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Starch and oil in the donor cow diet and starch in substrate differently affect the in vitro ruminal biohydrogenation of linoleic and linolenic acids

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ABSTRACT

Trans isomers of fatty acids exhibit different health properties. Among them, *trans*-10,*cis*-12 conjugated linoleic acid has negative effects on milk fat production and can affect human health. A shift from the *trans*-11 to the *trans*-10 pathway of biohydrogenation (BH) can occur in the rumen of dairy cows receiving high-concentrate diets, especially when the diet is supplemented with highly unsaturated fat sources. The differences of BH patterns between linoleic acid (LeA) and linolenic acid (LnA) in such ruminal conditions remain unknown; thus, the aim of this work was to investigate in vitro the effects of starch and sunflower oil in the diet of the donor cows and starch level in the incubates on the BH patterns and efficiencies of LeA and LnA. The design was a 4 × 4 Latin square design with 4 cows, 4 periods, and 4 diets with combinations of 21 or 34% starch and 0 or 5% sunflower oil. The rumen content of each cow during each period was incubated with 4 substrates, combining 2 starch levels and either LeA or LnA addition. Capillary electrophoresis single-strand conformation polymorphism of incubates showed that dietary starch decreased the diversity of the bacterial community and the high-starch plus oil diet modified its structure. High-starch diets poorly affected isomerization and first reduction of LeA and LnA, but decreased the efficiencies of *trans*-11,*cis*-15-C18:2 and *trans* C18:1 reduction. Dietary sunflower oil increased the efficiency of LeA isomerization but decreased the efficiency of *trans* C18:1 reduction. An interaction between dietary starch and dietary oil resulted in the highest *trans*-10 isomers production in incubates when the donor cow received the high-starch plus oil diet. The partition between *trans*-10 and *trans*-11 isomers was also affected by an interaction between starch level and the fatty acid added to the incubates, showing that the *trans*-10 shift only occurred with LeA, whereas LnA was mainly hydrogenated via the more usual *trans*-11 pathway, whatever the starch level in the substrate, although the bacterial communities were not different between LeA and LnA incubates. In LeA incubates, *trans*-10 isomer production was significantly related to the structure of the bacterial community.
Key words: rumen biohydrogenation, linoleic acid, linolenic acid, *trans*-10 shift

INTRODUCTION

Ruminal biohydrogenation (**BH**) intermediates of polyunsaturated fatty acids (**FA**) have important but contrasting effects on both dairy cows and human consumers. Among conjugated linoleic acids (**CLA**), *trans*-10,*cis*-12-CLA has strong negative effects on milk fat production by dairy cows (Baumgard et al., 2001) and can have adverse effects on human health (Tricon et al., 2004; Ip et al., 2007), but is usually present at very low concentrations in milk fat. On the contrary, *cis*-9,*trans*-11-CLA has positive effects on human health (Tricon et al., 2004; Ip et al., 2007). Milk *cis*-9,*trans*-11-CLA, which is usually the predominant CLA isomer, mainly originates from a mammary desaturation of *trans*-11-C18:1 (Griinari et al., 2000), another intermediate of polyunsaturated FA BH.

The most abundant BH intermediates in the rumen and in ruminant products are *trans*-11 isomers, but a shift from the *trans*-11 to the *trans*-10 pathway of BH can occur, with high-concentrate diets supplemented either with high linoleic acid (**LeA**; Roy et al., 2006) or high α-linolenic acid (**LnA**; Loor et al., 2004; Pottier et al., 2006) fat sources. This shift is, at least in part, linked to the low pH observed with high-concentrate diets (Piperova et al., 2002; Troegeler-Meynadier et al., 2003). The BH pathway leading to *trans*-10 isomers has been well described with LeA, but *trans*-10 isomer production during LnA BH has not yet been demonstrated. Besides, the *trans*-10 shift of BH pathway due to a high-starch diet is associated with a lowered milk fat content, and recent studies indicate specific changes of ruminal microbiota in cows exhibiting this milk fat

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Table 1. Ingredients and chemical composition of incubation substrates

	Incubation substrate[1]			
Item	LSLeA	HSLeA	LSLnA	HSLnA
Ingredient, g of DM per flask				
Corn leaves and stems	1.07	0.55	1.07	0.55
Corn grain	0.68	0.34	0.68	0.34
Alfalfa hay	0.39	0.00	0.39	0.00
Wheat	0.00	1.31	0.00	1.31
Soybean meal	0.44	0.35	0.44	0.35
99% pure free LeA[2]	0.09	0.09	0.00	0.00
99% pure free LnA[2]	0.00	0.00	0.09	0.09
Composition, % of DM				
NDF	37.8	26.5	37.8	26.5
Starch	18.3	40.9	18.3	40.9
C18:0	0.04	0.03	0.04	0.03
cis-9-C18:1	0.22	0.20	0.22	0.20
LeA	3.96	4.11	0.62	0.73
LnA	0.05	0.10	3.39	3.48

[1]LSLeA = low starch (LS) + free linoleic acid (LeA); HSLeA = high starch (HS) + free LeA; LSLnA = LS + free α-linolenic acid (LnA); HSLnA = HS + free LnA.
[2]Sigma Co., St. Louis, MO.

depression (Weimer et al., 2010). Moreover, dietary polyunsaturated FA also affect ruminal microbiota, decreasing the amount of some fibrolytic bacteria, including *Butyrivibrio fibrisolvens*, the main ruminal biohydrogenating bacterium, and the negative effects of linseed oil are more important than those of sunflower oil, suggesting that LnA is more inhibiting than LeA (Yang et al., 2009).

Relative effects of ruminal microbiota, dietary starch level and fermentation substrates in this *trans*-10 shift when diets are enriched with both concentrate and polyunsaturated FA have not been clearly separated. Moreover, the differences of BH patterns between LeA and LnA in a ruminal milieu inducing this *trans*-10 shift have not been elucidated. The aim of this study was to investigate this shift in vitro, using donor cows receiving different combinations of dietary starch level and oil addition, including a high-starch plus oil diet intended to result in a *trans*-10 shift, and culture substrates with different starch levels and either LeA or LnA as a BH substrate.

MATERIALS AND METHODS

In Vitro Cultures

Four dry Holstein cows (average BW 650 kg at the beginning of the experiment) equipped with a ruminal cannula and housed in individual stalls were assigned to a 4 × 4 Latin square design, with 4 diets and 4 periods. The 4 diets were based on corn silage and contained soybean meal and a mineral mixture: a low-starch diet (21.5% starch and 39.7% NDF, DM basis), containing alfalfa hay; a high-starch diet (34.8% starch and 30.9% NDF), containing 49% of a wheat/barley mixture; an oil diet (19.8% starch and 37.3% NDF) containing alfalfa hay and 5% sunflower oil; and a high-starch plus oil diet (33.1% starch and 28.6% NDF), containing 49% wheat/barley and 5% sunflower oil. Cows received 12.5 kg of DM daily, in 2 equal meals at 0800 and 1700 h. Water was available ad libitum. The experimental periods lasted 28 d. The cows received the control diet during the first 2 wk of each period and 1 of the 4 experimental diets during the last 2 wk.

Four incubation substrates were used: low starch with LeA, high starch with LeA, low starch with LnA, and high starch with LnA (Table 1). Because our objective was to study the BH pathways of LeA and LnA, we used free FA, not triglycerides, to avoid BH being affected by lipolysis.

In vitro incubations were performed on d 28. Ruminal fluid was taken from the 4 cows before the morning meal, strained through a metal sieve (1.6-mm mesh) and transferred quickly to the laboratory in anaerobic conditions at 39°C. One hundred milliliters of each sample of ruminal juice was stored at −18°C for subsequent analysis. A bicarbonate buffer solution (19.5 g of $Na_2HPO_4 \cdot 12H_2O$/L, 9.24 g of $NaHCO_3$/L, 0.705 g of NaCl/L, 0.675 g of KCl/L, 0.108 g of $CaCl_2 \cdot 2H_2O$/L, and 0.180 g of $MgSO_4 \cdot 7H_2O$/L) was prewarmed at 39°C, saturated with CO_2, and acidified to a pH of 6.0 with 6 *N* hydrochloric acid. The ruminal fluid from each cow was incubated with the 4 substrates, resulting in 16 incubation flasks that contained the substrate, 60 mL of ruminal fluid and 60 mL of buffer solution. The flasks were filled with CO_2 and placed in a water bath rotary shaker (Aquatron; Infors AG, Bottmingen, Germany) at 39°C. Flasks were then closed with a rubber cap

with a plastic tube leading into the water to vent fermentation gas without allowing the ingress of oxygen. Flasks were stirred at 130 rpm and kept safe from the light. After 5 h of incubation, flasks were placed into ice water to stop fermentations and the pH was measured. The contents of the flasks were then immediately frozen. Samples were freeze-dried (Virtis Freezemobile 25; Virtis Co. Inc., Gardiner, NY), weighed, ground and homogenized in a ball mill (Dangoumau; Prolabo, Nogent-sur-Marne, France), and kept at −18°C for later analysis.

FA Analysis

Substrates and non-incubated and incubated ruminal fluids were analyzed for FA contents and profiles. The FA were extracted and methylated using the procedure of Park and Goins (1994), except that the solution of 14% boron trifluoride in methanol was replaced by a solution of methanol-acetyl chloride (10:1 vol/vol). Nonadecanoic acid (Sigma Co., St. Louis, MO) was used as the internal standard at a dose of 0.8 mg.

Fatty acid methyl esters were quantified by GC (Agilent 6890N, equipped with a model 7683 auto injector, Network GC System; Agilent Technologies Inc., Palo Alto, CA) using a fused silica capillary column (100 m × 0.25 mm i.d., 0.20-μm film thickness; CPSil88; Varian Inc., Middelburg, the Netherlands). For analysis, the flame ionization detector temperature was maintained at 260°C and the injector at 255°C; the split ratio was 1:50. Hydrogen was used as the carrier gas with a constant flow of 1 mL/min. The samples were injected in 1 μL of hexane. The initial temperature of the oven was 60°C, which was held for 2 min; it was then increased by 8°C/min to 150°C, held at 150°C for 12 min, increased by 2°C/min to 175°C, held at 175°C for 20 min, increased by 5°C/min to 225°C, held at 225°C for 10 min, and finally increased by 10°C/min to 240°C and maintained at 240°C for 10 min. A second analysis was used to separate LnA from C20:1 and *trans*-13 + *trans*-14-C18:1 from *cis*-9-C18:1 with the same temperature of injector and detector as in the first analysis. The split ratio was 1:75 and hydrogen was the carrier gas with a constant pressure of 150 kPa. The samples were injected in 1 μL of hexane. The initial oven temperature was 60°C, which was held for 3 min; it was then increased by 8°C/min to 190°C, held at 190°C for 13 min, increased by 5°C/min to 225°C, held at 225°C for 10 min, increased by 10°C/min to a final temperature of 230°C, and maintained there for 10 min. Peaks were identified and quantified by comparison with commercial standards (Sigma Co.), except C18:1 FA other than *trans*-9-C18:1, *trans*-11-C18:1, and *cis*-9-C18:1, which were identified by order of elution. The major peak of conjugated linolenic acid (**CLnA**) was identified by order of elution (Akraim et al., 2007). Additionally, using a standard mixture of CLA isomers (*cis*-9,*trans*-11,*cis*-15; and *cis*-9,*trans*-13,*cis*-15), kindly provided by P. Y. Chouinard (Université Laval, Québec, Canada), the GC method adapted to the separation of these 2 isomers (Gervais and Chouinard, 2008) could not detect the *cis*-9,*trans*-13,*cis*-15 isomer in our samples, so that CLnA will refer to *cis*-9,*trans*-11,*cis*-15 CLnA in this paper.

Bacterial Community Analysis

Total DNA was extracted and purified with QIAamp DNA Stool Mini kit (Qiagen Ltd., West Sussex, UK) from approximately 0.2 g of sample with a previous bead-beating step in a FastPrep Instrument (MP Biomedicals, Illkirch, France). Extraction of DNA, PCR reactions, and capillary electrophoresis single-strand conformation polymorphism (**CE-SSCP**) procedures were based on the procedure described by Privé et al. (2010). The temperature program of the PCR reaction was different and consisted of 2 min at 94°C, 30 cycles of 30 s at 94°C, 15 s at 61°C, 15 s at 72°C, and a final extension of 7 min at 72°C.

The CE-SSCP data processing was computed with StatFingerprints software (Michelland et al., 2009), and CE-SSCP profiles were aligned using pairwise alignment of their internal standard with the same reference internal standard.

Calculations

The initial FA composition of each flask was calculated by adding the FA from ruminal fluids to the FA carried out by the substrates. All *trans* C18:1 isomers were summed to calculate *trans* C18:1, but only CLA isomers clearly known to be intermediates of LeA BH (i.e., *trans*-10,*cis*-12; *cis*-9,*trans*-11; and *trans*-9,*trans*-11-CLA) were summed to calculate total CLA. The balances (negative value for disappearances and positive values for productions) were calculated for each FA as the difference between final and initial percentages.

The calculations of the LeA and LnA isomerization efficiencies (**ELeA** and **ELnA**, respectively) and the calculations of the CLA, CLnA, *trans*-11,*cis*-15-C18:2, and total *trans* C18:1 reduction efficiencies (**ECLA**, **ECLnA**, **E*t*11*c*15**, and **E*t*18:1**, respectively) were adapted from Troegeler-Meynadier et al. (2006).

For ruminal LeA biohydrogenation,

$$\text{ELeA} = \text{LeAb}/\text{LeAi},$$

$$\text{ECLA} = (\text{LeAb} + \text{CLAb})/(\text{LeAb} + \text{CLAi}),$$

and for ruminal LnA biohydrogenation,

$$\text{ELnA} = \text{LnAb}/\text{LnAi},$$

$$\text{ECLnA} = (\text{LnAb} + \text{CLnAb})/(\text{LnAb} + \text{CLnAi}),$$

$$\text{E}t11c15 = (\text{LnAb} + \text{CLnAb} + t11c15\text{b})/(\text{LnAb} + \text{CLnAb} + t11c15\text{i}),$$

where the b suffix refers to the difference between initial and final proportions; the i suffix refers to the initial proportions of each FA; and CLA and $t11c15$ represent total CLA and *trans*-11,*cis*-15-C18:2, respectively.

The efficiency of the subsequent reduction, which is the same for the LeA and LnA BH, was calculated as

$$\text{E}t18{:}1 = (\text{LeAb} + \text{CLAb} + \text{LnAb} + \text{CLnAb} + t11c15\text{b} + t18{:}1\text{b})/(\text{LeAb} + \text{CLAb} + \text{LnAb} + \text{CLnAb} + t11c15\text{b} + t18{:}1\text{i}),$$

where $t18{:}1$ represents total *trans* C18:1.

To study the diversity of bacterial communities, the Simpson diversity index was calculated using the StatFingerprints software (Michelland et al., 2009) as

$$\text{Simpson index} = -\log \sum a_i^2,$$

where a_i corresponds to the relative abundance of each peak (i).

It is considered as a dominance index because it weights toward the abundance of the major species. The relative CE-SSCP sub-peak background area focuses on minor species and was calculated according to Loisel et al. (2006).

Statistical Analysis

The fatty acid profiles in the rumen fluids were analyzed by ANOVA, using the General Linear Model of SYSTAT (version 9; SPSS Inc., Chicago, IL), according to the following model:

$$Y = \mu + C + P + Sd + Od + Sd \times Od + \varepsilon,$$

where Y is the dependent variable; μ is the mean; C, P, Sd, and Od are the effects of cow, period, dietary starch level, and sunflower oil addition, respectively; and ε the residual error.

Fatty acid production or disappearance, efficiencies of BH reactions, Simpson index, and CE-SSCP background area were analyzed according to the following model:

$$Y = \mu + C + P + Sd + Od + Ss + FAs + Sd \times Od + Sd \times Ss + Sd \times FAs + Od \times Ss + Od \times FAs + Ss \times FAs + \varepsilon,$$

where Ss and FAs are the effects of starch level and FA source (LnA vs. LeA) in the in vitro substrates, respectively.

Because the initial amounts and balances of LnA, CLnA, and *trans*-11,*cis*-15-C18:2 were very low in cultures with added LeA, the statistical analysis of these balances, ELnA, ECLnA, and Et11c15 were only computed on data from LnA-enriched cultures, using the model

$$Y = \mu + C + P + Sd + Od + Ss + Sd \times Od + Sd \times Ss + Od \times Ss + \varepsilon.$$

Differences were declared significant at $P \leq 0.05$.

The structures of the bacterial communities were analyzed using the StatFingerprints software (Michelland et al., 2009). We compared the communities using the pairwise Euclidean distances of the CE-SSCP profiles. To explore this distance matrix, nonmetric multidimensional scaling (nMDS) was carried out. Pairwise analyses of similarities (**ANOSIM**) were performed on the distance matrix to test the effect of factors and their interactions. The test is based on the comparison of distances between groups corresponding to factors levels with distances within groups to produce the ANOSIM statistic R. The ANOSIM R value indicates the degree of similarity between the groups (R >0.75: well-separated groups; 0.50 < R <0.75: separated but overlapping groups; 0.25 < R <0.50: separated but strongly overlapping groups). We only considered effects of factors resulting in an ANOSIM R >0.25.

Additionally, we studied the relationship between some FA balances affected by dietary or incubation conditions and bacterial community. Correlation between the CE-SSCP profiles and FA were tested using redundancy analysis with 10,000 Monte Carlo permutations (Legendre and Legendre, 1998), and Pearson correlation coefficients between the Simpson index and CE-SSCP background area and FA balances were calculated.

RESULTS AND DISCUSSION

Fatty Acid Profile of Ruminal Fluid from Donor Cows

As expected, adding oil to the diets of donor cows increased the amount of total FA in the incubates (Table 2). Stearic acid was the major FA, whatever

the diet of dairy cows, but with a higher percentage when diets contained added oil than without dietary oil. Oil addition increased the proportions of total *trans* C18:1 but lowered the percentages of polyunsaturated FA. Actually, ruminal fluids were taken before the morning meal (i.e., 15 h after the previous meal), so that most added unsaturated FA from added oil had been at least isomerized, whereas BH was less complete when diets did not contain added oil, as unsaturated FA of vegetal cells were not as available for BH as FA of added oil. Increasing the dietary starch level strongly decreased *cis*-9,*trans*-11-CLA and *trans*-11-C18:1 proportions, especially in the high-starch plus oil diet. On the other hand, this diet resulted in the highest *trans*-10,*cis*-12-CLA and *trans*-10-C18:1 balances, due to a tendency toward an interaction between starch and oil additions ($P = 0.079$ and $P = 0.067$ for *trans*-10,*cis*-12-CLA and *trans*-10-C18:1, respectively), and the lowest *cis*-9,*trans*-11-CLA and *trans*-11-C18:1 balances. This demonstrates that this combination efficiently resulted in a *trans*-10 shift.

However, because samples were taken a long time after the meal, the variations of FA composition could fail to accurately reflect the ruminal effects of the diets, so that we will not further discuss these results.

Structure and Diversity of the Bacterial Community

Increasing dietary starch decreased the Simpson diversity index and the relative CE-SSCP sub-peak background area from 7.6 to 6.6 and from 0.91 to 0.85, respectively, indicating less numerous but more abundant major bacterial species. Dietary oil, starch level in the incubates, incubated FA, and interactions had no significant effect on the Simpson diversity index and the relative sub-peak background area.

The nonmetric multidimensional scaling (nMDS) plot (Figure 1) of the CE-SSCP profiles of the 5-h in vitro incubations showed that the structure of bacterial communities differed between the high-starch plus oil diet and the 3 other combinations (ANOSIM R = 0.43, $P < 0.01$). The starch level in the diets or the incubates, dietary oil, replacement of LeA with LnA and the other interactions between these factors did not modify the structure of the bacterial community. That diversity and structure of the bacterial communities only were affected by dietary starch and its interaction with dietary oil suggests that only the effects of the diet, but not the effects of starch amount and FA nature in the incubates, could have been mediated by a change of microbial population.

Effects of Donor Cow Diet and Starch in the Incubates

Increasing starch concentration from 21 to 34% of DM, on average, in the diet of donor cows had no significant effect on ELeA and ELnA (Table 3). Increasing the starch content from 18 to 41% in culture substrates significantly decreased ELeA from 42.8 to 36.9%, on average. However, this effect significantly interacted with the FA added to the cultures, only affecting cultures with added LnA, which had a low initial LeA amount. This shows that neither starch in the donor cows diet nor starch in the incubates affected the BH extent of added LeA. Literature data indicate that increasing the proportion of concentrates in the diet decreases the LeA BH extent in vivo (Loor et al., 2004; Glasser et al., 2008; Enjalbert and Troegeler-Meynadier, 2009), ant that a low pH decreases BH extent in vitro (Van Nevel and Demeyer, 1996; Troegeler-Meynadier et al., 2003). In our experiment, increasing dietary starch only

Table 2. Amount of total fatty acids (mg/flask) and fatty acid profile (% of total fatty acids) of ruminal fluids

Cow diet[1]	Low starch	High starch	Low starch + oil	High starch + oil	SEM	Significant effects[2]
Total FA	144.4	121.8	219.3	288.4	22.9	Od**
FA profile						
C18:0	44.48	41.97	49.57	54.62	3.15	Od*
*c*9-C18:1	2.04	2.89	2.14	1.49	0.20	Od*, Sd × Od*
*t*10-C18:1	0.48	0.46	1.15	11.49	2.32	Od*
*t*11-C18:1	3.86	4.05	13.07	1.31	0.93	Sd***, Od*, Sd × Od***
Total *t* C18:1	7.62	6.78	19.95	17.15	2.58	Od**
LeA	3.61	5.15	1.71	2.90	0.51	Sd*, Od**
*t*10,*c*12-CLA	0.02	0.01	0.02	0.03	0.01	Od*
*c*9,*t*11-CLA	0.06	0.04	0.18	0.01	0.02	Sd**, Sd × Od**
*t*9,*t*11-CLA	0.35	0.20	0.20	0.10	0.07	
LnA	0.79	0.32	0.27	0.27	0.13	
CLnA	0.045	0.016	0.023	0.005	0.007	Sd**, Od*

[1]FA = fatty acids; *c* = *cis*; *t* = *trans*; LeA = linoleic acid; CLA = conjugated linoleic acid; LnA = α-linolenic acid; CLnA = *cis*-9,*trans*-11,*cis*-15 conjugated linolenic acid.

[2]Od = dietary oil addition; Sd = dietary starch level; × indicates an interaction.

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

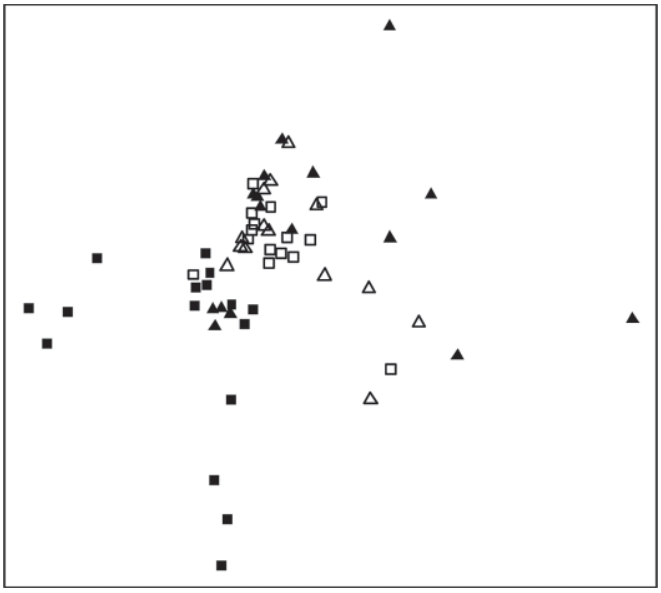

Figure 1. Nonmetric multidimensional scaling plot (nMDS) of the capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) profiles of 5-h in vitro cultures. The marks relate to the diets of donor cows: low starch without sunflower oil (Δ), low starch with sunflower oil (□), high starch without sunflower oil (▲), and high starch with sunflower oil (■).

decreased the final pH by 0.1 and increasing starch in substrates decreased ruminal pH by 0.3 (results not shown). A 0.3 pH unit decrease has already been shown to decrease ELeA disappearance (Van Nevel and Demeyer, 1996). These authors also showed that lipolysis is much more sensitive to low pH values than isomerization. In our experiment, in cultures with added LeA, most LeA was in a free form, so that its isomerization could not be precluded by a slow lipolysis. This could explain the lack of negative effect of starch level on ELeA.

A small effect of concentrates and pH on isomerization compared with lipolysis could also explain why, in our cultures with added LnA, increasing starch in the substrates did not negatively affect ELnA, as most LnA originated from added free LnA. Increasing the starch level in the substrate even increased ELnA by 10%; to our knowledge, no other published experiment has investigated the effect of starch level on the in vitro isomerization of free LnA.

High-starch diets slightly increased ECLA and ECLnA, resulting in a decreased CLnA balance (Table 4). Starch addition to the substrates did not affect ECLA but increased ECLnA, resulting in a decreased CLnA balance. Although statistically significant, these effects remained in a narrow range, as ECLA and ECLnA were near 90%, whatever the diet of the donor cow or the incubation substrate. The reduction of CLA is

Table 3. Effect of donor cow diet and culture substrate on the variations of efficiencies (E as %) of the reactions of biohydrogenation of polyunsaturated fatty acids during 5-h in vitro incubations

	Low-starch diet[2]				High-starch diet				Low-starch + oil diet				High-starch + oil diet					
Substrate[1]	LSLeA	HSLeA	LSLnA	HSLnA	LSLeA	HSLeA	LSLnA	HSLnA	LSLeA	HSLeA	LSLnA	HSLnA	LSLeA	HSLeA	LSLnA	HSLnA	SEM	Significant effects[3]
ELeA	44.1	44.8	32.8	23.3	47.7	51.5	39.9	23.8	49.8	55.1	40.9	26.7	53.3	52.4	33.8	17.7	3.2	Ss**, FAs***, Sd × Od*, Ss × FAs***
ECLA	95.9	96.8	90.0	87.4	96.9	97.6	94.2	91.2	94.0	96.9	88.5	87.0	96.3	96.1	93.5	88.3	1.7	Sd*, FAs***, Ss × FAs*
ELnA			55.7	61.9			55.0	64.9			60.9	68.5			61.7	63.3	2.9	Ss**
ECLnA			94.4	97.5			95.4	98.2			90.0	96.2			98.8	99.4	0.9	Sd***, Ss***, Sd × Od***, Sd × Ss*
E*t*11*c*15			93.4	94.3			91.3	87.9			94.1	95.0			85.1	87.3	3.7	Sd*
E*t*C18:1	60.6	67.5	61.5	60.1	57.6	58.4	49.3	46.6	41.1	48.3	41.2	40.9	44.0	46.9	38.5	42.5	3.8	Sd*, Od***, FAs**, Sd × Od*

[1]ELeA and ELnA = isomerization efficiencies of linoleic and linolenic acids, respectively; ECLA, ECLnA, E*t*11*c*15, and E*t*18:1 = efficiencies of reduction of total conjugated linoleic acids; *cis*-9,*trans*-11,*cis*-15 conjugated linolenic acid; *trans*-11,*cis*-15-C18:2; and total *trans* C18:1, respectively.

[2]LSLeA = low starch (LS) + free linoleic acid (LeA); HSLeA = high starch (HS) + free LeA; LSLnA = LS + free α-linolenic acid (LnA); HSLnA = HS + free LnA.

[3]Ss = starch level in the incubation substrate; FAs = fatty acid in the substrate (LnA vs. LeA); Sd = dietary starch level; Od = dietary oil addition; × indicates an interaction.

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

known to be very efficient even at low pH (Troegeler-Meynadier et al., 2006), which explains the very limited effect of starch in our experiment.

Starch addition to the diet decreased E*t*11*c*15 from 94 to 88% and E*t*18:1 from 53 to 48%, on average (Table 3), resulting in increased *trans*-11,*cis*-15-C18:2 and *trans* C18:1 balances (Table 4), but starch addition to the incubates did not affect E*t*11*c*15 and E*t*18:1. An increased duodenal flow of *trans* C18:1 has already been observed when increasing dietary concentrate, and buffer addition alleviated this effect, suggesting that ruminal pH played a major role in this accumulation (Kalscheur et al., 1997). In our experiment, donor cows receiving the high-starch diets did not receive a buffer, so that a starch effect on the ruminal pH and, consequently, the microflora could be expected, affecting E*t*11*c*15 and E*t*18:1 in vitro. On the other hand, the 0.3 final pH difference due to starch addition to the incubates could have failed to affect these efficiencies.

Oil addition to the diets marginally increased ELeA from 39 to 41% and did not affect ECLA, ELnA, ECLnA, and E*t*11*c*15. On the other hand, E*t*18:1 decreased from 58 to 43%, on average, when oil was added to the diets. Troegeler-Meynadier et al. (2006) previously reported that this BH reaction is easily saturated. Shingfield et al. (2008) observed an increased proportion of *trans* C18:1 among omasal FA when increasing dietary sunflower oil in the diet of lactating dairy cows, and modeling data from several in vivo experiments, Moate et al. (2004) established that the BH rate of *trans* C18:1 is negatively affected by the concentration of free FA in the rumen. Moreover, Lourenço et al. (2010) showed that *Butyrivibrio proteoclasticus*, which is the main bacteria responsible of this last reduction (Wallace et al., 2006), is more sensitive to unsaturated FA than *B. fibrisolvens* which is the main bacteria responsible for the previous BH steps. In spite of this negative effect on E*t*18:1, oil addition to the diets decreased the in vitro balances of most *trans* C18:1 BH intermediates, which could be due to the higher initial proportions of *trans* C18:1 (Table 2).

Taken together, our results relative to the effects of starch and oil addition to the diets of donor cows suggest that the last BH reduction was inhibited by either high-starch or oil-supplemented diets. The effect of dietary starch on E*t*18:1 interacted with dietary oil, being observed only when the diets did not contain added oil. This shows that starch and oil additions had no additive effect, as increasing the dietary starch level did not lower *trans*-C18:1 reduction when this reduction was already inhibited by oil addition to the diet, suggesting that they affected the same target.

The main isomer-specific effect of high-starch diets was observed on the equilibrium between *trans*-10- and *trans*-11 isomers in incubates (Table 4). High-starch diets decreased the cis-9,*trans*-11-CLA balance by 2.3 times, increased the *trans*-10,*cis*-12-C18:2 and *trans*-10-C18:1 balances by 2.2 and 1.8 times, respectively, but did not affect the *trans*-11-C18:1 balance. The effect of the starch content or forage-to-concentrate ratio of the production of *trans*-10-isomers in fat-supplemented diets has been previously observed in vivo with diets without (Piperova et al., 2002) or with (Loor et al., 2004) added fat. In vitro, Choi et al. (2005) reported that, whatever the culture pH, in vitro cultures contained more *trans*-10,*cis*-12-C18:2 when the donor cow received a high-concentrate diet.

As discussed later, we did not observe a *trans*-10 shift in LnA incubates; thus, we studied the relationship between *trans*-10 isomer (*trans*-10,*cis*-12-C18:2 + *trans*-10-C18:1) balance and bacterial community only on LeA incubates. Redundancy analysis showed that total *trans*-10 isomers balance explained 11% ($P = 0.005$) of the total inertia of CE-SSCP profiles. This is consistent with the results of Weimer et al. (2010) who observed that the bacterial community was affected in cows experiencing milk fat depression, usually associated with a *trans*-10 shift. In our experiment, with the same data set of LeA incubates, *trans*-10 isomer balance did not significantly correlate with the Simpson index and the CE-SSCP background area ($r = 0.33$, $P = 0.069$ and $r = 0.23$, $P = 0.21$, respectively).

Dietary starch interacted with dietary oil for cis-9,*trans*-11-CLA, *trans*-10,*cis*-12-CLA, and *trans*-11-C18:1 balances: the increase of *trans*-10,*cis*-12-CLA due to dietary starch addition was higher when the diets contained sunflower oil, and high-starch diets decreased the balance of *trans*-11 isomers only when oil was added to the diets, resulting in the highest *trans*-10,*cis*-12-CLA and lowest *trans*-11 isomer balances when donor cows received the high-starch plus oil diet (Table 4). This reveals a clear shift from the *trans*-11 to the *trans*-10 BH pathway. These changes could have been driven by the strong changes of bacterial community structure observed with the high-starch plus oil diet. Griinari et al. (1998), studying the effect of concentrate level and unsaturation level of dietary fat found a trend toward a similar interaction, with a strong decrease in *trans*-11-C18:1 in milk fat when increasing the concentrate level in diets containing unsaturated fat carried out by corn oil. Additionally, they described a significant interaction between dietary concentrate and unsaturated fat for milk *trans*-10-C18:1, the highest levels being observed with high-concentrate diets containing unsaturated fat. In our experiment, the trend toward this interaction could be observed on the ruminal fluid of donor cows (Table 2) but was not observed on in vitro balances: as discussed later, the

Table 4. Effect of donor cow diet and culture substrate on the variations of fatty acids profile (% of total fatty acids) during 5-h in vitro incubations

	Low-starch diet[2]				High-starch diet				Low-starch + oil diet				High-starch + oil diet					
Substrate[1]	LSLeA	HSLeA	LSLnA	HSLnA	LSLeA	HSLeA	LSLnA	HSLnA	LSLeA	HSLeA	LSLnA	HSLnA	LSLeA	HSLeA	LSLnA	HSLnA	SEM	Significant effects[3]
C18:0	9.9	10.2	9.3	8.0	11.2	10.9	7.2	4.6	9.4	10.6	8.9	7.8	9.1	9.0	6.5	6.7	1.3	FAs***
*c*9-C18:1	−0.43	0.28	0.68	0.24	−1.01	−0.51	−1.06	−1.91	−0.81	−0.41	−0.78	−0.26	−0.66	−0.39	−1.13	−0.80	0.35	Sd***, Sd × Od**, Sd × FAs*
*c*11-C18:1	0.12	0.19	0.14	0.26	0.10	0.29	0.18	0.39	0.07	0.13	0.08	0.19	0.09	0.24	0.11	0.26	0.03	Sd***, Od***, Ss***, FAs**, Sd × Ss**
*c*12-C18:1	0.38	0.39	0.24	0.42	0.51	0.95	0.41	0.64	0.15	0.19	0.08	0.17	0.27	0.24	0.19	0.17	0.08	Sd***, Od***, Ss**, FAs*, Sd × Od*, Od × Ss*
*c*15-C18:1	0.02	0.02	0.41	0.58	0.04	0.04	0.61	0.68	0.01	0.02	0.17	0.30	0.00	0.00	0.69	0.61	0.07	Sd***, Od*, FAs***, Sd × FAs***
*t*5-C18:1	0.02	0.02	0.02	0.02	0.03	0.04	0.01	0.02	0.01	0.01	0.01	0.01	−0.01	−0.02	−0.02	−0.03	0.01	Sd***, Od***, FAs*, Sd × Od***
*t*6 + *t*7 + *t*8-C18:1	0.27	0.28	0.26	0.28	0.37	0.56	0.33	0.37	0.18	0.22	0.19	0.28	0.12	0.07	0.06	0.02	0.05	Od***, Sd × Od***, Sd × FAs*
*t*9-C18:1	0.15	0.14	0.14	0.17	0.22	0.32	0.21	0.24	0.11	0.14	0.11	0.18	0.10	0.06	0.05	0.04	0.03	Od***, Sd × Od***
*t*10-C18:1	0.88	1.05	0.38	0.52	1.24	2.26	0.58	0.97	0.58	0.78	0.29	0.53	1.39	2.25	0.33	−0.18	0.27	Sd***, Ss*, FAs***, Sd × FAs**
*t*11-C18:1	1.93	1.23	2.03	1.90	2.67	2.34	3.44	4.34	1.90	1.20	1.42	2.09	1.05	0.25	0.46	0.34	0.33	Od***, FAs*, Sd × Od***, Od × FAs**, Ss × FAs**
*t*12-C18:1	0.38	0.37	0.47	0.56	0.54	0.73	0.61	0.49	0.21	0.27	0.30	0.40	0.18	0.07	0.22	0.21	0.07	Od***, Sd × Od***
*t*13+t14-C18:1	0.18	−0.16	0.41	1.94	0.91	1.02	2.66	3.42	0.35	0.39	1.07	1.66	0.34	0.19	1.88	1.73	0.37	Sd***, FAs***, Sd × Od**, Ss × FAs*
*t*15-C18:1	0.21	0.16	0.75	0.91	0.35	0.34	0.72	0.65	0.13	0.13	0.49	0.56	0.12	0.04	0.67	0.59	0.13	Od*, FAs***
*t*16-C18:1	0.25	0.24	0.61	0.66	0.35	0.34	0.54	0.35	0.16	0.20	0.48	0.50	0.12	0.09	0.45	0.40	0.08	Od**, FAs***
Total *t* C18:1	4.2	3.3	5.0	6.9	6.6	7.9	9.0	10.8	3.6	3.3	4.3	6.2	3.3	2.9	4.0	3.0	0.8	Sd**, Od***, FAs***, Sd × Od***
LeA	−18.7	−19.7	−2.7	−2.2	−21.2	−23.8	−3.6	−2.4	−15.7	−17.9	−2.4	−1.6	−14.8	−14.9	−2.0	−1.1	1.1	Od***, Lns***, Sd × Od**, Od × Lns***, Ss × Lns*
*t*10,*c*12-CLA	0.17	0.20	0.01	0.01	0.18	0.26	0.02	0.04	0.10	0.09	0.01	0.01	0.38	0.41	0.02	0.02	0.04	Sd***, FAs***, Sd × Od**, Sd × FAs***
*c*9,*t*11-CLA	0.33	0.13	0.06	0.02	0.30	0.10	0.07	0.04	0.52	0.21	0.05	0.02	0.05	0.02	0.01	0.00	0.04	Sd***, Ss***, FAs***, Sd × Od*** Sd × FAs***, Ss × FAs***
*t*9,*t*11-CLA	0.05	0.02	−0.01	0.00	0.05	0.08	0.01	0.01	0.07	−0.01	−0.01	−0.04	0.01	0.04	0.00	0.00	0.03	FAs**
*t*11,*t*13-CLA	0.02	0.02	0.27	0.39	0.02	0.02	0.36	0.67	0.00	0.01	0.08	0.10	0.01	0.00	0.22	0.19	0.05	Sd**, Od***, Lns***, Sd × Lns**, Od × Ss*, Od × Lns***, Ss × Lns*
Total-CLA	0.54	0.35	0.06	0.03	0.53	0.44	0.09	0.09	0.69	0.30	0.05	−0.01	0.44	0.47	0.04	0.02	0.05	Ss**, FAs***, Sd × Ss*, Ss × FAs*
*t*11,*c*15-C18:2			1.01	1.09			1.63	2.78			0.79	0.86			2.02	1.77	0.52	Sd**
LnA			−19.8	−22.3			−20.0	−24.1			−16.0	−18.4			−13.7	−14.3	1.5	Od***, Ss*
CLnA			1.08	0.48			0.91	0.42			1.58	0.65			0.17	0.08	0.14	Sd***, Ss***, Sd × Od***, Sd × Ss*

[1]*c* = *cis*; *t* = *trans*; LeA = linoleic acid; CLA = conjugated linoleic acid; LnA = α-linolenic acid; CLnA = *cis*-9,*trans*-11,*cis*-15 conjugated linolenic acid.
[2]LSLeA = low starch (LS) + free LeA; HSLeA = high starch (HS) + free LeA; LSLnA = LS + free LnA; HSLnA = HS + free LnA.
[3]FAs = fatty acid in the substrate (LnA vs. LeA); Sd = dietary starch level; Od = dietary oil addition; Ss = starch level in the incubation substrate; × indicates an interaction.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

production of *trans*-10-C18:1 in cultures incubated with LnA was low, which could have masked the effects on LeA of a *trans*-10-producing ruminal microflora.

Similar to high-starch diets, high-starch substrates decreased the *cis*-9,*trans*-11-CLA balance, which was numerically but not statistically observed by Fuentes et al. (2009), and increased the *trans*-10-C18:1 balance, whereas Fuentes et al. (2009) observed an opposite effect. Unlike dietary starch, increasing the starch content of substrates had no significant effect on *trans*-10,*cis*-12-CLA and *trans*-11-C18:1 balances.

Among BH intermediates that mainly result from LnA BH, high-starch diets strongly increased *trans*-11,*cis*-15-C18:2; *trans*-11,*trans*-13-CLA; and *trans*-13 + *trans*-14-C18:1. The high-starch substrates numerically increased *trans*-11,*trans*-13-CLA and *trans*-13 + *trans*-14-C18:1 balances ($P = 0.052$ and $P = 0.109$, respectively), which is consistent with the in vivo results of Loor et al. (2004). Increasing starch in the diets also increased *cis*-11, *cis*-12, and *cis*-15-C18:1 balances.

Effects of the Incubated Fatty Acid and Interactions with Starch in Diets and Substrates

On average, ELeA was 50% in the cultures with added LeA but only 30% in the cultures with added LnA (Table 3). In vitro, LnA addition has already been shown to inhibit LeA isomerization (Troegeler-Meynadier et al., 2003), which, at least in part, explains this very low LeA BH in our cultures with added LnA. In our experiment, most LeA in the cultures with added LeA was the pure free LeA added to the medium, whereas in the cultures with added LnA, the LeA originated from the ruminal fluid and the forage and concentrates used as a substrate, in an acylglycerol form, needing digestion of the vegetal structures and lipolysis before BH.

On average, ELnA was 61% in cultures with LnA, compared with 50% for ELeA in cultures with LeA (Table 3). This higher BH extent of LnA compared with LeA is consistent with literature data (Enjalbert and Troegeler-Meynadier, 2009). However, in our experiment, this higher extent of LnA than LeA BH was not necessarily due to the nature of the FA, but could also be due to different physical forms: 97% of LnA in cultures with added LnA originated from pure added LnA, compared with 79% of LeA originating from pure LeA in cultures with added LeA.

Adding LnA resulted in a 10 times lower increase in total CLA proportion than LeA addition (0.05 and 0.47%, on average, respectively), but resulted in high CLnA and *trans*-11,*cis*-15-C18:2 balances (Table 4), which is consistent with the present knowledge relative to LeA and LnA BH. Incubation of LnA resulted in a *trans*-11,*cis*-15-C18:2 balance that was 44 times greater than the *cis*9,*trans*-11-CLA balance, which is similar to the 47 ratio observed by Loor et al. (2004) in vivo when supplementing diets with linseed oil. On the contrary, incubation with added LeA resulted in negligible balances of CLnA, *trans*-11,*trans*-13-CLA and *trans*-11,*cis*-15-C18:2 (results not shown), but increased *trans*-10,*cis*-12-; *cis*-9,*trans*-11-; and *trans*-9,*trans*-11-CLA balances.

During incubations, the percentage of total *trans* C18:1 increased by 4.4%, on average, in cultures with added LeA compared with 6.2% in cultures with LnA, in relation to a higher E*t*18:1 than in cultures with LnA. The reduction of *trans*-C18:1 to C18:0 is inhibited in vitro by an LeA concentration over 1 mg/mL (Harfoot et al., 1973), and Troegeler-Meynadier et al. (2006) demonstrated that this effect was due to a saturation of the reduction when the concentration of *trans* C18:1 was high. A similar effect can be expected when LnA is incubated, as LnA BH also produces *trans* C18:1, but an inhibition of the last reduction by other LnA BH intermediates, or different inhibiting effects of different *trans* C18:1 isomers could have resulted in this lower E*t*18:1 with LnA than with LeA.

Compared with cultures with LeA, cultures with LnA had a quite different distribution of octadecenoic BH intermediates. Incubation of LnA instead of LeA increased the balances of *cis*-11-, *cis*-15-, *trans*-11-, *trans*-13 + *trans*-14-, *trans*-15-, and *trans*-16-C18:1; tended to increase the balance of *trans*-12-C18:1 ($P = 0.092$); and decreased the balances of *cis*-12-, *trans*-5-, and *trans*-10-C18:1. Most of these changes are consistent with the present knowledge on LeA and LnA BH, the latter being, in particular, known to result in *cis*-15-, *trans*-13 + *trans*-14-, *trans*-15-, and *trans*-16-isomers (Kemp and Lander, 1984; Jouany et al., 2007).

Destaillats et al. (2005) proposed a biohydrogenation pathway of LnA where LnA was, in part, isomerized to *cis*-9,*trans*-13,*cis*-15-C18:3, whose reductions produced *trans*-13-C18:1. Whatever the diets of cows and the incubation substrates, we did not find *cis*-9,*trans*-13,*cis*-15-C18:3, even in samples with high *trans*-13 + *trans*-14-C18:1 balances.

Formation of *trans*-10 isomers during LeA and LnA BH have been proposed by Griinari and Bauman (1999). This production from LeA has been clearly demonstrated in vivo with a corn oil-supplemented diet (Griinari et al., 1998), and in in vitro ruminal cultures with added LeA (Troegeler-Meynadier et al., 2003; Jouany et al., 2007). In the pathway proposed by Griinari and Bauman (1999), LnA could be hydrogenated via the *trans*-10 pathway instead of the more classical *trans*-11 pathway with *trans*-10,*cis*-12,*cis*-15-C18:3 and *trans*-10,*cis*-15-C18:2 as intermediates. As far as we are aware, only Kemp et al. (1975) reported traces of this

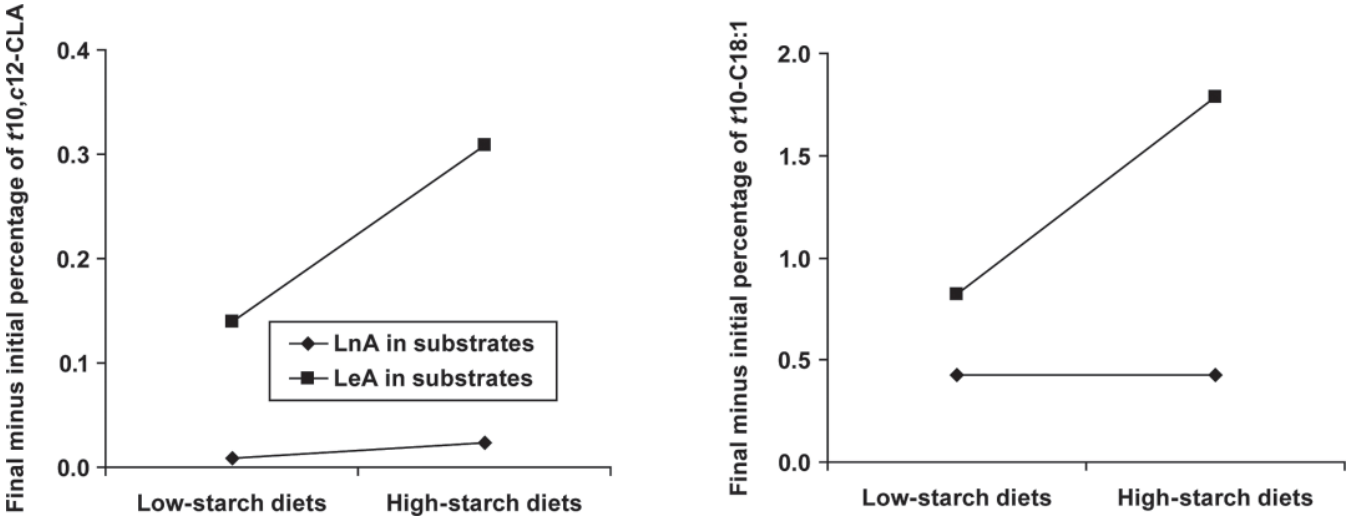

Figure 2. Effects of interaction between dietary starch level and the incubated fatty acid on the balances of *trans*-10,*cis*-12-conjugated linoleic acid (*t*10,*c*12-CLA) and *trans*-10-C18:1 in 5-h in vitro cultures. LnA = linolenic acid; LeA = linoleic acid.

latter isomer to be produced by a strain of ruminal bacteria. The *trans*-10,*cis*-12,*cis*-15-C18:3 isomer has been isolated in ewe cheese (Winkler and Steinhart, 2001), but it could result from a microbial isomerization of LnA or *cis*-9,*trans*-11,*cis*-15 CLnA in milk or cheese.

Increasing dietary starch strongly increased *trans*-10,*cis*-12-CLA and *trans*-10-C18:1 when LeA was incubated, but not when LnA was incubated (Figure 2). Using a high-concentrate diet, Loor et al. (2005) also observed 6.3 and 3.8 times lower proportions of *trans*-10,*cis*-12-CLA and *trans*-10-C18:1, respectively, in the duodenal flow of cows supplemented with linseed oil, whose main FA is LnA, compared with cows supplemented with sunflower oil, whose main FA is LeA.

Similarly, incubated starch interacted with incubated FA for *trans*-11-C18:1 and *trans*-13 + *trans*-14-C18:1 balances and a trend toward this interaction was observed for *trans*-10-C18:1 balance ($P = 0.073$). Actually, in cultures with LeA, increasing starch level in the substrate increased the *trans*-10-C18:1 balance by 55% but decreased the *trans*-11-C18:1 balance by 34%, clearly showing a shift in BH pathway (Figure 3). On the contrary, in cultures with LnA, increasing starch increased *trans*-10, *trans*-11, and *trans*-13 + *trans*-14-C18:1 balances by 15, 18, and 45%, respectively, showing no major shift in BH pathway.

This lack of *trans*-10 shift in cultures with LnA, whatever the starch content of the diets or the incubation

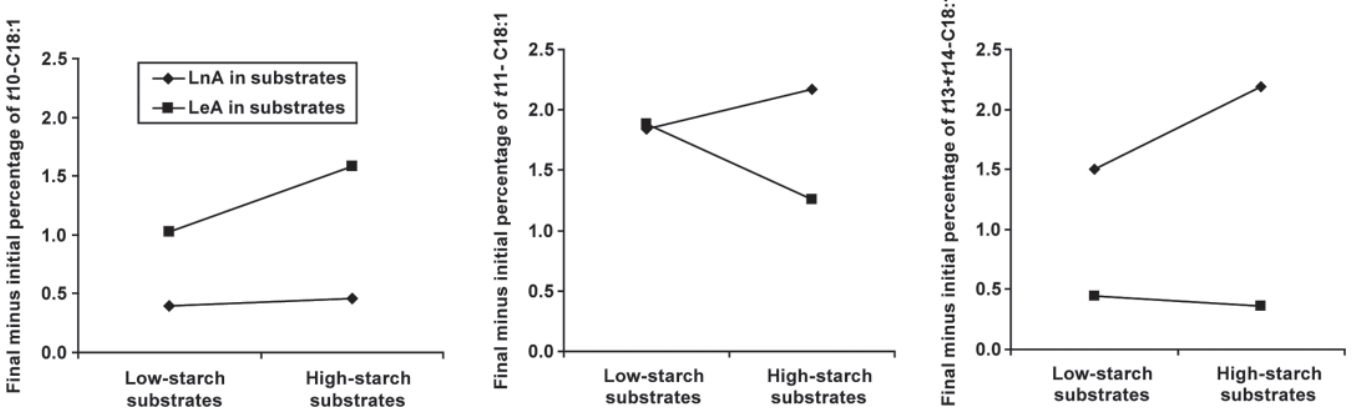

Figure 3. Effects of interaction between starch level in the incubation substrate and the incubated fatty acid on the balances of *trans*-10-C18:1, *trans*-11-C18:1, and *trans*-13 + *trans*-14-C18:1 in 5-h in vitro cultures. LnA = linolenic acid; LeA = linoleic acid.

substrates, strongly suggests that LnA BH does not produce *trans*-10 isomers. Moreover, the lack of effect of dietary starch on the isomeric profile of BH intermediates with LnA incubation, as opposed to the shift toward *trans*-10 isomers with LeA incubation, suggests that dietary conditions that result in a *trans*-10 shift of LeA BH do not produce this shift in LnA BH. This *trans*-10 shift has been described with high-starch diets without added fat (Griinari et al., 1998), with added sunflower oil (Roy et al., 2006), and also with diets supplemented with linseed oil (Loor et al., 2004; Pottier et al., 2006). However, LeA respectively represented 20 and 36% of total dietary FA in these experiments, making it impossible to ascertain what FA *trans*-10 isomers originated from.

That the incubated FA did not affect the structure and diversity of the bacterial community suggests that the differences of BH pathways between LeA and LnA observed in our incubations did not relate to differences of microbiota, but only to differences of BH substrate.

CONCLUSIONS

The high-starch plus oil diet resulted in a *trans*-10 shift of LeA BH in donor cows. This shift was also observed in vitro when ruminal fluids from cows receiving the high-starch plus oil diet were incubated with LeA, and it related to a change of structure of the bacterial community. Increasing the starch level in the incubates also increased the *trans*-10-C18:1 balance. On the contrary, no *trans*-10 shift was observed when LnA was incubated with the rumen fluid from cows receiving this high-starch plus oil diet or when incubated with high-starch substrate. Because LeA and LnA incubations resulted in similar bacterial communities at the end of the incubations, differences of BH pathways between these 2 FA when incubated with ruminal fluid from cows exhibiting a *trans*-10 shift are intrinsic to the nature of FA.

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