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In vitro ruminal biohydrogenation of eicosapentaenoic (EPA), docosapentaenoic (DPA), and docosahexaenoic acid (DHA) in cows and ewes: Intermediate metabolites and pathways

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ABSTRACT

A great deal of uncertainty still exists about intermediate metabolites and pathways explaining the biohydrogenation (BH) of 20- and 22-carbon polyunsaturated fatty acids (PUFA). Therefore, this study was conducted to provide further insight into the ruminal metabolism of 20:5 n-3 (EPA), 22:5 n-3 (DPA), and 22:6 n-3 (DHA), the main n-3 PUFA present in the marine lipids used in dairy ruminant feeding, and to examine potential differences between bovine and ovine. To meet this aim, we investigated the 20- and 22-carbon metabolites accumulated during in vitro incubation of EPA, DPA, and DHA with rumen inocula from cows and ewes. The PUFA were added at a dose of 2% incubated dry matter and digesta samples were analyzed after 24 h of incubation using complementary gas-liquid chromatography of fatty acid methyl esters and gas chromatography-mass spectrometry of 4,4-dimethyloxazoline derivatives. Results suggested that the main BH pathway of EPA and DPA would proceed via the reduction of the double bond closest to the carboxyl group (*cis*-5 in EPA and *cis*-7 in DPA); curiously, this mechanism seemed of much lower importance for DHA. Thus, DPA would not be a major intermediate product of DHA and their BH might actually follow separate pathways, with the accumulation of numerous unique metabolites in each case. A principal component analysis supported this hypothesis, with a clear separation between PUFA treatments in the score and loading plots. Within EPA and DPA groups, cow and ewe samples loaded separately from each other but not distant. No conjugated 20:5, 22:5, or 22:6 isomer compatible with the initial product of EPA, DPA, or DHA metabolism, respectively, was identified in the ruminal digesta, although this would not unequivocally exclude their transient formation. In this regard, results from DPA incubations provided the first indication that the metabolism of this very long chain PUFA may involve the formation of conjugated double bond structures. The BH of EPA, DPA, and DHA resulted in the appearance of several tentative *trans*-10–containing metabolites, showing a general trend to be more abundant in the digesta of ewes than in that of cows. This finding was speculated to have some relationship with the susceptibility of dairy sheep to marine lipid-induced milk fat depression. Differences in the relative proportion of intermediate products would also suggest an influence of ruminant species on BH kinetics, with a process that would likely be slower and less complete in cows than in ewes.

Key words: cattle, mass spectrometry, PUFA, ruminal lipid metabolism, sheep

INTRODUCTION

The effectiveness of nutritional strategies to enrich milk with very long chain n-3 PUFA is hindered by rumen microbial biohydrogenation (**BH**; Chilliard et al., 2007; Dewhurst and Moloney, 2013). For this reason, diet supplementation with fish oils or microalgae rich in these fatty acids (**FA**) has often been used to enhance milk CLA content (Offer et al., 1999; Chilliard et al., 2007). On the other hand, these strategies cause milk fat depression, a syndrome often encountered in modern dairy production (Gama et al., 2008; Bichi et al., 2013; Kairenius et al., 2015). Although its origin is still uncertain, some works suggest a putative contribution of very long chain n-3 PUFA or their BH intermediates (Loor et al., 2005; Dallaire et al., 2014; Kairenius et al., 2015).

Numerous studies have demonstrated the extensive ruminal disappearance of major 20- and 22-carbon

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PUFA from marine lipid supplements [i.e., eicosapentaenoic acid (**EPA**; 20:5 n-3) and docosahexaenoic acid (**DHA**, 22:6 n-3); Kim et al., 2008; AbuGhazaleh and Jenkins, 2004; Vlaeminck et al., 2014]. However, little is known about the intermediate products of their BH, as very few reports provide further description of this process (Toral et al., 2010; Kairenius et al., 2011; Jeyanathan et al., 2016).

A major constraint for unravelling the rumen metabolic fate of very long chain n-3 PUFA in in vivo studies is the complex composition of their sources (i.e., fish oils and microalgae; Or-Rashid et al., 2008; Toral et al., 2010; Shingfield et al., 2012). In vitro assays constitute a suitable alternative for characterizing the BH of pure 20- and 22-carbon PUFA, but the available literature is still very scant. Jeyanathan et al. (2016) reported a comprehensive description of in vitro DHA metabolism by *Butyrivibrio proteoclasticus* P18, but the role of *Butyrivibrio* species in ruminal BH is probably less relevant than initially thought (Kim et al., 2008; Huws et al., 2011; Shingfield et al., 2012).

As an analog with BH of 18-carbon PUFA, ruminal EPA and DHA metabolism is considered to involve a first isomerization step that would result in the formation of conjugated 20- and 22-carbon intermediates. Nevertheless, only very recently have minor conjugated 22:6 isomers been detected in digesta (Aldai et al., 2018), whereas other studies pointed to direct saturation as the predominant step in the initial BH of very long chain PUFA (Toral et al., 2010; Kairenius et al., 2011; Jeyanathan et al., 2016). Docosapentaenoic acid (**DPA**; 22:5n-3) might then be a major metabolite of DHA, which could explain the greater apparent transfer of the former from marine lipids into milk (Offer et al., 1999; Loor et al., 2005; Castañeda-Gutiérrez et al., 2007). In line with this, it may also be hypothesized that BH of both PUFA follows common pathways. However, a preliminary overview of GC-flame ionization detector (**GC-FID**) chromatograms from a recent in vitro study about ruminal responses to EPA, DPA, and DHA (Toral et al., 2017) revealed many unique unidentified peaks in each PUFA treatment, challenging that hypothesis. Complementary identifications would then be needed to confirm this finding.

The study by Toral et al. (2017) also demonstrated specificities in the ruminal response of cows and ewes to EPA, DPA, and DHA. Although the reasons for these variations are expected to derive from species differences in rumen microbial composition (Moon et al., 2010; Lee et al., 2012), the influence this may have on the pathways and intermediate products of 20- and 22-carbon PUFA metabolism is as yet unknown.

Our study was therefore conducted to provide further insight into the ruminal BH of very long chain PUFA and to examine potential differences between bovine and ovine. To meet this objective, we investigated the 20- and 22-carbon metabolites accumulated during in vitro incubation of EPA, DPA, and DHA with rumen inocula from cows and ewes.

MATERIALS AND METHODS

In Vitro Experiment and Samplings

All experimental procedures were approved and completed in accordance with the Spanish Royal Decree 53/2013 for the protection of animals used for experimental purposes.

Details of the experimental design and methodology were described in Toral et al. (2017). Briefly, the in vitro trial was conducted in batch cultures following a 2 × 4 factorial arrangement: 2 species (ovine and bovine) and 4 treatments (EPA, DPA, DHA, and a control without FA). In vitro incubations were performed in Hungate tubes using rumen inocula collected (before feeding) from cannulated cows (n = 2) and ewes (n = 2) fed a TMR formulated from alfalfa hay and a concentrate (50:50). They were repeated on 3 d (replicates). The TMR contained (per kg of DM) 187 g of CP, 311 g of NDF and 18 g of total FA, and the offer was fixed at estimated maintenance energy requirements (INRA, 2007) to work under similar conditions in both species. The 3 PUFA [10–2005–9 (EPA), 10–2205–9 (DPA), and 10–2206–9 (DHA); Larodan, Solna, Sweden] were dissolved in ethanol, dispersed with an ultrasonic device, and added at a dose of 2% substrate (the TMR fed to the animals) just before the incubation started. The control treatment was dosed only the corresponding amount of ethanol. Each tube contained 120 mg of DM of the TMR, which was incubated under anaerobic conditions at 39.5°C with 12 mL of a mix (1:4) of strained rumen fluid and artificial saliva, as detailed in Toral et al. (2017). After 24 h, the reaction was stopped by placing the vials into ice water for approximately 5 min. Samples were freeze-dried, and stored at −80°C until FA analysis.

FA Analysis

Lipid Extraction and Preparation of FAME. The lipids in ruminal digesta were extracted directly in the Hungate tubes, which contained approximately 200 mg of freeze-dried in vitro residue. The extraction procedure was repeated twice using 4 mL of a mixture (3:2, vol/vol) of hexane and isopropanol following the adjustment of digesta pH to 2.0 using 2 *M* hydrochloric acid (Shingfield et al., 2003). Organic extracts were combined and dried under nitrogen at 45°C. Lipid dis-

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