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In situ ruminal biohydrogenation of fatty acids from extruded soybeans: effects of dietary adaptation and of mixing with lecithin or wheat straw

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Abstract

Kinetics and intermediates of biohydrogenation of fatty acids were investigated in situ using extruded soybeans, a blend of extruded soybeans and lecithin (99:1), or a blend of extruded soybeans plus wheat straw (66:34). Two dry dairy cows received successively a diet with added palmitic acid and a diet with added extruded soybeans, and assays were completed after a 3-week adaptation to each diet. Adaptation of the cows to dietary polyunsaturated fatty acids suppressed the lag time before the beginning of biohydrogenation. Adaptation of cows, and mixing straw with soybeans, increased the rate of biohydrogenation of C18:2 and C18:3, resulting in less C18:2 and C18:3, and more *trans* C18:1 and C18:0 in the in situ bags. Lecithin did not affect the kinetics of biohydrogenation or the profil of fatty acids in the in situ bags. Differences in the rate of biohydrogenation, and profil of residual fatty acids in the bags were observed between the two cows. Even with a mixture of soybeans and straw in cows receiving dietary polyunsaturated fatty acids, biohydrogenation was slower and resulted in higher proportions of *trans*-C18:1 than expected from results of literature in vivo. Results

Abbreviations: BH, biohydrogenation; ES, extruded soybeans; ESL, extruded soybeans plus soy lecithin; ESS, extruded soybeans plus straw; FA, fatty acid; PUD, diet with added polyunsaturated FA; SD, diet with added saturated FA

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show that the biohydrogenation in situ is slow, highly dependent on experimental conditions, and that the use of several cows, adapted to the test fat source before the assay is initiated, is necessary in order to obtain a reliable estimate of kinetics parameters.

Keywords: Biohydrogenation; Soybeans; In situ; Adaptation; Wheat straw

1. Introduction

Dietary unsaturated fatty acids (FA) are extensively biohydrogenated in the rumen (Reiser, 1951). Knowledge, and control, of ruminal biohydrogenation (BH) are important in dairy cattle nutrition, because modification of dietary FA due to BH can affect milk fat content (Griinari et al., 1998), the ratio of saturated to unsaturated milk FA (Grummer, 1991; Kennelly, 1996), and/or result in the presence of FA with particular human diet effects, such as conjugated linoleic acids (Kelly et al., 1998; Chilliard et al., 2000).

Quantificatio of BH is usually performed by in vivo measurements, comparing the fl ws or profile of dietary and duodenal FA (Murphy et al., 1987; Enjalbert et al., 1997) or in vitro by measurement of the disappearance of unsaturated FA (Harfoot et al., 1973a; Gulati et al., 1997; Beam et al., 2000). The latter showed that the rate of BH of C18:2 depends on its concentration, but not on the presence of polyunsaturated FA in the diets of the donor animals. However in vitro results can vary largely as a function of fermentation conditions (Van Nevel and Demeyer, 1996). In situ methods have the ability to incubate substrates in a more physiological milieu than in vitro procedures, but have not been extensively studied for this purpose. Perrier et al. (1992) estimated that no distinction could be made between disappearance of unsaturated FA due to BH or due to loss from the bags. However, Enjalbert et al. (2003), incubating ground canola seeds in sterilised rumen flui with added pancreatin, demonstrated that all individual FA left the bags equally, so that the profil of FA remaining in the bags could be considered representative of BH inside the bags. Comparing in vitro and in situ methods, Enjalbert et al. (2003) demonstrated with canola seeds that both methods can ascertain the effects of processing feedstuffs, but that BH in situ is much slower than in vitro and only begins after a significan time lag. They hypothesised that hydrophobicity of a fat source containing about 200 g of FA/kg could slow the penetration of bacteria into the bags and their subsequent action.

The objectives of this study were to investigate the effects of mixing extruded soybeans with lecithin or straw, in order to decrease hydrophobicity and lipid content, and the effects of adaptation of cows to a diet containing polyunsaturated FA, on the kinetics of BH of polyunsaturated FA from extruded soybeans in situ.

2. Materials and methods

2.1. Description of the experiment

Two ruminally fistulate dry Holstein cows were successively fed two different diets with added saturated FA (SD), provided as palmitic acid, or polyunsaturated FA (PUD), provided

Table 1
Ingredients (kg as fed/day) and chemical composition of the diet (g/kg of dry matter)

	Saturated diet	Polyunsaturated diet
Ingredient		
Corn silage	10.60	10.60
Wheat straw	0.50	0.50
Wheat grain, ground	1.50	1.50
Soybean meal, solvent extracted	2.15	0.50
Extruded soybeans	0.00	2.00
Palmitic acid	0.35	0.00
Sodium bicarbonate	0.10	0.10
Mineral–vitamin mix[a]	0.10	0.10
Chemical composition		
Acid detergent fibr	170	171
Crude protein	138	135
Fatty acids	41	40
Net energy for lactation[b] (Mcal/kg)	1760	1750

[a] Contains: P (50 g/kg), Ca (140 g/kg), Na (60 g/kg), Zn (4 g/kg), Mn (3.2 g/kg), Fe (3 g/kg), Cu (0.8 g/kg), Vitamin A (250,800 IU/kg), Vitamin D_3 (62,700 IU/kg), and Vitamin E (112 IU/kg).
[b] Calculated from Andrieu et al. (1988).

as extruded soybeans. The diets were isoenergetic and isonitrogenous (Table 1), and were fed two times/day, at 08:00 and 16:00 h, as suggested for studies for in situ measurement of ruminal degradability of protein (Michalet-Doreau et al., 1987; NRC, 2001). The cows were adapted to the diets for 3 weeks before the in situ measurements.

Extruded soybeans and wheat straw were ground through a 5 mm screen, and sieved through a 0.125 mm screen for elimination of small particles. Heat-sealed nylon bags (11 cm × 6 cm; mean pore size 45 μm; Blutex, Tissage Tissus Techniques, Combles, France) were fille with 3 g of extruded soybeans (ES), or 3 g of extruded soybeans plus 30 mg of soy lecithin (ESL), or 2 g of extruded soybeans plus 1 g of straw (ESS). The FA composition of the extruded soybeans, straw and soy lecithin is in Table 2. Bags with incubation times of 2, 4, 8, and 24 h were introduced into the rumen before the morning meal, and 16 h incubation time bags were introduced before the evening meal, as suggested by Michalet-Doreau et al. (1987) for in situ measurement of ruminal degradability of protein. For each incubation time, there were two replicates in each cow and diet. After removal from the rumen, bags

Table 2
Dry matter and fatty acid composition of extruded soybeans, soy lecithin and straw[a]

	Extruded soybeans	Soy lecithin	Wheat straw
DM (g/kg)	897.5	966.6	919.6
Total C18[b] (g/kg DM)	176.8	228.2	45.9
C18:0 (g/kg of total C18)	49.8	50.2	99.1
cis-9-C18:1 (g/kg of total C18)	200.3	117.2	294.1
C18:2 (g/kg of total C18)	603.0	719.0	457.3
C18:3 (g/kg of total C18)	98.7	90.8	103.6

[a] Values represent means of three samples of each material.
[b] Fatty acids with 18 carbons.

were rinsed for approximately 2 min in cold water until no color appeared in the rinsing water, frozen at −18 °C over 24 h, machine washed (2 × 5 min) in cold water, dried (60 °C for 48 h), weighed and stored at −18 °C until analysis. Zero-hour bags were incubated for 1 h in water (38 °C), and rinsed, washed, dried and stored in a similar procedure as for the other bags.

2.2. Analysis of fatty acids

Samples were ground using a ball mill for 1 min (Dangoumau, distributed by Prolabo, Nogent-sur-Marne, France). The FA from feed samples and residues of incubation were extracted and methylated using a one-step procedure (Sukhija and Palmquist, 1988). The FA methyl esters were quantifie by GLC (Agilent technologies 6890N, Little Falls, DE, USA). The column was a fused silica capillary (CPSil188, 100 m × 250 μm ID, 20 μm fil thickness; Chrompack-Varian, Middlebourg, Netherlands). The carrier gas was He at a constant fl w of 1 ml/min. The flam ionisation detector was at 260 °C and the split ratio in the injector (255 °C) was 50:1. Oven temperature was initially 72 °C and was maintained at 72 °C for 15 min, then increased by 45 °C/min until 160 °C, maintained at 160 °C for 60 min, increased by 5 °C/min until 225 °C, and maintained at 225 °C for 10 min for a total duration of 100 min.

This method does not separate *cis*-9-C18:1 from some *trans* isomers, among them *trans*-15-C18:1. Because this isomer can be produced from C18:3 (Harfoot and Hazlewood, 1988) or possibly C18:2 (Loor et al., 2002), which are both abundant in soybeans, no results about *cis*-9-C18:1 could be determined. Moreover, methylation by the method of Sukhija and Palmquist (1988) leads to an underestimation of the main isomers of conjugated linoleic acid (Duckett et al., 2002), and so results will not discuss these FA.

The proportions of individual C18 FA were expressed as proportions of total C18 FA. For simplification FA designated as *trans*-C18:1, C18:2 and C18:3 refer to both *trans*-10-C18:1 and *trans*-11-C18:1, *cis*-9,cis-12-C18:2 and *cis*-9,*cis*-12,*cis*-15-C18:3, respectively.

2.3. Calculation and statistics

The following model, accounting for lipolysis of triacylglycerols and isomerization of PUFA, was used to describe the kinetics of BH (Enjalbert et al., 2003) as:

$$P = P_0\, \mathrm{e}^{(-c(t-l))}$$

where P is the proportion of the UFA at t h of incubation, P_0 the proportion of the UFA in the 0 h bags, c the rate of BH (per h), and l the lag time (h) before BH begins.

Data were computed with the nonlinear regression procedure of SYSTAT (Version 9; SPSS Inc., 1998, Chicago, IL, USA). The effects of cow, diet and bag contents on lag times and rates of BH were tested using the model:

$$P = P_0\, \mathrm{e}^{(-(\sum(c_{ij} D_i B_j) + a\,\mathrm{Cow})(t - (\sum(l_{ij} D_i B_j) + b\,\mathrm{Cow})))}$$

where i is SD or PUD, j the ES, ESL or ESS, $D_i B_j$ is coded 1 for diet i and bags material j and 0 in the other cases, c_{ij} the rate of BH for diet i and bags material j, l_{ij} the lag time

for diet *i* and bags material *j*, *a* and *b* are the effects of cow on the rate of BH and the lag time, respectively, Cow is coded 0 for the firs cow, 1 for the second. Lag times below zero were excluded. Mean values for each diet and bag material, and mean differences between diets and bag material, were computed and compared as function parameters, and were considered different from zero when their confidenc intervals did not contain zero.

Proportions of individual fatty acids in bags were analysed using the repeated measures procedure of the general linear model of SYSTAT.

3. Results

For both C18:2 and C18:3, PUD resulted in no lag time, as opposed to SD which did so (Table 3). As a consequence, after 2 h of incubation, the profil of FA was very close to the profil of the blanks with SD, whereas the profil of FA with PUD reflecte the start of BH (Fig. 1). In contrast, bag material had no effect on lag time.

Rate of BH was increased by 40 and 35% for C18:2 and C18:3, respectively, when the cows were fed PUD, compared to SD, resulting in lower proportions of C18:2 and C18:3, and significan effects of diet and interaction of diet by time of incubation. These significan effects were also observed for C18:0 and *trans*-C18:1 proportions, which were lower with SD than PUD except for *trans*-C18:1 at 24 h of incubation (Fig. 1).

Mixing lecithin with soybeans had no effect on the rate of BH of polyunsaturated FA from extruded soybeans, whereas mixing straw with soybeans increased the rate of BH by 89 and 98% for C18:2 and C18:3, respectively. As a consequence, profile of FA and evolution with incubation time were significantl affected (Table 4) by bags material: FA in ESS bags contained a higher proportion of C18:0 and *trans*-C18:1, and a lower proportion of C18:2 and C18:3, at all times of incubation (Fig. 1). For C18:0 and *trans*-C18:1, the effect of the inclusion of straw in the bags on the FA profile was more important with PUD, than with SD, resulting in interactions of diet by bags material, and this interaction depended on time of incubation for all FA (Table 4).

Table 3
Lag time and rate of biohydrogenation of polyunsaturated fatty acids from extruded soybeans

	Diet and bags material[a]						SEM	Effects, *P*			
	Saturated diet			Polyunsaturated diet				Diet	ES vs. ESL	ES vs. ESS	ESL vs. ESS
	ES	ESL	ESS	ES	ESL	ESS					
C18:2											
Lag time (h)	1.2	2.7[b]	3.0[b]	0.0	0.0	0.0	0.95	0.003	0.47	0.30	0.88
Rate of BH (/h)	0.031	0.028	0.063	0.044	0.044	0.083	0.006	<0.001	0.70	<0.001	<0.001
C18:3											
Lag time (h)	0.9	2.4	2.6[b]	0.0	0.0	0.0	1.07	0.02	0.49	0.39	0.95
Rate of BH (/h)	0.048	0.027	0.065	0.047	0.048	0.094	0.007	<0.001	0.78	<0.001	<0.01

[a] ES, extruded soybeans, ESL, extruded soybeans:soy lecithin (99:1), ESS, extruded soybeans:straw (66:34).
[b] Lag time significantl different from zero ($P < 0.05$).

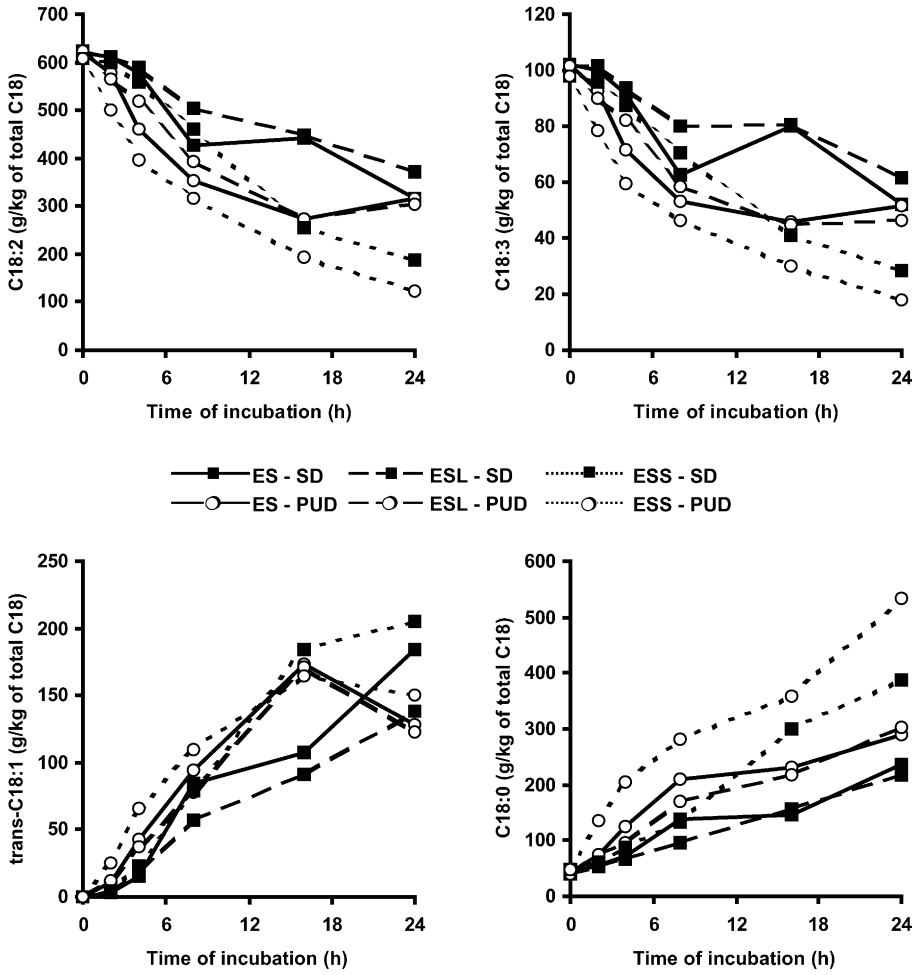

Fig. 1. Proportion of fatty acids in extruded soybeans alone (ES), or associated with lecithin (ESL) or straw (ESS), incubated in situ in cows receiving a diet with added saturated (SD) or unsaturated (PUD) fatty acids.

Table 4
Significanc levels of effects of bags material (B), diet (D), cow (C) and time of incubation (T) on the profil of fatty acids

	Effects											
	B	D	C	B·D	B·C	D·C	B·T	D·T	C·T	B·D·T	B·C·T	D·C·T
C18:2	<0.001	<0.001	<0.001	0.231	<0.001	0.157	<0.001	<0.001	<0.001	<0.001	<0.001	0.001
C18:3	<0.001	<0.001	<0.001	0.208	<0.001	0.011	<0.001	<0.001	<0.001	<0.001	<0.001	0.003
trans-C18:1	<0.001	<0.001	<0.001	0.006	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.006
C18:0	<0.001	<0.001	<0.001	0.007	<0.001	0.109	<0.001	<0.001	<0.001	0.021	<0.001	0.153

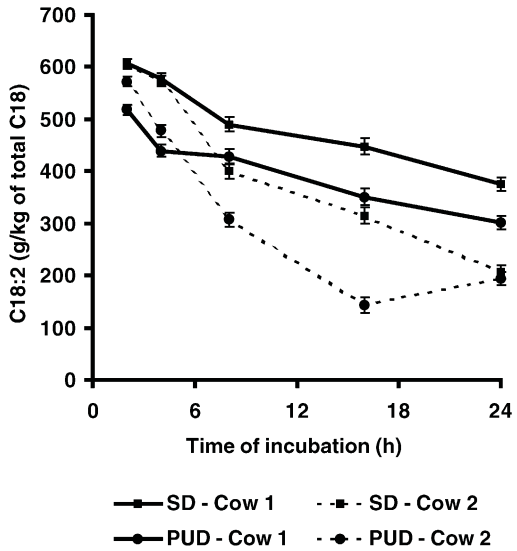

Fig. 2. Proportion of C18:2 in extruded soybeans incubated in situ in the two cows receiving a diet with saturated (SD) or unsaturated (PUD) added fatty acids. Error bars are ± S.E.

An effect of cow occurred for the rate of BH of both C18:2 and C18:3 ($P < 0.001$), but not for lag time. It resulted in an effect of cow on the profil of FA and its evolution during incubation (Table 4). Differences between cows could differ according to the diet (Fig. 2) or bag material, resulting in interactions of cow by diet or cow by bag material.

4. Discussion

4.1. Effect of adaptation of cows to dietary polyunsaturated fatty acids

The lag times observed in this experiment with SD were in the same range than those observed in situ by Enjalbert et al. (2003) with raw or extruded canola in cows eating a diet without added unsaturated FA. Adaptation to consumption of polyunsaturated FA with PUD suppressed lag time, as previously observed by Bayourthe et al. (2002), and increased rate of BH. In contrast, Beam et al. (2000) reported similar rates of BH of C18:2 in vitro with donor cows consuming, or not, soybean oil. Other in vitro studies have been completed with ruminal flui from unadapted animals (Van Nevel and Demeyer, 1996; Troegeler-Meynadier et al., 2003). For in vivo experiments, the necessity of adapting animals to diets with FA when studying the effects of added FA is a general consensus among authors, because all experiments on digestion, or milk FA profile include an adaptation period, as has been emphasised by Whitlock et al. (2002) who reported that the profil of FA in milk is stable only after 3 weeks of dietary fat addition. For in situ experiments measuring degradation of protein or fibre procedures do not explicitly recommend the adaptation of the animals to the studied feedstuffs (Vanzant et al., 1998; NRC, 2001). Because of the low level of

unsaturated FA in most standard diets, adaptation of animals can be more important when studying BH than when studying degradation of protein or fibre

To date, effects of the consumption of unsaturated FA on the populations of ruminal microbes, particularly microbes responsible for BH, remain poorly known. Dietary polyunsaturated FA decrease the number of protozoa and increase the number of bacteria in the rumen (Czerkawski et al., 1975). Bacteria are largely responsible for ruminal BH, whereas the protozoa are of minor importance (Harfoot and Hazlewood, 1988), so that an increased ratio of bacteria to protozoa in adapted animals could enhance the BH capacity of ruminal microorganisms. Moreover, unsaturated FA are known to be toxic to ruminal bacteria, resulting in decreased cellulolytic activity (Brooks et al., 1954), and hydrogenation could be a method of detoxication (Harfoot and Hazlewood, 1988; Bessa et al., 2000), which could explain an increased capacity of BH by ruminal bacteria during adaptation to unsaturated FA.

4.2. Effects of lecithin and straw

In this experiment, the lag time before BH began, and the rates of BH of C18:2 and C18:3, were not affected by addition of lecithin to the bags. Previous in vivo studies showed that lecithin does not affect the extent of BH of soybean oil (Enjalbert et al., 1994). The lack of effect of lecithin in the present study may be due either to a lack of intrinsic activity, either to an insufficien amount to promote penetration or dispersion of the bacteria responsible for BH, or perhaps to a rapid loss of lecithin from the bags. Similarly, lecithin did not affect the disappearance of DM (results not shown).

In contrast, addition of straw strongly enhanced rates of BH of both C18:2 and C18:3, with both SD and PUD. Several modes of action of straw can be hypothesised. First, straw could limit hydrophobicity and so hasten penetration of bacteria into the bags. However, such a mode of action should have decreased the lag time before BH began, which was not observed. Second, straw could improve the adsorption of lipids onto food particles, where BH occurs (Harfoot et al., 1973b). This effect could be of particular importance with extruded material, because extrusion results in rupture of cell membranes which in turn releases free oil. Third, dilution of the fat source with straw could decrease concentrations of C18:2 in the bags, and consequently decrease inhibitory effects of this FA on rate of BH (Beam et al., 2000). Under this hypothesis, the effect of mixing straw with the fat source would depend on the proportion of C18:2 in the fat source.

4.3. Extent of biohydrogenation

In situ, Perrier et al. (1992) used ground soybeans as a substrate to report a 40% decrease of the initial proportion of C18:2 after 8 h of incubation. In the present experiment, the corresponding value ranged from 19% with SD and ESL to 48% with PUD and ESS. In vivo, the extent of BH has been shown to be between 0.75 and 0.87 for C18:2, and between 0.83 and 0.93 for C18:3 (Murphy et al., 1987; Wu et al., 1991; Duckett et al., 2002). Calculations from BH kinetics parameters reported in Table 3 indicate that (1) in our experiment, these ranges of BH were not attained at 24 h of incubation, except with PUD and ESS, leading to these ranges of BH between 18 and 24 h of incubation, and (2) the outfl w rates consistent with the ranges of BH observed in vivo and the kinetic values

in Table 3 should be under 0.01/h for all bag materials with SD and bags ES and ESL with PUD, and under 0.03/h for PUD and ESS bags. These values showed that, even under the most favorable conditions of the present experiment, the BH in situ seems to be slower than in the whole rumen, because the outfl w rate of particles, onto which lipids are adsorbed in the rumen, is between 0.04 and 0.05/h for forages and over 0.05/h for concentrates in dairy cows (NRC, 2001).

Rates of BH reported by Enjalbert et al. (2003) with extruded canola seeds incubated without lecithin or straw in unadapted cows were 0.100 and 0.125/h for C18:2 and C18:3, respectively, compared with 0.031 and 0.048/h in this experiment with soybeans. This difference could be due to the higher concentration of C18:2 in soybeans compared to canola, because high concentrations of C18:2 have been shown to slow BH in vitro (Harfoot et al., 1973a; Beam et al., 2000; Troegeler-Meynadier et al., 2003). Under this hypothesis, the kinetics of BH in situ could be highly sensitive to C18:2 and because the concentration of C18:2 is always much higher in a bag than in a whole rumen, differences between the rates of BH in situ and in vivo could depend on the proportion of C18:2 in the fat source.

4.4. Individual effect of cow

Important individual variations in the composition of milk fat have been described, particularly for conjugated linoleic acid (Jiang et al., 1996; Peterson et al., 2002). This effect has been attributed to both ruminal BH and Δ9-desaturase activity in the mammary gland (Peterson et al., 2002). This variability implies another difficult for in situ studies, for which only two animals are recommended, when the purpose is measurement of protein degradation (Michalet-Doreau et al., 1987; NRC, 2001). Such a low number of animals will likely be insufficien to provide a reliable estimate of BH for any larger population of cows.

4.5. Intermediates of biohydrogenation

Incubating canola seeds in situ, Enjalbert et al. (2003) reported that proportions of *trans*-C18:1 increased until 24 h of incubation. In the present experiment, the proportions of *trans*-C18:1 increased until 24 h with SD, but decreased between 16 and 24 h with PUD. This decrease was probably related to the lower proportions of C18:2 and C18:3, both precursors of *trans*-C18:1, with PUD at 16 h, due to more rapid BH. A selection of bacteria able to reduce *trans*-C18:1 to C18:0 during adaptation is very improbable, because it would result in lower proportions of *trans*-C18:1 with PUD than SD even at 16 h of incubation.

Moreover, Enjalbert et al. (2003) with canola seeds reported that the proportion of *trans*-C18:1 was nearly as high as the proportions of C18:2 and C18:3 that had disappeared from the beginning of incubation. In the present experiment, disappeared C18:2 + C18:3 represented 377 and 430 g/kg of total C18 FA with SD and PUD, respectively, whereas *trans*-C18:1 represented only 175 and 138 g/kg of total C18 FA with SD and PUD, respectively. Because soybeans have much higher proportions of C18:2 than canola, this is in apparent contradiction with the results of Harfoot et al. (1973a), who reported that when the concentration of C18:2 increases in the culture media in vitro, *trans*-C18:1 accumulates. However, it is known that the BH of *trans*-11-C18:1 and *cis*-9-C18:1 are due to the same bacteria, which differ from bacteria that hydrogenate polyunsaturated FA to *trans*-11-C18:1

(Harfoot and Hazlewood, 1988). In the experiment of Enjalbert et al. (2003), use of canola seeds resulted in high concentrations of *cis*-9-C18:1 in the bags, which could have competed with *trans*-C18:1 for BH.

In vivo, the proportion of polyunsaturated FA that appear in the duodenum as *trans*-C18:1 ranges from 0.05 (Duckett et al., 2002) to 0.37 (Clapperton, 1980), which is much lower than in our in situ experiment. The ratio *trans*-10-C18:1/*trans*-11-C18:1 was under 0.1 with all diets or bag materials (result not shown), as observed with added polyunsaturated FA in vitro by Troegeler-Meynadier et al. (2003). Higher ratios have been measured in vivo in the rumen (Loor et al., 2002) or the duodenum (Duckett et al., 2002). Differences between results in our experiment and previous published in vivo studies suggest that the pattern of intermediates of BH in situ does not accurately reflec a whole rumen.

5. Conclusions

The BH of polyunsaturated FA in situ started earlier when cows were adapted to their consumption before the in situ trial, was more rapid when the source of fat was mixed with a source of fibre and varied between two cows. In addition, results suggest that the kinetics of BH and the proportions of *trans* intermediates of BH in situ could be affected by the relative proportions of the different FA in the fat source, particularly C18:2. Finally, whatever the diet of the cows and the material incubated, the BH was slow in situ and resulted in high proportions of *trans* intermediates, when compared to results obtained by others in vivo. These results suggest that the in situ studies can have a limited applicability to specify the BH of feedstuffs.

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