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Rumen ciliate protozoa contain high concentrations of conjugated linoleic acids and vaccenic acid, yet do not hydrogenate linoleic acid or desaturate stearic acid

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Conjugated linoleic acids (CLA) have been shown to improve human health. They are derived from the microbial conversion of dietary linoleic acid (*cis*-9,*cis*-12-18 : 2 (LA)) in the rumen. An investigation was undertaken to determine the role of ruminal ciliate protozoa *v.* bacteria in the formation of CLA and its precursor in animal tissues, vaccenic acid (*trans*-11-18 : 1 (VA)). Mixed protozoa from the sheep rumen contained at least two to three times more unsaturated fatty acids, including CLA and VA, than bacteria. Different species had different composition, with larger fibrolytic species such as *Epidinium ecaudatum caudatum* containing more than ten times more CLA and VA than some small species, including *Entodinium nanellum*. In incubations with ruminal microbial fractions (bacterial fraction (BAC), protozoal fraction (PRO)), LA metabolism was very similar in strained ruminal fluid (SRF) and in the BAC, while the PRO had LA-metabolising activity an order of magnitude lower. Using PCR-based methods, no genes homologous to fatty acid desaturase genes were found in cDNA libraries from ruminal protozoa. The absence of an alternative route of VA/CLA formation *via* desaturation of stearate was confirmed by incubations of SRF, BAC or PRO with [^{14}C]stearate. Thus, although protozoa are rich in CLA and VA, they appear to lack the ability to form these two fatty acids from LA or stearate. The most likely explanation is that protozoa preferentially incorporate CLA and VA formed by bacteria. The implication of the present findings is that the flow of unsaturated fatty acids, including CLA and VA, from the rumen could depend on the flow of protozoa rather than bacteria.

Biohydrogenation: Rumen protozoa: Conjugated linoleic acids: *Trans* fatty acids

Conjugated linoleic acids (CLA) is a collective term used to describe positional and geometric isomers of linoleic acid (*cis*-9,*cis*-12-18 : 2 (LA)). Animal studies and clinical trials indicate that CLA could be useful in improving human health in a number of areas, such as preventing carcinogenesis and atherosclerosis, controlling body fat gain and enhancing immunity while also decreasing inflammation (Belury, 2002; Pariza, 2004). Ritzenthaler *et al.* (2001) reported that beef and dairy products are the predominant sources of CLA in the human diet. CLA found in milk and beef originate from ruminal isomerisation of dietary LA. Only a small fraction of the CLA comes directly from ruminal CLA. Most CLA are in fact produced in the animal tissues by desaturation of vaccenic acid (*trans*-11-18 : 1 (VA)), which is a major product of the conversion of CLA in the rumen (Griinari *et al.* 2000).

It is usually considered that most of the biohydrogenation of LA occurring in the rumen is carried out by bacteria, predominantly belonging to *Butyrivibrio fibrisolvens* and related species (Polan *et al.* 1964; Harfoot & Hazlewood, 1997; van de Vossenberg & Joblin, 2003). Early studies by Wright (1959, 1960) concluded that both protozoa and bacteria were involved, but the extensive ingestion of bacteria by protozoa was considered by others (Dawson & Kemp, 1969) to cast doubt on this conclusion. Biohydrogenation in ruminal digesta was only slightly decreased following defaunation and the presence of protozoa was not necessary for biohydrogenation to occur (Dawson & Kemp, 1969). Girard & Hawke (1978) and Singh & Hawke (1979) also suggested that the small contribution of protozoa to the biohydrogenation process was due to the activity of ingested or associated bacteria. It has been known for a long time that protozoal lipids contain proportionally more unsaturated fatty acids than the bacterial lipids (Katz & Keeney, 1966; Harfoot & Hazlewood, 1997). However, these measurements were made before the significance of CLA and VA was understood, and the analyses do not tell us about CLA and VA concentrations. As up to half of the rumen microbial biomass may be protozoa (Williams & Coleman, 1992) and about 75 % of the microbial fatty acids present in the rumen may be of protozoal origin (Keeney, 1970), protozoa could represent a very important source of CLA and VA. The objectives of the present study were to compare the fatty acid composition of bacteria and protozoa, especially

Abbreviations: BAC, bacterial fraction; CLA, conjugated linoleic acids; LA, linoleic acid; NEFA, non-esterified fatty acids; PRO, protozoal fraction; SRF, strained ruminal fluid; VA, vaccenic acid (*trans*-11-18 : 1).

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their respective CLA and VA compositions, and to establish the role of ruminal protozoa, both mixed and individual species, in the biosynthesis of CLA and VA in the rumen.

Materials and methods

Animals and diets

All sheep used in these experiments were male, adult, with a body weight of around 70–80 kg. The animals were ruminally fistulated and all procedures had Home Office approval. Four normally faunated sheep received a mixed diet comprising grass hay, barley, molasses, soyabean meal, and minerals and vitamins at 300, 420, 100, 170 and 10 g/kg DM, respectively. Four defaunated sheep were obtained after complete removal of protozoa from the rumen of conventional sheep by a technique based on rumen emptying and successive washing (Jouany & Senaud, 1979). Monofaunated sheep, harbouring only one species of protozoa, were obtained by inoculating the rumen of defaunated sheep with single species of protozoa. The protozoal species *Isotricha prostoma* and *Epidinium ecaudatum caudatum* were provided by Dr Jean-Pierre Jouany (Institut National de la Recherche Agronomique, Clermont-Ferrand/Theix, France), the protozoal species *Entodinium nanellum* and *Entodinium furca monolobum* were provided by Dr Svetlana Kisidayova (Institute of Animal Physiology, Slovak Academy of Sciences, Kosice, Slovak Republic) and the species *Entodinium caudatum*, *Diplodinium denticulatum*, *Diploplastron affine* and *Ophryoscolex caudatus* were provided by Dr Tadeusz Michalowski (Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Jablonna, Poland). The sheep remained monofaunated for at least 1 year prior to the experiment. Monofaunated and defaunated sheep received a mixed diet comprising grass hay, barley, molasses, soyabean meal, and minerals and vitamins at 500, 300, 100, 90 and 10 g/kg DM, respectively.

Ruminal digesta samples were taken via the ruminal fistula 2 h after feeding unless specified in the experimental description. Ruminal digesta and its fractions were kept at 39°C and under anaerobic conditions until further use. Four animals were used per individual treatment except for the experiment with monofaunated sheep, where two animals per protozoal species were used.

Preparation of strained ruminal fluid, and bacterial and protozoal fractions

Strained ruminal fluid (SRF) was obtained after straining whole ruminal digesta through two layers of muslin in order to remove large (>2 mm) particles, and to allow bacteria attached to small feed particles and protozoa to pass through the filtrate. Ruminal fluid was also fractionated to bacterial and protozoal fractions. SRF was centrifuged at 500***g*** for 10 min to remove protozoa and large particles. Bacteria were recovered in the supernatant (bacterial fraction (BAC)), and protozoal fraction (PRO) was prepared using a sedimentation procedure, as previously described by Williams & Coleman (1992). Briefly, SRF (300 ml) was diluted with 300 ml Coleman's salts solution D (anaerobic, 39°C) and 1 g glucose/l was added (Coleman, 1992). The mixture was transferred to a separating funnel and incubated at 39°C for 30 min to allow the protozoa to settle at the bottom of the funnel. The settled material was passed through a 10 μm nylon filter, and the retained cells were washed with Coleman's salts solution D until the filtrate was clear. The retained cells were then resuspended in a small volume of Coleman's salts solution D and were used as the PRO.

Incubation of unlabelled linoleic acid with ruminal fluid in vitro

Incubations of SRF with LA were carried out as follows. SRF from conventional or defaunated sheep (5–15 ml, depending on the experiment) was aliquoted in a tube maintained under CO_2 and at 39°C. At time 0 of the incubation, 100 μl of a solution of LA (Sigma, Poole, UK) in ethanol was added to the ruminal fluid, in order to obtain a final concentration of LA of 500 or 1000 μg/ml, depending on the experiment. Immediately, an aliquot (1–5 ml) was removed and added to an equal volume of 0·5 M-H_3PO_4 to prevent further metabolism, and stored at −20°C under CO_2 until further use. The remaining suspension was kept under CO_2 and at 39°C, samples being collected at different times between 1 and 24 h. Three tubes were sampled at each sampling time for each sheep, and the results are mean values from three sheep. Incubations of the different microbial fractions were carried out with slight modifications compared to that described for SRF. After preparation of the SRF and the separation of the BAC and the PRO, protein was assayed and the concentration was adjusted to equal values for all by adding Coleman's salts solution D. Aliquots of the different fractions were then treated in the same manner described earlier for the incubations with SRF.

Incubations of labelled [^{14}C]*stearic acid with ruminal digesta fractions* in vitro

After adjusting protein concentrations in SRF, BAC and PRO, aliquots (5 ml) of the different fractions were transferred to tubes maintained under CO_2 and at 39°C. At time 0, 100 μl of a solution of [^{14}C]stearic acid (0·5 μg/ml, 4000 Bq/ml; Amersham Biosciences, Little Chalfont, UK) were added. Immediately, a 1 ml aliquot was removed and added to 1 ml 0·5 M-H_3PO_4, then stored at −20°C under CO_2. The remainder of the suspension was kept under CO_2 and at 39°C, and samples were collected after 6 h. Duplicate tubes were sampled at each time, and the experiment was carried out using digesta from three sheep.

Fatty acid extractions

Microbial lipids were analysed for both total and unesterified fatty acids. Extraction of total fatty acids was based on a method described by Folch *et al.* (1957). Sample (1 ml) was added to 0·67 ml 5 M-NaOH and 200 μg C19 : 0/ml (100 μl) as a first internal standard and 50 μl saturated methyl orange (as pH indicator), flushed with N_2 and incubated at 85°C for 30 min. Samples were allowed to cool then 0·67 ml 5·1 M-HCl was added. The pH indicator changed colour from orange to red, indicating a pH < 2. As a second internal standard 200 μg C17 : 0/ml (100 μl) was added, followed by 2·5 ml methanol. The mixture was vortexed for 1 min, then 2·5 ml

chloroform with added 0·2 mg butylated hydroxytoluene/ml was added and the mixture was vortexed again for 2 min. The upper layer was removed by aspiration. The lower layer was dried by passing through an anhydrous sodium sulphate column and solvent was evaporated in a centrifugal evaporator (Savant AES2010; Thermo Electron Corporation, Basingstoke, UK).

Unesterified fatty acids were obtained by solid-phase extraction using Varian Mega Bond Elut 500 mg aminopropyl cartridges (Crawford Scientific, Strathaven, UK). Standard 200 μg C19 : 0/ml (100 μl) was added to 1 ml PRO or BAC suspension in triplicate and the samples were then freeze dried. Dried samples were then resuspended in 1 ml methanol and vortexed for 1 min. Total fatty acids were extracted by the method described by Folch *et al.* (1957). To the organic phase containing the fatty acids, 2·25 ml 0·88 % KCl was added, vortexed for 1 min and the upper aqueous phase removed by aspiration and discarded. To the bottom phase 2·25 ml methanol–0·88 % KCl (50:50, v/v) was added. The mixture was vortexed for 1 min, the upper phase removed by aspiration and discarded, and the bottom phase was dried at 50 °C under N_2. The dried sample was resuspended in 0·25 ml chloroform before passing through the bonded phase columns according to Kaluzny *et al.* (1985). Non-esterified fatty acids (NEFA) were eluted using 4 ml acetic acid–diethyl ether (2:98, v/v) and the eluant was dried down at 50°C under N_2. Dried samples were resuspended in 1 ml water and fatty acids were extracted, as described earlier.

Fatty acid analysis

Derivatisation of the extracted fatty acids to methyl esters was required to analyse the samples by GC. This was carried out using a procedure that contained a short, mild esterification step that minimised isomerisation of CLA (Wasowska *et al.* 2006). The dried extract was resuspended in 0·5 ml toluene, the suspension was vortexed, then 1 ml H_2SO_4–methanol (1 %, v/v, concentrated H_2SO_4 in methanol) was added. As a third internal standard to monitor the recovery through the derivatisation procedure, 200 μg C15 : 0/ml (100 μl) was added. The tube was flushed with N_2 then closed with a glass stopper and incubated at 50°C for 1 h. Thereafter, the tube was cooled, opened, 2·5 ml 5 % (w/v) NaCl was added, the tube was vortexed, then 1 ml of isohexane was added and the tube was vortexed again. When layers had formed, sometimes aided by brief centrifugation, the upper layer was transferred to a fresh tube and the isohexane extraction was repeated twice on the lower phase. The isohexane fractions were pooled and 1·5 ml 2 % $KHCO_3$ was added. The mixture was vortexed and allowed to settle, once again aided by brief centrifugation if required. The upper layer was removed, and the lower layer was evaporated and resuspended in 0·2 ml isohexane/butylated hydroxytoluene, then transferred to a GC vial.

The gas chromatograph was an Agilent 6890 instrument (Agilent Technologies UK, Stockport, UK) equipped with a Varian CP Sil88 column, 50 m × 0·25 mm with a film thickness of 0·2 mm (Varian Analytical Instruments, Walton-on-Thames, UK). The temperature programme was as follows: 80°C for 1 min; increased at 25°C/min to 160°C which was held for 3 min; increased at 1°C/min to 190°C, maintained for 5 min; increased at 2°C/min to 230°C, maintained for 25 min. The carrier gas was helium and the column was operated at constant pressure (20 psi) with a flow rate of 0·5 ml/min. A 15:1 split injection mode was used and the injection volume was typically 1 μl. Injector and detector temperatures were maintained at 250 and 275°C, respectively. Peaks were routinely identified by comparison of retention times with authentic FAME standards obtained from Sigma and Matreya Inc. (Pleasant Gap, PA, USA). Fatty acids were quantified using C19 : 0 as the standard.

Thin-layer chromatography

TLC was used to analyse the metabolism of ^{14}C-labelled fatty acids after extraction of total fatty acids from the samples. TLC plates were Silica Gel 60 (20 cm × 20 cm × 250 μm) purchased from Merck (Darmstadt, Germany). The plates were sprayed with a solution of silver nitrate (5 % in 80 % methanol) and dried at 110°C for 30 min. Samples of extracted total fatty acids (20 μl) and labelled standards ([^{14}C]stearic acid and [^{14}C]LA, 1111 Bq/ml, 15 μl) were spotted on the plate. The solvent used for TLC was *n*-hexane–diethyl ether–acetic acid (90:9:3, v/v/v). After a first migration, the plates were dried and placed in the solvent for a second migration, improving the resolution of the fatty acid separation.

To visualise the ^{14}C-labelled fatty acids separated by TLC, the plates were exposed overnight to a Fuji IP plate (Raytek, Sheffield, UK). The IP plate was then analysed using a scanner (Fuji scanner FLA-3000; Raytek) and the image analysis was carried out using the software AIDA (Raytek).

Protein assay

Protein was measured by alkaline hydrolysis of samples followed by reaction with the Folin-Ciocalteu reagent (Herbert *et al.* 1971), using bovine serum albumin as a standard.

PCR amplification of desaturase gene

cDNA libraries previously constructed from different ruminal protozoal species (*Polyplastron multivesiculatum*, *Isotricha prostoma*, *Eudiplodinium maggii* and *Epidinium ecaudatum caudatum*) and described by Ricard *et al.* (2006) were screened for the presence of genes encoding desaturases. Whole library extracts were used as a template for PCR amplification. The screening for desaturase genes was based on the use of degenerate primers targeting conserved histidine boxes (Wongwathanarat *et al.* 1999). Two sets of primers were used to amplify the two distinct conserved sequences of desaturases, namely the DesFor/DesRev pair and the P3/P4 pair (Wongwathanarat *et al.* 1999). The conditions used for the PCR were as follows: an aliquot of cDNA library (1 μl) was added to 20 pmol each of forward and reverse primers, 0·2 mM-dNTPs, 1·5 mM-$MgCl_2$, 5 units Taq Polymerase (Promega, Southampton, UK) and the supplier's reaction buffer in a final volume of 50 μl. The PCR was initiated with a denaturation step (94°C for 5 min), followed by thirty cycles consisting of sequential denaturation (94°C for 30 s), annealing (46°C for 1 min 30 s) and elongation (72°C for 1 min), followed by a final elongation step (72°C for 10 min). Amplicons were analysed by

electrophoresis on a 1·0 % agarose gel. Plasmid DNA (pBK-CMV:Ma-*ole2*) containing the *Mortierella alpina ole2* fatty acid Δ^9-desaturase gene was kindly supplied by Dr Donald MacKenzie (Institute of Food Research, Norwich, UK) as a positive control. Primers EukFor and EukRev, targeting sequences of 18S rRNA genes were the positive control set of the PCR reaction (van Hoek *et al.* 1998).

Statistical analysis

Fatty acid composition of ruminal protozoa and bacteria was analysed by two-way ANOVA (factors time and microbial population) with a random factor for sheep. When a normal distribution could not be assumed, a Mann–Whitney test was used to assess the effect of microbial population.

Fatty acid metabolism experiments were replicated for two, three or four animals, the samples being analysed in triplicate. The results are given as means with their standard errors. Differences between means were compared using paired *t*-tests.

Results

Fatty acid composition of ruminal protozoa and bacteria

Ruminal digesta from four different sheep was collected 1 h before feeding and 6 h after feeding. SRF was fractionated into BAC and PRO for the determination of their respective total fatty acid compositions (Fig. 1). The average total fatty acid contents of the BAC and PRO were very similar, 50·0 (SE 1·2) and 52·2 (SE 1·5) μg fatty acids/mg protein, respectively. However, the PRO contained a much higher proportion ($P<0·001$) of unsaturated fatty acids than mixed ruminal bacteria. This was true for both total fatty acids and the NEFA fractions. The average proportion of C18 unsaturated fatty acids was 42 % of the fatty acids in the PRO, with a range of 40·8–43·3, whereas this percentage was 9·3–12·7 in the BAC. Protozoa contained a higher ($P<0·001$) concentration of MUFA, mainly oleic acid (*cis*-9-18 : 1) and VA, than the bacteria (Fig. 1(a)). Indeed, the percentage of oleic acid and VA measured in the total fatty acids of protozoa was three to four times higher than in bacteria (Fig. 1(a)). Ruminal protozoa were rich in LA and also in CLA compared to bacteria (Fig. 1(b)). The two major isomers of CLA detected in the PRO were *cis*-9,*trans*-11-18 : 2 and *trans*-9,*trans*-11-18 : 2. In contrast, no CLA were detectable in the rumen BAC (Fig. 1(b)). Linolenic acid (C18 : 3) was only detected in the PRO. The time of sampling had no effect on the fatty acid composition, including CLA and VA, of the PRO or BAC (Fig. 1).

The fatty acid composition of different species of protozoa was determined using PRO prepared from monofaunated sheep, each containing one of *Isotricha prostoma*, *Diploplastron affine*, *Ophryoscolex caudatus*, *Epidinium ecaudatum caudatum*, *Entodinium caudatum*, *Diplodinium denticulatum*, *Entodinium furca monolobum* and *Entodinium nannellum*. The proportion of unsaturated fatty acids in the total fatty acids varied from 28·7 to 48·8 %, with differences between species particularly noticeable for the percentages of CLA and VA (Fig. 2). All species contained VA, but CLA were undetectable in *I. prostoma*, *E. nanellum* and *E. furca monolobum*. The presence of CLA was generally accompanied by a high percentage of VA (Fig. 2). The CLA + VA content of the protozoal lipids was lower in small species in comparison to larger species, except for *I. prostoma* (Fig. 2).

Role of ruminal protozoa in the biohydrogenation of linoleic acid: faunated and defaunated sheep

The metabolism of LA by ruminal microorganisms was compared in SRF from three conventional sheep and three defaunated sheep. When the rate of LA metabolism was compared between ruminal samples from conventional sheep or from defaunated animals, no difference was observed, with activities of 17·3 and 14·9 μg LA metabolised/h per mg protein, respectively. The fatty acids produced were the same for the two groups, with CLA and C18 : 1 (mainly VA) being the major metabolites formed (Fig. 3). The accumulation of the intermediate CLA was higher in samples from defaunated sheep than in samples from conventional sheep. This difference was significant ($P<0·05$) at 8 and 20 h, and was particularly noticeable after 8 h of incubation, where the concentration of CLA in SRF from defaunated sheep was five times higher than the concentration in SRF from conventional sheep (Fig. 3). However, the rate of formation of C18 : 1 was similar in the two types of sample (Fig. 3).

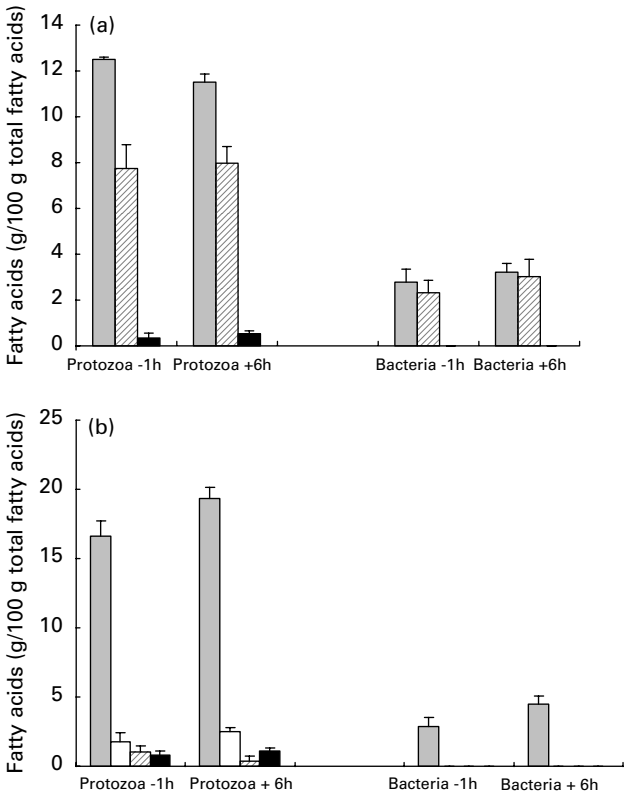

Fig. 1. Composition of the main unsaturated C18 fatty acids of mixed ruminal protozoa and bacteria isolated from the rumen of sheep 1 h before feeding and 6 h after feeding. For details of procedures, see p. 698. (a), C18 MUFA: *cis*-9-18 : 1 (▒), *trans*-11-18 : 1 (▨), *cis*-11-18 : 1 (■); (b), C18 PUFA: *cis*-9,*cis*-12-18 : 2 (▒), *cis*-9,*trans*-11-18 : 2 (□), other conjugated linoleic acids (▨), C18 : 3 (■). Values are means with their standard errors depicted by vertical bars (n 4). Each sample was analysed in triplicate.

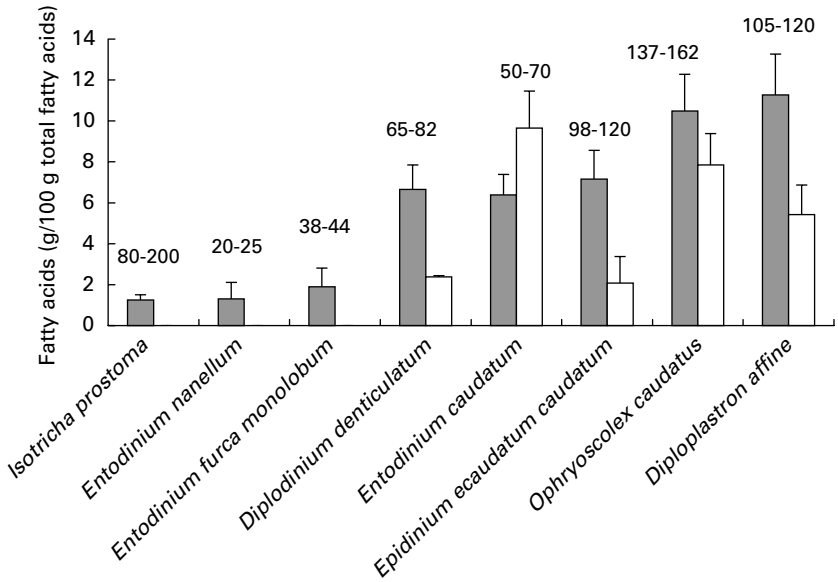

Fig. 2. Concentration of vaccenic acid (▩) and conjugated linoleic acids (□) in different species of ruminal protozoa isolated from the rumen of monofaunated sheep, 2 h after feeding. Values are means, with their standard errors depicted by vertical bars, of triplicate analyses from single monofaunated sheep. The numbers above the columns are the length range of the protozoal cell (μm), as described by Williams & Coleman (1992), for that protozoal species.

Role of ruminal protozoa in the biohydrogenation of linoleic acid: activities of different microbial fractions

Ruminal digesta from three conventional sheep was collected and fractionated into SRF, BAC and PRO. The fractions were incubated *in vitro* with LA and fatty acids were analysed at different incubation times. LA was metabolised at the same rate in SRF and BAC, at 18·2 (SE 3·2) and 17·5 (SE 2·1) μg LA metabolised/h per mg protein, respectively. In the PRO, the rate of LA metabolised was much lower ($P=0{\cdot}012$), at 2·5 μg (SE 0·9) LA metabolised/h per mg protein. The main products of LA metabolism were VA and CLA. The BAC showed very similar activity to SRF, with similar concentrations of VA and CLA formed. These concentrations were much higher ($P=0{\cdot}028$) than those observed with the PRO (Fig. 4). For example, the concentration of VA formed at the end of the incubation (after 24 h incubation) was 50·2, 55·1 and 5·0 μg/mg protein in SRF, BAC and PRO, respectively. No CLA was detectable during the incubation of the PRO with LA, whereas at least 33 μg CLA/mg protein was produced in SRF and the BAC.

Desaturation of stearic acid by ruminal micro-organisms

An alternative possible route for the formation of CLA and VA is the desaturation of stearic acid. Evidence for this reaction was sought by both genetic and metabolic experiments.

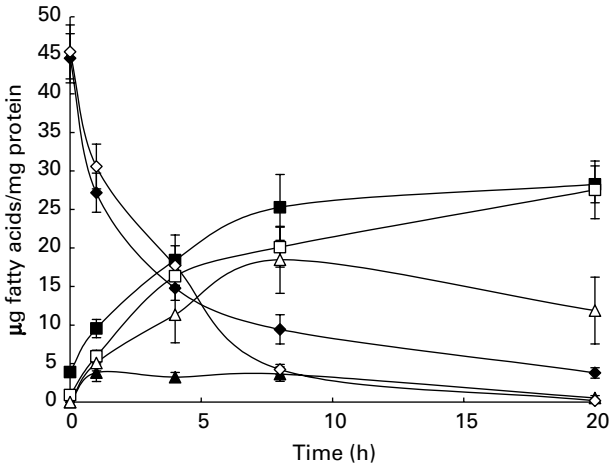

Fig. 3. Metabolism of linoleic acid (◆,◇) and formation of CLA (▲,△) and C18 : 1 fatty acids (■,□) in strained ruminal fluid from conventional (◆,▲,■) or defaunated (◇,△,□) sheep. For details of procedures, see p. 698. Values are means with their standard errors depicted by vertical bars (*n* 3).

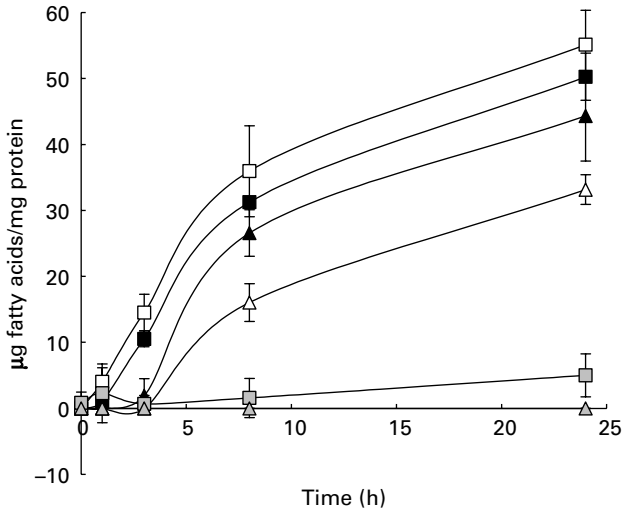

Fig. 4. Formation of conjugated linoleic acids (▲,△,◬) and vaccenic acid (■,□,▩) in strained ruminal fluid (▲,■), in the bacterial fraction (△,□) and in the protozoal fraction (◬,▩) incubated with linoleic acid. For details of procedures, see p. 698. Values are means with their standard errors depicted by vertical bars (*n* 3).

Using the conditions described earlier, degenerate primers, designed to target highly conserved regions of fatty acid desaturase genes, failed to amplify DNA in PCR reactions using cDNA libraries prepared from *E. ecaudatum caudatum*, *Eudiplodinium maggii*, *Isotricha prostoma* and *Polyplastron multivesiculatum*, whereas these conditions allowed amplification of gene fragments using the positive control *M. alpina* (results not shown). The quality of the DNA was confirmed to be satisfactory by the successful amplification of 18S rRNA gene sequences.

In metabolic experiments similar to those carried out before, SRF, PRO and BAC from three conventional sheep were incubated with [^{14}C]stearic acid for 6 h. Fatty acids were extracted and analysed by TLC. No label was found in positions corresponding to CLA or VA in any of the incubations, indeed all of the ^{14}C appeared to remain in the stearic acid spot (Fig. 5).

Discussion

It has been known for a long time that ruminal protozoa contain proportionally more unsaturated fatty acids than ruminal bacteria (Viviani, 1970; Emmanuel, 1974; Harfoot, 1978). The present study is consistent with these findings, in that unsaturated C18 fatty acids represented 42 % of the total fatty acids extracted from mixed protozoa, compared to 11 % from bacteria. What was not clear from the earlier analyses was whether these unsaturated fatty acids would include CLA, which was discovered more recently to have specific health implications, and its precursor in animal tissues, VA. The unsaturated fatty acids measured in protozoal lipids could have been derived directly from the feed. In contrast, CLA and VA are formed by microbial activity in the rumen and are not found in the feed. The present study has shown that CLA and VA, like unsaturated fatty acids in general, are present in much higher proportions in protozoa than in bacteria. Indeed, CLA were not detected in the samples of mixed bacteria, but reached 5 % of the total fatty acids in protozoal cells. The CLA and VA composition was not the same in different protozoal species, as determined from the analysis of protozoa isolated from monofaunated sheep. This appears to be the first time that differences in fatty acid composition have been found in different species of ruminal ciliate protozoa. For reasons that are unclear, the CLA and VA concentration in protozoal lipids corresponded roughly to the size of the protozoal cells, with the exception of *I. prostoma*. Ruminal protozoa comprise two main orders, *Entodiniomorphida* and *Vestibuliferida*, known trivially as entodiniomorphs and holotrichs (Williams & Coleman, 1992). *I. prostoma* was the only holotrich investigated here. In spite of its large size and generally high content of unsaturated fatty acids, similar to the entodiniomorphs (Harfoot, 1978), it had low VA and undetectable CLA concentrations.

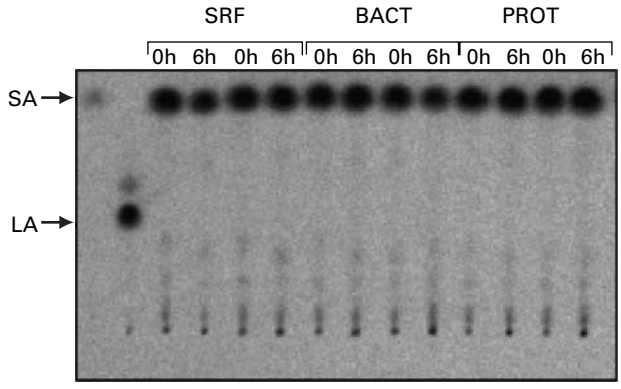

Fig. 5. Stearic acid (SA) metabolism in strained ruminal fluid (SRF), in the bacterial fraction (BAC) and in the protozoal fraction (PRO) from ruminal digesta. Fatty acids were extracted after 0 and 6 h of incubation with [^{14}C]SA, and analysed by TLC, with [^{14}C]SA and [^{14}C]linoleic acid (LA) as standards. The results are from duplicate samples from one sheep. Identical results were obtained with samples from two other sheep.

What, therefore, is the role of protozoa *vis-à-vis* bacteria in CLA and VA synthesis? SRF from defaunated sheep metabolised LA in a similar way to conventional sheep. Although in the present experiment the conventional and defaunated sheep were not matched perfectly because their diets were slightly different, the present result is entirely consistent with other studies of defaunated sheep (Dawson & Kemp, 1969). Defaunation experiments do not, however, tell us that protozoa have no role in LA metabolism or CLA/VA formation, because the bacterial community changes in response to defaunation (Williams & Coleman, 1992). Measurements made with BAC and PRO prepared from ruminal digesta provided better evidence that protozoa do not biohydrogenate LA. Even though a low activity was detected after a long incubation (>20 h), this is most likely explained by the presence of some bacteria still present intracellularly in the protozoal preparation. Furthermore, CLA formation actually increased when protozoa were removed, consistent with the observations of Jouany & Lassalas (2003). Incubations of mixed protozoa with LA in the presence of antibiotics had suggested that protozoa could be important for biohydrogenation but results were variable and inconclusive (Chalupa & Kutches, 1968; Wright, 1959). We conclude, therefore, that it is unlikely that CLA and VA are formed by protozoa via biohydrogenation of LA.

Another possible way for protozoa to form CLA and VA would be to desaturate stearic acid to VA then CLA. Fatty acid desaturases form a very large family of enzymes and some of those desaturases have been shown in primitive eukaryotes, such as protozoa or fungi (Pereira *et al.* 2003; Los & Murata, 1998). Δ^{11}-Desaturase activity has been reported in only a small number of organisms, mostly insects, and more recently in a microalga, *Thalassosira pseudonana* (Tonon *et al.* 2004). The gene sequence of desaturases contains three conserved histidine-rich motifs that were used to design primers which would target desaturase genes in protozoal cDNA libraries. No gene encoding desaturase was identified in any of the four libraries tested. Given the limited relatedness between rumen protozoa and other organisms where desaturase genes have been found, the results of the PCR were not completely conclusive. The possibility remained that the conditions for the PCR reaction were not optimal to amplify desaturase genes in protozoa. Also, it is possible that rumen protozoa desaturate stearic acid using a desaturase encoded by a gene dissimilar to the known desaturase genes. However, this was addressed in incubations of different fractions with [^{14}C]stearate. Labelled 18 : 1 and 18 : 2 products were not detected, further suggesting that VA

and CLA are not formed by desaturation of stearate in mixed ruminal digesta, ciliate protozoa or bacteria.

The mechanism whereby CLA and VA accumulate in the protozoa presents a conundrum, protozoa do not form CLA and VA yet contain high concentrations of these important fatty acids. It is possible that protozoa are simply more efficient in incorporating intermediates of bacterial biohydrogenation than are the bacteria themselves. CLA and VA could be formed in large quantity in the protozoal cells, because of a large volume of LA-containing external liquid exposed to high intracellular concentrations of bacteria. A protozoan can pass a volume of external medium many times its own volume each hour (William & Coleman, 1992). Protozoa are also considerable reservoirs of bacterial biomass, for example, Williams & Coleman (1992) calculated that 31 % of the total volume of *E. caudatum* could be occupied by engulfed bacteria. In the present study the low biohydrogenation in the PRO suggests that the washing procedure had been successful in removing biohydrogenating bacteria. Alternatively, protozoa may cause an inhibition of biohydrogenation in the mixture of bacteria and unsaturated fatty acids that they consume, making the unsaturated fatty acids more available for incorporation.

There could be important nutritional implications from the present findings. Ciliate protozoa, particularly holotrichs, are retained selectively within the rumen by a migration/sequestration mechanism that depends on chemotaxis (Abe *et al.* 1981; Ankrah *et al.* 1990; Martin *et al.* 1999). As a consequence, protozoa biomass reaching the duodenum is proportionally less than would be expected if they were to flow with the rest of the ruminal digesta (Hungate *et al.* 1971; Weller & Pilgrim, 1974). It might be imagined that this selective retention would be detrimental to the flow of CLA and VA into meat and milk. Much depends on the form in which CLA and VA flow from the rumen. If, as it has been reported for unsaturated fatty acids in general (Noble, 1981), CLA and VA flow from the rumen of grazing animals is in the form of NEFA, and these NEFA are not associated with microbial cells, the retention effect of protozoa would have little consequence. On the other hand, if the unsaturated fatty acids were predominantly in protozoa, enhancing protozoal flow would be beneficial. In defaunated sheep, the concentration of saturated fatty acids in the blood remains the same as in conventional sheep, whereas the concentration of C18 : 2 and C18 : 3 tends to increase when the protozoa are removed (Abaza *et al.* 1975; Klopfenstein *et al.* 1966), suggesting that the selective retention of unsaturated fatty acids in protozoa restricts their flow from the rumen. Whether this applies to CLA and VA as well remains to be seen, but a similar consequence might be expected. A further complication, in view of the different fatty acid composition of different protozoal species, could be that not all faunated animals would have similar restrictions, depending on the composition of their fauna.

In conclusion, the analysis of fatty acid composition indicates that most species of protozoa are rich in unsaturated fatty acids, especially CLA and VA. However, they do not form these fatty acids, but seem to play an important role in the uptake/protection of the substrate as well as the intermediates of biohydrogenation. Further experimentation needs to be done in digesta-flow experiments *in vivo* to ascertain the impact of protozoa on the flow of CLA and VA to meat and milk, and hence whether defaunated ruminants might form products with a healthier fatty acids profile than animals with protozoa.

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