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Effects of EPA and DHA on *in vitro* ruminal biohydrogenation of 18-carbon fatty acids in sheep

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Abstract. Marine lipid supplements have been used to inhibit the ruminal saturation of *trans*-11 18:1, with the final goal of enhancing *cis*-9 *trans*-11 conjugated linoleic acid (CLA) concentration in milk and meat. This response would be largely explained by the effects of n-3 very long chain polyunsaturated fatty acids (PUFA) on the last step of biohydrogenation (BH). In cows, docosahexaenoic acid (DHA, 22:6n-3) has been suggested to be a stronger inhibitor of *trans*-18:1 hydrogenation than eicosapentaenoic acid (EPA, 20:5n-3), but information about changes in individual 18:1 isomers is very limited, and no reports are available in sheep. This in vitro study was therefore conducted to compare the impact of EPA and DHA on the BH of 18-carbon fatty acids in ovine, using batch cultures of rumen microorganisms and cannulated ewes as inocula donors. The two PUFA were added at a dose of 2% incubated DM and effects were examined after 24 h of incubation. The DHA treatment led to the greatest concentration of *trans*-18:1 in digesta, but this was mainly accounted for by accumulation of metabolites from alternative BH pathways (e.g, *trans*-9, -10, -12 and -15 18:1), while the inhibition of *trans*-11 18:1 saturation was comparable with both PUFA. The saturation of *cis*-18:1 was constrained too, particularly by DHA, whereas EPA seemed to have specific effects on 18:3n-3 metabolism. Changes in oxo-FA concentrations suggested that ruminal hydration (an alternative metabolic pathway to BH) was also affected by PUFA treatments.

Keywords. Ewe – PUFA – Ruminal lipid metabolism – *Trans* fatty acid.

Effets de l'EPA et du DHA sur la biohydrogénation ruminale* in vitro *des acides gras à 18 carbones chez les ovins

Résumé. *Les suppléments lipidiques marins ont été utilisés pour inhiber la saturation ruminale du trans-11 18:1, dans le but final d'améliorer la concentration en acide linoléique conjugué (CLA) cis-9 trans-11 18:2 dans le lait et la viande. Cette réponse s'explique en grande partie par les effets des acides gras polyinsaturés n-3 à très longue chaîne (AGPI) sur la dernière étape de la biohydrogénation (BH). Chez le bovin, l'acide docosahexaénoïque (DHA, 22:6n-3) a été proposé comme un plus fort inhibiteur de l'hydrogénation des trans-18:1 que l'acide eicosapentaénoïque (EPA, 20:5n-3), mais il y a trop peu de données sur les variations des isomères 18:1 individuels et aucune étude n'est disponible chez les ovins. Cet essai in vitro a donc été réalisée pour comparer l'impact de l'EPA et du DHA sur la BH des acides gras à 18 carbones chez des moutons, en utilisant des cultures discontinues de microorganismes du rumen et des brebis canulées comme donneuses d'inoculum. Les deux AGPI ont été ajoutés à une dose de 2% de la matière sèche incubée et les effets ont été examinés après 24 h d'incubation. Le traitement DHA a induit la plus grande concentration en trans-18:1 dans les digesta, mais cela était principalement attribuable à l'accumulation des intermédiares de voies de BH alternatives (p. ex., trans-9, -10, -12 et -15 18: 1), alors que l'inhibition de la saturation du trans-11 18:1 était comparable pour les deux AGPI incubées. La saturation des cis-18:1 était également limitée, en particulier par le DHA, alors que l'EPA semblait avoir des effets spécifiques sur le métabolisme du 18:3n-3. Les changements des concentrations en céto-AG ont suggéré que l'hydratation ruminale (une voie métabolique alternative à la BH) a également été affectée par les traitements avec AGPI.*

Mots-clés. *Brebis – AGPI – Métabolisme ruminal des lipides – Acide gras trans.*

I – Introduction

Marine lipid supplements have been used to inhibit the ruminal saturation of *trans*-11 18:1, with the final goal of enhancing *cis*-9 *trans*-11 conjugated linoleic acid (CLA) concentration in milk and meat (Lee *et al.*, 2005; Toral *et al.*, 2012). This response would be largely explained by the effects of n-3 very long chain polyunsaturated fatty acids (PUFA) on the last step of biohydrogenation (BH). In cows, AbuGhazaleh and Jenkins (2004) suggested that docosahexaenoic acid (DHA, 22:6n-3) could be a stronger inhibitor of *trans*-18:1 hydrogenation than eicosapentaenoic acid (EPA, 20:5n-3), but we are not aware of similar works in sheep. Furthermore, their study did not report changes in *trans*-18:1 profile, although advances in the knowledge of the biological effects of fatty acids (FA) suggest relevant differences between individual isomers (Shingfield *et al.*, 2008; Wang and Proctor, 2013).

Trans-11 18:1, the predominant *trans*-18:1 in milk and meat, is a desirable FA that is desaturated to *cis*-9 *trans*-11 CLA in ruminant and human body tissues (Wang and Proctor, 2013). On the other hand, *trans*-9 and -10 18:1, more abundant isomers in industrial fats, might have potentially negative impact on consumer's health (Shingfield *et al.*, 2008; Wang and Proctor, 2013). In addition, the shift in ruminal BH pathways leading to *trans*-10 18:1 accumulation has been associated with the syndrome of milk fat depression in sheep fed fish oil or marine algae (Toral *et al.*, 2012, 2016). Providing further insight into the influence of specific n-3 PUFA on ruminal *trans*-18:1 profile may then contribute to develop feeding strategies that modulate ewe milk FA composition with the least side effects.

This in vitro study was therefore conducted to compare the impact of EPA and DHA, the major n-3 PUFA in marine lipids, on the BH of 18-carbon fatty acids in ovine.

II – Material and methods

Batch cultures of rumen microorganisms were conducted using 16-mL Hungate tubes and rumen fluid collected from 2 ruminally cannulated ewes fed a total mixed ration (forage:concentrate ratio 50:50). After an adaptation period of 2 weeks, the inocula (collected in three different days, each one corresponding to a replicate) were obtained before the morning feeding and mixed (1:4) with artificial saliva. The ration fed to the animals was used as the substrate for incubation (50 mg/mL of rumen fluid). The two PUFA were dissolved in ethanol 96% and added at a dose of 2% DM just before the incubation started. Only the ethanol was dosed to the control treatment. All vials were incubated under anaerobic conditions for 24 h at 39.5ºC.

At the end of the incubation, the reaction was stopped by placing the tubes into ice-water. They were then stored at -80ºC until FA analysis. The lipids in freeze-dried in vitro ruminal digesta were extracted and converted to FA methyl esters (FAME) by sequential base-acid catalysed transesterification (Toral *et al.*, 2010). Methyl esters were separated and quantified with a gas chromatograph (Agilent 7890A, Santa Clara, CA, USA) equipped with a flame-ionization detector and a 100-m fused silica capillary column (CP-SIL 88, Varian Ibérica S.A., Madrid, Spain). Total FAME profile was determined using a temperature gradient program and then isothermal conditions at 170ºC to further resolve 18:1 isomers (Shingfield *et al.*, 2003). Peaks were identified based on retention time comparisons with commercially available standard FAME mixtures and selected digesta samples for which the FA composition was determined based on GC analysis of FAME and GC-MS analysis of corresponding 4,4-DMOX derivatives (Toral *et al.*, 2010).

Statistical analyses were performed using the MIXED procedure of the SAS software package (version 9.4; SAS Institute Inc., Cary, NC, USA), with a model that included the fixed effect of treatments (control, EPA and DHA), and the random effect of the incubation run. Means were separated through the "pdiff" option of the "lsmeans" statement, and adjusted for multiple comparisons using Bonferroni's method.

III – Results and discussion

The DHA treatment led to the greatest concentration of total *trans*-18:1 in digesta ($P<0.001$), in agreement with earlier results in cows (AbuGhazaleh and Jenkins, 2004). However, this was mainly accounted for by accumulation of metabolites from alternative BH pathways (e.g, *trans*-9, -10, -12 and -15 18:1; $P>0.05$), while the inhibition of *trans*-11 18:1 saturation caused by EPA or DHA was similar ($P>0.10$). This is probably related to the toxicity of each PUFA for particular ruminal bacteria species (Maia *et al.*, 2007), and may have relevant implications due to the different EPA/DHA ratio of marine lipids (e.g., fish oils usually have greater proportions of EPA than DHA-rich algae). The first implication might be that increases in milk and meat *cis*-9 *trans*-11 CLA concentrations would be comparable at the same PUFA dose, as supported by the observed lack of significant variation in ruminal *cis*-9 *trans*-11 CLA ($P>0.10$). Secondly, based on the association between shifts in BH pathways and the low-fat milk syndrome (Kairenius *et al.*, 2015; Toral *et al.*, 2016), it could be expected that supplements rich in DHA (e.g., *Schizochytrium* sp. algae) would have the strongest negative effects on animal performance. In vivo research would be advisable to verify both points.

Table 1. Effect of EPA and DHA on some 18-carbon fatty acid concentration (% of total FA) after 24-h in vitro incubation with rumen inoculum from sheep

	Treatments				
	Control	**EPA**	**DHA**	**s.e.d.[1]**	***P*-value**
18:0	55.799^{a}	39.782^{b}	37.467^{b}	1.7526	<0.001
10-oxo-18:0	0.098^{b}	0.221^{a}	0.149^{ab}	0.0307	0.039
13-oxo-18:0	0.170^{ab}	0.173^{a}	0.126^{b}	0.0114	0.025
cis-9 18:1[2]	1.868^{b}	1.929^{ab}	2.698^{a}	0.2899	0.051
cis-11 18:1	0.254^{b}	0.399^{a}	0.500^{a}	0.0339	0.005
cis-12 18:1	0.170^{b}	0.240^{ab}	0.274^{a}	0.0337	0.054
cis-13 18:1	0.113^{b}	0.112^{b}	0.143^{a}	0.0069	0.017
trans-9 18:1	0.216^{c}	0.472^{b}	0.691^{a}	0.0552	<0.001
trans-10 18:1	0.238^{c}	0.599^{b}	1.025^{a}	0.0357	<0.001
trans-11 18:1	3.927^{b}	5.678^{a}	6.464^{a}	0.4309	0.003
trans-12 18:1	0.472^{c}	1.057^{b}	1.266^{a}	0.0511	<0.001
trans-13 18:1	0.586^{b}	1.434^{a}	1.610^{a}	0.0864	<0.001
trans-15 18:1	0.498^{b}	0.841^{a}	1.003^{a}	0.0310	<0.001
Σ *trans*-18:1	6.326^{c}	10.509^{b}	12.649^{a}	0.5622	<0.001
cis-9 *cis*-12 18:2	1.120^{a}	0.790^{b}	0.838^{ab}	0.0979	0.030
trans-11 *cis*-15 + *trans*-10 *cis*-15 18:2	0.097^{b}	0.492^{a}	0.397^{a}	0.0640	0.008
cis-9 *trans*-11 CLA	0.112	0.130	0.108	0.0134	0.321
trans-10 *cis*-12 CLA	0.028	0.035	0.022	0.0062	0.207
cis-9 *cis*-12 *cis*-15 18:3	0.208	0.126	0.113	0.0337	0.090
trans-9 *trans*-12 *cis*-15 18:3[3]	0.009^{b}	0.099^{a}	0.015^{b}	0.0160	0.002

$^{a-c}$ Within a row, different superscripts indicate significant differences ($P<0.05$) or a trend towards significance (in italics; $P<0.10$) due to the effect of treatment.

[1] s.e.d. = standard error of the difference. [2]Contains *trans*-14 18:1 as a minor component. [3]Coelutes with *cis*-9 *cis*-12 *trans*-15 18:3.

The ruminal BH of *cis*-18:1 was constrained too, consistent with previous investigations in cows and sheep (AbuGhazaleh and Jenkins, 2004; Toral *et al.*, 2012). Increases in *cis*-9, -12 and -13 18:1 accumulation would indicate a more pronounced response to DHA ($P<0.10$). On the contrary, EPA seemed to have specific, yet subtle, effects on 18:3n-3 metabolism, according to variation in minor intermediates (e.g., *trans*-9 *trans*-12 *cis*-15 + *cis*-9 *cis*-12 *trans*-15 18:3; $P<0.01$), although

numerical differences in the major metabolites (i.e., *trans*-11 *cis*-15 + *trans*-10 *cis*-15 18:2) between PUFA treatments did not attain statistical significance ($P>0.10$).

Finally, changes in oxo-FA concentrations (i.e., 10- and 13-oxo-18:0; $P<0.05$) suggested that ruminal hydration (an alternative metabolic pathway to BH) was differently affected by EPA and DHA. Given the limited information about the bioactivity of oxylipids in ruminants (Raphael *et al.*, 2014), the putative link between oxo-FA and milk fat depression (Kairenius *et al.*, 2015; Toral *et al.*, 2016) merits additional investigation.

IV – Conclusion

Sheep diet supplementation with EPA and DHA exerts some different actions on the in vitro ruminal BH of C18 FA (e.g., DHA promotes the accumulation of 18:1 metabolites from alternative BH pathways, such as *trans*-10 18:1, while EPA seems to specifically modify 18:3n-3 metabolism). However, both of them have a similar positive impact on *trans*-11 18:1 concentration, suggesting an equivalent potential to modulate ovine milk and meat FA profiles by improving *cis*-9 *trans*-11 CLA content.

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