

Mechanism of conjugated linoleic acid and vaccenic acid formation in human faecal suspensions and pure cultures of intestinal bacteria

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Faecal bacteria from four human donors and six species of human intestinal bacteria known to metabolize linoleic acid (LA) were incubated with LA in deuterium oxide-enriched medium to investigate the mechanisms of conjugated linoleic acid (CLA) and vaccenic acid (VA) formation. The main CLA products in faecal suspensions, rumenic acid (*cis*-9,*trans*-11-CLA; RA) and *trans*-9,*trans*-11-CLA, were labelled at C-13, as were other 9,11 geometric isomers. Traces of *trans*-10,*cis*-12-CLA formed were labelled to a much lower extent. In pure culture, *Bifidobacterium breve* NCFB 2258 formed labelled RA and *trans*-9,*trans*-11-CLA, while *Butyrivibrio fibrisolvens* 16.4, *Roseburia hominis* A2-183^T, *Roseburia inulinivorans* A2-192^T and *Ruminococcus obeum*-like strain A2-162 converted LA to VA, labelled in a manner indicating that VA was formed via C-13-labelled RA. *Propionibacterium freudenreichii* subsp. *shermanii* DSM 4902^T, a possible probiotic, formed mainly RA with smaller amounts of *trans*-10,*cis*-12-CLA and *trans*-9,*trans*-11-CLA, labelled the same as in the mixed microbiota. Ricinoleic acid (12-OH-*cis*-9-18:1) did not form CLA in the mixed microbiota, in contrast to CLA formation described for *Lactobacillus plantarum*. These results were similar to those reported for the mixed microbiota of the rumen. Thus, although the bacterial genera and species responsible for biohydrogenation in the rumen and the human intestine differ, and a second route of RA formation via a 10-OH-18:1 is present in the intestine, the overall labelling patterns of different CLA isomers formation are common to both gut ecosystems. A hydrogen-abstraction enzymic mechanism is proposed that may explain the role of a 10-OH-18:1 intermediate in 9,11-CLA formation in pure and mixed cultures.

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INTRODUCTION

Since Pariza and colleagues (Ha *et al.*, 1987; Pariza, 2004) first identified the anticarcinogenic component of beef as rumenic acid (*cis*-9,*trans*-11 conjugated linoleic acid, RA), a conjugated isomer of linoleic acid (*cis*-9,*cis*-12-18:2, LA), considerable research has been directed towards understanding the formation, physiological effects and potential health benefits of conjugated linoleic acid (CLA; of which RA is one stereoisomer) in a range of biological models (Kritchevsky, 2000; Wahle *et al.*, 2004; Bauman *et al.*, 2005). Isomers of CLA were first discovered as intermediates during the metabolism of LA to stearic acid (18:0, SA) by ruminal bacteria (Polan *et al.*, 1964; Harfoot

& Hazlewood, 1997). Subsequently it was found that LA was also converted to RA by human intestinal bacteria (Kamlage *et al.*, 1999; Coakley *et al.*, 2003; Devillard *et al.*, 2007). Thus, in addition to systemic health benefits from CLA in the diet, CLA formation in the human intestine could promote health. It is therefore important to understand how CLA is formed and metabolized by intestinal bacteria.

CLA formed in the intestine might be absorbed and contribute to systemic CLA. However, experiments with germ-free rats inoculated with a human faecal microbiota and fed a diet enriched with sunflower-seed oil indicated that no benefit accrued in terms of tissue concentrations of CLA (Kamlage *et al.*, 1999). Even if CLA absorption from the intestine is minimal, there may be *in situ* benefits from intestinal CLA production, however. In mouse models of inflammatory bowel disease, CLA was shown to exhibit anti-inflammatory properties via endoplasmic and nuclear mechanisms (Bassaganya-Riera *et al.*, 2002, 2004). Further studies have demonstrated that CLA exerts anti-carcino-

Abbreviations: CLA, conjugated linoleic acid; DMOX, 4,4-dimethyloxazoline; FAME, fatty acid methyl esters; HFA, hydroxy fatty acid; LA, linoleic acid; MPE, mol% excess; RA, rumenic acid; SA, stearic acid; VA, vaccenic acid.

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genic activity in the rat colon (Nichenamela *et al.*, 2004) and exhibits anti-proliferative properties on the growth of human colon cancer cells *in vitro* (Kemp *et al.*, 2003). Therefore, mechanisms by which CLA might be delivered to and formed in the intestine have important implications for long-term human health.

The precise isomer(s) that is formed has importance too, given the very different biological effects of RA and other isomers, particularly *trans*-10,*cis*-12-CLA (Pariza, 2004; Bauman *et al.*, 2005). Such information could have particular relevance to patients using the slimming drug tetrahydrolipstatin (orlistat; Hauptman *et al.*, 2000) or similar agents that prevent lipid absorption in the human small intestine. Large amounts of lipid reach the large intestine in patients using orlistat, sometimes with deleterious consequences (Chanoine *et al.*, 2005). Conversion of LA to RA under such circumstances might be beneficial, but the formation of 10,12 isomers detrimental (Pariza, 2004; Bauman *et al.*, 2005).

Given these concerns, we have re-examined the mechanism of CLA formation by intestinal bacteria, using methods employed recently to establish the mechanisms involved in CLA isomer formation by ruminal bacteria (Wallace *et al.*, 2007). The rumen seems to possess a single group of bacteria, closely related to *Butyrivibrio fibrisolvens*, that play a predominant role in biohydrogenation of dietary fatty acids (Polan *et al.*, 1964; Harfoot & Hazlewood, 1997; Wallace *et al.*, 2006). In contrast, several colonic species, of which *Roseburia* would usually be most numerous, form

RA and/or vaccenic acid (VA) in the human intestine (Devillard *et al.*, 2007). Furthermore, some *Roseburia* spp. form 10-OH-18:1 rather than RA from LA (Devillard *et al.*, 2007). This hydroxy fatty acid then serves as a substrate for RA synthesis by other species in the mixed microbiota. Thus, there appeared to be two potential routes of RA formation in the human intestine (Fig. 1). This investigation was undertaken to establish the relative importance of the different mechanisms of formation of different CLA isomers by mixed faecal microbiota and also pure cultures of intestinal bacteria, and to elucidate the molecular mechanism of formation of different CLA isomers.

METHODS

Incubations with suspensions of mixed human faecal bacteria.

Faecal samples were obtained from omnivorous volunteers A, C and D aged 31 to 39 years, all consuming Western-style diets, and a vegetarian, volunteer B, aged 55 years. Freshly voided faeces were collected in sterile containers, and stored on ice for a maximum of 1 h before being incubated with LA (Sigma). Faecal samples were homogenized, and subsamples (between 2 and 4 g wet weight) were transferred to sterile 50 ml tubes and diluted in 0.1 M potassium phosphate buffer, pH 7.0, made up in 40:50 (v/v) water/deuterium oxide (99.8% deuterium-enriched water; Norsk Hydro) to a final concentration of 10% (wet w/v). Incubations were carried out under CO₂ in 12.5 × 1.6 cm glass tubes, closed with screw caps fitted with butyl rubber septa (Bellco Biotechnology). An emulsion of LA in water (25 mg ml⁻¹) was prepared by sonication and 100 µl was added to each tube. Five millilitres of 10% (v/v) suspension was added, and the tubes were incubated at 37 °C. Subsamples (1 ml) were removed after 0, 1 and 4 h, transferred into clean tubes and heated at 100 °C

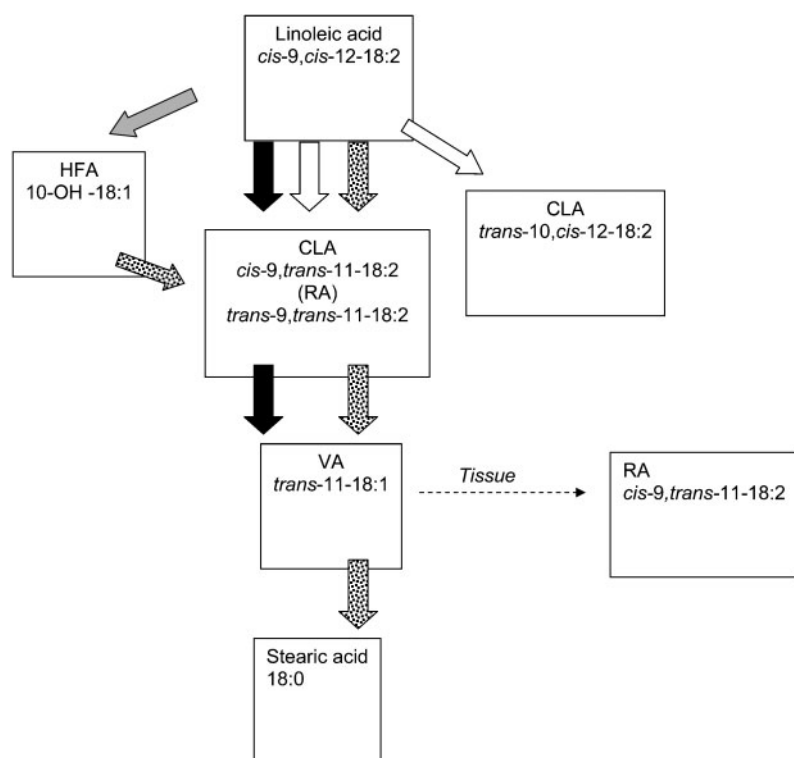


Fig. 1. LA metabolism by human faecal bacteria, adapted from Devillard *et al.* (2007). The white arrows represent the bacterial activity of *Lactobacillus*, *Propionibacterium* and *Bifidobacterium* species leading to the formation of CLA. The grey arrows represent the bacterial activity of some *Lactobacillus*, *Propionibacterium* and *Bifidobacterium* species, some *Clostridium*-like bacteria of the clusters IV (e.g. *Eubacterium siraeum*) and XIVa (e.g. *Roseburia intestinalis* and *Ros. faecis*) leading to the formation of HFA. The black arrows represent the bacterial activity of *Clostridium*-like bacteria of cluster XIVa leading to the formation of VA (e.g. *Ros. hominis* and *Ros. inulinivorans*). The dotted arrows represent activities observed in faecal microbiota, but for which the bacterial species responsible are still unknown. HFA, hydroxy fatty acid; CLA, conjugated linoleic acid, of which rumenic acid (RA) is one geometric isomer; VA, vaccenic acid.

for 10 min. Heated samples were stored at -20°C prior to lipid extraction and fatty acid determinations.

To establish the possible role of hydroxy fatty acids (HFA) as a precursor to RA formation and establish the ability of human microbiota to metabolize HFA, similar incubations were carried out with ricinoleic acid (*cis*-9,12-hydroxy-18:1).

Bacteria and growth conditions. The bacteria selected in this study are known to metabolize LA (Devillard *et al.*, 2007). *Bifidobacterium breve* NCFB 2258 was isolated originally from the human infant intestine (Coakley *et al.* 2003). *Butyrivibrio fibrisolvens* 16/4 was isolated from human faeces (Rumney *et al.*, 1995). *Roseburia hominis* A2-183^T and *Roseburia inulinovorans* A2-194^T were isolated in a study of butyrate producers from human faeces (Barcenilla *et al.*, 2000) and subsequently reclassified (Duncan *et al.*, 2006). Strain A2-162 was isolated in the same study; based on 16S RNA phylogenetic analysis it is closely related to *Ruminococcus obeum* (Duncan *et al.*, 2007). *Propionibacterium freudenreichii* subsp. *shermanii* DSM 4902^T is the type strain of this species (obtained from DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), originally isolated from cheese (Van Niel, 1928) and considered to be a potential probiotic organism.

The growth media were based on the liquid form of M2 medium (Hobson, 1969). All transfers and incubations were carried out under O_2 -free CO_2 at 37°C in Bellco tubes as described previously (Devillard *et al.*, 2007). Inoculum volumes were 5% (v/v) of a fresh culture into 5 ml of medium. One batch of medium was made up using unlabelled water, the other prepared with 50% (v/v) deuterium oxide. LA was added to a final concentration of 50 mg l^{-1} . In other cultures, ricinoleic acid (*cis*-9-12-OH-18:1) was added to the same concentration. Fatty acids were prepared as a separate solution, sonicated for 4 min in a small volume of medium and added to the medium before dispensing and autoclaving. Triplicate 5 ml cultures were grown for 24 h. Subsamples (1 ml) were removed for protein analysis (Herbert *et al.*, 1971) and analysis of the deuterium enrichment in water. Thereafter, $100\ \mu\text{l}$ of nonadecanoic acid (200 mg l^{-1} in methanol) was added and the tubes were stored at -70°C and subsequently freeze-dried.

Fatty acid extraction and analysis. Extraction of total fatty acids, preparation of fatty acid methyl esters (FAME) and 4,4-dimethyloxazoline (DMOX) derivatives and analysis by GC-MS were performed using standard procedures (Wallace *et al.*, 2007). Enrichment in the *m/z* ($m+1$), ($m+2$) and ($m+3$) isotopomers (molecular ion $+1$ $+2$ and $+3$, respectively) was determined by GC-MS of FAME (Wallace *et al.*, 2007). Analysis of DMOX derivatives by GC-MS was used to identify the position of deuterium labelling in the fatty acid moiety. Enrichment in water was determined by gas isotope ratio mass spectrometry (GIRMS) (Wallace *et al.*, 2007).

Data analysis. Three replicate measurements were made in incubations with faecal suspension samples from each of four human donors. Experimental data were analysed by ANOVA for repeated measures with a model that included the fixed effect of incubation time and random effects of volunteer assuming a compound symmetry covariance structure using the mixed linear model procedure of Statistical Analysis Systems software package version 8.2 (SAS Institute). This experimental design accounts for potential autocorrelation between sequential measurements. Measurements of fatty acid concentrations over time during incubation of LA with pure cultures of intestinal bacterial were analysed by ANOVA for repeated measures with a model that included the fixed effect of incubation time and random effects of replicate assuming a compound symmetry covariance structure. Least-square means \pm SE are reported and effects were considered significant at $P < 0.05$. Enrichment of ($m+1$) isotopomers was calculated from the *m/z* ratios at m , $m+1$, $m+2$ and $m+3$ by deconvolution according to Campbell (1974). Natural

abundance was calculated from the isotopomer distribution of LA in zero-time samples.

RESULTS

Metabolism of LA in faecal suspensions

The rate of disappearance of LA in faecal suspensions was similar during incubations with different samples, declining by about two-thirds in 4 h from an initial concentration of 500 mg l^{-1} , resulting in the accumulation of CLA isomers and smaller concentrations of VA (Fig. 2). However, the background concentration of SA varied between samples (Fig. 2). Concentrations of SA increased with time in the sample from volunteer B, the vegetarian, which also exhibited the highest zero-time SA concentrations.

Detailed analysis of the distribution of CLA isomers synthesized after 4 h incubations with LA revealed that RA was the most abundant isomer, with lower concentrations of *trans*-9,*trans*-11-CLA (on average 17% of RA concentrations) also being formed (Table 1). Trace amounts of *trans*-10,*cis*-12-CLA were also present, but at concentrations below 3% of total CLA.

Mass spectra of FAME prepared from samples collected after 4 h incubations indicated that 9,11 geometric isomers of CLA were labelled at $m+1$. Independent analysis of the enrichment in water allowed the ratio of moles per cent excess (MPE) in samples to be compared with the MPE of water. All samples of water had a similar enrichment (46.0%, SE 0.36%). Mean labelling (MPE sample/MPE water) of 9,11 geometric CLA isomers was 0.689, SE 0.042 (Table 1) whilst labelling at $m+2$ was not detected. In contrast, mean labelling at $m+1$ for *trans*-10,*cis*-12-CLA was 0.310, SE 0.056. There was no evidence of differences in labelling at $m+1$ in samples collected at 1 or 4 h (data not presented). Enrichment of VA and SA end products could not be determined due to their high concentrations in zero-time samples.

The mass spectrum of the DMOX derivative of RA (Fig. 3) indicated enrichment in ion fragments from the molecular ion above *m/z* 262. The occurrence of ion fragment isotopomers with *m/z* lower than 262 was comparable to the natural abundance of about 20% MPE. These data provide clear evidence that deuterium was labelled on C-13 of the fatty acid moiety (Fig. 3). Mass spectra of other 9,11 geometric CLA isomers revealed a similar pattern of enrichment as indicated by GC-MS analysis of the DMOX derivative of *trans*-9,*trans*-11 CLA (Fig. 3). Due to the low concentration of *trans*-10,*cis*-12-CLA following the conversion of FAME to DMOX derivatives, it was not possible to identify the position of the small amount of label.

Formation of CLA and VA by pure cultures of intestinal bacteria

Six bacterial species shown to metabolize LA (Devillard *et al.*, 2007) were grown in the presence of 50 mg LA l^{-1} in

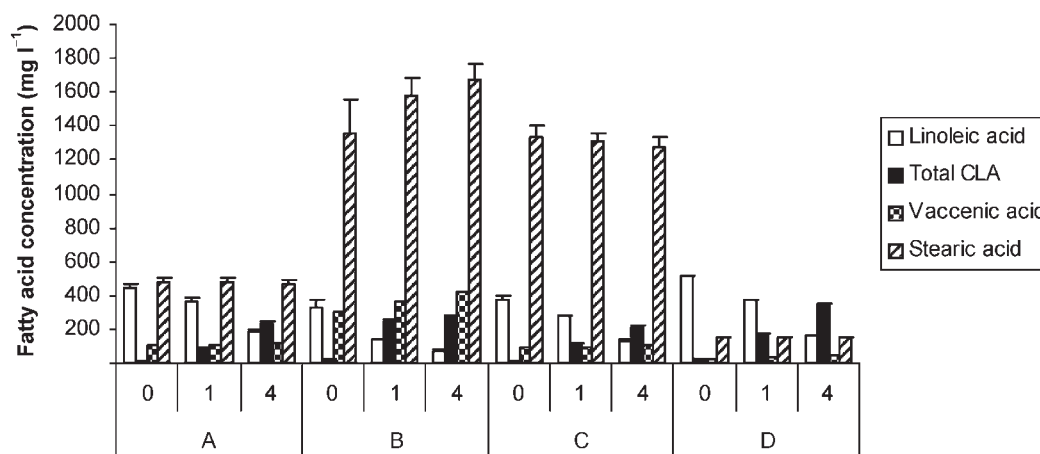


Fig. 2. LA metabolism in suspensions of faecal bacteria from four human volunteers, A–D. Samples were removed at 0, 1 and 4 h for fatty acid analysis. Mean incubation protein content 8.6, SE 2.1, mg ml⁻¹. Values represent the means of three incubations. Mean SE values were 15.2, 7.29, 12.3 and 59.4 (mg l⁻¹) for linoleic acid, total CLA, vaccenic acid, and stearic acid, respectively. The initial concentration of LA was 500 mg l⁻¹.

deuterium oxide-enriched medium and samples were collected for fatty acid determinations in the stationary phase. *Bif. breve* and *P. freudenreichii* subsp. *shermanii* produced RA as the predominant product, with smaller concentrations of *trans*-9,*trans*-11-CLA also being formed (Table 2). The latter species also produced a small quantity of *trans*-10,*cis*-12-CLA. LA was metabolized to VA in the four other species, with only minor amounts of CLA being detected. No SA was formed by any culture during incubations with LA.

The MPE enrichment of deuterium in the medium water was determined as 45.2%, SE 1.25%. The RA formed by *P. freudenreichii* subsp. *shermanii* was labelled 0.601, SE 0.025, in the *m*+1 isotopomer, while the *trans*-9,*trans*-11 isomer was also labelled to a similar extent (Table 2). Labelling was 0.011, SE 0.004, in the *m*+2 isotopomer. The other isomer formed, *trans*-10,*cis*-12-CLA, was unlabelled. Values in *Bif. breve* cultures were similar for RA, but a higher enrichment was determined for *trans*-9,*trans*-11-CLA (Table 2). The mass spectrum of labelled RA produced by pure cultures

Table 1. Concentrations and enrichment in *m*+1 isotopomers of CLA formed from LA in mixed faecal bacteria suspended in buffer containing deuterated water

The initial concentration of LA was 500 mg l⁻¹. The mean mol% excess (MPE) enrichment in water was 46.0, SE 0.36. Values are means ± SE (*n*=3) after 4 h incubations with LA. Mean ± SE concentration in 0 h incubations were 12.3 ± 3.00, 0.57 ± 0.176, 0.57 ± 0.060, 1.69 ± 1.883 and 2.40 ± 1.174 mg l⁻¹ for *cis*-9,*trans*-11 (RA), *trans*-9,*cis*-11, *trans*-10,*cis*-12, *cis*-9,*cis*-11 and *trans*-9,*trans*-11, respectively.

Volunteer	Concentration (mg l ⁻¹)									
	<i>cis</i> -9, <i>trans</i> -11 (RA)		<i>trans</i> -9, <i>cis</i> -11		<i>trans</i> -10, <i>cis</i> -12		<i>cis</i> -9, <i>cis</i> -11		<i>trans</i> -9, <i>trans</i> -11	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
A	185	14.5	4.4	0.53	6.1	0.36	7.8	0.51	30.5	2.76
B	201	14.5	5.4	0.90	2.4	0.09	15.6	1.43	44.5	6.78
C	176	9.0	3.2	0.37	1.6	0.09	6.1	0.32	23.6	2.05
D	286	5.6	4.9	0.58	2.5	0.06	9.0	0.93	45.1	3.87
MPE ratio (MPE in CLA isomer/MPE in water)										
A	0.746	0.0090	0.605	0.0028	0.143	0.0661	0.670	0.0689	0.740	0.0082
B	0.750	0.0065	0.700	0.0455	0.378	0.0264	0.453	0.0378	0.774	0.0147
C	0.758	0.0009	0.633	0.0218	0.291	0.0135	0.771	0.0198	0.762	0.0182
D	0.693	0.0208	0.612	0.0301	0.367	0.0172	0.678	0.0225	0.678	0.0225

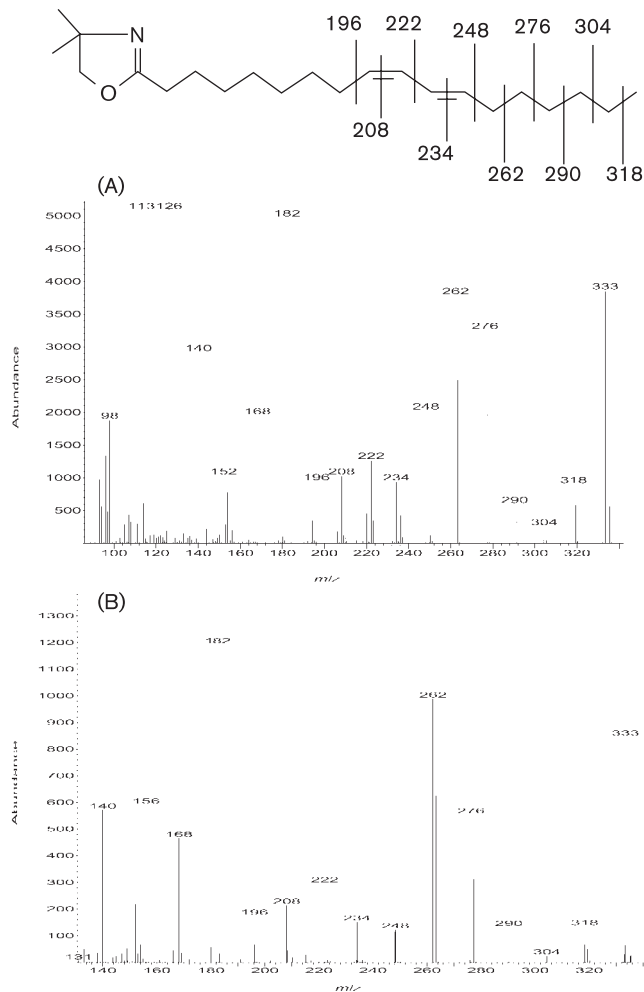


Fig. 3. Mass spectra of 4,4-dimethylloxazoline derivatives of (A) *cis*-9,*trans*-11 CLA (rumenic acid; RA) and (B) *trans*-9,*trans*-11 CLA synthesized from linoleic acid (LA) during 4 h incubations with suspensions of mixed faecal bacteria. Relative abundance of ion fragments at *m/z* 262 and 263 indicate that ^2H is located on C-13 of the fatty acid moiety.

was similar to that of mixed microbiota, indicating that labelling was located on C-13 (spectra not shown). However, the spectra of the DMOX derivative of *trans*-9,*trans*-11-CLA were less informative. Ion fragments corresponding to C-13 (*m/z* 262 and 263) appeared to indicate small amounts of label in the product from *P. freudenreichii* subsp. *shermanii*, whereas the *trans*-9,*trans*-11-CLA peak in *Bif. breve* samples contained a higher enrichment than RA. It is possible that, because *trans*-9,*trans*-11-CLA and *trans*-10,*trans*-12-CLA co-elute during GC analysis, the labelling pattern was due to the contributions of both CLA isomers.

Enrichment of VA was comparable for all four VA-producing species, *Bu. fibrisolvens*, *Ros. hominis*, *Ros. inulinivorans* and *Rum. obeum* (Table 2). The *m*+1

isotopomers had an MPE ratio of 0.879–0.941 relative to water, while the ratio for *m*+2 isotopomers varied between 0.214 and 0.303. There was no evidence of labelling at *m*+3. Mass spectra of DMOX derivatives of VA revealed a clear pattern of labelling in ion fragments of *m/z* 210 and 211 that was absent at *m/z* of 196 and 197 (Fig. 4). Low abundances of ions corresponding to C-10 to C-12 of the fatty acid moiety did not permit discrimination between singly- and doubly-labelled ion fragments. Thus, the DMOX spectrum in Fig. 4 clearly indicates a label located on C-9 of VA. Furthermore, the relative abundance of ions at *m/z* 264, 265 and 266 also suggests additional enrichment at C-13, which would have arisen from reduction of the labelled VA precursor. Comparison of the ratio of ions at *m/z* 211/210 and 265/264 is consistent with this conclusion.

Metabolism of ricinoleic acid in mixed and pure cultures

Ricinoleic acid was metabolized rapidly by mixed faecal bacteria, >80% of an initial concentration of 500 mg l^{-1} being lost in 8 h (not shown). No CLA isomers, VA or oleic acid were liberated during incubation with ricinoleic acid. Products formed eluted with a retention time close to that of ricinoleic acid. GC-MS analysis of FAME revealed the occurrence of abundant ion fragments consistent with the presence of a hydroxyl group, but due to the inability to detect the molecular ion or ion fragments in the high *m/z* region it was not possible to identify unequivocally the structure of the compounds formed. The pure cultures used in the present study did not metabolize ricinoleic acid.

DISCUSSION

Findings from this study advance the understanding of LA metabolism by human intestinal bacteria in three main respects. Firstly, RA is the main CLA formed in the mixed microbiota, while other geometric 9,11 isomers are also significant products but *trans*-10,*cis*-12-CLA is a minor metabolite. Secondly, the 9,11 isomers are formed in the mixed microbiota by a mechanism that results in the incorporation of a H atom from water at C-13. Thirdly, pure cultures produce RA and VA from LA by a mechanism similar to that in the mixed human intestinal microbiota, although the synthesis of minor CLA isomers may arise via different mechanisms for particular bacterial species. The results also indicate that, despite the large differences in the composition of the microbial communities in the human intestine and the rumen (Edwards *et al.*, 2004; Eckburg *et al.*, 2005), and the presence of an additional route of RA synthesis via a HFA in the human intestine (Devillard *et al.*, 2007), the final labelling patterns of CLA isomers formed by the two ecosystems are similar.

Evidence of CLA formation by intestinal bacteria was first obtained indirectly in the experiments of Chin *et al.* (1994), who noted that germ-free rats had a lower incorporation of

Table 2. Metabolism of LA and deuterium enrichment in products following growth of pure cultures of intestinal bacteria in medium enriched with deuterium oxide

The initial concentration of LA was 50 mg l⁻¹. No LA was detected after 24 h incubation in any sample. The mean MPE enrichment in water was 45.2, SEM 1.25. Values are means ± SE (n=3) after 24 h incubations with LA. Blank cells mean that, due to the low concentration of the fatty acid, the MPE value could not be measured.

Bacterium	Concentration (mg l ⁻¹)													
	<i>cis-9,trans-11</i> (RA)		<i>trans-9,cis-11</i>		<i>trans-10,cis-12</i>		<i>cis-9,cis-11</i>		<i>trans-9,trans-11</i>		VA			
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE		
<i>Bif. breve</i>	25.7	2.75	0.41	0.116	0.03	0.024	0.54	0.034	9.96	2.392	0	0		
<i>Bu. fibrisolvans</i>	0.37	0.108	0.0	0.00	0	0	0	0	0.54	0.211	28.2	0.64		
<i>P. freudenreichii</i> subsp. <i>shermanii</i>	37.6	0.96	0.18	0.018	3.17	0.109	0.43	0.028	2.26	0.098	0	0		
<i>Ros. hominis</i>	0	0	0	0	0	0	0	0	0.40	0.090	23.5	0.29		
<i>Ros. inulinivorans</i>	0.30	0.090	0.26	0.018	0	0	0	0	0.24	0.027	34.9	5.08		
<i>Rum. obeum</i>	0.67	0.052	0.0	0.00	0	0	0	0	0.74	0.186	20.9	3.10		
Bacterium	MPE ratio (MPE in CLA isomer/MPE in water)													
	<i>cis-9,trans-11</i> (RA)		<i>trans-10,cis-12</i>		<i>cis-10,trans-12</i>		<i>trans-9,trans-11</i>		VA, m + 1		VA, m + 2		VA, m + 3	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
<i>Bif. breve</i>	0.632	0.0370					0.990	0.0293						
<i>Bu. fibrisolvans</i>									0.941	0.0355	0.274	0.0259	0	0
<i>P. freudenreichii</i> subsp. <i>shermanii</i>	0.601	0.0254	0	0			0.595	0.0488						
<i>Ros. hominis</i>									0.915	0.0329	0.224	0.0084	0	0
<i>Ros. inulinivorans</i>									0.934	0.0053	0.303	0.0226	0	0
<i>Rum. obeum</i>									0.879	0.0116	0.214	0.0248	0	0



Fig. 4. Mass spectrum of 4,4-dimethyloxazoline derivative of VA produced from LA by *Ros. inulinovorans*. Relative abundance of ion fragments at m/z 210 and 211 indicates that ^2H is located on C-9 of the fatty acid moiety. Comparison of ion fragments at m/z 264, 265 and 266 reveals additional enrichment of ^2H on C-13 of the fatty acid moiety. *Bu. fibrisolvens*, *Ros. hominis* and *Rum. obeum* gave similar spectra.

CLA in liver, lung, kidney, skeletal muscle and abdominal adipose tissue than conventional animals. Kamlage *et al.* (1999, 2000) measured the ability of faecal suspensions to produce CLA from LA, but did not identify the structure of CLA isomers formed. This study provides unequivocal evidence that RA is the main CLA produced during LA metabolism by the human intestinal microbiota, with lower amounts of *trans*-9,*trans*-11-CLA also being formed. Other isomers, including *trans*-10,*cis*-12-CLA, were only minor products. This is a significant finding, since the anti-proliferative and anti-inflammatory properties of RA are well established (Ha *et al.*, 1987; Pariza, 2004; Wahle *et al.*, 2004; Bauman *et al.*, 2005), while the physiological effects of *trans*-10,*cis*-12-CLA on gut health are potentially less beneficial (Kritchevsky, 2000; Pariza, 2004). Far fewer data are available on the role of *trans*-9,*trans*-11-CLA, but *in vitro* studies with various human cell lines point towards this isomer exhibiting activity similar to or higher than RA (Coakley *et al.*, 2006).

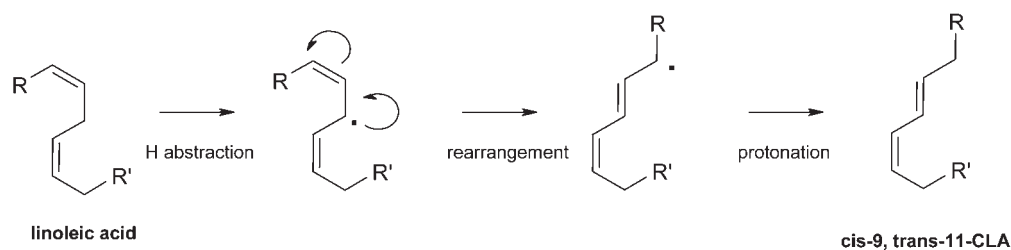
CLA formation has been studied in three other groups of bacteria, namely ruminal bacteria, lactic acid producers predominantly from dairy products, and *Propionibacterium acnes*. RA, *trans*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA were the major CLA intermediates formed from LA in ruminal digesta, with traces of *trans*-9,*cis*-11-CLA, *cis*-9,*cis*-11-CLA and *cis*-10,*cis*-12-CLA (Wallace *et al.*, 2007). In contrast, *trans*-10,*cis*-12-CLA was formed in only trace quantities by the human intestinal microbiota. In the rumen, the ratio of isomers formed seems to depend on pH (Choi *et al.*, 2005), possibly because different bacterial species producing various isomers have specific ranges in

pH for optimal growth. It is possible that this may also hold true for bacteria in the human intestine. The CLA isomers formed by human intestinal bacteria contrast even more with those produced during LA metabolism by *Lactobacillus plantarum* and related species, where *trans*-9,*trans*-11-CLA is often the most abundant isomer formed (Ogawa *et al.*, 2001, 2005). *P. acnes*, by contrast, produces only *trans*-10,*cis*-12 CLA (Liavonchanka *et al.*, 2006). The formation of CLA by *P. freudenreichii* subsp. *shermanii* reported here appears to be unique thus far, in that both 9,11 and 10,12 geometric isomers are formed, indicating that the two mechanisms of CLA formation are not mutually exclusive. It would be important to understand the regulation of both synthetic pathways in the same organism.

Mass spectrometric analysis indicated that a deuterium atom from deuterated water was incorporated into C-13 of RA and *trans*-9,*trans*-11-CLA in both mixed human microbiota and pure cultures. Bacteria that converted LA to VA had a labelling pattern suggesting that this was derived via the reduction of a C-13-labelled RA intermediate. Concentrations of *trans*-10,*cis*-12-CLA were too low in the mixed microbiota to make an assessment of labelling, but data from incubations of LA with pure cultures indicated that the labelling of *trans*-10,*cis*-12-CLA was much lower compared with RA. This is a pattern that has been observed previously in pure and mixed cultures of ruminal bacteria (Kepler *et al.*, 1971; Wallace *et al.*, 2007).

The labelling pattern provides an indication of the enzymic mechanism of CLA formation. CLA could be formed by a direct isomerization (Liavonchanka *et al.*, 2006), a hydration/dehydration mechanism (Ogawa *et al.*, 2001, 2005), or a hydrogen-abstraction mechanism involving a radical intermediate (Wallace *et al.*, 2007). From the data presented here and from elsewhere (Liavonchanka *et al.*, 2006; Wallace *et al.*, 2007), it seems clear that 10,12 CLA isomers are formed by an isomerase mechanism that does not involve proton exchange with water. The mechanism of RA formation remains uncertain, however. Our original interpretation of the labelling pattern from ruminal bacteria (Wallace *et al.*, 2007), which is very similar to that found here with human intestinal bacteria, was that RA synthesis proceeded with no hydroxy intermediate, resulting in labelling on C-13 (Fig. 5A). The fatty acid that we considered the most likely intermediate of a hydration/dehydration mechanism, ricinoleic acid (12-OH-18:1), was not converted to RA in the rumen. A hydrogen-abstraction mechanism with a radical intermediate was therefore proposed (Wallace *et al.*, 2007). That the same pattern occurs in the mixed intestinal microbiota, where some species form 10-OH-18:1 that in turn other species convert to RA, is consistent with two possible interpretations. One is that the direct mechanism predominated over the cross-species hydration/dehydration one, with the latter being undetectable. The other is that the 10-OH-18:1 is metabolized also by a hydrogen-abstraction mechanism (Fig. 5B). Indeed, it is tempting to speculate that, in those

A. Possible hydrogen-abstraction mechanism of ruminic acid formation



B. Possible hydration-dehydration mechanism of ruminic acid formation

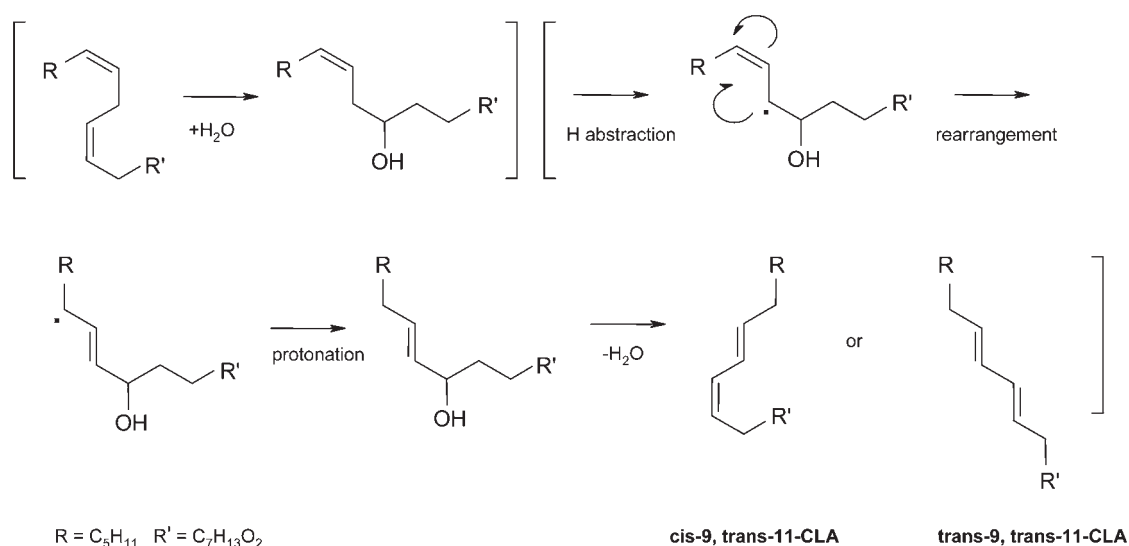


Fig. 5. Two potential mechanisms of RA formation. Both involve the abstraction of H from C-11, with a rearrangement that leads to the assimilation of H from water on C-13. The 'direct' mechanism (A) involves no hydroxy intermediate. The hydration/dehydration mechanism involves firstly the formation of 10-OH-18:1, from which H at C-11 is abstracted and hydration then occurs (B). The mechanisms could occur in two species (two sets of brackets), or possibly within a single species.

species that form RA and/or VA, 10-OH-18:1 is a transient intermediate that has not yet been detected. Thus, the unlikely scenario that some species of *Roseburia* metabolize LA by one route (the 'direct' route to RA then VA), while others metabolize LA by another (to 10-OH-18:1) (Devillard *et al.*, 2007), would no longer be required. In the latter case, all *Roseburia* spp. would form 10-OH-18:1, with only some species continuing the metabolism further by the rearrangement mechanism. However, the finding that VA-producing species do not metabolize 10-OH-18:1 to RA or VA tends to favour the former interpretation, of a predominant direct mechanism.

The major difference between the involvement of 10-OH-18:1 in CLA formation by *L. plantarum* and by human intestinal bacteria is that the 12-OH-18:1 fatty acid, ricinoleic acid, could be used to form RA in the former (Ando *et al.*, 2003) but not the latter species (Devillard *et al.*, 2007). Whether this indicates that the systems are fundamentally different, or only that *L. plantarum* has

additional enzymic potential to metabolize ricinoleic acid to another precursor of CLA formation, is unclear at this stage.

It is concluded that, even though the microbial species responsible for LA metabolism in the human intestine and the rumen are significantly different, and more than one route for RA synthesis is known in the former, the predominant mechanisms of CLA and VA formation in the two gut ecosystems are similar. RA formation in both ecosystems may involve the action of radical enzymes that abstract H atoms from the conjugated double bond system.

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