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Influence of fish oil on ruminal biohydrogenation of C18 unsaturated fatty acids

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Dietary *cis*-9, *trans*-11-conjugated linoleic acid (CLA) is generally thought to be beneficial for human health. Fish oil added to ruminant diets increases the CLA concentration of milk and meat, an increase thought to arise from alterations in ruminal biohydrogenation of unsaturated fatty acids. To investigate the mechanism for this effect, *in vitro* incubations were carried out with ruminal digesta and the main biohydrogenating ruminal bacterium, *Butyrivibrio fibrisolvens*. Linoleic acid (LA) or α-linolenic acid (LNA) was incubated (1·67 g/l) with strained ruminal digesta from sheep receiving a 50:50 grass hay–concentrate ration. Adding fish oil (up to 4·17 g/l) tended to decrease the initial rate of LA ($P=0·025$) and LNA ($P=0·137$) disappearance, decreased ($P<0·05$) the transient accumulation of conjugated isomers of both fatty acids, and increased ($P<0·05$) the accumulation of *trans*-11-18:1. Concentrations of EPA (20:5*n*-3) or DHA (22:6*n*-3), the major fatty acids in fish oil, were low (100 mg/l or less) after incubation of fish oil with ruminal digesta. Addition of EPA or DHA (50 mg/l) to pure cultures inhibited the growth and isomerase activity of *B. fibrisolvens*, while fish oil had no effect. In contrast, similar concentrations of EPA and DHA had no effect on biohydrogenation of LA by mixed digesta, while the addition of LA prevented metabolism of EPA and DHA. Neither EPA nor DHA was metabolised by *B. fibrisolvens* in pure culture. Thus, fish oil inhibits ruminal biohydrogenation by a mechanism which can be interpreted partly, but not entirely, in terms of its effects on *B. fibrisolvens.*

Biohydrogenation: Conjugated linoleic acid: Fish oil: *Trans* fatty acids

Clinical studies in human subjects have established that EPA (20:5*n*-3) and DHA (22:6*n*-3) exert anti-thrombotic and anti-arrhythmic properties (Williams, 2000). Furthermore, there is an increasing body of evidence that *cis*-9, *trans*-11-18:2 (conjugated linoleic acid; CLA) suppresses chemically induced tumour development in animal models (Parodi, 1999; Kritchevsky, 2000). Ruminant-derived foods make a major contribution to total fat consumption (Givens & Shingfield, 2004) and are the main source of CLA in the human diet (Lawson *et al.* 2001). In light of the potential benefits to long-term human health, there has been increased interest in enhancing the concentrations of potentially beneficial fatty acids in milk and meat. Inclusion of fish oil in the diet of ruminants has been shown to increase the concentrations of *cis*-9, *trans*-11-18:2, EPA and DHA in milk (Offer *et al.* 1999; Donovan *et al.* 2000), beef (Scollan *et al.* 2001*a*) and lamb (Wachira *et al.* 2002). The enrichment of the long-chain *n*-3 fatty acids can be attributed to the incorporation of small amounts of EPA and DHA in fish oil escaping metabolism in the rumen, but the increase in CLA appears to be related to an effect on the biohydrogenating activity and/or microbial community structure in the rumen. Fish oil is known to inhibit the reduction of *trans*-18:1 fatty acids to 18:0 *in vivo* (Wonsil *et al.* 1994; Scollan *et al.* 2001*b*). Studies in dairy cows have shown that fish oil has no effect on the amount of *cis*-9, *trans*-11-18:2 leaving the rumen (Shingfield *et al.* 2003; Loor *et al.* 2005), while Lee *et al.* (2005) reported an increase in steers fed a diet containing sunflower-seed oil. Most of the evidence thus far suggests that the increase in CLA in ruminant tissues and milk when fish oil is fed arises mainly from an increased supply of *trans*-11-18:1 (vaccenic acid; VA; Shingfield *et al.* 2003), which serves as a substrate for endogenous *cis*-9, *trans*-11-18:2 CLA synthesis (Griinari *et al.* 2000).

Several studies have attempted to explore the causes for the inhibitory effects of fish oil on ruminal fatty acid biohydrogenation. Fish oil is hydrolysed in the presence of mixed cultures of ruminal microbes and the non-esterified EPA and DHA released disappear over time, with the extent of metabolism being dependent on the initial concentration of added

Abbreviations: CLA, conjugated linoleic acid; DMOX, 4,4-dimethyloxazoline; FAME, fatty acid methyl ester; LA, linoleic acid; LNA, α-linolenic acid; SRF, strained ruminal fluid; VA, vaccenic acid.

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fish oil (Dohme *et al.* 2003; Chow *et al.* 2004). Further work has also shown that incubation of EPA and DHA causes the accumulation of *trans*-18:1 *in vitro* (AbuGhazaleh & Jenkins, 2004*a*), with VA being the major *trans*-18:1 isomer formed during incubation of DHA (AbuGhazaleh & Jenkins, 2004*b*).

The aim of the present study was to determine the influence of fish oil and its major constituent *n*-3 fatty acids on the biohydrogenation of C18 PUFA in ruminal fluid, coupled with studies of the effect of fish oil on the growth and isomerase activity of *Butyrivibrio fibrisolvens*, which is a key ruminal bacterium involved in the biohydrogenation of fatty acids in the rumen (Polan *et al.* 1964; Harfoot & Hazlewood, 1997; van de Vossenberg & Joblin, 2003). Part of the present study has been previously reported in preliminary form (Wąsowska *et al.* 2004).

Materials and methods

Animals and diets

Seven mature sheep, each fitted with a ruminal cannula, were fed (1 kg DM/d) a mixed diet comprising grass hay (500 g/kg DM), rolled barley (299·5 g/kg DM), molasses (100 g/kg DM), soyabean meal (91·0 g/kg DM) and minerals and vitamins (9·5 g/kg DM) as two equal meals at 08.00 and 16.00 hours. Samples of ruminal digesta were collected from each animal just before the morning feed. Digesta samples were maintained at 39°C and ruminal fluid was obtained by straining through two layers of linen cloth.

Incubations with ruminal fluid in vitro

Strained ruminal fluid (SRF) was incubated either alone or with a combination of fish oil and linoleic acid (LA; *cis*-9, *cis*-12-18:2) or α-linolenic acid (LNA; *cis*-9, *cis*-12, *cis*-15-18:3) to determine interactions between the metabolism of fish oil and the main PUFA in the ruminant diet. In general, 1 ml SRF was added under CO_2 to Pyrex tubes (120 × 11 mm) containing one of the following: 0·2 ml distilled water; 0·1 ml fish oil (50 g/l) + 0·1 ml water; 0·1 ml LA or LNA (20 g/l) + 0·1 ml water; 0·1 ml fish oil and 0·1 ml LA (or LNA). The fish oil (1812 TG; Napro Pharma AS, N-6270 Brattvaag, Norway) contained (% fatty acids) 18 EPA and 12 DHA (certified to contain less than 0·5 % of NEFA, actually containing about 0·1 %). Fish oil was suspended as an oil in water emulsion by sonication and added to incubation tubes according to the predefined concentration. The tubes were incubated under CO_2 at 39°C. Tubes were removed periodically, heated for 10 min in a block heater at 100°C and stored at −20°C before being submitted for determination of NEFA content. Samples of the original SRF were stored at −20°C for later protein analysis.

Similar incubations were carried out with SRF alone or SRF + LA in which the fish oil was replaced by 0·1 ml EPA (1·2 g/l) or DHA (0·6 g/l) or a mixture of the two. Lower concentrations of fish oil were examined for their effects on LA metabolism by decreasing the concentration of fish oil in the added solution to 30 and 15 g/l and maintaining the addition of 0·1 ml LA (20 g/l).

Influence of fatty acids on the growth and linoleic acid isomerase activity of Butyrivibrio fibrisolvens

B. fibrisolvens JW11 was originally isolated as a proteolytic species from the ovine rumen (Wallace & Brammall, 1985) and is held in the culture collection at the Rowett Research Institute. *B. fibrisolvens* JW11 was grown anaerobically at 39°C in M2 medium (Hobson, 1969) in 12·5 × 1·5 cm culture tubes closed with screw caps fitted with butyl rubber septa (Bellco Biotechnology, Vineland, NJ, USA). Fatty acids were prepared by sonicating for 4 min in water and adding to the medium before autoclaving to a concentration of 50 mg/l. Fish oil was added in the same way, up to a final concentration of 10 g/l. Growth was measured from the increase in optical density at 650 nm using a Novaspec II spectrophotometer (Amersham Pharmacia Biotech, St Albans, Herts, UK), from triplicate cultures, for incubation times up to 72 h.

LA isomerase activity was measured by a method derived from Kepler & Tove (1967). *B. fibrisolvens* JW11 was grown as before, cells were harvested by centrifugation (5000 ***g***, 10 min, 4°C), washed twice with 0·1 M-potassium phosphate buffer (pH 7·0), and re-suspended in one-fifth of the original volume. Whole-cell suspensions were stored at −20°C before use. Cell-free extracts were prepared by sonication of washed whole cells at 30 μm amplitude (MSE Soniprep 150; MSE UK, Beckenham, Kent, UK) for a total sonication time of 3 min on ice, in 60 s bursts with 90 s cooling intervals. Cell debris and intact cells were removed by centrifugation (10 000 ***g***, 4°C, 15 min). The assay, based on that developed by Kepler & Tove (1967), was carried out in a plate reader set to 233 nm and 39°C (Spectramax 190; Molecular Devices Ltd, Wokingham, Berks, UK). Each well contained 0·05 ml sample (diluted to contain 0·3–0·6 g protein/l in 0·1 M-potassium phosphate buffer, pH 7·0), 0·05 ml water or inhibitor and 0·10 ml reagent or control mixture. The reagent mixture was prepared by sonicating 5 mg LA in 5 ml 1,3-propanediol until the solution was optically clear, then mixing the sonicated solution with a further 10 ml propanediol and 55 ml 0·1 M-potassium phosphate buffer (pH 7·0). The control mixture was the same, with the exception that no LA was added. Formation of total CLA was calculated using an extinction coefficient of $2{\cdot}4 \times 10^4$/M per cm (Kepler & Tove, 1967), and activity was calculated from the rate of increase in A_{233} over the first 10 min. Fish oil, EPA and DHA were added as stable emulsions in water prepared by sonication.

Fatty acid extraction

Fatty acids were extracted and converted to methyl esters using procedures based on those described by Christie (2003). Samples of whole heated SRF suspension (1·2 ml) or pure culture (1·0 ml) were mixed with 1·25 ml acidified salt solution (17 mM-NaCl in 1 mM-H_2SO_4). We added 100 μl heptadecanoic acid in methanol (200 μg/ml) as an internal standard, followed by 2·5 ml methanol. The mixture was vortexed for 1 min, then 2·5 ml chloroform containing 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 anhydrous sodium sulfate, and solvent was removed by evaporation in a centrifugal

evaporator for 1 h at 43°C (Savant AES2010; Thermo Electron Corporation, Basingstoke, Hants, UK).

Extracted fatty acids were converted to fatty acid methyl esters (FAME) using a short, mild acid-catalysed esterification to minimise isomerisation of unsaturated fatty acids. Dried lipid extracts were re-suspended in 0·5 ml toluene, the suspension was vortexed, followed by the addition of methanolic H_2SO_4 (1 %, v/v, H_2SO_4 in methanol). We added 100 μl C15:0 in isohexane (200 μg/ml) as a second internal standard to account for potential lipid losses during esterification. 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 were added, the tube was vortexed for 30 s, then 1 ml 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. Organic fractions were combined and 1·5 ml 2 % (w/v) $KHCO_3$ were added. The mixture was vortexed for 30 s and allowed to settle, once again aided by brief centrifugation if required. The upper layer was removed, dried in a centrifugal evaporator as before and re-suspended in 0·2 ml of the isohexane–butylated–hydroxytoluene solvent and transferred to a GC vial.

Solid-phase extraction of non-esterified fatty acids

Concentrations of non-esterified EPA and DHA after 24 h incubation of fish oil (4·17 g/l) with SRF were determined using solid-phase extraction. A parallel incubation was done with 0·1 M-sodium phosphate buffer (pH 7·0). Extraction of lipid in boiled suspensions following 24 h incubations was carried out by the Folch *et al.* (1957) procedure. The dried lipid extract was fractionated according to Kaluzny *et al.* (1985) using 500 mg Bond Elut aminopropyl columns (Crawford Scientific, Strathaven, Lanarkshire, UK). Columns were conditioned by placing in a vacuum filtration device and rinsed with two 2 ml samples of hexane, and the dried lipid fraction was dissolved in 0·5 ml chloroform and applied on to the column. A vacuum was applied, then the tube was rinsed with another 0·5 ml sample of chloroform and the washings were applied to the column, at which time the vacuum was applied again. Neutral lipids were removed from the column by washing with 4 ml of a mixture of chloroform and propan-2-ol (2:1, v/v). The NEFA were obtained by eluting the column with 4 ml of a mixture of acetic acid and diethyl ether (2:98, v/v). Column effluents containing NEFA were dried by centrifugal evaporation and converted to methyl esters using 1 % H_2SO_4 in methanol and analysed by GC.

Fatty acid methyl ester analysis

FAME were separated and quantified using a gas chromatograph (model 6890; Agilent Technologies UK Ltd, Stockport, UK) equipped with a flame-ionisation detector, quadrupole mass selective detector (model 5973N), injection port and a 100 m fused silica capillary column (internal diameter 0·25 mm) coated with 0·2 μm film of cyanopropyl polysiloxane (CP-SIL 88; Varian Analytical Instruments, Walton-on-Thames, Surrey, UK). Total FAME profile in a 1 μl sample at a split ratio of 15:1 was determined using a temperature gradient programme (initial temperature 80°C for 1 min; increased at a rate of 25°C/min to 160°C, which was held for 3 min; increased at a rate of 1°C/min to 190°C, maintained for 5 min; increased at a rate of 2°C/min to 230°C, held for 25 min) and He as the carrier gas operated at constant pressure (20 pounds per square inch) and flow rate of 0·5 ml/min. 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 (Poole, Dorset, UK) and Matreya Inc. (Pleasant Gap, PA, USA). Identification was validated based on electron impact ionisation spectra of FAME obtained under an ionisation voltage of 1640 eV, and compared with authentic standards and an on-line reference mass spectra library (http://www.lipidlibrary.co.uk/masspec.html).

Structural analysis of fatty acid intermediates

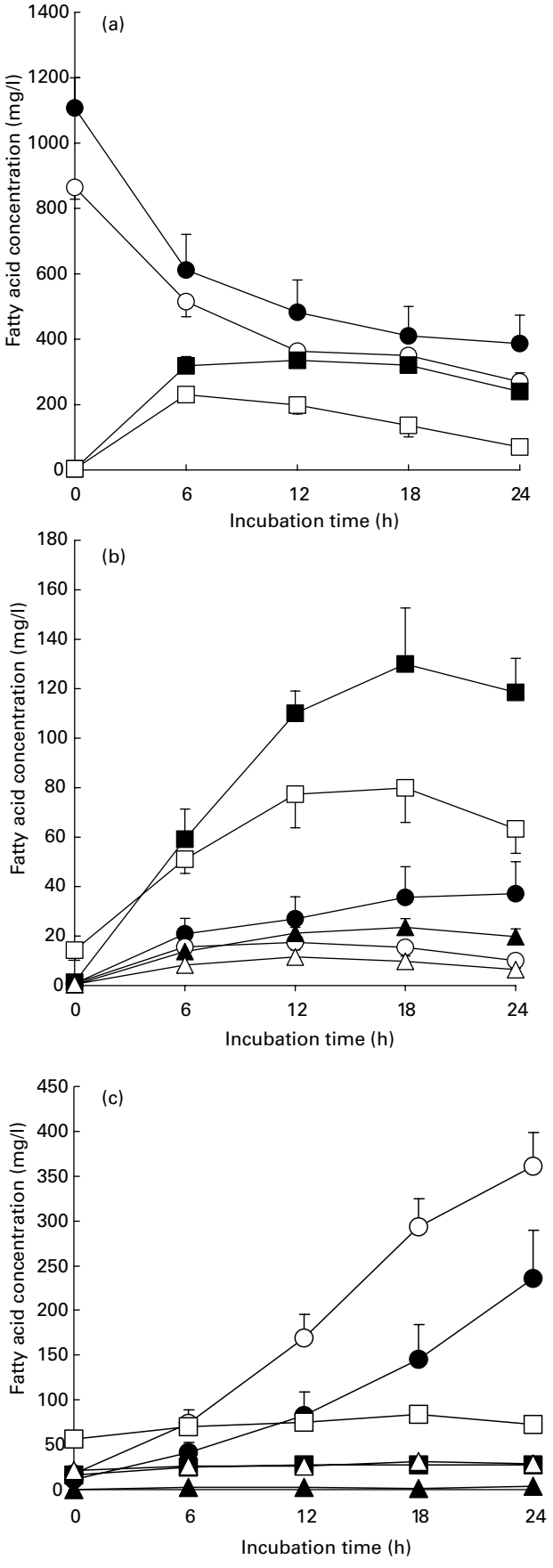
During incubation of LNA with SRF, several peaks were detected that were not present in commercially available authentic standards and unable to be identified unequivocally by GC-MS analysis of FAME. Formal structural identification of the major unidentified fatty acids was made based on GC-MS analysis of 4,4-dimethyloxazoline (DMOX) fatty acid derivatives. Selected samples of FAME were converted to DMOX derivatives through the addition of 2-amino, 2-methyl-1-propanol (500 μl) and heating for 18 h under an N_2 atmosphere according to Fay & Richli (1991), with the exception that a temperature of 150°C was used. GLC separation of DMOX derivatives was performed under isothermal conditions (170°C) and operating the mass spectrometer at an ionisation energy of 400 eV. Both the ion source and interface temperatures were maintained at 230°C. The electron impact ionisation spectra obtained were used to locate double bonds based on atomic mass unit distances, with an interval of 12 atomic mass units between the most intense peaks of clusters of ions containing n and n-1 carbon atoms being interpreted as cleavage of the double bond between carbon n and $n + 1$ in the fatty acid moiety.

Other analysis

Protein was measured by alkaline hydrolysis of samples followed by reaction with the Folin–Ciocalteu reagent (Herbert *et al.* 1971).

Experimental design and statistical analysis

Each set of incubations with mixed digesta was carried out using ruminal digesta from four sheep, incubated individually with fatty acids, fish oil or their mixture. Ruminal digesta was collected from a group of seven sheep managed under the same conditions and fed the same diet. Owing to differences related to animal health and age, the same sheep could not be used as donor animals for all experiments. Sheep 1–4 were used for studies of LA metabolism described by Fig. 1 and Fig. 2, sheep 2–5 for LNA and LA incubations reported in Fig. 3 and Table 1, respectively, and sheep 1, 5, 6 and 7 for experiments with EPA and DHA shown in Fig. 4. Single samples were taken from *in vitro* incubations at each time point for fatty acid extraction and analysis. Results were analysed by ANOVA, with the effect of treatment analysed for



significance at each time point or for concentration differences measured between time points using a paired *t* test. Growth experiments with *B. fibrisolvens* were replicated across three separate cultures, as were isomerase activities, and the results were compared using unpaired *t* tests.

Results

Influence of fish oil on metabolism of linoleic acid

SRF from four sheep was incubated *in vitro* with 1·67 g LA/l and 4·17 g fish oil/l and samples were removed for the analysis of NEFA at 6 h intervals. The initial decline in LA concentration was rapid, resulting in the accumulation of *cis*-9, *trans*-11-18:2 (Fig. 1 (a)) and *trans*-9, *trans*-11-18:2 (Fig. 1 (b)). *Trans*-10, *cis*-12-18:2 and *cis*-9, *cis*-11-18:2 were also produced but at much lower concentrations (Fig. 1 (b)). VA accumulated, while the concentration of other 18:1 fatty acids did not change with time (Fig. 1 (c)). Overall, fish oil caused a smaller loss of LA between 0 and 6 h ($P=0{\cdot}025$) and resulted in a lower ($P<0{\cdot}05$) accumulation of the *cis*-9, *trans*-11 isomer, *trans*-9, *trans*-11 isomer and *cis*-9, *cis*-11 isomer of CLA identified (Fig. 1(a) and (b)) and a doubling ($P<0{\cdot}05$) of the accumulation of VA, as well as higher ($P<0{\cdot}05$) concentrations of other 18:1 acids, principally *cis*-9-18:1 and *cis*-11-18:1 (Fig. 1(c)). The concentration of 18:0 increased throughout the incubations, corresponding to the complete metabolism of C18 unsaturated fatty acids added to ruminal digesta (data not shown). Between-animal variation in the concentration of metabolic intermediates was relatively high. CV for mean LA, *cis*-9, *trans*-11-18:2, *trans*-10, *cis*-12-18:2, *cis*-9, *cis*-11-18:2, VA, *cis*-9-18:1, EPA and DHA concentrations were 16, 28, 36, 37, 26, 7, 9 and 9 %, respectively.

A similar experiment was carried out with different concentrations of fish oil in a 6 h incubation. A decrease in the loss of LA was observed with increasing concentrations of fish oil (Table 1). Similarly, accumulation of both of *cis*-9, *trans*-11-18:2 and *trans*-9, *trans*-11-18:2 declined as the concentration of fish oil added to ruminal fluid increased (Table 1). VA accumulation increased as the concentration of fish oil increased (Table 1). Again, variation in rates of metabolism in samples from different animals was high, resulting in high standard error values.

Influence of fish oil on metabolism of α-linolenic acid

Samples of SRF were incubated with LNA, fish oil or a mixture of LNA and fish oil in a similar manner to the studies of LA metabolism (Fig. 3). LNA loss was most rapid over the first 6 h, followed by a slower rate of disappearance until 24 h. Several intermediates were found to accumulate. Methyl esters of one intermediate eluted 0·2 min after

Fig. 1. Influence of fish oil on metabolism of linoleic acid (LA; *cis*-9, *cis*-12-C18 : 2) in ruminal fluid from sheep receiving a mixed grass hay–concentrate diet. LA was added to an initial concentration of 1·67 g/l and fish oil to 4·17 g/l. (a) LA (●, ○), *cis*-9, *trans*-11-18 : 2 (■, □); (b) *trans*-10, *cis*-12-18 : 2 (●, ○), *trans*-9, *trans*-11-18 : 2 (■, or □), *cis*-9, *cis*-11-18 : 2 (▲, Δ); (c) *trans*-11-18 : 1 (●,○), *cis*-9-18 : 1 (■, or □), *cis*-11-18 : 1 (▲, Δ). Incubations were with LA alone (●, ■, ▲) or with LA + fish oil (○, □, Δ). Results are mean values for four sheep, with standard errors represented by vertical bars. For details of diets and procedures, see p. 1200.

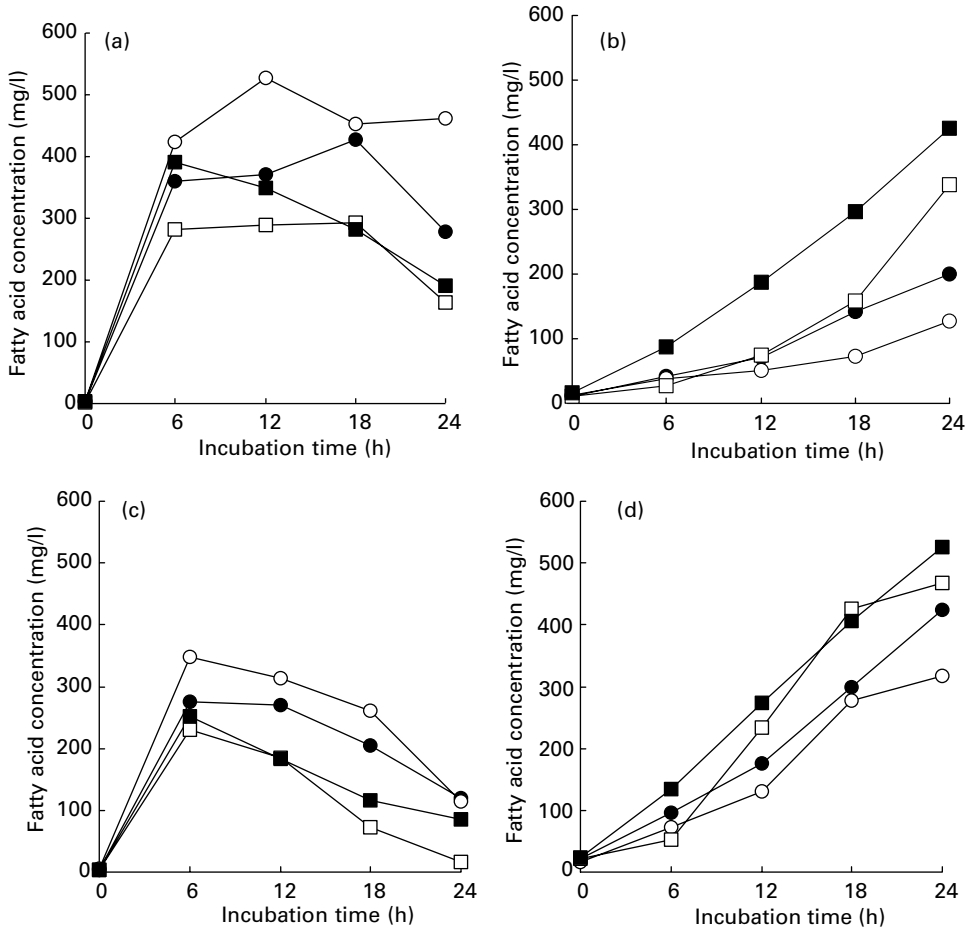


Fig. 2. Between-animal variation in biohydrogenating activity of ruminal fluid and on the effect of fish oil. (a) *Cis*-9, *trans*-11-18 : 2 formation in ruminal digesta from sheep 1 (●), sheep 2 (○), sheep 3 (■) and sheep 4 (□) resulting from incubation with linoleic acid (LA; 1·67 g/l) alone; (b) *trans*-11-18 : 1 formation in ruminal digesta resulting from incubation with LA alone; (c) *cis*-9, *trans*-11-18 : 2 formation in ruminal digesta resulting from incubation with LA with added fish oil (4·17 g/l); (d) *trans*-11-18 : 1 formation in ruminal digesta resulting from incubation with LA with added fish oil. The results are from individual animals in Fig. 1. For details of diets and procedures, see p. 1200.

methyl linoleate (retention time 49·0 min). GC-MS analysis confirmed a molecular ion at *m/z* 294 indicative of an octadecadienoic acid structure. Two other peaks were found to elute at 59·1 and 60·9 min. The electron impact ionisation spectra revealed a molecular ion at *m*/z 292 for both peaks, indicating that both were 18:3 intermediates.

Formal structural identification of the major intermediates formed during incubation of LNA and LNA plus fish oil with SRF was performed based on GC-MS analysis of DMOX derivatives, which were separated under isothermal conditions. Conversion to N-containing derivatives is a prerequisite for fatty acid structural determination, due to the susceptibility of the double bond of methyl esters to fragmentation and migration during exposure to ionisation in the mass spectrometer (Christie, 1998). All mass spectra of DMOX derivatives showed intense peaks at *m/z* 113 and 126 typical of oxazoline derivatives. The mass spectrum of the DMOX derivative of the fatty acid eluting at 49·0 min as a methyl ester indicated a molecular ion at *m/z* 333 confirming an octadecadienoic acid structure. An abundant ion fragment occurred at *m/z* 264, which is a characteristic feature indicative of a bis-methylene interrupted diene with double bonds in positions 11 and 15 (Christie, 1998). Distances of 12 atomic mass units between *m/z* 224 (C10) and 236 (C11) and 278 (C14) and 290 (C15) confirmed the location of double bonds at Δ^{11} and Δ^{15}, respectively, while sequential gaps of 14 atomic mass units from *m/z* 126 to 224 revealed the occurrence of methylene groups from C3 through to C10, allowing the fatty acid to be identified as *trans*-11, *cis*-15-18:2.

The mass spectra of DMOX derivatives of unidentified fatty acids, which eluted at 59·1 and 60·9 min during routine analysis of FAME, were similar and both exhibited a molecular ion at *m/z* 331, indicative of an octadecatrienoic acid structure. A key feature of both spectra was an intense fragment at *m/z* 262 that represents cleavage of the molecule between carbon 13 and 14, confirming the location of double bonds at Δ^{11} and Δ^{15}. Such an abundant ion fragment is a key diagnostic of cleavage at the centre of the *bis*-methylene-interrupted double-bond system, which facilitates the formation of two stabilised allylic radical fragments (Christie, 1998). Intervals of 12 atomic mass units between 196 (C8) and 208 (C9), 222 (C10) and 234 (C11) and 276 (C14) and 288 (C15) revealed double bonds at the Δ^{9}, Δ^{11} and Δ^{15} positions, respectively. The DMOX derivatives were prepared from FAME without prior fractionation, and therefore it is not

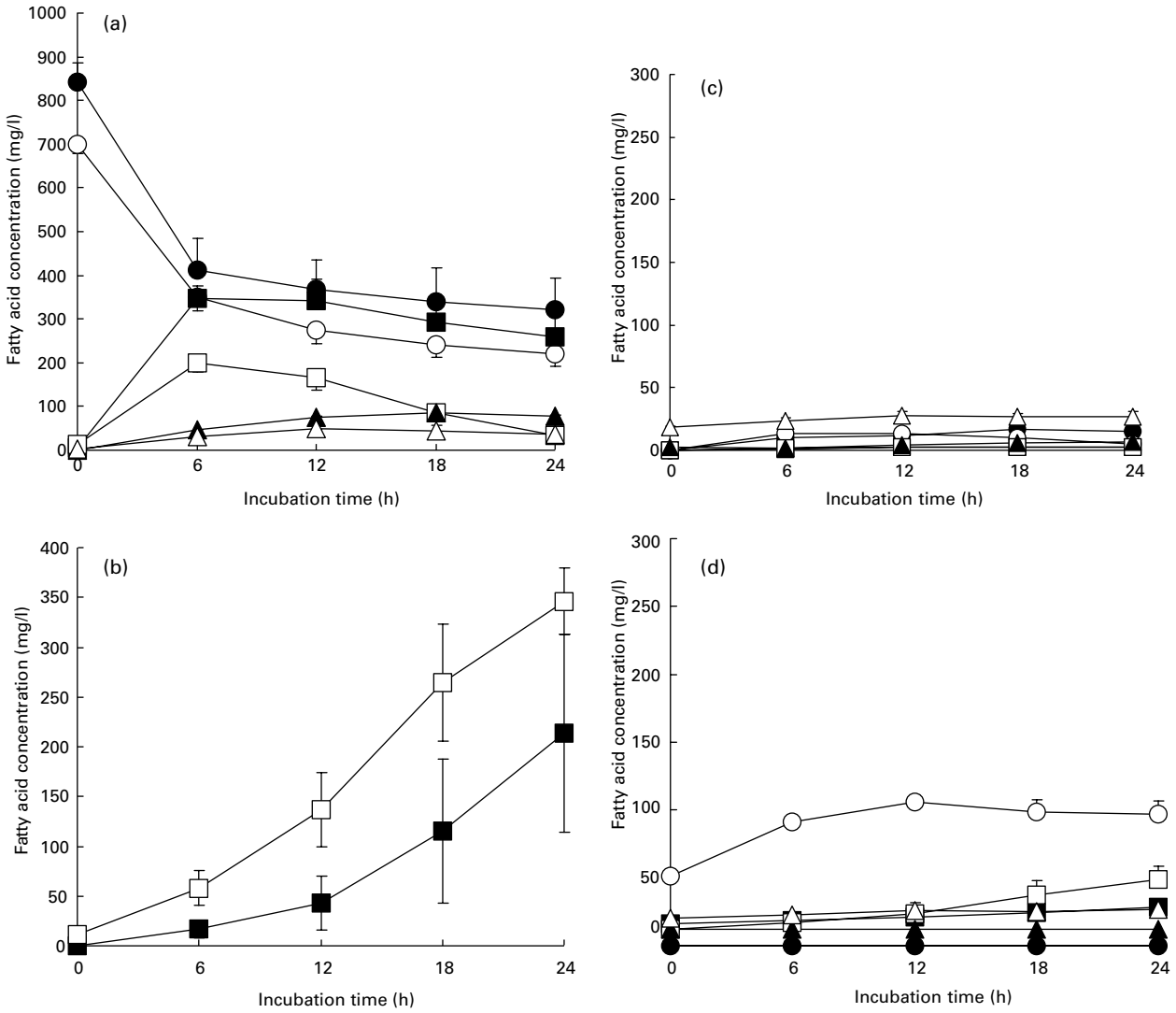


Fig. 3. Influence of fish oil on metabolism of linolenic acid (LNA; *cis*-9, *cis*-12, *cis*-15-C18 : 3) in ruminal fluid from sheep receiving a mixed grass hay–concentrate diet. LNA was added to an initial concentration of 1·67 g/l and fish oil to 4·17 g/l. (a) LNA (●, ○), *cis*-9, *trans*-11, *cis*-15-18 : 3 (■, □); *trans*-9, *trans*-11, *cis*-15-18 : 3, (▲, Δ); (b) *trans*-11, *cis*-15-18 : 2 (■, □); (c) *cis*-9, *trans*-11-18 : 2 (●, ○), *trans*-10, *cis*-12-18 : 2 (■, □), *trans*-9, *trans*-11-18 : 2 (▲, Δ); (d) *trans*-11-18 : 1 (■, □), *cis*-9-18 : 1 (●, ○), *cis*-11-18 : 1 (▲, Δ). Incubations were with LNA alone (●, ■, ▲) or with LNA + fish oil (○, □, Δ). Results are mean values for four sheep, with standard errors represented by vertical bars. For details of diets and procedures, see p. 1200.

possible to identify unequivocally the double bond geometry based on GC-MS analysis alone. However, the elution order of authentic standards of 9,11 geometric isomers of 18:2 methyl esters was determined, and by analogy, the larger peak eluting at 59·1 min during routine analysis was assigned the *cis*-9, *trans*-11, *cis*-15-18:3 structure, while that with a marginally longer retention time (60·9 min) was tentatively identified as *trans*-9, *trans*-11, *cis*-15-18:3. The mass spectra of the FAME and DMOX derivative of the more abundant intermediate, *cis*-9, *trans*-11, *cis*-15-18:3, are given in Fig. 5.

In the presence of fish oil, the initial rate of LNA loss tended to be decreased ($P=0·137$), while both *cis*-9, *trans*-11, *cis*-15-18:3 and *trans*-9, *trans*-11, *cis*-15-18:3 accumulated, but to lower concentrations than before (Fig. 3 (a)). Differences were significant ($P<0·05$) at all times for *cis*-9, *trans*-11, *cis*-15-18:3, but only at 6 and 18 h for *trans*-9, *trans*-11, *cis*-15-18:3. The concentrations of *trans*-9, *trans*-11, *cis*-15-18:3 were little changed, whereas the accumulation of *trans*-11, *cis*-15-18:2 more than doubled (Fig. 3 (a) and (b)). Due to between-animal variation, the last difference was significant ($P<0·05$) only at 6 and 12 h. No CLA or VA accumulated during LNA metabolism, either in the presence or absence of fish oil (Fig. 3 (c) and (d)).

Inter-animal variation

The high variation in fatty acid concentration at each time in Fig. 1 and Fig. 3 was caused by between-animal differences, illustrated for LA incubations by Fig. 2, in which data from each of the four sheep are presented. CLA (*cis*-9, *trans*-11-18:2) accumulated from LA to the greatest extent in sheep 2 (Fig. 2(a)), which also corresponded with the lowest accumulation of VA (Fig. 2 (b)). Sheep 3 showed the highest

Table 1. Influence of fish oil concentration on the metabolism of linoleic acid (LA) added to ruminal fluid *in vitro* and on the accumulation of conjugated LA isomers and vaccenic acid (difference in fatty acid concentration between 0 and 6 h incubation; mg/l)

(Mean values and standard errors of the differences of the means for four sheep per treatment)

	Concentration difference (mg/l)				
Fish oil concentration (g/l)...	0	1·25	2·5	4·17	SED
LA	−592[a]	−504[b]	−460[b]	−353[b]	122
Cis-9, *trans*-11-18 : 2	310[a]	238[a]	195[b]	169[a,c]	72
Trans-9, *trans*-11-18 : 2	133[a]	109[a]	82[b]	62[b]	51
Trans-11-18 : 1	84[a]	84[a,b]	123[c]	137[b,c]	56

[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P<0·10$).

For details of diets and procedures, see p. 1200.

accumulation of VA. Fish oil decreased the accumulation of CLA in all sheep (Fig. 2 (c)). The effects of fish oil on VA accumulation were minimal in sheep 3, but caused increased VA accumulation in the other samples, particularly from sheep 2 (Fig. 2 (d)). The pattern of metabolism in LNA incubations with digesta showed even greater variation between different animals, but the pattern was similar, in that fish oil decreased the transient accumulation of *cis*-9, *trans*-11, *cis*-15-18:3 and only increased the accumulation of *trans*-11, *cis*-15-18:2 when its concentration was low in the absence of fish oil (results not shown).

Concentration of non-esterified eicosapentaenoic acid and docosahexaenoic acid in incubations with fish oil

The method that was used routinely for lipid extraction and transmethylation of fatty acids enabled rapid processing of samples. However, these procedures do not involve extraction of total lipids from samples or saponification to release esterified fatty acids. Measurements of NEFA rather than total fatty acids has the advantage that this allows for a much clearer interpretation of the effects of adding LA, LNA or other fatty acids on the formation of biohydrogenation intermediates during *in vitro* incubations. The apparent concentration of EPA and DHA in samples taken between 6 and 24 h varied from 84–140 and 37–73 mg/l in incubations of ruminal digesta with fish oil (experiments described by Fig. 1 and Fig. 3). Incubation time had no influence on EPA and DHA concentrations, nor did the addition of either LA or LNA.

Solid-phase extraction of the 24 h incubation mixtures of fish oil with SRF indicated mean (n 4) concentrations of EPA and DHA of 30·0 (SD 11·6) and 17·0 (SD 5·6) mg/l, respectively. The concentration of both EPA and DHA was <3 mg/l at both 0 h and 24 h incubations with buffer and no EPA or DHA was detected in incubations without fish oil.

Incubations of strained ruminal fluid with linoleic acid, eicosapentaenoic acid and docosahexaenoic acid

Disappearance of LA was unaffected by the presence of non-esterified EPA and DHA at 100 and 50 mg/l, respectively, or a mixture of the two (Fig. 4). Similarly, the accumulation of CLA (Fig. 4(b)) and VA (Fig. 4(c)) was unaffected by EPA or DHA or their mixture. EPA (Fig. 4(d)) and DHA (Fig. 4(e)) were metabolised rapidly when added individually, but when added together with LA their rate of metabolism decreased by an order of magnitude ($P<0·05$; Fig. 4(d) and (e)). DHA inhibited the metabolism of EPA and *vice versa*, resulting in the rate of disappearance of each fatty acid decreasing by about half when both EPA and DHA were present (Fig. 4(d) and (e)). The effects of EPA on DHA loss was significant ($P<0·05$) at all times from 6 h, while the inhibition of EPA loss by DHA was significant only at 6 and 12 h.

Influence of fatty acids on growth and linoleate isomerase activity of Butyrivibrio fibrisolvens

LA was transiently toxic towards the growth of *B. fibrisolvens*, with the bacterium emerging from a lag phase 12 h after inoculation (Fig. 6 (a)). When LA and subsequently CLA were metabolised leaving low residual concentrations (Fig. 6 (b)), the culture grew to the same cell density as the controls. Thus, the effect of LA was bacteriostatic rather than bacteriocidal. Both EPA and DHA were much more toxic than LA. Cultures containing EPA and DHA did not grow up to 72 h incubation (Fig. 6 (a)), and neither EPA nor DHA was metabolised to a detectable extent (Fig. 6 (b)). Fish oil, when added to the cultures at a concentration of up to 10 g/l, had no influence on the growth of *B. fibrisolvens* (data not shown).

Linoleate isomerase activity was measured as a change in absorbance at 233 nm, characteristic of conjugated dienes. Thus, the formation of *cis*-9, *trans*-11-18:2 from LA leads to a rise in A_{233}. The results (Fig. 7) revealed a pattern of inhibition by fatty acids and fish oil similar to that found for the effects on growth. Linoleate isomerase in whole cells was unaffected by fish oil addition ($P>0·05$; Fig. 7). In contrast, EPA and DHA caused about a 50 % inhibition ($P<0·05$) of isomerase activity at a concentration of 50 mg/l. The results were similar when concentrations up to 200 mg/l were added (data not shown). Similar results were obtained with cell-free extracts of *B. fibrisolvens* prepared by sonication (data not shown).

Discussion

Fish oil is known to inhibit complete biohydrogenation of unsaturated fatty acids, causing an increase in the flow of *trans*-18:1 (Wonsil *et al.* 1994; Wachira *et al.* 2000; Scollan *et al.* 2001*b*; Lee *et al.* 2005) and *trans*-18:2 leaving the rumen (Shingfield *et al.* 2003; Loor *et al.* 2005). Several *in vitro* studies have explored the mechanism underlying the effects of fish oil (Gulati *et al.* 1999; Dohme *et al.* 2003; Chow *et al.* 2004) or the effects of the major long-chain *n*-3 fatty acids in fish oil, EPA and DHA (AbuGhazaleh & Jenkins, 2004*a*,*b*). Novel features of the series of experiments reported in the present paper include an investigation of fish oil, EPA and DHA alone or in combination with LA and LNA, using both mixed ruminal micro-organisms and pure cultures of *B. fibrisolvens*. The findings from these studies allow new inferences to be drawn on the mechanism by which fish oil inhibits biohydrogenation.

The concentrations of lipid added to mixed or pure cultures of ruminal bacteria are central to understanding and interpreting data from *in vitro* studies of lipid metabolism. Both the rate of lipolysis and biohydrogenation are known

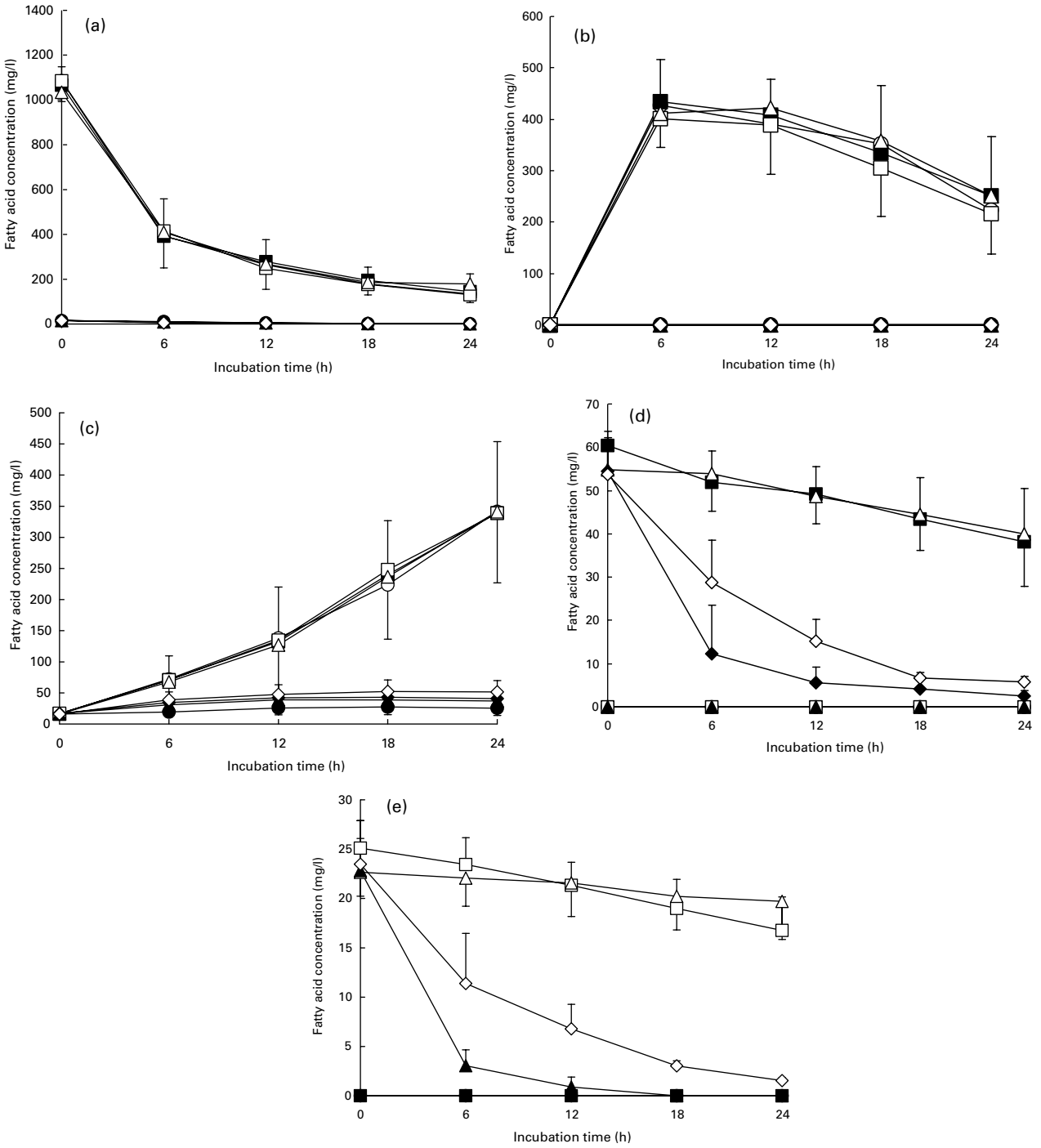


Fig. 4. Metabolism of (a) linoleic acid (LA), accumulation of (b) *cis*-9, *trans*-11-18 : 2 and (c) *trans*-11-18 : 1, and metabolism of (d) EPA and (e) DHA in ruminal fluid from sheep receiving a mixed grass hay–concentrate diet. LA, EPA and DHA were added alone or as mixtures, as follows: no addition (●); LA (○); LA + EPA (■); LA + DHA (□); EPA (◆); DHA (▲); EPA + DHA (◇); LA + EPA + DHA (Δ). LA was added at an initial concentration of 1·67 g/l, EPA at 0·10 g/l and DHA at 0·05 g/l. Results are mean values for four sheep, with standard errors represented by vertical bars. For details of diets and procedures, see p. 1200.

to be dependent on the type and concentration of added lipid (Noble *et al.* 1974; Gulati *et al.* 1999; Beam *et al.* 2000; Dohme *et al.* 2003; Troegeler-Meynadier *et al.* 2003; AbuGhazaleh & Jenkins, 2004*b*). It could be expected that the effects of fatty acid concentration on lipid metabolism *in vitro* would also hold true when a mixture of oils is incubated with mixed or pure cultures of ruminal bacteria. The highest concentration of fish oil added was just over 4 g/l. Supplementing the diet with 200–300 g fish oil/d has been shown to inhibit rumen biohydrogenation in dairy cows and steers, increasing the CLA content of milk and meat (Offer *et al.* 1999; Scollan *et al.* 2001*b*; Shingfield *et al.* 2003). Assuming a rumen pool size of 100 litres in dairy cows, *in vivo* ruminal concentrations of fish oil in earlier studies (Offer *et al.* 1999; Shingfield *et al.* 2003) would be expected to be about 2–3 g/l, comparable with the range of fish oil concentrations examined

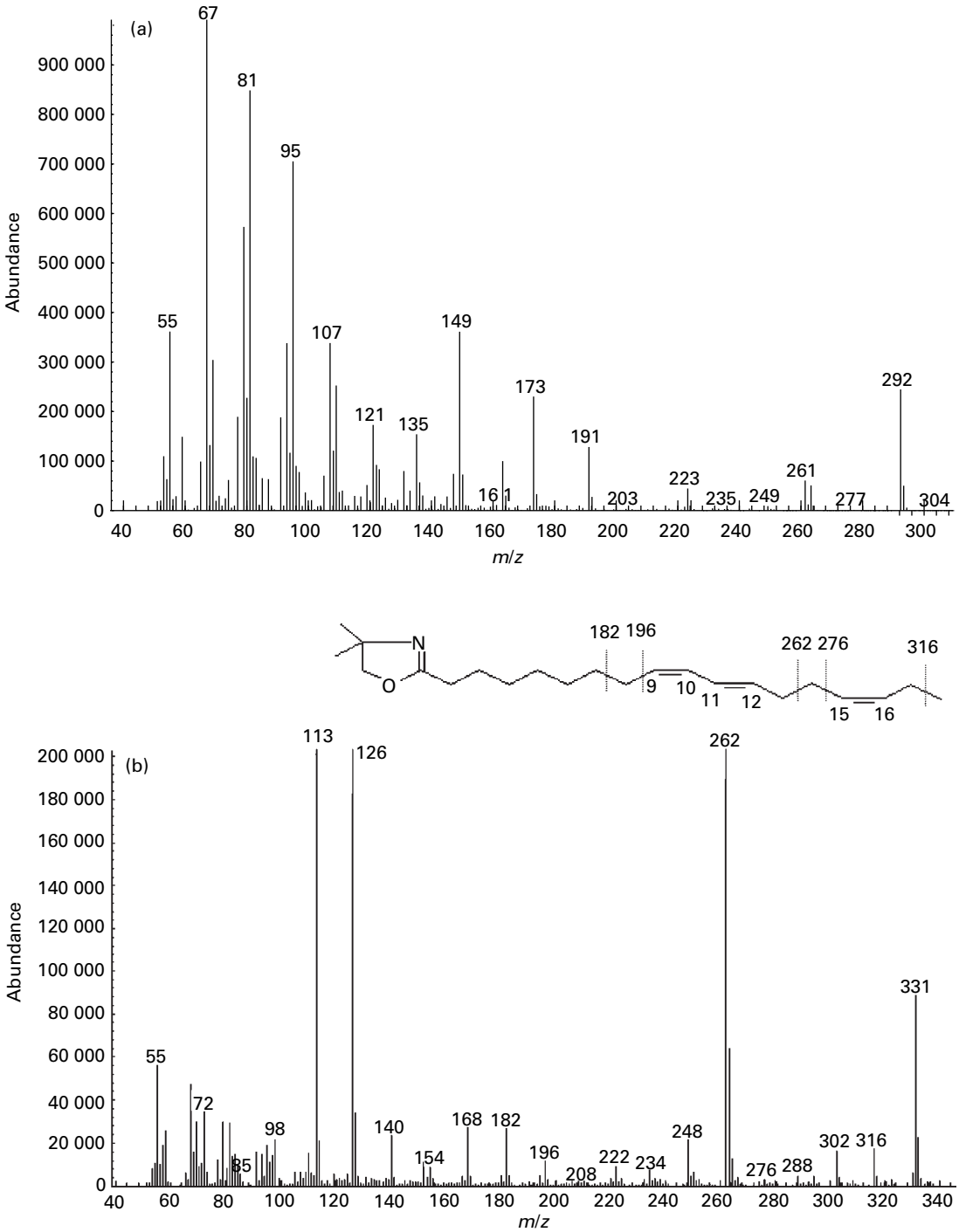


Fig. 5. Mass spectra of unidentified 18 : 3 fatty acid eluting at 59·1 min in fatty acid methyl ester GC, putatively *cis*-9, *trans*-11, *cis*-15-18 : 3, resulting from a 24 h incubation of strained ruminal fluid with linolenic acid. (a) Fatty acid methyl esters; (b) 4,4-dimethyloxazoline fatty acid derivative. For details of diets and procedures, see p. 1200.

in the present study. Concentrations of non-esterified EPA and DHA used in mixed- and pure-culture experiments (100 and 50 mg/l, respectively) were based on the concentrations of these fatty acids determined after incubation of fish oil with mixed ruminal micro-organisms (84–140 and 37–73 mg/l for EPA and DHA, respectively). The analytical procedures used did not involve saponification and lipids were recovered from digesta by simple solvent extraction. Furthermore, methyl esters were prepared by transesterification under mild acidic conditions to minimise isomerisation of unsaturated fatty acids. However, this procedure is known to cause a small amount of lipolysis resulting in the release of NEFA from esterified lipids (AJ Richardson, unpublished results), and as such the determination of fatty acid concentrations will almost certainly be overestimates. Separation of extracted lipids in a selected number of samples by solid-phase

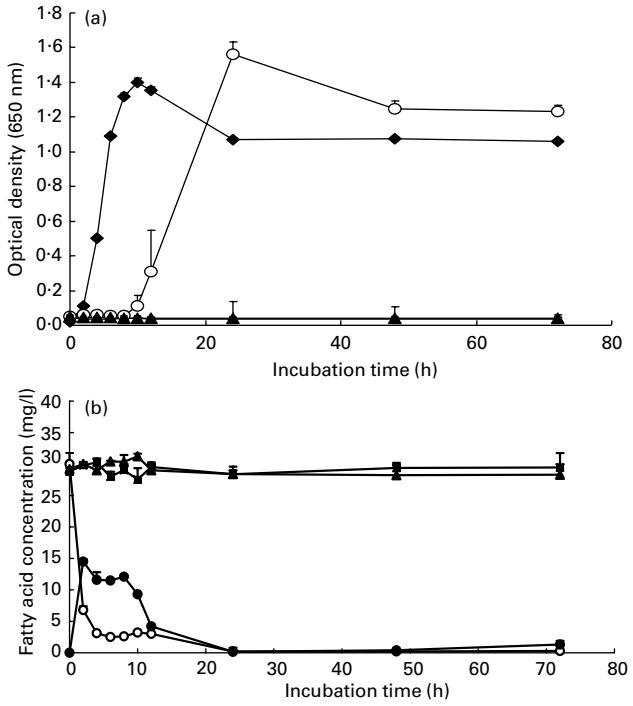


Fig. 6. (a) Influence of fatty acids (50 mg/l) on growth of *Butyrivibrio fibrisolvens* JW11: control (◆); linoleic acid (LA; ○); EPA (■); DHA (▲). (b) Influence of fatty acids on concentrations of fatty acids during growth: EPA in EPA-supplemented cultures (■); DHA in DHA-supplemented cultures (▲); LA (○) and *cis*-9, *trans*-11-18 : 2 (●) in LA-supplemented cultures. Results are mean values with their standard errors from three cultures. For details of diets and procedures, see p. 1200.

extraction indicated that the true concentration of non-esterified EPA and DHA could be less than half of these values. As a result there is the possibility that the EPA and DHA concentrations incubated in the present studies may be at the higher end of the range in concentrations of these fatty acids in ruminal digesta of ruminants fed fish oil. Nevertheless, the amounts of added lipid are in line with previous studies examining the effects on *in vitro* lipid metabolism in response to 0·25–5·0 g fish oil/l (Gulati *et al.* 1999; Dohme *et al.* 2003; Chow *et al.* 2004), 400 and 300 mg DHA and EPA/l, respectively (AbuGhazaleh & Jenkins, 2004*a*), or 100 mg DHA/l (AbuGhazaleh & Jenkins, 2004*b*). In addition, the concentration of LA and LNA added in the present study (1·67 g/l) is the same as that in earlier studies (Polan *et al.* 1964). Ingestion of LA and LNA in ruminant animals is highly variable, but in lactating cows fed grass silage and cereal concentrates, the intake of LA and LNA was 92 and 134 g/d, respectively (Shingfield *et al.* 2003). Based on a rumen volume of 100 litres, the mean maximum concentration of LA and LNA would be expected to approach 0·9 and 1·3 g/l, respectively. Grazing animals can have two- to three-fold higher PUFA intakes (Harfoot, 1981), indicating that the concentration of added fatty acids in the present series of experiments would fall in the middle of the range of expected initial concentrations of LA and LNA *in vivo*.

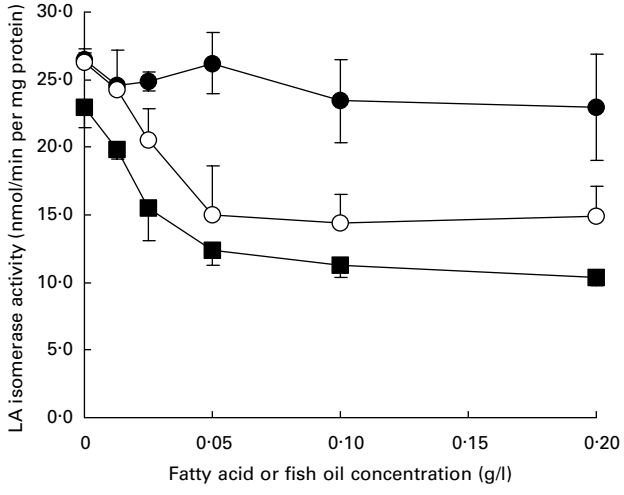


Fig. 7. Influence of fish oil and its main fatty acids on linoleate isomerase activity of *Butyrivibrio fibrisolvens* JW11. Washed whole cells of *B. fibrisolvens* were incubated with linoleic acid, and the influence of 50 mg/l additions of fish oil (●), EPA (○), and DHA (■) on the rate of formation of conjugated linoleic acid was determined by the increase in A_{233} associated with the formation of conjugated diene bonds. Results are mean values for three incubations, with standard errors represented by vertical bars. For details of diets and procedures, see p. 1200.

The pathway of LA metabolism, in which LA is initially isomerised to a mixture of conjugated dienes (predominantly *cis*-9, *trans*-11-18:2) that are hydrogenated to monoenoic acids (mainly VA; *trans*-11-18:1) and subsequently reduced to 18:0 is in line with previous studies (Polan *et al.* 1964; Noble *et al.* 1974; Fellner *et al.* 1995; Martin & Jenkins, 2002). The pathway of LNA metabolism is less well defined. By analogy with the metabolism of LA it has been assumed that the first step of LNA metabolism involves an isomerisation leading to the formation of a conjugated 18:3 intermediate (Wilde & Dawson, 1966). The evidence from the present studies confirms that *cis*-9, *trans*-11, *cis*-15-18:3 is the first intermediate formed during LNA metabolism (Wilde & Dawson, 1966), which is then reduced to *trans*-11, *cis*-15-18:2. Furthermore, the isomerisation step is not entirely specific and *trans*-9, *trans*-11, *cis*-15-18:3 is also a transient intermediate of LNA metabolism, which is also hydrogenated to yield *trans*-11, *cis*-15-18:2. Other intermediates identified included *trans*-11, *cis*-15-18:2. These observations may appear to contradict *in vivo* results with LNA-rich feed components, which have been observed to give rise to VA accumulation (Shingfield *et al.* 2003; Lee *et al.* 2005). However, the feeds were also rich in LA, so we would speculate that the VA that accumulated was derived from LA rather than LNA.

The effect of fish oil on biohydrogenation appeared, from incubations of digesta with LA and LNA (Fig. 1 and Fig. 3), to be most apparent in causing an accumulation of VA from LA and the corresponding *trans*-11, *cis*-15-18:2 from LNA. This would indicate that fish oil inhibits the reduction of these intermediates, consistent with the interpretation of findings from earlier studies (Shingfield *et al.* 2003; AbuGhazaleh & Jenkins, 2004*a*,*b*). However, incremental increases in fish oil addition to mixed digesta also resulted in a dose-dependent accumulation of conjugated C18:2 and C18:3 intermediates (Table 1), indicating that fish oil also inhibits the initial isomerisation of LA and LNA. Overall, it appears that fish oil in the diet can be expected to decrease the rate of all steps of the isomerisation and biohydrogenation of PUFA in the rumen.

It has often been assumed that the main fatty acid constituents in fish oil, EPA and DHA, are responsible for the inhibitory effects on biohydrogenation. It is interesting to note that both non-esterified EPA and DHA, but not fish oil, inhibited the growth and LA isomerase activity of *B. fibrisolvens*. EPA and DHA were much more toxic to *B. fibrisolvens* than LA. Incubating LA caused a lag phase, from which the bacteria recovered as LA was converted to CLA, then to VA. In contrast, EPA and DHA were not metabolised by *B. fibrisolvens* and the bacteria did not recover. Fujimoto *et al.* (1993) had previously noted that EPA was highly toxic to ruminal bacteria. In spite of these observations, adding non-esterified EPA and DHA to mixed ruminal digesta at similar concentrations to those measured in the fish oil incubations did not replicate the effects of fish oil on LA metabolism. The reason for the lack of effect was not that EPA and DHA were metabolised, because, although their concentrations declined when added alone, addition of these fatty acids in combination with LA prevented their metabolism. Even adding EPA and DHA together as a mixture appeared to stabilise each fatty acid to some extent. Many factors may be involved, but it is likely that these findings would also hold true for other fatty acids. It is possible that some fatty acids cause competitive inhibition of bacterial isomerases and reductases, the extent of which will depend on the relative concentration and affinity of specific fatty acids as demonstrated in early studies of biohydrogenation (Kepler *et al.* 1970). There may also be competition for reducing power, probably most important when considering the effects of adding different amounts of lipids or oils (Gulati *et al.* 1999; Beam *et al.* 2000; Dohme *et al.* 2003; Troegeler-Meynadier *et al.* 2003; AbuGhazaleh & Jenkins, 2004*b*). Another plausible explanation may relate to the effects of NEFA being potentiated in the presence of other lipids as observed in other studies (Dohme *et al.* 2003; AbuGhazaleh & Jenkins, 2004*b*). Fish oil is known to contain a complex mixture of long-chain PUFA and it has been speculated (Offer *et al.* 1999; Shingfield *et al.* 2003) that other fatty acids including 18:4*n*-3, 20:4*n*-6 or 20:4*n*-3, either acting alone or in a synergistic manner, may also be involved. Finally, the microbial cell density, which is much higher in mixed ruminal digesta than in pure culture, may also be important through absorbing or adsorbing fatty acids (Harfoot *et al.* 1974).

The experiments reported in the present paper have a number of shortcomings with regard to understanding the effects of fish oil on ruminal lipid metabolism. Principal among these is that adding fish oil to the diet may lead to changes in microbial ecology, which may take days or weeks to manifest themselves. The present experiments contain no element of adaptation. Second, the experiments were conducted using strained ruminal digesta, so any contribution of micro-organisms associated with large particles would not be seen. However, for sheep fed the experimental diet, most of the microbial biomass is associated with material that passes into strained ruminal digesta, whereas in cattle a high proportion of biomass is associated with large particles. The presence of food particles can have a significant influence on the availability of lipids for microbial metabolism (Harfoot *et al.* 1974; Legay-Carmier *et al.* 1989). In the present experiments, no additional substrate was added to digesta during *in vitro* incubations.

Biohydrogenation activity and responses to fish oil in these experiments were highly variable between individual animals. The *B. fibrisolvens* group of ruminal bacteria is fundamental to ruminal biohydrogenation, with the reduction of monoenoic acids being carried out solely by bacteria (previously named *Fusocillus*) that are found in a small branch of the *Butyrivibrio* phylogenetic tree (van de Vossenberg & Joblin, 2003; McKain *et al.* 2004). These bacteria are known to be much more sensitive than other *Butyrivibrio* isolates to the toxic effects of unsaturated fatty acids (Chaudhary *et al.* 2004). Thus, the digesta in which VA accumulated to the greatest extent might be expected to have the smallest population of '*Fusocillus*'. Ruminal fermentation would not be considered dependent on the presence of *B. fibrisolvens* or '*Fusocillus*', since these bacteria occupy a niche that, except for biohydrogenation, can be occupied by many others (Stewart *et al.* 1997). Thus, the between-animal variation may have been due to variation in the populations of *B. fibrisolvens* and '*Fusocillus*', and it may be possible to alter this population to decrease biohydrogenation without compromising other aspects of ruminal fermentation. It will now be important to use molecular methods for microbial community analysis (Zoetendal *et al.* 2004) to correlate population dynamics with biohydrogenating activity, and also to examine the ability of ruminal bacteria to adapt to fish oil in the diet. Exploration of the underlying causes of between-animal variation may also allow the factors affecting ruminal biohydrogenation to be identified and assist the development of nutritional strategies for manipulating this process to improve the health characteristics of ruminant-derived foods.

In conclusion, fish oil inhibited biohydrogenation of both LA and LNA, causing the accumulation of a number of intermediates, an effect that was variable between animals. The effects on biohydrogenation in the mixed population were not entirely consistent with the inhibitory effects being solely due to the concentrations of non-esterified EPA and DHA. If this mechanism can be identified, it may become possible to develop other dietary additives based on PUFA or other lipids to manipulate ruminal biohydrogenation in a manner favourable to the production of ruminant meats and milk enriched with fatty acids, which have the potential to improve long-term human health.

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