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Effects of pH and fermentative substrate on ruminal metabolism of fatty acids during short-term *in vitro* incubation

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Summary

The ruminal biohydrogenation of c9,c12-18:2 can be affected by the fibre/starch ratio of the diet and the ruminal pH. The objectives of this study were to examine independently *in vitro* the effects of fermentation substrate (hay vs. corn starch) and buffer pH (6 vs. 7) on the biohydrogenation of c9,c12-18:2 carried out by grape seed oil, focusing on its t11 and t10 pathways, using 6-h ruminal incubations. The experimental design was a 2 × 2 factorial arrangement. Fermentation substrate and pH affected the C18 fatty acid balance in incubated media, but few interactions were observed. Compared with starch, hay as the fermentation substrate favoured the production of 18:0 (×2.3), all *trans*-18:1 isomers (×12.6) and CLA (×6.1), except c9,t11-CLA, and the disappearance of unsaturated C18 fatty acids, but decreased the production of odd and branched chain fatty acids. Compared with pH 6 buffer, pH 7 buffer resulted in higher c9,c12-18:2 disappearance and CLA production. For c9,t11-CLA, an interaction was noticed between the two factors, leading to the highest production in cultures incubated on hay with the 7 pH buffer. Compared with starch, hay as fermentation substrate favoured the activity of t11 producers, which are fibrolytic bacteria, and the production of t10 isomers, possibly due to the presence of potential t10 producers in hay. Low pH resulted in a decreased t11 isomers production and in a slightly increased t10 isomers production, probably due to a modulation of enzymatic or bacterial activity.

Keywords ruminal biohydrogenation, *trans* fatty acids, linoleic acid, pH, hay, starch

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Introduction

Although early studies showed potential beneficial effects for humans of a mixture of conjugated linoleic acid (CLA) isomers containing c9,t11-CLA and t10, c12-CLA as major components, the physiological effects are isomer specific (Pariza et al., 2001). Specifically, t10,c12-CLA has been shown to exhibit possible detrimental effects on human health (Tricon et al., 2004; Wilson et al., 2006; Ip et al., 2007). Conversely, no detrimental effects have been demonstrated for c9, t11-CLA or its metabolic precursor (via tissue desaturation) t11-18:1. On the other hand, dairy milk fat content is specifically and strongly affected by t10, c12-CLA, which inhibits mammary fatty acid (FA) uptake and *de novo* synthesis, resulting in a milk fat depression (Shingfield and Griinari, 2007). The highest CLA contents in human food are found in ruminant products, mainly dairy products. Indeed, CLA

and t11-18:1 are formed during biohydrogenation (BH) of dietary unsaturated FA in the rumen (Enjalbert and Troegeler-Meynadier, 2009), and some of them are secreted into milk. Moreover, most milk c9, t11-CLA originates in a mammary desaturation of t11-18:1 (Enjalbert and Troegeler-Meynadier, 2009).

Attempts to increase milk CLA should focus on t11 isomers, as t10 isomers can be detrimental for human health and dairy cow milk fat production. *In vivo*, the t10 pathway is favoured by high-concentrate low-fibre diets (Flachowsky et al., 2006) inducing a low rumen pH, and the shift from t11 to t0 pathways, known as t10 shift, occurred mainly with fat-enriched diets (Loor et al., 2004). Although t10 shift is widely accepted to occur with high-concentrate diets supplemented with oils, as demonstrated *in vivo* (Roy et al., 2006; Zened et al., 2013) and *in vitro* (Zened et al., 2011), the key factor for its occurrence is not clear, as dietary starch and rumen pH are closely linked. Starch

was identified as the main factor for t10 shift in a long-term fermentation system (Maia et al., 2009), but starch and pH effects could not be completely separated. A low pH has been reported to induce t10 shift in batch (Choi et al., 2005) and continuous culture systems (Fuentes et al., 2009), although starch was also found to promote t10,c12-CLA formation in this latter study. The t10 isomers are thought to be produced from c9,c12-18:2 by *Megasphaera elsdenii* (Kim et al., 2002) and/or *Propionibacter acnes* (Verhulst et al., 1987), which are lactate-consuming bacteria, but the involvement of other bacteria that grow best on starch substrate and/or at low pH is possible. On the other hand, high-fibre diets favour the t11 pathway of BH by fibrolytic bacteria, among them *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticus* are reported to produce t11 isomers (Maia et al., 2007).

The objectives of this study were to investigate independently *in vitro* the effects of buffer pH (6 vs. 7) and fermentation substrate (starch vs. hay) and their interaction on the BH of linoleic acid (c9,c12-18:2), focusing on its disappearance and the production of t10 and t11 isomers, to reveal the contribution of each factor, since each could be corrected independently at the farm level, by dietary bicarbonate for example for rumen pH, and variation of the concentrates/forage ratio in the diet for starch and fibre levels.

Material and methods

In vitro cultures

The experiment was conducted in a 2 × 2 factorial arrangement (2 pH buffers × 2 fermentation substrates), using two ruminally fistulated dry dairy cows as donors of ruminal content. Cows received 8 kg dry matter (DM) per day of corn silage and concentrate-based diet (37/67 on a DM basis). Table 1 shows the

Table 1 Ingredients and chemical composition of the cows' diet (% of dry matter)

Ingredients (% of dry matter)	
Corn silage	37.3
Wheat flour	55.7
Soybean meal	5.6
Minerals and vitamins*	1.4
Nutrient content (% of dry matter)	
Crude protein	11.4
Neutral detergent fibre	18.1
Acid detergent fibre	7.2
Starch	39.2
Total fatty acids	2.3

*Contained: 50 g/kg of P, 140 g/kg of Ca, 60 g/kg of Na, 4 g/kg of Zn, 3.2 g/kg of Mn, 3 g/kg of Fe, 0.8 g/kg of Cu, 250 800 IU/kg of vitamin A, 62 700 IU/kg of vitamin D₃ and 112 IU/kg of vitamin E.

detailed composition of the diet, distributed in two equal meals per day.

Incubation substrates consisted of either 3.0 g of ground (1 mm screen) meadow hay (89% of DM; 8% of crude protein, 3% of crude fat, 8% of ash, 37% of crude fibre, 76% of neutral detergent fibre, 39% of acid detergent fibre and 3% of acid detergent lignin on DM basis) or 1.5 g of corn starch (purity 99%; Merck, Darmstadt, Germany). In both cases, substrates also contained 200 mg of grape seed oil, containing mainly c9,c12-18:2 (60% of total FA) and oleic acid (c9-18:1; 19% of total FA), and 0.15 g of urea (purity 99.5%; Prolabo, Nogent-sur-Marne, France). Fatty acids composition of the different media before incubation is shown in Table 2. The two buffer solutions were based on phosphate and bicarbonate, as described in a previous paper (Troegeler-Meynadier et al., 2003); after saturation with CO₂ at 39 °C, they had a pH near 6 (LpH, low pH buffer, pH = 6.15 on average, 64.12 mM of KH₂PO₄, 2.60 mM of Na₂HPO₄ and 27.50 mM of NaHCO₃) and 7 (HpH, high pH buffer, pH = 7.07 on average, 66.73 mM of Na₂HPO₄ and 11.00 mM of NaHCO₃) respectively. Buffers also contained minerals to sustain microbial growth: 12.06 mM of NaCl, 9.05 mM of KCl, 0.73 mM of CaCl₂ and 0.73 mM of MgSO₄. For each combination of fermentation substrate and pH buffer, six replicates with added oil were incubated for each of the two donor cow, for a total of 12 repetitions per treatment, and four control flasks, without added oil and not incubated, were prepared per donor cow (i.e. eight control flasks in total), which needed four series of *in vitro* incubations. Control flasks were immediately frozen.

Ruminal fluid was taken from each cow before each series of incubation with a vacuum pump 30 min after feeding and strained through a metal sieve (1.6-mm mesh). It was transferred to the laboratory under anaerobic conditions at 39 °C and then centrifuged (150 g, 5 min, 39 °C, CO₂) to produce low-fibre inoculums (<1% of NDF on a DM basis). The pH was measured, and a sample of centrifuged ruminal fluid was taken for FA analysis. Centrifuged ruminal fluid (80 ml) and 80 ml of the LpH or HpH solution, pre-warmed at 39 °C and saturated with CO₂, were placed in 250-ml Erlenmeyer flasks containing the fermentation substrates. Initial pH was measured, and flasks were placed in a water-bath rotary shaker (Aquatron; Infors AG, Bottmingen, Germany) at 39 °C and gassed for 1 h with CO₂. Flasks were then closed with a rubber cap, with a plastic tube leading into water to vent fermentation gas without oxygen ingress. Flasks were stirred at 130 rpm and kept in the dark during the 6-h incubation. Thereafter, incubations were stopped by

Fermentation substrate Buffer pH	Corn starch		Hay		SEM	p-value of FS
	LpH	HpH	LpH	HpH		
Total fatty acids (mg per flask)	259.36	255.04	273.38	273.63	7.07	0.02
Fatty acid quantity (mg per flask)						
10:0	0.05	0.05	0.05	0.05	0.01	0.37
11:0	0.02	<0.01	0.04	0.02	0.01	0.36
12:0	0.33	0.31	0.49	0.48	0.02	<0.01
13:0	0.04	0.02	0.17	0.11	0.01	<0.01
14:0	1.11	1.06	1.32	1.35	0.07	<0.01
15:0	0.84	0.81	0.90	0.91	0.06	0.15
anteiso-15:0	2.01	1.87	1.90	1.91	0.16	0.94
iso-15:0	0.73	0.69	0.68	0.68	0.06	0.65
16:0	28.05	27.15	32.36	32.49	1.32	<0.01
16:1+ anteiso-17:0	1.01	0.95	1.02	0.93	0.16	0.62
17:0	0.65	0.62	0.71	0.73	0.05	0.04
iso-17:0	0.53	0.47	0.59	0.57	0.05	0.10
18:0	43.74	41.49	43.29	43.74	4.03	0.80
c9-18:1	40.31	40.02	41.02	41.02	0.32	<0.01
c11-18:1	0.43	0.41	0.56	0.56	0.04	<0.01
c15-18:1	0.02	0.01	0.01	ND	0.01	0.11
Sum of <i>trans</i> -18:1	6.82	6.15	6.07	6.30	1.31	0.91
c9,c12-18:2	125.57	125.54	127.49	127.15	0.44	<0.01
Sum of CLA	0.35	0.25	0.38	0.57	0.16	0.26
t11,c15-18:2	0.05	0.01	0.06	0.13	0.05	0.09
c9,c12,c15-18:3	1.30	1.29	6.26	6.00	0.14	<0.01

HpH, high pH buffer; LpH, low pH buffer; FS, fermentative substrate; ND, non-detected.

placing the flasks in ice water and final pH was measured. The contents of the flasks were frozen, freeze-dried (Virtis Freezemobile 25; Virtis, Gardiner, NY, USA), weighed, ground and homogenized in a ball mill (Dangoumau, distributed by Prolabo), and kept at $-18\text{ }^{\circ}\text{C}$ until analysis.

Chemical analysis

The FAs were extracted and methylated *in situ* with the procedure of Park and Goins (1994), except that the solution of 14% of boron trifluoride in methanol was replaced by a solution of methanol-acetylchloride (10:1). Nonadecanoic acid (C19:0) was used as the internal standard at a dose of 0.8 mg. The FA methyl esters were then quantified by gas chromatography (Agilent 6890N, equipped with a model 7683 auto injector; Network GC System, Palo Alto, CA, USA), using a fused silica capillary column (CPSil88, 100 m \times 0.25 mm ID, 0.20 μm film thickness; Varian, Middelburg, The Netherlands).

For the analysis of samples, flame ionization detector temperature was maintained at $260\text{ }^{\circ}\text{C}$ and the injector at $255\text{ }^{\circ}\text{C}$, the split ratio was 1:75. Hydrogen was the carrier gas with a constant flow of 1 ml/min.

Table 2 Initial quantities (mg) of fatty acids in the different media before incubation ($n = 48$)

The samples were injected in $0.5\mu\text{l}$ of hexane. Initial temperature of the oven was $60\text{ }^{\circ}\text{C}$, held for 1 min, and then increased by $20\text{ }^{\circ}\text{C}/\text{min}$ to $150\text{ }^{\circ}\text{C}$, held at $150\text{ }^{\circ}\text{C}$ for 15 min, then increased by $2\text{ }^{\circ}\text{C}/\text{min}$ to $175\text{ }^{\circ}\text{C}$, held at $175\text{ }^{\circ}\text{C}$ for 20 min, then increased by $10\text{ }^{\circ}\text{C}/\text{min}$ to $225\text{ }^{\circ}\text{C}$, maintained at $225\text{ }^{\circ}\text{C}$ for 10 min, and increased by $10\text{ }^{\circ}\text{C}/\text{min}$ to reach a final temperature of $240\text{ }^{\circ}\text{C}$. This first method did not allow 16:1 to be separated from anteiso-17:0, t13+t14-18:1 from c9-18:1 and alpha-linolenic acid (c9,c12,c15-18:3) from 20:1, so a second analysis was carried out. Flame ionization detector temperature was maintained at $260\text{ }^{\circ}\text{C}$ and the injector at $255\text{ }^{\circ}\text{C}$; the split ratio was 1:50. Hydrogen was the carrier gas with a constant pressure of 160 kPa. The samples were injected in $0.5\mu\text{l}$ of hexane. Initial oven temperature was $60\text{ }^{\circ}\text{C}$, held for 3 min, increased by $8\text{ }^{\circ}\text{C}/\text{min}$ to $190\text{ }^{\circ}\text{C}$, held at $190\text{ }^{\circ}\text{C}$ for 13 min, increased by $5\text{ }^{\circ}\text{C}/\text{min}$ to $225\text{ }^{\circ}\text{C}$, held at $225\text{ }^{\circ}\text{C}$ for 10 min, increased by $10\text{ }^{\circ}\text{C}/\text{min}$ to a final temperature of $230\text{ }^{\circ}\text{C}$ and maintained there for 10 min.

Peaks were identified and quantified by comparison with commercial standards (Sigma, St. Louis, MO, USA), except 18:1 other than t9-18:1, t11-18:1 and c9-18:1, which were identified by order of elution.

Calculations and statistical analysis

The FA balance (expressed as mg per flask) during incubation of each flask was calculated as the amount assayed after incubation (final quantity) minus the initial amount, calculated as the quantity in the non-incubated flasks plus that carried out by the 200 mg of grape seed oil.

Sums of CLA and *trans*-18:1 were obtained by adding productions of all isomers measured; sums of t10 and t11 isomers were obtained by adding t10 and t11 isomers originating from c9,c12-18:2 BH:

$$\text{CLA} = \text{t10,c12-CLA} + \text{c9,c11-CLA} + \text{c9,t11-CLA} + \text{t9,t11-CLA}$$

$$\text{trans-18:1} = \text{t4-18:1} + \text{t5-18:1} + \text{t6+t7+t8-18:1} + \text{t9-18:1} + \text{t10-18:1} + \text{t11-18:1} + \text{t12-18:1} + \text{t13+t14-18:1} + \text{t15-18:1} + \text{t16-18:1}$$

$$\text{t10 isomers} = \text{t10,c12-CLA} + \text{t10-18:1}$$

$$\text{t11 isomers} = \text{c9,t11-CLA} + \text{t9,t11-CLA} + \text{t11-18:1}$$

The rates (mg/l/h) and efficiencies of the c9,c12-18:2 isomerization (v1, E1), CLA reduction (v2, E2) and *trans*-18:1 reduction (v3) were calculated as previously described by Troegeler-Meynadier et al. (2006):

$$v1 = ([c9,c12-18:2]b - [c9,c12-18:2]e)/6, \text{ with } 6 \text{ being the incubation time}$$

$$E1 = ([c9,c12-18:2]b - [c9,c12-18:2]e)/[C18:2]b,$$

where [c9,c12-18:2]b and [c9,c12-18:2]e represented the concentration of c9,c12-18:2 at the beginning and at the end of the 6 h incubation respectively.

$$v2 = ([c9,c12-18:2]b - [c9,c12-18:2]e + [CLA]b - [CLA]e)/6,$$

$$E2 = ([c9,c12-18:2]b - [c9,c12-18:2]e + [CLA]b - [CLA]e)/([c9,c12-18:2]b - [c9,c12-18:2]e + [CLA]b)$$

where [CLA]b and [CLA]e represented the concentration of total CLA isomers measured at the beginning and end of the 6 h incubation respectively.

$$v3 = ([c9,c12-18:2]b - [c9,c12-18:2]e + [CLA]b - [CLA]e + [trans-18:1]b - [trans-18:1]e)/6,$$

$$E3 = ([c9,c12-18:2]b - [c9,c12-18:2]e + [CLA]b - [CLA]e + [trans-18:1]b - [trans-18:1]e)/([c9,c12-18:2]b - [c9,c12-18:2]e + [CLA]b - [CLA]e + [trans-18:1]b),$$

where [trans-18:1]b and [trans-18:1]e represented the concentration of total *trans*-18:1 isomers measured at the beginning and end of the 6-h incubation period. As c9,c12,c15-18:3 was a minor unsaturated FA at the beginning of incubation (0.6% and 6.0% of total FA for cultures with starch and hay substrates

respectively) due to the great addition of c9,c12-18:2 by grape seed oil, the production of *trans*-18:1 from c9,c12,c15-18:3 was ignored (Troegeler-Meynadier et al., 2006).

All data were analysed using the GLM procedure of SYSTAT (version 9; SPSS, Chicago, IL, USA) and reported as mean values and their standard errors:

variable = mean + day of incubation effect + cow effect + buffer effect + substrate effect + buffer × substrate interaction + ε.

Differences were considered significant at $p \leq 0.05$, and trends were declared at $p \leq 0.10$.

Results

Ruminal cultures initially contained numerous *trans*-18:1 and CLA isomers, including 2.58 mg of t11 isomers and 1.62 mg of t10 isomers, on average. The most abundant FA was 18:0 (43.07 mg, on average), and c9-18:1 and c9,c12-18:2 quantities were similar among media (40.59 mg and 126.44 mg, on average respectively), but the quantity of c9,c12,c15-18:3 was higher in cultures with hay than in cultures with starch (6.13 mg vs. 1.30 mg on average respectively; Table 2). Hay also carried some other FA. Buffer addition had no effect on FA initial quantities of cultures.

The initial pH of incubates was significantly lower (by 0.5 units) in LpH than in HpH flasks (Table 3). The final pH of incubated media depended on the pH buffer but also on the fermentation substrate, with an interaction between these two factors, leading to the lowest final pH value (pH = 5.8) for cultures with corn starch and LpH (Table 3).

Considering FA with less than 18 carbons, iso-17:0 production was increased and 13:0 production was decreased by LpH. Compared with hay, starch as fermentation substrate favoured the production of more C10 to C17 FA (Table 3), including odd and branched chain FA. In particular, the productions of 12:0, 14:0 and 16:0 were 5.7, 2.9 and 2.4 times higher with starch than with hay as fermentation substrate respectively. A significant interaction between pH buffer and fermentation substrate was only observed for the 13:0 and iso-15:0 productions; however, no large differences between values were noticed. A trend towards an interaction between pH buffer and fermentation substrate was observed for anteiso-15:0 and 14:0, leading to the highest production for cultures incubated on starch with HpH.

For C18 FA, compared with starch, hay as fermentation substrate reduced c11-18:1 production but favoured the production of 18:0, c15-18:1, *trans*-18:1

Table 3 Initial and final pH, and production or disappearance (mg) of fatty acids (FA) assayed in the media after 6-h incubation with 200 mg of grape seed oil ($n = 48$)

Fermentation substrate	Corn starch		Hay		SEM	Effect, p-value		
	LpH	HpH	LpH	HpH		FS	pH	FS × pH
Initial pH	6.25	6.78	6.22	6.75	0.02	0.60	<0.01	–
Final pH	5.82	6.56	6.21	6.71	<0.01	<0.01	<0.01	0.02
Fatty acid production or disappearance (mg per flask)								
10:0	0.02	0.01	ND	0.01	<0.01	0.17	0.99	0.07
11:0	0.01	0.02	–0.01	0.01	<0.01	0.03	0.05	0.46
12:0	0.35	0.34	0.05	0.07	0.02	<0.01	0.86	0.72
13:0	0.06	0.08	<0.01	0.08	0.01	0.06	<0.01	0.05
14:0	0.71	0.86	0.31	0.23	0.03	<0.01	0.52	0.06
15:0	0.49	0.54	0.40	0.31	0.02	<0.01	0.68	0.13
anteiso-15:0	0.66	0.99	0.46	0.38	0.06	<0.01	0.29	0.08
iso-15:0	0.20	0.24	0.17	0.12	0.01	<0.01	0.75	0.03
16:0	2.54	2.75	1.09	1.07	0.12	<0.01	0.70	0.62
16:1+ anteiso-17:0	0.59	0.84	0.34	0.42	0.05	<0.01	0.08	0.37
17:0	0.35	0.35	0.30	0.28	0.01	<0.01	0.58	0.61
iso-17:0	0.15	0.10	0.12	0.09	0.01	0.22	0.02	0.73
18:0	8.84	10.65	20.62	24.91	0.94	<0.01	0.12	0.51
c9-18:1	–4.83	–5.28	–9.47	–10.98	0.23	<0.01	0.04	0.25
c11-18:1	1.82	2.02	1.38	1.35	0.03	<0.01	0.18	0.09
c15-18:1	<0.01	0.01	0.03	0.08	<0.01	<0.01	<0.01	0.09
Sum of <i>trans</i> -18:1	2.01	2.89	26.68	30.83	0.81	<0.01	0.11	0.37
c9,c12-18:2	–13.49	–18.69	–48.61	–60.83	1.21	<0.01	<0.01	0.16
Sum of CLA	0.57	3.03	8.39	13.65	0.41	<0.01	<0.01	0.10
t11,c15-18:2	–0.02	–0.01	0.05	–0.06	0.01	0.70	0.05	0.01
c9,c12,c15-18:3	–0.40	–0.49	–2.30	–2.03	0.07	<0.01	0.55	0.19
Percentage of disappearance of unsaturated fatty acids (%)								
c9-18:1	–12.00	–13.21	–23.14	–26.75	1.13	<0.01	0.03	0.27
c9,c12-18:2	–10.75	–14.91	–38.12	–47.84	1.95	<0.01	<0.01	0.15
c9,c12,c15-18:3	–30.95	–38.49	–36.08	–33.32	2.35	0.97	0.31	0.03

HpH, high pH buffer; LpH, low pH buffer; FS, fermentative substrate; ND, non-detected.

(all isomers, Tables 3 and 4) and CLA (all isomers except c9,c11-CLA, Table 4). Notably, the production of 18:0, *trans*-18:1 and CLA was 2.3, 12.6 and 6.1 times higher respectively, with hay than with starch. The productions of t10 and t11 isomers were 3.4 and 12.2 times higher with hay than with starch as substrate respectively. The quantity of unsaturated C18 FA that disappeared was significantly lower with starch than with hay, and the percentage of disappearance was also lower with starch than with hay except for c9,c12,c15-18:3.

Buffer pH significantly affected some C18 FA balances during incubation, although to a lesser extent than substrate (Table 3). The production of CLA was 1.2 times greater with HpH than with LpH. Moreover, the effects of buffer pH on CLA and *trans*-18:1 production concerned major isomers (Table 4): t9-18:1, t11-18:1, t16-18:1 and c9,t11-CLA productions were higher with HpH, and t10-18:1 production was higher with LpH. This resulted in a significant 1.5 times

increase in t11 isomers and a 1.4 times decrease in t10 isomers with HpH compared with LpH. HpH slightly but significantly increased c9-18:1 and c9,c12-18:2 disappearance (Table 3). Few significant interactions were observed (Tables 3 and 4): they concerned mainly CLA isomers, in particular c9,t11-CLA production, which was highest in cultures with hay and HpH, and lowest in cultures with starch and LpH (Table 4).

Kinetic parameters were affected by fermentation substrate and buffer pH (Table 5), but no interaction between these two factors was observed. Rate and efficiency of c9,c12-18:2 isomerization were increased by hay as substrate and by HpH. The rate of the first reduction was increased by single effects of hay and HpH, but the efficiency of this reduction was increased by single effects of starch and LpH. The last reduction was only slightly affected by fermentation substrate: hay as substrate increased its rate but decreased its efficiency.

Table 4 Production of *trans*-18:1 and CLA isomers (mg) in the media after 6-h incubation with 200 mg of grape seed oil ($n = 48$)

Fermentation substrate Buffer pH	Corn starch		Hay		SEM	Effect, p-value		
	LpH	HpH	LpH	HpH		FS	pH	FS × pH
<i>Trans</i> -18:1 isomers								
t4-18:1	-0.01	0.01	0.11	0.17	0.01	<0.01	0.03	0.39
t5-18:1	<0.01	<0.01	0.11	0.14	0.01	<0.01	0.27	0.10
t6+t7+t8-18:1	0.09	0.13	1.04	1.18	0.03	<0.01	0.12	0.44
t9-18:1	0.07	0.11	0.61	0.72	0.02	<0.01	0.04	0.31
t10-18:1	0.79	0.41	2.61	1.86	0.12	<0.01	0.03	0.46
t11-18:1	0.40	1.64	17.49	21.52	0.83	<0.01	0.10	0.45
t12-18:1	0.27	0.25	1.55	1.61	0.05	<0.01	0.79	0.66
t13+t14-18:1	0.15	0.13	2.04	2.24	0.06	<0.01	0.43	0.41
t15-18:1	0.16	0.14	0.63	0.71	0.04	<0.01	0.68	0.50
t16-18:1	0.07	0.08	0.50	0.67	0.03	<0.01	0.10	0.13
CLA isomers								
t10,c12-CLA	0.14	0.28	0.66	0.49	0.03	<0.01	0.62	0.02
c9,c11-CLA	ND	0.02	0.02	0.01	<0.01	0.09	0.38	0.06
c9,t11-CLA	0.39	2.52	7.34	12.87	0.39	<0.01	<0.01	0.04
t9,t11-CLA	0.03	0.22	0.37	0.27	0.03	<0.01	0.45	0.03
Sum of t11 isomers	0.83	4.38	25.19	34.67	1.08	<0.01	<0.01	0.20
Sum of t10 isomers	0.94	0.68	3.27	2.35	0.14	<0.01	0.04	0.24

HpH, high pH buffer; LpH, low pH buffer; FS, fermentative substrate; ND, non-detected.

Table 5 Rates (mg/l/h) and efficiencies of the three reactions of linoleic acid biohydrogenation for 6-h incubation with 200 mg of grape seed oil ($n = 48$)

Fermentation substrate Buffer pH	Corn starch		Hay		SEM	Effect, p-value		
	LpH	HpH	LpH	HpH		FS	pH	FS × pH
Isomerization of c9,c12-18:2								
Rate v1	14.06	19.47	50.63	63.37	1.26	<0.01	<0.01	0.16
Efficiency E1	0.11	0.15	0.38	0.48	0.01	<0.01	<0.01	0.15
Reduction in CLA								
Rate v2	13.46	16.31	41.90	49.15	1.02	<0.01	0.02	0.30
Efficiency E2	0.93	0.82	0.82	0.77	0.01	<0.01	<0.01	0.10
Reduction in <i>trans</i> -18:1								
Rate v3	11.37	13.30	14.11	17.04	0.92	0.01	0.22	0.73
Efficiency E3	0.59	0.52	0.26	0.25	0.02	<0.01	0.21	0.37

HpH, high pH buffer; LpH, low pH buffer; FS, fermentative substrate.

Discussion

Balance of FA with less than 18 carbons

In ruminants, C10 to C17 FAs are of interest because they could give some information about the bacterial community, in particular odd and branched FA, because these originate from bacterial synthesis (Vlaeminck et al., 2006a,b), and bacteria species differ in the FA they mainly produce (Ifkovits and Ragheb, 1968; Fievez et al., 2012). Moreover, some of them, that is, branched chain FA, may have a beneficial role in human health (Ran-Ressle et al., 2011), so that an increase in their ruminal production could increase their milk and meat content for human consumers.

Production of C10 to C17 FA, mainly 12:0, 14:0, 16:0 and anteiso-15:0, was higher with starch than hay as fermentation substrate. This is in agreement

with the study of Dewhurst et al. (2007) in which duodenal flows of 10:0, 12:0, 14:0, 16:0, and some odd and branched chain FA, that is, anteiso-15:0, iso-15:0, 17:0 and iso-17:0, increased linearly with the addition of concentrate to the diet. Indeed, some of these FAs predominate in amylolytic and lactate-utilizing bacteria, but are scarce in fibrolytic ones (Ifkovits and Ragheb, 1968; Vlaeminck et al., 2006a). In our experiment, all these FAs were produced in larger amounts in starch cultures, where amylolytic and lactate-consuming bacteria would be able to develop rapidly. On the other hand, iso-FAs are reported to be merely linked to fibrolytic bacteria (Fievez et al., 2012). In the present study, iso-17:0 production was not affected by fermentation substrate, and iso-C15:0 production was higher with starch than with hay. These FAs are both present in some amylolytic and

fibrolytic bacteria (Ifkovits and Ragheb, 1968). Hence, iso-FAs are difficult to attribute to specific bacterial species, as suggested by differences between studies, in particular between studies relative to digestive content and studies relative to milk FA (Dewhurst et al., 2007; Fievez et al., 2012). This could be explained by an important effect of the diet of cows on the microbiota, which makes comparisons between studies difficult, and by the origin of milk odd and branched chain FA, that could result from tissue synthesis and not only from rumen metabolism (Berthelot et al., 2001).

The buffer pH had little effect on C10 to C17 FA production in our study, except 13:0 and iso-17:0 productions that were only slightly affected. The pH range for bacterial growth is very wide: for example, 4.5–7.7 for the amylolytic bacteria *S. bovis* (with an optimum at 6.4), 4.6–7.8 for the lacticolytic bacteria *M. elsdenii* (with an optimum at 6.1), or 5.4–7.7 for the fibrolytic bacteria *B. fibrisolvens* (with an optimum at 6.3–6.5; Therion et al., 1982). So in our range of pH, a great variety of bacteria could develop, probably resulting in no clear selection of species by buffer pH.

Biohydrogenation steps of unsaturated C18 fatty acids

The efficiency of unsaturated C18 FA disappearance was significantly affected by fermentation substrate and pH buffer. The disappearances of c9-18:1 and c9, c12-18:2 were significantly higher with hay than starch substrate and were significantly greater with HpH than LpH. The percentage of disappearance of c9, c12, c15-18:3 was not affected by pH or by substrate, and its disappeared quantity was not affected by pH, but was higher in cultures with hay. This could be explained by the higher initial quantity of c9, c12, c15-C18:3 in these cultures, that is 6.1 mg per flask, compared with 1.6 mg in cultures with starch (Table 2). Hay cultures initially contained nearly the same quantities of c9-18:1 (41.0 vs. 40.2 mg per flask) and c9, c12-18:2 (127.3 vs. 125.6 mg) than starch cultures, so that the 2.0 and 3.4 times higher disappearances observed, respectively, for these FAs with hay than starch were due to higher BH efficiencies. Values obtained in the present study for kinetic parameters of c9, c12-18:2 BH (Table 5) were of the same order of magnitude as those obtained in a previous study (Troegeler-Meynadier et al., 2006).

The greater disappearance of unsaturated FA with hay than starch resulted in increased production of all c9, c12-18:2 BH intermediates or final products: CLA, *trans*-18:1 and 18:0. In fact, BH is mainly due to *B. fibrisolvens*, a fibrolytic bacterium that carries out

the two first reactions of c9, c12-18:2 BH, and secondly to *Butyrivibrio hungatei* (Van de Vossenberg and Joblin, 2003) and *B. proteoclasticus* (Wallace et al., 2006), other fibrolytic bacteria responsible for the last reduction to 18:0. Fibrolytic bacteria, including biohydrogenating ones, were more likely to develop on hay as the fermentation substrate than on starch, and so could achieve the three steps of c9, c12-18:2 BH, leading to higher rates for the three BH reactions and a higher efficiency for the first reaction of isomerization with hay.

In the present study, the accumulation of *trans*-18:1 and CLA obtained with cultures incubated on hay could not be linked to an inhibition of the reductases, as hay increased the rates of both reductions. The rapid disappearance of c9, c12-18:2, due to a high rate of isomerization observed in hay cultures, led to a great production of CLA, *trans*-18:1 and 18:0. In hay cultures compared with starch cultures, accumulation of CLA and *trans*-18:1 resulted from a saturation of the two reductions, leading to a decrease in their efficiencies in spite of increased rates. Such a saturation of the second reduction by a large quantity of its substrate or CLA had previously been hypothesized (Troegeler-Meynadier et al., 2006). In starch cultures, the productions of CLA and *trans*-18:1 were limited by a low isomerization rate.

Buffer pH did not affect c9, c12, c15-18:3 balance, as previously reported with low c9, c12, c15-18:3 level cultures (Fuentes et al., 2011). However, LpH slightly decreased the disappearance of c9-C18:1 and strongly decreased that of c9, c12-18:2. Such a protection of c9, c12-18:2 from BH by a low pH could firstly be explained by an inhibition of lipolysis as the growth of the main lipolytic bacteria, that is, *Anaerovibrio lipolytica*, has been shown to be decreased at low pH (Fuentes et al., 2011). Nevertheless, these authors observed that lipolysis was barely reduced at low pH as opposed to a strong BH reduction. In our study, the reduction in BH by low pH was linked to an inhibition of c9, c12-18:2 isomerization (Table 5), as previously reported (Troegeler-Meynadier et al., 2006). As a result, HpH resulted in a greater CLA production and a slight increase in *trans*-18:1 production. The reduction in CLA to *trans*-C18:1 had a higher rate but a lower efficiency with HpH than LpH, suggesting an inhibition by excess of substrate, originating in a high isomerization rate. Buffer pH did not significantly affect the kinetic parameters of the second reduction, so it did not affect 18:0 production. The slight accumulation of *trans*-C18:1 observed with HpH was probably due to the slowness of the third reaction compared with the previous reactions. Such results contrast with our

previous study (Troegeler-Meynadier et al., 2006), reporting an inhibition of the second reduction by low pH, but in this study, this inhibition only became significant at $\text{pH} \leq 5.5$, that is, lower than in the present study. Moreover, HpH cultures could have maintained a higher protozoa population (Dehority, 2005), which could have participated in the accumulation of *trans*-18:1 and CLA as they have high contents of CLA and *trans*-18:1 (Devillard et al., 2006).

Biohydrogenation pathways of unsaturated C18 fatty acids

Regarding the two main pathways of c9,c12-C18:2 BH, the t11 isomers production was much higher with hay than starch and higher with HpH than LpH buffer. Among fibrolytic bacteria, *B. fibrisolvans* is the main t11 isomers producer (Maia et al., 2007) and is able to develop on hay but not on starch (Klieve et al., 2003), so that hay as fermentation substrate offered better conditions for its growth and/or activity than starch.

Choi et al. (2005), Fuentes et al. (2011) and Troegeler-Meynadier et al. (2003, 2006) also noticed that t11 isomers production was favoured by a high pH. As the incubation duration in the present study was probably not sufficient to induce a notable modification of the microflora, the lower production of t11 isomers in cultures with LpH was probably due to an inhibition of the $\Delta 12$ isomerase, whose optimal pH is between 7 and 7.2 (Kepler and Tove, 1967). This inhibition begins at pH 6.3 (Choi et al., 2005) and would be very important below pH 6.0 (Troegeler-Meynadier et al., 2006). Indeed, a major inhibition of t11 isomers production was observed in the cultures with LpH. An interaction between pH and fermentation substrate was observed for c9,t11-CLA production: the HpH + hay combination led to the highest value and the LpH + starch combination to the lowest value. Hay was a good substrate for t11 isomers-producing bacteria and favoured an elevation of pH, and HpH favoured the activity of these bacteria, in particular that of the $\Delta 12$ isomerase they synthesized, unlike starch and LpH.

The surprising finding in the present study, contradicting previous research, was the higher values of t10 isomers production in cultures with hay as the fermentation substrate than in cultures with starch. Similarly, Fuentes et al. (2009) observed a higher t10 isomers production with their low-concentrate diet compared with their high-concentrate diet. Usually, t10 isomers are favoured by high-starch diets, whilst t11 isomers are favoured by high-fibre diets (Flachowsky et al., 2006; AbuGhazaleh and Jacobson, 2007).

Moreover, starch has been identified as the main factor for t10 isomers production in a long-term fermentation system (Maia et al., 2009). Among bacteria favoured by a high-starch diet, Kim et al. (2002) reported that *M. elsdenii*, a lactate-utilizing bacterium, is able to produce t10,c12-CLA from c9,c12-18:2. Nevertheless, the isomerization of c9,c12-18:2 into t10,c12-CLA by *M. elsdenii* still remains questionable, because Maia et al. (2007) did not observe a capacity of this bacterium to isomerize c9,c12-18:2. Moreover, in our experiment, the production of t10,c12-CLA by *M. elsdenii* in cultures with hay was probably low as this bacterium is unable to develop on a medium without grain (Klieve et al., 2003). Other species like *P. acnes* and *Propionibacter freudenreichii* are able to produce t10,c12-CLA from c9,c12-18:2 (Verhulst et al., 1987). *P. acnes* is also a lactate-consuming bacterium. As reported above, lactate-consuming and amylolytic bacteria probably grew and acted better on starch substrates, as suggested by the high production of some specific short and medium FA in starch cultures. However, hay could have provided these bacteria, as *Propionibacteria* that are similar to those isolated from rumen fluid of cattle can be found in hay (6.10^8 per gram of timothy hay, for example) as reported by Gutierrez (1953). Moreover, this author showed that *Propionibacteria* have the capacity to grow on hay. So in our experiment, *P. acnes* could have been responsible for the high t10 isomers production in the cultures incubated for 6 h on hay.

As expected, production of t10 isomers was higher with LpH than with HpH. On the other hand, Maia et al. (2009) did not observe an effect of pH on t10 isomers, but their low pH was 6.0, probably insufficiently acidic to promote t10 isomers production. Previous *in vitro* studies also reported a significant increase in t10 isomers (Troegeler-Meynadier et al., 2003) or t10,c12-CLA (Choi et al., 2005) when pH decreased from 6.6–6.5 to 5.6–5.4. However, as in the present study, values obtained for t10 isomers were very low and were largely below those of t11 isomers. AbuGhazaleh and Jacobson (2007) and Fuentes et al. (2009, 2011) obtained *in vitro* a ratio of t10/t11 isomers above 1, but the comparison with our experiment is difficult because they used continuous ruminal cultures over 10, 8 and 7 day at pH 5.6. The t10-producing bacteria probably had enough time to develop and be selected by the low pH and/or the high starch content of the substrates used in their study, but not in ours. As our 6-h incubation duration probably was not sufficient to induce a notable modification of the relative abundances of bacteria, the effect of low pH in our

experiment was more probably due to a greater $\Delta 9$ isomerase production by t10 biohydrogenating bacteria, or to the enhancement of the activity of the $\Delta 9$ isomerase. A greater activity of the $\Delta 9$ isomerase could be due to a better activity at low pH than $\Delta 12$ isomerase (optimal pH around 7.0; Kepler and Tove, 1967) and/or to an increased availability of its substrate, c9,c12-18:2, because of the inhibition of the $\Delta 12$ isomerase.

Conclusion

Ruminal c9,c12-18:2 BH was greatly affected *in vitro* by the fermentation substrate and buffer pH, but these factors essentially acted independently, and buffer pH,

within physiological limits, had smaller effects than fermentation substrate. So, to promote ruminal production of t11 isomers for human consumer, a high-fibre diet is necessary. Fiber mainly acts as a growth substrate for t11-producing bacteria. Low pH slowed $\Delta 12$ isomerase activity and so resulted in a decreased t11 isomers production, but addition of bicarbonate in high-starch diet was not efficient enough to restore t11 isomers production.

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