of the cases relatively more restricted in her feed intake. In other words, when fed ad libitum, the LSO diet could have resulted in a lower feed intake relatively to the CON diet. For *DGAT1*, we did not find an association between ad libitum feed intake and the *DGAT1* KK or AA genotype.

Additionally, we acknowledge that the absence of effects of *DGAT1* might be related to the number of animals used in the present study. Many genetic studies used hundreds to thousands of animals (e.g., Bovenhuis et al., 2016), whereas only 24 animals were used in the present study. It is known that *DGAT1* has major effects on milk yield and composition (such as milk fat content and milk protein content). The present study was able to confirm the difference in milk composition between the *DGAT1* KK and AA genotype, but for milk yield only a tendency was found. This shows that the number of animals in the present study might have been insufficient to find all known major effects of *DGAT1*. For marginal effects, such as CH_4 production, which differed 8 g/d between the *DGAT1* AA and KK genotypes (2% relative difference), this study did not have sufficient power because of the relative small number of animals. Furthermore, no dairy cows with the heterozygous *DGAT1* AK genotype were included in the present study. We assumed the AK genotype would be in between the homozygous *DGAT1* AA and KK genotypes, because there is little evidence for dominance effects of *DGAT1* (Bovenhuis et al., 2015). Whether this is also the case for other parameters, as measured in the current study, remains to be investigated.

Overall, the results of the present study suggest that *DGAT1* does not affect enteric CH_4 emission and production pathways, but that it does affect traits other than lactation characteristics, including metabolizability, N efficiency, and the relative abundance of *Bifidobacterium*. Additionally, linseed oil reduces CH_4 emission independent of *DGAT1* and affects the rumen microbiota and its fermentative activity.

CONCLUSIONS

Nutrient digestibility, CH_4 and H_2 emission, ruminal fermentation, rumen archaeal and bacterial concentrations, and the ruminal bacterial and archaeal com-

munity as a whole were not affected by *DGAT1*. The major effects of *DGAT1* on milk fat and protein content were independent of dietary linseed oil. Also, *DGAT1* affected other traits, including metabolizability, N efficiency, and the relative abundance of *Bifidobacterium*. Upon feeding linseed oil, H₂ emissions did not change, whereas CH₄ production (g/d) decreased with 8% (tendency only), CH₄ yield (g/kg of DMI) decreased with 6%, and CH₄ intensity (g/kg of FPCM) decreased with 11%, independent of *DGAT1*. In line with this, an increase in ruminal propionate proportion and a decrease in acetate proportion as well as acetate to propionate ratio was observed, and the archaea to bacteria ratio also decreased for the LSO diet. Linseed oil tended to affect the ruminal bacterial composition and affected the relative abundance of several bacterial genera.

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