J. Dairy Sci. 96:4100–4111
http://dx.doi.org/10.3168/jds.2012-6109

Grain-based versus alfalfa-based subacute ruminal acidosis induction experiments: Similarities and differences between changes in milk fatty acids

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ABSTRACT

Subacute ruminal acidosis (SARA) is one of the most important metabolic disorders, traditionally characterized by low rumen pH, which might be induced by an increase in the dietary proportion of grains as well as by a reduction of structural fiber. Both approaches were used in earlier published experiments in which SARA was induced by replacing part of the ration by a grain mixture or alfalfa hay by alfalfa pellets. The main differences between both experiments were the presence of blood lipopolysaccharide and *Escherichia coli* and associated effects on the rumen microbial population in the rumen of grain-based induced SARA animals as well as a great amount of quickly fermentable carbohydrates in the grain-based SARA induction experiment. Both induction approaches changed rumen pH although the pH decrease was more substantial in the alfalfa-based SARA induction protocol. The goal of the current analysis was to assess whether both acidosis induction approaches provoked similar shifts in the milk fatty acid (FA) profile. Similar changes of the odd- and branched-chain FA and the C18 biohydrogenation intermediates were observed in the alfalfa-based SARA induction experiment and the grain-based SARA induction experiment, although they were more pronounced in the former. The proportion of *trans*-10 C18:1 in the last week of the alfalfa-based induction experiment was 6 times higher than the proportion measured during the control week. The main difference between both induction experiments under similar rumen pH changes was the decreasing sum of *iso* FA during the grain-based SARA induction experiment whereas the sum of *iso* FA remained stable during the alfalfa-based SARA induction experiment. The cellulolytic bacterial community seemed to be negatively affected by either the presence of *E. coli* and the associated lipopolysaccharide accumulation in the rumen or by the amount of starch and quickly fermentable carbohydrates in the diet. In general, changes in the milk FA profile were related to changes in rumen pH. Nevertheless, feed characteristics (low in structural fiber vs. high in starch) also affected the milk FA profile and, as such, both effects should be taken into account when subacute acidosis occurs.
Key words: milk fatty acid, subacute ruminal acidosis

INTRODUCTION

Subacute ruminal acidosis is one of the most important metabolic disorders in intensive dairy farms and affects rumen fermentation, animal welfare, productivity, and farm profitability (e.g., increased veterinary costs and decreased fertility and productivity; Morgante et al., 2007). Several studies have investigated the etiology and pathophysiology of SARA (Gozho et al., 2005; Krause and Oetzel, 2005; Dohme et al., 2008). The main SARA induction protocol used in these studies relied on increasing the amount of quickly fermentable carbohydrates through increasing dietary proportions of grain. However, SARA can also be provoked by an insufficient amount of physically effective fiber in the diet (Mertens, 1997; Kleen et al., 2009). An experiment in which SARA was induced by the reduction of physically effective fiber was adopted by Khafipour et al. (2009b), in which alfalfa hay was gradually replaced by pellets consisting of ground alfalfa hay. Differences in blood parameters and rumen microbial population between the grain-induced and the alfalfa-induced SARA experiments have been described by Khafipour et al. (2009a,b,c). The main metabolic differences between the two protocols were the presence of blood LPS and higher numbers of *Escherichia coli* in the rumen of animals suffering from SARA in the grain-based SARA induction experiment. The lowest rumen pH levels were recorded in the alfalfa-induced acidotic animals, but disease-associated blood parameters, such as blood LPS, were not elevated in the latter situation.

Recent research has focused on the identification of biomarkers in milk for the detection of SARA. Milk fat depression has been associated with a decrease in rumen pH and the milk FA profile also showed potential as *trans* C18:1 FA and odd- and branched-chain

Received August 31, 2012.
Accepted March 9, 2013.
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FA (**OBCFA**) were related to SARA (Enjalbert et al., 2008; Colman et al., 2010). As the concentration in milk fat of these milk fatty acids depends on the ruminal microbial population, which differed between both acidosis induction experiments (Khafipour et al., 2009a, 2009b, 2009c), the goal of the current analysis was to assess whether these different microbial populations were associated with differences in the milk fatty acid profile.

MATERIALS AND METHODS

Sample Collection

The current study is based on milk samples collected during 2 different acidosis induction experiments, which were described by Khafipour et al. (2009a) and Khafipour et al. (2009b). Some of the data reported in the earlier publications were reanalyzed. Additionally, milk FA analyses were carried out. As the aim was to relate rumen parameters and milk FA, only the 4 rumen-fistulated animals were used in this manuscript, whereas data from the other 4 non-rumen-fistulated cows were discarded. This might result in a slight deviation from some of the data reported previously. Data from wk 5 and 6 of the grain-based SARA induction study were used for further analysis. In addition, rumen pH parameters of 1 d were linked to the pooled milk sample of that same day and not to the previously reported average values. A brief description of each experimental protocol is provided below.

Experiment 1. Four Holstein cows were used during 2 consecutive 6-wk periods. During wk 1 to 5 of each 6-wk period, cows received a TMR ad libitum with a forage-to-concentrate ratio of 50:50 (wt/wt on a DM basis). During wk 6 of both periods, a SARA challenge was conducted by replacing 21% of the DM of the TMR with pellets containing 50% ground wheat and 50% ground barley, resulting in a forage-to-concentrate ratio of 40:60 (wt/wt on a DM basis). Rumen pH was monitored continuously for 4 consecutive days during wk 5 and 6 of both experiments using indwelling pH probes. Cows were milked twice daily and milk samples were collected during 4 consecutive milkings during both sampling weeks. No preservative was used and milk samples were immediately frozen.

Experiment 2. Four Holstein cows were used in a 6-wk study. During wk 1, cows received a TMR that contained 50% of DM as concentrate and 50% of DM as chopped alfalfa hay. From wk 2 to 6, alfalfa hay was gradually replaced by alfalfa pellets at a rate of 8% per week to induce SARA. Rumen pH was monitored continuously for 5 consecutive days during each week of the experiment using indwelling pH probes. Cows were milked twice daily and milk samples were collected during 4 consecutive milkings during each sampling week. No preservative was used and milk samples were immediately frozen.

Sample Measurements

Milk Analysis. Milk samples of the evening and the morning after the pH registration day were sampled and pooled by volume for further analysis. In the first experiment, milk from 2 d in wk 5 (control) and wk 6 (SARA) of both periods was analyzed (n = 32). In the second experiment, milk was sampled weekly on 2 d (n = 48). Milk samples were stored at −20°C until being analyzed for FA composition. Milk FA were quantified by GC after extraction (Chouinard et al., 1997) and methylation (Stefanov et al., 2010) and were expressed as grams per 100 g of FA methyl esters. Tridecanoic acid (as triacylglyceride; Sigma, Bornem, Belgium) was added as internal standard to assess the accuracy of the chromatograms.

GC Analysis. Analysis of the FA was done by GC (HP 7890A; Agilent Technologies Belgium NV, Diegem, Belgium) equipped with a 75-m SP-2560 capillary column (i.d.: 0.18 mm; film thickness: 0.14 μm; Supelco Analytical, Bellefonte, PA) and a flame ionization detector. A combination of 2 oven temperature programs was used in this study to achieve separation of most *cis* and *trans* C16:1 and C18:1 isomers according to the method of Kramer et al. (2008) with modifications. A first temperature program was as follows: at the time of sample injection, the column temperature was 70°C for 2 min, which was then increased at 15°C/min to 150°C, followed by a second increase of 1°C/min to 165°C, which was maintained for 12 min, followed by a third increase at 2°C/min to 170°C, which was maintained for 5 min, and a final increase at 5°C/min to 215°C, which was maintained for 10 min. A second temperature program was used to separate most of the coeluting isomers: at the time of sample injection, the column temperature was 70°C, which was then increased at 50°C/min to 175°C and maintained isothermal for 13 min, followed by a second increase at 5°C/min to 215°C, which was maintained for 10 min. For both programs, inlet and detector temperatures were 250 and 255°C, respectively. The split ratio was 100:1. The flow rate for the hydrogen carrier gas was 1 mL/min. Most FA peaks were identified using quantitative mixtures of methyl ester standards (BR2 and BR3, Larodan Fine Chemicals AB, Malmö, Sweden; Supelco 37, Supelco Analytical; PUFA-3, Matreya LLC, Pleasant Gap, PA). Fatty acids for which no standards were available commercially were identified by order of elution according to Precht et al. (2001) and Kramer et al. (2008).

Statistical Analyses

One sample of the grain-induced SARA experiment and 2 samples of the alfalfa-induced SARA experiment were discarded from the data analysis due to faulty measurements of milk fat percentage (11%) and extremely low DMI (2 kg of DM/d). In total, 31 and 46 samples of the grain- and alfalfa-induced SARA experiment were kept for data analysis.

All statistical analyses were performed with SPSS 19.0 software (SPSS Inc., Chicago, IL) or R version 2.12 software (R Foundation for Statistical Computing, Vienna, Austria). Cow performance, rumen pH, and milk composition variables of the grain-induced SARA experiment were compared using the linear mixed model $Y_{ijkl} = \mu + A_i + B_j + C_k + \varepsilon_{ijkl}$, where Y_{ijkl} = response variable, μ = average, A_i = fixed effect of ration, B_j = random effect of cow, C_k = random effect of period, and ε_{ijkl} = residual error term. None of the interaction effects were significant and, hence, they were all excluded from the final model.

Cow performance variables, rumen pH variables, and milk composition variables of the alfalfa-induced SARA experiment were compared using the linear mixed model $Y_{ijk} = \mu + A_i + B_j + \varepsilon_{ijk}$, where Y_{ijk} = response variable, μ = average, A_i = fixed effect of week (diet change), B_j = random effect of cow, and ε_{ijk} = residual error term. The interaction effect was not significant and was left out from the final model. The Bonferroni post-hoc test was performed on both mixed models.

For each sample, during each experiment, time pH <5.0 to time pH <7.2 were calculated with 0.1-pH unit intervals and reported as minutes per day. This led to 23 new pH-related data points for each sample. Based on these data points, a logistic curve was fitted for each sample using R version 2.12, package drc, function drm (Colman et al., 2012). The upper limit was set to 1,440 min/d and 2 parameters for each sample were estimated: $\boldsymbol{\beta_0}$ and $\boldsymbol{\beta_1}$, which represent the slope and the inflection point of the logistic curve, respectively (Colman et al., 2012). The higher the value of β_0, the more stable the rumen pH is throughout the day. A higher value of β_1 indicates a greater average rumen pH.

Pearson correlation coefficients ($\boldsymbol{\tau_p}$) between pH parameters (time pH <5.8, β_0, and β_1) and milk FA were assessed by SPSS 19.0 software. Principal components (**PC**) analysis was performed based on the logistic parameters (β_0 and β_1), milk fat percentage (milk fat %), and milk FA, which are related to either the rumen microbial population or the rumen biohydrogenation (g/100 g of milk fat) and included *anteiso* C13:0, *iso* C13:0, *iso* C14:0, *anteiso* C15:0, *iso* C15:0, C15:0, *iso* C16:0, *iso* C17:0, *anteiso* C17:0, C17:0, *cis*-9 C17:1, *trans*-10 C18:1, *trans*-11 C18:1, *cis*-9,*trans*-11 C18:2, *trans*-10,*cis*-12 C18:2, and *trans*-11,*cis*-15 C18:2; and rumen parameters (minimum pH, average pH, area under the curve pH <5.8 or <6.0, and time pH <5.8 or <6.0) using SPSS 19.0 software (SPSS Inc.).

RESULTS

Generally, cow or period (experiment 1 only) effects on rumen pH parameters and milk FA were not significant and, hence, these results are not presented in detail.

Feed Intake and Milk Production

Dry matter intake increased in the grain-based SARA induction experiment from 15.4 to 18.1 kg/d as 21% of the DM of the TMR was replaced with pellets containing 50% ground wheat and 50% ground barley (Table 1). No effect on milk yield or milk fat content was recorded, but milk protein content tended to increase. No statistical difference in pH parameters was observed based on the pH measurements taken into account for the present data analysis, although time and area under the curve below pH 5.6, 5.8, and 6.0 numerically increased. Nevertheless, on average, SARA was not induced by the grain-based SARA induction experiment, based on the threshold values of Gozho et al. (2005; time pH <5.6 = 180 min) or AlZahal et al. (2007; time pH <5.8 = 475 min/d), although some cows did reach SARA threshold values (9/31 or 7/31 observations, based on threshold values of time pH <5.6 = 180 min/d or time pH <5.8 = 475 min/d, respectively). The alfalfa-based SARA induction experiment was more effective in decreasing rumen pH with the lowest average rumen pH (5.84) and the longest time below pH 5.8 (781 min/d) recorded in wk 6 (Table 2). As such, SARA was successfully induced during this experiment. The greatest shift in pH parameters was recorded when 48% of the alfalfa hay was replaced with pellets of ground alfalfa hay (wk 4). Although DMI increased during the alfalfa experiment compared with the control week, milk yield decreased. At the same time, the milk fat percentage decreased to 1.9% in wk 6.

Rumen pH and Milk FA Changes Related to the Acidosis Induction Experiment

Although the grain-based SARA induction experiment did not change average rumen pH measurements, shifts in the milk FA pattern were observed. As such, the proportion of short- and medium-chain FA (C6:0 to C14:0) increased in the milk fat, whereas the proportion of C18:0 and *iso* FA decreased as cows received

Table 1. Effect of a grain-based SARA induction diet on cow performance variables and rumen parameters

Variable	Mean	SEM[1]	Diet		*P*-value[2]
			Control	Acidosis	
n			16	15	
DMI (kg/d)	16.7	0.88	15.4	18.1	**
Milk yield (L/d)	29.1	4.72	30.3	27.8	0.16
Milk fat (%)	3.22	0.329	3.30	3.15	0.35
Milk protein (%)	3.44	0.210	3.34	3.55	†
FPCM[3] (L/d)	26.0	3.45	27.4	24.7	†
Average pH	6.13	0.042	6.15	6.11	0.39
Minimum pH	5.38	0.031	5.39	5.37	0.72
Maximum pH	6.94	0.034	6.98	6.90	0.22
Time pH <5.6[4] [min/d (no. of acidotic cases)]	144	45.3	118 (5)	171 (4)	0.33
Time pH <5.8[5] [min/d (no. of acidotic cases)]	312	59.3	274 (3)	350 (4)	0.32
Time pH <6.0 (min/d)	524	72.6	495	554	0.49
AUC pH <5.6[6] (Δ pH × min/d)	22.5	9.27	15.8	29.2	0.27
AUC pH <5.8 (Δ pH × min/d)	66.7	19.5	54.0	79.4	0.30
AUC pH <6.0 (Δ pH × min/d)	150	32.4	129	170	0.30
β_0[7]	5.09	0.307	5.02	5.17	0.80
β_1[7]	6.13	0.044	6.15	6.10	0.40

[1]Weighted SEM.

[2]*P*-values according to the linear mixed model $Y_{ijkl} = \mu + A_i + B_j + C_k + \varepsilon_{ijkl}$, where Y_{ijkl} = response variable, μ = average, A_i = fixed effect of ration, B_j = random effect of cow, C_k = random effect of period, and ε_{ijkl} = residual error term.

[3]Fat- and protein-corrected milk.

[4]Number of acidotic cases based on threshold value time pH <5.6 = 180 min/d (Gozho et al., 2005).

[5]Number of acidotic cases based on threshold value time pH <5.8 = 475 min/d (AlZahal et al., 2007).

[6]AUC = area under the curve.

[7]Parameters of the logistic curve: $y = 1{,}440/\{1 + \exp[-\beta_0 \times (x - \beta_1)]$, where β_0 is related to the rumen pH variation throughout the day and β_1 is related to average rumen pH.

**$0.001 < P < 0.01$; †$0.05 < P < 0.10$.

the grain-based diet (Tables 3, 4, and 5). On the other hand, C15:0 and *trans*-10 C18:1 proportions increased (Tables 4 and 5). In the case of the conjugated linoleic acids, *cis*-9,*trans*-11 C18:2 decreased, whereas *trans*-10,*cis*-12 C18:2 remained constant.

Similar milk FA changes of the OBCFA and the C18 biohydrogenation intermediates were observed in the alfalfa-based SARA induction experiment, although they were more pronounced (Tables 6, 7, and 8). The proportion of *trans*-10 C18:1 in the last week of the alfalfa experiment was 6 times greater than the proportion measured during the control week. Besides the changes reported for the grain-based SARA induction experiment, decreasing proportions of *trans*-11 C18:1 and increasing proportions of *trans*-10,*cis*-12 C18:2 were observed. In contrast to the grain-based SARA induction experiment, the sum of the short- and medium-chain FA (C6:0–C16:0) decreased during the alfalfa-based SARA induction experiment.

Changes in milk FA proportions were also compared across both experiments. To allow for a fair comparison, similar rumen pH changes should be considered. In this way, it could be assessed whether the acidosis induction strategy provokes shifts in the milk FA profile, irrespective of rumen pH. As rumen pH parameters of the acidotic week in the grain-based SARA induction experiment were similar to the pH parameters recorded in wk 2 and 3 of the alfalfa-based SARA induction experiment, the latter weeks were considered for comparison with wk 6 of the grain-based SARA induction experiment. Nevertheless, β_0 values were lower in the alfalfa-based SARA induction experiment compared with the grain-based SARA induction experiment, reflecting a greater daily rumen pH range. Not only rumen pH but also the diet has an influence on the milk FA profile, leading to a different control milk FA profile in each experiment. As such, relative changes in the milk FA to the control treatment of each experiment were compared. The most important difference between the grain- and alfalfa-based SARA induction experiments under similar pH changes was the decreasing sum of milk *iso* FA during the grain-based SARA induction experiment, whereas the sum of *iso* FA remained stable during the alfalfa-based SARA induction experiment. As for the other FA (i.e., odd-chain FA and C18 biohydrogenation intermediates), changes were similar across both experiments.

Principal Components Analysis

Principal components analysis was performed to study the relationship between rumen pH and milk

Table 2. Effect of an alfalfa-based SARA induction diet on cow performance variables and rumen parameters

Variable	Mean	SEM[1]	Week						*P*-value[2]
			1	2	3	4	5	6	
Alfalfa hay (% of DM)			50	42	34	26	18	10	
Alfalfa pellets (% of DM)			0	8	16	24	32	40	
n			8	8	6	8	8	8	
DMI (kg/d)	20.7	0.58	17.0^{a}	20.3^{b}	20.9^{b}	21.1^{b}	22.5^{b}	22.5^{b}	***
Milk yield (L/d)	33.3	2.87	36.2^{a}	34.6^{ab}	34.1^{ab}	33.1^{abc}	32.2^{bc}	30.0^{c}	***
Milk fat (%)	2.50	0.181	2.99^{a}	2.89^{ab}	2.54^{b}	2.57^{b}	2.09^{c}	1.90^{c}	***
Milk protein (%)	3.53	0.179	3.09^{a}	3.15^{a}	3.40^{ab}	3.55^{bc}	3.86^{cd}	4.12^{d}	***
FPCM[3] (L/d)	27.8	1.81	31.4^{a}	29.5^{ab}	28.4^{abc}	28.0^{bc}	25.8^{cd}	23.8^{d}	***
Average pH	6.08	0.041	6.30^{a}	6.29^{a}	6.30^{a}	5.89^{b}	5.87^{b}	5.84^{b}	***
Minimum pH	5.19	0.031	5.27	5.15	5.29	5.09	5.15	5.20	0.43
Maximum pH	6.98	0.038	7.06	7.06	7.07	6.84	6.94	6.92	*
Time pH <5.6[4] [min/d (no. of acidotic cases)]	337	45.3	135^{a} (4)	178^{ab} (3)	212^{abc} (3)	531^{c} (7)	476^{abc} (6)	490^{abc} (8)	**
Time pH <5.8[5] [min/d (no. of acidotic cases)]	538	47.4	238^{a} (0)	329^{a} (1)	348^{a} (3)	768^{b} (6)	764^{b} (7)	781^{b} (8)	***
Time pH <6.0 (min/d)	693	42.6	380^{a}	477^{a}	420^{a}	936^{b}	937^{b}	1,007^{b}	***
AUC pH <5.6[6] (Δ pH $\times$ min/d)	65.2	11.6	26.0	39.2	49.8	93.2	99.1	83.6	†
AUC pH <5.8 (Δ pH $\times$ min/d)	153	21.3	62.7^{a}	90.0^{ab}	106^{ab}	225^{b}	225^{b}	210^{ab}	**
AUC pH <6.0 (Δ pH $\times$ min/d)	277	29.9	124^{a}	171^{a}	182^{ab}	395^{b}	397^{b}	392^{b}	***
β_0[7]	4.16	0.382	5.12	3.00	4.31	4.32	3.79	4.44	0.40
β_1[7]	6.07	0.044	6.32^{a}	6.31^{a}	6.30^{a}	5.87^{b}	5.83^{b}	5.80^{b}	***

[a–d]Means within a row with different superscripts differ ($P < 0.05$).

[1]Weighted SEM.

[2]*P*-values according to the linear mixed model $Y_{ijk} = \mu + A_i + B_j + \varepsilon_{ijk}$, where Y_{ijk} = response variable, μ = average, A_i = fixed effect of week (diet change), B_j = random effect of cow, and ε_{ijk} = residual error term.

[3]Fat- and protein-corrected milk.

[4]Number of acidotic cases based on threshold value time pH <5.6 = 180 min/d (Gozho et al., 2005).

[5]Number of acidotic cases based on threshold value time pH <5.8 = 475 min/d (AlZahal et al., 2007).

[6]AUC = area under the curve.

[7]Parameters of the logistic curve: $y = 1{,}440/\{1 + \exp[-\beta_0 \times (x - \beta_1)]\}$, where β_0 is related to the rumen pH variation throughout the day and β_1 is related to average rumen pH.

*$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$; †$0.05 < P < 0.10$.

Table 3. Effect of a grain-based SARA induction diet on milk short- and medium-chain FA proportions (g/100 g of FA)

			Diet		
Variable	Mean	SEM[1]	Control	Acidosis	*P*-value[2]
n			16	15	
C4:0	3.00	0.127	3.19	2.80	**
C6:0	1.46	0.061	1.41	1.51	***
C8:0	0.812	0.052	0.733	0.891	0.23
C10:0	1.82	0.123	1.54	2.10	***
C12:0	2.24	0.163	1.92	2.56	**
C14:0	8.06	0.738	7.53	8.58	***
cis-9 C14:1	0.859	0.215	0.817	0.902	***
C16:0	22.2	0.689	20.9	23.6	†
cis-9 C16:1	1.20	0.158	1.11	1.29	***

[1]Weighted SEM.

[2]*P*-values according to the linear mixed model $Y_{ijkl} = \mu + A_i + B_j + C_k + \varepsilon_{ijkl}$, where Y_{ijkl} = response variable, μ = average, A_i = fixed effect of ration, B_j = random effect of cow, C_k = random effect of period, and ε_{ijkl} = residual error term.

**$0.001 < P < 0.01$; ***$P < 0.001$; †$0.05 < P < 0.10$.

FA. The first 3 PC of the grain-based SARA induction experiment explained 68.5% of the total variance (Figure 1). The main variables related to PC 1 were rumen pH characteristics (e.g., time pH <5.8, average pH, and β_1). Principal component 2 mainly consisted of biohydrogenation intermediates (*trans*-11,*cis*-15 C18:2 and *trans*-10,*cis*-12 C18:2) and long-chain OBCFA (>C15). Principal component 3 mainly distinguished between the latter and C15:0. Rumen parameters such as time pH <5.8 were negatively correlated with mainly *iso* C14:0 ($\tau_p = -0.363$; $P < 0.05$; Figure 1a) and *iso* C16:0 ($\tau_p = -0.497$; $P < 0.01$; Figure 1a) and positively correlated with *anteiso* C13:0 ($\tau_p = 0.370$; $P < 0.05$; Figure 1a). The FA *trans*-11,*cis*-15 C18:2 ($\tau_p = 0.038$; $P = 0.84$; Figure 1a) and *trans*-10,*cis*-12 C18:2 ($\tau_p = -0.058$; $P = 0.76$; Figure 1a) varied independently from rumen pH changes, as also illustrated by their high correlation with PC2. As parameter β_1 of the logistic curve was negatively correlated with time pH <5.8 ($\tau_p = -0.853$; $P < 0.001$; Figure 1a), correlations

Table 4. Effect of a grain-based SARA induction diet on milk C18 saturated and unsaturated FA proportions (g/100 g of FA)

			Diet		
Variable	Mean	SEM[1]	Control	Acidosis	*P*-value[2]
n			16	15	
C18:0	11.2	0.628	12.5	9.9	***
trans-6–8 C18:1	0.599	0.082	0.565	0.633	0.26
trans-9 C18:1	0.463	0.043	0.470	0.457	0.70
trans-10 C18:1	1.86	0.959	1.15	2.58	**
trans-11 C18:1	3.33	0.377	3.42	3.25	0.67
trans-12 C18:1	0.608	0.021	0.652	0.565	**
trans C18:1 + *cis*-14 C18:1	0.512	0.039	0.586	0.439	***
cis-9 C18:1	23.8	0.901	25.7	21.9	**
cis-11 C18:1	0.694	0.050	0.580	0.809	***
cis-12 C18:1	0.383	0.084	0.375	0.390	0.64
cis-13 C18:1	0.098	0.017	0.093	0.104	0.17
cis-15 C18:1	0.331	0.078	0.388	0.275	0.27
cis-9,*trans*-11 C18:2	1.68	0.156	1.82	1.53	**
trans-10,*cis*-12 C18:2	0.015	0.004	0.014	0.017	0.11
trans-11,*cis*-15 C18:2	0.283	0.075	0.244	0.323	†
C18:3n-3	0.520	0.077	0.497	0.544	0.10
C18:2n-6	3.03	0.399	2.72	3.34	***
C18:3n-6	0.028	0.005	0.027	0.030	0.33

[1]Weighted SEM.

[2]*P*-values according to the linear mixed model $Y_{ijkl} = \mu + A_i + B_j + C_k + \varepsilon_{ijkl}$, where Y_{ijkl} = response variable, μ = average, A_i = fixed effect of ration, B_j = random effect of cow, C_k = random effect of period, and ε_{ijkl} = residual error term.

**$0.001 < P < 0.01$; ***$P < 0.001$; †$0.05 < P < 0.10$.

Table 5. Effect of a grain-based SARA induction diet on milk odd- and branched-chain FA proportions (g/100 g of FA)

Variable	Mean	SEM[1]	Diet		*P*-value[2]
			Control	Acidosis	
n			16	15	
iso C13:0	0.026	0.002	0.029	0.024	**
iso C14:0	0.078	0.006	0.089	0.067	***
iso C15:0	0.148	0.005	0.167	0.129	***
iso C16:0	0.224	0.013	0.253	0.194	***
iso C17:0	0.296	0.036	0.320	0.272	**
iso C18:0	0.077	0.023	0.076	0.077	0.91
anteiso C13:0	0.009	0.001	0.009	0.009	0.47
anteiso C15:0	0.364	0.013	0.389	0.338	**
anteiso C17:0	0.401	0.033	0.420	0.382	†
C15:0	0.931	0.048	0.859	1.00	***
C17:0	0.620	0.101	0.643	0.597	0.34
cis-9 C17:1	0.195	0.049	0.195	0.196	0.97

[1]Weighted SEM.

[2]*P*-values according to the linear mixed model $Y_{ijkl} = \mu + A_i + B_j + C_k + \varepsilon_{ijkl}$, where Y_{ijkl} = response variable, μ = average, A_i = fixed effect of ration, B_j = random effect of cow, C_k = random effect of period, and ε_{ijkl} = residual error term.

**$0.001 < P < 0.01$; ***$P < 0.001$; †$0.05 < P < 0.10$.

of this parameter with milk FA were opposite to the ones mentioned before for time pH <5.8. Parameter β_0 was positively correlated with *iso* C16:0 ($\tau_p = 0.421$; $P < 0.05$; Figure 1c), *trans*-11 C18:1 ($\tau_p = 0.529$; $P < 0.01$; Figure 1a), and *trans*-11,*cis*-15 C18:2 ($\tau_p = 0.407$; $P < 0.05$; Figure 1b). The parameters of the logistic curve varied independently from each other ($\tau_p = 0.167$; $P = 0.37$).

In the case of the alfalfa-based SARA induction experiment, 71.1% of the total variance was explained with the first 3 PC (Figure 2). Similarly to the grain-based SARA induction experiment, PC1 correlated mainly with rumen parameters, whereas the main components of PC2 were *trans*-11,*cis*-15 C18:2 and *anteiso* C15:0. The main components of PC3 were *iso* branched-chain FA and parameter β_0. Similarly as for the grain-based SARA induction experiment, time pH <5.8 was negatively correlated with parameter β_1 ($\tau_p = -0.961$; $P < 0.01$; Figure 2a). Parameter β1 was mainly negatively correlated with *iso* C18:0 ($\tau_p = -0.561$; $P < 0.01$), *trans*-10 C18:1 ($\tau_p = -0.596$; $P < 0.01$), C15:0 ($\tau_p = -0.725$; $P < 0.01$; Figure 2a), and C17:0 ($\tau_p = -0.411$; $P < 0.01$; Figure 2a) and mainly positively correlated with *trans*-11 C18:1 ($\tau_p = 0.660$; $P < 0.01$; Figure 2a). Parameter β_0 correlated with *iso* C14:0 ($\tau_p = 0.499$; $P < 0.05$; Figure 2c). The parameters of the logistic curve

Table 6. Effect of an alfalfa-based SARA induction diet on milk short- and medium-chain FA proportions (g/100 g of FA)

Variable	Mean	SEM[1]	Week						*P*-value[2]
			1	2	3	4	5	6	
Alfalfa hay (% of DM)			50	42	34	26	18	10	
Alfalfa pellets (% of DM)			0	8	16	24	32	40	
n			8	8	6	8	8	8	
C4:0	2.75	0.296	3.47^{a}	3.29^{a}	2.85^{a}	2.98^{a}	2.18^{b}	1.73^{b}	***
C6:0	1.12	0.120	1.51^{c}	1.39^{bc}	1.15^{b}	1.18^{b}	0.832^{a}	0.669^{a}	***
C8:0	0.575	0.061	0.774^{c}	0.685^{bc}	0.588^{b}	0.611^{b}	0.440^{a}	0.355^{a}	***
C10:0	1.27	0.116	1.62^{c}	1.44^{bc}	1.31^{b}	1.36^{bc}	1.04^{a}	0.845^{a}	***
C12:0	1.74	0.102	1.91^{b}	1.72^{ab}	1.77^{ab}	1.82^{b}	1.70^{ab}	1.54^{a}	**
C14:0	6.93	0.398	7.30^{ab}	6.91^{ab}	7.08^{ab}	7.06^{ab}	6.93^{ab}	6.32^{a}	*
cis-9 C14:1	0.913	0.110	0.593^{a}	0.578^{a}	0.845^{ab}	0.884^{b}	1.23^{c}	1.35^{c}	***
C16:0	19.0	0.294	18.1^{a}	19.0^{ab}	18.5^{ab}	19.5^{b}	19.7^{b}	19.5^{b}	***
cis-9 C16:1	1.29	0.228	0.670^{a}	0.836^{ab}	1.10^{ab}	1.24^{bc}	1.70^{c}	2.21^{d}	***

[a–d]Means within a row with different superscripts differ ($P < 0.05$).

[1]Weighted SEM.

[2]*P*-values according to the linear mixed model $Y_{ijk} = \mu + A_i + B_j + \varepsilon_{ijk}$, where Y_{ijk} = response variable, μ = average, A_i = fixed effect of week (diet change), B_j = random effect of cow, and ε_{ijk} = residual error term.

*$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$.

Table 7. Effect of an alfalfa-based SARA induction diet on milk C18 saturated and unsaturated FA proportions (g/100 g of FA)

			Week						
Variable	Mean	SEM[1]	1	2	3	4	5	6	*P*-value[2]
Alfalfa hay (% of DM)			50	42	34	26	18	10	
Alfalfa pellets (% of DM)			0	8	16	24	32	40	
C18:0	10.8	0.55	13.4^{d}	12.4^{cd}	11.4^{bc}	10.2^{b}	8.96^{a}	8.36^{a}	***
trans-6–8 C18:1	0.974	0.062	0.749^{a}	0.740^{a}	1.01^{b}	0.815^{a}	1.14^{b}	1.38^{c}	***
trans-9 C18:1	0.724	0.041	0.585^{a}	0.541^{a}	0.727^{b}	0.635^{ab}	0.837^{c}	1.02^{d}	***
trans-10 C18:1	3.46	0.439	1.03^{a}	1.59^{a}	3.37^{b}	3.11^{b}	5.31^{c}	6.38^{c}	***
trans-11 C18:1	3.50	0.288	4.56^{b}	5.01^{b}	2.94^{a}	3.33^{a}	2.87^{a}	2.29^{a}	***
trans-12 C18:1	0.809	0.014	0.949^{b}	0.805^{a}	0.780^{a}	0.780^{a}	0.783^{a}	0.760^{a}	**
trans-16 C18:1 + *cis*-14 C18:1	0.551	0.012	0.659^{b}	0.554^{a}	0.516^{a}	0.547^{a}	0.531^{a}	0.501^{a}	***
cis-9 C18:1	23.5	0.45	23.6^{ab}	23.7^{ab}	25.2^{b}	23.6^{ab}	22.5^{a}	22.6^{a}	**
cis-11 C18:1	0.875	0.056	0.707^{ab}	0.670^{a}	0.936^{c}	0.831^{bc}	0.961^{c}	1.15^{d}	***
cis-12 C18:1	0.754	0.074	0.834^{ab}	0.869^{b}	0.591^{a}	0.876^{b}	0.716^{ab}	0.640^{ab}	**
cis-13 C18:1	0.128	0.009	0.091^{a}	0.096^{ab}	0.118^{bc}	0.128^{c}	0.159^{d}	0.178^{d}	***
cis-15 C18:1	0.375	0.026	0.273^{a}	0.263^{a}	0.404^{bc}	0.340^{ab}	0.457^{cd}	0.515^{d}	***
cis-9,*trans*-11 C18:2	1.84	0.111	1.96	2.11	1.64	1.79	1.89	1.67	*
trans-10,*cis*-12 C18:2	0.032	0.003	0.016^{a}	0.019^{a}	0.026^{ab}	0.029^{b}	0.047^{c}	0.056^{c}	***
trans-11,*cis*-15 C18:2	0.366	0.054	0.254^{a}	0.331^{ab}	0.381^{ab}	0.376^{ab}	0.417^{b}	0.440^{b}	**
C18:3n-3	0.859	0.073	0.732^{a}	0.763^{ab}	0.829^{abc}	0.903^{bcd}	0.922^{cd}	1.00^{d}	***
C18:2n-6	5.06	0.473	4.41^{a}	4.77^{a}	4.58^{a}	5.10^{ab}	5.61^{bc}	5.91^{c}	***
C18:3n-6	0.035	0.004	0.037	0.034	0.040	0.034	0.036	0.028	0.14

[a–d]Means within a row with different superscripts differ ($P < 0.05$).

[1]Weighted SEM.

[2]*P*-values according to the linear mixed model $Y_{ijk} = \mu + A_i + B_j + \varepsilon_{ijk}$, where Y_{ijk} = response variable, μ = average, A_i = fixed effect of week (diet change), B_j = random effect of cow, and ε_{ijk} = residual error term.

*$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$.

varied independently from each other ($\tau_p = 0.192$; $P = 0.20$).

DISCUSSION

The etiology and pathophysiology of SARA has been an important research topic for already quite some years. Khafipour et al. (2009a,b) investigated the effect of a grain- versus an alfalfa-based SARA induction experiment on blood parameters and the rumen microbial population. According to their results, SARA was successfully induced in both experiments, as in both cases the threshold value of Gozho et al. (2005; time pH <5.6 = 180 min/d) was reached. However, in the smaller data set used in the present analysis, no overall effect of

Table 8. Effect of an alfalfa-based SARA induction diet on milk odd- and branched-chain FA proportions (g/100 g of FA)

			Week						
Variable	Mean	SEM[1]	1	2	3	4	5	6	*P*-value[2]
Alfalfa hay (% of DM)			50	42	34	26	18	10	
Alfalfa pellets (% of DM)			0	8	16	24	32	40	
iso C13:0	0.025	0.001	0.031^{c}	0.030^{c}	0.029^{bc}	0.024^{abc}	0.021^{ab}	0.019^{a}	***
iso C14:0	0.062	0.006	0.069^{b}	0.067^{b}	0.065^{b}	0.063^{b}	0.056^{ab}	0.048^{a}	***
iso C15:0	0.126	0.006	0.158^{c}	0.145^{bc}	0.129^{bc}	0.131^{b}	0.101^{a}	0.158^{c}	***
iso C16:0	0.173	0.011	0.177	0.164	0.159	0.183	0.179	0.176	0.36
iso C17:0	0.310	0.014	0.291^{ab}	0.322^{ab}	0.306^{ab}	0.338^{b}	0.333^{b}	0.268^{a}	*
iso C18:0	0.071	0.003	0.059^{a}	0.060^{a}	0.062^{a}	0.070^{ab}	0.083^{bc}	0.092^{c}	***
anteiso C13:0	0.010	0.001	0.008^{a}	0.009^{a}	0.011^{ab}	0.009^{ab}	0.011^{ab}	0.013^{b}	**
anteiso C15:0	0.366	0.018	0.404^{c}	0.377^{bc}	0.366^{bc}	0.409^{c}	0.331^{ab}	0.311^{a}	***
anteiso C17:0	0.382	0.022	0.424^{b}	0.389^{ab}	0.339^{a}	0.401^{ab}	0.382^{ab}	0.357^{a}	**
C15:0	0.909	0.026	0.737^{a}	0.744^{a}	0.898^{b}	0.995^{bc}	1.02^{c}	1.06^{c}	***
C17:0	0.562	0.014	0.513^{a}	0.555^{abc}	0.526^{ab}	0.613^{c}	0.582^{bc}	0.581^{bc}	***
cis-9 C17:1	0.184	0.016	0.117^{a}	0.139^{a}	0.149^{a}	0.200^{b}	0.221^{b}	0.279^{c}	***

[a–c]Means within a row with different superscripts differ ($P < 0.05$).

[1]Weighted SEM.

[2]*P*-values according to the linear mixed model $Y_{ijk} = \mu + A_i + B_j + \varepsilon_{ijk}$, where Y_{ijk} = response variable, μ = average, A_i = fixed effect of week (diet change), B_j = random effect of cow, and ε_{ijk} = residual error term.

*$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$.

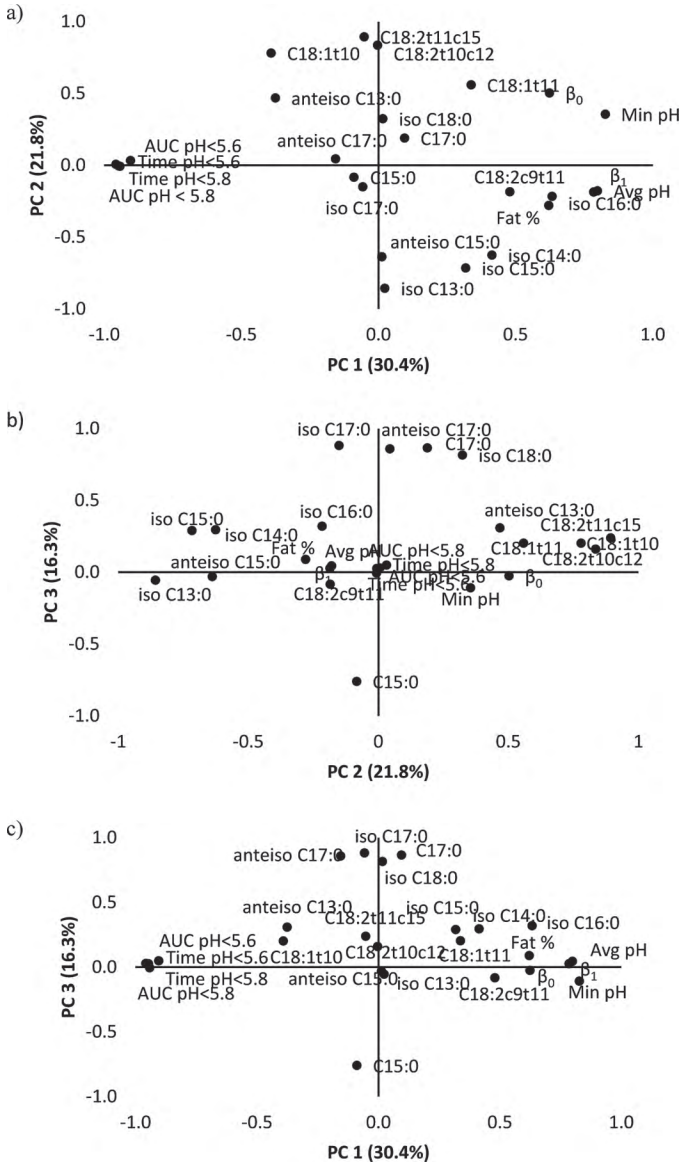

Figure 1. Plots presenting loadings of the first 3 principal components (PC) based on data from the grain-based SARA induction experiment based on rumen parameters [minimum pH (Min pH), average pH (Avg pH), area under the curve (AUC) pH <5.6 or <5.8, and time pH <5.6 or <5.8]; milk fat percentage (Fat %); FA proportions in milk fat (g/100 g of FA) of *anteiso* C13:0, *iso* C13:0, *iso* C14:0, *iso* C15:0, *anteiso* C15:0, C15:0, *iso* C16:0, *iso* C17:0, *anteiso* C17:0, C17:0, *iso* C18:0, *trans*-10 C18:1, *trans*-11 C18:1, *cis*-9,*trans*-11 C18:2, *trans*-10,*cis*-12 C18:2, and *trans*-11,*cis*-15 C18:2; and the parameters of the logistic curve (β_0 and β_1; n = 31). (a) PC 1 versus PC 2; (b) PC 2 versus PC 3; (c) PC 1 versus PC 3.

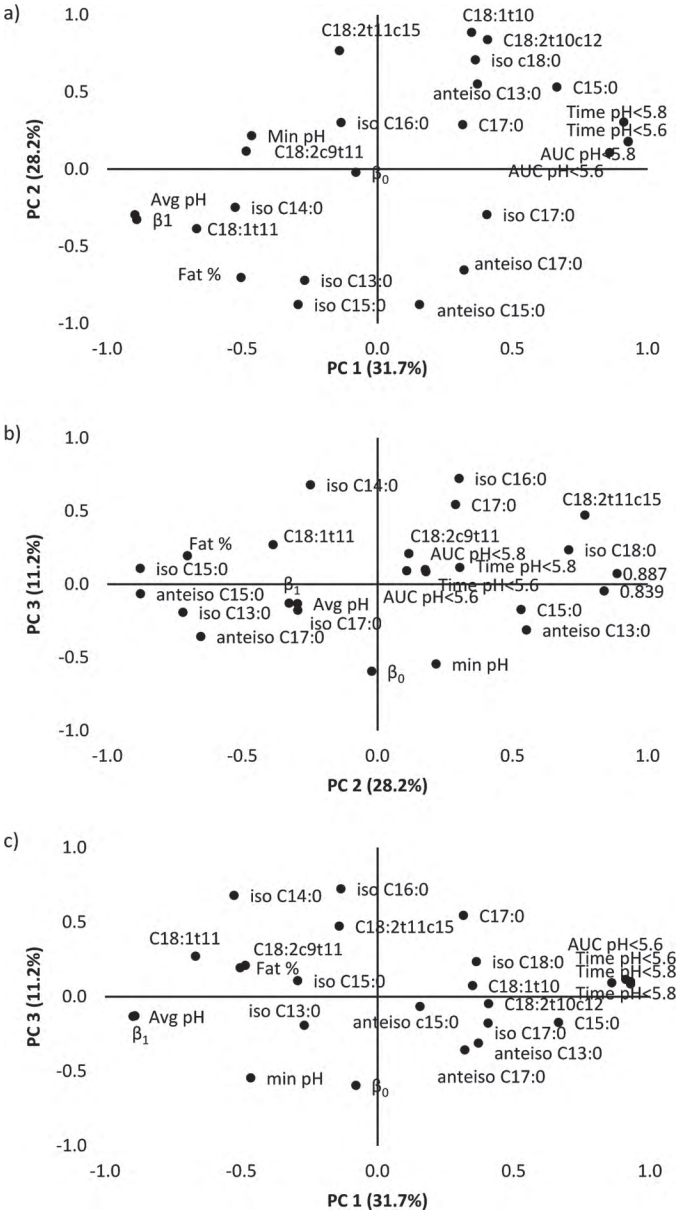

Figure 2. Plots presenting loadings of the first 3 principal components (PC) based on data from the alfalfa-based SARA induction experiment based on rumen parameters [pH minimum pH (Min pH), average pH (Avg pH), area under the curve (AUC) pH <5.6 or <5.8, and time pH <5.6 or <5.8]; milk fat percentage (Fat %); FA proportions in milk fat (g/100 g of FA) of *anteiso* C13:0, *iso* C13:0, *iso* C14:0, *iso* C15:0, *anteiso* C15:0, C15:0, *iso* C16:0, *iso* C17:0, *anteiso* C17:0, C17:0, *iso* C18:0, *trans*-10 C18:1, *trans*-11 C18:1, *cis*-9,*trans*-11 C18:2, *trans*-10,*cis*-12 C18:2, and *trans*-11,*cis*-15 C18:2; and the parameters of the logistic curve (β_0 and β_1; n = 46). (a) PC 1 versus PC 2; (b) PC 2 versus PC 3; (c) PC 1 versus PC 3.

the grain-based SARA induction diet was found on rumen pH parameters. Khafipour et al. (2009a) used pH measurements of 5 d per experimental week, whereas pH measurements of only 2 d, corresponding to the milk samplings, were used in the current data analysis, allowing linking both parameter sets.

Although the pH parameters did not change significantly during the days of the grain-based SARA induction experiment used for the purpose of this study, numerical differences of the pH parameters along with changes in the milk FA profile were recorded. Increas-

ing proportions of *trans*-10 C18:1 were observed, which are related to an incomplete and secondary ruminal biohydrogenation pathway (Martin and Jenkins, 2002; Enjalbert et al., 2008). Changes in the ruminal biohydrogenation pathway could be also associated with accumulation of conjugated linoleic acid intermediates (e.g., *trans*-10,*cis*-12 C18:2). However, no effect on *trans*-10,*cis*-12 C18:2 was observed in the grain-based SARA induction experiment, whereas *trans*-10,*cis*-12 C18:2 accumulated during the alfalfa-induced SARA experiment. Accumulation of *trans*-10 C18:1 was lower in the grain-based SARA induction experiment compared with the alfalfa-based SARA induction experiment. As such, acidosis does not only result in negative effects on the health of dairy cows, but also on the health of humans as the proportion of milk *trans* FA is higher in milk from acidotic cows.

Possibly, increasing amounts of *trans* MUFA, in particular *trans*-10 C18:1, in the rumen led to a retardation of the biohydrogenation process and, thus, accumulation of *trans*-10,*cis*-12 C18:2. This was suggested by Vlaeminck et al. (2008), who observed a delay in the formation of hydrogenation intermediates when *trans* FA accumulated in the rumen. Bauman and Griinari (2003) linked the presence of *trans*-10,*cis*-12 C18:2 in milk to the occurrence of milk fat depression, which is confirmed in both experiments, as an effect on the milk fat content was observed in the alfalfa-based SARA induction experiment but not in the grain-based SARA induction experiment. Shingfield et al. (2009) suggested that *trans*-10 C18:1 is also implicated in the onset of milk fat depression, although no association between *trans*-10 C18:1 and milk fat content has been suggested from the PC approach of Kadegowda et al. (2008). Nevertheless, the enrichment of *trans*-10 C18:1 in milk (0.47 to 1.11 g/100 g of FA) during postruminal infusions may have been too low to detect effects on mammary lipogenesis. This could explain the lack of effect on the milk fat percentage during the grain-based SARA induction experiment despite the increase in *trans*-10 C18:1.

The shift in the biohydrogenation pathway as observed from the milk FA profile coincided with shifts in the microbial population as reported earlier (Khafipour et al., 2009c). Interestingly, an increase was observed in the abundance of bacteria belonging to the phylum of *Actinobacteria* containing *Propionibacterium acnes*, one of the known producers of *trans*-10,*cis*-12 C18:2 (Lourenço et al., 2010).

In the case of the OBCFA, similar effects as in other experiments (Colman et al., 2010) were observed: decreasing *iso* branched-chain FA proportions and increasing odd-chain FA proportions were observed in both induction experiments, but were more pronounced in the alfalfa-based SARA induction experiment, which is in line with larger pH changes. Increasing proportions of C15:0 during the grain-based SARA induction experiment can be related to the higher abundance of *Megasphaera elsdenii* and *Streptococcus bovis* (Fievez et al., 2012) in the ruminal microbial population. Decreasing *iso* FA might originate from the decreasing abundance of the family *Ruminococcaceae* in the grain-based SARA induction experiment (Khafipour et al., 2009c; Fievez et al., 2012).

Escherichia coli was found in the ruminal microbial population of the grain-based SARA induction samples. However, the effect of *E. coli* in the rumen on the milk FA synthesis and profile has not yet been investigated. This could be due to the low abundance of *E. coli* in rumen samples and, as such, effects on milk FA profile would be rather indirect. As the presence of *E. coli* is associated with increasing amounts of starch in the diet (Diez-Gonzalez et al., 1998), it was not surprising that *E. coli* was found in the grain-based SARA induction experiment. Lipopolysaccharide originating from *E. coli* is known to have suppressive effects on FA synthetase and acetyl-CoA carboxylase, which are related to de novo FA synthesis in the mammary tissue (Pekala et al., 1983; Dong et al., 2011). Although LPS was found in the blood of the cows during the acidosis week in the grain-based SARA induction experiment, de novo synthesis seemed to increase compared with that in the control week. Increasing proportions of C4:0 to C14:0 in the milk when more starch was fed were also found in studies by Enjalbert et al. (2008) and Colman et al. (2010). However, no rumen or blood LPS was analyzed in these studies. The lack of effect of LPS on de novo FA synthesis during the grain-based SARA induction experiment could be due to the low amount of LPS found in the blood (0.043 ng/mL) compared with amounts used during in vitro experiments studying the LPS effect on lipogenic enzymes of the mammary gland (10,000 ng/mL; Pekala et al., 1983; Khafipour et al., 2009a). In the alfalfa-based SARA induction experiment, milk FA originating from de novo synthesis decreased when more alfalfa pellets were added to the diet. This is in accordance with situations of milk fat depression where de novo milk FA synthesis was more suppressed than the uptake of FA from the blood (Griinari et al., 1998).

For diagnosis of SARA, different criteria have been used based on the description of the ruminal pH. Average rumen pH and time rumen pH <5.6 or <5.8 have been regularly used. In a recent study by Colman et al. (2012), parameters of a logistic pH curve (β_0 and β_1) were determined to capture as much information as possible on the daily ruminal pH pattern. The β_0 is related to the rumen pH variation throughout the day,

whereas β_1 is related to the average rumen pH. Based on the correlation and PC analyses of data from the grain- and alfalfa-based SARA induction experiments, various milk FA were linked to these pH parameters. In the grain-based SARA induction experiment, decreasing average rumen pH (β_1) due to an increasing amount of starch in the diet was mainly related to decreasing proportions of *iso* C16:0 and *cis*-9,*trans*-11 C18:2. This is in accordance with the results reported in a grain-based SARA induction experiment performed by Colman et al. (2010). On the other hand, based on the correlation and PC analyses, low proportions of *trans*-11 C18:1 were found in situations with a highly variable pH (low β_0), which confirms the results of Colman et al. (2012). In the case of the alfalfa-based SARA induction experiment, low average rumen pH (low β_1) due to low amounts of dietary structural fiber was related to low proportions of *iso* C14:0 and *trans*-11 C18:1 in the milk. No effect of rumen pH variation, parameterized by β_0, on *trans*-11 C18:1 was observed, which contradicts the results of the grain-induced experiment and the results reported by Colman et al. (2012). In general, milk FA and rumen pH variation were not concomitantly varying, as milk FA were not related to rumen pH variation (β_0).

As differences in the rumen microbial population and blood parameters between grain- and alfalfa-based SARA induction experiments were found (Khafipour et al., 2009c), it was hypothesized that differences would exist in the milk FA profile between both experiments. In general, most differences between the experiments were in line with changes in rumen pH. However, no differences in the *iso* branched-chain FA were observed in the alfalfa-based SARA induction experiment under similar pH conditions as observed in the grain-based SARA induction experiment. This suggests that either the cellulolytic bacteria were more influenced by the starch content and the amount of quickly fermentable carbohydrates of the diet than by the physically effective fiber content or that rumen LPS and *E. coli* and associated changes had an effect on the cellulolytic population. Nevertheless, a decrease in milk *iso* C14:0 concentration was observed when the amount of physically effective fiber decreased further from wk 4 to 6 in the alfalfa-based SARA induction experiment, suggesting that the activity of the cellulolytic bacteria was affected.

CONCLUSIONS

Two acidosis induction experiments resulted in different effects on blood parameters and the rumen microbial population. Differences in the milk FA profiles of the 2 experiments were found, mainly caused by the larger extent of the rumen pH decrease in the alfalfa-based SARA induction experiment. The cellulolytic bacterial community seemed to be negatively affected by either the presence of *E. coli*, rumen LPS accumulation, and associated effects on the microbial population in the rumen or by the amount of starch and quickly fermentable carbohydrates in the diet. In general, milk FA could indicate the occurrence of acidosis and give an indication of the origin of acidosis (low in structural fiber vs. high in starch). However, this still needs to be confirmed by additional experiments.

ACKNOWLEDGMENTS

The doctoral research of E. Colman is financed by Institute for the Promotion of Innovation by Science and Technology (IWT) in Flanders (Belgium) and the Commission of the European Communities [Brussels, Belgium; FP7, KBB-2007-1 (Rednex)]. B. Vlaeminck is a postdoctoral fellow of the Fund for Scientific Research-Flanders (Belgium).

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