Metabolism of Linoleic Acid by Human Gut Bacteria: Different Routes for Biosynthesis of Conjugated Linoleic Acid

Estelle Devillard*, Freda M. McIntosh, Sylvia H. Duncan and R. John Wallace

Gut Health Division, Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, AB21 9SB, UK

Running title: Linoleic acid metabolism by gut bacteria

* Corresponding author: R.J. Wallace, Rowett Research Institute, Bucksburn, Aberdeen, AB21 9SB, UK, Tel: +44(0)1224-716656, Fax: +44(0)1224-716687, Email address: john.wallace@rowett.ac.uk.

Abbreviations: CLA, conjugated linoleic acid; FAME, fatty acid methyl ester; GC, gas chromatography; HFA, hydroxy fatty acid; LA, linoleic acid (cis-9,cis-12-18:2); LI, linoleate isomerase; SA, stearic acid (18:0); VA, vaccenic acid (trans-11-18:1).
Abstract

A survey of 30 representative strains of human Gram-positive intestinal bacteria indicated that Roseburia species were among the most active in metabolising linoleic acid (cis-9,cis-12-18:2). Different Roseburia spp. formed either vaccenic acid (trans-11-18:1) or a 10-hydroxy-18:1, precursors of the health-promoting conjugated linoleic acid (CLA), cis-9,trans-11-18:2 in human tissues and the intestine, respectively.
Linoleic acid (LA; cis-9, cis-12-18:2) is metabolised in the human colon via conjugated linoleic acid (CLA; mainly cis-9, trans-11-18:2, LA) to vaccenic acid (VA; trans-11-18:1), both of which are considered to be beneficial for health (5, 11, 27, 28, 31, 41), then to stearic acid (18:0) (17, 26). A similar pathway occurs in the rumen (12, 20), where this process, commonly known as biohydrogenation, carries important implications for the fatty acid composition of meat and milk (22, 38). The microbiology of biohydrogenation in the rumen has received a great deal of attention (12, 32, 40, 42), but similar investigations have not been carried out for the human intestinal microbiota. The aims of the present study were therefore to identify human gut bacteria that can undertake fatty acid biohydrogenation, and to assess their likely importance in the mixed intestinal ecosystem. It emerged that Roseburia spp. were probably of greatest importance, some species metabolising LA by the same pathway found in ruminal bacteria (20, 32), while others formed a hydration product that is a precursor of CLA in the mixed community.

Thirty bacterial strains (2 lactobacilli, 1 lactococcus, 5 propionibacteria, 3 bifidobacteria and 19 strains of the low G+C Clostridium cluster) isolated from or related to bacteria found in the human large intestine were studied for their ability to metabolise LA (Table 1). The bacteria were grown either in the liquid form of anaerobic basal M2 medium (13) or in the same medium supplemented with 50 µg/ml LA. For labelling experiments the medium was prepared using deuterium oxide, to provide an enrichment in the medium water of around 50%. Linoleate isomerase activity was measured in bacteria grown on unsupplemented M2 medium by the method described by Wąsowska et al. (43). Extraction and derivatization of the total fatty acids to fatty acid methyl esters (FAME) and identification methods were similar
to those described by Devillard et al. (7). In order to determine the fate of the hydroxy-18:1 fatty acid (HFA) produced by some of the *Roseburia* isolates, *Roseburia intestinalis* L1-952 was grown in M2 medium containing 50 µg/ml LA, the culture was centrifuged (10,000 g, 10 min, 4 °C), and the supernatant was used to prepare a M2-modified medium enriched in HFA. This medium was inoculated with freshly voided human feces from two omnivorous volunteers consuming a Western diet, which had been diluted (0.2 g in 1 ml) in sterile anaerobic 0.1 M potassium phosphate buffer, pH 7.0. Aliquots were taken in duplicate for each fecal sample after different times for fatty acid determination up to 72 h incubation.

Linoleate isomerase activity was >10 nmol CLA formed (mg protein)^−1 min^−1 in 8 of the 30 isolates (Table 1), with *Butryrivibrio fibrisolvens* 16.4 and two strains of *Roseburia inulinivorans* (A2-194 and L1-83) showing the highest activity. When inoculated into medium containing LA, most of the 30 strains showed a lag time before they started to grow (Table 1). The same 8 high-isomerase isolates also metabolised LA most extensively (Table 1). The final products for these strains were VA for the *Roseburia* and *B. fibrisolvens* isolates and a mixture of CLA, mainly cis-9,trans-11-18:2, trans-9,trans-11-18:2, and trans-10,cis-12-18:2, for *Propionibacterium freudenreichii* subsp.*shermani*, and cis-9,trans-11-18:2 and trans-9,trans-11-18:2 for *Bifidobacterium breve* (Table 1). Thus, it appears that, whereas other species produce as mixture of CLA products, bacteria from *Clostridium* Cluster XIVa formed one product, either VA or HFA. Thirteen strains from both phylogenetic groups metabolised LA (from 26% to 88%), despite low linoleate isomerase activity (Table 1). GC traces indicated that these strains all produced the same compound, which was subsequently identified by GC-MS (Fig. 1A). The main fragmentations in mass spectrometry led to the formation of the ions 169 and 201 characteristic of a 10-
HFA with the first ten bonds saturated. When *R. intestinalis* L1-952 was grown in the presence of 50 µg/ml LA and deuterium oxide, the two major peaks were shifted to ions 171 and 203 (Fig. 1B). This shift corresponded to an addition of deuterium atoms at the double bond between carbons 9 and 10, suggesting that hydration occurred on this double bond (Fig. 1B). The location of the double bond could not be determined by analysis of the FAME. However, the signature masses of 201 and 294 identified in 10-hydroxy-cis-12-18:1 by Schroepfer et al. (37) were present in the FAME spectrum.

This information, together with purely biochemical considerations and comparison of the elution time of the FAME with that of ricinoleic acid (12-hydroxy-cis-9-18:1) indicated that the product was most likely 10-hydroxy-cis-12-18:1.

M2-modified medium prepared from the culture supernatant of *R. intestinalis* L1-952 initially contained 5 µg/ml LA and 25 µg/ml HFA. The main fatty acids produced during incubation with diluted feces were cis-9,trans-11-18:2, VA and SA (Fig. 2). After few hours, LA was almost completely metabolised, but the synthesis of CLA, VA and SA continued and corresponded to the disappearance of HFA (Fig. 2). Thus, it was concluded that HFA is a precursor of cis-9,trans-11-18:2 in the mixed community. None of the pure cultures examined here metabolised the HFA further.

Animal studies and clinical trials indicate that CLA may be useful in improving human health (5, 27, 31). The uptake of CLA formed in the intestine seems to be minor (17). However, local effects on gut tissue might be anticipated. It is now well established that CLA have anti-proliferative and anti-inflammatory effects on colonocytes (4, 19), so the provision of CLA in the intestinal lumen would be considered beneficial, particularly for inflammatory bowel disease, such as ulcerative colitis and Crohn's disease (10). Bacteria from other ecosystems and from food products, but which are also found in the human gut, including strains of
Lactobacillus, Propionibacterium and Bifidobacterium (1, 6, 15, 23, 33, 34) have been known for some time to possess the ability to generate CLA. For the first time, here we show that the more abundant bacterial species belonging to clostridial clusters IV and XIVa also metabolise LA at among the fastest rates of all bacteria investigated, forming products that can be precursors of CLA (Fig. 3). Given the greater abundance of Clostridium-like bacteria present in the human intestinal microbiota (9) - lactobacilli, propionibacteria, bifidobacteria numbers are low, less than 5% of the total microbiota (2, 21) - it may be deduced that LA metabolism by this major group will be quantitatively more important than that of the Lactobacillus, Propionibacterium and Bifidobacterium groups.

The discovery that HFA is a precursor of cis-9,trans-11-18:2 in the mixed intestinal community is also new, leading to a likely scheme of CLA formation as described in Fig. 3. A similar importance of HFA was proposed for a Lactobacillus sp., involving a hydration/dehydration process (30). No similar role for HFA has been postulated for bacteria in the rumen, where biohydrogenation is a quantitatively highly important activity (12, 32).

The final product of LA metabolism by mixed fecal microbiota was shown here to be stearic acid, as already shown by Howard and Henderson (14), yet none of the strains tested here produced stearate from LA (Fig. 3). Searching for stearate producers in the rumen has proved difficult, largely because they are extraordinarily sensitive to the toxic effects of unsaturated fatty acids (25, 42). The same may be true of human intestinal bacteria. Thus, stearate producers and those species that convert HFA to CLA, both potentially very important reactions in the mixed ecosystem of the human intestine, remain to be identified.
The Rowett Research Institute receives funding from the Scottish Executive Environmental and Rural Affairs Department. We thank David Brown, Graham Calder and Maureen Annand for technical help and expertise. We thank Kevin Shingfield and William Christie for advice on fatty acid analysis. We are grateful to Harry Flint for the helpful criticism of this manuscript.
REFERENCES


21. **Lay, C., M. Sutren, V. Rochet, K. Saunier, J. Dore, and L. Rigottier-Gois.** 2005 Design and validation of 16S rRNA probes to enumerate members of the
*Clostridium leptum* subgroup in human fecal microbiota. Environ. Microbiol. 7:933-946.


LEGENDS

FIG. 1. Mass spectra of the methyl ester of the HFA produced by 13 of the 30 bacterial strains studied here when incubated with linoleic acid. A. Mass spectrum obtained with the unlabelled FAME derivative. B. Mass spectrum obtained with the FAME derivative obtained from cultures containing deuterium oxide, showing the m/z from 160 to 210, where the two characteristic fragments of HFA are present.

FIG. 2. Metabolism of HFA (■) and LA (◆) and the formation of products: CLA (△), VA (○) and stearic acid (□) by mixed fecal flora from volunteer 1 (Fig. 2A) or from volunteer 2 (Fig. 2B). Values are the means of duplicates and are expressed as µg of fatty acid/ml fecal suspension.

FIG. 3. Proposed pathways of LA metabolism by bacterial species isolated from the human gut. 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. E. siraeum) and XIVa (e.g. R. intestinalis and R. 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. R. hominis and R. inulinivorans). The dotted arrows represent activities observed in fecal microbiota, but for which the responsible bacterial species are still unknown.
<table>
<thead>
<tr>
<th>Species</th>
<th>Group</th>
<th>Source [reference]</th>
<th>LI activity (nmol CLA mg protein⁻¹ min⁻¹)</th>
<th>Lag time (h)</th>
<th>% LA metabolised</th>
<th>Main products formed⁹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus reuteri DSM 20016&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Firmicutes</td>
<td>Human adult intestine [18]</td>
<td>0.7 ± 0.1</td>
<td>0</td>
<td>28 ± 3</td>
<td>HFA</td>
</tr>
<tr>
<td>Lactobacillus delbrueckii subsp. bulgaricus DSM 20081&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Firmicutes</td>
<td>Bulgarian yoghurt [39]</td>
<td>0.6 ± 0.1</td>
<td>72</td>
<td>4 ± 5</td>
<td>None</td>
</tr>
<tr>
<td>Lactococcus lactis subsp. lactis DSM 20729</td>
<td>Firmicutes</td>
<td>Swiss cheese [35]</td>
<td>0.0 ± 0.0</td>
<td>0</td>
<td>4 ± 6</td>
<td>HFA</td>
</tr>
<tr>
<td>Propionibacterium freudenreichii DSM 20271&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Actinobacteria</td>
<td>Swiss cheese [16]</td>
<td>1.2 ± 0.1</td>
<td>72</td>
<td>1 ± 1</td>
<td>None</td>
</tr>
<tr>
<td>Propionibacterium freudenreichii subsp. shermanii</td>
<td>Actinobacteria</td>
<td>Cheese [36]</td>
<td>14.3 ± 1.1</td>
<td>24</td>
<td>96 ± 9</td>
<td>CLA (c9t11, t9t11, t10c12)</td>
</tr>
<tr>
<td>Propionic acid bacteria</td>
<td></td>
<td>NK [16]&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.0 ± 1.7</td>
<td>72</td>
<td>95 ± 7</td>
<td>CLA (c9t11, t9t11, t10c12)</td>
</tr>
<tr>
<td>Propionibacterium Jensenii DSM 20274</td>
<td>Actinobacteria</td>
<td>Silage [36]</td>
<td>2.4 ± 0.1</td>
<td>0</td>
<td>2 ± 1</td>
<td>None</td>
</tr>
<tr>
<td>Propionibacterium thoenii DSM 20276&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Actinobacteria</td>
<td>Emmental cheese [36]</td>
<td>3.5 ± 0.2</td>
<td>48</td>
<td>9 ± 3</td>
<td>HFA</td>
</tr>
<tr>
<td>Bifidobacterium adolescentis NCFB 2204</td>
<td>Actinobacteria</td>
<td>Human adult intestine [6]</td>
<td>0.7 ± 0.0</td>
<td>24</td>
<td>3 ± 0</td>
<td>HFA</td>
</tr>
<tr>
<td>Bifidobacterium breve NCFB 2258</td>
<td>Actinobacteria</td>
<td>Human infant intestine [6]</td>
<td>15.4 ± 0.9</td>
<td>0</td>
<td>95 ± 8</td>
<td>HFA</td>
</tr>
<tr>
<td>Bifidobacterium infantis NCFB 2256</td>
<td>Actinobacteria</td>
<td>Human infant intestine, [6]</td>
<td>3.6 ± 0.2</td>
<td>24</td>
<td>30 ± 9</td>
<td>HFA</td>
</tr>
<tr>
<td>Faecalibacterium prausnitzii L2-6</td>
<td>Cluster IV</td>
<td>Human feces [3]</td>
<td>0.5 ± 0.0</td>
<td>48</td>
<td>7 ± 0</td>
<td>HFA</td>
</tr>
<tr>
<td>Eubacterium straemum DSM 15702&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Cluster IV</td>
<td>Human feces [29]</td>
<td>2.7 ± 0.0</td>
<td>24</td>
<td>2 ± 3</td>
<td>HFA</td>
</tr>
<tr>
<td>Anaerostipes caccae L1-92&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Cluster XIVa</td>
<td>Human feces [24]</td>
<td>0.0 ± 0.0</td>
<td>0</td>
<td>5 ± 4</td>
<td>None</td>
</tr>
<tr>
<td>Eubacterium hallii L2-7</td>
<td>Cluster XIVa</td>
<td>Human feces [24]</td>
<td>3.5 ± 0.5</td>
<td>24</td>
<td>0 ± 2</td>
<td>None</td>
</tr>
<tr>
<td>Eubacterium ventriosum L2-12</td>
<td>Cluster XIVa</td>
<td>Human feces [3]</td>
<td>0.0 ± 0.0</td>
<td>24</td>
<td>7 ± 5</td>
<td>None</td>
</tr>
<tr>
<td>Eubacterium ruminantium L2-50</td>
<td>Cluster XIVa</td>
<td>Human feces [24]</td>
<td>0.0 ± 0.0</td>
<td>24</td>
<td>13 ± 3</td>
<td>HFA</td>
</tr>
<tr>
<td>Eubacterium rectale T1-815</td>
<td>Cluster XIVa</td>
<td>Human feces [24]</td>
<td>1.3 ± 0.0</td>
<td>24</td>
<td>11 ± 2</td>
<td>None</td>
</tr>
<tr>
<td>Eubacterium rectale A1-86</td>
<td>Cluster XIVa</td>
<td>Human feces [24]</td>
<td>3.5 ± 0.2</td>
<td>24</td>
<td>15 ± 1</td>
<td>None</td>
</tr>
<tr>
<td>Eubacterium rectale M104/1</td>
<td>Cluster XIVa</td>
<td>Human feces [3]</td>
<td>2.4 ± 0.2</td>
<td>24</td>
<td>10 ± 2</td>
<td>None</td>
</tr>
<tr>
<td>Roseburia inulinivorans A2-194&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Cluster XIVa</td>
<td>Human feces [8]</td>
<td>3.3 ± 1.6</td>
<td>24</td>
<td>100 ± 6</td>
<td>VA</td>
</tr>
<tr>
<td>Roseburia inulinivorans L1-83</td>
<td>Cluster XIVa</td>
<td>Human feces [8]</td>
<td>30.9 ± 2.6</td>
<td>24</td>
<td>100 ± 4</td>
<td>VA</td>
</tr>
<tr>
<td>Roseburia hominis A2-183&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Cluster XIVa</td>
<td>Human feces [8]</td>
<td>23.0 ± 1.0</td>
<td>24</td>
<td>100 ± 5</td>
<td>VA</td>
</tr>
<tr>
<td>Roseburia hominis A2-181</td>
<td>Cluster XIVa</td>
<td>Human feces [8]</td>
<td>11.6 ± 0.6</td>
<td>0</td>
<td>40 ± 8</td>
<td>VA</td>
</tr>
<tr>
<td>Roseburia intestinalis L1-82&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Cluster XIVa</td>
<td>Human feces [8]</td>
<td>0.4 ± 0.0</td>
<td>24</td>
<td>81 ± 11</td>
<td>HFA</td>
</tr>
<tr>
<td>Roseburia intestinalis L1-952</td>
<td>Cluster XIVa</td>
<td>Human feces [8]</td>
<td>0.5 ± 0.1</td>
<td>24</td>
<td>85 ± 13</td>
<td>HFA</td>
</tr>
<tr>
<td>Roseburia faecis M6/1</td>
<td>Cluster XIVa</td>
<td>Human feces [8]</td>
<td>0.9 ± 0.1</td>
<td>24</td>
<td>86 ± 9</td>
<td>HFA</td>
</tr>
<tr>
<td>Roseburia faecis M88/1&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Cluster XIVa</td>
<td>Human feces [8]</td>
<td>0.9 ± 0.0</td>
<td>24</td>
<td>76 ± 8</td>
<td>HFA</td>
</tr>
<tr>
<td>Roseburia faecis M72/1&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Cluster XIVa</td>
<td>Human feces [8]</td>
<td>1.0 ± 0.0</td>
<td>24</td>
<td>88 ± 12</td>
<td>HFA</td>
</tr>
<tr>
<td>Butyrivibrio fibrisolvens 16.4</td>
<td>Cluster XIVa</td>
<td>Human feces [24]</td>
<td>45.3 ± 3.1</td>
<td>24</td>
<td>100 ± 4</td>
<td>VA</td>
</tr>
</tbody>
</table>
When the OD$_{600}$ of the cultures reached 0.6, fatty acids were extracted from the cultures, the percentages of linoleic acid metabolised were calculated, and the products formed during the growth were identified. These analyses were conducted in triplicate.

\[^{\text{NK}}\text{ NK : not known.}\]

**TABLE 1. Metabolism of linoleic acid by different bacterial species.**
Fig. 2A  Devillard et al.

![Graph showing fatty acid concentration over time](http://jb.asm.org/)
Fig. 2B  Devillard et al.
Linoleic acid
cis-9, cis-12 C18:2

CLA
cis-9, trans-11 C18:2
trans-9, trans-11 C18:2

VA
trans-11 C18:1

CLA
trans-10, cis-12 C18:2

Tissue

CLA
cis-9, trans-11 C18:2

Stearic acid
C18:0

HFA
OH C18:1