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Original article

Effects of preconditioning and extrusion of linseed on the ruminal biohydrogenation of fatty acids. 1. In vivo studies

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Abstract – The extent and intermediates of ruminal biohydrogenation (BH) of fatty acids (FA) from a blend of linseed and wheat bran (70:30) were investigated in the rumen fluid, rumen particle phase and duodenal flow. The blend was ground through a 3 mm screen and used raw or extruded, or was ground through a 6 mm screen and preconditioned. Three dry Holstein cows fitted with ruminal and duodenal cannulas were used in a 3 × 3 Latin square design, with 18 days adaptation. The diet contained 20% (DM basis) of the linseed based blend. Twelve samples taken over 3 days were composited for analysis of rumen fluid, rumen particle phase and duodenal flow. The BH of FA from linseed resulted in the appearance of a great number of C18:1 intermediates, among which *trans*-10+11 to *trans*-16C18:1 were the most abundant. The proportion of *cis*-9,*trans*-11C18:2 was low. Preconditioning coarsely ground linseed resulted in a lower extent of C18:2 and C18:3 BH, and lower proportions of *trans*-12 to *trans*-16C18:1 intermediates than extrusion or a lack of processing of finely ground linseed. On the contrary, extrusion did not affect the extent of BH and had no significant effect on the proportions of *trans*-C18:1 intermediates, but increased the proportion of *cis*-9,*trans*-11C18:2 in both rumen phases. Different digesta types resulted in different estimates of BH. The extent of BH and the proportions of *trans*-C18:1 intermediates were lower in the rumen particle phase and higher in the rumen fluid than in the duodenum. Moreover, interactions between digesta type and treatment of linseed were observed.

biohydrogenation / linseed / preconditioning / extrusion

Résumé – **Effets du préconditionnement et de l'extrusion de la graine de lin sur la biohydrogénation ruminale des acides gras. 1. Études in vivo.** L'importance et les intermédiaires de la biohydrogénation ruminale (BH) des acides gras d'un mélange lin / son (70:30) ont été étudiés dans les phases solide et liquide du rumen et dans le contenu duodénal. Le mélange à base de lin

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était broyé à la grille de 3 mm et utilisé cru ou extrudé, ou broyé à la grille de 6 mm et préconditionné. Trois vaches Holstein taries, équipées de canules ruminale et duodénale, ont été utilisées dans un protocole en carré latin 3 × 3, avec 18 jours d'adaptation par période. La ration contenait 20 % (par rapport à la matière sèche) de mélange à base de lin. Douze échantillons de phases solide et liquide du rumen et de contenu duodénal, ont été prélevés sur 3 jours et mélangés avant analyse. La BH des acides gras du lin a conduit à l'apparition d'un grand nombre d'intermédiaires C18:1*trans*, parmi lesquels les *trans*-10+11 à *trans*-16 étaient les plus abondants. La proportion de C18:2*cis*-9,*trans*-11 était faible. Le préconditionnement du mélange grossièrement broyé a conduit à une BH de C18:2 et C18:3 et des proportions de C18:1*trans*-12 à *trans*-16 plus faibles que l'extrusion ou l'absence de traitement de mélange finement broyé. Au contraire, l'extrusion n'a pas modifié l'importance de la BH ou les proportions de C18:1*trans*, mais a augmenté la proportion de C18:2*cis*-9,*trans*-11 dans le rumen. Les différents types de prélèvement ont conduit à différentes estimations de la BH. L'importance de la BH et les proportions d'intermédiaires *trans*-C18:1 étaient plus faibles dans la phase solide et plus élevées dans la phase liquide du rumen que dans le contenu duodénal. En outre, des interactions entre type de digesta et traitement du lin ont été observées.

biohydrogénation / lin / préconditionnement / extrusion

1. INTRODUCTION

Adding fat to ruminant rations increases the energy density of the diet [22] and, when unsaturated fatty acids (FA) are used, improves the nutritional quality of milk fat [24] and meat [12, 29] by decreasing the ratio of saturated to unsaturated FA. The use of dietary fat with polyunsaturated FA (PUFA) can result in the enrichment of products with FA that have positive effects on the consumer's health [24, 36], and if C18:3 is fed to cows via linseed, to a decreased n-6/n-3 polyunsaturated FA ratio [17, 29, 36]. However, ruminal biohydrogenation (BH) is very extensive for C18:3 [10, 19], which is isomerised to conjugated C18:3, then hydrogenated to nonconjugated *cis,trans*-C18:2 [14, 18], subsequently to C18:1 FA and finally to C18:0 [35].

The effects of several methods of treatment of oilseeds on the flow of unsaturated FA to the intestine have been studied: chemical treatment [8], extrusion [6, 7, 30], roasting [30], or steam treating [12]. In the feed industry, extrusion is often preceded by preconditioning, which consists of preheating and premoistening a raw material by mixing it with steam and water for adjustment of moisture content [3]. To our knowledge, the single effects of this treatment have not been investigated.

The objectives of this work were to examine the impact of preconditioning or extrusion of linseed, focusing on the extent of BH, *cis*-9,*trans*-11C18:2 and *trans*-C18:1 isomers.

2. MATERIALS AND METHODS

2.1. Treatments of linseed

The linseed investigated was a commercial blend of 70% linseed and 30% wheat bran, and linseed will designate this blend. Three forms of linseed were compared: raw linseed crushed through a 3 mm screen (RL), linseed crushed through a 6 mm screen and preconditioned at 35 °C (CL), linseed crushed through a 3 mm screen, preconditioned at 50 °C, and extruded at 120 °C (EL). The composition of linseed is in Table I.

2.2. Experimental procedure

Three dry Holstein cows, fitted with ruminal and duodenal cannulas, were utilised. The diet was based on hay, soybean meal and 1 of the 3 forms of linseed, which supplied 86.1% of total FA with 18 carbon atoms in the diets. The amount distributed per cow, chemical composition

Table I. Chemical composition of diet ingredients.

Ingredients	RL[1]	CL[1]	EL[1]	Hay[2]	Soybean meal
DM (%)	92.3	89.3	93.5	92.0	89.1
			(% of DM)		
CP	20.8	21.7	21.0	8.6	47.4
NDF	30.8	33.1	32.5	65.7	16.3
ADF	14.5	13.6	13.2	34.4	11.3
Total C18[3]	25.1	24.3	25.9	1.0	2.1
			(% of total C18)		
C18:0	3.5	3.3	3.5	3.7	5.0
C18:1	18.0	16.6	18.1	10.5	18.3
C18:2	19.1	19.8	19.2	20.6	61.4
C18:3	58.1	59.1	57.9	59.5	11.8

[1] RL: raw linseed; CL: preconditioned linseed; EL: extruded linseed.
[2] Hay composed of *Dactylis glomerata* and *Festuca arundinacea*.
[3] Fatty acids with 18 carbon atoms.

of the diets and diet ingredients are presented in Tables I and II. The cows were housed in individual tie stalls, meals were at 08:00 and 18:00 h, and water was available ad libitum. The treatments were carried out in a 3 × 3 Latin square with 3 periods of 3 weeks. During the last 3 days of each period, rumen and duodenal samples were collected at 08:00, 14:00 and 20:00 h in the first day, 02:00, 06:00, 12:00, 18:00 and 24:00 h in the second day, and 10:00, 16:00, 22:00 and 04:00 h in the third day, this schedule representing a sample every 2 hours over a period of 24 hours. Rumen content was taken from the cows by a vacuum pump, and was strained through a metal sieve (0.25 mm) to separate rumen fluid and rumen particle phase. The samples were immediately frozen and kept at –18 °C until analysis.

Table II. Ingredient and proximate chemical composition of the diets.

Diets with	RL[1]	CL[1]	EL[1]
Amount (kg DM·d^{-1})			
Hay[2]	9.20	9.20	9.20
Soybean meal	0.89	0.89	0.89
Raw linseed	2.77	-	-
Preconditioned linseed	-	2.68	-
Extruded linseed	-	-	2.80
Mineral-vitamin mix[3]	0.085	0.085	0.085
Chemical composition (% of dry matter)			
CP	13.8	13.9	13.9
NDF	54.5	55.1	54.8
ADF	28.4	28.3	28.1
Total C18	6.3	6.0	6.5

[1] Diet with RL: raw linseed; CL: preconditioned linseed; or EL: extruded linseed.
[2] Hay composed of *Dactylis glomerata* and *Festuca arundinacea*.
[3] Complex containing 2% Ca, 7% P, 6% Mg, 1% Na, 300 000 IU per kg vitamin A, 60 000 IU per kg vitamin D3, 700 mg per kg vitamin E, and a blend of trace-elements.

2.3. Analytical procedures

Ruminal and duodenal samples were freeze-dried (Vitris Freezemobile 25; Vitris Gardiner, NY), and subsequently ground in a ball mill (Dangoumau, distributed by Prolabo, Nogent-sur-Marne,

France). Different forms of linseed and diet ingredients were sampled during each period and analysed for DM, crude protein, neutral detergent fibre, acid detergent fibre, and FA composition [2, 34].

Fatty acids in the samples were extracted and converted to methyl esters in one step, using sodium methoxide followed by boron trifluoride as described by Park and Goins [23]. This method allows a complete recovery of the main CLA isomers [11]. As mentioned by Precht and Molkentin [27], determination of *trans*-C18:1 isomers by GLC without a preliminary separation does not allow an exact quantification of *trans*-13C18:1,*trans*-14C18:1, and *trans*-15C18:1 due to overlap of *cis*-9C18:1. Hence, one part of the FA methyl esters of each sample was fractionated by argentation TLC (plates 20 × 20 cm, Silica gel 60, Merk KGaA, Germany) as described by Le Doux et al. [15].

Total and *trans*-C18:1 FA were analysed by GLC (Agilent 6890N, equipped with a model 7683 auto injector, Network GC System, Palo alto, California, USA). The column was a fused silica capillary (CPSil88, 100 m × 0.25 mm ID, Chrompack-Varian, Middleburg, Netherlands). The flame ionisation detector temperature was maintained at 260 °C and the injector at 255 °C with a split ratio of 1:50. Helium was the carrier gas with constant pressure (24.6 psi). The samples were injected in 0.5 mL of hexane. The initial temperature of the oven was 70 °C, held for 1 min, increased by 5 °C·min^{-1} to 100 °C, held at 100 °C for 2 min, increased by 10 °C·min^{-1} to 175 °C, held at 175 °C for 40 min, increased by 5 °C·min^{-1} to a final temperature of 225 °C and maintained at 225 °C for 15 min, as described by Loor et al. [16]. The identification and quantification of peaks was made by comparison with commercial standards when available (Sigma, St. Louis, USA). The standards were used for *trans*-9, *trans*-10 and *trans*-11C18:1, but the identification of *trans*-4 to *trans*-8C18:1 and *trans*-12 to *trans*-16C18:1 was made by comparison with published chromatograms [28]. *Trans*-10C18:1 and *trans*-11C18:1 were not completely separated, and were considered together and designated as *trans*-10+11C18:1. They were used as an internal standard to quantify *trans*-C18:1 FA determined by Ag-TLC.

2.4. Calculations and statistical analysis

Ruminal apparent BH of C18:2 and C18:3 in duodenal flow and the different rumen phases was calculated using the following formula: BH = 100 – 100 × (individual unsaturated C18 / total C18 in samples) / (individual unsaturated C18 / total C18 in the diet) [37].

The differences between linseed treatments and digesta types were assessed by analysis of variance using the general linear model of SYSTAT (version 9, SPPS Inc., 1998 Chicago). The model used was:

$$Y_{ijkl} = \mu + L_i + C_j + P_k + S_l + LS_{il} + \varepsilon_{ijkl}$$

where Y are the individual values for dependent variables, μ is the overall mean, L is the effect of the form of linseed, C is the cow effect, P is the period effect, S is the effect of digesta type, and LS is the effect of the form of linseed × digesta type interaction. A Tukey pairwise comparison test was used to compare the different forms of linseed or digesta types. The differences were declared significant at $P < 0.05$.

3. RESULTS AND DISCUSSION

The proportions of FA in the duodenum, the rumen fluid and the rumen particle phase are shown in Table III.

Table III. Effects of linseed treatment and digesta type on the profile of fatty acids and the extent of biohydrogenation.

	Digesta type									SE	*P*-values		
	Duodenum			Rumen fluid			Rumen particle phase				Linseed	Digesta	Linseed ×
Diets with	RL[1]	CL[1]	EL[1]	RL[1]	CL[1]	EL[1]	RL[1]	CL[1]	EL[1]		treatment	type	digesta
Profile of fatty acids (% of total C18)													
C18:0	67.1	66.0	67.7	59.2	69.9	63.0	54.9	44.1	52.9	4.1	0.49	< 0.01	0.17
total *trans*-C18:1	18.8	11.5	17.5	20.3	15.7	20.4	13.6	9.5	13.5	2.4	0.03	0.02	0.97
trans-4C18:1	0.09	0.08	0.03	0.11	0.13	0.08	0.09	0.08	0.13	0.02	0.43	0.05	0.06
trans-5C18:1	0.16	0.15	0.17	0.06	0.06	0.04	0.05	0.04	0.04	0.02	0.90	< 0.01	0.87
trans-6+7+8C18:1	0.71	0.46	0.59	0.38	0.27	0.49	0.38	0.09	0.37	0.09	0.02	< 0.01	0.71
trans-9C18:1	0.40	0.25	0.35	0.40	0.34	0.42	0.33	0.16	0.28	0.06	0.08	0.09	0.91
trans-10+11C18:1	8.96	5.27	8.13	10.50	7.52	10.04	6.67	4.94	6.64	2.00	0.22	0.17	0.99
trans-12C18:1	1.10	0.64	0.92	1.10	0.95	1.12	0.78	0.53	0.75	0.10	0.01	< 0.01	0.86
trans-13+14C18:1	3.71	2.07	3.55	4.09	3.26	4.17	2.76	1.83	2.64	0.32	< 0.01	< 0.01	0.69
trans-15C18:1	1.95	1.19	2.00	2.02	1.60	2.19	1.36	0.91	1.39	0.15	< 0.01	< 0.01	0.74
trans-16C18:1	1.79	1.36	1.82	1.68	1.60	1.84	1.18	0.94	1.24	0.14	0.03	< 0.01	0.80
cis-9,*trans*-11C18:2	0.21	0.24	0.20	0.24	0.19	0.50	0.46	0.60	1.24	0.12	< 0.01	< 0.01	0.04
C18:2	1.99	3.69	2.03	1.73	1.63	1.52	4.77	7.87	4.66	0.44	< 0.01	< 0.01	0.02
C18:3	4.29	10.22	4.71	3.63	3.66	3.09	12.85	24.40	12.90	1.26	< 0.01	< 0.01	< 0.01
Extent of biohydrogenation (%)[2]													
C18:2	90.2	82.4	90.1	91.5	92.3	92.5	76.6	62.6	77.1	2.16	< 0.01	< 0.01	0.03
C18:3	92.6	82.2	91.8	93.6	93.7	94.6	77.6	58.2	77.8	2.23	< 0.01	< 0.01	< 0.01

[1] RL: raw linseed; CL: preconditioned linseed; EL: extruded linseed.
[2] Biohydrogenation of dietary polyunsaturated fatty acids was calculated according to Wu et al. [37].

3.1. Effects of linseed treatments on apparent biohydrogenation of polyunsaturated fatty acids

The FA profile in the duodenal flow indicated that C18:2 and C18:3 BH were high, as previously observed with raw linseed in vivo [31] or with (^{14}C) linoleic and (^{14}C) linolenic acids in vitro [35]. CL resulted in a higher proportion and hence lower apparent BH of both C18:2 and C18:3. To our knowledge, the effect of preconditioning without extrusion on BH of unsaturated FA, at the low temperature utilised in our experiment, has not been studied. Although the increased temperature during the premoistening process might provoke partial protection of PUFA against BH [21, 25], it is unlikely that this explains the higher PUFA proportion in the digesta of cows fed CL in the current experiment because of the low preconditioning temperature and the reduced PUFA proportion in the digesta of animals fed EL, which was pretreated at a higher temperature. Current differences between CL, RL and EL were most probably due to the higher particle size of CL compared to the other linseed forms. The effects of oilseed particle size on ruminal BH have not been extensively studied, but a comparison between whole and ground seeds did not show significant differences on duodenal flow of PUFA from soybeans [32]. On the contrary, Pires et al. [26] mentioned that BH tended to be lower with ground than with whole cottonseed, and hypothesised that grinding could result in a lower residence time in the rumen, which could reduce BH extent. Indeed, a potential protective effect against rumen BH of preconditioning cannot be concluded from the current experiment and would need further experiments, specially designed, with different preconditioning temperatures, and similar particle size among treatments.

In our experiment, extrusion of linseed did not affect the BH of PUFA calculated from the FA profile in duodenal flow, rumen fluid or rumen particle phase. Several previous reports mentioned that extrusion does not protect PUFA from canola in vitro [13], from linseed or canola in situ [7, 13] or from soybeans in vivo [6]. On the contrary, others mentioned a decreased BH with rapeseeds in vivo [4] or with soybeans in vitro [30] or in situ [6]. Extrusion temperature was low in the present experiment, as opposed to the temperatures over 140 °C used in experiments showing a decreased BH [4, 30]. However, increasing extrusion temperature from 120 °C to 140 °C has only minor effects on PUFA proportions in ruminal bags or milk fat [6].

3.2. Biohydrogenation intermediates

The proportion of *cis*-9,*trans*-11C18:2 was low in rumen phases and the duodenum in our experiment. Similarly, the addition of linseed oil only resulted in a minor increase in *cis*-9,*trans*-11C18:2 in the duodenal flow [18], and this FA probably originated from the C18:2 of linseed. In the duodenum, *trans*-10+11C18:1 represented 48.1, 45.9 and 46.3% of total *trans*-C18:1 with RL, CL and EL, respectively, and were the most important isomers followed by *trans*-13+14C18:1. *Trans*-15-C18:1 and *trans*-16-C18:1 were also important BH intermediates in agreement with the results obtained by Loor et al. [18] with linseed oil. Similar patterns were observed in rumen samples.

In the rumen, extrusion of linseed resulted in twice higher proportions of *cis*-9,*trans*-11C18:2 than RL or CL. Chouinard et al. [5] observed 3 times higher proportions of *cis*-9,*trans*-11C18:2 in milk with dietary addition of extruded compared with raw soybeans. In vitro, the extrusion of canola strongly increased the proportion of *cis*-9,*trans*-11C18:2 [13]. The lower effect in the present experiment could be explained by an inhibition of the

isomerisation of C18:2 by high amounts of C18:3 provided by linseed [33].

Amongst all digesta types, CL resulted in lower proportions of total *trans*-C18:1, *trans*-6+7+8C18:1 and *trans*-12 to *trans*-16C18:1 intermediates, but *trans*-9 and *trans*-10+11C18:1 were not significantly affected by linseed treatments. These lower proportions of *trans*-C18:1 with CL are consistent with the lower PUFA disappearance.

Compared with RL, EL did not affect *trans*-C18:1 FA. An increased *trans*-11C18:1 proportion in response to extrusion has been mentioned in situ with soybeans [6] or canola [13], and in vivo with soybeans [6]. The effect of extrusion on *trans*-C18:1 and *cis*-9,*trans*-11C18:2 could be due to higher concentrations of free oil, available for isomerisation, resulting in an accumulation of *trans* intermediates because of the limited capacity of the two reduction steps of BH of C18:2 [33]. In our experiment, we observed a high accumulation of *trans*-10+11C18:1 in the ruminal and duodenal contents of the cow consuming RL during the first period, which resulted in a high variability and could have masked a possible effect of extrusion.

3.3. Effect of digesta type

The different digesta types resulted in different estimates of the BH extent or proportions of BH intermediates. The evaluation of the duodenal FA profile is the reference method to estimate rumen BH. Compared with duodenal flow, ruminal fluid contained lower proportions of PUFA. Opposite differences were observed in the rumen particle phase. Higher PUFA proportions in the rumen particle phase could be partly due to undigested feedstuff within this digesta type when sampling little after the meal [35]. Moreover, because fat inside particles is less accessible to rumen bacteria than in the rumen fluid, a slower BH could explain the higher proportion of the first intermediate of BH (*cis*-9,*trans*-11C18:2) and the lower proportions of later intermediates (*trans*-C18:1) or final BH products (predominantly C18:0).

The significant interaction between digesta type and linseed treatment for several FA indicates some differences between linseed treatments only to be apparent in specific digesta types: e.g. preconditioning and grinding to a higher particle size increased C18:2 and C18:3 proportions in the duodenum and the ruminal particle phase, but not in the rumen fluid, whereas extrusion increased rumen *cis*-9,*trans*-11C18:2 but not duodenal proportions.

Except for *cis*-9,*trans*-11C18:2, the results obtained from duodenal samples were intermediate between the results from the rumen fluid and the rumen particle phase, suggesting that a representative sample of these two ruminal phases could be accurate for studying BH. The proportion of *cis*-9,*trans*-11C18:2 in the duodenum was similar to that in the rumen fluid and much lower than in the rumen particle phase. This low proportion could relate to the fact that ruminal *cis*-9,*trans*-11C18:2 is mainly in protozoa [9], which sequesters in the rumen [1]. However, this role of protozoa cannot explain why differences between ruminal and duodenal *cis*-9,*trans*-11C18:2 were more important with EL, because due to free oil, extrusion is rather expected to reduce protozoa numbers [20].

4. CONCLUSIONS

Our results showed that BH of PUFA from linseed results in a low proportion of *cis*-9,*trans*-11C18:2, and that *trans*-10+11 to *trans*-16C18:1 are important BH intermediates. Compared to 3-mm screen ground raw linseed, 6-mm screen ground and preconditioned linseed exhibited a partial protection of PUFA from rumen BH

and lower proportions of *trans*-C18:1, especially *trans*-12 to *trans*-16C18:1. Extrusion did not result in significant modifications of the extent of BH, and increased the proportion of *cis*-9,*trans*-11C18:2 in the rumen but not in the duodenal flow. For most FA, the results obtained with the duodenal flow were intermediate between the results obtained from liquid and particle phases of the rumen.

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