Ruminal metabolism of flaxseed (*Linum usitatissimum*) lignans to the mammalian lignan enterolactone and its concentration in ruminal fluid, plasma, urine and milk of dairy cows

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Secoisolariciresinol diglucoside is the main flax (*Linum usitatissimum*) lignan that is converted to the mammalian lignans enterodiol (ED) and enterolactone (EL) by gastrointestinal microbiota. The objectives of the present study were to investigate the role of ruminal microbiota and the effects of flax oil on *in vivo* metabolism of flax lignans and concentration of EL in biological fluids. Four rumen-cannulated dairy cows were used in a 4×4 Latin square design. There were four periods of 21 d each and four treatments utilising flax hulls (1800 g/d) and oil (400 g/d) supplements. The treatments were: (1) oil and hulls administered in the rumen and abomasal infusion of water; (2) oil and hulls administered in the abomasum; (3) oil infused in the abomasum and hulls placed in the rumen; (4) oil placed in the rumen and hulls administered in the abomasum. Samples were collected during the last week of each period and subjected to chemical analysis. The site of supplementation of oil and hulls had no effect on ruminal EL concentration. Supplementing flax oil in the rumen and the abomasum led to similar EL concentrations in urine, plasma and milk. Concentrations of EL were higher in the urine, plasma and milk of cows supplemented with hulls in the rumen than in those placed with hulls in the abomasum. The present study demonstrated that ruminal microbiota play an important role in the metabolism of flax lignans.

Lignans: Antioxidants: Flaxseed

Flaxseed (*Linum usitatissimum*) is an excellent source of polyunsaturated oil in the form of α-linolenic acid and is the richest source of plant lignans[1]. Lignans are polyphenolic compounds with a range of biological activities, including antioxidant, anti-tumour, weakly oestrogenic and anti-oestrogenic properties, and they also inhibit enzymes involved in the metabolism of sex hormones[2,3]. Greater intakes of flaxseed by humans have been linked to potential health benefits, especially in the prevention of CVD, hypercholesterolaemia, menopausal symptoms, and breast and prostate cancers[4–6]. The study of Saarinen *et al.*[7] on the distribution of absorbed lignans in rat models fed [³H]secoisolariciresinol diglucoside (SDG) showed the presence of lignans in liver, kidney, breast tumours, spleen, skin, lung, brain, uterus and adipose tissues. This indicates that lignans may have local effects in tissues such as tumour tissue that may contribute to human breast cancer prevention.

SDG represents over 95 % of the total lignans in flaxseed[8]. Flax lignans are concentrated in the outer fibre-containing layers[9], thus resulting in higher concentration of SDG in hulls than seeds[10]. In non-ruminant animals, SDG is converted into secoisolariciresinol under the action of intestinal glycosidases and the colonic microbiota convert secoisolariciresinol to the mammalian lignans enterodiol (ED) and enterolactone (EL)[11,12]. Flaxseed also contains small quantities of the plant lignan matairesinol, which is also converted by the colonic microbiota to EL[13]. Mammalian lignans are absorbed by the intestine and, under the action of specific enzymes, they are conjugated as sulfate and glucuronide in the intestinal wall and liver[14,15]. They are excreted in physiological fluids (for example, plasma and urine) or returned to the intestinal lumen via enterohepatic circulation[16,17]. The conjugated forms of mammalian lignans are poorly absorbed by the intestine; deconjugation increases the hydrophobicity of lignans and allows their reabsorption. The mammalian lignans are more resistant to cellular enzymes but are readily cleaved by microbial enzymes such as β-glucuronidase. This enzyme is important for optimal absorption of mammalian lignans[18,19]. Variation in intestinal microflora plays an important role in the inter-individual variation of the metabolism of SDG[19]. Moreover, there is a five-fold increase in the urinary excretion of EL in rats fed pure EL

compared with those fed plant lignans[11], thus suggesting that absorption of mammalian lignans is higher than that of plant lignans. According to Saarinen *et al.*[11], deconjugated EL may be passively absorbed along the intestine while plant lignans must first be converted by microbes to EL in the colon before being absorbed. Therefore, milk with an elevated EL content may be interesting as a dietary source of EL and an excellent strategy to optimise the effects of lignans on human health.

Different studies have confirmed the presence of polyphenolic compounds such as equol, daidzein and genistein[20,21] and mammalian lignan EL[22] in the milk of dairy cows. Feeding flax meal to dairy cows resulted in a linear increase of EL concentration in milk but ED was not detected[23]. In non-ruminants animals, intestinal fermentation plays an important role in the metabolism of plant lignans to the mammalian lignans ED and EL. Recent *in vitro* results have shown that the main mammalian lignan metabolite produced from flax hulls by ruminal microbiota of dairy cows was EL, while faecal microbiota led mainly to the net production of ED[10]. Other *in vivo* results have reported that the ruminal microbiota of goats convert SDG to ED and EL, and that EL is the main lignan metabolite present in ruminal fluid and plasma[24]. However, feeding PUFA is known to modify ruminal microbiota[25,26], which could interfere with the ruminal metabolism of flax lignans, affecting the concentration of its metabolites in biological fluids. Therefore, the objective of the present study was to investigate the importance of the site of administration (rumen and abomasum) of flax oil and flax hulls on the conversion of plant lignans into the mammalian lignan EL and the consequent concentration of EL in blood, ruminal fluid, urine and milk of dairy cows. This was achieved by supplementing flax products (hulls and oil) in the rumen or the abomasum in four experimental treatments.

Materials and methods

Animals and experimental treatments

Four primiparous lactating Holstein cows fitted with ruminal cannulas (10 cm; Bar Diamond Inc., Parma, ID, USA) were used in a 4×4 Latin square design with four 21 d periods balanced for residual effect and four treatments. The cows averaged 92 (SE 12) d in milk at the start of the experiment with an average body weight of 575 (SE 25) kg and an average body condition score of 3·00 (SE 0·15; five-point scale)[27]. No antibiotics were given for at least 16 weeks before the initiation of the experiment. The cows were kept in individual stalls and had free access to water. Cows were milked twice per d at 08.30 and 20.00 hours. All cows were fed for *ad libitum* intake (10 % refusals on as-fed basis) twice per d (08.30 and 14.30 hours). The diet (Table 1) was formulated to meet requirements for cows that were 575 kg body weight and producing 35 kg milk/d with 3·8 % fat[28]. National guidelines for the care and use of animals were followed as recommended by the Canadian Council on Animal Care[29] and all experimental procedures were approved by the local Animal Care Committee 'Comité Institutionnel de Protection des Animaux'.

The four experimental treatments were: (1) oil and hulls administered in the abomasum (ABO/ABO); (2) oil placed in the rumen and hulls administered in the abomasum (RUM/ABO); (3) oil and hulls administered in the rumen

Table 1. Ingredients and chemical composition of the total mixed diet

	Amount
Ingredients (g/kg DM)	
Grass silage	320·0
Maize silage	322·0
Cracked maize grain	119·0
Ground barley	77·0
Soya meal	102·0
Protein supplement*	28·0
Calcium carbonate	7·0
Minerals and vitamins†	25·0
Chemical composition	
DM (g/100 g diet)	41·8
Crude protein (g/100 g DM)	16·0
Neutral-detergent fibre (g/100 g DM)	29·7
Acid-detergent fibre (g/100 g DM)	20·4
Total lipids (g/100 g DM)	2·33
Net energy for lactation (MJ/kg DM)‡	6·61

* Contained 20 % of rapeseed meal, 30 % of maize gluten meal, 20 % of soyabean meal and 30 % of brewer's corn.
† The premix contained (per kg diet): 2·3 g Ca; 1·2 g P; 1·2 g Mg; 0·4 g S; 3·5 g Na; 0·4 g K; 51·7 mg Fe; 68 mg Zn; 11·2 mg Cu; 45·4 mg Mn; 1·7 mg I; 0·2 mg Co; 0·5 mg Se; 113 00 IU vitamin A (118·4 µmol/l); 1450 IU vitamin D_3 (90 480 nmol/l); 67 300 IU vitamin E (1048 796 µmol/l).
‡ Calculated using published values of feed ingredients[28].

and abomasal infusion of water (RUM/RUM); (4) oil infused in the abomasum and hulls placed in the rumen (ABO/RUM). Abomasal infusions consisted of 20 kg tap water (RUM/RUM treatment), 400 g flax oil + 20 kg tap water (ABO/RUM treatment) or 1800 g flax hulls suspended in 18·2 kg tap water (ABO/ABO and RUM/ABO treatments). Flax oil (Brenntag Canada Inc., Montreal, QC, Canada) contained (expressed as a percentage of total fatty acids): 16 : 0, 5·3 %; 18 : 0, 3·8 %; *cis*-9–18 : 1, 18·4 %; *cis*-11–18 : 1, 0·8 %; *cis*-9, *cis*-12–18 : 2, 15·8 %; *cis*-9, *cis*-12, *cis*-15–18 : 3, 52·7 %; others, 3·2 %. Flax hulls (Natunola Health Inc., Nepean, ON, Canada) contained (expressed as a percentage of DM): crude protein, 23·5 %; neutral-detergent fibre, 19·4 %; acid-detergent fibre, 14·3 %; total lipids, 29·8 %; SDG, 0·99 %; 6·86 kJ/g DM. During the first 7 d of each 21 d period, only 30 % of the experimental dose of oil and hulls was administered in the abomasum over a 7 h period. From day 8 to day 21, infusion in the abomasum was conducted with 100 % of the experimental dose of oil and hulls over a 23 h period. Administration in the rumen was done by adding one-third each of oil and hulls three times daily (09.30, 14.30 and 21.30 hours) during all the experiment. Samples of the diet and of flax hulls were taken daily from day 14 to day 21 and pooled within period. All samples were frozen at −20°C for subsequent drying at 55°C and analysed according to the procedures used by Petit & Benchaar[30]. Infusates of flax products were prepared daily and the appropriate amount of infusate for each cow was weighed into tared buckets that were stirred continuously while being infused. To perform abomasal infusions, an infusion line was inserted through the rumen cannula and the sulcus omasi into the abomasum as described by Gressley *et al.*[31]. Placement of the infusion lines was monitored daily to ensure post-ruminal delivery. Suspensions were pumped into the abomasum by using peristaltic pumps (Masterflex L/S; Cole-Parmer Canada Inc., Montreal, QC, Canada). The daily amount of oil supplied by flax hulls was similar to that supplied by flax oil. Half of the lipids were provided by

flax oil and the other half by flax hulls for a total amount of 800 g daily, thus resulting in a fat intake below 6 to 7 % of the DM, which is known to have little effect on DM intake[28].

Sampling

Feed intake and milk yield were measured daily. Milk samples were taken twice daily from day 16 to day 21, pooled on a 6 d basis proportionally to the corresponding milk yield, and frozen at $-20°C$ for lignan analysis. On day 20, blood was withdrawn into K_3EDTA-vacutainer tubes (Becton Dickinson and Cie, Rutherford, NJ, USA) from the jugular vein 6 h after the morning meal. Plasma samples were kept frozen at $-20°C$ until lignan analysis. On day 21, a sample of urine was taken 2 h after the morning meal by hand stimulation of the perineal region and kept frozen at $-20°C$ for lignan analysis. Also, ruminal contents were collected 0, 2, 4 and 6 h after the morning meal from different locations within the rumen (the anterior dorsal, anterior ventral, medium ventral, posterior dorsal and posterior ventral locations) to obtain a representative sample. Ruminal pH was monitored immediately after sample collection with a portable pH meter (Oakton; Eutech Instruments Pte Ltd, Singapore). The ruminal contents were then strained through four layers of cheesecloth. A 350 ml sample of strained ruminal fluid was mixed with ruminal retentate (26 g) and frozen at $-20°C$ until assay for β-glucuronidase activity. One portion of filtered ruminal fluid was acidified to pH 2 with 50 % H_2SO_4 and frozen at $-20°C$ for later determination of volatile fatty acids (VFA) and ammonia-N concentrations. Concentrations of NH_3 and VFA in ruminal fluid were analysed, respectively, with the indophenol-blue method[32] and a GLC Waters Alliance 2695 system (Waters, Milford, MA, USA) fitted with an IR detector autosampler according to the procedure used by Petit & Benchaar[30]. Another portion of ruminal fluid was kept at $-20°C$ and freeze-dried for further analysis of lignans. As previous results (N Gagnon, C Côrtes, D da Silva, R Kazama and HV Petit, unpublished results) have shown that there is no variation in ruminal lignan concentration after feeding, ruminal samples for the three post-feeding times (2, 4 and 6 h) were pooled within cow and period to obtain only one composite sample for lignan analysis. Moreover, faecal grab samples (250 g) were collected directly from the rectum 2, 4, 6 and 8 h post-feeding on the same day (day 21). Faecal pH was monitored immediately after sample collection. Faecal samples were pooled and kept frozen at $-20°C$ for further β-glucuronidase analysis.

Lignan extraction

Lignans in ruminal fluid, plasma, urine and milk samples were hydrolysed and extracted according to the method of Frank & Custer[33] with some modifications. Freeze-dried samples of filtered ruminal fluid were re-suspended in Milli-Q purified water (20 mg/0·5 ml) as described by Heinonen et al.[34]. A quantity of 500 μl warmed (40°C) milk and re-suspended ruminal samples (0 h and pooled post-feeding samples) were mixed with 5 μl β-glucuronidase/arylsulfatase from Helix pomatia (Roche-Diagnostics, Laval, QC, Canada) while 500 μl plasma and urine were mixed with 500 μl 0·1 M-sodium acetate buffer (pH 5) and 5 μl β-glucuronidase/arylsulfatase. Milk samples were incubated

for 1·5 h while plasma, urine and ruminal samples were incubated overnight at 37°C in a shaking water-bath. After hydrolysis, all samples were acidified with 10 μl 6 M-HCl. Acidified milk samples were washed with 3 ml hexane before extraction to remove lipids[19]. All samples were extracted with 2 ml diethyl ether. The samples were vortex-mixed twice for 2 min. The organic layer was separated by freezing. The remaining liquid phase was submitted to a second extraction under the same conditions. The organic layers were pooled and evaporated by vacuum (Speed-Vac; Thermo Savant, Holbrook, NY, USA) at room temperature for 40 min. The dry extract was re-dissolved in 500 μl enzyme immunoassay (EIA) buffer and warmed at 37°C. Four serial dilutions were done in EIA buffer for EL analysis using an EIA kit (Cayman Chemical, Ann Arbor, MI, USA). The starting dilutions were 1:50, 1:200, 1:1600 and 1:60 000 for milk, plasma, ruminal fluid and urine samples, respectively. The kit is a competitive assay that recognises both enantiometric forms of EL and utilises a standard curve ranging from 15·6 to 2000 pg/ml. The assay exhibits a limit of quantification (defined as 80 % B/B_0; 80 % binding of samples) of 70 pg/ml and a half-maximal inhibitory concentration (IC_{50}; 50 % B/B_0) of 240 pg/ml. The recoveries of EL ranged from 85 to 104 %.

β-Glucuronidase activity

The determination of β-glucuronidase activity was based on a modified method of Jenab & Thompson[18]. Briefly, faecal samples (5 g) were homogenised with a Polytron (Kinematica AG, Lucerne, Switzerland) in a final volume of 20 ml cold KH_2PO_4 (pH 6·8) for 15 s while ruminal samples were homogenised using a stomacher (A. J. Seward & Co. Ltd, London, UK). Samples were then filtered through two layers of cheesecloth, sonicated on ice (two bursts, 1 min; Sonics and Materials Inc., Danbury, CT, USA) and centrifuged at 10 000 g for 15 min at 4°C. The supernatant fraction was stored at $-80°C$ until enzyme assay. Activity of the β-glucuronidase was quantified by mixing 25 μl of extracted sample with 125 μl 0·04 M-KH_2PO_4 (pH 6·8), 50 μl 0·5 mM-EDTA and 50 μl 5 mM-phenolphthalein diglucuronide (Sigma-Aldrich, Oakville, ON, Canada). The assay was performed in quadruplicate with one duplicate used as a baseline and the other one was incubated at 37°C for 60 min. The reaction was stopped by adding 1·25 ml 0·2 M-glycine buffer (pH 10·4) containing 0·2 M-NaCl. After 10 min of incubation at room temperature, 200 μl of each replicate was transferred to a ninety-six-well flat-bottomed plate and the plate was read on a Spectra Max 250 ELISA reader (Molecular Devices, Sunnyvale, CA, USA) at 540 nm. The absorbance values directly correlate to the amount of the phenolphthalein released based on a phenolphthalein standard curve. The specific activity of β-glucuronidase was calculated by the formula: (nmol phenolphthalein 60 min − nmol phenolphthalein 0 min)/(60 min × mg protein). Protein was determined by a bicinchoninic acid protein assay kit (Sigma-Aldrich) using bovine serum albumin as the standard.

Statistical analysis

All data were analysed using the MIXED procedure of SAS (SAS 2000; SAS Institute, Cary, NC, USA) according to the

model:

$$Y_{ijk} = \mu + a_i + \beta_j + \tau_k + e_{ijk},$$

where Y_{ijk} is the response variable, μ is the overall mean, a_i is the random effect of cow i, β_j is the effect of period j, τ_k is the effect of treatment k and e_{ijk} is the residual error. The residual effect was initially included in the model but was removed because it was not significant. Data on EL concentrations were transformed (log) as previously performed by Hausner *et al.* [35] and Nesbitt *et al.* [36] but the results in Table 2 are reported as the adjusted mean value (with CI) on the original scale of measurements. The model for specific β-glucuronidase activity and pH was augmented with time and time × treatment interaction for repeated measurements and values are reported with their adjusted mean values with standard errors. The two-sided level of significance was set at $P < 0.05$, although probability values up to $P < 0.1$ are shown in the text if the data suggest a trend. When a significant F test was detected, multiple comparisons were done using a Tukey's adjustment for the probability. A specific contrast was also used *a posteriori* to compare the site of administration of either oil or hulls.

Results

Oil supplementation and dry matter intake

The actual amounts of oil administered in the rumen and the abomasum averaged, respectively, 450 and 440 g/d. The total amount of oil provided by both flax products averaged 912 g/d and there was no difference ($P = 0.12$) among treatments. Intake of DM differed ($P = 0.002$) among diets and averaged, respectively, 13·3, 16·3, 15·4 and 13·8 kg/d for treatments RUM/RUM, ABO/ABO, RUM/ABO and ABO/RUM. Total input of DM (intake of DM + flax products added in the rumen or the abomasum) was also different ($P = 0.02$) among treatments with an average of 15·4, 18·5, 17·5 and 15·9 kg/d for the treatments RUM/RUM, ABO/ABO, RUM/ABO and ABO/RUM, respectively.

Concentration of enterolactone in ruminal fluid

Treatment had no effect ($P = 0.26$) on EL concentration of ruminal fluid collected before feeding (Table 2). Similarly, the site of administration of oil and hulls had no effect ($P = 0.18$) on EL concentration of post-feeding pooled ruminal samples.

Concentration of enterolactone in plasma

Concentration of EL in plasma was different ($P = 0.04$) among treatments (Table 2). Supplementation of flax hulls in the rumen (ABO/RUM and RUM/RUM) resulted in higher ($P = 0.008$) concentration of EL in the plasma than when hulls were supplemented in the abomasum (ABO/ABO and RUM/ABO). The site of oil infusion had no significant effect on plasma EL concentration.

Concentration of enterolactone in urine

Concentration of EL in urine was different ($P < 0.0001$) among treatments (Table 2). Cows supplemented with flax hulls in the rumen (RUM/RUM and ABO/RUM) had higher ($P < 0.0001$) EL concentration in urine than those receiving hulls in the abomasum (RUM/ABO and ABO/ABO). There was no difference ($P = 0.71$) between the RUM/RUM and ABO/RUM treatments. Moreover, there was no difference ($P = 0.18$) in urine concentration of EL between cows on the ABO/ABO and those on the RUM/ABO treatments.

Concentration of enterolactone in milk

Concentration of EL in milk differed ($P = 0.001$) among treatments (Table 2). Milk concentration of EL was similar ($P = 0.44$) for cows on the ABO/RUM and RUM/RUM treatments and it was significantly higher ($P = 0.0002$) than that of cows on the RUM/ABO and ABO/ABO treatments. There was no difference ($P = 0.90$) in milk EL concentration between cows on ABO/ABO and those on RUM/ABO.

Ruminal fermentation characteristics

There were interactions ($P = 0.01$) between sampling time and treatment for the acetate:propionate ratio and the (acetate + butyrate):propionate ratio in the rumen (data not shown). Ammonia-N concentration in the rumen was similar among treatments (Table 3). Concentration of total VFA in the rumen was higher ($P = 0.02$) for cows on the ABO/RUM and RUM/ABO treatments than for those on the RUM/RUM treatment. Cows supplemented with oil in the rumen (RUM/RUM and RUM/ABO) had lower ($P = 0.05$) concentration of total VFA in the rumen than those receiving oil in the abomasum (ABO/RUM and ABO/ABO). Cows on the ABO/ABO treatment had higher ($P = 0.02$) and lower ($P = 0.004$) proportions

Table 2. Concentration of enterolactone in biological fluids of Holstein cows receiving oil and hulls administered in the abomasum (ABO/ABO), oil placed in the rumen and hulls administered in the abomasum (RUM/ABO), oil and hulls placed in the rumen (RUM/RUM) and oil infused in the abomasum and hulls placed in the rumen (ABO/RUM)

(Adjusted mean values and 95 % confidence intervals on the original scale of measurements)

Treatment…	ABO/ABO		RUM/ABO		RUM/RUM		ABO/RUM		
	Mean	95 % CI	Mean	95 % CI	Mean	95 % CI	Mean	95 % CI	P
Ruminal fluid (μmol/l)									
0 h	15·1	8·92, 25·5	21·7	12·9, 36·7	28·3	16·7, 47·8	31·3	18·5, 52·9	0·26
Pool (2 h, 4 h, 6 h)	13·3	6·30, 28·2	19·2	9·07, 40·6	21·5	10·2, 45·5	35·4	16·7, 74·8	0·17
Urine (μmol/l)	34·4[b]	21·0, 56·3	67·5[b]	41·3, 111	540[a]	330, 884	735[a]	449, 1203	<0·0001
Plasma (μmol/l)	0·50	0·23, 1·07	0·48	0·22, 1·03	1·59	0·74, 3·41	1·78	0·83, 3·80	0·04
Milk (μmol/l)	0·04[b]	0·02, 0·08	0·06[b]	0·03, 0·10	0·52[a]	0·28, 0·96	0·29[a]	0·16, 0·54	0·001

[a,b] Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

Table 3. Ruminal fermentation characteristics of Holstein cows receiving oil and hulls administered in the abomasum (ABO/ABO), oil placed in the rumen and hulls administered in the abomasum (RUM/ABO), oil and hulls placed in the rumen (RUM/RUM) and oil infused in the abomasum and hulls placed in the rumen (ABO/RUM)

(Mean values with their standard errors)

	Treatment				SEM	P
	ABO/ABO	RUM/ABO	RUM/RUM	ABO/RUM		
Ammonia-N (mmol/l)	8·51	7·92	8·34	10·1	1·16	0·60
Volatile fatty acids (mmol/l)	107[a,b]	110[a]	100[b]	113[a]	1·91	0·02
Molar proportions (mmol/mol)						
Acetate	644[a]	626[a,b]	590[b]	625[a,b]	8·97	0·02
Propionate	202[b]	214[b]	266[a]	231[a,b]	7·23	0·004
Butyrate	94·5	97·8	90·8	89·8	2·74	0·27
Isobutyrate	7·34	5·78	6·96	6·17	0·47	0·18
Valerate	11·0	9·68	12·0	9·89	0·46	0·07
Isovalerate	15·5	15·0	20·8	15·1	1·47	0·08
Lactate	25·8	31·6	13·3	22·6	8·22	0·51
Molar ratios						
Acetate:propionate*	32·2[a]	29·6[a]	22·9[b]	27·8[a]	0·93	0·002
(Acetate + butyrate):propionate*	37·0[a]	34·1[a,b]	26·4[c]	31·8[b]	1·04	0·002

[a,b] Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).
* Interaction between treatment and hour ($P < 0.05$).

of acetate and propionate in the rumen, respectively, than those on the RUM/RUM treatment. Treatment had no effect on the proportions of butyrate ($P = 0.27$), isobutyrate ($P = 0.18$) and lactate ($P = 0.51$) in the rumen.

Activity of β-glucuronidase in ruminal fluid and faeces

There was no interaction ($P = 0.22$) between hour and treatment for specific β-glucuronidase activity in ruminal fluid and faeces. Therefore, only mean values for the 6 h ruminal sampling period are presented in Fig. 1(a). Cows on the RUM/ABO treatment tended to have lower ($P = 0.06$) ruminal β-glucuronidase activity than those on the ABO/RUM treatment. Infusing oil directly in the rumen (RUM/ABO and RUM/RUM) reduced ($P = 0.01$) ruminal β-glucuronidase activity compared with infusing oil in the abomasum (ABO/ABO and ABO/RUM). There was no difference ($P = 0.97$) between the ABO/ABO and ABO/RUM treatments, indicating that ruminal β-glucuronidase activity was not modulated by flax hull supplementation in the rumen. Treatment had no effect ($P = 0.46$) on faecal β-glucuronidase activity (Fig. 1(b)) although activity was on average five times higher ($P = 0.0001$) in faeces than in ruminal fluid (9·77 and 1·96 nmol phenolphthalein/min per mg protein, respectively).

Ruminal fluid pH

There was no significant interaction ($P = 0.43$) between hour and treatment for ruminal fluid pH. There was a decrease ($P < 0.0001$) in ruminal fluid pH after feeding for all treatments and the pH remained stable afterwards (Fig. 2(a)). Cows on the ABO/RUM treatment had lower ($P = 0.04$) pH values than cows on the ABO/ABO treatment. The ruminal fluid pH tended ($P = 0.07$) to be lower when hulls were supplemented in the rumen (ABO/RUM and RUM/RUM) than when they were administered in the abomasum (ABO/ABO and RUM/ABO).

Faecal pH

There was no interaction ($P = 0.20$) between hour and treatment and no effect ($P = 0.20$) of sampling time for faecal pH (Fig. 2(b)). However, faecal pH tended ($P = 0.07$) to differ among treatments. Supplementation of flax hulls in the
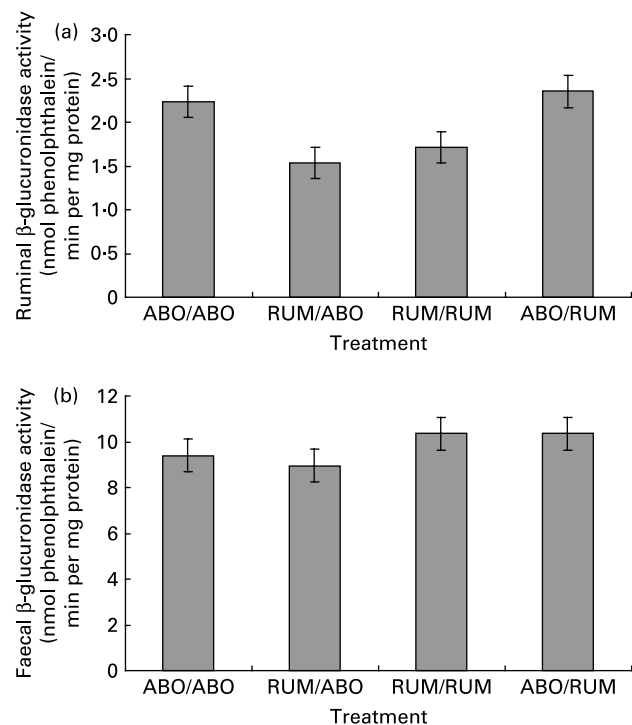

Fig. 1. Post-feeding activity of β-glucuronidase in ruminal fluid (a) and faeces (b) of dairy cows supplemented with flax hulls and flax oil. Treatments were: (1) oil and hulls administered in the abomasum (ABO/ABO); (2) oil placed in the rumen and hulls administered in the abomasum (RUM/ABO); (3) oil and hulls placed in the rumen (RUM/RUM); (4) oil infused in the abomasum and hulls placed in the rumen (ABO/RUM). Data are means, with standard errors represented by vertical bars. There was an effect ($P = 0.01$) of infusion site of oil on ruminal β-glucuronidase activity.
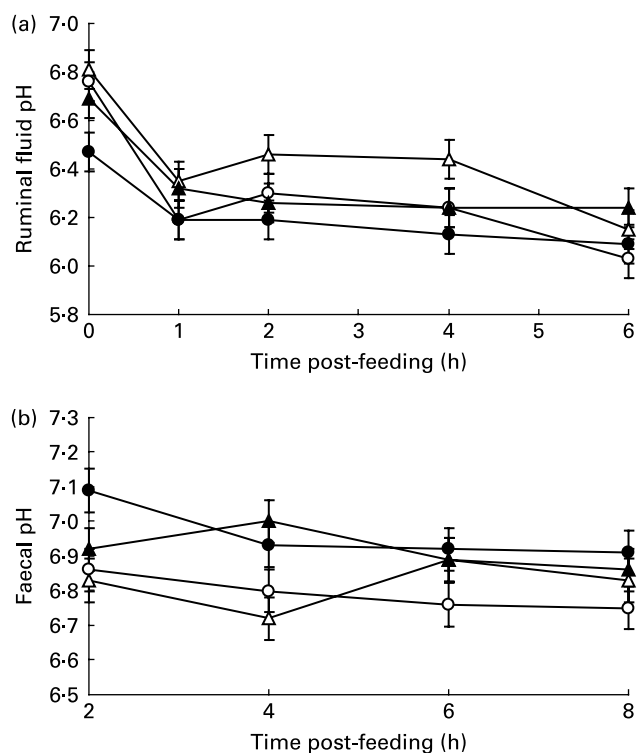
Fig. 2. Post-feeding pH in ruminal fluid (a) and faeces (b) of dairy cows supplemented with flax hulls and flax oil. Treatments were: (1) oil and hulls administered in the abomasum (−△−; ABO/ABO); (2) oil placed in the rumen and hulls administered in the abomasum (−○−; RUM/ABO); (3) oil and hulls placed in the rumen (−▲−; RUM/RUM); (4) oil infused in the abomasum and hulls placed in the rumen (−●−; ABO/RUM). Data are means, with standard errors represented by vertical bars. The effect of infusion site of flax hulls on ruminal pH tended ($P=0.07$) to be significant and this effect was significant for faecal pH ($P=0.015$).

abomasum (ABO/ABO and RUM/ABO) resulted in lower ($P=0.015$) faecal pH than when hulls were supplemented in the rumen (ABO/RUM and RUM/RUM).

Discussion

Many studies have reported that intestinal micro-organisms play an important role in phyto-oestrogen metabolism[37,38]. In ruminants, fermentation processes occur first in the rumen as opposed to non-ruminant animals, where they take place in the colon. Previous studies with ruminant animals have shown that lignans are metabolised by both ruminal and faecal microbiota[10,24] and lignan metabolites are present in biological fluids such as plasma, semen and milk[22,39,40]. To our knowledge, this is the first *in vivo* study investigating the importance of rumen microbiota in the conversion of flax lignans into the mammalian lignan EL and its concentration in physiological fluids of dairy cows.

According to our *in vitro* results[10], ruminal microbiota of dairy cows have the ability to metabolise lignans from flax products to mammalian lignan EL. Other dietary feed ingredients such as forages and cereals also contain lignans[41,42], which may lead to the production of EL and explain the presence of EL in the ruminal fluid of cows on all present treatments. The levels of EL in ruminal fluid were not significantly different among treatments, but numerically higher values of EL were observed in the ruminal fluid of cows supplemented with flax hulls in the rumen (ABO/RUM and RUM/RUM). Ruminal fluid pH tended to decrease when flax hulls were added in the rumen (ABO/RUM and RUM/RUM) compared with when they were administered in the abomasum (ABO/ABO and RUM/ABO). Similarly, infusing the plant lignan SDG into the rumen of goats led to increased ruminal EL concentration and decreased pH[24]. Ruminal fluid pH in the present experiment decreased within 1 h of feeding, probably as a result of the production of VFA, which are the endproducts of ruminal fermentation following the supply of substrate[10]. Flax hulls are a rich source of fermentable carbohydrates, which may contribute to the lower pH of the ruminal fluid of cows supplemented with flax hulls in the rumen (ABO/RUM and RUM/RUM) as compared with the abomasum (ABO/ABO and RUM/ABO) due to greater availability of carbohydrates in the rumen[43]. However, the concentration of VFA was not affected by the site of oil infusion when flax hulls were administered in the abomasum. On the other hand, in the presence of flax hulls in the rumen, VFA concentration decreased with oil administered in the rumen as compared with the abomasum, probably as a result of the oil modulating the microbial population. Moreover, flax hulls placed in the rumen may contribute to increase the proportion of propionate due to the presence of highly fermentable products. Results from a recent *in vitro* study[26] reported that growth of ruminal bacteria can be affected by PUFA such as *n*-3, although sensitivity differs among species.

In the present study, SDG metabolism in the rumen was not affected by flax oil, which is rich in *n*-3 fatty acids, as indicated by similar ruminal fluid EL concentrations among treatments. On the other hand, the ruminal fluid activity of microbial β-glucuronidase was influenced by the presence of flax oil in the rumen as shown by lower activity for cows supplemented with flax oil in the rumen (RUM/ABO and RUM/RUM) than for those with flax oil bypassing the rumen (ABO/ABO and ABO/RUM). The activity of β-glucuronidase has been attributed to bacteria belonging to the dominant human intestinal microbiota, such as *Ruminococcus*, *Bacteroides*, *Bifidobacterium* and *Eubacterium*[44]. Henderson[25] showed that the growth of some strains of important rumen bacteria such as *Butyrivibrio*, *Ruminococcus* and *Methanobrevibacter* is strongly inhibited by the presence of long-chain fatty acids. The data of the present study may indicate that species of ruminal microbiota with β-glucuronidase activity are more sensitive to the presence of PUFA than ruminal species involved in the conversion of plant SDG into the mammalian lignan EL. This may indicate that a diet rich in PUFA will not affect the conversion of SDG to EL in the rumen but will decrease β-glucuronidase activity. However, the importance of deconjugation in the rumen for further absorption of mammalian lignan is not well understood in ruminant animals.

In a study carried out with colonic microbiota from rats, Jenab & Thompson[18] observed a positive correlation between SDG dietary concentration and microbial β-glucuronidase activity. In goats, Zhou *et al.*[24] suggested that SDG supplementation stimulates the growth of *Ruminococcus gnavus*, which plays a role in glucuronidase activity of the rumen. In the present study, supplementation with the plant

lignan SDG had no effect on ruminal fluid and faecal β-glucuronidase activity of dairy cows. One explanation for the lack of an effect of flax hull supplementation on β-glucuronidase activity in ruminal fluid and faeces may be the small number of cows used in the present study. Microbiota play an important role in β-glucuronidase activity and there is a large variation in microbiota composition among cows[45,46], suggesting that a greater number of cows is required to detect any difference in β-glucuronidase activity. Moreover, differences in animal species (for example, non-ruminant v. ruminant animals) and plant lignan concentration used in experiments could explain discrepancies between results on the effect of flax hull supplementation on β-glucuronidase activity.

Specific β-glucuronidase activity was on average five times higher in faeces than in ruminal fluid, indicating that deconjugation activity may be more important in the large intestine than in the rumen of dairy cows. This is the first time that the activities of both faecal and ruminal microbial β-glucuronidase were compared in ruminants. Further investigations on microbial β-glucuronidase activity along the gastrointestinal tract of cattle are required to better understand the reabsorption of deconjugated metabolites such as mammalian lignans in dairy cows to improve the understanding of their metabolic pathway in dairy cows, in order to enable targeted manipulation of their quantities in milk. Microbial β-glucuronidase activity is important for the absorption of mammalian lignan in humans and this enzyme is inducible and positively correlated with the level of plant lignans and urinary excretion of mammalian lignans[18]. In the present experiment, milk EL concentration followed a profile similar to that of urinary EL excretion, which may suggest that microbial β-glucuronidase activity can contribute to increase the amount of lignans in milk as a result of a greater absorption of mammalian lignans in the body.

Lower faecal pH for cows supplemented with flax hulls in the abomasum compared with that of cows supplemented with flax hulls in the rumen may be the result of a greater amount of undigested plant lignans (dietary source of carbohydrates) reaching the colon and being fermented by intestinal microflora due to rumen bypass of flax hulls. Fermentation of carbohydrates in the colon results in the production of short-chain VFA that lower colonic pH and serve as an energy source for the colonocytes[43].

After absorption by the intestine, conjugated mammalian lignans return to the intestinal lumen via enterohepatic circulation or are excreted in physiological fluids. Many studies have shown the presence of lignans in the plasma and milk of dairy cows[40,42] but none has investigated the role of the rumen in the transfer of mammalian lignans in biological fluids such as plasma, urine and milk. The present study shows that the concentration of EL obtained in plasma, urine and milk depends on ruminal metabolism of plant lignans. A significant increase in EL concentration was observed in the plasma, milk and urine of cows when a source of lignans (flax hulls) was placed directly in the rumen compared with when lignans bypassed the rumen through administration in the abomasum. Therefore, it appears that the main site for metabolism of flax lignans in dairy cows is the rumen and that the small and large intestine are not as efficient in metabolising plant SDG into mammalian EL. The response to plant lignan SDG supplementation was more important in urine and milk than blood. Increases in EL concentration of milk and urine were, respectively, twelve and sixteen times higher for cows receiving both flax products (hulls + oil) in the rumen compared with those administered with flax products directly in the abomasum while plasma EL concentration was only three times higher. It appears that the best biomarkers for lignan metabolism of flax products in cattle may be obtained by measuring the concentration of mammalian lignan EL in urine and milk rather than in ruminal fluid and plasma. Ruminal fluid and blood are dynamic media with body exchanges such as absorption while milk and urine may accumulate over time (for example, in the mammary gland). As a result, the mammalian lignan EL is more likely to be concentrated in urine and milk than in ruminal fluid and blood.

It has been shown that individuals with higher blood concentrations of EL have lower incidence of CVD[47]. However, it is unknown if health effects of mammalian lignans are similar in dairy cows. Further investigations are required to better understand the impact of increasing mammalian lignans in biological fluids on reproduction, lactation and health of dairy cows. The protective effects of lignans in humans are well documented and production of value-added milk naturally enriched with mammalian lignans could then contribute to better human health.

In conclusion, flax oil supplementation has no influence on the ruminal metabolism of lignans supplied as flax hulls. Moreover, the results demonstrate that the main site for metabolism of flax lignans in dairy cows is the rumen. Therefore, ruminal microbiota may be the most important flora to target for plant lignan metabolism in order to increase the concentration of the mammalian lignan EL in the milk of dairy cows.

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British Journal of Nutrition