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Effects of pH and Concentrations of Linoleic and Linolenic Acids on Extent and Intermediates of Ruminal Biohydrogenation in Vitro

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ABSTRACT

Three experiments were conducted by in vitro incubations in ruminal fluid to investigate the effects of pH and amounts of linoleic and linolenic acids on the extent of their biohydrogenation, the proportions of conjugated linoleic acid (CLA) and *trans*-C18:1 as intermediates, and the ratio *trans*-10:*trans*-11 intermediates. The effects of pH and amount of linoleic acid were investigated in kinetic studies, and effects of the amount of linolenic acid were studied with 6-h incubations. With identical initial amounts of linoleic acid, its disappearance declined when the mean pH during incubation was under 6.0 compared with a mean pH over 6.5, and when the amount of linolenic acid increased from 10 to 180 mg/160-ml flask, suggesting an inhibition of the isomerization step of the biohydrogenation. Low pH decreased the ratio of *trans*-10:*trans*-11 intermediates. With initial amounts of linoleic acid increasing from 100 to 300 mg, the percentage of linoleic acid disappearance declined, but the amount that disappeared increased, without modification of the *trans*-10:*trans*-11 ratio, suggesting a maximal capacity of isomerization rather than an inhibition. Moreover, increasing initial linoleic acid resulted in high amounts of *trans*-C18:1 and an increase of C18:0 that was a linear function of time, suggesting a maximal capacity for the second reduction step of biohydrogenation. High amounts of initial linolenic acid did not affect the amounts of CLA, *trans*-C18:1, or the ratio *trans*-10:*trans*-11. Based on these experiments, a ruminal pH near neutrality with high amount of dietary linoleic acid should modulate the reactions of biohydrogenation in a way that supports CLA and *trans*-11C18:1 in the rumen.

(**Key words:** biohydrogenation, conjugated linoleic acid, in vitro, polyunsaturated fatty acids)

Abbreviation key: BH = biohydrogenation, **CLA** = conjugated linoleic acid, **FA** = fatty acids, **HpH** = high-pH buffer, **LpH** = low-pH buffer, **PUFA** = polyunsaturated fatty acids, **t10 FA** = *trans*-10C18:1 + *trans*-10, *cis*-12C18:2, **t11FA** = *trans*-11C18:1 + *cis*-9, *trans*-11C18:2.

INTRODUCTION

Conjugated linoleic acid (**CLA**) groups positional and geometric isomers of linoleic acid (*cis*-9,*cis*-12C18:2, which will be referred as *cis*-C18:2) (Ha et al., 1989). Among them, *cis*-9,*trans*-11C18:2 and *trans*-10,*cis*-12C18:2 are the most abundant in the human food (Chin et al., 1992), and have been shown to have specific dietetic properties: *cis*-9,*trans*-11C18:2 inhibits carcinogenesis (Parodi, 1999) and *trans*-10,*cis*-12C18:2 affects lipid metabolism (Park et al., 1999). Moreover, in laboratory animals, CLA has been shown to modulate atherogenesis (Nicolosi et al., 1993), diabetes (Houseknecht et al., 1998), and immunity (Yamasaki et al., 2000), but the responsible isomer(s) is (are) still unknown. The major sources of CLA for human are milk and dairy products (Chin et al., 1992), so that an increase of CLA content in cow's milk would increase CLA intake with possible beneficial effects on consumer's health.

Conjugated linoleic acid of milk originates from ruminal biohydrogenation (**BH**) of dietary *cis*-C18:2 and linolenic acid (*cis*-9, *cis*-12, *cis*-15C18:3, which will be referred as *cis*-C18:3). The BH of *cis*-C18:2 is divided into three steps: isomerization to CLA, reduction of CLA to *trans*-C18:1 by a first reductase, and reduction of *trans*-C18:1 to C18:0 by a second reductase. Initial

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isomerization produces *trans*-11 and presumably *trans*-10 isomers (Griinari and Bauman, 1999). The BH of *cis*-C18:3 comprises four steps (Griinari and Bauman, 1999): isomerization to conjugated *cis*-C18:3, and three successive reductions that produce C18:2 isomers other than CLA, *cis* or *trans* isomers of C18:1 and finally C18:0, respectively.

A minor part of CLA in milk comes directly from ruminal CLA, but most milk *cis*-9, *trans*-11C18:2 is synthesized in the mammary gland by desaturation of *trans*-11C18:1 (Griinari et al., 2000), which is the main *trans*-C18:1 produced by BH of *cis*-C18:2. Whatever the origin of milk CLA, factors affecting BH would be able to modulate CLA contents in milk. Ruminal pH and source and amount of polyunsaturated fatty acids (**PUFA**) are known to affect quantitatively the extent of BH (Van Nevel and Demeyer, 1996; Griinari and Bauman, 1999; Beam et al., 2000). From a qualitative point of view, Griinari et al. (1998) noticed that acid conditions in the rumen increase *trans*-10C18:1 in milk. However, the effects of pH and PUFA on the intermediates of BH in the rumen have not been extensively studied (Kucuk et al., 2001; Meynadier et al., 2002).

Our purpose was to investigate in vitro the effect of pH and PUFA source and amount on the extent of BH of PUFA, on the proportions of CLA and *trans*-C18:1, and on the equilibrium between (*trans*-10C18:1 + *trans*-10, *cis*-12C18:2) (**t10 FA**) and (*trans*-11C18:1 + *cis*-9, *trans*-11C18:2) (**t11FA**) during ruminal incubations.

MATERIALS AND METHODS

Description of Experiments

Three experiments were conducted in vitro to investigate the effects of pH, amount of *cis*-C18:2, and the amount of *cis*-C18:3 on BH of PUFA. Incubation times were 2, 4, 8, 16, and 24 h in Exp. 1 and 2, and 6 h in Exp. 3. The buffer solutions were based on phosphate and bicarbonate, which have both been previously used by others in experiments on BH (Kim et al., 2000; Martin and Jenkins, 2002; Meynadier et al., 2002; Wang et al., 2002; Enjalbert et al., 2003). In Exp. 1, the 2 buffer solutions resulted in an initial pH near 6 and 7, and were called low pH (**LpH**) and high pH (**HpH**), respectively.

Fatty acid (**FA**) compositions of added fat for the 3 experiments are shown in Table 1. In Exp. 1, added fat was soybean oil. In Exp. 2, 100 or 300 mg of *cis*-C18:2 was associated with a constant amount of other FA, and in Exp. 3, 10, 60, 120, or 180 mg of *cis*-C18:3 was associated with a constant amount of other FA; in both experiments, a mixture of oils (canola, linseed, olive, soybean, grapeseed), tripalmitin (Sigma-Aldrich Chimie, St. Quentin Fallavier, France), and tristearin (ICN Biomedicals Inc., Orsay, France) was used.

In Vitro Cultures

Incubations were accomplished in a waterbath rotary shaker (Aquatron; Infors AG, 4103 Bottmingen, Germany). Ruminal fluid was obtained from 2 fistulated dry cows receiving 8.5 kg of DM of a blend of corn silage and concentrates, and orchardgrass hay ad libitum. One liter of ruminal fluid was taken from each cow with a vacuum pump 1 h after feeding, and strained through a metal sieve (1.6 mm mesh). The ruminal fluids obtained from the 2 cows were mixed in a bottle and transferred (30 min) to the laboratory under anaerobic conditions at 39°C.

Eighty milliliters of this mixed ruminal fluid and 80 ml of a buffer solution were placed in a 250-ml Erlenmeyer flask containing added fat and 3 g of dehydrated alfalfa. All buffer solutions were prewarmed at 39°C and saturated with CO_2 when containing bicarbonate. The filled flasks were gassed with CO_2 and placed in the waterbath at 39°C. They were closed in order to clear out fermentation gas without entrance of oxygen and stirred at 130 rpm. They were kept from light during incubation.

Six replicates, in 2 or 3 series of incubations, were realized for each pH, each amount of PUFA, and each incubation time. For each incubation time and each buffer solution in each series, a control flask without added fat was incubated. Incubations were stopped by placing the flasks into iced water, and pH was then measured. The contents of the flasks were frozen, freeze-dried (Virtis Freezemobile 25; Virtis, Gardiner, NY), weighed, ground, homogenized in a ball mill (Dangoumau, distributed by Prolabo, Nogent-sur-Marne, France), and kept at –18°C until analysis.

Analysis of Fatty Acids

The FA of feed samples and residues of incubation were extracted and methylated with the 1-step procedure of Sukhija and Palmquist (1988), after addition of C19:0 as an internal standard. This method is known to lead to underestimation of *cis*-9,*trans*-11C18:2 and *trans*-10,*cis*-12C18:2 by 41 and 44%, respectively, due to isomerization during transmethylation (Duckett et al., 2002). Therefore, the values for CLA isomers presented in this article can only be used for comparisons within this experiment, not for comparisons with values obtained by others with different analytical methods. The FA methyl esters were then quantified by gas chromatography (Agilent 6890N,

Table 1. Amount of added fatty acids in Experiments 1, 2, and 3 (mg/flask).

Fatty acid	Experiment 1	Experiment 2	Experiment 3
C16:0	9.9	29.9	16.5
C18:0	3.7	17.0	11.6
cis-9C18:1	19.2	60.8	49.0
cis-9,*cis*-12C18:2	46.5	100 or 300	44.5
cis-9,*cis*-12, *cis*-15C18:3	6.4	1.5	10, 60, 120, or 180

equipped with a model 7683 auto injector, Network GC System, Palo Alto, CA). The column was a fused silica capillary (CPSil88, 100 m × 0.25 mm id, 0.20-μm film thickness; Chrompack-Varian, Middleburg, The Netherlands). A first analysis was made using the method described by Enjalbert et al. (2003). A second analysis, using different patterns of oven temperature, allowed the separation of *trans*-10C18:1 and *trans*-11C18:1; the oven temperature was 72°C for 15 min and then increased by 45°C/min until 160°C, maintained at 160°C for 60 min and then increased by 5°C/min until 225°C, and was then maintained at 225°C for 10 min. Peaks were identified and quantified by comparison with commercial standards (Sigma, St. Louis, MO).

Calculations and Statistics

Amount of added fat in each culture was computed as the difference in the amount of fatty acid between control and added fat cultures in the same series, measured after incubation. The amount of C18:0 resulting from BH of PUFA was calculated by subtracting from the C18:0 from added fat, the C18:0 initially present in added fat, and that resulting from BH of *cis*-9C18:1, which was the only other unsaturated FA present in added fats used in these 3 experiments. Although a small amount (about 3%) of *cis*-9C18:1 can be isomerized to *trans*-C18:1 (Ward et al., 1964; Loor et al., 2002; Mosley et al., 2002), we considered that all disappeared *cis*-9C18:1 was converted to C18:0.

The estimation of BH was based on the disappearance of the unsaturated FA (Beam et al., 2000; Enjalbert et al., 2003). For all experiments, this disappearance was expressed as flux of PUFA loss (mg/[ml·h]). For Exp. 1 and 2, we estimated the parameters of BH kinetics with the exponential model of Orskov and McDonald (1979), adapted for the BH of FA in vitro as described by Enjalbert et al. (2003). As previously described (Kepler and Tove, 1967; Harfoot et al., 1973) in our Exp. 1 and 2, a complete BH of PUFA could not be expected from the kinetics curves of BH, so we added a fraction escaping BH to the model. The final model was:

$$P_t = P_0 \times (1 - \text{FEBH}) \times e^{[-c \times (t - \text{lag})]} + P_0 \times \text{FEBH},$$

where P_t = proportion of the UFA at t h of incubation, P_0 = initial proportion of the UFA in the experimental substrate, FEBH = fraction of the UFA escaping BH, c = rate of BH, and lag = lag time before the BH begins.

Data were computed with the nonlinear regression procedure of SYSTAT (Version 9, SPSS Inc., Chicago, IL). The comparison between treatments of the fractions escaping BH, BH rates, and lag times was made as described by Enjalbert et al. (2003). These 2 estimations of BH took into account only the lipolysis of triacylglycerols and the isomerization step of BH. The activity of the reductases was estimated through the evolution of concentrations of the BH intermediates.

The proportions of each FA, the ratio t10FA:t11FA at each time of incubation and the flux of PUFA loss were compared between treatments using the general linear model of SYSTAT, followed by the Tukey's pairwise comparison test when more than two treatments were compared. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of pH on Biohydrogenation

During Exp. 1, the pH of HpH cultures decreased from 6.9 to 6.4, whereas pH in LpH cultures decreased from 6.2 to 5.4 so that the difference between treatments was always above 0.7 pH units (Figure 1).

The lag times were between 1 and 2 h except for *cis*-C18:3 with LpH (Table 2). This lag time could be due to the time necessary for lipolysis, which is between 2 and 4 h for soybean oil (Reddy et al., 1994), and/or the time necessary for a multiplication of rumen bacteria in order to have a sufficient amount of bacteria to lipolyze and hydrogenate fat. Indeed, bacteria are fixed onto feed particles, and strained ruminal fluid contains few feed particles. Beam et al. (2000), in their strained ruminal fluid cultures, also noticed a lag time between 1.39 and 2.47 h, which they attributed to the time necessary for lipolysis of soybean oil. Because in the present experiment, and with both treatments, the

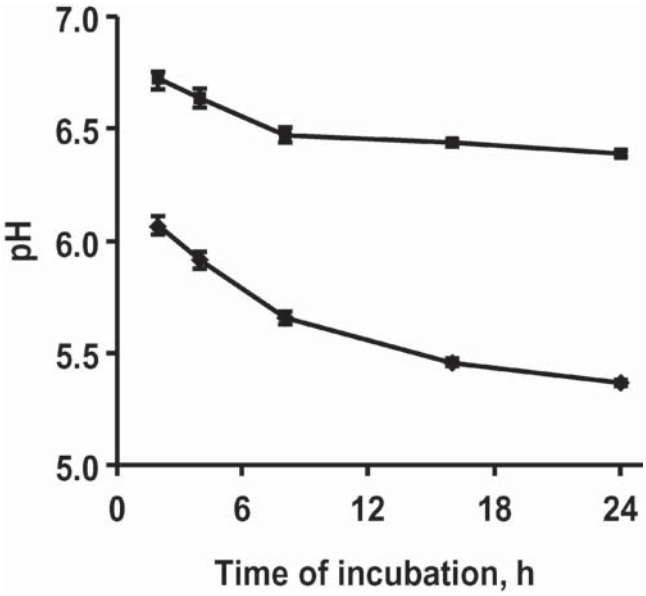

Figure 1. Evolution of pH during low-pH buffer (◆) and high-pH buffer (■) in vitro incubations in Experiment 1.

pH was over 5.9 at 2 and 4 h of incubation, there was no inhibition of lipolysis (Van Nevel and Demeyer, 1996).

The proportion of *cis*-C18:2 escaping BH was 4 times higher with LpH than with HpH. On the contrary, but with both buffers, the proportion of *cis*-C18:3 escaping BH was not different from zero (Table 2).

With HpH, the flux of disappearance of *cis*-C18:2 and *cis*-C18:3 were higher ($P > 0.01$ and $P > 0.05$, respectively) than with LpH (Table 3) after 2 h of incubation. Similarly, the rate of disappearance of PUFA, with LpH was about one-half that of HpH for *cis*-C18:2 and *cis*-C18:3 (Table 2).

Table 2. Fraction escaping biohydrogenation (FEBH), lag time, and rate of biohydrogenation of polyunsaturated fatty acids from soybean oil (mean ± SEM), Experiment 1.

Buffer	LpH[1]	HpH[1]
cis-9,*cis*-12C18:2		
FEBH, % initial *cis*-C18:2	20.90 ± 4.70[ab]	5.50 ± 2.40[ac]
Lag time, h	1.28 ± 0.34[a]	1.89 ± 0.13[a]
Rate of biohydrogenation, %/h	11.60 ± 2.00[ab]	20.70 ± 2.00[ac]
r^2	0.96	
cis-9,*cis*-12,*cis*-15C18:3		
FEBH, % initial *cis*-C18:3	8.30 ± 11.80	1.60 ± 4.20
Lag time, h	0.00 ± 0.96[d]	2.09 ± 0.20[ae]
Rate of biohydrogenation, %/h	9.50 ± 3.50[ad]	22.70 ± 3.60[ae]
r^2	0.89	

[1]LpH refers to low-pH buffer, HpH refers to high-pH buffer.

[a]Value significantly different from zero ($P < 0.05$).

[b,c]Means in the same row with unlike superscripts differ ($P < 0.01$).

[d,e]Means in the same row with unlike superscripts differ ($P < 0.05$).

The amounts of BH products were always significantly lower with LpH than with HpH beyond 2 h of incubation, and the amounts of intermediates were very close after 16 and 24 h of incubation (Figure 2). Wang et al. (2002) and Martin and Jenkins (2002) also noticed at low pH in vitro decrease of *cis*-C18:2 and *cis*-C18:3 BH with a decrease of *trans*-C18:1 and CLA amounts. Similarly, in vivo, an increase of the proportion of concentrates decreases milk CLA (Griinari et al., 1998; Dhiman et al., 1999).

Low amounts of CLA observed with LpH could be due either to low isomerase activity or to high reductase activity. Low amounts of *trans*-C18:1 and C18:0 from BH of PUFA with LpH did not suggest a high reductase activity. On the contrary, the low rate of disappearance of PUFA suggested that the inhibition occurred on the isomerase. Indeed, the optimal pH of the isomerase is between 7 and 7.2 (Kepler and Tove, 1967).

Griinari et al. (1998) found that lowering the fiber contents of a diet containing unsaturated FA reduced ruminal pH and resulted in a decreased proportion of *trans*-11C18:1 in milk and an increased proportion of *trans*-10C18:1. In the present experiment, LpH resulted in lower amounts of t11FA at all incubation times compared with HpH, but amounts of t10FA were higher only at 16 and 24 h of incubation. This resulted in a ratio t10FA:t11FA significantly different between LpH and HpH ($P < 0.01$), but with the same pattern during the 24 h of incubation (Figure 3). Differences between in vitro and in vivo results can be expected because the differences of pH in the present experiment were due to differences between buffers, not to differences between culture substrates. The *trans*-10 isomerase has recently been shown to be produced by *Megasphaera elsdenii* (Kim et al., 2002), a bacteria that utilizes lactate and develops with high-grain diets, such as the diet used by Griinari et al. (1998), but not with the diet or the culture media used in this in vitro experiment. On the contrary, a lower production of *trans*-11 intermediates of BH was observed both in the in vivo experiment of Griinari et al. (1998) and in this experiment; the *trans*-11 isomerase of *cis*-C18:2 is produced by *Butyrivibrio fibrisolvens* (Kepler and Tove, 1967; Kim et al., 2000), and the inhibition of cellulolytic bacteria can be mediated directly by low pH (Russell and Dombrowski, 1980).

The BH was not completely inhibited by LpH but was slowed down (Table 2 and 3). The inhibition could be reversible, either by an adaptation of the bacteria, mainly *B. fibrisolvens*, which would synthesize more enzyme, or by cell multiplication, since the CLA accumulation was shown to depend on *B. fibrisolvens* density (Kim et al., 2000).

Table 3. Flux of disappearance of polyunsaturated fatty acids from soybean oil, Experiment 1.

Item	Buffer			SEM
	Incubation time, h	LpH[1]	HpH[1]	
		mg/(L·h)		
cis-9,*cis*-12C18:2	2	20.80	27.62	3.09
	4	14.06[a]	24.70[b]	1.08
	8	15.29[a]	26.58[b]	1.02
	16	12.48[a]	16.37[b]	0.21
	24	8.43[a]	11.06[b]	0.11
cis-9,*cis*-12,*cis*-15C18:3	2	5.75	2.71	1.70
	4	1.98[b]	3.55[c]	0.50
	8	2.44[a]	3.83[b]	0.21
	16	1.91[a]	2.38[b]	0.05
	24	1.29[a]	1.58[b]	0.03

[1]See Figure 1.

[a,b]Means in the same row with unlike superscripts differ ($P < 0.01$).

[c,d]Means in the same row with unlike superscripts differ ($P < 0.05$).

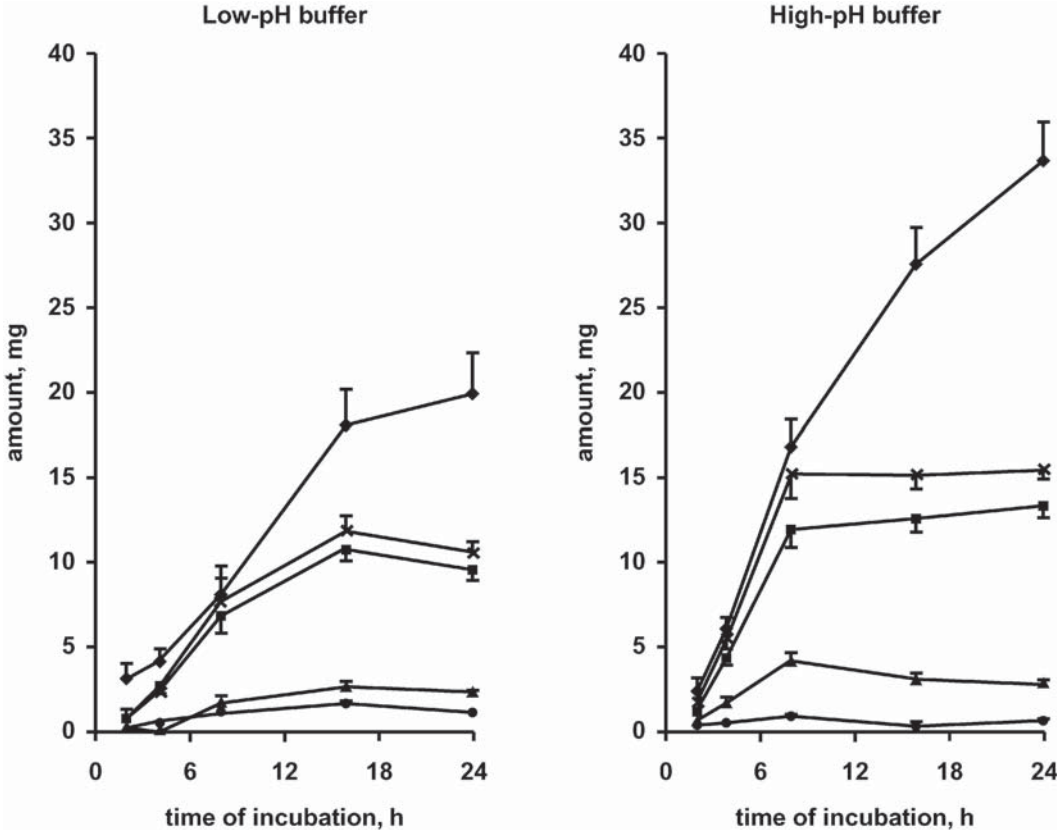

Figure 2. Effects of pH and incubation time on the products of biohydrogenation of polyunsaturated fatty acids: C18:0 from polyunsaturated fatty acids (◆), *trans*-11C18:1 + *trans*-10C18:1 (■), *cis*-9, *trans*-11C18:2 + *trans*-10, *cis*-12C18:2 (▲), *trans*-10C18:1 + *trans*-10, *cis*-12C18:2 (●), and *trans*-11C18:1 + *cis*-9, *trans*-11C18:2 (✖) in Experiment 1.

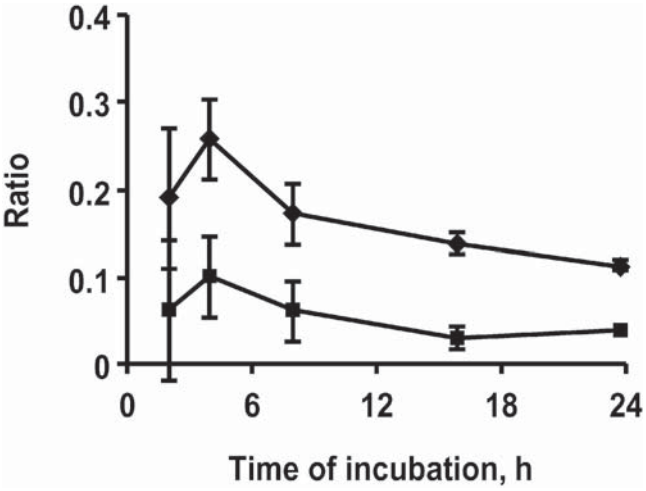

Figure 3. Effect of pH on the evolution over time of the (*trans*-10C18:1 + *trans*-10, *cis*-12C182):(*trans*-11C18:1 + *cis*-9, *trans*-11C18:2) ratio. Low-pH buffer (◆) and high-pH buffer (■). Experiment 1.

Effect of Amount of Linoleic Acid on Biohydrogenation

The lag time for BH of *cis*-C18:2 was the same with 100 or 300 mg of added *cis*-C18:2 (Table 4), and slightly higher than in Exp. 1, which could be due to the higher amount. The fraction escaping BH was not significantly different from zero with 300 mg of added *cis*-C18:2.

The flux of disappearance of C18:2 was twice as great ($P < 0.01$) with 300 as with 100 mg of added *cis*-C18:2 (Table 5). The rate of *cis*-C18:2 BH was twice as great ($P < 0.05$) with 100 as with 300 mg of added *cis*-C18:2 (Table 4). This means that, when increasing the initial amount of *cis*-C18:2, a lower proportion of the initial amount but a higher amount of *cis*-C18:2 disappeared due to isomerization. This suggested that the lipase or/and the isomerase, which are both necessary for disappearance of *cis*-C18:2 from oils, would have a maximal capacity that was reached with 300 mg of added *cis*-C18:2. Lipolysis has been shown to be slower in vitro when the amount of *cis*-C18:2 is high (Van Nevel and Demeyer, 1996). On the contrary, Beam et al. (2000) demonstrated that increasing *cis*-C18:2 does not lead to a saturation of the isomerase, but their maximal concentration of *cis*-C18:2 was much lower than in the present experiment (200 mg in 200 ml vs. 300 mg in 160 ml).

Table 4. Fraction escaping biohydrogenation (FEBH), lag time, and rate of disappearance of linoleic acid during incubations with 100 or 300 mg of added linoleic acid (mean ± SEM), Experiment 2.

Initial amount of linoleic acid, mg	100	300
FEBH, % initial *cis*-C18:2	11.25 ± 2.61[a]	2.21 ± 3.59
Lag time, h	3.09 ± 0.22[a]	3.29 ± 0.19[a]
Rate of biohydrogenation, %/h	22.52 ± 3.08[ab]	12.03 ± 1.28[ac]
r^2	0.98	

[a]Value significantly different from zero ($P < 0.05$).

[b,c]Means in the same row with unlike superscripts differ ($P < 0.01$).

Table 5. Flux of disappearance of linoleic acid, Experiment 2.

Incubation time, h	Initial amount of linoleic acid, mg		SEM
	100	300	
	mg/(L·h)		
2	87.71[a]	208.73[b]	16.108
4	59.90[a]	132.36[b]	8.497
8	53.34[a]	115.49[b]	4.173
16	33.92[a]	97.06[b]	1.338
24	23.41[a]	69.15[b]	0.364

[a,b]Means in the same row with unlike superscripts differ ($P < 0.01$).

Adding 300 mg of *cis*-C18:2 increased the amount of its BH intermediates or end-product (CLA, *trans*-C18:1, C18:0) beyond 4 h of incubation ($P < 0.01$, Figure 4), which is consistent with the increased amounts of CLA (Dhiman et al., 2000) and *trans*-C18:1 (Casper et al., 1988) in the milk from cows receiving dietary PUFA.

With 300 mg of added *cis*-C18:2, the ratio of C18:0:(*trans*-10C18:1 + *trans*-11C18:1) was low compared with 100 mg of added *cis*-C18:2. As shown in Figure 4, C18:0 amount increased linearly with incubation time (C18:0 = 3.37 T, $r^2 = 0.97$, $P < 0.0001$), whatever the concentration of *trans*-10C18:1 + *trans*-11C18:1, suggesting that the reductase transforming *trans*-C18:1 to C18:0 had reached a maximum capacity. Beyond this capacity, an increase of *trans*-C18:1 concentration would have no effect on the rate of production of C18:0 so that *trans*-C18:1 accumulated, reaching a maximum concentration at 16 h of incubation. Polan et al. (1964) and Harfoot et al. (1973) also reported that when the concentration of *cis*-C18:2 increases in the culture media, CLA and *trans*-C18:1 accumulate. They suggested that this accumulation was due to an inhibition of the transformation of *trans*-C18:1 to C18:0 by *cis*-C18:2, and Griinari and Bauman (1999) suggested that it would be a competitive inhibition for the hydrogenation of monoenoic acids.

With either 100 or 300 mg of *cis*-C18:2, from 16 h of incubation, the decreased amount of residual *cis*-C18:2 resulted in slower production and decreased concentration of CLA. With 100 mg of added *cis*-C18:2, this low amount of CLA limited the production of *trans*-10C18:1 and *trans*-11C18:1, whose concentrations decreased because reduction to C18:0 had not reached its maximum capacity. With 300 mg of added *cis*-

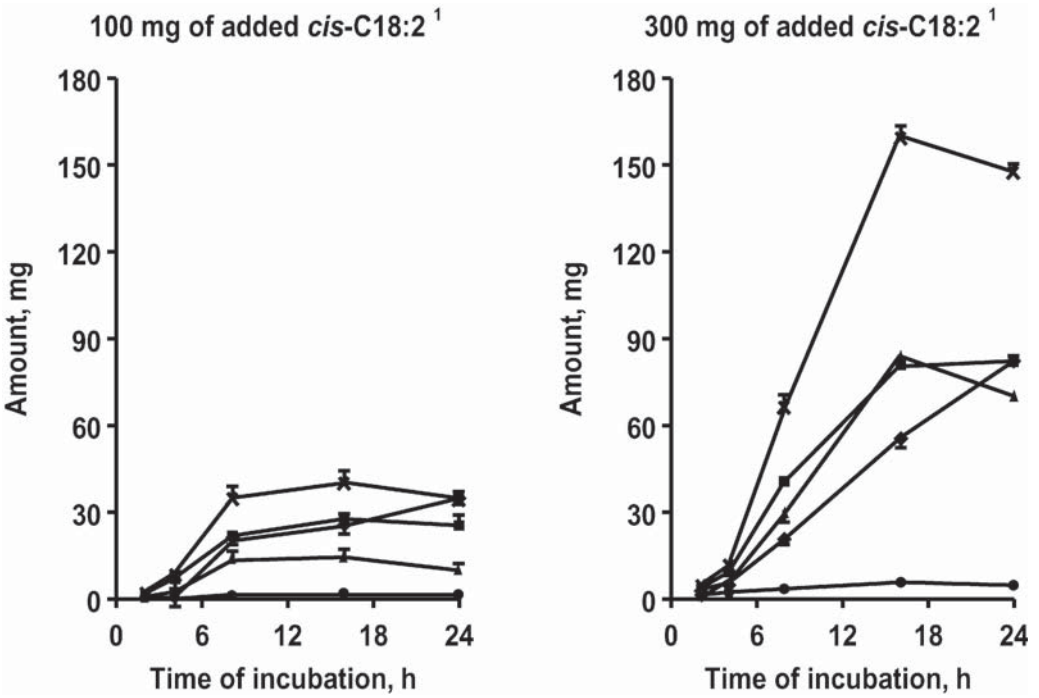

Figure 4. Effect of the amount of added linoleic acid and incubation time on the amount of its intermediates of biohydrogenation: C18:0 from polyunsaturated fatty acids (◆), *trans*-11C18:1 + *trans*-10C18:1 (■), *cis*-9, *trans*-11C18:2 + *trans*-10, *cis*-12C18:2 (▲), *trans*-10C18:1 + *trans*-10, *cis*-12C18:2 (●), and *trans*-11C18:1 + *cis*-9, *trans*-11C18:2 (✖); Experiment 2. [1]*cis*-C18:2 refers to *cis*-9,*cis*-12C18:2.

C18:2, the concentration of *trans*-10C18:1 + *trans*-11C18:1 did not decrease because of the higher concentration of CLA as a precursor and limited reduction of *trans*-C18:1 to C18:0 due to the limited capacity of the second reductase. The inhibition of this reductase when high concentrations of *cis*-C18:2 are incubated has been described as irreversible (Harfoot et al., 1973), which should result in lack of decrease of amount of *trans*-C18:1. Incubation times over 24 h should be necessary to ascertain this irreversibility. Until 16 h of incubation, the amount of *cis*-C18:2 did not affect the ratio t10FA:t11FA (Figure 5), which is consistent with the lack of effect of amount of *cis*-C18:2 on the isomerization step. At 24 h of incubation, this ratio was lower with 300 mg than with 100 mg of added *cis*-C18:2 due to the accumulation of *trans*-11C18:1 with 300 mg of *cis*-C18:2 at the moment when the *trans*-11C18:1 decreased with 100 mg of *cis*-C18:2.

Effect of Amount of Linolenic Acid on Biohydrogenation

As with *cis*-C18:2 addition in Exp. 2, increasing the initial amount of *cis*-C18:3 decreased only slightly but significantly ($P < 0.01$) the proportion of *cis*-C18:3 that disappeared due to BH, whereas the amount that was biohydrogenated increased nearly linearly with the initial amount (Figure 6). On the other hand, increasing the initial amount of *cis*-C18:3 from 10 to 180 mg

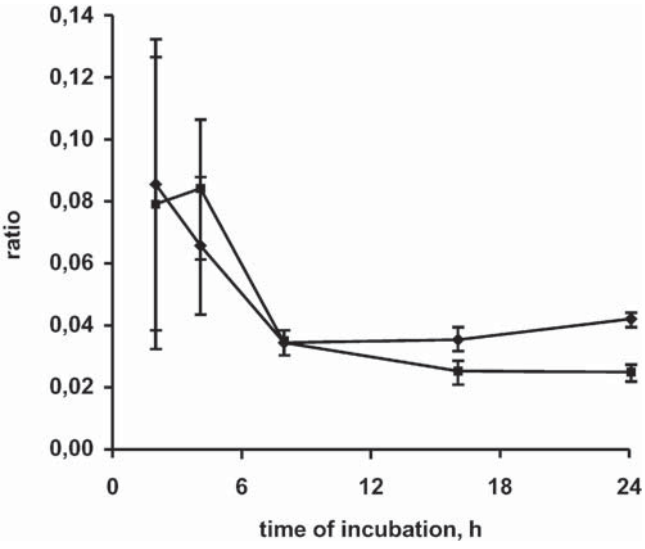

Figure 5. Effect of the addition of 100 (◆) or 300 mg (■) of linoleic acid on the evolution over time of the (*trans*-10C18:1 + *trans*-10, *cis*-12C182):(*trans*-11C18:1 + *cis*-9, *trans*-11C18:2) ratio; Experiment 2.

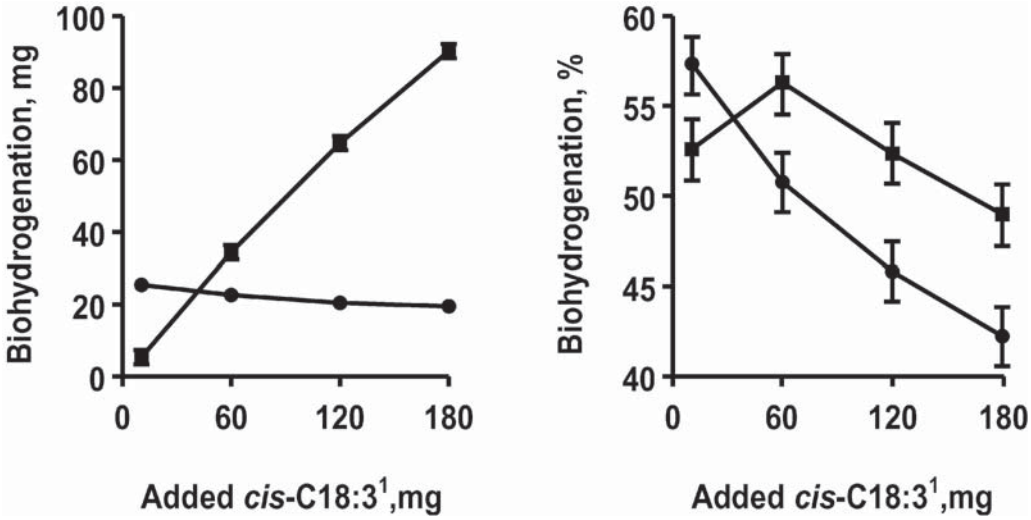

Figure 6. Effect of the amount of added linolenic acid on the amounts and percentages of biohydrogenation of linoleic (●) and linolenic (■) acids after 6 h of incubation; Experiment 3. [1]*cis*-9, *cis*-12, *cis*-15C18:3.

decreased by 23% the amount of *cis*-C18:2 that disappeared after 6 h of incubation (Table 6). This effect was much lower than the effect of pH in Exp. 1: LpH decreased BH (flux of *cis*-C18:2 disappearance) by about 44% compared with HpH, after 4 and 8 h of incubation (Table 3).

The addition of *cis*-C18:3 did not affect significantly the amount of CLA and *trans*-10C18:1 + *trans*-11C18:1 after 6 h of incubation (Figure 7), suggesting that BH of *cis*-C18:3 did not produce significant amounts of these intermediates. Indeed, CLA isomers are not intermediates of the BH of *cis*-C18:3 (Kepler and Tove, 1967; Griinari and Bauman, 1999). The *trans*-11C18:1 is one of the monoenoic intermediates of the BH of *cis*-C18:3 (Kepler and Tove, 1967), but not the most important compared with *trans*-15C18:1 and *cis*-15C18:1 (Loor et al., 2002), which were not identified by our method of analysis.

Decreased disappearance of *cis*-C18:2 when the amount of *cis*-C18:3 was high suggested an inhibition of *cis*-C18:2 BH at the isomerization step. Unchanged amounts of CLA and *trans*-C18:1 in spite of decreased *cis*-C18:2 disappearance suggested lower reductase activities. The ratio of t10FA:t11FA was between 0.019 and 0.036 and not significantly different among amounts of *cis*-C18:3, suggesting that both transformations to *trans*-10 and *trans*-11 isomers were inhib-

Table 6. Flux of linoleic acid disappeared after 6 hours of incubation with different initial amounts of linolenic acid (mean ± SEM), Experiment 3.

Initial amount of linolenic acid, mg/flask	Flux of *cis*-9,*cis*-12C18:2 loss, mg/(L·h)
10	26.38 ± 0.75[a]
60	23.48 ± 0.75[be]
120	21.25 ± 0.75[c]
180	19.74 ± 0.75[df]

[a]Differs from[b,c,d] ($P < 0.05$).
[e]Differs from[f] ($P < 0.01$).

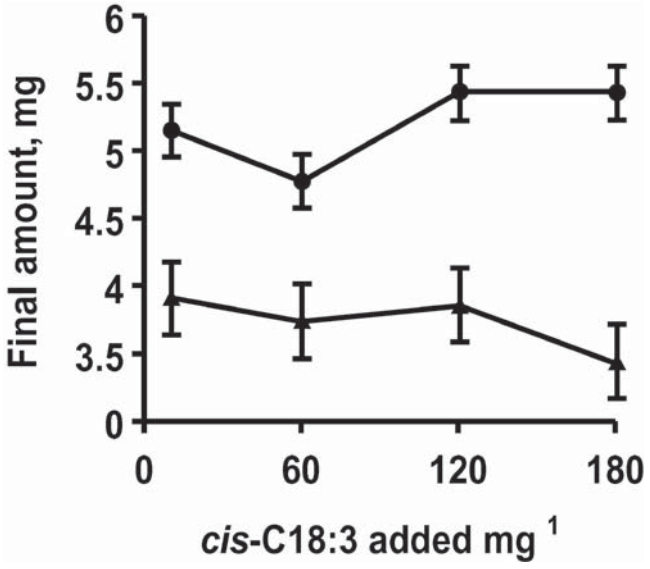

Figure 7. Effect of the amount of added linolenic acid on the amount of conjugated linoleic acid[1] (▲) and *trans*-C18:1[1] (●) after 6 h of incubation; Experiment 3. [1]*trans*-C18:1 refers to *trans*-11C18:1 and *trans*-10C18:1, CLA refers to *cis*-9, *trans*-11C18:2 and *trans*-10, *cis*-12C18:2, and *cis*-C18:3 refers to *cis*-9, *cis*-12, *cis*-15C18:3.

ited. These inhibitions of isomerization and reductions could be due to a competition between *cis*-C18:2 and *cis*-C18:3 for the fixation onto the envelope of the bacteria carrying isomerases, because the isomerizations of *cis*-C18:2 and *cis*-C18:3 are realized by the same bacteria and the same enzyme (Kepler et al., 1966; Kepler and Tove, 1967), and probably to other competitive inhibitions for the reductions between the intermediates of BH of *cis*-C18:2 and *cis*-C18:3.

CONCLUSIONS

Isomerization of *cis*-C18:2 to CLA was strongly inhibited by a low ruminal pH and by increasing concentrations of *cis*-C18:3 in the incubation media. On the contrary, increased concentrations of *cis*-C18:2 did not inhibit the BH of *cis*-C18:2, but isomerization and second reduction steps showed a limited capacity. These results suggest that optimizing the CLA contents in the milk could be obtained with diets leading to a ruminal pH that is nearly neutral, and containing high amounts of *cis*-C18:2. Further research, based both on enzymological and in vivo studies, will be necessary to ascertain these modes of action and their effects on the concentration of CLA in the milk of cows.

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