Distinct blood and milk 18-carbon fatty acid proportions and buccal bacterial populations in dairy cows differing in reticulorumen pH response to dietary supplementation of rapidly fermentable carbohydrates

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ABSTRACT

Nine Holstein dairy cows were fed diets with increasing proportions of rapidly fermentable carbohydrates (RFCH) to investigate the effect on reticular pH, milk fat content (MFC), 18-carbon fatty acid proportions in blood plasma and milk, and bacterial community in buccal swab samples. Inter-animal variation was expected in terms of reticular pH response upon higher RFCH proportions, which would be reflected in the occurrence or not of milk fat depression (MFD). Moreover, this variation in occurrence of MFD was hypothesized to be related to differences in blood and milk fatty acid proportions and in the bacterial community in buccal samples. Cows were fed a total mixed ration throughout the experiment, which consisted of 4 periods: adaptation (d 0–4) and low (d 5–18), increasing (d 19–24), and high RFCH (d 25–28). During the increasing RFCH period, the standard concentrate (211 g of starch/kg of dry matter) was gradually and partly replaced by a concentrate high in RFCH (486 g of starch/kg of dry matter). The reticular pH was measured using a bolus and the time below pH 6.00 was calculated on a daily basis. On d 13, 14, 25, 27, and 28, plasma and milk samples were collected and analyzed for 18-carbon fatty acid proportions, and buccal swabs were collected for bacterial community analysis based on 16S rRNA gene amplicon sequencing. Inter-animal variation was observed in terms of reticular pH, which allowed us to divide the cows into 2 groups: tolerant (time below pH 6.00 $\leq$ 0.1 h/d) and susceptible cows (time below pH 6.00 $\geq$ 1.26 h/d). The lower reticular pH of susceptible

cows was accompanied by lower MFC. Both groups already differed in reticular pH and MFC during the low-RFCH period. Furthermore, higher RFCH amounts did not decrease the reticular pH in either of the 2 groups. Nevertheless, MFD was observed in both groups during the high-RFCH period compared with the low-RFCH period. Lower MFC in animals with lower reticular pH or during the high-RFCH period was associated with a shift in 18-carbon fatty acids toward *trans*-10 at the expense of *trans*-11 intermediates, which was observed in plasma as well as in milk samples. Moreover, lower MFC was accompanied by shifts in the relative abundance of specific bacteria in buccal samples. Genera *Dialister*, *Sharpea*, *Carnobacterium*, *Acidaminococcus*, and uncultured genera belonging to the *Betaproteobacteria* were more abundant in situations with greater *trans*-10 proportions.

Key words: biohydrogenation, microbiome, milk fat depression, rapidly fermentable carbohydrate

INTRODUCTION

Diet-induced milk fat depression (MFD) in dairy cattle is characterized by a reduction in milk fat content and yield, and alterations in milk fatty acid (FA) composition (Bauman and Griinari, 2001). The biohydrogenation theory established that MFD is caused by alterations in rumen biohydrogenation of dietary PUFA. When high-starch diets are fed or rumen pH is low, typical pathways of rumen biohydrogenation are altered to produce unique FA intermediates that inhibit milk fat synthesis (Harvatine et al., 2009). These milk fat-depressing FA are mainly 18-carbon isomers (Baumgard et al., 2002; Saebø et al., 2005; Perfield et al., 2007). Under normal rumen conditions, 18:2n-6 is mainly isomerized to *cis*-9,*trans*-11 CLA, which is further hydrogenated to *trans*-11 18:1 and, ultimately, to 18:0 (Bauman and Griinari, 2003; Harvatine et al.,

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2009). The predominant biohydrogenation pathway of 18:3n-3 involves *cis*-9,*trans*-11,*cis*-15 conjugated linolenic acid (CLnA), *trans*-11,*cis*-15 18:2, and *trans*-11 18:1 as intermediates (Shingfield and Wallace, 2014). However, under milk fat-depressing conditions, a shift in biohydrogenation pathways occurs toward the formation of *trans*-10 intermediates (i.e., *trans*-10,*cis*-12,*cis*-15 CLnA, *trans*-10,*cis*-15 18:2, *trans*-10,*cis*-12 CLA, and *trans*-10 18:1), referred to as the *trans*-11 to *trans*-10 shift (Harvatine et al., 2009; Shingfield and Wallace, 2014).

Of extreme interest in this respect is the inter-animal variation in rumen pH response to dietary supplementation of rapidly fermentable carbohydrates (RFCH; Penner et al., 2007; Schlau et al., 2012; Nasrollahi et al., 2017). Animals that experience less severe decreases in rumen pH upon a dietary RFCH challenge might also be less susceptible to alterations in ruminal biohydrogenation pathways and the associated MFD.

Rumen bacteria play an important role in biohydrogenation of PUFA (Buccioni et al., 2012). Indeed, in vitro studies with pure cultures of bacteria from the gastrointestinal tract of both ruminants and humans revealed that (*Pseudo*)*butyrivibrio* spp. are capable of *trans*-11 formation (e.g., Kepler et al., 1966; McIntosh et al., 2009), and that *Megasphaera elsdenii* (Kim et al., 2002), *Propionibacterium acnes* (Wallace et al., 2007), and *Lactobacillus* spp. (Alonso et al., 2003; Renes et al., 2017) are capable of producing *trans*-10 isomers from 18:2n-6. However, the role of these microbes in ruminal biohydrogenation of 18-carbon FA is not well known. As such, the rumen microbial etiology of the *trans*-11 to *trans*-10 shift is not well understood yet.

The current study investigated the changes in 18-carbon FA proportions and alterations in bacterial populations of dairy cows upon a dietary RFCH challenge. We hypothesized that inter-animal variation would be observed in response to the diet in terms of reticular pH, and that this would be reflected in the occurrence or not of MFD. Moreover, this variation in occurrence of MFD was hypothesized to be related to differences in FA proportions and bacterial community. As such, we aimed to gain more insight into the microbiology of the *trans*-11 to *trans*-10 shift by investigating associations between specific 18-carbon FA proportions and bacterial populations.

MATERIALS AND METHODS

Animals, Experimental Design, Diets, and Management

All experimental procedures involving animals were approved by the Central Committee on Animal Ex-

periments (AVD24246002017848, appendix 2) and the Institute for Animal Welfare of the Schothorst Feed Research B.V. (RM17-15-LRA-57). Ten Holstein-Friesian dairy cows (1 primiparous, 9 multiparous; 638 ± 74 kg of BW; 157 ± 43 DIM; 27.43 ± 7.77 kg/d of milk yield) were selected from a herd of 125 cows based on their milk FA profile in early lactation (as described by Jing et al., 2018). Based on specific FA, which have been identified as biomarkers for SARA (i.e., *trans*-10 18:1, *trans*-11 18:1 and 15:0; Jing et al., 2018), it was hypothesized to observe distinct rumen pH profiles within those animals. In early lactation, half of the selected animals ($n = 5$) showed a high proportion in milk fat of *trans*-10 18:1 at 3 wk in milk (>0.31 g/100 g of FA), a high level of 15:0 (on average, ≥ 1.18 g/100 g of FA over the 4 wk in milk), and a sharp decrease of *trans*-11 18:1 ($\Delta \geq 0.25$ g/100 g of FA during the 4 wk in milk). Those animals were hypothesized to be more susceptible to increased amounts of RFCH. Their counterparts ($n = 5$) had a low milk fat proportion of *trans*-10 18:1 at 3 wk in milk (<0.23 g/100 g of FA), an average 15:0 proportion of 0.99 g/100 g of FA or lower, and a rather stable *trans*-11 18:1 proportion in milk fat during early lactation. In the current experiment, the selected animals were housed in a freestall barn and were subjected to increasing amounts of RFCH in the diet (explained below). Unfortunately, 1 cow had to be withdrawn from the experiment due to an accident unrelated to the experiment.

The experiment lasted for 28 d and was divided into 4 periods based on the relative amount of RFCH in the diet: (1) adaptation period (d 0–4); (2) low-RFCH period (d 5–18); (3) increasing RFCH period (d 19–24); and (4) high-RFCH period (d 25–28). Cows were fed a TMR (basal diet/concentrate, on average, 670/330, wt/wt DM basis) ad libitum with a limited amount of residual feed to prevent cows from feed selection. Refusals of TMR were weighed daily before the morning feeding and used to adjust the amounts of feed offered. The basal diet (Table 1) consisted of (g/kg of DM) grass silage (514), maize silage (367), soybean meal (56.5), rapeseed meal (56.5), and a mineral premix (6.0). Two different concentrates were used in our study: a standard concentrate (concentrate A) and a concentrate high in RFCH (particularly supplied through wheat; concentrate B; Table 1). Both concentrates were formulated to be isoenergetic and isoproteic. During the low-RFCH period, cows only received concentrate A, which was gradually and partly replaced by concentrate B during the increasing RFCH period. The ratio of concentrate A to concentrate B remained constant in the high-RFCH period. The experimental protocol is presented in Table 2. Cows were continuously monitored based on their milk production, reticular pH,

Table 1. Ingredients and chemical composition of the basal diet and concentrates A and B¹ (g/kg of DM)

Item	Basal diet	Concentrate A	Concentrate B
Ingredient, g/kg of DM			
Grass silage	514	—	—
Maize silage	367	—	—
Beet pulp	—	234.7	—
Wheat	—	183.4	705.0
Rapeseed meal	56.5	147.3	—
Maize gluten feed	—	140.2	—
Maize	—	130.2	—
Molasses	—	59.4	55.0
Soybean meal	56.5	49.9	—
Citrus pulp	—	21.7	—
Limestone	—	6.9	14.0
Premix ²	6.0	6.5	6.5
Salt	—	3.0	8.5
Palm oil	—	7.4	—
Peas	—	—	200.0
Urea	—	—	11.0
Chemical composition, g/kg of DM (unless noted)			
DM, g/kg	494	881	861
OM	—	814	811
CP	222	151	149
Ether extract	—	32	20
Crude fat	—	77	24
NDF	811	203	116
ADF	—	94	40
ADL	—	12	5
Starch	—	211	486
Sugar	—	105	55
NE _L , ³ MJ/kg of DM	—	6.7	6.7
Fatty acid composition, g/kg			
16:0	3.01	2.84	1.20
18:0	0.40	0.66	0.28
<i>cis</i> -9 18:1	2.71	4.82	0.99
18:2n-6	6.16	9.72	3.67
18:3n-3	6.27	0.97	0.35
Total fatty acids	19.0	19.0	6.35

¹Two different concentrates were used in our study: a standard concentrate (concentrate A) and a concentrate high in rapidly fermentable carbohydrates (particularly supplied through wheat; concentrate B)

²Contains: 140 g of Ca, 0 g of P, 14 g of Mg, 7.5 g of Na, 0.3 g of K, 11.5 g of Cl, 0.3 g of S, 2,000 mg of Cu, 4,025 mg of Zn, 3,050 mg of Mn, 40 mg of Se, 75 mg of Co, 124 mg of I, 600,000 IU of vitamin A, 120,000 IU of vitamin D₃, and 5,000 IU of vitamin E.

³NE_L calculated based on the Dutch net energy evaluation system (VEM; Van Es, 1975).

and occurrence of health problems. Some cows showed very low reticular pH values and were therefore fed less concentrate than originally planned. On the other

hand, other cows did not respond on increasing RFCH in the diet, according to the reticular pH monitoring, and extra concentrate B was provided to these animals.

Table 2. Experimental design and intended ratios (g/kg of DM) of basal diet, concentrate A, and concentrate B¹ during the low-, increasing, and high-RFCH² periods³

Day	Period	Basal diet	Concentrate A	Concentrate B
5–18	Low RFCH	676	324	0
19–24	Increasing RFCH	678	77	245
25–28	High RFCH	659	75	266

¹Two different concentrates were used in our study: a standard concentrate (concentrate A) and a concentrate high in RFCH (particularly supplied through wheat; concentrate B).

²RFCH = rapidly fermentable carbohydrates.

³Mean values are reported for the low- and high-RFCH period. The value of the last day is reported for the increasing RFCH period.

Hence, due to large variation between cows in response to increasing amounts of concentrate B, differences in the ratio of concentrate A and concentrate B within the same period occurred (Supplemental Table S1; <https://doi.org/10.3168/jds.2018-15823>).

Diets were offered as 2 equal meals at 0730 and 1500 h. Animals had access to a constant supply of fresh water and were milked twice a day at 0530 and 1600 h. Individual milk yield was recorded at each milking.

Measurement of Reticular pH and Grouping of Animals

During the whole experiment, the reticular pH was measured every 10 min using a SmaXtec Premium bolus (SmaXtec Animal Care GmbH, Graz, Austria). Boli were introduced before the experiment using an oral balling gun. Various reticular pH parameters were calculated on a daily basis during the low- and high-RFCH period (i.e., mean pH, pH nadir, time below pH 5.60, time below pH 5.80, and time below pH 6.00; Table 3). Based on the time below pH 6.00, the animals were divided into 2 groups; that is, tolerant ($n = 4$) and susceptible ($n = 5$) cows. During both the low- and high-RFCH periods, the reticular pH of tolerant cows hardly decreased below 6.00 (time below pH 6.00 ≤ 0.10 h/d), whereas the reticular pH of susceptible cows was lower than 6.00 at least during 1 hour per day, both in the high- as well as in the low-RFCH period (Table 3).

Sampling Procedures

As our cows were not ruminally cannulated and oral intubation or rumenocentesis could not be applied for ethical reasons, we collected alternative samples (i.e., milk, saliva, blood, and buccal swabs). As the diet of the cows changed daily during the increasing RFCH period, samples were only collected during the low- and high-RFCH periods, in which the ratio of basal diet to concentrate was more stable.

From d 11 to 18 (low-RFCH period) and 25 to 28 (high-RFCH period), milk samples were collected daily at each milking (morning and afternoon) and stored with preservative (Bronopol; Qlip, Zutphen, the Netherlands) at -20°C until analysis of long-chain FA (LCFA).

On d 13 and 14 (low-RFCH period) and 25, 27, and 28 (high-RFCH period), samples of saliva, blood plasma, and buccal swabs were collected 3 h after feeding (based on Kittelmann et al., 2015). Saliva was collected using a sponge (Koala Universal Sponge; Es-sel, Sint-Eloois-Winkel, Belgium), which was cut into smaller pieces (approximately $10 \times 5 \times 2$ cm). The sponge was inserted into the mouth of the animal for approximately 1 min using a forceps (~ 25 cm long). The collected saliva was then pressed out into plastic tubes, which were placed on ice and then transferred to -20°C until further analysis. After thawing, subsamples (1 mL) were collected in glass tubes, stored at -20°C , and freeze-dried before LCFA analysis. As ruminants regularly regurgitate large amounts of rumen material

Table 3. Lactation and mean reticular pH parameters of the individual cows that were subjected to increased amounts of rapidly fermentable carbohydrates (RFCH) in the diet during different periods (low and high RFCH)

Group ¹	Cow	Parity	DIM	RFCH period	Mean pH	pH nadir	Time below pH threshold (h/d)		
							pH 5.60	pH 5.80	pH 6.00
Tolerant	T1	3	175	Low	6.54	6.16	0.00	0.00	0.01
				High	6.60	6.24	0.00	0.00	0.00
	T2	3	183	Low	6.44	6.10	0.00	0.00	0.04
				High	6.53	6.11	0.00	0.00	0.04
	T3	1	147	Low	6.48	6.15	0.00	0.00	0.02
				High	6.88	6.56	0.00	0.00	0.00
	T4	4	87	Low	6.50	6.10	0.00	0.00	0.10
				High	6.60	6.16	0.00	0.00	0.00
Susceptible	S1	4	127	Low	6.43	5.99	0.00	0.14	1.26
				High	6.33	5.91	0.00	0.04	1.42
	S2	4	183	Low	6.07	5.53	1.30	4.69	9.57
				High	6.10	5.63	0.04	3.25	8.71
	S3	4	106	Low	6.27	5.69	0.11	0.64	3.36
				High	6.34	5.70	0.00	0.79	3.54
	S4	3	189	Low	6.11	5.71	0.05	1.25	6.73
				High	6.11	5.65	0.09	1.79	9.08
	S5	4	229	Low	5.87	5.40	3.07	9.88	16.77
				High	5.99	5.46	1.71	6.88	12.21

¹Cows were divided into 2 groups based on the time below reticular pH 6.00. Tolerant cows ($n = 4$): ≤ 0.10 h/d; susceptible cows ($n = 5$): ≥ 1.26 h/d.

to their oral cavities, those saliva samples were used as a noninvasive alternative to assess the 18-carbon FA composition of the rumen.

Blood was collected in lithium heparin-containing tubes. After collection, blood was centrifuged at $3,000 \times g$ for 10 min at room temperature. The supernatant was separated and transferred into plastic tubes and stored at -20°C until analysis of LCFA.

Buccal swabs were collected using a sterile cotton roll (10×38 mm; Alan dental rolls, Alan & Co., Verviers, Belgium) according to Kittelmann et al. (2015). The sterile cotton roll was held with a sterile forceps (~ 25 cm long) and inserted into the mouth of the animal and swabbed several times across the inner side of the left cheek. The cotton roll was then placed in a sterile glass tube, which was placed on ice and then transferred to -20°C until further analysis. These buccal swabs were used as a noninvasive proxy to assess the composition of the rumen bacterial community (Kittelmann et al., 2015; Tapio et al., 2016).

LCFA Composition

Preparation of FAME. Fatty acid methyl esters were prepared from freeze-dried saliva samples and plasma samples using a direct transesterification procedure as described by Vlaeminck et al. (2014). Briefly, toluene (2 mL) containing the internal standard (21:0; Sigma Aldrich, Diegem, Belgium) and methanolic NaOH (2 mL; 0.5 M) were added and the mixture was incubated at 70°C for 60 min. This was followed by 30 min of incubation at 50°C after addition of methanolic HCl (3 mL), prepared by dissolving acetyl chloride in methanol (5/1, vol/vol). The FAME were extracted with hexane.

In addition, total plasma lipids were extracted using the BUME method (adapted from Löfgren et al., 2012) before separation into 4 lipid classes (i.e., free FA, phospholipids, cholesterol esters, and triacylglycerols) using solid phase extraction columns according to Vlaeminck et al. (2015).

Total lipids from milk were extracted using the mini-Röse-Gottlieb method (Chouinard et al., 1997), after which methylation was performed according to Stefanov et al. (2010). The internal standard (TAG-13:0; Sigma Aldrich) was added before milk fat extraction to quantify the total fat content based on the sum of individual FA.

Composition Analysis

Analysis of FAME was carried out using a GC (HP7890A; Agilent Technologies, Diegem, Belgium) equipped with a fused silica capillary column (SP-2560; $75 \text{ m} \times 0.18 \text{ mm i.d.} \times 0.14 \text{ }\mu\text{m}$ film thickness; Supelco

Analytical, Bellefonte, PA) and a flame ionization detector. A combination of 2 oven temperature programs was used in our study to achieve separation of most 18:1 isomers (Kramer et al., 2008). For the first GC run, the temperature program was initially 70°C for 2 min, increasing by $15^{\circ}\text{C}/\text{min}$ to 150°C , followed by a second increase at $1^{\circ}\text{C}/\text{min}$ up to 165°C and holding for 12 min, followed by a third increase at $2^{\circ}\text{C}/\text{min}$ to 170°C , held at 170°C for 5 min, increased at $5^{\circ}\text{C}/\text{min}$ to 215°C , and held at 215°C for 20 min. For the second GC run, the temperature program was, at the time of sample injection, column temperature of 70°C , increased at $50^{\circ}\text{C}/\text{min}$ to 175°C and maintained for 13 min, followed by a final increase at $5^{\circ}\text{C}/\text{min}$ to 215°C , and maintained for 20 min. For both GC runs, inlet and detector temperatures were 250°C and 255°C , respectively. The injection volume and split ratio depended on sample type and GC run. Hydrogen was used as the carrier gas at a flow rate of 1 mL/min. Identities of peaks were determined using mixtures of methyl ester standards (GLC463, Nu-Chek Prep, Elysian, MN; *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA, Larodan 279, Fine Chemicals AB, Malmö, Sweden). Quantification of FA was based on the area of the internal standard and on the conversion of peak areas to the weight of FA by a theoretical response factor for each FA (Ackman and Sipos, 1964; Wolff et al., 1995).

Bacterial Community

Genomic DNA Extraction from Buccal Swabs.

Before genomic DNA extraction, the cotton roll was removed from the tube with a sterile forceps, and approximately one-third of the swab was cut off using a sterile pair of scissors and immediately submerged in 2 mL of PBS buffer (Dulbecco and Vogt, 1954) for approximately 18 h. After centrifugation at 4°C for 10 min at $14,000 \times g$, the supernatant was transferred into a sterile 7-mL screw-cap tube containing zirconia beads, after which genomic DNA extraction was performed using the repeated bead beating plus column purification (RBB+C) method (adapted from Yu and Morrison, 2004). The yield and quality of extracted DNA was determined using a NanoDrop spectrophotometer (VWR International BVBA, Leuven, Belgium).

Illumina Library Generation and Data Mining.

Bacterial 16S rRNA gene amplicon sequencing (V3-V4 region) using Illumina MiSeq (Illumina Inc., San Diego, CA) technology (2×300 bp) was performed by MacroGen Sequencing Service (MacroGen Korea, Seoul, Korea). Preparation of the amplicon barcoded library (primers: 344F and 806R; Klindworth et al., 2013) was based on the 16S metagenomic sequencing library preparation protocol provided by the manufacturer (<https://>

support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf).

The amplicon sequencing data set was demultiplexed and barcodes were clipped off by the sequencing service provider. Reads were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) bioinformatics pipeline, version 1.9.1 (Caporaso et al., 2010). Forward and reverse reads were merged using the fastq-join method (Aronesty, 2011), after which primer removal and quality filtering ($Q \geq 4$) was performed using QIIME (Caporaso et al., 2010). This resulted in an average of $76,142 \pm 11,216$ sequences per sample (mean $\pm$ SD). The subsequent analysis, picking operational taxonomic units (OTU), assigning taxonomy, inferring phylogeny, and creating OTU tables, were also performed by QIIME software (Caporaso et al., 2010). The sequences were clustered into OTU using the open-reference OTU picking workflow with a 97% similarity threshold using UCLUST (Edgar, 2010), and chimeras were removed using UCHIME (Edgar, 2010). Representative sequences from individual OTU were aligned using PyNAST (Caporaso et al., 2010), and a taxonomy identity was assigned to each representative sequence using UCLUST (Edgar, 2010) and the GreenGenes database for reference (v13_8; DeSantis et al., 2006).

Potential oral bacteria were manually removed based on Kittelmann et al. (2015). Afterward, all potential rumen bacteria were retained based on cow rumen samples from our laboratory (J. Jeyanathan, Ghent University, Ghent, Belgium, personal communication) and based on Henderson et al. (2015). The OTU with less than 0.005% of the total number of sequences were removed. To ensure the comparability between samples, rarefied OTU sets (2,227 sequences per sample) were used for further analysis.

Two measures of α diversity were calculated in QIIME (Caporaso et al., 2010): (1) Shannon entropy, an indicator of species richness and evenness in community structure, and (2) the number of OTU observed. Next to this, β diversity indices between samples were determined in QIIME (Caporaso et al., 2010) based on Bray-Curtis dissimilarity (Bray and Curtis, 1957). Sequence data have been deposited in the National Center for Biotechnology Information (NCBI; <https://www.ncbi.nlm.nih.gov/>) database under accession number SRP136158.

Statistical Analysis

DMI, Reticular pH, Milk Fat Content, Milk Production, and 18-Carbon FA. Data were ana-

lyzed using the MIXED procedure of SAS (version Enterprise Guide 7.1; SAS Institute Inc., Cary, NC) by the following model

$$Y_{ijkl} = \mu + S_i + Pe_j + D_k + T_l + S_i \times Pe_j + \varepsilon_{ijkl}$$

where Y_{ijkl} = response variable, μ = average, S_i is the fixed effect of animal group (i = tolerant or susceptible), Pe_j is the fixed effect of period (j = low or high RFCH), D_k is the random effect of sampling day, T_l is the random effect of time of sampling (l = morning or afternoon, only applicable for milk samples), $S_i \times Pe_j$ is the interaction between group and period, and ε_{ijkl} is the residual error term. Least squares means are reported with treatment effects declared significant at $P < 0.05$. A Tukey-Kramer multiple comparison test was used to evaluate significant differences.

Bacterial Community. Both α diversity indices were analyzed in SAS (version Enterprise Guide 7.1; SAS Institute Inc.) by the model described above. In addition, the nonparametric permutational MANOVA-based statistical test ANOSIM was used in QIIME (Caporaso et al., 2010) to determine differences in overall bacterial community between groups or periods based on β diversity indices. To analyze for differences in taxa relative abundance (at species level), a first screening was performed by the Kruskal-Wallis test in QIIME (Caporaso et al., 2010). Bacterial taxa that showed differences between groups or periods or both based on this analysis were considered for further analysis in SAS (version Enterprise Guide 7.1; SAS Institute Inc.) by the model described above. When assumptions of normality or constant variance were not fulfilled, a square root or a sin-square root transformation was performed before analysis. In addition, Spearman rank correlation was used to check the correlation between different milk and plasma parameters and the abundance of different bacterial taxa (at species level) using QIIME (Caporaso et al., 2010).

RESULTS

Reticular pH Parameters

The mean reticular pH parameters are presented in Table 4. Grouping of the animals was based on the time below pH 6.00. Moreover, the discrepancy in reticular pH between groups was observed in all pH parameters (group: $P < 0.05$): mean pH, pH nadir, and time below pH 5.60, 5.80, and 6.00. Unexpectedly, gradual shifting from the low- to the high-RFCH period did not decrease the mean or minimum reticular pH or increase the time below a certain pH threshold (period: $P > 0.05$) in both groups of animals. In tolerant cows, the

Table 4. Reticular pH variables from cows which were tolerant or susceptible¹ to increased amounts of rapidly fermentable carbohydrates (RFCH) in the diet during different periods (low and high RFCH)

Variable	RFCH period				SEM	P-value		
	Tolerant (n = 4)		Susceptible (n = 5)			Group (S)	Period (Pe)	S × Pe
	Low	High	Low	High				
Mean pH	6.49 ^b	6.65 ^a	6.15 ^c	6.17 ^c	0.031	<0.001	0.006	0.031
pH nadir	6.13	6.27	5.66	5.67	0.035	<0.001	0.051	0.074
Time below pH 5.60, h/d	<0.01	<0.01	0.90	0.37	0.216	0.006	0.236	0.236
Time below pH 5.80, h/d	<0.01	<0.01	3.32	2.55	0.541	<0.001	0.497	0.497
Time below pH 6.00, h/d	0.04	0.01	7.54	6.99	0.774	<0.001	0.722	0.751

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

¹Cows were divided into 2 groups based on the time below reticular pH 6.00. Tolerant cows (n = 4): ≤0.10 h/d; susceptible cows (n = 5): ≥1.26 h/d.

mean reticular pH was even higher in the high-RFCH period as compared with the low-RFCH period (group × period: $P = 0.031$).

DMI, Milk Fat Content, and Milk Production

The DMI, milk fat content, and milk yield in the low- and high-RFCH period are presented in Table 5. Susceptible cows showed a lower DMI (group: $P = 0.004$), milk fat content (group: $P = 0.004$), and milk production (group: $P = 0.010$) as compared with the tolerant ones. Irrespective of group of cows, the milk fat content decreased in the high-RFCH period (period: $P < 0.001$) without changing milk production (period: $P = 0.684$). In that period, the milk fat content of tolerant cows decreased to the level of the susceptible cows in the low-RFCH period, whereas the milk fat content of susceptible cows decreased further.

18-Carbon FA Composition

As mainly 18-carbon FA are known to induce MFD, we traced shifts within this group of FA in particular. Hence, we expressed the proportions of these FA

relative to the sum of all 18-carbon FA to avoid confounding factors when expressed relative to total FA (e.g., alterations in odd- and branched-chain FA, which might also be influenced by varying dietary RFCH).

Blood Plasma

Proportions of different 18-carbon FA and total 18-carbon FA in blood plasma are presented in Table 6. Total 18-carbon FA (group: $P < 0.001$) and the proportion of 18:2n-6 (group: $P = 0.072$) and 18:3n-3 (group: $P < 0.001$) were higher or tended to be higher in tolerant cows as compared with susceptible cows. Plasma proportions of total *trans*-11 (**t11**) and total *trans*-10 (**t10**) intermediates were higher (group: $P = 0.032$ and $P < 0.001$, respectively) in susceptible cows as compared with tolerant cows. As the difference between groups was greater for t10 intermediates than for t11 intermediates, the ratio of t10 to t11 intermediates was also higher in plasma of susceptible cows (group: $P = 0.002$). The higher proportion of t11 intermediates was mainly caused by a higher proportion of *cis*-9,*trans*-11 CLA (group: $P = 0.002$), as no differences were observed between groups in other t11 intermediates. The higher

Table 5. Dry matter intake, milk fat content, and milk production from cows that were tolerant or susceptible¹ to increased amounts of rapidly fermentable carbohydrates (RFCH) in the diet during different periods (low and high RFCH)

Variable	RFCH period				SEM	P-value		
	Tolerant (n = 4)		Susceptible (n = 5)			Group (S)	Period (Pe)	S × Pe
	Low	High	Low	High				
DMI, kg/d	23.64	22.74	21.78	20.76	0.624	0.004	0.146	0.929
Milk fat content, mg/g	40.11	36.63	37.38	33.50	3.871	0.004	<0.001	0.844
Milk production, kg/d	34.62	33.61	30.09	29.81	1.483	0.010	0.684	0.820

¹Cows were divided into 2 groups based on the time below reticular pH 6.00. Tolerant cows (n = 4): ≤ 0.10 h/d; susceptible cows (n = 5): ≥ 1.26 h/d.

Table 6. Proportions of different 18-carbon fatty acids (g/100 g of C18) in blood plasma collected from cows that were tolerant or susceptible¹ to increased amounts of rapidly fermentable carbohydrates (RFCH) in the diet during different periods (low and high RFCH)

Variable ^{2,3}	RFCH period				SEM	<i>P</i> -value		
	Tolerant (n = 4)		Susceptible (n = 5)			Group (S)	Period (Pe)	S × Pe
	Low	High	Low	High				
Total C18 fatty acids, mg/mL	2.05	1.90	1.45	1.30	0.080	<0.001	0.067	0.991
18:0	18.26	17.81	19.36	19.10	0.312	<0.001	0.272	0.758
<i>t</i> 6 18:1 + <i>t</i> 7 18:1 + <i>t</i> 8 18:1	0.17	0.19	0.18	0.20	0.014	0.382	0.273	0.931
<i>t</i> 9 18:1	0.11	0.09	0.11	0.10	0.010	0.167	0.268	0.562
<i>t</i> 10 18:1	0.09	0.10	0.13	0.16	0.016	0.006	0.252	0.475
<i>t</i> 11 18:1	0.53	0.40	0.50	0.45	0.027	0.767	0.002	0.155
<i>t</i> 12 18:1	0.53	0.55	0.62	0.61	0.024	0.004	0.808	0.667
<i>c</i> 9 18:1 + <i>t</i> 13 18:1 + <i>t</i> 14 18:1	10.02	8.80	10.26	9.90	0.426	0.128	0.072	0.325
<i>t</i> 15 18:1 + <i>c</i> 11 18:1	0.76	0.78	0.85	0.92	0.034	0.002	0.175	0.510
<i>c</i> 12 18:1	0.55	0.53	0.56	0.54	0.021	0.573	0.263	0.876
<i>c</i> 13 18:1	0.10	0.10	0.13	0.12	0.007	<0.001	0.888	0.596
<i>c</i> 14 18:1 + <i>t</i> 16 18:1	0.15	0.15	0.19	0.19	0.007	<0.001	0.907	0.393
<i>c</i> 15 18:1	0.08	0.11	0.13	0.13	0.007	<0.001	0.179	0.072
Sum 18:1	13.09	11.80	13.68	13.32	0.459	0.029	0.082	0.315
<i>t</i> 11, <i>c</i> 15 18:2	0.20	0.18	0.21	0.18	0.012	0.561	0.189	0.581
<i>c</i> 9, <i>t</i> 11 CLA	0.28	0.30	0.34	0.40	0.022	0.002	0.124	0.351
<i>c</i> 9, <i>t</i> 11, <i>c</i> 15 CLnA	0.06	0.05	0.06	0.06	0.008	0.377	0.827	0.799
<i>t</i> 10, <i>c</i> 12, <i>c</i> 15 CLnA	0.21	0.22	0.26	0.31	0.022	<0.001	0.186	0.386
18:2n-6	57.61	59.62	56.55	57.98	0.723	0.072	0.023	0.697
18:3n-3	10.12	9.83	9.36	8.43	0.286	<0.001	0.042	0.272
<i>t</i> 11	1.07	0.94	1.11	1.09	0.044	0.032	0.095	0.202
<i>t</i> 10	0.30	0.34	0.39	0.47	0.027	<0.001	0.035	0.486
<i>t</i> 10: <i>t</i> 11	0.28	0.36	0.36	0.43	0.022	0.002	0.002	0.747

¹Cows were divided into 2 groups based on the time below reticular pH 6.00. Tolerant cows (n = 4): ≤0.10 h/d; susceptible cows (n = 5): ≥1.26 h/d.

²*c* = *cis*; *t* = *trans*; CLnA = conjugated linolenic acid; *t*10 and *t*11 = sum of all 18-carbon biohydrogenation intermediates containing a double bond in the *trans* configuration either at the 10th or the 11th carbon atom (from the carboxyl end); *t*10:*t*11 = ratio of *trans*-10 intermediates to *trans*-11 intermediates.

³The results from 1 cow at 1 sampling time were omitted before statistical analysis (outlier).

proportion of *t*10 intermediates in susceptible cows was caused by higher proportions of *trans*-10,*cis*-12,*cis*-15 CLnA (group: *P* < 0.001) and *trans*-10 18:1 (group: *P* = 0.006). Plasma proportions of *trans*-10,*cis*-12 CLA were extremely low in all samples (<0.10 g/100 g of C18, data not shown). In addition, the proportions of total 18:1 FA and 18:0 were also higher in plasma of susceptible cows (group: *P* = 0.029 and *P* < 0.001, respectively).

Irrespective of group of cows, plasma proportions of *trans*-11 18:1 (period: *P* = 0.002) and 18:3n-3 (period: *P* = 0.042) were lower in the high-RFCH period, whereas proportions of 18:2n-6 (period: *P* = 0.023), total *t*10 intermediates (period: *P* = 0.035) and the ratio of *t*10 to *t*11 intermediates (period: *P* = 0.002) were higher during the high-RFCH period.

The effects of animal group and RFCH period on proportions of different 18-carbon FA in 4 blood plasma fractions (free FA, phospholipids, cholesterol esters, and triacylglycerols) are available as supplementary material (Supplemental Table S2; <https://doi.org/10.3168/jds.2018-15823>).

Milk

Proportions of different 18-carbon FA and total 18-carbon FA in milk fat are shown in Table 7. Total 18-carbon FA were higher in milk fat of susceptible cows as compared with tolerant cows (group: *P* < 0.001) and decreased in the high-RFCH period in both groups (period: *P* < 0.001). Both 18:2n-6 and 18:3n-3 proportions were lower in milk of susceptible cows (group: *P* < 0.05) and increased in the high-RFCH period (period: *P* < 0.001). The proportion of total *t*11 intermediates in milk was higher in tolerant cows at the beginning of the experiment, but decreased to the level observed in the susceptible cows during the high-RFCH period (group × period: *P* = 0.003). This interaction effect in total *t*11 intermediates was caused by both *cis*-9,*trans*-11 CLA (group × period: *P* = 0.003) and *trans*-11 18:1 (group × period: *P* = 0.009). The milk proportion of *trans*-11,*cis*-15 18:2 was also higher in tolerant cows (group: *P* = 0.014), but was not lower in the high-RFCH period. The total proportion of *t*10 intermediates and the ratio of *t*10 to *t*11 intermediates

were higher in milk from susceptible cows (group: $P < 0.001$) and increased in the high-RFCH period in both groups (period: $P < 0.001$). This was mainly caused by higher proportions of *trans*-10 18:1 (group: $P < 0.001$; period: $P < 0.001$), as the milk proportion of *trans*-10,*cis*-12 CLA was lower in susceptible cows (group: $P < 0.001$). However, the latter milk FA only represented very minor proportions in milk fat (on average, 0.05 to 0.06 g/100 g of C18; minimum = 0.00 g/100 g of C18; maximum = 0.09 g/100 g of C18). Total 18:1 FA increased in the high-RFCH period only in susceptible cows (group $\times$ period: $P = 0.037$), whereas 18:0 decreased during the high-RFCH period in both groups of cows (period: $P = 0.003$), although this decrease tended (group $\times$ period: $P = 0.059$) to be larger for susceptible cows.

Saliva

Proportions of different 18-carbon FA and total 18-carbon FA in saliva are presented in Supplemental Table S3 (<https://doi.org/10.3168/jds.2018-15823>).

Total 18-carbon FA and proportions of different isomers (i.e., total *t11* intermediates, *cis*-9,*trans*-11 CLA, *trans*-11 18:1, and *trans*-10,*cis*-12 CLA) decreased or tended to decrease in the high-RFCH period, irrespective of group (period: $P < 0.10$). The saliva proportion of total 18:1 FA tended to be higher in susceptible cows as compared with tolerant cows (group: $P = 0.068$).

Bacterial Community of Susceptible and Tolerant Cows Fed RFCH

No differences ($P > 0.05$) were observed in species richness (Figure 1A) or Shannon diversity (Figure 1B) between tolerant and susceptible cows or between the low- and high-RFCH period. In addition, the statistical test ANOSIM revealed no differences in overall bacterial community composition between susceptible and tolerant cows (group: $P = 0.255$) or between periods (period: $P = 0.392$).

Nevertheless, differences were observed in relative abundance of specific bacterial taxa between groups or periods or both (Table 8). The relative abundance

Table 7. Proportions of different 18-carbon fatty acids (FA; g/100 g of C18) in milk collected from cows that were tolerant or susceptible¹ to increased amounts of rapidly fermentable carbohydrates (RFCH) in the diet during different periods (low and high RFCH)

Variable ²	RFCH period				SEM	P-value		
	Tolerant (n = 4)		Susceptible (n = 5)			Group (S)	Period (Pe)	S × Pe
	Low	High	Low	High				
Total C18 FA, g/100 g FA	33.16	29.74	35.30	32.89	0.510	<0.001	<0.001	0.277
18:0	29.16	28.61	29.86	27.35	0.513	0.579	0.003	0.059
<i>t6</i> 18:1 + <i>t7</i> 18:1 + <i>t8</i> 18:1	0.75	0.66	0.75	0.72	0.020	0.165	0.007	0.095
<i>t9</i> 18:1	0.59 ^{ab}	0.53 ^c	0.58 ^b	0.63 ^a	0.013	0.002	0.574	<0.001
<i>t10</i> 18:1	0.88	0.99	1.06	1.39	0.058	<0.001	<0.001	0.063
<i>t11</i> 18:1	2.96 ^a	2.33 ^c	2.75 ^b	2.45 ^c	0.080	0.528	<0.001	0.009
<i>t12</i> 18:1	0.90 ^b	0.90 ^b	0.93 ^b	1.06 ^a	0.024	<0.001	0.006	0.005
<i>c9</i> 18:1 + <i>t13</i> 18:1 + <i>t14</i> 18:1	52.23	52.22	51.61	52.38	0.483	0.584	0.362	0.349
<i>t15</i> 18:1	0.82	0.86	0.85	0.95	0.018	<0.001	<0.001	0.099
<i>c11</i> 18:1	1.36	1.75	1.61	2.04	0.049	<0.001	<0.001	0.742
<i>c12</i> 18:1	0.71 ^c	0.77 ^b	0.70 ^c	0.83 ^a	0.017	0.226	<0.001	0.041
<i>c13</i> 18:1	0.18	0.20	0.21	0.21	0.007	0.010	0.294	0.222
<i>c14</i> 18:1 + <i>t16</i> 18:1	1.15	1.20	1.19	1.31	0.025	0.002	<0.001	0.169
<i>c15</i> 18:1	0.35	0.36	0.37	0.38	0.016	0.036	0.528	0.883
Sum 18:1	62.88 ^b	62.76 ^b	62.61 ^b	64.36 ^a	0.453	0.141	0.070	0.037
<i>t11</i> , <i>c15</i> 18:2	0.32	0.31	0.29	0.30	0.011	0.014	0.760	0.338
<i>c9</i> , <i>t11</i> CLA	1.33 ^a	1.11 ^b	1.21 ^b	1.22 ^b	0.038	0.897	0.007	0.003
<i>t10</i> , <i>c12</i> CLA	0.06	0.06	0.05	0.05	0.002	<0.001	0.363	0.098
18:2n-6	4.80	5.39	4.61	5.20	0.082	0.022	<0.001	0.989
18:3n-3	1.32 ^b	1.60 ^a	1.24 ^c	1.36 ^b	0.019	<0.001	<0.001	<0.001
<i>t11</i>	4.62 ^a	3.75 ^c	4.26 ^b	3.97 ^{bc}	0.104	0.491	<0.001	0.003
<i>t10</i>	0.94	1.05	1.11	1.44	0.058	<0.001	<0.001	0.071
<i>t10</i> : <i>t11</i>	0.21	0.29	0.26	0.36	0.014	<0.001	<0.001	0.503

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

¹Cows were divided into 2 groups based on the time below reticular pH 6.00. Tolerant cows (n = 4): ≤ 0.10 h/d; susceptible cows (n = 5): ≥ 1.26 h/d.

²*c* = *cis*; *t* = *trans*; CLnA = conjugated linolenic acid; *t10* and *t11* = sum of all 18-carbon biohydrogenation intermediates containing a double bond in the *trans* configuration either at the 10th or the 11th carbon atom (from the carboxyl end); *t10*:*t11* = ratio of *trans*-10 intermediates to *trans*-11 intermediates.

of the genus *Butyrivibrio* decreased during the high-RFCH period, irrespective of group (period: $P = 0.035$), whereas the genus *Pseudobutyrvibrio* did not differ between groups or periods ($P > 0.05$). Relative abundances of the genera *Dialister* and *Megasphaera* were both higher in buccal swab samples from susceptible cows as compared with tolerant cows (group: $P = 0.043$), whereas *Propionibacterium acnes* was more abundant in tolerant cows (group: $P = 0.007$). Several other taxa, mainly within the phylum *Firmicutes*, were also more abundant in buccal swabs from susceptible cows compared with tolerant cows (group: $P < 0.05$; e.g., *Staphylococcus epidermidis*, uncultured *Enterococcaceae*, uncultured *Lactobacillales*, and uncultured *Streptococcaceae*). In contrast, uncultured RFP12 within the phylum *Verrucomicrobia* was more abundant in tolerant cows (group: $P = 0.007$). Decreases in relative abundance upon increased fermentability of the diet (period: $P < 0.05$) were observed for taxa within the phyla *Bacteroidetes* (e.g., CF231, uncultured *Bacteroidales*), *Firmicutes* (e.g., *Ruminococcus*, uncultured *Erysipelotrichaceae*), and *Proteobacteria* (e.g., *Ruminobacter*). The genus *Carnobacterium* was more abundant in buccal fluid from susceptible cows (group: $P = 0.034$) and increased in the high-RFCH period (period: $P = 0.023$), whereas opposite effects were observed for uncultured RF32 (group: $P = 0.037$; period: $P = 0.023$). The genus *Anaeroplasma* was more abundant in buccal fluid from tolerant cows in the low-RFCH period, but decreased to the level of susceptible cows in the high-RFCH period (group $\times$ period: $P = 0.036$).

Associations Between 18-Carbon FA and Bacterial Taxa

Several genera within the phyla *Bacteroidetes* (i.e., CF231, uncultured p-2534-18B5, uncultured *Porphyro-*

monadaceae) and *Firmicutes* (i.e., *Oscillospira*, uncultured *Erysipelotrichaceae*) correlated positively with milk fat content and proportions of total t11 intermediates in both milk and plasma (Table 9). *Butyrivibrio* and *Pseudobutyrvibrio* only showed positive correlations with milk fat content and milk t11 proportion, respectively. In contrast, *Acidaminococcus*, *Dialister*, *Sharpea* p-3329-23G2, and uncultured *Betaproteobacteria* showed positive correlations with total t10 intermediates in milk, plasma, or both, whereas uncultured *Fibrobacter* species additionally correlated negatively with milk fat content. Notably, *Megasphaera* did not correlate with any of the discussed milk or plasma FA proportions.

DISCUSSION

When high-starch diets are fed to dairy cows, it is known that typical pathways of rumen biohydrogenation are altered to produce unique FA intermediates (e.g., *trans*-10,*cis*-12 CLA), of which some might inhibit milk fat synthesis when reaching the mammary gland (Harvatine et al., 2009). As a consequence, the milk fat content and yield decrease, which is known as MFD (Bauman and Griinari, 2001). High-starch diets are also associated with decreases in rumen pH in dairy cows (Plaizier et al., 2008); however, inter-animal variation exists in rumen pH response upon increased diet fermentability (Penner et al., 2007; Schlau et al., 2012; Nasrollahi et al., 2017). As the cows had been selected from a herd of 125 cows based on their milk FA profile in early lactation (Jing et al., 2018), we hypothesized to observe distinct reticular pH profiles within the limited number of cows that had been selected for the trial ($n = 10$, of which 1 died). As expected, inter-animal differences were observed, which allowed us to divide cows

Figure 1. Alpha diversity of bacterial communities by the number of observed species (A) and by Shannon diversity (B) in buccal swab samples collected from cows that were tolerant or susceptible to increased amounts of rapidly fermentable carbohydrates (RFCH) in the diet during different periods (low RFCH = black; high RFCH = gray). Cows were divided into 2 groups based on the time below reticular pH 6.00. Tolerant cows ($n = 4$): ≤ 10 h/d; susceptible cows ($n = 5$): ≥ 1.26 h/d. Data are presented as mean $\pm$ SD. No significant differences were observed (group: $P > 0.05$; period: $P > 0.05$; group $\times$ period: $P > 0.05$).

into 2 groups based on their reticular pH (i.e., tolerant and susceptible cows). Interestingly, the discrepancy in reticular pH between susceptible and tolerant cows was already observed during the low-RFCH period, whereas higher amounts of RFCH did not decrease the reticular pH in any of the 2 groups of cows. Tolerant cows even showed higher reticular pH values during the high-RFCH period. Similar effects were observed in the study of Colman et al. (2010), when considering individual cow data: cows experiencing SARA at the end of the SARA induction experiment already showed longer

periods of rumen pH below 5.60 during the preinduction period. On the other hand, the SARA-induction protocol failed to induce SARA (defined as pH <5.60 for more than 283 min/d) in cows that had less than 50 min/d below 5.60 during the preinduction period (Colman et al., 2010).

As a low rumen pH is often associated with MFD (Plaizier et al., 2008), we hypothesized that the inter-animal variation in reticular pH would be reflected in the occurrence or not of MFD. Indeed, in line with a lower reticular pH, the susceptible cows also showed a

Table 8. Average relative abundance (%) of different bacterial taxa in buccal swab samples collected from cows that were tolerant or susceptible¹ to increased amounts of rapidly fermentable carbohydrates (RFCH) in the diet during different periods (low and high RFCH)

Bacterial taxa	RFCH period				SEM	Group (S)	P-value	
	Tolerant (n = 4)		Susceptible (n = 5)					
	Low	High	Low	High			Period (Pe)	S × Pe
<i>Actinobacteria</i>								
<i>Propionibacterium acnes</i> ²	1.41	2.03	0.73	0.42	0.488	0.007	0.902	0.153
<i>Bacteroidetes</i>								
CF231 ²	1.00	0.19	0.79	0.17	0.190	0.650	0.004	0.539
Uncultured <i>Bacteroidales</i> ²	4.88	1.82	4.04	1.34	0.699	0.254	0.002	0.954
Uncultured RF16 ²	1.06	0.27	0.60	0.18	0.187	0.182	0.006	0.603
Uncultured S24-7 ²	0.60	0.41	0.66	0.12	0.199	0.936	0.027	0.299
<i>Firmicutes</i>								
<i>Butyrivibrio</i> ²	2.19	0.85	2.14	0.89	0.552	0.575	0.035	0.791
<i>Carnobacterium</i> ²	0.12	2.48	2.63	9.68	3.193	0.034	0.023	0.945
<i>Dialister</i> ³	<0.01	0.02	0.08	0.14	0.055	0.043	0.391	0.903
<i>Lactococcus garvieae</i> ²	<0.01 ^b	<0.01 ^b	0.08 ^a	<0.01 ^b	0.019	0.030	0.068	0.024
<i>Megasphaera</i> ²	<0.01	<0.01	0.14	0.12	0.076	0.043	0.928	0.928
<i>Pseudobutyrvibrio</i> ²	0.28	0.29	0.27	0.11	0.107	0.324	0.153	0.995
rc4-4 ²	0.02	<0.01	0.26	<0.01	0.081	0.126	0.027	0.114
<i>Ruminococcus</i>								
<i>Staphylococcus epidermidis</i> ²	<0.01	0.05	0.09	0.07	0.034	0.036	0.717	0.064
<i>Staphylococcus succinus</i> ²	0.01	0.03	0.10	0.10	0.035	0.015	0.936	0.662
Uncultured <i>Bacillales</i> ²	0.21	0.73	0.88	1.28	0.410	0.034	0.207	0.767
Uncultured <i>Enterococcaceae</i> ²	0.35	0.04	0.88	0.97	0.530	0.021	0.588	0.793
Uncultured <i>Erysipelotrichaceae</i> ²	0.31	0.10	0.20	0.07	0.077	0.654	0.015	0.886
Uncultured <i>Lactobacillales</i> ²	0.06	0.38	0.47	0.57	0.206	0.012	0.356	0.152
Uncultured <i>Planococcaceae</i> ²	0.08	0.24	0.43	0.34	0.139	0.023	0.517	0.545
Uncultured <i>Streptococcaceae</i> ²	<0.01	<0.01	0.39	0.17	0.141	0.006	0.613	0.808
<i>Proteobacteria</i>								
<i>Psychrobacter marincola</i> ²	<0.01	0.01	0.03	0.04	0.026	0.032	0.861	0.637
<i>Ruminobacter</i> ²	0.32	0.14	0.11	<0.01	0.092	0.147	0.042	0.912
Uncultured <i>Betaproteobacteria</i> ²	<0.01	0.01	0.07	0.04	0.022	0.013	0.613	0.418
Uncultured <i>Desulfovibrionaceae</i>	0.15	0.07	0.19	0.01	0.056	0.888	0.037	0.326
Uncultured RF32 ²	0.48	0.15	0.15	0.06	0.089	0.037	0.023	0.371
Uncultured <i>Rhodocyclaceae</i> ²	0.08	0.16	0.06	0.03	0.037	0.036	0.391	0.060
<i>Synergistetes</i>								
<i>Pyramidobacter</i> ²	0.02	<0.01	<0.01	n.d. ⁴	0.007	0.022	0.294	0.148
<i>Tenericutes</i>								
<i>Anaeroplasma</i>	0.58 ^a	0.12 ^b	0.10 ^b	0.11 ^b	0.109	0.033	0.046	0.036
<i>Verrucomicrobia</i>								
Uncultured RFP12 ²	0.30	0.27	0.09	0.03	0.097	0.007	0.268	0.943

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

¹Cows were divided into 2 groups based on the time below reticular pH 6.00. Tolerant cows (n = 4): ≤0.10 h/d; susceptible cows (n = 5): ≥1.26 h/d.

²Reported *P*-values are based on the square root transformation of the respective parameter.

³Reported *P*-values are based on the sin-square root transformation of the respective parameter.

⁴n.d. = not detected.

Table 9. Spearman rank correlations between relative abundance of bacterial taxa in buccal swab samples and milk fat content and total proportions of *trans*-11 and *trans*-10 intermediates in milk and plasma

Bacterial taxa	Milk fat content	Milk proportion		Plasma proportion	
		<i>trans</i> -11	<i>trans</i> -10	<i>trans</i> -11	<i>trans</i> -10
<i>Actinobacteria</i>					
<i>Bifidobacterium pseudolongum</i>	0.44**			0.40**	
<i>Propionibacterium acnes</i>					
<i>Bacteroidetes</i>					
CF231	0.46**	0.39**		0.34*	
<i>Odoribacter</i>				0.39**	
Uncultured p-2534—18B5	0.39**	0.42**		0.40**	
Uncultured <i>Porphyromonadaceae</i>	0.43**	0.41**		0.48***	
<i>Fibrobacteres</i>					
<i>Fibrobacter</i>	−0.40**		0.50***		
<i>Fibrobacter succinogenes</i>		0.39**			
<i>Firmicutes</i>					
<i>Acidaminococcus</i>			0.33*		0.40**
<i>Anaerostipes</i>	0.32*			0.40**	
<i>Butyrivibrio</i>	0.32*				
<i>Dialister</i>			0.43**		
<i>Erysipelothrix</i>				0.39**	
<i>Megasphaera</i>					
<i>Oscillospira</i>	0.33*	0.42**		0.38**	
<i>Pseudobutyrvibrio</i>		0.35*			
RFN20		0.42**			
<i>Sharpea</i> p-3329—23G2			0.37*		0.31*
<i>Tissierella Soehngenia</i>		0.53***		0.47**	
<i>Trichococcus</i>		0.35*		0.39**	
<i>Turicibacter</i>				0.48***	
Uncultured <i>Clostridiaceae</i>				0.40**	
Uncultured <i>Erysipelotrichaceae</i>	0.41**	0.51***		0.36*	
<i>Lentisphaerae</i>					
Uncultured <i>Victivallaceae</i>		0.39**		0.59***	
<i>Proteobacteria</i>					
<i>Arcobacter</i>				0.38**	
<i>Luteimonas</i>				0.46**	
<i>Sutterella</i>		0.40**		0.36*	
Uncultured <i>Betaproteobacteria</i>			0.46**		0.39**
Uncultured <i>Desulfovibrionaceae</i>		0.38**			
<i>Tenericutes</i>					
<i>Anaeroplasm</i>	0.31*	0.41**			
Uncultured ML615J-28				0.43**	

*** $P < 0.001$; ** $0.001 \leq P < 0.01$; * $0.01 \leq P < 0.05$; blank cells, $P \geq 0.05$.

lower milk fat content in comparison with the tolerant cows (35.44 vs. 38.37 mg/g, −7.64%). This lower milk fat content was not a dilution effect, as total milk yield was also lower in susceptible cows (29.95 vs. 34.12 kg/d, −12.22%), which might be related to the lower DMI of these cows (21.27 vs. 23.19 kg/d, −8.28%). This lower DMI of susceptible cows might be the result of reduced fiber digestibility occurring with lower rumen pH values (Plaizier et al., 2008), which increases the ruminal retention time and, thus, limits DMI. However, fiber digestibility was not measured in our study. In contrast to the lack of response of increasing amounts of dietary RFCH on reticular pH, MFD (i.e., decreased milk fat content without changes in milk yield) was observed when shifting from the low- to the high-RFCH diet in both groups of cows (35.07 vs. 38.75 mg/g, −9.50%). This 9.50% reduction in milk fat was lower than milk

fat reductions observed in other reports [e.g., −34%, from 40.3 to 28.3 mg/g in Rico and Harvatine (2013); −31%, from 33.4 to 22.9 mg/g in Toral et al. (2015)]. However, in the current study, the relative amount of RFCH was gradually increased over a period of 7 d, whereas cows were abruptly switched to a diet high in starch in the experiments of Rico and Harvatine (2013) and Toral et al. (2015). In addition, these cows were also supplemented with plant oils (i.e., soybean oil or sunflower oil), which was not the case in the current experiment. Hence, the response of the milk fat content to the increased amounts of dietary RFCH without a concomitant decrease in reticular pH suggests that a low rumen pH is not the main determinant of MFD.

Previous studies suggested that diet-induced MFD is caused by alterations in rumen biohydrogenation pathways of dietary PUFA, resulting in the formation

of specific FA isomers, of which some might inhibit milk fat synthesis (Harvatine et al., 2009). The milk fat-depressing FA that have been described before are mainly 18-carbon isomers (Baumgard et al., 2002; Saebø et al., 2005; Perfield et al., 2007); therefore, we focused on proportions of 18-carbon FA in the current study. Two distinct pathways of biohydrogenation have been described for 18:2n-6 and 18:3n-3 (Bauman and Griinari, 2003; Shingfield and Wallace, 2014), which are the majority of the dietary FA (Ferlay et al., 2017). Under normal rumen conditions, 18:2n-6 is mainly isomerized to *cis*-9,*trans*-11 CLA, which is further hydrogenated to *trans*-11 18:1 and, ultimately, to 18:0 (Bauman and Griinari, 2003; Harvatine et al., 2009). The predominant biohydrogenation pathway of 18:3n-3 involves *cis*-9,*trans*-11,*cis*-15 CLnA, *trans*-11,*cis*-15 18:2, and *trans*-11 18:1 as intermediates (Shingfield and Wallace, 2014). Under altered ruminal conditions (e.g., supply of marine products, a low rumen pH, high amounts of starch in the diet), a shift occurs toward the formation of *t*10 intermediates (i.e., *trans*-10,*cis*-12,*cis*-15 CLnA, *trans*-10,*cis*-15 18:2, *trans*-10,*cis*-12 CLA, and *trans*-10 18:1) at the expense of *t*11 intermediates (Harvatine et al., 2009). As the milk fat content was generally lower in susceptible cows compared with tolerant cows and decreased in both groups of cows during the high-RFCH period, differences in rumen proportions of *t*11 and *t*10 intermediates were expected (1) between susceptible and tolerant cows, irrespective of period, and (2) between the low- and high-RFCH period, irrespective of animal group.

As rumen samples were not available in the current trial, alternative samples were collected to assess possible shifts in the rumen FA metabolism. Fatty acid profiles of blood and milk samples might be acceptable proxies to assess a shift in the rumen biohydrogenation pathways, despite postabsorptive conversions that can take place. Indeed, in the mammary gland, *trans*-11 18:1 might be converted to *cis*-9,*trans*-11 CLA through the action of Δ^9 -desaturase (Griinari et al., 2000). In line with this, Δ^9 -desaturation of *trans*-11 18:1 might also occur in the intestine (Renaville et al., 2006), which affects the FA distribution of blood plasma. To bypass these issues, we focused on the sum of *t*11 and *t*10 intermediates in the current discussion, rather than specific *t*11 and *t*10 isomers. As expected, we found lower milk fat concentrations associated with a *t*11 to *t*10 shift both in milk as well as in blood plasma, which might be related to alterations in rumen biohydrogenation pathways. Total *t*10 intermediates and the ratio of *t*10 to *t*11 intermediates were higher in plasma and milk (1) from susceptible cows, irrespective of RFCH period, and (2) during the high-RFCH period, irrespective of animal group. On the other hand, total *t*11 intermedi-

ates were lower in milk (1) from susceptible cows and (2) during the high-RFCH period.

As ruminants regularly regurgitate large amounts of rumen material to their oral cavities, oral samples might also be an alternative to rumen sampling for the analysis of the rumen FA composition. To our knowledge, this is the first time that saliva samples were collected for this purpose. Unfortunately, proportions of particular *trans* FA (e.g., total *t*10 and *t*11 intermediates) in saliva did not reflect the differences that were expected based on milk and plasma FA proportions. Furthermore, the variation in saliva samples was higher as compared with plasma and milk samples. This suggests that saliva, collected using the current sampling procedure, could not represent an alternative to milk or blood and, ultimately, to rumen samples. To achieve representative saliva samples, rumen fluid should be present in the sample, which can be identified by its green-brown color. With the current sampling procedure, most cows were nervous at the time of sampling and were, as a consequence, not ruminating, which might be the main reason of limited coherence between FA profiles of saliva versus blood and milk FA profiles.

As bacteria play an important role in rumen biohydrogenation of PUFA (Buccioni et al., 2012), differences in bacterial composition might be related to the shift in 18-carbon FA metabolism from its main biohydrogenation pathway to the alternative pathway. In the current study, buccal swab samples were used as a noninvasive method to determine the rumen bacterial community (Kittelmann et al., 2015; Tapio et al., 2016). In contrast to the study of Plaizier et al. (2017a), we observed no differences in species richness and diversity of bacterial communities and no difference in overall bacterial community composition when gradually switching from the low- to high-RFCH period. However, at the species level, some differences in relative abundance were observed between susceptible and tolerant cows or upon increased amounts of dietary RFCH. This discussion will focus on changes in bacterial taxa that could potentially be involved in ruminal biohydrogenation of 18-carbon FA. Decreased abundance of the genus *Butyrivibrio* upon increasing the fermentability of the diet aligns with former observations of Fernando et al. (2010), in which decreases of *Butyrivibrio fibrisolvens* populations were observed when animals were adapted to a high-concentrate diet. This might indicate a potential role of *Butyrivibrio* spp. in ruminal *t*11 formation, as suggested by other reports (Kepler et al., 1966; Shingfield et al., 2012; Rico et al., 2015). However, as no difference was observed between susceptible and tolerant cows, no correlation between *Butyrivibrio* spp. and *t*11 intermediates was observed in our study, concurring with other reports (Zened et

al., 2016; Dewanckele et al., 2018). This might indicate that other bacteria are involved in ruminal *t*11 production, as suggested by Huws et al. (2011). In contrast to the genus *Butyrivibrio*, the genus *Pseudobutyrvibrio*, which also produces *trans*-11 18:1 from 18:2n-6 in vitro (Paillard et al., 2007), correlated positively with *t*11 intermediates in the current trial. An alternative explanation is that *Butyrivibrio* spp. are the main bacteria involved in ruminal *t*11 production, and low rumen pH, as encountered by susceptible cows, could decrease their activity (i.e., *t*11 production; Dewanckele et al., 2017), which is not reflected in relative abundance data.

The ruminal bacteria responsible for the formation of *t*10 intermediates are not well known. Increased concentrations in blood and milk of *t*10 intermediates in the susceptible cows in comparison with tolerant ones coincided with a higher relative abundance of *Megasphaera* spp. Others also observed such concomitant increases of these FA isomers and the relative abundance of *Megasphaera* spp. in rumen content (Rico et al., 2015; Dewanckele et al., 2018). However, in contrast to the observations of Fernando et al. (2010) and Plaizier et al. (2017b), in which higher amounts of *Megasphaera (elsdenii)* were observed in the rumen of cows upon a high-grain diet, the abundance of this genus did not increase when shifting from the low- to high-RFCH period in the current study. Furthermore, *Megasphaera* did not linearly correlate with *t*10 intermediates in milk or blood in the current study. Together with contrasting results about the capability of *Megasphaera* spp. to produce *trans*-10,*cis*-12 CLA in vitro (Kim et al., 2002; Maia et al., 2007), this questions the contribution of *Megasphaera* in the alternative biohydrogenation pathway. In vitro studies by Wallace et al. (2007) indicated that *Propionibacterium acnes* is a producer of *trans*-10,*cis*-12 CLA, which is the end product of its 18:2n-6 metabolism (McKain et al., 2010; Dewanckele et al., 2017); however, ruminal abundance of this species is very low (Shingfield et al., 2012; Dewanckele et al., 2018). Surprisingly, in the current study, *P. acnes* abundance was comparable with the abundance of *Butyrivibrio* spp., and its abundance was higher in tolerant cows compared with susceptible cows. This might indicate that *P. acnes* is also present in the oral cavity of cows, as observed in humans (Perry and Lambert, 2006). As such, buccal swab samples might not be a good alternative to assess the rumen abundance of this particular species. In a former experiment in our laboratory (Dewanckele et al., 2018), *Dialister* spp. and *Sharpea* spp. were more abundant in the rumen of goats in situations with greater *t*10 accumulation, which was confirmed in the current study; nevertheless, contrasting results were observed for *Lactobacillus* spp.

and *Bifidobacterium* spp. (Dewanckele et al., 2018). According to the current study, *Carnobacterium* spp., *Acidaminococcus* spp., and uncultured genera belonging to the *Betaproteobacteria* were more abundant in situations with greater *t*10 accumulation, potentially indicating a role of these bacteria in ruminal *t*10 formation. However, in vitro experiments with pure cultures are required to confirm the capacity of these bacteria to produce *t*10 intermediates.

CONCLUSIONS

Inter-animal variation was observed in our study in terms of reticular pH, which allowed us to divide cows into 2 groups (i.e., tolerant cows showing higher pH values and susceptible cows with lower pH values). In line with this, susceptible cows also showed lower milk fat concentrations in comparison with tolerant cows. This discrepancy in reticular pH between cows was observed throughout the experiment, irrespective of the dietary RFCH level. Even more, gradually switching from the low- to high-RFCH period did not decrease the reticular pH in either of the 2 groups. Nevertheless, MFD was observed in both groups of cows upon increasing RFCH amounts in the diet, which indicates that a low rumen pH is not the main determinant of MFD. Lower milk fat concentrations were associated with a shift in biohydrogenation pathways toward the formation of *t*10 intermediates at the expense of *t*11 intermediates. Moreover, lower milk fat concentrations were also accompanied by significant shifts in the relative abundance of specific bacterial taxa, which were correlated with either *t*10 or *t*11 biohydrogenation intermediates. *Dialister* spp., *Sharpea* spp., *Carnobacterium* spp., *Acidaminococcus* spp., and uncultured genera belonging to the *Betaproteobacteria* were more abundant in situations with greater *t*10 accumulation, suggesting their potential role in altered biohydrogenation pathways.

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