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Metabolism of polyunsaturated fatty acids and their toxicity to the microflora of the rumen

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Abstract Ruminal microorganisms hydrogenate polyunsaturated fatty acids (PUFA) present in forages and thereby restrict the availability of health-promoting PUFA in meat and milk. The aim of this study was to investigate PUFA metabolism and the influence of PUFA on members of the ruminal microflora. Eleven of 26 predominant species of ruminal bacteria metabolised linoleic acid (LA; *cis*-9,*cis*-12–18:2) substantially. The most common product was vaccenic acid (*trans*-11–18:1), produced by species related to *Butyrivibrio fibrisolvens*. α-Linolenic acid (LNA; *cis*-9,*cis*-12,*cis*-15–18:3) was metabolised mostly by the same species. The fish oil fatty acids, eicosapentaenoic acid (EPA; 20:5(n – 3)) and docosahexaenoic acid (DHA; 22:6(n – 3)) were not metabolised. Cellulolytic bacteria did not grow in the presence of any PUFA at 50 μg ml^{-1}, nor did some butyrate-producing bacteria, including the stearate producer *Clostridium proteoclasticum*, *Butyrivibrio hungatei* and *Eubacterium ruminantium*. Toxicity to growth was ranked EPA > DHA > LNA > LA. Cell integrity, as measured using propidium iodide, was damaged by LA in all 26 bacteria, but to different extents. Correlations between its effects on growth and apparent effects on cell integrity in different bacteria were low. Combined effects of LA and sodium lactate in *E. ruminantium* and *C. proteoclasticum* indicated that LA toxicity is linked to metabolism in butyrate-producing bacteria. PUFA also inhibited the growth of the cellulolytic ruminal fungi, with *Neocallimastix frontalis* producing small amounts of *cis*-9,*trans*-11–18:2 (CLA) from LA. Thus, while dietary PUFA might be useful in suppressing the numbers of biohydrogenating ruminal bacteria, particularly *C. proteoclasticum*, care should be taken to avoid unwanted effects in suppressing cellulolysis.

Keywords Biohydrogenation · Fatty acids · Linoleic acid · Linolenic acid · Rumen

Abbreviations
CLA Conjugated linoleic acid
DHA Docosahexaenoic acid

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DTT Dithiothreitol
EPA Eicosapentaenoic acid
LA Linoleic acid
LNA α-Linolenic acid
OD Optical density
PI Propidium iodide
PUFA Polyunsaturated fatty acids
VA Vaccenic acid

Introduction

Unsaturated fatty acids, particularly α-linolenic acid (LNA; *cis*-9,*cis*-12,*cis*-15–18:3) and linoleic acid (LA; *cis*-9,*cis*-12–18:2), are abundant in grass and other ruminant feedstuffs, yet are present at low concentrations in meat and milk. Furthermore, tissue lipids of ruminants have been known for a long time to be more saturated than those of non-ruminants (Banks and Hilditch 1931). As the consumption of dairy products and ruminant meats is often associated with an increased incidence of coronary heart disease in man (Menotti et al. 1999), the transformation of unsaturated fatty acids to saturated fatty acids, or biohydrogenation, in ruminants represents a major human health issue. The biohydrogenation process has long been known to occur in the rumen as the result of microbial metabolic activity (Shorland et al. 1955; Viviani 1970). Thus, if ruminal biohydrogenation of unsaturated fatty acids can be controlled, it may be possible to improve the healthiness of ruminant meats and milk by increasing their unsaturated fatty acids composition in general and conjugated linoleic acid (CLA) and the *n*-3 fatty acids in particular (Scollan et al. 2001).

A recent preliminary study concluded that the ruminal bacteria that form stearate were also highly sensitive to the toxic effects of LA (Chaudhary et al. 2004). Differential toxicity could be envisaged as a strategy for arresting biohydrogenation of vaccenic acid (*trans*-11–18:1), which would lead to ruminant products with a healthier fatty acid profile (Griinari et al. 2000). Although a few studies have examined the toxicity of saturated fatty acids, oleic acid and other lipids to ruminal bacteria (Henderson 1973; Marounek et al. 2002), none has related PUFA toxicity and biohydrogenation. The present study was undertaken to investigate the effects of LA and other PUFA on individual members of the ruminal microflora.

Materials and methods

Microorganisms and growth conditions

Bacteria and fungi were from the collection held at the Rowett Research Institute. The provenance of most of the bacteria has been given previously (Wallace and McKain 1991; Avguštin et al. 1997; Edwards et al. 2005). *Butyrivibrio hungatei* JK611 was a gift from J. Kopečný and is described by (2003). *Neocallimastix frontalis* RE1 was described by Stewart et al. (1987). *Piromyces communis* P was a gift from C.G. Orpin (Babraham, UK).

All transfers and incubations were carried out under O_2-free CO_2 and at 39°C in Bellco tubes (Bellco Biotechnology, Vineland, NJ 08380, US). Inoculum volumes were 5% (v/v) of a fresh culture. The media used in these experiments were the liquid form of M2 medium (Hobson 1969), which contains 70 mM sodium lactate, or the same medium with sodium lactate omitted (M2-L) Fatty acids were prepared as a separate solution, sonicated for 4 min in water and added to the medium before autoclaving.

In order to determine if bacteria metabolised LA (*cis*-9, *cis*-12–18:2), they were inoculated firstly into the liquid form of Bryant and Robinson (1961) medium containing 50 μg LA ml^{-1} and incubated for 48 h. Cultures were then extracted and analysed for free fatty acid concentrations. Where cultures failed to grow but grew on medium with no added LA, the culture was inoculated into medium containing 20 μg LA ml^{-1} and the process was repeated. Fermentation products were determined by derivatization and capillary gas-liquid chromatography (Richardson et al. 1989) of supernatants from cultures grown in the liquid form of M2 medium.

The metabolism of PUFA was determined using medium M2-L containing 50 μg PUFA ml^{-1}

for all species except *Anaerovibrio lipolytica*5S and *Veillonella parvula* L59, which required the addition of 10 g l^{-1} glycerol or sodium pyruvate respectively to support growth. Growth of bacteria was measured from the increase in optical density (OD) at 650 nm of the control tubes, in triplicate, using a Novaspec II spectrophotometer (Amersham Biosciences, UK). Growth was followed turbidimetrically at 12-h intervals to determine the length of the lag period and the cell density at stationary phase. Similar experiments were carried out using M2 medium, except that only the effects of LA, at 200 and 50 μg ml^{-1}, were tested and the growth measurement intervals were shorter. Where cultures failed to grow but grew on medium with no added LA, the culture was inoculated into medium containing 20 or 5 μg LA ml^{-1} and the process was repeated.

Ruminal fungi were grown in M2-L medium to which 0.05% agar had been added. Growth was assessed visually, and fatty acid concentration was determined on the total freeze-dried culture.

Measurement of cell integrity using propidium iodide

The methods used to assess the influence of LA on cell integrity of different species were based on the work of Ben Amor et al. (2002). One ml of overnight M2 culture was inoculated into 10 ml of M2 medium and incubated at 39°C until it reached mid-exponential phase (OD_{650} = 0.4, approx. 4 h). The bacterial culture was centrifuged (3,000 *g*, 10 min, 4°C) and the pellet was washed twice with anaerobic potassium phosphate buffer (100 mM; pH 7.0) containing 1 mM dithiothreitol (DTT). Anaerobic conditions were maintained by carrying out transfers in an anaerobic chamber, with a gas phase of 80% N_2, 10% CO_2 and 10% H_2. Cells were resuspended to an OD_{650} of 0.4 in the same buffer, then 0.05 ml of 1 mg ml^{-1} LA solution, also in the same buffer and prepared by sonicating for 3 min, was added to 0.95 ml of cell suspension. The suspension was incubated at 39°C for 15 min. Cells resuspended in buffer alone and heat-treated cells (100°C for 15 min) served as control samples.

Propidium iodide (PI) working solution (1.5 mM) was prepared in distilled water and stored at 4°C in the dark. Fifty μl of each sample were added to 149 μl of anaerobic potassium phosphate buffer (100 mM, pH 7.0, containing 1 mM DTT) in the presence of 1 μl of PI solution. The mixtures were incubated for 15 min at 39°C in the anaerobic chamber. Samples were kept in the dark on ice and used within 45 min for fluorescence analysis. Fluorimetry measurements were made using a Spectramax GeminiXS spectrofluorimeter (Molecular Devices, Wokingham, Berks, UK) set at λ_{EX} = 530 nm and λ_{EM} = 620 nm.

Influence of LA and sodium lactate on growth of *C. proteoclasticum* P-18:

Clostridium proteoclasticum P-18 was grown overnight in medium M2-L. Fresh tubes of the following media were then inoculated (5%, v/v): M2; M2-L; M2+50 μg LA ml^{-1}; M2-L+50 μg LA ml^{-1}. Similar experiments were done with vaccenic acid (*trans*-11–18:1). Growth was followed turbidimetrically and samples for fatty acid analysis were prepared as before.

Fatty acid extraction and analysis

Extraction, derivatization of fatty acids and GC analysis of methyl esters were carried out using procedures described by Wąsowska et al. (2006).

Results

Metabolism of linoleic acid by individual species of ruminal bacteria

The metabolism of LA and its influence on the growth of 26 culture-collection strains was determined in Bryant and Robinson (1961) complete medium containing 50 μg ml^{-1} LA (Table 1). Eleven strains metabolised LA to an extent easily detectable by GC. Most of these were *Butyrivibrio* and related strains, which showed an accumulation of vaccenic acid (VA; *trans*-11–18:1), but *Clostridium aminophilum* and *Mitsuokella multiacidus* formed oleic acid (*cis*-9–18:1), *Fibrobacter succinogenes* appeared

to form some C16:0, and *Streptococcus bovis* produced a C-18 hydroxy fatty acid product. Only *C. proteoclasticum* P-18 formed a CLA, *cis*-9,*trans*-11–18:2, as one of its end products. *C. proteoclasticum* B316^{T}and P-18 were the only bacteria to form stearate. The products of LA metabolism by *Lachnospira multipara* and *Peptostreptococcus anaerobius* were not determined; LA was used incompletely by these bacteria. No *trans*-10,*cis*-12–18:2 was produced by any culture-collection strain, including the two *Megasphaera elsdenii*. Five of the 26 bacteria suffered growth inhibition by 50 μg LA ml^{-1}. Of these, three were butyrate producers, viz. *B. hungatei*, *Clostridium sticklandii* and *Eubacterium ruminantium*, and the other two were cellulolytic *Ruminococcus* spp.

Influence of different PUFA on growth of predominant species of ruminal bacteria

Most species of ruminal bacteria grew in the presence of 50 μg ml^{-1} PUFA in M2-L medium (Table 2). In about half these species, there was no discernible lag phase induced by the LA, with a slight decrease in cell density being the only sign of toxicity. The most resistant species were members of the genera *Prevotella*, *Megasphaera*, *Selenomonas*, *Veillonella* and *Anaerovibrio*. The two cellulolytic *Ruminococcus* species were unable to grow in the presence of any PUFA in this medium, while the other cellulolytic species, *F. succinogenes*, grew to a much lower final cell density in LA and DHA and was unable to grow in medium containing

Table 1 Fermentation products and ability of predominant species of ruminal bacteria to metabolize LA (50 μg ml^{-1})

Bacterium	Main fermentation products	Use of LA	Growth inhibition >50% in 50 μg LA ml^{-1}	CLA formed	VA formed	Stearate	Other
Anaerovibrio lipolytica 5S	FAS	No					
Butyrivibrio fibrisolvens SH13	FAB	Yes			+		
Butyrivibrio fibrisolvens JW11	FAB	Yes			+		
Butyrivibrio hungatei JK611	FAB	Yes	Inhibited		+		
Clostridium aminophilum 49906	FB	Yes					*cis*-9–18:1
Clostridium proteoclasticum B316^T	FB	Yes			+	+	
Clostridium proteoclasticum P-18	FAB	Yes		*cis*-9,*trans*11–18:2	+	+	
Clostridium sticklandii 12662	APiBBiV	No	Inhibited				
Eubacterium pyruvativorans I-6	ABVC	No					
Eubacterium ruminantium 2388	FBL	No	Inhibited				
Fibrobacter succinogenes S85	FS	Yes					C16:0
Lachnospira multipara D15d	FAL	Yes					
Megasphaera elsdenii LC1	PiBBiVC	No					
Megasphaera elsdenii T81	PiBBiVC	No					
Mitsuokella multiacidus 46/5	APL	Yes					*cis*-9-18:1
Peptostreptococcus anaerobius 27337	AiBiVV	Yes					
Prevotella albensis M384	FAS	No					
Prevotella brevis GA33	FAS	No					
Prevotella bryantii $B_1$4	FAS	No					
Prevotella ruminicola 23	FAS	No					
Ruminobacter amylophilus 70	FAS	No					
Ruminococcus albus SY3	FAS	No	Inhibited				
Ruminococcus flavefaciens FD1	FAS	No	Inhibited				
Selenomonas ruminantium Z108	APL	No					
Streptococcus bovis ES1	AL	Yes					OH-C18:1
Veillonella parvula L59	AP	No					

A, acetate; B, butyrate; F, formate; iB, isobutyrate; iV, isovalerate; L, lactate; P, propionate; S, succinate

Table 2 Influence of polyunsaturated fatty acids (50 μg ml^{-1}) on growth of predominant species of ruminal bacteria in M2-L medium

Species	No addition t (h)[a]	LA		LNA		EPA		DHA	
		t (h)	Cell density[b]	t (h)	Cell density	t (h)	Cell density	t (h)	Cell density
R. flavefaciens FD-1	24	>96		>96		>96		>96	
B. hungatei JK611	12	>96		>96		>96		>96	
R. albus SY3	24	>96		>96		>96		>96	
E. ruminantium 2388	24	>96		>96		>96		>96	
C. proteoclasticum P-18	12	72	0.93	>96		>96		>96	
F. succinogenes S85	24	24	0.38	>96		>96		24	0.56
Pseudobutyrivibrio xylanovorans Mz5	12	24	1.11	96	0.99	>96		84	0.98
L. multipara D15d	12	36	0.80	48	0.54	96	0.51	48	0.67
Pseudobutyrivibrio ruminis A12-1	12	24	1.04	36	1.01	>96		48	0.69
E. pyruvativorans I-6	24	36	0.45	36	0.45	48	0.49	36	0.52
P. anaerobius 27336	12	24	0.78	12	0.62	12	1.45	12	1.51
C. aminophilum 49906	36	49	1.18	48	1.08	96	1.26	48	1.17
S. bovis ES1	12	24	0.97	24	1.00	96	0.66	24	1.01
B. fibrisolvens JW11	12	12	1.23	24	1.34	96	0.42	36	1.23
R. amylophilus WP225	24	24	0.85	24	0.88	24	0.77	24	0.90
P. bryantii $B_1$4	12	12	0.97	12	1.00	12	0.97	12	0.96
P. brevis GA33	12	24	1.01	24	1.04	24	0.97	12	1.05
P. albensis M384	12	24	0.71	12	0.90	24	0.86	24	0.80
P. ruminicola 23	24	24	0.70	24	0.97	24	0.88	24	0.67
M. multiacidus 46/5	24	24	1.05	24	1.07	24	1.01	24	1.09
S. ruminantium Z108	12	12	1.01	12	1.02	12	0.98	12	1.01
M. elsdenii LC1	12	12	0.97	12	0.93	12	0.97	12	0.99
V. parvula L59	24	24	0.98	24	0.96	24	1.02	24	1.10
A. lipolytica 5S	12	12	0.99	12	1.00	12	0.99	12	1.04

[a] Time to reach stationary phase, measured at 12-h intervals

[b] Cell density at stationary phase, proportionally to growth in medium with no added fatty acid. Results are means of three cultures

LNA or EPA. The other most sensitive bacteria were *B. hungatei*, *E. ruminantium* and *C. proteoclasticum*. The relative sensitivity of the different species to other PUFA was generally similar to their sensitivity to LA. LNA was more toxic than LA, while the fish fatty acids, EPA and DHA, were more toxic than LNA. There was a tendency for EPA to be more toxic than DHA. LNA was metabolised by the same strains that had been found to metabolise LA. Significant metabolism of EPA and DHA was not observed in any culture.

A third set of experiments was done with LA using M2 medium, which contains 70 mM sodium lactate (Table 3). Once again, members of the genera *Prevotella*, *Megasphaera*, *Selenomonas*, *Veillonella* and *Anaerovibrio* were insensitive to the fatty acids, and the most sensitive bacteria were the cellulolytic species, and *B. hungatei* and *C. proteoclasticum*. *Ruminococcus flavefaciens* remained unable to grow at 20 μg LA ml^{-1}, but the other sensitive strains grew, albeit, especially with *B. hungatei*, after a long lag phase. *B. hungatei* was not inhibited by 5 μg LA ml^{-1} (data not shown). Comparing Tables 2 and 3, the presence of sodium lactate in the medium influenced the sensitivity of several species to LA. *E. ruminantium*, which was insensitive to LA in the presence of sodium lactate (Table 3), became highly sensitive in its absence (Table 2).

Influence of PUFA on growth of ruminal anaerobic fungi

Two species of ruminal fungi were grown in M2-L medium to which 0.05% agar and 50 μg ml^{-1}

Table 3 Influence of linoleic acid on growth of predominant species of ruminal bacteria in M2 medium (containing 70 mM sodium lactate)

Species	Lag phase (h)			Final cell density as proportion of control	
	200 μg ml^{-1} LA	50 μg ml^{-1} LA	20 μg ml^{-1} LA	200 μg ml^{-1} LA	50 μg ml^{-1} LA
R. flavefaciens FD-1	>100	>100	>100		
B. hungatei JK615	>100	>100	84	–	–
C. proteoclasticum P-18	>100	>100	32	–	–
R. albus SY3	>100	24	12	–	0.47
F. succinogenes S85	44	40	15	0.58	0.72
Pseudobutyrivibrio xylanovorans Mz5	46	26		0.70	0.96
L. multipara D15d	41	19		0.44	0.77
P. ruminis A12-1	20	12		0.81	0.92
E. pyruvativorans I-6	34	7		0.53	0.56
P. anaerobius 27336	10	10		0.67	0.87
B. fibrisolvens JW11	10	10		0.97	1.00
C. aminophilum 49906	13	0		1.00	1.00
S. bovis ES1	9	7		0.71	0.91
Ruminobacter amylophilus 70	6	0		0.50	0.76
E. ruminantium 2388	0	0		0.79	1.00
P. bryantii $B_1$4	0	0		0.79	0.96
P. brevis GA33	8	8		0.77	1.00
P. albensis M384	0	0		0.85	0.88
P. ruminicola 23	0	0		0.87	0.94
M. multiacidus 46/5	0	0		0.85	1.00
S. ruminantium Z108	0	0		0.81	1.02
Megasphaera elsdenii J1	0	0		0.82	1.00
V. parvula L59	0	0		0.86	1.00
A. lipolytica 5S	0	0		0.80	0.96

Results are means of three cultures

PUFA had been added. At 96 h following a 5% inoculum, the only culture to have grown was *N. frontalis* in the LA-containing medium; no growth was observed with *P. communis* in the presence of fatty acids 1 week after a 10% inoculum had been added to the 50 μg ml^{-1} LA medium, both species grew. *N. frontalis* had metabolised about half of the LA in the medium, forming *cis*-9,*trans*-11–18:2. *P. communis* grew but did not metabolise LA.

Influence of LA on cell integrity of different species of ruminal bacteria

Fluorescence of PI was used to assess how LA affected the cell integrity of some of the most common species of ruminal bacteria (Fig. 1). PI fluoresces when in contact with DNA, but is normally unable to permeate across the cell membrane. Thus, only cells which have lost their cell membrane integrity fluoresce with PI. Fluorescence readings from treated cell suspensions were therefore compared with values obtained from the same culture which had been boiled for 10 min. *F. succinogenes* and *B. fibrisolvens* A38^T had a high permeability in the absence of LA, while others gave 20% or less of the values obtained with boiled cells. LA increased the fluorescence of all bacteria. The increase differed with different species and even among the same species or closely related bacteria, such as the *Prevotella* spp., sensitivity to LA varied considerably. The least sensitive species was *M. elsdenii*. Correlation analysis was carried out by grouping the species in five groups according to sensitivity to LA in Table 2 or Table 3, then comparing with the cell integrity data from Fig. 1. Correlation coefficients of 0.52 and 0.36 were found between the effects of LA on cell integrity (Fig. 1) and effects of LA on growth in the absence (Table 2) or presence (Table 3) of sodium lactate, respectively.

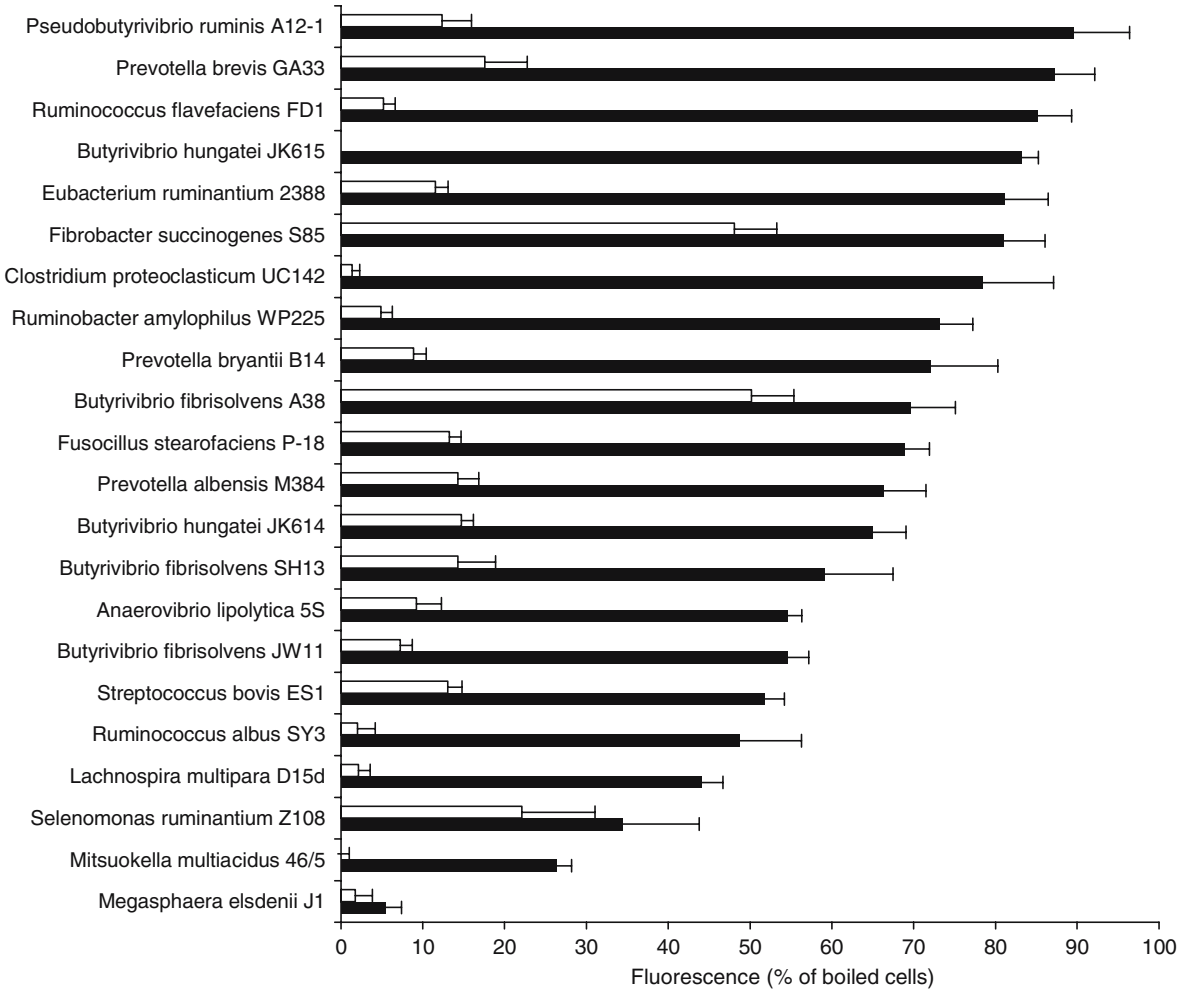

Fig. 1 Influence of LA (50 μg ml^{-1}) on membrane integrity of different strains of ruminal bacteria. Loss of cell integrity was determined by fluorescence in the presence of propidium iodide. 100% loss of cell integrity was taken as the fluorescence of the same culture which had been incubated at 100 °C for 15 min. No addition (□), LA (■). Results are means and SD from 3 cultures, each of which was subject to 8 replicate measurements (n = 24)

Influence of LA, VA and sodium lactate on growth of *C. proteoclasticum* P-18:

Growth of *C. proteoclasticum* was more sensitive to the addition of PUFA than most other bacteria (Tables 1–3). The presence of sodium lactate in M2 medium had no influence on the growth of *C. proteoclasticum*, however it tripled the lag phase in the presence of 50 μg LA ml^{-1} (Fig. 2a). The cause appeared to be a prolonged accumulation of CLA (Fig. 2b). Any influence of sodium lactate on LA isomerisation during early lag phase was slight (Fig. 2c). Growth was also inhibited slightly by VA (Fig. 3a), but the result was a slight lowering of the specific growth rate rather than an obvious effect on the lag phase. Sodium lactate slowed the specific growth rate further with little influence on the lag phase (Fig. 3a) or the rate of metabolism of VA (Fig. 3b). Sodium chloride added to the medium at the same concentration as sodium lactate had no effect on growth (not shown).

Discussion

There has been much recent interest in PUFA, particularly *n*-3 PUFA, and intermediates of the

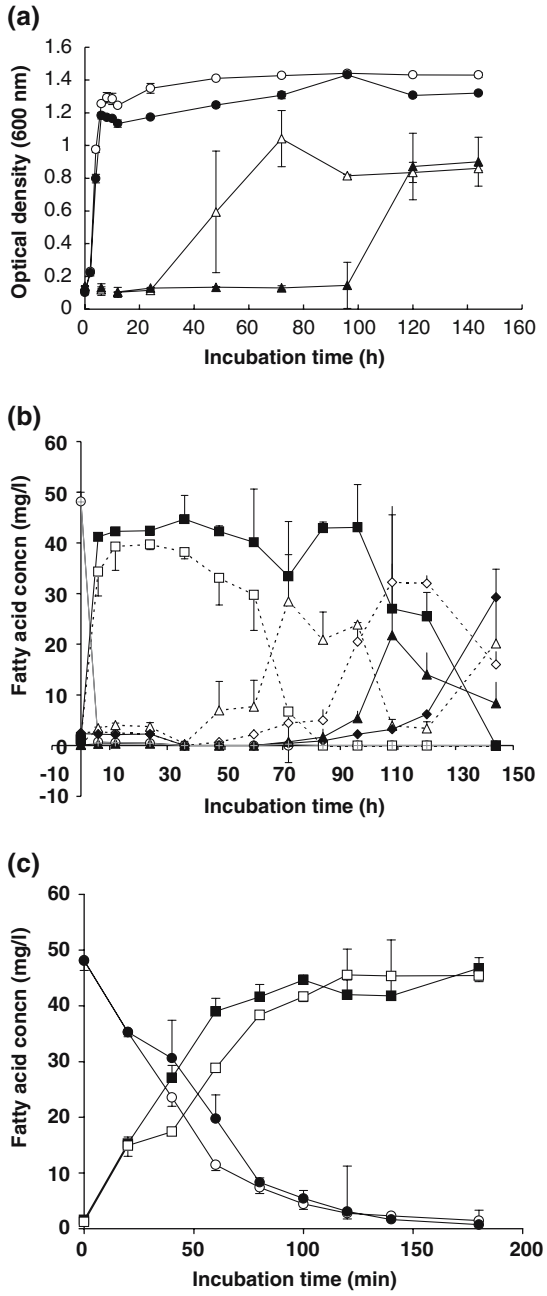

Fig. 2 Influence of sodium lactate on growth of *C. proteoclasticum* P-18 in the presence of 50 μg ml^{-1} LA. Medium M2 contains 70 mM sodium lactate, medium M2-L contains no added lactate. (**a**) Growth in M2-L (○), M2-L + LA (△), M2 (●), M2 + LA (▲); (**b**) fatty acid concentrations in M2-L medium (open symbols) and M2 medium (closed symbols) - LA (○, ●), *cis*-9,*trans*-11–18:2 (□, ■), *trans*-11–18:1 (△, ▲), 18:0 (◊, ♦); (**c**) fatty acid concentrations during initial phase in M2-L medium (open symbols) and M2 medium (closed symbols)—LA (○, ●), *cis*-9,*trans*-11–18:2 (□, ■). Results are means and standard deviations from three cultures

biohydrogenation pathway from LA as possible health-enhancing metabolites in man (Pariza 2004). Thus, it is timely to revisit the microbial basis of this pathway in ruminal bacteria. Previous microbiological studies (Polan et al. 1964; Kemp et al. 1975; Hazlewood et al. 1976) were carried out before the significance of *n*-3 PUFA and CLA were known. They also used radiolabelled fatty acids at low concentrations (2 μg ml^{-1}) that make comparing the activity and quantitative significance of different bacteria difficult. Here, higher concentrations of fatty acids were used with bacteria representative of the wide range of ruminal

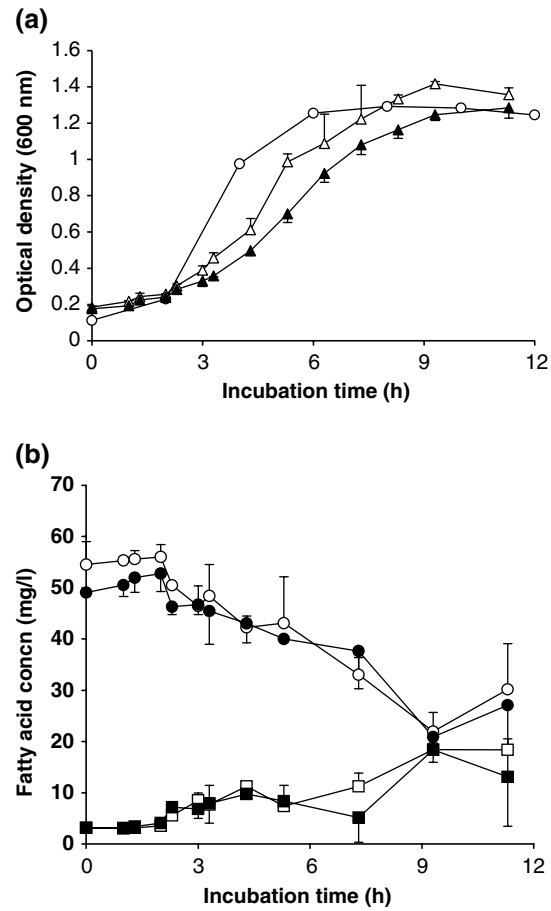

Fig. 3 Influence of sodium lactate on growth of *C. proteoclasticum* P-18 in the presence of 50 μg ml^{-1} *trans*-11–18:1. (**a**) Growth in M2-L (○), M2-L + *trans*-11–18:1 (△), M2 + *trans*-11–18:1 (▲); (**b**) fatty acid concentrations: *trans*-11–18:1 (○,●), 18:0 (□, ■), open symbols—M2-L medium, closed symbols—M2 medium. Results are means and standard deviations from three cultures

bacteria (Edwards et al. 2004), revealing patterns not only of metabolism but toxicity that are relevant to microbial ecology and nutrition. The concentrations used (up to 200 μg ml^{-1}) were chosen for practical reasons, mainly to provide optically clear suspensions when sonicated. These were nevertheless lower than might occur transiently in ruminal digesta in cows grazing fresh grass, where the concentration might reach a peak of 3 mg ml^{-1} (Wąsowska et al. 2006). Other issues, such as fatty acid solubility and compartmentation of bacterial populations, mean that it is very difficult to predict the true concentration of PUFA that bacteria might actually encounter in vivo.

Several species metabolised LA, by at least four possible routes. The most common product was vaccenic acid (VA; *trans*-11–18:1), which was produced by three *Butyrivibrio* strains and two strains of *C. proteoclasticum*. Only *C. proteoclasticum* P-18 produced the CLA, *cis*-9,*trans*-11–18:2 in a 48-h incubation, along with VA and stearic acid. However, as VA is formed via *cis*-9,*trans*-11–18:2 as an intermediate (Harfoot and Hazlewood 1997), the bacteria forming VA might be considered CLA producers that had completed its conversion to VA. Thus, as *C. proteoclasticum* is related closely to *Butyrivibrio* (Kopečný et al. 2003; van de Vossenberg and Joblin 2003), these results are in agreement with the original results of Polan et al. (1964), who investigated more than 20 strains of ruminal bacteria. The most abundant CLA isomer usually found in milk is *cis*-9,*trans*-11–18:2 (Offer et al. 2001; Shingfield et al. 2003). The *cis*-9,*trans*-11 isomer is generally considered to be the main health-promoting CLA for human consumption (Pariza 2004). VA is also desirable as a product flowing from the rumen because VA acts as a substrate for the formation of *cis*-9,*trans*-11–18:2 in the animal's own tissues (Griinari et al. 2000). Thus, the VA producers can be considered beneficial for producing ruminant meat and milk with a healthy fatty acid profile. The finding that *N. frontalis* produces CLA is the first published report of a fungal contribution to CLA formation; however, a preliminary report to this effect was presented by I.S. Nam and P.C. Garnsworthy at the 26th World Congress of the International Society for Fat Research, Prague, 25–28 September 2005. The activity of *N. frontalis* in forming CLA from LA was very small in comparison with *B. fibrisolvens*: about half a 50 μg ml^{-1} concentration of LA was converted to CLA after 96 h. A similar conversion by a culture of *B. fibrisolvens* takes a few minutes.

LA was metabolised in different ways by other bacteria, reflecting a diversity in the way in which different species deal with LA. Oleic acid (*cis*-9–18:1) was formed from LA by *C. aminophilum* and *M. multiacidus*. Ruminal oleic acid producers were not identified previously (Harfoot and Hazlewood 1997). *F. succinogenes* formed a product that appeared to be 16:0, though this was not confirmed by mass spectrometry. Presumptive hydroxy acids were formed by *S. bovis*, which would be consistent with the conclusions of Hudson et al. (2000).

Many isomers of C18:2 other than *cis*-9,*trans*-11–18:2 appear in digesta and milk too (Shingfield et al. 2003). The source of these is not known, either biochemically or microbiologically, nor are their likely effects on health. For example *trans*-11,*cis*-13–18:2, *trans*-7,*cis*-9–18:2 and *trans*-11,*trans*-13–18:2 were the next most abundant CLA isomers found in milk in the Shingfield et al. (2003) study. None of the cultures tested here formed *trans*-11,*cis*-13–18:2, *trans*-7,*cis*-9-18:2 or *trans*-11,*trans*-13–18:2; it is possible they are formed from *cis*-9,*trans*-11-CLA by species other than *Butyrivibrio*. The *trans*-10,*cis*-12 isomer is usually present at low concentrations in digesta and milk (Offer et al. 2001; Shingfield et al. 2003), yet it, or its derived *trans*-10–18:1 biohydrogenation product, can accumulate under certain dietary circumstances, causing milk fat depression, which can be an important problem in the dairy industry (Griinari and Bauman 1999). No isomerisation of LA to the *trans*-10,*cis*-12-CLA isomer was found in any of the bacteria investigated here. *Bifidobacterium*, *Propionibacterium, Lactococcus*, *Streptococcus* and *Lactobacillus* isolates from other habitats have been shown to form *trans*-10,*cis*-12-CLA (Jiang et al. 1998; Ando et al. 2004; Coakley et al. 2003). In the rumen, Kim et al. (2002) found that two strains of ruminal *M. elsdenii* produced the*trans*-10,*cis*-12 isomer from LA. In contrast, neither of the *M. elsdenii* strains analysed here, which included the T81 strain tested by Kim et al.

(2002), formed *trans*-10,*cis*-12-CLA. Our initial purchase of *M. elsdenii* T81 from ATCC produced *trans*-10,*cis*-12-CLA, but it was contaminated with *Streptococcus gordonii*; the negative test reported is from a pure strain supplied subsequently. Thus, the bacteria responsible for *trans*-10,*cis*-12-CLA formation in the rumen must be considered unknown at this time.

The present results therefore appear to confirm what was previously thought by others to be the dominant role of *Butyrivibrio* and related bacteria in fatty acid biohydrogenation, although some questions remain. In addition to the examples of CLA production by unknown organisms just mentioned, DHA and EPA are metabolized in ruminal digesta (AbuGhazaleh and Jenkins 2004; Wąsowska et al. 2006), yet none of the microorganisms tested here had any effect on their concentration. There are many species of bacteria that have been isolated from the rumen (Stewart et al. 1997) which were not screened here. Furthermore, those bacteria whose 16S rRNA genes have been recovered from ruminal contents but which have not yet been cultivated (Whitford et al. 1998; Tajima et al. 1999; Edwards et al. 2004) may be of significance in this respect. Studies are under way in our laboratories to try to correlate ruminal bacterial populations, particularly the *Butyrivibrio* group, with biohydrogenating activity of the mixed population. The protozoa have also been considered to be candidates for biohydrogenation because of their high content of CLA and VA, although no truly protozoal (i.e., not due to associated bacteria) biohydrogenation activity has been demonstrated (Devillard et al. 2006).

It is well known that fatty acids have bacteriostatic and bacteriocidal effects, indeed man has been utilising these properties for centuries. The present study compares for the first time the toxicity of PUFA across a range of the ruminal microflora, including the fungi. Henderson (1973) examined the effects of fatty acids on ruminal bacteria. A *Butyrivibrio* sp. was generally most sensitive to fatty acids, but only saturated and monoenoic acids were included in the study. Oleic acid was much more toxic than the saturated fatty acids. Marounek et al. (2002) found that C-12 and C-14 fatty acids were more toxic to ruminal and rabbit caecal bacteria than other chain lengths, but again the study was of saturated acids and oleic acid. In non-ruminal bacteria, LA and LNA were much more toxic than saturated or monoenoic acids (Galbraith et al. 1971). The present paper covers the effects of several PUFA on a range of ruminal bacterial and fungal species. It emerged that the most sensitive of all were cellulolytic bacteria and fungi, *B. hungatei* and the closely related *C. proteoclasticum*. Henderson (1973) also found that a *Ruminococcus* sp. was sensitive to fatty acids.

There are several possible reasons why unsaturated fatty acids are more toxic than saturated fatty acids. The double bonds alter the shape of the molecule, such that kinked unsaturated fatty acids disrupt the lipid bilayer structure (Keweloh and Heipeiper 1996). Whether multiple double bonds caused more toxicity because they are more sterically disruptive, or because it takes longer to biohydrogenate the whole molecule, is not clear from this study. Also it is not clear that the toxicity was necessarily a membrane effect. The extent to which cell integrity was disrupted by LA, as measured by PI fluorescence, in different species did not correlate with the inhibition of growth. An enhanced toxicity of PUFA in the presence of sodium lactate was found here in *C. proteoclasticum*. This was obvious from the growth curve (Fig. 2) even though it had not been apparent by comparing Tables 2 and 3 because of the timing of the observations. Similar increases in lag phases when LA and sodium lactate were combined were reported in other strains related to *Butyrivibrio* (McKain et al. 2004). Lactate appears to have a significant role in *E. ruminantium* too, effectively protecting the bacterium from the toxic effects of LA. White et al. (1970) found that one of the original '*Fusocillus*' isolates hydrogenated oleic acid more extensively when grown in a lactate-containing medium. In common with *Butyrivibrio*, *E. ruminantium* and *C. proteoclasticum* are butyrate producers. Thus, there appears to be a metabolic link between lactate, butyrate metabolism and fatty acid biohydrogenation. Perhaps the different sensitivities are due to different mechanisms of butyrate production (Diez-Gonzalez et al. 1999). Lactate appeared to inhibit hydrogenation of CLA, but not VA, in *C. proteoclasticum*, indicating that

these two reductase activities may be carried out by different enzymes and that only the former is affected by sodium lactate. It is worth noting that all the experiments were done here with growing bacteria and fungi. Stationary-phase bacteria were much less active in biohydrogenation than growing cells (Chaudhary et al. 2004).

Major advances have been made in achieving the desired changes in fatty acid content of meat and milk experimentally, via dietary manipulation in ruminants, generally by adding oils containing unsaturated fatty acids to the diet (Jenkins 1994; Offer et al. 2001; Scollan et al. 2001; Shingfield et al. 2003). Often, benefits can be attributed directly to the oils which were added to the diet escaping the rumen without being hydrogenated by ruminal microorganisms. However, other dietary changes, such as the inclusion of fish oil, seem to alter the fatty acid composition of the products via a direct effect on biohydrogenating activity in the rumen. This paper now indicates that the basis of these effects may be a differential toxicity of different PUFA to ruminal bacteria, particularly those in the *Butyrivibrio* group. It will be important to determine the mechanism by which PUFA exert their toxicity in *C. proteoclasticum* because selective suppression of this species might be expected to decrease the degree of saturation of fatty acids leaving the rumen and being incorporated into milk and meat. Furthermore, all the main species that comprise the ruminal cellulolytic flora, i.e., *F. succinogenes*, *Ruminococcus* spp. and the anaerobic fungi, appear vulnerable to inhibition by PUFA. Any strategy for manipulating biohydrogenation using PUFA would therefore have to take care that fibre digestion was not compromised.

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